

Running Head: Transport in Neurodegeneration

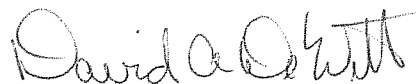
Clustering of the Endoplasmic Reticulum in Aluminum Induced Apoptosis

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A Senior Thesis submitted in partial fulfillment  
of the requirements for graduation  
in the Honors Program  
Liberty University  
Spring 2003

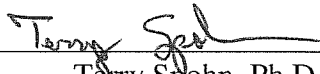
Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial fulfillment of the requirements for graduation from the Honors Program of Liberty University.



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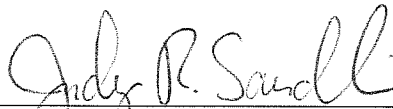
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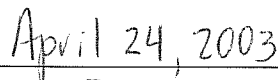
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Abstract

Disruption of the cytoskeleton and axonal transport has been implicated in certain neurodegenerative diseases such as Alzheimer's disease. It is not clear whether cytoskeletal and transport abnormalities play a causative role in neurodegeneration or whether they are simply byproducts of disrupted metabolism and signal transduction pathways in neurons undergoing apoptosis. Aluminum has been used to induce apoptosis in both *in vivo* and *in vitro* models of apoptosis that share many biochemical and pathological similarities to Alzheimer's disease. The specific aim of this project was to examine the effect that aluminum-induced apoptosis has on intracellular distribution of the endoplasmic reticulum. The results indicate that following aluminum treatment the endoplasmic reticulum is abnormally clustered around the nucleus, consistent with disruption of kinesin-dependent transport.

### Clustering of the Endoplasmic Reticulum in Aluminum Induced Apoptosis

Intracellular transport is fundamental to cellular activity and functioning.

Virtually all eukaryotic cells organize their cytoplasm by translocating various membrane-bound vesicles and macromolecular complexes to specific subcellular locations. The intracellular traffic is not random, but rather is polar transport along a microtubule network. Microtubules serve as the tracks on which molecular motor proteins, such as kinesin and dynein, carry their cargo. Transport along this cytoskeletal infrastructure is critical, as materials, such as proteins, produced in specific regions must be transported to maintain other regions of the cell.

Proteins targeted for vesicular transport are synthesized by cytosolic ribosomes and transported into the endoplasmic reticulum (ER). In the ER, proteins are assembled and folded into polypeptide chains in preparation for their secretion or distribution to various subcellular compartments. From the ER, proteins are packaged into vesicles and transported to the Golgi complex. Proteins undergo sorting and processing in the Golgi before being transported along microtubules to their targeted destinations.

#### *Axonal Transport*

Neurons are specialized cells for communication by electrical conduction. The axon, the main cellular process of the neuron, is designed to conduct electrical signals away from the cell body to adjacent neurons. The axon is supported by cytoskeletal neurofilaments and a microtubule network that provides the tracks by which proteins and vesicles are transported along the axon.

The length of the axon provides the neuron with unique dimensions beyond comparison to any other cells in the body. Individual axons can reach a length of over one meter in humans and other large animals and the extreme length-to-diameter ratios can be as high as  $1^7$  in some cases.<sup>57</sup> The volume of cytoplasm in these long axons accounts for approximately 99% of the cell's volume, while the cell body supplies virtually all of the cell's proteins, lipids, and other materials.<sup>7</sup> Branching and small diameters in parts of the axon further add to the challenge of transporting the materials necessary for cellular function and maintenance in axons. Such challenges place remarkable demands on the neuronal cytoskeleton and associated axonal transport to develop and maintain the axon. This critical function has led to increasing research focusing on the role of axonal transport defects in neurodegenerative diseases such as Alzheimer's disease.<sup>8</sup>

With the recognition that the neuronal cell body is the site of synthesis supplying the necessary materials to the axon, mechanical models for transport of these materials were proposed. Based on his observation of axoplasm oozing from the proximal ends of freshly cut giant nerve axons, in 1944 J.Z. Young proposed one of the first models of mechanical transport.<sup>50</sup> He suggested that a constant production of axoplasm from the cell bodies creates a pressure forcing the fluid axoplasm down the length of the axon. He proposed that the axon wall, by exerting an opposite pressure due to surface tension of its lipid myelin sheath, maintains the cylindrical shape of the axon.

The first major advancement to the modern concept of axonal transport came in 1948, when Paul Weiss and H.B. Hiscoe performed their "damming" experiment and

described what would later be termed slow axonal transport.<sup>50</sup> They ligated a nerve trunk with thread and noticed several days later that the region of the axon before the ligation was bulging from what they concluded was damming of axoplasm, while the region of the axon beyond the ligation was narrowing. When the ligature was removed, they noted that the material proceeded down the axon at the rate of 1-2 mm per day. This experiment demonstrated the directional flow of axon-maintaining material from the cell body.

The slow axonal transport observed by Weiss can be subdivided into two components: slow component A [0.2-1 mm/day] and slow component B [2-8 mm/day]. Slow transport is well developed in axons and provides the means by which cytoskeletal materials required for maintaining the axonal cytoskeleton are transported.<sup>36</sup> Slow transport is also responsible for the locomotion of the axon in periods of growth and regeneration.<sup>34</sup>

Whether growing or not, slow axonal transport keeps the axonal polymers in continuous motion.<sup>36</sup> Slow axonal transport is responsible for moving large amounts of cytoskeletal materials; however, it operates on a local level by moving individual polymers within the axon.<sup>34</sup> The net result of the movement of individual polymers is a translocation of the entire axonal cytoskeleton towards the terminus of the axon.

Later experiments with radioactively-tagged amino acids provided evidence for a second type of axonal transport, one that moves at a much faster rate.<sup>39</sup> Fast axonal transport, as it was termed, travels at a rate of 200-400 mm per day. Video microscopy

has allowed researchers to directly view vesicles in giant squid axons undergoing fast axonal transport.<sup>41</sup>

Fast axonal transport accounts for vesicular traffic within the axon.<sup>1</sup> Enzymes, lipids, and membrane glycoproteins are among the materials carried along the axonal microtubules via fast axonal transport. Unlike slow axonal transport, fast axonal transport has been experimentally shown to operate in the anterograde and retrograde directions.<sup>57</sup>

Slow axonal transport has been the subject of some controversy in recent years. Recent studies have examined neurofilament proteins tagged with green fluorescent protein (GFP) in cultured rat sympathetic neurons.<sup>61</sup> The axons of these neurons contain relatively few neurofilaments and frequently have gaps lacking neurofilament in their axonal cytoskeletal network. Time-lapse imaging of these gaps has allowed the observation of axonal transport without the normal photobleaching or photoactivation approaches. These studies have produced some surprising results. Contrary to the standard association of cytoskeletal materials with “slow” transport, neurofilaments have been observed to move rapidly in the axon, with rates as high as 3  $\mu\text{m}$  per second.<sup>62</sup> This rapid movement is interrupted by long pauses, however. Comparing these rapid bursts to the net movement of neurofilament proteins at 0.3-3 mm per day, it can be estimated that neurofilaments spend 83-99% of their axonal transport in these long pauses.<sup>10</sup> The mechanistic reasons behind these pauses has not been determined.

In 1986, Nixon and Logvinenko used radioisotopic pulse labeling in mouse optic nerves to propose a model of two kinetically distinct types of neurofilaments.<sup>39</sup> They

suggested that neurofilament polymers transition between moving and stationary phase as they travel along the axon. Lasek and his associates, however, challenged this model of two kinetically different neurofilament populations.<sup>35</sup> Lasek suggested that there is a single population of neurofilaments in axons and they are all in a continuous state of movement, albeit at a broad range of rates. Results showing GFP-tagged neurofilaments moving in alternating fits bursts and pauses do not provide conclusive evidence for either one of these models. The fits and starts could represent a transition between two distinct phases or it could represent irregular movements of a single population of neurofilaments.

Another point of controversy in slow axonal transport has been the structural forms in which the cytoskeletal and cytosolic proteins move. Slow axonal transport consists not only of cytoskeletal proteins, but a wide range of additional cytosolic proteins. Lasek proposed a model where these cytosolic proteins are transported by physically associating with moving cytoskeletal polymers.<sup>34</sup> The relatively simple protein composition that makes up slow component A indicates that microtubules and neurofilaments could act as the only structural carriers for proteins in this component.<sup>10</sup> Proteins in slow component A are either integral parts of the cytoskeletal polymers or have been shown to associate with these polymers *in vivo*. Slow component B consists of a much more diverse and complex protein composition. It is likely that the carrier structures for slow component B are likewise complex and may consist of several macromolecular complexes.



It has been generally held that slow axonal transport occurs only in the anterograde direction. However, observations of GFP-tagged neurofilaments show that up to 20-30% of the filaments move towards the cell body in a retrograde fashion.<sup>62</sup> Supporting this, *in vitro* studies have shown neurofilaments moving towards the minus ends, as well as the plus ends, of microtubules.<sup>48</sup> Retrograde movement of neurofilament could indicate a distinct population or transient reversals of filaments that have a net movement in the anterograde direction.

Mitochondria display unique motility within axons of nerve cells. Unlike many other types of organelles, mitochondria undergo net movement of both anterograde and retrograde transport within the axon.<sup>27</sup> Mitochondria serve essential functions of aerobic ATP production and intracellular calcium regulation to maintain the axon. Movement of mitochondria is distinguished by three key elements: saltatory movement, with frequent starts and stops; true bidirectional movement; and prolonged stationary phases.<sup>27</sup>

Rapid-freezing electron microscopy demonstrated cross bridges between microtubules and mitochondria, indicating that microtubules serve as the tracks for mitochondrial axonal transport.<sup>25</sup> Observation of mitochondrial movement in the growth cone region of growing axons of sympathetic neurons in culture demonstrated that microfilaments can also serve as mitochondrial tracks.<sup>16</sup> Mitochondria can move bidirectionally on either set of cytoskeletal tracks, but there are key differences in motility on the different tracks. Mitochondria are transported at a much higher rate on microtubules, implying that molecular motors with different characteristics are associated with mitochondrial transport on the different tracks.<sup>38</sup>

*Molecular Motors*

To achieve directional transport within the cell, vesicles and organelles must associate with motor proteins that simultaneously attach to the microtubule network and provide energy for the movement. These motor proteins move unidirectionally along microtubules by coupling ATP hydrolysis to force generation. This force allows motor proteins to carry their cargo to various destinations in the cell.

Evidence for a differential transport of components, and the discovery of a fast transport in axons, led to a search for molecular models of transport. In 1964, Lubinska observed that acetylcholinesterase not only moved anterogradely to accumulate above a nerve ligation, but also accumulated below a second ligation made more proximal to the cell body.<sup>50</sup> While the total amount of acetylcholinesterase remained the same in a segment of axon isolated in such a way, it became redistributed as a result of two-way transport. The presence of both anterograde and retrograde transport provided evidence that there is a structure within the neuron's axon that is able to selectively guide axonal transport. Based on evidence that microtubules are present in the axon and implicated in the transport process, a basic model was established in which carriers moved along microtubules using the energy provided by ATP.

The motor proteins associated with the microtubules during intracellular transport were not discovered until the mid-1980's.<sup>58</sup> Evidence that these proteins required association with both the microtubules and adenosine triphosphate (ATP) pointed to mechanochemical ATPases as prime candidates. Dynein, like that which operates in the movement of cilia and flagella, had been discovered in the 1960's and was considered for

some time.<sup>37</sup> However, advances in technology led to the discovery of the motor protein kinesin, and two years later, cytoplasmic dynein.<sup>58</sup>

Molecular motor proteins function by converting the chemical energy of ATP into a force that propels molecular motion. Association of the motor proteins with the microtubules and ATP causes a conformational change in the motor head regions of the proteins. This conformational change propels the motor proteins and their associated vesicular cargo along the microtubules.

Kinesin is a 380 kilo Dalton (kD) dimer consisting of two 120-kD heavy chains and two 64-kD light chains.<sup>26</sup> Kinesin has a rod-like structure with two large globular heads, made up of the heavy chains, connected by a stalk region to a fan-like tail, comprised of the light chains. While the head region is directly associated with the microtubules and ATP, the tail region associates with the membrane vesicles.<sup>19</sup> The head region of kinesin generates force for movement and is a highly conserved domain.<sup>26</sup> On the contrary, the tail region has a highly variable amino acid sequence. Kinesin is an anterograde motor protein that moves its cargo towards the plus-end of the microtubule, or the cell periphery.

Dynein is the second group of motor proteins and transports its cargo along the microtubules toward the minus-end. Cytoplasmic dynein is a member of the dynein family that is involved in the movement of vesicles within the cell. It is a huge multisubunit protein complex (1.2 mega Daltons) and is found in all eukaryotic cells.<sup>26</sup> It consists of two heavy chains (~530 kD), three intermediate chains (74 kD), and four light intermediate chains (~55 kD). Like kinesin, the two heavy chains comprise two large

globular heads that associate with the microtubules and ATP. However, dynein cannot directly associate with its vesicular cargo. Vesicular attachment is thus mediated by another protein complex known as dynactin.<sup>18</sup>

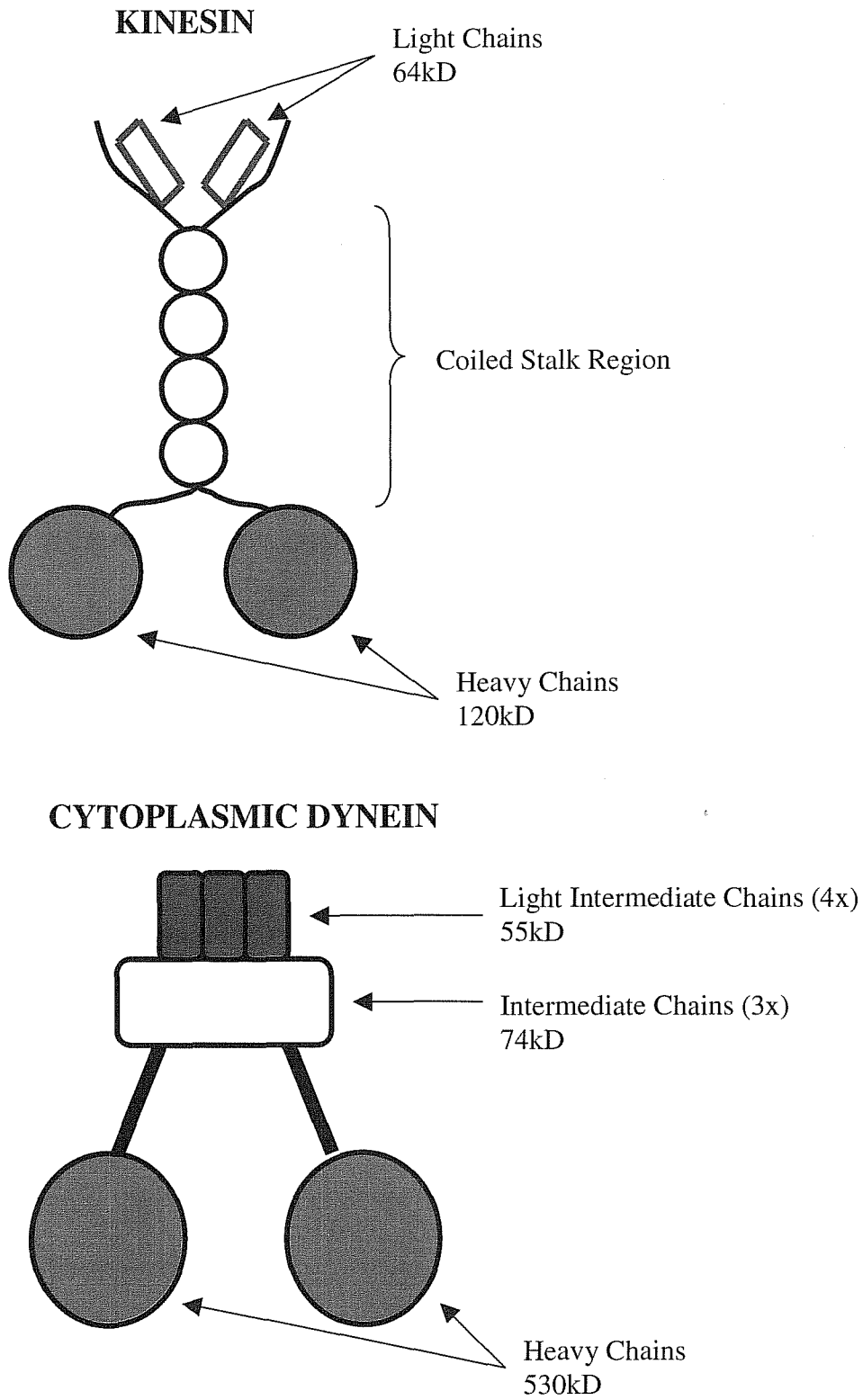


Figure 1. Structural representation of kinesin and cytoplasmic dynein motor proteins.

*Transport Between ER and Golgi*

A unique form of intracellular transport of vesicles occurs between the endoplasmic reticulum and the Golgi complex. Proteins are assembled and folded in the ER, packaged into vesicles, and shipped to the Golgi for processing and transport. Transport between these two structures involves an intermediate compartment and is somewhat, though not completely, dependent on microtubules. This specific intracellular pathway is critical, as disruption of this transport could result in an accumulation of proteins that could elicit cellular stress responses.

Proteins that are destined for vesicular traffic in secretory and endocytotic pathways are synthesized by ribosomes in the cytosol. These proteins are then co-translationally transported into the lumen or membrane of the ER. After proper folding and oligomer assembly, the proteins are incorporated into vesicles for transport to the Golgi complex. The Golgi complex is responsible for performing various tasks such as glycosylation of many proteins, sorting of proteins into vesicles, and transport of these vesicles to various destinations throughout the cell.<sup>2</sup>

Transport of proteins from the ER to the Golgi complex involves transport vesicles that bud from the ER membrane and fuse with the membrane of the Golgi apparatus. Early studies using pancreatic acinar cells revealed that vesicle budding occurs in specialized regions of the ER known as transitional elements.<sup>60</sup> This transport has been demonstrated *in vitro* using permeabilized cells with added cytosolic fraction, ATP-regenerating system, and GDP-mannose.<sup>4</sup>

Genetic approaches in *Saccharomyces cerevisiae* have been important in identifying many proteins involved in ER to Golgi transport.<sup>60</sup> A group of *sec* genes- *sec12*, *sec13*, *sec16*, and *sec23*- seems to be involved in vesicle formation. Another group of *sec* genes- *sec17*, *sec18*, and *sec22*- appear to be involved in the fusion of these vesicles to their targets, such as the Golgi membrane. Another group of proteins, small GTP binding proteins, play an important role in the ER to Golgi transport.<sup>60</sup> One such protein is Sar1p, which interacts with *sec12* and *sec23* proteins and the endoplasmic reticulum membrane. Sar1p, in conjunction with Sec12p, seems to associate with the endoplasmic reticulum and promote the formation of transport vesicles by hydrolyzing GTP. The ARF (ADP-ribosylating factor) family of proteins also seems to be involved in ER to Golgi transport and appears to function in the maintenance of the Golgi apparatus.<sup>12</sup>

*Table 1.* Protein families involved in transport between the endoplasmic reticulum and the Golgi apparatus.

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**Table 1**

*Proteins Involved in ER to Golgi Transport*

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Family of Proteins	Function
<b>Sec proteins</b>	
- <i>sec12, sec13, sec16, sec23</i> proteins	Involved in vesicle formation
- <i>sec17, sec18, sec22</i> proteins	Involved in fusion of vesicles to targets
<b>Small GTP binding proteins</b>	
-Sar1p	In association with ER and Sec12p, hydrolyzes GTP to promote formation of transport vesicles
<b>ARF proteins</b>	Involved in maintenance of Golgi structure



Studies have shown that ER to Golgi transport in mammalian cells involves an intermediate compartment (IC) between the endoplasmic reticulum and the Golgi.<sup>60</sup> When cells infected with a temperature-sensitive virus were incubated at 15°C, viral glycoproteins accumulated in morphologically distinct structures about 300 nm in diameter.<sup>45</sup> Evidence supports the idea that the IC is a distinct intermediate in ER to Golgi transport, with no direct continuity with either the ER or the Golgi. The IC appears to be a dynamic collection of ER transitional elements and vesicular-tubular clusters (VTC)<sup>3</sup>. The IC compartment is involved in the anterograde transport of proteins from the ER to the Golgi and the recycling of proteins retrogradely to the ER.

The retrograde transport of proteins from the Golgi back to the ER has been another subject of study in ER to Golgi transport. The anterograde transport pathway is mediated by vesicles covered by a protein coatamer, referred to as COPII.<sup>46</sup> Association of COPII with vesicles targeted for the ER requires GTP and may involve the GTP-binding protein ARF. In the retrograde pathway, uncoated, Golgi-derived tubules are seen and their presence has been demonstrated to be dependent on microtubules.<sup>13</sup> Tubular budding, rather than vesicle mediation, has been implicated in retrograde transport.<sup>31</sup> This difference in mediation appears evident in experiments that block anterograde transport using the drug Brefeldin A. Treatment with Brefeldin A results in the disappearance of the Golgi apparatus and subsequent increase in size of the endoplasmic reticulum, suggesting that the retrograde pathway dominates.<sup>2</sup>

It has recently been clearly demonstrated that transport between the intermediate compartment and the Golgi apparatus occurs along microtubules.<sup>42,46</sup> Evidence came

from cells transfected with VSVG-GFP, a chimaeric protein containing the COOH-terminal region of a temperature-sensitive vesicular stomatitis virus glycoprotein fused to green fluorescent protein. Fluorescence microscopy was used to observe synchronous transport of the fusion protein through the secretory pathway. IC membranes containing VSVG-GFP were seen to move along microtubules to the Golgi.<sup>42,46</sup> If the microtubules are depolymerized, rates of Golgi-dependent protein processing drop dramatically, indicating that the transport from the IC to the Golgi has been inhibited. If the microtubules are disrupted by a drug such as nocodazole, the microtubule-dependent movement from the IC to Golgi is disrupted. However, removal of nocodazole after brief exposure can lead to the resumption of normal IC to Golgi transport.<sup>6</sup>

Interestingly, disruption of microtubules does not always result in decreased rates of Golgi-based protein processing or secretion.<sup>32</sup> In these cells that maintain normal processing or secretion rates there are considerable changes in the distribution of the Golgi, however. In these cells, the Golgi fragments into ministacks which are distributed to locations adjacent to endoplasmic reticulum exit sites, at a distance less than 1  $\mu\text{m}$ .<sup>11</sup> It seems that diffusion-based membrane trafficking is then used and operates at an efficient level. In contrast, in cells with a functional microtubule network, the Golgi can be a distance of dozens of micrometers away from the endoplasmic reticulum.<sup>44</sup> Diffusion would not operate at an efficient enough level at such distances.

#### *Effects of Aluminum on Transport*

When studying changes in the cytoskeleton and axonal transport in certain neurodegenerative diseases, it is useful to establish a biochemical and pathological model

of the disease. If one is able to trigger similar changes in a system, whether it is *in vivo* or *in vitro*, the model may provide clues to the pathological causes of the disease.

Aluminum can be used to induce a model of apoptosis that shares certain characteristics with neuronal death in Alzheimer's disease.

Aluminum is an environmental neurotoxin that has been shown to have an inhibitory effect on axonal transport.<sup>29</sup> Aluminum has been shown to cause accumulation of neurofilaments in neuronal perikarya of certain vertebrate species *in vivo* and *in vitro*.<sup>49</sup> Intrathecal injection of aluminum salts into rabbits induces accumulation of neurofilaments, associated with dysfunctional metabolic activity and impaired axonal transport of neurofilaments.<sup>17</sup> Chronic exposure of primary cultured neurons to aluminum salts also leads to similar neurofilament accumulations in the neuronal perikaryon.<sup>49</sup> In addition, pulse exposure of cultured rat cortical neurons to aluminum-maltol causes neurofilaments and fast axonal transported proteins to be abnormally distributed.<sup>29</sup>

Neurofilaments are critical to the integrity of a functional cytoskeleton. Thus, aluminum-induced abnormalities in neurofilaments would have definite repercussions for microtubule-dependent axonal transport processes. The impairment of axonal transport by accumulating neurofilaments is thought to be the mechanistic cause of aluminum toxicity.<sup>59</sup>

Accumulation of neurofilament proteins and abnormal axonal transport are also neuronal characteristics of certain neurodegenerative diseases such as Alzheimer's disease.<sup>8</sup> The neurofibrillary tangles in AD display a similar, though not identical,

accumulation pattern of phosphorylated neurofilament immunoreactivity.<sup>56</sup> Thus, there may be a similar pathological process causing accumulation of neurofilaments and disrupted axonal transport in both neurodegeneration and aluminum neurotoxicity. Inducing such effects using aluminum may then provide a useful model for elucidating clues about Alzheimer's pathology.

#### *Alzheimer's and Intracellular Transport*

Alzheimer's disease is characterized by two chief pathological manifestations. The first is the presence of extracellular senile plaques composed primarily of amyloid- $\beta$ , a peptide derived from amyloid precursor protein ( $\beta$ PP).<sup>61</sup> The second of the hallmark characteristics is formation of neurofibrillary tangles. The neurofibrillary tangles consist of bundles of ~10 nanometer paired helical filaments believed to be composed primarily of hyperphosphorylated tau.<sup>9</sup> It is not clear whether these pathological characteristics are causes or simply markers of Alzheimer's disease.

#### *The Role of Tau in Neurodegeneration*

As researchers try to determine the biochemical and pathological causes of Alzheimer's disease, they have recognized several proteins as key players in the process. Tau is a microtubule-associated protein that seems to be critical to the integrity of the cytoskeletal network. Tau is associated with the neurofibrillary tangles characteristic of Alzheimer's disease.

Microtubules are stabilized by a group of microtubule-associated proteins (MAPs) that includes MAP2, MAP4, and tau.<sup>55</sup> These proteins seem to play an important role in

cytoskeletal organization by binding to the surface of the microtubules and promoting microtubule assembly.<sup>30</sup> Tau is especially prevalent in the axons.

Paired helical filaments in tangles consist of hyperphosphorylated tau. As discussed, microtubules compose the cytoskeleton of the cell and are involved in various processes within the cell such as transport of vesicles and organelles. Tau normally stabilizes microtubules, but when it is abnormally phosphorylated it forms tangles.<sup>23</sup> Accumulation of paired helical filaments is believed to disrupt the cytoskeleton and axonal transport, affecting cell metabolism and leading to neuronal death.<sup>51</sup>

There seems to be a critical regulated balance of tau phosphorylation, distribution, and concentration within the neuron. Kinases and phosphatases seem to regulate this balance, which is disturbed in Alzheimer's disease.<sup>8</sup> In AD, tau concentrations are elevated and tau becomes localized in the somatodendritic portion of the neuron<sup>22</sup>

Elevation of tau concentrations in AD could be attributed to overcompensation by the cell. Upon hyperphosphorylation, tau has reduced affinity for the microtubules<sup>23</sup> The cell may try to compensate by producing more tau. Research shows that overexpression of tau results in change in cell shape, blocking of kinesin movement, and altered distribution of organelles and vesicles.<sup>51</sup> Following tau overexpression, there is perinuclear clustering of the mitochondria at the microtubule-organizing center. Also, the ER becomes less dense and moves away from the periphery of the cell. Overexpression of tau and subsequent disruption in axonal transport are similarly observed in the neurons of Alzheimer's patients.<sup>8</sup>

The role of microtubule transport in abnormal distribution of organelles following tau expression has been verified by using drugs that affect the microtubule network. Tau overexpression induces clustering of organelles, however, upon treatment with nocodazole, a drug that leads to the disassembly of the microtubule network, mitochondria redistribute themselves throughout the cell, seemingly by diffusion.<sup>14</sup> Removing nocodazole reconstitutes the microtubule network and again leads to mitochondrial clustering around the microtubule-organizing center. These results demonstrate that the process of mitochondrial clustering is indeed dependent on microtubule transport.

Distribution of the ER is also dependent on the microtubule network and kinesin transport. The distribution behavior of the ER, however, seems to involve more than the mitochondria. Treatment with nocodazole does not cause the ER to expand to its normal distribution to the cell periphery. The ER is not able to simply diffuse to normal distribution throughout the cell in the same way that the mitochondria are able to. These results suggest that distributional transport of the ER is dependent on a functional microtubule network.

Changes in the distribution of organelles such as the mitochondria and ER could have important impacts on the cell. Irregular distribution of the mitochondria could lead to deficient glucose metabolism and ATP synthetase throughout the axon, as mitochondria may become concentrated in one region and lacking in another. Abnormal distribution of the ER and disruption of its microtubule-dependent secretory pathways

could disrupt calcium homeostasis and lipid synthesis. These changes could lead to neuronal cell death.

### *The Role of $\beta$ PP in Neurodegeneration*

Another protein that has been the focus of much Alzheimer's research is amyloid precursor protein ( $\beta$ PP). While its function in the cell is still in question, it seems to bind to kinesin-I and to mediate axonal transport.  $\beta$ PP is associated with the other hallmark characteristic of Alzheimer's disease, A $\beta$  senile plaques.

Kinesin-I is suggested to be the motor protein responsible for fast anterograde axonal transport.<sup>58</sup> This motor protein has not been clearly linked to a particular type of vesicular cargo, nor has a protein receptor assisting in kinesin-I's binding to its vesicular cargo been definitely identified. One of the prime candidates, however, is  $\beta$ PP.  $\beta$ PP has been found to mediate the axonal transport of presenilin-1 and  $\beta$ -secretase.<sup>53</sup>  $\beta$ PP has been linked to the initiation and/or progression of Alzheimer's disease and has been a target of much neurodegenerative research.

$\beta$ PP is an integral transmembrane protein whose normal role in cellular function has not been firmly established.  $\beta$ PP proteins mature in the ER and Golgi apparatus and undergo post-translational modification such as glycosylation and phosphorylation.  $\alpha$ -,  $\beta$ -, and  $\gamma$ -secretases are the three putative proteases that have been implicated in the cleavage of mutant  $\beta$ PP.<sup>15</sup> Proteolytic cleavage of  $\beta$ PP results in the formation of A $\beta$  peptides of various lengths, though typically consisting of 40 or 42 residues. A $\beta$  peptides normally exist as soluble monomers, but aggregate into insoluble, fibrillar plaques in the brains of AD patients.<sup>63</sup>

Fragments of  $\beta$ PP are abundant in the senile plaques characteristic of AD.<sup>53</sup>

Some forms of familial Alzheimer's disease are caused by mutants of the  $\beta$ PP gene.

Research shows that these mutants lead to an increase in the number of senile plaques and an increase in the proteolytic  $\beta$ PP fragments in these plaques.<sup>47</sup> Abnormal trafficking of  $\beta$ PP has also been suggested as an initiation factor of AD. Evidence of  $\beta$ PP's direct binding to kinesin-I further links it to the disruption of axonal transport characteristic of AD.

While there is controversy over the role of axonal transport defects in the initiation of AD, evidence suggests that  $\beta$ PP's role in transport could be related to initiation of AD.  $\beta$ PP appears to bind directly to kinesin-I and thus is in intimate contact with the axonal transport network. Organelle and vesicle accumulations are early changes in morphology in mouse, and perhaps human, models of Alzheimer's disease.<sup>20</sup> Overexpression of a  $\beta$ PP homolog, APPL, in *Drosophila* results in similar axonal clogs and disrupts axonal transport.<sup>54</sup> Also, while  $\beta$ PP is widely expressed in the body, cellular characteristics of Alzheimer's disease are primarily limited to the neurons. Long axonal processes are a distinguishing characteristic of neurons and the critical function of transport along these long axons may be important to the development of AD.

The idea that altered neuronal transport is critical in inducing neuronal death is further supported by experiments with axonal blockages. It has been found that reducing the amount of kinesin in the axons made blockages worse. On the other hand, reducing the amount of dynein or presenilin, a protein responsible for cleavage of  $\beta$ PP, somewhat relieved the axonal blockages.



Improperly processed  $\beta$ PP may accumulate and lead to axonal clogging.

Increased  $A\beta$  secretion as a function of stressed neurons may lead to the aggregation of amyloid- $\beta$  ( $A\beta$ ) in senile plaques. This may further disrupt axonal transport and act a causative role in neurodegeneration.

Research shows that  $\beta$ PP causing neuronal death contains two key regions: the amyloid- $\beta$  region and the C-terminus. Enzymatic cleavage of the C-terminus liberates kinesin because the C-terminus includes the kinesin-binding site. The C-terminus may carry a cell-death signal that is released when axonal transport is disrupted.

Evidence from other models has indicated that defects in transport play a critical role in neurodegeneration. Apolipoprotein E (ApoE) is a protein that plays an important role in lipid transport and is produced in a variety of cells, such as kidney, liver, and fat cells. In the brain, ApoE is mainly produced by astrocytes and is important in lipid transport within the nervous system. ApoE gene has been linked to increased plaque formation in Alzheimer's disease and the early stages of neurofibrillary tangles.<sup>52</sup> Neurons undergo uptake and synthesis of ApoE and the regions of the brain where ApoE is present are shown to be more susceptible to neurofibrillary tangles.

Expression of human ApoE4 in the neurons of mice produced axonal transport abnormalities, such as accumulation of organelles, and resulted in decreased sensorimotor abilities.<sup>52</sup> Expression of ApoE4 also results in the hyperphosphorylation of the protein tau in the brain and spinal cord, though the mechanism tying these two together is not known. This hyperphosphorylation affects the stability of the microtubules and disrupts axonal transport.

The cytoskeleton and its associated network of intracellular transportation are a critical area of study and research, particularly for their role in neurodegenerative diseases such as Alzheimer's disease. It is clear that the integrity of the cytoskeleton and transport mechanisms is required for function and survival of neurons. Not surprisingly, many neurodegenerative diseases are characterized by abnormalities in the cytoskeleton and accompanying intracellular transport malfunction. In Alzheimer's disease, cytoskeletal abnormalities are so integral that they serve as the basis for the classification of the progressive stages of the disease.<sup>8</sup>

Although cytoskeletal and transport abnormalities provide the hallmarks of many neurodegenerative diseases, the role they play in the development of the disease is not clear. Specifically, it is not clear whether these abnormalities play a causative role in neurodegeneration or whether they are simply byproducts of disrupted metabolism and signal transduction pathways in degenerating neurons. Thus, continued research in the role of changes in the cytoskeleton and transport is critical, as they may actively contribute to neuronal death and may serve as potential therapeutic targets.

## Introduction

Changes in the cytoskeleton and intracellular transport were observed in an *in vitro* model of apoptosis. Al-maltolate was used to induce apoptosis in cultured human neuroblastoma cells. Specifically, staining techniques were used to observe the effect that Al-maltolate treatment has on the intracellular distribution of the endoplasmic reticulum. If Al-maltolate affects the intracellular transport of the ER, observed changes in the density, brightness, and branching of the ER would be expected. The ER is normally highly branched and distributed throughout the cell. If the ER is retracted toward the nucleus following treatment, however, this would provide evidence that Al-maltolate affects kinesin dependent transport

Cell counts were used to establish aluminum's toxicity in the *in vitro* model. To verify that transport effects are specific to Al-maltolate, hydrogen peroxide was also used trigger oxidative stress and apoptosis. Examining changes in such representative models may provide clues to the underlying pathological causes of certain types of neurodegenerative diseases.

## Methods

### *Cell Culture*

Human neuroblastoma (NT2) cells (Stratagene) were grown on glass coverslips in D-MEM/F-12 (Gibco) growth medium with 10% (v/v) FBS, 2mM L-glutamine and 1% (v/v) penicillin-streptomycin and kept in 5% CO<sub>2</sub> at 37°C and 95% humidity. Cells were grown at a density of  $1 \times 10^5$  / mL on the glass coverslips and kept in 6-well plates.

### *Aluminum Treatment*

Fresh aluminum maltolate was prepared as a stock solution of 25 mM in sterile water and filtered through a 0.2 mm filter. Cells were allowed to adhere for 24 hours in media containing 0 to 500  $\mu$ M Al-maltolate before the media was replaced.

### *Hydrogen Peroxide Treatment*

A solution of 0.03% hydrogen peroxide was prepared in sterile water. Cells were incubated in the hydrogen peroxide solution for 30 minutes before the solution was replaced with fresh media.

### *Visualization of the Endoplasmic Reticulum*

For each six-well plate, three wells containing NT2 cells were incubated in 500  $\mu$ M Al-maltolate solution and three wells were incubated in fresh media as controls. To visualize the effects on the endoplasmic reticulum, ER-Tracker™ Blue-White DPX (Molecular Probes) was added. This is a fluorescent dye that specifically detects ER. CMXRos Mitotracker Red was added 2 $\mu$ L/10mL for 20 minutes, in order to visualize the mitochondria. Following the appropriate incubation, cells were fixed in 4% formaldehyde for 15 minutes and then washed with PBS three times. This solution was removed with three 1x PBS washes. Coverslips were mounted onto glass slides using VectaShield (Vecta Laboratories) and cells were viewed with an Olympus microscope and pictures were taken with a digital camera.

### *Cell Counts*

Using the Olympus microscope and a digital camera, pictures of ten random, but representative, fields were taken for each coverslip. Using Image Pro Plus software, the

number of cells per viewing field was calculated by counting the number of nuclei stained with Hoechst 33258. Apoptotic cells were identified by strongly condensed or fragmented nuclei. Using the total number of cells per coverslip and the corresponding number of apoptotic cells, a percentage of apoptotic cells were calculated for each coverslip.

## Results

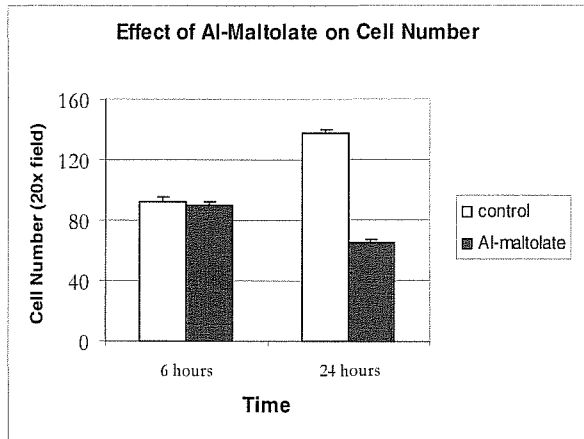
### *Cell Count- % Apoptotic Cells*

Hoescht-stained cells were identified as apoptotic based on several criteria, including: cell shrinkage, strongly condensed nuclei, and fragmented/irregular nuclei. Percentage of apoptotic cells were calculated for Al-maltolate treated and control cells at 6- and 24-hour incubation periods. Percentages of apoptotic cells in the Al-treated cells for 6- and 24-hour incubation periods were 10.16% and 30.82%, respectively, in the first trial. The second trial yielded percentages of apoptotic cells for 6- and 24- hour incubations of 33.4% and 45.4%, respectively. Results for the control and Al-treated cells in both experimental trials are summarized in Figure 2.

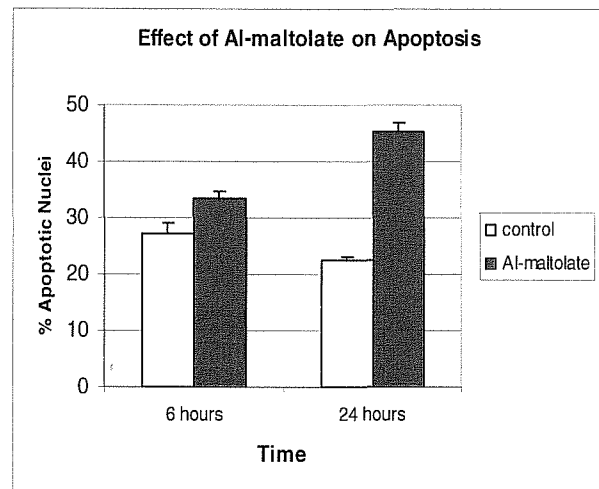
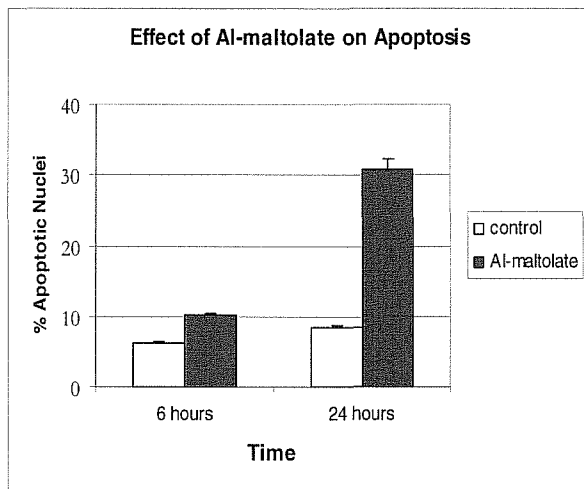
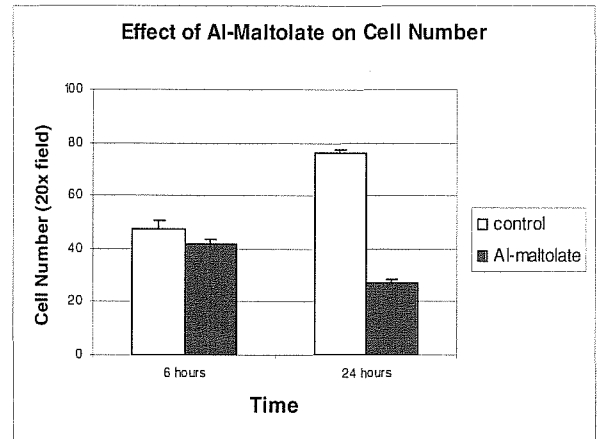
### *Al-induced Changes in the Endoplasmic Reticulum*

To visualize the effects of aluminum treatment on the cells, an ER-Tracker™ Blue-White DPX (Molecular Probes) was used. The ER appeared dense and branched in control cultures, and was extended throughout the cell to the cell's periphery (Figure 3). In Al-treated cells, however, the ER appeared less dense and clustered around the nuclei of the cells (Figure 3).

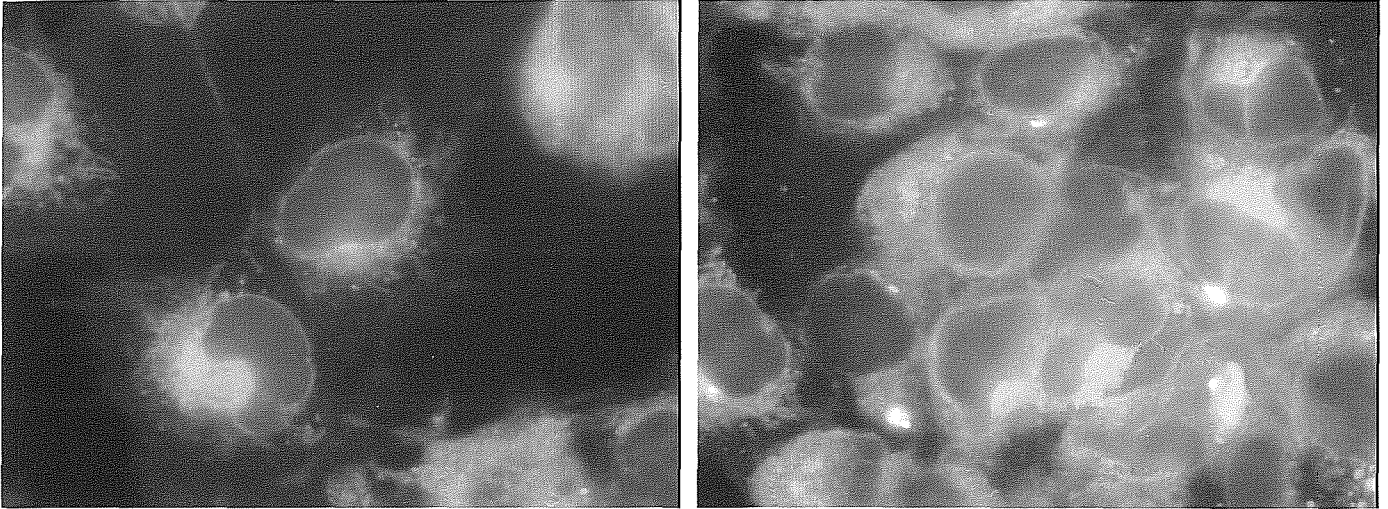
**Trial 1**



**Trial 2**



*Figure 2.* Cell number decreased significantly over 24 hours with Al-maltolate treatment (top). In addition, a significant increase in the percentage of strongly condensed, fragmented, and/or irregular nuclei was observed following incubation with 500  $\mu$ M Al-maltolate (bottom).

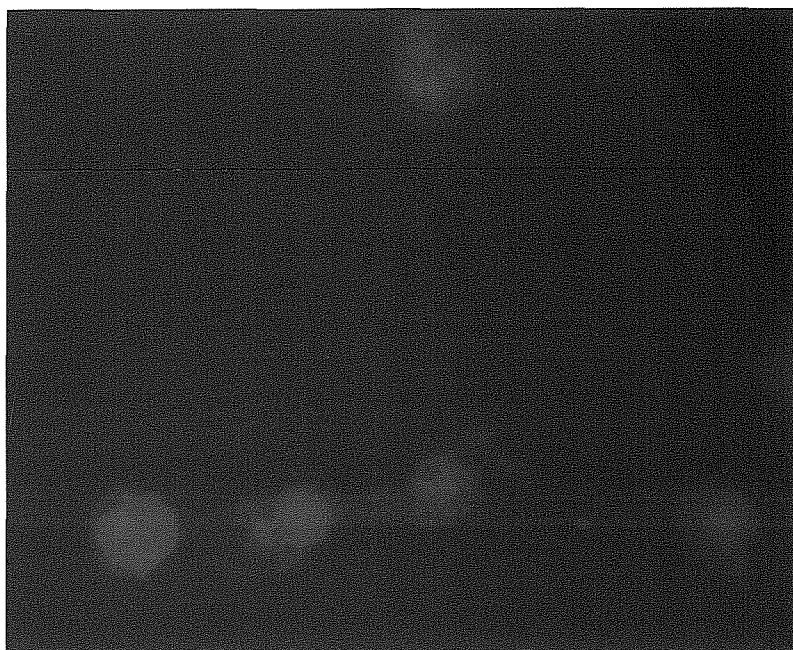


*Figure 3.* ER-Tracker™ Blue-White DPX reveals clustering of the endoplasmic reticulum around the nucleus in NT2 cells incubated in Al-maltolate for 6 hours (left). The endoplasmic reticulum of NT2 cells in control media is highly branched and extended to the cell periphery (right).

*Hydrogen Peroxide-induced Changes in the Endoplasmic Reticulum*

The effects of hydrogen peroxide were also visualized using ER-Tracker™ Blue-White DPX. The ER appeared dense and branched in control cultures, and was extended throughout the cell to the cell's periphery (Figure 3). In cells treated with hydrogen peroxide, however, the ER appeared decreased and clustered around the nucleus (Figure 4). Additionally, the cell itself was greatly reduced in size after hydrogen peroxide treatment.





*Figure 4.* NT2 cells incubated in hydrogen peroxide have endoplasmic reticulum clustered around the nucleus, as revealed by ER-Tracker™ Blue-White DPX. In addition, the cell itself was greatly reduced in size following hydrogen peroxide treatment.

## Discussion

For this study, an effective model of apoptosis was produced using Al-maltolate. The results indicate that a significant percent of cells were apoptotic following aluminum treatment based on certain apoptotic criteria: cell shrinkage, strongly condensed nuclei, and fragmented/irregular nuclei. Apoptosis has been implicated Alzheimer's disease and our model shares many biochemical and pathological characteristics with this neurodegenerative disease.

This study specifically examined the effect of aluminum treatment on intracellular distribution of the endoplasmic reticulum. The ER is the primary site of protein synthesis and assembly in the cell.<sup>2</sup> However, the ER is also involved in controlling intracellular  $\text{Ca}^{2+}$  levels and regulating cellular responses to stress. Disruption of  $\text{Ca}^{2+}$  homeostasis, inhibition of protein glycosylation, and accumulation of misfolded proteins in the ER can all result in ER stress that ultimately leads to apoptosis.<sup>43</sup>

While mitochondria has received much attention for its role in apoptosis and neurodegeneration, studies of the involvement of the ER in the pathogenesis of neurodegenerative diseases have been relatively few. Research has suggested that ER may also play an important role in stress-mediated cell death induced by a wide variety of insults. Prolonged ER stress and associated misfolded proteins are linked to the pathogenesis of certain neurodegenerative diseases such as Alzheimer's.<sup>43</sup> However, the role of the ER in initiating cell death after prolonged stress is poorly understood.

The Golgi apparatus is involved with the ER in the vesicular traffic of proteins. Transport between these two structures is at least partially dependent on microtubules.<sup>46</sup>

Disruption of this microtubule-mediated transport may lead to the fragmentation of the Golgi apparatus. Treatment with the microtubule-disrupting agent, nocodazole, causes Golgi proteins to be redistributed to the exit sites of the ER.<sup>21</sup> Disruption of ER to Golgi transport could cause an accumulation of proteins and lead to stress-mediated cell death.<sup>43</sup>

The results of this study show that treatment with A $\beta$ -maltolate has significant effects on intracellular transport of the ER. In treated cells, the ER appeared to be less dense and retracted around the nucleus. These results are consistent with the idea that transport along the microtubules in the kinesin-dependent, plus-end direction is disrupted. The inhibition of kinesin-dependent transport of the endoplasmic reticulum indicated by our results is reminiscent of the morphological effects of tau and A $\beta$  overexpression.<sup>14</sup>

One of the hallmarks of Alzheimer's disease is the presence of cytoskeletal abnormalities such as neurofibrillary tangles.<sup>9</sup> This study indicates that an A $\beta$ -induced model of apoptosis produces pathological changes in the cytoskeletal transport system similar to those found in such neurodegenerative diseases. Understanding the underlying pathological processes may help us to develop treatments that target the cause of the disease, rather than the effects.

Acknowledgements

I would like to give special thanks to Nena Fox, M.D. for help with cell culture. I would like to thank Drs. John Savory and Othman Ghribi for their generosity and insightful discussions. I would also like to thank Dr. David DeWitt for the opportunity to be involved in his research and for his support and assistance. This research was supported by the Jeffress Memorial Trust and the NIH grant # AG20996-01.

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