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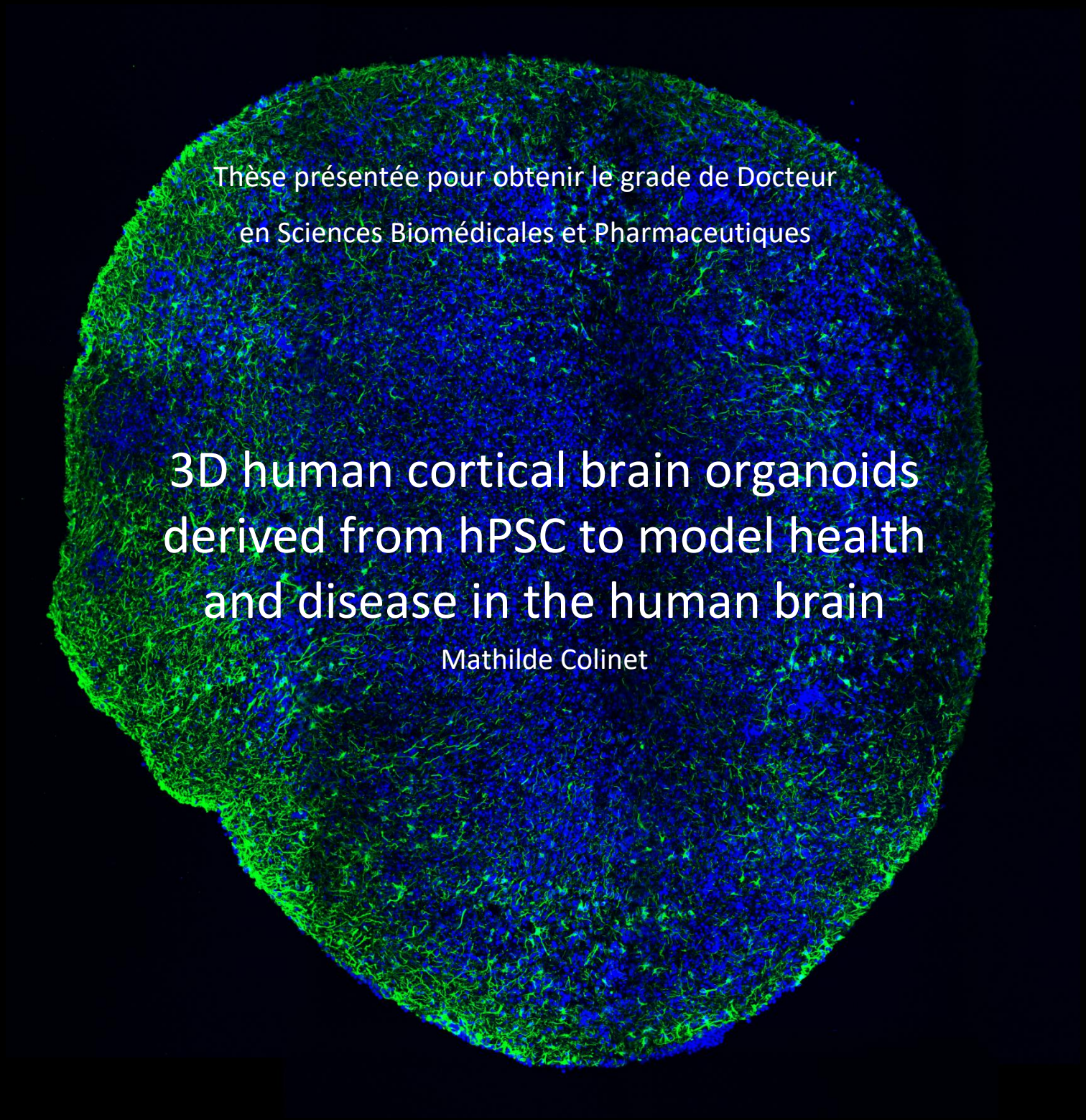

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3D human cortical brain organoids derived from hPSC to model health and disease in the human brain

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List of publications

Ph.D work, University of Liège:

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Table of abbreviations

4R tau:	4-repeat tau
β-AR:	Beta-adrenergic receptor
a.a.:	Amino acids
Aβ:	Amyloid beta
ACHA7:	Neuronal acetylcholine receptor subunit alpha-7
ACE2:	Angiotensin-converting enzyme 2
ACSF:	Artificial cerebrospinal fluid
AD:	Alzheimer's disease
ADHA:	Attention-deficit/hyperactivity disorder
ADN:	Acid deoxyribonucleic
AGTR2:	Angiotensin II receptor type 2
ALIX:	ALG-2 interacting protein X
AMPA:	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPs:	Antimicrobial peptides
AMPK:	Adenosine monophosphate-activated protein kinase
APOE:	Apolipoprotein E
APP:	Amyloid precursor protein
ARN:	Acid ribonucleic
ATAT1:	Alpha tubulin acetyltransferase 1
AXL:	Tyrosine-protein kinase receptor UFO
BAALC:	Acute leukemia cytoplasmic protein
BBB:	Blood-brain-barrier
BDNF:	Brain-derived neuro-trophic factor
BMP:	Bone morphogenetic protein
BOs:	Brain organoids
Brn2:	Brain-2
BSG:	Basigin
CAA:	Cerebral amyloid angiopathy
CALB:	Calbindin
CASP3:	Caspase 3
Caspases:	Cysteine-dependent aspartate specific proteases
CATD:	Cathepsin D
cDNA:	Complementary acid deoxyribonucleic
CD147:	Cluster of differentiation 147
CD44:	Cluster of differentiation 44
CDR:	Clinical dementia rating

CERAD:	Consortium to establish a registry for Alzheimer’s disease
CFI:	Gene complement factor 1
c-MYC:	MYC proto-oncogene
CNS:	Central nervous system
COVID-19:	Coronavirus Disease 2019
CRB:	Cerebellum
CSF:	Cerebrospinal fluid
CTF:	C terminal fragment
CTL or CTRL:	Control
CTIP2:	COUP-TF-interacting protein 2
CTSB:	Cathepsin B
CTSL:	Cathepsin L
CUX1:	Cut-like homeobox 1
CUX2:	Cut-like homeobox 2
DMEM-F12:	Dubelcco's modified eagle medium mixture F-12
DNA:	Deoxyribonucleic acid
DPP4:	Dypeptidylpeptidase 4
DR4/5:	Death receptor 4/5
DRG:	Dorsal root ganglia
DYRK1A:	Dual specificity tyrosine phosphorylation-regulated kinase 1A
EGFR:	Epidermal growth factor receptor
EPHR:	Erythropoietin-producing hepatoma cell line receptor
ER:	Endoplasmic reticulum
FAD:	Familial Alzheimer’s disease
FASR:	FAS cell surface death receptor
FDA:	Food and drug administration
fMRI:	Functional magnetic resonance imaging
FOXP1:	Forkhead box protein P1
$GABA_BR1a$:	Gamma-aminobutyric acid type B receptor subunit 1a
GEO:	Gene expression omnibus
GFAP:	Glial fibrillary acidic protein
GLUR1:	Glutamate receptor 1
GLUR2:	Glutamate receptor 2
GMR5:	Glutamate metabotropic receptor 5
GRIN2A/B/C/D:	Glutamate ionotropic receptor NMDA type subunit 2A/B/C/D
GRP78:	Glucose-regulated protein 78
GSEA:	Gene set enrichment analysis
GSK-3β:	Glycogen-synthase kinase-3β

GWAS:	Genome-wide association studies
H2AX:	H2A histone family member X
h:	Hours
hCO:	Human cortical organoids
hESC:	Human embryonic stem cells
HGF:	Hepatocyte growth factor
hiPSC:	Human induced pluripotent stem cells
hPSC:	Human pluripotent stem cells
HSPA5:	Heat shock protein family A member 5
IBA1:	Ionized calcium-binding adaptor molecule 1
IL:	Interleukins
IL13RA1:	Interleukin 13 Receptor subunit Alpha 1
ITPR3:	Inositol 1,4,5-trisphosphate-gated calcium channel
IWR-1:	Inhibitors of Wnt Response 1
IP:	Intermediate progenitor
JAK:	Janus kinase
KI67:	Kiel 67
KLF4:	Krueppel-like factor 4
LAMP1:	Lysosomal-associated membrane protein 1
LC3:	Microtubule-associated protein 1 light chain 3 positive
LHX2:	LIM homeodomain 2
LIF:	Leukemia inhibitory factor
LRP:	Low-density lipoprotein receptor-related protein
LRP5/6:	Low-density lipoprotein receptor-related protein5/6
LS:	Long-stage
M:	Months
MAPs:	Microtubule associated proteins
MAPT:	Microtubule associated protein tau
MASP:	Membrane-associated serine proteinase
MBD:	Microtubules binding domain
MBP:	Myelin basic protein
MCS:	Mild cognitive symptoms
MEA:	Multielectrode array
MGE:	Medial ganglionic eminences
MOI:	Multiplicity of infection
MRI:	Magnetic resonance imaging
NC:	Nucleocapsid
NEUN:	Neuronal-nuclei

NEAA:	Non-Essential Amino Acids
MLKL:	Mixed lineage kinase domain-like pseudokinase
NF-κB:	Nuclear factor kappa B
NFIA:	Nuclear factor-I A
NFT:	Neurofibrillary tau tangles
NG2:	Neural/glial antigen 2
NLF:	Neurofilament light chain
NMDA:	N-methyl-D-aspartate
NMDAR:	N-methyl-D-aspartate receptor
NRP1:	Neuropilin 1
NRP2:	Neuropilin 2
NTRK2:	Neurotrophic receptor tyrosine kinase 2
mTORC1:	Mammalian target of rapamycin complex 1
OLIG2:	Oligodendrocyte transcription factor 2
OPC:	Oligodendrocytes precursors cells
P75NRT:	p75 neurotrophin receptor
p-tau:	Phosphorylated tau
PAD:	Phosphatase-Activating Domain
PAMPs:	Pathogen-associated molecular patterns
PAX6:	Paired box 6-positive
PCR:	Polymerase chain reaction
PDGFRα:	Platelet derived growth factor receptor alpha
PHF:	Paired helicoidal fragments
PIP:	Phosphatidylinositol
PIRB:	Paired immunoglobulin-like receptor B
PLCD1:	Phospholipase C delta 1
PLCD3:	Phospholipase C delta 3
PLP:	Proteolipid protein
PrP^{c}:	Cellular prion protein
PSEN1:	Presenilin 1
PSEN2:	Presenilin 2
PTM:	Post translational modifications
qPCR:	Quantitative polymerase chain reaction
REST:	Repressor element-1 silencing transcription factor
RG:	Radial glia
RIN:	RNA integrity number
RILP:	RAB7 interacting lysosomal protein
RIPK:	Receptor-interacting protein kinase 1

RNA:	Ribonucleic acid
RNAseq:	RNA sequencing
ROI:	Region of interest
RPM:	Revolution per minute
RT:	Room temperature
RYRs:	Ryanodine receptors
S100A10:	Calcium binding protein A10
S100B:	S100 calcium binding protein B
SARS-CoV2:	Severe acute respiratory syndrome coronavirus 2
SATB2:	Special AT-rich sequence-binding protein 2
SB:	SB431542
SCN2A:	Sodium channel protein type 2 subunit alpha
SERPINA3:	Alpha 1 antichymotrypsin
SNP:	Single nucleotide polymorphism
SOD2:	Superoxide dismutase 2
STAT:	Signal transducer and activator of transcription
TFEB:	Transcription factor EB
TGFβ:	Transforming growth factor β
THL:	Thalamus
TLR:	Toll like receptor
TMPRSS2:	Transmembrane serine protease 2
TNF:	Tumor necrosis factor
TNFA or TNFα:	Tumor necrosis factor α
TNFR1:	Tumor necrosis factor receptor 1
TTL:	Tubulin-tyrosine ligase
TTX:	Tetrodotoxin
ULK1:	Unc-51 like autophagy activating kinase 1
VGLUT1:	Vesicular glutamate transporter 1
VIM:	Vimentin
W:	Weeks
WT IPS:	Wild-type induce pluripotent stem cell

Summary

The human brain is the most complex organ in the body, yet many aspects of its development and the cellular and molecular mechanisms triggered following viral infections and neurodegenerative diseases remain poorly understood.

The brain is a tissue that remains difficult to access, and due to this limitation, studies of brain maturation have predominantly relied on rodent models. Whereas much of our current understanding on brain development, as well as key aspects of brain diseases, has been revealed in such models, they may mask important human specific features. For instance, it has been described that the human brain contains higher proportion of certain cell types such as basal radial glia during development and astrocytes in adulthood. Important differences between mice and humans also are evident in disease modeling, for example, in the context of Alzheimer's disease, where mice cannot naturally develop disease features without the introduction of human-specific genes. As a result, specific morphological and functional aspects of the human brain such as the density of different cortical layer neurons, changes in calcium dynamics related to synapse formation and axonal growth remain poorly understood across different developmental stages. Similarly, the role of axonal transport, an important mechanism for neuronal homeostasis and axonal growth, has yet to be thoroughly investigated in the context of human brain maturation at different developmental stages.

The global outbreak of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV2) in 2020 led to a variety of medical symptoms affecting a variety of organs such as lung, blood vessels, liver and kidney. The emergence of various neurological symptoms in patients, raised concerns about the neurotropic potential of the virus. The origin of these symptoms remains unclear, as whether they result from a direct viral invasion of the brain or from indirect consequences of a systemic infection. Although epithelial cells from the choroid plexus surrounding parts of the brain, astrocytes and neurons have been identified as targets of SARS-CoV2, the relative infection capacity of the virus and its downstream effects concerning cell death mechanisms are still debated.

Alzheimer's disease (AD) is one of the most common forms of dementia, however, the mechanisms driving its onset and progression remain poorly understood. Among the early phenotypes observed in patients, neuronal hyperactivity has been reported in preclinical stages of the disease. Although several hypotheses have been proposed to explain this phenomenon, the underlying mechanisms are still not yet fully established. In addition, defects in axonal transport and endolysosomal-autophagy pathway have also been associated to early stages of AD. Similarly to calcium dysregulation, the chronological appearance and contribution of those early defects to the disease initiation and progression are not yet fully understood.

Human brain organoids derived from patient cells offer a powerful model to study human brain development and neurological pathologies, including viral infections and neurodegenerative disorders, in a system that retains the patient's genetic background. These 3D structures can mimic some aspects of brain complexity, including cellular diversity, and human specific timeline of development. In the context of Alzheimer's disease, brain organoids can be generated directly from patient-derived induced pluripotent stem cells (iPSCs), providing a unique opportunity to investigate early cellular and molecular events involved in disease initiation.

1. Chapter 1: General Introduction and objectives

1.1 Human brain development

The adult human brain contains more than 100 billion neurons, each forming an average of 7,000 synaptic connections with other neurons, resulting in an extremely complex and highly interconnected network[1]. The human brain is organized into several regions derived from three primary vesicles: forebrain, mid-brain and hindbrain[2]. The forebrain subdivides into the telencephalon and the diencephalon, while the hindbrain gives rise to the metencephalon and the myelencephalon[2]. The telencephalon notably forms structures such as the cortex, the hippocampus, and the striatum, whereas the diencephalon contains important nuclei including the thalamus and hypothalamus[3]. The mid-brain houses nuclei involved in processing visual and auditory information and it is involved in motor processes. The metencephalon develops into the cerebellum, and the myelencephalon forms the medulla oblongata[3]. Among those unique regions, we chose to focus our study on the cortex which is one of the brain regions that has undergone the highest expansion in size through evolution[4]. The folded structure of our brain maximizes cortical surface while fitting within the limited volume of our brain skull[4]. Brain maturation is a multi-step process that can be broadly divided in 6 major steps; i) neurogenesis, characterized by the birth and migration of neurons to their final destination; ii) gliogenesis which gives rise to astrocytes and oligodendrocytes; iii) neuronal maturation/axonal growth, characterized by the development of the neuronal dendritic tree; iv) synaptogenesis, or the formation of synapses, v) myelinization processes to enhance the axonal conduction of neurons; and finally, vi) pruning of excess of neurons and synapses to refine neuronal circuits[5].

1.1.1 Neuronal progenitor amplification phase

Neurogenesis in humans is preceded by a phase of progenitor amplification that ensures the expansion of the precursor pool[1,4]. The neuroepithelium is composed by a monolayer of neural stem cells (neuroepithelial cells) which proliferate by symmetric division to expand their pool and form the ventricular zone[4,6]. Around 5-6 gestational weeks in humans, these cells turn into apical radial glia cells (aRGs)[1,4] paired box 6-positive (PAX6+)[7] and sex

determining region Y-box 2 positive (SOX2+)[8] (Figure 1). aRGs initially divide symmetrically to increase their own numbers during a short phase, and then switch to asymmetric divisions that produce intermediate progenitor cells (low level PAX6+)[9], basal (or outer) radial glia (bRGs) SOX2+[8], or postmitotic neurons, while maintaining the apical progenitor pool[1,6,10]. Intermediate progenitors (IPs) T-box brain protein 2 positive (TBR2+)[8] have limited proliferative capacity and differentiate into immature neurons[4,9]. In contrast, basal radial glia cells have a higher proliferative potential that maintain the pool of progenitors by asymmetric divisions, while giving rise to immature neurons[4,10]. In addition, they are also able to produce two neurons via symmetric divisions[10]. Proliferating cells can be identified using the antigen Kiel 67 (KI67) which is expressed during active phases of the cell cycle[7,11]. IPs and bRGs form the subventricular zone[1]. Radial glia cells do not only have the capacity to give rise to progenitors and neurons but they also give rise to astrocytes and oligodendrocytes through a neurogenic-to-gliogenic fate switch during late neurogenesis[10].

These progenitor dynamics contribute to cortical expansion and vary significantly between species, particularly in terms of subventricular zone size and progenitor composition, which may help explain differences in cortical size and complexity[10]. In the developing human brain, about half of the basal progenitors are bRGs, whereas this proportion drops to only around 10% in the embryonic mouse brain[12].

1.1.2 Neurogenesis

The neurogenesis is the emergence and migration of neurons towards their final location in the brain[1]. In most mammalian species, pyramidal neurons are born during embryonic development from progenitor populations residing in the ventricular and subventricular zone[1,4]. In humans, neurogenesis begins around gestational weeks 5 to 6, marked by the transition of neuroepithelial cells into radial glial cells, which gives rise to various progenitors and postmitotic neurons[1,4,6,10]. Newborn neurons emerge from the ventricular and subventricular zone, where the radial glia cells are localized and they migrate towards the cortical plate[1]. Radial glial cells extend processes to the pial surface which constitute a scaffold for neurons to migrate, contributing to the organization of the cortical layers and the marginal zone (MZ), situated at the top of the cortical plate[7]. The neuronal migration process starts at

the intermediate zone (IZ), located below the cortical plate, and continues towards the correct positioning of each cortical layer subtype within the cortical plate[1,7].

Early in neurogenesis, neurons can reach their final localization by moving their soma along radial glia (RG) basal processes[1,4]. The neuronal migration follows an inside-out pattern with the earliest born neurons forming the deeper layer (VI)[4,7]. As neurogenesis progresses, the neuronal migration distance increases and neurons have to migrate and cross the subventricular and intermediate zones to reach the cortical plate[1,4]. Their migration follows 4 main steps: 1) they acquire a bipolar morphology to travel outside the ventricular zone, 2) their morphology switches to multipolar to cross the subventricular and intermediate zones, 3) they switch to bipolar to move along the RG scaffold and reach the cortical plate, 4) they make contact with the marginal zone[4] (Figure 1).

Layer I, located at the MZ, is composed by Cajal-Retzius cells (reelin positive)[7,13], interneurons[7,13] and contains dendritic arborizations[7]. Upper layers of the cortex are composed by layer II, III and IV[14] pyramidal neurons which are notably cut-like homeobox 1 positive (CUX1+)[14,15], cut-like homeobox 2 positive (CUX2+)[14,16] and LIM homeodomain 2 positive (LHX2+)[16]. Neurons from layer II and III are brain-2 positive (BRN2+)[14,16] and neurons from layer IV are RAR-related orphan receptor B positive (RORB+)[16]. Deep layers of the cortex are composed by layers V and VI neurons. Layer V neurons are positive for COUP-TF-interacting protein 2 (CTIP2) whereas the levels of expression are lower in neurons from layer VI[15,16] developing cortex. Deep layer VI cortical neurons also express notably TBR1[14,16] or forkhead box protein P1 (FOXP1)[14,15], which levels are reduced in layer V cortical neurons in the developing cortex. Some markers such as special AT-rich sequence-binding protein 2 (SATB2) are specific to callosal cortical neurons and are located in a subset of neurons located in layer V, but also in neurons from II to IV cortical layers[14].

A mature cortex can be divided into grey and white matter. The grey matter contains 6 layers of mature projection neurons which are positive for the neuron-specific nuclear protein (NEUN+)[17] generated during the neurogenesis phase, while the white matter contains mostly neuronal fibers[1,4].

1.1.3 Gliogenesis

During late neurogenesis, around post gestational weeks 20-22, RGs progressively switch from producing neurons to producing first astrocytes and then oligodendrocytes[5,18] (Figure 1). The neurogenesis to astrogliogenesis transition is mediated by the activation of the janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway which triggers expression of astrocyte associated genes[18,19]. Notably, the transcription factor nuclear factor-I A (NFIA) binds to the promoter regions of gliogenic genes such as glial fibrillary acidic protein (*GFAP*)[19]. Astrocyte generation continues postnatally to reach a ratio 4 to 1 of astrocytes to neurons in the human brain[1,5,20].

Human astrocytes are involved in the processes of synaptogenesis and synapse pruning, they are capable of modulating neuronal networks and influence neuronal plasticity[21,22]. They have been reported to recycle released neurotransmitters to the synaptic cleft such as glutamate[21] and they are also essential to maintain homeostatic ion levels[20]. The main functions of astrocytes consist also in the regulation of the blood flow and the glymphatic system with their processes, which role is the exchange of fluids and the elimination of waste products such as misfolded proteins[23]. Astrocytes are also able to sense nutrients such as glucose and fatty acids, to store them and to deliver them to neurons to support the high demand of energy of those cells[24]. Based on anatomical and morphological analysis, different types of human astrocytes have been characterized among different cortical regions: interlaminar astrocytes located in layers I and II, protoplasmic astrocytes found in layers III and IV, astrocytes with varicose projections described in layers V and VI and fibrous astrocytes present in the white matter[20].

At the onset of oligogenesis, RGs from the cortex produce progenitors expressing epidermal growth factor receptor (EGFR) which subsequently generate mitotic oligodendrocytes precursors cells (OPC) positive for oligodendrocyte transcription factor 2 (OLIG2), platelet derived growth factor receptor alpha (PDFGRα) and neural/glial antigen 2 (NG2) proteoglycans[25]. The generation of OPC has been extensively studied in rodents which has shown three temporal waves originated first from subcortical ganglionic eminences (waves 1 and 2) followed by a third wave originated from RGs from the ventricular/subventricular zone from the dorsal cortex[26,27], which is conserved in humans[25,28]. OPC progressively differentiate

into pre-myelinating oligodendrocytes and then mature myelinating oligodendrocytes[29]. Mature oligodendrocytes produce the myelin sheath that insulate the neuronal axon, a multilamellar structure notably composed by proteins such as myelin basic protein (MBP) and proteolipid protein (PLP)[29]. The neuronal myelin sheath enables the saltatory conduction of the nerve impulse and enhances the conduction properties of neurons, ensuring a faster delivery of action potentials[30].

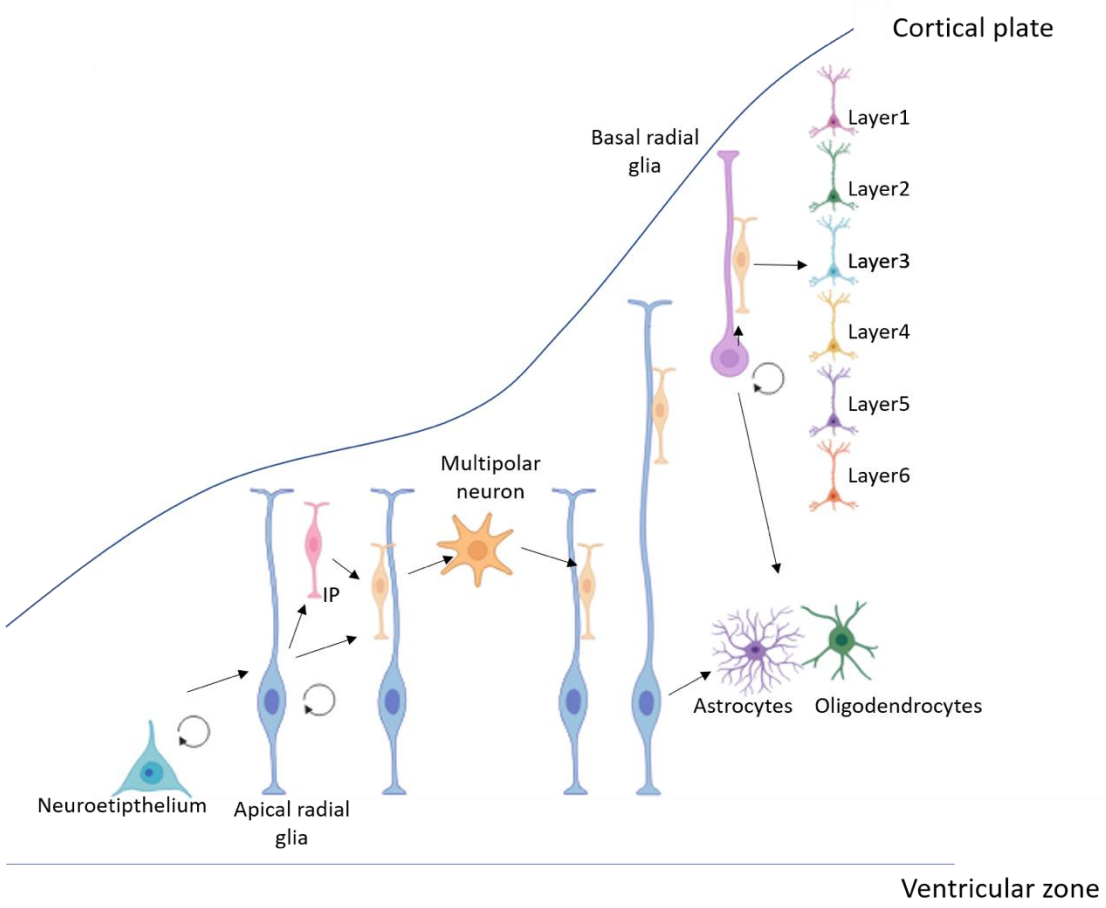


Figure 1: Neurogenesis and gliogenesis.

Neuroepithelial cells proliferate and form the ventricular zone before becoming aRGs, which generates progenitors and neurons through asymmetric divisions. Neurons migrate through intermediate zones, changing morphology before reaching the cortical plate. During late neurogenesis, RGs give rise to astrocytes and oligodendrocytes. Created via Biorender.

1.1.4 Microglia cells

Microglia are considered as brain macrophages that are present in a broad range from 5 to 15% in different regions of the adult human brain. Contrary to astrocytes and oligodendrocytes, microglia cells are not derived from the neuroectoderm but from the yolk-

sac mesoderm[5,31]. Studies on human fetuses have shown that at 4.5 gestational weeks (GW), amoeboid microglia Ionized calcium-binding adaptor molecule 1 positive (IBA1+) start to penetrate the developing brain, and localize at the leptomeninges near blood vessels[5,31,32]. Microglia start to proliferate and migrate towards the cortical plate during brain development[31,32]. Around 9-13 GW they reach the cortical plate and continue their proliferation[32]. At 22-23 GW the morphology of the microglia changes from an amoeboid shape to a more ramified morphology with long processes, however most of microglia at this stage are not yet ramified to the extent found within the adult brain[33,34].

Different populations of microglia with different morphologies are present within the brain[31,34]. Microglia are highly dynamic cells and their morphology and function are both dependent on the health status and the age of the organism[35]. Some regions of the brain will also harbor specific phenotypes of microglia such as ramified, amoeboid, rod, hypertrophic, dystrophic or satellite[5,36]. It has been shown that microglia play important roles in neuronal differentiation and proliferation, notably by clearing dead cells and cellular debris through their phagocytic activity, but also in the establishment of neuronal networks by contributing to synaptic pruning[1,37].

1.1.5 Axonal growth and axonal transport

The proper functioning of the brain depends on the formation of an efficient neuronal network, which requires neurons to establish specific connections with specific target neurons and to receive inputs through their developed dendritic trees[38,39]. To achieve this, neurons need to extent their axons and navigate through the complex cellular environment of the developing brain. Axonal pathfinding involves dynamic processes, including directional changes and transients pauses to interpret and react to environmental cues[38].

Axonal elongation implies that the neurons grow in size, which requires the synthesis and delivery of lipids for membrane expansion, as well as a plethora of proteins necessary for instance for cytoskeletal assembly[38]. This delivery is mediated by axonal transport, a bidirectional process that moves cargo (organelles, vesicles) along microtubules[40]. Anterograde transport moves materials from the soma to the tip of the axon, supplying

essential components for the cell homeostasis. Among these, mitochondria support local ATP production and ensure energy delivery to the distal part of the neurons, which is necessary for axonal growth or synapse formation[40,41]. Additionally, anterograde transport provides essential components such as neuropeptides and neurotrophins, which play important roles in synapse formation and function[40,42]. Conversely, retrograde transport moves material towards the soma of the neuron. This process is essential for recycling cellular components such as defective organelles and for the clearance of protein aggregates and misfolded proteins, helping maintain neuronal health and proteostasis[40,43].

Axonal transport relies on a dynamic network of microtubules, key components of the cytoskeleton that serve as tracks for motor proteins[44]. These motors move cargo anterogradely, retrogradely or bidirectionally along microtubules in an ATP-dependent manner[40,43,45]. Two main classes of motor proteins are involved; i) kinesins which primarily drive anterograde transport and ii) dyneins which mediate retrograde transport[46]. Kinesins represent a superfamily of proteins, containing more than 45 different members specialized for specific cargos and functions[40,45,47]. While dynein is encoded by a single gene producing multiple splicing variants of the protein, that associates with the 23 subunit dynactin protein complex and with one of the adaptor protein family members to transport specific cargoes[40,47]. Vesicles and organelles are often bound to multiple motors from both families simultaneously, resulting in a competitive tug between motors[40,45].

Microtubules themselves are not uniform tracks, they undergo various post-translational modifications (PTMs) such as acetylation, detyrosination, and glutamylation, which regulate their stability and interactions with motor proteins[44,47,48]. Additionally, they possess specific regions called GTP islands which are known to influence microtubule dynamics and motor attachment. For instance, kinesin-3 shows reduced affinity for GTP-bound tubulin, which can lead to cargo release at these sites[47]. Acetylation of α-tubulin is known to modulate motor proteins involved in axonal transport[49] and it appears to enhance microtubule stability, possibly by increasing resistance to mechanical disruption[47,48]. The detailed pathways through which it influences brain development and axonal growth are still under investigation[47]. However, it has been shown *in vivo* that preventing tubulin acetylation leads to cortical axon overgrowth, a phenotype associated with anxiety[47]. Tyrosination status of α-tubulin also

varies along the axon, with tyrosinated tubulin sites enriched at the dynamic growth cone, while detyrosinated tubulin predominates in more stable microtubule segments[44,47].

Beyond these PTMs, the expression of different isoforms of microtubule-associated proteins (MAPs) further modulates microtubule properties and transport efficiency during brain development. tau is a MAP protein existing in multiple isoforms produced through the alternative splicing of exons 2,3 and 10[50]. The 4-repeat (4R) tau isoform, generated through inclusion of exon 10 of microtubule associated protein tau (*MAPT)*, is characterized by an additional microtubule binding domain and a postnatally pattern of expression[51]. This isoform switch correlates with changes in axonal transport dynamics that could be linked to neuronal maturation[52,53]. Other MAPs such as MAP2 also undergo isoform transitions during brain maturation that influence microtubule dynamics[54].

Beyond the cytoskeletal and transport mechanisms, axonal growth is also regulated by extracellular signals and intracellular ion dynamics. Among these, peptide trophic factors such as brain-derived neuro-trophic factor (BDNF) plays a central role to trigger axonal growth[38]. The release of BDNF is dependent on membrane depolarization and increases with high intracellular calcium[55]. Calcium oscillations in the growth cone have been recognized as regulators of axonal growth[56]. More recently, calcium influx through L-type voltage-gated ion channels has been demonstrated to influence axonal growth dynamics, with channel blockade resulting in a shorter growth period of time and decreased axon size[57].

1.1.6 Synapse formation

Once axons reach their target areas, the establishment of functional synaptic connections becomes essential for the establishment of neural networks. Cortical synapses can be either excitatory, among glutamatergic projection neurons, or inhibitory, as those established between interneurons and excitatory neurons[58]. In glutamatergic synapses, the transmission of the action potential depends mostly on the release of glutamate[59]. In inhibitory synapses, GABA is the major neurotransmitter used[60]. The balance between excitatory and inhibitory synapses is essential for the correct function of neuronal networks in the brain[60].

Studies on post mortem human brain have revealed that synaptogenesis occurs from embryonic stages till late childhood (10 years old), which peaks at around 2-3 years of age followed by a phase of pruning of unused synapses[5,61]. Synapses pruning lasts till late adolescence to reach the synapse density observed in the adult brain[61]. Dendritic spines are small protrusions present on dendrites which are mostly the location of excitatory synapses[61]. During human development, the formation of dendritic spines is modulated by environmental factors such as extracellular components and neuronal activity, as well as by intrinsic factors such as actin cytoskeleton dynamics and the expression of synaptic proteins[62]. However, this plasticity is known be to also present in adult subjects in response to several stimuli such as hormone variation or other sensory information[61].

Axonal transport and synaptic function are closely linked by the need of proteins which are synthetized at the soma of the neuron to be delivered at pre-synaptic terminals. Kinesin proteins, which are the molecular motors responsible for anterograde transport have a crucial role in synapse function. It has been shown that increased expression of kinesins in the mouse brain led to an increase in cognitive capacity *in vivo* (e.g. spatial exploration)[61].

Postnatal development is also marked by isoform switches in proteins involved in synaptic activity and neuronal excitability. For instance, expression of the glutamate ionotropic receptor NMDA type subunit 2A (*GRIN2A*), encoding a subunit of the N-methyl-D-aspartate receptor (NMDAR), increases after birth, contributing to changes in synaptic transmission dynamics[63]. Similarly, *SCN8A*, encoding voltage-gated sodium channels critical for action potential propagation, undergoes an isoform switch postnatally at the position 7 of exon 5 position (from an asparagine to an aspartic acid residue), altering the electrophysiological properties of the channel[64].

1.1.7 Neuronal myelinization and Cell and synapse pruning

Neurons have different axonal lengths depending on their connectivity and function. Projection neurons which extend axons to distant subcortical brain regions have typically long axons that require myelination to ensure fast, precise and efficient signal conduction[61,65]. The likelihood of myelinization of neurons has been described to be correlated to the diameter of

the axon but also to the neuronal subtype[65]. Myelination is mediated by oligodendrocytes, whose processes wrap around axons to form insulating myelin sheaths[66]. This process, largely occurring postnatally in humans, extends over decades and is influenced by neuronal electrical activity and increased firing, which in turn promote oligodendrocyte maturation and myelin production[1,5]. The establishment of these sheaths is critical for enhancing the conductive properties of axons and enabling long-range communication within the brain[61].

In parallel with myelination, synaptogenesis continues after birth and is followed by extensive synaptic pruning, which reduces the number of synaptic connections in the brain[5,61]. During this step, an excess of early-formed synapses is selectively eliminated, while others are stabilized and strengthened, contributing to the fine-tuning of neuronal circuits[67]. This process is dependent on neuronal activity and the expression of specific markers, tagging synapses for elimination[67]. Microglia play a central role in this remodeling by phagocytosing unnecessary synapses[67]. Neuronal network remodeling also includes the pruning of axons and dendrites, as well as the programmed cell death of excess or improperly integrated neurons[68].

1.2 Human stem cell derived models for the study of the human brain

Understanding the complex and prolonged maturation of the human brain has long relied on animal models and post-mortem tissues[69]. However, these approaches face important limitations either in recapitulating human-specific developmental features (differences in cell populations, timing of development and gene expression)[70,71] or in tissue availability. In this context, the emergence of brain organoid models has opened new avenues to investigate human neurodevelopment in a controlled, accessible, and physiologically relevant system[72].

From antiquity, humans have been interested in learning about the functioning of the human body and have used tissue derived from humans or animals to try to understand its basic physiology. However, major scientific discoveries such as the functioning of the human vascular system, was only achieved in the mid-16th century through the dissection of postmortem human corpses[73]. The use of animals, mostly rodents, as models for research increased from beginning of the 20th century. Genetically modified mice, such as transgenic mice that express exogenous genes or knockout mice for the deletion of one or several genes, were first generated in 1980[73]. Human and mouse species share major similarities, with 80%

of the human genes being conserved in the mouse genome, and a 40% of sequence homology, at the nucleotide level[74] between both species. However, human and mice diverged 65–110 million years ago[74] and possess species-specific features, such as a specific developmental time clock and different complexity which raises the need of complementing our knowledge through the use of human specific systems. Protocols for the culture of neurons *in vitro* were established early in the 20th century but they were greatly improved from the 80's through large scale production and standardization of the main components needed for the medium culture. Related to this, human neuronal cell lines were first derived from tumors in 1970 that could be further differentiated into neurons. However, the accuracy of these cultures was approximative as it was not possible to generate specific subtypes, in contrast to neurons derived from rodent tumor cell lines. These human tumor-derived cultures were neuroblastoma cell lines and teratocarcinomas, the latter a kind of tumor which is composed from different types of tissue and developed from gonads[75]. These tumors contain also specific structures such as hairs and teeth but also proliferative undifferentiated cells that hold the properties to generate all the multilineage tissues characteristic of the teratocarcinoma if injected in another organ of an healthy individual[76]. Mouse and human embryonic pluripotent stem cells were first isolated in 1981 and 1998 from mouse and human blastocysts, respectively[69]. By definition, pluripotent stem cells have the ability to derive any tissue of the body of the individual[75,76].

Stem cells (SC) are a powerful tool since they can be kept indefinitely in culture in an undifferentiated state while keeping pluripotency, which means that they have the capacity to spontaneously differentiate into the three germ embryonic layers: endoderm, mesoderm and ectoderm[75,76]. They also maintain a stable diploid karyotype throughout the culture process, ensuring overall genetic integrity over time, although chromosomal aberrations are known to commonly arise during extended culture[75]. Several transcription factors have been associated with the state of pluripotency such as SOX2, octamer-binding transcription factor 4 (OCT4) and NANOG, which are commonly used as molecular markers to assess their pluripotency *in vitro*[69,76]. Human embryonic stem cells are also characterized by a specific morphology, defined as flat or multilayer colonies with distinct borders, whereas mouse ES are forming round aggregates without distinct borders which are difficult to dissociate[75]. Other differences between mouse and human ES reside in the use of different reagents to

keep their undifferentiated state, such as the need of leukemia inhibitory factor (LIF) for the culture of mouse ES cells, and their relative state of pluripotency[77,78]. In fact, mouse ES cells have been described as a "naïve" multipotent-like state that corresponds to a pre-implantation blastocyst stage, whereas human ES are associated to a "prime" pluripotency state related to a post-implantation phase[78,79]. The difference between both pluripotency states resides in the capacity of differentiation into embryonic and extraembryonic tissues (mouse ES), or a more limited potency to generate solely embryonic tissues (human ES)[78,79].

In 2006, Takahashi and Yamanaka successfully reprogrammed adult mouse fibroblasts into induced pluripotent stem cells (iPS) with a combination of OCT4, SOX2, Krueppel-like factor 4 (KLF4), and MYC proto-oncogene (c-Myc) transgenes. The confirmation of pluripotency was assessed by the formation of teratomas containing mesoderm, endoderm and ectoderm[76]. Using a similar protocol, but adapted conditions for the culture, they published one year after the reprogramming of human somatic cells into hiPS cells. This discovery also showed a conserved mechanism for reprogramming, dependent on the presence of the "Yamanaka factors", in mouse and human cells[80]. Other groups simultaneously or shortly after confirmed the capacity of reprogramming factors to induce a pluripotent state from human somatic cells. For instance, the group of Thomson used a different combination of factors, OCT4, SOX2, NANOG, and LIN28, for reprogramming hiPS[81], and the group of Melton used OCT4, SOX2 and valproic acid (an histone deacetylase inhibitor) for the generation of hiPS from adult somatic cells[82]. Histone deacetylases have been shown to be involved in the regulation of genes involved in pluripotency such as OCT4[83]. Nowadays, human somatic cells such as fibroblasts or peripheral blood mononuclear cells (PBMCs) are commonly reprogrammed into hiPS using either the Yamanaka factors (OCT4, SOX2, Klf4, and c-Myc) or Thomson factors (OCT4, SOX2, NANOG, and LIN28)[69]. The pluripotency is usually assessed by testing the expression of makers for pluripotency by immunofluorescence, western blot or qPCR, and validating the capacity of the cells to spontaneously differentiate into the 3 germ layers of the embryo: mesoderm, endoderm and ectoderm by teratoma formation (*in vivo*) or embryoid body formation (*in vitro*)[76]. Following these key discoveries numerous protocols were published for the differentiation of human and mouse iPS into different types of tissues, such as those from the neuroectodermal lineage, with different type of neurons generated such as glutamatergic excitatory neurons and GABAergic inhibitor neurons in a 2D fashion[84,85].

1.2.1 Generation of 3D brain and cortical organoids

Human brain organoids recapitulate certain aspects of the developing human brain, including the formation of 3D self-organized, polarized structures. Brain organoids can also recapitulate complex cellular populations, including both neurons and glial cells, as well as structural aspects with a spatial separation between progenitor proliferative regions and neuronal derivatives in a rudimentary cortical plate-like organization, that more closely resembles the *in vivo* human brain than 2D differentiation systems[72,86]. Generation of cerebral organoids involve a neuroectodermal cell fate specification from iPS. To enrich for neuroectoderm fate and a specific brain regional identity, such as the cortex, specific morphogens which are present in a gradient concentration in the developing brain, are used for the generation of directed differentiation protocols from stem cells[87].

One way to achieve a high neuroectoderm cell fate differentiation is the inhibition of the SMAD signaling through both bone morphogenetic protein (BMP) and transforming growth factor beta (TGFβ) pathway inhibition (dual-SMAD)[88]. The name SMAD is coming from a contraction of the *Caenorhabditis elegans Sma* genes and the *Drosophila Mad* (mothers against decapentaplegic) genes[89]. SMAD molecules are involved in a plethora of cellular activities such as cell division, migration, organization and adhesion[89]. These proteins are composed by two domains, one which is able to bind to DNA (MH1), and another (MH2) which can interact with cytoplasmic retention proteins, DNA binding co-factors and nucleoporins. The main function of SMAD1, 5, and 8 is to notably bind to the BMP receptors, whereas SMAD 2 and 3 bind to the TGFβ receptors and SMAD4 is a partner for other SMADs[89] (Figure 2). The phosphorylation of the ligands SMAD1, 5, and 8 and SMAD 2 and 3 by their receptors (BMP receptor or TGFβ receptor) initiate the pathway. Phosphorylated SMADs form a heterotrimer with two phosphorylated SMADs and one SMAD4 and translocate to the nucleus where they activate or repress the expression of certain genes (Figure 2)[89]. The addition of molecules such as noggin or SB431542 (SB) block the binding of the ligand to the receptor effectively blocking these pathways and resulting in an enhanced neuronal differentiation and rostral forebrain-cortical identity of the organoids[88,89]. It has been shown in *Xenopus* that the joint inhibition of BMP and Wnt signaling pathways is sufficient to induce the formation of forebrain structures, whereas the activation of Wnt represses anterior neuronal markers in favor of posterior markers of the neural tube[90].

Experiments using human stem cells also showed that Wnt activation promotes the derivation of a more posterior identity[91]. The term Wnt is also a fusion of two genes names, the gene *wingless* from *Drosophilia* and its homolog *integrated* present in vertebrates[92]. The binding of Wnt to its receptor and co-receptor, low-density lipoprotein receptor-related protein5/6 (LRP5/6) and the 7-pass transmembrane protein frizzled, induces the translocation of axin to the membrane, and more precisely to associate with LRP5/6 (Figure 2). The protein dishevelled is then recruited to LRP5/6, axin and frizzled site. Activated dishevelled inhibits the activity of glycogen-synthase kinase-3β (GSK-3β). One of the targets for phosphorylation of GSK-3β is β-catenin, and β-catenin phosphorylation induces also its ubiquitination, leading to its degradation through the proteasome (Figure 2). When GSK-3β is inhibited by dishevelled, β-catenin is not degraded and instead accumulates in the cytoplasm where it pairs with other transcription factors to enter the nucleus and activate the transcription of target genes[93] (Figure 2). Inhibitors of Wnt response 1 (IWR-1) block the Wnt pathway by preventing the turnover of the axin, therefore allowing the phosphorylation of β-catenin and its subsequent degradation[93] (Figure 2). To generate patterned cortical organoids through morphogen exposure, some groups have used dual-SMAD inhibition[58,88], TGFβ and Wnt[86,94] inhibition, BMP and Wnt inhibition[85] or dual-SMAD in addition to Wnt inhibition paradigms[95,96]. These directed protocols enrich for neuroectodermal cells and increase the proportion of cortical identity cells among organoids[97].

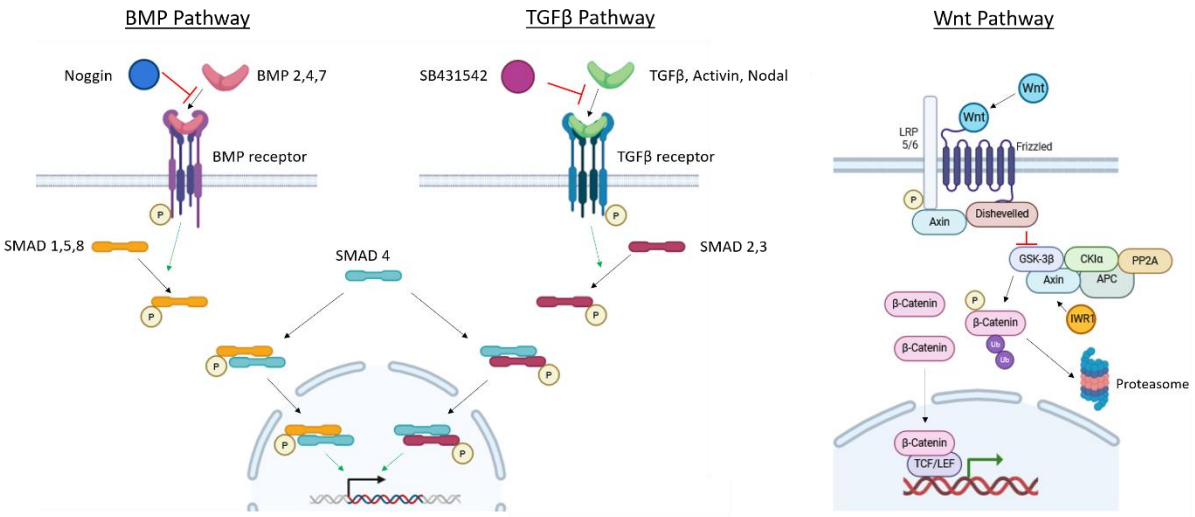


Figure 2: Pathways inhibited to induce neuronal differentiation and forebrain identity in organoids.

BMP, TGFβ, and Wnt signaling pathways regulate gene transcription through phosphorylation of SMAD proteins or stabilization of β-catenin. Ligand binding to specific receptors triggers intracellular cascades that modulate

target gene expression. Inhibitors such as noggin, SB431542, and IWR-1 block these interactions, thereby preventing pathway activation. Created via Biorender.

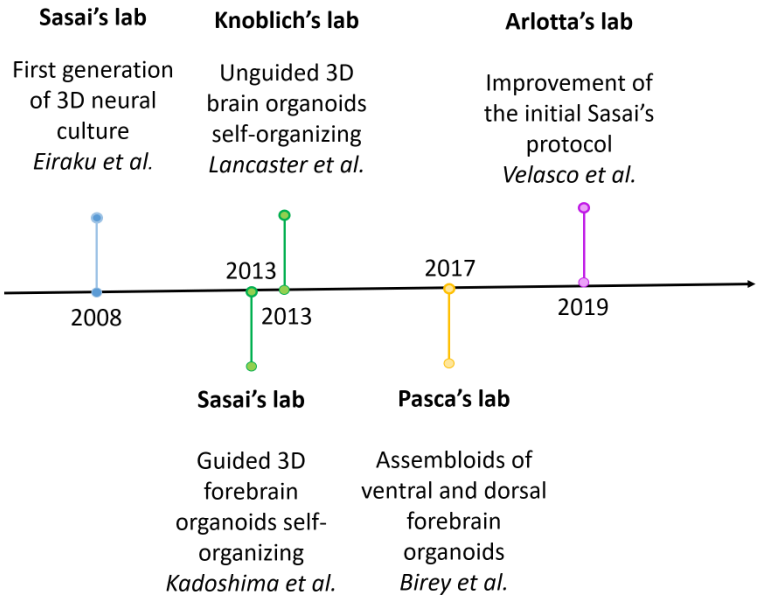
1.2.2 Major breakthroughs in the field of cortical organoid models

The first generation of 3D neuronal structures was reported in 2008 by Sasai's team, who demonstrated efficient corticogenesis from both mouse and human ESC[85]. These pioneering studies laid the foundation for the development of brain organoids, a term that entered the scientific literature in 2013. That year was marked by two landmark publications: the study by Lancaster from Knoblich's team and the work of Kadoshima from Sasai's group (Figure 3)[72,86]. Following these pioneering studies, various protocols were developed to generate brain organoids, differing in the degree of guidance and the signaling pathways manipulated. The laboratory from Sasai used a guided differentiation approach, combining Wnt and TGFβ inhibitors to directed neuroectodermal fate (Figure 3)[86]. In contrast, Knoblich's group, developed an unguided protocol, which stem cells are dissociated into single cells, aggregated, and cultured in medium supporting neuronal fates without preventing mesoderm or endoderm formation (Figure 3)[72]. Organoids are embedded in matrigel for structural support and grown in spinning bioreactors to enhance nutrient and oxygen supply. This unguided method generates heterogeneous organoids in terms of size, brain regional identity, and germ layer composition, including few non-neuroectodermal cells such as mesoderm-derived microglia[72].

These methodological advances enabled researchers to model aspects of human brain development and disease. For instance, Knoblich's group used brain organoids to model viral infections affecting the brain such as the Zika virus and herpes simplex virus. They showed viral replication and associated cell death, leading to microcephalic-like features. Comparisons between 3D and 2D systems revealed differences in initial viral load and replication rates, suggesting a role for cell-to-cell communication and variations in inflammatory responses, with some phenotypes occurring only in 3D structures[98].

In 2017, Knoblich's and Pasca's group, introduced the concept of assembloids by fusing ventral and dorsal forebrain organoids (Figure 3)[99,100]. They demonstrated that assembloids recapitulated interneuron migration patterns from the ventral telencephalon to cortical

structures. They identified interneuron migration defects in Tymothy syndrome patient-derived interneurons, using functional analyses including calcium dynamics to assess neuronal activity[101]. Follow up studies by the same teams, and others, generated assembloids combining additional brain regions, such as thalamic-cortical like structures or striatal-cortical structures[102,103]. Recent studies, such as the one from Arlotta's group in 2019, that followed the Sasai guided differentiation protocol, showed that long-term hCOs recapitulate the cellular diversity from the human brain (Figure 3). Moreover, single cell transcriptomics revealed molecular heterogeneity among individual brain organoids similar to the variability found in individual brains[94]. Together, these studies highlighted the potential of brain organoids to model human neurodevelopment and disease.



Lab	Morphogens used	Used of bioreactors	Identity	Reproducibility	Reference
Sasai	Wtn and TGFβ inhibitors	No	Dorsal forebrain	Moderate	Kadoshima et al., 2013
Knoblich, Lancaster	NA	No	Multiple brain regions	Low	Lancaster et al., 2013
Pasca	BMP and TGFβ inhibitors	No	Dorsal forebrain	High	Pasca et al., 2015 Birey et al., 2017
Arlotta	Wtn and TGFβ inhibitors	Yes	Dorsal forebrain	High	Velasco et al., 2019

Figure 3: Majors breakthrough in the field of cortical organoids.

Timeline of the major breakthroughs in the field of cortical organoids and comparison of the protocols used.

1.2.3 Advantages and disadvantages of *in vivo* animal models

Mouse models have been used for several decades and are very useful to study the functioning of the brain in an *in vivo* context. The biggest advantage of working with a mouse model is to have an integrative method to evaluate effects at the organism level, which has for instance a vascular and immunity systems. With the generation of transgenic mice, it became possible the study of the function of human specific genes and the study of human specific diseases. Examples of these are SARS-CoV2 infected mouse models, thanks to the expression of the human angiotensin-converting enzyme 2 receptor, ACE2, which is the entry point of SARS-CoV2 in the cell, in contrast to the murine ACE2 form which is not targeted by SARS-CoV2[104]. Other interesting examples are the use of transgenic mice expressing human mutated genes which are associated with inherited genetic forms of diseases (e.g. Alzheimer's disease)[105].

However, a major disadvantage of mice studies is the lack of human genetic background when applying it to the study of specific human diseases or brain development. Even following the expression of human genes in transgenic mouse models, we must consider that differences in the genetic background may impact the result of these studies. To support this, AD transgenic mouse models have shown that they cannot recapitulate all features of the disease, such as the formation of tau tangles and neuronal loss[106]. This suggests that some additional human specific factors are playing a role in the development of the disease that are absent in mice. In addition, timing is profoundly different between mouse and human at all stages of brain development, including brain maturation. As such, mice cannot reproduce the longer time needed for the development of the human brain and they can neither reproduce the generation of the exact proportion of some specific cell populations such as outer radial glia cells (oRGs) from the cortex present in primates and human[12] or neither the process of brain folding present in gyrencephalic species[4]. Moreover, there is also the issue of the variability in reproducibility from some mice strains[107].

1.2.4 Advantages and disadvantages of human 2D and 3D stem cell culture models

The isolation and discovery of human ESC and the generation of reprogrammed hiPS lines have opened the possibility to work in a system that carry a human genetic background.

On the other hand, the use of hESC, isolated from human embryos, has as a disadvantage that only few fully characterized lines are available and that they have a non-described clinical profile. Therefore, we cannot exclude or predict the possibility that some hESC cell lines could react differently in the case of some specific diseases due to inherent genetic background that could protect or worsened a define pathology[69]. Another disadvantage of the use of hESC is the ethical issues that can arise from the use of cells isolated from the destruction of human embryos *in vitro*[69,80]. This disadvantage can be overcome by the use of hiPS reprogrammed from somatic cells which possess the genetic background of the patient, allowing as well to perform personalized medicine[69].

Neurons can be directly differentiated from hESC and hiPS lines in 2D which are easy to culture, contrary to 3D organoids. However, the major disadvantage of 2D neuronal cultures is the time constriction of the culture system and the lack of other cell types generated in the culture, which precludes maturation and absence of a complex neuronal network[107,108]. By definition, 2D neuronal cultures do not possess a 3D structure which can be restrictive in some context, notably to model AD. For instance, 2D models have been shown to reduce the deposition of amyloid beta due to media change[109]. On the contrary, organoid models have a 3D structure which favors cell-to-cell interaction and *in vivo* cytoarchitecture and can recapitulate the presence of different cell types, neurons, progenitors and glia cells[107]. However, major disadvantages of the use of *in vitro* brain organoids are the lack of the brain vasculature and absence of immune system cell types which are both currently topics under investigation to be implemented by many laboratories[110]. Related to this, absence of brain vasculature has been related to the observation of necrotic cores in the center of long-term brain organoids due to the low penetrance of nutrients and oxygen inside the organoids[111]. Applying quality control techniques in the laboratory, such as close monitoring of brain organoid size and transferring brain organoids to adequate culture area plates and/or using a spinning device allows to minimize the presence of this necrotic core and improve general quality of the tissue. In addition, absence of immune cell types and endothelial cells surrounding the brain may mask the normal cellular interactions and environment of neurons and glia in the brain in these models[112]. Another limitation of brain organoids is the intrinsic variability across different cell lines, and even among batches of cells from the same cell line, resulting in differences in cell-type generation[113]. Such variability could be reduced by

implementing quality control measures to verify proper cell-type differentiation, employing methods such as qPCR.

1.2.5 Advantages and disadvantages of hPSC-derived xenotransplantation models

Human stem cell-derived neurons have also been xenotransplanted into the mouse brain, resulting in human-mouse chimeric brain models[107]. This technique offers the advantage of enabling the study of human cell pathophysiology within an *in vivo* context, including the presence of a vascular and immune system[107]. It has been demonstrated that xenotransplanted human neurons not only integrate into the mouse neuronal network but also continue to mature and develop complex dendritic morphology after several month post-transplantation[14]. Beyond neurons, other glial cell types such as microglia[114], astrocytes[115] and oligodendrocytes[116] have also been successfully xenotransplanted. These human-mouse chimeric brain model further allows the investigation of therapies for human disease by using the patient-derived iPSC and studying the development of the disease or drugs treatment within a *in vivo* system[107].

However, most of these studies involved the transplantation of a single cell type into the mouse brain and therefore cannot recapitulate different brain cell types from a human genetic background. Besides, some studies have reported inefficient neuronal maturation and differentiation and a dysregulation in the expression of certain disease-associated genes[117,118]. They have also observed discrepancies in the proportion of transplanted cell between different animals[119]. Several authors also raised ethical concerns regarding the degree of humanization of the target tissue[102,119]. Specifically, questions remain about the extent to which human neurons and glia in a mouse brain might improve the cognitive abilities of the animal such as of learning and memory[107].

1.3 Effects of SARS-Cov2 on the human brain

SARS-CoV-2 is a large RNA virus responsible of COVID-19 disease which rose initially in 2019 in China and led to a global pandemic in 2020 with more than 6.75 million of deaths[120]. The main symptoms associated to SARS-CoV2 are respiratory symptoms (cough), fever, myalgia, headache, fatigue and diarrhea. Most of the people infected were mildly or moderately

affected with symptoms restricted to their upper airways but, however, between 10 and 30% of the infected cases required hospitalization, resulting in a saturation of hospitals and health care systems[121]. Beyond these acute clinical manifestations, SARS-CoV2 infection also raised concerns in specific vulnerable populations, including pregnant women and their developing offspring. *In utero* exposure to SARS-CoV2 occurs as a consequence of maternal infection during pregnancy. However, the impact of such exposure on early human development remains poorly understood[122]. Some studies have reported cerebral hemorrhages and disrupted vascularization in fetuses exposed *in utero* to SARS-CoV2, but it remains unclear whether these effects are caused directly by the virus or indirectly by maternal systemic inflammation, and whether these observations are transient or permanent[123]. Moreover, clinical studies have reported conflicting outcomes, with some detecting neurodevelopmental alterations and others finding no significant effects[122,124]. Children, have been reported to experience predominantly mild symptoms compared to adults following SARS-CoV2 infection[125]. Although the underlying mechanisms are not fully understood, several hypotheses have been proposed, including a more efficient innate immune response, lower levels of the ACE2 receptor and increase competition with other respiratory pathogens frequently present in children[125].

About one third of the adult patients reported neurological symptoms, among those, headache, dizziness, brain fog and in some rare situations, stroke or encephalitis[126]. The neurological symptoms appeared either prior to the first main symptoms of the disease, concomitantly or *a posteriori*[126]. The disease is transmitted through aerosols and droplets that can last for about 9 to 14 days depending on the variants[127]. However, it has been found that some symptoms could persist longer than 6 months after the initial infection, which led to the term of long COVID-19[128]. About 10 to 60% of the infected cases reported long COVID-19symptoms such as loss of smell, short breath and myalgia but also neurological symptoms like insomnia, fatigue, cognitive impairment and anxiety[128,129]. Although currently COVID-19 is not causing any major waves of case hospitalization, there are still some remaining questions on the underlying process behind the neurological symptoms associated to COVID-19 and long COVID-19 such as the tropism of the virus for brain cell types and the downstream effects of the infection in the brain[129]. *In vivo* studies on SARS-CoV2 neurotropism are limited since mouse models require forced expression of human ACE2 receptors to become susceptible to

infection[104]. This reliance on a single-entry pathway overlooks the possibility that the virus may also use alternative receptors, which are more abundantly expressed in other cell types such as astrocytes. Human brain organoids, composed of neurons and glia cells carrying the human genetic background and expressing a broader repertoire of SARS-CoV2 (co)receptors, may provide a more relevant model to investigate the impact of SARS-CoV2 infection on the human brain.

1.3.1 SARS-CoV2 structure and mechanism of cell infection

SARS-CoV2 belongs to the family of coronaviruses which contain 7 human coronaviruses, from which among 3 of them are causing severe acute respiratory syndrome (SARS), SARS-CoV, middle east respiratory syndrome (MERS) and SARS-CoV2. Interestingly, it has been shown that SARS-CoV2 can infect a variety of species such as cats, dogs, bats, ferrets, primates and humans, but cannot infect others such as pigs, cattle, poultry and rodents[130,131]. It is believed that SARS-CoV2 was originated from the infection of an animal recipient, as most of the coronaviruses are zoonoses. In fact, it has been hypothesized that SARS-CoV2 could have been originated in bats, as it shares 96,2 and 94,5% of homology with the RaTG13 and RpYN06 bat coronaviruses[132]. It is also suggested that there was probably an intermediate animal host between the bat and the human in the origin of the pandemic[133]. SARS-CoV2 genome shares about 80% of homology with SARS-CoV virus.

The SARS-CoV2 is a positive single-strand RNA virus encapsulated with a membrane and enveloppe[130]. Its genome of about 30kb contain 4 majors genes encoding for proteins which have a structural function: the nucleocapsid (NC), the protein E, the spike and the protein M[130]. Non-structural and unknown function proteins are also present, 16 for the first category and 6 for the second[130]. The nucleocapsid structural protein encapsulates and compacts the viral RNA inside the membrane. The protein E is an ion channel involved in establishing the curvature of the membrane of the virus and the release from host cell. The protein M is a transmembrane glycoprotein acting as a scaffold and interacting with the other 3 structural proteins of the virus[130]. Finally, the spike is involved in binding and fusion of the virus to the host cell via specific receptors. The spike is composed of 2 subunits (S1 and S2), the S1 binds to the receptor and the S2 anchors the virus to the host cell[134] (Figure 4). The main receptor

attributed for the entry of the SARS-CoV2 inside the cell is the ACE2, however, other receptors or co-receptors have been described to facilitate the entry of the virus, such as neuropilin-1, angiotensin II receptor type 2 (AGTR2), basigin/ cluster of differentiation 147 (BSG/CD147)[135]. The *ACE2* is highly expressed in the lungs compared to other tissues such as the brain[136]. In order to successfully penetrate the cells, the spike needs to be cleaved by proteases to trigger fusion of the virus membrane with the cell membrane[134] (Figure 4). Inside the cell, the virus first needs to convert the positive single strand RNA genome into a negative single strand to be able to replicate itself using the host machinery. The new genetic copies are encapsulated at the interface between the ER and Golgi and virions are released from the host cell by exocytosis[126].

SARS-CoV2 is prone to mutations. Over 12 thousand sequences analyzed, more than 7 thousands single nucleotide polymorphisms (SNP) have been listed[130]. Five main different variants have been discovered (alpha, beta, delta, gamma and omicron), plus current variants from 2024 and 2025 ongoing in new infections among the population. These variants have been associated with different mutations, the vast majority, notably, in the spike gene, which increases the affinity for the receptors. Some variants have been associated with increased infectivity but also higher propension to escape the immune system[120].

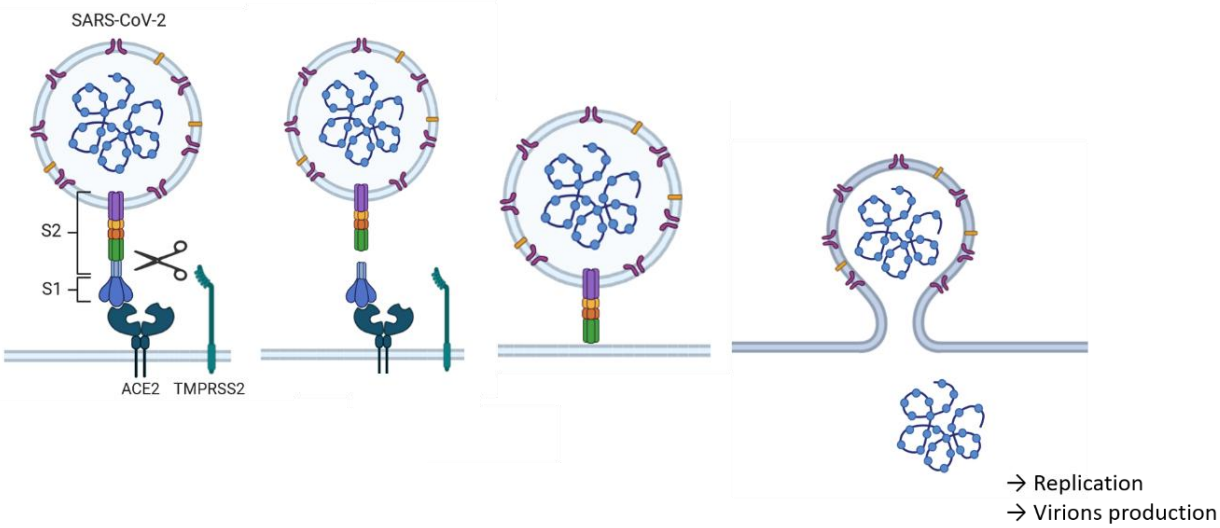


Figure 4 : Mechanism of entry of the SARS-CoV2 in cells.

The S1 subunit of the spike binds to host receptors such as ACE2, while the S2 subunit facilitates anchoring of the virus to the host cell. Host proteases, such as TMPRSS2, cleave the spike protein, triggering the fusion of the viral and cellular membranes. This process allows the release of the SARS-CoV2 genetic material into the host cell, where it can replicate, produce viral proteins, and ultimately assemble and release new virions. Created via Biorender.

1.3.2 Hypothesis for SARS-CoV2 entry to the brain

The neurological symptoms of SARS-CoV2 could be explained by a direct infection of the brain or through an indirect effect of the infection from peripheral tissues. Assessing the direct infection of SARS-CoV2 in postmortem brain patient samples is challenging due to the high risk of cross-contamination with other body tissues leading to false positive results. In fact, several studies reported the presence of viral particles in post-mortem brain[104,137,138]. Whereas other failed to detect the presence of virus in post-mortem brain or CSF[139,140]. Presence of SARS-CoV2 has been detected using qPCR on post-mortem brain samples, however, risk of contamination during autopsy cannot be excluded[137,141]. Electron microscopy has also been used to assess the SARS-CoV2 presence in the brain of patients, but results can be difficult to interpret due to low rate of infectivity and/or proximity to surrounding tissues of the brain[142]. Finally, magnetic resonance imaging studies (MRI) on COVID-19 patients have assessed the structural effects on the brain following recovery from COVID-19. These studies reported the presence of subtle anomalies in the brain, suggesting low infection rate of SARS-CoV2 in the brain and possibly mild post-infection downstream effects in the brain in most cases[143,144].

Several hypotheses exist to explain the direct infection of the brain by the virus or the indirect effects from peripheral tissue infection. Among the possible routes of direct infection, the olfactory pathway has received particular attention. Given the high viral load observed in the nasal cavity, it has been suggested that the virus may enter through olfactory sensory neurons and reach the brain via retrograde axonal transport[145,146] (Figure 5). This hypothesis has been supported by studies in transgenic mice expressing the human *ACE2* receptor, in which intranasal infection led to the detection of viral particles in brain tissue[104,147]. Other neuronal routes have also been considered, including peripheral nerves innervating organs such as the lungs and the gut[148,149]. Additionally, a hematogenous route has been proposed, supported by

the presence of viral RNA in the blood of infected individuals[150,151] and reports of SARS-CoV2 infection of endothelial cells from the wall of blood vessels[152,153] (Figure 5). Finally, systemic inflammation and the associated cytokine storm, could fragilize the integrity of the blood-brain barrier (BBB), potentially facilitating viral entry into the brain[126] (Figure 5).

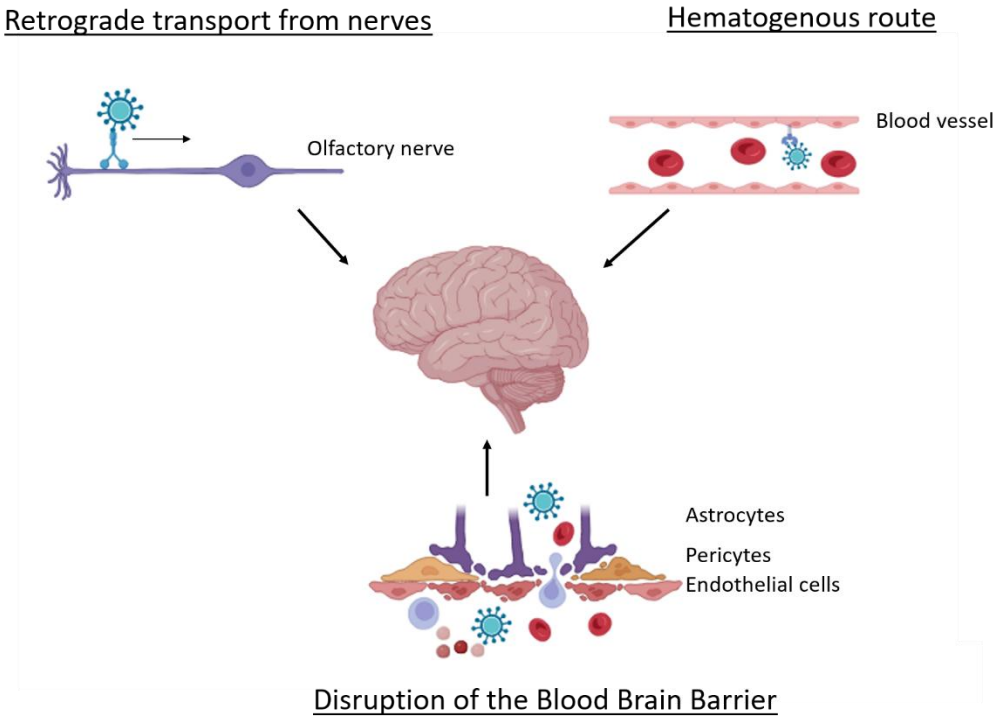


Figure 5: Hypothesis of the mechanisms of SARS-CoV2 brain invasion.

One hypothesis for direct viral infection involves retrograde transport along nerves, such as the olfactory nerve. Indirect infection is also believed to occur via the hematogenous route, where SARS-CoV2 enters through ACE2 receptors expressed on endothelial cells lining the blood vessels. Additionally, systemic inflammation may disrupt the blood-brain barrier, compromising its integrity and allowing viral access to the brain. Created via Biorender.

1.3.3 Neuroinflammation

Neuroinflammation can be defined as an inflammation of the central nervous system due to, for instance, infection, traumatic brain injury or ischemic stroke, but also to neurodegenerative and metabolic disorders[154]. This inflammation is often characterized by the released of different cytokines such as IL-6, IL-18 and tumor necrosis factor (TNF), that are pro-inflammatory, and by the production of different reactive oxygen species such as nitric oxide (NO)[155]. Inflammation needs to be counteracted in the brain to avoid an escalade of inflammation-related effects, which is driven by the release of anti-inflammatory cytokines[155].

In the brain, the inflammation is mediated by microglia (microgliosis) and astrocytes (astrogliosis) with changes in their morphology, proliferation rate and activation state.
In physiological conditions, microglia exhibit a highly ramified morphology consistent with a continuous surveillance behavior in which they extend and retract processes to monitor the surrounding environment[36]. In the context of viral infection, microglia will adopt an amoeboid morphology, associated with increased phagocytosis activity[36] and the release of pro-inflammatory cytokines such as interleukins (IL) IL-1b, IL-6, and tumor necrosis factor alpha (TNFα)[156]. For instance, studies using transgenic mouse models expressing the human *ACE2* receptor have reported increased activation of microglia following SARS-CoV2 infection, accompanied by elevated cytokine levels, infiltration of peripheral immune cells, and increased apoptotic cell death in the brain[157]. Increased levels of IL-6 have also been reported in serum of COVID-19 patients[158].

In the context of neuroinflammation, reactive astrocytes present a defined morphology characterized by an enlarged soma, a highly ramified structure and the presence of enlarged processes[24,159]. They also present molecular changes with upregulation of some key genes such as *GFAP* and vimentin (*VIM*) but also S100 calcium binding protein B (*S100B*), the cell-surface glycoprotein cluster of differentiation 44 (*CD44)*[159,160], and secreted proteins such as the complement factor C3, the alpha 1 antichymotrypsin (SERPINA3). Reactive astrocytes can contribute to the inflammation of the brain, like microglia, by releasing inflammatory factors and cytokines (IL-6, TNFα, IL-1) and producing reactive oxygen species[161,162]. Therefore enhancing the death of neurons[24]. However, reactive astrocytes can also have a neuroprotective role. In fact, they have been described to be involved in the repair of damage to the BBB, and they can also reduce the inflammation through the release of factors such as TGFβ[163]. Clinical data has shown that about 50% of COVID-19 patients showed astrogliosis and microgliosis with a 44% reported T-cell lymphocyte invasion in the brain tissue[141]. Astrocytes have also been reported to be infected by SARS-CoV2 in the brain of some infected patients[138]. Previous studies on *in vitro* human astrocytes suggested that SARS-CoV2 infected astrocytes led to an increase in the metabolism (glycolysis) as well as in the oxidative response in astrocytes, suggested to cause the observed cell death[138].

1.3.4 Apoptosis

Historically, necrosis was the first described mechanism of cell death, characterized by cellular swelling, lysis and release of intracellular contents into the extracellular environment, thereby triggering inflammation[164]. Necrosis typically occurs in acute non-physiological contexts such as exposure to toxins[164]. In contrast, apoptosis is a form of programmed cell death characterized morphologically by chromatin condensation and nuclear fragmentation, membrane blebbing (caused by the delamination of the plasma membrane from the cortical cytoskeleton), cell shrinkage, and the formation of apoptotic bodies (membrane-bound fragments resulting from the breakdown of the cell)[165,166].

The major driver of apoptosis are cysteine-dependent aspartate specific proteases (caspases)[165,166]. Extrinsic cues like the binding of ligands such as TNF ligand, FAS/CD95 ligand or Apo 2 ligand (TRAIL/Apo-2L) to its death receptors: tumor necrosis factor receptor 1 (TNFR1), FAS cell surface death receptor (FASR/CD45) or death receptor 4/5 (DR4/5)[165,167], induce the activation by cleavage of pro-caspase 8 into active caspase 8[165,166] (Figure 6). The intrinsic pathway depends on the mitochondria and the release to the cytoplasm of caspase-activating proteins such as the cytochrome c[167], which induces the cleavage and activation of pro-caspase 9 into active caspase 9[165,166] (Figure 6). Both caspase 8 and 9 have been shown to be activated by caspase 3 among other effector caspases[165,166], which leads to the apoptosis of the cell via activation of gelsolin (associated to membrane blebbing) and endonuclease (DNA fragmentation)[168] (Figure 6). Other caspases, such as caspases 1, 4, 5, 11 and 12 are classified as inflammatory caspases. They are recruited by inflammasomes, heterologous oligomeric protein complexes activated in response to microbial infections, leading to a form of programmed inflammatory cell death known as pyroptosis[169,170]. Unlike, necrosis and pyroptosis, apoptosis does not trigger an inflammatory response[164,169,170]. Among the protective apoptotic pathways, TNFα signaling-activated nuclear factor kappa B (NF-κB) pathway has been reported to have protective effects against apoptosis via an anti-apoptotic function of NF-κB[171,172]. Superoxide dismutase 2 (SOD2) has also been shown to have a protective role against apoptosis[173]. Indeed, upregulation of SOD2 is associated to decreased levels of cytochrome c and apoptotic cells[173]. In contrast, downregulation of SOD2 is associated to higher leakage of cytochrome c into the cytoplasm and increased number of apoptotic cells[174].

Apoptosis has been linked to various diseases such as neurodegenerative diseases[175], including Alzheimer's disease, which involves the apoptotic loss of cortical and hippocampal neurons[176]; Parkinson's disease, characterized by the apoptosis of dopaminergic neurons in the substantia nigra[177]; and amyotrophic lateral sclerosis (ALS), marked by the apoptosis of motor neurons[178]. Apoptosis has also been related to central nervous system (CNS) cancers, acute injury such as stroke and ischemia and to autoimmune diseases[179].

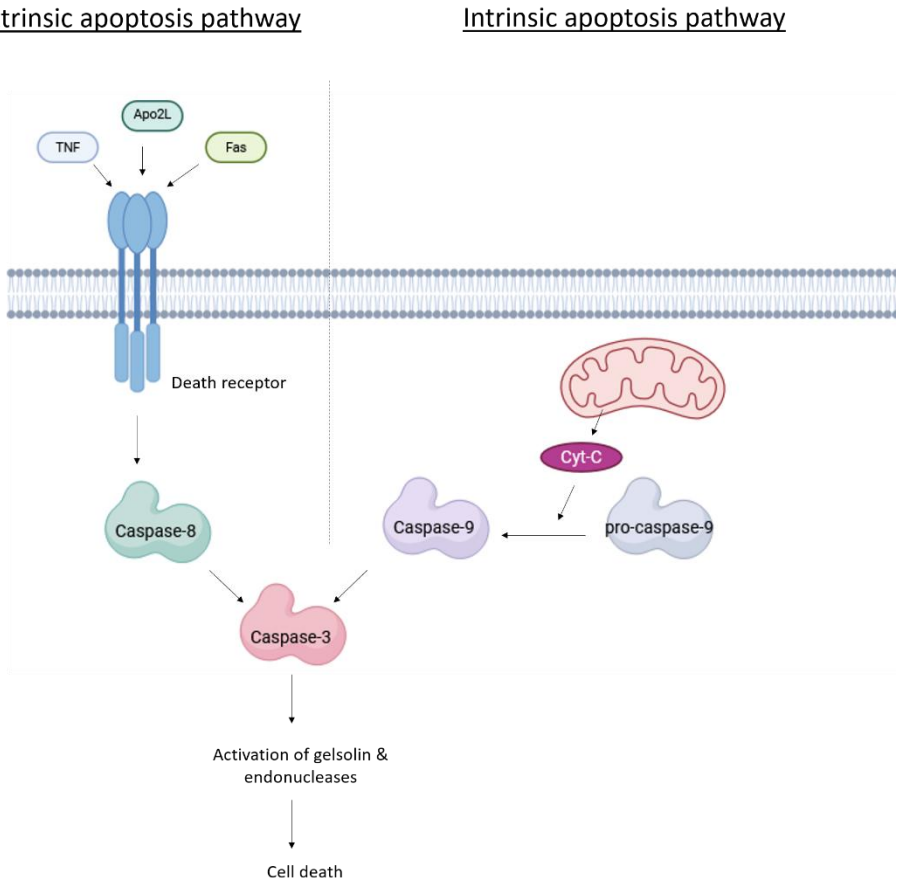


Figure 6: Extrinsic and intrinsic apoptosis pathways.

In the extrinsic pathway, the binding of ligands such as TNF, Apo-2L or FAS to their respective death receptor triggers the activation of caspase 8 which in turn activates caspase 3 among other caspase effectors. In the intrinsic pathway, the release of cytochrome c from the mitochondria cleaves the pro-caspase 9 into active caspase 9 which in turn activates caspase 3 among other caspase effectors. Once activated, caspase-3 cleaves various cellular substrates such as gelsolin and endonucleases, ultimately leading to the controlled death of the cell. Created via Biorender.

1.3.5 Necroptosis

Necroptosis is another form of programmed cell death which leads to increased cellular volume, translucent cytoplasm, swollen organelles and disruption of the membrane. Importantly, necroptotic cells keep their nuclei almost intact[180].

Mechanistically, necroptosis is also initiated by death receptors such as TNF receptor 1 which activates receptor-interacting protein kinase 1 (RIPK1) and will lead to the formation of the necrosome, a multi-protein complex[180,181] (Figure 7). The phosphorylation of the downstream effector mixed lineage kinase domain-like pseudokinase (MLKL) by RIPK1 and RIPK3 induces its translocation to the cell membrane where its binds to phosphatidylinositol (PIP) and impairs the membrane permeability[181] (Figure 7).

In the brain, necroptosis has been associated to several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and ALS[182] but also to ischemia and viral infections[180].

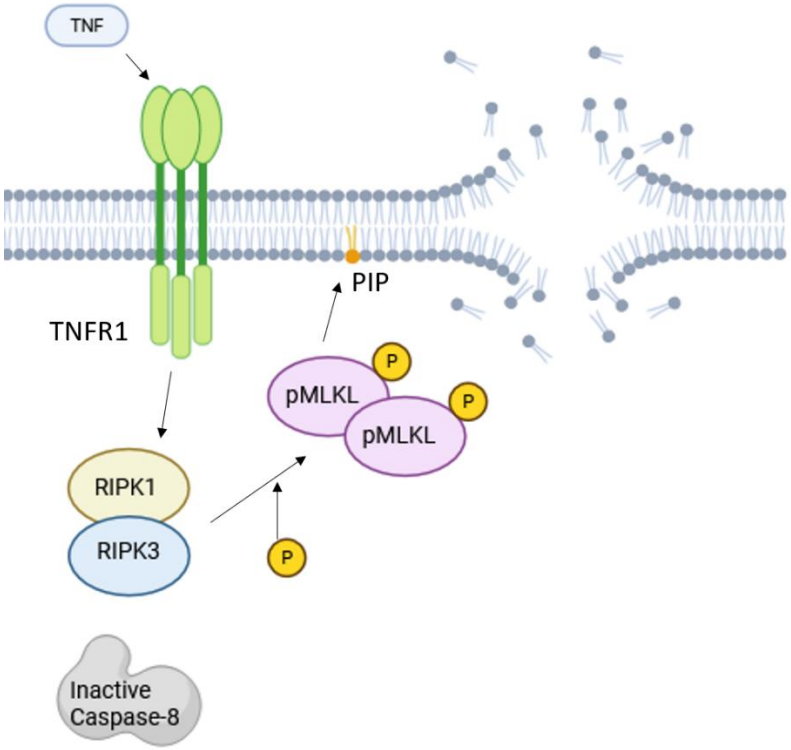


Figure 7: Necroptosis pathways.

In the necroptosis pathway, the binding of ligands such as TNF to death receptors such as TNFR1 triggers the activation of RIPK1 which in turn activates the formation of the necrosome, a multiprotein complex including notably RIPK1 and RIPK3. The necrosome will phosphorylate its effector MLKL which will form oligomers of pMLKL and bind to PIP, leading to disruption of the cell membrane. Created via Biorender.

1.4 Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease affecting around 50 million people across the world (2010 data) with so far no understanding of the full underlying molecular

mechanisms[183]. It was first described by Dr. Alois Alzheimer in 1906[184]. Patients suffering from this disease show signs of dementia; chronic loss of memory (for most of the cases) and decline of other cognitive abilities (e.g. speech)[185]. Dementia can be defined as a strong impairment of cognitive function affecting the independence and the daily life of the patient[183]. In general, the onset of the disease occurs after 65 years old[186]. Two forms of the disease can be distinguished, the familial form (FAD) and the sporadic form (SAD). The majority of patients suffer from the sporadic form (around 95% which mostly occurs later in the life of the patient and is due to a combination of genetic risk factors and environmental factors[187].

FAD patients present typically an earlier onset of the disease (before age of 65) and represent less than 5% of the total AD cases. Dominant mutations in either, the amyloid precursor protein (*APP*), the presenilin 1 (*PSEN1*) or in the presenilin 2 (*PSEN2*) have been found to be the underlying causes of FAD. There are about 35 to 50 pathogenic mutations identified within *APP*, among those, the *APP* V717I G>A, exon 17 (London) mutation was the first one described, and it remains the one most extensively characterized to date[188,189]. The *APP* KM670/671NL (Swedish mutation) is a double mutation found in few Swedish families[188,189], characterized by a general brain atrophy, typical AD pathology and cerebral amyloid angiopathy (CAA)[190,191]. The London mutation is associated with a highly variable neuropathological phenotype with abundant amyloid deposition but with marked inter-individual differences in the distribution of amyloid and the presence of additional pathologies. In addition, more than 300 FAD mutations have been detected within *PSEN1*. Among those, *PSEN1* M146L A>C, exon 5, is an aggressive early onset disease mutation (around 30-40 years old), whereas *PSEN1* A246E C>A, exon 7 is a milder early onset mutation (around 50 years old). Both *PSEN1* mutations M146L and A246E are associated with Alzheimer-type neuropathology, including amyloid plaques, neurofibrillary tangles, neuronal loss and gliosis.

The histopathological hallmarks of AD are the presence of intracellular tangles of hyperphosphorylated tau and the accumulation of extracellular amyloid beta (Aβ) plaques in the brain[192]. Disease progression can be assessed in patients by MRI[193,194] or analysis of different biomarkers such as concentration of $A\beta_{42}$, total tau and phosphorylated tau in cerebrospinal fluid[195]. Positron emission tomography can also be used to detect amyloid

plaques and glucose activity in the brain. In addition, levels of brain atrophy can be assessed by magnetic resonance imaging[185]. More recently, the first FDA approved *in vitro* test for AD biomarker analysis has been released, which assesses the levels of phosphorylated tau (p-tau) 217 in blood[196]. On post-mortem brain tissues, amyloid beta plaques can be detected using thioflavin dye, which is a chemical staining detecting the conformational change of beta-sheet structure of amyloid proteins within plaques[197]. Besides these major AD hallmarks, the disease is also characterized by neuroinflammation and neuronal loss[198] resulting in an atrophy of the patients' brain[199,200].

1.4.1 Clinical characterization of AD

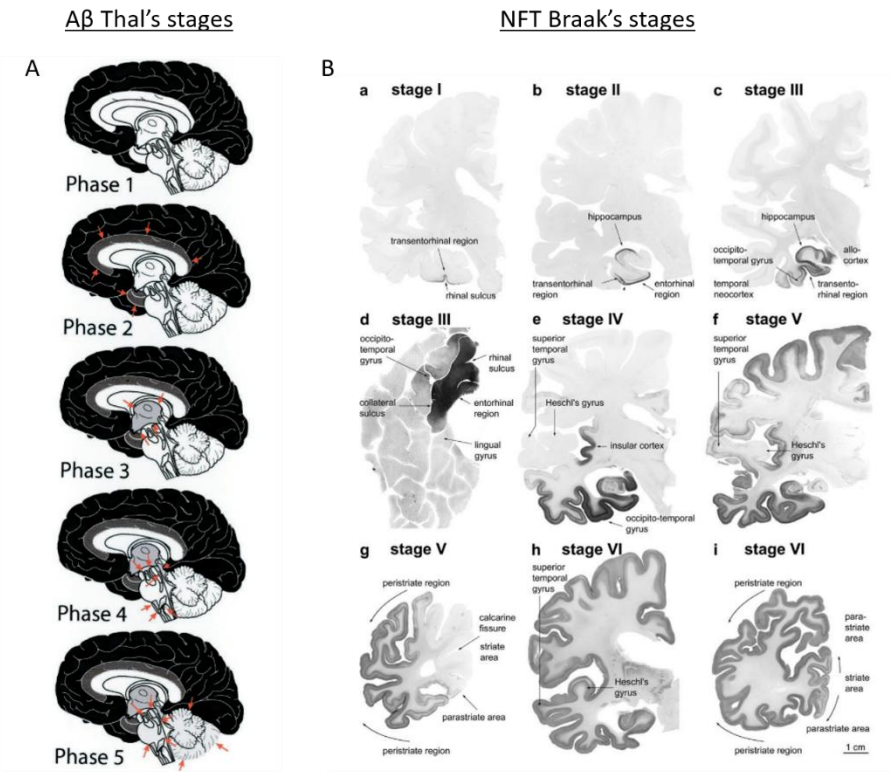
From a clinical point of view, it has been established that the process leading to AD starts to develop in the brain of the patients about 10 to 20 years before the onset of any symptoms[201]. Nowadays, the gold standard for AD identification and disease staging is based on post-mortem semi-quantitative evaluation of the anatomical distribution of amyloid beta plaques, tau tangles and neuritic plaques[201].

The amyloid component is assessed using the Thal's classification[202]. Briefly, Aβ deposits are labeled by immunohistochemistry methods and by silver staining that assess disease stage based on the brain location of aggregates in the post-mortem brain. A stage 1 corresponds to the presence of Aβ deposits only in the neocortex; stage 2 is defined by the presence of additional Aβ in the allocortex; stage 3 by additional Aβ in diencephalic nuclei and striatum; stage 4 includes the presence of additional Aβ in some brainstem nuclei and stage 5 is characterized by additional Aβ in more brainstem nuclei as well as in cerebellum (Figure 8A)[202].

Neurofibrillary tau tangles (NFT) are evaluated using the Braak's stages: stage 1 (I) corresponds to NFT localized in the transentorhinal region of the cortex; stage 2 (II) is defined by additional NFT is the transentorhinal region in entorhinal region and in the CA1 region of the hippocampus. Stage 3 (III) is characterized by increased NFT in the transentorhinal and entorhinal regions, presence of NFT in the subiculum and CA2 of the hippocampus, mild changes can be observed at the thalamus, and amygdala and the basal portions of frontal, temporal and occipital association areas for some individuals. Stage 4 (IV) includes the presence of additional NFT in the transentorhinal and entorhinal regions, CA1 and CA2 regions

from the hippocampus with the presence of NFT also in the regions CA3 and 4, NFT also start to extent in the neocortex. Stage (V) presents NFT throughout the hippocampus and neocortex, the frontal, superolateral, and occipital regions and they start to reach the peristriate region. In stage 6 (VI), most regions of the neocortex contain NFT, which are present in almost all neuronal layers, and in occipital lobe. NFT reaches the parastriate and striate areas (Figure 8B)[203].

Neuritic plaques are Aβ plaques surrounded by tau aggregates in degenerating neurons. Based on their density in the neocortex, there have been ranging from none, sparse, moderate and frequent by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD)[201]. The combination of Aβ plaques (A), NFT (B) and neuritic plaques (C) phenotypes are used to form an "ABC" score, ranging from A0B0C0 to A3B3C3 (Figure 8C). Each 4 categories correspond to; (0) absence, (1) low, (2) intermediate and (3) high AD neuropathologic changes[204]. The levels of amyloid beta deposition in the brain of the patient do not always correlate with their cognitive state[155]. On the contrary, patients exhibiting advanced Braak changes (based on NFT) were most of the time, presenting cognitive impairment, suggesting a closer relationship of the latter with onset of symptoms[201].



C "ABC" score

"A"	Thal Phase for Aβ plaques [57]	"B"	Braak and Braak NFT stage [14,15]	"C"	CERAD neuritic plaque score [41]
0	0	0	None	0	None
1	1 or 2	1	I or II	1	Sparse
2	3	2	III or IV	2	Moderate
3	4 or 5	3	V or VI	3	Frequent

Figure 8: Visualization of the location of Aβ deposits used to define the Thal's stages and the NFT used to define the Braak's stages and the "ABC" score.

A. Modified from Thal et al.,2002 B. Modified from Braak et al., 2006. C. Modified from Montine et al., 2012.

Whereas these classifications have been a major advance in AD staging, however, there is also a need to be able to track the evolution of the disease in patients which are still alive through uniform methods that serve as a reference standard. Such a standard should allow clinicians to communicate using the same reference for staging the disease and could be used to classify patients for their adequacy as recipients of therapies targeting specific phases of the disease[201]. Several standards have been developed, notably the Clinical Dementia Rating (CDR) that ranges AD patients from score 0 (none), 0.5 (questionable), 1 (mild impairment), 2 (moderate) and 3 (severe)[205]. This CDR is assessed in the clinics by questioning directly the patient but also, if available, a knowledgeable informant and is based on cognitive and behavioral tests[201]. The final score depends on scores from 6 different categories in which

memory is the main one next to the 5 other secondary categories: orientation, judgement and problem solving, community affairs, home and hobbies and personal care. The CDR final score will be equal to the partial memory score if at least 3 secondary categories have the same score as the memory score. If 3 or more secondary categories have a score lower or higher than the memory score, the CDR score will be the value corresponding to the majority of the secondary categories, independently of the value of the memory score. In the case the secondary categories show a combination of higher and lower scores than the memory score, only the latter is taken into account[205].

More recently, in 2021, the National Institute on Aging – Alzheimer's Association (NIA-AA), proposed a NIA-AA scoring method for patients which already have biomarkers associated to AD[206]. This scoring method is based on 4 components: the objective cognition (OBJ); subjective cognitive decline (SCD); neurobehavioral symptoms (NBS) and impact on daily life (FXN) and requires 3 medical visits spred over ± 30 months. The OBJ is composed of the current cognitive performance (determined on visit 3) and the decline in cognition which englobes all visits. The SCD is based on the level of independence to execute daily life cognition related tasks. NBS encompasses anxiety and clinical depression levels and FXN is the ability of the individual to perform independently daily life tasks[206]. By taking this 4 components into account, patients can be assessed as stage 1, which is defined by normal cognitive function, stage 2 associated to normal cognitive function but marked by a decline from previous measurements, stage 3 correspond to lower cognitive function but with the ability to perform daily tasks, and stage 4, 5 and 6 with respectively mild, moderate and severe dementia[201]. Positron Emission Tomography (PET) imaging of Aβ deposits and tau give the spatial location of these features and are powerful tools to follow disease progression, even in the absence of cognitive symptoms. Novel methods, such as the detection of biomarkers in cerebrospinal fluid (CSF) and even plasma of patients such as Aβ42 and p-tau 217 or p-tau 181 can also be used to monitor the risk of developing AD and the progression of the disease allowing for a less invasive method to monitor disease stage in patients[195].

Notably, recent studies have shown that a significant number of AD patients present features from other dementia linked pathologies such as Lewis bodies or TDP-43 characteristic of Lewy

body dementia (LBD) and TDP-43 proteinopathies[207]. The presence of several markers linked to multiple pathologies has been suggested to be a characteristic of AD[207].

1.4.2 Amyloid precursor protein (APP) processing

In the context of FAD, around 14% of the patients carry dominant mutations in *APP*[208], localized on the chromosome 21. Individuals harboring a trisomy of the chromosome 21 or a duplication of *APP* have been shown to be more prone to develop Alzheimer's disease[209]. *APP* can be spliced into 3 majors isoforms: APP695, APP751 and APP770, with the APP695 being the form the most expressed in neurons[210] and APP751 and APP770 the forms the most expressed in astrocytes[211]. APP is a type I transmembrane protein composed of an extracellular N-terminal domain, a hydrophobic transmembrane domain, and a short C-terminus intracellular domain[212].

APP can be cleaved following both, the amyloidogenic and non-amyloidogenic pathways. In the amyloidogenic pathway, APP is first cleaved by a β-secretase (β-site APP cleaving enzyme 1 or 2 (BACE1 or BACE2))[213] that leads to the production of the secreted APP fragment (sAPP-β) which is released outside of the cell and to a fragment attached to the inner cell membrane, the C terminal fragment (CTF-β) or c99 fragment. Consecutively to the first cleavage, the CTF fragment is cut by the γ-secretase, leading to the production of Aβ peptides that are released to the external environment of the cell and the generation of the APP intracellular domain (AICD) fragment (Figure 9A)[212]. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase enzymes (notably members of the A disintegrin and metalloproteinase (ADAM) proteins)[213] which produce the secreted APP fragment (sAPP-α) which is released externally and the CTF-α or c83 fragment that remains intracellular. CTF-α is further cut by the γ-secretase, resulting in the production of the p3 fragment which will be released in the external environment and the AICD fragment (Figure 9A). After the release to the extracellular environment, Aβ peptide monomers can aggregate and form a structure called oligomers. Further, oligomers can organize themselves into fibrils, that with time may form the senile plaques, which can be classified into two main types, diffuse and dense-core (associated with inflammation)[214,215]. Patients with an advanced stage of the disease exhibit more than 20 plaques per mm^2 in the brain[216].

In the context of FAD, there is an imbalance between the amyloidogenic and the non-amyloidogenic pathways, in which FAD mutations promote the generation and aggregation of amyloidogenic Aβ peptides. For instance, the *APP* V717I London mutation alters γ-secretase processing of APP, increasing the Aβ42/Aβ40 ratio and favoring longer Aβ species through changes in APP-PS1 interactions and cleavage dynamics[217,218]. *APP* KM670/671NL, Swedish mutation, is located just before the N-terminus sequence of the Aβ peptide, where the β-secretase cleavage occurs. In the presence of this mutation, β-secretase activity is enhanced and results in increased production of Aβ peptides[217,219]. On the other hand, in sporadic forms of the disease, Aβ aggregation may be mediated by defects in the proteostasis pathways that can impair the clearance of Aβ peptides[214].

Lastly, APP can also be processed by other non-canonical pathways such as the η-secretase which produces a CTF-η that is enriched in dystrophic neurites in AD brain and *in vivo* models[220] (Figure 9B). Following this first cleavage, the protein can either be cleaved by α-secretase (generating Aη-α) or β secretase (generating Aη-β) (Figure 9B). Aη-α peptides have been found to reduce calcium activity and long-term potentiation (involved in memory processes) in hippocampal neurons[220]. Due to the various enzymes which can cleave APP, and the resulting production of various metabolites, the understanding of the role of APP processing is not fully understood[218,221].

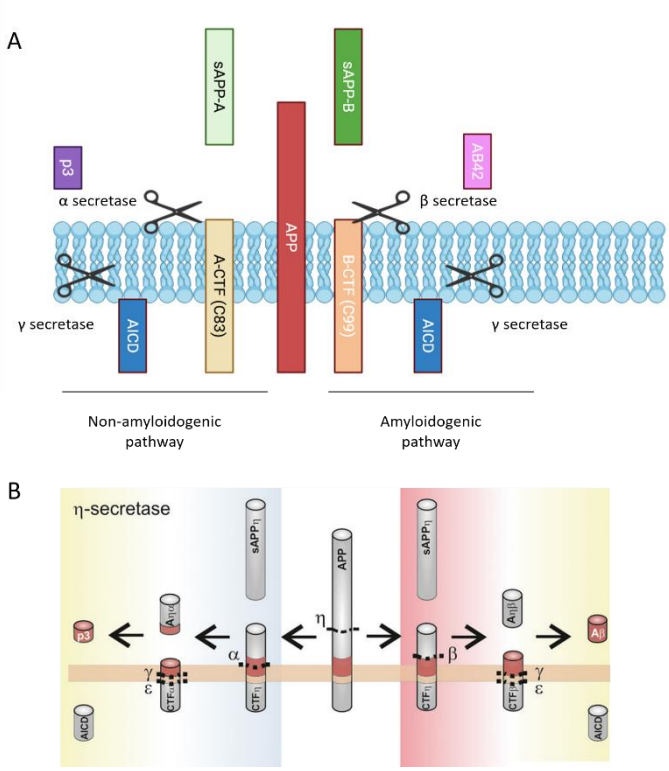


Figure 9: APP processing through non-amyloidogenic and amyloidogenic pathways.

A. In the amyloidogenic pathway, APP is first cleaved by a β-secretase that leads to the production of sAPP-β and the CTF-β or c99 fragment. Consecutively to the first cleavage, the CTF fragment is cut by the γ-secretase, leading to the production of Aβ peptides and the generation of the AICD fragment. In the non-amyloidogenic pathway, APP is first cleaved by α- which produce the sAPP-α and the CTF-α or c83 fragment. CTF-α is further cut by the γ-secretase, resulting in the production of the p3 fragment and the AICD fragment. Created via Biorender. B. APP can also be processed according to other non-canonical pathways such as by a η-secretase which produces a CTF-η. Following this cleavage, the protein can either be cleaved by α-secretase (generating Aη-α) or β secretase (generating Aη-β) fragments. Figure from Eggert et al., 2018.

1.4.3 Roles of APP metabolites in the cell

1.4.3.1 Secreted APP fragments

The role of full-length APP has not been completely elucidated but several roles related to cell surface receptor, cell adhesion, neuronal growth and axon guidance, synaptogenesis and synaptic function, among others, have been described to date[210,218,221]. Among those, notably, a role of secreted APP fragments binding the gamma-aminobutyric acid type B receptor subunit 1a ($GABA_BR1a$) receptor and modulating interneuron function has been described recently[222]. In this paper the authors found that sAPPα can bind to $GABA_BR1a$ and modulate synaptic plasticity by decreasing the recycling of synaptic vesicles and their release probability[222]. Moreover, APP expression is ubiquitous, and as such it is also expressed in other tissues such as liver, lungs and heart, suggesting additional roles for APP and/or its fragments

in various tissues outside the brain[212]. Additionally, there is a broad subcellular localization of APP which includes the cell membrane[221], ER[218], Golgi[218], endosomes[218], lysosomes[218] and also mitochondria[223]. These findings may suggest multiple organelle-dependent phenotypes following APP processing in the cell[218,221].

1.4.3.2 Aβ fragments

Aβ fragments derived from APP processing following the amyloidogenic pathway, are commonly a mixture of several (length) species of different amino acid (a.a) sizes ranging from 37 to 49 a.a with the longer Aβ peptides being reported to be more amylogenic than shorter ones due to reduced solubility, favoring the aggregation[214]. In addition to its pathological roles, Aβ may act as an antimicrobial peptide, binding microbial surfaces and fibrils to limit infection and biofilm formation[224]. Like classical pathogen-associated molecular patterns (PAMPs), Aβ can activate innate immune receptors such as Toll like receptor (TLRs) on microglia, triggering pro-inflammatory responses and enhancing phagocytosis[225]. These observations suggest that Aβ aggregation may represent an ancient, protective defense mechanism against pathogens.

Aβ may also be necessary for synaptic plasticity and memory, and its depletion leads to reduced LTP and short- and long- term memory deficits in mice[226]. Additionally, exogenously added picomolar concentration of Aβ42 led to enhanced memory in mice[227]. On the other hand, Aβ may exert neuronal toxicity by binding to a variety of cellular receptors: p75 neurotrophin receptor (P75NRT), LRP, cellular prion protein (PrP^c), glutamate metabotropic receptor 5 (GMR5), neuronal acetylcholine receptor subunit alpha-7 (ACHA7), NMDAR, beta-adrenergic receptor (β-AR), erythropoietin-producing hepatoma cell line receptor (EPHR) and paired immunoglobulin-like receptor B (PIRB)[228] (Figure 10). The binding of soluble Aβ to these receptors initiates a cascade of intracellular events that lead to the generation of reactive oxygen species, synaptic dysfunction, DNA damage, and endoplasmic reticulum (ER) stress. It also promotes tau protein hyperphosphorylation and triggers inflammatory responses[228]. Collectively, these pathological processes may drive neuronal degeneration and contribute to the onset of Alzheimer's disease (Figure 10).

On the other hand, downstream effects of Aβ peptides comprise the modulation of calcium levels inside the neurons through either its direct binding to NMDAR[21,229] or through the formation of intramembrane channels permissive to calcium[230]. Binding of Aβ peptides to NMDA receptors may lead to increased neuronal activity[21,229]. In addition, Aβ has also been suggested to enhance neurotransmitter release[231], which could further contribute to neuronal hyperactivity.

Moreover, mitochondria function has been demonstrated to be modulated by Aβ peptides[223]. Aβ fragments disrupt the electron transport chain and interact with various mitochondrial enzymes, thereby reducing the transfer of hydrogen from the mitochondrial matrix to the intramembrane space[232]. As a consequence, the mitochondria membrane potential decreases, impairing the ATP production and leading to increase levels of reactive oxygen species (ROS)[232]. Although most of the literature reports a decrease in mitochondrial activity in response to Aβ exposure, it has also been shown that Aβ can initially increase mitochondrial activity by increased calcium concentration within the mitochondria[233]. However, this hyperactivity can subsequently impair mitochondrial function, leading to increased production of ROS and potentially triggering the release of pro-apoptotic factors, ultimately resulting in cell death[234]. Aβ peptides have also been implicated in endolysosomal dysfunction, a topic that will be discussed later in detail see Section 1.4.12.

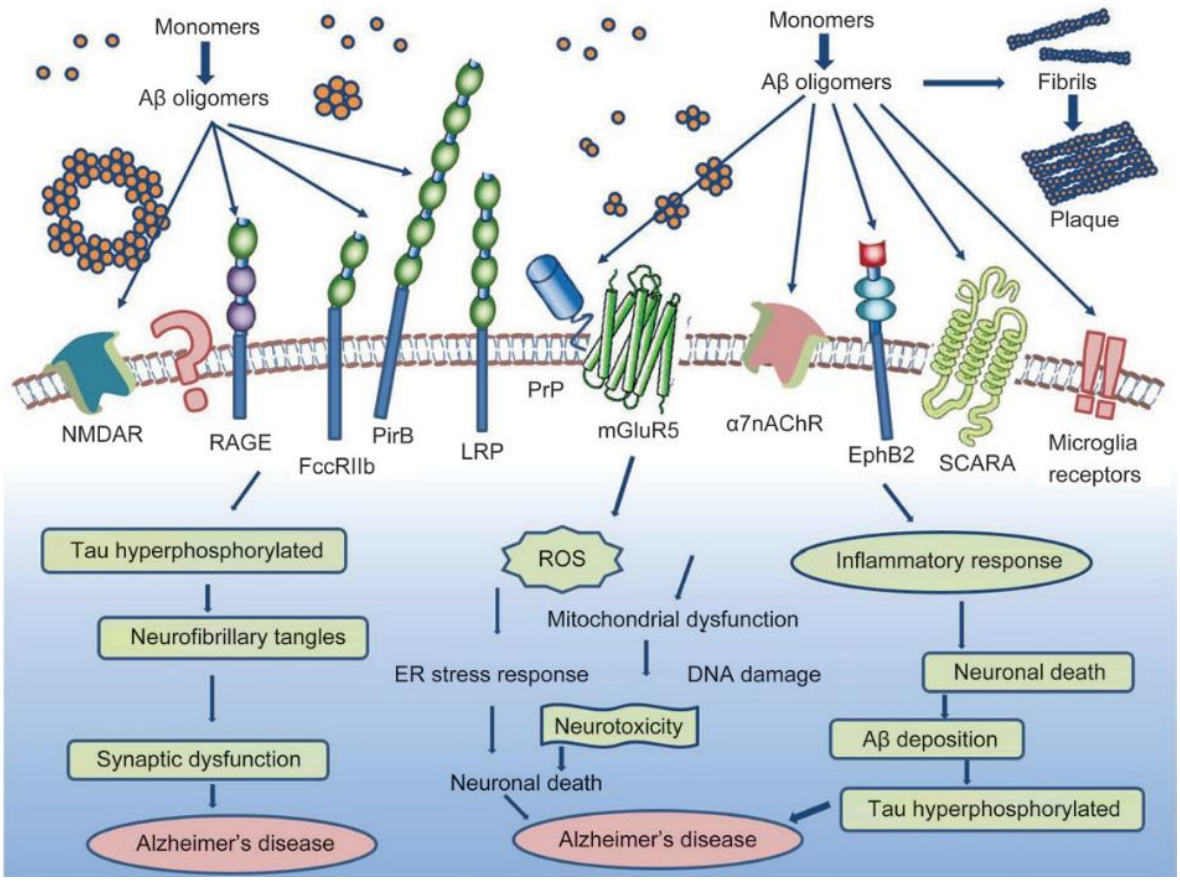


Figure 10: Representation of the different binding Aβ/ Aβ receptors and their downstream effects.

Soluble Aβ can bind to specific cellular receptors and trigger downstream signaling pathways that produce reactive oxygen species, disrupt synaptic function, induce DNA damage and ER stress response, promote tau protein hyperphosphorylation, and induce inflammatory responses. These processes may contribute to neuronal death and the development of Alzheimer's disease. Figure from Chen et al., 2017.

1.4.3.3 CTF fragments

Membrane bound CTF-α fragments have shown a neuroprotective role, facilitate memory, synapse plasticity and promote cell survival[221,235]. On the other hand, CTF-β may impair memory and cognitive function[235]. In addition, one study also highlighted the presence of endolysosomal defects caused by both CTF-α and CTF-β fragments[236]. CTF-α and CTF-β have also been hypothesized to influence the levels of p-tau in the cell. In this report the authors measured changes in p-tau levels which were correlated to the ratio of C99/C83 fragments. Although the mechanisms underlying this effect were not further investigated[237].

1.4.3.4 AICD fragment

The AICD fragment has been hypothesized to play a role in transcriptional regulation. *In vitro* studies indicate that AICD interacts with the adaptor protein Fe65 in the cytoplasm[238,239]. The AICD-Fe65 complex may subsequently translocate to the nucleus, where Fe65 could recruit the histone acetyltransferase TIP60, leading to the formation of a transcriptional regulatory complex[238,239]. This complex has been proposed to regulate gene expression and modulate several pathways, such as cell death, cellular trafficking, neuronal guidance, neurogenesis, synaptogenesis, ER calcium homeostasis and genes involved in APP processing such as BACE1[238]. It has also been suggested that the soluble AICD fragment could influence p-tau by modulating the levels of expression of GSK-3β (one of the kinases that phosphorylate the tau protein)[239]. In addition, the soluble AICD has been proposed to inhibit Wnt signaling, thereby promoting neuronal differentiation and proliferation, and to modulate intraneuronal calcium homeostasis[238,239]. However, these hypotheses remain difficult to demonstrate, as the soluble AICD fragment has a short half-life and most reports have relied on non-physiological conditions (APP/Fe65 overexpression, γ-secretase inhibitors)[238].

1.4.4 Presenilins

Most of the familial Alzheimer's disease (FAD) dominant mutations have been linked to *PSEN1* (80%), and a small percentage of FAD patients carry mutations within *PSEN2* (5%)[208]. PS1 and PS2 proteins share 66% of identity[240] and constitute the catalytic part of the γ-secretase complex[241]. The γ-secretase complex is able to interact and cleave various substrates (more than 30): APP, as described above, but also NOTCH, beta-catenin, N-cadherin, E-cadherin, ERBB4, LRP, CD43, CD44 and tyrosinase proteins among others[208,242]. *PSEN1* and *PSEN2* are predominantly expressed in neurons but are also expressed in glial cells to a lower extent[243]. PS1 subcellular localization comprises the cell membrane, early endosomes as well as recycling endosomes, whereas PS2 has been detected mainly in early and late endosomes[208,240,244].

FAD mutations in *PSEN* may increase the production of $A\beta_{42}$ and $A\beta_{43}$ species over shorter species such as $A\beta_{40}$[245] or $A\beta_{38}$, being the former more prone to aggregation and therefore enhancing the formation of Aβ plaques[246,247]. For instance, the *PSEN1* A246E and M146L

mutations lead to altered γ-secretase processing, with a shift towards longer, more pathogenic Aβ species[248].

Nowadays it is accepted that this shift in Aβ peptide length, from shorter to longer peptides, is the result of a loss-of-function of the γ-secretase complex[246,247]. In addition, PS1 has been shown to have γ-secretase independent roles, such as controlling cell adhesion through the regulation of β-catenin levels and Wnt signaling and regulating calcium homeostasis in the ER[234]. In the latter, PS1 function has been linked to increased activity or expression of calcium release channels (such as ryanodine receptors (RYRs) and inositol 1,4,5-trisphosphate-gated calcium channel (ITPR3), modulation of the smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, but also a direct role in the formation of calcium leak channels[249]. Interestingly, the presence of mutations within *PSEN1* has been shown to lead to abnormal Ca^{2+} levels and its massive release to the cytoplasm of the cell[249].

1.4.5 Tau (*MAPT*)

MAPT can produce 6 alternative isoforms of the tau protein in the adult brain. Three of these isoforms contain 3 microtubule binding domains (called 3R tau isoforms), whereas the other three contain an additional fourth domain 4-repeat tau (4R tau isoforms)[250]. Inclusion or exclusion of exon 10 of *MAPT* results in the production of 4R tau or 3R tau, respectively[250]. In humans, 4R tau isoforms are only present postnatally, whereas 3R tau forms are expressed both during embryonic development and adulthood[50]. Tau is a microtubule-associated protein that stabilizes microtubules upon binding and thereby modulates their dynamics, a process essential for axonal growth and guidance during neuronal maturation[50].

Interactome studies have revealed tau interactions with various signaling pathways involved in cell differentiation, survival and synaptic plasticity[251]. For an example, the N-terminal phosphatase-activating Domain (PAD) of tau can activate PP1–GSK-3β, leading to kinesin-1 phosphorylation and its subsequent detachment from cargo[251]. Tau has also been reported to interact with post-synaptic kinases such as FYN, mediating its localization to post-synapses where it phosphorylates the NMDAR subunit 2b, enhancing its stabilization[251]. In oligodendrocytes, FYN-mediated phosphorylation of ROA2 decreases MBP mRNA transport,

thereby enabling its local translation. This process requires tau, as its silencing blocks MBP mRNA transport[251]. In addition, tau has been reported to promote signal transduction in insulin and neurotrophic factor pathways[251].

While most studies have focused on tau association with microtubules, approximately 16% of total tau localizes within the nucleus and interacts with nuclei acids[252]. Under stress conditions, tau is translocated to the nucleus, through a protective process against DNA damage[252]. Nuclear tau has also been implicated in heterochromatin regulation, and tau depletion disrupts the distribution of epigenetic marks and protein involved in gene silencing, correlating with higher levels of heterochromatin[252]. This phenotype can be rescued by the overexpression of nuclear-targeted tau[252]. Conversely, tau phosphorylation prevents its nuclear localization, precluding its protective and regulatory functions[252].

Tau protein can undergo various PTMs such as phosphorylation, ubiquitination, sumoylation, glycosylation, methylation, etc. and tau (hyper)phosphorylation results in microtubules detachment[253]. In AD brain, tau is highly phosphorylated at multiple sites, such as serine 202 and 214, as well as at threonine 205, 212 and 217 (phosphorylation sites recognized by the AT8 and AT100 antibodies)[254]. Notably, tau hyperphosphorylation also occurs in a non-pathological context during brain development[254].

Hyperphosphorylation of tau may impair microtubule stability by favoring depolymerization and leading to defects in transport of organelles such as mitochondria, autophagic and synaptic vesicles[255]. In addition, tau (hyper)phosphorylation and aggregation not only impacts microtubule stability and axonal transport, but reduces the number of synapses and impairs mitochondria function in mice[256,257]. Tau pathology is a hallmark of several neurodegenerative disorders including AD and frontotemporal dementia (FTD), but the mechanisms and isoform involvement differ across diseases[250]. The process of tau aggregation and spreading has been related to prion propagation, in which misfolded aberrant tau triggers the recruitment of normally folded tau proteins to seed and extend fibrils[51,258,259]. In FTD, mutations in *MAPT* often lead to an imbalance in tau isoform expression, particularly an increase in 4R tau, which has been associated with enhanced aggregation propensity[250,260]. In contrast, AD typically does not clearly involve *MAPT* mutations, nor a clear overrepresentation of 4R tau, although

both 3R and 4R isoforms can be found in tau aggregates[260]. Studies using 2D hiPSC derived neurons that lack 4R tau expression present very few intraneuronal tau accumulations compared with 4R tau expressing neurons, suggesting that 4R tau is necessary for the formation of tau aggregates[259]. Further, these models showed that human neurons expressing 4R tau and containing p-tau inclusions exhibit reduced calcium peaks, suggesting an impact on neuronal activity[259].

1.4.6 Aβ and tau interactions

In cognitively normal older adults, tau pathology initially appears in the entorhinal cortex, and the presence of cortical Aβ plaques increases the likelihood of tau spreading to additional cortical regions[261]. Similarly, in AD patients, tau propagation tends to occur preferentially in brain regions already affected by Aβ plaques, suggesting interactions between amyloid beta plaques and tau pathology[261]. The association of Aβ plaques and tau has also been linked to cortical hypometabolism (reduced glucose metabolism), a predictor of memory decline. Additionally, in patient exhibiting Aβ deposition, cognitive performance has been reported to correlate with total tau and p-tau levels in the CSF[261].

At the molecular levels, Aβ has been reported to increase cytosolic Ca^{2+}levels, leading to the activation of calcium-dependent kinases such as GSK-3β, which in turn phosphorylates tau and contributes to the increase of p-tau[234]. tau phosphorylation can also be triggered by Aβ binding to several receptors, including RAGE, which will activates downstream kinase signaling pathways[228]. In addition, Aβ and tau proteins can directly interact, promoting conformational changes that facilitate tau aggregation and the formation of mixed Aβ-tau oligomers or granular aggregates. These aggregates have been shown to be toxic, either through loss of function of tau or through the intrinsic toxicity of the aggregates themselves[262].

1.4.7 AD hypothesis

More than a century after its initial description by Alois Alzheimer, the pathological mechanisms underlying Alzheimer's disease remain incompletely understood. One major challenge lies in the long preclinical phase of the disease: neuropathological changes begin to accumulate 10 to 20 years before the appearance of cognitive symptoms and AD diagnosis[263].

As a result, the majority of our current understanding is based on late-stage observations, complicating the distinction between causal mechanisms and late downstream consequences. Furthermore, a plethora of intracellular pathways has been implicated as components of AD pathogenesis[264]. This complexity has given rise to multiple hypotheses aimed at explaining the etiology of AD (Figure 11)[187,231,234].

One of the main hypothesis that was postulated to explain the origin of the disease is the amyloid cascade deposition hypothesis, which places Aβ at the center of the pathological events in AD[265]. In support of this hypothesis, a percentage of FAD patients carry mutations within the *APP* gene, *APP* duplication and trisomy of chromosome 21 are related to AD[209,218]. This hypothesis was born in 1992 after the discovery by Yanker's group and others of the neurotoxic effects of some amyloid peptides in cell culture leading to neurodegeneration[266], also supported by more recent work[265,267]. The hypothesis assumed that accumulation of amyloid beta deposits in the brain would lead to neurodegeneration and neuronal loss but also to the formation of tau tangles[265,268]. Later, *in vivo* models showed that injection of Aβ species triggered increased formation of tau tangles in the brains of tau mutant (P301L) mice[269].

Another major hypothesis posits hyperphosphorylation of tau and formation of neurofibrillary tau tangles as the key elements triggering the disease. This hypothesis strongly relies on the observation of a closer correlation between the spreading of tau pathology (PET data) and cognitive function decline compared to Aβ deposition in the brain. Indeed tau tangles have been detected in the brain of the patients years after the detection of Aβ plaques[270]. However, the presence of solely tau tangles is not restricted to AD as they are present in other neurodegenerative pathologies such as FTD, but in the absence of amyloid beta plaques, contrary to AD.

Changes in calcium homeostasis, such as abnormal increase of intracellular, mitochondrial and ER calcium detected in AD models have also led to the hypothesis of calcium signaling defects at the origin of the disease[234]. Mitochondria function alterations in the cell have also been described as possible causes of AD[187]. Another hypothesis state that neuroinflammation, mostly mediated by active microglia releasing cytokines, will be the main responsible for the

changes in the brain leading to neurodegeneration and AD[271]. In addition, the cholinergic hypothesis, postulates that there is a reduction in acetylcholine levels and choline acetyltransferase activity in the brain that may lead to the origin of the disease[187]. Other hypothesis link metabolism changes or vascular changes to the origin of AD[187].

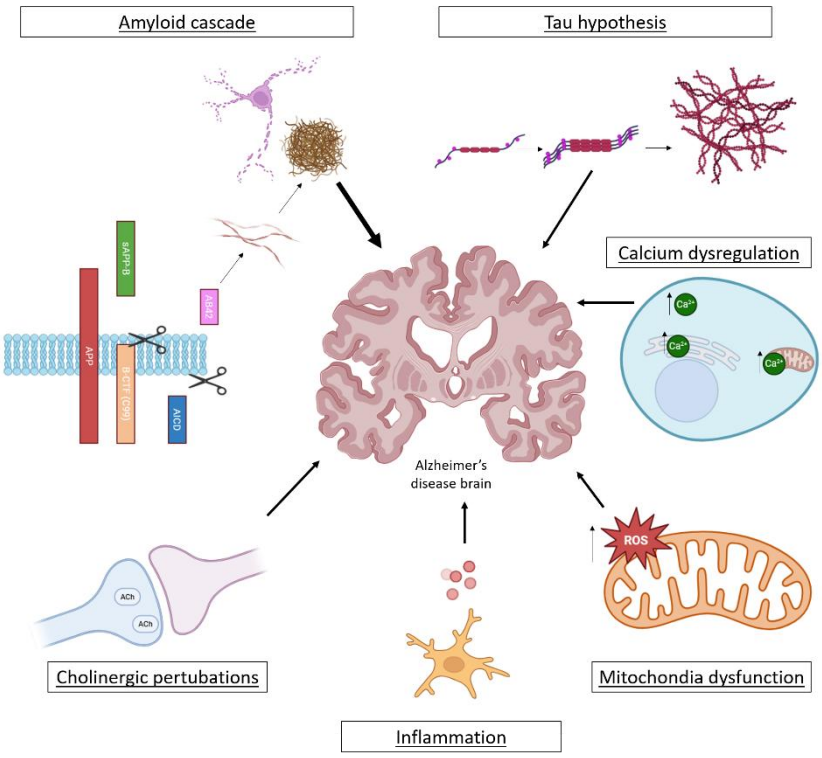


Figure 11: Current hypothesis to explain Alzheimer's disease.

Scheme of the major current theories explaining disease initiation. Created via Biorender.

1.4.8 Overview of the major past and current therapeutic strategies to slow down AD

Numerous hypotheses have been proposed to explain the pathogenesis of AD, some of which have served as the foundation for therapeutic development and clinical trials[231,272]. Early efforts were focused on the cholinergic hypothesis, with the generation of tacrine, donepezil, Rivastigmine and galantamine cholinesterase inhibitor drugs which were approved by the Food and Drug Administration (FDA) in 1993, 1996, 2000 and 2001, respectively[273,274]. The use of these drugs lead to increased levels of acetylcholine in the brain. The four of them reported similar effects providing symptomatic relief for cognitive symptoms but they do not slow down or halter the progression of the disease. In addition, they showed minor adverse effects at the gastrointestinal level and in a smaller proportion, dizziness[272,275]. Memantine is an NMDA

receptor antagonist which reduces the stimulation of the glutamatergic system in order to reduce excitotoxicity of the neurons due to a constant influx of calcium ions. It was approved by the FDA in 2003 and is currently prescribed in combination with cholinesterase inhibitors for mild to severe AD patients[272,275]. However, as for the cholinesterase inhibitors, memantine offers only symptomatic relief in the patient's cognitive decline and as such cannot modify the course of the disease.

Based on the amyloid cascade hypothesis, drugs have been developed to target and inhibit the activity of the β- and γ- secretases, the enzymes cleaving APP through the amyloidogenic pathway, to reduce the levels of Aβ in the brain. However those treatments are accompanied by severe deleterious effects as these enzymes have additional substrates which cleavages are essential for specific tissues of the body[231]. The β-secretase is a type I membrane anchored aspartyl protease found in endosomes and the Golgi with an optimal pH of 4.5[276]. Besides APP, it has numerous substrates such as SEZ6, neurotrimin and neurexin-1a among others which are associated to neurite growth and synapse formation, pointing out that β-secretase is also playing an important role in non-pathological conditions[277]. The γ- secretase also cleaves other proteins besides APP, such as the Notch signaling proteins[231] which are essential during neuronal development for cell proliferation and differentiation, but also in the adult, controlling the proliferation of cells in the epidermis and the hematopoietic system, among others[278].

Based as well on the amyloid cascade hypothesis immunotherapies were developed aiming at triggering an immune system response to enhance the clearance of Aβ plaques. For instance the AN1792 vaccine, which targets $A\beta_{42}$[231,279] lead to a reduction of Aβ plaques, tau phosphorylation levels and microglia activation as well as an improvement of neuritic plaques ("amyloid core and a feltwork of changed neurites"[280]) and a decrease in cognitive decline[279]. Moreover, brain volume measured 3.5 years after treatment showed a similar decrease between the treated and placebo groups[279]. However, a small percentage of the patients developed meningoencephalitis[279], which led to a search for new vaccines to target Aβ. New generations of Aβ vaccines were developed to target 1-6 amino acid fragment of the Aβ sequence, with several clinical trials currently ongoing[279]. Therapeutic strategies targeting tau, including both vaccines designed to elicit an immune response and monoclonal antibodies,

are currently being tested in clinical trials, but results to date remain preliminary and inconclusive regarding their efficacy on cognitive decline and tau pathology[281,282].

Similarly, passive immunotherapy strategies were also developed based on the amyloidogenic cascade hypothesis which focused on the development of Aβ monoclonal binding antibodies, such as bapineuzumab, Crenezumab, solanezumab and ponezumab, most of those directed towards the C-terminus of the central region of the Aβ peptide sequence[283]. However, despite showing potential to clear Aβ plaques, these therapies failed to show any improvement in patients in phase 3 clinical studies and/or showed serious adverse effects[231,281]. In fact, clinical trials conducted from 2012 to 2017 in mild to late-stage AD showed no significant effects on cognition with any of these treatments. Aducanumab, a monoclonal antibody targeting a specific conformation of Aβ to recognize oligomers and plaques was approved by the FDA[272,281,284]. Data from two parallel large phase 3 clinical trials in 2019 showed conflictive opposite results, with one of the studies showing a modest but significant effect on cognitive decline whereas the other one showed no measurable effects[283]. The compound however, reduced amyloid beta deposition in the brain of the patients and was approved by the FDA against the advice of its own advisory committee[272,281]. The treatment was also associated to cerebral edema and hemorrhages and was finally discontinued around February-May 2024[284]. Lecanemab, developed after aducanumab, is also a monoclonal antibody that binds to Aβ protofibrils and has also showed decrease in amyloid beta plaques, tau aggregation and a modest improvement in cognitive condition in phase 3 clinical trials[283]. It was also approved by the FDA in 2023[281,284]. Contrary to the aducanumab, the adverse effects were milder and is currently approved for the treatment of early stage AD[284,285]. In July 2024, the use of Donanemab antibodies targeting pyroglutamate Aβ, which is a modified truncated form of Aβ present in amyloid beta plaques, was also approved by the FDA[283]. Phase 2 and 3 clinical trials showed an effective clearance of amyloid beta plaques after one year of treatment and a significant effect slowing down cognitive decline. The treatment however, showed similar side effects as Lecanemab[272].

Current efforts on therapeutic research and development are still ongoing based on the amyloid cascade hypothesis, but also additional hypothesis that focus on other mechanisms,

such as the inflammatory responses mediated by microglia, or tau pathology[272] are being tested in clinical trials[286].

1.4.9 Cell types affected in AD

There is multiple evidence of a variety of brain cell types being affected in AD. Neuronal defects have been reported across various models of AD, including *in vivo* rodent models, human *in vitro* systems and post-mortem human brain tissue. These alterations are detectable from early stages of the disease, preceding the deposition of amyloid beta plaques and persist at later stages of the disease, as revealed in analysis of post-mortem human brains. In different experimental models, some of the earliest neuronal impairments involve disruptions of the endolysosomal-autophagy pathway, as reported in rodent brains, in human 2D neuronal cultures and in postmortem human AD brain[209,287–289]. In parallel, neuronal hyperactivity has also been detected at early AD stages both in human and mouse models, *in vivo* and *in vitro*[21,234,290]. Functionally, increased levels of amyloid aggregates have been shown to decrease synaptic transmission and reduce the presence of dendritic spines[291,292] *in vivo*. Structural alterations have also been described in rodents models, synapses are frequently absent from the vicinity of amyloid plaques[293], and neuronal loss has been observed to follow synaptic loss[294,295], both contributing to brain atrophy[193,200]. Studies using human neurons in an AD chimeric model and from human postmortem AD brain also reported aberrant accumulation of presynaptic vesicles and loss of postsynaptic material around Aβ plaques[106]. Comparable to rodents studies, synaptic loss was also detected in AD patient brains and was correlated to the patient's score at different cognitive tests[295].

Astrocytes are the main cell type in the brain expressing the main risk factor gene linked to SAD, the apolipoprotein E (*APOE*), which may suggest an important participation of this cell line into the pathology[296]. In rodents models, both atrophic and reactive astrocytes have been described to appear before amyloid plaque formation[297]. Atrophic astrocytes are characterized by reduced volume, fewer processes and diminished function[297] whereas reactive astrocytes are characterized by hypertrophy of soma and main processes, and overexpression of intermediated filaments[159]. Reactive astrocytes in rodent models have been shown to release a neurotoxin which leads to the death of neurons and oligodendrocytes at

later stages of the disease[298]. However, *in vivo* studies further show that reactive astrocytes can phagocytose dystrophic neurites and clear Aβ deposits, indicating a potentially protective role[299]. Nevertheless, their accumulation around Aβ plaques might also suggest an impairment in their protective degradative abilities over time[155,300]. Reactive astrocytes have been detected in post-mortem AD brains[296,297]. Similarly, hiPSC derived astrocytes from AD patients have shown an overall atrophic phenotype, marked by a reduced morphological complexity compared to control cells[301]. However, unlike in rodent models, their reactive profile does not differ significantly from the control derived astrocytes[301].

Myelinization alterations have also been observed in AD patient brain and were found to correlate with the level of cognitive impairment[302]. In postmortem AD brain, DNA damage has been reported at early symptomatic stages, even before the onset of amyloid pathology[303]. These damages have been associated with oligodendrocyte degeneration, suggesting that DNA damage could contribute to the loss of oligodendrocytes[303]. Induction of DNA double strand breaks by etoposide in *in vitro* rodent cultures leads to loss of MBP-expressing oligodendrocytes, suggesting a link between DNA damage and oligodendrocyte degeneration[302]. In rodent models, a decrease in the number of MBP+ oligodendrocytes has been observed prior to the onset of changes in levels of APP, $A\beta_{42}$ and human tau[304]. Disruption in neuronal myelination was also detected before the appearance of cognitive impairment[305]. *In vitro* rodent studies have also shown that the addition of Aβ peptides reduces the survival of mature oligodendrocytes and impairs the formation of the myelin sheath[306,307]. On the contrary, an increase in OPC proliferation has been observed in rodents both *in vivo* and *in vitro*, which might suggest the presence of compensatory mechanisms to early myelin damage[305,306].

Microglia is the brain cell type which shows the highest enrichment in most risk factor genes associated to AD, suggesting an essential role in the disease[308]. It has been suggested that at early stages of AD, microglia could undergo a process of activation, characterized by an amoeboid morphology and overexpression of specific markers such as CD68[309]. In this early phase, microglia migrate and accumulate around Aβ plaques, where they may initially exert a protective role by phagocytosing and clearing Aβ deposits, as shown both in *in vivo* and *in vitro* studies[310,311]. Then, with the progression of the disease, microglia could contribute to the

inflammatory environment, notably mediating astrocytic activation, as suggested by a study in mice[298]. The inflammation could damage oligodendrocytes and contribute to their loss and mediated neuronal death[308]. In a pro-inflammatory state, microglia release pro-inflammatory cytokines that reinforce this detrimental environment and simultaneously downregulate genes involved in amyloid clearance, thereby reducing their phagocytic capacity and leading to increased levels of Aβ deposits[312].

Finally, single-cell RNA sequencing studies using brain tissue from AD patients have also reported a wide variety of cell types being affected in the context of the disease[313–315]. They have revealed cell-type-specific gene expression changes, notably involving *APOE* and other Genome-wide association studies (GWAS) risk genes across distinct cellular populations. These studies also identified sex-specific transcriptional responses, and highlighted both shared and unique molecular alterations contributing to AD pathology[313–315].

1.4.10 Neuronal hyperexcitability, an early AD phenotype

Release of the neurotransmitter glutamate from pre-synaptic excitatory glutamatergic neurons activate the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in postsynaptic neurons. This activation is followed by entry of Na^+ to the cell which will further activate the voltage-dependent NMDAR leading to influx of Na^+ and Ca^{2+} inside the neuron (Figure 12)[316]. This increase in calcium can activate the activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), triggering signaling pathways involved in long term potentiation or depression, which are the underlying mechanisms of memory[317].

Neuronal hyperexcitability is defined as the increased probability that a certain stimulus will trigger the activation of a neuron[21]. In the context of AD development, it has been observed by functional magnetic resonance imaging (fMRI) that patients with mild cognitive symptoms (MCS) showed increased activation of some specific cortical and hippocampal areas. On the contrary, patients with higher MCS or with AD reported an hypoactivation of these brain zones[194]. Other patients that present neuronal hyperexcitability are for instance epileptic, schizophrenic and attention-deficit/hyperactivity disorder (ADHD) patients. Interestingly, all these different populations exhibit deficits in cognitive function, notably in attention or in

executive functions. Interestingly, a study performing electrical stimulation of the motor cortex in healthy patients showed reduced cognitive aptitudes to perform specific tasks, suggesting that hyperexcitability is deleterious for cognitive associated tasks[60]. Moreover, late-onset epilepsy has been associated with a threefold increased risk of developing AD, and seizures have also been reported in AD patients, with higher prevalence in those with FAD compared to sporadic cases[318,319]. Mice studies have further reported that epileptiform activity can occur prior to Aβ deposition, and that *in vivo* exposure to Aβ oligomers triggers epileptiform activity, suggesting an early AD phenotype[318].

Hyperexcitability and seizures could be mediated by Aβ peptides. It has been reported that Aβ can to bind to the NR1, NR2A or NR2B subunits of the NMDAR, resulting in its activation[320]. It has also been shown that Aβ can form pores in the cell membrane which are permeable for calcium (Figure 12). Strengthening this hypothesis of hyperexcitability mediated by amyloids, the exposure to β and γ- secretase inhibitors in mouse models prevents neuronal hyperexcitability[321]. Hyperexcitability in AD could also be due to reduced levels of the glutamine synthetase in astrocytes which catabolizes glutamate into glutamine, ensuring homeostatic conditions[21]. Aβ itself could also increase the levels of glutamate, related to this, a study on the rat magnocellular nucleus basalis showed that infusion of $A\beta_{42}$ increases glutamate release[322]. Another report showed the binding of Aβ to astrocyte acetylcholine receptor subunit alpha-7 leading to glutamate release[323]. Higher levels of glutamate would lead to increased intracellular calcium, triggering an increase in mitochondrial calcium, which will first lead to increased ATP production (Figure 12)[324].

However, elevated calcium may also lead to ROS overproduction and provoke membrane permeabilization and release of both ROS and pro-apoptotic factors within the cell, resulting in cell death (Figure 12)[324]. In addition, hyperexcitability may worsen AD pathological outcomes, as it has been shown that increases in neuronal activity by applying glutamate or picrotoxin are able to increase the secretion of tau to the extracellular medium, leading to increased tau accumulation[325], but also increase amyloid beta production in human embryonic kidney 293 cells[326].

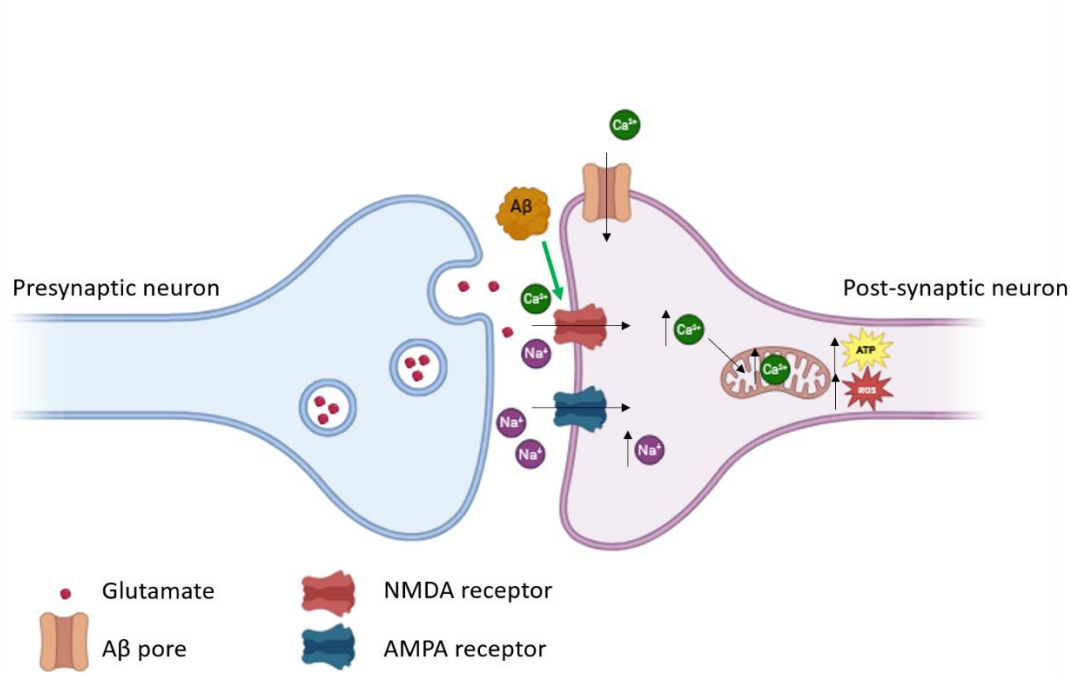


Figure 12: Impact of Aβ on neuronal hyperactivity and mitochondrial function.

Intracellular calcium concentration may be increased by i) Aβ pores in the cell membrane which permit the entry of calcium inside the cell, and ii) presence of Aβ at the synapse where activates NMDA receptors. This activation leads to an entry of Na^+ to the cell which will further activate the voltage-dependent NMDA receptor leading to influx of Na^+ and Ca^{2+}. Increased intracellular calcium concentration promotes calcium entry inside the mitochondria, resulting first in increased ATP production and then in increased ROS production. Created via Biorender.

1.4.11 Endo-lysosomal – autophagy pathways

Autophagy is a pathway which enables the degradation of cytosolic components (typically misfolded proteins and damaged organelles) that will be engulfed in autophagosomes that will ultimately fuse with lysosomes[327,328]. The formation of the autophagosome membrane involves multiple sources, including components of both the endocytic and secretory pathways[327]. Autophagy can be further classified in selective and non-selective autophagy depending on their preference for specific cargos. An example is the specific autophagy of mitochondria which is called mitophagy[327]. Autophagy is an important mechanism for the cell homeostasis and is known to be involved in cancer, aging and neurodegeneration[327]. Inhibiting autophagy leads to aggregates of ubiquitinated proteins inside neurons and might result in cell death[329].

Autophagy can be subdivided into 4 steps: i) the initiation of autophagy by stress signals; ii) the formation of autophagosomes; iii) the fusion of autophagosomes with lysosomes; and iv)

the degradation of autolysosomes[328]. The initiation can be triggered by various signals such as low levels of nutrients or low levels of energy available in the cell and is characterized by the inhibition of the mammalian target of rapamycin complex 1 (mTORC1) and the activation of adenosine monophosphate-activated protein kinase (AMPK) that phosphorylates and activates the Unc-51 like autophagy activating kinase 1 (ULK1) complex[328,330]. Several protein complexes are recruited to form the double membrane structure of the phagophore (first step of the autophagy before autophagosome formation)[328] (Figure 13). The phagophore will elongate and form the autophagosome (microtubule-associated protein 1 light chain 3 positive, LC3+) which will ultimately fuse to lysosomes to degrade its content (Figure 13)[328]. mTORC1 has also a role at the late stage of autophagy where it controls the phosphorylation of the transcription factor EB (TFEB). When mTORC1 is active, TFEB is phosphorylated and trapped in the cytoplasm through interactions with cytoplasmic proteins. In contrast, inhibition of mTORC1 allows the dephosphorylation and translocation of TFEB to the nucleus where it promotes the transcription of autophagy and lysosome related genes[327,328].

1.4.11.1 Endosomes

Endosomes are vesicles formed through the process of endocytosis in the cell that will fuse to lysosomes to degrade and/or recycle its components[331]. Early endosomes are characterized by the presence of RAB5, a small GTPase bound to the endosome surface. Early endosomes either undergo recycling of their components at the cell membrane or are sent for degradation. In the latter, endosomes undergo a process of maturation from early endosomes (RAB5+) to late endosomes (RAB7+) (Figure 13)[329]. RAB7 proteins are the main driver for the maturation of early endosomes into late endosomes by initiating the recruitment of RAB7 interacting lysosomal protein (RILP)[327]. Hydrolases, notably cathepsins, are incorporated into late endosomes where they become active through catalytic processing, however their degradative potential is not optimal due to the pH 5-5.5 of late endosomes[332].

1.4.11.2 Multivesicular bodies

Multivesicular bodies (MVBs) are organelles characterized by 1 outer layer membrane containing several vesicles (between 2 and several dozen)[333]. MVBs inner vesicles are formed by invagination of the membrane of a late endosome, or derived from early endosomes

(Figure 13)[327,331]. Similarly to lysosomes and late endosomes, they also present a low pH around 5.5[334]. Ultrastructural morphology analysis is needed to distinguish MVB from other types of vesicles such as endosomes or phagocytic vacuoles. Their content is composed of various proteins such as growth factors, receptors or exogenous proteins. Their function could be linked to the degradative pathway and/or the exocytic pathway and they could have a role in the sorting of macromolecules that they harbor for degradation, secretion and recycling[333]. Accumulation of MVB in the axon could be caused by a reduction in the transport of smaller vesicles[333]. One way to labelled MVB but also late endosomes is to use phospholipid lysobisphosphatidic acid (LBPA) which is typically found in the membrane of these structures[335]. LBPA has been shown to promote invagination of the membrane to create the vesicles inside MVBs. ALG-2 interacting protein X (ALIX), is notably one protein important for the formation of MVBs. Present in excess, ALIX prevents the generation of MVBs[335]. Contrary, using an FGFR1 inhibitor like PD173074, the formation of MVBs is impaired and it leads to a reduction in the secretion of extracellular vesicles in the cell[336].

1.4.11.3 Lysosomes

Lysosomes are one layer membrane organelles present in the cell which are responsible for the degradation of material coming from outside the cell (through endocytosis) and inside the cell (through autophagy). They can perform their degradative function thanks to their acidic pH 3.8-5 and the presence of 50 enzymes responsible for the cleavage of different macromolecules[337]. Among these enzymes, they contain proteases (e.g. cathepsins) which are able to breakdown proteins, such as misfolded proteins. Cathepsin D is an aspartic protease with a catalytic site containing 2 aspartic acid side chains[338]. One degradative pathway in the cell is via the lysosome-autophagy system, and an alternative one is the ubiquitin-proteasome system. Through their ability to cleave proteins, the lysosomes play an important role in the homeostasis of the cell and the proper cell function[337]. Lysosomes can be considered as the last step for the autophagic and endocytic pathway[339]. A mature lysosome is an organelle characterized by the presence of active hydrolases able to hydrolyze their substrates due to an optimal pH but also by the presence of glycosylated membrane associated protein (lysosomal-associated membrane protein 1, LAMP1+) and the absence of non-lysosomal proteins[339]. Lysosomes associated membrane proteins (Lamp) are not only found in lysosomes

but also in endosomes and in the plasma membrane. Lysosomes are able to degrade proteins with long half-lives, aggregates of proteins, organelles, external material as well as membrane components[329]. Whereas ubiquitinated proteins with short half-lives are preferentially degraded by the proteasome. An inhibition of the proteasome results in accumulation of ubiquitinated proteins that are taking up by autophagic vesicles[329]. As ubiquitination is used for both lysosome and proteasome degradation, defects in ubiquitination may have an impact on both mechanisms[329].

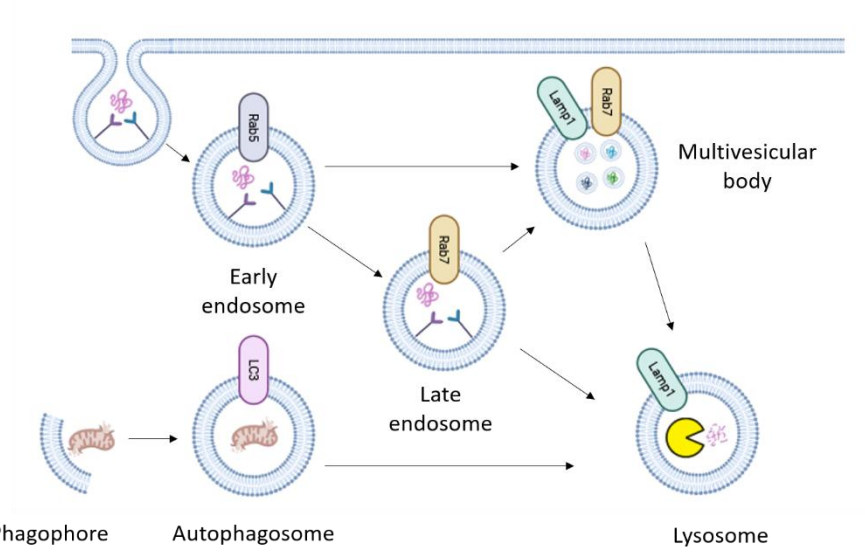


Figure 13: Scheme of autophagy and endolysosomal pathways.

During autophagy, defective or aged organelles are enclosed by a phagophore, forming a double-membrane autophagosome marked by LC3. The autophagosome fuses with lysosomes to degrade its content. Endocytic material is first internalized into early endosomes (RAB5-positive), which mature into late endosomes (RAB7-positive) or multivesicular bodies (RAB7- and LAMP1-positive). Both late endosomes and multivesicular bodies fuse with lysosomes for degradation. Created via Biorender.

1.4.12 Early defects in endo-lysosomal – autophagy pathways in AD

Recent studies have highlighted defects in the endolysosomal and autophagy pathways in Alzheimer's disease. Evidence from post-mortem brain tissue analysis revealed accumulation of enlarged early endosomes (RAB5+), late endosomes (RAB7+)[340,341], autophagosomes (LC3+)[342,343] and lysosomes (LAMP1+)[343,344]. Transgenic mouse models also recapitulated these features, showing an increase in LC3+ vesicles[287,342], lysosome accumulation[287,288,342] and enlarged endosomes RAB5+[345]. Similarly, 2D neuronal cultures derived from AD patients display an increase in both size and/or number of endosomes and lysosomes[209]. In the hippocampus of AD patients there is increased expression of mTORC1 and reduced levels of

ULK1, suggesting defects in lysosome biogenesis and autophagy initiation[328]. Other studies reported elevated levels of ESCRT proteins and galectins, both markers of damaged lysosomes[289], as well as abnormal cytoplasmic accumulation of cathepsin D in AD patient post-mortem brain[343]. Importantly, endolysosomal system dysfunction has been observed in mouse models before the appearance of amyloid plaques, tau tangles, and neuronal loss, indicating that these alterations may occur at very early stages of AD pathogenesis and before the onset of symptoms[209,328].

Impairment in axonal transport has been proposed as a contributing factor to the accumulation of lysosomal and autophagic vesicles in Alzheimer's disease (AD). Mutations in *APP* and *PSEN1* have been shown to reduce axonal transport efficiency, characterized by an increase in stationary vesicles and a decrease in the percentage of moving vesicles in cultured neurons derived from AD patients[209,346]. In addition, exposure of neurons to $A\beta_{42}$ has been associated with the formation of tubulin clusters, giving the characteristic beading morphology in neurons in the pathology[346]. Studies in both AD mouse models and AD post-mortem human tissue have reported the presence of dystrophic axons surrounding Aβ plaques. These dystrophic neurites exhibit disrupted or absent microtubules, which likely impair axonal transport[346]. Furthermore, mis-localization of motor proteins, such as dynein and kinesin has been observed, further indicating disruption of the axonal transport in some stages of the disease[106,346].

Another defect described in AD model is the deacidification of lysosomes[287,330,347]. In fact, the presence of *PSEN1* mutations has been linked to acidification defects[347,348]. PS1 may act as a chaperone protein, for instance for the proton pump responsible of the acidification of the lysosome, the vATPase V0a1 sub-unit[347,348]. Under normal conditions vATPase V0a1 sub-unit, is correctly folded and glycosylated, which enhances its stability[329]. However, in the absence of PS1, the vATPase V0a1 sub-unit is not correctly folded which prevents its correct glycosylation and results in defects in its lysosome acidification function[329,347,348]. Importantly, endosomes and lysosomes are central to APP metabolism, and disruptions in the endolysosomal pathway can result in APP accumulation, shifts between non-amyloidogenic and amyloidogenic processing, and increased production of Aβ peptides[209]. Indeed, endosomes and lysosomes provide a favorable environment for amyloidogenic APP cleavage,

as they contain the β- and γ- secretase enzymes and a relatively low pH, that allows the production of both β-CTFs and Aβ peptides[349]. In addition, changes in the lysosome acidification may provoke the loss of cathepsin D function involved in the degradation of p-tau, APP and its metabolites[330]. In agreement with that, increased levels of β-CTFs have been observed co-localizing with cathepsin B positive structures in lysosomes at early stages of AD in 3xTgAD mice, prior to Aβ plaque deposition[350]. Increased β-CTFs levels have also been observed in the context of γ-secretase inhibition, exposure to alkaline conditions and lysosomal protease inhibition[350]. Additionally, the accumulation of β-CTFs is not only a consequence of an impaired lysosomal function, but also it may actively contribute to lysosomal dysfunction[350–352]. Interestingly, it has been suggested that β-CTFs compete with the V1 subunit of the vATPase pump for binding to the V0a1 subunit, thereby reducing the levels of fully assembled and active vATPase pump enzymes (Figure 14)[352,353]. This results in defects in vATPase function with increased lysosomal pH which impaired the activity of pH sensitive proteases such as cathepsin D (CATD) in human fibroblasts derived from Down syndrome patients[353] (Figure 14). Therefore, higher APP amyloidogenic processing rate and lower lysosomal degradative function may synergistically contribute to the building up of β-CTFs within lysosomes, consequently exacerbating lysosomal dysfunction[352].

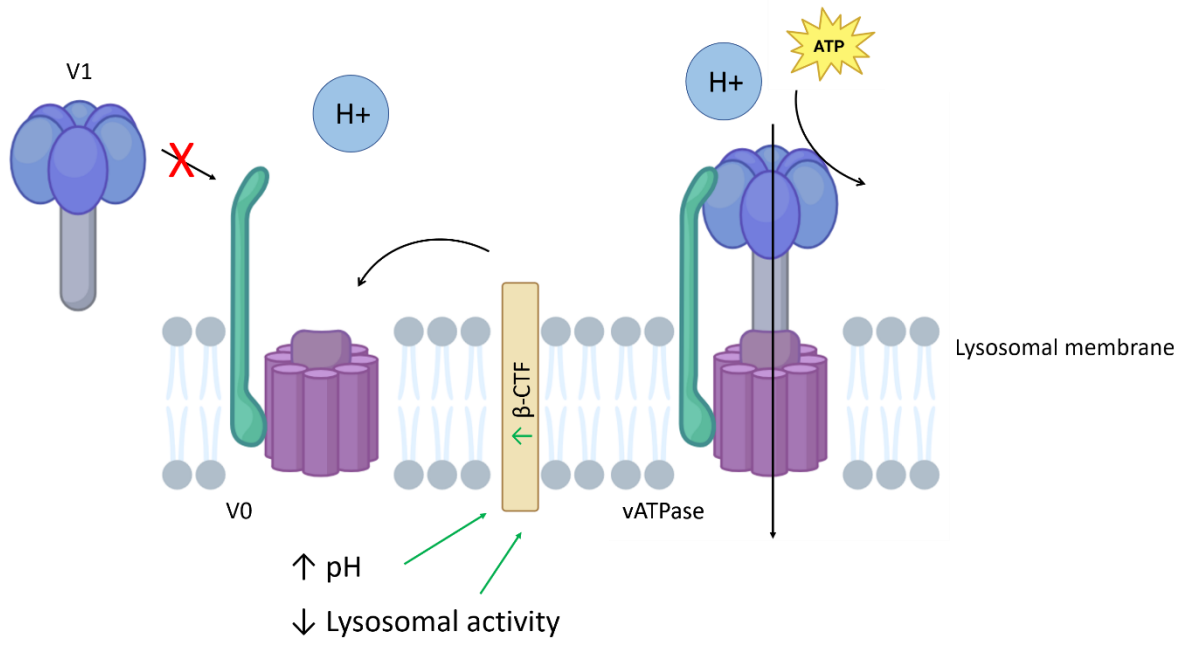


Figure 14: Effects of β-CTFs on lysosomal impairment.

Increased levels of β-CTFs lead to competition with the V1 subunit of the v-ATPase for binding to the V0 subunit. When β-CTFs bind to V0, proton translocation into the lysosome is impaired, resulting in elevated lysosomal pH and reduced enzymatic activity. In contrast, proper V1–V0 assembly enables ATP-dependent proton transport and acidification of the lysosomal lumen. Created via Biorender.

1.5 Objectives

In this thesis, we aim to generate a matured long-term cortical brain organoid model to investigate features of human brain maturation as well as its alterations in the context of infectious and neurodegenerative diseases. To achieve these objectives, the thesis will be organized into three sections:

- In Chapter 2, we intended to decipher cellular, molecular and functional changes accompanying human cortical organoid maturation *in vitro* to set the basis for a model to study human brain maturation and its diseases. To achieve this aim, we thoroughly characterized the human cortical organoid model: the time-dependent appearance of neuronal and glial populations as well as its temporal molecular changes. We also aimed at characterizing the changes in neuronal function upon time using calcium dynamics *in vitro*. We completed these studies by analyzing time-dependent changes in axonal transport dynamics, as this mechanism is a key player in neuronal homeostasis linked to neuronal maturation.
- In Chapter 3, we sought to understand the pathological consequences of SARS-CoV2 infectivity in the brain. We questioned first whether levels of SARS-CoV2 infectivity and cell tropism would be dependent on the maturation stage of human cortical organoids *in vitro*. Then, we focused on the analysis of pathological downstream effects of SARS-CoV2 infection in terms of neuronal death mechanisms and broad transcriptomic changes revealed by bulk RNA sequencing. In addition, we analyzed long-term effects of SARS-CoV2 infection in human cortical organoids.
- In Chapter 4, we aimed to unravel a timeline for early mechanisms of AD in brain cells and their cause-dependent effect to one of the main AD hallmarks, the Aβ aggregation in the brain. Our first question was to unravel the timeline of appearance of cellular pathological phenotypes, such as Aβ aggregates and tau phosphorylation in our *in vitro* model. Next, we investigated if our "early *in vitro* AD model" would be sufficient to reproduce defects in neuronal activity, axonal transport and alterations in the endolysosomal pathway, reported in patients and mouse models. Finally, we interrogated our model for the presence of Aβ-dependent or independent early AD phenotypes by the use of γ-secretase and β-secretase inhibitors.

2 Chapter 2: Modeling human brain development using hESC and hiPSC -derived cortical organoids

2.1 Abstract

In this first chapter, we used human cortical organoids (hCOs) to study maturation features of the human brain. Access to human brain samples is rare which poses limitations to the study of human brain development and its underlying functional changes. We showed here that hCOs recapitulate the appearance of various cortical neuronal subtypes, such as deep layer neurons (CTIP2+), upper layer neurons (CUX1+) and calbindin+ (CALB+) interneurons, but also glia cells, such as GFAP+ astrocytes and OLIG2+ oligodendrocytes in a time dependent manner. We observed an increase in calcium oscillations in 6 months (6M) hCOs when compared to 3.5M hCOs, suggestive of increased synaptic activity upon time, supported by transcriptomic data. We then investigated the dynamics of axonal transport, an important mechanism of the neuron supporting the establishment of synapses, axonal growth and recycling of cellular material. We observed an increase in anterograde axonal transport speed in 6M hCOs compared to 3.5M, suggestive of neuronal maturation and active axonal growth processes at the latest stage *in vitro*, a feature which was not yet described in human *in vitro* systems. Altogether, these results highlight the potential of hCOs to model human brain maturation over time and uncover dynamic functional changes associated with this process.

2.2 Introduction

Among all organs, the brain is possibly one of the most complex and evolved structures, as it holds the distinctive cognitive traits that define the human species. The cerebral cortex undergoes a process of maturation that encompasses a variety of processes and extends during a large time period, from embryonic stages to the 2 first decades of life[354]. The first step of the brain maturation that can be detected is the neurogenesis, the generation of neurons from progenitors and their migration towards their final location into the cortex[4]. The migration of projection neurons follows[1,4]. After the production of neurons, RGs switch their potential to generate glial subtypes, such as astrocytes[1], followed later by the production of oligodendrocytes[29]. Neurons extend their processes and increase their morphological

complexity through a process of axonal growth to reach their specific synaptic targets[38]. Concomitantly to astrocytes and oligodendrocytes generation, synapses are formed and its number is increased until childhood[355]. Around the time of birth, when the first oligodendrocyte cells are generated in the cortex, neurons will start to be myelinated[5]. The final step of cortical development corresponds to the postnatal pruning of cells and synapses[5].

The maturation of the cortex is not only accompanied by changes in cell type diversity but also by changes in the expression pattern of different genes. Genes linked to progenitor cell identity and to control/repression of neurogenesis such as *PAX6*[356] and the repressor element-1 silencing transcription factor (*REST*)[357], respectively, decrease their expression upon time, whereas genes linked to brain maturation, such as the subunits of the *NMDAR*, (*GRIN2A*)[356,358] and the glutamate ionotropic receptor NMDA type subunit 2B (*GRIN2B*)[356,358], the subunits of the AMPA receptor such as the glutamate receptor 2 (*GLUR2*) and the glutamate receptor 1 (*GLUR1*) subunits[359], and genes important for synapse formation such as neurexin 1 (*NRXN1*) and neuroligin 1 (*NL1*)[356,360] increase their expression with time *in vivo*[360–362]. Several studies have also shown a switch of expression of different splicing isoforms between prenatal and postnatal stages in the brain that is kept through adulthood[50,54,64]. Among these splicing variants there are notably the 3R and 4R isoforms of tau which are developmentally regulated in the brain. Whereas 3R tau is the only isoform expressed in the embryonic brain, the adult human brain presents a ratio 1:1 of 3R and 4R tau isoforms[41,50,250]. The difference between these isoforms resides in the exclusion (3R) or inclusion (4R) of the exon 10 of the tau gene, encoding for one of the microtubule binding domains[50]. 4R tau proteins are therefore able to enhance microtubule stability and influence directly microtubule growth and shortening[363].

Current knowledge on the maturation features of the human brain is primarily coming from rodent studies, as human brain tissue is hardly accessible. Although they share common biological processes, human and mice differ in several important features such as the abundance of specific cortical cell types[364,365], the timeline for human brain maturation[14,70,366] and the expression of important regulated genes[71,367–369] during brain development. Therefore, focusing solely on rodent models might mask unique human specific features of the human cortex. The possibility to generate iPSCs and the subsequent development of brain organoid models[370], has allowed the generation of specialized cells and tissue-like structures

derived from human donors. Although several studies have shown that brain organoids still present some limitations, they can partially recapitulate aspects of brain maturation[112,371].

Today, most *in vitro* studies using human cortical organoids have focused on a descriptive characterization of cortical cell types compared to what is found in human embryonic and post-embryonic brain samples, as well as a comparison of gene expression patterns between human brain organoids and the human brain[356,358,371]. However, very few of these studies have investigated the functional maturation of human brain cortical organoids as a model to study the development of the human brain. Among the functional studies developed to study brain models, the study of calcium dynamics is a well know tool to assess changes in brain cultures related to the neural transmission of action potentials[372] but also related to axonal growth, axonal guidance and branching processes[57,373]. Disruption in calcium homeostasis can have crucial outcomes, for instance, an increase in internal Ca^{2+} concentration that can lead to seizures, stimulate the production of reactive oxygen species (ROS) and activate Ca^{2+}-dependent apoptosis cell death mechanisms[374]. To date, only few studies have focused on the evolution of calcium dynamics linked to cell maturation in cortical brain organoids[375,376]. Few brain organoid studies investigated neuronal activity through the use of multielectrode array (MEA)[377] or electrophysiology techniques[378], but most of these studies focused on functional changes in the context of diseases such as epilepsy[379], psychiatric diseases[380] or neurodegenerative diseases such as Alzheimer's disease[381] or Parkinson's disease[382]. Therefore, there is currently a need to assess calcium dynamics in cortical organoids upon time to understand how *in vitro* maturation may impact calcium oscillations as well as to understand which biological mechanisms are implicated in these changes.

Another important functional feature, not previously addressed in human neurons, is the dynamic changes in axonal transport linked to brain maturation. Axonal transport involves the coordinated interaction of several key components, the microtubules which constitute the tracks for the transport along the axons, the motor proteins that bind and move cargoes along axons, and the different proteins associated with axonal transport regulation[47]. Axonal transport encompasses the transport of organelles and vesicles inside the cell and can be unidirectional or bidirectional[46]. Anterograde transport delivers cargos from the soma to the tip of the axon through the motor protein kinesin[383]. Anterograde transport is essential for

the growth of the axon with the delivery of proteins such as neurofilaments[46] for axonal growth[46,49,384] and for the formation of synapses by delivering synaptic material at the location of synapses[46]. Retrograde transport is the reverse movement, starting from the tip of the axon to the soma of the cells and is mediated by dynein motor proteins[383]. Retrograde transport is important for the recycling of misfolded or damaged proteins and aging organelles transported by endosomes and/or autophagosomes that will fuse to lysosomes containing key enzymes for degradation of their content[46,385].

Axonal transport will not be possible without the microtubule forming "highways" or tracks on which motor proteins "walk" to reach their final destination. Microtubules exist in a dynamic state in which they are constantly growing and shrinking. Thanks to this dynamic system, axons may elongate or retract to reach precise target locations at the cell periphery[44]. Microtubule stability and capacity to elongate in a fast or slow fashion, is modulated among other mechanisms, by PTMs of microtubules. The main PTMs involved in microtubule regulation are acetylation, polyglutamylation and tyrosination[47]. Regions of tyrosinated tubulins in microtubules, for example, have been linked to an increase in the dynamics of microtubules to grow and shrink and will be mostly found at the tip of the growing axon[44,47]. Microtubule stability is also modulated by MAPs, such as tau or MAP2[52,54,386]. These proteins present different isoforms which contain different number of microtubule binding domains (MBD). Interestingly, the isoforms 4R tau and MAP2A have both an extra MBD, and are associated with a reduction in microtubule dynamics[54]. Both isoforms are absent in the embryonic brain but expressed postnatally[50,54]. Blocking microtubule extension whether pharmacologically, or by antibodies targeted to specific proteins involved in microtubule extension, has been shown to lead to a reduction or total inhibition of axonal growth[44]. Axonal transport defects during brain maturation are mainly described as arising from mutations located in genes linked to axonal transport components, from the tubulin gene to the motor proteins (dynein and kinesin). Axonal transport defects are linked to phenotypes related to mental retardation, microcephaly and to cognitive disabilities[387].

Among the type of cargoes that undergo axonal transport along microtubules are late endosomes and lysosomes, mitochondria and mRNA vesicles[40,47,388] The transport of lysosomes has been shown to be essential for neuronal homeostasis for the efficient

degradation of proteins and organelles along the axon[389,390]. It is well characterized that in the context of neurodegenerative diseases, such as Alzheimer's disease, the endolysosomal pathway is altered, with lysosomes accumulating in neurites around amyloid beta plaques[288,389,390], which suggests that lysosomes may be a key player in the development of the disease[209,328]. In contrast, there are just a few reports on the behavior of endosomes and lysosomes in the context of the maturation of the human brain and their contribution to neuronal homeostasis and neuronal function. Therefore, we decided to focus on its characterization during brain maturation using human brain cortical organoids[391]. Axonal growth has been shown to be dependent on axonal transport of late endosomes. For instance, under conditions such as nutrient stress, late endosomes detach from microtubules and axonal elongation is impaired[384]. In addition, BDNF signaling, which plays a key role in synaptic transmission, requires efficient anterograde transport for the delivery of both the ligand and its receptor to specific brain regions[392]. Together, these findings support the idea that a proper endosomal and lysosomal trafficking is essential for neuronal connectivity and the development of neuronal dendritic trees. Disruption of this transport can impair axon elongation and synaptic function, highlighting the importance of intracellular trafficking in brain maturation. While the role of endosomal and lysosomal trafficking in brain maturation has been characterized, their axonal transport dynamics during neuronal maturation in human models remain poorly characterized.

Here in this study, our aim is first to characterize cell type composition changes in a long-term human cortical organoid (hCOs) culture model to understand the cell diversity present in hCOs *in vitro*, as well as their timeline of appearance. We also aimed to investigate the maturation processes occurring in hCO models during long-term culture, by detecting the expression of different brain maturation markers upon time. Next, we focused on the characterization of neuronal maturation from a functional point of view by analyzing calcium dynamics in hCOs and their time-dependent evolution. Finally, our last objective was the study of axonal transport changes of late endosomes and lysosomes in our model upon hCOs maturation *in vitro*. The quantification of parameters such as axonal transport speed, size, and density of late endosomes and lysosomes along axons provides new insights into how the endolysosomal transport evolves as neurons mature, building a reference framework, and identifies potential key maturation-dependent regulatory changes that may be altered early during

developmental diseases. Overall, our results on brain maturation gene expression coupled to functional brain maturation processes adds valuable information to the current gap in the literature concerning the understanding of calcium dynamics as well as axonal transport changes during human cortical brain organoid maturation *in vitro*.

2.3 Conclusion

Features characteristic of the development and maturation of the human brain are difficult to study due to limited access to human brain samples. Brain organoid models allow to study brain maturation in a human context and to capture human specific features. We show that our hCO model derived from human stem cells can recapitulate the time-dependent appearance of neuronal populations and glial cells. We also reported specific maturation features in hCOs from 1M to 6M *in vitro* such as an increase in the expression of genes associated to axon guidance (*ROBOs*), expression of neurotransmitter vesicles (*SYP*), maturation of the NMDAR subunits, detection of synapses and the presence of the postnatal 4R tau isoform in 6M hCOs. Our calcium analysis revealed an increase in calcium dynamics at 6M that could reflect both an increase in synaptic activity and axonal growth upon maturation in hCOs. The presence of increased mechanisms of axonal growth in 6M hCOs are also supported by specific changes in lysosome axonal transport dynamics which favor axonal growth processes. In the future, hCO neuronal arborization could be analyzed to measure axonal growth upon maturation *in vitro*. In addition, the expression of other specific isoforms which are associated to postnatal brain stages, besides 4R tau, could be investigated to clarify the stage of maturation reached by hCOs upon time in our system.

3 Chapter 3: SARS-CoV2 infection triggers inflammatory conditions and astrogliosis-related gene expression in long-term Human Cortical Organoids

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4 Chapter 4: Modeling early Alzheimer's disease phenotypes using hESC and hiPSC FAD patient cell-derived 3D cortical organoids.

4.1 Abstract

In this chapter, we used human cortical organoids (hCOs) to study pathological phenotypes associated with early stage of Alzheimer's disease (AD). This neurodegenerative disorder, which affects over 50 million people worldwide, remains currently unresolved in terms of which factors lead to its initiation and progression. From a histopathological point of view, AD is characterized by the accumulation of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles composed of phosphorylated tau (p-tau) protein. To unravel some of these questions we generated human cortical organoids from familial AD (FAD) patient cells. We successfully recapitulated Aβ aggregation and increased levels of p-tau in FAD hCOs. We also observed elevated calcium dynamics and mitochondrial activity in FAD hCOs, suggestive of neuronal hyperexcitability, previously reported at early phases of the disease in AD mouse models and brain patient material. Treatment with β- and γ-secretase inhibitors efficiently lowered Aβ and p-tau levels to control values, suggesting a causal relationship between Aβ accumulation and tau pathology. We then investigated the endolysosomal pathway, which has been reported to be disrupted in early AD. While no defects were detected in axonal transport of lysosomes/late endosomes, we observed the presence of aberrant lysosomes with reduced levels of CATD. Our preliminary results also suggest an increase in the proportion of acidic lysosomes. Interestingly, CATD had a higher tendency to be localized to lysosomes in FAD hCOs, possibly reflecting a compensatory mechanism. This phenotype was not rescued by reduction in Aβ production or secretion, suggesting either an Aβ-independent mechanism, or a non-reversible effect triggered by Aβ at an earlier time window. These results may help explain the limited success of current therapies that aim to reduce Aβ burden to halt disease progression in the patients.

4.2 Introduction

Alzheimer's disease is the most common forms of dementia, characterized by progressive cognitive decline associated with the accumulation of Aβ peptides and hyperphosphorylated tau within the brain[183]. It is believed that pathological processes in the brain start decades before the onset of the first clinical cognitive symptoms, defining a long preclinical phase during which cellular dysfunction gradually develops[201]. Investigating the initial pathological alterations occurring at early stages of AD is essential to better understand its development and to support the design of effective therapeutic strategies to prevent the initiation/progression of the disease.

The amyloidogenic processing of APP, is mediated by a first cleavage enacted by the β secretase, followed by the cleavage by the γ secretase complex, resulting in the production of amyloidogenic Aβ peptides[213]. Familial inherited forms of Alzheimer's disease result from dominant mutations in *APP*[208] or in genes encoding the catalytic subunit of γ secretase; *PSEN1*[242] and *PSEN2*[508]. However, the incidence of FAD is relatively low, accounting for less than 5% of cases, with some estimates as low as 2%[509]. Despite their low prevalence, experimental models based on these mutations represent powerful tools to study AD. Moreover, the emergence of hiPSC have allowed the generation of human specific brain models derived from FAD patients to study AD-related mechanisms.

One of the earliest functional alterations observed in Alzheimer's disease is neuronal hyperexcitability, defined as an increased likelihood of neurons to fire in response to stimuli[21]. This phenomenon has been reported in patients and is thought to precede cognitive symptoms by decades[199,234]. Neuronal hyperactivity has been associated to alterations in intracellular calcium homeostasis, a tightly regulated process involving calcium influx through membrane channels, and calcium release from intracellular storages such as the ER and mitochondria[234]. While mechanisms have been studied in murine models and 2D cell cultures, their investigation in patient-derived 3D organoid models remains limited.

Defects in axonal transport represent another early pathological phenotype observed in AD. As evocated in the first chapter of this thesis, axonal transport of late endosomes and lysosomes plays an important role in the homeostasis of the cell, notably in the destruction of

misfolded proteins and aged organelles[46,385]. Similarly to the study of calcium disruption, defects in axonal transport in organoid models derived from patients have not yet been extensive described. Lastly, changes in the endolysosomal-autophagy pathway represent another early phenotype of Alzheimer's disease pathology. Vesicles from both pathways progress through distinct stages characterized by specific markers and converge into a common late stage for degradation by fusion to lysosomes: early endosomes (RAB5+), late endosomes (RAB7+), autophagosomes (LC3+), and finally fusion with lysosomes (LAMP1+) for degradation[329,339]. Notably, the subcellular localization of the β- and γ-secretase enzyme complexes raised the possibility that endolysosomal-autophagy dysfunction may enhance Aβ peptide accumulation[209,287,288]. Although co-localization of $A\beta_{42}$ with lysosomal markers has been observed in several studies[510,511], the exact relationship between Aβ accumulation and lysosomal impairment remains to be fully elucidated. Studying the evolution of the endolysosomal-autophagy pathway in human brain organoid models, in relation to Aβ accumulation, may provide new insights into the mechanisms driving early defects in these pathways in Alzheimer's disease.

While many of these early phenotypes have been characterized in murine models and 2D cell cultures, such systems present limitations. Rodent models do not spontaneously develop Alzheimer's disease and require the (over)expression of several human mutations associated to familial AD to be able to recapitulate some of the hallmarks, such as Aβ plaques, but they lack the formation of tau tangles and major cell loss[106,381]. Models of 2D cultures allow to work with a human genetic background and from cells originated from patients but they lack cellular diversity and robust Aβ deposition might be impaired by media changes[509]. Human derived 3D cortical organoids provide a promising alternative to study the emergence and chronology of early phenotypes in a patient-specific context, offering an intermediate level of complexity between simplified *in vitro* systems and *in vivo* models. This technology may help bridge the gap in our understanding of how early pathological events initiate and evolve in Alzheimer's disease.

Here, we used human stem cell-derived cortical organoid models whether carrying a transgene for the expression of FAD mutations or derived from FAD hiPS cells to recapitulate major features of early-stage AD. First, we assessed whether neuronal calcium oscillations and

axonal transport dynamics were altered in “early stage” *in vitro* AD human cortical organoid models. Next, we asked whether “early stage” *in vitro* AD cortical organoid models present endolysosomal pathway alterations. Finally, we evaluated whether early AD phenotypes could be rescued by pharmacological inhibition of Aβ production or secretion in human cortical organoids.

4.3 Conclusion

We successfully generated hCO models derived from a transgenic hESC line expressing *APP* carrying FAD mutations and from hiPSC FAD patient cells, which recapitulate main AD hallmarks such as increased levels of Aβ peptides and p-tau when compared to healthy donor hiPS-derived hCOs. We observed an early increase in calcium dynamics that we hypothesize could be involved in the mechanism for phosphorylation of tau by enhancing the activity of several kinases such as GSK-3β in the cell. Aβ inhibitors could efficiently rescue the early AD p-tau phenotype, which suggests that p-tau and calcium activity defects might be modulated by an Aβ-dependent mechanism. We hypothesize that an increase in calcium activity may reflect a hyperexcitability state of the neurons in an early AD context in our AD hCO models. Analysis on the endo-lysosomal pathway revealed no changes in the transport of these vesicles along neuronal axons in FAD compared to control hCOs. We did not observe accumulation of early or late endosomes, nor autophagosomes, but a trend for accumulation of lysosomal structures. Strikingly, we detected a reduction in the percentage of lysosomes containing CATD, highlighting lysosomal functional defects in AD hCOs. This phenotype could not be rescued by the inhibition of Aβ generation or secretion in the cell, suggesting an Aβ-independent mechanism or an irreversible phenotype triggered by Aβ at an earlier time window. Overall, our work has identified the presence of divergent early AD phenotypes, with Aβ-dependent *vs* Aβ-independent response phenotypes in human hCO models. Although our work leads to the discovery of several interesting phenotypes connected to early stages of AD, it contains nevertheless several open questions and preliminary data which need to be further completed to better understand the earlier pathological states of AD in the brain. Overall, our work supports the important value of human cortical brain organoids to study the early development of neurodegenerative diseases such as Alzheimer’s disease, opening future

venues of this model for pharmacological- and/or genetic related approaches to unravel better therapeutic strategies to treat this disease.

5 Chapter 5: General discussion and conclusion

5.1 Studying human cortical brain maturation with cortical brain organoids

Human brain organoids have been shown to recapitulate certain aspects of human brain development, such as a 3D self-organized formation of polarized structures which make them a good model for this topic[551]. As previously discussed in the first chapter, our brain organoid model is able to recapitulate the time dependent appearance of neuronal progenitors, neurons and glia *in vitro*, similar to the *in vivo* situation[94]. Cortical organoids also offer the possibility to study the functionality of the developing brain. We have shown here that we were able to highlight differences in calcium activity upon organoid maturation *in vitro*. Although we cannot discriminate if this increased activity is linked to neuronal activity or axonal growth, or both, complementary experiments like patch-clamp would allow to prove changes linked to synaptic activity. Our group is also performing neuronal Sholl analysis to assess the complexity of neurons in brain organoids upon maturation. Our data supports changes in axonal transport dynamics correlated with the maturation stage of hCOs *in vitro*. This conclusion is supported by previous data showing changes in the dynamics of dense core vesicle transport upon maturation in mice[436].

We specifically detected an increase in lysosome anterograde speed at 6M when compared to 3.5M, which could underly a link to axonal growth and building up of synapses, both processes connected to brain maturation. In addition, we reported an increase in 4R tau isoform expression at 6M compared to 3.5M, which suggests that long-time brain organoids acquire important features of the postnatal brain. Further studies on the analysis of tau isoform expression and their role on axonal transport in our system could allow us to discriminate if the axonal transport changes observed upon maturation can be caused by the presence of the 4R tau isoform in 6M hCOs, as previously suggested[41,53].

A side-to-side time comparison between the *in vitro* hCOs model and the *in vivo* human brain has been long debated using for instance comparative transcriptomic analysis. These studies suggested that organoids of about 6 months resemble a mid-embryonic human brain stage[356,358,371,552]. These studies found that the enrichment of certain populations, such as

oRGs (basal progenitors) and upper layer neurons of the cortex was suboptimal in hCOs, or that showed a slowdown in their maturation capacity. This difference in cell type percentage was mostly attributed to the presence of a necrotic core in hCO models[371]. This conclusion is somehow in disagreement with our findings, where we observe expression of 4R tau, a neonatal isoform of *MAPT*, from 6M in hCOs. Expression of 4R tau is usually absent from *in vitro* models derived from hESC or hiPS cells, however, the use of specific culture media such as BrainPhys, which may promote neuronal networks in 2D cultures, has been shown to promote 4R tau expression[553]. We hypothesize that maturation marks may be heterogenous throughout hCOs with some regions more advanced and other less advanced in their maturation. For instance, in our experiments we detected the presence of OPC but very few or no cells positive for MBP, a marker for mature myelinated oligodendrocytes inside 6M hCOs[554]. However, we detected the expression of the 4R tau isoform and an increase in the expression of synaptic proteins, such as *SYP*, *SYN1*, *SYPL2*, *HOMER1*, *HOMER2*, neurotrophic factors such as *BDNF*, vesicular transporters *VGAT* and *VGLUT* necessary for inhibitory and excitatory neuronal activity, and the mature subunit of the NMDAR, *GRIN2A*. In the future our team will characterize the expression pattern of other genes, such as *MAP2* and *SCN2A*, which undergo a switch in splicing forms postnatally, to better understand the corresponding *in vivo*-like maturation stage of long-term hCOs. In the context of this thesis, the maturation stage of hCOs was essential to be described prior to its use as a model to study pathological conditions, such as SARS-CoV2 infection and Alzheimer's disease. Besides, the expression onset of 4R tau in 6M hCOs could be instrumental to recapitulate hallmarks of adult brain diseases such as Alzheimer's disease or frontotemporal dementia (FTD) *in vitro*.

5.2 Effects of SARS-CoV2 infection in cortical brain organoids

Our study of the effects enacted by SARS-CoV2 infection in the brain using hCOs, revealed low but reproducible levels of infectivity, fitting with previous results showing low levels of viral particles present in brain tissue from infected patients. Our model shows that SARS-CoV2 viral infection of the brain is possible, even without the presence of endothelial cells which express high levels of *ACE2*[152], the main receptor for entry of the SARS-CoV2 virus.

Similar to previous studies using brain organoids, we also observed infection of neurons[460,470,494,555] and astrocytes by SARS-CoV2[460,470,472,494,556]. Infectivity of progenitors has

led to conflicting results, with some studies reporting infection[494,498] while others, like us, showed absence of infectivity of this cell type[460,469,555]. We therefore hypothesize that neuronal progenitors are not preferentially targeted by the virus, in agreement with studies that suggested absence of infectivity of progenitors and/or major downstream effects in embryos[124] in *in vivo* studies. Most studies investigating viral infection using brain organoids have focused on Zika virus (ZIKV), which leads to secondary microcephaly upon infection during pregnancy, and on Herpes simplex virus type 1 (HSV-1), which is responsible for encephalitis[557]. Brain organoids infected with ZIKV display high infectivity of neural progenitors and astrocytes and low infectivity of neurons as well as increased cell death[557], whereas brain organoids infected with HSV-1 show infectivity of astrocytes but no increased cell death[558]. Brain organoids infected with varicella-zoster virus (VZV) showed infectivity of astrocytes without inducing the release of pro-inflammatory cytokines[560]. In addition, no cell death was observed, but formation of stress granules, which may have a protective effect. This was suggested to result from the ability of VZV to evade the innate immune response[560].

Our results suggest that infection of astrocytes by SARS-CoV2 leads to a global response of inflammation with increased presence of inflammatory pathways that is nevertheless not sufficient on its own to trigger cell death, but rather compensatory mechanisms promoting cell survival (increased expression of *SOD2*, decreased presence of H2AXγ in infected organoids). Although only a small fraction of the cells was infected, these transcriptional and phenotypic responses demonstrate that even limited infection rate can elicit biological changes in neurons and astrocytes. Based on observations made with other neurotropic viruses, it would be likely that the innate immune response varies according to the virus infecting brain organoids. In this context, our results seem closely related to those reported for VZV. In both cases, astrocytes were infected, however, the downstream consequences do not include cell death pathways but instead involve protective or compensatory mechanisms.

The inflammatory pathways triggered upon SARS-CoV2 infection in our model are likely linked to changes occurring in astrocytes upon infection by the virus. These astrocytic changes include increased expression of astrogliosis markers, such as CD44, SERPINA3 and S100A10. Taking into account the low number of differentially expressed genes that we detected by bulk RNA sequencing analysis, it is likely that only a subpopulation or a fraction of the astrocytes

within hCOs would react to the infection, consistent with the low level of infectivity detected. We could hypothesize that if the levels of astrogliosis would be higher, we would be able to detect broader changes such as a bigger number of differentially expressed genes associated to reactive astrocytes such as *GFAP*, *VIM*, s*ynemin*[472,561], among others. Besides, a broader pro-inflammatory reaction could lead to broader pathological effects, such as an increase in cell death. The inflammatory conditions observed in post-mortem brain of infected patients in some studies[562] would certainly be mediated by microglia, which are the immune cell of the brain[563]. Microglia are absent from our hCO model and therefore we could hypothesize that adding microglia to our hCOs could increase the global inflammatory response and activate astrocytes, potentially leading to pathological downstream effects such as cell death. However, studies on HSV-1 have shown that although microglia activation are observed in 3D organoids, this does not necessarily result in of cell death[559]. This suggests that cell death induction may be dependent on the type of virus rather than on the presence of microglia.

We want to highlight that previous studies drew conflictive results with some reporting cell death[460,470,555], while others, like us, showed absence of cell death mechanisms following SARS-CoV2 infection in cortical organoids[556]. Besides, minor alterations have been detected by MRI in surviving COVID-19 patients, mostly in the white matter, which may suggest in general limited downstream effects of the virus in the brain in the general population[564,565], in agreement with the low infectivity and lack of cell death mechanisms reproduced in hCOs from our data. Although our results demonstrate that our model can be infected by SARS-CoV2, they do not allow us to determine whether neurological symptoms in patients are caused by direct viral infection or by indirect systemic inflammation. Investigating systemic contributions would require models incorporating vascularization, immune components, or interactions with peripheral organs, which remain a challenge in current organoid systems. Addressing this question has important clinical implications, if symptoms arise from direct infection, targeted therapies to preventing SARS-CoV2 entry in the cells could be appropriate, whereas, if they are mediated by systemic inflammation, anti-inflammatory treatments may be more relevant.

It has notably been reported that patients suffering from AD were more susceptible to SARS-CoV2 infection and over 60%[566,567] of these patients developed neurological symptoms.

However, there was a mismatch in terms of age between control and infected subjects, with the latter being older than control individuals, which is a factor in favor of increased pathological effects and mortality[141]. We have however showed absence of cell death upon infection of SARS-CoV2, even when using higher amounts of the SARS-CoV2 virus or prolonged time post-infection of hCOs. Following log-term SARS-CoV2 post infection we found similar percentage of infected astrocytes among the total population (around 40%), but a higher total number of infected astrocytes compared to short-term SARS-CoV2 post infection. It would have been interesting to analyze from a transcriptomic point of view the response to long term post infection in hCOs, to understand the balance between cell survival genes and pro-apoptotic pathways upon longer time points.

It has also been suggested that patients suffering from SARS-CoV2 could be more prone to develop AD[567]. It has been reported that long COVID-19 patients that were infected with the initial SARS-CoV2 variant or with the α variant, presented reduced cognitive function compared to controls[568,569]. On a period of one year, the risk of new onset dementia was also higher in patients infected with SARS-CoV2 compared to the control group[570]. GFAP, total tau, p-tau181 and the intermediate filament neurofilament light chain (NLF) are biomarkers for the detection of neurological diseases such as Alzheimer's disease in blood samples. Interestingly, the levels of NLF and GFAP were found to be significantly higher in severe COVID-19 patients than in patients suffering from AD. The authors also analyzed Aβ42 levels but showed no correlation with the severity of COVID-19[571]. Another study, published in 2025 reported opposite results, with a reduced $A\beta_{42}/A\beta_{40}$ ratio in the plasma of patients suffering from COVID-19, similarly to the reduction that can be observed in AD preclinical stage. They also described an increase in p-tau181 in blood samples in some participants infected by SARS-CoV2 but they did not observe a net increase in the levels of GFAP and NLF, but only an initial increase that dropped back to baseline within 6 months[572].

These studies suggest a higher tendency of patients infected by SARS-CoV2 to present AD biomarkers and cognitive decline, but there is currently no causal link between the infection and the apparition of a form of dementia such as AD. It has also been suggested that this correlation might not be SARS-CoV2 specific, but could rather be applied to any infection reaching the brain tissue[572]. It would be interesting in the future to analyze AD hallmarks in

SARS-CoV2 infected hCOs to test the putative link between both diseases using our model *in vitro*. For instance, it would be interesting to analyze the Aβ levels in our SARS-CoV2 infected hCOs as they showed upregulation of the hypoxia pathway following SARS-CoV2 infection, which was previously linked to APP processing. Indeed, it has been reported that the hypoxia-inducible factor-1α (Hif-1α) can upregulate both β- and γ-secretase activities, leading to an increase in Aβ levels[573].

5.3 Deciphering early AD phenotypes with cortical brain organoids

Alzheimer's disease is a multifactorial complex disease, with a high variety of cellular pathways and brain cell types being altered at certain level[264]. We could argue that this is an important reason why we still do not know the exact initial causes of a disease with so many different components affected at some level, much like navigating a maze without a map, unsure of which path to take. Organoids are useful reductionist models, much simpler than the human brain but still retaining a human background and recapitulating key features of the disease such as Aβ deposition and p-tau. Given the simplicity of the system, we can use external sources of Aβ or other stimuli to test different hypothesis and analyze downstream changes in Aβ load, p-tau and other early AD phenotypes. Interestingly, our hCO model can recapitulate the presence of 4R tau expression, which suggests that neurons may have mature features of the adult brain, and therefore be more susceptible to degeneration. Our FAD hCOs show a time dependent increase in Aβ deposits, however, this model cannot recapitulate the presence of Aβ plaques or the presence of Aβ fibrils (results not shown from my host team). This is an interesting finding because it highlights the fact that our FAD hCOs may represent a very early stage of the pathology prior to the formation of Aβ plaques, and therefore before the onset of symptoms in patients.

In the present study, we unraveled several defects connected with the endolysosomal pathway in FAD hCOs. Since we are able to recapitulate a decrease of lysosomal CATD in AD brain organoids, this suggests that the model may be suitable to study in depth this pathway and to identify potential targets to restore CATD levels to prevent AD progression. Related, one study in AD transgenic mice reported that increased expression of ADAM30, a metalloprotease involved in the cleavage of APP, resulted in restored CATD activity and

reduction in the secretion of Aβ peptides[574]. In addition, we postulate that our FAD hCOs may present defects in lysosomal pH, which could contribute in the long-term to defects in axonal transport and neuronal function. Lastly, our data highlights that lysosomal defects may be caused by either an Aβ-independent mechanism or may be triggered at an early stage and be irreversible for the cell. In any of the two events, it highlights the limitation of AD therapeutic approaches aiming solely at reducing the levels of Aβ in the brain. Studying these earliest cellular changes in AD is crucial to identify which features can be targeted therapeutically to slow or prevent disease progression.

Concerning therapeutic strategies to combat AD, the use of antibodies directed against amyloid beta deposits in AD mouse models has shown to lead to lower levels of Aβ aggregates, but worsen neuronal hyperactivity effects, even at stages preceding the apparition of plaques[575]. However, these studies lacked a thorough analysis of the Aβ species present following treatment. It is therefore difficult to determine whether the failure of antibody treatment was linked to an inability to reduce soluble Aβ species which may be directly causing hyperactivity in the cells. The use of NMDAR antagonists, such as NitroSynapsin, a derivative from memantine, a FDA approved drug for AD treatment, have been shown to reduce neuronal hyperexcitability assessed by patch-clamp and to reduce the levels of intracellular calcium in human AD organoids[576]. However, this study did not analyze the effect of the drug on the levels of Aβ peptides. It is interesting that disregard less from the levels of Aβ, blocking the NMDAR could be sufficient to decrease the hyperactivation of the system, implying that Aβ peptides may bind and activate the receptor in the cell. Another study showed a reduction in hyperactivity following β- or γ-secretase inhibitor treatment in brain organoids[290], however, this study did not analyze further the causes of the underlying mechanisms linking Aβ and hyperactivity. Nevertheless, these studies re-enforce the fact that *in vitro* brain organoids are a good model to study early features of AD such as hyperactivity. Using these simple systems, one could dissect the mechanisms underlying these pathological effects and find new targets to combat disease.

5.4 Brain development, infection, and neurodegeneration

Although the three projects included in this thesis address distinct aspects of human brain biology (forebrain maturation, viral infection, and neurodegenerative disease development), they nonetheless share few similarities among them.

First, we highlight the importance of using matured cortical organoid to study of the last stages of human brain development, its susceptibility to viral infection and the mechanisms involved in neurodegenerative diseases that strike the adult brain. Second astrocytes appear to play a central role across all models. In the maturation project, we identified the developmental window at which astrocytes emerge in our cortical organoids, and we hypothesize that this increasing presence may contribute to the higher neural activity observed in long term hCOs. Astrocytes were also the primary cell types infected in 6M hCOs exposed to SARS-CoV2, they showed reduced size and higher levels of astrogliosis markers. Although astrocytic reactivity was not assessed in our AD hCO models, this would be an important direction for future work, given the well-established association between AD, neuroinflammation and reactive astrocytes. Lastly, future transcriptomic analyses of AD hCOs could further allow a direct comparison of genes and pathways differentially regulated with those identified during brain maturation and following SARS-CoV2 infection.

5.5 Limitations of the model and prospects

In general, the human brain organoid field is challenging to develop from an economical and technical point of view, especially when ensuring a high level of quality of the model used, and a high number of replicates to enhance the strength of the results (several cell lines from the same genotype, several experiments performed, several organoids per experiment and several slices of the same organoids,…)[416]. This is a major issue due to the time, money and personal resources needed to achieve this goal, especially for small labs. Protocols maintaining cortical organoids in culture for 6–7 years, as in Arlotta's work, raise practical challenges due to the high maintenance and resources required, and also pose questions regarding inter-laboratory reproducibility and their suitability for studying disease mechanisms. Extended culture beyond one year could be necessary to increase specific cell type population and their maturation to more closely resemble the adult human brain, but it would also increase the

likelihood for contaminations or technical/mechanical problems related to the culture of 3D organoids. Developing reproducible cortical organoid protocols across laboratories is essential to allow meaningful comparisons of data and to advance the field. This is particularly important for patient-derived cell lines, where it is desirable that similar cellular and functional features are observed across different laboratories.

In the future, with the development of imaging and artificial intelligence, this method could be faster thanks to the ability of advanced software versions to rapidly scan slides, image them autonomously, plus pipelines for downstream analyses. This will allow for a considerable time and money saving, and likely more objective results independent from individual biases. The automatization of the analysis will also help to go through big data, amplifying the power of the analysis. The development of automated approaches also highlights the importance of sharing underlying codes and protocols, as detailed documentation is essential to ensure reproducibility and standardization across laboratories. Ethical considerations, such as the use of embryonic stem cells and the degree of humanization in rodent models, should be taken into consideration, as mentioned in the introduction (Section 1.2.3). Beyond these ethical aspects, cortical organoids also hold translational potential, bridging preclinical models and human disease. Using patient-derived cell lines allows the development of personalized therapies based on the patient's genetic background. Cortical organoids also provide a reductionist human-based model enable to study human specific disease such as AD and SARS-CoV2 infection, potentially revealing human-specific features underlying disease development and progression.

We are using an adapted version of the protocol from Sasai and modified by Arlotta by using bioreactors and monitoring organoids' size[86,94]. From Arlotta's modifications, we kept the use of atmospheric O_2 concentrations, the use of bioreactors and monitoring organoids size to transfer them to plates with larger areas ensuring an optimal organoid density per media volume[94]. This helps to provide sufficient oxygen and nutrients to promote robust growth. However, we modified the protocol by slightly changing media composition, and reducing the matrigel concentration in the medium, added it only from day 70 at 1%, and removing heparin and FBS, as reported in protocols derived from Pasca's lab[577]. We chose the Sasai/Arlotta-

derived protocol because it yields organoids with consistent forebrain identity and improve survival, reproducibility, and culture standardization.

In the adult brain, neurons and glia are present in approximately equal proportion (50% each)[578], whereas in our model at 6M, we can recapitulate the neuronal fraction but we fail to reach the expected proportion of glia. Similarly, It has recently been shown by others that the percentage of astrocytes present in brain organoids at 6M[577,579] is lower than the expected 20-40% ratio in the human adult brain[578], mainly due to the fact that longer time frames would be needed for the gliogenesis phase to be completed *in vitro*[160]. Astrocytes have been reported to arise in brain organoids at around 3 months, independent on the differentiation protocol used[160,371]. Although oligodendrocytes are qualitatively observed in our hCOs, their abundance do not reach the 45-75% of glia cells reported in the adult brain[578]. Microglia, which normally represent less than 10% of the glia population[578], are absent from our model. Our model also does not recapitulate the presence of endothelial cells or pericytes.

Future hCO models including endothelial and pericyte cells could promote the formation of a BBB-like structure through their interaction with astrocytes. Indeed, pathways associated with BBB development, such as “retinoic acid metabolism” and “maintenance of the BBB” have been observed following the incorporation of endothelial cells and pericytes into brain organoids, suggesting the emergence of a nascent BBB[110]. However, a fully functional and mature BBB has not yet been achieved[110]. The incorporation of endothelial and pericyte cells into our hCOs could also provide a more physiologically relevant system to study for instance the combined effects of viral infection and neuroinflammation. Endothelial cells and pericytes express high levels of ACE2 receptors[580] and can interact with microglia and astrocytes, thereby exacerbating inflammatory responses[581]. Their presence could therefore increase viral infectivity within brain organoids and potentially amplify downstream neuroinflammation, especially in the context of neurotropic viruses, where endothelial infection may facilitate viral entry, spread, and inflammation[581].

Implementing microglia into hCOs could enhance the formation of neuronal networks and increase neuronal activity[35,563,582], getting one step closer to physiological conditions *in vitro*. It would be interesting to analyze the level of maturation reached by hCOs containing

microglia to understand if microglia can potentiate neuronal function during developmental stages, as suggested previously[583–585]. Important concerns would be to implement microglia at the right timepoint into the organoids without inducing deleterious effects for the cells, and in the right proportion to result in homeostatic conditions. Besides, for practical reasons, it would be desirable to generate large stocks of microglia cells that could be used for different experiments to minimize batch-dependent effects (for instance by using frozen microglia stocks). In addition, microglia should be analyzed for their level of activation inside the organoids which might have adverse effects for cell survival.

This *in vitro* model would be important to study AD, as most AD genetic risk factor genes are enriched in microglia. And given the fact that these immune cells are key players in disease development, particularly at early stages[308]. Microglia cells have been shown to phagocytose Aβ deposits and trigger inflammatory responses, and their activation can be induced by both Aβ and tau[261,311]. Incorporating microglia into our organoid models would allow us to assess their activation in a time-dependent manner, in relation to increasing levels of Aβ and p-tau. Moreover, integrating microglia derived from iPSCs carrying AD-associated risk variants into healthy organoids could help elucidate the impact of these variants specifically on the immune cell type of the brain and their contribution to disease. Conversely, adding microglia from healthy donors to AD hCOs could enable us to test whether they can mitigate Aβ deposition and delay the emergence of our observed early pathological phenotypes or on the contrary, trigger cell death in our *in vitro* system.

The biggest disadvantage on the use of brain organoids to study neurodegenerative diseases, such as AD, is the fact that their maturation may be closer to an embryonic stage rather than that from the adult brain[356,358]. Indeed, several groups are working on acceleration of the maturation process in organoids[586,587]. Age is one of the major risk factors to develop AD, especially important for sporadic AD[187,588]. Aging can be characterized by several processes such as dysfunction of mitochondria, altered nutrient sensing properties, shortening of telomers, impaired proteostasis, among others[589]. One of the most well-known drivers of aging is cellular senescence, which are non-proliferative cells which exit cell cycle permanently, present DNA damage and secret proinflammatory molecules[589].

Several groups have tried to push the aging of 3D brain organoids or 2D cells, such as for instance the group of Lorenz Studer who used a model with overexpression of the progeria gene (a short spliced variant of the nuclear envelope protein lamin A) in 2D neuronal cultures. These experiments revealed increased DNA double strand breaks, shorter telomers, increased production of ROS by mitochondria and degenerating neurons[590]. However, we must point out that progeria is a pathological cleaved protein form present only in the body of patients that suffer from accelerated aging disease Hutchinson-Gilford Progeria, and that these patients do not experience brain aging due to the fact that progeria is spared from the brain[590].

Therefore, alternative models showing advanced physiological maturation should be developed. The presence of senescent cells and its increase with time in culture has been reported in brain organoids[591]. Senescence can also be induced with specific chemicals[592], inhibitors of the telomerase[593] besides overexpression of progeria[590]. Some studies have also tried to recapitulate an aging phenotype in organoids by inducing mutations in mitochondria to mimic the phenotypes observed in aged individuals[588]. However, recapitulating the physiological aging process that occurs in the *in vivo* human brain using brain organoids is challenging, notably due to the numerous changes that it involves. The possibility to generate brain organoids that resemble the aged brain would be an advantageous system to model AD and other neurodegenerative diseases, for which aging is an important factor. In addition, it would be instrumental to understand the role of some of the key aspects of aging in the disease process.

Future approaches could generate more complex, yet still reductionist, in vitro models by using reprogrammed AD patient hiPS cells combined with protocols enhancing brain maturation and co-cultured with patient-derived microglia and vascular components, thereby building a stronger model to decipher early AD phenotypes and their causal links.

Conclusions and translatability

This work showed that long-term hCOs *in vitro* system can model certain aspects of the late developing human brain: (i) time-dependent emergence of neuronal and glial populations; (ii) increases in calcium activity; (iii) changes in axonal transport that can be linked to axonal growth and the formation of synapses, suggesting important changes in the physiology of neurons at this maturation stage; (iv) expression of the neonatal 4R tau isoform from the MAPT gene, which highlights important molecular changes related to a post-embryonic stage. This study supports the use of cortical organoids to model the human brain through the last stages of brain development and early postnatal life. This model could be an interesting paradigm to study neurodevelopmental but also neurodegenerative diseases.

The long-term hCO model was challenged with SARS-CoV2 virus to study its pathological effects in the brain. SARS-CoV2 reproducibly infected hCOs at low levels at all the development stages tested *in vitro*. The major cell types infected were astrocytes and to a lower extent neurons, with more than 1% of the total astrocyte population being infected. SARS-CoV2 infection was associated with changes in astrocyte morphology and increased expression of astrogliosis related genes. At 6 months, the infection led to a global inflammation with the upregulation of proinflammatory and astrogliosis related pathways. However, those changes were also accompanied by an upregulation of genes involved in cell survival. In agreement with this, we did not observe global, nor localized cell death, suggesting that SARS-CoV2 infection triggers compensatory mechanisms to the inflammation which favor cell survival. This study showed the potential of long-term cortical organoids to study human brain viral infections and could be used for the study of other viruses to understand their impact on the brain and its downstream effects.

Finally, we showed that long-term cortical organoids derived from AD patient reprogrammed induced pluripotent stem cells can successfully recapitulate main hallmarks of Alzheimer's disease such as amyloid accumulation and tau phosphorylation. Long-term hCOs can also be used to study early phenotypes associated to a preclinical phase of the disease such as hyperactivity. Our model showed that increased levels of p-tau were directly dependent on

the presence of Aβ aggregates, highlighting a cause-effect link of Aβ. Although we did not observe any alteration in the axonal transport of late endosomes/lysosomes, neither structural differences in early or late endosomes, or autophagosomes, we observed a tendency for increased presence of lysosomal structures and a decrease in the percentage of lysosomes containing the protease CATD in FAD hCOs. Our preliminary data also suggest an increase in the percentage of acidified lysosomes, and increased localization of CATD inside lysosomes in FAD compared to control hCOs. We hypothesize that the decrease in the population of lysosomes containing CATD+ implies defects in the degradative function of lysosomes, which may be partially compensated in the cell by mechanisms for increased lysosomal localization of CATD and increased acidification. This defective lysosome phenotype could not be rescued by reducing the levels of Aβ through, which implies that this phenotype could be either independent from amyloid deposition, or could have been triggered at an earlier stage which could not any longer be reversed.

This work shows that long-term cortical organoids derived from AD patient cells allow to study the early phases of AD and could further allow the discovery of novel early features linked to the onset of AD. Although this model could be further improved, for instance by adding a human vascular and immune system components and promoting an aged-like environment, human cortical organoids represent a promising model to study neurodegenerative diseases *in vitro*.

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