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Mateo
Barro Fernández

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DEVELOPMENT OF A NOVEL IN VITRO MODEL OF ALZHEIMER´S DISEASE AND ITS APPLICATION TO HIGH-THROUGHPUT SCREENING ASSAYS

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DEVELOPMENT OF A NOVEL IN VITRO MODEL OF ALZHEIMER´S DISEASE AND ITS APPLICATION TO HIGH-THROUGHPUT SCREENING ASSAYS

Author

Mateo Barro Fernández

Supervisors: María Isabel Loza García; José Manuel Brea Floriani

Tutor: María Isabel Loza García

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Conflict of interest statement

I hereby declare that I have not incurred in any conflict of interest during the development and completion of this thesis. The research presented herein was conducted with full academic integrity, and no external influences, personal relationships, financial interests, or affiliations affected the design, execution, interpretation, or reporting of the findings.

All resources used during this research were acquired in accordance with institutional policies and ethical guidelines.

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ABBREVIATION LIST

5-HT2 5-Hydroxytryptamine receptor 2 (Serotonin receptor)

AAV Adeno-associated virus

α2 Adrenergic receptor alpha 2

Aβ Amyloid-beta

Aβ1-42 Amyloid-beta 1-42 peptide

ACID APP intracellular domain

AchE Acetylcholinesterase

AD Alzheimer's disease

ADAM10 A disintegrin and metalloprotease 10

AI Artificial intelligence

AKT Protein kinase B

AMPAR Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ApoE Apolipoprotein E

APP Amyloid precursor protein

AR Adrenergic receptor

ATP Adenosine triphosphate

BAD BCL2-associated agonist of cell death

BACE Beta-site APP cleaving enzyme

BAX BCL2 associated X protein

BBB Blood-brain barrier

BCL2 B-cell lymphoma 2

BDNF Brain-derived neurotrophic factor

BIN1 Bridging integrator 1 protein

BPSD Behavioural and psychological symptoms of dementia

BSA Bovine serum albumin

BuChE Butyrylcholinesterase

°C Degrees Celsius

Ca^{2+} Calcium cation

CALHM Calcium homeostasis modulator

CA1 Cornu ammonis 1 region of the hippocampus

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CAA Cerebral amyloid angiopathy

CAMKII Calcium/Calmodulin-dependent protein kinase II

cAMP Cyclic adenosine monophosphate

CBD Cortico-basal degeneration

CCO Cytochrome C oxidase

CD33 Cluster of differentiation 33

Cdk5 Cyclin-dependent kinase 5

cDNA Complementary DNA

ChAT Choline acetyltransferase

ChEIs Cholinesterase inhibitors

Clu Clusterin

c-MYC Cellular myelocytomatosis oncogene

cNOS Constitutive nitric oxide synthase

CNS Central nervous system

COX-2 cyclooxygenase-2

CREB cAMP response element-binding protein

CR1 Complement receptor 1

CSF Cerebrospinal fluid

CTF-β C-Terminal fragment beta

CV Coefficient of variation

DAT Dopamine transporter

DAMPs Damage-associated molecular patterns

DBS Deep brain stimulation

dcAMP Dibutyryl cyclic adenosine monophosphate

DMSO Dimethyl sulfoxide

DMTs Disease-modifying therapies

DNA Deoxyribonucleic acid

dNTPs Deoxynucleotide triphosphates

EC_{50} Half maximal effective concentration

EOAD Early-onset Alzheimer's disease

ER Endoplasmic reticulum

ERs Estrogen receptors

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ERK Extracellular signal-regulated kinase

ESCs Embryonic stem cells

fAD Familial Alzheimer's disease

FBS Foetal bovine serum

FDA *Food and Drug Administration*

FRET Fluorescence resonance energy transfer

FTD Frontotemporal dementia

FTDP Frontotemporal dementia with Parkinsonism

GABA Gamma-aminobutyric acid

GAP43 Growth associated protein 43

GFAP Glial fibrillary acidic protein

GLP-1 Glucagon-like peptide 1

GPx1 Glutathione peroxidase 1

GRP78 Glucose-regulated protein 78

GSK-3β Glycogen synthase kinase 3 beta

H2 Histamine receptor subtype 2

HBSS Hank's balanced salt solution

HDAC6 Histone deacetylase 6

HEPES 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

HGF Hepatocyte growth factor

HP1α Heterochromatin protein 1 alpha

HSPGs Heparan sulphate proteoglycans

HSV-1 Herpes simplex virus type 1

HTS High-throughput screening

IgG Immunoglobulin G

IL Interleukin

IL-R Interleukin receptor

iNOS Inducible nitric oxide synthase

INPP5D Inositol polyphosphate-5-phosphatase D

iPSC Induced pluripotent stem cells

K^+ Potassium cation

KDa Kilodalton

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KCl Potassium chloride

KH_2PO_4 Potassium dihydrogen phosphate

LB Lysogeny broth

LOAD Late-onset Alzheimer's disease

LPS Lipopolysaccharides

LRP1 Low-density lipoprotein receptor-related protein 1

LTD Long-term depression

LTP Long-term potentiation

MAPs Microtubule associated proteins

MAP2 Microtubule associated protein 2

MAPK Mitogen-activated protein kinase

MAPT Microtubule associated protein Tau

M-EOAD Mendelian early-onset Alzheimer's disease

MBD Microtubule binding domains

MCI Mild cognitive impairment

MCP-1 Monocyte chemoattractant protein-1

MEK Mitogen-activated protein kinase

$MgCl_2$ Magnesium chloride

$MgSO_4$ Magnesium sulphate

MLKL Mixed lineage kinase domain-like protein

MMSE Mini-mental state examination

MoCA Montreal cognitive assessment

MRI magnetic resonance imaging

MTs Microtubules

mtHSP 60/70 Mitochondrial heat shock protein 60/70

mTOR Mechanistic target of rapamycin

MTx Melatonin receptor X

NaCl Sodium chloride

NADPH Nicotinamide adenine dinucleotide phosphate

Na_2HPO_4 Disodium hydrogen phosphate

Na_2SO_4 Sodium sulphate

NF-κB Nuclear factor kappa B

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NFTs Neurofibrillary tangles

NGF Nerve growth factor

NM-EOAD Non-mendelian early-onset Alzheimer's disease

Nrf2 Nuclear factor erythroid 2-related factor 2

OA Okadaic acid

OPCs Oligodendrocyte precursor cells

OCT4 Octamer-binding transcription factor 4

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate buffered saline

PCR Polymerase chain reaction

p53 Tumour protein p53

P150 150 mm culture dish

PARP-1 Poly (ADP-ribose) polymerase 1

PD-1 Programmed cell death protein 1

PDPKs Proline-directed protein kinases

PET Positron emission tomography

pGlu-Aβ Pyroglutamate Amyloid-beta

PHFs Paired helical fragments

PI3K Phosphatidylinositol 3 kinase

Pin1 Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1

PKA Protein kinase A

PKC Protein kinase C

PMA Phorbol 12-myristate 13-acetate

PMCA Plasma membrane calcium ATPase

PP1 Protein phosphatase 1

PP2A Protein phosphatase 2A

P2Y12/X7R Purinergic receptor P2Y12/X7R

PSEN1 Presenilin **1**

PSEN2 Presenilin 2

PSP Progressive supranuclear palsy

RA Retinoic acid

RAR Retinoic acid receptor

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RFU Relative fluorescence units

RIPK1 Receptor-interacting protein kinase 1

RNA Ribonucleic acid

RNS Reactive nitrogen species

ROS Reactive oxygen species

Rpm Revolutions per minute

Ru(III) Ruthenium(III)

RXR Retinoid X receptor

sAD Sporadic Alzheimer's disease

sAPPα Soluble APP alpha

sAPPβ Soluble APP beta

SDS Sodium dodecyl sulphate

SIRT-1 Sirtuin 1 protein

SNPs Single nucleotide polymorphisms

SOD1 Superoxide dismutase 1

SORL1 Sortilin-related receptor **1**

SPI1 Spleen focus forming virus proviral integration oncogene

STZ Streptozotocin

TCA Tricyclic antidepressant

TGFβ Transforming growth factor beta

TH Tyrosine hydroxylase

TNF-α Tumour necrosis factor alpha

TMED10 Transmembrane P24 trafficking protein 10

TREM2 Triggering receptor expressed on myeloid cells 2

TRPA1 Transient receptor potential ankyrin 1

UNC5 Uncoordinated-5 protein family

VMAT Vesicular monoamine transporter

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RESUMO EN GALEGO

A enfermidade de Alzheimer (EA) foi descrita por primeira vez en 1906 por Alois Alzheimer, quen documentou os síntomas dunha paciente con perda severa de memoria, cambios de comportamento e deterioración cognitiva. Hoxe en día, a EA constitúe unha das enfermidades neurodexenerativas máis prevalentes e devastadoras a nivel global, afectando actualmente a aproximadamente 50 millóns de persoas en todo o mundo, cunha proxección que apunta a que este número se triplique para o ano 2050. Esta enfermidade caracterízase por un deterioro progresivo e irreversible das capacidades cognitivas e comportamentais, que culmina nunha perda completa da capacidade para realizar tarefas diarias básicas. Entre os síntomas máis comúns atópanse a perda de memoria, as dificultades na linguaxe, a desorientación e os cambios de comportamento. Este deterioro está relacionado con cambios patofisiolóxicos no cerebro, como a perda neuronal e sináptica, a neuroinflamación e a acumulación de proteínas mal pregadas, como a beta-amiloide e a proteína tau hiperfosforilada.

A EA pode dividirse en dous tipos principais: a enfermidade de Alzheimer de inicio temperán (*EOAD*, polas súas siglas en inglés) e a enfermidade de Alzheimer de inicio tardío (*LOAD*, polas súas siglas en inglés). A *EOAD* representa unha porcentaxe minoritaria (1-5%) dos casos e está asociada a mutacións xenéticas herdadas que afectan a proteínas como a proteína precursora amiloide (*APP*) e as presenilinas 1 e 2, implicadas na produción e eliminación da proteína amiloide. As mutacións coñecidas nestes xenes favorecen a acumulación de agregados de beta-amiloide, promovendo a formación de placas e outros cambios a nivel celular, acelerando desta forma o deterioro cognitivo e progresión da enfermidade. Por outro lado, a *LOAD* é a forma máis común da enfermidade, representando entre o 95% e o 99% dos casos. A diferencia da de inicio precoz, non segue un padrón hereditario claro, pero varios factores, como o envellecemento, mutacións en xenes como ApoE, o historial de patoloxías previas e factores ambientais, están implicados no seu desenvolvemento. O ApoE4, por exemplo, incrementa ata doce veces o risco de desenvolver EA en individuos homocigotos.

Ademais da clasificación segundo a idade de inicio, a etiopatoxenia da EA é altamente complexa e multifactorial. Diversas hipóteses tentaron explicar os mecanismos subxacentes da enfermidade durante as últimas décadas, pero segue sen haber un consenso claro sobre a súa etioloxía. A hipótese da cascada amiloide sostén que a acumulación de peptídos Aβ, derivados do metabolismo aberrante da proteína *APP*, é o desencadéante inicial. Estas acumulacións provocan unha serie de eventos patóxenos, incluíndo a hiperfosforilación da tau, o estrés oxidativo e a neuroinflamación. Outra teoría, a hipótese da tau, centra a súa atención na formación de enredos neurofibrilares debido á hiperfosforilación da tau, que interfire na estabilidade dos microtúbulos, esenciais para o transporte intracelular e o mantemento do funcionamento das sinapses. Por outra banda, as hipóteses colinérxica e glutamatérxica salientan a perda de neuronas e a desregulación destes neurotransmisores como un evento crucial no inicio do deterioro cognitivo. Ademais, nos últimos anos, a inflamación neuroxénica

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gañou relevancia como un factor que non só acompaña a progresión da enfermidade, senón que contribúe activamente á patoloxía.

A pesar da falta de consenso sobre o inicio da enfermidade, tanto a proteína amiloide como a proteína tau, son amplamente descritas na bibliografía como principais marcadores e promotores de progreso da enfermidade, así como de desencadear moitos dos procesos patolóxicos que culminan na perda sináptica, a morte neuronal e en cambios nas propiedades fiolóxicas neuronais. A patoloxía amiloide comeza coa acumulación do péptido beta-amiloide, xerado na proteólise amiloidoxénica da proteína precursora amiloide. Esta glicoproteína transmembrana altamente conservada exprésase principalmente no cerebro e cumpre funcións fisiolóxicas esenciais, como a plasticidade sináptica e o crecemento neuronal. Os péptidos Aβ sofren un proceso de agregación que comeza coa formación de oligómeros pequenos, seguidos de protofibriñas e fibriñas maduras, culminando na formación de placas insolúbeis. Estas placas inducen danos celulares significativos mediante mecanismos como o estrés oxidativo, a desregulación do calcio ou a indución do procesamento patoxénico doutras proteínas. Tau, outra proteína clave na EA, forma parte da familia das proteínas asociadas aos microtúbulos. En condicións normais, tau estabiliza os microtúbulos e regula o transporte intracelular. Porén, a hiperfosforilación de tau reduce a súa capacidade de unirse aos microtúbulos, favorecendo a formación de oligómeros e enredos neurofibrilares, así como a súa propagación cerebral seguindo un patrón similar ao rexistrado nas enfermidades priónicas. A neurotoxicidade de tau maniféstase en varios procesos intracelulares. A desestabilización dos microtúbulos altera o transporte axonal, mentres que a interacción de tau coas membranas celulares e mitocondriales provoca disfuncións na sinalización do calcio, na homeostase iónica e no metabolismo enerxético. Ademais, os oligómeros de tau interfiren coa membrana e poros nucleares, aumentando a susceptibilidade ao dano no ADN.

Dentro das mutacións asociadas ao Alzheimer, ou ao desenvolvemento de tauopatías similares ás exhibidas polos pacientes desta enfermidade, destacan entre outras, por prevalencia, tanto a mutación *London* na *APP* (APP V717I) e na proteína asociada a microtúbulos tau (MAPT; MAPT P301L). A mutación APP V717I provoca un cambio crítico na dinámica do seu procesamento. Esta mutación altera a unión da zona de corte das secretasas, favorecendo a produción do péptido de maior lonxitude. Estes péptidos pola súa natureza hidrofóbica, teñen unha maior tendencia a agregarse, o que leva á formación de oligómeros tóxicos dunha forma máis rápida. Por outra banda, a mutación MAPT P301L afecta á proteína tau, e aínda que non está considerada como factor de risco único para a EOAD, si é unha das máis prevalentes na demencia frontotemporal hereditaria, cunha tauopatía e síntomas comparables aos de Alzheimer. Esta mutación substitúe unha prolina por unha leucina na posición 301 da tau, o que altera a súa capacidade para unirse aos microtúbulos e favorece a hiperfosforilación da tau na serina 262, inducindo a súa polimerización e agregación en enredos neurofibrilares intracelulares.

A neuropatoloxía característica do Alzheimer, á que contribuen os depositos destas proteínas, inclúe a perda progresiva de sinapses e neuronas, acompañada dunha ampla atrofia

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cortical. Este proceso comeza nas áreas implicadas na memoria, como o hipocampo, estendéndose progresivamente ás cortizas frontotemporais e outras rexións cerebrais. A excitabilidade neuronal é outro factor clave na EA na que contribúen tanto tau como a proteína amiloide e segue un patrón bifásico ao longo da enfermidade. Nos estadios iniciais, obsérvase unha hiperactividade cerebral, especialmente nas rexións do hipocampo e a corteza entorrinal. Coa progresión da enfermidade e a formación de oligómeros insolubéis esta hiperactividade evoluciona cara a unha hipoactividade xeneralizada, resultado dos cambios estruturais e funcionais, conducindo a unha perda sináptica e a desregulación do calcio.

A nivel socioeconómico, o impacto da EA é inmenso. A enfermidade provoca custos directos e indirectos asociados á atención médica e á perda de produtividade tanto dos pacientes como dos coidadores, estimándose un custo global superior a 950.000 millóns de dólares anuais. Ademais, a carga emocional e psicolóxica é significativa, afectando tanto aos pacientes como aos seus familiares e coidadores, quen adoitan sufrir altos niveis de estrés, ansiedade e depresión. Do mesmo xeito, o Alzheimer tamén representa unha enorme carga para a saúde pública xa que as opcións farmacolóxicas existentes son fundamentalmente paliativas e centran a súa actividade terapéutica en aliviar algúns dos síntomas cognitivos e comportamentais da enfermidade durante os estadíos iniciais da enfermidade, non se dirixen ás causas subxacentes nin proporcionan unha cura definitiva para os pacientes. Fármacos como os inhibidores da acetilcolinesterasa (donepezil, galantamina e rivastigmina) buscan mellorar a sinalización colinérxica, que está deteriorada na maioría dos pacientes. Además, o antagonista do receptor NMDA, memantina, utilízase para reducir a excitotoxicidade asociada á enfermidade. Recentemente, e despois de máis de dúas décadas sen aprobacións farmacolóxicas por parte das principais axencias de medicamentos, tres novos fármacos, aducanumab, lecanemab e donanemab, foron aceptados para o seu uso en clínica. Estes tres antibióticos contra as formas solubles e insolúbeis de beta-amiloide, supuxeron un gran paso cara a diante no avance do descubrimento de fármacos para o Alzheimer, aínda que con resultados modestos nos ensaios con pacientes. Esta falta de opcións terapéuticas, o enfoque limitado das mesmas, e a gran taxa de fracaso que presentan os ensaios clínicos para esta enfermidade, destaca a necesidade de desenvolver novos tratamentos que orienten a súa actividade as múltiples bases patofisiolóxicas da EA.

O avance no estudo e descubrimento temperá de fármacos para a EA depende en gran medida do desenvolvemento de modelos experimentais que permitan reproducir os principais aspectos patofisiolóxicos da enfermidade. Os modelos in vivo tradicionais, como os ratos transxénicos, foron e son un pilar fundamental no estudo do Alzheimer, sendo cruciais para moitos dos descubrimentos realizados no campo da EA nas últimas décadas. Aínda así, presentan limitacións significativas na súa aplicación ao campo da farmacoloxía, como as limitacións éticas, a imposibilidade de empregar estes modelos en cribados de moléculas e a falta de translacionalidade dos resultados obtidos con eles. Por este motivo, os modelos in vitro cobraron gran importancia nas últimas décadas. Entre eles destacan as liñas celulares inmortalizadas, como a SH-SY5Y, derivada de neuroblastomas humanos, que pode diferenciarse en neuronas maduras baixo condicións específicas. Estas células ofrecen unha

alternativa reproducible, rápida e de alto custo-beneficio para estudar o comportamento neuronal baixo a influencia de características neurodexenerativas da EA. Ademais, as células pluripotentes inducidas representan unha opción máis personalizada, ao permitir a xeración de neuronas a partir de células somáticas de pacientes, conservando o seu contexto xenético. Non obstante, o custo elevado, a variabilidade, e as dificultades técnicas para producir liñas celulares consistentes, limitan o seu uso en campañas de descubrimento de fármacos novidosos para á enfermidade.

O desenvolvemento de modelos in vitro orientados ao cribado de fármacos é un obxectivo estratéxico na investigación da EA. A metodoloxía de cribado de alto rendemento (*HTS*, polas súas siglas en inglés) permite a potencial identificación de novos compostos con potenciais actividades terapéuticas ao permitir analizar grandes librarías de compostos de maneira rápida e escalable. Os ensaios de HTS facilitan o desenvolvemento de estratexias terapéuticas innovadoras, como o reposicionamento de fármacos, que resulta imprescindible para as enfermidades cunha etioloxía difusa, que permiten a análise de moléculas sen establecer de antemán unha diana farmacolóxica clara. Ademais, o Alzheimer presenta unha taxa de compostos reposicionados en fase clínica de arredor dun 30 %, reforzando a estratexia de orientar aproximacións deste tipo ao descubrimento de fármacos para a enfermidade. Así, o HTS combinado con outras técnicas como a intelixencia artificial, a transferencia de enerxía por resonancia de fluorescencia ou o cribado virtual emerxen como unha opción indispensable para desenvolver estratexias para o descubrimento de fármacos que reduzan significativamente os tempos e custos asociados ao desenvolvemento preclínico.

Neste contexto, a presente tese propón o desenvolvemento dun modelo translacional, robusto e innovador da EA empregando a liña celular SH-SY5Y diferenciada en neuronas maduras e transfectada con mutacións xenéticas na *APP*(*APP V717I*) e tau (*MAPT P301L*) relacionadas coa *EOAD*. Este modelo busca reproducir aspectos patofisiolóxicos clave da enfermidade, como a acumulación de Aβ e a hiperfosforilación da tau, coa intención de obter unha base biolóxica que permita estudar os cambios fenotípicos xurdidos destas. Finalmente, pretendeuse a aplicación deste modelo a ensaios de HTS que permitan a identificación de *hits* cunha acción neuroprotectora significativa ante o dano neuronal morfolóxico e fisiolóxico xurdido das modificacións xenéticas anteriormente mencionadas.

Para o desenvolvemento do modelo de Alzheimer, optouse pola liña celular SH-SY5Y, derivada de neuroblastoma humano, debido á súa capacidade de diferenciación en fenotipos neuronais específicos e á súa ampla utilización en estudos no campo da neurociencia. A diferenciación neuronal foi inducida mediante un protocolo en dúas etapas, empregando na primeira ácido retinoico (*RA*), un composto coñecido por promover o crecemento de neuritas e potenciar as características dopaminérxicas inherentes ás células SH-SY5Y. Ademáis, incorporáronse na segunda etapa da diferenciación dous compostos específicos: un péptido similar ao glucagón-1 (*GLP-1*) ou o Forbol 12-miristato 13-acetato (*PMA*), para inducir diferentes fenotipos neuronais, glutamatérxicos ou dopaminérxicos, respectivamente, adaptando protocolos experimentais previos descritos na bibliografía. O proceso de

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diferenciación complementouse con técnicas de mutaxénese dirixida e transfección química, que permitiron introducir mutacións específicas en xenes asociados ao Alzheimer de inicio temperán, *APP* V717I e MAPT P301L, para estudar os seus efectos sobre a morfoloxía celular e en procesos de fisioloxía neuronal. Para caracterizar estes cambios empregáronse técnicas de microscopía de alto contido, que permite as medidas automatizadas das variables seleccionadas, viabilidade celular, arborización dendrítica e o acurtamento das neuritas, empregadas para avaliar a neurotoxicidade inducida pola expresión de estes xenes mutados. Para validar a eficacia do modelo e a súa aplicación a ensaios de HTS, realizáronse estudos empregando compostos de referencia neuroprotectores como melatonina e resveratrol, coñecidos polas súas propiedades antioxidantes e antiinflamatorias. Adicionalmente, realizouse o desenvolvemento dun ensaio primario baseado na medida de calcio intracelular en resposta ao KCl. Este ensaio empregouse para avaliar a capacidade excitabilidade neuronal dos cultivos, coa finalidade de demonstrar a adquisición de características neuronais depois da diferenciación, de observar os cambios fisiolóxicos surxidos das mutacións introducidas e de avaliar a acción dos compostos de referencia máis aló da neuroprotección morfolóxica. O modelo integrouse finalmente no cribado de alto rendemento da quimioteca Prestwick® para identificar *hits* cunha acción neuroprotectora e potenciais candidatas ao tratamento da enfermidade de Alzheimer. Co obxectivo de validar a reproducibilidade e robustez dos ensaios, empregáronse métricas e condicións restritivas como a concentración de compostos (1 µM), as métricas como o factor Z' e a xanela de sinal (> 0,5; > 2), e un coeficiente de variabilidade dos controis inferior ao 20%, enmarcándonos así nas garantías de calidade de ensaios de HTS establecidas neste campo de investigación.

Polo tanto, na presente tese doutoral:

i) Desenvolveuse un modelo miniaturizado e escalable con fenotípico neuronal a partir da diferenciación da liña celular SH-SY5Y mediante RA e GLP-1, obtendo un fenotipo neuronal maduro caracterizado por un maior número e densidade de neuritas en comparación aos obtidos coa diferenciación con RA e PMA.

ii) Puxéronse a punto os protocolos de transfección celular, permitindo a introdución das mutacións xenéticas *APP* V717I e MAPT P301L de forma transitoria nas células diferenciadas. Estas mutacións induciron cambios morfolóxicos significativos: diminución do número de células por pozo ($p<0,01$ e $p<0,0001$), redución na lonxitude media das neuritas ($p<0,0001$) e menor arborización dendrítica ($p<0,001$ e $p<0,01$). A co-transfección con ambas mutacións intensificou estes efectos en tódalas variables ($p<0,0001$), reproducindo características similares ás rexistradas nos procesos de neurodexeneración observadas na EA.

iii) Demostrouse que o tratamento dos cultivos celulares cos compostos de referencia neuroprotectores, melatonina e resveratrol, produciron unha reversión significativa do dano morfolóxico inducido polas mutacións. A exposición a 1 µM de melatonina resultou nun aumento significativo dos valores de tódalas variables morfolóxicas, mentres que 1 µM de resveratrol tamén revertíu significativamente os cambios neurodexenerativos, excepto no número de células e na arborización dendrítica das células transfectadas con *APP* V717I.

iv) Desenvolveuse un ensaio fisiolóxico primario baseado na medición de calcio intracelular, optimizado para estudar cambios de excitabilidade en resposta a KCl derivados das mutacións *APP* V717I e MAPT P301L. A liña celular diferenciada mostrou unha maior excitabilidade ($p<0,0001$) en resposta a KCl 30 mM respecto á liña celular sen diferenciar, destacando as propiedades neuronais adquiridas máis aló das propiedades fenotípicas. Pola contra, as células co-transfectadas presentaron un comportamento hipoexcitatorio ($p<0,0001$), que foi revertido significativamente por melatonina e resveratrol ($p<0,0001$).

v) Adaptáronse os ensaios de calcio intracelular a un cribado de alto rendemento, utilizando a quimioteca Prestwick® para avaliar a acción neuroprotectora de compostos contra a hipoexcitabilidade rexistrada post-transfección (Factor Z' = 0,642 ± 0,069; Xanela de sinal = 7,413 ± 1,555). Identificáronse oito compostos (dapsona, mianserina, etosuximida, doxepina, rifampicina, canavanina, betazol dihidrocloruro e a metolazona) con capacidade para reverter a hipoexcitabilidade neuronal inducida polas mutacións. Estes hits demostraron eficacia terapéutica con valores de EC50 entre 4 e 220 nM.

Dos oito compostos, avaliáronse as dianas e mecanismos de acción dos *hits* segundo as propiedades farmacolóxicas e a bibliografía para identificar a características neuroprotectoras destes fármacos. Entre eles, tres teñen usos terapéuticos clínicos para doenzas do sistema nervioso central (doxepina, mianserína e etosuximida), dous son antibióticos con efectos neuroprotectores descritos e ensaios clínicos na EA (rifampicina e dapsona), e os outros tres non teñen unha actividade terapéutica directa descrita no SNC, pero demostraron efectos protectores no modelo desenvolvido. A doxepina é un antidepresivo tricíclico amplamente utilizado no tratamento de depresión e trastornos de ansiedade. A súa acción terapéutica é multifuncional, involucrando receptores serotoninérxicos, adrenérxicos, glutamatérxicos e GABAérxicos, así como a modulación de canais iónicos de sodio e calcio. Ademais, posúe propiedades neuroprotectoras demostradas en modelos de enfermidades neurodexenerativas, como a redución do estrés oxidativo, a modulación da toxicidade neuronal inducida por beta-amiloide e a regulación de xenes apoptóticos. A mianserina, outro antidepresivo, actúa sobre receptores como os 5-HT_2 e α_2, mellorando a sinalización de serotonina e noradrenalina. Además das súas propiedades antidepresivas, mostrou un carácter neuroprotector a través de propiedades anti-apoptoticas, protexer contra o estrés oxidativo e recuperar a transmisión sináptica en modelos de neurodexeneración. A etosuximida é un anticonvulsivante utilizado clinicamente para tratar crises de ausencia na epilepsia infantil. O seu mecanismo neuroprotector inclúe a inhibición de canais de calcio tipo T e a activación da vía PI3K-AKT, protexendo contra a neurodexeneración mediada por beta-amiloide e reducindo a hiperexcitabilidade precoz no Alzheimer. Os antibióticos dapsona e rifampicina, usados principalmente no tratamento da lepra, posúen propiedades antioxidantes, antiinflamatorias e antiapoptóticas, sendo capaz de regular a homeostase iónica e mitigar o estrés oxidativo. A metolazona, un diurético usado para tratar hipertensión e insuficiencia cardíaca, mostrou efectos protectores indirectos, potencialmente inducidos a través da regulación de chaperonas mitocondriais como mtHSP70 e HSP60, que prevén a disfunción mitocondrial e a apoptose. A canavanina, un análogo da arginina, foi un dos resultados máis inesperados do estudo. Aínda

que hai literatura contraditoria sobre o seu impacto neurotóxico, neste modelo demostrou propiedades neuroprotectoras, probablemente relacionadas coas accións descritas noutros modelos como a inhibición de especies reactivas de nitróxeno e a súa interacción con receptores imidazolina. Por último, o betazol, un derivado do pirazol empregado como estimulante gástrico. Aínda que, como molécula individual, carece de literatura sobre actividade neuroprotectora ou interaccións no sistema nervioso, a súa estrutura química baseada no pirazol está ben caracterizada para paliar a patoloxía do alzheimer en modelos preclínicos. Así, exemplos como o riluzol ou o aminopirazol demostraron propiedades antioxidantes significativas, como a eliminación de especies reactivas de osíxeno e a inhibición da peroxidación lipídica, ademais de mostrar eficacia na prevención da fibrilación de beta-amiloide e a agregación de tau.

En conclusión, esta tese doutoral contribuíu ao desenvolvemento dun novo modelo in vitro de Alzheimer, baseado no uso da liña celular diferenciada SH-SY5Y para a adquisición dun fenoripo neuronal maduro. Este modelo demostrou ser aplicable a ensaios morfolóxicos e fisiolóxicos escalables e automatizables para a caracterización dos cambios patolóxicos asociados á enfermidade. Mediante estes ensaios foi posible caracterizar o dano citotóxico e a hipoexcitabilidade provocados pola expresión, tras a transfección, coas mutacións xénicas APP V717I e MAPT P301L, directamente relacionadas coa variante familiar do Alzheimer. Ademais, validouse a compatibilidade de este modelo para a súa aplicación a ensaios de HTS mediante o emprego de compostos de referencia, melatonina e resveratrol, que teñen acción neuroprotectora previamente descrita noutros modelos. Para rematar, o modelo aplicouse a un cribado de moléculas pertencentes á quimioteca Prestwick®, que permitíu atopar 8 *hits* con propiedades neuroprotectoras.

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ABSTRACT

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1. ABSTRACT

Alzheimer's disease (AD) is the leading neurodegenerative disorder worldwide and the most prevalent cause of dementia, accounting for 60–80% of all cases. With an estimated 50 million individuals affected globally and the incidence projected to triple by 2050, AD represents a growing public health and socioeconomic burden. A small percentage of AD cases (1–5%) are familial in origin, caused by mutations in genes such as APP, PSEN1/2, and MAPT, which influence the amyloidogenic pathway or tau protein processing. Conversely, the sporadic form of AD, which constitutes the majority of cases, is linked to complex genetic and environmental interactions, including aging, previous pathologies, oxidative stress, and chronic inflammation. Alzheimer's pathology is characterized by a progressive cognitive decline driven by the loss of synaptic integrity, neuronal death, and chronic neuroinflammation. These neurodegenerative changes and progression of the disease are underpinned by key hallmark features such as amyloid-β and tau deposits. Despite extensive research over the past few decades, the pathogenesis of AD remains incompletely understood, primarily due to its multifactorial nature and the interplay of genetic, environmental, and biochemical risk factors. Despite Alzheimer's undeniable impact in current society, it remains as one of the diseases with the highest rate of failure in clinical trials and which therapeutic interventions remain palliative, addressing early-stage symptoms without halting disease progression. Taking in consideration these necessities, there is an unmet demand to develop translational models that can accelerate the pre-clinical drug discovery, enabling the detection of synergies and new mechanisms of action that are currently challenging to identify using traditional models. Therefore, the use of the differentiated SH-SY5Y neuroblastoma-derived cell line differentiated into a mature neuronal phenotype and transfected with fAD mutations could constitute an innovative biological platform for developing a scalable and automatable in vitro model for its application to high-throughput screening assays.

Therefore, in this doctoral thesis: i) A miniaturized and scalable neuronal phenotypic model was developed from the differentiation of the SH-SY5Y characterized by an increased number and density of neurites; ii) The transfection with APP V717I and MAPT P301L into induced significant morphological changes, including a reduction in the number of cells per well, a decrease in average neurite length, and reduced dendritic arborization; iii) It was demonstrated that treating the cell cultures with the reference neuroprotective compounds, melatonin and resveratrol, significantly reversed the morphological damage induced by the mutations; iv) A primary physiological assay based on intracellular calcium measurement was developed and optimized to study excitability changes in response to a depolarizing agent, registering a hypo-excitation in transfected cultures that melatonin and resveratrol were able to revert; v) It was confirmed that the model is suitable for high-throughput screening assays, by using the Prestwick® chemical library to evaluate the neuroprotective action of compounds against the hypo-excitability observed post-transfection (Z' factor > 0.5). Eight compounds were identified for their ability to reverse the neuronal hypo-excitability with EC50 values ranging from 4 to 220 nm.

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In conclusion, this doctoral thesis contributed to the development of a novel in vitro Alzheimer's model, based on the use of the differentiated SH-SY5Y cell line to achieve a mature neuronal phenotype. This model proved to be applicable to scalable and automatable morphological and physiological assays for the characterization of neurodegenerative derived from cell transfection with APP V717I and MAPT P301L. Through these assays, it was possible to characterize the cytotoxic damage and hypo-excitability which were comparable to the neurodegenerative processes observed in AD. Furthermore, the compatibility of this model for application in high-throughput screening assays was validated using melatonin and resveratrol as reference compounds. Lastly, the model was applied to a screening of molecules from the Prestwick® chemical library, leading to the identification of eight hits with neuroprotective properties.

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BACKGROUND, HYPOTHESIS AND OBJECTIVES

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2. BACKGROUND, HYPOTHESIS AND OBJECTIVES

Alzheimer´s disease (AD) is the most prevalent neurodegenerative disease in the world and the most common form of dementia, affecting currently around 50 million people, and showing a rising tendency that is thought to triplicate the cases by 2050 (Dos Santos Picanco et al., 2016). AD is characterized by a slow progression and an escalated behavioural and cognitive decline due to the pathophysiological changes produced in patient´s brain, presented as synaptic and neuronal loss, neuroinflammation and misfolded protein aggregation, particularly involving amyloid-beta (Aβ) and microtubule associated protein tau (MAPT) (Monteiro et al., 2023).

■ Primarily associated with aging and environmental factors as main risks for the sporadic onset of the disease, a small percentage of cases (≈ 1-5%) are considered to be familial and driven by inheritable genetic mutations in proteins involved in the metabolic and clearance processes of Aβ and MAPT (i.e. APP, PSEN 1-2, ApoE4) (Shea et al., 2016; Andrade-Guerrero et al., 2023). In addition to its high impact, AD is a disease that imposes a huge socio-economic burden prolonged on time by the impeding and long-lasting nature of the disease that involves intensive care of the patients both from healthcare systems and families to improve their quality of live (Tay et al., 2024). Despite the necessity and efforts made in the past decades, available pharmacological treatments for the pathology only target the symptomatic relief for the early stages of the disease, with no cure or preventive option available.

■ Alzheimer´s disease etiology remains not fully dilucidated due to the complexity and multifactoriality of AD´s pathophysiology. Although, the extra and intra-cellular deposits of misfolded Aβ, in plaques, and Tau, in neurofibrillary tangles, have been the most described, studied and described as the most recognizable pathological hallmarks in the patients´ brain (Sheppard & Coleman, 2020). These deposits are mainly located in the hippocampal and cortical areas, and lead to a severe synaptic dysfunction and neuronal loss in those regions, which play a critical role at memory and cognition processes (Cai et al., 2023). Over the years, many hypotheses have tried to elucidate if these deposits are the initiators of the disease or just a consequence of other involved biochemical pathways involved in the onset and course of the pathology. Amongst all these plausible theories, the most prominent and accepted to explain the underlying mechanisms involving Alzheimer´s pathophysiology is the amyloid cascade hypothesis. This suggests that the aggregation of long amyloid-β peptides (Aβ-42) initiates the series of neuropathological occurrences such as the aberrant tau hyperphosphorylation, the neuroinflammation and the oxidative stress. Other propositions focus on the abnormal hyperphosphorylation of Tau protein or the loss of cholinergic neurons to assess the onset and cognitive decline symptoms of AD (Terry & Buccafusco, 2003; Ricciarelli & Fedele, 2017; Paroni et al., 2019; Arnsten et al., 2021). This lack of consensus on the understanding of the mechanisms involved in all of the diseases stages, the intrinsic difficulty that neurodegenerative diseases present for their study in human patients combined with the lack of new effective treatments from the early 2000s until 2023, with the approval of anti-amyloid-β antibodies, highlight Alzheimer´s disease as a primary focus for breakthrough advances in the neuroscience field that can be translational to the pharmaceutical industry (Cummings et al., 2023; Ameen et

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al., 2024). This new class of drugs (anti-beta amyloid and anti-tau antibodies), that are commercially available or in late stages of clinical trials, directly correlates with the reduction of protein deposits, aiming to address one of the most common clinical hallmarks of AD, thus emphasizing a shift in the trend of drug discovery research for the disease.

■ In Alzheimer's early-stage research, there have been many developed and described methods and models of study that could be used as a representative base for drug discovery. Various types of in vivo models, using invertebrates, zebrafish and even primates (Banik et al., 2015). However, the majority of in vivo model´s described in both basic and pharmacological research use transgenic mouse models that overexpress mutated genes discovered in familial variant AD patients (Drummond & Wisniewski, 2016). Amongst these, the most complete for the study of the disease has been the triple transgenic mouse, which includes mutant forms of APP, PSEN and Tau genes, producing both amyloid plaques and neurofibrillary tangles in the same model (Oddo et al., 2004). Despite the ever-increasing complexity of animal models, including more pathophysiological characteristics of AD, such as neuroinflammation or vascular pathologies, the application of these models for translational research has been limited due to the numerous obstacles that neurodegenerative disease study presents on animal models (Dawson et al., 2018). Beyond the ethical and economic concerns associated with animal experimentations, developed in vivo models often focus on the final stages of the disease, preventing the study of the initiation and progression of the disease, and base the representation of AD on the overexpression of human genes in a completely different species that can hinder the translation of findings as well as constituting an impossible model type in which to screen large libraries of compounds that could potentially lead to new pathological insights of the protein deposits' interactions and role in the pathophysiology of the disease (LaFerla & Green, 2012; Andersen & Winter, 2019; Vangala et al., 2022).

Over the past two decades, with the development of somatic cell reprogramming into induced-pluripotent stem cells (iPSCs), in vitro models for the study of neurodegenerative diseases have gained a lot of attention and popularity. In vitro cell models have allowed researchers to investigate and dilucidated many underlying mechanisms of AD´s pathology as well as evaluate potential new treatments by screening drug candidates (Choi & Tanzi., 2012; Kondo et al., 2017; Arber et al., 2019). Taking into consideration how these models mimic key pathological features of AD, the mostly used and simplest method involves cell cultures exposed to synthetic peptides of Aβ and tau, focusing on exclusively studying the toxic effects of the various amyloid peptides involved in the disease (D'Errico & Meyer-Luehmann, 2020; Ulm et al., 2021). More advanced and closely related to the pathophysiology of AD are the models that use genetic modification of proteins involved in the familial variant, in order to build a more progressive and complex representation of the disease on human cells. These modifications lead to an increased production of beta amyloid peptides and hyperphosphorylation of Tau and allow to replicate the accumulation process and other derived

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such as neuroinflammatory processes or cellular damage over a given timespan (Bassil et al., 2021; Lu et al., 2021; Xie et al., 2023).

Immortalized cell lines have become an essential in the development of Alzheimer´s models as they represent an easy maintenance, reproducible and scalable tool for pharmacological assays that also have a great cost-effectiveness when compared to other types of cell models. Amongst the cell lines used in Alzheimer´s research. The SH-SY5Y cell line, derived from neuroblastoma, is amongst the most frequently employed, especially for its ability to differentiate in various types of mature neuron-like cells under specific conditions. Through these differentiation processes, cells can form synaptic structures and express specific neuron proteins, making them valuable for the research of neuronal behaviour under AD´s pathophysiology (Lopez-Suarez et al., 2022; Targett et al., 2024). In contrast, iPSCs offer a more complex alternative to build AD models. By reprogramming somatic cells, like fibroblasts, from AD patients or those at risk, iPSCs can be differentiated into neurons that maintain the donor's genetic background, allowing for a personalized exploration of AD mechanisms (Arber et al., 2019). However, challenges with iPSC models include phenotypic variability among patients, the potential persistence of epigenetic mutations, and difficulties and costs in generating cell lines that can be stored and reproduced for other studies (Yagi et al., 2011; Israel et al., 2012; Shi et al., 2012). Additionally, to overcome the issues that 2D monocultures present at representing the complex structures and interactions of the brain, complex models have increasingly adopted co-culture or three-dimensional cell culture models, which allow to grow cells in structures that more closely resemble human brain´s in vivo conditions. These types of cultures allow for a spatial and temporal study of the pathophysiology and allow for the interaction of glial and neuronal cells, opening for a more comprehensive view of the role of glial and support cells in AD (Pereira et al., 2021; Barak et al., 2022; Shi et al., 2024). Despite the undeniable advantages that these more complex models present for developing an Alzheimer´s model, their high costs, technical difficulty and lack of reproducibility and stability for screening assays, making them a non-viable option for early-stage pharmacological research (Akhtar et al., 2022). This context highlights the current need to develop new disease models that are compatible with drug discovery, while also enabling the reproduction and evaluation of key pathophysiological hallmarks of Alzheimer's disease like neuronal death, neuronal excitability, and synaptic damage.

■ Alzheimer´s disease presents one of the highest rates of failure rate on drug development during clinical trials (Cummings et al., 2019). Development and advances in drug screening techniques have significantly accelerated in the past decade the advances made in Alzheimer's research. These techniques allow for a rapid, scalable and automated testing of large compound libraries that may potentially lead to the identification of new pathophysiological pathways or viable treatment candidates (Zhang et al., 2024). This tool has been proven instrumental in pharmacological research for AD for identifying new molecules that can prevent amyloid-β aggregation, reduce tau hyperphosphorylation or even modulate the neuroinflammatory processes. High-throughput screening (HTS) assays have also facilitated the development of novel therapeutic strategies, such as the repositioning of existing drugs for other

neurodegenerative diseases and the discovery of new pathological pathways and protein-protein interactions relevant for the onset and advancement of the disease (Aldewachi et al., 2021). Overall HTS combined other modern techniques such as artificial intelligence (AI), fluorescence resonance energy transfer (FRET) or virtual screening have emerged and postulated as indispensable options to develop new therapies and understanding of the complex pathophysiology involving this devastating disease (Kale et al., 2024; Kumar et al., 2024b; Zhou et al., 2024).

Having discussed these background considerations, in this doctoral thesis we propose the development of a novel translational in vitro model of Alzheimer´s disease using the immortalized cell line differentiated into mature neuron-like cells and transfected with AD-related mutations. The development of these new model is oriented towards obtaining a reproducible and scalable in vitro representation of various pathophysiological hallmarks of Alzheimer´s. So, it could be used for HTS assays for drug discovery that may lead to the identification of potential new *hits* that alleviate the morpho- physiological neuronal damage resulting from the protein deposits.

HYPOTHESIS

"*The development of a novel neuron-like phenotypical SH-SY5Y Alzheimer´s model could constitute a robust biological basis for HTS assays that lead to the identification of potential new drugs for the disease".*

MAIN OBJECTIVE

Develop a new *in vitro* translational model based on familial Alzheimer's disease for its use in high-throughput screening assays to be able to identify novel hits for new therapeutic approaches.

SPECIFIC OBJECTIVES

1. Design and develop a miniaturized and scalable model of mature phenotypic neurons resulting from the differentiation of the SH-SY5Y cell line.
2. Induce the elected familial AD genetic modifications (APP V717I and MAPT P301L) in the differentiated SH-SY5Y cell line to replicate pathophysiological-like characteristics of AD.
3. Characterize the pathophysiological hallmarks resulting from the introduced mutations and their direct effects on cell viability, neurite shortening and arborization in image-based assays.

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4. Study the response of this model to reference compounds with a described neuro-protective action *in vitro*.
5. Development of a primary assay based on the measurement of intracellular calcium for high throughput screening that correlates with neuronal excitation differences in Alzheimer's.
6. Explore the application of this novel Alzheimer's **in vitro** model in high-throughput screening assays using the Prestwick® chemical library to identify potential hits for Alzheimer's drug discovery programme.

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INTRODUCTION

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3. INTRODUCTION

3.1 Alzheimer´s disease

Alzheimer´s Disease (AD) is defined as a progressive and irreversible neurodegenerative process that leads to a severe cognitive and behavioural decline, eventually resulting in the inability of the patient to carry out basic tasks (Knopman et al., 2021). Alzheimer´s disease is named after the psychiatrist and neurologist Alois Alzheimer, who first described it in 1906. Dr. Alzheimer did so after studying a patient's who suffered from symptoms including language impairment, memory loss and unstable behaviour (Hippius & Neundörfer., 2003). Posthumously, he discovered in the patient's brain tissue anomalous protein accumulations (Amyloid-β plaques) and tangled protein fibres (Neurofibrillary Tau Tangles). These two are still considered, after more than a century later, two main pathological hallmarks to define the pathophysiology of AD (Gulisano et al., 2018).

3.1.1 Types of Alzheimer´s disease

AD is a heterogeneous complex disease that have shown a great variability in the clinical presentation and the age of onset since the early reports. Based on the age of onset and etiology of the disease, Alzheimer can be classified into two different types: Early-onset Alzheimer's Disease (EOAD) or Late-onset Alzheimer's Disease (LOAD). Despite the mentioned differences, both manifestations of AD share the same characteristic symptomatology and pathophysiological lesions commonly associated with the condition (Monteiro at al., 2023).

3.1.1.1 Early-onset Alzheimer´s disease

Early-onset Alzheimer´s disease, also known as the familial variant (fAD), is rarer that LOAD, affecting between 1-5% of the total patients (Andrade-Guerrero et al., 2023). EOAD typically manifests at younger ages, generally between the ages of 35-50 (Ringman et. al., 2015). Over the past four decades, research on fAD has highlighted more than 300 distinct mutations closely related to protein accumulation (Petit et al., 2022). Depending on the type of inheritance pattern, EOAD can be classified into two different subtypes.

- **Mendelian EOAD**

Mendelian EOAD (M-EOAD) follows an autosomal dominant inheritance pattern and is linked to mutations in the APP, PSEN1 and PSEN2 genes. Despite the well described pathogenesis of these mutations, they only account for around 10% of the familial cases (Sirkis et al., 2022). The APP V717I was the first discovered mutation associated to the late onset variant and is still the one that has affected most patients. These mutations primarily affect the amyloidogenic pathway, increasing the production of insoluble large amyloid-β peptides and

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therefore favouring its aggregation. APP registered to cluster around the Aβ sequence whilst those in PSEN genes are found more widely within the genome (Muratore et al., 2014; Goate et al., 1991). Presenilin mutations follow a similar function at inducing AD due to their direct relationship with Aβ cleavage by β- and γ-secretases (Both are components of γ-secretase). Notably, PSEN1 and PSEN2 mutations differ in their pathogenicity, being PSEN1 the one leading to an earlier and more severe disease (Lanoiselée et al., 2017; Xiao et al., 2021; Bagaria et al., 2022).

Besides the amyloidogenic pathway, the microtubule associated protein Tau (MAPT) is the other recognizable component of pathomorphological changes occurring at a microscopic level in the brain. Despite the close relationship of this protein and Alzheimer´s pathophysiological impairments associated with it, mutations in this protein have not been commonly associated with the onset of the disease, but rather linked to other neurodegenerative diseases like Frontotemporal Dementia linked with parkinsonism (FTDP) or Progressive Supranuclear Palsy (PSP) (Strang et al., 2019). Regardless of mutations not being directly linked by themselves to the onset of fAD, there have been multiple recent studies indicating a direct link or a pleiotropy of mutations such as P301L or R406W for the early onset variant of Alzheimer's (Nakamura et al., 2019; Li et al., 2022).

- **Non-Mendelian EOAD**

A significant portion of the totality of EOAD patients cannot be attributed to the known mutations that give rise to M-EOAD, and most of those englobed under Non-mendelian EOAD (NM-EOAD) do not exhibit an autosomal dominance inheritance. This suggests that this variant is an even more complex version of the disease in which rare genetic and common variants alongside environmental factors may have a determinant role for the onset at young ages (Ayodele et al., 2021). Over the past decade genetic studies have found rare genetic variants in a wide variety of genes not classically associated to AD. Noteworthy, are the mutations on Sortilin-related receptor (SORL1), which is involved in the degradation of amyloid-β peptides through its internalization in lysosomes or the Golgi apparatus. These rare variants reduce the affinity with peptide monomers, resulting in a reduced targeting and elimination of β-amyloid. Carriers of certain mutations of this gene have been demonstrated to have a five time more likelihood on developing EOAD (Barthelson et al., 2020; Mishra et al., 2022). Similarly, triggering receptor expressed on myeloid cells 2 (TREM 2) have recently been associated with this variant of the disease. TREM2 is a modulator of myeloid cells, but certain mutations have shown a direct increase of Aβ deposition, increased uptake of Aβ by the microglia and the lead to neuroinflammatory processes (Zhao et al., 2018; Qin et al., 2021). Finally, environmental factors are also though to play a complementary role in this variant, which could act through CG sites, affecting DNA methylation levels similarly to those observed with age, and ultimately resulting in an impaired gene expression (Reitz et al., 2020).

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3.1.1.2 Late-onset Alzheimer´s disease

Late-onset Alzheimer´s disease, also known as the sporadic variant, is the most common form of the disease, accounting for 95-99 % of all the cases. It typically affects individuals over the age of 65, and its incidence rises exponentially with age, affecting nearly 50% of the population aged 85 or higher (Andrade-Guerrero et al., 2023). LOAD does not show a clear familial inheritance pattern like its counterpart, and despite age being the main risk factor associated to this variant, a grand variety of genetic and environmental factors have been identified as determinant for the onset of AD (Silva et al., 2019). These risk factors play a complex interplay role, making the sporadic variant a multifactorial disease with an elusive etiology for the majority of the patients. Amongst the most relevant risk factors for this variant is Apolipoprotein E (ApoE). This is a glycoprotein mainly expressed in glial cells within the brain and although it plays a main role in lipid metabolism, it also actively mediates in the metabolism of Amyloid-β peptides and neuroinflammatory processes (Qu et al., 2024a).

- **Genetic risk factors**

While this variant does not follow a dominant autosomal mendelian inheritance pattern, genetics are closely related for the development of the disease. Recent studies of the human genome have served to identify genetic mutations in over 20 genes expressed both in neurons and glial cells that have associated to the onset and progression of the pathology (Naj & Schellenberg, 2017). ApoE has three distinct isoforms ε2, ε3, ε4, being this last one the highest genetic risk factor associated to the sporadic variant. Individuals with one copy of this gene have a three-time more likelihood to develop Alzheimer's, while those with both copies of the ApoE ε4 are 8-12 times more propense. However, the presence of this variant, is not by itself determinant for the onset of AD, which indicates that other gene and environmental interactions are also required (Silva et al., 2019; Serrano-Pozo et al., 2021). The other relevant genes for the sporadic variant are either linked to specific mutations that; promote the oligomerization and deposition of β-amyloid (Clu), lead to abnormal and extended microgliosis (CR1), produce and impaired endocytosis of proteins and precursors related to the disease (BIN1), limit the axonal transport (UNC5) and, finally, those who impede the correct calcium homeostasis (CALHM) (Dreses-Werringloer et al., 2009; Efthymiou & Goate, 2017; Robinson et al., 2017; Foster et al., 2019; Zhang et al., 2021b).

Finally, it is worth mentioning that, even though sporadic AD affects both men and women, almost two thirds of the diagnosis are registered on females, which also experience a much more severe neurodegeneration than men (Zhang et al., 2021d). From a genetically standpoint, ApoE4 allele significantly increasing AD risk in women, oestrogen through its effects through oestrogen receptors (ERs) is described to play a critical role at attenuating protein aggregation and protecting against oxidative stress (Uddin et al., 2020). Studies have shown single nucleotide polymorphisms (SNPs) in both the α and β oestrogen receptors (i.e. PvuII) that induce cognitive aging and constitute risks factors alongside the loss of oestrogen during menopause (Corbo et al., 2006; Lee et al., 2014).

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- **Environmental risk factors**

Although aging and genetics significantly influence in the development of the AD's sporadic variant, they do not account for the entirety of the cases. A wide range of environmental agents have also been identified as contributors, frequently through the induction of oxidative stress and neuroinflammation (Wainaina et al., 2014).

Air pollution is increasingly recognised as a risk for Alzheimer´s. Exposure to air pollutants like ozone, lead, sulphur dioxide and carbon dioxide has been linked to many cardiovascular and respiratory disease, it has been recently reported the connection between these molecules and both neuroinflammation and the increase in abnormal protein deposits (Moulton & Yang, 2012; Croze & Zimmer, 2018). Dietary habits have also an impact on AD. Diets high in antioxidant and essential nutrients have demonstrated a reduction in the onset of the pathology, contrary to those rich in calories and saturated fats (Hu et al., 2013). Additionally, previous clinical conditions and infections heighten the chances of suffering from Alzheimer's. Herpes simplex virus 1 (HSV-1) together with obesity, cardiovascular diseases and diabetes mellitus are the most common health disorders associated with neurodegenerative processes related to Alzheimer's such as: an impaired neurovascular system, protein deposits and inflammation linked to oxidative stress (Piacentini et al., 2014; Santos et al., 2017; Alford et al., 2018; Lee et al., 2018) (Figure 1.).

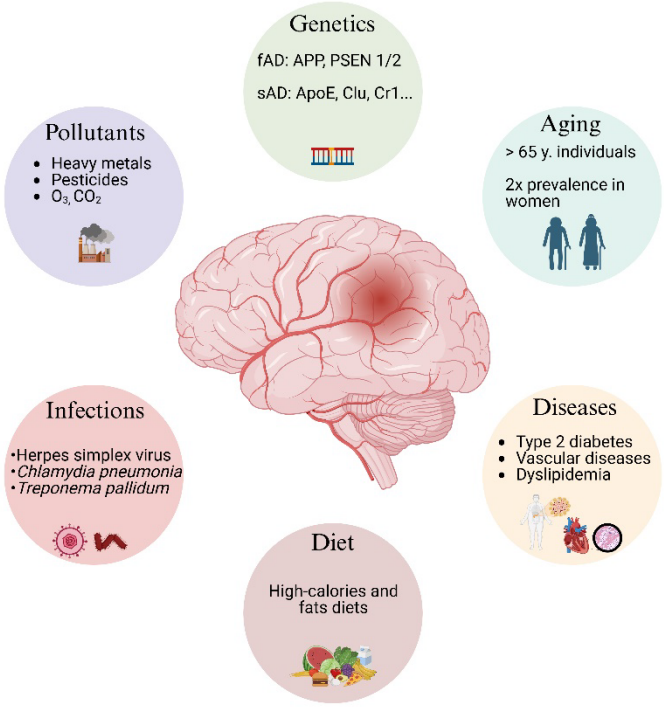


Figure 1. Risk factors for Alzheimer's disease. Alzheimer's multi-factorial etiology is also accompanied by a

wide variety of risk factors. Genetic risk factors for both variants are amongst the most studied at driving the onset of both fAD and sAD patients. Environmental factors such as air pollutants of previous pathologies are now highly linked to the sporadic variant. Created in BioRender. Barro, M. (2024).

3.1.2 Epidemiology and socioeconomic impact

With the rise in the numbers of aging population worldwide, so have the Alzheimer's patients, rapidly setting the disease as one of the top public health concerns globally alongside cardiovascular diseases and cancer. Alzheimer's is the most prevalent neurodegenerative disease globally and represents today the most common cause of dementia, accounting for between 60-80 % of the cases (Haque & Levey, 2019). Therefore, AD's epidemiology is closely related to all-cause dementia. The prevalence of dementia is thought to affect around 57 million worldwide and expected to triplicate by 2050 (Nichols et al., 2022). The incidence of dementia rises sharply from age 65 individuals, and so does clinical Alzheimer´s, which affects 3-4 % of adults in their retirement ages and increments to a staggering 35.8% of the population above 85 (Tahami Monfared et al., 2022). As mentioned above, AD is more common in women, showing a prevalence higher than double the one registered in men (Niu et al., 2017). Although, AD and dementia cases have been rising in both high- middle- and low-income countries at alarming rates, recent studies have shown a slight decrease in some high-income countries such as the United States or the United Kingdom. This decrease is often related to the socioeconomic, health and educational changes that have taken place in the past decades (Roehr et al., 2018). Dementia (all-cause) is also in the top of the charts of deaths globally, occupying the fifth place and accounting for around 4.4 percent of total deaths. This number of deaths attributed to dementia, including AD cases, has more than doubled in the past 3 decades. In Europe, deaths for individuals over 50 years old have followed a similar trend standardizing at a mortality rate of 45.2 per 100,000 (Nichols et al., 2019; Li et al., 2022). These crude data highlight the critical need for targeted therapies and an early detection of the disease, as the rising impact and mortality rates are only worsening the huge consequences that Alzheimer's represents for modern society.

AD follows a progressive and long-lasting course that after years of cognitive decline and behavioural changes, incurs in a total disability. This prolonged debilitation creates an overwhelming financial burden due to the direct medical and caregiving costs as well as the indirect costs that result from the loss of productivity of the patients and their relatives who are responsible of their supervision. The estimated costs of this disease are estimated to be more than 950 billion dollars worldwide and expected to increase exponentially with the rising tendency of patients expected in the upcoming decades (Tahami Monfared et al., 2022). The relationship between AD severity and costs also follows a growing pattern, with estimations of direct costs being a few hundred euros per month at the early stages of the disease, climbing almost to the two thousand by the later phases (Mauskopf et al., 2010; Rapp et al., 2012; Darbà et al., 2014). Complementary to the economic burden associated to the disease, Alzheimer´s also provokes an enormous human component concern for both patients, relatives and caregivers. The cognitive decline of the disease severely limits the patient´s ability to perform

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instrumental daily activities such as personal hygiene, feeding, mobility or management of medication and finances. These functional loses severely affect que quality of life of the patient and an emotional and psychological burden for the families. The physical, emotional and financial challenges that close relatives suffer, especially as the cognitive decline worsens, exacerbate the levels of stress, anxiety and mild depression of caregivers (Huang, 2022; Tahami Monfared et al., 2022; Velilla et al., 2022).

3.2 Etiology and pathophysiology of Alzheimer´s disease

Alzheimer´s disease is a complex condition, with an elusive etiology due to its multifactorial pathophysiology. Given the intricacies that neurodegenerative disease show, the lack of standardized research tools and the absence of known cures for the disease, have kept the precise onset mechanisms unclear (Marques et al., 2010). Several hypotheses have been formulated in the past, to aid with setting a target for pharmacological research, and are based on attribution the development of the disease to each of the main pathophysiological hallmarks of AD. These can be classified into four major hypotheses: Aβ-amyloid cascade, Tau, Cholinergic and oxidative stress and inflammatory (Figure 2.).

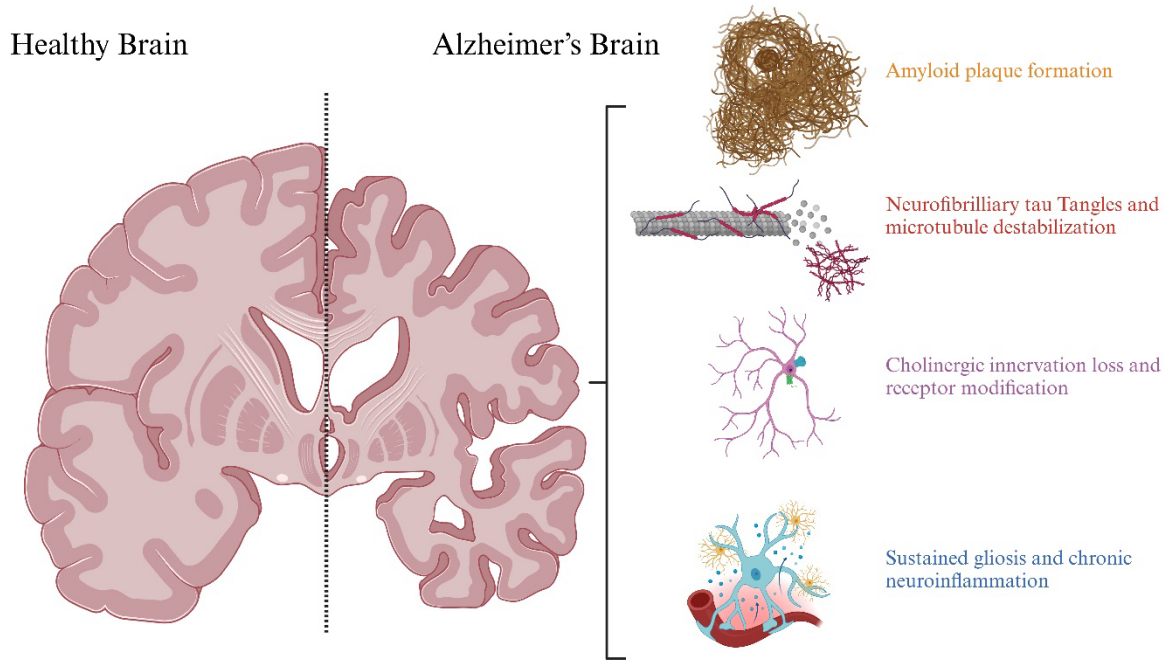


Figure 2. Alzheimer's etiology hypotheses. Alzheimer's disease non-dilucidated etiology has led to numerous hypotheses over time. Protein deposition and cholinergic signalling impairment have been the most accepted for AD research assays, although others like chronic neuroinflammation have gained recognition in the past decades for their presence at the onset and progression of the disease. Created in BioRender. Barro, M. (2024).

- **Amyloid cascade hypothesis:**

The most recognizable pathological feature of AD are the protein deposits and has been that way since the first description of the disease by Dr. Alzheimer. Amongst these deposits, the amyloid plaques have been one of the main targets for the pharmacological industry, with efforts focusing on reducing the Aβ production, modulating β- and γ-secretase or targeting Aβ clearance through immunotherapy (Ricciarelli & Fedele, 2017). Despite the efforts, clinical trials have failed over and over, raising concern about putting the amyloid protein in the centre of the pathological cascade (Karran & De Strooper, 2022). Although, the approval of aducanumab, lecanemab and donanemab (monoclonal antibodies against soluble and insoluble forms of Aβ) between 2021 and 2024 has proven efficacy for patients in early stages, all these setbacks have affected the established correlation between amyloid deposits and the disease onset (Kepp et al., 2023; Ameen et al., 2024). This suggests indeed that Aβ plays as a necessary initiator, but not solely role on the onset of the disease and progressive cellular decline.

- **Tau hypothesis:**

Similarly to the amyloid protein, the tau hypothesis focuses as the main contributor of the disease, the protein deposits in form of neurofibrillary tangles. Tau undergoes various post-translational modifications in neurons that can severely affect its behaviour. Hyperphosphorylation of tau is the one that provokes a more prone aggregation, affecting the axonal transport and neuronal plasticity (Arnsten et al., 2021). Tau therapies gained interest after the numerous amyloid targeting fails from the past decades, but despite the efforts, either aggregation blockers, tau-targetting vaccines and modulators of phosphatases have failed to show benefits in clinical trials (Congdon & Sigurdsson, 2018; Ceyzériat et al., 2020). However, extensive data on patient´s human brains have showed that Tau pathology starts as early as a decade before the first amyloid plaques are formed which combined with extensive cortical grey matter loss present in AD, strongly reinforce the importance of abnormal phosphorylated Tau in the disease (Arnsten et al., 2021).

- **Cholinergic hypothesis:**

Acetylcholine (ACh) is a neurotransmitter produced by cholinergic neurons and is involved in essential functions within our brain. Ach is present in the neuronal circuits responsible of the memory, attention, learning or sensory processing, therefore loss and damage of cholinergic neurons have always been identified as a key pathological step in Alzheimer's (Terry & Buccafusco, 2003). In AD, there is a progressive cholinergic loss due to the neurodegenerative processes in the Basal nucleus of Meynert, which is the source of cortical cholinergic innervation and its projections (Ferreira-Vieira et al., 2016). Changes also occur in nicotinic and muscarinic acetylcholine receptors, as well as presynaptic deficits related to the enzyme

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choline acetyltransferase, leading to a progressive loss of basal cholinergic neurons and anterograde loss of their connections to the cortex, hippocampus, and amygdala (brain structures associated with memory and learning) (Vereecken et al., 1994; Hampel et al., 2018). The development of this hypothesis, led to clinical testing with cholinesterase (AchE) inhibitors. These constitute nowadays the most widely available therapeutic drugs for Alzheimer's, and although have proven useful for a symptomatic relief during the first years of mild cognitive decline, fail to provide a cure for the disease (Sharma, 2019). This ultimately has led to the change in focus of novel therapeutic approaches and leave acetylcholine as a major dysfunction in AD but not an etiological key factor.

- **Neuroinflammation:**

Over the past decade another core pathological process has been gaining interest to provide a new insight into the complexity of Alzheimer's etiology and pathophysiology. Many studies, both in pre-clinical trials and postmortem have identified evidence of a prolonged abnormal gliosis and neuroinflammation alongside the protein deposits (Kinney et al., 2018). This defence mechanism of the brain through activation of microglia and release of cytokines was believed for a long period of time to be a reaction against the insults provoked by amyloid plaques, NFTs and oxidative/nitrosative stress. However, with the recent discovery of the exacerbation effect that this process exhibits on both amyloid and tau pathologies associated with AD, combined with the neurodegenerative effects that sustained neuroinflammation involves, have support the major role that this has on developing LOAD (Thakur et al., 2023; Amelimojarad et al., 2024). Consideration of neuroinflammation as a therapeutical target emerges as a solid option to open new combinatory treatments of the disease, especially taking into consideration the promising results that therapies like the immune checkpoint blockade against programme death-1 (PD-1) pathway have shown on in vivo models (Baruch et al., 2016; Rosenzweig et al., 2019; Zou et al., 2021).

All of these hypotheses converge in Alzheimer's pathophysiology to create an intricate network of molecular mechanisms driving the progression and neurodegenerative processes attached to the pathology. In the following sub-sections, the main hallmarks of Alzheimer´s pathophysiology will be delved individually with the objective of establishing the essential characteristics necessary to replicate the disease with available research tools.

3.2.1 Amyloid pathology

The accumulation of amyloid peptide, forming plaques, in a wide variety of organs of is a clinical and pathological condition known as amyloidosis (Ma et al., 2022). This abnormal buildup of beta-sheet amyloid leads to severe cellular damage and eventually resulting in the failure of the organ. AD is the most common type of amyloidosis and belongs to the localized type of the pathology, mainly affecting the patient's brain. These deposits that may or may not have a hereditary origin, are alongside NFTs, one of the most important hallmarks leading to

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the direct and indirect neuronal damage present in Alzheimer's (Ow & Dunstan, 2014; Ma et al., 2022).

3.2.1.1 Amyloid precursor protein

Amyloid precursor protein (APP) is a highly conserved type I transmembrane glycoprotein that is expressed in various organs throughout the body, but at especially high concentration in the brain. APP is encoded by a gene on chromosome 21, which consists of 18 exons that undergo alternative splicing to produce 10 different isoforms (Chen et al., 2024). Out of these, the most prevalent in the nervous system is the APP695 isoform, although during amyloidogenic pathologies like AD, other isoforms like APP751 and APP 770 are particularly upregulated (Nguyen, 2015). Isoforms of the APP family are highly versatile proteins that participate in important physiological roles in the brain, including the synaptic plasticity, neuronal growth and intercellular communication and exert their effects acting like receptor-like proteins, as ligands or by the release of proteolytic fragments (Müller et al., 2012). Newly synthesized APP produced in neurons is transported to the synapse areas through axonal transport, where it undergoes a rapid metabolism (Koo et al., 1990). Its proteolysis can occur through different pathways that produce different peptide fragments. Such process is influenced by the intracellular location of the protein, whereas the accumulation in the cell surface favours the non-amyloidogenic processing pathway by transmembrane secretases and the presence in acidic compartments, the amyloidogenic (Chen et al., 2017). In the amyloidogenic pathway, APP is first cleaved by the beta-site APP cleaving enzyme 1 (BACE-1; β-secretase), releasing to the extracellular region a soluble N-Terminus fragment (sAPPβ) and leaving a C-terminal fragment bound to the cell membrane (CTF-β). This fragment is then processed by the γ-secretase complex, composed of both Presenilin proteins, along with other subunits, which results in the generation of amyloid-β peptides and APP intracellular domains (ACID). The cleavage by γ-secretase can be produced at ranging positions ranging from +40 to +44 aminoacids, resulting in the productions of different length peptides (most commonly Aβ1–40 and Aβ1–42) which greatly affects their solubility and neurotoxicity (Zhang et al., 2012; Chen et al., 2017; Hampel et al., 2021). In contrast, the non-amyloidogenic pathway involves cleavage by A desintegrin and metalloprotease 10 (ADAM10; α-secretase), which cuts APP within the Aβ region (Within aminoacids 16-17), preventing Aβ formation. This cleavage releases the soluble ectodomain sAPPα, which has neuroprotective and neurotrophic functions. An alternative recently identified pathway involves η-secretase, which also prevents Aβ generation by producing different non-amyloidogenic fragments like CTF-η and Aη-α (Chen et al., 2017; Hampel et al., 2021) (Figure 3.).

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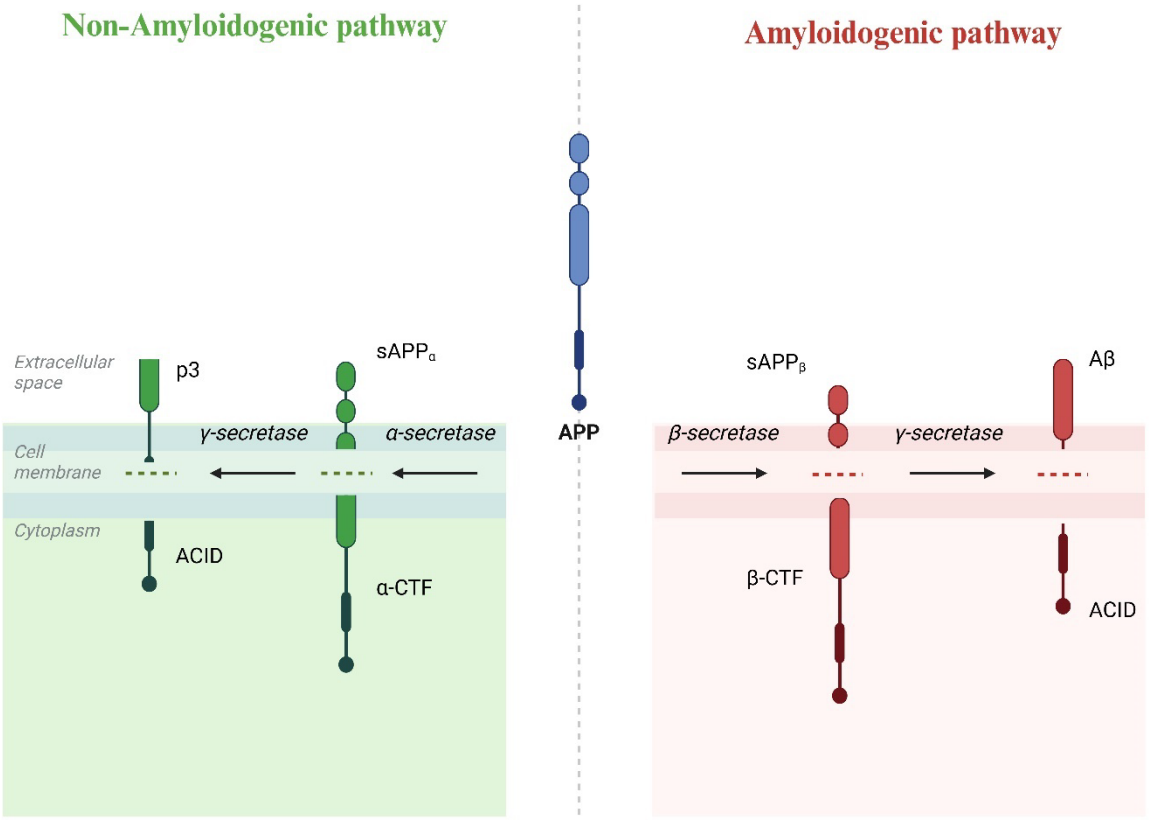


Figure 3. Amyloid precursor protein processing pathways. Non-amyloidogenic processing involves the cleaveage by α- y γ-secretase, producing the fragments $sAPP_{\alpha}$, p3 and ACID. Contrary, processing via the action of β- y γ-secretase produces amyloid-β peptides, $sAPP_{\beta}$, and ACID. Created in BioRender. Barro, M. (2024).

Some of the biggest advancements in the amyloidosis ligated to AD began with the discovery of autosomal dominant mutations in the APP. Currently, there are more than 40 mutations in APP considered to be pathogenic and more than 70 identified as contributors to the pathophysiology of AD (Mutations | ALZFORUM). Notable mutations by their frequency of report and severity, include the London mutation (APPV717I), the Swedish mutations (K595N/M596L) or the trisomy 21 (Down's syndrome) induce the amyloidogenic processing pathway, rise the synthesis levels of Aβ, increase the Aβ-42 production, favour the aggregation/plaque formation and accelerate cognitive decline (Lloyd et al., 2020; Fortea et al., 2021; Zhou et al., 2022). Contrary to these genetic mutations, there are others with a protective effect over the development of AD. Variant of APP A673T have demonstrated a reduction in amyloid production and aggregation, delaying the cognitive decline associated with age (Jonsson et al., 2012).

3.2.1.2 Amyloid-β peptide aggregation

Amyloid-β is a short 4.2 KDa protein consisting of a length between 37-49 amino acid residues. The accumulation of this peptide typically begins in cerebral regions with an elevated

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bioenergetic activity, such as the association cortices, and gradually spreads from the neocortex to allocortex and the brainstem, ultimately reaching the cerebellum (Nunan & Small, 2000; Thal et al., 2002; Chen et al., 2017). After the production Aβ monomers in neuronal cells, these undergo different aggregation stages until the formation of the characteristic plaques. These include dimers, trimers, oligomers, proto-fibrils and fibrils (Hampel et al., 2021). This process starts with a series of monomer association and nucleation process in which small nuclei (Aβ oligomers) are formed through thermodynamically unfavourable reactions, which represent the most limiting stem in Aβ aggregation process. Aβ-42 peptide is key to the primary nucleation step on AD, as the presence in their structure of two additional hydrophobic residues (Ile-41 and Ala-42) makes this variant much greater propense to rapid and robust aggregation due to its folding in an antiparallel β-hairpin configuration. The oligomers formed after nucleation can vary in size, ranging from dimers to dodecamers, which by their own exhibit substantial neurotoxicity dysregulating memory-related receptor signalling, reducing dendritic density and promoting synaptic loss. The transition of oligomers to protofibrils emerges through the addition of individual Aβ monomers into the already formed nuclei structures by an elongation process. These larger structures often accumulate within glial cells such as microglia and astrocytes, inducing an inflammatory response. The aggregation process culminates in the assembly of these fibrils into amyloid structure in a concentration-dependant pattern, eventually forming the characteristic cross-β-sheet plaques, which represent the most insoluble and toxic form of these aggregations. Additionally, fibrils can undergo a secondary nucleation process which consists of the formation of new oligomeric nuclei from critical concentration accumulates of fibrils. This monomer-dependent oligomerization differs from the classical described nucleation, as the pre-existing fibres act as a catalysing agent for the assemble on their surface of different Aβ monomers. This process requires a much smaller energy-dependant reaction compared to primary nucleation, which rapidly further increase the presence of toxic aggregates in the affected areas (Schmit et al., 2011; Arosio et al., 2015; Man et al., 2019; Almeida & Brito, 2020; Michaels et al., 2020; Seuma et al., 2022).

3.2.1.3 Amyloid-β toxicity

The role of APP in Alzheimer's disease is closely bound to the toxicity of Aβ aggregates. This pathogenesis that amyloidosis exhibits in AD can be explained by analysing some of the most important pathophysiological traits observed in patients' brains including oxidative stress, aberrant phosphorylation of Tau, neuronal damage and neuroinflammation (Sun et al., 2015).

Oxidative stress is a term used to describe the damage derived from an excess of reactive oxygen species, which affects a wide variety of cellular components' functions. This pathological hallmark is registered early in the development of AD, and specifically in zones that are linked to Alzheimer's amyloidosis such as the hippocampus and cortical regions of the brain whereas lower Aβ-level regions such as the cerebellum do not show oxidative stress markers (Chen & Zhong, 2014; Huang et al., 2016). Amyloid aggregation favours the production of ROS through several distinct mechanisms. Primarily, it has been demonstrated

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that these peptides conforming the plaques show a high affinity for metal ions such as copper (Cu) and iron (Fe). These interactions make the metal molecules alternate between different oxidation states and facilitating the reduction of molecular oxygen to form ROS that include hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) (Cheignon et al., 2018). It has also been demonstrated the interaction between Aβ fibrils and mitochondrial membrane, blocking the protein transport, disrupting the electron transport chain and leading to an increase in ROS production and energy metabolism failure (Sharma et al., 2021a; De la Cueva et al., 2022). Studies have also shown that the upregulated activity of NADPH oxidase (NOX) is also mediated by Aβ in astrocytes, further increasing the formation of superoxide radicals by reduction of molecular oxygen (Ganguly et al., 2021).

In addition to the fomentation of ROS creation, Amyloid-β peptides also play a role in the cleavage and phosphorylation of Tau. These mechanisms regulated by several proteins and kinases such as GSK3β, Cdk5, calpain or caspase 3, have been described to be abnormally activated by extracellular Aβ species (Zhang et al., 2021a). Sustained Aβ accumulation also triggers a chronic gliosis response, activating microglia and leading to neuronal loss through direct phagocytosis and the release of inflammatory mediators (cytokines, eicosanoids, chemokines) that exacerbate neuronal damage and impair Aβ clearance (Leng & Edison, 2020). Finally, these plaques also disrupt calcium homeostasis. The binding of Amyloid peptides to the neuronal membranes and intracellular organelles induces the formation of pores, provoking an impaired inward flow of ions and an aberrant release of intracellularly stored Ca^{2+} ions, resulting in synaptic failure and neurotransmitter depletion (Chami & Checler, 2020; Sciaccaluga et al., 2021; Viles, 2023) (Figure 4.).

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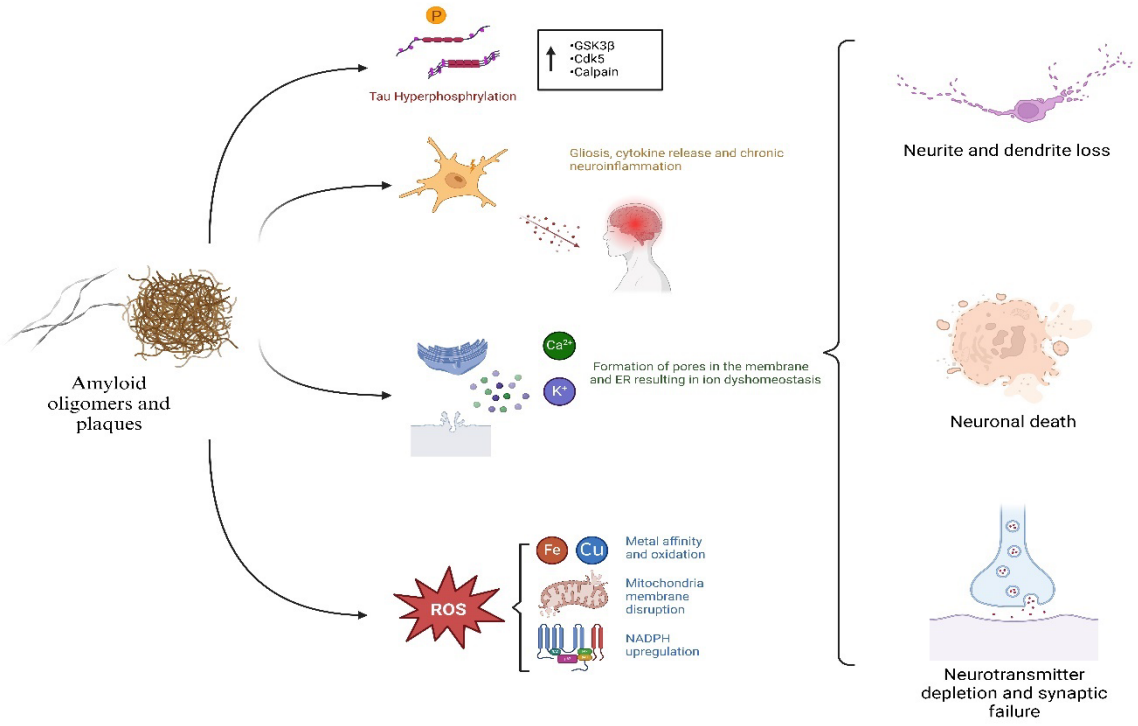


Figure 4. Neurotoxicity of amyloid aggregates in AD. Schematic representation of the neurotoxic pathways exerted by amyloid species intracellularly and extracellularly. Amyloid peptides interact with signalling pathways, phusiological membranes and organelles leading to ROS generation, morphological irreversible damage, neuroinflammatory processes and ion dysregulation. Created in BioRender. Barro, M. (2024).

3.2.2 Tau tangle pathology

Tau is a soluble protein that belongs to the microtubule-associated proteins family (MAPs), and in humans, is primarily expressed in neurons. Tau is encoded by the MAPT gene located on chromosome 17, which consists of 16 exons. Through alternative splicing of exons 2, 3 and 10, six distinct isoforms can be generated, differing by the presence or absence of N-terminal inserts (0, 1 or 2) and by the number of tubulin-binding repeats located in the C-terminal region (Kent et al., 2020; Sinsky et al., 2021). The six configurations of Tau in adult human brains are 3R0N, 3R1N, 3R2N, 4R0N, 4R1N, and 4R2N. In a healthy adult brain, there is approximately an equal ratio of ·R and 4R isoforms, but this balance is often disrupted in neurodegenerative diseases linked to tauopathies (Gao et al., 2018).

Tau's primary function in neurons is the stabilization of microtubules (MTs), especially in the distal segments of axons and dendrites. By promoting the polymerization of tubulin, tau ensures that the MTs remain intact and properly oriented, which is vital for transporting essential cellular components like organelles and signalling molecules along the axons and dendrites (Kent et al., 2020; Muralidar et al., 2020). Tau also influences this transport by regulating motor-proteins like kinesin and dynein along MTs (Dixit et al., 2008). Another

emergingly studied function of Tau is the prevention of microtubule cleavage by protein katanin (Qiang et al., 2006). Tau is not only involved in maintaining MT dynamics and genesis but also participates in critical cellular processes such as protecting DNA integrity, myelination, facilitating iron export, and insulin signalling in the brain (Tuo et al., 2017; Lopresti, 2018; Mangiafico et al., 2023). Importantly, tau's functions are regulated by their post-translational modifications, which profoundly affects its ability to bind MTs and perform its other roles (Alquezar et al., 2021).

Despite tau's prominent role in the neurodegenerative processes related to AD, mutations in this protein are classically associated with other tauopathies such as frontotemporal dementia (FTD), progressive supranuclear palsy (PSP) or cortico-basal degeneration (CBD) rather than fAD (Samudra et al., 2023). Both FTD and AD are neurodegenerative diseases that result in dementia, and the early symptomatology and tauopathy distribution is comparable between them. While none of these genetic variations have been identified as causative, many have produced in vitro similar aggregation patterns or are considered as risk modifiers for AD. Mutations like P301L and P301S, some of the most common associated to FTD, lead to the hyperphosphorylation of tau and enhance its aggregation into paired helical filaments (PHFs), similar to those observed in AD brains (Rademakers et al., 2004). The P301L mutation has also been extensively studied in animal models where it triggers significant tau hyperphosphorylation and aggregation, leading to neurodegeneration (Lewis et al., 2000; Wenger et. al., 2023). Despite, its unlikelihood of provoking the onset of EOAD, there have been some registered cases attributable to the mutations on tau protein as described previously. Other relevant tau mutations for the study of AD are V337M and R406W, that have shown to produce tau folding and aggregation resemblant to that found in AD (Qi et al., 2024). Additionally, in humans, the H1/H1 genotype of tau protein has been identified as a risk factor for LOAD due to the increased expression of longer 4R tau peptides and generates a significantly higher concentration of soluble and insoluble species compared to the individuals with the H2 haplotype (Strang et al., 2019).

3.2.2.1 Tau hyperphosphorylation pathways

Tau is, natively, an unfolded protein that does not show propensity to aggregation due to its conformation in a hairpin structure conformed by the interaction between its N-terminal, C-terminal and microtubule-binding domains (MBD). However, Tau can undergo through a variety of post-translational modifications, being phosphorylation, the one causing the protein to be more prone to accumulate into paired helical filaments (PHFs). Tau structure shows numerous threonine, tyrosine and serine residues near the MBDs that are susceptible to phosphorylation (Guo et al., 2017). While phosphorylation is necessary for tau's normal function, excessive phosphorylation of reduces its biological activity and truncates microtubule stabilization. For instance, phosphorylation at residues Ser202/208 and Thr205 promote Tau fibrilization and abnormal migration to the soma (Neddens et al., 2018; Xia et al., 2020). This hyperphosphorylation process is mediated by a balance between protein kinases and

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phosphatases. Amongst these, we can identify proline-directed protein kinases (PDPKs), non-PDPKs, and tyrosine kinases such as FYN. Over 20 kinases, including glycogen synthase kinase 3 (GSK-3β) and cyclin-dependent kinase 5 (Cdk5) and cAMP-dependent protein kinase (PKA) (Alquezar et al., 2020). Inhibition of phosphatases, especially protein phosphatase 2A (PP2A), which accounts for over 70% of tau dephosphorylation, further contributes to tau hyperphosphorylation in AD as its activity is notably decreased during the development of the pathology (Wang et al., 2015). Post-translational modifications other than phosphorylation, also play a role in tau aggregation. Glycosylation, acetylation, and ubiquitination have all been implicated in modulating tau's conformation and its propensity to aggregate (Guo et al., 2017). Acetylation, for instance, inhibits tau's normal degradation by the proteasome, allowing for the accumulation of pathological forms of tau (Min et al., 2010).

3.2.2.2 Tau aggregation and neurofibrillary tangles

In Alzheimer's disease, the aggregation of phosphorylated tau is central for the development of the pathology. As the protein becomes hyperphosphorylated, it detaches from microtubules and leading to a mislocalization from axons to the soma and dendrites. This unbound state tau is more prone to aggregation into neurotoxic dimers and trimers, which are considered to appear earlier than the first symptoms, and to be the key co-contributors alongside insoluble forms of tau for AD (Gong & Iqbal, 2008; Niewidok et al., 2016; Rawat et al., 2022). This is then followed by the grouping of small oligomers into larger twisted-strand insoluble species of the protein in the form of paired-helical filaments, which ultimately form the well-recognised neurofibrillary tangles (Mroczko et al., 2019). In AD, the development of tau aggregation follows a pattern similar to the one observed for amyloidosis, starting from the entorhinal cortex and progressively spreading to the limbic system, hippocampus and neocortical areas (D'Errico & Meyer-Luehmann, 2020). Tau pathology is believed nowadays to spread similarly to prion-derived diseases, where pathological proteins spreading from affected areas can influence those present on healthy areas to adopt pathological conformations (Ayers et al., 2019). Soluble toxic conformation of tau can be propagated by different mechanisms including its direct release to the extracellular space via exosomes or ectosomes, secretion across the plasma membrane, mediated by interactions with lipids and heparan sulphate proteoglycans (HSPG) or even between cells via tunnelling nanotubes (intercellular channels formed of actin filaments) (Abounit et al., 2016; Katsinelos et al., 2018).

Once released into the extracellular space, tau is internalized by recipient cells through various endocytic processes. Bulk-endocytosis and micropinocytosis, facilitated by HSPGs and other receptors such as LRP1 and amyloid precursor protein (APP), have been identified as major mechanisms of tau uptake (Rauch et al., 2020). After internalization, tau is accumulated in the cytoplasm, where it acts as a template for further tau oligomerization. Glial cells, in particular microglia, is also believed to play a major role in tau propagation, as its inability to fully digest hyperphosphorylated aggregates, leads to the release of small oligomers through exosomes, further propagating and accelerating the tauopathy (Amro et al., 2021).

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3.2.2.3 Molecular pathogenesis of tau

Neurotoxicity of tau is based on its interference on a broad spectrum of intraneuronal function such the microtubules structural integrity, mitochondrial functioning, genome stability, calcium homeostasis and synaptic integrity. All these processes are dependent on the form of tau aggregation and its localization within the cell (Niewiadomska., 2021).

Tau protein plays a multifaceted and crucial role in neuronal genomic stability, chromatin organization, and the protection of both DNA and RNA from oxidative stress. For instance, Tau protein has shown to interact directly with DNA, forming complexes that are crucial for maintaining the integrity of chromatin, particularly in the pericentromeric heterochromatin (PCH) regions. These regions are densely packed with chromatin substructures which play essential roles in regulating gene expression and maintaining genome stability. Aggregation of the protein leads to the disruption in the distribution of H3K9me3 and HP1α, key components of heterochromatin, which increase the vulnerability to DNA damage (Violet et al., 2014; Mansuroglu et al., 2016). Tau oligomers are also implicated in the direct regulation of gene expression, by its interactions with the tumour suppressor protein 53 (p53). In AD, tau oligomers have been shown to directly influence the misfolding of p53, preventing it from entering the nucleus and carrying out its regulatory functions (Farmer et al., 2020). Pathological forms of tau also affect the structure of the nuclear membrane and the nuclear pore complex. Tau oligomers can bind to nucleoporins, such as Nup98 and Nup62, thus altering permeability of the nuclear membrane, disrupting the transport of molecules between the nucleus and the cytoplasm (Eftekharzadeh et al., 2018).

Mitochondrial impairment in Alzheimer's disease (AD) is also closely linked to tau pathology. Tau impairs mitochondrial distribution by destabilizing microtubules, particularly affecting axonal terminals and synapses, where energy is most required. The co-localization of Tau with mitochondrial membrane marker porin and interactions with cardiolipin, also suggests that tau morphologically disrupts the membrane integrity, leading to cytochrome c, pro-apoptotic caspases and ROS release. Elevated levels of hyperphosphorylated tau have also been related to mitochondrial inability to produce ATP, through the reduction in levels of complex V ATP synthase (Quntanilla & Tapia-Monsalves, 2020; Trease et al., 2022).

Oligomeric forms of tau are also crucial for the synaptic dysfunction in AD and other tauopathies. Accumulation in the synapse leads to a reduction in the concentration of synaptic vesicles, GABA release, electrophysiological changes such as increased depolarization and input resistance or a decrease in the speed of action potential decays (Niewiadomska et al., 2021). Synaptic loss is also related to the impaired microtubule stabilization occurring from the detachment of tau. This disrupts the axonal transport through the disruption of PP1-GSK-3β signalling cascade and the inactivation of c-Jun N-terminal kinase-interacting protein 1 (JIP1), both pivotal for the motor protein complex in the axon (Morfini et al., 2009; Moreno et al., 2016). The disruption of axonal transport of peroxisomes, neurofilaments, Golgi-derived vesicles, neurotrophins receptors, amyloid precursor protein (APP), and other organelles into

neurites poses a serious threat to neurons, potentially leading to their degeneration and eventual death (Stamer et al., 2002; Reddy, 2012). Finally, hyperphosphorylated tau also disrupt ion homeostasis in the pre-synaptic and post-synaptic areas. Aggregation proximate to neuronal and mitochondrial membranes are thought to disrupt calcium homeostasis by interaction with voltage-gated channels, abnormal release and uptake of Ca^{2+} by the mitochondria, evoking a deficit in K^{+} - dependent glutamate release and by the over-stimulation of muscarinic receptors (Gómez-Ramos et al., 2009; Florenzano et al., 2017) (Figure 5.).

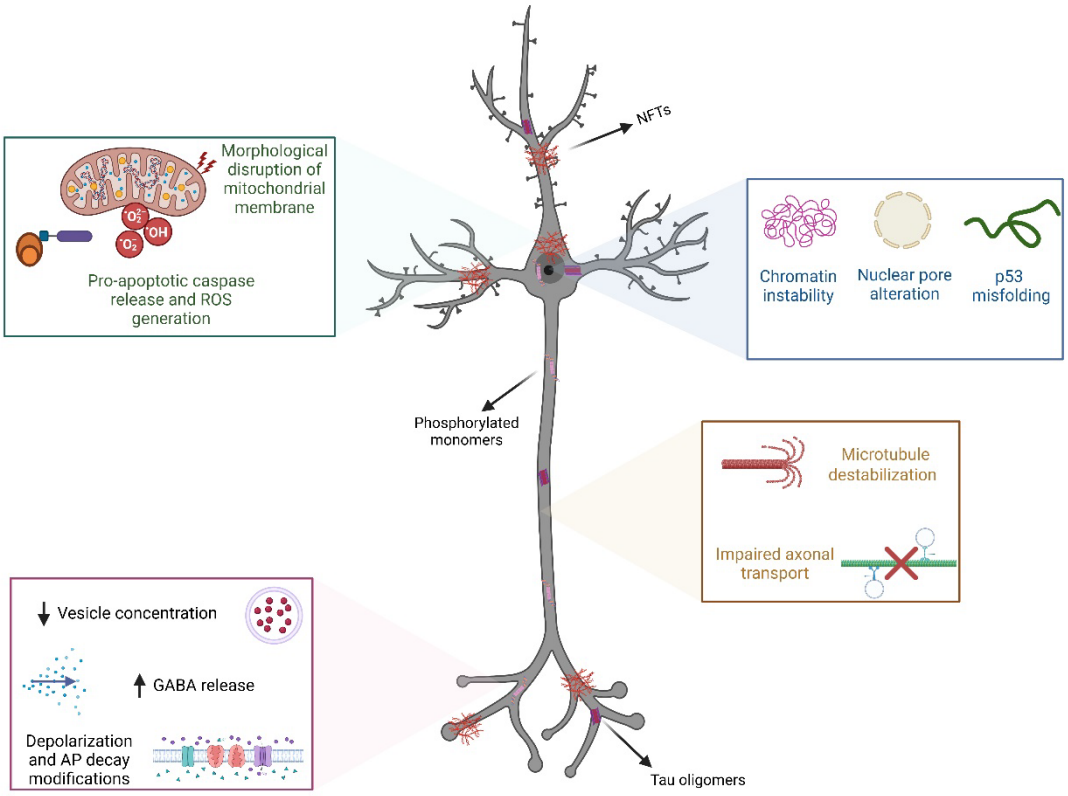


Figure 5. Intracellular processes affected by Tau hyperphosphorylation and oligomerization. Schematic illustration of the events affected by toxic tau intracellularly. Oligomers and NFTs disrupt metabolic events, neuronal/organelles morphological integrity and the physiological/electrical capabilities of neurons. Created in BioRender. Barro, M. (2024).

3.2.3 Neuropathology

The neurodegenerative process of Alzheimer´s is marked by progressive synapse loss, neurite retraction followed by widespread neuronal atrophy throughout the cerebral cortex. Neuropathological processes typically begin in the hippocampus and entorhinal cortex, two key areas responsible for memory processing, and spreads progressively through the fronto-temporal cortices. In more advanced stages, the degeneration can extend to regions such as the striatum and thalamus (Deture & Dickson, 2019).

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At a macroscopic level, MRI scans of AD patients consistently show shrinkage in these regions, reflecting the underlying neuronal and glial damage. In particular, pyramidal cells within the CA1 region of the hippocampus are highly vulnerable to morphological changes and eventual cell death. This vulnerability is closely associated with memory loss, which often appears in the early stages of the disease (Serrano-Pozo et al., 2011). At a microscopic level, we can identify by the early synapse loss, followed by a progressive neuronal death and chronic neuroinflammation events. Additionally, the accumulation of Aβ plaques and NFTs, the two hallmark protein lesions of AD, precedes the appearance of clinical symptoms. This suggests that by the time symptoms such as memory impairment and cognitive decline emerge, years of pathological changes have already taken place. This extensive preclinical phase makes early intervention particularly challenging, as it is difficult to detect and address the disease in its initial stages (Dubois et al., 2019; Lloret et al., 2019; Calabrò et al., 2020).

3.2.3.1 Neuronal and synapse loss

Synapses are specialized and intricate structure essential for neuronal communication within the brain and crucial their normal functioning. Synaptic activity is supported by the highly complex and adaptable in shape, size, and internal organization of synapses. The ongoing process of synaptic regulation is vital for learning and memory, particularly through mechanisms like long-term potentiation (LTP) and long-term depression (LTD) (Citri and Malenka, 2008). Any disruption to these properties, which affects normal synaptic physiology, can result in significant neurophysiological impairments. Brain disorders characterized by synaptic dysfunction are referred to as synaptopathies, in which AD is often recognized (Lepeta et al., 2016).

Analyses of post-mortem brain tissue from Alzheimer's disease patients have identified synapse loss as the strongest link to cognitive decline (DeKosky et al., 1996; Scheff et al., 2006; De Wilde et al., 2016). It has been identified that even before that the neuronal loss takes place, patients lose an average of 30 % of the synaptic connections in the entorhinal and hippocampal areas. Mouse models with familial AD mutations affecting amyloid precursor protein and presenilin, which increase amyloid levels, or those with mutated Tau that leads to neurofibrillary tangles are used to study mechanisms behind synapse loss related to prominent AD pathologies (Jankowsky & Zheng, 2017). Research has shown that these mutations result in synaptic dysfunction, including both impaired plasticity and increased synapse loss (Selkoe, 2002; Spires-Jones and Knafo, 2012).

While postmortem analyses provide a snapshot of pathology, they miss the dynamic processes occurring in the brain. In-vivo and animal studies capture these dynamics but do not reflect the entirety neural circuits of a living brain. Through the employment of these research tools, it has been demonstrated that Aβ, Tau and neuroinflammation play a key role in this loss (Chen & Yu, 2023; Zhang et al., 2023b). Amyloid soluble oligomers and plaques are thought to decrease long term potentiation by inducing neuronal hyperactivity, altering the NMDA: AMPA receptor expression ratio, favouring the release of neurotransmitter GABA, thus reducing the

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paired-pulse ration of neurons (Parodi et al., 2009). Tau does so, specially altering the transport of necessary receptors and organelles needed for the correct functioning of both ends of the synapsis, which results in retrograde degeneration. Tau is also capable of binding to synaptic vesicles, contributing to presynaptic dysfunction (Salvadores et al., 2020). The mechanism behind this involves a dual action by Tau: it binds to synaptic vesicles with its N-terminal while simultaneously promoting the polymerization of presynaptic actin. This actin polymerization crosslinks the vesicles, restricting their mobilization in the pre-synaptic area. Increases in Ca^{2+} concentration also leads to compensatory changes and defects in neuronal Ca^{2+} signalling. These altered Ca^{2+} signals disrupt the balance between Ca^{2+}-dependent phosphatase calcineurin (CaN) and its antagonist, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which are highly concentrated at synaptic sites. This imbalance between CaMKII and CaN activity impair long-term potentiation (Oka et al., 2017; Zhou et al., 2017; Ge et al., 2022). Finally, activated microgliosis is also related to early synaptic loss in Alzheimer through direct phagocytosis of synaptic components marked by the abnormally upregulated complement binding 1 (C1q) (Carvalho et al., 2019).

In Alzheimer's disease, neuronal loss is another neuropathological hallmark that progressively affects specific brain regions and cell types. This neuron death predominantly impacts areas related to cognitive functions such as the CA1 region of the hippocampus and the layer II of the entorhinal cortex. Other affected areas include the olfactory bulb and anterior olfactory nucleus, the amygdala, the basal nucleus of Meynert, and the raphe nuclei (Vogels et al., 1990; Aletrino et al., 1992; Gómez-Isla et al., 1996; Price et al., 2001). Apoptosis and necrosis, along with necroptosis, pyroptosis, and ferroptosis, represent various forms of cell death that contribute to neurodegeneration in Alzheimer's disease (Mangalmurti & Lukens, 2022). Apoptosis is the most characterized form of cell death in AD and is a non-inflammatory regulated process that removes damaged cells without releasing intracellular contents, maintaining tissue homeostasis. It is triggered by intrinsic and extrinsic pathways leading to caspase activation, particularly Caspase-3, resulting in cell shrinkage and membrane blebbing (Nichani et al., 2020). On the other hand, necrosis is an uncontrolled form of cell death caused by external damage caused by the explained toxicity of protein aggregations. Necrosis is pro-inflammatory and is marked by cell lysis and the release of damage-associated molecular patterns (DAMPs). Evidence suggests necrosis plays a significant role in AD, as seen in increased RIPK1 and MLKL levels in AD brains (Koper et al., 2020; Zhang et al., 2022). Similarly, pyroptosis, another inflammatory form of cell death, involves inflammasome assembly, particularly the NLRP3 and NLRP1 inflammasomes, which leads to caspase-1 activation and secretion of pro-inflammatory cytokines, culminating in the activation of protein Gasdermin-D, which results in pore formation and cell lysis (Tan et al., 2014; Broz & Dixit, 2016; Stancu et al., 2019; Ising et al., 2020). Lastly, parthanatos is a recently discovered cell death form linked to AD, caused by the overactivation of PARP-1 by ROS generated from protein aggregation, leading to mitochondrial membrane depolarization and the release of apoptotic inducing factors (Mangalmurti & Lukens, 2022).

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3.2.3.2 Neuronal excitability

Hyper- and hypo-excitability are two physiological changes in the neurons observed in Alzheimer's patients during different stages of the disease. Clinical research has demonstrated that early-stage patients show an elevated cortical and hippocampal activity, which gradually shifts to a diminished activity in the entire brain as the disease progresses, protein deposit neurotoxicity worsens, and the neuron death is pronounced. Neuronal hyperactivity has been identified in patients with mild cognitive impairment (MCI) during memory tasks, while advanced AD patients typically show reduced activity in these areas. Research also suggests that hyperactivity in the brain occurs before hypoactivity in AD progression (Targa Dias Anastacio et al., 2022). A study on individuals genetically predisposed to late-onset Alzheimer's disease (LOAD) found elevated brain activity in frontal and temporal lobes years before symptoms emerged (Basset et al., 2006). Additionally, AD patients, particularly those with the familial variant, have a higher risk of epilepsy and seizures, supporting the notion that brain hyperactivity is an early feature of the disease (Friedman et al., 2011). Amyloid-beta pathology is typically associated with neuronal hyperactivity in early stages of Alzheimer's disease. However, as plaques are formed, neurons undergo structural and functional changes that impair their ability to fire, leading to synaptic loss, impaired calcium homeostasis, and membrane depolarization, which together "silence" neuronal firing (Zott et al., 2019). Contrary, tau pathology has been recognized as a key driver of AD's hypo-excitability from early-stages, suggesting that soluble tau is as responsible as NFTs for this physiological impairment. Studies using transgenic mouse models expressing human tau mutations, such as the P301L mutation or hyperphosphorylation on residues T231/S262, have demonstrated reduced neuronal firing (Hatch et al., 2017; Busche & Hyman, 2020). These findings highlight that tau hyperphosphorylation destabilizes the microtubule network, particularly in regions like the axon initial segment (AIS), where action potentials are initiated. This disruption causes the AIS to shift further from the soma, leading to decreased neuronal firing (Hatch et al., 2017). Microglia is another important component of hyper- and hypo-excitability as it can sense these stages and act as a mediator. During hypoactivity, microglia become more active to tissue damage and participate with astrocytes in the drainage of debris during normal biological hypoactive stages such as sleep, playing a key role in neuronal plasticity. Prolonged phases of hypoactivity as those registered in advance AD could promote the gliosis also observed during the late stage of the pathology. During hyper-activity, microglia typically reduce neuronal activity by displacing GABAergic synapses through P2Y12 signalling. In AD, the expression of this receptor is downregulated in regions with increased protein depositions, which leads to worsened seizure outcomes, further affecting LTP and neuroinflammatory responses (Eyo et al., 2014).

3.2.3.3 Cerebral vasculature in Alzheimer's

Functional and structural impairment of the brain's vasculature contribute vastly to the cognitive decline and the progression of Alzheimer's pathology. Most AD patients, exhibit

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mixed vascular pathologies, including cerebrovascular disease and in around 80% of the cases present cerebral amyloid angiopathy (CAA) (Jäkel et al., 2021). Imaging analysis of patients suggests that vascular dysregulation may be an early and potentially initiating factor in sAD pathology. Cerebral hypoperfusion and the disruption of the blood-brain barrier are observed even before of the symptomatology, and it is more pronounced than that occurring from aging (Apátiga-Pérez et al., 2021). In familial AD, it is particularly common the pericyte damage and reductions in glucose uptake and cerebral blood flow before clinical diagnosis. The disruption of a normal vasculature structure due to localized cerebral infarcts, haemorrhages, retraction of astrocytes or altered vascular density are mainly attributed to the amyloid deposition in the arterioles and perivascular spaces. This vascular damage impedes the periarteriolar clearance of Aβ and impedes receptor-mediated removal of Aβ peptides across the blood-brain barrier, which accelerates Aβ buildup. Reduced oxygen delivery to the brain, caused by diminished blood flow and impaired neurovascular coupling, also promotes the amyloidogenic pathway of APP processing, further increasing the pathogenic cycle. Aβ peptides can exhibit vasoactive properties, leading to the contraction of pericytes and vascular smooth muscle cells, worsening hypoperfusion (Thal et al., 2008; Salminen et al., 2017; Sweeney et al., 2019). Additionally, vascular dysfunction has been strongly correlated with the accumulation of hyperphosphorylated Tau. Tau is present in the bloodstream as monomers, oligomers and fibrillar structures in AD. It is released from neurons due to damage, inflammation, or active secretion. Emerging research shows that tau in the blood may influence blood vessel permeability by exerting its toxicity pathways (i.e. ROS generation, mitochondrial dysfunction…) on endothelial cells, leading to leaky vessels and continuing to process such as inflammation, vasoconstriction, and the BBB breakdown (Miyakawa, 2010; Rather et al., 2024) (Figure 6.).

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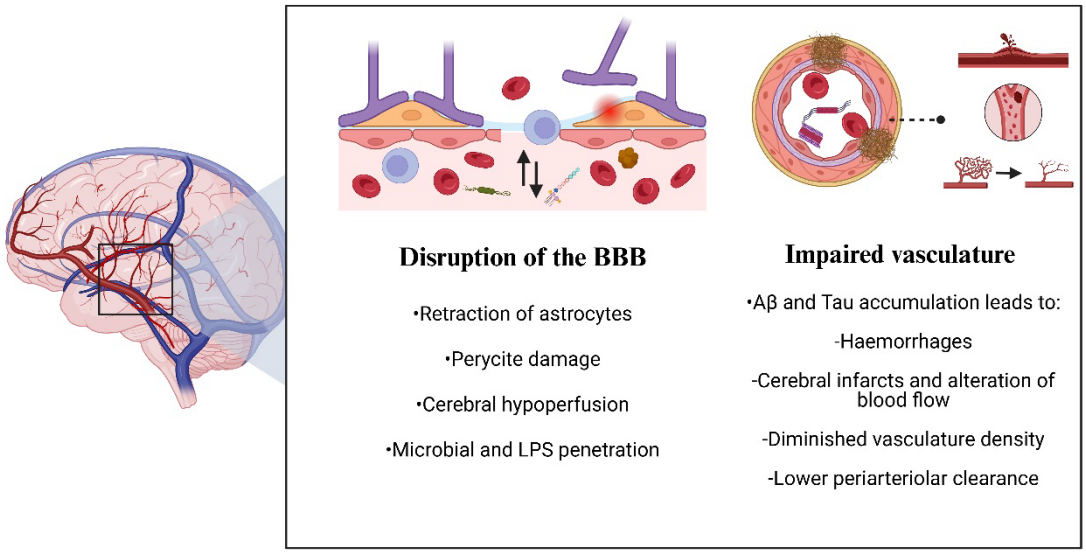


Figure 6. Alzheimer's related events in human brain vasculature. Schematic diagram of the morphological and functional vascular pathogenesis observed in AD brains. Mediated by toxic tau and amyloid forms aggregating in blood vessel walls and perivascular spaces. Created in BioRender. Barro, M. (2024).

3.2.4 Neuroinflammation

Neuroinflammation is an inherent host defence mechanism that aims to protect the central nervous system (CNS) and restore its function following infections, injuries, or other pathological insults. This response involves several types of cells, including microglia, astrocytes, and neurons, and is driven by the release of biochemical molecules such as cytokines, chemokines, reactive oxygen species (ROS), and other inflammatory mediators (Thakur et al., 2023). These molecules are primarily produced by immune cells in the CNS, including microglia and astrocytes, though capillary endothelial cells and infiltrating blood cells also contribute, especially when the blood-brain barrier (BBB) is damaged. While this immune response is essential for clearing infections or repairing injuries, chronic or unresolved inflammation can result in synaptic dysfunction, neuronal death, and neurodegeneration (Leng & Edison, 2020). For many years, the chronic gliosis associated to AD was perceived as a secondary reaction to the main pathological hallmarks, the protein aggregations. However, recent advances in AD research, have set neuroinflammatory processes in a more proactive role for the disease's progression, being considered nowadays as the third main hallmark in Alzheimer's pathology. In fact, many genetic variants related to neuroinflammation have been identified as risks for the development of LOAD, such as TREM2, CD33, SPI1, INPP5D or

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CR1 which are either mainly or highly expressed on microglia (Efthymiou & Goate, 2017; Leng & Edison, 2020; Zhang et al., 2023b). For instance, mutations in TREM2 induce overexpression of receptor IL-3R, which makes microglia overresponsive to Interleukin-3 secreted by astrocytes (Li et al., 2023).

Several key pathways are involved in AD-related neuroinflammation. The NF-κB pathway, a central regulator of inflammation, is activated by amyloid-beta via microglial activation of P2X7R. Once activated, NF-κB modulates the expression of various proinflammatory genes and cytokines, contributing to microglial activation and neuronal death (Messemer et al., 2013; Thakur et al., 2023). The mTOR pathway also plays a significant role in AD, particularly in cerebrovascular dysfunction and impaired clearance of Aβ. Increased signalling of mTOR reduces autophagy, further exacerbating the accumulation of misfolded proteins (Mueed et al., 2019). The upregulation of pro-inflammatory factors like interleukins 1 and 6 or tumour necrosis factor alpha (TNF-α) and the downregulation of anti-inflammatory cytokines such as interleukins 4 and 10 or tumour necrosis factor beta (TNF-α), are the main catalysts of neurodegenerative processes (Chang et al., 2017; Amelimojarad et al., 2024). IL-1 actively participates in the generation and processing of amyloid-beta peptides through augmented γ secretase activity or in the aberrant phosphorylation of Tau by activation of p38 MAP kinase or GSK3β (Mrak et al., 2000). TNF-α contributes by promoting neuronal death and negatively affecting the normal levels of nerve growth factor. In recent years, the gut microbiota differences between AD patients and healthy individuals have been also extensively related to explaining the development of chronic neuroinflammation (Ekert et al., 2018). Gut dysbiosis leads to systemic inflammation by activation of inflammasomes like NLRP3, or the secretion to the bloodstream of microbial metabolites such as lipopolysaccharides (LPS) and amyloids entering the brain through an already debilitated BBB and triggering inflammatory responses in new areas (Shen et al., 2020) (Figure 7.).

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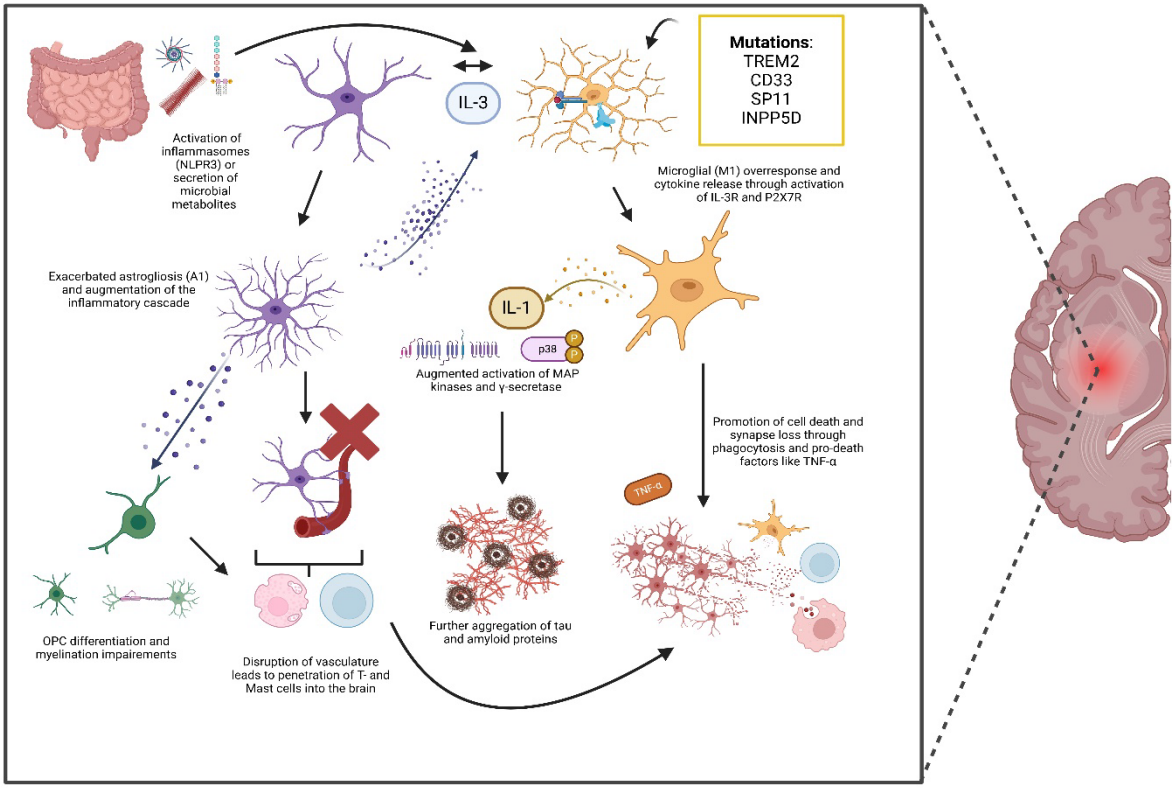


Figure 7. Neuroinflammatory mediators and processes in Alzheimer's. Graphical diagram of the cells and pathogenic pathways involved in sustained neurodegeneration. Reactive microglia and astrocytes mediate the production of pro.inflammatory factors and recruitment of immune cells, further exacerbating the neuropathology of AD. Created in BioRender. Barro, M. (2024).

3.2.4.1 Glial cells and their role in Alzheimer´s pathology

-Microglia:

Microglia, the immune cells of the central nervous system, play a multifaceted role in Alzheimer's, acting as both protectors and perpetrators of neuroinflammation depending on the area and stage of the pathology. These cells, derived from monocytes, are dynamic and exhibit behavioural and morphological changes in response to environment modifications. In the early stages of AD, microglia can clear amyloid-beta (Aβ) through mechanisms like phagocytosis and enzyme-mediated degradation, providing neuroprotection. Microglia can show 3 different phenotypes within a healthy brain that are classified as resting (M0) or activated (M1 & M2). The M1 phenotype is associated with pro-inflammatory actions that can lead to neuronal loss and sustained cytokine release, whereas the M2 phenotype is linked to anti-inflammatory responses and phagocytosis of debris. Typically, M2 microglia are more prevalent during the initial stages of inflammation, but as inflammation persists, the M1 phenotype becomes more dominant (Peng et al., 2021). In the context of Alzheimer's disease, there is a notable shift from a predominantly M2 phenotype to M1 as the disease progresses (Amelimojarad et al., 2024). As aging progresses, microglia become dystrophic and much less branched, affecting its area

of reach. This is only exacerbated by the high concentrations of misfolded proteins, reactive oxygen and nitrogen species and pro-inflammatory molecules (Muzio et al., 2021). Under these conditions, the interactions between the cells and Aβ activates pro-neuroinflammatory processes without the ability of metabolizing the aggregates or compacting and isolating the plaques. Furthermore, defective microglia can significantly influence tau pathology as it facilitates its propagation between neurons through endocytosis and the release of exosomes (Asai et al., 2015; Wang et al., 2017).

-Astrocytes:

Astrocytes, also known as astroglia, are highly specialized glial cells derived from ectodermal and neuroepithelial tissues and constitute the most present type of glial cell within the CNS. They are involved in numerous essential functions such as neurogenesis, neurotransmission, and plasticity, while also regulating cerebral blood flow, maintaining the blood-brain barrier (BBB), and supporting neuronal metabolism and ion homeostasis (Peng et al., 2021). In Alzheimer's, astrocytes undergo a pathological transformation known as reactive astrogliosis. This process is characterized by increased cell volume, thicker processes, and elevated levels of glial fibrillary acidic protein (GFAP). Reactive astrocytes can be classified into two types: A1 and A2 (Escartin et al., 2021). A1 astrocytes, which are neurotoxic, are upregulated by neuroinflammation and enhance the expression of pro-inflammatory cascade genes (Hinkle et al., 2020). Conversely, A2 astrocytes, which have a protective role, promote the expression of neurotrophic genes. Similarly to the microglia, astrocytes contribute to chronic neuroinflammation by releasing cytokines, interleukins, ROS and other prejudicial molecules (Hayakawa et al., 2015). Additionally, given the close relationship between astrocytes and the BBB, severely increases the entry of immune cells and undesirable molecules into the brain at the same time that reduces the clearance of pathological proteins from the parenchyma to the perivenous drainage (Manu et al., 2023).

-Other cell types:

Despite the major role that astrocytes and microglia plays in the onset and development of chronic gliosis in AD patients, as the pathology progresses and the brains vasculature is degraded, other nervous and peripheral cells contribute to neurodegenerative damage (Peng et al., 2021). Oligodendrocytes, the myelinating cells of the CNS, are traditionally known for their role in axonal insulation and support. Recent evidence suggests that oligodendrocytes may also play a role in neuroinflammation. Oligodendrocyte precursor cells (OPCs) can respond to inflammatory cues by proliferating and differentiating into mature oligodendrocytes, which may attempt to repair damaged myelin (Zou et al., 2023). However, in AD, these cells exhibit signs of dysfunction, including impaired differentiation, reduced capacity to myelinate axons, and can even act as phagocytes or modulators of T-cell activation (Kalafatakis & Karagogeos, 2021; Zou et al., 2023). The disruption of the blood-brain barrier in AD allows immune cells

from the periphery to enter the brain, where they may contribute to the inflammatory response. In particular, macrophages can migrate to sites of Aβ deposition, where they participate in Aβ clearance. However, like microglia, peripheral macrophages can also release pro-inflammatory cytokines and contribute to neurotoxicity when their clearance capacity is overwhelmed (Gate et al., 2010). T-cells, particularly CD4+ and CD8+, have been identified in AD brain tissue and can exacerbate neuroinflammation by releasing cytokines such as IL-10, TNF-α, IL-6, IL-1β, and monocyte chemoattractant protein-1 (MCP-1), mobilizing and activating both microglia and astrocytes (Leclercq-Cohen et al., 2023). Finally, mast cells, a haematopoietically-derived immune cell are though to play also a modulatory role in AD. Typically found in the perivascular zone of the microvasculature, these cells can migrate to injured zones releasing pro-inflammatory cytokines and other molecules such as histamine or prostaglandins, causing vasoconstriction and oedema in the surrounding vasculature, further aggravating the microenvironment and inflammatory responses (Shaik-Dasthagirisaheb & Conti, 2016).

3.3 Clinical presentation and diagnosis

Alzheimer's disease is a chronic, progressive neurodegenerative condition that manifests with a characteristic pattern of cognitive and functional decline. The initial and most prominent clinical feature is memory impairment, especially affecting the ability to retain and recall recent information. This early memory loss is often subtle and mistaken for normal aging, but it gradually worsens, leading to more profound deficits as the disease progresses. As Alzheimer's advances, other cognitive domains like visuospatial disfunction and executive tasks become impaired (Lam et al., 2013; Tahami Monfared et al., 2022). In addition to cognitive impairment, Alzheimer's disease is often accompanied by behavioural and psychological symptoms such as irritability, aggression episodes and social withdrawal. Motor symptoms, while less prominent in the early stages, are more evident as the disease progresses. Patients may experience difficulties with balance, coordination, and eventually, a loss of motor control, which, coupled with cognitive decline, further impairs their ability to perform essential daily activities (Atri, 2019b).

Clinically, Alzheimer's can be categorized into distinct clinical phases depending on the disease progression. These stages of Alzheimer's disease are directly linked to the pathological changes occurring in the brain, such as amyloid plaques, neurofibrillary tangles, synaptic loss, and neuroinflammation. The first phase, known as the preclinical or asymptomatic stage, can last for many years without obvious symptoms. During this period, subtle memory issues may arise, and early pathological changes begin to occur in the hippocampus and cortex. However, there is no significant impact on daily functioning, and no clinical signs of Alzheimer's are apparent. The second phase, termed the mild or early stage, is when more noticeable symptoms start to emerge. These include difficulties in daily tasks, lapses in concentration and memory, disorientation in time and place, mood swings, and the potential onset of depression. In the third phase, known as the moderate stage, the disease advances to broader regions of the cerebral

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cortex. At this point, memory decline worsens, making it harder for individuals to recognize loved ones. Patients may experience a lack of impulse control and struggle with tasks such as reading, writing, and speaking. The final stage, severe or late-stage Alzheimer's, is marked by the disease spreading throughout the entire cortex, resulting in a significant accumulation of amyloid plaques and neurofibrillary tangles. This leads to severe cognitive and functional deterioration, where individuals lose the ability to be able to take care of themselves, may become bedridden, and experience difficulties on crucial tasks such as swallowing or bladder and bowel functions. Ultimately, these complications lead ultimately to the patient's death, being the most common causes secondary complications derived from Alzheimer's such as aspiration pneumonias, urinary tract infections, heart complications or thromboembolisms (Kalia, 2003; Breijyeh & Karaman, 2020; Zupanic et al., 2021; Kumar et al., 2024a; Lai et al., 2024).

3.3.1 Alzheimer's disease heterogenicity

Alzheimer's disease phenotypic variability has been described from as early as 1969, assessing the differences between memory, parietal function, pace of disease progression and aphasia between dementia patients (McDonald, 1969). Unfortunately, this early recognition of AD heterogeneity did not lead to widespread acknowledgment of subtype variability, largely due to the dominant theory at the time, the phase hypothesis, which suggested that the clinical differences were due to observing different stages of disease progression, rather than distinct subtypes of AD (Lam et al., 2013).

Later, early positron emission tomography (PET) studies provided further evidence supporting the subtype hypothesis. These studies revealed distinct topographic patterns of brain hypometabolism, mirroring the clinical diversity seen in AD patients. For instance, asymmetries in PET scans showed that greater impairment in language occurred when the left hemisphere was predominantly affected, whereas visuospatial deficits were more pronounced when right parietal hypometabolism was observed (Foster et al., 1983; Haxby et al., 1985; Duara et al., 1986).

Alzheimer's can be presented in various subtypes, each with distinct clinical and pathological features The most common form or "typical Alzheimer's" is characterized by late-onset amnestic impairment. This subtype is associated with hippocampal and temporal-parietal atrophy or decreased perfusion/metabolism, leading to progressive memory decline alongside impairments in other cognitive domains. In typical AD, there is a relatively symmetrical and generalized pattern of brain atrophy, distinguishing it from more focal variants such as temporal, visuoperceptive or language. The rate of progression in typical AD is slower than in other non-amnestic variants (Lam et al., 2013).

The "temporal variant" is one example of these focal subtypes. This late-onset subtype is characterized by isolated episodic memory impairment and a slow rate of cognitive decline. Unlike typical AD, the pathology in this variant is largely confined to the mesial temporal lobes,

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with minimal spread to the neocortex. Despite significant memory issues, other cognitive functions like visuospatial and executive abilities are relatively preserved (Butters et al., 1996; Cappa et all., 2001; Lam et al., 2013). The "language variant" is an early-onset form marked by non-fluent speech, while memory remains relatively intact. Atrophy is typically located in the left perisylvian region, and neurofibrillary tangles are predominantly confined to the left neocortex (Galton et al., 2000). The "frontal variant" is a rare subtype, distinguished by significant frontal lobe-related cognitive and behavioural changes. Pathologically, this variant shows a higher concentration of neurofibrillary tangles in the frontal regions (Johnson et al., 1999). Finally, the "visuoperceptive variant" is characterized by early visuospatial dysfunction, often linked to deposition of NFTs and amyloid plaques in the right hemisphere (Lam et al., 2013).

3.3.2 Diagnostic techniques

Alzheimer's complexity is also translatable to its diagnosis, where often a multifaceted approach is often employed integrating clinical evaluation, cognitive testing, neuroimaging techniques and biomarker analysis. The diagnostic path typically begins with a clinical assessment, where a detailed patient clinical history, interviews with close family members or caregivers and physical and neurological inspections are performed. This initial assessment focuses on identifying memory deficits, difficulties with problem-solving, and daily functioning challenges, which are hallmark indicators of Alzheimer's disease symptomatology (Dubois et al., 2021; Jack et al., 2024). However, clinical assessment alone is insufficient for a conclusive diagnosis, as these symptoms can be common in other dementias or neurodegenerative diseases. To supplement the clinical evaluation, standardized cognitive tests are also performed. The Mini-Mental State Examination (MMSE) and the Montreal Cognitive Assessment (MoCA) are widely used tools to assess cognitive functioning. These tests examine key areas such as memory, attention, language, and visuospatial skills, providing scores that can be tracked over time to monitor disease progression. Although helpful, these tests are not specific to Alzheimer's disease, further pushing the modernization of new diagnostic methods (Atri et al., 2019b).

Neuroimaging plays a pivotal role in the modern diagnosis of AD, particularly in distinguishing it from other neurodegenerative disorders. Magnetic Resonance Imaging (MRI) is commonly used to assess structural brain changes. In Alzheimer's disease, there is often pronounced atrophy in the hippocampus and medial temporal lobes, regions associated with memory formation and storage. MRI helps visualize this degeneration, providing crucial information on the extent of brain shrinkage, which correlates with disease progression. Positron Emission Tomography (PET), on the other hand, offers functional imaging by detecting amyloid plaques and tau tangles in the brain (Zhang et al., 2017). In recent years, biomarker analysis has revolutionized the diagnostic approach to AD. Cerebrospinal fluid (CSF) biomarkers, such as beta-amyloid 42 (Aβ42), total tau (t-tau), and phosphorylated tau (p-tau), offer insight into the molecular changes driving Alzheimer's pathology (Clifford et el.,

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2024). Typically, AD patients show reduced Aβ42 and elevated tau forms in the CSF, accounting for the presence of amyloid plaques and tau tangles in the brain (Campbell et al., 2021). Blood-based biomarkers, though less established than CSF tests, have shown significant promise. Blood tests like p-tau 181 can detect Alzheimer's pathology with greater convenience and less invasiveness compared to lumbar punctures (Lantero Rodriguez et al., 2020). Another important aspect of the diagnostic process is genetic testing, particularly in cases of early-onset AD. Mutations in genes such as APP, PSEN1, and PSEN2 and APOE are strongly associated with familial Alzheimer's disease as discussed earlier. While these mutations account for a minority of cases, their detection provides certainty in diagnosis (Lam et al., 2013).

3.4 Treatment of Alzheimer´s disease

In today's medical practice, the treatment for Alzheimer's disease has shifted to a multi-modal approach, combining both pharmacological and non-pharmacological interventions (Atri et al., 2019a). This shift from an exclusive pharmacological approach is due to no new FDA approved AD medications from 2002 to 2021, with the exception of a combining treatment of two existing drugs. Additionally, these treatments have demonstrated modest success in slowing cognitive decline and managing symptoms (Atri et al., 2019a; Esang & Gupta, 2021).

Currently, there is no available cure for AD, and it is widely acknowledged that the disease process begins decades before clinical symptoms appear. By the time memory loss and cognitive decline become evident, substantial and likely irreversible damage has already occurred at the molecular, cellular, and systemic levels. As a result, the current treatment paradigm revolves around managing symptoms, maintaining quality of life, and slowing disease progression (Alhazmi & Albratty, 2022) (Figure 8.).

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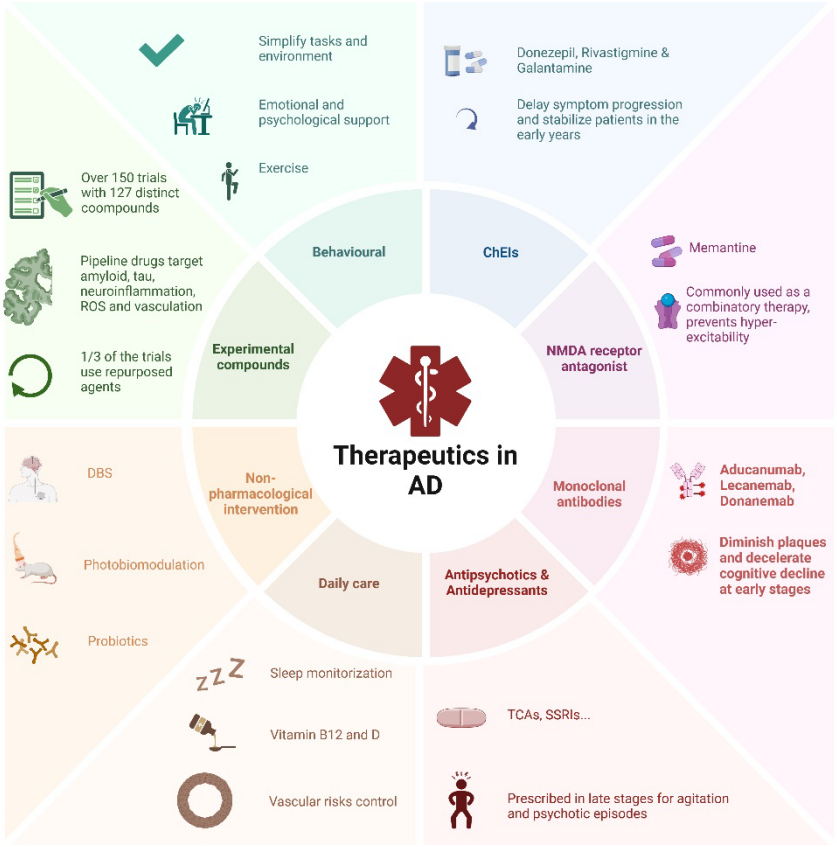


Figure 8. Present and future therapeutic interventions for Alzheimer's disease. Graphical representation of the currently available and foreseeable therapeutic approaches for AD. Current management of AD includes a multi-modal approach mixing pharmacological symptom treatment with behavioural and psychological complementary therapies Created in BioRender. Barro, M. (2024).

AD treatment often starts with communication amongst doctors, caregivers, and patients thus establishing concise parameters on the identification of symptoms, accurate evaluation and diagnosis, and appropriate guidance tailored to individual needs (Yiannopoulou & Papageorgiou, 2020). Behavioural approaches play a crucial role in the care of individuals with Alzheimer's. Reducing the complexity of the environment and tasks can help reduce confusion and anxiety, while established routines promote stability and predictability in daily activities. Communication strategies are also essential; caregivers are encouraged to engage in calm interactions, provide pleasurable activities, and use simple language (Atri et al., 2019). Cognitive therapy can also be beneficial for addressing specific emotional and psychological challenges, while complementary therapies, including exercise can enhance overall well-being and quality of life (Choi & Twamley, 2014). In addition to behavioural and supportive strategies, pharmacological interventions may also be appropriate for managing symptoms and improving quality of life. Pharmacotherapy typically begins with monotherapy using a cholinesterase inhibitor (ChEIs), eventually progressing to combination therapy with memantine (Atri et al., 2019a; Amat-Ur-Rasool et al., 2021).

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3.4.1 Clinical pharmacological medicine

The FDA approved drugs for the treatment of Alzheimer's disease (AD), have traditionally consisted of acetylcholinesterase inhibitors (AChEIs) (donepezil, galantamine, and rivastigmine) and the NMDA receptor antagonist memantine. AChEIs function by inhibiting the enzyme acetylcholinesterase, which is responsible for breaking down acetylcholine. By preventing this breakdown, these drugs increase cholinergic activity in the brain, helping to slow cognitive decline, especially during the first year of treatment (Atri et al., 2019a). Clinical studies have consistently demonstrated the benefits of AChEIs in delaying symptom progression, stabilizing cognitive function, and even enhancing the ability to perform necessary daily activities for patients with mild AD (Small, 2005). Long-term studies reinforce their value, showing sustained benefits when the medications are continued beyond one year (Rountree et al., 2012; Tricco et al., 2017). Pharmacologically, there are differences between the drugs: donepezil and galantamine are metabolized mainly by the liver, while rivastigmine is metabolized in both the liver and intestines. Additionally, rivastigmine differs in its inhibition of acetylcholinesterase in an almost irreversible manner (Rountree et al., 2012; Yiannopoulou & Papageorgiou, 2020).

Memantine, an NMDA receptor antagonist, works by blocking these receptors, which are involved in glutamatergic neurotransmission. By regulating this process, memantine helps prevent excessive neuronal damage due to hyperexcitability of neurons caused by the glutamate excess in the synaptic cleft. Memantine is approved for moderate to severe stages of AD, where it can be used either alone or commonly in combination with an AChEIs. Research has demonstrated its effectiveness in improving cognitive function, enhancing daily activities, and managing behavioural symptoms associated with later stages of the disease (Matsunaga et al., 2015).

The oligomerization of Aβ represents the earliest histopathological marker of Alzheimer's disease (AD), and therefore has been one of the main targets for the pharmaceutical industry for the past decades (Zhang et al., 2023c). The past few years have seen the breakthrough of three humanised IgG1 monoclonal antibodies against amyloid-β into the clinical prescriptions (aducanumab, lecanemab and donanemab). These are intended to mild-cognitive impairment associated to AD, as manage and slow the amyloid deposition at early stages. As anti-β amyloid antibodies, these reduce both soluble and insoluble forms of Aβ by targeting these depositions and triggering microglial removal. All these novel therapeutical options have shown a significant effect on the diminishing of plaques and yielded a significant deceleration of cognitive and functional decline of patients (Cummings et al., 2023; Ameen et al., 2024).

In addition to these core medications, behavioural and psychological symptoms of dementia (BPSD) are often treated with antipsychotics and antidepressants. These drugs are typically used when the patient poses a safety risk to themselves or caregivers due to aggressive behaviour in the later stages of the disease. Atypical and tricyclic antidepressants as well as selective serotonin reuptake inhibitors (SSRIs), such as sertraline, citalopram, mianserin and doxepin, are commonly prescribed to manage depression and anxiety in AD patients (Dudas et

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al., 2018). Managing underlying medical conditions is equally important in the care of Alzheimer's patients. Proper control of vascular risk factors, such as hypertension, diabetes, and hyperlipidaemia, is essential. Additionally, regular monitoring of hydration, sleep, and nutrition is necessary, along with addressing deficiencies in vitamins B12 and D (Atri et al., 2019a).

3.4.2 Non-pharmacological interventional therapies

The lack of novel pharmacological options for so many years promoted the search of non- and invasive non-pharmacological treatments that aim to mitigate the disease symptoms and improve cellular and neuronal functioning in Alzheimer's patients. An example of non-invasive techniques that reached clinical trials is photobiomodulation. This technique also known as low-level laser therapy, uses near-infrared light to induce cellular processes for therapeutic effects. Light at this wavelength activates chromophores in cells, particularly cytochrome c oxidase (CCO), to enhance mitochondrial function, ATP production, and reduce oxidative stress as well as inducing expression of BDNF. Photobiomodulation has shown promise in reducing amyloid-beta and tau pathology in Alzheimer's disease models but applying it to the nervous system poses a serious challenge (Hamblin, 2019; Enengl et al., 2020; Huang et al., 2024). As mentioned in a previous section, dysbiosis, or imbalances in intestinal microbiota, is linked to various pathogenic processes in AD. Following this hallmark, now only dysbiosis has been studied as a biomarker but also as a treatment target. Interventions targeting intestinal microbiota, such as probiotics and microbiota transplantation, have shown promise in reducing AD symptoms. Probiotics have demonstrated cognitive benefits and reductions in inflammation in both animal and human studies while microbiota transplantation has shown a decrease amyloid deposition and improved cognitive skills on *in vivo* models (Naomi et al., 2021).

Similarly to other neurodegenerative diseases, following the successful outcomes of Parkinson's, Alzheimer has also been subject of more invasive techniques like the deep brain stimulation (DBS) surgical procedure (Luo et al., 2021). DBS involves surgically implanted electrodes that deliver electrical pulses to specific brain regions, aiming to modulate neural activity. Implementation of DBS on Alzheimer's patients have shown that high-frequency stimulation significantly reduces Aβ levels, induces expression of neurotrophic factors and increase cerebral glucose metabolism, helping with the metabolic deficiencies associated to AD. Results obtained with these techniques summed to the multi-faceted pathology of Alzheimer's suggests that integrative approaches targeting each of the main pathological hallmarks are needed for finding a cure for this disease at its various stages (Aldehri et al., 2018; Majdi et al., 2023).

3.4.3 Experimental medications for Alzheimer´s disease

Despite the lack of new treatments for over two decades, the orientation of the available treatments to slow the disease progression at early-stages and the failure or resign of more than 200 clinical trials in that timespan, Alzheimer's drug development remains one of the main

targets for the pharmaceutical industry (Yiannopoulou & Papageorgiou, 2020). Despite the precarious situation that pharmacological management of the disease has went through, the recent approval of disease-modifying therapies (DMTs) and treatments for neuropsychiatric symptoms associated with Alzheimer's disease (AD) like the mentioned anti-amyloid monoclonal antibodies or the antipsychotic brexpiprazole approved for agitation associated with AD have marked significant progress in this field, laying the groundwork for future and current compounds in clinical trials (Cummings et al., 2024). As of 2024 there are 164 clinical trials composed of 127 distinct compounds targeted at AD. These drugs in the pipeline target various pathological processes of Alzheimer's like neuroinflammation, amyloid beta proteins, synaptic plasticity, protein tau, metabolism and bioenergetics, oxidative stress, vasculature and lipoprotein receptors. Noteworthy, a third of all of the current trials are currently being developed with repurposed agents, highlighting the importance of already available compounds in diseases with a pharmacological necessity (Cummings et al., 2024; ClinicalTrials.gov).

Amyloid-β remains as one of the main targets amongst compounds in advanced clinical trials (Cummings et al., 2024). In addition to the already mentioned commercialized antibodies and the clinical trials of analogous compounds by other pharmaceutical companies, the most promising compounds are:

- **Remternetug**: a monoclonal antibody specifically designed to target a pyroglutamated form of amyloid-beta (Aβ) that contributes to the formation of amyloid plaques. It is currently undergoing evaluation in Phase 3 clinical trials (Cummings et al., 2024; ClinicalTrials.gov).

- **Solanezumab**: a humanized monoclonal antibody that targets the mid-domain of the Aβ peptide, aiming to enhance the clearance of Aβ from the brain. It is also in Phase 3 trials (Cummings et al., 2024; ClinicalTrials.gov).

- **Simufilam**: a drug binds to filamin, which stabilizes the interaction between soluble Aβ42 and the α7 nicotinic acetylcholine receptor. There are three active Phase 3 trials assessing its effectiveness (Cummings et al., 2024; ClinicalTrials.gov).

- **Varoglutamstat**: a glutaminyl cyclase inhibitor, varoglutamstat reduces the production of toxic pyroglutamated Aβ (pGlu-Aβ), a key component of amyloid plaques. It is currently involved in two open Phase 2 clinical trials (Cummings et al., 2024; ClinicalTrials.gov).

The other major DMT agents in the final phases of clinical trials are those aiming to prevent the phosphorylation and fibrillization of Tau protein:

- Bepranemab: a monoclonal IgG4 antibody is designed to target a central epitope of tau, demonstrating stronger binding to the protein compared to antibodies aimed at the N-terminal region. It is currently in Phase 2 trials, with plans to progress to Phase 3 in 2024 (Cummings et al., 2024; ClinicalTrials.gov).

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- E2814: a monoclonal IgG1 antibody that focuses on an HVPGG epitope within the microtubule-binding domain of tau, E2814 prevents the propagation of pathogenic tau and facilitates its clearance. There are currently two active Phase 3 clinical trials for this drug (Cummings et al., 2024; ClinicalTrials.gov).

- LY3372689: a small-molecule inhibitor of O-GlcNAcase enhances tau glycosylation and inhibits tau aggregation by preventing its phosphorylation. It is presently undergoing evaluation in an open Phase 2 trial (Cummings et al., 2024; ClinicalTrials.gov).

- BIIB080: a tau-targeting DNA/RNA-based antisense oligonucleotide, which is intended to inhibit the translation of tau mRNA thereby reducing tau expression. It is currently in Phase 2 trials (Cummings et al., 2024; ClinicalTrials.gov).

Other interesting compounds in advanced stages of clinical trials include those that act as neuroprotectors and cognitive enhancers, target neuroinflammatory processes or have been repurposed due to their beneficial effects on Alzheimer's pathology:

- Fosgonimeton: an active metabolite that acts as a positive modulator of hepatocyte growth factor (HGF)/MET signaling, which promotes survival and proliferation of neurons. Two clinical trials active in phase 3 (Cummings et al., 2024; ClinicalTrials.gov).

- Buntanetap: a novel translational inhibitor of multiple neurotoxic proteins, including amyloid precursor protein, tau, and α-synuclein. Active trial in Phase 3 (Cummings et al., 2024; ClinicalTrials.gov).

- Metformin: a widely used antidiabetic medication, has shown potential in improving cognition or mood across various neurological disorders including AD. In phase 3 clinical trial (Cummings et al., 2024; ClinicalTrials.gov).

- Masitinib: a tyrosine kinase inhibitor that inhibits microglial and mast cell activity and shows high CNS penetration. Currently undergoing phase 3 trials (Cummings et al., 2024; ClinicalTrials.gov).

- Sodium oligomannate: an oligosaccharide, that can ameliorate neuroinflammatory processes such as infiltration of immune cells into the brain driven by bacterial metabolites, inhibit the formation of amyloid-β fibrils, and ability to restore gut microbiota on *in vivo* studies. It is the studied compound in two phase four clinical trials (Cummings et al., 2024; ClinicalTrials.gov).

- Spironolactone: an aldosterone mineralocorticoid receptor antagonist, that was commonly used for cardiovascular conditions. In neurodegenerative models, it has demonstrated anti-inflammatory properties in the CNS and upregulation of BDNF. It is currently in the last phase of clinical trials (Cummings et al., 2024; ClinicalTrials.gov).

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Aside the compounds being already tested in the different clinical trials, the approach for the developing and testing of new potential compounds have shifted over the last few years. Selective inhibitors have gained attention over the past few years due to the limitations and failure of pan-inhibitors due to cytotoxicity and adverse effects. These newly explored inhibitors target specific categories, subtypes, and structural domains, offering improved efficacy, safety, and tolerability. The development of selective inhibitors focuses on targeting less conserved binding sites, such as ATP-independent sites, to enhance drug specificity and minimize off-target effects (Zhang et al., 2024). For example, kadsuranin, a stereoisomer, have shown potential in inhibiting GSK-3β and reducing tau phosphorylation in AD models. Selective inhibition of GSK-3α is another promising strategy, as it reduces tau phosphorylation without overactivating the wnt/β-catenin pathway, which could cause unwanted cell proliferation (Hu et al., 2019). Histone deacetylase 6 (HDAC6) inhibitors, which modulate gene expression, also play a role in AD pathology. Compounds with improved HDAC6 selectivity, such as melatonin and derivatives, exhibit neuroprotective effects and improve memory in AD models (Wang et al., 2018; Wang et al., 2021). Multi-target inhibitors are also being explored to ameliorate the multifactorial pathological processes with a synergistic effect, while prolonging potential therapeutic length and reducing side effects and required dosage. For instance, cholinesterase inhibitors, such as those targeting both acetylcholinesterase and butyrylcholinesterase (BuChE), not only alleviate cognitive impairment in AD patients but may also delay the formation of Aβ plaques. Drawing the successful results in cancer therapy that covalent inhibitors have shown, its application and development for AD has also yielded promising results (Miao et al., 2022). The Ru(III) anticancer agent KP1019 have demonstrated protective effects in cortical neurons due to amyloid toxicity in vitro, or the 6H8 fragment, which targets the pathological substrates of γ-secretase (Jones et al., 2015). Finally, are the proteolysis-targeting chimeras aim to modulate the impaired ubiquitin-proteasome system (UPS) in Alzheimer, to selectively target the degradation of the pathological proteins. The discovery of molecules such as PT-65, which significantly degrades the GSK-3 protein family or phosTAC7 which induces dephosphorylation of tau, have managed to show lowered aggregation and more prolonged effects compared to traditional GSK-3 inhibitors (Qu et al., 2021; Sibley & Schneekloth, 2022).

All of the exposed current research invites to think on a positive and fast evolving landscape for the future of Alzheimer's management, specially taking into consideration the rise of distinct in phase trials involving DMTs and the prioritization status that the FDA has given the disease (*FDA Grants Accelerated Approval for Alzheimer's Drug | FDA, n.d.*). The recent breakthrough of the technological tools associated to Artificial Intelligence (AI) has already been proven beneficial when incorporated into clinical AD. AI data learning models have already been able to accurately predict AD from biomarker and neuroimaging, predict the conversion of patients from MCI to AD at high rates characterize patients into sub-groups depending on the biological variables observed amongst them and even repurpose drug candidates for AD which altogether represent promising results for the early-diagnose,

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personalized therapies and a huge reduction in the costly and time-consuming process of novel drug discovery (Fabrizio et al., 2021).

3.5 Alzheimer´s disease models

During the past few decades, many advances have been made in the understanding of Alzheimer's various pathological pathways and their intricate interactions. This research has heavily relied on the development of ever more complex models that represented the characteristics of this disease and are essential before progressing to human clinical trials in drug research (King, 2018) (Figure 9.).

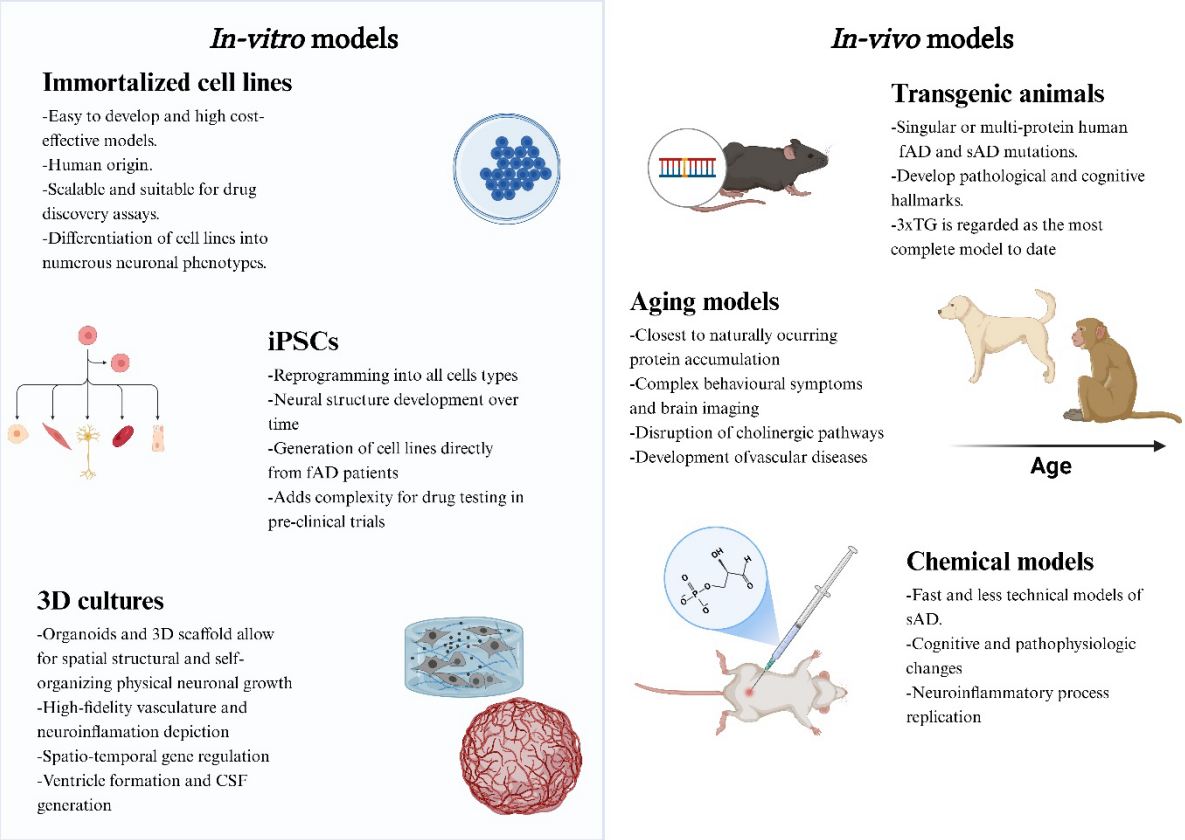


Figure 9. Research models of Alzheimer's disease. Schematic summary of the distinct animal and cellular models developed for Alzheimer's and pharmacological research. Created in BioRender. Barro, M. (2024).

The first models developed to study Alzheimer's disease were rudimentary and focused primarily on inducing brain lesions or pharmacologically disrupting neuronal function in areas related to cognitive decline, but they lacked the disease's key pathological features, such as amyloid plaques and NFTs. Aging models also provided some insight, as advanced age is a significant risk factor for AD, but these models failed to fully recapitulate the multifactorial nature of AD pathology (Scearce-Levie et al., 2020). The discovery of autosomal dominant mutations in the amyloid precursor protein, presenilin 1, and presenilin 2 genes, which cause

familial forms of EOAD), led to the next significant leap in experimental modelling. These genetic mutations were found to accelerate the production of amyloid-β (Aβ) peptides, key components in the formation of amyloid plaques (Myers & Mcgonigle, 2019). In 1995, the first transgenic mouse model was developed by inducing the expression of Indiana mutation form of human APP, resulting in the formation of amyloid plaques that closely resembled those seen in human AD. This model marked a turning point in AD research, as it allowed for the examination of plaque formation, neuronal dysfunction, and cognitive decline in a living organism (Games et al., 1995).

Since the development of the first transgenic mouse, the field has seen a rapid proliferation of animal and in vitro models, each designed to mimic different aspects of AD pathology. By inducing the replication of protein variants associated to AD, such as APP, PSEN1, PSEN2, and MAPT, researchers have created a wide variety of models that exhibit amyloid plaque accumulation, tau pathology, neuroinflammation, synaptic loss, and cognitive deficits. However, despite their utility, these models have significant limitations in terms of their ability to fully replicate human AD, often raising concern about the validity of translating preclinical findings to clinical success (Dawson et al., 2018).

Alzheimer's pathology replication both in animal and cellular models is typically induced through genetic manipulation or by directly introducing toxic agents, such as amyloid-β peptides or neurotoxins. The most common methods include:

1. Genetic manipulation

Genome editing

- CRISPR-Cas9, electroporation or chemical transfection are the most popular techniques to produce robust in vitro model, allowing researchers to induce Alzheimer's characteristics by selectively editing genes involved in AD pathology to mimic the fAD pathogenic variants known in the disease. These mutations lead to abnormal processing of amyloid precursor protein or aberrant phosphorylation of Tau. With these techniques is possible to generate multi-genomic editing and produce transient or stable models depending on the specific research aims (Bassil et al., 2021; Lu et al., 2021; Xie et al., 2023).

Transgenic techniques (Gene overexpression/Knock-in/-out models)

- Transgenic animals are created by inserting human genes associated with familial Alzheimer's disease into the animal's genome. Overexpression of these genes leads to Alzheimer's-like pathology. Common genes used include human APP, PSEN1, and MAPT, leading to an overproduction of Aβ or tau hyperphosphorylation. This technique

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allows for a stable, progressive development of AD-like pathology in animal models (Myers & McGonigle, 2019; Sanchez-Varo et al., 2022).

2. Pharmacological induction of AD pathology

Amyloid-β synthetic peptide

- This methodology consists of injecting synthetic Aβ peptides (such as Aβ1-42) directly into the brain (often into the hippocampus or cortex) or intravenously in *in vivo* models or add them to cell culture media on *in vitro* assays. These peptides aggregate, forming plaques that resemble those found in AD, inducing synaptic dysfunction, oxidative stress, and inflammation processes which lead to cognitive defects in animal models (Ulm et al., 2021).

Tauopathy induction via tau oligomers/fibrils

- To model tau pathology, researchers introduce synthetic tau oligomers or fibrils into the brain (via direct injection) or into neuronal cultures. These tau aggregates can seed the formation of aggregation and induce tau hyperphosphorylation, mimicking spread of tau pathology found in AD (Iba et al., 2013; D'Errico & Meyer-Luehmann, 2020).

Neurotoxic agents (Streptozotocin, Okadaic Acid, etc.)

- Neurotoxic compounds are used to induce specific Alzheimer's-like characteristics, such as metabolic disfunction, microtubule destabilization, neuronal death and tau hyperphosphorylation (Rapaka et al., 2022). Some of the most used ones are:

- **Streptozotocin (STZ)**: Injected into the brain or administered systemically to induce brain insulin resistance, mimicking aspects of sporadic AD and leading to cognitive deficits, protein aggregation, and oxidative stress (Kamat, 2015; Park et al., 2020; Gáspár et al., 2022).

- **Okadaic Acid (OA)**: Inhibits protein phosphatases (PP1 and PP2A), which control tau dephosphorylation. When administered to neuronal cultures or injected into animal brains, this toxin induces tau hyperphosphorylation, formation of amyloid plaques, oxidative stress and cognitive decline (Kamat et al., 2013; Chen & Zhang, 2022).

- **Colchicine**: This anti-inflammatory compound is used in AD models as a microtubule-disrupting agent. Upon injection in the hippocampus, colchicine induces cell death and neurite loss through depolarization of microtubules and disruption of axonal transport

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as well as neurotoxicity through oxidative stress (Kumar et al., 2007; Sil et al., 2014; Akhtar et al., 2022).

- **Scopolamine:** Scopolamine, an anticholinergic agent, inhibits muscarinic cholinergic receptors, causing excessive acetylcholine release, which ultimately leads to morpho-physiological damage in key brain regions such as the hippocampus, cortex, and nucleus basalis which play critical roles in memory and cognitive functions (Bajo et al., 2015; Chen & Yeong, 2020).

3. Environmental and stress-based techniques

High-fat diets and metabolic stress models

- High-fat diets and high-glucose diets are used in animal models to induce metabolic dysfunctions such as insulin resistance and obesity, both of which are risk factors for sporadic AD. These diets lead to cognitive decline, metabolic dysfunctions, brain insulin resistance, and amyloidosis (Kothari et al., 2016; Akhtar et al., 2022; Amelianchik et al., 2022).

Chronic stress models

- Chronic stress is a complementary method to further replicate the complexity AD-like pathology in *in vivo* models. By subjecting animals like glucocorticoids, electrophysiological and behavioural stressors researchers had described the potentiation that these have in cognitive decline and exacerbate amyloid and tau pathologies (Green et al., 2006; Milligan Armstrong et al., 2021).

4. Inflammatory techniques

Lipopolysaccharide (LPS) / IL-1β

- LPS, a component of bacterial cell walls, is injected into the brains or systemically to induce an immune response and chronic neuroinflammation, which can lead to neuronal damage and cognitive deficits. IL-1β sustained overexpression is another common way to develop *in vivo* models employed to study neuroinflammatory processes and the study of anti-inflammatory treatments (Shaftel et al., 2007; Batista et al., 2019; Ludwig et al., 2022).

Physiological models of sporadic AD are regarded as some of the most accurate representations of the disease due to the complexity that animal models offer, but they come

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with notable practical and scientific limitations. For example, many of these models have long lifespans and exhibit variable pathology between individual specimens. This not only makes experiments costly and time-consuming but also complicates preclinical testing due to the difficulty in standardizing disease onset, progression and dose efficacy across studies. Furthermore, cognitive testing in these models is less standardized, posing another challenge for consistent experimental outcomes (Drummond & Wisniewski, 2016; Chen & Zhang, 2022, Zhang et al., 2024). Animal models of familial AD also demonstrate limitations, primarily due to the overexpression of human Aβ and tau, which leads to non-human physiological downstream effects. These transgenic models also exhibit solubility differences in plaque cores compared to human AD, which may explain why amyloid-targeting therapies often show greater efficacy in mice than in humans (Granzotto et al., 2024). Moreover, mouse models expressing human PSEN1/2 fAD mutations fail to develop plaques, highlighting species-specific differences that limit the translatability of results (Kitazawa et al., 2015). The orientation towards individual specific pathophysiological characteristics, despite significant homology with their human counterparts, is another major issue. This provokes the absence of physiological interactions between Aβ and tau in these models, which complicates the study of crosstalk between these proteins and their contributions to neurodegeneration (Drummond & Wisniewski, 2016).

In addition to animal models, human cell-culture models also face significant challenges. One of the main issues is the lack of standardized protocols for generating and maintaining induced cell lines, which can result in phenotype variability, lack of translational ability and scalability for their use in drug discovery and repurposing assays. Moreover, these models do not exhibit long-term progression of the disease and how risk factors like dietary modifications, stress or aging alter the onset or course of the disease (Shree Sharma et al., 2021b; Pereira et al., 2023). Although advancements in 3D cell culture models, such as iPSCs and organoid cultures, are beginning to address the morphological limitations of 2D cell models, these systems still do not fully replicate the brain's complex cellular variety, environment, vascularity and biological barriers, which play critical roles in the disease (Logan et al., 2019). Finally, most of these models mainly represent fAD due to the ease of representing protein depositions and other related pathways through genomic editing while preserving the initiator of the disease. While both variants share the majority of the biological and molecular changes, there are fundamental differences between fAD and sAD. For instance, fAD tends to exhibit more amyloid deposition in subcortical regions and more grey matter atrophy compared to sAD, while sAD is associated with poor Aβ clearance rather than overproduction (Condello et al., 2017; Jorge et al., 2021). These differences have raised concerns about whether therapeutics that perform well in these models will translate effectively to sAD patients.

Despite these limitations, current models of AD remain invaluable tools for exploring disease mechanisms and testing potential treatments and advances towards their translation potential, their multi-faceted approach to the large number of pathophysiological changes occurring in an Alzheimer’s brain and the presentation of reproducible cost-effective

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methodologies will still serve as the foundation for future basic and pharmacological research towards the find of a definitive cure for the pathology.

3.5.1 Animal models

Historically, the utilization of animal models in biomedical research has been a critical aspect of understanding human diseases. The evolutionary relationship between humans and other species, particularly mammals, has allowed scientists to investigate physiological and pathological processes in ways that are often not feasible in human subjects. While the practice of using animals for research dates back centuries into the Greek civilization, it gained significant momentum from the nineteenth century onwards when they became integral to the fields of pharmacology, toxicology, and basic research disease modelling (Mukherjee et al., 2022).

In the context of Alzheimer's disease, animal models have emerged as invaluable tools for dissecting the molecular mechanisms that contribute to disease pathogenesis. As introduced before, these models, through a wide variety of mechanisms are normally oriented towards replicating individual, in most cases, or mixed pathophysiological characteristics of AD. In Alzheimer's research, hundreds of models have been proposed over the decades, using many mammalians and invertebrate species, being transgenic mice the most widespread one (Trujillo-Estrada et al., 2021; Castillo-Rangel et al., 2023). Rodents, particularly mice, have become the predominant species used in AD research due to their genetic similarity to humans, relatively short lifespan, and well-characterized genomes, allowing for age-dependent accumulation of proteins and the progressive cognitive deterioration associated with it (Chen & Yang, 2022). Apart from the rodents' models, in the past few years, invertebrate species such as *Drosophila melanogaster* (fruit flies) and *Caenorhabditis elegans* (nematodes) have emerged as alternative and commonly employed biological models (Mukherjee et al., 2022). These organisms provide distinct advantages, including shorter lifespans and simpler nervous systems, which enable rapid genetic manipulation and high-throughput screening of potential therapeutics without the ethical concerns that more complex species share. Despite the greater evolutionary differences between these organisms with humans, invertebrates can effectively replicate the amyloid and tau pathologies although cognitive and behavioural symptoms are limited (Nichols, 2006; Tan & Azzam, 2017; Cook et al., 2019; Griffin et al., 2019). In addition to rodents and invertebrates, larger mammalian models, such as dogs, pigs, and non-human primates, have been explored for their potential in AD research. Old canine and primate models have been of particular interest due to their natural development of age-related cognitive decline that are key to sAD. These more sophisticated models are able to naturally produce amyloidosis and tauopathies, as well as develop similar CSF biomarkers and cerebrovascular pathologies that are involved in human AD (Schmidt et al., 2015; Schütt et al., 2016; Rusbridge et al., 2018; Chen & Yang, 2022; Mukherjee et al., 2022).

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Even though mammalian models are, on paper, the ones that can more accurately mimic the multi-factorial etiology of Alzheimer's, their use is accompanied by several significant

setbacks and ethical concerns. This feeling is further exacerbated by the huge translational gap between pre-clinical studies on animals and their reach towards practical clinical use (LaFerla & Green, 2012). This discrepancy raises questions about the appropriateness of the models used, as no single animal model can fully replicate the complex human disease spectrum both physiologically or behaviourally. However, alongside this lack of progress from a pharmacological perspective, recent public and governmental concern have increased the awareness about animal welfare and rights, shifting the experimentation for its adequation of the reduction, refinement and replacement of animal models in favour of cellular *in vitro* assays or less complex species (Andersen & Winter, 2019; Vangala et al., 2022).

3.5.1.1 Transgenic models

Transgenic models of Alzheimer's disease (AD) have become in the past 30 years, the most employed tool in understanding the disease's complex pathology and developing potential therapeutic interventions. Since the first described mutations associated with fAD in the early 90s, the research field on animal models saw an enormous progression with the development of the first transgenic mouse models using mutations on the APP gene. Similarly, by 1999, PSEN1 was firstly described as a protein with mutations linked to AD, and the transgenic mouse models followed (Myers & McGonigle, 2019; Sanchez-Varo et al., 2022). Focusing on a single mutation inevitably led to very one-sided models where pathological and behavioural characteristics of AD were not fully represented. The desire to build even more complex models in individual animals accelerated the creation of multi-transgenic models at the start of the 2000s, which also included tau pathology generating mutations (Oddo et al., 2003b). Regardless of the numerous advantages that rats offer over mice, their contribution to transgenic models is very scarce. Rats are generically and physiologically closer to humans, exhibit more complex social behaviours and express all isoforms of tau but the longer gestation and costly periods, the more demanding genetic manipulation challenges and the research focus on standardizing mouse lines had relegated rat models to the ostracism, being the first generated one as late as 2007 and being less complex to current existing mice models (Flood et al., 2007; DoCarmo & Cuello, 2013; Myers & Mcgonigle, 2019).

Generation of these transgenic models has involved a wide variety of genetic engineering techniques that allowed the manipulation of rodent´s genome to express the human variant of mutated proteins associated to AD. Including fAD-linked mutated genes has been the foundation of in-vivo models because rodents, as they constitute the majority of the more than 200 developed models in the past three decades, do not have the capacity to naturally develop AD (Myers & Mcgonigle, 2019). In fact, concentrations of Aβ in rodents' brains is found in picomolar concentrations, whereas in human AD patients, reaches nanomolar concentrations. Additionally, rodent Aβ only differs from its human counterpart in three amino acids, but these are significantly enough to prevent the aggregation of the protein (Teich et al., 2013). The primarily used strategies, alongside with the more recent artificial bacterial/yeast chromosomes and CRISPR, to generate these transgenic organisms have commonly involved:

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- Pronuclear Injection: This technique is one of the oldest and consists of microinjecting complementary DNA (cDNA) containing the transgene into the pronucleus of fertilized zygotes. The transgene typically consists of the gene of interest along with a promoter that controls its expression. DNA is inserted randomly into the host genome, often leading to the integration of multiple copies of the transgene. The injected zygotes are then implanted into female mice to complete gestation. This method commonly results in the overexpression of the introduced gene (Ittner & Götz, 2007; Chen & Yang, 2022).

- Gene targeting via Embryonic Stem Cells: In this approach, a targeting vector containing the desired mutation is introduced into embryonic stem (ES) cells, which modifies a specific endogenous gene through homologous recombination. The gene-targeted ES cells are then injected into wild-type blastocyst-stage embryos, which are implanted into female specimens. This method is typically used to generate knock-in or knock-out mice and have become the staple method as it allows for more precise genetic alterations and "natural" expression of the proteins compared to previous techniques (Esquerda-Canals et al., 2017).

- Viral vector mediated transgenesis: Through this strategy, transgenes are delivered into embryonic cells or fertilized embryos by using viral vectors like lentiviruses and adeno-associated vectors (AAV) to create a stable expression in the offspring. Viral transgenesis allows for larger constructs and multiple transgenesis, making it particularly useful in more complex models (Dawson et al., 2018; Ittner et al., 2019).

As previously mentioned, APP-based transgenic models were the first introduced in the mid-1990s with the development of the PDAPP mouse, followed by widely used models like Tg2576, APP23, TgCRND8, and J20. These models overexpress human APP with either the Indiana (V717F) or Swedish (KM670/671NL) or London mutation APP(V717I), crucial for mimicking Alzheimer's disease pathology. These APP-based models show key AD-like features, such as amyloid-β (Aβ) deposition, neuroinflammation, synaptic dysfunction, neuronal loss, and cognitive deficits from young ages. A key limitation of APP-based models is the lack of tau pathology. Although some models, like TgCRND8, APP23, and APP(V717I), exhibit tau hyperphosphorylation, they do not develop NFTs (Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Mucke et al., 2000; Chishti et al., 2001; Esquerda-Canals et al., 2017). Taking this drawback into consideration besides the lack of 3R isoforms of Tau in murine brains, in 2003, the 8c mouse line, was the first to express all six human isoforms of tau. This model developed Alzheimer's-like pathology, including hyperphosphorylated tau forming paired helical filaments, along with deficits in spatial and recognition memory (Andorfer et al., 2003). From this point onwards, other models created from mutations linked to FTD were created to

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accelerate the cognitive decline related to tauopathies. For instance, the rTg4510 model or the tau line 19, which carry the mutations MAPT P301L and P301S respectively express high levels of mutated tau, resulting in progressive NFTs, neuronal loss, and cognitive/behavioural impairments as well as overexpressing human tau at higher rates than the endogenous murine Tau (Ramsden et al., 2005; DeVos et al., 2017; Gamache et al., 2019). The biggest breakthrough in transgenic Alzheimer's modelling with the first double transgenic models including both APP and PSEN1 mutated genes. These models, such as APPswe/PSEN1dE9, demonstrated a more pronounced and accelerated pathology compared to individual APP models (Jankowsky et al., 2004). Amongst these, probably the most advanced model is the 5x fAD that combines the London, Swedish and Florida mutations in the APP genes and M146L and L286V in the PSEN protein. Consequently, this model shows Aβ accumulation as early as 6 weeks of age and plaque formation by 2 months (Oakley et al., 2006). In addition to these fast-paced amyloidosis models, combinatory tau and APP/PSEN models have also been developed to further push the representation of AD's multi-factorial pathology. The 3x TG model, considered the most comprehensive *in vivo* Alzheimer's model currently available, is the most widely used in research and it contains the Swedish APP mutation, the M146V mutation in PSEN1, and the P301L MAPT mutation. In this model, Aβ accumulation is observed as early as 3-4 months of age plaque development by 6 months, and NFTs by 12 months, with cognitive impairments observable around the 6-month mark (Oddo et al., 2003a; Drummond & Wisniewski, 2016; Chen & Zhang, 2022). Aside from the most traditionally known models, others such as the the TgSweDI model incorporating the Swedish, Dutch and Iowa mutations, which lead to development of cerebral amyloid angiopathy or the APP E693 model which carries the Osaka mutations and only produce smaller oligomeric forms of Aβ, may constitute instrumental options for assessing specific AD related pathogenic pathways (Xu et al., 2007a; Umeda et al., 2014; Tan et al., 2023). Models incorporating mutations in ApoE or TREM2 proteins have also emerged over the past few years in response to the lack of animal models representing the sporadic variant of the disease (McKean et al., 2021).

3.5.1.2 Other in-vivo models

Transgenic rodent models of Alzheimer's disease are widely used but come with significant limitations in the form of primarily representing familial AD rather than sAD, and the development of pathology in these models is often non-naturally. Because of these limitations, there is growing interest in naturally occurring models of AD, which may better reflect the changes that occur in sAD. In contrast to transgenic models, non-transgenic models allow researchers to study not only amyloid and tau pathology but also other critical pathological states, such as oxidative stress, synaptic dysfunction, apoptosis, and gliosis. These include models replicating conditions like type 3 diabetes (insulin resistance), neuroinflammation, gut microbiota-brain axis disruptions, lipid metabolism abnormalities, autophagy dysfunction, and metal ion disorders (LaFerla & Green, 2012; Akhtar et al., 2022; Rapaka et al., 2022). Various non-rodent models, such as cholesterol-fed rabbits, aged dogs, and non-human primates, have been used to study these aspects of sAD. Depending on the mechanisms of Alzheimer's

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replication, non-transgenic in vivo models can be categorized into physiological and chemically induced models (Akhtar et al., 2022).

Regarding naturally occurring AD characteristics, non-human primates are the most well characterized model. Primates provide the closest available naturally occurring model due to their genetic and physiological similarities to humans, complex behaviour, possibility of brain imaging and 100% homology sequence of proteins such Aβ. Age-related neurodegeneration in primates also shares many morpho-physiological changes with human AD, including accumulation of amyloid-β from age 25, brain shrinkage, the presence of amyloid plaques, tau-related changes, and the loss of cholinergic neurons (Geula et al., 1998; Wallace et al., 2015; Vitek et al., 2021). Studies show that key proteins, such as amyloid precursor protein, apolipoprotein E, and complement components like C1q and C3c, exhibit similar cellular distribution to the human one (Heuer et al., 2013; Braidy et al., 2015). Electron microscopy has revealed that amyloid plaques in older macaques resemble those seen in AD, with dense amyloid cores, though they lack the twisted filaments often found in human plaques. Aged monkeys also display alterations in both monoaminergic and cholinergic pathways. Tauopathies have also been observed in the hippocampus in several species, including baboons or lemurs, although the formation of NFTS is absent. However, unlike in humans, these tangles can be found in glial cells (Gandy et al., 2004; Lemere et al., 2004; Bons et al., 2006). Another prominent species used in physiological studies are canines. Cognitive decline in dogs begins in middle age and progresses similarly to early-stage AD in humans and is closely related to the spread of amyloid plaques from the pre-frontal cortex. Dogs also develop cerebral amyloid angiopathy, similarly to humans, where Aβ accumulates in blood vessels, leading to vascular dysfunction and microhaemorrhages. However, dogs do not develop tauopathies or cholinergic disfunction, although this is not an impediment to show significant decline at spatial learning and memory tasks (Uchida et al., 1990; Head et al., 2000; Head et al., 2010; Head, 2013). Finally, another non-animal physiological model has also been more frequently used due to its availability and shorter lifespan making it more feasible for experimentation. The *Octodon degus,* a rodent native to Chile*,* shares a high degree homology with human amyloid-beta, differing by only one amino-acid and being able to develop both protein aggregation hallmarks seen in AD. The extracellular deposits are age-dependent and widespread across cortical layers, while intracellular Aβ aggregates are more localized, particularly in specific cortical and hippocampal regions. Additionally, *Octodon degus* shows degeneration of cholinergic pyramidal neurons that is accompanied by memory deficits and cognitive decline. Moreover, like humans with AD, aged *O. degus* show increased levels of glial fibrillary acidic protein (GFAP)-positive astrocytes in affected brain regions, such as the cortex and hippocampus, reflecting pronounced astrogliosis (Inestrosa et al., 2004; Castro-Fuentes & Socas-Pérez, 2013; Mugnaini et al., 2020).

The other main portion of non-transgenic *in vivo* models tries to replicate the main hallmarks of AD by employing synthetic compounds that mimic the risk factors associated with the pathology in a shorter time span. Intracerebral injections are the main way of inducing amyloidosis and tauopathies in non-transgenic models by employing synthetic human mutated

fibrils. These models offer shorter action time and immediate neurodegenerative processes such as synapse loss and neuronal death within the perfusion site. Cognitive impairments can be observed as early as seven days post-infusion, making it a time-efficient alternative to transgenic models. Non-amyloid/tau models often include a diversity of chemical treatments that lead to other pathological hallmarks of AD such as oxidative stress, neuroinflammation or cholinergic disfunction (Rapaka et al., 2022; Xu et al., 2023; Valentin-Escalera et al., 2024). An example of these techniques is Streptozotocin. This compound is administered intracerebroventricularly to mimic systemic diabetes and study the role of insulin mechanisms in AD. Administration of Streptozotocin results in morphological changes in the brain, including ventricular enlargement and thickening of the corpus callosum. These changes are associated with reduced neurogenesis and increased neuroinflammation in periventricular areas and the dorsal hippocampus. It has also been demonstrated to induce oxidative stress, disrupt cholinergic transmission, alter insulin receptor signalling, and cause memory impairments (Gáspár et al., 2022; Kadhim et al., 2022; Silva et al., 2023). For the study of ROS species, D-galactose, an aldohexose, is commonly used. D-galactose administration incurs in brain maturation and "aging", also modelling hallmarks like mitochondrial dysfunction, oxidative stress, neurodegeneration and memory impairment. This model is favoured for its mild side effects and high survival rate, being often combined with other non-transgenic models to exacerbate different pathologies (Haider et al., 2015; Shwe et al., 2018; Azman & Zakaria, 2019). Additionally, for inflammatory processes, besides the injections of inflammatory compounds like the previously mentioned LPS or the polyriboinosinic-polyribocytidilic acid, exposure to heavy metals forms like aluminium chloride are also beginning to be explored. Heavy metals pose significant toxicity risks for various health disorders including AD. Usage of these compounds on *in vivo* models produce exacerbated oxidative stress, increased inflammation and enhanced dendritic loss in the hippocampal area (Walton & Wang, 2009; Erazi et al., 2010; Krstic et al., 2012).

3.5.2 Cellular models

The limited success of translation that classic animal models have shown for drug development in AD, combined with the ethical concerns and the high costs associated to *in vivo* research have highlighted the inadequacies of animal modelling for drug discovery in neurodegenerative diseases. This has prompted the demand for more variety of physiologically relevant tools that can be used to bridge the gap between basic research and clinical studies. These models offer a more controlled environment, enabling precise manipulation of cellular and molecular components while offering powerful biological tools for high-throughput screening and other drug discovery techniques that are of great value for multi-factorial and complex diseases with uncertain etiologies (Dubey et al., 2019; Sharma et al., 2021b; Xie et al., 2023).

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The development of in vitro models for neurodegenerative diseases such as AD or PD started rudimentary with traditional organotypic cultures from brain slices which attempted to

recreate tissue architecture and function of areas such as the hippocampus and with models of Aβ oligomerization in vitro (Fitzjohn et al., 2008; Sharma et al., 2021b). A huge leap took place with the appearance of the first in vitro models based on immortalized cell lines in the 90s such as SH-SY5Y or PC-12. These allowed to be induced into neuronal phenotypes, stable biological systems and a huge reduction in cost-effective experimentation, converting them in a staple for neurodegenerative disease research in past and modern times (König et al., 1990; Neill et al., 1994; Peng et al., 2021). The advent of human embryonic stem cells (ESCs) and later, human-induced pluripotent stem cells, also supposed a huge revolution in the field by providing a tool for generating various cell types with the same genetic background (Thomson et al., 1998; Takahashi et al., 2007). iPSCs derived from patients with fAD mutations have since become a powerful platform for modelling disease processes in a patient-specific context. iPSCs also offer a significant advantage in the form of its ability to differentiate in every neural cell type. Additionally, iPSC-derived neurons have been instrumental in modelling synaptic dysfunction, neuronal death, and tauopathies in a human context, offering new insights into disease mechanisms and screening options that were previously unattainable using animal models (Choi & Tanzi., 2012; Kondo et al., 2017). Another promising breakthrough in AD modelling came from the development of of three-dimensional (3D) cultures, particularly brain organoids and spheroids. These miniaturized, self-organizing tissues, which are derived from iPSCs or embryonic stem cells (ESCs), exhibit a higher level of cellular diversity and organization, closely resembling the human brain's structural and functional features. Brain organoids have been used to model amyloid plaque formation, tau tangle pathology, and neuroinflammatory responses (Raja et al., 2016). These models represent an exciting step forward, as they can recapitulate key aspects of AD in a system that allows for more physiologically relevant observations compared to traditional two-dimensional cultures although pose a difficult model to perform drug discovery due to their complex creation and rate of production. Complexity of two-dimensional models has also advanced in recent times with co-culture systems that can hold multiple cell types in a single culture. For example, studies utilizing neuron-microglia-astrocyte co-cultures have provided valuable insights into the inflammatory aspects of AD, such as the role of glial activation in response to amyloid neurotoxicity (Park et al., 2018; Luchena et al., 2022). Despite the many advances in cell modelling of AD, these lack the full diversity and structure of human brains and the inability to replicate key interactions with other body regions such as the gut or the systemic vasculature. Nevertheless, in vitro models are gaining increasing recognition as complementary tools to in vivo studies, allowing for a fast and accessible biological model that is now key for early-stage drug discovery campaigns. Furthermore, recent advancements in gene-editing technologies, such as CRISPR-Cas9, have greatly augmented the utility of in vitro systems, allowing for precise manipulation of AD-related genes to also model sporadic forms of the disease (Paquet et al., 2016).

The mechanisms behind the modelling of the pathological hallmarks of AD are also very diverse. Besides the previously described genetic modification techniques and the reprogramming of fAD patients' somatic cells into stable iPSCs cell lines, in vitro studies allow for a much quicker interactions with environmental and chemical agents that may pose risks in

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the real world but require much more lengthy experiments when testing in animals (Huang et al., 2021). Following the trend of aluminium in animals, there are many examples of other heavy metals involved in the onset presentation of certain Alzheimer's characteristics in vitro. For instance, Manganese induces oxidative stress and apoptosis by activating MAPK/ GSK3-β signalling, leading to tau hyperphosphorylation and reduces viability while impairing APP processing in N2a od PC-12 cell models (Cai et al., 2011; Yang et al., 2021). Lead and arsenic are another two metals with described neurodegenerative potential. The first demonstrated increased amyloid-beta levels, morphological neurodegenerative-like changes, and reduced amyloid degradation on SH-SY5Y models while the second is used to increase ROS production and the study of them with mitochondrial disfunction and apoptosis (Huang et al., 2011; Rahaman et al., 2021). Other novel environmental models of Alzheimer include the use of hypoxia to mimic the deprivation of vascular oxygen in AD brains, which further exacerbates the amyloid oligomerization (Lall et al., 2019); air pollutants in the form of particulate matter composed of molecules such as carbon, hydrogen ions, water and sulphates which results in a ROS increase, neuronal death and neuroinflammatory processes (Jang et al., 2018; Lee et al., 2021); and derivatives of cholesterol like 27-Hydroxycholesterol, which reduces glucose uptake in neurons, impairing insulin signalling, contributes to synaptic dysfunction and increases neuroinflammation through the TGFβ/ NF-κB pathway (Loera-Valencia et al., 2021; Wang et al., 2020). Regarding chemical-inducing agents, many are shared with *in vivo* modelling such as the human synthetic fibrils and compounds like STZ or OKA. Particularly worth mentioning in this section are those that are considered risk factors for AD yet can and could be found in everyday products. Formaldehyde, an environmental agent commonly found in household products like cosmetics, has been found to form neurotoxic amyloid complexes and induce the formation of tau aggregates, exacerbating neuronal death in SH-SY5Y cells (Nie et al., 2007; Lu et al., 2013). Herbicides and insecticides like paraquat or dichlorodiphenyltrichloroethane, have also shown in SH models and H4 cells, a promotion of oxidative stress, neuronal death and increased amyloid levels (McCarthy et al., 2004; Li et al., 2015). Finally, Perfluorooctanesulfonic acid, found in commercial products as *Teflon* of *Gore-Tex* has demonstrated on SH-SY5Y models to trigger tau hyperphosphorylation and increasing amyloid precursor protein and apolipoprotein E levels (Basaly et al., 2021; Darbre, 2022).

All in all, in vitro models of Alzheimer's disease offer several advantages, such as providing a controlled environment to study specific pathophysiological aspects in a simplified form. These models also enable high-throughput drug screening, accelerating the identification of potential therapeutic agents, as little to-none is known about the target receptors, aiding in drug repurposing and shortening pre-clinical trial research. Additionally, they offer high flexibility on the approaches to represent both fAD and sAD and disease progression in much shorter time spans (Sharma et al., 2021b; Prajapat et al., 2023). However, in vitro systems also face notable challenges. They often lack the full reproducibility and standardized protocols, technical challenges in complex models and less complete disease recapitulation of AD than animal models, which poses *in vitro* research as a critical complementary tool but not exclusive, in advancing the understanding of AD pathogenesis and therapies (De Vitis et al., 2024).

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Following this trend, institutions such as the Food and Drug administration, have recently taken steps towards recognizing and validating non-*in vivo* tools such as cell-based assays, organoids and advanced artificial intelligence algorithms as specific alternatives for animal testing for assessing drug safety and effectiveness during pre-clinical trials (Zushin et al., 2023).

3.5.2.1 Immortalized cell lines

Drug screening with immortalized, undifferentiated cells sits at the intersection of medicinal chemistry and biology and serve as a launchpad for clinical trials of AD treatments. This phase is crucial for gathering initial data on the molecule's toxicity and its efficacy in a simplified cellular environment. For a compound to be considered for further testing, it must exhibit minimal intrinsic cytotoxicity promote high cell survival rates and efficacy at reverting the pathological changes of the given model. Immortalized cell lines offer several advantages for this process, including their availability, ease of use, consistent proliferation, and cost-effective maintenance. Additionally, many of these cell lines originate from human tissues, which increases the relevance of the results to human biology (Aldewachi et al., 2021; Maniam et al., 2024).

The most employed cell line in CNS research is the SH-SY5Y cell line. In its undifferentiated state, SH-SY5Y cells resemble immature catecholaminergic neurons, possessing incipient neurite structures and expressing early-stage neuronal markers. SH-SY5Y cells are derived from neuroblastoma, a cancer of immature neuronal cells in the embryonic stage and obtained through subcloning the parent line SK-N-SH (Dubey et al., 2018; Giri et al., 2018; D'Aloia et al., 2024). SH-SY5Y cells, in their undifferentiated form, can be differentiated into various neuronal phenotypes depending on the type of differentiating agent used. For example, treatment with compounds such as retinoic acid (RA), BDNF, Glucagon-like peptide 1 (GLP-1) or phorbol esters induces differentiation into a cholinergic, dopaminergic, glutamatergic, noradrenergic or mixed neuron phenotype (Kovalevich & Langford, 2013; Elnagar et al., 2018; Jun et al., 2020; D'Aloia et al., 2024). As such, SH-SY5Y cells are more suitable for neurodegenerative disease research compared to other non-neuronal widely used cell lines, like HeLa, HEK293, and PC12 cells. This cell line has been employed to model various aspects of Alzheimer's disease pathology, including amyloid-β toxicity, tau phosphorylation, oxidative stress, mitochondrial dysfunction, and neuroinflammation, as well as serving as a validation tool for compounds with neuroprotective described effects *in vitro* (Tauffenberger et al., 2019; Liu et al., 2020b; Hu et al., 2021; Peng et al., 2021; De la Cueva et al., 2022). Another two examples of cholinergic precursor cells used in Alzheimer are the also neuroblastoma derived SK-N-MC and the precursor SK-N-SH. These have also proven their utility for studying cholinergic dysfunction, amyloid toxicity, tauopathies and neuroprotective strategies in AD, although have lost interest in favour of the SH-SY5Y due to their higher tumorigenic characteristics and their limitations at neuronal differentiation (Dubey et al., 2019; D'Aloia et al., 2024). In addition to neuroblastoma derived cells, human neuronal progenitor cells (hNPCs) are also an option of modelling Alzheimer *in vitro* with a human biological base.

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These have the potential of differentiating into various CNS cell types and naturally express neurotransmitter such as GABA and glutamate receptors, as well as be biologically responsive to them, making them a candidate for more robust and complex in vitro models (Fukusumi et al., 2016; Kang et al., 2017). Aside from human-origin cells, others like the pheochromocytoma rat medulla derived PC-12 cells have also been widely explored in AD research. PC-12 cell line mimics a differentiation process into neuron-like cells and develops neurite outgrowth upon exposure with nerve growth factor (NGF). This cell model has been widely used for the study of amyloidosis, oxidative stress and evaluation of neuroprotective and antioxidant agents like resveratrol (Jang & Surh, 2003; Xie et al., 2023). Another group of cells widely used in AD pharmacological research are the vasculature endothelial cells. Human (HBMECs, hCMEC/D3, BMVEC) or animal (bEnd3, RBE4, BCECs) derived, they serve the necessary purpose of mimicking physiological similarities to the BBB such as the tight junction proteins (claudins, occludins, and junctional adhesion proteins) and transporters. Often co-cultured with other neuronal or astrocyte cell lines, these have allowed the study of processes such as the relationship between barrier integrity and amyloidosis, vascular inflammation and drug transport and delivery through this biological obstacle (Daniels et al., 2013; McCarthy and Kosman., 2014; Katt et al., 2016; Qosa et al., 2016). Finally, although not sharing a neural origin, classic cell lines like HEK293 cells have been also extensively use in AD modelling. These provide a viable and very strong model with transfection capabilities that ease the generation of stable cell lines as well as expression of various neurogenic factors, including neurofilament subunits and show similar morphology to immature neurons (Gordon et al., 2013; Lin et al., 2014b). HEK293 have provided valuable studies that have focused on AD-protein deposition, cytotoxicity and even screening of potential anti-AD pathogenesis drugs (Dubey et al., 2019).

Neuroinflammation is currently one of the most explored mechanisms for potential treatments in AD. Thus, its replication on *in vitro* models is also on the rise. Currently, the most used in vitro representation of gliosis is achieved by microglial culturing. This is performed through the use of primary microglial cultures derived from rodents and immortalized cell lines. Primary microglia cultures are widely used in neuro-inflammatory studies because of their phenotypic similarities to in vivo cells. These cultures are typically obtained from the cortex of neonatal rats or mice and are useful due to their more advanced functional and secretory abilities upon aberrant insults, being able to phagocyte Aβ oligomers or cytokine release (Peng et al., 2022). An alternative to these time-consuming cultures is the use of immortalized microglial cell lines. Two of the more extended lines are BV2 and N9, which are retroviral immortalized murine macrophages and microglial cells respectively. These types of cells have also exhibited a dose-dependent increase in IL-1 secretion and displayed phagocytic capabilities for Aβ-42 fibrils, which also occurred in a time- and dose-dependent manner upon LPS treatment (Hickman et al., 2008; Fleisher-Berkovich et al., 2010; Guo et al., 2019; Liu et al., 2019). Despite the challenging ethical and legal considerations, the representation of human microglial systems is also possible through the use of cell lines such as HMO6. Created in 2001 from primary human embryonic microglia, this cell line maintains phagocytic capabilities and

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production of cytokines (Nagai et al., 2001). Besides microglial representation *in vitro*, astrocytes also play a major role in neuroinflammatory, ion homeostasis and cell-cell interactions in AD. For this reason, astrocytes derived from human iPSCs and 3x-Tg iAstro cells have demonstrated their usefulness at studying potassium and calcium homeostasis and their role in lipid signalling as well as serve as platforms for novel therapeutic approaches through, for example, modulation of retinoid and liver receptor agonists for Aβ clearance or reduced astrogliosis (Tapella et al., 2022; Gong et al., 2023).

3.5.2.2 Induced pluripotent stem cells

Since its discovery in 2007, the reprogramming of terminally differentiated cells into a pluripotent state by using the set of transcription factors, OCT4, KLF4, SOX2, and c-MYC, induced pluripotent stem cells supposed a huge leap on some of the limitations linked to traditional in vitro models of many diseases, including AD. iPSCs allow for their differentiation into multiple neuronal phenotypes and neural cells, therefore allowing for the study of different mechanisms involved in all of the Alzheimer's stages. Additionally, the ability to generate neurons from individual Alzheimer's patients offers a novel platform for mechanistic research and drug discovery due to their human origin and disease-specific context (Takahashi et al., 2007; Arber et al., 2017; Barak et al., 2022). In the past decade, stem cell differentiation into disease-relevant neurons has advanced significantly, allowing for the creation of highly pure and active neuronal populations, in which cortical layers develop in a time-dependent manner resembling the biological neurogenesis. In addition to cortical neurons, protocols have also been established for differentiating stem cells into other AD-relevant cell types, such as cholinergic neurons, cortical interneurons, astrocytes and microglia (Maroof et al., 2014; Liao et al., 2016; Xu et al., 2020). Despite the advances of 2D cultures, these still lack the cellular diversity and spatial complexity seen in vivo. To overcome these limitations and similarly to the ones developed with other cell types, 3D scaffolds made from various materials, including hydrogels, polydimethylsiloxane, and nanofibers, have been developed. These structures provide a more accurate representation of the brain's architecture by allowing neurons to grow in all directions, mimicking in vivo neurite growth. Rigid scaffolds offer structural support and guidance for cell growth, while softer encapsulating matrices replicate also the extracellular matrix (Hejazian et al., 2012; Bhatia & Ingber, 2014; Bosi et al., 2015; Li et al., 2020a).

The first application of iPSCs in AD modelling was performed from familial AD patients carrying mutations in the presenilin-1 (A264E) and presenilin-2 (N141I) genes. This AD cell line cultures exhibited elevated levels of extracellular Aβ-42 which was significantly reduced with a γ-secretase inhibitor (Yagi et al. 2011). From that first study until today, research models using iPSCs to represent both sAD and fAD have been constantly growing. Studies of fAD classic mutations such as APP V717I, Swedish mutation or V717F have found elevated Aβ-42 ratios, higher total and phosphorylated tau, loss of synapses and endosome enlargement and dysfunction (Muratore et al., 2014; Kwak et al., 2020; Zhou et al., 2022). Regarding APP, there have been numerous studies using iPSCs from Down's syndrome patients. The extra copy of

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APP has been shown to cause increased intra- and extracellular location of Aβ-42, phosphorylated tau located on dendrites and somas as well as an overexpression of genes associated to Alzheimer's progression (i.e. BACE2, TMED10) (Shi et al., 2012; Chang et al., 2015; Penney et al., 2019). Studies exploring mutations of the TAU protein, have also shown deficiencies in the neurite outgrowth, differentiation potential and electrophysiological activity when using iPSCs from FTD patients. sAD representation of Alzheimer's in stem cell models either by the expression of mutations in the ApoE protein or directly reprogrammed from patients of this variant have also provided valuable insights for the pathological differences between both variants (Lin et al., 2019; Parra Bravo et al., 2024). Additionally, iPSC models have also shown usefulness in drug discovery as they conform a much more complex environment in which to further validate compounds with promising characteristics observed in other models. For example, γ-secretase and β-secretase inhibitors have managed to reduce the total levels of Aβ and modulate GSK3-β activity or non-steroidal anti-inflammatory drugs that have also proven efficacy against the naturally occurring pathological processes in fAD patient cells (Yahata et al., 2011; Israel et al., 2012; Barak et al., 2022).

Despite the groundbreaking improves of modelling with iPSCs, their employment still faces several challenges. Variability between cell lines requires large-scale replication across biological and experimental settings which is further exacerbated in the case of cell lines generated from fAD patients. Technical complexity is another major drawback as protocols for the generation of mature cortical tissues can take up to 100 days and require complex culturing protocols that make them a difficult biological base to scale down for screening assays. Finally, another major complication that in vitro models have not seemed to bypass just yet, is the biological "aging", in which complex neurodegeneration, cell spatial redistribution and risk factors that contribute not only to the onset but to the development are still major accomplishments that remain without suitable modelling in vitro (Arber et al., 2017; Arber et al., 2019; Barak et al., 2022). Three-dimensional organoid and spheroid iPSC-derived cultures are expected to further enhance the field of AD modelling in the following years. These newly developed models advance in the replication of cellular diversity, structural complexity, and physical architecture resembling closely those found in vivo. Organoids are self-organizing tissues that allow for spatio-temporal gene regulation, signalling events and even replicate features such as cerebrospinal fluid or ventricle formation and shrinkage. Additionally, organoids represent the present and future of AD drug discovery in vitro as they also allow for complex cell-cell interaction, a high-fidelity depiction of neuroinflammatory processes and the maturation of the tissue for up to 6-12 months with today's protocols, thus allowing for valuable assays that were unthinkable of in the past decades of pharmacological research (Arber et al., 2017; Koo et al., 2019; Qian et al., 2019; Shi et al., 2024).

3.5.3 Future model approaches

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Drug discovery for brain disorders, has been exceptionally challenging due to the brain's complex structure, the intrinsic difficulties of studying the diseases in human brains and the

limited experimental models available to fully represent the disease environment and pathology. In this context, in vitro cell models and artificial cell cultures offer a faster, more efficient means of understanding disease mechanisms, drug responses, and the overall pathophysiology of brain disorders. These models provide a valuable alternative but complementary to animal studies which help reduce the reliance on animal testing in the early stages of drug development (Zhao, 2023; Stresser et al., 2023).

Looking forward, future AD disease modelling must focus on refining cell culture conditions and protocols to overcome the challenges previously mentioned, while further excelling to replicate the brain's native architecture, cell variety and complex tissue interactions. Innovations in brain organoids and spheroids will clearly come in the form of standardized and simplified protocols that allow for modelling more complex immune and vascular systems, while keeping the tissue conditions intact for longer time spans (Barak et al., 2022; Shi et al., 2024). Regarding in vitro models, the progress in tissue engineering also provide valuable tools for more complete neuronal networks and cell encapsulation. Modelling with hydrogel biomaterials, nanofibrillar and peptide-based scaffolds more closely mimic the extracellular matrix as well as favouring accelerated maturation, culture stability and synaptic connectivity of neurons, enhancing traditional culturing techniques (Muratore, et al., 2014; Roth et al., 2021; Pereira et al., 2021). Microfluidic assays also provide a faster and more efficient alternative to monitor the aggregation and metabolic removal of proteins associated to AD in the present of chemical risk factors, as well as provide an interconnected and variable multi-cell sub-culture environment allowing for the study of characteristics such as BBB permeability and morphology changes or more "real" distribution on screening assays (Prasanna et al., 2021). Amongst the fabrication techniques for developing *in vitro* models, 3D printing has emerged as an innovative and valuable tool for producing scaffolds. With this technique cells and materials like hydrogels are printed together, enabling precise control over the deposition of both to recreate brain tissue architecture in almost one to one form to human counterparts (De la Vega et al., 2021; Benwood et al., 2023). Similarly, to the evolution of *in vitro* techniques, novel animal models are also oriented towards a more specific representation of both sporadic and familial AD features. New approaches, like combining multiple pathways or mimicking metabolic disorders linked to AD, offer promising directions for novel model development. Dual models like the innovative STZ-APP/PSEN1 model have been able to simulate overlapping characteristics of both forms of the disease, showing the main pathological hallmarks in a single *in vivo* model (Kelliny et al., 2021). Hypertension has also begun to be included in animal models as a form of further exacerbate cognitive decline and provide insights of the vascular contribution to the progression of this disease (Coatl-Cuaya et al., 2022). Additionally, other chemicals such as alloxan or AchE activators could also serve as valuable models to represent diabetogenic properties and the role of Acetylcholine as initiators of Alzheimer's pathology (Akhtar et al., 2022). Ultimately, AI has also proven particularly useful for AD modelling and pharmacological research. With this technology is possible to track and characterize in vitro models by how well mimic cerebral structures and identify potential

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repurposing of drug candidates by expressed mutations or targets in a much more efficient and less time-consuming way than ever before (Rodriguez et al., 2021; Qiu et al., 2024).

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MATERIALS AND METHODS

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4. MATERIALS AND METHODS

4.1 Cell line

Human immortalized neuroblastoma derived SH-SY5Y was the selected cell line for the development of this study. This line was originated as a thrice-clone of the SK-N-SH line, which was produced in the 70s from the biopsy of a 4 y.o. patient of neuroblastoma and presents two diverse phenotypes: neuroblast-like cells (N-type) and epithelial-like cells (S-type) that shares with the clone lines. SH-SY5Y N-type presents flattened, polygonal and small cell bodies with very short neurite-like processes (Biedler et al., 1973; Encinas et al., 2000; Lopez-Suarez et al., 2022). Commercial cell line was obtained from *Innoprot* (Bizkaia, Spain).

4.1.1 Cell line maintenance

Cell line culturing conditions were addressed following the supplier's recommendations, keeping the cells at a 37 °C with 5% CO_2 and saturating humidity. Cells were cultured on 150mm culture dishes (P150; *Corning*) using RPMI 1640 medium with GlutaMAX™ (*Gibco*) supplemented with 10% foetal bovine serum (FBS; *Sigma-Aldrich*) to provide necessary nutrients and growth factors for cell proliferation, and a combination of antibiotics composed of both 1% Penicillin-Streptomycin (*Milipore*) and 50 µg/mL Gentamicin (*Sigma-Aldrich*) to avoid potential bacterial contaminations.

4.1.2 Thawing of the cell line

Thawing of the cells was carried out by placing the frozen cryovials (-80°C) (*Thermo-Fisher*) at 37°C in a thermostatic bath during a 5-minute period. Subsequently, cells were transferred into a 15 mL Falcon® tube (*Corning*) and centrifuged at 1000 Rpm for 10 minutes. Supernatant was discarded, and the cell pellet resuspended in 5 mL culture medium and seeded in p150 cell plates.

4.1.3 Cell line expansion

At 70-80 % confluence, cells were passaged, transferring around 20% of the total cells to new culture dishes. This was performed to both maintain cell stock at low passage stages, preventing slow-progression and growth issues, and to prevent cell overgrowth into subsequent layers leading to culture death and phenotypic differences. Cell cultures were split three times per week by aspirating the old culture media and performing a wash with 10 mL Phosphate-buffered saline (PBS; 140 mM NaCl; 10 mM Na_2HPO_4; 2.7 mM KCl; 2 mM KH_2HPO_4). Right after the wash, cells were treated with 1.5 mL of 0.25% Trypsin (*Gibco*), to lyse the cell-cell and cell-plate adhesions, and left at 37 °C for 5 minutes. Plate was washed with 10 mL of fresh media to dilute the cell suspension and transfer 2 mL of this into each of the new p150 plates,

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previously filled with 18 mL of new media. Across all the experiments performed, cell line was always used and kept at a low passage number (<18) and recurrently tested for mycoplasma contaminations.

4.1.4 Cryopreservation of the cell line

To freeze cryogenically, the cell media was aspirated, and the culture was washed with 10 mL PBS. Subsequently 1.5 mL of trypsin were added, and cells were centrifuged for 10 minutes at 1000 Rpm. Supernatant was discarded, and pellet was resuspended in a solution of 10% (v/v) of dimethyl sulfoxide (DMSO) (*Sigma-Aldrich*) in non-dialyzed FBS. DMSO is added as a cryoprotectant, as it can prevent ice crystal formation in the membranes and promotes cell viability (Kaiser et al., 2021). Cell suspension is then transferred into cryotubes, and these placed in a Frosty® container (*Nalgene*). Cryotubes spent 24 hours in a -80 ºC freezer before being stored into liquid nitrogen containers.

4.1.5 Cellular seeding

High-content microscopy and intracellular calcium assays were performed using cultures in 96-well and 384-well plates (*Greiner*) respectively. Previously to cell seeding, plates were pretreated with laminin (*Sigma-Aldrich*) for 2 hours at 37 ºC. Laminin was prepared by dilution in sterilized water for a final concentration of 100 µg/mL, and further diluted in sterile PBS for a final concentration of 2 µg/mL.After the incubation with laminin, wells were rinsed with sterile PBS and left to dry at room temperature inside a laminar flow hood. Trypsinization and centrifugation of cell cultures was performed as described in the Cell expansion sub-section. Once the cell pellet was resuspended in 3 mL of fresh media, 0.5 mL were deposited into a plastic container (*Thermo-Fisher*, KA1938) suitable for the automated counter (Vi-Cell CR Cell, *Beckman-Coulter*) and analysed with an adjusted algorithm. Once concentration was determined, cells were subsequently diluted to achieve the desired final concentrations per well: 7500 cells in 96-well plates and 2500 cells in 384-well plates in 100 and 50 µL respectively.

4.1.6 Cell differentiation

Cell differentiation protocols were performed on either 96-well or 384-well plates depending on the assay requirements. Plates were pre-treated with 2µg/mL laminin (*Sigma-Aldrich*) to enhance cell adhesion. During the cell passage, a portion of the cells were separated, and their density was quantified using the Vi-CELL XR Cell Analyzer. Subsequently, cells were seeded on either 96w or 384w plates respectively following the previously introduced densities.

After the initial 24 hours, a change of media is performed to both start the differentiation and prevent further growth of the cells (Day 1). As differentiation of SH-SY5Y relies on gradual serum privation, the medium used in this step (Differentiation Medium 1) is the same as the previously described but with a 2% concentration of FBS (Shipley et al., 2016). Retinoic acid

(RA; *Sigma-Aldritch*) was also added to the medium for a final concentration of 10 µM. Cells were kept under this growing conditions for 6 days, performing a media change between day 3-4. RA has been extensively employed for SH-SY5Y as it manages to promote neurite growth, enhance cell survival and accentuate the dopaminergic features that the cell line shows even at undifferentiated states (Cheung et al., 2009; Lopes et al., 2017).

On day 7, culture medium is replaced once again for the differentiation medium 2 (No FBS, supplemented with B27 serum (1:50)) (*Gibco*), for enhanced cell survival and neurite outgrowth in the absence of serum. Depending on the protocol and secondary differentiation compound, either Glucagon-like Peptide 1 (GLP-1; *Sigma-Aldrich*) [100 nM] or Phorbol 12-Myristate 13-Acetate (PMA; *Sigma-Aldrich*) [10 nM] were added as supplements. Cultures treated with GLP-1 were maintained until day 10, whilst PMA-differentiated cells were kept until day 12 which marked the end for both differentiation protocols. GLP-1 is an incretin hormone secreted in the intestines and primarily recognized as an insulin secretor promotor. However, in recent years, many studies have shown the potential neurotrophic function of this compound, promoting neuronal survival and neurite growth during memory-related processes (Erbil et al., 2019; Grieco et al., 2019). Treatment with GLP-1 yields SH-SY5Y cultures with a mature glutamatergic phenotype via the PI3K-AKT axis (Yang et al., 2020). PMA is one of the first compounds used to induce SH-SY5Y differentiation, it promotes cell growth and drives in combination of RA a differentiation process with a pure dopaminergic phenotype with adrenergic features (Xie & Hu, 2010). Key data regarding both differentiation protocols is summarized in *Table 1*.

Protocol	Duration	Plate treatment	[] final	Phenotype
RA + PMA	10 days	30 µg/mL poli-D-lysine	·10 µM RA ·10 nM PMA	Dopaminergic & adrenergic
RA + GLP-1	12 days	2µg/mL laminin	10 µM RA 100 nM GLP-1	Dopaminergic & glutamatergic

Table 1. SH-SY5Y differentiation protocols conditions tested in this study.

All the compounds mentioned in this section were stocked by diluting them on DMSO and kept at -20 °C following the manufacturer's guidelines. Laminin was also kept at -20 °C and diluted using sterilized Elix® purified water. Both protocols were adjusted and modified from the ones described by Presgraves & colleagues. and Yang & colleagues (Presgraves et al., 2004; Yang et al., 2020).

4.2 Site-directed mutagenesis

Site-directed mutagenesis by polymerase chain reaction (PCR) is a widely used method for introducing precise mutations into a specific DNA sequence. This technique uses PCR to

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amplify DNA fragments containing the desired mutations. Oligonucleotide primers, designed with the intended mutation, guide the replication process, allowing for targeted sequence changes within a single- or double-stranded DNA plasmid containing the interest sequence. The resulting mutated fragment can be further expanded and purified for its use in transfection assays. An overview of the steps involved in the technique can be consulted in *Figure 10*. This approach provides a reliable and efficient means of studying gene function, protein interactions, and other molecular mechanisms in biological systems, and has been applied for Alzheimer's models (Zheng et al., 2004; Dinoto et al., 2005; Liu & Naismith, 2008; Park et al., 2019).

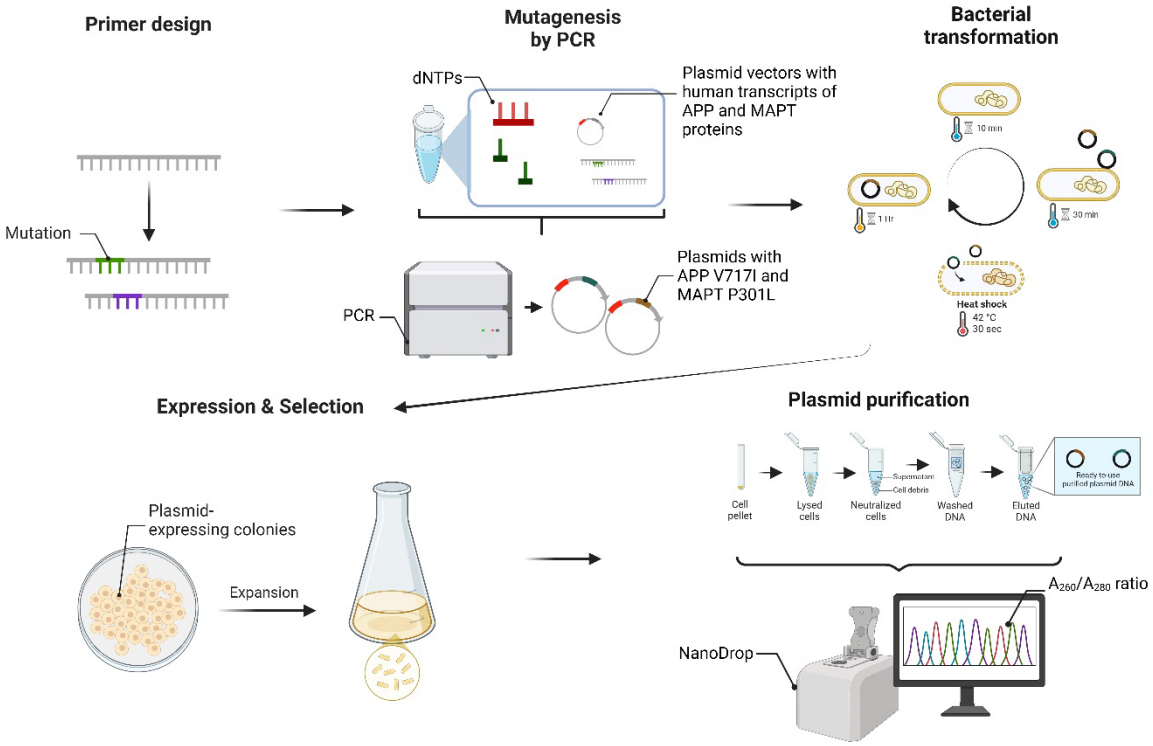


Figure 10. PCR mutagenesis construction of APP V717I and MAPT P301L expression plasmids. Graphical representation of the multi-step process followed for the creation of two expression pcDNA3.1^{+} plasmids containing the desired fAD mutations. Created in BioRender. Barro, M. (2024)

4.2.1 Plasmid and primer design

Amyloid Precursor Protein (APP) and Microtubule Associated Protein Tau (MAPT) Plasmids (pcDNA3.1^{+} backbone) were purchased from *GenScript* (Oxford, UK) containing human transcript variant 1 and 2 of each gene and a length of 2256 and 1326 base pairs respectively. These variants were selected taking in consideration the sequence of amino acids and their positions, in order to be able to produce the APP V717 and MAPT P301L mutations. Primers employed over the mutagenesis assay were designed with the software tool OLIGOARCHITECT® by *Sigma-Aldrich*. For each of the plasmids, a pair of primers was constructed containing the required sequence to induce the mutations APP V717I and MAPT

P301L on each of the transcript's variants. Plasmids were obtained through *Sigma-Aldrich*, pre-purified by polyacrylamide gel electrophoresis (PAGE) methodology and received in dry powder. Upon receival, primers were diluted in distilled water to a final concentration of 100 ng/µL. Primer sequences used for APP V717I were: Forward (F) 5' TCATAGCGACAGTGATCATCATCACCTTGGTGATG 3'; Reverse (R) 5'CATCACCAAGGTGATGATGATCACTGTCGCTATGA 3', and the ones designed for MAPT P301L were: F 5' TATCAAACACGTCCTGGGAGGCGGCAGTG 3'; R 5' CACTGCCGCCTCCCAGGACGTGTTTGATA 3'. These were designed to meet the required criteria of having a melting temperature of ≥ 78°C and a length between 25-45 bases.

4.2.2 Mutagenesis by PCR

In vitro site-directed mutagenesis was performed using the QuickChange II XL Site-Directed Mutagenesis Kit (*Agilent*) following manufacturer's instructions both for PCR cycling conditions and DNA template, restriction enzyme (Dpn1) and *PfuUltra* DNA polymerase concentrations.

For each of the individual preparations, to introduce the mutations in the DNA plasmid backbone, 5 µl of 10x reaction buffer, 10 ng of DNA plasmid template, 125 ng of oligonucleotide primer 1 (forward) and oligonucleotide primer 2 (reverse), 1 µl of dinucleotide triphosphate (dNTP) mix, 3 µl of QuickSolution® and distilled water to reach a final volume of 50 µL were added to a 0.1 mL *Eppendorf* tube. After properly mixing the contents, 1 µl of *PfuUltra* high-fidelity DNA polymerase (2.5 U/µl) was added. Each reaction was then cycled in a thermal cycler following the conditions outlined in *Table 2*.

Segment	Nº of cycles	Temperature	Time
1	1	95ºC	1 minute
2	18	95ºC 60ºC 68ºC	50 s 50 s 1 minute/kb of plasmid length
3	1	68ºC	7 minutes

Table 2. Thermocycling conditions set for the mutagenesis assays. APP plasmid 2256 bp ≈ 3-minute at 68ºC per cycle. MAPT plasmid 1326 bp ≈ 2-minute at 68ºC per cycle.

Right after thermocycling process, tubes were placed in ice for two minutes to drop the reactions below 37 ºC. Following this ice incubation, 1 µl of Dpn I restriction enzyme (10 U/µl) was added to each of the samples. Tubes were immediately centrifuged for 1 minute at 5000 Rpm and incubated at 37ºC for 1 hour to digest the parental supercoiled double-stranded DNA (non-mutated). Each of the PCR reactions was stored at -20 °C and sent for sequencing through *Secugen* (Madrid, Spain) to verify that the mutations and correct positioning were performed in a satisfactory manner.

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4.2.3 Agar plate preparation

Agar plates were made in batches of 10 units (200 ml LB agar) to avoid waste as they can only be stored for a month at 4ºC while keeping their properties. Preparation started by weighing and adding 2 g of NaCl, 2 g of tryptone, 1 g of yeast extract, and 3 g of agar into a beaker with distilled water. Distilled water was added to reach a total volume of 200 ml, and the mixture was stirred with a magnetic bead until all components were fully dissolved. The final volume was measured with a graduated cylinder and adjusted as needed before the solution was poured into an autoclave-safe bottle. The bottle was autoclaved at 120°C to ensure sterility. After autoclaving, the bottle was allowed to cool until the temperature of the solution reached approximately 45-55°C. In a sterile area, 400 µl of ampicillin (*Thermo-fisher*) was added to achieve a final concentration of 100 µg/ml. Approximately 20 ml of the LB agar solution was poured into each Petri dish (*Corning*), taking care to avoid bubbles. Plates were left at room temperature inside a laminar flow hood for around 20 minutes to solidify and then inverted to prevent condensation on the agar surface. Finally, the plates were sealed with parafilm, placed in a plastic bag, and stored at 4°C.

4.2.4 Bacterial transformation

For bacterial transformation, the kit-provided XL10-Gold ultracompetent cells (*Agilent*) were employed. These exhibit high transformation efficacy for large DNA constructs while maintaining the fast growth and big colonies. Firstly, aliquots of XL10-Gold cells were thawed on ice for 10 minutes to ensure maximum cell survival. For the transformation of each reaction, 25 µl of the ultracompetent cells were aliquoted into a 15 ml Falcon tube (*Corning*), and 1 µl of the β-mercaptoethanol mix provided in the kit was added. The contents of the tubes were mixed and incubated on ice for 10 minutes, with mixing every 2 minutes. β-mercaptoethanol is used in bacterial transformation for its ability to increase transformation efficacy as it increases the membrane permeability while neutralizing the oxidative stress during this process (Gonzalez et al., 2007; Tu et al., 2018). Consecutively, 2 µl of Dpn-I treated DNA from each sample were transferred to the aliquoted cells, mixed thoroughly and incubated on ice for 30 minutes. Meanwhile, filter-sterilized NZY+ broth (10 g/L casein hydrolysate; 5 g/L yeast extract; 5 g/L NaCl [pH 7.5]; 10 mM $MgCl_2$ & $MgSO_4$ and 20 mM glucose) was preheated in a water bath at 42ºC, and the tubes were placed on a 42ºC heat block for 30 seconds, followed immediately by ice incubation for 2 minutes. Next, 0.5 ml of preheated NZY+ medium was added to each tube and incubated at 37ºC for 1 hour with shaking at 225-250 Rpm. Finally, 50 µl of each transformation reaction was plated onto agar plates containing the appropriate antibiotic (ampicillin) for the plasmid vector set resistance.

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4.2.5 Bacterial expansion and plasmid purification

A colony of XL10-GOLD cells was picked from a transformed agar plate (after overnight incubation at 37 ºC) and added to a volumetric flask with 1 litre of sterilized LB broth. This culture was shaken at 37°C and 300 Rpm for 12-16 hours. The sample was then centrifuged in a a conical Falcon® tube (*Corning*) at 4500 g for over 10 minutes at 4°C, and the supernatant was discarded. At this point, pellet was either ultra frozen at -80 ºC or used for extraction and purification. The plasmid extraction and purification were performed using the NucleoBond Xtra Midi kit for transfection-grade plasmid DNA (*Macherey-Nagel*), following the protocol and materials provided by the manufacturer.

To perform a plasmid DNA purification, the cell pellet was first completely resuspended in 8 mL resuspension buffer (RES) with RNase A, to degrade any RNA leftovers, by pipetting up and down, ensuring that no cell clumps remained for efficient lysis. In the next step, 8 mL of lysis buffer (LYS) were added to the cell suspension, and gentle mixing by inverting and incubated at room temperature for 5 minutes, avoiding prolonged exposure to alkaline conditions to preserve plasmid DNA integrity. LYS buffer uses a combination of sodium hydroxide and sodium dodecyl sulphate (SDS) to degrade cell membranes. During lysis incubation, the NucleoBond Xtra column was equilibrated by applying Equilibration Buffer (EQU) along the rim of the column filter ensuring the entire filter was saturated in order to avoid potential residual molecules out of the filter. Once the lysis has been completed neutralization buffer (NEU) was added to the lysate, which was immediately mixed by inverting the tube until the blue colour of LYS buffer had completely disappeared. NEU contains potassium acetate, which makes SDS to precipitate, pulling down the proteins, chromosomal DNA and cell debris. Contents of the tube were transferred into the column and allow to pass it by gravity without clogging. The column was discarded afterward to improve washing efficiency, and the column was washed with wash buffer (WASH) for increased purity. The plasmid DNA was then eluted with elution buffer, preheated to 50 ºC, (ELU) into a centrifuge 15 mL Falcon® tube, used to improve yields for larger DNA constructs. ELU buffer changes the filter conditions to an alkaline pH, which produces a neutralization of the resin and allowing the plasmid DNA to be released from it. For DNA precipitation, room-temperature isopropanol was added to the eluate, mixed thoroughly, and centrifuged at 12000 x g for 1 hour at 4°C, and the supernatant was carefully discarded. The pellet was washed with 70% ethanol at room temperature, centrifuged for 5 minutes at 5000 Rpm, and the ethanol was carefully removed, allowing the pellet to air-dry. Once dry, pellet was reconstituted and diluted using 100 µL of sterilized water. The performance of purification was assessed using a NanoDrop spectrophotometer (*Thermo-Fisher*), considering values of A_{260}/A_{280} ratio over 1.8.

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4.3 Cell chemical transfection

Chemical transfection was performed on day 7 of the differentiation protocol, just before switching to the GLP-1 differentiation factor in the cell cultures. Lipofectamine LTX™ (*Thermo-Fisher*) was employed as the transfection agent for this protocol. This reactive was chosen for its low cytotoxicity and while offering a high transfection rate and simple protocols for sensitive cell lines and primary neuronal cultures (Ishiyama et al., 2010; Rust et al., 2015).

Transfection was performed by mixing 2.5 µg of each protein's expression plasmid, 125 µL of OPTI-MEM (*Gibco*) and 2.5 µL of Plus Reagent® in an Eppendorf tube® (*PortoMedica*) [1] and left for a 5-minute incubation at room temperature. Meanwhile, 5 µL of Lipofectamine LTX and 125 µL of OPTI-MEM were mixed in a separate tube [2], and after the incubation, contents of the Eppendorf tube 1 were transferred to the second and allowed for a 20-minute incubation at RT. This was performed in such way to avoid Lipofectamine LTX™ liposomes loss due to adhesion to the Eppendorf tube® walls. After this period, sample was transferred to a 15 mL Falcon tube and mixed with 2.5 mL of OPTI-MEM medium. In the case of double transfection assays employing both plasmids, volumes used were also doubled, excluding only the OPTI-MEM. Differentiation medium in the culture plate wells was removed and substituted for either 20 (384w) or 50 µL (96w) of the transfection mixture. After 5-6 hours, transfection media was replaced for the differentiation medium containing GLP-1 as previously described in section 4.1.6. In experiments that used the Aβ-42 synthetic peptide (*Bachem*) as a control to assess efficacy of APP transfection and biological changes upon expression, 10 µM concentration of this peptide diluted in DMSO was added to GLP-1 differentiation media.

Untransfected cell wells were used as normal controls across all experimental part. For each experiment condition, at least 24 biological replicates were performed across multiple assays. When given experiments required cells to be treated with compounds, these were included in the differentiation media after transfection.

4.3.1 Neuroprotective compound-based assays

To validate the proposed model as a valid biological platform for drug discovery assays, the following compounds, known for their neuroprotective effects against various morpho-physiological changes associated with Alzheimer's disease, were added to the differentiation medium with GLP-1 (day 7; post-transfection): melatonin (1 µM, *Sigma-Aldrich*) and resveratrol (1 µM, *Abcam*). Compounds were allowed for at least 72-hour incubation with the transfected cultures. Melatonin, a hormone mainly secreted by the pineal gland, is a well characterized antioxidant and neuroprotective compound against ROS, RNS and neuroinflammatory processes (Cardinali et al., 2014). Moreover, melatonin has also shown inhibition of amyloidosis progression and tau hyperphosphorylation in a wide variety of *in vivo* and *in vitro* models (Pappolla et al., 1998; Poeggeler et al., 2001; Montilla et al., 2003; Li et al., 2005). These pathogenic processes ultimately lead to neuronal death, neurite degeneration and ion dysregulation, events at which melatonin has also shown efficacy at reverting (Matsubara

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et al., 2003; Chen et al., 2020; Leung et al., 2020; Li et al., 2020b; Wang et al., 2020). Resveratrol is another extensively tested compound in neurodegenerative diseases for its antioxidant properties but also for its anti-inflammatory activity and anti-beta aggregation (Granzotto & Zatta, 2011; Rahman et al., 2020). In vitro, resveratrol has shown efficacy at preventing intracellular calcium elevation, reduce cell death and hyperphosphorylation as well as prevent axonal damage and promote neurite outgrowth (Han et al., 2004; Dasgupta & Milbrandt, 2007; Ban et al., 2008; Shindler et al., 2010; Loureiro et al., 2017).

4.4 High-content microscopy assays

High-content microscopy is an advanced approach use for cell morphology analysis through a multi-channel fluorescence automated process. Use of this technique allows for sophisticated algorithm-based analysis of hundreds of morphological variables and phenotypic assessment in cell 96- 384 cell well plates, thus allowing for a faster experiment with more biological replicates. All these benefits with respect to more traditional microscopy approaches have set high-content microscopy as a pilar for *in vitro* modelling and pharmacological discovery pipeline (Way et al., 2023). Cell cultures in this section were imaged using an Operetta CLS™ high-content analysis microscope (*Perkin-Elmer*). These were obtained by registering 9 fields per well using a 20x objective and the analysis software Harmony® 4.3 (*Perkin-Elmer*).

4.4.1 Pathologic morphological variables

Cell differentiation into a mature neuronal phenotype and the morphological changes arising from the transfection and expression of fAD mutated proteins were assessed by measuring three different variables. These were: Number of cell bodies per well (registering this way cell death) and the neurodegenerative processes of neurite shortening and loss of dendritic arborization as a direct consequence of Tau and Aβ accumulation. Arborization was presented as the number of cells presenting ≥ 2 processes emerging from their soma. Experimentation for the study of these variables required from fluorescent compounds that stained the cellular regions of interest. To identify neurites, cultures were labelled with a 1:500 dilution of anti-β-tubulin class III antibody conjugated to Alexa Fluor® 488 (*BD Pharmigen*), while cell somas were stained using 1:2000 dilution of nuclear and cytoplasm marker DRAQ5® (*Abcam*).

4.4.2 Neurite morphology and cell survival quantification

For cell imaging, upon day 10 of the differentiation stage, cells in culture plates (96w) were fixed by removing the culture media and adding half the volume of 4% paraformaldehyde (PFA; *Santa Cruz Biotechnologies*) diluted in an equal volume of Hank's buffer saline solution

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(HBSS; *Thermo-Fisher*) for a final concentration of 2% PFA in each of the wells and incubated for 20 minutes at 4 °C. Paraformaldehyde is used in cell fixation as it causes cell death while maintaining the cell morphology and keeping them immobile (Kim et al., 2017). Next, PFA solution was removed and two 10-minute washes with HBSS were performed at RT. Cells undergone a third and final wash for 30 minutes at room temperature, with a solution that consisted of HBSS, 5% of Bovine Serum Albumin (BSA; *Roche*) and 0.1 % Triton® X-100 (*Sigma-Aldrich*). BSA is used to prevent the non-specific binding of anti-bodies by occupying the remaining spaces on a solid surface after a capture biomolecule has been immobilized, thus reducing unintended interactions (Ahirwar et al., 2015). Triton® X-100 is employed in blocking solute for its cell permeability properties as a detergent, thus favouring tint penetration (Jamur & Oliver, 2010). After the final incubation, cells were treated with a dilution of 1:2000 of nuclear marker Draq-5 (Abcam) and a dilution of 1:500 of the antibody Alexa Fluor® 488 anti-β-tubulin Class III (BD Pharmigen) in blocking solution over a 1h timespan while keeping the plate from light sources. Assessment of both fluorophores was performed with the Operetta CLS™ at a wavelength excitation of 490 nm for Alexa Fluor® 488 and 547 nm for DRAQ5®. Images were taken using a 20x objective, analysing 9 fields per well through at least three independent assays with 6 biological replicates each. Harmony®4.3 software was employed for the quantification of neurite length and cell nucleus by adjusting the pre-existing algorithms for this matter. Quantification of arborization was performed by developing an algorithm to detect those cell bodies with 2 or more intersecting neurites.

4.5 Intracellular calcium assays

Intracellular calcium measures are a key component for model development as it allows for the study of many biological processes and the screening of compounds that modulate these physiological pathways. This technique requires from a fluorophore attached to a calcium ion chelator (Ca^{2+}), which will excite at various wavelengths and exhibiting dynamic ranges, thus allowing for the quantification of absolute or relative calcium concentrations (Wu et al., 2019). This study was carried out using the FLIPR® fluorescence assay kit containing the Calcium- 6® fluorophore (*Molecular devices*). An overview of this physiological process can be observed in *Figure 11*.

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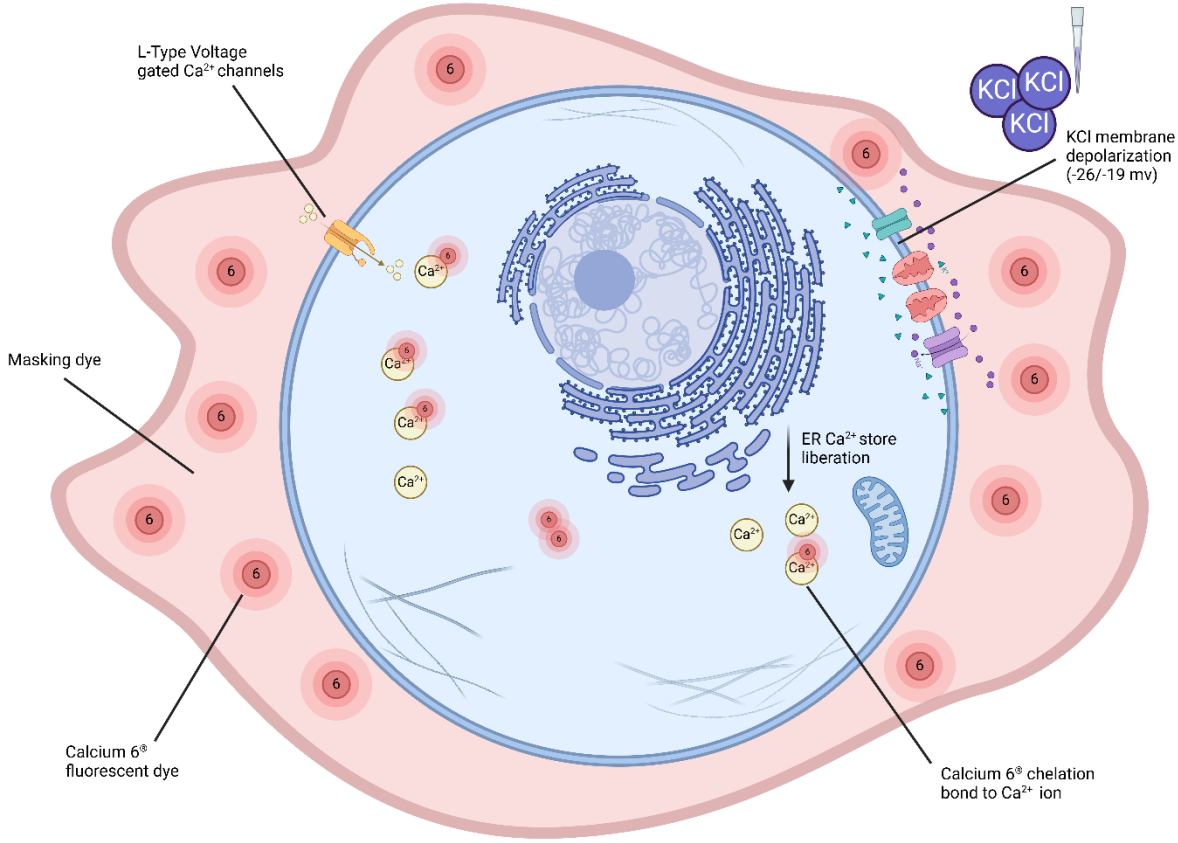


Figure 11. KCl excitation effects on intracellular calcium mobilization. Graphical summary of the physiological pathways that follow 30mM KCl administration. Neuronal membrane potential is sensitive to extracellular potassium levels; increasing potassium depolarizes the neuron by reducing the potassium gradient, raising the equilibrium potential. This depolarization allows calcium influx through channels like NMDA receptors, AMPA receptors, VSCCs, and ER calcium stores. Created in BioRender. Barro, M. (2024)

4.5.1 Exogenous stimulation of intracellular calcium mobilization

Intracellular calcium assays were performed on black 384-well plates with transparent flat bottom, seeding 2500 cells per well. The culture, differentiation, and transfection method used was identical to that used in previous assays. Incubation with fluorescence probe was performed on the last day of the differentiation protocol (day 10). Previously to plate analysis, media was removed and replaced with 25 µL of fresh media without any additives and 25 µL of the Calcium- 6® probe reconstituted in 10 mL of the assay buffer provided with the kit (20 mM HEPES buffer+ 1x Hank's Balanced Salt solution (HBSS), pH 7.4). This was left for a two-hour incubation at 37 ºC in light privation conditions. To induce cellular excitation, KCl was diluted at 330 mM in a buffer composed of HBSS and Hepes (20 µM; *Sigma-Aldrich*) and added (25 µL per well) to an intermediate compound plate. KCl was added to cell cultures by an automated process using the kinetic plate imager FDSS 7000EX® (*Hamamatsu Photonics*, Barcelona, Spain) for a final concentration in each well of 30 mM. Increasing the concentration of extracellular potassium chloride is a common method for inducing membrane depolarization in cultured neurons which provides valuable insights into the mechanisms of calcium entry and

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release from intracellular compartments as well as other cellular responses to depolarization (Rienecker et al., 2020). Fluorescence values were measured with the FDSS 7000EX®, using the provider's proprietary software FDSS v3.4 with excitation parameters of 470-495 nm and emission parameters of 515-575 nm.

Obtained data was processed using the software FDSS7000EX/μcell® (*Hamamatsu Photonics*) in relative fluorescence units (rfu) and the ratio taking in consideration the maximum fluorescence values minus the basal signal values (Max-Min) recorded before the addition of KCl and normalized to a baseline.

4.5.2 Prestwick® chemical library screening

Drug discovery assays were performed using the high-throughput screening approach to both find potential molecules that could be used on AD research and to further validate the biological model as a solid base in which to run larger screening campaigns. Compound screening assays were performed on 4 384-well plates cell cultures, assessing the significative changes in calcium mobilization assays and validating the hits through a secondary replication assay. For the testing, 1120 compounds conforming the Prestwick® Chemical library were dissolved in DMSO at a concentration of 10 mM. On day 8 of differentiation, culture media was replaced, and in between changes, 5 nL of each of the compounds were dispensed per well using an Echo 525 liquid handler (*Labcyte inc*., Staffordshire, UK) for a final concentration of 1 μM. Molecules were allowed in the culture for a minimum of 72 hours across all different experiments. Results were obtained performing and following the protocol presented in *section 4.5.1*.

4.6 Other laboratory reagents

In addition to the previously mentioned materials, buffer preparation was performed using the salts KH_2PO_4 and KCl obtained from *Panreac* as well as Na_2HPO_4 and NaCL purchased through *Sigma-Aldrich*. Likewise, reagents for the making of agar plates, tryptone, yeast extract and agar were supplied by *VWR chemicals*, *Affymetrix* and *Sigma-Aldrich* respectively. NZY+ broth's casein hydrolysate, $MgCl_2$, $MgSO_4$ and glucose were obtained through and *Panreac*. Isopropanol used in the final steps of plasmid purification was provided by *Galmedic* while ethanol was purchased from *VWR chemicals*. GLP-1 and RA were aliquoted in DMSO at 1 mM and 5 mM concentrations respectively for assays but stored as dry powder at -20 ºC. Weighing of these reagents was performed on a nitrogen-filled atmosphere to prevent oxidation and moisture absorption. All non-specified aliquots were performed using sterilized water using an Elix® purification filter by *Milipore*.

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4.7 Data analysis

Data analysis is presented as the mean ± standard deviation with a dot cloud for all the morphological variables assessed and the comparative analysis of intracellular calcium between different cellular conditions. Analysis was performed on raw data by using the Graphpad Prism® version 10 for *Windows* software (*Graphpad Software*, CA, USA). Comparative data analysis was performed with the student's *t*-test, setting statistical significance at $\alpha > 0{,}05$.

The response value percentage exhibited by the compounds in the Prestwick® chemical library was calculated using ActivityBase version 9.5 for *Windows* (*IDBS*, Woking, UK) by applying the following equation:

$$\%\ Response\ = \frac{X - C_{min}}{C_{max} - C_{min}}\ x\ 100$$

In which X represents the rfu (Max-Min) value for a given tested compound. C_{Max} represents the average value of rfu (Max-Min) for the control differentiated cells and C_{min} does so for the average value of rfu (Max-Min) for the control transfected cells.

The fitting of dose-response correlation to a sigmoidal curve, a non-linear regression fit was used using the three-parameter log(agonist) vs. response equation, assuming a slope factor of 1.0. Equation for the fit is represented as:

$$Y\ =\ Z_{min} + \frac{Z_{max} - Z_{min}}{1 + 10^{logEC_{50} - x}}$$

In which Z_{max} and Z_{min} represent the plateaus in the Y axis values. The EC_{50} represents the concentration of agonist that produces a response halfway between the maximum and minimum response levels.

Data variability of the positive and negative controls was calculated using the coefficient of variation (CV%). The acceptance criteria require that the coefficient of variation (CV) for each signal is less than or equal to 20% (Iversen et al., 2012). Equation employed was:

$$CV\% = \frac{\sigma_c}{\mu_c}\ x\ 100$$

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In which μ represents the mean for either positive and negative controls and σ the standard deviation.

To determine the suitability of the assay window for measuring excitability through intracellular calcium concentration, the Z' factor and signal window were used. The calculation of these factors was performed using the following equations (Zhang et al., 1999; Iversen et al., 2012):

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

$$SW = \frac{(\mu_{c+} - 3\sigma_{c+}) - (\mu_{c-} + 3\sigma_{c-})}{\sigma_{c+}}$$

In which μ represents the mean for positive and negative controls and σ the standard deviation. Z′ represents the theoretical gap between positive and negative controls, setting a value of 1 to the ideal assay. Assays scoring between 0 and 0.5 are considered to have limited utility, while those with a Z-prime greater than 0.5 are deemed highly effective (McClure et al., 2019). Signal window values are considered acceptable from values ≥ 2 in every single plate (Iversen et al., 2012).

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RESULTS

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5. RESULTS

5.1 SH-SY5Y cell line differentiation yielded cell cultures with mature neuron-like cell morphology

SH-SY5Y cell line is commonly used in the neurobiology field for the design of functional neuronal models to study neurodegenerative diseases (Lopez-Suarez et al., 2022). But the raw cell line lacks the neuronal phenotype both morphologically and physiologically as we present in this study. Thus, making the differentiation process a critical and necessary step towards obtaining a reproduceable and viable neuron-like cultures that proportionate an accurate environment for subsequent morphological and biochemical assays. Two differentiation protocols were adapted and optimized to yield neuron-like cultures that exhibited morphological characteristics of this cell types. Neuron-like characteristics were considered taking into account the density of cell cultures, the neurite arborization and the cells that contain a projection lengthier than 30 μm.

5.1.1 Qualitative and quantitative analysis of mature neuron phenotypic characteristics of differentiated cultures

Undifferentiated SH-SY5Y cells show a flat epithelial-like phenotype with short and truncated neurites whilst when employing both differentiation methods, cells drastically changed their morphology into neuron-like shaped cells (Figure 12. A-C). In the present study, the cell line was exposed to two different combinations of differentiation-inducing factors: 10 μM RA + 10 nM PMA; 10 μM RA + 100 nM GLP-1. Upon exposure of the cells to both differentiation protocols, cells exhibited a greater number of neurites longer than 30μm and arborization in comparison to those undifferentiated (Figure 13.B-C) ($p<0.0001$). Regarding the average number of cells per well, cultures exposed to RA and GLP-1 showed the highest density, while those exposed to RA and PMA showed a lower average number of cells compared to undifferentiated cells ($p<0.001$) ($p<0.0001$) (Figure 13.A). The medium supplemented with RA and GLP-1 resulted in cultures with the highest arborization, cell density and number of cells with neurites longer than 30μm comparison to both undifferentiated and PMA treated cultures ($p<0.0001$) (Figure 13.D-F). In both differentiation protocols, a change into neuron-like cells was registered in comparison to those cultures with undifferentiated SH-SY5Y cells (Figure 12.A-C; Figure 13.A-C). Obtained statistical data complemented by the homogeneity of the cultures exposed to GLP-1 compared to the clumpy masses registered upon the usage of PMA, made the combination of RA and GLP-1, the most adequate and the selected protocol in which to develop the *in vitro* model (Figure 12.A-B; Figure 13.A-C).

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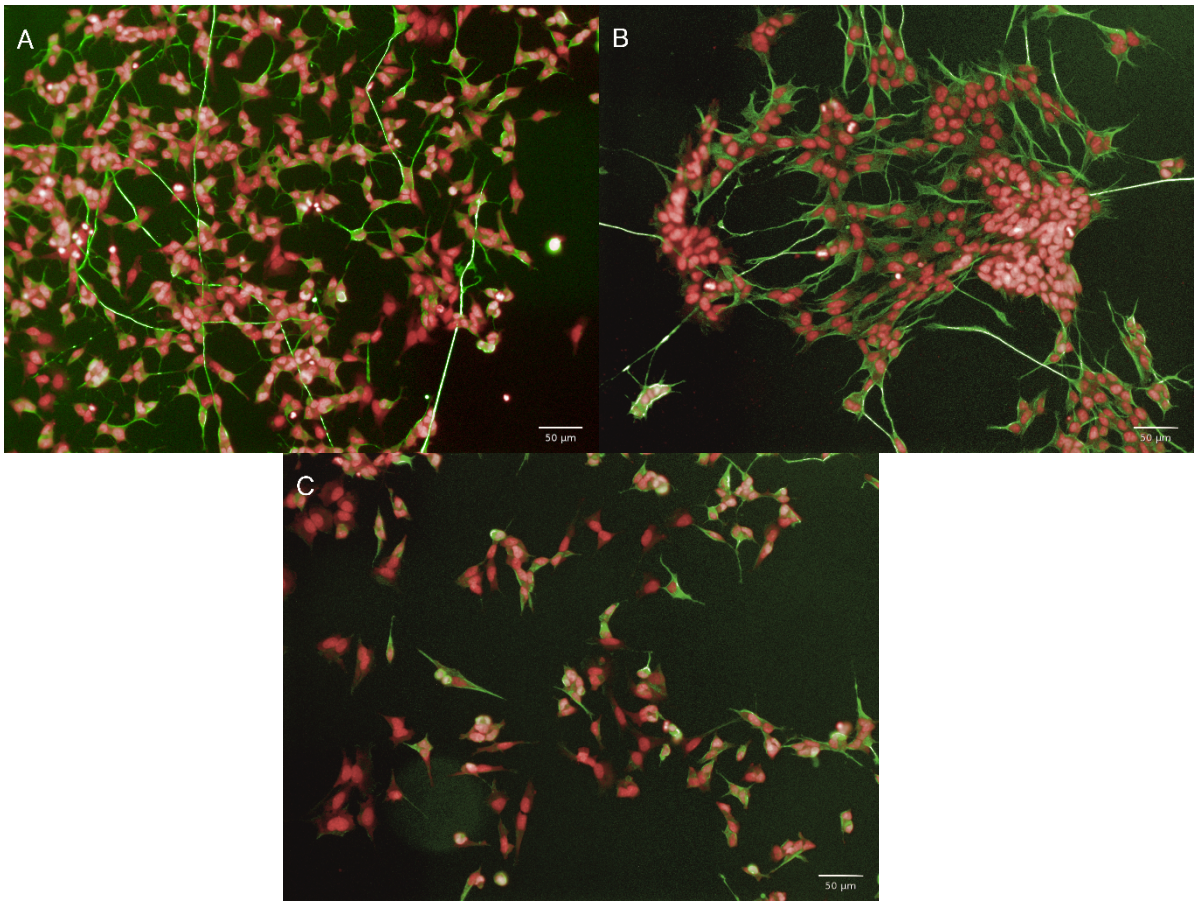
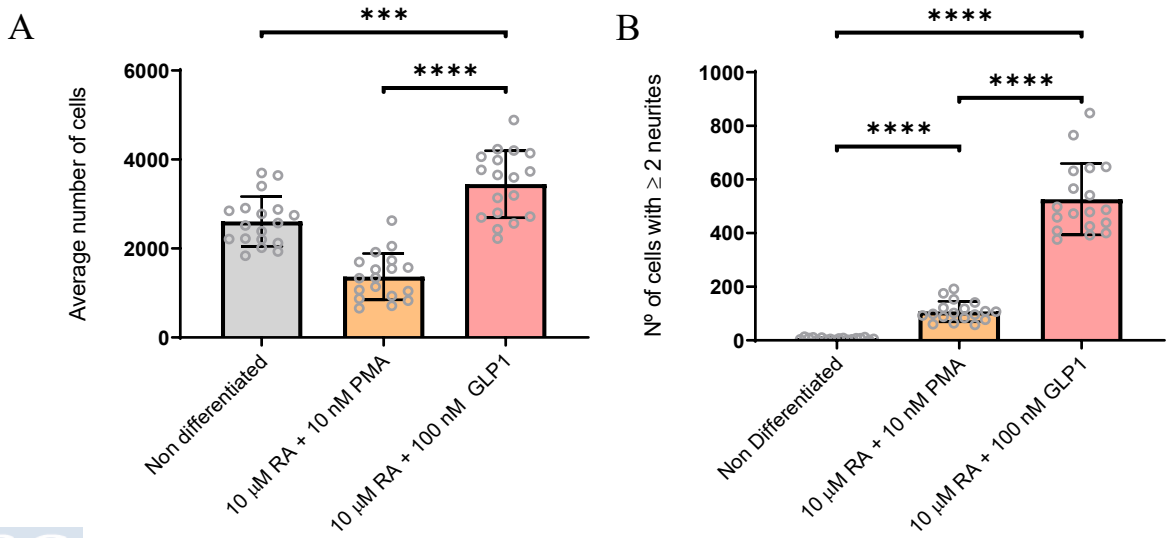


Figure 12. SH-SY5Y cell line morphology with different culturing treatments. Microphotographs representative of the neuroblastoma-derived cell line SH-SY5Y, subjected to different differentiation protocols. A) 10 µM retinoic acid and 100 nM GLP-1; B) 10 µM retinoic acid and 10 nM PMA; C) Control (Untreated). Red: DRAQ5®; Green: Anti-β Tubulin Class III Alexa 488®. Scale bar = 50 µm.



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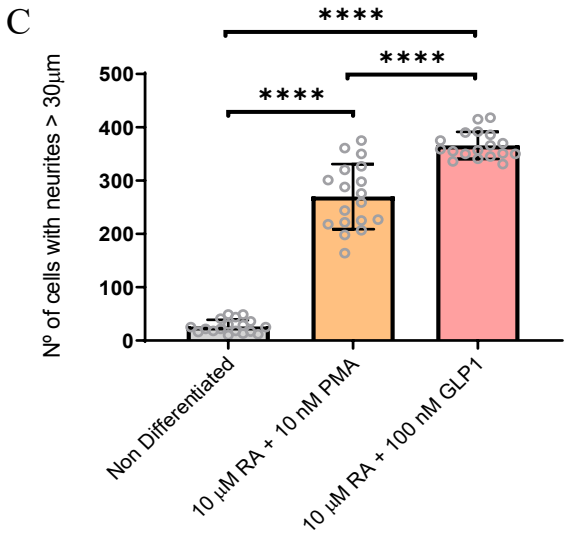


Figure 13. Statistical graph representation of different variables assessed to study the differentiation process of SH-SY5Y line. A) Average number of alive cells per well; B) Dendrite arborization assessed as number of cells with 2 or more neurites; C) Number of cells with neurites > 30 µm. (■) Undifferentiated; (■) RA + PMA; (■) RA + GLP-1. *** $p < 0.001$; **** $p < 0.0001$ (Student t test).

5.2 SH-SY5Y cell line transfection with mutations in APP and MAPT proteins produced significant neurodegenerative-like morphological changes.

Amyloid and Tau proteins misfolding and deposits are both main pathophysiological hallmarks in AD, and, as previously indicated, drivers of many other disease-involved molecular and biological pathways (See introduction for details). Amyloid precursor protein V717I mutation is involved with the favouring the amyloidogenic processing pathway, resulting in increased levels of Aβ42, the most insoluble isoform and related to most of the molecular events leading to neurodegeneration in the disease. Aβ deposits are involved in neuronal death, synapse and dendritic spines loss as well as axonal damage (Wei et al., 2009; Marin et al., 2016). MAPT P301L gene mutation is associated with the generation of Tau proteins that are more prone to be phosphorylated in Serine 262, resulting in higher polymerization and aggregation rates (Wang et al., 2023). Misfolded and aberrant phosphorylation of tau in the axon and soma, contributes to cell death and projections loss (Liu et al., 2020b; Wang et al., 2023). This peptide isoform was selected for being the most common present in AD brains, as well as being one of the most common mechanisms employed to produce amyloid pathologies on *in vitro* models (Fontana et al., 2020). Taking in consideration these effects on neurodegeneration, morphological changes directly derived from the expression of the impaired tau and amyloid proteins occurring in the cells 72 hours post-transfection in the form of neurite damage, loss and neuronal death.

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5.2.1 MAPT P301L mutation derived into cell cultures with affected cellular survival and neurite properties.

Upon transfection with mutation MAPT P301L, cell cultures shown morphological changes resemblant of a neurodegenerative-like process. Transfected cell cultures (Figure 14.B) showed a significantly lower number of cells compared to those differentiated, used as controls ($p<0.0001$) (Figure 14.A). Additionally, cells transfected with the mutation also exhibited a significantly impact on the cytoskeleton of the SH-SY5Y cell line, registering a significant reduction in the neurite arborization and the average neurite length ($p<0.01$; $p<0.05$) (Figure 14.A-B; 15. A-C).

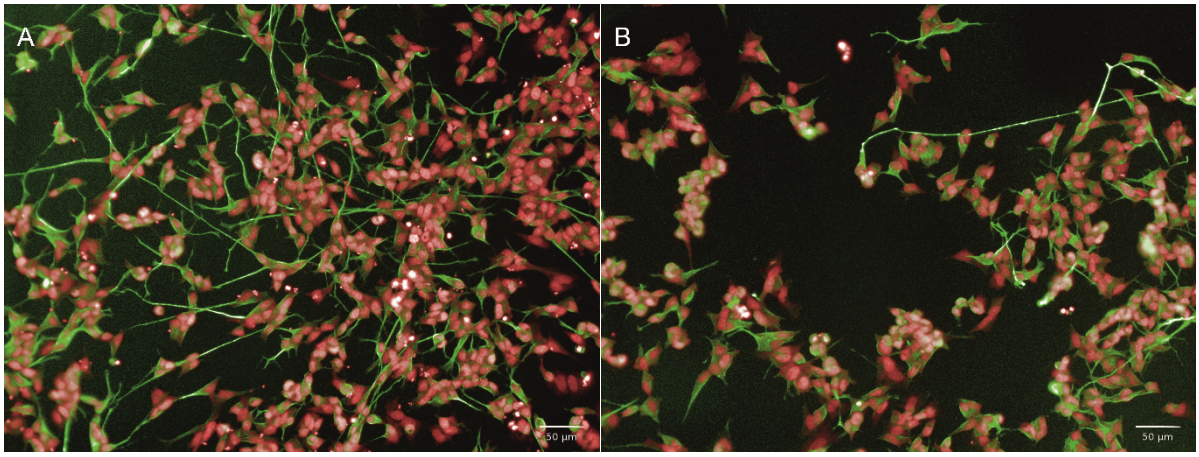
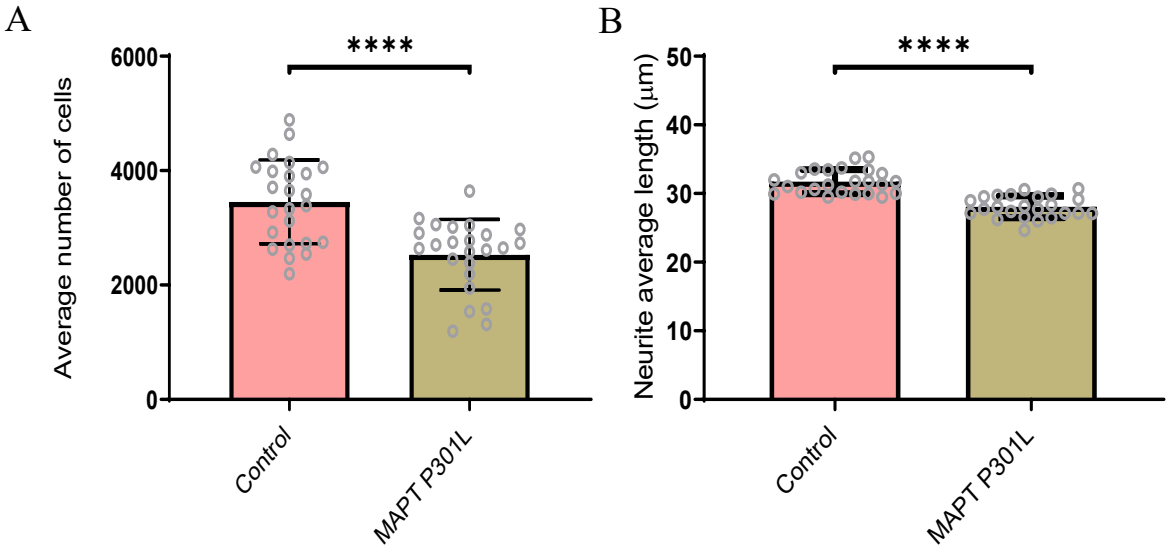


Figure 14. Effects of MAPT P301L mutations and neuroprotective compounds on neurite attributes and cell survival. Microphotographs representative of the neuroblastoma derived cell line SH-SY5Y subjected to different treatments. A) Control differentiated cells; B) Cells transfected with MAPT P301L. Red: DRAQ5®; Green: Anti-β Tubulin Class III Alexa 488®. Scale bar = 50 µm.



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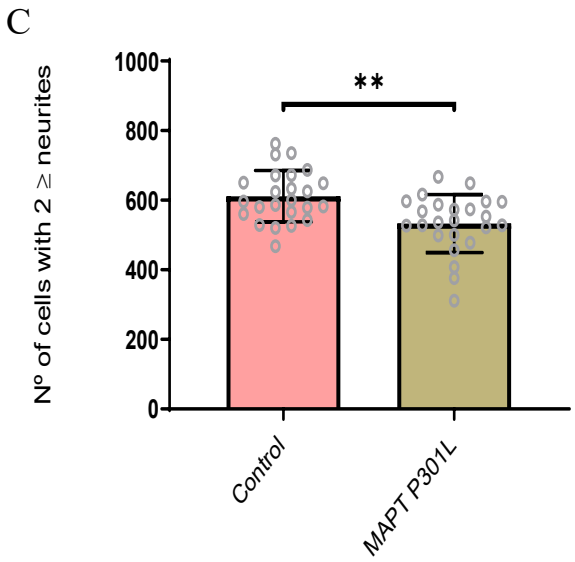


Figure 15. Statistical graph representation of different variables assessed to study the effects on cell morphology of MAPT P301L mutation. A) Average number of alive cells per well; B) Neurite average length; C) Dendrite arborization assessed as number of cells with 2 or more neurites. (■) RA + GLP-1; (■) MAPT P301L. ** $p < 0.01$; **** $p < 0.0001$ (Student t test).

5.2.2 The inclusion of the mutated protein APP V717I and exposure to synthetic amyloid-β 1-42 peptide produced a significant decrease on all of the morphological variables assessed and cell survival.

Similarly to the neurodegenerative resemblant changes observed upon transfection with Tau protein, cultures expressing the London mutation presented a significantly decrease in the number of viable cells ($p<0.001$), the neurite arborization ($p<0.001$) and average neurite length ($p<0.0001$) (Fig 16. A-B; Figure 17. A-C). Synthetic human recombinant amyloid-β 1-42 were used as a control to assess the morpho-pathological changes exhibited upon transfection and cell translation of mutation in protein APP V717I. The exposure of the cell line to 10 µM of Aβ 1-42 also produced a significant reduction in all morphological variables assessed, to an even higher degree than those registered with inclusion of the APP mutation, due to the immediate toxicity that this concentration of amyloid peptide exerts to the cell line (Fig 16. A-C; Figure 17. A-C).

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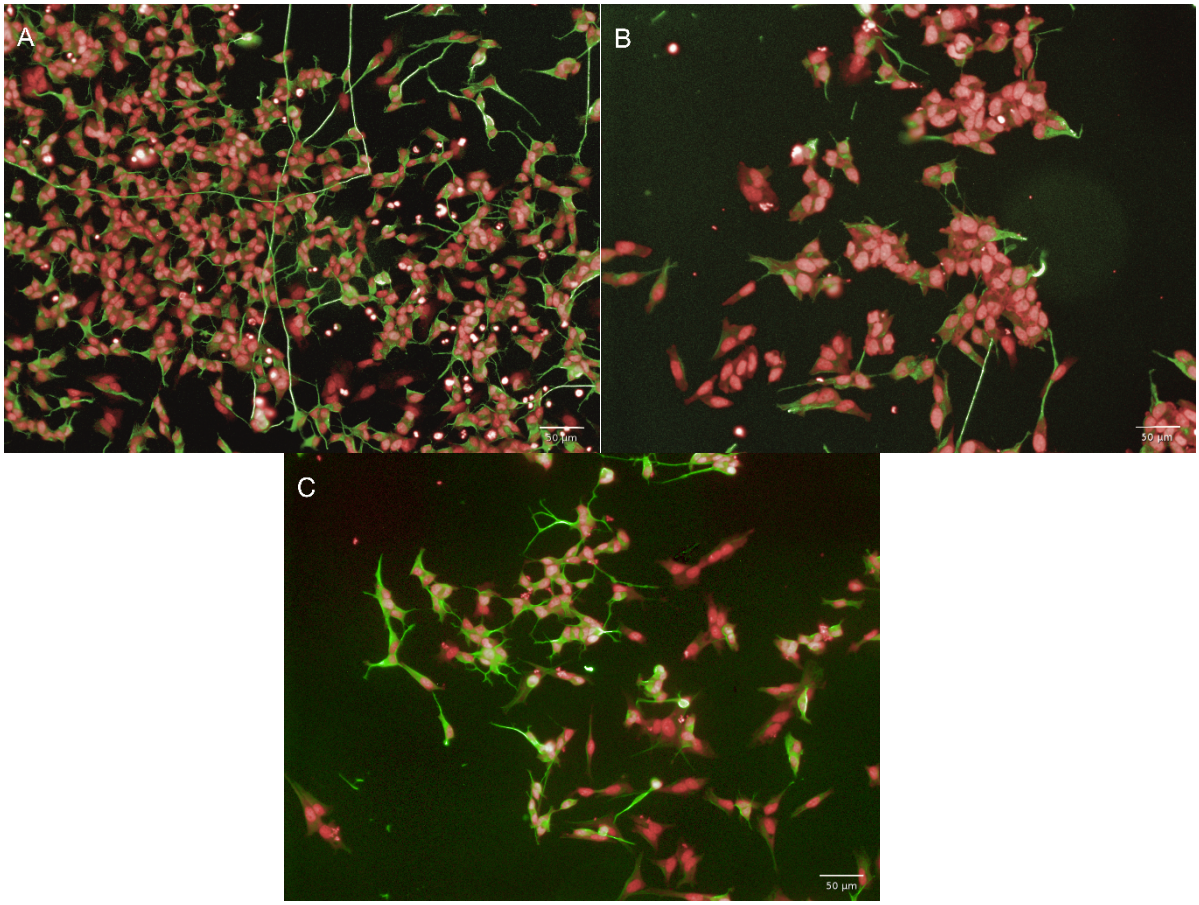
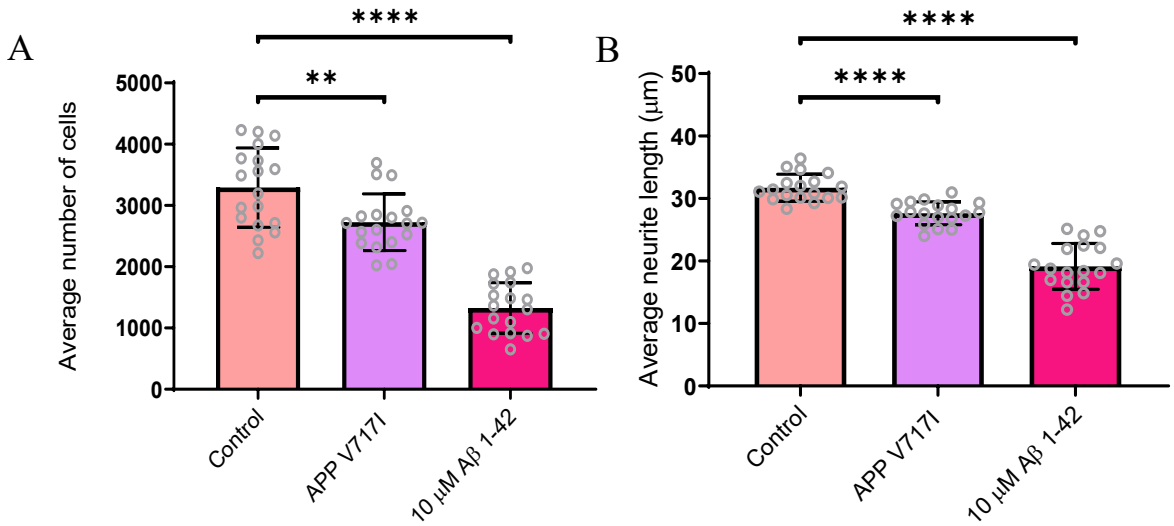


Figure 16. Effect of APP V717I mutation and neuroprotective compounds on neurite attributes and cell survival. Microphotographs representative of the neuroblastoma derived cell line SH-SY5Y subjected to different treatments. A) Control differentiated cells; B) Cells transfected with APP V717I; C) 10 µM Aβ 1-42 peptide. Red: DRAQ5®; Green: Anti-β Tubulin Class III Alexa 488®. Scale bar = 50 µm.



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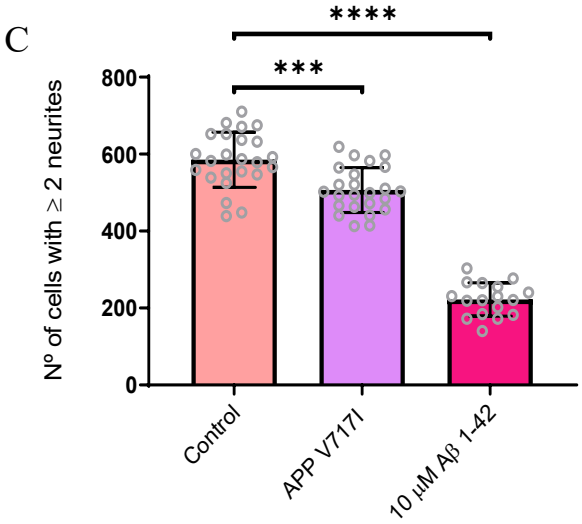


Figure 17. Statistical graph representation of different variables assessed to study the effects on cell morphology of APP V717I mutation A) Average number of alive cells per well; B) Neurite average length; C) Dendrite arborization assessed as number of cells with 2 or more neurites. (■) RA + GLP-1; (■) APP V717I; (■) Aβ 1-42. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Student t test).

5.2.3 Co-transfection with both APP V717I & MAPT P301L also showed a significant and greater impact than individual mutation across all the morphological parameters assessed.

In line with the findings indicative of neurodegeneration observed upon transfection with both proteins individually, the co-transfection of the cell line yielded cultures with a significative decline in the number of cells per well, the average neurite length and the neurite arborization ($p<0.0001$) (Figure 18. A-B; Figure 19. A-C). Absolute values for all of the variables were quantitively lower than those registered in cultures singularly transfected, with the greatest level of significance across all measured variables.

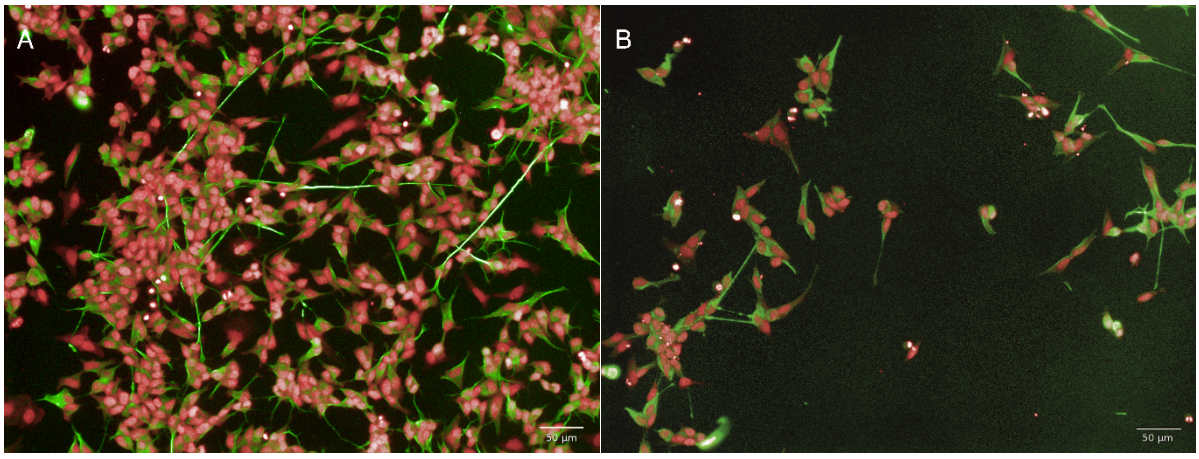


Figure 18. Co-transfection with MAPT P301L & APP V717I mutations effect on neurite attributes and cell survival. Microphotographs representative of the neuroblastoma derived cell line SH-SY5Y subjected to different

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treatments. A) Control differentiated cells; B) Cells co-transfected with APP V717I & MAPT P301L. Red: DRAQ5®; Green: Anti-β Tubulin Class III Alexa 488®. Scale bar = 50 µm.

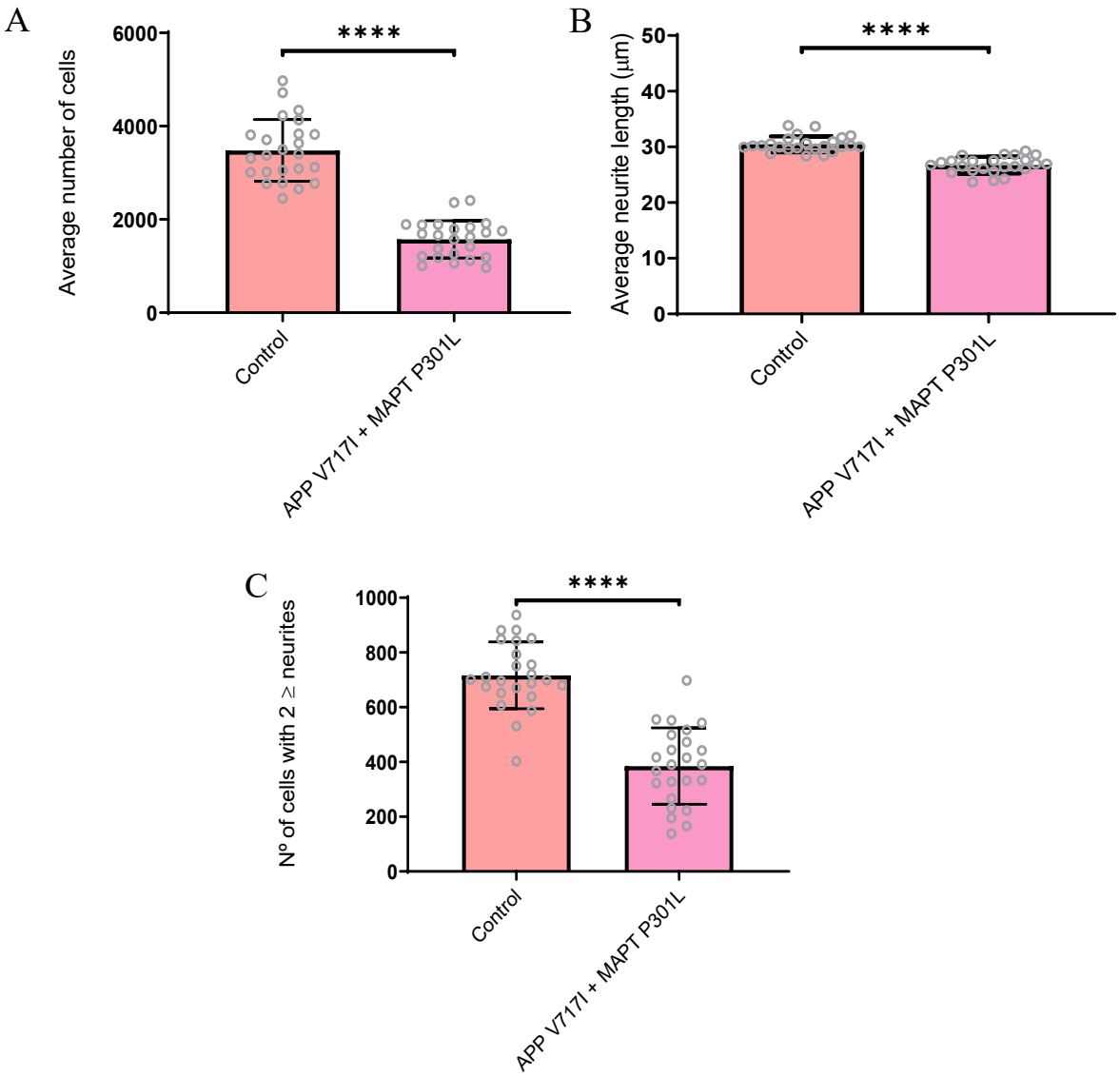


Figure 19. Statistical graphical representation showing the effects of co-transfection with MAPT P301L and APP V717I mutations on various morphological variables. A) Average number of alive cells per well; B) Neurite average length; C) Dendrite arborization assessed as number of cells with 2 or more neurites. (■) RA + GLP-1; (■) APP V717I + MAPT P301L. **** $p < 0.0001$ (Student t test).

5.3 Melatonin and resveratrol showed significant neuroprotective effect against all morphological variables assessed

With the objective of assessing the suitability of the developed model not only to replicate morphological characteristics resemblant of AD but to also be able to serve as a biological base for drug discovery, melatonin and resveratrol at 1 µM were included to cell cultures. These

compounds have an already described neuroprotective effect on *in vitro* studies, so its characterization in this neurodegenerative model supported by previous bibliography will help validate the model's relevance and reliability.

5.3.1 Exposure to both 1 µM melatonin and 1 µM resveratrol significantly reverted the neurodegeneration-like pathogenesis for all the variables studied upon transfection with MAPT P301L.

The exposure of the MAPT P301L transfected cell line to 1 µM melatonin over 48 hours significantly reverted the decrease in the number of viable cells ($p<0.001$), the lowered neurite arborization ($p<0.01$) and the reduced average neurite length ($p<0.05$) that were registered post-transfection (Figure 20. A-B; Figure 21. A-C). The addition of 1 µM of resveratrol induced similar effects to those observed with melatonin, as it managed to significantly revert the changes observed upon transfection for the number of viable cells, neurite arborization ($p<0.05$) and neurite length ($p<0.0001$) (Figure 20. A-C; Figure 21. A-C).

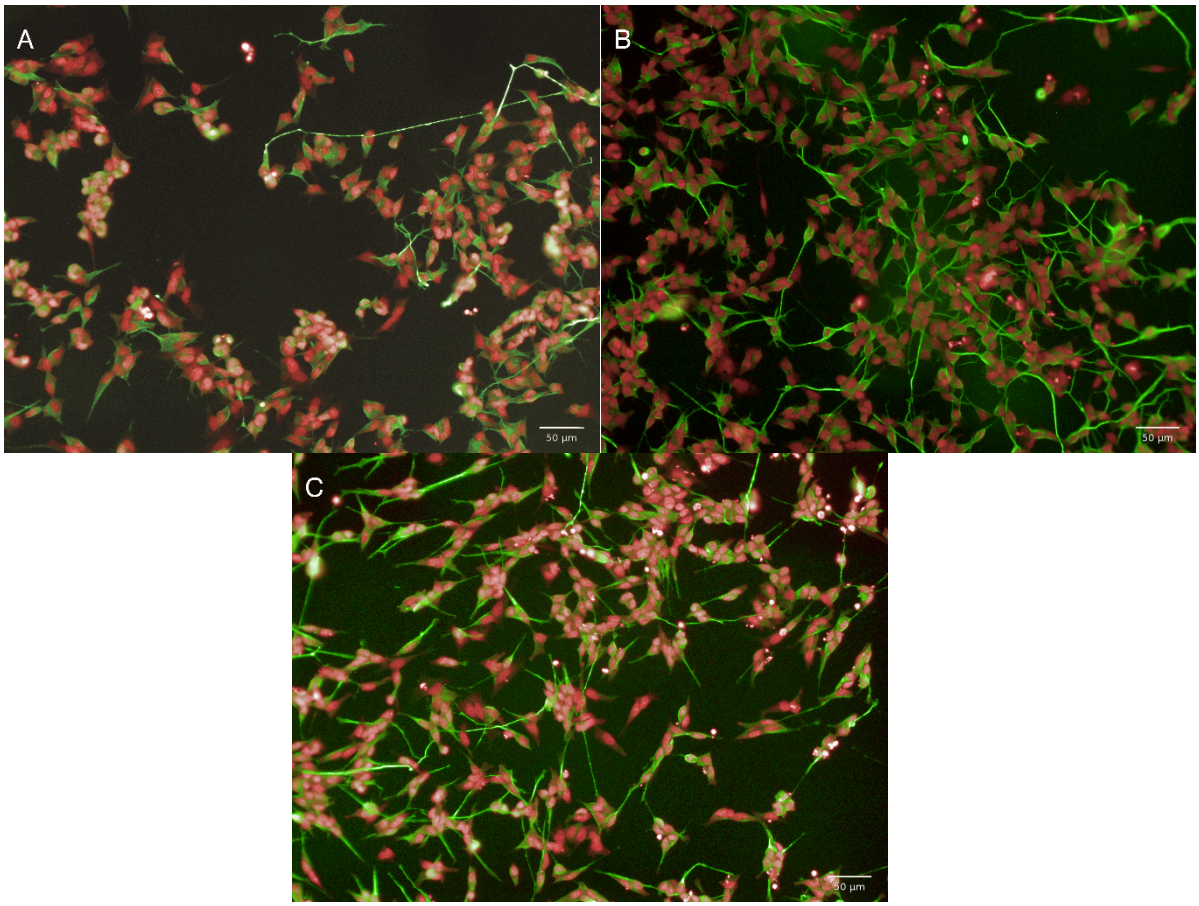


Figure 20. Neuroprotective effects of melatonin and resveratrol on neurite attributes and cell survival in MAPT P301L cell cultures. Microphotographs representative of the neuroblastoma derived cell line SH-SY5Y subjected to different treatments. A) Cells transfected with MAPT P301L B) MAPT P301L + 1 µM Melatonin C) MAPT P301L + 1µM Resveratrol. Red: DRAQ5®; Green: Anti-β Tubulin Class III Alexa 488®. Scale bar = 50 µm.

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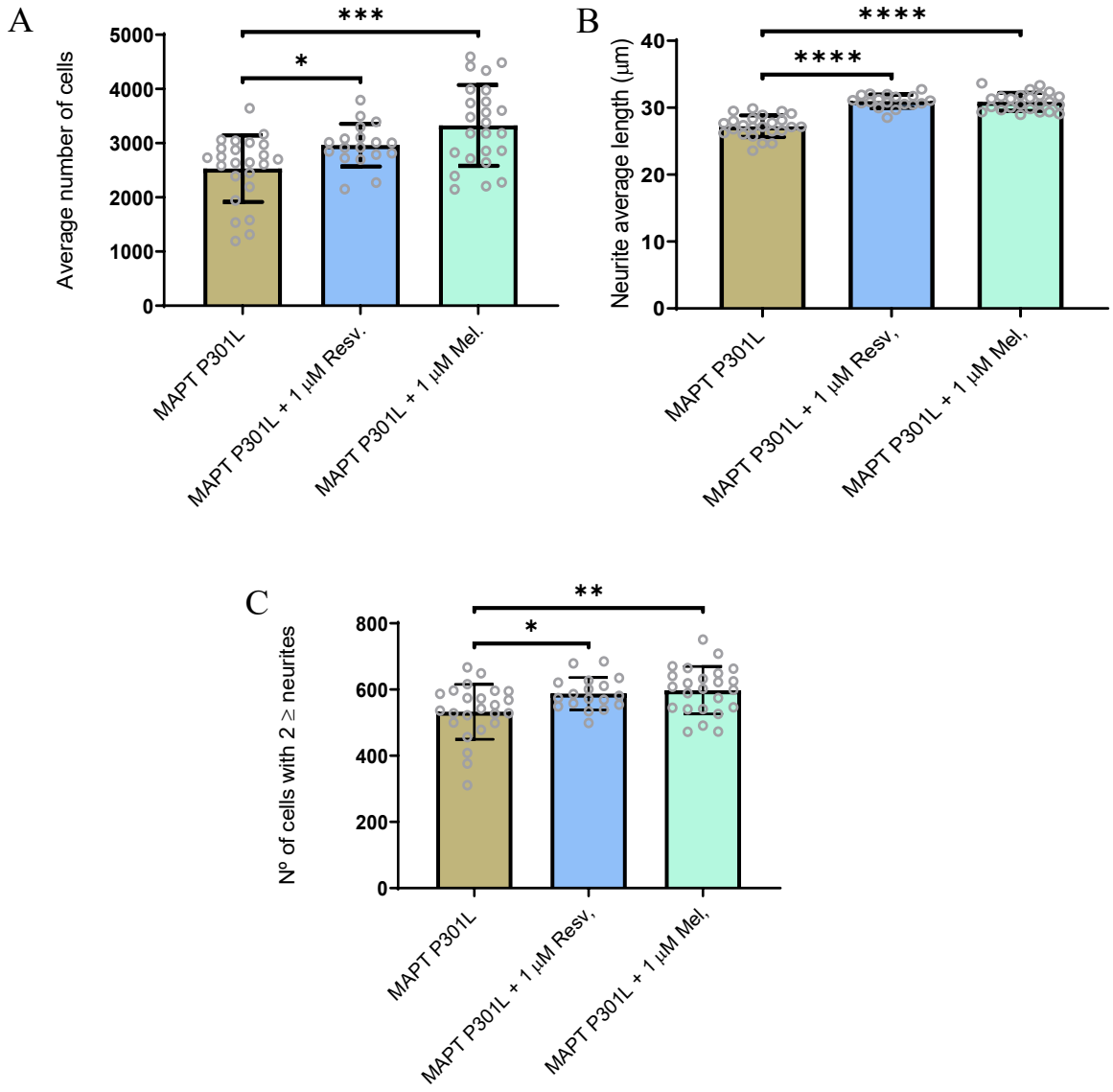


Figure 21. Statistical graph representation of different variables assessed to study the effects on cell morphology of MAPT P301L mutation and neuroprotective agents' melatonin and resveratrol. A) Average number of alive cells per well; B) Neurite average length; C) Dendrite arborization assessed as number of cells with 2 or more neurites. (■) MAPT P301L; (■) Resveratrol; (■) Melatonin. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Student t test).

5.3.2 Treatment of APP V717I transfected cultures with melatonin resulted in a significant increase for all variables assessed, whilst resveratrol only did so for the average neurite length.

Following the treatment of APP V717I transfected cultures with 1 µM of melatonin, a significant reversion was observed for all assessed variables, thus reaffirming its efficacy as a neuroprotective compound for cell survival, neurite structure density and length ($p<0.0001$; $p<0.05$; $p<0.001$) (Fig 22. A-B; Figure 23. A-C). In this case, the addition of significantly reverted the neurite shortening ($p<0.0001$), but did not show any effect in the other variables

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(Fig 22. A-C; Figure 23. A-C). These results suggest that melatonin exhibits neuroprotective properties against both amyloid and tau deposits, but resveratrol does so mainly for tau toxicity. Therefore, at 1 µM, the effects on resveratrol on SIRT-1 receptor are sufficient to moderate the toxicity effects of tau but lacks the ability of modulating β-secretase activity at the used concentration.

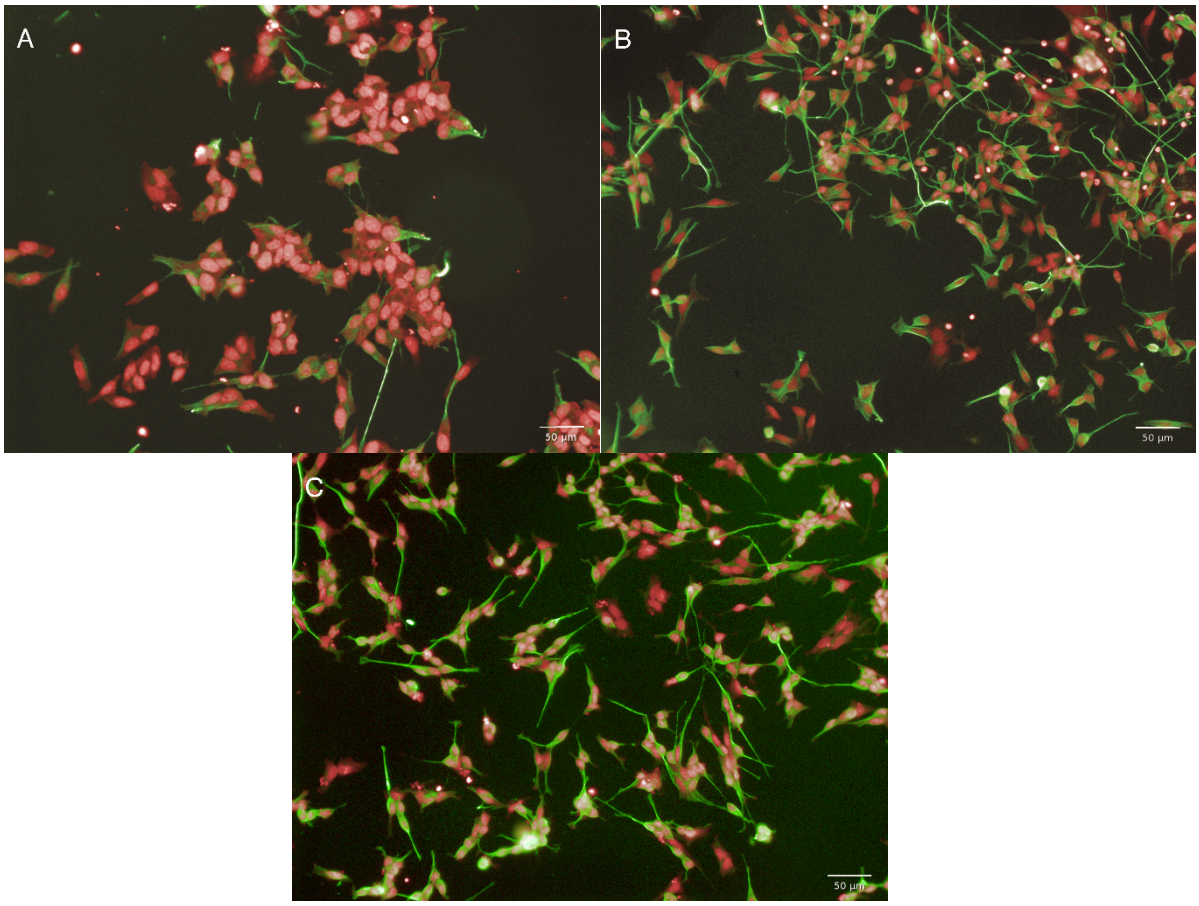


Figure 22. Neuroprotective effects of melatonin and resveratrol on neurite attributes and cell survival in APP V717I cell cultures. Microphotographs representative of the neuroblastoma derived cell line SH-SY5Y subjected to different treatments. A) Cells transfected with APP V717I B) APP V717I + 1 µM Melatonin C) APP V717I + 1µM Resveratrol. Red: DRAQ5®; Green: Anti-β Tubulin Class III Alexa 488®. Scale bar = 50 µm.

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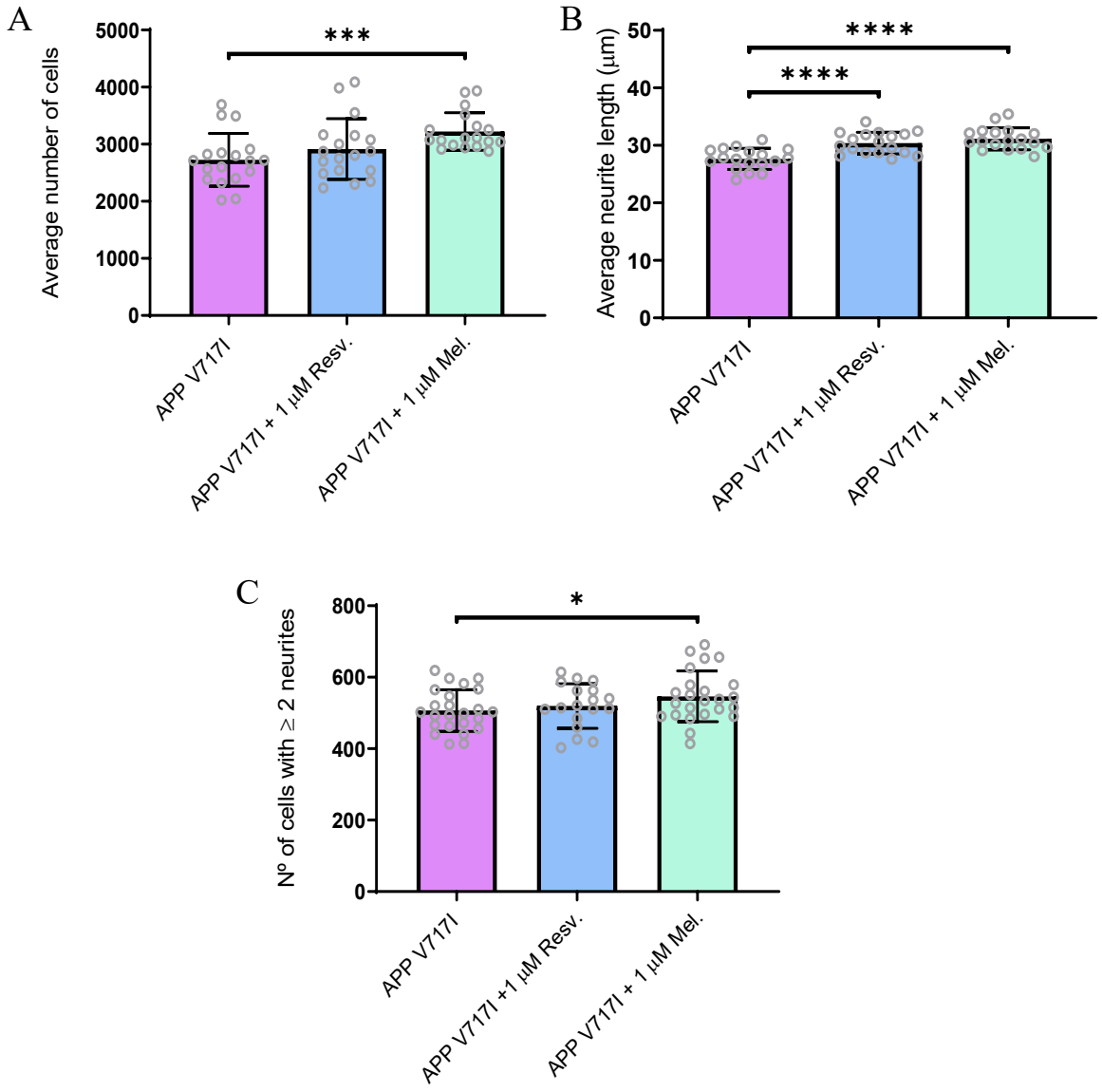


Figure 23. Statistical graph representation of different variables assessed to study the effects on cell morphology of APP V717I mutation and neuroprotective agents' melatonin and resveratrol. A) Average number of alive cells per well; B) Neurite average length; C) Dendrite arborization assessed as number of cells with 2 or more neurites. (■) APP V717I; (■) Resveratrol; (■) Melatonin. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$ (Student t test).

5.3.3 Addition of 1 µM melatonin and 1 µM resveratrol to co-transfected cultures significantly reverted the reduction of all the studied morphological variables.

Regarding neuroprotective effects on cultures transfected with both APP V717I and MAPT P301L, treatment with 1 µM melatonin, also managed to significantly revert all of the assessed variables: average neurite length ($p<0.001$), neurite arborization ($p<0.0001$) and number of cell nucleus ($p<0.0001$) (Figure 24. A-B; Figure 25. A-C). The inclusion of resveratrol in the cell culture media provoked a significant reversion on the changes registered post-transfection for

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average neurite length (p<0,05), number of cells (p<0.05) and neurite arborization (p<0.01), in a similar way and efficacy to the observed for MAPT P301L individual transfection (Figure 24. A-C; Figure 25. A-C).

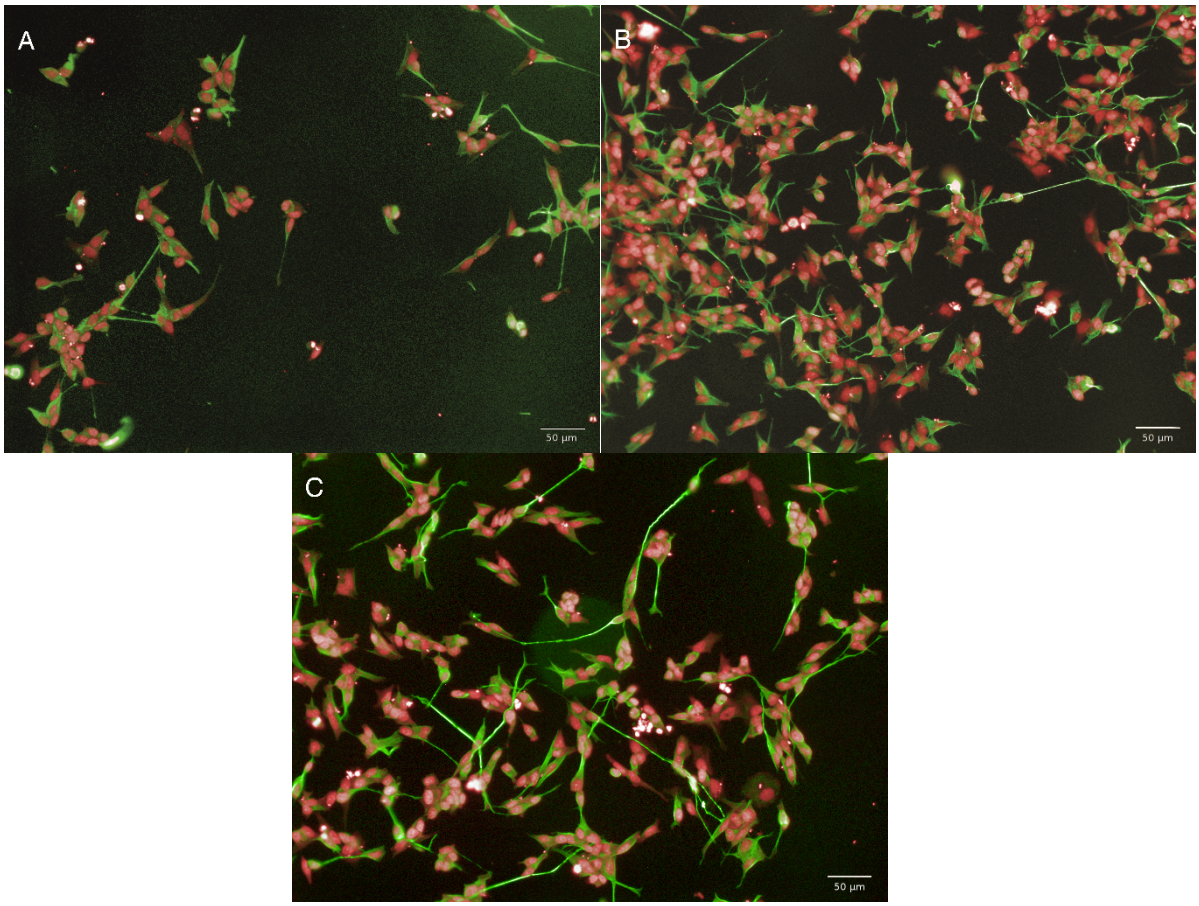


Figure 24. Neuroprotective effects of melatonin and resveratrol on neurite attributes and cell survival in co-transfected cell cultures. Microphotographs representative of the neuroblastoma derived cell line SH-SY5Y subjected to different treatments. A) Cells co-transfected with APP V717I & MAPT P301L B) APP V717I & MAPT P301L + 1 µM Melatonin C) APP V717I & MAPT P301L + 1µM Resveratrol. Red: DRAQ5®; Green: Anti-β Tubulin Class III Alexa 488®. Scale bar = 50 µm.

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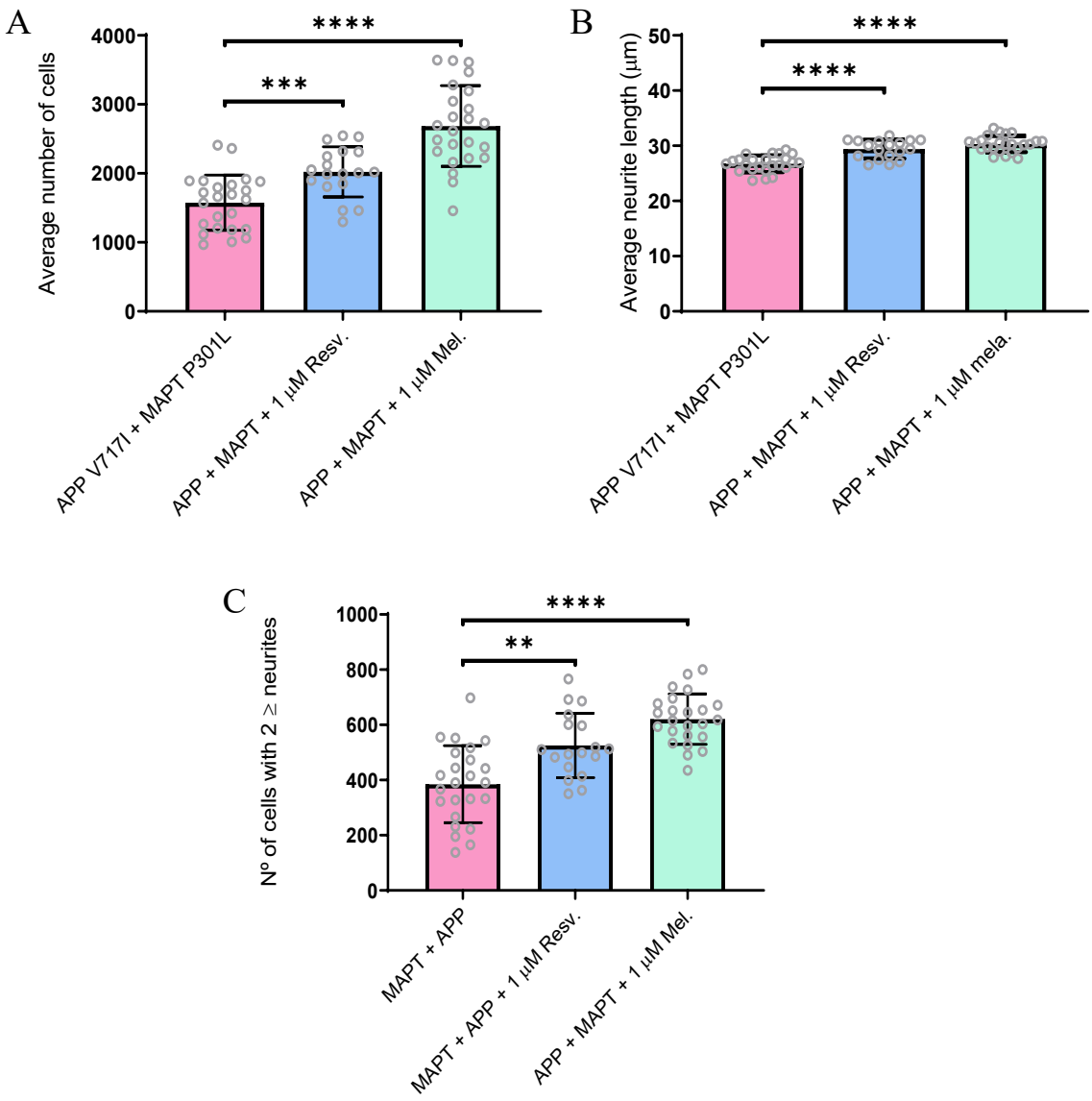


Figure 25. Statistical graphical representation showing the effects of co-transfection with MAPT P301L and APP V717I mutations alongside neuroprotective agents' melatonin and resveratrol on various morphological variables. A) Average number of alive cells per well; B) Neurite average length; C) Dendrite arborization assessed as number of cells with 2 or more neurites. (■) APP V717I + MAPT P301L; (■) Resveratrol; (■) Melatonin. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Student t test).

5.4 MAPT P301L and APP V717I induced the SH-SY5Y to show a hypo-excitatory response to KCl

In Alzheimer's disease pathology, the excitatory response of neurons is notably altered due to the misfolded amyloid and Tau proteins, contributing to synaptic dysfunction and excitotoxicity. Under excitatory conditions, such as exposure to potassium chloride (KCl), neurons transfected with the APP V717I and MAPT P301L mutations exhibit disrupted ionic homeostasis and impaired excitability, which intensify cellular stress and neurodegenerative

outcomes (Busche et al., 2012; Angulo et al., 2017; Busche et al., 2018; Targa Dias Anastacio et al., 2022).

Given that KCl stimulation elevates intracellular calcium and triggers excitatory signalling pathways, the response in these transfected cells offers insight into how amyloid and Tau pathologies affect synaptic response and respond to excitatory stimuli. The results observed in this experimental setup reflect how AD-associated mutations can modulate also physiological properties of the cell line in the form excitatory thresholds and further represent functional impairments tied to disease progression.

5.4.1 Co-transfection with both APP V717I & MAPT P301L induced significant hypo-excitability in cultured cells. The treatment post-transfection with 1 µM melatonin and 1 µM resveratrol was able to significantly revert the hypo-excitability.

Differentiated and un-differentiated cell cultures were exposed to 30 mM KCl in order to assess how differentiation and the subsequent transfections affected to a key physiological property of neurons, the excitatory response. With this assay, a greater excitatory response was registered within cell cultures that underwent the differentiation process versus those that were undifferentiated, which showed an almost an imperceptible response, as expected due to their non-neuronal nature ($P<0.0001$) (Figure 26.; Figure 27.; Table 3.). The co-transfection of the cell line with both of previously mentioned mutations led to a significantly fall in the response registered, following the neurodegeneration-like pattern that these mutations showed in the morphological study ($p<0.0001$) (Figure 26. Table 3.). Both 1µM of melatonin and resveratrol exhibited a significantly protective effect at maintaining the response of cells to higher excitatory responses than those obtained post-transfection ($p<0.0001$) (Figure 26. Table 3.).

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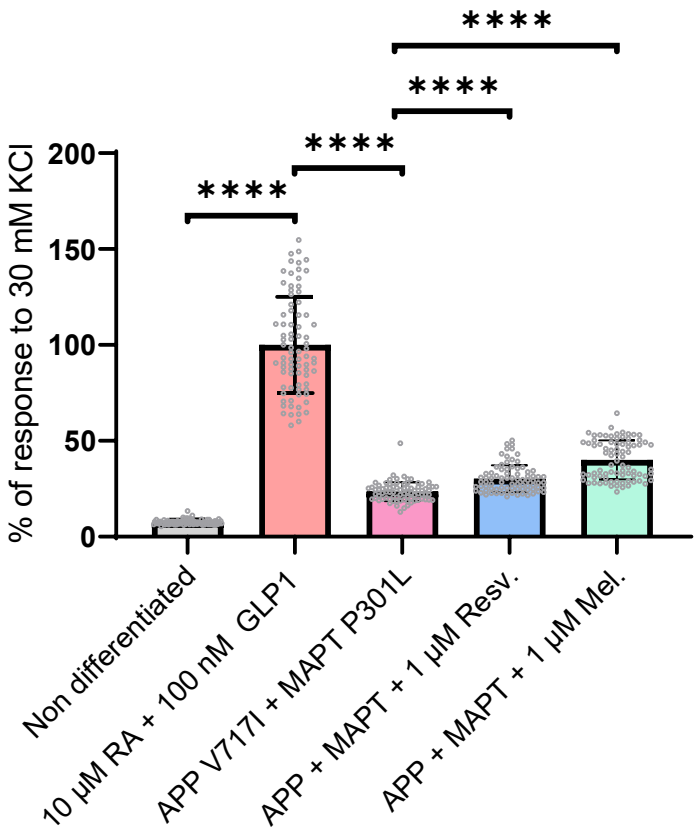


Figure 26. Statistical graphical representation showing the impact of co-transfection with MAPT P301L and APP V717I mutations alongside neuroprotective agents' melatonin and resveratrol on excitation properties. Response upon treatment with 30 mM KCl of 1) Undifferentiated cell cultures; 2) Differentiated cell cultures; 3) Co-transfected with MAPT P301L and APP V717I; 4) Co-transfected and treated with 1µM of Resveratrol 5) Co-transfected and treated with 1µM of Melatonin. (■) Undifferentiated; (■) RA + GLP-1; (■) APP V717I + MAPT P301L; (■) Resveratrol; (■) Melatonin. **** $p < 0.0001$ (Student t test).

Cellular culture	Response (Mean ± SD)
10 µM RA + 100 nM GLP-1	100 ± 25.13 %
Undifferentiated SH-SY5Y cells	7.59 ± 1.29 %
APP V717I + MAPT P301L	23.51 ± 4.59 %
Co-transfected + 1 µM Resv.	30.24 ± 7.06 %
Co-transfected + 1 µM Mel.	40.08 ± 10.14 %

Table 3. Data of the response to excitation with 30 mM KCl on different SH-SY5Y culturing conditions. Response was calculated from normalizing absolute RFU values registered in respect to those obtained for differentiated cells.

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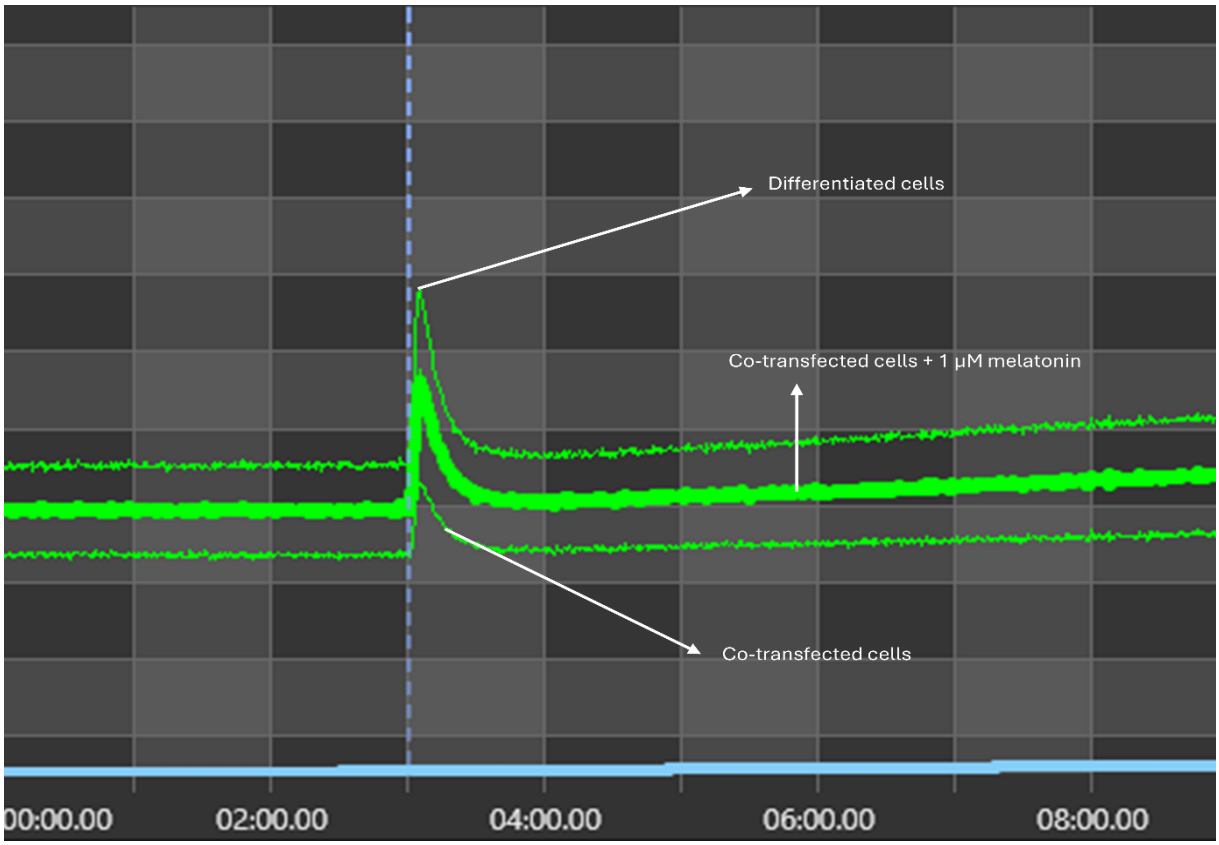


Figure 27. Representative image of the changes in fluorescence relative to intracellular calcium concentrations. Graphical plot taken during an intracellular calcium assay using FDSS v3.4 representing the curves for three independent conditions. Vertical line (- -) represents the moment in which 30 mM KCl is introduced in the cell cultures.

5.5 Identification of novel compounds with neuroprotective properties through Prestwick® chemical library screening

High-throughput screening assays represent a promising approach in Alzheimer's disease (AD) research, offering the capacity to systematically evaluate thousands of compounds for therapeutic potential in a disease-relevant context. This rapid and high-result volume technique allows to provide not only therapeutic options, but new insights on novel molecular pathways. Given the complexity of AD, with its numerous hallmarks, and the still under debate multi-etiology, HTS offers a bridge to clinical assays and the repurposing of drug compounds with a research background in human physiology.

The integration of HTS assays in this study underscores the importance of efficient, biologically relevant models in drug discovery, especially for neurodegenerative diseases where traditional drug development faces high attrition rates. Application of this methodology has provided not only potential candidates for further testing in more complex environment down

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the drug discovery pipeline, but also a scalable and reliable biological model in which to perform larger screening campaigns.

5.5.1 Activity evaluation of 1,120 compounds from the Prestwick® chemical library.

The evaluation of 1120 compounds from the Prestwick® chemical library at a concentration of 1 µM was performed taking in consideration the compounds' capacity to palliate the hypo-excitability observed in co-transfected cultures. This was done by exposure of cell cultures to 30 mM KCl after 72 hours post-transfection and allowing the compounds to exert their effect for at least 48 hours. Due to the dispersion observed, the efficacy threshold was set at the mean inhibition value plus two times the standard deviation (Figure 28.; Table 4). Forty-five compounds were regarded as potential candidates after this first screening, and further studied in dose-response assays. Quality control of the screening assay was performed for each individual plate taking in consideration the Z´ factor, signal window and coefficient of variation (Table 5). DMSO tolerance was tested to a maximum of 1% without significant differences for the assessed variables and kept at 0.1% in the screening's final volume.

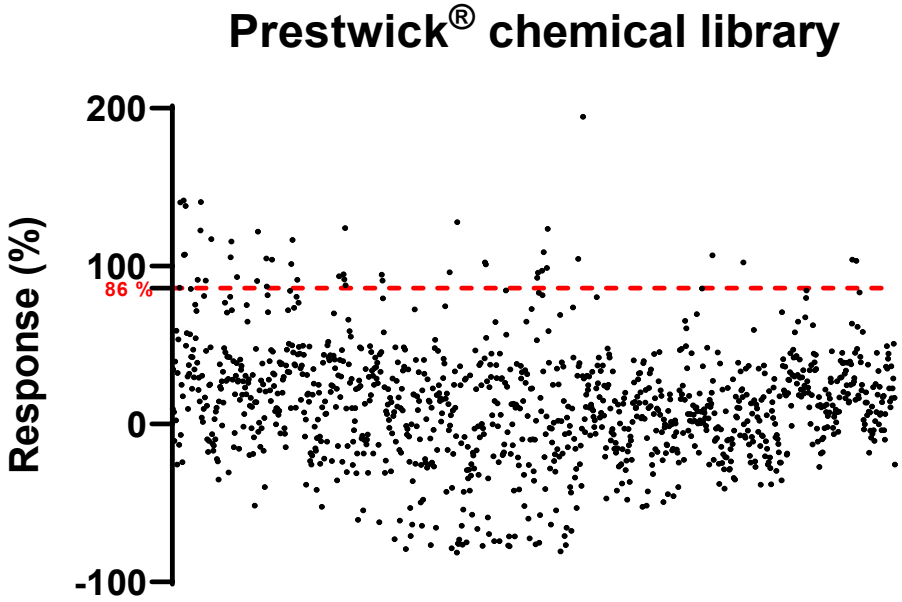


Figure 28. Activity of SH-SY5Y cultures in the presence of every 1120 compounds present in Prestwick® chemical library. Percentage of response measures the intracellular calcium rfu values considering the mean registered for transfected cultures (0%) and the mean obtained in differentiated cultures (100%). Presented values are from single replicates. On the y-axis, the cutoff point marked (- -), delimitates those compounds that were selected for a secondary assessment (86%).

Mean % Response	12.96 ± 36.41
Y-Axis Cutoff (Mean + 2 x SD)	86 %
Std. Error of Mean	1.08

Table 4. Statistical data of the screening representation of 1120 compounds present in the Prestwick® chemical library.

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Plate	CVc_+	CVc-	Z´factor	Signal window
1	4.68 %	3.99 %	0.61	6.30
2	5.60 %	3.03 %	0.64	8.14
3	5.42 %	5.32 %	0.58	5.96
4	5.39 %	3.82 %	0.74	9.25

Table 5. Statistical data of the quality control performed on individual plates during Prestwick® chemical library screening

5.5.2 Validation of *hits* with a dose-response relationship

To validate that the compounds of interest derived from the first screening assay were indeed capable of enhancing the cell excitation above the cutoff and exhibit neuroprotective activity, a concentration-response correlation assay was designed. Serial dilutions of each compound were tested in replicates at concentrations ranging from 100 pM to 10 µM in tenfold increments. Amongst the forty-five compounds, twelve showed a correlation between compound concentration and the increased response induced by 30 mM KCl after being co-transfected with proteins APP V717I and P301L (Figure 29.; Table 6).

Compound	**E_{max} (Mean)**
Doxepin	136 %
Metolazone	107 %
Mianserin	105.5 %
Ethosuximide	94.9 %
Canavanine	94.5 %
Betazole	85.5 %
Dapsone	86.1 %
Ajmaline	85.3 %
Rifampicin	74.5 %
Picotamide	73 %
Bacampicillin	72.7 %
Dicyclomine hydrochloride	65%

Table 6. Compound activity of reverting the hypo-excitation observed upon co-transfection with APP V717I and MAPT.P301L Efficacy of each drug (E_{max}) is presented as the % of recovery response at the highest registered values for one of the six tested concentrations in respect to the average differentiated cell value. The twelve *hits* that showed a correlation between dosage and response are represented.

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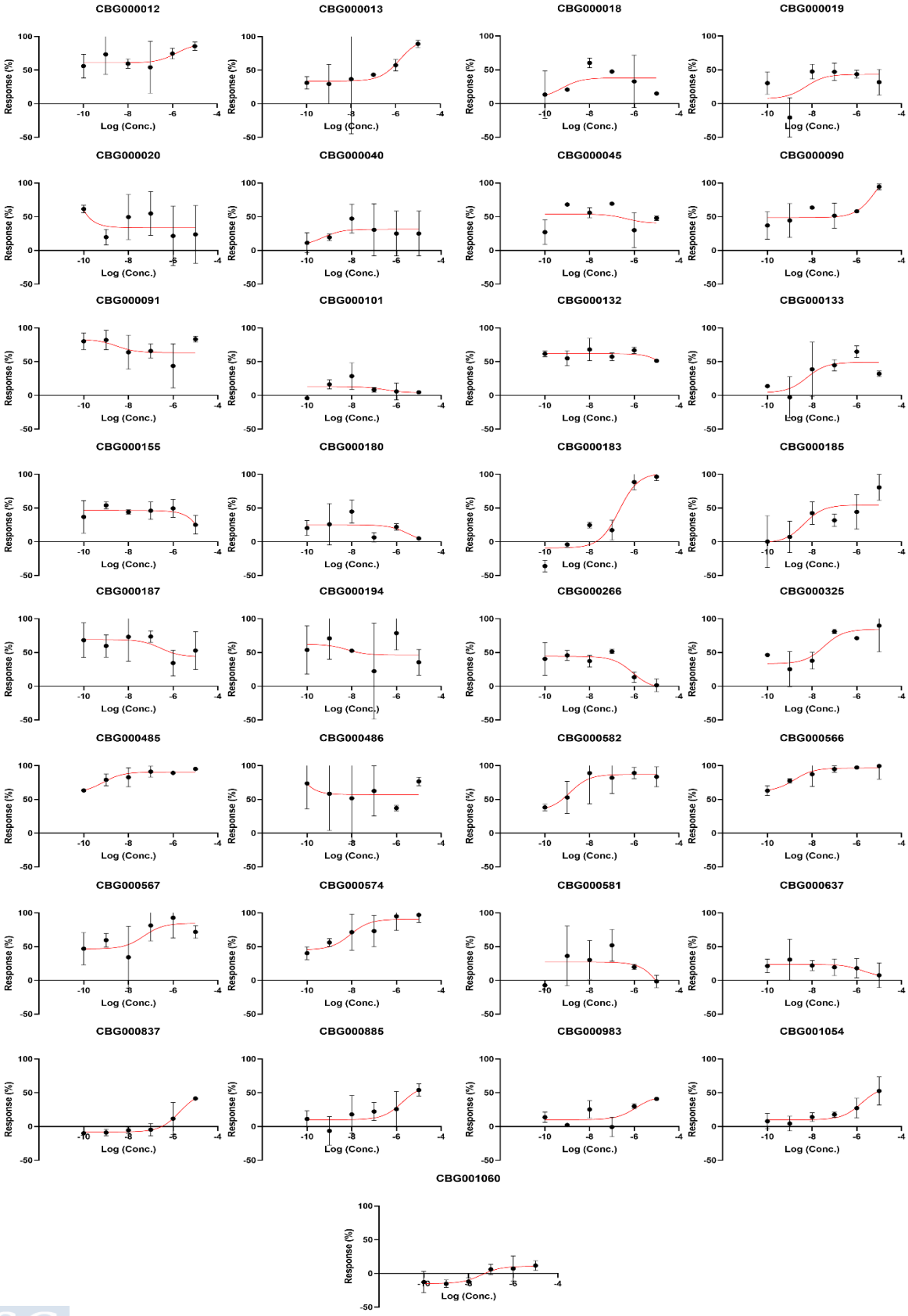


Figure 29. Graphical layout of the thirty-three compounds without dose-response activity. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are

standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

5.5.3 Confirmation of *hits* with neuroprotective efficacy

In the confirmation assay, the twelve hit compounds identified from the secondary validation were further tested to confirm their neuroprotective effects. Each compound was evaluated across the previous range of concentrations to determine a precise dose-response relationship and EC_{50} efficacy, specifically assessing their capacity to mitigate the KCl-induced hypo-excitatory response when co-transfected with APP V717I and P301L proteins. Out of the twelve compounds, eight displayed consistent dose-response neuroprotective activity, suggesting their potential efficacy in modulating physiological activity under Alzheimer's disease model conditions. This stage also reinforced the model suitability for further investigation in therapeutic development with larger drug discovery assays and translation to other superior models. Data and dose-response adjusted curves for all eight *hits*: Dapsone, Mianserin, Ethosuximide, Doxepin, Rifampicin, Canavanine, Betazole and Metolazone can be found on *Table* 7 and *Figures* C.30-37.

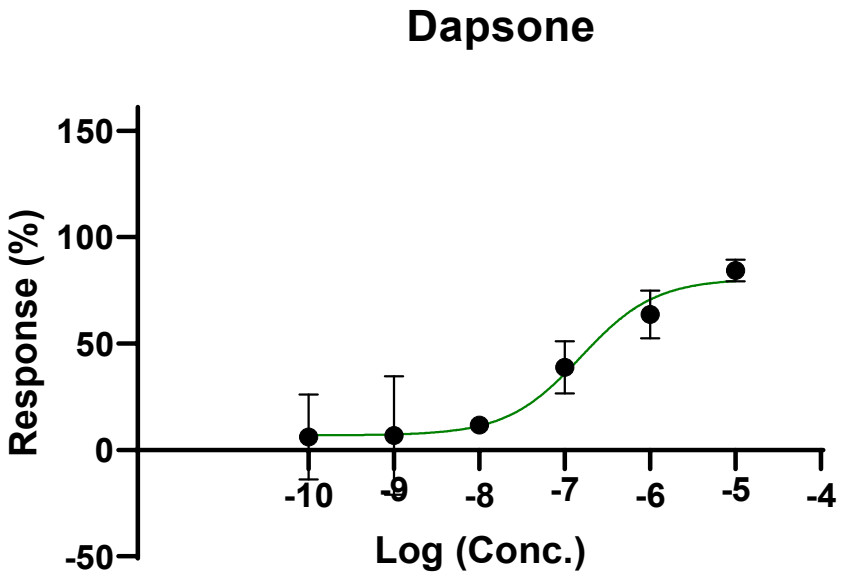


Figure 30. Measurement of intracellular calcium after treatment of co-transfected SH-SY5Y cell line with 1 pM to 10 µM Dapsone. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

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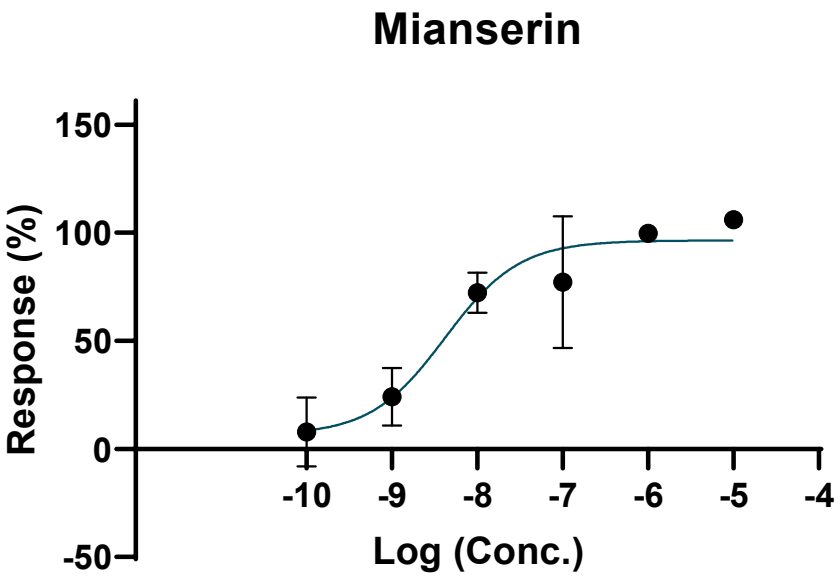


Figure 31. Measurement of intracellular calcium after treatment of co-transfected SH-SY5Y cell line with 1 pM to 10 µM Dapsone. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

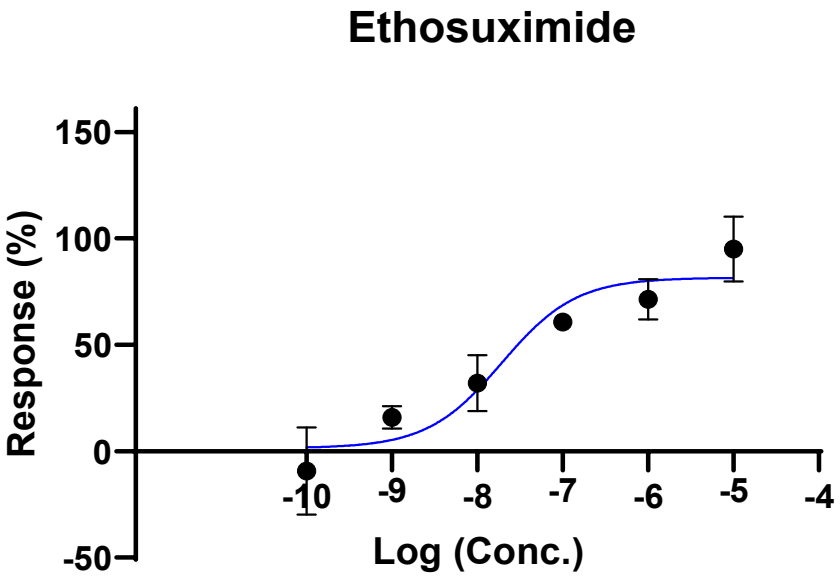


Figure 32. Measurement of intracellular calcium after treatment of co-transfected SH-SY5Y cell line with 1 pM to 10 µM Ethosuximide. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

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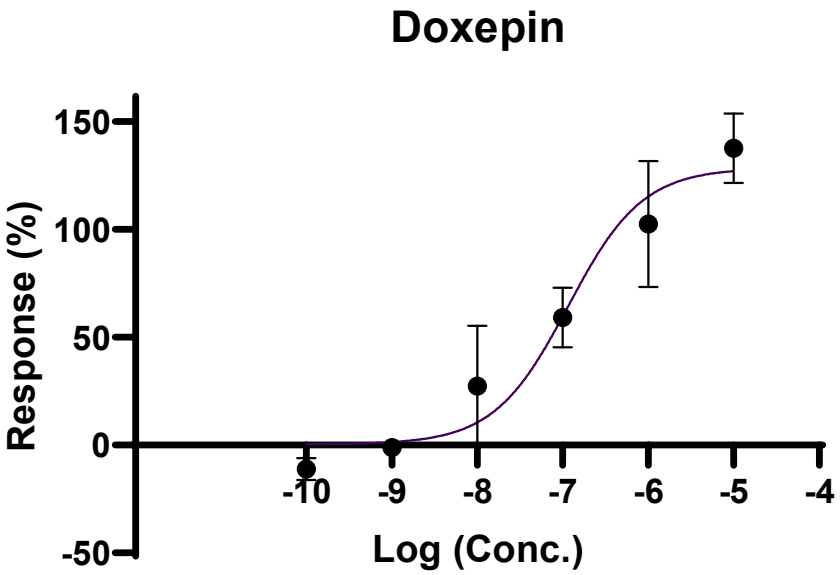


Figure 33. Measurement of intracellular calcium after treatment of co-transfected SH-SY5Y cell line with 1 pM to 10 µM Doxepin. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

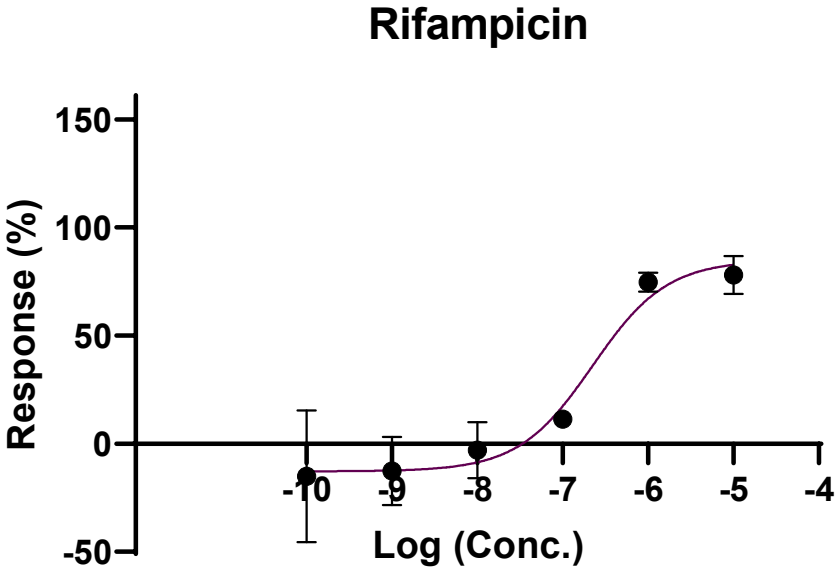


Figure 34. Measurement of intracellular calcium after treatment of co-transfected SH-SY5Y cell line with 1 pM to 10 µM Rifampicin. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

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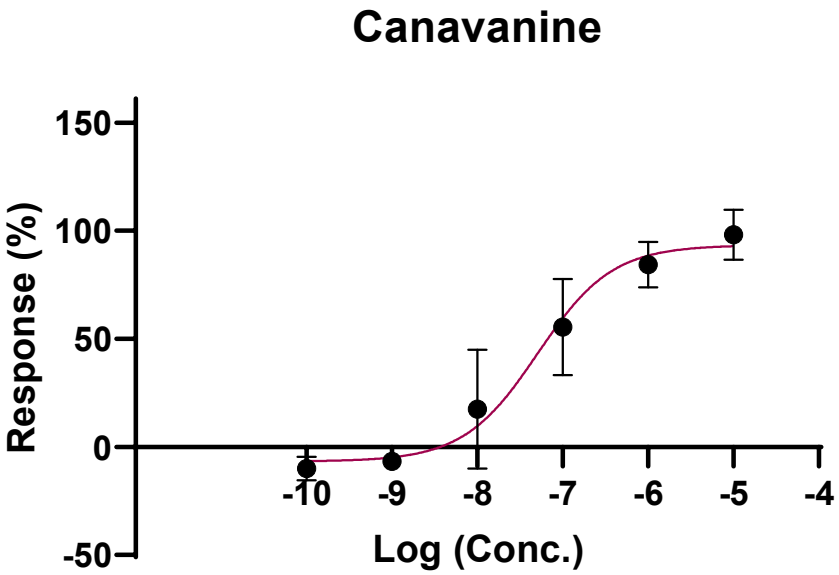


Figure 35. Measurement of intracellular calcium after treatment of co-transfected SH-SY5Y cell line with 1 pM to 10 µM Canavinine. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

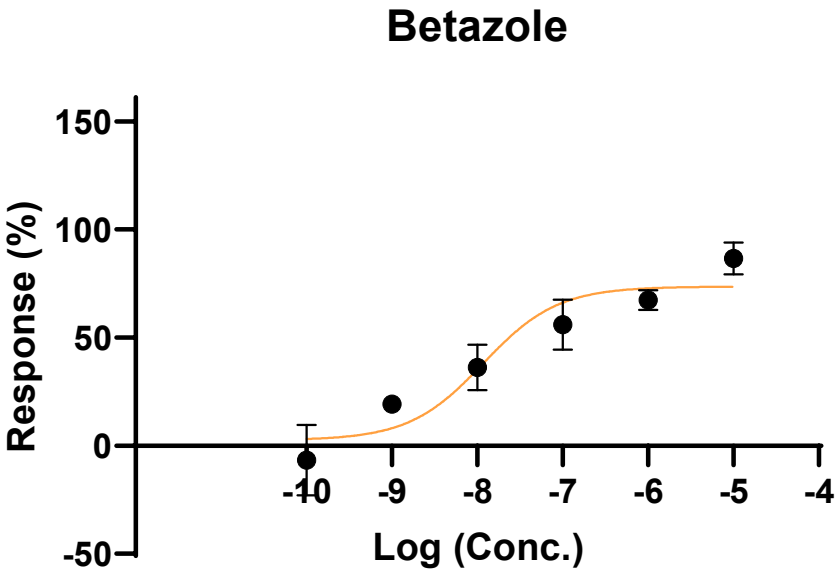


Figure 36. Measurement of intracellular calcium after treatment of co-transfected SH-SY5Y cell line with 1 pM to 10 µM Betazole. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

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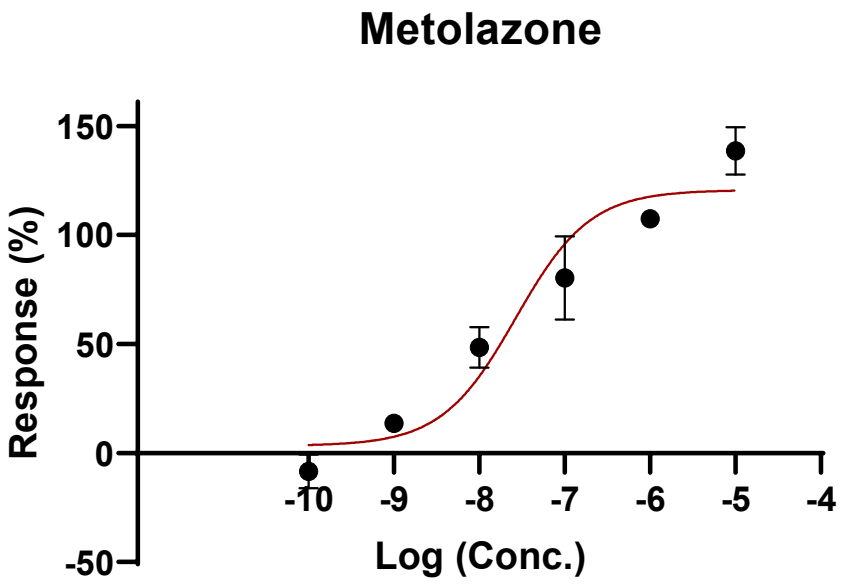


Figure 37. Measurement of intracellular calcium after treatment of co-transfected SH-SY5Y cell line with 1 pM to 10 µM Metolazone. Mean ± Standard deviation is represented per point and response values are plotted as response to 30 mM KCl. Response values are standardized against the mean response values of differentiated cells. Sigmoidal curve adjusted using the non-linear fit with three parameters.

Compound	EC_{50}
Dapsone	0.15 ± 0.017
Mianserin	0.004 ± 0.0007
Ethosuximide	0.02 ± 0.002
Doxepin	0.11 ± 0.014
Rifampicin	0.22 ± 0.015
Canavanine	0.05 ± 0.004
Betazole	0.01 ±0.001
Metolazone	0.02 ± 0.002

Table 7. EC_{50} values of the eight compounds from Prestwick® chemical library that showed correlation between concentration and efficacy.

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DISCUSSION

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6. DISCUSSION

This doctoral thesis presents the development of a novel, robust, and translational in vitro model of phenotypically differentiated neurons, incorporating Alzheimer's disease mutations, and validated for HTS. This model was based on the employment of the SH-SY5Y neuroblastoma-derived cell line, differentiated to acquire both a morphological and physiological phenotype of mature neurons. To replicate the neurodegeneration observed in familial Alzheimer's brain, two mutations in key pathological proteins involved in AD, APP V717I and MAPT P301L, were integrated in the cell line. Neuroprotective compounds melatonin and resveratrol that have been previously studied and characterized in other Alzheimer's *in vitro* and *in vivo* models, were used for reverting the pathophysiological neurodegenerative-like hallmarks present in this model. In fact, both compounds have proven significant efficacy at modulating the hypo-excitability, neuronal death and neurite loss and shortening present post-transfection, all of which are manifested clinically in AD patients. The achievement of similar results with these neuroprotective compounds validated this model for its application in HTS assays, enabling the identification of *hit* compounds and paving the way for Alzheimer's drug discovery.

Alzheimer's disease remains a significant unmet therapeutic need, with a global prevalence that continues to rise, especially amongst aging populations (Prince et al., 2016). Despite numerous clinical trials and high-failure rates in the past two decades, current treatments demonstrate limited efficacy in slowing disease progression and are often associated with important adverse effects (Hoffman & Bloermer, 2021). Drug discovery efforts have traditionally relied on in vitro models or transgenic animals that mimic single specific pathological features of AD, such as protein deposition or neuroinflammation, by genetically or synthetically inducing the expression of associated targets. While this target-centric approach has led to important discoveries in biomolecular pathways associated to AD, it inherently predefines molecular targets, constraining the search for novel therapeutic mechanisms and often failing to address the complex interactions happening on AD, ultimately leading to novel compounds that have largely failed in human clinical trials. (LaFerla & Green, 2012; Drummond & Wisniewski, 2016; King, 2018; Blaikie et al., 2022).

This highlights the urgent need for translational models that can more closely replicate human AD pathophysiology. Advances in human-derived cell models, such as iPSCs and 3D cultures, offer promising alternatives, as do animal models incorporating sporadic AD features and modifiable environmental risk factors, but technical complexity and scalability of these models suppose a serious challenge for early drug discovery (Choi et al., 2014; Conrado et al., 2020). Simpler models that retain multi-target key aspects of the disease could then bridge the gap between preclinical and clinical studies, ultimately supporting the identification of compounds and biomolecular that address the multifaceted nature of AD and that are translational to more complex environments (Blanchard et al., 2021; Blaikie et al., 2022).

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There is a growing number of molecules from diverse origins, amounting to millions of potential drug candidates, that lack translational assays capable of evaluating them efficiently

at a large scale, and predicting their potential application to diseases such as Alzheimer's. This necessity directly aligns with the main objective of this study, that focuses in providing a novel translational model for HTS that can replicate key pathological hallmarks in AD in the form of cell death, loss of neurite arborization, neurite shortening and neuronal impaired excitability. Employment of the SH-SY5Y cell line allowed for fast-growing and short-differentiation protocols cultures, thus overcoming main limitations of primary cultures and iPSCs in their application to drug discovery (Cetin et al., 2022; Voloshin et al., 2023). As a result, the 2D monolayer model presented in this study exhibited both morphological and physiological characteristics suggestive of a neurodegeneration upon transfection. Our model also was established as solid base for compound testing that may have a protective effect against the observed changes, being able to replicate the results observed in previous models for compounds with neuroprotective properties. Application to high-throughput screening assays for drug discovery led to the identification and validation of 8 novel *hits* that countered the hypo-excitability registered in cell cultures post-transfection, all of them having a described theoretical background that supports these results.

6.1 Differentiation of the SH-SY5Y with a combination of RA and GLP-1 endowed them with a mature neuron-like phenotype

The first objective of this piece of work consisted in the differentiation of a neuroblastoma-derived cell line into the acquisition of neuron-like phenotype characteristics, aiming to establish a reliable biological base for the study of Alzheimer-induced pathophysiology and its use in drug discovery campaigns. Initially, we compared two differentiation protocols, both based on the induction of neuronal phenotypes using all-trans retinoic acid in combination with either GLP-1 or PMA.

The SH-SY5Y cell line is widely used in neurobiological research due to its capacity to differentiate into cells with neuron-like characteristics with multiple outcome phenotypes under specific conditions (Lopez-Suarez et al., 2022; Targett et al., 2024). Differentiating SH-SY5Y neuroblastoma cells into neuron-like cells by adjusting the culture medium has become a valuable tool in neuroscience research. This approach offers several benefits, including the capacity to expand the cell line on a large scale before differentiation, relatively low costs, simplified culture protocols compared to primary neuron cultures and bypassing the ethical concerns associated to animal models and other human-derived cell cultures (Kovalevich & Langford, 2013). Additionally, SH-SY5Y cells are derived from human tissue, so they express human-specific proteins and protein variants that rodent primary neurons lack (Ross et al., 1983). Differentiation of SH-SY5Y line induces key neuronal properties, including the extension of neurites, increased electrical excitability, the formation of functional synapses, and the expression of neuron-specific enzymes, neurotransmitters, and receptors (Påhlman et al., 1981; Påhlman et al., 1984; Lopes et al., 2010).

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In the context of neuronal differentiation, retinoic acid, a synthetic derivative of vitamin A, is the most widely described and employed factor to induce differentiation. In timespans of 3-7 days, it induces a morphological shift from neuroblasts to neuron-like cells and significantly reducing proliferation rates (Encinas et al., 2000; Lopez-Suarez et al., 2022). RA-differentiated SH-SY5Y cells generally adopt a dopaminergic phenotype that is also mixed with a cholinergic phenotype marked by choline acetyltransferase (ChAT) and vesicular monoamine transporter (VMAT) activity (Presgraves et al., 2004; Lopes et al., 2010). Retinoic acid drives differentiation in SH-SY5Y cells through multiple mechanisms, being the most characterized one the gene reprogramming through activation of nuclear receptors (RARs/RXRs) (Girardi et al., 2019). Other nongenomic pathways include the CREB sustained phosphorylation and ERK1/2 stimulation, PI3K/Akt signalling pathway activation, mitochondrial remodelling and cytoskeleton reorganization, collectively promoting a mature neuron-like phenotype and cell survival (Masiá et al., 2007; Simões et al., 2021; Kaya et al., 2024). Despite these advantages, differentiation with RA has limitations, particularly concerning cell population heterogeneity. For this reason, in the past two decades though, there has been a shift to the co-differentiation protocols with other factors to avoid the presence of epithelial cells and enhance neuronal characteristics of the cell line (Lopez-Suarez et al., 2022).

Phorbol esters, such as PMA, represent another old and well-characterized approach to induce differentiation in SH-SY5Y neuroblastoma cells. PMA co-treatment, often applied posteriorly to RA differentiations in concentrations ranging from 10^{-8} to 10^{-7} M for 3–5 days, prompts SH-SY5Y cells differentiation containing distinct features such as extended, uneven neurite outgrowth and prominent varicosities (Påhlman et al., 1981; Kovalevich & Langford, 2013). PMA-induced differentiation also significantly slows cell proliferation, leading to partial growth arrest that reinforces the maturation-like state of the cells into a dopaminergic phenotype with adrenergic characteristics (Murphy et al., 1991; Presgraves et al., 2004). At the mechanistic level, PMA drives differentiation by activating protein kinase C (PKC). PKC activation initiates a cascade of phosphorylation events that engage several cellular pathways, including the mitogen-activated protein kinase MEK/ERK pathway leading to a genetic upregulation of important genes for neuronal differentiation such as β-tubulin, MAP2 or the dopamine transporter (DAT) (Wu et al., 2007; Zogovic et al., 2014).

Apart from retinoic acid and phorbol esters, several other factors are described to induce differentiation in SH-SY5Y neuroblastoma cells. For instance, dibutyryl cyclic AMP (dbcAMP), a cyclic AMP analog, promotes neurite extension and upregulates growth-associated protein 43 (GAP43), an indicator of mature neuronal development. Treatment with dbcAMP also increases tyrosine hydroxylase (TH) activity and noradrenaline content via the protein kinase A (PKA) pathway, producing a largely adrenergic neuron-like phenotype (Sánchez et al., 2004; Kume et al., 2008). Other methods include staurosporine, a protein kinase C (PKC) inhibitor that induces neuritogenesis but makes cells more susceptible to cytotoxic agents (Tieu et al., 1999). Growth factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) promote synaptogenesis, neuronal polarization and axonal

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outgrowth (Hromadkova et al., 2020; Dravid et al., 2021). Finally, neurobasal medium with B27 supplement is also used for promoting neuronal growth (Kovalevich & Langford, 2013).

All of the mentioned factors have been commonly used over the past thirty years for modelling neurodegenerative diseases in the SH-SY5Y cell line, but in the past few, a novel approach has been described for a novel differentiation process of this line. Glucagon-like peptide-1 is an incretin hormone produced by enteroendocrine L-cells in the small intestine, initially identified in the early 1980s (Bell et al., 1983). GLP-1 plays a crucial role in regulating blood glucose by stimulating insulin release from pancreatic β-cells, slowing gastric emptying, reducing food intake, and inhibiting glucagon release from α-cells (Drucker & Nauck, 2006). These properties have made GLP-1 agonists a main target for diabetes, hyperglycaemia and obesity treatments but has also shown potential as a neuroprotective agent (Smilowitz et al., 2014; Müller et al., 2019; Li et al., 2021b). Besides the common role of GLP-1, it has also exhibited trophic characteristics. In a wide variety of cell types, GLP-1 treatment induces a gene-dependant differentiation, by its binding to GLP-1 receptor (GLP-1R) initiates two primary signalling pathways: the phosphatidylinositol 3 kinase (PI3K) - protein kinase B (AKT) pathway and the protein kinase A (PKA) - mitogen-activated protein kinase (MEK)-extracellular-signal-regulated kinase (ERK/MAPK) pathway (Campbell & Drucker, 2013; Yang et al., 2020). Additionally, the glycogen synthase kinase 3β (GSK-3β) pathway is implicated in GLP-1R signalling, ultimately leading to phosphorylation of cyclic AMP response element-binding protein (CREB) for transcriptional regulation (Chen et al., 2013). Differentiation of the SH-SY5Y cell line with GLP-1 induces a mixed glutamatergic and dopaminergic phenotype, yielding cultures of neuron-like cells with morphological and physiological functionalities resembling those of mature neurons (Luciani et al., 2010; Yang et al., 2020). A representation of the main pathways of action followed by the used factors in this work is summarized in *Figure 38*.

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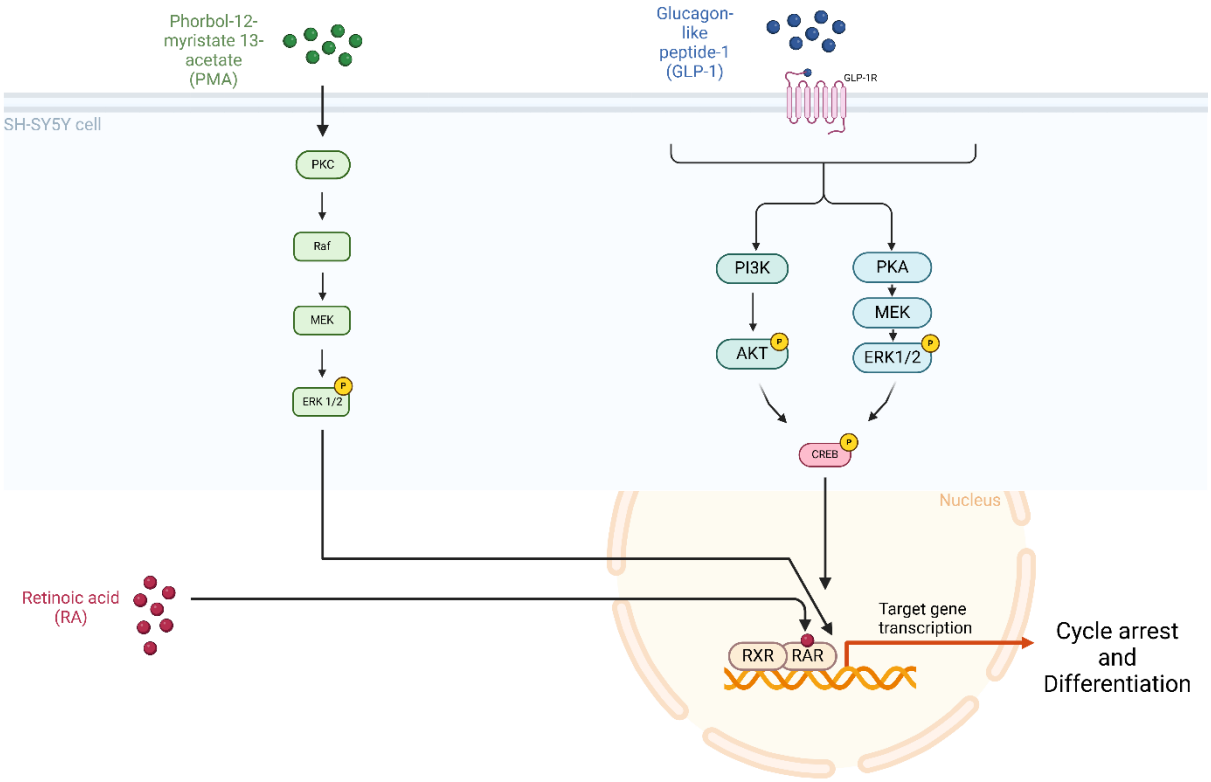


Figure 38. The neurotrophic function of differentiation factors used for cell line SH-SY5Y. Schematic view of the roles and main pathways of RA, PMA and GLP-1 to induce differentiation on *in vitro* cultures of the SH-SY5Y cell line. Retinoic acid exerts its main activity through the gene transcription regulation mediated from the nuclear receptors RAR and RXR. GLP-1R activation mediates the activation of the PI3K-AKT and the PKA-ERK pathways resulting in CREB phosphorylation. PMA involves gene transcription regulation through the activation of protein kinase C cascade. Created in BioRender. Barro, M. (2024).

In this study, taking into account the acquisition of neuronal phenotypic characteristics (neurite arborization and the number of cells with a projection > 30 µm), the homogeneity of cell cultures and the significant promotion in cell survival and proliferation, GLP-1 combinatory treatment was significantly superior across all the variables assessed. PMA also induced neurospheres and clustered population of cells in our test, making it inviable for a scalable and reproducible model. Therefore, in line with the first objective of this thesis, we have proposed a model of SH-SY5Y cells differentiated into mature phenotypic neurons using 10 µM RA and 100 nM GLP-1 over a period of ten days, in preparation for subsequent experiments aimed at applying the model to screening programs.

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6.2 Mutations APP V717I and MAPT P301L induced neurodegenerative-like morphological changes and a hypo-excitatory response in differentiated cells

Over the course of the last 4 decades of fAD research, more than 200 singular mutations have been discovered across the APP, the PSEN 1-2 and the ApoE genes, all closely related to the accumulation of Amyloid-β. APP V717I was the first mutation to have been linked with the late-onset variant of the disease and remains one the most prevalent mutation for the given gene (Lloyd et al., 2020). The overexpression of this mutation has showed an increase in the Aβ42/40 ratio, attributable to Amyloid-β deposition, both on *in vitro* and *in vivo* studies (Muratore et al., 2014; Arber et al., 2019; Lopes van den Broek et al., 2022). Accumulation of this protein in the early stages is predominantly located extracellularly where are mainly found in the synaptic spaces of the hippocampal and cortical neurons in the brain causing synaptic loss, a neuroinflammatory response and impairing neuronal communication (Hampel et al., 2021; Orobets & Karamyshec, 2023). Intracellular accumulation, although less prominent, plays also a key role in the neurodegeneration process as the formation of these deposits in the mitochondrial and cellular membrane, the endoplasmic reticulum (ER) and its interaction with microtubules, contribute to the creation of reactive oxygen species and energetic deficits, impaired axonal transport, calcium dysregulation and receptor modulation (Bayer & Wirths, 2010; Gallego Villarejo et al., 2022). In vitro models using various forms of amyloid, ranging from fAD patient´s cell lines to cell transfection and the use of synthetic oligomers, have been explored over the years to explore the main pathophysiological changes associated to this protein's deposition within the brain (Muratore et al., 2014; Arber et al., 2019; Fontana et al., 2020).

The next set objectives were the induction and characterization of the pathological changes observed upon transfection with either individual APP V717I and MAPT P301L or co-transfected with both after 72 hours. Waiting 72 hours post-transfection with Lipofectamine products before checking results is a standard practice in cell biology, as it provides adequate time for both gene expression and cellular recovery after transfection. Following transfection, cells undergo a period of stress and allowing a full 48–72 hours is recommended to ensure peak expression of the target gene and to stabilize cellular functions before assessment (Sariyer, 2016; Jamour et al., 2024). These effects were measured taking in consideration the morphological variables: average number of cells in order to assess cellular death, average neurite length and neurite arborization to study the neurite shortening and loss observed in AD. Finally, a cell excitability assay using 30 mM KCl was also implemented in the developed model to assess cellular responses post-transfection by measuring intracellular calcium concentration. Measuring intracellular calcium concentration is particularly relevant as it is a secondary messenger that is impaired in Alzheimer's, leading to synaptic dysfunction and encourage the buildup of Aβ plaques and neurofibrillary tangles (Ge et al., 2022) (See introduction for details).

In the developed model, upon transfection with the APP *London* mutation we were able to verify a significant effect on cell death, decreasing neurite arborization and neurite shortening.

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Transfection of SH-SY5Y with APP V717I translates to an increased concentration of long forms of amyloid peptides that are more prone to aggregation (Guyon et al., 2021). Oligomerization of these peptides on in vitro models have seen to initiate a cascade of intracellular stress responses, including oxidative stress, mitochondrial dysfunction, and activation of apoptotic pathways, all of which contribute to neuronal death (De Felice et al., 2007; Querfurth & LaFerla, 2010). Specifically, amyloid-beta can induce mitochondrial dysfunction by impairing electron transport, creating membrane pores and increasing reactive oxygen species (ROS) production, which leads to cell death and neurite damage (Du et al., 2009; Lustbader et al., 2004). Moreover, amyloid-beta oligomers are known to activate caspases, particularly caspase-3, which directly cleaves cytoskeletal proteins, leading to neurite collapse and apoptotic cell death (D'Amelio et al., 2012; Chang et al., 2016). Additionally, amyloid-beta oligomers interact with receptors and create pores on the neuronal membrane, such as the cellular prion protein and glutamate receptors, leading to calcium dysregulation and excitotoxicity, which exacerbates neuronal injury and promotes apoptosis (Lacor et al., 2007). These interactions also destabilize cytoskeletal elements, particularly microtubules, by promoting tau hyperphosphorylation through GSK-3β activation, a process that impairs axonal transport and contributes to neurite retraction and fragmentation (Ittner et al., 2010; Zempel et al., 2013). These severe effects resemblant of neurodegenerative processes align with those previously observed and described in a wide range of in vitro biological models expressing or exposed to pathological forms of Aβ show significant neuronal death and morphology impairments such as neurite density (Stoppelkamp et al., 2011; Mckoy et al., 2012; Yang et al., 2017; Sardar Sinha et al., 2018; Kono et al., 2019; Esselun et al., 2022; Haque et al., 2022; Mak et al., 2024; Qu et al., 2024b; Sebastijanović et al., 2024).

Microtubule-associated protein TAU is the other major protein linked to the aberrant protein aggregates observed in Alzheimer´s patients. Due to its critical role play in maintaining the structure and stabilization of microtubules, this protein has been a target of particular interest for the study of many neurodegenerative pathologies, from frontotemporal dementia to Parkinson´s and Alzheimer´s (Barbier et al., 2019). Despite the close relationship of this protein and Alzheimer´s progression and pathophysiological impairments associated with it, mutations in this protein have not been associated with the onset of the Alzheimer's, but rather linked to other tauopathies like Frontotemporal Dementia linked with parkinsonism (FTDP) or Progressive Supranuclear Palsy (PSP) (Strang et al., 2020). Regardless of mutations not being directly linked by themselves to the onset of fAD, there have been multiple studies over the past decade indicating a direct link or a pleiotropy of mutations such as P301L, R406W or ΔK280 for the early and late onset of the disease (Momeni et al., 2010; Li et al., 2022; Qi et al., 2024). Nevertheless, mutations in tau protein represent a solid form of representation of the aggregation process *in vitro*, as the pattern of hyperphosphorylated TAU is very similar for both diseases, located intracellularly both in the soma and dendrites of neurons. Additionally, both diseases involve a similar change in neuronal and brain tissue morphology and the disruption of correct physiological function (Frost, 2023). The P301L mutation is one of the most common variants associated with FTD involves a significantly increase in Tau aberrant phosphorylation,

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promoting its aggregation in neurofibrillary tangles, which lead to a disruption in the axonal transport and resulting in synaptic dysfunction. The formation of this deposits intracellularly, also contribute to the creation of reactive oxygen species and neuroinflammatory cascade responses, contributing to cell death (Dumont et al., 2011; Didonna, 2020; Al Kabbani et al., 2024). Numerous studies on this particular mutation have shown the influence of it in neurodegenerative processes analogous to those observed in the pathophysiology of Alzheimer´s such as promoting prominent cell death, impaired neurite outgrowth, neurite loss and impaired calcium signalling (Verstraelen et al., 2017: Minaya et al., 2023; Wenger et al., 2023; Wu et al., 2023). Therefore, a solid link is established for AD and FTD between the role and behaviour of Tau protein both at a macro and microscopic level in the brain, making the P301L mutations a good candidate to study and develop new therapeutic options that may be able to mitigate tau-induced neurodegeneration and its interaction with amyloid-β in Alzheimer´s pathology.

Comparably to the morphological changes observed upon transfection with APP V717I, the P301L MAPT mutation elicited significant morphological disruptions, consistent with the effects of pathological tau modifications observed in tauopathies. Cytotoxic effects of MAPT mutations were slightly higher than those obtained for mutation APP V717I, which is probably due to the dependency on tau presence that APP needs to induce drastic neurotoxic effects (Rapoport et al., 2002). The P301L mutation is known to increase tau's tendency to detach from microtubules, which destabilizes the cytoskeleton and disrupts axonal transport (Hutton et al., 1998). This mutation also induces the transcription of a tau protein more prone to phosphorylation (Wang et al., 2023). Hyperphosphorylated tau forms insoluble aggregates within neurons, impairing cytoskeletal structure by detaching from tubulin and leading to impaired axonal transport neurite fragmentation and neuronal death (Liu et al., 2020b; Sone et al., 2020; Buchholz & Zempel, 2024). In our transfected SH-SY5Y cells, we observed a reduction in neurite arborization and neurite shortening, mirroring early findings in similar cellular models and clinical studies where tau pathology was shown to compromise neurite integrity (Shelton & Johnson, 2001; Wen et al., 2021; Verstraelen et al., 2017). Mechanistically, P301L mutant tau aggregates exert neurotoxicity through multiple pathways that result in neuronal death. Notably, are impairing mitochondrial function, disrupting cellular energy balance, and promoting oxidative stress ultimately leading to apoptosis (Alavi Naini & Soussi-Yanicostas, 2015). Hyperphosphorylated tau has also been related with the promotion of necroptotic processes through the activation of NF-κB pathway (Dong et al., 2022). Finally, tau has also been shown to exert heterochromatin de-condensation allowing aberrant transcription of silenced genes that lead to neuronal death (Frost, 2023). Taken together, these results highlight the role of MAPT P301L mutation in driving multiple, interrelated neurotoxic mechanisms, providing a basis for further understanding tau's contributions to Alzheimer's disease neurodegeneration.

In Alzheimer's disease, amyloid and tau pathologies are interconnected, evidenced by neuritic plaques. These protein aggregations are formed from extracellular deposits of amyloid-β and intracellular forms of tau protein that are mixed upon neurite fragmentation. The

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reciprocal interactions of these proteins are supported by genetic studies, in which fAD patients develop both hallmarks from mutations affecting the APP and PSEN 1/2 at a younger age than sporadic patients (Spires-Jones et al., 2017). Aβ can directly influence and exacerbate tau aggregation through mechanisms like cross-seeding or caspase-mediated tau cleavage (Gamblin et al., 2003; Ferrari et al., 2004). Biological models have also demonstrated this aggravation of tau pathology and aggregation due to APP mutations, and therefore a lessened tau toxicity in the absence of amyloid mutations (Oddo et al. 2004a; Choi et al., 2014; Umeda et al., 2014; Walls et al., 2014). The complex synergy between amyloid-beta and tau in Alzheimer's has become a focal point for understanding disease progression and explore novel multi-target therapeutic approaches. Firstly, Amyloid-β deposits greatly increase the phosphorylation of tau and its aggregation through the GSK3β pathway (Zhang et al., 2020a). Synergistic effect of both has also been related to exhibit physical interaction behaviours related to neuronal loss and synaptic dysfunction through glial and kinase activation and further modulating a reduction in protein degradation (Ittner et al., 2010; Manczak et al., 2013; Ittner et al., 2016). Additionally, when both protein deposits are present, a tau-dependant neuronal hypo-excitability is dramatically accelerated in comparison with amyloid based pathologies that exhibit an early hyper-excitability (Busche & Himan, 2020). Physiological models have also been used to express this relationship. When present combined, cytotoxic effects on cells have been proven to be much more intensified compared to amyloid-only models, as well as the microtubule disassembly, mitochondrial damage, severe dendritic loss and the trigger of ectopic cell cycle re-entry, which can lead to exacerbated neuronal death (Götz et al., 2001; King et al., 2006; Caccamo et al., 2007; Zhu et al., 2007; Ittner & Götz, 2010; Quintanilla et al., 2014; Seward et al., 2013; Bloom, 2014; Rajmohan & Reddy, 2017; Habekost et al., 2021; Haque et al., 2022). This synergistic effect between both proteins was replicated in our model, obtaining significant reductions for neurite density, length and cell survival but quantitively higher than those obtained in the cultures singularly transfected. This way, we developed an *in vitro* model capable of expressing two mutations and replicate the cytotoxic combined effect of both on neuronal architecture.

Co-transfection of APP V717I and MAPT P301L led to a significant reduction in the response to KCl-mediated excitation, further integrating a critical aspect of Alzheimer's pathophysiology into the model. This highlights that, in addition to causing morphological damage, these mutations also disrupt essential physiological functions, such as neuronal excitability, which are key to understanding and developing treatments targeting both the morphological and physiological aspects of AD. Hypo-excitatory responses of cells have also been characterized on severe Alzheimer's patients as previously described. Intracellular Aβ has previously been shown to raise membrane potential followed by lack of response to chemical stimuli and induction of rapid apoptotic cell death (Hou et al., 2009; Stoppelkamp et al., 2011). This effect is mediated through the action of amyloid depositions in the cell and ER membranes, affecting voltage-gated calcium channels, NMDA receptor-mediated signalling and intracellular deposits of calcium and other ions (Shankar et al., 2007; Ferreiro et al., 2008). Protein tau has also been linked to calcium dysregulation associated to neurodegenerative

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diseases, by disrupting the functionality of plasma membrane Ca^{2+}-ATPase (PMCA) transport proteins and muscarinic receptors, promoting the liberation without stimuli of intracellular calcium liberation (Berrocal & Mata, 2023). Thus, making it likely that both the registered cell death and hypo-excitation registered in our model are probably related to the disruption of calcium homeostasis.

Taking into consideration all the presented results and in alignment with objectives 2 and 3, we developed a novel in vitro model capable of representing key aspects of Alzheimer's disease pathology. Upon co-transfection, this model exhibits neurodegenerative-like deterioration in both morphological and physiological hallmarks of AD, including reduced cell viability, neuronal shortening, loss of arborization, and hypo-excitability. This model not only mimics AD-like pathology but also provides a biological platform to characterize and study the interactions between the two main protein deposits.

6.3 Melatonin and resveratrol managed to significantly revert the cytotoxic damage induced by mutations APP V717I and MAPT P301L

Previous to the application of the model to drug screening assays, we used melatonin and resveratrol as reference compounds on both image-based and intracellular calcium concentration for the validation of this model and its suitability to high-throughput screening compounds. Melatonin and resveratrol were selected taking in consideration their well described and characterized neuroprotective action in both *in vivo* and *in vitro* models of neurodegenerative diseases. Both compounds were used at a concentration of 1 µM. Melatonin concentration was decided given the range of 10^{-3} M – 10 µM at which it has shown significant observable effects over impaired morphology and viability in neuronal cell models and analysing previous studies on SH-SY5Y cells (Pappolla et al., 1999; Olivieri et al., 2000; Olivieri et al., 2001; López-Iglesias et al., 2014; Buendía et al., 2015; Song et al., 2015; Chinchalongporn et al., 2018; Figueiro-Silva et al., 2018; Shu et al., 2018; Li et al., 2019; Pachón-Angona et al., 2019; Wang et al., 2019; Liu et al., 2020a; Gu et al., 2024). Resveratrol concentrations for in vitro assays have been tested in a wider range than melatonin, exhibiting neuroprotective effects in a concentration of 25 mM – 100 µM (Dos Santos et al., 2022). 1 µM concentration of resveratrol was selected by analysing assays performed on SH-SY5Y cell line (Chao et al., 2008; Chao et al., 2010; Xu et al., 2021) and the cytotoxicity impacting morphophysiological characteristics and viability that increasing concentrations of resveratrol from 1 µM onwards have in this cell line (Chao et al., 2008; Chao et al., 2010; Xu et al., 2021; Yuan et al., 2024). The 48-hour incubation period with resveratrol and melatonin was chosen to allow sufficient time for their bioactive effects to manifest on the SH-SY5Y transfected cell line. This timeframe aligns with previous studies investigating these neuroprotective agents, providing an adequate window for the compounds to modulate cellular pathways such as oxidative stress reduction, mitochondrial protection, and amyloid-beta aggregation inhibition

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(Pappolla et al., 1999; Wu et al., 2011; Wu et al., 2013; Lin et al., 2014a; Chen et al., 2020; Maleki et al., 2024).

Melatonin is a neurohormone produced by the pineal gland and other tissues, and although there is evidence of its localization through the whole body, its large implication with fomentation and maintenance of sleep has prevailed over many decades as its main function within the body (Radogna et al., 2010). In recent years, melatonin´s neuroprotective effect evidence has been widely described for a large variety of neurodegenerative diseases (Gunata et al., 2020). Due to its antioxidant and anti-inflammatory properties, melatonin is directly involved in the scavenge of ROS and reactive nitrogen species (RNS) through melatonin receptor 3 activation (MT3), thus protecting neurons from direct damage occurring from these types of molecules vastly present in Alzheimer´s (Reiter et al., 2010; Tordjman et al., 2017; Kamfar et al., 2024). Additionally, it is also demonstrated the efficacy of melatonin at enhancing the Aβ clearance and reducing amyloid beta production through the transcription regulation of proteins and enzymes like like neprilysin, APP, β-secretase or A Disintegrin and Metalloproteinase 10 (ADAM10, α-secretase) through MT1 and MT2 receptor activity (Li et al., 2020b). Melatonin is also linked to increments in the production of brain-derived neurotrophic factor, further supporting the neuronal growth and survival (Luo et al., 2017; Miranda-Riestra et al., 2022). Regarding neurodegenerative diseases characterized by an abnormal hyperphosphorylation of Tau, such as AD, PD or FTD, melatonin has also showed its influence in numerous key molecular pathways that lead to neuroprotection. One of these main pathways is the active down-regulation and inhibition of GSK-3β and CDK5, two enzymes that play a key role in tau phosphorylation (Das et al., 2020; Chen et al., 2022). Melatonin also contributes to Tau elimination by promoting autophagy and increasing the expression of certain ubiquitin ligases and chaperones (Zhu et al., 2024). Finally, melatonin has also proven to mitigate phosphorylation and aggregation of pathologic tau through upregulation of phosphatase 2A and Peptidyl-Prolyl cis-trans Isomerase NIMA-Interacting 1 (Pin1) (Lu et al., 1999; Ling et al., 2004). The multifaceted nature of melatonin has been thoroughly tested in many *in vitro* and *in vivo* models, providing this way a neuroprotective solid foundation to revert amyloid and tau changes on pre-clinical models, that unfortunately failed at clinical trials due to its inability to dissolve plaques despite its efficacy at inhibiting small oligomeric forms of Aβ (Roy et al., 2022). A summarized schematic view of neuroprotective biomolecular pathways of melatonin can be observed in *Figure 39*. In this model, melatonin follows the trend of showing a neuroprotective effect against damage induced by pathological forms of tau and amyloid-β. At 1 μM concentration, the addition of this compound to cell cultures managed to significantly revert the damage caused by the accumulation of hyperphosphorylated tau and β-amyloid, across all morphological and physiological variables assessed, upon transfection with the previously described mutations. These results align with those described previously on *in vitro* studies, in which melatonin is able to significantly palliate neurodegenerative processes such as neurite formation and microtubule enlargement (Olivieri et al., 2000; Benitez-King, 2006; Olivieri et al., 2008; Ortiz et al., 2008; Panmanee et al., 2015; Shi et al., 2018; Panyatip et al., 2020; Ozansoy et al., 2020).

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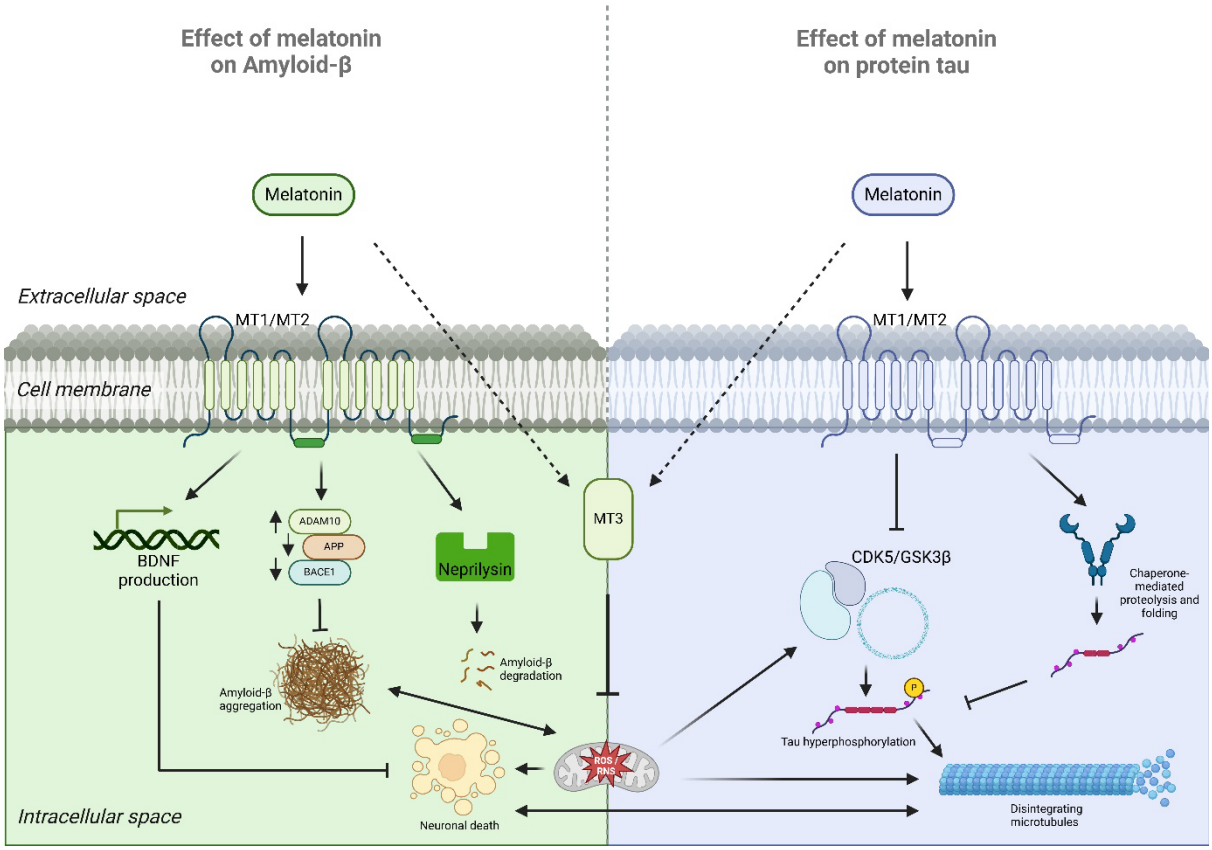


Figure 39. The neuroprotective biomolecular pathways of melatonin. Graphical representation of the melatonin's neuroprotective actions against proteins Amyloid-β and tau-induced damage. MT3 activation (Quinone reductase 2) mediates the scavenging of ROS and RNS, thus alleviating consequential events such as neuronal death and disintegrating microtubules. MT1 and MT2, two g-protein coupled receptors, mediate neuroprotective actions such as promoting the non-amyloidogenic APP synthesis pathway or promoting amyloid and tau proteolysis through genetic regulation of key proteins. Created in BioRender. Barro, M. (2024).

Resveratrol is a natural polyphenolic compound that can be predominantly found in berries and grape skin and peanuts. Similarly to melatonin, it is another compound that has gained the attention of the neuroscientific community over the past decade due to its promising results at preventing neuronal damage and arising as a therapeutic option for many neurodegenerative diseases, including AD (Zhang et al., 2021c). Resveratrol shares with melatonin being an antioxidant and anti-inflammatory molecule with an action range that covers many different pathological cascades and targets involved in the progression of Alzheimer´s. The scavenging of ROS and RNS and the upregulation of antioxidant enzymes such as superoxide dismutase 1 (SOD1) or glutathione peroxidase 1 (GPx1), aid in palliating the oxidative damage in many diseases including AD (Spanier et al., 2009). Resveratrol has also been found to reduce ROS and microglial activation through the downregulation of NADPH oxidase (Yao et al., 2015). Targeting amyloid-β, resveratrol has shown to reduce the levels of the amyloidogenic β-secretase and to have an hydrophobic interaction with Aβ-42, leading to the formation of less toxic fibrils and contributing to an increased cell survival (Jia et al., 2017; Sharma et al., 2023). Another major key points of resveratrol activity is its ability to activate the nicotinamide

adenine dinucleotide NAD (+)-dependent histone deacetylase Sirtuin-1 (SIRT-1). SIRT-1 activation is demonstrated to improve cell survival and reduce neuroinflammation by a direct modulation in the expression of p53 and nuclear factor-κB (Manjula et al., 2021). Moreover, SIRT-1 activation is thought to reduce the Aβ oligomerization by promoting a non-amyloidogenic processing pathway through ADAM 10 expression and also reduce the β-catenin degradation by Aβ and allowing its translocation to the nucleus for gene regulation (Jia et al., 2020; Surya et al., 2023). Resveratrol, via activation of SIRT-1, is also important in aiding the removal of hyperphosphorylated Tau, as the deacetylation that this produces in the protein, helps with an easier degradation (Min et al., 2018). Resveratrol also aids in the tau ubiquitination and its degradation by proteasome 20s (Ashrafizadeh et al., 2020). Finally, resveratrol has also shown its capability of reducing the phosphorylation levels of tau at AD specific regions of the protein by inducing an enhanced activity of protein phosphatase 2 A (PP2A) (Schweiger et al., 2017; Wiciński et al., 2020). Schematic view of the different neuroprotective pathways proposed for resveratrol is presented in *Figure* D.3. Despite resveratrol´s undeniable neuroprotective properties, its efficacy has been mainly demonstrated in vitro, but the issues observed in clinical trials, its low intestinal absorption rate and the poor bioavailability, have limited its therapeutic usage (Islam et al., 2022).

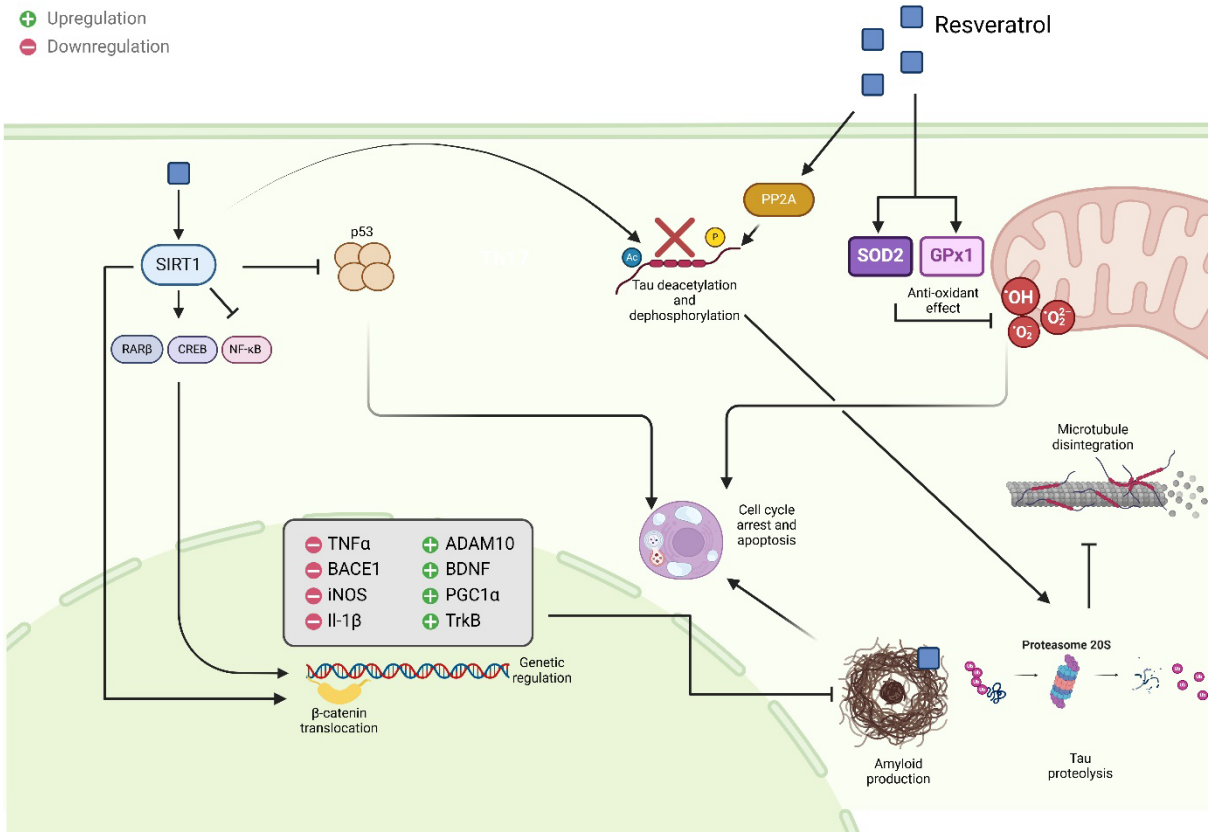


Figure 40. The neuroprotective biomolecular pathways of resveratrol. Schematic representation of the neuroprotective functions of resveratrol in AD pathophysiology. SIRT1 activation induces gene regulation processes, the inhibition of apoptosis through downregulation of p53 and also is involved tau deacetylation. Antioxidant effects of resveratrol are mediated though upregulation on enzymes SOD2 and GPx1 as well as

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downregulation of iNOS. Dephosphorylation of tau through upregulated enzyme PP2A aids with its proteolysis and prevent microtubular damage. Created in BioRender. Barro, M. (2024).

Upon treatment of the mutated cell line with 1 μM of resveratrol, significant neuroprotective effect at preventing the effects of MAPT mutation across all morphological variables studied was observed. Same results were obtained in the co-transfection assays in which resveratrol showed a significant protective effect at those morphological changes occurring post-transfection as well as improving cell survival. These results follow the neuroprotective role that different forms of resveratrol have demonstrated in palliating a wide variety of neurotoxic insults in the SH-SY5Y cell line (Wang et al., 2004; Marambaud et al., 2005; Rigolio et al., 2005; Lee et al., 2007; Chao et al., 2010; Laudati et al., 2019; Hu et al., 2021; Xu et al., 2021; Zhang et al., 2021e; Donozo et al., 2023; Mahamud et al., 2023). Regarding the neurodegeneration-like alterations induced by mutated protein APP V717I, resveratrol only managed to be significantly effective on palliating the decrease on the average neurite length. The lack of significant attenuating effects on Aβ damage, suggests that higher concentrations may be necessary to replicate those effects observed on hyperphosphorylated tau. Additionally, it might also highlight the ineffectiveness of resveratrol to prevent Amyloid-β oligomerization, being the 24-hr timespan of APPV717I expression without treatment enough to induce the cytotoxic damage observed (Feng et al., 2009; Granzotto & Zatta, 2011). Another factor to highlight to understand this result is that amyloid deposition has been characterized to be over an order of a magnitude faster than those of tau in vitro or in mice transgenic models, making the amyloid-related cytotoxicity to appear faster than tau-derived pathophysiological changes (Meisl et al., 2021). Furthermore, the lowered neuroprotective effect of resveratrol in comparison with that exhibited by melatonin might be due to the small cytotoxic effects that resveratrol exhibits even at 1μM on the SH-SY5Y cell line, despite its clear prevalent neuroprotective properties (Yuan et al., 2024).

Melatonin and resveratrol also managed to significantly revert the lowered response registered in transfected cells upon their stimulation with 30 mM KCl. Besides the clear neuroprotective role that melatonin exhibits at reverting morphological damage, and thus maintaining more alive cells and affecting the overall excitability registered in each well, it has also been demonstrated its efficacy at reverting early neuronal death related to hyperexcitation of neurons and to regulate intracellular calcium levels (Cardinali et al., 2014). This is probably mediated in this cell line by the reduction in reactive oxygen species, lowered Ca^{2+} intracellular levels observed upon melatonin treatment as well as the decrease mitochondrial depolarization through transient receptor potential cation channel A1 (TRPA1) (Naziroğlu, 2015; Özşimşek & Övey, 2022). Resveratrol has also been studied for its abilities to counteract AD-related excitatory impairments on *in vitro* and *in vivo* models successfully. Resveratrol has the capability of reverse the Aβ inhibition of potential dependant K^+ channels, protect the activity of Na^+/K^+ ATPase pump and also regulate the Na^+- dependant intracellular calcium overload (Zhang et al., 2008; McCalley et al., 2014; Yin et al., 2017). Excitability impairment in AD, is mainly mediated by Aβ deposits and therefore, maybe the reason for resveratrol lowered

neuroprotective action against this physiological change in our model, as it follows the trend observed against APP V717I-derived morphological insults (Yan et al., 2020).

In summary, following objective 4, the standard compounds melatonin and resveratrol showed the previously described neuroprotective properties of these molecules in our model, thereby validating the designed and proposed model as a tool in which pathophysiological neurodegenerative processes can be reverted with neuroprotective compounds, and therefore justifying its use as a suitable platform in which to perform drug discovery assays. Thus, making this model, a unique platform for conducting HTS assays for large number of compounds that could provide new insights on the functioning and interaction between these two proteins in AD, as well as serving as a valuable tool for identifying pharmacological *hits* with a potential therapeutic benefit at aiding with the neurodegeneration derived from protein deposits.

6.4 A neuronal excitability assay was developed for high-throughput screening of neuroprotective compounds in differentiated and co-transfected SH-SY5Y cells

Excitability is a critical aspect of understanding Alzheimer's because it reflects the functional state of neuronal networks, which are highly sensitive to the balance between excitatory and inhibitory inputs. Neuronal excitability is tightly regulated by ion channels, neurotransmitters, and calcium signalling, which together shape synaptic communication and brain network dynamics (D'Amelio & Rossini, 2012). In the context of AD, in early stages, hyperexcitability is frequently observed, driven by neurotoxicity on inhibitory interneurons and changes in glutamate and acetylcholine homeostasis. This hyperexcitability can lead to synaptic dysfunction and loss, ultimately contributing to neuronal damage and vice versa. However, as the disease progresses, shifts in the protein deposition and excitotoxicity resulting in hypo-excitability (Hampel et al., 2021; Lauterborn et al., 2021; Targa Dias Anastacio et al., 2022). Hypo-excitability results from various pathological events, including disruptions in calcium homeostasis that impair mitochondrial buffering capacity and lead to energy deficits; prolonged excitotoxicity, which causes synaptic pruning and neuronal loss; and chronic stress from pathological protein aggregates in neuronal membranes, impairing ion channel function (Targa Dias Anastacio et al., 2022; Verma et al., 2022).

Therefore, the following objective set for this thesis was the development of a biochemical assay based on the intracellular calcium measurement post-excitation with KCl to identify compounds with different action mechanisms performing a high-throughput screening assay. Intracellular calcium measurement was selected due to the advantages this technique offers in terms of automation and miniaturization for drug discovery assays in comparison to a phenotypic screening (Monteith & Bird, 2005). For assay development, cell cultures were differentiated and co-transfected with APP V717I and MAPT P301, to induce the hypo-excitability and cytotoxicity addressed in previous sections. Upon co-transfection, cultures exhibited a significant hypo-excitability to KCl stimuli compared to differentiated cells. Despite

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the reduced response and due to the acquisition of neuronal properties during differentiation, co-transfected cultures still shown a higher signal than that registered in undifferentiated cell cultures. Assay validation for its application to HTS assays was performed by using the same standard neuroprotective controls melatonin and resveratrol, that also managed to significantly revert the impaired excitability in co-transfected cultures. This assay, developed in line with objective 5, represents a reliable tool for high-throughput screening to identify compounds that modulate neuronal excitability in Alzheimer's disease. By correlating intracellular calcium dynamics with neuronal excitation, the assay bridges the gap between phenotypic observations and mechanistic insights. The successful validation using standard neuroprotective agents, melatonin and resveratrol, highlights the assay's sensitivity in detecting neuroprotective effects in the context of impaired excitability caused by pathological co-expression of APP V717I and MAPT P301L mutations.

Application of our developed model to HTS assays was performed using the Prestwick® chemical library. This chemical library provides the opportunity of testing 1120 compounds that have been already approved for its use in humans by the main drug administrations, thus allowing for an easier translational approach of repurposed compounds. Compounds were added following the same methodology as for melatonin and resveratrol and keeping the 1 µM concentration. The Prestwick® chemical library was screened at a concentration of 1 µM, which falls within the commonly used concentration range of 1–10 µM for HTS. This concentration offers a balanced but restrictive approach to capture potential therapeutic action while minimizing cytotoxicity, off-target effects and the presence of false-positive artifacts. Additionally, this concentration has been used in HTS assays with this chemical library in neurodegenerative and other diseases, ensuring compatibility with established protocols with other studies in the field (Brownjohn et al., 2017; Panda et al., 2019; Ghorbanalipoor et al., 2024). During the screening assays, differentiated cell cultures were used as positive controls and co-transfected cultures as negative controls, taking in considerations their mean values to assess the 100% and 0% response in our evaluation rubric. Idoneity of controls reproducibility and their quality, was measured using Z′ factor and signal window, that were calculated for every plate used during the screening process, with values superior to 0.5 and 2 respectively ($Z'_{mean} = 0.642 \pm 0.069$; $SW_{mean} = 7.413 \pm 1.555$), which are the standardized values for assay reliability and robustness (Iversen et al., 2012; Murray & Wigglesworth, 2016). A total of 45 *hits* were identified from the Prestwick® screening, defined by mean activity + 2SD. The use of mean + 2 SD instead of the more restrictive mean + 3 SD was chosen to enhance the sensitivity of the screening process and ensure that a broader range of potential hits were identified. This strategy is beneficial for HTS assays with wider range of compound activity, where the goal is to maximize the discovery of candidates that warrant further validation. After a dose-response characterization and validation of these compounds with replicate assays, eight compounds were confirmed as *hits* against the reduced hypo-excitability observed as a consequence of transfection with mutated proteins APP V717I and MAPT P301L. From the eight confirmed compounds, three, doxepin and mianserin, both antidepressants, and ethosuximide, an anticonvulsant, have described therapeutic activity in the CNS and are commercially available

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for these neurological conditions. Two of the remaining five, rifampicin and dapsone, are antibiotics commonly used in the treatment of leprosy, and have also shown potential therapeutic use in a range of neurodegenerative models and trials. Amongst the remaining compounds, metolazone, a diuretic, canavanine, an arginine analogue with potential antioxidant effects, and betazole, a pyrazole derivative, do not have a direct described therapeutic activity within the CNS but have also demonstrated significant protective activity in our developed model and may hold therapeutic potential in treating neurodegenerative diseases.

Doxepin is a tricyclic antidepressant (TCA) widely used to treat depression and anxiety disorders in clinic. Similarly to the rest of of TCAs, it exerts its therapeutic role in a multi-target mechanism of action involving serotonergic, adrenergic, glutamatergic and GABAergic receptors as well as modulate the sodium and calcium voltage-gated channels (Harmer et al., 2017). Up to now various other therapeutical properties of doxepin have been reported, such as anti-inflammatory, anticonvulsant effects and even neuroprotective effects (Reisi et al., 2017). Doxepin's neuroprotective role has been related to a wide variety of pathways. Experimental data on *in vitro* and *in vivo* models of neurodegenerative diseases has demonstrated its protection against oxidative stress, through the reduction of intracellular calcium overload and increasing the activity of antioxidant enzymes, its ability to modulate amyloid-β induced memory deficits and neuronal toxicity as well as promote the downregulation in the expression of pro-apoptotic genes BAD and BAX (Ji et al., 2004; Bu & Zu, 2017; Reisi et al., 2017). In the present study, doxepin managed to revert the response to KCl with a EC_{50} potency of 110 nM. Therefore, doxepin was found as one of the strongest candidates at reversing hypo-excitability derived from both mutations. This aligned with the previously detailed studies, as preventing apoptosis and maintaining the intracellular calcium homeostasis are two key variables impacting the response of cells to KCl.

Mianserin is a second-generation tetracyclic antidepressant that enhances the serotonin and norepinephrine signalling acting on receptors such as 5-HT_2 and α_2 (Marek et al., 2002). Beyond its antidepressant role, mianserin has also demonstrated sedative, anxiolytic, antiemetic, and appetite-stimulating activities, making it versatile in treating related conditions. Its diverse receptor interactions provide therapeutic benefits across several psychiatric and physical health domains (Fasipe, 2018). Mianserin has demonstrated neuroprotective properties *in vitro* through the anti-apoptotic effect by the inhibition of TNF- α pathway (Olianas et al., 2018). Moreover, mianserin just like the other antidepressant *hit* doxepin, has proven to be a direct protector against oxidative stress damage and enhancer of synaptic transmission in an aging *C.elegans* model (Rangaraju et al., 2015). In addition to studies with mianserin, others using closely related molecules both chemically and in pharmacological targets such as mirtazapine, have found that this compound is able to recover the vesicular traffic previously inhibited by Aβ and even recover neuronal polarity and neurite atrophy and arborization in primary rat neuronal cultures at 10 μM concentration (Fabbretti et al., 2021). These results align with the registered neuroprotective effect shown by mianserin in this particular study, being able to preserve cell and neurite integrity in vitro, it is also expected to rise the excitability

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levels. Mianserin showed a EC_{50} potency of 4 nM, the lowest registered amongst all compounds, highlighting the efficacy of this anti-depressant even at low concentrations.

Ethosuximide is used clinically as an anti-convulsant antiepileptic drug by inhibiting T-type voltage-gated Ca^{2+} channels and suppressing low-threshold Ca^{2+} currents, specifically for absence seizures, a children's idiopathic epilepsy form (Kostyuk et al., 1992; Hwang et al., 2012). Similarly to metolazone and mianserin, ethosuximide has gain interest and relevance as a potential neuroprotective compound due to its prolongation of life span and reversal of age-related neurodegeneration in *C.elegans* models (Collins et al., 2008; Rangaraju et al., 2015; Ito et al., 2021). Mechanistically, ethosuximide has shown to inhibit Aβ-mediated neurodegeneration, providing neuroprotection and higher cell survival rates through the activation of PI3K-AKT pathway involving the translocation of catenin into the nucleus in a similar manner to the explained for resveratrol (*Section* 6.3) (Tiwari et al., 2015). Furthermore, the activation of this pathway also involves the inactivation of GSK-3β, and theoretically reducing the phosphorylation levels of tau protein, for which ethosuximide has shown significant efficacy at neuroprotection in tau-related neurodegenerative phenotypes (Tiwari et al., 2015; Chen et al., 2018). *In vitro*, at a 10 μM and higher concentrations, ethosuximide has reportedly been able to significantly prevent cell death upon cytotoxic insults (Sahin et al., 2021). In line with these results, ethosuximide managed to revert the hypo-excitatory response seen in our Alzheimer's model with an EC_{50} potency of 20 nM, probably through the neuroprotective properties previously described and hypothetically through the ability to prevent early hyper-excitatory behaviour seen in AD as well as protect from Aβ oligomer production through the blockage of voltage-dependent calcium channels that are expressed in the SH-SY5Y cell line (Wareski et al., 2009; Wen et al., 2011; Tan et al., 2012).

Dapsone and rifampicin are two antibiotics and major treatments of choice either as a mono or multi-therapy for leprosy and some forms of dermatitis (Guragain et al., 2017; Kim et al., 2017; Kurien et al., 2024). Both compounds have been thoroughly studied for Alzheimer's and other neurodegenerative diseases, showing promising neuroprotective efficacy and even reaching clinical trial stages for Alzheimer in the case of rifampicin (*Clinicaltrials.gov*. Available at: https://clinicaltrials.gov/search?cond=Alzheimer+Disease&intr=Rifampicin (Accessed: 26 November 2024)). Beside dapsone's antibiotic properties, its anti-inflammatory potential is also well characterized through its application to dermatological diseases. In recent years, new attributes such as being antioxidant, anti-excitotoxic and antiapoptotic have been demonstrated in a wide variety of neurodegenerative models (Diaz-Ruiz et al., 2021). Regarding Alzheimer's disease, dapsone is particularly interesting due to its ability to cross the BBB and have bioactive concentrations in the CSF, alongside being effective countering the progression of MCI, dementia and AD on numerous trials with patients (Nau et al., 2010; Lee et al., 2022). Although it does not seem to have a direct effect on amyloid-β accumulation, dapsone exerts its neuroprotective role through a wide variety of pathways (Endoh & Tabira, 1999; Diaz-Ruiz et al., 2022). Its antiexcitotoxic properties involve the regulation of glutamate release, thereby preventing the activation of metabotropic and ionotropic glutamate receptors. This regulation prevents membrane aberrant depolarization in early AD, effectively modulating

signaling cascades and ion homeostasis (Diaz-Ruiz et al., 2021). Additionally, dapsone has significant antioxidant effects, reducing the formation of superoxide radicals and hydrogen peroxide generated during excitotoxic processes, while also decreasing nitric oxide levels to mitigate oxidative stress and cell death through the inhibition of myeloperoxidase and peroxidase (Bozeman & Thomas et al., 1992; Shayesteh et al., 2022; Lee et al., 2022). Other critical mechanisms of dapsone include its modulation of inflammasomes and pro-inflammatory signalling cascades as well as demonstrating anti-apoptotic efficacy (Khalilzadeh et al., 2022). Dapsone regulates cell death through the reduction of TNF-α, NF-kB and caspase levels, while promoting the expression of BDNF (Ríos et al., 2015; Mahale et al., 2020).

Rifampicin shares some similarities with dapsone's properties such as the neuroinflammation against inflammatory and oxidative pathogenic pathways besides being able to cross the BBB but differs in key aspects regarding AD (Yulug et al., 2018). Mechanistically, rifampicin has shown to promote neuronal survival through the stabilization of the mitochondria and the endoplasmic reticulum under stressors through the modulation of key cytoprotective proteins, such as glucose-regulated protein 78 (GRP78) and Bcl-2 (Xu et al., 2007b; Yulug et al., 2018). Rifampicin's antioxidative properties, mediated by its naphthol-hydroquinone ring, have been shown to promote radical-scavenging activity, particularly at the C-1 hydroxyl group (Tomiyama et al., 1996). Other described pathway of modulating oxidative stress include the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) with the consequential inhibition of COX-2 involved in tau and amyloid accumulation in Alzheimer's (Chen et al., 2016; Guan & Wang, 2019). Probably the major difference between both compounds is the ability of rifampicin to directly physically interact with low-*n* β-amyloid fibrils, thus preventing its aggregation and the associated neurotoxicity induced by interactions of amyloid oligomers with intracellular organelles and the cell membrane. Tau dephosphorylation and inhibition of pro-apoptotic caspase-based cascades are another described and tested mechanisms *in vitro* in which rifampicin has demonstrated efficacy (Umeda et al., 2014; Iizuka et al., 2017; Umeda et al., 2018; Yulug et al., 2018). Despite promising preclinical data, clinical trials on rifampicin for Alzheimer's disease have shown inconsistent results, with some promising findings suggesting cognitive benefits at higher doses (450 mg/day) over extended periods, but later trials failing to replicate these outcomes, likely due to suboptimal dosing and most importantly late intervention, highlighting the difficulties of AD treatment once the disease has progressed to mild and severe stages (Endoh et al., 1999; Loeb et al., 2004; Meng et al., 2008; Molloy et al., 2013; Iizuka et al., 2017).

In our developed Alzheimer's model both compounds showed a dose-response effect, with EC_{50} potencies of 150 nM for dapsone and 220 nM for rifampicin, at reverting hypo-excitatory signal registered in co-transfected cells. This lowered maximum signal of rifampicin and with respect to other compounds could be a direct consequence of the maximum used concentration (10 μM) as similar previous studies for rifampicin have been performed using concentration between 100 and 300 μM (Umeda et al., 2014; Wei et al., 2014; Acuña et al., 2019). Dapsone has also been recently tested in the SH-SY5Y cell line against cytotoxic insults, showing the best results with concentrations of 20 μM onwards, making these increased concentrations an

interesting variable to include in further research studies using the proposed neurodegenerative model in this doctoral thesis (Islas-Cortez et al., 2024).

Regarding metolazone, this compound is used clinically as a thiazide-class diuretic and for the treatment of hypertension and congestive heart failure (Bond et al., 2024). Neuroprotective effects of these compounds type are limited and scarce, although its use in recent years have shown a significant reduction in risk of AD development patients using these medications, in particular thiazide diuretics were the ones showing the greatest efficacy (Chuang et al., 2014; DeLoach & Beall, 2018). At a molecular level, metolazone has proven *in vitro* to increase the expression levels of mitochondrial chaperones mtHSP70 and HSP60 (Ito et al., 2021). Overexpression of mtHSP70 has been shown to prevent damage, apoptosis, and cytotoxicity caused by amyloid-beta, while also reversing amyloid-beta-induced mitochondrial dysfunction. The protective effects on mitochondrial function were linked to the inhibition of the mitochondrial permeability transition pore, a reduction in caspase activation, lower free radical levels, and decreased cytosolic calcium release (Bahr et al., 2021). HSP60 normal functioning has also been related to the inhibition of Aβ fibrilization and aggregation (Mangione et al., 2016). Potentially the efficacy of metolazone at reversing the hypo-excitatory effect in out model, exhibiting an EC_{50} potency of 20 nM, comes for these neuroprotective effects arising from the maintenance of mitochondrial functioning and this preventing oxidative damage and apoptotic events leading to the loss of overall signal upon excitatory inputs.

Canavanine or L-canavanine is perhaps the most enigmatic and unexpected hit from the drug screening. This molecule is a potent antagonist of L-arginine, discovered in the jack bean, and registered to be synthesized by vegetables, in specific leguminous seeds, has been tried clinically for its anti-carcinogenic properties (Rosenthal, 1982; Fried et al., 2021). Bibliography for this compound in relation to its mechanism of action is varied and often contradictory. On one hand, canavanine has shown a neurotoxic behaviour, resulting in significant neuronal and astrocytic death (Dasuri et al., 2011). In addition to the cytotoxicity, canavanine has also been related to critical impairment of mitochondrial function, protein homeostasis and mitoribosome performance on the SH-SY5Y cell line exposed to a high concentration of 20 mM of canavanine (Konovalova et al., 2015). On the other hand, there are studies showing a neuroprotective action through the inhibition of enzyme iNOS and cNOS, lowering the pathological forms of nitrogen species in endotoxemia and LPS treatments treatment models (Fatehi-Hassanabad et al., 1996; Abd El-Gawad, Khalifa, 2001). Canavanine also interacts with imidazoline receptors, which upon activation, have demonstrated to ameliorate AD pathophysiology related to amyloidosis, tau hyperphosphorylation and oxidative stress processes (Chang et al., 2015; Abás et al., 2017; Griñán-Ferré et al., 2019; Vasilopoulou et al., 2021). Furthermore, canavanine has also been registered as a *hit* against ganglioside GM1 accumulation on a neuronal stem cell model (Kajihara et al., 2020). Ganglioside GM1 accumulation has been described as a promotor through co-assembly of Amyloid-β depositions, further exacerbating the pathology (Matsuzaki et al., 2020; Hu et al., 2023). In the present model, canavanine was able to revert the hypo-excitability of cells with an EC_{50} potency of 50 nM. The maximum response was registered at 10 μM concentration, which is significantly lower than the described neurotoxic concentrations

previously commented, and probably enough to exert an antioxidant effect on the cell line without exhibiting cytotoxicity, resulting in a higher rate of cell survival and therefore signalling of intracellular calcium.

Betazole or betazole-hydrochloride is a pyrazole-derived molecule with agonist properties for H_2 histamine receptor that has been employed clinically as a gastric stimulant for the production of gastric inhibitory peptide in diagnostic process for gastric secretory functioning (Spitz et al., 1982; Ghosh et al., 2011). Betazole, as an individual molecule, lacks published literature regarding its potential neural activity and interactions, but so does its molecular backbone pyrazole. Pyrazoles have garnered significant interest in the past decade for their therapeutic applications in inflammation, cancer, and neurodegenerative diseases, owing to their ability to modulate oxidative stress, inflammation, and enzyme activity (Küçükgüzel & Şenkardeş, 2015). Mechanistically, pyrazole derivatives exhibit antioxidant properties by scavenging reactive oxygen species and inhibiting lipid peroxidation, contributing to cellular resilience against oxidative damage (Jayaraj et al., 2014; Ahsan et al., 2015; Silva et al., 2018). Pyrazoles are also known to interact with multiple receptor systems and ion channels, suggesting a capacity to modulate synaptic activity and neuronal excitability (Qi et al., 2011). Furthermore, pyrazoles such as riluzole, trimeric aminopyrazole or curcumin pyrozole derivatives have demonstrated a significant efficacy at preventing amyloid fibrilization and tau aggregations (Hochdörffer et al., 2011; Ahsan et al., 2015; Okamoto et al., 2018; Li et al., 2021a). In this study, betazole successfully reversed the hypo-excitability of transfected cells with an EC_{50} potency of 10 nM, indicating restoration of intracellular calcium dynamics and ion channel function. A potential explanation to this neuroprotective behaviour for betazole might involve the activation of cAMP by H2 pairing with betazole, thus leading to a cascade of intracellular signalling involving the activation of protein PKA which induces CREB transcriptional signalling (Timmerman & Van der Goot, 2009; Zhang et al., 2023a). This particular pathway has been a therapeutical target of Alzheimer's for a long time and demonstrated to be dysregulated in AD, and its activation to enhance synaptic plasticity, reducing inflammation, regulate voltage-gated ion channels and inhibit amyloid deposition, potentially alleviating AD-associated neuropathology (Murphy et al., 2014; Sharma & Singh, 2020; Zhang et al., 2020b).

Overall, in line with objective 6, it was demonstrated that the developed model allows for its application to HTS assays, allowing the identification of novel compounds that can significantly revert the pathological characteristics derived from the co-transfection of mutated amyloid precursor protein V717I and tau protein P301L. Eight total *hits* were confirmed for the reversion of AD-associated hypo-excitability, mainly supported with previous literature on pre-clinical animal and cellular models, refuting the suitability of this model for its use in larger screening assays, thus helping with translationability of *in vitro* models for this particular disease.

In conclusion, this doctoral thesis contributes to the development of a novel in vitro Alzheimer's model, based on the use of the differentiated SH-SY5Y neuroblastoma-derived

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cell line, that is applicable to scalable and automatable morphological and physiological assays for the characterization of pathological hallmarks. These assays allowed for the characterization of cytotoxic damage and hypo-excitability arising from the expression, upon transfection, of mutated proteins APP V717I and P301L, closely related to the familiar variant of AD. Therefore, paving the way for the inclusion of representative hallmarks of the disease (neuronal death, impaired neuronal excitability, and synapse dysfunction) in our developed model. This model was also tested and resulted compatible with high-throughput screening campaigns for the identification of novel molecular pathways and therapeutic compounds that can be translated into more complex pre-clinical testing.

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CONCLUSIONS

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7. CONCLUSIONS

1. A new neuron-like phenotypic miniaturized and scalable model was developed from the differentiation of the neuroblastoma derived cell line SH-SY5Y with retinoic acid and glucagon-like peptide 1 into a mature neuronal phenotype, characterized by the neurite number and density.
2. Culture transfection protocols were developed and optimized, allowing for the transitory induction of the genetic mutations APP V717I and MAPT P301L in the differentiated cell line.
3. Transfection of the cell line with mutation APP V717I induced a significant decrease in average number of cells per well ($p<0.01$), average neurite length ($p<0.0001$) and neurite arborization ($p<0.001$). Mutation P301L in the MAPT gene also produced significant decrements in average number of cells per well ($p<0.0001$), average neurite length ($p<0.0001$) and neurite arborization ($p<0.01$). Co-transfection with both APPV717I and MAPT P301L also yielded cultures with significantly reduced values for all the variables assessed ($p<0.0001$). This data represents hallmarks compatible with the neurodegenerative morphological changes observed in AD patients.
4. Treatment of cell cultures with reference neuroprotective compounds on i*n vitro* assays, managed to accomplish significant efficacy against the pathophysiological damage induced by both mutations: exposure of transfected cultures to 1 μM of melatonin resulted in a significant increase of all variables measured, thus reverting the effects of both mutations studied over the cells´ morphology. Use of 1 μM of resveratrol also show a significant effect reverting the morphological changes observed post-transfection, with the exception of arborization and number of cells in cells transfected with APP V/17I.
5. A primary physiological assay based on intracellular calcium measurement was developed and optimized for the study of excitability changes arising from the mutations APP V717I and MAPT P301L.
6. Differentiated SH-SY5Y cultures showed a significantly higher excitability ($p<0.0001$) in response to 30 mM KCl than those undifferentiated, reinforcing the acquisition of neuron-like properties aside from the morphological phenotype. Co-transfected cells showed a hypo-excitatory behaviour as a response to 30 mM KCl stimulation in comparison to differentiated cultures ($p<0.0001$). The treatment with 1 μM of Melatonin or 1 μM of Resveratrol significantly reverted the hypo-excitability observed upon co-transfection ($p<0.0001$).
7. Intracellular calcium assays were adapted and fine-tuned for its application to a high-throughput screening study by using the Prestwick® chemical library.
8. The Prestwick® chemical library screening, for the validation of the model as a biological base for drug discovery, led to the identification of eight *hits* that were able to revert the neuronal hypo-excitability derived from the mutations APP V717I and MAPT P301L. The eight pharmacological *hits*: Dapsone, mianserine,

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ethosuximide, doxepin, rifampicin, canavanine, betazole and metolazone, exerted their therapeutic action with EC_{50} values between 4 and 220 nM. For these compounds, both the pharmacological mechanisms and the bibliographical background are compatible with their potential neuroprotective properties.

In summary, in this study, a novel translational model for HTS was developed based on familial Alzheimer's disease using SH-SY5Y cell line differentiated into a neuron-like phenotype and transfected with the genetic mutations: APP V717I and MAPT P301L. The neurodegenerative-like pathological hallmarks derived from the mutations were characterized using image-based and intracellular calcium measurement assays to assess both morphological and physiological variables of interest. Reference compounds, melatonin and resveratrol, both with previously described neuroprotective activity on in vitro Alzheimer's models, managed to significantly revert the pathological differences observed in transfected cells. The developed model was validated for early drug discovery assays by its employment in a high-throughput screening assay using the Prestwick® chemical library.

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Alzheimer's disease (AD) is the leading neurodegenerative disorder and the most common cause of dementia worldwide, accounting for 60–80% of cases. Its pathology is characterized by progressive cognitive decline, synaptic loss, neuronal death, and neuroinflammation, associated with amyloid-β and tau deposits. Despite its impact, clinical trials have a high failure rate, and current therapies are only palliative. This thesis developed an in vitro model using differentiated SH-SY5Y cells to achieve a mature neuronal phenotype, transfected with APP V717I and MAPT P301L mutations, reproducing morphological damage and hypo-excitation similar to AD processes. Furthermore, reference neuroprotective compounds, melatonin and resveratrol, managed to reverse these damages, allowing the model to be applied to HTS assays.