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# TUBULIN ISOFORMS:
# INVOLVEMENT IN THE FORMATION OF STABLE MICROTUBULE ARRAYS DURING NEURAL DIFFERENTIATION

by

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*A peasant can stand on a hillside, with his mouth open, for a long time before a roast duck flies in.*

Chinese proverb

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# ABSTRACT

Pluripotent P19 embryonal carcinoma (EC) cells can be induced to differentiate along neural or muscle pathways by addition of the appropriate morphogen. This culture system has an advantage in that uncommitted cells can be compared to differentiated cells and that both commitment-related and differentiation related events can be observed. In this thesis, commitment is defined as the events that constrain a cell (and its progeny) to follow a particular developmental path, while differentiation is narrowly considered to involve events that result in attainment of a specific cell shape and does not consider functional or other characteristics. Incorporation of P19 EC cells into all tissues of chimeric mice indicates that this tissue culture system is a good model for differentiation events which occur *in vivo*. Therefore the P19 culture system was used to investigate the involvement of alpha and beta tubulin isoforms in formation of the stable microtubule (MT) array during neural differentiation.

Two posttranslational modifications of alpha tubulin, acetylation and detyrosination, are associated with stable MT populations including those of neural processes. Uncommitted EC cells have minimal arrays of acetylated and detyrosinated MTs. Following neural induction with retinoic acid (RA), indirect immunofluorescence microscopy shows that the first MT modifications occur during commitment, before any morphological change is observed. RA-induced cells initially polymerize a temporarily enlarged

population of MTs. Included in this population is a new array of acetylated MTs arranged in a bundle of parallel MTs. This bundle is colchicine stable and only MT-associated protein (MAP) 1B is detected in association with this bundle. In cells which proceed to differentiate directly after commitment, this bundle apparently extends to form a neural process. MAP2C is first detected at about the time of neurite extension but is not limited to dendritic processes. During early neural differentiation, changes in vimentin intermediate filament and MAP2 distributions were analyzed with respect to the changes in the acetylated MT array. During a brief period early in differentiation, indirect immunofluorescence staining shows the colocalization of colchicine stable acetylated MTs, vimentin and MAP2C. Using acrylamide to disrupt the organization of vimentin intermediate filaments and estramustine to disrupt the binding of MAP2C to MTs, this thesis shows that acetylated MTs, MAP2 and vimentin intermediate filaments are arranged in an interdependent cytoskeletal array. This array may serve to stabilize processes in neural stem cells thereby allowing the stem cell to receive signals which influence the final decision to differentiate into neurons or glia.

Finally, the involvement of beta tubulin isotypes in formation of the stable MT array during neural differentiation in P19 cells is analyzed by indirect immunofluorescence staining and by immunoblotting. Beta tubulin isotypes are defined by variation in the amino acid sequence at the carboxy terminus of the molecule. Antibodies have been raised to peptides representing these variable regions and classified as beta I, II, III, IV, V and

VI. Class VI beta isotype is specific to erythrocytes and no antibody could be obtained to Class V, which is present in all tissues, therefore only Class I, II, III and IV beta isotypes were analyzed in this study. Class III is neuron specific, all other isotypes are present to some degree in all tissues.

During neural commitment, there is an increase in the relative percentage of beta II and a decrease in the percentage of beta IV isotypes. Indirect immunofluorescence staining shows beta II but not beta III is present in the early stable MT bundle. As neural differentiation proceeds, both beta II and beta III increase in a subset of cells and this increase is concommitant with expression of MAP2C. MAP2C colocalizes on a 1:1 basis with cells containing stable MTs. Immunoblotting indicates that there is preferential inclusion of beta II in the stable MT array and preferential exclusion of beta III from this array.

To see if brain-specific MT-associated proteins (MAPs) are important in this segregation of isotypes, P19 cells were induced to differentiate into muscle cells which lack these MAPs. A comparison of the beta tubulin isotypes in differentiated muscle and neural cells indicates that the preferential inclusion of beta II in stable MTs occurs only in neural cells and suggests that MAPs are important in this subcellular sorting.

# RÉSUMÉ

Les cellules de carcinome embryonnaire (EC) pluripotentes P19 peuvent être induites à se différencier selon les lignées neurale ou musculaire, en ajoutant le morphogène approprié. Ce système de culture a l'avantage de permettre la comparaison entre les cellules non-engagées et les cellules différenciées, ce qui permet donc l'observation d'évènements liés à l'engagement ainsi qu'à la différenciation même. Dans cette thèse, l'engagement est défini comme étant les évènements qui contraignent une cellule et sa progéniture à suivre une voie développementale particulière, tandis que la différenciation est considérée comme impliquant des évènements qui lui permettent d'acquérir d'une morphologie cellulaire spécifique. L'incorporation de cellules EC P19 dans tous les tissus de souris chimèriques indique que cette lignée de cellules constitue un bon modèle pour la différenciation *in vivo*. Par conséquent, le système de culture P19 a été utilisé pour étudier le rôle des isoformes de tubuline alpha et beta dans la formation de microtubules (MT) stables pendant la différenciation neurale.

Deux modifications post-traductionnelles de la tubuline alpha, l'acétylation et la détyrosination, sont associées avec des populations de MTs stables, incluant celles des extensions neurales. Les cellules EC indifférenciées ont très peu de MTs acétylés et/ou détyrosinés. Suivant l'induction avec à l'acide rétinoïque (RA), la microscopie par immunofluorescence indirecte montre que les premières modifications des MTs ont lieu durant l'engagement, avant qu'aucun changement

morphologique ne soit observable. Les cellules induites à l'RA polymérisent initialement une population de MTs temporairement élargie. Au sein de celle-ci se trouve un nouvel ensemble de MTs acétylés formant un faisceau de MTs parallèles les uns aux autres. Ce faisceau est stable aux effets de la colchicine, et seule la MAP1B y est détectée. Dans les cellules qui se différencient directement après l'engagement, ce faisceau s'allonge apparemment pour former l'extension neurale. La MAP2C est alors détectée à peu près au moment de l'extension neuritique, mais n'est pas limitée aux extensions dendritiques.

Tôt durant la différenciation neurale, les changements dans la distribution de la vimentine et de la MAP2 ont été analysés par rapport aux changements subis par les MTs acétylés. Pendant une brève période tôt durant la différenciation, le marquage par immunofluorescence indirecte montre la co-localisation de MTs acétylés stables à la colchicine, de vimentine, et de MAP2C. En utilisant l'acrylamide pour modifier l'organization des filaments intermédiaires de vimentine, et l'estramustine pour affecter l'attachement de la MAP2C aux MTs, cette thèse montre que les Mts acétylés, la MAP2C, et les filaments intermédiaires de vimentine forment un complexe cytosquelettique interdépendant. Ce complèxe pourrait servir à stabiliser les extensions dans les cellules-souches neurales, permettant à celles-ci de recevoir les signaux qui influencent la décision finale à se différencier en neurones ou en cellules gliales.

Enfin, l'implication d'isotypes de tubuline bêta dans la formation des MTs stables durant la différenciation neurale des cellules P19 est analysée

par marquage pour l'immunofluorescence indirecte, et par la technique d'immunoblotting. Les isotypes de tubuline bêta sont définis par des variations dans la séquence d'acides aminés à l'extrémité carboxylique de la molécule. Des anticorps ont été produits contre des peptides représentant ces régions variables, classifiées comme bêta I, II, III, IV, V, et VI. Or, l'isotype bêta de classe VI est spécifique aux érythrocytes, tandis qu'aucun anticorps n'a pu être obtenu pour la classe V, qui est présente dans tous les tissus, et conséquemment, seules les isotypes bêta de classe I, II, III, et IV ont été analysés dans cette étude. La classe III est spécifique aux neurones, tandis que tous les autres isotypes sont présents dans d'autres tissus à différents degrés.

Durant l'engagement neural, il y a une hausse dans le pourcentage relatif de la bêta II et une baisse dans le pourcentage de la bêta IV. Le marquage par immunofluorescence indirecte montre que la bêta II mais non la bêta III est présente dans le faisceau stable de MTs. Tandis que la différenciation neurale se poursuit, la bêta II ainsi que la beta III augmentent dans une sous-population de cellules, et ceci coïncide avec l'expression de la MAP2C. Celle-ci est alors strictement co-localisée avec les cellules qui contiennent des MTs stables. L'immunoblotting indique qu'il y a une inclusion préférentielle de bêta II dans les MTs stables, et une exclusion préférentielle de bêta III de ceux-ci.

Pour voir si les protéines associées aux MTs (MAPs) spécifiques au cerveau sont importantes dans cette ségrégation d'isotypes, des cellules P19 ont été induites à se différencier en cellules musculaires qui, elles, n'ont pas

ces MAPs. Une comparaison des isotypes de tubuline bêta dans des cellules différenciées musculaires et neurales indique que l'inclusion préférentielle de bêta II dans les MTs stables a lieu seulement dans les cellules neurales, suggérant que les MAPs sont importantes dans cet arrangement subcellulaire.

# LIST OF FIGURES

## CHAPTER 1:

## CHAPTER 2:



## CHAPTER 3:

# ABBREVIATIONS

| | | |
|---|---|---|
| ACET | : | acetylated |
| CaCAMdpK | : | calcium calmodulin dependent protein kinase |
| cAMPdpK | : | cyclic adenosine monophosphate dependent protein kinase |
| cDNA | : | complementary DNA |
| CEF | : | chick embryo fibroblasts |
| CRABP | : | cellular retinoic acid-binding protein |
| DABCO | : | 1,4-diazabicyclo(2.2.2)octane |
| DIC | : | differential interference contrast microscopy |
| DMSO | : | dimethyl sulfoxide |
| EC | : | embryonal carcinoma |
| EDTA | : | ethylene diamine tetra-acetic acid |
| EGTA | : | ethylene glycol bis(β-aminoethylether) N,N,N',N'-tetra-acetic acid |
| FBS | : | fetal bovine serum |
| GDP | : | guanosine diphosphate |
| GFAP | : | glial fibrillary acid protein |
| GLU | : | detyrosinated |
| GTP | : | guanosine triphosphate |
| IF | : | intermediate filament |
| IgG | : | gamma chain immunoglobin |
| kDa | : | kilo Dalton |
| MAP | : | microtubule-associated protein |
| MEM | : | modified Eagle's medium |
| mRNA | : | messenger ribonucleic acid |
| MT | : | microtubule |
| MTOC | : | microtubule organizing center |
| NRP | : | nuclear receptor protein |
| PAGE | : | polyacrylamide gel electrophoresis |
| PBS | : | phosphate buffered saline |
| PC12 | : | pheochromocytoma cell culture |
| PEM | : | PIPES-EGTA-magnesium buffer |
| PIPES | : | piperazine N, N'-bis(2-ethane sulfonic acid) |
| RA | : | retinoic acid |
| RAR | : | retinoic acid receptor |
| STOP | : | stable tubule only protein |
| TRIS | : | tris(hydroxymethane)aminomethane |
| TYR | : | tyrosinated |

# LITERATURE SURVEY

## MICROTUBULES

Microtubules (MTs) are ubiquitous organelles present in all eukaryotic cells. They participate in vital cell processes including generation and maintainance of cell shape, mitosis, cell motility, and intracellular transport. In spite of such diverse functions, at the ultrastructural level, MTs present a surprisingly uniform picture - that of a tube, 25 nm in diameter.

MTs are reversible polymers composed of tubulin and microtubule-associated proteins (MAPs) (reviewed by Engelborghs 1990). Tubulin, the basic building block of MTs, is an heterodimer consisting of two globular protein subunits, alpha tubulin and beta tubulin. Both subunits have a molecular weight of 50 kDa but differ slightly in electrophoretic mobility resulting in two closely spaced bands when separated on polyacrylamide gels (reviewed in Dustin 1984).

In the electron microscope, MTs can be seen to contain 13 protofilaments arranged to form a cylinder. Each protofilament is formed by regular alternation of alpha and beta tubulin subunits. This results in a polarized structure with alpha subunits exposed at one end of the MT and beta subunits at the opposite end (Dustin 1984).

Two guanosine nucleotide binding sites are present on each tubulin molecule. The beta tubulin subunit contains an "exchangeable" or E-site where GTP is rapidly bound and then rapidly hydrolyzed to GDP during MT assembly (Geahlen and Haley 1979). The alpha subunit binds GTP in a non-exchangeable form, presumably because the GTP site is buried in the interior of the molecule after polymerization (Spiegelman et al. 1977).

MTs can be assembled from tubulin *in vitro* in the absence of calcium and the presence of GTP and magnesium (Weisenberg 1972, Herzog and Weber 1977). *In vitro* polymerization demonstrates that the two ends of microtubules have different assembly rates at a given concentration of tubulin (Summers and Kirschner 1979, reviewed by Engelborghs 1990). At a given tubulin concentration, C, addition of tubulin occurs preferentially at the "+" or growing end of the MT. The opposite end is defined as the "-" end and may be either growing slower than the "+" end or shrinking, depending upon the tubulin concentration. At a critical concentration of tubulin ($C_c$), the addition at the "+" end will be balanced by loss at the "-" end, a condition known as treadmilling (Wegner 1976).

Margolis and Wilson (1978) used $^3$H-GTP to show that incorporation and hydrolysis of GTP takes place rapidly and preferentially at the "+" end of the molecule. However, at very high tubulin concentrations, hydrolysis of GTP does not keep pace with addition of tubulin subunits to a microtubule (Carlier and Pantaloni 1981). This results in formation of a GTP "cap" at the "+" end of an assembling microtubule. The size of this cap will

fluctuate with the available amount of free tubulin. Below a critical tubulin concentration, addition of new GTP-containing subunits will be slower than hydrolysis, resulting in a MT containing only GDP-tubulin. GDP MTs are rapidly depolymerized (Hill 1984) for reasons not yet determined although Kirschner and Mitchison (1986) postulate that hydrolysis of GTP to GDP confers a conformational change upon GDP tubulin which lessens intersubunit bonds. The growing and shrinking of this GTP "cap" may regulate both MT length and stability (Hill and Carlier 1983).

## DYNAMIC INSTABILITY MODEL

Mitchison and Kirschner (1984 a,b) analyzed the polymerization of MTs using purified centrosomes as MT nucleating sites. At high tubulin concentrations (above $C_c$), MTs grow from all possible nucleating sites on the centrosome, but below $C_c$, some MTs rapidly depolymerize from the free end while others unexpectedly continue to grow. The shrinking MTs provide free tubulin subunits which are assembled onto the remaining, growing MTs or are used to assemble new MTs from open nucleating sites on the centrosome. MTs, threfore, can exist in two populations, one steadily growing, the other rapidly shrinking, with little conversion between the two states.

The paradoxical lengthening of MTs "anchored" in a MT organizing center (MTOC), when tubulin is below the critical concentration, is termed "MT dynamic instability" (reviewed in Kirschner and Mitchison 1986). The best explanation for such MT behavior is the presence of a GTP stabilizing

cap on the growing MT. Loss of the GTP cap, through hydrolysis, exposes the MT GDP core and results in catastrophic depolymerization of a MT.

In a cell, most MTs arise from the centrosome which acts as a cap for the "-" end. The free MT ends extend towards the plasma membrane and are of uniform polarity, with the "+" end distal to the centrosome (Euteneuer and McIntosh 1981). Growing ends of MTs can be identified by microinjection of fluorescein-labeled tubulin into interphase fibroblasts. Incorporation of injected tubulin occurs only at the distal ends of MTs (Soltys and Borisy 1985) and that growth is very rapid - on the order of 3.7 μm/min (Schulze and Kirschner 1986).

Video-enhanced differential interference contrast microscopy (DIC) of live cells shows that individual MTs undergo periods of elongation followed by rapid shortening (Cassimeris et al. 1988, Walker et al. 1988). Unlike the *in vitro* results, in live cells about 70% of depolymerizing MTs are "rescued" before depolymerization is complete and those MTs resume elongation. In addition, a portion of the MT population is stable and does not change in length.

Exposure of the MT GDP core is suggested as the cause of catastrophic MT depolymerization. Laser transection of MTs should expose unstable GDP tubulin at both cut ends. However when this is done in living cells, neither the remainder of the transected MT nor the isolated distal portion of this MT depolymerizes catastrophically (Tao et al. 1988). These

results indicate that the tempered dynamic instability seen *in vivo*, may be due to factors in addition to GDP cap instability.

Turnover rates for tubulin in MTs have been established by microinjecting fluorescein-labeled tubulin and allowing the injected tubulin to come to equilibrium. In this technique, a small area containing several MTs is laser photobleached and recovery of fluorescently labled MTs within this area is observed. In a spindle, the MTs recover fluorescence within 26 seconds, while in an interphase cell most MTs recover within 10 minutes after photobleaching (Salmon et al. 1984, Schulze and Kirschner 1986). Thus the MT population is constantly depolymerizing and repolymerizing with rapid turnover of most MTs. However, there is a small population of sinuous MTs which shows no incorporation of fluorescein-labeled tubulin when observed over a period of up to 24 hours. These MTs are termed "stable" MTs indicating they are not actively turning over tubulin subunits.

## STABLE MICROTUBULES IN NEURITES

The MTs in neuronal processes exhibit quite different organization and stability compared to those in proliferating cell populations. In the axon, MTs are present as short, overlapping segments, which are not associated with any visible MTOC (reviewed by Diaz-Nido 1990). In neurons from mature bovine brain, approximately half of the MTs are stable to cold- or calcium-induced depolymerization (Brady et al. 1984) as well as to colchicine-induced depolymerization (Donoso 1986). The stable MTs often

are present as short, stable regions embedded within longer, less stable MTs (Baas and Heidemann 1986, Sahenk and Brady 1987).

The relative stability of axonal MTs depends upon the source and relative maturity of the axon as well as the proximity to the cell body. In squid axons, essentially all MTs are stable and do not show dynamic length changes (Seitz-Tutter et al. 1988). In differentiated pheochromocytoma (PC 12) neurons grown in tissue culture, approximately 57% of MTs are stable to colchicine (Black and Greene 1982). However, in dorsal root ganglion neurons growing in culture, a large proportion of axonal MTs are dynamic and interchange tubulin subunits with the surrounding cytoplasm (Okabe and Hirokawa 1990). In addition, distal regions of the axon have fewer stable MTs than the regions more proximal to the cell body (Donoso 1986).

Stable MTs which are present as short regions in labile MTs (Joshi et al. 1986) may serve as nucleating sites for MT polymerization (Baas and Heidemann 1986). The mechanism that confers stability on axonal MTs is not known. Tubulin isoforms in stabile and labile MTs present different profiles and suggest that biochemical properties of tubulin may be involved in MT stabilization (Brady et al. 1984, Binet and Meininger 1988).

Interaction with MAPs also may contribute to MT stability. Binding of MAPs increases MT stability *in vitro* (reviewed by Olmstead 1986). Neuronal MTs have a complement of high molecular weight MAPs as well as tau proteins, which may be involved in MT stabilization (reviewed by Matus 1988). This is supported by experiments in which transfection or

microinjection of tau into non-neuronal cell lines results in increased stability of MTs (Drubin and Kirschner 1986, Kanai et al. 1989). Other MAPs which may stabilize MTs include the "STOP" (Stable Tubule Only Proteins) proteins which confer stability on cold labile MTs *in vitro* (Job et al. 1982).

Kirschner and Mitchison (1986) suggested a model in which dynamic instability, combined with changes in MT stability, plays a role in establishing cell polarity. In this model, the undifferentiated cell is constantly probed by rapidly polymerizing and depolymerizing MTs. When an external signal is received by the cell, it causes a change in membrane proteins. Probing MTs encounter the changed membrane region and are "caught and capped" becoming stabilized and inducing a polarization of the cell. This initial stabilization can be modified by other changes leading to even greater stability (Mitchison and Kirschner 1988). In developing neurites, initial stabilization may be reinforced by MAPs, providing a population of highly stable axonal MTs.

## TUBULIN GENES IN HIGHER EUKARYOTES

The multiple roles of MTs often seem to require MTs that are functionally quite different. The discovery of tubulins with differing isoelectric points raises the possibility that different functions may be carried out by MTs composed of a particular tubulin isoform. This theory has been articulated by Fulton and Simpson (1976) as the "multi-tubulin hypothesis".

The multiple tubulins, which are identified by isoelectric focusing or two-dimensional polyacrylamide gel electrophoresis, are often referred to as "isotubulins", "tubulin isoforms" or "tubulin isotypes". Tubulin isotypes are generally understood to be the primary translation products of multiple genes, each having a specific amino acid variation(s). Tubulin isoforms include both tubulin isotypes and the posttranslational modifications of these isotypes such as acetylation, detyrosination and phosphorylation. In this thesis, the term "isoform" will be used to describe all tubulin variations while "isotype" will be used to refer specifically to tubulins which are primary gene products. Moreover, in this thesis, "isoform expression" will include gene expression but will also include the appearance of a posttranslationally modified tubulin.

In higher eukaryotes, there are multiple functional genes for both alpha and beta tubulin which govern both the composition of the encoded tubulin and the regulation of tubulin expression (reviewed by Cleveland and Sullivan 1985, Sullivan 1988, Lewis and Cowan 1990). In chicken, six alpha and seven beta genes and in mouse, six alpha and six beta genes encode tubulin polypeptides. The genes are widely dispersed in the genome and alpha and beta tubulin genes in higher organisms are unlinked. Alpha and beta tubulins show about 40% sequence homology, indicating a common ancestral gene. There are regions that are stringently conserved between the two gene families, including the sites for GTP binding.

A large percentage of the encoded genetic variation for both alpha and beta isotype diversity is found in the region of the carboxy terminal. Classification systems for alpha and beta tubulin isotypes are based on the characteristic sequences of the final 15 carboxy terminal amino acids. The variable region of each isotype as well as the pattern of isotype expression has been rigidly conserved since the mammalian radiation. There is 97-99% amino acid homology of beta tubulin isotypes between mammals and birds (Sullivan 1988). Beta tubulin isotypes are more conserved than alpha. Alpha tubulin isotypes are conserved between mammalian species but, unlike beta isotypes, there is minimal conservation between mammals and birds.

There is greater conservation of individual beta isotypes across species lines than between isotypes of beta tubulin within a single species. Within a given species, the amino acid homology between beta isotypes ranges from 78-97% while across species lines the homology for specific isotypes is 97-99% (Monteiro and Cleveland 1988, Sullivan 1988, Lewis and Cowan 1990). This extremely high degree of isotype conservation across species lines suggests that isotypes may have specific functions.

## ISOFORM CLASSIFICATION

Tubulin isoforms have been identified by different laboratories and direct comparison is difficult. Isoform nomenclature is based upon numbering of bands or spots separated by one- or two-dimensional polyacrylamide gel electrophoresis (PAGE). Depending upon the tubulin

source and upon techniques, the beta 1 of one group may be a different isoform than the beta 1 of another laboratory. This has resulted in confusing nomenclature making it difficult to compare data from different laboratories.

Using nucleotide sequences, it should be possible to reconcile these nomenclature systems. Virtually the complete nucleotide sequences are known for all functional genes encoding beta tubulin isotypes in three vertebrate species: mouse, chicken and human (reviewed in Cleveland and Sullivan 1985, Sullivan 1988, Lewis and Cowan 1990). In addition, a partial characterization of beta tubulin genes is available for several other species.

Since the variable carboxy terminal regions are conserved across species, Cleveland has used the amino acid sequence from these regions to classify beta tubulin isotypes into six major classes identified by Roman numerals: I, II, III, IV, V, VI (Lopata and Cleveland 1987, Sullivan 1988). Lewis and Cowan (1990) used the same conserved, variable amino acid sequences to classify beta tubulin into six types identified by Arabic numbers: 1, 2, 3, 4, 5, 6. The rational for the two classification schemes is identical but comparison between the two is difficult because the categories do not contain the same isotypes. Both classification systems are currently in use.

Classification of alpha tubulin isotypes also is based on the variable region of the carboxy terminus. However, to date only a single classification

system has been put forth, thereby eliminating the confusing situation present in the beta tubulin system (Lewis and Cowan 1990).

## ANTIBODIES TO TUBULIN ISOTYPES

Both polyclonal (Lopata and Cleveland 1987) and monoclonal (Lewis et al. 1987, Banerjee et al. 1988, Asai and Remolona 1989) antibodies have been raised to synthetic peptides which match the variable carboxy terminal region of beta tubulin isotypes. Gu et al. (1988) also generated antibodies to alpha tubulin isotypes using the same methods. These antibodies have proven useful in immunolabelling studies of isotype localization, at both the light and electron microscope level.

## EXPRESSION Of TUBULIN ISOFORMS

Expression of tubulin isoforms has been determined by numerous methods including: two-dimensional gels, Northern blots of mRNA using various tissues as source material, *in situ* hybridization of mRNA and indirect-immunostaining of cells. Multiple isoforms are expressed by a single cell (Gozes and Littauer 1978). However, light and electron microscope immunostaining indicate that all MTs are copolymers of available isoforms although the percentages of a particular isoform within a given MT can differ (Geuens et al. 1986, Gu et al. 1988, Joshi and Cleveland 1989).

Examination of tubulin mRNA from tissue samples assayed during development shows that each gene has a unique pattern of expression and that alpha and beta tubulin are not expressed in pairs (Lewis and Cowan 1990). Virtually all isotypes are present in all tissue samples, although levels differ greatly between tissues. For a given isotype, the concentration also varies within a single tissue during differentiation.

The number of tubulin isoforms increases during brain development and the level for each isoform is developmentally regulated (reviewed by Morrison and Griffin 1986). Although most of the 12 beta tubulins identified in brain are generated by posttranslational modifications (Sullivan 1988), most of the information about the beta tubulins concerns those which are generated as primary transcripts of beta tubulin genes. Only one

posttranslational modification of beta tubulin, phosphorylation, has been characterized in detail.

The situation regarding information about alpha tubulins is exactly the reverse. There is only limited data on the isotypes which result from expression of alpha tubulin genes. Most information regarding alpha tubulins has been gathered using antibodies to posttranslational modifications.

## β-TUBULIN ISOFORM EXPRESSION

Luduena and co-workers (Banerjee et al. 1988) examined the β-tubulin isoforms in bovine brain and found that it contains 3% Class I, 58% Class II, 25% Class III and 13% Class IV beta tubulin.

When bovine brain tubulin is separated on polyacrylamide gels, two electrophoretic variants can be detected, $\beta_1$ and $\beta_2$. The $\beta_1$ band contains isotypes from Classes I, II and IV while $\beta_2$ consists of only of Class III isotype. Functionally active dimers containing 1 of 3 isotypes of alpha tubulin combined with a beta tubulin of class II, III or IV have been purified by antibody affinity columns and tested for *in vitro* polymerization. Isotypically-pure tubulin assembles faster and the resulting MTS are more stable compared to MTs from isotypically-mixed tubulin pools (Banerjee et al. 1989).

Beta tubulin in the major bovine isoelectric variant, $\beta_1$, contains two cysteines that may play a role in MT assembly. The brain specific $\beta_2$ isoelectric variant lacks one of these cysteines and cannot be cross-linked by

a sulfhydryl reaction (Little and Ludueña 1985). The authors suggest that cross-linking of $\beta_1$ may suppress polymerization of this isotype and be one way to generate subcellular sorting of this isotype.

The suggestion that tubulin isoforms may contribute to MT functions is strengthened by the observation that neurospecific forms of tubulin appear sequentially during fetal and postnatal brain development (Gozes and Littauer 1978, Denoulet et al. 1982). In a series of papers, Denoulet and Eddé (1986, 1987, 1989) examined the expression of tubulin isoforms during neuronal differentiation of the embryonal carcinoma cell line 1003. In those cells, beta tubulin isoforms are differentially regulated with one isoform, $\beta$'1, expressed very early, during neuronal commitment. Other isoforms are expressed later in differentiation.

Evidence that particular beta isoforms are expressed during neurite extension indicates that MTs containing these isoforms may also have a particular function. Expression of the gene encoding beta II is induced to high levels during development and axonal regeneration of rat sciatic nerve (Hoffman and Cleveland 1988). Beta II and beta III isotypes are induced sequentially during neuronal differentiation in PC 12 cells and beta II is preferentially polymerized into axonal MTs (Joshi and Cleveland 1989).

In the axon, tubulin isoforms have been localized to different regions. During axonal transport, tubulin is moved at two rates, in slow component a (SCa) and in slow component b (SCb). Denoulet et al. (1989) examined tubulin isoforms in rat sciatic nerve and found that two neuron specific

isoforms, β'1 and β'2, were preferentially moved in SCb. They suggest that recruitment of isoforms into distinct MT subsets may be involved in establishing stability or other functional properties of these MTs. For example, in lobster axons, a distinct population of MTs is associated with vesicle transport (Miller et al. 1987).

The suggestion that MT isoforms may be involved in MT stability is supported by the presence of different proportions of beta tubulin isoforms in cold stable and cold labile MTs in cultured cells (Joshi et al. 1987). Similarly, mouse brain cold stable MTs have a characteristic isoform pattern with predominance of one beta and two alpha tubulin isoforms (Binet and Meininger 1988).

Burgoyne et al. (1988) used antibodies to mouse beta tubulin isotypes to stain sections of adult cerebellum. One isotype, mB1 gave no staining. Three other isotypes, Mβ2, Mβ3/4 and Mβ5 were present in both neuronal and non-neuronal cells but showed specific cell type staining within the cerebellum. The Mβ6 isotype was limited to neurons. The expression patterns of Mβ6 and Mβ2 were similar to the patterns of expression of MAP1A and MAP3 respectively indicating that tubulin isotypes may be involved in sorting of MAPs and thereby influencing MT function. Additional evidence that tubulin isoforms may be associated with particular MAPs is shown in squid neurons (Arai and Matsumoto 1988). Regional stability of MTs within the squid neuron colocalizes with particular tubulin isoforms and MAPs.

In the brain, 12 beta isoforms can be seen by isoelectric focusing (Sullivan 1988). Five of these isoforms result from translation of tubulin genes and the remaining seven are produced by posttranslational modifications of these.

**Glutamylation of beta tubulin**

Recently the addition of 1 to 5 glutamic acid residues on glu residue 438 in the carboxy terminal of the class III beta tubulin has also been reported although it is not yet known if other beta tubulins also undergo this posttranslational modification (personal communication from Dr. A. Frankfurter, Joshi and Cleveland 1990). These polyglutamyl residues extend outward and result in highly negatively charged regions which may be involved in MT assembly/disassembly and/or interaction with MAPs.

Phosphorylation of beta tubulin is the best known of these posttranslational modifications. The progressive acetylation of six isoforms of beta tubulin during culture of fetal mouse brain has also been reported (Eddé et al. 1989). Phosphorylation may be involved in axonal vesical transport. A protein (NP185) has been found which is associated only with neuronal clathrin-coated vesicles. However, the affinity of this protein for tubulin is greatly enhanced when tubulin is phosphorylated by a clathrin-coated vesicle-associated kinase II during neurite development. Thus phosphorylation of tubulin may play a role in axonal transport (Kohtz and Puszkin 1989).

During neuronal differentiation, phosphorylation occurs on the neuron-specific tubulin isotype, beta III (Díaz-Nido et al. 1990). In neuroblastoma cells, beta III is first expressed during neuronal commitment but phosphorylation of this isotype occurs later, during axonal outgrowth (Gard and Kirschner 1985, Eddé et al. 1987, Denoulet et al. 1989). Brain tubulin is phosphorylated exclusively at a serine residue on beta tubulin which probably is located in the carboxy terminal region (reviewed by Serrano and Avila 1990).

Phosphorylation occurs preferentially on the polymer. Although the role of phosphorylation remains unknown, phosphorylated beta (and alpha) tubulin has been found associated with coated vesicles from brain and may play a role in binding these vesicles or in vesicle transport. *In vitro*, a CaCAMdpK can phosphorylate both alpha and beta subunits, producing a conformational change which greatly reduces the ability of this tubulin to polymerize and which may be involved in the association of tubulin with membranes (Serrano and Avila 1990).

## α-TUBULIN ISOTYPE EXPRESSION

Isotypes which result from expression of alpha tubulin genes are, like beta isotypes, under developmental control. The alpha isotype variable region, although smaller than that of beta tubulin, also is located predominantly at the carboxy terminal (reviewed in Sullivan 1988). Alpha

tubulin genes have a complex pattern of expression with most isotypes expressed in all tissues but at varying levels.

Although antibodies have been raised against synthetic peptides representing the conserved carboxy terminal regions of alpha tubulin isotypes (Gu et al. 1988), little data has been published using these antibodies. One investigation of alpha and beta tubulin isotypes during differentiation of testis and muscle cells indicates there is no segregation of isotypes into particular MTs although the isotypes are expressed in a complex pattern which is characteristic of the cell type (Lewis and Cowan 1988). However, an antibody to a highly divergent alpha tubulin isotype has identified an isotype which is present in all MT structures within the spermatid but is absent from brain, so some tissue specificity does exist (Hecht et al. 1988).

During mouse embryogenesis, *in situ* hybridization to mRNA for murine alpha tubulin isotypes indicates that one isotype, Mα2, is expressed in all cells at fairly uniform levels while Mα1 is limited to growing neurons of the central and peripheral nervous system. The Mα1 isotype appears to be the major alpha tubulin mRNA expressed during axonal growth (Miller et al. 1988).

## POSTTRANSLATIONAL MODIFICATIONS OF TUBULIN

Posttranslational modifications to tubulin gene products provide another mechanism for altering MT characteristics and thereby possibly altering MT functions. Four forms of posttranslational tubulin modification

have been described: alpha tubulin detyrosination/re-tyrosination and acetylation, and beta tubulin phosphorylation and acetylation. The development of antibodies which identify tyrosinated, detyrosinated and acetylated alpha tubulin as well as antibodies which recognize all alpha or beta tubulins, have been instrumental in analyzing the occurence and possible significance of these alpha tubulins. To date there are no antibodies which are specific for beta tubulin posttranslational modifications and these modifications are less well characterized than those of alpha tubulin.

## Tyrosination/detyrosination of alpha tubulin

The carboxy terminus of most alpha tubulin isoforms can undergo enzymatic removal and (re)addition of the final encoded tyrosine residue at the carboxy terminal (reviewed in Sullivan 1988). Two tubulin-specific enzymes which participate in these reactions have been identified, a carboxy peptidase which removes the tyrosine and a tubulin:tyrosine ligase which catalyzes the re-addition of tyrosine to the carboxy terminal.

Most alpha tubulins are translated with a final tyrosine at the carboxy terminus. These subunits are assembled into MTs which contain predominantly tyrosinated (TYR) tubulin, i.e. TYR MTs (Burns 1987). The polymerized TYR MT is the preferred substrate for the tubulin carboxy peptidase which removes the final tyrosine, exposing the penultimate glutamic acid and thereby creating a MT in which most of the alpha subunits have been modified, i.e. a GLU MT (Kumar and Flavin 1981). This process has been variously termed detyrosination, detyrosylation or detyrosinolation.

Tubulin:tyrosine ligase is preferentially active on tubulin monomers (Kumar and Flavin 1981). Following depolymerization of GLU MTs, the GLU tubulin subunits are rapidly retyrosinated by the tubulin:tyrosine ligase. This creates a pool of TYR tubulin which is then assembled into new TYR MTs thus completing the cycle of detyrosination-tyrosination (Gundersen et al. 1984, Wehland and Weber 1987b).

Gundersen and Bulinski (Gundersen et al. 1984) raised TYR and GLU polyclonal antibodies to polypeptides representing the final seven C-terminal sequences of alpha tubulin and terminating in a tyrosine or glutamic acid respecitvely. Double label immunofluorescent staining using TYR or GLU antibodies and an antibody which recognizes all alpha tubulin, YOL 1/34 (Kilmartin et al. 1982, Wehland et al. 1983), shows that there are two distinct populations of MTs in cells (Gundersen et al. 1984). TYR MTs, identified by the TYR antibody, are highly dynamic. They constitute most of the interphase MT network and are rapidly turning over subunits. GLU MTs, as identified by the GLU antibody, are a distinct subset of relatively stable MTs with low tubulin turnover rates. In many cells, GLU MTs are largely restricted to the centrosomal region (Webster et al. 1987). MTs with a high GLU content are present in a variety of structures with stable MTs including neurites, flagella and marginal bands (Cambray-Deakin and Burgoyne 1987a, Gundersen et al. 1984). In most cells, GLU MTs are resistant to drug-induced depolymerization but not resistant to cold-induced depolymerization (Wehland and Weber 1987a).

**Acetylation of α-tublin**

A second reversible posttranslational modification of alpha tubulin consists of acetylation of lysine-40 (L'Hernault and Rosenbaum 1985a,b; LeDizet and Piperno 1986). The acetyl-transferase is highly specific for alpha tubulin and acts preferentially on polymerized MTs. After

depolymerization of acetylated (Acet) MTs, a deacetylase acts preferentially on monomeric tubulin, restoring the tubulin pool to its non-acetylated form and completing the cycle.

A monoclonal antibody, 6-11B-1, which identifies acetylated alpha tubulin has been useful in determining the presence and stability of acetylated MTs (Piperno and Fuller 1985). Acetylated MTs constitute a distinct subset of relatively stable, interphase MTs which have a much slower tubulin turnover than do the majority of MTs (Webster and Borisy 1988). Although stable MTs are often acetylated, acetylation does not confer stability (Schulze et al. 1987). There are cell lines ($PtK_2$) which have no acetylated MTs but nevertheless do have stable MTs (Piperno et al. 1987).

The population of acetylated MTs is often, but not always, the same as the GLU MT population. Both modifications occur on relatively stable MTs (Schulze et al. 1987, Bulinski et al. 1988). Neither modification is dependent upon the other and both can occur independently.

In fibroblasts, individual stable MTs have been seen to have acetylated regions flanking non-acetylated regions, indicating that acetylation can occur in discrete domains of the MT (Webster and Borisy 1988). Acetylated MTs in rat fibroblasts were identified as cold stable, while TYR MTs were cold labile and the two isotypes were often segregated to different MTs (Cambray-Deakin and Burgoyne 1987b).

Acetylated MTs from many cell lines are resistant to drug-induced depolymerization (LeDizet and Piperno 1986). However, resistance to cold-

induced depolymerization varies with the cell line. The acetylated MTs in neuronal cells are cold stable (Cambray-Deaken and Burgoyne 1987a) while acetylated MTs in *Chlamydomonas*, 3T3 and HeLa cells are not (LeDizet and Piperno 1986, Piperno et al. 1987).

## POSTTRANSLATIONAL MODIFICATION OF TUBULIN IN NEURONS

### Glutamylation of alpha tubulin

Glutamylation is a major modification in adult brain with 40 to 50% of alpha tubulin showing the addition of glutamic acid residues at the carboxy terminus. Between 1 and 5 residues are added to the glutamic acid at position 445 causing a large increase in the negative charge in the region where MAPs bind (Eddé et al. 1990). This modification probably occurs on essentially all neuronal alpha tubulins and may play an important role in regulating MT dynamics and functions (Joshi and Cleveland 1990).

### Localization of acetylated alpha tubulin during neurogenesis

Acetylation of alpha tubulin is among the earliest changes in neuronal differentiation. Nerve growth factor induces PC 12 cells to extend neurites and causes acetylation of alpha tubulin (Black et al. 1986) which correlates with the appearance of acetylated MTs in the neurite (Lim et al. 1989). In embryonal carcinoma (EC) cells induced to differentiate into neurons, a population of stable, acetylated MTs appears within 24 hours after neural induction. A subpopulation of the acetylated MTs is present as a colchicine stable bundle of MTs which may extend to form the early neurite (Falconer

et al. 1989a). A similar population of colchicine stable, acetylated MTs was reported in the developing axons of cerebellar macroneurons in culture (Ferreira and Cáceres 1989). High levels of acetylated alpha tubulin also appear in neural folds and neuronal processes during stages 16 to 19 in *Xenopus* development (Chu and Klymkowsky 1989).

Recent evidence, however, suggests that acetylation is a relatively minor posttranslational modification in adult brain with less than 5% of brain acetylated at lysine 40 (Eddé et al. 1990).

**Isoforms of alpha tubulin in neurons**

Tyrosinated alpha tubulin is transiently expressed by developing axons (Cumming et al. 1984). Very young neurites from differentiating EC cultures contain tyrosinated, detyrosinated and acetylated forms of alpha tubulin. However, mature neurons are characterized by loss of the dynamic, tyrosinated MTs and retention of a population of stable, acetylated and detyrosinated MTs (Falconer et al. 1989). In sections of mature rat cerebellum, axons and dendrites contain both acetylated and detyrosinated alpha tubulin although acetylated tubulin is preferentially localized to axons (Cambray-Deakin and Burgoyne 1987a).

Indirect immunofluorescent staining indicated that tyrosinated MTs were present in the cell body and growth cone, regions with rapid tubulin turnover (Robson and Burgoyne 1989). Acetylated and detyrosinated MTs were not found in these regions (Lim et al. 1989). However, acetylated and

detyrosinated MTs were present in regions of slow tubulin turnover, specifically in the neurite.

To examine tubulin turnover rate in neurons, PC 12 neurons were microinjected with rhodamine labeled tubulin followed by laser bleaching (Lim et al. 1989). Recovery of fluorescence was most rapid at the cell body and the growth cone, with markedly slower recovery in neurites.

Acetylated MTs may be linked to intermediate filament proteins in neural cells. Acetylated, but not tyrosinated MTs have been shown to colocalize with glial filament bundles in astrocytes (Cambray-Deakin et al. 1988) and with vimentin in differentiating neural cells (Falconer etl al. 1989).

## NEURONAL MICROTUBULE-ASSOCIATED PROTEINS (MAPs)

Microtubule-associated proteins, MAPs, are a collection of various proteins which are defined on the basis of: a) binding to MTs through cycles of MT depolymerization -repolymerization, b) ability to promote tubulin assembly and stabilize MTs, and c) colocalization with MTs at the light and electron microscope level (reviewed by Olmstead 1986).

In brain tissue, up to thirty five MAPs, or isoforms of MAPs, can be separated by PAGE (reviewed by Nuñez 1986). Functional differences in MTs may result from association of tubulin isoforms with particular MAPs and MAPs, in turn, may preferentially asssociate with a particular tubulin isoform. MAPs can undergo posttranslational modifications and

phosphorylation of MAPs has been shown to influence MAP interactions with other cytoskeletal elements (reviewed by Wiche 1989).

Although MAPs have been identified in many cell types, brain MAPs are the best characterized. When separated by PAGE, brain MAPs fall into two categories: high molecular weight MAPs (270-350 kD) which include MAP1 and MAP2, and MAPs with molecular weight between 55-82 kD which include the tau proteins, STOP proteins and chartins. Homologous genes for MAP1 and 2 have been detected in *Xenopus* (Viereck et al. 1988), mammals and chicken but not in *drosophila* or sea urchin (Lewis et al. 1986). During brain development, MAPs show differential expression suggesting that they may be involved in the development of various cell types and or the acquisition of new MT functions.

Immunofluorescent staining of differentiating neurons in mammalian brain shows the developmental appearance of MAPs. The earliest MAPs detected are MAP1B, MAP2C, and juvenile tau. MAPs present during the final stages of maturation and/or in the adult brain include MAP1A, MAP2A, and adult tau. MAPs which are present both during some period of differentiation as well as in the mature brain include MAP2B and MAP3 (Tucker and Matus 1988).

**MAP1**

MAP1 is comprised of three polypeptides, MAP1A, MAP1B and MAP1C with apparent molecular weights of 350, 320 and 300 kD

respectively, which can be separated into closely spaced bands by PAGE techniques. MAP1A and MAP1B are transcribed from separate genes which are differentially regulated (Garner et al. 1990). Both MAPs can be phosphorylated (reviewed by Díaz-Nido et al. 1990). Both MAP1A and MAP1B have wide distribution outside the brain (reviewed in Díaz-Nido et al. 1990). The somewhat conflicting reports concerning immunolocalization of these two MAPs may be due to the use of monoclonal antibodies which recognize different isoforms of the same MAP or, in the case of non-neuronal cells, the same epitope on different proteins. Immunostaining of neurons shows MAP1A stains cell bodies, dendrites, and axons, although dendrites have the most intense labelling (Peng et al. 1986). Expression of MAP1A is developmentally regulated and increases as the brain matures. One report suggests that during neuronal differentiation, MAP1A is initially present in axons but later in development localizes preferentially to dendrites (Bernhardt et al. 1985).

MAP1B is also referred to as MAP-1X (Calvert and Anderton 1985) or MAP 5 (Riederer et al. 1986). Antibodies to MAP1B stain axons, dendrites and cell bodies (Peng et al. 1986). MAP1B is more abundant in immature neuronal processes and has been reported to be preferentially localized to axons (Riederer and Matus 1985). It is especially prominent during neuronal outgrowth in PC 12 cells and neuroblastoma cells when it is phosphorylated (Brugg and Matus 1988, Díaz-Nido et al. 1988).

MAP1B is encoded by a single gene (Lewis et al. 1986, Safaei and Fischer 1989). The complete sequence of MAP1B has been deduced from overlapping cDNA clones and contains two unusual sequences (Noble et al. 1989). The first is a highly basic region between amino acids 589 and 786 which is responsible for binding MAP1B to MTs *in vivo,* probably by interacting with the negatively charged carboxy terminal on alpha and/or beta tubulin. This tubulin binding region bears no similarity to the binding regions which have been described for tau, MAP 2 or kinesin. The second unusual region is a set of 12 imperfect repeats for which no function is yet apparent. Transfection and expression of MAP1B, in cells which do not normally express MAP1B, has no effect on the organization or stability of the interphase MT network (Noble et al. 1989).

MAP1B has been found in all tissues of the body and may play a role in MT assembly (Díaz-Nido and Avila 1989). Although it is present in non-neuronal tissues at lower level than in brain, the relative ratio of MAP1B to tubulin is the same. The expression of both the protein and the mRNA of MAP1B are developmentally regulated in differentiating brain, with maximum expression at the time of neurite growth and may be associated with MT polymerization (Díaz-Nido and Avila 1989, Safaei and Fischer 1989). The levels are subsequently reduced at a period coincident with dendritic and axonal maturation and synaptogenesis. Expression of MAP1B does not show similar regulation in non-neuronal tissues.

MAP1C is a 300 kD protein, unrelated to MAP1A or MAP1B. It is a dynein-like ATPase which supports retrograde movement (towards the "-" end) on MTs and is suggested as the possible force generator for retrograde transport in the axon (Paschal et al. 1987, Vallee et al. 1988).

**MAP2**

MAP2, the most abundant MAP in brain, is an heat stable protein which has an apparent molecular weight of approximately 270 kD on polyacrylamide gels. It is a strong promoter of MT polymerization and enhances MT stability. *In vitro* experiments indicate that MAP2 can bind to, and possibly link MTs to, intermediate filaments, neurofilaments and actin microfilaments (reviewed by Olmstead 1986). The protein has an "L" shape which can be cleaved by proteolysis into a short, MT binding region of about 35 kD and a long, projection arm of about 240 kD (Vallee 1980).

The complete amino acid sequence was derived from a series of overlapping cDNA clones (Lewis et al. 1988). At the carboxy terminal there is a MT binding region which consists of three imperfect repeats and which has high homology to a similar MT binding region in tau (Aizawa et al. 1989). When peptides corresponding to the three regions were synthesized and added individually to pure, unpolymerized tubulin, only the peptide representing the second repeat region proved capable of stimulating MT nucleation and elongation (Joly et al. 1989). However all three peptides

show binding to polymerized MTs (Lewis et al. 1989). In addition, the MT binding region of MAP2 can also bind to mitochondria and may be implicated in moving mitochondria into the axon or dendrite (Lindén, et al. 1989, Jancsik et al. 1989). It was initially proposed that the bundling properties of MAP2 resides in the final 18 amino acids at the C-terminal (Lewis et al. 1989). A subsequent report indicates amino acids 1758-1780, just 3' to the binding region, are either directly or indirectly involved in MT bundling (Lewis and Cowan 1990). However, the inter- MT distance reported in these bundles in less than that reported for MT to MT spacing in the dendrite and this bundling may not represent the *in vivo* situation.

MAP2 is a major substrate for kinase activity and sites for phosphorylation are located both on the short, tubulin binding region and on the projection arm. A cAMPdpK, tightly bound to the projection arm (Vallee et al. 1981), can phosphorylate both the tubulin binding region and the projection arm (Hernandez et al. 1987). Phosphorylation within the tubulin binding region reduces the affinity of MAP2 for tubulin in proportion to the level of phosphorylation (Burns et al. 1984). Phosphorylation in the projection arm may modify cross linking other proteins (reviewed by Olmstead 1986).

Transfection of MAP2-encoding cDNA into cell lines which do not express MAP2 results in dramatic alteration of the interphase MTs. Dense parallel bundles of MTs are observed. These MTs are not associated with

the centrosome and are stable against MT depolymerizing drugs (Lewis et al. 1989).

MAP2 is considered a dendritic MAP. *In situ* hybridization shows that MAP2 mRNA is present in dendrites where is is synthesized on polyribosomes however no ribosomes are present in axons and no proteintranslation occurs there (Garner et al. 1988). Immunolocalization of adult forms is largely restricted to dendrites and cell bodies (Peng et al. 1986). However, staining of the proximal portion of the axon as well as staining of the entire axon in unipolar neurons has been reported (Higgins et al. 1988). When biotinylated MAP2 is microinjected into mature neurons, labelled MAP2 is distributed in both axons and dendrites. However, after 3 days, the biotin-labelled MAP2 decreases dramatically in axons indicating that axonal MAP2 is not strongly associated with the axonal cytoskeleton and undergoes rapid turnover (Okabe and Hirokawa 1989). This experiment provides an indication that compartmentalization of MAP2 may occur by selective loss of MAP2 from the axon.

In adult brain, MAP2 consists of two closely related polypeptides, MAP2A and MAP2B. A cDNA probe constructed by Lewis et al. (1986) detects a mRNA of about 9 kb, indicating the presence of a single gene encoding MAP2 in mouse. This heterogeneity in MAP2A and 2B is probably due to posttranslational modification(s) or alternative splicing or both. Cross hybridization to other species indicates there is significant species divergence although antibody recognition has been demonstrated in

species as diverse as rat, quail and *drosophila* (Tucker et al. 1988). During brain differentiation, MAP2A and 2B are under developmental control. In rats, MAP2B is present at birth while MAP2A does not appear until the second postnatal week (Binder et al. 1984).

Juvenile brain contains a protein, MAP2C with a molecular weight of 70 kD. Southern blot analysis indicates that MAP2C is derived from the same gene as MAP2A and 2B, but is encoded by its own 6 kb mRNA and has the same $NH_3$ and COOH termini as MAP2A and B. It is suggested that MAP2C lacks the extended arm portion of adult MAP2, while retaining the MT binding portion. Although the function of the extended arm remains unknown, it may be involved in spacing MT-MT distances, which are greater in the dendrite than in the axon (Vallee 1990). Thus in developing neurites, MAP2C can act to polymerize and stabilize MTs without extensively cross-linking (Garner and Matus 1988). There is no evidence that MAP2 cross-links MTs *in situ*.

Two antibodies, one which recognizes only the adult forms of MAP2 (AP-14) and another which recognizes both juvenile and adult forms of MAP2 (AP-18) have been useful in analyzing developmental patterns of MAP2 (Tucker et al. 1988, Viereck et al. 1988). MAP2C appears before the adult forms of MAP2 and is present in axons and glia (Tucker et al. 1988). MAP2C, but not the adult form(s) of MAP2, is a major component of newborn rat brain. Its expression is greatly reduced by 15 days after birth at the time when the adult form(s) are present (Rieder and Matus 1985).

## MAP3, MAP4 and CHARTINS

Relatively little is known about these MAPs which, in comparison with MAPs 1 and 2, are minor components of recycled brain tubulin. MAP3 consists of a pair of closely spaced polypeptides with apparent molecular weight of about 180 kD (reviewed by Olmstead 1986, Díaz-Nido et al. 1990). MAP3A is present in rat embryo brain while MAP3B is expressed after birth. Expression of both is reduced after postnatal day 10 with the exception of proliferating astrocytes. In neurons, MAP3 is restricted to neurofilament-rich axons.

MAP4 consists of a group of polypeptides with apparent molecular weight of about 210 kD which are present in glial cells (astrocytes and oligodendrocytes) in adult brain (Parysek et al. 1985). HeLa cells contain a 210 kD homolog of MAP4. Immunolocalization indicates MAP4 is present in a wide range of tissues including heart, liver, lung, and brain but is absent from mature sperm and all types of blood cells (Parysek et al. 1985, Olmstead et al. 1986).

The chartins are a family of related proteins which have apparent molecular weight of 69 to 80 kD, in the same range as the tau proteins, but unlike tau, chartins are heat labile. Tissue and/or cellular distribution of the chartins has not been determined. Biochemical data suggest they are present in many cell types and the low antigenicity of these proteins suggests they are highly conserved (Magendantz and Solomon 1985).

## TAU PROTEINS

The tau proteins are present in neuronal tissue and constitute a family of heat stabile polypeptides with apparent molecular weight between 55 and 65 kD. A 48 kD juvenile form of tau also is seen in newborn rat (reviewed by Olmstead 1986, Díaz-Nido et al. 1990). Peptide mapping and shared reactivity to monoclonal antibodies indicates the tau proteins are closely related and are expressed only in neuronal cells (Cleveland et al. 1979, Binder et al. 1985). The tau proteins are highly conserved across species lines with mouse, bovine and human tau cDNA sequences showing 82% homology at the nucleotide level (Himmler et al. 1989). Tau is a short, rodlike molecule that forms short cross-bridges between MTs *in vitro* (Hirokawa 1988).

Tau proteins can be phosphorylated *in vitro* by cAMPdpK and by CaCAMdpK I which contributes to formation of isoforms of tau (reviewed by Díaz-Nido et al. 1990). Dephosphorylation of tau from bovine brain reduces the number of isoforms and indicates that at least two of the bands result from varying degrees of phosphorylation. This confirms an earlier study which showed that the dephosphorylation of tau decreased the number of tau variants by half. At that time the authors suggested that the two phosphorylated states may represent axonal versus cell body tau (Butler and Shelanski 1986).

Phosphorylated and non-phosphorylated tau can be distinguished by two monoclonal antibodies: tau-1 recognizes an epitope which can be blocked by phosphorylation while tau-2 recognizes an epitope present on both the phosphorylated and non-phosphorylated forms of tau (Papasozomenos and Binder 1987). The non-phosphorylated form of tau is localized to the axon; the phosphorylated form is present in axons, dendrites and cell bodies. The presence of the phosphorylated form in the cell body where tau is synthesized indicates that dephosphorylation of tau occurs upon transit into the axon and may confer enhanced stability upon the MTs that it binds to (Papasozomenos and Binder 1987).

Microinjection of tau into cells which do not normally express this protein increases tubulin polymerization and stabilizes MTs against depolymerization but does not cause major changes in cell morphology (Drubin and Kirschner 1986.) However, transfection of a cDNA expressing tau causes a reorganization of the MTs into thick bundles, the formation of cell extensions and a twofold increase in the amount of tubulin (Kanai et al. 1989). Immunoblotting of transfected cells reveals 3 bands originating from a single transfected cDNA. The lack of bundling by microinjected tau may be due to the difference in the amount of tau present in comparison to that produced by the transfected gene.

*In vitro* phosphorylation of tau paracrystals shows that the elasticity of tau is related to the state of phosphorylation (Hagestedt et al. 1989). When tau is dephosphorylated by alkaline phosphatase, it becomes shorter and more

elastic. It is this non-phosphorylated form of tau which is present in dendrites. When tau is phosphorylated, it becomes longer and stiffer. It is this phosphorylated, less plastic form of tau which is localized to axons.

Current information suggests the existence of only one tau gene although six or more isoforms can be identified by PAGE techniques (Drubin et al. 1984). The molecular basis for this heterogeneity is not completely understoood. Multiple tau mRNAs have been identified and may result from alternative RNA splicing (Goedert et al. 1989). Screening of several tau cDNA clones has shown that regions of heterogeneity are present at the carboxy terminal (Lee et al. 1988) and at the amino terminal (Himmler et al. 1989, Lee et al. 1989).

All tau sequences reported to date contain a characteristic three or four tandem repeat in the carboxy terminal half which has extensive homology to the tubulin binding region of MAP2 (Lee et al. 1988, Lewis et al. 1989). For a recent review see Lee (1990). Radioactively labeled peptides representing these repeats were generated and shown to bind to MTs *in vitro* (Himmler et al. 1989). The basic tubulin binding unit for tau protein is a single repeat (Lee et al. 1989), although the 50 amino acids preceeding the repeats contribute to MT binding as well (Lewis et al. 1989).

Axonal MTs which are associated with tau protein, are more closely spaced than those in dendrites which are associated with MAP2. Lewis et al. (1989) suggest the reason for this inter-MT distance lies in the length and functional properties of the N-terminal arms. Unlike MAP2, tau does not

have an extended projection of the amino terminal portion which may function to physically prevent close apposition of MTs.

MT bundling properties of MAP2 and tau are not in the extended amino terminal region but reside at the extreme carboxy terminus (Lewis et al. 1989). Isoforms of tau which encode an hydrophobic α-helix in this region, similar to that of MAP2, are able to bundle MTs using a leucine-zipper like interaction to cross-bridge to another tau (Lewis et al. 1989). In some isoforms of tau, a short C-terminal extension contributes to MT binding but also prevents bundling (Himmler et al. 1989). These forms may be segregated between the axon and the dendrite by as yet unknown mechanisms.

Goedert et al. (1989) have isolated cDNA clones encoding two isoforms of tau which differ in the number of repeats contained in the MT binding region. Type I encodes tau with three tandem repeats and Type II encodes an isoform with four repeats. These two isoforms are differentially expressed in the human brain. Type I and Type II are present in pyramidal calls while only Type I is expressed in granule cells of the hippocampus (Goedert et al. 1989). The isoforms are also developmentally expressed. In fetal brain, only Type I mRNA is present, while adult brains express both Type I and Type II. Type I may correspond to juvenile tau, which appears on gels as a single protein band of approximately 48 kD. Juvenile tau may have lower binding and/or bundling abilities which could allow for more plasticity in developing neurons. These forms are developmentally regulated

(Kosik et al. 1989). The transition from the juvenile form of tau to adult forms begins at a time coincident with axonal maturation and synaptogenesis (Couchie and Nunez 1985).

## MICROTUBULE / MAP INTERACTION

There are two regions in alpha and two in beta which contain most of the amino acid substitutions that occur between tubulin isotypes. In alpha tubulins these regions occur between residues 265-273 and at amino acid 340. In beta tubulins the regions of diversity are found between residues 48-57 and at the carboxy terminal (reviewed by Morrison and Griffin 1986). It has been suggested that these regions may be important in binding particular MAPs to particular tubulin isoforms.

All MAPs do not interact with tubulin with the same degree of affinity (Carlier et al. 1984). Both alpha and beta subunits have multiple binding sites with different affinities for MAPs (Littauer et al. 1986). These binding sites may be more or less conserved in the various tubulin isoforms. This raises the possibility that different MT functions could result from the expression of particular tubulin isoforms in concert with specific MAPs (Cowan et al. 1988).

The binding of MAP2 and tau to tubulin has been investigated *in vitro* (Littauer et al. 1986, Maccioni et al. 1988). Both tau and MAP2 preferentially bind to beta tubulin although binding sites also are present on the alpha tubulin subunit between amino acids 430-441 (Maccioni et al. 1988). Radioactively labeled MAP fragments bind to several positions on the beta tubulin molecule (Littauer et al. 1986). Using five synthetic peptides of various sizes, the beta tubulin region for binding MAP2 and tau

has been located between amino acid residues 422-440 at the carboxy terminal. This region includes the variable region for beta tubulin isotypes and is not at the extreme C-terminal but is within the final 4 kb and is external on the tubulin molecule (Maccioni et al. 1988).

The carboxy termini of both alpha and beta tubulin carries a negative charge with the tau binding region of tubulin particularly rich in glutamic acid (Lee et al. 1989). The multiple repeats present in MAP2 and tau, which represent the tubulin binding region, are positively charged. This suggests that electrostatic interactions are important in binding MAPs and MTs (Vallee 1982, reviewed by Serrano and Avila 1990).

## MODELS FOR NEURONAL DIFFERENTIATION

The object of this thesis is to examine the role of tubulin isoforms in the formation and function of stable MT arrays during neural differentiation. However, such a study is difficult to carry out in a mammalian embryo and therefore *in vitro* cell cultures provide good model systems to examine events which occur during embryogenesis. Three of the most commonly used tissue culture models of neurogenesis are: a) pheochromocytoma (PC 12) cells which differentiate into neurons in the presence of nerve growth factor (Greene and Tischler 1976); b) primary cultures of hippocampal neurons from embryonic brain (Bartlett and Banker 1984); c) neuroblastoma cell lines (Ross et al. 1975). All of these systems have provided useful information about neuronal differentiation. However, all of these systems

share at least one disadvantage in studying the very early events of neurogenesis - the cells in all of the systems are neuronally committed and therefore the earliest events of neural induction and commitment cannot be studied.

**Embryonal carcinoma (EC) cultures, a model for neurogenesis**

In this thesis use is made of a fourth model system - a continuous culture of pluripotent embryonal carcinoma cells. After addition of the appropriate morphogen, retinoic acid (RA), these cells undergo neural commitment followed by differentiation, thus making them a useful model to study events which occur in both early and later stages in neural differentiation (Jones-Villeneuve et al. 1982).

Embryonal carcinoma cell lines are derived from spontaneously arising teratocarcinomas or from teratocarcinomas which are induced by transplanting an early embryo to an extra-uterine site, often the testis or adrenal glands (McBurney and Rogers 1982, Rossant and McBurney 1982). These tumors contain both differentiated cell types and undifferentiated stem cells. Under appropriate culture conditions, the stem cells can be maintained as an exponentially growing cell line which consists of undifferentiated cells. Cultured EC cells retain the ability to differentiate into a variety of cell types when given an appropriate morphogen and/or put into the proper environment (Edwards and McBurney 1983). When EC cells are injected into an embryo at the blastocyst stage, the cells incorporate into the embryo and result in a

chimeric mouse in which the injected cells contribute to all tissues (Rossant and McBurney 1982) (for reference manual on EC cells see E.J. Robertson 1987).

The EC cell line used in this thesis was derived from a 7 day mouse embryo which was grafted to the testis of a mature male mouse to generate a teratocarcinoma (McBurney and Rogers 1982). The stem cells of the teratocarcinoma were subsequently established as a permanent tissue culture line, P19, and found to have a normal, male euploid karyotype (McBurney 1976, McBurney and Rogers 1982). This cell line can differentiate into neurons, glial cells, fibroblast-like cells and muscle cells depending upon the concentration of RA (Jones-Villeneuve et al. 1982, Edwards and McBurney 1983). High doses of RA ($10^{-5}$ to $10^{-7}$ M) induce neurons and glial cells while RA at ($10^{-9}$ M) induces muscle differentiation (Jones-Villeneuve et al. 1982, Edwards and McBurney 1983). Muscle can also be induced by addition of 1-2% dimethylsulfoxide (DMSO) to growth medium (Edwards et al. 1983, Smith et al. 1987, Rudnicki et al. 1990).

The neurons differentiated from P19 EC cells contain many of the neuronal markers that are associated with neurons *in vivo*, indicating that this is a good model system (Jones-Villeneuve et al. 1982, Levine and Flynn 1986, McBurney et al. 1988, Sharma and Notter 1988). Neurons in P19 cultures show synapse-forming neurites and synthesize acetylcholine suggesting that there is a population of cholinergic neurons (McBurney et al.

1988). In addition, there is evidence of another, as yet unidentified type of neuron in differentiating P19 cultures (Sharma and Notter 1988).

**Retinoic acid (RA) and DMSO - morphogens for neural and muscle differentiation of P19 cells**

Retinoic acid is a 300 Da, lipid-soluble molecule which belongs to the class of vitamin A derivatives, the retinoids. These compounds have been implicated in differentiation, cell communication, and proliferation. They are highly teratogenic and cause defects in limbs, the face and central nervous system (Summerbell and Maden 1990).

Embryologists have shown that RA can modify the pattern of differentiation by duplicating parts of the embryo when applied to regenerating limb buds (Maden et al. 1989). In undifferentiated EC cells, RA acts to induce expression of a gene, or a sequence of genes, that results in cell differentiation (Summerbell and Maden 1990).

The mode of action of RA in inducing gene transcription is partially understood. Many cells, including the P19 cells, contain a protein, cellular retinoic acid binding protein (CRABP) which specifically binds RA with high affinity (Jones-Villeneuve et al. 1982, Chytil and Ong 1984). RA enters the cell by diffusing across the membrane and is bound by the CRABP. This binding serves one of two purposes: either CRABP transports RA to the nucleus where it is picked up by nuclear RA binding proteins (NRP), or the CRABPs bind excess RA in the cytosol, thereby acting as a buffer so that comparatively little RA reaches the nucleus and is bound by NRPs. After RA enters the nucleus, it binds to the NRP, probably causing a

conformational change which allows the RA:NRP complex to bind to regions on the gene termed RA-receptor regions (RAR). This binding signals for transcription of the gene. (Summerhill and Maden, 1990). The NRP has many of the properties of steroid hormone receptors, and like them, plays a role in transcriptional mediation of some fundamental differentiation processes (Petkovich et al. 1987).

In the nucleus, 3 RA-receptors have been identified, $\alpha$, $\beta$ and $\gamma$ (Petkovich et al. 1987). The $\alpha$ and $\beta$, but not the $\gamma$, receptor genes are expressed in P19 cells (personal communication from Dr. M.W. McBurney). However, in a P19 clone which does not differentiate into neurons in the presence of RA, RAC 65, $\beta$ and $\gamma$ RA-receptors, and not the $\alpha$ receptor, are expressed. This strongly suggests that it is the $\alpha$ RA-receptor which is responsible for turning on genes that result in neuronal differentiation.

The mode of action of dimethyl sulfoxide (DMSO) in inducing muscle differentiation is less well understood. When DMSO is added to P19 cultures, neuronal differentiation does not occur, instead muscle cell differentiation is induced. DMSO may act by enhancing the activity of a differentiation-inducing substance which is secreted as extracellular material (Campione-Piccardo et al. 1985, Smith et al. 1987). DMSO may also induce protein kinase activity, resulting in a cascade of events which trigger gene activation (personal communication, Dr. M.W. McBurney). Although the role of DMSO in muscle cell differentiation remains largely unknown, it provides a method of inducing muscle cell formation without simultaneous induction of any neuronal differentiation.

# INTRODUCTION

During the 1960's and early 1970's, the common structure exhibited by MTs at the electron microscope level suggested that all MTs were identical. This hypothesis was troublesome because of the wide diversity of MT arrays and functions in cells. With the advent of isoelectric focusing and polyacrylamide gel electrophoresis, many isoforms of tubulin were identified. This led to the proposition of the "multi-tubulin hypothesis" by Fulton and Simpson in 1976. Briefly stated, this hypothesis suggests that the isoform composition of MTs is responsible for the multiple functions of MTs. However, evidence to support this hypothesis has been difficult to gather and the fundamental question regarding the function of tubulin isoforms remains unsettled. Nevertheless, the existence of variable regions in the tubulin molecule, which are highly conserved across species lines, is a strong indication that there must be some degree of function associated with isoform variation. This is further bolstered by findings which show that tubulin isoforms are differentially expressed during development and that some isoforms are tissue specific (reviewed by Sullivan 1988).

Investigation of tubulin isoforms was aided by the isolation of cDNA clones of alpha and beta tubulin mRNAs and by subsequent development of

antibodies recognizing the specific variable region of each genetically encoded isotype. Initially, it seemed that the question of isotype function would be resolved shortly. Instead, investigations in lower eukaryotic cells, which have fewer tubulin isotypes, showed that all tubulin isotypes were interchangeable in all MTs (reviewed by Raff 1984, Sullivan 1988). As well, immunoelectron microscopy showed that MTs are copolymers of all available isotypes (Geuens 1986). Moreover, even in tissue culture cells derived from higher eukaryote tissue, there was no evidence of subcellular segregation of tubulin isotypes (Lopata and Cleveland 1987). However, evidence is now emerging which shows that subcellular sorting of tubulin isoforms does occur and that the isoform composition of MTs within a cell can vary (Joshi and Cleveland 1989). Determining the function of isoforms is turning out to be very complicated. Tubulin function almost certainly is influenced by MAPs. In turn, the association and function of MAPs may be related to the presence of particular tubulin isoforms. This is further complicated by the fact that both alpha and beta tubulins have multiple binding sites for each MAP and that these sites bind with differing affinities (Littauer et al. 1986, Maccioni et al. 1988). This raises the possibility that a particular tubulin isoform may, under normal circumstances, bind a given MAP, but if expression of the tubulin isoform (or the MAP) is interferred with, an alternative isoform may be able to serve the same purpose. In such a case all tubulin isoforms could be interchangeable, but one is maximally efficient. This makes it difficult to determine any direct relationship between

a particular tubulin isoform and a particular MT function. It appears that the best chance to determine the function of tubulin isoforms/MAPs is in highly differentiated systems where there is cell-specific expression of developmentally expressed isoforms.

The pluripotent P19 EC system appears to be an almost ideal system to investigate the function(s) of tubulin isoforms. It consists of undifferentiated cells, which might be expected to have minimal specialization of MT functions and isoforms. At least two distinct differentiation pathways can be induced, neural and muscle, allowing observation and comparison of changes in tubulin isoforms during differentiation. In addition, the earliest stages of induction and commitment to a specific cell type can be explored. Finally, the P19 cells can differentiate into fully functional cell types as shown by production of normal tissues in chimeric mice (Rossant and McBurney 1982).

Therefore, the P19 EC system was chosen to investigate the involvement of tubulin isoforms in MT arrays which are specific to neurally differentiating cells. Moreover, I chose to analyze a distinct array of MTs characterized by low turnover rate and stability to colchicine induced depolymerization.

In neurons, up to 50% of the MTs are resistant to depolymerizing agents such as cold and colchicine. The function(s) of these stable MTs include structural stability of an extended neurite, possible nucleation sites for addition of new tubulin dimers or oligomers, and a role in axonal transport

(reviewed in Diaz-Nido et al. 1990). However, the role of tubulin isoforms in the establishment and/or function of the neurite stable MT array has not been determined and is therefore the focus of this thesis.

# FOREWORD

The following three sections of the thesis: Chapters 1, 2 and 3, are written in journal format. Most of the materials and methods are described in Chapter 1, with only materials and methods that are new to each section described thereafter. References for the entire thesis are assembled at the end of the thesis.

The text of Chapter 1 is a revised version of a recently published article, "Establishment of a stable, acetylated microtubule bundle during neuronal commitment" by M.M. Falconer, U. Vielkind, and D.L. Brown, published in *Cell Motility and the Cytoskeleton*, vol. 12, pp. 169-180, 1989. I have added additional data on MAP1B which was obtained after publication of this article (page 71) and have updated information on detection of MAP2C (page 72). I also have added a graph which was not included in the original manuscript (Fig. 5e) as well as additional information on continuing neural differentiation (pages 75, 77 and Fig. 9). The materials and methods section has been changed only as necessary to accomodate this additional information. The discussion section has been revised to incorporate the new data and relevant articles published since this paper appeared.

Chapter 2 also was published recently as "Association of acetylated microtubules, vimentin intermediate filaments and MAP 2 during early neural differentiation in EC cell culture" by M.M. Falconer, U. Vielkind, D.L. Brown, in *Biochemistry and Cell Biology*, vol. 67, pp. 537-544, 1989. It is presented here in essentially the same form as it was published with the exception that materials and methods previously described in Chapter 1 have been omitted from this chapter and there is addition of a paragraph on page 96 describing the detection of MAP2C.

Chapter 3 is written in journal format although the introduction is longer than usual to accomodate extra background material. It is still in preparation and has not been submitted for publication.

# CHAPTER 1: Establishment of a stable, acetylated microtubule bundle during neuronal commitment.

## INTRODUCTION

Microtubules (MTs) can be classified as dynamic or stable according to the rate at which they add and subtract tubulin dimers (Schulze and Kirschner 1987). In most cells, the MT population consists predominantly of dynamic MTs containing tyrosinated alpha tubulin (TYR MTs) (Webster et al. 1987). However, many cell types also have a subpopulation of less dynamic, more stable MTs in which two posttranslational modifications of alpha tubulin, detyrosination and acetylation, are known to occur. A tubulin-specific carboxy peptidase acts preferentially on the MT polymer to remove the final encoded tyrosine from the alpha tubulin carboxy terminus. This exposes the penultimate glutamic acid along the MT, hence the name GLU MTs (Kumar and Flavin 1981, Schulze et al. 1987, Webster et al. 1987). In a similar modification, alpha tubulin in MTs is subject to acetylation of lysine 40 which results in acetylated MTs (ACET MTs) (L'Hernault and Rosenbaum 1985b). Both posttranslational modifications can occur on the

same MT (Schulze et al. 1987). Although these modifications do not confer stability upon MTs, they are often present on colchicine resistant MTs as well as in specialized, stable MT arrays such as flagella (Schulze et al. 1987, Webster et al. 1987, LeDizet and Piperno 1986, Piperno et al. 1987).

Posttranslational modifications to MTs may play a role in the development of neuronal processes. Immature axons in rat brain contain TYR and ACET MTs which subsequently are modified to GLU and ACET MTS during differentiation. (Cambray-Deakin and Burgoyne 1987a). Stable MTs are an integral part of highly polarized structures such as axons, which can extend great distances from the cell body and contain MTs that persist for months (Sahenk and Brady 1987). These stable MTs are involved in maintaining the elongated morphology and in axonal transport (Brady et al. 1984) and may also act as nucleating sites for MT assembly within the axon (Heidemann et al. 1984, Sahenk and Brady 1987).

The availability of antibodies specific for tyrosinated alpha tubulin (Kilmartin et al. 1982, Wehland et al. 1983), detyrosinated alpha tubulin (Gundersen et al. 1984), and acetylated alpha tubulin (Piperno and Fuller 1985) has stimulated examination of possible stable MT involvement in cell differentiation and specialization. We have used these antibodies to examine the changes in MT dynamics and stability which occur during neuronal differentiation in the pluripotent, embryonal carcinoma (EC) cell line, P19 (McBurney and Rogers 1982).

When P19 cells are maintained in the proliferative state, they remain uncommitted and pluripotent. By addition of varying concentrations of the morphogen, retinoic acid (RA), P19 EC cells can be induced to form either neurons and glial cells, skeletal muscle and beating cardiac muscle, or fibroblast-like cells (Jones-Villeneuve et al. 1982, Edwards and McBurney 1983).

This P19 cell line is particularly valuable for studying the process of neuronal differentiation because, unlike PC 12 or neuroblastoma cell lines, P19 cells have not undergone commitment to the neural line of differentiation. This allows an investigation of MT modifications which occur during the earliest period of commitment as well as those which occur later, during differentiation.

This chapter shows there are changes in MT dynamics and stability during commitment, 24 hours after addition of the morphogen, RA. There also is establishment of an acetylated, colchicine-stable MT bundle which may be important in establishing polarity in differentiating cells. Although not directly tested, we suggest in cells which proceed to differentiate immediately, this MT bundle may extend to form the neurite. Finally, a model is presented which describes the possible involvement of acetylated alpha tubulin in neuronal differentiation.

# MATERIALS AND METHODS

## Cell Culture

The P19 mouse EC cell line (McBurney and Rogers 1982) was the kind gift of Dr. M. McBurney, Univ. of Ottawa. The P19 cells were maintained at 37°C and 5% $CO_2$ in alpha-MEM (Flow Laboratories, Mississauga, Ont.), supplemented with 10% heat-inactivated FBS (Flow Lab.), and passaged every two days using 0.25% trypsin (Flow Lab.) and 1 mM EDTA (Sigma Chemical Co., St. Louis, Mo.) in calcium-free and magnesium-free PBS.

For RA (Sigma) induced neuronal differentiation, cells were aggregated for 1 hour in alpha-MEM plus 10% FBS then plated on 22 x 22 cm coverslips that had been coated with sterile 0.1% gelatin (Sigma) diluted w/v in distilled water. The plating density of cells was 1 X $10^5$ cells per coverslip in experiments requiring only one day RA differentiation. When longer periods of RA-induced differentiation were required, plating density was lowered to 2 x $10^4$ cells per coverslip to prevent crowding. The coverslips were placed in 35 mm tissue culture dishs in alpha-MEM plus 10% FBS. After 24 hours, the medium was replaced with alpha-MEM plus 2% FBS containing $10^{-6}$ M RA to induce neuronal differentiation. RA-containing medium was replaced every second day when differentiation was continued for three or more days. For muscle differentiation, cells were

aggregated for 1 hour then plated at 1 x $10^4$ cells per 22 x 22 mm coverslip in alpha MEM plus 10% FBS. One day after plating, the medium was removed and replaced by alpha MEM plus 8% FBS containing $10^{-9}$ M RA. The medium was replaced every two days thereafter with identical medium containing RA. A $10^{-2}$ M stock solution of RA was prepared in ethanol, stored at -80° C, and diluted to $10^{-4}$ M in medium. Appropriate amounts were added to the cultures for a final concentration of $10^{-6}$ M RA.

To determine if later rounds of neural differentiation require the continuous presence of RA, parallel cultures were grown as detailed above except that 1 culture was treated with $10^{-6}$ M RA for 5 hours followed by washing 3X with PBS and then grown in medium without RA while the parallel culture was grown in the continuous presence of $10^{-6}$ M RA as described above. The cultures were examined for differentiation at 4, 5 and 6 days after initial addition of RA.

To examine MT stability, MTs were treated with 1 μg/ml colchicine (Sigma) in growth medium for 45 minutes to 1 hour at 37° C followed by rapid fixation. Colchicine was made up as a stock solution of 1 mg/mL in sterile distilled water and stored at -80° C.

**Immunofluorescence Techniques**

Cells were routinely fixed according to the following protocol. Cells attached to coverslips were briefly washed in calcium-free and magnesium-free PBS and then simultaneously fixed and extracted in 3.7% formalin plus 0.25% glutaraldehyde (both from J.B. EM Services) (v/v) in PEM buffer pH 6.8 (80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$, and 1% Triton X-100 [all purchased from Sigma]), for 5 minutes. The coverslips were rinsed 3 x 3 minutes in PBS, postfixed for 3 minutes in ice-cold 95% ethanol, followed by 3 x 3 minute rinses in PBS. Cells were then processed for immunofluorescence microscopy. When cells were to be stained with anti-MAP antibodies or with a muscle specific anti-actin antibody, the following fixation method was used. Cells attached to coverslips were washed in PBS as previously described, then fixed in methanol at -20° C for 20 minutes followed by 3 x 5 minute rinses in PBS.

After fixation, staining for immunofluorescence microscopy was done according to the following method. Primary antibody was diluted in PBS and incubated with cells attached to the coverslip for 45 minutes, at room temperature. Double immunofluorescence staining was done by simultaneous incubation in two primary antibodies for 45 minutes at room temperature. Coverslips were then rinsed 3 x 3 minutes in PBS followed by incubation with the appropriate secondary antibody for 45 minutes at room temperature.

For double labeling, the two secondary antibodies were applied simultaneously. Coverslips then were stained for 1 minute with Hoechst dye no. 33258 (Calbiochem-Behring Corp., LaJolla, Calif.) diluted 1:10000 (w/v) in PBS, to visualize DNA. This was followed by 3 x 3 minute rinses in PBS. Coverslips were mounted in mounting medium containing 2.5% (w/v) of 1,4-diazabicyclo(2.2.2)octaine (DABCO) (Aldrich Chem. Corp., Milwaukee, Wis.) dissolved in 9 parts glycerol to 1 part Tris (Bio-Rad) buffer, pH 8.6, for observation. All cells were examined with either a Zeiss Axiophot microscope or a Zeiss Universal microscope with epifluorescence optics. Micrographs were taken on Ilford XP1-400 ASA film.

The following primary antibodies were used: 6-11B-1 mouse monoclonal antibody against acetylated alpha tubulin (Piperno and Fuller 1985), a gift from Dr. G. Piperno (Rockefeller Univ.); YOL 1/34, a rat monoclonal which stains most forms of alpha tubulin (Kilmartin et al. 1982), purchased from Dimension Laboratories, Mississauga, Ont.; YL 1/2, a rat monoclonal which stains tyrosinated alpha tubulin (Wehland et al. 1983) (Dimension Lab.); GLU rabbit polyclonal antibody which recognizes detyrosinated alpha tubulin, the gift of Dr. G. Gundersen and Dr. C. Bulinski.

The following mouse monoclonal antibodies to MAPs were the gift of Dr. L.I. Binder: MAP1A (clone 1WM-4C2), MAP1A (clone 1WM-4C8), MAP1B (clone 1mW-6E2,4), MAP1B (clone 1W-6D4), MAP2 (clone AP14), MAP2 (clone AP-18), tau protein (clone Tau-1) and (clone Tau-2). A mouse

monoclonal antibody, MA-931, recognizing alpha and gamma muscle actin (Tsukada et al. 1987) was purchased from Enzo Biochem. Inc., N.Y., N.Y.

The following secondary antibodies were used: FITC-conjugated rabbit anti-rat IgG cross-absorbed against mouse (Zymed, Dimension Lab.); FITC-conjugated goat anti-rabbit IgG (Miles-Yeda Ltd., Research Prod. Elkhart, Ind.); FITC-conjugated goat anti-mouse IgG (Cappel, Organon Teknika, West Chester, Pa.); rhodamine-conjugated rabbit anti-mouse IgG cross-absorbed against rat (Zymed, Dimension Lab.).

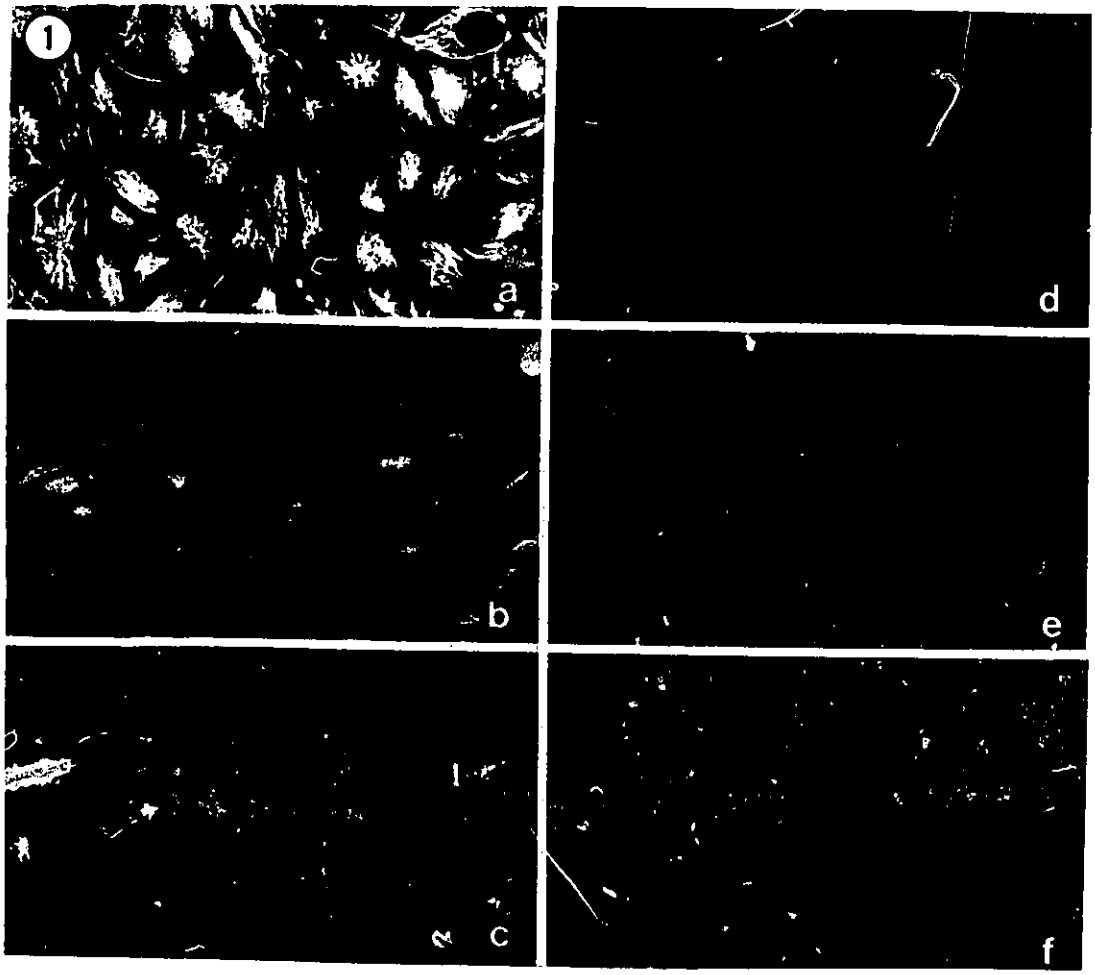
# RESULTS

This article is primarily concerned with changes in MT *arrays* during neural differentiation, as opposed to changes at the level of individual MTs. These changes in MT organization, composition and stability were monitored by indirect immunofluorescence microscopy using antibodies recognizing acetylated alpha tubulin (ACET), tyrosinated alpha tubulin (TYR) and detyrosinated alpha tubulin (GLU). A fourth antibody, YOL 1/34, which recognizes most forms of alpha tubulin was used as a general tubulin stain.

## MT Arrays in Uncommitted EC Cells

The MT population in uncommitted P19 EC cells consists predominantly of MTs that stain with the TYR antibody (Fig. 1a) plus a smaller array GLU-stained MTs (Fig. 1b). Uncommitted EC cells have a minimal subset of centrosome-associated MTs that stain with the ACET antibody (Fig. 1c). For ease in description, MTs visible with TYR, ACET or GLU antibody staining will be called TYR, ACET or GLU MTs, respectively. This is not to imply that individual MTs contain only tyrosinated, acetylated or detyrosinated alpha tubulin. Geuens et al. (1986), using immuno-electron microscopy, have previously demonstrated that all MTs are co-polymers of available tubulin isotypes, although the isotype amounts vary between MTs and within regions of the same MT.

Fig. 1. Immunofluorescence staining of MT arrays in uncommitted EC cells. **a:** YL 1/2 antibody to tyrosinated (TYR) alpha tubulin. **b:** GLU antibody to detyrosinated (GLU) alpha tubulin. **c:** 6-11B-1 antibody to acetylated (ACET) alpha tubulin. **d to f:** Immunofluorescence staining of colchicine stable MT arrays in uncommitted EC cells using (d) TYR, (e) GLU, and (f) ACET antibodies. **d:** No stable TYR MTS are visible. Cytoplasmic stain in (d) is due to YL 1/2 staining of incompletely extracted tubulin monomers. Nuclei appear as dark areas with the cells. **e:** Few stable GLU MTs and **f:** Few stable ACET MTs are present in undifferentiated EC cells.
Mag. = 500 X.


1
a
d
b
e
c
f

Most MTs in uncommitted P19 EC cells are not stable against colchicine-induced MT depolymerization. After treatment of the cells with 1 μg/ml colchicine for 1 hour, all TYR MTs, with the exception of midbodies and a few, sinuous MTs, are depolymerized (Fig. 1d). Essentially all GLU and ACET MTs are also depolymerized under these conditions, again with the exception of midbodies and a small, stable MT array (Fig. 1e,f).

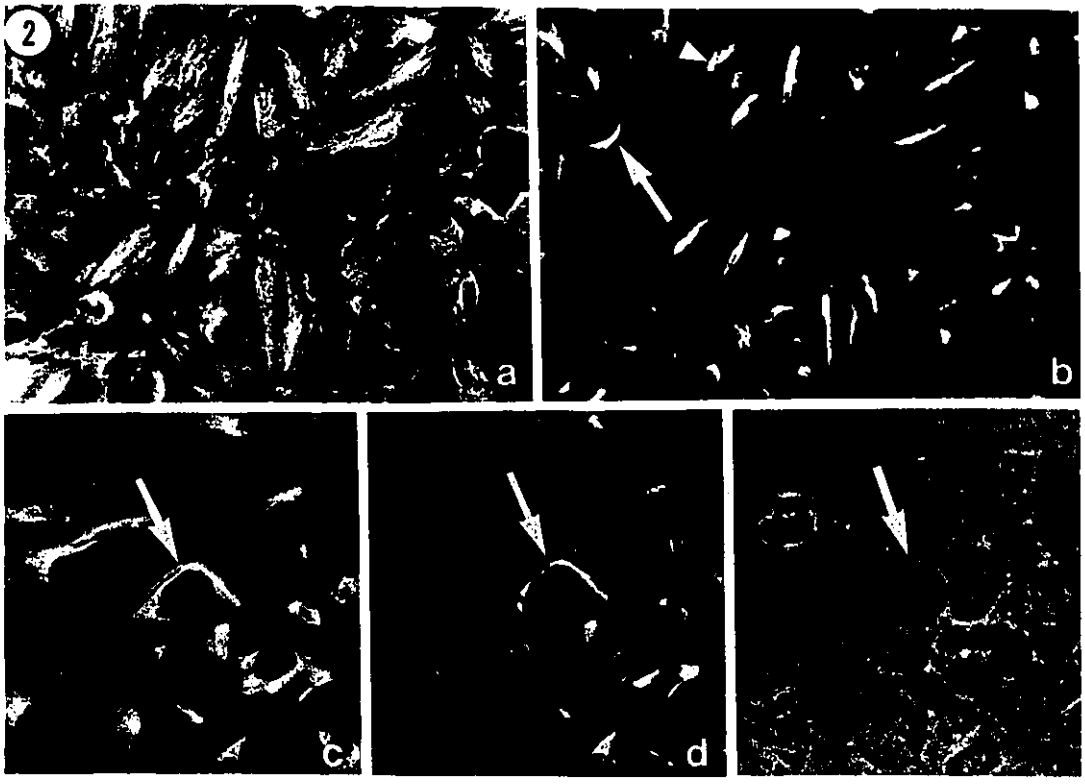
**Increase in MT Array Size and Stability During Neural Commitment**

The initial 24 hour period after addition of $10^{-6}$ M RA (1 day RA) is referred to as the period of neural commitment. During this time, there is a temporary increase in the MT population stained with TYR, ACET, and GLU antibodies (Fig. 2a-c). At the same time, a new MT array can be seen although it is difficult to detect in cells stained with the TYR antibody because of the numerous MTs (Fig. 2a). However, in ACET stained cells, the newly enlarged MT array is present as a distinct subset (Fig. 2b), in which MT array patterns are easily classified.

Approximately 50% of ACET stained cells have small "centrosomal arrays" of MTs. The remaining 50% of cells have MT arrays classified as "MT bundles".

Fig. 2. MT arrays increase in extent after 1 day retinoic acid (RA) (compare with Fig. 1 a,b, and c). Double label with (a) TYR and (b) ACET antibodies. **a:** Increase in TYR MTs. Note that ACET MT arrays consist of loose MT bundles (arrowhead), tight MT bundles (arrow), and centrosomal arrays. Double label with GLU (c) and ACET (**d**) of a cell with a MT bundle. Phase contrast of same cell (arrows in c, d, and e locate the same cell) indicates cell does not have any extensions at this time.

Mag. = 500 X.


2
a
b
c
d

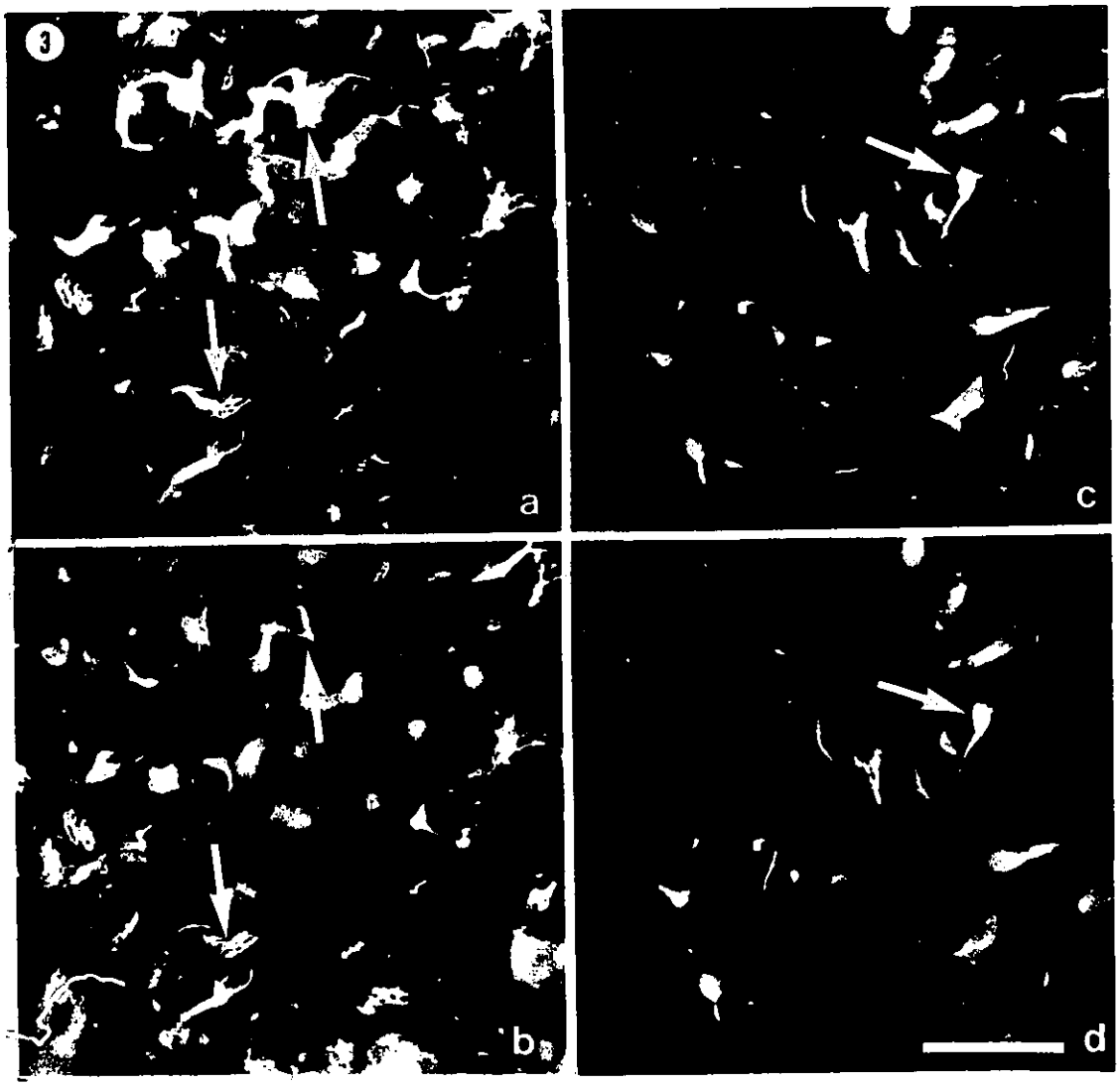
This category can be further subdivided into "loose bundles" (arrowhead in Fig. 2b), and "tight bundles" (arrows in Fig. 2b). The distinction between loose and tight is based upon the amount of space visible between MTs (at the resolution level of the light microscope). Additionally, tight bundles, but not loose bundles, often appear to taper at the end farthest from the cell nucleus and are not more than 1 to 2 cell bodies in length.

GLU MT arrays can also be classified as centrosomal arrays or bundles (Fig. 2c), although the distinction is made difficult by the presence of a greater number of MTs not contained within the bundles. Double staining with GLU (Fig. 2c) and ACET antibodies (Fig. 2d) illustrates that the ACET MT arrays are contained within the GLU MT arrays, but there are GLU MTs that are not acetylated. Phase contrast images of cells containing MT bundles show that most cells have no elongated processes at this time (Fig. 2e).

Virtually all cells acquire a subpopulation of colchicine-stable MTs by 24 hours after addition of RA (Fig. 3). Although individual colchicine-stable TYR, ACET and/or GLU MTs are present, it is the bundled array of stable MTs that is the dominant feature of the differentiating cell culture. Double labeling of colchicine-treated cells with ACET and TYR antibodies (Fig. 3a and b) and with ACET and GLU antibodies (Fig. 3c and d) shows that these bundles of stable MT contain all three alpha tubulin isotypes.

Fig. 3. MT bundles in 1 day RA cells treated with μg/ml colchicine are colchicine stable and contain ACET, TYR and GLU MTs. Double label with (a) ACET and (b) TYR antibodies. Note the colocalization of stable ACET and TYR MT bundles (arrows). Double label with (c) ACET and (d) GLU antibodies. Note identical staining patterns of ACET and GLU stable MT bundles (arrow).

Mag. = 500 X.


3
a
c
b
d

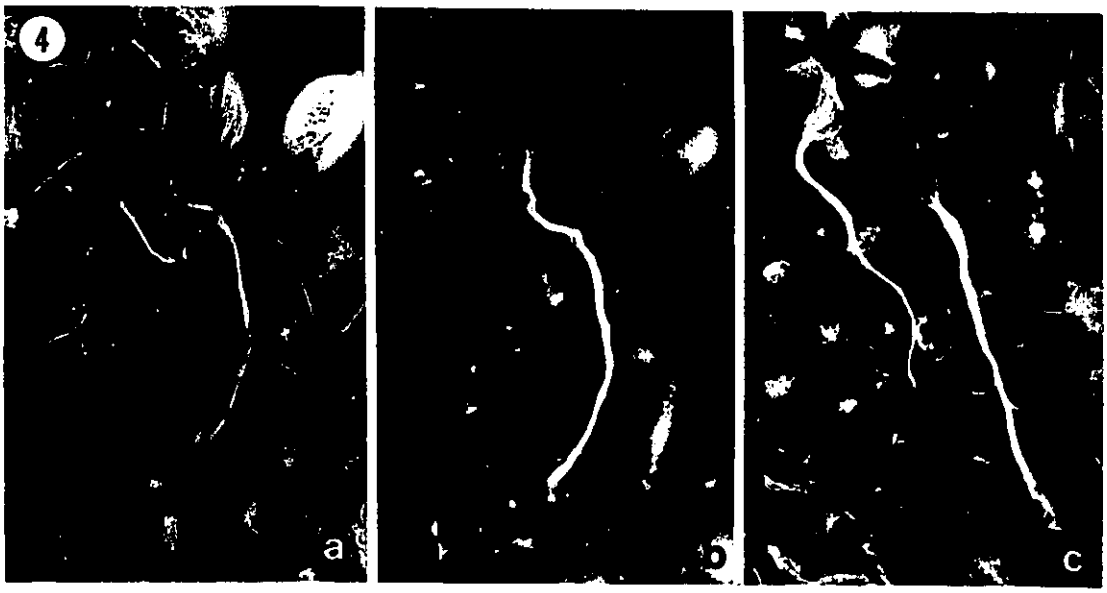
Although we have not tested this directly, repeated observations of bundles of varying length suggest that the short, stable MT bundles may elongate into neurites. These early neurites are short, less than 5 cell bodies in length, and like the stable MT bundles, contain TYR, ACET, and GLU MTs (Fig. 4). In cells stained with the TYR antibody, neurite extensions can be difficult to detect (Fig. 4a). However, in cells stained with the ACET or GLU antibodies, the elongating neurite stands out clearly against the MT arrays found in nonneural cells (Fig. 4b,c).

The increase in polymerized MTs, which occurs at 1 day RA, is a transient event. In the period between 24 and 48 hours after RA addition (i.e. between 1 day RA and 2 days RA), the TYR, ACET and GLU MT arrays in 75% or more of cells are reduced to levels approximating those in uncommited EC

cells. This reduction can be seen by comparing the MT arrays in the non-neural cells in Figure 4 with those in Figure 2a-c. Since many MT bundles are also depolymerized, we wondered if the bundles were, in fact, important in neurite formation. Evidence to support the importance of MT bundles can be obtained by exploiting the fact that EC cultures are pluripotent.

Fig. 4. Neurite extension in 2 day RA double labelled cells contain (**a**) TYR MTs and (**b**) ACET MTs. Similar neurite extensions in other cells also contain GLU MTs (**c**). TYR, ACET, and GLU MT arrays in non-neuronal cells in the monolayer have decreased to approximately the level present in uncommitted EC cells (compare with Fig. 1a and b).

Mag. = 650 X.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Jones-Villeneuve et al., (1982) have shown that differentiation pathways in P19 cells can be altered by changing the concentration of RA. Neurons are induced in the presence of $10^{-6}$ M RA, while muscle cells (but no neurons) are induced at $10^{-9}$ M RA. These results were obtained using a slightly different method of culturing. We have confirmed these results in our culture system.

We examined cultures induced with $10^{-6}$ M and with $10^{-9}$ M RA at two time points: after 1 day RA and after 6 days RA. To ascertain if stable MT bundles were present, the 1 day RA cultures were treated with 1 μg/ml colchicine for 45 minutes immediately before fixation. There was a large increase in acetylated centrosomal MT arrays at 1 day RA in the cultures treated with $10^{-9}$ M RA compared to the cultures treated with $10^{-6}$ M RA. Parallel cultures, grown in $10^{-6}$ M or $10^{-9}$ M RA for 6 days, were scored for presence or absence of neuron-like morphology by staining with either antibody to acetylated alpha tubulin or the YOL 1/34 antibody to all alpha tubulin.

Bundles of stable ACET MTs were present in the cultures which were exposed to $10^{-6}$ M RA (Fig. 5a), and after 6 days, parallel cultures had a large network of well-developed neurons as shown by YOL 1/34 anti-tubulin staining (Fig. 5b). However, cells induced with $10^{-9}$ M RA had no stable ACET MT bundles after 24 hours of RA (Fig. 5c). Parallel cultures, exposed for 6 days to $10^{-9}$ M RA, had no neurons as shown by YOL 1/34 anti-tubulin staining (Fig. 5d).

Fig. 5. **a:** Bundles of ACET stable MTs are present in cells grown for 1 day in $10^{-6}$ M RA and treated with 1 μg/ml colchicine for 1 hour immediately before fixation. **b:** Parallel culture grown for 6 days in $10^{-9}$ M RA (not treated with colchicine) and stained with YOL 1/34 to visualize extent of neuronal differentiation. **c:** Only small centrosomal arrays of ACET stable MTs are present in cells grown for 1 day at $10^{-9}$ M RA and treated with 1 μg/ml colchicine for 1 hour. **d:** Parallel culture grown in $10^{-9}$ M RA for 6 days (but not treated with colchicine) has no neurons, as shown by staining with YOL 1/34 antibody.

Mag. in a and c = 600 X. Mag. in b and d = 225 X.

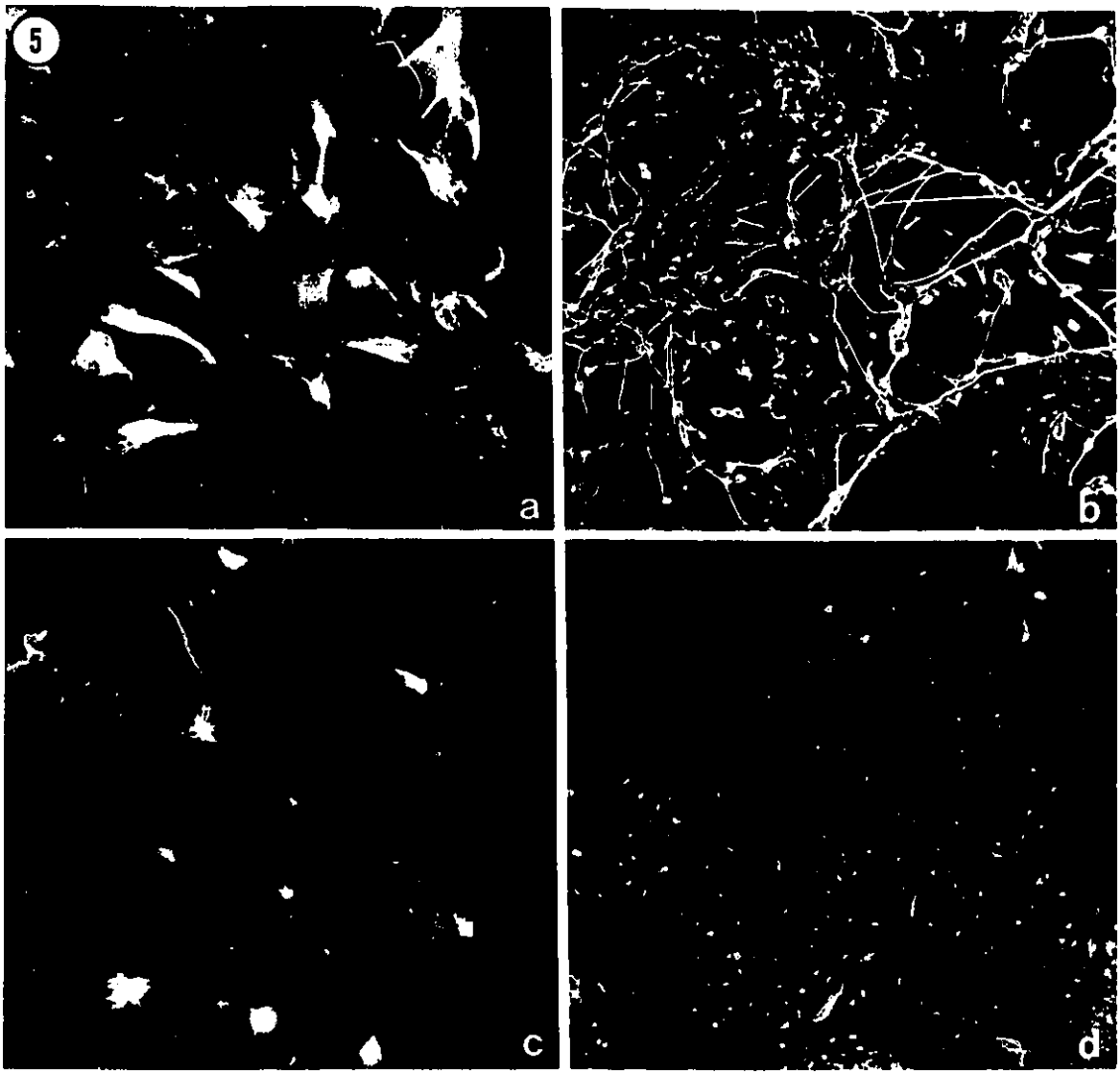
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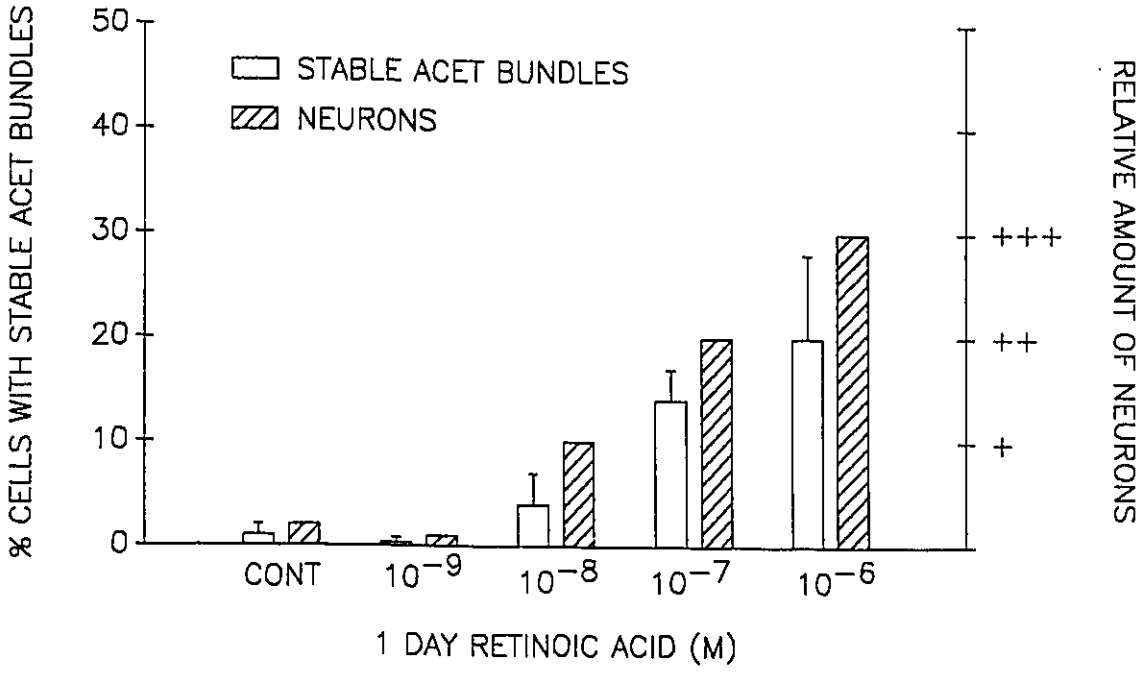
Fig. 5e. Superimposed graphs showing: 1) Left axis - the percent of cells with stable ACET MT bundles after 1 day in varying amounts of RA and, 2) Right axis - the relative amount of neurons which differentiated in parallel cultures after 6 days RA. Control cultures received no RA. Mean and standard deviation for each treatment based on counts of 500 cells in each of 4 independent samples. The relative amount of neurons is a value judgement obtained by scanning samples at each RA level in a "blind" assay and assigning the samples to categories of: essentially no neurons; few neurons (+), more neurons (++), most neurons (+++) - where most neurons represents neuronal differentiation of about 75% of the total cell population.


% CELLS WITH STABLE ACET BUNDLES
50
40
30
20
10
0
STABLE ACET BUNDLES
NEURONS
+++
++
+
RELATIVE AMOUNT OF NEURONS
CONT
$10^{-9}$
$10^{-8}$
$10^{-7}$
$10^{-6}$
1 DAY RETINOIC ACID (M)

Due to branching and anastomosing of neurite extensions, it is not possible to count differentiated neurons. However, by inducing cultures with RA levels between $10^{-6}$ M and $10^{-9}$ M, states intermediate in number can be reached. Cultures with a relatively low number of stable MT bundles, (determined by counting cells using low-power, wide-field observation) subsequently differentiated into cultures with correspondingly fewer neurons (Fig. 5e). Staining with an antibody specific for alpha and gamma muscle actins, MA-931, showed that after 6 days RA, cultures treated with $10^{-8}$ and $10^{-9}$ M RA had increased muscle cell differentiation in comparison with cells grown in the presence of $10^{-6}$ M RA (data not shown).

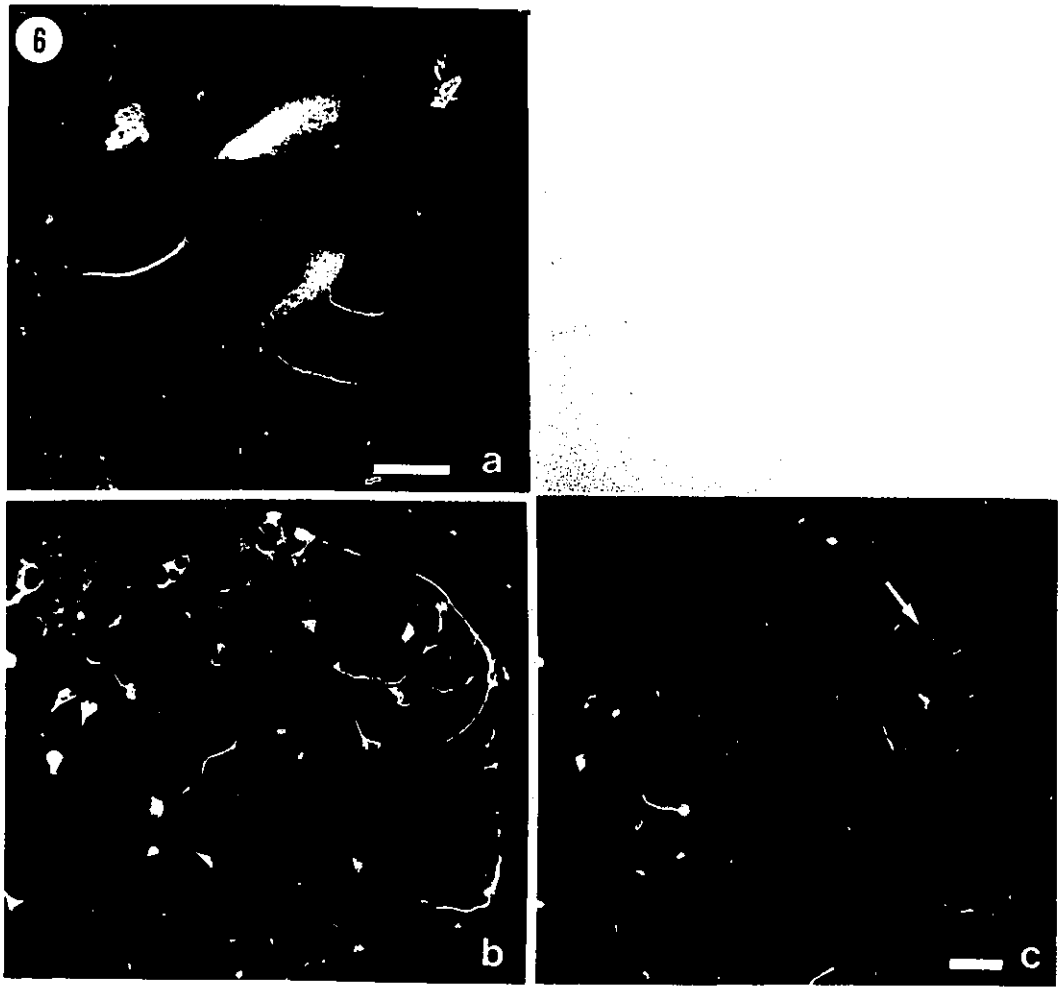
**Presence of MAPs in Stable MT Arrays**

To determine whether MAPs are involved in the colchicine resistance of the ACET MT bundle, colocalization of MAPs with the stable MT bundle at 1 day RA was examined by indirect immunofluorescence staining. Only MAP1B staining is detected in the early stable MT bundle that appears at 1 day RA. MAP1B is present in numerous non-neural cells (reviewed by Diaz-Nido et al. 1990). In uncommitted EC cells, the MAP1B staining pattern is coincident with most MTs and is present in all cells (data shown in Chapter 3, Fig. 8). After 1 day RA, MAP1B staining increases in about 15% of differentiating cells and appears to be preferentially localized to MT bundles although this has not been demonstrated by immuno-electron microscopy. In colchicine treated cells, MAP1B colocalizes with the stable

MT array including the stable MT bundle, suggesting that MAP1B, or a posttranslationally modified form of MAP1B, could be involved in stabilization of the bundle.

The earliest neuron-specific MAP to appear is MAP 2C, the juvenile form of MAP2, which is first detected between 2 and 3 days RA. The specific presence of MAP2C is detected by a process of elimination using two MAP2 antibodies; clone AP-18 which recognizes the 270 kDa adult forms (MAP2A and MAP2B) as well as the 70 kDa juvenile MAP2C, and clone AP-14 which recognizes only the high molecular weight adult forms (Binder et al. 1986, Tucker et al. 1988). Between 2 and 3 days RA, a subset of cells are stained only by clone AP-18 indicating that it is the juvenile MAP2C which is present. This subsequently was confirmed by immunoblotting (see Chapter 3, Fig. 16). MAP2C is present in the cell body as a diffuse, cytoplasmic stain and is not restricted to a particular developing neurite process (Fig. 6a). If a cell has two processes, MAP2C is present in both (Fig. 6a) and MAP2C can also be found in cells that have no neurite extensions (Fig. 6c). MAP2C staining colocalize on a 1:1 basis with cells containing colchicine stable MT arrays (compare Figs. 6b and c). Although

Fig. 6. Immunofluorescence stain of MAP2C first appears 2 days after addition of RA. **a:** Two cells with MAP2C; in a cell with two developing neurites, MAP2C is present in both. Mag. = 675 X. **b and c:** Cells treated with 1 μg/ml colchicine for 1 hour and double labelled with (b) YOL 1/34, an antibody that recognizes most isotypes of alpha tubulin and (c) antibody to MAP2C. All cells with colchicine stable MTs also have MAP2C. Note that MAP2C is also present in some cells without neurites (arrow). The cytoplasmic stain in (b) is due to YOL 1/34 staining of incompletely extracted tubulin monomers. Mag. b and c = 300 X.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it is not possible to determine if all cells with MAP2C will have stable MTs after colchicine treatment, it is possible to demonstrate that all cells with colchicine stable MTs do have MAP2C at this stage of differentiation.

**ACET and TYR MTs in Neurites Are Differentially Stable to Colchicine**

By 3 days after RA induction of differentiation, clusters of neurons can be seen on top of the cell monolayer. Although both ACET and TYR MTs are present in short neurites (see Fig. 4), double labeling of longer neurites with ACET (Fig. 7a,) and TYR antibodies (Fig. 7b) indicates that not all neurites have a population of TYR MTs. In many neurons, ACET MTs are found along the length of the entire neurite, while TYR MTs are present only in sections of the neurite.

When 3 day RA cultures are treated with colchicine and double labeled for ACET (Fig. 8a) and TYR (Fig. 8b), ACET MTs in long neurites remain colchicine-resistant while the TYR MTs are now completely depolymerized by colchicine. Thus, unlike short neurites that have stable ACET *and* TYR MT populations, long, colchicine-treated neurites do not have any regions that label with *both* ACET and TYR antibodies (Fig. 8a and b).

During days 3, 4 and 5 RA, neural differentiation continues. Now organized *groups* of cells that contain a particular alpha tubulin isotype, or combination of isotypes, can be identified. Low-magnification, wide field

microscopy shows cluster of cells containing ACET MT arrays, arranged in a radial pattern (demarcated by brackets in Fig. 8a). Although more difficult to distinguish, a TYR MT array is also present in these cells, as shown by a double label with TYR antibody (Fig. 8b). Higher magnification of a similar group of cells (Fig. 8c and d) confirms that at this time during differentiation, new short bundles of MTs contain both ACET MTs (arrows, Fig. 8c) and TYR MTs (corresponding arrows, Fig. 8d).

Another type of neural differentiation occurs within the period of 4 to 5 days RA. Islands of cells with increased ACET MTs appear within the monolayer (Fig. 9a), although the cells do not show increased TYR MTs (Fig. 9b). These cells have ACET MT bundles (Fig. 9c) but unlike the ACET bundles in 1 day RA cells, they are not colchicine stable (data not shown).

To determine if these cells represent later rounds of neural differentiation resulting from the long-term presence of RA, uncommitted EC cultures were treated only for a 5 hour period with $10^{-6}$ M RA and then grown in medium without RA. Duplicate cultures were maintained under standard conditions with RA present for the entire period of differentiation. Results indicate that later rounds of differentiation do not depend upon the continuous presence of RA.

In "5 hour pulsed-RA" cultures (Fig. 9d) and in "continuous RA" cultures (Fig. 9a), islands of cells with increased ACET MTs appear. Late differentiating cells arranged in a radial pattern (Figs. 8a and c) also were

present in the "5 hour pulsed-RA" cultures. These two types of differentiating cells may represent development of other neural cell types.

Fig. 7. Low magnification showing double label with (a) ACET and (b) TYR antibodies of neurons 3 days after RA addition. **a:** A group of neurons with MTs containing ACET alpha tubulin. **b:** Tyrosinated alpha tubulin is present in some MT regions at this stage of differentiation. Arrow in (a) points to neurite with ACET MTs and corresponding arrow in (b) identifies the same region, which has no TYR-stained MTs. Mag. = 225 X.

Fig. 8. Very low magnification showing: **a:** Colchicine treated cells initiating differentiation after 4 days RA and stained with antibody to acetylated tubulin. Neural cells appear as foci of short, colchicine stable, ACET MT bundles (brackets). **b:** Double label of same cells with TYR antibody. Cytoplasmic staining in **b** due to label of monomeric alpha tubulin makes it difficult to identify colchicine stable TYR MTs in these developing bundles. **c** and **d:** Higher magnification of a similar, colchicine treated area double labelled with ACET (c) and TYR (d) antibodies. Both long, older neurites and short MT bundles in developing neurites (arrows) contain colchicine stable ACET MTs (c); colchicine stable TYR MTs are found only in short MT bundles (arrows) (d).
Mag. a and b = 50 X. c and d = 225 X.

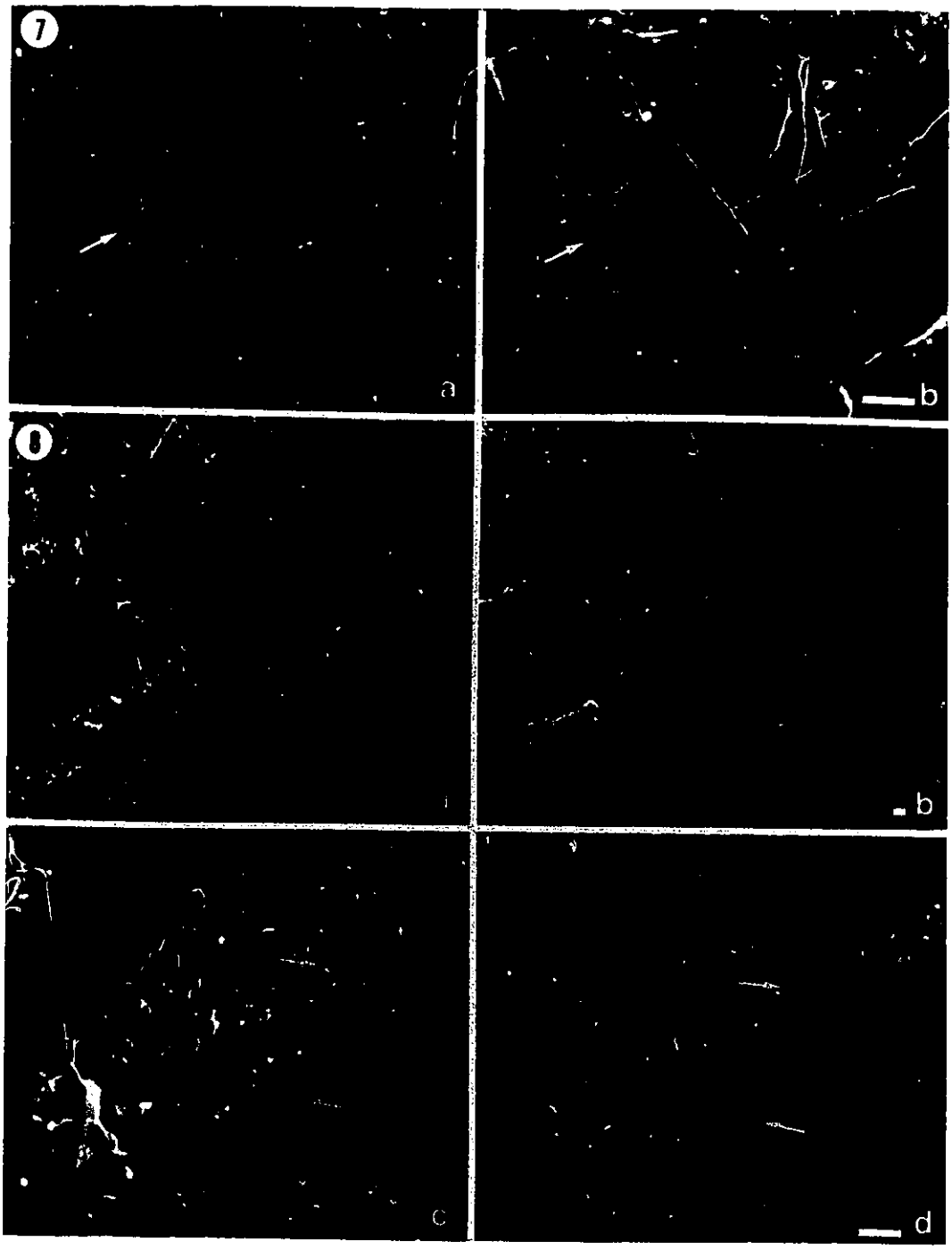
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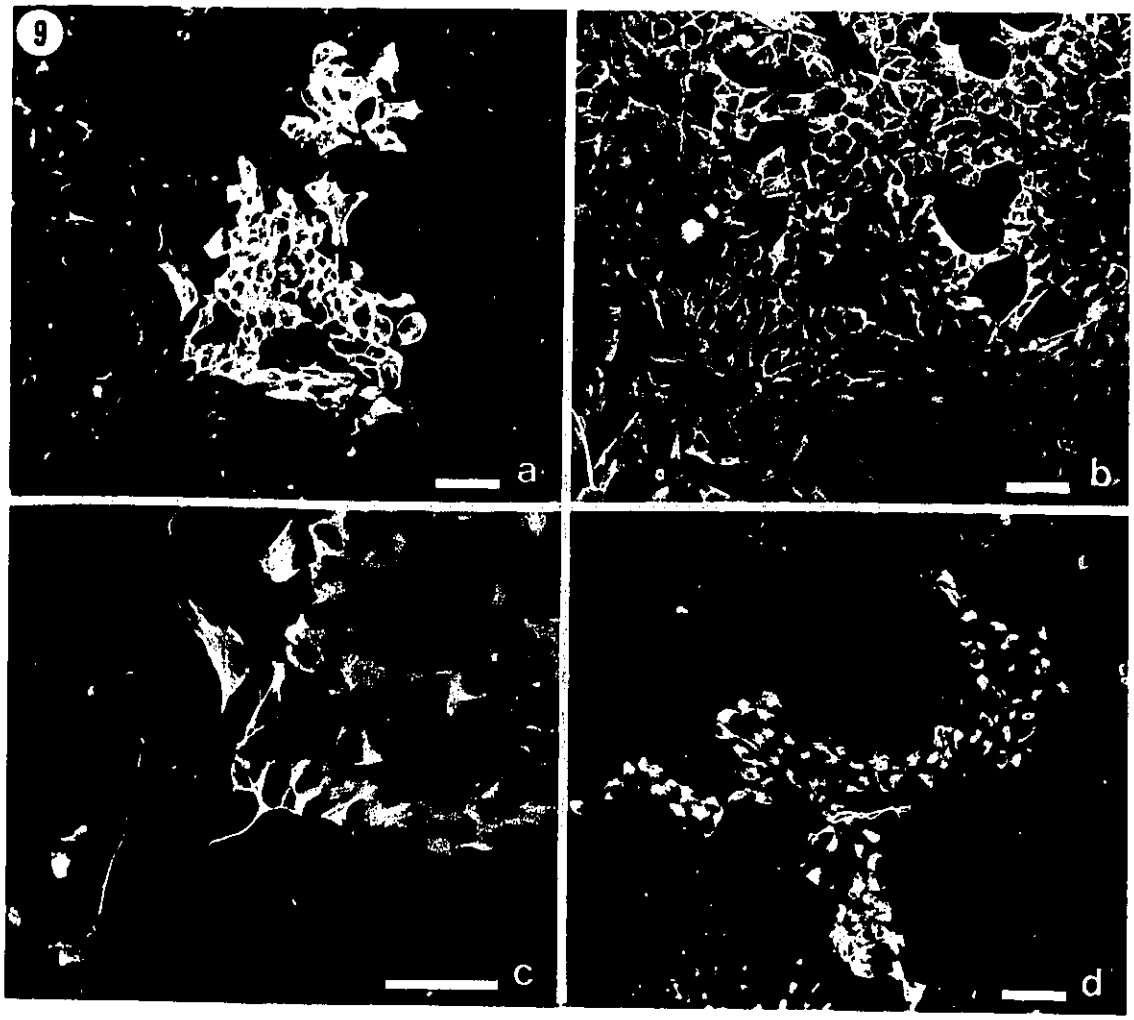
Fig. 9. **a:** Late differentiating cells stained with anti-acetylated antibody show an increase in acetylated alpha tubulin. **b:** The same cells double labelled with TYR antibody, no increase in tyrosinated alpha tubulin can be detected. **c:** Higher magnification of (a) shows ACET MTs are present in cell bodies and in processes. **d:** Late differentiation also occurs in cells exposed to RA for only 5 hours as shown by staining with anti-acetylated antibody.

Mag.a, b and d = 300 X, c = 600 X.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# DISCUSSION

## Neural Induction, Commitment, and Differentiation

In the embryo and in cell culture, induction of a pluripotent cell by a morphogen is followed by a period of commitment and differentiation. For the purposes of this discussion commitment is defined as the events that constrain a cell (and its progeny) to follow a particular developmental path, while differentiation is considered to involve events that result in attainment of a specific cell shape and/or function. Using this definition, commitment and differentiation can be separable events. A cell can undergo commitment without immediately proceeding to undergo morphological and/or functional differentiation.

To simplify description of neurogenesis in P19 cells, post-induction changes in MT arrays have been divided into stages.

### Stage 1: neural commitment.

Stage 1 (24 hours RA) is characterized by a transient increase in MTs; initiation of an ACET MT array; increased MT stability and formation of a bundle of stable MTs containing tyrosinated, detyrosinated and acetylated alpha tubulin isotypes. MAP1B is present in all cells but is found at higher levels in a small percentage of cells where it preferentially labels

MT bundles. No other brain-specific MAPs are detectable in cells containing colchicine stable MT bundles.

**Stage 2: events of early differentiation.**

Stage 2 (48 hours RA) is characterized by the elongation of neurite extensions from cells which now lie atop the cell monolayer. The first neuron-specific MAP, the juvenile form of MAP2 (MAP2C), is found in newly elongating neurites but is not yet restricted to a specific process (i.e. is not yet dendrite-specific). MAP2C is present in all cells with colchicine stable MTs. During Stage 2, ACET MT arrays in nondifferentiating cells in the monolayer return to levels approximating those in uncommitted EC cells.

**Stage 3: neurite specialization**

The long neurites present in stage 3 (72+ hours RA) are characterized by progressive loss of tyrosinated alpha tubulin. Although long neurites show some TYR staining, after colchicine treatment, long neurites do not stain for TYR MTs indicating a probably loss of TYR MT stability to colchicine. New rounds of differentiationg are initiated. Tau and the adult form of MAP2, now appear (see Chapter 3, Fig. 16).

TYR-staining MTs constitute a population of dynamic MTs (Webster et al. 1987, Gundersen et al. 1987), while acetylation is a posttranslational modification present on stable MTs (Piperno et al. 1987). Axonal MTs of mature neurons are noted for stability (Black et al. 1984, Brady et al. 198[illegible]

and Cambray-Deakin and Burgoyne (1987a) demonstrated, in sections of rat cerebellum, that mature axonal MTs are enriched in acetylated and detyrosinated (GLU) alpha tubulin, while immature axons contain acetylated and tyrosinated alpha tubulin. Our observations are similar, except that in very early neurons we detect not only acetylated and tyrosinated but also detyrosinated MT staining.

**The Stable, Acetylated MT Bundle, An Early Event in Neural Commitment**

We suggest that establishment of a stable, acetylated MT bundle takes place very early in neurogenesis, before morphological change has occurred. The transient increase in polymerized MTs, which occurs during commitment, could result in bundle formation in at least two possible ways: 1) enough MTs may be polymerized to encourage lateral interaction between MTs, thereby forming a stabilized structure which is subsequently acetylated; and/or 2) under optimal polymerizing conditions, MTs may elongate until they reach a structure (such as the plasma membrane) and are "end-stabilized" by this structure (Kirschner and Mitchison 1984). These end-stabilized MTs would then be acetylated and detyrosinated, which may confer other stabilizing effects upon them. The stabilized MTs subsequently are bundled by some unknown mechanism such as MAPs, lateral interaction of MTs, membrane involvement, etc. The exact mechanism of bundle formation and the function of this bundle is unknown.

Although there is continuing formation of new stable MT bundles throughout the period examined, counts at 1 day RA and 2 day RA do not show increased numbers of stable MT bundles, suggesting that some of these bundles are depolymerized. It may be that if process extension or neurite growth does not immediately take place, the commitment-specific MT array largely depolymerizes. The formation of such a transient MT array has not been previously reported and appears to be specific to neural differentiation. Similar stable MT bundles do not appear when P19 cells are induced to differentiate along the muscle pathway.

The appearance of an acetylated array of stable MTs early in neuronal differentiation also has been shown in cerebellar macroneurons from 15 day old rat embryos (Ferreira and Caceres 1989). In these cells, acetylated MTs initially are present only as a small, centrosomal array. After 1 day in culture, the cells extend several processes, one of which is significantly longer than the rest and is presumed to be the nascent axon. Only this process contains an array of colchicine stable, acetylated MTs. Unlike the cerebellar macroneurons, P19 cells require close association with other cells to differentiate. Therefore it is difficult to ascertain if short processes are also present in neurally differentiating P19 cells.

The appearance of a transient array of acetylated MTs during neural differentiation is seen in *Xenopus* embryos, at the neural fold stage (stage 17/18) (Chu and Klymkowsky 1989). At this time, cells which are perpendicular to the axis of the neural tube have long processes containing

acetylated alpha tubulin. The staining of these fibers disappears following neural tube closure (stage 19) while within the neural tube itself, acetylated MTs appear in short neuronal cell processes and cell bodies. It is possible that the transient acetylated MT array in neurally differentiating P19 cells may be analogous to the array in *Xenopus*.

**Acetylated MTs in Neurons**

The establishment and growth of neurites requires MT stability of varying degree. Acetylated MTs are more stable and hence more resistant to depolymerization by colchicine than are non-acetylated MTs (Sale et al. 1988). Microinjection of labeled tubulin followed by laser photobleaching of differentiating PC 12 cells indicates that neurites show increasing stability as they mature (Lim et al. 1989). Two studies (Robson and Burgoyne 1989, and Lim et al. 1989) examine the localization of acetylated MTs in mature neurons and find they are localized to regions of greatest stability. GLU and ACET MTs are present in the axon but in general do not extend into growth cones. This is in agreement with the localization of GLU and ACET MTs in stage 3 neurons in differentiating P19 cultures.

**Changes in Tubulin Isotypes**

Eddé et al. (1987) examined expression of tubulin isotypes during neuronal differentiation in an EC cell line, 1003. The expression of a new beta tubulin isotype was seen during the period they define as commitment,

and the appearance of a posttranslationally acetylated form of alpha tubulin was found at the time neurite extension. Their methods and sampling times differ from those reported here and therefore they may have missed the advent of acetylated MT arrays during the earliest stages of commitment. However, our findings of acetylated MTs in short neurites during Stage 2 does aggree with their report of the appearance of acetylated alpha tubulin.

Neurite extension in embryonic rats correlates with high expression levels of a new alpha tubulin mRNA (Miller et al. 1987). These authors suggest that the protein encoded by this mRNA probably differs from the protein coded for by constitutive mRNA. Among other possibilities, this could allow for MT modifications, including formation of new MAP binding sites. If a similar event occurs in P19 cells, then the initial round of acetylation, during commitment, may take place on unspecialized MTs containing alpha tubulin coded for by the constitutive form of mRNA. During neurite extension, many of the added tubulin subunits would presumably contain alpha tubulin coded for by the newly expressed mRNA. These modified MTs in extending neurites may then be capable of binding MAP2C better than MTs with a predominance of other alpha tubulin isoforms.

The particular MAP associated with neurite outgrowth may vary with cell type or may arise from early neuronal specialization (Peng et al. 1986, Nunez 1986). Drubin et al. (1985) report that MAP1 and tau increase coordinately with neurite extension in PC 12 cultures stimulated with nerve

growth factor. MAP2 has also been reported to be present in differentiating PC 12 cells (Black et al. 1986). Some conflicting results may reflect the limits of detection with the techniques employed and/or reflect real differences in tissue culture systems.

**Later Rounds of Neural Differentiation**

Not all committed neurons in the P19 cell culture proceed to differentiate immediately, a situation analogous to events within the embryo. Commitment and morphological differentiation can be separated in time. Late differentiating cells do not retain the large acetylated MT array found during commitment which may indicate that, although the enlarged array is associated with neural differentiation, it is not necessary for neurite extension. During subsequent differentiation, there appears to be recapitualtion of events similar to those described in Stages 2 and 3 implying that at least some of the stable MT bundles present in 4 or 5 day RA cultures will continue to differentiate along the neural line.

In differentiating P19 cultures two or more types of late differentiation occur. After 3 days RA, very short processes, which resemble the early Stage 2 neurites can be seen in the monolayer. These may represent cells which underwent commitment during the first 24 hours of RA, but which are only now differentiating into neurons or they may represent a completely different type of neural development such as radial glial cells.

In addition, another late differentiating cell type contains an increased acetylated array and a short extending process. However, the acetylated MTs in these cells are not stable to colchicine and these cells may represent differentiation along some as yet unknown pathway, or alternately they may represent an artifact of tissue culture.

# CHAPTER 2. Association of acetylated microtubules, vimentin intermediate filaments, and MAP2 during early neural differentiation in EC cell culture.

## INTRODUCTION

The elongated processes of neural cells contain MTs aligned parallel to the long axis of the process. Up to 60% of these MTs resist depolymerization by cold or colchicine and can be described as "stable MTs" (Sahenk and Brady 1987, Brady et al. 1984, Black and Greene 1982). These MTs contain acetylated α tubulin, a posttranslational modification that is associated with stable MT arrays (Piperno et al. 1987, Cambray-Deakin and Burgoyne 1987a, Sale et al. 1988, Lim et al. 1989, Robson and Burgoyne 1989, Ferreira and Caceres 1989b).

Chapter 1 documents the induction of a stable, acetylated MT array during neural differentiation in pluripotent P19 EC cell cultures (Jones-Villeneuve et al. 1982, Edwards and McBurney 1983). Changes in the acetylated MT array are used to classify neural differentiation into stages.

In the first stage, which occurs before extension of processes is seen, there is formation of a stable, acetylated MT bundle. Only MAP1B was detected in apparent association with all MTs, both labile and stable, at this stage. In the second stage, extension of neurites occurs and MAP2C now colocalizes to cells with stable MT arrays. In the third stage, further neurite specialization is seen including staining of neurofilaments, adult MAP2, and tau.

In this paper the interactions between stable MTs and IFs are examined. These investigations focus on vimentin, which is the first IF protein found during differentiation in EC cells (Paulin et al. 1982) and is also the first IF protein seen during neuronal differentiation in embryos (reviewed by Fedoroff et al. 1982).

This information suggests that vimentin IFs are unlikely to play a role in stabilizing the early, stage 1 MT bundle. However, during stage 2, vimentin IFs are part of a cytoskeletal complex present in the newly extending neural process. By using two cytoskeletal disrupting agents, acrylamide, which acts by disrupting IF organization (Eckert 1985, Sager 1989), and estramustine, which binds to MAPs resulting in MT depolymerization (Stearns and Tew 1985, Stearns and Tew 1988, Stearns et. al 1988), the existence of an interdependent cytoskeletal complex, consisting at least of acetylated MTs, MAP2C and vimentin IFs is demonstrated. It is suggested that this complex can serve to stabilize a newly extended process in a cell. We suggest that one function for such a complex is to stabilize a

cell extension in a cell committed to the neural pathway but whose final differentiation into either neurons or glial cells is not yet specified.

# MATERIALS AND METHODS

Culture and differentiation of P19 cells with $10^{-6}$ M RA were previously described in Chapter 1. Only methods and materials that were specific to these experiments will be described here.

Experiments using cytoskeleton disrupting agents were performed on stage 1 (24 hours after addition of RA), stage 2 (48 hours after addition of RA), and stage 3 (72 or more hours after addition of RA) cells, grown on coverslips. Colchicine (Sigma) was made up as a stock solution of 1 mg/ml in sterile distilled water, stored at -80° C, and used at a final dilution of 1 μg/ml in culture medium for 45 minutes. Estramustine (a gift from Mark Stearns) was freshly prepared for each experiment as a 10mM stock solution in dimethyl sulfoxide (DMSO) (Sigma) and used at a final dilution of 150 μM for 1 hour. Acrylamide (Bio-Rad Laboratories, Richmond, Calif.) was freshly made up as a 4 M stock solution in sterile distilled water and used at a final dilution in culture medium of 4 mM for 5 hours. When both acrylamide and colchicine were used, 1 μg/ml colchicine was added after 4 hours and 15 minutes of exposure to 4 mM acrylamide, and the cells were fixed 45 minutes later.

Methods for indirect immunofluorescence staining were described in Chapter 1. The following primary antibodies were used and previously described in Chapter 1: 6-11B-1 to acetylated α tubulin, YOL 1/34 to all α tubulin, MAP2 antibody, clones AP-18 and AP-14. In addition in these experiments a rabbit polyclonal antibody to vimentin, a gift from V. Kalnins, was used (Fedoroff et al. 1982).

The secondary antibodies used were previously described in Chapter 1. In addition, FITC-conjugated goat anti-rabbit IgG (Miles-Yeda LTd. Research Products, Elkhart, Ind.) was used either alone or in combination with the anti-mouse IgG or anti-rat IgG antibodies previously described.

# RESULTS

Uncommitted P19 cells were induced to differentiate along the neural pathway by addition of $10^{-6}$ M RA and surveyed at 24 hour intervals by indirect immunofluorescence for the distribution of vimentin IFs, MAP2C, and colchicine stable acetylated MTs. Most uncommitted P19 cells contain no filamentous vimentin, but do have a diffuse intracellular stain (Fig. 1a). Only a few, colchicine stable acetylated MTs are present in uncommitted cells (Fig. 1b) (Falconer et al. 1989a).

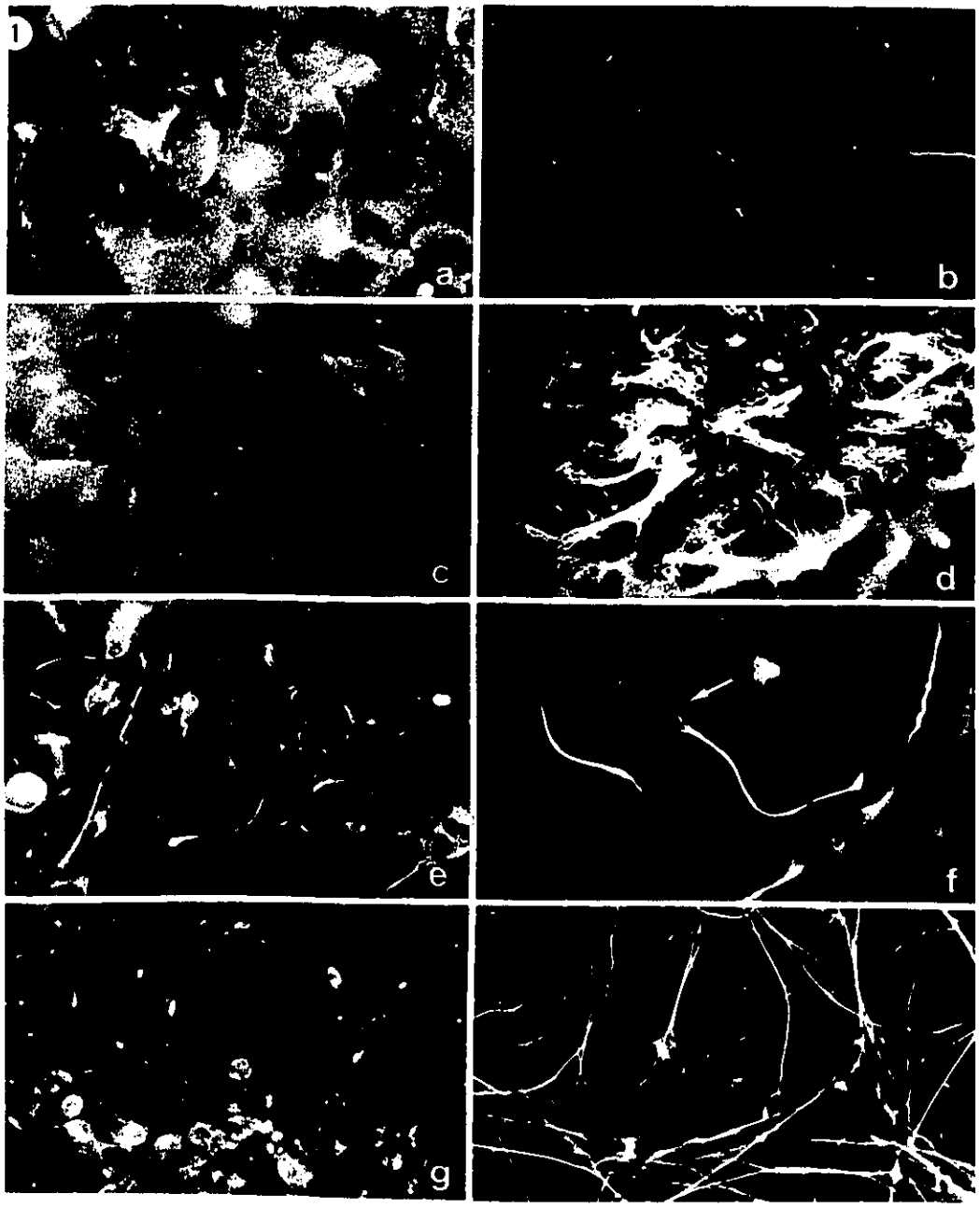
During the 24 hour period after addition of RA, stage 1 differentiation, staining with an antibody recognizing vimentin remains diffuse (Fig. 1c). However, at this time, short bundles of colchicine stable, acetylated MTs are induced in most cells although no cytoplasmic extensions longer than 1 cell body in length are visible (Fig. 1d).

During stage 2, about 48 hours after RA addition, filamentous vimentin becomes a prominent feature (Fig. 1e). Also at this time, cytoplasmic processes are extended and are easily seen by staining the stable, acetylated MT population (Fig. 1f).

By stage 3, from 72 hours after RA addition and onward, a complex pattern of neurons is present. When cells are double stained for vimentin (Fig. 1g) and acetylated MTs (Fig. 1h), very little colocalization of staining is observed.

Fig. 1. P19 cell stained for vimentin (**a,c,e,g**) and for acetylated MTs (**b,d,f,h**) during stages of neural differentiation. Uncommitted EC cells are shown in Figs. 1a and 1b. **a:** EC cells show diffuse staining by antibody to vimentin. **b:** Colchicine treated EC cells have few stable, acetylated MTs. Differentiating stage 1 cells (24 hours after RA addition) are shown in Figs. 1c and 1d. **c:** Staining with antibody to vimentin remains diffuse. **d:** Colchicine treated cultures now show bundles of stable, acetylated MTs. Stage 2 cells (48 hours after RA addition) are shown in Figs. 1e and 1f. **e:** Filamentous vimentin is now present. **f:** In colchicine treated cultures, the first processes containing acetylated stable MTs can be identified. Note the growth cone (arrow). Stage 3 cells (3 days post RA) are shown, double labelled for vimentin (**g**) and for acetylated MTs (**h**). Very few processes contain vimentin (g), although a complex network of neurons containing acetylated MTs is evident (h).

Mag. in a to f = 650 X. Mag. in g and h = 350 X.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h

In most colchicine treated cells, vimentin IFs collapse around the nucleus. However, in a small but constant proportion of colchicine treated cells (less than 5% of the differentiating cells), the vimentin IF network does not collapse (Fig. 2a), but, instead, is similar in distribution to the acetylated stable MT array (Fig. 2b). This colocalization is limited to the brief period of stage 2 differentiation. The appearance of acetylated MTs in the growth cone of the cell shown in Fig. 2a is unusual and may reflect drug treatment and/or culture conditions.

MAP 2C first appears during stage 2 differentiation. MAP2C, the 70 kDa juvenile form of MAP2, is identified by positive staining with anti-MAP2 clone (AP-18), which recognizes both adult and juvenile forms and by lack of staining with anti-MAP2 clone (AP-14), which recognizes only the 270 kDa adult forms of MAP2A and MAP2B (Tucker et al. 1988). In differentiating P19 cells, AP-18 but not AP-14 stained stage 2 cells indicating that AP-18 staining recognizes MAP2C. Double labeling of colchicine treated cells shows that all cells with stable MTs (Fig. 3a) also stain for MAP2C (Fig. 3b). MAP2C can be identified in cells without processes, in cells with a single process, and in cells with two or more processes

Fig. 2. A stage 2, colchicine treated cell, double labelled for vimentin (**a**) and for stable acetylated MTs (**b**). A growth cone is visible (arrow). Mag. = 350 X.

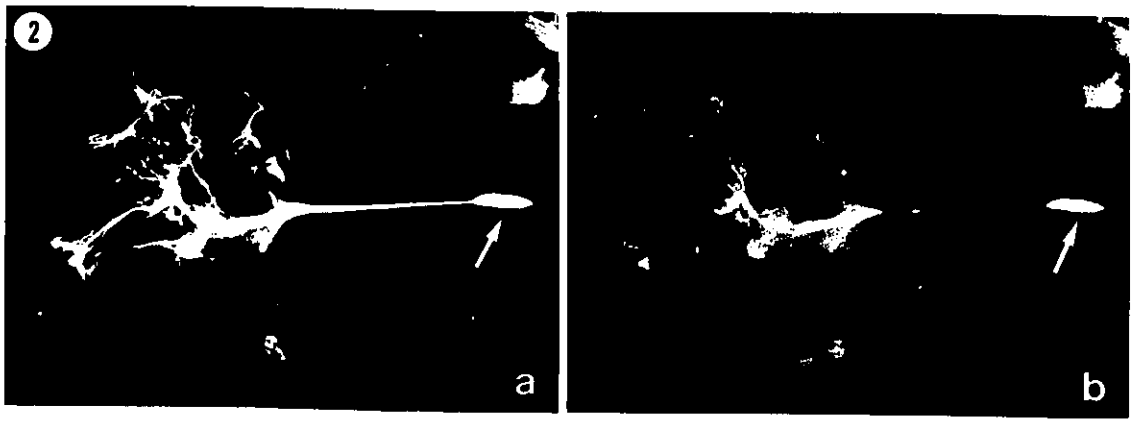
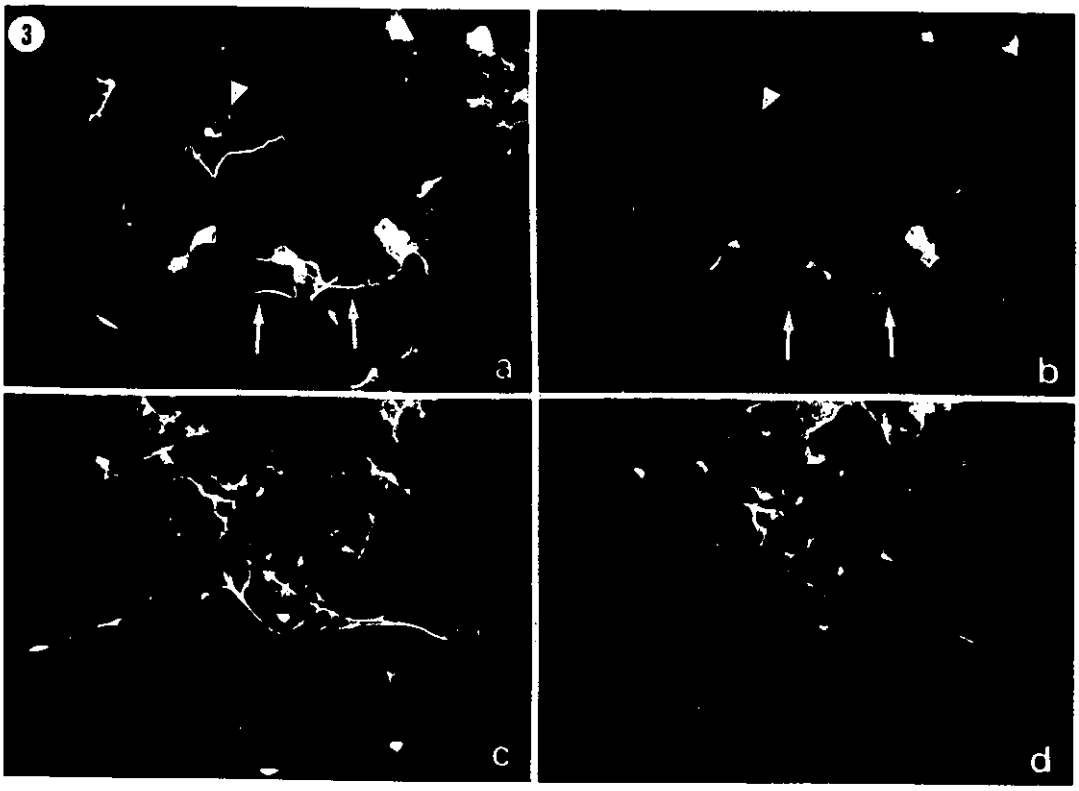

2
a
b

Fig. 3. Stage 2, colchicine treated cells, double labelled for stable MTs (**a**) and for MAP2C (**b**). Colocalization of MAP2C and stable MTs can be seen in cells with no processes (arrowhead), in cells with a single process, and in cells with two processes (arrows). By stage 3, (Figs. 3c and 3d), colocalization of stable MTs (**c**) and of MAP2C (**d**) in colchicine treated cells is only partial.

Mag. in a and b = 300 X. Mag. in c and d = 250 X.

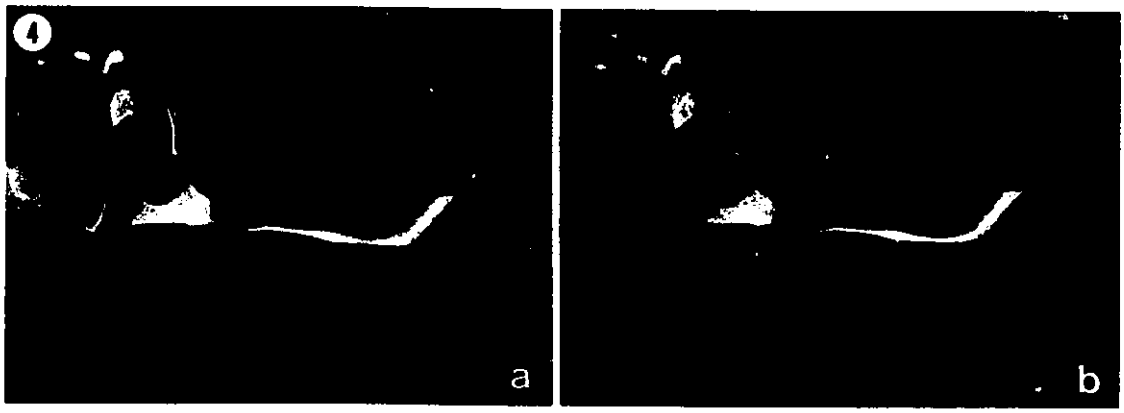

3
a
b
c
d

(Fig. 3b). However, by stage 3, double labeling of acetylated MTs (Fig. 3c) and MAP2 (Fig. 3d) shows MAP2 is now localized to only some of the neurites which contain stable, acetylated MTs. The MAP2 positive processes have, presumably, differentiated into dendrites (Peng et al. 1986).

Since some stable acetylated MTs colocalize with vimentin IFs and with MAP2C, vimentin IFs should also colocalize with processes containing MAP2C. Double staining with vimentin and MAP2 antibodies confirms this (Figs. 4a and 4b).

These results suggest that during stage 2, stable, acetylated MTs, MAP2C and vimentin IFs are linked together to form a stabilizing structure in the newly extended processes. If this linkage exists, disruption or elimination of one of the components should affect the others. To test this hypothesis, we analyzed the effect on the colchicine stable cytoskeleton complex of two disrupting agents, 4 mM acrylamide and 150 μM estramustine. Although the mechanism of action is not understood, acrylamide induces collapse of the IF network, resulting in a "cap" of IFs near the nucleus without concommitant MT changes (Eckert 1985, Sager 1989). Estramustine, a drug used in cancer therapy, binds preferentially to

Fig. 4. Stage 2 colchicine treated cell double labelled for vimentin (a) and for MAP2C (b). Mag. = 900 X.


4
a
b

MAPs, including MAP2, inhibiting MT assembly and thereby resulting in net depolymerization of MT arrays (Stearns and Tew 1985, 1988).

Exposure to acrylamide results in loss of vimentin IFs from the processes and formation of a diffusely staining vimentin "cap" near the nucleus (Fig. 5a) although acetylated MTs remain in the process (Fig. 5b). MAP2C is not detectable or is present only as a faint, diffuse cytoplasmic stain (Fig. 5c). To test if these remaining acetylated MTs are still stable against colchicine induced depolymerization, a combined drug treatment was used. Stage 2 cells were exposed to acrylamide for 5 hours and colchicine was added during the last 45 minutes of treatment. With this procedure, vimentin IFs were collapsed to the cell body (Fig. 5d), no colchicine stable acetylated MTs remained in the process (Fig. 5e), and MAP2C could not be detected. In addition, the processes in this combined treatment were very short (Fig. 5f) and few in number implying that many had completely retracted and were no longer detectable.

In estramustine treated cells, double labeled for vimentin and acetylated tubulin, most vimentin IFs are absent from the process. The vimentin staining pattern shows a diffuse stain in the cell body and a few discrete filaments near the nucleus (Fig. 6a). No acetylated MTs remain in the process and the few MTs present are colocalized to the remaining vimentin filaments (Fig. 6b).

Fig. 5. Acrylamide treatment of stage 2 cells. Double label of same cell (Figs. 5a and 5b). **a:** Vimentin IFs have collapsed to the region near the nucleus. **b:** Acetylated MTs remain in the processes and cell body. **c:** After acrylamide treatment, no MAP2C staining is visible in the process (arrow). To ascertain if the actylated MTs are still stable to colchicine after collapse of the vimentin IFs, colchicine is added for the final 45 min. of acrylamide treatment (Figs. 5d, 5e, and 5f). After this combined treatment, **d:** vimentin IFs collapse (arrow). **e:** No acetylated MTs remain in the process (arrow). **f:** Phase contrast indicates the process is quite short. Mag. = 750 X.

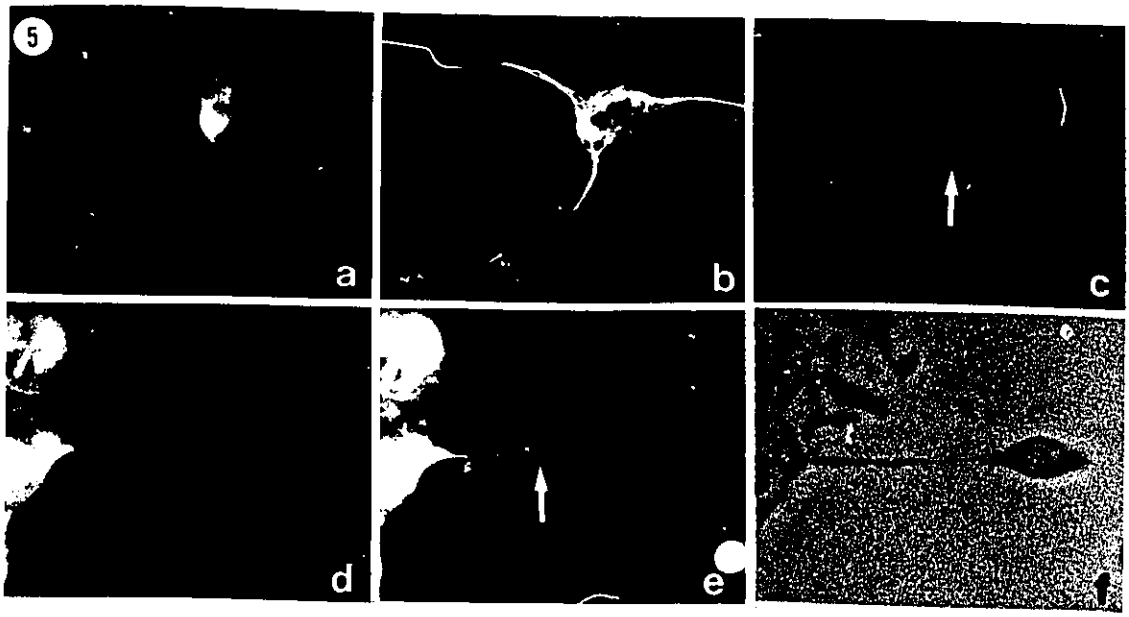
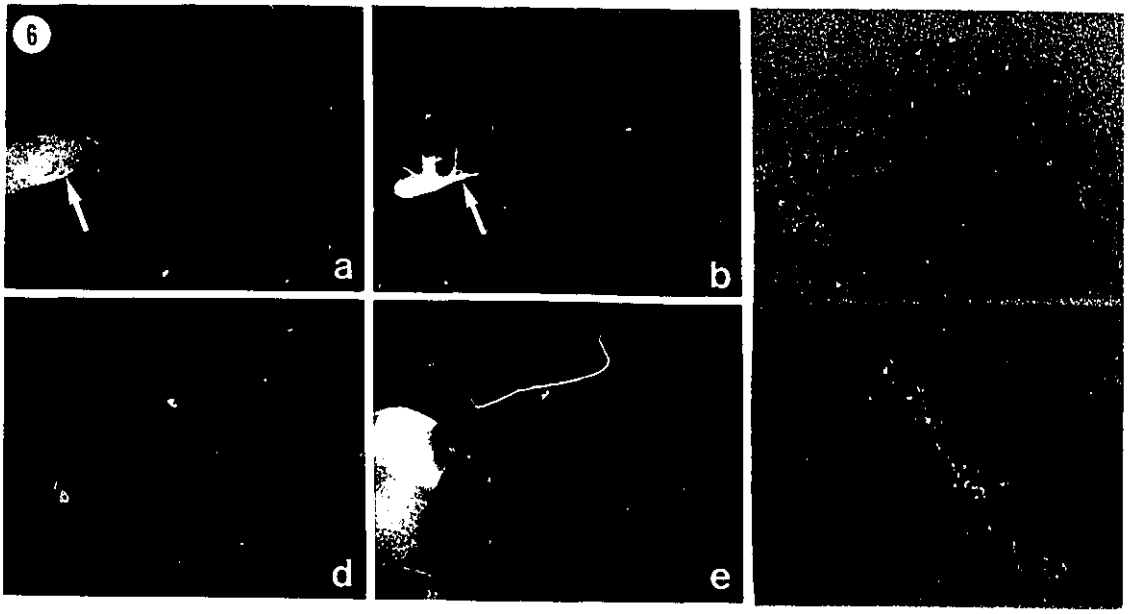

5
a
b
c
d
e
f

Fig. 6. Estramustine treatment of stage 2 cells. Double label of a cell for vimentin (a) and acetylated MTs (b). **a:** Vimentin IFs collapsed near the nucleus with only a few filaments visible (arrow). **b:** The few remaining acetylated MTs (arrow) colocalize with the vimentin IFs. **c:** Phase contrast indicates the process is quite short. Double label of a cell for MAP2C (**d**) and vimentin (**e**) shows that little MAP2C remains in the process and the vimentin IFs are collapsed near the nucleus. **f:** Phase contrast of the same cell. Mag. = 750 X.


6
a
b
d
e

The process itself is quite short (Fig. 6c). When cells are double labeled for MAP2C and vimentin, little or no MAP2C is present in the process (Fig. 6d) and the vimentin IFs have collapsed and are not present in the process (Fig. 6e). The processes shown here (Fig. 6f) had not completely retracted, although in estramustine treated cells, most processes are short and few in number implying that many have retracted.

# DISCUSSION

In P19 cells, during the first 24 hours of neural differentiation (stage 1), a population of colchicine stable, acetylated MTs appears (Falconer et al. 1989). It is not known what stabilizes these MTs, but stable MTs are often posttranslationally modified by acetylation of α tubulin (L'Hernault and Rosenbaum 1985a) and acetylation itself may play some role in MT stabilization (Piperno et al. 1987).

The initial stabilization of MTs may be enhanced at stage 2 differentiation (48 hours after induction with RA) by the appearance of MAP2C which colocalizes with colchicine stable MTs. *In vitro* studies have shown that MAPs increase MT stability (Kirschner 1978,) and influence spacing between adjacent MTs (Brown and Berlin 1985). This MAP 2C mediated stabilization of differentiating cells is apparently also a transient phenomenon as shown by the results in this article.

In P19 cultures induced to differentiate along neural pathways, the colocalization of stable acetylated MTs, MAP2C, and vimentin IFs is seen only during the brief period of stage 2, when process outgrowth first appears. When this cytoskeletal complex is treated with disrupting agents, perturbation of one element of the complex results in reorganization or altered stability of the other elements. This suggests that the three elements are associated together, with MAP2C as the probable linking agent between MTs and vimentin IFs, although inclusion of other linking elements in addition to

MAP2C cannot be ruled out. MAP2 has been suggested to be a cross-linking protein between MTs and IFs (reviewed by Matus 1988). Recently MAP2 has been shown to be a component of crossbridges between MTs and NFs in frozen sections of rat neurons (Hirokawa et al. 1988). An association of MAP2 with MTs and vimentin in flat cells from primary cultures of mature rat brain also has been demonstrated (Bloom and Vallee 1983).

In differentiated cells *in situ*, vimentin is characteristic of cells of mesenchymal origin, but is also expressed by and considered a marker of cells derived from the neuroectoderm, including astrocytes and radial glial cells (Schnitzer et al. 1981). Most continuous cell lines express vimentin; however, filamentous vimentin in P19 cells probably is related to differentiation and not a result of culturing the cells *in vitro*. In uncommitted EC cultures, only about 15% of cells have some filamentous vimentin staining. The remaining cells have a diffuse intracellular stain and significant amounts of filamentous vimentin are not seen until 48 hours after addition of RA. Paulin et al. (1982) document the occurrence of filamentous vimentin in 17 different EC cell lines (not including the P19 line) and find that the percentage of cells containing vimentin varies from line to line, but all lines express more vimentin after treatment with RA. We have confirmed these results in differentiating P19 cells (data not shown).

During differentiation, vimentin is the first IF protein expressed in P19 cells (Levine and Flynn 1986). Neurofilament (NF) staining is not seen until 3 or more days after addition of RA (Levine and Flynn 1986), while

GFAP arises at least several days after NFs are seen (Jones-Villeneuve et al. 1982). Since it is NF and glial fibrillary acidic proteins (GFAP) proteins that are considered definitive markers for neurons and glial cells, respectively, it is not possible to predict what will be the final differentiated state of stage 2 cells.

During development of the mouse embryo nervous system, vimentin is first detected on the 9th day of gestation (E9), in processes arising from the basal plate of neuro-epithelium of the neural tube (Houle and Fedoroff 1983). However, neither NFs nor GFAP are expressed at this stage of mouse embryo development (Schnitzer et al. 1981, Bignami et al. 1982). Houle and Fedoroff (1983) and Schnitzer et al. (1981) suggest that the first cells to express vimentin may be ventricular cells, a neural stem cell which retains the capacity for multipotential differentiation along either neuronal or glial pathways. The period of colocalization of vimentin IFs with acetylated MTs and with MAP2C in newly extending processes of P19 cells may correspond to the time when filamentous vimentin appears in processes extended by E9 ventricular cells.

The vimentin IF-MAP2C-acetylated MT complex may be present in a relatively unspecified stem cell which retains the ability to modify various components during subsequent differentiation. Thus, it is suggested that acetylated MTs, found in both neurites (Cambray-Deakin and Burgoyne 1987a) and in glial processes (Sale et al. 1988, Cambray-Deakin and Burgoyne 1988), will remain in the process, regardless of the subsequent

differentiation pathway. If the cell commits to the glial lineage, acetylated MTs and vimentin IFs will remain and GFAP will be subsequently added. However, MAP2C will be replaced by MAP1A, or MAP1B. If the neuronal lineage is chosen, vimentin IFs first will coexist with NF protein and then will be replaced by NF protein, as seen during neuronal differentiation in embryos (Bignami et al. 1982, Ziller et al. 1983). MAP2C will remain and MAP2A and MAP2B will be found in dendritic processes. In axons, MAP2C will remain in the proximal end of the process but tau and other MAPs will also be added.

In differentiating P19 cells, and possibly during embryogenesis as well, the stabilization of a newly extended process may itself be important in determining the final differentiation path, particularly if surrounding cells provide a signal that is perceived by the extension.

These results also substantiate that the P19 EC cell culture system is a good model for the study of neurogenesis. Unlike neuroblastoma and pheochromocytoma (PC 12) cultures, which derive from neurally committed cells, the P19 culture consists of pluripotent cells (Jones-Villeneuve et al. 1982, Edwards and McBurney 1983). In addition, differentiating neurons in P19 cultures have the same alterations in IF population and distribution as that reported *in vivo* (Levine and Flynn 1986). However, neurogenesis in neuroblastoma and PC 12 cultures is atypical with neurons retaining both vimentin IFs and neurofilaments throughout differentiation (Shea and Nixon 1988).

# CHAPTER 3.

# Changes in beta tubulin isotypes of total and stable MT arrays during neural differentiation

## INTRODUCTION

In the mouse, cDNA cloning has revealed the presence of five functional beta tubulin genes, each encoding a distinct beta tubulin polypeptide (Lewis et al. 1985, Wang et al. 1986). Within the amino acid sequences for these isotypes, regions of strict conservation can be seen associated with GTP binding sites (Sternlicht et al. 1987). However, at the carboxy terminus, and to a lesser extent, at the amino terminus, there are regions of major variability. In the polymerized state, both of these regions are exposed on the outside of the MT and therefore may play a role in MT functions. An indication that amino acid variation at the carboxy terminus may be important, is the strict conservation of these variations across species lines. In four of the isotypes, the COOH amino acid sequences for a particular isotype are completely conserved between various mammalian

species and are 97-99% conserved between mammals and birds (reviewed by Sullivan 1988).

Beta tubulin isotypes have been classified according to the variations in the final 15 amino acids and polyclonal antibodies generated to synthetic peptides, representing the variable carboxy terminus region of each isotype (Lopata and Cleveland 1987). However, it must be pointed out that the peptide which represents the carboxy fragment of Class IVa (brain specific beta tubulin) is identical to the peptide which represents Class IVb (major testis isotype, varying levels in other tissues). Therefore the antibody generated by this peptide, and used in the following studies, should not be considered as brain-specific.

Expression patterns of each isotype have been investigated by assaying the mRNA level of a given isotype in different tissues (see reviews by Lewis and Cowan 1990, Sullivan 1988). A general pattern of isotype expression emerges:

Class I: Many tissues

Class II: Major brain isotype, in both neurons and glia and many other tissues

Class III: Neuron specific in vertebrate brain, also in Sertoli cells of testis and transformed cell lines

Class IVa: Brain specific

Class IVb: Major testis isotype, varying levels in other tissues

Class V: All tissues except brain, not expressed in mouse

Class VI: In erythrocytes only

The expression of beta tubulin (and alpha tubulin) isotypes is regulated during development as shown by measuring the relative abundance of the isotype-specific mRNA in different tissues at different times (Denoulet et al. 1986, reviewed by Lewis and Cowan 1990).

When beta tubulin isotype staining was examined in 3T3 and CEF cells, no isotype-specific MT array was detected (Lopata and Cleveland 1987). However, these are relatively undifferentiated cells and may not have the specialized functions which require a particular isotype and/or the cells also may lack a complementary MAP which might be needed for isotype function and/or segregation. Therefore, a highly differentiated cell, such as a neuron, might be a better place to look for tubulin isotype segregation and/or function.

In an article published while this research was in progress, it was shown that neurons in differentiating PC12 cells express all five classes of beta tubulin isotypes, although only Classes II and III show significant increase during neurogenesis (Joshi and Cleveland 1989). There appears to be preferential incorporation of some isotypes into neuronal MTs, with 70% of Class I and Class II isotypes found in the polymerized MT fraction. However, Class III is preferentially retained in the unpolymerized state, with only 50% of Class III present in MTs. Immuno-gold staining of PC12

neurons and examination by electron microscopy, shows that all MTs are co-polymers of isotypes present in the cell examined (Joshi et al. 1989)

Other results indicate that some MTs in neurites may have specialized states and/or functions. MTs with an abundance of two alpha tubulins and one beta tubulin have been shown to be cold stable in neurons from the CNS and PNS of adult rat (Binet and Meininger 1988). In lobster axons, there is evidence that only about 25% of MTs are substrates for transport of vesicles (Miller et al 1986). These MTs may be both specialized for transport and less dynamic to act as "tracks". These findings indicate that the isotype profile of stable MTs may differ from that in the more dynamic population.

Isoelectric focusing of tubulin in uncommitted cells from the embryonal carcinoma line, 1003, shows the presence of one alpha tubulin, a major beta tubulin band, $\beta_3$, and two minor beta bands, $\beta_4$ and $\beta_5$ (Eddé et al. 1987). During neuronal commitment there is expression of a new beta isotype, $\beta'_1$, which is encoded by a newly expressed mRNA (Denoulet et al. 1986). The $\beta'_1$ isotype has been suggested to be identical to the Class III, neuron specific isotype identified by Cleveland (Diaz-Nido et al. 1990, Avila et al. 1988). The other beta tubulin isoforms reported by Eddé et al. (1987) have not yet been classified according to the Cleveland nomenclature system.

The above results indicate that the P19 embryonal carcinoma cell culture might be a good model system to investigate beta tubulin isotype occurence and function in differentiating cells. We have previously used this

system to document the appearance of a new, bundled array of acetylated, colchicine stable MTs after 1 day of neural differentiation (Falconer et al. 1989a).

The goal of the present work is first to establish if there are changes in the expression of beta tubulin isotypes during neural differentiation in P19 cells. If there are such changes, the next question to be addressed is to establish if the beta tubulin isotypes in the stable MT array is the same as or different than the beta tubulin isotypes in the dynamic MT array. In addition, an investigation of the expression of brain-specific MAPs is done to see if this can be correlated with any changes in beta tubulin isotypes. Finally, the third goal of the work is to compare the beta tubulin isotype profiles in differentiating neural cells with the beta tubulin profiles of cells differentiating along the muscle line.

Using indirect immunofluorescent staining, changes in beta II and beta III isotype staining patterns can be seen during neural development. In addition, variations in the staining intensity of MTs in the total array and the stable array indicate that there are isotypic differences in these two MT populations. To examine these differences more closely, cell extracts containing tubulin from either the total or the stable MT array were analyzed by immunoblotting using antibodies to Class I, II, III and IV beta tubulin isotypes. The data indicate there is a progressive change in the beta tubulin isotype profile as uncommitted EC cells differentiate along the neural line

and that, in neurally differentiated cells, the beta tubulin profile of the stable MTs differs from the beta tubulin profile of the total MTs.

To ascertain if changes in the beta tubulin isotype profile are specific to neural cells, the pluripotent aspect of P19 cells was exploited. P19 cells can be induced to differentiate along neural or muscle pathways depending upon the morphogen (Jones-Villeneuve et al. 1982, Edwards and McBurney 1983) making it possible to compare the beta tubulin profiles of the total and stable MT populations of neural cells with that of muscle cells. The results of this comparison indicate that the subcellular sortin of Class II beta isotype into stable MTs and the subcellular sorting of Class III beta tubulin into dynamic MTs is specific to cells differentiating along the neuronal line and is not present in muscle cells. This is the first report of such subcellular sorting of beta tubulin isotypes during very early neural differentiation.

# MATERIALS AND METHODS

## Cell culture

P19 cells were cultured as described in Chapter 1. Conditions for neural differentiation in the continuous presence of $10^{-6}$ M RA (Sigma) also were described previously. For muscle differentiation, P19 cells were aggregated, as described in Chapter 1, and plated at a density of 1 x $10^4$ cells per 22 x 22 mm coverslip in alpha MEM plus 10% FBS (both from Flow Lab.). One day after plating, the medium was removed and replaced by alpha MEM plus 8% FBS containing 2% (v/v) dimethyl sulfoxide (DMSO) (Sigma). The medium was replaced every two days thereafter with identical medium containing DMSO.

## Preparation of cell extracts

To prepare cell extracts containing tubulin from the total polymerized MT population, a 100 mm tissue culture dish containing a semi-confluent monolayer of either untreated EC cells, RA treated cells at 2 days RA, 4 days RA, or 6 days RA, or 6 day DMSO treated cells, was washed once with 37°C PBS and twice with 37°C MT stabilizing PEM buffer (80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$, [all from Sigma] pH 6.8). The cells were then permeabilized for 3 to 5 minutes in PEM buffer plus 0.75% Triton X-100 (Sigma) at 37°C to extract non-polymerized tubulin and other soluble

proteins. The remaining cytoskeleton was solubilized in 1 ml of 0.5% SDS (Bio-Rad) in 25 mM Tris (Bio-Rad) pH 6.8 and 2 M glycerol, by boiling for 10 minutes in a microfuge tube. This extract was centrifuged at 13,000 rpm for 3 minutes, and the supernatant frozen at -20° C. The sample was subsequently thawed and centrifuged again at 13,000 RPM for 3 minutes and total protein in the supernatant was determined using the bicinchonic acid assay (Smith et al. 1985). Samples were stored frozen at -20°C. Samples of tubulin from colchicine stable MTs (i.e. extracts containing tubulin from the stable MT population only) were obtained exactly as described above except that the cells were treated with 1 μg/ml colchicine (Sigma) for 45 minutes to depolymerize the colchicine labile MT population before preparation of the cell extracts.

**Gel electrophoresis and immunoblotting**

The 7.5% polyacrylamide gels for analysis of protein samples were performed as described (Laemmli 1970) using colored high molecular weight markers as standards (Bio-Rad). Gels typically were run at 175 volts using a Bio-Rad Mini-gel system. Proteins were electrophoretically transferred to nitrocellulose membrane (BA 85; Schleicher & Schuell, Inc.) in Laemmli gel running buffer containing 20% methanol. Transfer was done overnight at a constant current of 30 milli-amps, using a Bio-Rad Mini-blot system. The nitrocellulose filters containing the transferred samples were stained for 5 minutes with Ponceau red (0.2% Ponceau S dye [Sigma] w/v in 3 %

trichloroacetic acid [Sigma]) to visualize sample lanes. The positions of the sample lanes were marked on the filter and the filters were destained in PBS. Filters were blocked in 5% (w/v) skim milk powder (Carnation) dissolved in PBS for 1 hour at room temperature then washed 3 x in PBS, 15 minutes per wash.

For Western immunoblotting, a Miniblotter 28 (Immunetics, Cambridge, Mass.) apparatus was used. Primary antibodies were diluted in PBS and incubated on a blot overnight. The blot was then washed 4 times, one hour per wash, and incubated with $^{125}$I-labeled anti-rabbit IgG secondary antibody (specific activity 7.71 μCi/μg) or $^{125}$I anti-mouse IgG secondary antibody (specific activity 8.81 μCi/μg) (both from NEN Research Products, DuPont). $^{125}$I was used at 5 x $10^5$ cpm/ml and incubated overnight followed by 4 washes, 20 minutes each, in PBS plus 0.1% Tween-20. Secondary controls, in which primary antibody was omitted, were included in each experiment. Binding was detected by autoradiography using Kodak X-OMAT film (Kodak, Rochester, N.Y.) and exposing at -80°C for 3 days before developing the film. The X-ray films were scanned using an LKB Ultroscan XL laser densitometer which computed the area under the curve representing the density of the radioactive secondary binding. Each band was scanned a minimum of 5 times at 800 μm intervals to determine the value for that band.

A series of tubulin dilutions, from 0.1 ug to 10 ug was immunoblotted and the autoradiogram scanned to ensure that experimental immunoblot densitometer readings were in the linear range.

The following number of samples was examined by Western blotting:

| | Total MTs | Stable MTs |
|---|---|---|
| EC | 5 | 4 |
| 2 Days RA | 2 | 2 |
| 4 Days RA | 3 | 3 |
| 6 Days RA | 4 | 3 |
| Muscle | 3 | 3 |

**Calculation of relative percentage of an isotype**

The relative percentage of any isotype "Y" in a given sample is calculated as follows, where "Area Y" is the computed area under the curve for that isotype (as found by laser densitometric scanning of the autoradiogram):

(Area Y x 100%) / (Area isotype I) + (Area isotype II) + (Area isotype III) + (Area isotype IV) = relative percentage of isotype "Y".

Mean and standard deviation were calculated from three or more independent samples. To ascertain if sample means were significantly different, a Student's t test was done (Williams 1977).

**Primary antibodies used**

Rabbit polyclonal antibodies specific to three vertebrate beta tubulin isotypes, Classes I, II and IV, (Lopata and Cleveland 1987) were the kind

gift of Dr. D. W. Cleveland. These antibodies were raised against the final 9 to 15 amino acid peptides corresponding to sequences at the carboxy terminus of the respective vertebrate beta tubulin isotypes. All antibodies had been affinity purified by chromatography of each antiserum on a column produced by covalently linking the respective carboxy-terminal peptide to Sepharose as detailed in Lopata and Cleveland (1987). The amount of these antibodies was limited and therefore they were used only for immunoblotting.

Mouse monoclonal IgG antibody to Class II beta tubulin, the gift of Dr. R. L. Luduena, was raised against a synthetic peptide representing the final amino acids of the carboxy terminal for this isotype and subsequently the cell culture supernatant was affinity purified (Banerjee et al. 1989). This antibody was used for immunofluoresence staining.

The mouse monoclonal IgG antibody to Class III beta tubulin, TUJ1, the gift of Dr. A. Frankfurter, was raised by Dr. L.I. Binder against an extract of taxol stabilized MTs from 10 day postnatal mouse brain tubulin and specificity was determined by specific recognition of a cloned fusion protein representing the Class III isotype (personal communication, Drs. Binder and Frankfurter) (Gass et al. 1990). This antibody was used both for immunoblotting and immunostaining.

Rabbit polyclonal antibody to detyrosinated alpha tubulin (GLU) (Gundersen et al. 1984) was the kind gift of Dr. G. Gundersen and Dr. C. Bulinski. Mouse monoclonal antibodies to Tau, MAP1B and to MAP2 (clone AP-18) (Viereck et al. 1988) were the kind gift of Dr. L. I. Binder.

Mouse monoclonal antibody to neurofilament 160 was purchased from Amersham Corp. The YOL 1/34 antibody (Dimension Lab.) was used as a general tubulin stain for indirect immunofluorescence staining (Kilmartin et al. 1982, Cumming et al. 1983). Mouse monoclonal antibody which recognizes alpha and gamma muscle actin, MA-931, (Tsukada et al. 1987) was used to identify muscle-differentiated cells (Enzo Biochem. Inc.).

**Secondary antibodies used**

The secondary antibodies for immunoblotting have been described above. The following secondary antibodies were used for indirect immunofluoresence staining: FITC-conjugated rabbit anti-rat IgG cross-absorbed against mouse (Zymed, Dimension Lab.); FITC-conjugated donkey anti-rabbit IgG cross-absorbed against rat and mouse (Jackson Lab.); FITC-conjugated goat anti-mouse IgG cross absorbed against rat (Zymed, Dimension Lab.); Rhodamine-conjugated rabbit anti-mouse IgG cross absorbed against rat (Zymed, Dimension Lab.); Rhodamine-conjugated donkey anti-rabbit IgG cross absorbed against rat and mouse (Jackson Lab.); Rhodamine conjugated donkey anti-mouse IgG cross absorbed against rat and rabbit (Jackson Lab.).

**Indirect immunofluorescence microscopy**

The techniques for indirect immunofluoresence microscopy were previously described (Falconer et al. 1989a) with the exception that cells

stained for MAP1B were fixed with ice-cold methanol at -20°C for 20 minutes, rehydrated and rinsed 3 times in PBS, 5 minutes each rinse, before staining with primary antibodies.

# RESULTS

**Indirect immunofluorescence staining patterns of beta II and beta III tubulin isotypes in neurally differentiating EC cells**

Undifferentiated EC cells and cells induced along the neural pathway by $10^{-6}$ M RA were stained with antibodies to Class II beta and Class III beta tubulin isotypes and changes in expression during neural differentiation were monitored by indirect immunofluorescence microscopy. Both beta II and beta III showed changes in staining patterns during neural differentiation in cultured P19 cells. Beta II is a major brain isotype and is associated with early stages of neurogenesis and/or neuronal regeneration of rat sciatic nerve (Hoffman and Cleveland 1988) while beta III is a neuron specific beta tubulin isotype (Lewis and Cowan 1990).

## Uncommitted EC cells

**Beta II and Beta III staining of EC cells** - Virtually all cells in undifferentiated P19 cultures show low to moderate levels of MT staining with antibodies to both beta II and beta III isotypes which may represent constitutive levels of expression in pluripotent cells. The few stable MTs that remain after colchicine treatment of EC cells show faint staining with beta II and no staining with beta III antibodies (data not shown).

## 1 Day RA

**Beta II staining** - the intensity of beta II staining is increased in about 15% of the total cell population. In these cells there is, at the level of the light microscope, an apparent preferential incorporation of beta II into the MTs present in bundles (Fig. 1A and B).

In colchicine treated cultures, staining with the general anti-alpha tubulin antibody, YOL 1/34, identifies the most MTs as shown by double labelling with antibodies to tyrosinated, detyrosinated and acetylated alpha tubulin (see Chapter 1). Double labelling of colchicine treated 1 day RA cells with beta II and YOL 1/34 antibodies indicates that most stable MTs contain beta II and that these stable MTs are often present as bundles (Fig 1C and D).

**Beta III staining** - In 1 day RA cells, the level of beta III staining remains unchanged from that of EC cells. In colchicine treated cells, beta III staining is not present in the stable MT bundle (Fig. 2).

## 2 Days RA

**Beta II staining** - The intensity of beta II staining is increased in all 2 day RA cells compared to that seen in EC

Fig. 1. Double labelling of 1 day RA cells with; antibody to beta II (**A and C**), and the YOL 1/34 antibody (**B and D**). MTs of some cells show increased levels of beta II with preferential incorporation of beta II into the bundled MT array. In colchicine treated 1 day RA cells (C and D), stable MT bundles contain beta II. All stable MTs stained by YOL 1/34 are also stained by beta II.

Mag. A,B,C,D = 650 X.

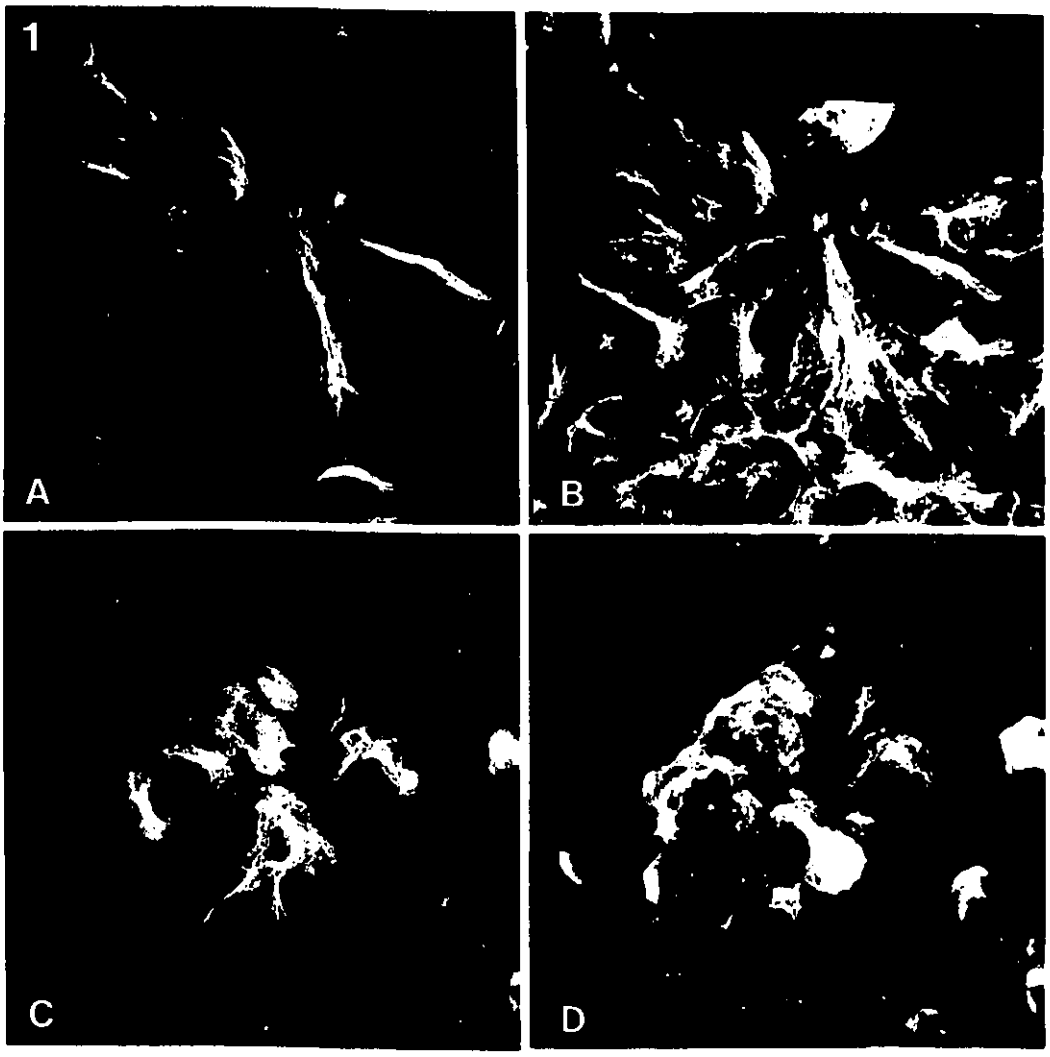
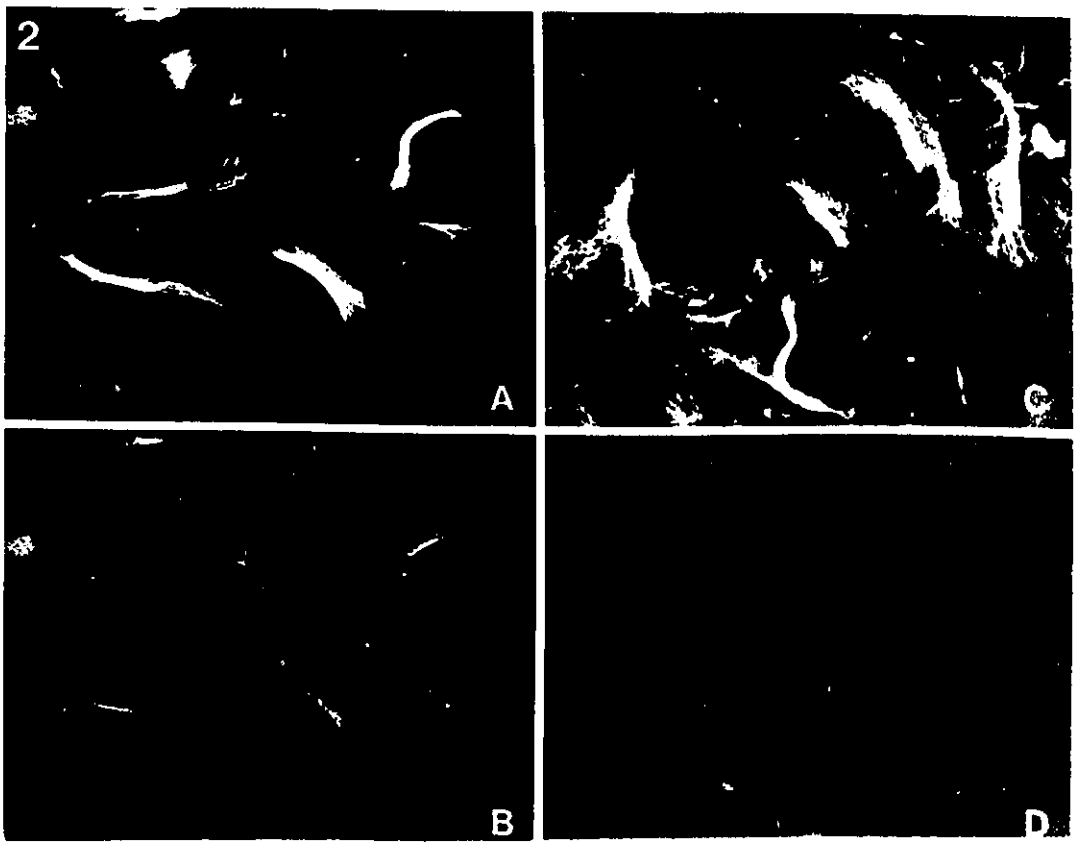

1
A
B
C
D

Fig. 2. Stable MT bundles in 1 day RA, colchicine treated cells double labelled for: **A:** detyrosinated alpha tubulin (GLU) and **B:** beta II tubulin. Note the presence of some beta II staining in the stable MT bundles. Colchicine treated 1 day RA cells double labelled for **C:** detyrosinated alpha tubulin (GLU) and **D:** beta III tubulin. Stable MT bundles do not stain with beta III.

Mag. A,B,C,D = 650 X.


2
A
C
B
D

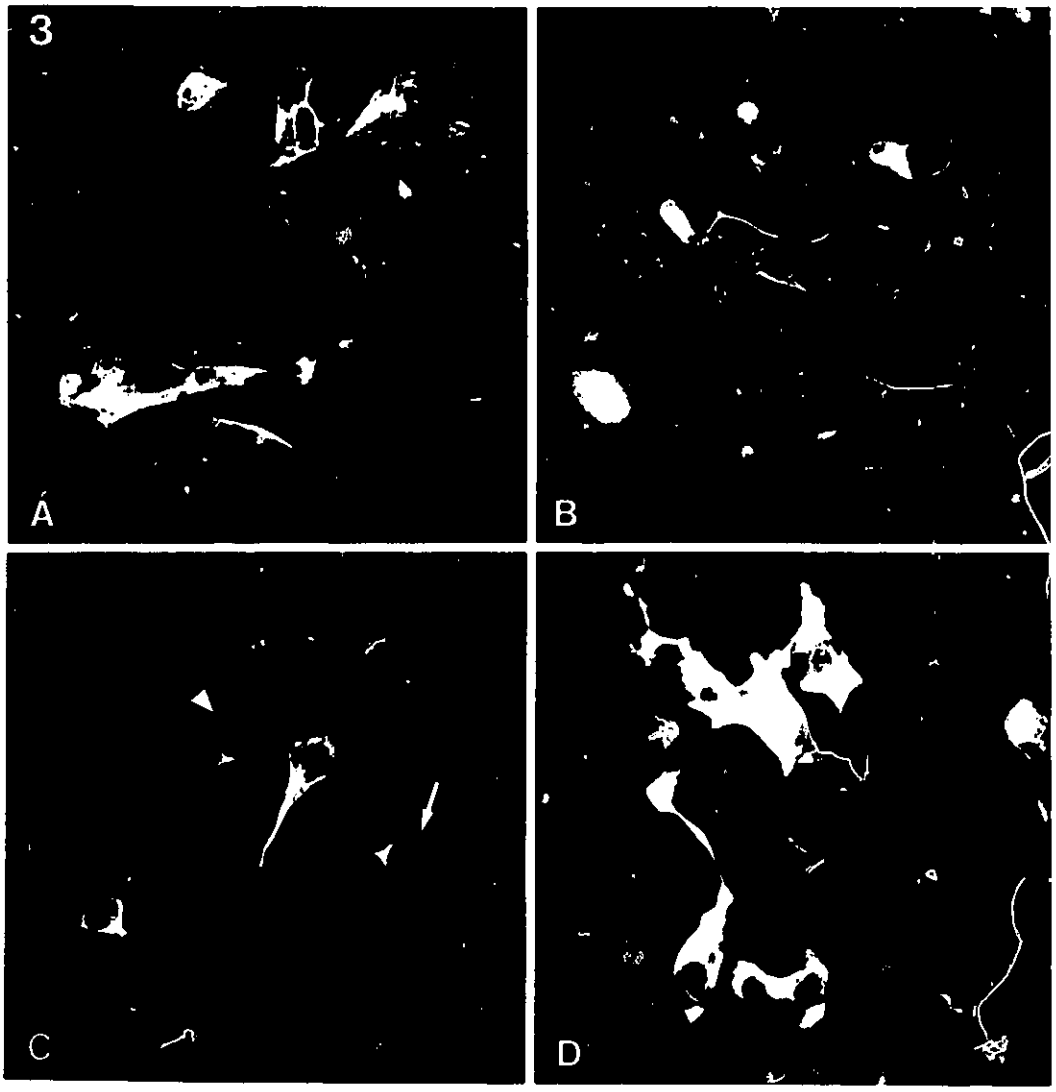
and 1 day RA cells. Moreover, a subpopulation of cells (less than 15%) on top of the cell monolayer, shows increased staining intensity compared to cells in the monolayer. Some of these brightly staining cells have neurite-like processes (Fig. 3A).

In colchicine treated 2 day RA cultures, beta II staining is present in stable MT bundles similar to those of 1 day RA cultures as well as in short neurites (Fig. 3B).

**Beta III staining** - For the purposes of this thesis, cells which show intense staining with the beta III antibody will be described as neurons. It must be pointed out that no other neuronal markers were used to confirm this description. At 2 days RA beta III is induced in a subset of cells as shown by increased staining of these cells with (Fig. 3C). All beta III positive cells are on top of the cell monolayer and some display short neurites. However many of the beta III positive cells either have no cytoplasmic extensions or have one or more short cytoplasmic "spikes", i.e. very thin extensions that are about one cell body in length. The beta III positive cells also have a diffuse intracellular stain indicating the presence of unpolymerized beta III tubulin (Fig. 3C). Also at 2 days RA, there is a reduction of the beta III level in the non-neuronal cells as shown by decrease in the staining intensity of beta III in the general cell population of the monolayer.

Fig. 3. Staining of beta II (**A and B**) and beta III (**C and D**) in 2 day RA cells. **A:** A subset of cells shows increased beta II staining with preferential incorporation into MT bundles and/or neurites. Beta II increases in the cells of the monolayer compared to 1 day RA. **B:** In colchicine treated 2 day RA cells, antibody to beta II stains stable MTs in cells with processes (neurites) and without processes. Bundles similar to 1 day RA cells are also stained. **C:** There is induction of beta III staining at 2 days RA in a subset of cells always present on top of the monolayer. These cells may have no processes (arrowhead), short "cytoplasmic spikes" (arrow) or short neurites. Diffuse intracellular stain may be due to unpolymerized beta III. **D:** In colchicine treated 2 day RA cells stained for beta III, strong intracellular fluorescence may conceal staining of polymerized, stable MTs.

Mag. A,B,C = 350 X, D = 650 X.


3
A
B
C
D

Colchicine treated 2 day RA cells have high beta III intracellular fluorescence due to unpolymerized tubulin not extracted during fixation (Fig. 3D).

## 4 Days RA

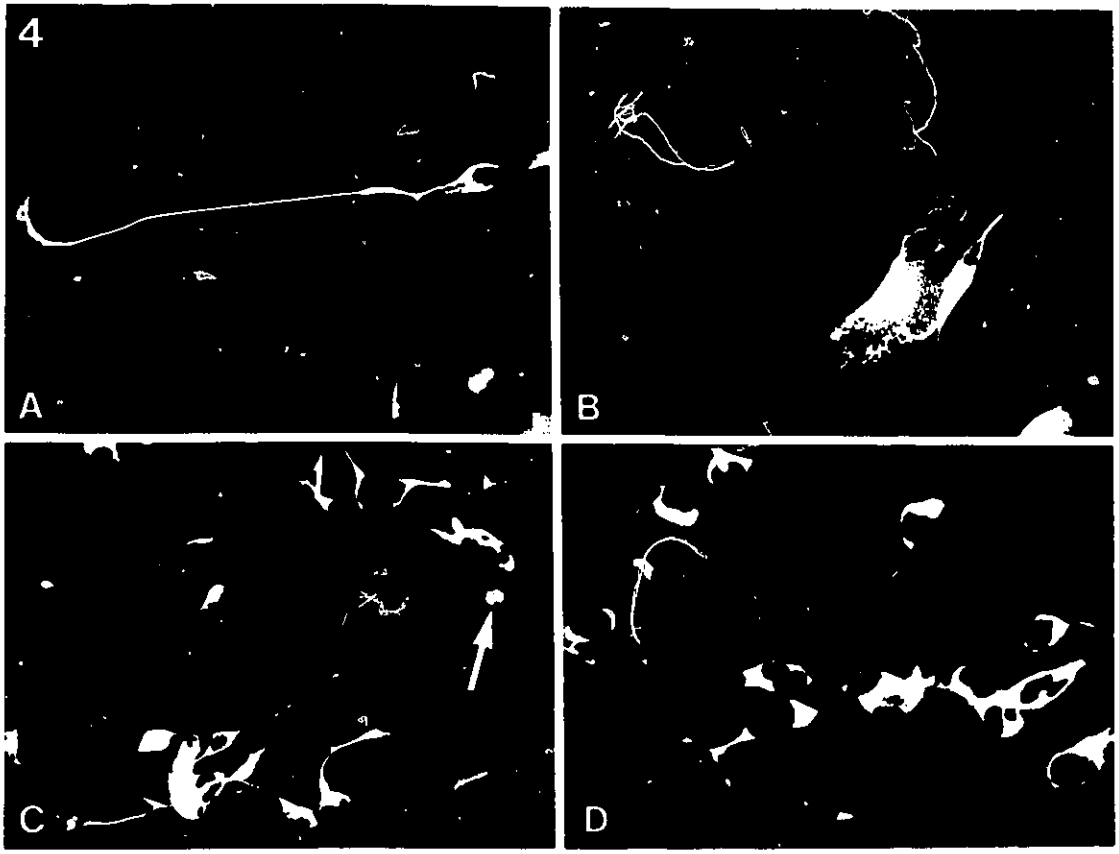
**Beta II staining** - During this period, there is a rapid extension of neurite-like processes. Beta II staining is reduced in the underlying monolayer cells and becomes more intense in the neural cells atop the monolayer. Neural extensions are easily visualized by beta II staining (Fig. 4A). There are also clusters of flat cells which are beta II positive and may represent the low-level differentiation of muscle cells which can be found in cultures grown in $10^{-6}$ M RA (see Fig 7).

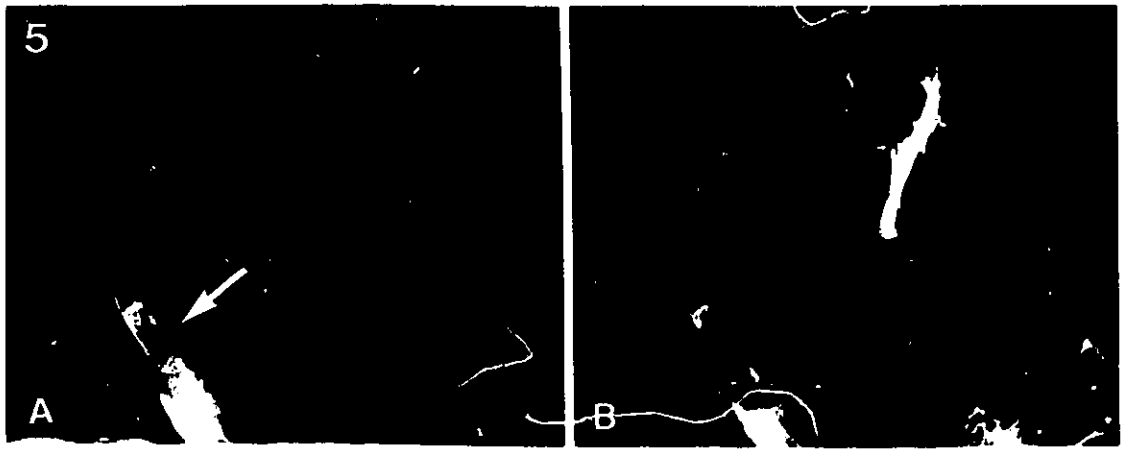
In colchicine treated cells, anti-beta II labels stable MTs in processes and in cell bodies of neurally differentiating cells. No diffuse cellular staining is observed with beta II (Fig. 4B).

**Beta III staining** - Beta III staining is confined to neurons which are found only on top of the cell monolayer (Fig. 4C). Cells with extensions which do not stain for beta III are considered to be non-neuronal although we cannot rule out the possibility that not all neurons express beta III. The EC-like and fibroblast-like cells of the underlying monolayer also do not stain for beta III. Individual beta III MTs can be identified in differentiating

Fig. 4. Stain of differentiating cells for beta II **(A and B)**, and for beta III **(C and D)**. **A:** Beta II staining is present in the cell body, neurite and the growth cone. In the cells of the monolayer there is a decrease in the level of beta II staining compared to 2 day RA cells. **B:** In colchicine treated cells, beta II staining is seen in stable MTs in the cell body and in neurites (out of the plane of focus). **C:** Antibody to beta III stains MTs in rapidly extending neurites but also shows a diffuse intracellular stain. **D:** In colchicine treated cells, the diffuse fluoresence of beta III staining may conceal stable MTs which are present.
Mag. A,D = 350 X, B = 600 X, C = 250 X.

Fig. 5. Double labelling of colchicine treated 4 day RA cells for (A) beta III (B) all alpha tubulin (YOL 1/34). At 4 days RA there are stable MT bundles, similar to those seen at 1 day RA, which do not stain for beta III even though there are beta III positive cells present at this time (arrow).
Mag. = 600 X.


4
A
B
C
D


5
A
B

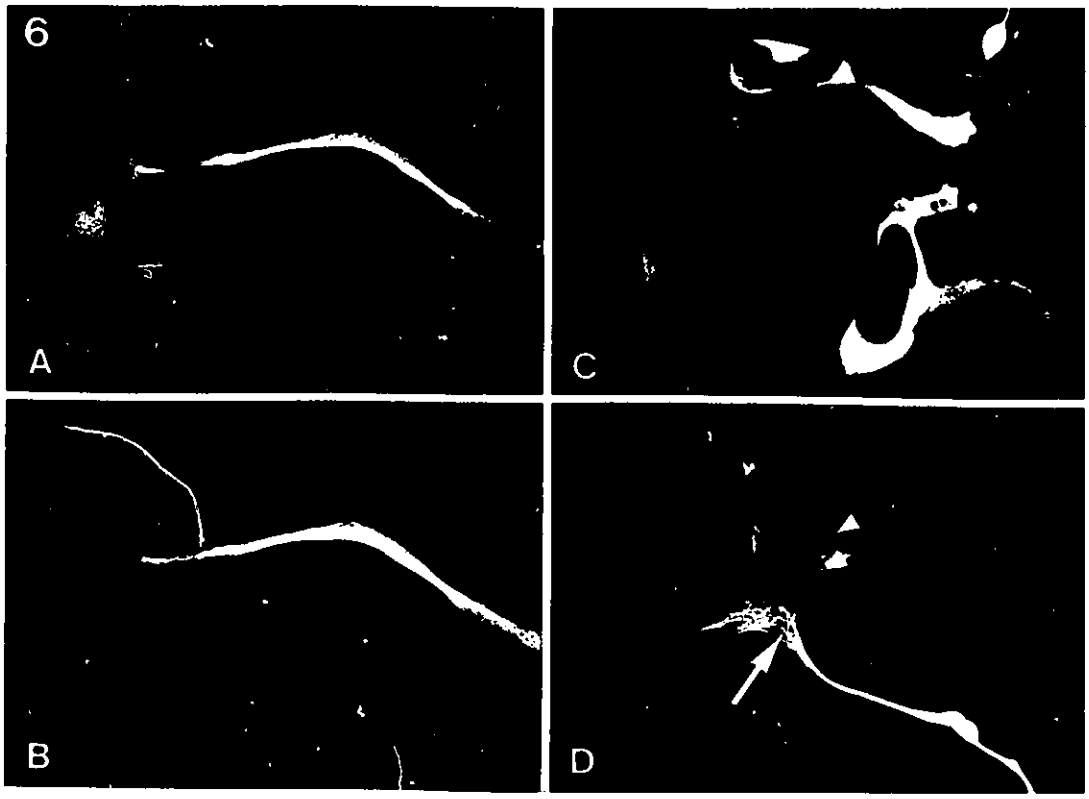
neurons, but there also is a high level of diffuse intracellular fluorescence in these cells indicating the presence of unpolymerized beta III tubulin (Fig. 4D).

In colchicine treated cultures, double labelled for beta III and YOL 1/34, one can identify bundles of stable MTs which are similar to those found after 1 day RA. As occurs at 1 day RA, these stable MT bundles do not stain with the beta III antibody although there are other cells present which have beta III stained stable MTs (Fig. 5).

In colchicine treated 4 day RA cells, double labelling indicates that the stable MT array in differentiating neurons is stained less intensely by the beta III antibody than it is by YOL 1/34 (Fig. 6A and B). However, cells stained for beta III show intense intracellular fluorescence which somewhat obscures the stable MT staining. In cells lacking neurite processes (Fig. 6C), staining for beta III primarily shows a brilliant, diffuse intracellular stain which may obscure a weakly stained stable MT population.

In colchicine treated neurons with an identifiable growth cone, stable MTs can be seen extending just into the base of the growth cone

Fig. 6. (A) and (B) Colchicine treated cell at 4 days RA, double labelled for beta III (A), and all alpha tubulin (YOL 1/34) (B). Stable MTs have a lower level of fluorescence when stained for beta III than they do when stained by YOL 1/34. (C) Staining of beta III in cells without processes. The high intracellular fluorescence completely conceals any stable MTs. (D) In 4 day RA neurons treated with colchicine and stained for beta III, some stable MTs can be detected at the base of the growth cone (arrow), but most beta III staining does not show a typical MT pattern (arrowhead).
Mag. = 650 X.


6
A
B
C
D

(arrow Fig. 6D). However, beta III staining of non-microtubular structures, or short MTs, in the growth cone is also evident (arrowhead).

## 6 Days RA

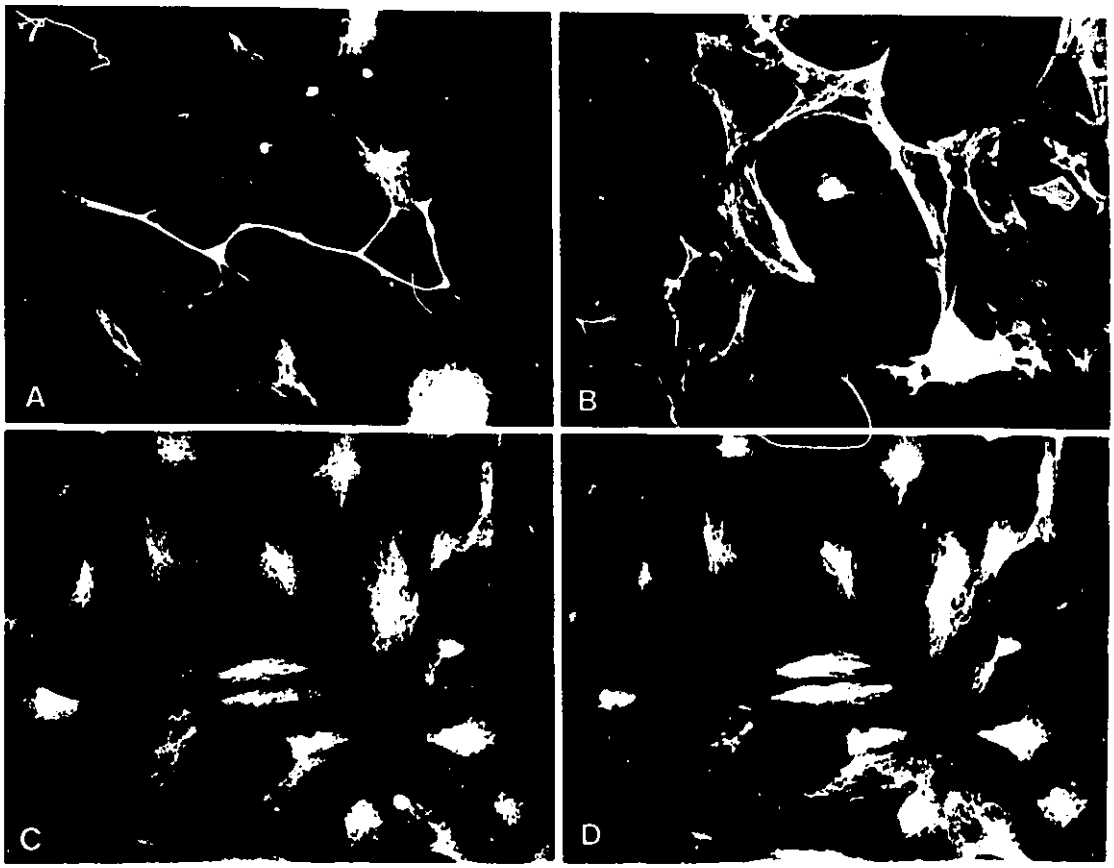
**Beta II staining** - Neurons, and possibly other cells with processes, stained for beta II form a network of anastomosing neurites and processes. These extensions often are atop a monolayer of flat cells which also stain with beta II (Fig.7A). Staining with an antibody that recognizes alpha smooth muscle actin, indicates that many of these flat cells may be differentiating muscle cells (Fig. 7B).

Double labelling with beta II and YOL 1/34 antibodies of 6 day DMSO cultures induced to differentiate along the muscle pathway shows that there is a relatively high level of beta II staining in all MTs (Fig. 7C and D). Antibody to beta III does not stain flat cells in cultures grown in DMSO.

**Beta III staining** - Neurons stained for beta III form a large network on top of a monolayer of flat cells. The neuronal staining patterns of both beta II and beta III are identical (data not shown). As at 4 days RA, colchicine

Fig. 7. (A) By 6 days RA, beta II stained neurons are visible atop a monolayer of flat cells which also show beta II staining. (B) In a 6 day RA culture, cells with similar "flat" morphology show staining with an antibody specific for alpha and gamma muscle actins. (C) Flat, muscle-like cells differentiated in DMSO for 6 days show a high level of beta II staining. (D) Compare with the same cells double labelled with YOL 1/34 to visualize all MTs.

Mag. = 600 X.


A
B
C
D

treatment shows beta III staining in stable MTs, but there is a diffuse intracellular stain indicating the presence of unpolymerized tubulin.

**MAP1B and beta II staining patterns are similar**

The antibodies which recognize MAP1B and beta II are both mouse IgGs, thereby preventing double indirect immuno-labelling with these two antibodies. However, single labelling of EC, 2 day RA and 6 day RA cells with antibody to MAP1B (Fig. 8) shows a staining pattern remarkably similar to that of beta II. In the EC cells, all cells show some degree of MAP1B staining. At 2 days RA, a subset of cells shows increased MAP1B staining, particularly in the MT bundles of these cells (compare Figs. 1A and 8B). By 6 days RA, a large network of processes and neurites is stained with anti-MAP 1B (Fig. 8C).

**Non-neuronal differentiation in the presence of $10^{-6}$ M RA**

Differentiation of neurons and muscle cells, in $10^{-6}$ M RA, can be demonstrated by staining with cell type-specific antibodies: anti-beta III tubulin for neurons and anti-myosin and/or anti-muscle actin for muscle cells (Jones-Villeneuve 1982, Edwards et al. 1983). In addition there are fibroblast-like cells, found during embryonic development and which resemble myofibroblastic or myoepithelial cells, that also express alpha

Fig. 8. MAP1B stain in differentiating neural cells. (A) MAP1B stain in undifferentiated EC cells. (B) Increase in MAP1B stain in a subset of cells at 2 days RA. Note preferential incorporation into MT bundles. (C) MAP1B in neurites and perhaps also in other processes after 6 days RA. Mag. A,B = 600 X, C = 250 X.

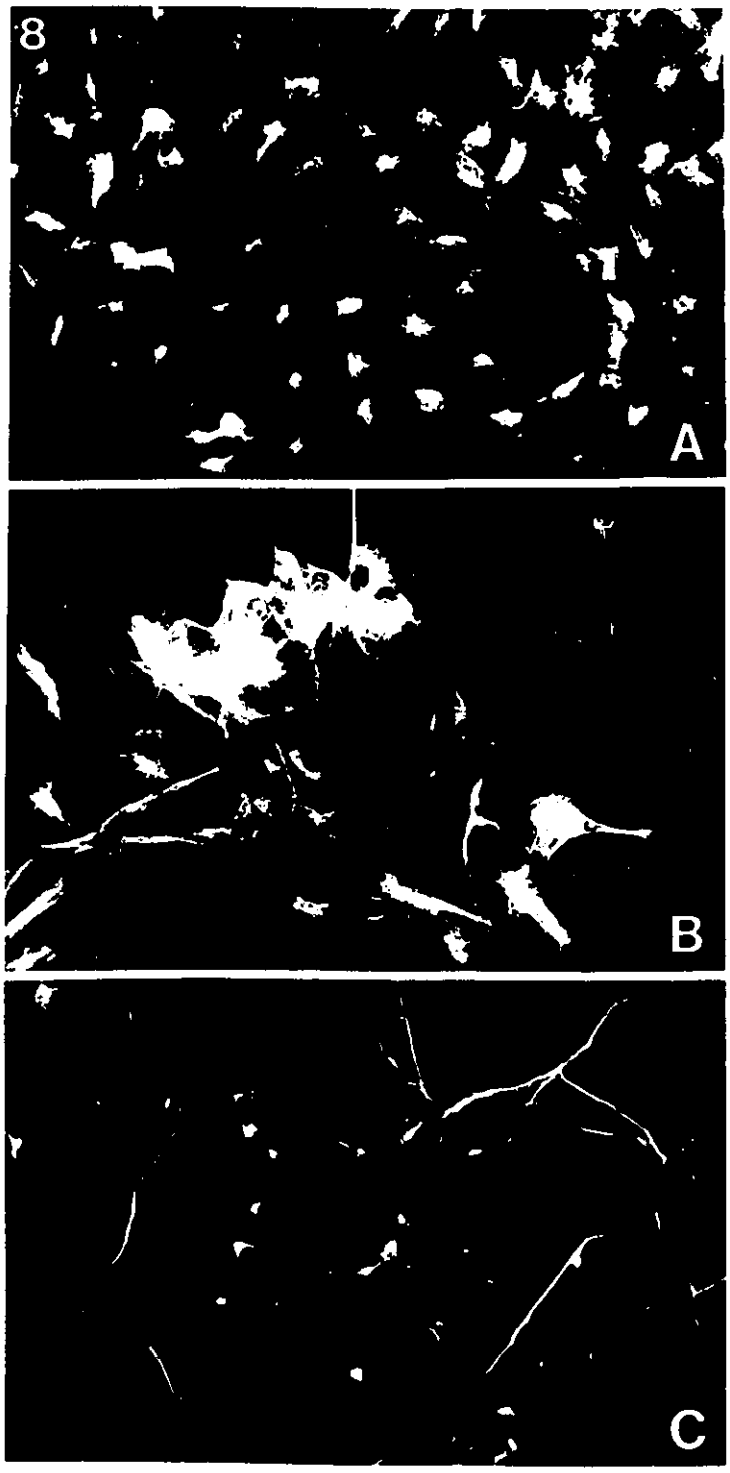
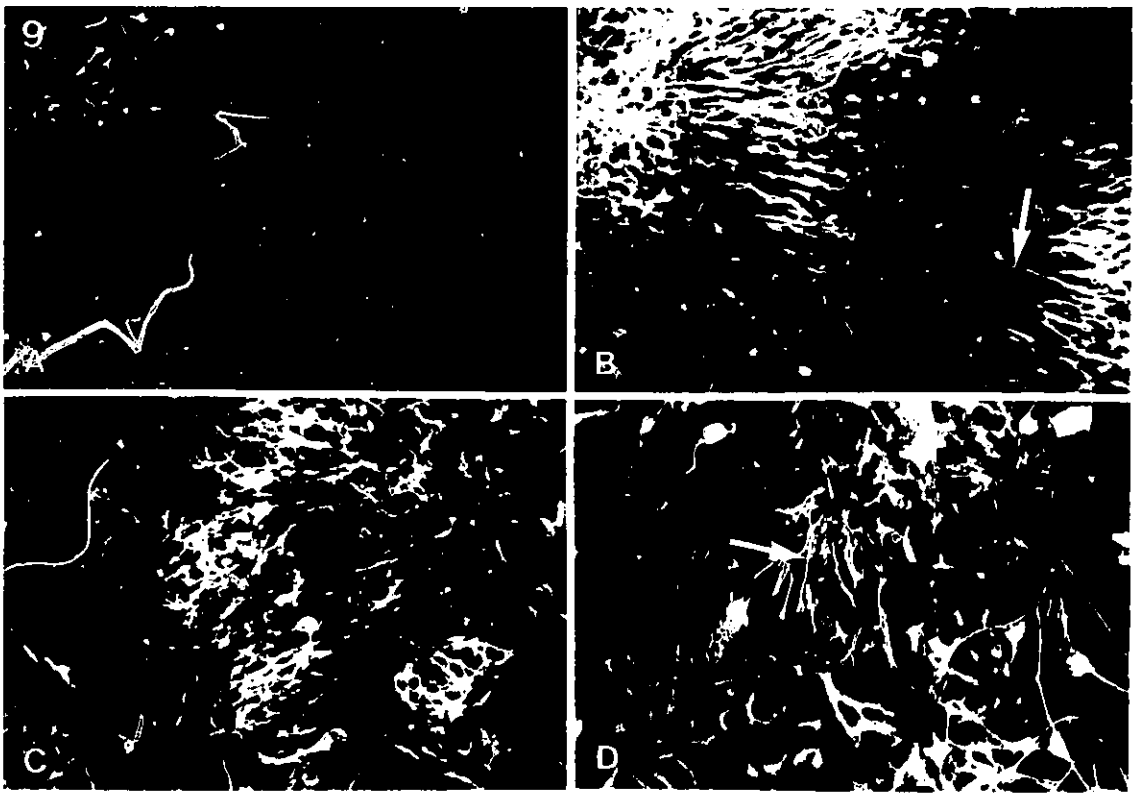

8
A
B
C

Fig. 9. Differentiation of two types of cells containing processes after 4 to 6 days of RA. Double label of cells for (A) Beta III and (B) YOL 1/34. (A) Antibody to beta III stains only neurons while in (B) YOL 1/34 stains all cells including cells with processes which do not stain with beta III (arrow). (C) Group of morphologically similar cells stained with antibody to vimentin (magnification is lower than in A and B)(Micrograph courtesy of Dr. U. Vielkind). (D) Cells morphologically similar to those in (B and C) stained with beta II antibody (magnification is higher than in B and C). Mag. A,B,C = 250 X, D = 350 X.


9
B
C
D

smooth muscle actin forms (Rudnicki et al. 1990). However, there are other differentiating cells for which we have no specific identifying marker. These cells have long processes which do not stain with anti-beta III indicating that they are not neurons (Fig 9A and B). Moreover, the locations of these differentiated cells and of neurons appear to be mutually exclusive as indicated by the double labelling in Fig. 9A and B. In a micrograph of a 6 day RA culture stained with anti-vimentin antibody (courtesy of Dr. U. Vielkind), a morphologically similar group of differentiated cells have processes containing higher levels of vimentin than the surrounding cells (Fig. 9C). Staining of a similar appearing group of cells with anti-beta II antibody shows that they are beta II positive (Fig. 9D). The above results suggest that these differentiated cells may be radial glial cells. However, antibodies against the marker protein, glial fibrillary acidic protein (GFAP), do not show positive staining until at least 10 to 12 days RA and thus cannot be used to identify these cells at day 6 RA.

**Analysis of beta tubulin isotypes by Western blotting**

To further characterize the involvement of beta tubulin isotypes, extracts of total and stable MT arrays were examined by Western blotting with antibodies to beta tubulin isotypes Class I, II, III and IV. The results are expressed as relative percentages and plotted as histograms. The isotype "profile" of a given sample is portrayed by an histogram of all four isotypes in that sample, and this profile then can be compared with similar profiles from other samples.

**Sequential changes in beta tubulin isotypes during neural differentiation**

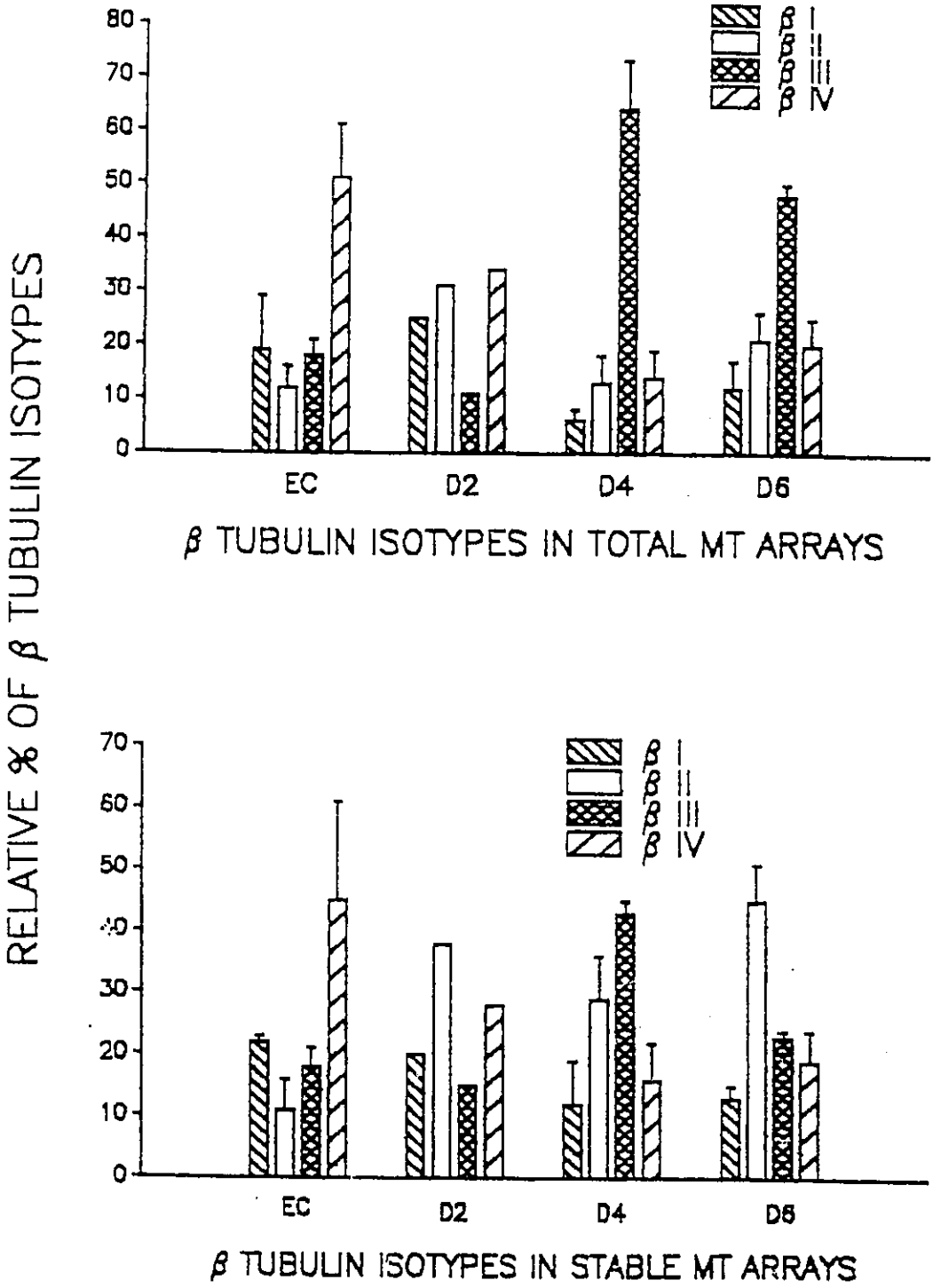
A comparison of the 2 days RA and uncommitted EC beta tubulin profiles shows the largest changes are an increase in the relative percentage of beta II and a decrease in beta IV (Fig. 10). The relative percentages of beta I and III change only slightly. (Due to sample size limitations, standard deviations could not be calculated for 2 days RA samples.)

In the stable MT sample at 2 days RA, beta II is the predominant isotype and the relative percentage of beta II is greater in this sample than it is in the total MT sample. The predominance of beta II in the stable MT sample initially occurs at the time that MAP2C first can be detected by either indirect immunofluoresence staining (Falconer et al. 1989) or immunoblotting (see Fig. 16).

At 4 days RA, there is a large increase in beta III (Fig. 10) which occurs simultaneously with the rapid extension of neurites. In the total MT sample, the relative percentage of beta III is 64% (+/- 9%), while in the stable MT sample, beta III constitutes only 43% (+/- 2%). By the Student's t test, these two percentages are significantly

Fig. 10. Isotype profiles showing sequential changes in the relative percentages of beta tubulin isotypes in total and in stable MT arrays of neurally differentiating cells.

Mean and standard deviation based on 5 samples for EC total MTs, 4 samples for EC stable MTs, 2 samples for 2 day RA total MTs and 2 samples for 2 day RA stable MTs, 3 samples for 4 day RA total MTs and 3 samples for 4 day RA stable MTs, 4 samples for 6 day RA total MTs and 3 samples for 6 day RA stable MTs.


RELATIVE % OF β TUBULIN ISOTYPES
80
70
60
50
40
30
20
10
0
β I
β II
β III
β IV
EC
D2
D4
D6
β TUBULIN ISOTYPES IN TOTAL MT ARRAYS
70
60
50
40
30
20
10
0
β I
β II
β III
β IV
EC
D2
D4
D6
β TUBULIN ISOTYPES IN STABLE MT ARRAYS

different at the 95% confidence level. (Hereafter "significant" refers to 95% confidence level or better unless otherwise stated.) There is less Beta III, in the stable MT array indicating some degree of exclusion from these MTs or some degree of inclusion in the non-stable MTs.

At 4 days RA, the relative percentages of beta II in the total and stable MT samples, respectively, are 13% (+/-5%) and 29% (+/-7%). At the 90% confidence level, there is significantly more beta II in the stable MT sample than in the total MT sample.

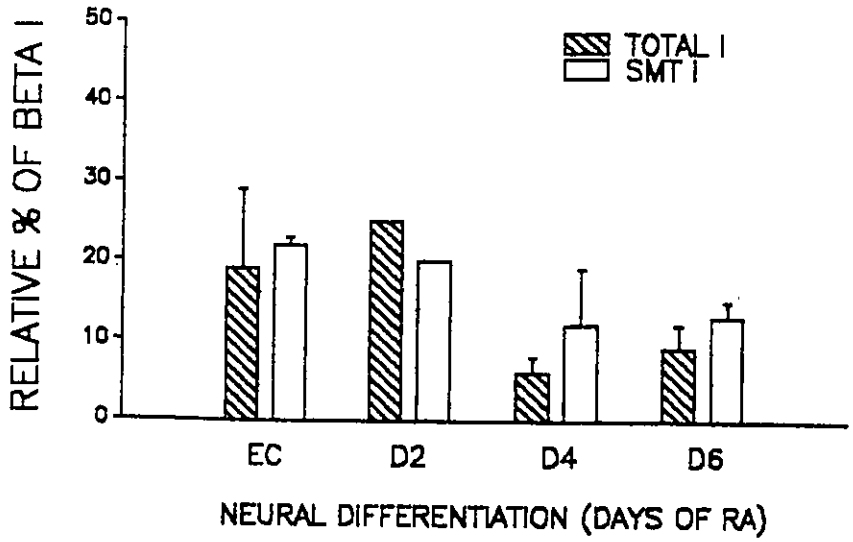
By 6 days RA, the total and stable MT profiles are distinctly different. In the total MT sample, beta III continues to show the highest relative percentage, although this percentage has decreased since 4 days RA. There is significantly less beta III in the stable MT sample, than in the total MT sample. In the stable MT sample, beta II constitutes the predominant isotype and there is a significantly higher relative percentage of beta II in the stable MT sample compared to the total MT sample. Beta I and beta IV, respectively, do not show significant differences between total and stable MT arrays.

Figures 11 and 12 show the same data as that in Fig. 10 but present separately the changes in the relative percentages of a single isotype which occur during neural differentiation.

The relative percentage of isotype I in the total MTs (Fig. 11) decreases during differentiation from 19% (+/-10%) in EC cells to 9% (+/-3%) at 6 days RA. The reduction is not significant at this small sample size. There is a small but significant reduction in beta I in stable MTs with the percentage reduced from 22% (+/-1%) in EC cells to 13% (+/-2%) at 6 days RA.

The relative percentage of beta II in neurally differentiated cells is significantly increased compared to that in undifferentiated EC cells (Fig. 11). In total MTs, the relative percentage of beta II increases from 12% (+/-4%) in undifferentiated EC cells to 21% (+/-4%) at 6 days RA. In the stable MT population this increase is even greater, rising from 11% (+/-5%) in EC stable MTs to 45% (+/- 6%) in neurally differentiated cells at 6 days RA.

Fig. 11. Changes in the relative percentages of Class I and Class II beta tubulin isotypes during neural differentiation. Mean and standard deviation based on same samples as in Fig. 10.


RELATIVE % OF BETA I
50
40
30
20
10
0
TOTAL I
SMT I
EC
D2
D4
D6
NEURAL DIFFERENTIATION (DAYS OF RA)

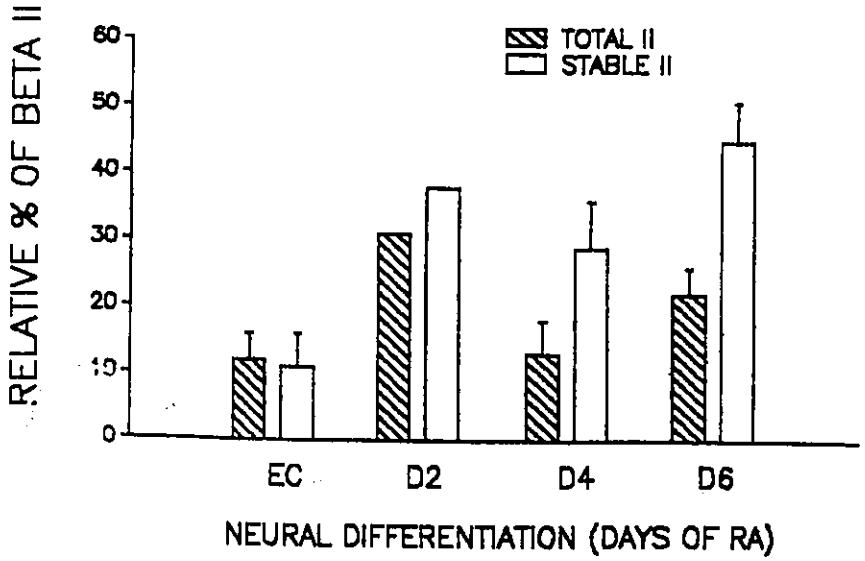
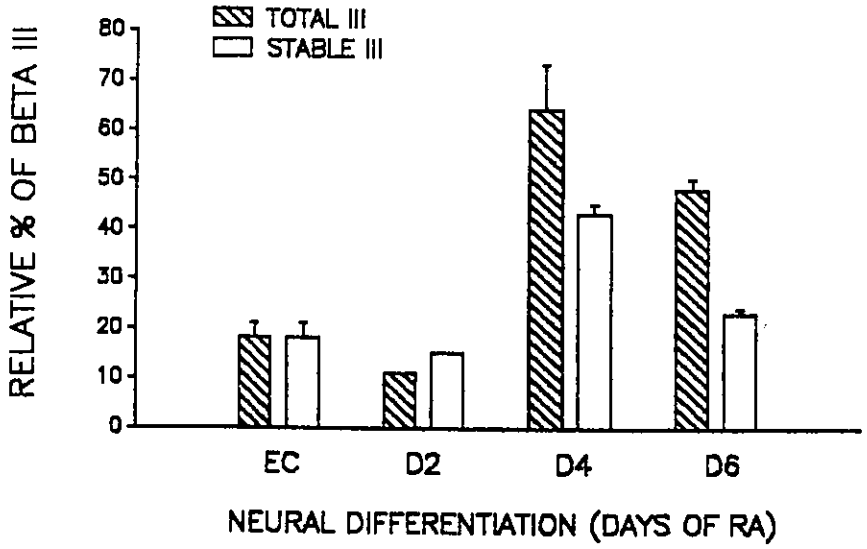
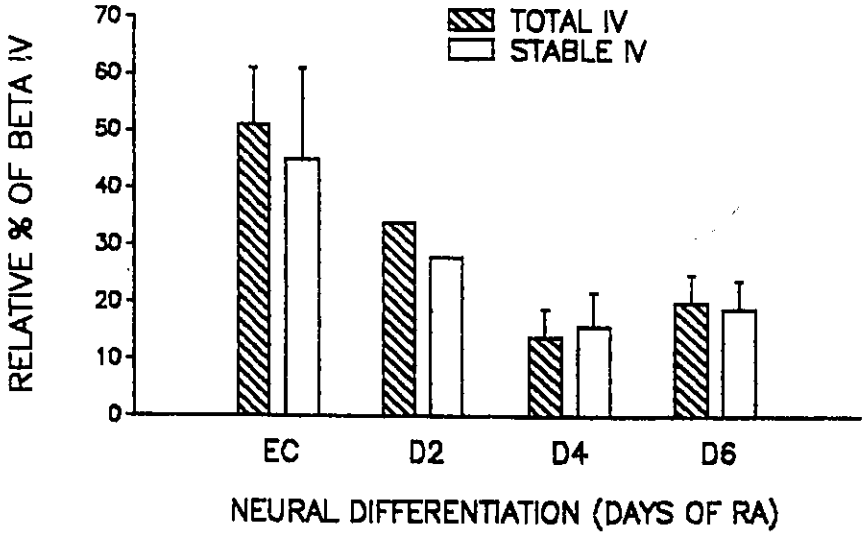

RELATIVE % OF BETA II
60
50
40
30
20
10
0
TOTAL II
STABLE II
EC
D2
D4
D6
NEURAL DIFFERENTIATION (DAYS OF RA)

Fig. 12. Changes in the relative percentages of Class III and Class IV beta tubulin isotypes during neural differentiation. Mean and standard deviation based on same samples as in Fig. 10.


TOTAL III
STABLE III
RELATIVE % OF BETA III
80
70
60
50
40
30
20
10
0
EC
D2
D4
D6
NEURAL DIFFERENTIATION (DAYS OF RA)


TOTAL IV
STABLE IV
RELATIVE % OF BETA IV
70
60
50
40
30
20
10
0
EC
D2
D4
D6
NEURAL DIFFERENTIATION (DAYS OF RA)

The increase in beta II begins with the 2 day RA sample and the relative percentage is always highest in the stable MT array. The highest relative percentage of beta II is seen at 6 days RA and coincides with the appearance of tau protein as detected by immunoblotting (see Fig. 16).

The relative percentage of beta III shows a striking increase in the total MT array between 2 and 4 days RA, which then decreases somewhat by 6 days RA (Fig. 12). Thus cells from both 4 and 6 days of neural differentiation show some degree of subcellular sorting with less beta III present in the stable MT array.

The relative percentage of beta IV at 6 days RA is 20% (+/-5%), down from the 51% (+/-10%) in undifferentiated EC cells (Fig. 12). At all sampling days, beta IV is present in approximately equal percentages in both total and stable MT samples and there is no significant difference between the relative percentages of beta IV in these two MT populations.

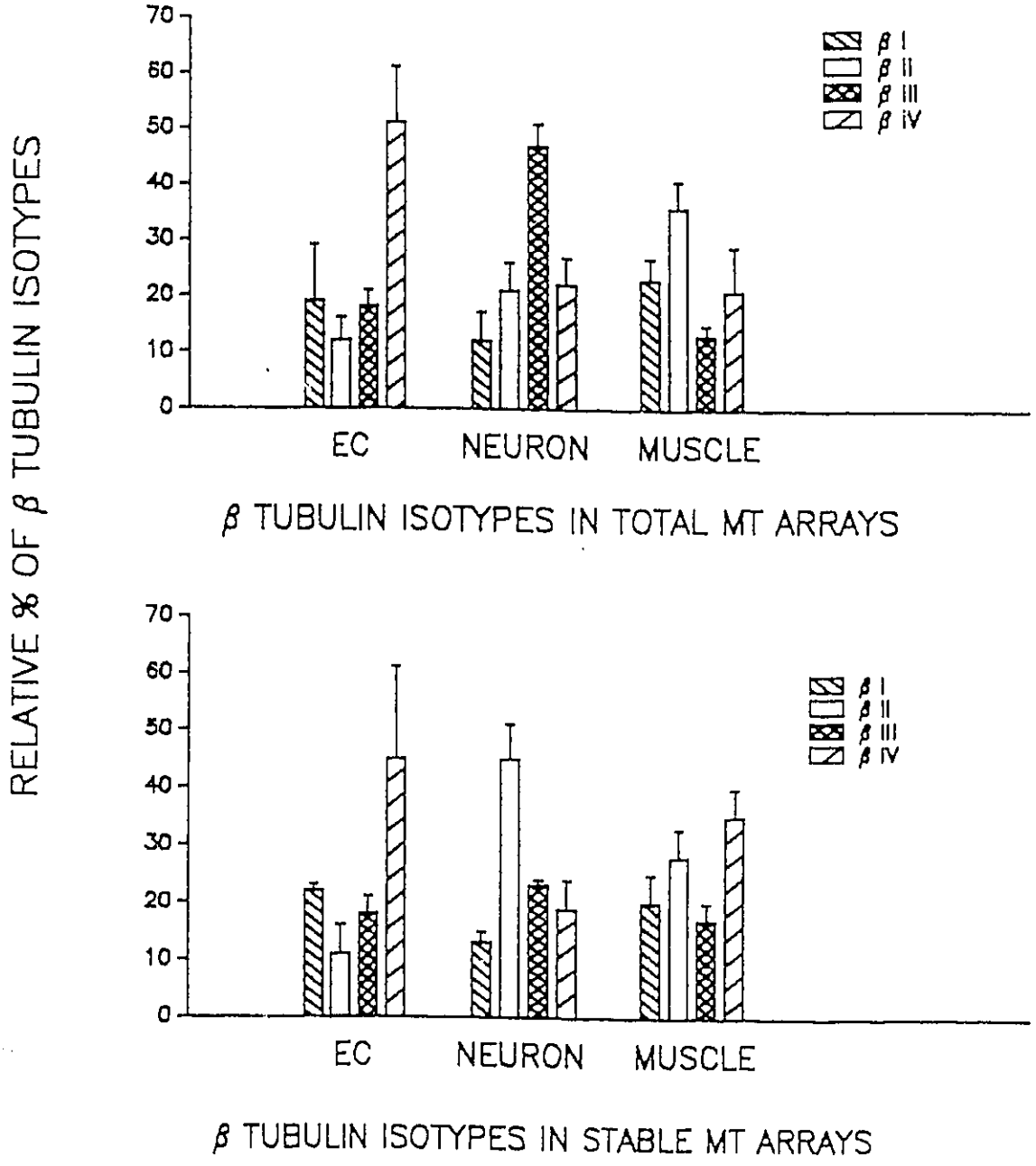
**Analysis of isotype profiles in EC, neural and muscle differentiated cells**

The above results indicate that one major change during neural differentiation is the subcellular sorting of beta II into the stable MT array accompanied by subcellular sorting of beta III to the dynamic MT array. It has been shown that MAPs play a role in stabilizing MTs (Lewis et al. 1989, Ferreira et al. 1989, Kanai et al. 1989). Therefore interaction between MAP(s) and a specific tubulin isotype, or conversely, lack of interaction between MAP(s) and a specific tubulin isotype, may result in selective sorting of these isotypes into particular MT arrays. One possibility is that beta II interacts with MAP2 and/or tau in a manner that stabilizes MTs and allows further incorporation of beta II which further stabilizes these MTs, etc. in a self-perpetuating cycle.

This hypothesis was tested by inducing differentiation of P19 cells along two separate pathways, neural and muscle. The beta tubulin isotype profiles of neural and muscle differentiated samples then can be identified and compared. If brain specific MAP(s) are important for the preferential

Fig. 13. Beta tubulin isotype "profiles" of total and stable MT arrays in uncommitted EC, neural and muscle differentiated cells.

Mean and standard deviation based on: 5 samples for EC total MTs and 4 samples for EC stable MTs, 4 samples for neural total MTs and 3 samples for neural stable MTs, 3 samples for muscle total MTs and 3 samples for muscle stable MTs.


RELATIVE % OF β TUBULIN ISOTYPES
70
60
50
40
30
20
10
0
β I
β II
β III
β IV
EC
NEURON
MUSCLE
β TUBULIN ISOTYPES IN TOTAL MT ARRAYS
70
60
50
40
30
20
10
0
β I
β II
β III
β IV
EC
NEURON
MUSCLE
β TUBULIN ISOTYPES IN STABLE MT ARRAYS

incorporation of beta II into stable MTs, then the muscle samples should not show such preferential incorporation of beta II. The absence of MAP2 and tau in P19 cells differentiated along the muscle pathway was demonstrated by indirect immunofluoresence staining and immunoblotting (data not shown).

A comparison of the beta tubulin isotype profiles in EC, neural and muscle cells is seen in the histogram of Fig. 13. The total MT profiles of EC, neural and muscle cells are distinct from each other. However, in uncommitted EC cells, the profiles in samples of total and stable MT arrays are essentially identical. Similarly, the isotype profiles of the total MT array and of the stable MT array in muscle-differentiated samples are similar, although there is a significant increase in the percentage of isotype IV in the stable MT array (at the 90% confidence level). In neurally differentiated cells, the isotype profiles of total MTs and stable MTs are quite different, with preferential inclusion of beta II in stable MTs and preferential inclusion of beta III in total MTs.

Fig. 14. Relative percentages of Class I and Class II beta tubulin isotypes in total and stable MT arrays of uncommitted EC cells, neurally differentiated and muscle differentiated cells. Mean and standard deviation based on same samples as in Fig. 13.

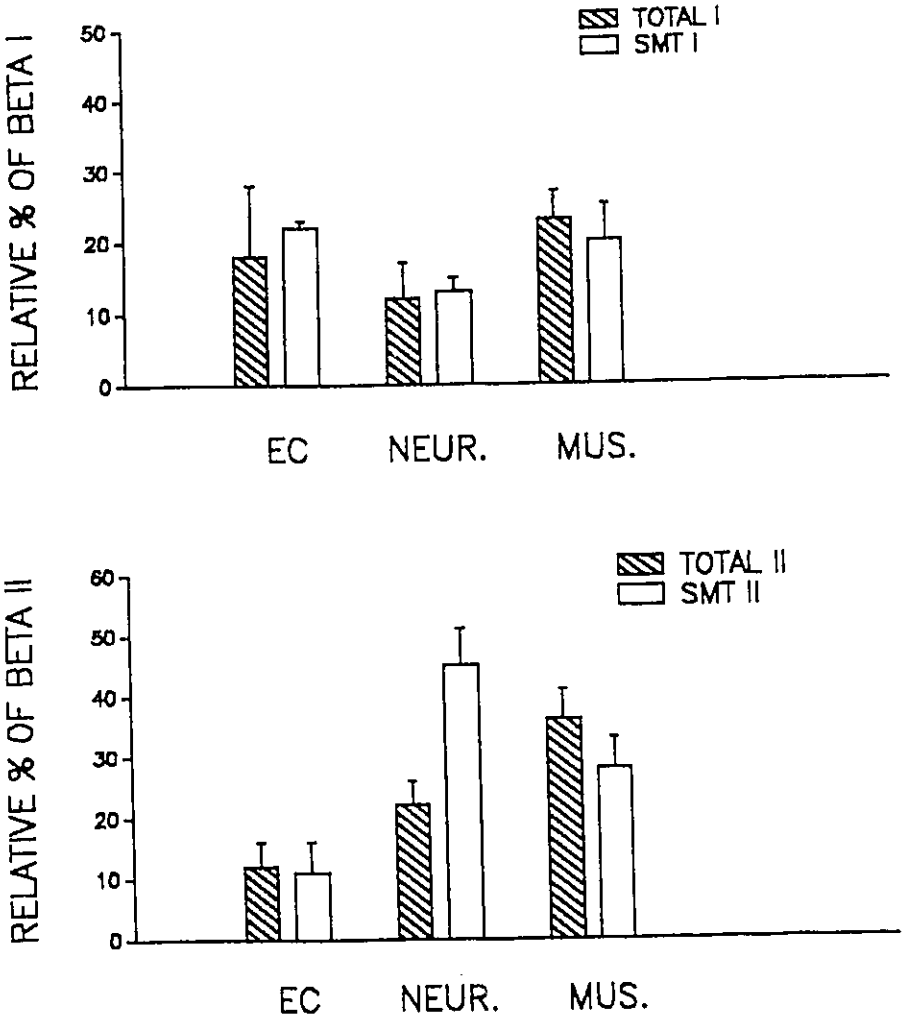
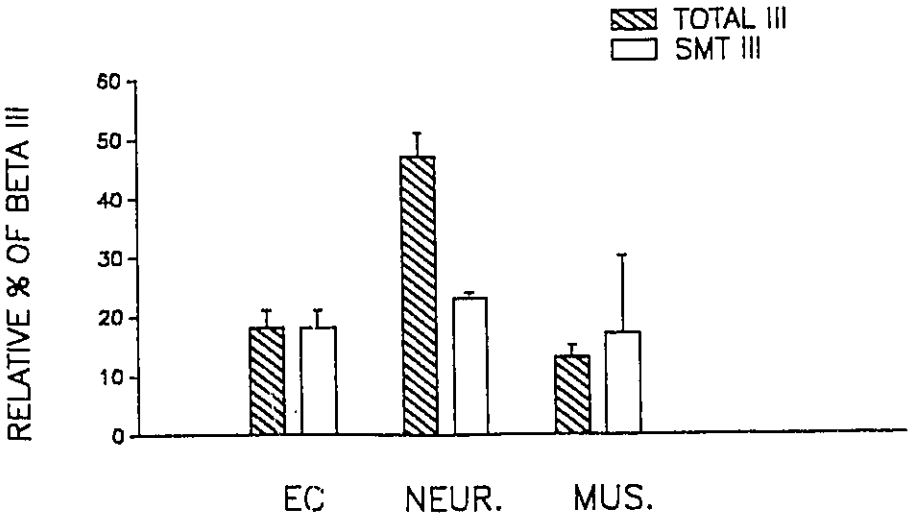
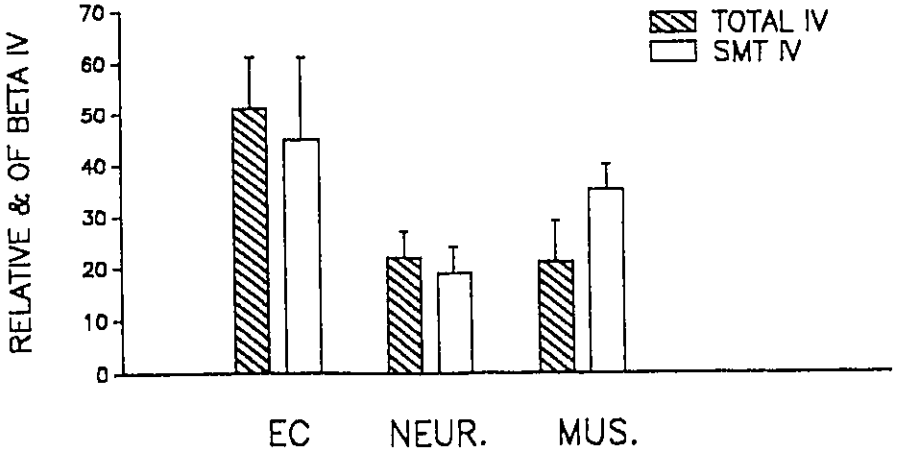

TOTAL I
SMT I
RELATIVE % OF BETA I
50
40
30
20
10
0
EC
NEUR.
MUS.
TOTAL II
SMT II
RELATIVE % OF BETA II
60
50
40
30
20
10
0
EC
NEUR.
MUS.

Fig. 15. Relative percentages of Class III and Class IV beta tubulin isotypes in total and stable MT arrays of uncommitted EC cells, neurally differentiated and muscle differentiated cells. Mean and standard deviation based on same samples as in Fig. 13.


TOTAL III
SMT III
RELATIVE % OF BETA III
60
50
40
30
20
10
0
EC
NEUR.
MUS.


TOTAL IV
SMT IV
RELATIVE % OF BETA IV
70
60
50
40
30
20
10
0
EC
NEUR.
MUS.

In Figures 14 and 15, this same data is presented in a format which depicts the changes in each isotype separately. Isotype I shows relatively minor changes between EC, neural and muscle samples although the relative percentage of beta I in the neural total MT sample is significantly less than that in the total muscle sample. The difference between the percentage of beta I in the stable MT sample of neural versus muscle cells also is significant (at the 85% confidence level).

The relative percentage of beta II in the stable MT sample of neural cells is significantly higher than that in either the EC stable MT sample or the muscle stable MT sample. This is in spite of the fact that there is a higher relative percentage of beta II in the muscle total MT sample (36% +/- 5%) than there is the neural total MT sample (21% +/- 4%).

The relative percentage of the neuron-specific isotype, beta III, increases only in the total MT sample from neural differentiated cells (Fig. 15). All other values are essentially unchanged from those of the uncommitted EC cells. Finally, the relative percentage of beta IV, decreases in neural and muscle samples of both total and stable MTs compared to the EC values (Fig. 15). However, there is a somewhat larger percentage of beta IV in the stable MT sample of muscle than in the total MT sample.

**Increase in tubulin during differentiation**

Under ideal conditions, up to 80% of cells show long neurite-like processes after 6 days RA. These processes contain bundles of MTs which should result in an increase in the amount of tubulin per μg of protein during differentiation. Therefore 100 μg of total protein from EC, 2, 4 and 6 day RA samples was subjected to PAGE and stained with Coomasie blue to show that there is an increase in tubulin/μg protein (Fig. 16 top).

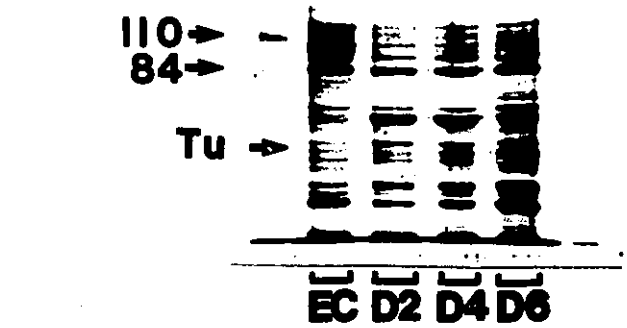
**Appearance of tau and MAP2 during differentiation**

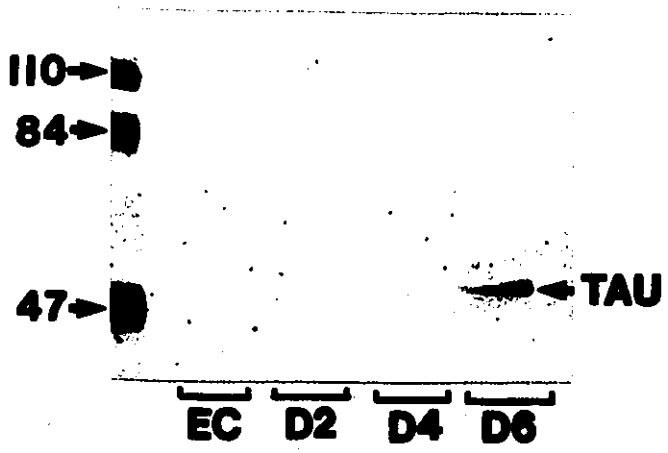
Indirect immunofluorescence staining indicates that tau is absent from EC cells and appears first between 4 and 6 days RA. Immunoblotting of EC, 2, 4 and 6 day RA total MT samples confirms this observation and also shows that the form of tau which appears by 6 days RA is the 48 kD juvenile tau (Fig. 16 middle). Immunoblotting of these same samples using the AP-18 antibody shows that there is no MAP2 in undifferentiated cells and that low levels of MAP2C are detected by 2 days RA and increase during the next 4 days. High molecular weight adult MAP2 can first be detected at low levels after 6 days of differentiation (Fig. 16 bottom).

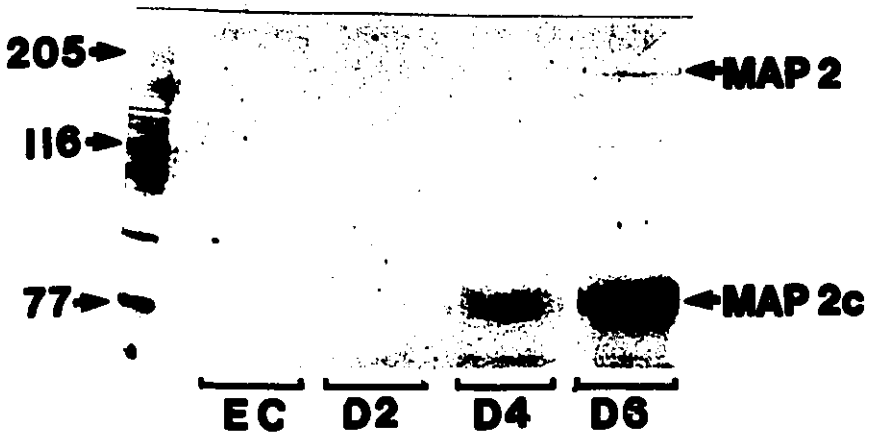
Fig. 16. (A) Polyacrylamide gel stained with Coomassie blue to visualize loading of 100 μg of total protein in samples from EC, 2, 4, and 6 days RA. Position of tubulin is marked by an arrow (Tu arrow). Note the increase in the amount of tubulin as differentiation proceeds.

(B) Juvenile tau appears at 6 days RA as shown by a Western blot stained with antibody to Tau-1. 2 μg of 4 times cycled brain tubulin was added to the molecular weight standards lane as a positive control for tau staining, note the faint bands of multiple tau isotypes.

(C) Juvenile MAP2C appears faintly at 2 days RA and adult, high molecular weight MAP2 first appears at 6 days RA. 2 μg of 4 times cycled brain tubulin was added to the molecular weight standards lane as a positive control for MAP2 staining, but MAP2 had degraded causing numerous bands to appear.


110
84
Tu
EC D2 D4 D6


110
84
47
TAU
EC D2 D4 D6


205
116
77
MAP 2
MAP 2c
EC D2 D4 D6

# DISCUSSION

During neural differentiation, both indirect immunofluoresence microscopy and immunoblotting indicate that significant changes occur in expression patterns of Class II and Class III beta tubulin isotypes, the most obvious of which is the time of expression. Beta II expression increases before 2 days RA as indicated by increased staining of total and stable MT arrays in about 15% of cells and by immunoblotting. During this time, beta III expression is very low and is not detected in the newly induced arrays of stable MT bundles.

The first increase in expression of beta III, at 2 days RA, occurs only in a subset of cells on top of the cell monolayer while the low-level staining of undifferentiated cells in the monolayer disappears. This increase in beta III can be observed by immunofluoresence microscopy but is not detected by immunoblotting. The discrepancy in detection probably results from two factors or a combination of these factors. On the one hand the increase in beta III occurs in only a few cells while all other cells have decreased beta III expression. Thus there is a change in the distribution of the isotype while the total amount of beta III may remain relatively constant. On the other hand, much of beta III remains unpolymerized (Joshi and Cleveland 1989) and would be extracted in the permeabilization step of sample

preparation. The loss of this tubulin will not affect the "total MT" sample since the tubulin in this sample represents only that polymerized into MTs. Thus an increase in unpolymerized beta III would not be detected in immunoblots but will be detected by the immunofluorescence staining since fixation procedures can trap some monomeric tubulin.

A major difference in the expression patterns of beta II and beta III is detected by immunoblotting neural total and stable MT samples. Beta II apparently is incorporated preferentially into the stable MT population beginning at 2 days RA and continuing until the end of the sampling period at 6 days RA (Fig. 10). During this same period, the highest percentage of beta III is in the total MT array while it is preferentially excluded from the stable MT array. This is confirmed by indirect immunofluoresence microscopy. Antibody to beta II brilliantly stains all stable MTs in neurally differentiating cells, including arrays of stable MTs which are not stained by beta III. However, the staining pattern of the beta III antibody is markedly dimmer in stable MT arrays than it is in total MT arrays.

Although immunoblotting indicates that beta II is preferentially found in the stable MT array beginning with samples from 2 days RA, this analysis is complicated by the fact that the EC culture does not differentiate simultaneously. Thus undifferentiated EC cells have less beta II than the neurally differentiating cells. In the total MT sample, which includes both cell populations, the EC cells will serve to lower the relative percentage of beta II. Conversely, differentiating cells have more stable MTs than EC

cells. Therefore most stable MTs will be derived from differentiating cells which have more beta II and this will raise the proportion of beta II in the stable MT sample. Although indirect immunofluorescence staining indicates that beta II is preferentially incorporated in neural stable MT arrays before 2 days RA, on the basis of this evidence we cannot conclude this with certainty. By 4 days RA, fewer uncommitted EC cells remain and immunofluorescence staining indicates that the level of beta II in non-neural cells is greatly diminished compared to EC and 2 day RA levels. Therefore the effect of lower beta II in undifferentiated cells is minimized. Similarly, more than 50% of the cells are differentiating along the neural pathway and have increased stable MTs, therefore the stable MT sample will begin to reflect the isotype compositon of stable MTs in the culture as a whole.

By 6 days RA, about 80% of cells are neural cells with extended processes and, since neurite-like processes contain large numbers of MTs and will have more tubulin per cell, more than 80% of the tubulin is derived from neural cells. Therefore the MT population which contributes to the total and the stable MT samples is essentially identical. At this point we can confidently say that beta II is preferentially included in the stable MT array.

Samples from differentiating muscle cells do not have the same complications since only the 6 day sample is analyzed and indirect immunofluorescence assay indicates that all cells have approximately the same amount of stable MTs. The lack of subcellular sorting of beta II into

the muscle stable MT sample suggests that an abundance of beta II is not sufficient to induce this subcellular sorting.

Unlike beta II, beta III is expressed at high levels ONLY in neurons thus the relative percentage of beta III in total and stable MT samples reflects the relative presence or absence of this isotype in neural cells. Therefore we can confidently say that immunoblotting indicates that there is subcellular sorting of beta II into the stable MT array and subcellular sorting of beta III into the dynamic array.

Beta III is present only in neurons and is the earliest marker of strictly neuronal differentiation that we have used. The neuronal specificity of beta III in brain tissue has been noted by many researchers in neurons from a variety of sources (Gass et al. 1990, Asai and Remolona 1989, Burgoyne et al. 1988, Eddé et al. 1987). However, beta III is also expressed by transformed cells (personal communication Dr. Binder) and this expression correlates with the invasiveness of the cell (personal communication Dr. Mark Stearns). This may explain the low-level staining in undifferentiated P19 cells and in 6 day RA or DMSO samples which contain tubulin from some undifferentiated EC cells as well.

Unlike beta III, in differentiating P19 cultures, beta II is present in neurons, muscle cells and other cells of the monolayer, including what may be differentiating glial processes. In sections of developing and adult mouse cerebellum, beta II isotype has been observed in Bergmann glial cells, and in astrocytes (Burgoyne et al. 1988). The cells with processes which show beta

II, but not beta III, staining (see Fig. 9) may represent differentiating radial glial cells. However at this stage of development, the marker protein, GFAP, is not yet expressed and so a definite lineage cannot be determined.

Although not specific to neurons, Class II beta tubulin is associated with neuronal differentiation and regeneration and is selectively induced during axonal growth (Hoffman and Cleveland 1988) while Class IVa beta tubulin is expressed later in neuronal development (Sullivan 1988).

Beta II is the major isotype in bovine brain and is expressed in multiple cell types in the brain including neurons and glia (Burgoyne et al. 1988). About 58% of bovine brain tubulin is beta II, while the remaining isotypes make up the remainder: 3% beta I, 25% beta III, and 13% beta IV (Banerjee et al. 1988). However, in 6 days RA neural samples from P19 cells, the beta III isotype has the highest relative percentage, although beta II is the predominant isotype in the stable MT array (see Fig. 10). In adult brain, 58% is beta II (Banerjee et al. 1988). We cannot rule out the possibility that further changes in isotype distribution may occur later in differentiation. Beta II may become the predominant isotype later in differentiation since an increase in beta II and a decrease in beta III takes place between 4 and 6 days RA and this trend may continue.

Immunoblots of differentiating P19 cells indicate muscle cells contain a high percentage of beta II. Previous examination of beta II mRNA in muscle from adult mouse showed only low level expression of this isotype (Lewis and Cowan 1990). However, differentiating muscle cells in P19

cultures may have high expression of beta II and this may reflect beta II expression in muscle derived from neural crest cells and associated with cranial development (Lumsden 1989).

Tubulins from different sources have different assembly characteristics (Baker et al. 1990) which suggests that it is possible that MTs with high levels of one tubulin isotype may be less dynamic and therefore more resistant to depolymerization by colchicine. The high levels of beta II which are present in muscle cells, however, are not sufficient to increase resistance to colchicine induced depolymerization since the highest relative percentage of beta II is found in the dynamic MT sample. A high relative percentage of beta II also is not sufficient to induce subcellular sorting of beta II into the stable MT array. In differentiating P19 cultures, the MTs in muscle cells contain a higher relative percentage of beta II than do MTs in neural cells. However, all MTs in muscle cells are not colchicine stable and, in fact, in muscle cells there is no preferential subcellular sorting of beta II into the colchicine stable MTs. Brain-specific MAPs, which are postulated to interact with beta II and/or beta III are absent from muscle differentiated cells. This indicates that the subcellular sorting of beta II into the stable MT population as well as the increased colchicine resistance of these MTs may result from interaction between beta II and brain-specific MAP(s).

Neurally differentiating P19 cells show asequential increase in expression of beta II and beta III which is similar to that seen in

differentiating PC12 cultures (Joshi and Cleveland 1989). In the PC12 system,about 50% of beta III remains unpolymerized while beta I and II are preferentially used for assembly of neurite MTs. In addition, beta II and beta III increase concomitantly with the increase in levels of tau and MAP2.

A relationship between MAP2 and/or tau and MT stability has been shown during neurogenesis in another model system. In cerebellar macroneurons, tau expression and accumulation follows a time course identical to that of the induction of stable MTs and tau seems to be associated with stable MTs at all stages of neurite differentiation and growth (Ferreira et al. 1989b). Both MAP2 and tau can stabilize and bundle MTs. Transfection of a MAP2-encoding cDNA into cell lines which do not express MAP2 results in formation of bundled MTs which are stable to colchicine (lewis et al. 1989). Transfection studies introducing tau into cells which do not normally express this protein also result in bundling and stabilization of MTs along with formation of elongated cell processes (Kanai et al. 1989).

Both alpha and beta tubulins have regions that bind MAPs. However, there is preferential binding of MAP 2 and tau to the beta subunit in the domain containing amino acid residues 422-434 (Maccioni et al. 1988). This overlaps the variable region used to classify beta tubulin isotypes which begins at residue 430 and extends to the end of the molecule. Within the overlapping regions, at positions 430 and 432, there are two amino acids which are particular to the Class II isotype, aspartic acid and glutamine (Sullivan 1988). The addition of aspartic acid to the already acidic COOH

terminus may enhance charge interactions between beta II and the repeated sequences of MAP2 and tau. These homologous repeats are basic and are implicated in binding the MAPs to MTs (reviewed by Vallee 1990).

Interaction with MAP(s) also may influence selective incorporation of beta II into stable MTs. One possibility is that induction of neural differentiation increases expression of beta II, initially creating higher levels of unpolymerized beta II tubulin in the cytoplasm. This results in increased incorporation of beta II into MTs. Preferential incorporation of a single isotype, from a mixture of isotypes, has been demonstrated *in vitro* although the mechanism of tubulin subunit sorting is not known (Murphy 1988). If preferential incorporation also occurs *in vivo*, MTs with higher levels of beta II may bind more MAP1B (and later in differentiation bind more MAP 2 and/or tau) thereby increasing MT stability and/or assembly. This is suggested by the increased intensity of MAP1B staining in some differentiating cells at 1 Day RA.

Kirschner and Mitchison (1986) suggest that cell polarity arises from selective stabilization of a subset of MTs. Subsequent modifications to these stable MTs then result in a further increase in stability. Thus beta II/ MAP1B interactions may provide the initial stability. These stabilized MTs remain polymerized longer and therefore can incorporate more beta II. Later addition of MAP 2 and then tau further stabilizes MTs during the consolidation phase of neuron maturation.

Subcellular sorting of beta III may depend upon another mechanism. *In vitro* experiments indicate that the inherent differential stability of a particular isotype the dissociation rate constant which governs the rate at which subunits are released from MTs and made available for reassembly may be important in subcellular isotype sorting (Murphy 1988). In neurons, beta III is expressed after beta II, at a time when the stable MT population has a high percentage of beta II isotype. The increased expression of beta III will result in the more dynamic MTs acquiring higher levels of beta III compared to the stable MT population. Once this sorting occurs, it will tend to be self-perpetuating, particularly if beta III interacts with MAPs with somewhat less efficiency than beta II (Carlier et al. 1984).

Until recently, all MTs were assumed to be composed of equal amounts of all available tubulin isoforms. No segregation of isoforms into any subset of MTs was observed (Lewis et al. 1987, Geuens et al. 1986). However, within the past year, four examples of subcellular tubulin sorting have emerged. In neurons of PC 12 cells, Class I, II and IV beta isotypes are preferentially incorporated into neurite MTs while Class III is preferentially found in the unpolymerized tubulin fraction (Joshi and Cleveland 1989). Also in PC 12 neurites, indirect immunofluorescence staining of Class III beta tubulin shows beta III is excluded from MTs in the soma of neurons but instead is present as a nonfilamentous, granular pattern. Beta III is present, however, in the polymerized MTs of axons (Asai and Remolona 1989). Another example of sorting can be seen in axonal MTs in

differentiating EC cells, where the levels of beta III are significantly raised in the axonal compartment designated as slow component b (SCb) (Denoulet et al. 1989). Finally, in squid axons, the colchicine stable MT population contains a beta tubulin isoform, $\beta_1$ and a high molecular weight MAP, axolinin, which are not present in the colchicine labile MT population (Arai and Matsumoto 1988).

The data presented here provide the first evidence that subcellular sorting of beta isotypes takes place during formation of stable MTs early in neural differentiation. The lack of this sorting in the stable MT population of cells differentiated along the muscle line suggests that this sorting as well as stability of MTs may arise from interaction of tubulin isoforms with brain-specific MAPs. The formation of stable MTs early in neural differentiation may be important for establishing and maintaining processes. Stable MTs are also thought to act as nucleating sites for insertion of new MTs along the neurite and may have an additional function to serve as "tracks" for guided transport of vesicles.

# CONCLUSIONS

The goal of this thesis is to examine the tubulin isoforms present in the stable MT array during neural differentiation. Stable MT arrays are present in both neurons and glial cells, where they contribute to maintainence of the elongated cellular processes and may also have additional functions. Tubulin isoforms, especially those which are developmentally regulated, may be involved in these functions, probably in coordination with specific MAPs.

To investigate the formation of the stable MT array, the pluripotent P19 EC culture system is employed. Two specific advanatges of this system are that the cells are uncommitted and therefore the earliest stages of neural induction and differentiation can be observed, and that two or more differentiation pathways, can be induced thereby allowing comparison of tubulin isotypes in neural and non-neural cell types.

The first two chapters of this thesis investigate the involvement of three isoforms of alpha tubulin: acetylated, tyrosinated and detyrosinated in formation and function of the initial stable MT array. The results show that the first changes in the isoform composition of MT arrays occur during the period of neural induction, within 24 hours after addition of RA, and include the appearance of an enlarged, colchicine stable, acetylated MT array. In

about 20 to 30% of these cells, there is formation of a tightly bundled array of stable MTs which contains all three alpha tubulin isoforms. MAP1B, but no other brain-specific MAPs, colocalizes to this array as well as to MTs that are not colchicine stable. This stable MT array is specific to cells undergoing neural induction and does not occur when EC cells are induced to differentiate along the muscle pathway. The function of this stable MT bundle is not known and in some cells, appears to be a transient array which disappears after about 24 hours. Although stable MT bundles are found in some cells 4 to 5 days after the initial addition of RA, we do not know if these bundles are reappearing in cells which previously displayed them or if they arise *de novo* in late differentiating cells. At the same time that this information was published (Falconer et al. 1989) another report appeared describing acetylated, colchicine stable MTs which are present in the early axonal processes of cerebellar macroneurons (Ferreira and Caceres 1989). The events described in this paper may be analogous to those reported here.

The formation of an increased acetylated MT array at 1 day RA, but not the formation of the stable MT bundle, may be an effect of RA which is unrelated to neural differentiation. Although not described in this thesis, a clone of the P19 cells, RAC D+, which does not differentiate into neurons in the presence of $10^{-6}$ M RA, was also examined. At 1 day RA the RAC D+ cells showed an increase in acetylated MTs similar to the increase shown by the P19 cells under the same conditions. However, these acetylated MTs were not resistant to colchicine and no bundled arrays were detected.

Approximately two days after addition of RA, there is extension of neural processes and the juvenile form of MAP2, MAP2C is expressed. At this time, an interdependent cytoskeletal complex is established which consists, at least, of acetylated MTs, MAP2C and vimentin intermediate filaments. This is a transient array and is suggested to play a role in the differentiation of neural stem cells. Stabilization of the process may allow interaction with extracellular signals which determine if the stem cell differentiates into a neuron or into a glial cell. This is the first time that such a complex has been demonstrated during neural differentiation.

Finally, after three or more days of RA, neuron maturation begins as shown by the appearance of high molecular weight MAP2 and tau. At this time tyrosinated alpha tubulin is no longer found in extended neurites, but is detyrosinated to form GLU MTs while acetylated alpha tubulin remains in all processes.

In the third chapter of the thesis, the involvement of beta tubulin isotypes in formation and maintenance of the neural stable MT array is investigated. As in the previous two parts, extensive use is made of antibodies recognizing tubulin isotypes.

The data in chapter 3 show that beta II but not beta III isotype is present in the stable MT bundle which is formed at 1 day RA. Beta III is a neuron specific isotype and appears first in a subset of cells at 2 days RA. Throughout neural differentiation, beta II is the major tubulin isotype in the neural stable MT array while Class III beta tubulin is partially excluded from

this array. Both indirect immunofluorescence microscopy and immunoblotting indicate that the preferential inclusion of beta II begins early in neural differentiation and continues throughout the 6 day period analyzed. This is the first indication that subcellular sorting of tubulin isotypes occurs in stable MT arrays early in neural differentiation.

In addition, this section shows that beta tubulin isotype profiles change as EC cells differentiate along either the neural or the muscle pathway. Muscle cells do not show the preferential incorporation of beta II into the stable MT array. Muscle cells also do not have the complement of brain-specific MAPs present in neuronally differentiated cells. Therefore, it is possible that subcellular sorting of beta II, as well as the increase in MT stability to colchicine depolymerization, depends upon association with brain-specific MAPs.

Colocalization of staining patterns in stable MTs indicates that the alpha tubulin which is paired with beta II is probably an acetylated, detyrosinated isoform. Recently, preferred combinations of alpha and beta tubulin isoforms in bovine brain have been examined *in vitro* by immunoaffinity chromatography (Luduena et al. 1989). Three different alpha isoforms were found, each in a preferred combination with a particular beta isotype. Beta II was preferentially paired with an acetylated alpha. This particular combination of tubulins may be an important factor in interaction with MAPs, explaining why beta II is the major isotype in stable MTs.

Alpha and beta tubulins have specific sites on the exterior of the tubulin molecule that interact with MAPs and in beta tubulin these sites overlap with residues which define the beta tubulin isotypes (Littauer et al. 1986, Maccioni et al. 1988, Sullivan 1988). Only beta II contains an aspartic acid within the MAP2 binding region which might be important in charge interactions with the basic MT binding region(s) of MAP2. Alpha tubulin also has specific sites for MAP2 and tau binding and there may be similar preferential interactions (Littauer et al. 1986, Maccioni et al. 1988, Sullivan 1988). Thus the particular isotype composition of the alpha:beta dimer probably influences the binding of MAPs which in turn influences such properties as stability.

During neural differentiation, the earliest indication of isoform preference in a stable MT array is seen at 1 day RA. At this time, beta II and acetylated alpha tubulin colocalize, indicating the probable polymerization of MTs with increased levels of acetylated alpha:beta II tubulin dimers. Also at this time, increased MAP 1B staining is found in the bundled MT array. This increase may result from charge interactions of MAP 1B with MTs containing increased amounts of the acetylated alpha:beta II dimer, thereby adding to the stability of the MT array.

At 2 days RA, the neuron specific isotype, beta III, is expressed only in a subpopulation of cells on top of the monolayer. MAP 2C is also expressed and shows a diffuse staining pattern in cells with stable MTs in differentiating cells. These two antibodies provide the first, certain

identification of cells differentiating into neurons. At this time, most of these cells have no processes.

The lack of short neurites in neuronally committed cells suggests that the hypothesis put forward in Chapter 1 of the thesis may need to be modified. This hypothesis states, in part, that the early stable MT bundle is the first evidence of a neuronal stable MT array and that this bundle extends to form short neurites. In this hypothesis, the stable MT bundle is important in establishing polarity of the neuron - extending, first to form a short process, and then to form a mature neurite. Establishment of a similar stable MT array which extends to form a neurite, is described in differentiating hippocampal neurons and cerebellar macroneurons (Dotti et al. 1988, Ferreira and Caceres 1989). However, there is a question as to when this stable bundle appears relative to the expression of beta III isotype.

During neurogenesis in hippocampal neurons, the axon is established by a sequence of three events (Dotti et al. 1988). There is initial formation of lamellipodia, followed by outgrowth of relatively stable minor processes which persist for days until one eventually is transformed into the axon. Similarly, in primary cultures of cerebellar macroneurons, the neuroblasts first extend lamellipodia, then display 4 or 5 short processes (Ferreira and Caceres 1989). Eventually one of these processes extends to form the axon. The cerebellar macroneurons have acetylated MT arrays similar to those in differentiating P19 cells. Newly explanted neuroblasts have only a small, centrosomal array of acetylated MTs. During differentiation, an enlarged

array of colchicine stable, acetylated MTs appears in only one of the multiple processes and this process subsequently extends to become the axon.

The P19 cells do not differentiate as isolated cells, therefore it is difficult to establish if multiple, short processes are present at 1 day RA. However, double staining with the beta III and YOL 1/34 antibodies shows that, at 2 days RA, most cells have numerous short processes but do not have a stable MT bundle. This brings into question the significance and the fate of the 1 day RA stable MT bundle which was initially thought to extend into a neurite.

There are several alternative hypotheses to describe the function and fate of the 1 day RA stable MT bundle:

a) The stable MT bundle is a transient array present in neuroblasts and plays some role in the migration of these cells to a position on top of the monolayer. Migrating neuroblasts in the avian forebrain have elongated processes (Alvarez-Buylla and Nottebohm 1988). While nothing is known about the MTs in these cells, the stabilization of a bundled array of MTs preceeding cell migration and oriented in the direction of cell migration has been demonstrated in 3T3 cells (Gundersen and Bulinski 1988).

b) The stable MT bundle is the first step in establishing polarity in a neural cell which is not yet committed to either neuronal or glial differentiation. The MT bundle stabilizes a process which may be important

in detecting signals that determine the subsequent differentiation path. This would be in agreement with results in chapter 2 of this thesis.

c) The stable MT bundle is present in cells which are differentiating along the glial pathway and the bundle extends to form radial glial processes. The differentiation of cells with processes which contain vimentin and beta II but not beta III indicates that glial differentiation may be taking place.

With the information currently available, I cannot determine the fate of the early stable MT bundle or the ultimate differentiation pathway of the cells which contain the bundle. New methods of research will be required to discriminate among these possibilities.

**Direction of future research**

The results of this thesis suggest two main avenues for future research: examination of the exact location and function of Class II beta tubulin isotype in the stable MT array, and determination of the role of the stage 1 stable MT bundle.

To determine if beta II is required for the stabilization of MTs, anti-sense oligodeoxyribonucleotides can be used to inhibit beta II synthesis. It will then be possible to determine if cells without beta II, or with very low levels of beta II, have stable MTs. Inhibition of beta II synthesis will also determine if beta II and/or a combination of beta II and MAP(s) is necessary for neurite outgrowth. Inhibition of axonal extension by tau antisense oligonucleotides has been recently demonstrated in primary cerebellar neurons in culture (Caceres and Kosik 1990).

To examine the fate of the early stable MT bundle and to determine the cell type in which it is found, microinjection of fluorochrome-labelled tubulin followed by time-lapse video microscopy should be undertaken. Cells with labelled MT arrays can be directly observed. This will show if there are short processes preceding the establishment of the stage 1 stable MT bundle. It will also establish if this bundle extends directly to form a neurite. Labelled cells can be fixed and stained with anti-beta III and/or anti-GFAP antibodies to discriminate between neuronal and glial processes.

In conclusion, this thesis provides the first examination of changes in the total and stable acetylated MT arrays which are associated with neural differentiation. It has established the existence of an interdependent network of cytoskeletal elements early in neural differentiation. It is suggested this process may play a role in determining the final lineage of a stem cell. This thesis also provides the first evidence that subcellular sorting of beta tubulin isotypes is involved in establishment of the stable MT array and occurs very early in neural differentiation.

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