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Synapse-to-neuron ratios in rat cerebellar cortex following lengthy periods of undernutrition

M. A. WARREN AND K. S. BEDI*

*Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, England and * Department of Anatomy, University of Queensland, St Lucia, Brisbane* 4067, *Australia*

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INTRODUCTION

The brain is vulnerable to even relatively short periods of undernutrition during early life, causing deficits and distortions of brain structure (Bedi, 1987; Bedi & Warren, 1988). This is especially so if these periods coincide with the 'brain growth spurt', i.e. at a time when the brain is going through its rapid phase of growth (Dobbing, 1981). However, as there is considerable inter-regional variation in the timing of the growth spurt, a given period of undernutrition can cause differential effects in the various brain regions.

In recent years there have been a number of quantitative light and electron microscopic studies on the effects of undernutrition during early life on the neurons, glial cells and synaptic populations of various brain regions (Dobbing, Hopewell & Lynch, 1971; Cragg, 1972; Siassi & Siassi, 1973; Dyson & Jones, 1976; Clos, Favre, Selme-Matrat & Legrand, 1977; Thomas, Bedi, Davies & Dobbing, 1979; Bedi, Hall, Davies & Dobbing, 1980; Thomas *et al.* 1980; Warren & Bedi, 1984; Ahmed, Bedi, Warren & Kamel, 1987). Some of these studies (see Bedi, 1984, 1987 for reviews) have been concerned particularly with quantifying synapse-to-neuron ratios, as these can be regarded as a measure of inter-neuronal connectivity (Cragg, 1972; Bedi, 1984).

The brain regions examined in most of these studies have been from the cerebral hemispheres. Yet it is known that undernutrition during early life usually causes the cerebellum to have a more marked deficit in weight than the rest of the brain (Bedi, 1984). There are relatively few studies on the effects of undernutrition during early life on the inter-neuronal connectivity of the cerebellar cortex. This is despite the fact that probably more information is available about the development, cytoarchitecture and cellular connectivity of the cerebellum than for any other brain region (see Jacobson, 1978 for a review).

In a previous study on the cerebellar cortex we (Bedi *et al.* 1980) found that 30 days old rats, undernourished from birth, had a large and significant deficit in the synapse-to-granule cell ratio. This deficit disappeared by 160 days of age following nutritional rehabilitation.

However, it was uncertain from this experiment whether or not the period of nutritional rehabilitation was an important factor in contributing to the 'catch-up' observed in the synapse-to-neuron ratio. It is possible that 'catch-up' may have occurred without the need for a period of nutritional rehabilitation. Also, a much longer period of undernutrition may have additional or more permanent effects on the synapse-to-neuron ratio within the cerebellar cortex. This is likely to be an important scientific and medical question; undernutrition of human populations is not merely

restricted to a short period of time during early life but may often extend into, or recur in, adult life. There is no other study currently reported in the literature which has examined this question in relation to the cerebellum.

METHODS

Animals and tissue preparation

Virgin Black and White hooded Lister rats were mated and housed in individual cages. Eighteen days after conception, these rats were assigned randomly to either control or experimental groups. Both groups were fed the same good quality food pellets (Oxoid breeding diet, Oxoid Ltd, UK) throughout the pregnancy and the suckling period. However, the experimental dams received about half the quantity of food eaten by the controls, which were fed *ad libitum*.

At birth the size of all litters was standardised to contain eight pups, with as many males as possible up to a maximum of six. At 21 days of age the male pups from both the well-fed and undernourished mothers were separated and housed in individual cages; female pups were discarded. Undernutrition of these post-weaning rats was continued by feeding them about half the quantity of the same good quality food eaten by age-matched controls fed *ad libitum*. Rats were undernourished until either 75 or 150 days of age. One group was undernourished until 75 days of age and then nutritionally rehabilitated until 150 days by allowing them unrestricted access to food. All rats were kept in a room maintained at 20 ± 1 °C and illuminated on a 12 hour red/white light cycle. Fresh water was freely available to all animals throughout the experiment.

Eight rats, which were not siblings, from each group were anaesthetised by intraperitoneal injection of sodium pentobarbitone (Sagatal, May & Baker Ltd, UK) at each of 21, 75 and 150 days of age and killed by intracardiac perfusion. The perfusate contained 2·5% sodium cacodylate-buffered glutaraldehyde (pH 7·3) pre-warmed to body temperature. The brains were removed and the cerebellum was dissected from the rest of the brain by making a vertical cut between the superior and inferior colliculi (Zeman & Innes, 1963).

A thin slice of tissue cut in the parasagittal plane, approximately 1 mm thick, was taken from the right paravermal region of each cerebellum. Lobes 4, 5 and 6 were dissected from each slice of tissue and each lobe was further subdivided into two approximately equal-sized pieces. These pieces of tissue were processed for electron microscopy by routine procedures and embedded in resin.

Stereological procedures

Numerical density of granule cells (N_{Vg})

For each animal two or three blocks of tissue were selected at random and from each a 0·5 μm thick toluidine blue-stained section was prepared. Each section contained the entire depth of the cortex cut in the parasagittal plane. The numerical density of granule cells was estimated from these sections by using the procedures outlined in detail in a previous paper (Bedi *et al.* 1980). Briefly, these procedures involved measuring the major (a) and minor (b) axes of several hundred granule cell nuclear profiles for each animal. These measurements were used to estimate the diameters (d) of the profiles by using the relationship $d = \sqrt{ab}$. The measurements were accomplished by using a light microscope fitted with a drawing tube which was projecting over a computer-linked digitising tablet. Profile diameters were corrected for the effects of sectioning by using the Schwartz–Saltykov unfolding procedure

(Underwood, 1970) and assuming that the granule cell nuclei were spherical. The numerical density of granule cell nuclei (and hence granule cells) was estimated by using the formula (Underwood, 1970):

$$N_{Vg} = N_{Ag}/\bar{D}_g + t,$$

N_{Ag} = the number of granule cell nuclear profiles per unit area of section, $\bar{D}_g$ = mean granule cell nuclear diameter and t = section thickness.

Numerical density of synapses (N_{Vs})

The same blocks of tissue as those used for the granule cell measurements were trimmed and sectioned for electron microscopy on a Reichert ultramicrotome. The sections were picked up on 400 mesh copper grids and examined with a Philips 301 electron microscope operated at an accelerating voltage of 80 kV. About 15 electron micrographs were taken at random from within the neuropil of the granular layer in one section from each block. The micrographs were taken at a nominal magnification of $\times 11000$ and printed to a final magnification of about $\times 35000$. A micrograph of a grating replica having 2160 lines/mm was taken as a magnification standard on each film used.

The counting unit used to estimate synapses was the synaptic membrane thickenings. It was assumed that each synapse possessed one such thickening and that it was approximately disc-shaped (subsequently referred to as synaptic discs). The diameters of all the synaptic disc profiles appearing within the counting frame (Gundersen, 1977) of electron micrographs from a given animal were measured and counted. These data were analysed by an unfolding procedure (identical to that used for the granule cell nuclei) in order to estimate the mean diameter of the synaptic discs. The numerical density of the synaptic discs (and hence synapses) was estimated by using the formula (Underwood, 1970):

$$N_{Vs} = N_{As}/\bar{H} + t,$$

where N_{As} = the number of synaptic disc profiles per unit area, t = section thickness and $\bar{H} = \pi/4 \times \bar{D}_s$ ($\bar{D}_s$ = mean synaptic disc diameter).

These estimates of synaptic numerical density were on a per unit volume of neuropil basis. They were converted to estimates on a per unit volume of granular cell layer by taking account of the volume proportions of granule cells and neuropil per granule cell layer. These estimates of volume proportions were derived by point count analysis (Weibel, 1979) of the granule cell layer in the light microscope sections described above.

Synapse-to-neuron ratios were obtained by dividing the numerical density of synapses per granular cell layer with the numerical density of granule cells per granular cell layer.

Statistics

Initially all values were calculated for each individual animal. These were then pooled and the mean and standard errors calculated for each group. Results were analysed with two-way analysis of variance procedures (Sokal & Rohlf, 1981) with *post hoc* tests where appropriate. Some group differences were investigated by using Student's t test.

Table 1. *Mean and standard error body (g) and cerebellar (mg) weights of control and experimental rats*

Age (days)	Feature	Control	Under-nourished	Previously undernourished
21	Body weight	42·8 ± 1·3	17·5 ± 0·3**	—
	Cerebellar weight	219 ± 7	158 ± 5**	—
75	Body weight	337·4 ± 9·4	167·1 ± 3·2**	—
	Cerebellar weight	329 ± 12	248 ± 6**	—
150	Body weight	460·0 ± 15·4	242·6 ± 2·9**	***345·7 ± 4·6**
	Cerebellar weight	388 ± 10	309 ± 8**	***352 ± 4**

There were 8 rats in each group. ** $P < 0.01$ compared with controls. *** $P < 0.01$ compared with undernourished.

Table 2. *Data on granule cells in the cerebellar cortex of control and experimental rats*

Age (days)	Feature	Control	Under-nourished	Previously undernourished
21	Nuclear diameter (μm)	5·64 ± 0·06	5·59 ± 0·06	—
	Numerical density[a] ($\times 10^6$ mm^{-3})	3·17 ± 0·11	3·52 ± 0·09**	—
75	Nuclear diameter (μm)	5·43 ± 0·06	5·24 ± 0·10	—
	Numerical density[a] ($\times 10^6$ mm^{-3})	2·82 ± 0·12	2·89 ± 0·18	—
150	Nuclear diameter (μm)	5·37 ± 0·11	5·52 ± 0·08	5·74 ± 0·07*
	Numerical density[a] ($\times 10^6$ mm^{-3})	2·45 ± 0·13	2·66 ± 0·09	2·26 ± 0·12***

Results are means and standard error. There were 8 rats in each group. * $P < 0.01$ compared with control. ** $P < 0.05$ compared with control. *** $P < 0.05$ compared with undernourished.

[a] Numerical densities of granule cells estimated on the basis of the number of granule cells per unit volume of granule cell layer.

RESULTS

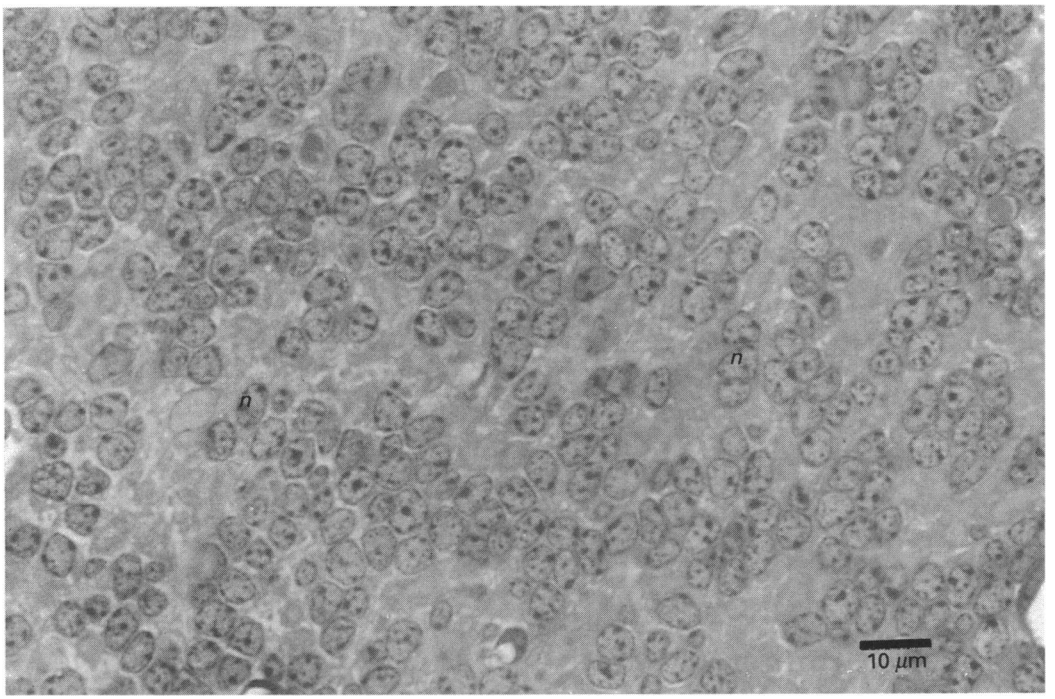
Body and cerebellar weights

Table 1 shows that undernourished rats had substantial deficits in both body and cerebellar weights compared with age-matched well-fed controls. These deficits persisted even in those animals allowed a period of nutritional rehabilitation. The ANOVA tests showed significant ($P < 0.01$) effects of age and nutrition, for both body and cerebellar weights (Table 5). The interaction component was also statistically significant for body, but not cerebellar, weight.

Granule cells

Figure 1 shows the granule cell layer of the cerebellar cortex taken from a 21 days old control animal. Comparison of many such micrographs did not reveal any obvious qualitative differences between the different groups of animals in this study.

Two-way ANOVA tests on the granule cell nuclear diameter data (see Tables 2 and 5) from 21, 75 and 150 days old control and undernourished rats revealed a significant effect of age but not of nutrition. The interaction component was not significant. However, 150 days old rats, previously undernourished from birth until 75 days of age, had granule cell nuclei that were significantly ($P < 0.01$) larger than age-matched controls.



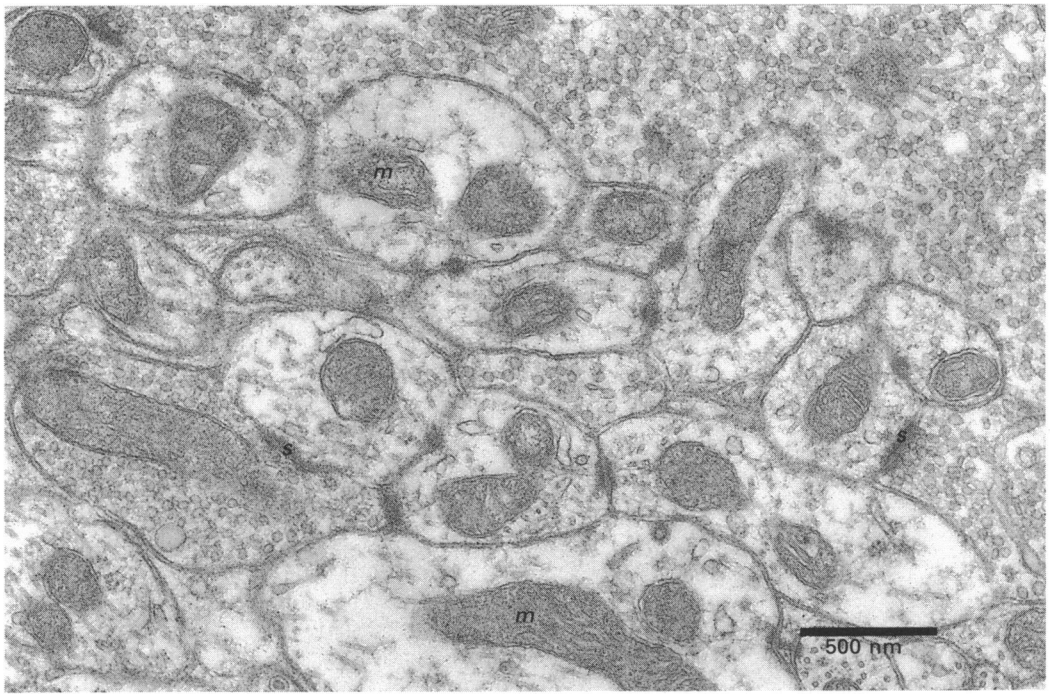


Fig. 1. Light micrograph of a 0·5 μm thick, toluidine blue-stained section of cerebellar cortex from a 21 days old control rat. *n*, granule cell nucleus.
Fig. 2. Electron micrograph of cerebellar cortex from a 21 days old control rat. *s*, synaptic thickening; *m*, mitochondria.

Table 3. *Data on synapses in the cerebellar cortex of control and experimental rats*

Age (days)	Feature	n	Control	n	Under-nourished	n	Previously undernourished
21	Disc diameter (nm)	7	211·0±5·8	6	208·4±6·6		—
	Numerical density ($\times 10^9$ mm^{-3})	7	1·037±0·037	6	0·894±0·064		—
75	Disc diameter (nm)	7	177·7±4·6	8	176·9±4·0		—
	Numerical density ($\times 10^9$ mm^{-3})	7	1·990±0·098	8	1·760±0·128		—
150	Disc diameter (nm)	7	201·4±4·3	8	192·5±2·9	8	194·0±3·4
	Numerical density ($\times 10^9$ mm^{-3})	7	1·335±0·078	8	1·594±0·061*	8	1·461±0·068

Results are means and standard errors. n, number of rats. * $P < 0.05$ compared with controls.

Table 4. *Synapse-to-neuron ratios in the granular layer of the cerebellar cortex of control and experimental rats*

Age (days)	n	Control	n	Under-nourished	n	Previously undernourished
21	7	338±18	6	258±20*		—
75	7	707±52	8	626±53		—
150	7	539±27	8	600±17	8	655±38*

Results are means and standard errors. n, number of rats. * $P < 0.05$ compared with controls.

Table 5. *Two-way ANOVA on data from* 21, 75 *and* 150 *days old control and undernourished rats*

Source	F-values					
	D.F.	Nutrition	D.F.	Age	D.F.	Interaction
Body weight	1,42	355·36**	2,42	766·71**	1,42	73·30**
Cerebellar weight	1,42	118·44**	2,42	193·52**	1,42	0·85
Granule cell nuclear diameter	1,42	0·15	2,42	5·96**	1,42	2·74
Numerical density of granule cells	1,42	4·27*	2,42	21·02**	1,42	0·64
Synaptic disc diameter	1,37	0·75	2,37	26·22**	1,37	0·71
Numerical density of synapses	1,37	0·21	2,37	52·94**	1,37	4·73*
Synapse-to-neuron ratio	1,37	1·11	2,37	53·14**	1,37	2·62

D.F., degrees of freedom. * $P < 0.05$. ** $P < 0.01$.

The numerical density of granule cells per unit volume of granular cell layer was significantly ($P < 0.05$) greater in 21 days old undernourished rats than in age-matched controls (Table 2). No such differences between control and undernourished rats were observed in 75 or 150 days old animals. These differences were reflected in the ANOVA test on these data by significant effects of age and nutrition (Table 5).

One hundred and fifty days old rats, previously undernourished until 75 days of age, had fewer granule cells per unit volume than rats continually undernourished until 150 days of age (Table 2).

Synapses

Figure 2 shows an electron micrograph taken in the neuropil of the granular layer. This shows the profiles of several post-synaptic densities (synaptic discs). Once again examination of many such micrographs did not reveal any obvious qualitative differences between the various groups of animals in this study.

Table 3 shows that the mean diameter of these synaptic discs did not differ between control and experimental animals, at any of the ages examined in this study. However, in the ANOVA test on these data there was a significant ($P < 0{\cdot}01$) effect of age (Table 5).

Table 3 also shows the data on the numerical densities of synapses per unit volume of granular layer. There were no significant differences between control and undernourished rats at 21 and 75 days of age. The 150 days old undernourished rats had a significantly ($P < 0{\cdot}05$) greater numerical density of synapses than age-matched controls. This difference was also reflected in the ANOVA test on these data, where there was a significant ($P < 0{\cdot}05$) interaction component between age and nutrition (Table 5).

The numerical density of synapses in 150 days old rats, previously undernourished until 75 days of age, was not significantly different from that observed in either age-matched controls or rats undernourished continually for 150 days (Table 3).

Synapse-to-neuron ratios

At 21 days of age undernourished rats had, on average, about 258 synapses per granule cell compared with a value of 338 for age-matched controls (Table 4). This difference was significant at the 5% level. By 75 days of age the mean synapse-to-granule cell ratio had increased in both control and undernourished rats to values of 707 and 626 respectively (Table 4); these were not significantly different. The value for well-fed controls declined to about 539 by 150 days of age. In contrast, synapse-to-granule cell ratios remained steady at about 600 for rats undernourished from birth to 150 days of age. These changes were reflected in the ANOVA test by a highly significant ($P < 0{\cdot}01$) effect of age (Table 5). There was no significant overall effect of nutrition, although the interaction component was significant at the 5% level (Table 5).

The average synapse-to-granule cell ratio in 150 days old rats, previously undernourished from birth until 75 days of age, was 655 (Table 4). This was significantly ($P < 0{\cdot}05$) larger than that observed in the well-fed age-matched controls, but not greater than in the rats continuously undernourished until 150 days of age (Table 4).

DISCUSSION

In our experiments, the synapse-to-neuron ratio in the granular layer of the cerebellar cortex showed a significant deficit in undernourished rats compared with well-fed controls at 21 days of age. This finding confirms the results of our previous experiment where we found that 30 days old rats, undernourished from birth, had a substantial deficit in this ratio (Bedi *et al.* 1980). In this previous study we found that the deficit disappeared in 160 days old rats which had been nutritionally rehabilitated from 30 days of age. However, we were unsure whether or not this period of nutritional rehabilitation was essential to remove the deficit. The results of our present experiment show that nutritional rehabilitation is not necessary to remove the deficit in the synapse-to-neuron ratio induced by undernutrition during early postnatal life.

The deficit disappeared by 75 days of age despite the continuing undernutrition of the rats. Furthermore, even when the period of undernutrition was extended up to 150 days of age, the synapse-to-neuron ratio appeared to remain approximately the same as in control animals matched for age. The estimates of synapse-to-neuron ratios in the present study were based on traditional stereological methods. More recent counting techniques (e.g. Gundersen, 1986) are likely to prove the method of choice in future work, but were not in common use when the present study was begun.

It is tempting to conclude that undernutrition during early life merely delays the formation of the synaptic connections in this brain region and causes no permanent effects. However, some caution should be exercised before accepting such a conclusion. It seems that a period of undernutrition from birth to 75 days of age does have some long lasting effects on the development of the interneuronal connections. These appear to manifest themselves, at least in quantitative terms, only during a subsequent period of nutritional rehabilitation. Thus, the 150 days old nutritionally rehabilitated animals which had been subjected to undernutrition from birth until 75 days of age had a significantly higher synapse-to-neuron ratio than the age-matched controls. Such a finding is not unique; we have obtained similar results for the synapse-to-granule cell ratio in the hippocampal formation (Ahmed *et al.* 1987) and the synapse-to-neuron ratio in the visual cortex (Warren & Bedi, 1984).

It is possible that the capacity to increase the degree of interneuronal connectivity during a period of nutritional rehabilitation following undernutrition during early life is a general phenomenon. It may reflect an adaptive potential of the central nervous system to ameliorate sub-optimal environmental conditions. It also suggests that considerable synaptogenesis in the brain can continue well into adult life and gives an indication of the degree of morphological remodelling possible. The exact mechanisms involved which govern and control the morphological plasticity are, at present, unknown.

It should also be emphasised that the functional implications of an initial deficit and subsequent 'catch up' in the synapse-to-neuron ratio are unknown. The methods used in this study give an estimate of the average number of synapses associated with a neuron; no information is available on the qualitative distribution of synapses in control and undernourished animals nor of their functional capacity. It is easy to imagine that the apparent delay in the development of the synapse-to-neuron ratio observed in the undernourished rats could be part of a series of changes including alteration in the qualitative distribution of synapses within the brain region. This could of course result in subtle changes in the interneuronal connections and pathways which, in turn, could affect the normal functioning of the brain.

The changes in synapse-to-neuron ratio with age in both control and undernourished rats are worthy of further comment. Both groups showed a marked increase in this ratio between birth and 75 days of age. Thereafter, the ratio appeared to level off in the undernourished rats whereas it decreased in the control animals. This finding of an initial overproduction and a subsequent loss of synapses has also been observed in other brain regions (Warren & Bedi, 1984; Ahmed *et al.* 1987). It may therefore be a general phenomenon during brain development and of some considerable functional importance.

In conclusion, our results have shown that nutritional rehabilitation is not a prerequisite to restore the deficit in the cerebellar synapse-to-granule cell ratio induced by a period of undernutrition during early postnatal life. However, the observation that a period of nutritional rehabilitation of rats, undernourished from birth until 75 days of age, can result in a significantly greater synapse-to-granule cell ratio than in

control animals indicates that such undernutrition can have subtle and long lasting effects on the development of the interneuronal connectivity of the cerebellum. Although the functional significance (if any) of these changes is unknown at present, as similar findings have been reported for other brain regions, it is possible to speculate that they represent a general adaptive response to the effects of prolonged periods of undernutrition during early life.

SUMMARY

Black and white hooded Lister rats were undernourished for various times up to 150 days of age; some of them were nutritionally rehabilitated from 75 days. Undernourished rats weighed significantly less than well-fed controls at all ages studied. After embedding in resin, sections of cerebellar cortex were cut and examined at the light and electron microscopical levels using traditional morphometric methods.

Undernourished rats showed significant deficits in synapse-to-neuron ratio, compared with controls, at 21 days of age. This deficit disappeared by 75 days despite continued undernutrition. Indeed, there was no alteration in this ratio even when undernutrition was extended up to 150 days even though the ratio for the controls decreased after that period. Rats undernourished from birth to 75 days and subsequently rehabilitated to 150 days had significantly more synapses per neuron than controls. The functional sequelae of these morphological changes remain unknown.

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