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The effects of a lengthy period of undernutrition from birth and subsequent nutritional rehabilitation on the granule-to-Purkinje cell ratio in the rat cerebellum

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

*Department of Anatomy and Cell Biology, University of Sheffield, Western Bank, Sheffield S10 2TN and * Department of Anatomy, University of Aberdeen, Marischal College, Aberdeen AB9 1AS*

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INTRODUCTION

In previous experiments (Bedi, Hall, Davies & Dobbing, 1980) we have shown that the cerebella of rats undernourished for the first 30 days of postnatal life have permanent deficits in their granule-to-Purkinje cell ratios. The question arises whether or not much longer periods of undernutrition have any additional effects on this ratio.

There are no previously published reports on the effects of a lengthy period of undernutrition on the numerical densities of nerve cells in the cerebellum. Most reports to date have been concerned with rats which have been undernourished for relatively short periods of time before 35 days of postnatal age, either during the suckling period (e.g. Neville & Chase, 1971; Altman & McCrady, 1972; Dobbing, Hopewell & Lynch, 1971; McConnel & Berry, 1978*a*,*b*, 1981; Bedi *et al.* 1980), or during gestation and suckling periods (e.g. Barnes & Altman, 1973*a*,*b*; Clos, Favre, Selme-Matrat & Legrand, 1977; but see also Hillman & Chen, 1981).

The present paper reports an investigation into the effects of undernutrition from just before birth until 150 days of age, with some rats being nutritionally rehabilitated from 75 days of age. Particular attention has been paid to studying the numerical densities of cerebellar granule and Purkinje cells as well as granule-to-Purkinje cell ratios.

MATERIALS AND METHODS

Black and white hooded Lister rats were used in this study. Virgin females were mated and housed singly in standard plastic cages in an animal house kept at $20° \pm 1$ °C and illuminated on a 12 hours red/white light cycle. Some of these rats were undernourished from the 18th day of gestation through to the end of their lactation period, whilst others were allowed to feed *ad libitum*. At birth litters were standardised to contain eight pups with as many males as possible up to a maximum of six. The undernourished dams received an amount of diet (Oxoid Breeding Diet, Oxoid Ltd., Hampshire) corresponding to half that eaten by control mothers fed *ad libitum*. This amounted to 15 g from the 18th day of pregnancy until 6 days *post partum*, 20 g from Day 7 to Day 15, and 25 g from Day 16 to Day 25. All male pups from both control and undernourished mothers were weaned into separate cages on Day 21.

The pups raised by the undernourished mothers were further deprived of food by giving them only half the amount of normal diet eaten by age-matched controls. The

actual amounts of food given to the undernourished rats have been described in detail previously (Ahmed, Bedi, Warren & Kamel, 1987). The period of undernutrition for some pups was continued until 150 days of age. Other pups were nutritionally rehabilitated between 75 and 150 days of age. Nutritional rehabilitation was achieved by allowing the previously undernourished rats access to an unlimited supply of the normal food pellets. Drinking water was available to all rats throughout the experiment.

Groups of control and experimental rats were killed at 21, 75 and 150 days of age. There were 8 control and 8 experimental rats in each age group. These animals were randomly selected from eight separate litters for each age group. Animals were killed by intracardiac perfusion with 2·5% cacodylate-buffered glutaraldehyde, after being anaesthetised with sodium pentobarbitone (Sagatal; May & Baker Ltd, England). The brains of these animals were removed between two and five hours after killing. The cerebellum of each brain was separated from the cerebral hemispheres and brainstem by cutting between the superior and inferior colliculi in the transverse fissure (Zeeman & Innes, 1963). The weights of the individual cerebella were determined using a Sartorius balance capable of measuring with a precision of 0·1 mg.

An approximately 1 mm thick coronal slice of tissue located in the paravermal region of the right hemisphere was obtained from each cerebellum. From this, six pieces of cortical tissue, taken from lobes IV, V and VI, were obtained such that each piece contained the entire depth of the cortex. This tissue was then washed in several changes of buffer, postfixed in 1% osmium tetroxide for about two hours and processed for embedding in Spurr's resin (Spurr, 1969). This resulted in six blocks of tissue being available for each animal.

A 0·5 μm thick section which included the whole cortical depth was cut from each block and stained with toluidine blue. These sections were used to estimate the numerical densities of both Purkinje and granule cells as well as granule-to-Purkinje cell ratios. The stereological procedures used to make these estimates have been described in detail in our previous papers (e.g. Bedi *et al.* 1980) and so are given only in outline form here.

These procedures involved measuring the profile diameters of randomly selected samples of granule and Purkinje cell nuclei which were used as the counting units for these cells. It was assumed that these nuclei were spherical. Several hundred nuclear profiles of each cell type were measured for each animal. The major (a) and minor (b) axes of each profile were measured and the diameter computed by calculating $\sqrt{a \cdot b}$. All measurements were conducted with the aid of a camera lucida attachment fitted to a light microscope and a digitising tablet linked to a BBC microcomputer.

In order to estimate the true mean diameter of the granule cell nuclei from measurements of the profile diameters the Schwartz–Saltykov 'unfolding' procedure was used as described previously (Bedi *et al.* 1980). For Purkinje cells it was assumed that their nuclei contained a centrally located nucleolus. Measurements of Purkinje cell nuclear profiles showing a nucleolus were thus regarded as yielding the true nuclear diameter directly (Bedi *et al.* 1980).

Numerical densities of granule and Purkinje cells were calculated by the formula (Underwood, 1970):

$$Nv = Na/\bar{D} + t,$$

where Nv = number of cells per unit volume of tissue, Na = number of nuclear profiles per unit area of section, $\bar{D}$ = mean nuclear diameter, and t = section thickness.

The estimates of numerical density for both granule and Purkinje cells were made

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

Age (days)		Control	Undernourished	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 control, ‡‡ $P < 0·01$ compared with undernourished.

on the basis of the number of cells per unit volume of whole cortex as described previously (Bedi *et al.* 1980). Granule-to-Purkinje cell ratios were estimated simply by dividing the numerical density of granule cells by that of Purkinje cells.

Statistics

Initially weights, numbers and ratios were calculated for each individual animal. These were later pooled to estimate the mean ± S.E. for each of the control and experimental groups at every age examined. Differences between groups were tested using a Student's *t*-test, and the data were also analysed by two-way analysis of variance (ANOVA) procedures (Sokal & Rohlf, 1981). Two-way ANOVA tests were carried out on data from 21, 75 and 150 days old control and undernourished rats to determine the overall effects of age and nutrition on each variable measured.

RESULTS

Body and cerebellar weights

The mean ± S.E. body and cerebellar weights are given in Table 1. Undernourished rats had substantial deficits in body weight compared to well-fed controls at all ages examined. These deficits ranged from about 58% at 21 days of age to about 47% at 150 days of age. One hundred and fifty days old rats, previously undernourished until 75 days of age, also showed persisting deficits in terms of body weight. Two-way ANOVA tests on the body weight data from 21, 75 and 150 days old control and undernourished rats revealed significant main effects of nutrition and age as well as a significant interaction between them (Table 3).

The mean cerebellar weights of undernourished rats were also significantly smaller than age-matched controls at 21, 75 and 150 days of age (Table 1). Once again this deficit persisted, even in those rats allowed a period of nutritional rehabilitation between 75 and 150 days of age. Two-way ANOVA tests on the cerebellar weight data revealed significant main effects of nutrition and age but no significant interaction between these factors (Table 3).

Numerical densities of granule and Purkinje cells

Data on the numerical densities of granule and Purkinje cells are shown in Table 2. At 21 days of age undernourished rats had significantly more granule and Purkinje cells per unit volume of cerebellar cortex than well-fed controls. By 75 days these differences had disappeared despite the continuing undernutrition. However, 150 days

Table 2. *Data on cerebellar granule and Purkinje cells in control and experimental rats*

Age (days)		Control	Undernourished	Previously undernourished
21	N_{vg}	1·51 ± 0·08	1·74 ± 0·07*	—
	N_{vp}	4603 ± 239	5630 ± 284*	—
	G:P	336 ± 30	312 ± 14	—
75	N_{vg}	1·27 ± 0·06	1·33 ± 0·09	—
	N_{vp}	3068 ± 172	3229 ± 179	—
	G:P	428 ± 39	413 ± 25	—
150	N_{vg}	1·15 ± 0·08	1·19 ± 0·06	1·01 ± 0·08
	N_{vp}	2952 ± 182	3681 ± 132**	‡3220 ± 145
	G:P	392 ± 19	327 ± 21*	315 ± 27*

N_{vg} = Numerical density of granule cells ($\times 10^6$/mm^3), N_{vp} = Numerical density of Purkinje cells (/mm^3), G:P = Granule-to-Purkinje cell ratios.

Each value represents the mean ± S.E. of 8 animals.

* $P < 0·05$ compared with controls, ** $P < 0·01$ compared with controls, ‡ $P < 0·05$ compared with undernourished.

Table 3. *Two-way ANOVA on data from* 21, 75 *and* 150 *days old control and undernourished rats*

Source	F-Values		
	Nutrition (D.F. = 1, 42)	Age (D.F. = 2, 42)	Interaction (D.F. = 2, 42)
Body weight	355·36**	766·71**	75·30**
Cerebellar weight	118·44**	193·52**	0·85
Numerical density of granule cells	4·15*	21·36**	0·84
Numerical density of Purkinje cells	14·71**	57·14**	2·33
Granule-to-Purkinje cell ratio	2·65	7·14**	0·54

D.F. = degrees of freedom, * $P < 0·05$, ** $P < 0·01$.

old rats, undernourished from birth, had a significantly greater density of Purkinje cells, although not granule cells, than age-matched controls (Table 2). One hundred and fifty days old rats, undernourished until 75 days of age and then rehabilitated, had a significantly lower density of Purkinje cells compared with rats undernourished continuously until 150 days of age, but not when compared to age-matched controls (Table 2). Two-way ANOVA tests on the data for both the granule and Purkinje cell numerical densities showed significant main effects of both nutrition and age but no significant interaction between them (Table 3).

Granule-to-Purkinje cell ratios

There were no significant differences in the granule-to-Purkinje cell ratios between control and undernourished rats at either 21 or 75 days of age (Table 2). At 150 days of age control rats had 392 ± 19 (mean ± S.E.) granule cells per Purkinje cell. This was significantly greater than the values of 327 ± 21 and 315 ± 27 observed in age-matched undernourished rats and in rats previously undernourished until 75 days of age

respectively (Table 2). Two-way ANOVA tests on the granule-to-Purkinje cell ratio data showed a significant main effect of age but not of nutrition and with no significant interaction between them (Table 3).

DISCUSSION

Estimates of numerical density such as the number of granule or Purkinje cells per unit volume of tissue are difficult to interpret as they can be influenced both by a change in the number of cells and/or by a change in the volume of the tissue (Bedi, 1984, 1987). However, the observation made in the present experiments that undernourished rats can have a greater numerical density of granule and Purkinje cells than age-matched controls confirms many previous findings made in this and other laboratories. The explanation usually offered for this is that undernutrition during early life delays the maturation of the dendritic arborisation of neurons, hence causing a deficit in the cortical volume. This results in the neurons being packed into a smaller volume of tissue, thus giving an increased numerical density of neurons in the undernourished animals.

These problems of interpretation can be overcome to some extent by using estimates of numerical density to calculate ratios such as the granule-to-Purkinje cell ratio. Changes in this ratio then show relative alterations of the two components, independently of changes in the volume of the cortex (Bedi, 1984).

In the present experiments we have found that rats undernourished from about birth until either 21 or 75 days of age had no significant differences in their granule-to-Purkinje cell ratios compared with age-matched controls. However, 150 days old rats which had been undernourished from birth did have a significant deficit in this ratio. Rats which had been undernourished until 75 days of age and then nutritionally rehabilitated until 150 days also showed a significant deficit in the average number of granule cells associated with each Purkinje cell.

This last finding is of particular interest as it occurred despite the fact that 75 days old undernourished rats had no significant deficit in the ratio. This indicates that undernutrition during early postnatal life can cause long term changes in the morphological development of the cerebellum, some of which may become apparent after, rather than during, the actual period of undernourishment.

Purkinje cells in the rat cerebellum arise mainly, if not exclusively, during the prenatal period (Jacobson, 1978). It is therefore unlikely that undernutrition during postnatal life could actually cause an increase in their number. Therefore, the deficit in the granule-to-Purkinje cell ratio seen in our 150 days old rats suggests strongly that the period of undernutrition imposed in the present experiments caused a reduction in the number of granule cells. This reduction could have been due either to an actual loss of granule cells and/or to a partial failure in the production of these cells. In the rat, granule cells are known to be produced during the first few weeks of postnatal life (Jacobson, 1978).

The observation that the 75 days old undernourished rats did not have a significant deficit in the granule-to-Purkinje cell ratio is at variance with some of our own previous work as well as that of other researchers. For example in other experiments we found that undernutrition from birth to just 30 days of age was sufficient to cause fairly substantial deficits in the granule-to-Purkinje cell ratio (Bedi *et al.* 1980). Other researchers have made similar observations (e.g. Clos *et al.* 1977; McConnel & Berry, 1981). The exact reasons for these discrepancies are unknown but they may be related to the severity and duration of the periods of undernutrition imposed in the separate

Table 4. *Some estimates of granule-to-Purkinje cell ratios in normal rat cerebellum published in the literature*

Author(s)	Age of rats (days)	Mean ± S.E.M. Granule:Purkinje cell ratio
Clos *et al.* (1977)	35	328 ± 18
Bedi *et al.* (1980)	30	395 ± 34
	160	335 ± 28
McConnel & Berry (1981)	10	126 ± 13
	15	308 ± 14
	20	493 ± 25
	30	427 ± 18
	80	454 ± 45
Hillman & Chen (1981)	60	356
Warren & Bedi (present paper)	21	336 ± 30
	75	428 ± 39
	150	392 ± 19

experiments. The deficits in body and cerebellar weights achieved in our previous study (Bedi *et al.* 1980) were greater than those in the present experiment because of some differences in the feeding regimes adopted.

Values for granule-to-Purkinje cell ratio obtained in the present experiments are in general agreement with those published elsewhere in the literature. For example Table 4 gives values for this ratio obtained by several groups of researchers on well-fed rats of various ages. This shows that, on average, 20 to 30 days old rats have between 300 and 400 granule cells associated with each Purkinje cell. The present experiments and the results published by McConnel & Berry (1981) indicate the possibility that this ratio may actually increase slightly but significantly between 30 and 80 days of age. The exact explanation for this possible increase is unclear at present. Cerebellar granule cells arise mainly during the first three weeks of postnatal life, but it is possible that they go on increasing in number at a slow rate after this period. On the other hand it is possible that some Purkinje cells may be lost during normal development after 30 days of age. Either of these possibilities could explain the observed increase in the granule-to-Purkinje cell ratio between about 30 and 80 days of age.

In conclusion we have found substantial deficits in the average number of granule cells associated with each Purkinje cell in rats subjected to a lengthy period of undernutrition beginning at birth. The deficit was also observed in nutritionally rehabilitated rats which had been previously subjected to a period of undernutrition. Analysis of the present evidence seems to suggest that these deficits in the granule-to-Purkinje cell ratios are due to a reduction in the number of granule cells.

SUMMARY

Male rats were undernourished for various lengths of time between birth and 150 days of age, with some rats being nutritionally rehabilitated between 75 and 150 days of age. Eight control and eight experimental rats were anaesthetised and perfused with 2·5% glutaraldehyde at each of 21, 75 and 150 days of age. Stereological procedures were used to estimate granule-to-Purkinje cell ratios in lobes IV, V and VI, using 0·5 μm thick toluidine blue-stained sections.

Undernourished rats had significantly lower body and cerebellar weights than

controls at all ages examined. These deficits persisted even after a period of nutritional rehabilitation. The granule-to-Purkinje cells ratio did not differ between control and experimental groups at 21 or 75 days of age. However, at 150 days both undernourished and rehabilitated groups of animals had significant deficits in this ratio compared with age-matched controls. These results suggest that undernutrition can have profound effects on brain development in later life even if the effects are not apparent during the period of undernutrition.

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