

Selective Destruction of Precursors of Microneurons of the Cerebellar Cortex with Fractionated Low-Dose X-Rays

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The study was undertaken to determine the amount of irradiation needed to selectively destroy certain postnatally-forming neural elements of the brain. The heads of kittens during their first 2 weeks of life were irradiated unilaterally with five repeated doses of x-ray, single doses ranging from 50 to 400r; the animals were killed on the sixteenth day. The effects of radiation were evaluated quantitatively on the irradiated and control sides in the ansiform lobule of the cerebellar cortex. Repeated doses of 50 to 100r reduced the migratory cell population of the external granular layer; 150r produced a subtotal, and 200r a total destruction of the layer. With 150r and higher doses the total area and the cell population of the internal granular layer of the ansiform lobule were appreciably reduced; with 200r and higher doses the cell packing density of the granular layer was also affected. Irradiation with 150r or higher doses reduced the ratio of granule cells to Purkinje cells. Although x-ray irradiation up to 200r had no effect on the control side, effects were produced with 300 and 400r. The Purkinje cells were adversely affected only with 400r. We concluded that 200r is the optimal dose in kittens for selective destruction of the precursors of the postnatally-forming granule cells in circumscribed parts of the cerebellar cortex.

Introduction

A large body of experimental evidence is available (8) indicating that most if not all mature cells in the mammalian body are "radioresistant," that is, are not seriously harmed either morphologically or functionally by exposure to low or intermediate doses (a few hundred roentgen units) of ionizing radiation. The fact that adult organisms, nevertheless, can be adversely affected by low-level irradiation is attributed to the selective vulnerability of the undifferentiated or immature cells of various organs that depend on continual or periodic renewal of their cell populations. The extreme radiosensitivity of embryonic tissues has been known for long, but this view is supported more directly by the selective sensitivity of portions of the adult body (e.g., the

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hemopoietic system) where cellular constituents are in a continual turnover, lost as a consequence of use and replaced through cell proliferation and differentiation. In agreement with this view, organs that show minimal cell turnover are highly resistant to low-level irradiation, and are seriously affected only when exposed to excessive doses of ionizing radiation (several thousand roentgen units). An oft-mentioned example of the latter is the nervous system. Until recently the brain was considered to be among the most radio-resistant organs of the body, and this was attributed, first, to the fact that the differentiated cell population of the nervous system is a stable one, and second, to the generally held belief that the brain is devoid of undifferentiated cells that are capable of multiplication, migration and differentiation.

Some recent studies have cast doubt on the generalization that the nervous system is insensitive to radiation. It is true, as many studies have shown (12, 13), that morphological changes (at least immediate ones, as opposed to delayed, secondary ones) are not demonstrable in nerve cells in most regions of the brain of experimental animals unless relatively high doses are administered. But evidence has been accumulating that some regions of the nervous system are functionally quite responsive to low-level radiation, as determined by physiological and behavioral testing criteria (11, 15, 18), and that even gross morphological changes may be produced in certain parts of the brain by such a procedure. As a corollary, the idea that cell multiplication is absent in the mammalian brain also requires revision. Recent studies indicate that periventricular proliferative zones in the forebrain of mammals persist for a long time after birth (1, 24), and that many neuroglia cells of the forebrain and certain small neurons of various brain regions are formed after birth in rats (2-5), mice (7), guinea pigs and cats (Altman, unpublished observations).

This concept is based on studies in which the DNA of multiplying cells was tagged with intraperitoneally-injected thymidine- H^3 and the migration, remultiplication and differentiation of the tagged cells were traced in animals surviving for different periods after injection, using serial brain sections with autoradiography. Certain very small neurons, which we call "microneurons,"² form discrete granular layers in various brain regions in rodents, as in the cerebellar cortex, dentate gyrus of the hippocampus, and olfactory bulb. In these structures the majority of cells in the granular layers in-

² Microneurons are defined as small nerve cells whose axons terminate within the structure in which their cell bodies are located. That is, from a functional point of view, microneurons are interneurons with restricted local output. Examples are the stellate neurons of the cortex, and the granule cells forming the granular layers of the olfactory bulb, cochlear nucleus, hippocampal dentate gyrus, etc. In the cerebellar cortex the term includes not only the granule cells of the granular layer but also the stellate and basket cells of the molecular layer.

corporate thymidine- H^3 injected systemically after birth, indicating that they are of postnatal origin.

We have elsewhere postulated (2, 4) that the postnatally-formed micro-neurons have a major role in the adaptive (environmentally-conditioned, or input-dependent) organization of the maturing brain. If the multiplying, undifferentiated cells of the body are selectively susceptible to ionizing radiation, irradiation could provide us with a tool for selectively extirpating the precursors of the postnatally-forming cells, and thus enable us to examine the role of these elements in the organization of brain and behavior.

In this study we have undertaken a dosimetric investigation of the vulnerability of the precursors of cerebellar granule cells, located in the external granular layer of the cerebellar cortex. The external granular layer is a subpial germinal zone, composed of cells undergoing rapid and continuous mitosis. As earlier studies have established (2, 5, 19, 20, 24), the cells formed in this layer move inward through the gradually developing molecular layer, migrate past the Purkinje cells and become differentiated into small nerve cells, forming the internal granular layer.

Materials and Methods

Irradiation. Eight kittens from two litters were used. Prior to irradiation, the animals were placed in a holder which immobilized their heads. The radiation source was a 2-million-volt Van de Graaff x-ray unit (HVEC, Model AM). The target to skin distance was 125 cm; dose rate 50 r/min. In all instances only the left half of the skull was irradiated, with the medial edge of the field defined by a high density absorber block located about 50 cm from the skull. The animals were irradiated from a single aspect, variation in the depth of the cerebellum amounting to a few per cent. The dose specified was the equilibrium value as measured by a Victoreen "R" meter. Xeroradiography was used to check the defined field prior to irradiation. (Due to penumbra and scattered radiation the dose does not cut off totally at the medial edge of the field.)

Essentials of the irradiation schedule are given in Table 1. The experimental animals were irradiated five times with 50, 100, 150, 200, 300 and 400r; with respective total doses of 250, 500, 750, 1000 and 2000r. (One animal exposed to 500r on two successive occasions died.) In addition, an unirradiated control animal was placed in the holder each time its littermates were irradiated. While the repeated doses were delivered in all instances between birth and the sixteenth day (the age at which the animals were killed), there were important differences in the irradiation schedules for the two litters. The initial irradiation of the kittens of the first litter occurred 2 days after their birth, that of the second litter 1 day after birth. The interval between successive irradiations varied differentially in the two litters

TABLE 1
IRRADIATION SCHEDULE

Animal No.	Litter No.	Dose		Age when irradiated (days)					Age when killed (days)
		Single r	Total r						
C661	2	0	0	1	3	8	10	14	16
C658	2	50	250	1	3	8	10	14	16
C654	1	100	500	2	4	7	9	11	16
C659	2	150	750	1	3	8	10	14	16
C655	1	200	1000	2	4	7	9	11	16
C656	1	300	1500	2	4	7	9	11	16
C657	1	400	2000	2	4	7	9	11	16
C660	2	500	2500	1	3	^a	—	—	—

^a Kitten died.

between 2 and 5 days, and the time elapsing between the last irradiation and perfusion was 5 days for the first litter, only 2 days for the second litter. In all other respects an attempt was made to give identical treatments to the animals exposed to different doses of x-ray.

Histology. All the animals were killed at 16 days by cardiac perfusion with 10% neutral formalin. After removal of the brains and further fixation in formalin, the tissue blocks were embedded in Paraplast, and serial coronal sections were cut at 10 μ , with two sections or sets of sections preserved out of every forty. Of the pairs of preserved sections, alternate ones were stained with cresyl violet for cells and Weil's technique for myelinated nerve fibers.

Evaluation of the Material. The laterally situated, paired ansiform lobules (Fig. 1) of the cerebellum were selected for quantitative evaluation of histological and cytological differences in the irradiated and nonirradiated halves of the cerebellum. The homologous appearance of the underlying medulla oblongata (which was not visibly affected by radiation) was used as a landmark to ensure that symmetrical halves of the ansiform lobule were compared on the irradiated and nonirradiated side. An attempt was also made (never quite successful) to compare homologous portions of the ansiform lobule in different animals.

Areal changes in the ansiform lobule were determined by making tracings of this structure at 50 \times magnification with a modified Leitz projection apparatus and measuring the outlines drawn on a paper with a planimeter (Ott, type 10). In a similar manner, planimetric measurements were also taken of tracings of the internal granular layer in the same structure. The width of the external granular layer was measured in homologous parts of the second sublobule of the ansiform lobule on the ground-glass face of a Zeiss projection apparatus (equipped with a calibrated grid) with ten replica-

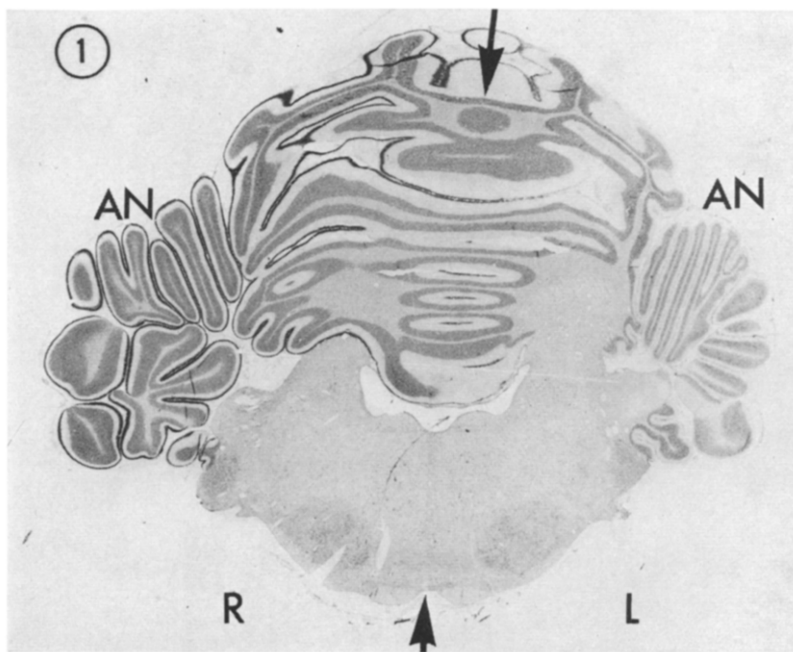


FIG. 1. Photomicrograph of a section through the cerebellum from a rat whose skull was irradiated on the left with five repeated doses of 200r. AN, ansiform lobule; R, right, nonirradiated side; L, left, irradiated side. Note total absence of external granular layer over entire left cerebellum. Arrows point to approximate midline. Magnification about $\times 5$; cresyl violet.

tions. The same technique was also used (at $500\times$ magnification) to count Purkinje cells. Finally, the packing density of granule cells in the internal granular layer was determined by counting the number of cells in rectangular areas of $200 \times 20 \mu$ with an ocular grid at $500\times$ magnification. With the data available, we could estimate the granule cell to Purkinje cell ratios.

Results

Figure 1 illustrates the radical size reduction in the cerebellar cortex of the developing kitten on the irradiated side following exposure to five repeated doses of 200r. The progressive reduction in the ansiform lobule in the irradiated half of the cerebellum, in animals exposed to 50, 100, 300 and 400r, is illustrated in Fig. 2. Planimetric measurements of changes in the area of the ansiform lobule between the control and irradiated sides in all the experimental animals, and between the right and left sides in the unirradiated control animal, are summarized in Fig. 3. Considerable variability may be seen in the measured area of the ansiform lobule on the nonirradiated side

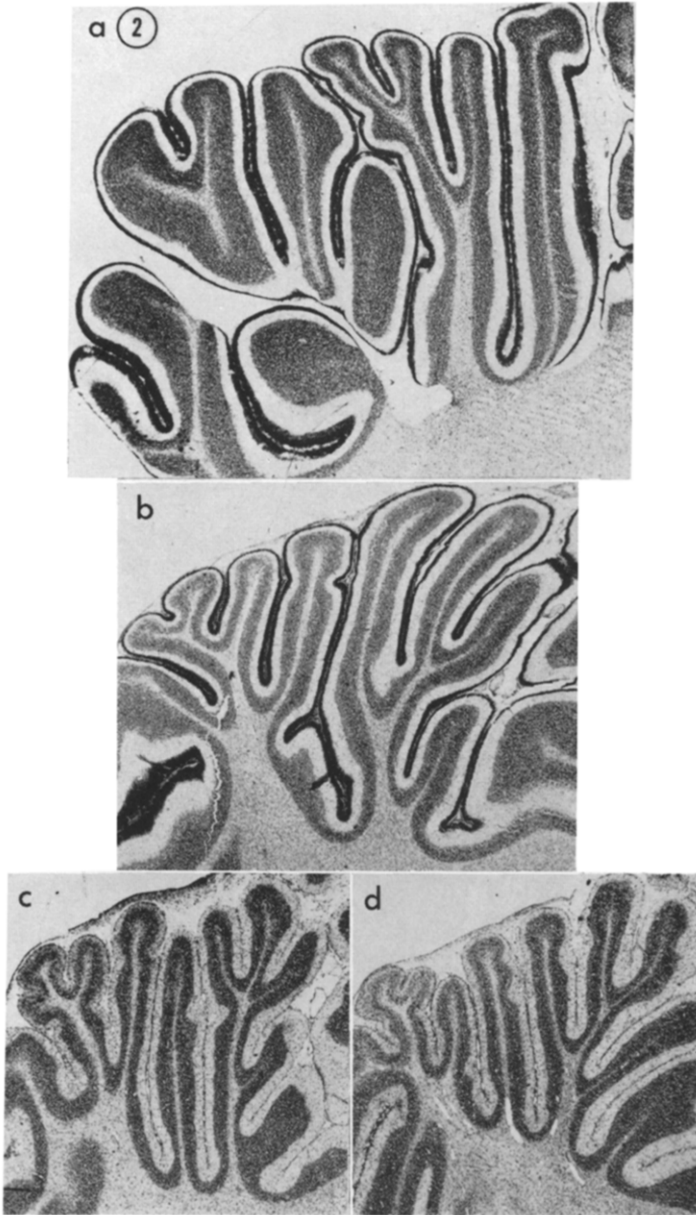


FIG. 2. Reduction in the size of the ansiform lobule on the irradiated side in animals exposed to *a*, 50r; *b*, 100r; *c*, 300r; *d*, 400r, on five successive occasions. Magnification $\times 11$; cresyl violet.

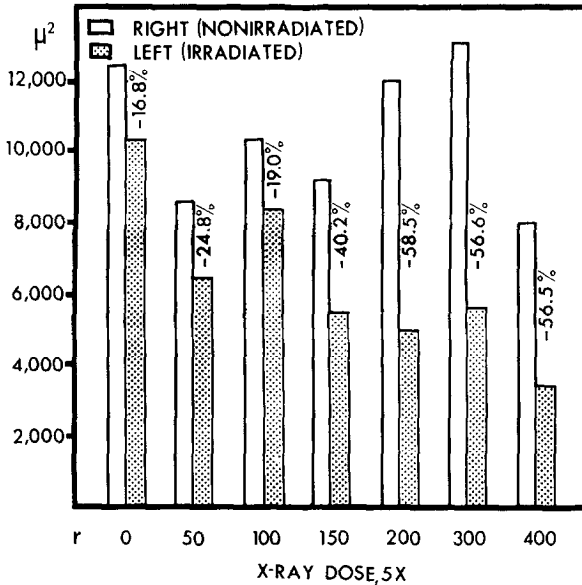


FIG. 3. Area of ansiform lobule measured by planimetry in homologous sections in different animals on the right (control) and left (irradiated) side. Reduction in area of section on irradiated side given in percentages.

among all the animals. This may have been due partly to differences in the sampled regions of the ansiform lobules in the different animals, but it may also reflect individual variations. In all animals, including the nonirradiated control, the area of the right ansiform lobule always exceeded its counterpart on the left side. In the nonirradiated animal the left ansiform lobule was 16.8% smaller than the right, and comparable values were obtained in the animals irradiated with 50 or 100r (the left side smaller by 24.8 and 19.0%, respectively). However, the difference between the nonirradiated right and irradiated left ansiform lobules increased considerably in the animals exposed to 150, 200, 300 and 400r (the reduction on the exposed side being 40.2, 58.5, 56.6 and 56.5%, respectively). Comparable differences were also obtained in the surface area of the internal granular layer in the same region, and the results are summarized in Fig. 4. Irregular reductions were also observed in the width of the sublobules of the ansiform lobule with increased doses of x-ray. These changes, as our measurements showed, could be attributed to reductions in the width of both the internal granular layer and the molecular layer (both were evident with 100r or more).

Figure 2 shows changes in the external granular layer of the ansiform lobule. The external granular layer was still conspicuous as a dark band in the animal irradiated with 50r, but there was an apparent reduction in its

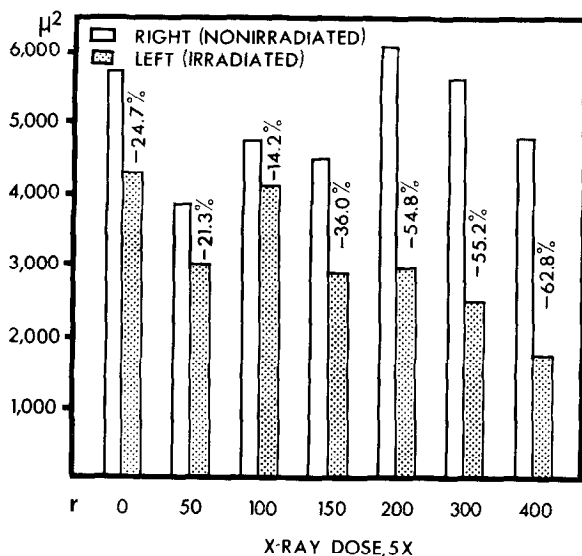


FIG. 4. Area of the internal granular layer in homologous sections of the ansiform lobule.

width in the one exposed to 150r. The external granular layer was altogether absent on the irradiated side in the animals exposed to 300 or 400r (Fig. 2c, d). The reduction in the size of the external granular layer can be more clearly seen in Figs. 5 and 6. The external granular layer was quite prominent in the 16-day old animal on the nonirradiated side following repeated doses of 50r. The thick subpial proliferative zone (round cells, many showing mitotic activity) and, below it, the migratory zone of similar width (composed of spindle-shaped cells) were evident (Fig. 5a). In the same animal there appeared to be a slight reduction in the width of the external granular layer on the irradiated side (Fig. 5b). The destructive effect of repeated doses of 50r appeared more pronounced on the migratory zone of this layer than on the proliferative zone. Figure 6a shows more pronounced reduction in the width of the external granular layer on the irradiated side in the animal exposed to repeated doses of 100r, and the width of the migratory zone of the external granular layer was drastically reduced. Irradiation with repeated doses of 200r (Fig. 6b), totally eliminated the external granular layer, and a considerable reduction in the width of the sublobules of the ansiform lobule was also evident.

Measurements of changes in the width of the external granular layer in the second folium of the ansiform lobule are summarized in Fig. 7. In the animal exposed to 50r, there was no significant decrease in the mean width of the external granular layer on the irradiated side. The external granular

layer was clearly reduced on the irradiated side in the animal exposed to 100r, and drastically reduced to a layer 1 or 2 cells thick in the animal exposed to 150r. It was absent on the irradiated side in those exposed to 200, 300 and 400r. Whereas the width of the external granular layer was unaffected on the nonirradiated side with doses up to 200r, there was a suggestive reduction on the nonirradiated side in the animal irradiated with 300r (reduction to 45 μ), and the layer was absent on the control side (indeed, over the entire cerebellum) in the one whose half head was exposed to repeated doses of 400r (Fig. 7).

With the total area of the granular layer on both sides for each animal and the cell packing density known, we could estimate the total number of cells of the granular layer in the sampled sections of the ansiform lobule. These data are summarized in Fig. 8. The estimated granule cell population (plus some neuroglia cells) was appreciably lower on the left side than the right side in the nonirradiated control (less by 23%), and the reduction was significant only in the animals in which the left cerebellum was irradiated with repeated doses of more than 150r (Fig. 8). With exposure of the left cerebellum to higher doses of x-ray, there was a further reduction in the cell population of the granular layer: a reduction of 36% with 150r; 63% with 200r; and 69% with 300r. The reduction with 400r was 64%, but with this level of radiation a reduction was also observed in the cell population of the control side.

A large proportion of all proliferative and migratory neuroblasts of the external granular layer was destroyed with repeated doses of 150r or more. This, in turn, led to a reduction in the number of differentiating granular neurons and a retarded growth in the size of the exposed cerebellar cortex. In contrast to this destructive effect on the postnatally-forming elements of the cerebellar cortex, the prenatally-formed and partially differentiated Purkinje cells were not visibly affected. There was no clearly discernible difference in respect to the stainability or size of Purkinje cells on the irradiated side (Fig. 9).

If the radiation level used does not affect the Purkinje cells, it should follow that the total number of Purkinje cells should not be lower in the greatly reduced ansiform lobule on the irradiated side than in the larger area on the control side. Indeed, an increase might be expected within a single plane, due to the diminished three-dimensional spacing out of the Purkinje cells in the reduced volume of the lobule. This expectation was borne out by counts of Purkinje cells in the ansiform lobule on both sides in all the animals (only those with visible nucleoli were counted). A slightly lower number of Purkinje cells was counted on the left than the right side in the non-irradiated control animal and in the animal exposed to 50r. Then, with exposure ranging between 100 and 300r, more were encountered in the ever-decreasing area of the irradiated, left ansiform lobule. However, with re-

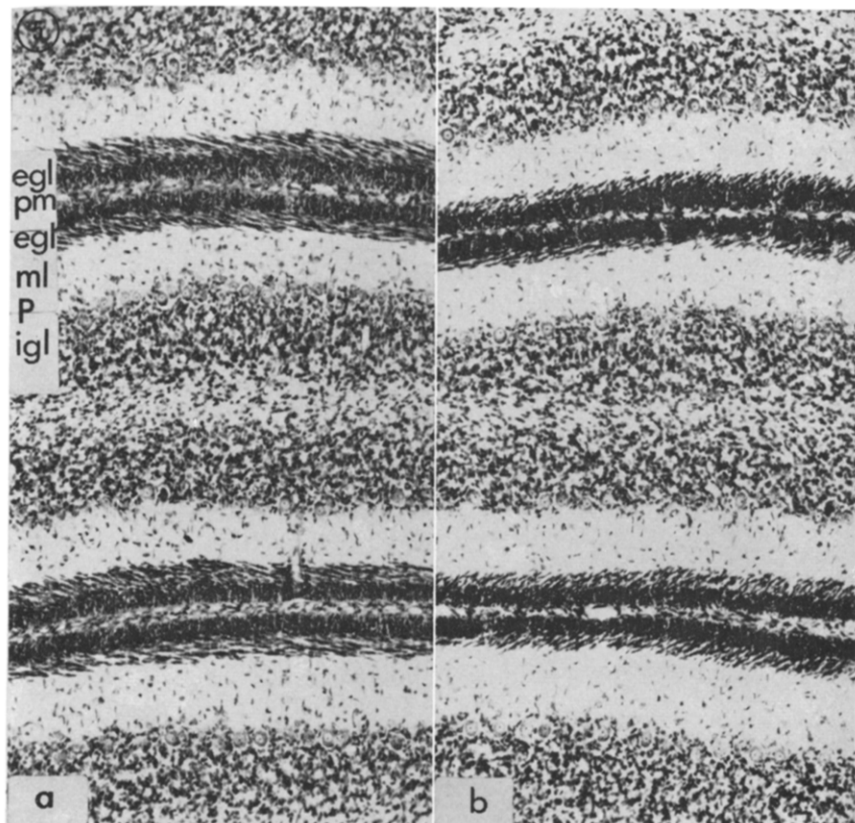
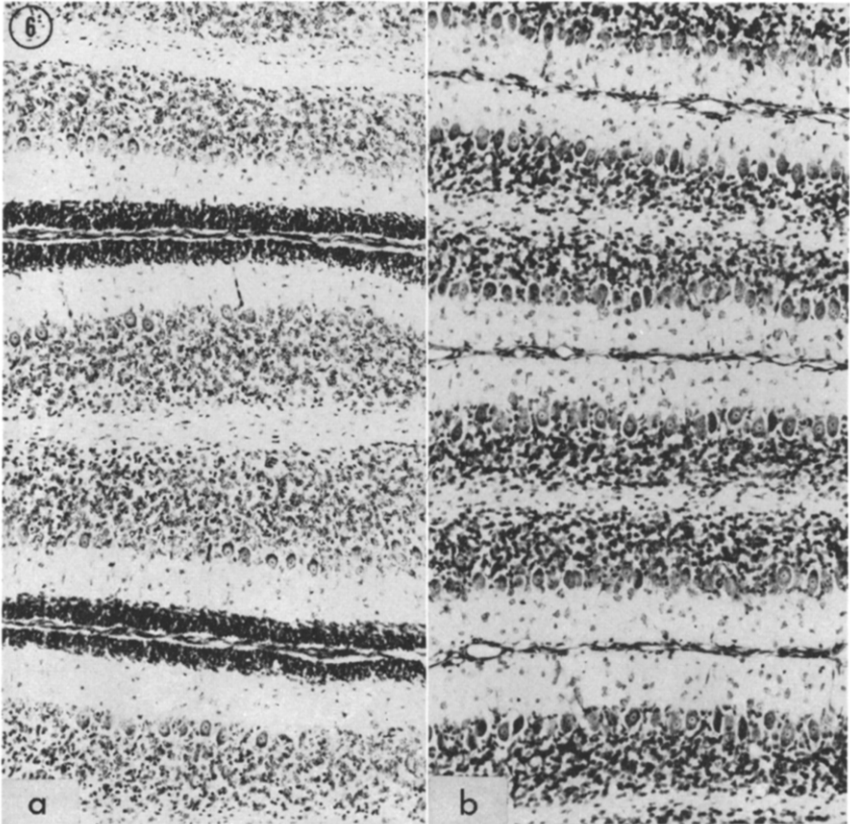


FIGURE 5

peated doses of 400r there was a decrease in the total number of Purkinje cells, indicating that with this dosage a small proportion of these cells may also have been lethally affected.

With an estimate available of the total number of granule cells in the sampled ansiform lobules and the count of the Purkinje cells, we could compute the changing ratio of granule cells to Purkinje cells in all the sections studied (Fig. 10). This ratio, indicating the number of granule cells structurally and functionally associated with each Purkinje cell, was lower on the left side in the control animal (172 granule cells for every Purkinje cell) than on the right (151 to 1), the difference being 12%. The values were near this range on the right and left side in the animals unilaterally exposed to 50 or 100r, with a difference of 23 and 19% between the two sides. With doses of 150, 200 and 300r, there was a considerable drop in the ratio of granule cells to Purkinje cells on the irradiated side (44, 70 and 69%, respectively). In the animal irradiated with 300r, this ratio was slightly affected also on the



FIGS. 5 & 6. High-power photomicrographs from matched portions of the ansiform lobule. *5a*, unirradiated side and *5b*, irradiated side, from a kitten exposed five times to 50r. *6a*, irradiated side in a kitten exposed to repeated doses of 100r; *6b*, irradiated side in a kitten exposed to repeated doses of 200r. Note total disappearance of the external granular layer in *6b*. Abbreviations: egl, external granular layer; igl, (internal) granular layer; ml, molecular layer; P, Purkinje cells; pm, pial membrane. Magnification, $82\times$; cresyl violet.

nonirradiated side, and this was more pronounced on the control side in the one irradiated with 400r. In the latter, there was no further decline in this ratio on the irradiated side, which may be attributed to a concomitant reduction in the number of Purkinje cells.

Discussion

This study establishes the optimal dose of x-rays necessary for the selective destruction of the postnatally-forming cell components of a selected region in the kitten brain. The optimal single dose is 200r. With repeated doses of 200r all the cells of the external granular layer were destroyed on the irradi-

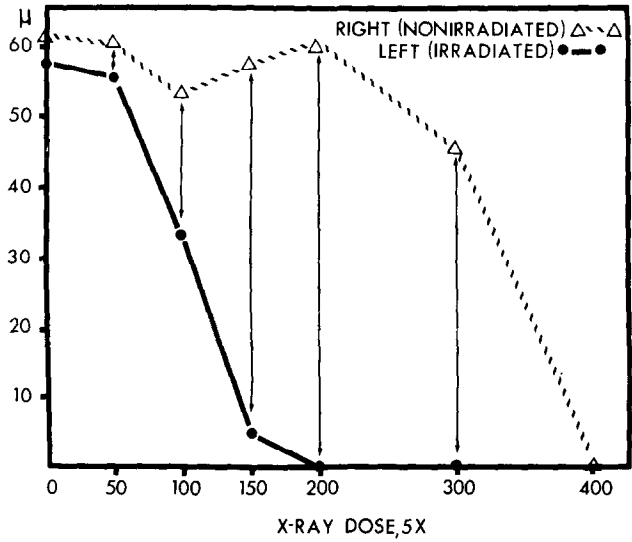


FIG. 7. Mean width of external granular layer on the irradiated and control side.

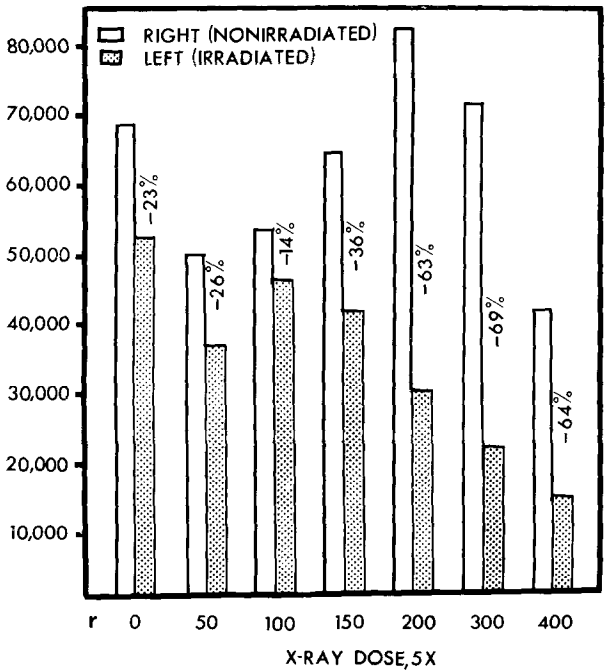


FIG. 8. Estimated total number of granule cells in sections of the ansiform lobule in different animals, with reduction on the left (irradiated) side given in percentage.

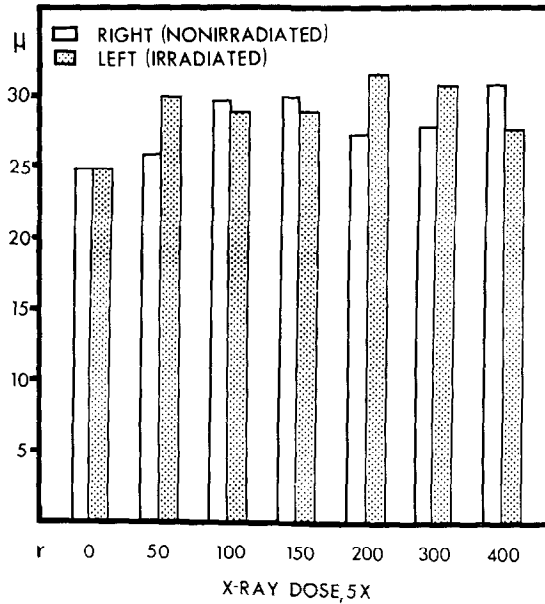


FIG. 9. Mean size (length of long axis) of Purkinje cells in the ansiform lobule.

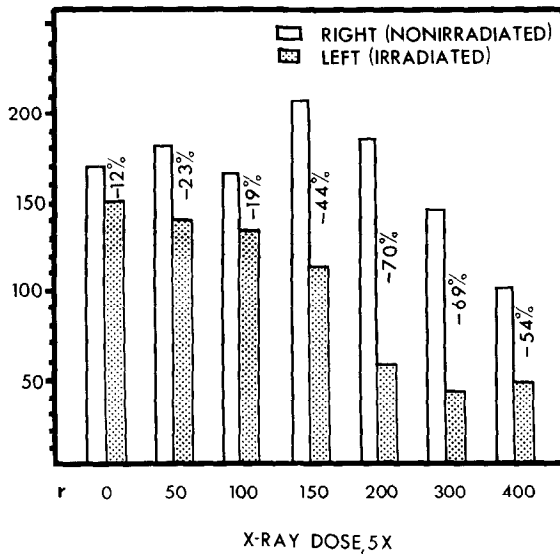


FIG. 10. Granule-cell/Purkinje cell ratio in the ansiform lobule, with percentage reduction on the irradiated side.

ated side, without visibly harming the same type of cells on the control side; and this dose did not detectably affect the Purkinje cells. With repeated doses of 150r the destruction was subtotal in the external granular layer on the irradiated side, and with repeated doses of 300r, the external granular layer (and also the total volume of the ansiform lobule) was also affected on the nonirradiated side. Repeated doses of 400r apparently reduced the number of Purkinje cells; but we have no information about possible harmful effects produced by low-dose irradiation on the dendritic development of Purkinje cells or other elements. Furthermore, since the kittens were permitted to survive only for a short period after irradiation, we could not investigate possible secondary effects (such as transneuronal) on the Purkinje cells.

The differential effects of fractionated and equivalent single doses of ionizing radiation on brain tissue have not been resolved, though most studies appear to suggest that fractionated doses are much less harmful than single equivalent doses (17). The rationale of the use of repeated doses in this study was the observation of Hicks (14) that the mitotic cells situated in the ependymal wall of the neural tube (or the cerebral ventricles) are more resistant to radiation than the postmitotic cells that are migrating from the subependymal layer toward their destinations. Few of these postmitotic cells escaped destruction with 200r, whereas mitotic cells of the ependyma were not regularly killed until the dose was raised to 400r or higher. By irradiating the animals repeatedly with lower doses we were hoping to destroy the postmitotic cells *en route* from their site of production, and thus lower the single dose level necessary for the selective extirpation of the granule-cell system. Our findings add support to the conclusions of Hicks, since the spindle-shaped cells of the migratory zone of the external granular layer were affected by lower doses (50-100r) than the cells of the subpial proliferative zone (150r and up). We cannot resolve the question whether it is necessary to use repeated doses of 200r to obtain the effects observed in this experiment or whether the same result could have been achieved with a single dose of 200r administered soon after birth. Nor can we determine from the data available why, in spite of the total extirpation of the external granular layer with 200r or more, there was a remaining cell population in the internal granular layer. Our failure to accomplish total extirpation of the internal granular layer may have been due to three factors: (a) there may be an appreciable complement of granule cells in the cerebellar cortex which are of prenatal origin; (b) since the initial irradiation in one litter group began 1 day after birth, and in the other 2 days after birth, the cells multiplying and migrating during this period could have escaped destruction; finally (c) a single dose of 200 to 400r may not be sufficient to destroy all the proliferating cells in the subpial zone of the external granular layer and, therefore, with 2 to 5 days elapsing between the irradiation sessions, some of these cells could have had a chance

to migrate through the molecular layer into the internal granular layer and become differentiated there.

The selective vulnerability of the cerebellum, more specifically its granular layer, is known. Brunner and Schwartz (10) and Brunner (9) reported that the granule cells of the cerebellum of young cats and dogs were, of all structures in the brain, most easily affected by radiation. More recently Schmidt (21) found in neonatal mice, and Shofer, Pappas and Purpura (23) in neonatal kittens, that intermediate doses of radiation destroy the external granular layer of the developing cerebellar cortex. Yamazaki, Bennett and Clemente (28) irradiated rats, aged 8 hours to 15 days, with graded doses of x-ray up to 1000r and found the most severe neurologic deficits at 3 or 4 days, and with doses of 300 to 500r. (During this period 1000r proved to be fatal to practically all the animals.) The incidence of neurological deficits was less in those irradiated at a later date, and by the fifteenth day, irradiation with 1000r failed to produce any detectable deficits. Among the most commonly observed deficits were tremor, paresis, incoordination and abnormal gait. In conformity with this neurological finding, the most common and outstanding gross morphological change was a reduction in the size of the cerebellum. Abnormal development of the cerebellar cortex in rats irradiated with 200r within a few days after birth was also reported by Hicks, D'Amato and Falk (16).

Several reports suggest that the microneurons composing the granular layer of the cerebellum retain their selective radiosensitivity into adulthood, though the doses which affect these cells in adults are higher than in neonates and infants. Alvord and Brace (6) reported pyknosis of granule cells in guinea pigs within 8 hours after exposure to 7500r whole-body x-ray irradiation. This effect was not obtained if the cerebellum was shielded, and could be demonstrated also when the cerebellum alone was exposed; suggesting that the effect of irradiation was a direct one on the granule cells. Similar results were reported by Vogel (26, 27) using gamma rays with a dose range of 5000 to 10000r in macaque monkeys. With these doses no vascular damage was evident in the cerebellum nor were the Purkinje cells visibly affected. In another study with adult mice, Schumelfeder (22) found that 12 hours after irradiation with x-ray doses of 4000 and 5000r granule cells with pyknotic nuclei were seen scattered in the granular layer. By the fifth day after irradiation almost the entire superficial part of the granular layer had undergone partial dissolution. With this dose, the Purkinje cells were killed only in the most severely affected parts of the cerebellar cortex, and the cells of the molecular layer and the medullary layer were largely spared. These studies, and incidental observations by others (12, 13), suggest that the granule cells of the cerebellum are relatively more radiosensitive even in adults than other type of nerve cells.

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