Contract AT(11-1)-2000

ANNUAL PROGRESS REPORT

September 1, 1970-August 31, 1971

Title: LOW-LEVEL X-IRRADIATION OF THE BRAIN DURING DEVELOPMENT:
MORPHOLOGICAL, PHYSIOLOGICAL AND BEHAVIORAL CONSEQUENCES.

Principal Investigator: JOSEPH ALTMAN, Ph.D. Professor
Department of Biological Sciences

Institution: PURDUE UNIVERSITY
Lafayette, Indiana 47907

CONTENTS

1. ABSTRACT
2. MORPHOLOGICAL EFFECTS OF IRRADIATION OF THE CEREBELLUM
 - (a) Histological and Cytological Results
 - (b) Histochemical Results
 - (c) Ultrastructural Results
3. BEHAVIORAL EFFECTS OF IRRADIATION OF THE CEREBELLUM
4. NORMATIVE STUDIES OF THE DEVELOPMENT OF THE CEREBELLUM
 - (a) Autoradiographic Study of the Chronology of Cell Acquisition
 - (b) Histological, Histochemical and Ultrastructural Studies
5. PROFESSIONAL AFFILIATES, CONSULTANTS
6. BIBLIOGRAPHY

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

1. ABSTRACT

(a) In 10, 30 and 90 day old rats we examined the morphological consequences of focal irradiation of the cerebellum during infancy with successive daily doses of low-level X-ray (1-10 x 200 r from birth onward). In animals exposed to 1-2 x 200 r the subtotally eliminated external granular layer was reconstituted by 10 days and there was little or no reduction in the total area of the cerebellar cortex and its various layers at 30 and 90 days. With higher number of successive daily doses there was decreasingly less regeneration of the external granular layer at 10 days, and there was a proportional reduction in the areas occupied by the molecular and internal granular layers at 30 and 90 days. In adult rats the reduction in the population of granule cells was also proportional to the number of daily exposures during infancy. These lasting effects were attributed not to greater damage done to the germinal layer by the higher number of daily exposures but to a postponement of recovery and hence reduction in the time left for regeneration when cerebellar neurogenesis comes to an end (about 21 days). Even with the highest number of exposures, the number of Purkinje cells was not affected. In the animals exposed to more than two doses of 200 r lasting morphological and cytological changes were produced (abnormal folial patterns, streaks and islands of molecular layer, abnormally oriented and misshapen Purkinje cells). These abnormalities could be traced to events associated with the time course of cell death and regeneration in the external granular layer and to the autonomous but disoriented growth of the Purkinje cells.

(b) The skulls of 9-day old rats were irradiated with 100 r X-ray and they were allowed to survive for varying intervals, ranging from 10 minutes to 24 hours. Mitotic activity, cell pyknosis and phagocytosis were examined in the external granular layer of the cerebellar cortex with light and electron microscopy. Mitotic activity was reduced within 1 hour after irradiation, ceased altogether by the 3rd hour, and was resumed again by the 6th hour. Cell pyknosis became first evident morphologically by the 3rd hour. Several phases of imminent cell death were distinguished. The first sign was the darkening of the nucleus, and the condensation and peripheral displacement of nuclear chromatin, with or without cytoplasmic involvement. The total condensation and rounding up of the nucleus was the second phase. The disappearance of nuclear membrane, the coalescing of nucleus and cytoplasm and the rounding up of the entire cell signalled cell death. Astrocytic reaction to tissue damage was evident by the 3rd hour, phagocytosis was first seen after 6 hours. Two types of phagocytic cells were identified, one that resembled Bergmann astrocytes and another that was indistinguishable in appearance from the typical undifferentiated cells of the external granular layer.

(c) Groups of rats were injected with thymidine- H^3 on 2 or 4 successive days between birth and 19 days of age covering the entire time span of postnatal neurogenesis. At maturity the concentration of cells that had their last mitosis soon after the injections (intensely-labeled cells) were counted in different layers and sublayers of the cerebellar cortex in order to determine the time of origin of the different microneurons. The following sequence was indicated: low granule cells $\rightarrow$ basket cells $\rightarrow$ low and high granule cells $\rightarrow$ stellate cells $\rightarrow$ high granule cells.

(d) The cerebellum of rats was irradiated daily with 150 r X-ray from birth for 2, 4, 6, 8 and 10 days. This led to a proportional decrease in the mature weight of the cerebellum due to interference with the acquisition of cerebellar microneurons. The motor development of these animals was observed from 11-30 days in an open field situation. It was also assessed in tests in which they had either to climb up on a rod or descend on a rope. The animals exposed to the highest number of doses persisted longer in displaying "infantile" motor patterns and showed a reduced frequency or duration in the exhibition of various motor skills. They also tended to drag their hindfeet, fell during locomotion and were severely handicapped in climbing. The deficits of the intermediate radiation groups was of a lower magnitude, and the low radiation groups were handicapped only in tasks which depend predominantly on the coordination of the hindlimbs.

(e) The distribution of coated vesicles was examined by electron microscopy in the cerebellar cortex of rats aged 0, 3, 5, 7, 10, 12, 15, 21 and 30 days. Coated vesicles with a honey-comb-like external covering were identified in closed (spherical) and open (flask-like) forms. Closed coated vesicles were seen in large numbers in the vicinity of the Golgi apparatus, and sometimes in direct continuity with its cisterns, in rapidly growing regions of immature neurons, such as the apical cones of Purkinje cells in 3-7 day old animals, and in the dendrites of Purkinje cells in the 10-15 day old animals. Open coated vesicles were seen on the surface of cells, and in neurons these were frequently opposite cell processes with which synapses are formed, such as in the dendrites of Purkinje cells opposite parallel fibers. Occasionally, these open coated vesicles were situated opposite cell processes which had dense membranes, and sometimes they were continuous with the dense membranes of identified early or mature synapses. In the mossy axon terminals in the glomeruli, granular coated vesicles were common in the 21 and 30 day old rats. It was postulated that the coated vesicles are precursors of the dense membranes of synapses, and the following sequence of events was suggested. Through a budding process spherical, coated dense membranes are formed from the membranes of the cisterns of the Golgi apparatus. These spherical vesicles migrate to the surface of cells and after attachment to the membrane open up and unfold over its surface. When this unfolding becomes complete the flat dense membranes form desmosomes or synaptic membranes. Coated vesicles may also contribute to the expansion of the dense membrane surfaces of already-formed synapses. The relation between these vesicles and micropinosomes was discussed.

2. MORPHOLOGICAL EFFECTS OF IRRADIATION OF THE CEREBELLUM

(a) Histological and Cytological Results

In a study using gross-morphological measurements, we established (1) that irradiation of the cerebellum on the day of birth with a single dose of 200 r hard X-ray led to an appreciable reduction in the length of the cerebellum at 10 days of age. This developmental retardation was attributed to the decimation of the cells of the external granular layer (2,3,4). We also found in rats that received such a dose, or even two successive daily doses of 200 r, that the reduction of cerebellar length was no longer evident at 30 and 90 days of age (1). This recovery we could ascribe to the regeneration of the external granular layer after irradiation (4-5). However with higher number of successive daily irradiations permanent growth retardation was produced, the magnitude of which was a function of the number of exposures. In rats that received 4-5 x 200 r, cerebellar length at 30 and 90 days was comparable to that of control animals 10 days of age, and following irradiation with 10 x 200 r the length of the adult cerebellum was comparable to that of normal neonates.

This developmental retardation was expected to occur on the basis of two known facts: (a) that the precursor cells of the granule, basket and stellate cells in the cerebellar cortex, constituting the transient external granular layer, are multiplying and migrating after birth (6-11) and (6) that multiplying (12) and migrating (13) cells are extremely radiosensitive. That, indeed, the cells of the external granular layer are destroyed by low and intermediate doses of X-ray is established (3-5, 11, 13-15) though its consequences are complicated by the observation referred to that the external granular layer can recover after irradiation (4-5, 14-15). In a recent study (16) we evaluated Nissl-stained and Golgi-impregnated sections of cerebella from animals that were irradiated with different doses of X-ray (1-10 x 200 r) and were killed at 10, 30 and 90 days of age. We paid attention to qualitative changes in the histology of the cerebellar cortex and to the cytology of surviving elements, particularly the Purkinje cells. The quantitative studies were aimed at establishing a correlation between radiation schedules and histological alterations, particularly areal reductions of the different cortical layers, reduction in the population of granule cells, and consequent changes in granule cell/Purkinje cell ratios.

-
1. J. Altman, W. J. Anderson and K. A. Wright, Exp. Neurol., 1968, 21:69.
 2. J. Altman, W. J. Anderson and K. A. Wright, Exp. Neurol., 1967, 17:481.
 3. J. Altman, W. J. Anderson and K. A. Wright, Exp. Neurol., 1968, 22:52.
 4. J. Altman, W. J. Anderson and K. A. Wright, Anat. Rec., 1969, 163:453.
 5. J. Altman, W. J. Anderson and K. A. Wright, Exp. Neurol., 1969, 24:196.
 6. I. L. Miale and R. L. Sidman, Exp. Neurol., 1961, 4:277.
 7. J. Altman and G. D. Das, J. Comp. Neurol., 1966, 126:337.
 8. J. Altman, J. Comp. Neurol., 1966, 128:431.
 9. J. Altman, J. Comp. Neurol., 1969, 136:269.
 10. S. Fujita et al, J. Comp. Neurol., 1966, 128:191.
 11. E. J. Ebels, De Rijping van het Cerebellum. Groningen, 1969.
 12. V. P. Bond, et al, Mammalian Radiation Lethality, New York, 1965.
 13. S. P. Hicks and C. J. D'Amato, In D. H. M. Woollam, Advances in Teratology. London, 1966.
 14. R. D. Phemister et al, J. Neuropath. Exp. Neurol., 1969, 28:119.
 15. R. D. Phemister et al, J. Neuropath. Exp. Neurol., 1969, 28:128.
 16. J. Altman and W. J. Anderson, Exp. Neurol., 1971, 30:492.

Qualitative evaluation of Nissl-stained sections of the vermis of 10 day old animals indicated that with irradiation restricted to the first days after birth (1-2 x 200 r) the external granular layer recovered and appeared normal by the 10th day, and only the maturation of the molecular and internal granular layers was retarded. With increasing number of successive daily irradiations (3-5 x 200 r) the reconstituted external granular layer became more abnormal in appearance and its cells, as judged by the absence of molecular and internal granular layers, were not yet differentiating. With maximal number of irradiations (8-10 x 200 r) the recovery of the external granular layer was prevented altogether in the affected regions. These effects are easily understood in terms of what we already know about the immediate consequences of irradiation and of the time course of the subsequent regeneration. Irradiation with 1 or more daily doses of 200 r drastically reduces or subtotally eliminates the multiplying cells of the external granular layer (3) but enough radioresistant cells remain and these begin to repopulate the external granular layer (to a greater or lesser degree as a function of the number of irradiations) by the 4th day after irradiation (4-5). The presence of a normal external granular layer in the rats that received 1-2 x 200 r is due to the circumstance that more than a week elapses between the cessation of irradiation and removal of the cerebellum to permit the total reconstitution of this germinal layer. The retardation in the development of the internal granular and molecular layers reflects the delay produced in the differentiation of the cells of the external granular layer while regeneration is in progress.

Little difference was seen within treatment conditions between 30 and 90 day old animals. The cerebellum of adult rats that received a single dose of 200 r on the day of birth was indistinguishable from that of normal animals. Likewise, in the majority of rats that received an additional dose of 200 r on the next day (2 x 200 r) the cerebellum appeared entirely normal. In the animals that received 3-4 x 200 r several abnormalities were noted. In general, the folial pattern was distorted; the molecular layer was thin and had a reduced concentration of cells; the internal granular layer had an irregular shape and was occasionally studded with cell-sparse streaks and islands; the Purkinje cells were scattered throughout the the depth of the internal granular layer; an occasional Purkinje cell had a misshapen nucleus; and the relative concentration of Purkinje cells was extremely high.

Similar observations were made in the animals that received 5 x 200 r, but the abnormalities noted were more pronounced and more common. Thus, there was a greater reduction in the concentration of granule cells, there were more streaks and islands in the internal granular layer, and a greater frequency of Purkinje cells with twisted nuclei. In the animals that were exposed to 8-10 x 200 r the molecular layer was essentially obliterated over the surface of the anterior lobes indicating that the formation of basket and stellate cells was prevented. Few granule cells were present in the "internal granular layer", as the latter was composed almost entirely of Purkinje cells which in some regions were concentrated 3-6 cells deep. The Purkinje cells were of normal size but in many regions, particularly where the folia were folded abnormally, the nuclei of many Purkinje cells were twisted. Even in the most affected cerebellum (in which the molecular and internal granular layers were essentially absent) no abnormalities could be detected in the neurons of the deep cerebellar nuclei.

In Golgi-impregnated cerebella of unirradiated 10 day old rats there was considerable variability in the appearance of Purkinje cells in different lobules and also within the same lobule. Immature Purkinje cells were seen that were covered with many perisomatic processes, and were lacking in clearly recognizable primary or secondary dendrites. More frequent were maturing Purkinje cells that no longer had perisomatic processes and had secondary and some tertiary dendrites which reached to the border of the external granular layer and were moderately covered with spines. Also in the irradiated cerebella, Purkinje cells were seen that fell within the range of those from normal cerebella. But in the animals that received 2 or a higher number of daily doses of 200 r, many Purkinje cells showed clear signs of abnormality. Common among these was the disorientation of the primary dendrites which were oriented obliquely or parallel to the cerebellar surface. Often these dendrites had long straight or tortuous shafts and were devoid of branches. Dendritic branches that penetrate the external granular layer were seldom seen in normal animals, in the irradiated animals Purkinje cells with thick branches were observed that penetrated deep into the external granular layer. These branches had no spiny branchlets whereas those that reached the molecular layer had branchlets and spines.

In the 30-90 day old rats that received 2 x 200 r many normally-oriented Purkinje cells were seen with a rich dendritic plexus in the molecular layer. But many were also seen which had extremely long and bare primary dendrites embedded in the internal granular layer. Normal Purkinje cells were less frequently seen in the animals that received 5 x 200 r and few, if any, in those that received 8-10 x 200 r. The following distortions were noted in Purkinje cells. A long, slender and bare primary dendrite oriented either obliquely or parallel to the surface of the molecular layer. In many cells several "primary" dendrites were seen, apparently abortive ones without any branching terminals and others terminating in tufts with dendritic branchlets and spines. These multiple main dendrites were often oriented in different directions and planes. In other instances the main dendrites lay at the interface of the internal granular layer and molecular layer with branches absent in the former and rich in the latter. The radial streaks or circular islands of cell-sparse areas that were seen in Nissl-stained material in the cerebellum of animals that received 5-10 x 200 r were identified in Golgi material as parts of the molecular layer in which the Purkinje cell dendrites were located. Their branchlets often followed the tortuous outlines of the streaks or islands, with occasional semi-circular dendrites. In general, the total area of the arborizing branchlets of these abnormal Purkinje cells was far below that of typical normal Purkinje cells. We established in this study that the Purkinje cells are not destroyed by single or multiple doses of 200 r and that their perikarya grow normally in terms of size. However, the direction of growth of their apical cone, which gives rise to the dendrites, is affected (4-5). In normal cerebella the apical cones of the Purkinje cells are oriented at a right angle to the surface, but when the external granular layer is destroyed, they are oriented randomly. This was also observed in hamsters in which the external granular layer was destroyed by administration of methylazoxymethanol (17). This random orientation of the outgrowing Purkinje cell primary dendrite

might be due to the removal of some guiding influence emanating from the cells of the external granular layer or the parallel fibers that they form before descending. But a simpler explanation is that because of the retarded growth of the irradiated cerebella, the Purkinje cells cannot all distribute themselves over the surface (which occurs at about 3-5 days) and therefore there is no room for apical cones to grow in the preferred direction. In any case the crowded Purkinje cells grow apical cones and primary dendrites in all directions and the molecular layer, which is the junctional region of Purkinje cell dendrites and parallel fibers, assumes an abnormally folded, streaky appearance as differentiation proceeds. The changes that are seen in the cytological differentiation of the Purkinje cells are, accordingly, secondary effects of the morphological abnormalities produced by the destruction of the multiplying precursors of the cerebellar interneurons.

Some of the highlights of the quantitative determinations were the following. There was a 40% areal reduction in the 10 day old animals that received 1-2 x 200 r, more than 60% reduction in those that received 3-10 x 200 r. The relatively lower loss in the former group was due to recovery of the external granular layer and commencement of differentiation of these cells; the uniformly greater reduction in all the others reflects the absence of appreciable recovery by 10 days of age. In the 30-90 day old rats there was minimal (if any) areal reduction in those exposed to 1 x 200 r indicating total recovery, but ambiguous results were obtained in the two age groups in the animals that received 2 x 200 r. In general, there was a gradual areal reduction as a function of number of irradiations, but this was more consistent in the 30 day than 90 day old animals.

There was considerable variability in the total number of Purkinje cells with nucleoli in the scanned sections but there were no systematic differences attributable either to age or radiation treatment. It was concluded, therefore, that the number of the prenatally-formed Purkinje cells was not reduced by radiation up to 10 x 200 r. Because there was no change in the number of Purkinje cells due to radiation and no change in the size of their perikarya, the reduction in the total area of the cerebellum was attributed to changes in the Purkinje cell dendrites and other components of the cerebellum. Determination of the area occupied by the internal granular layer and the sampling of the packing density of granule cells provided an estimate of the changes in the granule cell population of the sampled sections. Changes in total number of granule cells, and the granule/Purkinje cell ratios paralleled closely the reduction in the total area of the sections. Comparable changes were seen in the area occupied by the molecular layer.

In conclusion the evidence indicated that the progressive reduction seen in the total volume of the cerebellar cortex with increasing number of daily irradiations is not due to greater direct damage done to the external granular layer. Rather the supplementary daily doses (after the initial ones on the first and second days after birth) prevent the reconstitution of the external granular layer and postpone the production of the germinal cells and their differentiation. Since cerebellar neurogenesis comes to an end at about 21 days of age in normal as well irradiated animals (unpublished observations) the longer cellular reproduction and differentiation are delayed the less ultimate regeneration can take place.

(b) Histochemical Results

We have recently examined the changing pattern of cholinesterase staining in the cerebellar cortex of rats age 0-30 days (18). In agreement with the results of other investigators (e.g. 19, 20) we found no staining in the molecular layer at any age, indicating that the climbing fibers, parallel fibers and the basket and stellate cells are noncholinergic. In the internal granular layer the Golgi cells were lightly staining by the end of the first week, and the mossy fibers in some lobules stained lightly by 5 days, and at 10 days the glomeruli were intensely outlined by the staining reaction. When the external granular layer was destroyed by repeated doses of 200 r X-ray, the glomerular accumulation of cholinesterase was prevented. Instead, staining appeared now in the molecular layer above the Purkinje cells. This result suggested that the elimination of descending granule cells with which the mossy fibers synapse in the glomeruli caused the continued growth of the mossy fibers toward the molecular layer. But it could not be determined with what cellular elements, if any, these mossy fibers established synaptic contacts. This was among the first of the evidence suggesting a change in the circuitry of the cerebellar cortex following elimination of the precursors of its microneurons by irradiation, and it pointed to the desirability of examining this and some related problems with electron microscopy.

(c) Ultrastructural Results

In our first study using electron microscopy (21) we examined some aspects of cell necrosis produced by 100 r X-ray in the external granular layer of infant rats. The animals were allowed to survive for varying intervals from 10 minutes to 24 hours. Mitotic activity, cell pyknosis and phagocytosis were examined with light microscopy and electron microscopy. In particular we wished to determine what changes occur after irradiation in the structure of multiplying and migrating cells before cell death becomes obvious and also the ways whereby necrotic cells are eliminated. Mitotic activity was reduced within 1 hour after irradiation, ceased altogether by the 3rd hour, and was resumed again by the 6th hour. Previous studies (4,13) indicated that cell death following irradiation was a protracted process, becoming manifest as pyknosis 4 hours after irradiation. This study showed that the latency is somewhat shorter with many cells showing morphological signs of imminent cell death 3 hours after irradiation. No information has been available hitherto about the phases of radiation-induced cell pyknosis. We could distinguish several phases in the process. Among the first affected cells were those that showed condensation and peripheral displacement of the nuclear chromatin, with or without evident cytoplasmic involvement. This was judged the first phase of lethal cell damage. We could not identify any organelles that were particularly affected. Affected cells whose nucleus became spherical and opaque were

-
- 18. J. Altman and G. D. Das, Exp. Neurol., 1970, 28:11.
 - 19. B. Csillik et al, J. Histochem. Cytochem., 1963, 11:113.
 - 20. C. C. D. Shute and P. R. Lewis, Nature, 1965, 205:242.
 - 21. J. Altman and J. L. Nicholson, Radiation Research, 1971 (in press).

judged to represent the second phase in the process and we assumed that this reflected nuclear death. The cells in which the nuclear membrane disappeared and in which the cytoplasm became also rounded were judged to have died altogether, and these were considered to represent the third phase in cell pyknosis.

In a previous study (3) we concluded that migratory proliferative cells are more radiosensitive than the stationary proliferative cells. This was based on computing pyknosis index/labeling index ratios in material prepared with thymidine- H^3 autoradiography. We also concluded that migratory, postmitotic cells are also extremely radiosensitive, and this was based on the observation that pyknotic cells were present in the nonproliferative, migratory, or inner, zone of the external granular layer. However, the present study showed clearly that initially pyknotic cells were concentrated in the outer, proliferative zone. Their subsequent appearance in the migratory zone could have been due, therefore, either to the migration or the passive displacement of the dying cells from the proliferative zone by the multiplying cells. It is interesting to note that the arrest in mitotic activity was evident before cell pyknosis was observed and by the time cell pyknosis was pronounced, mitotic activity was resumed. While some relationship must exist between the intense nuclear activity of these proliferative cells and their radiosensitivity, cell death apparently is not directly due to mitotic death. The relationship between cell cycle and cell death and the reason for the apparent asynchrony in cell death (cells in the first phase of pyknosis were seen even after 24 hours) remain to be determined.

The final phase of cell pyknosis was marked by phagocytosis and digestion of the cell debris. We identified two types of phagocytic cells. One with light nucleus and cytoplasm, resembling Bergmann astrocytes, the other with darker nucleus and cytoplasm, similar to the typical cells of the external granular layer. The increase in the area occupied by lightly staining cell processes among the cells of the external granular layer were noted as soon as pyknotic cells appeared, suggesting early astrocytic reaction to tissue damage. In time these tissue processes engulfed the dead cells. If the other cell types represented external granule cells rather than histiocytes, this would suggest that primitive cells destined to differentiate into neurons have phagocytic potency before their differentiation begins.

3. BEHAVIORAL EFFECTS OF IRRADIATION OF THE CEREBELLUM

It was noted by several investigators that the duration of postnatal cerebellar neurogenesis (as determined by the dissolution of the external granular layer) coincides in various mammalian species with the maturation of their motor, especially locomotor, skills. In the rat the external granular layer disappears at 21 days and it is at this age that rats are weaned, suggesting a sufficient degree of behavioral self-sufficiency. Is the acquisition of the interneurons of the cerebellar cortex and their interdigitation into its circuitry essential for the development of locomotor skills?

Evidence has been available for some time that some of the motor deficits that result from damage to or destruction of the cerebellum as a whole, such as tremor and ataxia, are also present when merely the micro-neurons of the cerebellar cortex fail to develop. The ataxia produced in mice and rats by whole-body or whole-head irradiation has for a long time been associated with cerebellar hypoplasia (23, 24, 25) and we showed recently (26, 27) that ataxia and tremor is produced by selective irradiation of the cerebellum, with a correlation between the severity of motor abnormalities and the reduction in the estimated number of cerebellar microneurons. Likewise, examination of certain neurological mutants in mice showed an association between ataxia (and other motor disorders) and cerebellar hypoplasia attributable to paucity of granule cells (28). Ataxia in cats and ferrets was traced to a viral agent that has affinity for dividing cells and prevents the acquisition of granule cells (29).

The intent of a new study (30) was to examine in greater detail the behavioral consequences of the graduated reduction of the cerebellar interneurons on the development of motor capacity in young rats. The observations and quantitative tests were similar to those that we used in another series of studies (31) in which we examined the effects of mild and severe undernutrition upon the development of the cerebellum and motor performance. General motor activity, as assessed by the frequency of time spent in ambulation was higher in most of the irradiated animals than in the controls for some time after the irradiations. This is in agreement with our earlier results, in which a different assessment technique was used (27) and may be related to the higher "emotionality" of the irradiated animals. But the "effective" locomotion of the irradiated animals,

-
22. J. Altman, in The Neurosciences (G. D. Quarten, eds.) New York, 1967.
 23. H. Brunner, Arch. Klin. Chirur., 1920, 144:332.
 24. S. P. Hicks and C. J. D'Amato, Advances in Teratology (D. H. M. Woollam, ed.). London, 1966.
 25. J. N. Yamazaki et al, Response of the Nervous System to Ionizing Radiation. New York, 1962.
 26. R. B. Wallace and J. Altman, Devel. Psychobiol., 1969, 2:257.
 27. R. B. Wallace and J. Altman, Devel. Psychobiol., 1969, 2:266.
 28. R. L. Sidman et al, Catalog of the Neurological Mutants of the Mouse. Cambridge, 1965.
 29. L. Kilham and G. Margolis, Amer. J. Pathol., 1966, 48:991.
 30. J. Altman, W. J. Anderson and M. Strop, Physiol. Behav., 1971 (in press).
 31. J. Altman, et al, Devel. Psychobiol., 1971 (in press).

judged by the number of squares traversed by crawling or walking, was not higher in the irradiated animals, indeed it was reduced in the high irradiation groups. These results suggested that cerebellar irradiation did not diminish the animals' readiness for ambulation but it retarded slightly, at least in the more severely affected animals, its effectiveness.

More profound deficits were seen in the irradiated animals in the abandoning of "infantile" locomotor patterns (pivoting and descending on a rope backward) and in the display of emerging more complex ones. In some of these observations only the high irradiation groups (that is, those with maximal cerebellar damage) were affected, in others the intermediate group was also handicapped, and only in one (standing on hindlegs without forelimb support) were even the low irradiation animals retarded. Thus, the ineffective pivoting, which is replaced by crawling and walking, persisted longer in the high irradiation groups than in the controls, but the other experimental groups were not affected. Moreover, this pattern disappeared at the end of the observation period even in the animals that received the highest number of irradiations. Sitting without support was reduced in frequency only in the high radiation groups, standing on the hindlegs with forelimb support decreased also in the intermediate group, and standing on the hindlegs without forelimb support was reduced even in the lowest radiation group.

Comparable results were obtained in the tests in which the animals were called upon to display their skills in climbing up a rod to a platform to avoid falling into the water or climbing down a rope to reach the ground. Generally speaking in these tests only the high and intermediate radiation groups were affected. None of the 10 x 150 r animals, and few of those receiving 8 x 150 r, could reach the platform in the allotted time to escape the punishment of falling into the cold water. This skill was delayed in the intermediate group and their latency was much longer than that of the control or low radiation animals. The transient and infrequent pattern of climbing down backward (head upward) was seen earlier in the latter groups and it appeared in the intermediate and high radiation animals after this "infantile" pattern was replaced in the controls by the consistent response of turning around the rope and climbing down "forward" (head downward). This was seldom displayed by the high irradiation animals even at the end of the testing period and they fell down often or climbed down very slowly.

The experiments that were performed do not permit us to specify with certainty the underlying causes of these motor deficits. General observation indicated that one of the most pronounced deficits of the irradiated animals was an incoordination of the hindlimbs. The longer persistence of pivoting (which is caused by the abdomen and passive hindlimbs acting as pivots when the forelimbs are moving); the handicap shown by all the irradiated animals in standing on the hindlegs without forelimb support; the difficulty of the irradiated animals in supporting the weight of their body with the hindlimbs when getting off the rod to reach the platform or when turning around for descent, all of these observations indicate severe deficits in hindlimb coordination and less (if any) loss in the control of the forelimbs. It was these observations that suggested the possibility that cerebellar control in the rat is greater for the predominantly "ambulatory" hindlimbs than for the partially "manipulatory" forelimbs. The latter are presumably to a larger extent under cerebral control which makes more restitution possible when the development of the cerebellum is retarded.

4. NORMATIVE STUDIES OF THE DEVELOPMENT OF THE CEREBELLUM

(a) Autoradiographic Study of the Chronology of Cell Acquisition

The cells of the proliferative external granular layer give rise to three types of microneurons, the stellate cells of the upper molecular layer, the basket cells of the lower molecular layer and the granule cells of the internal granular layer. Physiological evidence (32) indicates that the stellate cells exert inhibitory influence on the dendrites of Purkinje cells, the basket cells inhibit their soma and the granule cells through the parallel fiber, excite the Purkinje cell dendrites. Because of their differential role in the operation of the circuitry of the cerebellum, it appeared worthwhile to establish the time course of the differentiation of these cell types with the aim of using timed X-irradiation for the selective elimination of one or the other of these cell types.

In a previous study (33) evidence was obtained that the cells of the lower molecular layer (where the basket cells are located) differentiate before the cells of the upper molecular layer (which include the stellate cells) and that the time of differentiation of the granule cells overlaps with the other two. This conclusion was based on counting the number of intensely-labeled cells in autoradiograms of the cerebellum of adult rats that were injected with thymidine- H^3 at 0, 2, 6 and 13 days of age. The rationale of the method was that intense nuclear labeling (maximal concentration of tagged DNA) indicates that the cell was formed at the time of injection but that it had not undergone further subdivisions afterwards (which leads to the dilution of the tagged DNA) because it has commenced to differentiate. Maximal number of intensely-labeled "basket cells" were found in the animals injected at 6 days of age, with very few at 2 or 13 days, intensely-labeled "stellate cells" were seen in highest number at 13 days. In the internal granular layer the concentration of heavily tagged cells was highest at 13 days but there was also a moderate concentration of these at 6 days.

In the study described, the animals received a single injection of thymidine- H^3 on a specified day and only a few groups of injected rats were used. These drawbacks made it difficult to give an accurate estimate of the time of origin of the different cell types. In a recent study (34) the entire period of postnatal cerebellar neurogenesis was covered in two groups of animals. In one group, pairs of Long-Evans hooded rats were injected with thymidine- H^3 on two successive days at the following ages: 0-1, 2-3, 4-5, 6-7, 8-9, 10-11, 12-13, 14-15, 16-17, and 18-19 days. In the other, pairs of animals were injected daily on four successive days at the ages of 0-3, 4-7, 8-11, 12-15 and 16-19 days. For microscopic examination of the autoradiograms matched regions of the pyramis of the posterior vermis were selected. The encountered intensely-labeled cells

-
- 32. J. C. Eccles et al, The Cerebellum as a Neuronal Machine. New York, 1967.
 - 33. J. Altman, J. comp. Neurol., 1969, 136:269.
 - 34. J. Altman and G. D. Das, Brain Research, (submitted for publ.)

(those that ceased to multiply soon after the injection) in the folded cortex of the pyramis were assigned to the following laminar subdivisions in the path of scan: upper and lower molecular layer; upper and lower granular layer; medullary layer; lower and upper granular layer; lower and upper molecular layer. Cells in the upper molecular layer (which include an unknown proportion of stellate cells and glia cells) are referred to as "stellate cells"; the cells of the lower molecular layer as "basket cells". The cells of the internal granular layer (which are predominantly granule cells but also include some Lugaro and some glia cells) are referred to as "granule cells".

The highest concentration of intensely-labeled "basket cells" was seen in the animals injected at 4-7 days in the group receiving 4 successive daily injections, and at 6-7 days in the group that received 2 injections. The fact that nearly as many intensely-labeled cells were seen in the latter group as in the former indicates that the injections on days 4-5 contributed few cells to the "basket cell" population in the group receiving 4 injections (this is indicated directly for these days in the double-injection group). Therefore, it is concluded that the bulk of the "basket cells" in the pyramis are formed on days 6-7.

In the group that received 4 successive daily injections, the highest concentration of "stellate cells" was seen in the animals injected at 8-11 days. In the group injected with 2 successive daily doses a comparable concentration of intensely-labeled cells were obtained in both the animals injected at 8-9 days and at 10-11 days. However, in these animals the number of such cells was less than half of that counted in those that received 4 successive injections on days 8-11. This difference permits several interpretations. First, the "stellate cells" are formed over the protracted period of 4 days. Second, the "stellate cells" are formed over a 2-day period on days 9-10, with injection on day 8 in the 8-9 day group and on day 11 in the 10-11 day group contributing few intensely-labeled cells. The third possibility is that the "stellate cells" are composed of two populations and those in the lower aspect of the upper molecular layer are formed earlier (8-9 days) than those in the upper aspect (10-11 days). This possibility was supported by the observation of a gradient in the molecular layer, with the cells situated near the surface being among the last labeled.

Counts of intensely-labeled cells in the lower and upper zone of the internal granular layer in the group that received 4 successive injections showed maximal concentration of such cells in the animals injected at 8-11 days; but the concentration of these cells was also high in the animals injected at 12-15 days. In the group that received 2 successive daily doses of thymidine- H^3 , highest concentration of intensely-labeled cells was obtained at 10-11 days, but the concentration of these cells was also high at 8-9, 12-13 and 14-15 days. From this it was concluded that the bulk of the "granule cells" are formed between 7-15 days, that is, over the long time span of the second week of life. Interestingly, of the small proportion of "granule cells" formed during the first week more were situated in the lower half of the internal granular layer than in the upper half, and the reverse held for the "granule cells" formed during the third week. This suggested that the granule cells that will become affiliated with the basket cells (and with the lower dendritic zone of the Purkinje cells) differentiate

first, whereas those that will become affiliated with the stellate cells (and with the upper dendritic zone of the Purkinje cells) differentiate last.

In this study we also observed several larger cells situated below the Purkinje cells with intense-labeling toward the end of the second week. On the basis of electron microscopic studies (unpublished) we identify these with Lugaro cells. Furthermore, we also observed smaller intensely-labeled cells in the lower half of the molecular layer at the beginning of the third week (these are conceivably glia cells but the identification must remain tentative). The definitive identification of the cells of the cerebellar cortex during postnatal development will require additional tools, in particular electron microscopic autoradiography. In summary, the suggested tentative sequence in the acquisition of the microneurons of the cerebellar cortex is as follows: (1) low granule cells; (2) basket cells; (3) low and high granule cells; (4) stellate cells; (5) high granule cells.

(b) Histological, Histochemical and Ultrastructural Studies

During this contract year we prepared and have been examining with electron microscopy an extensive number of cerebella from rats aged 0, 3, 5, 7, 10, 15, 21 and 30 days of age. Matching these ages we also prepared and examined cerebella prepared with several histochemical procedures (AChE, SDH, LDH, CYO, NAD, NADP) and with Golgi impregnation. The evaluation of this material, which consists of several hundred specimens, is now in progress and will be hopefully completed early next year. This material, and what we learn from it, will serve us as a standard against which we will compare the material that is being collected from animals that are exposed at different ages during infancy to different irradiation schedules for the purpose of producing cerebella with different cell constituents and different neuronal "circuits" (see RENEWAL REQUEST, 1971-1972). Here we describe the first result of our electron microscopic developmental study, concerned with some hitherto unreported findings relating to cerebellar synaptogenesis.

Synaptogenesis is a major event in the ontogenetic development of nerve cells. On the basis of logical considerations it is assumed that the formation of adhesion sites is the first step in synaptogenesis, and this is thought to be based on some chemical recognition process, or affinity, between cells or cell processes that move past each other in the course of development. Some empirical evidence is available in support of this view (35) but others have claimed that the dense membranes and synaptic vesicles are formed at the same time to produce a functional synapse (36,37). Some of our recent observations (38) offer clues about the possible origin of dense membranes, together with some data relating to the sequence of events in synaptogenesis in the developing cerebellar cortex of the rat.

-
- 35. L. M. H. Larramendi, in Neurobiology of Cerebellar Evolution and Development (R. Llinas, ed.). Chicago, 1969.
 - 36. D. Bodian, J. comp. Neurol., 1968, 133:113.
 - 37. J. Ochi, Z. Zellforsch., 1967, 76:339.
 - 38. J. Altman, Brain Research, 1971 (in press).

Round vesicles, 400-1400 Å in diameter and furnished with an outer coat, were seen in various parts of the cerebellar cortex in all the animals, but in particularly large numbers in the animals aged 0-15 days. The outer coats of these vesicles had often a fuzzy or spiky appearance; in other instances they could be clearly seen to consist of circular discs. In immature nerve cells these coated vesicles were seen in highest concentration in a perinuclear position in the vicinity of the Golgi apparatus. Occasionally there was a continuity between the coated vesicles and the cisterns of the Golgi apparatus or the cisterns of the agranular reticulum, giving the impression of their production at these sites by "pinching-off" or budding.

These coated vesicles were also seen singly or occasionally in pairs, in other regions of the cytoplasm, particularly in the vicinity of the cell membrane. In the 3-7 day old animals this was frequent in the apical cone of the Purkinje cells; in the 10-15 day old animals in the smooth and spiny branchlets of the Purkinje cell dendrites. But they were also seen in many other regions, including occasionally in parallel fibers. Coated vesicles that were in contact with the cell membrane often had an opening into the extracellular space or were in various shapes of apparent folding or unfolding, resembling flask-shaped pinocytosomes. Significantly, these open "coated vesicles" were often situated opposite cell processes with which synaptic relation is common in older animals, such as in the dendrites of Purkinje cells opposite parallel fibers.

The open coated vesicles seen in maturing nerve cells were in some cases situated opposite cell processes with a simple membrane, or opposite cell processes with partially or fully developed dense membranes. In the latter instances dense material of low opacity was occasionally seen in the intercellular cleft. Rarely, paired open coated vesicles were situated apposed on both sides of the intercellular cleft. In the same position where open coated vesicles were often seen in contiguity with other cell processes, flat dense membranes were also seen resembling desmosomes. When these desmosome-like contacts were between nerve processes which have synapses in older animals, such as between the spines of Purkinje cells and parallel fibers, they were identified as "early synapses." The possibility that the dense membranes of early synapses were flattened or transformed open coated vesicles was suggested by the frequent presence of closed or open coated vesicles near or in contiguity with the postsynaptic dense membrane of identified synapses. Less frequently this was also seen in the presynaptic membrane. The dense membranes of the coated vesicles and that of the synapse were often continuous.

The foregoing results suggested the following sequence of events in the formation of the pre- and postsynaptic dense membranes. Through a budding process, spherical coated dense membranes are formed from the cisterns of the Golgi apparatus and the associated agranular reticulum and these take the shape of closed, or spherical, coated vesicles. These spherical coated vesicles migrate to the surface of the cell and after their attachment to the cell membrane, open up and unfold over its surface. At this stage they resemble micropinosomes, but when the unfolding becomes complete the flat membranes become indistinguishable from desmosomes or synaptic dense membranes. Coated vesicles may also contribute to the extension of the dense membrane surfaces of already-formed synapses.

5. TRAINING AND PROFESSIONAL AFFILIATES

1970-1971

Professional Affiliates

Das, G. D. Ph.D. Associate Professor, Purdue University
 Ebels, E. J. M.D., Ph.D. Associate Professor (returned to University
 of Groningen, October, 1970)
 Wallace, R. B. Ph.D. Assistant Professor, Hartford University
 Freeman, J. M.D., Ph.D. Major, USAF (Consultant, 1970)
 Hoffer, B. M.D., Ph.D. N.I.M.H.
 Woodward, D. Ph.D. Assistant Professor, Rochester University
 Anderson, W. J. B.A. Instructor, Chief Technician, Purdue University

Degree Candidates

J. L. Nicholson (Ph.D.)*	H. Nornes (Ph.D.)*
F. Horgos (M.Sc.)*	R. Hine (Ph.D.)
C. B. Moffett (Ph.D.)	F. Bulut (Ph.D.)
N. Sievers (Ph.D.)	E. Sheehy (M.Sc.)
S. A. Bayer (Ph.D.)	D. Barnes (M.Sc.)
K. Sudarshan (Ph.D.)	G. Carayanapoulos (Ph.D.)
J. Deblek (M.Sc.)	M. Pfaffenroth (Ph.D.)

Honors (thesis) Students and Others

V. Hinemann*	M. Ritchie
L. Misner	K. Miller
M. Harschman	G. Greenman
L. Berte	

* Graduating, 1971

6. BIBLIOGRAPHY 1970-1971

1. Altman, J. 1970 Postnatal neurogenesis and the problem of neural plasticity. In W. A. Himwich, (ed.), Developmental Neurobiology, P. 197-237. Springfield, Thomas.
2. Altman, J. and G. D. Das 1970 Postnatal changes in the concentration and distribution of cholinesterase in the cerebellar cortex of rats. Exp. Neurol. 28: 11-34.
3. Das, G. D. and J. Altman 1970 Postnatal neurogenesis in the caudate nucleus and nucleus accumbens septi in the rat. Brain Res. 21: 122-127.
4. Wallace, R. B., and J. Altman 1970 Behavioral effects of neonatal irradiation of the cerebellum. I. Qualitative observations in infant and adolescent rats. Develop. Psychobiol. 2: 257-265.
5. Wallace, R. B. and J. Altman 1970 Behavioral effects of neonatal irradiation of the cerebellum. II. Quantitative studies in young-adult and adult rats. Develop. Psychobiol. 2: 266-272.
6. Altman, J. and W. J. Anderson 1971 Irradiation of the cerebellum in infant rats with low-level x-ray: histological and cytological effects during infancy and adulthood. Exp. Neurol. 30: 492-509.
7. Altman, J., G. D. Das and K. Sudarshan 1971 The influence of nutrition on neural and behavioral development. I. Critical review of some data on the growth of the body and the brain following dietary deprivation during gestation and lactation. Devel. Psychobiol. 3: 281-301.
8. Altman, J., G. D. Das, K. Sudarshan and J. B. Anderson 1971 The influence of nutrition on neural and behavioral development. II. Growth of body and brain in infant rats using different techniques of undernutrition. Devel. Psychobiol. (in press).
9. Altman, J. and B. McCrady 1971 The influence of nutrition on neural and behavioral development. IV. Effects of infantile undernutrition on the growth of the cerebellum. Devel. Psychobiol. (accepted for publication).
10. Altman, J., K. Sudarshan, G. D. Das, N. McCormick and D. Barnes 1971 The influence of nutrition on neural and behavioral development. III. Development of some motor, particularly locomotor patterns during infancy. Devel. Psychobiol. (in press).
11. Altman, J. 1971 Environmental influences on the development of the cerebellum and motor performancy in rats. (in press).
12. Altman, J. 1971 Coated vesicles and synaptogenesis. A developmental study in the cerebellar cortex of the rat. Brain Research. (in press).
13. Altman, J. 1971 Nutritional deprivation and neural development. In Sterman, McGinty, and Adinolfi (eds.), Neural Ontogeny and Behavior. New York, Academic Press. (in press).

14. Altman, J., W. J. Anderson, and M. Strop 1971 Retardation of cerebellar and motor development by focal x-irradiation during infancy. Physiol. Behavior. (in press).
15. Altman, J. 1971 Environmental influences on the development of the cerebellum and motor performance in rats. (in press).
16. Altman, J. 1971 Autoradiographic examination of behaviorally induced changes in the protein and nucleic acid metabolism of the brain. In J. Gaito (ed.), Macromolecules and Behavior. Appleton-Century-Croft. 2nd ed. (in press).
17. Altman, J. and G. D. Das Sequential differentiation of cerebellar micro-neurons during postnatal development: an autoradiographic study. Brain Research. (submitted for publication).
18. Wallace, R. B., J. Altman and C. E. Daniels 1971 Behavioral effects of neonatal irradiation of the cerebellum: III. Qualitative observations in aged rats. Devel. Psychobiol. (in press).
19. Altman, J. and J. L. Nicholson 1971 Cell pyknosis in the cerebellar cortex of infant rats following low-level x-irradiation. Rad. Research. (in press).
20. G. D. Das and J. Altman 1971 Postnatal neurogenesis in the cerebellum of the cat and tritiated thymidine autoradiography. Brain Research. (in press).