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Short Communication

Evidence for Participation of Aluminum in Neurofibrillary Tangle Formation and Growth in Alzheimer's Disease

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

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Abstract. This study examines hippocampal CA1 cells from brains of aged humans, with and without Alzheimer's disease, for hyperphosphorylated tau and aluminum during early neurofibrillary tangle (NFT) formation and growth. A very small proportion of hippocampal pyramidal cells contain cytoplasmic pools within their soma that either appear homogeneous or contain short filaments (i.e., early NFTs). The cytoplasmic pools are aggregates of an aluminum/hyperphosphorylated tau complex similar to that found in mature NFTs. The photographic evidence presented combines with existing evidence to support a role for aluminum in the formation and growth of NFTs in neurons of humans with Alzheimer's disease.

Keywords: Aluminum, Alzheimer's disease, hyperphosphorylated tau, neurofibrillary tangles

Alzheimer's disease (AD) is a neurodegenerative condition identified approximately 100 years ago [1]. AD now affects more than 21 million people worldwide [2], particularly those living in industrialized countries [3]. Identical twin epidemiological studies demonstrate AD causality is partly genetic and partly environmental (e.g. [4]). Severe dementia is the primary clinical feature of AD, and amyloid plaques and neurofibrillary tangles (NFTs) are its most prominent neuropathological hallmarks. Fewer NFTs occur in brain tissue of younger old (∼75 years) non-demented humans but NFTs gradually increase, approaching equivalence in brains of non-demented and demented humans around age 95 [5].

*Correspondence to: Associate Professor J.R. Walton, Research and Education Centre, 4–10 South St, Kogarah (Sydney) NSW 2217, Australia. Fax: +61 2 9113 3967; E-mail: j.walton@unsw.edu.au.

Alzheimer used Bielschowsky staining and described NFTs at early, mature, and extracellular stages in AD pyramidal cells [6]. Development of antibody immunostaining techniques has since enabled identification of a pre-tangle stage [7,8], marked by cells containing cytoplasmic granules that immunostain for hyperphosphorylated tau. Under transitional conditions yet to be defined, the hyperphosphorylated tau of these granules eventually assembles into a protease-resistant linear polymer of truncated tau protein that constitutes the main structural unit of AD NFT filament cores [9,10]. Once formed, intracellular NFTs continue to grow [11], becoming sufficiently large in some cases to enucleate their host cell [12].

Aluminum (Al) has been demonstrated in AD NFTs by histological and spectroscopic techniques [12–16]. The present communication provides photographic evidence of pre-tangle and early NFT stages characterized by one or more cytoplasmic pools of an

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Table 1
Demographics of cases included in this study

Case	Age	Gender	Ethnicity	PMI	Diagnosis	Yrs since diagnosis	Brain wt
1	74	Male	Caucasian	24	AD	unknown	1265
2	81	Male	Caucasian	21	AD	8	1480
3	84	Female	Caucasian	24	AD	12	883
4	86	Male	Caucasian	48	AD	3	1425
5	88	Male	Caucasian	72	AD	unknown	1180
6	90	Female	Caucasian	3	Con	–	1200
7	70	Male	Caucasian	14.5	Con	–	1370
8	81	Male	Caucasian	24	Con	–	1240
9	82	Male	Caucasian	36	Con	–	1300
10	82	Male	Caucasian	23.5	Con	–	1320

Al/hyperphosphorylated tau complex within the soma. The findings support a role for Al in NFT formation.

This study was based on 10 μm paraffin sections of corticolimbic tissue from five humans with AD and five non-demented controls, autopsy-confirmed with CERAD criteria [17] and anonymized for ethical reasons (Table 1). Tissue sections were provided by the NSW Tissue Resource Centre under human research ethics approval.

Sections from each case were histologically stained for Al or immunostained with anti-PHF-1 antibody, using an antigen retrieval avidin-biotin procedure described elsewhere [18], to examine for a possible relationship between Al and hyperphosphorylated tau protein in AD hippocampal pyramidal cells at early stages of NFT formation. The Walton method for identifying endogenous Al in biological tissues was previously validated by tests for sensitivity and specificity [19]. Phloxine B binds to Al in biological tissue, staining it pink, magenta, or purple, depending on Al concentration and binding substrate. Al-stained NFTs are positively identified using a 100x oil immersion objective. The counterstains provide a context for Al, coloring nuclear membranes gray, cytoplasm blue, and endothelium green. The modified Walton method [20,21] utilizes a different nuclear counterstain from the hematoxylins (which contain Al salts) originally used. This modification increases its similarity to Lapham's stain for myelin [22]. Al's high affinity for myelin [23–26] may explain why the Lapham method effectively stains myelin.

Al-stained hippocampal CA1 fields were carefully studied and cells of interest photographed, using vernier scale readings for the x- and y-axes of the microscope stage to record their locations. Some of these sections were de-stained and subsequently immunostained for hyperphosphorylated tau. The slides were put into xylene for several days to remove the coverslip, followed by 70% ethanol for one hour to remove dyes. The previously photographed cells were located in the immunostained sections using vernier readings and morphological features to enable re-photography. This de-staining/re-staining process is unidirectional because the antigen retrieval step of the immunohistochemical procedure chelates and removes Al.

Sections from 9/10 aged cases confirmed the presence of pre-tangle cells in the hippocampal CA1 field. Case 3 (the exception) exhibited extensive CA1 cell loss. Pre-tangle cells contain small cytoplasmic granules, diffusely-distributed throughout their soma, that immunostain for hyperphosphorylated tau (Fig. 1A) [7, 8]. The cytoplasmic granules lack membranes, unlike those in granulovacuolar degeneration (GVD). GVD granules also stain for hyperphosphorylated tau, and for Al if sufficiently large [12]. The cytoplasmic granules shown in Fig. 1B are larger and partially aggregated. They appear intermediate between the stages shown in Figs 1A and 2B.

One Al-stained CA1 pyramidal cell from Case 9 (control) and four from Case 2 (AD) were observed to contain distinctive purple cytoplasmic pools within their soma. One Case 2 cell had a purple cytoplasmic pool with a slightly grainy texture that otherwise appeared homogeneous (Fig. 2A). After de-staining and immunostaining for hyperphosphorylated tau, the cytoplasmic pool was dark brown, retaining its homogeneous appearance (Fig. 2B). The darkest brown regions of this cytoplasmic pool are super-imposable over the purple Al-stained cytoplasmic pool up to its boundary. Cells with the characteristics shown in Figs 1B and 2 are seldom observed.

Four cells (that from case 9 and the remaining three from case 2) exhibited early (stage 1) NFTs, having just discernable filamentous structure within their cytoplasmic pools when stained for Al (Fig. 3A) and hyperphosphorylated tau (Fig. 3B). The filament color under stain resembles that of the cytoplasmic pools from which they precipitate.

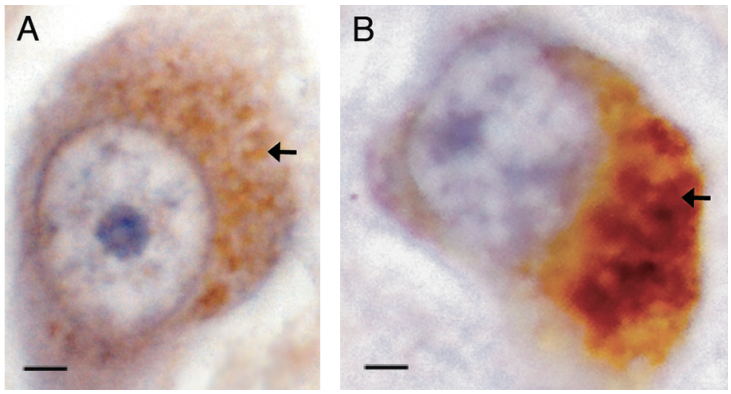

Fig. 1. Pre-tangle pyramidal cells (stage 0) immunostained for hyperphosphorylated tau. Al-stained images of these pre-tangle cells are unavailable since their positive identification requires immunostaining for hyperphosphorylated tau and the de-staining/re-staining procedure is unidirectional (Al stain to immunostain). A) Early pre-tangle phase cell (Case 6) is identifiable by small cytoplasmic granules that immunostain brown (arrow) for hyperphosphorylated tau and surround the membrane-bound nucleus that contains a blue (hematoxylin-counterstained) nucleolus. Such cells are common in aged neocortex and hippocampus, both in demented and non-demented humans. B) In this pre-tangle cell (Case 5), the cytoplasmic granules of hyperphosphorylated tau stain orange (arrow), are larger, and aggregated. Magnification bar (MB) = 2.5 μm.

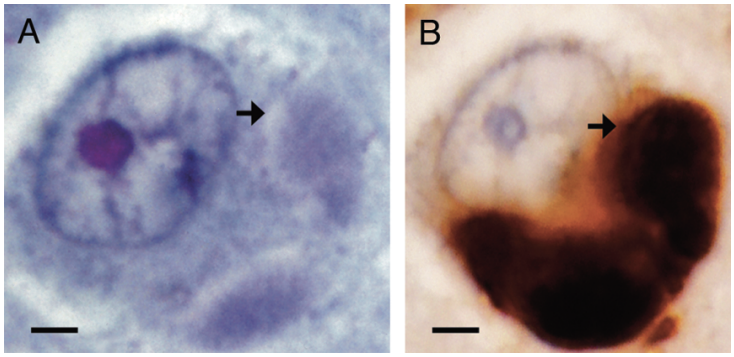

Fig. 2. A late pre-tangle stage CA1 cell (Case 2) stained for Al and then de-stained and re-stained for hyperphosphorylated tau. This seldom-observed stage is characterized by its grainy-textured cytoplasmic pools that are otherwise featureless. A) This cell stains purple for Al in its cytoplasmic pools and magenta for Al in its nucleolus. The arrow denotes a margin of the cytoplasmic pool that lacks stain for Al. B) Upon de-staining this cell and re-staining it for hyperphosphorylated tau, the cytoplasmic pool stains dark brown where it formerly stained purple. Less strongly immunostained margins (i.e., arrow) probably represent thinner or less dense edges of the cytoplasmic pool. Taken together, the results suggest that the concentration of Al has to exceed a threshold level in order to stain. MB = 2.5 μm.

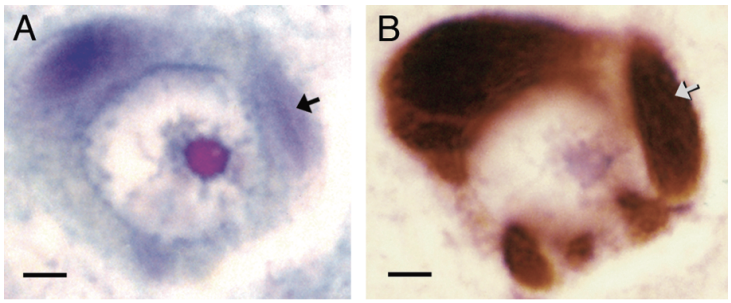

Fig. 3. A CA1 cell containing an early (stage 1) NFT. This cell (Case 2) was (A) stained for Al and then (B) immunostained for hyperphosphorylated tau. In (A), an arrow indicates just visible filament structure in a cytoplasmic pool. In (B), the immunostain reveals filaments (arrowed) that are slightly more pronounced. Early (stage 1) NFTs are infrequently seen. MB = 2.5 μm.

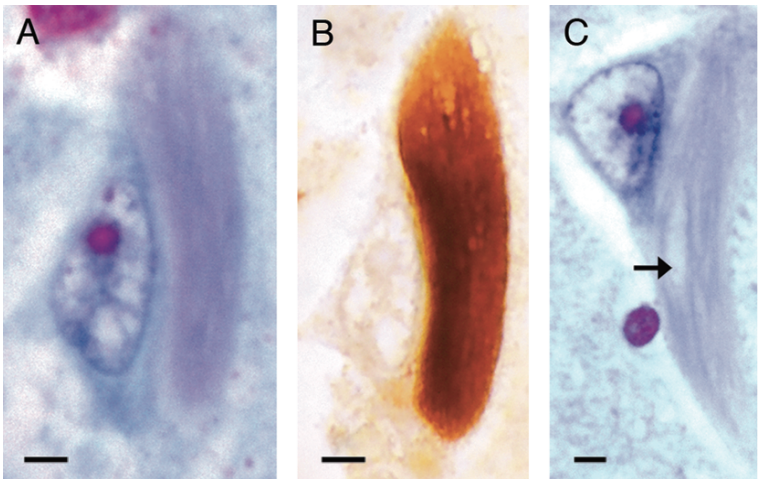

Fig. 4. Mature (stage 2) NFTs. A,B) This cell (Case 2) has a large NFT and its nucleus has been displaced to the cell periphery. A) Al-stained image. B) Same cell immunostained for hyperphosphorylated tau. The NFTs are superimposable. C) Another Al-stained cell (Case 1) has a large NFT that appears to have fewer and relatively thick purple fibrils, indicating filament aggregation. The absence of the cytoplasmic pool substance between fibrils (i.e., arrow) suggests it was consumed during filament growth. CA1 cells with mature (stage 2) NFTs are frequently seen in AD and in fewer numbers in aged controls. MB = 2.5 μm.

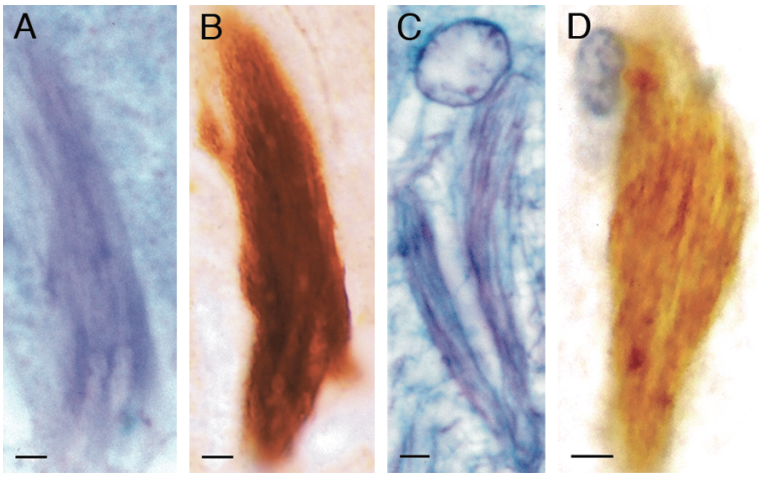

Fig. 5. Extracellular (stage 3) NFTs. One tangle (Case 2) is (A) stained for Al and (B) immunostained for hyperphosphorylated tau. These NFT images are superimposable. C,D) Other extracellular NFTs appear more degraded. C) This Al-stained extracellular NFT (Case 3) has fibrils that stain non-uniformly. D) An immunostained example (Case 8) shows patchy immunostaining for hyperphosphorylated tau. A glial cell appears to the left of this extracellular NFT. Stage 3 NFTs are commonly seen in AD cases, less so in controls. MB = 2.5 μm.

Mature (stage 2) NFTs were prominent in all AD cases plus 3/5 controls, comprising densely-packed filaments that stain for Al (Fig. 4A) and hyperphosphorylated tau (Fig. 4B). The cytoplasmic pool substance is no longer visible between the fibrils (Fig. 4C) of some cells with very large NFTs. Extracellular (stage 3) NFTs also stain for both Al (Figs 5A and C) and hyperphosphorylated tau (Figs 5B and D). Glial cells are commonly associated with extracellular NFTs and are believed to modify their structural and immunostaining characteristics (Fig. 5D).

Humans are routinely exposed to Al from dietary and other sources [reviews [27,28]]. Such exposure gradually increases brain Al levels, with rising age, in humans with normal cognition [29–31] and more so with dementia [reviews [32–34]]. Al particularly accumulates in pyramidal cells (of Al-exposed dialysis patients, rats and rabbits) in the same brain regions that are susceptible to damage in AD [12–14,20,35–38], altering their tau structure and function [13,21,37].

Normal tau function depends on phosphate addition by kinases and phosphate removal by protein phosphatase 2A (PP2A), the main phosphatase in mammalian brain responsible for tau dephosphorylation at serine and threonine residues [39–41]. AD cortex and hippocampus exhibit low PP2A activity and expression [39,42,43], depletion of normal tau, and elevated levels of hyperphosphorylated tau [44] with the additional phosphates accumulating on serine and threonine residues [45]. Al inhibits PP2A activity, disrupting the tau kinase/phosphatase balance and inducing tau hyperphosphorylation *in vivo* and *in vitro* [18,46]. For example, low PP2A activity and hyperphosphorylated tau occur in brain tissue of an aging rat model for AD that exhibits cognitive deterioration after chronically ingesting Al throughout most of the lifespan in amounts equivalent to Al additive ingestion by many humans living in contemporary urban society [18,20,47].

Al can induce the Alz-50 epitope [48,49], an early event in AD tau change [50], and activate caspase *in vivo* and *in vitro* [51–54]. Caspase activation leads to the truncation of hyperphosphorylated tau at its Glu-391 residue [55]. Truncated hyperphosphorylated tau is already evident in granules of early pre-tangle neurons in sporadic AD cases and aged controls [10,56]. Analogous changes in tau truncation, and in levels of normal and hyperphosphorylated tau, occur in brains of long-term dialysis patients at an earlier age, their magnitudes correlating with brain Al accumulation [37].

Findings from the present study indicate that in AD the pre-tangle stage has an early phase of long duration and a brief late phase marked by aggregation of cytoplasmic granules and formation of grainy-textured cytoplasmic pools. Advancement to the late pre-tangle phase may relate to intraneuronal Al and truncated hyperphosphorylated tau levels and dementia progress. NFT formation within cytoplasmic pools is consistent with Alzheimer's illustrations of early stage NFTs where filaments are confined to the soma [1,6]. Other authors have depicted early stage NFTs as further advanced with fibrils already extending well into the apical dendrite [[7] Figs 1A, 2B].

Absence of Al staining in early pre-tangle granules, along some edges of Al-stained cytoplasmic pools, in small GVD granules, and in some sectioned NFTs, could either indicate Al absence or Al presence at concentrations below the staining threshold. If, as is the case, the centrally-located nucleolus is the only Al-positive component in many aged Al-stained pyramidal cells then, logically, some Al must be or have been present in the cytoplasm and nucleoplasm at sub-visible levels.

Experimental studies show that Al binds to phosphoproteins and changes their conformation [57,58]. Formation of stable cross-links between Al and phosphate groups on hyperphosphorylated tau isolated from AD brain tissue and neurofilament protein from animal brain results in their aggregation [13,46,59–65], which could reasonably account for the formation of pre-tangle granules and cytoplasmic pools. The superimposability of the stained images of Al and hyperphosphorylated tau in Figs 2–5 demonstrates their co-localization in an AD-vulnerable region of the human brain, at least by the late pre-tangle stage and at early, mature and extracellular NFT stages. Furthermore, co-injection of rat brains with Al and hyperphosphorylated tau dramatically increases that protein's resistance to *in vivo* proteolysis [59]. As the Al/hyperphosphorylated tau complex is the major constituent of NFTs, the co-injection finding could explain why NFTs can outlast the cells in which they grow.

The growing NFT may protect cell viability for a time [66,67] by binding and sequestering Al in the cytoplasm, thereby slowing Al accumulation in the nucleus, nuclear oxidative reactions, and cross-linkage of nuclear proteins and nucleic acids [68–70].

Present and previously-published data are consistent with the following sequence:

- Al induces hyperphosphorylation of tau by inhibiting PP2A activity in pyramidal cells.
- Hyperphosphorylated tau levels greatly increase in these cells.
- Al activates caspase which truncates hyperphosphorylated tau.
- Al binds to the truncated hyperphosphorylated tau, aggregating it into granules. When confluent, the aggregates appear as cytoplasmic pools. Confluence, associated with high local concentrations of Al/truncated hyperphosphorylated tau, may provide the trigger for polymerization.
- NFT filaments polymerize within the Al/truncated hyperphosphorylated tau complex of the cytoplasmic pools. NFT formation pre-dates AD clinical symptoms that become more severe as NFT numbers increase.
- Some NFTs become extracellular after growing sufficiently large to enucleate, and kill, their host cell.

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