Introduction

Chapter 1

1. Introduction

1.1. Alzheimer's disease

Alzheimer’s disease (AD) is a multifactorial progressive neurodegenerative disorder characterized by gradual loss of neurons and synapses, particularly within the brain’s cholinergic system, resulting in loss of memory and of other cognitive functions [1]. The symptoms associated with AD involve impairment in memory, language skills, personal behavior, and thinking [2]. These symptoms appear due to the neuronal damage not only limited to the brain's region responsible for the cognitive function but may extend to neurons in different parts of the brain. As the disease advances, psychiatric symptoms and behavioral disturbances, e.g. depression, hallucinations, delusions, agitation collectively termed non-cognitive symptoms develop. 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 acetylcholine (ACh), amyloid-beta (Aβ) aggregates, oxidative stress (OS), and the concentrations of metals interdependently play a key role in the neurodegeneration process [3-8].

AD was described for the first time in 1907 by German psychiatrist Alois Alzheimer. Alzheimer's study case of the patient Auguste D who had extreme memory loss, unfounded suspicions about her family, and gradually worsening psychological changes [9]. In performing a histopathology study of her brain, Alzheimer saw a dramatic shrinkage and abnormal deposits in and around nerve cells. He brought to light the two types of lesions in the brain: (a) senile plaques, (b) neurofibrillary tangles. He concluded a distinct disease of the cerebral cortex [10].

Acetylcholine (ACh) and butyrylcholine (BCh) are critical neurotransmitters involved in learning and memory. The evidence from the literature and growing research findings strongly indicated

these neurotransmitters' play a crucial role in AD etiology [11]. AD is associated with a decrease in both the concentration and function of ACh. The metabolism of ACh takes place in the synaptic gap by acetylcholinesterase (AChE), which regulates the dynamic equilibrium of ACh in the healthy brain. Thus, inhibition of AChE and BChE can effectively induce symptomatic relief in the treatment of 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 have 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 responsible for memory, learning, and emotional behaviors [13].

Oxidative stress (OS) and excessive amounts of iron can lead to generation 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, however, age and genetic factors also play a very critical role in the disease process [14].

1.2. Statistics of AD

AD is the most common 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. As per 2020 AD facts and figures, the mortality rate in the last two decades due to stroke, HIV, and heart disease has decreased significantly, while a 146.2% increase in deaths from AD has been reported [16]. As per-WHO, around 50 million people worldwide live with dementia, and every year this number is escalating at a rapid rate [17]. The overall burden is estimated to grow to nearly $1.1 trillion by

2050 for all people with AD and other dementias. Age is the best-known risk factor for Alzheimer's disease [18]. The risk of developing the disease doubles every five years after age 65. AD becomes increasingly common as people reach their 80s, 90s, and beyond. These facts are significant because the number of older adults is growing. About 20-40% of healthy people between 60 and 78 years old experience discernable decrements in cognitive performance in several domains, including working and spatial memory [19]. Further, it is estimated that around 68% of the projected increase in the global prevalence and burden of AD-related dementia will take place in low and middle-income countries. The social and economic burden of AD is very high; the current annual cost of dementia is estimated to be US $1 trillion, which is projected to be more than double by 2030.

1.3. Pathophysiology of AD

1.3.1. Role of acetyl and butyrylcholines in AD

The brain is composed of highly organized neurons that form a neural network, and it communicates among itself by neurotransmitter [20]. Cholinergic neurons secrete acetylcholine (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 level has been considered a viable therapy for AD [21, 22]. Acetylcholine (ACh) and butyrylcholine (BCh) are critical neurotransmitters involved 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 AD theory states the cortical deficit of cholinergic neurotransmission is one of the major contributing factors to the characteristic cognitive impairment in the patient. AD is associated with a decrease in both the concentration and function of ACh. The synthesis of ACh

occurs in presynaptic nerve terminals through condensation of choline and acetyl coenzyme A with co-enzyme acetyltransferase (CAT). CAT is the rate-limiting enzyme for the synthesis of ACh. Thus, synthesized ACh remains stored in the presynaptic vesicles, and it gets released into the synaptic gap and acts on the postsynaptic target cell, which further mediates nerve-to-nerve signaling.

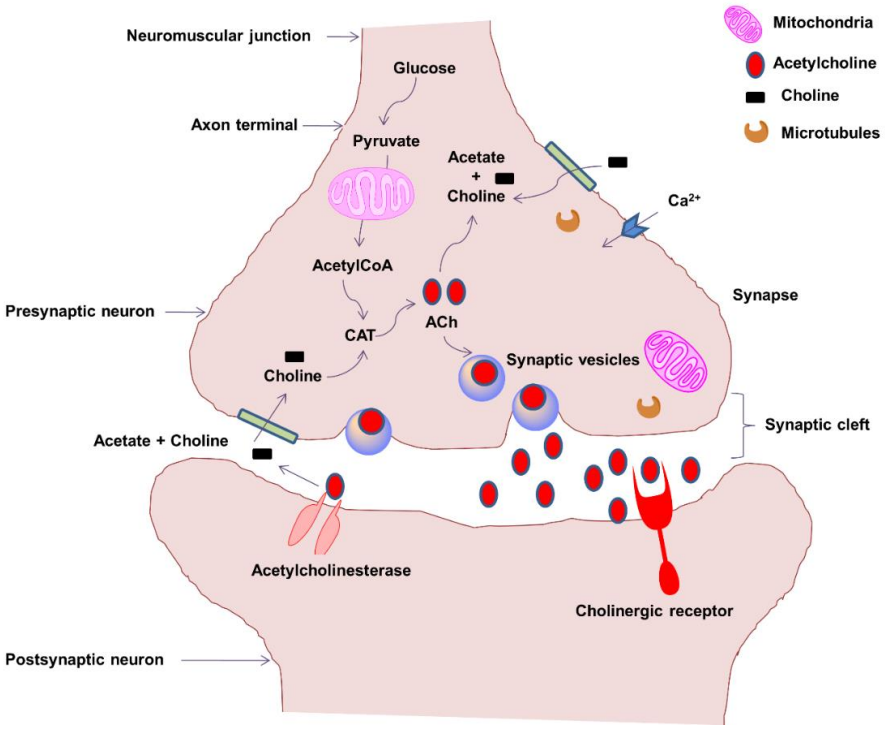

Figure 1.1. Diagrammatic representation of ACh synthesis, metabolism, and its mode of action. Reproduced with permission from ref. [213]. Copyright 2021 Y. P. Singh *et al.* Reprinted with the permission from European journal of medicinal chemistry, Elsevier.

The metabolism of ACh takes place in the synaptic gap by acetylcholinesterase (AChE), which regulates the dynamic equilibrium of ACh in the healthy 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). Upon entering the active site, the hydroxyl anion attacks the carbonyl carbon of ACh, resulting in the acylation of the serine residue and release of choline from

the active site. This is immediately followed by the de-acylation of the serine residue from the nucleophilic attack by water molecules, resulting in the liberation of acetic acid and active site regeneration [29].

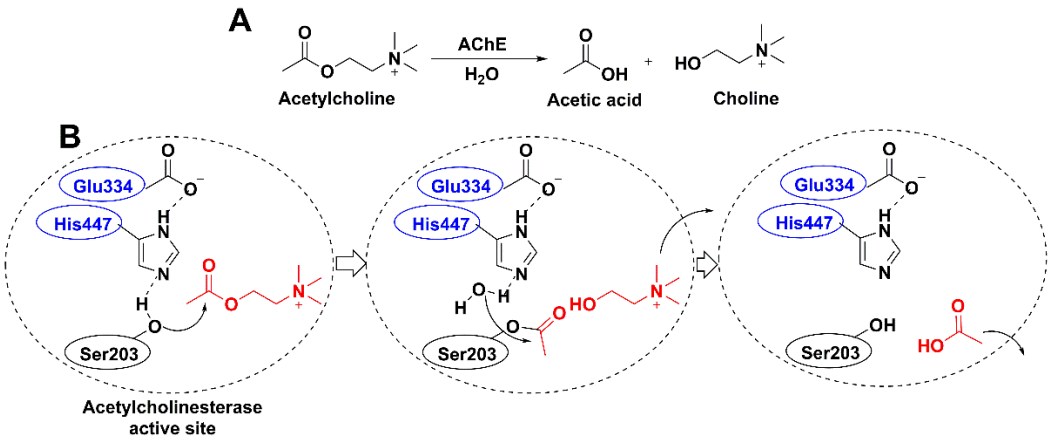

Figure 1.2. Mechanism of substrate cleavage through AChE (**A**&**B**).

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

AD's pathological hallmarks are characterized by the accumulation of amyloid β (Aβ) plaques outside the neurons and neurofibrillary tau protein tangles (NFTs) inside the diseased neurons. In recent years, mounting evidence have driven the hypothesis that these markers probably appear many years before the onset of cognitive symptoms of AD [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 transmembrane glycoprotein, known as an amyloid precursor protein (APP; 695 amino acid residues), involves a fragmentation process to produce toxic Aβ peptides of variable length (39-42 AAs). Among these, $A\beta_{1\text{-}42}$ peptides are more hydrophobic and tending to self-aggregate into more neurotoxic soluble misfolded Aβ-aggregates (dimers and oligomers) protofibrils, fibrils and, ultimately into insoluble fibrils (senile plaque) [31]. The oligomeric isoforms are considered to be principle 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 inside the mitochondria, disrupt its normal functioning and upregulates Janus kinase (JNK), Cdk5, dual-specificity tyrosine-phosphorylation regulated kinase-1 A (Dyrk1A), and mitogen-activated protein kinase (p38MAPK) activity by promoting various types of cellular stress such as oxidative stress, reduce glucose intake and calcium dyshomeostasis [33]. It can also enhance the production of proinflammatory cytokines [tumor necrosis factor (TNF∝), interleukin 6 (IL-6), and interleukin-1β (IL-1β), etc.], and subsequent formation of ROS [34]. The Aβ mediated microglial cells activation induces a neuronal inflammatory response by producing a variety of inflammatory factors, which includes TNFα, monocyte chemotactic protein 1 (MCP-1), IL-6, and ROS, which triggers a long-standing reactive cycle to microglial cells and eventually leads to the development of AD *via* promoting neurotoxicity [35, 36].

The elevated Aβ level activates a complex cascade of events, including NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) mediated transcription of proinflammatory cytokines and chemokines in astrocytes. This may stimulate neuronal cellular toxicity or even over-production of Aβ in astrocytes [37]. There is number of experimental pieces of evidences from *in-vitro* and preclinical studies that implicated the release of proinflammatory cytokines. The ROS accelerate tau pathology and NFT formation by upregulating Cdk5, JNK, Dyrk1A, and p38MAPK activity, which exacerbates hyperphosphorylation of axonal microtubule-associated tau proteins by stimulating tau kinases activity and inactivation of phosphatases [38-41]. These anomalous tau proteins lose their tubulin-binding capacity, contributing to the destabilization of microtubules, and serve as precursors for the formation of paired helical filaments (PHFs) or straight, helical filaments (SFs). These filamentous tau proteins undergo self-polymerization and

produce NFTs [42]. Despite significant advances in AD pathogenesis, its primary causes are still unclear; however, it is hypothesized that accumulation of the Aβ may be one of the early events that can trigger a complex cascade of events and the signaling process involved in tauopathies. The average duration of clinically apparent dementia is 8-10 years, which is generally preceded by preclinical and prodromal stages that typically extend over two decades [13].

1.3.3. Role of metals in AD

Small amounts of certain metals, such as zinc, copper, and iron, are necessary for our body to function correctly. The biometals zinc, copper, and iron, play an important role in the brain, including cell signaling and neuroplasticity [43]. However, excessive amount of free metals may be harmful to our health. Abnormally high concentrations of the metals above have been found inside of Aβ plaques, the toxic protein deposits that are the hallmark of the AD [44]. Copper, and iron are also believed to contribute to oxidative stress.

Iron plays a vital role in several cellular functions, including mitochondrial oxidation, cell growth, synthesis, and metabolism of neurotransmitters such as dopamine (DA) [45]. The iron content in the brain gradually increases with age. Iron is essential for neuronal growth; however, excess iron can cause the demise of the neuron [46]. Therefore, an optimal concentration of iron should always be maintained in cellular compartments to avoid the iron-associated toxic effects such as reactive oxygen species (ROS) generation. The iron storage proteins like ferritin and neuromelanin get saturated under an overload condition; thereby, an increase in the labile iron pool leads to neurodegeneration [47].

Aβ binds to iron through three histidine residues and one tyrosine residue in the hydrophilic N-terminal region of the peptide, which helps to stabilize these iron ions [48]. Furthermore, studies also found that the binding of Fe^{2+} ion to Aβ reduced the peptide helix structure and increased the

β-sheet content of the peptide, indicating that Fe^{2+} ion promotes Aβ monomers to form oligomers and fibrils by enhancing the interaction between peptide-peptides [49]. The high binding affinity of Aβ towards metals and its ability to reduce the Fe^{2+}, is leading cause for the generation of ROS. Iron is also responsible for tau phosphorylation and aggregation. Hyperphosphorylated tau accumulates in NFTs, thereby, induces antioxidant heme oxygenase-1 (HO-1) protein. Although HO-1 is antioxidant, on the other hand, it causes Fenton reaction *via* the release of Fe^{2+} (Figure 1.3). Iron overload inhibits the expression of furin, which favors the activation of β-secretase, thereby promoting the production of Aβ from the amyloid pathway [50]. Thus, it seems that iron can stimulate Aβ aggregation but also reduces the toxicity of these aggregates [51] (Figure 1.4).

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

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

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

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

Copper is a necessary trace metal in nervous system development since disruption of its homeostasis leads to neurodegenerative disorders like AD. Cu^{2+} ions bind to Aβ peptides with greater affinity and increase the percentage of β-sheet and α-helix structures in Aβ peptides, which ultimately initiate Aβ aggregation [52]. Increased concentrations of Cu^{2+} ions enhance Aβ fibril formation while binding of Cu^{2+} ions to Aβ noticeably increases its toxicity for cells. Copper ions in complex with Aβ fibrils produce H_2O_2 in the presence of reducing agents. When the ratio of Cu^{2+} to peptide increases, H_2O_2 levels and the production of OH^- radicals increase, and the morphology of aggregates changes from fibrillar to amorphous [53].

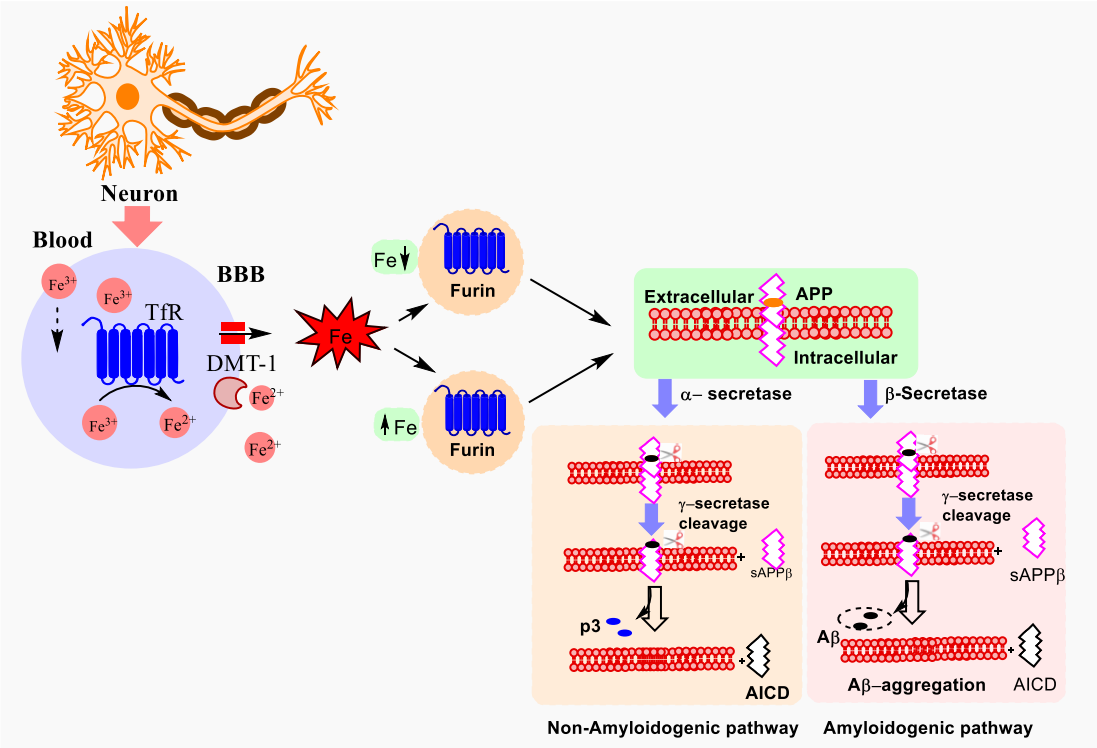

Figure 1.4. Schematic representation of role of iron in AD. Reproduced with permission from ref. [214]. Copyright 2021 Y. P. Singh *et al.* Reprinted with the permission from molecular diversty, Springer-Nature.

1.3.4. Role of oxidative stress (OS) in AD

Oxidative stress is defined as dyshomeostasis between ROS/RNS and the cells' antioxidant ability to neutralize them. One of the early events in the neurodegeneration pathway related to AD is increased oxidative stress. The ROS is mainly generated due to the imbalance in the redox system of the mitochondria [54]. The electrons leaked from the inner membrane react with oxygen to form superoxide anions ($O_2^{\cdot-}$). These superoxide radicals further react and generate other ROS forms like hydrogen peroxide (H_2O_2) and hydroxyl ion (OH^-). Whereas ROS like superoxide ($O_2^{\cdot-}$) and H_2O_2 interacts with nitric oxide (NO) to generate peroxynitrite anion (RNS) [55, 56]. The oxidative stress is also mediated by metals, especially copper and iron, as shown in figure 1.5. Iron generates free radicals through the Fenton reaction. Overproduction of reactive species

(ROS/RNS) leads to compromised antioxidant function and induces toxicity *via* lipid peroxidation, oxidation of proteins, DNA and RNA [57]. Hydroxyl radicals formed from the reaction of highly diffusible H_2O_2 reacts with redox-active metals (Cu and Fe) is the most probable source of oxidized nucleotides (DNA and RNA) [58]. The oxidative damage to neuronal DNA and RNA can cause transcription and replication of essential genes like nucleoside guanosine [59].

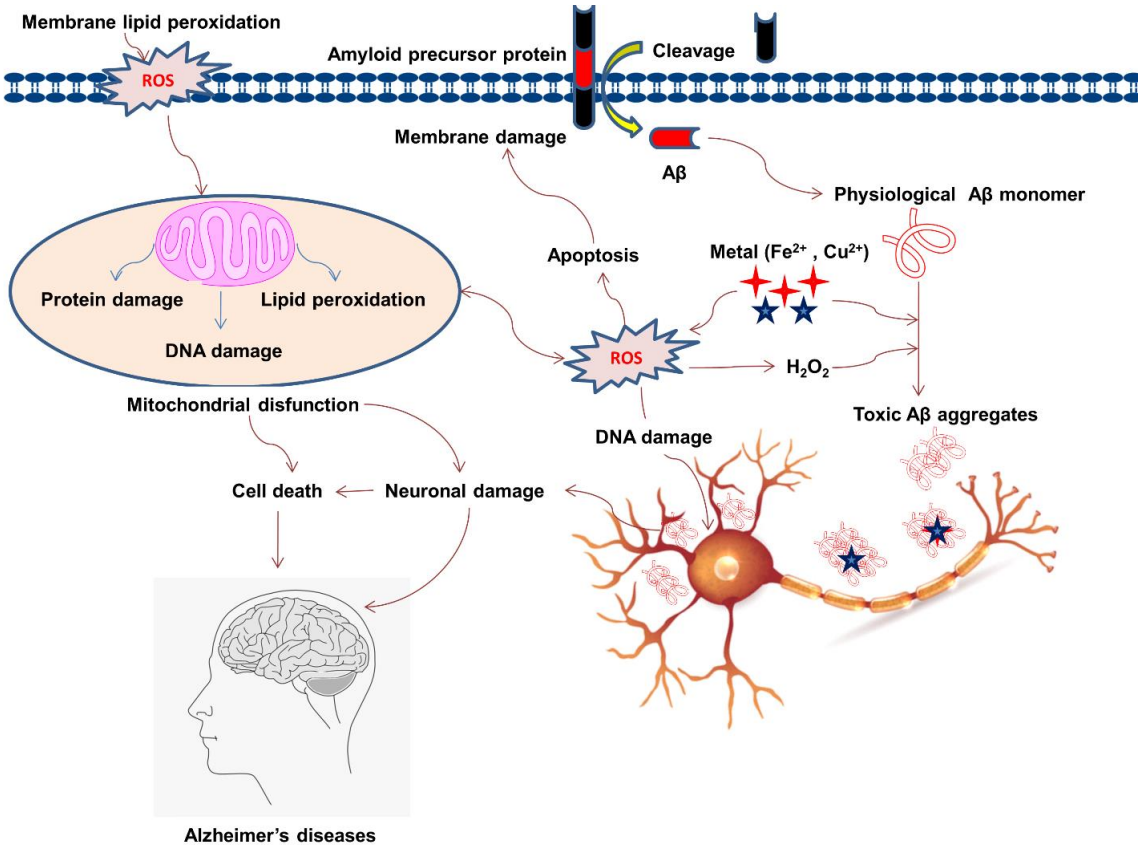

Figure 1.5. Diagrammatic representation indicating the role of ROS and metals in AD progression. Reproduced with permission from ref. [213]. Copyright 2021 Y. P. Singh *et al.* Reprinted with the permission from European journal of medicinal chemistry, Elsevier.

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

The amyloid precursor protein (APP) is one member of a large family of type 1 transmembrane protein that includes the amyloid precursor-like proteins (APLP1 and APLP2) in mammals and the amyloid precursor protein-like (APPL) in Drosophila [60]. APP is produced in considerable

amounts in neurons and is metabolized very speedily. The exact biological function of APP is not well established and remains one of the significant issues in this field. Aβ is produced by the endoproteolysis of APP. On the cell surface, APP can be proteolyzed by two proteases, the α, and β-secretases. The cleavage initiated by α-secretase is non-amyloidogenic, which does not generate toxic Aβ [61]. While cleavage started by β-secretase generate the N terminus of Aβ, thus producing a membrane-bound C-terminal fragment called C99 [62]. Then, γ-secretase cleaves C99 to generate the mature toxic Aβ peptide. However, β-secretase cleavage occurs precisely at Asp+1 and Glu+11 of Aβ, indicating that β-secretase is a site-specific protease. Significantly, therapeutic inhibition of β-secretase would decrease the production of all forms of Aβ, including the pathogenic $A\beta_{42}$ (Figure 1.6).

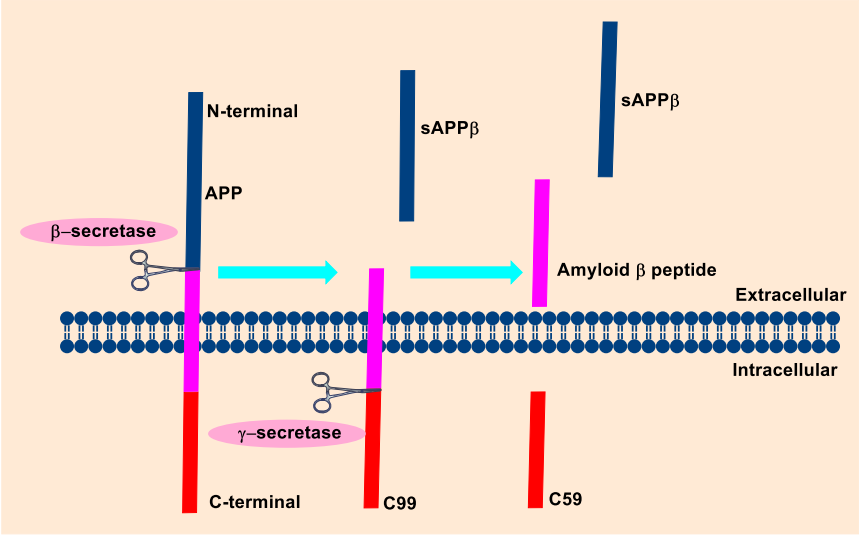

Figure 1.6. Diagrammatic representation indicating the role of β-secretase in Aβ aggregation.

1.4. Current drug targets for AD

The currently available target for AD includes AChE and NMDA receptor. Acetylcholinesterase (AChE) is classified as a serine protease responsible for the degradation of acetylcholine [63]. Therefore, it plays a very critical role in cholinergic neurotransmission. The active site can be divided into anionic site, catalytic triad or esteratic site (ES), oxyanion hole, selectivity

determinant acyl pocket, and peripheral anionic site (PAS) [64]. Next, butyrylcholinesterase (BChE), an isomer of cholinesterase, is also present in the brain responsible for ACh cleavage. Recent studies suggest that brain-targeted BChE lowers Aβ levels in transgenic mice and improves cognitive performance in animals. The levels of both AChE and BChE change dramatically as the disease progresses. Therefore, both enzymes have been explored for neuroprotective and disease-modifying therapy for AD.

1.4.1. Cholinesterases (ChEs)

Cholinesterases (ChEs) are responsible for the hydrolysis of ACh into choline and acetic acid. The two main types of ChEs: (i) acetylcholinesterase (AChE), and (ii) butyrylcholinesterase (BChE) are reported in the literature [65]. ChE is one of the key enzymes that play a significant role in synaptic transmission by hydrolyzing the neurotransmitter ACh. Therefore, an increase in ACh level by inhibiting AChE activity could ameliorate cognitive and mental abilities in AD patients. Intriguingly, in the advanced stages of AD, the level of AChE goes down by 90% compared to the normal brain, which has grave concern about its utility as a target for therapeutic agents in the later stages of AD [25]. The recent biological studies implicated that inhibition of AChE alone cannot escalate ACh level; therefore, ChE inhibitors have favorable outcomes that last for a shorter time, about 1 to 3 years, without altering disease progression [66]. Evidence from clinical studies is in substantial agreement with AChE inhibitors' ineffectiveness in the appropriate management of moderate to severe stages of AD [67-69].

AChE is a monomer in a natural state with an MW around 60,000. It contains 537 amino acid residues which are arranged as 12 stranded mixed β-sheet surrounded by 14α-helices. AChE contains a hydrophobic active site which can be divided into two subunits catalytic active site (CAS) and peripheral anionic site (PAS). CAS (Ser200, Glu327, and His440) is situated at the

base of a deep and narrow gorge (around 20 Å long and 4.5 Å wide), lined with 14 aromatic residues [29]. The active site additionally contains a subsite (the "anionic subsite"), which has Trp84 as an essential residue to interact with the quaternary ammonium group in the substrate (ACh) and different ligands utilizing cation π-interaction, situated close to the bottom of the cavity in addition to the Phe330. The PAS or β-anionic site of AChE is not very well characterized and is located at the catalytic gorge's entrance and is roughly 20 Å away from the active center. The presence of a ligand changes the conformation of the active containing Tyr70, Asp72, Tyr121, Trp279, and Tyr334. Trp279 among these is a crucial residue involved in the adhesive action of the AChE (Figure 1.7). The PAS binds with the substrate temporarily as the initial phase and improves the catalytic efficiency trapping the substrate on its way to the active site [70].

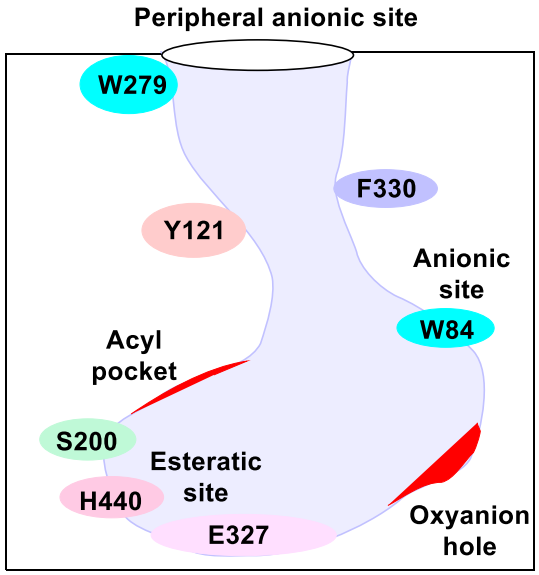

Figure 1.7. Schematic view of the active site of AChE. The bottom of the gorge is characterized by an anionic site, which contains esteratic site, acyl pocket, an oxyanion hole, and PAS is located 20 Ȧ above the active site.

On the other hand, BChE is an ά-glycoprotein found in the central and peripheral nervous systems. It is a nonspecific or pseudocholinesterase or serum cholinesterase, hydrolyzing both choline and aliphatic esters [71]. CAS of *h*BChE is composed of Ser198, His438, and Glu325 residue. The

exact role of BChE in AD is still under investigation; however, it can compensate the role of neuronal AChE in progressive neurodegeneration in AD and takes over the function of AChE [72]. Thus, inhibition of AChE and BChE can effectively induce symptomatic relief in AD treatment [73]. Therefore, dual and selective AChE/BChE inhibitors can be an effective therapeutic approach for the effective management of AD.

1.4.2. NMDA receptor in AD

The N-methyl-D-aspartate receptor (also known as NMDAR), is an ionotropic glutamate receptor and ion channel found in neurons (Figure 1.8). NMDA plays a pivotal role in the synaptic transmission, and synaptic plasticity is thought to underlie learning and memory, which is not only central to the function of the nervous system but also to neurotoxicity. Overactivation of the NMDA receptor, causing an excessive influx of Ca^{2+} can initiate excitotoxicity which leads to neurodegenerative disorders including AD [74]. The main problem with the utilization of NMDA receptor antagonists for neuroprotection is that the physiological actions of the NMDA receptor are essential for normal neuronal function [75].

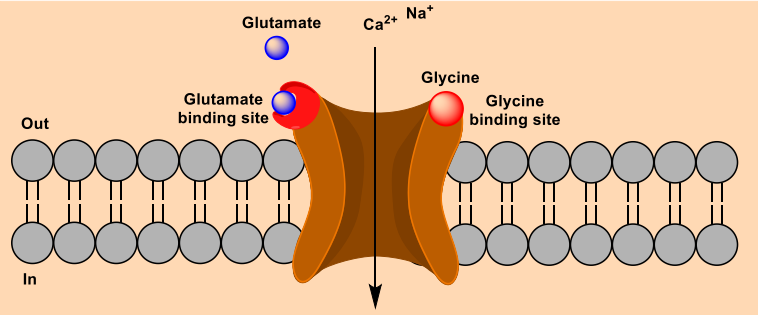

Figure 1.8. NMDA receptor complex as a therapeutic target in AD.