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NUTRITIONAL RECOVERY DOES NOT REVERSE THE ACTIVATION OF BRAIN SEROTONIN SYNTHESIS IN THE ONTOGENETICALLY MALNOURISHED RAT

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Abstract—In the present work we confirm that gestational malnutrition affects body and brain composition and results in an activation of the synthesis of the brain neurotransmitter 5-hydroxytryptamine. These results also demonstrate more activity of the rate-limiting enzyme tryptophan hydroxylase in the malnourished fetal and postnatal brain. However, the activity of this enzyme remains increased in the brain of nutritionally recovered animals accompanied by an increase in the synthesis of 5-hydroxytryptamine. We therefore suggest that, in the nutritionally recovered animal, the mechanism of activation of this biosynthetic path in the brain may be not dependent on the increased availability of free L-tryptophan observed in malnourished animals, but might be due to a specific change in the enzyme complex itself. This hypothesis is supported by the fact that plasma free and brain L-tryptophan return to normal in the recovered animal.

Key words: tryptophan hydroxylase, 5-hydroxytryptamine, ontogenetic malnutrition, nutritional rehabilitation.

We have described important changes induced by early malnutrition on the brain serotonergic system.[24–27,33,34] Brain 5-hydroxytryptamine (5-HT, serotonin), a specific neurotransmitter, is found in increased amounts.[24–27,33,34] This increase seems to be directly related to an increase in the free fraction of plasma L-tryptophan (L-Trp), the metabolic precursor of 5-HT.[7,11,16,19,30,36,41,45] Plasma L-Trp is transported to the brain where it is hydroxylated in serotonergic neurons by the action of the enzyme tryptophan hydroxylase (EC 1.14.16.4, TrpOH).[3,23,28,39] 5-Hydroxytryptophan is then decarboxylated to 5-HT.[4,14,31] We have reported evidence that, in rats malnourished during gestation or lactation, there is an acceleration in the synthesis of brain 5-HT, starting in the fetal period, coincident with an increase of both the concentration of brain L-Trp and the activity of TrpOH, which suggests that an activation of the serotonergic system occurs during this period of nutritional stress.[24–27,33,34]

The increase in the concentration of L-Trp and in the activity of TrpOH in the malnourished brain, and the concomitant acceleration of the synthesis of the neurotransmitter 5-HT, prompted us to search for more information on the mechanism implicit in the activation of TrpOH. The cellular mechanism involved seems not to be as simple as an increase of L-Trp, because we observed a change in the kinetic behaviour of the enzyme. The affinity of TrpOH for L-Trp is higher and its activation by various phosphorylating mechanisms, such as cyclic-adenosine monophosphate (cAMP), inositol-1,4,5-triphosphate (IP_3), diacylglycerol and calcium/calmodulin-dependent protein kinase II, are also augmented in the malnourished brain.[34] These findings lead us to hypothesize that gestational malnutrition might induce a structural change in the TrpOH complex. It is not known what happens to these changes when the ontogenetically malnourished animals are nutritionally recovered, except that we know plasma free and brain L-Trp return to control amounts. If the accelerated 5-HT synthesis in the malnourished brain was only dependent on increased plasma L-Trp passing through the BBB, it should also return to normal values in the nutritionally recovered animals. In the present study we tested this hypothesis.

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EXPERIMENTAL PROCEDURES

Albino female Wistar rats were used, weighing an average of 220 ± 10 g at initiation of these experiments. The rats were adapted for 2 weeks to the following environmental conditions: temperature 22 ± 2°C; 12-hr periods of light and darkness; light from 07:00 to 19:00 hr and darkness from 19:00 to 07:00 hr; manipulation and noise were minimal, relative humidity oscillated between 50 and 60%. During this period of adaptation, the rats were fed Purina chow and water *ad libitum*. At the end of this period the rats were divided into the following experimental groups: a group with protein-calorie malnutrition denoted (group PCM), whose food intake was limited to 50% of that consumed *ad libitum* by the rats in the control group (C) in 24 hr. After 2 weeks on this nutritional regime, the female rats of both groups were paired to normal males and only in this mating period were allowed a normal food intake. Gestational age was assessed taking the presence of a vaginal plug as the first day of gestation. During the gestational period, the animals were returned to the respective conditions described above. The amount of food that C rats consumed before gestation was 25 ± 3 g/day and during gestation 32 ± 2 g/day. The malnourished group thus received 12.5 ± 2 and 16 ± 2 g/day of food, respectively, in each period.

At birth, the offspring from each group were mixed and randomly redistributed in groups of eight members to mothers of the same PCM or C groups. In addition, a crossover of the offspring of these groups was made to give a final total of four experimental groups, as follows: (a) group PCM consisted of the offspring malnourished during gestation and when born they were fed by the same malnourished mothers, so they remained undernourished, both in the fetal stage as well as in the immediate postnatal stage; (b) a nutritionally recovered group (NR) consisted of the offspring from gestationally malnourished mothers, which when born were shifted to normally fed mothers of group C; (c) another group included pups malnourished only during lactation (MDL) formed by offspring of mothers from group C that from birth were fed by malnourished mothers from group PCM; (d) group C consisted of group C offspring, fed by their own normal mothers. The daily consumption of food by the control mothers during this lactation stage was 40 ± 3 g/day so the mothers of groups PCM and MDL were fed 20 ± 2 g/day during the following 21 postnatal days.

At ages 1, 10, 15 and 21 days after birth, animals from at least three different litters from each experimental group were killed, and their brains were dissected without cerebellum on an ice-cold plate, weighed and homogenized in the appropriate cold solution for each biochemical trial.

The criterion for malnutrition was a body weight significantly less than 10% of the control group. In order to decrease any possible variations in the circadian rhythm, collection of the brain tissue was carried out between 09:00 and 11:00 hr. In addition, body and brain weight and crown–rump length (CRL) were measured in all experimental groups.

Biochemical assays

The activity of the enzyme TrpOH was determined by the method of Gál and Patterson.[17] Briefly, it consisted of incubating 1.5 mg of protein from the supernatant solution of brain homogenate at $30\,000 \times g$ for 30 min, in the presence of 1 mM 2-mercaptoethanol, 0.3 mM pargyline, 0.16 mM 2-amine-4-hydroxy-6-methyl tetrahydrobiopterine (6-MPH4), 0.088 mM L-Trp, 10 μg catalase and 50 mM trisacetate buffer. After 30 min of incubation at 37°C, the reaction was stopped by heat (95°C). The hydroxyindoles formed were then measured spectrofluorometrically by means of the orthophthaldialdehyde reaction.

The 5-HT content was measured spectrofluorometrically by the method of Curzon and Green[10] after separation on columns of Bio-Rad 70, cationic exchange resin (200–400 mesh in the sodium form), at pH 6.1.

Brain L-Trp was determined after homogenizing the tissue in 10% cold trichloroacetic acid and two centrifugations in a Sorvall RC5C centrifuge at 4°C by the spectrofluorometric method of Denckla and Dewey[12] modified by Bloxam and Warren.[2]

Brain proteins were determined by the spectrophotometric method of Lowry *et al.*[32] using bovine serum albumin as standard.

Analysis of variance showed homogeneity among the groups, thus the significance of the differences among groups was determined by Student's *t*-test for sets of independent data with a significance level of $P < 0.05$.

RESULTS

In groups PCM and MDL, a significant decrease in body weight was seen, in the former at birth and from day 10 in the latter, relative to C group ($P<0.001$). On day 21, the decrease in weight was 50% in group PCM and 46% in group MDL (Fig. 1A). Notice in the same figure the developmental pattern shown by the nutritionally recovered group, NR, which gained weight from day 10, reaching group C on day 15, without statistical differences from controls up to day 21.

The developmental pattern of brain weight is shown in Fig. 1B. There was a significant difference in the PCM and MDL groups compared to the C group ($P<0.001$), the deficit of brain weight at day 21 of life was 19% in group PCM and 18% in group MDL. In addition, in the same figure, the NR pattern recovered to control values after day 10, in the same way as body weight.

Figure 1C shows the developmental pattern of the CRL, which was significantly less at birth in both groups PCM and NR ($P<0.001$), starting on day 10 in the MDL group ($P<0.001$). In groups PCM and MDL, the deficit persisted up to 21 days of age in relation to group C ($P<0.001$). In the same way as the body and brain weights, the CRL in group NR showed a recovery with age, reaching the control values on day 15.

The tissue proteins for the various groups are shown in Table 1. A developmental deficit is present in both group PCM and develops for MDL and a recovery of NR is also noticed from day 15.

The L-Trp brain concentration is shown in Fig. 2. As can be seen, group C showed a decrease after birth from 45.5 μmol to 2.61 μmol/g wet tissue at the tenth day, stabilizing thereafter. In group PCM, the pattern was very similar to group C, with a decrease with age, yet its concentration was always significantly greater compared to the control group ($P<0.05$). In the same figure, the pattern for the MDL group also showed a greater concentration of brain L-Trp at all ages studied in comparison to the control ($P<0.05$). In addition, brain L-Trp of the NR group also showed a decrease with age, but it was higher than controls ($P<0.01$) with normal levels on day 21.

The specific activity of TrpOH showed an ascending age pattern, from 0.25 nmol at birth to 0.40 nmol/mg protein/hr at 21 days in group C (Fig. 3). In the same figure, it can be seen that

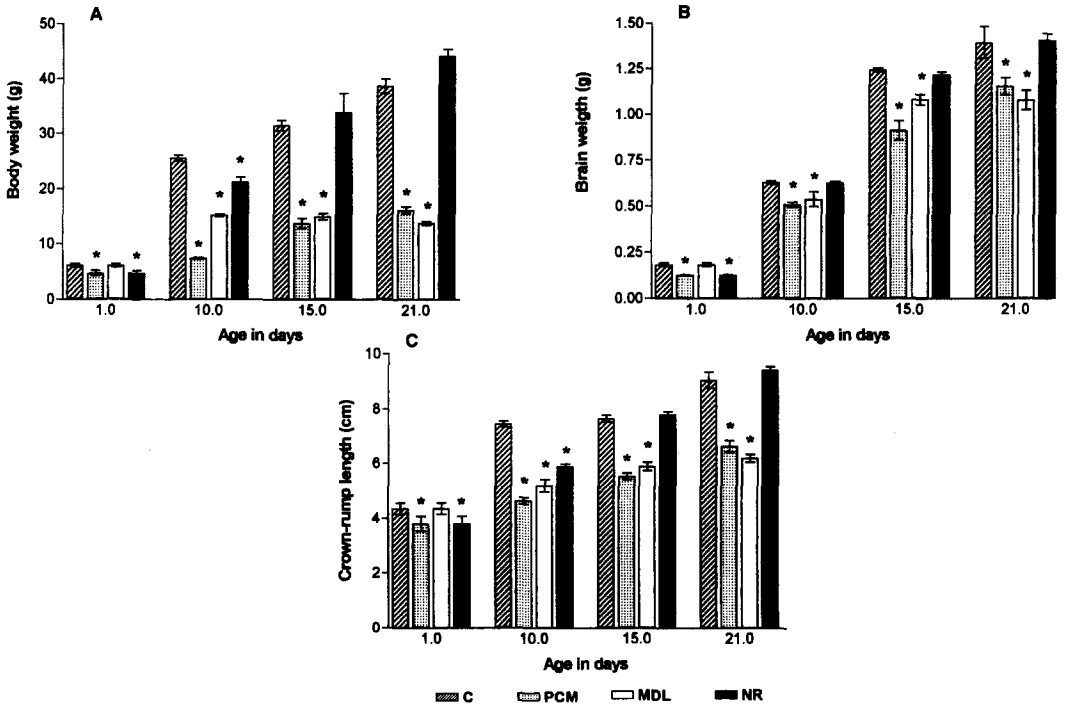

Fig. 1. Somatometric data. A, Body weight; B, brain weight and C, crown rump length. ▨ C control; ▩ PCM protein-calorie malnutrition; □ MDL malnourished during lactation; ■ NR nutritionally recovered. Each bar represents the mean value ± S.D. of 13 subjects. F = 189.10, DFn = 9, DFd = 64. *$P<0.001$, ANOVA, Student's t-test.

Table 1. Brain protein concentration (mg/g wet tissue)

Age (days)	C	PCM	MDL	NR
1	24.40 ± 0.86	20.50 ± 1.17[a]	25.40 ± 0.86	20.90 ± 1.07[a]
10	49.45 ± 1.04	45.20 ± 0.73[a]	51.46 ± 0.60	42.99 ± 1.96[b]
15	93.29 ± 0.81	65.10 ± 4.14[b]	67.06 ± 5.44[b]	84.96 ± 3.00[a]
21	91.33 ± 3.00	69.35 ± 2.98[b]	72.84 ± 1.05[b]	93.54 ± 2.20

C, Control; PCM, protein-calorie malnutrition; MDL, malnourished during lactation; NR, nutritionally recovered. Each point represents the mean value from four experiments in duplicate ± S.D. [a]$P<0.001$; [b]$P<0.01$; C vs. PCM, C vs. MDL and C vs. NR; F = 5.86, DFn = 9, DFd = 48, ANOVA, Student's *t*-test.

groups' PCM and NR specific activity is significantly elevated at birth, with a level of activity seen in the control group at the tenth day of life. After day 10 TrpOH activity remained significantly elevated in groups PCM and MDL up to day 21, and interestingly the enzyme activity in the nutritionally recovered NR group remained significantly increased and did not return to normal values.

In Fig. 4, the developmental pattern of brain 5-HT is illustrated. As can be seen in group C there is an increase of the neurotransmitter with age from 0.720 nmol at birth to 1.833 nmol/g wet tissue at 21 days of life. In group PCM, there is an increase of this neurotransmitter during the first day of postnatal life, with a concentration corresponding to that observed for group C on day 10. The same significant increase in the concentration of L-Trp and in the enzyme activity was seen for brain 5-HT in groups PCM and MDL ($P<0.001$). The pattern of 5-HT in the NR group is similar to that shown by the non-recovered groups and it is noteworthy that, just as for the TrpOH activity, a significant increase in brain 5-HT concentration was still present on day 21 in the nutritionally recovered group ($P<0.05$).

DISCUSSION

The results of the different somatometric measurements carried out in the animals used for this study confirm that gestational malnutrition produces a retardation in physical growth. It was

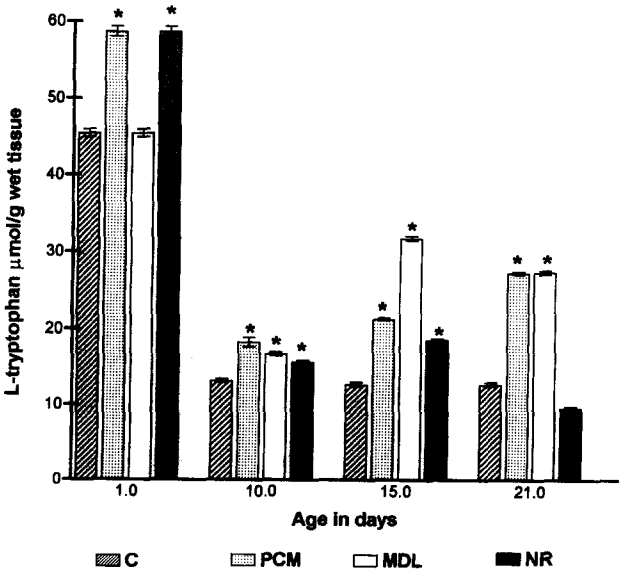

Fig. 2. Brain L-Trp concentration. ▨ C control; ▩ PCM protein-calorie malnutrition; □ MDL malnourished during lactation; ■ NR nutritionally recovered. Each bar represents the mean value from four experiments in duplicate ± S.D. F = 50.39, DFn = 9, DFd = 48. *$P<0.001$, ANOVA, Student's *t*-test.

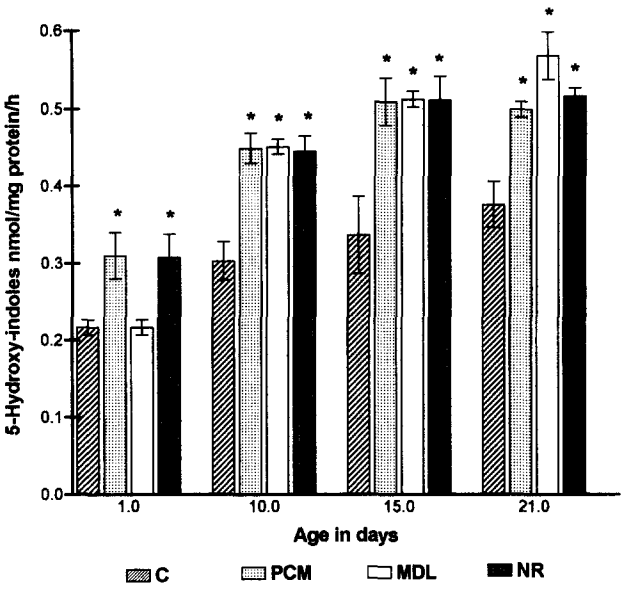

Fig. 3. Activity of TrpOH. ▨ C control; □ PCM protein-calorie malnutrition; □ MDL malnourished during lactation; ■ NR nutritionally recovered. Each bar represents the mean value from four experiments in duplicate ± S.D. F = 4.53, DFn = 9, DFd = 48. *$P < 0.001$, ANOVA, Student's t-test.

demonstrated that body weight changes more easily than length and brain weight, which agrees with previously reported findings.[8,20,22,35,40] It was also observed that animals malnourished during gestation, but submitted at birth to a normal scheme of nutrition (NR group), presented sufficient catch-up in growth which allowed them to reach the rate of the control group. The same was true for protein concentration. It can therefore be concluded that an adequate and early nutritional therapy is capable of accelerating growth of the body in rats undergoing rehabilitation after a period of food restriction. An increased rate of growth was associated with greater feed intake and higher efficiency of utilization, enough to correct the differences in tissue protein.[1,15,21,29,38]

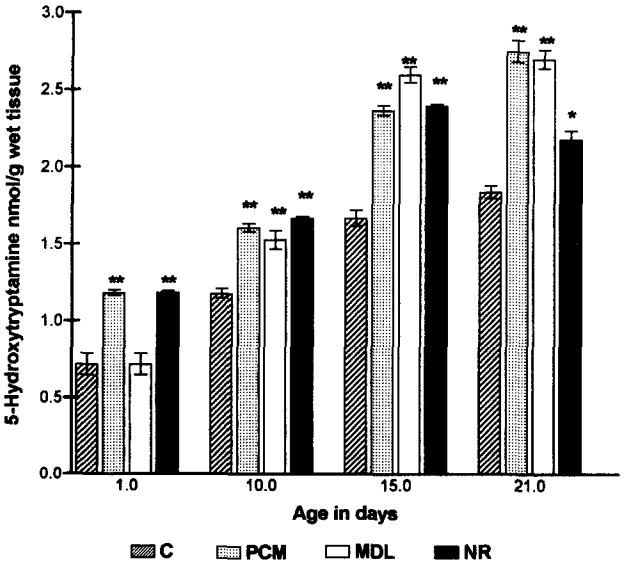

Fig. 4. Brain 5-HT concentration. ▨ C control; ▩ PCM protein-calorie malnutrition; □ MDL malnourished during lactation; ■ NR nutritionally recovered. Each bar represents the mean value from four experiments in duplicate ± S.D. F = 5974, DFn = 9, DFd = 48. *$P < 0.001$, ANOVA, Student's t-test.

In addition to the general changes, ontogenetic malnutrition is capable of producing more specific brain changes. One of these is demonstrated for the serotonergic system during development, which consists of an activation in the synthesis of this neurotransmitter.[6,9,24–27]. The current study confirms, and adds to previous results, that an increase in 5-HT content, in L-Trp concentration and in the activity of TrpOH in brains of malnourished rats occurs not only during gestation but also during the lactation period.[24–27,33,36,37,42–44]

The increase in the activity of TrpOH observed starting during the fetal stage and continuing to postnatal life, seems to be secondary to an increased transport of plasma L-Trp to the malnourished brain.[3,7,19,33,37,41] However, the persistence of accelerated serotonin synthesis in the nutritionally recovered brain is suggestive that other mechanisms may be involved, particularly in the activation of TrpOH. Besides, L-Trp loading experiments in gestating rats not malnourished activated brain TrpOH in the fetal brain, remaining active up to the lactational period, suggesting that in this particular case the activation mechanism could be different from that involved in ontogenetic malnutrition.[5] We have recently observed that gestational malnutrition produces a change in TrpOH kinetics with an increase in its affinity for L-Trp and more activity of the enzyme by different phosphorylating mechanisms.[34] These findings allow us to suggest that ontogenetic malnutrition may produce a structural change in the TrpOH enzyme complex as the main mechanism of the chronic enzyme activation and the persistence of an acceleration in the biosynthesis of the neurotransmitter in the brain.

The gestationally malnourished animals submitted at birth to nutritional rescue showed a complete recovery in physical growth and brain biochemical composition and a return of their brain concentration of L-Trp to normal. But, in spite of the physical and biochemical recovery, the activity of TrpOH remained increased and an increase in the concentration of the neurotransmitter persisted. It is interesting to see that the activation mechanism in the rehabilitated animal seems not to be due to the changes in the metabolism of the synthesis of 5-HT observed earlier in the malnourished animal because brain L-Trp returned to normal levels and seems no longer to be the predominating factor in the activation of the enzyme, although it remains activated. This increase of TrpOH activity would favour the view that in the nutritionally recovered brain the changes in the metabolism of L-Trp that are observed during malnutrition are no longer responsible for the activation of the enzyme in the recovered brain. Indeed, preliminary data from our laboratory point to the fact that, in nutritionally recovered animals, the plasma free L-Trp concentration and its binding constants to albumin tend to be normal.[18] Therefore, it seems that the mechanism which keeps the biosynthetic path of this important brain neurotransmitter activated, in the nutritionally recovered animal, differs from that in the malnourished animal. We already mentioned the changes in affinity and phosphorylation capacity of TrpOH in the malnourished brain,[34] which suggest the possibility of structural modifications in the enzyme complex, induced by chronic malnutrition that may persist in the recovered animal. This hypothesis remains to be proved. There is also evidence that the serotonergic activation is also present in discrete regions of the brain, such as the cerebral cortex and the hypothalamus, in rats that present an abnormal feeding behaviour,[13] suggesting that these changes may be of functional relevance.

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