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Studies on Ectopic Granule Cells in the Cerebellar Cortex

III. An Investigation into the Restoration of the External Granular Layer after Partial Destruction

Ebel J. Ebels, Ieteke Peters, and Aaltje Thijs

Division of Neuropathology, Department of Pathology, University of Groningen, Netherlands

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Summary. In order to strengthen a hypothesis concerning the occurrence of ectopic granule cells, one of the assumptions made was tested systematically. The reaction of the EGL to partial destruction by various single doses of hydroxyurea at various ages was followed. Under all conditions examined, re-population of the EGL takes place—rapidly after lower doses, slowly after high doses of HU. The phenomena observed are discussed with a view on the hypothesis mentioned. Re-population is beneficial, but may itself be a major pathogenetic factor in certain developmental malformations by upsetting the "time-schedule". The results are also of potential interest for an analysis of the regulation of normal cerebellar morphogenesis.

Key words: Cerebellar Development — Experimental Malformations — Regeneration — Hydroxyurea.

Introduction

In 1972, Ebels suggested a hypothesis to explain the reproducible occurrence of ectopic granule cells in the molecular layer of the cerebellum. A delay in granule cell development, due to restoration of the external granular layer of the cerebellum (EGL) after partial destruction was postulated as an important pathogenetic factor. That such a re-population is possible is known from various authors for various conditions: after low level X-irradiation (Altman *et al.*, 1969; Ebels, 1969; Korogadina *et al.*, 1969; Phemister *et al.*, 1969); after the administration of methylazoxymethanol (Shimada and Langman, 1970a; Chanda *et al.*, 1973) and fluorodeoxyuridine (Shimada and Langman, 1970b; Langman *et al.*, 1972) and ethylnitrosourea (in unexplained contrast with methylnitrosourea: Bosch *et al.*, 1973).

In this paper one factor, the restoration of the matrix, is subjected to systematic investigation. For this purpose hydroxyurea (HU) was preferred to X-irradiation. HU is known selectively to kill proliferating cells (Philips *et al.*, 1967) and has no alkylating effects. HU was shown by us lethally to damage cells in the EGL and—in suitable doses—to give rise to ectopic cells. In view of the dose-dependency of the occurrence of ectopic granule cells (after X-irradiation as well as after HU) we decided to study the effect of various single doses of HU; we also wanted to know whether the age of the animals at the time of the administration had any influence on the course of events.

Abbreviations. EGL = external granular layer of the cerebellum; HU = hydroxyurea.

Materials and Methods

Inbred Wistar rats were used throughout the experiments. 6-day old rats were injected with 0.5, 1 or 4 mg hydroxyurea, 8- and 12-day old animals with 1 or 4 mg HU per gram body weight. HU (Hydroxyharnstoff obtained from Schuchardt, München) was made up fresh when needed by dissolving it in isotonic saline in a concentration of 50 mg/ml, 100 mg/ml or 400 mg/ml. Each rat received an intraperitoneal injection of 0.02 ml/g body weight + 0.025 ml to compensate for leakage.

Pairs of rats were killed at various ages up to 21 days. The brain was removed, fixed in Bouin or Bouin d'Hollande fluid and embedded in paraffin. 6 μ midsagittal sections were stained with H&E. The thickness of the EGL was determined at various (at least 10) sites along the fissura prima. The value indicated in the figures is the mean of those obtained for each of the two animals.

Results

The results of the experiments are graphically shown in Figs. 1—3.

The effect of the administration of HU to 6-day old rats (Fig. 1) varied with the dose given. With all three, however, a rapid, severe loss of cells ensued, reducing the thickness of the EGL very considerably (to 1 cell after 4 mg HU, to 2.5 after 0.5 mg). Lethal damage of cells was evident from severe nuclear changes. After about 2 days these necrotic cells had disappeared without any visible reaction. By that time the EGL was at its smallest. Thereafter re-population occurred; it was much more rapid after the lower (0.5 or 1 mg) than after the highest (4 mg) dose. After 0.5 and 1 mg HU normal thickness was reached again in another 2 days, after 4 mg after 8 days only. There was a distinct "overshoot" at 16 days after 4 mg and at 12 days after 1 mg.

The effect of 1 mg HU, administered at various ages, is shown in Fig. 2. At all ages, there was a rapid reduction of the EGL with a minimum thickness being reached after 2 days. Recovery to normal thickness occurred within 2—4 days. The overshoot on day 12 after 1 mg at 6 days has already been mentioned.

After 4 mg HU (Fig. 3) given at the same ages, the damage produced was much greater than by 1 mg. The minimum thickness reached was smaller and recovery much slower, with a distinct overshoot on day 16 after administration at 6 days of age and a smaller one after injection at 8 days.

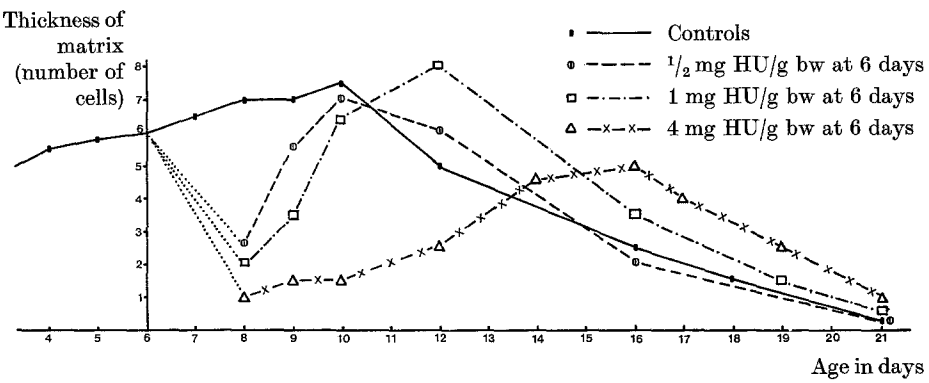

Fig. 1. The effect of various doses of hydroxyurea given to 6-day old rats on the thickness of the external granular layer

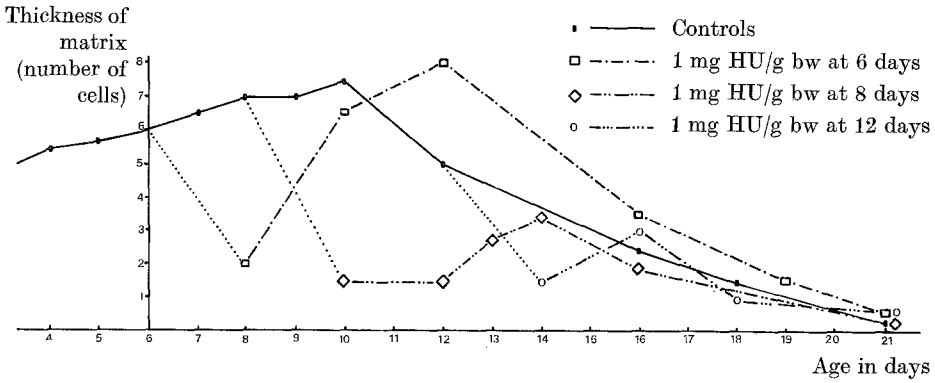

Fig. 2. Thickness of the external granular layer at various intervals after the administration of 1 mg hydroxyurea per gram body weight to 6-, 8- or 12-day old rats

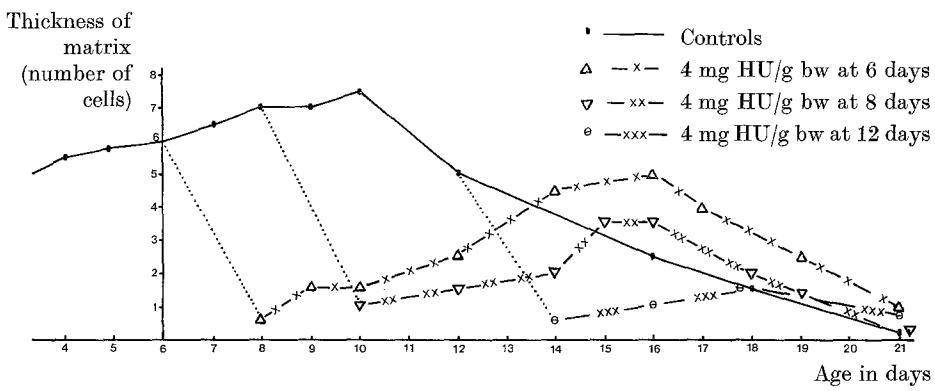

Fig. 3. As Fig. 2, but after 4 mg hydroxyurea per gram body weight

In all animals, regardless of the dose given and the age at the time of the injection, the decline of the EGL followed the normal pattern: at 21 days of age the EGL was reduced to 0—1 cell in thickness.

Discussion

The results of our experiments clearly show that under the conditions of the experiments[1] marked recovery of a matrix, even when severely damaged, may occur. This accords well with the data presented in the Introduction, especially with the findings of Chanda *et al.* (1973), who investigated DNA, RNA and protein in the cerebellum of rats after early postnatal administration of methylazoxymethanol. For those ages investigated, the speed of recovery seemed independent of the age of the animal at the time of the administration of HU. But it was clearly influenced by the dose of HU given—being much slower after 4 mg than

[1] The direct effect of HU itself may be assumed to last for a very limited period (some hours) only; the sequence of events occurring afterwards can be interpreted as resulting from the reduction of the matrix.

after 0.5 or 1 mg HU. An important finding seems to be that after the lower doses the time needed for reaching the "normal" thickness of the EGL was very much smaller than the period needed for a similar increase in thickness during normal development. Faster than normal increase in the amount of DNA was (also) observed by Chanda *et al.* (1973).

Usually, once the normal thickness had been reached, the EGL started to decline. In some instances however there was an "overshoot" as the result of re-population, the thickness of the EGL surpassing that seen in normal animals of the same age. This was most conspicuous on day 16 after 4 mg HU, at 6 days, and on day 12 after 1 mg HU at 6 days. A similar overshoot was referred to by Chanda *et al.* (1973) for the EGL after damage by methylazoxymethanol, and was also described by Poulakos and Osborne (1973) after X-irradiation of the rat ileum and after the administration of HU in the duodenal crypts of the C3H mouse by Dethlefsen and Riley (1973).

In all instances, once the maximum thickness of the EGL was reached, the decline of the matrix showed the same slope as during undisturbed maturation—with a near complete disappearance at 21 days of age.

These results are of wider interest if conclusions could be drawn that might throw some light upon the way in which the rise and decline of the matrix is controlled and regulated during normal development. Although it is not yet possible (it will be extremely difficult to do so) to establish "hard" facts, some interesting suggestions emerge from our data that may possible serve as starting points for further investigation.

The rapid re-population of the matrix after not too severe damage—much faster, as we noticed, than its growth during normal development—seems to point to an age-dependent (or, more cautiously: age-related) optimum thickness of the matrix. Under normal conditions this thickness is the result of balanced proliferation of matrix cells and migration of cells out of the matrix—the balance varying with the age of the animal. After partial destruction the first need seems to be for restoration of the appropriate thickness. The fact that re-population usually (with the exception of the instances of overshoot mentioned) continues until the age-appropriate thickness is reached, is another argument in favour of the postulated age-dependent optimum thickness. The much slower restoration of the EGL after the highest dose of HU may be due to too small a number of cells remaining which are capable of proliferation.

The normal slope of the curves of the EGL after regeneration and the disappearance of the EGL at 21 days show that the "biological clock" involved is not interfered with by the conditions of the experiments; this again points to age-related regulation of the thickness of the matrix. The way in which this relation is obtained remains unsettled. From our data it can be tentatively concluded that it is independent of the developmental processes in the cerebellum that are not directly altered by the administration of HU.

The regenerative potential of the matrix is certainly beneficial: it reduces the ultimate deficit of cells produced from the matrix. But at the same time our assumption that it contributes to the occurrence of certain developmental malformations seems strengthened by the results of our experiments. From the

figures it is evident that even after low doses of HU at least 4 days elapse before the normal thickness of the matrix is reached again.

Ebels (1972) postulated that this would result in delay of the morphogenetic processes originating from this matrix, thereby upsetting the "time-schedule" of cerebellar development. That both the regenerative processes and the occurrence of the ectopic neurons are dependent on the dose of the noxious agents is another (indirect) argument in favour of their being related phenomena.

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Dr. E. J. Ebels
Pathologisch-Anatomisch Laboratorium
Oostersingel 63
Groningen
Netherlands