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Review

Aluminium in Alzheimer's disease: are we still at a crossroad?

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Abstract. Aluminium, an environmentally abundant non-redox trivalent cation has long been implicated in the pathogenesis of Alzheimer's disease (AD). However, the definite mechanism of aluminium toxicity in AD is not known. Evidence suggests that trace metal homeostasis plays a crucial role in the normal functioning of the brain, and any disturbance in it can exacerbate events associated with AD. The present paper reviews the scientific literature linking aluminium with AD. The focus is on aluminium levels in brain, region-specific and subcellular distribution, its relation to neurofibrillary tangles, amyloid beta, and other metals. A detailed mechanism of the role of aluminium in oxidative stress and cell death is highlighted. The importance of complex speciation chemistry of aluminium in relation to biology has been emphasized. The debatable role of aluminium in AD and the cross-talk between aluminium and genetic susceptibility are also discussed. Finally, it is concluded based on extensive literature that the neurotoxic effects of aluminium are beyond any doubt, and aluminium as a factor in AD cannot be discarded. However, whether aluminium is a sole factor in AD and whether it is a factor in all AD cases still needs to be understood.

Key words. Alzheimer's disease; neurofibrillary tangles; amyloid beta; aluminium; oxidative stress; cell death; genetics.

Introduction

Alzheimer's disease (AD) is a neuropsychiatric disorder affecting elderly people, as described by Alois Alzheimer in 1906 [1, 2]. AD is a progressive mental deterioration manifested by memory loss, inability to calculate, visual-spatial disturbances, confusion and disorientation. The neuropathological characteristics include cortical and subcortical atrophy, formation of intraneuronal neurofibrillary tangles (NFTs), deposition of amyloid beta peptide (Aβ) in neuritic plaques or senile plaques (SPs), formation of neuropil threads, loss of synaptic function, oxidative stress and apoptosis, leading to neuronal loss. These events are observed mostly in the hippocampal and corti-

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cal regions of AD brains. The etiological factors of AD are not clearly known, although unproven hypotheses have included genetics, head trauma, oxidative stress, infectious agents, and environmental factors including aluminium (Al) toxicity.

Al has never been demonstrated to have any definite biological function, suggesting that the element possesses properties which are neutral or incompatible with fundamental life processes. Many scientific studies have brought to light the potential toxicity of Al in experimental animal models and in humans under different clinical conditions [3, 4]. The pioneering studies on Al neurotoxicity in experimental animals were initially described in 1886 by Siem and Dollken [5]. Most of the understanding of Al toxicity in humans was established as a result of studies of disorders experienced by dialysis patients when the dialysis fluid contained Al at or above 0.5 µmol/l. In such patients, Al accumulated in various tissues, including kidney, liver, bone and heart [6, 7], giving rise to pathological conditions such as (i) dialysis encephalopathy that can lead to dementia and death and (ii) dialysis osteomalacic osteodystrophy. Al as a neurotoxic metal was initially established in the early 1970s after years of uncertainty. The Al hypothesis in AD came to light following the extraordinary discovery of Klatzo et al. [8], who showed that injections of Al salts into rabbit brain led to the formation of NFTs which appeared similar to the NFTs of AD [8, 9]. These results were replicated in cats by Crapper et al. [10], and they also evidenced the increased level of Al in the brain of AD patients, which was the first report for its linkage with AD. Since then numerous reports have prompted the suggestion that Al is a possible cause of AD [10–17]. Subsequent studies using a low dose of Al salts inoculated intracisternally into rabbits led to the formation of NFTs and the first chronic neurotoxicity model of Al [18, 19].

In this commentary, we have tried to argue that the Al hypothesis continues to survive for the following reasons: (i) there is a definite toxic action of Al in brain; (ii) Al levels are elevated in the brain of patients with AD; and (iii) the incidence of AD is increased in regions where people are more exposed to Al. However, the Al hypothesis has been disputed based on the following features: (i) not all patients with AD have high brain levels of Al, and the SPs that are common in AD are not seen in experimental Al toxicity [20]; (ii) it has been shown that NFTs in AD are made up mainly of abnormal tau proteins, in contrast to Al-induced NFTs, which are made up of normal neurofilaments [21]; and (iii) the incidence of cognitive impairment and AD symptoms is not increased, but only transit dialysis dementia in renal patients is observed with increased Al levels [22]. However, the neurotoxic effects of Al have been repeatedly demonstrated and shown to interfere with a variety of cellular and metabolic processes in the nervous system as well as several other systems. After decades of research towards resolving Al toxicity, the exact mechanism of Al neurotoxicity and its complex biology still remain unanswered. A plethora of studies on Al toxicity and neurodegeneration are still being undertaken with a promise to generate more scientific controversies in the future.

Al hypothesis in AD: putting together pieces of puzzles

The relevance of Al in AD is highlighted by discussing the Al load in the brain, Al and NFTs, Al and Aβ formation, and Al in relation to other metals.

Brain Al load

The possible role of Al in AD gives rise to the important question whether Al can enter the brain, and if it does, what the mechanism(s) of entry are. Three routes have been proposed by which Al could enter the brain from systemic circulation: blood-brain barrier (BBB), nasal-olfactory pathway and cerebrospinal fluid (CSF) [23, 24]. More rapid exchange is possible through the BBB, as many carriers of Al have been identified at the BBB. Transferrin (Tf)-mediated transport of Al has been suggested to be one of the mechanisms [25]. Another important carrier for brain Al influx may be monocarboxylate transporter (MCT), a proton co-transporter which is located at both the luminal and abluminal surfaces of the BBB [26].

Despite its ubiquitous presence, only 0.06–0.1% of ingested Al is absorbed across the gastrointestinal tract [27]. Al uptake is limited by the presence of certain other dietary components such as citrate, which forms a complex with it [28], and its competition with other elements such as Ca, Mg, and Si [29]. Vitamin D and parathyroid hormone are also presumed to affect Al absorption [30]. Involvement of a genetic variant of Tf, TfC2, has been found to be responsible for the excess transport of Al into the brain [31]. Once in the blood, the BBB comes into picture, which restricts Al entry into the brain. It is hypothesized that changes in the structure and function of the BBB underlie various findings related to Al accumulation in AD [32]. Thus, even though there is selective uptake of Al, the question is why we find an increased concentration of Al in the brain of AD patients.

Al is available to humans through drinking water, Al vessels, Al foils used in food packaging, and higher levels of Al in food and beverages such as tea [33–35]. It is also present in higher quantity in certain drugs, such as antacids. Al is a common flocculent used in water treatment plants, leaving open the possibility of considerable contamination in drinking water [36]. In one of the studies

carried out by McLachlan et al. [37], it was found that a significant relationship existed between the number of AD cases and the level of Al present in drinking water [37]. A similar study carried out by Jacqmin et al. [38] showed a relationship between Al and AD when pH was taken into consideration [38]. Studies of long-term exposure to different forms of Al in drinking water and AD suggested that a sizable relationship exists between Al and AD, involving increased risk in populations exposed to Al concentrations in drinking water higher than 0.1 mg/l [39]. A recent study carried out in mice shows that there is an increase in inflammatory processes in the brain following chronic exposure to Al in drinking water [40]. Many controversial reports have also appeared, questioning the link between AD and exposure to Al in drinking water. However, drinking water confuses the issue, because it contributes only a minor portion of the total daily oral intake of Al.

Here we have tried to summarize region-specific and intracellular localization of Al in the brain. To date, the distribution of Al in brain is debatable because of its complexity and the analytical problems in Al quantitation. Limited studies have been done on Al distribution in the brain. The concentration of Al varied (58–196 μg/g wet weight) in different brain regions (frontal cerebrum, temporal cerebrum, parietal cerebrum, somatosensory cortex, occipital cerebrum, cerebellum mid-brain, pons, hypothalamus, thalamus, hippocampus and medulla oblongata) of the normal brain. The reasons for the higher levels of Al were dietary habits and higher usage of Al cookware [41]. Two regions, namely temporal cortex and hippocampus, were found to have high Al content, and these two regions are also known to be significantly involved in AD. Xu et al. [42] reported 6.2–9.8 μg/g (dry mass brain) of Al in human brain. Crapper et al. [10] reported elevated concentrations of Al (9.0–11.0 μg/g dry weight) in some regions (cortex, mesial temporal and temporal cortex) of the brain of AD patients [10]. In case of Al encephalopathy, significant Al content was observed in hippocampus, occipito-parietal cortex, cerebellum and striatum. This topographic distribution correlates with the clinical defects in higher cortical functions, including aphasia [43, 44]. It is interesting to note in the above studies that Al tends to accumulate more in the cortex and hippocampus, both in normal and AD brains [45]. At the subcellular level, the distribution of Al is more selective to lipofuscin, cytosolic, mitochondrial, lysosomal and nuclear compartments [46]. Schuurmans et al. [46] studied the subcellular localization of Al in rat cerebral organotype cultures using electron probe X-ray microanalysis (EPXMA). A major concentration (60%) of Al was found in lysosomes, followed by mitochondria. They found Al has low DNA binding affinity, while it has higher affinity towards RNA. They also found low Al levels in the nucleus. The Al affinity for DNA is still debated. However, Lukiw et al. [47] have shown increased amounts of Al in chromatin, and they detected a high concentration of Al (885.4 μg/g DNA) in DNA isolated from the neuronal nuclei of AD [47]. Recent studies from our lab showed that Al not only strongly binds to DNA but also causes helicity change in DNA [48, 49]. Further, brain cell-specific studies using nuclear magnetic resonance (NMR) indicated that astrocytes were more sensitive to Al binding compared to neuronal cells [50]. Further studies in this direction are needed to understand the complex neurobiology of Al. Studies on subcellular localization and cell-specific interactions may also provide new directions in understanding Al toxicity.

Evidence from clinical and animal model studies demonstrate that brain Al content increases with age, suggesting increased exposure with age or a decreased ability to remove Al from the brain with age [51, 52]. A very detailed study by Savory et al. [53] clearly showed that aged rabbits are more susceptible to Al toxicity compared to young rabbits. Al-induced neuropathological events (alteration in Bcl-2:Bax ratio, oxidation, apoptosis, redox-active iron (Fe) etc.) in aged rabbits mimic AD neuropathology [53]. It has been demonstrated by Taylor et al. [54] that less Al is absorbed by younger individuals when compared to older people, indicating that aging makes a person more susceptible to Al accumulation in the brain. Furthermore, studies have been done to determine whether patients with dementia show a propensity to absorb more Al from their diet than healthy volunteers without dementia. The findings suggest an almost threefold increased absorption of Al in AD patients compared to healthy controls [55]. It was also found that patients with AD are exposed to a greater body burden of Al, implying that such exposure may be an important factor in the etiology of the disease [55]. It has been hypothesized that Al may act by affecting the number or distribution of cell surface charges, hence altering BBB activity [56]. Based on the above observations, we have outlined a few possible pathways leading to Al accumulation in AD brain in figure 1.

Al and NFTs

NFTs are the most consistent post-mortem characteristic of AD, consisting of phosphorylated fibrillary proteins aggregated within the neuronal cytoplasm. They are composed of paired helical filaments (PHFs) of tau proteins. Tau is bound by microtubules in healthy neurons, and is essential to the form and function of the neuronal cytoskeleton. Al is thought to be involved in the formation of PHFs [57, 58]. Al-ATP, like glutamate, stimulates glutamate receptor activity and leads to an increased level of neuronal tau [59]. Stimulation by Al-ATP results in high concentration of tau that is not bound by microtubules, and self-assembly of free tau leads to formation of PHFs. So, it is clear that Al plays a role both in the abnormal

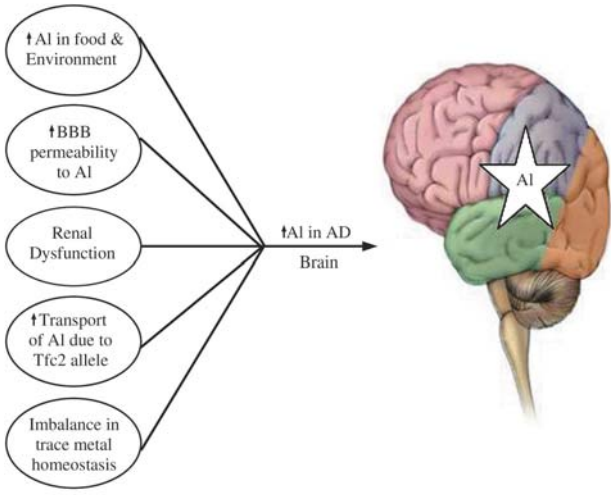

Figure 1. Different pathways attributed to Al accumulation in the brain of AD patients. Al bioavailability through food and environment is moderate. AD patients are known to have altered permeability of the BBB, permitting more Al entry into brain. Further, people suffering from renal dysfunction accumulate Al in their brain due to its insufficient removal from the body. Accumulation of Al is seen more in people having the Tfc2 allele of transferrin. Apart from the above, imbalance in trace metal homeostasis in the brain may lead to Al deposition.

expression of tau and in the intraneuronal deposition in NFTs. In vivo studies carried out by Shin et al. [57] showed that PHF tau co-injected with $AlCl_3$ in rodent brain appears uniquely capable of inducing co-deposits of a number of proteins found in authentic AD SPs and NFTs, namely co-deposits of Aβ, ubiquitin, α1-antichymotrypsin and apolipoprotein E (ApoE). Injections of $AlCl_3$ alone as well as injections of normal adult and fetal CNS tau, several different synthetic peptides, neurofilaments, α1-antichymotrypsin, heparan sulphate proteoglycan or ApoE with and without $AlCl_3$ failed to induce co-deposits of Aβ or alter the immunoreactivity of tau [57]. It has also been suggested that binding of Al to PHF tau induces aggregation and retards its proteolysis. Al concentrations of 15–80 ppm were detected in NFTs [57, 60]. In the case of acute encephalopathy, Al-induced tangles are due to the accumulation of 10-nm neurofilaments in the cell body and processes (axon and dendrites) of neurons [10]. Neurofilaments begin to accumulate a few hours after intracisternal injection of Al [61]. The diffuse distribution of neurofibrillary pathology suggests that nearly all nerve cell populations are prone to the toxic action of Al, the most vulnerable being neurons of the anterior horns of the spinal cord, of the reticular formation and the basal forebrain, the purkinje cell system and the pyramidal neurons of the cerebral cortex. These studies have supported the theory concerning Al as an etiological agent for cerebral changes in AD [62]. Intracisternal administration of Al has been shown to induce a number of other biochemical and histological changes in the rabbit brain. In response to this, intense argyrophilic masses of fibrillar material were observed [19]. It is clear from silver-staining techniques and electron microscopic studies that Al induces normally present protein subunits in neurons to assemble into masses of organized filaments. It has been demonstrated that the process of NFT formation can be partially reversed by initiating intramuscular desferroxamine (a metal chelator) treatment, even after the process of NFT has begun [63]. Al-induced neurofibrillary degeneration in rabbits is accompanied by reduction in serotonin and noradrenaline. In addition up to a 40% reduction in choline acetyl transferase activity in the hippocampus, suggesting that neuronal regions preferentially affected in AD are correspondingly affected in Al-induced neurofibrillary degeneration [64].

Although the neurotoxic effect of Al leading to neurofilamentous accumulation in neurons is fairly well established, the etiological role in initiation and progression to dementing pathology, recapitulating the classical pathology of AD, is doubtful. The presence of Si and Al in the cores of amyloid-rich, argyrophilic plaques has been described with variable consistency [65, 66]. However, the increased content of Al in NFTs has been established [67, 68], proving interest in the possible association of Al toxicity in the evolution of Alzheimer's dementia. The NFTs and neurofilamentous accumulation induced in rabbits by intracisternal administration of $AlCl_3$ may superficially resemble those seen in aged human brain. However, these are relatively less compact, rich in neurofilament protein and made up of 12-nm straight filaments [69], unlike the irreversible nature of NFTs, ultrastructurally made up of PHFs and rich in phosphorylated tau and polymerized ubiquitin. The neuropathology of the often quoted dialysis dementia, with high levels of Al in dialysate, does not replicate Alzheimer's pathology. Although there is evidence for neurotoxicity of Al in humans following chronic ingestion, its causative role for AD or any other specific dementing illness has yet to be established [70].

The accumulation of phosphorylated neurofilaments in motor neurons in amyotropic lateral sclerosis, NFTs in AD and Al-induced encephalopathies reflect an anomaly of neurofilament processing, inefficient degradation by proteases, enhanced and aberrant phosphorylation and protein misfolding. All of these features are also noted in the normal aging brain, without manifestation of dementia. Insidious accumulation of these aberrant misfolded proteins in large quantities interfering with synaptic transmission in critical topographic brain areas probably manifests as motor neuron disease or AD-type dementia. Al and other metals in the environment may well be one of the initiating factors for the formation of intraneuronal filamentous protein tangles in humans, but for unrelentlessly progressive, debilitating neurological disorders, yet unidentified cofactors may be needed. It is also essential

to realize that AD-like illness can be seen in the absence of NFTs and SPs, and centurions with numerous NFTs and SPs may not manifest cognitive deficits characteristic of AD. Al could be one of the 'flagpoles' in the evolution of AD.

Al and Aβ

Aβ, which is the major component of SPs in the brain of AD patients, has an intrinsic tendency to form insoluble aggregates. Aβ is a 39–43-amino acid long peptide derived from a larger transmembrane protein, the amyloid precursor protein (APP). A large number of studies have focused on the structure, aggregational properties and neurotoxicity of Aβ, and their roles in AD [71–73]. Al, a constituent of SPs, influences the aggregation and toxicity of Aβ [74, 75]. Neurofibrillary lesions in experimental Al-induced encephalomyelopathy and AD share immunoreactivity for APP, Aβ, α1-chymotrypsin and ubiquitin-protein conjugates [15]. In addition, an increased burden of amyloid plaques was observed in patients with renal failure, which involves accumulation of Al in the brain [76]. In physiological buffers, Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Na^{+} or K^{+} at 10-mM concentration had no effect on the rate of Aβ aggregation. In sharp contrast, Al, Fe and Zn under the same conditions strongly promoted aggregation (a rate enhancement of 100–1000-fold) [77, 78]. Al, a non-transition metal, is also known to enhance the processing of APP. It has been shown by Clauberg and Joshi [79] that Al accumulated in AD brain accelerates the generation of Aβ due to the faulty proteolysis of normal APP [79]. It has been shown that APP has a domain homologous to bovine pancreatic trypsin inhibitor, and the activity of serine protease inhibitors is inhibited by Al. Thus Al is indirectly involved in activating serine proteases such as α-chymotrypsin, enhancing processing of APP and leading to accumulation of Aβ and plaque formation [79]. Studies carried out by Cherny et al. [80] have shown that metals potentiate Aβ aggregation in vitro and associated neurotoxicity. In their recent experiments on transgenic mice developing Aβ plaques, they have shown that the metal chelator clioquinol can reduce Aβ plaques by solubilizing them.

In response to altered metal ion metabolism, there is a change in the concentration of soluble and deposited Aβ [81]. Aβ is a metalloprotein that can bind transition metal ions such as Zn, Fe and Cu [82]. This in turn promotes the aggregation of Aβ, which deposits as amyloid plaques. The chelating properties of Aβ for Cu, Zn and Fe explain why there is a high concentration of these metals in amyloid plaques [82]. Binding of redox-active Cu or Fe to Aβ produces H_2O_2, which involves the reduction of these metal ions [83–86]. Hence, it is likely that binding of redox metal ions to Aβ is essential for the redox activity and neurotoxicity of the peptide. Therefore, Aβ may not be directly toxic but acts toxically via the generation of H_2O_2, which causes oxidative damage and neuronal dysfunction. In support of these results, it can be argued that amyloid plaques may be formed as a compensatory response to reduce oxidative stress [83]. Hence, Aβ production appears to be a regulatory response that helps the brain to tackle abnormal metabolism of these metal ions. Metals were always thought to play an enhancing role in Aβ toxicity. However, recent studies [87] infer that metals such us Zn^{2+} and Cu^{2+} may hinder the formation of Aβ aggregates, thus playing a protective role against Aβ toxicity. These metals disrupt β-sheet formation and induce the formation of non-fibrillar aggregates, which are considered less toxic.

Al and other metals

Assessment of the progressive accumulation of Al and other metals in the AD brain revealed that Cu, Fe and Zn increase in the early phase of AD, while Al starts increasing significantly in the later phase of AD. Studies by our team showed that there is progressive deposition of Al in AD brain [88]. Al level is expressed in mole percentage (the mole percentage is calculated as [elemental concentration in mole % = elemental concentrations (µmol/ml) × 100/total elemental concentration (µmol/ml) of analyzed elements in each sample] for the analyzed elements, and the relative distribution based on the mole percentage was computed. Mole percentage calculations are essential to understand the relative distribution of each element in relation to other elements in the biological matrix, and this will also help to normalize the data of different samples to arrive at a clear interelement relationships. See [88]). In normal brain, the mole percentage of Al was 2.8%, while in moderate AD it was 4.6% and in severe AD 65.5% in the frontal cortical region. In the hippocampus, however, the mole percentage was 1.6% in control, 8.1% in moderate AD, and 48.4% in severe AD [88]. These data clearly indicate progressive deposition of Al, as shown in figure 2. It was also observed by our team that Al concentration does not vary substantially in AD CSF compared to control in young and aged patients [89]. Roberts et al. [55] reported that Al increases by ~3.2% in AD serum. Comparison of Al levels in serum, CSF (both young and aged) and hippocampus of control and AD samples is shown in figure 3. Moreover, from our previous studies on trace elemental homeostasis, we have hypothesized that irrespective of metals being a primary risk factor or consequence of the disease mechanism, a moderate change in a single metal ion will upset the whole elemental homeostatic pool, resulting in a significant imbalance in elemental levels in the body (serum, CSF and brain). The effect of an increase or decrease of a single metal is not restricted to the initiating metal alone, and such changes will affect the total elemental and

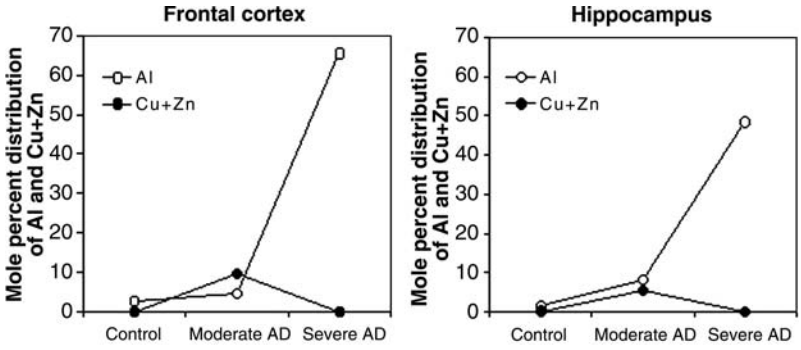

Figure 2. Levels of Al and Cu + Zn during the progression in AD in frontal cortex and hippocampus. Al starts accumulating in moderate AD along with Cu and Zn, while Al is significantly deposited in severe AD. However, Cu and Zn levels were decreased in both frontal cortex and hippocampus [88].

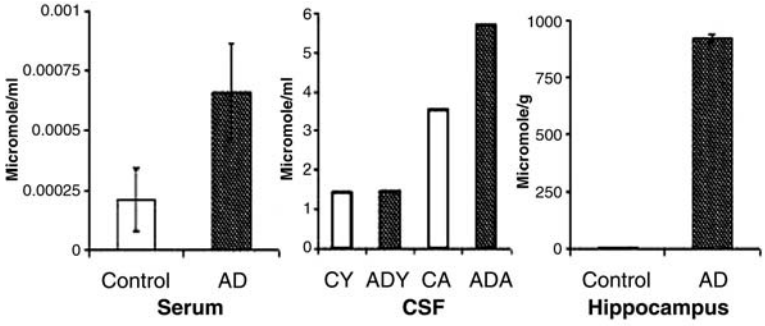

Figure 3. Comparison of levels of Al in serum, CSF (both young and aged) and hippocampus of control and AD samples. CY, control young; ADY, Alzheimer's disease young; CA, control aged; ADA, Alzheimer's disease aged). Serum Al levels in AD and control subjects reveal that Al is increased threefold in AD patients. However, Al concentration does not vary significantly between CY and ADY CSF, but in CA and ADA, the CSF Al level increased significantly. There was a manifold increase in Al concentration in AD hippocampus compared to control [55, 88, 89].

charge distribution pattern in the nervous system. Hence, trace elemental homeostasis plays a crucial role in the normal functioning of the human brain. The rise in total metal concentration in the AD brain is mainly due to the elevation of Al and Fe.

With reference to Al and Fe, an inverse correlation exists between the two metals in a normal individual's brain, while both metals increase simultaneously in the AD brain [88]. However, the differential mechanism of Al and Fe localization in normal and AD remains puzzling, though it has been attributed to a similar charge-to-ionic radius ratio of Al and Fe (Al: 0.16; Fe: 0.169) [88]. We feel that efforts should be geared towards understanding why AD brain cells selectively accumulate both Al and Fe in high concentration as well as the reason for region-specific metal buildup in the AD brain.

Al has also been reported to displace other ions of physiological significance. Al is a small ion with an ionic radius of 54 ppm and can replace divalent metals such as Ca, Mg and Zn, whose ionic radii are 72, 74 and 100 ppm, respectively, and hence is thought to be responsible for executing various toxic effects [90, 91]. This displacement of divalent metals by Al is likely a result of differences in the metal-ligand affinity exchange rates. Al can penetrate into the minor grooves of biomolecules and replace other elements due to its higher metal-ligand affinity compared to other essential metals. An interesting speculation is that Al is able to exchange Ca, Mg and Zn from binding sites necessary for normal molecular genetic functions of transcription factor proteins, thereby playing an ancillary role in further disrupting gene transcription mechanisms [58]. Another mechanism which might explain the neurotoxic actions of Al is its interaction with calmodulin, a Ca^{2+} binding protein [92]. Calmodulin couples with intracellular Ca^{2+} signal, which modulates different proteins and enzymes in a Ca^{2+}-dependent manner [93]. The calmodulin-Ca complex modulates a number of different enzymes and cellular processes. Al inhibits Ca-dependent inactivation of the N-methyl-D-Aspartate (NMDA) receptor channel. The NMDA receptor mediates synaptic transmission and plasticity in the CNS [92].

Al hinders the binding of metal ions like $Mg^{2+}$and Ca^{2+} with proteins, thereby acting as an inhibitor of many metal ion-dependent enzymes, such as hexokinase [94]. On the other hand, it can also interfere with the Kreb's cycle by activating certain enzymes such as α-ketoglu-

tarate dehydrogenase and succinate dehydrogenase, and inhibiting others such as aconitase [95]. Al is also known to inhibit the activity of certain proteases, such as trypsin and chymotrypsin [96]. It also alters the activity of monoamine oxidase type B [97] and acetyl cholinesterase [98]. Al^{3+} binds almost 10^7 times more strongly to ATP than does Mg^{2+} [99] and forms a complex that is more stable than the complex with Mg [99]. Mg^{2+} is associated with phosphate groups, and Al^{3+} can compete with Mg^{2+} for phosphate sites. In the brain, ATP acts on extracellular ionotropic (P2X) and metabotropic (P2Y) receptors to optimize the activities of neurotransmitters, including glutamate, gamma aminobutyric acid (GABA) and acetylcholine [99]. Al, in competition to form Al-ATP instead of Mg-ATP, might act upon muscarinic receptors to potentiate the negative feedback controlling the release of acetylcholine in the synaptic cleft, causing deficits in neurotransmitter stimulation [92].

In the normal brain, Zn is thought to provide highly specialized neuromodulatory, neuroprotective and neurosecretory functions and is intimately involved with structural, regulatory and enzymatic proteins that provide these functions [93]. Al has the potential to interact with many important Zn-containing elements of the brain's gene expression system [58]. The exact role of Zn in AD pathogenesis remains unclear despite various hypotheses which attempt to link Zn and AD. Studies that attempt to quantify cerebral Zn levels in AD produce highly variable results. These differences may be due to the differences in the methodology employed, technical difficulties encountered during tissue processing and small sample size [100]. It has been hypothesized that Zn may be linked to AD pathogenesis by its ability to precipitate Aβ [101 – 104].

Si has been shown to have a considerable effect on Al homeostasis in humans. Si reacts antagonistically to Al. Bellia et al. [105] reported that Si favored Al urinary excretion in renal failure patients. In another study, it has been shown that intake of Si Silicic acid by healthy volunteers resulted in rapid excretion of Al and Si [106]. Carlisle and Curran [107] showed that dietary Si supplements slowed down Al deposition in the aging rat brain. The toxic effect of Al has been shown to be reduced in the presence of silicic acid due the formation of hydroxyaluminosilicates. It has been also found that sodium-orthosilicates could reverse Al^{+3}-induced β-sheet conformation in neurofilament protein (NF-M17) [108]. This has been attributed to the high binding constant of SiO_4^{-4} with Al^{+3}. Based on the contributions, it has been hypothesized that silicates may serve as a possible preventive treatment for individuals showing early symptoms of AD or as a method of retarding the advance of the disease [108]. Thus it seems that metal-metal interrelations play a significant role, but further peer-reviewed data are needed in this area.

Al and oxidative stress

The role of oxidative stress in neuronal degeneration is a widely discussed concept, and the arguments on the role of Al in mediating neuronal oxidative stress may help in understanding the role of Al in AD. Neurons appear to be particularly vulnerable to free radicals for the following reasons: (i) their glutathione content, an important natural antioxidant, is low; (ii) their membranes contain a high proportion of polyunsaturated fatty acids; and (iii) the brain requires substantial quantities of oxygen for its metabolism.

Oxidative stress is one of the critical features in the pathogenesis of AD. Evidence of oxidative damage has been demonstrated in brain tissue from AD patients by Smith et al. [109]. In their subsequent study, it was shown that oxidative damage is linked to the accumulation of redox-active Fe [110]. The role of oxidative stress in AD has been reviewed [111, 112]; the debate, however, is on the initiator of reactive oxygen species (ROS). Furthermore, Al is considered one of the contributing factors to oxidative stress, as it generates ROS. Al as a non-redox active metal has been shown to cause oxidative damage to neurons through Fe [113, 114]. It has been shown that Al stabilizes ferrous (Fe^{2+}) ion by reducing its rate of oxidation. Fe^{2+} is potent in promoting the generation of oxidative species, as it actively catalyzes the Fenton reaction [115]. The Fenton reaction leads to the formation of OH*, OH^- and Fe^{3+} from the non-enzymatic reaction of Fe^{2+} with H_2O_2. Because Al is an activator of superoxide dismutase (SOD) and an inhibitor of catalase, superoxide radical is readily converted to H_2O_2, and the breakdown of H_2O_2 to H_2O and O_2 by catalase is slowed down [88]. Excess accumulation of H_2O_2 further leads to the production of OH* radicals, which in turn damage various proteins, DNA and membrane lipids [116, 117]. In 1985, it was first reported that Al^{3+} ions enhance membrane oxidative damage [113]. At an acidic pH, Al salts accelerate peroxidation of membrane lipids in the presence of Fe^{2+}. Alteration in cell membranes in AD has been reported in several studies [118, 119]. Al has been shown to modify the biophysical properties of membranes, making it certain that Al^{3+} interacts with cell membranes. These are the primary targets of Al^{3+} to cause structural and functional impairments in neurons [118]. Increased platelet membrane fluidity has been reported in certain AD patients. Al treatment of platelet membranes in vitro, caused an increase in the fluidity of membranes similar to that observed in AD platelets [119]. Other cellular models on which experiments have been carried out include neurons and synapses of invertebrates and vertebrates. Disturbances in resting membrane potential, voltage-activated ionic channels, transmitter secretion as well as transmembrane potential differences were reported. It was suggested that these alterations in cell membranes may be due either to direct interaction of Al with membrane proteins, or to induction of

alterations in the lipid matrix. Al appears to bind to the membrane polar heads, resulting in injurious consequences for biological transport processes and cellular metabolism [119]. It was also suggested that Al^{3+} ions produced a rearrangement in the membrane structure that facilitated the oxidative action of Fe [113]. Al^{3+} and Fe^{2+} compete for negatively charged oxygen groups of membranes, and hence the effect of Al mainly depends on the individual concentrations of the two metals [120].

Savory et al. [53] proposed that aging is an important factor in the susceptibility of neurons to oxidative stress and to subsequent apoptosis. Aged rabbits treated with Al-maltolate exhibit intense intraneuronal silver positivity indicative of the formation of oxidative stress. These changes occur in the CA1 region of the hippocampus, as well as in the cerebral cortical areas. Apoptosis was co-localized with oxidative stress. Young animals treated with Al show few of these alterations, while age-matched controls are essentially negative. A time course study reveals that oxidative stress and redox-active Fe accumulation occur in the hippocampus within a period of 3 h and increase in intensity at 72 h. Changes suggestive of apoptosis are even seen by 24 h and are pronounced at 72 h. In aged animals there is an initially intense immunopositivity at 3 h for Bcl-2, with negative staining for Bax. By 72 h, when apoptosis is strongly evident, Bcl-2 is negative and Bax is strongly positive. In contrast to the aged rabbits, young animals treated similarly with Al exhibit much less oxidative stress with no apoptosis and negative Bax staining. These findings strongly support the key role of oxidative damage in the process of neurodegeneration and in the increased vulnerability to Al-induced injury in the aged animals. These findings have important implications in aiding our understanding of the pathogenesis of the neurodegeneration occurring in AD.

ROS have been observed in rat glial and neuronal cells after treatment with Al. The level of ROS generated by glial predominant or neuronal predominant cells was 1.5 or 1.8 times higher than that of each of the untreated control cells. The ROS level in neuronal predominant cells was 2.5 times higher than that of glial predominant cells [121]. In another separate study on rats, Al-induced pro-oxidative effects were demonstrated in liver and brain (cortex, hippocampus and cerebellum) tissues by examining oxidative stress markers – glutathione transferase, reduced glutathione, SOD, glutathione reductase, glutathione peroxidase, thiobarbituric acid reactive substances, as well as protein content [122]. Hence, both in vitro and in vivo studies indicate that Al is a definite promoter of oxidative stress. The complex biology of Al induced oxidative stress is highlighted in figure 4.

Al and cell death

Apoptosis is one of the mechanisms contributing to neuronal loss in AD, as suggested by Cotman et al. [123].

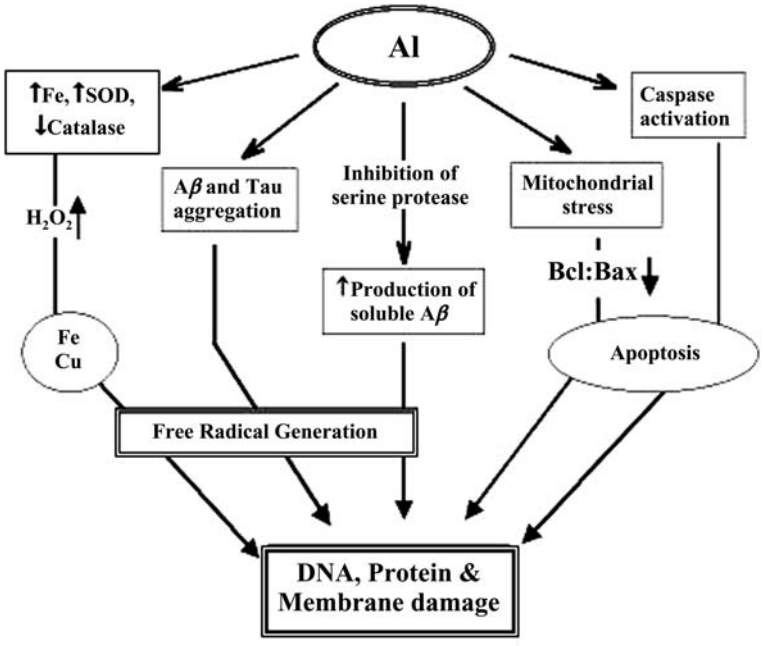

Figure 4. Possible ways of Al toxicity to drive free-radical generation leading to neuronal cell death. Al contributes to oxidative stress through the Fenton reaction, which leads to proteins, DNA and membrane damage. Al also influences the aggregation of Aβ, thus contributing to the toxicity. Further, Al is known to enhance the processing of APP, causing the accumulation of abnormal Aβ. Al contributes to mitochondrial dysfunction, leading to alteration in the Bcl-2:Bax ratio, which favors apoptosis. In addition, Al acts as a stress-inducing agent in the ER by activation of caspases, a step essential for cell death events.

Neurons in susceptible regions (cortex and hippocampus) of the AD brain show evidence of DNA damage, nuclear apoptotic bodies and chromatin condensation.

Ample studies have shown that Al induces cell death stimulus similar to that of AD [53, 124–126]. Al leads to the alteration of the Bcl-2:Bax ratio in the brain (anti-apoptotic:proapoptotic) [125]. This alteration is an important indication in the development of extensive apoptosis. Apoptosis under mitochondrial control has been implicated in the neuronal death process and involves the release of cytochrome c into the cytoplasm and initiation of the apoptosis cascade. However, a growing body of evidence suggests an active role for the endoplasmic reticulum (ER) in regulating apoptosis, either independent of mitochondria, or in concert with mitochondrial initiated pathway. A study on New Zealand White rabbits, an animal system relevant to study of human neurodegenerative diseases in that it reflects many of the histological and biochemical changes associated with AD, has shown that Al induces neuronal apoptosis by its effects on the functioning of both the ER and mitochondria [125]. At the ER level, Al leads to the activation of caspase-12, which in turn can activate the effector caspase-3. Al, which is a stress-inducing agent in ER, has been shown to activate the expression of various genes, such as those coding for the transcription factors gadd 153, important in growth arrest and DNA damage induction, and NF-κB, which initiates apoptosis. Stress in mitochondria leads to opening of the mitochondria permeability transition pore (MTP), release of cytochrome c, activation of caspase-9 and of caspase-3 [125]. Stress in both mitochondria and ER leads to downregulation of the anti-apoptotic protein Bcl-2, increase in the level of the pro-apoptotic Bax, and activation of the effector of apoptosis, caspase-3. Stress in the ER may also lead to perturbation of the Ca^{2+} stores and incorrect folding of proteins, resulting in an increase in cytosolic Ca^{2+} concentration. Subsequent to this increase in cytosolic Ca^{2+} levels, mitochondrial Ca^{2+} also rises. A rise in mitochondrial Ca^{2+} is also observed when Al inhibits Na^{+}/Ca^{2+} exchange by accumulating in neurons following cell depolarization [127]. Since mitochondria have a limited capacity for storing Ca^{2+}, when this capacity is exceeded, the excess Ca^{2+} is released back into the cytosol, a consequence of which is the opening of the MTP and release of cytochrome c – an apoptogenic factor. Cytochrome c complexes to Apaf-1 in cytoplasm and activates the initiator caspase-9, which in turn activates the effector caspase-3. Therefore, these proteins and proteases in the apoptotic pathway hence are directly or indirectly affected by Al [126].

When astrocytes and neurons were incubated with Al, it accumulated both in neurons and astrocytes and caused strong changes in the morphology of astrocytes, including shrinkage of cell bodies and retraction of processes after 8–12 days of exposure. Exposure over 15–18 days reduced astrocyte viability by 50%. The Al-induced degeneration of astrocytes involved DNA fragmentation characteristic of apoptosis, and staining of Al-treated astrocytes with DNA-binding fluorochrome Hoechst 33258 revealed the apoptotic condensation and fragmentation typical of chromatin. Al neurotoxicity occurred in neuroglial cultures containing 10% astrocytes but not in near-pure neuronal cultures containing only 1% astrocytes. Staining of cells co-cultured with Hoechst 33258 showed apoptotic condensation and fragmentation of chromatin in Al-treated astrocytes but not in co-cultured neurons. This study demonstrates that Al can induce the apoptotic degeneration of astrocytes, and that this toxicity is critical in determining neuronal degeneration and death [128]. In analogy to this, Su et al. [129] evidenced apoptosis during neuronal DNA damage as an early event preceding NFT formation in AD. Subsequent to this study, Kitamura et al. [130] provided evidence of alteration in the Bcl-2:Bax ratio, supporting the similar role played by Al in AD.

The multifaceted pattern of Al-induced cell death through Aβ-mediated pathway is highlighted in figure 5.

Al speciation: a fundamental prerequisite for understanding Al biology

Al still remains a puzzling question even after nearly 4 decades of research because of the inherent difficulties in understanding the role of chemical speciation of Al in biological systems. Hence, we need to look deeply into the speciation chemistry of Al. The effects of different inorganic salts of Al in causing neurodegeneration have been studied by a number of groups [131]. All these studies have clearly shown that most of the inorganic Al salts could cause only localized effects at the site of injection. Al is prevented from entering the CNS through the BBB. However, an increased amount of Al has been found in the brain under pathological conditions [131]. Hence, it is important to understand the complex hydrolysis chemistry of Al as a function of pH [90, 132]. The reactions of Al are complicated by the existence of Al as different species in aqueous solution. Thus, it is essential to know the effects of different Al species present under a given set of conditions. The speciation chemistry of Al is especially important in the experimental design of research investigations into Al toxicity. Hardly any attention has been paid to metal speciation while carrying out Al toxicity studies. It is known that different ligands vary in their ability to solubilize and transport Al^{3+} to critical target sites [133]. Hence, some Al complexes are more toxic than others. Al speciation in the stock solutions must be evaluated, since it hydrolyzes readily and at pH 7.0 there is a strong tendency for precipitation of $Al(OH)_3$, which makes the preparation of Al stock solu-

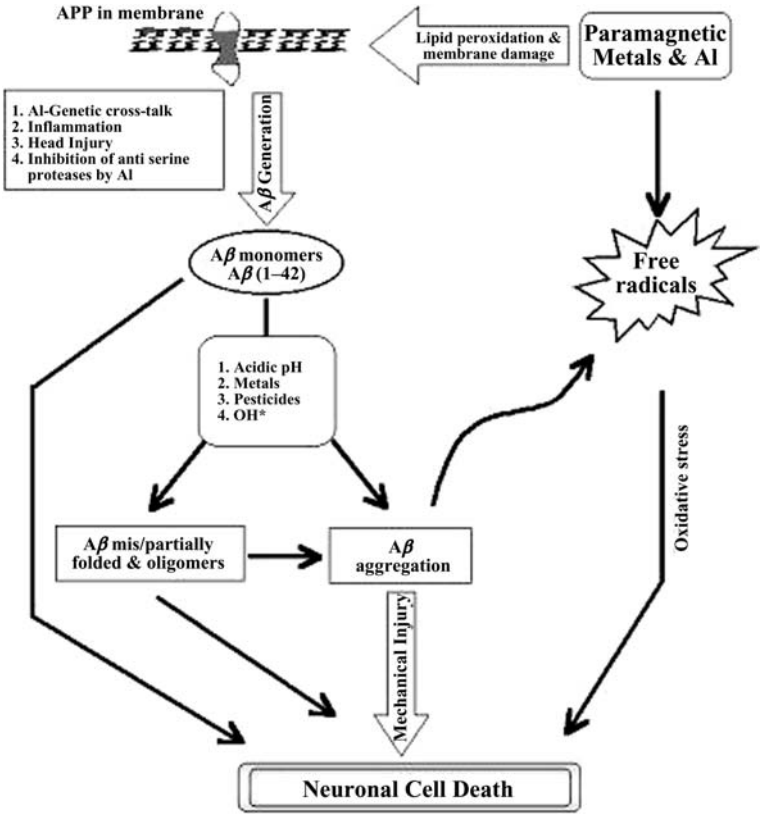

Figure 5. Complexity of Al involvement, and various targets of its action in causing neuronal cell death. Al may directly affect processing of APP by inhibiting antiserine proteases. Accumulation of paramagnetic metals is also known to cause Aβ generation. This phenomenon leads to accumulation of excess and abnormal Aβ, which may mis/partially fold, forming oligomers that exhibit toxicity. Some of these may aggregate due to various factors, finally leading to mechanical and oxidative damage, and cell death.

tions difficult. Al is a strong hydrolyzing element and is generally insoluble at neutral pH. Its solubility is enhanced under acidic or alkaline conditions, and in the presence of complexing agents. In aqueous solution at pH < 5.0, Al exists as an octahedral hexahydrate, $[Al(H_2O)_6]^{3+}$, usually abbreviated as Al^{3+}. Under acidic conditions $[Al(H_2O)_6]^{3+}$ undergoes successive deprotonations to yield different species such as $[Al(OH)]^{2+}$, $[Al(OH)_2]^{+}$ and $Al(OH)_3$. Neutral solutions produce $Al(OH)_3$ precipitate, which redissolves because of the formation of tetrahedral aluminate, $[Al(OH)_4]^{-}$, the primary soluble Al(III) species at a biological pH > 6.2. The overall deprotonation reactions will take place according to the following equation:

$$[Al(H_2O)_6]^{3+} \Leftrightarrow [Al(H_2O)_5(OH)]^{2+} \Leftrightarrow [Al(H_2O)_4(OH)_2]^{+} \Leftrightarrow Al(OH)_3 \Leftrightarrow [Al(OH)_4]^{-}$$

Hydrolysis reactions of Al have to be taken into consideration while computing the soluble Al concentration of the solution. For example, when Al inorganic salts such as chloride, sulphate, hydroxide or perchlorite are dissolved in water at a calculated concentration of 10 mM, the exact Al concentration is about 50 µM. The use of Al-lactate or Al-aspartate, however, increases the soluble Al concentration to ~55–330 µM, and the use of Al-maltolate or gluconate increases the soluble Al concentration to 4–6 mM. Al-maltolate has been particularly suitable for toxicological studies because of its neutral charge, high solubility and hydrolytic stability over a wide range of pH, rendering it the most efficient compound for biological studies [131]. A majority of studies carried out using different Al compounds at their autogenous pH with enzymatic systems report a general inhibitory effect. However, reinvestigation of the effect of Al on acetylcholinesterase, hexokinase and Na^{+}/K^{+}/ATPase using the protocol reported by Zatta and co-workers [98] showed a strong activation of all three enzymes. Since the Al-speciation chemistry is pH dependent, it is important to know the exact ionic species of Al at physiological pH. Al^{3+} has very minimal existence at neutral pH, and the other dominant species at this pH are ligand dependent as studied by different Al complexes used for the toxicity study. Hence, reexamination of the exact ionic species of Al in physiological conditions may be required to draw any relevance to its toxicity, and more interest has to be invested in this direction. We feel this can answer the inconsistency in reproducing the manifestation of Al in Al-treated animal models. The methodology followed for the estimation of Al has also been a matter of

great concern, and no two teams follow the same protocol. We strongly feel that a definite strategy for the estimation of Al should be followed by different groups, which would minimize the confusing states of Al levels.

Furthermore, we do not know what happens to Al speciation once it passes through the body, through different biological system with pH and chemical interactions in vivo. This, too, is an area for intensive investigation. Therefore, in addition to the solution chemistry of Al, we need to understand the biochemistry of Al in vivo. There is a great need to understand the complex biology of Al.

Can the sole involvement of Al in AD be accepted?

Although there is abundant evidence that implicates Al in the progression of events leading to AD, some of the evidence available remains controversial. A number of studies found elevated levels of Al in the brain of AD patients [42, 134], whereas a number of other groups have failed to find any difference between the levels of Al in AD brain compared with age-matched controls [45, 135]. This inconsistency in the results has been used to argue against the pathogenic involvement of Al in AD [136]. These discrepancies are almost certainly due to the use of bulk tissue for analysis. This problem has been overcome by resorting to the microanalysis techniques of EPXMA, laser microprobe mass spectrometry and microdetection, which have demonstrated Al deposits to be located in tangle-bearing neurons [100].

A major argument against Al causing neurodegeneration in humans has been the failure to find NFTs in the brain of patients with dialysis encephalopathy resulting from hyperaluminemia. However, NFTs have been observed in four such patients [100]. Studies carried out in patients with chronic renal failure using imaging secondary ion mass spectrometry (SIMS) have revealed extensive intraneuronal accumulation in cases which fall within the normal range of Al content as judged by atomic absorption spectroscopy (AAS) [100]. Quantitative investigations using SIMS have indicated that these intracellular concentrations can exceed 500 ppm of Al, and it is thus relevant to consider whether pathological changes are associated with intraneuronal accumulation. Studies in patients with dialysis encephalopathy have shown the presence of NFTs [62, 129, 137] and of spongiform changes in the neuropil [43]. Nuclear immunostaining with an antibody to the N-terminal region of the APP has been shown in patients with chronic renal failure. Five of these patients had amorphous SP in the frontal, temporal, parietal and occipital cortices. The plaques were identical to the immature plaques seen in AD and Down's syndrome individuals. In dialysis patients intracellular staining predominantly occurred in the pyramidal neurons, which selectively accumulate Al [138].

Despite the controversies over the Al hypothesis, researchers have not been able to disprove the involvement of Al in AD. This is primarily because there are strong arguments in support of Al as a factor or cofactor in AD as follows:

1) Al is highly neurotoxic at picomolar concentrations unlike Cu, Zn and Fe, which are biologically very essential (see commentary by Savory et al. [139]).

2) It has been observed that in inherited diseases attributed to elevated Cu (Wilson's disease) or Fe (Hallervorden-Spartz disease) [76], there is no evidence of increased formation of Aβ plaques. However, an increased burden of amyloid plaques was observed in human disorders that involve the accumulation of Al in the brain, such as long-term hemodialysis where Al-contaminated water was used to prepare dialysate solutions, hence supporting our Al hypothesis [62].

3) The intracisternal injection of Al maltolate and $AlCl_3$ into rabbits induced the formation of intensely argyrophilic intraneuronal neurafilamentous aggregates which are present in the NFTs of AD [15, 18–19]. This also prevents us from discarding Al as a possible cause because no such similar findings are observed with Cu, Fe and Zn. In support of this, treatment with an Al-chelating agent will cause the bulk of these intraneuronal protein accumulations to disappear [140].

4) As already reviewed, Al induces stress in the ER of rabbit hippocampus, manifesting in a decrease in antiapoptotic and an increase in proapoptotic proteins, caspase -3 activation, and finally apoptosis [125]. No such similar cascade of events leading to apoptosis is seen with regard to other metals.

5) Finally, Al might exert its toxic effects by disrupting mechanisms which control iron homeostasis. There is increased concentration of Fe in proportion to the Al loading, thereby possibly indicating a similar pathway of uptake into the cell. Animal experiments have established that the presence of Al in the brain will increase Fe content, and cell cultures incubated with Al showed a higher number of surface Tf receptors [141]. These results indicate that brain regions of AD patients may not necessarily show significant increase in Al content; hence, just a small increase in Al in specific brain regions becomes a stimulus for alterations in brain Fe homeostasis, which can participate in events contributing to AD.

Cross- talk between genetic susceptibility and Al in AD

It is now considered that AD is possibly the result of a multifactorial process involving genetic and environmental components [142–144]. It has long been thought that a genetic susceptibility to Al loading and clearance may increase one's risk not only to AD but also to devel-

opmental problems associated with Al loading in infants and children, even those with normal renal function [145]. There are few studies in the literature addressing this issue. Recent progress in genetic analysis has made it possible to identify three genes that cause familial AD, which are the genes for APP on chromosome 21 [146, 147], presenilin 1 (PS-1) on chromosome 14 [148, 149] and presenilin 2 (PS-2) on chromosome 1 [150, 151]. In addition, the ε4 allele of the ApoE gene present on chromosome 19 is found to be an important risk factor for senile [152, 153] and presenile [154] AD. The genetic components account for only a part of AD cases [155], suggesting that interaction of environmental risk factors and genes could be a possible hallmark in the etiology of AD. Gauthier et al. [156] reported a significant association between water Al exposure and the development of AD, if the presence of the ApoE4 allele is considered. Recently a genetic variant of transferrin, namely TfC2, was identified which may be involved in aberrant transport of Al. This variant occurs in high frequency in patients with AD compared with non-demented controls [157–160]. It has also been shown that early onset of AD is observed in patients with both ApoE4 and TfC2 alleles [161]. Further, and that Al uptake occurs more in neurons of cortex and hippocampus, which are rich in Tf receptors [31]. Further work in this direction is needed to validate the Al-ApoE4-TfC2 interrelationships. Fosmire et al. [162] suggested that there are genetic differences in the permeability of the BBB and lent support to the hypothesis that variability in Al loading and toxicity may, in part, be genetically determined. They examined this hypothesis using five strains of mice tested for genetic differences in exposure to moderate levels of Al. The authors concluded that such differences do exist in response to a dietary aluminum challenges resulting in permeability of the BBB and subsequent differential loading of aluminum. Matsuzaki et al. [163], reported that chronic Al exposure has accelerated PS2V (the aberrant splicing isoform generated by skipping exon 5 of the PS-2 gene) production through hypoxia mechanism. HMGA1a, a mediator of PS2V production was shown to be induced by Al as well as by hypoxia. These studies provide an insight into the cross-talk between Al, ApoE4, PS2V and TfC2, showing that Al has a potential role in familial AD as well as an independent part to play in sporadic AD.

Conclusion

Based on the above data and arguments, the neurotoxic effects of Al are beyond any doubt, and Al as a factor in AD cannot be discarded. However, whether Al is a sole factor in AD and whether it is a factor in all AD cases is still debatable. This is mainly because AD is multifactorial disease, and to date the specific etiology(s) of AD are unknown. Thus, the Al hypothesis, along with other hypotheses, continues to survive. Since the accumulation of Al may occur in later stages of AD, we propose that Al may not initiate the AD disease process, but suspect that at the very least it exacerbates the neurodegenerative process. Moreover, the question of why Al accumulates in some, but not all, degenerating brain [Traub R. D. et al. (1981) Neurology **31:** 986–990]. Al can be thought of as one of the etiological factors along with other factors combined with a differential genetic susceptibility that may be crucial to the pathology of AD. Future efforts should systematically track the changes in Al and other transition elements during the progression of AD in order to better understand its involvement in the disease process.

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