1 Introduction

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that leads to the gradual loss of neurons and synapses, particularly affecting the brain's cholinergic system. [1]. AD is characterized by cognitive impairments such as memory loss, difficulties with language, reasoning deficits, and changes in personal behavior. [2]. As AD progresses, non-cognitive symptoms become more prominent, including behavioral disturbances and psychiatric manifestations such as agitation, hallucinations, depression, and delusions. [3]. The precise molecular pathways driving neurodegeneration in AD remain elusive. Still, extensive evidences suggest that low levels of neurotransmitters, especially acetylcholine (ACh), amyloid-beta (Aβ) aggregates, oxidative stress, and abnormal metal concentrations are critical contributing factors. [4-14]. While the etiology of AD is not fully understood, age-related brain changes, along with genetic, environmental, and lifestyle factors, are likely involved.

In 1907, German psychiatrist Alois Alzheimer was the first to describe AD after observing a patient named Auguste D, who experienced profound memory loss, irrational fears, and psychological issues. [15]. The histopathological examination of her brain revealed significant atrophy and the presence of abnormal deposits in and around nerve cells, leading to the identification of two distinct types of brain lesions: senile plaques and neurofibrillary tangles. These discoveries revealed that a particular disease impacted the cerebral cortex. [16]. Amyloid-beta (Aβ) plaques accumulating extracellularly, and neurofibrillary tau protein tangles (NFTs) accumulating intracellularly are the pathological hallmarks of AD. [17].

Decreased acetylcholine (ACh) levels, a neurotransmitter crucial for memory and learning, are linked to cognitive decline in AD [18]. Acetylcholinesterase (AChE) and butyrylcholinesterase

(BChE) break down ACh, reducing cholinergic transmission and exacerbating cognitive symptoms [19, 20]. Current AD management strategies focus on inhibiting cholinesterase enzymes to maintain ACh levels.

Oxidative stress is a significant contributor to AD pathology. [21-23]. It arises when the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) exceeds the body's antioxidant capacity, resulting in cellular damage. [24-26]. Factors such as inflammation, metal dyshomeostasis, and mitochondrial dysfunction can increase ROS production. [27].

Metal dyshomeostasis is another critical factor in AD. [28]. Metals are essential for brain function, but excess of these metals can lead to neurodegeneration. [29]. The metal content in the brain naturally increases with age. The elevated levels of metals such as copper, iron, and zinc were found in the postmortem report of the brain of an AD patient, suggesting a role in the disease's pathogenesis. [29-31].

The amyloid hypothesis remains a crucial area of research in AD [32, 33]. Sequential processing of amyloid precursor protein (APP) by β- and γ-secretases produces Aβ peptides, particularly $A\beta_{1-42}$, which form toxic plaques. These plaques induce inflammatory responses and generate ROS, further damaging neurons [32, 34].

NFTs and Aβ plaques are the two main pathogenic characteristics of AD. These disease markers primarily localize in areas important for memory, learning, and emotional regulation, such as the entorhinal cortex, basal forebrain, hippocampal, and amygdala [35]. These markers appear several years before the onset of cognitive decline[36].

1.2 Statistics of AD

AD is the leading cause of dementia and the sixth-leading cause of death in the United States. Approximately one in ten Americans aged 65 and older is affected by AD. According to AD facts and **Figure**s (2023), mortality rates from stroke, HIV, and heart disease have significantly decreased over the past two decades. In contrast, deaths from AD have increased by 145%. Globally, approximately 55 million people live with AD and related dementias, and this number is projected to exceed 152 million by 2050 without effective treatments[37]. India is expected to experience a notable increase in dementia cases due to its aging population and decreasing fertility rates [38]. By 2050, 19.1% of India's population is expected to be 60 years or older. The Dementia in India 2023 report estimates that in 2020, 8.8 million Indians over the age of 60 had dementia, with projections suggesting this **Figure** will increase by 16.9 million by 2036 [39]. Regional variations in dementia incidence are evident across India, with higher rates observed in southern India compared to rural northern India. The rising prevalence of dementia in India highlights the critical need for comprehensive epidemiological studies across different regions. As per the report of AD International, the global annual cost of dementia currently exceeds US$ 1.3 trillion and is projected to reach 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 complex organ comprised of organized networks of neurons that communicate through neurotransmitters. Cholinergic neurons, which release the neurotransmitter acetylcholine (ACh), play a pivotal role in processes related to learning and memory [40]. In AD, the regions of the brain most affected by neuronal loss are primarily composed of cholinergic neurons, indicating that restoring normal levels of ACh could be a promising

therapeutic approach [41, 42]. Research supports the cholinergic theory of AD, which hypothesizes that a deficit in cholinergic signaling in the cortex significantly contributes to the cognitive decline seen in AD patients. A reduction in ACh's concentration and function characterizes the disease [43]. ACh is synthesized in presynaptic nerve terminals through the combination of choline and acetyl coenzyme A (acetyl-CoA), a process facilitated by choline acetyltransferase (ChAT), the rate-limiting enzyme for ACh synthesis. Once synthesized, ACh is stored in presynaptic vesicles and released into the synaptic gap during neural communication. Upon release, ACh binds to receptors on postsynaptic target cells, facilitating the transmission of nerve impulses and mediating communication between neurons. ACh metabolism occurs in the synaptic gap and is primarily regulated by acetylcholinesterase (AChE), an enzyme responsible for breaking down ACh and maintaining its dynamic balance in the healthy brain [40]. As shown in **Figure** 1.1, the active site of AChE contains a serine residue that participates in a series of hydrogen bonding interactions to create a highly nucleophilic environment. This environment enables the hydroxyl anion to attack the carbonyl carbon of ACh, leading to the acylation of the serine residue and the release of choline. The subsequent deacylation of the serine residue is facilitated by water molecules, releasing acetic acid and restoring the enzyme's active site. This process ensures that ACh levels remain balanced and neural signaling can continue efficiently [44]. Understanding and exploring this pathway has potential therapeutic implications for managing AD symptoms and slowing disease progression.

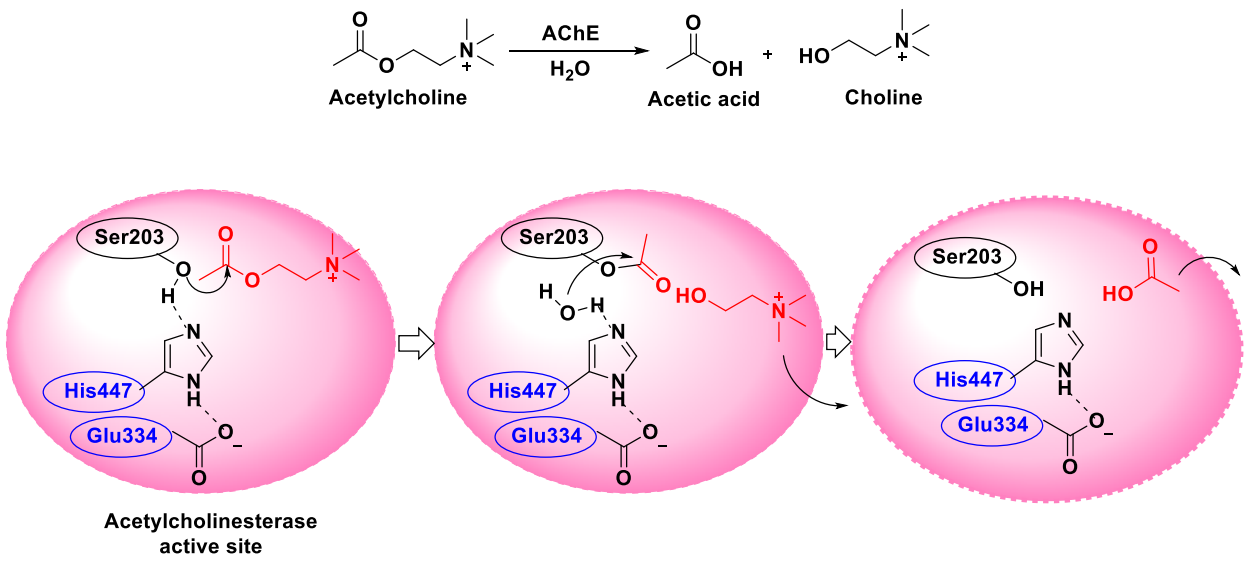


Figure 1.1: Mechanism of breakdown of acetylcholine by AChE

1.3.2 Role of Oxidative Stress in AD

Normal aerobic oxygen metabolism produces ROS as a natural by-product essential in cell signaling and homeostasis [7]. However, elevated levels of ROS can damage cellular components such as proteins and DNA by oxidizing them, impairing their normal functions. ROS levels can increase significantly due to environmental stressors such as infections, UV or heat exposure, or pathogenic conditions such as inflammation, metal dyshomeostasis, or mitochondrial dysfunction [45]. Oxidative stress arises from an imbalance between ROS production and cells' antioxidant capacity. In AD, an increase in oxidative stress is considered one of the early events in the neurodegenerative pathway. [46]. This stress results from redox system imbalances within the mitochondria, where leaked electrons react with oxygen to form superoxide anions (O2•−). These superoxide radicals generate other forms of ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl ion (OH−), while interacting with nitric oxide (NO) to produce peroxynitrite anion (RNS). Oxidative stress is also influenced by metals,

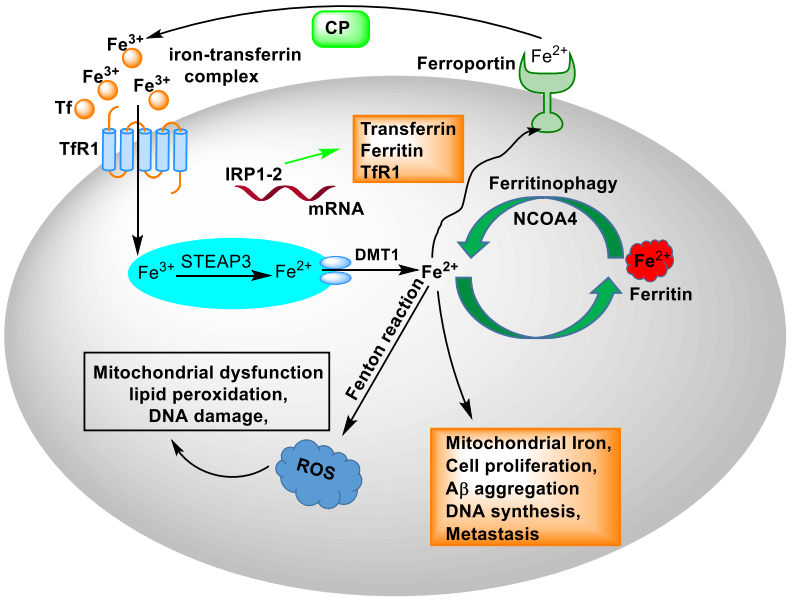
particularly copper and iron [45]. Copper mediates hydroxyl radicals, while iron generates free radicals through the Fenton reaction [27].

Overproduction of ROS and RNS leads to compromised antioxidant function and induces toxicity via lipid peroxidation and the oxidation of proteins, DNA, and RNA. Hydroxyl radicals formed, when H_2O_2 react with redox-active metals like copper and iron are a significant source of oxidized nucleosides (DNA and RNA). Oxidative damage to neuronal DNA can lead to the transcription and replication of crucial genes, such as nucleoside guanosine, which produces 8-hydroxydeoxyguanosine (8-OH-dG), a key biomarker of DNA oxidation. Oxidation of RNA yields 8-OH-dG and 8-hydroxyguanosine (8-OHG), given its proximity to sites of ROS generation within the cell. This can lead to nucleotide strand breakage and cellular toxicity from ribosomal dysfunction [47]. Indeed, oxidative RNA damage has been described in the early stages of prevalent neurodegenerative diseases such as AD, Lewy body dementia, and Parkinson's disease. In AD, oxidative stress-induced protein carbonylation has been observed in the frontal and parietal cortices and hippocampus but not in the cerebellum, indicating specific regional vulnerability [48].

1.3.3 Role of Metals in AD

Aβ and tau proteins are well recognized as metalloproteins due to their ability to bind with metals. The neuropathy study of AD brains has found elevated levels of zinc, copper, and iron within the Aβ aggregates and neurofibrillary tangles characteristic of the disease [49]. The increased copper and iron levels in the aging brain can lead to an overproduction of amyloid precursor protein (APP) and $A\beta_{1\text{-}42}$ peptides. Copper ions (Cu^{2+}) promote Aβ aggregation by facilitating the formation of β-sheet structures, while iron (Fe) contributes to the formation of senile plaques [50, 51]. Additionally, high concentrations of zinc ions in synapses can

precipitate soluble Aβ, which catalyzes amyloid plaque formation [52]. These metals also interact with tau proteins. Zinc and copper ions play roles in the hyperphosphorylation of tau proteins by activating various signaling pathways such as kinases and phosphatases [28, 53]. Iron activates kinases that lead to the aggregation of phosphorylated tau proteins, contributing to the development of neurofibrillary tangles [54]. Hyperphosphorylated tau accumulates in NFTs and triggers the production of the antioxidant protein heme oxygenase-1 (HO-1). Although HO-1 has antioxidant properties, it can also promote the Fenton reaction (**Figure** 1.2) by releasing Fe^{2+}. Excess iron levels inhibit furin expression, activating β-secretase and increasing Aβ production from the amyloid pathway. Therefore, iron can stimulate Aβ aggregation and decrease the toxicity of these aggregates [55].



$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + OH^- + Fe^{3+}$$

$$Fe^{3+} + O^{2-\cdot} \longrightarrow Fe^{2+} + O_2$$

$$O_2^{-\cdot} + H_2O_2 \longrightarrow O_2 + OH^- + OH^-$$

Figure 1.2: Iron transport and its metabolism. Iron catalyzed Fenton reaction.

The interaction of Aβ plaques and neurofibrillary tangles with these metals (Cu^{2+}, Zn, and Fe) also creates sites for redox reactivity. This redox activity is catalyzed by iron-binding, which stimulates the production of hydrogen peroxide (H_2O_2) and myeloperoxidase. Hydrogen peroxide, being membrane-permeable, can enter intracellular spaces and react with copper and iron ions, forming hydroxyl free radicals [56].

These hydroxyl free radicals can oxidize nucleic acids, proteins, and lipids, contributing to cellular damage and oxidative stress, key features of AD pathology. This cascade of events involving metal ions, Aβ, tau proteins, and redox reactions underscores the complex interplay of factors in AD progression and presents potential targets for therapeutic intervention [57].

1.3.4 Role of amyloid beta (Aβ) and tau proteins in AD

AD is characterized by the pathological accumulation of Aβ plaques outside neurons and neurofibrillary tau protein tangles (NFTs) inside neurons. Emerging evidence suggests that these markers can develop many years prior to the onset of cognitive symptoms, affecting brain regions critical for memory, learning, and emotional behaviors, such as the hippocampus, amygdala, entorhinal cortex, and basal forebrain [33, 58, 59]. The cleavage of amyloid precursor protein (APP) generates toxic Aβ peptides of varying lengths (39-42 amino acids). The $A\beta_{1\text{-}42}$ peptides are mainly hydrophobic and prone to self-aggregation into neurotoxic forms such as soluble misfolded aggregates, including dimers, oligomers, protofibrils, fibrils, and insoluble senile plaques. These insoluble oligomers are the primary neurotoxins, interacting with various synaptic receptors and triggering innate immune responses [60].

The accumulation of Aβ aggregates within mitochondria disrupts normal function and activates pathways such as Janus kinase (JNK), CDK5, dual-specificity tyrosine-phosphorylation regulated kinase-1 A (Dyrk1A), and mitogen-activated protein kinase

(p38MAPK) [61]. These pathways contribute to oxidative stress, reduced glucose uptake, calcium imbalance, and increased production of proinflammatory cytokines, including TNF-α, IL-6, and IL-1β, leading to reactive oxygen species (ROS) production [62]. Aβ-induced activation of microglial cells triggers a chronic inflammatory response, releasing inflammatory factors such as TNF-α, monocyte chemotactic protein 1 (MCP-1), IL-6, and ROS. This ongoing inflammatory cycle exacerbates neurotoxicity and furthers AD progression [63, 64].

Elevated Aβ levels stimulate astrocytic transcription of proinflammatory cytokines and chemokines through NF-κB activation, potentially increasing neuronal toxicity and Aβ production [65]. ROS also accelerates tau pathology and NFT formation by upregulating kinases such as CDK5, JNK, Dyrk1A, and p38MAPK, contributing to tau hyperphosphorylation. This hyperphosphorylation destabilizes microtubules and promotes the self-polymerization of tau into NFTs [34, 66]. Despite advances in understanding AD pathogenesis, the primary causes remain illusive, though Aβ accumulation was expected to be an early trigger for the complex cascade of events leading to tauopathies. The duration of clinically evident dementia is typically 8-10 years, preceded by preclinical and prodromal stages that can span up to two decades. Understanding these early pathological changes is essential for developing interventions to slow or prevent the progression of AD.

1.3.5 Role of β-secretase (BACE) in AD

Amyloid precursor protein (APP) is a transmembrane protein that is part of a family of proteins that includes amyloid precursor-like proteins (APLP1 and APLP2) in mammals, as well as amyloid precursor protein-like (APPL) in Drosophila [67]. Although APP is predominantly localized in neurons and undergoes rapid metabolism, its precise physiological function remains largely unexplored, posing a significant challenge for researchers. APP can be

processed through two distinct pathways: one mediated by α-secretase and the other by β-secretase [68]. When APP is cleaved by α-secretase, the process follows a non-amyloidogenic path, which prevents the generation of toxic amyloid-beta (Aβ) peptides [33]. Conversely, β-secretase cleavage initiates the amyloidogenic pathway by producing the N-terminus of Aβ and leaving a membrane-bound C-terminal fragment known as C99 [69]. This fragment is then further processed by γ-secretase to yield mature, toxic Aβ peptides. β-secretase exhibits site-specific activity, targeting specific amino acid residues, Asp+1 and Glu+11, within the Aβ sequence. This precise cleavage pattern underscores the enzyme's role as a site-specific protease[70]. Therapeutic inhibition of β-secretase is considered a promising approach to reduce the production of all forms of Aβ, particularly the pathogenic Aβ42 variant, which is implicated in the pathogenesis of AD.

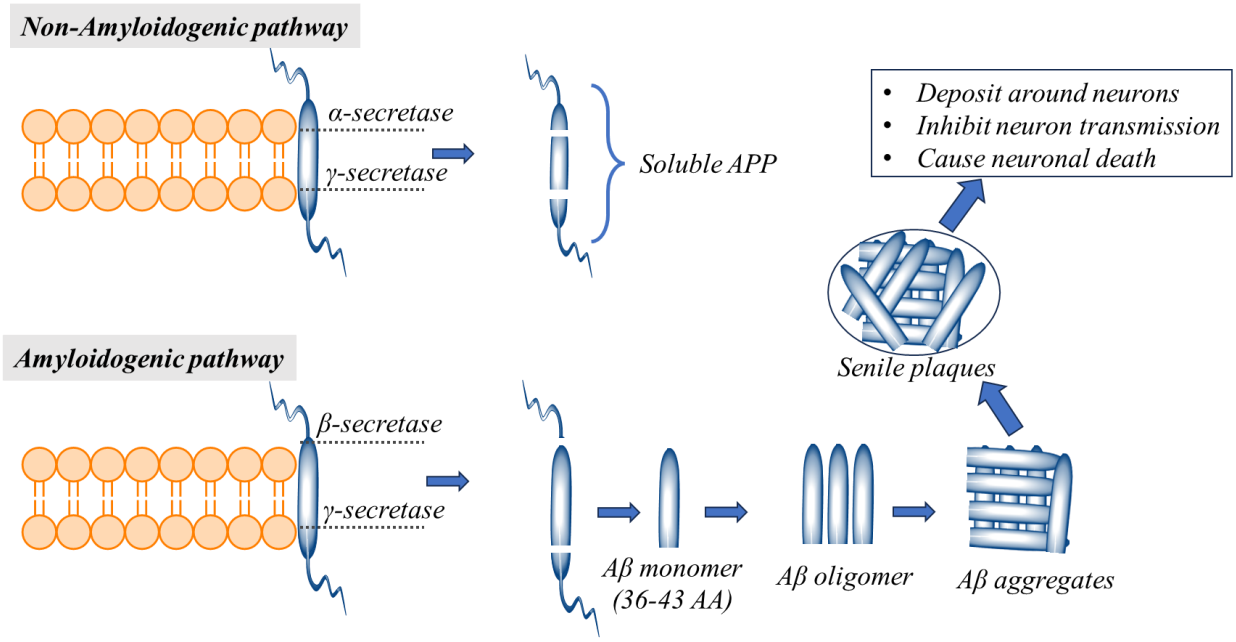


Figure 1.3: The role of β-secretase (BACE) in the cleavage of APP to generate Aβ monomer.

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

The NLRP3 inflammasome is a crucial component of the innate immune system, functioning as a multiprotein complex that detects cellular stress and triggers the release of pro-inflammatory cytokines, notably interleukin-1β (IL-1β) and interleukin-18 (IL-18). Various stimuli, including pathogens, tissue injury, and metabolic disturbances can activate it. Dysregulation of the NLRP3 inflammasome is linked to the development of several diseases, including Alzheimer's disease, cancer, and autoimmune disorders, highlighting its importance in maintaining immune homeostasis. Neuroinflammation, mainly mediated by the NLRP3 inflammasome, is critical to AD pathogenesis. This process starts as a defense mechanism during acute infection but becomes harmful when it transitions to chronic inflammation[71]. In AD, the NLRP3 inflammasome is activated by Aβ aggregates, including oligomers, fibrils, and sometimes tau protein, leading to a cascade of detrimental effects in the brain. Aβ fibrils act as damage-associated molecular patterns (DAMPs) that are recognized by pattern recognition receptors such as TLRs and NLRs, signaling the presence of cellular damage [72]. Upon recognition of Aβ, the NLRP3 inflammasome is activated in microglia, triggering the release of pro-inflammatory cytokines IL-1β and IL-18. NLRP3 activation leads to caspase-1-mediated pyroptosis, a form of programmed cell death in neurons, contributing to cognitive decline and disease progression. Activated inflammasomes also influence Aβ deposition and clearance [73]. NLRP3 activation can hinder glial cells' phagocytic abilities, increasing Aβ accumulation and feeding into a positive feedback loop that exacerbates AD. Tau aggregates can provide the priming signal for NLRP3 inflammasome activation, similar to Aβ fibrils. After being internalized by microglia, Tau aggregates stimulate NLRP3-mediated expression of pro-inflammatory cytokines, worsening neuroinflammation [74]. NLRP3 activation can

increase tau hyperphosphorylation and aggregation by influencing tau kinases and phosphorylases. This interaction creates a feedback loop that promotes the spread and severity of tau pathology [72].

In summary, the NLRP3 inflammasome acts as a central mediator in AD, with both Aβ and tau aggregates playing significant roles in its activation [75]. This dual relationship where the inflammasome's activation by Aβ and tau contributes to neuroinflammation and neuronal damage while being influenced by the buildup and spread of these proteins highlights the potential of targeting the NLRP3 inflammasome as a therapeutic strategy in AD[76]. By disrupting this cycle, there may be opportunities to mitigate AD progression and improve patient outcomes.

1.4 Current Drug Targets for AD

Current therapeutic targets for AD include acetylcholinesterase (AChE) and N-methyl-D-aspartate (NMDA) receptors. AChE plays a vital role in cholinergic neurotransmission by breaking down acetylcholine, which is crucial for memory and cognitive functions. AChE inhibitors maintain higher acetylcholine levels, potentially improving cognitive function in AD patients. AChE's active site consists of multiple regions, including the anionic site, catalytic triad (or static site), oxyanion hole, selectivity determinant acyl pocket, and peripheral anionic site (PAS) [77]. These regions are critical for the enzyme's function in hydrolyzing acetylcholine. By inhibiting AChE, these drugs can preserve acetylcholine levels in the synaptic cleft, which may benefit patients with AD by enhancing communication between neurons.

In addition to AChE, butyrylcholinesterase (BChE) is another brain enzyme involved in acetylcholine hydrolysis. Targeting BChE may offer additional therapeutic benefits by

lowering beta-amyloid (Aβ) levels and improving cognitive performance in animal models[78]. AChE and BChE levels fluctuate significantly during AD progression, prompting interest in exploring these enzymes as targets for neuroprotective and disease-modifying therapies. By supporting cholinergic function and impacting Aβ accumulation, these therapies aim to slow AD progression.

1.4.1 Cholinesterase enzymes as target in AD treatment

Cholinesterases (ChEs) break down acetylcholine (ACh) into choline and acetic acid. The two main types of ChEs are AChE and BChE. These enzymes play a crucial role in synaptic transmission by hydrolyzing acetylcholine, a neurotransmitter essential for cognitive and mental functions. Inhibiting AChE can raise acetylcholine levels, potentially enhancing cognitive function in AD patients. However, as AD advances, AChE levels can significantly drop by up to 90% in later stages, making it less effective as a therapeutic target in these stages. AChE inhibitors alone may not sufficiently increase acetylcholine levels for sustained cognitive benefits, providing only temporary improvements lasting 1 to 3 years without altering the course of the disease [79]. Clinical studies support the limited efficacy of AChE inhibitors in treating moderate to severe AD [60, 80].

AChE is a monomeric protein with approximately 60,000 molecular weight and 537 amino acids, structured with 12 stranded mixed β-sheets and 14 α-helices. Its hydrophobic active site is divided into two regions: the catalytic active site (CAS) and the peripheral anionic site (PAS). The CAS is at the base of a deep, narrow gorge (around 20 Å long and 4.5 Å wide) lined with 14 aromatic residues. The critical residues in CAS include Ser203, Glu334, and His447 (**Figure** 1.4), which are crucial for the enzyme's function. The anionic subsite in CAS

includes Trp84 and Phe330, interacting with acetylcholine and other ligands via cation-π interactions [20].

The PAS, located at the entrance of the catalytic gorge, about 20 Å from the active center, contains residues like Tyr70, Asp72, Tyr121, Trp279, and Tyr334. Trp279 is essential for AChE binding. The PAS temporarily holds the substrate during initial catalysis, enhancing efficiency and guiding the substrate to the active site [14].

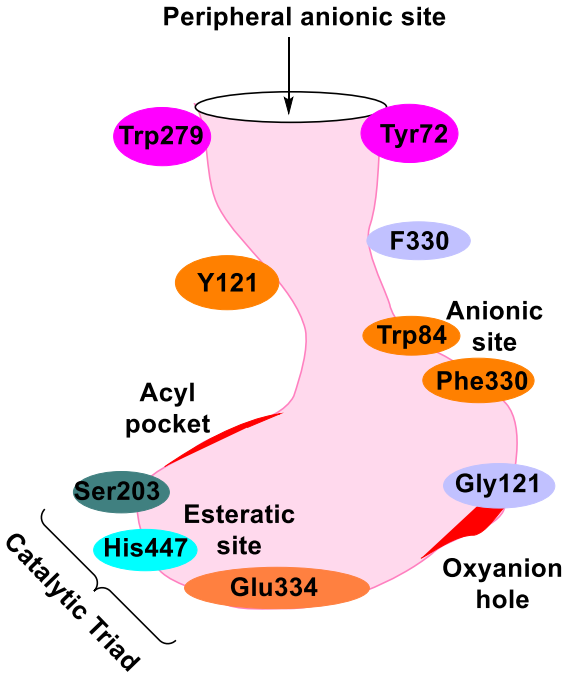


Figure 1.4: The active sites of AChE enzyme.

On the other hand, BChE is an alpha-glycoprotein enzyme present in the central and peripheral nervous systems. It is commonly called pseudocholinesterase or serum cholinesterase due to its ability to hydrolyze choline and other aliphatic esters [19] . The CAS of human BChE includes the amino acid residues Ser198, His438, and Glu325. Although the exact function of BChE in AD remains under investigation, it is thought to compensate for the loss of AChE

function in the disease and substitute the roles of AChE in progressive neurodegeneration [78]. This understanding supports the notion that simultaneous inhibition of AChE and BChE could provide symptomatic relief in AD management. Thus, developing dual and selective inhibitors targeting AChE and BChE presents a promising therapeutic strategy for managing AD more effectively [81]. These drugs could help maintain higher levels of acetylcholine, potentially supporting cognitive function and moderating disease progression.

1.4.2 NMDA receptor in AD

The N-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor and ion channel found in neurons, where it plays a pivotal role in synaptic transmission and plasticity, key processes underlying learning and memory. These functions are integral to the overall performance of the nervous system [82]. Overstimulation of the NMDA receptor can result in an excessive influx of calcium ions (Ca2+), potentially leading to excitotoxicity (**Figure** 1.5) and contribute to neurotoxicity [83]. A significant challenge in employing NMDA receptor antagonists for neuroprotection is that these receptors are crucial for normal neuronal functions. Therefore, inhibiting NMDA receptors may disrupt necessary physiological processes within the nervous system [84]. Balancing the prevention of excitotoxicity with the preservation of essential receptor activities is vital in the development of effective NMDA receptor-targeted therapies for AD and other neurodegenerative disorders.

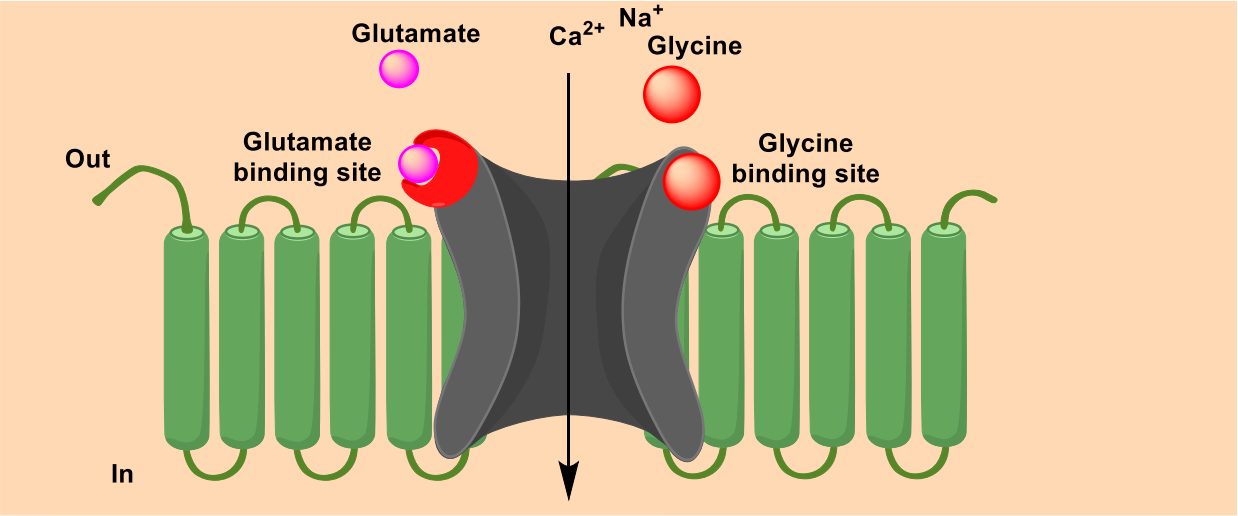


Figure 1.5: The diagrammatic representation of NMDA receptor.

1.4.3 Monoclonal antibodies targeting Amyloid beta (Aβ aggregates)

The FDA approval of anti-amyloid monoclonal antibodies (MABs) such as lecanemab (Leqembi®) and aducanumab (Aduhelm®) represents a significant advancement in the treatment of AD [85]. These agents target the fundamental biological mechanisms of AD and have the potential to slow the progression of the disease, thereby transitioning AD therapy from symptom management to disease modification. Clinical trial results validate the amyloid hypothesis, supporting the approach of targeting amyloid in developing drugs for AD. The success of these MABs demonstrates the impact of applied neuroscience in addressing complex medical challenges and opens a new paradigm for more innovative treatments targeting amyloid and other aspects of AD biology [86]. Despite their potential, these therapies come with risks, such as amyloid-related imaging abnormalities (ARIA) and infusion reactions, necessitating careful monitoring during treatment initiation [87]. ARIA are changes seen on MRI scans in patients with AD receiving anti-amyloid therapies. These abnormalities are often asymptomatic, tend to resolve on their own, and are usually only detectable through

MRI [88]. Introducing these agents signifies a new era in AD therapy and sets the stage for future therapeutic advances.