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Sub-lethal concentrations of Aβ oligomers and glutamate induce ROS formation largely by calcium-mediated activation of the NADPH oxidase

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To those who have always been there, to those who have left,
to those who have crossed my path, to those who have met my gaze,
to all who have enriched my soul.

To my beloved grandparents,
Gabriella, Ivo, Olivano, Adriana

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Abstract

Neuronal hyperactivation has emerged as an early and critical feature of Alzheimer's Disease (AD), increasingly recognized as a potential contributor to disease onset and progression. Evidence from clinical studies, animal models, and induced pluripotent stem cell (iPSC)-derived neuronal systems indicates that hyperactivation can manifest well before the accumulation of amyloid-β (Aβ) plaques, even preceding the preclinical stage associated with AD, either in the traditional staging scheme or new AT(N) framework. Understanding the cellular and molecular consequences associated with this early hyperactivity is therefore essential, as it may reveal biomarkers for early diagnosis and targets for therapeutic intervention.

Among the proposed responses associated with driving early hyperactivation, dysregulated intracellular Ca^{2+} signalling induced by Aβ oligomers and excessive glutamate (Glu) neurotransmission are central. Both Aβ oligomers and elevated extracellular Glu can induce local increases in intracellular Ca^{2+}, perturbing neuronal excitability and synaptic function. However, the precise sequence of events and the specific cellular pathways involved in the earliest stages of AD, when Aβ overproduction and Glu levels increase are sub-threshold, remain incompletely understood. In particular, it is unclear how subtle changes in neuronal signalling translate into early pathological outcomes without immediately triggering cell death or other overt signs of neurodegeneration.

To explore these questions, we established *in vitro* models using human neuroblastoma SH-SY5Y cell line and primary neuron cultures exposed to sub-threshold concentrations of $Aβ_{42}$ oligomers and Glu. This approach allowed us to replicate early-stage pathological conditions while avoiding high-level toxicity. Under these conditions, ionotropic N-methyl-D-aspartate receptors (NMDARs) were locally activated, but without altering the global intracellular Ca^{2+} levels that remained unchanged. Importantly, canonical pathological outcomes associated with $Aβ_{42}$ oligomers or Glu exposure, such as mitochondrial dysfunction, metabolic alterations, or cell death, were absent.

Despite the absence of overt toxicity, neurons progressively accumulated reactive oxygen species (ROS). The results showed that the ROS production was primarily mediated by NADPH oxidases (NOXs) in a Ca^{2+}-dependent manner, indicating a direct link between localized Ca^{2+} signalling and oxidative stress. In particular, ROS generation in SH-SY5Y cells was mainly driven by a Ca^{2+}-dependent activation of NOXs, with no

detectable mitochondrial contribution, whereas in primary neurons ROS production was still largely mediated via a Ca^{2+}-dependent activation of NOXs and, to a significant extent, mitochondrial ROS generation. This oxidative profile aligns with recent proteomic studies in individuals carrying AD-causing mutations, which report upregulation of proteins involved in Ca^{2+}-dependent NOX activation detectable years before clinical onset.

These convergent lines of evidence support the notion that early NOX activity is a critical molecular event in preclinical AD and highlight the potential of NOX regulatory proteins as early biomarkers.

Abbreviation list

Aβ Amyloid-β

AA Arachidonic acid

Ac Anionic region

AD Alzheimer’s Disease

ADAM A Disintegrin And Metalloproteinase

ADAS-Cog Alzheimer’s Disease Assessment Scale - Cognitive Subscale

ADDLs Aβ$_{42}$-derived diffusible ligands

AFM Atomic force microscopy

AICD Intracellular C-terminal domain

AMPARs α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

APH1 Anterior pharynx-defective 1

APP Amyloid Precursor Protein

ATD Amino-terminal domain

AS-IV Astragaloside IV

BACE β-site APP-cleaving enzyme

CAA Cerebral amyloid angiopathy

CDR Clinical Dementia Rating

CNS Central nervous system

CuBD Copper/zinc-binding domain

DMSO Dimethyl sulfoxide

EAATs Excitatory amino acid transporters

EOAD Familial early-onset AD

ER Endoplasmic Reticulum

ETS Electron transfer system

FAQ Functional Activities Questionnaire

fMRI Functional MRI

FTIR Fourier-transform infrared spectroscopy

GFLD Growth factor-like domain

Glu Glutamate

HBD1 Heparin-binding domain 1

HBD2 Heparin-binding domain 2

HFIP Hexafluoro-2-propanol

iGluRs Ionotropic Glutamate receptors
iPSC Induced pluripotent stem cell
JM Juxtamembrane
KARs Kainate receptors
KPI Kunitz protease inhibitor
LBD Ligand-binding domain
LTD Long-term depression
LTP Long-term potentiation
MCI Mild cognitive impairment
MEG Magnetoencephalography
mGluRs Metabotropic Glutamate receptors
MMSE Mini-Mental State Examination
MoCA Montreal Cognitive Assessment
MRI Magnetic resonance imaging
NCT Nicastrin
NCX Na^+-Ca^{2+} Exchanger
ND Neurodegenerative disease
NFTs Neurofibrillary tangles
NMDARs N-methyl-D-aspartate receptors
NMR Nuclear magnetic resonance
NOXs NADPH oxidases
PEN2 Presenilin enhancer 2
PHFs Paired helical filaments
PMCA Plasma Membrane Ca^{2+} ATPase
PSEN1 Presenilin-1
PSEN2 Presenilin-2
qEEG Quantitative electroencephalography
ROS Reactive oxygen species
ROX Residual oxygen consumption
SERCA Sarco-Endoplasmic Reticulum Ca^{2+} ATPase
SFs Straight filaments
TEM Transmission electron microscopy
TM Transmembrane

1. Introduction

1.1 Epidemiology and social impact of Alzheimer’s Disease (AD)

Alzheimer’s Disease (AD) is the most prevalent neurodegenerative disease (ND), accounting for approximately 60-80% of all dementia cases and remains one of the leading causes of death and disability among older adults globally (Alzheimer’s & Dementia, 2025; Z. He et al., 2025). Over 55 million people worldwide were living with dementia in 2020, with projections reaching 78 million in 2030 and 139 million in 2050; approximately 60% of these individuals reside in low- and middle-income countries, a proportion expected to rise to 71% by 2050 (Alzheimer’s & Dementia, 2025; Kamatham et al., 2024; www.who.int, 2025). Understanding the epidemiology and pathophysiology of AD is crucial for public health planning, early intervention strategies, and the development of novel therapeutics. The increasing prevalence of AD, driven by global aging trends, underscores the urgent need for preventive measures, improved diagnostic tools, and a deeper understanding of disease mechanisms.

AD exerts a substantial impact not only on patients but also on their families, caregivers, and society at large. Informal caregivers, most often family members, provide comprehensive day-to-day support, including assistance with personal care, medication management, and supervision (Alzheimer’s & Dementia, 2025). The chronic and progressive nature of AD places increasing demands on healthcare systems, with hospitalizations, long-term care, and specialized interventions contributing to considerable economic costs. Globally, the total societal burden of dementia, including direct medical expenses as well as indirect costs such as lost productivity, is estimated to rise sharply in the coming decades (Alzheimer’s & Dementia, 2025; S. Chen, Cao, et al., 2024). Beyond economic and healthcare considerations, AD profoundly affects social and psychological well-being, compromising quality of life, social engagement, and the mental health of both patients and their families.

1.2 Alzheimer's Disease (AD): from discovery to histopathology, biochemistry, and clinical staging

1.2.1 General clinical presentation, discovery and history

AD is characterized by significant atrophy of the cerebral cortex and loss of cortical neurons, resulting in deficits in memory, communication, judgment, reasoning, and behaviour, including apathy and psychosis (Blennow et al., 2006; Samanta et al., 2006; Tenchov et al., 2024). Several studies based on magnetic resonance imaging (MRI) have highlighted a reduction in the volume of the hippocampus, which plays an important role in the consolidation of information from short-term memory to long-term memory, and in spatial memory, and of the amygdala, which is involved in memory and emotional responses (Basso et al., 2006; Den Heijer et al., 2006). Volumetric reductions were also found in other regions such as in the temporal lobe, important in memory and language comprehension, in the parietal lobe, involved in tactile and visual perception, and in the frontal lobe, which is implicated in behaviour and problem solving (Ramos Bernardes Da Silva Filho et al., 2017). In addition to these structural changes, activated microglia can induce reactive astrocyte phenotypes, which may impair synaptic function and promote neuronal loss, thereby accelerating neurodegenerative progression (Ferrari-Souza et al., 2025; Kwon & Koh, 2020). Although the precise pathophysiology is not fully elucidated, AD is primarily associated with the accumulation of extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein (Breijyeh & Karaman, 2020; Selkoe & Hardy, 2016). Additional factors, including neuroinflammation, oxidative stress, and vascular alterations, also may contribute to the progression of the disease (Dash et al., 2025; Mekala & Qiu, 2025).

AD was first described by Alois Alzheimer in 1906 through his detailed clinical and pathological examination of a 51-year-old woman, Auguste D., who presented with profound cognitive and behavioural decline, including severe memory loss, language disturbances, and marked changes in personality and social functioning. Alzheimer's pioneering observations not only documented the clinical manifestations of a previously unrecognized disorder but also revealed its underlying neuropathology the defining histopathological hallmarks of the disease (Alzheimer, 1911; Stelzmann et al., 1995). Notably, he also noted the presence of so-called "adipose saccules" within glial cells, a finding that would be largely overlooked for decades. Modern research has revisited these early observations, proposing that dysregulation of glial lipid metabolism and neuroinflammatory processes may contribute to AD pathogenesis alongside classical neuronal pathology (Morrone et al., 2020; Moulton et al., 2021; Steinman et al., 2021). These insights underscore the multifactorial nature of AD, highlighting that its development involves not only neuronal degeneration but also complex interactions with astrocytes and glial cells, vascular components, and the wider neural environment (J. Chen et al., 2025). In this sense, Alzheimer's original work provided a framework that continues to inform contemporary research over a century later.

Throughout the following decades, neuropathological research expanded substantially, refining our understanding of how AD-related lesions develop and spread throughout the brain. Building on Alzheimer's original observations, subsequent post-mortem studies systematically characterized the progression of AD pathology across brain regions and disease stages. The pioneering work of Braak and Braak 1991 established a comprehensive framework for tau pathology, describing its stereotypical spread across specific and interconnected brain regions. Building on these concepts, Thal et al. 2002 introduced a complementary model for Aβ, delineating distinct phases of deposition that reflect a gradual and regionally ordered propagation throughout the brain parenchyma. Understanding these patterns is critical for contextualizing the mechanisms of neurodegeneration and provides the basis for examining the detailed histopathological characteristics of Aβ and tau deposition.

1.2.2 Histopathological patterns of amyloid-β (Aβ)

Aβ deposition is the central pathological feature of AD and one of the earliest detectable events in the disease continuum (Pemberton et al., 2022). Histopathological studies classify extracellular Aβ deposits into two principal forms: diffuse and neuritic plaques (Almeida et al., 2025; Neri et al., 2025). Diffuse plaques are composed of non-fibrillar or loosely aggregated Aβ, lack a compact amyloid core and provoke minimal neuronal or glial reaction. They are frequently detected in presymptomatic individuals or in the earliest phases of AD, representing an initial stage in the plaque maturation process (Rahman & Lendel, 2021; Selkoe, 2001; J. Sun et al., 2025). In contrast, neuritic plaques are characterized by a dense amyloid core, surrounded by dystrophic neurites, reactive astrocytes, and activated microglia, underscoring their strong association with synaptic dysfunction and neurotoxicity (DeTure & Dickson, 2019; Selkoe, 2001).

To systematically describe the spatial and temporal progression of amyloid pathology, a five-phase model of Aβ deposition based on extensive post-mortem analyses was developed (Thal et al., 2002). In this framework, Aβ deposits first appear in neocortical association areas (phase 1), which are critical for higher-order cognitive functions such as reasoning, planning, and language. Deposition then progresses to the allocortex and hippocampal formation (phase 2), key regions for learning and episodic memory, followed by involvement of the basal ganglia and diencephalic nuclei (phase 3), which contribute to motor control, attention, and affective regulation. In subsequent stages, brainstem structures become affected (phase 4), playing roles in autonomic functions, arousal, and motor coordination, and finally, the cerebellum is reached (phase 5), a region primarily governing motor control. This phase-based model provides a structured framework for understanding the ordered and region-specific propagation of Aβ pathology across the central nervous system (CNS). This hierarchical sequence reflects a predictable anatomical pattern that parallels, but is distinct from, the previously described six-stage Braak scheme for tau (Braak & Braak, 1991). Together, these staging systems provide a complementary basis for understanding the topographical and temporal dynamics of AD pathology and remain essential references for correlating post-mortem findings with *in vivo* biomarker and clinical data. In addition to parenchymal deposits, Aβ also accumulates in cerebral blood vessel walls, causing cerebral amyloid angiopathy (CAA) (Aivalioti et al., 2025; Greenberg et al., 2020; Thal et al., 2025; Vonsattel et al., 1991). CAA is present in most AD patients and contributes to vascular dysfunction and increased risk of intracerebral haemorrhage.

1.2.3 Amyloid Precursor Protein (APP) and Aβ processing: the amyloidogenic and non-amyloidogenic pathways

Aβ is an intrinsically disordered protein involved in neuronal development, synaptic plasticity, learning and memory and is normally expressed ubiquitously in neurons (Wells et al., 2021). Aβ derives by sequential mechanism of extramembrane and intramembrane mechanisms of proteolysis of the Aβ precursor protein (APP, about 750 amino acid residues), whose coding gene is located on chromosome 21q21.3, and its alternative splicing generates 10 different isoforms in different tissues such as brain, heart, spleen and kidneys (Haass & Selkoe, 2007; Urban et al., 2021). APP695 predominates in neurons, whereas the longer isoforms containing a Kunitz protease inhibitor (KPI) domain are expressed in non-neuronal tissues (Dawkins & Small, 2014; J. Kang et al., 1987). Far from being a simple substrate for Aβ generation, structurally, APP is a type I multidomain protein with a single transmembrane (TM) span, composed of several domains: the E1 subunit consisting in a heparin-binding domain together with a cysteine-rich growth factor-like domain (HBD1/GFLD) and a copper/zinc-binding domain (CuBD); it also contains an anionic region (Ac) and a KPI domain (absent in APP695 isoform which is prevalent in neuronal tissue); the E2 subunit with a second heparin-binding domain (HBD2), a random coil juxtamembrane (JM) region, a TM domain containing the Aβ sequence, and an intracellular C-terminal domain (AICD) which contains YENPTY motifs crucial for adaptor protein binding, including Fe65, Mint/X11, and Dab1 (Ng et al., 2024). Furthermore, in the N-terminal E1 region, the link between the HBD1/GFLD and CuBD induces the lateral homodimerization of APP by the formation of stable disulfide bridges; in addition, JM and TM participate in homodimerization (Urban et al., 2021).

Under physiological conditions, APP is implicated in synaptic formation and plasticity, neurite outgrowth, and axonal transport (Chau et al., 2023; Reinhard et al., 2005; Sirisi et al., 2024). Soluble APP fragments (sAPPα and sAPPβ), released by secretase cleavage (see below), exhibit neurotrophic and neuroprotective properties, promoting long-term potentiation (LTP), regulating Ca^{2+} homeostasis, and modulating neurotransmitter release (Plummer et al., 2016). APP also participates in cell adhesion and intracellular signalling through its interaction with G proteins and cytoskeletal components, influencing neuronal migration and network stability (Sosa et al., 2017; Z. Zhou et al., 2011).

The first extramembrane cleavage of APP can be mediated by one of two different membrane-anchored proteases: α-secretase, which is a member of the A Disintegrin And Metalloproteinase (ADAM) or β-secretase, which is an aspartyl protease also called β-site APP-cleaving enzyme (BACE). The second and intramembrane cleavage is performed by γ-secretase complex, an aspartyl protease composed of presenilin-1 (PSEN1) or presenilin-2 (PSEN2), nicastrin (NCT), anterior pharynx-defective 1 (APH1) and presenilin enhancer 2 (PEN2). In the non-amyloidogenic pathway, α-secretase cleaves APP producing a large soluble ectodomain fragment, named sAPPα, which is an important factor involved in the development of the neural system enhancing proliferation of neuronal cells and modulating synaptic plasticity, which is released in the extracellular environment (Gralle et al., 2006; Scheidig et al., 1997). The complementary fragment is named CTFα fragment (C83) (Gralle et al., 2006; Scheidig et al., 1997), further processed with a second cleavage exerted by the γ-secretase, within the transmembrane domain, into the short non-pathogenic P3 peptide and AICD (**Fig. 1.1**) (Bachurin et al., 2017; Krawczuk et al., 2025; Urban et al., 2021). sAPPα secretion is stimulated by neuronal activity, protein kinase C activation, and glutamatergic signalling (Avramovich et al., 2002; Camden et al., 2005). The non-amyloidogenic pathway is, therefore, considered a physiological processing route, contributing to homeostasis and synaptic resilience. Conversely, within the amyloidogenic pathway, which predominates under pathological conditions, APP is cleaved by β-secretase producing an sAPPβ, lacking the Aβ metal-binding domain and neuroprotective functions of the corresponding sAPPα, and a CTFβ fragment (C99), that undergoes additionally cleavage by the γ-secretase complex. The γ-secretase complex can cleave at multiple sites in the TM domain of CTFβ, namely at the ε-site, ζ-site, and γ-site. The ε-cleavage at position 49 on the TM domain releases the AICD in the cytosol; the remaining fragment undergoes a subsequent ζ-cleavage at position 46; whereas the final cleavage at γ-site can occur at position 38, 40 or 42, resulting in the release of $A\beta_{38}$, $A\beta_{40}$ or $A\beta_{42}$, respectively, in the extracellular environment, leading to Aβ oligomerization (**Fig. 1.1**) (Bachurin et al., 2017; Krawczuk et al., 2025; Urban et al., 2021).

All Aβ isoforms are known to be able to associate forming cytotoxic oligomers interacting with neuronal membranes and membrane receptors and further aggregate into diffuse plaques or fibrils, resulting ultimately in senile plaques observed at the last stages of AD progression (Ashraf et al., 2014; G. Chen et al., 2017). The onset of AD is attributable to a general increase in the production of all Aβ isoforms and a variation of the distribution between $A\beta_{42}$ and $A\beta_{40}$ which are present in larger amount than the other isoforms in brains of AD patients (Urban et al., 2021). Under normal conditions, Aβ peptides are cleared efficiently, but when production exceeds clearance or degradation fails, soluble oligomers form, initiating synaptic toxicity and network hyperexcitability (Gu & Guo, 2021; Itoh et al., 2022; Portelius et al., 2010).

Some studies indicate that the molar ratio between the two $A\beta_{40}$ and $A\beta_{42}$ isoforms does not remain constant and it may be involved in the AD onset (Oren et al., 2021; Urban et al., 2021). It has been shown that $A\beta_{42}$ and $A\beta_{40}$ isoforms may affect the aggregation of one another; in fact, recent evidence demonstrated that at high $A\beta_{40}/A\beta_{42}$ ratio, $A\beta_{40}$ acted as an inhibitor, and vice versa, and cross-seeding of $A\beta_{40}$ with pre-formed $A\beta_{42}$ seeds accelerated the aggregation of $A\beta_{40}$ (Oren et al., 2021). Other studies have supposed a protective function of $A\beta_{40}$ oligomers by their interference with the nucleation of $A\beta_{42}$ oligomer and a consequence reduction in Aβ deposition (Wells et al., 2021).

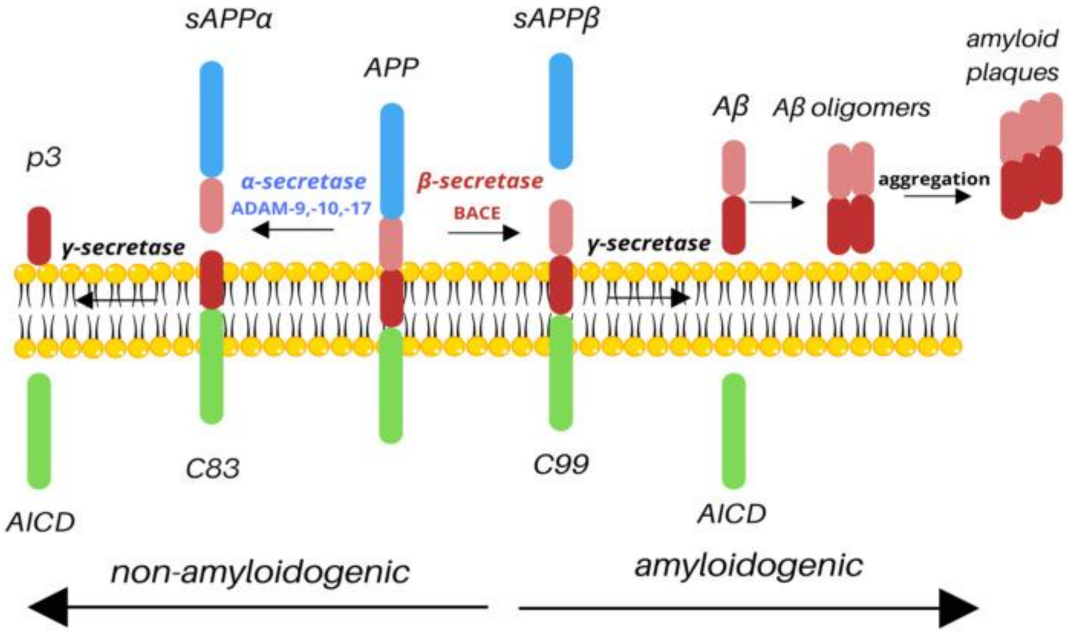

Figure 1.1. Schematic representation of Aβ pathways. In the amyloidogenic pathway, on the right, the cleavage of APP by β-secretase and subsequently by γ-secretase results in the production of an Aβ peptide which self-assembles into amyloid plaques. The pathogenic pathway initiates by β-secretase and results in sAPPβ release externally. The remaining CTFβ fragment is cleaved by γ-secretase and releases $A\beta_{38}$, $A\beta_{40}$ or $A\beta_{42}$ amino acid amyloid monomers. The externally released Aβ fragment forms toxic oligomers and eventually packaged in amyloid plaques. In the non-amyloidogenic pathway, on the left, APP in processed by α-secretase releasing sAPPα into the intracellular space. The resulting CTFα fragment is cleaved by γ-secretase in the intermembrane space resulting in both the AICD and P3, both non-plaques forming elements. Figure taken from Krawczuk et al. 2025.

1.2.4 Genetic mutations and their role in Alzheimer's Disease (AD)

Mutations in APP or in either PSEN1 or PSEN2 enhance the cellular production of Aβ and increase the cleavage of γ-secretase and β-secretase. Nowadays, about 26 different mutations in the gene of APP, all of which are located within or near the region coding for $A\beta_{42}$, are known but not all of them are characterized (Chiti & Dobson, 2017). As an example, one double mutation K670N-M671L (also known as Swedish mutation), located immediately before the cleavage site for the β-secretase at the N-terminus, the A673V mutation, and the A692G mutation render APP a better substrate for the BACE1 and

BACE2 resulting in an overproduction of both Aβ_{40} and Aβ_{42} (Chiti & Dobson, 2017; Di Fede et al., 2009; Farzan et al., 2000). The Swedish mutation KM670/671NL and in mutations in PSEN1 or in PSEN2 genes are involved in familial early-onset AD (EOAD) (György et al., 2018; Varte et al., 2025) Furthermore, 14 mutations are downstream of the cleavage sites for γ-secretase that generate the C-terminus, other 11 are within the Aβ_{42} itself (Chiti & Dobson, 2017). The mutations following the C-terminus were found to increase the specificity of γ-secretase resulting in an increased release of Aβ_{42} isoform relative to Aβ_{40} (Ancolio et al., 1999; Chiti & Dobson, 2017; De Jonghe, 2001). Mutations such as E693Q, E693G, E693K, ΔGlu693, D694N and A713T have been found to have no effect on the specificity of the secretases (Armstrong et al., 2004; Chiti & Dobson, 2017; De Jonghe et al., 1998; Nilsberth et al., 2001; Tomiyama et al., 2008), whereas the effects of other three mutations have not yet been reported, but it is supposed that these nine mutations are involved in the increase of aggregation rate of the intrinsically disordered Aβ peptides (Chiti & Dobson, 2017; Miravalle, 2000; L. Zhou et al., 2011).

Interestingly, in a recent study, a novel mutation, namely A673T, was identified in the Icelandic population, and located at the same site as A673V (Xia et al., 2021). Unlike the A673V mutation, however, the A673T substitution is protective (Xia et al., 2021). This mutation is believed to reduce the generation and the aggregation level of Aβ in SH-SY5Y cells, by increasing APP processing by α-secretase, and reducing both of sAPPβ and C99 levels (Xia et al., 2021).

While Aβ_{40} is the most abundant species, Aβ_{42} is more hydrophobic and fibrillogenic and forms oligomers at a faster rate than Aβ_{40}, making it the principal neurotoxic form (Bagheri & Saso, 2025; W. Huang et al., 2025). This assessment is corroborated by the fact that Aβ_{42} has been found to be the major or even the sole component of neuritic plaques, suggesting its pathogenic and neurotoxic role in AD etiology and progression and consequently receiving more attention in the literature (Bitan et al., 2003; Selkoe, 2001; Wells et al., 2021). The higher predisposition of Aβ_{42} peptide to aggregate and form Aβ fibrils is due to the presence of extra two or three hydrophobic amino acid residues at the C-terminus (Jarrett et al., 1993; Otzen et al., 2000; Salahuddin et al., 2021; Soto et al., 1995). Furthermore, Aβ peptide assembles into extracellular aggregates because of the formation of β-sheet structures which are strongly stabilized by numerous interchain hydrogen bonds and give rise to Aβ fibrils and plaques formation (Gaggelli et al., 2006; Salahuddin et al., 2021).

1.2.5 Histopathological patterns of tau

Tau accumulation is a defining feature AD and of a range of other tauopathies. In AD, tau aggregates, typically hyperphosphorylated, primarily accumulate in neurons as NFTs and related hallmark lesions, whereas in some primary tauopathies, tau also aggregates in glial cells (e.g., astrocytic plaques, tufted astrocytes, and oligodendroglial coiled bodies) (Braak & Braak, 1991; Goedert et al., 2017; Goedert & Spillantini, 2017; Neri et al., 2025).

In AD, tau pathology typically begins in the transentorhinal/entorhinal cortex and hippocampus and thereafter spreads to associative neocortical areas and, in later stages, to primary sensory and motor cortices (Braak & Braak, 1991; Braak & Del Tredici, 2015). Within affected neurons, NFTs are accompanied by neuropil threads and dystrophic neurites, and extracellular "ghost tangles" remain as remnants of degenerated neurons (Braak et al., 2006; Iqbal et al., 2005). Importantly, the spatial distribution and burden of aggregated tau have been shown to correlate more closely with clinical measures of cognitive decline than the presence of Aβ plaques, although the emergence of clinical symptoms likely reflects the interaction of multiple pathological processes rather than tau burden alone (Brier et al., 2016; Pontecorvo et al., 2017).

1.2.6 Tau protein: structure, function, and pathological alterations

Tau is a microtubule-associated protein that plays a central role in stabilizing and assembling axonal microtubules, protecting them from depolymerization, and is involved in regulating intracellular transport, essential for neuronal function and structural integrity. In adult human brain six isoforms of tau are expressed, which are produced by alternative mRNA of its gene (*MAPT*) located on the long arm of chromosome 17q21.1, and differ in the number of N-terminal inserts, *i.e.* 0, 1, or 2, and microtubule-binding repeats 3R or 4R (Goedert & Spillantini, 2017; Spillantini & Goedert, 1998).

Under normal conditions, tau binds along the microtubules via its C-terminal microtubule-binding domains (Barbier et al., 2019). Phosphorylation regulates its binding affinity; a balanced phosphorylation state ensures dynamic microtubule remodelling necessary for synaptic plasticity and axonal transport (Cario & Berger, 2023; X. Hong et al., 2025). Tau also participates in neuronal signalling by interacting with kinases, phosphatases, and scaffold proteins, influencing processes such as LTP and memory consolidation (Fan et al., 2022; Pallas-Bazarra et al., 2016; Robbins et al., 2021). Moreover, tau is implicated in dendritic spine function, nuclear signalling, and DNA protection, highlighting its multifunctional role beyond cytoskeletal stabilization (Asada-Utsugi & Urushitani, 2024; Robbins et al., 2021; Diez & Wegmann, 2020).

Within AD and tauopathies, tau undergoes hyperphosphorylation, truncation, and conformational alterations that reduce microtubule binding, leading to dissociation and mislocalization from axons to somatodendritic compartments (Mandelkow et al., 1995), and then leading to tau oligomerization and filament formation in NFTs in the cytoplasm of affected neurons, dendrites, and dendritic spines (Grundke-Iqbal et al., 1986). This phenomenon may explain why NFTs are strongly correlated with synaptic dysfunction and cognitive decline, as well as contributing to cell death (Wells et al., 2021). NFTs consist of paired helical filaments (PHFs) or straight filaments (SFs). PHFs are approximately 20 nm in width, composed of two ~10 nm protofilaments twisted around each other in a periodic fashion with a crossover distance of 65-80 nm, whereas SFs exhibit less periodicity with a longer crossover distance and a width of about 15 nm (Crowther, 1991; DeTure & Dickson, 2019; Spillantini & Goedert, 1998). PHFs and SFs are made of the same protofilament pairs, and their cores are composed of eight β-strands adopting a C-shaped architecture that is perpendicular to the axial direction of the filament (Goedert, 2021). Five of the eight β-strands give rise to two antiparallel β-sheets, whereas the other three β-strands form a β-helix which confers filament stability and resistance to degradation (Goedert, 2021).

Furthermore, fibrillar tau clusters at excitatory synapses, leading to redistribution of AMPA receptors and disruption of synaptic organization, thereby perturbing Ca^{2+} homeostasis, excitatory signalling, and dendritic spine morphology (Shrivastava et al., 2019). Mutations in *MAPT* have been linked to familial primary tauopathies but not to AD (Gallo et al., 2023). Dysregulation of kinases (GSK3β, CDK5) and phosphatases (PP2A) contributes to tau hyperphosphorylation, while oxidative stress and mitochondrial dysfunction further exacerbate misfolding and neuronal vulnerability (Alavi Naini & Soussi-Yanicostas, 2015; Duan et al., 2012; G. Ganguly et al., 2017; Guha et al., 2020).

1.2.7 Alzheimer’s Disease (AD) traditional clinical stages

The clinical progression of AD is typically described in a series of stages that reflect the gradual evolution and worsening of cognitive, functional, and behavioural abilities. From the earliest subtle changes in memory and executive function to the profound impairments observed in advanced dementia, each stage is characterized by a distinct pattern of symptoms and impact on daily living. This stepwise deterioration, cognitive decline, neuropsychiatric symptoms, and functional impairment, mirrors the underlying neurodegenerative process and provides a useful framework for clinical assessment, prognosis, and management. Understanding the general course of these stages is essential for clinicians and researchers alike, as it informs diagnostic criteria, therapeutic strategies, and the development of supportive care plans throughout the disease trajectory (Alzheimer’s & Dementia, 2025; Jack et al., 2018; G. M. McKhann et al., 2011; Tahami Monfared et al., 2022).

The clinical course of AD is typically conceptualized in three main different stages. The first stage is the preclinical phase, in which individuals typically maintain normal cognitive function, and standard neuropsychological tests fail to detect significant deficits. Subtle changes in episodic memory and executive functioning may already be present, detectable only through sensitive assessments or advanced imaging techniques. This asymptomatic period can span several years, offering a theoretical window for early intervention that may slow down or prevent progression to symptomatic disease (Jack et al., 2018; Sperling et al., 2011; Villemagne et al., 2013). As the pathological process continues, individuals often develop mild cognitive impairment (MCI), in which cognitive deficits become more apparent, yet still do not significantly impair daily functioning. Memory, attention, and executive functions are most commonly affected, and patients may notice difficulties in complex tasks such as managing finances or following multi-step instructions. Importantly, MCI represents a transitional stage in which early recognition and potential therapeutic intervention can still influence the trajectory of disease (Albert et al., 2011; Brodaty et al., 2013; Petersen, 2016).

Finally, when the disease reaches its clinically manifest stage, dementia emerges with progressively severe cognitive and functional impairments. In this phase of clinical AD, patients experience increasing difficulties in memory, language, decision-making, and orientation, accompanied by neuropsychiatric symptoms such as apathy, agitation, depression, and anxiety. Functional dependence grows, and everyday activities, including personal care, mobility, and social interactions, become progressively compromised. This decline is influenced not only by the progression of cognitive impairment but also by neuropsychiatric symptoms and individual differences in cognitive reserve (Binetti et al., 1998; Cummings et al., 2015; Galasko et al., 1997; Geda et al., 2013; Stern, 2012). Clinical AD dementia is typically subdivided into various sub-stages depending on its severity (Albert et al., 2011; Brodaty et al., 2013; Burns & Iliffe, 2009). In mild AD, patients experience noticeable deficits in memory, language, and complex decision-making, yet retain partial independence in daily activities. In moderate AD, disorientation, impaired judgment, and neuropsychiatric symptoms emerge, often necessitating substantial caregiver support. In severe AD, profound cognitive deterioration occurs, resulting in complete loss of independence in activities of daily living and, in later stages, motor impairments (Burns & Iliffe, 2009; N. K. Zhang et al., 2024).

These clinical manifestations underscore the need for standardized cognitive and functional assessments to monitor disease progression and guide intervention. Accordingly, a range of validated assessment tools is employed across AD in both clinical and research settings (Dubois et al., 2021; Jack et al., 2018). These tests allow clinicians to quantify the severity of cognitive deficits, track changes over time, and inform diagnostic and treatment decisions. Among the most widely used is the Mini-Mental State Examination (MMSE), which provides a rapid evaluation of global cognitive function and allows clinicians to quantify overall impairment (Arevalo-Rodriguez et al., 2015; Folstein et al., 1975). The Clinical Dementia Rating (CDR) offers a more nuanced staging of cognitive and functional abilities, assessing domains such as memory, orientation, and the capacity to perform everyday activities (H. Huang et al., 2021; Morris, 1993). For a more detailed analysis of domain-specific cognitive deficits, the Alzheimer’s Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) is often utilized, particularly in research and clinical trials, it evaluates domain-specific cognitive deficits (Kueper et al., 2018; Rosen et al., 1984; N. Verma et al., 2015). Additionally, instruments like the Montreal Cognitive Assessment (MoCA) and the Functional Activities Questionnaire (FAQ) have been developed to increase sensitivity to early and subtle deficits, capturing impairments that may be missed by broader screening tools (González et al., 2021; Nasreddine et al., 2005; Pfeffer et al., 1982). The combined use of these assessments provides a comprehensive overview of a patient’s cognitive profile and functional status, facilitating early detection, monitoring of disease progression, and evaluation of therapeutic interventions.

In parallel, quantitative electroencephalography (qEEG) provides a non-invasive approach to evaluate altered neural synchrony in AD. Characteristic slowing of dominant rhythms and impaired network connectivity may help differentiate individuals with MCI from those with overt AD dementia (Babiloni et al., 2009; Jeong, 2004). Other emerging neurophysiological and neuroimaging modalities, such as magnetoencephalography (MEG) and functional MRI (fMRI), are being actively investigated for their ability to detect early network disruptions and improve disease staging. Recent studies indicate that MEG, with its high temporal resolution, can identify dynamic alterations in functional connectivity during the preclinical and MCI stages of AD (Jin et al., 2023). Similarly, functional connectivity analyses using fMRI, combined with advanced machine learning approaches, have demonstrated high accuracy in classifying early stages of AD (Matthews & Jezzard, 2004; Warren & Moustafa, 2023). Taken together, these complementary approaches provide a more comprehensive and sensitive understanding of AD-related brain changes, potentially supporting earlier and more targeted clinical decision-making.

1.3 Biomarker-based framework AT(N) System

1.3.1 Advances in Alzheimer’s Disease (AD) Staging

Over the past two decades, AD staging criteria have undergone a profound conceptual transformation. Once defined primarily by its clinical manifestations, as described in *Section 1.2.7*, it is now increasingly recognised as a biological continuum driven by a cascade of molecular and cellular events that begin decades before the appearance of overt symptoms (Dubois et al., 2021; Jack et al., 2018).

Although classical clinicopathological staging systems have been instrumental in elucidating the anatomical progression of AD, they have several intrinsic limitations when applied to living individuals or to dynamic disease assessment (Therriault, Gauthier, et al., 2022; Therriault, Pascoal, et al., 2022). These systems are inherently static, providing only a snapshot of the pathology and failing to capture disease evolution over time. Moreover, inter-individual variability and atypical progression patterns can lead to under- or overestimation of disease severity in specific cases (Putra et al., 2021). Clinical classifications based primarily on cognitive symptoms and neuropsychological assessments contribute to delayed detection, as they identify the disease only after substantial neuropathological burden has accumulated, thereby limiting opportunities for early intervention (Dubois et al., 2021). Symptom-based criteria also fail to differentiate individuals with similar cognitive profiles but distinct underlying pathophysiological processes, such as variations in Aβ deposition, tau aggregation, or neuroinflammatory status. Furthermore, traditional staging does not reflect the heterogeneous and multifactorial nature of AD, as key modifiers of disease trajectory, including genetic risk, vascular comorbidities, cognitive reserve, and lifestyle factors, are not captured by standard clinical classifications (Sperling et al., 2011). Consequently, patient stratification for personalized interventions or clinical trials remains challenging, as classical staging approaches do not adequately capture individual differences (Abdelnour et al., 2022; Pascoal et al., 2024).

Neuropathological and *in vivo* molecular imaging studies, as well cerebrospinal fluid (CSF) evaluation of the contents of Aβ and tau, have demonstrated that the accumulation of Aβ and tau pathology begins many years, often more than two decades, before the clinical onset of cognitive impairment (Cohen & Villemagne, 2025; Georgakas et al., 2023; Ravikumar et al., 2024; Serrano-Pozo et al., 2011). These findings have redefined the temporal dynamics of the disease, revealing that Aβ deposition represents an early initiating event, followed by the deposition and spread of tau and hyperphosphorylated tau and the subsequent emergence of neurodegeneration (Gallego-Rudolf et al., 2024; Giorgio et al., 2025; Jack et al., 2018; Jagust et al., 2021; Serrano-Pozo et al., 2011; Vermunt et al., 2019).

The AT(N) system, proposed by the National Institute on Aging and the Alzheimer's Association (NIA-AA), has become a cornerstone of the modern understanding of AD as a progressive continuum of biological alterations, rather than a series of discrete clinical stages (Jack et al., 2018; Neri et al., 2025). The AD continuum can be divided into three main stages: the preclinical stage, during which pathological changes associated mainly with Aβ occur in the absence of clinical symptoms, with early biomarker evidence of cerebral Aβ accumulation, as detected by Aβ-PET imaging or reduced CSF $A\beta_{42}$ levels (Jack et al., 2018; Jansen et al., 2015; Parnetti et al., 2019; Rafii & Aisen, 2023; Sperling et al., 2011); the prodromal stage, corresponding to early symptomatic stage or MCI, characterized by subtle cognitive deficits detectable with sensitive assessment, together with the increase of t-tau and p-tau in the CSF and detected tau-PET imaging in the CNS (Aisen et al., 2022; Albert et al., 2011; Porsteinsson et al., 2021; Vermunt et al., 2019); and finally, the dementia stage, which reflects progressively severe cognitive and functional impairments, associated with widespread neurodegeneration reflected in brain atrophy, hypometabolism, with a plateau accumulation of Aβ plaques and NFTs in the CNS (Grassi et al., 2019; Jack et al., 2018; G. McKhann et al., 1984; Winblad et al., 2016). Recognising this temporal hierarchy is fundamental to the biological definition of AD and underpins the logic of the AT(N) framework in which A stands for Aβ, T for tau, and N for neurodegeneration, each representing a core aspect of AD (Gauthier et al., 2018; Jack et al., 2018). In other words, the AT(N) framework represents a major step toward understanding AD from a biological perspective. By linking CSF-based biomarkers, brain imaging, and clinical symptoms, it provides a unified approach that helps both research and patient care (Jack et al., 2018).

1.3.2 Biomarker progression in Alzheimer's Disease (AD)

Following the establishment of the AT(N) framework, examining the dynamics of CSF biomarkers and imaging findings across the stages of AD provides valuable insights into disease progression (**Fig. 1.2**). In the preclinical stage, Aβ (A) is the earliest detectable signal in the disease cascade. Reduced concentrations of $A\beta_{42}$, or a decreased $A\beta_{42}/A\beta_{40}$ ratio in CSF, reflect the sequestration of Aβ into plaques, while PET imaging with radioligands such as $[^{11}C]$PiB or $[^{18}F]$florbetapir reveals fibrillar Aβ deposition across cortical regions. Remarkably, these changes can be observed in cognitively unimpaired individuals, illustrating that Aβ pathology begins silently, years before tau accumulation or cognitive decline become evident (Blennow et al., 2010; Georgakas et al., 2023; Palmqvist et al., 2017). At this stage, tau (T) and neurodegeneration (N) markers remain largely within normal ranges, and structural MRI shows no evident atrophy, corresponding to an $A^{+}T^{-}N^{-}$ profile (Jack et al., 2013; Jansen et al., 2015; Rafii & Aisen, 2023). Although tau levels are generally within normal limits, subtle increases may already be detectable in some individuals, reflecting early neuronal stress. The preclinical stage thus represents a window of silent pathology, where molecular changes precede any clinical manifestation.

As the disease progresses into the prodromal stage, corresponding to MCI in the classical staging scheme, tau pathology becomes detectable. CSF analysis shows continued reduction in Aβ_{42}, alongside elevated phosphorylated tau species, including p-tau181, p-tau217, and p-tau231 (Holper et al., 2022; Janelidze et al., 2020; Kurihara et al., 2023; Leuzy et al., 2021; Llibre-Guerra et al., 2019; Nabizadeh et al., 2022; Visser et al., 2022; M.-Y. Wang et al., 2025). Concurrently, t-tau levels increase, alongside structural MRI evidence of hippocampal or entorhinal atrophy although in later stages of the disease t-tau may stabilize or decline due to neuronal loss (Leuzy et al., 2021; W. Xu et al., 2019). Tau-PET imaging has revolutionized our understanding of the spatiotemporal dynamics of tau aggregation. Radiotracers such as [^{18}F]flortaucipir, MK-6240, and RO-948 enable direct visualization of tau burden *in vivo*, highlighting focal deposition in medial temporal regions that closely correlates with cognitive performance and emerging clinical symptoms (Gogola et al., 2022; Leuzy et al., 2021). Depending on the degree of neurodegeneration, individuals may present an $A^{+}T^{+}N^{-}$ or $A^{+}T^{+}N^{+}$ profile, reflecting the transition from subtle molecular changes to measurable structural and functional impairment. Interestingly, among these tau biomarkers, p-tau217 has demonstrated accuracy in distinguishing AD from non-AD neurodegenerative disorders, including primary tauopathies such as progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (Gonzalez-Ortiz et al., 2023).

The progression to the dementia stage is marked by pronounced cognitive and functional deterioration. Neurodegeneration or neuronal injury (N) reflects the structural and functional brain changes resulting from the accumulation of Aβ and tau (Agnello et al., 2025; Cacciaglia et al., 2025; Kamatham et al., 2024; Sperling et al., 2011). This neurodegeneration involves structural, metabolic, and functional brain alterations, including synaptic loss, neuronal death, and network disruption, ultimately translating into measurable cognitive and behavioural decline (Busche & Hyman, 2020; DeTure & Dickson, 2019). Structural MRI provides high-resolution assessment of cortical and subcortical atrophy, with the earliest and most prominent changes typically involving the medial temporal lobe, including the hippocampus, entorhinal cortex, and parahippocampal gyrus, regions essential for episodic memory (Ali Zarad et al., 2025; Frisoni et al., 2010; Suchy-Dicey et al., 2023). As the disease progresses, atrophy extends to temporoparietal and frontal association areas, correlating with broader cognitive and functional deficits. Fluorodeoxyglucose positron emission tomography (FDG-PET) complements MRI by measuring cerebral glucose metabolism, a proxy for synaptic activity, revealing characteristic hypometabolism in the posterior cingulate, praecuneus, and temporoparietal cortices, which distinguishes AD from other dementias and correlates strongly with cognitive impairment (Bouter et al., 2025; B. Huang et al., 2024; Shivamurthy et al., 2015). At this stage, CSF biomarkers show persistently low $A\beta_{42}$, accompanied by elevated p-tau and t-tau levels, reflecting extensive neuronal and synaptic dysfunction (Leuzy et al., 2021; Mattsson-Carlgren et al., 2022). Complementary imaging studies further confirm the widespread nature of the disease; indeed, tau-PET reveals diffuse cortical and hippocampal deposition, and Aβ-PET confirms widespread Aβ deposits (Lagarde et al., 2021; Ossenkoppele et al., 2016). In parallel, CSF Neurofilament Light chain (NfL) provides additional evidence of axonal degeneration, correlating with MRI-detected atrophy and FDG-PET hypometabolism (Mattsson-Carlgren et al., 2022; Zetterberg et al., 2016). Collectively, these findings correspond to an $A^+T^+N^+$ profile, confirming that prodromal changes have progressed to full-blown dementia (Jack et al., 2018; G. M. McKhann et al., 2011).

Taken together, tracking biomarker changes from the preclinical to prodromal and dementia stages provides a clear view of AD progression (**Fig. 1.2**), enabling biological staging independent of symptoms, supporting monitoring over time, patient selection for clinical trials, and evaluation of interventions (Blennow et al., 2010; Jack et al., 2018; Palmqvist et al., 2017). Combining CSF biomarkers with advanced imaging allows precise tracking of disease evolution, earlier detection, and more personalized therapeutic strategies.

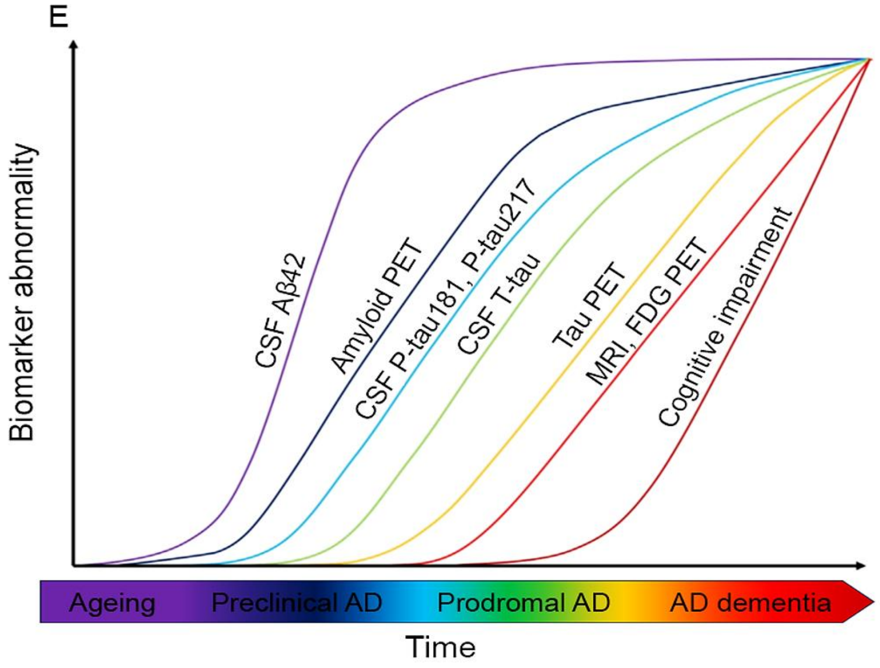

Figure 1.2. Temporal evolution of biomarkers across the AD continuum.

1.3.3 Towards the expansion of the AT(N) Framework

While the AT(N) system has been essential for biologically defining AD, it only captures part of the picture. Beyond Aβ, tau, and neurodegeneration, AD is shaped by a dynamic and heterogeneous inflammatory response within the brain. This process, driven mainly by microglia and astrocytes, plays a key role in triggering, propagating, and modulating neuronal injury, influencing both the onset and progression of the disease (Jack et al., 2024; Janelidze et al., 2018).

Neuroinflammation in AD is characterized by chronic glial activation, reactive astrocytes, alterations in cytokine signalling, oxidative stress, and disruption of homeostatic glial-neuronal communication (Fakorede et al., 2025; Luca et al., 2018; F. Zhang & Jiang, 2015). Experimental and human imaging studies reveal that neuroinflammation begins early in the disease course, sometimes preceding neurodegeneration (Fakorede et al., 2025; Heneka et al., 2025; Stoessl, 2012). It has been observed that activated microglia cluster around Aβ plaques, initially attempting to contain and clear Aβ aggregates (Öztürk et al., 2025), but may later adopt a proinflammatory phenotype that accelerates synaptic and neuronal injury, and enhance tau phosphorylation and propagation, linking immune dysregulation directly to classical AD pathology (Z. Sun et al., 2024). Neuroinflammation biomarkers can be assessed through imaging and CSF samples. For example, TSPO-PET imaging allows the detection of activated microglia, with ligands ([^{11}C]PBR28, [^{18}F]DPA-714, [^{18}F]GE-180) improving both sensitivity and quantification (Rossano et al., 2024; Vomacka et al., 2017; R. Zhou et al., 2021). Another biomarker that has received recent attention is the soluble microglial marker TREM2 (sTREM2), which reflects microglial reactivity and can be measured in CSF (Morenas-Rodríguez et al., 2022; Pereira et al., 2022; Suárez-Calvet et al., 2016). Clinically, incorporating neuroinflammatory biomarkers may improve patient stratification and trial design.

In addition to inflammation, growing evidence supports the integration of vascular (V) and α-synuclein (α-syn) (S) co-pathologies into the AT(N) framework, acknowledging the multifactorial nature of AD (Jack et al., 2024). Cerebrovascular dysfunction, including small vessel disease, blood-brain barrier (BBB) disruption, and microinfarcts, may contribute to neuronal injury and in accelerate cognitive decline (Y. Chen et al., 2023; Y. Liu et al., 2018; Santisteban et al., 2023). The assessment of these vascular contributions has been facilitated by the identification of specific imaging biomarkers, such as white matter hyperintensities (WMH) on structural MRI (Gaubert et al., 2021; Y. Liu et al., 2018; Vemuri et al., 2021), cerebral microbleeds detectable with susceptibility-weighted imaging (SWI) (Basselerie et al., 2017; Chin et al., 2024; Sparacia et al., 2017), and cerebral perfusion alterations revealed by arterial spin labelling (ASL) or [^{18}F]-FDG-PET (Binnewijzend et al., 2016; Dolui et al., 2020; Thropp et al., 2024; Verclytte et al., 2016).

Interestingly, α-syn aggregation is increasingly recognized as a co-pathology in AD, particularly among patients exhibiting parkinsonian features or showing a more rapid clinical progression (Franzmeier et al., 2025). This phenomenon highlights the overlap between AD and synucleinopathies, further emphasizing the biological heterogeneity of AD. Similarly to other biomarkers, total and phosphorylated α-syn can be measured in the CSF (Constantinides et al., 2021). In experimental settings, highly sensitive α-syn seed amplification assays (α-syn-SAAs), such as real-time quaking-induced conversion (RT-QuIC), enable the detection of misfolded α-syn aggregates at extremely low concentrations, providing a reliable approach for identifying syn-related pathology (Arnold et al., 2022; Hall et al., 2022; Rissardo & Fornari Caprara, 2025).

Adding biomarkers for inflammation (I), vascular brain injury (V), and α-synuclein (S) expands the AT(N) framework into a more complex, yet complete model of AD (Jack et al., 2024). This broader approach allows for better disease staging, helps explain differences between patients, and improves selection for clinical trials targeting specific disease processes. It also supports the combined use of imaging and fluid biomarkers for a clearer understanding of the disease in the living brain (Jack et al., 2024).

1.3.4 Blood biomarkers in Alzheimer's Disease (AD)

While CSF provides a direct window into brain pathology, its invasiveness restricts its widespread use. The search for reliable blood biomarkers has emerged as one of the most recent and significant challenges in AD research and diagnosis. This ongoing effort reflects the growing need for minimally invasive, cost-effective, and scalable diagnostic tools that can overcome the limitations of CSF analysis, PET, and other neuroimaging techniques (Hampel et al., 2018; Leuzy et al., 2022; Ray et al., 2007; Rembach, 2014; Teunissen et al., 2022). Blood is easily accessible, but early studies faced substantial methodological challenges, including the need for highly sensitive assays, the influence of peripheral confounders, and the demonstration of clinical relevance (Ray et al., 2007; Therriault et al., 2024). Despite these obstacles, research increasingly focused on detecting AD-related pathological changes even in cognitively unimpaired individuals, aiming to identify brain alterations before the onset of clinical symptoms (Hampel et al., 2018; Leuzy et al., 2022; Ray et al., 2007; Rembach, 2014; Zeng et al., 2024).

Recent technological advances in ultrasensitive analytical platforms, such as single-molecule array (Simoa) (Álvarez-Sánchez et al., 2022; Truffi et al., 2023), immunoprecipitation-mass spectrometry (IP-MS) (Y. Chen et al., 2024), and electrochemiluminescence (ECL) immunoassays (Fatima et al., 2024; Kivisäkk et al., 2023), allow for precise and reproducible detection of brain-derived proteins in plasma, overcoming the sensitivity limitations that previously constrained peripheral biomarker research (Pacoova Dal Maschio et al., 2025).

New studies highlighted that plasma $A\beta_{42}/A\beta_{40}$ ratios, measured via MS (Y. Chen et al., 2024; Janelidze et al., 2021; Jang et al., 2021; Schindler et al., 2019; D. M. Weber et al., 2024) or automated immunoassays (Bun et al., 2023; Palmqvist et al., 2019; Vogelgsang et al., 2024; Yamashita et al., 2022), show strong correlations with Aβ-PET positivity and mirror the reduction in CSF $A\beta_{42}$ observed in Aβ-positive individuals. Similarly, plasma p-tau181, p-tau217, p-tau231 demonstrate remarkable diagnostic specificity for AD (Bayoumy et al., 2021; Lombardi et al., 2024; Mielke et al., 2021). Specifically, it has been individuated that p-tau181 correlates with clinical severity and shows strong associations with both tau and Aβ-PET measures, reflecting ongoing neurofibrillary pathology even in early disease stages (Mielke et al., 2018; X.-N. Shen et al., 2021). P-tau217 exhibits the closest correspondence with CSF and tau-PET assessments and is a robust predictor of conversion from mild cognitive impairment to dementia (Ossenkoppele et al., 2025; Palmqvist et al., 2020; Rissman et al., 2024). Meanwhile, p-tau231 may capture even earlier Aβ-induced tau dysregulation, potentially identifying pathological changes before the onset of clinical symptoms (Ashton et al., 2021; González-Escalante et al., 2025; Suárez-Calvet, 2022). Combining measurements of these plasma phosphorylated tau isoforms with $A\beta_{42}/A\beta_{40}$ ratios further enhances diagnostic accuracy, achieving performance comparable to that of multimodal imaging approaches (Ashton et al., 2022; H. Kang et al., 2025; Kritikos et al., 2023; Lehmann et al., 2025; J. Wang et al., 2025).

As markers of neurodegeneration are concerned, it has been assessed that plasma NfL levels correlate strongly with CSF concentrations and with imaging measures of cortical atrophy on structural MRI and cerebral hypometabolism on FDG-PET, enabling longitudinal tracking of neurodegenerative progression and cognitive decline (Abukuri, 2024; Benedet et al., 2019; Hofmann et al., 2024; Q. Hu et al., 2024; Mattsson et al., 2017; Mielke et al., 2019). In more recent studies, NfL has also shown associations with diffusion tensor imaging (DTI) (Jung & Damoiseaux, 2024), reflecting white matter integrity, and with tau-PET measures, indicating its close relationship to neuroaxonal damage (Benedet et al., 2020).

Reflecting the increasing recognition of neuroinflammation in AD pathophysiology, blood-based assays of glial activation have also gained growing prominence. Plasma GFAP is strongly associated with Aβ positivity and correlates with cortical atrophy and tau burden even in preclinical stages (Benedet et al., 2021; Y. He et al., 2025; Ingannato et al., 2024; K. Y. Kim et al., 2023; Lee et al., 2025; Pereira et al., 2021; Peretti et al., 2024). Furthermore, sTREM2 may reflect an early microglial response to pathological changes in AD, potentially representing a compensatory immune mechanism (Qi et al., 2025; G. E. Weber et al., 2022), while inflammatory markers such as IL-6, TNF-α, and YKL-40 (CHI3L1) provide additional information on neuroinflammatory processes, although they are less specific (Heneka et al., 2025; Muszyński et al., 2017; Vergallo et al., 2020). These blood-based measures can therefore offer complementary insights into the role of immune activation and inflammation in the progression of AD.

A major milestone in blood-based biomarker development for AD occurred in 2025, when the US FDA authorized the Lumipulse G p-tau217/$A\beta_{42}$ Plasma Ratio for clinical use in adults with cognitive impairment suggestive of AD. This minimally invasive test quantifies plasma p-tau217 and $A\beta_{42}$ and computes their ratio as a surrogate for cerebral amyloid burden, providing a cost-effective and scalable alternative to PET imaging (Wisch and Ances 2025; www.fda.gov). In a multi-centre study of 499 participants, the assay achieved 91.7% positive concordance with Aβ-PET/CSF positivity and 97.3% negative concordance with Aβ-negative cases (www.fda.gov).

1.4 The two-phase model within the Alzheimer's Disease (AD) continuum

1.4.1 Overproduction of amyloid-beta (Aβ) and glutamate (Glu) before the preclinical phase

Converging evidence from neurophysiological, molecular, and neuroimaging studies suggests that distinct alterations in neuronal activity may emerge upstream of these canonical stages, revealing an earlier sequence of functional dysregulation (Aizenstein & Klunk, 2015; Cox et al., 2022; Findley et al., 2019; Huijbers et al., 2015; J. Li et al., 2024; Olney, 1997; C. G. Parsons et al., 2007; Targa Dias Anastacio et al., 2022). From this mechanistic perspective, AD is thought to be initiated by overproduction of Aβ and its oligomerisation, which disrupt glutamatergic neurotransmission and induce excitotoxicity through sustained production of the Glu neurotransmitter and stimulation of neuronal ionotropic Glu receptors, primarily N-methyl-D-aspartate receptors (NMDARs) (Abdul et al., 2009; Olney, 1997; Talantova et al., 2013). These events mark the onset of a two-stage model in which AD has been proposed to consist of a first earlier functional stage characterized by a neuronal hyperactivity phase (phase of glutamatergic hyperactivity, or NMDAR hyperexcitability or NRHyper), followed by a second stage, the neuronal hypoactivity phase (phase of glutamatergic hypoactivity or NRHypo), in which chronic overstimulation results in NMDAR downregulation, synaptic depletion, and neuronal loss (Cox et al., 2022; Findley et al., 2019; Olney, 1997).

1.4.2 Neuronal hyperactivity (NRHyper)

The NRHyper phase represents the first of a two-phase model of AD, describing a functional and biochemical state in which excessive excitatory neurotransmission precedes Aβ plaque accumulation and neurodegeneration, driven by disruption of glutamatergic homeostasis induced by soluble forms of Aβ, particularly $A\beta_{40}$ and $A\beta_{42}$ oligomers, which interfere with neuronal and astrocytic signalling (Abdul et al., 2009; Acosta et al., 2017; Findley et al., 2019; Olney, 1997; Talantova et al., 2013). At the synaptic level, soluble Aβ species interact with NMDARs leading to their persistent and

and chronically stimulation and altered receptor kinetics (Cox et al., 2022; Fani et al., 2021, 2022; Fani & Chiti, 2022; Findley et al., 2019; Olney, 1997). This abnormal stimulation enhances presynaptic Glu release while impairing astrocytic Glu uptake via excitatory amino acid transporters (EAATs), particularly EAAT2 (GLT-1), resulting in extracellular Glu accumulation. In addition, Aβ oligomers stimulate NMDARs by interacting with the cell membrane through a mechanosensitive transduction (Fani et al., 2021; Fani & Chiti, 2022) The consequent and sustained NMDAR overactivation determines a Ca^{2+} influx heading to mitochondrial dysfunction, oxidative stress and ROS increasing, and intracellular Ca^{2+} dyshomeostasis, which collectively bring to excitotoxicity and early synaptic instability (Abdul et al., 2009; Acosta et al., 2017; Aizenstein & Klunk, 2015; Cox et al., 2022; Findley et al., 2019; Han et al., 2016; E. R. Hascup et al., 2019; K. N. Hascup & Hascup, 2016; Huijbers et al., 2015; J. Li et al., 2024; Lovell et al., 2012; Olney, 1997; C. G. Parsons et al., 2007; Qin et al., 2006; Schallier et al., 2011; Scimemi et al., 2013; Sokolow et al., 2012; Talantova et al., 2013; Targa Dias Anastacio et al., 2022; X.-K. Yang et al., 2019; Zumkehr et al., 2015).

These biochemical alterations induced by soluble Aβ and excessive glutamategic signalling, lead to the NRHyper phase that many different clinical and animal model studies have shown to overlap with the prodromal/MCI phase of AD, thus as a very early event in AD (J. Li et al., 2024; Targa Dias Anastacio et al., 2022). Indeed, it has been highlighted by multiple studies that NRHyper overlaps temporally with the prodromal or MCI stage and may even precede the earliest pathologic change detected by classical biomarkers, such as Aβ-PET detection and CSF $Aβ_{42}$ or $Aβ_{42}/Aβ_{40}$ ratio reduction (Cox et al., 2022; Findley et al., 2019; J. Li et al., 2024; Targa Dias Anastacio et al., 2022). Consistent with this interpretation, several functional neuroimaging studies have documented neuronal hyperactivation in cognitively normal individuals genetically predisposed to AD, well before measurable Aβ or tau biomarkers in fluids. Indeed, it has been observed by Reiman et al. 2012 in young human PSEN1 mutation an increased activation in hippocampal and parahippocampal regions and reduced deactivation in the precuneus and posterior cingulate cortex compared to non-carriers, as assessed by functional MRI (fMRI), even prior $Aβ_{42}$ impoverishment and tau enrichment in the CSF. Likewise, healthy non-demented offspring of early- or late-onset familial AD cases, who were 10-30 years younger than their parents when they developed AD, display higher hyperactivity in hippocampal, frontal, and temporal lobes relative to age-matched controls (Bassett et al., 2006; Mondadori et al., 2006). Moreover, young adult human carriers of the APOE ε4 risk factor allele showed hyperactivation activity in hippocampal, parietal, and prefrontal regions (Bookheimer et al., 2000; Dennis et al., 2010; S. C. Johnson et al., 2006; Sinha et al., 2018). Fischer et al. 2025 demonstrated that cognitively normal older APOE ε4 carriers showed an increased praecuneus activation during episodic memory retrieval, which positively correlated with cortical Aβ burden, indicating a compensatory overactivity in regions highly vulnerable to AD despite the absence of cognitive decline, as an initial attempt to maintain memory performances despite emerging Aβ associated synaptic dysfunction (Fischer et al., 2025). Corriveau-Lecavalier et al. 2024 demonstrated through recent fMRI meta-analytic evidence an inverted U-shaped trajectory of cerebral activation across the AD continuum, with early hyperactivation in memory-related regions such as the hippocampus and praecuneus during subjective cognitive decline and early MCI, followed by progressive hypoactivation in later symptomatic stages, suggesting that neuronal hyperactivity is an intrinsic component of the AD cascade closely linked to Aβ and tau accumulation (Corriveau-Lecavalier et al., 2024).

In addition, experimental findings from transgenic models support these observations. In APP/PS1 mice, both soluble $A\beta_{40}$ and $A\beta_{42}$ levels and stimulus-evoked Glu release in specific area of the hippocampus are significantly elevated at 2-4 months of age compared to genetically similar but nontransgenic C57BL/6J control mice (K. N. Hascup et al., 2020; M. Wang et al., 2023). These changes precede the formation of Aβ deposits in the APP/PS1 mice that remain absent before 4 months and gradually increase over the 4- to 10-month period (Garcia-Alloza et al., 2006; M. Wang et al., 2023) and also precede the cognitive impairment, which remains absent at 6 months of age and progressively worsen up to 18 months (Cox et al., 2022; Savonenko et al., 2005). Similarly, very young transgenic APP23×PS45 mouse models of AD showed hippocampal CA1 hyperactivated neurons, Aβ accumulation, and elevated Glu levels well before Aβ plaque formation (Busche & Hyman, 2020; Zott et al., 2019). Interestingly, APOE4-knock-in mice, in the absence of Aβ plaques or tau tangles, develop hippocampal/entorhinal hyperactivity driven by reduced GABAergic inhibitory tone (Nuriel et al., 2017). iPSC-derived neurons from a sporadic AD patient also offered evidence in this direction as they showed hyper-excitable Ca^{2+} signalling compared to control neurons (Balez et al., 2016), and iPSC-derived neurons from a sporadic APOE4/4 AD patient showed increased synapse network, $A\beta_{42}$ secretion and frequency of miniature excitatory post-synaptic currents, relative to isogenic APOE3/3 control neurons (Lin et al., 2018). This evidence may contribute to the conceptualization of a further phase characterized by subtle biochemical and metabolic perturbations emerging in individuals with genetic susceptibility (e.g., APOE ε4 carriers) even before biomarker positivity.

Together, these findings demonstrate that neuronal hyperactivation driven by soluble Aβ oligomers and glutamatergic dysregulation occurs well before Aβ plaque formation and clinical symptom onset, representing one of the earliest and potentially reversible hallmarks of AD. Within this early temporal window, neuronal hyperactivity may constitute a measurable functional signature of the AD continuum, preceding classical Aβ and tau abnormalities and reflecting an initial imbalance in excitatory-inhibitory dynamics. This stage likely occurs before the biomarker-defined pre-symptomatic ($A^{+}T^{-}[N]^{-}$) phase of the AT(N) framework. Detecting and therapeutically targeting this precocious imbalance could help preserve normal neuronal activity, protect synaptic integrity, and delay the onset of neurodegeneration.

1.4.3 Neuronal hypoactivity (NRHypo)

Following the initial phase of NRHyper, AD progression transitions toward a state of neuronal NRHypo, which is the second of the two-phase model of AD. This stage marks a functional decline characterized by reduced excitatory neurotransmission, widespread synaptic dysfunction, and progressive neurodegeneration. Mechanistically, NRHypo arises as a consequence of the chronic glutamatergic overstimulation occurring during the NRHyper phase, which leads to NMDAR desensitization, receptor downregulation, and cumulative excitotoxic injury (Cox et al., 2022; Findley et al., 2019; Olney, 1997). Persistent exposure to elevated extracellular Glu induces sustained Ca^{2+} influx, oxidative stress, and mitochondrial impairment, ultimately resulting in synaptic exhaustion and neuronal loss (E. R. Hascup et al., 2019; Talantova et al., 2013). Concurrent glial dysfunction further exacerbates hypoactivity, astrocytic atrophy and microglial activation impair Glu clearance, amplifying neurotoxicity and synaptic disconnection (Lovell et al., 2012; Zumkehr et al., 2015).

Within this framework, the NRHypo phase can associated to the neurodegenerative (N) component of the AT(N) model, reflecting the downstream outcome of sustained excitotoxic stress and impaired synaptic signalling (Jack et al., 2018, 2024). The reduction in network activity matches the onset of MCI and the progression to dementia, linking molecular and synaptic dysfunction with measurable cognitive decline. This stage is characterized by decreased hippocampal activation and reduced glucose metabolism, reflecting the underlying loss of synaptic integrity (Ali Zarad et al., 2025; Bouter et al., 2025; Busche & Hyman, 2020; Cox et al., 2022; DeTure & Dickson, 2019; Findley et al., 2019; Frisoni et al., 2010; B. Huang et al., 2024; Suchy-Dicey et al., 2023; Shivamurthy et al., 2015). This hypoactivation coincides with Aβ- and tau-PET positivity, reduced $A\beta_{42}/A\beta_{40}$ ratios and elevated t-tau and p-tau levels in CSF, indicating that the NRHypo phase temporally aligns with the fully established biochemical stage of AD (Lagarde et al., 2021; Leuzy et al., 2021; Mattsson-Carlgren et al., 2022; Ossenkoppele et al., 2025; Zetterberg et al., 2016). In line with this molecular evidence, functional imaging studies further clarify how Aβ and tau exert opposite effects on brain network activity. It was shown that in cognitively normal older adults, Aβ accumulation is associated with increased functional connectivity, whereas tau correlates with reduced connectivity in overlapping regions (Sepulcre et al., 2017). These findings suggest that Aβ-driven hyperactivity and tau-related hypoactivity may coexist during early disease stages, marking the gradual shift from compensatory to degenerative network dysfunction (Gallego-Rudolf et al., 2024).

The NRHypo phase therefore represents both a marker of advanced functional decline and the culmination of hyperexcitability-driven damage. Understanding the temporal transition between the NRHyper and NRHypo phases could be essential for developing stage-specific therapeutic strategies to slow the progression from reversible synaptic dysfunction to irreversible network collapse.

1.5 Molecular mediators of the two-phase model

1.5.1 Structure and toxic nature of Aβ oligomers

The two-phase model of AD progression can be mechanistically framed through the interaction of three principal molecular players, Aβ oligomers, Glu, and NMDARs, which together orchestrate the occurrence of the NRHyper phase and then the transition from NRHyper to NRHypo. Soluble Aβ oligomers disrupt synaptic homeostasis by altering glutamatergic signalling, inducing aberrant receptor activation and inhibiting Glu reuptake (Abdul et al., 2009; Aizenstein & Klunk, 2015; Findley et al., 2019; Huijbers et al., 2015; Olney, 1997; Talantova et al., 2013). This interference compromises the delicate excitatory-inhibitory balance within neural circuits, promoting sustained Glu release and excessive NMDAR stimulation (Acosta et al., 2017; Bassett et al., 2006; Bookheimer et al., 2000; Cox et al., 2022; E. R. Hascup et al., 2019; K. N. Hascup et al., 2020; K. N. Hascup & Hascup, 2016; Mondadori et al., 2006). The resulting excitotoxic cascade triggers Ca^{2+} dyshomeostasis, oxidative stress, and synaptic dysfunction, setting the foundation for the early hyperactive state that ultimately evolves into chronic synaptic depression and neuronal loss (Balez et al., 2016; J. Li et al., 2024; Lin et al., 2018; Nuriel et al., 2017; C. G. Parsons et al., 2007; Targa Dias Anastacio et al., 2022). Recent neuroimaging evidence further supports this interplay, showing that subtle Aβ-associated synaptic stress correlates with regional hyperactivation in cognitively normal individuals, especially in areas vulnerable to AD pathology (Corriveau-Lecavalier et al., 2024; Fischer et al., 2025). Understanding how these three interconnected components, Aβ oligomers, Glu, and NMDARs, contribute to this biphasic progression is crucial for identifying early therapeutic targets capable of restoring network stability and preventing irreversible neurodegeneration (Busche & Hyman, 2020; Cox et al., 2022; Findley et al., 2019).

Using techniques such as transmission electron microscopy (TEM), atomic force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR), the structures of Aβ oligomers, protofilaments, protofibrils, and fibrils were characterized. Most large oligomers are either globular or annular in shape, often forming ring-like structures (Janson et al., 1999; Lashuel et al., 2002). The β-sheet structure of both $A\beta_{40}$ and $A\beta_{42}$ oligomers are antiparallel, whereas Aβ protofilaments and fibrils possess parallel β-sheet arrangements. In addition, these oligomers show higher diffusibility, hydrophobicity, and ability to interact with membranes (Caughey & Raymond, 1993; Miura et al., 2007; Nguyen et al., 2014), which could explain how they cause toxicity. Additionally, other aggregates, such as annular protofibrils, are composed of multiple oligomers arranged in a ring-like structure that encloses water molecules. Notably, the structure of annular protofibrils differs from that of mature fibrils and protofilaments (Lashuel et al., 2002; Salahuddin et al., 2021). Protofibrils appear with an elongated and linear structure, without the higher-order structure and periodicity present in mature fibrils; in fact they appear curvilinear, thinner, and shorter than fibrils and protofilaments (Goldsbury et al., 2000; Petkova et al., 2002; Saiki et al., 2005; Walsh et al., 1999).

Aβ fibrils adopt a parallel arrangement and are composed of 2-8 protofilaments, each approximately 27 nm in diameter (Chiti & Dobson, 2017). These protofilaments often twist around one another to form a 7-13 nm supercoiled, rope-like structure (Chiti & Dobson, 2017; Sunde & Blake, 1997). They can also laterally associate to create long ribbon-like structures, measuring 2-7 nm in thickness and up to 30 nm in width (Bauer et al., 1995; Chiti & Dobson, 2017; Saiki et al., 2005). Typically, the core of most amyloid fibrils is dehydrated due to the presence of hydrophobic residues within the fibrils (Eisenberg & Sawaya, 2017).

According to the various models so far proposed, native proteins that undergo conformational changes into misfolded monomers can, upon reaching a critical concentration, form oligomers that assemble into early oligomers and eventually mature fibrils (**Fig. 1.3**) through primary nucleation. Some oligomers, however, do not proceed to fibril formation, as in the case of off-pathway oligomers. Mature fibrils can also fragment to produce shorter fibrils, which can re-aggregate into additional mature fibrils (**Fig. 1.3**). Furthermore, through secondary nucleation (**Fig. 1.3**), pre-existing mature fibrils catalyze the generation of diffusible nuclei, resulting in a significant increase in the levels of toxic oligomers (Arosio et al., 2015; Chiti & Dobson, 2017; Knowles et al., 2009, 2014; M. Verma et al., 2015). Secondary nucleation processes amplify oligomers with distinct biophysical properties that may preferentially target synapses and microvascular structures, linking fibril dynamics to early synaptic and vascular dysfunction in AD (Taddei & E. Duff, 2025; Tang et al., 2025).

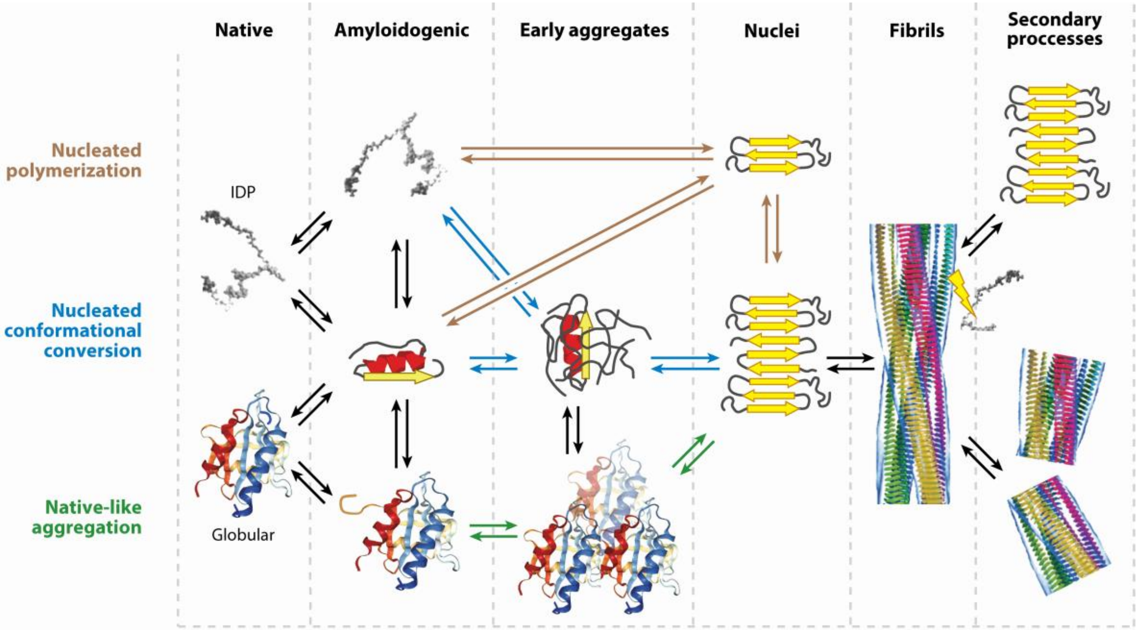

Figure 1.3. Schematic illustration of protein aggregation in amyloid disorders. Figure taken and modified from Chiti and Dobson 2017.

In recent years, increasing evidence has supported the idea that soluble Aβ oligomers, rather than fibrils, are the primary neurotoxic species driving AD (Benilova et al., 2012; Cheng et al., 2007; Chiti & Dobson, 2017; Diociaiuti et al., 2021; Gonzalez-Garcia et al., 2021; Siddiqi et al., 2019; Tolar et al., 2021; M. Verma et al., 2015; Walsh et al., 2002; Wells et al., 2021; J. Yang et al., 2020), in fact, experiments have shown that oligomers are toxic entities both *in vivo* (Ono et al., 2009) and *in vitro* (Bitan et al., 2003), and that learning, and memory deficits caused by oligomers in transgenic mouse models can be reduced when oligomer levels are decreased by accelerating fibril formation (Cheng et al., 2007). Moreover, studies have shown that AD animal models without brain Aβ plaques, but still containing oligomers, exhibited disease symptoms (Meilandt et al., 2009). Interestingly, even when levels of the Aβ-degrading enzyme neprilysin, which is a zinc metalloprotease, were increased, oligomer levels and the severity of memory impairments remained unchanged, despite neprilysin effectively reducing overall Aβ levels, Aβ monomers, and plaque burdens (Meilandt et al., 2009). Moreover, in APP transgenic mice, plaques in situ were found to be surrounded by a ‘penumbra’ of soluble Aβ oligomers, where the number of synapses was reduced, while synapse density increased at the margin of the plaque core (Koffie et al., 2009). Another study found that post-mortem brain tissue from clinically normal subjects had a much lower oligomer-to-plaque ratio compared to mildly demented subjects (Esparza et al., 2013). Based on these findings, authors have hypothesized that plaques may sequester soluble oligomers up to a physical limit, beyond which excess Aβ oligomers can diffuse, interact with, and damage surrounding synapses and cell membranes (S. Hong et al., 2014; Selkoe & Hardy, 2016). Furthermore, oligomers have been shown to induce microglial activation and neuroinflammatory responses, linking synaptic dysfunction to broader neuroimmune mechanisms (Tolar et al., 2021), and have been implicated in cerebrovascular dysfunction, inducing pericyte-mediated endothelial damage and contributing to AD-related microvasculopathy (S. Chen, Guo, et al., 2024; Morrone et al., 2020; Steinman et al., 2021).

A few studies have suggested that the toxicity of oligomers may result from their structural flexibility and exposed hydrophobic regions (Campioni et al., 2010; Capitini et al., 2018; Chiti & Dobson, 2017), which enable them to interact with membrane proteins and lipids, causing oxidative stress and altering membrane electrical properties and ion permeability (Benilova et al., 2012; Evangelisti et al., 2016; Tang et al., 2025). It has been found that Aβ oligomers induce an influx of Ca^{2+} across the cell membrane by activating cell-surface receptors such as NMDARs (Fani et al., 2021; Snyder et al., 2005), which are Glu-gated ion channels involved in synaptic plasticity and memory consolidation, and postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Fani et al., 2021; Yamin, 2009), as well as through nonspecific lateral destabilization of the lipid bilayer, leading to elevate intracellular Ca^{2+} and ROS levels (Fani et al., 2021, 2022; Fani & Chiti, 2022). Hyperstimulation of NMDARs by Aβ oligomers were shown to induce an excessive production the nitric oxide (NO) inducing oxidative stress causing a damage of neuronal synapsis (Wells et al., 2021).

Furthermore, the size of Aβ aggregates plays a crucial role in determining their neurotoxicity (Benilova et al., 2012). Smaller and soluble oligomeric species are often more toxic than larger fibrillar aggregates, as they can more readily interact with neuronal membranes and synaptic receptors, including NMDARs, owing to their higher diffusibility and structural flexibility. In contrast, mature Aβ fibrils, although more abundant at advanced stages of the disease, are generally considered less harmful at the synaptic level (Benilova et al., 2012; Chiti & Dobson, 2017). Therefore, the size-dependent toxicity of Aβ aggregates suggests that at early-stage, small oligomers are thought to contribute to synaptic dysfunction, Ca^{2+} dyshomeostasis, oxidative stress, and neuronal damage (Evangelisti et al., 2016).

Other studies proposed the formation of Aβ ion channel or pores in the membrane determining ion deregulation and the onset of the ND (Arispe, 2004; Ciudad et al., 2020; Darling & Shorter, 2020; Quist et al., 2005). Furthermore, Aβ oligomers were also found to interact with the metabotropic Glu receptor 5 (Benilova et al., 2012; Chiti & Dobson, 2017), the nicotinic acetylcholine receptor α7-nAcChR (Dineley et al., 2001) and with the insulin receptor (Giuffrida et al., 2010).

Increasing evidence suggests that the interaction of misfolded protein oligomers with cell membranes is a primary event causing cytotoxicity (Evangelisti et al., 2012). Furthermore oligomer-mediate cytotoxicity does not depend simply on the type, structure, size and physicochemical properties of the protein aggregate themselves, but also on the chemical composition and physiochemical features of the cell membrane with which the oligomers interact (Evangelisti et al., 2012, 2016). Supporting the hypothesis that lipid membrane composition plays a crucial role in oligomer toxicity, it has been shown that altering the levels of cholesterol and monosialotetrahexosylganglioside 1 (GM1) in the plasma membrane of SH-SY5Y cells changes the cytotoxicity of $A\beta_{42}$ oligomers (Evangelisti et al., 2012). Specifically, it has been reported that an increase of cholesterol content, obtained by incubating cells cultures with different concentrations of soluble cholesterol, determined a decrease of Aβ_{42} oligomers toxicity, whereas an increase of the toxicity was obtained by reducing cholesterol treating cells with β-CD or Mevastatine. Conversely, GM1 had the opposite effect: increasing GM1 content in the plasma membrane enhanced cytotoxicity, whereas reducing it decreased the toxic effects of Aβ_{42} oligomers (Evangelisti et al., 2012). Recent lipidomic analyses have highlighted that alterations in phospholipids, gangliosides, and cholesterol levels in the AD brain may create a permissive environment for Aβ oligomer-membrane interactions, amplifying synaptic and cellular damage (S. He et al., 2025).

1.5.2 Glutamate (Glu): physiological and pathological role

Glu is the primary and major fast excitatory neurotransmitter in the mammalian CNS, especially in cortical and hippocampal regions, playing a pivotal role in synaptic transmission, plasticity, learning, and memory (A. Rahn et al., 2012; Y. Zhou & Danbolt, 2014). Approximately 80-90% of all excitatory synapses in the CNS utilize Glu as a neurotransmitter (Mutluay & Karataş, 2022). Beyond its classical neurotransmitter role, it also exerts critical trophic and autocrine functions in non-neuronal cells, influencing neuronal metabolism, gene expression, and neurodevelopmental processes (Teng et al., 2023). Dysregulation of these pathways in the mature brain could therefore contribute to maladaptive plasticity and neurodegenerative processes, as observed in AD and other Glu-mediated neuropathologies or neuropsychiatric disorders (Egerton et al., 2020; Gupta et al., 2013; Teng et al., 2023; W. Zhang et al., 2022).

Neurons store Glu in synaptic vesicles via VGLUT1-3, which ensure its rapid release upon membrane depolarization, while neurons themselves, astrocytes and glial cells maintain extracellular homeostasis by efficiently removing Glu from the synaptic cleft (Borghans et al., 2025; Eriksen et al., 2020; Sidoryk-Węgrzynowicz et al., 2024). Glutamatergic transmission occurs within the so-called tripartite synapse, involving the presynaptic neuron, the postsynaptic neuron, and surrounding astrocytic processes that dynamically regulate extracellular Glu concentration and receptor activity. Within this tripartite organization, astrocytes play an active role in shaping synaptic plasticity, not only through Glu uptake via EAATs, but also through the release of gliotransmitters and modulation of metabotropic Glu receptors (mGluRs). EAAT1 and EAAT2 are responsible for clearing the majority of synaptic Glu, thereby preventing excitotoxicity and maintaining low micromolar extracellular concentrations (Cheong & Lee, 2025; Ortega et al., 2025; Parpura & Zorec, 2010).

Upon neuronal activation, Glu is released into the synaptic space, where it binds to ionotropic Glu receptors (iGluRs), including NMDARs, AMPARs, and kainate receptors (KARs), and metabotropic Glu receptors (mGluRs), initiating postsynaptic signalling cascades (Crupi et al., 2019; Ferraguti & Shigemoto, 2006; Hansen et al., 2021; Reiner & Levitz, 2018; Traynelis et al., 2010). NMDARs are particularly critical for Ca^{2+} influx, triggering downstream processes involved in synaptic plasticity, LTP, and memory formation. AMPARs mediate fast excitatory transmission, while KARs contribute to slower excitatory currents and modulate both pre- and postsynaptic activity, thereby fine-tuning synaptic strength and excitability (Alberdi et al., 2002; Danysz & Parsons, 2012; Furukawa et al., 2005; Gan et al., 2015; Negrete-Díaz et al., 2022; R. Wang & Reddy, 2017). mGluRs, in contrast, regulate neuronal excitability and neurotransmitter release through G-protein-coupled pathways. The precise spatiotemporal coordination of glutamatergic signalling is essential for maintaining network stability and preventing hyperexcitability, with dysregulation leading to excitotoxic neuronal injury (Crupi et al., 2019; Ferraguti & Shigemoto, 2006; Hansen et al., 2021; Reiner & Levitz, 2018; Traynelis et al., 2010).

Given the multiplicity of biological systems and processes in which Glu is involved, its concentration in the mammalian CNS is highly compartmentalized and tightly regulated. Intracellular neuronal Glu in the brain, including cortex and hippocampus, typically ranges from 5-15 mM (Krzyżanowska et al., 2014; Pál, 2018; Y. Zhou & Danbolt, 2014). Within synaptic vesicles in the hippocampus and cortex, Glu is highly concentrated, generally 60-100 mM, ensuring rapid and efficient synaptic release (Bai & Zhou, 2017; Cooper & Jeitner, 2016; Maio, 2019; Scimemi & Beato, 2009). Extracellular concentrations measured electrophysiologically in rodents are in the nanomolar range (~25-32 nM) in the Nucleus Accumbens and hippocampus (Chiu & Jahr, 2017; Herman & Jahr, 2007), whereas microdialysis studies of the extracellular space in hippocampus, nucleus accumbens, and prefrontal cortex, report concentrations in the low micromolar range (~0.02-20 µM) (Moussawi et al., 2011; Pál, 2018). Glial uptake and the activity of EAATs maintain tonic extracellular Glu levels in the range of approximately 0.18-2.12 µM, while localized microdomains near synapses or glial processes can reach 4-45 µM, reflecting spatial heterogeneity in Glu clearance and uptake

(Krzyżanowska et al., 2014; Murphy-Royal et al., 2017; Pál, 2018; Sheldon & Robinson, 2007). Transient synaptic peaks of extracellular Glu during vesicular release can reach the millimolar range, as shown by optical sensors like iGluSnFR (a fluorescent Glu indicator), highlighting the steep concentration gradients between vesicles, cytosol, synaptic cleft, and ambient extracellular space (Aggarwal et al., 2023; Herman & Jahr, 2007). In humans, plasma Glu concentrations are significantly higher, ranging from ~38.8 μM in healthy controls to 49.7-58.7 μM in patients with episodic or chronic migraine (C. G. Park & Chu, 2022).

Impairments in EAAT function, whether due to oxidative stress, aging, or Aβ pathology, result in elevated extracellular Glu, leading to overactivation of NMDARs, Ca^{2+} dysregulation, mitochondrial dysfunction, and increased production of ROS (Coxon et al., 2010; Findley et al., 2019; Wu et al., 2025). In AD, EAAT2/GLT-1 is consistently downregulated, correlating with amyloid burden and cognitive deficits. EAAT2 is particularly sensitive to oxidative modifications and inflammatory cytokines, making glial Glu uptake vulnerable and further compromising extracellular homeostasis (Fontana et al., 2023; Lauderback et al., 2001; Takahashi et al., 2015; Wood et al., 2022). Soluble Aβ oligomers exacerbate this dysregulation by impairing Glu uptake in the neurons and astrocytes, stimulating excessive release by the astrocytes, and enhancing NMDAR-mediated Ca^{2+} influx, ultimately causing synaptic toxicity, dendritic spine loss, and neuronal death (Findley et al., 2019; Lei et al., 2016; Takahashi et al., 2015; H. Zhang et al., 2022).

Glu spillover from synaptic clefts can activate extrasynaptic NMDARs, often containing GluN2B subunits, which preferentially trigger cell death pathways. The activation of these receptors initiates neurodegenerative cascades distinct from those mediated by synaptic NMDARs, tipping the balance toward neuronal death rather than survival (Bordji et al., 2010; Chalifoux & Carter, 2011; Yu et al., 2023). Dysregulation of synaptic versus extrasynaptic Glu signalling has been proposed as a unifying mechanism in several neurodegenerative and neuropsychiatric disorders, including AD, Parkinson's Disease (PD), and schizophrenia (Capó et al., 2025; Hardingham & Bading, 2010). Furthermore, elevated extracellular Glu can promote microglial activation and pro-inflammatory signalling, establishing a deleterious feedback loop that amplifies neuronal injury (Haroon et al., 2017; Lull & Block, 2010; Y. Zhang et al., 2022).

1.5.3 NMDA receptor (NMDARs) subunits and function

NMDARs are Glu-gated ion channels critical for synaptic plasticity, learning, and memory, and have also been implicated in neurodegeneration and excitotoxicity (Capó et al., 2025; Escamilla et al., 2024; Geoffroy et al., 2022; Hansen et al., 2018; J. Liu et al., 2019; Yu et al., 2023; K. Zhang et al., 2025; Y. Zhang et al., 2016). Their most remarkable feature is their ability to function as highly refined coincidence detectors. In fact, Glu binding alone is insufficient to open the channel of synaptic NMDARs unless the postsynaptic membrane is sufficiently depolarized, typically via AMPAR-mediated currents, to relieve the voltage-dependent Mg^{2+} block (Di Maio et al., 2016; Hansen et al., 2018; Mayer et al., 1984). This gating requirement ensures that physiologically relevant Ca^{2+} influx occurs only during meaningful patterns of synaptic activity, supporting LTP, long-term depression (LTD), synaptogenesis, and activity-dependent refinement of neural circuits (Luscher & Malenka, 2012; K. Zhang et al., 2025). However, their high Ca^{2+} permeability also makes NMDARs potent mediators of excitotoxicity when dysregulated, particularly extrasynaptic NMDARs (Capó et al., 2025; Escamilla et al., 2024; J. Liu et al., 2019; R. Wang & Reddy, 2017; Wu et al., 2025; Yu et al., 2023; K. Zhang et al., 2025). The delicate balance between physiological plasticity and pathological degeneration relies on receptor subunit composition, developmental regulation, structural organization, spatial localization, intracellular coupling, and dynamic trafficking, all of which are profoundly disrupted in AD (Danysz & Parsons, 2012; Hardingham & Bading, 2010; Sheng et al., 2012).

Structurally, NMDARs are tetramers composed of two obligatory GluN1 subunits and two GluN2 (A-D) or GluN3 (A-B) subunits (Capó et al., 2025; Escamilla et al., 2024; Geoffroy et al., 2022; Hansen et al., 2018; J. Liu et al., 2019; Yu et al., 2023; Y. Zhang et al., 2016; S. Zhu & Paoletti, 2015). While canonical NMDARs are diheteromers (GluN1/GluN2), recent evidence indicates that most native NMDARs are triheteromers, containing two GluN1 subunits paired with a combination of GluN2 and GluN3 subunits (Capó et al., 2025; Hansen et al., 2018; J. Liu et al., 2019; Yu et al., 2023). Triheteromeric NMDARs display intermediate sensitivity to Glu and Gly, reflecting differences in channel conductance and gating kinetics imparted by subunit composition (Capó et al., 2025; Hansen et al., 2018; J. Liu et al., 2019; Yu et al., 2023). GluN3 subunits are distinguished by a positive charge in their pore-lining sequence, which reduces Ca^{2+} permeability, attenuates Mg^{2+} block at hyperpolarized potentials, and acts as a dominant-negative modulator in triheteromeric receptors (Chatterton et al., 2002; Kehoe et al., 2013; Pachernegg et al., 2012; Xiong et al., 2025; Yu et al., 2023). GluN1/GluN3 diheteromers can form glycine-activated receptors essentially insensitive to Glu, highlighting the functional diversity imparted by GluN3 (Chatterton et al., 2002; Grand et al., 2018; Hemelikova et al., 2019; Kehoe et al., 2013; Osorio et al., 2023; Otsu et al., 2019; Xiong et al., 2025; K. Zhang et al., 2025).

Each subunit contains four semi-autonomous domains that collectively orchestrate receptor function: the amino-terminal domain (ATD), the ligand-binding domain (LBD), the transmembrane domain (TMD), and the intracellular carboxyl-terminal domain (CTD) (Bender et al., 2024; Hansen et al., 2018; J. Liu et al., 2019; Y. Zhang et al., 2016) (**Fig. 1.4**). The extracellular ATD regulates subunit assembly, receptor clustering, and allosteric modulation by endogenous and pharmacological agents, including Zn^{2+}, polyamines, and protons. Physiological extracellular pH (~7.3-7.4) tonically inhibits NMDARs, and small decreases further enhance this inhibition; Zn^{2+} binds to produce potent allosteric inhibition, and polyamines such as spermine and spermidine enhance gating of GluN2B-containing receptors mediating positive modulation (Capó et al., 2025; Geoffroy et al., 2022; Hansen et al., 2018; Kehoe et al., 2013; J. Liu et al., 2019; Pachernegg et al., 2012; Xiong et al., 2025; Yu et al., 2023; K. Zhang et al., 2025; Y. Zhang et al., 2016; S. Zhu & Paoletti, 2015). Furthermore, redox-sensitive extracellular cysteine pairs form reversible disulfide bonds that shift receptor conformation and modulate channel open probability in response to the synaptic oxidative environment (Talukder et al., 2011). The LBD, formed by S1 and S2 lobes, binds Glu (GluN2) or Gly/D-serine (D-Ser) (GluN1), inducing the conformational rearrangements required to open the pore (Capó et al., 2025). The TMD contains M1, M3, M4, and the re-entrant M2 loop, which forms the channel's selectivity filter, determines divalent cation permeability, and mediates voltage-dependent Mg^{2+} block (Geoffroy et al., 2022; Kehoe et al., 2013; J. Liu et al., 2019; Y. Zhang et al., 2016). Finally, the intracellular CTD is a large, intrinsically disordered protein-interaction hub that binds scaffolding proteins (PSD-95, SAP102, GKAP, Shank), signalling enzymes (CaMKII, PKC, Src family kinases, ERK modules, nNOS), trafficking regulators, and components of apoptotic pathways (Capó et al., 2025). These CTD interactions are key determinants of whether NMDAR activation engages pro-survival or pro-death cascades (Cui et al., 2007; Fan et al., 2014; Shu et al., 2014; Hardingham et al., 2002; Escamilla et al., 2024; Hansen et al., 2018; Geoffroy et al., 2022; Kehoe et al., 2013). Both Glu binding to the GluN2 subunit and Gly/D-Ser binding to GluN1 are strictly required for NMDAR activation, as neither agonist alone is sufficient to gate the channel. At resting membrane potentials, a voltage-dependent Mg^{2+} block occludes the pore and prevents ion flow, ensuring that NMDARs conduct Ca^{2+} only when presynaptic Glu release coincides with postsynaptic depolarization (Hansen et al., 2018; Paoletti et al., 2013; Traynelis et al., 2010).

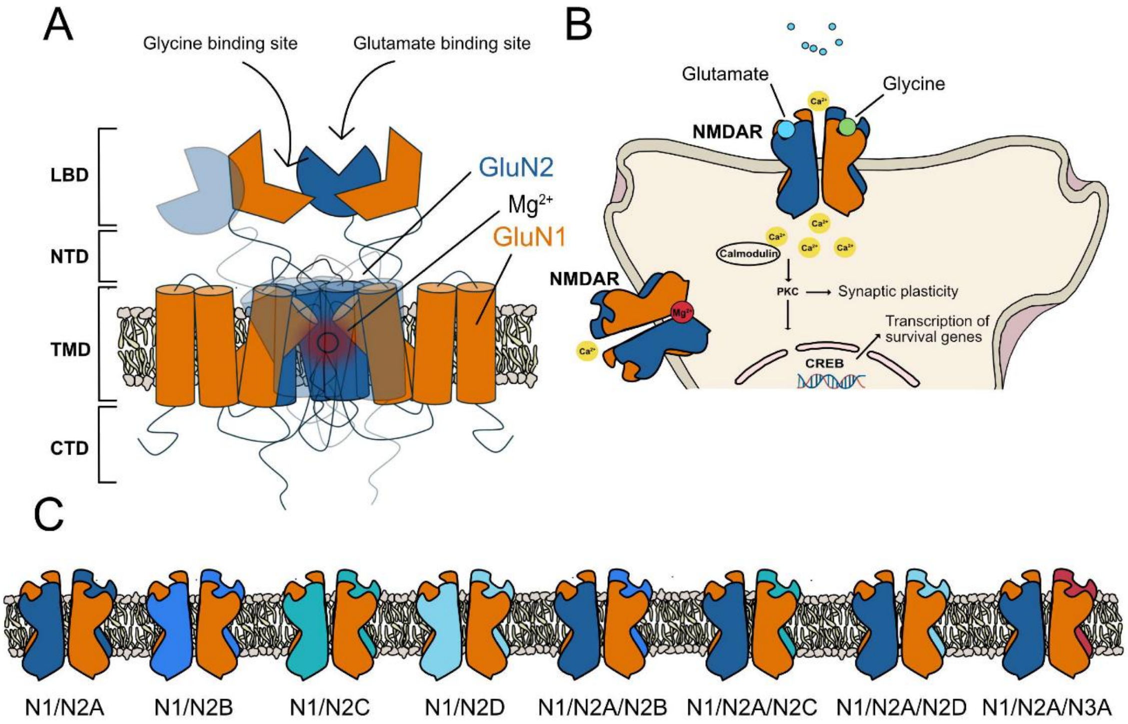

Figure 1.4. NMDAR structure, function, and subunit diversity. (A) Structure of the NMDAR, showing the ligand-binding domain (LBD) and the N-terminal domain (NTD), where several allosteric modulators bind, altering the NMDAR functionality, the transmembrane domain (TMD), and the C-terminal domain (CTD). **(B)** The activation of the NMDAR has multiple physiological effects, as this receptor plays a key role in neurotransmission, synaptic plasticity, and neuronal survival. **(C)** NMDARs are tetrameric receptors formed by the combination of different subunits: the GluN1 subunit, four different GluN2 subunits (GluN2A, GluN2B, GluN2C, and GluN2D), and two GluN3 subunits (GluN3A and GluN3B), all of them encoded by different genes. Figure taken from Capó et al. 2025.

Spatiotemporal changes in NMDAR subunit expression critically regulate synaptic maturation and circuit function by shaping receptor signalling during development (Paoletti et al., 2013; S. Zhu & Paoletti, 2015). GluN1 is expressed from prenatal stages and maintained into adulthood (Henson et al., 2008). During early postnatal development, GluN2B predominates in cortical and hippocampal synapses, supporting enhanced plasticity, whereas maturation is marked by a progressive increase in GluN2A expression, resulting in the canonical GluN2B-to-GluN2A switch that refines receptor kinetics and stabilizes adult synaptic plasticity (Kelsch et al., 2014; Sanz-

Clemente et al., 2013; Sinclair et al., 2016). Other NMDAR subunits show more restricted expression patterns: GluN2C and GluN2D are enriched in specific neuronal and glial populations, while GluN3 subunits modulate synaptic maturation in a development-dependent manner, with GluN3A acting as a negative regulator early in life and GluN3B persisting in select motor circuits into adulthood (González-González et al., 2023; Kehoe et al., 2014; Nishi et al., 2001). The coordinated expression of NMDAR subunits establishes the biophysical and signalling properties necessary for synaptic plasticity, dendritic maturation, and circuit stability (Baez et al., 2018; Gambrill & Barria, 2011; Keith et al., 2024). Disruption of these developmental trajectories can have profound pathological consequences (Baudouin et al., 2012; Beaurain et al., 2024; Tumdam et al., 2024; Vieira et al., 2020). Interestingly, recent findings show that Aβ oligomers have been shown to preferentially bind GluN2B-containing NMDARs and mGluR1, forming aberrant receptor complexes that alter downstream signalling and promote synaptic dysfunction (Taniguchi et al., 2022). Moreover, the interaction of Aβ oligomer with the tyrosine kinase EphB2, which normally stabilizes GluN2B receptors at the synaptic membrane, appears to contribute to their depletion: increased EphB2 expression in murine AD models restores GluN2B receptor localization and function and improves memory (R. Hu et al., 2017). Finally, studies in late-onset AD patients have identified a rare variant in the GRIN2C gene (Rubino et al., 2025). Collectively, these data indicate that the balance of NMDAR subunits, both genetically determined and modulated by toxic factors such as Aβ, represents a critical node of synaptic instability in neurological disorders, spanning from neurodevelopmental conditions to neurodegeneration.

The precise subcellular localization of NMDARs, at synaptic, perisynaptic, extrasynaptic, or presynaptic sites, shapes the downstream signalling pathways they engage, from pro-survival and plasticity-promoting cascades to excitotoxic mechanisms (Escamilla et al., 2024; Geoffroy et al., 2022; Grand et al., 2018; Osorio et al., 2023; Paoletti et al., 2013; Yu et al., 2023; S. Zhu & Paoletti, 2015).

High Ca^{2+} permeability, long open times, slow deactivation, and agonist/co-agonist cooperativity are key biophysical properties of NMDARs, fundamentally determined by their subunits. GluN2A-containing receptors exhibit relatively rapid deactivation and higher open probability, whereas GluN2B-containing receptors show slower deactivation and lower open probability (Erreger et al., 2005; Paoletti et al., 2013; Vicini et al., 1998). NMDARs incorporating GluN2C or GluN2D exhibit low open probability, reduced single-channel conductance, and minimal Mg^{2+}-dependent block, allowing activation at relatively hyperpolarized potentials, features well suited to cerebellar and thalamic computation (Hansen et al., 2018; Swanger et al., 2015). A further regulatory layer arises from GluN3 subunits. Incorporation of GluN3A or GluN3B generates atypical receptors with reduced Ca^{2+} permeability, attenuated Mg^{2+} sensitivity, and distinct gating properties (Chatterton et al., 2002; Grand et al., 2018; Kehoe et al., 2013; Xiong et al., 2025).

In terms of subcellular localization, GluN2A receptors are predominantly synaptic, whereas GluN2B, GluN2C/D, and GluN3-containing receptors are largely extrasynaptic, with minor synaptic fractions for GluN2C/D and transient synaptic presence of GluN3A during early postnatal development (Escamilla et al., 2024; Paoletti et al., 2013). This differential distribution is reflected in the distinct functions of presynaptic, postsynaptic, and extrasynaptic NMDARs, which mediate pro-survival or pro-death signalling depending on their compartmental localization (Shu et al., 2014; Yu et al., 2023; K. Zhang et al., 2025).

Excitotoxicity, first described by John Olney (Olney, 1969, 1971, 1997; Olney et al., 1972; Olney & Ho, 1970; Olney & Sharpe, 1969), is a pathological process in which excessive activation of Glu receptors, particularly extrasynaptic NMDARs, drives neuronal dysfunction and death through sustained intracellular Ca^{2+} influx and aberrant downstream Ca^{2+} signalling. Initially identified in acute neuronal injury, excitotoxicity is now recognized as a central mechanism in both rapid-onset and slowly progressive neurodegenerative conditions, including ischemic stroke and AD (Wu et al., 2025; Yu et al., 2023). As already described in the previous section, synaptic NMDAR activation supports neuronal survival, synaptic plasticity, and metabolic homeostasis under physiological conditions. In contrast, when extracellular Glu accumulates, due to impaired clearance, excessive release, or synaptic spillover, NMDARs become chronically overstimulated, leading to disrupted Ca^{2+} homeostasis and a shift from pro-survival to pathological signalling pathways (Hardingham & Bading, 2010; Wu et al., 2025; Yu et al., 2023).

A major advance in the understanding of excitotoxicity is the spatial and functional segregation of synaptic and extrasynaptic NMDARs (Hardingham & Bading, 2010; Pál, 2018). Synaptic NMDARs, predominantly containing GluN2A subunits, generate transient Ca^{2+} signals that activate pro-survival pathways, including CaMKII, Ras-ERK, and PI3K-Akt, leading to CREB phosphorylation, BDNF expression, and the stabilization of dendritic spines and LTP, thereby reinforcing neuronal resilience (Corlew et al., 2007; Franchini et al., 2020; Yu et al., 2023). In contrast, extrasynaptic NMDARs enriched in GluN2B preferentially couple to neurotoxic signalling cascades (Hardingham et al., 2002; Rischka et al., 2022). Ca^{2+} influx through these receptors suppresses CREB activity via phosphatase activation, reduces neurotrophic support, and compromises synaptic maintenance (Sala et al., 2000; H. Wang et al., 2018). Concurrently, extrasynaptic NMDAR activation engages calpains, caspases, and stress-activated kinases such as p38 MAPK, driving cytoskeletal breakdown, mitochondrial dysfunction, and apoptotic gene programs (M. P. Parsons & Raymond, 2014; Talantova et al., 2013). These processes are further amplified by Ca^{2+}-dependent activation of nNOS and NADPH oxidases, leading to excessive ROS/RNS production, lipid peroxidation, impaired Glu uptake, and sustained extrasynaptic receptor activation (Brandes et al., 2014; Maccallini & Amoroso, 2016). Mitochondrial Ca^{2+} dysfunction, oxidative damage, and accumulation of toxic lipid peroxides, among other effects, ultimately promote irreversible neuronal loss through excitotoxic degeneration and ferroptosis, mechanisms of particular relevance in AD and chronic ischemia (Ayton et al., 2021; Dixon et al., 2012; Zhao et al., 2023).

In AD, excitotoxicity primarily arises from Aβ-driven glutamatergic dysregulation. Soluble Aβ oligomers, in equilibrium with monomeric Aβ, enhance extracellular Glu spillover, impair the function of astrocytic Glu transporters such as EAAT2/GLT-1, and promote the accumulation of GluN2B-containing NMDARs at extrasynaptic sites, thereby sustaining chronic excitotoxic signalling at extrasynaptic NMDARs and promoting Ca^{2+}-dependent synaptic dysfunction (Han et al., 2016; Takahashi et al., 2015; Taniguchi et al., 2022; Wood et al., 2022). The overall effect is a self-reinforcing cycle of excitotoxic neurodegeneration, driven by persistent extrasynaptic NMDAR activation, abnormal Ca^{2+} signalling, mitochondrial dysfunction, and oxidative/nitrosative stress. In this environment, ongoing lipid peroxidation and iron-dependent reactions increase neuronal vulnerability to cell death. This cascade ultimately leads to progressive dendritic spine loss, synaptic breakdown, and neuronal degeneration, as observed in AD. Understanding the distinction between harmful extrasynaptic NMDAR signalling and normal synaptic receptor activity is crucial for the development of targeted neuroprotective therapies.

1.6 NADPH Oxidases (NOXs) in Alzheimer's Disease (AD)

1.6.1 Sources of reactive oxygen species (ROS)

ROS are highly reactive molecules, including superoxide anion (O_2•⁻), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), singlet oxygen ($^1O_2$), NO•, and ONOO⁻ generated both endogenously and from environmental sources (Begum et al., 2022; De Almeida et al., 2022; Martemucci et al., 2022). Endogenously, ROS arise as byproducts of aerobic metabolism, particularly during mitochondrial oxidative phosphorylation. Mitochondria constitute the primary intracellular source of ROS, generating an estimated 90% of the total, as electron leakage from complexes I and III of the electron transport chain produces superoxide, which can then be converted into other ROS species (Jomova et al., 2023; Tirichen et al., 2021). Additional intracellular sources include peroxisomes, where ROS are produced during β-oxidation of very long-chain fatty acids, the endoplasmic reticulum (ER), where ROS are generated during protein folding and Ca^{2+} dysregulation, and various oxidase enzymes such as xanthine oxidase, nitric oxide synthase (NOS, especially when uncoupled), cytochrome P450s, lipoxygenases (LOXs) and cyclooxygenases (COXs), NADH-cytochrome b5 reductase (c5br), dihydroorotate dehydrogenase (DHODH), succinate dehydrogenase (complex II), monoamine oxidases (MAO), xanthine oxidoreductase (XOR), urate oxidase (UO), and NADPH oxidases (NOXs), which generate ROS as their primary function (De Almeida et al., 2022; Di Meo et al., 2016; Egea et al., 2020). Even exogenous factors such as environmental pollution, cigarette smoke, radiation, certain foods, and drugs can also contribute to ROS generation, highlighting the dual origin of these reactive molecules. At moderate levels, ROS play essential physiological roles, including intracellular signalling, gene expression regulation, immune defence, apoptosis, autophagy, and interactions with the extracellular matrix (ECM) (Begum et al., 2022; Bhattacharyya et al., 2014; Jomova et al., 2023). However, excessive ROS accumulation leads to oxidative stress, causing damage to lipids, proteins, and nucleic acids, thereby contributing to aging and the development of neurodegenerative diseases (Chaudhary et al., 2023; Korovesis et al., 2023; Üremiş & Üremiş, 2025). ROS levels are tightly regulated by enzymatic and non-enzymatic antioxidant systems. Key enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, whereas non-enzymatic antioxidants include vitamins A, C, and E, flavonoids, and carotenoids, all of which convert ROS into stable, non-toxic molecules (Begum et al., 2022; De Almeida et al., 2022).

1.6.2 NADPH Oxidases (NOXs): structure and regulation

Among enzymatic sources, NOXs stand out as the only family of dedicated ROS-producing enzymes. Unlike mitochondria, peroxisomes or the ER, where ROS are largely metabolic byproducts, NOX isoforms are specifically designed to generate superoxide or hydrogen peroxide in a regulated manner. They participate in redox signalling, immune responses, and maintenance of cellular homeostasis. The discovery of NOX enzymes developed progressively over the twentieth century. The NOX family comprises seven isoforms involved in various essential human physiological functions, NOX1 through NOX5 and the dual oxidases DUOX1 and DUOX2 (Begum et al., 2022; Brandes et al., 2014; Vermot et al., 2021) (**Fig. 1.5**).

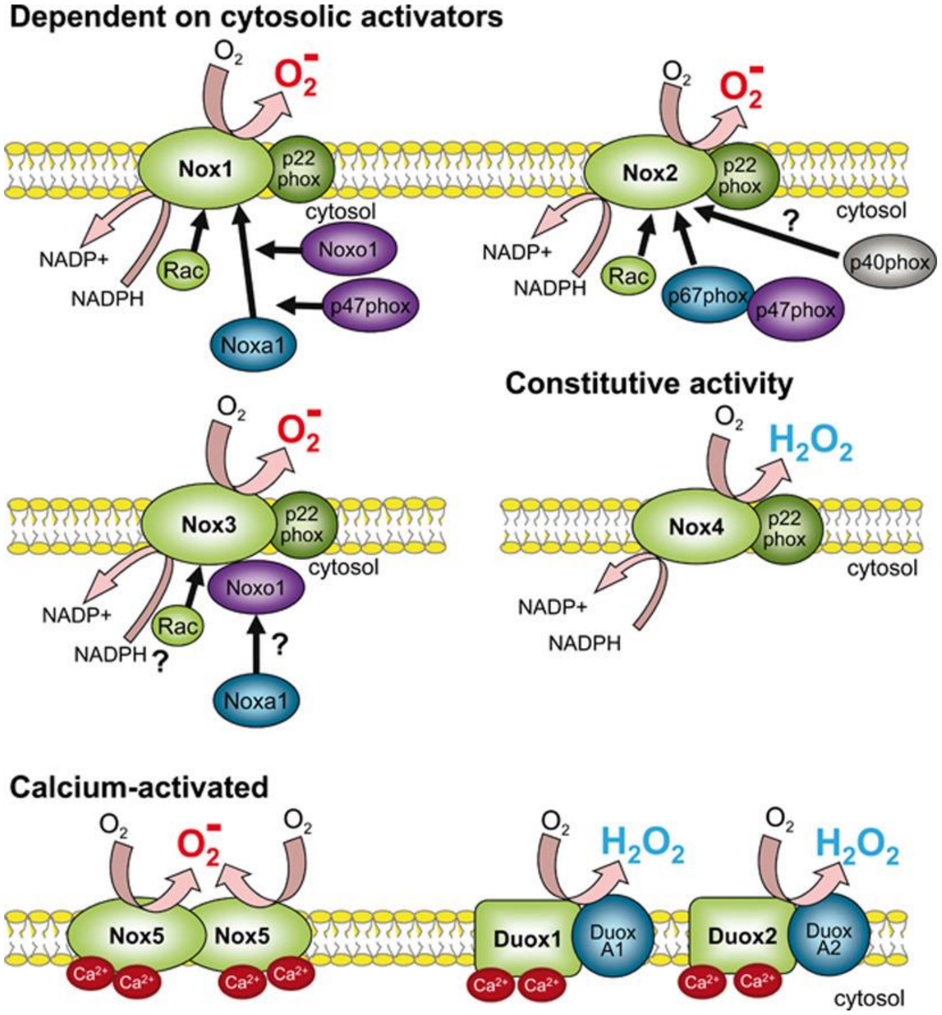

Figure 1.5. Composition of Nox enzyme complexes. Figure taken from Brandes et al. 2014.

Structurally, despite their functional diversity, all NOX isoforms retain a highly conserved catalytic, membrane bound subunit, named after its catalytic homolog, with isoform-specific subunits conferring unique regulatory and functional properties. This subunit consists of a transmembrane domain containing two b-type heme-binding regions and a cytosolic C-terminal dehydrogenase (DH) domain that binds NADPH, enabling electron transfer to molecular oxygen (O_2) and the generation of $O_2{\bullet}^-$, with FAD acting as an essential prostetic group coenzyme for the transfer (Vermot et al., 2021). The stoichiometry of the reaction is $2O_2 + NADPH \rightarrow 2\ O_2{\bullet}^- + NADP^+ + H^+$ (Wakeyama et al., 1982). The transmembrane subunit of all isoforms has six or seven transmembrane helices that form the scaffold for the heme groups and electron transfer. These superoxide radicals can subsequently dismutate to form hydrogen peroxide (H_2O_2), although certain isoforms, such as NOX4, DUOX1, and DUOX2, are thought to produce H_2O_2 directly, bypassing superoxide as an intermediate (Begum et al., 2022; Brandes et al., 2014; Vermot et al., 2021).

The classical NOX complex, best studied in neutrophils as NOX2, is organized into membrane and cytosolic compartments. At rest, the NOX complex is divided between the membrane and cytosolic compartments (Belambri et al., 2018; Nocella et al., 2023; Rastogi et al., 2017; Vermunt et al., 2019). The membrane portion forms the flavocytochrome b_{558}, composed of gp91^{phox}, which is the catalytic NOX2 subunit, and p22^{phox}. The following activation of the entire complex requires the translocation of cytosolic subunits, which are p47^{phox}, p67^{phox}, and p40^{phox}, as well as the involvement of the Rho-family GTPase Rac, which is essential for catalytic activation. Furthermore, Additional small GTPases, such as Rap1A, have been reported to modulate membrane assembly and fine-tune NOX2 activity in specific cellular contexts (H. E. Johnson et al., 2025). The cytosolic subunits are regulated by the phosphorylated form p47^{phox}, which acts as the main organizer by exposing binding regions that recruit p67^{phox} and p40^{phox}, forming a trimeric complex that translocate to the membrane. Within this complex, p47^{phox} undergoes phosphorylation-dependent activation that exposes SH3 domains enabling binding to the proline-rich region of p22^{phox}; p67^{phox} serves as the activator by interacting with the transmembrane subunit gp91^{phox} and Rac-GTP to enhance catalytic activity; while p40^{phox} helps stabilize the cytosolic complex and mediates membrane targeting through its PI3P-binding Phox homology (PX) domain. This hierarchical, multi-step activation allows precise regulation of NOX2 activity (Belambri et al., 2018; Nocella et al., 2023; Rastogi et al., 2017; Vermunt et al., 2019).

While NOX2 represents the canonical model, other NOX isoforms operate according to similar principles but with isoform-specific variations. NOX1 and NOX3 rely on the homologous subunits NADPH oxidase organizer 1 (NOXO1) and NADPH oxidase activator 1 (NOXA1) in place of $p47^{phox}$ and $p67^{phox}$. Notably, NOXO1 lacks the autoinhibitory region present in $p47^{phox}$ and does not require phosphorylation for translocation, enabling NOX1 to associate with the membrane constitutively and maintain a higher basal activity (Begum et al., 2022; Rastogi et al., 2017). NOX4, although forming a heterodimer with $p22^{phox}$, is constitutively active and does not require cytosolic subunits. Its activity is primarily regulated at the transcriptional level or through interacting partners such as Polymerase δ-Interacting Protein 2 (Poldip2), which enhances enzymatic activity and stabilizes ROS production (Lyle et al., 2009; Vukelic et al., 2018). NOX5, as well as DUOX1 and DUOX2, which also contain N-terminal peroxidase-like domains, bypass the need for $p22^{phox}$ and cytosolic organizer subunits, relying instead on N-terminal EF-hand Ca^{2+}-binding domains for rapid, Ca^{2+}-dependent activation (Begum et al., 2022; U. Ganguly et al., 2021; Vermot et al., 2021). Furthermore, DUOX1 and DUOX2 require the maturation factors DUOXA1 and DUOXA2 to ensure proper folding, trafficking to the plasma membrane, and acquisition of full enzymatic competence, thereby enabling efficient ROS production (Brandes et al., 2014; Díaz & Courtneidge, 2012; Grasberger & Refetoff, 2006; Luxen et al., 2009; Morand et al., 2009).

1.6.3 Physiological functions, tissue and cellular localization of NADPH Oxidases (NOXs)

The structural and functional diversity of NOX isoforms enables them to perform distinct roles in physiology and pathology. Each isoform exhibits its own pattern of tissue distribution, subcellular localization, and regulatory mechanisms. NOX1 is primarily expressed in the colonic epithelium and vascular smooth muscle, but its presence extends to endothelial cells, the uterus, placenta, osteoclasts, retinal pericytes, neurons, astrocytes, and microglia (Begum et al., 2022). It localizes mainly to the plasma membrane, where it supports extracellular or membrane-proximal ROS signalling, although some evidence suggests that a small fraction may be transiently internalized into endosomal compartments, with the predominant activity remaining at the plasma membrane (Miller et al., 2007). NOX2 predominates in phagocytes, including monocytes, macrophages, and neutrophils, and is also present in the brain (neurons and microglia), cardiovascular tissues (including vascular endothelial cells, smooth muscle cells, and cardiomyocytes), kidney, gastrointestinal tract, liver, and pancreas (Begum et al., 2022). Its distribution spans both the plasma membrane and intracellular vesicles, particularly endosomal and phagosomal compartments (Dingjan et al., 2017), enabling ROS production in extracellular and vesicular microenvironments. NOX3 is largely restricted to the inner ear, where its localization at the plasma membrane supports specialized redox signalling required for otoconia formation in the vestibular system; however, low expression is also detectable in the liver, lungs, spleen, and fetal kidney (Begum et al., 2022). NOX4 is abundantly expressed in the kidneys and in a wide range of cell types, including mesangial and smooth muscle cells, fibroblasts, osteoclasts, endothelial cells, neurons, and hepatocytes (Begum et al., 2022). Its subcellular distribution is broader than that of the other isoforms and includes the plasma membrane, ER, mitochondria, and nucleus; mitochondrial localization appears primarily associated with mitochondria-associated membranes (MAMs), supporting compartmentalized ROS signalling for migration, differentiation, and transcription (Beretta et al., 2020). NOX5 is expressed in the spleen, testis, heart, and endothelial cells (Begum et al., 2022) and localizes predominantly to the plasma membrane, with additional intracellular localization in the ER reported in some studies, consistent with its rapid Ca^{2+}-dependent activation via N-terminal EF-hand domains (Touyz et al., 2019). DUOX1 and DUOX2 are expressed in the thyroid, respiratory epithelium, gastrointestinal tract and brain (Begum et al., 2022; Damiano et al., 2012) and are targeted to the apical plasma membrane in epithelial cells where they produce H_2O_2 (Kohda et al., 2024).

Physiologically, NOX-derived ROS act as tightly regulated second messengers in redox-sensitive signalling pathways, modulating the activity of transcription factors, kinases, and phosphatases through reversible oxidative modifications. By doing so, specific NOX isoforms influence key cellular processes, including proliferation, differentiation, migration, and survival, and contribute to broader physiological functions such as innate immunity, cardiovascular homeostasis, neuronal signalling, tissue repair, and hormone biosynthesis (Bedard & Krause, 2007; U. Ganguly et al., 2021; Vermunt et al., 2019).

In host defence, NOX1 is expressed in the colon epithelium and may participate in mucosal defence (Rokutan et al., 2006; Szanto et al., 2005). NOX2 plays a central role in phagocytes, generating ROS during the respiratory burst to kill pathogens and mediate inflammatory signalling (Bedard & Krause, 2007; Winterbourn et al., 2016). NOX4 can interact with TLR4 and, under LPS stimulation, contribute to redox-dependent activation of NF-κB, highlighting a broader immunoregulatory role of NOX in innate immunity (H. Park et al., 2006). Meanwhile, DUOX1 and DUOX2 contribute to innate immunity by producing H_2O_2 at mucosal surfaces, which is used by lactoperoxidase to generate antimicrobial compounds in respiratory and gastrointestinal epithelia (Forteza et al., 2005; Geiszt et al., 2003; Grasberger et al., 2013; Sarr et al., 2018).

NOX-derived ROS contribute significantly to cardiovascular function. NOX1, NOX2, NOX4, and NOX5 are expressed in vascular endothelial cells, smooth muscle cells, and cardiomyocytes, where they regulate processes such as proliferation, migration, and angiogenesis in response to stimuli like VEGF, hypoxia, FGF-2, Ang1, or hypertrophic stress (Rajaram et al., 2019; Sirker et al., 2011). In particular, NOX4 in cardiomyocytes modulates mitochondrial function and mediates adaptive cardiac remodelling in response to pressure overload, preventing maladaptive hypertrophy and fibrosis (Ago et al., 2010; M. Zhang et al., 2010).

In the CNS, NOX enzymes support normal neuronal and glial function. NOX1, although less abundant, is expressed in brain endothelial cells and in neurons, and may participate in redox-mediated endothelial signalling (Chrissobolis & Faraci, 2008; Hernandes et al., 2022). NOX2, present in neurons, astrocytes and microglia, participates in synaptic plasticity, axonal guidance, and modulation of neurotransmitter receptor activity (Terzi et al., 2021; De Pasquale et al., 2014; Haslund-Vinding et al., 2017; Yi et al., 2018). NOX3, largely restricted to the inner ear, supports vestibular function and balance (Bedard & Krause, 2007; Herb, 2024; Paffenholz et al., 2004). NOX4 contributes to neuronal redox homeostasis: in neurons and glial cells, NOX4-derived H_2O_2 regulates Ca^{2+} signalling, and in some stem cell models supports neuronal differentiation (Gola et al., 2023; Tarafdar & Pula, 2018). Overall, NOX-derived ROS in the CNS act as essential redox messengers for neurodevelopment and neuronal communication (Bedard & Krause, 2007; J. Fang et al., 2021; Haslund-Vinding et al., 2017; Nayernia et al., 2014; Terzi & Suter, 2020).

At the cellular level, NOX-dependent ROS regulate signalling cascades by reversible oxidation of cysteine residues in phosphatases and kinases, influence MAPK/ERK, JNK, and p38 pathways, and modulate Ca^{2+} flux through plasma membrane channels, ER release channels, and Ca^{2+} pumps (Vermunt et al., 2019). ROS thus orchestrate cell growth, survival, and apoptosis in a spatially compartmentalized manner.

Moderate NOX activity contributes to redox homeostasis and the regulation of physiological senescence, supporting tissue repair and cellular resilience (Bedard & Krause, 2007). NOX-derived ROS activate redox-sensitive transcription factors and adaptive signalling pathways, promoting cell survival and longevity, whereas excessive or dysregulated ROS can accelerate premature senescence, causing oxidative damage, inflammation, and tissue dysfunction, highlighting NOX enzymes as key regulators of both normal cellular function and controlled senescence (Bedard & Krause, 2007; Vermot et al., 2021).

1.6.4 The role of NADPH Oxidases (NOXs) in Alzheimer's Disease (AD)

NOXs have gained increasing attention in recent years due to their proposed involvement in neurodegenerative diseases such as AD (J. Kim & Moon, 2024; S. Hernandes & R.G. Britto, 2012; Tarafdar & Pula, 2018). NOX enzymes mediate the production of $O_2{\bullet}^-$ and amplify oxidative stress, acting as central effectors of neuronal damage. In AD, the accumulation of extracellular soluble Aβ oligomers appears to trigger NOX activation through both direct and indirect mechanisms (Begum et al., 2022; Bruce-Keller et al., 2010; U. Ganguly et al., 2021; Shelat et al., 2008; Simonyi et al., 2010; Wyssenbach et al., 2016; Zilberter et al., 2023). Aβ oligomers stimulate microglia, provoking NOX2-dependent ROS generation (Cipriano et al., 2023; Wilkinson & Landreth, 2006). Furthermore, activation of NMDARs has been observed to trigger NOX activation, suggesting a functional interplay between receptor-mediated excitatory signalling and ROS production (Brennan et al., 2009). In this context, soluble Aβ oligomers can exacerbate excitotoxicity by enhancing glutamatergic transmission, which further potentiates NMDAR-dependent Ca^{2+} influx. This creates a synergistic loop in which Aβ-induced glutamatergic activity and NMDAR signalling converge on NOX activation, amplifying oxidative stress, synaptic dysfunction, and neuronal vulnerability (Begum et al., 2022; Cipriano et al., 2023; Wilkinson & Landreth, 2006). Interestingly, in cerebral endothelial cells, $A\beta_{40}$ has been shown to induce H_2O_2 production via NOX4, which paradoxically can improve endothelial function, suggesting a complex role for NOX4 in cerebrovascular dynamics (Heller et al., 2025). By integrating excitotoxic, inflammatory, and redox signals, NOX contributes to synaptic dysfunction, neuroinflammation, and neuronal degeneration, highlighting its potential as a therapeutic target.

Postmortem analyses of AD brains have demonstrated the translocation of NOX2 subunits $p47^{phox}$ and $p67^{phox}$ from cytosol to the membrane, predominantly in activated microglia (J. Kim & Moon, 2024; Ma et al., 2017). Clinical studies comparing no cognitive impairment (NCI), preclinical AD, MCI, and early to moderate AD have shown that NOX activity is significantly elevated in MCI and remains high in AD, with increased levels of NOX2 cytoplasmic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$) and a strong inverse correlation with cognitive performance (Ansari & Scheff, 2011; Bruce-Keller et al., 2010). In MCI patients, in which $gp91^{phox}$ is expressed in both microglia and neurons, the toxic effects of soluble $A\beta_{42}$ oligomers on neurons are mitigated by the NOX inhibitor apocynin, demonstrating that NOX2 is a central mediator of neurodegeneration (Ansari & Scheff, 2011; Bruce-Keller et al., 2010). Microglial NOX2 activation is further modulated by ATP release and purinergic P2X7 receptor signalling, which requires Ca^{2+} influx and leads to ROS production. *In vivo*, inflammatory responses triggered by intracerebrovascular injection of LPS or Aβ oligomers are attenuated in NOX-deficient mice or following NOX inhibition, favouring the M2 microglial phenotype associated with tissue repair. Co-culture experiments of APP-overexpressing neuroblastoma cells with microglia confirm that neuronal degeneration is largely dependent on NOX-derived ROS, an effect reduced by DPI or radical scavengers (Parvathenani et al., 2003; Zheng et al., 2024). NOX1 and NOX4 are also upregulated in AD animal models (Ma et al., 2017; M. W. Park et al., 2021).

A growing body of evidence indicates that Aβ, in its various aggregated forms, is a potent activator of NOX in microglia and other brain-resident cells. Foundational studies demonstrated that Aβ directly triggers NOX2 activation by promoting the translocation of cytosolic regulatory subunits, especially $p47^{phox}$, to the plasma membrane, enabling assembly of the active oxidase complex and superoxide production (Della Bianca et al., 1999). Fibrillar Aβ species, such as $A\beta_{40}$, similarly induce microglial ROS generation via NOX-dependent mechanisms (Jekabsone et al., 2006). Recent works have shown that oligomeric Aβ not only acutely activates NOX2 but also upregulates the expression of its core components in astrocytes and in hippocampal tissue of AD models and human frontal cortex post-mortem samples (Wyssenbach et al., 2016) and in cultured microglia, where it increases levels of $gp91^{phox}$ and $p47^{phox}$, amplifying oxidative capacity and downstream inflammatory responses (Geng et al., 2020). Pharmacological interventions, such as resveratrol, reduce Aβ-induced NOX2 activation and ROS production, confirming its central role in oligomer-driven neuroinflammation (Yao et al., 2015). *In vivo*, mouse models exposed to oligomeric Aβ exhibit coordinated upregulation of multiple NOX subunits, including gp91^{phox}, p47^{phox}, p22^{phox}, and p67^{phox}, and NOX inhibition with Astragaloside IV (AS-IV) attenuates microglial activation, reduces oxidative damage, and improves cognitive function, further supporting a central role of NOX2 in Aβ-driven neuroinflammation and neurodegeneration (F. Chen et al., 2021). Beyond microglia, Aβ also activates NOX2 in cerebrovascular endothelial cells, where ROS mediate vascular dysfunction (L. Park et al., 2005, 2008). Inhibition of NOX2 using the specific peptide inhibitor gp91ds-tat prevents Aβ-induced endothelial impairments, demonstrating the enzyme's role in cerebrovascular oxidative stress and endothelial dysfunction (L. Park et al., 2005). In addition, genetic deletion of NOX2 in AD transgenic mice attenuates ROS production and preserves neurovascular function, highlighting the enzyme relevance in AD (L. Park et al., 2008). *In vitro*, Aβ$_{25\text{-}35}$ treatment increases gp91^{phox} expression and ROS production in mixed cortical cultures, and neuronal death is attenuated by NOX inhibitors such as apocynin and AEBSF (Chay et al., 2017). Aβ oligomers also activate β1-integrin receptor-mediated signalling in astrocytes, causing cytosolic Ca^{2+} elevation and activation of the PI3K/PKC/Rac pathway, which upregulates NOX2 expression (Wyssenbach et al., 2016). Aβ peptides have been shown to induce ROS production in neurons and glia through the activation of NOXs as well as via crosstalk with NMDA receptors and PLA2 signalling. In cortical neurons, Aβ oligomers stimulate NOX-dependent ROS generation, an effect potentiated by NMDA receptor activation, which increases intracellular Ca^{2+} and further promotes NOX enzymatic activity. Concurrently, Aβ and NMDA activate cytosolic PLA2, leading to the release of arachidonic acid (AA), a lipid mediator that can amplify oxidative stress and inflammatory signalling (Shelat et al., 2008; Simonyi et al., 2010).

Glu-induced excitotoxicity is closely associated with the activation of NOXs, particularly NOX2 and NOX4, in neurons. Both iGluRs and mGluRs contribute to NOX2 activation, triggering translocation and phosphorylation of regulatory subunits, such as p47^{phox}, and assembly of the active oxidase complex, ultimately leading to ROS generation and neuronal damage (Guemez-Gamboa et al., 2011). NMDAR stimulation specifically increases ROS production via NOX2 and NO, with Ca^{2+} influx acting as a critical upstream signal that engages downstream kinases responsible for p47^{phox} phosphorylation (Girouard et al., 2009). In this context, PKC and Rac1 have been implicated in mediating NOX2 activation in response to Glu, further linking excitatory signalling to oxidative stress and neuronal death (Guemez-Gamboa et al., 2011).

Moreover, chronic Glu exposure engages NOX4 in primary cortical neurons, contributing to sustained H_2O_2 production and prolonged oxidative stress (Ha et al., 2010). These findings demonstrate that multiple NOX isoforms act as essential mediators of Glu-induced ROS generation, establishing a direct mechanistic link between excitatory signalling and ROS-driven neuronal damage.

Beyond the direct activation of NOX by Aβ oligomers, Glu released from neurons and astrocytes may further enhance oxidative stress through Ca^{2+}-dependent mechanisms. Both Glu and Aβ oligomers can promote Ca^{2+} influx via NMDARs, which in turn may engage multiple downstream signalling cascades. These include the activation of specific PKC isoforms (α, βII, δ, ζ) via PLCβ1 or PI3K pathways (Brennan-Minnella et al., 2013; Choi et al., 2008; Fontayne et al., 2002; Kelher et al., 2017; Teshima et al., 2014), as well as p38 MAPK following activation of Ca^{2+}/CaM-dependent CaMKII (Kyrmizi et al., 2018; Lu et al., 2013; Yamamori et al., 2000; L. J. Zhu et al., 2014). These convergent pathways can facilitate the phosphorylation of $p47^{phox}$ and assembly of the active NOX complex, while parallel Ca^{2+}-dependent transcriptional regulators, such as NFAT activated by calcineurin, may enhance the expression of NOX subunits (Williams & Gooch, 2014), supporting sustained ROS generation and pro-inflammatory gene transcription. Additional modulators, including Rac and $cPLA_2$/AA signalling (Shelat et al., 2008; Simonyi et al., 2010), may further amplify oxidative stress and neuronal vulnerability.

Proteomic and transcriptomic analyses of human AD brains and CSFs from familial AD cohorts reveal early upregulation of proteins involved in Ca^{2+}-dependent NOX activation, such as PKCγ (PRKCG), PLCβ1 (PLCB1), calcineurin subunits (PPP3R1/PPP3CA), multiple CaMKII isoforms, and p38 MAPKs, detectable up to fifteen years before symptom onset. Remarkably, these changes occur without modifications in ionotropic Glu receptor expression, suggesting that dysregulated Ca^{2+} signalling and NOX2 priming are early contributors to preclinical oxidative stress and synaptic vulnerability (Y. Shen, Ali, et al., 2024; Y. Shen, Timsina, et al., 2024).

Although NOX2 remains the primary isoform implicated in AD models, NOX1 and NOX4 are also upregulated, correlating with Aβ accumulation, oxidative damage, and cognitive decline. APP/PS1 double transgenic mice show elevated expression of both NOX2 and NOX4 in hippocampal and cortical regions, associated with increased oxidative stress and lipid peroxidation. Treatment with the antioxidant tert-butylhydroquinone (tBHQ) suppresses NOX2 expression and reduces lipid peroxidation, highlighting the contribution of NOX2-derived ROS to acute oxidative damage (Akhter et al., 2011). NOX4 upregulation in these mice has been linked to astrocyte ferroptosis and chronic oxidative stress, suggesting that its constitutive H_2O_2 production may drive persistent redox imbalance independently of acute stimuli (M. W. Park et al., 2021). Furthermore, NOX4 expression increases with age, correlating with progressive cognitive deficits (Bruce-Keller et al., 2011). Overall, these findings support a model in which NOX2 mediates acute ROS production and lipid peroxidation, whereas NOX4 contributes to sustained oxidative stress and metabolic damage (Hernandes & Britto, 2012). Bilateral cerebral artery occlusion models demonstrate significant upregulation of NOX1 mRNA in the CA1 hippocampal region, correlating with increased superoxide production and oxidative stress (Ma et al., 2017). Tg2576 mice lacking the catalytic subunit of NOX2 do not develop oxidative damage, neurovascular dysfunction, or cognitive deficits despite similar levels of Aβ, confirming the central role of NOX2-derived ROS in AD-related pathology (L. Park et al., 2008).

Table 1.1 NOX distribution, physiological functions and role Alzheimer’s disease (AD)

NOX Isoforms	Tissue and Cellular Localization	Subcellular Localization	Physiological Functions	Role in AD
NOX1	Colonic epithelium, vascular smooth muscle, endothelial cells, uterus, placenta, osteoclasts, retinal pericytes, neurons, astrocytes, microglia	Plasma membrane, endosomal compartments	Mucosal defence, proliferation, migration, angiogenesis	Upregulated in AD animal models, contributes to Glu-induced excitotoxicity, correlates with cognitive decline
NOX2	Monocytes, macrophages, neutrophils, neurons, astrocytes, microglia, endothelial cells, vascular endothelial cells, smooth muscle cells, and cardiomyocytes, kidney, gastrointestinal tract, liver, pancreas	Plasma membrane, endosomal and phagosomal vesicles	Defence and inflammatory signalling, synaptic plasticity, axonal guidance, neurotransmitter modulation receptor activity, proliferation, migration, angiogenesis	Upregulated in AD animal models, activated by Aβ oligomers and Glu, elevated activity in MCI and AD, mediates acute ROS production, lipid peroxidation, synaptic and vascular dysfunction, neuronal damage and death
NOX3	Inner ear, low expression in liver, lungs, spleen, fetal kidney	Plasma membrane	Signalling in inner ear for otoconia formation and vestibular function system	Not specifically mentioned
NOX4	Kidney, mesangial cells, smooth muscle cells, fibroblasts, osteoclasts, endothelial cells, neurons, hepatocytes	Plasma membrane, ER, mitochondria, MAMs, nucleus	Migration, differentiation, transcription, immunoregulatory role in innate immunity	Upregulated in AD animal models, contributes to Glu-induced excitotoxicity and sustained ROS production, lipid peroxidation, ferroptosis, metabolic damage, correlates with cognitive decline
NOX5	Spleen, testis, heart, endothelial cells	Plasma membrane, ER	Proliferation, migration, and angiogenesis	Not specifically mentioned
DUOX1 **DUOX2**	Thyroid, respiratory epithelium, gastrointestinal tract, brain	Apical plasma membrane in epithelial cells	Mucosal defence	Not specifically mentioned

1.7 Aim of the thesis

This thesis aims to investigate the cellular and molecular mechanisms associated with the earliest phase of AD, with particular focus on the so-called neuronal hyperactivity, which can be observed even before the appearance of Aβ plaques and classical biomarker alterations, therefore before the preclinical phase of the AT(N) framework. Clinical studies, animal models, and iPSC-derived systems indicate that this hyperactivity, characterized by persistent stimulation of NMDARs, represents an early phenotypic feature potentially predictive of disease progression. Although multiple glutamatergic receptors contribute to neuronal excitability, including AMPARs and KARs receptors, the present work specifically focuses on NMDAR-mediated signalling, given the well-established role of NMDAR overactivation and Ca^{2+} dysregulation in excitotoxic processes and early hyperexcitability in AD models.

To this end, we established *in vitro* models using human neuroblastoma SH-SY5Y cells and primary rat cortical neurons, which were treated with sub-threshold concentrations of $A\beta_{42}$ oligomers and Glu *i.e.* concentrations that did not cause an apparent increase of intracellular Ca^{2+} levels. These models aim to recapitulate key aspects of the earliest preclinical stages of AD, a phase in which neuronal hyperactivation is already present but classical CSF and imaging biomarkers remain undetectable. Using these systems, we investigated the cellular responses triggered by sub-threshold stimuli, focusing on conditions that do not induce an apparent increase of intracellular Ca^{2+} levels, mitochondrial dysfunction, overt toxicity or cell death, while observing the production of ROS mediated by ionotropic NMDARs. We also analyzed the relative contributions of mitochondrial and non-mitochondrial sources of ROS and explored potential metabolic alterations, particularly those related to NADPH flux and the pentose phosphate pathway.

Through this approach, the thesis has the purpose to investigate and clarify the cellular and molecular responses and consequences associated with neuronal hyperactivity in response to sub-lethal $A\beta_{42}$ and Glu exposure in SH-SY5Y cells and primary rat cortical neurons, providing insights into the early events that precede and may contribute to AD onset.

2. Materials and methods

2.1 Cell culture

Authenticated human neuroblastoma SH-SY5Y cells (A.T.C.C. CRL-2266, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; D6429, Sigma-Aldrich, St. Louis, MO, USA) and F-12 HAM (N4888, Sigma-Aldrich) with 25 mM N-2-hydroxyethylpirazine-N-2-ethansuofonic acid (HEPES) and $NaHCO_3$ (1:1) and supplemented with 10% Fetal Bovine Serum (FBS; F2442, Sigma-Aldrich), 1 mM glutamine (G7513, Sigma-Aldrich), and 1% penicillin and streptomycin solution (P0781, Sigma-Aldrich). Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C and grown until they reached 80% confluence for a maximum of 20 passages. The cells were tested to be free from mycoplasma contaminations using the MycoGenic Rapid Detection Kit (MORV0011, AssayGenie, Dublin, Ireland).

Primary rat cortical neurons (A1084002, Thermo Fisher Scientific, Eugene, OR, USA) were seeded on 18 mm diameter glass coverslips in a 12-well plate at a density of 120,000 cells per well and maintained in Neurobasal™ Plus Medium (A3582901, Thermo Fisher Scientific) supplemented with 0.5 mM GlutaMAX™ (35050061, Thermo Fisher Scientific) and 2% (v/v) B-27™ serum-free complement (17504044, Thermo Fisher Scientific) in 5% CO_2 humidified atmosphere at 37 °C. Every 4 days the medium was partially replaced with a fresh one. All the experiments were performed 12-16 days after plating. The cells were tested to be free from bacteria and fungi using optical microscope inspection and mycoplasma contaminations using the MycoGenic Rapid Detection Kit (MORV0011, AssayGenie).

2.2 Preparation of $A\beta_{42}$-derived diffusible ligands (ADDLs)

Lyophilised $A\beta_{42}$ (4014447, Bachem, Bubendorf, Switzerland) was dissolved in hexafluoro-2-propanol (HFIP) to 1 mM and incubated for 1 h at room temperature to allow the complete $A\beta_{42}$ peptide monomerization and then stored at -20°C until use. $A\beta_{42}$-derived diffusible ligands (ADDLs) were prepared as previously described (Lambert et al., 2001). Specifically, HFIP was removed with a gentle flow of N_2 and the dried protein was resuspended to 5 mM with anhydrous dimethyl sulfoxide (DMSO) and then diluted with F-12 HAM (N4888, Sigma-Aldrich) to 100 µM and incubated at 4 °C for 24 h. The sample was then centrifuged at 12,000*g* for 10 min to collect the supernatant.

2.3 Measurement of intracellular Ca^{2+} levels

SH-SY5Y cells were seeded on 18 mm diameter glass coverslips in a 12-well plate at a density of 120,000 cells per well and cultured for 24 h at 37 °C before each treatment. The cytosolic Ca^{2+} levels were measured in living SH-SY5Y cells washed with PBS (D8537, Sigma-Aldrich) and then loaded with 4 µM Fluo-4 AM fluorescent probe (F14201, Thermo Fisher Scientific) for 10 min after each treatment. Fluorescence was recorded upon excitation at 488 nm by TCS SP8 scanning confocal microscopy system (Leica Microsystems, Mannheim, Germany) equipped with an argon laser source. A series of 1 µm thick optical sections (1024 × 1024) was taken through the cell depth for each sample using a Leica Plan Apo 63× oil immersion objective and projected as a single composite image by superimposition. Ca^{2+}-derived fluorescence was then analysed using ImageJ software and expressed as the percentage of that observed in untreated cells, taken as 100%.

In a first set of experiments, SH-SY5Y cells were treated with decreasing concentrations of $A\beta_{42}$ ADDLs (1.0, 0.50, 0.25, 0.10, 0.050, 0.025 and 0.010 µM, monomer equivalents) or Glu (100, 80, 60, 40 and 60, 100 mM) (49621, Sigma-Aldrich) for 10 and 60 min at 37 °C. In another set of experiments, SH-SY5Y cells were treated with 0.25 µM ADDLs (monomer equivalents), 60 mM Glu, or both for 10, 60, 120 and 180 min at 37 °C.

In a separate series of experiments, SH-SY5Y cells were treated with 0.25 µM ADDLs or 60 mM Glu for 10 min at 37 °C, with or without a 5 min pre-treatment with 1 µM Thapsigargin (SERCA inhibitor; T9033, Sigma-Aldrich), or 3 µM SN-6 (NCX inhibitor; SML1937, Sigma-Aldrich), or 5 µM Vanadate (PMCA inhibitor; S6508, Sigma-Aldrich), individually or in pairs (Thapsigargin and SN-6, Thapsigargin and Vanadate, or SN-6 and Vanadate, respectively). Inhibitors alone were also evaluated as a control.

In another set of experiments, SH-SY5Y cells were treated with 50 µM AMPA or 1 mM NMDA with or without a 60 min pre-treatment with 5 µM CNQX (AMPAR inhibitor; C127, Sigma-Aldrich) and 10 µM Memantine (NMDAR inhibitor; M9292, Sigma-Aldrich), respectively.

In a final set of experiments, primary rat cortical neurons were treated with decreasing concentrations of ADDLs (1.0, 0.50, 0.25, 0.10, 0.050 and 0.025 µM, monomer equivalents) for 60 min at 37 °C.

2.4 Measurement of intracellular ROS levels

SH-SY5Y cells were seeded on 18 mm diameter glass coverslips in a 12-well plate at a density of 120,000 cells per well and cultured for 24 h at 37 °C before each treatment. The cytosolic ROS levels were measured in living SH-SY5Y cells loaded with 5 µM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; C6827, Thermo Fisher Scientific) in the last 15 min of the different treatments or with 5 µM CellRox™ Deep Red Reagent (C10522, Thermo Fisher Scientific) in the last 30 min of the different treatments. Fluorescence was recorded after excitation at 488 or 633 nm, respectively, by the TCS SP8 scanning confocal microscopy system described above. Cells were then analysed using ImageJ software and the fluorescence was expressed as the percentage of that observed in untreated cells, taken as 100%.

In a first set of experiments, SH-SY5Y cells were treated with 0.25 µM Aβ_{42} ADDLs (monomer equivalents) or 60 mM Glu for 10, 60, 120 and 180 min at 37 °C. Cells were then loaded with 5 µM CM-H_2DCFDA or with 5 µM CellRox™ Deep Red Reagent.

In another set of experiments, SH-SY5Y cells were treated with 0.25 µM ADDLs (monomer equivalents), 60 mM Glu for 180 min at 37 °C, with or without a 30 min pre-treatment with 5 µM APX115 (pan-NADPH oxidase inhibitor; HY-120801, MedChemExpress, Monmouth Junction, NJ, USA). Cells were then loaded with 5 µM CM-H_2DCFDA.

In a separate set of experiments, SH-SY5Y cells were treated with 0.25 µM ADDLs (monomer equivalents), or 60 mM Glu for 60, 120 and 180 min at 37 °C, or 500 µM H_2O_2 for 60 min (as a positive control) with or without a 60 min pre-treatment with 5 µM CNQX and 10 µM Memantine. The same treatments were also performed in a Ca^{2+}-free medium containing the Ca^{2+} chelator 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA; 14510, Sigma-Aldrich) at 10 µM. Cells were then loaded with 5 µM CM-H_2DCFDA.

In another set of experiments, primary rat cortical neurons were treated with 0.050 μM ADDLs (monomer equivalents) for 10, 60, 120 and 180 min at 37 °C, or with 5 μM Rotenone for 60 min (as a positive control) with or without a 30 min pre-treatment with 5 μM APX115 or with or without a 60 min pre-treatment with 5 μM CNQX and 10 μM Memantine. Cells were then loaded with 5 μM CM-H_2DCFDA.

2.5 Measurement of mitochondrial superoxide ions

SH-SY5Y cells were seeded on 18 mm of diameter glass coverslips in a 12-well plate at a density of 120,000 cells per well and cultured for 24 h at 37 °C before treatment. SH-SY5Y cells were treated with either 0.25 μM $A\beta_{42}$ ADDLs (monomer equivalents) or 60 mM Glu for 60 and 180 min at 37 °C, 50 μM Rotenone for 180 min (as a positive control for mitochondrial ROS production; R8875, Sigma-Aldrich), and then incubated with 5 μM MitoSOX^{TM} Red Mitochondrial Superoxide Indicator (TM36008, Thermo Fisher Scientific) in the last 10 min of the different treatments. Cells were then washed twice with PBS and fixed with 2% (v/v) paraformaldehyde for 10 min at room temperature. Cells were again washed twice with PBS and mounted with VECTASHIELD® antifade mounting medium (H-1000, Vector Laboratories, Newark, CA, USA). MitoSOX-derived fluorescence was detected after excitation at 550 nm by the TCS SP8 scanning confocal microscopy system described above and then analysed using ImageJ software. MitoSOX-derived fluorescence was expressed as the percentage of that observed in untreated cells, taken as 100%.

MitoSOX-derived fluorescence was detected also in primary rat cortical neurons treated with 0.25 μM ADDLs (monomer equivalents) for 10 and 60 min, or with 5 μM Rotenone for 60 min (as a positive control).

2.6 Measurement of NMDARs and AMPARs expression

SH-SY5Y cells were seeded on 18 mm of diameter glass coverslips in a 12-well plate at a density of 120,000 cells per well and cultured for 24 h at 37 °C before treatment. After 24 h, SH-SY5Y cells were washed with PBS, fixed with 2% (v/v) paraformaldehyde for 10 min at room temperature, and then incubated for 60 min at 37 °C in PBS and 1% FBS with 1:150 diluted rabbit polyclonal antibody against the subunit 2A of AMPAR (NR2A; AGC-002-AG, Alomone Lab, Jerusalem) or with 1:100 diluted rabbit polyclonal antibody against the subunit 2A of the NMDAR (GluN2A; AGC-005-AG, Alomone Lab), both labelled with ATTO488. Fluorescence was detected after excitation at 488 nm by the TCS SP8 confocal microscopy system described above and analysed using ImageJ software.

In another set of experiments, SH-SY5Y cells were transfected using 25 nM Stealth RNAi siRNA against NMDAR subunit GluN2B, or 25 nM Stealth RNAi siRNA negative controls (HSS104474 and 12935110, respectively, Thermo Fisher Scientific) or vehicle (transfection mix without siRNA), 7 µL of Lipofectamine, 10 µL of 5 mg/L transferrin in DMEM for 3 h in a 5% CO_2 humidified atmosphere at 37 °C. 3 h after transfection the DMEM was replaced with fresh complete medium, and the cells were incubated for 72 h. The cells were then fixed with 2% (v/v) paraformaldehyde for 10 min ar toom remperature, washed and incubated for 60 min at 37 °C with 1:400 diluted mouse monoclonal anti-NMDAR GluN2B antibody (320700, Thermo Fisher Scientific) in PBS and 1% FBS and for 90 min with 1:1000 diluted Alexa Fluor 488-conjugated anti-mouse secondary antibodies (A11001, Life Technologies/Thermo Fisher Scientific) in PBS and 1% of FBS. Fluorescence was detected using the TCS SP8 confocal microscopy described above and analysed using ImageJ software.

2.7 Measurement of NMDAR membrane exposure

SH-SY5Y cells were seeded on 18 mm of diameter glass coverslips in a 12-well plate at a density of 120,000 cells per well and cultured for 24 h at 37 °C before treatment. Cells were treated with 0.25 µM $A\beta_{42}$ ADDLs (monomer equivalents) or 60 mM Glu for 10, 60, 120 and 180 min at 37 °C. After washing two times with PBS, cells were fixed with ice-cold 100% methanol at -20 °C for 15 min and then blocked with PBS, 5% (v/v) FBS and 0.1% (v/v) Triton^{TM} X-100 (X100, Sigma Aldrich) for 60 min at room temperature. After washing in PBS, cells were stained with 1:300 diluted mouse monoclonal anti-GluN2B labelled with Alexa Fluor 488 (sc-36559, Santa Cruz Biotechnology, Dallas, TX, USA) in PBS and 0.1% (v/v) Triton^{TM} X-100 overnight at 4 °C. Cells were then rinsed three times with PBS and mounted with the VECTASHIELD® antifade mounting medium. The fluorescence emission was detected after excitation at 488 nm by the TCS SP8 scanning confocal microscopy system described above. Cells were then analysed using ImageJ software and the fluorescence was expressed as the percentage of that observed in untreated cells, taken as 100%.

2.8 MTT reduction assay

SH-SY5Y cells were seeded in 96-well plate at a density of 15,000 cells per well and cultured for 24 h at 37 °C before treatment. Cells were then treated with Aβ_{42} ADDLs at 0.25 and 1 μM (monomer equivalents) or Glu at 60 and 100 mM, respectively. 180 min and 24 h after treatment the cells were washed with PBS and incubated at 37 °C with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; M5655, Sigma-Aldrich) solution in RPMI for 4 h and then incubated for 1-3 h with the lysis buffer (20% SDS, 50% *N*,*N*-dimethylformamide, pH 4.7). Cell viability was determined by measuring the absorbance at 595 nm and expressed as the percentage of MTT reduction relative to the untreated cells, taken as 100%.

2.9 Cell count

SH-SY5Y cells were seeded into a 12-well plate at a density of 120,000 cells per well. After 24 h at 37 °C they were treated with 0.25, 1 μM Aβ_{42} ADDLs (monomer equivalents) or 60, 100 mM Glu and incubated for 180 min at 37 °C. Then, the cells were detached with trypsin-EDTA solution (T4049, Sigma-Aldrich) and counted with a Bürker counting chamber. The total cell number was expressed as the percentage of that of untreated cells, taken as 100%.

2.10 Radiolabelled Glucose Uptake assay

SH-SY5Y cells were seeded in a 12-well plate at a density of 200,000 cells per well. After 24 h at 37 °C they were treated with 0.25 μM Aβ_{42} ADDLs (monomer equivalents) or 60 mM Glu and incubated for 180 min at 37 °C. Cells were then incubated with 20 mM HEPES, 140 mM NaCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM KCl, pH 7.4 containing 0.1 μCi [U-^{14}C] radioactive glucose (NEC042B005MC, PerkinElmer, Waltham, Massachusetts, USA) for 15 min at 37 °C. Cells were subsequently washed with cold PBS and lysed with 0.1 M NaOH. The incorporated radioactive glucose-derived signal was measured by liquid scintillation counting and normalized on protein content, measured using BCA assay (1003290033, Sigma-Aldrich).

2.11 Oroboros O2k Analysis

Oxygen consumption was analysed in 2 mL glass chambers at 37 °C using the Oroboros Oxygraph-2K high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) and the substrate, uncoupler, inhibitor, titration (SUIT) protocol (D009). The oxygen flux normalized on the number of cells was calculated as the negative time derivative of oxygen concentration, measured in sealed chambers, and normalized on the instrumental background (measured in a dedicated experiment before assaying the cells). SH-SY5Y cells were seeded in Petri dishes (60 mm) at a density of 3,000,000 cells per Petri dish. After 24 h at 37 °C they were treated with 0.25 μM $A\beta_{42}$ ADDLs (monomer equivalents) or 60 mM Glu and incubated for 180 min at 37 °C. After instrumental air calibration, cells were detached with trypsin-EDTA solution, counted with a Bürker counting chamber, resuspended in a complete cell medium, and introduced into the chambers of the instrument. The basal respiratory activity was measured as routine respiration (R). The LEAK state (L) represents the non-phosphorylating state of uncoupled respiration due to proton leak, proton and electron slip, and cation cycling and was measured after ATP synthase inhibition by 5 nM oligomycin. The capability of the electron transfer system (ETS) was measured by uncoupler titrations using the uncoupler Carbonyl Cyanide 3-ChloroPhenylhydrazone (CCCP; 1.5 μM/titration step) as the readout of the maximal capacity of oxygen utilization (E). The residual oxygen consumption (ROX) that remains after the inhibition of ETS was determined by 2.5 μM antimycin A. Data acquisition and analysis were performed using DatLab software (Oroboros Instrument). The oxygen fluxes measured during individual titration steps were corrected for ROX, which serves as a proxy for residual respiration from oxidative reactions persisting after ETS inhibition. Correcting for ROX is essential to accurately assess mitochondrial respiration, as it represents the baseline state.

2.12 ^{1}H NMR Spectroscopy

SH-SY5Y cells were seeded in Petri dishes (60 mm) at a density of 4,000,000 cells per Petri dish. After 24 h at 37 °C they were treated with 0.25 µM Aβ_{42} ADDLs (monomer equivalents) or 60 mM Glu and incubated for 180 min at 37 °C. Then, cell media were collected and immediately stored at -80 °C. SH-SY5Y cells were detached by scraping and resuspended in PBS in the presence of 0.1X protease inhibitors (P8340, Sigma-Aldrich) and 0.1 X phosphatase inhibitors (524629, Sigma-Aldrich) cocktails and lysed with five freeze-thaw cycles (-80°C/room temperature) and a subsequent sonication at 50% amplitude (pulsed on-time 15 s, off-time 15 s for five cycles). Cell lysates were then centrifuged at 14,000*g* for 30 min at 4 °C and the supernatant was immediately stored at -80 °C. Six independent biological replicates were prepared for each of the three conditions.

Frozen cell lysates were thawed at room temperature and for each sample. A total of 60 µL of deuterated water (D_2O), containing 0.58 mM 3-(Trimethylsilyl)propionate-2,2,3,3-d4 as NMR reference was added to 540 µL of cell lysate and the mixture was transferred into a 5 mm NMR tube. Frozen cell media were thawed at room temperature. A total of 300 µL of 75 mM Na_2HPO_4 buffer, 20% (v/v) D_2O, 4.6 mM 3-(Trimethylsilyl)propionate-2,2,3,3-d4, 6.1 mM NaN_3, pH 7.4, was added to 300 µL of cell medium and the mixture was transferred into a 5 mm NMR tube. ^{1}H NMR spectra were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin) operating at 600.13 MHz proton Larmor frequency equipped with an automatic and refrigerated (6 °C) sample changer (SampleJet, Bruker BioSpin). The spectra of cell lysates were acquired at 300 K using the Carr-Purcell-Meiboom-Gill (CPMG) one-dimensional spin-echo sequence (Bruker sequence cpmgpr1d) (Ghini et al., 2023) with water presaturation, 512 scans, 73 728 data points, a spectral width of 12 019 Hz and a relaxation delay of 4 s. The spectra of the cell media were acquired at 310 K with a 1D CPMG pulse sequence (Bruker sequence cpmgpr1d) with water presaturation, 64 scans, 73 728 data points, a spectral width of 12 019 Hz and a relaxation delay of 4 s. Before applying Fourier transform, free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated to the anomeric glucose doubled at δ 5.24 ppm. NMR signals of metabolites were manually assigned by Chenomx NMR suite 10.0, freely available databases, and published literature. The identified metabolites were quantified (in arbitrary units) by the integration of the NMR region of interest using an R script developed in-house. A panel of 41 and 32 metabolites were quantified in cell lysates and cell media, respectively. To reduce batch effects, the matrices of quantified metabolites in cell lysates and cell media were processed using the Harmony algorithm (Korsunsky et al., 2019). Harmonized data were scaled without centering.

2.13 Pentose Phosphate Pathway (PPP) flux radioactive assay

SH-SY5Y cells were seeded in Petri dishes (35 mm) at a density of 350,000 cells per Petri dish. After 24 h at 37 °C, they were treated with 0.25 μM Aβ_{42} ADDLs (monomer equivalents) or 60 mM Glu and incubated for 180 min at 37 °C. Measurement of CO_2 released from radioactive glucose was used as a readout of the oxidative phosphorylation and the PPP activity and was evaluated by using radioactive glucose labelled at position 1 [1-^{14}C] at position 6 [6-^{14}C] (NEC043X050UC, and NEC045X050UC, respectively, PerkinElmer, Waltham, Massachusetts, USA). Two different plates of the same sample were treated in parallel with 0.1 μCi [1-^{14}C]-glucose or 0.1 μCi [6-^{14}C]-glucose for 15 min. Each dish had a taped piece of Whatman paper facing the inside part of the dish wetted with 200 μL of phenylethylamine-methanol (1:1) to trap the CO_2. After the incubation, 200 μL of 0.8 M H_2SO_4 in water were added to the cells for 1 h. Finally, Whatman paper was removed and transferred into scintillation vials for counting. The radioactive signal was measured by a liquid scintillation counter (Hidex 300 SL, Hidex Oy, Turku, Finland) and normalized on protein content. $^{14}CO_2$ released from [1-^{14}C]-glucose oxidation originates from the PPP and/or the TCA cycle, whereas $^{14}CO_2$ derived from [6-^{14}C]-glucose originates only from the TCA cycle. Therefore, PPP CO_2 production was revealed by subtracting the radioactive signal derived from [6-^{14}C]-glucose from that of [1-^{14}C]-glucose.

2.14 Statistical Analysis

All data were expressed as means ± S.E.M. (standard error of the mean). Comparisons between the different groups were performed by Student's t-test. The single (∗; §), double (∗∗; §§) and triple (∗∗∗) asterisks or simbols refer to p values <0.05, <0.01 and <0.001, respectively. In NMR, Student t-test was used to point out metabolite differences among the three conditions of interest.

3. Results

3.1 Aβ_{42} oligomers and Glu cause an increase of intracellular Ca^{2+} levels in a dose-dependent manner

The investigation began by identifying the highest concentrations of Aβ_{42} ADDLs and Glu that did not appear to affect Ca^{2+} ion homeostasis in human neuroblastoma SH-SY5Y cell cultures, which is a widely used cell model for studying *in vitro* the fundamental biochemical changes upon Aβ and Glu exposure, can be easily cultured and possess only extrasynaptic ionotropic Glu receptors (Dong et al., 2009; Fani et al., 2021; Giordano et al., 2014; Kritis et al., 2015; Rezvani Boroujeni et al., 2020; Sakono & Zako, 2010; Y. Zhang et al., 2016). To this aim, we treated SH-SY5Y cells with decreasing concentrations of ADDLs (1.0-0.010 µM, monomer equivalents) or Glu (100-20 mM) and monitored the increase of intracellular Ca^{2+} ions by loading the cells with the specific Ca^{2+} indicator Fluo-4 AM probe. Glu, which is the main excitatory neurotransmitter in the central nervous system, directly activates metabotropic and ionotropic Glu receptors (Crupi et al., 2019; Ferraguti & Shigemoto, 2006; Hansen et al., 2021; Reiner & Levitz, 2018; Traynelis et al., 2010), but only the ionotropic receptors allow an influx of Ca^{2+} ions and they include NMDARs and AMPARs (Alberdi et al., 2002; Danysz & Parsons, 2012; Furukawa et al., 2005; Gan et al., 2015; Negrete-Díaz et al., 2022; R. Wang & Reddy, 2017). It should be noted that SH-SY5Y cells are a human neuroblastoma cell line and therefore do not fully reproduce the physiological properties of primary neurons. For this reason, the concentrations used in this study were not intended to mimic extracellular Glu levels in the brain, but rather to identify sub-threshold conditions within this cellular model capable of reproducing excitatory stimulation conditions comparable to those observed in neurons. On the other side, ADDLs determine an intracellular increase of Ca^{2+} levels in neuronal cells through the rapid activation of AMPARs and, in particular, NMDARs via a Glu-independent mechanism, by interacting with the lipid bilayer of the cell membrane, causing a change of its mechanical properties (Fani et al., 2021; Fani & Chiti, 2022).

We identified 0.25 µM ADDLs and 60 mM Glu as the highest concentrations of these two species that did not cause a significant rise of intracellular Ca^{2+}, both after 10 and 60 min (**Fig. 3.1**).

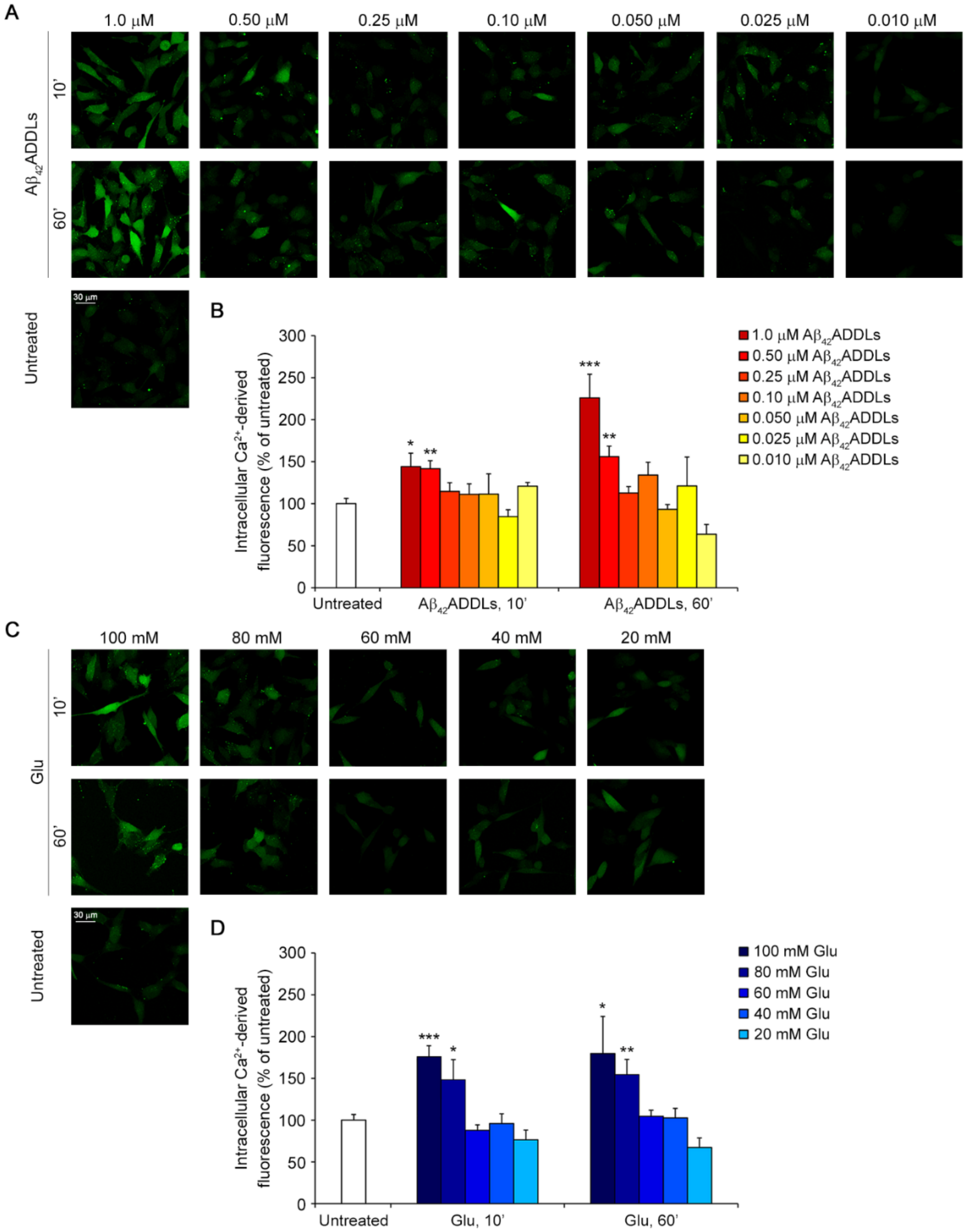

Figure 3.1. Intracellular Ca^{2+} levels in SH-SY5Y cells treated with Aβ_{42} ADDLs or Glu. (A,C) Representative confocal scanning microscopy images of untreated cells and cells treated with decreasing concentrations of **(A)** Aβ_{42} ADDLs (1.0-0.010 µM, monomer equivalents) or **(C)** Glu (100-20 mM) for 10 and 60 min. **(B,D)** Semiquantitative analysis of intracellular Ca^{2+}-derived fluorescence. Data are represented as the mean ± SEM (n=3). The single (∗) double (∗∗) and triple (∗∗∗) asterisks refer to p values of <0.05, <0.01, <0.001 relative to untreated cells, respectively.

We also observed that these concentrations of ADDLs and Glu, either individually or in combination, did not provoke a significant Ca^{2+} influx within the cells even at prolonged times of exposure and even when they were used together, up to 180 min (**Fig. 3.2**). We will refer to these two concentrations as sub-threshold conditions hereafter in the text.

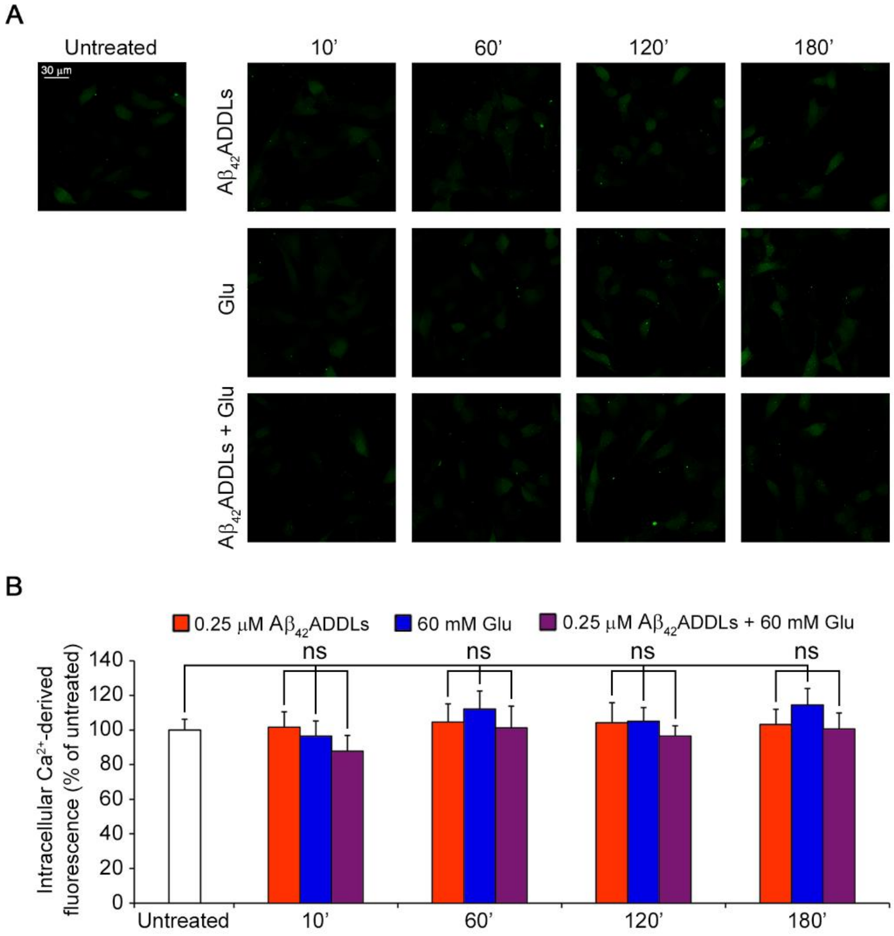

Figure 3.2. Intracellular Ca^{2+} levels in SH-SY5Y cells treated with $A\beta_{42}$ ADDLs or Glu. (A) Representative confocal scanning microscopy images of untreated cells and cells treated with 0.25 µM $A\beta_{42}$ ADDLs (monomer equivalents), 60 mM Glu, or both for 10, 60, 120 and 180 min. **(B)** Semiquantitative analysis of intracellular Ca^{2+}-derived fluorescence. Data are represented as the mean ± SEM (n=3). ns means non-significant.

Although NMDARs and AMPARs are commonly studied in SH-SY5Y cells (Y. Fang et al., 2014; H. Xu et al., 2019) earlier reports suggested that they might be absent (Jantas et al., 2008). We therefore checked their expression in our cell line using fluorescently labelled primary antibodies against NMDARs or AMPARs. High NMDAR- and AMPAR-derived fluorescence was observed in the cells (**Fig. 3.3A**), whereas cells pre-treated with the anti-GluN2B siRNA exhibited significantly reduced fluorescence (**Fig. 3.3B**). Moreover, treatment with the agonists NMDA or AMPA caused a significant increase of intracellular Ca^{2+} ions, relative to untreated cells, which was significantly reduced with a 60 min pre-treatment with the NMDAR inhibitor Memantine or the AMPAR inhibitor CNQX (**Fig. 3.3C,D**), confirming that these receptors were present and functional in this cell line.

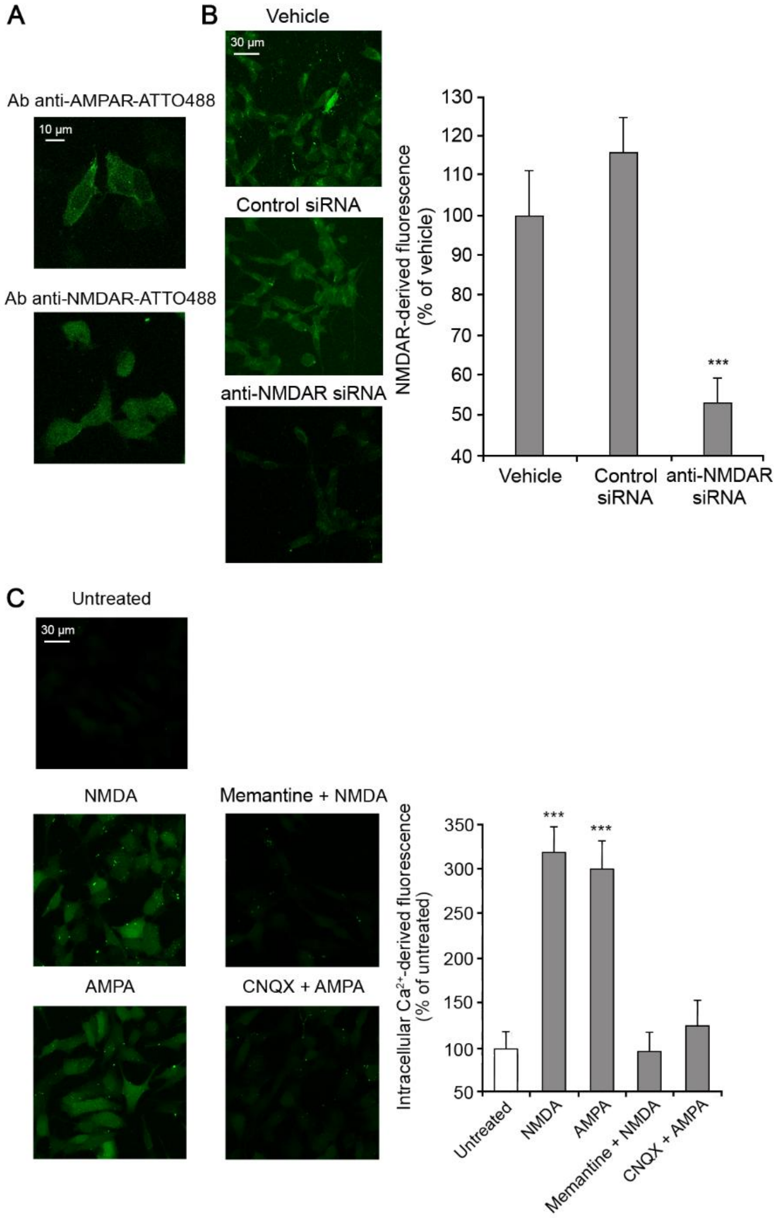

Figure 3.3. **Functional expression of AMPARs and NMDARs in SH-SY5Y cells. (A)** Representative confocal scanning microscopy images of SH-SY5Y cells following the treatment with primary antibody against AMPARs and NMDARs, both labelled with fluorescent ATTO488. **(B)** Representative confocal scanning microscopy images following pre-treatment with vehicle, 25 nM negative control siRNA and 25 nM anti-NMDA siRNA, and associated semi-quantitative analysis of NMDAR-derived fluorescence. **(C)** Representative confocal scanning microscopy images of Ca^{2+} levels following no treatment, and treatment with 1 mM NMDA, 50 µM AMPA, 1 mM NMDA preceded by pre-treatment with 10 µM Memantine, and 50 µM AMPA preceded by pre-treatment with 5 µM CNQX. The histogram reports the associated semi-quantitative analysis of intracellular free Ca^{2+}-derived fluorescence. Variable numbers of cells (12-22) in three different experiments were analysed for each condition. The triple (∗∗∗) asterisks refer to p values <0.001 relative to untreated cells.

3.2 Ca^{2+} influx triggered by sub-threshold concentrations of $A\beta_{42}$ oligomers and Glu is counteracted by Ca^{2+} pumps

The lack of detectable increase of intracellular Ca^{2+} levels upon exposure to the selected sub-threshold concentrations of $A\beta_{42}$ ADDLs and Glu may be due to an effective absence of Ca^{2+} influx under these conditions of treatment, or to the presence of an influx that is efficiently buffered by Ca^{2+} pumps. To address this issue, we evaluated the effective capability of both ADDLs and Glu at the selected sub-threshold concentrations to induce a Ca^{2+} influx within the cells using the three main Ca^{2+} pumps inhibitors: 1 μM Thapsigargin, inhibitor of the Sarco-Endoplasmic Reticulum Ca^{2+} ATPase (SERCA); 3 μM SN-6, inhibitor of the Na^{+}-Ca^{2+} Exchanger (NCX); and 5 μM Vanadate, inhibitor of the Plasma Membrane Ca^{2+} ATPase (PMCA). We observed that 0.25 μM ADDLs (monomer equivalents) or 60 mM Glu for 10 min were able to increase the intracellular Ca^{2+} levels in the presence of the inhibitors used individually (**Fig. 3.4**). Hence, even in the presence of sub-threshold concentrations of ADDLs or Glu, there is an entry of Ca^{2+} ions that is usually physiologically buffered by the cells through internalization in intracellular stores or extrusion in the extracellular space thanks to the activation of membrane Ca^{2+} pumps. As a control, inhibition of individual Ca^{2+} pumps in the absence of ADDLs/Glu did not modify the intracellular Ca^{2+} level, which is comparable to that of untreated cells (**Fig. 3.4**).

The concomitant inhibition of two different Ca^{2+} pumps caused a significant increase of the intracellular Ca^{2+} levels, even in the absence of ADDLs/Glu (**Fig. 3.4**). This is probably due to a spontaneous and energetically favourable entry of Ca^{2+} in the cytosol from the extracellular space or intracellular stores that is not effectively pumped away when at least two pumps are inhibited. When Thapsigargin and Vanadate are used together to inhibit the PMCA and SERCA, the highest increase of Ca^{2+} ions was observed, which was further exacerbated in the presence of ADDLs or Glu (**Fig. 3.4**). By contrast, the treatment with the selected subthreshold concentrations in the presence of the inhibitor pairs SN-6/Vanadate and Thapsigargin/SN-6 led to intracellular Ca^{2+} levels that did not differ significantly from those observed in the presence of the inhibitor pairs alone (**Fig. 3.4**). This phenomenon could be explained by the fact that ATP, which was not used by the inhibited PMCA and SERCA pumps, interferes with the inhibitory effect of SN-6 on NCX (Dipolo & Beaugé, 2006; Iwamoto et al., 2004).

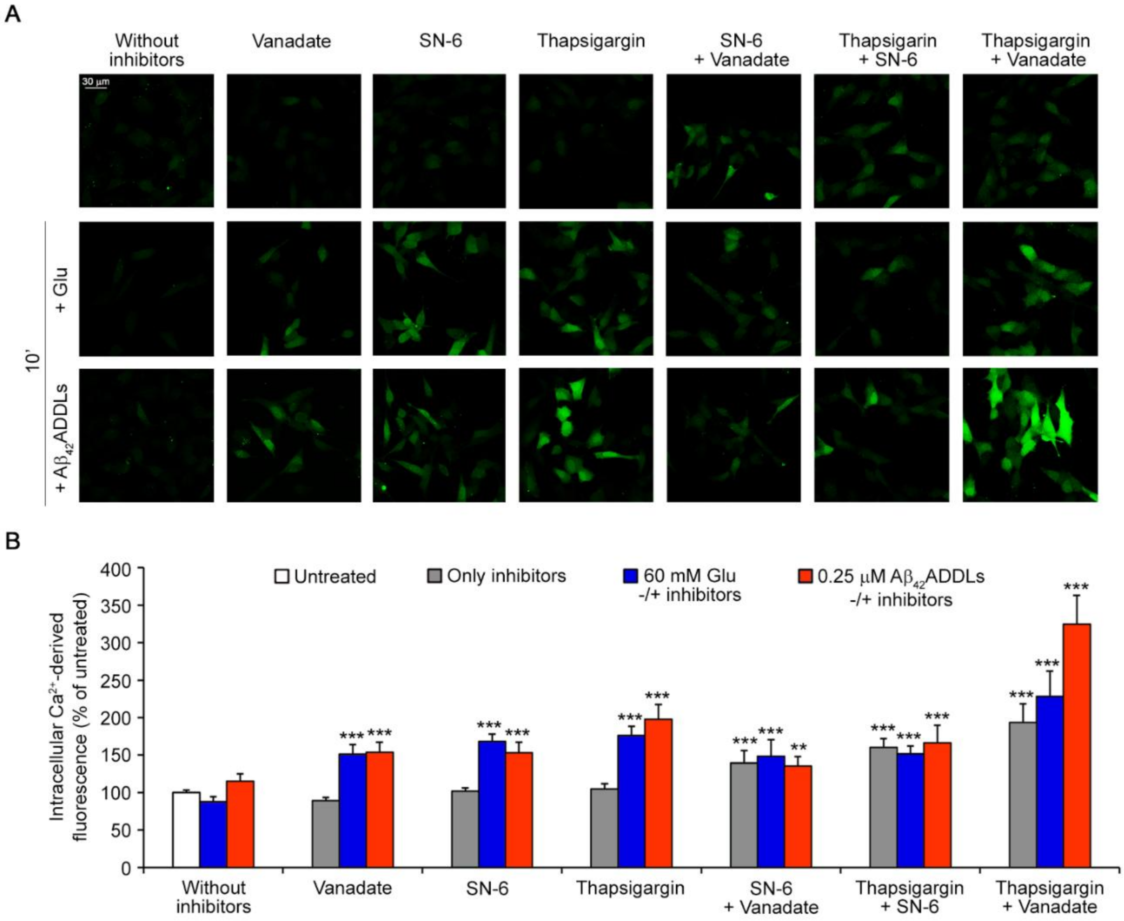

Figure 3.4 Intracellular Ca^{2+} levels in SH-SY5Y cells treated with Aβ42 ADDLs or Glu, after the inhibition of Ca^{2+} pumps. (A) Representative confocal scanning microscopy images following the treatment with 0.25 µM Aβ42 ADDLs (monomer equivalents) or 60 mM Glu for 10 min, after pre-treatment with 5 µM Vanadate (PMCA inhibitor), 3 µM SN-6 (NCX inhibitor), or 1 µM Thapsigargin (SERCA inhibitor) for 5 min. **(B)** Semiquantitative analysis of intracellular free Ca^{2+}-derived fluorescence. Data are represented as the mean ± SEM (n=3). The double (∗∗) and triple (∗∗∗) asterisks refer to p values of <0.01 and <0.001 relative to untreated cells, respectively.

3.3 Sub-threshold concentrations of Aβ42 oligomers and Glu did not modify NMDAR membrane exposure

We then questioned whether prolonged treatment of SH-SY5Y cells with sub-threshold concentrations of Aβ42 ADDLs and Glu could trigger any change in the membrane exposure of NMDARs. The hypothesis arose from observations that Aβ causes down-regulation of synaptic NMDARs in cortical neurons, by increased endocytosis and reduced expression (Snyder et al., 2005; Y. Zhang et al., 2016). Cells were treated with sub-threshold concentrations of ADDLs and Glu for varying time lengths (10, 60, 120, and 180 min) and then the membrane expression of NMDARs was monitored, using an antibody specifically targeting the NMDAε2 receptor subunit (GluN2B), conjugated with the Alexa Fluor 488 dye. A decrease in the NMDAR-derived fluorescence with time was not observed (**Fig. 3.5**), indicating that sub-threshold concentrations of ADDLs/Glu induce a significant Ca^{2+} influx that remains undetectable due to the compensatory activity of Ca^{2+} pumps, rather than changes in NMDAR membrane expression.

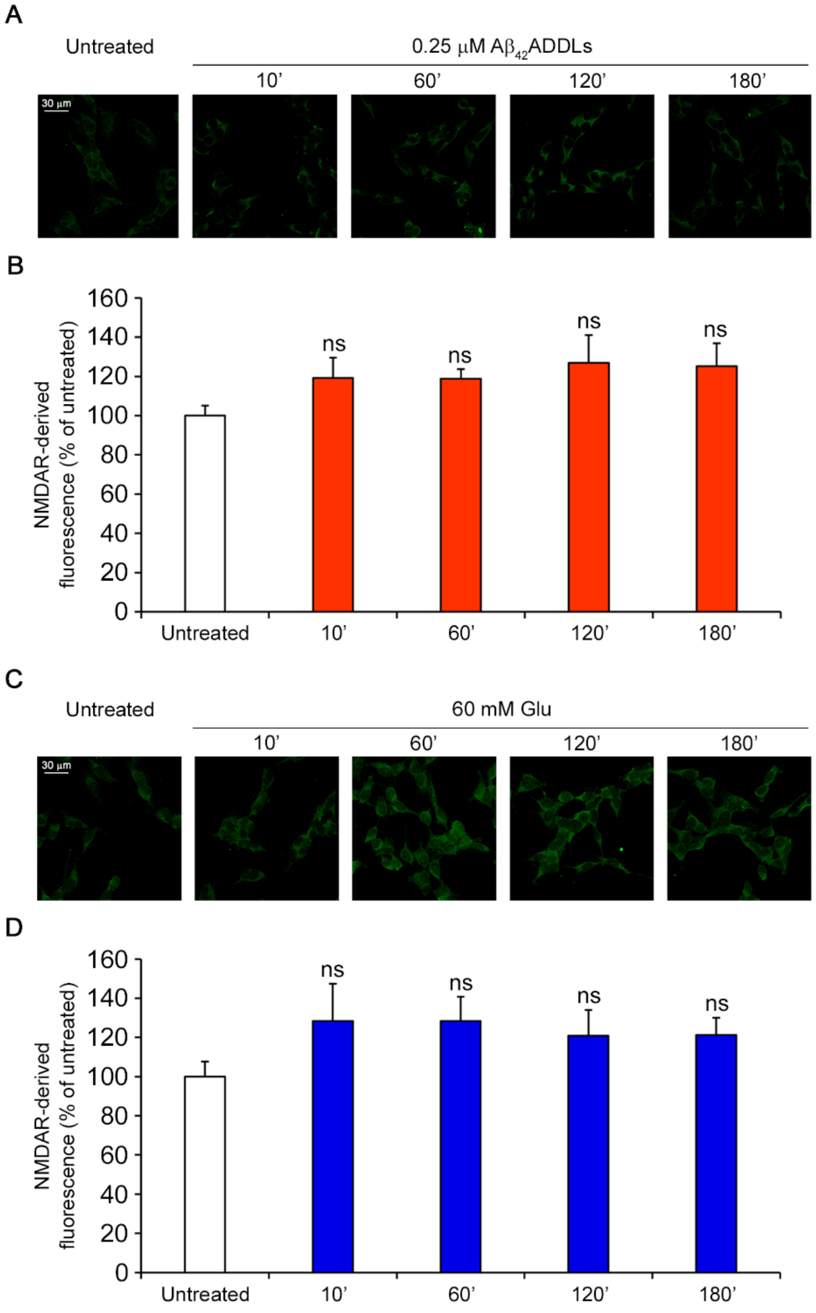

Figure 3.5. NMDAR membrane exposure in SH-SY5Y cells treated with $A\beta_{42}$ ADDLs or Glu. (A,C) Representative confocal scanning microscopy images of cells treated with primary antibody against NMDAR labelled with fluorescent ATTO488 after treating cells with **(A)** 0.25 µM $A\beta_{42}$ ADDLs (monomer equivalents) or **(C)** 60 mM Glu for 10, 60, 120 and 180 min. **(B,D)** Semiquantitative analysis of NMDAR-derived fluorescence. Data are represented as the mean ± SEM (n=3). ns means non-significant.

3.4 Sub-threshold concentrations of Aβ_{42} oligomers and Glu cause a Ca^{2+}-dependent increase in intracellular ROS levels

We then evaluated whether the selected sub-threshold concentrations of Aβ_{42} ADDLs and Glu could induce an increase in the intracellular ROS levels. This was measured using the CM-H_2DCFDA probe, which detects ROS independently of their subcellular site of formation (Deb et al., 2024; Oparka et al., 2016; Wojtala et al., 2014). Interestingly, both ADDLs (monomer equivalents) and Glu induced an enhancement of ROS production in a time-dependent manner, with intracellular ROS levels starting to increase already after 10 min, until reaching significantly higher values at 60, 120 and 180 min of treatment (**Fig. 3.6A,B**).

To assess whether the grossly undetectable Ca^{2+} influx observed by treating cells with sub-threshold concentrations of ADDLs and Glu could be effectively responsible for the enhanced ROS levels, we treated cells with ADDLs/Glu for 60, 120 and 180 min, after a concomitant pre-treatment with 5 µM CNQX (AMPAR inhibitor) and 10 µM Memantine (NMDAR inhibitor) for 60 min, or in a Ca^{2+}-free extracellular medium. The results showed that in the presence of both NMDAR/AMPAR inhibitors or in a Ca^{2+}-free medium, the ADDL- and Glu-induced production of ROS was completely prevented (**Fig. 3.6C,D**), strongly suggesting that the Ca^{2+} entry into the cells mediates ROS generation. As a positive control, cells were treated with H_2O_2, that induced a marked increase in intracellular ROS which was not reduced by the Memantine/CNQX pre-treatment or in the presence of a Ca^{2+}-free medium (**Fig. 3.6C,D**). Taken together, these results shed light on the biochemical events triggered in these cells by sub-threshold concentrations of ADDLs and Glu.

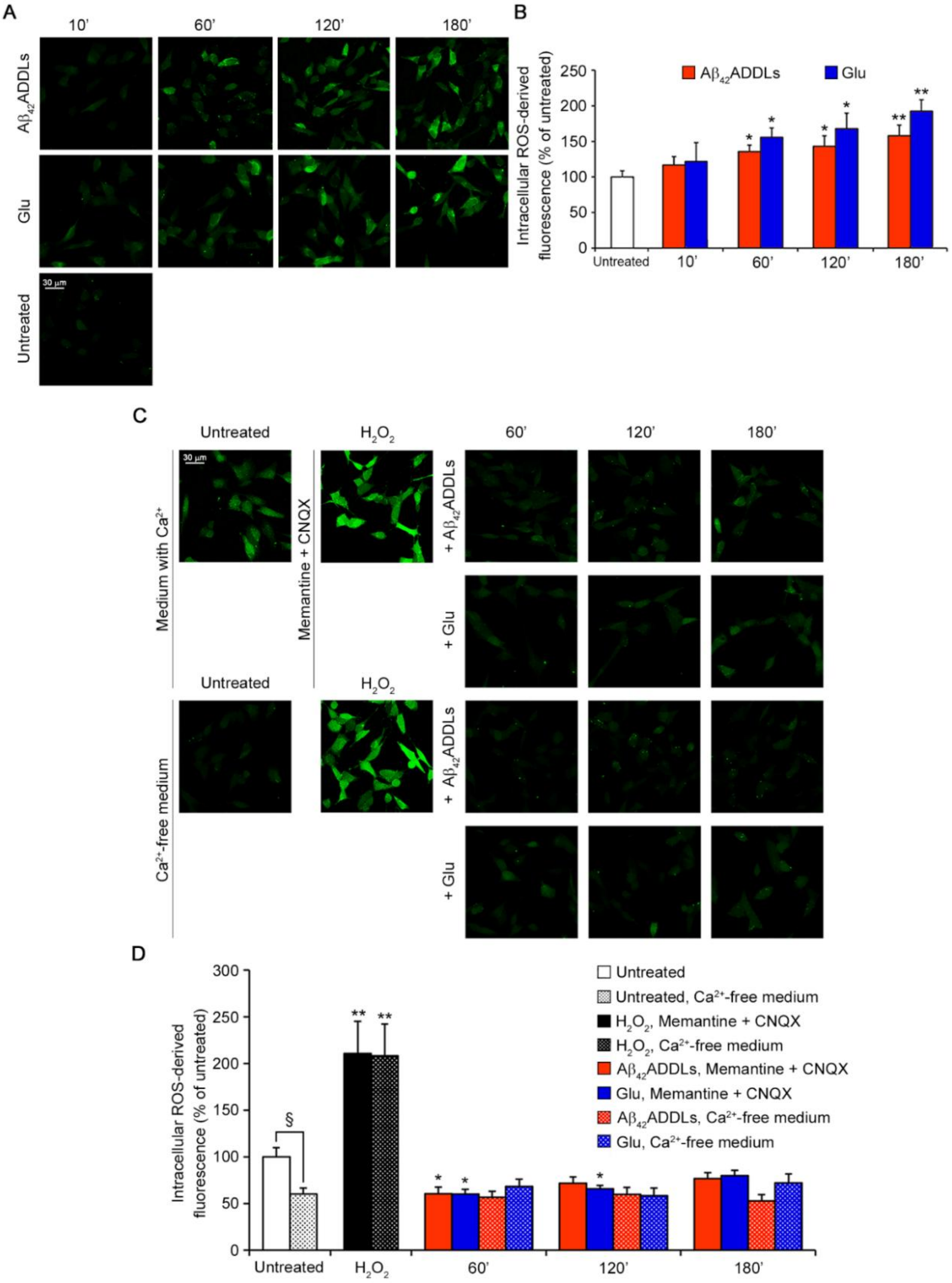

Figure 3.6. Intracellular ROS levels in SH-SY5Y cells treated with $A\beta_{42}$ ADDLs or Glu, probed CM-H_2DCFDA. (A) Representative confocal scanning microscopy images of untreated cells and cells treated with 0.25 µM $A\beta_{42}$ ADDLs (monomer equivalents) or 60 mM Glu for 10, 60, 120 and 180 min. **(B)** Semiquantitative analysis of intracellular ROS-derived fluorescence. Data are represented as the mean ± SEM (n=3). The single (∗) and double (∗∗) asterisks refer to p values of <0.05 and <0.01 relative to untreated cells, respectively. **(C)** Representative confocal scanning microscopy images following the treatment with 0.25 µM ADDLs or 60 mM Glu for 60, 120 and 180 min, and 500 µM H_2O_2 for 60 min as a positive control, after pre-treatment with 5 µM CNQX (AMPAR inhibitor) and 10 µM Memantine (NMDAR inhibitor) for 60 min, or in Ca^{2+}-free medium. **(D)** Semiquantitative analysis of intracellular ROS-derived fluorescence. Data are represented as the mean ± SEM (n=3). The single (∗) and double (∗∗) asterisks refer to p values of <0.05 and <0.01 relative to the corresponding untreated cells in medium with or without Ca^{2+}, respectively. The single (§) symbol refers to p values of <0.05 relative to untreated cells in medium with Ca^{2+}.

The increase of ROS was further confirmed in cells treated with Glu using the CellRox™ Deep Red Reagent probe (**Fig. 3.7**), which detects all forms of cellular ROS (Eruslanov & Kusmartsev, 2010; Fallico et al., 2020; Tkachev et al., 2015).

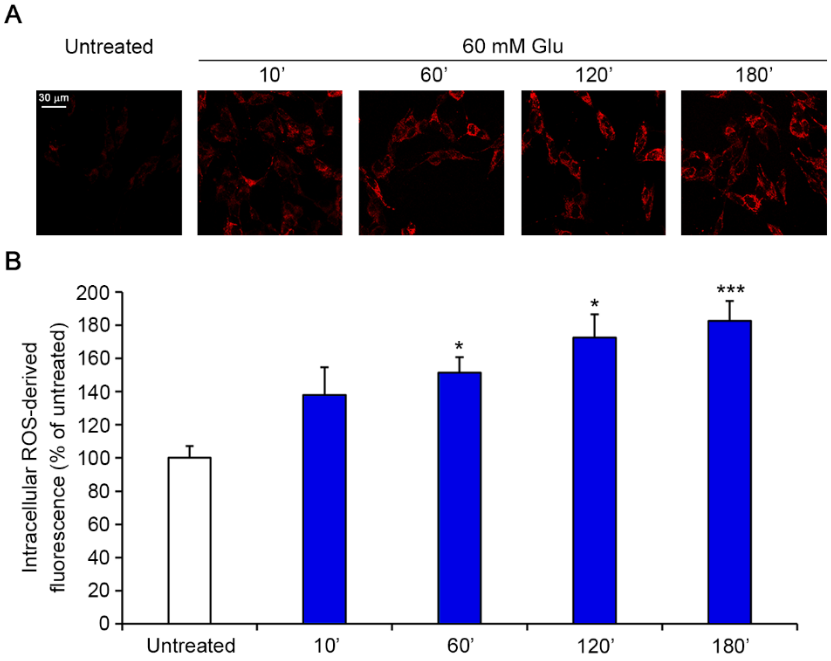

Figure 3.7. Intracellular ROS levels in SH-SY5Y cells treated with Glu, probed with CellRox™ Deep Red reagent. (A) Representative confocal scanning microscopy images of untreated cells and cells treated with 60 mM Glu for 10, 60, 120 and 180 min. **(B)** Semiquantitative analysis of intracellular ROS-derived fluorescence. Data are represented as the mean ± SEM (n=3). The single (∗) and triple (∗∗∗) asterisks refer to p values of <0.05 and <0.001 relative to untreated cells, respectively.

3.5 Sub-threshold concentrations of Aβ42 oligomers and Glu increase non-mitochondrial oxygen consumption without affecting glucose uptake and oxidative catabolism

Following the significant time-dependent and Ca^{2+} mediated increase in ROS levels upon $A\beta_{42}$ ADDL/Glu treatment, we hypothesized that this elevation could be attributed to intensified cellular respiration linked to enhanced catabolic activity, particularly related to glucose metabolism, which is a key molecule supporting the metabolism of SH-SY5Y cells (Kronenberger et al., 2024; Murphy et al., 2022). However, we did not observe any significant changes in glucose uptake of SH-SY5Y cells cultured upon treatment with sub-threshold ADDLs or Glu concentrations, as determined by radioactive tracing analysis using ^{14}C-uniformly labelled glucose (**Fig. 3.8A**).

We then measured O_2 consumption in real-time using the Oroboros oxygraph-2K high-resolution respirometer. After 180 min of treatment with sub-threshold concentrations of ADDLs and Glu, both basal (Routine) and maximal (E) respiration rates remained unaffected relative to the condition without these treatments (**Fig. 3.8B**). This suggests that ADDLs and Glu, at these sub-threshold doses, do not alter mitochondrial O_2 consumption, thus excluding the possibility that the excess of ROS observed in cells treated with Glu/ADDLs arise from an overactive respiratory chain. Importantly, an analysis of non-mitochondrial residual O_2 consumption (ROX) revealed increased ROX levels in both ADDL- and Glu-treated cells (**Fig. 3.8C**), indicating notable O_2 consumption at non-mitochondrial sites within the cell and suggesting a non-mitochondrial source of ROS.

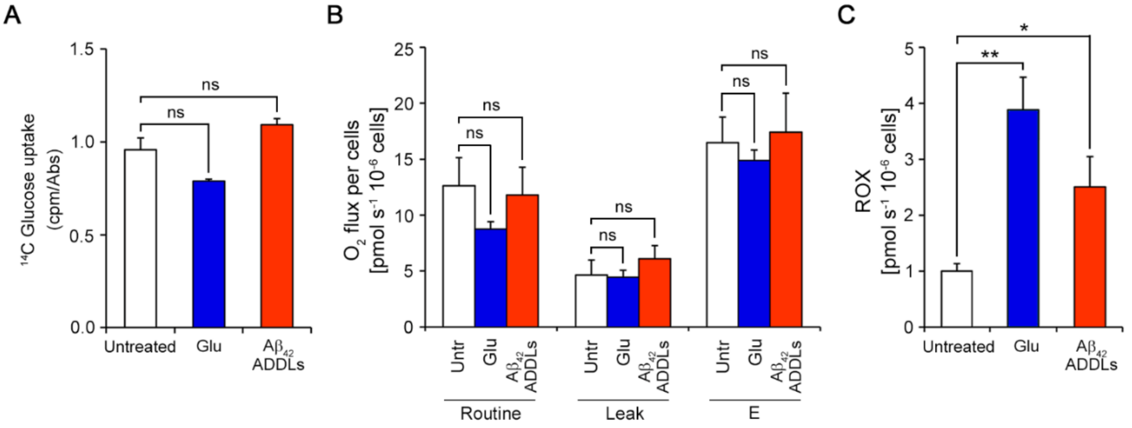

Figure 3.8. Glucose uptake and oxidative catabolism in SH-SY5Y cells treated with Aβ_{42} ADDLs or Glu. (A) ^{14}C-glucose uptake was measured in SH-SY5Y cells left untreated or treated with sub-threshold Aβ_{42} ADDLs or Glu concentrations for 180 min. Data are represented as the mean ± SEM (n=3). **(B)** SH-SY5Y cells were left untreated or treated with sub-threshold ADDLs or Glu concentrations for 180 min. After detachment, cells were subjected to high-resolution respirometry analysis by Oroboros-O2K instrument. The oxygen consumption (O_2 flux per cells) was measured before and after the serial injections of oligomycin, uncoupler CCCP, and antimycin A. Bar chart graph of basal oxygen consumption (Routine), proton leak achieved with oligomycin (Leak) and maximal oxygen consumption achieved with uncoupler CCCP (E) values minus residual oxygen consumption measured with antimycin A (ROX) is shown. Data are represented as the mean ± SEM (n=4). **(C)** ROX was extrapolated from the Oroboros analysis and compared between SH-SY5Y cells left untreated and treated with sub-threshold ADDL or Glu concentrations for 180 min. Data are represented as the mean ± SEM (n=4). The single (∗) and double (∗∗) asterisks refer to p values of <0.05 and <0.01 relative to untreated cells, respectively. ns means non-significant.

3.6 Sub-threshold concentrations of Aβ_{42} oligomers and Glu cause a mitochondria-independent increase of intracellular ROS levels and no mitochondrial dysfunction

In principle, the rate of mitochondrial ROS formation may also increase under conditions of non-intensified cellular respiration, particularly if the mitochondrial function is impaired (Murphy, 2009). To assess this hypothesis, we probed directly mitochondrial ROS using MitoSOX, a probe that emits red fluorescence upon reacting with mitochondrial superoxide ions (Kauffman et al., 2016; Little et al., 2020; Murphy et al., 2022; Robinson et al., 2006). Unlike SH-SY5Y cells incubated with 50 µM Rotenone as a positive control of mitochondrial ROS production (Katila et al., 2021; N. Li et al., 2003), SH-SY5Y cells incubated with either 0.25 µM ADDLs (monomer equivalents) or 60 mM Glu for 60 and 180 min did not show any significant increase in MitoSOX fluorescence (**Fig. 3.9**). This suggests that mitochondria are not responsible for the ROS production

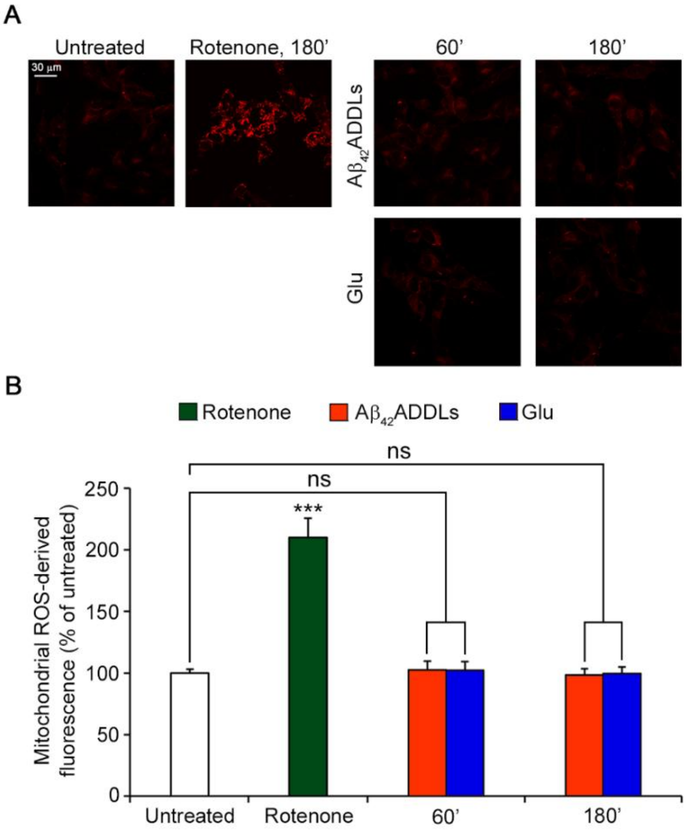

Figure 3.9. Mitochondrial ROS levels in SH-SY5Y cells treated with Aβ_{42} ADDLs or Glu. (A) Representative confocal scanning microscopy images of untreated cells and cells treated with either 0.25 µM Aβ_{42} ADDLs (monomer equivalents) or 60 mM Glu for 60 and 180 min, or 50 µM Rotenone for 180 min as a positive control for mitochondrial ROS formation, revealed with the MitoSOX probe. **(B)** Semiquantitative analysis of mitochondrial ROS-derived fluorescence. Data are represented as the mean ± SEM (n=3). The triple (∗∗∗) asterisks refer to p values <0.001 relative to untreated cells. ns means non-significant.

observed in our conditions of treatment and revealed with the CM-H_2DCFDA probe that detects ROS independently of their subcellular site of formation.

We further investigated whether these sub-threshold treatments could induce mitochondrial dysfunction after 180 min by utilizing the MTT assay, which is dependent on the activity of mitochondrial dehydrogenases present in functional mitochondria (Mosmann, 1983). Neither sub- nor super-threshold concentrations of the two species caused mitochondrial impairment within 180 min (**Fig. 3.10**). Mitochondrial dysfunction became apparent only after 24 h, showing a non-significant or mild effect with sub-threshold treatments and a more pronounced effect with super-threshold treatments (**Fig. 3.10**).

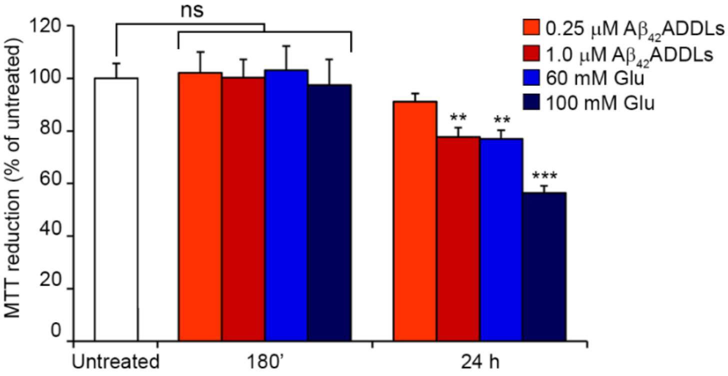

Figure 3.10. Toxicity of sub-threshold concentrations of Aβ$_{42}$ ADDLs and Glu. MTT reduction assay in SH-SY5Y cells treated with 0.25 and 1 µM Aβ$_{42}$ ADDLs (monomer equivalents) or 60 and 100 mM Glu for 180 min and 24 h. Data are represented as the mean ± SEM (n=3). The double (∗∗) and triple (∗∗∗) asterisks refer to p values of <0.01 and <0.001 relative to untreated cells, respectively. ns means non-significant.

Moreover, counting the number of cells using Bürker counting chamber, neither sub- nor super-threshold concentrations of ADDLs and Glu led to significant cell death within 180 min of treatment, excluding cell loss (**Fig. 3.11**). For this reason, we will refer to "sub-threshold" concentrations of these agents also as sub-lethal".

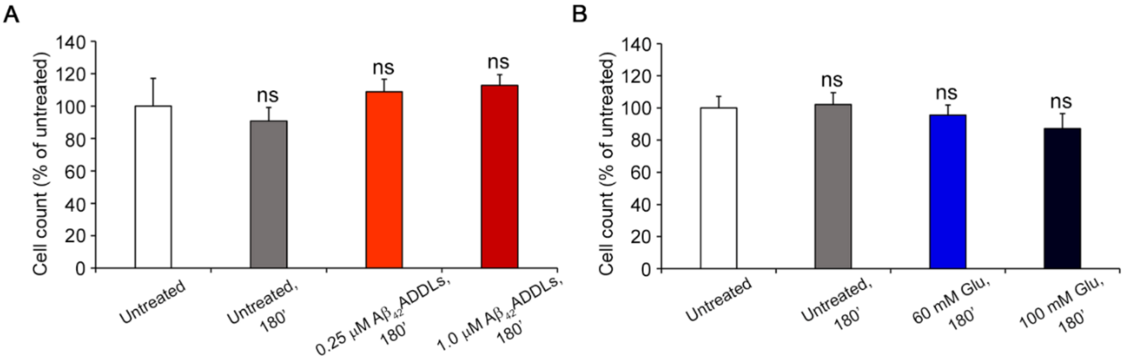

Figure 3.11. Cell count of SH-SY5Y cells treated with Aβ$_{42}$ ADDLs or Glu. (A,B) Quantitative analysis of cells treated with **(A)** 0.25 and 1 µM Aβ$_{42}$ ADDLs (monomer equivalents) or **(B)** 60 and 100 mM Glu for 180 min. Data are represented as the mean ± SEM (n=3). ns means non-significant.

3.7 NADPH oxidases (NOXs) are involved in ROS production induced by sublethal $A\beta_{42}$ oligomers and Glu concentrations

Besides mitochondria, another major source of cellular ROS is the family of NOX enzymes (NOXs), particularly relevant in the context of aging and AD (Brennan et al., 2009; De Almeida et al., 2022; U. Ganguly et al., 2021; Krause, 2007; Shimohama et al., 2000). These cell membrane multi-subunit proteins catalyse the oxidation of NADPH + H^+ and reduction of O_2 with the subsequent formation of O_2^- or H_2O_2 (Begum et al., 2022). A 30 min pre-treatment of cultured SH-SY5Y cells with 5 µM APX115, which is a broad-spectrum pan-inhibitor of all known NOXs, effectively prevented ADDL- and Glu-induced ROS production (**Fig. 3.12**). By contrast, the same 30 min pre-treatment with 5 µM APX115 did not have any effect on the ROS production induced by 50 µM Rotenone incubated for 180 min, which was used as a ROS-inducer independent of NOXs and rather acting on the mitochondrial respiratory chain (**Fig. 3.12**). These findings suggest that NOXs are the primary enzymes responsible for the ROS increase induced by sub-lethal concentrations of ADDLs/Glu in neuroblastoma cells.

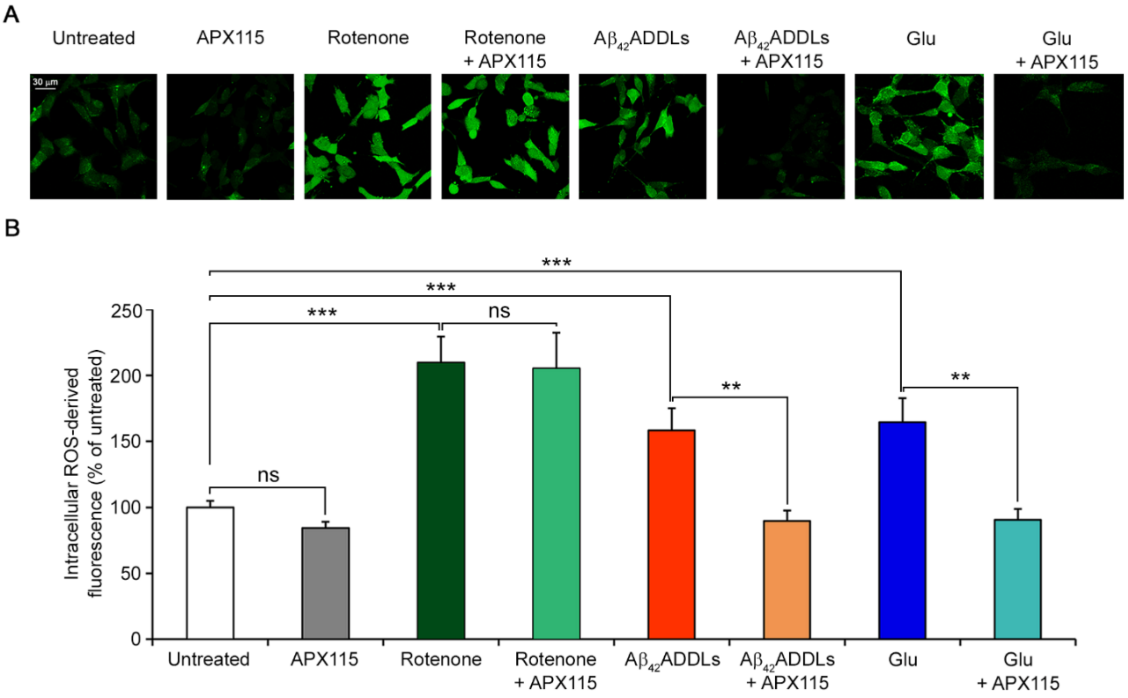

Figure 3.12. Intracellular ROS levels in SH-SY5Y cells treated with $A\beta_{42}$ ADDLs or Glu, after the inhibition of NADPH oxidases (NOXs). (A) Representative confocal scanning microscopy images of untreated cells and cells treated for 180 min with 0.25 µM $A\beta_{42}$ ADDLs (monomer equivalents), or 60 mM Glu, or 50 µM Rotenone as a positive control for mitochondrial ROS formation, with or without pre-treatment with 5 µM APX115 (pan-NOX inhibitor) for 30 min. **(B)** Semiquantitative analysis of intracellular ROS-derived fluorescence. Data are represented as the mean ± SEM (n=3). The double (∗∗) and triple (∗∗∗) asterisks refer to p values of <0.01 and <0.001, respectively. ns means non-significant.

3.8 Effects of sub-threshold concentrations of Aβ_{42} oligomers and Glu on cell metabolomic profiles

Our data presented so far indicate that the upstream Ca^{2+} rise induced by ADDLs/Glu causes a NOX-dependent ROS production in the cells. This may result from either the Ca^{2+}-mediated downstream activation of NOX enzymes (NOXs) or a Ca^{2+}-dependent shift in cellular metabolism leading to excessive production of NADPH, which is the substrate of NOXs (see *Discussion* for details). We therefore investigated with nuclear magnetic resonance (NMR) spectroscopy the possible changes of the metabolic profile of SH-SY5Y cells treated with either ADDLs or Glu at sub-threshold concentrations, for both the cell medium (exometabolome) and cell lysate (endometabolome) following these treatments. In particular, we recorded the ^{1}H one-dimensional NMR spectra for each treatment. The various NMR resonances of each spectrum were assigned to well defined metabolites and their relative concentrations quantified, as described in the *Materials and Methods* section. A panel of 41 and 32 metabolites were identified and quantified in cell lysates and cell media, respectively, as shown here for the two representative spectra in untreated cells (**Fig. 3.13-3.14**).

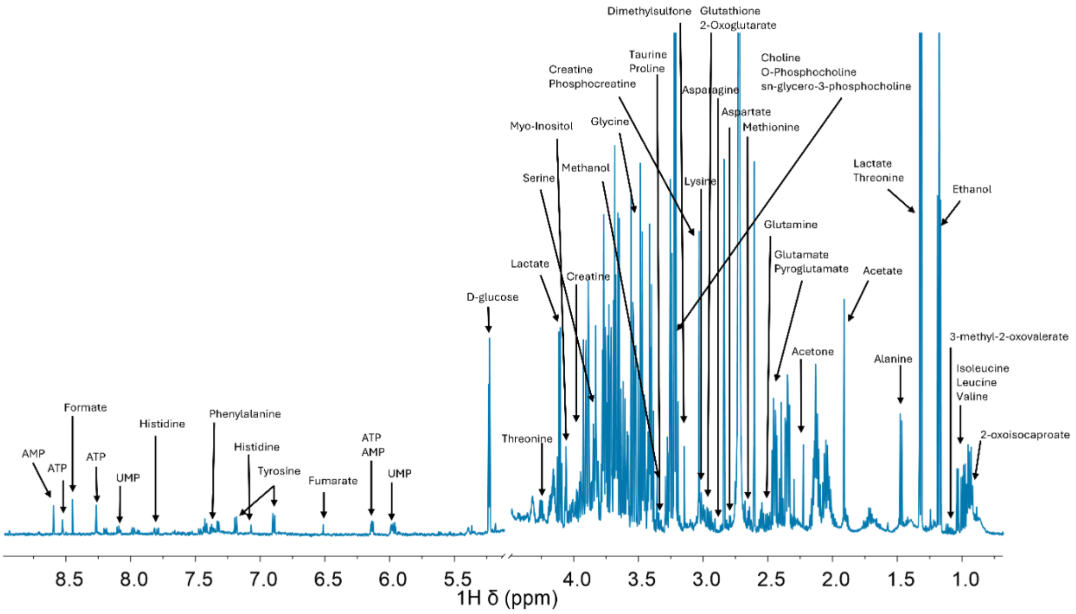

Figure 3.13. Representative ^{1}H CPMG NMR spectrum of the endometabolome (cell lysate) of an untreated SH-SY5Y cell line. Metabolites assigned and quantified are reported.

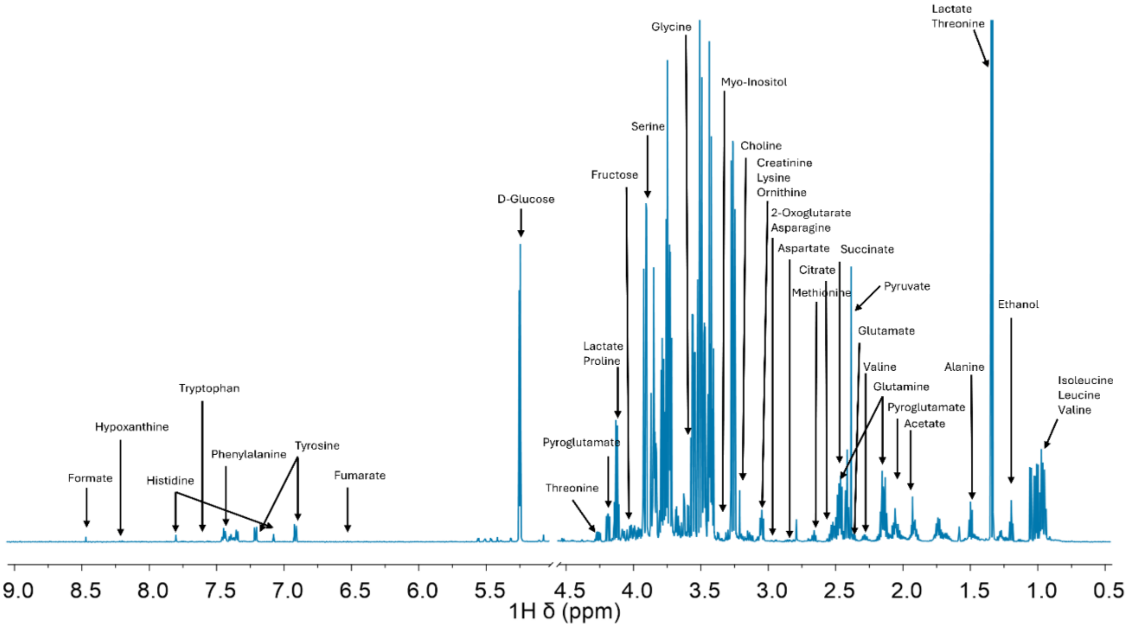

Figure 3.14. Representative ^{1}H CPMG NMR spectrum of the esometabolome (cell medium) of an untreated SH-SY5Y cell line. Metabolites assigned and quantified are reported.

The ADDL treatment did not induce any significant change in the monitored metabolites, relative to untreated cells, in either the endo- or exometabolome (**Fig. 3.15A,B**). Only extracellular levels of phenylalanine were found to be significantly increased with respect to untreated cells. This indicates that the treatment with sub-lethal concentrations of ADDLs does not alter cell metabolism to any significant extent.

The Glu treatment induced significant alterations of cell metabolism altering the levels of several intra- and extracellular metabolites as compared to both ADDLs and untreated cells (**Fig. 3.15A,B**). The intracellular levels of 15 amino acids appeared to be significantly increased in Glu treated cells (**Fig. 3.15A**), whereas the extracellular levels of 5 amino acids appeared decreased (**Fig. 3.15B**). Furthermore, in Glu treated cells we observed reduced intracellular levels of 2-oxoisocaproate and 3-methyl-2-oxovalerate, two metabolites involved in the catabolism of leucine and valine, respectively. Both the intra- and extracellular levels of alanine and α-ketoglutarate (or 2-oxoglutarate) were significantly higher in the Glu treatment as compared to both ADDLs and untreated cells (**Fig. 3.15**), probably because cells take advantage of the high availability of Glu to convert it directly into Ala and α-ketoglutarate through transamination. Glu treatment is also associated with significantly reduced intracellular levels of ATP, AMP, glutathione and fumarate as well as reduced extracellular levels of citrate, fumarate, lactate and fructose. Furthermore, in Glu treated cells we observed significantly decreased extracellular levels of choline and myo-inositol and increased intracellular levels of sn-glycero-3-phosphocholine, taurine and phosphocreatine.

Overall, a significant alteration of the cell metabolome is observed only following treatment with 60 mM Glu, most probably because of the high availability of this metabolite and the intermediates represented by Glu and its derivatives in many metabolic pathways. However, none of these metabolic changes are found to occur following ADDL treatment, ruling out their involvement in the NOX-mediated ROS production that is, by contrast, observed after both treatments.

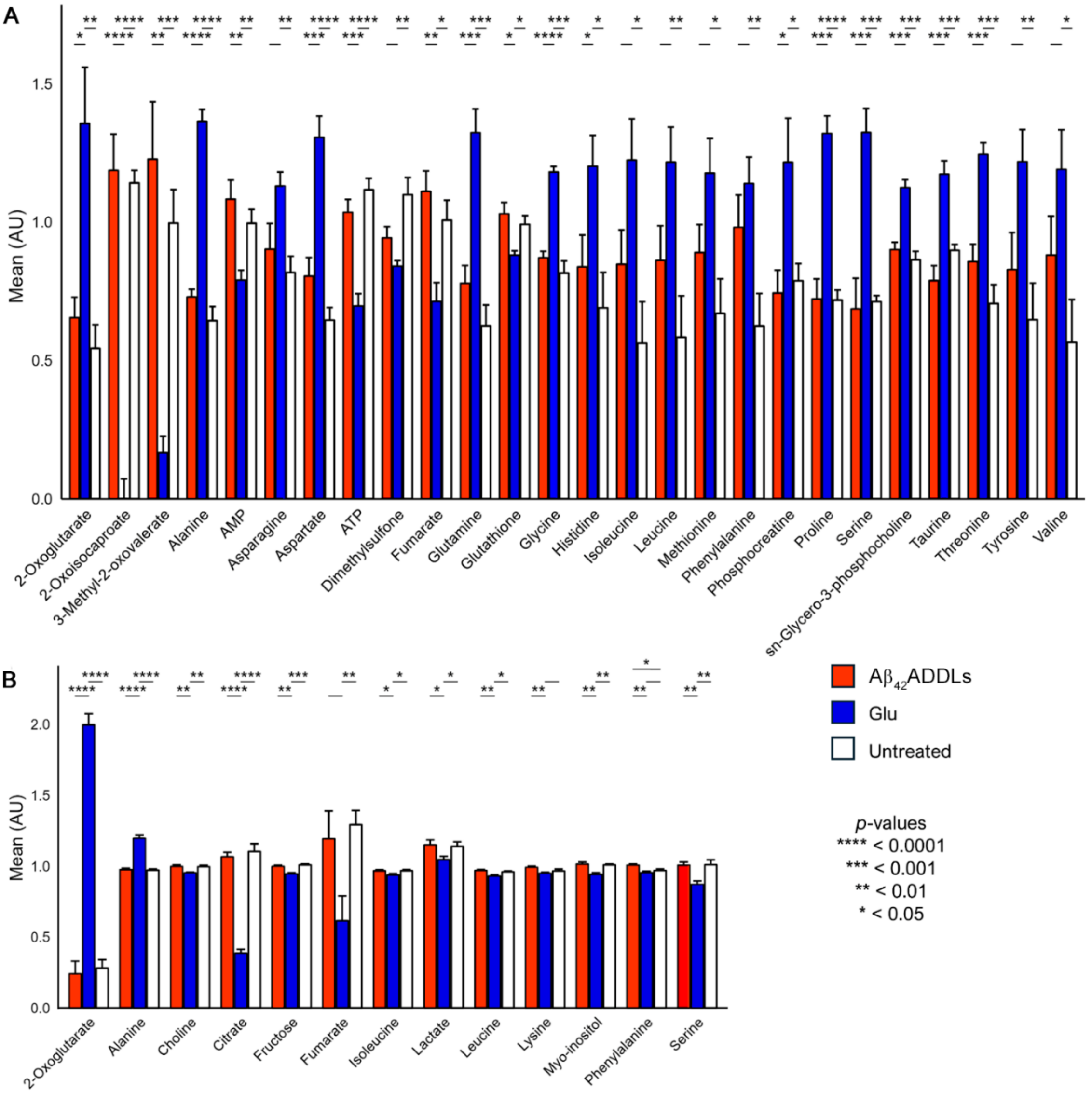

Figure 3.15. NMR spectroscopy of the metabolic profile of SH-SY5Y cells treated with Aβ_{42} ADDLs or Glu. NMR-determined relative concentrations of metabolites in untreated cells or cells treated with 0.25 µM Aβ_{42} ADDLs (monomer equivalents) or 60 mM Glu in comparison to untreated cells, for both cell lysates (intracellular, top; **A**) and cell media (extracellular, bottom; **B**), after 180 min of treatment.

Moreover, treatment with ADDLs or Glu did not lead to significant changes of the pentose phosphate pathway (PPP) flux, which is the main metabolic pathway responsible for NADPH production (**Fig. 3.16**). In particular, although non statistically significant, Glu administration shows a reduced PPP flux trend. Since Glu can take part in multiple enzymatic reactions in these cell models, including those catalyzed by amino transferases enzymes and Glu dehydrogenase, it is difficult to speculate the potential metabolic destiny. However, the alteration of the metabolome observed after Glu administration did not suggest a higher exploitation of the PPP, ruling out the production of NADPH by a stimulated PPP.

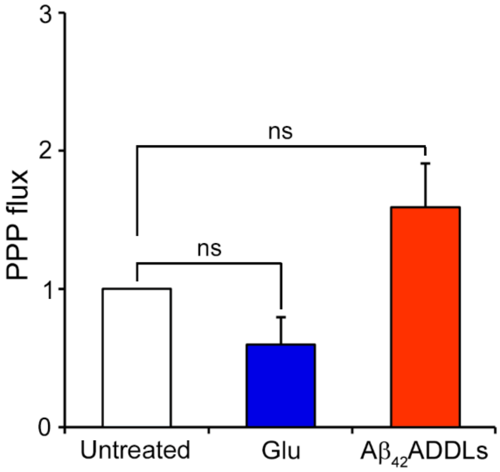

Figure 3.16. PPP flux of SH-SY5Y cells treated with Aβ$_{42}$ ADDLs or Glu. PPP flux was measured in SH-SY5Y cells left untreated or treated with sub-threshold of Aβ$_{42}$ ADDLs or Glu concentrations for 180 min. PPP flux was calculated by measuring $^{14}CO_2$ derived from ^{14}C radioactive glucose as detailed in the Materials and Methods section. Data are represented as the mean ± SEM (n=3). ns means non-significant.

3.9 Sub-threshold concentration of Aβ$_{42}$ oligomers causes a Ca^{2+}-mediated, partial NOX-dependent, increase of intracellular ROS levels in primary neurons

All results presented so far were obtained on human neuroblastoma SH-SY5Y cells. Although they are widely employed for studying *in vitro* neurotoxicity and neurodegenerative diseases (Capitini et al., 2014; Y. Fang et al., 2014; Fani et al., 2021, 2022; H. Xu et al., 2019), they are not able to reproduce the complexity of neurons. For this reason, we repeated key experiments on primary rat cortical neurons focusing on the action of the Aβ$_{42}$ ADDLs.

We first identified 0.050 μM ADDLs (monomer equivalents) as the highest sub-threshold concentration that did not cause a significant rise of intracellular Ca^{2+} (**Fig. 3.17A,B**). This was much lower than that identified on SH-SY5Y cells, emphasizing the higher sensitivity of primary neurons as compared to SH-SY5Y cells. We also observed that 0.050 μM ADDLs induced an enhancement of ROS production in primary rat cortical neurons in a time-dependent manner, as assessed with the CM-H_2DCFDA probe (**Fig. 3.17C,D**).

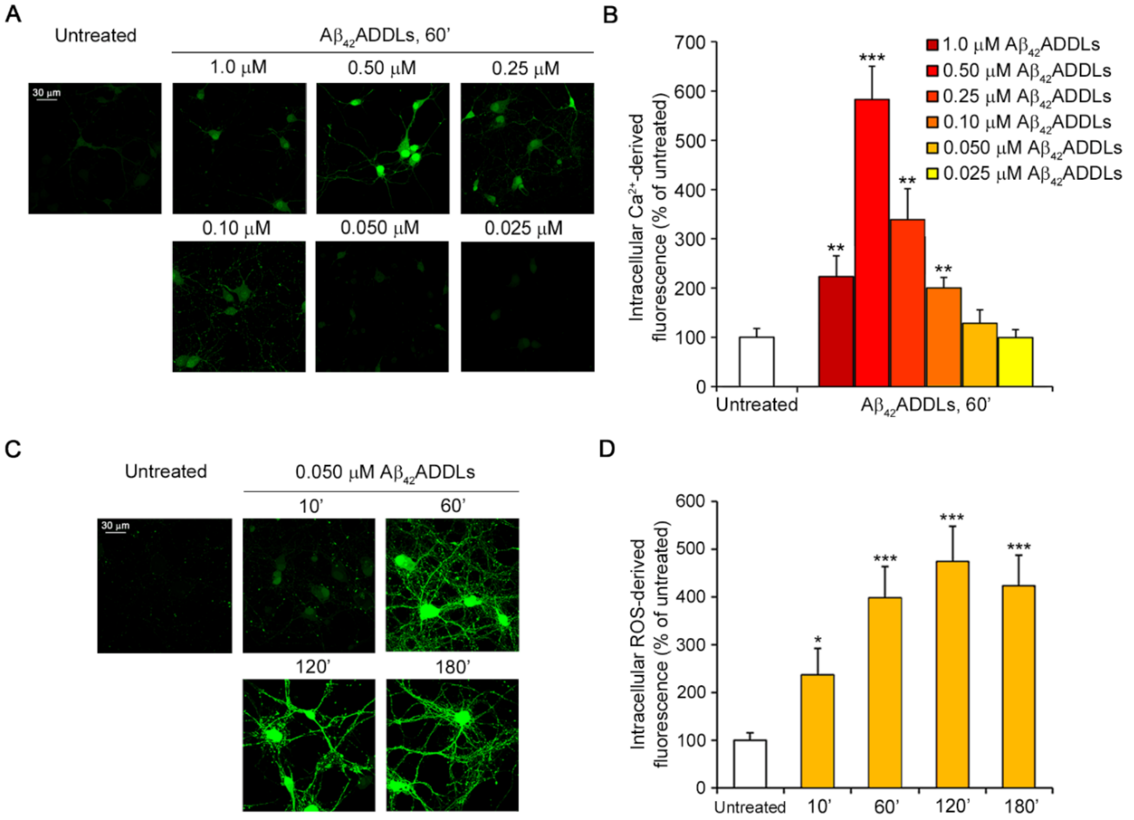

Figure 3.17. Intracellular Ca^{2+} and ROS levels in primary rat cortical neurons treated with $A\beta_{42}$ ADDLs. (A) Representative confocal scanning microscopy images of untreated cells and cells treated with decreasing concentrations of $A\beta_{42}$ ADDLs (1.0-0.025 μM, monomer equivalents) for 60 min. **(B)** Semiquantitative analysis of intracellular Ca^{2+}-derived fluorescence shown in panel A. **(C)** Representative confocal scanning microscopy images of untreated cells and cells treated with 0.050 μM ADDLs for 10, 60, 120 and 180 min. **(D)** Semiquantitative analysis of intracellular ROS-derived fluorescence shown in panel C. In both histograms, data are represented as the mean ± SEM (n=3). Single (∗), double (∗∗) and triple (∗∗∗) asterisks refer to p values of <0.05, <0.01 and <0.001 relative to untreated cells, respectively.

Next, we treated the cultured neurons with 0.050 μM $A\beta_{42}$ ADDLs for 10 and 60 min after a pre-treatment with 5 μM APX115 for 30 min or with a concomitant pre-treatment with 5 μM CNQX and 10 μM Memantine for 60 min (**Fig. 3.18A,B**). Pre-treatment with APX115 decreased significantly ROS production, as indicated by the CM-H_2DCFDA probe, but did not lead to background levels of untreated cells (**Fig. 3.18A,B**). By contrast, pre-treatment with Memantine and CNQX showed a complete reduction of

ROS levels, indicating that the effect was mediated by a Ca^{2+} influx mediated by AMPARs and NMDARs (**Fig. 3.18A,B**).

We then treated primary rat cortical neurons with 0.050 µM ADDLs for 10 and 60 min and monitored ROS formation with the MitoSOX probe, specific for mitochondrial superoxide ion production, observing a significant rise of its fluorescence, although not as marked as that induced by rotenone (**Fig. 3.18C,D**). These findings indicate a mitochondrial involvement, together with NOXs, in the production of ROS in primary rat cortical neurons under these conditions of treatment, still mediated by the Ca^{2+} influx.

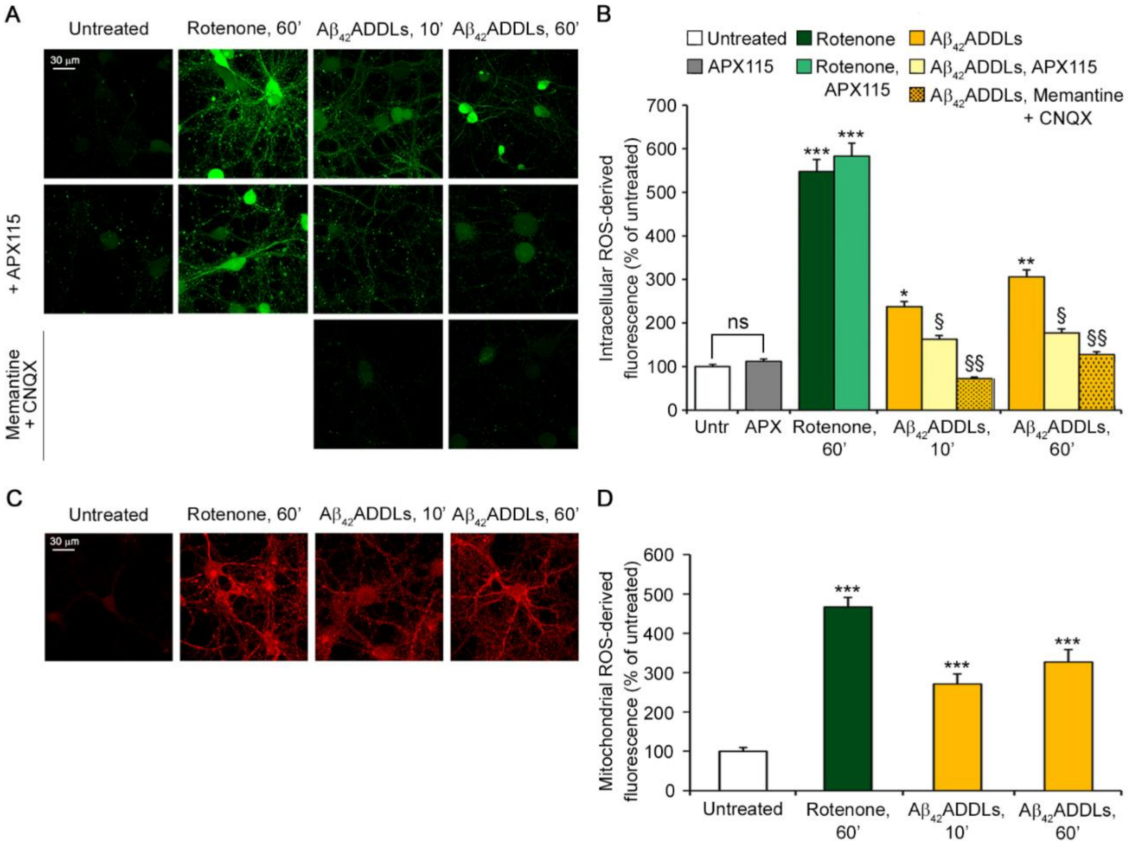

Figure 3.18. Total and mitochondrial ROS levels in primary rat cortical neurons treated with Aβ$_{42}$ ADDLs and specific inhibitors. (A) Representative confocal scanning microscopy images following the treatment with 0.050 µM Aβ$_{42}$ ADDLs (monomer equivalents) for 10 and 60 min, and 5 µM Rotenone for 60 min as a positive control, with or without pre-treatment with 5 µM APX115 (pan-NADPH oxidase inhibitor) for 30 min, or with or without pre-treatment with 5 µM CNQX (AMPAR inhibitor) and 10 µM Memantine (NMDAR inhibitor) for 60 min. Green fluorescence indicates intracellular ROS detected with the CM-H$_2$DCFDA probe. **(B)** Semiquantitative analysis of intracellular ROS-derived fluorescence. Data are represented as the mean ± SEM (n=3). **(C)** Representative confocal scanning microscopy images of cells treated with 0.050 µM ADDLs for 10 and 60 min, or 5 µM Rotenone for 60 min as a positive control for mitochondrial ROS formation. Red fluorescence indicates mitochondrial ROS detected with MitoSOX probe. **(D)** Semiquantitative analysis of mitochondrial ROS-derived fluorescence. Data are represented as the mean ± SEM (n=3). In all panels, the single (∗), double (∗∗) and triple (∗∗∗) asterisks refer to p values of <0.05, <0.01 and <0.001 relative to untreated cells, respectively. The single (§) and double (§§) symbol refers to p values of <0.05 and <0.01 relative to cells treated with ADDLs without inhibitors. ns means non-significant.

3.10 Contributions and collaborations

The Oroboros O2k Analysis and the Radiolabelled Glucose Uptake assay described in *Section 3.5*, and the Pentose Phosphate Pathway (PPP) flux radioactive assay described in *Section 3.8* were conducted in cooperation with the research group led by Prof. Andrea Morandi, with the assistance from Dr Marina Bacci and Dr Nicla Lorito, of the Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Section of Biochemistry, University of Florence, Viale Morgagni 50, Florence, Italy.

The ^1H NMR Spectroscopy experiments described in *Section 3.8* were conducted in cooperation with the research group led by Prof. Leonardo Tenori, with the assistance from Dr Alessia Vignoli, of the Department of Chemistry "Ugo Schiff", University of Florence, Sesto Fiorentino, Italy, and at the Magnetic Resonance Center (CERM), University of Florence, 50019 Sesto Fiorentino, Italy.

4. Discussion

Hippocampal hyperactivity has been assessed as a key emerging biological trait in clinical cases, animal models and iPSC-derived neurons in prodromal AD and even in preceding non-symptomatic and biomarker-free phases, even before Aβ plaque deposition (Aizenstein & Klunk, 2015; Bookheimer et al., 2000; Busche et al., 2012; Cox et al., 2022; Dennis et al., 2010; Findley et al., 2019; Garcia-Alloza et al., 2006; K. N. Hascup et al., 2020; Huijbers et al., 2015; S. C. Johnson et al., 2006; Lin et al., 2018; Olney, 1997; Savonenko et al., 2005; Sinha et al., 2018; M. Wang et al., 2023; Zott et al., 2019). Hyperactivity is characterized, among other factors, by a persistent and chronic activation of NMDARs (NRHyper phase). Both soluble Aβ oligomers and Aβ-induced aberrant Glu signaling are involved in the NRHyper phase (Abdul et al., 2009; Acosta et al., 2017; Han et al., 2016; E. R. Hascup et al., 2019; K. N. Hascup & Hascup, 2016; Lovell et al., 2012; Qin et al., 2006; Schallier et al., 2011; Scimemi et al., 2013; Sokolow et al., 2012; Talantova et al., 2013; X.-K. Yang et al., 2019; Zumkehr et al., 2015). Here we developed two cell culture model systems using, first, immortalised and undifferentiated SH-SY5Y cells and, then, primary rat cortical neurons to investigate how cells respond to the hyperexcitation induced by sub-lethal concentrations of $A\beta_{42}$ oligomers and Glu, trying to mimic the pathological conditions observed in the early stages of AD and even before. We also referred to these sub-lethal concentrations as "sub-threshold", implying that they do not lead to a gross and observable increase of intracellular Ca^{2+} concentration and other dysfunctional events.

In the first model system, the sub-threshold $A\beta_{42}$ ADDLs/Glu treatments did not lead to an increase of intracellular Ca^{2+} concentration, mitochondrial dysfunction, cell loss, or substantial metabolic changes over the 3h time scale investigated, unlike normally used doses. A significant alteration of the cell metabolome is observed only with Glu, most probably because of the high availability of this amino acid and its derivatives in many metabolic pathways. Primary rat cortical neurons also showed the absence of a rise of intracellular Ca^{2+} concentration, although neurons appeared to be more sensitive to ADDLs than SH-SY5Y cells, requiring lower doses as sub-effective concentrations, that is 0.050 μM rather than 0.25 μM ADDLs (monomer equivalents). Nevertheless, despite this apparent lack of effects, sub-threshold concentrations of $A\beta_{42}$ oligomers and Glu do induce an influx of Ca^{2+} ions within SH-SY5Y neuroblastoma cells by activating NMDARs and possibly other receptors, such as AMPARs and KARs, but this influx is effectively counteracted by the three major Ca^{2+} pumps, such as PMCA, SERCA and NCX, which restore the physiological intracellular Ca^{2+} concentration. Indeed, it is just by inhibiting these pumps that we could see a significant increase in the intracellular Ca^{2+} levels under these conditions of sub-threshold treatment.

In both cell models, we observed a time-dependent increase of intracellular ROS levels under these conditions of treatment, which appears to be mediated by NMDARs and AMPARs and their consequent Ca^{2+} influx, as their respective inhibitors Memantine and CNQX, or a Ca^{2+}-free cell medium, were able to inhibit ROS production. The observed ROS increase upon ADDL/Glu treatments may be due to enhanced mitochondrial respiration required to satisfy an increased ATP need by Ca^{2+} pumps to restore Ca^{2+} homeostasis. Surprisingly, mitochondrial respiration did not appear to be the source of ROS in SH-SY5Y cells under these conditions, because ROS remained undetected with the MitoSOX probe specific for mitochondrial ROS and basal or maximal mitochondrial respiration was found to be unaffected as measured with Oroboros O2k high-resolution respirometry. Accordingly, non-mitochondrial residual oxygen consumption (ROX) was significantly increased with both ADDL/Glu treatments, suggesting a non-mitochondrial source of ROS.

Possible sources of extramitochondrial ROS within cells may be NADPH oxidases (NOXs), xanthine oxidase, cytochrome P450, cyclooxygenase, lipoxygenases, microsomal enzymes, nitric oxide synthase, ER, and peroxisomes (De Almeida et al., 2022; Holmström & Finkel, 2014; J. Kim & Moon, 2024). NOXs appeared to be the main source of ROS in our SH-SY5Y cell system under these conditions of treatment, following the observation that pre-treatment of the cells with the NOX pan-inhibitor APX115 suppressed completely ROS generation in response to sub-threshold ADDL/Glu treatments. In cortical rat primary neurons, NOXs were also found to be largely involved, although in this case the APX115 inhibitor did not completely cancel ROS production and the MitoSOX assay was positive, indicating additional mitochondrial ROS production in neurons, which are indeed metabolically more active than neuroblastoma cells. However, our studies on neuroblastoma cells have allowed us to identify the contribution of NOXs in ROS production under this regime of sub-threshold treatment, whose presence was then confirmed and validated in primary neurons.

NOX enzyme family has gathered significant attention in the pathophysiology of neurodegenerative diseases including AD (Brennan et al., 2009; U. Ganguly et al., 2021; Ha et al., 2010; Harraz et al., 2008; Hou et al., 2019; Krause, 2007; Ma et al., 2017;

Minnella et al., 2018; Shimohama et al., 2000; Tarafdar & Pula, 2018; Y. Zhang et al., 2016). The Ca^{2+} dependence of NOX activation observed here can be explained by the close linkage between NOXs and Ca^{2+} signalling, through multiple regulatory mechanisms. Ca^{2+} can activate the CaM/CaMKII/p38MAPK pathway (Kyrmizi et al., 2018; Lu et al., 2013; Roe & Ren, 2013; L. J. Zhu et al., 2014), with the latter p38MAPK kinase activating directly, by phosphorylation, the cytosolic NOX2 $p47^{phox}$ subunit, which is a key regulator of NOX activation and complex assembly (Lu et al., 2012). Ca^{2+} can also bind to and activate PKC and its regulating enzyme phospholipase C (Brennan et al., 2009; Choi et al., 2008), with the α, βII, δ, γ and ζ isoforms of PKC activating directly, by phosphorylation, the p47phox subunit of NOX2 (Fontayne et al., 2002; Kelher et al., 2017). Ca^{2+} ions can activate the Ca^{2+}- and calmodulin-dependent serine/threonine protein phosphatase calcineurin (CaN), leading to the activation of nuclear factor of activated T cells (NFAT), which induces the overexpression of the NOX2/4 genes (Williams & Gooch, 2014). Finally, Ca^{2+} ions are activators of three isoforms of NOXs (NOX5/DUOX1/DUOX2) by direct binding to EF-hand motifs, which are Ca^{2+}-binding domains located in the N- and C-terminal cytoplasmic regions (Bánfi et al., 2004; Faria & Fortunato, 2020). Among them, NOX5 is present in SH-SY5Y cells (Yamamuro-Tanabe et al., 2024), and DUOX1 and DUOX2 are present in the brain (Damiano et al., 2012).

The Ca^{2+} dependent activation of NOXs, induced by the typical sub-lethal rise of Aβ/Glu associated with AD-preceding neuronal hyperactivation, can explain very recent observations on the increase of key proteins involved in NOX regulation by many years before AD. In fact, in a very recent large proteomic study carried out on the CSFs of 291 familial AD and 185 age-matched control cases, over 500 proteins were identified with significantly different levels ($p<0.01$) in the two groups starting fifteen-to-five years before clinical onset (Y. Shen, Ali, et al., 2024; Y. Shen, Timsina, et al., 2024). Among them were two protein subunits of the calcineurin complex (PPP3R1, PPP3CA, $p<10^{-17}$), PKCγ (gene name PRKCG, $p<10^{-5}$), PLC-beta-1 (PLCB1, $p<10^{-7}$), three isoforms of CaMKII (CaMK2A/B/D, $p<10^{-3}$) and four isoforms of p38MAPK (MAPK1/9/10/12, $p<10^{-2}$). As mentioned above, these proteins are factors involved in the Ca^{2+}-dependent activation of the NOXs class and, in all cases, protein levels were higher, rather than lower, in the AD group relative to the control one. By contrast, none of the protein subunits of the ionotropic Glu receptors (NMDARs/AMPARs/KARs) or NOX isoenzymes had different levels. It therefore appears that in the presence of neuronal hyperactivation preceding AD, when concentrations of Glu and Aβ oligomers (but not plaques) are already high and Ca^{2+} signaling is already dysfunctional, upregulated concentrations of factors involved in the Ca^{2+}-dependent NOX activation may contribute to the production of NOX-dependent ROS, which appears a key biochemical change in the two cell models studied here under conditions that are aimed at mimicking this regime.

We can exclude that the NOX-mediated oxidative stress induced by $A\beta_{42}$ oligomers and Glu is caused by an overproduction of the NOX substrate NADPH, because the metabolic profile of SH-SY5Y cells was not significantly affected in our conditions of treatment. The PPP pathway, which is the main producer of NADPH, was also unaffected. Additionally, using NMR spectroscopy we observed that, while Glu exposure led to significant changes in the amino acid metabolite profiles, probably due to the large availability of Glu, ADDL exposure alone did not cause significant metabolic alterations. Thus, NOX activation in our cell system and conditions of treatment is not associated with modifications of cellular metabolism on the short term. Indeed, most of the experiments focused on relatively short exposure times (up to 3 h), which were specifically selected to investigate the earliest cellular responses to sub-threshold excitatory stimulation.

In conclusion, we have set up neuroblastoma and primary neuron cell cultures treated with sub-threshold concentrations of $A\beta_{42}$ ADDLs and Glu as model systems to study biochemical, metabolic and signal transduction changes occurring in the earliest phases preceding preclinical AD. These changes occur even before the first classical CSF and imaging biomarkers of AD are detectable with the AD continuum of the AT(N) scheme, when neuronal hyperactivation is already present.

Our findings demonstrate that even minor variations of Ca^{2+} homeostasis induced by sub-lethal concentrations of these agents, in which overall intracellular Ca^{2+} concentration remains unaffected, trigger a slow time-dependent ROS production. This response is primarily driven by NOX enzymes (NOXs) through Ca^{2+}-activated regulation pathways.

These findings raise the hypothesis that an early, often undetectable, imbalance in neuronal excitability, mediated by Ca^{2+} dysregulation, triggers a cascade of oxidative stress events that may contribute to neurodegeneration progression. Supporting this hypothesis, proteins involved in Ca^{2+}-dependent NOX regulation are largely represented among those found to be upregulated in carriers of AD-causing mutations before disease onset. This underscores the potential role of these processes/pathways in AD pathophysiology and highlights their relevance for early biomarker identification.

More generally, the cell culture models and conditions of treatment established in this study offer a framework for investigating the long-term biological, molecular, metabolic, and signalling effects of NOX-mediated ROS production in neurons, and for providing insight into the early molecular events preceding MCI/AD, even before the preclinical biomarker-positive phase. Future studies may extend these investigations to longer exposure and further explore the effects of sub-threshold concentrations of Glu and Aβ oligomers in iPSCs-derived neurons, in order to better characterise the chronic consequences of this early hyperexcitability-associated oxidative response.

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