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The effects of undernutrition on connectivity in the cerebellar cortex of adult rats

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ABSTRACT

The effects of a 30 d period of undernutrition, followed in some animals by nutritional rehabilitation, on neuronal connectivity in adult rat cerebellum were investigated using the disector method. There was no significant difference between well fed (719 ± 74, mean ± S.E.) and undernourished (709 ± 53) synapse-to-neuron ratios in 134-d-old rat cerebellar cortex, nor was there a significant difference in synapse-to-neuron ratios between control animals (941 ± 71) and previously undernourished rats (813 ± 42) at 175 d of age. However, the age-related changes were significant ($P < 0.05$) in the controls, but not in the experimental group. It may be that the period of undernutrition caused subtle changes in the rehabilitating group which reduced the capacity for growth seen in well fed, matched control animals.

INTRODUCTION

Undernutrition during early life can have profound effects on the body and brain growth of rats (Dobbing et al. 1971; Cragg, 1972; Clos et al. 1977; Bedi et al. 1980; Berry et al. 1981; Hillman & Chen, 1981; McConnel & Berry, 1981; Warren & Bedi, 1985; Bedi, 1987). Some of the effects are permanent, whereas others disappear following lengthy periods of nutritional rehabilitation. The adult brain is thought to be resistant to the effects of undernutrition (Dobbing, 1980), although Warren et al. (1989) have shown that neuronal connectivity (measured as synapse-to-neuron ratios; Cragg, 1967) in the visual cortex of rats can be affected by a short period of undernutrition in early adult life. Despite its relatively simple structure, there is little published information on the effects of undernutrition on cerebellar connectivity. Thomas et al. (1979) reported significant deficits in the synapse-to-granule cell ratio in the cerebellar cortex of 30-d-old rats undernourished from birth, although this deficit disappeared following 130 d of nutritional rehabilitation (Thomas et al. 1980).

Warren & Bedi (1990) found that even though this early deficit in synapse-to-neuron ratio disappeared, even if undernutrition was extended up to 150 d, rats undernourished for 75 d from birth and rehabilitated to 150 d had significantly more synapses per neuron than controls well fed throughout life (Warren & Bedi, 1990).

In an attempt to investigate whether or not this apparent plasticity exists in the cerebella of adult rats, we have undernourished sexually mature rats for a short period of time and then allowed some of them unrestricted access to food to rehabilitate them nutritionally. Cerebellar connectivity (estimated by the synapse-to-granule cell ratio; Bedi et al. 1980; Warren & Bedi, 1990) was compared with age-matched controls which were well fed throughout life.

MATERIALS AND METHODS

Animals and tissue preparation

A total of 34 adult male Wistar rats were used. At birth litters were standardised to 8 pups (6 males, 2 females) and the male pups were weaned at 25 d of age when they were caged in randomly assigned pairs. By about 100 d rats were housed singly and all animals were allocated to 1 of 4 groups, 2 control and 2 undernourished. From 105 to 134 d, the 17 rats in the undernourished groups were fed 50% of the same

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Table 1. *Body and cerebellar weights of control and experimental rats at different ages*

Feature	Age (d)	n	Control	n	Experimental	% difference
Body weight (g)	134	8	401.6±17.5	9	245.7±7.3*	−39
	175	8	466.3±18.9†	9	417.8±9.4*	−10
	% difference		+16		+70	
Cerebellar weight (mg)	134	8	224.3±16.3	9	199.6±6.1	−11
	175	8	281.0±8.90††	9	268.6±5.0††	−4
	% difference		+25		+35	

Results are means±S.E.M.; n, number of rats; * $P < 0.01$ compared with controls; † $P < 0.05$ compared with 134 d value; †† $P < 0.01$ compared with 134 d value.

good-quality feed (CRM diet) as that eaten by the matched controls which were fed ad libitum. At 134 d, 9 undernourished and 8 well fed rats were selected at random; anaesthetised with chloroform and killed by transcardiac perfusion of 2.5% cacodylate-buffered glutaraldehyde prewarmed to body temperature and with a pH of ~ 7.3. The remaining animals were allowed unrestricted access to food until d 175 when they were anaesthetised and killed. All animals were housed in standard conditions where temperature was maintained at 22.5±1.5 °C and with a 12 h dark/light cycle. Fresh water was available to all animals throughout the experiment.

Four groups of animals were thus obtained: (1) C1 – well fed, killed at 134 d, n = 8; (2) C2 – well fed killed at 175 d, n = 8; (3) Un – undernourished, killed at 134 d, n = 9; (4) PU – previously undernourished and subsequently nutritionally rehabilitated, killed at 175 d, n = 9. After perfusion the brain of each rat was removed and the cerebellum separated from the rest of the brain by a cut in the transverse fissure between the superior and inferior colliculi (Zeeman & Innes, 1963). A horizontal cut separated the cerebellum from the rest of the hindbrain. Each cerebellum was weighed (after removal of the flocculonodular nodes) and then bisected by a sagittal cut. A thin (about 1 mm) parasagittal slice was taken from the right paravermal region of each cerebellum. From each of these slices lobes 4, 5 and 6 were removed and each lobe was further divided by cutting along the white matter to give a total of 6 pieces of tissue for every animal; that is 2 from lobe 4, 2 from lobe 5 and 2 from lobe 6. These regions of the cerebellum were the same as those taken in previous studies on the effects of undernutrition on the cerebellum (Warren & Bedi, 1990) and so enabled direct comparison of results from the current experiment to be made with published data. However, these regions are a small part of the whole cerebellum and it remains to be seen whether or not other areas of the cerebellum behave in the same way as lobes 4, 5 and 6 following nutritional intervention. The 6 pieces of cerebellum from each animal were embedded in Spurr's resin (Spurr, 1969) and sectioned for both light and electron microscopy. Following embedding and sectioning, 3 of the 34 rats anaesthetised and perfused (2 controls and 1 experimental from the 134-d-old group) were found to be inadequately fixed and so were rejected before performing any quantitative histological analysis. The n-values given in Table 1 (body and cerebellar weight data) therefore represent all 34 rats killed. The n-values in Tables 2, 3 and 4 (quantitative histological results) represent the 31 rats which were considered to be technically adequate for subsequent quantitative histological examination.

Estimates of granule cell density

Of the 6 blocks of tissue available from each animal 2 were chosen by lottery (Bedi et al. 1980; Warren and Bedi, 1990). From each of these, 4 serial semithin sections were cut in order to obtain the numerical density of granule cells (N_{Vg}). Each block face was lightly scored so that 2 sections were cut from each cutting cycle (Bedi, 1988). The larger of these was stained with toluidine blue, mounted in DPX and used for cell counts, while the other was air-dried, left unmounted and used to estimate section thickness using a Vickers M86 scanning and integrating microinterferometer (Goldstein & Hartmann-Goldstein, 1974). This method of estimating section thickness uses phase changes in light (from a laser, wavelength 632.8 nm) transmitted through the section and is reported to be both accurate and precise (Goldstein & Hartmann-Goldstein, 1974). However, it can be used only on unmounted sections owing to the effects of the different refractive indices of mountant and section. Therefore the section thicknesses obtained from the unmounted sections (a typical mean and S.E.M. value from a random selection of 6 sections is 0.42±0.01 μm)

may be different from the thickness of the mounted and stained sections used for counting. Even so, such differences are likely to be small and be constant for all groups, making these estimates useful for a comparative study such as the one reported here.

The stained sections were used to obtain estimates of granule cell numerical density using the disector (Sterio, 1984; Gundersen, 1986). Images were projected on 2 video screens of a Quantimet 970 image analyser at a final magnification of ×1900 (Cambridge Instruments, UK) and granule cell profiles present in the 1st section, but missing from section 4 were counted (ΣQ^-) if they fell within the unbiased counting frame (Gundersen, 1977). Counts were also made in the reverse direction so that at least 20 disectors were taken from each block, giving a minimum of 40 dissectors per cerebellum. Numerical density was found from:

$$N_{Vg} = \frac{\Sigma Q^-}{ND.A.h}$$

where ND = number of disectors used (at least 20 per block), A = frame area corrected for magnification and h = the distance between the 1st and 4th sections (sum of the section thickness). An estimate of mean granule cell nuclear 'height' ($\bar{H}$) was obtained from:

$$\bar{H} = \frac{\Sigma Q.h}{\Sigma Q^-}$$

where ΣQ = number of granule cells seen in the counting frame over the 1st section. In nonisotropic sections, such as those used in the present study, this value of 'height' is not equivalent to the traditional 'mean projected height' reported previously (e.g. Bedi et al. 1980), but is the mean length of nuclei perpendicular to the surface of the section (Sterio, 1984).

The same blocks of tissue were subsequently sectioned for electron microscopy after trimming to reveal only the granular layer. Two adjacent sections were cut, collected on Pioloform-coated slot grids and stained with uranyl acetate and lead citrate. The thickness of ultrathin sections can be measured using the scanning and integrating microinterferometer in a way similar to that described earlier for semithin sections. However, measurements of ultrathin sections, which are not usually surrounded in a mounting medium, can be made directly under the microinterferometer. A random sample of 6 such sections measured in this way gave a mean and S.E.M. of 57.7 ± 0.8 nm. Ten micrographs were taken in a random manner from the nuclear-free neuropil of the granule layer using the paramembranous density as the counting unit. The disector was used, as described above, to obtain estimates of numerical density of synapses. The magnification used was ×29000 with at least 20 disectors being used for each block, that is at least 40 disectors per cerebellum. These estimates gave the numerical density of synapses per unit volume of neuropil. They were converted to estimates on a per unit volume of granular cell layer basis by combining with estimates of granule cell nuclei per granule layer obtained by point counting (Weibel, 1979; Bedi et al. 1980). Synapse 'height' was obtained in a manner similar to that used for granule cell nuclei, and is equivalent to the mean length of synaptic densities perpendicular to the section surface.

Synapse-to-neuron ratios were obtained by dividing the numerical density of synapses per granular layer (N_{Vs}) by the numerical density of granule cells per granular cell layer (N_{Vg}) and may be regarded as a measure of neuronal connectivity (Bedi, 1984).

Statistics

Initially all values were calculated for each individual. These were then pooled to give means and standard errors for each group. Data were analysed using Student's t test with prior testing for normality and, if necessary, were transformed to render them suitable for analysis (Sokal & Rohlf, 1981). Two-tailed tests were considered most suitable since, although undernutrition has been reported to produce changes which may be predicted in some circumstances (e.g. Bedi et al. 1980; Warren & Bedi, 1990) unexpected changes have also been seen (Warren et al. 1989).

RESULTS

Rats undernourished for about 30 d weighed substantially and significantly ($P < 0.01$) less than matched controls at 134 days of age (Table 1). Even after about 40 days of unlimited access to food, previously undernourished rats weighed less ($P < 0.05$) than age-matched controls (Table 1). Furthermore both control ($P < 0.05$) and experimental ($P < 0.01$) rats showed significant increases in body weight between 134 and 175 d of age. In contrast, cerebellar weights were unaffected by undernutrition (Table 1). However the cerebella of 175-d-old rats were heavier than those from 134-d-old animals.

The numerical density of granule cells did not differ between the well fed and Un or PU groups (Table 2). Although there was no difference in N_{Vg} between C1

Table 2. *Data on granule cells from control and experimental rats at different ages*

Feature	Age (d)	n	Control	n	Experimental	% difference
Numerical density of granule cells (mm^{-3})	134	6	1795000±120600	8	1785000±75700	−1
	175	8	2025000±72900	9	2150000±83900†	+6
	% difference		+13		+20	
'Diameter' of granule cell nuclei (μm)	134	6	6.6±0.3	8	6.7±0.4	+1
	175	8	6.7±0.2	9	6.5±0.3	−3
	% difference		+1		−2	

Results are means±S.E.M.; n, number of rats; † $P < 0.01$ compared with 134 d value.

Table 3. *Data on synapses from control and experimental rats at different ages*

Feature	Age (d)	n	Control	n	Experimental	% difference
Numerical density of synapses ($\times 10^9$ mm^{-3})	134	6	1.25±0.08	8	1.26±0.10	+1
	175	8	1.88±0.10†	9	1.73±0.07†	−8
	% difference		+50		+37	
'Diameter' of synaptic discs (nm)	134	6	307.7±9.2	8	323.0±13.8	+5
	175	8	283.3±8.4*	9	297.2±10.4	+5
	% difference		−8		−8	

Results are means±S.E.M.; n, number of rats; * $P = 0.078$; † = $P < 0.01$ compared with 134 d value.

Table 4. *Synapse-to-neuron ratios in the granular cell layer of control and experimental rats at different ages*

Age (d)	n	Control	n	Experimental	% difference
134	6	719±74	8	709±53	−1
175	8	941±71*	9	813±42	−14
% difference		+31		+15	

Results are means±S.E.M.; n, number of rats; * $P < 0.05$ compared with 134 d value.

and C2, those rats previously undernourished and subsequently rehabilitated (PU) had about 20% ($P < 0.01$) more granule cells per cubic mm of granular layer than those killed immediately after undernutrition (Un; Table 2). It can be seen from Table 2 that neither age nor nutrition caused any alteration in the projected height ('diameter') of granule cell nuclei.

Neither undernutrition nor previous undernutrition followed by nutritional rehabilitation had any significant effect on the numerical density of synapses, compared with matched controls (Table 3). However, it can be seen that the well-fed rats had a much increased ($P < 0.01$) N_{Vs} at 175 d compared with 134 d (Table 3). In contrast neither age nor nutrition affected the mean projected height ('diameter') of synaptic discs, although the 8% decline apparent between C1 and C2 gave a probability level of 0.078 (Table 3).

Table 4 shows synapse-to-neuron values for all 4 groups of rats. The values (mean±S.E.M.) of 719±74 for C1 and 709±53 for Un show remarkable similarity. By 175 d of age the values of C2 had increased ($P < 0.05$) to 941±71 synapses per neuron; however, since the PU also showed an apparent age-related increase (to 813±42), this numerical difference between PU and C2 animals was not significant (Table 4).

DISCUSSION

Our results show that the synapse-to-neuron ratio in the granular layer of the cerebellar cortex of rats undernourished for about 30 d during early adult life is not significantly different from matched controls. Furthermore, animals previously undernourished and subsequently allowed unrestricted access to food did not differ significantly from age-matched controls well-fed throughout life. However, controls showed a significant increase in synapse-to-neuron ratio between 134 and 175 d of age whereas previously undernourished animals did not.

Rats previously undernourished for about 30 d from birth have been shown to have substantial deficits in their synapse-to-neuron ratio in the cerebellar granular layer (Bedi et al. 1980). This deficit disappeared following 130 d of rehabilitation. We later showed that this period of rehabilitation was not

necessary to remove the deficit, since in rats undernourished continuously from birth to 150 d of age the deficit also disappeared (Warren & Bedi, 1990). Rats undernourished for about 30 d during early adult life are reported to have more synapses per neuron in their visual cortex than matched controls (Warren et al. 1989), probably due to a delay in the normal pattern of synapse elimination (Warren & Bedi, 1984; Ahmed et al. 1987). Our present results suggest that this is not the case in the granular layer of the rat cerebellum: this may reflect the very different growth patterns of the 2 brain regions. Although the body weight of undernourished rats was substantially reduced compared with matched controls, the cerebella of experimental rats appeared to be remarkably resistant to the nutritional treatments. Indeed, the cerebellar weight, numerical density and size of both neurons and synapses remained unaffected by either undernutrition or rehabilitation.

Although the period of undernutrition had no significant effect on neuronal connectivity in the cerebellar granular layer, some relatively subtle alterations may have occurred. This may be inferred from the different pattern of synapse-to-neuron growth between the well fed and previously undernourished rats. The controls showed a significant increase from 719 ± 74 (mean $\pm$ S.E.M.) synapses per neuron at 134 d of age to 941 ± 71 by 175 d. The numerical change in the previously undernourished group between these times was not significant. It may be that the previous 30 d period of undernutrition up to 134 d of age caused subtle changes which, although not severe enough to result in an alteration in synapse-to-neuron ratio, did affect the capacity of the tissue to grow in later life. Therefore the period of undernutrition could have had subtle deleterious effects on the growth potential of the cerebellar granular layer which only subsequently became apparent. It may be speculated that such changes would reduce the brain's potential for recovery from other insults from which it might otherwise recover. Even more subtle behavioural and functional changes cannot be ruled out.

Perhaps surprisingly the largest differences observed were between control animals between the ages of 134 and 175 d of age. Synapse-to-neuron ratios increased by over 30%, principally due to the large increase in synaptic numerical density. The reason for these changes remains unclear, but similar increases were seen in neuronal and synaptic density estimates in rehabilitating rats between the same ages. The main differences were that granule cell numerical density increased significantly in the experimental group (but not in the controls) whereas the increase in synaptic numerical density was smaller (37%) than in the controls (50%). The combination of these 2 features resulted in a numerically (but not significantly) smaller synapse-to-neuron ratio at 175 d compared with well fed control animals. It is possible that these differences could become significant if the undernutrition was more severe. Recently Bedi et al. (1992) have implied that there may be a threshold below which undernutrition does not cause permanent changes in the brain, although further work is required to confirm this hypothesis.

Finally, the results obtained in the present study, which were obtained using the unbiased disector method, agree very well with those previously reported using traditional methods (e.g. Warren & Bedi, 1990).

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