Effects of Postnatal Zinc Deficiency on Cerebellar and Hippocampal Development in the Rat

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Because the majority of hippocampal and cerebellar development occurs postnaturally, these brain regions might be expected to be very sensitive to zinc deficiency during this period. Rat pups suckled for 21 days by dams fed a zinc-deficient diet demonstrated impaired body growth and had smaller cerebella compared to pups from pair-fed or ad libitum-fed controls given adequate zinc and had smaller hippocampi compared to the ad libitum-fed controls. In histologic preparations, there was a marked retention of the external granular layer of the cerebellum in response to zinc deficiency; the appearance of the hippocampus did not seem to be altered by the dietary treatments. Determinations of DNA, RNA, and protein indicated that both brain regions failed to maintain a normal rate of development. Pups from dams pair-fed with the zinc-deficient dams also displayed alterations in the morphology of the cerebellum and in the composition of both hippocampus and cerebellum, but those differences were not as severe as those seen in the zinc-deficient pups. The results indicate additional effects due to zinc deficiency which could not be explained solely on the basis of the undernutrition associated with zinc deficiency.

INTRODUCTION

Regions of the brain and different cell types within these regions develop at various stages in the fetal and neonatal rat (18). The cerebellum and hippocampus, which serve as integrative centers for motor and emotional activity respectively, develop later relative to other brain regions.

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199
(2). The cerebellar cortex of the rat has been found to acquire virtually all its cellular constituents except Purkinje cells in the first three weeks after birth (1). The hippocampus has been shown to acquire up to 85% of its cellular population postnatally (6). This pattern of late development might be expected to render those structures vulnerable to adverse environmental conditions during early postnatal life. In support of this suggestion are findings of Brunner and Altman (7), who described histologic abnormalities in the cerebellum and hippocampus, and abnormalities in motor and emotional activity of rats exposed to x-irradiation during the first three postnatal weeks. Studies of postnatal undernutrition in rats have shown retarded cerebellar development (3, 4, 15).

It was shown that zinc deficiency adversely affects rapidly proliferating tissues (5, 26, 28). Zinc deficiency in suckling rats resulted in decreased body weights and brain weights (22, 25). In addition, impaired uptake of thymidine by the brain (27), lower cerebellar weight, and decreased RNA,³ DNA, and protein content in the cerebellum (13) have been observed. Furthermore, rehabilitated adult rats which were exposed to zinc deficiency from birth to age 21 days displayed impaired maze acquisition (20). Measurement of zinc concentrations in various regions of the brain revealed that the distribution of zinc is uneven and that amounts are highest in the hippocampus (9, 10). It was reported (9) that the large deposits of zinc in the rat hippocampus accumulate postnatally, with the heaviest deposition occurring during the third week of life.

This investigation, utilizing histological, histochemical, and biochemical techniques, was an extension of previous work on the effects of zinc deficiency on brain development (13, 27). In addition we felt that the role of zinc in the hippocampus might be further elucidated by using dietary zinc deficiency as a model for study. On histological examination, the cerebellum from the zinc-deficient rats appeared immature, and biochemical alterations were observed which suggested an overall failure to maintain a normal rate of development in the cerebellum and hippocampus.

**MATERIALS AND METHODS**

Nulliparous hooded Long–Evans rats were mated and fed a commercially available laboratory rat food throughout gestation. At parturition, the dams and pups were moved to clean Plexiglas cages and divided into three dietary groups. One group was fed a Zn-deficient (< 1 µg Zn/g), biotin-enriched, sprayed egg white diet (21) (which was modified to include inositol (1 mg/kg) and to exclude the antibiotic) *ad libitum* and given distilled, demineralized drinking water. Animals of a second group

³ Abbreviations: RNA—ribonucleic acid; DNA—deoxyribonucleic acid.
were individually pair-fed an amount of the diet equal to that consumed by a Zn-deficient rat and given drinking water supplemented with 25 μg Zn/ml. The third group was fed the diet ad libitum and given the zinc-supplemented drinking water. Litters were equalized to eight pups early in the first week. On the 21st day, pups of litters maintained at seven or eight pups were taken for analysis. One or two were used for histological preparation, two for dry tissue weight determination, and four for biochemical analysis. For histological study, pups were anesthetized with sodium pentobarbital and perfused with formalin. Their brains were removed, sectioned with a cryostat at 24 μm, and stained with cresyl violet acetate. Other pups were perfused with a sodium sulfide solution. Brains were removed, cryostat-sectioned at 40 μm, and stained according to Haug’s modification (17) of Timm’s silver sulfide method (29) for heavy metal visualization. Cerebellar sections were taken in a midsagittal plane through the vermis. Tracings of these sections were made in triplicate with the aid of a photo enlarger. The tracings were cut out and weighed, and the weights were averaged. Photomicrographs of the sections were taken at the external surface of lobule V. Hippocampal sections were taken in a horizontal plane, and the aqueduct of Sylvius and the inferior aspect of the frontal cortex in the medial plane were used as landmarks. Tracings of these sections were also made. Pups used for dry tissue weight determination were decapitated. Their cerebella and hippocampi were excised, pooled, weighed, frozen, lyophilized, and reweighed.

Pups used for biochemical analysis were decapitated. Blood for hematocrits was taken from the trunk blood, and the cerebellum and hippocampus were excised, pooled, and weighed in cold buffered sucrose (0.25 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 50 mM Tris–HCl, pH 7.5). Samples were homogenized and precipitated with an equal volume of 0.4 N HClO₄, and the precipitates were washed three times with 0.2 N HClO₄. Separate aliquots were taken for analysis of DNA, RNA, and protein content. DNA was measured by Burton’s method (8) involving a diethylamino reaction, RNA by Fleck and Munro’s technique (12) for measuring nucleic acids by ultraviolet absorption, and protein by Hartree’s modification (16) of the method of Lowry et al. (19) which uses the Folin–Ciocalteu reagent. Samples were read on a Cary 118 spectrophotometer. Statistical analysis was performed by single classification analysis of variance (ANOVA) and by Student’s t test. Values cited in text reflect the means ± SE.

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RESULTS

At 21 days of age, pup weights were severely depressed by zinc deficiency or pair feeding. Pups of the zinc-deficient dams were smaller (25.2 ± 0.8 g) than those from both the pair-fed (30.4 ± 3.1 g) and *ad libitum*-fed (46.5 ± 1.2 g) dams. Hematocrits of Zn-deficient and pair-fed pups (30.1 ± 1.5 and 29.2 ± 0.9, respectively) were greater than those of the *ad libitum*-fed pups (25.5 ± 0.6).

Cerebellum. The cerebella of the Zn-deficient and pair-fed pups were smaller than those of *ad libitum*-fed pups. A comparison of weighed tracings of midsagittal sections of the vermis showed the cross-sectional area in the pair-fed pups to be 24% smaller and the cross-sectional area of Zn-deficient pups to be 37% smaller than comparable values for *ad libitum*-fed pups (Fig. 1A, C, E). The shape of the vermis in the deficient pups was basically triangular and had a dorso rostral prominence. In the vermis of pair-fed animals, the width of the dorsal region was greater in a rostral—caudal direction, giving the cerebellum a more rectangular outline. The vermis of the *ad-libitum*-fed pups had the usual outline present in normal pups; the ventral aspect of the vermis was more flattened where it covered the fourth ventricle, and the entire dorsal aspect appeared rounded and well proportioned; the dorsocaudal region was developed to such an extent that the prominence found in the zinc-deficient pups and the rectangular appearance seen in the pair-fed pups were not evident (Fig. 1A, C, E).

In histological preparations the external granular layer was very prominent in the Zn-deficient pups (Fig. 1F). It was five to eight cells in thickness and appeared densely packed with darkly stained cells. The cells were similar in appearance to the cells of the internal granular layer with heavily stained round nuclei. The molecular layer contained cells with darkly stained fusiform and rounded nuclei heavily concentrated near the external granular layer but less frequent near the internal granular layer. Fusiform cells were oriented parallel to the pial surface in the outer zone and perpendicular to the surface in the deeper molecular layer. Stellate and glial cells in the background of the molecular layer were of normal appearance.

There was also a persistence of the external granular layer in the pair-fed pups, but to a lesser degree (Fig. 1D). Although the thickness of the layer was comparable to that for the Zn-deficient pups, the cellularity was diminished. In the molecular layer there were more cells with darkly stained round and fusiform nuclei than in that of the Zn-deficient pups. In the *ad-libitum*-fed pups, the external granular layer was almost completely dispersed, with an occasional granule cell among the cells of the molecular layer (Fig. 1B). The histology of the cerebellum did not appear
Fig. 1. A, C, E—Line drawings made from tracings of photo enlarger-projected images of midsagittal sections of the cerebellar vermis of 21-day-old rat pups. Asterisks indicate lobule V. B, D, F—Photomicrographs taken at the external granular layer (EGL) of lobule V. Note smaller overall dimensions (E) and persistence of EGL (F) in zinc-deficient specimens. Pair-fed specimens (C, D) appear intermediate between ad libitum-fed (A, B) and zinc-deficient specimens. Sections were stained with cresyl violet acetate. Magnification of B, D, and F. ×200.
otherwise affected. The size and number of Purkinje cells and thickness of the cerebellar layers appeared similar in all three groups.

The weights of the cerebella of the Zn-deficient pups were significantly less than those of the pair-fed and ad libitum-fed pups (Table 1). The cerebella of the pair-fed pups also weighed less than those of the ad libitum-fed. When corrected for body weight, the cerebella of pair-fed and Zn-deficient pups were relatively larger than the cerebella of the ad libitum-fed, but the cerebella of pair-fed animals, although intermediate, did not differ significantly from those of Zn-deficient animals. The percentage of water was not affected by dietary treatment. The concentrations of DNA showed a tendency toward higher values in the pair-fed and Zn-deficient pups, but they did not differ significantly from the concentration in the cerebella of the ad libitum-fed group. It was found, however, that total DNA was significantly less in the cerebella of Zn-deficient than of ad libitum-fed pups. Pair-fed pups were intermediate with respect to total cerebellar DNA but did not differ significantly from either ad libitum-fed or Zn-deficient pups. There was a trend toward higher concentrations of RNA in the cerebella of pair-fed and Zn-deficient pups, but the differences did not achieve statistical significance. Total RNA was less in the pair-fed than in the ad libitum-fed cerebella and lower yet in Zn-deficient cerebella, which did not differ significantly from the pair-fed in total cerebellar RNA. The ratio of RNA to DNA was unaffected by dietary treatment. Cerebellar protein concentration was somewhat higher in the Zn-deficient than in the ad libitum-fed pups, with pair-fed values intermediate, but the differences were not significant. Total protein was lower in the cerebella of Zn-deficient pups than in those of the ad libitum-fed. Pair-fed pups were intermediate with respect to cerebellar protein content and did not differ significantly from ad libitum-fed or Zn-deficient pups. No trend in the ratio of protein to DNA was observed.

Hippocampus. In cresyl violet-stained material, the hippocampi did not appear affected by dietary treatment. The cross-sectional area of the hippocampus in the plane examined was constant among the three dietary groups. The zone of cell migration at the medial aspect of the lateral ventricle was lightly populated with immature granule cells. In the pyramidal cell layer of Ammon's horn, Nissl substance, pyramidal cell size, and cell packing density were similar among dietary groups.

The Timm's stain for heavy metals showed no differences between dietary groups. The hippocampi stained heavily along the pyramidal cell layer where the terminal boutons of the granular layer synapse on the pyramidal cell bodies. There was also heavy staining in the facia dentata of the dentate gyrus. The staining of these regions and of lighter strata observed throughout the entire hippocampal formation were consistent with
<table>
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<th>Parameter</th>
<th>Cerebella&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hippocampi&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>AL 8&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> Values are means ± standard errors.
<sup>b</sup> Number of litters.
<sup>c</sup> Differs from *ad libitum*-fed, $P < 0.01$.
<sup>d</sup> Differs from *ad libitum*-fed, $P < 0.05$.
<sup>e</sup> Differs from *ad libitum*-fed, $P < 0.10$.
<sup>f</sup> Differs from pair-fed, $P < 0.05$.
<sup>*</sup> Significant difference by Student's $t$ test only.
those described by Gesner-Jensen et al. (14). Coded photographs could not be assigned a dietary group when examined for pattern or intensity of stain deposition, or for thickness of strata.

The weights of the hippocampi were significantly affected by dietary treatment (Table 1). The hippocampi of the Zn-deficient pups were smaller than those of the ad libitum-fed pups, and pair-fed pups were intermediate with respect to hippocampal weight. When corrected for body weight, hippocampal weight was relatively higher in both the pair-fed and the Zn-deficient pups. DNA concentration was significantly higher in the hippocampi of Zn-deficient pups, and total DNA was lower. DNA concentration in the hippocampi of pair-fed animals was intermediate and did not differ significantly from the hippocampi of either the ad libitum-fed or the Zn-deficient pups. RNA concentration was not affected but total RNA was lower in Zn-deficient and pair-fed than in ad libitum-fed pups. The ratio of RNA to DNA tended to be smaller in pair-fed and smaller yet in Zn-deficient pups but did not differ significantly. There was significantly less total protein in the Zn-deficient hippocampi and the pair-fed were intermediate in protein content and did not differ from either ad libitum-fed or Zn-deficient hippocampi. In the hippocampi of the deficient pups, the ratio of protein to DNA was lower than that of the ad libitum-fed pups; the ratio for the pair-fed pups was intermediate.

DISCUSSION

Morphologic alterations in the cerebellum suggest that the effects of zinc deficiency and restricted feeding are related to the rate but not to the pattern of development. The persistence of the external granular layer and the general outline of the vermis in sagittal section are very similar to what is seen in adequately nourished pups at a much earlier stage in development (1) and in pups which suffered postnatal undernutrition (15). It should be noted that the cerebellar vermis develops earlier in the postnatal period than do other cerebellar regions (1) and that it is more severely affected by transient nutritional insult (23). The magnitude of the effects due to dietary treatment seen in the midsagittal plane of the cerebellum must therefore be viewed with caution. Nonetheless, it appears that the mechanism whereby the proliferative zone normally disappears early in the fourth week of life is in some way sensitive to the number of cells or the size or shape of the cerebellum and not only to the age of the animal. Nutritional inadequacy results in the proliferative zone remaining in place. It is unknown whether it retains the capacity for subsequent normal development if nutrients become available. Studies on the effects of hypo- and hyperthyroidism have shown that, although the rate of cell division can fluctuate in the face of fluctuating hormone levels, cell
differentiation goes forward at its own intrinsic pace (24). The cerebellar complement of a given cell type could suffer permanent damage if adverse environmental conditions occur at the time of its normal genesis. Thus the prognosis for full recovery following nutritional rehabilitation seems poor even with the persistence of the external granular layer. It appears that pair feeding slows the rate of development to a lesser degree than does zinc deficiency. Results of cerebellar tissue analysis are consistent with this interpretation. The concentrations of water, RNA, and protein were not significantly affected, indicating that accumulation of these components was proportional to overall development. The slower rate of cerebellar growth resulted in fewer cells overall in the zinc-deficient pups, as indicated by the decrease in the total amount of DNA compared to the ad libitum-fed controls. The trend toward higher DNA concentrations is suggestive of more closely packed cells in the cerebella of zinc-deficient pups than of ad libitum-fed controls.

Histologically, the effects of dietary treatment are not evident in the rat pup hippocampus. Although the vast majority of histogenesis of the hippocampus takes place postnatally, there was no sign of deviation from the normal rate and pattern of growth and differentiation, in contrast to the changes seen in the cerebellum. The appearance of the zone of cell proliferation and migration along aspects of the lateral ventricle and the overall shape and size were similar in the three groups. It was noted earlier that the cerebellar vermis might not be representative of the cerebellum as a whole with respect to vulnerability to nutritional insult. Therefore, other regions of the brain, including the hippocampus, might not be expected to manifest such dramatic signs of retardation. It is also possible that the hippocampus was not affected in its cross-sectional diameter but in its total longitudinal dimension as it courses underneath the cortex. It also seems possible that the hippocampus has a high priority for zinc at the expense of other tissues.

Timm's stain also failed to demonstrate differences between dietary groups, despite the fact that the deposits of zinc in the hippocampus associated with this stain accumulate in the postnatal period (9). The results of the staining might also be due to the fact that the hippocampus is high in other heavy metals including lead (11), and this might have a masking effect on any change in zinc levels. Second, there is no evidence that Timm's stain reacts quantitatively, i.e., changes in zinc concentration within certain limits might not be reflected in the stain intensity. Finally, it is possible that the changes in hippocampal weight and composition in response to dietary treatment may be due to decreased levels of circulating zinc or to a generalized decrease in brain growth rate rather than to a local decrease in hippocampal zinc content.
Tissue analysis of the hippocampus yielded results comparable to those found in the cerebellum. Concentrations of water, RNA, and protein remained relatively constant whereas total amounts of these components and of DNA were significantly lower in the zinc-deficient animals. The slightly higher concentrations of DNA and lower protein-to-DNA ratio are suggestive of smaller, more closely packed cells. As in the cerebellum, the effect seems to be a reduction in the rate of cell proliferation or accumulation, rather than a drastic change in the pattern of hippocampal development. Histological examination of the longitudinal dimensions of the hippocampus and of the germinative cells in the floor of the lateral ventricle might reveal the nature of the morphological effects of zinc deficiency on hippocampal development.

The histologic data from this investigation are consistent with previous studies in which retarded cerebellar development was found to result from postnatal undernutrition (3, 4, 15). We observed an additional effect in the zinc-deficient animals which cannot be explained solely on the basis of undernutrition associated with zinc deficiency. The compositional data also indicate that zinc deficiency affects the developing cerebellum to a greater extent than does undernutrition alone. Although morphologic alterations were not detected in the hippocampus of the zinc-deficient pups, the effects of zinc deficiency on DNA, RNA, protein, and water content were similar to those seen in the cerebellum. Again, the effects of zinc deficiency were more severe than those of pair feeding and thus indicate that inanition is not the sole causative factor. The precise mechanism whereby the effects of dietary zinc deficiency are mediated in such a manner as to disturb development of the central nervous system remain yet unclear.

REFERENCES


