Chapter II

Review of Literature
2.1 Introduction of Alzheimer Disease (AD)

Alzheimer’s disease (AD) was first described in 1907 by German physician, Alois Alzheimer (Stelzma et al., 1995). It is a most common form of dementia which emerged as a major public health issue throughout the world. An estimated 5.2 million American people aged over 65 suffered from Alzheimer’s disease and total cost of care was reported to be $200 billion in 2012 (Alzheimer’s Association, 2012). In India for the year 2010, 3.7 million Indian people aged over 60 had been identified with dementia and estimated cost for taking care was about $3.417 billion (Alzheimer’s & Related Disorder Society of India, 2010). AD is an irreversible, progressive neurodegenerative disorder (Khachaturian and Radebaugh, 1996). It is characterized by a group of symptoms such as cognitive dysfunction and non-cognitive dysfunctions. The cognitive dysfunction includes memory loss, language difficulties and executive dysfunction and non-cognitive dysfunctions such as psychiatric symptoms and behavioral disturbances, depression, hallucinations, delusion agitation (Burns et al., 1990). The symptoms of AD progress from mild to moderate which lead to increase in disease severity, including disordered cognitive function and non-cognitive function (Bali et al., 2010). The cognitive performance declines to such an extent that patients need constant support for daily activities in the final stage of disease (Burns et al., 2009; Bali et al., 2010). AD is a complex neurodegenerative disorder probably caused by copathogenic interactions between various factors such as genetic, epigenetic and environmental factors (Huang and Mucke, 2012).

Advances in human genetic research have identified two distinct forms of AD such as Familial Alzheimer’s Disease (FAD) and Sporadic Alzheimer’s disease (SAD) (Ling et al., 2003; Bertram et al., 2010). The total percentage of FAD has been found nearly about 4-8% or less than the overall AD cases. In humans FAD is caused due to autosomal dominant mutations in APP (Goate et al., 1991), PSEN1 and PSEN2 genes (Campion et al., 1995; Cruts et al., 1995; Sherrington et al., 1996). These genes are represented as a major genetic risk factor for AD and are inherited in Mendelian fashion. The sporadic AD is the most common form accounting for 90% of all AD cases (Goedert and Spillantini, 2006). It has also been suggested that the synergistic action of genetic and environmental factors could be responsible for the FAD and SAD forms (Huang and Mucke et al., 2012). So far the pathological or clinical features have not been identified to distinguish Familial and Sporadic AD pathogenesis (Strimatter and Roses, 1995; Wahlster et al., 2013). Furthermore, AD can be classified according to the age of onset into early (<60 years) and late (≥60 years) onset forms (Blennow et al., 2006;
Bertram et al., 2010). Generally, FAD has an earlier age of onset as compared to SAD. This lack of distinguishing features suggests that distinct factors initiate similar disease processes.

2.2 Hypothesis of AD pathogenesis

Despite these complexities, extensive research has laid the foundation of our current understanding of the etiology and pathogenesis of AD (Tanzi, 1999; Tanzi and Bertram, 2001). During the past decades many hypotheses have been put forward for AD pathogenesis (Hardy and Higgins, 1992) as described below;

2.2.1 Cholinergic hypothesis

It is an oldest hypothesis based on cholinergic dysfunction (Contestabile, 2011). The currently available drug therapies are based on this hypothesis (Lleo et al., 2006; Reitz et al., 2011). The biochemical investigation of biopsy tissue (Francis et al., 1993) and Post-mortem brain tissues from AD patients showed reduced choline acetyl transferase activity (Wilcock et al., 1982), acetylcholine synthesis (Sims et al., 1983), choline uptake (Rylette et al., 1983) and acetylcholine release (Nilsson et al., 1986). These remarkable observations constituted that degeneration of cholinergic neurons and associated loss of cholinergic neurotransmission in the cerebral cortex and other areas contributed significantly to impairment of cognitive functions in Alzheimer’s disease (Bartus et al., 1982). To improve cholinergic neurotransmission, different therapeutic approaches used to develop potentially useful drugs for symptomatic treatment in Alzheimer’s disease (Lleo et al., 2006; Contestabile, 2011; Reitz et al., 2011).

Over the last two decades, the development of different strategies including cholinesterase inhibitors, choline precursor, postsynaptic and presynaptic cholinergic stimulation with muscarinic agonists and nicotinic agonist has been investigated (Hebert et al., 2003). However, clinical studies showed that the use of precursor for a presynaptic releasing agent and muscarinic agonists are not effective because of lack of efficacy and unacceptable side effects (Doody et al., 2001; Giacobini, 2000, 2001, 2002). But other studies have depicted beneficial effects of cholinesterase inhibitors on cognitive, functional and behavioral symptoms in AD (Rogers et al., 1998; Corey-Bloom et al., 1998; Tariot et al; 2000; Cummings, 2000). Four cholinesterase inhibitors have been approved by FDA for treatment of mild to moderate AD such as tacrine, donepezil, rivastigmine and galantamine. Tacrine was first widely used inhibitor but later on abandoned due to short half life, hepatotoxicity and cholinergic side effects (Hong-Qi et
al., 2012). Now, second generation inhibitors include donepezil, rivastigmine and galantamine have fewer side effects, longer half lives, and greater efficacy (Giacobini, 2000, 2002). It has been reported that donepezil is a piperidine derivative which can non-competitively and reversibly inhibits acetylcholinesterase (Seltzer, 2007). Galantamine is a tertiary alkaloid agent and allosterically binds to nicotinic receptors to improve cholinergic function (Scott and Goa, 2000). Rivastigmine is a carbamate derivative that inhibits acetylcholinesterase and butyrylcholinesterase (Onor, 2007).

Unfortunately, the data from clinical trials based on long-term administration of cholinesterase inhibitors to patient with Mild Cognitive Impairment revealed that they fail to reduce the risk or delay the onset of Alzheimer’s disease (Rashetti et al., 2007; Contestabile, 2011). Moreover, the adverse effects like gastrointestinal, cardiovascular, neuromuscular risks associated with these inhibitors are not negligible (Francis et al., 1999; Rashetti et al., 2007).

2.2.2 Tau hypothesis

Neuropathologically Alzheimer’s disease is defined as the presence of intraneuronal neurofibrillary lesions made of tau protein (Trojanowski et al., 2003; Forman et al., 2004). Tau proteins are mainly found in neurons and belong to the family of microtubule-associated proteins (Tucker et al., 1990). In the adult brain six isoforms of tau protein have been identified which are derived through alternative splicing from single gene located on the long arm of chromosome 17 (Neve et al., 1986; Andreadis et al., 1992). They play an important role in microtubule assembly and stabilization of neuronal microtubules network (Lee et al., 1989). The most striking feature of tau protein is the presence of a microtubule binding domain which is composed of highly conserved three or four repeats of 18 amino acids located in the carboxy terminal (C-terminal) half of the protein (Fig. 2.2.1). This microtubule binding domain is involved in microtubule polymerization and stabilization (Goedert et al., 1989). The amino terminal part (N-terminal) of tau consists of highly acidic amino acids followed by basic proline-rich region which normally referred as the projection domain. The projection domain may interact with cytoskeleton element and plasma membrane (Brandt et al., 1995).

The phosphorylation of tau protein is developmentally regulated from fetal to adult stage (Butler et al., 1986). The immature brain tau has been phosphorylated at six to eight sites present on shortest isoforms whereas, in adult brain two to three phosphorylation sites are present in six isoforms (Ksiezak-Reding et al., 1992). The
microtubule binding capability of tau protein can be effectively regulated post translationally by modulating serine/threonine phosphorylation (Fig. 2.2.1). The microtubule binding domain (MBD) of tau consists of four sequence repeats (R1-R4) consist of Serine (S) and Threonine (T) followed by proline (P). These amino acids are hyperphosphorylated by glycogen synthase kinase 3 (GSK-3β), cyclin-dependent kinase (cdk5) and its activator subunit p25 or mitogen-activated protein kinase (MAPK). Similarly, nonproline directed kinases such as Akt, Fyn, protein kinase A (PKA), calcium-calmodulin protein kinase 2 (CaMKII) and microtubule affinity regulating kinases (MAPK) are also involved in hyperphosphorylation of tau protein (Fig. 2.2.1). The hyperphosphorylation of tau reduces its affinity towards microtubules (Mazanetz et al., 2007).

Under pathological conditions there is an abnormal increase in the levels of hyperphosphorylated tau protein in cytosol (Kuret et al., 2005). This hyperphosphorylated tau polymerized into paired helical filaments (PHF) and straight filaments (SF) referred as neurofibrillary tangles (Kopke et al., 1993). The accumulation of abnormally hyperphosphorylated tau not only found in neurofibrillary tangles but also in the cytosol of AD brain (Iqbal et al., 1986). The loss of normal tau function leads to a pathological disturbance in structural and regulatory functions of the cytoskeleton. These affect normal cellular functions of neurons such as maintenance of appropriate morphology, axonal transport, synaptic dysfunction and neurodegeneration (Roy et al., 2005). The several pathogenic events contribute to hyperphosphorylation, misfolding and aggregation of tau.

The genetic studies demonstrate that mutation in tau gene cause frontotemporal dementia and parkinsonism (FTDP) linked to chromosome 17 (Poorkaj et al., 1998; Goedert and Jakes, 2005). All cases of FTDP-17 have shown filamentous inclusions made up of hyperphosphorylated tau which undergo rapid fibrillization (Nacharaju et al., 1999). The mutant tau protein is readily phosphorylated and less prone to dephosphorylation. This has led to impairment in microtubule binding properties (Hong et al., 1998; Dayanandan et al., 1999). Furthermore, intronic mutations and coding region mutations may alter the alternative splicing of tau which can change the normal ratio of tau isoforms (Ballatore et al., 2007). Additional relationships have been observed between tau hyperphosphorylation and regulation of tau kinases and phosphatases. Several tau kinases have been capable for phosphorylating tau in vitro such as glycogen synthase kinase 3β (GSK-3β), cyclin-dependent protein kinase 5 (cdk5), cAMP-dependent protein kinase (PKA) and stress-activated protein kinase (Ferrer et al., 2005; Mazanetz and Fischer, 2007).
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Figure 2.2.1: Showing structure of microtubule binding domain of tau protein with phosphorylation sites (Inset). The hyperphosphorylation of tau by glycogen synthase kinase 3β (GSK-3β), cyclin-dependent kinase (cdk5) resulted into destabilization microtubules followed by detachment of tau and self-aggregation into Paired helical filament (Henry et al., 2010).

Therefore, inhibition of such kinases which are responsible for tau hyperphosphorylation would be an interesting strategy to block tau deposition. It has been observed that lithium is the most studied compound to inhibit the GSK-3β enzyme, but several other compounds are under development stage such as pyrazolopyrazines, pyrazolopyridines, aminothiazole AR-A014418, aloisines, flavopiridol and sodium valproate. Similarly, several cdk5 inhibitors such as purine olomocine, flavopiridol, aloisines, and indirubins are under active investigation and development (De Azevedo et al., 1997; Churcher, 2006; Mazanetz and Fischer, 2007). The kinases are dynamically regulated and are vital in many signaling pathways so efforts must be made to focus on the side effects caused by using these kinase inhibitors as reported in several studies (Wang et al., 2007; Gong and Iqbal, 2008).
Unlike the protein kinases, number of phosphatases (PP) 1, PP2A, PP2B and PP2C drives dephosphorylation of tau (Tian et al., 2002). The Upregulation of tau phosphatases is another approach to drive dephosphorylation of tau. The phosphatases have much broader substrate specificity which leads to adverse side effects (Goedert et al., 1995; Sontag et al., 1996; Gong and Iqbal, 2008). However, the role of these kinases and phosphatases under pathophysiological conditions has not yet clear. Despite extensive studies, the physiological and pathophysiological conditions regarding abnormal hyperphosphorylation of tau have not fully understood yet.

2.2.3 Amyloid cascade hypothesis

The amyloid cascade hypothesis postulates that the neurodegeneration in Alzheimer’s disease caused by abnormal accumulation of amyloid beta (Aβ) plaques in various areas of brain (Hardy and Higgins, 1992; Evin and Weidemann, 2002). According to the amyloid hypothesis accumulation of amyloid beta (Aβ) plaques acts as a pathological trigger for a cascade that includes neuritic injury, formation of neurofibrillary tangles via tau protein leads to neuronal dysfunction and cell death in AD (Fig. 2.2.2) (Hardy and Higgins, 1992; Dickson, 1997; Selkoe, 1999). Genetic, biochemical and pathological evidences support amyloid cascade hypothesis which postulates that accumulation and aggregation of Aβ plaques is the primary cause of AD (Goate et al., 1991; Chen et al., 2000; Kayed et al., 2003).

The Aβ plaques are composed of principle proteinaceous component called Aβ peptides (Masters et al., 1985). Amyloid beta peptides are 39-43 amino acid residue peptides proteolytically derived from the sequential enzymatic action of β-secretase and γ-secretase on widely distributed transmembrane amyloid precursor protein (APP) (Kang et al., 1987; Coulson et al., 2000). The length of Aβ peptide varies at C-terminal according to the cleavage pattern of APP. The Aβ1-40 isoform is the most prevalent, followed by Aβ1-42 isoforms which is hydrophobic in nature and aggregates at a faster rate than Aβ1-40 (Walsh and Selkoe, 2007; Perl, 2010). Within plaques, Aβ peptides in β-sheet conformation assemble and polymerizes into structurally distinct forms including fibrillar, protofibers and polymorphic oligomers (Glenner et al., 1984; Selkoe et al., 1994). The Aβ deposition and diffused plaque formation lead to local microglial activation, cytokine release, reactive astrocytosis and a multi-protein inflammatory response (Fig. 2.2.2) (Eikelenboom et al., 1994; McGeer and McGeer, 1995; Rogers et al., 1996). Also, the multifaceted biochemical and structural changes in surrounding axons, dendrites and
neuronal cell bodies that are characterized by synapse loss, neuron loss and gross cerebral atrophy in AD (Terry et al., 1981, Terry et al., 1991; Braak and Braak 1994).

**Figure 2.2.2:** The amyloid (Aβ) cascade hypothesis (Haass and Selkoe, 2007)

Despite these, three decades of genetic research in deciphering AD have substantially broadened our understanding of AD genetics. These studies have suggested that genetic factor plays a major role in the development and progress of AD (Ling et al., 2003; Bertram et al., 2010). The autosomal dominant mutations in the APP (Zhang et al., 2011), PSEN1 and PSEN2 gene (Campion et al., 1995; Cruts et al., 1995; Sherrington et al., 1996) causing early-onset familial AD followed by APOE, a major genetic risk factor for the disorder in the typical late-onset period (Strittmatter et al., 1993). The genetic studies suggest that neurodegenerative processes in AD are the consequences of an imbalance between Aβ peptide production and clearance.
**2.2.3.1 Genetic studies provide evidence for amyloid cascade hypothesis**

The genetic studies had given important breakthrough to understand gene mutations associated with Alzheimer’s disease. The autosomal dominant mutation in *APP, PSEN-1* and *PSEN-2* genes lead to abnormal Aβ peptide production (Selkoe, 1994; Bertram et al., 2010). Similarly, ApoE increases Aβ peptide aggregation and impair its clearance in the brain (Ma et al., 1994; Wisniewski et al., 1994; Castano et al., 1995). These genes have been firmly implicated in the pathophysiology of AD.

**2.2.3.1.1 Amyloid precursor protein (APP)**

Amyloid precursor protein (APP) is a type I membrane glycoprotein having multiple isoforms derived by alternative splicing. However, physiological functions of APP are poorly understood (Yoshikai et al., 1990; Ling et al., 2003; Muller and Zheng, 2012). The *APP* gene is located on chromosome 21 and those individuals suffer from Down’s syndrome (trisomy 21) with an extra copy of this chromosome develops an early-onset familial AD (EOFAD) (Yoshikai et al., 1990; Ling et al., 2003).

![Figure 2.2.3](image-url)

**Figure 2.2.3:** Overview of dominantly inherited mutations clustered around the α-secretase, β-secretase and γ-secretase sites of the amyloid beta (Aβ) peptide sequence. The observed mutation (White circle) and resulting amino acid substitution (red font) (Bateman et al., 2011).
The discovery of the first specific genetic cause of AD was the occurrence of autosomal dominant mutation in the APP gene (Goate et al., 1991). Several missense mutations have been clustered around the three cleavage sites (Fig. 2.2.3). The Swedish mutation in APP gene leads to changes in the amino acids at 670 and 671 from Lys-Met to Asp-Leu which increases Aβ peptide generation by increasing β-secretase processing activity while other mutation changes biophysical properties of Aβ peptide as indicated in Fig. 2.2.3 (Mullan et al., 1992; Citron et al., 1992).

Figure 2.2.4: The structure and processing of APP showing amyloidogenic and non-amyloidogenic pathways. Aβ (purple box) constitutes part of the transmembrane domain and an adjacent short fragment of the extracellular domain (Vardy et al., 2005).

There are two proteolytic processing pathways of APP as shown in Fig. 2.2.4. In the non-amyloidogenic pathway, APP cleaved by α and γ secretase resulting in the production of soluble form of APP (sAPPα). The sAPPα has several neuroprotective properties and AICD has nuclear signaling functions (Kojiro et al., 2001; Selkoe and Schenk, 2003). In amyloidogenic pathway, APP cleaved by β-secretase generate membrane bound C-terminal fragment (C99) which subsequently cleaved by γ secretase and produces Aβ peptide (Nunan and Small, 2000; Selkoe and Schenk, 2003). Another two enzyme capable to cleave APP at β site called as BACE and BACE2 (Dingwall, 2001). It was shown that γ secretase cleaves APP near the boundary of the cytoplasmic
membrane and middle of the membrane called ε-cleavage and γ-cleavage respectively (Weidemann et al., 2002).

2.2.3.1.2. Presenilins (PSEN)

The presenilins 1 and presenilins 2 proteins are predominantly present in neuron encoded by PSEN1 and PSEN2 genes present on chromosomes 14 and 1 respectively (Levy-Lahad et al., 1995a; Levy-Lahad, 1995b; Sherrington et al., 1995). PSEN gene encodes nine transmembrane domains that are subsequently cleaved by presenilinase and form N- and C- terminal fragment which remain associated with each other (Fig. 2.2.7) (Doan et al., 1996; Haass and De Strooper, 1999). The PSEN1 and PSEN2 are homologous proteins that provide the active site aspartate residues required to the catalytic active site of γ-secretase (Fig. 2.2.7) (Wolfe et al., 1999). The PSEN1 and PSEN2 are mainly localized to the endoplasmic reticulum and Golgi Subcellular compartment. It is also found in the nucleus, endosomal system and the plasma membrane (Walter et al., 1996; Kovacs et al., 1996; De Strooper et al., 1997). The functions of the presenilins have not yet understood clearly. But based on most informative observation it is involved in protein trafficking and notch signaling (L’Hernault et al., 1992; Li et al., 1996; Mercken et al., 1996; Wong et al., 1997). To date, >100 mutations in PSEN1 and 11 mutations in PSEN2 have been identified as an autosomal dominant in which highly penetrant mutations cause early-onset AD (Bertram et al., 2008). The PS1-D9 and PS1-L166P mutations caused a reduction in γ-secretase activity which lead to decreased proportion of Aβ1-42 whereas PS1-G384A mutant significantly increased production of the more pathogenic Aβ1-42 which cause early-onset AD (Bentahir et al., 2006).

Specifically, it has been observed that PSEN1 gene mutation altered cleavage pattern of γ-secretase which has resulted into higher Aβ1-42 production and loss of PSEN dependent function (Bentahir et al., 2006; Shen et al., 2007). However, PSEN2 containing γ-secretase complexes does not have a major role in the processing of amyloid precursor protein and therefore only fewer PSEN2 mutations that lead to FAD (Herreman, et al., 1999). The pathogenic mechanism of presenilin mutations that alter APP metabolism which would be responsible to elevate levels of Aβ peptides.

2.2.3.1.3 Apolipoprotein E (ApoE)

ApoE is a lipoprotein of 299 amino acids encoded by APOE gene present on chromosome 19 and has three major alleles, designated as ε2, ε3, and ε4. The three major
isoforms of ApoE: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), and ApoE4 (Arg112, Arg158) differ by 1 or 2 amino acids, which are encoded by ε2, ε3, and ε4 alleles respectively (Zannis et al., 1982; Mahley, 1988). Human ApoE is expressed in several organs, with the highest expression in the liver followed by brain (Elshourbagy et al., 1985). ApoE has a key role in lipid metabolism by directing their transport, delivery and distribution from one tissue or cell type to another through ApoE receptors and associated proteins (Mahley, 1988; Mahley and Rall, 2000). Biochemical, cell biological and animal studies suggest that ApoE4 can increase Aβ peptide aggregation and impair Aβ clearance in the brain which acts as driving force for pathogenesis of AD (Ma et al., 1994; Wisniewski et al., 1994; Castano et al., 1995).

In almost all populations, APOE ε3 allele is present in 50-90% of people, whereas APOE ε4 and APOE ε2 allele account for only 5-35% and 1-5% respectively (Weisgraber et al., 1981; Mahley, 1988). It has been identified that APOEε4 is a major risk factor associated with AD than ε3 and ε2 (Corder et al., 1993; Saunders et al., 2000).

Figure 2.2.5: Role of ApoE on Aβ peptide metabolism and deposition (Kim et al., 2009).

Recent studies have explored Aβ dependent and independent roles of APOE in AD pathogenesis. ApoE is primarily produced by both astrocytes and microglia and is subsequently lipidated by ABCA1 to form lipoprotein particles. In the extracellular space,
lapsed ApoE binds to soluble Aβ in an isoform-dependent pattern (E2 > E3 >E4) and influence the formation of parenchymal amyloid plaques and transport of Aβ within the CNS (Fig. 2.2.5). ApoE is endocytosed into various cell types within the brain by different members of the LDL receptor (LDLR and LPR1) and facilitate the cellular uptake of Aβ through the endocytosis of a complex of ApoE containing lipoprotein particles bound to Aβ peptide. Furthermore, ApoE has been shown to directly enhance both the degradation of Aβ within microglial cells and the ability of astrocytes to clear Aβ deposits (Fig. 2.2.5). Aβ associated with ApoE containing lipoprotein particles may also retain within the CNS through their binding to heparin sulfate proteoglycan (HSPG) moieties present in the extracellular space. At the BBB, soluble Aβ is predominantly transported from the interstitial fluid into the blood stream via LRP1 and P-glycoprotein (Fig. 2.2.5) (Kim et al., 2009). In vitro and in vivo studies showed that ApoE3 and ApoE4 form a stable complex with Aβ peptides while ApoE4 forming complexes with Aβ peptides more rapidly and effectively (Jiang et al., 2008; Huang, 2010; Verghese et al., 2011).

However, ApoE4 secreted by neurons in response to brain stress and brain injury is more susceptible to proteolytic cleavage than ApoE3. The proteolytic cleavage of ApoE4 resulted into the C-terminal truncated neurotoxic fragment leads to tau phosphorylation and neurodegeneration (Huang et al., 2001; Huang, 2010). Accumulating evidence suggests that ApoE4 is a major risk factor for AD, although further studies are needed to understand the possible association of ApoE with the rate of disease progression (Strittmatter and Roses, 1995).

2.3 Amyloid based therapeutic approaches

The tremendous efforts have been undertaken in clinical research to develop novel AD therapeutics (Fig.2.2.6). All of the approved drugs provide symptomatic relief but do not alter the progression of disease (Scarpini et al., 2003; Stone et al., 2011). However, these drugs are associated with serious side effects and their use is only advised in severe cases. Therefore, current research progress in the areas of amyloid based therapies will provide effective treatment to cure this devastating disease. The amyloid hypothesis has continued to gain support over the last two decades, particularly from genetic studies. So accumulation of Aβ peptide in brain play pivotal role in the pathogenesis of AD (Tanzi and Bertram, 2005). Many efforts are currently undertaken to develop appropriate treatment strategies to decrease Aβ production or enhance Aβ clearance. The numbers of
therapeutic strategies have been developed to block effect of Aβ peptide and they broadly divided as follows;

**Figure 2.2.6:** Timeline of the emergence of various experimental and clinical therapies for Alzheimer’s disease (Stone et al., 2011).

### 2.3.1 Decreasing Aβ production

The reduction of Aβ production, either through inhibition of β and γ secretase or activation of α-secretase has been attractive approach in the development of therapeutics for AD.

#### 2.3.1.1 Inhibition of β-secretase

β-secretase is novel transmembrane aspartic protease BACE-1, also called Asp2 and memapsin2 (Vassar et al., 1999). This enzyme is responsible for cleaving APP at the β-secretase site leading to the production of Aβ peptide in the brain (Sinha et al., 1999; Citron, 2004). It has been found that there is increased β-secretase activity in sporadic AD. However, BACE knockout mice that appear normal, healthy, fertile and have no consistent phenotypic differences from their wild-type littermates and produced much less Aβ from APP (Vassar et al., 1999; Vassar, 2002). Therefore, β-secretase is a major therapeutic target for the development of inhibitor drugs (Vassar, 2002; Ghosh et al., 2008). Since the discovery of β-secretase more than 400 publications and patents have been appeared in the last eight years focusing on β-secretase inhibitors (Ghosh et al.,...
The first potent inhibitor OM99-2 created based on substrate followed by phenylnorstatine moiety as the transition-state isostere (Ghosh et al., 2012). Subsequently, Hydroxyethylene-based inhibitors, Hydroxyethylamine-based inhibitors, Carbinamine-based inhibitors, Macrocyclic inhibitors, Non-peptidomimetic β-secretase inhibitors are developed as explained by Ghosh and co-workers in 2012 (Ghosh et al., 2012). Unfortunately, only few β-secretase inhibitors have entered in Phase I clinical trial. The first publicly announced phase I clinical trial on a β-secretase inhibitor CTS-21166 was conducted by CoMentis (Hey et al. 2008; Hsu, 2010). Although, β-secretase inhibitors lowers the Aβ concentration in both animal model and cell level but their ability to penetrate blood-brain barrier is susceptible (Hong-Qi et al., 2012). Furthermore, β-secretase inhibitors not entirely specific to β-secretase they could inhibit other aspartic proteases also (Gruninger-Leitch et al., 2002). However, there is a need to develop an effective and safe β-secretase inhibitor through the structure based design which ultimately fulfills its promise as a valuable therapeutic target for the treatment of Alzheimer’s disease.

2.3.1.2 Inhibition of γ secretase

γ-secretase is membrane embedded aspartate protease complex consists of four subunits namely Aph1, Pen2, glycosylated nicastrin, and endo-proteolyzed presenilin as the catalytic core (Fig. 2.2.7) (Mangialasche et al., 2010; De Strooper and Annaert, 2010). Presenilin undergoes endoproteolysis into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) that remains associated with each other. Two conserved aspartates (D) within adjacent transmembrane domains are essential for both presenilin endoproteolysis and γ-secretase activity (Fig. 2.2.7) (Wolfe et al., 1999).

![Components of the γ-secretase complex](image-url)
Presenilins are expressed in the brain and most peripheral tissues which are known to be involved in Notch signaling, cell adhesion and cell signaling (Levitan and Greenwald, 1995; Ling et al., 2003). Approximately, 185 mutation in PSEN1 and 13 in PSEN2 gene have been reported to be linked with FAD (Bertam and Tanzi, 2008; Lessard et al., 2010). These mutations can alter the active site of γ-secretase, resulting in a potential shift in the cleavage site of substrate (De Strooper et al., 2012). The altered APP processing causes increased production of the more pathogenic Aβ1-42 peptide (Tsai et al., 2002; De Strooper et al., 2012). Thus, inhibition of γ-secretase could provide an effective therapy for AD.

The sulfonamide based inhibitors selectively inhibit PSEN1 and PSEN2, whereas DAPT and L685458 showed minimal selectivity (Tsai et al., 2002; Zhao et al., 2008). The γ-secretase inhibitors BMS-299897 and MRK-560 were considerably more potent in lowering Aβ level. Another γ-secretase inhibitor LY45139 was studied in a randomized and controlled trial on patients with mild to moderate Alzheimer which showed reduced Aβ level (Borgegard et al., 2012; Zhao et al., 2008). But the γ-secretase appears to have more than a single function, γ-secretase inhibitors interference with numerous physiological processes most notably the notch signaling pathway (Kreft et al., 2009; Mangialasche et al., 2010). Subsequently, the development of γ-secretase modulators that can change the ratio between Aβ1-38, Aβ1-40 and Aβ1-42 production while sparing notch activity is inadequate.

Some nonsteroidal anti-inflammatory drugs (NSAIDs), like ibuprofen, indomethacin, and sulindac sulfide can decrease the production Aβ1-40 but increase production of Aβ1-38 (Weggen et al., 2003). At present, a detailed 3D structure of this complex not yet solved. Other undesirable consequences with the notch signaling pathway might arise from inhibition and modulation of γ-secretase (Netzer et al., 2003; Wolf, 2008).

### 2.3.1.3 Activation of α-secretase:

The cleavage of amyloid precursor protein (APP) in its transmembrane region by an enzyme or family of enzymes collectively known as α-secretases (Kojro and Fahrenholz, 2005). Several proteases such as, ADAM family (ADAM17, ADAM10, ADAM9 and tumour necrosis factor-α convertase (TACE)) fulfills some of the criteria of α-secretases (Black et al., 1997; Kojro and Fahrenholz, 2005). α-secretase cleavage site is within the Aβ domain of the amyloid
precursor protein (APP) and releases soluble APP which has neuroprotective and memory enhancing effects (Robert et al., 1994; Mattison et al., 1997). Thus, upregulation of α-secretase activity would preclude the formation of Aβ which is considered as a therapeutic approach for AD (Fahrenholz, 2007). Over expression of ADAM10 in transgenic mice showed less amyloid deposition in the hippocampus and improved neurological function (Postina et al., 2004). The PKC activator TPPB [(2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam] could increase α-secretase activity and decrease Aβ production (Yang et al., 2007). Sirtuin-1 which increases ADAM10 expression which promotes α-secretase cleavage and lowered Aβ levels as explained by Donmez and Guarente in 2010 (Donmez and Guarente, 2010). The activation and increased expression ADAM 17 may be caused by M1 muscarinic agonists such as AF267B in an AD mouse model showed increased α-secretase cleavage of APP (Caccamo et al., 2006). Like these, many other methodologies are used for regulation of α-secretase cleavage of APP at the transcriptional, translational and posttranslational level.

Alternatively, ADAM 10 involved in different physiological and pathophysiological processes such as embryonic development, cell adhesion, signal transduction, cancer and AD (Edwards et al., 2008; Reiss and Saftig, 2009). But we do not have clear information about the cellular mechanisms of α-secretase cleavage. So, increasing the expression or activity of α-secretase should also be tested for their therapeutic potential in AD (Lichtenthaler, 2011).

2.3.2 Inhibitors of Aβ oligomerization or fibrillation

After γ-secretase cleavage of APP the released Aβ peptide monomers present in α-helical conformation followed by extensive conformational transitions from α-helix to β-sheet (Kirkitadze et al., 2001; Xu et al., 2005). These Aβ peptides rapidly self aggregates to form oligomers, protofibrils, fibrils and leads to deposition of Aβ plaques associated with AD (kirkitadze et al., 2001; Xu et al., 2005; Mahiuddin et al., 2010). Considerable effort is currently undertaking for the development of compounds that interfere with Aβ polymerization and fibrillogenesis to prevent or treat AD (De Felice et al., 2002). Several diverse molecules have been identified showing capability either to prevent Aβ aggregation and fibrillogenesis or inhibit Aβ toxicity. The Aβ fibril binding dye congo red reduces fibril formation and neurotoxicity (Lorenzo and Yankner, 1994). Subsequently, the group of sulfonated dyes such as benzofuran-based compounds (Allsop et al.,
Structural significance of Endothelin Converting Enzyme and its role in Alzheimer's disease

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1998), sulfonated anions (Kisilevsky et al., 1995) and amphiphilic surfactants (di-C6-PC and di-C7-PC) (Wang et al., 2005) have been used as Aβ aggregation inhibitors (Ono et al., 2003). Similarly, the identification of melatonin (Pappolla et al., 1998), nicotine (Salomon et al., 1996), estrogen (Mook-Jung et al., 1997) and anthracycline 49-iodo-49-deoxydoxorubicin (Merlini et al., 1995) that inhibit Aβ aggregation or reduce Aβ neurotoxicity.

Furthermore, the development of specific peptide ligands having self recognition ability of Aβ such as, N-methylated (NMe) derivatives of Aβ (25–35), Aβ (16–20) (Hughes et al., 2000; Gordon et al., 2002) and lysine hexamer (KLVFFKKKKK) (Pallitto et al., 1999) have ability to bind full length Aβ which alters fibril morphology and reduce Aβ cellular toxicity. Even though the recent developed drug iAβ5p is a β-sheet breaker which decreases Aβ plaque deposits and improves spatial memory (Hong-Qi et al., 2012). However, the major disadvantage of small molecule as aggregation inhibitors includes lack of specificity, toxicity and unknown mechanism of action whereas, in case of peptide derivatives various problems associated with administration, delivery and its rapid degradation (Estrada and Soto, 2007). Thus, efforts should be made to search for a safe antifibrilizing agents and minimize weaknesses of earlier discovered drugs.

2.3.3. Aβ degradation and clearance

The imbalance between production and removal of the Aβ peptide is responsible for complex pathological events in Alzheimer’s disease (AD) (Kurz and Perneczky, 2011). The increased level of Aβ peptides within the brain is caused by over production or impaired removal of Aβ (Bates et al., 2009). The clearance can be reduced by several reasons which include increased aggregation, defective degradation and disturbed transport across the blood brain barrier or inefficient peripheral removal of the peptide (Mawuenyega et al., 2010; Sagare et al., 2012). Therefore, over the past several years these mechanisms have been targeted for pharmacological interventions. Although, several compounds have limited clinical efficacy in Aβ clearance mechanism (Tanzi et al., 2004; Kurz and Perneczky, 2011).

Altering the catabolism is another way to decrease Aβ levels in AD brain (Iwata et al., 2001; Vardy et al., 2005; Miners et al., 2008). In recent year, a new line of attack has been emerged and that is nothing but Aβ peptide degradation and clearance which may provide an alternative therapeutic target in Alzheimer’s disease treatment. In this regard most attention has been focused on the identification of proteases such as Neprilysin.
(NEP) (Howell et al., 1995) Insulin Degrading Enzyme (IDE) (Kurochkin et al., 1994), Endothelin Converting Enzyme (ECE-1) (Eckman et al., 2001; Barage and Sonawane, 2014; Barage et al., 2014), plasmin (Verde et al., 1988), Angiotensin Converting Enzyme (ACE) (Hu et al., 2001; Jalkute et al., 2013) cathepsin B (Mueller-Steiner et al., 2006; Wang et al., 2012), and aminopeptidase (Sevalle et al., 2009; Dhanavade et al., 2014) that degrade Aβ peptide intracellularly or extracellularly. The over expression of these enzymes would be an alternative approach to reduce Aβ peptide concentration. Previous studies have been identified NEP (Howell et al., 1995; Iwata et al., 2000; Iwata et al., 2001), IDE (Kurochkin and Goto, 1994; McDermott and Gibson, 1997; Qiu et al., 1998), Human Endothelin Converting Enzyme (hECE-1) (Eckman et al., 2001), ACE (Hu et al., 2001), uPA/tPA-plasmin system (Sasaki et al., 1988; verde et al., 1988), cathepsin B (Mueller-Steiner et al., 2006; Wang et al., 2012), gelatinase A (Yamada et al., 1995), matrix metalloendopeptidase-9 (Backstorm et al., 1996), coagulation factor Xia (Saporito-Irwin and Van Nostrand, 1995), antibody light chain c23.5 and hk14 (Rangan et al., 2003) and α2-macroglobulin complexes (Qiu et al., 1996) implicated in Aβ degradation.

These Aβ degrading enzymes can cleave Aβ peptides at single or multiple sites. There are probably other proteases with ability to degrade Aβ peptide if all peptide bonds are taken into consideration, but only physiologically or pathologically relevant enzymes are discussed. These include cleavage products are less neurotoxic and less likely to aggregate than Aβ itself. These enzymes are known to maintain Aβ homeostasis in neurons (Wang et al., 2006). The enzymes like NEP, IDE, ACE and ECE are well accepted Aβ degrading enzymes. Nevertheless further studies assessing the relative contribution of each of these enzymes to understand the key players involved in the amyloid cascade to develop more adequate therapies against AD.

2.3.3.1 Neprilysin (NEP)

NEP (EC 3.4.24.11) is also known as neutral endopeptidase enkephalinase, neutrophil cluster-differentiation antigen 10 or common acute lymphoblastic leukemia antigen which belongs to M13 zinc metallopeptidase subfamily of type II integral membrane protein (Turner, 1990; Turner and Tanzawa, 1997; Oefner et al., 2004). NEP is located at the cell surface which is composed of three domains, a short N-terminal cytoplasmic tail, a membrane-spanning domain, and a large C-terminal extracellular catalytic domain containing typical HExxH zinc-binding motif. Therefore, functions as an ectoenzyme catalyzing peptide hydrolysis outside of the cell (Turner, 1990; Turner,
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2001; Oefner et al., 2004). It is expressed in many normal tissues including brush-border of intestinal and kidney epithelial cells, neutrophils, thymocytes, lung, prostate, testis and brain (Erdos and Skidgel, 1988; Pierart et al., 1988).

NEP is a zinc dependent endopeptidase which cleaves peptides within hydrophobic residues (Turner, 2001; Oefner et al., 2004). It hydrolyzes and inactivates several peptides such as enkephalin, atrial natriuretic peptide, endothelin, and substance P (Pierart et al., 1988; Erdos and Skidgel, 1989). It was first time showed that NEP could cleave Aβ peptides by Howell et al. in 1995 (Howell et al., 1995). NEP can degrade Aβ peptides in vivo and when NEP inhibitors injected in brains of rats led to a significant increase of Aβ plaques in the brain region (Iwata et al., 2000). The injection of NEP expressing lentivirus in hippocampus of transgenic mice (hAPP-human amyloid precursor protein) led to reduction in Aβ plaque load (Marr et al., 2004). Similarly, the increased level of endogenous Aβ1-40 and Aβ1-42 were observed in NEP deficient mice (Carson and Turner, 2002). Moreover, the reduced expression and activity of NEP have been reported in AD brain (Yasojima et al., 2001). Despite this, the elucidation of regulatory mechanism of NEP may be valuable to manipulate its level in vivo to prevent progression of AD.

2.3.3.2 Insulin Degrading Enzyme (IDE)

IDE (EC 3.4.24.56) is a zinc-metalloendopeptidase also known as insulysin or insulinase. IDE is expressed in all tissues but highly expressed in liver, testis, muscle and brain (Kuo et al., 1993). Several peptides serve as substrate of the IDE including insulin, glucagon, amylin, insulin-like growth factors I and II and others (Song et al., 2001). IDE has ability to degrade Aβ both in vivo and in vitro (Vekrellis et al., 2000; Farris et al., 2003). IDE deficient mice with homozygous deletions of the IDE gene shows that increased cerebral accumulation of Aβ, hyperinsulinemia and glucose intolerance (Farris et al., 2003). Upregulation of IDE in transgenic mice have shown decreased levels of Aβ peptide, amyloid plaque and associated cytopathology (Leissring et al., 2003). It has been shown that purified IDE from rat liver and brain efficiently degrades synthetic Aβ peptide in vitro (McDermott and Gibson, 1997). Studies of cultured neurons also show that extracellular IDE as well as IDE on the cell surface degrade Aβ peptide (Vekrellis et al., 2000).

Thus, upregulation and increased activity of IDE could be considered as a new therapeutic approach in AD. Insulin and insulin receptors are found in large quantities in
the brain. Several studies have explored that both insulin and its receptor have been implicated in AD pathology (de la Monte and Wands, 2005). Furthermore, hyperinsulinemia, insulin resistance and type 2 diabetes mellitus and IDE gene variation are associated with the risk of AD (Ho et al., 2004). Based on current evidence upregulation of the IDE is an effective therapeutic strategy in AD, but physiological consequences of increased proteolytic activity should be determined.

2.3.3.3 Angiotensin Converting Enzyme (ACE)

ACE (EC 3.4.15.1) is a membrane bound zinc-metalloprotease also known as dipeptidyl carboxypeptidase (Yang and Erdös, 1967; Yang et al., 1971). It is widely expressed in the blood vessels throughout the body (Erdos and Skidgel, 1987; Corvol et al., 1995). ACE plays an important role in blood pressure regulation by converting angiotensin I to the potent vasoconstrictor angiotensin II (Reid, 1992; Corvol et al., 1995). Mammalian ACE has two distinct isoforms, somatic (sACE) and testis (tACE) in which Somatic ACE consists of two identical catalytic domains (C- and N-domains) which are proteolytically active (Wei et al., 1991) whereas, testis ACE consists of only single domain corresponding to the C-terminal domain of sACE (Howard et al., 1990). The highest levels of ACE have been detected in the circumventricular organs of brains such as subfornical organs, area postrema and the median eminence (Miners et al., 2008).

Human genetic studies have been suggested potential relationship between ACE and AD (Elkins et al., 2004). It has been found that ACE significantly inhibits the aggregation, deposition and cytotoxicity of Aβ in vitro (Hu et al., 2001). A recent study reported that mouse and human brain homogenates exhibit Aβ1-42 to Aβ1-40 converting activity mediated by the ACE. The purified ACE degrades Aβ peptide as well as decrease ratio of Aβ1-42 /Aβ1-40 (Zou et al., 2007). ACE inhibition results in increased Aβ levels in APP- and ACE-transfected cells (Hemming and Selkoe, 2005). Few studies have shown that inactivation of the mouse ACE gene or inhibition of ACE in mice does not affect Aβ level (Eckman et al., 2006; Hemming et al., 2007). The role of ACE inhibitors on Aβ level is still controversial. Therefore, additional studies are required to determine their effect. The upregulation of ACE is another therapeutic strategy for AD patients without hypertension.

2.3.3.4 Cathepsin B

Cathepsin B belongs to cysteine protease family and carries out a variety of physiological and pathological processes (Mort et al., 1997; Knop et al., 1993). It
degrades proteins which are entering the endolysosomal system (Knop et al., 1993). The cathepsin B degrades Aβ by truncating Aβ1-42 at C-terminal which significantly reduces Aβ peptide deposition. Therefore, the enhanced activity of endogenous cathepsin B may decrease Aβ1-42 peptide level in AD (Mueller-Steiner et al., 2006; Sun et al., 2008). Sun et al. in 2008 have reported that the amyloid beta plaque load was reduced by increased cathepsin B concentration in transgenic mice with overexpression of human APP (Sun et al., 2008). Enhancing Cathepsin B activity by deletion of endogenous inhibitor cystatin C (CysC) significantly lowered total Aβ1-40 and Aβ1-42 levels in mice expressing wild type hAPP (Wang et al., 2012). The molecular modeling study of Cathepsin B revealed that Aβ peptide accommodate properly in the active site and Cys 17 catalytic residue form interaction with Lys 16 of Aβ peptide (Dhanavade et al., 2013).

2.3.3.5 Endothelin Converting Enzymes (ECE)

Human Endothelin Converting Enzymes, hECE-1 and hECE-2 (EC 3.4.24.71) are classified as a type II integral membrane zinc metalloendopeptidases belongs to M13 subfamily (Xu et al., 1994). They are expressed in endothelial cells of the aorta, lungs, ovary and testis (Davenport et al., 1998). hECE-1, unlike neprilysin was found to degrade a variety of biologically active large peptides instead of the smaller one such as big endothelin-1, neurotensin, substance P, bradykinin, and the oxidized insulin B chain (Johnson et al., 1999). hECE-1 cleaves big ET at a Trp21-Val/Ile22 specific site and forms ET-1 which can act as a most potent vasoconstrictor (Yanagisawa et al., 1988b). Immunolabeling studies revealed that ECE-1 present in neurons, specifically within pyramidal neurons of the hippocampus and neocortex of rat and bovine brain (Barnes et al., 1997; Sluck et al., 1999). Immunocytochemical studies of the human brain also showed expression of hECE-1 in various regions including hippocampus as well as pyramidal neurons of layers III and V of neocortex (Funalot et al., 2004). Several studies have showed that the ECE-1 and ECE-2 mRNAs are localized in neurons of rat brain (Barnes et al., 1997; Nakagomi et al., 2000). Previous experimental studies have shown that, the treatment of metalloprotease inhibitor phosphoramidon to endogenous ECE-expressing cell lines increased 2-3 fold elevation in extracellular Aβ concentration.

Furthermore, the overexpression of ECE-1 resulted in a striking 75-90% reduction in Aβ1-40 and 45–60% reduction in Aβ1-42, which indicate that hECE-1 is capable to hydrolyze Aβ peptide both in vitro and in vivo (Eckman et al., 2001). The increased Aβ levels were observed in the brain of ECE-deficient mice as compared with age-matched
littermate controls (Eckman et al., 2003). Eckman and coworkers show that oral administration and intracerebroventricular (ICV) injection of selective ECE inhibitor along with a dual NEP-ECE inhibitor which caused a rapid increase of plasma Aβ concentration in mice. After few days also this has caused a small but significant increase in brain Aβ concentration (Eckman et al., 2006). The hECE-2 mRNA level increased in temporal cortex of AD brain and upregulated in SH-SY5Y cells after 24 hours treatment with both monomeric Aβ1-42 and oligomeric Aβ1-42 (Palmer et al., 2009).

The hECE-1 mRNA was found upregulated after the treatment of HNE and Aβ peptide. The double fluorescence staining followed by confocal analysis of oxidatively stressed SH-SY5Y cells showed that hECE-1 detected in both cytosol and membrane similar to previous reports (Wang et al., 2009). The induction of ECE-1 activity by transgenic overexpression of protein kinase C strongly reduced plaque and Aβ deposit area (Choi et al., 2006). ECE participates in regulation of Aβ level intracellularly by endosomal-autophagic-lysosomal pathways (Pacheco-Quinto and Eckman, 2013). An intracranial administration of recombinant adeno-associated viral vector (rAAV) of ECE1 synthetic gene into anterior cortex and hippocampus of 6 month old transgenic mice with amyloid precursor protein (APP) plus presenilin-1 (PSEN1) resulted in the decreased Aβ level in these areas as detected by immunohistochemical and congo red staining analysis (Carty et al., 2008).

Genetic association study identified a functional genetic variant (hECE-1b C-338A) located in a regulatory region of the ECE1 gene. The increased ECE-1 mRNA expression in human neocortex reduced risk of AD observed in a large case control study having homozygous carriers of the A allele (Funalot et al., 2004). The CpG-CA repeat within the human hECE-1c promoter is highly polymorphic. CpG-CA repeat composition of AD patients and controls is distinct which contributes to the pathogenesis of AD (Li et al., 2012). These findings indicate that hECE-1 is an enzyme that plays an important role in the Aβ degradation in AD (Eckman and Eckman, 2005; Ito et al., 2013; Pacheco-Quinto et al., 2013). However, the limited information available related to genetic variation of hECE-2 in the development of AD. In fact, only two studies have been conducted which shows the controversial result. In the first report, the hECE-2 gene is downregulated as well as protein levels highly decreased in AD patient whereas, second report indicates that ECE-2 mRNA level increased in the cortex of AD patient (Weeraratna et al., 2007; Palmer et al., 2009). Further studies are needed to address the possible role of ECE-2 in AD (Pacheco-Quinto et al., 2013).
Indeed, the results of in vitro, in vivo and animal model studies suggest that hECE-1 activity could prevent or delay progression of AD (Eckman and Eckman, 2005; Ito et al., 2013; Pacheco-Quinto et al., 2013). Alternatively, the use of allosteric activators or gene therapy may become realistic in the near future. These entire opportunities make hECE-1 is a promising therapeutic target for the treatment of AD. Despite this, the proteolytic role of hECE-1 in degradation of Aβ is still to be uncover at the molecular level. Thus, the identification of hECE-1 and Aβ interactions would be very helpful to expand our knowledge about structural determinants of hECE-1 that contributes to substrate specificity. In addition, in silico studies extend our views of Aβ peptide recognition and its cleavage by hECE-1. Therefore, computational studies will offer a wealth of fairly reliable information regarding the potential of hECE-1 as a therapeutic target for AD.

2.4 ECE isoforms and subcellular localization

ECE is a key enzyme in biosynthesis pathway of endothelins (Xu et al., 1994; Emoto and Yanagisawa, 1995). Extensive research in this area from past three decades resulted into identification of two major forms of ECE; i.e. ECE-1 and ECE-2 with their isoforms and subcellular localization (Turner and Tanzawa, 1997; Schweizer et al., 1997; Valdenaire et al., 1995).

2.4.1 Endothelin Converting Enzyme-1 (ECE-1)

The hECE-1 gene localized on chromosome 1p36 which is composed of 19 exons and that span more than 68 kb (Valdenaire et al., 1995). It has been identified that hECE-1 has diversity in both human and rat species (Valdenaire et al., 1999b). The hECE-1 has four isoforms (ECE-1a-d) derived from the same gene via the use of four different promoters and they are most active at neutral pH (Schweizer et al., 1997; Orzechowski, 1997; Valdenaire et al., 1999a). The main role of hECE-1 is to convert big ET-1 into ET-1. The four human ECE-1 isoforms, termed as hECE-1a (758 residues), hECE-1b (770 residues), hECE-1c (754 residues) and hECE-1d (767 residues) having similar catalytic properties but distinct subcellular localization and tissue distribution (Schweizer et al., 1997; Valdenaire et al., 1999a). The N-terminal region controls the specific cellular and subcellular localization of hECE-1 isoforms (Schweizer et al., 1997; Goettsch et al., 2008) The immunofluorescence analysis revealed that hECE-1a and hECE-1c are localized at the cell surface whereas, hECE-1b was found to be an intracellular compartment and
trans-Golgi Network (Schweizer et al., 1997). The hECE-1d was detected at the cell surface and intracellularly in Golgi and endosomal structures however in comparison with three other isoforms hECE-1d is more strongly expressed at the cell surface than hECE-1c but less than hECE-1a (Valdenaire et al., 1999b).

Mutational study revealed that di-leucine (LL and LV) motif of the N-terminal region controls the subcellular localization of hECE-1b and hECE-1c (Valdenaire et al., 1999b). The hECE-1 exists as a disulfide linked dimer which may be either homodimer or heterodimer. Cys 412 and Cys 428 contribute in formation of the dimeric structure of ECE-1 in rat and human respectively (Shimida et al., 1996; Schulz, et al., 2009). The hECE-1 is composed of three domains such as short N-terminal cytoplasmic domain, followed by a single transmembrane helix, and a larger C-terminal extracellular domain that contains the active site. The four isoforms differ only in the cytoplasmic and transmembrane domain whereas extracellular C-terminal domains are identical (Xu et al., 1994; Turner et al., 1997). The extracellular C-terminal domain of hECE-1 has 10 conserved cysteine residues and sequence similarity with catalytic domains of hECE-2, hNEP, hKELL and hPHEX suggesting that these enzymes share common origin and similar fold (Turner et al., 1997; Schulz, et al., 2009). hECE-1 is highly glycosylated protein having 10 possible consensus sequences for N-linked glycosylation (Turner et al., 1997). Furthermore, the structural information of hECE-1 is essential to understand the exact mechanism of action in the treatment of Alzheimer’s and cardiovascular diseases.

2.4.1.1 Crystal structure study of hECE-1

The three dimensional structure of monomeric hECE-1 (C428S) ectodomain complex with phosphoramidon has been solved by X-ray crystallography at 2.38 Å resolution (Schulz, et al., 2009). The ecotodomain consist of two large α-helical domains termed as C-terminal and N-terminal domain connected by interdomain linker fragment (Schulz, et al., 2009). The C-terminal domain contains substrate recognition subsites (S1, S1’ and S2’) and zinc binding site. The highly conserved consensus sequence motifs $^{607}$HExxH$^{611}$, $^{667}$ExxD$^{671}$, $^{542}$NAYY$^{545}$ and $^{565}$VNA$^{567}$ are present in catalytic C-terminal domain of hECE-1 (Turner et al., 1997; Johnson et al., 2002; Schulz, et al., 2009). X-ray crystallography uses a single set of coordinates and B factors to describe macromolecular conformations and its function. But detailed characterizations like conformation flexibility, enzymatic reaction, interaction of substrate and inhibitors with buried active
site by experimental technique remains laborious and limited in applicability (Lindoff-Larsen et al., 2005; Furham et al., 2006; Levin et al., 2007).

Therefore, molecular modeling techniques are essential to understand the structure function relationship of proteins. Recently, molecular dynamic simulation was performed on available crystal structures of hECE-1 in order to understand the conformational flexibility, subsites and their specificity and dynamic nature of active site residues and zinc catalytic site at the molecular level (Ul-Haq et al., 2012; Barage et al., 2013; Barage and Sonawane, 2014; Barage et al., 2014). Further computational studies are needed for characterization of disulphide-linked dimeric hECE-1 structure and dynamics.

2.4.2 Endothelin Converting Enzyme-2 (ECE-2)

hECE-2 gene localized on chromosome 3q28-q29 was assessed using radiation hybrid (RH) and fluorescence in situ hybridization (FISH) (Lorenzo et al., 2001). hECE-2 shares approximately 59% overall homology with ECE-1 (Emoto and Yanagisawa, 1995; Turner et al., 1997). hECE-2 efficiently cleaves big ET-1 and has a similar substrate affinity like ECE-1. However, hECE-2 has acidic pH optima at around pH 5-5.5, which strongly suggest that it is an intracellular processing enzyme (Emoto and Yanagisawa, 1995). The four ECE-2 subisoforms have been reported such as ECE-2a-1, ECE-2a-2, ECE-2b-1 and ECE-2b-2. They differ in N-terminal cytoplasmic tail (Lorenzo et al., 2001; Ikeda et al., 2002).

These subisoforms exhibit distinct subcellular localization and tissue distribution. The tissue distribution pattern of ECE-2 subisoforms in bovine examined by RT-PCR revealed that ECE-2a-1 and ECE-2a-2 expressed in liver, kidney, adrenal gland and vascular endothelial cell whereas, ECE-2b-1 and ECE-2b-2 are found in neuronal tissues like cerebrum, cerebellum, and adrenal medulla (Ikeda et al., 2002). The examination of human tissue for ECE-2 mRNA variants showed that ECE-2A, ECE-2B-1, and ECE-2B-2 are expressed in brain, adrenal gland and lung (Lorenzo et al., 2001).

The immunohistochemistry studies in mice show ECE-2 present in pyramidal neurons of hippocampus and neocortex as well as astrocytes and microglia of AD brains, while ECE-2 mRNA was localized to neurons in the rat brain (Barnes et al., 1997; Nakagomi et al., 2000; Palmer et al., 2009). The limited data available regarding subcellular localization of ECE-2 sub isoform, single report shows that ECE-2a-1 and ECE-2b-2 were localized to intracellular compartments (Ikeda et al., 2002). Further studies
with several complementary methods are required to understand ECE-2 isoforms. Moreover, the cellular and subcellular localization studies of ECE-2 are needed to resolve discrepancies and to evaluate the relative role in various diseases.

2.5 ECE in other diseases

Endothelin converting enzyme cleaves biologically inactive intermediate of 37 to 41 amino acid peptide termed big endothelins (big ETs) at the Trp$^{21}$-Val/Ile$^{22}$ bond to form the 21 amino acid final products as active endothelin (Inoue et al., 1989a). This active endothelin peptide acts as most powerful vasoconstrictor with a hydrophobic C-terminal and 2 cysteine bridges at N-terminal (Yanagisawa et al., 1988b). The four active ET isopeptides termed as endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), endothelin-4 (ET-4), each containing 21 amino-acids encoded by different genes (Inoue et al., 1989b; Saida et al., 1989). Endothelins are expressed by a variety of cell types including endothelial cells, macrophages, astrocytes, fibroblast, cardiac myocytes and brain neurons (Kedzierski and Yanagisawa, 2001; Luscher and Barton, 2000). The endothelin isoforms exert their vasoconstrictive effect via receptor mediated fashion, the endothelin A receptor (ET_A) and endothelin B receptor (ET_B) (Fig.2.2.8) (Henry and Goldie, 2001). The ET_A and ET_B receptor belongs to the G-protein coupled receptor family and contain seven transmembrane domains (Arai et al., 1990; Sakurai et al., 1990).

ET_A receptor has higher affinity to ET-1, ET-2 and 100 fold lower affinity for ET-3 whereas ET_B receptors has the equal affinity for all endothelin peptides (Luscher and Barton, 2000). ET_A receptors are present on vascular smooth muscle cells when activated leads to phospholipase C activation followed by accumulation of inositol triphosphate and intracellular calcium which results in long lasting vasoconstriction (Fig 2.2.8) (Goto et al., 1989; Yang et al., 1990; Pollock et al., 1995). In contrast, ET_B receptors are located on endothelial cells and to some extent on smooth muscle cell. The activation ET_B receptors results in the release of NO and prostacyclin that prevents apoptosis and inhibits ECE-1 expression as well as the clearance of pulmonary ET-1 and uptake of ET-1 by endothelial cells (Warner et al., 1989; Hirata et al., 1993; Fukuroda et al., 1994). Most of the cardiovascular diseases such as arterial hypertension, atherosclerosis, restenosis, heart failure, idiopathic cardiomyopathy, pulmonary hypertension, and renal failure are associated with the activation of endothelin and endothelin receptor (Luscher and Barton, 2000).
Therefore, inhibition of ECE or blockade of ET receptors has been suggested as an attractive target in a number of cardiovascular diseases (Barton and Yanagisawa, 2008; Kawanabe and Nauli, 2011; Ohkita et al., 2012). Over the past 20 years, several ET receptor antagonists (peptides and nonpeptide compounds) have been developed. These ET receptor antagonists can block either ET\textsubscript{A} or ET\textsubscript{B} receptors or both (Barton and Yanagisawa, 2008). However, the endothelin receptor antagonists are contraindicated during pregnancy and their teratogenic effects lead to craniofacial and organic malformations (Kurihara et al., 1994; Fleish et al., 2000). So, therapeutic applications of the endothelin receptor antagonist must be carefully assessed.

The ECE represents as a plausible target for pharmacological intervention to the ET biosynthesis (Jeng et al., 2000; Loffler et al., 2000). To date, majority of ECE inhibitors was discovered, they are classified into metal chelators, peptidic inhibitors, natural products and low molecular weight organic compounds (Jeng et al., 2000). The efficacies of these inhibitors were determined by different enzyme assays as well as animal studies (Dickstein et al., 2004; Cerdeira et al., 2008). However, ECE-1 shares high degree of homology with other metalloprotease such as NEP and ACE. Therefore, several ECE-1 inhibitors have lower potency towards ECE and shows inhibitory activity against other metalloprotease (Wallace et al., 1998; Jeng et al., 2000; Lombaert et al., 2000). The inhibition of other metalloproteases might be responsible for the unwanted side effect.

**Figure 2.2.8:** Endothelin-1 (ET-1) production and their receptor mediated action (Kawanabe and Nauli, 2011).
such as dry cough and angioedema (Daull et al., 2007). The inhibition of NEP may not be always necessary under some pathological condition e.g. asthma (Hay and Goldie, 1998).

However, to design potent selective inhibitor further studies are needed to understand active site and subsite specificities of hECE-1. Recently, computational studies were used to identify selective ECE-1 inhibitors by virtual screening technique (Babu et al., 2012; Barage et al., 2013). In order to gain structural insights, molecular modeling studies found useful to generate multiple conformations and orientations of ligands within the active site of hECE-1. These findings are likely to give an extended view of hECE-1 catalytic subsites and their specificities which lead to demarcation of differences in substrate specificity and to uncover its physiological function. Furthermore, these compounds can be used in future studies as leads for the development of potent and selective inhibitors and will aid in the elucidation of the physiological functions of the hECE-1.

2.6 Scope of the thesis

hECE-1 is known to degrade Aβ peptides at multiple sites between residues Lys$^{16}$-Leu$^{17}$, Leu$^{17}$-Val$^{18}$ and Phe$^{19}$-Phe$^{20}$. These finding suggest that hECE-1 is physiologically relevant Aβ degrading enzyme having potential as therapeutic target in Alzheimer’s disease. Hence, the present computational study is employed to gain structural information of hECE-1 active site residues and pockets associated with it. The MD simulation of hECE-1 crystal structure with phosphoramidon as well as virtual screening of other protease inhibitors provides wealth of useful information regarding the active site architecture of hECE-1 such as substrate and inhibitor interacting residues, subsite and their specificities. The lead inhibitors obtained using virtual screening technique could also be useful to study the pathophysiological role of endothelin in cardiovascular diseases. The molecular docking and MD simulation studies of hECE-1 with different Aβ peptide conformation help to uncover the binding affinity of different Aβ peptide conformations towards the hECE-1 active site.

The positioning and orientation of the side chain of wild type Aβ peptide conformation makes a significant contribution to provide structural stability to the enzyme-substrate complex which would be helpful in degradation process. Furthermore, the novel catalytic mechanism of hECE-1 in which carboxyl group of E 608 acts as a proton shuttle in catalysis of Aβ peptide. This elucidation of the Aβ peptide binding and its cleavage by hECE-1 was the key factor to understand the role of hECE-1 in AD. The
structural significance of hECE-1 dimer embedded in lipid bilayer along with Aβ1-42 peptide has also been studied. The homology modelling, molecular docking and MD simulation studies of hECE-1 dimer and Aβ1-42 peptide would be helpful to design new hECE-1 based therapies to control AD. Thus, the obtained information would be important to design novel hECE-1 based therapies in the treatment of Alzheimer’s disease. The objectives of present thesis are given below;

**Objectives**

- Collection of sequences to predict three-dimensional structure of ECE.
- Homology modeling and molecular dynamic simulation of ECE complexed with known inhibitor.
- Identification of ECE subsites and their specificities with other inhibitors using virtual screening and MD simulation.
- Investigate the binding mode of different conformations of Aβ peptides with ECE by molecular docking and MD simulation techniques.
- Role of ECE in the degradation of Aβ peptides.
- Explore dynamic features of membrane bound ECE dimer.