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Review

Chronic Aluminum Intake Causes Alzheimer's Disease: Applying Sir Austin Bradford Hill's Causality Criteria

J.R. Walton*
Faculty of Medicine, University of New South Wales, St George Hospital, Sydney, Australia

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Abstract. Industrialized societies produce many convenience foods with aluminum additives that enhance various food properties and use alum (aluminum sulfate or aluminum potassium sulfate) in water treatment to enable delivery of large volumes of drinking water to millions of urban consumers. The present causality analysis evaluates the extent to which the routine, life-long intake, and metabolism of aluminum compounds can account for Alzheimer's disease (AD), using Austin Bradford Hill's nine epidemiological and experimental causality criteria, including strength of the relationship, consistency, specificity, temporality, dose-dependent response, biological rationale, coherence with existing knowledge, experimental evidence, and analogy. Mechanisms that underlie the risk of low concentrations of aluminum relate to (1) aluminum's absorption rates, allowing the impression that aluminum is safe to ingest and as an additive in food and drinking water treatment, (2) aluminum's slow progressive uptake into the brain over a long prodromal phase, and (3) aluminum's similarity to iron, in terms of ionic size, allows aluminum to use iron-evolved mechanisms to enter the highly-active, iron-dependent cells responsible for memory processing. Aluminum particularly accumulates in these iron-dependent cells to toxic levels, dysregulating iron homeostasis and causing microtubule depletion, eventually producing changes that result in disconnection of neuronal afferents and efferents, loss of function and regional atrophy consistent with MRI findings in AD brains. AD is a human form of chronic aluminum neurotoxicity. The causality analysis demonstrates that chronic aluminum intake causes AD.

Keywords: Aluminum, Alzheimer's disease, amyloidogenesis, animal disease models, causality, disconnection, entorhinal cortex, microtubules, neurofibrillary tangles, transferrin receptors

INTRODUCTION

Alzheimer's disease (AD) begins with a long clinically-silent period [1] that leads to mild cognitive impairment that subsequently progresses to irreversible and incapacitating memory loss, accompanied by additional cognitive and behavioral impairments including progressive loss of self-help skills. These features combine to make AD one of the most dreaded and tragic medical conditions of our time. The number of people affected by dementia is currently estimated at 36 million worldwide [2]. Since 85% of dementia cases meet CERAD (Consortium to Establish a Registry for Alzheimer's Disease) criteria for "definite AD" [3], the current worldwide estimate of AD prevalence should be approaching 30 million cases or about 0.4% of the world population. The prevalence of this disease suggests a pervasive yet, possibly in the end, simple cause. The social, emotional, and financial burdens that AD imposes on society oblige us to identify the cause of AD and take action to the fullest extent possible.

The present causality analysis reviews evidence relevant to the hypothesis that AD is a human form of chronic aluminum neurotoxicity; in particular,

*Correspondence to: Dr. J.R. Walton, Faculty of Medicine, University of New South Wales, St George Hospital, Sydney, Australia. E-mail: j.walton@unsw.edu.au.

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focusing on chronic aluminum intake. Oral uptake is the main route by which most humans are exposed to aluminum additives [4] in the forms of aluminum chloride, aluminum citrate, aluminum maltolate and other aluminum-food acid complexes, aluminum phosphate, aluminum silicate, aluminum sulfate and other aluminum species. Some over-the-counter pharmaceuticals and other sources of aluminum with different routes of aluminum intake also contribute to the human body's aluminum burden. Aluminum is absorbed through the skin from aluminum-based topical applications (including sunscreens and deodorants), from injected vaccines that contain adjuvant aluminum, and from certain medical treatments and surgical products.

The properties of aluminum compounds are multifarious and versatile, allowing them to fulfil many functions in foods and beverages. Aluminum compounds, hitherto generally regarded as safe, are included as additives in processed foods and water for many reasons [5, 6]. Aluminum-mordanted dye lakes color snacks and desserts such as corn chips, ice cream, cakes, candies, and jams as well as coatings for vitamins, pharmaceutical tablets, and medicinal capsules. Aluminum is used to prevent caking (salt, cocoa powders, milk powders), for emulsifying and improving the meltability of cheeses, as a rising agent (breads, cakes, other baked goods), to thicken gravies and sauces, as meat-binders (sausages and luncheon meats), for pickling vegetables and candying fruits, as carriers, dough strengtheners, stabilizing agents, buffers, neutralizing agents, texturizers, and curing agents. Some food items have very high aluminum content. For example, one slice of processed cheese (particularly the pre-sliced, individually wrapped type) is allowed by US and Canadian food regulators to contain as much as 50 mg aluminum [7]. Alum is widely used to clarify public drinking water supplies [4, 5]. At the time of writing, new aluminum-containing products intended for human ingestion routinely enter the marketplace.

Humans are estimated to consume between 2 and 14 mg aluminum per day, depending on their age and gender, according to an FDA study [8]. Greger [5] estimates that Americans consume from 1 to 10 mg/day from fresh food (fruits, vegetables, unprocessed meat and fish). In addition, 50% of Americans consume up to 24 mg aluminum in the form of aluminum additives each day; 45% take in between 24 and 95 mg aluminum and about 5% ingest more than 95 mg aluminum each day. Greger's figures [5] are probably the most accurate of available estimates because only those figures take account of aluminum amounts that food manufacturers report to have included in their products [9].

In 2007, the Joint FAO/WHO Expert Committee on Food Additives (Food and Agriculture Organization (of the United Nations)/World Health Organization) reduced the provisional tolerable weekly intake for aluminum in humans from 7 mg to 1 mg Al/kg bodyweight (bw) [10]. Many humans routinely exceed this lower amount. Paradoxically, the US FDA continues to allow aluminum salts to be ranked as Generally Recognized as Safe (GRAS). This in turn allows increasing numbers of food manufacturers to include the aluminum additives in their products while exempting the need for listing aluminum quantities (in mg amounts) on food labels. In many countries, aluminum additives are indicated on food product labels only in the form of coded numbers [6]. Currently, it is difficult for any consumer to monitor the extent of their aluminum exposure.

Aluminum is the third most abundant element in the earth's crust. Life evolved at a time when most aluminum was locked up in rocks and clays. Animals developed a mucus lining of their gastrointestinal tract, and plants mucins at their roots, that trapped the relatively small amounts of aluminum and other toxins fortuitously ingested. Thus, the long-held assumption was that ingested aluminum is not absorbed, or, any small amount of aluminum absorbed would be removed by the kidneys and precluded from uptake into the brain [11].

Mankind has discovered how to refine alumina extracted from the earth to form aluminum oxide from which many aluminum compounds have been synthesized. Numerous uses have been found for aluminum compounds and their versatile properties in a wide array of products, including many intended for ingestion. While most ingested aluminum becomes trapped in the mucus lining of the intestinal tract, and is excreted from the body along with undigested food [12, 13], a small amount of aluminum is absorbed from each intake. Some aluminum species (e.g., aluminum sulfate) dissociate more readily and are more absorbable than others (e.g., aluminum oxide). A fraction of the absorbed aluminum is taken up into the brain, even from the minute amounts of aluminum contained in alum-treated drinking water [14–16]. Importantly, more aluminum enters the brain than can exit, resulting in a net increase in the brain's aluminum content that correlates with increasing age ($r = 0.226$, $p < 0.01$) (Fig. 1) [17], even in brains of non-demented controls [17–20].

Aluminum is formally classified as a definite neurotoxicant [21]. Aluminum particularly accumulates in the large highly active neurons of brain regions most prone to damage in AD. This warrants a brief consideration as to how the aluminum ion (Al^{3+})

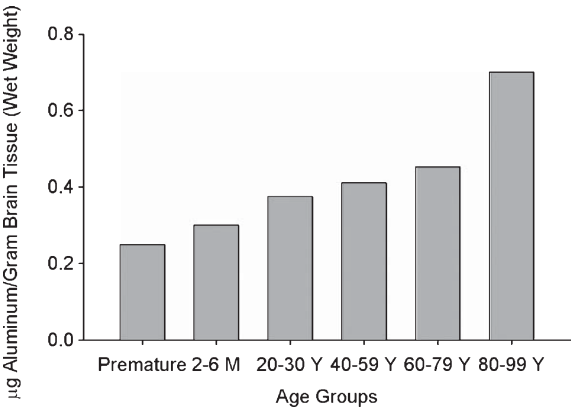

Fig. 1. Aluminum levels progressively accumulate in the brain with increasing age (20 year increments). Sourced from [17] with permission from Elsevier.

behaves in biological tissues. Size similarity is more important than charge similarity for metal ion substitutions [22]. Al^{3+} is a small ion with a high fixed charge. The effective ionic radii for Al^{3+}, ferric iron (Fe^{3+}), and magnesium (Mg^{2+}) are 0.54, 0.65, and 0.72 angstroms, respectively, for their favored six-fold coordination number. The physical properties of Al^{3+} allow its substitution in enzymes and structural proteins for the essential metals, magnesium (Mg^{2+}) and ferric iron (Fe^{3+}) [22]. Al^{3+} substitution for Mg^{2+} in ATPases and other of the 300 Mg^{2+}-dependent proteins alters their activity [23–25].

The similarity between the ionic radii of Al^{3+} and Fe^{3+} can account for Al^{3+} binding to transferrin, an iron transport protein that ferries 80–90% of plasma aluminum throughout the circulatory system. The remainder binds to plasma albumin and low molecular weight species such as citrate [26]. Protein-bound Al^{3+} is excluded from filtration into the urine and transferrin facilitates aluminum transport across the blood-brain barrier and into cells bearing transferrin receptors on their surface [27]. Al^{3+} dysregulates iron metabolism in cells [28, 29] of AD-vulnerable brain regions and also disrupts intracellular magnesium metabolism, as mentioned. Al^{3+} also disturbs calcium (Ca^{2+}) metabolism by interfering with Ca^{2+} signaling pathways, blocking Ca^{2+} channels and competing with Ca^{2+} for phosphates and other small ligands [30]. The near maximal charge:size ratio of Al^{3+} causes Al^{3+} to dissociate from cell ligands 10^4 times more slowly than Mg^{2+} and 10^8 times more slowly than Ca^{2+} [22]. Aluminum's slow dissociation rates rule out Al^{3+} as an effective participant in normal cellular functions as they depend on rapid association and dissociation reactions.

Moreover, Al^{3+} is a pro-oxidant, producing oxidative damage in its own right and synergistically with iron. Al^{3+} binds to the superoxide ion, forming aluminum superoxide, a species more reactive than the superoxide radical [31]. Al^{3+} stabilizes ferrous ion (Fe^{2+}), preventing its oxidation to Fe^{3+} [32]. Fe^{2+} drives the highly toxic Fenton reaction in cells. This synergism between Al^{3+} and Fe^{2+} results in significantly elevated peroxide levels in cells, peroxidative damage, and oxidative stress [31].

Other properties of aluminum are its interference with brain electrophysiology [33] and its ability to catalyze polymerization reactions [34]. Ziegler shared a Nobel Prize in Chemistry in 1963 for discovering that aluminum alkyl catalyzes polymerization in polyethylene production, without the need for high pressure and high temperature as previously required [34]. Moreover, the resulting polyethylene polymer consists of straight chain (unbranched) highly-ordered molecules unlike previously synthesized polymers. Whether aluminum also catalyzes polymerization in biological systems remains to be examined. However, in AD, intracellular aluminum associates with tau and amyloid-β (Aβ) peptides that polymerize to form highly-ordered straight chain paired helical filaments (PHFs) and Aβ filaments, respectively. The role of aluminum as a catalyst in pathobiological polymerization reactions is an interesting research topic in itself.

Aluminum has caused aluminum encephalopathy (dialysis dementia) in renal failure patients [35] and is associated with several other dementias that develop in humans with certain occupations or with particular ethno-geographic backgrounds [36, 37]. However, this paper focuses on AD, a slowly-developing dementia that greatly increased in industrialized societies, particularly after World War II in the mid-20th century.

The aluminum/AD relationship is examined in the light of the epidemiological and experimental criteria for causality set out by Sir Austin Bradford Hill [38], and takes into account opinions expressed by Robert Van Reekum et al. in regard to the application of Bradford Hill's criteria for causation to neuropsychiatric conditions, including AD [39]. Briefly stated, these criteria for causality are (1) strength, (2) consistency, (3) specificity, (4) temporality, (5) biological gradient, (6) biological plausibility, (7) coherence, (8) experimental evidence, and (9) analogy. Studies based on large numbers of identical twin pairs have consistently determined that AD has mixed causality; that is, AD is caused by a combination of environmental and genetic susceptibility components [40–44].

The resulting analysis indicates that chronic aluminum intake is the environmental cause not only of AD but also the trigger for the AD hallmarks assumed by many to cause AD.

CRITERION I: STRENGTH OF THE ASSOCIATION

Population and experimental studies

Bradford Hill writes, "First upon my list I would put the strength of the association". "We must not be too ready to dismiss a cause-and-effect hypothesis merely on the grounds that the observed association appears to be slight. There are many occasions in medicine when this is in truth so." Earlier he had given the following example. "The death rate from coronary thrombosis in smokers is no more than twice, possibly less, the death rate in non-smokers" [38].

Van Reekum adds, "Clearly if condition A causes outcome B, then it must be that A and B can be demonstrably associated with each other. The association has to be strong enough to be judged clinically significant by the reader of the argument. This is a necessary, but not sufficient, criterion in establishing an argument of causation" [39]. That is, where a group of individuals have had considerable exposure to a putative cause (in this case, chronic aluminum intake), producing a significant clinical outcome (AD) to a greater extent than in a minimally-exposed group of individuals, then the cause (chronic aluminum intake) and effect (AD) can be shown to be associated with each other.

AD prevalence and incidence rates are higher in populations exposed to high aluminum levels than in populations exposed to low aluminum levels

AD has been described as a disease of developed industrialized societies [45]. This concept, that developing regions have a lower prevalence of dementia, has more recently been reinforced by the consensus judgment of a Delphi consensus panel [46]. Industrialization involves a population shift from farming communities to cities, mass production of food and water, and an increasing population density prone to the spread of disease and the subject of mass public health policy in the form of disease control, vaccinations, and other measures. Industrialized societies are the geographic regions with greatest access to sources of aluminum compounds in processed foods, public drinking water supplies, pharmaceuticals, topical applications, and other treatments. With globalization, AD, processed foods, and alum-treatment of drinking water supplies are becoming ubiquitous. Human populations without significant exposure to bioavailable aluminum are, by now, difficult to locate. Human populations in industrialized societies are all exposed to significant sources of dietary aluminum.

Pockets remain in developing regions, including parts of Africa and India, where people have had less exposure to aluminum in their food and/or drinking water than in contemporary westernized societies. The inhabitants of village societies in these regions mainly rely on wells and streams for their drinking water and on market gardens for their vegetables.

AD in Nigeria

In the 1980s, AD was virtually unknown amongst people living in Nigeria. Community-based door-to-door surveys were carried out to determine the prevalence of neurological disorders, including one in an urban population of 9,000 people [47]. This survey was based on a sample of 930 of the 9,000 residents living in Idikan, a ward of Ibadan City, including 295 subjects aged 65 years or older. All subjects were assessed for dementia with a modified Mini-Mental State Examination (MMSE) administered by trained medical students. The subjects showed no evidence of dementia as defined by the DSM-IIIR (Diagnostic & Statistical Manual of Mental Disorders – 3rd Edition Revised). "Benign forgetfulness" was reported for 34 older subjects classified as having age-dependent cognitive impairment that was insufficient to interfere with their day-to-day activities, such as problem-solving and use of money. This study concluded that none of the people interviewed exhibited more than "questionable dementia" on the severity score [47, 48].

An autopsy study was undertaken on brains from consecutive deceased non-demented Nigerians older than 40 years who died in the University College Hospital of Ibadan. None of the 198 brains examined, including 45 brains from humans over the age of 65 years, satisfied the criteria for AD in relation to senile plaques, neurofibrillary tangles (NFTs), and granulovacuolar degeneration [49], the three main neuropathological hallmarks of AD.

Immunocytochemical examinations of tissue sections of entorhinal cortex, hippocampus, inferior, middle, superior frontal gyrus, and inferior parietal gyrus obtained from necropsied brains of 111 non-demented Nigerians, from the University College Hospital in Ibadan, were also carried out in Australia [50]. This study confirmed that Aβ deposition was significantly less in Nigerian brain tissues than in brain

tissue from an equivalent non-demented, age-matched Australian population in which 11% of cases displayed high plaque loads, showing grade 3+ immunostaining indicative of heavy Aβ deposition [51]. The findings indicate that pre-clinical evidence of AD was still low in Nigerians in the early 1990s, even in an urban region.

A comparative study of AD prevalence, reported in 1995 [52], was also performed in two groups with similar ethnic backgrounds residing in very different environments: 2,494 Yoruba living in Ibadan, Nigeria and 2,212 Afro-Americans in Indianapolis, USA. The latter group included 106 people with AD living in nursing homes [52]. One group of investigators conducted both arms of the study, using the same criteria to determine age-adjusted prevalence rates for AD and dementia in the two population groups. The age-adjusted prevalence rates were significantly lower at 1.41% for AD and 2.29% for dementia in the Ibadan sample whereas the Afro-American sample living in the community and nursing homes of Indianapolis had prevalence rates of 6.24% for AD and 8.24% for dementia.

In 2001, the same group of researchers reported on a 5-year follow-up study that compared annual incidence rates for AD and dementia in non-demented community-dwelling Yorubas ($n = 2459$) of Ibadan and Afro-Americans of Indianapolis ($n = 2,147$) [53]. They reported that the annual incidence rates were 1.15% (95% CI = 0.96%–1.35%) for AD and 1.35% (95% CI = 1.13%–1.56%) for dementia in the Ibadan Yoruba ($n = 2,459$), which were significantly lower than the annual incidence rates of 2.52% (95% CI = 1.40%–3.64%) for AD and 3.52% (95% CI = 2.11%–4.38%) for dementia in the Afro-American sample ($n = 2,147$), despite their shared genetic backgrounds. The authors stated that methodological issues were unlikely to have contributed to the observed differences because the same protocols had been applied to both groups [53].

As of 2000, approximately 1/3 of Ibadan City was already supplied by alum-treated drinking water and, due to the city's modernization, processed food products with aluminum additives were becoming increasingly available, but most Yoruba preferred their traditional meals (Ogunniyi, personal communication, 2000).

AD in rural India

Similarly, a comparison was made of AD incidence rates of persons living in Ballabgarh, India with a reference US population in the Monongahela Valley of Pennsylvania. This study showed an overall incidence rate of 0.47/100 person-years for the group in India and 1.75/100 person-years for the Pennsylvanian group [54].

These observations support Henderson's view [45] and the conclusion from twin studies that AD has a significant environmental component [40–44]. However, these studies leave the environmental component of AD undefined. If AD were present to the same extent in developing societies as in developed societies, or if chronic ingestion of aluminum-containing foods and water were as widespread in developing countries as in developed countries, the likelihood that aluminum is the environmental cause could be ruled out. Instead, AD rates tend to correlate with aluminum usage. The hypothesized cause and effect coincide in their distribution.

Increased risk for AD in humans that routinely consume processed foods with aluminum additives than those that prefer a fresh food diet

One preliminary epidemiological study has examined the relationship between the risk for AD and ingestion of processed foods that contain aluminum additives [55]. This retrospective case-control study found that the crude odds ratio for AD in humans whose dietary history indicates they routinely consumed foods high in aluminum additives was 2.0 and the odds ratio for AD increased to 8.6 when adjusted for covariates ($p = 0.19$), compared to those who preferred a fresh food diet. This small preliminary study should be repeated with a large population base.

AD risk is higher in human populations living in regions that supply water with aluminum levels above 100 μg/l than in regions with lower water aluminum levels

Many epidemiological studies have been carried out to investigate a possible link between the risk for AD and aluminum in drinking water supplies [56–73], with some more rigorously performed than others. Most of these studies have shown a positive relationship between aluminum exposure from drinking water and AD. Eight of them also measured silica levels in the drinking water in view of evidence that oligomeric silica reduces aluminum absorption and bioavailability by reacting with the aluminum to form aluminosilicates in the gastrointestinal tract [74].

A meta-analysis [75] which integrated results from all aluminum in drinking water/AD studies performed prior to 2001 reveals a significant association

between drinking water aluminum levels at or exceeding 100 μg/l and the risk for developing AD [76]. These findings are intriguing, considering that drinking water contains a relatively small percentage of the total amount of aluminum that humans ingest. They support the suggestion that aluminum dissolved in water is more easily absorbed than aluminum contained in food [57, 77, 78]. Aluminum absorption studies have provided evidence that both support [77, 78] and counter [79] this suggestion. A logical explanation may be that water drunk on an empty stomach has a lower pH than water in a stomach containing food which tends to increase the pH. Aluminum is more soluble at lower pH values, increasing aluminum's absorbability [22].

Ganrot regards aluminum epidemiological studies as almost impossible to perform because all humans (in developed countries) are abundantly exposed to potentially bioavailable aluminum from diverse sources [80]. The examples which follow illustrate complex epidemiological studies that have controlled for important potential confounding factors, to obtain meaningful data from defined geographic populations, incorporating, where appropriate and possible, equivalent follow-up data. The most extensively controlled retrospective study of AD and drinking water aluminum had a case-control experimental design based on autopsy-verified brains from non-demented controls and AD subjects donated to the Canadian Brain Bank between 1981 and 1991 [68]. These brains were from subjects who had lived in 162 geographical localities of Ontario with recorded aluminum levels for their drinking water supplies. Histopathological examination confirmed that 287 subjects were AD cases, 125 were controls without neuropathology, and 167 were controls with non-AD neuropathology. All neuropathological and clinical reports were reviewed by the same neurologist to ensure universal application of diagnostic criteria.

Next-of-kin telephone interviews (blind to the purpose) established the subjects' places of residence during the decade prior to AD diagnosis as well as their educational, medical, and occupational histories. These data enabled estimates of the aluminum levels in the subjects' drinking water supplies over the entire decade preceding overt AD. Such information is important for internal validity in this type of study because many people with AD are relocated to nursing homes as the disease progresses. Consequently, their final address may differ from the community where they had prolonged exposure to a particular water supply.

The results from this aluminum in drinking water study showed that the estimated relative risk for subjects who drank water containing an aluminum level at or higher than 100 μg/l, for at least 10 years, was 2.6 (95% CI = 1.2–5.7) times greater than for those who drank water containing less than 100 μg aluminum/l [68]. A dose-dependent response was shown for water containing aluminum levels above 100 μg aluminum/l. As might be expected, by taking into account the long-term residential history and histopathological verification of the AD and control categories, the study produced a higher relative risk for AD than retrospective studies that were less controlled [68].

A large prospective study of 3,777 subjects aged 65 years or older (the PAQUID cohort), was carried out to determine AD incidence in people living in regions of France with high and low aluminum levels in their drinking water [63]. Mean exposures to aluminum and silica from bottled waters and public drinking water supplies were estimated for each geographical region. In total, 182 cases with probable or possible AD, giving an AD incidence estimated at 1.22 per 100 person-years, were identified after 8 years of follow-up [71]. The PAQUID results also show that subjects exposed to water containing more than 100 μg aluminum/l (adjusted for age, gender, educational level, place of residence, and wine consumption) had a relative risk of 2.14 for developing AD (95% CI: 1.21–3.80), compared to those who drank water containing less aluminum ($p = 0.007$) [71].

At the 15-year follow-up, the PAQUID cohort [72], 364 of 461 subjects diagnosed with dementia were classified as having probable or possible AD. The incidence rate for AD at the 15-year follow-up was estimated at 1.92 per 100 person-years. The relative risk for dementia in subjects with a high aluminum intake was 2.26 (CI: 1.00 - 5.07; $p = 0.049$). The authors determined the participants that ingested 100 μg aluminum or more per day from drinking water had a relative risk of AD = 3.35 (CI: 1.49 - 7.52; $p = 0.003$), after adjusting for age and gender, compared to those that ingested less than 100 μg aluminum per day from their drinking water [72].

Another drinking water aluminum study [73] aimed to assess long-term exposure to different species of aluminum in drinking water. This study was based on 1,924 participants from which 86 cases of AD were diagnosed and the final sample contained 68 AD cases who were willing to provide blood samples. These were matched to 68 controls of the same gender and age (±2 years). The subjects' residential histories were taken into account and aluminum speciation was determined for the relevant water supplies.

The authors found an association between AD and, on average, 44 years of exposure to organic monomeric aluminum in drinking water, which includes some of the most soluble and toxic aluminum species. Adjustments were made for education level, family history of AD, and the presence of the ApoE4 allele, a risk factor for AD discussed below. Taking these factors into account, the adjusted odds ratio for developing AD was 2.67 (95% CI 1.04–6.90) for subjects who had long-term exposure to organic monomeric aluminum [73].

With the passage of time, these retrospective and prospective epidemiological studies have been able to show a consistent pattern of increased relative risk for AD, in the band of 2.3- to 2.6-fold, for human populations who drink from urban water supplies containing aluminum at a concentration $\geq$0.1 mg/l compared to those who drink water with less aluminum than 0.1 mg/l. However, human studies are fraught with confounding factors, such as high aluminum content in processed foods and some bottled waters, that tend to lower the true risk for AD from exposure to aluminum in a public drinking water supply.

The dilemma of prospective interventional randomized controlled longitudinal human studies and a solution

A prospective, randomized controlled longitudinal trial that could provide a dose-response effect from humans that routinely ingest standardized amounts of aluminum in their food and water for a significant part of their life, might be an ideal way to examine for the potential neurotoxicity of bioavailable aluminum. Realistically, as Van Reekum notes [39], it would be grossly unethical to carry out a prospective human study that tests the long-term outcomes from ingestion of a neurotoxicant, even if it were technically possible to administer and obtain participant compliance.

The WHO [10] has recognized the limitations on performing an epidemiological study such as this in humans and called for well-designed long-term animal studies which focus on aluminum neurotoxicity with suitable endpoints that could provide a dose-response relationship for aluminum exposure by either the oral route or inhalation.

Such a protocol was applied to an aging wild-type rat model in lieu of any human or animal studies that had previously compared the long-term consequences of chronic aluminum consumption, of amounts equivalent to those within the human total dietary aluminum range, in a dose-dependent manner. Total aluminum consumption was controlled in the rats after they completed their main growth phase at six months until the end of their natural lifespan [81, 82]. The aim of these longitudinal studies was to learn whether rats could grow old and maintain healthy brain function (i.e., in the absence of a dementia-like condition) despite routinely consuming bioavailable aluminum in their food and water at human-relevant levels. The choice of animal used for these studies was based on the need to have healthy animals that age in a natural manner, demonstrating normal brain function pre-treatment and, with hybrid vigor, surviving well into old age. The investigator had prior knowledge that brains of normal rats and mice are unable to form Aβ plaques and/or NFTs for species reasons.

Old age is the main risk factor for AD. The WHO noted that AD research had been hampered by the lack of a suitable experimental animal model and recommended the use of aged amyloid-β protein precursor (AβPP)-transgenic mice for this purpose. Since then, numerous studies have used AβPP-transgenic mice as a model for amyloidogenesis. Most of these studies have used young or middle-aged AβPP-transgenic mice even though the aged brain is very different from the younger brain with regard to neuronal redundancy and capacity for neuroplasticity and repair.

The results of transgenic mouse models depend on overexpression of AβPP transgenes with familial AD (FAD) mutations expressed at non-physiological levels. Elder et al. comment that many proteins will be toxic at some point if overexpressed at sufficient levels [83]. Transgenic mice are inbred and therefore more uniform than outbred animals, but with risk of compromises to their health. Human AβPP-transgenic mice are descendants of genetically modified mice that grew from a fertilized egg transfected by a cosmid, a small strand of DNA capable of replicating independently of chromosomes, that carries genes (recombinant DNA) into living tissue where the genes can replicate and become over-expressed in the tissue when driven by a promoter sequence.

The AβPP transgene containing the Swedish FAD mutation (tg 2756) is driven by a hamster prion (PrP) promoter that stimulates AβPP gene expression widely throughout the brain [84]. AβPP expression of the PDAPP transgene is driven by platelet-derived growth factor [85]. The type of promoter determines which brain region(s) will be affected and the strength of AβPP overexpression. These are the two main mouse models for amyloidogenesis although many others have been produced. The models show high levels

of Aβ, dystrophic neurites, and gliosis compared to non-transgenic controls.

In the case of the PDAPP transgene, forty copies are integrated into brain cells, representing an almost 18-fold increase of AβPP RNA and 10-fold increase of (human) AβPP protein in the mouse brain in addition to mouse AβPP protein [85]. Aβ plaques promoted by platelet-derived growth factor appeared in transgenic mouse brains from six months onwards [85]. In mice, six months of age is equivalent to a human age approximating 17.5 years, given that rats and mice age almost 35 times faster than humans [86].

Homozygous strain PDAPP mice show 70% (male) and 60% (female) mortality rates between 12 and 21 months of age [87]. Tg 2576 mice have considerably better survival, declining by 25% at age 22 months [87]. Westerman et al. indicate that some Tg 2576 mice may attain the age of 25 months [88]. The maximum longevity of the transgenic mice is apparently in the range of 62 to 73 years in human equivalent terms [88]. Another study of transgenic mice with the Swedish mutation reported lower survival at 9 months than for non-transgenic mice (84.5% of transgenic animals were still alive by 9 months compared to 95.8% in non-transgenic controls) [89].

A study by King et al. [89] examined for early cognitive and sensorimotor deficits in AβPP transgenic mice. The authors reported female transgenic mice were cognitively impaired at 3 months of age compared to non-transgenic females in all circular platform measures whereas transgenic male mice showed progressive cognitive impairment on circular platform tasks between ages 3 and 9 months (equating to almost 9 and 26 human years, respectively) [89]. The 3-month transgenic males were hyperactive compared to controls. Other authors have noted that transgenic mice were similarly impaired at ages 3 to 4 months in Y-maze spontaneous alternation [90]. In view of their ages, this indicates a developmental problem with cognition in at least some strains of transgenic animals.

Most AβPP-transgenic animal studies focus on amyloidogenic changes that occur during the developmental stages of youth, young adulthood, and early middle age. The present author's concerns about the suitability of transgenic mice for long-term aging studies, led to using wild-type rats that proved to have a mean lifespan of 31 months (90 years in human terms) for the survivors to at least age 28 months. To this author, the term of natural life outweighed the need for animals to have brains that exhibit Aβ plaques. Nevertheless, transgenic animals can and do provide interesting and informative results.

A rat model for chronic aluminum neurotoxicity provides a translational animal model for chronic aluminum neurotoxicity and AD-type dementia

Two longitudinal studies were performed with outbred germ-free male Wistar rats chronically exposed to dietary concentrations of aluminum in their diet throughout most of their adult lifespan [81, 82]. The aluminum levels given to these animals were based on total amounts of aluminum (mg/kg body weight (bw)/day) that Americans are estimated to consume daily from their diet, drinking water, and aluminum additives [5, 9]. Chronic aluminum neurotoxicity with cognitive deterioration was the toxicological endpoint of the studies.

Between weaning and age 6 months (their main growth phase), the young rats consumed an *ad libitum* diet. From age 6 months, the rats were given measured rations (20 g/day) of pelleted feed containing a low base level of aluminum (at 9 ppm/kg feed) twice weekly, amounting to 0.4 mg aluminum/kg bw/day. Their weights were maintained at $500\,g \pm 50\,g$ and, between 6 and 12 months of age, they were given ultrapure water to drink with no graphite furnace atomic absorption spectroscopy (GFAAS)-detectable aluminum.

In both studies, aluminum supplementation was intentionally delayed until middle age in order to ensure that the rats' brains had time for normal development. A major advantage of using laboratory rats for chronic aluminum intake experiments is that their life-long aluminum intake can be rigorously controlled and accurately monitored over their three-year lifespan. The studies were performed under the auspices of an animal care and ethics committee and are referred to as "the longitudinal studies".

The first report [81] described a small longitudinal pilot study in which six rats having served as their own controls for T-maze testing in middle age versus old age, received supplementary aluminum in their drinking water from age 16 months until the end of their natural lifespan in an amount equivalent to the high end of the human total dietary aluminum range (totaling 1.6 mg/kg bw/day). This timing mimicked that of middle-aged urban Americans who began consuming processed foods after World War II when the use of aluminum additives was becoming more widespread, and who continued to consume food and drink water containing aluminum additives into their old age. The first study found two of the six aluminum-treated rats developed chronic aluminum neurotoxicity with cognitive deterioration in

old age. These two rats obtained significantly lower T-maze performance scores in old age than in middle age and displayed AD-like behaviors, including poor attention span, confusion, incontinence (while in the T-maze), and perseverative activities. These aluminum-induced changes are more similar to AD than those produced from greater aluminum exposure such as intracerebral injection. The four remaining rats were asymptomatic, showing no significant difference in their T-maze scores between middle age and old age.

The second report [82] describes the main longitudinal study, a prospective controlled trial that compared three groups of male germ-free Wistar rats randomly assigned to treatment groups that consumed aluminum at different levels (low, intermediate, and high), from age 12 months (onset of middle age) onwards, in amounts equivalent to those within the total dietary aluminum range consumed by many Americans from food, drinking water, and aluminum additives [5, 9]. In the main study, the animals' kidney and liver functions were determined in middle age and old age and were found to be normal for each test except for one animal. The average lifespan of all rat groups in the main longitudinal study was 31 months [82].

The main study confirmed the results of the pilot study and provided further information on chronic aluminum neurotoxicity [91–94]. The rats were trained to perform a rewarded continuous alternation T-maze task commonly used to assess memory performance [95, 96] between 5 and 6 months of age. The rats had to survive well into old age and perform the T-maze task within five minutes for inclusion in the experimental analysis. The rats of both studies were tested weekly, from age 9 months until a terminal physical condition was evident, at which time they were euthanized.

The low aluminum control group, receiving 0.4 mg/kg bw/day entirely from their food, had a NOEL level in that all rats in the low aluminum control group continued to score as well or better in old age as in middle age [82]. The lowest observable adverse effect level (LOAEL) for aluminum was an amount given to the intermediate group in which 20% (2/10) of rats developed chronic aluminum neurotoxicity with cognitive deterioration after chronically consuming 0.5 mg aluminum/kg bw/day. The LOAEL may reflect either a greater bioavailability of aluminum contained in drinking water or a threshold amount attained in some animals. The low and intermediate dose levels were intentionally close (at 0.4 mg and 0.5 mg/kg bw/day, respectively) to investigate whether a small additional amount of aluminum provided in the drinking water might affect the results, despite some evidence that absorption levels from food and water are approximately the same.

In the high aluminum group, 70% (7/10) of rats had a significantly lower mean T-maze performance score in old age than in middle age [82]. The rats with chronic aluminum neurotoxicity, like those without this condition, were all given opportunities to perform the T-maze task until the end of their natural lifespan. The higher proportion of aluminum-affected rats in the main study was attributed to the earlier commencement of aluminum supplementation (at age 12 months instead of 16 months) and, hence, longer aluminum exposure with more time for aluminum to accumulate in the brain to toxic levels. This proportional difference shows that the aluminum exposure period must be sufficiently long in such experiments for aluminum to accumulate to toxic levels in the brain. Premature termination of an experiment, use of a too high dose level (causing aluminum precipitation), or a dose level too low to allow sufficient accumulation in the available time, may prevent the availability of observable effects.

Laboratory animals share many biochemical similarities with humans, but also differ in some important respects that need to be recognized and taken into account when interpreting the extent to which such translational animals may serve as models for human disease. For example, transgenic mice that express human AβPP have significantly contributed to an understanding of amyloidogenesis but fail to show an irreversible dementia-like condition or AD neuropathology involving NFTs. On the other hand, brains of a significant proportion of aged wild-type rats, chronically exposed to aluminum at human-relevant levels, develop an irreversible dementia-like condition with AD-related neuropathology, showing aluminum accumulation without NFTs in cells homologous to those of AD patients that are most prone to contain NFTs. These aluminum-exposed rats show incipient stages of plaques and tangles. The probable reason for plaque absence is that the structure of the rat/mouse Aβ sequence differs from the human sequence by three amino acid residues [97]. Rat Aβ is more resistant to metal-induced oxidation than soluble human Aβ which fibrillizes and forms Aβ deposits in the presence of pro-oxidant metals [98, 99].

The reason why wild-type rat and mouse brains are unable to form NFTs is less well understood. A possible explanation may relate to the observation by Goedert et al. [100] that adult rat neurons are only able to express 3-repeat tau isoforms whereas adult human neurons express all six tau isoforms: three that have 3-repeats of the microtubule-binding domain and three

that have 4-repeats. Whether the hyperphosphorylated tau that forms in wild-type rat and mouse neurons is appropriately truncated for NFT formation, as in human NFTs which contain truncated versions of all six tau isoforms, is a matter for further research.

Aluminum-fed rats exhibited subtle neuropathology, which also occurs in AD, that may account for dementia to a greater extent than either plaques or tangles, as described later in this analysis. The aluminum-based aging rat model for chronic aluminum neurotoxicity is proposed as the best available model for studying the change from normal brain function to AD-type dementia; that is, for understanding how AD dementia develops and spreads throughout the brain in old age, and for testing possible interventions. The human AβPP transgenic mouse model is an excellent model for studies of amyloidosis, and has also been used to examine the effects of aluminum on amyloidogenesis [101]. Both animal models require the aluminum dosage to be in balance with the duration of the experiment. AD brain tissue has also been valuable for analyzing aluminum involvement in NFT formation [102].

A video clip, comparing the maze performances of a rat in middle age and the same rat in old age, before and after developing chronic aluminum neurotoxicity with cognitive deterioration, abnormal behaviors, and a maze performance by an extremely old low aluminum control rat can be viewed as a supplementary file to this causality analysis (http://dx.doi.org/10.3233/JAD-132204).

CRITERION II: CONSISTENCY OF THE ASSOCIATION

Important findings pertaining to the putative causal relationship have been consistently confirmed in independent investigations

Bradford Hill asked "Has [the observed association] been repeatedly observed by different persons, in different places, circumstances and times?" [38] Van Reekum et al. define this criterion as "consistency of the findings across research sites and methodologies." [39] Important associations have been observed between aluminum and AD, and its neuropathological and behavioral correlates, by many investigators working in different research institutes, using different instrumentation and various aluminum species while testing humans or various experimental animal species. In this section, we describe critical studies that have contributed toward understanding the association between chronic aluminum intake and AD that demonstrate this consistency.

Aluminum absorption rates vary among apparently healthy individuals

Aluminum ions are small and, at the relatively low concentrations (μM or less) found in brain tissue, can be difficult to measure by conventional techniques. Modern aluminum absorption experiments have increasingly utilized a technique that involves ingestion of small amounts of a stable radioactive aluminum isotope (^{26}Al) which can be detected at very low levels in blood plasma and other tissues with accelerator mass spectrometry (AMS) [103]. ^{26}Al/AMS studies have provided clear evidence that small amounts (~0.1% to 0.3%) of dietary aluminum are routinely absorbed across the gastrointestinal tract lining and into the blood [104]. The ^{26}Al isotope of aluminum is synthetic and, as such, ^{26}Al is naturally absent from biological tissues. Hence, the presence of any ^{26}Al in biological tissues, of humans and experimental animals that have been intentionally exposed to ^{26}Al, must derive from that exposure. ^{26}Al/AMS experiments independently performed in Australia, the USA, and France have unambiguously shown that measurable amounts of ^{26}Al are detectable in brain tissue of animals two weeks after they have consumed an equivalent amount of aluminum to that consumed by humans drinking a single glass of alum-treated water [14–16].

A range of values is consistently seen in aluminum measurements made on blood samples taken from a group of subjects that have consumed a standardized amount of either ^{26}Al or natural aluminum (^{27}Al). At any one time, some subjects (whether humans or laboratory animals) absorb significantly more aluminum than others [104–108]. High and low aluminum absorbers are clearly evident even among healthy controls tested after consuming a standardized aluminum dose (Fig. 2) [104]. The high absorbers have plasma or serum aluminum values that peak at two or three times higher than the peak for the lowest aluminum absorber in the same test [104–108]. This indicates that some individuals are innately more efficient at absorbing aluminum than others; or, alternatively, less able to exclude aluminum from absorption.

Conceivably, the high aluminum absorbers could be those most prone to develop AD after decades of aluminum exposure. The reason for increased aluminum absorption in some individuals more than others needs to be examined in order to more fully understand the

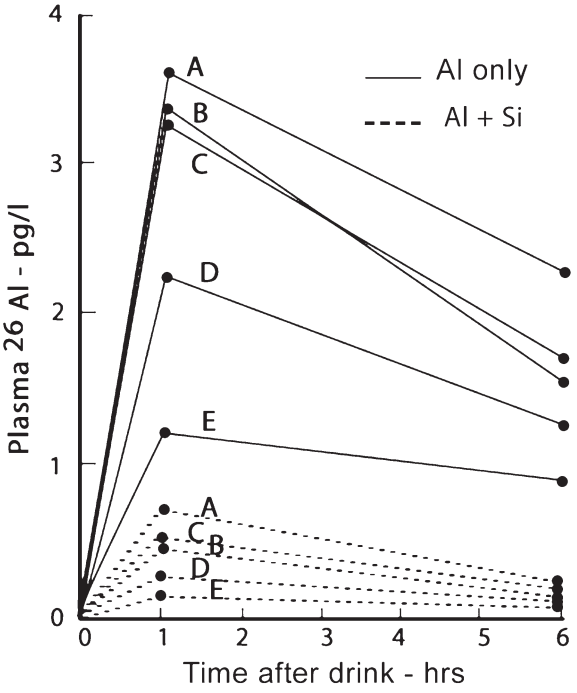

Fig. 2. Aluminum absorption in humans showing plasma ^{26}Al levels after five subjects (A–E) ingested an ^{26}Al dose on two occasions, with silica (dotted line) and without silica (solid line). Note that the values for A, B, and C peak almost 3 times higher than for E. Also, the subjects rank in almost the same order for both experiments despite difference in the form of ^{26}Al they consumed. Reprinted from [104] with permission from Elsevier.

genetic component of AD causality, and to determine whether the rate of aluminum absorption may change over time. If high aluminum absorption were known to be predictive for AD, those at risk could adjust their dietary habits by consuming a fresh food (low aluminum) diet before aluminum has a chance to accumulate to a neurotoxic level in AD-vulnerable brain regions.

Humans with AD absorb about 64% more aluminum, and have higher serum/plasma aluminum levels, than non-demented controls

An ^{26}Al/AMS experiment has shown that subjects with AD absorb approximately 64% more ^{26}Al than non-demented age-matched controls from a standardized ^{26}Al dose in an orange drink [109]. This increase in aluminum absorption can explain the significantly higher (by two- or three-fold) increase in plasma/serum aluminum levels observed in AD patients, relative to non-demented controls, in nine of ten studies [110–118]. The small exceptional study [119] has low statistical power, making it susceptible to a type II statistical error; i.e., failing to observe a difference when one actually exists.

AD patients also exhibit a difference in their serum ferritin levels compared to controls. Ferritin, the main iron storage protein, forms a hollow sphere that can store up to 4500 iron atoms [120]. In addition, ferritin binds other metals including aluminum and zinc. Serum ferritin of AD patients was much higher in aluminum content, having 35% iron:3% zinc:62% aluminum in contrast with the equivalent ratio from non-demented controls (in this case, 4 pools of plasma obtained from 200 randomly selected blood donors) that had a ratio of 50% iron:13% zinc:37% aluminum. Hence, aluminum significantly increases in serum ferritin of AD patients whereas iron and zinc lack statistically significant change.

The same study showed that serum ferritin from patients with mild AD binds considerably more aluminum (25% iron:2% zinc:73% aluminum) than serum ferritin from patients with moderate AD (40% iron:4% zinc:56% aluminum), leading to the interpretation that plasma/serum ferritin contains a high proportion of aluminum in early AD but eventually loses its capacity to contain so much aluminum [120]. This finding suggested to the authors that ferritin may eventually release aluminum into the blood during later life, making the aluminum more bioavailable for uptake into the brain and increasing AD severity [120].

Standardized tests for measurements of aluminum absorption into the plasma or into serum ferritin-bound iron:zinc:aluminum ratios could open the way for new diagnostic tests with regard to the risk for AD and the presence of incipient AD, respectively. Through these and other mechanisms, higher blood aluminum levels give rise to higher brain aluminum levels, as observed in dialysis patients [121]. As described below, aluminum accumulation to toxic levels in neurons leads to AD neuropathology, including upregulated expression of AβPP [122], the formation of Aβ plaques [123], NFTs [102], and granulovacuolar degeneration (GVD) in specific brain regions of AD-affected humans. These neuropathological effects are also induced in the same brain regions of aluminum-exposed laboratory animals [92, 101, 124, 125].

Rats that develop cognitive deterioration after chronic aluminum exposure have higher serum aluminum levels than low aluminum controls that remain cognitively-intact

The low aluminum group, of the three rat groups described above, chronically consumed 0.4 mg

aluminum/kg bw/day, entirely contained in their measured feed rations. The intermediate aluminum group ingested 0.5 mg aluminum/kg bw/day, consisting of the same amount of aluminum in their feed rations as the low aluminum dose group plus drinking water containing a small amount (0.12 mg/kg bw/day) of additional aluminum [82]. The high aluminum group also consumed 0.4 mg aluminum/kg bw/day from their feed. Their drinking water contained ten times more aluminum than that given to the intermediate group, so their aluminum intake totaled 1.6 mg aluminum/kg bw/day.

Six significant observations were made about serum aluminum levels in the rat model for chronic aluminum neurotoxicity [82]:

(1) Serum aluminum levels for the three rat groups correlated with their aluminum dose levels ($r_s = 0.56$, $p < 0.01$; Spearman's correlation coefficient) (Fig. 3).

(2) Rats in the high aluminum group (consuming aluminum at a level equivalent to the high end of the human dietary aluminum range) had a significantly higher median serum aluminum level (35.1 μg/l) than the low aluminum group (14.9 μg/l) that served as controls (ANOVA on ranks, Dunn's *post-hoc* test; $p = 0.015$) (Fig. 4A). The intermediate aluminum group had a median serum aluminum level of 16.2 μg/l, similar to that of the low aluminum group, reflecting the small difference in their aluminum dose levels.

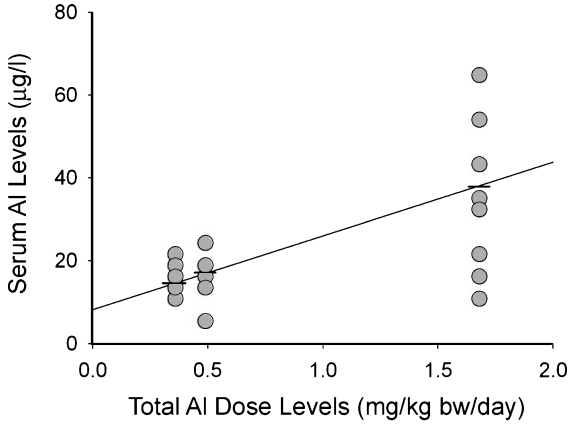

Fig. 3. Scatter plot showing the relationship between the aluminum dose levels (0.4 mg, 0.5 mg and 1.6 mg aluminum/kg bodyweight/day) consumed by the three aged rat groups and their serum aluminum levels. $r_s = 0.56$, $p < 0.01$. Reprinted from [82] with permission from Elsevier.

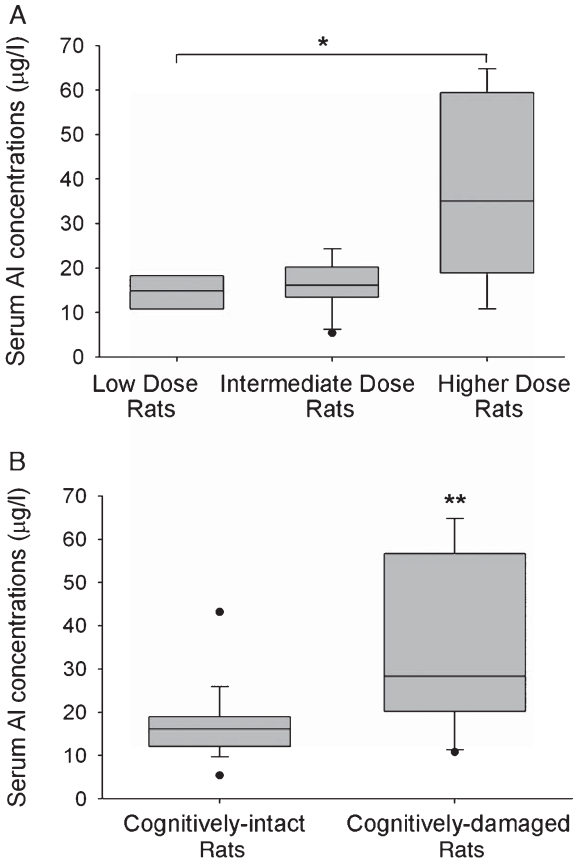

Fig. 4. Boxplots showing serum aluminum levels of rat groups in old age. A) Rats that consumed the highest aluminum dose had significantly higher aluminum levels and a larger range of values than those in the low aluminum dose group (ANOVA on ranks, $p < 0.05$). The two rats in the intermediate dose group that developed chronic aluminum neurotoxicity account for the slight rise in their median serum aluminum level. B) Serum aluminum levels of rats that developed chronic aluminum neurotoxicity ("cognitively-damaged") were significantly (75%) higher than those of the low aluminum ("cognitively-intact") rat group (Mann-Whitney, $p < 0.01$) (* = $p < 0.05$; ** = $p < 0.01$). Reprinted from [82] with permission from Elsevier.

(3) As previously mentioned, some rats absorb aluminum more efficiently than others in their treatment group. This is particularly evident for rats that consumed the intermediate aluminum dose level. Two rats, that had higher serum aluminum levels (21.8 μg/l) than the rest of the intermediate aluminum dose treatment group, were the only rats in their group to develop chronic aluminum neurotoxicity with cognitive deterioration [82]. This indicates the two rats probably had a genetic predisposition for higher aluminum absorption.

Table 1
Brain aluminum measurements from humans with AD and non-demented controls [127, 129]

Brain Aluminum Measurements	Controls (μg/g brain tissue) dry weight	AD (μg/g brain tissue) dry weight	Significance level (p-values)
Study by Andrási et al. [127]			
Entorhinal cortex	1.5 ± 0.9	10.2 ± 9.0	–
Hippocampus	1.4 ± 0.6	4.9 ± 3.0	–
Frontal cortex (parietal)	1.8 ± 0.6	6.8 ± 4.3	–
Frontal cortex (basal)	2.5 ± 0.7	6.4 ± 2.9	–
Study by Xu et al. [129]			
Superior and medial temporal gyrus	1.4 ± 1.1	2.9 ± 1.3	not significant
Inferior parietal lobule	1.7 ± 1.4	3.8 ± 2.5	$p = 0.03$
Hippocampus	1.5 ± 0.9	2.7 ± 1.6	$p = 0.05$
Middle frontal gyrus	1.7 ± 0.8	2.1 ± 1.1	$p = 0.02$

(4) The group of seven animals that exhibited cognitive deterioration in old age (by obtaining significantly lower T-maze performance scores in their old age than in middle age, and displaying abnormal behaviors such as confusion, indecision, standing in their urine, and repetitive behaviors) after consuming aluminum in an amount equivalent to the high end of the human total dietary aluminum range, had a significantly higher median serum aluminum level than the cognitively-intact control group that consumed only the aluminum contained in their food (28.4 μg/l versus 14.9 μg/l; $p = 0.010$) (Fig. 4B) [82]. Humans could also be expected to show similar results for comparable groups, in view of the concordance between human and animal effects produced by metal toxicants [126].
(5) Individuals who are high absorbers and who consume aluminum in an amount equivalent to the high end of the human dietary aluminum range are likely to have even higher serum aluminum levels, more extensive numbers of neurons with high-stage aluminum accumulation and to exhibit early-onset chronic aluminum neurotoxicity. These conditions may be relevant to families with a history of early-onset AD.

Aluminum accumulates with age in the human brain, even in non-demented controls

Several research groups have reported that aluminum accumulates in human brains with increasing age [17–20]. The effect of aging on brain aluminum levels has been studied by ranking data from human brain aluminum measurements into age groups based on 20-year increments (Fig. 1) [17]. This revealed that brain aluminum levels significantly increase with rising age ($r = 0.226$, $p < 0.01$).

Aluminum concentrations are higher in gray matter of brain regions that become damaged in humans with AD and rats with chronic aluminum neurotoxicity

Improvements have been made in aluminum measurement techniques, particularly with respect to eliminating interference from magnesium and phosphorus [127–129]. In 2005, a study by Andrási et al. [127] used inductively-coupled plasma atomic emission spectrometry to measure aluminum values in gray matter of five brain regions from three non-demented controls and three AD cases. This group measured brain aluminum levels in the entorhinal cortex as well as the hippocampus and frontal cortex (Table 1). The region with the overall highest aluminum measurements was the entorhinal cortex [127]. This region is amongst the earliest brain regions to develop NFTs (as markers of toxicity) in AD and is ultimately the most severely damaged region in the AD brain [130–133].

Rusina et al. [128] report their 2011 hippocampal results (expressed in wet weight) were similar to those of the Andrási et al. study [127]. Rusina measured aluminum and mercury values in samples from the hippocampus and associative visual cortex of 27 controls and 29 histologically-confirmed AD cases. They found that AD cases showed a significant (4-fold) increase in aluminum content in hippocampal tissue samples compared to controls ($p = 0.039$) whereas mercury levels were not significantly different between these groups [128].

A study performed in 1992 by Xu et al. [129], using graphite furnace atomic absorption spectroscopy and correcting for phosphorus, reported mean (±SD) values (dry weight (d.w.)) for aluminum in gray matter of the superior and middle temporal gyri, inferior parietal lobule, hippocampus, and middle frontal gyrus of AD patients versus controls (Table 1). These AD values were lower than those observed more recently [127]

but were approximately twice as high in the AD brains as in brains of controls [129]. Brain aluminum levels in AD could be expected to increase or decrease according to severity of the AD cases examined.

Xu et al. [129] reported that a proportion of their measurements concerned "hot spots" that measured 3 SD or more above their mean aluminum value. The highest brain aluminum concentration observed in their study was 8 μg/g in the inferior parietal lobule. Similar hot spots have also been reported by earlier investigators who measured aluminum in AD-affected brains with GFAAS [134]. This feature demonstrates the patchy, non-uniform distribution of aluminum within a region and between regions of AD-affected brains. Hot spots in human brains typically exceed 4 μg aluminum/g brain tissue (d.w.) [129, 134]. They are likely to contain large numbers of NFTs, (staining for aluminum), or large pyramidal cells with high-stage nuclear aluminum accumulation in human brain sections. Hot spots are absent at the aluminum concentrations found in brain tissue samples from aged controls [129, 134].

It should be noted that aluminum levels greater than 4 μg/g brain tissue are neurotoxic in cat brains [33]. Electrophysiological defects occur [33] and NFTs begin to form in brain tissues around that aluminum level [134, 135]. Moreover, the LD_{50} brain aluminum concentration for rabbits given systemic aluminum injections is also at 4 or 5 μg/g brain tissue [135].

Aluminum accumulation in human and rat brains affects the same types of neurons in memory processing regions of the brain

Aluminum accumulates in the same types of large neurons, in memory-processing brain regions of humans using chronic renal dialysis and humans with AD [136–140] and in homologous brain regions of rats with chronic aluminum neurotoxicity that chronically ingested aluminum at human-relevant levels throughout their middle age and old age [82] and of cats and rabbits given intracerebral aluminum injection [33, 134, 135].

As human and rat neurons become increasingly aluminum-laden in AD and chronic aluminum neurotoxicity, their neurons undergo similar morphological changes. Rat and human pyramidal and stellate neurons, in which aluminum has slowly but progressively accumulated, can be classified by optical microscopy according to their stage of aluminum accumulation and accompanying morphology (Fig. 5) [91, 136].

Many cells with high-stage aluminum accumulation, in brain sections from rats with cognitive deterioration, appear in the form of lesions as do NFTs [91, 94]. Processed with the Walton stain, groups of adjacent cells, in clusters or bands, appear shrunken and stain bright magenta, flanked by neurons with a more normal appearance (Fig. 5C). These lesions of cells probably equate to the hot spots of gray matter, measured and shown to have high aluminum content [129, 134]. Some lesions have been observed to be very large. In a serially-sectioned cerebrum of a rat with chronic aluminum neurotoxicity, from the intermediate aluminum dose group, a lesion of cells with high-stage aluminum accumulation was found to extend the entire anteroposterior length of the dorsal hippocampus [91]. All rats that developed chronic aluminum neurotoxicity had at least one substantial aluminum-rich lesion in their hippocampus or subiculum whereas all rats that remained cognitively intact lacked equivalent lesions [91].

Aluminum has been shown to transfer between olfactory and limbic regions, in anterograde and retrograde directions, via long-projecting axons [141]. Brain regions that contain large neurons with high-stage aluminum accumulation in rats with chronic aluminum neurotoxicity include the entorhinal cortex, hippocampus, subiculum, amygdala, cortical layers III and V of the association areas of the temporal and parietal cortices, frontal and posterior cingulate cortices, olfactory bulb, locus coeruleus, dorsal raphe nucleus, and the basal nucleus of Meynert [82]. These brain regions are all interconnected with each other, facilitating anterograde and retrograde transport of aluminum [141] in addition to their ability to take up aluminum via the transferrin receptors in their plasma membranes.

It is reasonable to question why aluminum accumulates in AD-vulnerable brain regions. There are some common denominators for the most vulnerable cell types in brains of (1) AD cases, (2) long-term dialysis cases, (3) Down's syndrome (DS) cases, (4) cats and rabbits that have had intraventicular aluminum-injection, and (5) the rat model for chronic aluminum neurotoxicity [33, 82, 121, 134].

Transferrin-bound aluminum in plasma has to cross the blood-brain barrier to enter the brain. Cerebral capillary endothelial cells have transferrin receptors on their luminal surface that aid the removal of transferrin-bound aluminum and iron from blood [142]. The cells most vulnerable to aluminum accumulation are large corticocortical and corticosubcortical neurons that project over great distances, generate large amounts of ATP, have high iron utilization and high oxygen requirements [137–139, 143]. These highly active neurons normally have high densities of transferrin receptors on their surfaces that facilitate their uptake

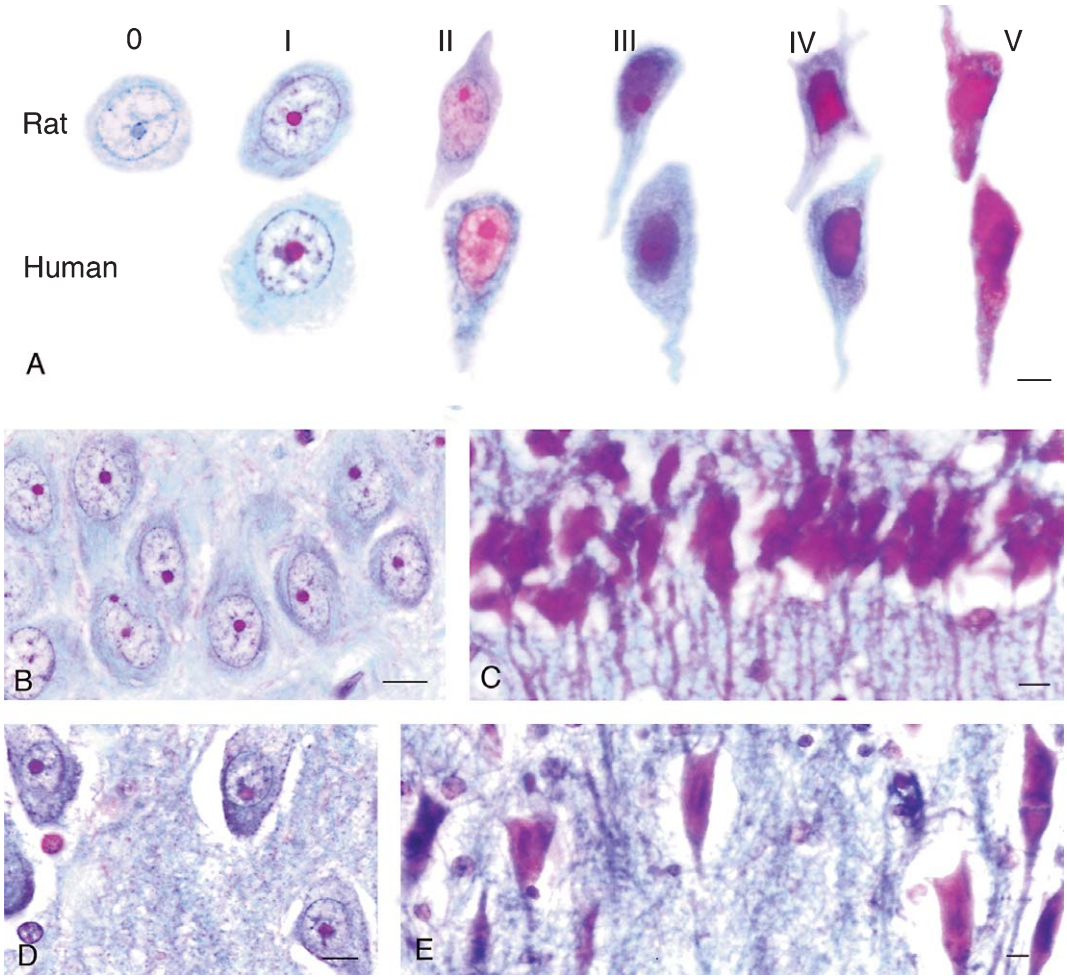

Fig. 5. Aged rat and human hippocampal neurons stained for aluminum with the Walton method. A) Staging of rat (upper row) and human (lower row) pyramidal cells shows progressive aluminum accumulation, pink to purple or magenta, accompanied by cytopathological change. (Left to right) Stage 0: the entire cell appears aluminum-negative and has normal morphology. This stage has yet to be observed by the author in aged human cortical and hippocampal pyramidal neurons. Stage I: magenta nucleolus stains for aluminum; otherwise normal morphology. Stage II: magenta nucleolus in pink nucleoplasm with increased heterochromatin; cytoplasm remains blue, cell shrinkage already evident. Stage III: magenta nucleolus in an elongated or irregularly shaped, purple nucleus; the cytoplasm is blue. Apical dendrite may have serpentine appearance. Stage IV: magenta staining of the elongated nucleus which now shows less structural detail; dendrites show irregularity; cytoplasm remains blue. Stage V: purple to magenta staining appears throughout the nucleus and cytoplasm. Cell shape is shrunken, cell processes exhibit dieback. B) Stage I hippocampal cells from a control rat brain. C) A lesion consisting of stage V hippocampal cells from a rat with chronic aluminum neurotoxicity. Abnormal neurites associated with these deformed hippocampal cells are interpreted as neuropil threads. D) Stage I hippocampal cells from a non-demented human brain. E) A lesion consisting of stage V hippocampal cells from a human with severe AD. Magnification scale bars: A = 5 μm; B–E = 10 μm. Reprinted from [91] with permission from Elsevier.

of transferrin-bound iron and aluminum [27, 144]. Together, these findings can help to explain why aluminum preferentially deposits in large corticocortical projection neurons. In contrast, interneurons involved in the local circuitry of AD-vulnerable brain regions are relatively resistant to aluminum uptake and AD-type changes [145].

The rat brain regions that accumulate aluminum are homologous to the regions that exhibit NFT damage in brains of humans with AD [82, 136–139]. Aluminum ingested in the form of aluminum-based phosphate binders, by renal patients on long-term dialysis therapy, also accumulates in large cells of the same brain regions where aluminum accumulates in AD [137–139]. The motor cortex, caudate nucleus, and putamen are less susceptible to aluminum accumulation in brains of dialysis patients, rats with chronic aluminum neurotoxicity, and humans with AD.

Kowall et al. [145] have shown that aluminum injection into rabbit brains induces early NFTs in the same

brain regions that are NFT-affected in AD [146–148]. Aluminum injection directly into the cerebrum or cerebral ventricles rapidly elevates the aluminum concentrations in brains of laboratory cats and rabbits to toxic levels, inducing early NFTs to form in large neurons within three to four days [33, 124, 125, 145]. This rapid response to injected aluminum supports the suggestion that NFTs form as a protective mechanism for trapping and sequestering cytoplasmic aluminum, and possibly other toxic metals, thereby slowing aluminum entry into the nucleus [102]. NFT formation is absent in rat brains, even in those that have received aluminum by direct intracerebral injection [149].

Humans with AD and rats with chronic aluminum neurotoxicity show high-stage aluminum accumulation in the cells of origin for the perforant pathway

The entorhinal cortex, hippocampus, amygdala, and neocortical association areas are major memory-processing regions in mammalian brains. Rats that develop chronic aluminum neurotoxicity show significantly more neurons with high-stage aluminum accumulation in these brain regions than the low aluminum controls [91, 94]. The entorhinal cortex is the most severely affected brain region in rats with chronic aluminum neurotoxicity as in humans with AD. Aluminum particularly concentrates in the cells of origin for the perforant pathway of the rat superficial entorhinal cortex [91, 94]. The equivalent cells in humans are extensively affected by NFT formation at an early stage of AD [130–133]. NFTs also form in the superficial entorhinal cortex of aged non-demented humans but to a lesser degree [150].

Brain sections from rats with chronic aluminum neurotoxicity revealed that $60\% \pm 7\%$ (mean $\pm$ SEM) of the large cells of origin for the perforant pathway display stage IV aluminum accumulation (Fig. 6A). In contrast, $23\% \pm 7\%$ of the same cells in the same brain region of the low aluminum controls were at stage IV aluminum accumulation (Fig. 6B) ($p < 0.001$), almost a 3-fold difference (Fig. 6C) [91].

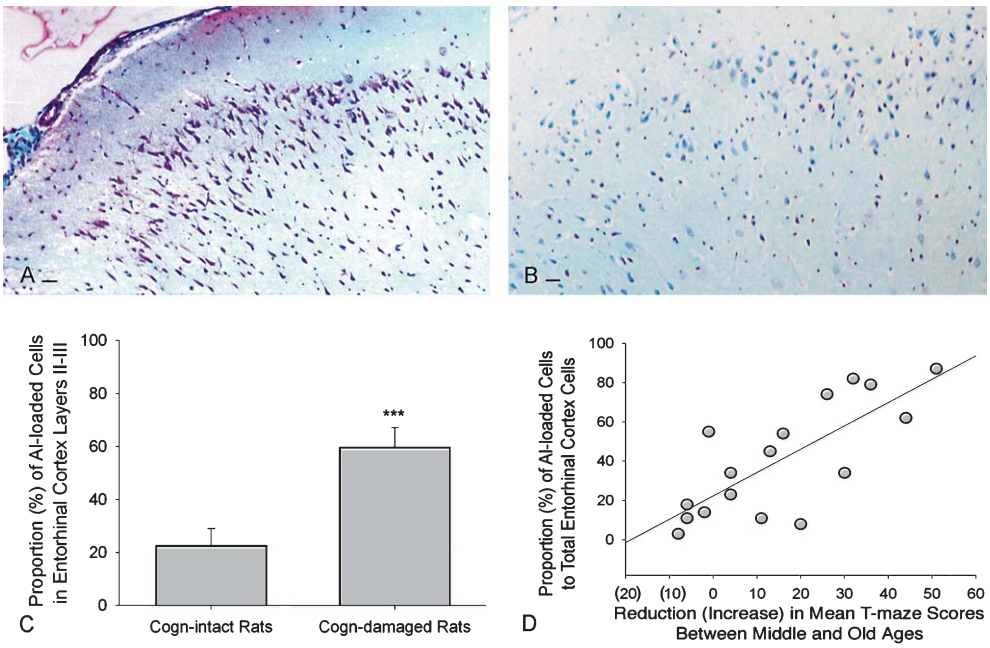

Fig. 6. Cells of origin for the perforant pathway of the entorhinal cortex. A) The Walton stain reveals aluminum (magenta or purple staining) in many large pyramidal and stellate cells of origin for the perforant pathway, and layer IV neurons, from an aged rat with chronic aluminum neurotoxicity and cognitive deterioration. B) Walton-stained entorhinal cortex from an age-matched low aluminum control rat shows little evidence of aluminum in the cells of origin for the perforant pathway, and deeper cell layers. The neurons stain blue, indicating they are aluminum-negative. In this case, aluminum staining is mostly confined to small glial cells and erythrocytes. Magnification bars for A and B = 50 μm. C) The rats with chronic aluminum neurotoxicity and cognitive damage have a significantly higher proportion of cells that exhibit high-stage aluminum accumulation than the low aluminum, cognitively-intact, rat controls ($p < 0.001$). D) The difference between the rats' mean T-maze performance scores correlates strongly with the estimated percentage of entorhinal cortical neurons (cells of origin for the perforant pathway) that stain for high-stage aluminum accumulation in brains of individual rats. $r = 0.76$, $p < 0.0005$. D is reprinted from [91] with permission from Elsevier.

A similar but less extensive pattern of pyramidal cells with high stage aluminum accumulation was observed in the temporal association cortex [91]. On average, 40% ± 7% of pyramidal neurons stained for high-stage (IV) aluminum accumulation in layers III and V of the temporal association cortex in brain sections from rats with chronic aluminum neurotoxicity. Stage IV aluminum accumulation affected 13% ± 3% of the pyramidal cells in the same layers in sections of temporal association cortex from the low aluminum controls ($p < 0.01$). Here again, the two rat groups showed almost a three-fold difference in their numbers of aluminum-damaged cells.

Aluminum accumulation in the cells of origin for the perforant pathway, whether in the form of NFTs in humans with AD or throughout neuronal nuclei of rats with chronic aluminum neurotoxicity, damages the perforant pathway cells and their axons that connect the hippocampal formation to the neocortex and conduct bi-directional information between these brain regions.

Hippocampal CA1 cells, and the large neurons of the subiculum, are also prone to high-stage aluminum accumulation in humans. Aluminum-affected cells in the hippocampal CA2 field can be seen in the form of toxic lesions comprised of adjacent shrunken cells with aluminum accumulation either at stage IV or stage V (Fig. 5E). NFTs in human brains also take the form of lesions. At first, NFTs form singly, then in clusters, and eventually as bands of increasing size. According to Armstrong, some lesions of NFTs measure 12,800 μm or longer [151].

Extensive damage to the cells of origin for the perforant pathway, together with one or more lesions in the hippocampal CA1/subiculum zones, result in isolation of the hippocampal formation from the neocortex, accompanied by severe loss of recent memory in humans with AD and rats with chronic aluminum neurotoxicity [91, 94, 130–133]. The progression of AD and chronic aluminum neurotoxicity involves aluminum accumulation in the large corticocortical cells of additional interconnected regions that subsequently show neuropathology and functional loss. The same fundamental sequence of events describes the pathoetiology of both chronic aluminum neurotoxicity and AD. It seems reasonable to consider the concept that AD could be a human form of chronic aluminum neurotoxicity.

Aluminum exposure impacts on learning and memory processing

Studies involving intentional chronic exposure of humans to aluminum are rare for ethical reasons. However, more than 95% of northern Ontario gold miners were directed to inhale poorly soluble McIntyre powder (aluminum oxide and pulverized metallic aluminum) prior to entering the mines, ostensibly for prophylactic reasons on the assumption that these forms of aluminum would be inert in the body and should trap the silica they inhaled [152]. Such exposure was an attempt to prevent silicosis of the lung by aluminosilicate formation. This practice continued from 1944 to 1979.

Subsequent cognitive testing in 1988–1989, that compared 261 men in an aluminum-exposed group with 346 men in an unexposed group, found that 13% of the aluminum-exposed miners showed cognitive impairment by obtaining lower scores on cognitive state examinations, compared to 5% of the 346 unexposed controls, and were more likely to be in the cognitively-impaired range than unexposed workers. Also, the risk for obtaining scores in the cognitively-impaired range correlated with the duration of the miners' exposure to McIntyre powder [152]. Importantly, those exposed to McIntyre powder for 10–20 years were 3.1 times more likely to score poorly on cognitive function tests. Those exposed for 20 years or more were 4.5 times more likely than controls to show this effect. Thus, length of exposure to this aluminum powder significantly correlates with cognitive impairment in humans.

The author has already described how rats with chronic aluminum neurotoxicity lost their ability to continuously alternate in a T-maze task in old age as a result of chronic aluminum neurotoxicity [82]. This rat model for chronic aluminum neurotoxicity has shown that ingestion of less than 2 mg aluminum/kg bw/day caused AD-type damage in brains of aged rats after 16 months of aluminum exposure, at human-relevant levels, throughout middle age and old age [82]. There are many other examples of aluminum-based animal models that developed disruptions to their memory and learning abilities [153–166] after exposure over shorter periods of time to higher aluminum concentrations. Aluminum reliably interferes with learning and memory functions of the brain as long as the duration of exposure is sufficient for the aluminum dose that is given. However, the use of excessively high concentrations of aluminum in experimental animals, in either food and/or water, is counter-productive in toxicity tests since absorption of oral doses of elemental aluminum above 8 mg/l (given as aluminum sulfate) is non-linear [6]. Higher concentrations of aluminum form colloids that precipitate and are absorbed less efficiently than soluble aluminum supplied at lower concentrations.

CRITERION III: SPECIFICITY OF THE ASSOCIATION

Specific classifications of aluminum neurotoxicity

The criterion of specificity comes from old beliefs that one disease results in one outcome. Bradford Hill states, "One reason...is the specificity of the association ... We must not ... over-emphasize the importance of the characteristic ... if specificity exists we may be able to draw conclusions without hesitation; if it is not apparent, we are not thereby necessarily left sitting irresolutely on the fence" [38]. Van Reekum et al. note that this criterion is invalid for toxic exposures that can produce a number of outcomes [39].

This section describes the three major types of aluminum neurotoxicity (acute, sub-acute, and chronic). These three types are distinguished by their plasma and brain aluminum levels and the duration of aluminum exposure required to show pathological effects.

Other proposed causes of AD are also considered.

Acute aluminum neurotoxicity

Acute aluminum neurotoxicity occurs when a large bolus of aluminum (as much as 500 μg/l or more) enters the circulation. Acute aluminum neurotoxicity can affect humans with normal kidney function as well as those with chronic renal failure, resulting in an encephalopathy that typically involves grand mal seizures and culminates in coma and death within days or several weeks [167, 168].

Sub-acute aluminum neurotoxicity: Dialysis encephalopathy syndrome (dialysis dementia)

Chronic dialysis patients have impaired kidney function and are unable to efficiently excrete the aluminum they routinely acquire, from contaminant aluminum, if present, in the dialysate fluid, and from ingesting aluminum-based antacids, or phosphate binders. Phosphate binders provide up to 4000 mg aluminum per day, equating to 100 times more aluminum than most humans ingest daily [169].

Renal failure patients may develop a form of sub-acute aluminum neurotoxicity unless their serum aluminum levels are adequately monitored and, if necessary, chelation-controlled. If too much aluminum enters the brain, dialysis encephalopathy syndrome (DES) or dialysis dementia can result. This form of sub-acute aluminum neurotoxicity involves medium to high aluminum levels in blood and/or cerebrospinal fluid for a period sufficiently long to produce high aluminum levels in the brain (e.g., ranging from 12.4 μg to 33.0 μg/gram brain tissue (d.w.)) [35, 166, 170], progressing over many months or several years.

The onset of DES is insidious, often involving problems with speech. Seven to nine months after symptoms first appear, the patient becomes totally mute, unable to perform purposeful movements and soon dies. DES epidemics have previously occurred in patients with chronic renal failure who shared a source of aluminum-contaminated dialysis water [171, 172]. The high brain aluminum concentrations characteristic of DES result in aluminum precipitate in the brain in the form of numerous solid granular deposits in lysosomes within the choroid plexus, cortical glia, and neurons [170]. The granular aluminum precipitates probably explain why DES patients seldom exhibit NFTs [170] that typically develop slowly and at lower aluminum concentrations in human and non-human primate brains.

DES is a progressive encephalopathy that occurs less frequently now than in the past due to better dialysis management. Removal of aluminum from dialysis equipment and dialysis water, careful monitoring of the patient's plasma aluminum levels, and intervention with desferrioxamine (DFO), a chelating agent that removes aluminum in a timely manner, have helped to prevent many adult deaths from DES [173].

Intracerebral or intraventricular aluminum injection into rabbit and cat brains is sometimes high enough for the animals to develop a DES-like encephalopathy that results in death, making the animals a better model for DES than AD. Results of such treatments are probably dose-dependent. Aluminum-induced NFTs form in AD-relevant regions of cat brains that contain brain aluminum levels between 4 and 6 μg/g brain tissue (d.w.) [33].

Chronic aluminum neurotoxicity

The present article investigates the evidence that AD is a form of chronic aluminum neurotoxicity that may occur in humans that routinely ingest dietary aluminum in the range of 35 mg to 119 mg/day for an average-sized (70 kg) human. This range is equivalent to 0.5 mg/kg bw to 1.6 mg/kg bw/day [82].

Other human dementias associated with chronic aluminum neurotoxicity are the premature AD of DS and amyotrophic lateral sclerosis/parkinsonism dementia of Guam (ALS/PD) which has specifically affected the Chamorro people of Guam and some other indigenous Pacific islanders. ALS/PD of Guam and AD share two

major neuropathological hallmarks: NFTs [174, 175] and hippocampal granulovacuolar degeneration [176, 177]. Studies, involving histological stains that reveal aluminum in tissue sections and aluminum measurements, have shown that NFTs in brains of humans with AD and ALS/PD of Guam contain aluminum in abundant amounts [102, 178–181], as much as 250 ppm [182]. These NFTs are also reported to contain calcium. Despite this overlap, different brain regions are affected in ALS/PD of Guam and AD and they are distinctly different diseases.

Non-DES dialysis patients serve as a human model for the prodromal phase of chronic aluminum neurotoxicity

Microtubules are an important part of the neuronal cytoskeleton, providing structure and transport throughout the cell and its processes. Microtubules have been likened to railroad tracks that allow the conveyance of synaptic neurotransmitter vesicles, mitochondria, and other cellular components from the nucleus to the synapses. Normal tau is a soluble microtubule-associated protein. Kinases are the main enzymes that add phosphates to tau and protein phosphatase 2A (PP2A) is the main enzyme that removes phosphates from tau, which requires reversible phosphorylation for its function. Tau is primarily responsible for assembling and stabilizing microtubules in neurons. Most normal tau in adults is associated with axonal microtubules in white matter [183]. Changes in white matter tau occur at an early stage of tau pathology [184].

PP2A activity is abnormally low in the AD brain [185], causing an imbalance between phosphorylation and dephosphorylation. Low PP2A activity in AD brains results in depletion of normal tau and widespread accumulation of hyperphosphorylated tau [186]. Superfluous phosphate groups on tau, particularly in and near its microtubule-binding domain, prevent tau from binding to microtubules and performing its normal functions. Eventually, microtubules become depleted in neurons which develop axonopathy and failure of axonal transport.

Aluminum is an inhibitor of PP2A activity in brains of experimental animals and *in vitro* [92, 187]. Aluminum accumulation in neurons of AD-vulnerable regions of human brains probably accounts for the low PP2A activity and hyperphosphorylated tau found in brains of AD-affected humans. Most hyperphosphorylated tau accumulates in the cytoplasm of pyramidal cells, in gray matter, where the tau participates in the formation of NFTs. About 8–11% of hyperphosphorylated tau in AD remains in the white matter [184].

Aluminum interaction with hyperphosphorylated tau causes the tau to aggregate into granules of increasing size that eventually fuse to form cytoplasmic pools in human neurons [102]. These pools consist of an aluminum/hyperphosphorylated tau complex (Fig. 7). Unbranched highly-ordered filaments polymerize within this cytoplasmic complex, in certain large hippocampal and cortical neurons of AD brains. The filaments have two distinct domains: a protease-sensitive fuzzy coat and a protease-resistant segment of tau that forms the filament core [188]. As the filaments grow and increase in numbers they ultimately develop into NFTs.

A study by Harrington et al. [121] identified early AD-type changes in tau protein processing and Aβ formation in brains of (non-DES) renal dialysis patients with prolonged sub-acute exposure to the high aluminum levels contained in phosphate binders. This study was based on brains from 15 renal dialysis patients with a mean age of 57.1 ± 2.2 years, compared with five AD cases (age 61.8 ± 2.2 years) and six healthy controls without any neurological disorder (59.8 ± 5.0 years).

Brain aluminum levels of the study groups

For some analyses, brains from the 15 dialysis cases were assigned to two sub-groups: a higher brain aluminum group ($n = 6$) averaging $11.4\ \mu g \pm 1.1\ \mu g$ aluminum/g brain tissue (d.w.) and a lower brain aluminum group ($n = 9$) with an average aluminum value of $4.7\ \mu g \pm 0.6\ \mu g$/g brain tissue [121]. These aluminum measurements, based on gray matter dissected from the frontal cortex, were performed with GFAAS. Brains from the controls had considerably lower aluminum levels, averaging 1.95 μg/g brain tissue. Brain aluminum measurements were only available for 2/5 AD brains; these averaged 2.55 μg aluminum/g brain tissue. Brain aluminum levels of the dialysis patients correlated with their serum aluminum levels ($r = 0.772$, $p = 0.008$), prepared from blood samples taken during the dialysis period [121].

Hyperphosphorylated tau accumulation in brains of dialysis patients

Depletion of normal tau and accumulation of hyperphosphorylated tau correlated with brain aluminum levels [121]. The high aluminum group of dialysis patients showed significantly greater depletion of normal tau from gray matter than the low aluminum group ($p = 0.047$) and controls ($p = 0.011$). Normal tau in gray

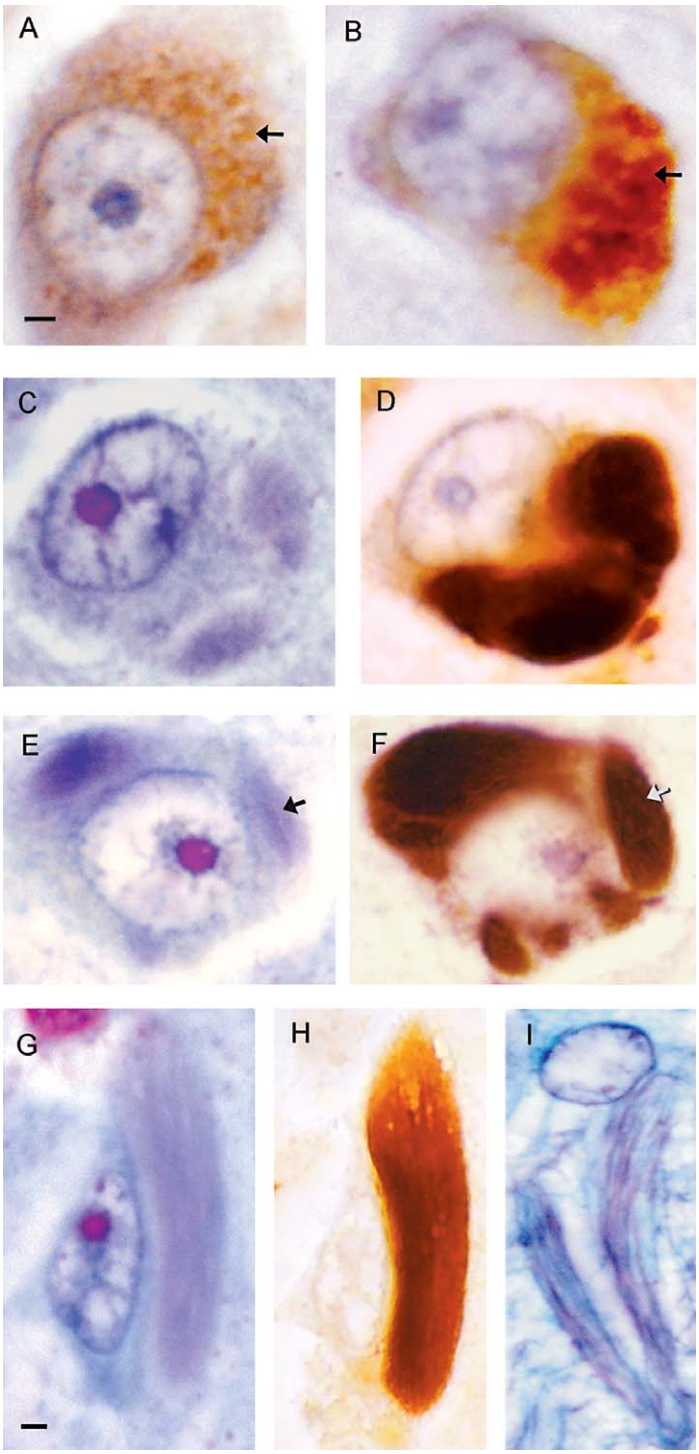

Fig. 7. Formation and growth of NFTs in AD hippocampal neurons. A, B) Pre-tangle pyramidal neurons stained for hyperphosphorylated tau show small (A) and larger, fused (B) granules (arrows). C, D) Cytoplasmic pools that form from fused granules stain for (C) aluminum and (D) hyperphosphorylated tau. E, F) Nascent filaments (arrows) are just visible in cytoplasmic pools stained for (E) aluminum and (F) hyperphosphorylated tau. G, H) As the filaments increase and grow they form recognizable NFTs that continue to stain for aluminum (G) and hyperphosphorylated tau (H). I) A ghost (extracellular) NFT stained to show aluminum (purple filaments). Magnification bars = 2.5 μm for A–F, 2 μM for G and H, 2.5 μm for I. Reprinted from [102] with permission from IOS Press.

matter of the high aluminum dialysis group amounted to 1.21 units (U)/g brain tissue, compared to 6.02 U/g in the low aluminum dialysis group and 8.10 U/g in the control group.

Five of the six brains in the high aluminum group and two of the nine brains in the low aluminum group exhibited strong bands for hyperphosphorylated tau in their gray matter, confirming that hyperphosphorylated tau increases with rising aluminum levels in brains from dialysis patients ($p = 0.034$), provided that the rate of aluminum accumulation is sufficiently low to avoid precipitation. Aged rats that chronically consumed dietary aluminum throughout their adult life likewise developed hyperphosphorylated tau in their cortical and hippocampal neurons [92].

Tau truncation in brains of dialysis patients

In AD, the hyperphosphorylated tau becomes pathologically truncated near its C-terminus by the enzyme caspase [189, 190]. Accumulation of truncated tau protein is a prominent AD-type change in tau that also occurs in dialysis cases [121]. Aluminum activates types of caspase involved in tau truncation [191]. Accumulation of C-terminally truncated tau in white matter strongly correlates with brain aluminum levels ($r = 0.753$, $p = 0.001$) [121] with 2.47 U of truncated tau protein/g white matter for the high brain aluminum group and 0.97 U/g in the low brain aluminum group ($p = 0.037$). Accumulation of truncated tau further correlates with patient age but not with durations of dialysis treatment or uremia.

Filament core tau accumulates in gray matter, correlating inversely with normal tau depletion from white matter ($r = -0.573$; $p = 0.025$). Filament core tau in white matter also shows strong inverse correlation with normal tau depletion from white matter ($r = -0.802$; $p < 0.001$) [121]. Overall, most truncated tau is found in gray matter in AD with about 10% in white matter [184].

NFT filaments in brains of dialysis patients

Transmission electron microscopy revealed twisted ribbon filaments in the supernatant fraction of brain homogenates from two dialysis patients (i.e., one in the high and the other in the low aluminum groups) who had the highest levels of truncated tau and hyperphosphorylated tau in their brains. These filaments were indistinguishable from the twisted ribbon filaments that compose NFTs of AD brains [121]. Brain tissue from the low aluminum case that had twisted ribbon filaments also exhibited NFTs.

Aβ deposition in brains of dialysis patients

The study by Harrington et al. examined for Aβ in the two dialysis groups, the AD group and the control group [121]. Eight of the 15 brains from dialysis patients exhibited immunoreactivity for fibrillar Aβ, including 5/9 in the low aluminum group and 3 out of 6 in the high aluminum group.

Six dialysis brains showed diffuse Aβ deposits: five in the low brain aluminum group and one in the high aluminum group.

Comparison of AD and dialysis patient neuropathology

AD-type changes observed in the brains of dialysis patients took place over a much shorter period of time and at higher levels of plasma and brain aluminum than those that occur in AD patients. The observed changes relate both to the formation of NFTs and Aβ plaques. Depletion of normal tau and accumulation of hyperphosphorylated tau as well as tau truncation are well-documented features of NFT formation in brains of AD cases. Hyperphosphorylation and truncation of tau occurred in the brains of dialysis cases in an aluminum-dependent manner. That is, the tau changes significantly increased with rising levels of brain aluminum. As for Aβ, brains of the AD group had significantly more Aβ content (197 ng/g) than the entire dialysis group (3.61 ng/g; $p < 0.001$), which, in turn, had significantly more Aβ content than the control group (0.15 ng/g; $p = 0.036$). Aβ deposits were qualitatively similar but were smaller in brains from dialysis cases compared to Aβ deposits in brains from AD cases [121].

The observed frequency for AD-type changes in the brains of dialysis patients, whose ages ranged from 38 to 68 years, are reported to significantly exceed the expected frequency of changes (at 0 to 1%) in the 38 to 68 year age group ($p < 0.0001$). The observed AD-type changes in the dialysis groups correspond to the expected frequency for AD cases above age 80. Thus, the findings by Harrington et al. [121] support a role for aluminum in AD pathogenesis.

Other hypotheses proposed to cause AD

Viruses and prions

Conventional viruses, prions, and soluble forms of metal neurotoxicants have previously been proposed as the environmental component of AD causality. The arguments for and against the putative infective causes have been reviewed at some length in another open access publication [143]. As at time of writing, there is

more evidence against these proposals than for them, making them unlikely candidates for the role of the environmental component.

The essential metals iron, copper, and zinc are crucial to human survival and they have numerous and redundant controls over their content and activities. They are subject to states of dietary deficiency and excess and can be corrected with restoration of normal conditions. Lead, mercury, and aluminum are non-essential neurotoxicants that cause tissue damage.

Iron

Many researchers have observed that iron changes in AD, in its distribution and concentrations in the AD-affected brain [192]. Iron, like copper, is a redox metal that, if uncontrolled, generates reactive oxygen species (ROS) that cause free radical damage. As will become evident in this paper, iron becomes dysregulated in AD and iron levels rise in cells of AD-affected brain regions. Excess iron and aluminum, co-localized in the brain, interact in a number of ways, producing synergistic effects on oxidative stress, AβPP upregulation, and Aβ aggregation. The author regards the metabolic activities of aluminum and iron as too interwoven to effectively dissociate their separate contributions to AD. Moreover, iron might continue to be controlled in AD were it not for aluminum interference with iron homeostasis.

Zinc

Zinc also has numerous essential functions in the brain, in health, and in disease. Zinc modulates NMDA and AMPA receptors and is critical in the non-amylodogenic processing of AβPP and in the enzymatic degradation of the Aβ peptide. According to Watt et al. [193], there is no consensus on whether zinc concentrations are elevated, depressed, or unchanged in brains of AD patients. Zinc's role in Aβ aggregation has been described as the most well-established contribution that zinc may make to AD pathogenesis [193].

Copper

Copper has also been proposed as a candidate for AD causality. Copper is important in many enzymes for normal brain function such as Cu/Zn-superoxide dismutase (SOD1), an antioxidant defense enzyme. Moreover, BACE1 (responsible for the second cleavage that releases Aβ) has a copper-binding site. Whether or not this is problematic in AD is unknown. Uncontrolled copper produces ROS and oxidative stress.

Copper dysregulation has been described as a candidate cause of neurodegenerative disorders on the basis that copper is a redox metal required for cellular respiration that may be involved in free radical production via the Haber-Weiss reaction and cause mitochondrial damage [194]. Most evidence for copper toxicity in AD relates to its effects on amyloidogenesis, which is also stimulated by iron and aluminum.

Mice were dosed with 0.13 mg/L copper sulfate for 90 days. The dosed aged mice were reported to have a four-fold increase in copper levels in brain capillaries and a 1.5-fold increase in brain $A\beta_{40}$ and $A\beta_{42}$ levels [195]. The authors noted that excess copper interacts with AβPP and Aβ and may contribute to AD via cellular stress. Paradoxically, low copper levels have been suggested by others to disrupt brain Aβ by altering its production and slowing Aβ clearance [195]. Also, copper deficiency activates microglia and astrocytes and elicits glial and neuronal responses, mostly in the cerebral cortex and thalamus [196]. Brain levels of copper in AD patients are yet to be established before determining whether human copper levels are raised, lowered or unchanged in AD. Nevertheless, evidence is lacking that copper has any role in NFT formation, AD progression, or AD-type dementia.

Lead

Weuve and Weisskopf [197] pose the question, "Does exposure to lead contribute to the risk of dementia?" Lead is a potent neurotoxicant that has been shown to damage the nervous system, particularly during development. Lead accumulation in the brain has decreased the intelligence quotient in children [198], especially those who have eaten flakes of lead paint. Animal-based evidence indicates that exposure to lead in early life may increase expression of the AβPP gene in later life [197]. Individuals who survive lead poisoning in childhood could suffer neuronal death at the time of poisoning, thereby reducing their neuronal redundancy in a way that compromises the brain, and increasing their vulnerability to rapid cognitive decline in later life. Lead also produces oxidative damage, mitochondrial dysfunction, and brain inflammation [199].

Lead exposure is associated with impaired cognition and accelerated cognitive decline. Human exposure to lead was greatest between 1925 and 1980, from leaded gasoline in motor exhaust and lead-based paints. Lead in fuel was phased out between 1976 and completely banned by 1995 in the US so lead levels from that source should be declining in the population. Lead paint can contain up to 50% lead (by weight) so paint removal still poses a risk of lead exposure for some

workers. Also, lead in plumbing, and the use of lead crystalware and lead-glazed pottery can contaminate drinking water and other beverages. Almost all brain lead level studies have focused on children. Whether lead accumulates in the aging brain is uncertain. Lead produces toxic effects on the cardiovascular system.

Weuve and Weisskopf conclude that the data needed to answer whether lead exposure increases the risk for dementia is absent because high quality lead exposure has seldom coincided with high quality dementia assessments and the studies have been underpowered to detect subtle effects [197]. However, lead is a pro-oxidant that causes oxidative stress, erodes antioxidant activity in the brain, increases AβPP expression, and induces glial cell reactivity [197], a hallmark of brain inflammation, as in AD.

Mercury

A systematic review with a comprehensive search strategy was carried out in 2010 to investigate whether there is sufficient published data to determine whether inorganic mercury plays a role in AD [200]. Out of 1041 articles initially identified, 88 met the authors' inclusion criteria. Forty studies were based on mercury-exposed humans, either from workers with occupational exposure or amalgam bearers. The most widely-cited study was based on gold miners in the Philippines who use mercury without protection. The gold is extracted by adding liquid mercury to form gold-amalgam and separated from the mercury, in most cases, by heating the gold-amalgam in the open with blow-torches. The miners had significantly worse scores for attention span and memory tests that always correlated well with greater mercury excretion into the urine. Another group of studies involved follow-up, ranging from 5 to 30 years, of workers with past exposure to mercury. The study that investigated workers 30 years after their exposure to mercury found significantly more tremor and lack of coordination in ex-workers compared to controls without reporting any increase in dementia over non-exposed controls.

Other studies examined for mercury in AD patients (either living patients or autopsy studies). One showed significantly higher mercury in plasma of AD patients than controls and another found significantly more mercury in brain samples from 14 AD patients compared to controls [201]. Mercury was found to accumulate in the cerebellum, putamen, thalamus, upper parietal and occipital lobes of the AD brains. A recent study [128] observed no difference in the amount of mercury in hippocampal samples from AD patients and controls.

The systematic review included five animal studies that showed the mercury level in brain tissue was higher in an experimental group of rats exposed to mercury vapors than in controls. Two of these found mercury in the cerebellum after exposure.

Some *in vivo* and *in vitro* studies have shown that mercury interferes with microtubule polymerization. One reported abnormal mercury interaction with the metal/GTP/tubulin complex similar to that which occurs in microtubules with aluminum exposure [24]. Aluminum could substitute for Mg^{2+}, the physiological regulator of microtubule assembly, in the metal/GTP/tubulin complex, but substitution by mercury for Mg^{2+} would be thermodynamically unfavorable, given that mercury is much larger than Mg^{2+}.

Mercury is a pro-oxidant that causes oxidative stress, diminishing the brain's antioxidant activity, increasing AβPP expression, and inducing glial cell reactivity. Mercury interferes with membrane structure and leads to neurofibrillary aggregates and axonal degeneration [200]. Mercury exposure at 50 μg/L (a relatively high level) is reported to induce a 30% reduction in glutathione levels of neuroblastoma cells within 30 minutes, increases the release of $A\beta_{40}$ and $A\beta_{42}$ in up to 6 hours, and doubles tau phosphorylation over a 9-hour exposure period whereas pre-incubation with melatonin reverses these effects.

The systematic review concluded that mercury produces paradoxical findings [200]. Epidemiological and other human studies suggest a weak relationship between mercury and AD, noting that the mild cognitive deficits observed in the miners' attention span and memory ability did not necessarily transition to dementia. Contrastingly, much of the evidence from the experimental studies was consistent with neuropathological features of AD.

A comparison of aluminum and other metal toxicants proposed to cause AD

Some AD traits can be induced by mercury and lead as well as aluminum. Aluminum parallels all of the lead and mercury-induced AD neuropathological features, and others, without elevating lead or mercury levels in the brain. The shared AD changes may represent common tissue responses to neurotoxic metals, but there are notable differences. These involve different magnitudes of bioavailability and different distributions in the brain.

Firstly, mercury accumulates in the cerebellum, putamen, thalamus, upper parietal and occipital lobes of AD patients [201]. It seems that mercury-induced

neuropathological features would only be relevant to AD if mercury preferentially accumulated in the hierarchy of AD-affected brain regions as does aluminum, in particular, the entorhinal cortex, hippocampus, amygdala, olfactory lobe, neocortical association areas, basal nucleus of Meynert, dorsal raphe nucleus, and locus coeruleus [82].

Another distinguishing feature between aluminum and any of the other proposed candidates for AD causality discussed above is that aluminum is the only one of these metals that is routinely consumed by many humans in processed foods and municipal water supplies [5, 6], providing a continuous source of bioavailable aluminum for uptake into the blood and brain, and enabling aluminum to progressively accumulate to toxic levels in AD-vulnerable brain regions of many humans. The ubiquity of aluminum salts as food and water additives, combined with their GRAS status, could prevent the recognition that each individual dose, while apparently safe, may cumulatively contribute to disease in almost 35 million people, the number currently estimated to have AD.

The amyloid cascade hypothesis

The present author acknowledges the research effort by the scientific community over the decades exploring Aβ and its role in the brain. The amyloid hypothesis posits that a pathological cascade culminating in AD starts when a mutation in AβPP leads to Aβ deposition in the brain, causing tau hyperphosphorylation and NFT formation that in turn cause neuronal death and dementia [202]. These brief statements hardly do justice to that scientific effort, but for present purposes will have to suffice.

The amyloid cascade hypothesis is essentially genetically based, and in the presumed absence of an environmental component. The hypothesis has yet to explain how a genetic condition could spread so rapidly throughout the world population within one century. On the other hand, accepting the presence of an environmental component may bring logic to the amyloid hypothesis.

Despite evolving over the years, the amyloid cascade hypothesis [202] has a number of shortcomings [203–208] yet to be addressed. (1) The human genome contains numerous gene mutations without accounting for the upregulation of AβPP gene expression observed in sporadic AD. (2) Some AβPP-transgenic mice develop more plaques per unit of brain tissue than aged humans without developing a progressive AD-like dementia. (3) Demonstration of $A\beta_{42}$ toxicity primarily depends on cultured cells exposed to commercial supplies of $A\beta_{42}$.

Significant concentrations of aluminum, iron, copper, and zinc co-localize with $A\beta_{42}$ in senile plaques [209]. The addition of either iron or aluminum to an $A\beta_{42}$ solution promotes the formation of β-pleated sheets of $A\beta_{42}$ and the aggregation of $A\beta_{42}$ to form fibrils [210]. These fibrils dissolved, or were prevented from forming, when incubated with the chelator DFO. On the other hand, zinc inhibited, and copper prevented, the formation of β-pleated sheets when incubated with $A\beta_{42}$.

House et al. [210] observed that a commercial preparation of $A\beta_{42,}$ dissolved in ultrapure water to make up a 1 μM $A\beta_{42}$ solution, contained 0.72 μM of aluminum and 0.39 μM of iron. The combined presence of these two metals in the $A\beta_{42}$, as provided by the supplier, was almost in an equimolar concentration to the $A\beta_{42}$. Measurable amounts of copper or zinc were absent. This finding suggests that the purported *in vitro* self-aggregation of $A\beta_{42}$ (alone), after being allowed to age for many months, may in fact require the presence of aluminum and/or iron ion catalysts [210], and raises doubt about the validity of other *in vitro* findings concerning Aβ that closely resemble metal toxicity, where commercial supplies of $A\beta_{42}$ may need optimal quality control.

Hardy [211] described how the amyloid cascade hypothesis came into being, admitting that adjudication of whether the amyloid cascade hypothesis is sustainable will depend on the application of successful therapy. As at the time of writing, attempts to block the formation of Aβ plaques by vaccines have failed in humans, ending in encephalopathy and death for more than a few AD patients. According to Castellani and Smith, many more human Aβ-blocking vaccine trials have been scheduled [203]. On the other hand, pharmaceutical companies were reported to be withdrawing funding for Aβ human vaccination trials with at least one research group having concluded that the science of the amyloid cascade hypothesis needed more critical examination [212].

Alzheimer himself rejected Aβ as a cause of AD [213], well before the amyloid cascade hypothesis was proposed. Upon observing the plaques he concluded they were an "accompanying phenomenon" to the disease on the basis that Aβ plaques were often absent from brain regions that showed obvious damage and were present in some undamaged regions. Chronic aluminum neurotoxicity can produce the symptomatic phenomena which have hitherto been interpreted by others as the causes in themselves.

CRITERION IV: TEMPORALITY

The temporal sequence: Chronic aluminum intake precedes chronic aluminum neurotoxicity and AD

Bradford Hill states, "My fourth characteristic is the temporal relationship of the association" [38]. Dietary aluminum leads to AD, a disease of the long-lived, rather than the young. Van Reekum et al. list the temporality criterion as "the demonstration of the appropriate temporal sequence, so that the causative agent occurs prior to the outcome" [39]. Exposure always precedes the outcome in causality analyses [214]. Chronic aluminum exposure must precede AD if chronic aluminum intake is the environmental cause of AD.

Aluminum uptake into the brain

Aluminum uptake into the brain begins early in antenatal life as aluminum is transferred from the maternal circulation to the fetal circulation via the placenta [215–217]. In one study, brain aluminum values in a human fetus and a 13-month old infant averaged 0.70 μg/g brain tissue (d.w.) [134]. In another, brain aluminum measurements were (mean ± SD) 0.30 μg ± 0.05 μg/g brain tissue (wet weight), approximately equal to a mean of 1.2 μg/g brain tissue (d.w.), in seven very young humans (three fetuses, one full-term infant, and three infants from ages two to six months). The blood-brain barrier is incompletely developed at birth and is even less mature in the fetus [218]. The rate of aluminum uptake into the brain dramatically slows (10^4 times) with maturation of the blood-brain barrier [219].

Aluminum is routinely absorbed from the contemporary urban diet throughout the lifespan. Many infants consume formulas with high aluminum levels. The aluminum content of reconstituted infant formulas from eight manufacturers averaged 226 μg/l, differing markedly between them with one product having a particularly high aluminum content of 551 μg/l [220, 221]. Cow's milk had a mean aluminum concentration of 70 μg/l. The aluminum content of human breast milk in the colostrum, intermediate, and mature stages remained stable at 23.4 ± 9.6 μg/l [221]. However, this level is expected to increase in mothers with higher plasma aluminum levels.

Aluminum is usually present in blood so minute amounts of orally ingested aluminum can continuously enter the brains [14–16] of aluminum-exposed subjects and accumulate with age [17–20]. As mentioned, aluminum and iron that cross the gastrointestinal and blood-brain barriers preferentially deposit in large pyramidal neurons of brain regions that are highly active with substantial iron requirements and have a high density of transferrin receptors on their surface [136–139]. Aluminum and iron also enter cells by transferrin-independent means [222, 223].

Four sub-cellular changes in brain physiology are known to occur in the prodromal phase that precedes the appearance of overt AD in humans and overt chronic aluminum neurotoxicity in rats. These include: progressive aluminum accumulation in neuronal cells, inhibition of PP2A activity, hyperphosphorylation of tau, and oxidative stress. Aluminum accumulation in neuronal cells is causal to all three of the other subcellular changes that occur at an early stage of AD.

Chronic aluminum intake precedes AD

Aluminum accumulates in the human brain from the fetal stage to old age [17–20]. Hence, humans living in industrialized societies accumulate aluminum in certain regions of their brains decades before reaching toxic levels and showing evidence of AD (Fig. 8, row 1). Aluminum can be observed at an early stage of its accumulation in the nucleolus of Walton-stained human cortical and hippocampal neurons before they show any trace of NFT formation [91, 136]. As aluminum accumulation progressively increases to toxic levels (stages IV and V), the cells develop morphological changes including NFT formation, granulovacuolar degeneration, and/or further intracellular aluminum accumulation. Cells with high-stage aluminum accumulation appear shrunken with collapsed structure, remaining alive in brain tissue without evidence of necrosis but are microtubule-depleted and too deteriorated to function as neurons.

PP2A activity is inhibited, resulting in hyperphosphorylated tau, at an early stage of aluminum accumulation

PP2A activity is inhibited in AD brains [185]. Low PP2A activity upsets the balance between phosphate addition to, and removal from, the microtubule protein tau [224] and other cellular proteins that require phosphorylation and de-phosphorylation for their function. Low PP2A activity thereby leads to hyperphosphorylation of tau and neurofilament proteins in neurons [185, 224].

Low PP2A activity also occurs in brains of rats with chronic aluminum neurotoxicity, accounting for the presence of hyperphosphorylated tau granules in their hippocampal and cortical neurons [92]. Small perinuclear granules that stain with PHF-1 for

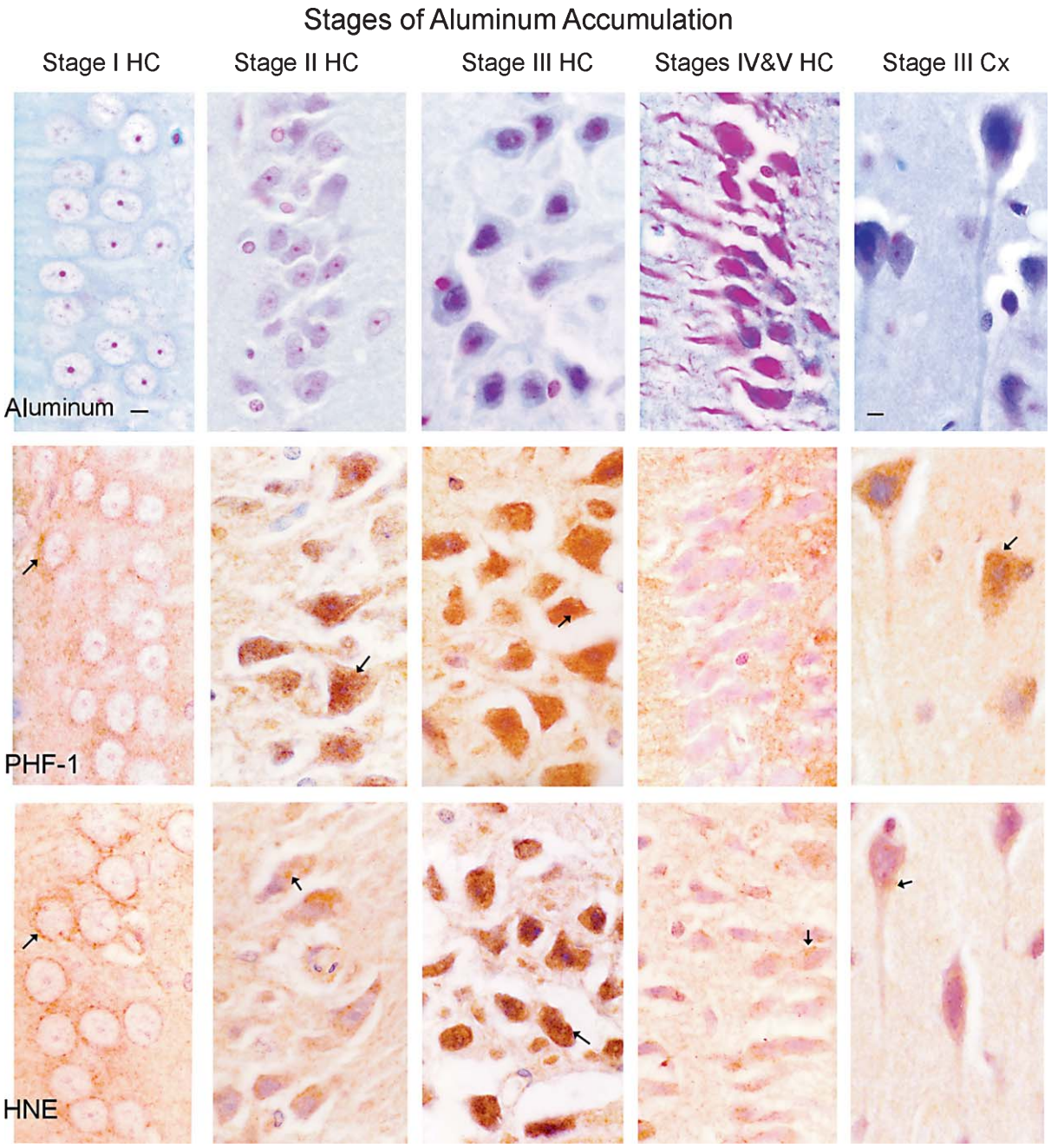

Fig. 8. Stages of aluminum accumulation in rat hippocampal and cortical neurons correlate with quantitative changes in hyperphosphorylated tau and 4-hydroxynonenal (HNE) adducts of proteins in brains of rats that chronically consumed aluminum-supplementation in their diet. (Row 1): Hippocampal cells (HC) at increasing stages of aluminum accumulation, processed with the Walton stain for aluminum; (row 2) HCs immunostained with the antibody PHF-1 for hyperphosphorylated tau (arrows); (row 3) HCs immunostained with the 4-hydroxynonenal antibody to reveal HNE adducts on proteins (arrows). HNE is a marker of oxidative stress. Columns 1–4 (left to right) HC CA1 cells; column 5: Pyramidal cells of layer V cortex (Cx). Micrographs in rows 2 and 3 illustrate equivalent cells in adjacent sections to those shown in row 1 demonstrating increase in antibody immunostaining with increasing aluminum accumulation but decrease from stage IV onwards due to deterioration of the cell structure. Some cells at stage I of aluminum accumulation (row 1, column 1) already show small amounts of immunoreactivity for hyperphosphorylated tau (brown perinuclear granules) and HNE (faint yellow-brown granules) indicating oxidative stress. Magnification bars = 10 μm, columns 1–4 and 5 μm, column 5. Reprinted from [92] with permission from Elsevier.

hyperphosphorylated tau can be seen in rat neurons that exhibit stage I aluminum accumulation (Fig. 8, row 2), evidence that low concentrations of intraneuronal aluminum are sufficient to inhibit PP2A activity and result in hyperphosphorylation. Increasing numbers of the hyperphosphorylated tau granules appear in neurons at intermediate stages (II and III) of aluminum accumulation [92]. By stages IV and V, the cell structure has deteriorated and these cells are grossly altered, showing little if any evidence of immunoreactivity for tau or tubulin [91, 92], appearing similar to the shrunken hippocampal cells seen in AD and rat brains stained for aluminum (e.g., Figs. 5C and E [91, 136]).

The sequence of events that leads from inhibition of PP2A to the formation of hyperphosphorylated tau, and ultimately to NFTs, occurs over years or decades in humans, commencing in the prodromal phase at a time when neurons are still at low stages of aluminum accumulation. Tau hyperphosphorylation affects increasing numbers of neurons in AD-vulnerable brain regions as more accumulate aluminum to stages where aluminum can be visualized. By stages IV and V, the aluminum has accumulated to toxic levels, and NFTs form in cat and AD brains containing aluminum at or more than 4 μg/g brain tissue [33, 134]. NFT formation occurs much more slowly in AD brains than in cat and rabbit brains directly injected with soluble aluminum. NFT formation is reported to take more than 380 days to form in brains of non-human primates directly injected with soluble aluminum [225] compared to 3–4 days in cat and rabbit brains [33, 124, 125].

Oxidative damage occurs early in AD and precedes amyloidogenesis

Oxidative damage has been called the "earliest event in AD" [226, 227]. Oxidative damage, like hyperphosphorylated tau, occurs at an early stage of aluminum accumulation in rats as indicated by the presence of 4-hydroxynonenal (HNE) adducts, a marker of oxidative stress, on neuronal protein at early stages of aluminum accumulation (Fig. 8, row 3) [92]. Faint yellow granules are still visible in some HNE-immunostained stage IV cells.

Oxidative stress precedes, and stimulates, amyloidogenesis with Aβ plaque formation in AβPP-transgenic mice [101, 227]. Amyloidogenesis occurs at some stage of the prodromal phase of AD because many non-demented controls show Aβ deposits [150]. Also, chronic aluminum intake has been shown to precede and increase the oxidative stress that modulates amyloidogenesis in brain tissue [101].

Length of the prodromal phases in AD and chronic aluminum neurotoxicity

AD and chronic aluminum neurotoxicity are each preceded by a long prodromal phase. The commencement of the prodromal phase for AD in humans and chronic aluminum neurotoxicity in rats depends on how the prodromal phase is defined. Hyperphosphorylated tau has been observed in the brain of an 11-year old child [228]. It is unknown whether this phenomenon heralds the beginning of the prodromal phase of AD or is a protective response to sequester incoming aluminum that appears and then resolves. This uncertainty makes the significance of hyperphosphorylated tau observed in a child's brain difficult to interpret.

The time at which the prodromal phase for AD commences may vary between individuals in accordance with the rates at which their neurons accumulate aluminum. The prodromal phase would start earlier, and be shorter, for those with higher than normal aluminum absorption and/or those that consume very high aluminum levels in their diet.

Historical perspective of aluminum usage

Since ancient classical times, alum deposits (aluminum sulfate, aluminum potassium sulfate) have been mined to obtain a chemical that can set bright dyes, tan leather, and clarify water. The earliest record of alum usage in water clarification was circa AD 77 when Pliny wrote that the argilla (ground alum-containing clay) of Italy could be used to make bitter water suitable for drinking [229].

Historically, water was cleaned in early urban drinking water treatment plants by slow filtration through sand. Reports indicate that nineteenth century French and English water engineers were reluctant to add alum or other aluminum compounds to drinking water supplies [230, 231]. Due to population growth, many of the world's cities converted their public drinking water supplies to rapid filtration that requires a coagulant, in the majority of cases alum [230]. The practice of using alum as a coagulant for rapid water filtration was instituted during the latter part of the nineteenth century at locations in Western Europe and the USA. The 20th century saw the establishment of numerous drinking water treatment plants, using rapid water filtration with alum as the coagulant. This process has provided clear drinking water over the long term to expanding urban populations on all five continents [230]. Some major cities continued to use slow water filtration well into the

20th century. For example, the McMillan Sand Filtration site supplied safe drinking water for Washington, DC, from 1905 to 1985 [232].

Coagulants aggregate aluminosilicate clay particulates that harbor microbes, into larger particles that settle out and can be removed by rapid filtration [230]. In principle, this should be an effective way to remove contaminants, including particulate aluminum, from drinking water. However, since the mid-1990s, alum treatment has been known to change the composition of aluminum in water, removing poorly-absorbed clay particulates that pass through the gastrointestinal tract largely unchanged while adding a small monomeric inorganic species of aluminum that can be more easily absorbed [233, 234]. Water data show that with alum treatment there is a 40–50% likelihood that the total aluminum concentration in clarified water will increase above the raw water aluminum concentration [235].

Most drinking water treatment plants continue to utilize rapid water filtration with alum-induced coagulation to clarify drinking water despite aluminum's classification as a proven neurotoxicant [21, 35] that is able to enter the brain from drinking water [14–16]. Articles are now appearing in the literature about the potential usage of aluminum nanoparticles for use in water clarification. Little is known about the body's ability to excrete aluminum nanoparticles. A few cities, such as Sydney, Australia [236] and the City of Paris, France [237], have chosen to exercise prudent avoidance of aluminum in their drinking water treatment, electing to utilize a low concentration of ferric salts that the body can metabolize.

In addition to alum treatment of drinking water, aluminum additives have been increasingly incorporated into growing numbers of convenience foods since the end of World War II. By 1982, almost 2 million kilograms of aluminum per year were used in the USA for all food additives [238]. In the year 2000, almost two million kilograms of aluminum were added to food in the USA for the sole purpose of binding dyes to foods, for coloring snacks and desserts [239]. The growth of additive use in the food industry [238, 239] saw an array of products containing bioavailable aluminum routinely consumed, for the first time in our evolutionary history.

Historical perspective of AD and other dementias

Reference was made to dementia more than 1700 years ago during the time of the Roman empire [240]. Throughout history, various types of dementia have been associated with (1) infections, e.g., neurosyphilis [241], kuru [242], Creutzfeldt-Jakob disease [243], and HIV-AIDS [244]; (2) genetic mutations, e.g., Huntington's disease [245], DS [246]; (3) vascular deterioration in the form of multi-infarct dementia [247]; (4) traumatic head injury, e.g., post-traumatic dementia [248] and dementia pugilistica [249]; and (5) environmental or lifestyle factors, e.g., dialysis dementia [35], Balint's syndrome (an occupational dementia) [36], ALS/PD [250], and Wernicke-Korsakoff Syndrome or alcohol-related dementia [251]. In 1979, AD accounted for closer to 50% of all dementia cases [252]. Currently, AD is the most common form of dementia in westernized societies, accounting for up to 85% of all dementia cases [2].

One feature that distinguishes AD from most other dementias is evidence that AD could be a new disease that developed throughout the 20th century [253, 254]. AD was first described at a scientific meeting in 1906 and published in 1907 [255]. In 1901, Alois Alzheimer, a neuropathologist then based in Frankfurt-am-Main, Germany, observed the progressive decline of a 51-year old female patient with pre-senile dementia in the municipal mental asylum. Following her death, Alzheimer analyzed her brain. In a report to the Southwest German Society for Psychiatrists, he stated "This is the first time I have seen this disease, either in the clinic or in the laboratory" [255]. He referred to the NFTs as "chemically changed neurofibrils" in the neurons of her brain and to the extracellular Aβ plaques as "milia". The words and tone of Alzheimer's report suggest that the woman's behavioral and neurochemical characteristics distinguished her condition from the more ubiquitous dementias of the day such as those associated with vascular deterioration, syphilis, and alcohol abuse. In 1911, Alzheimer concluded that the disease initially recognized in the middle-aged woman had the same neuropathology and represented the same disease that can occur in elderly humans [213].

There is evidence that AD was rare, if extant at all, during the 19th century [256]. A British Medical Association Health Survey, published at Cambridge in 1889, reported the results of medical assessments based on nearly 900 persons who had already lived to 80 years or older, including 74 centenarians. This survey reported that only two of 74 UK centenarians (2.7%) were affected by any form of dementia [256], stating "[Dementia, the] saddest state of all, was witnessed only in two of our centenarians ... Indeed, the brain in many held out as well or better than other organs – which may be regarded one of the bright rays, if not the brightest, in the centenarian landscape".

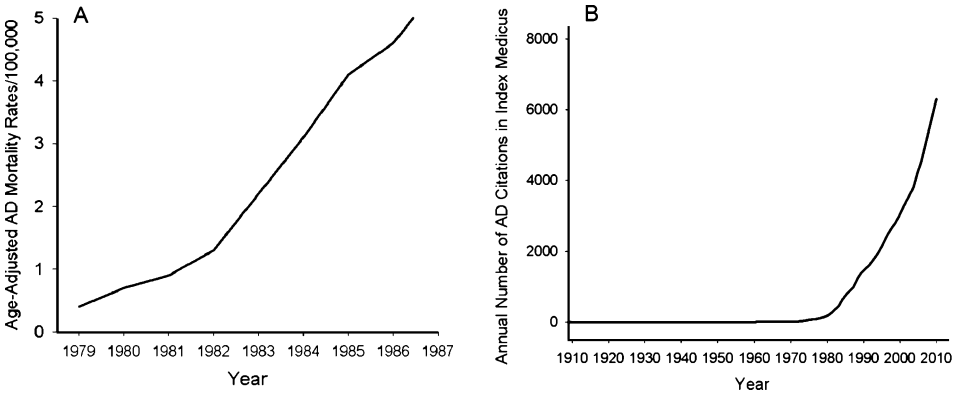

Fig. 9. AD historical statistics. A) Annual age-adjusted mortality rates for AD as the underlying cause of death in the US, 1979–1987. CDC, National Center for Health Statistics [263]. B) Annual proportion of AD citations per total number of citations in Medline between 1969 and 2012 on the subjects "Alzheimer's disease"/"presenile dementia"/"senile dementia"/"senile psychosis".

In view of the subjects' ages, one might reasonably assume that either or both of the two cases with dementia could have resulted from other dementias prevalent at that time. By the year 2000, 18 of the 20 centenarians in three towns in the Netherlands, with populations exceeding 25,000, were diagnosed with dementia, mostly AD, and the other two centenarians could not be interviewed [257]. A Fisher exact test indicates that the proportion of demented centenarians in these studies significantly increased between 1889 and 2000 ($p < 0.001$).

Brewer [254] uses US population statistics from 1900 and notes that 3,184,363 people were over age 60 at that time. He calculates that, at today's rate of AD prevalence, there should have been 36,000 people in the US suffering from AD around the turn of the previous century. Instead, he notes that other authors who published comprehensive medical books on neurological diseases and pathology around that time make no mention of a dementia with AD clinical characteristics and neuropathological features characterized by plaques and tangles. These include *The Complete Psychological Works of Sigmund Freud* in 24 volumes written between 1895 and 1939; William Osler's *Modern Medicine in Theory and Practice* (1910); William Richard Gowers' *A Manual of Diseases of the Nervous System* (1888); and William Boyd's *A Textbook of Pathology: An Introduction to Medicine* (1938).

Fifteen years after Alzheimer's 1911 paper, AD was described in *The Lancet* as a rare disease [258]. The reported number of known AD cases rose from one in 1907 to 14 by 1912 [259], 49 by 1931 [260] and more than 90 by 1935 [261]. Fred Plum, the founding President of the Society for Neuroscience, predicted in 1979 that an epidemic of AD was imminent [252]. AD was still described as an obscure condition in 1980.

Subsequently, the age-adjusted death rate from AD exponentially rose in the USA from 0.4 per 100,000 in 1979 to 25.1 per 100,000 in 2010 [263, 264] (Fig. 9A). In the 25-year span from 1980 to 2004 inclusive, the annual US death rate from AD, for persons aged 65 years and older, increased from 1,037 to 65,313 per year [263]. The annual number of published research papers on AD cited between 1969 and 2010 in the *Medline* database, searched on the categories "pre-senile dementia," "senile dementia," "senile psychosis" and "Alzheimer's disease", reflects the impact of the exponential increase of AD and public awareness of this condition from 1969 when publications on these topics were scarce, up to the present time (Fig. 9B).

The percent change between 2000 and 2010 in age-adjusted death rates showed almost a 40% increase in AD (Fig. 10) [264]. Over the same time interval, age-adjusted deaths from heart disease and stroke declined by 30.5% and 35.8%, respectively. The exponential increase in AD prevalence since 1979 may be partially explained by several contributing factors.

(1) Clinical criteria and diagnostic methods for AD improved.
(2) Adoption of the 10th Revision of the International Classification of Diseases (ICD-10) in 1999 enabled the US National Center for Health Statistics to name AD as a cause of death instead of other conditions that often end an AD patient's life such as pneumonia or stroke.
(3) Life expectancy increased greatly over the last 110 years and care for the elderly has increased.

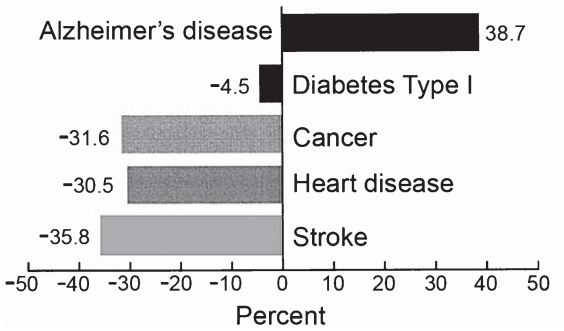

Fig. 10. Percent change in age-adjusted mortality rates for AD and other major causes of death in the US from 2000 to 2010. Reprinted from [264].

(4) Growth of the oldest sector of the US population has undoubtedly accelerated AD incidence and affected AD death rates [263].
(5) Martin Roth initiated clinico-pathological studies on the elderly in the 1960s and demonstrated that AD severity relates to its neuropathology in 1966 [265].
(6) Research interests change. The appearance of AD citations reflects the recognition in the US of AD as a disease rather than as a normal part of the aging process. This recognition, presented by Katzman in 1976 [266], stimulated research funding for AD in the years that followed.
(7) The range of tools for studying dementia is now larger and more readily available than previously. Sensitive antibodies detect the presence of Aβ and tau protein and techniques have been developed that provide images from inside the living human brain. Many research teams are refining cognitive assessments for AD and other forms of dementia.
(8) Pathological criteria for characterizing AD are also improving. There is much greater awareness among practitioners, researchers, and carers. AD is now fully recognized as a disease, albeit one without effective treatments.

During the same timeframe, aluminum's presence in AD brains and to a smaller extent in brains of aged controls has been verified in neurons by histological stains and microscopic techniques [136, 178] as well as analytical instruments (as in Fig. 11) [134, 175, 181]. Chronic aluminum neurotoxicity is involved in the neuropathology and altered behaviors [91–94, 121–124, 153–165, 175, 267–269] and in the progression of AD [143].

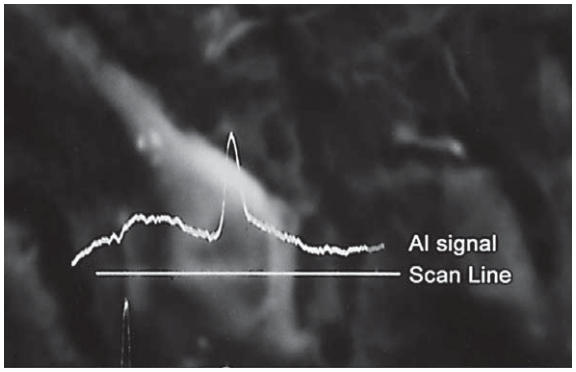

Fig. 11. Scanning electron micrograph of an NFT-bearing neuron viewed with back-scatter imaging, showing a spectrum generated from a line scan that reports wave-length dispersive X-ray data specific for the presence of aluminum. Note the aluminum peak as the scan line passes through the cell nucleus. Reprinted from [175] with kind permission from Dr. Perl and the AAAS.

Temporality of experimental evidence

The only definitive way to determine the temporal sequence is to conduct a prospective randomized controlled trial [39]. This allows the subjects to be studied both prior to and after exposure to a noxious agent. It would be highly unethical and impractical to carry out a prospective and interventional randomized controlled trial involving chronic aluminum addition to the diet of humans throughout most of their life, with one human group given aluminum at the high end of the human range for dietary aluminum to determine the outcomes. The only way the temporal sequence can be observed in this instance is by carrying out a randomized controlled trial with laboratory animals. As previously noted, humans and other mammals show remarkable similarities in their responses to metal toxicants [126].

In the main longitudinal study [82], healthy outbred wild-type (non-transgenic) male rats were allowed to develop normal brain function over their first year of life. All animals were trained to perform a T-maze task commonly used to test memory performance [95, 96] and performed this task with 70% to 100% accuracy prior to commencing aluminum supplementation. At age 12 months, the animals were given three different amounts of aluminum in their drinking water as described earlier. The aluminum contained in their feed was unchanged. Throughout their old age (24 months and older), the rats that consumed aluminum at the lowest level were able to maintain or improve the scores they achieved during middle age, indicating the amount of aluminum they orally consumed was insufficient to

accumulate to toxic levels in their brains. In contrast, 20% of the rats that consumed the low intermediate aluminum level and 70% of those at the high aluminum level obtained significantly lower mean T-maze performance scores in old age compared to their mean scores obtained in middle age. Thus, nine rats in the two experimental groups exhibited chronic aluminum neurotoxicity in old age with progressive cognitive deterioration and were eventually unable to perform the T-maze task.

The rats had consumed aluminum at human-relevant levels for at least 16 months (between ages 12 and 28 months) by the time their performance scores showed deterioration. The 16-month period that elapsed since commencing aluminum supplementation in the rats' diet until they exhibited cognitive deterioration was equivalent to approximately 47 human years [86].

The rats that exhibited cognitive deterioration also had higher mean serum aluminum levels and more brain cells that stained for high-stage aluminum accumulation than others in their treatment group that continued to perform the T-maze task as well in old age as in middle age [81, 82, 91]. An additional rat in the high aluminum group had an elevated serum aluminum level and exhibited declining scores but failed to survive enough weeks for his mean score in old age to be significantly lower than his mean score in middle age. Another in the high aluminum dose group developed cognitive deterioration in late middle age (at 58 years in human age equivalence). As will become evident, the author proposes that most brains of rats with chronic aluminum neurotoxicity lose their capacity around 27–28 months of age to undergo effective neural plasticity and compensatory repair for the functional loss induced by damage from aluminum that had accumulated in the brain by that age.

CRITERION V: BIOLOGICAL GRADIENT

Dose-response effects can be demonstrated in AD and chronic aluminum neurotoxicity

Bradford Hill states [38], "If the association is one which can reveal a biological gradient, or dose-response curve, then we should look most carefully for such evidence ... The clear dose-response curve admits of a simple explanation and obviously puts the case in a clearer light" [38]. Van Reekum et al. list this criterion as "The demonstration of a biological gradient, in which more of the causative agent leads to a poorer outcome" [39]. As will be shown, aluminum dose-response effects are demonstrable in AD and chronic aluminum neurotoxicity.

Dose-response effects in epidemiological studies of aluminum in drinking water and risk for AD

McLachlan et al. [68] observed an aluminum dose-response effect for increased relative risk of AD in humans who live in areas where aluminum concentrations in the water supply range between less than 100 μg/l up to 175 μg/l. The subjects' residential history for the 10-year period preceding their deaths was taken into account. A single pathologist made a histopathological assessment of neuritic plaques and NFTs in all 614 brains included in this study, taking into account the cases' clinical history of dementia, or lack of dementia, in order to ensure their correct assignment to AD and control categories.

McLachlan's study shows the estimated relative risk (RR) for AD, in subjects who drank water containing aluminum at or higher than 100 μg/l for at least 10 years before they died, was 2.6 (95% CI = 1.2–5.7) times greater than for those who drank water containing less than 100 μg aluminum/l [68]. Drinking water concentrations higher than 100 μg aluminum/l resulted in higher estimates of relative risk for AD. At 125 μg aluminum/l, the RR for AD was 3.6 (95% CI = 1.4–9.9). At 150 μg aluminum/l, the RR for AD rose to 4.4 (95% CI = 0.98–20) and, at 175 μg aluminum/l, rose further to 7.6 (95% CI = 0.98–61). Community water supplies, with high aluminum levels at 150 μg/l or more, have population bases too small to demonstrate statistically significant increases [68]. Regardless, this study shows a dose-response relationship between aluminum intake from drinking water and risk of AD.

Other epidemiological studies of drinking water and AD risk have also shown dose-response effects [58, 61].

Dose-response effect in the rat model for chronic aluminum neurotoxicity

In the main longitudinal rat study, in which three groups chronically consumed different aluminum dose levels, in amounts equivalent to those within the human total dietary aluminum range, nine rats exhibited chronic aluminum neurotoxicity in old age in an aluminum dose-response manner [82]. All (10/10) of the rats that consumed the low aluminum dose (contained entirely in their feed rations) performed their T-maze task as well or better in old age than in middle

age. In contrast, twenty percent (2/10) of the rats that consumed the intermediate aluminum dose and seventy percent (7/10) that consumed an aluminum dose equivalent to the middle and high end of the human total dietary aluminum range, respectively, attained significantly lower T-maze performance scores in old age than in middle age ($p = 0.015$).

CRITERION VI: BIOLOGICAL PLAUSIBILITY

The demonstration of a biological rationale

Bradford Hill notes [38], "It will be helpful if the causation we suspect is biologically plausible" and "the demonstration of a biological rationale, such that it makes sense that the causative agent causes the outcome" [38]. In the words of Van Reekum et al., "There is a greater likelihood of a causative relationship being present if it makes biological sense that A causes B" [39]. This section demonstrates biological plausibility for the contention that chronic aluminum intake can cause chronic aluminum neurotoxicity in humans with AD, and that aluminum indeed also causes the Aβ, Aβ plaques, and NFTs that have been presumed to cause AD. Thus, the animal model for cognitive deterioration from chronic aluminum neurotoxicity gives rise to a sequence of neurotoxic events that parallels those that occur in AD.

Aluminum bioavailability from the diet

Since the industrial revolution, humans have been, and are continuing to be, exposed to increasing levels of bioavailable aluminum. Aluminum is classified as a definite neurotoxicant [21], and yet, the US FDA allows aluminum salts to have a GRAS rating which in turn allows manufacturers of convenience foods and municipal drinking water suppliers to add aluminum compounds to foods and drinking water to enhance certain properties or fulfil certain functions [5, 6, 9].

Many residents of industrialized societies consume aluminum additives, from ingesting processed foods and drinking alum-treated tap water, in amounts that exceed the current provisional tolerable weekly limit set by the WHO, lowered in 2007 by the WHO to 1 mg aluminum/kg bw/week or 0.14 mg/kg bw/day [10]. Much ingested aluminum is trapped in the mucus lining of the gastrointestinal tract so it could be said that aluminum is poorly absorbed [12, 13], which may have been the basis for aluminum's GRAS rating. Nevertheless, humans (and experimental animals) do absorb a fraction of the ingested amount [14–16] and some individuals absorb 2–3 times more aluminum than others from a standardized aluminum dose (Fig. 2) [104–108].

Ingestion of a single aluminum dose, of the magnitude contained in processed foods and drinking water, might be insignificant if human consumption of aluminum were rare. On the contrary, aluminum additives are contained in a wide selection of convenience foods that are routinely consumed throughout the lifespan. Most people living in an industrialized society would have measureable plasma aluminum levels. The cumulative dosage of aluminum thus becomes important.

AD patients are among the "high" aluminum absorbers as they have been shown to absorb significantly more ^{26}Al dose than age-matched cognitive-intact controls from a standardized ^{26}Al dose contained in an orange drink [109]. Also, those with AD typically have significantly higher plasma/serum aluminum levels than controls [110–118]. The circulatory system of AD patients shows abnormally high aluminum levels in plasma ferritin that change with AD severity [120]. Plasma aluminum levels of humans correlate with their brain aluminum levels [121].

The minute amounts of aluminum that continuously enter the brain throughout life, are supplied by dietary aluminum and other sources of bioavailable aluminum. Aluminum enters the brain more readily than exits, leading to a net accumulation of aluminum in the brain with increasing age. This is even the case for aged non-demented controls which generally contain about 1.5 μg aluminum/g brain tissue [17–20]. There is a small margin of safety between tolerable and intolerable neurotoxic aluminum levels. The gray matter of AD-vulnerable regions of AD-affected brains contains a modest 2- to 3-fold increase in their aluminum levels compared to the same regions of brains from controls [127–129]. Brain aluminum levels greater than 4 μg/g brain tissue are sufficient to induce electrophysiological defects and NFT formation in brains of cats and rabbits [33, 134, 135].

Aluminum's preferential accumulation in AD-affected brain regions

Animal studies and dialysis patients have shown that brain aluminum preferentially accumulates in the entorhinal cortex (Fig. 6A) and, to a lesser extent, in other brain regions particularly susceptible to damage in AD [91, 94, 145]. Studies of AD in humans also indicate that the entorhinal cortex is damaged earlier, and ultimately more severely, than other brain regions [130–133, 277–279].

The preferential deposition of aluminum in brain regions susceptible to damage in AD relates to the aluminum ion's physical resemblance to ferric iron ions, in that cellular molecules and cells cannot readily make a distinction. Both types of ions have similar ionic radii and the same (3+) charge [22]. Aluminum-binding to transferrin, an iron transport protein, enables 90% of the aluminum absorbed into plasma to circulate throughout the body without rapid filtration into the urine [26]. Transferrin-bound iron attaches to transferrin receptors on capillary endothelium to move across the blood-brain barrier and to transferrin receptors on the neuronal surface that are taken into the interior of the cell [142, 144]. Transferrin-bound aluminum crosses the blood-brain barrier and enters neurons by the same mechanisms [27] even though neurons have no known need for aluminum.

Aluminum particularly accumulates in the large corticocortical and corticosubcortical neurons of memory-processing regions that project to certain other AD-affected brain regions [91, 94, 145]. These highly active cells have great demands for iron and, to accommodate their needs for iron importation, have a high density of transferrin receptors on their plasma membrane [137–139]. Aluminum enters neurons by transferrin-dependent means, transferrin-independent means, and by anterograde and retrograde transfer from large corticocortical neurons in other brain regions to which neurons in memory-processing regions are connected [141].

Aluminum causes damage upstream of Aβ, NFTs, and GVD and significantly contributes to their formation [91, 92, 102, 160, 210, 269, 270–276]. AD-damaged brain regions are generally marked by NFTs and Aβ plaques with GVD occurring mostly in hippocampal neurons. NFTs form in the aluminum-affected cytoplasm of cells whereas Aβ plaques commonly form in the extracellular matrix where synapses are degenerating [132]. These AD hallmarks can also be viewed as indicators of aluminum's presence in human brain tissue.

A major characteristic of AD progression in humans and progression of chronic aluminum neurotoxicity in experimental animals is that both have a long prodromal phase during which time aluminum is gradually accumulating in neurons, particularly in the hierarchy of AD-vulnerable brain regions. The prodromal phase leads to a relatively brief period of amnestic mild cognitive impairment, during which time memory problems are becoming evident. Atrophy is already detectable at this stage upon comparing serial magnetic resonance images (MRIs) of the human entorhinal cortex and hippocampus [277–279]. This stage is followed by overt AD in humans and cognitive deterioration in rats with chronic aluminum neurotoxicity that increase in severity over time.

The entorhinal cortex is at the top of the hierarchy of AD damage, followed by the hippocampus and other limbic regions [91, 94, 130–133]. The entorhinal cortex is, by far, the region in AD brains observed to contain the most NFTs, based on NFT counts made in 39 cortical areas of brains from AD cases and controls [130]. The next brain regions to be affected in the AD hierarchical order are the neocortical association areas. These are followed by sub-cortical regions including the basal nucleus of Meynert, locus coeruleus, and dorsal raphe nuclei [146–148]. There is a certain amount of heterogeneity in the order of AD-affected brain regions from one case to another, particularly after the neocortical association areas have been affected [143].

The entorhinal cortex normally relays information between the hippocampal formation and neocortical association areas through its perforant pathway that bi-directionally connects the hippocampal formation with the neocortex, thus playing crucial and pivotal roles in memory-processing in normal individuals. The entorhinal cortical cells of origin for the perforant pathway become irreversibly damaged in humans with AD and in rats with chronic aluminum neurotoxicity, showing parallel pathology [132, 143].

Loss of microtubule support

Cells with NFTs and cells with high-stage nuclear aluminum accumulation contain substantial amounts of aluminum. Cells that exhibit either of these conditions are depleted of their microtubules [91, 153, 280, 281]. Microtubules normally provide structure to cell bodies, their dendrites, and axons and are crucial for intracellular communication and transport of organelles, neurotransmitter vesicles, and other substances, necessary for normal cell function, between the cell body and its distant synapses. Microtubule depletion results in collapse of the cell body and dieback of the cell processes. The consequences of microtubule depletion are more devastating than those of the traditional AD hallmarks, Aβ plaques, NFTs, and GVD because cells without microtubules are unable to function as neurons.

Aluminum accumulation in AD-vulnerable brain regions has many AD consequences of which microtubule depletion is central to brain atrophy and functional failure. The neurotoxic sequence of effects that stems from microtubule depletion (Fig. 12) include

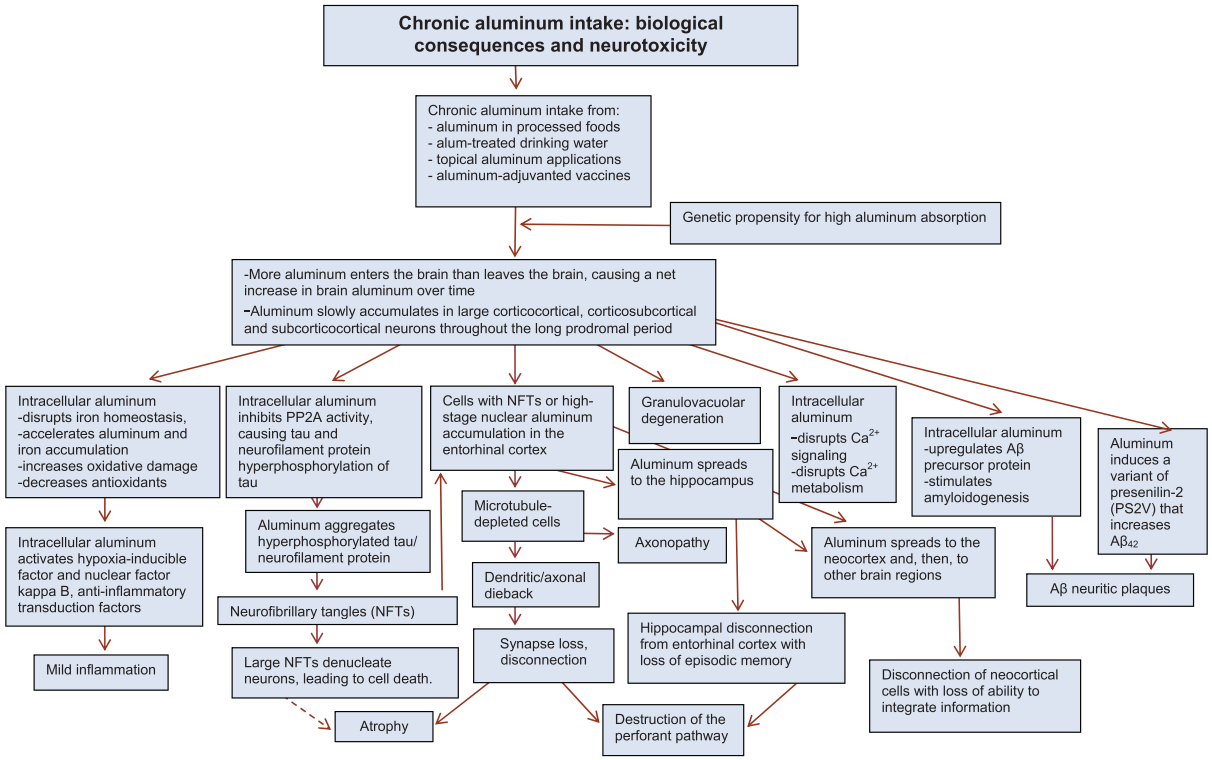

Fig. 12. Flowchart showing toxic and pathological consequences of chronic aluminum intake in brains of humans and some experimental animals.

(1) axonopathy with failure of axonal transport [93, 269]; (2) dendritic and axonal shriveling or dieback [282–284]; and (3) loss of synapse density [91, 285, 286] due to synapse breakdown as the cell processes shrivel, retract and disintegrate. These processes culminate in (4) disconnection [287] that, in turn, results in (5) atrophy and (6) loss of the affected brain region's function [91, 94, 131, 132]. Depleted of their microtubules, neurons are reduced to poorly-differentiated cells that survive in the tissue, sometimes for decades [288].

The hippocampus, in AD, is affected by aluminum accumulation in the same manner as the entorhinal cortex. Entire bands of adjacent neurons in the CA1 field, or subiculum, become aluminum-rich and microtubule-depleted, presenting in brain tissue as a substantial lesion or scar amidst the delicate neuronal network [91, 151]. These cells undergo the same neurotoxic sequence of effects that occurs in the entorhinal cortex. All rats in the longitudinal studies that developed cognitive deterioration had at least one substantial lesion consisting of aluminum-rich, microtubule-depleted cells in the hippocampal CA1 field or subiculum. Conversely, lesions are absent in these brain regions from all rats that remained cognitively intact [91]. AD brains generally have lesions consisting of clusters or bands of contiguous cells that contain NFTs [151], which show positive staining for aluminum when appropriately stained [102, 136].

Progressive dissociation and atrophy of brain regions

The first disconnections between brain regions in AD are likely to isolate the hippocampal formation from the entorhinal cortex [131, 132]. Isolation of the hippocampal formation occurs at a relatively early stage of AD in humans and cognitive deterioration in rats, resulting in loss of episodic memory [91, 94, 130–133, 143] (Fig. 12).

The entire neurotoxic sequence is repeated in the amygdala and other limbic regions and in neocortical association areas [147, 287]. Aluminum's spread to, and accumulation in, the neocortical association areas results in microtubule depletion, NFT formation, and disconnection, diminishing the ability of the neocortical association areas to receive and integrate incoming information. The same process, subsequently repeated in the basal nucleus of Meynert, results in attentional deficits and a sharp decrease in cholinergic neurotransmission.

The process repeats as aluminum continues its spread throughout the AD hierarchy of affected brain regions [141]. Additional cortical and sub-cortical regions, particularly those that project heavily to the cortex, are similarly affected, displaying the same pathological sequence that culminates in disconnection, atrophy, and functional loss of the affected brain region [143, 287], thereby intensifying AD severity. Eventually, AD patients lose all types of memory except those for motor skills [132]. Brain regions least affected in AD are the motor cortex and primary sensory areas.

Global loss of neurons is not a feature of AD [289]. Instead, cell death mainly occurs in specific brain regions in cells that form large NFTs [136]. Cortical atrophy largely derives from widespread dieback of the dendritic tree that accounts for 95% of the neuronal volume [290–293].

CRITERION VII: COHERENCE

The relationship between cause (chronic aluminum intake) and effect (AD) are fundamentally compatible with existing knowledge

Hill states, "The cause-and-effect interpretation of our data should not seriously conflict with the generally known facts of the natural history and biology of the disease." Van Reekum et al. restate Bradford Hill's seventh criterion as "Coherence of the findings, such that the causation argument is in agreement with what we already know." [39].

Now that the biological rationale has been described for chronic aluminum intake as a cause of AD, it is necessary to anchor the features of the rationale to known aspects of AD. There is a greater likelihood that chronic aluminum intake causes AD if this putative causal relationship coheres with existing knowledge about the behavior of aluminum in biological tissues and the natural history and biology of AD, including its progression and neuropathology. This section also discusses the most salient features of AD and chronic aluminum neurotoxicity that indicate AD is a human form of chronic aluminum neurotoxicity.

The prodromal phases of AD and chronic aluminum neurotoxicity show striking similarity

Aluminum slowly accumulates in the brain throughout life [17–20]. AD in humans and chronic aluminum neurotoxicity in rats are both slowly-developing conditions preceded by a long clinically-silent prodromal phase that often continues into old age [81, 82, 228]. The silence is broken by a mild cognitive impairment that intensifies, and often converts within a few years to overt AD in humans [277–279] and in an equivalent time (several weeks) to overt chronic aluminum neurotoxicity in rats [82]. Both conditions are characterized by irreversible memory impairment that becomes increasingly severe as other cognitive abilities are lost [82, 277–279].

The slow rate of aluminum uptake into the brain could account for the long prodromal phase required for aluminum to accumulate to toxic levels in neurons of aged experimental animals that develop chronic aluminum neurotoxicity in old age and in elderly humans that develop AD [1, 82]. By the time behavioral change is evident in affected individuals of both species, significant deterioration has already occurred in neurons of the entorhinal cortex, hippocampus, and other limbic regions [91, 94, 131, 132, 277–279]. Neuronal damage in the entorhinal cortex and hippocampus is sufficient to result in their disconnection, resulting in hippocampal isolation accompanied by loss of episodic memory (memory for recent events) [132].

Serial MRIs reveal prodromal atrophy in these brain regions. In one MRI study, cognitively-intact individuals, some with and others without a maternal history of AD, were given MRI on trial entry and again on a 2-year follow-up visit [277]. When compared, MRIs of the subjects with a maternal history of AD indicated measurable gray matter atrophy, particularly affecting the precuneus, parahippocampal gyrus (including the entorhinal cortex), and hippocampal region, with cerebrospinal fluid expansion.

A reason that neuronal damage can be masked for a considerable time in the prodromal phase is that neuroplasticity is stimulated in healthy neurons to compensate for damage occurring in neighboring neurons [290]. Axons of the neurons that have remained healthy branch and send out collaterals. These collaterals reinnervate other neurons in their vicinity that have become denervated as a result of damage in neural cells to which the denervated neurons normally connect. However, compensatory neuroplasticity eventually exhausts in brains of humans with AD [290, 291].

Aluminum accumulation in aged human neurons is very common (Figs. 5, 7). The author has yet to observe a single large corticocortical neuron in any aged human cortical or hippocampal section that has stage 0 aluminum accumulation; i.e., that is aluminum-negative when processed with the Walton stain for aluminum. Most large neurons of aged non-demented controls are

at stage 1, staining magenta for aluminum only in the nucleolus. Cortical and hippocampal regions from gray matter of aged non-demented controls have mean aluminum levels that measure around 1.5 μg/g brain tissue (d.w.) [127–129, 134]. Their aluminum values tend to be relatively uniform in multiple samples taken from the same region. In contrast, mean aluminum values for gray matter taken from the same regions of aged AD brains are more variable. On average, they generally measure around 3.5 to 4 μg/g brain tissue (d.w.) or higher, due to the presence of toxic lesions ("hot spots") in some gray matter samples [127–129, 134].

Sub-cellular changes associated with the prodromal phase are: (1) progressive aluminum accumulation in the brain; (2) inhibition of PP2A activity; (3) appearance of hyperphosphorylated tau, a precursor to NFTs; and (4) oxidative stress that gives rise to ROS and precedes amyloidogenesis; (5) cortical atrophy can also be seen at a relatively early stage. It is noteworthy that features (2) to (5) are all consequences of intracellular aluminum accumulation [31, 92, 93, 187, 294, 295].

The animals of the two longitudinal studies were entering old age, by convention, at 24 months. This is approximately equivalent in age to a 70-year-old human [86]. The rats that were ultimately affected by chronic aluminum neurotoxicity were still in the prodromal phase at age 24 months, looking physically sleek, alert, and continuing to perform their T-maze task skilfully, showing no outward difference from the low aluminum controls in their appearance or behaviors.

This phase of chronic aluminum neurotoxicity drew to a close around 27 months of age for eight rats that began to make more mistakes than usual in their weekly T-maze performances [82], presumably reflecting mild cognitive impairment. By 28 months, it became noticeable that these rats were increasingly choosing the wrong arm of the T-maze when seeking rewards, indicating damage to their short-term (working) memory. They also made procedural errors, indicative of damage to their long-term (reference) memory [82], by stopping prematurely and searching for the reward in the wrong part of the maze arm. Each of the aluminum-affected rats obtained a significantly lower mean score in old age than in middle age. Eventually, the rats looked confused when placed in the T-maze as if they no longer knew what to do. They formerly refrained from urinating or defecating in the T-maze, possibly because of the association with food rewards but once cognitively-affected were incontinent in the T-maze.

Chronic aluminum neurotoxicity was overt in eight of the nine aluminum-affected rats by age 28 months as they obtained significantly lower T-maze performance scores in old age than those obtained in middle age [82] and demonstrated behavioral changes. One other rat with chronic aluminum neurotoxicity had a shorter prodromal phase. This rat stopped performing the T-maze task in late middle age when 20 months old, reminiscent of the 5–10% of humans that develop AD in middle age. A possible explanation is that this rat developed an early form of chronic aluminum neurotoxicity because of its routinely high aluminum intake (as a member of the high aluminum group), coupled with a genetically higher rate of aluminum absorption. This rat's serum aluminum level was high (64.8 μg/l±) compared to the mean (±SD) value (14.8 ± 4.1 μg/l) of low aluminum controls and compared to other members of the high aluminum group (34.8 μg/l ± 18.7 μg/l). All rats with chronic aluminum neurotoxicity exhibited behaviors consistent with AD-type cognitive deterioration [81, 82]. Examples of these behaviors, an aluminum-affected rat's T-maze performances in middle age and old age, and a maze performance from an extremely old rat are documented in a supplementary video clip accompanying this article.

With the passage of time, aluminum continued to accumulate in, and spread to, increasing numbers of neurons in additional interconnected brain regions of the AD hierarchy [143]. Chronic aluminum neurotoxicity in rats without therapeutic intervention is irreversible as is currently AD. All rats were given an opportunity to perform the T-maze task each week until their terminal condition was observed [82] but the rats with chronic aluminum neurotoxicity failed to improve. All rats in the main longitudinal study except one retained normal kidney and liver functions in old age and had lifespans averaging 31.3 months [82]. This approximates 91 human-equivalent years [86].

Aluminum accumulates to toxic levels in AD-affected brain regions of humans with AD and rats with chronic aluminum neurotoxicity

Overt chronic aluminum neurotoxicity and AD coincide with toxic levels of aluminum in foci or toxic lesions consisting of groups of large aluminum-rich microtubule-depleted cells located in AD-vulnerable brain regions [91, 129, 134]. Some groups cite, in addition to the mean aluminum value plus standard deviation (SD), the percentage of samples that were measured and found to contain toxic aluminum levels. The percentage of such samples depends on the

severity of the AD cases examined [129, 134]. Toxic aluminum levels in various mammalian species are associated with additional neuropathology including NFTs, neuropil threads, nuclear aluminum accumulation, Aβ deposits, and hippocampal GVD [91, 101, 124]. Chronic aluminum neurotoxicity in transgenic mice whose neurons express high levels of human mutant AβPP, contain sufficiently high levels of brain aluminum to provide the oxidative environment necessary for Aβ formation [101, 296]. Sub-cellular neuropathology associated with toxic lesions includes NFTs and neuropil threads, microtubule depletion, dendritic dieback, synapse breakdown, and altered electrical activity [33, 91, 94]. The toxic lesions are surrounded by cells with a normal appearance [91]. Brain aluminum levels correlate well with NFT levels [134].

Aluminum is most prone to accumulate in brain regions particularly susceptible to damage in AD and chronic aluminum neurotoxicity

Neuropathological examination indicates brains of rats with chronic aluminum neurotoxicity show parallel pathology to brains of humans who have died with overt AD except for species-specific differences. Examination of completely-sectioned rat brains revealed aluminum staining in large corticocortical, corticosubcortical, and subcorticocortical neurons of specific brain regions homologous to those that are NFT-damaged in humans with end-stage AD [82, 297]. These rat brain regions include the entorhinal cortex, septum, hippocampus, subiculum, amygdala, temporal and parietal association areas, frontal and cingulate cortices, olfactory bulb, piriform cortex, basal nucleus of Meynert, substantia nigra, dorsal raphe nucleus, and the locus coeruleus [82].

There are reasons why aluminum specifically deposits in these particular cell types of the same regions—in particular, memory-processing regions—of human and rat brains that are affected by AD and chronic aluminum neurotoxicity. These cells have very high energy needs to perform their functions and send information via their axons over great distances to other brain regions. The high rate of energy utilization increases the demand for imported iron. These cells have a high density of transferrin receptors on their surface to facilitate iron uptake. However, since aluminum uses the same mechanisms as iron for entry into neurons, aluminum is also taken up by these cells even though they have no need for aluminum.

Neurotransmission declines in AD and chronic aluminum neurotoxicity

AD is accompanied by reduced levels of acetylcholine, norepinephrine, serotonin, glutamate, and aspartate neurotransmission [298–305]. Loss of cortical cholinergic innervation has been widely reported in AD [306]. However, NFT numbers are variable in cholinergic neurons of the nucleus basalis of Meynert, being plentiful in some AD brains and sparse in others [130].

Tetrahydrobiopterin (BH4) deficiency is characteristic of late-stage AD [303, 307]. BH4 is a co-factor required for the hydroxylation of phenylalanine, tyrosine, and tryptophan in the synthesis of the monoaminergic neurotransmitters dopamine, norepinephrine, and serotonin, respectively [308]. Furthermore, BH4 modulates the release of neurotransmitters. Impaired synthesis of catecholamine neurotransmitters may result from tetrahydrobiopterin inhibition [303].

Many experiments have shown that aluminum-exposed animals have reduced levels of acetylcholine, catecholamines, and other neurotransmitters [309–318]. Aluminum injection into the lateral ventricles of rabbit brains rapidly elevates their aluminum levels to toxic levels observed in AD brains and results in NFT formation [124, 145]. The same treatment significantly reduces the levels of serotonin in rabbit brain by 43% in the frontal pole, 40% in the posterior parietal cortex, and 50% in the entorhinal cortex [311]. Rabbit brain glutamate levels reduce by 21% in the frontoparietal cortex and 33% in the posterior parietal cortex. Norepinephrine declines by 5% in the frontal pole, 42% in the entorhinal cortex, and 20% in the hippocampus. Aspartate concentrations fall by 42% and taurine levels by 35% in the posterior parietal cortex. Choline acetyltransferase, a marker for acetylcholine activity, is depressed by 14% and 27% in the hippocampal and entorhinal cortex, respectively [311], coinciding with the appearance of NFTs in these AD-vulnerable brain regions [145]. Another experiment showed that intraventricular-injected aluminum damages the cingulate bundle in rat brains, resulting in severe anterograde degeneration of cholinergic terminals in the hippocampus and cerebral cortex [319].

Chronic aluminum intake inhibits BH4 synthesis in experimental animals [77], depressing the levels of serotonin and its 5-hydroxyindole acetic acid metabolite [316]. Overall, results from these animal studies indicate that neuronal populations responsible for neu-

rotransmitter generation are susceptible to damage in AD and in animal studies designed to mimic brain aluminum levels.

Oxidative damage is a feature of AD and chronic aluminum neurotoxicity

AD brain tissue exhibits relatively high levels of oxidant activity [320–324]. Oxidative damage commences relatively early in the prodromal phase of AD [226, 324], probably preceding the formation of plaques and tangles. Evidence for oxidative damage in prodromal brain tissue includes numerous reports of increased lipid peroxidation and decreased antioxidant levels, in particular, decreased levels of superoxide dismutase and glutathione peroxidase [321, 324].

The oxidative properties of aluminum are well-established [31, 32, 76, 294, 295]. Aluminum, as a strong Lewis acid, is capable of producing oxidative stress [31, 92, 101, 293]. Aluminum is a pro-oxidant on its own [31, 294, 295] and synergistically with iron [31, 32, 76, 294, 325, 326]. Oxidative stress arising from aluminum treatment of animals and cell cultures modifies neuronal proteins and fatty acids by inducing HNE and isoprostane adducts, respectively [92, 101], increasing membrane lipid peroxidation [165] and elevating hydroxylation of nucleic acid bases as indicated by the marker 8-hydroxy-2-deoxyguanosine (8-OHdG) [327]. Chronic aluminum exposure exhausts both superoxide dismutase and glutathione peroxidase levels in brains of experimental animals [29, 165, 295]. Aluminum's oxidative properties contribute to neuroinflammation by upregulating genes that have promoters enriched in binding sites for stress-inducible transaction factors; namely, the nuclear factor for kappa B (NFκB) and hypoxia inducible factor (HIF-1) [122].

Attempts have been made to counteract oxidative damage in AD patients by supplementing the diet with vitamin E. The natural form of vitamin E available in foods (γ-tocopherol) shows more promise for this purpose than α-tocopherol [328, 329].

Neuroinflammation in AD and chronic aluminum neurotoxicity

Marked cortical gliosis is a prominent feature of AD neuroinflammation [330]. Gliosis is marked by elevated levels of glial fibrillary acidic protein (GFAP) in reactive astrocytes [330]. The AD brain is associated with activated microglia, reactive GFAP-producing astrocytes, and pro-inflammatory cytokines [331–335]. Gene microarray experiments that have profiled the expression of 12633 genes in AD hippocampal CA1 tissue showed generalized depression in normal neuronal gene transcription and 3-fold or more increases in mRNAs for apoptotic genes, pro-inflammatory signaling genes, including the NFκB and HIF-1 transduction factors, AβPP, and cytokines [336, 337].

Similarly, gene microarrays of normal human neural cells, exposed to aluminum in primary cultures, showed upregulated gene expression for NFκB, AβPP, cytokines, and other inflammatory proteins [122]. In fact, 18/24 (75%) of pro-inflammatory and pro-apoptotic genes, that are either upregulated or down-regulated by a factor of 3-fold or more in AD brain tissue [336] are also altered in aluminum-exposed human neural cells [122]. Exposure of human neural cells to both aluminum and iron produces a synergistic effect on their gene expression [338]. Aluminum also down-regulates gene expression by condensing chromatin in the cell nucleus [339].

Mice chronically exposed to 0.01, 0.1, or 1 mM aluminum lactate in their drinking water show a neuroinflammatory response, exhibiting increased expression of NFκB, AβPP, and inflammatory cytokines in their brains [267, 340–345]. The lowest of these levels is less than aluminum levels that increase AD prevalence in geographical regions where aluminum concentrations are elevated in residential drinking water [68, 71, 72, 75]. Microglia become activated in the presence of aluminum [319, 346] and astroglia become reactive and immunopositive for GFAP, as in AD [319, 347].

Axonopathy and metabolic changes that occur in AD and chronic aluminum neurotoxicity

Aβ plaques have long been regarded as a marker of AD and are important for AD diagnosis. Axonopathy and altered metabolism of AβPP and Aβ products are likewise important features of AD [336, 337, 348–351]. Stokin's group [352] link axonopathy and failure of axonal transport to Aβ plaques, neuropil threads, and NFTs, observing that axonopathy precedes Aβ formation by at least one year in a mouse model for amyloidogenesis. They suggest that reductions in microtubule-dependent axonal transport may stimulate AβPP cleavage in cells, leading to senile plaques and AD.

Brains of rats chronically fed human-relevant dietary aluminum levels [93] and brains of rabbits exposed to aluminum via intracerebral injection [269] both exhibit hippocampal and cortical axonopathy

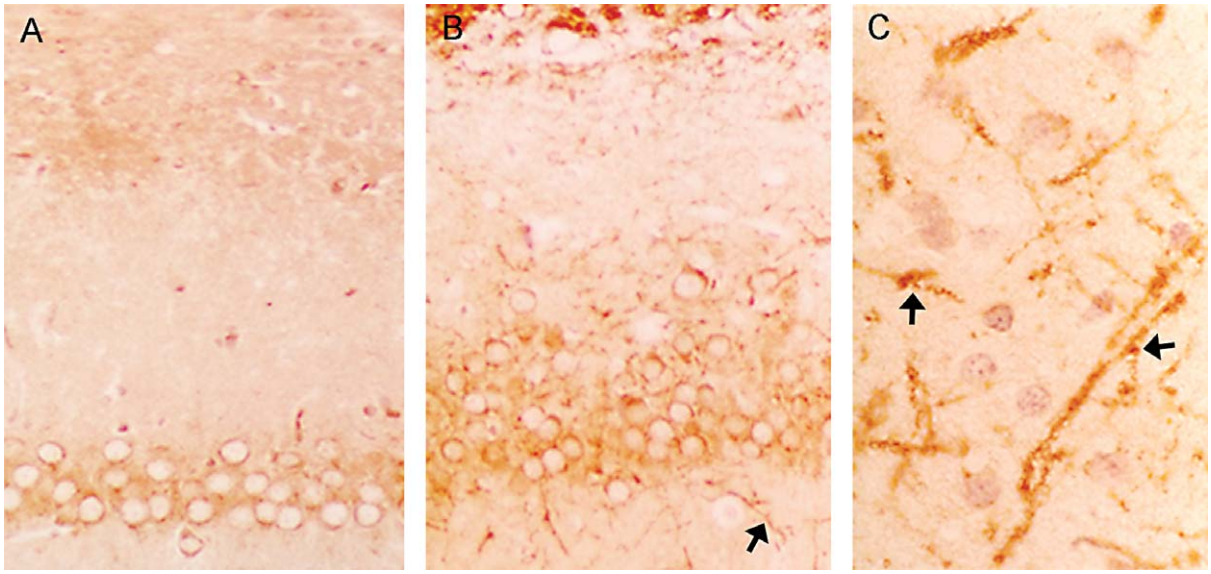

Fig. 13. AβPP immunoreactivity in brains of rats without and with chronic aluminum neurotoxicity. A) Hippocampal cell field from an aged cognitively-intact low aluminum control. Note absence of swollen neurites (axonopathy) in and around the multilayered CA1 pyramidal cells. AβPP immunoreactivity is weak in white matter (at top of micrograph). B) Axonopathy, demonstrated by AβPP immunoreactivity, can be seen in a hippocampal cell field from a rat with chronic aluminum neurotoxicity. Swollen and distorted neurites, in and around the hippocampal CA1 cells, are immunoreactive for AβPP. AβPP reactivity is intense in white matter (top). C) Cortical region from a rat with chronic aluminum neurotoxicity shown at higher magnification. Swollen neurites, showing axonopathy, are immunoreactive for AβPP, with irregular diameters and knotted or varicose appearance. Arrows: varicosities and constrictions. Magnification bars: A, B = 10 μm; C = 5 μm. Reprinted from [93] with permission from Elsevier.

whereas intrathecal injection of aluminum in rabbits produces axonopathy in motor neurons [353]. Immunostained brain tissue of the aluminum-exposed rats and rabbits reveals AβPP immunoreactivity in deformed axons of hippocampal and cortical pyramidal cells [93, 269], distinguished by varicosities and constrictions that indicate congestion of axonal flow and impaired delivery of AβPP along the length of axons (Fig. 13) [93]. Axonopathy is likely to result from aluminum-induced microtubule depletion.

Several types of experimental systems have demonstrated aluminum involvement at various stages of amyloidogenesis [91, 93, 98, 101, 210, 270–276]. Aluminum is present in aged human neurons of AD-relevant brain regions [136] and in Aβ plaques of AD brains [123, 209, 354]. In neurons, nanomolar amounts of aluminum are sufficient to upregulate expression of the human AβPP gene, in response to oxidative stress mediated by NFκB and HIF-1 transduction factors [122]. The addition of iron further enhances this effect [338].

AβPP mRNA expression increases in brains of rats chronically exposed to dietary aluminum compared to low aluminum controls ($p < 0.01$) (Fig. 14) [93]. AβPP has been intensively studied but its functions are still poorly understood. This integral membrane protein is a

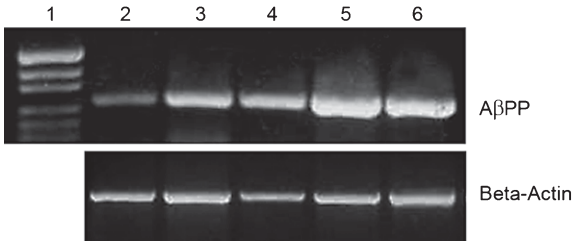

Fig. 14. RT-PCR analysis for AβPP mRNA in rat brain. Lane 1, pUC19; lane 2, negative control (macrophage cell line RAW 264.7 mouse macrophage negative control); lanes 3 and 4, brain tissue from aged low aluminum rat controls; lanes 5 and 6, brain tissue from aged rats that exhibited chronic aluminum neurotoxicity. β-Actin bands are shown as controls. Reprinted from [93] with permission from Elsevier.

member of a large family that includes AβPP-like proteins with functions sufficiently similar to substitute for AβPP where deficient. Brain AβPP has been associated with neuronal migration and brain development; the formation and maintenance of synaptic structure and synaptic transmission; neurite outgrowth, increasing spine density, dendritic lengthening, and dendritic arborization; and neuronal repair. Most research attention has been given to AβPP cleavages and its cleavage products, including Aβ.

sAβPPα, a secreted non-amyloidogenic cleavage product of AβPP, is important for promoting neurite growth and maintaining brain tissue. Nanomolar concentrations of aluminum redirect AβPP cleavage from its non-amyloidogenic pathway to its amyloidogenic pathway by inhibiting 90% of the phosphorylation activity of protein kinase C (PKC) [270] which is required for AβPP cleavage along its non-amyloidogenic pathway [271]. Aluminum's interference with PKC phosphorylation thus favors AβPP cleavages that result in an amyloidogenic product (Aβ). Aluminum and iron both oxidize soluble human Aβ, changing its structure from an α-helical random coil conformation of soluble Aβ to a fibrillar beta-sheet conformation that precipitates and forms plaques in brains of humans and certain other species [98, 99, 210].

Transmission electron microscopy, combined with energy-dispersive X-ray spectroscopy, reveals aluminum's presence (Fig. 15) in an individual Aβ fiber of an AD plaque core and its absence in extracellular matrix surrounding the plaque. This ultra-high resolution technique demonstrates aluminum's presence in Aβ fibers of AD brain and shows aluminum is well-placed in proximity to Aβ to affect its metabolism in various ways: by (1) increasing Aβ oligomerization and (2) stabilizing Aβ oligomers [272]; (3) aggregating Aβ fibrils [210, 355, 356]; and (4) forming Aβ deposits that stain for thioflavin S as do Aβ plaques [273]. Also, (5) aluminum forms Al/Aβ complexes with plasma Aβ that increase Aβ penetration of the blood-brain barrier [357] and are purported to be more toxic than aluminum or Aβ on their own [272, 296, 357].

Two of the many important studies that relate aluminum to AβPP and Aβ deserve special mention: AβPP gene expression was upregulated in the brains of human mutant AβPP-transgenic (Tg 2576) mice fed a diet supplemented with aluminum for nine months [101]. The aluminum treatment resulted in oxidative stress which in turn accelerated and increased the formation of Aβ plaques in brains of the aluminum-supplemented group compared to a cohort of human AβPP-transgenic mice fed a diet without aluminum supplementation. Logically, aluminum could produce the same effects on AβPP and Aβ in human brains as the effects produced by aluminum on human AβPP and Aβ metabolism in brains of AβPP-transgenic mouse brains [101].

The brains of a third cohort, that consumed a diet supplemented with aluminum plus vitamin E, resembled control brains in regard to their Aβ plaque content [101], suggesting that vitamin E is protective against aluminum-induced Aβ increase.

Secondly, $A\beta_{42}$ levels are increased in AD brain tissue by mutant presenilins [358]. $A\beta_{42}$ is the type usually found in plaques and it is generally regarded as more toxic than smaller Aβ peptides. Interestingly, chronic aluminum exposure, followed by a short hypoxic period, induces a variant form of presenilin 2 (PS2V) in human neuroblastoma cells [274]. The same PS2 variant occurs in AD-affected brain regions of humans with AD, particularly in the hippocampal CA1 field, and is diagnostic for sporadic AD. PS2V interferes with AβPP maturation, impairs the unfolded protein response signaling pathway, and significantly increases $A\beta_{42}$ formation [275, 276]. These results indicate that PS2V and its deleterious effects are also induced by intraneuronal aluminum in brains of humans with sporadic AD.

Oxidative stress, axonopathy, and even apoptosis (programmed cell death) have been attributed, by some authors, to the Aβ peptide. However, aluminum and its oxidative stress occur in cells prior to upregulation of AβPP gene expression. Therefore, aluminum either on its own or as an Al/Aβ complex is a likely cause of the oxidative stress attributed to Aβ. This section shows that intraneuronal aluminum is influential in AβPP metabolism and $A\beta_{42}$ formation.

Low PP2A activity, hyperphosphorylated tau, and NFTs in AD and chronic aluminum neurotoxicity

NFTs represent another neuropathological hallmark of AD important for the diagnosis of AD [297, 359]. NFTs increase in numbers as AD progresses, correlating well with AD duration and severity [360–362]. The low PP2A activity that occurs in some aged brains, particularly those affected with AD [185], gives rise to hyperphosphorylation of tau and neurofilament proteins that are important components of NFTs in human brains [92, 102, 121, 185, 228, 363–366]. Normal neurofilament and tau proteins decrease as their hyperphosphorylated forms increase in brain regions of humans with AD and aluminum-exposed animals [363, 367]. Pre-tangle cells in human brains as well as in rabbit brains contain neurofilament protein that is gradually replaced by tau protein [124, 125, 364, 365]. Hyperphosphorylated tau eventually predominates and is integral to the mature structure of NFTs in AD brains [92, 102, 121, 187–190, 365].

Aluminum progressively accumulates in neurons of AD-affected brain regions [136–139]. Also, aluminum is an inhibitor of PP2A activity [92, 187]. Since

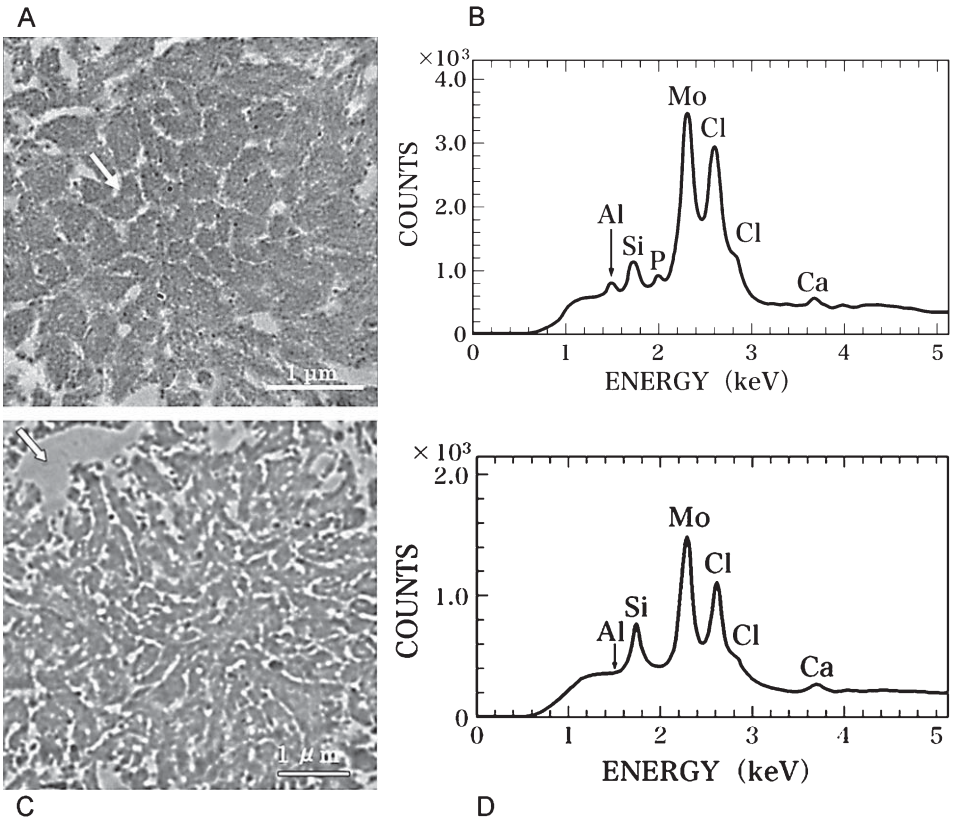

Fig. 15. Aluminum presence in an Aβ plaque core. A) An Aβ fiber is designated by an arrow in the core of a senile Aβ plaque examined by transmission electron microscopy by energy-dispersive X-ray spectroscopy. B) A small peak for aluminum is clearly visible in the Aβ fiber shown in (A) at the position in the spectrum corresponding to elemental aluminum. C) Surrounding extracellular space examined by the same technique. D) An aluminum peak is undetectable in the spectrum corresponding to the surrounding extracellular material shown in (C). Reprinted from [123] with kind permission from Dr. Yumoto and Elsevier.

aluminum preferentially accumulates in neurons of vulnerable regions of AD brains, and aluminum has been shown to be an inhibitor of PP2A activity that results in hyperphosphorylation of proteins such as tau, aluminum that has accumulated in neurons could well account for the low PP2A activity and hyperphosphorylated tau found in brains of AD-affected humans [185].

Aluminum has been shown to participate in NFT formation and growth, both in pre-tangle and tangle-bearing cells of AD brains [102]. Intraneuronal aluminum interacts with hyperphosphorylated tau in AD hippocampus and cortex prior to NFT formation, aggregating the hyperphosphorylated tau into granules that grow by fusion [102, 366–368]. Aluminum also interacts with hyperphosphorylated tau during NFT formation and growth since a cytoplasmic complex of aluminum/hyperphosphorylated tau gives rise to the NFT filaments (Fig. 7) [102].

NFTs form in the brains of cats and rabbits given an intraventricular or intracerebral injection of aluminum and in the spinal columns of those given an intracisternal aluminum injection [33, 124, 145, 353]. Aluminum injected into cat and rabbit brains rapidly raises brain aluminum levels, and induces the formation of immature NFTs in regions of their brains homologous to regions of AD brains where NFTs are most prone to form [33, 124, 135, 145]. The NFTs induced by aluminum in cat and rabbit brains form within a few days after aluminum injection [33, 369]. The composition of the immature NFTs begins to change shortly thereafter, by acquiring tau. Aluminum also induces hyperphosphorylated neurofilament and tau proteins in cultured neuroblastoma cells and *in vitro* systems [370–372].

Newly-induced NFTs are primarily composed of straight filaments of neurofilament protein so they differ from mature human NFTs in ultrastructure as well as composition. Mature human NFTs comprise a mix-

ture of straight, twisted, and half-straight/half-twisted filaments with a core of hyperphosphorylated tau [188, 373]. The individual filaments of mature NFTs are commonly known as PHFs. PHFs viewed with atomic force microscopy appear to be single, ribbon-shaped filaments with twists having a periodicity at about 80 nm intervals, so it is unnecessary for two filaments to form and then intertwine with each other [374].

There is sufficient evidence to indicate that immature, newly-induced (nascent) NFTs should be regarded on a continuum with mature NFTs since the immature form evolves to resemble the mature form over time [124, 125]. Experiments using multiple antibody stains reveal that nascent NFTs of humans and rabbits have a hyperphosphorylated neurofilament protein phase that precedes their hyperphosphorylated tau phase [124, 125, 364, 375, 376]. Nascent human NFTs and rabbit NFTs gradually undergo compositional changes as they develop into their mature forms [143]. The molecular structure of maturing filaments in NFTs probably determines the stage at which the tau core polymer develops twists.

Granules that immunostain for hyperphosphorylated tau are seen in neurons of aged rats that have chronically consumed human-relevant aluminum levels (Fig. 8) [92]. These granules differ from granules that stain for hyperphosphorylated tau in the cytoplasm of human neurons [92, 102] (Fig. 7) that fuse and eventually form cytoplasmic pools of hyperphosphorylated tau with aluminum that give rise to NFT filaments in AD pyramidal and stellate cells [102]. In contrast, hyperphosphorylated tau that forms in rat neurons has less tendency to fuse and some of it appears in the nucleus as well as cytoplasm (Fig. 8) [92]. These species differences in the hyperphosphorylated tau produced by rats may interfere with their ability to form NFTs.

Another possibility relates to the observation of Goedert et al. [100] that adult rat neurons are only able to express 3-repeat tau isoforms whereas adult human neurons express all six tau isoforms, three with 3-repeats of the microtubule-binding domain and three with 4-repeats. All six tau isoforms are present in human NFTs [188].

Results from the two longitudinal studies of rats [81, 82] that develop chronic aluminum neurotoxicity in old age suggest that if rabbits, which can be induced by aluminum to form NFTs, were maintained to the end of their natural lifespan on an equivalent (in mg/kg bw) oral aluminum protocol, rather than giving them an intracerebral injection, the rabbit brains could be predicted to develop AD-like hyperphosphorylated tau in old age, as did the rats when they were old. If so, mature rabbit NFTs could be expected to resemble mature human NFTs.

Fine neuropil threads are also seen in AD brains. These dystrophic neurites are scattered throughout the extracellular matrix of AD-vulnerable brain regions [359]. Similarly, neuropil threads that stain for aluminum are seen in brain sections magnified to show extracellular matrix in regions homologous to AD-vulnerable brain regions from rats with chronic aluminum neurotoxicity (Fig. 5C) [91].

Hippocampal granulovacuolar degeneration is an AD hallmark inducible by aluminum

GVD is prominent in large hippocampal neurons in AD [359, 377–379]. This AD hallmark was first reported by Simchowicz [176], one of Alzheimer's students. Aluminum is the only agent reported to induce hippocampal GVD in experimental animals. GVD is induced by chronically feeding rats aluminum at human-relevant dietary levels throughout their middle age and old age [92] or by giving rats repeated intraperitoneal injections of aluminum over 60 days, a treatment that raises hippocampal aluminum levels [160, 268].

Microtubule depletion in AD and chronic aluminum neurotoxicity

Neuronal structure and function is dependent on their microtubules. Microtubules are a specialized part of the cytoskeleton that provide strength and rigidity to the cell body, its axon and dendrites. Microtubules provide a means for transporting cell constituents between the nucleus of the cell and its synapses (Fig. 16A).

Pyramidal cells that exhibit high-stage nuclear aluminum accumulation are consistently microtubule-depleted in brains of rats that show cognitive deterioration after chronically consuming aluminum at human-relevant levels (Fig. 16B and C) [91]. These pyramidal cells show an absence of tubulin immunoreactivity and failure of axonal transport [93]. Some human pyramidal cells also contain high-stage nuclear aluminum accumulation (Fig. 5E) or NFTs that stain for aluminum (Fig. 7) [175, 182]. Human cells in both conditions are microtubule-depleted (Fig. 16D and E) [280, 281].

Microtubule depletion can be studied by immunostaining with appropriate antibodies, or with transmission electron microscopy. Aluminum-induced microtubule depletion was reported as early as 1973

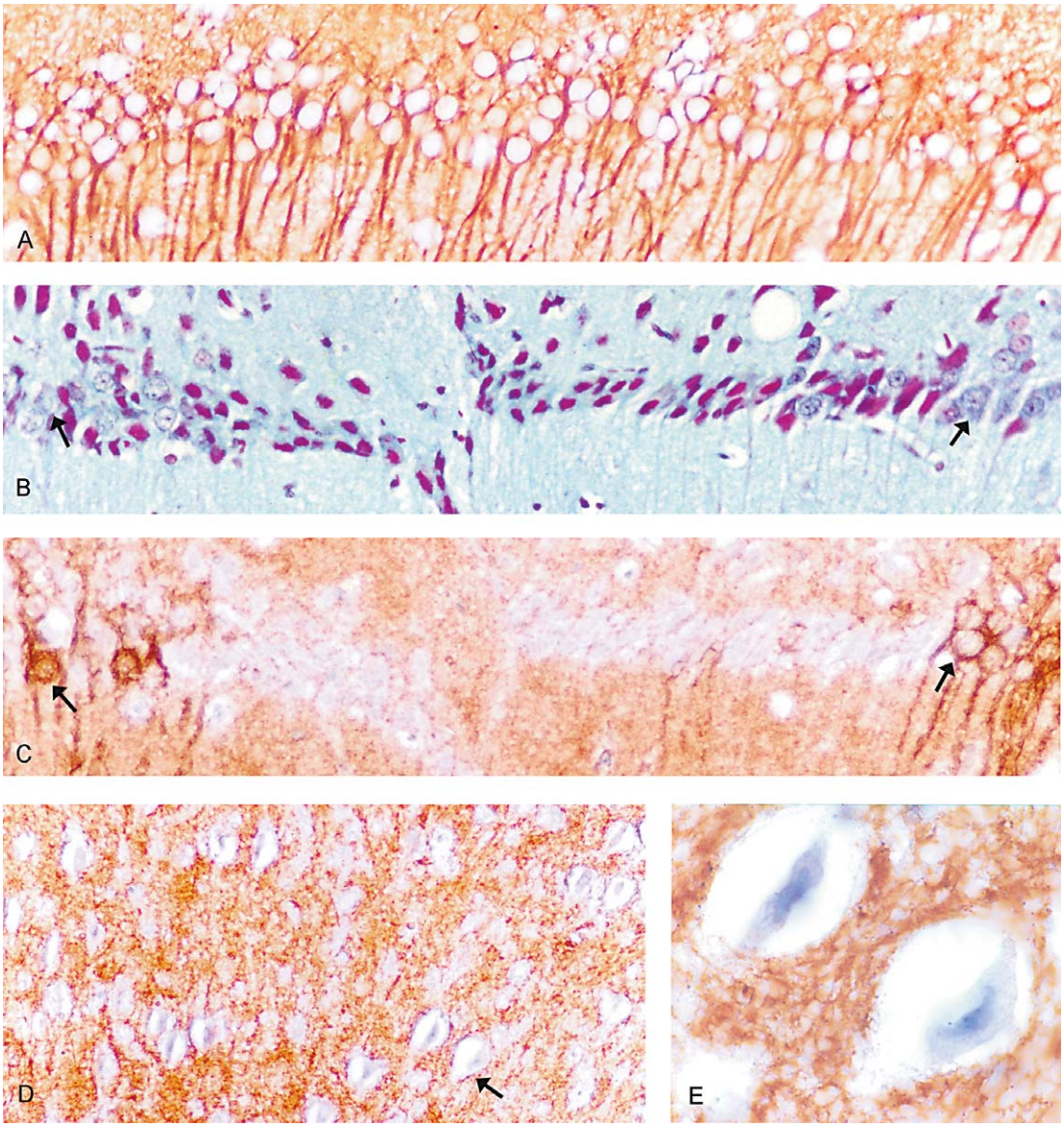

Fig. 16. Microtubule depletion occurs in all large rat and human corticocortical cells examined that exhibit either high stage (IV or V) nuclear aluminum accumulation or NFTs. A) Low magnification appearance of hippocampal CA1 pyramidal cells from the brain of an aged low aluminum control rat immunostained to show microtubules. B, C) Micrographs of adjacent sections from a rat brain with a distinctively-shaped lesion in the hippocampal CA1 field. B) The Walton stain for aluminum reveals a lesion, at center of the micrograph, consisting of CA1 pyramidal cells with Stage IV aluminum accumulation (magenta nuclei). Normal-appearing cells at the periphery of the lesion are designated by arrows. C) The section adjacent to (B) is immunostained for acetylated-α-tubulin. Microtubules are depleted from cells in the lesion, at center of micrograph, that correspond to pyramidal cells with stage IV aluminum accumulation in (B). Cells with stage I aluminum accumulation (arrows in B) stain for acetylated-α-tubulin, indicating they have microtubules (arrows in C). D) Human hippocampal CA2 cells immunostained for acetylated-α-tubulin intended to reveal microtubules (low magnification). Human hippocampal CA2 cells are more dispersed than rat CA2 cells that, like rat CA1 cells, form a compact band. Microtubules are absent from this lesion shown at low magnification (D) and high magnification (E). Aluminum-staining of the lesion shows microtubule-depleted cells at stage V of aluminum accumulation (Fig. 5E). Magnification bar = 20 μm for A and D, 10 μm for B and C, 2.5 μm for E. Reprinted from [91] with permission from Elsevier Press.

when electron micrographs of aluminum-injected cat brains showed that neurons containing early NFTs were either devoid of microtubules or had an average of three microtubules or less per μm^2 compared to a range of 30 to 130 microtubules per μm^2 in neurons without NFTs [153].

Neuronal aluminum accumulation and microtubule depletion of neurons have been invisible to many

Fig. 17. Camera lucida drawings of AD Golgi-stained pyramidal cells, illustrating a progression of mild to severe dendritic dieback (left to right). The severely deteriorated cell at right resembles aluminum-rich cells in the lesion of Figs. 5C and 16B. Redrawn from [292] with permission from Elsevier.

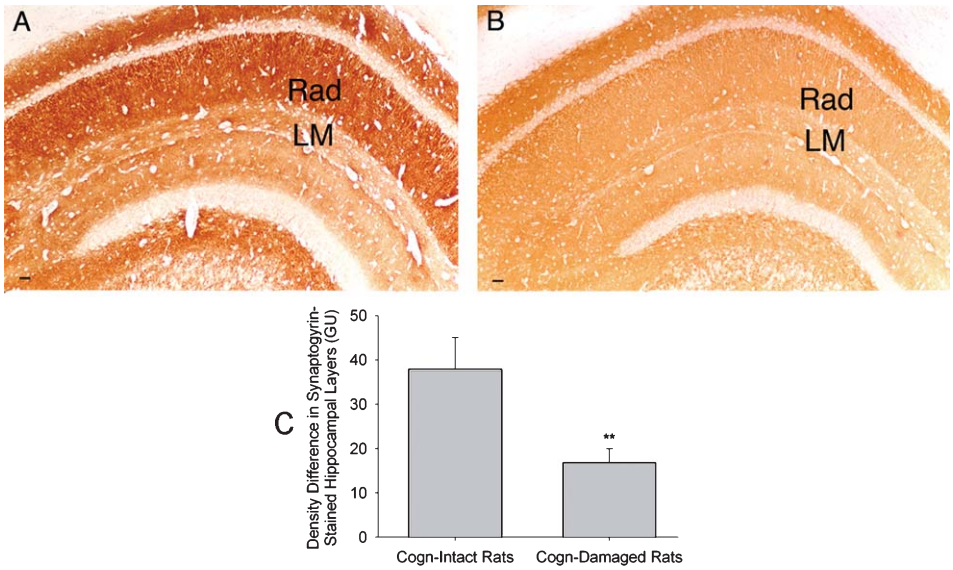

Fig. 18. Hippocampal sections from a control rat and a rat with chronic aluminum neurotoxicity. Synapse density in sections of rat hippocampus immunostained for synaptogyrin. A) Hippocampal section from a control rat. B) Hippocampal section from a rat with chronic aluminum neurotoxicity and cognitive deterioration. Note the contrast difference between the stratus radiatum (Rad) versus the stratum lacunosum moleculaire (LM) in (A) compared to (B). Scale bars are A and B = 50 μm. C) The bar graph shows mean density difference in synaptogyrin-stained brain sections from control rats at left (37.91 ± 7.11 Gray Units (GU)) and rats with chronic aluminum neurotoxicity and cognitive deterioration at right (16.83 ± 3.14 GU, $p > 0.01$). Reprinted from [91] with permission from Elsevier.

AD researchers whose studies have relied on silver-staining methods to reveal Aβ deposits and NFTs in AD brain tissue. In this author's view, the relevance of microtubule depletion to AD has been generally underestimated and is probably more fundamental to AD neuropathology than Aβ oligomers, Aβ plaques, or NFTs, given that microtubule depletion is much more damaging to neuronal connectivity and neural function than these more visible AD hallmarks that may represent protective cell responses [102, 204].

Aluminum-rich microtubule-depleted cells appear shrunken and exhibit axonal/dendritic dieback as a result of the microtubule depletion, progressing in a way similar to that illustrated in Fig. 17 [292]. This pathology [282–284] is consistent with AD being a disconnection syndrome [287]. Furthermore, aluminum-induced microtubule depletion and axonal/dendritic dieback lead to synapse breakdown and loss of synapse density (Fig. 18) [91, 285, 286, 380]. Microtubule depletion can also explain why pyramidal cells in brains of aluminum-exposed rats with

chronic aluminum neurotoxicity and humans with AD have axonopathy and impaired axonal transport with AβPP congestion in defunct axons [93, 269, 348, 352].

Several mechanisms have been proposed to explain microtubule depletion but these require further elucidation. Microtubule depletion may begin with aluminum-induced inhibition of PP2A activity, since such inhibition causes normal tau to convert to hyperphosphorylated tau in rat neurons at relatively low stages of aluminum accumulation (Fig. 8) [92]. Hyperphosphorylated tau is unable to bind to tubulin if the PP2A activity is too low to remove phosphate from the microtubule-binding region of tau [381]. This step is required by tau in order to perform its microtubule assembly and stabilization functions.

By way of complication, hyperphosphorylated tau sequesters normal tau (and other microtubule-binding proteins), further inhibiting microtubule assembly [382]. Addition of hyperphosphorylated tau to a mixture of normal tau and tubulin causes hyperphosphorylated tau to bind to normal tau instead of tubulin [382], consequently blocking microtubule assembly.

Alternatively, microtubule depletion may occur from direct interference of aluminum with microtubule assembly. Picomolar concentrations of Al^{3+} stimulate *in vitro* assembly of tubulin sub-units into microtubules, because Al^{3+} competes with Mg^{2+}, the physiological mediator of microtubule assembly [24]. Al^{3+} has an association constant 10^7 times stronger than Mg^{2+} for the Mg^{2+}-binding site in the metal/GTP/tubulin ternary complex. This allows aluminum, at Al^{3+} concentrations as low as 4×10^{-10} M, to effectively compete with intracellular mM concentrations of Mg^{2+} to polymerize tubulin subunits for microtubule formation. This high association constant may also explain why aluminum-catalyzed microtubules, which appear to have an identical ultrastructure to normal microtubules, are functionally defective as demonstrated by their inability to respond to Ca^{2+}-regulated depolymerization [24]. Microtubules are virtually absent at higher *in vivo* aluminum concentrations. Aluminum also causes neuronal euchromatin to condense to heterochromatin [339], interfering with cellular ability to synthesize specialized neuronal proteins. Aluminum's pathological consequences cripple differentiated cell functions rather than killing cells outright.

It is currently unknown which one, or combination, of these suggested mechanisms, or a mechanism yet to be described, is responsible for depleting microtubules in AD and in the animal model for chronic aluminum neurotoxicity. However, high-stage aluminum accumulation in rat brain tissue strongly correlates with microtubule depletion. Given that microtubule depletion is central to loss of cell function and dieback in AD, microtubule depletion is worthy of further research.

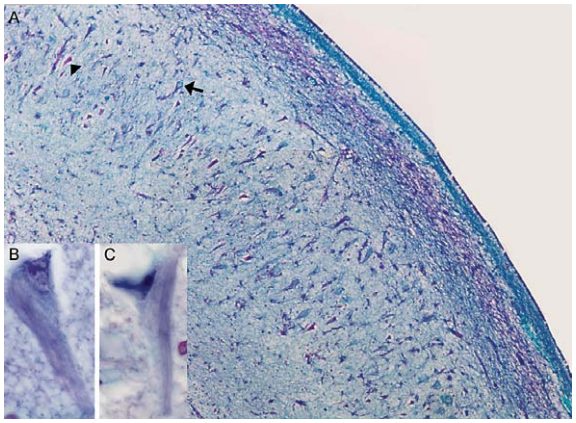

Fig. 19. Hippocampal CA1 cell enucleation and death. A) Montage of a devastated AD hippocampal CA1 field containing numerous (blue- or blue and purple-stained) ghost NFTs (e.g., arrow). The relatively few intact pyramidal cells all stain purple for aluminum (e.g., arrowhead). B) The NFT of this AD hippocampal cell has displaced the nucleus to the cell periphery. C) AD hippocampal cell in the process of denucleation. The nucleus is evaginating from this cell. Magnification bars = 20 μm for A, 1.5 μm for B and C. B is reprinted from [136] with permission from Elsevier.

Neuronal death in AD and relationship to chronic aluminum neurotoxicity

Neuronal death is increased in certain regions of the AD brain. This is evident either by observing numerous extracellular NFTs in certain neuroanatomical regions such as the hippocampal CA1 field (Fig. 19A) or by stereological counts [383]. Neuronal death particularly affects large pyramidal neurons that accumulate substantial amounts of aluminum and form large NFTs.

Large intracellular NFTs tend to displace the nucleus to the cell periphery (Fig. 19B, C). This can result in denucleation of pyramidal cells, leaving behind the NFT in membrane-bound cytoplasm, which survives in that form for a short time [136]. The denucleated cell is unable to effectively renew its membrane proteins and other cellular constituents. Eventually, the cell membrane ruptures and leaves behind an extracellular or ghost NFT (Fig. 7I). Human CA2 and rat CA1/CA2 hippocampal neurons, which are less able than human CA1 cells to form NFTs, survive in the tissue for a long time even at high stages of nuclear aluminum accumulation. However, neurons with high-

stage aluminum accumulation or with NFTs both lack microtubules and are incapable of neuronal function.

Immunochemical evidence indicates that apoptosis has a role in neuronal death in AD [384]. The extent of its importance in AD is still unclear. Aluminum induces apoptosis in neurons and astrocytes by a process preceded by autophagy that involves endoplasmic reticulum stress and impairment of protein-folding chaperones [385, 386].

Altered cell metabolism in AD and chronic aluminum neurotoxicity

Early stages of AD are accompanied by decrease in regional cerebral glucose metabolism [387–391] and low cytochrome oxidase activity in mitochondrial respiratory chains [392–394]. Aluminum depresses cerebral glucose metabolism by inhibiting the glycolytic enzyme hexokinase and the citric acid cycle enzyme α-ketoglutaric acid [310, 395, 396]. Aluminum also decreases mitochondrial respiration and inhibits cytochrome oxidases [397, 398].

Calcium homeostasis and signaling are disrupted in AD and chronic aluminum neurotoxicity

Calcium metabolism [349, 399–401], calcium signaling [402, 403], and calmodulin binding [404] are dysregulated in AD. There are at least six aspects of Ca^{2+} homeostasis and signaling in AD brain tissue that differ from those of normal age-matched controls [30]. Aluminum contributes to this disruption because Al^{3+} ions compete with Ca^{2+} and Mg^{2+} ions and disrupt calcium homeostasis in ways consistent with those that occur in AD. Evidence for these modes of aluminum disruption is reviewed more fully elsewhere [30].

(1) Al^{3+} blocks Ca^{2+} influx into neurons through plasma membrane Ca^{2+} channels [405–408].
(2) Al^{3+} disrupts the phosphoinositide and c-AMP signaling pathways and their downstream effects on PKC activity [270, 409–413].
(3) Al^{3+} impairs Ca^{2+} signaling [313, 408, 414–421] and calmodulin-mediated signal transduction [23, 178, 422–430]. Al^{3+} interferes with Ca^{2+} signaling by restricting both inositol triphosphate- and caffeine-evoked Ca^{2+} release from endoplasmic reticulum stores [313].
(4) Al^{3+} disrupts synaptic plasticity and memory-processing [162, 163, 416].
(5) Al^{3+} interferes with restoration of the resting Ca^{2+} level in neurons [419, 431, 432].
(6) Al^{3+} exposure also increases resting and peak cytoplasmic Ca^{2+} levels in neurons [407, 408].

Iron dysregulation in AD and chronic aluminum neurotoxicity

Iron is essential for neurons, and excess iron is detrimental, unless controlled, since excess free iron causes oxidative stress [144]. Iron homeostasis involves two proteins with opposing functions: transferrin receptors that stimulate iron uptake into cells and ferritin that stores and detoxifies excess iron. In brain, neurons are the main cells with transferrin receptors whereas oligodendrocytes and microglia are the main cells that contain iron, ferritin, and transferrin [433]. Transferrin receptor synthesis, iron uptake and storage are controlled by iron regulatory proteins (IRP1 and IRP2) [144], being intracellular iron sensors. Figure 20 depicts how the IRPs respond under intracellular conditions of iron deficiency and iron sufficiency. When cellular iron stores are low, IRPs bind to the 3' end of transferrin receptor mRNA, resulting in transferrin receptor synthesis and increased iron uptake. In cells with sufficiently high iron levels, free Fe^{3+} binds to Fe^{3+}-binding sites on the IRP2s and signals for IRP2 degradation. This causes the IRP2s to detach from transferrin receptor mRNA which exposes that mRNA to degradation by nucleases. Consequently, transferrin receptor synthesis and iron uptake both cease [144].

A characteristic of AD and chronic aluminum neurotoxicity is that IRP2 becomes stabilized and prevented from degrading [29, 434]. IRP2 stabilization leads to dysregulation of iron metabolism [435], causing the cells to behave as though they were permanently iron-deficient. The cells continue to synthesize transferrin receptors and import iron (as in Fig. 20B) even though their iron levels are already high. As a result of IRP2 stabilization, iron and aluminum levels are abnormally high in vulnerable regions of AD brains [127, 436–438]. Iron levels that rise in AD brains do so without the usual age-related rise in ferritin [439], probably as a consequence of the IRP2 stabilization. This imbalance is expected to increase oxidative stress in brain cells [434, 440].

Aluminum that accumulates in vulnerable regions of the AD brain interferes with iron regulation. Aluminum has greater affinity than iron for the iron-binding site(s) on IRP2 [28]. Aluminum substitution for iron at this site blocks iron from binding to IRP2, preventing both IRP2 detachment from transferrin receptor mRNA and IRP2 from signaling for its own degradation. Aluminum-binding to IRP2 effectively

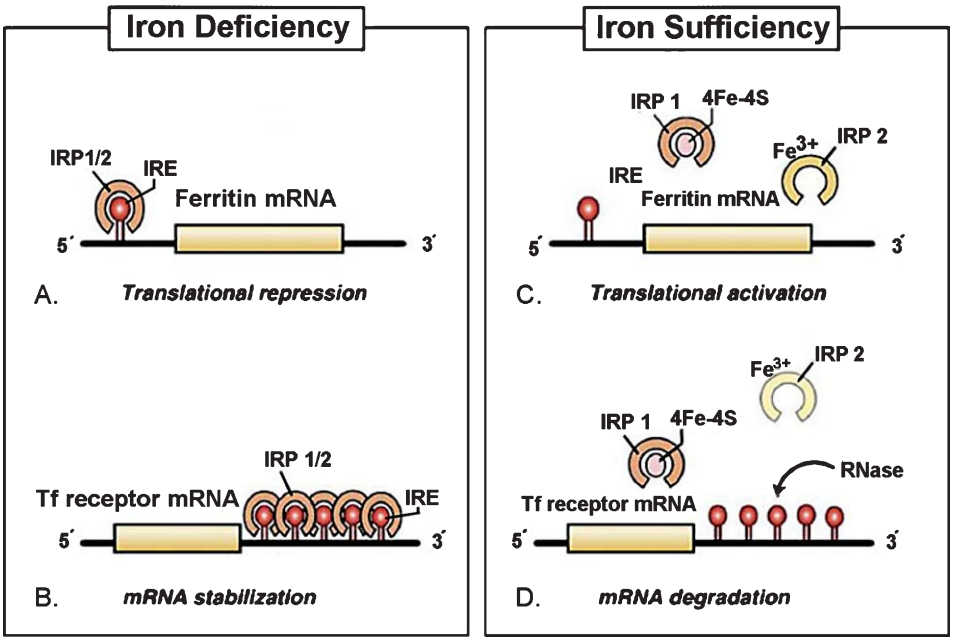

Fig. 20. Diagram showing how iron regulatory proteins 1 and 2 control intracellular iron content under low and high iron conditions in cells. A) When intracellular iron content is low, IRPs 1 and 2 bind with high affinity to the iron regulatory element (IRE) in the 5′ UTR of ferritin mRNA, repressing its translation. B) IRPs simultaneously bind to the IREs in the 3′ UTR of transferrin receptor mRNA, initiating and stabilizing its translation by protecting the mRNA from RNase degradation. This allows the continuation of transferrin receptor synthesis and iron uptake. C) When the iron supply is replete, free iron (Fe^{3+}) binds to iron-binding sites on the IRP2s, causing them to lose their affinity for the IRE and signaling for IRP2 degradation by the ubiquitin-proteasomal pathway. Free Fe^{3+} also changes the conformation of IRP1 from an RNA-binding protein to an inactive form that binds an iron-sulfur cluster (4Fe-4S). IRPs detach from the 5′ end of ferritin mRNA, activating ferritin translation. D) IRP2 degradation allows the mRNA for transferrin receptors to be degraded by RNase. Transferrin receptors are no longer synthesized and iron uptake ceases. Reprinted from [144] with permission from Elsevier.

stabilizes IRP2 activity, thereby maintaining translation of transferrin receptor mRNA with continuing synthesis of transferrin receptors as well as iron and aluminum uptake into vulnerable regions of the AD brain [28].

Aluminum-dysregulated iron metabolism has been demonstrated in cultured cells and in brains of iron-loaded animals [28, 29, 441]. Aluminum-loaded rats acquire iron concentrations up to three-fold higher than controls in regions of their brain homologous to human brain regions that are damaged in AD; namely, temporal, parietal, and frontal cortices and the hippocampus [29]. Aluminum and excess free iron produce synergistic increases in ROS and oxidative stress in cells [31, 325, 326].

Aluminum is present in greater amounts in aged human brains [17–20]. Much of this aluminum is contained in NFTs [102, 136]. Aluminum accumulation in AD-affected brain regions and its ability to dysregulate iron metabolism can explain why iron levels rise in the same regions of AD brains where aluminum preferentially accumulates [29].

ApoE-dependent neuroplasticity in AD and chronic aluminum neurotoxicity

Neuroplasticity is the brain's mechanism for creating new neural pathways to compensate for cell dysfunction, cell denervation, and cell death that occur in the AD brain and under other conditions. Astrocytes enable neuroplasticity by synthesizing and secreting apolipoprotein E (ApoE) [442], the main lipid carrier in the mammalian brain [443]. Astrocyte-secreted ApoE stimulates compensatory neuroplasticity by recycling cholesterol and cholesterol esters from neurons with deteriorating synapses for delivery to other neurons that use this cholesterol to sprout buds, grow new axonal branches, undergo synaptogenesis, and reinnervate de-afferented neurons [443].

Humans express three forms of this protein: ApoE2, ApoE3, and ApoE4 [444]. ApoE2 and ApoE3 are capable of stimulating neuroplasticity in response to injury-induced denervation. The molecular structure of ApoE4 is defective in that ApoE4 is unable to hold and transport cholesterol. This, in turn, severely

impairs ApoE4's ability to promote sprouting and re-innervation [445].

As AD progresses, damage to neurons of the entorhinal cortex, hippocampus, and other AD-vulnerable brain regions evokes vigorous sprouting by less affected neurons to form synapses with the de-afferented target cells. The effectiveness of ApoE-dependent neuroplasticity is to postpone overt AD. The earlier age at which AD occurs in humans with ApoE4 alleles reflects ApoE4's inability to bind and transport the cholesterol and cholesterol esters required for neuroplasticity and re-innervation.

Neuroplasticity and re-innervation have been demonstrated in rats with unilateral lesions of the entorhinal cortex. These rats temporarily lose their ability to perform continuous alternation in the T-maze in the days immediately after surgery [446]. Compensatory sprouting from the contralateral entorhinal cortex offsets damage to the perforant pathway by re-innervating the de-afferented hippocampal formation and restoring the rat's ability to perform the continuous alternation T-maze task. Rats that receive bilateral lesions of the entorhinal cortex develop neuropathology and behaviors similar to those observed in humans with AD [447]. The bilaterally-lesioned rats experience sprouting but the sprouting is from regions with different functions and interconnections. This inappropriate attempt at compensatory neuroplasticity is unable to restore the rat brains' original connections and hence their T-maze performance. These rats are left with severe memory deficits.

The rat model for chronic aluminum neurotoxicity showed remarkable similarities in neuropathology and continuous alternation T-maze performance to rats with bilateral lesions of the entorhinal cortex [447]. However, chronic aluminum exposure damages the entorhinal cortex over a long prodromal phase whereas lesioned animals rapidly develop almost-equivalent pathology. Thus, chronic aluminum neurotoxicity more closely resembles the slowly developing process of cognitive deterioration that occurs in AD.

Rats that developed chronic aluminum neurotoxicity from the aluminum-supplemented diet showed cognitive deterioration by age 27 months (equivalent to about 82 human-equivalent years) [86]. ApoE mRNA was significantly higher in their cortical and hippocampal tissue than for the same brain regions from low-aluminum controls. This suggests that ApoE-dependent neuroplasticity was stimulated in the brains of rats with chronic aluminum neurotoxicity but not in the low aluminum controls (Fig. 21) [293]. However, axonal sprouting, re-innervation and synaptogenesis is

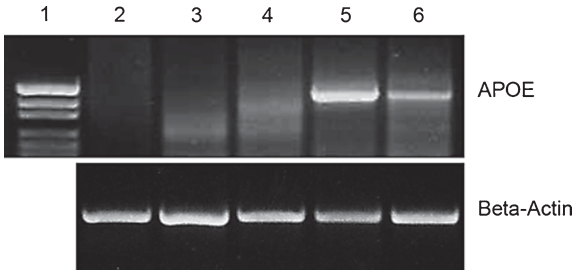

Fig. 21. RT-PCR analysis for ApoE mRNA in rat brain. Lane 1, pUC19; lane 2, negative control (macrophage cell line RAW 264); lanes 3 and 4, aged brain tissue from aged low aluminum control rats; lanes 5 and 6, brain tissue from aged rats that exhibited chronic aluminum neurotoxicity. β-Actin bands are shown as controls. Reprinted from [293] with permission from the Royal Society of Chemistry.

eventually inadequate to compensate for the damage caused by continuous aluminum insult that leads to microtubule depletion and dieback in pyramidal cells of the entorhinal cortex, hippocampal formation, neocortex, and other AD-vulnerable brain regions, as in AD [448].

Progression of AD and chronic aluminum neurotoxicity

AD and chronic aluminum neurotoxicity appear to progress in similar ways to that described in Fig. 12. The progression of AD and of chronic aluminum neurotoxicity involves a hierarchy of specific brain regions that become increasingly damaged over time as aluminum continues to be taken up into these regions. The same characteristics affect other brain regions to which the entorhinal cortex and hippocampus normally connect. Aluminum has been shown to spread from one brain region to another by both retrograde and anterograde transport within axons of large corticocortical and corticosubcortical cells [141] while these cell processes are still capable of axonal transport. Also, transferrin-bound aluminum crosses the blood-brain barrier [142] and is taken up into the same neurons by their transferrin receptors [27]. Expanding numbers of neurons develop NFTs in some brain regions and high-stage aluminum accumulation in other regions, as aluminum accumulates to toxic levels in increasing numbers of neurons. AD severity also increases in these brain regions over time [143].

According to Braak and Braak [297], the transentorhinal and entorhinal cortex are already damaged during the clinically-silent "entorhinal phase". The next stage, the "limbic phase" or amnestic mild cognitive impairment, is characterized by damage in the

hippocampus, amygdala, and other limbic structures. The limbic phase occurs in brains of humans, on average, about 20 years later than the transentorhinal phase [297]. In the "neocortical phase", dementia is overt, becoming more severe with progressive destruction of the association cortices.

CRITERION VIII: EXPERIMENTAL EVIDENCE

Indications that AD and/or AD-related pathology can be relieved, reversed, or prevented by appropriate experimental regimens

According to Bradford Hill, "Occasionally it is possible to appeal to experimental, or semi-experimental, evidence. For example, because of an observed association some preventive action is taken. Does it in fact prevent? ... Is the frequency of the associated events affected? Here the strongest support for the causation hypothesis may be revealed" [38]. This section describes studies in which the cognitive deterioration and neuropathology of AD cases and animal models are either reduced or prevented by chelating agents that lower the brain aluminum concentration, by antioxidants that counteract oxidative effects of aluminum and by avoidance or minimization of aluminum exposure.

Chelation studies

Chelation of aluminum slows neurodeterioration in AD patients

After years of intensive research into AD, one human study stands out, as the only, to date, to have significantly slowed the rate of AD neurodeterioration [449, 450]. This human study used chelation by DFO, a natural chelator produced by a bacterium, which has been successfully employed since 1979 to reduce and control aluminum levels in patients with chronic kidney disease (e.g., [173]). DFO also chelates iron. In humans and experimental animals, DFO treatment significantly mobilizes aluminum for excretion, both in iron-normal [451, 452] and iron-overload [453] patients.

In order to investigate whether aluminum removal might slow the progress of AD, 48 human subjects with probable AD were randomized to one of three treatment groups [449, 450]. In this double-blind, placebo-controlled clinical study, Group 1 was given intramuscular injections of DFO twice daily for 5 days/week over 2 years. Group 2 was given an oral placebo and Group 3 had no treatment. The only observed side effects from the DFO treatment were appetite reduction and weight loss.

Videotapes of the patients' daily living skills were recorded and assessed at the time of their entry into the trial and at their 6, 12, 18, and 24-month follow-up visits [449, 450]. The videotapes showed the AD patients performing routine activities of daily living in their own homes. The tapes documented that the rate of deterioration of 25 AD patients given DFO injections twice daily for 5 days/week was at half the rate of deterioration of the 20 AD patients who either consumed a lecithin placebo or received no treatment [449, 450].

At the trial onset, the 48 participants showed no significant differences with respect to age or their videotaped home behavior (VBH), Wechsler Adult Intelligence Scale-Revised (WAIS-R), Wechsler Memory Scale (WMS), or Western Aphasia Battery (WAB) scores. The VHB strongly correlated with the WAIS-R ($r = 0.6$, $p = 0.0001$) at baseline. The 25 DFO-treated subjects, 23 oral placebo and no-treatment controls were re-tested for all of these outcome measures at 6-month follow-up visits over 2 years.

At the two-year follow-up, the VHB scores showed significant correlation with the patients' performance scores on the standardized clinical measures, including the WAIS-R, WMS, and WAB. These correlations ranged from $r = 0.443$ ($p = 0.002$) to $r = 0.712$ ($p < 0.000$). Six AD patients in the trial died from causes unrelated to the DFO treatment (coronary atherosclerosis, pneumonia, sepsis, peritonitis from diverticulosis, and carcinoma of the bowel) [449, 450]. Autopsies performed on their brains verified that all showed the hallmarks of AD. Twenty-two samples of neocortical gray matter, weighing 20 to 40 mgs, were dissected from comparable regions of seven gyri from each of the six brains. These brain samples were coded to enable evaluation of their aluminum content by a trace metal analyst in a blinded manner, unaware of their source.

Three of the brains were from AD patients who died after receiving less than 3.5 g total of DFO. These, designated as "minimal DFO treatment" had a mean aluminum content of 4.09 μg/g brain tissue (d.w.) [449]. Brains from the three other AD cases that died were designated as "extended DFO treatment" because those patients had received between 23.5 g and 54 g of DFO. Those brains were found to have a lower mean aluminum content of 2.69 μg/g, which approaches the aluminum concentrations found in brains of non-demented controls. The mean aluminum concentration in brains of the extended DFO treatment group was significantly lower than the mean aluminum concentrations of brains from the minimal

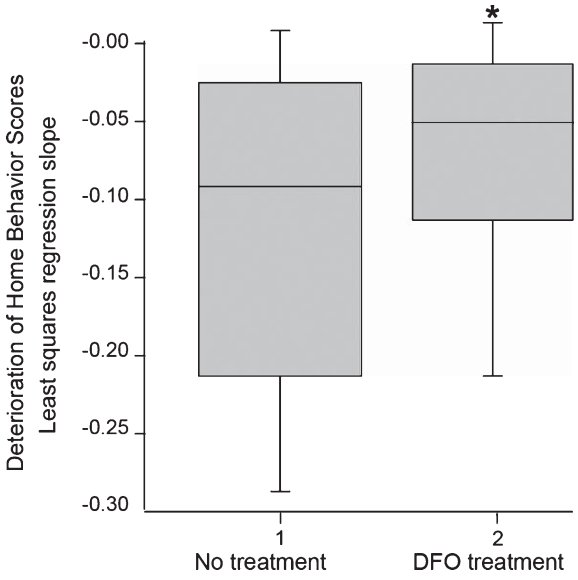

Fig. 22. Box plots showing that the least squares regression slope for videotaped home behavior scores (daily living skills) of the no-treatment AD groups (including the lecithin placebo group) is significantly steeper than for scores of the DFO-treated group whose home behaviors deteriorated at half the rate ($p = 0.03$) and exhibited significantly less variance within the group ($p = 0.04$). Redrawn from [449] with permission from Elsevier.

DFO treatment group (*t*-test, $p = 0.008$). This study showed that 500 mg DFO given intramuscularly, twice daily for 5 days/week over 28–70 days, can lower brain aluminum in AD patients to near normal levels; i.e., similar to the mean aluminum level found in brains of elderly cognition-intact controls [449, 450].

Deterioration rates in the patients' videotaped home behavior scores were shown with box plots that compared the slope of scores for the videotaped behaviors of the DFO-treatment groups and the no-treatment groups (including the placebo controls) (Fig. 22) [449]. The mean slope of the deterioration rate for the no-treatment groups' scores, was significantly steeper (ANOVA, $p = 0.038$) than that of the DFO treatment group. The DFO treatment significantly slowed the rate of decline for the AD patients' daily living skills compared with those of the placebo group ($p = 0.03$). In fact, the placebo group and no-treatment group lost these skills twice as rapidly as the DFO-treatment group [449, 450], indicating that aluminum removal by chelation slows cognitive decline in AD patients.

The protocol demonstrates substantial potential for the principle of brain aluminum removal in AD treatment [449, 450]. This is the only human treatment that has, as of this date, shown a significant reduction in the loss of daily living skills of AD patients.

Chelation reduces the formation of aluminum-induced NFTs in rabbit brains

An animal study was performed by other investigators [454] to learn whether the injection regimen could be simplified for DFO treatment of humans with AD [449, 450]. For this, rabbits were injected with a single dose of 50 mM aluminum maltolate, contained in 100 ml of sterile saline, directly into the cisterna magna. Intramuscular DFO injections were then given for two days before the animals were euthanized on days four, six, or eight.

NFTs occurred widely in all aluminum-injected brains and were absent from saline-injected brains of control rabbits. Monoclonal antibodies for hyperphosphorylated tau, including Tau-2, AT8, PHF-1, and Alz-50, all of which characteristically immunostain NFTs in AD cases, strongly labeled the aluminum-induced NFTs [454]. NFT numbers in brains of rabbits that received aluminum followed by DFO were much fewer than in rabbits that received aluminum alone. The authors concluded, on the basis of their animal experiments, that the frequency of DFO injections for AD treatment [449, 450] could probably be substantially reduced without detracting from the benefits of the protocol [454].

Chelation reverses an aluminum-induced learning deficit in rat brains

Several experimental studies of aluminum-loaded animals have demonstrated that DFO increases aluminum excretion and enhances their survival [452–455]. A further behavioral trial showed DFO reversal of an aluminum-induced learning deficit in rats [456]. The rats were put on a lit runway separated from a black Plexiglas® box by a manually operated guillotine door. They were allowed to enter the dark chamber of the Plexiglas box. The guillotine door closed behind the rat, keeping it in the dark apparatus for three minutes. Twenty-four hours later, this procedure was repeated except that all rats were given a 4-second, 0.8 mA foot shock administered through the chamber's electrified floor grid 40 seconds after closing the guillotine door. Thirty seconds later, the subjects were returned to their home cage. Animals were tested at 24-hour intervals until they learned to avoid entering the chamber where they were given the electric foot shock.

Acquisition and extinction criteria were evaluated by the length of time required by the rat to enter the dark box. Five minutes was the maximum latency time allowed for entry. The acquisition criterion was reached when the rat refused to enter the dark box for

the maximum latency period: that is, how many days the rats took to learn and remember that they would receive a foot shock after entering the dark chamber within the five-minute period allowed for the test. The extinction criterion was achieved when the rat entered the dark chamber in less than one minute on three out of four consecutive days [456], indicating the rat had forgotten, and lost fear of, the foot shock.

On day one, six groups of eight rats each, 48 in all, learned to avoid entry of the dark box. Thereafter, 32 rats were given 0.3% aluminum sulfate in their drinking water for four weeks except for two control groups that both drank ordinary tap water: one with and the other without DFO injections in week five. At the end of four weeks, one group of eight aluminum-treated rats and one group of eight controls were re-tested. This experiment showed a striking difference between the various treatment groups in the number of days the animals required to lose their fear of the electric shock (over-all group effect $F(6, 41) = 22.7$, $p < 0.0001$) [456]. The eight aluminum-treated rats tested immediately after continuous aluminum intake lost fear of the electric shock within ten days, significantly faster than the rats in all other treatment groups ($p < 0.05$).

Three groups of aluminum-loaded rats (24 rats in all), and a control group of eight rats, were given ordinary tap water to drink during weeks five and six. One group of the aluminum-loaded rats was given four intraperitoneal injections consisting of 75 mg DFO every other day during week five. Two other groups of aluminum-loaded rats were also given four intraperitoneal injections on the same occasions: one containing a lower (30 mg) DFO dose and the other containing only saline solution. The 24 aluminum-loaded rats and eight controls were re-tested again at the end of week six.

The loss of fear in eight aluminum-loaded rats was completely reversed by giving them four high (75 mg) DFO injections, making their fear levels equivalent to those of the controls. The lower DFO dose significantly improved the performance of eight other aluminum-treated rats but failed to return their fear levels to those of the control rats. Thus, the DFO injections reversed the aluminum-induced behavioral impairment in a dose-dependent manner [456].

Chelation lowers brain aluminum levels and reduces GFAP, a marker for neuroinflammation

AD is mildly neuroinflammatory, marked by enhanced expression of gliosis (indicated by increased cortical GFAP) in AD patients compared to controls, particularly in the temporal cortex [457]. A high GFAP level in the brain is also a feature of systemic aluminum intoxication [347].

Rabbits were aluminum-loaded by 20 intravenous injections of 100 μM aluminum lactate into the ear vein, given five days per week for four weeks [347]. The aluminum treatment increased GFAP levels by 80% over that of controls. A control group of eight rabbits was given sodium lactate injections. Starting ten days after the last injection, the aluminum-loaded rabbits were treated 12 times in one month with chelating agents: either with 150 μM DFO/kg bw by injection or with one of six types of hydroxypyridin-4-ones (CPs), including doses of 450 μM. These new-generation chelating agents are effective by the oral route.

Three of the most lipophilic CP chelating agents (CP24, CP52, and CP93) significantly lowered GFAP levels in rabbit frontal cortex. Under the conditions of this study, CP24 was the only chelating agent that significantly reduced the level of brain aluminum [347]. This study suggests a protective role for CPs against gliosis and, possibly, other adverse effects of AD.

Chelation of aluminum improves neurotransmitter biosynthesis in vitro

In humans, two catecholamines (norepinephrine and dopamine) act as neuromodulators in the central nervous system. These are produced from phenylalanine and tyrosine. Tetrahydrobiopterin (BH4), a central cofactor in the synthesis of catecholamine neurotransmitters, is at abnormally low levels in AD brains [458] and in brains of animals exposed to soluble aluminum [77]. An experimental treatment to improve these low levels is based on the capacity of transferrin to bind aluminum and iron. Homogenates of AD brains were treated with transferrin. The transferrin treatment increased BH4 synthesis in the homogenates which in turn improved neurotransmitter biosynthesis *in vitro* [459].

Antioxidant studies

Free radical scavengers and chelators of aluminum lower ROS levels, reduce neuroinflammatory cytokines, and improve antioxidant status in brain cells

All cells generate ROS, including hydrogen peroxide, hydroxyl radicals, peroxyl radicals, and superoxide anions, during normal metabolic reactions. These ROS are usually neutralized by intracellular antioxidants [460]. When cultured human neural cells were exposed to iron sulfate their ROS levels increased 1.5-fold. Their ROS levels increased by 2.9-fold when exposed to alu-

minum sulfate. Exposure to aluminum sulfate and iron sulfate increased the cells' ROS levels by 5.9-fold and induced expression of the inflammatory genes COX-2 (cyclooxygenase-2) and $cPLA_2$ (cytosolic phospholipase A_2). This larger ROS burden overwhelmed the neutralizing effects of intracellular antioxidants.

Five agents, either free radical scavengers or aluminum chelators, were tested for their ability to reverse the elevated ROS levels and inflammatory gene expression in the human neural cells, testing them on their own and in combination. The antioxidants included ascorbate, folate, and phenyl butyl nitrone (PBN). The chelating agents were DFO and Feralex-G (a chelator synthesized from maltol, glycine, and glycosamine). A combination of PBN and Feralex-G was found to be the most effective means for quenching aluminum and iron-induced ROS and repressing the aluminum and iron-induced inflammatory genes [460].

In another animal experiment, rats were given 0.2% aluminum nitrate in their drinking water for eight months. This treatment significantly increased prooxidant activity and reduced antioxidant activity in rat brain cells [461]. The rats were then given oral citric acid with intraperitoneal ethylene diamine tetraacetic acid (EDTA) for five days. This combined treatment, which lowers brain aluminum content, moderately improved the level of antioxidant activity in the rat brains.

Antioxidant protection against AD and aluminum damage

Oxidative damage is an early feature of AD [226]. Combined use of the antioxidant vitamins E and C has been associated with reduced prevalence (−78%) and incidence (−64%) of AD in an elderly population [462]. Appropriately chosen antioxidants protect against free radical-mediated oxidative damage that stems from the aluminum that accumulates over time in aged neurons. Vitamin E is reported to reduce aluminum levels in sera and brain tissue of aluminum-loaded rodents [463] and protect brain tissue against aluminum-induced ROS [464].

Antioxidant treatment reduces lipid peroxidation in synaptosomes

Synaptosomes are isolated synaptic terminals, often used to investigate synaptic biology. Synaptosomes were experimentally oxidized by incubation with $FeCl_3$ and ascorbic acid, as indicated by their increased contents of malondialdehyde and 4-hydroxyalkenyl [465]. These oxidation products were elevated even further, and more significantly, by the addition of 0.0001–1.0 mmol/L $AlCl_3$. Upon inclusion of the antioxidants melatonin or pinoline in the synaptosomal preparations, each reduced lipid peroxidation in a concentration-dependent manner for aluminum, ferric iron, and ascorbic acid [465].

Vitamin E supplementation counteracts aluminum-induced oxidative stress which leads to amyloidogenesis in transgenic mice

A study of a transgenic mouse model for AD-type amyloidogenesis [101] in which mice were genetically engineered to overexpress human mutant AβPP, one cohort of the mice was fed an aluminum-supplemented diet between the ages of three and twelve months whereas a control cohort was fed a diet without aluminum supplementation. The aluminum-supplemented cohort exhibited higher levels of oxidative stress, showing elevated levels of brain, urinary, and plasma 8,12-*iso*-$iPF_{2\alpha}$-VI, an isoprostane marker of oxidative stress. The aluminum-supplemented cohort also showed increased and accelerated Aβ plaque development compared to the transgenic cohort that had no added aluminum [101].

The third arm of this mouse study involved a cohort of transgenic mice that ingested a diet supplemented with both aluminum and vitamin E. This cohort showed significantly less oxidative stress, with urinary isoprostane levels significantly lower than in the animals that received only aluminum (2.8 ± 0.1 ng/mg creatinine versus 5.3 ± 0.2 ng/mg creatinine, $p < 0.01$). Plasma levels of vitamin E were well above control values by the end of the study (65 ± 4 versus 2 ± 2 μM vitamin E in controls; $p < 0.001$). The brains of mice with vitamin E supplementation also had lower isoprostane levels and smaller Aβ plaque sizes and burdens, resembling plaques in brains of the control group without aluminum added to their diet [101].

This study confirms that aluminum increases oxidative stress and Aβ plaque levels and accelerates the time at which Aβ plaques appear in brains of transgenic mice expressing a human mutant AβPP gene. The study also furnishes evidence that increased plaque formation is a response to aluminum-induced oxidative stress and free radical damage in mice that carry a human gene for AβPP. All of these effects were blocked by vitamin E.

Aluminum avoidance reduces the risk for chronic aluminum neurotoxicity

Rats from the longitudinal studies that exhibited chronic aluminum neurotoxicity in old age, after

ingesting aluminum at human-relevant dietary levels throughout most of their life, represent an aluminum-inducible rat model for AD that develops severe memory impairment and other AD-type behaviors in old age [81, 82]. This neurodegenerative condition develops from progressive aluminum accumulation in the brain, particularly in the entorhinal cortical cells of origin for the perforant pathway and hippocampal CA1 cells.

AD-like cognitive deterioration, that occurred in rats with chronic aluminum exposure to aluminum in their drinking water, was prevented in a control population that had a relatively low level of total dietary aluminum throughout the duration of the study by consuming aluminum-free drinking water. Chronic aluminum avoidance is preferable to chronic aluminum exposure and a subsequent need for pharmaceutical treatments that remove aluminum.

CRITERION IX: ANALOGY

Down's syndrome (DS) demonstrates certain characteristics that reinforce the relationship between AD and chronic aluminum neurotoxicity in humans

"In some circumstances, it would be fair to judge by analogy." Bradford Hill [38]. The reasoning of the criterion of analogy for causality is as follows. "If some condition similar to A causes an outcome similar to B, then this is evidence that A causes B." Van Reekum et al. [39]. That is, chronic aluminum ingestion that results in higher than normal serum aluminum levels—whether in rats fed aluminum at the high end of the human dietary aluminum range, dialysis patients, DS patients, or AD patients—eventually results in loss of episodic memory that originates from sufficient damage to the cells of origin for the perforant pathway, to isolate the hippocampal formation from the entorhinal cortex and neocortical association areas.

AD in DS patients serve as an analogous model for chronic aluminum neurotoxicity

AD is a heterogeneous disease with respect to age of onset, its neuropathology, and cognitive syndrome [466]. According to Folstein, amnesia, apraxia, aphasia, and agnosis in AD relate to different distributions of plaques and tangles. Also, AD has different ages of onset. Early-onset AD affects at least two groups of people. Those with DS are one group and the small numbers of families with mutations in the AβPP, presenilin-1, or presenilin-2 genes form the other early-onset group. The neuropathology of AD also shows heterogeneity with approximately 10% of AD cases displaying cortical atrophy in the absence of significant numbers of plaques and tangles [466].

DS is a congenital disorder that develops in humans whose cells have acquired a third copy of chromosome 21, or at least a part of chromosome 21, as a result of chromosomal non-disjunction; i.e., failure of the chromosome pairs to completely separate during germ cell development. People with DS generally develop AD neuropathology at a relatively early age and have a higher risk for developing AD-type dementia [246, 467–470]. Those with DS who develop AD can be viewed as an analogous model for other humans who develop chronic aluminum neurotoxicity. People with DS absorb much greater amounts of aluminum than age-matched non-DS controls [471]. Higher plasma aluminum levels correlate with higher aluminum levels in human brains [121].

Aluminum absorption levels in DS patients

As illustrated in Fig. 2, aluminum absorption experiments carried out in humans and other mammalian species consistently show a range of responses, with some individuals having aluminum absorption values that peak in the plasma at two or three times higher than others after consuming a standardized aluminum dose [104]. The extent of this increase depends, in part, on the individual's genetic ability for aluminum absorption or, alternatively, their inability to exclude aluminum from absorption. Aluminum absorption levels also depend on the amount of aluminum consumed, the form of ingested aluminum (e.g., aluminum citrate, aluminum chloride, and other aluminum salts), the intestinal pH, and other stomach contents co-present with the aluminum, such as food acids, that form salts with aluminum and increase or decrease aluminum absorption in the duodenum [472].

In 1997, Moore et al. [471] showed that humans with DS absorb much more aluminum than age-matched, non-DS controls from a standardized aluminum dose consumed and tested under prescribed conditions. The first part of this study involved giving 15 people with DS and 15 controls, aged 36 to 46 years, a citrus drink containing a pharmacological-sized (280 mg) dose of aluminum, equivalent to that contained in one aluminum-based antacid tablet [473]. Blood samples from the 15 DS subjects and 15 gender- and age-matched non-DS controls were compared for their aluminum levels before and after consuming the alu-

minum citrate drink. Baseline aluminum levels of both groups were similar and normally distributed. Blood aluminum levels of the DS group, measured one hour after ingesting the aluminum citrate drink, peaked four times higher than the aluminum levels in post-ingestion blood samples taken from controls at the same time. The aluminum levels were 77 ± 62 μg/l for the DS patients and 19 ± 12 μg/l for the controls ($p < 0.001$). The aluminum measurements for this trial were made using GFAAS [471].

For the second part of this study, five fasted DS patients and five fasted non-DS controls, at mean ages of 43 and 41 years, respectively, drank 140 ng of ^{26}Al diluted in 100 ml of an orange drink [471]. This is equivalent to a dietary concentration of aluminum as opposed to a pharmacological concentration. Baseline aluminum measurements were unnecessary for this test because ^{26}Al is naturally absent from individuals prior to ^{26}Al exposure. The DS group's ^{26}Al concentrations reached a peak (mean ± SD) of 86.8 ± 41.6 pg/l in plasma samples taken one hour after the drink. This was six times higher than the plasma ^{26}Al concentration of the control group taken at the same time (13.8 ± 1.4 pg/l; $p = 0.02$). The dietary ^{26}Al levels were measured with AMS [471]. Another study by the same authors showed that middle-aged (35–46 years old) DS patients have higher aluminum absorption than younger (18–25 years) DS patients [474].

DS patients develop NFTs at an earlier age than AD patients

The high rate of aluminum absorption observed in DS patients could be expected to elevate their plasma aluminum levels, correlate with higher brain aluminum levels, as observed in AD and dialysis patients [121, 134], and increase the development of AD hallmarks in the brain since aluminum participates in their formation. Intraneuronal aluminum could lower the PP2A activity that occurs in DS brains as in rat brains [92] and an *in vitro* system [187]. Inhibition of PP2A activity leading to accumulation of hyperphosphorylated tau [92, 187], could explain observations that hyperphosphorylated tau accumulates in neurons of DS patients at younger ages than neurons of age-matched controls [475]. These changes precede and result in NFT formation which occurs in DS brains as much as 30 years earlier than in brains affected by sporadic AD [467, 468]. By old age, brains from some of the oldest DS patients (up to age 74 years) were observed to have higher NFT densities in the entorhinal cortex than in the same region of brains from elderly AD cases [476]. Many DS patients show evidence of chronic aluminum neurotoxicity, by formation of plaques and tangles in their brains during early middle age (35–40 years) [467, 468].

The cells of origin for the perforant pathway in the entorhinal cortex specifically show NFTs earlier than other brain regions in DS [467] as in AD [130–133]. The NFT-affected stellate-shaped cells of origin for the perforant pathway of DS and AD brains are homologous to the stellate-shaped cells of origin for the perforant pathway in the superficial entorhinal cortex of the rat model for chronic aluminum neurotoxicity that show high-stage nuclear aluminum accumulation [91]. The entorhinal cortex is ultimately the most severely-damaged brain region in aged DS [477] and AD subjects [130–133], and the rat model for chronic aluminum neurotoxicity [91, 94].

NFTs continue to grow with time. By old age, many cells containing large NFTs become enucleated by the NFTs and destined for cell death [136]. The remaining neuronal populations in the entorhinal cortex and hippocampus of DS patients are only 10% and 50%, respectively, of the expected normal number for their decade [476, 478]. Also, the volume of the entorhinal cortex in DS brains diminishes by 40% [476]. These findings are consistent with accelerated NFT growth.

The progression of AD-type neuropathology in DS cases follows a similar pattern to that which occurs in AD cases apart from an earlier time of onset. The hippocampus is the next NFT-affected region after the entorhinal cortex, followed by neocortical association areas [476] as in AD and the rat model for chronic aluminum neurotoxicity. DS patients also have decreased levels of acetylcholine and other neurotransmitters as in AD and the rat model for chronic aluminum neurotoxicity [479, 480]. The main differences in neurotoxicity between DS and AD brains are revealed by quantitative analyses of lesion densities. DS brains frequently display higher regional densities of Aβ plaques compared to plaque densities in AD brains. The superior frontal cortex and inferior temporal cortex of most DS patients have fewer NFTs than in AD [477].

The search for mutant genes on chromosome 21

Proponents of the amyloid cascade hypothesis have looked to DS for clues as to how DS can help to explain that hypothesis. AβPP is located on chromosome 21 [481], the chromosome that has an additional copy in DS, and for many years there has been an intensive search for mutations in the AβPP gene on chromosome 21 of sporadic AD cases as observed in early-onset familial cases. These searches have

also been extended to mutations in presenilin genes, on other chromosomes, that affect AβPP processing. Dozens of mutations have been identified but none are common to the large numbers of individuals with sporadic AD who comprise up to 95% of all AD cases.

DS patients have higher brain aluminum levels than controls and a higher risk for developing AD at earlier ages

The unusually high level of aluminum absorption in DS patients shortens the time required for aluminum to reach toxic concentrations in brain tissue. The individual's ability to absorb aluminum, and the amount of aluminum routinely consumed, determine the time required for neurons in AD-vulnerable regions to attain toxic aluminum concentrations and explain why brains of middle-aged DS cases contain as much aluminum as brains of sporadic AD cases decades older at time of death [134].

A disproportionately large percentage of individuals with DS are diagnosed with AD-type dementia by 55 years of age [246, 469]. Estimates of dementia in people with DS vary from 13% to 45% [469]. Dementia occurred at a mean age of 54.7 years, with a prevalence of 13.3% in a population of 235 people with DS, which is significantly greater than for age-matched controls [246]. In a group of 98 people with DS, dementia was reported to increase rapidly from age 40 years and to peak at 30% between 51 and 55 years before declining [469], with an earlier onset in DS females than males.

In general, DS neuropathology and cognitive deterioration parallel those that occur in sporadic AD but at an accelerated rate. Furthermore, the cognitive deterioration that develops from both human conditions is comparable to the cognitive deterioration that occurs in rats with chronic aluminum neurotoxicity induced from chronic dietary aluminum intake at human-relevant concentrations.

CONCLUSIONS

Applying Bradford Hill's nine causality criteria

I. Strength of the aluminum/AD association

- Epidemiological studies based on identical and non-identical twin pairs have consistently shown that AD has prominent environmental and genetic components.
- The environmental component of AD is associated with living conditions in industrialized countries.
- AD prevalence and incidence are increased in populations exposed to high aluminum concentrations compared to those exposed to low aluminum levels.
- Large and rigorously-controlled prospective and retrospective human studies have examined the aluminum levels in drinking water supplied to geographic regions. Those studies have shown significantly increased risk for AD in human populations that routinely consume water containing $\geq$0.1 mg/l aluminum compared to those that routinely consume water in regions with aluminum levels below 0.1 mg/l.
- The toxic nature of aluminum precludes its use in prospective interventional randomized controlled trials (RCTs) on humans to test chronic aluminum intake outcomes.
- An RCT was carried out in the main longitudinal study [82] using laboratory rats that mimicked exposure to total dietary aluminum in amounts that humans ingest from food, water, and aluminum additives.
- The protocol for the main longitudinal study provided sufficient aluminum to accumulate to toxic levels in neurons of a substantial proportion of the rats' brains, during a long clinically-silent prodromal phase, eventually causing overt cognitive deterioration with severe impairment of memory and appearance of abnormal AD-like behaviors when the rats were in old age.
- The results indicate that long-term aluminum ingestion in an amount equivalent to a relatively low level in the total dietary aluminum range that most humans ingest from food, water, and aluminum additives, is sufficient to increase the risk for cognitive deterioration in old age in susceptible individuals that efficiently absorb aluminum, as indicated by a relatively high plasma or serum aluminum level.
- The longitudinal animal studies showed increased risk for cognitive deterioration in old age in the majority of those that routinely ingested aluminum at the high end of the total dietary aluminum range for humans, regardless of their genetic disposition.

Taken together, these factors are evidence for an aluminum/AD association.

II. Consistency of the observed aluminum/AD association

- Several studies have shown orally-consumed aluminum (^{26}Al) enters the brain, even from trace aluminum levels [14–16].

- In industrialized societies, aluminum accumulates in the brain with advancing age, including healthy people without dementia [17–20].
- Humans with AD absorb more ^{26}Al from a standardized ^{26}Al dose [109] and also have higher plasma/serum aluminum levels than controls [109–118].
- A hierarchy of affected regions in AD brains has higher aluminum content in the gray matter than in that of the same brain regions of controls [127–129, 134].
- Aluminum preferentially accumulates in human brain regions which are vulnerable to AD and in homologous brain regions of rats, and can reach levels that are toxic to neurons in humans and other mammalian species [33, 91, 127–129, 134, 135].
- Toxic aluminum levels in brain cells are associated with NFT formation, high-stage nuclear aluminum accumulation, and other important subcellular changes [33, 91, 124, 134].
- Aluminum particularly accumulates in, and damages, the cells of origin for the perforant pathway of the entorhinal cortex, and cells of the hippocampal CA1/subiculum zone, in rats with chronic aluminum neurotoxicity. This results in microtubule depletion, dieback, and disconnection of the hippocampal formation from the neocortex by the same mechanism that occurs in AD [131, 132].
- Hippocampal disconnection provides a structural basis for the early memory changes that occur in AD, manifesting as confusion and inability to recall new and changing episodes [91, 94, 130–133].
- Many investigators have shown that aluminum exposure interferes with learning and memory processing under a range of experimental conditions [153–166].

Thus, key findings that implicate a causal role for aluminum in AD have been verified by multiple investigators, working in different countries, using various species of aluminum, in several mammalian species, meeting the test of consistency.

III. Specificity of the aluminum/AD association

- The three major categories of aluminum neurotoxicity are (1) acute aluminum neurotoxicity, (2) sub-acute aluminum neurotoxicity, and (3) chronic aluminum neurotoxicity. The categories are distinguishable by their plasma and brain aluminum levels and the duration of aluminum exposure required to result in neuropathological effects.
- AD closely resembles the category of chronic aluminum neurotoxicity.
- Neuropathology observed in renal failure patients on long-term dialysis, in the sub-acute aluminum neurotoxicity category, with brain aluminum levels less than 7 μg/gram brain tissue (frontal cortex), resembles stages of AD neuropathology that occur in the AD prodromal phase.
- The specificity criterion requires comparison of aluminum with other environmental agents proposed to cause AD.
- Iron, copper and zinc have been proposed as causes of AD but are essential metals subject to inherent cellular controls necessary for their participation in metabolic reactions.
  - Aluminum actually dysregulates iron metabolism, causing abnormally high levels of iron to accumulate in memory-processing regions. Excess iron and aluminum cause synergistic oxidative damage and interact in other ways where co-present in AD.
  - Metabolic activities of iron and aluminum, when co-present, are too intertwined to dissociate and quantify their individual contributions to neurotoxicity.
  - Evidence for copper causality is mainly concerned with amyloid metabolism, lacking effect on NFTs and clinical dementia. Evidence for zinc causality of AD is limited.
- Lead and mercury produce some effects in cells apparently equivalent to those produced by aluminum, suggesting that upregulation of AβPP, oxidative stress, and hyperphosphorylated tau may be protective cell responses to toxic metals. Lead and mercury experiments should be repeated and the histopathology stained for aluminum to verify the absence of aluminum (or otherwise) to know whether lead and/or mercury can produce AD neurotoxic effects. Aluminum produces AD neurotoxic effects without elevating lead and mercury levels.
- Two major differences between aluminum on the one hand and lead and mercury on the other are:
  - Aluminum is available for brain uptake due to its inclusion in processed foods, alum-treated drinking water, and other sources of bioavailable aluminum such as aluminum-adjuvanted vaccines, deodorants, suntan lotions, and aluminum-based antacids.
  - Aluminum accumulates in brain regions that are susceptible to damage in AD whereas lead and mercury and the other metals proposed to cause AD distribute in different brain regions.

Hence, the aluminum/AD relationship fulfils the specificity criterion.

IV. Temporal relationship for the aluminum/AD association

- Aluminum intake is a life-long process in industrialized societies, beginning at birth, or prior to birth in fetuses of pregnant women who either consume a fast food diet rich in aluminum additives, ingest antacids, or receive aluminum-adjuvanted vaccines during pregnancy.
- Low concentrations of aluminum inhibit PP2A activity, which results in hyperphosphorylation of tau and neurofilament proteins and induces oxidative stress which increases amyloidogenesis in brains of susceptible species.
- The available evidence indicates that AD is a relatively new neurodegenerative disease, and that increases in aluminum exposure and usage have contributed to the brain's aluminum burden and the increasing prevalence of AD throughout the 20th century, particularly since the 1980s.
- Long-term exposure of experimental animals to aluminum compounds in amounts equivalent to the human range of total dietary aluminum results in an AD-like animal-equivalent condition, described in this paper as chronic aluminum neurotoxicity with cognitive deterioration, provided that the duration of exposure is appropriate for the aluminum dose level so the aluminum has time to accumulate in the brain to toxic levels.

The exposure of aluminum occurs before AD or cognitive deterioration develop, indicating that the temporality criterion is satisfied.

V. Biological gradients in the aluminum/AD association

- Dose-response effects have been demonstrated in prospective and retrospective epidemiological studies of aluminum in drinking water and risk for AD [58, 61, 68].
- Dose-response effects were observed in the main longitudinal study where three groups of rats that chronically consumed different amounts of total dietary aluminum at human-relevant levels developed cognitive deterioration in a dose-dependent manner. None of the rats in the 0.4 mg/kg bw/day group, 2 of 10 in the 0.5 mg/kg bw/day group, and 7 of 10 rats in the 1.6 mg/kg bw/day group developed a form of chronic aluminum neurotoxicity.

Experiments designed to test the relationship between aluminum and AD have shown significant results with dose-response effects.

VI. Plausibility of chronic aluminum intake as a cause of AD

A plausible biological rationale detailed in Figure 12 explains how chronic aluminum intake can cause AD in older humans:

- A sequence of neurotoxic events occurs in AD-affected and interconnected brain regions that accumulate aluminum to toxic levels.
- Aluminum transfers between interconnected AD-vulnerable brain regions. This transferability allows aluminum to replicate the same sequence of neurotoxic events in those other brain regions.
- Aluminum-induced microtubule depletion results in a neurotoxic sequence involving axonopathy, dendritic/axonal dieback, synapse loss, cortical atrophy, a disconnection syndrome and loss of neurotransmission, and attentional and memory dysfunctions within the affected brain regions.

VII. Coherence: relationship between cause (chronic aluminum intake) and effect (AD) compatible with existing knowledge

The proposed cause and effect should relate to the scientific literature on AD, showing coherence with what is already known about AD. The most salient features of AD in humans and chronic aluminum neurotoxicity in rats show the two conditions are similar with a high degree of probability:

- A long prodromal phase precedes the appearance of cognitive deterioration in humans and experimental animals.
- Aluminum accumulates in the same brain regions in animals, that are susceptible to damage in AD.
- Aluminum inhibits PP2A activity, giving rise to hyperphosphorylated tau in mammalian neurons.
- Aluminum interacts with hyperphosphorylated tau to form NFTs in brains of humans with AD.
- Aluminum produces oxidative stress which upregulates AβPP and increases amyloid formation.
- Aluminum induces a variant form of presenilin 2 which occurs in aged patients with sporadic AD, and which increases the formation of $A\beta_{1\text{-}42}$.
- Aluminum accumulation in pyramidal cells favors the amyloidogenic pathway.
- Aluminum depletes cells of their microtubules, resulting in axonopathy or failure of axonal transport.

- Microtubule depletion results in dendritic/axonal dieback, with synapse breakdown and loss as the cell processes shrivel.
- Over time, the neurotoxic events occur in increasing numbers of cells, ultimately leading to disconnection of brain regions from each other, brain atrophy and loss of abilities for memory processing.

Therefore, the concept of chronic aluminum intake as a cause of AD coheres with existing knowledge about the behavior of aluminum in biological tissues and the natural history and biology of AD, including AD development, progression and neuropathology. The evidence supports the concept that AD is a human form of chronic aluminum neurotoxicity.

VIII. Experimental evidence

The criterion of experiment calls for studies that either prevent or partially reverse the effects of aluminum on AD:

- The main longitudinal rat study has shown that rats that chronically drink aluminum-free water and consume foods with relatively low aluminum levels remain cognitively-intact whereas rats with greater chronic aluminum intake have an increased risk for developing chronic aluminum neurotoxicity with cognitive deterioration.
- Where cognitive deterioration from AD is already apparent, McLachlan's studies [449, 450] indicate that aluminum chelation by frequent intramuscular DFO injections significantly reduce the amount of brain aluminum in humans and halve the rate of AD neurodeterioration. Aluminum chelation is the only therapeutic approach to date that has proven effectiveness, particularly if treatment can be commenced at a relatively early stage of overt AD.
- DFO injections into rabbits partially reversed NFT formation in their brains.
- New-generation chelating agents for aluminum (Feralex-G and CPs), used with or without antioxidants, have already helped to suppress oxidative stress and neuroinflammation induced by aluminum in brains of experimental animals.

It is suggested that Bradford Hill's criterion for experimental evidence has been met.

IX. Analogy

DS patients with early-onset AD provide a model for chronic aluminum neurotoxicity in humans. DS patients absorb 6-fold more aluminum than age-matched non-DS controls at the dietary aluminum level and 4-fold more at the pharmacological level. Analogous consequences occur:

- DS patients exhibit chronic aluminum neurotoxicity at an early age, having low PP2A activity and high levels of hyperphosphorylated tau.
- A disproportionately large number of individuals with DS have been diagnosed with AD-type dementia.
- The high rate of aluminum absorption in DS subjects accelerates the time required for aluminum accumulation to toxic levels in neurons of AD-vulnerable brain regions.
- DS brains exhibit large amounts of senile plaques and NFTs. The accelerated appearance of AD hallmarks in DS brains is likely due to the unusually high levels of aluminum that people with DS absorb.
- Dementia outcomes in DS are similar to those of AD and both are similar to the outcomes resulting from cognitive deterioration in the rat model for chronic aluminum neurotoxicity.
- Findings indicate that DS, prolonged dialysis treatment, and rats that chronically consume high aluminum levels, are all analogies of chronic aluminum neurotoxicity. In the words of Van Reekum et al., "If some condition similar to A causes an outcome similar to B, then this is evidence that A causes B". The essence of the analogy is demonstrated in Table 2. Hence, the criterion of analogy has been satisfied.

This review has addressed the nine criteria prescribed by Austin Bradford Hill when studying the aluminum/AD relationship before claiming causality

On its own, a single criterion is insufficient to provide indisputable evidence for a cause-and-effect hypothesis. Taken together, a group of criteria can help us to decide whether chronic aluminum intake is the best explanation for AD. The author welcomes a similar analysis from anyone who can provide a better explanation for AD in terms of Bradford Hill's causality criteria. "The evidence is there to be judged on its own merits and the judgment ... should be utterly independent of what hangs on it" [38]. In the author's opinion, other hypotheses for AD fall short on these criteria. All hypotheses for AD have their critics, and rebuttals [75, 80, 94, 135, 143, 482–486], including the amyloid cascade hypothesis [203–208]. The author

Table 2
Analogies as Evidence of Causation (Criterion 9). If a condition similar to A (chronic aluminum intake and/or high aluminum absorption) causes an outcome similar to B (upper box), this provides evidence that A (chronic aluminum intake and/or enhanced aluminum absorption) causes B (Alzheimer's disease) (lower box)

Condition similar to A	Via	Outcome similar to B
High chronic aluminum intake and/or absorption in rats [81, 82]	High serum aluminum level and brain lesions [82, 91, 94]	AD-equivalent behaviors and species-specific AD neuropathology [91, 94] (hyperphosphorylated tau, oxidative stress, microtubule depletion)
High sub-acute aluminum intake in dialysis patients [121]	High plasma and brain aluminum levels [121]	Early signs of AD neuropathology [121]
High chronic aluminum absorption in Down's syndrome [471]	High plasma aluminum levels and brain lesions [134, 471]	Premature AD dementia and AD neuropathology [246, 470]
Condition A	Via	Outcome B
Chronic aluminum intake and/or high aluminum absorption in humans [104]	Chronic aluminum neurotoxicity resulting in high plasma levels [110–118] and brain lesions [91, 94, 134, 143]	Alzheimer's disease, with attendant behaviors and neuropathology

also believes that the present causality analysis takes into account the subsequent discussion of the merits of Bradford Hill's methodology [487].

AD is a human form of chronic aluminum neurotoxicity and the aluminum-inducible rat model for chronic aluminum neurotoxicity is a translational model for AD

The effects of chronic aluminum neurotoxicity and AD are numerous, and too strikingly similar to each other to be coincidental. These data indicate that the considerable length of the prodromal phase in AD is primarily due to the time required for sufficient aluminum to cross the gastrointestinal and blood-brain barriers to accumulate to toxic levels in AD-vulnerable neuronal populations, comparable to that which occurs in rats with chronic aluminum neurotoxicity. A threshold effect would explain the narrow margin of difference in brain aluminum concentrations that distinguishes people with AD from other residents of industrialized societies still in the prodromal phase [80].

AD and chronic aluminum neurotoxicity are progressive, and they are irreversible once overt, after the prodromal phase required for the slow intraneuronal aluminum accumulation to toxic levels in the large corticocortical cells of the entorhinal cortex, hippocampus, amygdala, and olfactory bulb. Substantial numbers of cells in these regions either exhibit NFTs or staining for high-stage (IV and V) nuclear aluminum accumulation [33, 91, 136], demonstrable with the Walton histological stain for aluminum [91]. Cortical atrophy dynamically spreads from entorhinal and limbic regions into neocortical association areas and beyond, affecting the same regions in AD and chronic aluminum neurotoxicity [82, 91, 143].

The longitudinal aluminum-inducible rat model is a translational animal model for AD dementia [94] as well as a model for chronic aluminum neurotoxicity. Apart from species difference, this animal model replicates a mechanism that occurs early in AD (hippocampal isolation) and is a basis for investigations into the spread of AD to other brain regions as AD increases in severity.

A reliable animal model for AD is also important for developing interventional treatments to slow AD deterioration or provide earlier detection. The rat model for chronic aluminum neurotoxicity suggests that a diagnostic test for a moderate rise in aluminum absorption from a standardized aluminum dose could serve as an early warning for AD risk well before cortical atrophy, NFTs, and Aβ deposits occur. The animal model also opens the way for analyzing AD progression by examining a temporal sequence of effect on brain regions under various parameters, and for testing the effectiveness of new treatment protocols, such as the new generation oral chelating agents described in this causality analysis.

Importantly, the main longitudinal study showed that a lowest observed adverse effect level (LOAEL) was in the rat group in which 20% of the animals developed cognitive deterioration after chronically consuming an intermediate dose of 0.5 mg/kg bw/day [82]. All rats in this study were weight-controlled at 500 g ± 50 g. When converted to a LOAEL by surface area, as recommended by the FDA [488], the equivalent dosage for humans is at 0.12 mg aluminum/kg bw/day, a dose level that most residents of contemporary industrialized societies routinely exceed. Interestingly, this

dose limit is similar to the daily level (0.14 mg aluminum/kg bw/day) of the WHO's provisional tolerable weekly intake level for humans, which was lowered to 1 mg aluminum/kg bw/week in 2007 [10].

Overall, the evidence indicates that AD is a human form of chronic aluminum neurotoxicity. Many aspects of AD can now be answered by the growing knowledge of chronic aluminum neurotoxicity in translational animals, dialysis patients, and DS patients.

The case for action

Bradford Hill's causality treatise ends with the case for action [38]. He writes, "in passing from association to causation I believe in 'real life' we shall have to consider what flows from that decision ... Our object is usually to take action. If this be operative cause and that be deleterious effect, then we shall wish to intervene to abolish or reduce death or disease ... If we are wrong in deducing causation from association no great harm will be done. The good lady [i.e., the patient] and the ... industry will doubtless survive."

Bradford Hill makes the point that "All scientific work is incomplete – whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have, or to postpone the action that it appears to demand at a given time" [38]. Given the evidence presented in the current analysis, the balance of probability supports a central role for chronic aluminum intake and, hence, chronic aluminum neurotoxicity in AD causality.

The way humans may be able to protect themselves from AD is by consuming a fresh food diet and trying to avoid, or at least minimize, routine exposure to products that contain potentially bioavailable aluminum compounds.

The actions proposed are:

1. Reassessment of the GRAS rating for all potentially bioavailable aluminum substances that may contribute to the body's aluminum burden.
2. Label the amounts of aluminum contained in processed food products, bottled water, and any other product that contains potentially bioavailable aluminum.
3. Facilitate urban water suppliers to convert from use of aluminum salts to ferric salts, or other acceptably safe compound for coagulation so that drinking water may be consistently delivered at a specification well below 0.1 mg aluminum/liter.
4. Develop standardized tests for plasma or serum aluminum content so that individuals can know whether they are high, intermediate, or low absorbers of aluminum.
5. Develop policies that could help people at high risk for AD to avoid products that contain aluminum compounds.
6. Resume chelation studies designed to reduce aluminum in the brain to an acceptable level.

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The author's disclosure is available online (http://www.j-alz.com/disclosures/view.php?id=2071).

SUPPLEMENTARY MATERIAL

A QuickTime video clip of rats with and without cognitive deterioration is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-132204.

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