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Some Biochemical Changes of Growth Cones During Synaptogenesis of Rat Brain

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The formation, maturation and specialization of the synaptic membrane are reflected by the changes occurring in the lipid and protein composition of growth cones. Here we present data obtained during our studies on the changes occurring in the free amino acid and free fatty acid pools, as well as in the content of tubulin and in the content and composition of τ-proteins in growth cones during synaptogenesis. All of the studied moieties are important factors partaking in a number of vital developmental processes.

Key words: synaptogenesis, free amino acids, free fatty acids, microtubulin, τ-protein.

Introduction

During the past years great progress has been made in the study of the morphological and biochemical basis of synaptogenesis. The studies by Tennyson [15], Bunge [3], Fox et al. [4], Knyihar-Csillic et al. [11] were carried out on structures, actively participating in outgrowth of neurites and in the formation of the synaptic complex.

The mechanisms governing the development of the specific components of the synaptic structures are not clear (see Blue and Parnavelas [2]). The studies by Surchev et al. [13] revealed changes of integral membrane proteins, cholesterol, carbohydrate residues and anionic sites in the growth cones (GC) during the early postnatal period - 1 to 15 postnatal days (PD).

The reports by Gonatas et al. [7], Gordon-Weeks and Lockerbie [8], Venkov et al. [17] describe the first methods for isolation of enriched fraction of growth cones. The enriched fractions are important tools for studying of the quantitative changes of the biochemical composition of GC during synaptogenesis.

We report here that during synaptogenesis there are biochemical cascades of changes in the pools of free amino acids (FAA), free fatty acids (FFA), tubulin and τ-protein.

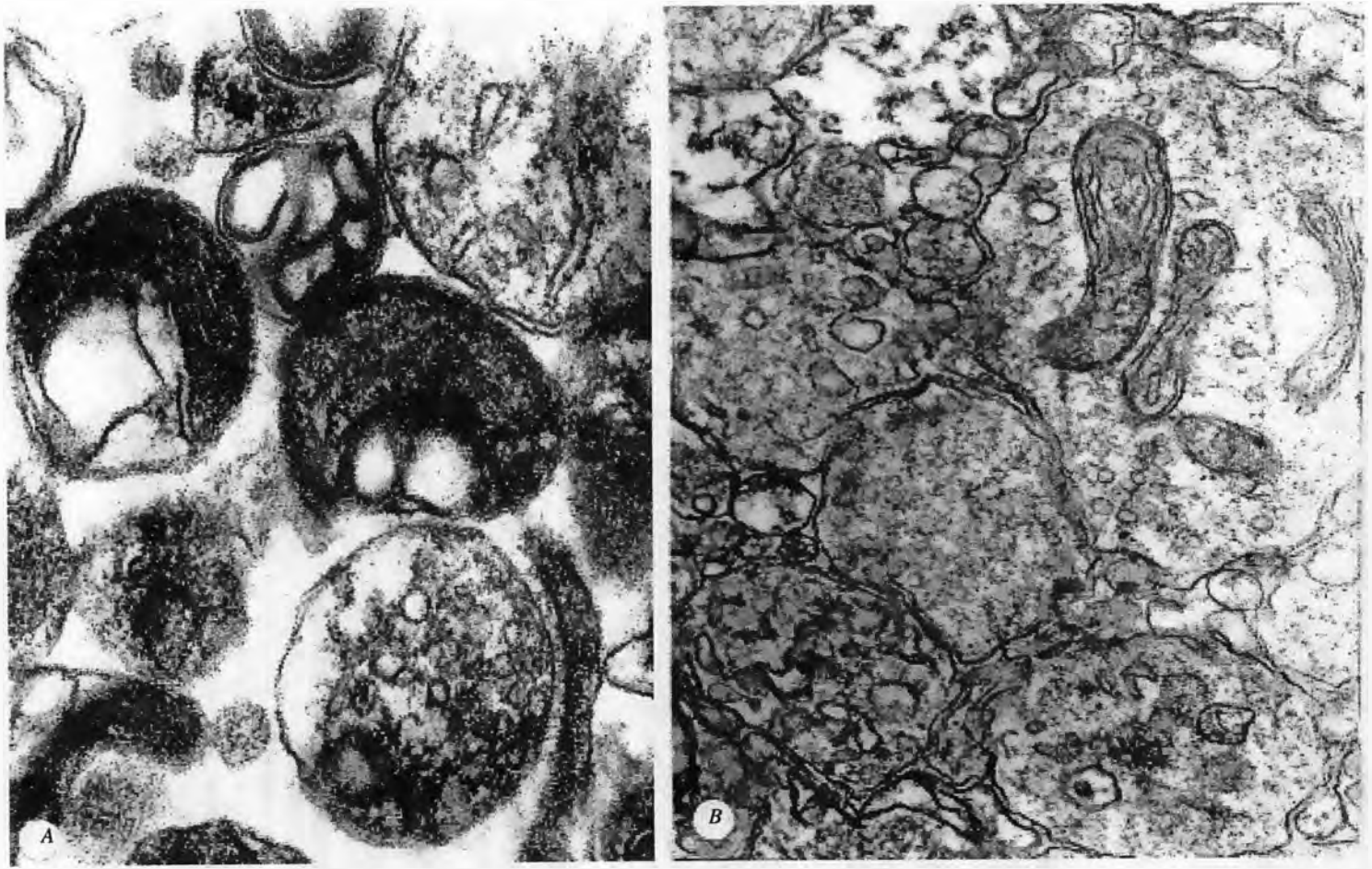

Fig. 1. Electron micrographs of growth cones (*A*) and synaptosomes (*B*)

Materials and Methods

Animals

Wistar albino rats aged 1 to 15 days were used in this study. Ten to 15 brains were used for each experiment. The forebrains were dissected and then processed for isolation of GC and synaptosomal (Sy) fractions.

Isolation of growth cones and synaptosomes

GC enriched fractions were prepared from the forebrains 1 to 10 day-old rats, essentially as described in [17]. 10 to 15 animals, depending on their age were used in a typical experiment. The brains were homogenized in 8 cm^3 of 0.32 M sucrose/g tissue, 2 mM HEPES, pH 7.5, and the homogenate was centrifuged at 1200g for 5 min. The pellet was washed with the same volume of buffer and the supernatants were combined. The combined supernatant was centrifuged at 13 300g for 15 min and the resulting pellet was resuspended in 4 cm^3 HEPES per 6 brains. 6 cm^3 samples were layered over 54 cm^3 7% Ficoll and spun at 30 400g for 20 min in a 3x60 MSE swing-out rotor. The interphase between the sample and 7% Ficoll was collected and made up to 30 cm^3 with buffer and 14% Ficoll. 30 cm^3 of the resulting mixture were layered on top of a 30 cm^3 glycerol cushion and after centrifugation at 18 000g for 60 min in a 3x60 swing-out rotor the Ficoll phase was collected in three equal parts, mixed with three volumes of Krebs solution and centrifuged at 30 000g for 20 min to yield the final GC fraction (Fig. 1 – A).

Sy were isolated by the method described by V e n k o v and V e l i c h k o v a [16] from the forebrains of 15-day-old rats (Fig. 1– B).

Determination of free amino acids

FAA were extracted from GC- and Sy-fractions in chloroform-methanol-water (65:25:4 v/v) according to K a t e s [10]. Stable esters of the amino acids contained in the water-soluble phase were obtained as described by G e r h k e [6]. After concentration and subsequent silanization the extracts were analyzed by gas chromatography employing the method of F u l e r et al. [5].

Determination of free fatty acids

FFA were extracted according to K a t e s [10]. The methyl esters of the extracted FFA were purified by two passes through an exchange resin (IRA-400, Fluka). The volume of the extract was reduced to 1 cm^3 under a stream of nitrogen. Samples from the concentrated extracts were analyzed by gas chromatography.

Electrophoresis and blotting of tubulin and τ-protein

The qualitative and quantitative changes of these cytoskeletal proteins were studied by isoelectric focusing - S v e n s s o n [14], SDS-polyacrylamide electrophoresis according to L a e m m l i [12], immunoblotting and quantitative densitometry.

Results and Discussion

Free amino acids

There is subcellular compartmentation of a number of amino acids – alanine, proline, phenylalanine and arginine in addition to the compartmentation of the amino acid transmitters glutamine (GLU) and glycine (GLY), as illustrated in Fig. 2.

Three types of developmental changes were found in the FAA: 1. Peak values at PD3 and PD7 - cysteine (CYS), tyrosine (TYR), lysine (LYS), histidine (HIS), aspargine (ASN), tryptophan (TRY), methionine (MET); 2. Peak values at PD3 alone - aspargine (ASP), arginine (ARG), serotonin (SER), valine (VAL), leucine (LEU); 3. Fluctuation of some FAA - glutathione (GLU), proline (PRO), alanine (ALA), glutamine (GLN), phenylalanine (PHE), glycine (GLY), as shown in Fig. 3.

The content of the nonpolar ALA in GC is high, which might reflect the increased synthesis of membrane proteins with hydrophobic domains in all probability important for the fine specialization of the synaptic regions. The peak at PD3 coincides with the cessation of neuronal cell proliferation and the appearance of glial cells. The changes of FAA at PD7 coincide with the period of most active division of glial cells and highest rates of brain weight and DNA-content increase (Table 1).

Free fatty acids

During the early postnatal periods (up to PD 6) the amount of short chain saturated FFA decreases. The quantity of long chain C-18:0 and the long chain unsaturated C-20:4

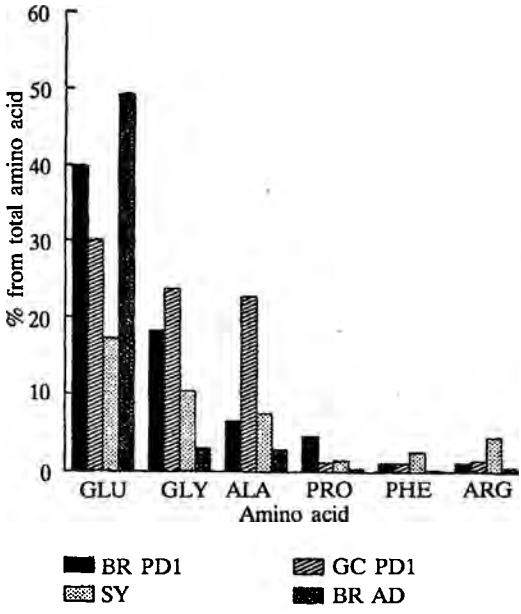

Fig. 2. Compartmentation of FAA in rat brain. The content of the individual FAA is calculated as % from the total. BR PD1 – whole rat brain at postnatal day 1; GC PD1 – growth cones from PD1 rat brain; SY – synaptosomes isolated from adult rat brain; BR AD – whole brain of adult rat

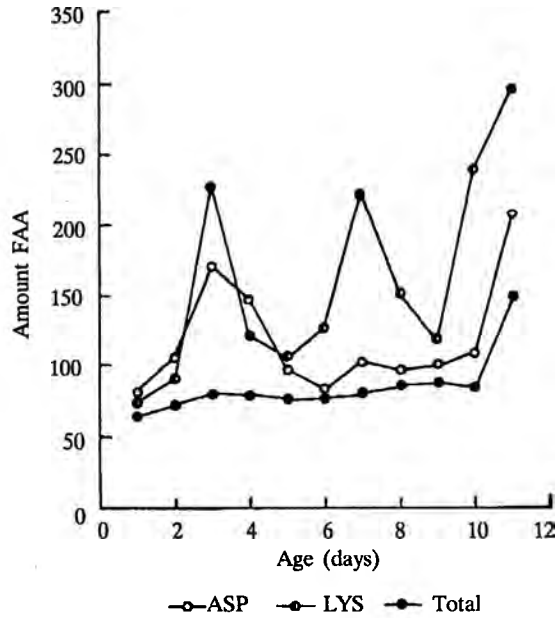

Fig. 3. Typical changes of FAA in growth cones from developing rat brain. Examples of the general types of developmental changes of individual FAA are observed in the FAA-pool of growth cones. For a more comprehensive representation the amounts of the shown FAA were scaled as follows: Lys × 10; Total × 10^{-1}

Table 1. Changes of the amounts of FAA in growth cones during early postnatal development

FAA/day	1	2	3	4	5	6	7	8	9	10	Sy
Ala	107.8 ±2.4	90.5 ±2.0	56.6 ±1.7	58.8 ±1.3	64.6 ±1.2	36.7 ±1.0	40.2 ±1.2	56.7 ±1.2	76.6 ±1.4	65.3 ±1.6	103.1 ±2.1
Gly	112.3 ±2.5	97.3 ±2.0	102.6 ±2.1	91.6 ±2.1	71.8 ±1.9	59.3 ±2.0	68.4 ±1.8	45.9 ±1.0	28.5 ±0.8	81.4 ±1.4	144.5 ±2.3
Val	7.3 ±1.6	7.9 ±0.4	18.1 ±1.0	10.5 ±1.0	6.6 ±0.7	8.5 ±0.4	11.1 ±0.9	11.5 ±0.6	11.0 ±0.7	25.5 ±1.0	28.8 ±0.9
Thr	3.7 ±0.1	3.9 ±0.1	18.8 ±0.8	6.2 ±0.2	5.1 ±0.2	12.3 ±0.6	23.1 ±0.8	13.9 ±0.7	18.9 ±0.4	18.6 ±0.6	20.5 ±1.0
Ser	115.9 ±1.1	3.4 ±0.1	78.2 ±1.7	53.2 ±1.4	23.7 ±0.9	39.3 ±1.1	55.3 ±1.1	51.5 ±0.9	44.9 ±0.9	45.6 ±0.6	94.1 ±1.2
Leu	5.2 ±0.2	4.4 ±0.1	11.9 ±0.4	10.6 ±0.5	8.5 ±0.4	9.5 ±0.3	9.8 ±0.6	8.5 ±0.4	12.1 ±0.7	17.3 ±0.7	28.8 ±0.9
Ile	–	4.1 ±0.1	–	–	4.3 ±0.1	7.3 ±0.2	6.9 ±0.1	6.5 ±0.2	12.7 ±0.4	8.2 ±0.2	15.9 ±0.5
Pro	5.5 ±0.1	7.1 ±0.2	12.3 ±0.4	9.7 ±0.3	11.1 ±0.3	9.2 ±0.2	15.2 ±0.6	7.9 ±0.2	11.9 ±0.4	12.1 ±0.5	20.1 ±0.7
Cys	3.4 ±0.1	3.8 ±0.1	8.1 ±0.2	7.4 ±0.2	6.1 ±0.2	16.1 ±0.6	22.7 ±0.8	3.3 ±0.1	21.1 ±0.7	15.9 ±0.7	19.0 ±0.6
Met	3.8 ±0.1	4.6 ±0.1	14.6 ±0.5	9.4 ±0.3	5.7 ±0.2	7.1 ±0.2	11.2 ±0.4	4.3 ±0.1	11.7 ±0.6	8.2 ±0.3	14.7 ±0.7
Asn	5.7 ±0.1	6.6 ±0.1	18.2 ±0.5	7.1 ±0.2	6.1 ±0.3	8.6 ±0.3	10.8 ±0.4	6.6 ±0.2	11.6 ±0.3	20.0 ±0.1	59.1 ±2.0
Asp	80.7 ±1.7	106.4 ±2.0	169.6 ±2.5	146.4 ±2.7	97.3 ±1.3	83.1 ±1.4	102.8 ±2.1	97.3 ±1.6	99.7 ±1.6	109.4 ±1.7	205.5 ±2.4
Phe	4.9 ±0.1	6.2 ±0.1	12.3 ±0.4	11.4 ±0.4	12.2 ±0.4	17.7 ±0.6	10.6 ±0.4	14.4 ±0.5	12.7 ±0.6	28.8 ±0.8	33.9 ±0.6
Gln	102.8 ±1.9	131.1 ±2.4	92.9 ±1.3	106.3 ±1.2	129.4 ±1.5	124.1 ±1.4	151.5 ±1.9	127.0 ±2.0	180.0 ±2.4	186.9 ±2.5	292.0 ±2.9
Glu	142.2 ±1.6	206.1 ±2.5	91.2 ±1.0	205.1 ±2.0	253.7 ±2.7	292.5 ±2.6	185.2 ±2.1	337.8 ±3.4	240.3 ±2.5	121.1 ±1.9	242.0 ±3.2
Lys	7.5 ±0.2	9.1 ±0.3	22.8 ±0.5	12.1 ±1.7	10.5 ±0.4	12.6 ±0.7	22.2 ±0.7	15.1 ±0.6	11.9 ±0.4	23.9 ±0.8	45.2 ±1.0
Tyr	4.1 ±0.3	11.5 ±0.4	25.5 ±0.6	17.6 ±0.5	14.9 ±0.3	13.9 ±0.4	29.4 ±0.6	12.2 ±0.4	22.4 ±0.7	20.0 ±0.7	20.3 ±0.8
Arg	6.8 ±0.4	6.6 ±0.4	14.1 ±0.8	8.3 ±0.3	7.2 ±0.2	6.1 ±0.2	7.6 ±0.2	9.2 ±0.3	10.3 ±0.1	17.1 ±0.5	61.7 ±0.9
His	3.9 ±0.4	4.3 ±0.3	9.2 ±0.5	7.4 ±0.3	6.0 ±0.3	4.8 ±0.1	9.0 ±0.1	7.6 ±0.4	12.8 ±0.7	19.7 ±1.0	29.9 ±1.1
Try	3.9 ±0.4	4.7 ±0.3	16.7 ±0.9	6.5 ±0.3	12.3 ±0.7	14.2 ±0.6	21.1 ±0.4	6.5 ±0.4	22.0 ±1.0	11.5 ±0.6	21.4 ±1.3
Total	637.9	715.5	793.7	785.6	752.8	775.6	796.4	837.2	860.4	843.3	1484.6

FFA increased throughout the studied period (Fig. 4). After PD DIED 6 a number of long chain unsaturated FFA become evident (Table 2).

It can be suggested that the changes, occurring in the composition of the FFA-pool of growth cones, reflect to a considerable extent the processes of growth and maturation of the synaptical neuronal membrane, as well as the formation of specialized structures, namely the synapses.

Tubulin and τ-protein

The pronounced increase of tubulin content after PD6 coincides with the period of both glia proliferation and the outgrowth of neuronal processes. During this period microtu-

Table 2. Changes of the amounts of FFA in growth cones during early postnatal development (mg FFA/g dry tot. lip. extr. × 10^{-8})

FFA/day	1	2	3	4	5	6	7	8	9	10	Sy
$C_{14:0}$	39.9 ±1.3	22.2 ±1.1	6.7 ±0.3	9.8 ±0.3	8.9 ±0.4	8.7 ±0.2	6.9 ±0.2	–	–	8.7 ±0.3	13.3 ±0.3
$C_{16:0}$	65.4 ±1.5	46.1 ±1.4	39.5 ±1.1	40.8 ±1.4	36.4 ±0.8	60.0 ±1.0	187.0 ±2.5	48.0 ±1.1	68.7 ±1.1	66.7 ±1.1	84.5 ±1.3
$C_{16:1}$	–	–	–	–	7.8 ±0.4	9.4 ±0.2	0.5 ±0.1	6.5 ±0.1	9.7 ±0.2	9.3 ±0.2	–
$C_{18:0}$	10.4 ±0.9	70.9 ±2.0	56.9 ±1.7	47.3 ±1.1	50.4 ±1.2	110.3 ±2.0	43.0 ±0.3	74.2 ±1.8	67.0 ±1.0	104.6 ±1.8	161.9 ±2.4
$C_{18:1}$	68.7 ±2.0	89.5 ±2.0	65.9 ±1.7	82.8 ±2.0	106.6 ±2.4	102.4 ±1.9	46.8 ±0.3	82.2 ±1.5	115.0 ±2.0	161.6 ±2.1	255.0 ±3.3
$C_{18:2}$	79.8 ±1.9	84.5 ±2.1	58.0 ±1.5	55.8 ±1.3	39.2 ±1.2	55.6 ±0.7	7.8 ±0.1	70.3 ±1.4	19.8 ±0.8	33.9 ±0.9	49.5 ±1.0
$C_{18:3}$	–	–	–	–	–	–	–	–	–	–	12.4 ±0.4
$C_{20:0}$	–	5.4 ±0.3	6.8 ±0.3	8.1 ±0.4	6.4 ±0.1	4.9 ±0.1	10.7 ±0.2	9.1 ±0.2	4.3 ±0.2	9.1 ±0.3	20.4 ±0.3
$C_{20:2}$	–	–	4.7 ±0.2	–	5.1 ±0.1	4.3 ±0.1	–	12.7 ±0.3	6.2 ±0.1	10.4 ±0.2	7.8 ±0.2
$C_{20:4}$	5.3 ±0.3	8.2 ±0.2	19.4 ±0.7	21.3 ±0.7	26.8 ±0.7	32.3 ±0.4	32.5 ±0.4	40.0 ±1.0	30.9 ±1.0	30.7 ±1.0	54.2 ±1.0
$C_{20:5}$	–	–	–	–	–	–	–	–	–	–	18.0 ±0.3
$C_{22:0}$	–	–	–	–	5.9 ±0.2	7.4 ±0.1	7.9 ±0.1	4.8 ±0.1	5.3 ±0.1	–	8.6 ±0.2
$C_{22:2}$	–	–	–	–	–	–	–	–	–	–	25.1 ±0.4
$C_{22:4}$	–	–	–	–	–	12.3 ±0.2	3.8 ±0.1	–	8.1 ±0.1	15.1 ±0.2	10.2 ±0.2
$C_{22:6}$	–	–	–	–	–	–	–	–	–	–	13.9 ±0.2
Total	269.5	326.0	257.9	265.9	293.5	407.6	381.6	347.0	335.0	457.0	734.8

bules furnish a multitude of functions – from serving as a mechanical backbone of the developing cellular skeleton to determining and maintaining the order of the intracellular events, needed for the upkeep and extension of neurites. During these stages the tubulin pool consists of dynamic subpopulations required to satisfy the need for relatively fast alterations of the length, stability and the function(s) of microtubules in the growing neuronal tip [9]. The lower tubulin content in the mature synapses (synaptosomes) reflect a poorer microtubulin pool, serving the major purpose of maintaining the stability of existing microtubules with more clearly defined function (Fig. 5).

From the immunoblots it becomes evident that until PD2 the two τ-isoforms with lower molecular mass are equally represented. Traces of the two lower molecular mass isoforms appear on the blot on PD5 and can be resolved already on PD6. Since in whole brain homogenates the adult τ-pattern (four major bands) becomes evident only on PD21 it can be suggested that the higher molecular mass τ-forms are characteristic for the specialized functions, acquired by neurons at later stages of development. The specialized functions of the two higher molecular mass τ-isoforms may well be related to the functioning of the synapse.

The formation and restructuring of the specialized morphological elements building up the central nervous system are developmental processes, which are characterized by their specific biochemical dynamics. The synaptic regions formed on differen-

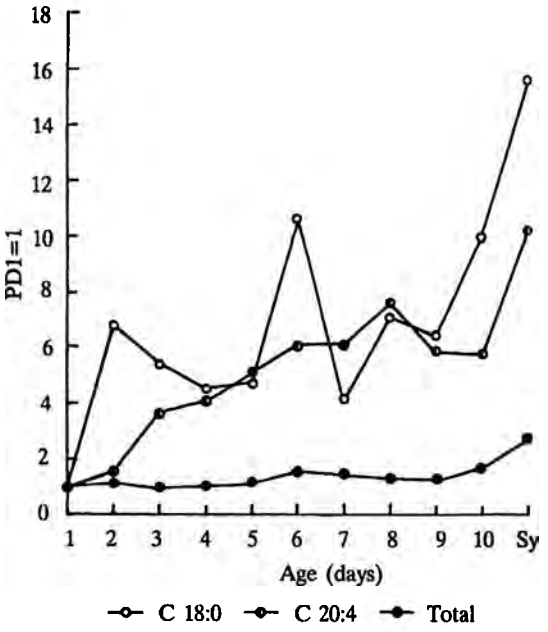

Fig. 4. Free fatty acids in growth cones from developing rat brain. The age of the animals is given in postnatal days; Sy – synaptosomes; the amount of FFA on PD1 is taken as 100% and the amounts on subsequent days are calculated therefrom

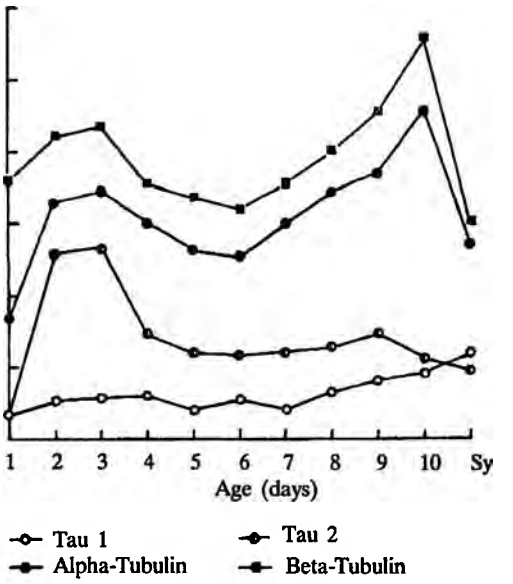

Fig. 5. Changes of α- and β-tubulin and τ-protein in GC during development. GC proteins were separated in 7% PAA slabs and stained with Coomassie Brilliant Blue R250, followed by quantitative densitometry

tiating neuronal membranes consist of numerous biochemical components, which change specifically during postnatal development (see e.g. [1]). The changes occurring in the composition of the free amino and free fatty acid pools, as well as in the content of tubulin and composition of τ-proteins reflect to a considerable extent the processes of growth and maturation of the neuronal membrane, as well as the formation of specialized structures, namely the synapse.

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