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Brain cholinesterases: II. The molecular and cellular basis of Alzheimer's disease

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Summary Currently available evidence demonstrates that cholinesterases (ChEs), owing to their powerful enzymatic and non-catalytic actions, unusually strong electrostatics, and exceptionally ubiquitous presence and redundancy in their capacity as the connector, the organizer and the safeguard of the brain, play fundamental role(s) in the well-being of cells, tissues, animal and human lives, while they present themselves adequately in quality and quantity. The widespread intracellular and extracellular membrane networks of ChEs in the brain are also subject to various insults, such as aging, gene anomalies, environmental hazards, head trauma, excessive oxidative stress, imbalances and/or deficits of organic constituents. The loss and the alteration of ChEs on the outer surface membranous network may initiate the formation of extracellular senile plaques and induce an outside–in cascade of Alzheimer's disease (AD). The alteration in ChEs on the intracellular compartments membranous network may give rise to the development of intracellular neurofibrillary tangles and induce an inside–out cascade of AD. The abnormal patterns of glycosylation and configuration changes in ChEs may be reflecting their impaired metabolism at the molecular and cellular level and causing the enzymatic and pharmacodynamical modifications and neurotoxicity detected in brain tissue and/or CSF of patients with AD and in specimens in laboratory experiments. The inflammatory reactions mainly arising from ChEs-containing neuroglial cells may facilitate the pathophysiologic process of AD. It is proposed that brain ChEs may serve as a central point rallying various hypotheses regarding the etio-pathogenesis of AD.

Introduction

The year 2007 will be the centenary since the German psychiatrist, Alois Alzheimer [1], first described the clinico-pathological correlation of symptoms of dementia with the neuropathological findings of intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques (SPs) in the brain of a 51-year-old patient. The disease bears Alzheimer's name and draws tremendous concern and interest from the public and scientific communities, notably in the recent two to three decades, worldwide.

Remarkable progress has been made ever since, particularly in the recognition that Alzheimer's disease (AD) is not rare and peculiar anymore but is one of the most common threats in late life in modern society. The gradual weakening of memory and the severe ravages in AD is no longer considered a normal consequence of aging. There is seemingly an increasing demand to develop pharmacological agents enhancing learning and

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Abbreviations: ChEs, Cholinesterases; AChE, Acetylcholinesterase; BChE, Butyrylcholinesterase; AD, Alzheimer's disease; SP, Senile plaque; NFT, Neurofibrillary tangle.

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memory, even in the absence of specific anatomically evident pathology in the brain [2].

A definitive diagnosis of AD still depends on counting the numbers of NFTs and SPs relative to a certain amount of postmortem brain tissue, because the two pathologic hallmarks are not qualitative for patients suffering from AD, age-matched non-demented individuals, and subjects with other brain disorders. A clinical diagnosis of AD will not be made until cognitive impairment is severe enough to compromise the activities of daily living — a condition of irreversible cognitive deficit. However, there is a growing awareness among medical professionals that the traditional clinicopathological disease concept cannot promote our understanding of the preclinical pathogenetic processes and their interaction, and may have outlived its usefulness, if new molecular biological discoveries cannot be integrated with the traditional clinico-pathological model [3].

Research on AD has been extensively undertaken with rapidly advancing technology in epidemiology, genetics, molecular—cellular biology, longitudinal clinico-pathological relationship and brain imaging techniques that relate structure to function. It is now more than ever recognized that only with a better understanding of the anatomical, biochemical and molecular—cellular mechanisms of memory formation, accompanied by a solution to the century-old mystery of SPs and NFTs formation, may the effective prevention and treatment of AD be attained.

Current theories of AD etiology and pathogenesis

Several theories concerning the etiology and the pathogenesis of AD have been put forward and their principles have been tested and evaluated. Among these include hypotheses implicating roles of "cholinergic deficiency" [4], "free radicals" [5], "apolipoprotein E" [6], "β-amyloid/presenilins" [7], "tau" [8], "cobalaminergic (vitamin B12) deficiency" [9] and "inflammation" [10].

The cholinergic hypothesis of AD was established 20 years ago. The therapeutic results achieved from the early clinical trials employing cholinergic precursors in the 1980s to the recent use of AChE inhibitors based on the hypothesis have been dubious [4,11,12].

The mainstream "β-amyloid", "presenilins (PSs)" and "tau" hypotheses have suffered from lack of clear evidence in both pertinent transgenic mice and in vitro experiments that any one of the gene abnormalities alone is able to induce all the known neuropathological, morphometrical and behavioral features seen in AD [7,8,13—15]. Further disapproval of β-amyloid hypothesis of AD came from the recent Aβ vaccination treatment for AD patients in the clinical trial, which caused some unacceptable inflammatory reactions of the brain [7]. The monoclonal antibodies targeting the β-amyloid peptide in mutant APP transgenic mice, while reducing the numbers of plaques, increased more than 2-fold the likelihood of severe cerebral hemorrhage, compared with mice that did not receive the immunotherapy [16].

The apolipoprotein E (ApoE) has been shown to be an important risk factor for AD, but it is concluded as neither essential for the development of the disease nor specific for AD [17].

The cobalaminergic (vitamin B12) deficiency has been repeatedly confirmed in considerably large numbers of patients with AD [18]. The vitamin B12-deficient animal models have displayed abnormal metabolism of neurotransmitters [19]. Vitamin B12 deficiency could be a primary or a secondary event in AD [18]. How or why vitamin B12 deficit is related to dementia is still not known.

Oxidative stress and inflammatory factors have been demonstrated to play very important roles in the pathological development of AD [20,21]. However, it has not been established why or how oxidative stress and inflammatory reactions are elevated, so as to cause brain damage in a selective manner.

Recently, alternative hypotheses trying to bridge the gap between plaques and tangles theories include neuroplasticity failure [22], perturbations in "Wnt" signaling pathway [23], aging-induced protein degradation inefficiency [24] and aging-induced destruction of brain self-organization or loss of connectivity [17]. The vulnerability to developing AD pathology in certain brain regions has been ascribed to poor regional myelination [25].

AChE hypothesis of AD

Apart from the above hypotheses, the acetylcholinesterase (AChE) hypothesis of AD pathogenesis [26] has received due attention [27—29]. The AChE hypothesis emphasizes the basic anatomical association of AChE with the histopathological characteristics of AD. Alterations in its intrinsic functions and characteristics in its differential distributions represented by a three-grade staining pattern account for the vulnerability and selectivity of AD pathology [26].

The progress achieved in the past several years substantiates the AChE hypothesis of AD further. AChE has been found not only to consistently colocalize with all pathological lesions of AD brain [26,30]; but importantly, AChE purified from bovine brain, and human and mouse recombinant AChE, have all been demonstrated to be able to convert the α-helical, inert, wild form of Aβ (1—40 and 1—42) peptides as well as two mutant fragments (Aβ 12—28 Val 18 → Ala and Glu → Gln) into the β-pleated Aβ fibrils, which stain positively for both Thioflavin-T and AChE. The aggregations start as early as 30 min after incubation through binding to each other, thereby forming stable AChE—Aβ macromolecular complexes [23,27—28,31—34], whilst Aβ species alone only produce a few fibrils under identical conditions or aggregated 2-fold less than they do in the presence of AChE after 5-h incubation [23,27—28,31—34]. The formed AChE—Aβ complexes are more neurotoxic than Aβ alone, and the AChE—Aβ 1—40 complex is more toxic than AChE—Aβ 1—42. The neurotoxicity depends on the amount of AChE bound to the complexes. The AChE—Aβ complexes generated in the laboratory setting display similar modifications detected in brain tissue of AD [26,27,29—34] including resistance to low pH, a higher K_m and V_{max} values, and physical hindrance in both peripheral and active sites, relative to the free enzyme [23,27—28, 31—34].

When the AChE—Aβ complex is injected stereotaxically into the CA1 region of the rat hippocampus, neuronal loss is observed, astrocytic responses are detected, endogenous Aβ deposition is triggered and Aβ deposit is found after 2 weeks of injection, as confirmed by Thioflavine-S staining [34]. This phenomenon is highly remarkable compared with the negative results obtained by injecting synthetic Aβ alone into the hippocampus and cortex of adult rats over a wide range of concentrations and by a variety of vehicles, in which no Aβ deposition is generated, as examined 2—35 days following the implant [35]. The cause for the two contrasting results apparently has to do with the presence or absence of AChE in the experimental approach. It is well known that rat Aβ peptide has three amino acids substitutions in contrast to the human Aβ peptides and because of this difference, rats never form amyloid plaques [34]. The structural motif of AChE for the interaction has been identified as the 35-residue hydrophobic peptide close to the peripheral anionic binding site [36].

The considerable consensus that biochemical and pathological changes have occurred long before the clinical manifestations of AD [37,38] is consistent with the notion that an altered AChE can be found in subjects with or without clinical symptoms of AD in biochemical and pathological examinations [26].

By means of positron emission tomography (PET), AChE activity can now be measured in the brains of living individuals. The results confirmed that AChE activity was preserved in aged normal cerebral cortex [39,40], but significantly reduced in the entire neocortex and the hippocampus of AD brains, even at the mild and moderate stages of the disease [40,41]. A continued loss of AChE activity was detected as the disease progressed, and the lower cortical k3 values correlated significantly with the poorer memory and cognitive performances [41].

These exciting and much appreciated progresses certainly encourage a continuing search for the role (s) of cholinesterases (ChEs) in the normal brain and in the pathogenesis of AD, specifically in the connection with the formation of senile plaques (SPs) and neurofibrillary tangles (NFTs), so that the nature of AD would be better understood, and the treatment strategies would be accordingly justified. Hopefully, a unifying hypothesis of AD pathogenesis could be eventually reached by an in-depth study of the role of ChEs.

Biochemical physiology of ChEs

In naturally serving the triple roles of organizer, connector and safeguard for the brain, AChE and BChE have been documented to have a wide spectrum of properties and functions.

Unlike ChAT, which has an extremely low content in mammalian brain (estimated at 0.0001% of brain protein) and intrinsic instability [42], both AChE and BChE are of extremely high abundance and great redundancy in the brain and are extremely stable [26,30,43—46]. Purified human BChE is stable for years [47], and boiled AChE can still influence the behaviors of surrounding neurons [48].

A single gene on chromosome 7q22 encodes human AChE. A single gene for human BChE is located on chromosome 3q26 [46,47]. Each gene is surprisingly simple and small compared to their exceptional ubiquity and redundancy; and compared with their close partners — the less ubiquitous muscarinic and nicotinic receptors, which are encoded by a total of 19 human genes [49]. The alternative splicing, transcriptional, post-transcriptional and post-translational modifications give rise to the unusually rich molecular polymorphism of AChE/BChE, as illustrated in Fig. 1 in the

part I of this review. These forms, however, are specific for the species, cell-types, cell ultrastructures, states of differentiation, physiological conditions and response to external stimulation. They are characterized by distinct quaternary structures, mode of ionic, lipid and protein interactions, hydrodynamic and catalytic properties, allosteric modifications, electrostatic forces, glycosylations and phosphorylations [46,47,49–52].

AChE/BChE has been established as belonging to the superfamily of α/β-fold proteins. Several members of the family that display substantial sequence and structure alignments with AChE or BChE are identified to be either non-catalytic proteins such as thyroglobulin, glutactin, neurotactin, neuroligins, gliotactin and β-neurexin, which have been demonstrated to possess transmembrane or extracellular domains and are essential to neuronal adhesion, axon guidance, neuritogenesis, synaptogenesis and neuron-glia interactions in diverse species and cell types [46,49,53], or hydrolases such as trypsin, aryl acymidase and peptidase [46,48,53], and insulin degrading enzyme (IDE) [54] which degrades APP and insulin [7,54]. The triads of AChE or BChE, however, show both features, respectively [46,49,50,53,54]. ChEs own an unusually strong electric field due to the asymmetric charge distribution [49,50,53]. AChE subunits also possess a flexible conformation [51]. BChE exhibits strong similarities to AChE in protein and gene sequences but differs mainly in the size of the anionic site [43,50]. In humans, BChE subunit has acquired nine carbohydrates chains, while AChE has only three potential N-linked glycans [43,47,50,51].

Controlling concentrations of ACh and scavenging a variety of poisons

Both AChE and BChE are best known to hydrolyze acetylcholine (ACh) rapidly. Their joint efforts are to effectively prevent the levels of ACh from rising to neurotoxic levels.

AChE and BChE alone or together hydrolyze or scavenge not only ACh but also a wide range of choline and non-choline esters and toxic compounds that are eaten or inhaled, stoichiometrically, such as pesticides, nerve agents, cocaine, acetate, mivacurium, heroin, aspirin, etc. [43,44,46,47] in order to protect animals and human beings from poisoning [46,47].

AChE is also secreted from neuronal structures in several areas of the brain, and the secreted enzymes have been demonstrated to hydrolyze substrates and influence cellular behaviors within the region of the synaptic cleft more effectively than membrane-bound ones, since they are able to diffuse freely [46,48,55].

Non-catalytic functions of ChEs

Currently available evidence obtained form in vivo and in vitro experiments has shown that AChE and BChE possess many functions that do not seem to be solely related to their hydrolytic properties. These include neurotogenesis and its development, synaptogenesis and its development, neurotrophic activity, cell proliferation, cell differentiation, cell adhesion, cell plasticity, signal transduction, regulation of the blood–brain barrier, glucose/energy metabolisms, stress response and motor/behavior activities [46,48,52–53,55–59]. AChE is also expressed in retinal photoreceptors throughout their life. The photoreceptors are known to remodel on a daily basis [46], thus a novel morphogenic role for AChE in the continuous cytoarchitectural changes of adult photoreceptors has been suggested [46].

Soluble monomeric "readthrough" AChE-R variant has been demonstrated to appear in embryonic and tumor cells and is induced under psychological, chemical and physical stress [46,53].

Implications of animal model of AChE

A point mutation in AChE gene in zebrafish embryos showed defects in muscle fiber formation and innervation, and primary sensory neurons die prematurely, demonstrating that AChE is required for development and maintenance of the axial muscle apparatus and for survival of primary sensory neurons [60].

The AChE gene knockout mice survived at birth but about 50% of them lived only 14 days and 100% died by day 21 [61]. The AChE-deficiency mice that lived to the age of 12 days exhibited translucent cerebral hemispheres, whereas the brains of normal littermates were opaque [61]. The AChE−/− mice also displayed retarded physical development, persistent body tremor, circling while walking, were unable to open their eyes, lacked righting reflex and were highly vulnerable to toxic compounds [61].

Feeding on the "Ensure" — a complete food for human beings, the AChE−/− mice had an extended survival to an average life span of only 100 days. The adult AChE deficient mice had smaller body size, lower body weight, splayed feet, hunched

back and pinpoint pupils that failed to respond to light. They continued to suffer whole body tremor, lower body temperature, absent grip strength, inability to eat solid food and limited locomotor activity. They were susceptible to seizures and convulsions in normal environment, unable to learn to urinate and defecate outside the nest, lacking defensive and territorial aggressive behaviors, in addition to poor pain perception, poor vocalization, overreaction to stress, sexual dysfunction and postnatal development delay [62].

The transgenic mice overexpressing human AChE gene (hAChE) or AChE variants demonstrated an upper limit of 1.5—2.0-fold higher enzyme level in the brain over controls, a level which was only compatible with life [63,64]. The hAChE transgenic mice showed learning and memory impairment shortly after early adulthood and became progressively incapacitated compared with control mice [46,63,64]. An attenuation of dendritic branching and depletion of dendritic spines harboring synapses of cortical neurons was also discovered [65].

When they were hybridized with transgenic mice overexpressing APP gene mutations (hAPP) (Tg 2576), SPs were detected at the age of 3 months. The exhibition of SPs in the parental hAPP (Tg2576) mice was at 9-months old. The hybrids of hAChE and hAPP mice displayed 50% more SPs than non-hybrid hAPP mice at 9 and 12 months of age.[1]

The altered ChE metabolism may underlie the formation of SPs

Extensive research on β-amyloid (Aβ) hypothesis has determined that the amyloid fragments (1—40 and 1—42, 43) are derived from an abnormally processed amyloid precursor protein (APP), which is larger in size. The normal APP is an integral membrane glycoprotein, which is generated from intracellular compartments, the Golgi/endoplasmic reticulum, and delivered to the cell membrane [15,23]. Aβ is found to be a major component of senile plaques (SPs). Its initial deposition in extracellular space is designated as the first stage in its evolution.

Two necessary conditions for plaque formation must be kept in mind while searching for its probable cause and mechanism. One is that in order for extracellular Aβ deposition to be stainable with Aβ antibodies the membrane must be damaged [66] and the other is that an intracellular mis-processing of the precursor must take place [15,66].

Loss of extracellular membrane-bound ChEs may initiate the formation of SPs

In mammalian brains including human brain, the predominant AChE form is membrane-bound amphiphilic globular tetramer ($G4^a$) [27,50], which makes up the largest proportion (~70—75%) of the total brain AChE activity [50]. Membrane-bound AChE $G4^a$ is attached to the outer surface of the cell's basal lamina including axonal, dendritic membranes and the vicinity of synaptic junctions [27,30,43—46,50,53]. The AChE $G4^a$ protein acts as the first line of defenders and protectors for the membrane gateway physically and biochemically.

As illustrated in Fig. 3(a) and (g) in the part I of this review, an extensive network of AChE membrane-bound fibers and neurons is characteristically present and/or well preserved in normal mature brains of young and old individuals, whereas in AD this network is profoundly depleted and replaced by prominent SPs, NFTs, NTs and DNs (Fig. 3(b) and (h) in the part I of this review) [26,28—30,67—70]. The fine network of neuronal AChE is best revealed at pH 8.0 in histochemical staining [26,30]. In AD only a few remaining normal fibers and neurons are stained with unaltered enzymatic characteristics. The neurophils are scarce; the axons become virtually empty; SPs are associated with remnant, distorted fibers and the number of NFTs is correlated with fiber loss [26,30,67—70].

Recent studies have further demonstrated that diffuse plaques are associated with a loss of AChE fibers up to 30% in density in the inferior temporal gyrus and to 50% in the entorhinal cortex, in individuals dying with diffuse plaques but without a history of dementia, e.g., preclinical stage of AD, versus old and young subjects who are free of diffuse plaques. Staining for AChE that is associated with Thioflavin-S and Congo red-positive diffuse plaques is no longer sensitive at pH 8.0, but sensitive at pH 6.8—7.0 [71].

Consistent with the histochemical observations, there are significant reductions in the total AChE activity (reduced to 55—67% of normal levels in specific brain regions), and/or the membrane-bound AChE G4 forms, determined in vivo and in vitro in affected brain areas and in cerebro-spinal fluid (CSF) of aged non-demented subjects, early and advanced AD patients, relative to normal controls [26,29,30,39—41,50]. Taken together they prove a gradual and selective, but constant ex-

[1] Rees T, Soreq H, Younkin S, Brimijoin S. Acetylcholinesterase Promotes Amyloid Beta Plaque Deposition In Vivo. Neurobiol Aging 2002;23(Suppl 1):S243.

haustion of the AChE-G4 membrane pool throughout the course of AD.

The relationship between the alteration and injury of membranes in cell bodies and axons due to the chronic loss of membrane-bound AChE and the formation of SPs and NFTs is vividly illustrated in Figs. 3(c)—(f) and 4(a)—(f) in the part I of this review by AChE staining [67]. The general inference drawn from the figures may be that membranes on cell bodies, processes and vessel walls stained for AChE lose integrity in showing initially mild swelling of the membranes. The swelling then begins to enlarge and to balloon resulting in membrane breaching and ragging. The AChE-positive material and Aβ peptide (s), whose precursor APP is partially extruded into extracellular space [15,23], with or without membrane debris, escape and/or are abnormally cleaved (Aβ fragments) from the leaky and/or partially breached membranes, and seeded into the surrounding extracellular space. Once Aβ peptide encounters the AChE, they directly interact with each other and form the AChE—Aβ complex, e.g., the diffuse plaque. This explains why an intimate relationship exists between AChE and Aβ, and why both AChE and Aβ stain positively for diffuse plaques in perineuronal, perivascular and subpial distributions, and are aligned along axons, dendrites and their terminals in human brains [67,68,70,71], and in brains of transgenic mice, which are overexpressing APP gene mutations [72].

Inducement of subsequent chain reactions

The deterioration of the safeguard, the membrane-bound AChE G4[a], due to the gradual loss of AChE G4 forms, prior to the evident membrane damage, may conceivably trigger a series of abnormal chain reactions.

The reactions from intracellular network of AChE and/or BChE have been demonstrated by a small but significant increase of soluble or hydrophobic G1 and/or G2 of AChE and/or BChE and a low G4/G1 ratio for both AChE and BChE in certain affected areas of AD brains, compared with controls [26,29,30,50]. The patterns of glycosylation of AChE G1[a] and G2[a] and unspecified forms of BChE are also altered, when examined by lectin binding in the affected frontal cortex and in CSF of patients with AD vs. controls [73].

It is recognized that the stable and correct functioning of AChE and BChE is dependent on a correct organization and glycosylation. Changes in the sugar residues of the enzymes may result in altered tertiary structures in the enzyme subunits, which may interfere with the oligomerization process or affect the pH preferences and inhibitor selectivity. The molecular weight, charge- and size-based heterogeneity in glycans is important for effective biosynthesis, secretion and stability [46,50,53,74]. As a result, the AD-bound ChEs show lower sensitivity to traditional inhibitors and require excess substrate for inhibition versus those of normal neuronal elements [26,29,30,50,75]. These enzymatic and pharmacodynamical changes are recently reproduced by in vitro experiments, when AChE is bound to Aβ forming AChE—Aβ complexes [28,33].

The immunocytochemistry of AChEs in AD lesions are also altered, as antibodies against normal brain AChE are no longer able to label those AChEs associated with AD pathology [30]. The rich AChE in SPs can only be partially extracted as G4 form using collagenase, protease or high-salt buffers plus detergent digestion, indicating that the AChE of SPs is mainly of type A, which is different from the AChE in normal brain or skeletal muscle [75].

The altered patterns of ChEs glycosylation associated with the increased light forms have also been detected in brains of transgenic mice (Tg 2576) overexpressing Swedish APP mutations as early as at 4 months of age [76]. These abnormalities are concomitant with the emergence of the soluble human-sequence Aβ, prior to amyloid plaque depositions, which can only be detected at the age of 12 months [76].

Other subsequent reactions may be related to the alterations in the membrane phospholipid, oxidative and high energy phosphate metabolisms [20,21,77], signal transductions [24,38], glucose/energy regulation and control [77], calcium distribution [24], cytoskeletal stability [7,8,13] and APP processing [7,15,23,66], as demonstrated in AD-related in vitro and in vivo studies.

The occurrence of an intracellular misregulation in the trafficking of APP together with depositions of APP and Aβ at membranous compartments predating the diffuse plaque formation seen in brains of humans and transgenic mice overexpressing APP/PS-1 gene mutations [15,78,79] may reflect this mechanism and indicate an impairment(s) in the integrity of the intracellular network.

Altered metabolism of intracellular ChEs may initiate the formation of NFTs

The abnormally phosphorylated protein tau is the initial sign of intracellular structures undergoing NFT degeneration [8,13]. Tau glycosylation has

recently been discovered to occur early and may facilitate its abnormal phosphorylation [80]. Glycosylated and phosphorylated taus are major constituents of the paired helical filament (PHF) and straight filament (SF) that lead to the formation of neurofibrillary tangles (NFT) in perikarya, neuropil thread (NT) in fibers and dystropic neurites (DN) surrounding SPs [8,13,80].

It is proposed that the alteration in intracellular AChE and/or BChE may be directly linked to tau glycosylation and/or its abnormal phosphorylation. ChEs, especially AChE, has been clearly demonstrated to be present in cellular membranous profiles, such as the nuclear envelope, the Golgi apparatus, the mitochondria and cisternae of the rough endoplasmic reticulum [26,27,30,44, 45,75,81]. The mitochondria are normally evenly dispersed throughout the cytoplasm. The intracellular AChE and/or BChE delineate the shapes of cellular compartments and may safeguard them from various insults. When human brains are examined ultrastructurally at the pretangle stage, the membranes of cellular organelles are frequently dilated, forming vacuoles of variable morphology, which are partially studded with ribosomes positive for tau [82]. Meanwhile, the apical dendrite is delicately immunoreactive for tau, while the AChE or BChE reaction product that label the perikarya in a diffuse way colocalize with tau [81].

Tau filaments have been noticed frequently to colocalize with cellular membrane structures in lamprey central neurons (ABCs), when they are made to overexpress human tau isoform [83]. The tau-membrane interaction in ABCs also appears to be strongly associated with localized degenerative changes, with "beaded" dendritic regions frequently containing nothing but scraps of membranous material interspersed with bundles of tau filaments [83]. PHFs can also be seen arising at, or from the surfaces of cytomembranes; membranes in perikarya or dendrites often form irregular stacks, clumps, or lamellated bodies in the very early stage of afflicted neurons in AD brain [82,84]. The AChE and/or BChE reaction product is closely associated with these filaments [26,30,81].

These observations suggest that the intracellular AChE and/or BChE may have been altered, and have leaked from the dilated membranes and stuck to tau protein, while the plasmic transport between organelles and cytoskeleton is taking place, and subsequently forms AChE and/or BChE-tau complexes. In consequence the tau protein is glycosylated and abnormally phosphorylated. The complex first appears as granular material, and slowly aggregates into short and disordered PHFs and SFs. These filaments become elongated into bundles along with the longitudinally oriented fibrillar cytoskeletal organelles (unbranched cylinders) that extend throughout the neuron, including its processes. Other elements, such as glycosyltransferases that transfer sugar moieties to proteins, could also leak from the afflicted endoplasmic reticulum and Golgi apparatus, and participate in the formation of AChE and/or BChE-tau complexes.

The reported defects in mitochondrial respiratory chain complex activities, the fragmentation of Golgi apparatus, the perturbed endoplasmic reticulum functions, and the impairment in the fast and slow axonal and dendritic transport, detected in vivo or in vitro studies, in non-demented subjects with family history of AD and in aging human brain tissue [77,85—87], may happen when the intracellular organelles are affected.

Currently the precise forms and characteristics of the intracellular membrane-bound AChE/BChE in each type of organelles are not elucidated. Theoretically, amphiphilic or non-amphiphilic globular monomer ($G1^a$ or $G1^{na}$), dimer ($G2^a$ or $G2^{na}$) and tetramer ($G4^a$ or $G4^{na}$) of AChE and BChE could constitute the sources. It has been pointed out that AChE $G1^a$ and $G2^a$ probably represent intracellular precursors of the mature physiologically active $G4^a$ species [50], and the synthesis of AChE G forms and their further processing and assembly take place in the rough endoplasmic reticulum and the Golgi apparatus [27]. Membrane-bound or soluble intracellular AChE/BChE forms can be distributed and released normally through the cytoskeletal transport network, and can leak from the dilated membranes of organelles in pathologic conditions.

Maturation of SPs and NFTs may be facilitated by ChEs-neuroglia

Once the AChE—Aβ diffuse plaque and AChE and/or BChE-tau complexes are formed, conformational changes of both substances may have already taken place. But they are largely non-β-pleated sheet structures, not able to bind sufficient amount of dyes to be visualized by conventional staining methods, such as Thioflavin-S and Congo red, but they can be detected by AChE enzyme histochemistry at pH 8.0 [68] — used for normal neuronal AChE visualization [30], and by antibodies against Aβ peptides and modified tau proteins [68,69]. As the diffuse plaques and AChE and/or BChE-tau complexes gradually change the extracellular and intracellular microenvironment by self-aggregation

and by incorporating other proteins, proteoglycans and metals from the surroundings [26], they grow and gain more β-pleated sheet structures, and become Thioflavin-S and Congo red positive. The optional pH for detecting ChEs in the lesions at this stage shifts from 8.0 to 6.8—7.0, as demonstrated repeatedly by independent research groups [26,30,68—71,81].

The maturation of SPs and NFTs may be facilitated initially by the significant activation and participation of the brain's defense system and later by the action of acquired immunity [10,21]. The so-called inflammatory reactions are doing their natural defense duties in phagocytizing, removing and repairing the injured neuronal membranes, when the oligodendrocytes and the astrocytes are under direct stress in the presence of injured myelin sheaths and envelope-like sheets. Fig. 2 in the part I of this review which illustrates perfectly the close relationship of a neuron and neuroglial cells and provides the histo-anatomic basis for the mechanism(s) of inflammatory reactions in AD. Neuroglial capability of developing chronic inflammation may largely depend on the cellular intensity of their ChEs expression.

Microglia are few in the normal brain, but in brains of AD and transgenic mice of APP they increase locally and enter into the center of diffuse plaques, while hypertrophic astrocytes surround the peripheries of plaques and NFTs [10,21, 30,67,72]. AChE and/or BChE believed to come from local neuroglia are detected invariably as soon as β-pleated fibrillar structure is being formed in diffuse SPs [26,30,67,71,88]. The repairing processes, which are to reduce and/or stop the AChE-positive material discharge, also produce an AChE-intense core and an expanding halo surrounding the discharged substance [67]. The alteration in membrane repairing process measured by 31^P nuclear magnetic resonance (NMR) that exists in normal aging brain and more markedly in AD [89] is consistent with the histochemical description [67]. Glial AChE and/or BChE are present at almost all stages of SPs and NFTs even in the burnt-out SPs and extracellular ghost tangles [26,30,81,88].

Neuroglia and neurons together with the cells of the acquired immune system such as the AChE-containing lymphocytes and BChE containing serum also secrete a variety of inflammatory mediators and other injury response factors [10,21]. The uncontrolled release of such inflammatory factors is potentially toxic to both the diseased and the adjacent still healthy tissues, creating a more complicated and prolonged pathophysiological process.

A unifying hypothesis of AD pathogenesis by ChEs

It appears that the widespread brain ChEs network is constantly subject to insults from various risk factors, such as aging, gene anomalies, environmental hazards, head trauma, excessive oxidative stress, imbalances and/or deficits of organic constituents such as vitamin B12, calcium and so on [5—7,9,24]. When the network adjusts to or overcomes the insults by responsive plasticity [22] or by enhancing optimal protein degradation [24], the cell membranes will remain intact (see Fig. 3(a) and (g) and Fig. 4(a) in the part I of this review) and the cell, the tissue and the organ can survive with normal functions. As a result normal memory and intelligence can be well maintained even to a very old age. The presence of a normal or slightly increased AChE activity in both the brain tissue with no evidence of decline even during advanced senescence [26], and in CSF of normal controls, but not in subjects with mild cognitive impairment, age-related change, or early AD, serves as the best evidence for such an assumption [26,39,40].

ChEs' outside—in cascade in relation to APP, PS-1 and PS-2 gene anomalies

Due to various insults, the ChEs network is chronically losing its capacity of first line defender on the extracellular membranes, because of diminishing plasticity and efficiency in chemical degradation, which slowly and ultimately lead to the injury and damage of membrane-bound AChE and cause an outside—in cascade as described in the sections "Loss of extracellular membrane-bound ChEs may initiate the formation of SPs" and "Inducement of subsequent chain reactions". Those expressing weak AChE on the surface membranes of the cell will be particularly vulnerable to the insults. When the membranes become wounded and AChE—Aβ complexes are formed, inflammatory reactions are subsequently triggered. These events influence each other and interact with surrounding substances in extracellular space, resulting in further deterioration in local conditions. As the integrity of extracellular membrane-bound AChE network is breached, the intracellular cascade is induced as described.

The recent discovery that most (90%) AChE and BChE $G4^a$ are anchored to the outer membranes by the type 1 transmembrane protein — proline-rich membrane anchor (PRiMA) in brain cells of mice and humans [90] — may provide further clues to the

molecular—cellular basis for the outside—in cascade. It is conceivable that the initial loss of AChE G4 molecules at the outer membranes may disturb the homeostasis of their membrane anchor protein — the proline operation within the cell membranes. Proline has been implicated in the wnt/wingless signal pathway, especially in tau abnormal phosphorylation and tau structural conformation, protein—protein interaction, APP processing, Aβ aggregation, cell cycling and transcription [91,92]. The conditional transgenic mouse model overexpressing GSK-3β, a proline-directed serine/threonine kinase in the brain, has shown decreased levels of nuclear β-catenin and hyperphosphorylation of tau in hippocampal neurons, the latter resulting in pretangle-like somatodendritic localization of tau with abnormal morphologies and detachment from the surrounding neuropil. Reactive astrocytosis and microgliosis are also detectable and neuronal stress and death are confirmed by TUNEL examination [93].

It is recognized that genes seldom act alone to cause a disease. A genetic disease is often a network problem [94]. The brain ChEs on all accounts may serve as the brain's network. Proteins produced by gene mutations or gene duplications, such as APP, PS-1 and PS-2, may primarily interact and affect the extracellular AChE $G4^a$ network, especially those expressing weak AChE, and induce the outside—in cellular cascade. This may explain why patients with Down's syndrome, and other familial forms of dementia, display SPs first and then NFTs [7,15].

However, damage to the extracellular AChE network alone can also cause dementia symptoms if the destruction is sufficiently severe, as seen in some AD patients with cortical SPs only [7]. The genotype/phenotype of transgenic mice overexpressing wild [95] and mutant APP [72], producing no NFTs but diffuse and/or neuritic SPs, may serve as convincing evidence of the direct interaction between APP and extracellular ChEs network.

ChEs inside—out cascade in relation to tauopathies

The ChEs also form intracellular membranous network in the mitochondria, nuclear envelope, endoplasmic reticulum (ER) and Golgi apparatus, although the network is smaller in size compared with the extracellular one. The intracellular network is also subject to constant insults from various risk factors as mentioned earlier. These insults affect the intracellular ChEs network, especially those expressing weak AChE, which in turn alters the cytoskeletal network and causes an inside—out cascade as described in the section "Altered metabolism of intracellular ChEs initiates the formation of NFTs". The characteristic initial, pretangle, somatodendritic tau localization observed in both brains of humans and animal models [8,81,82,93] is interesting since only dendrites, not axons, contain the same cytoplasmic organelles as perikarya do, confirming the primary involvement of intracellular compartmental ChEs network in the formation of NFTs.

The demonstration of less-pronounced cytoplasmic AChE staining often extending into the apical dendrites in the neurons of subiculum and prosubiculum [96] provides evidence of the presence of a somatodendritic AChE network, which may explain why tau, found most prominently in the axons of neurons in normal adult brain, noticeably "translocated" from axons to somatodendritic compartments and deposited there in brains of patients and animal models with tauopathies [8,82,83,93].

The dynamic intracellular cytoskeleton is known to be associated with plasma membranes and is the substrate for numerous protein kinases in vivo and in vitro, and can be regulated by such modifications. On the other hand the cytoskeletal protein(s), for instance, tau protein, is also able to direct numerous protein kinases [97], thereby influencing the regulation of intracellular and extracellular signal transduction. The tauopathy theory of AD may represent the inside—out cascade of the pathogenetic process, based on the observation that neurofibrillary degeneration of cell bodies and their fibers increases gradually with age in humans and that the emergence of plaques is a relatively later event [8,13].

In gene mutations encoding intracellular substances, such as cytoskeletal compounds, the products may originally and severely interfere with perikaryal AChE and/or BChE network. Consequentially they cause deposition of filamentous cell inclusions, perturbation of signal transduction, destruction of cytoskeletal trafficking, dysfunction of organelles and death of neurons and neuroglia. This may explain why patients with tau gene anomalies display abundant intracellular NFTs in the brain and the spinal cord, which by themselves are sufficient to cause symptoms of dementia as described in the clinico-pathological findings of tauopathies [8,13]. The transgenic mice overexpressing human mutant tau gene and exhibiting only NFT-like pathology [98] may serve as evidence of the direct interaction between tau and intracellular ChEs network.

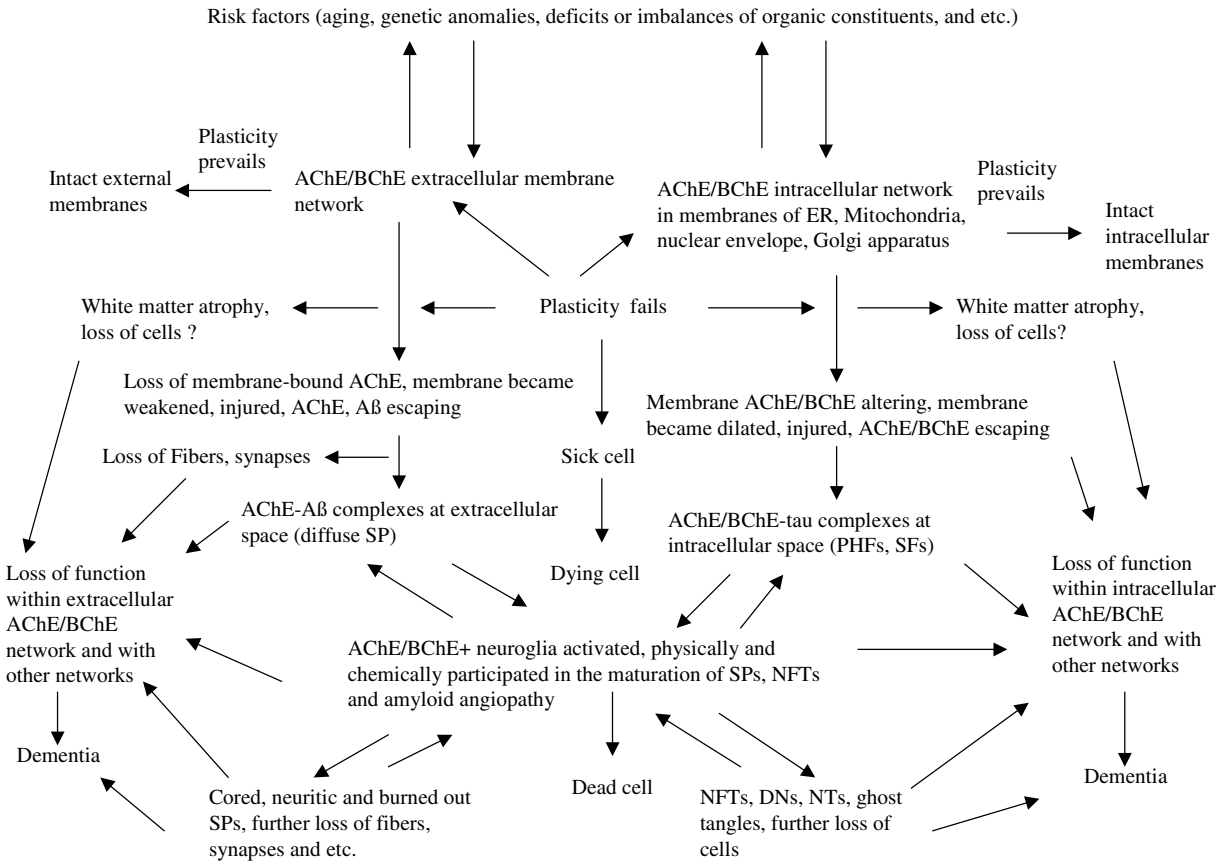

Figure 1 Schematic model of ChEs cascades of Alzheimer's disease. The majority of extracellular membrane-bound AChE and BChE are G4 forms. The rest remains to be determined. Intracellular membrane-bound AChE and/or BChE forms have not been fully identified. The impaired integrity of extracellular or intracellular membrane-bound AChE/BChE causes the sickness and death of the cell, which is represented by structural, metabolic and functional alterations, and its demise. The loss of connections within the network of ChEs themselves and with other networks ultimately leads to the clinical manifestation of dementia. The vulnerability and selectivity of AD pathology correspond to the intensity of AChE and or BChE expression. The most vulnerable part of a cell or a region in the course of AD usually expresses the least AChE activity and vice versa.

The newly created transgenic mouse model overexpressing both mutant APP and mutant tau, and having both plaques and tangles [99], illustrates how specific gene mutations can cause specific extracellular and intracellular ChEs network problems simultaneously.

Sporadic AD cascade

The cascade for sporadic AD cases could be either outside—in or inside—out or both, largely depending on the intensity of AChE expression and plasticity on the intracellular or extracellular membranes of a given neuron. Predictably, the weaker the cellular portion expresses AChE, the more susceptible to degeneration the portion becomes.

Various deficits and/or imbalances in organic constitution also play important roles in the etiology and pathogenesis of AD through mainly affecting ChEs. For instance, vitamin B12 deficiency can be both the cause and the consequence of dementia [18]. Vitamin-B12-defect animal model shows significantly lower serum AChE and BChE levels than controls [19], demonstrating its detrimental influence on ChEs system.

The loss of extracellular membrane-bound AChEs together with an altered glycosylation in ChEs precursor forms may in part explain why the AD brain suffers from neuroplasticity failure [22], the failure to synthesize AChE G4 forms which are lost, and the alterations in enzymatic and pharmacological properties as well as immunocytochemistry [26,29,30,75].

The intense AChE and/or BChE reactivity in SPs and NFTs, which once puzzled researchers [100,101]

may be very likely due to at least two determinants in the diseased environment, which has lower pH in contrast to the normal environment. The lower pH can cause wild-type and mutant AChE to behave like BChE, thus instead of the defined property of substrate inhibition, substrate activation occurs [102]. The other factor may be the higher ACh level resulting from the decreased AChE catalytic activity and the upregulated ChAT, HAChAT and VAChT [17,103]. The higher level of ACh can activate AChE and BChE trapped in SPs, and NFTs.

When Alzheimer reported the prototypic case, he prophetically maintained that in the neuron there was the storage of an as yet undefined pathological product of metabolism, possibly an allusion to the abnormal fibrils, which survived the destruction of the cell [1]. Credible evidence presented here strongly indicates that the brain cholinesterases (ChEs) may be what Alzheimer alluded to. The proposed pathogenesis of AD, such as the neuronal plasticity failure [22]; the destruction of brain self-organization or the loss of connectivity [11,22]; the aging-induced inefficiency in protein degradation [24]; the gene mutations [6—8,13]; the perturbation of signal transduction [23]; the oxidative stress and inflammation [5,10]; the abnormalities in neurotransmitters/neuropeptides [17] and the vitamin B12 deficiency [9] may all be related to the ChEs network. The brain ChEs play a pivotal role(s) in the pathogenesis of AD, as illustrated in Fig. 1, and have been implicated in many neurological, psychiatric, neuromuscular and other disorders [26,46,47,53] owing to their ubiquitous presence in the brain and throughout the body, their functions being active for a life time.

Conclusion

Consistent evidence obtained from in vitro and in vivo studies demonstrates that cholinesterases (ChEs), in serving as the connector, the organizer and the safeguard for brain histo-anatomic and biochemical architecture, possess a wide spectrum of properties and functions crucial to the well-being of cells, tissues, animal and human lives, while they exist adequately in quality and quantity. The loss and the alteration of ChEs on the outer surface membranes and/or the membranes of the intracellular compartments together with their modification due to glycosylation and conformation change may give rise to the development of extracellular senile plaques, intracellular neurofibrillary tangles and many other AD-related abnormalities. The inflammatory reactions mainly arising from ChEs-containing neuroglial cells may facilitate the pathophysiologic process of Alzheimer's disease. The mechanisms of the outside—in and inside—out cascades of AD in relation to the abnormal metabolism of ChEs proteins on the extracellular and intracellular networks are discussed. The brain ChEs by all means play a pivotal role(s) in the etio-pathogenesis of AD.

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