Elevated Ferric, Calcium and Magnesium Ions in the Brain Induce Protein Aggregation in Brain Mitochondria

T Alleyne[1], N Mohan[1], A Adogwa[2]

ABSTRACT

Objective: *Alzheimer's disease and Parkinson's disease are two of several neurodegenerative disorders that affect the elderly. Although their aetiology remains uncertain, studies suggest that elevated aluminium or other metal ions in the brain directly influence the development of the histological abnormalities normally associated with these diseases; other investigations suggest that metal-ion-induced-dysfunction of mitochondria might be a critical factor.*

Methods: *In this study, the impact of elevated aluminum (Al^{3+}), ferric (Fe^{3+}), calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions on brain histology and on the protein composition of brain mitochondria were evaluated. Rabbits were injected intra-cerebrally with 1.4% solutions of either aluminium chloride ($AlCl_3$), ferric chloride ($FeCl_3$), calcium chloride ($CaCl_2$) or magnesium chloride ($MgCl_2$) and sacrificed 10 days later.*

Results: *Histological analysis revealed that Al^{3+} but not the other ions induced neurofibrillary degeneration within the midbrain and medulla. Alternatively, SDS-PAGE revealed that Fe^{3+}, Ca^{2+} and Mg^{2+} but not Al^{3+} induced alterations to the distribution of brain mitochondrial proteins. Both Fe^{3+} and Ca^{2+} triggered decreased concentration of three low molecular weight proteins (~7−14 kd) but Ca^{2+} precipitated their total absence. Both ions led to increased concentration of a high molecular weight protein (~ 110 kd). In contrast, Mg^{2+} led to the total absence of the protein of lowest molecular weight (~7 kd) and increased concentration of a ~36 kd protein.*

Conclusion: *These results suggest that elevation of some metal ions in the brain induces protein aggregation with the nature of the aggregation being highly ion dependent. The results also point toward major differences between the histopathological effect of Al^{3+} and other ions.*

Keywords: Alzheimer's, metal ions, neurodegenerative disorder, protein aggregation

El Aumento de Iones Férricos, Calcio y Magnesio en el Cerebro Induce la Agregación de Proteínas en las Mitocondrias del Cerebro

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RESUMEN

Objetivo: *La enfermedad de Alzheimer y la enfermedad de Parkinson son dos de los varios trastornos neurodegenerativos que afectan al anciano. Aunque su etiología sigue siendo incierta, los estudios sugieren que el aumento de los iones de aluminio, influyen directamente sobre el desarrollo de las anormalidades histológicas asociadas normalmente con estas enfermedades. Otras investigaciones sugieren que la disfunción de las mitocondrias, inducida por iones metálicos, pudiera ser un factor crítico.*

Métodos: *Este estudio evalúa el impacto del aumento de los iones de aluminio (Al^{3+}), los iones férricos (Fe^{3+}), y los iones de calcio (Ca^{2+}) y magnesio (Mg^{2+}) sobre la histología del cerebro y la composición proteica de las mitocondrias del cerebro. Un número de conejos recibieron inyecciones intracerebrales de soluciones al 1.4% de soluciones de cloruro de aluminio ($AlCl_3$), cloruro ferroso*

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($FeCl_3$), cloruro de calcio ($CaCl_2$), o cloruro de magnesio ($MgCl_2$), y fueron sacrificados después de 10 días.

Resultados: *El análisis histológico reveló que el Al^{3+} indujo una degeneración neurofibrilar dentro del mesencéfalo y la médula, Sin embargo, esto no ocurrió con los otros iones. Alternativamente, la técnica de electroforesis SDS-PAGE reveló que los iones Fe^{3+}, Ca^{2+} y Mg^{2+}, a diferencia del ión Al^{3+}, inducían alteraciones de la distribución de las proteínas mitocondriales cerebrales. Tanto el Fe^{3+} como el Ca^{2+} desencadenaron una disminución de la concentración de tres proteínas de bajo peso molecular (~7−14 kd) pero Ca^{2+} precipitó su ausencia total. Ambos iones condujeron a un aumento de una proteína de peso molecular alto (~ 110 kd). En cambio, Mg^{2+} llevó a la ausencia total de la proteína de más bajo peso molecular (~7 kd) y al aumento de la concentración de una proteína de ~36 kd.*

Conclusión: *Estos resultados parecen sugerir que la elevación de algunos iones de metal en el cerebro induce la agregación de la proteína, siendo la naturaleza de la agregación altamente dependiente de los iones. Los resultados también apuntan a grandes diferencias entre el efecto histopatológico del Al^{3+} y otros iones.*

Palabras claves: Alzheimer, iones de metal, trastorno neurodegenerativo, agregación proteica

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INTRODUCTION

In the last decade, Alzheimer's disease (AD) and Parkinson's disease (PD), two of the more common neurodegenerative disorders, have emerged as major causes of death and morbidity in the aged (1, 2). At the pathological level, AD is characterized by the presence of neurofibrillary tangles and amyloid plaques (3, 4) while biochemically, the disease is characterized by decreased cytochrome c oxidase (COX) activity (5−7) and by increased activity of cathepsin D (8).

Whereas, there is consensus on what constitutes the pathological and biochemical characteristics of AD, there is no such consensus with regard to its aetiology. A number of studies have reported the presence of decreased magnesium [Mg^{2+}] (9) but elevated aluminum (Al^{3+}), ferric (Fe^{3+}) and calcium (Ca^{2+}) ions (10−12) in the brains of persons with AD. Presently, however, it is not clear whether these altered levels cause the disease or occur as a result of it (9, 13). Querfurth and Selkoe (14), using cultured cells, have demonstrated that the presence of Ca^{2+} ionophores can lead to increased production of amyloid beta peptides, while Emilsson *et al* (15) have reported the presence of altered Ca^{2+} signalling genes in AD patients. To add to the state of confusion, Murayama *et al* (16) have shown that neurofibrillary degeneration of the type seen in AD can be caused by interaction of aluminium ions with paired helical filament tau proteins.

Parkinson's disease is characterized pathologically by the presence of Lewy bodies and Lewy neurites in the brain (17, 18) but like AD, its aetiology is unresolved. A number of studies have detected elevated Fe^{3+} in the brain of patients with PD (19) while others have shown that Al^{3+} and other heavy metals increase the production of α-synuclein, the major constituent of Lewy bodies (20, 21).

In recent times, Büeler (22) has suggested that mitochondrial dysfunction might be playing a critical role in the pathogenesis of PD. Similarly, a number of studies have led to the conclusion that inhibitor-induced alterations to mitochondrial function may be a critical factor in the pathogenesis of AD (23).

In an attempt to get better insight into the role played by metal ions and mitochondria in the pathogenesis of neurodegenerative disorders, we decided to extend the rabbit studies of Klatzo *et al* (24). In their study, Klatzo *et al* (24) reported that the injection of aluminium salts into the cerebral region of the rabbit brain caused the animals to develop ataxia after about ten days. They further reported that by such time, the treatment had also induced neurofibrillary degeneration and other pathological changes similar to those seen in the brain of patients with AD. Our objective in this study was to determine if other valency two and three metal ions had similar effects on rabbits and what effects if any the injected metal ions had on brain mitochondrial protein composition. We injected aluminium chloride ($AlCl_3$), ferric chloride ($FeCl_3$), calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) and compared their effects on the physical behaviour, brain histology as well as the difference spectra and protein composition of brain mitochondria.

SUBJECTS AND METHOD

New Zealand white rabbits were used throughout the study; only alert, active animals were employed. Six groups of rabbits, two controls and four experimental, were studied; groups comprised five rabbits each. All protocols were approved by The University of the West Indies (UWI), St Augustine, Ethics Committee.

Metal ion injection: Solutions of $AlCl_3$, $FeCl_3$, $CaCl_2$ and $MgCl_2$, each 1.4%, were prepared in 2.5% sodium phosphate buffer, pH 6.5. Using the method described by Klatzo *et al* (24), one each of the metal ions was injected into the cerebral hemisphere of a selected group of rabbits. Two groups of control animals were employed. For one group of

controls, the animals were injected with the phosphate buffer *minus* the added metal ions; the second control group received no injections. All animals were sacrificed ten days after injections were performed and the brains removed. The left hemispheres of each brain were dissected and stored at -70°C to be used for biochemical studies. Tissue from the brainstem region was harvested to be used for histological studies; the tissue was immersed in 10% buffered formal saline and stored for approximately one month before processing.

Histological analysis: Samples from the medulla and midbrain of the two groups of controls and four groups of metal-ion-injected rabbits were prepared for histological examination as described by Luna (25). The sections were stained with Mitchell's silver stain for viewing (26).

Biochemical Methods

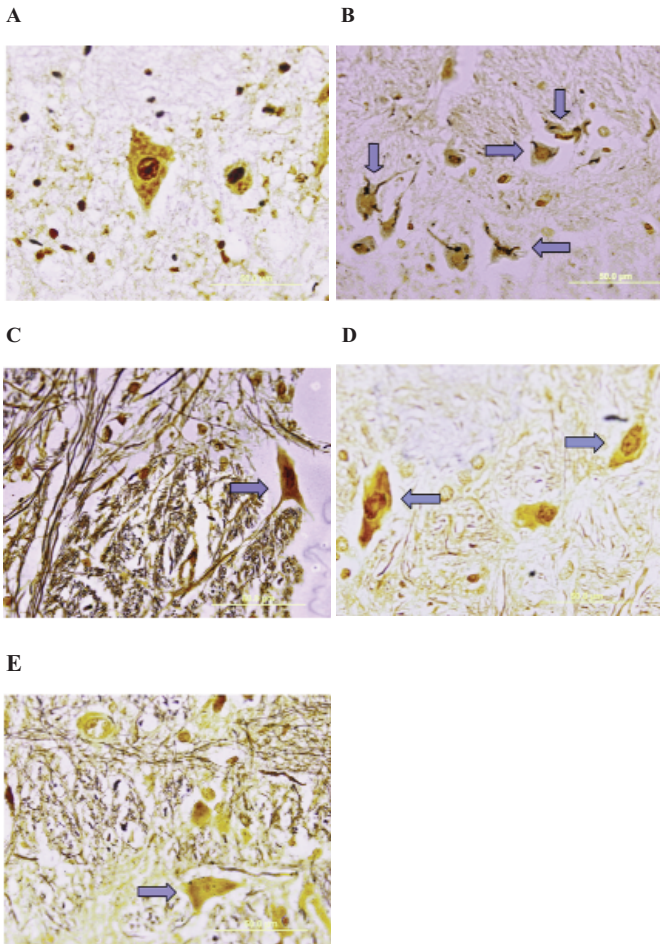
Mitochondria were isolated from the brains of controls and treated animals by the method of Yonetani (27) and stored at -70°C until needed. In the studies conducted, there was no pooling of tissue; the tissue from each rabbit was processed and studied independently. For each preparation of mitochondria, the difference spectra and protein composition were determined.

Difference spectra: Difference spectra, reduced *minus* oxidized, 400−630 nm, were recorded for mitochondria isolated from the six different groups of animals. Spectra were recorded in phosphate buffer 0.1 M, pH 7.4, containing 0.3% Tween 80 as previously described (28).

SDS-PAGE: Following solubilization of the mitochondrial membrane using a buffered solution of 2% sodium cholate, the mitochondrial proteins from the six groups were compared using SDS-PAGE. Subsequently, densitometric analysis of the stained gels was performed as described elsewhere (29).

RESULTS

The injection of sodium phosphate, $FeCl_3$, $CaCl_2$ or $MgCl_2$ had no visible immediate or long-term effect on the behaviour of the rabbits. For those injected with the $AlCl_3$ solution, however, while there was no immediate effect, the animals started to show signs of lethargy and ataxia in the hind legs by day nine, consistent with previous reports (24). Histological analysis of the midbrain revealed that the $AlCl_3$-injected rabbits exhibited the presence of neurofibrillary tangle-like structures, at the level of the rostral colliculi as previously reported (24). Similar degeneration was found in large multipolar neurons of the midbrain, particularly those of the red nucleus, occulomotor nucleus and lateral vestibular nucleus, as well as neurons of the facial nucleus and in neurons of the medulla. However, none of the other ions led to the development of such histological changes (Figs. 1: A−E).



Figs. 1 (A−E): Mitchell's silver stain of medulla sections: (A) control rabbits, (B−E) metal-ion-injected rabbits.

A – Control (un-injected) rabbits
B – Aluminium chloride ($AlCl_3$)-injected rabbits
C – Ferric chloride ($FeCl_3$)-injected rabbits
D – Magnesium chloride ($MgCl_2$)-injected rabbits
E – Calcium chloride ($CaCl_2$)-injected rabbits

Rabbits injected with $AlCl_3$ showed evidence of neurofibrillary degeneration within the neurons. None of the other ions led to similar neurofibrillary degeneration. Neurons are highlighted by blue arrows. Magnification x 100.

Unlike the case of rabbits raised on a Cu-cholesterol rich diet (30), the difference spectra of mitochondria isolated from the brain of the metal-ion injected animals appeared normal. All six groups of rabbits (the two control and four experimental) produced difference spectra that were typical, with the major peak centred around 434 nm and two smaller peaks centred at 550 nm and 604 nm, respectively (Fig. 2). For each spectrum, the ratio of the area under the curve of the major and minor peaks was calculated. The calculations detected no significant differences between the spectra produced by the different cohorts.

SDS-PAGE combined with densitometric analysis revealed that the protein composition of mitochondria isolated from the brains of rabbits injected with Al^{3+} was

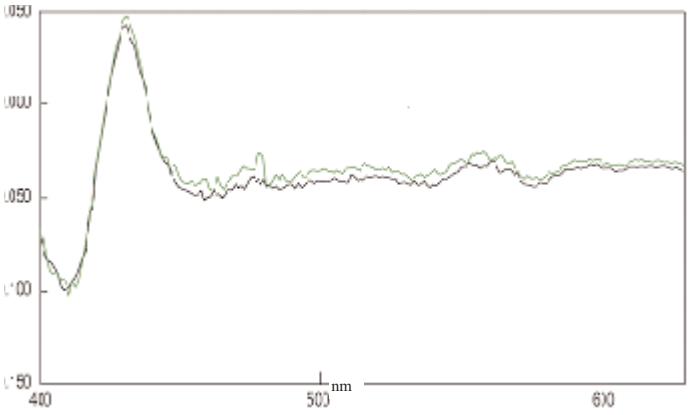
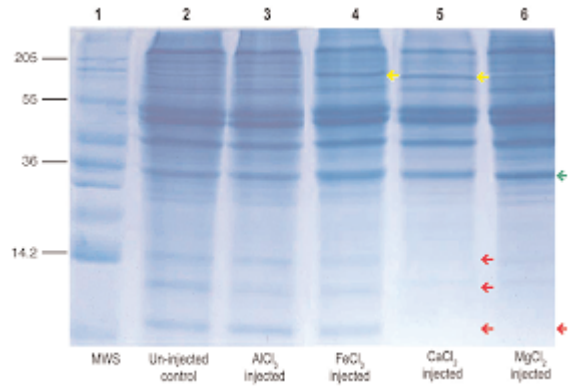


Fig. 2: Difference spectra, 400−630 nm, of brain mitochondria from rabbits.

The difference spectra of brain mitochondria (oxidized minus reduced) of the controls (black) and the aluminium chloride injected animals (green) were typical with the major peak centred around 434 nm and two smaller peaks centred at 550 nm and 604 nm, respectively. Spectra for the ferric chloride ($FeCl_3$), magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) [not shown] were all identical.

very similar if not identical to the controls (Figs. 3A and 3B). For mitochondria from the Fe^{3+} and Ca^{2+} injected animals,

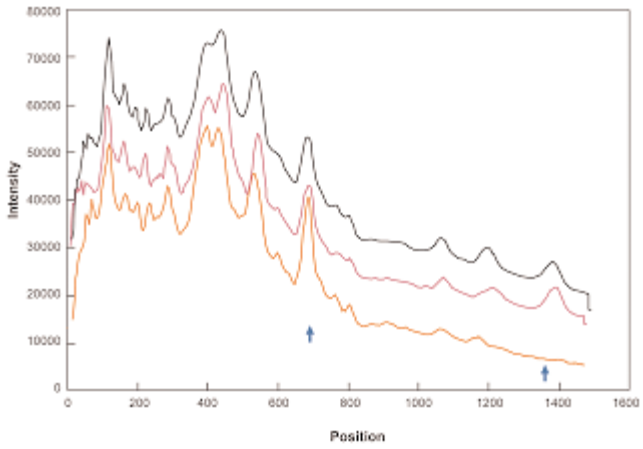
3A



Figs. 3 (A−C): SDS-PAGE and densitometric analysis of lipid depleted rabbit brain mitochondria.

(A) SDS-PAGE: The mitochondria from the aluminium chloride ($AlCl_3$) injected rabbits (lane 3) produced a banding pattern that was identical to that of the control (lane 2). For the calcium chloride ($CaCl_2$) injected (lane 5) there was total absence of three low molecular weight proteins (see red arrows) and increased concentration of a 110 kd protein (yellow arrow). For the ferric chloride ($FeCl_3$) injected (lane 4) there was decreased concentration of the three low molecular weight proteins (follow red arrows) and increased concentration of the 110 kd protein (yellow arrow). While for magnesium chloride ($MgCl_2$) [lane 6], there was absence of the protein of lowest molecular weight (red arrow) and increased concentration of a 36 kd protein (green arrow). Molecular weight standards ranging from 6.5−205 Kda are shown in lane 1.

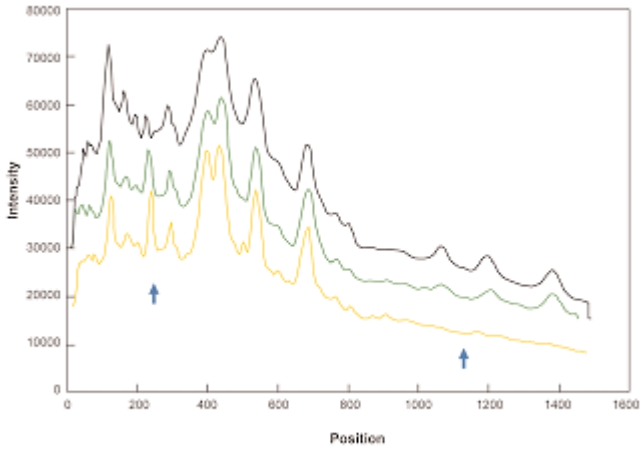
3B



(B and C) Densitometric scan, from top to bottom, of the stained SDS gel shown in (A).

B: control (black), $AlCl_3$ injected (purple) and $MgCl_2$ injected (orange).

3C



C: control (black); $FeCl_3$ injected (green) and $CaCl_2$ injected (yellow).

there was increased concentration of a high molecular weight protein (~ 110 kd) and decreased concentration of three low molecular weight proteins (~7−14 kd) [Figs. 3A and 3C]. In the case of Ca^{2+}, the three low molecular weight proteins appeared to be totally absent. For Mg^{2+}, there was also decreased concentration of the three low molecular weight proteins but for this ion, there was total absence of the protein of lowest molecular weight (~7 kd). The Mg^{2+} results differed in one other important way. Whereas Fe^{3+} and Ca^{2+} led to increased concentration of a 110 kd protein, Mg^{2+} led to increased concentration of a protein of ~36 kd (Figs. 3A and 3B).

DISCUSSION

Consistent with the reports of Klatzo *et al* (24), the present study showed that rabbits injected with $AlCl_3$ into the cerebral hemisphere displayed lethargy and ataxia of the hind legs after about ten days. In addition, these rabbits also developed neurofibrillary degeneration within neurons of the midbrain and medulla (Fig. 1). The fact that none of the other salts injected ($FeCl_3$, $CaCl_2$ or $MgCl_2$) exerted a similar effect indicates that the chloride ions, common to all four salts, did not contribute toward the development of the ataxia or neurodegeneration. The results further suggest that the alterations to the animals' state of physical health was most probably linked to the neurodegeneration and that both the physical ill health and the neurodegeneration were probably linked specifically to the presence of Al^{3+} ions in the brain; none of the other ions produced such effects, at least after ten days. It seems, therefore, that Fe^{3+}, Ca^{2+} and Mg^{2+} either have no direct effect on the neurodegeneration of neurons or that if they do, they act much more slowly than Al^{3+}.

In contrast to their lack of impact on the animals' mobility and neuropathology, the results of the SDS-PAGE in combination with densitometry showed that after ten days Fe^{3+}, Ca^{2+} and Mg^{2+} had already induced changes to the protein composition of the brain mitochondria of the rabbits. Surprisingly, Al^{3+}, which had a profound effect on the animals' neuropathology and physical activity, induced no changes to the protein composition of mitochondria. These results appear to suggest that elevated metal ions in the brain precipitate a range of changes with different metals inducing different initial effects. This conclusion is consistent with the finding of our earlier study which investigated the impact of these injected ions on brain COX activity. Then, it was found that all four ions led to decrease enzymatic activity but to differing extents (31). When the latter results are recalculated so that the ions are equimolar rather than being of equal percentage, the inhibition by Fe^{3+} is twice that of Mg^{2+} and three times that of Al^{3+} and Ca^{2+}.

Returning to the impact of the injected ions on the structure of brain mitochondria, two of the metals, Fe^{3+} and Ca^{2+} appeared to induce similar kinds of changes, in which there was decreased concentration of three low molecular weight proteins (7−14 kd) and increased concentration of a protein with an approximate weight of 110 kd. However, whereas Fe^{3+} merely led to decreased protein concentration, Ca^{2+} led to the total disappearance of the three low molecular weight proteins (Fig. 2). This result appeared to suggest that Fe^{3+} and Ca^{2+} might be activating a common pathway, with the effects of Ca^{2+} being much more pronounced. Alternatively, since the method used in this study (24) employed percentages rather than molar concentrations, the difference in the extent of change induced by the two ions could be reflecting their difference in molar concentration; $FeCl_3$, 0.086 mol L^{-1} compared to $CaCl_2$, 0.126 mol L^{-1}. The other ion studied, Mg^{2+}, appeared to be acting in a way that was completely different to Fe^{3+} and Ca^{2+}. In the case of Mg^{2+}, there was decreased concentration of the low molecular weight proteins but only the protein of lowest molecular weight (~7 kd) disappeared totally while there was increased concentration of a 36 kd protein (Fig. 2).

Although there might be a small concentration effect, the overall picture emerging from this study is one of a complicated sequence of biochemical events, the outcomes of which are ion specific. It has long been established that there are at least three different types of amyloid beta plaques found in the brains of patients with AD (32−34). It has also been shown that formation of these plaques result from aggregation of fragments of amyloid precursor protein, abnormal neurites and other structures formed as a result of ion-induced (35, 36) or enzyme catalysed processes (37−39). Similarly, metal-ion-induced aggregation of α-synuclein has been linked to the pathogenesis of PD (21). Our present results appear to be suggesting that increases of certain metal ions in the brain also induce aggregation of some mitochondrial proteins, with the nature of the aggregation being highly dependent on the properties of the specific ion. These results are consistent with the findings from several studies which have shown that elevated Al^{3+}, Fe^{3+} and Ca^{2+} in the brain are associated with mitochondrial dysfunction and that the latter plays a critical role in the aetiology of AD (23) and PD (22). Moreover, our earlier finding that these injected ions also lead to low brain COX activity (31) may have unearthed the link between mitochondrial dysfunction and AD. We have proposed that low COX activity could trigger the release of oxygen free radicals and that these free radicals could cause damage to cell organelles. We speculate that the aggregation of proteins reported here may be linked to increased oxidative stress which was precipitated by ion-induced low COX activity. Establishing the identity of the low molecular weight proteins involved in this aggregation process should provide some very useful information.

Finally, a number of studies (9, 40) have shown that decreased rather than increased Mg^{2+} in the brain is a factor in the pathogenesis of AD. Our finding that within mitochondria, elevated Mg^{2+} induces structural changes that are different to those promoted by Al^{3+}, Fe^{3+} and Ca^{2+} is, therefore, noteworthy. Whereas low Mg^{2+} is likely to impair the activity of magnesium dependent enzymes, we have shown that elevated Mg^{2+} inhibits COX (31); it would seem therefore that either below normal levels or above normal levels of brain Mg^{2+} could be factors in the pathogenesis of AD. Equally noteworthy is the fact that over the ten-day period of the study, Al^{3+} induced neurodegeneration but no protein aggregation, while the other ions did the exact reverse; they induced protein aggregation but no neurodegeneration. This finding appears to set Al^{3+} apart from other ions.

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