Characterization of Tau Isoforms Expressed in Cultured Human Cortical Neurons on Poly-L-lysine and Laminin Substrates

Introduction

Alzheimer's disease is characterized by two main lesions, extra-cellular beta-amyloid plaques and intracellular neurofibrillary tangles (NFTs). NFTs are composed of hyperphosphorylated tau proteins dimerized into paired helical filaments (PHFs). Tau is a neuron-specific microtubule-associated protein, which lost its microtubule-binding ability upon PHF and NFT formations. Since beta-amyloid proteins are observed prior to neurofibrillary tangles in Alzheimer's brains (Spillantini and Goedert, review 1998), it is postulated that the former induces the formation of NFTs via hyperphosphorylation (Busciglio 1995). Hyperphosphorylated tau is also found in aberrant neuronal processes in the Alzheimer's brain (neuronal dystrophy, Grace and Busciglio 2003), which may lead to neuronal death and memory loss. Oxidation (Schweers et al. 1995, Horiguchi et al. 2003), glycation (Nacharaju et al.1997) and genetic mutations (Pigino and Busciglio 2003) are also involved in the development of tau pathology.

There are six isoforms of tau in the human adult brain. They are produced by alternative splicing of RNA molecules during neuronal development (Friedhoff et al. 2000). Isoforms are categorized as three-repeat (3R) or four-repeat (4R). As the terms imply, 3R has three tandem repeats near the carboxy-end whereas 4R has four repeats. 3R and 4R are in turn classified according to the number (0, 1 or 2) of 31 amino acid inserts near the amino end (Fig. 1). Therefore, the six different tau isoforms in the human adult brains vary according to the number of repeats and/or inserts.

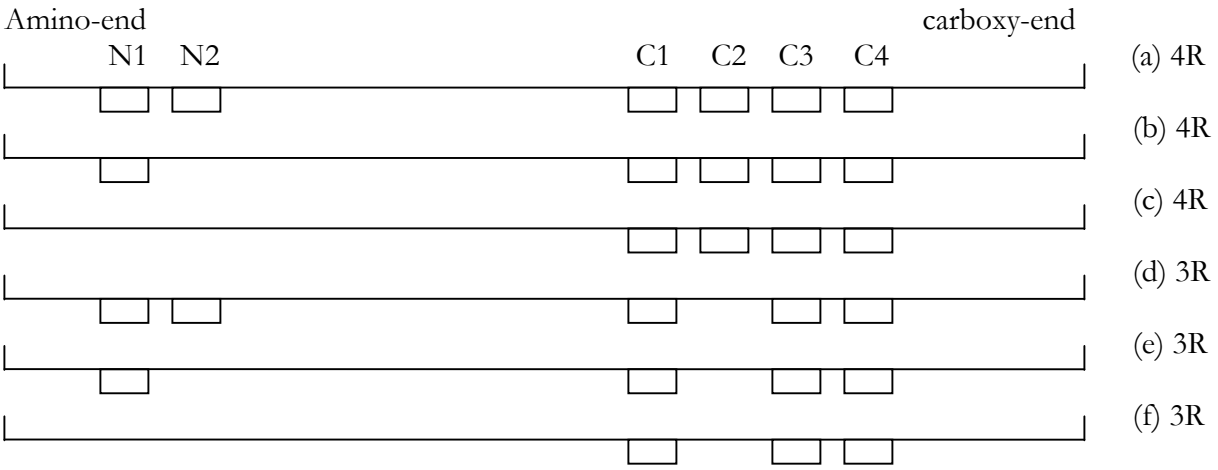

Fig.1: *The six human tau isoforms. N1-N2 represents near amino-end inserts. C1-C4 represents near carboxy-end repeats. (a-c) Four-repeat tau isoforms with 0, 1 or 2 amino-end inserts respectively. (d-f) Three-repeat tau isoforms with 0, 1 or 2 amino-end inserts respectively.*

Tau is a protein molecule with multiple functions in neurons. Its functions include microtubule binding and stabilization (Drubin and Kirschner, 1997), and mediating the interaction of microtubules with the cytoskeleton (Cunningham et al., 1997). Some or all of those functions are either disrupted or completely impaired in the neurons of Alzheimer's disease and other neurodegenerative diseases, such as Down Syndrome, prion diseases with tangles, and Niemann-pick disease type C. (Spillantini and Goedert, 1998). For instance, 4R tau isoforms appear to have higher microtubule-binding abilities than 3R isoforms (Goode et al., 2000). Therefore, 4R and 3R tau isoforms may have different roles in promoting PHF formation under pathological condition. As a result, the proportion of tau isoforms changes, causing alterations in neuronal functioning.

In this regard, the balance of the six tau isoforms is likely to be essential for proper microtubule-binding in neurons. Most research on tau pathology has been performed on animal models with a single isoform of human recombinant tau, or using neuroblastoma cell lines, which may not reflect accurately the expression of tau isoforms in mature human brains. Therefore, the goal of this project is to characterize the expression of tau isoforms in human cortical neurons developed in culture. The long-range goal is to establish an accurate experimental model for future studies of molecular mechanisms involving tau proteins in neurodegenerative diseases.

The summer project will establish the expression of tau proteins in human neurons developed on both laminin- and polylysine-coated plates. Laminin is an extracellular matrix protein found in basement membranes of human brains. Its function is to activate focal adhesion proteins for neuronal development. Laminin may accelerate the expression of tau isoforms, and/or stimulate the development of longer neuronal processes than polylysine. Polylysine, on the other hand, is a poly-cationic compound that is used to cover the surface to which neurons attach. The binding of neurons to polylysine is based on the negative charges of cell membranes. Therefore, the binding of neurons on laminin is more specific physiologically than it does on polylysine.

Specific Aims

1) To determine the expression of tau isoforms in human cortical neurons developed in culture growing on laminin- and polylysine-coated surfaces. (The presence of all six isoforms renders an accurate model to study tau pathology.)

2) To determine the level of microtubule-bound tau during neuronal development in polylysine and laminin substrates. (Tau proteins are in equilibrium between microtubule-binding state and soluble state; therefore, increased levels of microtubule-bound tau indicate increased stabilization and maturation of neuronal processes.)

3) To establish the sub-cellular localization of tau in neurons growing on laminin and polylysine substrates. (Tau also becomes increasingly compartmentalized upon maturation of neurons, thereby found preferentially in axons and very small amounts in neuronal bodies and dendrites.)

Materials and Methods

These procedures are routinely utilized in our laboratory.
Appropriate sterile techniques are applied to all procedures, and universal precautions and lab safety protocols are followed.

Coating Petri dishes with poly-L-lysine or laminin. Poly-L-lysine is prepared by diluting a 20x stock solution in borate buffer. Laminin solution is used at the final concentration of 5ug/ml to cover tissue culture dishes. The dishes are left to be coated overnight before they are washed with sterile distilled water.

Cell culture. Human cortical tissue is dissociated into small pieces with needles and treated with 0.25% trypsin and approximately 40 μg/ml of DNase. PR 10% media (D-MEM nutrients + 10% serum + antibiotics including penicillin, streptomycin, and fungizone) is then added. It is washed before centrifugation, and the pellet is resuspended in PR 10%. Neurons are further dissociated into individual cells using small-diameter polished Pasteur pipettes, then rewashed, re-centrifuged, and the pellet re-suspended. The number of cells per millilitre is counted on a haemocytometer under a microscope. The cells are plated at the following densities: 4 E5 cells per 60 mm Petri dish, and 1-1.5 E5 cells per 35 mm dish. The cultures are incubated at 37 °C humid chamber for 60 min before switching to a serum-free medium containing appropriate nutrients (neurobasal + N2 + B27 + antibiotics including penicillin, streptomycin, and fungizone).

Immunocytochemistry and Microscopic Imaging.

Cell Fixation: Cells are fixed with 4% paraformaldehyde in PBS (phosphate buffer saline) for 30 min, and then washed three times with PBS for 15 min each. They are fixed at various timepoints after plating, 3, 5, 8, 12, 15, 25 and 30 days, in culture for immunocytochemistry and Western Blotting.

Immunostaining: 20 mm-diameter glass coverslips are used to plate the cells for immunocytochemical analysis. The fixed coverslips are treated with 0.2% PBX-Triton X 100 (a biochemical detergent that increases membrane permeability of the cells) for 5 min. The detergent is then washed off three times with PBS for 5 min each, and the cells are blocked with 5%BSA (bovine serum albumin) for 30 min. The primary antibodies are applied before incubating the fixed coverslips overnight at 4 °C. Another three washes each with PBS is carried out, before and after the application of secondary antibodies. The coverslips are mounted on a glass microscopic slide with immu-mount solution, and let dry. The slides are now ready to be observed under a microscope.

Western Blot Analysis. Neuronal cultures are washed with PBS. Scraped cell samples from cultured dishes are suspended in SDS- (Sodium dodecyl sulphate) reducing buffer, and heated for 5 min at boiling temperature. The sample is centrifuged at 100,000 × g for 30 min to obtain supernatants. The amount of protein in the culture is determined by the Bio-Rad protein assay. Protein is applied to SDS-polyacrylamide gels for electrophoresis, and blotted onto nitrocellulose. The protein sample on nitrocellulose is then blocked with 5% nonfat dry milk. The primary antibody and then secondary antibody containing peroxidase (1:1000 dilutions from Amersham) are used to incubate the blot for 12 hr at 4 °C, and for 1 hr at 25 °C respectively. Enhanced chemiluminescence (from Amersham) is utilized to capture banding pattern. (Busciglio et al., 1995)

Applications of techniques for individual analyses:

1. *Cytoskeletal preparation.* The neuronal cytoskeleton for measuring the amount of microtubule-bound tau is prepared as follows. Living neuronal culture is treated with a warm microtubule-stabilizing buffer (HEPES, $MgCl_2$, and EGTA). The 0.2% Triton X-100 is then added for 2 min incubation at 37 °C. (Busciglio et al., 1995)
    a) *For immunochytochemistry*. The culture is fixed and immunostaining is prepared with the neuronal cytoskeleton on the fixed coverslips. (Busciglio et al., 1995)
    b) *For Western blot analysis*. The microtubule and its binding tau are treated with SDS-reducing buffer followed by boiling. The Triton-resistant fraction is centrifuged at 100,000 × g for 30 min to obtain supernatant for Western blotting. (Busciglio et al., 1995)

2. *Detection of human tau isoforms, 3R and 4R tau.*
    a) *Antibodies to be used with immunocytochemistry.* CP27 (IgG2B) recognizes all human tau isoforms, ET2 (IgG1) and ET3 (IgG2B) specifically recognize 4R tau isoforms.
    b) Western Blotting techniques will be utilized to separate all six tau isoforms according to their molecular masses, and the same antibodies as above, CP27, ET2 and ET3, will be utilized for recognition.

3. *Sub-cellular localization of tau.* Immunocytochemistry will be performed with the following primary antibodies: the polyclonal antibody against tau from Dako (1:1000 dilutions with 1%BSA), and the monoclonal antibody against tubulin (1:500 dilutions with 1%BSA) from Sigma. Secondary antibodies conjugate with green and red fluorophores will be used for double immuno-fluorescence. The preparation will be observed under a fluorescent microscope using appropriate filters.

4. *Image analysis.* Digital images of microscopic fields will be captured, and image analysis software (Axiovision) will be used to measure cell morphology, sub-cellular localization of tau, and fluorescent intensity.

Expected Results

1) All six human tau isoforms are expressed in mature cultures
2) Neurons grown on laminin develop longer processes at an accelerated speed, and express mature tau isoforms earlier than neurons growing on polylysine.
3) Tau proteins become compartmentalized with maturity in culture.
4) Tau proteins attach to microtubules with greater affinity as neurons mature in culture.

Current Results

We have initiated this project with two neuronal cultures. Cortical neurons have been fixed at 3, 5 and 8 days for immuno-fluorescent analysis. The preliminary results indicate that tau proteins are compartmentalized between 5 to 8 days after plating (Fig.2), and they express a complex pattern of tau isoform that will be further analyzed. The number of neuronal processes as well as their lengths increases drastically with each time-point as well.

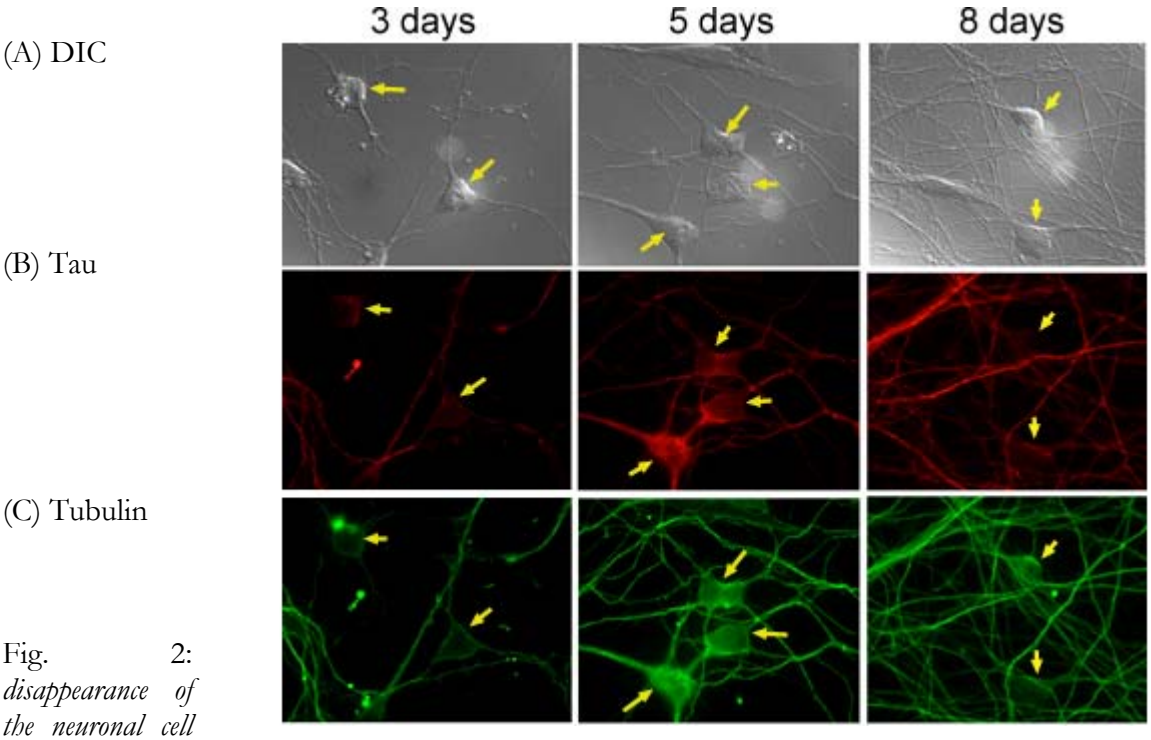

Fig. 2: *Increasing disappearance of tau proteins in the neuronal cell bodies 3, 5 and 8 days after plating. (A) Neurons under the white light. (B) Tau proteins under red fluorescent light. (C) Tubulin under green fluorescent light. Arrows are pointed at where neuronal cell bodies lie. Tau proteins are disappearing in cell bodies of 8-day culture.*

Project Timeline

Analysis of tau isoform expression, sub-cellular localization and binding to microtubules will be performed during the three month period of the summer. The last three to four weeks will also be utilized for image analysis and organization of scientific data. The experiments will be repeated as necessary to minimize random errors and to achieve statistical significance.

Student's Level of Participation

I have been involved in the preparation and coating surfaces (Petri dishes and coverslips) with polylysine and laminin, and in the preparation of tissue culture materials. I have also observed and familiarized myself with neuronal culture procedure. I will be attending the training course on handling of bloodborne pathogens before performing the neuronal culture procedure. I have further been trained to perform immunofluorescent technique and microscopic imaging. I am currently learning the Western Blotting technique. Therefore, I will be fully prepared to perform the experiments required for the project under the direct supervision of my faculty mentor.

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