MASTER

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COMPREHENSIVE REPORT

June 15, 1962 - August 31, 1971

Title: LOW-LEVEL X-IRRADIATION OF THE BRAIN DURING DEVELOPMENT:
MORPHOLOGICAL, PHYSIOLOGICAL AND BEHAVIORAL CONSEQUENCES.
Former title: AUTORADIOGRAPHIC INVESTIGATION OF BRAIN
METABOLISM UNDER NORMAL, EXPERIMENTAL AND PATHOLOGICAL
CONDITIONS.

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1. RESEARCH ACCOMPLISHMENTS

First Phase: General Autoradiographic Studies

The original objective of this research program was to investigate with autoradiography, 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:

- (a) 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).
- (b) 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 (21); handling of rats during infancy (14); raising weaned or young adult rats in restricted or enriched environments (20). 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.
- (c) 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.
- (d) 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.
- (e) 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).
- (f) 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.)

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 (12,17,18,24,38,39) 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 (14,29).

(g) 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.

(h) This hypothesis was confirmed and extended in a subsequent large-scale study (17,18,38,39) 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.

(i) 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 (42,44), 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).

(j) 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.

(k) 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).

(l) In another study (39) 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.

Third Phase: Vulnerability of the Postnatally Formed Neurons. Focal Irradiation with Low-level X-ray

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 (31). 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 warrants 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.

(m) 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 50-400 r hard X-ray, several doses of 150 r subtotally and of 200 r 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.

(n) In a combined histological and autoradiographic study (33) the heads of 3-day old rats were irradiated with a single dose of 200 r 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.

(o) In a related study (37) we examined the time course of cell death in 3-day old rats irradiated with a single dose of 200 r, 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.

(p) In a subsequent study (41) the regeneration of the external granular layer was studied in rats in which the cerebellum was focally irradiated with 200 r 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 sub-totally destroyed when exposed to two successive doses. In animals irradiated with daily doses of 200 r 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.

(q) In a related study (32) the animals whose cerebellum was irradiated from birth onward with 1-10 X 200 r 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 1-2 X 200 r, but with exposure to 4-5 X 200 r, cerebellar length was reduced to the level of control animals 10 days of age, with 10 X 200 r to the level of neonates.

(r) In a recent quantitative histological study (49) 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 1-2 X 200 r. 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 200 r, 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.

(s) In a series of behavioral studies we subsequently examined the early motor development of rats whose cerebellum was exposed to 1-10 X 200 r during infancy and we assessed qualitatively and quantitatively their motor deficits during adulthood and senescence. During the preweaning and postweaning periods (47) 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 8-10 X 200 r. Spontaneous locomotion in activity wheels in 50 day old animals (48) 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 1-4 X 200 r 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.

(t) In a follow-up study (61) 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.

(u) In a recent study (57) motor development was studied with quantitative techniques in rats whose cerebellum was irradiated with 2, 4, 6, 8 and 10 X 150 r 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.

Auxiliary Studies: Vulnerability of Postnatally
Formed Neurons. Effects of Undernutrition,
Hormonal Treatments and other Noxious Agents.

We have also been carrying out research on the effects of other environmental variables on the postnatal development of the mammalian brain. These included graded undernutrition (50,51,52,53,56), experimental hypo- and hyperthyroidism (in progress), the administration of alcohol to lactating mothers (in progress) and several behavioral manipulations. (This research program is supported by a program grant from the National Institute of Mental Health.) Unlike focal X-irradiation of the cerebellum which selectively retards the development of the cerebellar cortex, undernutrition and the other treatments referred to have a widespread effect on the development of the body and the brain, although the cerebellum appears more vulnerable during this period than other brain structures. Accordingly, in our efforts to analyze the morphological and behavioral deficits produced by the various global manipulations, we have made comparisons with the data obtained from the X-ray studies and attempted to parcel out the contributions made by cerebellar retardation from the many other variables. For instance, the different degrees of cerebellar retardation produced by different schedules of undernutrition have been related to comparable magnitudes of retardation produced by different schedules of irradiation. However, the behavioral consequences of the two procedures are different. Both the undernourished and irradiated animals show motor deficits of comparable nature, but the undernourished animals are abnormally hypoactive, the irradiated animals are abnormally hyperactive. Thus, the program dealing with the effects of different environmental variables on cerebellar and motor development has been aided by the results of the studies of X-irradiation. But the converse has also been the case. The large number of brains that we have been able to examine in conjunction with this auxiliary program, with histological, histochemical, Golgi and, more recently, electron microscopic techniques, and the various behavioral tests that have been applied, have greatly aided us in interpreting the data obtained with X-irradiation.

2. RESEARCH IN PROGRESS AND PLANNED

Much of the work that has been in progress in our laboratory during the last year has been carried out in preparation for an intensive and extensive study of changes produced in the morphological, chemical, physiological and functional maturation of the cerebellar cortex as a result of focal, low-level X-irradiation during different periods of postnatal development. This preparation included the following. (a) A detailed ultrastructural study of the development of the cerebellar cortex under normal conditions (55; in progress). (b) Examination of a large series of histochemically processed cerebellar sections for the reconstruction of aspects of the chemical maturation of the normal cerebellum (45; in progress). (c) An autoradiographic study of cell proliferation and migration, with multiple injection of thymidine- H^3 that covered the entire time-span of postnatal cerebellar neurogenesis (60). This will allow us to arrive at a more exact time table of the sequential acquisition of cerebellar microneurons. (d) A pilot study was carried out in which the cerebellum was irradiated at different periods and for different intervals. The purpose was to eliminate selectively the different postnatally acquired cell components of the cerebellum.

(e) We have spent considerable effort on the design, assembling and testing of a series of behavioral testing devices which will make possible a more detailed and accurate analysis of the postural and motor deficits produced by retardation of cerebellar development. (f) Recently we have begun the ultrastructural and histochemical examination of cerebella that were irradiated with different number of successive doses from birth onward (62; in progress). In the near future we hope to examine with the same techniques the cerebella in which basket, stellate and granule cells were selectively eliminated, or granule cell ectopia was produced, by timed irradiations. (g) The electrophysiological and pharmacological examination of these different cerebellar "species" with altered circuitries was recently begun with the collaboration of Dr. Donald Woodward of Rochester University and Dr. Barry Hoffer of the National Institute of Health, Washington, D.C. (h) Finally, the possibility of replacing the eliminated cells of the external granular layer by transplanting cells harvested from unirradiated animals will be examined in collaboration with Dr. Gopal Das. The attempt of transplanting radioactively labeled external granule cell from donor infant rats has been successful.

These plans are described in detail in the attached Renewal Request, 1971-1972.

3. 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 granular 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 200 r, indicate that certain brain regions remain radiosensitive for some time after birth. Our behavioral tests, which are still quite crude, have shown that focal irradiation of the cerebellum may produce lasting motor deficits. It will be especially important that more sensitive tests are designed if we are to establish the transient and lasting consequences of low-level irradiation of different brain regions. The animal that "looks" normal in its home cage may prove to be seriously defective when adequately tested in various complex test situations.

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? The possibility of "re-seeding" the external granular layer by transplantation from normal donors is being examined in our laboratory.

Cellular Dissection of the Cerebellar Cortex. In addition to the described practical considerations, our newly developed technique of timed irradiations will make it possible to selectively eliminate most of the neuronal components of the cerebellar cortex. 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.

4. STUDENTS TRAINED, POSTDOCTORAL AFFILIATES

Postdoctoral

Present 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.	Assistant Professor, Hartford University
D. W. Pfaff, Ph.D.	Assistant Professor, Rockefeller University
E. J. Ebels, M.D., Ph.D.	Associate Professor, University of Groningen

Doctorates Granted

Masters Granted

G. D. Das, 1966	A. Taylor, 1968
D. W. Pfaff, 1967	R. Gaumond, 1970
H. Nornes, (1971)	F. Horgos, (1971)

Graduate Students now in Training

J. L. Nicholson (Ph.D.)	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. Carayanopoulos (Ph.D.)

Honors (Thesis) Students Trained

J. Chang (1967)	M. Cowan (1967)
P. Phillipps (1968)	J. Hokanson (1968)
A. J. Wenzel (1968)	D. King (1969)
N. Sievers (1970)	V. Hineman (1971)

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