Inhibition of Anterograde Microtubule Transport Leads to Mitochondrial Transit Deficiency and Alzheimer’s-Like Pathology

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Abstract

Alzheimer’s disease is a neurodegenerative disorder providing a wide variety of molecular topics for investigation. Although apoptosis pathways have been the particular focus in recent years, identifying the mechanisms that stimulate intracellular transport disruption could provide the key to understanding the disease and potentially being able to prescribe remedies that will prevent the biochemical malfunctions. Disruption of mitochondrial transport is a highlight of the transport research focus and the focus of this project. By integrating immunocytochemistry, fluorescent microscopy, and the real-time video image capabilities of MEDIC software, the transport of mitochondria and other vesicles in control and Staurosporine-treated cells was examined. Although mitochondria could not be distinguished as well as originally hoped, this project paved the way for future studies using combined fluorescence and MEDIC tracking to follow the pathways of particular vesicles through a cell, and provides groundwork for assessing the cause of the peri-nuclear clustering effect evident in tissues following apoptotic or Alzheimer’s-mimicking tissues.
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Introduction

Much has been established about the adverse transport effects caused by neurodegenerative disorders, but progressive research requires novel models that are both functional and informative. The purpose of this research project establishes such a model for specifically investigating the kinesin-mediated transport of mitochondria by incorporating a unique combination of motion-enhanced differential interference contrast microscopy with standard fluorescent microscopy techniques. The ability to pinpoint mitochondrial location and observe live imaging of intracellular trafficking allows inspection of the process of perinuclear clustering which will lead to more accurate characterization of the mechanism by which it occurs.

Developed by Drs. George Holzwarth and Keith Bonin (Wake Forest University, Physics), motion-enhanced differential interference contrast (MEDIC) microscopy is an enhance version of the DIC concept and a significant aspect of this project. MEDIC is improved to allow 50X the contrast of regular DIC imaging for moving vesicles. An additional component of the program, motion-enhanced cross-correlation algorithm (MECCA) software, traces the pattern of an image and uses this pattern to track the image through a series of frames with a high degree of accuracy (~10nm). Together, MEDIC and MECCA provide invaluable tools for observation and tracking of mitochondrial movement through the axons of neuronal processes. These programs have already proven successful with honey bee Kenyon cells as well as cells from the PC12 line (personal communication). This project successfully extended the experiment using
differentiated NT2 cells, a pluripotent line from human testicular embryonal carcinoma
that extends thin neuritic processes conducive to examining fast vesicle transport.

Review of Literature

*Fundamentals of Intracellular Transport*

Transport is vital to a cell. While some essential molecules can be adequately
distributed by diffusion, the extensive length of axons requires directed transport. This is
accomplished by intracellular trafficking through an axonal region in order for essential
proteins, membrane components, and organelles to reach their destination.\textsuperscript{51} Any factor
adversely affecting this transport system would, in principle, result in degeneration of the
neurosystem, and provides, therefore, a possible starting point for investigating the
initiation event of multiple neurodegenerative symptoms.\textsuperscript{19}

In order to assemble this intracellular trafficking system, a cell lays a foundation
of microtubule tracks which are polarized to provide the capability of directing forward
and reverse transport, a property which is essential to bidirectional transport of important
organelles such as mitochondria. A central organization center directs the formation of
microtubules from tubulin subunits, such that the fast-growing plus end is directed
toward the growth cone (axonal terminus) and the slower growing minus end aids
transport of cargo back towards the cell body.\textsuperscript{20}

A second essential component to a functional trafficking system is molecular
motor proteins.\textsuperscript{20} It has been established that retrograde (reverse) and anterograde
(forward) transport mechanisms have their own unique motors: kinesin and dynein.
Confirmation that bidirectional transport functions by using two distinct motors is based
on observations of different maximum velocities, rapid directional oscillation of
mitochondrial movement, and independent regulation of anterograde transport.\textsuperscript{34} Dynein serves as the minus-end directed, or retrograde transport motor, while kinesin is a plus-end directed motor and powers anterograde transport toward the growth cone or synapse\textsuperscript{19,25} (Figure 1). Once these microtubule tracks are organized, cargo can be transported along them to the most distal ends of axons or back to the cell body.

Axonal transport can be categorized as either fast or slow, depending on its rate. Both categories require a unique mechanism to function. Slow transport carries tubulin, the basic subunit of microtubules, as well as other cytoskeletal elements such as neurofilaments (NFs) and actin.\textsuperscript{53} Fast transport rapidly transfers mitochondria, vesicles, and membrane components at a rate of 50-200 mm/day compared to the 0.5-2 mm/day of slow transport.\textsuperscript{24} Fast transport also carries membranous proteins in a bidirectional intermittent manner, while slow transport is specific for cytoskeletal proteins.\textsuperscript{7}

Individually, mitochondria move at a pace of 0.3-2.0 μm per second, excepting one quarter to one half of the mitochondria in a single neuron which are stationary. This allows them to be distributed relatively uniformly throughout neurons. If the axonal transport by which they normally move is disrupted, this uniform distribution is lost. This occurrence is evident in neurodegenerative diseases such as Alzheimer’s.\textsuperscript{32}

\textit{Axonal Transport Defects: Implications for Alzheimer’s Disease}

The most widely recognized pathologies of Alzheimer’s disease (AD) include neurofibrillary tangles, which are composed of the microtubule-associated protein tau, and senile plaques, which are aggregates of amyloid-β (Aβ) protein. Both of these neurodegenerative effects are axonal pathologies, potentially linked to transport.\textsuperscript{47} Yet another typical pathological symptom is axonal swellings, which appear as bulges
trapping mitochondria and other fast axonal transport cargos such as vesicles. Stokin et al. report that axonal swellings increase in direct correlation to kinesin reduction.47

Figure 1. Intracellular trafficking inside an axon. (left) Drawing of a typical neuron. (right) Kinesin (purple) and dynein (blue) directs anterograde and retrograde transport of mitochondria along a microtubule.

Similarly, Hurd and Saxton demonstrate that axonal swellings are caused by a kinesin mutation and not affected by slow axonal transport; neither are the slow transport cargos tubulin or actin found in the swellings.24 Mitochondria trapped by kinesin reduction and swellings present a particular problem. Although, as stated previously, some cells rely on diffusion for molecular transport, even small cells require a specialized mechanism of mitochondria distribution. It is vital to a cell’s metabolic demands that these ATP-generating powerhouses be delivered in a timely, efficient manner.24 In neurons, this is particularly important due to the vast ATP requirement at the growth cone, the region from which the axon extends.5 These reports provide significant insight into synaptic loss evident in AD as it pertains to dementia symptoms.46 Mitochondria hindered from
reaching the synapse cannot generate energy (ATP) necessary for transmission of a neurotransmitter signal. In such a case, the synapse function collapses, and the progress of the essential cargo of microtubules would halt like a train stopped on the tracks right before a washed out bridge. Interestingly, axonal swelling morphologies are some of the earliest cellular symptoms, present in both mouse and human AD pathology. Axonal swelling observations have been reported up to a year in advance of Aβ plaque formation.\textsuperscript{47}

Additional factors have also been identified as contributing to this dangerous inhibition of mitochondrial transport. One of these warning signals is hyperphosphorylated tau. Tau is one of a group of microtubule-associated proteins (MAPs) that plays important roles in the construction and maintenance of microtubules tracks.\textsuperscript{10,18} As a neuronal MAP, tau is expressed in a wide variety of cell lines, and functions to stabilize microtubules, promote neurite growth, and support transport of cargo.\textsuperscript{29} Like other MAPs, tau seems to cause cargo to cluster in the perinuclear region of the cell by interfering with anterograde transport.\textsuperscript{29,45} It has been proposed that this transport inhibition is effected by bound (unphosphorylated) tau creating an obstacle to motor proteins. In principle, then, removal of bound tau by increasing phosphorylation would free motors to proceed along the microtubules.\textsuperscript{3,10} Because hyperphosphorylated tau is a characteristic condition of Alzheimer's pathology, the mechanisms of tau regulation via phosphorylation are of particular interest to studying this neurodegenerative disease.
Mitochondrial Distribution

The transport system of mitochondria is particularly fascinating because of its unique aspect. Mitochondria are transported bidirectionally, both from and to neuronal cell bodies via axons in order to supply ATP to synapses and other areas of high metabolic demand. Trafficking velocities are not constant, and difficult to classify as fast or slow, because of selected stationary mitochondria and a plethora of regulatory signals. Though saltatory, mitochondria movement is remarkably coordinated and complex.\textsuperscript{22}

Mitochondria are essential to the normal dynamic state of neurons, which undergo outgrowth at the growth cones for both normal development and regeneration.\textsuperscript{27} This elongation process is intermittent, but generally does not completely cease unless in cases of extreme nutritional deficiencies in the cell or severely interrupted transport. Surprisingly, rather than transport inefficiency or an inadequate supply of structural components, neuronal elongation is challenged by an unclear inefficiency in the tension forces that cause outgrowth. Lamoureux et al. report that maximal outgrowth is not usually achieved due to this inefficiency.\textsuperscript{27} Regardless of the rate of outgrowth, or the efficiency in tensile forces that extend neurites, mitochondria are strategically located in regions of high ATP consumption in order to maximize efficiency by locally providing energy as needed. By distributing mitochondria where ATP is most needed, a cell eliminates the time-response factor that would be expected from relying on power from a distant location.\textsuperscript{16} During axonal elongation, a great amount of ATP is required at the growth cone; to support this energy need, mitochondrial density at the growth cone increases.\textsuperscript{28, 34} Morris et al. showed, however, that once the axon ceases growing, mitochondria are redistributed uniformly in as little as one hour. This research implies a
growth-dependent mitochondrial distribution gradient rather than a distribution based
solely upon other physiological factors that would shift membrane potential or organelle size.\textsuperscript{35} Ligon and Steward propose that mitochondrial distribution variability may even
be regulated in a unique manner for different types of neurons.\textsuperscript{28} While this theory
requires more investigation, several possible qualifications of a general regulatory system
have been suggested. First of all, a sensor would be required, triggered by ionic change,
potential, protein conformation, or some other pertinent flux. Second, coordination
between anterograde and retrograde transport would need to be included in order to
balance distribution.\textsuperscript{35}

\textit{Mitochondrial Rate of Motion}

Rate of mitochondrial movement is also correlated with growth. Anterograde
transport of mitochondria is slowed near the growth cone of growing axons, and rapid
near the cell body. Retrograde transport, on the other hand, proceeds quickly near the
growth cone of \textit{non}-growing axons.\textsuperscript{20} These experiments further imply individual,
primary molecular motors that direct movement in an either anterograde or retrograde
pathway.\textsuperscript{28} In general, observations by Ligon and Steward suggest that individual
mitochondrial movement is both rapid and saltatory, with a great deal of stops, starts, and
overall fluctuations in velocity.\textsuperscript{28} Because of these fluctuations, translocation progresses
by a sustained series of rapid motions rather than continual translocation. Faster-
traveling mitochondria usually exhibit unidirectional motion, while slower-traveling
mitochondria tend to travel both anterogradely and retrogradely. Although mitochondria
are frequently seen reversing direction, net transport is anterograde. Ligon and Steward
report that as much as 72\% of nearly all net axonal transport observed was anterograde.\textsuperscript{28}
Dendrites expressed slightly lower, but comparable percentages of anterograde transport. Interestingly, both Ligon and Steward, and Morris and Hollenbeck show that while anterograde transport is most prominent, retrograde transport is faster.  

Docked Mitochondria: Stationary or Low Velocity Transport?

Mitochondrial docking has been mentioned in several studies, with reports that approximately a quarter of the mitochondria in a given cell are stationary.  “Docking” refers to the temporary binding of mitochondria to a structural component of the axon such as microtubule, microfilament, or actin filament along the anterograde route through an axon. In a recent investigation, Miller and Sheetz observed a peculiar motion pertinent to mitochondria which were previously thought to be molecularly tagged as stationary. This research suggested a form of motility that represented neither fast nor slow transport. Termed low velocity transport (LVT) by the investigators, it applies specifically to temporarily docked mitochondria moving anterogradely and may help explain the overall consistency in mitochondrial flux (mitochondria per hour).

Contrary to intuitive implications that both axonal elongation and LVT are caused by distally-directed forces such as pushing or motor activity, Miller and Sheetz discovered that LVT and axonal elongation continued even during growth cone pauses, leading them to believe that both elongation and LVT result from specialized viscoelastic stretching.

To maintain a net uniform distribution throughout an axon and satisfy increased metabolic demand as the axon grows, mitochondria proceed by fast axonal transport near the proximal region. Flux (mitochondria per hour) decreases down the axon as mitochondria are docked more frequently in regions that have been depleted by LVT. In distal regions (last 150 µm of axon), LVT increases where stretching due to tension
forces are greatest, thereby supplying mitochondria for growth cone elongation. This novel mitochondrial motility, while not fully understood yet, contributes to a mechanism of intercalated addition of mitochondria that necessarily compensates for decreased flux, keeping the axon supplied with ATP. In other words, fast anterograde transport decreases in distal regions of the axon as mitochondrial transit pace slows until it is just fulfilling demand. This prevents surplus transport of mitochondria that could cause a traffic jam in the mid-region of the axon. These results implicate the existence of an ATP gradient, since energy would be lower near the growth cone where it is consumed more rapidly. Because ATP levels would naturally be higher near the somatic region of the cell where energy demand is not as great as at the growth cone, LVT is a sensible explanation of how a cell compensates for ATP being depleted faster in one region than another.

In another relevant study prior to classifying LVT, Miller and Sheetz statistically examined organization of mitochondria, and found compelling evidence for uniform distribution, maintained by non-random stopping and docking events. Stopping appeared to occur with a high probability in regions between already stationary mitochondria, thereby filling gaps left by nonmoving mitochondria. In some cases, nevertheless, the high stopping probability declined, permitting mitochondria to proceed to regions of higher metabolic demand. The mechanism implies highly specific regulation of mitochondrial location and, in hindsight, may be described by LVT.

**Microtubule Dependency**

Microtubules are the structural components underlying cellular morphology. As such, they are essential to intracellular transport (trafficking), neurite outgrowth, and
cellular polarity. Importantly, microtubules are involved in mitochondrial transport. Based on observations of their alignment with microtubules, mitochondria appear to be organized and distributed in a microtubule-dependent manner.\textsuperscript{11} Through interaction with the molecular motor protein kinesin, mitochondria are transported forward, along axons, toward the growth cone via microtubule tracks\textsuperscript{11} (\textit{Figure 2}). Although microfilaments also form part of the cellular transport framework, Morris and Hollenbeck established that microfilaments alone are not sufficient to direct mitochondrial transport, at least in the anterograde direction.\textsuperscript{34} Cells possessing

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{kinesin-mitochondria.png}
\caption{Motor activity of kinesin along microtubules can be envisioned like a train moving on railroad tracks. Kinesin represent the engine, and the cars would then be membrane bound organelles with their respective cargo. Breaks in the microtubule tracks cause kinesin motors to halt or derail.}
\end{figure}
microfilaments—but not microtubules—exhibit an inability of mitochondria to leave the cell body (implying inhibited anterograde transport), while in the absence of microfilaments, mitochondrial movement is sufficiently conducted along microtubules.\textsuperscript{34}

Because net transport along microfilaments is retrograde, cells with a disrupted or nonexistent microtubule pathway would not be able to overcome this net directional effect to sufficiently transport mitochondria from the cell body to neurites. Results from this study strongly implicate the role of microfilaments in mitochondrial clustering in cases where microtubules are no longer intact. Morris and Hollenbeck further suggest that microfilaments, as a localized transport system, may be responsible for re-associating displaced organelles back on microtubules.\textsuperscript{34} Although microtubules are capable of facilitating functional bidirectional transport, anterograde transport is favored, particularly in instances where microfilaments are present and intact.\textsuperscript{34}

*Kinesin Directs Anterograde Transport*

Kinesin has been identified as the essential molecular motor for anterograde transport. Present in neural cells, this enzyme moves cargo in a polarity-dependent manner via ATPase activity.\textsuperscript{12} (Figure 3) Muto et al. report that kinesin’s directionality may be established by ATP hydrolysis creating a conformational change in microtubules as it binds, increasing microtubule-kinesin binding affinity.\textsuperscript{12} This concept of an identifiable molecular motor for forward transport, with its directionality contingent upon the polarity of the microtubule tracks, seems sensible and simple enough. But as is the case with most studies of proteins and enzymes, the “simple” protein turns out to be a complexity of subunits and isoforms and family members that challenges researchers’ ability to pinpoint an individual kinesin derivative responsible for transport. In all
probability, various kinesins function simultaneously and cooperatively to ensure transport with a varying combination of proteins for each species or cell line. Indeed, it has been found that kinesin is not just one molecular motor but rather a group of superfAMILY proteins with different roles in directing transport. For instance, kinesin-II is present in the green alga *Chlamydomonas reinhardii*, where it aids flagella assembly by moving flagellar construction components to distant parts of the cell. Kinesin-II is also present in mammals, where it is composed of KIF3A and KIF3B subunits, and as a mammalian motor, it is implicated in left-right axis determination. Mutations in this motor result in normal ciliary and flagellar dysfunction, causing sperm motility defects, bronchial abnormalities, and left-right body axis determination failure. Additionally, if kinesin-II is removed from vertebrate photoreceptors, opsin and arrestin accumulate, leading to possible involvement with human retinitis pigmentosa. Other studies demonstrate that kinesins bind to specific cargoes. The kinesin KIF1B associates with mitochondria, while KIF1A does not, suggesting that KIF1B may be a motor specific to mitochondria. Yet another kinesin family member, KIF2, is associated with fast axonal transport of membrane-bound organelles, providing further confirmation for the theory that certain cargos are bound and conveyed only by specific motor proteins.

Whatever the function of the individual kinesin may be, this collective group of motors is essential to anterograde transport system in non-neuronal as well as neuronal cells. These motors assume a tedious task in traveling great distances along axons, conveying cargo consisting of critical cellular components to various positions along neurons. Yet this tedious system is indispensable to the cell. Considering the multitude
of factors that could impair it, it is easy to see why Goldstein refers to intracellular trafficking as the “Achilles heel” of a cell.\textsuperscript{17}

Kinesin I was the first of this important motor family to be discovered. Initially, it was thought to be a component of the fast axonal transport system in which it was found—squid.\textsuperscript{51} Kinesin I consists of two chains, the kinesin heavy chain (KHC), and kinesin light chain (KLC).\textsuperscript{17} It is likely that there are two KHCs and two KLCs, forming a tetramer.\textsuperscript{16} Though mutations in the KHC domain cause transport disruption, dystrophic neuron development, and distal paralysis in \textit{Drosophila}, it is unclear whether the KHC or KLC actually binds to cargo. In fact, a separate set of proteins, the kinesin-related proteins (KRP), probably serve this cargo-binding function.\textsuperscript{24} In a recent study, Glater et al. performed experiments on a possible mitochondrial cargo recruiter.\textsuperscript{16} This protein, milton, was implicated as a recruiter that binds mitochondria to the KHC.

Interestingly, in \textit{Drosophila}, the milton-KHC complex is independent of KLC, and milton appears to displace KLC. This result implies that KHC is the more important of the two chains when considering adaptors for mitochondrial cargo.\textsuperscript{16}

As has been already mentioned, mutations in kinesin cause axonal swellings that are detrimental to transport. Also of relevance is an accumulation of organelles associated with fast axonal transport at the proximal region of the cell following kinesin mutations. This morphology, described as the perinuclear clustering effect, is observed in numerous other studies of AD, and will be discussed in greater detail later.

\textit{Tau Interacts with Microtubules}

Tau, included in a family composed of six identified isoforms, is a microtubule-associated protein which has surfaced in pathological studies of AD in the form of paired
helical filaments (PHFs). The formation of these insoluble PHFs seems strange as tau is easily soluble; nevertheless, these aggregations form in AD and are associated with microtubule interactions. These PHFs are major components of neurofibrillary tangles, a prominent histopathological effect of AD. Eventually, PHF and tangles actually displace the cytoskeleton. As a surface protein, tau can be seen as a microscopically fuzzy coating on the outside of microtubules. In its normal functioning capacities, tau stabilizes the subunits of microtubules by binding to tubulin or reinforcing protofilaments into raft-like structures. Its interaction with tubulin is also dynamic, even after the axonal framework is set up. Tau binds and unbinds tubulin, creating a continuous stabilization process. Interestingly, when too much tau has been phosphorylated, only the outer microtubule surface is blocked. Also of significance is the observation that kinesin binding exhibits selectivity over tau binding, and can even partially—though not fully—displace tau. Tau binds more selectively to α-tubulin while kinesin’s affinity is higher for β-tubulin.

Experiments have confirmed that tau does not influence velocity and therefore does not facilitate a kinesin motor gear shift that would reduce anterograde transport. Rather, kinesin and dynein have a different mode of walking. The motion of kinesin is processive, much like walking rather than running, where one foot must be kept on the ground—or in this case the microtubule track—at all times. Dynein is said to tumble around the microtubule instead. Therefore, according to Trinczek et al., dynein’s apparently more random motion is less likely to bump into and be impaired by tau roadblocks than kinesin’s methodical manner of walking. Dynein may simply be more adept at avoiding tau obstacles, which would explain the shift in transport direction that
leads to perinuclear clustering. Additionally, Trinczek et al. reports that molecular motors are prone to slipping off tracks when tau is present. They suggest that the presence of tau may influence motor binding sites, increasing predominance of dynein binding.\textsuperscript{50}

As could be deduced from results implying a tau-mediated kinesin inhibition, tau suppresses anterograde transport.\textsuperscript{50} This significantly affects mitochondrial distribution throughout the cell, decreasing mitochondrial-occupied area as much as 45%. The resulting perinuclear clustering effect can be temporarily reversed by drugs that randomize or destabilize the microtubule tracks, such as a nocodazole or taxotere, but re-clustering occurs in as little as five hours. In cells expressing tau, mitochondrial transport speed is not affected, though run length does decrease. However, the clustering effect cannot be attributed to this shortened run length, as it applies to both retrograde and anterograde transport. To further investigate what factors might be causing the clustering, Trinczek et al. examined whether or not tau affected the binding of motors to mitochondria by quantifying actively transported organelles in tau-stable and control cells. This parameter was found to decrease in tau-expressing cells, with anterograde transport being affected significantly more than retrograde transport. It would seem that tau interferes with the binding of motors that direct anterograde transport of mitochondria. As confirmation of this theory, taxotere-treated cells do not display the clustering effect and anterograde and retrograde transport are more closely equivalent.\textsuperscript{50}

An interesting investigation by Santwana et al. observed competitive binding of tau and taxol. Taxol reduces tau’s ability to bind to tubulin, and in fact, it seems that the binding motifs for tau and tubulin overlap.\textsuperscript{44} Another intriguing discovery is that tau
normally binds to the inside surface of microtubules. When it is present in excess, however, it binds to the outside. At even higher concentrations, tau can bind to itself, accumulating in clumps on the microtubule.\textsuperscript{44}

\textit{Amyloid Precursor Protein Role in Axonal Swellings and Association with Kinesin}

In looking at the aspects of disrupted intracellular trafficking on a biochemical and genetic level, it is necessary to consider gene products and proteins that interact, directly or indirectly, with kinesin and have been found abnormally in AD biopsies. Two genes have thus far been implicated in pathological effects of AD. One of these genes encodes the amyloid precursor protein (APP) which is processed by proteolysis and plays a role in cell growth regulation and axonal structure.\textsuperscript{46} Of particular interest is the interaction of APP with kinesin-I and dynein in the cytoplasm.\textsuperscript{19} Though the exact function of APP (sometimes called the AD protein) remains to be elucidated, Kamal et al., have suggested that APP is a receptor for kinesin-I, and associates with the KLC.\textsuperscript{27} One such receptor for kinesin-I has already been identified, but this protein, kinecotin, is not present in axons and is therefore insufficient to mediate the fast axonal transport of either APP or other proteins and vesicles.\textsuperscript{49} In addition, Kamal et al. found evidence that kinesin-I movement requires the C-terminal end of APP.\textsuperscript{26} Another protein, called Sunday driver (SYD), has also been found to associate with kinesin-I and may be an additional receptor.\textsuperscript{25} Further investigation will be needed to establish any existent connection between Sunday driver and APP.\textsuperscript{26} In the meantime, it appears that a membrane-associated receptor is necessary for KLC-dependent transport, while KHC-cargo interactions occur adequately without a receptor. Without a membrane receptor for KLC, active transport is hindered. Coordination of specific and non-specific cargo
binding between KHC and KLC activates and deactivates axonal transport of cargoes. It is tempting to speculate that mis-interactions involving these specific components of axonal trafficking machinery are involved in AD pathogenesis.\textsuperscript{26}

Loss of the APP gene in Drosophila results in obstacles to transport such as axon blockage and organelle accumulations. Such blockage is sufficient to trigger cell death.\textsuperscript{3} Similarly, removing or mutating this gene in mice is severely detrimental to normal synaptic functions and results in a reduction of both heavy and light chains of kinesin in sciatic nerves.\textsuperscript{25}

APP has also been found to associate with tau. Both APP and tau adversely affect axonal transport when examined alone, but coexpression of the two further enhances the effect.\textsuperscript{36}

\textit{Amyloid-β Promotes Anterograde Hindrance}

An important aim of this study is to establish that the accumulation of mitochondria and other vesicles and organelles distributed by fast axonal transport around the nuclear region is due to inhibited anterograde transport rather than shoving inward from pressure as the cellular membrane shrinks. Because amyloid-β (Aβ) plaques are implicated in the initiation of apoptosis characteristics such as membrane blebbing and cell shrinkage, the effects of this protein on anterograde transport cannot be overlooked.

Aβ has been extensively studied and is the primary peptide component of senile plaques\textsuperscript{38} (Figure 3) and is produced from kinesin-transported proteins in an axonal compartment identified by Kamal et al.\textsuperscript{25} This compartment holds APP processing proteins such as PS1, β- and γ-secretases, as well as APP. Because all the necessary
components are present, the compartment is the mostly likely location for proteolytic
lysis of APP.\textsuperscript{36} Numerous studies have established that presenilins and secretase activity
are necessary for APP cleavage, releasing Aβ.\textsuperscript{21,54} APP vesicles also carry the β-site
APP cleaving enzyme (BACE) another enzyme contributing to the secretase activity by
which Aβ is formed.\textsuperscript{19} If transport of these APP cleavage/Aβ-producing vesicles is
hindered, Aβ would accumulate into the typical AD senile plaques.

\textbf{Figure 3.} Contrast between neurons in normal brain tissue and Alzheimer's brain
morphology showing amyloid plaque obstructions, which form when beta and
gamma secretases cleave the C- and N-terminal ends of the amyloid precursor
protein.

It is undeniable that the plaques present abnormalities sufficient to trigger apoptosis, and
that neurons are more susceptible to apoptosis when presenilin genes are mutated.\textsuperscript{8}
However, the timeframe of these events seems to be secondary to initial AD symptoms
such as axonal swellings, which are caused by transport defects. Stokin et al. confirms
that vesicle and organelle-filled swellings arise from kinesin impairment long before Aβ
plaques form. Furthermore, anterograde APP transport can be effectively halted by
kinesin mutations with increased retrograde transport prior to Aβ deposition.\textsuperscript{47} Stokin
makes an important suggestion: in that axonal swellings are the site of Aβ processing
from APP, inducing kinesin-I release from vesicles. Decreased transport would evoke continued processing of an APP and Aβ, intensifying the problem.\textsuperscript{47}

\textit{Presenilin Mutations and Transport}

The presenilin genes, both PS1 and PS2 have been found in mutated forms in cases of familial AD. These genes, located on chromosomes 14 and 1, respectively, encode proteins which are commonly found in neurons of AD brain.\textsuperscript{8} These proteins indirectly regulate APP by constructing fragments that interact with secretase proteins to form complexes which lyse the APP intramembrane.\textsuperscript{42,46,47} In laboratory conditions, an apoptotic environment can be accentuated by overexpression of PS2, as shown by Cotman.\textsuperscript{8} Interestingly, when apoptosis was not induced by initiators such as hydrogen peroxide or staurosporine, excessive amounts of the presenilin protein do not appear to be detrimental.\textsuperscript{8} Because this overexpression factor merely contributes to cell sensitivity to apoptotic factors (such as those induced by Aβ buildup), but does not initiate cell death, it seems another mechanism must be involved to trigger apoptosis. Furthermore, this insinuates that transport defects may play an earlier role in AD than Aβ toxicity. Studies indicate that kinesin-mediated transport inhibition of APP must precede Aβ accumulation.\textsuperscript{11,46} This sequence of events, though far from being fully characterized, increasingly suggest a significant role of kinesin-directed transport leading to AD pathology.

Presenilins are involved in disrupted transport by their involvement with the tau-regulator Gsk-3β and Aβ plaques. In fully functional neurons, PS1 allows kinesin-I to be released from the membrane or membrane-bound organelles. This release from the membrane could very well correspond to normal APP processing which provides an open
kinesin receptor away from the membrane. Conversely, AD-linked mutations in PS1 could interfere with kinesin-propelled transport by creating abnormally processed APP. Because APP is transported via kinesin, but also may act as a KLC receptor allowing kinesin’s release from the membrane, abnormal processing due to presenilin mutations coincides reduced transport of APP as well as inhibited kinesin motor activity.42

Gsk-3β and PS1 Conflict

Gsk-3β has become an exceedingly important protein in AD research because of the variety of AD pathological aspects it is involved in. With kinesin-I as one of its substrates, Gsk-3β plays an important role in intracellular trafficking control, and has been specifically associated with anterograde transport regulation. When the light chain of kinesin-I is phosphorylated, it releases membrane-bound vesicles so they are unable to be transported forward. PS1 mutations tend to increase this dissociation of kinesin motors from their tracks by further activating Gsk-3β. This finding fits in perfectly with theories behind the loss of axonal transport in AD. Additionally, this increased activity causes hyperphosphorylation of tau, another hallmark biochemical symptom of AD.42 Thus far, research has traced mis-regulation of Gsk-3β activity to PS1 mutations, though the mechanism by which this occurs is not yet known.

Gsk-3β is a highly complex protein, and though many studies have investigated its roles in phosphorylation, it seems to play a diverse role in cells and its full function is not yet known. Of interest is the property of Threonine181 and numerous serine binding sites of tau which share the feature of being phosphorylated (and thus regulated) by Gsk-3β.42 When phosphorylated on its N-terminus, Gsk-3β is rendered inactive, a process which appears to be somewhat self-regulated. The active kinase interacts with a protein
phosphatase I inhibitor, I2, hindering it from its inhibitory function and thus allowing the protein phosphatase (PP1) to dephosphorylate Gsk. Conversely, inhibited Gsk cannot hinder I2 from inhibiting PP1, and Gsk is further inactivated because PP1 cannot dephosphorylate it. Thus Gsk regulates itself, but is also sensitive to cellular environmental factors such as levels of other phosphatases and kinases as well.\textsuperscript{13} From this involved regulatory process alone, it is easy to see why Gsk-3β’s multiple roles have posed problems to researchers implicating it with a single function or pathway characteristic of disease. Fortunately, recent advances have at least narrowed the pathway to describe a probable mechanism for neurodegenerative disorders, which includes the kinase Akt, in active form, as an inhibitor of Gsk-3β. Akt is suggested rather than other Gsk kinases because it has been shown to be highly reactive with Gsk.\textsuperscript{41}

In consideration of the many proteins involved in the cellular AD pathway, as well as all their substrates and functions, keeping in mind an overview of the activation requirements of the various proteins along the way is helpful to understanding the experiments behind each further mechanistic step that is elucidated. Beginning with the presenilin gene in this phosphatidylinositol 3-kinase signaling pathway, presenilin proteins are encoded which are involved in the downstream activation of Akt by PI3K kinase. Activated Akt phosphorylates—and thus deactivates—Gsk-3β. Since only activated Gsk-3β can phosphorylate tau and regulate its dissociation from microtubules, tau is unphosphorylated when Akt is activated. In cases such as those mentioned where familial AD is correlated with PS1 mutations, Akt is left inactivated and phosphorylation of tau by activated Gsk-3β unregulated.\textsuperscript{5}
Aside from their γ-secretase activity, which cleaves the terminal end of APP, the presenilins have been shown to colocalize with the glycogen synthase kinase, Gsk-3β. Gsk-3β is extremely important to this research, as it phosphorylates PS1, the light chain of kinesin-I, and the MAP tau. In AD, this PS1/Gsk-3β relationship seems to be affected. PS1 mutations linked to AD, discussed in the previous section, interfere with appropriate regulation of phosphorylation by Gsk-3β. These mutations increase Gsk activity rather than decrease it, leading to further phosphorylation of tau and upsetting ratios of phospho-tau to unphosphorylated tau.\textsuperscript{42}

\textit{Kinase-Related Tau Pathology}

Tau pathology in AD in somewhat puzzling and contradictory. AD pathology exhibits both phosphorylated tau which forms NFTs, and unphosphorylated tau which binds microtubules and interferes with kinesin transport, suggesting that both active and inactive Gsk-3β are involved in AD pathology. Or, in a somewhat more complicated manner, several kinases could act together to regulate—or mis-regulate, rather—tau phosphorylation. Another kinase, the stress activated kinase, c-Jun N-terminal kinase (SAPK/JNK) has been implicated in tauopathies, can phosphorylate tau, and colocalizes with tau around sites in the region of amyloid depositions.\textsuperscript{13}

Research has not yet succeeded in elucidating these factors, and scientists still must wonder if perhaps hyperphosphorylated tau is a survival response to axonal transport blockage due to bound tau. Alternatively, as suggested by Ferrer, hyperphosphorylated tau could trigger a survival pathway via Gsk-3β activation to attempt to halt NFT formation and repair destabilized microtubules. This theory could possibly extend to selective survival of cells with tau.\textsuperscript{13, 22, 34}
It might appear that the majority of reports agree that tau is detrimental and hinders transport, evoking a host of detrimental cellular consequences. While this is true, tau is still essential to stabilization of microtubules in neurons; notably, the ratios required to satisfy this stabilization are very low. Thus when tau is elevated in any system, whether it be an increase in phosphorylated tau or unphosphorylated tau, normal ratios between the MAP and tubulin become unbalanced and problems result.\textsuperscript{45} Tauopathies from mutations in the tau gene on chromosome 17 have been linked to Parkinson’s and other degenerative diseases, and hyperphosphorylated tau seems to be an effect of this mutation.\textsuperscript{43} In light of this, it would be interesting to investigate whether or not tau gene mutations account for abnormal levels of phosphorylated tau in AD or promote tau kinase activity.

\textit{Gsk-3\textbeta And the Amyloid Hypothesis}

Observed influence of Gsk activity by A\textbeta may provide insight into this increased Gsk-3\textbeta conundrum. Previous studies have determined that addition of A\textbeta to embryonic neurons resulted in neurotoxicity related to elevated Gsk activity. At the time this study was performed by Takashima et al., the assumption made was that increased Gsk activity led to an increase in tau phosphorylation, which led to PHF/NFT formation.\textsuperscript{48} This all seemed to relate to A\textbeta dependent apoptosis, and has been called the amyloid hypothesis.\textsuperscript{40} How the amyloid deposits regulate Gsk is another matter for investigation. Because A\textbeta increases levels of calcium ions, it is conceivable that, through calcium, it interacts with intracellular signals that trigger Gsk activation.\textsuperscript{48} Hoshi et al. acquired similar results implicating A\textbeta both to increased Gsk activity and neurotoxicity resulting
in neurodegeneration such as seen in AD. By treating neurons with lithium, which inhibits Gsk-3β, they were able to reduce apoptosis.\textsuperscript{23}

\textit{Mitochondrial Distribution: Perinuclear Clustering Effect}

Although the normal function of tau corresponds to microtubule stabilization, enzyme anchoring, and other membrane interactions, studies examining the role of tau in fibroblasts demonstrated tau’s detrimental ability to reduce transport, causing a net distribution of cellular components away from the cell periphery\textsuperscript{45} (\textit{Figures 4-5}). If this effect is mimicked in neurons, it could provide a credible explanation for neurodegeneration and dementia due to a loss of ATP at synapses and throughout neuritic processes. In fact, this is what has been shown in experiments modeling AD with neuronal-like cell lines. Cells abnormally expressing tau demonstrated significant clustering of mitochondria around the mitochondrial organizing center and the nucleus.\textsuperscript{9,11,45} Mitochondria are not the only cellular components to cluster, however. Stamer et al. reports clustering of peroxisomes, depleting axons of these important detoxification organelles and thereby increasing sensitivity to oxidative stress, which leads to apoptosis.\textsuperscript{45} This effect suggests that transport inhibition may precede apoptosis in AD cases. In a recent study, Fransson et al. reported the discovery of another factor of mitochondrial transport. These Rho GTPases, known as Miro-1 and Miro-2, disrupt normal axonal transport and induce perinuclear clustering of mitochondria when atypically expressed.\textsuperscript{14}
Figure 4. Pictorial representation of perinuclear clustering. The neuron on the left has vesicles dispersed relatively evenly throughout the cell. The middle neuron typifies the initial response of a neuron to a damaging agent or oxidative stress by evacuation from the outermost boundaries and processes. The neuron on the right shows advanced stages of clustering.

Figure 5. Mitochondrial clustering in NT2 cFells. (Left) Clustering induced by Aluminum maltolate treatment results in significant accumulation of mitochondria (red) around the nucleus (blue). (Right) Perinuclear clustering of mitochondria is seen additionally with staurosporine treatment. Mitochondria appear as bright red spots near the nucleus.⁹
Another implication for the clustering lies in the observation that retrograde transport increases in response to an interruption in mitochondrial ATP production. This strongly supports the theory that age-dependent or trauma-related apoptosis instigates an increase in retrograde transport. It has been suggested that apoptotic cells, with loss of potential and ATP production, precede the perinuclear clustering effect.

However, because tau is also known to affect the perinuclear clustering morphology, another sequence of events is possible. If transport were blocked by tau or microtubules destabilized by hyperphosphorylated tau, mitochondria ATP production in the growth cones would diminish from inability to transport the energy supplier, and retrograde transport would stimulate movement back towards the perinuclear region.

*Neurodegenerative Disorders: Apoptosis Or Transport Malfunction?*

Previous studies have focused on the role of apoptosis in Alzheimer’s disease (AD). However, certain observations have led a group of scientists to believe that transport malfunction is the main factor causing neurodegeneration. Because disease morphology implies both apoptosis (programmed cell death) and transport malfunction, it is difficult to determine which abnormality initiates the other. Perhaps they occur simultaneously; perhaps abnormal transport leads to apoptosis or vice versa. At present, this cannot be determined. While both affects are worthy of reviewing in depth, transport malfunction will be discussed here, in particular pertaining to mitochondria.

**Materials and Methods**

*Cell Culture*

Human teratocarcinoma (NT2) cells were differentiated by treatment with 10μM retinoic acid (Sigma, St. Louis, MO) for 6 weeks, then cyropreserved with DMSO in
liquid nitrogen prior to experimental use. Frozen cells were plated on Fluorodishes (World Precision Instruments, Sarasota, FL) coated with Poly-D-lysine and laminin, and allowed to adhere at least 24 hrs. at 37°C in 5% CO₂. Growth control media contained DMEM/F-12 with 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) penicillin-streptomycin.

**Cell Treatments**

*Staurosporine.* As a broad-spectrum kinase inhibitor and potent apoptotic agent, staurosporine effectively induces transport interference and perinuclear clustering. Cells were treated with 500 µM staurosporine (Sigma) pre-diluted in DMSO. Treated cells were stained with either JC-1 or CMXRos to indicate polarized and depolarized mitochondria, and incubated 1-2 hr. Cells were observed during treatment, alternating between fluorescent and DIC/MEDIC microscopy. Control plates were fluorescently stained and received fresh media.

*Peroxide.* Peroxide causes rapid cell death and extreme membrane shrinkage, and was thus used as a comparison for reagents that cause slower cell death/membrane shrinkage and clearer transport defects. A stock solution of 30% H₂O₂ (Sigma) was prepared and filter sterilized. Cells were incubated for 30 min. in stock solution diluted to 0.03% with growth media. Media was replaced with control media and cells examined both during and after treatment to visualize any apparent transport interference effects.

**Bead Experiments**

MEDIC images of 0.1 and 0.2 µm beads were taken using the linear function and an LUT slope of one. Fluorodishes containing the beads were attached to a piezoelectric stage (Wye Creek Instruments, Frederick, MD) to induce internal vibrations, generating a moving background. The stage was aligned at a 45° angle and images taken with
movement in both the NW-SE and NE-SW directions. Imaging was stopped when beads had switched direction once and had translated approximately halfway back across the frame. Linescans of individual beads using ImageJ software revealed S-curves that could be categorized based on peak height. Peak height was then graphed versus size (in inches rather than pixels), displaying a linear relationship. An in vivo experiment based on the same techniques found height versus width for vesicles in NT2 cells in order to identify a calibration point for assessing vesicle size.

Tracking Vesicles In Vivo

Using 8-frame per minute MEDIC microscopy, images of vesicles within cultured NT2 cells were taken. Cells were stained with mitochondrial dyes JC-1 (Sigma), diluted to 2µg/ml in DMSO (Sigma), or CMXRos (Sigma), to pinpoint typical mitochondrial locations and verify organelle shape and size.

Results

Determination of a Size/Intensity Correlation for Small Vesicles

A previous investigation by graduate student David Hall (Wake Forest University) established that beads and vesicles with a diameter greater than 0.4µm generated S-curves. Analysis of the width of the curve from peak to peak demonstrated a valid correspondence of to bead diameter only above 0.4 µm. Preliminary experiments of the current project revealed that the majority of vesicles in NT2 cells fell below the 0.4 µm diameter range, posing the problem of no previously identified correlation between size and intensity of small and large vesicles. This project investigated linescans of individual vesicles, which supplied S-curves with a seemingly consistent trend of intensity ranges, suggesting the possibility of categorization based on peak height.
Categories were chosen from trends visually apparent on the graphs with peak heights ranging from 50-200, 25-225, or 0-250 pixels (Figure 6). Due to camera limitations, larger vesicles having intensities above the 250 pixel point were saturated and intensity could not be accurately determined.

Figure 6. S-curves generated from linescans through NT2 vesicles. Peak width was found to be less than 0.4 μm, at which point the previously determined size/intensity correlation breaks down. Vesicles were categorized, based on peak height rather than width, as either small (50-200), medium (25-225), or large (0-250). Individual linescans were averaged and graphed as shown.

In order to determine if vesicle peak height could indeed be related to vesicle size, cellular vesicles were modeled using diluted beads of known sizes 0.4 μm and less in diameter (Figure 7). Linescans of individual beads revealed a linear correlation between peak height and size. Intensity neared 190 units for 0.2 μm beads, while 0.1 μm beads corresponded to between 150 and 160 intensity units. Beads having a 0.4 μm diameter were also observed (data not shown), but peak height augmented to extremes above 255 intensity units, preventing accurate measurements due to the 250-255 light saturation range.
Figure 7. (A,C) Beads were diluted in sterile H2O, placed on Fluorodishes, and imaged by MEDIC microscopy. Background translocation was generated with aid of a piezoelectric stage. A total of 200 images were saved in an 8-second timeframe. (B,D) Llinescans of individual beads were taken using ImageJ software. The resulting S-curves are shown, depicting a distinct peak height difference dependent on bead size. Smaller beads (0.1 μm) correspond with peak height of ~160 intensity units, while larger beads (0.2 μm) correspond to a peak height of ~190 intensity units.
In Vivo Vesicle Imaging/Tracking

Experiments performed using MEDIC software and the MECCA tracking program provided a valuable method for observing motion within a cell. In particular, we were able to note changes in velocity and direction of vesicles, and watch changes in the shape of cellular membrane and variations in extent of cell boundaries. Microscopically viewing various cells just from one small fluorodish revealed incredible variety in a cell’s motive behavior. Some cells appeared to have more vigor than others and exhibited thriving traffic patterns of vesicular motion. Some cells had wildly undulating extensions of fillopodia, while others had a calmer surface that lacked these extensions. For some cells, motion was most prevalent within the boundaries of the cell, consisting of transportation of vesicles or Brownian motion, while for other cells, vesicles moved lethargically and most of the motion observed was limited to a rapidly changing cell membrane. Our previous research has implications a compelling lack of anterograde transport in degenerating neurons. When looking at the one-time-frame images of fixed cells, this malfunction appears to cause a drastic clustering of mitochondria, peroxisomes, endoplasmic reticulum, and Golgi, to the nucleus. Using MEDIC for in vivo imaging and trafficking showed us the labyrinth of cellular transportation live rather than at one fixed time frame. In Figure 8, eight consecutive images from a live video depict the progress of vesicular motion through an NT2 cell. Typically in any given cell, some vesicles were moving while other remained stationary.
Figure 8. Vesicle tracking. NT2 cells viewed with MEDIC. Black and white arrows mark the moving and stationary vesicles, respectively.

Figure 9. The MECCA tracked trajectory of the same vesicle. Stars mark ends of the 8 segments used to fit the data. This number of segments represents the best fit to the data as measured by a reduced chi-squared minimization (inset). The maximum velocity is 3.1 μm/s (V1) over 2.5 frames (0.3 s).
While no single or multiple frame images can quite capture the intriguing ability to visualize vesicles in motion \textit{in vivo}, \textit{Figure 10} depicts the enhanced ability to detect moving particles within a cell compared to older DIC imaging techniques. With DIC imaging, motion is not detected and unless moving vesicles are very large, intracellular transport cannot be detected. MEDIC, however, proved highly sensitive in its ability to detect trafficking. In some cases, trafficking was so chaotic it was difficult to separate individual vesicles to track for the purpose of calculating velocity. \textit{Figure 10} points out some of the vesicles which were successfully able to be tracked using MECCA. NT2 vesicles tracked by MECCA provided a database of trajectories typical of that particular cell line. Similar trajectory databases, for honeybee Kenyon cells and the PC12 cell line, were collected in earlier experiments by our collaborator Dr. Macosko and his colleagues at Wake Forest University. Extending the variety of cell lines used in these tracking experiments lent credibility to the developing theory of processitivity, or cooperation, between kinesin motor function \textit{in vivo}. 
Figure 10. Three views of NT2 cells. (A) DIC image of a neurite process. (B) Image of the same region with motion-enhanced DIC. Arrows indicate vesicles that are easily tracked. (C) Fluorescent image of a cell body and neuritic process with CMXRos- stained (red) mitochondria.

Trajectories Determine Individual Vesicle Velocities

Following tracking with MECCA, each trajectory (distance over time) was analyzed and a trend line added to fit the data points. Trend lines for several consecutive trajectories of a vesicle were fitted together to form a larger trajectory showing more extensively the movement of a vesicle over time. Slopes of the best fit lines, which are equivalent to velocity, were added at points along the large trajectory as shown in Figure 9. Because each velocity was affected by differing drag forces exerted on vesicles, all velocities were normalized by dividing by the lowest velocity (smallest slope). Normalized velocities were binned to generate a histogram which showed quantized velocity peaks (Data not shown). Histograms were surprisingly inconsistent with results using PC12 cells, which were used in previous experiments to investigate the
cooperativity theory of kinesin motors. If this cooperativity theory is indeed accurate, each addition of a molecular motor per vesicle would increase the velocity of the vesicle it carried in a quantized manner. Thus the first peak of the histogram would represent one motor, the second peak at twice $V_0$ would represent two motors, and so forth. While more experiments are currently underway to investigate this developing hypothesis, it is impossible at present to conclude that the NT2 cell line will be the desired addition to the library of cell lines which exhibit consistent trends in velocity peaks depending on the number of kinesin motors.

*Observations of Intracellular Transport And Membrane Fluctuation in NT2 Cells*

A comparison of the cells shown in Figures 11-12 demonstrates the variation in vesicle motility. Consistently, cells removed from optimum CO$_2$ and temperature conditions displayed a decline in both velocity and occurrence of trafficking in as little as an hour of observation. Also, we found that Brownian motion became more evident than directed movement after an extended period of observation (~3 hr).
Figure 11. Cellular region depleted of organelles and vesicles. Culture plates removed from the incubator were maintained at room temperature, to keep cells viable as long as possible during imaging. After nearly an hour, however, most cells began to display characteristics of apoptosis. This cell exhibits clustering resulting in a marked depletion of organelles and vesicles from outer cellular regions.

Figure 12. Peripheral cell region with abundant vesicular trafficking. Cell very recently removed from optimal conditions displays significantly more trafficking nearer the boundaries of the cell than the less viable cell depicted in Figure 16. Vesicles are distributed evenly and move rapidly.
As shown in *Figure 13*, rapid membrane retraction occurred visibly, as well as the decline in trafficking. In some cases, the membrane retractions and fluctuations were more drastic and more pronounced than changes in vesicle motion. Membrane retraction was observed in real time with MEDIC imaging, but for better visualization purposes here, is shown in fixed time frames using the raw (DIC) images. In *Figure 14*, both MEDIC and DIC images are shown to illustrate membrane motility. Both viable, and unhealthy cells displayed this type of membrane motility, expressed in constantly growing or shrinking of portions of the cell, change in boundary shape, or rapid extension or retraction of processes.
Figure 13. Rapid membrane retraction and vesicle clustering occurs in cells removed from optimum conditions. (A,B) Images taken several minutes apart of healthy NT2 cells demonstrate the rapidity by which the membrane can be extended or retracted. Vesicles observed did not appear to halt or diminish in motility during the retraction process. (C,D) Cells removed from viable conditions for a longer time reveal both membrane retraction and clustering confined to inner cellular regions. (E) Vesicles dotting distinct track disclose range of vesicle motility in waning cells.
Figure 14. Membrane motility in differentiated NT2 cells. First three columns display background subtracted images from three consecutive 200-frame movies (A, B, and C, respectively) with an approximate one and half minute time lapse between each movie. The first three images in each column are 99-frames apart. The far right hand column shows raw DIC images corresponding to each movie. Even in the short time frame represented, there are obvious membrane changes including both progressive shrinking and fading of processes.
Figure 15 focuses on the long, thin neuritic processes that are characteristic of NT2 cells. Although the vesicles within these processes were too minute to be distinguished for individual tracking, net motion could be easily detected in healthy cells. In less viable cells, the processes appeared void of motion. Bulges in these axonal processes, proved to be sites of particular interest for observation. Motion was readily detected in these bulges, but appeared confined to them. Similar to these bulges are the axonal blebbings shown in Figures 16-17.

Figure 15. DIC images of neuritic processes extended from four differentiated NT2 cells. (A,B) Long, flat processes are ideal for MEDIC microscopy. Although movement can be easily detected with background subtraction, vesicles traveling within these processes appear smaller than those seen in wider portions of the cell body, and are difficult to distinguish and track. (C) Motion in cells such as this one which have just begun to extend neuritic processes provide little resource for tracking, but display a metropolis of net motion as cellular components busily transport cargo for the new extensions. (D) The wider of the two processes in this cell is more ideal for background subtraction imaging and vesicle tracking than the processes in A-B.
Figure 16. DIC and background subtracted images of axonal processes disclose progression toward vesicle clustering (top) Axonal swelling indicated by the arrow restricts movement of vesicles. Fine lines in DIC image that appear as faint protrusions form tracks over which the majority of trafficking occurs. (bottom) Cell decreasing in viability with the greater part of its trafficking occurring in or directed towards the cell body (indicated by arrows)
Figure 17. Axonal blebbings in differentiated NT2 cells are seen to retract in background subtracted imaging (A,B) DIC images showing typical axonal processes. Arrows indicate a swollen area that undergoes rapid retraction. Images are ~1 min apart (C-E) Background enhanced images from an 8-second movie. Cell membrane retracts as the movie progresses and visibly changes shape and size at axonal bulges.
While membrane shape fluctuations were the most prominently visible observations of these experiments, some vesicle clustering was observed as well. In particular, cell periphery became almost completely devoid of moving vesicles if the cell was observed for an extended period of time. As the cell was exposed for longer amounts of time to the suboptimal environment of microscopy, motion was diminished and localized to the perinuclear region. This is consistent with the perinuclear clustering reported with fluorescent images of fixed cells. Figure 18 illustrates cells which have sparse vesicle distribution and severely diminished motion in the outer boundaries of the cell. Cells such as these appeared lethargic, lacking the vital energy that properly dispersed mitochondria would provide. Figure 19 focuses in on the somatic portion of the NT2 cells. Any detectable movement was observed closely here to see whether or not there was a noticeable decline in anterograde vesicle transport. Motion was detected in this region, but the processes proved too thin to detect actual direction of motion through them.
Figure 18. Clustering in the vicinity of the nucleus results in sparse vesicle trafficking near cell periphery. (A,B) The boundary of the cell is faint but still visible, and almost no vesicles can be seen outside the nuclear region. Real time images reveal mostly Brownian motion near the nucleus and very few vesicles that could be tracked. (C) Clear outline of the cell shown by DIC microscopy. The arrow indicates the nucleus.
Figure 19. Process extending from the somatic portion of a differentiated NT2 cell. Background subtracted images from two separate movies of the same cell region. Vesicular motion is easily observed at the portion where cell body extends into the process but less easily tracked within the process, where vesicles appear smaller and are less distinguishable from one another.
Discussion

Perinuclear clustering is a troublesome problem for proper cell function. Mitochondria that cannot reach axonal peripheries or synapses create a lack of ATP and inability to power signal transduction. Peroxisomes accumulate near the nucleus and can no longer provide the necessary detoxification throughout the cell, causing net increased sensitivity to oxidative stress and apoptosis. While perinuclear clustering is implicated in AD using the NT2 cell line, the problem it presents extends to other cell lines and disease histopathies as well. To many branches of research, therefore, an understanding of the mechanism producing this clustering effect is crucial.

There are several possible hypotheses to consider. The first involves membrane shrinkage and dissolution of the cytoskeleton, creating an inward force much like a carpet being rolled up. Caught up with this cytoskeletal “carpet” are organelles and other vesicles that have been distributed to the boundaries of the cell. There are several problems with this hypothesis. Our previous research project implicates precedence of perinuclear clustering to membrane blebbing and shrinkage, characteristics that become evident in cells as they undergo apoptosis. Therefore the initial nuclear-directed force cannot be accounted for by the membrane shoved everything in its pathway to a more central location. If this were the case, we might also except to see a similar distribution of organelles throughout the cells, taking on a squished appearance as the ratio of cell volume to membrane expanse diminished. Furthermore, experiments by Stamer et al. established that cells exhibiting the clustering effect maintain an intact microtubule array. A dissolved cytoskeleton is not consistent with this result. Further methods of investigating this hypothesis might include immunostaining cells for presence of actin. It
would be interesting to see the distribution of actin and other cytoskeletal elements near the membrane of the cell, and compare peripheral actin location with kinesin distribution. Although kinesin travels along microtubules normally, it is also associated with an actin motor that is involved in mitochondrial transport along actin filaments and perhaps actin transportation is an alternative method of mitochondrial translocation when microtubules are obstructed.51

A second hypothesis involves trafficking inhibition by biochemical regulators such as Gsk-3β which phosphorylate tau. Previous results in this lab investigated the effects of Gsk-3β inhibition on NT2 cells, establishing that the apoptotic-inducer staurosporine initiated localization of Gsk-3β from the cytosol to the nucleus, and caused its inhibition. Gsk inhibitors also led to mitochondrial depolarization.4 The reasons for this have yet to be resolved. Our results also implicated Gsk-3β inhibition in direct correlation to perinuclear clustering in a concentration-dependent manner. A possible reason for Gsk inhibition may occur, as suggested by Ferrar, as a cell survival response to reduce accumulation of hyperphosphorylated tau into neurofibrillary tangles.13

The third hypothesis to explain the perinuclear clustering effect implicates anterograde transport inhibition as the earliest effect of neurodegenerative pathology. Several factors could account for this, such as competitive binding between kinesin and tau, mutations in genes regulating the amount of tau, or the inability of kinesin to bind to microtubules or mitochondria. This theory also involves misregulation of Gsk-3β, but, at least initially, in an opposite role. The effect of overexpressed tau accumulating on microtubules would provide a definite obstacle to kinesin-mediated transport, as kinesin moves processively along the β-tubulin portion of microtubule dimers and cannot tumble
about α- and β-tubulin as retrograde motor dynein does. An imbalance of tau, which is not normally found on the outer surface of microtubules, tends to promote motors slipping off their tracks. It has been suggested that this shifts normal binding ratios to predominance of dynein binding and decreased ability of kinesin to remain attached to the microtubules.\(^{49}\) Activated Gsk-3β could assist this problem by phosphorylating tau, releasing it from the microtubules. Though this would partially remove the obstacles along microtubules, it would not remove tau from the cell, and could be the cause for hyperphosphorylated tau accumulation in neurodegenerative disorders. Ackmann et al. concur with this theory, reporting that AD cases are typified by lack of tau affinity for microtubules.\(^1\) This study also suggested the possibility that overexpressed tau accumulates loosely on the microtubules surfaces, and factors instigating a decrease in binding affinities result in detached tau forming PHFs. Intriguingly, accumulated tau could actually extend into PHFs right on the microtubule surface, presenting a huge hindrance to transport.\(^{1,11}\)

Anterograde transport of mitochondria could also be hindered by inability of kinesin to bind to membrane bound organelles. This could be the result of misregulation of kinesin kinases such as Gsk-3β, which also phosphorylates the kinesin light chain and specifically inhibits KLC function.\(^{32}\) It is possible that apoptosis and Gsk-3β inhibition, observed in AD and modeled by staurosporine treatment of NT2 cells, occurs subsequent to this initial transport inhibition mediated by active Gsk-3β. Alternatively, or perhaps cooperatively, kinesin binding to mitochondria could be influenced by the GTPases Miro-1 and Miro-2, which have been observed to interact and interfere with GRIF-1 and
OIP06, two mammalian homologues of the milton linker protein which binds kinesin to mitochondria.\textsuperscript{14}

It was determined that while MEDIC provides useful information regarding velocity and traffic patterns, a more conclusive means of observing mitochondria pathways in dying cells would be by videos taken with normal fluorescent microscopy. This method could be varied by staining for mitochondria to assess net movement, tubulin to visualize whether or not microtubule tracks remained intact, or actin to ascertain the exact role of actin in the retractive movements of the cytoskeleton and membrane. This project also determined that DIC is a valuable tool for observations of minute extensions of the cellular network that are not as easily visible without the interference contrasting component of the program. Overall, although results regarding simultaneous observation of stained mitochondria and their movement in staurosporine treated cells were not as conclusive as we had hoped, this project established the feasibility of several methods that allow more detailed observation of NT2 cells than had previously been reported. The model described here opens a new avenue for combing fluorescent, DIC, and background-subtracted imaging.

The results from these experiments provide foundational information to further test the mechanisms responsible for perinuclear clustering of mitochondria and other organelles, essentially by establishing a functional model of transport inhibition whereby the halts and breaks in intracellular trafficking can be observed. We were successful in determining a linear relationship between vesicle size and intensity curves, which provides a standard for identifying large and small vesicles. This relationship was further extended to force-velocity curves which can later be used to determine processitivity of
kinesin motors. Additionally, visualization of intracellular trafficking using MEDIC provides a valuable method of portraying the process of perinuclear clustering over a continuous period of time. Previously, the clustering effect had only been observed at one time frame, making it impossible to determine how it was prompted. We were also able to observe significant membrane changes as cells progressed through apoptosis. Somewhat unexpectedly, clustering did not always precede membrane shrinking, and some cells displayed more evidence of shrinkage and blebbing than perinuclear clustering. Because the insight provided by observing intracellular trafficking in real time rather than at a fixed point did not further confirm our previous studies, our hypothesis must now be more carefully considered. There may be another possible mechanism that more extensive live imaging will elucidate.
References


