Chapter 1
(Introduction)

1.1 Alzheimer's disease

Alzheimer's disease (AD) is an enormous healthcare burden caused by loss of neurons and synapses, particularly in the neocortex and hippocampus [Katzman and Saitoh 1991]. AD causes substantial structural and functional damage to the brain, which results in severe behavioral alterations and cognitive dysfunction [Kim et al. 2020]. The loss of cognitive abilities is one of the most demoralizing factors of AD that implicates the massive financial and emotional burden on the patients and their families [Cherian and Mathew 2019]. AD was first diagnosed and notified by German psychiatrist Dr. Alois Alzheimer in 1901. After an autopsy of patient brain suffering from AD, Alzheimer discovered and described the histological alterations later known as plaques, and neurofibrillary tangles (NFTs). He also identified nerve cells atrophies with shrinkage of the brain and termed this illness as "Alzheimer's disease" [Hippius and Neundörfer 2003].

The etiology of AD indicates three main phases: mild, moderate, and severe. Each phase presents with different sets of symptoms. The first phase lasts for 2–4 years eliciting mild symptoms such as mood swings, lack of interest, troubled writing and driving, language problems, depression, etc. Also, these symptoms may precipitate owing to other medical conditions; but does not confirm that patients are actually suffering from AD. In the moderate phase of AD, which can last for 2–10 years, memory loss begins to worsen, affecting daily chores. In the severe AD phase, symptoms of previous phases get combined, and the person also face some other severe complications such as hallucinations, weight loss, seizures, skin infections, extreme mood swings, motor dysfunctions, etc, which lasts for 1–3 years, and finally patient deceased [Birks and Harvey 2018, de Oliveira Silva et al. 2019].

A recent report of 2018 from the World Health Organization (WHO) accounts for 50 million cases of AD worldwide, and this figure is estimated to be tripled by 2050 [Patterson 2018]. AD is eighth topmost health complication that progresses symptomatically from mild to severe and is most prevalent amongst aged population [Pan and Nicolazzo 2018, van der Lee et al. 2018]. The estimated prevalence in low and middle-income countries is 58%, rising to 63% in 2030 and 68% in 2050. In India, four million peoples are currently reported to be suffering from this devastating and terrible disease [Sathianathan and Kantipudi 2018].

1.2 Pathophysiological mechanisms involved in AD

There are several underlying pathophysiological mechanisms involved in the progressive cognitive deficits of AD such as, decline in acetylcholine (ACh) levels, amyloid-beta (Aβ) aggregation and deposition, activation of *N*-methyl-D-aspartate receptor (NMDAR), oxidative stress, tau hyperphosphorylation and generation of NFTs, apolipoprotein E4 (APOε4) gene transcription, cyclic-AMP-response element-binding protein (CREB) signaling pathways, etc.

1.2.1 Cholinergic hypothesis

The cholinergic hypothesis states that cholinergic neurons are affected in the early stage of the AD with the loss of cholinergic functions involved in the synthesis of ACh in basal forebrain resulting in cognitive dysfunction. This hypothesis came in existence in mid-1970s with the biochemical investigation of brain ACh levels in AD patients [Bohnen et al. 2018, Perry et al. 1977, Perry et al. 1978]. These studies have observed profound loss of choline acetyltransferase (CAT) responsible for the synthesis of ACh. Further, CAT was found to be explicitly reduced in hippocampal and neocortical regions, which are the prime sites of neurodegeneration in AD [Perry 1986]. The studies on AD patients and animals have also suggested that cholinergic abnormalities such as

choline transport, ACh release, nicotinic and muscarinic receptor expression, and axonal transport can contribute significantly to behavioral dysfunctions associated with the AD [Sultzer 2018]. These findings revealed that cholinergic replacement therapy should be the primary approach to address the treatment of AD.

The profound loss of cholinergic neurons is the most accepted and established pathophysiology of AD, which subsequently transforms into progressive decline in the later stage of the disease. ACh neurotransmitter plays a pivotal role in the regulation of cognitive functions, which is hydrolyzed by acetylcholinesterase (AChE) into choline and acetic acid. The low levels of ACh in cholinergic synapse causes cognitive dysfunction [Simpraga et al. 2018]. The detailed cholinergic transmission is discussed in Figure 1.1 [Sharma et al. 2019].

Cholinesterase (ChE) is of two types: AChE and butyrylcholinesterase (BChE). AChE exists in two isoforms: asymmetric and globular. There is a selective loss of globular G4 tetrameric form in AD. Therefore, predominantly available G1 monomeric form could be the more potential target for AChE inhibitors [Sakayanathan et al. 2019]. Another esterase enzyme is known as pseudo or non-neuronal cholinesterase termed as BChE, which is the non-specific type of serine hydrolase enzyme catalyzes the choline ester hydrolysis [Massoulié et al. 1993]. Clinical evidence has suggested the significant role of BChE in the regulation of ACh and maintaining normal cholinergic functions, which makes BChE an additional promising target against AD [Knez et al. 2018, Kumar et al. 2018, Lu et al. 2018]. Therefore, dual targeting of both ChEs could render a therapeutic advantage in the advanced and late-phase AD. Moreover, both ChEs are acknowledged for their substantial role in Aβ aggregation [El-Sayed et al. 2019, Husain et al. 2018].

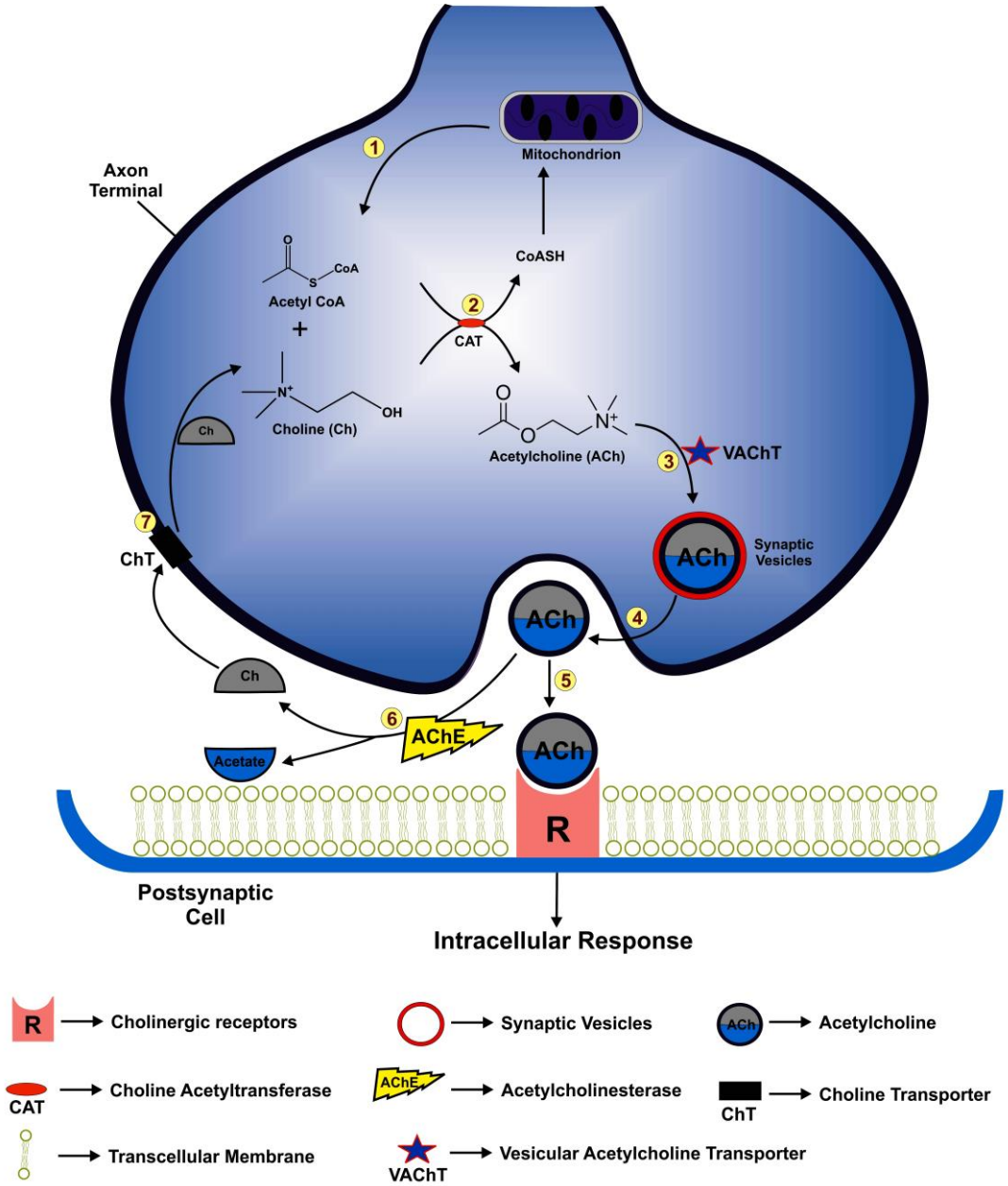


Figure 1.1. Synthesis of ACh and cholinergic neurotransmission. (1) Firstly, acetyl coenzyme A is made available through glycolysis in mitochondrion; (2) Choline acetyltransferase (CAT) mediated transfer of acetyl group from acetyl CoA to choline; (3) Vesicular ACh transporter (VAChT) facilitates the storage of ACh into the synaptic vesicles; (4) Increase Ca^{2+} influx facilitates the release of ACh from synaptic cleft; (5) Binding of ACh to the cholinergic receptors; (6) Hydrolytic degradation of ACh by AChE to choline and acetic acid; (7) Re-uptake of choline by presynaptic neuron [Sharma et al. 2019].

1.2.2 Aβ hypothesis

The deposition of toxic Aβ aggregates in nerve cells is the mainstream hypothesis of AD for the last two decades. The Aβ is a small fragment formed after the proteolytic

cleavage of an amyloid precursor protein (APP) [Vassar et al. 1999]. The APP is a transmembrane protein extended from inside (C-terminal) to outside (N-terminal) of the fatty acid membrane [Kienlen-Campard et al. 2008]. The proteolytic breakdown of APP processed through two different pathways: amyloidogenic and non-amyloidogenic. The alpha- (α-) secretase acts in non-amyloidogenic pathway and believes to have neuroprotective activity with the formation of APP-α precursor and C83 α-subunit [Kojro and Fahrenholz 2005]. On the contrary, the β-secretase enzyme also known as β-amyloid cleaving enzyme forms APP-β along with C99 β-subunit in amyloidogenic pathway [Lin et al. 2000]. Further, by the action of γ-secretase, the β-subunit forms Aβ peptides, which get accumulated extracellularly and leads to the deposition of toxic Aβ plaques [Menting and Claassen 2014]. Progressive cerebral accumulation of Aβ aggregates leads to senile plaque formation followed by a complex cascade of events such as inflammatory responses, microglia activation, cytokine release, and astrocytosis. Altogether these reactions are responsible for progressive neuritic injury, neuronal deficits and cognitive dysfunctions [Hardy and Higgins 1992] (Figure 1.2).

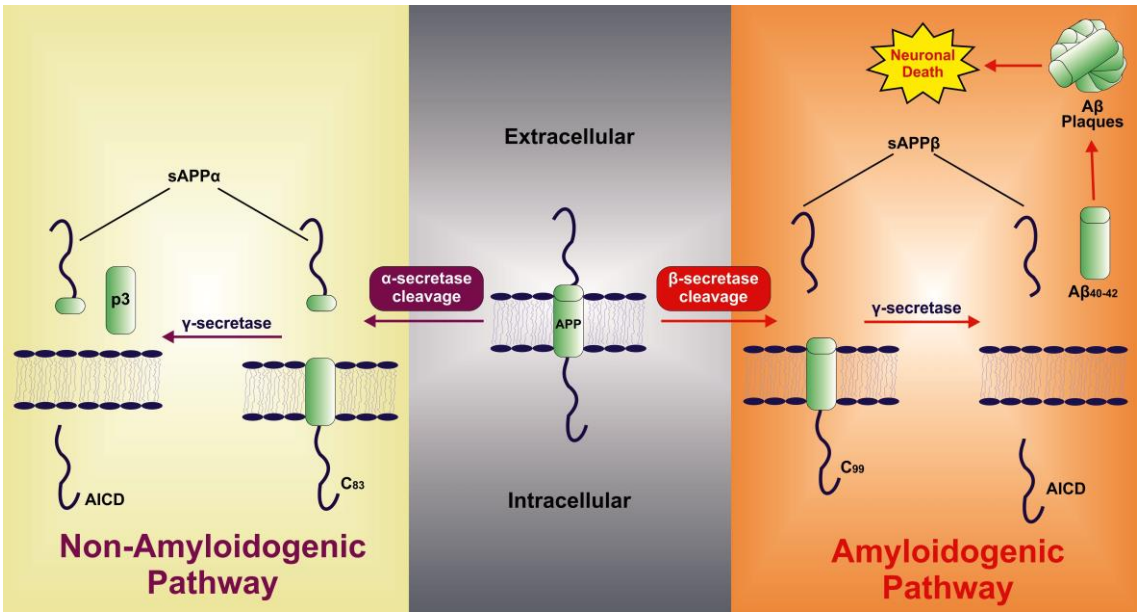


Figure 1.2. Amyloidogenic (β-secretase) and non-amyloidogenic (α-secretase) pathways [Sharma et al. 2019].

1.2.3 Excitotoxic hypothesis

The glutamate is an excitatory neurotransmitter, found abundantly in the hippocampus and cortex and acts via glutamate receptors: (1) Ionotropic and (2) Metabotropic receptors. The glutamate mostly binds to ionotropic receptor subtypes, i.e., NMDAR, and promotes depolarization, followed by magnesium ions mediated closing of the cationic channel to prevent the entry of calcium ions in resting stage. In AD, there is an excessive activation of NMDA type glutamate receptor in neuronal cells that causes the release of bound magnesium ions and allows the entry of calcium ions into the neuronal cells. The excessive influx of calcium ions hampers the neuronal transmission, damages the nerve cells, and is responsible for neurodegeneration and cell death [Olney et al. 1997].

1.2.4 Oxidative stress hypothesis

The role of free radicals in neurodegeneration is well recognized in aging and AD. Neuronal cells are more vulnerable to free radicals damage due to high oxygen consumption and lack availability of antioxidant enzymes compared to other organs [Coyle and Puttfarcken 1993]. An imbalanced production of the free radicals, i.e., reactive oxygen and nitrogen species (ROS and RNS), and poor availability of antioxidant enzymes are the major detrimental factors leading to oxidative stress. The release of disproportionate free radicals prompts protein oxidation and lipid peroxidation, which subsequently results in impaired cognitive functions [Allan Butterfield et al. 2002]. There are several factors that initiate the oxidative damage in the brain, such as defective mitochondrial energy metabolism [Beal et al. 1993, Lin and Beal 2006], aggregation of Aβ peptides [Butterfield et al. 2001], DNA oxidation [Lyras et al. 1997], lipid peroxidation, and presence of excessive trace elements and transition

metals (aluminum, mercury, copper, zinc, and iron) [Ercal et al. 2001, Lyras et al. 1997, Stohs and Bagchi 1995].

The Aβ could enter the mitochondria to increase the generation of free radicals and might induce oxidative stress during the early stage of AD [Markesbery 1997, Zhao and Zhao 2013]. The study of AD post-mortem brain showed increased Aβ and APP levels in the mitochondrial membranes, which lead to disruption of electron transport chain (ETC), and irreversible neurodegeneration [Reddy and Beal 2008]. Several environmental factors such as UV and ozone, pollutants and smoking significantly potentiate the formation of ROS and RNS [Kandola et al. 2015]. There are several consequences of these stress conditions that include reduced endogenous antioxidant capacity, mitochondrial dysfunction, altered Ca^{2+} homeostasis, abnormal protein accumulation, altered proteasome functions, and membrane damage [Poprac et al. 2017]. Various cytosolic and membranous enzymes such as cytochrome P450 (CYP) [Hrycay and Bandiera 2015], manganese superoxide dismutase (MnSOD) [Indo et al. 2015], xanthine oxidase (XO) [Jaeschke and Mitchell 1989], myeloperoxidase (MPO) [Al Ghouleh et al. 2011], lipoxygenases and cyclooxygenases [Adibhatla and Hatcher 2008] constitute the primary source of generation of ROS, while NADPH oxidase (NOx) [Daiber et al. 2017] and nitric oxide synthase (NOS) [Hsieh et al. 2014] are mostly responsible for RNS generation, leading to oxidative or nitrative stress, respectively [Pratico 2008].

1.2.5 Tau hypothesis

The tau protein plays a significant role in the stability of microtubules, which is an essential factor in sustaining cell integrity [Drubin and Kirschner 1986]. Tau proteins exist in six isoforms having three major binding domains: N-terminal projection, C-terminal microtubule-binding domain and shorter tailing sequence [Wang and

Mandelkow 2016]. In normal condition, a tau protein stabilizes the microtubules by phosphorylation process in the axonal membrane. There are 85 phosphorylation sites have been identified in the tau protein. Protein phosphorylation is the addition of a phosphate group by esterification at three types of amino acids: serine, threonine and tyrosine [Ittner and Götz 2011, Mietelska-Porowska et al. 2014].

Several studies indicated that overactivation of kinases and inactivation of phosphatases significantly induces tau hyperphosphorylation. The kinases such as glycogen synthase kinase-3β (GSK-3β), CDK-5, tau protein kinase-I (TPKI), microtubule affinity-regulating kinase (MARK), extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), mitogen-activated protein kinase (MAPK), AMP-activated protein kinase (AMPK), dual-specificity tyrosine-phosphorylation-regulated kinase-1A (Dyrk1A), Src family non-receptor tyrosine kinase (SFKs), protein kinase-A (PKA), Ca^{2+}/calmodulin dependent kinase (CaMK), and Janus kinase (JNK) are responsible for tau hyperphosphorylation (Figure 1.3) [Coman and Nemeş 2017]. In the AD, the tau proteins get hyperphosphorylated into insoluble intracellular NFTs and lose the tenacity to bind to the microtubules of brain cells [Šimić et al. 2016]. These hyperphosphorylated forms bind to each other, tying themselves in knots called NFTs that disrupt neurological plasticity and lead to neurodegeneration [Grill and Cummings 2010].

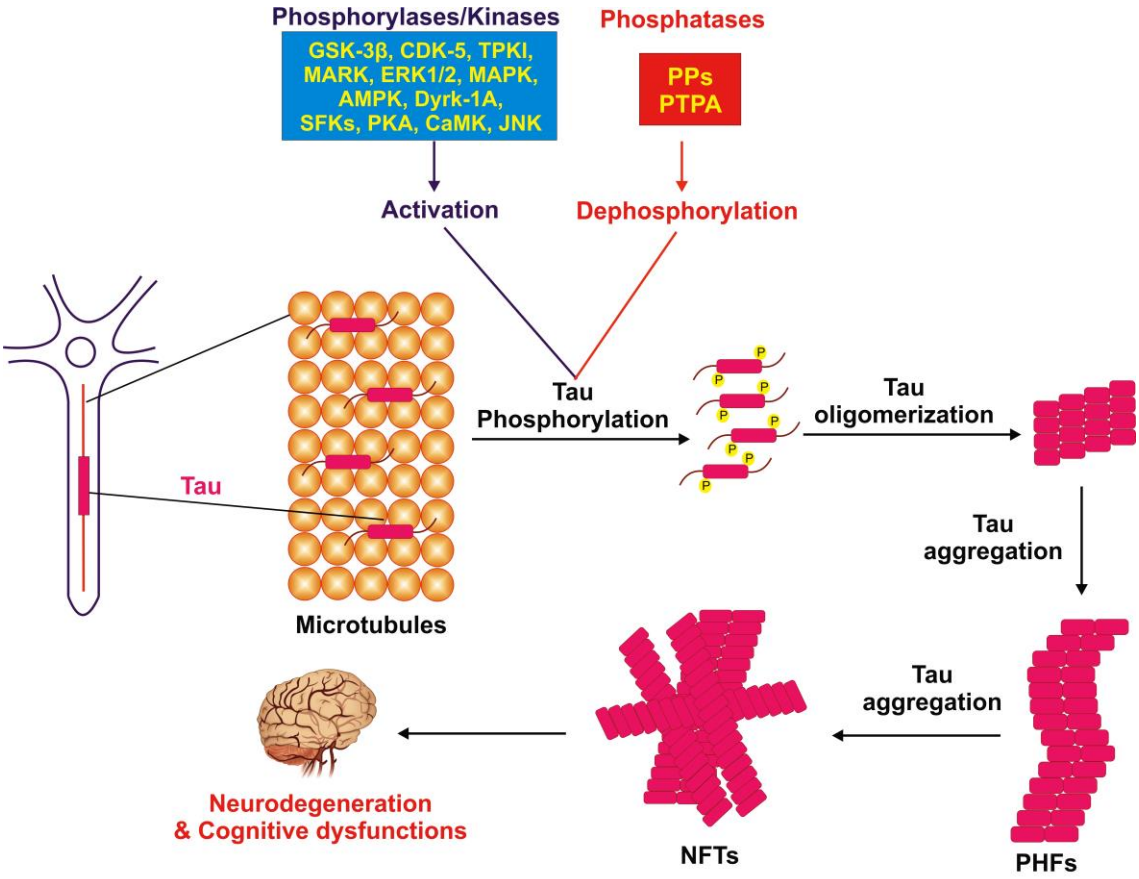


Figure 1.3. The process of tau aggregation, formation of NFTs, and neurodegeneration. GSK-3β: glycogen synthase kinase-3β; CDK-5: cyclin-dependent kinase-5 ; TPKI: tau protein kinase-I; MARK: microtubule affinity-regulating kinase; ERK1/ 2: extracellular signal-regulated protein kinases 1 and 2; MAPK: mitogen-activated protein kinase; AMPK: AMP-activated protein kinase; Dyrk1A: dual-specificity tyrosine-phosphorylation-regulated kinase-1 A; SFKs: Src family non-receptor tyrosine kinases; PKA: protein kinase-A; CaMK: Ca^{2+}/calmodulin dependent kinase; JNK: Janus kinase; PPs: protein phosphatases; PTPA: protein tyrosine phosphatase-A; PHFs: paired helical fibrils; NFTs: neurofibrillary tangles [Sharma et al. 2019].

1.2.6 APOε4 hypothesis

The ApoE is a glycoprotein comprising of 299 amino acid residues. ApoE is mainly produced by astrocytes and plays role in cholesterol transportation through ApoE receptors [Guo et al. 2018]. ApoE gene is located on chromosome 19 having three most common types of polymorphic alleles: ApoE2 (ε2), ApoE3 (ε3) and ApoE4 (ε4) [Dai et al. 2018]. Several studies have confirmed that the ε4 allele is the most commonly associated genetic risk factor linked with the late onset of the AD [Glorioso et al. 2019,

Michaelson 2014, Saunders et al. 1993], whereas the ε2 allele decreases the risk of the AD [Sinclair et al. 2017]. Women with APOE4 are more likely to develop mild cognitive impairment or Alzheimer's disease than men with APOE4 [Altmann et al. 2014, Müller-Gerards et al. 2019]. In APOE4 carriers with mild cognitive impairment, women have higher levels of biological markers associated with Alzheimer's than men [Liu et al. 2019].

The Apoε4 allele increases Aβ production with reduced Aβ clearance [Corona et al. 2016, Qi and Ma 2017]. There are high affinity and specific binding of Aβ peptide with the ApoE [Garai et al. 2018]. Other mechanisms involved to be associated with ε4 allele are tauopathy [Shi et al. 2017], neuroinflammation [Kloske and Wilcock 2020] and reduced rate of glucose metabolism at the parietal, temporal, and prefrontal regions of the brain in AD condition [Bussy et al. 2019, Karim et al. 2019]. In response to injury or neuroinflammation, Apoε4 undergoes neuron-specific proteolysis, which results the formation of its toxic bioactive fragments that are responsible for disruption of mitochondrial energy balance leading to neuronal death [Jembrek et al. 2018].

1.2.7 CREB signaling pathways

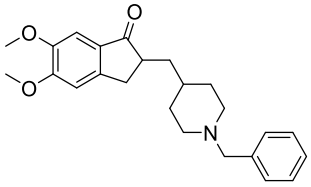
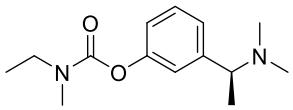
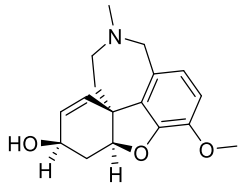
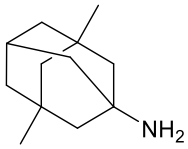
The CREB regulates several downstream genes such as somatostatin [Gonzalez and Montminy 1989], enkephalin [Mayr and Montminy 2001], corticoliberin [Kageyama et al. 2007], and period circadian protein homolog 1/2 [Dibner et al. 2010]. CREB is abundantly expressed in the hippocampal and neocortex region, center of neurodegeneration in the AD [Ettcheto et al. 2018]. CREB-dependent genes are regulated by phosphorylation, and significantly controlled by kinases [Bitner 2012]. CREB expression is found to be considerably downregulated in several mouse models of the AD [Pugazhenthi et al. 2011]. It is also evident that the effect of Aβ induced memory and synaptic loss is also mediated by CREB signaling pathway [Saura and

Valero 2011]. Thereby, increasing expression of CREB could be one of the potential therapeutic approaches in the treatment of AD.

1.3 Available neurotherapeutics for the treatment of AD

Currently, there are four drugs approved for the treatment of AD. Among them, three are cholinesterase inhibitors: Donepezil, Rivastigmine and Galantamine, and one is a NMDA receptor antagonist, memantine. The structures, mechanism, and adverse effects of available therapeutics are tabulated in Table 1.1. All the currently available therapeutics only provide symptomatic relief, and are unable to halt the progression of AD.

Table 1.1. Chemical structures, mechanism, and adverse effects of USFDA approved drugs for the treatment of AD.

Name	Structure	Mechanism	Adverse effects	Ref.
Donepezil		Cholinesterase inhibitor	Nausea, vomiting, loss of appetite/weight loss, diarrhea, weakness, dizziness, drowsiness, tremor, slow/irregular heartbeat, fainting, black stools, vomit that looks like coffee grounds, severe stomach/abdominal pain, seizures, trouble urinating.	[Kudo 2016]
Rivastigmine		Cholinesterase inhibitor		[Gonzalez et al. 2016]
Galantamine		Cholinesterase inhibitor		[Janssen and Schäfer 2017]
Memantine		NMDA receptor antagonist	Body aches, dizziness, constipation, headache, and trouble breathing	[Wilkinson 2016]

1.4 Novel therapeutic strategies for the development and discovery of compounds for the treatment of AD

Over the past couple of decades, several efforts have been made to develop novel therapeutics for the effective treatment of AD. Several new drug candidates were

identified to treat AD, but had to be eventually withdrawn from the clinical trials owing to their toxicity at a higher dose and lack of clinical efficacy. These include metrifonate, tesofensine, velnacrine, eptastigmine, and huperzine A [Imbimbo et al. 2000, López-Arrieta and Schneider 2006, Murphy et al. 1991, Yaari and Hake 2015]. Additionally, there are various disease-modifying candidates such as gantenerumab, crenezumab from Roche, solanezumab from Eli Lilly, and bapineuzumab from Pfizer have been reported to be unsuccessful in clinical trials [Mehta et al. 2017]. The diminished pipeline of new molecules presents an urgent need to identify novel therapeutic strategies for the development of neurotherapeutics against the progression of AD.

1.4.1 Multitarget approach

The AD is multifaceted in nature with the involvement of diverse pathophysiological mechanisms, such as decreased ACh levels, augmentation in Aβ aggregation and plaque deposition, and increased expression of several factors such as BACE-1, tau hyperphosphorylation, oxidative stress, monoamine oxidase-B (MAO-B), phosphodiesterase (PDE) and cyclooxygenase 2 (COX-2) [Ibrahim and Gabr 2019]. These diversified targets prompted the researchers to shift their focus from single target to multiple targets. The multitarget strategy involves simultaneous targeting at multiple pathogenic mechanisms by a single molecule. The multifactorial targeting through multifunctional enzymes inhibition might be successful in targeting the actual cause of neuronal damage rather than only providing symptomatic relief [Hughes et al. 2016]. The available medications could be taken in combination to affect several targets, but combination therapy is also marred with several pitfalls such as drug-drug interactions, bioavailability issues, metabolism, and patient compliance [Rochais et al. 2015].

The multitargeted therapeutics bind to several targets simultaneously and affecting multiple pathways at single point of time. For example, ligand bound to peripheral

anionic and catalytic anionic sites (PAS and CAS) of AChE could effectively inhibit the BACE-1 and Aβ aggregation [Chen et al. 2014]. Also, compounds with cholinesterase inhibition along with antioxidant and anti-Aβ aggregatory activities were observed intensively in the recent past [Jiang et al. 2011].

1.4.2 Computer-aided drug design approach

The computer-aided drug design (CADD) approach is extensively explored by drug discovery units of leading pharmaceutical and biotechnological companies. These approaches provide superiority and reliability to investigate and identify the promising drug candidate among the library of databases [Nadendla 2004]. The initial screening using computational methods can reduce the time and minimize the cost of overall process. These computational methods can be divided into two broad classes: structure- and ligand-based drug design (SBDD and LBDD) also known as direct and indirect approaches, respectively.

The direct approach, i.e., SBDD is based upon the known 3D structure of the biological targets and discovering the molecules that satisfy some geometric constraints. The molecular docking and dynamics, X-ray diffraction (XRD), nuclear magnetic resonance (NMR), homology modeling, molecular mechanics generalized Born surface area (MM-GBSA) are the tools can be used in SBDD.

The indirect drug design approach, known as LBDD involves creating a lead molecule by comparing various structural characteristics of known active and inactive molecules. LBDD involves several tools, such as quantitative structure-activity relationship (QSAR), comparative molecular field analysis (CoMFA), comparative molecular similarity indices analysis (CoMSIA), pharmacophore modeling, high throughput virtual screening (HTVS) and combinatorial chemistry [Bacilieri and Moro 2006].

Indeed molecular modeling helps in the identification of moieties involved in the interaction with a particular protein and permits to understand the underlying molecular mechanisms responsible for specific biological activity. This knowledge of CADD helps to expedite the development of new active molecules to be successful drug candidates. However, as simulation accuracy is limited to the precision of the constructed models, computational simulations have to be evaluated against *in vitro*/*in vivo* experimental outcomes to confirm the accuracy of the model and modify them if necessary, to yield better representations of the system.

1.4.3 Molecular hybridization

The molecular hybridization approach is a novel concept in drug design and discovery, which involves hybridization of two or more pharmacophores in a single molecule with enhanced biological activity compared to individual parent molecules. The concept of molecular hybridization approach is now well adopted by medicinal and biological chemists to design novel compounds to improve affinity and efficacy toward biological targets, and reduced toxic effects [Viegas-Junior et al. 2007].

The AD is multifactorial disease involves several pathophysiological mechanisms, and very few commercial drugs are available for the treatment, which could provide symptomatic relief only rather than affecting the progression of AD. Therefore, pharmacophoric moieties of available therapeutics could be hybridized to improve the biological activity toward multiple targets, reduce toxicity, and improve pharmacokinetic profile compared to parent molecule.

1.5 Design hypothesis in the present study

The hypothesis of current work is based on designing the compounds based on the structural framework of donepezil, which is the first-line drug for the treatment of the AD. The detailed structure activity relationship (SAR) study also indicated that *N*-

benzylpiperidine is the basic pharmacophoric moiety in donepezil and extend deep into the CAS of AChE. Also, *N*-benzylpiperidine possesses basic nitrogen, which gets protonated at physiological pH, and would be beneficial in charged interaction toward aspartate dyad of BACE-1. Therefore, *N*-benzylpiperidine was selected as a primary pharmacophoric moiety for designing the series of ligands. In the first part of the thesis (Series I), the potential hits were identified using several computational methods, such as, e-pharmacophore modeling, HTVS, docking-post processing (DPP), MM-GBSA, molecular docking and molecular dynamics simulations. On the basis of Series I outcomes, part two of thesis was envisaged with the designing of Series II-V ligands using molecular hybridization approach by tethering several pharmacophoric moieties.