Introduction

1. Introduction

Chapter 1

1.1. Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the gradual loss of neurons and synapses, particularly affecting the brain's cholinergic system (1). AD is marked by cognitive deficits, including memory loss, language difficulties, reasoning impairments, and changes in personal behavior (2). The symptoms of AD that appear due to neuronal damage are not limited to the brain's region responsible for cognitive function but may extend to neurons in different brain parts. As the disease advances, non-cognitive symptoms, such as behavioral disturbances and psychiatric issues like agitation, hallucinations, depression, and delusions, become more pronounced (3-8).

In 1907, German psychiatrist Alois Alzheimer was the first to document AD after studying a patient named Auguste D, who exhibited severe memory loss, irrational fears, and psychological disturbances (9). A histopathological analysis of her brain revealed significant atrophy and the presence of abnormal deposits in and around nerve cells, which led to the identification of two distinct types of brain lesions: senile plaques and neurofibrillary tangles. These findings demonstrated that a specific disease affected the cerebral cortex. (10).

The exact molecular mechanisms responsible for neurodegeneration in AD are not precise yet; however, there is a plethora of evidence, including publications from our research group, that low levels of neurotransmitters, especially ACh, amyloid-beta (Aβ) aggregates, oxidative stress (OS), and the concentrations of metals interdependently play a key role in the neurodegeneration process.

ACh and BCh are essential neurotransmitters in learning and memory. Evidence from the literature and ongoing research strongly suggests that these neurotransmitters play a significant

role in the pathogenesis of AD (11). A decline in both the levels and functionality of ACh characterizes AD. The metabolism of ACh occurs in the synaptic cleft, where it is regulated by acetylcholinesterase (AChE), maintaining its balance in a healthy brain. Therefore, inhibiting AChE and butyrylcholinesterase (BChE) can provide symptomatic relief and serve as an effective strategy in treating AD.

AD's pathological hallmarks are characterized by the accumulation of Aβ plaques outside the neurons and neurofibrillary tau protein tangles (NFTs) inside the diseased neurons. In recent years, mounting evidence has driven the hypothesis that these markers probably appear many years before the onset of cognitive symptoms of AD (12). These plaques and neurofibrillary tangles are mainly found in the hippocampus, amygdala, entorhinal cortex, and basal forebrain, which are responsible for memory, learning, and emotional behaviors (13).

Oxidative stress (OS) and excessive amounts of iron can lead to the generation of reactive oxygen species (ROS). These ROS inhibit mitochondrial respiration and promote the aggregation of Aβ plaques in the form of intracellular plaques and extracellular neurofibrillary tangles. Although the specific reason for AD is unknown, age and genetic factors also play a very critical role in the disease process (14).

1.2. Statistics of AD

AD is the leading cause of dementia and the sixth-leading cause of death in the United States (15). One in ten people older than 65 in the USA is suffering from AD. Approximately one in ten Americans aged 65 or older is affected by AD. According to the *Alzheimer's Disease Facts and Figures (2023)* report, while mortality rates for stroke, HIV, and heart disease have significantly declined over the past two decades, deaths due to AD have risen by 145% (16). Globally, an estimated 55 million people live with AD and related dementias, a number projected to exceed

152 million by 2050 if effective treatments are not developed (17). India is also expected to face a significant rise in dementia cases due to its aging population and declining fertility rates (18). By 2050, 19.1% of India's population is expected to be aged 60 or older. According to the *Dementia in India 2023* report, 8.8 million Indians aged 60 and above were living with dementia in 2020, with this figure projected to rise to 16.9 million by 2036 (19). Regional variations in dementia prevalence have been observed, with higher rates reported in southern India compared to rural northern regions. The increasing prevalence underscores the urgent need for comprehensive epidemiological studies across different parts of India. According to the *Alzheimer's Disease International* report, the global annual cost of dementia currently exceeds US$ 1.3 trillion and is expected to rise to US$ 2.8 trillion by 2030.

1.3. Pathophysiology of AD

1.3.1. Role of acetyl and butyrylcholines in AD

The brain is a highly intricate organ composed of organized networks of neurons that communicate via neurotransmitters. Cholinergic neurons, which release the neurotransmitters acetylcholine (ACh) and butyrylcholine (BCh), play a crucial role in learning and memory processes (20). Cholinergic neurons secrete ACh as their neurotransmitter. ACh is one of the major neurotransmitters involved in learning and memory. The brain regions most affected by neuronal loss in AD are essentially made of cholinergic neurons; therefore, restoring physiological ACh levels have been considered a viable therapy for AD (21, 22). ACh and BCh are critical neurotransmitters in learning and memory. The evidence from the literature and growing body of research findings strongly indicated these neurotransmitter's crucial role in AD etiology (23-26). The cholinergic hypothesis of AD posits that a deficit in cholinergic signaling within the cortex significantly contributes to the cognitive decline observed in AD patients. A

reduction in the concentration and function of ACh marks the disease. ACh is synthesized in presynaptic nerve terminals through the combination of choline and acetyl coenzyme A (acetyl-CoA), a reaction catalyzed by choline acetyltransferase (ChAT), the rate-limiting enzyme in ACh synthesis. Once produced, ACh is stored in presynaptic vesicles and released into the synaptic cleft during neuronal communication. Upon release, ACh binds to receptors on postsynaptic target cells, facilitating nerve impulse transmission and inter-neuronal communication.

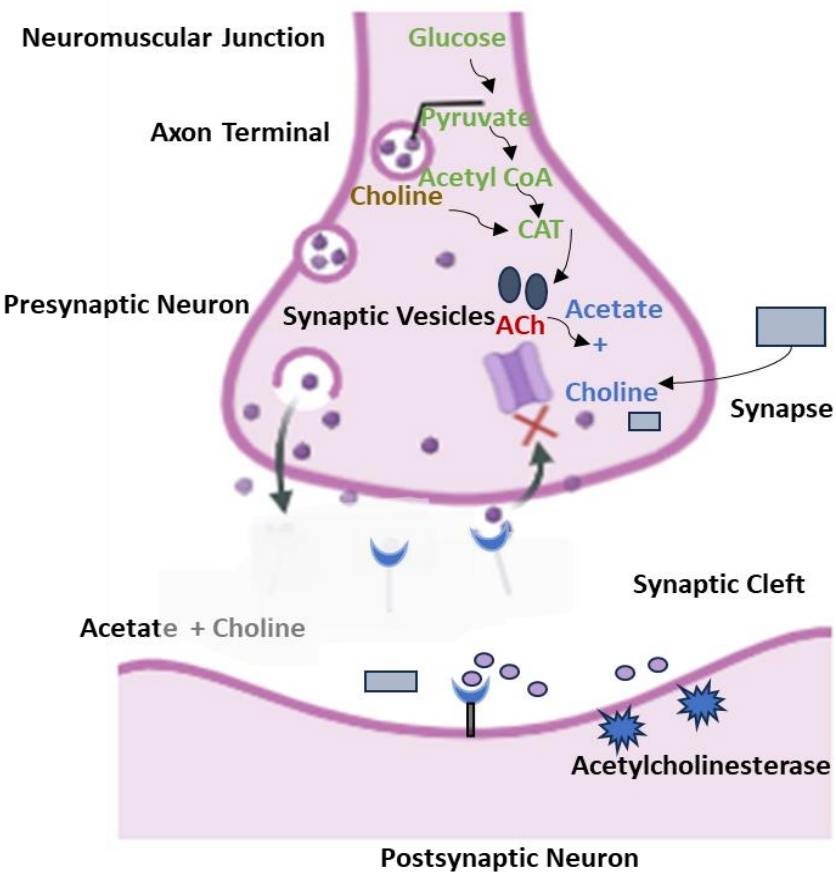

Figure 1.1: Diagrammatic representation of ACh synthesis, metabolism, and its mode of action.

The metabolism of ACh occurs in the synaptic cleft and is primarily regulated by AChE, an enzyme responsible for breaking down ACh and maintaining its balance in the brain (**Figure 1.1**) (27). The serine residue in the active site is rendered highly nucleophilic through a charge

relay system involving hydrogen bonding among the glutamate carboxyl, the histidine imidazole (His447), and the hydroxyl of the serine (Ser203) (28) (**Figure 1.2**). This environment allows the hydroxyl anion to attack the carbonyl carbon of ACh, resulting in the acylation of the serine residue and the release of choline. Deacylation of the serine residue is facilitated by water molecules, releasing acetic acid and restoring the enzyme's active site. This enzymatic process ensures the dynamic regulation of ACh levels, enabling efficient neural signaling (29). Understanding this pathway offers potential therapeutic insights for managing AD symptoms and slowing disease progression.

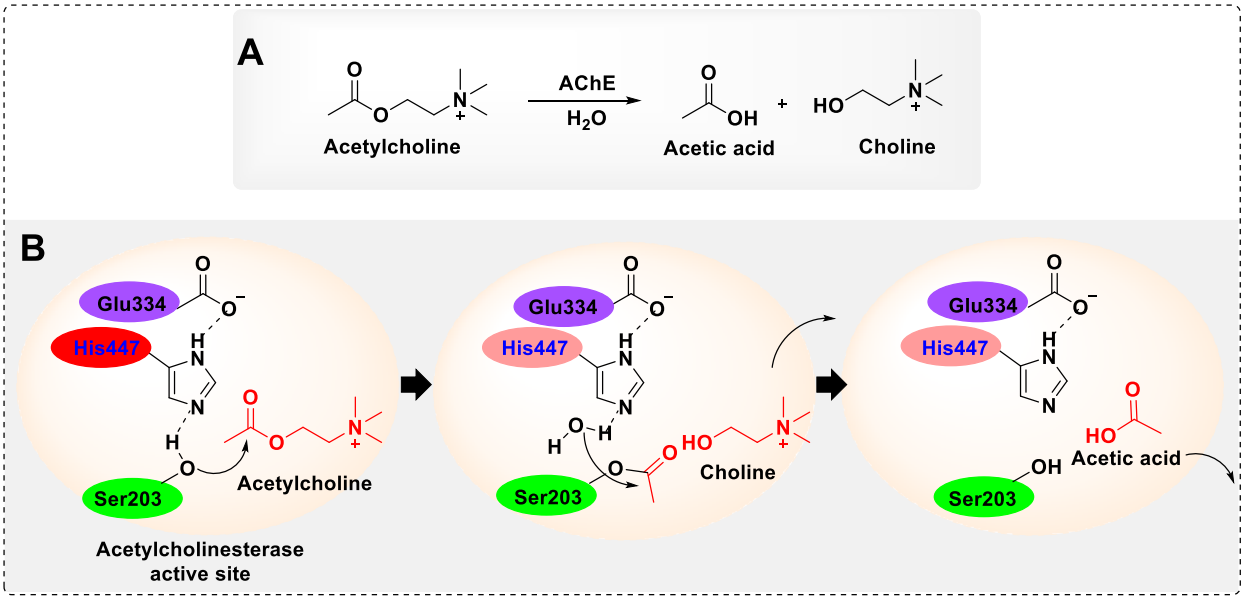

Figure 1.2: Mechanism of substrate cleavage through AChE.

1.3.2. Role of amyloid beta (Aβ) and tau proteins

Amyloid-beta (Aβ) and tau proteins are recognized as metalloproteins due to their capacity to bind metal ions. Neuropathological studies of AD brains have revealed elevated levels of zinc, copper, and iron within Aβ aggregates and neurofibrillary tangles, hallmark features of the disease (12, 30). These plaques and neurofibrillary tangles are mainly found in the

hippocampus, amygdala, entorhinal cortex, and basal forebrain, responsible for memory, learning, and emotional behaviors (13). The β-amyloidogenic processing of the transmembrane glycoprotein amyloid precursor protein (APP), which consists of 695 amino acid residues, involves fragmentation that generates toxic amyloid-beta (Aβ) peptides of varying lengths (39-42 amino acids). Among these, $A\beta_{1-42}$ peptides are more hydrophobic and prone to self-aggregation, forming highly neurotoxic soluble misfolded aggregates such as dimers, oligomers, protofibrils, and fibrils, which eventually develop into insoluble fibrils (senile plaques) (31). The oligomeric isoforms are considered to be the principal neurotoxin [32], which, upon interaction with several synaptic receptors (e.g., NMDAR, PRPc, FPRL1, RAGE, and P75NTR), induces activation of pattern recognition receptors (PRRs) of the innate immunity system.

The accumulation of Aβ aggregates within mitochondria disrupts their normal function. It activates signaling pathways such as Janus kinase (JNK), cyclin-dependent kinase 5 (Cdk5), dual-specificity tyrosine-phosphorylation-regulated kinase-1A (Dyrk1A), and mitogen-activated protein kinase (p38MAPK). Various cellular stress factors drive this activation, including oxidative stress, impaired glucose uptake, and calcium imbalance (32). It can also stimulate the production of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), as well as the subsequent generation of reactive oxygen species (ROS) (33). Aβ-induced activation of microglial cells triggers a neuronal inflammatory response, releasing various inflammatory mediators such as TNF-α, monocyte chemotactic protein-1 (MCP-1), IL-6, and ROS. This activation establishes a persistent feedback loop in microglial cells, ultimately promoting neurotoxicity and contributing to the progression of AD (34, 35).

Elevated levels of Aβ trigger a complex cascade of events, including the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which drives the transcription of proinflammatory cytokines and chemokines in astrocytes. This process can lead to neuronal toxicity and may even result in the overproduction of Aβ within astrocytes (36). Numerous experimental findings from in vitro and preclinical studies have highlighted the role of proinflammatory cytokine release. Reactive oxygen species (ROS) accelerate tau pathology and neurofibrillary tangle (NFT) formation by activating kinases such as Cdk5, JNK, Dyrk1A, and p38MAPK. This upregulation enhances the hyperphosphorylation of axonal microtubule-associated tau proteins by stimulating tau kinase activity and inhibiting phosphatase function (37-40). These abnormal tau proteins lose their ability to bind to tubulin, leading to microtubule destabilization and acting as precursors for forming paired helical filaments (PHFs) or straight filaments (SFs). These filamentous tau proteins undergo self-polymerization, forming neurofibrillary tangles (NFTs) (41). Despite considerable progress in understanding the pathogenesis of AD, its primary causes remain uncertain. It is hypothesized, however, that the accumulation of Aβ may be an early event that initiates a complex cascade of processes and signaling pathways involved in tauopathies. Clinically apparent dementia typically lasts 8-10 years and is often preceded by preclinical and prodromal stages that may span over two decades (13).

1.3.3. Role of metals in AD

Small amounts of metals like zinc, copper, and iron are essential for proper bodily function. These biometals play critical roles in the brain, including facilitating cell signaling and supporting neuroplasticity (42). However, excessive levels of free metals can be detrimental to health. Abnormally high concentrations of these metals have been detected within Aβ plaques,

the toxic protein deposits characteristic of AD (43). Moreover, copper and iron are thought to contribute to oxidative stress.

Iron is crucial for various cellular processes, such as mitochondrial oxidation, cell growth, and synthesizing and metabolizing neurotransmitters like dopamine (DA) (44). Brain iron level naturally increases with age. While iron is indispensable for neuronal growth, excessive iron can lead to neuronal damage (45). Therefore, maintaining an optimal iron concentration within cellular compartments is vital to prevent iron-induced toxic effects, including generating reactive oxygen species (ROS). In conditions of iron overload, storage proteins such as ferritin and neuromelanin become saturated, causing an increase in the labile iron pool and ultimately contributing to neurodegeneration (46).

Aβ binds to iron through three histidine residues and one tyrosine residue located in the hydrophilic N-terminal region of the peptide, stabilizing these iron ions (47). Additionally, studies have shown that binding of Fe^{2+} ions to Aβ reduces the peptide's helical structure while increasing its β-sheet content. This structural alteration facilitates the conversion of Aβ monomers into oligomers and fibrils by enhancing peptide-peptide interactions (48). Iron also contributes to tau phosphorylation and aggregation. Hyperphosphorylated tau accumulates in neurofibrillary tangles (NFTs) and induces the expression of the antioxidant enzyme heme oxygenase-1 (HO-1). Although HO-1 has antioxidative properties, it paradoxically promotes the Fenton reaction by releasing Fe^{2+} (Figure 1.3). Excess iron inhibits the expression of furin, favoring the activation of β-secretase and enhancing Aβ production through the amyloidogenic pathway (49). Thus, iron appears to promote Aβ aggregation while simultaneously reducing the toxicity of these aggregates (50) (Figure 1.4).

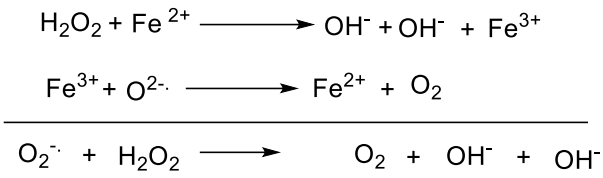

Figure 1.3: Fenton reaction by which H_2O_2 forms hydroxyl radical in an iron-rich environment.

Copper is an essential trace metal critical for the development of the nervous system; however, disruptions in its homeostasis are linked to neurodegenerative diseases such as AD. Cu^{2+} ions exhibit a high affinity for binding with Aβ peptides, which increases the proportion of β-sheet and α-helix structures in these peptides, ultimately triggering Aβ aggregation (51). Elevated Cu^{2+} concentrations accelerate the formation of Aβ fibrils, and the binding of Cu^{2+} ions to Aβ significantly heightens its cellular toxicity. Copper ions complexed with Aβ fibrils can generate hydrogen peroxide (H_2O_2) in the presence of reducing agents. As the Cu^{2+}-to-peptide ratio increases, the production of H_2O_2 and hydroxyl (OH^-) radicals also rises. This shift alters the morphology of Aβ aggregates from fibrillar to amorphous structures (52).

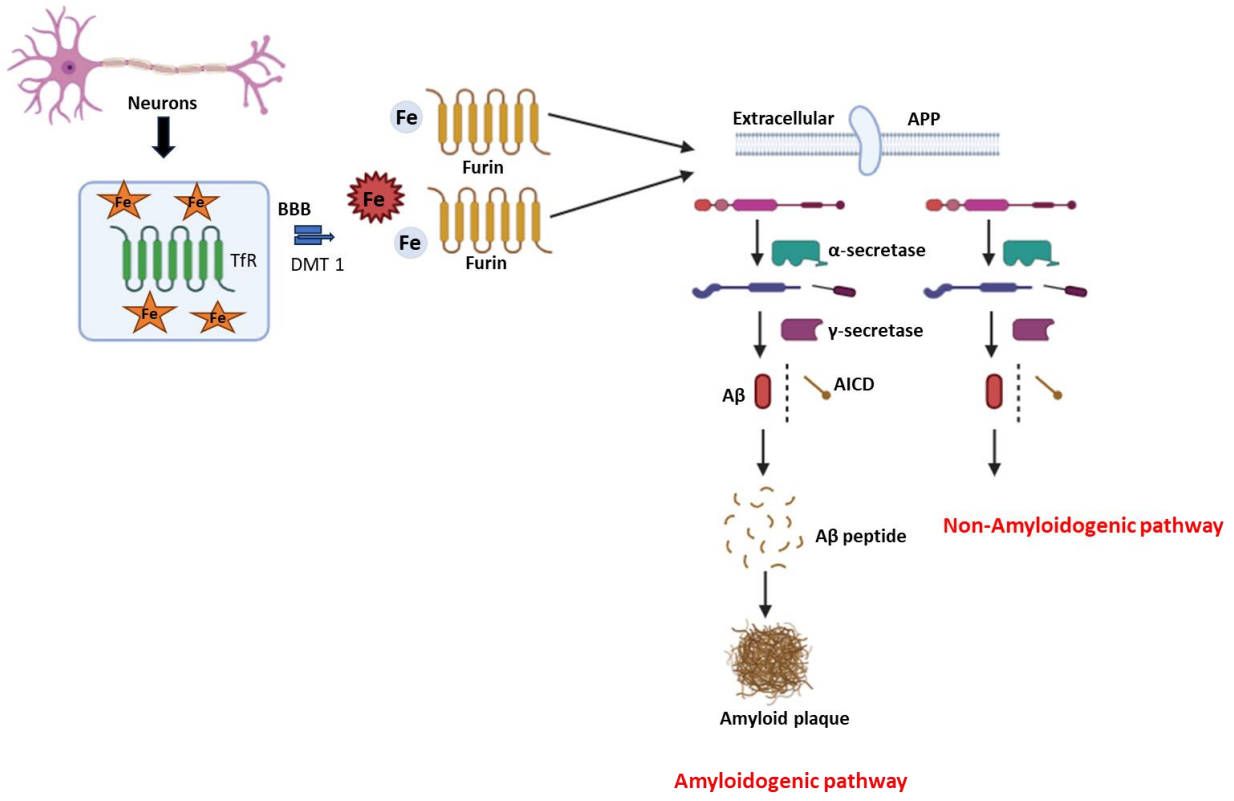

Figure 1.4: Schematic representation of the role of iron in AD.

1.3.4. Role of oxidative stress (OS) in AD

ROS are naturally produced as by-products of normal aerobic metabolism and play crucial roles in cellular signaling and maintaining homeostasis. However, when ROS levels become excessive, they can oxidize and damage cellular components like proteins and DNA, disrupting their normal functions. Environmental stressors such as infections, UV radiation, heat exposure, and pathological conditions like inflammation, metal imbalance, or mitochondrial dysfunction can significantly increase ROS levels (53). This imbalance is primarily caused by dysfunction in the mitochondrial redox system, where leaked electrons react with oxygen to form superoxide anions ($O_2^{\bullet-}$). These anions generate additional ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl ions (OH^-), and can interact with nitric oxide (NO) to form peroxynitrite anions (RNS) (54, 55). Metals, especially copper and iron, also contribute to oxidative stress;

copper aids in the formation of hydroxyl radicals, while iron generates free radicals through the Fenton reaction. Excessive production of ROS and RNS can overwhelm antioxidant defense, causing cellular toxicity through lipid peroxidation and the oxidation of proteins, DNA, and RNA (56). Hydroxyl radicals, generated when H_2O_2 reacts with redox-active metals like copper and iron, are major contributors to nucleic acid oxidation (57). Oxidative damage to neuronal DNA can impair the transcription and replication of critical genes, such as nucleoside guanosine, which produces 8-hydroxydeoxyguanosine (8-OH-dG), a key biomarker of DNA oxidation. Similarly, RNA oxidation results in 8-OH-dG and 8-hydroxyguanosine (8-OHG) due to its proximity to ROS generation sites in the cell, potentially causing nucleotide strand breaks and cellular toxicity from ribosomal dysfunction (58).

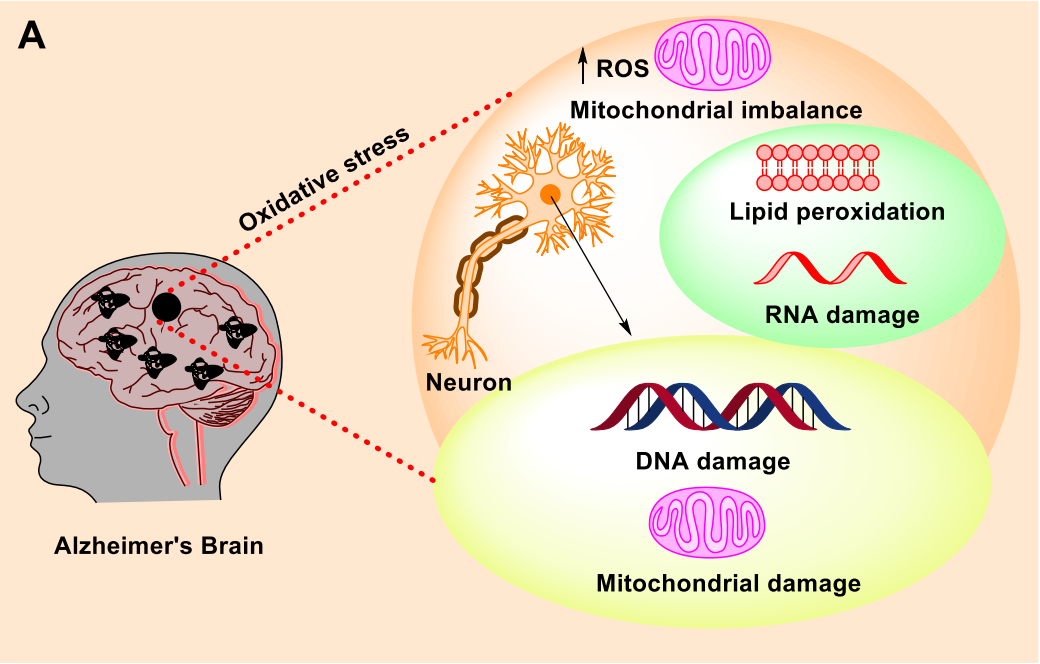

Figure 1.5: Diagrammatic representation indicating the role of ROS and metals in AD progression.

1.3.5. Role of β-secretase (BACE) in AD

The amyloid precursor protein (APP) is part of a large family of type 1 transmembrane proteins, which also includes amyloid precursor-like proteins (APLP1 and APLP2) in mammals and amyloid precursor protein-like (APPL) in *Drosophila* (59). APP is produced in large quantities in neurons and is metabolized rapidly. The precise biological function of APP remains unclear and is one of the key unresolved issues in the field. Aβ is generated through the endoproteolysis of APP. APP can be cleaved on the cell surface by two proteases: α-secretase and β-secretase. Cleavage by α-secretase is non-amyloidogenic and does not produce toxic Aβ (60). In contrast, β-secretase cleavage generates the N terminus of Aβ, resulting in a membrane-bound C-terminal fragment known as C99 (61). Subsequently, γ-secretase cleaves C99 to produce the mature, toxic Aβ peptide. Notably, β-secretase cleavage occurs specifically at Asp+1 and Glu+11 of Aβ, indicating that β-secretase is a site-specific protease. Significantly, therapeutic inhibition of β-secretase would reduce the production of all forms of Aβ, including the pathogenic Aβ42 (Figure 1.6).

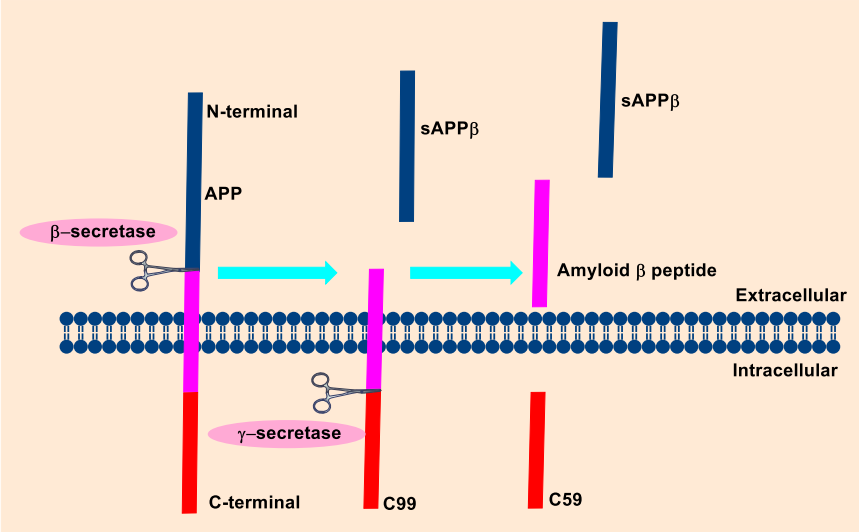

Figure 1.6: Diagrammatic representation indicating the role of β-secretase in Aβ aggregation.

1.3.6. Role of NLRP3 (NOD-like receptor family pyrin domain containing 3) Inflammasome in AD

The NLRP3 inflammasome is an essential part of the innate immune system, acting as a multiprotein complex that detects cellular stress and triggers the release of pro-inflammatory cytokines, particularly interleukin-1β (IL-1β) and interleukin-18 (IL-18)(62, 63). Various stimuli, including pathogens, tissue injury, and metabolic disturbances, can activate it. Dysregulation of the NLRP3 inflammasome has been linked to the development of several diseases, such as AD, cancer, and autoimmune disorders, underscoring its role in immune system regulation. In AD, neuroinflammation, mainly mediated by the NLRP3 inflammasome, plays a central role in disease progression. Initially, this process serves as a defense mechanism during acute infection, but it becomes harmful when it shifts to chronic inflammation (64, 65). In AD, the NLRP3 inflammasome is activated by Aβ aggregates, including oligomers, fibrils, and, in some cases, tau protein, leading to a cascade of damaging effects in the brain. Aβ fibrils function as damage-associated molecular patterns (DAMPs), recognized by pattern recognition receptors such as TLRs and NLRs, signaling cellular damage (66). When Aβ aggregates, it activates NLRP3 inflammasome in microglia, triggering the release of pro-inflammatory cytokines IL-1β and IL-18. This activation leads to caspase-1-mediated pyroptosis, a form of programmed cell death in neurons, contributing to cognitive decline and disease progression. Additionally, activated inflammasomes impact Aβ deposition and clearance (67). NLRP3 activation can impair the phagocytic function of glial cells, promoting further Aβ accumulation and reinforcing a positive feedback loop that worsens AD. Like Aβ fibrils, Tau aggregates can act as priming signals for NLRP3 inflammasome activation. When tau aggregates are internalized by microglia, they stimulate the NLRP3-mediated release of pro-inflammatory cytokines, exacerbating

neuroinflammation (68). NLRP3 activation can also promote tau hyperphosphorylation and aggregation by affecting tau kinases and phosphatases. This interaction generates a feedback loop that accelerates tau pathology spread and severity (66).

In conclusion, the NLRP3 inflammasome is a key mediator in AD, with both Aβ and tau aggregates playing crucial roles in its activation (69). This dual relationship, where the inflammasome's activation by Aβ and tau promotes neuroinflammation and neuronal damage, while also being influenced by the accumulation and spread of these proteins, highlights the potential of targeting the NLRP3 inflammasome as a therapeutic strategy in AD (70). Disrupting this cycle could offer opportunities to slow AD progression and improve patient outcomes.

1.4. Current drug targets for AD

Currently, the primary therapeutic targets for AD include AChE and the NMDA receptor. AChE, a serine protease, plays a crucial role in the breakdown of ACh and is essential for cholinergic neurotransmission. (71). Its active site consists of several components, including the anionic site, catalytic triad or esteratic site (ES), oxyanion hole, selectivity-determining acyl pocket, and the PAS (72). BChE, an isozyme of cholinesterase (ChEs), is also in the brain and is involved in ACh hydrolysis. Recent studies suggest that targeting brain-specific BChE can reduce Aβ levels in transgenic mice and improve cognitive performance in these animals. As AD progresses, both AChE and BChE levels undergo significant changes. Consequently, both enzymes have been investigated as potential targets for neuroprotective and disease-modifying treatments for AD.

1.4.1. Cholinesterases (ChEs)

Cholinesterases (ChEs) hydrolyze, ACh into choline and acetic acid. The two main types of ChEs are acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), as reported in the

literature (73). ChE plays a crucial role in synaptic transmission by breaking the neurotransmitter ACh. Therefore, inhibiting AChE activity to increase ACh levels could improve cognitive and mental functions in AD patients. Interestingly, in the later stages of AD, AChE levels decline by about 90% compared to normal brain levels, raising concerns about its effectiveness as a therapeutic target in these stages (25). Recent studies have shown that inhibiting AChE alone cannot raise ACh levels significantly, so ChE inhibitors produce short-term benefits (1 to 3 years) without altering disease progression (74). Clinical evidence suggests that AChE inhibitors are ineffective in managing the moderate to severe stages of AD (75-77).

AChE is naturally a monomer with a molecular weight of approximately 60,000 and contains 537 amino acid residues, arranged in a 12-stranded mixed β-sheet structure surrounded by 14 α-helices. The enzyme has a hydrophobic active site divided into two subunits: the catalytic active site (CAS) and the peripheral anionic site (PAS). The CAS, consisting of Ser200, Glu327, and His440, is located at the base of a narrow, deep gorge (approximately 20 Å long and 4.5 Å wide), lined with 14 aromatic residues (29). The active site also contains an "anionic subsite" with Trp84, an essential residue that interacts with the quaternary ammonium group in the ACh substrate and other ligands via cation-π interactions. The PAS, located near the entrance of the catalytic gorge, about 20 Å away from the active center, is less well characterized. The binding of a ligand to the PAS alters the enzyme's conformation, involving residues such as Tyr70, Asp72, Tyr121, Trp279, and Tyr334. Trp279 is key in AChE's adhesive action (**Figure 1.7**). The PAS temporarily binds with the substrate, enhancing catalytic efficiency and trapping the substrate as it moves toward the active site (78).

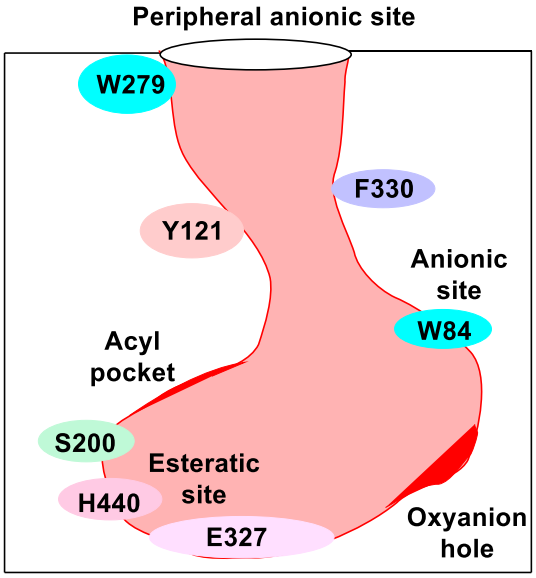

Figure 1.7: Schematic view of the active site of AChE. The bottom of the gorge is characterized by an anionic site, which contains an esteratic site, an acyl pocket, and an oxyanion hole, and PAS is located 20 Å above the active site.

BChE, a glycoprotein found in the central and peripheral nervous systems, is a nonspecific pseudocholinesterase, also known as serum cholinesterase, that hydrolyzes choline and aliphatic esters (79). The catalytic active site (CAS) of human BChE comprises Ser198, His438, and Glu325 residues. While the precise role of BChE in AD remains under investigation, it is believed to compensate for the loss of neuronal AChE function during neurodegeneration in AD, essentially taking over the role of AChE (80). Therefore, inhibiting AChE and BChE can provide symptomatic relief in AD treatment (81). Consequently, dual and selective inhibitors of AChE and BChE may represent an effective therapeutic strategy for managing AD.

1.4.2. NMDA receptor in AD

The N-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor and ion channel located in neurons (Figure 1.8). NMDAR plays a crucial role in synaptic

transmission and plasticity, which are essential for learning and memory. These functions are central to the nervous system's normal operations and its potential for neurotoxicity. Overactivation of the NMDA receptor, leading to excessive calcium (Ca^{2+}) influx, can trigger excitotoxicity, contributing to neurodegenerative disorders, including AD (82). A major challenge in using NMDA receptor antagonists for neuroprotection is that the physiological functions of the NMDA receptor are vital for normal neuronal activity (83).

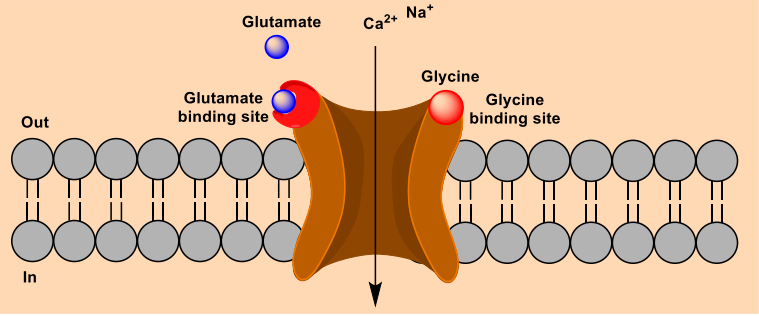

Figure 1.8: NMDA receptor complex as a therapeutic target in AD.

1.4.3. Monoclonal antibodies targeting Aβ aggregates

The FDA's approval of anti-amyloid monoclonal antibodies (MABs), such as Lecanemab (Leqembi®) and Aducanumab (Aduhelm®), marks a significant milestone in AD treatment (84). These therapies target the underlying biological mechanisms of AD, offering the potential to slow the disease's progression and shift treatment from merely managing symptoms to modifying the disease itself. Clinical trial outcomes support the amyloid hypothesis, reinforcing the strategy of targeting amyloid for drug development in AD. The success of these MABs highlights the influence of applied neuroscience in solving complex medical issues. It paves the way for innovative treatments aimed at amyloid pathway and other aspects of AD biology (85). Despite their potential, these therapies come with risks, such as amyloid-related imaging abnormalities (ARIA) and infusion reactions, necessitating careful monitoring during treatment initiation (86).

However, these therapies carry risks, such as amyloid-related imaging abnormalities (ARIA) and infusion reactions, requiring careful monitoring during treatment initiation [87]. ARIA refers to changes detected in MRI scans of AD patients undergoing anti-amyloid therapy. These abnormalities are typically asymptomatic, resolve independently, and can only be identified through MRI (87). Introducing these agents marks a new phase in AD treatment and lays the groundwork for future therapeutic advancements.