Author

Khin Win's advice to students interested in conducting research is to "be optimistic." Her optimism has helped her keep an open mind through her research—seeing even unexpected results as valuable information. She is particularly interested in Alzheimer's Disease and its gradual degradation of human ability and dignity. She hopes that her research may help with future understanding of this disease. In addition, she expects her research experience to help her become more knowledgeable in patient care as she moves on to medical school in the Fall of 2006. In her spare time, Khin enjoys meditation and swimming.

Environmental Regulation of Neurite Growth and tau Expression in Cultured Human Cortical Neurons

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Abstract

Neurofibrillary tangles (NFTs) are intraneuronal accumulations of fibrillar proteins—composed of hyperphosphorylated tau protein—found in several neurodegenerative diseases, including Alzheimer's disease. Rodents have been used to study NFT pathology, but significant differences exist between human and rodent tau. This research aims to characterize the expression of tau variants (isoforms) in cultured human cortical neurons (HCNs) grown on two substrates commonly used to stimulate neuronal attachment and growth *in vitro*. The goal is to establish an experimental model to study molecular mechanisms involving tau in neurodegenerative diseases. Since tau plays a role in early neuronal development, we monitored neurite outgrowth during active neurite elongation. We analyzed the subcellular distribution of tau, quantified the length of neuronal extensions, and characterized the expression of tau variants. Results indicate that tau becomes progressively compartmentalized in axons during neuronal development, and that laminin significantly stimulates neurite extension. We found that all six isoforms of tau in the adult human brain are expressed in HCNs growing on both PLL and laminin substrates. Tau isoforms with four microtubule-binding tandem repeats near the carboxy terminus are subject to developmental regulation and modulation by laminin. Thus, HCNs in culture may represent a useful model for studies of tau pathology and NFT formation.

Faculty Mentor

The quest for effective therapies to treat Alzheimer's disease (AD) is one of the major scientific challenges faced by biomedical investigators. To achieve this goal, development of appropriate experimental models is critical to determining the molecular mechanisms relevant to the disease process. In this study, Khin Win used human cortical neurons, one of the main cell types affected in AD, to characterize the changes in the expression of tau protein variants in response to environmental cues (e.g. extracellular matrix molecules). These results represent a first step in further characterizing the modifications in tau that lead to neurofibrillary pathology in AD. Undergraduates obtain numerous benefits from participating in independent research, most notably the value of teamwork and the discovery of their own potential as future researchers and/or health care providers.

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Key Terms

- Alzheimer's Disease
- Immunofluorescence
- Laminin
- Neurofibrillary Tangles
- Neuronal Cultures
- Tau
- Western Blot

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative condition affecting the elderly. There are approximately 4.5 million AD patients in the U. S.; thus, finding an effective therapy for AD is a major public health priority (Alzheimer's Association). AD is characterized by the presence of two main pathological features in the brain of affected individuals: amyloid plaques in the extracellular space and neurofibrillary tangles (NFTs) inside brain cells. Since the appearance of amyloid plaques precedes the appearance of NFTs in AD brains, researchers have postulated that plaques may trigger tangle formation (Spillantini and Goedert, 1998).

NFTs comprise bundles of hyperphosphorylated helical filaments (PHFs) of tau proteins. Tau is a microtubule-associated protein found specifically in neurons. Its association with microtubules is important for neuronal function and survival. Upon hyperphosphorylation and assembly into PHFs and NFTs, the microtubule-binding ability of tau becomes diminished. In the AD brain, hyperphosphorylated tau is also found in aberrant neuronal extensions called dystrophic neurites, which have been implicated in the loss of neuronal connections (synapses), leading to the cognitive decline observed in AD patients (McKee et al., 1991). One possibility for the loss of synapses is that tau hyperphosphorylation and NFT formation impair the ability of tau to perform its normal role of stabilizing the neuronal cytoskeleton (Drubin and Kirschner, 1986; Cunningham et al., 1997). This may compromise the neuron's ability to maintain axonal structure and function, leading to the loss of synaptic contacts.

Current experimental models for studying tau pathology include different animal models and cultured neuroblastoma cells. While these experimental systems are of value, they may not accurately replicate the expression of tau isoforms (the tau protein variants in neurons) in the adult human brain. For instance, rodent and human tau isoforms are different, and tau expression in neuroblastoma cells is limited to tau isoforms only present in immature neurons (Smith et al., 1995). There are six isoforms of tau in the human brain; all are generated by alternative splicing during development (Friedhoff et al., 2000). These isoforms are composed of three (3R) or four (4R) tandem repeats of microtubule-binding domains near the carboxy-terminus of the protein, and a varying number (0, 1 or 2) of 31-amino acid sequences near the amino-terminus (Figure 1). The structural differences are likely responsible for functional variations. For example, 4R isoforms appear to have signif-
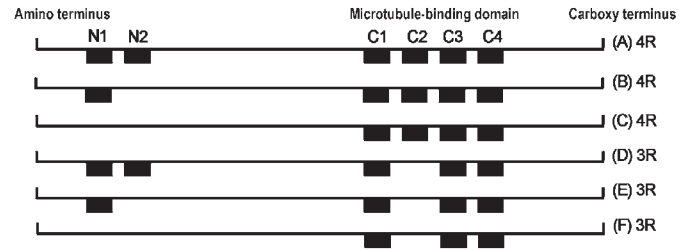

Figure 1

Schematic representation of the six isoforms of human tau. N1 and N2 represent splicing inserts near the amino terminus. C1–C4 represent tandem repeat sequences near the carboxy terminus. (A–C) Four-repeat tau isoforms with 0, 1 or 2 amino terminus inserts respectively. (D–F) Three-repeat tau isoforms with 0, 1 or 2 amino-terminus inserts respectively.

icantly higher microtubule-binding affinity than 3R isoforms (Goode et al., 2000). Thus, 4R and 3R tau isoforms may have different roles in promoting PHF formation and tangle aggregation under pathological conditions.

An experimental system that can faithfully reflect the molecular changes involved in the assembly of NFTs is necessary for understanding the alterations in tau that lead to pathology. The goal of this research is to establish an accurate experimental model for the study of molecular mechanisms involving tau proteins in neurodegenerative diseases. The first step toward this goal is to characterize the expression of tau isoforms in cultured human cortical neurons (HCNs).

Materials and Methods

Cell Culture

Tissue samples from the cortical region of human fetal brain were obtained from the Department of Pathology, Albert Einstein College of Medicine. The tissue was dissociated, and cells were plated on culture dishes or glass coverslips. The dishes and coverslips were coated overnight with 5 µg/ml of poly-L-lysine (PLL), a synthetic molecule; half of the dishes were also coated with 20 µg/ml of laminin, an extracellular matrix protein known to stimulate both neuronal maturation and the outgrowth of neuronal extensions. The cells were plated at densities of 400,000 cells per 60 mm dish and 100,000 cells per 35 mm dish in Dulbecco's Modified Eagle Medium (DMEM) plus 10% calf serum. After cell attachment, the medium was switched to a serum-free formulation, neurobasal medium plus 1% N2 and B27 supplements, to prevent the proliferation of non-neuronal cells. The cells growing on laminin substrate were also treated with soluble laminin (20 µg/ml) every three days.

Antibodies

The following antibodies were used: polyclonal rabbit antibody against tau (Dako); mouse monoclonal antibody against dephosphorylated tau at amino acids Ser-199/Ser-202 (Chemicon); mouse monoclonal antibody against neuronal beta-tubulin isotype III (Sigma); mouse monoclonal antibody against MAP2, a microtubule-associated protein found in neuronal cell bodies and dendrites (Chemicon); and a mouse monoclonal antibody that specifically recognizes 4R tau (Et2, provided by Dr. P. Davies, Albert Einstein College of Medicine). Secondary antibodies conjugated with green and red fluorophores or peroxidase (Molecular Probes) were used for double immuno-fluorescence and Western blot, respectively.

Immunocytochemistry

At three time points (3–5 days, 7–10 days, and 25 days), the cells were fixed with warm 4% paraformaldehyde for 30 min and washed with phosphate buffer saline (PBS); the cellular membranes were permeabilized with 0.2% PBS-Triton X-100 for 5 min, washed, and blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. Then, the cells were incubated overnight in primary antibodies at 4 °C before they were incubated in secondary antibodies tagged with fluorescent molecules for 30 min at room temperature. The preparations were mounted on glass slides and observed under a fluorescent microscope with filters. Digital images were captured using Axiovision Image analysis software.

Image Analysis

For quantitative image analysis, three independent sets of experiments in 4–5 day old cultures, each from different brain specimens, were performed. For each experiment, 30 random neurons were measured at each time point. NIH image software was used to obtain average neuritic lengths. The longest neurite (putative axon) in each cell, the average neurite length, and the average number of neurites per cell were compared between laminin-treated and non-treated groups. The results were expressed as the average values ± standard error of the mean (SEM) for each group. Statistical analysis was performed with In-Stat Analysis software. Statistical significance was assessed as $*p<0.05$.

Western Blot Analysis

For the Western blot analysis, cell cultures were harvested in cold RIPA buffer (for cell lysis) at various stages of development. The samples were centrifuged at 100,000 x g for 30 min to obtain a clear protein homogenate. The total protein concentration in each sample was determined using a commercial kit (Bio-Rad) following the manufacturer's instruc-

tions. Twenty μg of protein from each sample was denatured and reduced in SDS-PAGE sample buffer at 100 °C for 5 min. The proteins were separated by molecular weight using SDS-polyacrylamide gel electrophoresis in 10% linear gels before transfer onto a PVDF membrane. The membranes were then washed and blocked with 5% nonfat dry milk in Tris/saline buffer to prevent non-specific binding of other proteins to the particular antibody used. The blot was incubated in primary antibody overnight at 4 °C and then in secondary antibody for 1 hr at room temperature. The chemiluminescent reaction was performed using the Immuno-Star HRP substrate kit (Bio-Rad). To quantify protein content, the intensity of the reaction was assessed using NIH Image software.

Results

Tau is Compartmentalized in Axonal Processes in Differentiated HCNs

Cortical neurons were fixed at 3, 8 and 25 days in culture for immunofluorescence analysis. A progressive increase in the density of neuronal processes was evident at each time point (Figure 2). Tau expression in all neuronal compart-
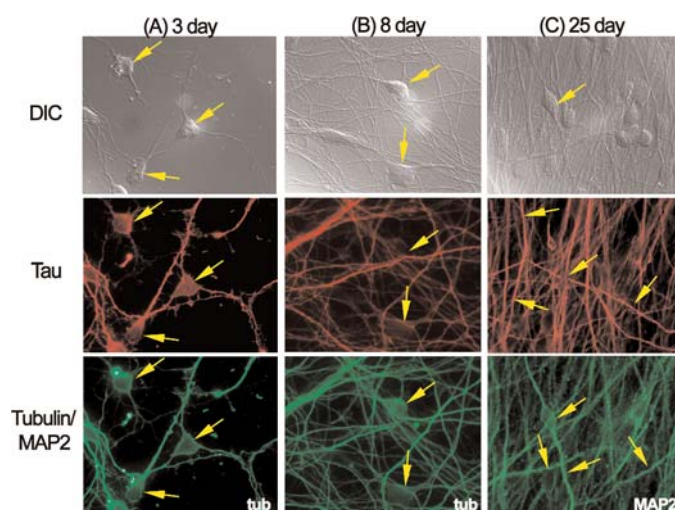

Figure 2

Tau compartmentalization during HCN differentiation in culture. HCN cultures were fixed after 3, 8 and 25 days. Each column represents the same microscopic field. The first row is a differential contrast interference image (DCI) that enables us to view the entire cell surface. The second row shows immunoreactivity with anti-tau antibody (red fluorescence), showing the location of tau proteins. The third row illustrates immunoreactivity to neuron-specific tubulin type III, which comprises the cytoskeleton in human neurons (3 and 8 days), or MAP2 immunoreactivity (25 days) (green fluorescence). Tau disappears from cell bodies by day 8. At 25 days, tau is completely excluded from several MAP-2 positive neurites.

ments was observed at 3 days. By 8 days, tau had started to disappear from neuronal cell bodies, and by 25 days, it was mostly localized in axonal processes. The axonal localization of tau was confirmed by the lack of co-localization with the neuronal-specific, somatodendritic protein MAP2. The co-staining of tau and MAP2—the latter found only in dendrites and cell body in mature neurons—did not overlap in these neuronal cultures. Thus, tau gradually disappeared from neuronal bodies between days 3 and 8 in culture, and by 25 days, tau was exclusively localized in axonal processes.

Laminin Stimulates Neurite Growth in HCNs

Examination of the time course of HCN differentiation indicates that significant axonal growth occurs over days 4 and 5 in culture. Since previous work in rodent neurons suggests a strong effect of laminin on axonal growth, we measured neuritic length in HCNs growing on laminin or PLL at 4–5 days. Cultures were fixed at days 4 and 5, and the images of the neurons were captured under a microscope after immunofluorescence staining with neuronal-specific tubulin type III. Approximately 100 neurons were analyzed from laminin and PLL cultures by measuring the lengths of their neurites using NIH Image software. The results indicate that the average length of the longest neurites (putative axons) were 109 ± 5.36 (SEM) μm and 142 ± 5.11 μm (p value = 0.0116) for neurons treated with PLL and laminin respectively (Figure 3A). The average neuritic length per neuron was determined and again compared between the two experimental groups. The average length for the PLL group was 70 ± 1.77 μm, while that for the laminin group was 86 ± 3.01 μm (p value = 0.0112) (Figure 3B). The average number of neurites per cell was not significantly different between PLL and laminin groups: 2.75 ± 0.23 for the PLL group and 2.66 ± 0.11 for the laminin group (p value = 0.7389) (Figure 3C). Thus, laminin significantly increased the length of both the longest neurite in each cell and the total neuritic length per cell, but did not significantly change the number of neurites per cell.

Tau Expression during HCN Differentiation in Culture

Western blot analysis of tau proteins shows that all six isoforms of tau in the mature human brain are expressed in HCN in culture (Figure 4). The intensity of the immunostaining progressively increased and correlated with the growth and maturation of HCNs. Marked increases in tau expression were observed between 5 and 10 days and between 15 and 20 days, and the expression of higher molecular weight isoforms of tau (V and VI) was specifically observed at 20 days. We also examined the expression
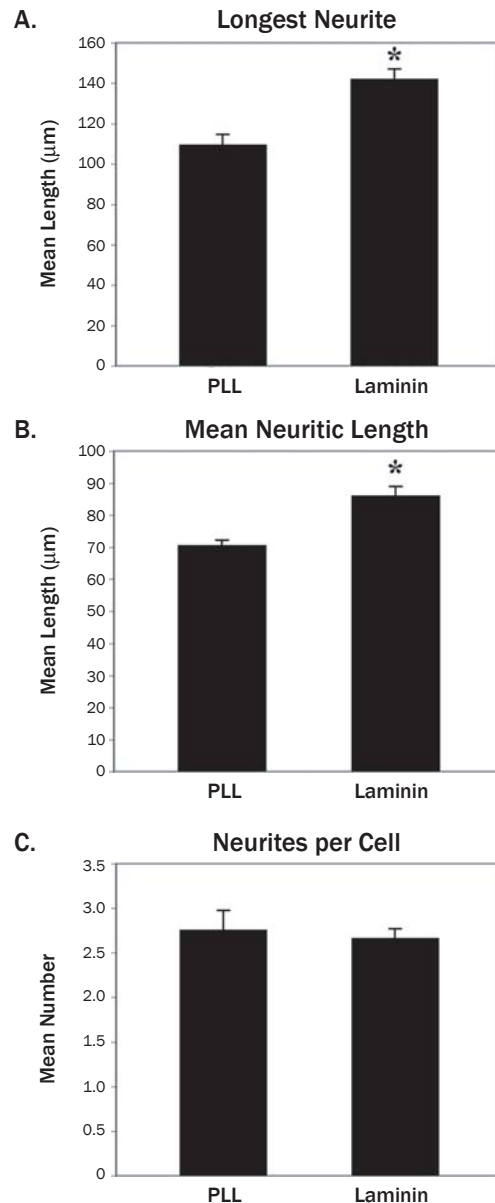

Figure 3

Comparison of mean lengths of longest neurites (A), mean total neurite length/cell (B), and total number of neurites/cell (C) between PLL- and laminin-grown HCNs after 4–5 days in culture. The values are presented as the mean ± SEM. Differences between PLL and laminin are significant by Student's t test (*$p < 0.02$).

of 4R tau using the 4R-specific antibody Et2 (Fujino et al., 2005). Western blots with Et2 revealed a gradual increase in both the level of expression and the complexity of 4R tau isoforms, which correlated with neuronal maturation. Specifically, a 38 kDa band showed increased expression at 15 days compared to 6-day sample homogenates. In addition, laminin appeared to enhance the expression of the 38 kDa isoform at both 6 and 15 days (Figure 5). By 25 days,
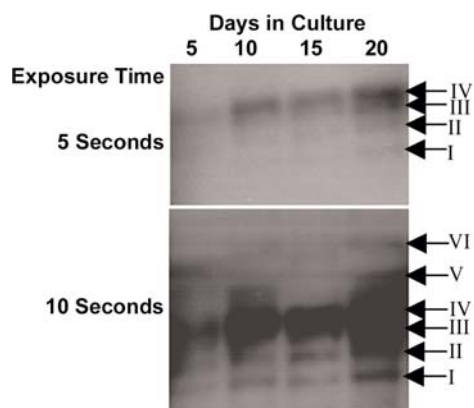
Figure 4

Six tau isoforms are expressed in HCN cultures. Cultures were harvested at the indicated time points, proteins were separated on a 10% SDS polyacrylamide gel, and transferred onto a PVDF membrane before immunochemistry against tau. The chemoluminescent reaction was detected by film images, which were scanned and converted to digital files. The increase in band intensity represents an increase in tau expression over time.
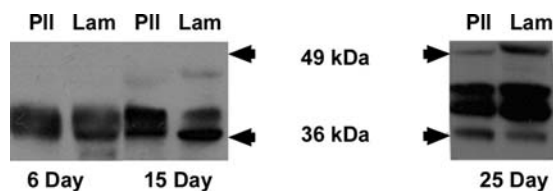

Figure 5

4R tau isoforms are differentially regulated during neuronal growth and by laminin. At the indicated time points, the cells were harvested, their proteins extracted and separated on a 10% SDS-PAGE, and transferred onto a PVDF membrane before staining with a specific anti-4R tau monoclonal antibody (Et2). The bands increased with time in culture and there was a differential expression of 4R isoforms in HCNs grown on laminin compared to PLL.

the expression of the 38 kDa 4R isoform decreased, while higher molecular weight isoforms appeared. The 49 kDa 4R isoform was clearly enhanced in HCNs growing on laminin. The band intensities were measured and the ratio between PLL and laminin samples for each isoform was calculated. At day 6, the ratios were 1.2 and 1, from low to high molecular weight bands respectively, while at 15 days, the ratios were 0.5 and 5, respectively. Four visible bands were present at 25 days, with ratios of 1.14, 0.75, 0.66, and 0.25 from 38 to 49 kDa. Thus, the pattern of expression of 4R isoforms differs in HCNs developed in PLL and laminin.

Discussion

To establish the role of the environment in the expression of tau isoforms, we analyzed neurite growth and tau expres-

sion in human cortical neurons grown on poly-L-lysine, a synthetic substrate for neuronal attachment, or laminin, one of the most abundant basement membrane proteins in the mammalian brain. In rodent neurons, laminin activates cell adhesion to the substrate and stimulates axonal growth, possibly by accelerating the expression of specific tau isoforms (DiTella et al., 1996). During morphological differentiation, rodent neurons undergo a series of characteristic changes (Dotti and Banker, 1988). We found a similar sequence of morphological differentiation in human cortical neurons: 1) formation of lamellipodia, 2) outgrowth of minor processes, 3) development of axon and major processes, and 4) elongation into dendrites. One of the characteristic features of such differentiation, particularly of the later stages, is the compartmentalization of tau in axonal processes and its disappearance from dendrites and neuronal bodies.

The development of useful experimental models for the study of neurodegenerative diseases is a topic of high priority in the health sciences. We initiated experiments to characterize the expression of tau proteins in cultured HCNs and to study how this expression is modulated by environmental cues in the human brain. Our results indicate that all six tau isoforms found in the adult human brain are also present in HCN cultures. Moreover, tau expression appears to be tightly regulated during development, acquiring an axonal-specific distribution of tau and differentiation of neuronal processes into axons and dendrites. In fact, tau expression increased as neurons underwent morphological differentiation in both PLL and laminin substrates.

The results also show that laminin, one of the most common extracellular matrix proteins in the mammalian brain, significantly enhances neurite elongation during the stages of active neurite growth in cultured HCNs. This enhancement in neurite growth correlates with the increased complexity in the expression of tau isoforms. In particular, laminin appears to enhance the expression of specific 4R isoforms at different stages of neuronal development, suggesting that the expression of 4R tau is associated with an increased rate of neurite outgrowth. In this regard, increased laminin expression of 4R tau, which exhibits increased microtubule binding affinity, is consistent with increased stabilization of microtubular structures required for faster axonal growth, as observed on the laminin substrate (Goode et al., 2000).

These results demonstrate that cultured HCNs represent a valuable experimental model, which supports molecular aspects of cortical neurons in the human brain. As such,

HCNs may prove to be a useful tool for the study of molecular mechanisms relevant to neurodegenerative diseases.

Acknowledgments

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