Reconstitution of the External Granular Layer of the Cerebellar Cortex in Infant Rats after Low-level X-irradiation

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ABSTRACT The heads of three-day old rats were irradiated with a single dose of 200 r x-ray and the animals were killed afterwards at intervals ranging from ten minutes to five days. Necrosis in the external granular layer of the cerebellum was evident by the fourth hour and the pyknotic cells increased in number up to 12 hours after irradiation. Between 24 to 48 hours all the pyknotic cells disappeared and the width of the layer was drastically reduced. By the third day after irradiation the external granular layer began to increase in width, and by the fourth day it was indistinguishable from normal. In adults of this group the cerebellum appeared structurally normal. In another experiment the cerebellum of rats was exposed from birth onward to 200 r on five successive days. In the animals killed immediately or one day after the last radiation session the external granular layer was totally or subtotally eradicated. In the animals surviving for four days the external granular layer reappeared over many regions of the cerebellum, and by the sixth day after irradiation it was present over its entire surface. In the latter group in animals that survived to 30 and 90 days of age the cerebellum contained a large, though subnormal, population of granule cells, indicating that the reconstituted cells were able to differentiate. These results suggested that the proliferative matrix of the postnatally developing cerebellum may be endowed with regenerative capacity.

Exposure of the developing cerebellum of infant rats (Altman, Anderson and Wright, '68b) and kittens (Altman, Anderson and Wright, '67) to single or multiple doses of 200 r x-ray kills a considerable proportion of the cells of the external granular layer, the multiplying and migrating precursor cells of the postnatally-forming basket, stellate and granule cells of the cerebellar cortex. (For a detailed description of the chronology of the postnatal recruitment of these cerebellar microneurons, see Altman, '69.) We have recently undertaken an extensive study in which the cerebellum of rats was irradiated with x-ray from the day of birth, with daily doses ranging from 50 to 200 r, number of exposures from one to ten days, and survival after irradiation ranging from one hour to over one year. The purpose was to destroy selectively a specifiable proportion of the precursor cells of the postnatally-forming cerebellar microneurons (Altman, Anderson and Wright, '68a) and study the consequences of reduction in the number, or total elimination, of cerebellar microneurons (a) on the morphological development of the cerebellar cortex and (b) on the development of locomotor capacity.

In our first analysis of this material, concerned with the quantitative, gross-morphological effects of this radiation schedule (Altman, Anderson and Wright, '68a) we found that after exposure of the cerebellum to a single dose of 200 r x-ray, its size was above normal (see in Altman, Anderson and Wright, '68a, especially figs. 5b, 8-11) and its morphology apparently normal (to be published). This appeared paradoxical because we also observed that six hours after irradiation a large proportion of the cells of the external granular layer were destroyed after exposure to a single dose of 200 r x-ray (Altman, Anderson and Wright, '68b). Accordingly, we have undertaken a pilot study to examine the possibility that the proliferative matrix of the cerebellum, the external granular layer, has reconstitutive capacities. A more detailed

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examination of this problem, with improved methodology, is in progress.

MATERIALS AND METHODS

In the first part of this pilot study two litters of Long-Evans hooded rats from a laboratory-raised colony were used. At three days of age the heads of all the pups, one litter at a time, were irradiated with 200 r of 2 MEV x-ray. The radiation procedure, dosimetry and the positioning of the heads as targets were described earlier (Altman, Anderson and Wright, '68a). Pairs of animals were allowed to survive for 10 and 60 minutes, 2, 4, 8 and 12 hours after irradiation; animals with six hours' survival were available from a previous study (Altman, Anderson and Wright, '68b). Material from this group was used to establish the time course of cell pyknosis following irradiation. Animals from another litter were assigned to the second part of the experiment, concerned with the fate of pyknotic cells and the problem of developmental regeneration. In this group pairs of animals were allowed to survive for 1, 2, 3, 4 and 5 days after irradiation.

Supplementing this material we also used the brains of rats whose cerebellum was exposed to 200 r on five successive days from birth onward, as described earlier (Altman, Anderson and Wright, '68a). Pairs of animals survived after the last irradiation (at the age of 4 days) for the following periods: 1 to 2 hours; 1, 4 and 6 days; and to the ages of 30 and 90 days. Finally, a large number of brains from rats of the same colony, ranging in age from birth to 30 days, were used to obtain data on normal cerebellar development.

All the animals were killed by cardiac perfusion with 10% neutral formalin. The removed brains were further fixed in formalin, dehydrated, and embedded in Paraplast. Sagittal sections were cut at 6 μ and were stained with cresyl violet.

Matched sagittal sections of the cerebellum were evaluated qualitatively and quantitatively. Among the qualitative observations were the presence or absence of mitotic and pyknotic cells in, and reduction or increase in the thickness of, the external granular layer over the entire cerebellum. (Cells that were spheroid in appearance, greatly reduced in size and darkly-stained, were classified as pyknotic.) Quantitative measurements were carried out in the anterior cerebellum in the ventral portion of lobus centralis, or Larsell's lobe II (Larsell, '52). (No measurements were taken in the caudal aspects of the posterior cerebellum where, presumably due to variations in the positioning of the animals' heads under the x-ray beam, there was sparing of cells in the external granular layer in some animals.) In this region the number of pyknotic cells was counted in strips of external granular layer 130 μ in length, and the means of ten counts tabulated. The "cell-thickness" of the external granular layer was determined by positioning the center line of an ocular grid of a microscope at 640 × magnification at random intervals perpendicular to the surface of the cerebellar cortex and counting the number of bisected cells. We chose this method of estimating the thickness of the external granular layer to eliminate possible variations due to differential shrinkage of tissue. In each section the means of ten determinations were tabulated.

RESULTS

Time course of radiation-induced cell-death and disintegration

In the rats killed ten minutes after irradiation only an occasional pyknotic cell was seen in the external granular layer of the cerebellum. There was no evident increase in the number of pyknotic cells in this layer in the animals that survived for one or two hours after irradiation (fig. 1). Many pyknotic cells were present in the external granular layer in the animals that survived for 4 hours, with the majority of these cells located in the upper half, the proliferative zone, of the layer. Pyknotic cells were present in the external granular layer in the animals that survived for four hours, with the majority of these cells located in the upper half, the proliferative zone, of the layer. Pyknotic cells were present in increasing numbers in the animals that survived for 8 and 12 hours (figs. 2, 3). In the animals surviving for 12 hours after irradiation as many as half of the cells of the external granular layer were pyknotic in many regions of the cerebellum, with many of these located in the lower portion, or migratory zone, of the layer. The thickness of the external granular layer was greatly reduced in these animals, which was partly due to the volume shrinkage of pyknotic cells. The
Figs. 1–4 Photomicrographs of the external granular layer over the lobus centralis ventralis in animals exposed to a single dose of 200 r x-ray, with survival after irradiation ranging from 60 minutes to 24 hours. Small, dark, circular dots are pyknotic cells; egl, external granular layer; pa, pia-arachnoid membrane. Cresyl violet, × 400.
total number of cells, including pyknotic cells, was reduced in the animals that survived for 24 hours after irradiation (fig. 4). Maximal shrinkage in the thickness of the external granular layer was seen 24 to 48 hours after irradiation with variability among animals (in one animal there was an increase in the width of the layer by the forty-eighth hour, as shown in fig. 6). Two days after irradiation only an occasional pyknotic cell remained. The few pyknotic cells that were seen were often located in the molecular layer or in the vicinity of Purkinje cells, indicating that these cells succeeded in migrating some distance before they were killed. Quantitative data on the time course of cell death from ten minutes to 48 hours after irradiation in the lobus centralis is summarized in figure 5 and its effect on the "cell-thickness" of the external granular layer in figure 6.

These results indicate that radiation-induced cell injury that leads to necrosis is a protracted process that requires about 4 to 12 hours to become manifest as cell pyknosis. The pyknotic cells disappeared 24 to 48 hours after irradiation. The fact that 24 hours after irradiation there was maximal reduction in the cell-thickness of the external granular layer (fig. 6) indicates that the pyknotic cells were eliminated. During the entire period after irradiation, even in the greatly reduced or fragmented external granular layer, mitotic cells were encountered in variable numbers.

It may be noted that a comparable cycle was also observed in the proliferative subependymal layer of the lateral ventricle where an even higher proportion of cells were pyknotic 8 to 12 hours after irradiation and where we observed a subtotal eradication of cells in this proliferative zone.

Recovery of the external granular layer

The external granular layer was reduced to a two-cell thick zone in the lobus centralis 24 hours after irradiation (fig. 4), in some other regions (in the anterior cerebellum) it altogether disappeared as a coherent layer and was merely present in the form of scattered cells or fragments of cell aggregates in a zone overlying the layer of Purkinje cells. In the two animals that were permitted to survive for two days after irradiation divergent results were obtained. In one animal the external granular layer was either absent or was thinner than 24 hours after irradiation, while in the other it was of considerable width and was normal in appearance. In both animals that survived for three days (fig. 5) the external granular layer appeared re-
Figs. 7-10. Photomicrographs of the external granular layer over the lobus centralis ventralis in animals exposed to a single dose of 200 r x-ray, with survival after irradiation ranging from two to five days. Arrows in figure 7 point to surviving isolated cells or cell clusters representing the decimated external granular layer; pa, pia-arachnoid membrane. Cresyl violet, X 400.
generated, although as indicated by the curve obtained from a limited number of normal animals (fig. 6), its width may still have been subnormal. These observations indicated that by the third day after irradiation the proliferative compartment of the cerebellar cortex was reconstituted. Further increases were observed in the width of the external granular in the animals that survived for four and five days after irradiation (figs. 9, 10), with indications that by the latter day it surpassed the declining width observed in normals (fig. 6). The cycle of degenerative and regenerative changes following irradiation with a single dose of 200 r is illustrated in the low-power photomicrographs in figures 11 to 14.

The problem of developmental reconstitution of cells of the external granular layer was also studied in a group of rats whose cerebellum was exposed to doses of 200 r from birth onward on five successive days (0-4 days of age) and were killed 1 to 2 hours and 1, 4 and 6 days after irradiation, and at the ages of 30 and 90 days. Figure 15 illustrates the appearance of the external granular layer over the surface of lobus centralis, showing the presence of a thick external granular layer, in an unirradiated rat at four days of age. The appearance of the lobus centralis in an animal of the same age after exposure of the cerebellum to 200 r on five successive days is illustrated in figure 16. The external granular layer has disappeared but the scattered cells over the layer of Purkinje cells may represent some surviving elements of this germinal matrix. It may be noted that the ependyma of the fourth ventricle was not visibly affected and that many cells survived in the leptomeninges (pia-arachnoid membrane). Little change may be seen in the animal that survived for one day after the last irradiation (5 days of age), as shown in figure 17, although some increase in the width of the cerebellar cortex and the size of Purkinje cells was noted. Clumps of cells, suggesting re-formation of the external granular layer, were seen in the animals that survived for four days after the last irradiation (8 days of age), as shown in figure 18. In the animals that survived for six days after the last irradiation (fig. 19), the external granular layer formed a continuous, though somewhat disorganized sheet of cells over the cerebellar cortex.

These degenerative and reconstitutive changes in animals surviving for different periods after irradiation on five successive days are illustrated in low-power photomicrographs in figures 21 to 24. It may be noted that vestiges of the external granular layer survived in the posterior cerebellum in the animal killed one day after irradiation ((fig. 22). This was commonly observed in our material and we attributed it to sparing of cells as a result of incomplete exposure of the posterior portion of the cerebellum to the x-ray beam. Another possibility is that because cell proliferation is less brisk in the external granular layer of the posterior cerebellum during the first week of life (granule cells in the uvula, pyramis, tuber and decline are formed later than in the nodulus or in the vermis lobules of the anterior cerebellum; Altman, '69) its cells are more radioresistant. Cell recovery in the external granular typically commenced in the posterior cerebellum as shown in figure 23 in the animal that survived for four days after the last irradiation session. However, by the sixth day after the last irradiation (10 days of age) the external granular layer was reconstituted over the entire surface of the cerebellar cortex, with the exception of the nodulus (fig. 24). The corrugated appearance of the external granular layer is an abnormal phenomenon and the cells appear to be disoriented in many regions. Figure 20 illustrates the appearance of the lobus centralis in a 30 day old animal after exposure to $5 \times 200$ r. The external granular layer, which disappears normally at about 20 to 21 days of age is no longer present. Granule cells are seen in large, though subnormal number, suggesting that the reconstituted cells of the external granular layer migrated and differentiated. But unlike in animals that were exposed to a single dose of 200 r, many abnormalities can be observed in the animals exposed to $5 \times 200$ r. Conspicuous among these is the sparsity of cells in the molecular layer, irregular scattering of Purkinje cells in the granular layer, and the presence of cell-free islands in the granular layer.
DISCUSSION

Our observation that pyknotic cells accumulate in the cerebellar cortex of infant rats between 4 to 12 hours after irradiation with 200 r is in agreement with the earlier observation of Hicks and his associates (Hicks et al., ’61; Hicks and D’Amato, ’66) in the nervous system and retina of rat embryos exposed to the same dose of x-rays. No satisfactory explanation is available at present for this long and synchronized delay in cell death. The concept that this delay is tied to the mitotic process, that is, that proliferating cells have to reach a stage in the cell generation cycle before the radiation-produced injury becomes manifest as cell pyknosis, has never been satisfactorily formulated. Doubt is cast on such a theory by the observation (Hicks et al., ’61; Altman, Anderson and Wright, ’68b) that necrosis is highest among primitive migratory cells, including those of the migratory zone of the external granular layer of the cerebellum which do not multiply.

The regenerative capacity of the retina of young rat embryos following x-irradiation was observed by Rugh and Wolff (’55a,b) and Hicks and D’Amato (’61). The results of our experiments indicate that in the postnatally developing cerebellum the external granular layer is endowed with considerable restitutive power. In the first experiment, in which the heads of infant rats were exposed to a single dose of 200 r, the destruction of the external granular layer was incomplete, with a one to two cell-thick fragmented layer remaining in most regions, and an even thicker one in others. In this experiment the recovery of the external granular layer a few days after irradiation could be attributed to an enhanced rate of proliferation in the surviving cell population. In the second experiment, in which the cerebellum was exposed to 200 r on five successive days after birth, the destruction of the external granular layer was more drastic. In some animals the external granular layer disappeared altogether as a distinct layer by the fourth or fifth day of life over the entire surface of the cerebellum, in others only vestiges remained in isolated parts of the posterior cerebellum. The observation that recovery commenced in the posterior cerebellum lends support to the idea that residual elements are responsible for the recovery of the entire proliferative layer. The assumption must be made either that these remaining islands of cells in the posterior cerebellum produce daughter cells in prodigious number and that these migrate eventually over the entire surface of the cerebellum or, else, that recovery proceeds simultaneously everywhere. In the latter case, the occasional remaining cells over the layer of Purkinje cells that were seen throughout the cerebellum presumably repopulated the external granular layer by local proliferation. A third possibility is that the cells of the ependyma of the fourth ventricle, which were apparently unaffected by radiation, provided stem cells for the regeneration of the external granular layer. The resolution of this question should be possible with the use of thymidine-H3 autoradiography.

Both experiments indicated that the germinal compartment of the cerebellum has a capacity to repair cell losses. This recovery process might account for the development of a cerebellum with apparently normal morphology, and supernormal size (Altman, Anderson and Wright, ’68a), in the animals exposed to a single dose of 200 r. The mature cerebellum of the animals exposed to $5 \times 200$ r was not normal, but this may be attributed in part to the structural disorganization produced by the ongoing development of other cell constituents, such as the disoriented growth of Purkinje cells (fig. 17) during the first week of life. In this context the question may be raised whether the restitutive ability observed in the developing cerebellum of rats also applies to the postnatally developing human brain in which the external granular layer of the cerebellum persists up to 20 months of age (Raaf and Kernohan, ’44).

These findings, which suggest the possibility of developmental regeneration in the cerebellum, are being re-examined in a more extensive study with improved histological techniques.

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LITERATURE CITED

PLATE 1
EXPLANATION OF FIGURES
Low power photomicrographs of midsagittal sections of the cerebellum. Cresyl violet, × 40.

11 Cerebellum of a rat irradiated with 200 r x-ray at three days of age and killed one hour later. The external granular layer is seemingly unaffected. a, anterior cerebellum; lcv, lobus centralis ventralis; p, posterior cerebellum.

12 Cerebellum of a rat that survived for one day after irradiation. The thickness of the external granular layer is greatly reduced in the anterior cerebellum, destructive effect is less severe in the posterior cerebellum, particularly the uvula (u).
PLATE 2
EXPLANATION OF FIGURES

Low power photomicrographs of midsagittal sections of the cerebellum. Cresyl violet, × 40.

13  Cerebellum of a rat irradiated with 200 r x-ray and killed four days after irradiation. The external granular layer appears normal in most regions.

14  Cerebellum of a rat that survived for five days after irradiation. It is morphologically indistinguishable from normal.
PLATE 3
EXPLANATION OF FIGURES

High power photomicrographs of matched regions of the lobus centralis ventralis. Cresyl violet, × 256.

15 The external granular layer in a normal rat, four days of age. eg, external granular layer; ep, ependymal wall of the cerebellar recess of the fourth ventricle; pa, pia-arachnoid membrane; Pu, layer of Purkinje cells.

16 The cerebellum of this rat was exposed to 200 r on days 0, 1, 2, 3, and 4 and the animal was killed about two hours after the last irradiation at the age of four days. Arrow points to a row of cells that may represent surviving elements of the eradicated external granular layer. The row of elongated cells below the ependyma probably are mesenchymal elements of the pia-arachnoid membrane.

17 The irradiation of the cerebellum of this rat was similar to that in figure 16; the animal survived for one day after the last irradiation. Few if any cells of the external granular layer are present. Note apparent increase in the spacing between and in the size of Purkinje cells. Also note that the apical cone of many Purkinje cells, which in a normally developing cerebellum is typically oriented toward the surface of the cerebellar cortex (that is, toward the external granular layer) are randomly oriented after destruction of the external granular layer, with many of them pointing abnormally in the opposite direction (arrows).
RECOVERY OF EXTERNAL GRANULAR LAYER
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PLATE 3

15
16
17

ep pa
eg
Pu

465
PLATE 4
EXPLANATION OF FIGURES

High power photomicrographs of matched regions of the lobus centralis ventralis. Cresyl violet, × 256.

18 The cerebellum of this rat was exposed to 200 r on days 0, 1, 2, 3, and 4 and the animal was allowed to survive for four days after the last irradiation. Arrows point to reappearing clusters of cells representing the regenerating external granular layer.

19 The cerebellum of this rat was treated as those in figures 16 to 18, the animal survived for six days after the last irradiation. The external granular layer forms a continuous sheet of cells, though the layer is somewhat fragmented and the orientation of the cells is irregular.

20 The appearance of a portion of the lobus centralis ventralis in a rat that survived to 30 days of age after it was subjected to radiation as described above. igl, internal granular layer; ep, ependyma of the fourth ventricle (broken); mo, molecular layer. At this age the external granular layer is no longer present. Many granule cells may be seen in the granular layer, which is spotted with cell-free islands. The location of Purkinje cells within the granular layer, and the scarcity of cells in the molecular layer, are abnormal.
PLATE 5

EXPLANATION OF FIGURES

Low power photomicrographs of midsagittal sections of the cerebellum. Cresyl violet, × 40.

21 Appearance of the external granular layer over the surface of the cerebellum in a normal four day old rat. IV, fourth ventricle.

22 The external granular layer was eradicated over most regions in this animal exposed on successive days to five doses of 200 r and killed about two hours after the last irradiation. Patches of the external granular layer remain in the uvula in this animal. Note the dense packing of Purkinje cells in the anterior cerebellum, with scattered small, dark cells that may represent surviving elements of the external granular layer.
Low power photomicrographs of midsagittal sections of the cerebellum. Cresyl violet, × 40.

The external granular layer begins to reappear in the posterior cerebellum in this animal whose cerebellum was exposed on successive days to five doses of 200 r and was killed four days after the last irradiation.

The external granular layer is present over the entire surface of the cerebellum (portion of the nodulus excepted), this animal survived for six days after the last irradiation. Note the fragmented appearance of the external granular layer and some rosette formation.
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Plate 6

Images 23 and 24 show sections of the brain with the external granular layer.