



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file / Votre référence

Our file / Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

**TAU PROTEIN EXPRESSION AND THE DEVELOPMENT OF STABLE
MICROTUBULES DURING NEURONAL DIFFERENTIATION OF D310 CELLS**

by

LIJUAN SUN

**Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfilment of the requirements for the
M.Sc. degree in the**

Ottawa-Carleton Institute of Biology

© Copyright by Lijuan Sun 1993



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file *Vostra référence*

Our file *Notre référence*

THE AUTHOR HAS GRANTED AN
IRREVOCABLE NON-EXCLUSIVE
LICENCE ALLOWING THE NATIONAL
LIBRARY OF CANADA TO
REPRODUCE, LOAN, DISTRIBUTE OR
SELL COPIES OF HIS/HER THESIS BY
ANY MEANS AND IN ANY FORM OR
FORMAT, MAKING THIS THESIS
AVAILABLE TO INTERESTED
PERSONS.

L'AUTEUR A ACCORDE UNE LICENCE
IRREVOCABLE ET NON EXCLUSIVE
PERMETTANT A LA BIBLIOTHEQUE
NATIONALE DU CANADA DE
REPRODUIRE, PRETER, DISTRIBUER
OU VENDRE DES COPIES DE SA
THESE DE QUELQUE MANIERE ET
SOUS QUELQUE FORME QUE CE SOIT
POUR METTRE DES EXEMPLAIRES DE
CETTE THESE A LA DISPOSITION DES
PERSONNE INTERESSEES.

THE AUTHOR RETAINS OWNERSHIP
OF THE COPYRIGHT IN HIS/HER
THESIS. NEITHER THE THESIS NOR
SUBSTANTIAL EXTRACTS FROM IT
MAY BE PRINTED OR OTHERWISE
REPRODUCED WITHOUT HIS/HER
PERMISSION.

L'AUTEUR CONSERVE LA PROPRIETE
DU DROIT D'AUTEUR QUI PROTEGE
SA THESE. NI LA THESE NI DES
EXTRAITS SUBSTANTIELS DE CELLE-
CI NE DOIVENT ETRE IMPRIMES OU
AUTREMENT REPRODUITS SANS SON
AUTORISATION.

ISBN 0-315-95984-3

Canada



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ACKNOWLEDGEMENTS

My first, deepest gratitude goes to Professor David L. Brown, my academic advisor, for his constant and worthwhile guidance, for his encouragement, patience and support to my research and my thesis, without which this study would have been impossible.

I am indebted to all my supervisory committee members, Professors N. Chaly, M. McBurney, and M. Paulin-Levasseur, for their help in overseeing my degree program and the research.

I am equally indebted to Mrs. Bea Valentine for her constant technical guidance and for her valuable comments and suggestions during the writing of the thesis. My appreciation is also extended to Ms. Judy Little for her lab assistance needed in the experiments.

I share a lot of wonderful memories with Andrew Vaillant, Nicole Laferriere and Srabani Banerjee with whom I shared an office during this study and with whom I could exchange intellectual comments and ideas as well as hearty feeling on most subjects. Their comments, and suggestions on my research and thesis writing were extremely valuable.

My special thanks should go to my husband, Xiaoping Yuan, and my son, David, who gave their moral support, understanding, and encouragement needed for this tedious and

emotional experience. Thanks to my friend, Dr. Zhendong Liu and his family, for their enthusiastic help, to each of you for making the program and this study possible.

TABLE OF CONTENTS

	Page
TITLE PAGE	i
ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iv
LIST OF FIGURES	vi
ABBREVIATIONS	viii
ABSTRACT	x
RÉSUMÉ	xii
INTRODUCTION	1
1.1 Microtubules	3
1.2 Posttranslational Modifications of Tubulin	5
1.3 Neuronal Microtubules	10
1.4 Microtubule-Associated Proteins (MAPS)	14
1.5 Embryonal Carcinoma P19 Cells	21
PURPOSE OF THE THESIS	24
MATERIALS AND METHODS	26
2.1 Cell Cultures	26
2.2 Immunofluorescence Techniques	27
2.3 Electrophoresis and Immunoblotting	29
2.4 Enzyme Linked-Immunsorbent Assay (ELISA)	32
2.5 Immunogold Labelling for Light and Electron Microscopy	34
RESULTS	37
3.1 RA-induced Neuronal Differentiation	37
3.2 Immunofluorescence Observations of Tau Expression	41
3.3 Enzyme-linked Immunosorbent Assay	48
3.4 Tau Expression and Microtubule Stability	49
3.5 Localization of Tau Protein on Microtubules at EM Level	60
DISCUSSION	67
4.1 RA-induced neurally differentiated D310 EC cells	67

4.2	Developmental Regulation of Tau Expression	68
4.3	Tau Expression and Neurite Outgrowth	73
4.4	Tau Expression and Microtubule Stability	74
4.5	Association of Tau and Stable Microtubules	78

REFERENCES	80
-------------------	----

LIST OF FIGURES

	Page
Figure 1 The morphology of undifferentiated D310 EC cells.	38
Figure 2 Double immunofluorescence of tubulin and class III β -tubulin in 2 day RA-induced D310 cells.	39
Figure 3 Neuronal markers expressed in 6 day RA cells.	40
Figure 4 Double immunofluorescence staining of tubulin and tau in undifferentiated EC cells and 3 day RA cells.	43
Figure 5 Double immunofluorescence staining of tubulin and tau in 6 day and 12 day RA cells.	44
Figure 6 Double immunofluorescence staining of β III-tubulin and tau in undifferentiated EC cells, and in 3 day and 6 day RA cells.	46
Figure 7 Co-localization of β III-tubulin and tau in day 6 and day 12 RA cells.	47
Figure 8 Change in total cellular tubulin during neuronal differentiation.	50
Figure 9 Change in the relative amount of β III-tubulin during neuronal differentiation.	51
Figure 10 Change in the relative amount of tau expression during neuronal differentiation.	52
Figure 11 Double immunofluorescence staining of tubulin and tau in colchicine-treated day 0 and day 3 RA cultures.	54
Figure 12 Double immunofluorescence staining of tubulin and tau in colchicine-treated day 6 and day 12 RA cells.	55
Figure 13 Immunoblotting results.	58
Figure 14 Immunogold bright field light microscopy.	61
Figure 15 Immunogold dark field light microscopy.	62
Figure 16 Electron micrographs of 6 day RA cells.	64

- Figure 17 Ultrastructure of the cell body of a 6 day RA cell. 65
- Figure 18 Immunogold electron microscopy of day 6 RA cells. 66

ABBREVIATIONS

BSA	:	Bovine Serum Albumin
DMSO	:	Dimethylsulfoxide
DTAF	:	DichloroTriazinyl Amino Fluorescein
EC	:	Embryonal Carcinoma
EDTA	:	Ethylenediaminetetraacetic acid
EGTA	:	Ethylene glycol-bis (β -aminoethyl ether) N, N, N', N'-tetraacetic acid
ELISA	:	Enzyme-Linked Immunosorbent Assay
FCS	:	Fetal Calf Serum
GLU	:	Glutamic acid (refers to terminal amino acid of modified α -tubulin)
HEPES	:	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HRP	:	Streptavidin biotinylated Horseradish Peroxidase
MAPS	:	Microtubule-associated proteins
MEM	:	Minimum Essential Medium
MSB	:	Microtubule Stabilizing Buffer
NORS	:	Nucleolar organizer regions
OPD	:	O-Phenylenediamine
PBS	:	Phosphate Buffered Saline
PC	:	Phosphocellulose Chromatography
PEM	:	Pipes, EGTA, MgCl ₂
PHEM	:	Pipes, Hepes, EGTA, MgCl ₂

PIPES : Piperazine -N, N'-bis (2-ethanesulfonic acid)
PMSF : Phenylmethylsulfonylfluoride
PNS : Peripheral nervous system
RA : Retinoic Acid
TYR : Tyrosine (refers to terminal amino acid of
unmodified α -tubulin)

ABSTRACT

The D310 EC cell line is multipotent and differentiation can be directed along the neuronal pathway by addition of retinoic acid (RA). The correlation between the expression of tau protein and the development of stable microtubule arrays during neuronal differentiation was studied by indirect immunofluorescence, ELISA immunoblotting and Immunogold labelling techniques. By immunofluorescence staining tau protein is not detected in undifferentiated EC cells, but it starts to be expressed at day 3 of RA induction and shows a beaded morphological appearance. The expression of tau increases steadily during neuronal differentiation. Double immunofluorescence labelling demonstrates that the expression of tau protein correlates well with the development of colchicine-resistant microtubules.

The levels of total cellular tubulin, total class III β -tubulin and total tau protein were measured by ELISA. The amount of class III β -tubulin and tau protein were expressed as a ratio of the relative amount of class III β -tubulin or tau protein/mg total tubulin. ELISA results support the observations of immunofluorescence staining. The relative amount of tau protein expressed after RA induction increases steadily and parallels the increased expression of class III

β -tubulin, an indicator of neuronal differentiation.

Immunoblotting shows that both juvenile and mature isoforms of tau protein are present in RA-induced EC cells. Two isoforms are detected in day 3 RA-induced cells and four isoforms are detected in day 6 RA cells. All four isoforms associate with colchicine-stable microtubules.

A beaded morphological distribution of tau along neurite processes seen by immunofluorescence staining suggests that tau protein associates with some, but not all microtubules in neurite processes. The prediction that tau associates with colchicine-resistant microtubules was tested by immunogold labelling at the light and electron microscopic levels.

RÉSUMÉ

La cellules des D310 EC sont multiplicitées et leur différenciation peut être menée le long du chemin neuronal par l'addition d'acide rétinoïque (AR). La corrélation entre la production de la protéine tau et le développement de microtubules stables durant la différenciation neuronale a été étudiée par immunofluorescence indirecte, par les techniques immuno-enzymatiques (ELISA) et par la localisation d'or immunocytochimique. Par la coloration d'immunofluorescence, la protéine tau ne peut pas être détectée dans des cellules EC nondifférenciées, mais est exprimée à partir du troisième jour d'induction à l'AR et a un aspect en chapelet. L'expression de la protéine tau augmente de façon régulière durant la différenciation neuronale. Le marquage par double immunofluorescence démontre que l'expression de la protéine tau est bien corrélée avec le développement de microtubules résistants à la colchicine.

Les niveaux totaux de tubuline cellulaire, de β -tubuline de classe III et de la protéine tau ont été mesurés par les tests ELISA. Les quantités de β -tubuline de classe III et de la protéine tau sont exprimées sous la forme du rapport entre la quantité relative de β -tubuline de classe III ou de protéine tau et la quantité totale de tubuline. Les résultats des tests ELISA appuient les résultats de

coloration d'immunofluorescence. La quantité relative de protéine tau exprimée après l'induction à l'AR augmente régulièrement et parallèlement à l'augmentation de l'expression de la β -tubuline de classe III, un indicateur de différenciation neuronale.

On a démontré, par immuno-transfert, que les isoformes jeunes et vieilles de la protéine tau sont toutes deux présentes dans les cellules EC induites par l'AR. Deux isoformes sont détectées au sixième jour de l'induction à l'AR, et quatre isoformes sont détectées au troisième jour de l'induction à l'AR. Ces quatre isoformes sont associées aux microtubules stables à la colchicine.

Une distribution de tau en forme de chapelet lors du processus neuronal, révélée par la coloration d'immunofluorescence, suggère que la protéine tau s'associe à quelques microtubules, mais pas tous, lors de ce processus. L'hypothèse que tau s'associe aux microtubules résistants à la colchicine a été vérifiée par marquage à l'or immunocytochimique en microscopie photonique et électronique.

INTRODUCTION

The cytoskeleton is an extensive array of fibrous elements, which defines the shape of cells and organizes their cytoplasm to carry out a variety of metabolic and motile processes which are important to life (Bershadsky and Vasiliev, 1988). The cytoskeleton is composed of three major classes of filaments: microtubules, intermediate filaments and microfilaments.

Microtubules are a major component of the cytoskeleton and are present in all eukaryotic cells. They are hollow rod-like structures with a diameter of approximately 24 nm. Microtubules are polarized structures which have a "+" end radiating toward the cell membrane and the "-" end facing toward the centrosome. Microtubules elongate by adding tubulin subunits to the "+" ends. *In vitro*, microtubules can be depolymerized and repolymerized depending upon the environmental conditions. In the presence of GTP and magnesium and the absence of calcium, tubulin dimers are added to the "+" (growing) end (reviewed by Gelfand and Bershadsky, 1991). Real time observation of microtubule dynamics in tissue culture demonstrates that there are two populations of microtubules in cells: a dynamic population in which individual microtubules elongate then shrink rapidly and a stable population which shows a

slower turn over rate of tubulin subunits (Schulze and Kirschner, 1987; Sammak and Borisy, 1988; Cassimeris et al., 1988). Most interphase microtubules are very dynamic and turn over quickly, whereas most microtubules in neurons are more stable and turn over more slowly. What makes microtubules, which are indistinguishable in their ultrastructure, behave so differently? Are there regulatory mechanisms controlling microtubule organization and stability? These questions initiated the study of the biochemical characterization of microtubules, their posttranslational modifications, and their interactions with microtubule-associated proteins (MAPs).

Neurons are highly specialized cells which generally have a small cell body, several branched dendrites, and a single long axon. Axons and dendrites contain a very high concentration of microtubules as well as MAPs. Microtubules in neurons are more stable than those in other cell types and the stability of microtubules to depolymerization varies according to the location of the microtubules within the neuronal cells (Lim et al., 1989). These qualities make the neuronal cells a good model system for investigating the temporal and spatial relationships between microtubule stability and MAP expression.

1.1 Microtubules

Composition and Structure

Microtubules are composed principally of 100 kD heterodimers of the protein tubulin. The tubulin heterodimer is formed by the noncovalent, hydrophobic interaction of α - and β -tubulin subunits. Both α - and β -tubulins are highly flexible, globular proteins, each with a predicted 25% α -helical and 40% β -pleated sheet content (Audenaert et al., 1989). Microtubules are formed when tubulin heterodimers self-assemble by a head-to-tail interaction to form 4-5 nm diameter linear protofilaments which then interact laterally with other protofilaments to form the microtubule wall. Microtubules typically contain 13 protofilaments.

Heterogeneity

Most tissues contain several α - and β -tubulin proteins. Vertebrates have 6 expressed α -tubulin genes which encode 6 different tubulin isotypes (Lewis and Cowan, 1988). There are also 6 vertebrate β -tubulin isotypes which are derived from 6 functional genes (Wang et al., 1986;

Lewis and Cowan, 1988; Monteiro and Cleveland, 1988). Tubulin heterogeneity is the result of multigene families of tubulin in most organisms and post-translational modifications of both α - and β -tubulins (Banerjee and Luduena, 1987). For example, in mammalian brain there are only five β -tubulin genes expressed, but as many as 14 electrophoretic variants of β -tubulin have been detected in bovine brain tubulin (Field et al., 1984).

The functional significance of tubulin isotypes and isoforms is not known. It has been proposed that tubulin isotypes are functionally differentiated such that each is specialized for different microtubule arrays (Fulton and Simpson, 1976; Murphy, 1988). Different isotypes often occur in different tissues (reviewed by Luduena, 1993). For example, in mammals and birds β I is found in most tissues, β II in most tissues but more abundant in the brain, β III localized in neurons and the Sertoli cells of the testis (Lewis and Cowan, 1988). There are also examples where different isotypes are segregated (spatial distribution) in the same cell. Some segregation of β II and β III was noted in pheochromocytoma cells (Asai and Remolona, 1989). Falconer and colleagues (1992) also demonstrated that during the early days of RA-induced neuronal differentiation of P19 EC cells there was a preferential incorporation of β II and β III into colchicine-stable and colchicine-labile microtubules respectively. Both β II and β IV are expressed

in bovine retinal rod cells and tracheal cilia, but β IV is present preferentially in the axonemal microtubules, while β III is associated largely with non-axonemal microtubules in both cell types (Renthal et al., 1993).

1.2 Posttranslational Modifications of Tubulin

There are four known types of posttranslational modifications of tubulin. Three affect α -tubulin and two β -tubulin. With the exception of Lys⁴⁰ acetylation, these modifications occur on residues within the carboxyl-terminal domain.

Acetylation

Acetylated α -tubulin, first discovered by L'Hernault and Rosenbaum (1985), is one form of posttranslational modification that may be important in determining microtubule organization and function. This α -tubulin has Lys⁴⁰ whose ϵ -amino group is acetylated in many organisms (L'Hernault and Rosenbaum, 1985; LeDizet and Piperno, 1987). The acetylation of α -tubulin occurs in different populations of microtubules and in a variety of eukaryotic cells. Acetylated α -tubulin is found in more stable microtubules.

It occurs in the flagellar axoneme (Maruta et al., 1986), in basal bodies (LeDizet and Piperno, 1987), among the interphase microtubules that are more stable in cultured mammalian cells (Bulinski et al., 1988), and in stable microtubule arrays of neurons (Black and Keyser, 1987; Black et al., 1989). Cytoplasmic microtubules containing acetylated α -tubulin in *Chlamydomonas* were found to be more resistant than other cellular microtubules to the effects of antimitotic drugs (LeDizet and Piperno, 1987). In addition, acetylation of α -tubulin has been observed in nerve growth factor (NGF)-treated PC12 cells (Black and Keyser, 1987) and in neuroblastoma cells (Edde et al., 1987). In mature neurons, acetylated microtubules are localized to the axon and thick dendritic trunks (Ferreira and Caceres, 1989). This observation was also supported by studies in rat cerebellum (Martin et al., 1987), and NB2a/d1 neuroblastoma cells (Shea et al., 1990).

Acetylated α -tubulin is polymer-specific since quantitative analyses revealed that about 99% of the acetylated tubulin partitioned with the cytoskeleton (Black et al., 1989). However, the acetylation of α -tubulin does not affect the *in vitro* assembly-disassembly kinetics of microtubules (Maruta et al., 1986). Therefore, there must be some other mechanism responsible for the enhanced stability of microtubules containing acetylated tubulin.

Tyrosination/detyrosination

Tyrosination/detyrosination also modifies α -tubulin. A tyrosine residue can be reversibly removed and added to the carboxyl-terminus of α -tubulin. After tyrosine is removed by a unique carboxypeptidase, the next residue exposed as the terminal amino acid in vertebrates is glutamic acid (Raybin and Flavin, 1977; Argarana *et al.*, 1980; Kumar and Flavin, 1981).

Tyrosinated (Tyr) tubulin is present in very dynamic microtubule populations. The microtubules containing predominantly Tyr tubulin turn over with a half-life measured in minutes, whereas the half-life of microtubules enriched in detyrosinated (Glu) tubulin is on the order of several hours (Kreis, 1987; Webster *et al.*, 1987). In some cultured cells, microtubules enriched in Glu tubulin are also enriched in acetylated α -tubulin (Bulinski *et al.*, 1988). Glu α -tubulin is more concentrated in neurites (Cambray-Deakin and Burgoyne, 1987), whereas tyrosinated α -tubulin is localized within more motile cellular areas such as growth cones (Gordon-Weeks and Lang, 1988). Stable microtubule arrays contain detyrosinated as well as acetylated α -tubulin, and acetylation and detyrosination appear to be associated with the stabilization of microtubule arrays during cell differentiation (Shea *et al.*, 1990). But neither modification has been shown to confer

microtubule stability. Both detyrosination and acetylation modifications occur in early neuronal differentiation of cultured EC cells (Falconer et al. 1989a) and are also present in processes of mature neurons (Baas and Black, 1990).

Glutamylation

Glutamylation is a posttranslational modification consisting of the successive addition of glutamyl units on the γ carboxyl group of a glutamate residue (Glu⁴⁴⁵ on α -tubulin). Glutamylation occurs on both α - and β -tubulin. During neuronal development, from one to six glutamate residues may be added in the mouse M α 1 and M α 2-tubulin isotypes (Edde et al., 1990). Class III β -tubulin isotype is modified by the addition of 1-6 Glu residues to Glu⁴³⁸ (Alexander et al., 1991). Approximately 85% of the class III β -tubulin isotype in the mature bovine brain is glutamylated. Interestingly, the class III β -tubulin isotype is expressed in both the brain and the testis in mammals, but glutamylation only occurs in the brain (Alexander et al., 1991). Class II β -tubulin, the major β -tubulin isotype of adult brain, is also glutamylated on glutamic acid residue 435 (Rudiger et al., 1992). It has been suggested that glutamylation plays a role in regulating

microtubule dynamics, because of the location of the modification within a region that is important in the interactions of tubulin with MAPs (Edde *et al.*, 1990).

Phosphorylation

Class III β -tubulin has been shown to be phosphorylated on both Ser⁴⁴⁴ and Tyr⁴³⁷ residues (Gard and Kirschner, 1985; Luduena *et al.*, 1988; Edde *et al.*, 1989). Of the 85% of glutamylated class III β -tubulin present in brain, 10% is also phosphorylated (Alexander *et al.*, 1991). Phosphorylation of the Ser⁴⁴⁴ residue is found only on neuronal class III β -tubulin (Lee *et al.*, 1990b; Alexander *et al.*, 1991). Glutamylation and phosphorylation occur progressively during neuronal development. The function of these posttranslational modifications is not clear. It is possible that these modifications to specific isotypes in developing brain favour the interactions between the microtubules and specific MAPs.

1.3 Neuronal Microtubules

Neurons have long cytoplasmic extensions, termed axons and dendrites, which contain large numbers of microtubules. The neuronal microtubules show differences in both composition and stability from the microtubules in non-neuronal cells.

The properties and composition of microtubules in neurons

Microtubules are one of the main cytoskeletal elements in neurons. In axons all microtubules are oriented in the same direction ("- end toward the cell body and "+" end away from the cell body), while in dendrites microtubules have an antiparallel orientation. In axons the microtubules form tight parallel arrays with cross bridges cross-linking the microtubules (Hirokawa, 1982). In contrast, microtubules in dendrites are quite loosely packed and have widely spaced parallel arrays (Black, 1987). Mature neurons are enriched in stable populations of microtubules, although the degree of microtubule stability varies according to the location of the microtubules within the cell (Lim et al., 1989). Microtubules in the neurite processes are more stable to depolymerizing agents than the microtubules in the cell body and at the growth cone (Cadrin

et al., 1988). Microtubule stability also increases during neuronal differentiation induced by RA in cultured P19 EC cells (Falconer *et al.*, 1992).

Several tubulin isotypes and isoforms have been found in brain. Isoelectric focusing shows that all the brain tubulins have a much larger degree of heterogeneity than that predictable from the number of genes coding for the α and β isotypes (Denoulet *et al.*, 1982; Wolff *et al.*, 1982; Field *et al.*, 1984). In addition, during brain development there is an increase in the number as well as a change in the distribution of the various tubulin isoforms (Gozes and Littauer, 1978; Dahl and Weibel, 1979). Neuronal microtubules also differ in the type of posttranslationally modified tubulin that they incorporate. Dendritic microtubules contain tyrosinated α -tubulin, while axonal microtubules contain little or no tyrosinated α -tubulin and are instead rich in posttranslationally detyrosinated and acetylated α -tubulin. Work by Ferreira and Caceres (1989) has shown that in cerebellar macroneurons maintained in culture, the acetylation of α -tubulin is developmentally regulated and that its levels and distribution vary with axonal and dendritic growth. A subset of stable, colchicine-resistant, microtubules that are acetylated is exclusively localized in the axons during early neuronal development. On the other hand, in mature neurons acetylated microtubules display a more widespread

distribution being localized in the axons and thick dendritic trunks. Acetylation also increases during neuronal differentiation in cultured EC cells (Falconer et al., 1989a).

The stability of microtubules in neurons

Development of the nerve cells requires that the neuronal cytoskeleton be endowed with the seemingly incompatible properties of stability and plasticity. Stability is critical for maintenance and proper function of neuronal circuits, while plasticity is required for cell growth and remodelling of cell processes during response to environmental inputs or injury (Diaz-Nido et al., 1990).

Microtubule formation is required for the growth and structural integrity of neuronal processes (Daniels, 1972). Neuronal microtubules exhibit unusual stability properties. Several studies have shown that *in vitro* a significant fraction of tubulin in brain is not solubilized by conventional methods of temperature-reversible, assembly-disassembly procedures (Webb and Wilson, 1980; Black et al., 1984; Brady et al., 1984; Heideman et al., 1984; Sahenk and Brady, 1987). When PC 12 cells are induced to extend neurites by treatment with nerve growth factor (NGF), there is an accompanying stabilization of their microtubule

network of microtubules to the depolymerizing action of colchicine and low temperature (Black and Greene, 1982). Also, in a variety of neurons, many microtubules are relatively insensitive to microtubule depolymerizing agents (Morris and Lasek, 1982; Brady et al., 1984).

Biochemical studies indicate that axonal tubulin is composed of at least two distinct pools that differ in cold solubility and biochemical composition (Brady et al., 1984). These two classes of microtubule polymers, stable and labile, differ in their sensitivity to nocodazole, a microtubule depolymerizing agent, by roughly 35-fold (Baas and Black, 1990). The stable and labile polymer fractions represent long-lived and recently assembled polymer, respectively. When visualized by immunoelectron microscopy, the labile polymers are stained densely with antibody to tyrosinated α -tubulin, while the stable polymers are not stained (Baas and Ahmad, 1992). The electron microscopic studies also indicate that stable microtubule segments are portions of longer microtubules containing both stable and labile regions (Joshi et al., 1986; Sahenk and Brady, 1987). The tyrosinated or labile domain is situated at the "+" end of the microtubule, whereas the detyrosinated or stable domain is situated at the "-" end of the microtubule (Baas and Black, 1990).

It is unclear what factors are responsible for the formation of microtubules with different stabilities in

neurons, but it is likely that tubulin isotypes and isoforms and MAPs are important. It has been shown that in nerve cells microtubules are differentiated in terms of the binding of MAPs; dendritic microtubules contain MAP-2, while their axonal counterparts are enriched in tau protein (reviewed by Matus, 1988). More importantly, the patterns of intracellular distribution and levels of these proteins are developmentally regulated (reviewed by Matus, 1988).

1.4 Microtubule-Associated Proteins (MAPs)

The microtubule-associated proteins (MAPs) are a family of molecules which co-purifies with tubulin when microtubules are repolymerized from brain homogenates. Up to 35 MAPs can be separated by polyacrylamide gel electrophoresis (Murphy *et al.*, 1977). Among these MAPs, two major groups, i.e., a group of high molecular weight proteins known as MAP1 (350kD), and MAP2 (220kD) (Sloboda *et al.*, 1976; Kuznetsov *et al.*, 1984) and a group of proteins known as tau (50-70 kD) (Cleveland *et al.*, 1977a, 1977b) have been purified and tested for their ability to promote tubulin polymerization *in vitro*.

The structure and properties of tau protein

Tau protein was first purified from microtubules of porcine brain (Weingarten et al., 1975). This protein was initially considered to be a proteolytic fragment of high molecular weight MAPs. It is now known that tau represents a family of microtubule binding proteins (reviewed by MacRae, 1992). This protein is associated with brain microtubules and induces assembly of microtubules from tubulin (Cleveland et al., 1977a).

Tau protein, as characterized by SDS/polyacrylamide gel electrophoresis, consists of four closely spaced bands of apparent molecular weights from 55 to 62 kD that have been shown to be closely related by both peptide sequence and amino acid composition (Cleveland et al., 1977b). Tau protein is heat stable (Weingarten et al., 1975). In adult porcine brain, tau is composed of at least four related phosphoproteins, while there are two isoforms present in mouse brain (Lee et al., 1988) and six isoforms in human (Goedert et al., 1989). The highly phosphorylated form of tau is a major component of paired helical filaments in Alzheimer's disease (Wischnik et al., 1988).

In structure, tau is a short, rod-like molecule that forms cross-bridges between microtubules *in vitro* (Hirokawa et al., 1988). Tau is elastic and can be induced to form paracrystals by end to end or lateral associations with each

other (Lichtenberg *et al.*, 1988). This property is related to the state of phosphorylation. When tau is dephosphorylated by alkaline phosphatase, it becomes shorter and more elastic, while when it is phosphorylated by Ca⁺⁺/calmodulin-dependent kinase, it becomes longer and stiffer (Hagestedt *et al.*, 1989).

Tau protein sequences have been determined from mouse, human, bovine, and rat, and conservation between species is high in the carboxy terminal half of the molecule (Lee, 1990; Andreadis, *et al.*, 1992). The family of tau proteins within each species is generated from alternatively spliced mRNA originating from one gene (Himmler *et al.*, 1989). Tau isoforms vary both in the N-terminal region and in the C-terminal region. An 18-amino acid residue stretch that is imperfectly repeated three or four times is located in the COOH-terminal half of the molecule. The repeats are separated by 13- and 14-residue stretches (Lee *et al.*, 1988). Each repeat resides in a different exon. These three or four repeats are considered to represent microtubule binding domains based on experiments using tau fragments synthesized *in vitro* and synthetic peptides (Himmler *et al.*, 1989; Lee *et al.*, 1989). Six isoforms identified from full-length cDNA clones are present in human brain (Goedert *et al.*, 1989). The shortest human form is 352 amino acids in length and contains three repeats, whereas the largest form is 441 amino acids in length and

contains four repeats.

In addition to the tau isoforms of molecular weight 48-65 kD expressed in the immature and adult brain, other proteins of higher molecular weight (110-125 kD) reacting with tau antibodies have been detected in the peripheral nervous system (PNS) (Oblinger *et al.*, 1991). cDNAs encoding these PNS taus have been cloned and sequenced in rats (Goedert *et al.*, 1992) and in mice (Couchie *et al.*, 1992). It has been suggested that PNS tau may play a role in the specialization of the adult peripheral nervous system (Couchie, *et al.*, 1992). More interestingly, the presence of tau in the nuclei of primate cells in culture has also been reported (Loomis *et al.*, 1990; Wang *et al.*, 1993). Nuclear tau was associated with both the fibrillar regions of interphase nucleoli and the nucleolar organizer regions (NORs) of mitotic chromosomes. Indirect immunofluorescence and Western blotting have demonstrated that nuclear and cytoplasmic tau proteins are present in human neuroblastoma cell lines. Immunofluorescence showed that the cytoplasmic tau was not localized to microtubules, suggesting non-microtubule functions of tau in both the cytoplasm and the nucleus (Wang *et al.*, 1993).

Developmental expression of tau proteins

The composition and expression of tau proteins are developmentally regulated (Francon et al., 1982; Drubin et al., 1984; Matus, 1988; Kosik et al., 1989). The transition in expression from juvenile tau to mature tau isoforms coincides with the period of formation of stable neurites (Ferreira et al., 1989). The composition of these tau isoforms changes during brain development. In embryonic rat brain only one tau isoform (MW 48kD) is present, while in mature rat brain six isoforms (MW 55-68 kD) are observed (Goedert et al., 1989). mRNAs encoding three-repeat containing isoforms are found both in fetal and adult brain, whereas mRNAs encoding four-repeat containing isoforms are found in adult but not in fetal brain (Goedert et al., 1989; Garcini et al., 1992).

Numerous studies of tau expression and localization in neuronal cells both *in situ* and in culture have been done. Initially, tau protein was found to be localized to axons in the central nervous system (Binder et al., 1985). Subsequent immunocytochemical studies indicated that tau was localized within neurons and was concentrated in axons in hippocampal pyramidal neurons in the rat *in situ*, but in cultured neurons tau was present throughout the dendrites, axons and somata (Dotti et al., 1987). This study also suggested that the biochemical maturation of tau that occurs

in situ failed to occur in culture (Dotti *et al.*, 1987). Further light and electron microscope localization by Migheli *et al.* (1988) showed that tau protein is mainly present in axons in white matter areas of rat brain, but is also localized in the cell bodies in the brain stem and basal ganglia and in the cell bodies of interfascicular oligodendrocytes. This study also showed that tau protein is colocalized with microtubules in axons and in the cytoplasm of cell bodies.

The functions of tau protein

Tau proteins are generally considered to be involved in microtubule stabilization. Early studies have demonstrated that tau is a stimulatory agent for *in vitro* microtubule assembly (Weingarten *et al.*, 1975; Goedert and Jakes, 1990). Subsequently, microinjection of tau protein into a cell type that has no endogenous tau protein showed that the injected tau protein associated specifically with microtubules, increased tubulin polymerization, and stabilized microtubules against depolymerization (Drubin and Kirschner, 1986). A recent study by Kanai *et al* (1992) revealed that the N-terminal region of the tau molecule is responsible for microtubule bundling *in vivo*.

Tau mRNA is abundantly expressed at the stage of

neurite formation in developing rat brain (Takemura et al., 1991). Inhibition of tau expression in primary cerebellar neurons by addition of antisense oligonucleotides to the culture media blocked the formation of the neurite asymmetry and resulted in the failure to elaborate an axon (Caceres and Kosik, 1990). In addition, tau is also believed to have some role in the development of polarity. The study by Kosik and Caceres (1991) has demonstrated that the inhibition of tau expression by antisense oligonucleotides resulted in the failure of a single minor neurite to elongate and form an axon-like neurite. The requirement of tau expression for axonal neurite elaboration was also examined in neuroblastoma cells. This study indicated that both the initiation and the continued outgrowth of neurites are dependent on tau proteins (Shea et al., 1992).

Analysis of tau protein isoforms in NGF-induced PC12 cells shows a transition from immature to mature tau isoforms, thus relating the appearance of the latter with the stabilization step of neurite outgrowth. This suggests that *in vivo* the mature tau isoforms are involved in the transition from unstable to stable neurites, which is a key step in neuronal development (Hanemaaijer and Ginzburg, 1991).

Transfection studies using rat tau cDNA show dramatic bundling of microtubules in fibroblasts expressing tau protein (Kanai et al., 1989; Bass et al., 1991). After

infection with tau-expressing virus, Sf9 cells from a moth ovary elaborated very long processes that resemble axons morphologically and were densely packed with microtubules (Knops *et al.*, 1991). This supports the hypothesis that tau functions in the formation of neurite processes.

1.5 Embryonal Carcinoma P19 Cells

Embryonal carcinoma (EC) cells are the stem cells of murine teratocarcinomas and are morphologically and functionally similar to the inner cell mass of mammalian embryos (Graham, 1977; Jetten, 1986).

The P19 clone with a euploid chromosome complement is one of the EC cell lines established by McBurney and Rogers in 1982. These cells are easily maintained in culture, they remain developmentally multipotential, and they can be chemically induced to differentiate terminally. Dimethylsulfoxide-induced differentiation proceeds along a mesodermal lineage, resulting in the formation of muscle cells (Edwards *et al.*, 1983). Retinoic acid (RA) at a concentration of 10^{-6} M induces the EC cells to follow a neuroectodermal pathway resulting in terminally differentiated neurons, glia, smooth muscle, and fibroblast-like cells (Jones-Villeneuve *et al.*, 1983). The neuronal differentiation in RA-induced P19 EC cells appears to be

similar to that in brain. These neurons possess a small cell body with several branched neurite extensions, express several neurotransmitters and can form synapses (McBurney et al., 1988).

Earlier studies by Falconer et al (1989a, 1989b, 1992) have documented that after RA-induced neuronal differentiation P19 EC cells express several neuronal-specific proteins, such as class III β -tubulin, neurofilament protein, MAP2 and tau protein. Class III β -tubulin was detected by the TuJ1 antibody as early as 60 hours after RA addition, before the appearance of neurite extensions (Falconer et al., 1989a; 1989b; 1992). Microtubule stability to colchicine depolymerization was also tested in the P19 cell line (Falconer et al., 1989a; 1992). The increase of microtubule stability starts within the first 24 hours of differentiation as demonstrated by the formation of a subpopulation of colchicine-stable acetylated microtubules. Differential β -tubulin isotype sorting also occurs in the P19 culture (Falconer et al., 1992). In the early stage of neuronal differentiation class II β -tubulin is preferentially incorporated into stable microtubules, while class III β -tubulin is incorporated into the dynamic portion of the microtubule population (Falconer et al., 1992; Falconer et al., in press). The increased stability of microtubules in differentiating neurons is correlated with changes in MAP expression similar to that observed in

developing brain (Falconer et al., in press).

The D310 cell line is a subclone of P19 cells. This cell line is incapable of muscle development under DMSO induction, but it does form neurons and glial cells when exposed to high RA concentrations. It is suggested that D310 EC cells differentiate into more neuronal cells than the P19 cell line does (Edwards et al., 1983). Thus, this cell line will generate a less heterogenous culture and is an attractive model system for the study of some early developmental events during neurogenesis.

PURPOSE OF THE THESIS

The work in David Brown's laboratory is focused on the investigation of the development of stable microtubules during neuronal differentiation of P19 EC cells. It has been demonstrated that tubulin and MAP expression in these neuronally differentiated cells is similar to that in brain and that tubulin isotype sorting into stable microtubules occurs during differentiation. However, the mechanism by which tubulin isotypes are differentially sorted into stable microtubule arrays remains to be answered. It has long been hypothesized that MAPs play a role in microtubule stabilization during neuronal differentiation since their expression is developmentally regulated.

Tau protein is one of MAPs which has been considered to be involved in microtubule stability. The studies of microinjection (Drubin and Kirschner, 1986), antisense oligonucleotide inhibition (Kosik and Caceres, 1991), and transfection (Knops et al., 1991) all provided evidence that tau is important in the establishment and maintenance of neurite extensions.

The objectives of this thesis are: (1) to characterize the expression of tau protein during RA-induced neuronal differentiation of EC cells; (2) to examine the temporal relationship between tau expression and the development of colchicine-stable microtubules; (3) to

determine whether or not tau protein associates with colchicine-stable microtubules; and (4) to examine any correlation between the expression of tau protein and the expression of individual tubulin isoforms during neuronal development.

MATERIALS AND METHODS

2.1 Cell Cultures

EC cells of the mouse D310 line (Edwards *et al.*, 1983) were obtained from Dr. M. McBurney, University of Ottawa. The cells were grown in tissue culture grade petri dishes in α -MEM medium: Minimum Essential Medium with L-glutamine without ribonucleosides and deoxyribonucleosides (Flow Laboratories, Mississauga, Ont.) supplemented with 10% heat-inactivated fetal calf serum (FCS, Flow Lab.). The cultures were maintained at 37°C in a 5% CO₂ atmosphere. To maintain the cells in the uncommitted, proliferative state, the cultures were passaged every two days using 0.025% trypsin (Flow Labs) and 1mM EDTA in calcium- and magnesium-free phosphate-buffered saline (PBS: 0.1309 M NaCl, 5.1 mM Na₂HPO₄, 15.6 mM KH₂PO₄, pH 7.4), and replated at approximately 3 X 10⁵ cells per dish.

For RA-induced neuronal differentiation, the cells were first dissociated using 0.025% trypsin and permitted to aggregate in α -MEM medium with 10% FCS for at least two hours in a bacterial grade petri dish, then seeded on to coverslips in a 35 mm Corning tissue culture dish (Canlab) in α -MEM plus 10% FCS. After 24 hours, the medium was replaced with α -MEM plus 4% FCS, and 10⁻⁶ M RA was added to

induce neuronal differentiation. A 10^{-2} M stock solution of RA was prepared in ethanol, stored at -80°C , and diluted to 10^{-4} M in medium for use. An appropriate amount was added to the cultures for a final concentration of 10^{-6} M RA. After that, the medium was changed every 2 days with α -MEM medium supplemented with 4% FCS.

Experiments using colchicine were performed at days 0, 3, 6, 9 and 12 after RA induction. Cells, grown on coverslips, were treated with $1\ \mu\text{g}/\text{ml}$ of colchicine (Sigma Chemical Co., St. Louis, MO) in culture medium for 45 min at 37°C . Colchicine was made up as a stock solution of $1\ \text{mg}/\text{ml}$ in sterile distilled water and was stored at -80°C .

2.2 Immunofluorescence Techniques

For immunofluorescence microscopy, the cells attached to coverslips were briefly washed in PBS, fixed in 4% paraformaldehyde (BDH Chemicals; Toronto, Ont.) plus 1% picric acid in 0.5 M dibasic sodium phosphate (pH 6.9) for 1 hour, and then permeabilized with 0.5% Triton X-100 (Pierce; Rockford, IL) in PBS for 5 min. After 3 X 5 min washes in PBS the cells were incubated in the appropriate primary antibody solution for 45 min. Following 3 X 5 min PBS washes, cells were incubated with the appropriate fluorochrome-conjugated secondary antibody for 45 min,

washed in PBS for 3 X 5 min, and stained for 2 min in 1 μ g/ml Hoechst dye #33258 (Calbiochem; LaJolla, CA) at 1:5000 dilution in PBS. Coverslips were then rinsed 3 X 5 min in PBS, and mounted on glass slides in mounting medium containing 0.1% p-phenylene diamine to retard fluorescence bleaching during observation. All processing steps for immunofluorescence were at room temperature. The samples were examined and photographed on a ZEISS Axiophot microscope equipped with phase contrast and epifluorescence optics. All photomicrographs were taken on Ilford XP-1 400 ASA film.

For double immunofluorescence staining, the two primary antibodies were sequentially applied, each for 45 min, and after 3 X 5 min PBS washes the two appropriate secondary antibodies were mixed and applied at the same time. Appropriate controls were done to ensure that no cross-reaction among secondary antibodies occurred. These included two non-mixed secondary antibodies applied separately.

The following primary antibodies were used for immunofluorescence. YOL 1/34 (Dimension Lab. Mississauga, Ontario, Canada), a rat monoclonal antibody (Kilmartin et al., 1982), was used as a general tubulin stain at a 1:200 dilution. A mouse monoclonal, tau-1, (Binder et al., 1985) and a rabbit polyclonal antibody (Sigma) which recognize all tau isoforms were used at a 1:80 and 1:300 dilution

respectively. TuJ1, the kind gift of Dr. A. Frankfurter, University of Virginia, is a mouse monoclonal antibody (Lee, *et al.*, 1990a) which recognizes β III-tubulin and was used as a neuronal marker at 1:300 dilution. Mouse monoclonal antibodies to MAP2 (clones AP-14 and AP-18) from L. I. Binder were used at 1:100 dilution (Binder *et al.*, 1985). A rabbit polyclonal antibody to neurofilament 150 kD protein was purchased from Amersham, Inc. and used at a 1:200 dilution.

The following secondary antibodies were used: Texas Red-conjugated donkey anti-rat IgG (1:80) cross-absorbed against mouse and rabbit (Jackson Research, Westgrove, PA); DTAF (dichlorotriazinyl amino fluorescein)-conjugated donkey anti-rabbit IgG (1:200) cross-absorbed to rat and mouse (Jackson Research); Rhodamine-conjugated donkey anti-rabbit IgG (1:200) cross-absorbed to mouse (Chemincin, International Inc) and DTAF-conjugated donkey anti-mouse IgG (1:200) cross-absorbed to rabbit (Jackson Research).

2.3 Electrophoresis and Immunoblotting

Two types of cell extracts were prepared for immunoblotting: the detergent-insoluble proteins containing the polymeric tubulin, and the detergent-soluble protein containing the soluble, unpolymerized tubulin. Uncommitted

EC cells or 3, 6, 9 and 12 day RA-treated cells were cultured in 100 mm tissue culture dishes to prepare cell extracts. To prepare the soluble and polymeric fractions of tubulin from colchicine-treated cells, uncommitted EC cells and 3, 6, 9, and 12 day after RA induction cells were pretreated with 1 μ g/ml colchicine for 45 minutes at 37°C.

To prepare the soluble and polymeric fractions of cellular tubulin from both colchicine-treated and non-colchicine-treated cultures, cells were washed twice very gently with warm PEM (80 mM PIPES, 5 mM EGTA, 1 mM MgCl₂, pH 6.8) buffer. Soluble proteins were extracted at 37°C for 5 min in 500 μ l/100 mm dish of microtubule stabilizing buffer (MSB: 0.1M N-morpholinoethanesulfonic acid, 1mM MgSO₄, 2 mM EGTA, 0.1 mM EDTA, 4 M glycerol, pH 6.75) containing 0.5% Triton X-100. The soluble extract was removed and centrifuged for 3 minutes at 1000 g in order to pellet any cytoskeletal material dislodged from the culture dish during extraction. The remaining cytoskeletal fraction in the culture dish was dissolved in 500 μ l of 25 mM Tris (pH 6.8) plus 0.5% SDS, and combined with the pellet from the soluble extraction. Both soluble and polymeric fractions were boiled in a water bath for 10 min, vortexed thoroughly, and either used immediately or stored at -80°C. All buffers for protein extractions contained 1mM PMSF (phenylmethylsulfonylfluoride), 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin. Protein concentration

was determined by the bicinchoninic acid assay (Smith et al., 1985) using BSA (bovine serum albumin) as the standard.

Polyacrylamide gels for analysis of protein samples were performed according to Laemmli (1970) using the BioRad mini-gel apparatus. Equal amounts of protein of the polymeric and soluble fractions from the non-colchicine-treated and colchicine-treated samples were loaded in each lane and electrophoresed on a 7.5% SDS polyacrylamide separating gel and 4% stacking gel at a constant voltage of 200 volts in Laemmli gel running buffer. Following electrophoresis the proteins were electrophoretically transferred to a nitrocellulose membrane in transfer buffer containing 20% methanol for 1 hour at a constant voltage of 100 volts or overnight at a constant voltage of 30 volts. The membranes containing transferred proteins were transiently stained with 0.2% Ponceau S in 3% trichloroacetic acid to identify the positions of protein samples and the molecular weight standards, then washed and dried. The lanes of protein samples were marked around their entire perimeter for use in a Miniblotter. After the membranes were destained in PBS, they were incubated in 5% (w/v) powdered skim milk in PBS overnight to block the non-specific binding sites and then mounted on a Miniblotter 28 (Immunitics; Cambridge, MA). Each sample was then probed with the following primary antibodies: DM1B (1:5000), a mouse monoclonal antibody, which recognizes all β -tubulin

(Amersham, Inc.); 6-11B-1 (1:10), a mouse monoclonal antibody, which recognizes all acetylated α -tubulin (Piperno and Fuller, 1985) (the kind gift of Dr. Piperno); and tau-1 (1:80), a mouse monoclonal antibody, which recognizes all tau isoforms, (the kind gift of Dr. L. I. Binder, University of Alabama). All antibodies were diluted in 2.5% powdered skim milk in PBS and incubations were for 1 hour at room temperature. After incubation with the primary antibodies, the blots were washed with PBS 3 x 5 min each. The blots were then incubated with a biotinylated goat anti-mouse IgG secondary antibody (Amersham) at a dilution of 1:5000 in 2.5% powdered skim milk in PBS for one hour. Following 3 x 5 min washes in PBS the blots were again incubated with a streptavidin biotinylated complex (HRP) (Amersham) at a dilution of 1:5000 for one hour, washed three times in PBS, and detected by chemiluminescence.

2.4 Enzyme Linked-Immunesorbent Assay (ELISA)

To prepare whole cell protein extracts for ELISA cells were rinsed briefly in warm (37°C) PBS, washed twice with warm PEM buffer, scraped into 500 μ l of PEM buffer using a rubber policeman and sonicated for one minute on ice at a low speed to break the cells. Protein concentrations were again determined by the bicinchoninic acid assay. The

samples were either used immediately or stored at -80°C .

To quantify the total amount of tubulin and the relative amounts of class III β -tubulin and tau protein expressed during neuronal differentiation, the indirect ELISA technique was applied (Binder *et al.*, 1985). Whole cell proteins were diluted in 0.1 M sodium carbonate buffer at pH 9.6. Immulon 1 ELISA plates (Dynatech Lab. Inc.) were coated with 15 $\mu\text{g}/\text{well}$ of whole cell protein and kept overnight at 37°C . The plates were washed once with washing buffer (PBS containing 0.05% Tween-20 and 2.5% powdered skim milk) for three minutes, incubated in blocking buffer (PBS containing 0.05% Tween-20 and 5% powdered skim milk) for 30 minutes at room temperature, and again washed once for 3 min. Plates were then incubated in primary antibody for two hours at 37°C . After 3 X 3 min washes, an appropriate secondary antibody conjugated with biotin was added and incubated for one hour at 37°C . The plates were washed with 0.2% Tween-20 in PBS for 3 X 3 minutes and incubated with biotinylated horseradish peroxidase complex (HRP) at a dilution of 1:750 for 45 minutes at 37°C . After 3 X 3 min washes with 0.2% Tween-20 in PBS, the plates were incubated with 100 μl substrate solution containing 50 ml citric acid/ Na_2HPO_4 buffer at pH 5.0, 17 mg o-phenylenediamine (OPD), and 20 μl 30% H_2O_2 . The reaction was stopped after 5 minutes by adding 100 μl 1 N H_2SO_4 . The absorbance values were read on a BIOTEK EL310 autoreader (Mandel Scientific)

at 490 nm. Purified tubulin from bovine brain prepared by the phosphocellulose chromatography (PC) method (Weingarten *et al.*, 1975) was used to generate a standard curve and the amount of total tubulin in the sample was determined within the linear range of the PC-tubulin standard. The absorbance values for β III-tubulin and tau protein were expressed as a ratio of relative amount of β III-tubulin or tau protein/mg of total tubulin.

The following primary antibodies were used for ELISA experiments: DM1B (1:500) for total β -tubulin; TuJ1 (1:1000) for class III β -tubulin; and tau (1:500), a polyclonal antibody to recognize all tau isoforms. The secondary antibodies were biotinylated goat anti-mouse IgG at 1:1000 dilution, and biotinylated goat anti-rabbit IgG (Vector, Inc.) at 1:1000 dilution.

2.5 Immunogold Labelling for Light and Electron Microscopy

For light microscopic immunogold localization of tau and β III-tubulin, cells were differentiated on coverslips as described for the immunofluorescence samples. At day 6 after RA induction, the cells were rinsed in PHEM buffer (containing 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgCl_2 \cdot H_2O$, pH 6.9), simultaneously fixed and permeabilized

with 0.5% glutaraldehyde and 0.2% Triton X-100 in PHEM for 10 minutes, then further permeabilized with 0.5% Triton X-100 in PHEM for 5 minutes. After 20 minutes washing in 20 mM glycine in PHEM to quench free aldehyde groups, the cells were incubated in blocking solution (5% goat serum in washing buffer containing 0.8% BSA and 0.1% gelatin in PBS) for 30 minutes, washed in washing buffer for 5 minutes, and incubated in the primary antibody for one hour at room temperature. The primary antibodies were TuJ1, at 1:300 dilution and tau, a rabbit polyclonal antibody at 1:300 dilution. After 3 X 10 min washes in washing buffer, the cells were incubated for one hour in either biotinylated goat anti-mouse IgG secondary antibody (Amersham) or biotinylated goat anti-rabbit IgG secondary antibody (Amersham) depending upon the primary antibodies used. Following 3 X 10 min washes, the cells were incubated in AuroProbe One streptavidin (Amersham) at 1:50 overnight at 4°C. Following 3 X 10 min washes in washing buffer, the cells were post-fixed with 2% glutaraldehyde for 10 min and silver enhanced (IntenSE M kit, Amersham) for 7-10 min. After thorough washing in distilled water, the coverslips were mounted on glass slides and visualized by both bright and dark field optics using a Zeiss Universal microscope. Micrographs were taken on Ilford XP 1-400 ASA film.

For electron microscopic immunogold localizations, the cells were differentiated as aggregates in 100 mm

bacterial grade petri dishes for two days in α -MEM medium containing 10% FCS plus 10^{-6} M RA to induce the neuronal differentiation and then seeded onto plastic films (Bellco Glass Inc.). The medium was changed every two days, and six days after plating the cells on the plastic support were fixed and permeablized, blocked and immunostained as described above with 5 min silver enhancement. After 3 X 10 min washes in distilled water, the cells were postfixed sequentially with 2% glutaraldehyde in PHEM buffer for 10 minutes and 4% osmium tetroxide for 30 minutes. The samples were dehydrated in 30%, 50%, 70%, and 90% ethanol for 10 min respectively, 100% ethanol for 2 X 10 min, and then in propylene oxide for 2 X 15 min. The samples were placed in a mixture of 1:1 propylene oxide: resin overnight. The resin was Epon-Araldite containing 15 gm of DDSA, 5.5 gm of Araldite, 7.75 gm of Epon and 0.7 gm of DMP-30. The second day, the mixture of propylene oxide and resin was replaced with pure Epon-Araldite for 4 hours, and then the samples were embedded in pure Epon-Araldite and polymerized for 36 hours at 60°C. Thin sections (silver-grey) were cut on an ultramicrotome using a glass or diamond knife and collected on copper grids. The grids were stained with 4% aqueous uranyl acetate for 40 minutes followed by lead citrate for 10 minutes, and observed on the Phillips EM 201 electron microscope. Micrographs were taken on Kodak Electron Image Film S0-163.

RESULTS

3.1 RA-induced Neuronal Differentiation

In my initial studies, immunofluorescence staining was used to monitor the morphological changes of the D310 line of EC cells during neuronal differentiation and to assay for the expression of neuronal-specific cytoskeletal proteins.

Fig. 1. shows the morphology of D310 cells before neuronal differentiation. These undifferentiated EC cells are irregularly shaped, but have no extended processes (Fig. 1a). Tubulin antibody staining shows that microtubules in the cytoplasm form a radial array extending from the centrosome (Fig. 1b). The undifferentiated EC cells were allowed to aggregate in bacterial grade petri dishes for 2 hours and the aggregates were then plated on glass coverslips in the presence of RA for neuronal induction. At 2 days after RA induction, a few cells extend short unbranched processes (Fig. 2a) and start to express the earliest neuronal marker I have monitored, class III β -tubulin (Fig. 2b). At 6 days, many cells show a neuronal morphology with a small cell body and long thin processes (Fig. 3). At this time, in addition to β III-tubulin (Fig. 3a), other neuronal markers like neurofilament (150kD)

Figure 1. (see the back of the page).

Figure 1. General morphology of undifferentiated EC cells. (a) Phase contrast showing irregular morphology. (b) tubulin antibody (YOL 1/34) staining showing microtubules radiating from centrosome in each cell. Magnification: 600X.

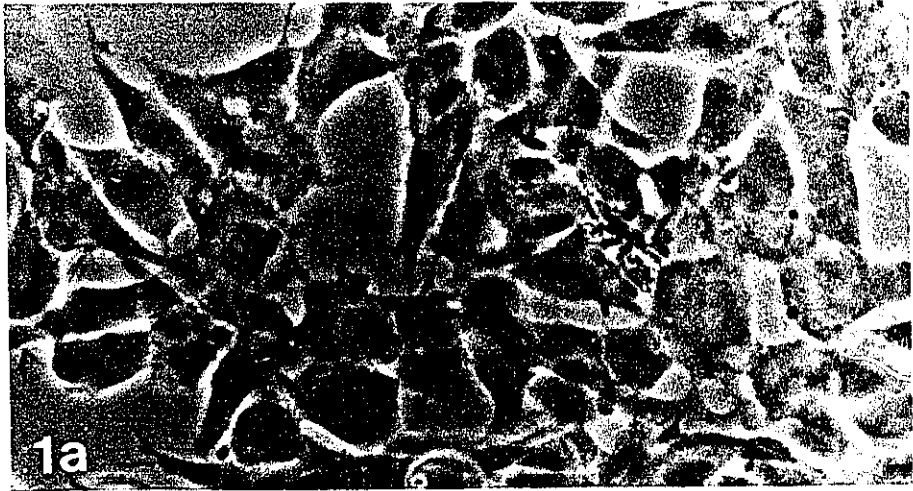


Figure 2. (see the back of the page).

Figure 2. Double immunofluorescence staining showing β III tubulin expression in day 2 RA-induced cells. (a) The general tubulin antibody (YOL 1/34) stained all cells in the culture. (b) Some cells were labelled by the antibody (TuJ1) to β III-tubulin. Note that the cells stained by the antibody to β III-tubulin also were stained more intensely by the general tubulin antibody YOL 1/34. (c) Control for YOL 1/34 using only the Texas Red-labelled donkey anti-rat secondary antibody. (d) Control for TuJ1 using only the DTAF-labelled donkey anti-mouse secondary antibody. Magnification: 400X.

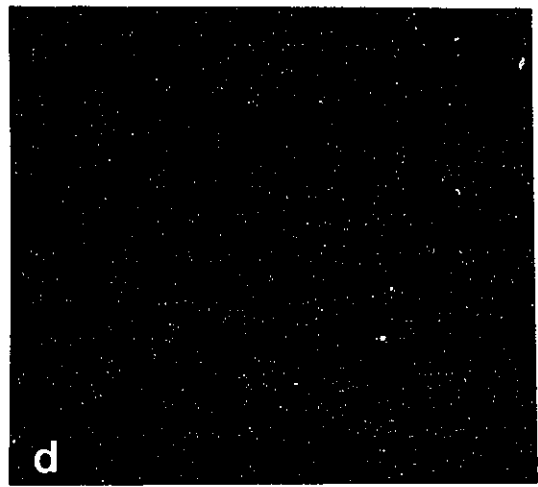
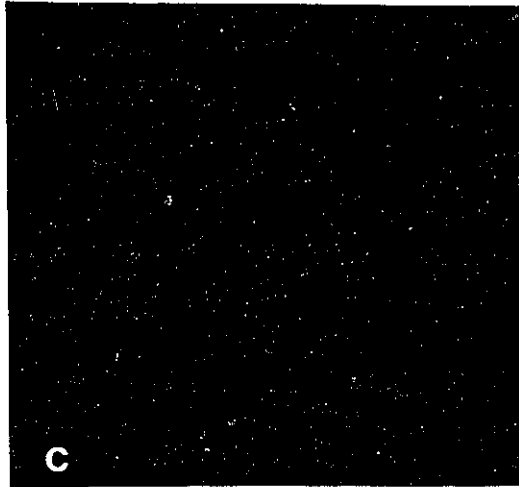
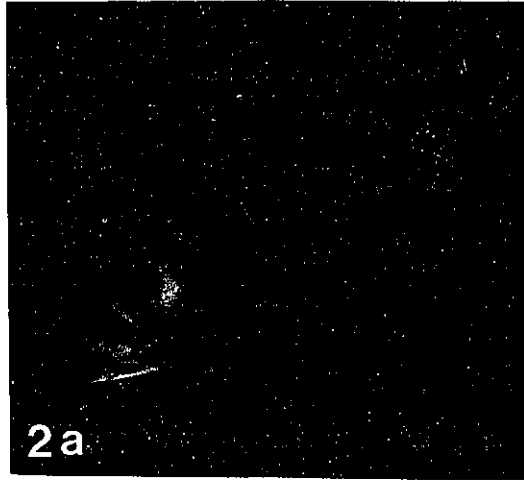
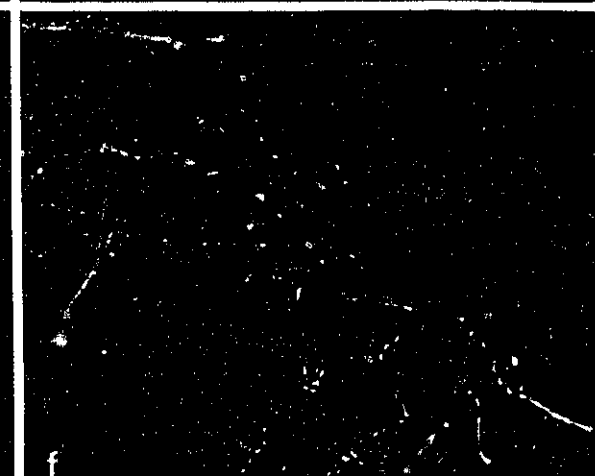
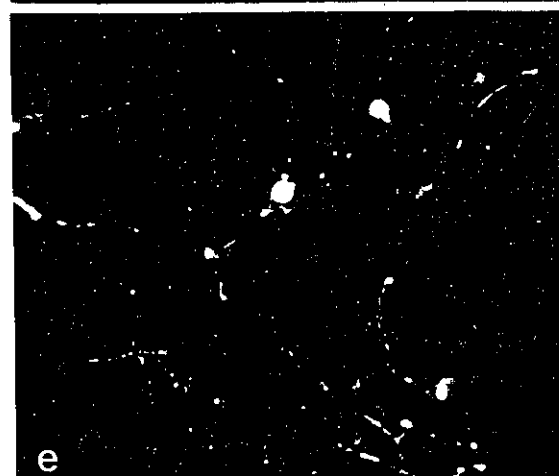
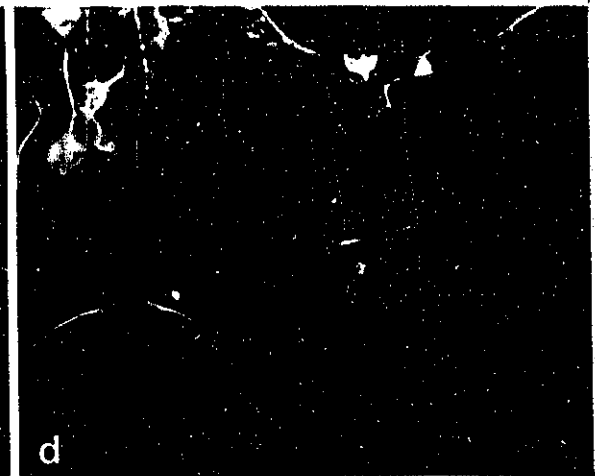
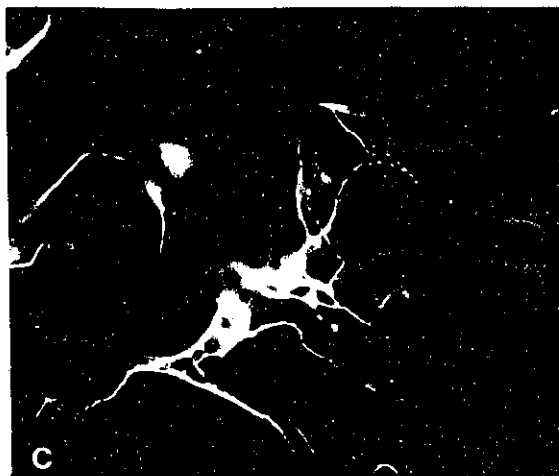
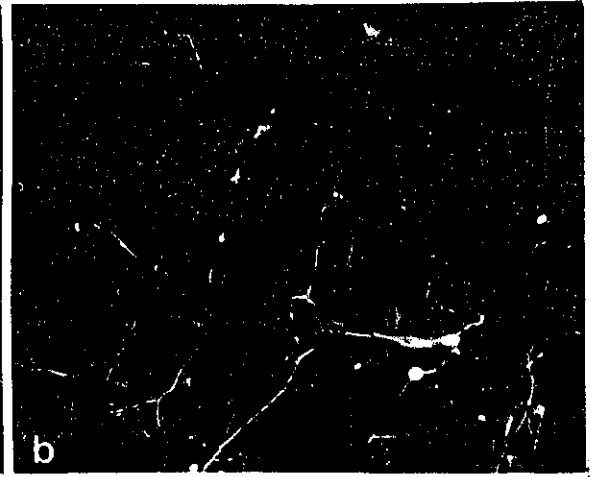
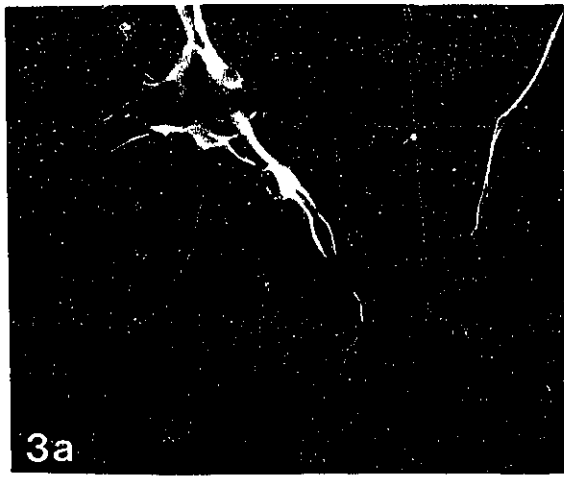


Figure 3. (see the back of the page).

Figure 3. Single immunofluorescence staining showing different neuronal markers expressed in day 6 RA-induced cells using antibodies to: (a) β III-tubulin labelled by TuJ1; (b) Neurofilament (150 kD) protein labelled by the antibody to neurofilament protein; (c) MAP2 labelled by AP-14; (d) MAP2c labelled by AP-18; (e) Tau protein labelled by monoclonal tau-1 antibody; (f) Tau protein labelled by polyclonal tau antibody. Note smooth and uniform staining for β III and neurofilament proteins and non-uniform staining for MAPs. Magnification: 400X.



protein (Fig. 3b), MAP2 (Fig. 3c and 3d) and tau (Fig. 3e and 3f) are expressed. The antibodies to β III-tubulin (Fig. 3a) and neurofilament protein (Fig. 3b) stain the processes uniformly, whereas the antibodies to MAPs (Fig. 3c, 3d, 3e, and 3f) stain the processes unevenly, giving the neurites a beaded appearance.

3.2 Immunofluorescence Observations of Tau Expression

Expression of tau protein is developmentally regulated during neuronal development in brain (Matus, 1988) and has been reported to be localized in axons in mature rat brain (Binder *et al.*, 1985). To determine whether tau protein is expressed in RA-induced neuronally differentiated EC cells, and if so, the time course of its expression, double immunostaining of tubulin and tau protein was applied.

Double immunostaining of tubulin and tau protein

Undifferentiated EC cells (Fig. 4a) show centrosomal microtubule arrays by tubulin antibody staining (Fig. 4b), whereas no staining is observed with tau antibody (Fig. 4c).

After 3 days of differentiation (Fig. 4d-4f), the

culture contains a small population of cells with short neurite processes while the rest of the cells still have the undifferentiated cell morphology (Fig. 4d). All cells in the culture are stained by antibody to tubulin (Fig. 4e), with the small number of cells with neurite processes staining more intensely than the undifferentiated cells. Some of these brightly stained cells also express tau protein (Fig. 4f). The antibody to tau protein stains both the cell body and the neurite processes. However, not all neurites are stained by the antibody to tau.

After 6 days of differentiation (Fig. 5a and 5b), the cells have a small cell body and long, thin neurite processes (Fig. 5a). They are stained by both anti-tubulin (Fig. 5a) and anti-tau (Fig. 5b). Tubulin staining of neurite processes is very smooth and uniform, whereas tau staining of extensions shows a beaded morphology.

The cells after 12 days of differentiation have long branched processes (Fig. 5c and 5d). In samples stained for tubulin, the tubulin staining is evenly distributed along the extensions (Fig. 5c). In contrast, the staining of tau protein shows a beaded morphology (Fig. 5d) that is more prominent than in the earlier days of differentiation. It appears that the antibody to tau only labels the long processes, and the short neurites are not labelled.

Figure 4. (see the back of the page).

Figure 4. Double immunofluorescence staining of tubulin and tau in undifferentiated cells and day 3 RA-induced cells. (a) Phase contrast of undifferentiated EC cells. (b) Anti-tubulin staining (YOL 1/34) of the same cells. (c) Anti-tau staining (polyclonal antibody) of the same cells. (d) Phase contrast of day 3 RA-induced cells. (e) Anti-tubulin staining (YOL 1/34) of the same cells. (f) Anti-tau staining (polyclonal antibody) of the same cells. Note tau protein is not expressed in undifferentiated EC cells as shown in (c), but in day 3 RA-induced culture, some cells with short processes start to express tau as shown in (f). Tau co-localizes with tubulin, but not all the processes are labelled by anti-tau. Magnification: 400X.

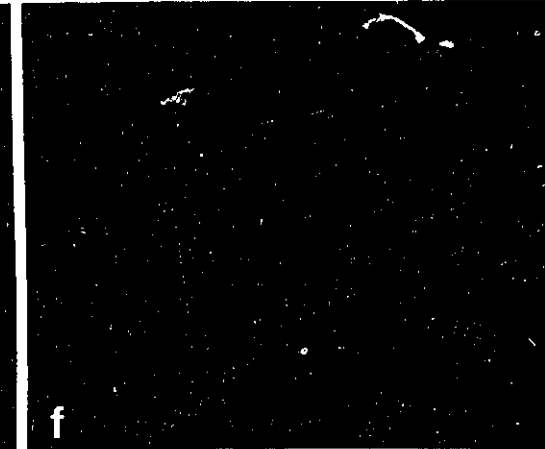
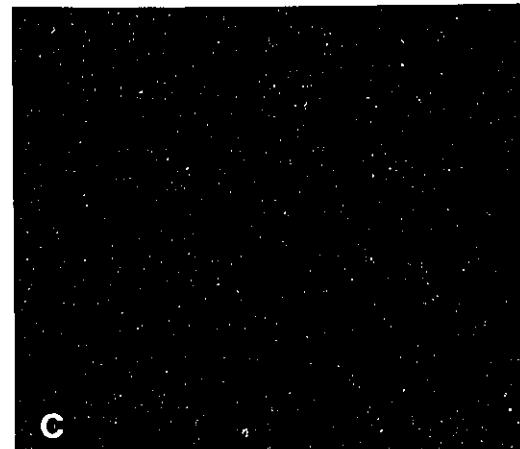
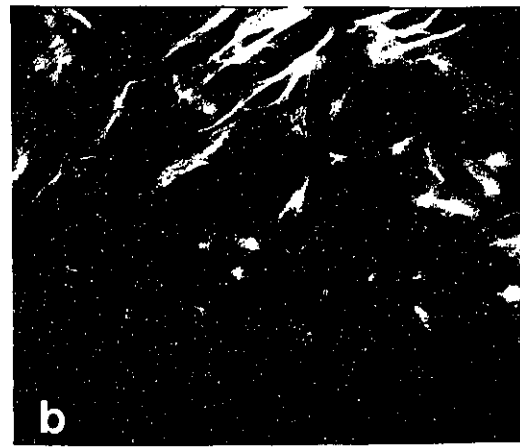
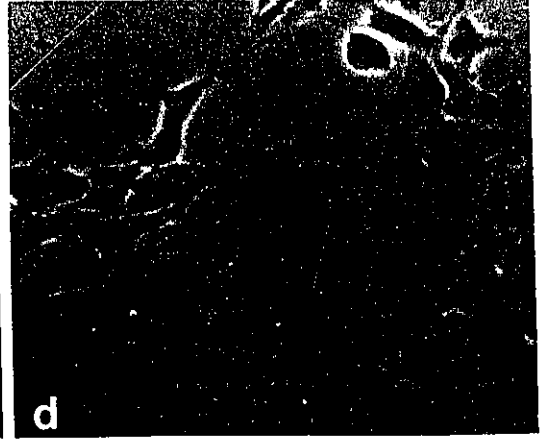
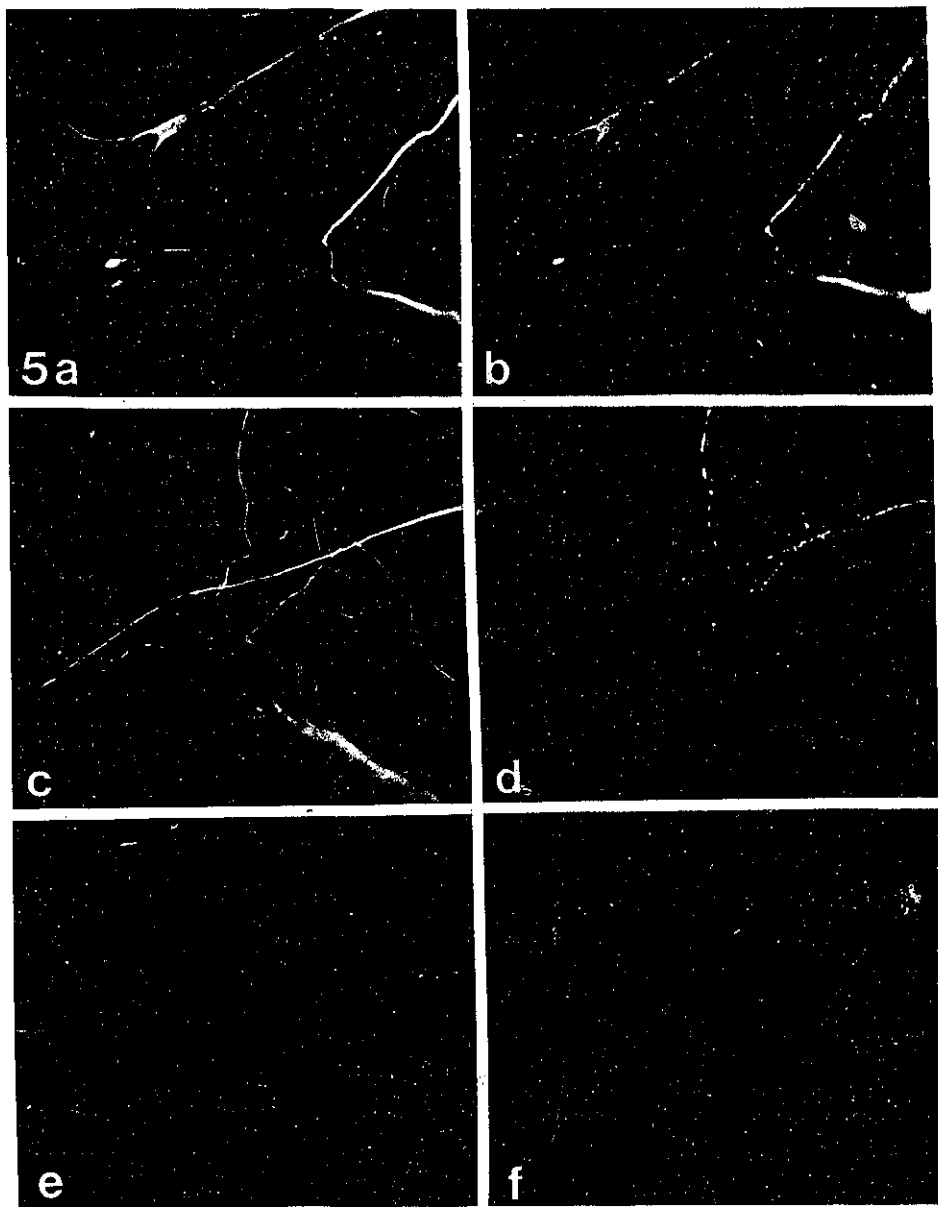


Figure 5. (see the back of the page).

Figure 5. Double immunofluorescence staining of neurite processes in day 6 and day 12 RA-induced cells. (a) Anti-tubulin staining (YOL 1/34) of day 6 RA-induced cells. (b) The same field as (a) stained with anti-tau (polyclonal antibody). (c) Anti-tubulin staining (YOL 1/34) of day 12 RA-induced culture. (d) The same field as (c) stained with anti-tau (polyclonal antibody). (e) Control for anti-tubulin using Texas Red-labelled secondary antibody alone. (f) Control for anti-tau using DTAF-labelled secondary antibody alone. Note smooth and uniform staining of tubulin and beaded appearance of tau protein. Magnification: 400X.



Double immunostaining of class III β -tubulin and tau protein

Observations of the developing central nervous system in chicken (Lee *et al.*, 1990b) have indicated that class III β -tubulin is present in all neurons during differentiation, but is not present in undifferentiated neural precursors. They concluded that β III-tubulin was expressed coincidentally with the final mitosis of neuroblasts and with the initial stages of neurite outgrowth. Therefore, class III β -tubulin is one of the earliest markers for neuronal differentiation. Double immunostaining was used to examine the expression of tau protein relative to this neuronal marker to study the early events during neuronal differentiation.

In undifferentiated EC cells, neither β III (Fig. 6a) nor tau (Fig. 6b) is expressed. β III starts to be expressed at day 2 of neuronal differentiation as shown in Fig. 2, whereas tau starts at day 3 (Fig. 4). At 3 days of differentiation, β III shows smooth and bright immunostaining in the cell body and along the neurite extensions (Fig. 6d). Double labelling shows that tau is co-expressed with β III in the cell body and neurite extensions (Fig. 6e). As noted earlier, the staining of tau gives a beaded morphology to the processes.

By 6 days of differentiation, all cells with long processes express β III (Fig. 6f), as well as tau (Fig. 6g).

Figure 6. (see the back of the page).

Figure 6. Double immunofluorescence staining of undifferentiated EC cells, day 3 and day 6 RA-induced cells with anti- β III tubulin (TuJ1) and anti-tau (polyclonal antibody). (a) Anti- β III staining of day 0 cells. (b) Anti-tau staining of the same cells. (c) Phase contrast of the same field as (a) and (b). (d) Anti- β III tubulin staining of day 3 RA-induced culture. (e) The same field as (d) stained with anti-tau. (f) Anti- β III staining of day 6 culture. (g) the same field as (f) stained with anti-tau. Note anti- β III shows a uniform staining pattern, whereas anti-tau gives a beaded morphology. Magnification: 400X.

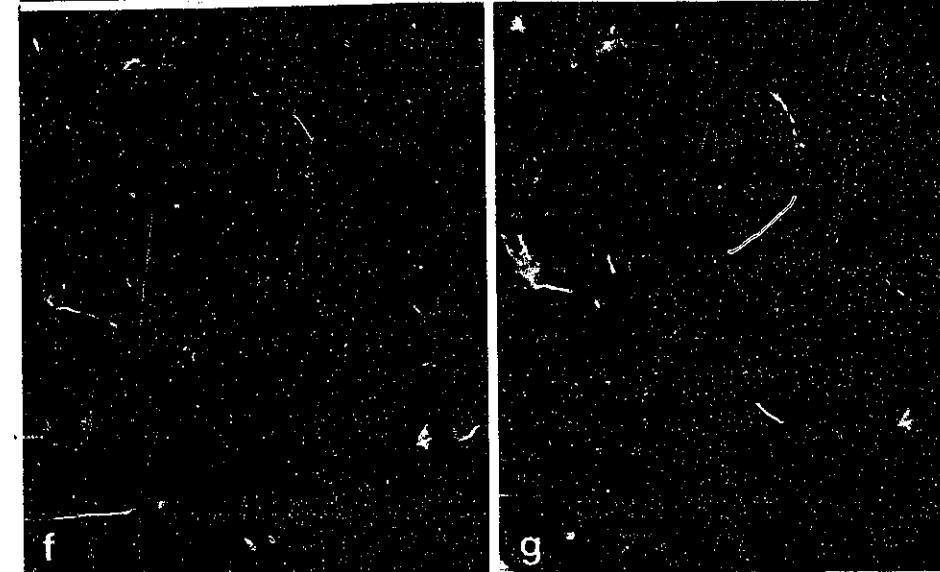
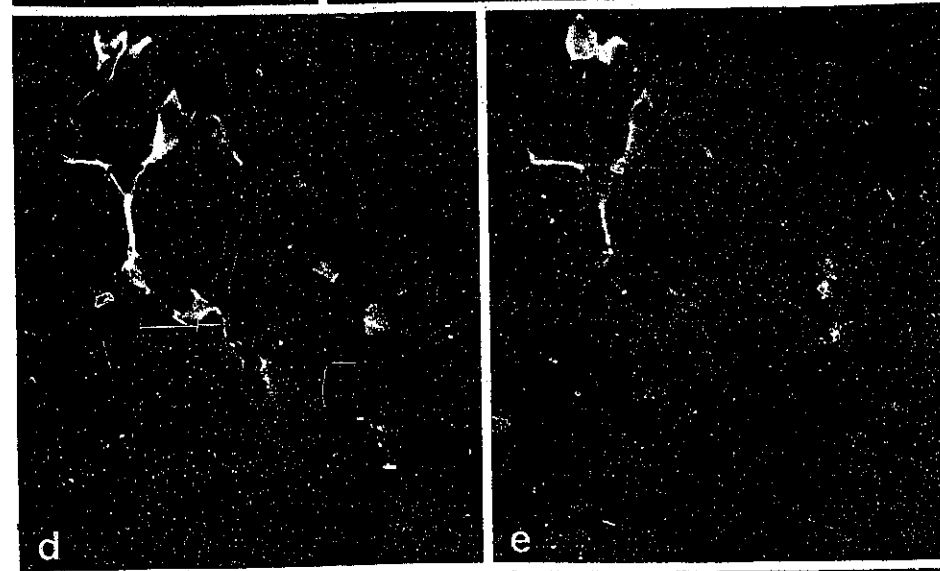
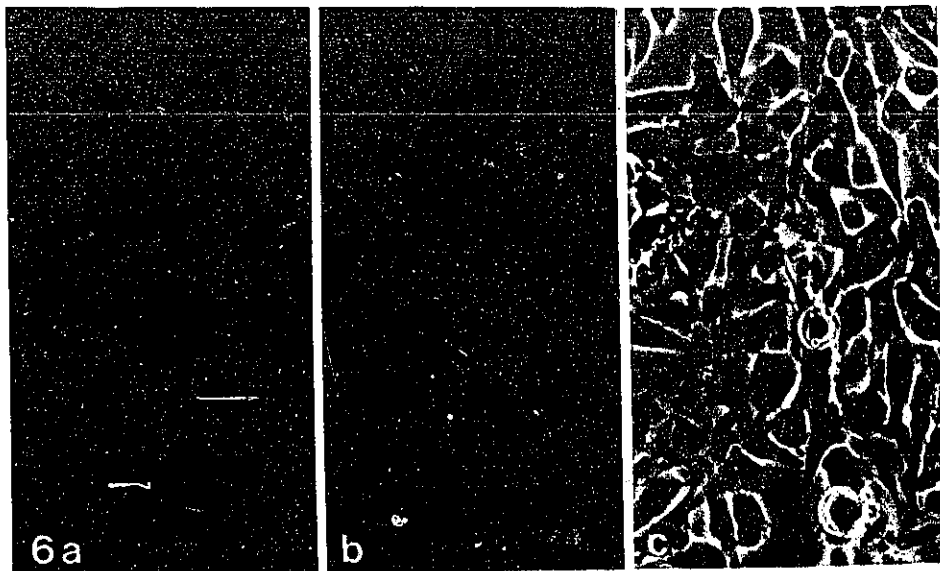
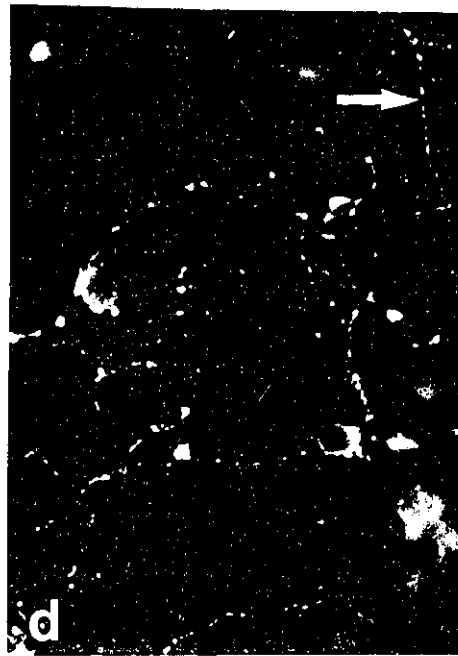
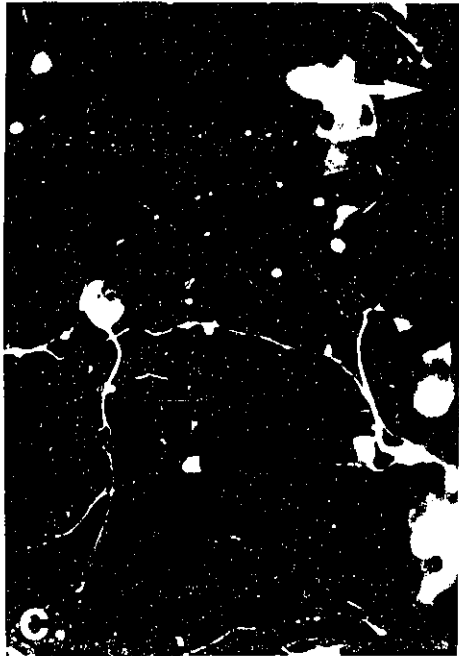
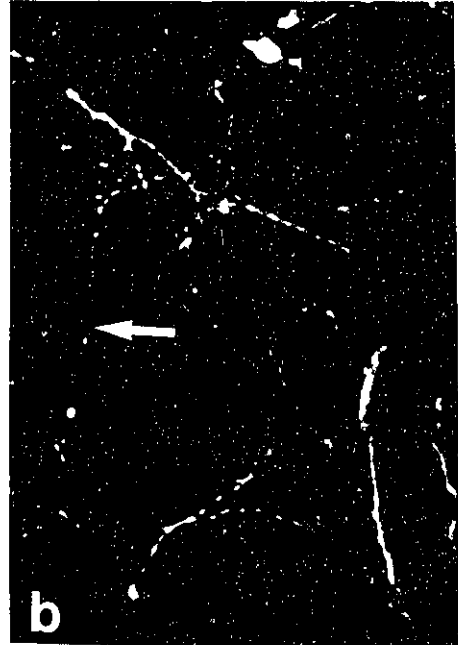
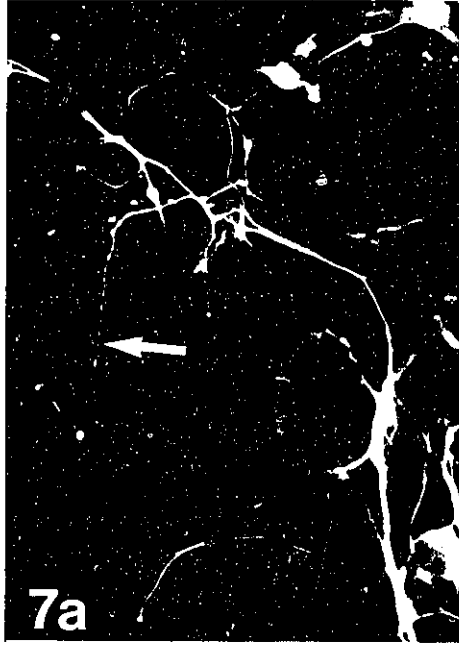


Figure 7. (see the back of the page).

Figure 7. Double immunofluorescence staining of day 9 and day 12 RA-induced cells with anti- β III tubulin (TuJ1) and anti-tau (polyclonal antibody). (a) Anti- β III of day 6 culture. (b) Anti-tau of day 6 culture. (c) Anti- β III of day 12 culture after RA induction. (d) Anti-tau of day 12 culture after RA induction. Note some beaded morphology is also shown by anti- β III staining (arrows in a and c). Magnification: 400X.

•



The cells at day 9 (Fig. 7a and 7b) and day 12 (Fig. 7c and 7d) of differentiation are very similar to the cells at day 6, except longer branched processes are observed. Some processes stained by the antibody to β III also show the beaded morphology (arrows in Fig. 7a and 7c), but this is not as obvious as in the samples stained by the antibody to tau (arrows in Fig. 7b and 7d).

3.3 Enzyme-linked Immunosorbent Assay

The immunofluorescence staining results in section 3.2 indicated that there was an increase in total tubulin and new expression of β III-tubulin and tau protein during neuronal differentiation of EC cells. To quantify these changes, enzyme-linked immunosorbent assays (ELISA) of total cell protein were carried out. Fig. 8 shows the change in the ratio of total cell tubulin to total cell protein in undifferentiated cells and up to 10 days of differentiation. In undifferentiated EC cells tubulin makes up about 0.5% of total cell protein. After RA induction, there is a gradual increase of tubulin to about 0.75% of total protein by day 10 of differentiation.

The change in relative amount of β III tubulin expressed during neuronal differentiation to total cell tubulin is shown in Fig. 9. In undifferentiated cells there

is only a background level of β III-tubulin detected. Although by immunofluorescence a few cells can be seen to express β III-tubulin after 2 days of differentiation, no increase is observed by ELISA. However, a significant increase in the amount of β III-tubulin is observed at day 4, and there is continued increase at the later days of differentiation.

The accumulation of tau protein during neuronal differentiation (Fig. 10) occurs coincident with the increase in the level of β III-tubulin. Tau protein is at background levels in the undifferentiated and 2 day RA-induced cells. The amount of tau protein then increases greatly at day 4 and continues to increase from day 4 to day 10 of differentiation.

3.4 Tau Expression and Microtubule Stability

Although it has been suggested in several studies (Drubin *et al.*, 1986; Kosik and Caceres, 1991; Knops *et al.*, 1991) that tau plays a role in stabilizing microtubules, the correlation between tau expression and the development of stable microtubules during neuronal differentiation has not been examined. In this section immunofluorescence and immunoblotting were used to investigate this proposed temporal correlation. Stability of microtubules was tested

Figure 8. (see the back of the page).

Figure 8. ELISA results showing total cellular tubulin in undifferentiated EC cells and in cells undergoing RA-induced neuronal differentiation. The amount of total tubulin was derived from the linear range of a standard curve generated using PC-tubulin of bovine brain. The values for each day are derived from triplicate samples from three separate experiments. Standard deviations are indicated by error bars.

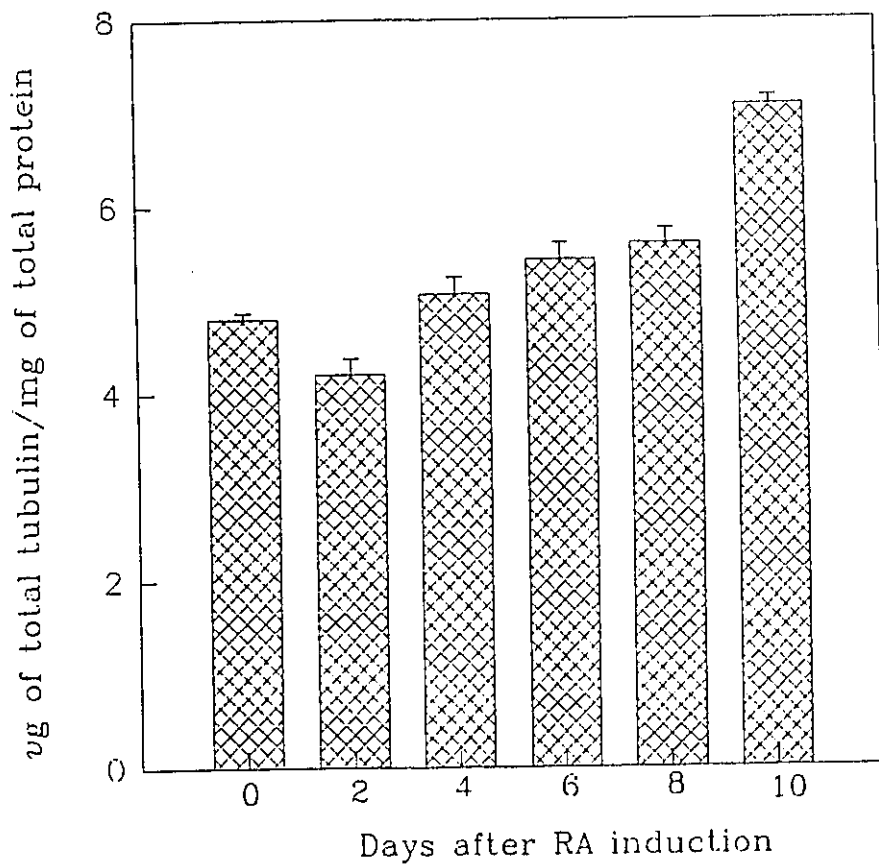


Figure 9. (see the back of the page).

Figure 9. ELISA results showing the change in the relative amount of β III-tubulin in the total microtubule array during neuronal differentiation. The values for each day are derived from triplicate samples from three separate experiments. Standard deviations are indicated by error bars.

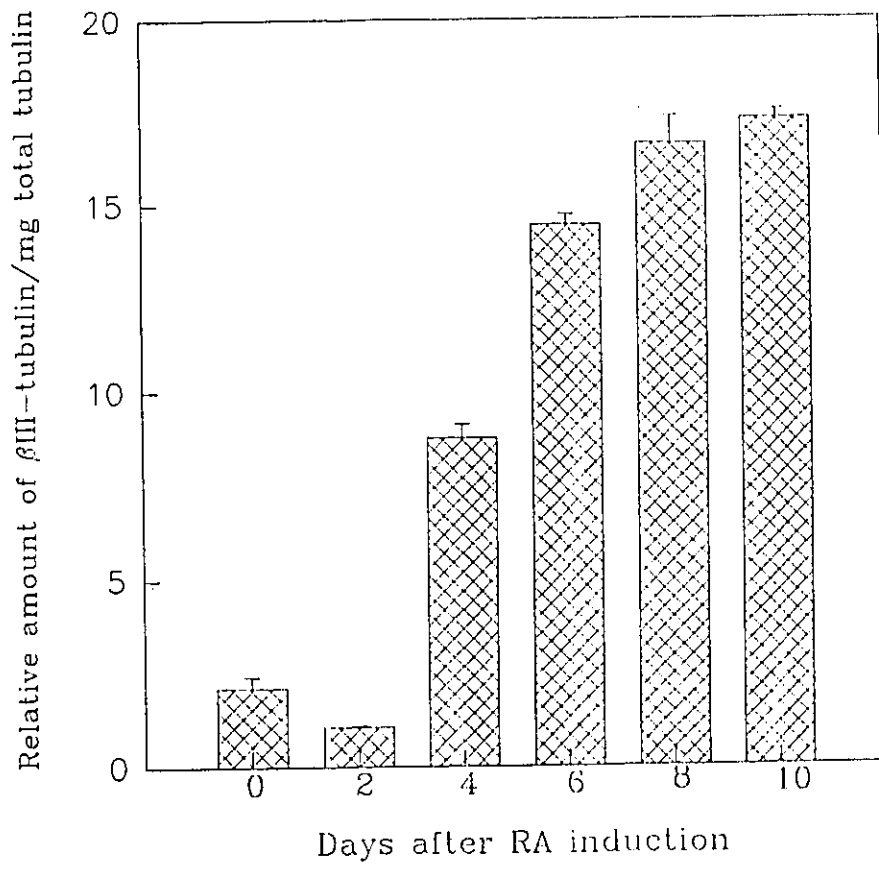
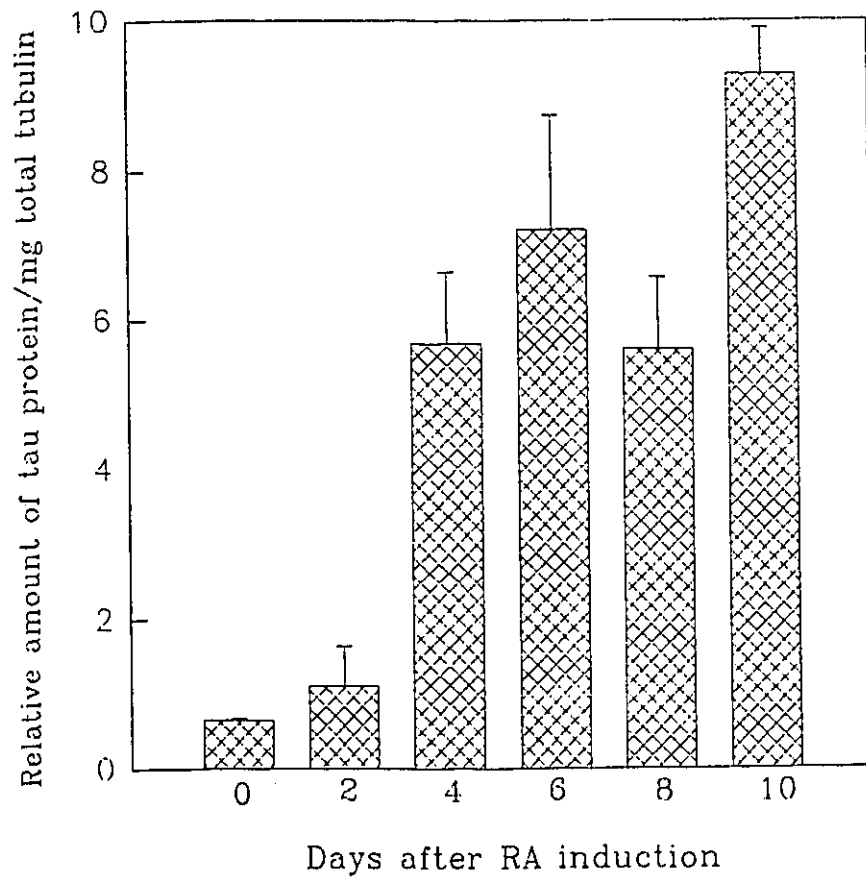


Figure 10. (see the back of the page).

Figure 10. ELISA results showing the change in the relative amount of tau protein in the total microtubule array during neuronal differentiation. The values for each day are derived from triplicate samples from three separate experiments. Standard deviations are indicated by error bars.



by treatment of cells with the microtubule depolymerizing drug colchicine.

Immunofluorescence

Fig. 11a-c shows double immunostaining of tubulin and tau in undifferentiated EC cells after 45 minutes of 1 $\mu\text{g/ml}$ colchicine treatment at 37°C. Most microtubules in undifferentiated cells (Fig. 11a) are depolymerized after 45 minute treatment with colchicine (Fig. 11b). Tau is not expressed in undifferentiated cells, therefore, no staining is observed for tau protein in the colchicine-treated samples (Fig. 11c).

At 3 days of differentiation (Fig. 11d-f), microtubules in non-neuronal cells disappeared following colchicine treatment, but the cells with short processes are still stained by the antibody to tubulin (Fig. 11e) and by the antibody to tau protein (Fig. 11f).

At 6 days (Fig. 12a-c) and 12 days (Fig. 12d-f) after RA induction, many cells with extensions contain microtubules which are colchicine resistant (Figs. 12b and Fig. 12e), and these cells also express tau protein (Figs. 12c and Fig. 12f). After colchicine treatment, the staining pattern with anti-tubulin becomes similar to the staining

Figure 11. (see the back of the page).

Figure 11. Double immunofluorescence staining of day 0 undifferentiated D310 EC cells and day 3 RA-induced cells after 45 minutes colchicine treatment. (a) Phase contrast of day 0 cells. (b) Anti-tubulin (YOL 1/34) staining of tubulin in same cells as (a). (c) The same cells as in (b) labelled with anti-tau (polyclonal antibody). (d), (e), and (f) show colocalization of tau protein and colchicine stable microtubules in day 3 neurally differentiated cells. (d) Phase contrast of day 3 cells. (e) Anti-tubulin staining of same day 3 cells. (f) Anti-tau staining of same day 3 cells. Note tau protein colocalizes with stable microtubules in the cells with neurite extensions. Magnification: 400X.

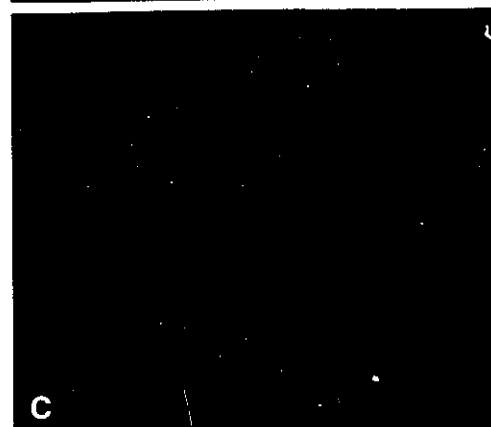
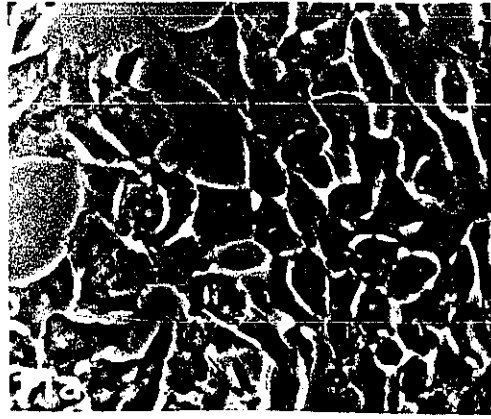
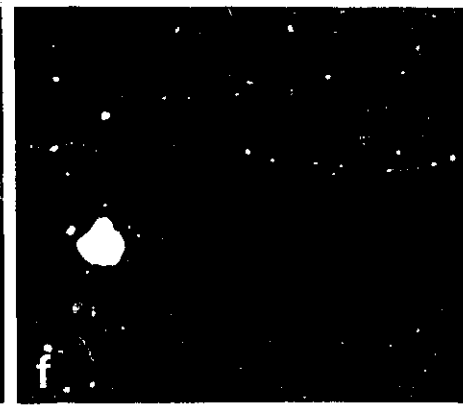
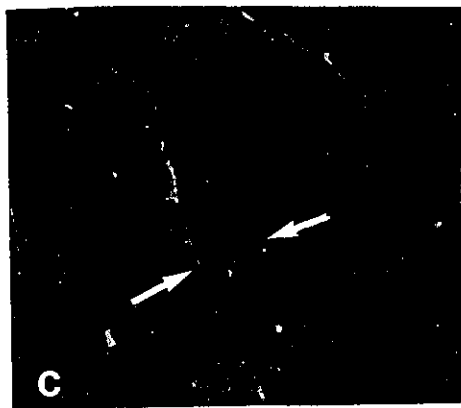
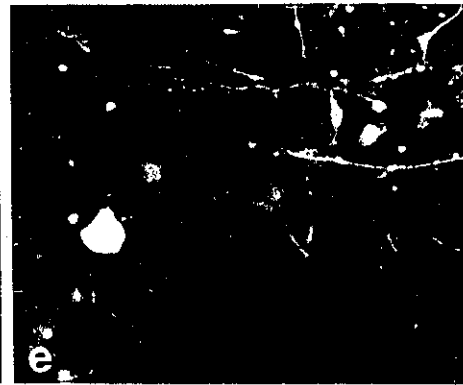
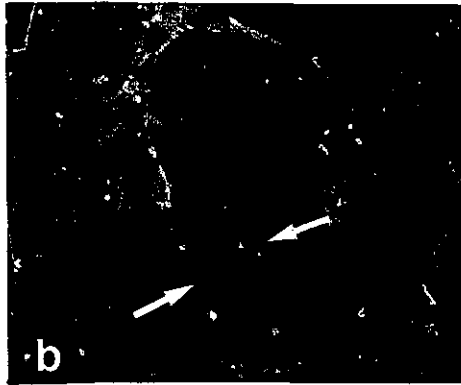
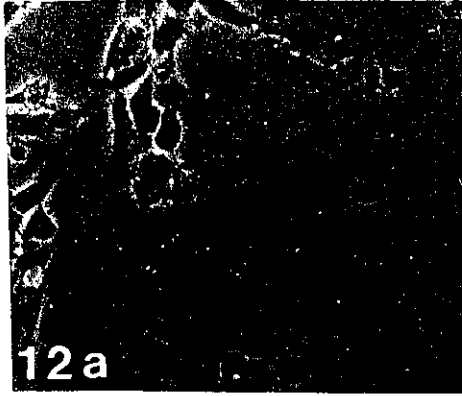


Figure 12. (see the back of the page).

Figure 12. Double immunofluorescence staining showing the colocalization of tau protein and colchicine stable microtubules in day 6 and day 12 RA-induced cells. (a) Phase contrast of day 6 colchicine-treated cells. (b) Anti-tubulin (YOL 1/34) staining of same cells as in (a). (c) Anti-tau staining of same cells. (d) Phase contrast of day 12 colchicine-treated cells. (e) Anti-tubulin staining of same cells as in (d). (f) Anti-tau staining of the same day 12 colchicine-treated cells. Note the beaded morphology is shown by the anti-tubulin staining (arrows in b and c). Magnification: 400X.



with anti-tau showing the beaded morphology (arrows in Fig. 12).

Immunoblotting

Immunofluorescence microscopy shows that the stability of neuronal microtubules to the depolymerizing drug colchicine increases during RA-induced neuronal differentiation. This increased stability of microtubules is accompanied by induction of tau expression. To determine whether the tau protein present is associated with assembled microtubules and remains associated after colchicine treatment, immunoblotting was carried out on unassembled (soluble) and assembled (polymeric) tubulin fractions from control and colchicine-treated cells (Fig. 13).

In undifferentiated EC cells, tubulin was identified in both polymeric and soluble fractions. At 3 days of neuronal differentiation, more tubulin assembles into microtubules which are present in the polymeric fraction and these are resistant to colchicine. At 6 and 12 days of differentiation, the majority of tubulin is present in the polymeric fraction of the untreated and colchicine-treated cells.

The changes in amount of acetylated α -tubulin in soluble and cytoskeletal fractions are also shown in Fig.

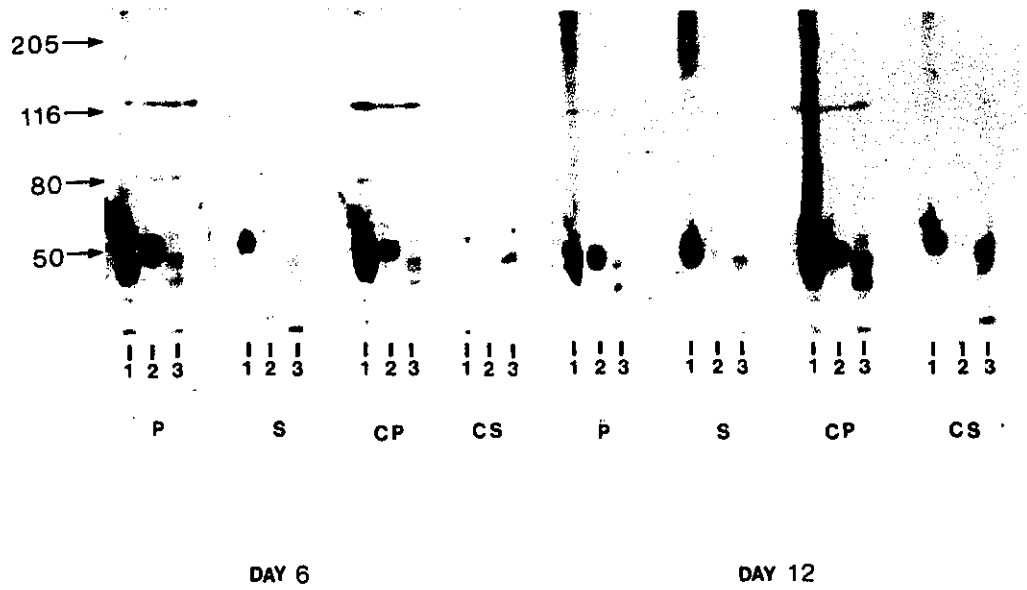
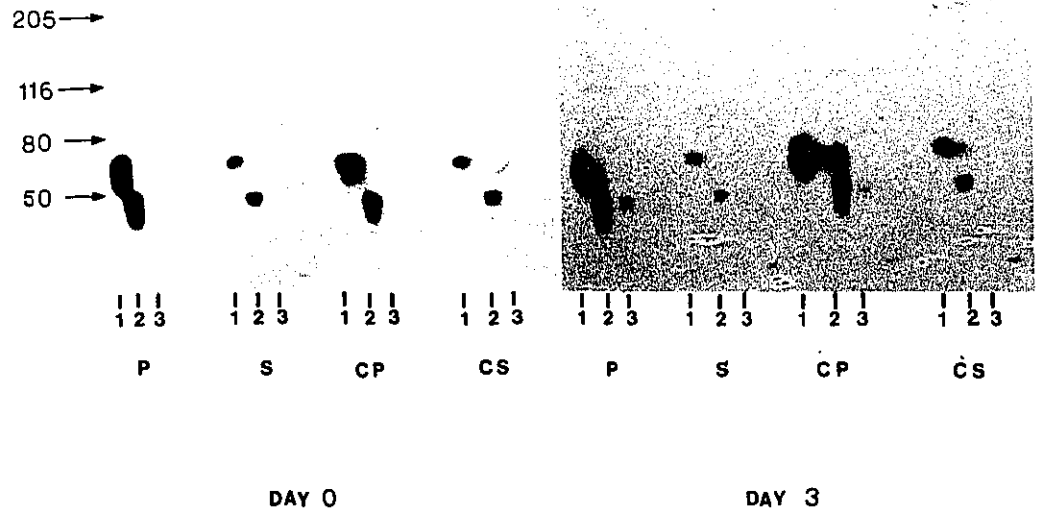
13. In undifferentiated EC cells, most acetylated α -tubulin is polymer associated, but there is a small proportion present in the soluble fraction in both untreated and colchicine-treated samples. At 3 days of differentiation, the amount of acetylated α -tubulin in the polymer fraction increases, but some is still detectable in the soluble fraction. At this time, although most acetylated α -tubulin is resistant to colchicine, a small proportion of acetylated α -tubulin appears in the soluble fraction after colchicine treatment. At day 6 and day 12 of differentiation, acetylated α -tubulin is no longer detectable in the soluble fractions of both colchicine treated and non-colchicine treated samples. The mobility of the acetylated α -tubulin changes during differentiation and was at a lower apparent molecular weight than the β -tubulin in the day 0 and day 3 samples.

In agreement with the immunofluorescence observations, tau starts to be expressed at 3 days of differentiation. Two low molecular weight isoforms are detected in the polymer fraction and these appear to be present in the polymer fraction after colchicine treatment. At days 6 and 12 of differentiation, at least four isoforms are observed in the polymer fractions and some of the two isoforms expressed at day 3 start to be detected in the soluble fraction in both the untreated and colchicine-treated samples.

Figure 13. (see the back of the page).

Figure 13. Immunoblotting analysis of time course expression of tau protein during neuronal differentiation and of the partitioning of tau protein into the soluble and polymeric tubulin fractions of untreated and colchicine-treated cells. P, polymer fraction and S, soluble fraction from untreated cells. CP, polymer fraction and CS, soluble fraction from colchicine-treated cells. 50 μ g protein were loaded per well for each sample. 1, tubulin probed with DM1B antibody; 2, acetylated α -tubulin probed with 6-11b-1 antibody; 3, tau protein probed with tau-1 antibody. Positions of molecular weight standards are indicated on the left.

Fig. 13



The bands on about 116 kD position in polymer fractions from all day 0, day 3, day 6, and day 12 samples and the bands on about 80 kD position in polymer fractions from day 6 samples are caused by the non-specific secondary antibody binding.

Immunogold labelling of light microscopy

To confirm that the beaded morphology of tau protein observed by immunofluorescence was not due to the fixation procedure, several other fixation protocols were tried, but they failed to show immunofluorescence staining with anti-tau antibody. In contrast, immunogold light microscopic labelling using a different fixation protocol was positive. One nm gold particles were silver enhanced and the samples were observed by bright-field and dark-field microscopy. The day 6 culture was chosen since the majority of cells in the culture showed neuronal processes and expressed all the neuronal specific proteins examined.

Fig. 14 shows the immunogold labelling of β III-tubulin (Fig. 14a, 14c and 14e) and tau (Fig. 14b, 14d and 14f) in untreated (Fig. 14a and 14b) and colchicine-treated (Fig. 14c and 14d) cells. In these bright-field micrographs, gold particles labelling tau and β III are localized in both the cell body and neurite processes (Fig.

14a and 14b), but are more concentrated in the processes. The gold particles over the processes show a discontinuous pattern similar to the beaded morphology seen by immunofluorescence (arrows in Fig. 14a and 14b). After colchicine treatment, gold particles are still detected in the cell body and neurite processes (Fig. 14c and 14d) and the discontinuous labelling (arrows in Fig. 14c and 14d) is more evident.

By dark-field microscopy (Fig. 15), the discontinuous distribution of gold particles detecting β III-tubulin and tau protein along neurite processes was even more evident. The labelling of the cells after colchicine treatment shows that gold particles are more densely packed in some parts of the processes, whereas in other parts there are no gold particles at all (arrows in Fig. 15c and 15d). The staining patterns for β III and tau are very similar at this point.

3.5 Localization of Tau Protein on Microtubules at EM Level

The beaded morphology of neurite processes immunolabelled for tau protein was observed by immunofluorescence and was confirmed by immunogold light microscopic labelling. To study the distribution of tau more precisely, immunogold labelling at the electron

Figure 14. (see the back of the page).

Figure 14. Immunogold bright-field light microscopy showing the localization of tau and β III-tubulin in day 6 RA-induced cells. (a) β III-tubulin labelled with TuJ1 antibody. (b) Tau protein labelled with anti-tau polyclonal antibody. (c) β III-tubulin in colchicine-treated cells. (d) Tau protein in colchicine-treated cells. (e) and (f) Controls for anti- β III tubulin and anti-tau staining in which the primary antibodies were omitted. Note the beaded morphology shown for both β III and tau (arrows in (a) and (b)). Arrows in (c) and (d) show discontinuous labelling of gold particles. Magnification: 800X.

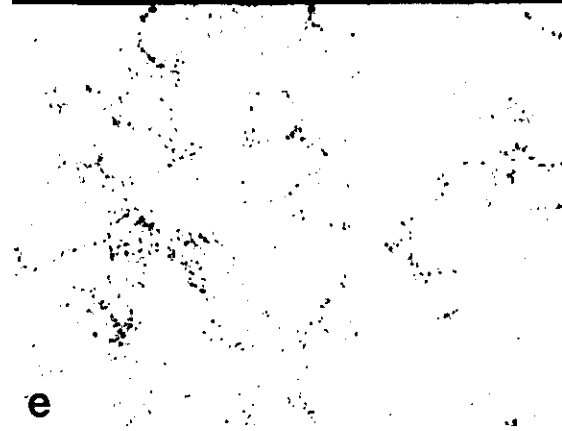
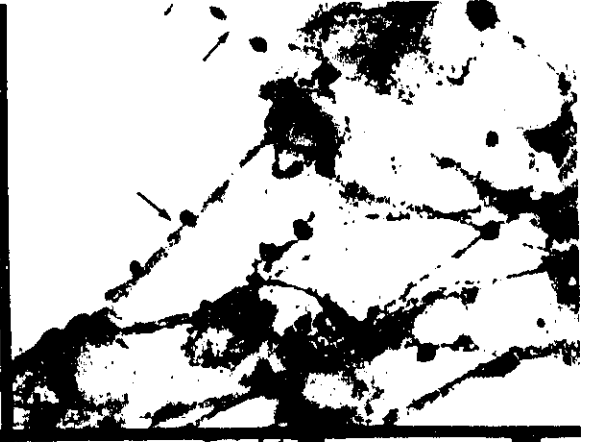
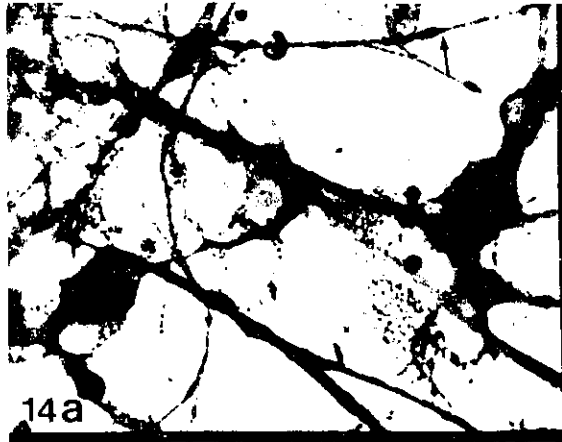
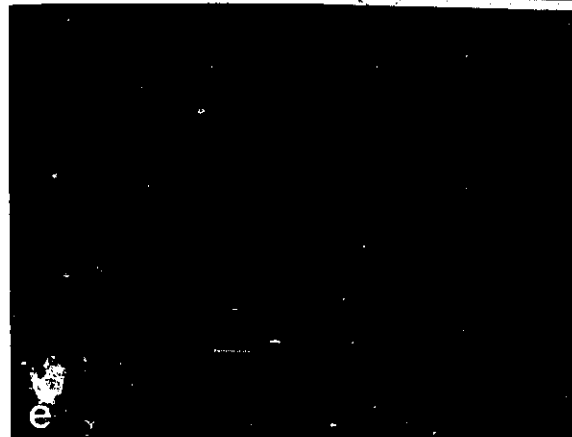


Figure 15. (see the back of the page).

Figure 15. Immunogold dark-field microscopy showing the localization of β III-tubulin and tau protein in 6 day RA-induced cells. (a) β III-tubulin labelled with TuJ1 antibody; (b) tau protein labelled with anti-tau polyclonal antibody; (c) β III-tubulin in colchicine-treated cells; (d) tau protein in colchicine-treated cells. (e) and (f) controls for anti- β III tubulin and anti-tau staining in which the primary antibodies were omitted. Arrows in (c) and (d) show discontinuous labelling of gold particles. Magnification: 800X.



microscopic level was performed on day 6 neuronally differentiated cultures. Figs. 16 and 17 show the general morphology of a cell body (Fig. 16a, 17) and neurite processes from 6 day RA-induced cells (Fig. 16b and 16c). These cells were grown on plastic coverslips and were simultaneously fixed and permeablized with glutaraldehyde and Triton x-100 using the same protocol as for immunogold labelling. The preservation of the structure of the nucleus, nuclear envelope, and cytoplasmic organelles, such as the mitochondria, is good. Within the neurites, some organelles are present.

Immunogold labelling of tau protein is shown in Fig. 18. One nm gold particles were silver-enhanced. In many fields of view, gold particles are showing a discontinuous or non-uniform distribution along the microtubules of the neurite processes (Fig. 18a). After colchicine treatment (Fig. 18b), anti-tau labelling remains localized on microtubules, although the number of gold particles appears fewer than on the samples that were not colchicine-treated.

Figure 16. (see the back of the page).

Figure 16. Electron micrographs of day 6 differentiated EC cells. (a) Region of a cell body (Cb) showing part of a nucleus, mitochondria and ribosomes. (b) and (c) Regions of neurite (N) processes showing large numbers of filaments running parallel to the long axis of the processes. Magnification: 11250X.

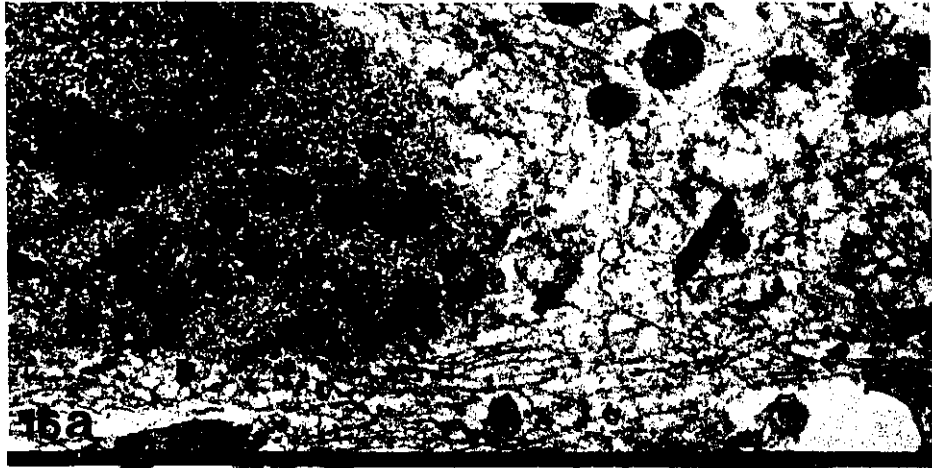


Figure 17. (see the back of the page).

Figure 17. Higher magnification of the cell body in Fig. 16a region from the day 6 differentiated EC cell showing nucleus (N), nucleolus (Nu), mitochondria (M), and rough endoplasmic reticulum (Er). Magnification: 17500X.

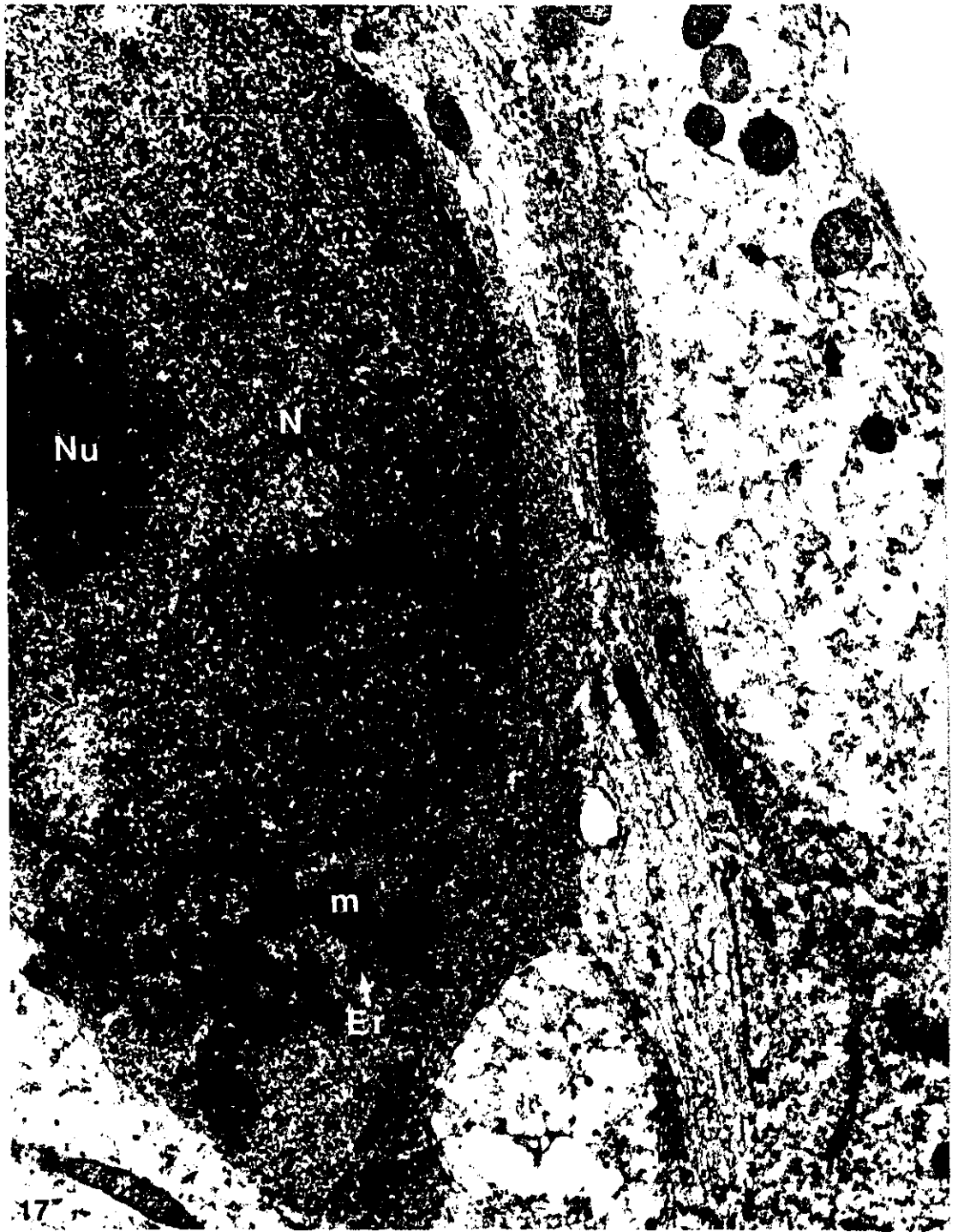
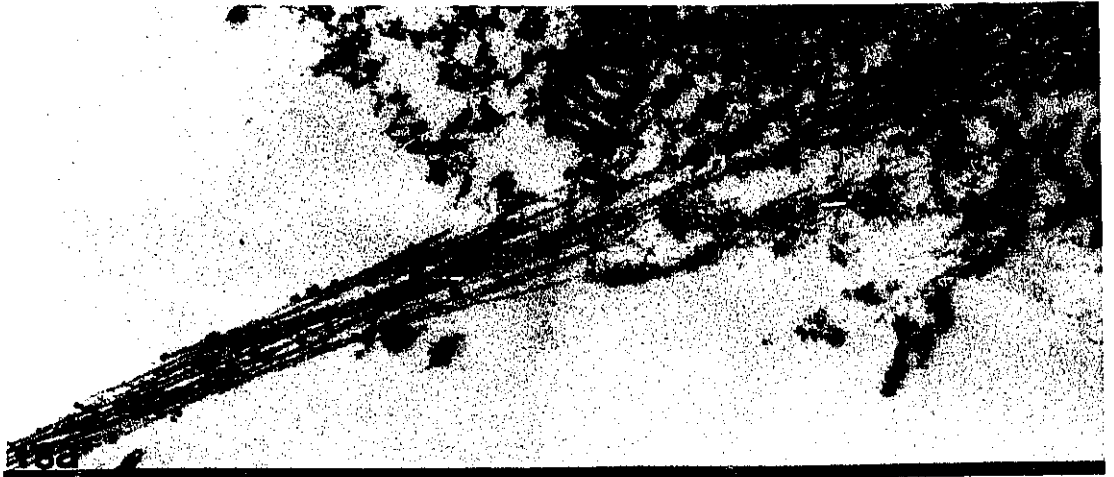


Figure 18. (see the back of the page).

Figure 18. Electron micrographs showing the localization of tau on microtubules in neurite processes of day 6 differentiated cells. (a) Tau protein labelled by gold particles is localized on a bundle of microtubules in a non-colchicine treated neurite process. (b) Tau protein labelled by gold particles is still seen on microtubules in a neurite extension after colchicine treatment. (c) Control sample in which the primary antibody was omitted. Magnification: (a), 25000X; (b), 37500X; (c), 37500X.



DISCUSSION

In the present study D310 EC cells were used to explore the relationship between tau expression and the development of stable microtubules during neurogenesis. In these neurally differentiated EC cells, tau expression is developmentally regulated. Also, the increase of microtubule stability and tau expression is highly correlated.

4.1 RA-induced neurally differentiated D310 EC cells

The results of this study demonstrate that D310 EC cells are capable of differentiating into neurons after RA-induction and express several neuronal markers including class III β -tubulin, juvenile MAP2c, high molecular weight MAP2, neurofilament protein, and tau protein. Previous studies by Falconer *et al.* (1989a, 1989b, 1992) had already documented that in early neurally differentiated cells induced by RA, P19 cells also formed neurons and expressed the same cytoskeletal neuronal markers. However, P19 cultures induced by RA are a heterogeneous mixture of cells which includes neurons, glial cells and fibroblast-like cells. The D310 cell line, which is a subclone of P19

cells, still gives a mixed population of cells, but it has been suggested (M. McBurney, personal communication) that this cell line differentiates into more neurons than the P19 cells do after RA induction. In addition, preliminary ELISA experiments that I carried out using β III-tubulin expression as a marker for neuronal differentiation also showed that D310 cells produce more neurons than P19 cells. This provides a good model system for the study of tubulin isoforms and their developmental regulation during neurogenesis.

4.2 Developmental Regulation of Tau Expression

Tau protein is not expressed in undifferentiated EC cells, starts to be expressed at 3 days of RA induction, and the amount of tau increases about two-fold from day 4 to day 10 of differentiation as the number of neurons increases in the culture. During RA-induced neuronal differentiation in P19 culture the expression of MAP2 also showed developmental regulation (Falconer *et al.*, 1992, 1993). Juvenile MAP2c is first detected at day 2 of neuronal differentiation and expression of MAP2c increases through day 6 after RA addition. High molecular weight MAP2 appears by day 6. These changes in MAP expression in neuronally differentiating EC cells are similar to that in brain.

The study by Binder et al. (1985) concluded that tau protein is localized in axons in the central nervous system of rat brain. Although it has not yet been well established that the neurons derived from EC cells have dendrites and axons, double immunofluorescence staining with anti-tubulin and anti-tau does show that anti-tau antibody only labels some neurite processes. This suggests that the neurite extensions expressing tau protein may be developing axons, whereas those not expressing tau may be dendrites. The study by Falconer et al. (1989a) demonstrated that MAP2c first was detected at day 2 of RA-induced cells and at later development, adult MAP2 starts to be expressed and was localized to only some of the processes. This suggests that MAP2 positive processes may differentiate into dendrites. Previous study concluded that MAP2 localized to dendrites while tau localized to axons (Hirokawa, 1982).

ELISA data provided in this study also support the immunofluorescence observations of the increase in tau expression during neuronal differentiation. The amount of total tubulin in undifferentiated EC cells is about the same as the level of tubulin in fibroblast-like cells. The statistical analysis (T-test) showed that there was no significant difference between the amount of total tubulin in day 2 RA cultures and the amount of total tubulin in day 0 cultures. The amount of total tubulin starts increasing after day 4 of RA induction, and slowly reaches about 0.75%

of total protein at 10 days of differentiation. This level of tubulin is much lower than 11-12% of tubulin in adult chick brain (Bamburg et al., 1973). It is not clear why the amount of tubulin in RA-induced neuronally differentiated cultures is so much lower than that in brain. However, there are a few possibilities which may contribute to the low level of tubulin. First, there are several types of cells in the culture, such as the fibroblast-like cells which probably have lower amount of microtubules than neuronal cells. Second, the differentiation is not synchronous, some neurons are more mature than the others, and the immature neurons contain less microtubules. Furthermore, the neurites differentiated from EC cultures are smaller and thinner in comparison to those in mature brain.

Class III β -tubulin is an indicator of neuronal differentiation (Lee et al., 1990b). The TuJ1 antibody to class III β -tubulin was used in this study to identify neurons from a background of fibroblast-like cells. This antibody can detect differentiating neurons as early as 2 days after addition of RA before the extension of neurites occurs. In neuronally differentiated D310 EC cells, as soon as tau starts to be expressed, the increase in the levels of tau protein parallels the accumulation of class III β -tubulin. During the period of 10 days after RA induction, both class III β -tubulin and tau increased about two-fold.

During early neuronal development class III β -tubulin is differentially incorporated into colchicine-labile microtubules (Facolner et al., 1993). Since β III-tubulin is an indicator of neuronal differentiation, the increase in the amount of β III-tubulin indicates the increase in the numbers of neurons as well as the maturation of neurons in the culture. Therefore, the paralleled increase between the amount of tau protein and β III-tubulin expressed during neuronal differentiation demonstrates that the expression of tau is developmentally regulated in RA-induced EC cells. This observation is also similar to that in PC12 cells differentiated by NGF (Drubin et al. 1985). Following long-term NGF treatment, tubulin levels increased by about three-fold, while the increase in the levels of the various MAPs ranged between three- and 15-fold. The low level of class III β -tubulin detected in undifferentiated EC cells is probably due both to the background caused by the secondary antibody binding and the presence of some spontaneously differentiated cells which express class III β -tubulin in the culture. The apparent decrease in tau expression at day 8 of differentiation may be result from the presence of rapidly dividing glial cells in the culture (M. McBurney, personal communication) which would cause an increase in total tubulin, but the relative amount of tau remains the same.

Four isoforms of tau proteins were detected in RA-

induced neuronally differentiated D310 cells by immunoblotting. Two isoforms appeared at 3 days after RA induction, and four isoforms were detected after 6 days of differentiation. The changes from two isoforms to four isoforms of tau proteins indicate that the transition from juvenile isoforms to adult isoforms occurred during neuronal differentiation of D310 EC cells, as well as the heterogeneity of tau increased during neuronal development. This correlates well with the developmental changes of tau in brain (Couchie and Nunez, 1985; Riederer and Innocenti, 1991; Takemura et al., 1991; Larcher et al., 1992). In newborn rat brain, the tau complex consists of two major components, whereas in adult tissue six components are present. The transition from juvenile tau to the adult isoforms begins around postnatal day 15 and is completed by day 35 (Couchie and Nunez, 1985). The developmental changes of tau expression are also observed in developing cat cerebral cortex and *corpus callosum*. During the postnatal development of cat visual cortex and *corpus callosum*, the molecular composition of tau protein changes between postnatal day 19 and 39 from a set of two juvenile forms to a set of at least two adult variants (Riederer and Innocenti, 1991). Tau protein heterogeneity increases dramatically during the second week of mouse brain development (Larcher et al., 1992). The study of *in situ* hybridization of tau mRNA in rat central nervous system

shows that the expression of tau mRNAs is abundant during the first postnatal week, but is reduced by the third postnatal week in neural cells throughout the cortical plate of the cerebral cortex (Takemura *et al.*, 1991). The increased heterogeneity of tau protein during neuronal differentiation observed in this study is also similar to that in PC12 cells induced to differentiate by NGF, in which both immature and mature tau isoforms were detected and the transition from the immature to the mature isoforms occurred after 1-2 days in culture (Hanemaaijer and Ginzburg, 1991).

4.3 Tau Expression and Neurite Outgrowth

In neurons derived from D310 EC cells by RA induction, the short neurite processes start to be observed at day 3, although some cells start to express class III β -tubulin before any obvious morphological change. The expression of tau protein occurs at the time when neurite processes appear and the level of tau increases as neurite processes become branched and extended. Although this observation does not provide a direct evidence for the role of tau protein in neurite outgrowth, the strong correlation between tau expression and neurite outgrowth is consistent with tau playing a role in the establishment of neurite

processes during neuronal development.

It has been suggested that tau functions in neurite elongation (Drubin *et al.*, 1985; Drubin *et al.*, 1988). When expressed in Sf9 cells from the moth ovary via a baculovirus, tau induced the outgrowth of long processes (Knops *et al.*, 1991). Tau may also have a role in the generation of asymmetric neuronal morphologies, because tau antisense oligonucleotide exposure in neuronal cell culture specifically inhibits the elaboration of an elongated, nontapering axon-like structure (Caceres and Kosik, 1990; Kosik and Caceres, 1991; Caceres *et al.*, 1991).

4.4 Tau Expression and Microtubule Stability

Neurite outgrowth is accompanied by an increase in the microtubule population, as well as an increase in microtubule stability (Matus, 1988).

The data presented in this study shows that during RA-induced neuronal differentiation the microtubule population increases steadily, and microtubule stability to colchicine depolymerization also increases. Both of these increases are accompanied by the increase in tau protein expression.

Immunofluorescence staining demonstrates that in undifferentiated EC cells almost all microtubules

disappeared after 45 min colchicine treatment, whereas the microtubules in RA-induced cells become more and more resistant to colchicine depolymerization during differentiation along the neuronal pathway. An early study by Falconer *et al.*, (1989a) also found that a population of colchicine-stable, acetylated microtubules appeared during early neuronal differentiation in P19 EC cells. In undifferentiated EC cells, most of microtubules are labile and are depolymerized by colchicine treatment (Fig. 11b). The tubulin from these depolymerized microtubules should be detected in the soluble fraction extracted from colchicine-treated cells. This is not seen in the day 0 samples of Fig. 13. The reason is probably caused by the percentage of tubulin in whole cell proteins. In EC cells about 70% of total cell protein is present in the polymer fraction and 30% is in the soluble fraction. It is possible that colchicine treatment has an effect on protein solubility which results in an increase in the percentage of soluble protein in the colchicine-treated samples. It is also possible that the extraction procedure applied in the experiments is not effective in removing all colchicine-labile tubulin from the polymer fraction. In the immunoblotting experiments an equal amount of protein from each sample was loaded. Therefore the amount of tubulin from colchicine-treated soluble fraction was actually less than that from untreated soluble fraction. At day 6 which

is the best stage for neuronal cell production, tubulin becomes prominent in the colchicine-treated polymer fraction. The soluble fraction contains very little tubulin after colchicine treatment. Again at day 12, more tubulin appears in the soluble fraction after colchicine treatment compared to that at day 6. This could be caused by the increase of glial cells in the cultures since glial cells start to proliferate at about day 8 after RA addition (Jones-Villeneuve *et al.*, 1982).

In RA-induced D310 cells, after tau protein is expressed, it appears that tau protein is consistently colocalized with colchicine-resistant microtubules, suggesting that tau is important in the formation of stable microtubules during neuronal differentiation. This is also supported by immunoblotting results. Tau protein started to be detected in 3 day RA cells which agreed with the immunofluorescence staining. Furthermore, tau appeared to be present in colchicine-treated polymer fractions which provided more evidence that tau colocalized with stable microtubules. At later days of differentiation, both the heterogeneity and the amount of tau protein increased. Taken together, these results agreed with the early studies in the developmental regulation of MAP expression in brain (reviewed by Matus, 1991), which further supports the hypothesis that tau protein is important in the development of stable microtubules during neuronal development.

Interestingly, the acetylated α -tubulin recognized by the antibody 6-11b-1 ran faster than β -tubulin at day 0 and day 3 and then shifted up at day 6 and day 12. α - and β -tubulin are not usually separated on SDS/polyacrylamide gel. To separate α - and β -tubulin, the proteins need to be carboxymethylated, and subjected to electrophoresis in 8M urea-acrylamide gels and usually β -tubulin runs faster than α -tubulin (Bryan and Wilson, 1971). Although it is not clear why α - and β -tubulin were separated in these experiments, the change in mobility of α -tubulin at day 6 and day 12 RA cells suggests that posttranslational modifications may have occurred. It has been suggested that posttranslational modifications of tubulin isotypes play a role in the formation of stable microtubules during neuronal differentiation (reviewed by Falconer *et al.*, in press). Preliminary isoelectric focusing data (Laferriere and Brown, 1992) indicate that undifferentiated P19 cells have a single, non-phosphorylated form of class III β -tubulin. In 3 day RA cells, two isoforms are present, and by day 6 this increases to six or more isoforms. One phosphorylated isoform is detected and the other isoforms are presumed to be due to polyglutamylation. To determine whether this change in mobility of α -tubulin position is caused by posttranslational modifications or not, isoelectric focusing technique should be applied.

4.5 Association of Tau and Stable Microtubules

In D310 cultures induced to differentiate along the neural pathway, the beaded-morphology by anti-tau immunofluorescence staining is consistently observed. Preliminary immunogold electron microscopy indicated that tau appeared to be unevenly distributed on microtubules. An alternative explanation is that labelled portions of the microtubule bundles were out of the plane of section. Examination of thick sections or of serial sections would have eliminated this possibility. Co-expression of tau protein with colchicine-stable microtubules is also demonstrated by both immunofluorescence and immunoblotting, thus suggesting that tau protein associates with colchicine-stable microtubules. Although the time course of tau expression was examined in neurally differentiated D310 EC cells and the correlation between tau expression and the development of stable microtubules during neuronal differentiation was explored, many questions regarding the functions of tau protein in stabilizing microtubules still remain to be answered. For example, is tau responsible for the formation of the stable segments of microtubules in axons or does it just passively associate with the stable segments; are there any tubulin isoforms preferentially associated with tau in the stable microtubules; and are any posttranslational modifications of both tubulin and tau

protein involved in the development of stable microtubules.

REFERENCES

- Alexander, J. E., Hunt, D. F., Lee, M. K., Shabanowitz, J., Michel, H., Berlin, S. C., Macdonald, T. L., Sundberg, R. J., and Frankfurter, A. (1991). Characterization of posttranslational modifications in neuron-specific class III β -tubulin by mass spectrometry. *Proc. Natl. Acad. Sci. USA* 88:4685-4689.
- Andreadis, A., Brown, W. M., and Kosik, K. S. (1992). Structure and novel exons of the human τ gene. *Biochem.* 31:10626-10633.
- Argarana, C. E., Barra, H. S., and Caputto, R. (1978). Release of ^{14}C tyrosine from tubulinyl- ^{14}C tyrosine by brain extract. Separation of a carboxypeptidase from tubulin tyrosine ligase. *Mol. Cell Biochem.* 19:17-22
- Argarana, C. E., Barra, H. S., and Caputto, R. (1980). Tubulinyl-tyrosine carboxy peptidase from chicken brain: properties and partial purification. *J. Neurochem.* 34:114-118.
- Asai, D. J., and Remolona, N. M. (1989). Tubulin isotype usage in vivo: a unique spatial distribution of the minor neuronal-specific β -tubulin isotype in pheochromocytoma cells. *Dev. Biol.* 132:398-409.
- Audenaert, R., Heremans, L., Heremans, K., and Engelborghs, Y. (1989). Secondary structure analysis of tubulin and microtubules with Raman spectroscopy. *Biochim. Biophys. Acta* 996:110-115.
- Bamburg, J. R., Shooter, E. M., and Wilson, L. (1973). Developmental changes in microtubule protein of chick brain. *Biochemistry* 12:1476-1481
- Banerjee, A., and Luduena, R. F. (1987). Kinetics of association and dissociation of colchicine-tubulin complex from brain and renal tubulin. Evidence for the existence of multiple isotypes of tubulin in brain with differential affinities for colchicine. *FEBS Lett.* 219:103-107.
- Banerjee, A., and Luduena, R. F. (1991). Distinct colchicine binding kinetics of bovine brain tubulin lacking the type III isotype of β -tubulin. *J. Biol. Chem.* 266:1689-1691.
- Baas, P. W., and Ahmad, F. J. (1992). The plus ends of

- stable microtubules are the exclusive nucleating structures for microtubules in the axon. *J. Cell Biol.* 116:1231-1241.
- Bass, P. W., and Black, M. M. (1990). Individual microtubules in the axon consist of domains that differ in both composition and stability. *J. Cell Biol.* 111:495-509.
- Bass, P. W., Pienkowski, T. P., and Kosik, K. S. (1991). Processes induced by tau expression in Sf9 cells have an axon-like microtubule organization. *J. Cell Biol.* 115:1333-1344.
- Bershadsky, A. D., and Vasiliev, J. M. (1988). *Cytoskeleton*. Plenum Press. New York and London.
- Binder, L. I., Frankfurter, A., and Rebhun, L. I. (1985). The distribution of tau in the mammalian central nervous system. *J. Cell Biol.* 101:1371-1378.
- Black, M. M. (1987). Comparison of the effects of microtubule-associated protein 2 and tau on the packing density of in vitro assembled microtubules. *Proc. Natl. Acad. Sci. USA* 84:7783-7787.
- Black, M. M., Bass, P. W., and Humphries, S. (1989). Dynamics of α -tubulin deacetylation in intact neurons. *J. Neurosci.* 9:358-368.
- Black, M. M., Cochran, J. M., and Kurdyla, J. T. (1984). Solubility properties of neuronal tubulin: evidence for labile and stable microtubules. *Brain Res.* 295:255-263.
- Black, M. M., and Greene, L. A. (1982). Changes in the colchicine susceptibility of microtubules associated with neurite outgrowth: studies with nerve growth factor-responsive PC12 pheochromocytoma cells. *J. Cell Biol.* 95:379-386.
- Black, M. M., and Keyser, P. (1987). Acetylation of α -tubulin in cultured neurons and the induction of α -tubulin acetylation in PC12 cells by treatment with nerve growth factor. *J. Neurosci.* 7:1833-1842.
- Bond, J. F., Fridovich-Keil, J. L., Pillus, L., Mulligan, R. C., and Solomon, F. (1986). A chicken-yeast chimeric β -tubulin protein is incorporated into mouse microtubules in vivo. *Cell* 44:461-468.
- Brady, S. T., Tytell, M., and Lasek, R. J. (1984). Axonal

tubulin and axonal microtubules: biochemical evidence for cold stability. *J. Cell Biol.* 99:1716-1724.

- Bryan, J., and Wilson, L. (1971). Are cytoplasmic microtubules heteropolymer? *Proc. Natl. Acad. Sci. USA* 68:1762-1766.
- Bulinski, J. C., Richards, J. E., and Piperno, G. (1988). Posttranslational modifications of α -tubulin: detyrosination and acetylation differentiate populations of interphase microtubules in cultured cells. *J. Cell Biol.* 106:1213-1220.
- Caceres, A. and Kosik, K. S. (1990). Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. *Nature* 343:461-463.
- Caceres, A., Potrebic, S., and Kosik, K. S. (1991). The effect of tau antisense oligonucleotides on neurite formation of cultured cerebellar macroneurons. *J. Neuroscience* 11:1515-1523.
- Cadrin, M., Wasteneys, G. O., Jones-Villeneuve, E. M. V., Brown, D. L., and Reuhl, K. R. (1988). Effects of methylmercury on retinoic acid-induced neuroectodermal derivatives of embryonal carcinoma cells. *Cell Biol. Toxicol.* 4:61-80.
- Cambray-Deakin, M. A., and Burgoyne, R. D. (1987). Posttranslational modifications of α -tubulin: acetylated and detyrosinated forms in axons of rat cerebellum. *J. Cell Bio.* 104:1569-1574.
- Cassimeris, L., Pryer, N. K., and Salmon, E. D. (1988). Real-time observation of microtubule dynamic instability in living cells. *J. Cell Biol.* 107:2223-2231.
- Cleveland, D. W., Hwo, S-Y., and Kirschner, M. W. (1977a) Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. *J. Mol. Biol.* 116:207-225.
- Cleveland, D. W., Hwo, S-Y., and Kirschner, M. W. (1977b). Physical and chemical properties of tau factor and the role of tau in microtubule assembly. *J. Mol. Biol.* 116:227-247.
- Couchie, D., Mavilia, C., Georgieff, I. S., Liem, R. K. H., Shelanski, M. L., and Nunez, J. (1992). Primary structure of high molecular weight tau present in the peripheral nervous system. *Proc. Natl. Acad. Sci.*

USA. 89:4378-4381.

- Couchie, D., and Nunez, J. (1985). Immunological characterization of microtubule-associated proteins specific for the immature brain. *FEBS Lett.* 188:331-335.
- Dahl, J. L. and Weibel, V. J. (1979). Changes in tubulin heterogeneity during post-natal development of the brain. *Biochem. Biophys. Res. Commun.* 86:822-828.
- Daniels, M. P. (1972). Colchicine inhibition of nerve fiber formation *in vitro*. *J. Cell Biol.* 53:164-176.
- Denoulet, P., Jeantet, C., and Gros, F. (1982). Tubulin microheterogeneity during mouse brain development. *Biochem. Biophys. Res. Commun.* 105:806-813.
- Diaz-Nido, J., Hernandez, M. A., and Avila, J. (1990). Microtubule proteins in neuronal cells, In "Microtubule proteins". Ed. J. Avila. CRC. Press, Inc. Boca Raton, Florida.
- Dotti, C. G., Banker, G. A., and Binder, L. I. (1987). The expression and distribution of the microtubule-associated proteins tau and microtubule-associated protein 2 in hippocampal neurons in the rat *in situ* and in cell culture. *Neuroscience* 23:121-130.
- Drubin, D. G., Caput, D., and Kirschner, M. W. (1984). Studies on the expression of the microtubule-associated protein, tau, during mouse brain development, with newly isolated complementary DNA probes. *J. Cell Biol.* 98:1090-1097.
- Drubin, D. G., Feinstein, S. C., Shooter, E. M., and Kirschner, M. W. (1985). Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. *J. Cell Biol.* 101:1799-1807.
- Drubin, D. G. and Kirschner, M. W. (1986). Tau protein function in living cells. *J. Cell Biol.* 103:2739-2746.
- Drubin, D. G., Kobayashi, S., Kellogg, D., Kirschner, M. W. (1988). Regulation of microtubule protein levels during cellular morphogenesis in nerve growth factor-treated PC12 cells. *J. Cell Biol.* 106:1583-1591
- Edde, B., De Nechaud, B., Denoulet, P., and Gros, F.,

- (1987). Control of isotubulin expression during neuronal differentiation of mouse neuroblastoma and teratocarcinoma cell lines. *Dev. Biol.* 123:549-558.
- Edde, B., de Denoulet, P., Nechaud, B., Koulakoff, A., Berwaldnetter, Y., and Gros, F. (1989). Posttranslational modification of tubulin in cultured mouse brain neurons and astroglia. *Biol. Cell.* 65:109-117.
- Edde, B., Rossier, J., Le Caer, J.-P., Desbruyeres, E., Gros, F., and Denoulet, P. (1990). Posttranslational glutamylation of α -tubulin. *Science* 247:83-85.
- Edwards, M. K. S., Harris, J. F., and McBurney, M. W. (1983). Induced muscle differentiation in an embryonal carcinoma cell line. *Mol. Cell. Biol.* 3:2280-2286.
- Falconer, M. M., Echeverri, C. J., and Brown, D. L. (1992). Differential sorting of beta tubulin isotypes into colchicine-stable microtubules during neuronal and muscle differentiation of embryonal carcinoma cells. *Cell Motil. Cytoskel.* 21:313-325.
- Falconer, M. M., Vaillant, A., Reuhl, K. R., Laferriere, N., and Brown, D. L. (1993). The molecular basis of microtubule stability in neurons. (*Neurotox.* in press).
- Falconer, M. M., Vielkind, U., and Brown, D. L. (1989a). Establishment of stable, acetylated microtubule bundle during neuronal commitment. *Cell Motil. Cytoskel.* 12:169-180.
- Falconer, M. M., Vielkind, U., and Brown, D. L. (1989b). Association of acetylated microtubules, vimentin intermediate filaments, and MAP2 during early neural differentiation in EC cell culture. *Biochem. Cell Biol.* 67:537-544.
- Ferreira, A., Busciglio, J., and Caceres, A. (1989). Microtubule formation and neurite growth in cerebellar macroneurons which develop in vitro: evidence for the involvement of the microtubule-associated proteins, MAP-1a, HMW-MAP2 and tau. *Dev. Brain Res.* 49:215-228.
- Ferreira, A. and Caceres, A. (1989). The expression of acetylated microtubules during axonal and dendritic growth in cerebellar macroneurons which develop in vitro. *Dev. Brain Res.* 49:205-213.

- Field, D. J., Collins, R. A., and Lee, J. C. (1984). Heterogeneity of vertebrate brain tubulins. *Proc. Natl. Acad. Sci. USA* 84:4041-4045.
- Francon, J., Lennon, A. M., Fellous, A., Mareck, A., Pierre, M., and Nunez, J. (1982). Heterogeneity of microtubule-associated proteins and brain development. *Eur. J. Biochem.* 129:465-471.
- Fulton, C. and Simpson, P. A. (1976). Selective synthesis and utilization of flagellar tubulin: the multitubulin hypothesis. In *cell Motility*, ed. R. Goldman, T. Pollard, J. Rosenbaum, pp. 987-1005. New York: Cold Springs Harbor Lab.
- Garcini, E. M., Corrochano, L., Wischik, C. M., Nido, J. D., Correas, I., and Avila, J. (1992). Differentiation of neuroblastoma cells correlates with an altered splicing pattern of tau RNA. *FEBS LETTERS* 299:10-14.
- Gard, D. L., and Kirschner, M. W. (1985). A polymer-dependent increase in phosphorylation of β -tubulin accompanies differentiation of mouse neuroblastoma cell line. *J. Cell Biol.* 100:746-774.
- Gelfand, V. I., and Bershadsky, A. D. (1991). Microtubule dynamics: mechanism, regulation, and function. *Ann. Rev. Cell Biol.* 7:93-116.
- Goedert, M. and Jakes, R. (1990). Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. *The EMBO J.* 9:4225-4230.
- Goedert, M., Spillantini, M. G., and Crowther, R. A. (1992). Cloning of a big tau microtubule-associated protein characteristic of the peripheral nervous system. *Proc. Natl. Acad. Sci. USA.* 89:1953-1957.
- Goedert, M., Spillantini, M. G., Potier, M. C., Ulrich, J., and Crowther, R. A. (1989). Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four random repeats: differential expression of tau protein mRNAs in human brain. *EMBO J.* 8:393-399.
- Gordon-Weeks, P. R., and Lang, R. D. A. (1988). The α -tubulin of the growth cone is predominantly in the tyrosinated form. *Dev. Brain Res.* 42:156-160.
- Gozes, I. and Littauer, V. Z. (1978). Tubulin microheterogeneity increases with rat brain

- maturation. *Nature* 276:411-413.
- Graham, C. F. (1977). Teratocarcinoma cells and normal mouse embryogenesis. In *Concepts in Mammalian Embryogenesis* (M.I. Sherman, Ed.), M.I.T. Press, Cambridge, M.A. pp.315-397.
- Hagestedt, T., Lichtenberg, B., Wille, H., Mandelkow, E-M., and Mandelkow, E. (1989). Tau protein becomes long and stiff upon phosphorylation: correlation between paracrystalline structure and degree of phosphorylation. *J. Cell Biol.* 109:1643-1651.
- Hanemaaijer, H. and Ginzburg, I. (1991). Involvement of mature tau isoforms in the stabilization of neurites in PC12 cells. *J. Neuroscience. Res.* 30:163-171.
- Heidemann, S. R., Hamborg, M. A., Thomas, S. J., Song, B., Lindley, S., and Chu, D. (1984). Spatial organization of axonal microtubules. *J. Cell Biol.* 99:1289-1295.
- Himmler, A., Drechsel, D., Kirschner, M. W., and Martin, D. W. (1989). Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. *Mol. Cell. Biol.* 9:1381-1388.
- Hirokawa, N. (1982). The crosslinker system between neurofilaments, microtubules and membranous organelles in frog axons revealed by quick freeze, freeze-fracture, deep-etching method. *J. Cell Biol.* 94:129-142.
- Hirokawa, N., Shiomura, Y., and Okabe, S. (1988). Tau proteins: the molecular structure and mode of binding on microtubules. *J. Cell Biol.* 107:1449-1459.
- Jacobs, J. R., and Stevens, J. K. (1986). Changes in the organization of the neuritic cytoskeleton during nerve growth factor-activated differentiation of PC12 cells: a serial electron microscopic study of the development and control of neurite shape. *J. Cell Bio.* 103:895-906.
- Jetten, A. M. (1986). Induction of differentiation of embryonal carcinoma cells by retinoids. In *"Retinoids and Cell Differentiation"* (M.I. Sherman, Ed.), CRC Press, Boca Raton, FL. pp.105-135.
- Jones-Villeneuve, E. M. V., McBurney, M. W., Rogers, K. A., and Kalnins, V. I. (1982). Tetinoic acid induces

- embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* 94:253-262.
- Joshi, H. C., Bass, P., Chu, D. T., and Heidemann, S. R. (1986). The cytoskeleton of neurites after microtubule depolymerization. *Exp. Cell Res.* 163:233-245.
- Kanai, Y., Chen, J., and Hirokawa, N. (1992). Microtubule bundling by tau proteins *in vivo*: analysis of functional domains. *EMBO J.* 11:3953-3961.
- Kanai, Y., Takemura, R., Oshima, T., Mori, H., Ihara, Y., Yanagisawa, M., Masaki, T., and Hirokawa, N. (1989). Expression of multiple tau isoforms and microtubule bundle formation in fibroblasts transfected with a single tau cDNA. *J. Cell Biol.* 109:1173-1184.
- Kilmartin, J. V., Wright, B., and Milstein, C. (1982). Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576-582.
- Kirschner, M. and Mitchison, T. (1986). Beyond self-assembly: from microtubules to morphogenesis. *Cell* 45:329-342.
- Knops, J. Kosik, K. S., Lee, G., Pardee, J. D., Cohen-Gould, L., and McConlogue, L. (1991). Overexpression of tau in a nonneuronal cell induces long cellular processes. *J. Cell Biol.* 114:725-733.
- Kosik, K. S. and Caceres, A. (1991). Tau protein and the establishment of an axonal morphology. *J. Cell Science* 15:69-74.
- Kosik, K. S., Orecchio, L. D., Bakalis, S., and Neve, R. L. (1989). Developmentally regulated expression of specific tau sequences. *Neuron* 2:1389-1397.
- Kreis, T. E. (1987). Microtubules containing detyrosinated tubulin are less dynamic. *EMBO J.* 6:2596-2606.
- Kumar, N., and Flavin, M. (1981). Preferential action of a brain detyrosinolytic carboxypeptidase on polymerized tubulin. *Proc. Natl, Acad. Sci. USA* 256:7678-7686.
- Kuznetsov, S., Rodinov, V., Gelfand, V., and Rosenblat, V. (1984). Microtubule associated protein 2 competes with microtubule associated protein 1 for binding to microtubules. *Biochem. Biophys. Res. Commun.* 119:173-178.

- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Laferriere, N., and Brown, D. L. (1992). Expression and posttranslational modification of beta III tubulin during differentiation of embryonal carcinoma cells. 5th Intl. Congress Cell Biol. Abstract 199.
- Larcher, J. C., Boucher, D., Ginzburg, I., Gros, F., and Denoulet, P. (1992). Heterogeneity of tau proteins during mouse brain development and differentiation of cultured neurons. *Dev. Biol.* 154:195-204.
- LeDizet, M., and Piperno, G. (1987). Identification of an acetylation site of Chamydomonas α -tubulin. *Proc. Natl. Acad. Sci. USA* 84:5720-5724.
- Lee, G. (1990). Tau protein: an update on structure and function. *Cell Motil. Cytoskel.* 15:199-203.
- Lee, G. and Rook, S. L. (1992). Expression of tau protein in non-neuronal cells: microtubule binding and stabilization. *J. Cell Science* 102:227-237.
- Lee, G., Cowan, N., and Kirschner, M. (1988). The primary structure and heterogeneity of tau protein from mouse brain. *Science* 239:285-288.
- Lee, G., Neve, R. L., and Kosik, K. S. (1989). The microtubule binding domain of tau protein. *Neuron* 2:1615-1624.
- Lee, M. K., Rebhun, L. I., and Frankfurter, A. (1990). Posttranslational modification of class III β -tubulin. *Proc. Natl. Acad. Sci. USA* 87:7195-7199.
- Lee, M. K., Tuttle, J. B., Rebhun, L. I., Cleveland, D. W., and Frankfurter, A. (1990). The expression and posttranslational modification of a neuron-specific β -tubulin isotype during chick embryogenesis. *Cell Mot. Cytoskeleton* 17:118-132.
- Lewis, S. A., and Cowan, N. J. (1988). Complex regulation and functional versatility of mammalian α - and β -tubulin isoforms during the differentiation of testis and muscle cells. *J. Cell. Biol.* 106:2023-2033.
- Lewis, S. A., Gu, W., and Cowan, N. J. (1987). Free intermingling of mammalian β -tubulin isoforms among functionally distinct microtubules. *Cell* 49:539-548.

- Lichtenberg, B., Mandelkow, E.-M., Hagestedt, T., and Mandelkow, E. (1988). Structure and elasticity of microtubule-associated protein tau. *Nature* 334:360-362.
- Lim, S.-S., Sammak, P. J., and Borisy, G. G. (1989). Progressive and spatially differentiated stability of microtubules in developing neuronal cells. *J. Cell Biol.* 109:253-263.
- Loomis, P. A., Howard, T. H., Castleberry, R. P., and Binder, L. I. (1990). Identification of nuclear tau isoforms in human neuroblastoma cells. *Proc. Natl. Acad. Sci. USA.* 87:8422-8426.
- Luduena, R. F. (1993). Are tubulin isotypes functionally significant. *Mol. Biol. Cell.* 4:445-457.
- Luduena, R. F., Zimmermann, H., and Little, M. (1988). Identification of the phosphorylated β -tubulin isotype in differentiated neuroblastoma cells. *FEBS. LETT.* 230:142-146.
- L'Hernault, S. W., and Rosenbaum, J. L. (1985). *Chlamydomonas* α -tubulin is posttranslationally modified by acetylation of the-amino group of a lysine. *Biochemistry* 24:258-263.
- MacRae, T. H. (1992). Microtubule organization by cross-linking and bundling proteins. *Biochem. Biophys. Acta* 1160:145-155.
- Martin, A., Deakin, C., and Burgoyne, R. D. (1987). Posttranslational modifications of α -tubulin: acetylated and detyrosinated forms in axons of rat cerebellum. *J. Cell Biol.* 104:1569-1574.
- Maruta, H., Greer, K., and Rosenbaum, J. L. (1986). The acetylation of α -tubulin and its relationship to the assembly and disassembly of microtubules. *J. Cell Biol.* 103:571-579.
- Matus, A. (1988). Microtubule-associated proteins: their potential role in determining neuronal morphology. *Annu. Rev. Neurosci.* 11:29-44.
- Matus, A. (1991). Microtubule-associated proteins and neuronal morphogenesis. *J. Cell Biol. Supplement.* 15:61-67.
- McBurney, M. W., Reuhl, K. R., Ally, A. I., Nasipuri, S., Bell, J. C., and Craig, J. (1988). Differentiation and maturation of embryonal carcinoma-derived neurons

- in cell culture. *J. Neurosci.* 8:1063-1073.
- McBurney, M. W. and Rogers, B. J. (1982). Isolation of male embryonal carcinoma cells and their chromosome replication patterns. *Dev. Biol.* 89:503-508.
- Migheli, A., Butler, M., Brown, K., and Shelanski, M. L. (1988). Light and electron microscope localization of the microtubule-associated tau protein in rat brain. *J. Neuroscience* 8(6):1846-1851.
- Monteiro, M. J., and Cleveland, D. W. (1988). Sequence of chicken β 7 tubulin. Analysis of a complete set of vertebrate β -tubulin isotypes. *J. Mol. Biol.* 199:439-446.
- Morris, J. R., and Lasek, R. J. (1982). Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* 92:192-198.
- Murphy, D. B., Johnson, K. A., and Borisy, G. G. (1977). Role of tubulin-associated proteins in microtubule nucleation and elongation. *J. Mol. Biol.* 117:33-52.
- Murphy, D. B. (1988). Tubulin subunit sorting: analysis of the mechanisms involved in the segregation of unique tubulin isotypes within microtubule copolymers. *Protoplasma* 145:176-181.
- Oblinger, M. M., Argasinski, M. A., Wong, J., and Kosik, K. S. (1991). Tau gene expression in DGR neurons during development and regeneration. *J. Neurosci.* 11:2453-2459.
- Piperno, G., and Fuller, M. T. (1985). Monoclonal antibodies specific for an acetylated form of α -tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* 101:2085-2094.
- Piperno, G., LeDizet, M., and Chang, X. (1987). Microtubules containing acetylated α -tubulin in mammalian cells in culture. *J. Cell Biol.* 104:289-302.
- Pratt, L. F., and Cleveland, D. W. (1988). A survey of the α -tubulin gene family in chicken: unexpected sequence heterogeneity in the polypeptides encoded by five expressed genes. *EMBO J.* 7:931-940.
- Raybin, D., and Flavin, M. (1977). Modification of tubulin tyrosylation of the α chain of tubulin. *J. Cell Biol.* 73:492-504

- Renthal, R., Schneider, B. G., Miller, M. M., and Luduena, R. F. (1993). β IV is the major β -tubulin isotype in bovine cilia. *Cell Motil. Cytoskeleton* (in press).
- Riederer, B. M. and Innocenti, G. M. (1991). Differential distribution of tau proteins in developing cat cerebral cortex and corpus callosum. *Eur. J. Neurosci.* 3:1134-1145.
- Rudiger, M., Plessman, U., Kloppel, K-D., Wehland, J., and Weber, K. (1992). Class II tubulin, the major brain β -tubulin isotype is polyglutamylated on glutamic acid residue 435. *FEBS* 308(1):101-105.
- Sahenk, Z., and Brady, S. T. (1987). Axonal tubulin and microtubules: Morphologic evidence for stable regions on axonal microtubules. *Cell Motil. Cytoskeleton* 8:155-164.
- Sammak, P. J., and Borisy, G. G. (1988). Detection of single fluorescent microtubules and methods for determining their dynamics in living cells. *Cell Motil. Cytoskel.* 10:237-245.
- Schulze, E., and Kirschner, M. (1986). Microtubule dynamics in interphase cells. *J. Cell. Biol.* 102:1020-1031.
- Schulze, E., and Kirschner, M. (1987). Dynamic and stable populations of microtubules in cells. *J. Cell. Biol.* 104:277-288.
- Shea, T. B., Beermann, M. L., and Nixon, R. A. (1990) Post-translational modification of α -tubulin by acetylation and detyrosination in NB2a/d1 neuroblastoma cells. *Dev. Brain Res.* 51:195-204.
- Shea, T. B., Beermann, M. L., Nixon, R. A., and Fischer, I. (1992). Microtubule-associated protein tau is required for axonal neurite elaboration by neuroblastoma cells. *J. Neuro. Res.* 32:363-374.
- Sloboda, R. D., Dentler, W. L., and Rosenbaum, J. L. (1976). Microtubule-associated proteins and the stimulation of tubulin assembly in vitro. *Biochemistry* 15:4497-4505.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76-85.

- Takemura, R., Kanai, Y., and Hirokawa, N. (1991). In situ localization of tau mRNA in developing rat brain. *Neuroscience* 44:393-407.
- Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, R. J., Kirschner, M. W., and Cleveland, D. W. (1981). Nucleotide and corresponding amino acid sequence of α and β tubulin mRNA. *Nature* 289:650-655
- Wang, D., Villasante, A., Lewis, S. A., and Cowan, N. J. (1986). The mammalian β -tubulin repertoire: hematopoietic expression of a novel, heterologous β -tubulin isotype. *J. Cell Biol.* 103:1903-1910.
- Wang, Y., Loomis, P. A., Zinkowski, R. P., and Binder, L. I. (1993). A novel tau transcript in cultured human neuroblastoma cells expressing nuclear tau. *J. Cell Biol.* 121:257-267.
- Webb, B. C. and Wilson, (1980). Cold stable microtubules from brain. *Biochem.* 19:1991-2001.
- Webster, D. R., Gundersen, G. G., Bulinski, J. C., and Borisy, G. G. (1987). Differential turnover of tyrosinated and detyrosinated microtubules. *Proc. Natl. Acad. Sci. USA* 84:9040-9044.
- Weingarten, M. D., Lockwood, A. H., Hwo, S., and Kirschner, M. W. (1975). A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA* 72:1858-1862.
- Wischik, C. M., Novak, M., Thogersen, H. C., Edwards, P. C., Runswick, M. J., Jakes, R., Walker, J. E., Milstein, C., Roth, M., and Klug, A. (1988). Isolation of a fragment of tau derived from the core of the paired helical filaments of Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 85:4506-4510.
- Wolff, A., Denoulef, P., and Jeantet, C. (1982). High level of tubulin microheterogeneity in the mouse brain. *Neurosci. Lett.* 31:323-328.