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

Comprehensive Progress Report

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1. RESEARCH ACCOMPLISHMENTS

A. FIRST PHASE: GENERAL AUTORADIOGRAPHIC STUDIES

The original objective of this research program was the autoradiographic examination of protein, RNA and DNA metabolism in the brains of experimental animals under normal conditions, and following behavioral manipulations and pathological treatments. The following were the main accomplishments of the initial set of experiments:

(i) Tritium-labeled amino acids were injected systemically into rats and the regional incorporation of the radiochemical was studied at different brain sites by preparing serial autoradiographic maps and by making microdensitometric measurements of uptake by single nerve cells (5).

(ii) Alterations in the regional incorporation of labeled amino acids were investigated in rats which were manipulated behaviorally. The manipulations included forced running in an activity wheel, with or without adaptation (6, 14, 19); prolonged auditory or visual stimulation (13, 14); prolonged training on visual discrimination tasks (20); handling of rats during infancy (14); raising weaned or young adult rats in restricted or enriched environments (21). The results indicated that stress or conditions that tended to promote stress in experimental situations led to significant increases in the incorporation of the administered radiochemicals. However, selective enhancement of protein synthesis in different brain regions by the different manipulations could not be demonstrated.

(iii) RNA metabolism was studied in the hypothalamus of sated or starved cats following injection of adenine- H^3 and uracil- H^3 into the third ventricle (7). Good uptake was demonstrated by single neurons in the vicinity of the ventricle but because of the sluggish diffusion of the radiochemicals from the site of injection it was concluded that this procedure was not suitable for the study of changing regional metabolism produced in different regions by differing physiological states.

(iv) Changes in the bilateral distribution of systemically injected leucine- H^3 were studied in pigeons and rats following unilateral enucleation (3, 9, 14). The drastic increases on the degenerated side were attributed mainly to the rapid proliferation of glia cells.

(v) Cell proliferation was studied in the visual pathway of adult rats following stereotaxic lesions in the lateral geniculate body and subsequent injection of thymidine- H^3 into the lesion site (2). The study showed a rapid and high rate of production of glia cells in the entire visual system, associated with degenerative and regenerative processes. But the study also indicated the formation of new glia cells in presumably unaffected brain regions. It also raised the possibility of the formation of new nerve cells in some brain regions in adult rats (1).

(vi) In pilot studies (11) tritium-labeled testosterone and estradiol were injected into male and female rats respectively and the regional binding of these sex hormones in the brain were investigated. (This study is now pursued at the Rockefeller University by Dr. Donald Pfaff and his collaborators.)

B. SECOND PHASE: STUDIES OF POSTNATAL NEUROGENESIS

The autoradiographic evidence for continued turnover of glia cells (4, 15) and the subsequent demonstration of the formation of new nerve cells (granule cells) in the hippocampus of young adult and mature rats (11) led us to a systematic examination of the extent and nature of postnatal neurogenesis. We set out to examine (12, 17, 18, 24, 37, 38) the regional patterns of cell proliferation in the brain during different stages of postnatal development and the possible effects of different treatments during maturation on the rate of cell multiplication (14, 32).

(i) In the first study of this series, rats were injected with thymidine- H^3 at different ages from birth onward and were killed at different intervals (from 4 days to 8 months) after injection. It was established that the majority of the granule cells of the dentate gyrus of the hippocampus are formed after birth (11). Most of the granule cells come into existence during infancy but a few are continuously added even during adulthood. Cell proliferation was traced to the subependymal layer of the lateral ventricle and it was assumed that the undifferentiated cells migrate to the hippocampus by way of the fimbria.

(ii) This hypothesis was confirmed and extended in a subsequent large-scale study (17, 18, 37, 38) in which rats of different ages were injected with thymidine- H^3 and were killed at frequent intervals (1 hour to 180 days) after injection in order to establish the sites of cell proliferation, the routes of cell migration and the mode of differentiation of granule cells in the hippocampus.

(iii) This study also established that granule cells in the olfactory bulb, granule cells in the cochlear nucleus, and basket, stellate and granule cells in the cerebellar cortex are largely or exclusively formed after birth in the rat. It was proposed that short-axoned nerve cells, or "microneurons" are of postnatal origin in phylogenetically old cortices of the nervous system (24). These microneurons are formed in secondary germinal matrices (38, 40), such as the subependymal layer of the lateral ventricle and the external granular layer of the cerebellum, and migrate by various routes to sites of differentiation. Migration time varies from 3 days (cerebellar cortex) to about 1 week (olfactory bulb).

(iv) A comparative study showed that postnatal neurogenesis is more pronounced in altricial species (rats and cats) than in a precocial one, the guinea pig (27). In the latter, postnatal neurogenesis is nearly completed at birth in the cerebellar cortex; however, it is pronounced and prolonged in the hippocampal dentate gyrus.

(v) Evidence was obtained of the continued migration of the precursors of glia cells in adolescent rats from the subependymal layer of the lateral ventricle into the neocortex by way of the corpus callosum and white matter (15). Pilot studies indicated that the rate of glial increase is differentially affected in young adult rats by rearing in restricted or enriched environments (10).

(vi) In another study (38) the properties and fate of the cells of the subependymal layer of the anterior lateral ventricle and its extension into the olfactory bulb were examined in young-adult rats. It was established that the cells that multiply at a high rate in this region of the subependymal layer migrate by way of the "rostral migratory stream" into the olfactory bulb and subsequently differentiate into granule cells. It was postulated that the function of cell migration to the olfactory bulb in young-adult rats is the renewal of its cell population.

C. THIRD PHASE: EFFECTS OF FOCAL X-IRRADIATION OF THE BRAIN AFTER BIRTH: THE CEREBELLUM

(a) Pilot Studies

Since it is well established that multiplying cells

are highly radiosensitive, it should follow that the precursors of microneurons and glia cells will be destroyed if the infant animal is exposed to relatively low doses of ionizing radiation (29). From a practical point of view, the demonstration that these cells are of postnatal origin implies that the nervous system is more sensitive after birth to ionizing radiation (and other noxious agents) than was hitherto assumed and warranted a thorough examination. From a theoretical point of view, this property of the postnatally-formed microneurons appeared as a useful experimental tool for their selective elimination and offered the possibility that we could study their role in the structural and functional organization of the brain and in the coordination of behavior. For several reasons the cerebellar cortex was chosen as the first target and the effect of selective irradiation of the cerebellum with low-level X-ray has been examined in great detail.

(i) In agreement with this expectation (and some earlier reports) we found in a pilot study (26) that in kittens whose skull was unilaterally exposed during the first two weeks of life to repeated doses of 50-400r hard X-ray, several doses of 150r subtotally and of 200r totally destroyed the germinal matrix, the external granular layer. The population of granule cells was greatly reduced by this procedure, whereas the number and appearance of the prenatally-formed Purkinje cells were not affected.

(ii) In a combined histological and autoradiographic study (31) the heads of 3-day old rats were irradiated with a single dose of 200r X-ray. It was established that the proliferative premigratory cells of the external granular layer of the cerebellum are killed in large numbers; the stationary proliferative cells were more radioresistant; the stationary nonproliferative cells were not affected.

(iii) In a related study (41) we examined the time course of cell death in 3-day old rats irradiated with a single dose of 200r, with survival after irradiation ranging from 10 minutes to 5 days. Cell death became first evident 4 hours after irradiation, and a maximal concentration of pyknotic cells was seen 12 hours after irradiation. There was a decline in the number of necrotic cells 24 hours after irradiation and by the 48th hour the killed cells have disappeared. Regeneration was indicated 2 days after irradiation and by the 4th day the depth of the external granular layer was normal or supernormal.

(b) Light Microscopic Studies of the Effects of
Cerebellar X-irradiation

(i) The regeneration of the external granular layer was studied (42) in rats in which the cerebellum was focally irradiated with 200r from the day of birth, with the number of successive daily exposures ranging from 1-10. The cell population of the external granular layer was drastically reduced 24 hours after irradiation with a single dose; it was subtotally destroyed when exposed to two successive doses. In animals irradiated with daily doses of 200r up to 5 times, regeneration of the external granular layer was evident 4 days after the last exposure and the extent of recovery was inversely related to the number of doses delivered. This study established that during this phase of cerebellar development, the proliferative matrix that gives rise to its microneurons can be reconstituted.

(ii) In an other study (30) the animals whose cerebellum was irradiated from birth onward with 1-10X 200r were allowed to survive to 10, 30 and 90 days of age. Because the external granular layer disappears under all circumstances by 21 days of age, it should be possible to delay or prevent recovery by increasing the number of exposures and this should lead to a graduated reduction in the final number of granule cells. Indeed, earlier measurements indicated proportional reduction in the weight of cerebellar blocks as a function of increased number of exposures. Linear measurements indicated that the length of the cerebellum was not affected by exposure to 1-2X 200r, but with exposure to 4-5X 200r, cerebellar length was reduced to the level of control animals 10 days of age, with 10X 200r to the level of neonates.

(iii) In a later quantitative histological study (54) we found that there was no reduction in the total area of the cerebellar cortex or its different layers at 30 or 90 days in rats exposed to 1-2X 200r. With higher number of successive daily doses there was a proportional reduction in the areas of the molecular and internal granular layers and the number of granule cells. 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 200r, lasting morphological and cytological changes were produced. These 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.

(c) Electron Microscopic Studies of the Effects
of Cerebellar X-irradiation

Because of a lack of systematic information about the ultrastructure of the developing cerebellar cortex in normal animals our first task was to establish the course of cytogenesis, morphogenesis and synaptogenesis. In the cerebellar cortex of rats aged 0, 3, 5, 7, 10, 12, 15, 21 and 30 days the different morphological events leading to the organization of the adult pattern were studied with histological, histochemical, autoradiographic and electron microscopic techniques. The results were published in a series of three papers (61, 62, 63).

(i) The first paper was concerned with transformations in the external germinal layer and in the transitional molecular layer (61). Among the highlights may be mentioned the demonstration that the parallel fibers are progressively formed on the surface of others from the bottom upward by a stacking process and that the external germinal layer is continually pushed upward. Likewise there is a stacking of the nonmigratory cells of the molecular layer from the bottom upward as a function of the time of the onset of their differentiation.

(ii) The second paper (62) described the growth of Purkinje cells. Five phases were distinguished. Of interest was also the demonstration of the "march" of synaptogenesis in the molecular layer from the bottom upward which was characterized by three successive events; an initial gradient in the appearance and disappearance of coated vesicles, heralding synaptogenesis; a similar subsequent trend in the formation of synapses; and finally, the interposition in the same sequence of glial processes between Purkinje cell dendrites and parallel fibers, marking the cessation of synaptogenesis.

(iii) The third paper (63) was concerned with the maturation of the granular layer. It was established that the bulk of the granule cells are formed during the second week, with a peak at 10-11 days. But due to the time required for their migration and the lag in the formation of dendrites, few glomerular synapses with mossy fibers are formed before the beginning of the third week and the process is still in progress at 30 days, long after the dissolution of the external germinal layer. Concluding these normative studies it was suggested in a recent review (82) that there are five successive phases in cerebellar neurogenesis which we termed cytogenesis, morphogenesis, synaptogenesis, gliogenesis and myelogenesis. We have as yet to study in any detail the latter two events.

These normative studies were followed by an electron microscopic examination of the effects of different schedules of X-irradiation on the developing cerebellum. The results were summarized in a series of four papers (65, 74, 75, 76).

(iv) In the first study of this series (65) the heads of Long-Evans rats were irradiated from birth with a schedule of repeated doses of low-level X-ray which prevented the acquisition of the postnatally-forming basket, stellate and granule cells. The absence of these microneurons led to several changes in the organization of the cerebellar cortex, studied at 30 days of age with light and electron microscopy. The somata of Purkinje cells were not strung out in a monolayer. The primary dendrites were randomly oriented and arborized differently depending on the position of their somata in the depth of the cortex. Many Purkinje cell perisomatic processes, normally seen only in infants, were retained. Climbing and mossy fibers formed inconspicuous, symmetrical synapses with the somata and conspicuous, asymmetrical synapses with the perisomatic processes and dendritic thorns of Purkinje cells. Massive Purkinje cell dendrites with innumerable thorns made up much of the neuropil of the cortex. The dendritic thorns, in addition to forming true synapses, also established pseudosynapses with glial processes and synapses devoid of vesicles with the surface or the interior of Purkinje cell dendrites. The latter type of contact apparently led to autolysis of part of the dendrite, which was the only clear pathological phenomenon observed. These results indicated considerable autonomy in the growth of Purkinje cell dendrites which arborized richly and sprouted innumerable small processes in the absence of parallel fibers, and formed postsynaptic dense membranes where contiguous with inappropriate processes, such as those of Purkinje cells or astrocytes.

(v) In the second study of this series (76) the heads of Long-Evans rats were irradiated from the 4th day after birth with a schedule of repeated doses of low-level X-ray which essentially prevented the formation of basket, stellate and granule cells in all except the earliest-forming lobules (nodulus and uvula). The morphogenic and synaptogenic effects of this treatment were examined with light and electron microscopy in 30 day old animals, with particular attention paid to the pyramis. Although at the time of the commencement of irradiation the Purkinje cells formed a monolayer and had upward oriented apical poles, they became scattered later and had randomly oriented dendrites. This secondary disorientation was attributed to insufficient space available in the arrested cerebellum for the rapidly expanding Purkinje cells. Due to the scarcity of basket cell terminals, basket cell-like terminals were formed on the soma of Purkinje cells apparently by recurrent axon collaterals of these cells.

The most common synapses with the thorns of Purkinje dendrites were formed by climbing fibers but also other elements, including glial processes, were in contact with postsynaptic loci on the thorns. Many mossy fiber terminals reached the surface. Where parallel fibers were present they were often thicker than in unirradiated animals and contained neurofilaments. There were no pathological changes seen in these cerebella, with the possible exception of excessive lobulation of the nuclei of many Purkinje cells.

(vi) In the third study (74) a different effect of early X-irradiation was investigated. The heads of Long-Evans hooded rats were irradiated daily from day 3, 4 or 6 onward with schedules of low-level X-ray which differentially delayed the regeneration of the subtotally eradicated external germinal layer or prevented its regeneration altogether. Most schedules that made regeneration possible led to the arrest of many granule cells in the molecular layer where they formed an ectopic zone. The dendrites of ectopic granule cells had synapses with mossy fibers located in their vicinity in the molecular layer. The position of the ectopic zone varied in different lobules as a function of the time of the commencement of regeneration and the estimated date of the descent of granule cells, and as a function of regional differences in cortical maturation and in the estimated upward growth of mossy fibers. Within the same lobule, the longer regeneration was delayed the higher in the molecular layer the differentiating and descending granule cells were "captured" by the apparently autonomously ascending mossy fibers. If regeneration started too early and allowed the descent of granule cells into the presumptive granular layer (early irradiation) or if regeneration of granular cells was prevented altogether (late irradiation) an ectopic zone was not formed. The technique was used for estimating the normal growth pattern of mossy fibers in the different lobules.

(vii) In the fourth study (75) attention was paid to the observation that in the regenerated molecular layer the parallel fibers were often reoriented. In some cerebella this was restricted to a single lobule, frequently several lobules were affected, rarely almost the entire vermis. It was postulated that the reorientation of parallel fibers was determined by the invasion route of multiplying cells from spared foci of the external germinal layer. Where parallel fibers were rotated transversely, the axons of basket cells and the arborizing dendrites of Purkinje cells became oriented longitudinally. This, and other considerations indicated that parallel fibers exert a guiding influence on the pattern of growth of the spiny branchlets of Purkinje cells.

These studies have indicated profound alterations in the

morphological organization of the cerebellar cortex following irradiation. The changes were drastic when the schedule of irradiation was such that the regeneration of the external germinal layer was prevented. But many changes affecting granule cells, parallel fibers and Purkinje cells were noted when the external germinal layer regenerated after irradiation. In the animals in which regeneration did not take place, the behavioral deficits (tremor, ataxia, etc.) were so severe that they were not suitable for behavioral testing. The motor tests were generally applied to groups of animals in which retardation of cerebellar development was less severe.

(d) Behavioral Studies of the Effects of X-irradiation

(i) In an initial series of studies (49, 50) we examined the early motor development of rats whose cerebellum was exposed to 1-10X 200r during infancy and we assessed qualitatively and quantitatively their motor deficits during adulthood and senescence. During the preweaning and postweaning periods (49) the presence or intensity of tremor and ataxia correlated well with the retardation of cerebellar growth produced by the graduated irradiation schedules. Tremor and ataxia declined in the low irradiation groups, but persisted for 30 days after irradiation in the animals exposed to 8-10X 200r. Spontaneous locomotion in activity wheels in 50 day old animals (50) declined sharply as a function of number of daily irradiations, and the interaction of treatment, age and prior experience was also investigated. Weight pulling capacity, as determined by running time in an automated alleyway for food reward was not affected by irradiation with 1-4X 200r with loads equalling the rats' own body weights. However, in these latter animals the ease of climbing up a rope was inversely related to the number of daily irradiations in terms of climbing time, number of shocks received, falls off the rope and other criteria.

(ii) In a follow-up study (73) of 2-2 1/2 year old rats it was noted that many of the behavioral abnormalities which were evident during the early period after irradiations, but which then declined in severity or disappeared, had reappeared again in old age.

(iii) In another exploratory study (55) motor development was studied with quantitative techniques in rats whose cerebellum was irradiated with 2, 4, 6, 8 and 10X 150r X-ray. Spontaneous motor

activity was assessed in an open field situation. The results indicated persistence of infantile motor patterns (e.g., pivoting) in the animals that received higher number of doses, and a delay in the display of more advanced patterns (e.g., standing on hindlegs without support). The more affected animals tended to drag their hindfeet and lost their balance during locomotion. In test situations where the animals had to climb up on a rope to an escape platform, the intermediate groups were handicapped; the high irradiation animals persistently failed. There was a proportional deficit produced as a function of number of irradiations in a test requiring the animals to climb down a rope. Analysis of the motor deficits indicated that the coordination of the hindlimbs was much more severely affected by retardation of cerebellar development than the coordination of the forelimbs.

(iv) In a new set of studies, which are still in progress, we have been examining locomotor behavior in adult rats that had received either two or four exposures to low-level X-ray of the cerebellum during infancy which led to moderate and massive retardation of cerebellar development. The broad purpose of this series of studies is to analyze, in a systematic fashion, the relationship between cerebellar interneuron loss and lasting motor performance deficits. The first paper of this series was recently published (81). In the two experimental groups used, the animals that received 2 daily focal cerebellar exposures on days 4 and 5, the population of granule cells was reduced by 49%, while in the animals that received 2 additional doses on days 7 and 9, the reduction of granule cells was 72%.

Irradiated groups equaled controls in swimming speed and on endurance in running on a treadmill. Swimming, like running, requires the coordinated movement of forelimbs, hindlimbs, head and tail, for effective forward propulsion and turning. It differs from locomotion on land in that the antigravity reactions are essentially suspended because rats tend to float in water. Moreover, because of invariant hydrodynamic conditions on the surface of calm water, the need for the utilization of exteroceptive and proprioceptive feedback cues for adjusting the endogenous rhythmicity of swimming movements to changing external conditions may be minimal. From this the inference follows that a pattern of rhythmic progression which requires minimal concurrent postural adjustments and exteroceptive and proprioceptive feedback may be executed successfully by an animal with an underdeveloped cerebellum.

(v) The task of running on a wide and level surface with good traction involves an additional performance variable, namely, the activation of antigravity responses to sustain the quadruped stance during ambulation. This did not handicap the 2Xr group either in terms of running speed or endurance. Even when another performance variable was added when running on inclines the

performance of 2Xr group was not affected. These experiments suggest that the postural and feedback requirements in a simple running situation place minimal demands on cerebellar contribution and, therefore, moderate cerebellar retardation has no effect on running performance and, severe retardation has only a slight effect.

(vi) Accordingly, in the next series of tests the demands for sensory and postural control were progressively increased and the deficits of the 4Xr animals and then also of the 2Xr group became apparent. Rats were required to cross rods of different width, texture and speed of rotation to reach a goal box for food reward. On the rough-surfaced, stationary rods there were essentially no falls in any of the experimental groups at any of the widths tested and only in terms of crossing speed did the handicaps of the 4Xr group and then of the 2Xr group become evident. The combination of reduced width and slippery surface led to occasional falls and disqualifications in the 2Xr animals, to more in the 4Xr group. On the narrowest slippery rod the 2Xr group was as badly affected as the 4Xr animals in terms of speed of crossing. Rotation of the rough and wide rod at a low speed did not lead to falls or disqualifications in the majority of the 4Xr, and a high proportion of the 2Xr rats. Because of the poor performance of both experimental groups on the narrowest slippery rod while stationary, rotation was not attempted. In summary, the locomotor tests used in this study suggest that full cerebellar involvement is not essential for successful progression if there is no need for precise concurrent proprioceptive and exteroceptive control. As the need for these sensory controls increases, the performance deficit of animals with severe and then moderate cerebellar retardation becomes manifest.

(e) Physiological Studies of the Effects of
Cerebellar X-irradiation

This work was carried out in collaboration with Dr. Donald Woodward of the University of Rochester and Dr. Barry Hoffer of St. Elizabeth's Hospital, Washington, D.C.

(i) It was determined (92) that in rats in which the acquisition of the bulk of granule, stellate and basket cells was prevented by X-irradiation, the Purkinje cells exhibited sustained spiking activity. Mean firing rates of 35-40/sec. were comparable to that obtained in normal cerebella.

(ii) In the irradiated cerebella spontaneous bursts, presumed to be generated by climbing fiber synaptic activity differed from normal by consisting of full sized spikes rather than characteristic inactivation responses. Intracellularly observed correlates of bursts consisted of EPSP's of several discretely different amplitudes appearing independently in time .

(iii) Stimulation of white matter revealed evidence for, (a) graded synaptic excitation of Purkinje cells indicating more than one converging excitatory synapse, and (b) inhibitory actions on Purkinje cells either through a few remaining inhibitory interneurons or through Purkinje cell recurrent collaterals.

These studies indicate considerable autonomy of Purkinje cell ontogenesis in the absence of normal interneuronal input. A unique synaptic relation not found in normal cerebellum is an innervation of single Purkinje cells by more than one climbing fiber.

D. FOURTH PHASE: EFFECTS OF FOCAL X-IRRADIATION OF THE BRAIN AFTER BIRTH: THE HIPPOCAMPUS

It was established with thymidine- H^3 autoradiography that a large proportion of the granule cells of the hippocampal dentate gyrus are formed after birth (11, 17, 18). The proliferating precursors of granule cells were located in the hilus of the dentate gyrus and at the base of the granular layer. The cells located in the latter region have a reduced proliferative capacity, and within about two to three weeks after arrival at this site they become differentiated into granule cells. The proliferation of neuroblasts in the dentate gyrus and their differentiation into granule cells continue late into adulthood in the rat. The significance of the persisting neurogenesis of the hippocampus has not been determined. That it may be involved in "behavioral maturation" was put forward in a recently published review paper on the subject (77). Since most granule cells of the dentate gyrus of the hippocampus originate postnatally, irradiation of the hippocampus during infancy in the rat with a schedule derived from previous autoradiographic and irradiation studies of the cerebellum should reduce the population of granule cells without directly harming the prenatally-formed pyramidal cells of Ammon's horn.

(a) Pilot Studies

(i) In a pilot study (80) the location of the hippocampus with respect to external landmarks was determined in rats aged 2-18 days, and 200r was aimed at this region on days 2 and 3, followed by 150r on days 5, 7, 9, 13 and 15. The irradiated and control animals were killed at 30, 60 and 90 days. Quantitative histological analysis indicated that the number of granule cells in the irradiated group was 17% of control levels at 30 days, 15% at 60 days and 18% at 90 days. The missing cells may represent the entire population of postnatally formed granule cells. In accordance with results using a similar schedule of irradiation of the cerebellar cortex, which led to no reduction in the prenatally-formed Purkinje cells, there was no significant difference in the numbers of prenatally-formed pyramidal cells between the groups at any age. Preliminary examination of Golgi-impregnated sections has shown no distinct morphological differences in the surviving granule and pyramidal cells. The overlying cortex and underlying thalamus and hypothalamus also appeared normal in the irradiated areas.

(ii) In the behavioral aspect of this study (80) four tests were selected which have repeatedly been found to be sensitive to surgical destruction of the hippocampus. It is well established that rats with hippocampal lesions display a high level of locomotor activity during the exploration of an open field or maze. In agreement with this, the irradiated group had a higher activity level than the control groups. An analysis of variance showed that the enhanced activity was reflected both in squares traversed ($p < 0.001$) and in rearing responses ($p < 0.001$).

(iii) The second test was spontaneous alternation, which refers to a rat's tendency to enter opposite goal arms during two successive non-rewarded explorations of a T maze with a probability of between 0.7 and 0.9. The observation that rats with hippocampal lesions repeat or alternate previous goal arm entries on a chance basis have been consistently reported. While control rats displayed a significant alternation tendency ($\chi^2=9.2$, $p < 0.01$) the irradiated rats alternated at a chance level ($\chi^2=0.09$).

(iv) In the third experiment 70 day old rats of each group were tested for the acquisition and retention of a passive avoidance response. Passive avoidance, the withholding of responses that lead to punishment, is severely disturbed by lesions involving the hippocampus. Rats reduced to 85% ad lib body weight were

trained to run for wet mash in a straight alley for five trials per day on three consecutive days. Latencies to eating were recorded and rats were allowed to feed for 10 seconds per trial. On the fourth day, rats were given a single non-shock trial followed by trials on which each contact with the food cup closed a shock circuit between the metal food cup and the grid floor. Groups did not differ prior to shock. Irradiated rats required an average of 1.7 trials to reach the 120 second avoidance criterion during training. The control group had a mean of 1.1 trials before reaching criterion. These scores were highly reliable and the differences between irradiated and control animals were significant ($p < 0.005$, t-test).

(v) It has been reported that hippocampal ablated rats are superior to controls in acquiring a two-way (shuttle) conditioned avoidance response. In the final experiment we tested irradiated and control rats in a fully automated two-way avoidance apparatus. A motor driven door and house light served as the conditioned stimulus (CS), five seconds following the CS, a 2.5ma scrambled footshock was delivered through the floor and walls until the rat escaped to the other compartment. Five rats of each group were given a single block of 100 trials with an inter-trial interval of 30 seconds. The irradiated rats made many more avoidances and because of no overlap between the control and irradiated rats no statistical test was performed.

(vi) In a subsequent study by members of this laboratory (90) the behavioral similarities noted between radiation-induced granule cell agenesis and conventional hippocampal lesions were demonstrated in four additional experiments. Irradiated and control rats were alike in the acquisition of a one-way avoidance response with a trend of greater resistance to extinction in the irradiated group. The irradiated animals displayed facilitated acquisition of an escape-from-fear response. When one-way avoidance was preceded by inescapable shock, the irradiated group was superior, suggesting that granule-cell loss, like hippocampal ablation, disrupts a tendency to remain immobile in the presence of stimuli related to inescapable punishment.

These data show that the selective prevention of the acquisition of dentate granule cells by irradiating the hippocampus in infant rats results in behavioral changes that closely resemble those observed following surgical destruction of the hippocampus in adults. These results implicate an important role for the granule cells in hippocampal function and point to the lasting deficit produced by early retardation of hippocampal development produced by X-irradiation.

E. AUXILIARY STUDIES: EFFECTS OF HARMFUL
AGENTS OTHER THAN X-IRRADIATION

In addition to its radiation-sensitivity, the postnatally-developing cerebellar cortex is harmed by a variety of environmental variables and we have the opportunity to compare the effects of such treatments as hypo- and hyperthyroidism, and undernutrition with the effects of focal irradiation of the cerebellum (this research is supported by a grant from the National Institute of Mental Health).

(a) Effects of Hormonal Treatments

(i) Both hypo- and hyperthyroidism cause a reduction in the number of synapses in the molecular layer of the cerebellar cortex (69) but for different reasons. One of the effects of thyroid treatment is on cell proliferation (70) the other on cell differentiation (71). Hyperthyroidism led to decreased body, brain, and cerebellar weight; early termination of cell proliferation in the external germinal layer accompanied by early disappearance of this layer; early cell differentiation in the molecular and internal granular layers; and terminal decrease in granule cells, basket cells, and astrocytes. Hypothyroidism caused decreased body, brain, and cerebellar weights; prolonged cell proliferation in the external granular layer and retarded disappearance of this layer; retarded cell differentiation in the molecular and internal granular layers; and terminal increase in granule cells and astrocytes, and decrease in basket cells.

(ii) With regards to cell differentiation hypothyroidism caused a transient developmental retardation in the width and area of the molecular layer, with normal levels being approached by 30 days; a slight, but not significant, increase in packing density of granule cells; probably reflecting a reduction in glomeruli or synaptogenesis in the internal granular layer, as well as crowding produced by increased numbers of granule cells; retardation of synaptogenesis in the molecular layer, especially in the outer half, as late as 55 days. This retardation was manifested by a decrease in the synaptic content of the Purkinje cell dendritic domain, and decreased size of the parallel fiber synaptic domain, resulting (along with the increase in granule cells) in a greater packing density of parallel fibers in the molecular layer. In contrast, hyperthyroidism caused an accelerated increase in width

of the molecular layer from day 10 on, such that by 30 days the molecular layer was wider than in controls. This was accompanied by only a small, transient increase in the area of the molecular layer and a sharp leveling off by 15 days such that the area was greatly decreased by 30 days compared to controls. This was interpreted to mean that the length of the molecular layer was decreased in the sagittal plane, resulting in taller and narrower Purkinje cell dendritic trees. Hyperthyroidism also caused a significant decrease in packing density of granule cells, probably reflecting both the decrease in the number of granule cells and an accelerated development of glomeruli; and an acceleration of synaptogenesis in the inner and outer halves of the molecular layer and an increase in the number of synaptic profiles/cell at 30 days. This probably is the result of accelerated growth of the Purkinje cell dendritic tree and the deficit in granule cells and basket cells described previously. Total synaptic profiles were decreased, however, due to the decreased sagittal length and area of the molecular layer. Thus it was shown that early hypo- and hyperthyroidism both cause a terminal deficit in total synaptic profiles in the rat cerebellar cortex, but for different reasons.

(b) Effects of Undernutrition

(i) The effects of undernutrition during gestation and lactation (separately or in combination) were examined in a series of past studies (56, 58, 66) in which the treatments involved either the allocation of an excessive number of rat pups to a dam or a reduction of her diet by specified amounts. The effects of such treatments on the development of the cerebellar cortex (66) and on the development of motor performance (58) were examined.

(ii) In a recent series of studies (78, 79) dams were fed during gestation and/or lactation 75% or 50% of their normal food intake. Mild (75%) or severe (50%) undernutrition during gestation alone had only transient effects on the preweaning weight of the brain or number of cells in the cerebellum. Mild or severe undernutrition during lactation had appreciable effects on body and brain growth. Severe lactational undernutrition resulted in a reduction in the number of cells in both the external germinal layer and in the molecular and internal granular layers. In the severely undernourished animals the reduction in the number of stellate, basket and granule cells was permanent.

The deficits produced by hypo- and hyperthyroidism, and undernutrition are much milder than those produced by X-irradiation,

but this study has provided us with a methodology which we hope to employ in the examination of the effects of very low doses of single X-ray exposure.

2. SIGNIFICANCE OF RESEARCH

Vulnerability of the Nervous System. It was believed for a long time that the nervous system is a relatively radioresistant part of the body. The demonstration by others and ourselves that cell proliferation remains brisk in many brain regions after birth, dispelled this belief and made it necessary that we re-examine the vulnerability of different brain regions at different periods of pre- and postnatal development. In this context it should be remembered that the proliferative external germinal layer of the cerebellar cortex is present in man up to 20 months of age and that the proliferative subependymal layer of the inferior horn of the lateral ventricle persists into adulthood. The demonstrations that these proliferative sites in animals give rise to nerve cells, and that they can be subtotally destroyed by exposure to a single dose of less than 200r, indicate that certain brain regions remain radiosensitive for some time after birth. Our behavioral tests have shown that postnatal X-irradiation of the cerebellum or hippocampus produces deficits that closely resemble the destruction of these brain structures (even though only the granule cells are directly affected).

Rehabilitation and Regeneration. It has been known for a long time that the deficits produced by trauma to the nervous system tend to subside and may altogether disappear with time. To what extent this is due to dissipation of the initial "shock", to "functional recovery", "neural reorganization" and possibly some degree of morphological "re-wiring" is a matter of speculation. In our studies, and those of others (Ebels, Phemister et al., Langman) evidence was obtained that the external granular layer of the cerebellar cortex is reconstituted after subtotal destruction, and there is suggestive evidence that its rate of cell production may be actually accelerated. This indicates some degree of functional regulation of cell proliferation but the mechanisms of this regulation remain to be determined. Is the regeneration of the external granular layer facilitated by some form of "physical rehabilitation"? Can it be usefully enhanced by pharmacological or other agents?

Cellular Dissection of Different Brain Regions. In addition to the described practical considerations, our newly developed technique of timed irradiations will make it possible to selectively eliminate some of the neuronal components of selected brain regions. For instance, in the cerebellum, if irradiation is started at birth and continued to prevent regeneration, a

cerebellar cortex is produced with all its Purkinje cells present (with input from and output to other brain structures maintained) but without a laminar organization and with all the microneurons absent. If the irradiation is started at 4-5 days, near-normal lamination is produced with subtotal elimination of all the microneurons. If the irradiation is started at 3-4 days and is continued to eliminate the cells that form the basket and early granule cells, but discontinued early to permit regeneration, it will acquire the late forming stellate cells and a large population of the late granule cells. Conversely, if irradiation is not started until the basket and early granule cells were formed and then it is continued to prevent regeneration, a cortex is formed which has the early-forming microneurons without the late-forming ones. Such an experimental tool for the selective elimination of different cell types has not been hitherto available and it promises to become a powerful tool in neurobiological research.

The Cerebellum as a Model Brain Structure. There are few brain regions that are as well understood, and which are as extensively and intensively being investigated at present as the cerebellum. It is a complex neural structure but experimental work on it is fostered by the circumstance that the cerebellum has a limited number of cell types; a limited number of input lines and only a single output line; a relatively stereotyped, geometrically organized circuitry; and that it controls motor activities which are easier to assess than some other behavioral functions such as perception or learning. It is not unlikely that the cerebellum will be among the first brain structures that neuroscientists will begin to be able to understand in some depth -- an optimism which is supported by the great advances that are now being made in the unraveling of its circuitry with morphological and electrophysiological techniques and its chemistry by the application of microchemical, histochemical and pharmacological techniques. Our effort is directed at obtaining information of how the cerebellar cortex is being assembled during development and what happens if this is disturbed by such agents as X-irradiation. The cerebellum is particularly suited for this task due to the favorable circumstance that it develops mostly postnatally when it has become more accessible for experimental manipulation.

The Hippocampus as a Model Brain Structure. The hippocampus, like the cerebellum, is a laminated structure, components of which develop largely postnatally. There is considerable amount of information about its structural and physiological properties and a wealth of behavioral data. However, unlike the cerebellum, it is involved in an as yet unspeciable series of "mental" functions.

It thus represents another model system that may allow the examination of the development and vulnerability of a brain structure implicated in "higher" processes.

3. STUDENTS TRAINED, POSTDOCTORAL AFFILIATESPostdoctoralPresent Position

G. Kreutzberg, M.D.	Chief, Neuropathology, Max Planck Institut, Munich
G. D. Das, Ph.D.	Associate Professor, Purdue University
R. B. Wallace, Ph.D.	Associate Professor, Hartford University
D. W. Pfaff, Ph.D.	Associate Professor, Rockefeller University
E. J. Ebels, M.D., Ph.D.	Professor, University of Groningen, Holland
H. Nornes, Ph.D.	Assistant Professor, Colorado State University
J. L. Nicholson, Ph.D.	Assistant Professor, University of Connecticut
P. J. Peters, Ph.D.	Associate Toxicologist, Midwest Research Institute
R. L. Brunner, Ph.D.	Assistant Professor, Purdue University

Doctorates GrantedMasters Granted

G. D. Das, 1966	A. Taylor, 1968
D. W. Pfaff, 1967	R. Gaumond, 1970
H. Nornes, 1971	F. Horgos, 1971
J. L. Nicholson, 1972	

Graduate Students now in Training

D. Barnes	Ph.D. Candidate
S. A. Bayer	Ph.D. Candidate
F. Bulut	Ph.D. Candidate
Z. Y. Chen	M.Sc. Candidate
R. A. Gazzara	M.Sc. Candidate
J. Gruner	M.Sc. Candidate
C. Moffett	Ph.D. Candidate
J. Moore	M.Sc. Candidate
E. Sheehy	M.Sc. Candidate
N. A. Spivack	Ph.D. Candidate
P. Strang	M.Sc. Candidate

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