Malnutrition increases neurofilament subunits concentration in rat cerebellum

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We reported previously that the expression of neurofilaments in rat cerebral cortex is sensitive to early malnutrition. Different cerebral structures respond differently to the same stimulus, therefore we undertook the present study to determine whether early malnutrition affects the expression and in vitro incorporation of ^{32}P in neurofilament subunits from rat cerebellum. Wistar rats were fed a normal protein (25% casein) or an isoenergetic low protein (8% casein) diet from the day of parturition to the day pups were weaned. Some litters were sacrificed at weaning; others (both normal and malnourished animals) received a 25% protein diet until day 90, when they were sacrificed. Intermediate filament preparations were obtained by extraction of the tissue with a high salt phosphate-buffered solution containing 1% Triton X-100. The pellet containing the bulk of neurofilament proteins identified as 200, 150, and 68 kDa subunits was scanned following one-dimensional gel electrophoresis. In vitro ^{32}P incorporation was measured in cytoskeletal proteins obtained by the method of axonal flotation. Total protein concentration in the cerebellum was not altered with malnutrition. Conversely, the concentration of the 200 and 150 kDa subunits increased in young rats, but this effect was absent in adults. However, in vitro ^{32}P incorporation into cytoskeletal proteins was not altered in malnourished animals. Early malnutrition seems to interfere with mechanisms that regulate neurofilament formation during the period of maximal cerebellum development. However, this effect was absent after the resumption of a normal diet, suggesting transient alterations probably not related to in vitro ^{32}P incorporation. (J. Nutr. Biochem. 4:644–650, 1993.)

Keywords: malnutrition; neurofilament; cerebellum; rat.

Introduction

In animals such as rodents, the cerebellum is immature at birth and its histogenesis and morphogenesis mainly occur during early postnatal life.[1] Nutritional deficiency appears to be particularly harmful when it occurs during phases of maximal rate development of the central nervous system (CNS).[1] Experimental undernutrition in lactating rats can be produced by different methods,[2] but the alterations observed strongly indicate that the crucial factor is not the method for imposing malnutrition, but the time when malnutrition is imposed.[1]

The cytoskeleton of all cells is formed by microtubules, intermediate filaments, and microfilaments, which are ultrastructurally distinct elements consisting of fibrous macromolecules.[3]

The neuronal intermediate filaments, also termed neurofilaments, are distinct from the intermediate filaments found in other cells. They are composed of three proteins with molecular weights of 200, 150, and 68 kDa, weights referred to as NF-H, NF-M and NF-L, respectively.[4] It has been demonstrated that NF-L constitutes the core of the filament, while NF-M and NF-H are peripherally distributed[5] and participate in the interactions between neurofilaments and other cytoskeletal elements.

Despite their poor solubility properties in physiological buffers, neurofilaments are dynamic structures. In the neuron, neurofilaments participate in events such as axonal extension or regeneration by assembly and disassembly.[6] Furthermore, neurofilaments function as a support for nerve axons.[7] They determine axonal caliber[7] and participate in the axonal transport of metabolites from cell bodies to synapses.[8]

Neurofilaments are among the most highly phosphorylated neuronal proteins.[9] Neurofilament subunits con-

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tain different numbers of phosphate residues[10] and are phosphorylated in vivo and in vitro by various protein kinases.[11] The role of phosphorylation of NF subunits is still unclear, but it has been suggested that this post-translational modification may regulate their role and interactions with other cytoskeletal proteins within the neuron.[10]

Alterations in neurofilaments are recognized as being of importance in several pathological disorders, including Alzheimer's disease,[12] experimentally induced diabetes,[13] and experimentally induced phenylketonuria.[14] Neurotoxins such as aluminum and B,B'-iminodipropionitrile (IDPN)[15] alter neurofilament phosphorylation. In addition, we have reported previously that postnatal malnutrition imposed during the period of brain growth spurt increased the expression of NF-L and NF-M proteins in the cerebral cortex of adult rats.[16]

These findings indicate that neurofilaments are sensitive to external factors. In view of our previous report showing that the expression of neurofilaments in the rat cerebral cortex is sensitive to early malnutrition and considering that different cerebral structures respond differently to the same stimulus,[1] we undertook the present study to determine whether malnutrition affects protein concentration and phosphorylation level of neurofilament subunits in the cerebellum of young and adult rats. High salt-insoluble fractions were prepared from normal and malnourished animals. Cytoskeletal proteins obtained from myelinated axons were phosphorylated in vitro by incubation with [γ^{32}P] ATP. The results demonstrated a marked increase in NF-M and NF-H subunits in young rats that was absent in adult animals. However, in vitro phosphorylation of cytoskeletal proteins was not altered as a consequence of the nutritional restriction imposed during the brain growth spurt period.

Methods and materials

Animals and diets

Pregnant Wistar rats from our breeding colony were maintained in individual cages. Diets containing 25% protein (control group) or 8% protein (malnourished group) *(Table 1)* were offered in pellets to the dams starting on the day the pups were born. Food intake in week 3 was significantly greater in well-nourished than in malnourished dams.[16] On the day of birth the litter size was culled to eight pups (males and females). Litters smaller than eight pups were not included in the experiments. At weaning (day 21), some litters were sacrificed; other control and malnourished rats received the 25% protein diet until day 90, when all animals were sacrificed. The animals were weighed at 21 and 90 days of age.

Preparation of neurofilament proteins for the quantitative study

The preparation of total neurofilament protein from cerebellum of 21- or 90-day-old rats for the quantitative study was performed in a high-salt buffer containing 5 mM KH_2PO_4 (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM ethylene glycol-bis (B-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM EDTA, and 1% Triton X-100, as described previously.[16]

Table 1 Composition of diets

Components	25% Casein diet	8% Casein diet
	g/100 g diet	
Casein*	28.7	9.2
Soybean oil	15.0	15.0
Corn starch	50.15	69.65
Salt mix†	4.0	4.0
Vitamin mix‡	1.0	1.0
Non-nutritive fiber	1.0	1.0
	Kj/100 g diet	
Energy	18	18

The control group consisted of rats whose dams received a 25% protein diet during lactation. The malnourished group consisted of rats whose dams received an 8%-protein diet during lactation. Diets were supplemented with 0.15% L-methionine from Merck (Rio de Janeiro, RJ, Brasil)

*From B. Herzog (São Paulo, SP, Brasil). Casein contains 87% protein.

†From Roche (São Paulo, SP, Brasil). Composition (mg/Kg diet): NaCl, 5570; KI, 32: KH_2PO_4, 15.560; $MgSO_4$, 2290; $CaCO_3$, 15.260; $FeSO_4.7H_2O$, 1080; $MnSO_4.7H_2O$, 160; $ZnSO_4.7H_2O$, 22; $CuSO_4.5H_2O$, 19; $CoCl_2.6H_2O$, 0.9.

‡From Roche. Composition (mg/Kg of diet): retinyl acetate, 6; cholecalciferol, 0.5; all-rac-α-tocoferol, 100; menadione, 5; choline, 1478; p-aminobenzoic acid, 100; inositol, 100; riboflavin, 8; thiamin.HCl, 5; pyridoxine.HCl, 5; folic acid, 2; biotin, 0.4; vitamin B-12, 0.03; niacin, 40; Ca D-pantothenate, 40.

This method was based on the insolubility of these proteins in high-salt buffers in the presence of Triton X-100. All steps were performed in the presence of the following protease inhibitors: 1 mM phenyl-methylsulphonyl fluoride (PMSF), 1 mM benzamidine, 1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin, and 0.7 pM chymostatin (Sigma Chemical, St. Louis, MO USA).

Intermediate filaments of the tissue were obtained after centrifugation in the final insoluble fraction. Protein was dissolved in 1% sodium dodecyl sulfate and stored frozen at −20° C until required.

Protein determination

Protein concentrations for the quantitative analysis were estimated by the method of Lowry et al.[17] using bovine serum albumin (Sigma Chemical Company) as the standard. Protein content from isolated axonal cytoskeleton obtained from flotation of myelinated axons was measured by the method of Bradford.[18]

Polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was performed on 10% acrylamide according to the discontinuous system of Laemmli.[19] Gels were stained with 0.25% Brilliant Blue R-250 (Sigma Chemical), 50% methanol and 10% acetic acid and destained overnight in 50% methanol and 10% acetic acid. Molecular weight standards are described in *Figure 1.* For autoradiography, the gels were dried and exposed at −70° C to Kodak X Omat K film (Kodak, Rochester, NY USA) using Cronex Xtra Life (E.I. Du Pont De Nemours & Co., Wilmington, DE USA) intensifying screens.

Quantitation procedure

Neurofilament proteins were quantified following SDS-PAGE as described by Rubin et al.[14] Destained dried gels were

scanned in a densitometer (Hoefer Scientific Instruments GS 300 Transmitance/Reflectance Scanning Densitometer, San Francisco, CA USA) equipped with a chart recorder. Each band within a slab gel was measured at least twice. The difference between two measurements did not exceed 5% for each protein band. Previous studies have demonstrated that the recorder intensity of all protein bands quantitated by densitometry fall within the linear range of the technique. The total area of the densitometric scan was related to the protein concentration of the intermediate filament-enriched fraction, estimated by the method of Lowry, as described above. The relative distribution of intermediate filament proteins was calculated by cutting out and weighing the area under each peak of the densitometric scan and calculating their percentage of contribution to the total area. Therefore, cytoskeletal protein concentrations were calculated from percentage values using the total protein concentration as 100%.

Preparation of cytoskeletal proteins for the in vitro incorporation of ^{32}P

A cytoskeletal-enriched fraction was prepared by the flotation method for myelinated axons, as described by Toru-Delbauffe and Pierre.[20] Briefly, 1 gram of tissue was homogenized in 15 volumes of 10 mM sodium phosphate buffer (pH 6.5) containing 100 mM NaCl, 1 mM EDTA, and 0.85 M sucrose in glass-teflon homogenizer.

The homogenate was centrifuged in a Sorvall SS 34 rotor at 27,000 *g* for 15 minutes. After collection of the flotation pad of myelinated axons, the flotation was repeated three times. The myelinated axons were strongly homogenized in 1% Triton X-100 and 10 mM sodium phosphate buffer (pH 6.5).

The solution was stirred overnight at 4° C to remove myelin and then centrifuged through a layer of 0.85 M sucrose in 10 mM sodium phosphate (pH 6.5), at 120,000*g* for 1 hr (Model L5-75 B, SW 50.1 rotor, Beckman Instruments, Palo Alto, CA USA). The final pellet, which represented the neurofilament preparation, was used immediately. All steps were performed in the presence of the protease inhibitors described above.

Assay of in vitro ^{32}P incorporation

A cytoskeletal-enriched fraction obtained from myelinated axons, prepared as described above, served as protein substrate and source of endogenous protein kinases. Each assay mixture contained 10 μg of protein. Phosphorylation was carried out in 60 μL of assay mixtures at pH 6.5 in buffer containing 50 mM (2-[N-morpholino] ethane-sulfonic acid) (MES); 10 mM $MgCl_2$, as described by Toru-Delbauffe and Pierre.[20] The reaction was started by adding 5 pM [γ ^{32}P]-ATP (5.5×10^{-4}Bq) (16.6×10^{-10}Bq/mmol) (ICN-Radiochemicals, Irvine, CA USA). After a 5-min incubation at 30° C, the reaction was stopped by adding Laemmli sample buffer, and the samples were boiled for 3 minutes. The ^{32}P incorporated into cytoskeletal proteins were measured by liquid scintillation counting in 5.06 M toluene, 2.05 M ethanol, 12.4 mM PPO (2,5-Diphenyloxazole) (Sigma Chemical Company), 0.6 mM POPOP (1,4-bis [5-Phenyl - 2-oxazoyl]benzene; 2,2 - p-Phenylene - bis [5-phenyloxazole]) (Sigma Chemical Company), and 34.5% Triton X-100. Proteins were analyzed by SDS-PAGE, and total radioactivity incorporated in the cytoskeleton fraction was measured by removing a 20 μL aliquot from each assay just before stopping the reaction. This aliquot was applied to 2 × 2 cm filter papers previously soaked in 20% trichloroacetic acid. The ^{32}P-labeled proteins precipitated onto the paper were washed twice in 10% trichloroacetic acid containing excess nonradiolabeled ATP, twice in 5% trichloroacetic acid, and twice in ethanol to remove non-protein-bound radioactivity before scintillation counting.[13]

Statistical analysis

Data were analyzed by two-way analysis of variance and Student's *t* test, with the level of significance set at $P < 0.05$. In some analyses, males and females were considered separately.

Results

Table 2 shows that at the end of the period of protein restriction (day 21) the malnourished pups weighed about 40% less than the well-nourished controls ($F(1,66) = 671.5$; $P < 0.001$). The animals subjected to nutritional rehabilitation after weaning presented a weight deficit of about 15% on day 90 ($F(1,59) = 107.1$; $P < 0.001$). Cerebral weight was not affected to the same extent as body weight, presenting a deficit of approximately 15% on day 21 ($F(1,66) = 531.5$; $P < 0.001$) and 6% on day 90 ($F(1,59) = 31.4$; $P < 0.001$). Conversely, cerebellar weight was more affected at the end of the food-restriction period, presenting a deficit of about 30% ($F(1,66) = 131.2$; $P < 0.001$) and recovering to a deficit of only 7% after nutritional rehabilitation ($F(1,59) = 7.97$; $P < 0.001$).

However, malnutrition had no effect on total protein concentration in the cerebellum at either age studied *(Table 3)*.

Intermediate filament preparations used in the quantitative study were obtained by extraction of the tissue with a high salt phosphate-buffered solution containing

Table 2 Effects of postnatal malnutrition imposed on dams on the body weights, cerebral weights, and cerebellar weights of their pups at 21 and 90 days of age

	Control	Malnourished
Body weight (g)		
day 21 males	43.7 ± 3.36 (37)	26.0 ± 2.69 (24)*
females	42.8 ± 3.01 (20)	25.3 ± 2.10 (20)*
day 90 males	328.1 ± 23.7 (20)	268.4 ± 18.2 (25)*
females	218.5 ± 17.1 (27)	188.4 ± 12.1 (16)*
Cerebral weight (mg)		
day 21 males	997.0 ± 30 (27)	854.0 ± 20 (22)*
females	985.0 ± 29 (20)	840.0 ± 20 (20)*
day 90 males	1242.0 ± 50 (22)	1172.0 ± 30 (10)*
females	1190.0 ± 40 (20)	1130.0 ± 50 (13)*
Cerebellar weight (mg)		
day 21 males	205.0 ± 30 (15)	143.0 ± 10 (30)*
females	204.1 ± 25 (15)	140.9 ± 11 (15)*
day 90 males	260.0 ± 30 (25)	240.0 ± 10 (15)#
females	248.0 ± 20 (25)	231.0 ± 20 (14)#

Each value is the mean ± SEM for the number of animals indicated in the parentheses. Data for 21-day-old rats were from five litters. The control group consisted of rats whose dams were fed a 25%-protein diet; the malnourished group consisted of rats whose dams were fed an 8%-protein diet. Comparisons between groups at each age were performed by ANOVA followed by Student's *t* test.
*, Significant at $P < 0.001$.
#, Significant at $P < 0.05$.

Table 3 Effects of postnatal malnutrition imposed on dams on the protein concentrations of the homogenate and of the intermediate filament-enriched fraction of the cerebellum of their pups at 21 and 90 days of age

	Control	Malnourished
	mg protein/g tissue	
day 21 HOM	75.8 ± 7.9 (9)	77.8 ± 8.1 (8)
FI	4.4 ± 0.6 (9)	4.2 ± 1.0 (5)
day 90 HOM	91.7 ± 13.8 (14)	86.3 ± 11 (11)
FI	5.5 ± 0.8 (15)	5.5 ± 0.6 (9)

Each value is the mean ± SEM for the number of animals indicated in parentheses. HOM, homogenate from cerebellum. IF, intermediate filament-enriched fraction obtained by tissue extration with a high-salt solution. Comparison between groups at each age was performed by Student's *t* test.

1% Triton X-100 in the presence of several protease inhibitors. The insoluble material obtained constituted the intermediate filament-enriched fraction. *Table 3* shows that the total protein content of this intermediate filament-enriched fraction from 21- and 90-day-old rats was not altered by protein restriction.

Figure 1 shows the electrophoretic pattern of the cerebellum proteins at each step of the extraction of intermediate filaments. Soluble fractions contained the largest number of proteins from the homogenate but only negligible amounts of the intermediate filament proteins.

The pellet contained the bulk of the cytoskeletal proteins we previously identified[16] as the neurofilament subunits NF-H, NF-M and NF-L, a 66 kDa neurofilament-associated protein,[21] a 57 kDa intermediate filament protein described for the central and peripheral nervous system,[22] and a 50 kDa glial fibrillary acidic protein.[16] A 43 kDa protein comigrating with actin[21] was the major contaminant of our preparations.

Intermediate filament-enriched fractions from control and malnourished rats at both day 21 and day 90 were scanned following SDS-PAGE. A representative electrophoretic pattern and the corresponding densitometric scan is shown in *Figure 2*. Comparison of cytoskeletal preparations from 21-day-old rats showed that malnutrition imposed on dams during lactation caused a marked increase in the concentration of the peripheral subunits NF-H and NF-M in pups as compared with control animals *(Figure 3)*. Conversely, 90-day-old pups submitted to nutritional rehabilitation

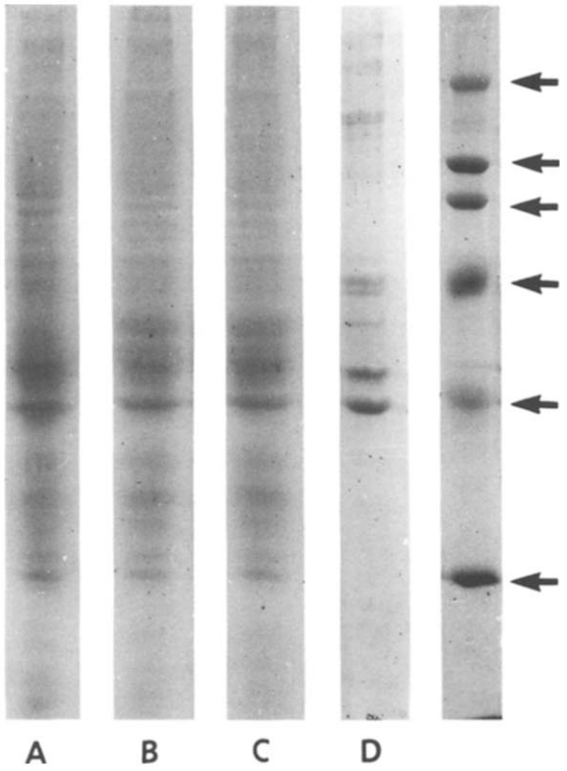

Figure 1 SDS-PAGE (10% acrylamide) of the successive steps in the preparation of intermediate filament-enriched fraction. Each sample corresponds to 40 μg of protein. (A) Whole cerebellum proteins, (B) first supernatant, (C) second supernatant, (D) high salt-insoluble fraction containing 200, 150, 68, 57, 50, and 43 kDa proteins. Molecular weights (kDa) are, from top to bottom: 205,000 (myosin), 116,000 (B-galactosidase), 97,400 (phosphorylase B), 66,000 (bovine albumin), 45,000 (egg albumin), and 29,000 (carbonic anhydrase).

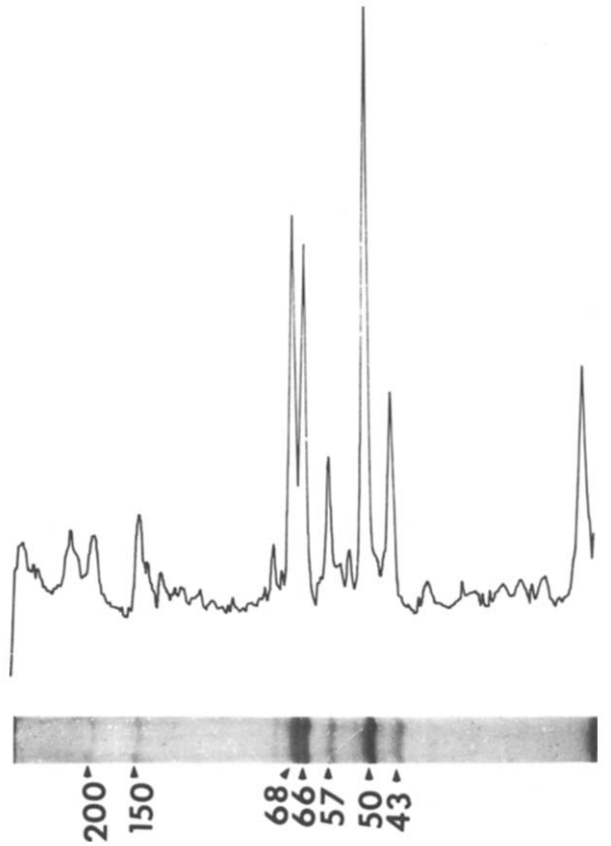

Figure 2 A representative electrophoretic pattern (SDS-PAGE, 10% acrylamide) and densitometric scan of the high salt-insoluble fraction for the quantitative study. The 200, 150, 68, 66, 57, 50, and 43 kDa proteins can be observed. The gel was stained with Brilliant Blue R-250.

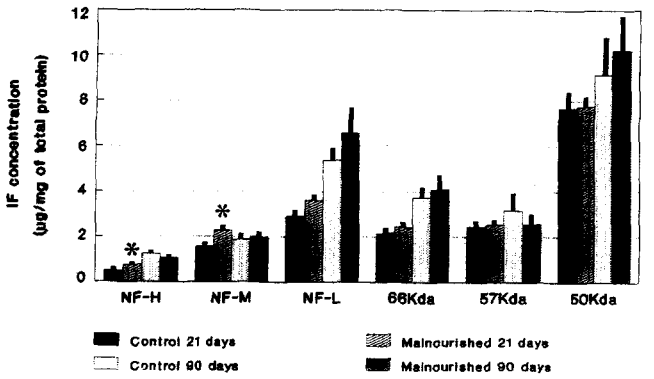

Figure 3 Effect of postnatal malnutrition on intermediate filament (IF) protein concentration of 21- and 90-day-old rats. Intermediate filament-enriched fractions were analysed by SDS-PAGE. Destained dried gels were scanned in a densitometer. Each experiment is a pool of two cerebella and each experimental point represents the mean ± SEM for 7–9 experiments. NF-H, 200 kDa neurofilament (NF) subunit; NF-M, 150 kDa NF subunit; NF-L, 68 kDa NF subunit; 66 kDa, NF-associated protein; 57 kDa, intermediate filament protein; 50 kDa, glial fibrillary acidic protein. Comparisons between control and malnourished groups at each age were performed by Student's *t* test. *, Significantly different at $P < 0.001$. #, Significantly different at $P < 0.01$.

after weaning presented similar concentrations of these subunits as compared with control animals. Also, we studied the effect of early malnutrition imposed on dams on the in vitro incorporation of ^{32}P into cytoskeletal proteins of 21- and 90-day-old pups. *Figure 4* shows the electrophoretic pattern of the cytoskeletal proteins from cerebellar myelinated axons obtained by the axonal flotation method. Among the proteins of this fraction we identified the three neurofilament subunits (NF-H, NF-M, and NF-L) and tubulin using monoclonal antibodies (not shown). *Figure 4* shows the autoradiographic pattern of the same proteins phosphorylated by endogenous kinases after incubation with [γ ^{32}P]-ATP. Tubulin and NF-M were very good substrates for endogenous protein kinases, although the relative ^{32}P incorporation into tubulin was considerably higher than that observed for NF-M. Under our experimental conditions, NF-H and NF-L were much poorer substrates than NF-M. Values of in vitro incorporation of ^{32}P into tubulin and NF-M are shown as relative incorporation in *Table 4*. Statistical analysis of these data indicated that early malnutrition imposed on dams had no effect on in vitro ^{32}P incorporation into these cytoskeletal proteins either in 21- or 90-day-old pups.

Discussion

In the present report we describe the effect of early malnutrition on the concentration and in vitro phosphorylation of cytoskeletal proteins from cerebellum of 21- and 90-day-old rats.

The quantitative study focused on the concentration of the neurofilaments in the cerebellum. Electrophoretic analysis of soluble fractions obtained during the different steps of the intermediate filaments extraction showed that small amounts of the intermediate filament

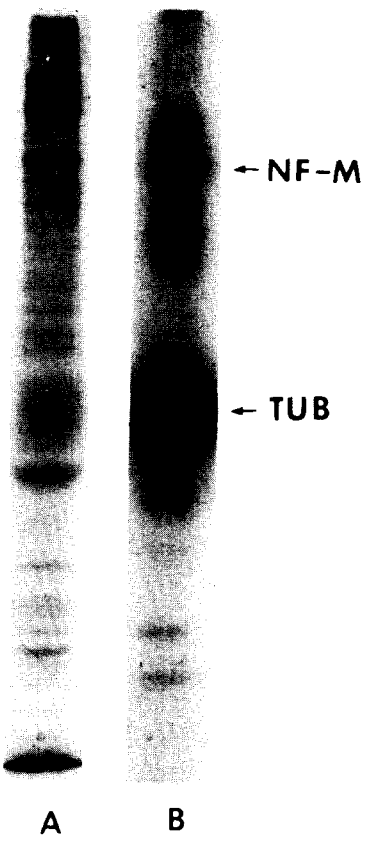

Figure 4 Electrophoretic pattern and the corresponding autoradiograph of the cytoskeletal proteins obtained by the axonal flotation method and incubated with [$\gamma^{32}P$]-ATP. Proteins were analysed on SDS-PAGE (10%) and stained with Brilliant Blue R-250 before autoradiographic exposure. (A) Electrophoretic pattern. (B) Autoradiograph of same fraction. NF-M, 150 kDa neurofilament subunit; TUB, tubulin.

Table 4 Effect of postnatal malnutrition imposed on dams on in vitro relative incorporation of ^{32}P in cytoskeletal proteins of their pups at 21 and 90 days of age

	Control	Malnourished
	21 days old	
NF-M	6.01 ± 1.6 (9)	6.63 ± 1.6 (10)
TUB	29.64 ± 4.1 (9)	23.40 ± 9.4 (10)
	90 days old	
NF-M	5.32 ± 1.6 (7)	5.88 ± 1.4 (5)
TUB	25.83 ± 6.3 (7)	21.55 ± 6.9 (5)

Cytoskeletal proteins were phosphorylated in vitro and separated by SDS-PAGE. The protein bands, identified as the 150 kDa NF subunit (NF-M) and tubulin (TUB), were excised, and incorporation of [$\gamma^{32}P$] phosphate was measured by radiation counting. Results are expressed as percentage values considering the radioactivity incorporated into total protein of cytoskeletal fraction as 100%. Each value is the mean ± SEM for the number of animals indicated in parentheses. The incorporation of ^{32}P in control incubations was typically 1400 cpm into NF-M and 6000 cpm into tubulin.

proteins were detected in these fractions, corresponding to unassembled neurofilament subunits.[23] The pellet fraction from tissue contained the bulk of polymerized cytoskeletal proteins. Our quantitative data showed that malnutrition imposed during the period of the brain growth spurt augmented the concentration of peripheral subunits NF-H and NF-M assembled into neurofilaments in the cerebellum of 21-day-old rats. This effect was absent in 90-day-old animals following postweaning nutritional rehabilitation. These findings indicate that our experimental conditions seem to interfere with the mechanisms that regulate neurofilament formation during the period of maximal cerebellum development. However, this effect can be reversed, suggesting transient alterations.

In view of the importance of neurofilament for neuronal activity, we propose that alterations in cytoskeletal elements contribute to chemical[24] and behavioral[25] alterations known to accompany protein restriction. We have previously reported that the concentration of neurofilament subunits extracted from the cerebral cortex of malnourished rats was not altered at weaning, but appeared to be augmented in adult animals after nutritional rehabilitation.[16] This finding emphasizes the high sensitivity of cytoskeletal constituents to early malnutrition. Furthermore, it suggests that the effects observed could be controlled by developmentally regulated factors acting differently in distinct cerebral structures.

The mechanisms responsible for an increase in neurofilament peripheral proteins in the cerebellum of malnourished animals are unknown. In addition to the involvement of regulatory factors as mentioned above, we may consider several other possibilities, such as a direct effect on the expression of neurofilament subunits causing an alteration in enzymatic activities involving stimulated intermediate filament protein synthesis or decreased intermediate filament degradation. An effect on posttranslational mechanisms influencing NF-H and NF-M incorporation into pre-existing immature filaments, consisting only of the 68 kDa subunit, is another possibility. Both tubulin and neurofilament protein undergo posttranslational modifications that play a role in regulating the dynamics of the axonal cytoskeleton.[8] The major modification of neurofilament proteins is phosphorylation.[26] To verify the probable role of phosphorylation in the increase in insoluble neurofilament protein, we undertook in vitro phosphorylation of cytoskeletal proteins obtained by extraction of myelinated axons with Triton X-100 at 4° C. The Triton insoluble fraction contained the three neurofilament subunits and the cold insoluble tubulins identified by monoclonal antibodies (not shown).

Cytoskeletal proteins can be phosphorylated in vitro by associated endogenous kinases.[4,20] The phosphate groups on mammalian neurofilament undergo considerable turnover. Differences in the extent of phosphate turnover on each neurofilament subunit indicate that dephosphorylation of neurofilament proteins is a selective process[27] and could reflect continuous alterations of phosphate topography that are functionally important. In our experimental conditions the in vitro phosphorylation of neurofilament proteins was predominantly in the NF-M subunit. Nevertheless, tubulin was the most extensively phosphorylated cytoskeletal protein. We ascribe the fact that NF-H is a poor substrate for our isolated kinase activity in vitro to the low phosphate turnover of this subunit in vivo.[27] Our pattern of in vitro ^{32}P incorporation into cytoskeletal proteins is in agreement with Leterrier et al.,[28] who described in the bovine brain an endogenous cyclic AMP-activated kinase activity dependent on microtubule proteins that predominantly phosphorylates tubulin and the NF-M subunit.

Analysis of our results indicates that malnutrition did not alter in vitro ^{32}P incorporation into these cytoskeletal proteins. This finding suggests that, at least in the case of NF-M, the mechanism involved in the increased concentration of this subunit after early malnutrition seems not to be related to its capacity of in vitro incorporation of ^{32}P. The molecular basis responsible for the increase in neurofilament peripheral subunit concentration in the cerebellum of rats subjected to early malnutrition remains to be clarified.

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