MICROTUBULES AND BRAIN DEVELOPMENT

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1. Introduction

There has been considerable interest in the past few years in brain neurotubules: the concentration of microtubule protein is much higher in central nervous system cells than in other tissues [1]; neurotubules are components of the axon and are believed to play an important role in axon function and in brain differentiation [2]. Thus neurotubules and neurotubulin might be good markers of axon differentiation. Bamburg et al. [3] using a time-decay colchicine binding assay and quantitative gel electrophoresis have found that microtubule brain protein concentration increases approximately two-fold between 5–7 days and 13 days of development in chick embryo brain, then returning slowly to the initial value in the adult. However these results do not provide an answer to the following questions: is neurotubulin progressively assembled to neurotubules as soon as synthesized? In this case the rate limiting factor would be the amount of tubulin present in the brain at each stage of development. Another possibility would be that all the neurotubulin would be formed before a given period of development without undergoing massive polymerisation, a specific signal inducing, at a precise stage of development, its polymerisation to neurotubules followed by rapid axon growth. An attempt is made here to answer these questions.

2. Materials and methods

2.1. *Preparation of supernatants*

Sprague Dawley rats at different stages of development were killed by decapitation. The brains were removed, immediately chilled in the reassembly buffer of Weisenberg [4] containing 0.1 M 2-(*N*-morpholino) ethanesulphonic acid (MES), 1 mM EGTA, 1 mM GTP, 0.5 mM $MgCl_2$ (pH 6.5) at 4°C, and homogenized in the same buffer at 4°C (1 v/w). The homogenate was centrifuged at 105 000 *g* for 1 hr at 4°C. The supernatant was mixed with an equal volume of MES buffer containing 8 M glycerol to give a final concentration of 4 M glycerol. This supernatant was directly used for the colchicine binding assays and for the in vitro polymerisation kinetic studies.

2.2. *Purification of tubulin*

Tubulin was purified from this supernatant using the assembly–disassembly procedure described by Shelanski et al. [5].

2.3. *Colchicine binding assays*

Aliquots of supernatant, or of purified tubulin, were incubated for 2 hr at 37°C (final vol. 0.5 ml of Weisenberg's buffer) in the presence of 2.5×10^{-5} M colchicine labelled with 0.2 μCi of [^{3}H]colchicine per assay. Bound colchicine was measured according to the procedure described by Weisenberg [6]. Binding values thus obtained with different colchicine concentrations (10^{-6} M to 2.5×10^{-5} M) were plotted by Scatchard's method, which showed that saturation was obtained with 2.5×10^{-5} M colchicine.

2.4. *Turbidimetry measurements*

Turbidimetry measurements during tubulin polymerisation at 37°C were performed at 345 nm using a Gilford 200 Spectrophotometer with an automatic thermostated four sample changer.

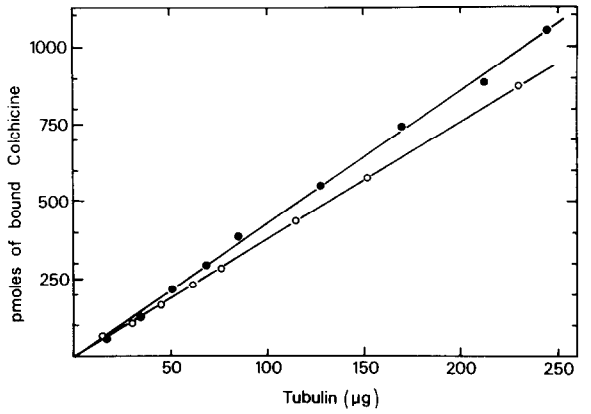

Fig. 1. Relationship between tubulin concentration and colchicine binding activity. Purified tubulin (10–250 μg) was incubated with [^{3}H]colchicine (2.5×10^{-5} M) in the conditions described in the Materials and methods section. Binding activity of tubulin prepared from 9-day old rat brain (○–○) and from 30-day old rat brain (●–●).

3. Results and discussion

Previous publications have shown that the colchicine binding activity of microtubule protein may be used to determine the concentration of neurotubulin [7,8]. However, in the usual buffers the colchicine binding activity decays in an apparent first order manner [9]; a number of factors (pH, ionic strength, temperature and microtubule protein concentration) markedly affect the decay rate [10]. Thus in these conditions the values obtained for colchicine binding must be corrected for the decay. In this work colchicine binding assays were performed in the medium described by Weisenberg [4] or Shelanski [5], which allows in vitro polymerisation of tubulin to microtubules. In these media the rate of decay of colchicine binding activity was very slow (about 4 days) and the binding capacity was dependent only on the concentration of microtubule protein. Fig.1 shows that the relationship between the amount of purified [4] brain microtubule protein and colchicine binding was linear. Using this assay the content of colchicine binding tubulin during brain development was determined. Fig.2 depicts the results obtained and shows that 6 days before birth rat brain contains as much colchicine binding tubulin as 30-day old rat brain. From this fetal stage till 9–10 days after birth the colchicine binding activity markedly increases and then decreases gradually to half of the 9–10 days value in the adult. However, further examination of fig.1 revealed that not all the tubulin protein present in the microtubules isolated from rat brain was actually estimated by the colchicine binding assay. Assuming that one mole of tubulin binds one mole of colchicine [11] it is clear from fig.1 that only 45%–50% of the molecules of neurotubulin present in the assay were able to bind colchicine. Kirshner et al. [12] have recently found that tubulin is present in brain in two forms: one of these forms (X) binds colchicine but is unable to polymerise in vitro to microtubules; the second form (Y) binds colchicine very poorly, copolymerises in vitro with the X form and is present as 36 S structures in the depolymerised preparation. In the electron microscope 36 S tubulin appears as discs or spirals. These structures might be nucleating centers [13] in the polymerisation process. Thus it may be assumed that neurotubulin present in the preparation which was not evaluated by the colchicine binding assay was at least partly in the Y form i.e. a nucleating, initiator form. In vitro polymerisation experiments were therefore performed in order to see if the initiation and initial rate of tubulin polymerisation vary during development. Crude brain soluble fractions were prepared in the polymerisation medium

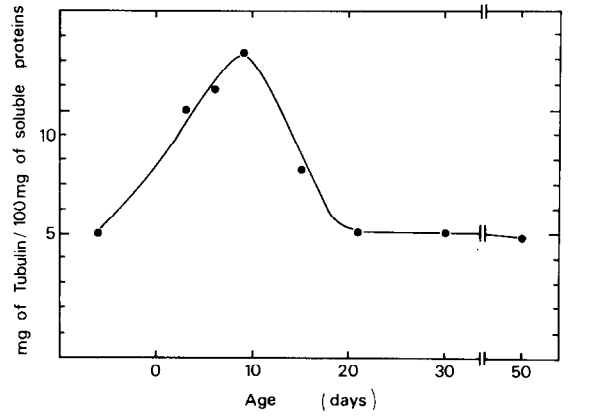

Fig. 2. Changes in colchicine binding capacity in supernatant fractions of rat brain at different stages of development. Aliquots of the different 105 000 *g* supernatants obtained as described in the Materials and methods section, were incubated in the conditions described in the fig.1 for purified tubulin. For each age it was acertained that linearity was obtained between colchicine binding activity and protein concentration.

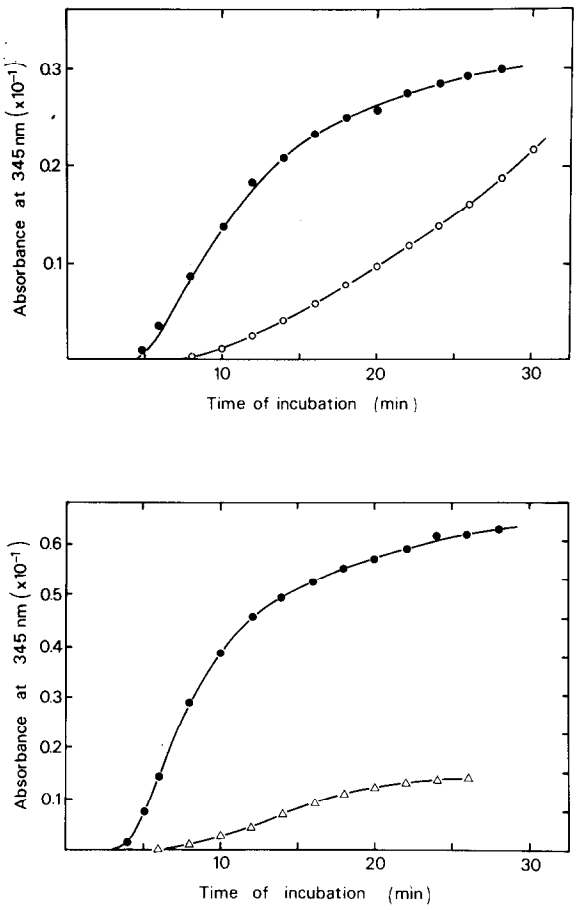

Fig.3. Turbidimetry time course at 37°C. The measurements were performed in the conditions described by Gaskin et al. [12] at 37°C and at 345 nm. In fig.3a, 30-day (●—●) and 9-day (○—○) old rat brain supernatants were compared; an identical amount of colchicine binding activity was used for the two ages (1177 pmol). In fig.3b, 30-day old (●—●) and fetal (15 days of gestation) rat brain supernatants, (△—△) were compared (2100 pmol of colchicine binding activity).

described by Shelanski [5] from brain of different ages and then incubated at 37°C. Fig.3a depicts the tissue dependence of turbidimetry for assembling tubulin in the supernatant from 30-day and 9-day old rat brains. Identical amounts of colchicine binding neurotubulin were used for the two ages. Fig.3a clearly shows that both the initial rate and maximal turbidimetry after 30 min of incubation were lower for the 9 day old preparation. In addition the lag period for polymerisation was longer; Gaskin et al. [13] have shown that both the lag period and the initial rate of polymerisation probably depend on the amount of initiator present; addition of small amounts of sonicated fragments of microtubules to tubulin preparations resulted in a shorter lag period and in an overall increase in the rate of assembly; when sufficient quantities of tubule fragments were added these authors noted that the rate of polymerisation approached a saturation value. These data suggested that initiation of the polymerisation process is governed by a specialised initiator (which might be the Y form [12]). Thus it may be assumed that the soluble fraction from adult brain contains a higher proportion of the initiator form of tubulin than the preparations obtained from younger rats. Gaskin et al. have also studied the effect of protein concentration on equilibrium turbidimetry; they found that below a certain concentration (critical concentration C_c) turbidimetry is the same as for the unpolymerized protein. From different kinetic experiments performed with several dilutions of the brain soluble fraction prepared at different ages, initial rates were calculated and plotted as a function of the colchicine binding. Fig.4 shows that with the 30-day old brain soluble fraction

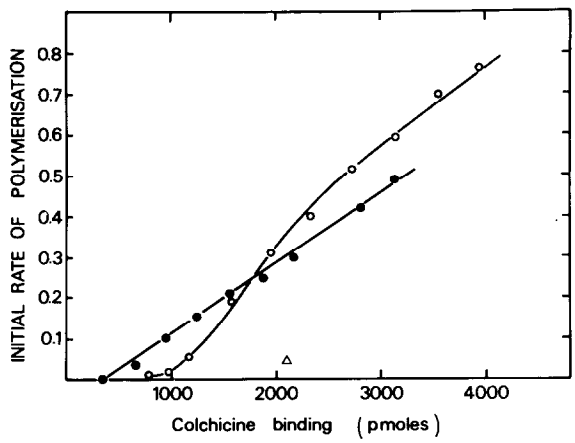

Fig. 4. Relationship between initial rates of polymerisation and colchicine binding activity. Initial rates [(△ absorbance at 345 nm/5 min) × 10^{-1}] were deduced from several kinetic experiments performed as described in fig.3 with various dilutions of the supernatants. Supernatants from 30-day (●—●), and 9-day (○—○) old rat brains. Initial rate for undiluted fetal supernatant (2100 pmol of colchicine binding activity) is also represented (△).

the apparent value of C_c at 37°C was in the range found by Gaskin et al. At 9 days the apparent C_c was three times higher than for the 30-day old fraction. These results suggest again that the amount of initiator is limiting in the young brain preparations. In addition fig.4 shows that the relationship between initial rates of polymerisation and the colchicine binding activity was linear for the adult supernatant but sigmoid for the 9 days old preparation. Thus, although the relative concentration of initiator is likely to be lower in the young brain the efficiency of polymerisation seems better as soon as the tubulin concentration is increased. To appreciate the meaning of these findings it is important to know the actual concentration of free tubulin in the brain at different stages of development.

Finally, at a very early stage, 15 days of gestation, tubulin polymerized very slowly (fig.3b); the lag was very long and the initial rate very slow; C_c at this stage was approximately 6–7 times higher than for the 30-day old preparation. Thus fetal tubulin, although its concentration is not limiting when compared to that measured 30 days after birth (fig.2), seems unable to undergo in vitro polymerisation efficiently (fig.3b). These results therefore suggest that in the process of neurotubule formation the concentration of colchicine binding tubulin is not a limiting factor. They rather favour the second hypothesis i.e. a specific signal induces at a precise stage of development neurotubule polymerisation from a pre-existing pool of neurotubulin.

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