Early-life undernutrition causes deficits in rat dentate gyrus granule cell number

K. S. Bedi

Department of Anatomy, University of Queensland, St Lucia, Brisbane (Australia 4072)
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Abstract. Recently developed stereological methods have been used in experiments to examine the effects of two levels of undernutrition during early postnatal life on the total number of rat dentate gyrus granule cells. This study has shown that previously undernourished rats have significant deficits in the total number of this particular type of neuron.
Key words. Malnutrition; hippocampus; stereology.

Undernutrition of rats during early life is known to cause permanent deficits in total brain cell number [1, 2] as determined by biochemical assays of brain DNA content. Whether this is due to a loss of neurons or a failure of glial cell proliferation remains uncertain from these biochemical measurements.

The question of whether undernutrition during early life causes a specific deficit in total neuron cell number in defined brain regions is important for a full understanding of the possible mechanisms behind the associated functional deficits. A number of quantitative histological studies of the brains of adult rats undernourished for a period during early life has revealed that deficits in brain weight are accompanied by histological changes in brain structure [3 – 6]. Unfortunately, histological investigations hitherto, have been unable to unequivocally resolve the question of whether undernutrition causes a deficit in total neuron cell number. This has been due to the lack of simple and reliable methods suitable for this purpose. During the last 6 years a range of new stereological methods such as the 'disector' [7], the 'selector' [8] and the 'fractionator' [9] have been described. These advances in modern morphological methods have made it possible, for the first time, to make relatively unbiased estimates of such things as tissue volumes, cell numerical densities and total (neuron) cell numbers. I have used these new stereological methods to examine the effects of two levels of undernutrition during early postnatal life on the total number of rat dentate gyrus granule cells.

The rat dentate gyrus has a morphologically distinct and simple histological structure having only three layers. The central layer is a curved cellular layer packed with granule cell neurons. The dendritic trees of these granule cells arborize superficially into the adjacent molecular layer and form synaptic contacts with incoming fibers from other brain regions. The granule cell axons pass through the more deeply situated polymorphic cell layer and form mossy fiber contacts with the dendritic trees of the pyramidal cells in the hippocampal formation [10, 11]. The polymorphic zone contains local and projection interneurons [12]. Autoradiographic studies have shown that, in the rat, about 80% of the granule cells arise after birth [13, 14]. More recent evidence [15 – 17] has shown that granule cells of the rat dentate gyrus, in fact, continue to be produced well into adulthood.

The rats used in this study were of an outbred hooded Long Evans strain. Rats were undernourished between the 16th day of gestation and the 19th postnatal day of age by restricting the amount of food given to their mothers during gestation and suckling. Male rats were weaned at 19 days of age; undernutrition of these pups was continued to 30 days of age by directly restricting their food intake. Three groups of rats were raised, namely well-fed controls, level-1 and level-2 undernourished. The daily food intake of the level-1 and level-2 rats represented about 60% and 40% respectively, of that eaten by well-fed controls. Nutritional rehabilitation of the rats was commenced when they had reached 30 days of age by placing them on an ad libitum diet.

Groups of control and experimental rats were killed at 70 and 212 days of age by perfusion with about 350 ml of a modified Karnovsky fixative [18]. The entire right hippocampus from each brain was removed and completely sectioned on a vibrotome (Oxford Instruments, England) to yield serial sections with a nominal thickness of 100 μm. To ensure that all regions of the hippocampus had the same chance of being sliced, the position of the first cut was randomised along the interval 0 to 100 μm where 0 represents the tangent to the portion of tissue being cut. The first section to be picked up was chosen by lottery, between the first two sections cut containing hippocampal tissue. Thereafter, every alternative section in any given series of sections from a given animal was picked up. These sections were mounted on clean glass histology slides and stained with 0.1% toluidine blue. They were used to make an unbiased estimate of the volume of the granule cell layer using the Cavalieri principle [19]. This involved estimating the area of granule cell layer profile in the sampled sections using a point counting procedure [9, 20]. This together with a knowledge of the mean distance between serial sections (measured by a resectioning technique [21]) was used to make an unbiased estimate of the volume of the granule cell layer compartment.

A random sample of the vibrotome sections not used for the estimation of compartment volume was embedded in

resin. The tissue shrinkage due to this embedding procedure was assessed and found to be negligible. These blocks were cut on an ultramicrotome to yield three serial 2-μm-thick semithin sections which were stained with toluidine blue. The 'disector' method [7] was used on these sections to make an unbiased estimate of the numerical density of granule cell neurons contained within the granule cell layer. This method involves examining serial sections of known distance apart (h) and counting neuronal profiles (Q^-) which appear in one section but not the adjacent serial section. The formula for the disector method is:

$$Nv = Q^- / a \cdot h$$

where a = the area of section examined. The actual thickness of the serial sections used for the 'disector' method was determined by a resectioning technique [21] in order to determine h.

Table 1 shows the unbiased estimates of the total numbers of granule cell neurons within the granule cell layer. There was between 260 and 320 thousand granule cells within the dentate gyrus of 70-day-old control and experimental rats. However, by 212 days of age the previously undernourished rats had between 500 and 600 hundred thousand granule cell neurons. This was significantly fewer than well-fed age-matched controls. A two-way analysis of variance for unequal sample sizes showed significant main effects of nutrition and age as well as a significant interaction between them (table). Post-hoc analysis showed that there were no significant differences between control and level-1 or level-2 undernourished rats at 70 days of age. However by 212 days of age both previously undernourished groups were significantly lower than age-matched controls. There was also a significant increase with age in the total number of granule cell neurons for all nutritional groups of rats, the greatest effect being observed in the control animals (table).

These results provide strong and unequivocal evidence, for the first time, that a period of undernutrition during early life causes a long-term deficit in the total number of neurons in a well-defined and circumscribed region of the brain. Furthermore, the results confirm the observation that dentate gyrus granule cells continue to be produced in adult life. In this study more than half of the adult cell number seemed to arise after 30 postnatal days of age. This lengthy period of neurogenesis makes granule cells of the dentate gyrus unusual compared with the majority of other neuronal types. It may be that the period of undernutrition imposed during early life causes a permanent change in the duration of the cell cycle of dentate gyrus granule cell neurons, causing fewer of them to be produced in adult life than would otherwise be the case. There is good evidence that this may occur. Lewis et al. [22] has used tritiated thymidine autoradiography to show that undernutrition during early life can cause the total cell cycle time of the dentate gyrus granule cells to be increased. This therefore offers a reasonable explanation for the observed deficit in the total number of dentate gyrus granule cells as a result of undernutrition.

Mean ± SE of the estimates of granule cells in the dentate gyrus of control and experimental rats

Age (days)	n	Controls	n	Level-1	n	Level-2
70	8	302,600 ±25,000	8	261,400 ±20,400	8	321,700 ±26,800
212	9	834,000 ±98,000	7	515,000 ±51,000	6	595,000 ±76,000

Results of two-way analysis of variance of above data

Factor	df	F	p<
Nutrition	(2,40)	3.98	0.0265
Age	(1,40)	55.89	0.0001
Interaction	(2,40)	4.53	0.0169

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