EFFECTS OF LATE PRENATAL IRRADIATION ON THE DEVELOPMENT OF THE CEREBELLAR CORTEX IN THE RAT

by

TERESA ANNA MARIA RALCEWICZ

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DEDICATION

I thank God for two important people in my life to whom I dedicate this thesis. Throughout the duration of my doctoral thesis they have believed in me, provided support and encouragement, and have always been there for me.

To my mother Antonina,

for "All I am, or hope to be, I owe to my angel Mother"

A. Lincoln.

and

To my brother Henryk,

who has reaffirmed in me that
"Dreams are the touchstones of our character"

H.D. Thoreau

and

"A journey of a thousand miles
must begin with a single step"

Lao-Tzu

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ABSTRACT

The effects of maternal exposure to a single dose of whole body irradiation (0.5 Gy) on gestational days (GD) 17, 18, 19, or 20 on the development of the cerebellum was examined in the offspring of Sprague Dawley rats at 21 and 28 days postnatally. No gross cerebellar anomalies were observed in the irradiated animals. However, rats irradiated on each of GD-17, 18, 19 and 20 showed a significant incidence ($p < 0.05$) of circumscribed cerebellar lesions (CL) distributed in the inner granular layer of the anterior and posterior lobes. These lesions were characterized by a loss of granule cells and atrophied and/or reduced number of Purkinje cells. In 21 day old rats, irradiation on GD-17 resulted in more CL anteriorly (75%) and in the vermis whereas on GD-20, the CL predominated posteriorly (100%) and in the lateral hemispheres. In 28 day old rats, following irradiation on each of GD-17 and GD-20, there was an equal distribution of CL in both the anterior and posterior lobes. However, with irradiation on both GD-17 and GD-20, CL occurred more frequently in the anterior lobe of the lateral hemispheres, whereas in the posterior lobe they predominated in the vermis.

The laterolateral numbers of both granule and Purkinje cells in the pyramis were significantly reduced ($p < 0.001$) from controls in rats irradiated on each of GD- 17, 18, 19 and 20. There was a greater deficit in granule cell

number with irradiation on GD-20 than on GD-17 ($p < 0.05$). Purkinje cells were reduced in number with irradiation on GD-17 and GD-20; however, the decrease did not correspond to the degree of reduction in the number of granule cells. There was a greater reduction of both granule and Purkinje cells in the vermis with irradiation on GD-17, whereas on GD-20, both granule ($p < 0.05$) and Purkinje cells ($p < 0.001$) were more reduced in the lateral hemispheres. The GC/PC ratio was smaller in rats irradiated on GD-20 than on GD-17. The GC/PC ratio between the irradiated animals and the controls were relatively similar.

The results from this study suggest that a direct relationship exists between the proliferation, migration, development, and maturation of granule cells and their induction by Purkinje cells. The findings also support the view that both cell death and the regulation of granule cells by Purkinje cells maximize the effective development and organization of the cerebellum.

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ABBREVIATIONS

GD	Gestational Day
CL	Cerebellar Lesion
PC	Purkinje Cells
GC	Granule Cells
PN	Postnatal
ED	Embryonic Day
PCL	Purkinje Cell Layer
IGL	Inner Granular Layer
ML	Molecular Layer
MC	Medullary Center

1.0 HISTORICAL PERSPECTIVE AND CURRENT INVESTIGATION

The reporting by Roentgen in 1895 to the Wurzburg Physico-Medical Society on a new form of radiation and the discovery of radioactivity by Becquerel in 1896 resulted in the birth of radiobiology. Radiobiology is a branch of science concerned with the action of light, ultraviolet and ionizing radiation on living organisms (Dorlands 1982). It is also characterized by the 4 R's: 1) repair of sublethal damage, 2) repopulation and regrowth of cells, 3) redistribution of cells within the cell cycle, and 4) reoxygenation (Hall 1978m).

After the discovery of X-rays, many of the investigators in radiotherapy who exposed their hands to irradiation for several hours developed radiation induced cancers and died. The one survivor, Hall-Edwards, was the first to record carcinomas as a direct result from exposure to X-rays (Furth & Lorenz 1954). In earlier years, radiation was used to perform therapeutic abortion. The center of the uterus was irradiated with 360 rad no later than the fourteenth week of gestation (Yamazaki 1966, Mayer et. al. 1936). In 90% of the cases, the conceptus aborted spontaneously.

During the late 1910's and throughout the 1920's and 1930's, several investigators reported a variety of fetal abnormalities in rats and rabbits with exposure to prenatal irradiation (Bagg 1922, Murphy & de Renyi 1930, Hanson 1923). The earliest animal work lacked the precise timing of the

stage of development when irradiation was done. In 1935, Job, Leibold, and Fitzmaurice were the first to determine critical periods in development and were able to correlate the defects with the time of irradiation. The discovery of critical periods, prompted other investigators including Kosaka (1927), Warkany and Schraffenberger (1947), Wilson and his co-workers (1951, 1953, 1954) to study the relation between the timing of irradiation and the resultant abnormalities.

In an extensive series of experiments, Russell (1950) defined the critical periods for the induction of malformations by radiation in mice. She correlated the induction of external and gross visceral changes with the precise timing of irradiation during a particular stage of development. Through an extensive series of experiments, Altman, Hicks and each of their co-workers, have used radiation to study the progressive stages of cerebellar neurogenesis in rats from birth to adolescence. They have used radiation as an experimental tool to study radiation induced morphogenetic changes and re-organization of cerebellar constituents following irradiation during specific stages in development (Altman, Altman et. al. 1965-69, 1971-73, 1976-78; Hicks, Hicks et. al. 1950, 1952-58, 1980). Using radiation as an experimental tool, has allowed them to draw inferences about normal patterns of neurological development of the nervous system in mammals based on their studies of the malformation processes.

Radiation is an established teratogen (Brent 1979-80, Hicks et. al. 1980, Schull et. al. 1990), and both animal and human data have shown that radiation has a direct effect on the brain. It is well documented that in animals, prenatal exposure to ionizing radiation (> 1 Gy) results in intrauterine growth retardation, genetic defects, anomalies of the developing nervous system, deficits in motor coordination, hyperactivity and a hopping gait, increased emotional behavior, deficits in maze learning, death of neuronal or glial precursors, altered migratory pathways, degeneration of post-mitotic neurons, altered cell surface contact and synaptogenesis, as well as disoriented dendritic arborizations to name a few (D'Amato & Hicks 1980, Fowler et. al. 1962, Hicks & D'Amato 1980, Jensh & Brent 1986-88, Jensh et. al. 1987, Kimler & Norton 1988, Mullenix et. al. 1975, Norton 1986, 1989, Norton et. al. 1976, Schneider & Norton 1979, Werboff, 1962).

Concerning human studies, the Japanese atomic bomb survivors in Hiroshima and Nagasaki provide the best information on the risk estimation and the effects of in utero exposure to radiation during various developmental phases on the developing nervous system (Blot & Miller 1973, Hashizume et. al. 1973, Miller 1956, Otake & Schull 1984, Schull et. al. 1988, Tabuchi et. al. 1967, Wood et. al. 1965-67, Yamazaki et. al. 1954). In humans, in utero exposure to ionizing radiation results in growth and mental retardation, and

microcephaly. Apart from the Japanese experience, there is little information concerning human exposure or gestational age during exposure.

The developing nervous system has a long period of sensitivity to radiation which is altered with age as is its capacity for repair. The induction of gross brain anomalies is correlated with the precise timing of irradiation relative to the stages of development, dose and dose-rate (Russell 1954).

The cerebellum is an ideal structure to study for several reasons. Its location allows for easy accessibility. At birth, the cerebellum is largely immature and postnatal development is characterized by intense cell proliferation, migration and differentiation. Because the development of locomotory skills is correlated with postnatal cerebellar neurogenesis, any prenatal insult can be manifested visibly postnatally by deficits in motor function. Any alterations from norm, such as growth, cerebellar lesions or hypoplasia as a result from exposure in utero to irradiation will be characterized by ataxia; loss of motor co-ordination as a result of the paucity of granule cells or interference of connections between Purkinje and granule cells (Altman et. al. 1968).

There is little information concerning the effects of maternal exposure to low levels of ionizing radiation (< 1 Gy), during late gestation on the postnatal development of the cerebellum. The current investigation examines the effects of late in utero exposure to a single dose of 50 cGy gamma

radiation on gestational days (GD) 17, 18, 19, and 20 on the development of the cerebella in offspring of Sprague-Dawley rats at 21 and 28 days of life.

2.0 REVIEW OF THE LITERATURE

2.1 Radiobiology and Ionizing Radiation

2.1.1 Radiation

Radiation is electromagnetic waves. A source is considered ionizing if the energy absorbed by the biological material is sufficient enough to interact with its atoms, causing the ejection of one or more orbital electrons leaving the atom ionized; an unbalanced charge (Hall 1978m). An important characteristic of ionizing radiation is the release of localized "packets" or photons of large amounts of energy (Hall 1978m).

The units used to express the measurement of a quantity of radiation is the roentgen (R) and the rad. Since 1985, the rad unit has been replaced by the gray (Gy), where 1 gray = 100 rad. The roentgen is the unit of exposure, quantity of X-ray or gamma radiation that will ionize air (Dorlands 1982, Hall 1978m). The rad is the unit of an absorbed dose corresponding to 100 ergs/g of absorbing material (Dorlands 1982, Hall 1978m).

2.1.2 Types of Radiation

There are many sources of electromagnetic radiation but not all are ionizing. Sources of non-ionizing radiation include visible light, radiowaves, radar and radiant heat (Hall 1978m). Ionizing radiation falls into two categories: 1) the earth's natural background and 2) man-made, a result

of modern technology (Hall 1978m). Natural background radiation includes cosmic rays (which varies with latitude and altitude above sea level), radioactivity in the earth's crust and materials used in building houses (Hall 1978m). Man-made radiation includes diagnostic radiology (X-rays) and radiotherapy (X-rays and gamma rays), as well as others (Hall 1978m).

2.1.3 Absorption

The absorption of photons depends upon two factors: 1) the energy of the photon and 2) the absorbing material's chemical composition (Hall 1978m). The Compton process is dominant, where high energy photons are used, such as gamma rays from the Cobalt 60 Radiotherapy Unit. The photoelectric process predominates, where photons are utilized in the lower energy range, such as diagnostic X-rays (Hall 1978m). The difference between these two processes is that in the Compton process, absorption is independent of the atomic number of the absorbing material, whereas, in the photoelectric process, the absorption varies with the atomic number (Hall 1978m). Irrespective of the absorption process, in both processes most of the energy that is absorbed by the biological material interacts with the orbital electrons of an atom, and is converted into kinetic energy to produce fast moving electrons (Hall 1978m).

2.1.4 Action - Direct and Indirect

The effects of radiation fall into two categories; 1) directly or 2) indirectly ionizing (Hall 1978m, Patt & Brues 1954). In direct ionization, there is sufficient kinetic energy in fast moving electrons which directly disrupts the DNA of the absorber, resulting in chemical and biological change. In indirect ionization, such as by gamma or X-rays, the fast moving electrons interact with water molecules in the absorbing tissue, producing free radicals which interact with different cellular constituents, resulting in chemical and biological changes (Hall 1978m, Furth & Lorenz 1954).

2.1.5 Classification of Damage

Damage incurred by radiation has been classified into three categories: 1) lethal; the damage is irreversible, irrepairable and results in cell death, 2) sublethal; damage can repair within hours unless another dose is administered soon after or 3) potentially lethal; the damage that occurs with post-irradiation environmental conditions (Hall 1978g). These are operational terms because it is still uncertain how the forms of damage relate.

In humans, exposure to a specific high dose of radiation produces varying symptoms termed the "radiation syndrome" and results in early lethal effects. There is no record of a human surviving a dose in excess of 1000 rad (Hall 1978b). Exposure in the range of 250 to 500 rad results in death after several weeks from hematological effects, 0 to 60 days; with 500 to 1200 rad,

the gastrointestinal syndrome is exhibited, death occurs in 3 to 10 days; with 10,000 to 15,000 rad, the CNS syndrome is exhibited resulting in death (Hall 1978b).

2.2 Radiosensitivity and the Cell Cycle

2.2.1 Radiosensitivity and Threshold

In 1906, Bergonie and Tribondeau postulated that rapidly proliferating tissues are radiosensitive and this sensitivity varies inversely with the degree of differentiation (Bergonie & Tribondeau 1906, Patt & Brues 1954). From these radiosensitivity studies arose the concepts of the action of radiation. With exposure to the same dose and time, it was shown that the action of radiation in different cells or different parts of the same cell was selective. It was also shown that a relationship existed between radiosensitivity and mitotic ability and the degree of differentiation of tissues (Patt & Brues 1954). These studies concluded that the responses of cells to the same amount of radiation depends upon two factors: 1) the stage of the mitotic cycle and 2) the degree of their differentiation.

The threshold dose in rats and mice when necrosis or noticeable change is first observed in radiosensitive cells is between 30 and 40 rad, whereas a dose of 200 rad kills radiosensitive cells in the germinal epithelia (Hicks 1952). Some of the factors which influence threshold for different

radiobiological effects include: 1) the intensity or rate of radiation 2) manner of exposure; local, whole body, single, continuous, fractionated, internal or external and 3) the quality of ionizing radiation (Patt & Brues 1954). Both X-rays and gamma rays produce similar physiological effects, however the efficiency to produce damage depends on the absorption of radiation per unit volume and its distribution (Patt & Brues 1954).

The relationship that exists between radiation dose and the induced biological changes is dependent upon two factors: 1) the extent of recovery between treatments and the latency in the appearance of effect (Carlson 1954). Differences in response to radiation varies with age, sex and the type of radiation. The very young and old are more radiosensitive than middle-age individuals and young adults and females express a higher degree of tolerance to radiation than males (Hall 1978b). It has also been shown in the survivors of the atomic bombing of Hiroshima and Nagasaki, that males were more susceptible to irradiation than females (Hall 1978j). Females are more resistant by approximately 0.5 Gy than males but this sex difference characteristic has not been noted in all species or in different strains of the same species (Patt & Brues 1954, Abrams 1951).

Other factors which have been found to increase susceptibility to radiation include: trauma, protein deficiency, infection, adrenal insufficiency, vitamin deficiency and exhaustive exercise (Patt & Brues 1954). Individuals

who have been previously exposed to gamma irradiation have acquired some degree of radioresistance but there is no adequate explanation for this (Patt & Brues 1954, Raper 1947, Cronkite et. al. 1950).

2.2.2 Radiation Sensitizers

Radiation sensitizers potentiate the effects of radiation. For sparsely ionizing radiation, the oxygen effect is large and important (Hall 1978a, Hall 1978i). The presence of oxygen potentiates the effect of a dose of radiation; it renders the tissue radiosensitive. Oxygen must be present during radiation exposure since before or after it is ineffective (Hall 1978i). In the presence of oxygen, the survival curves show greatly diminished shoulder widths and the slopes are slightly steeper (Hall 1978a). The decreased shoulder width in the survival curve indicates a decrease in the cell's capacity to repair the sublethal radiation damage (Hall 1978e). Decreasing the oxygen levels in the tissues results in reduced radiation effects (Patt & Brues 1954) and hypoxic cells show a greater resistance to radiation. Other radiation sensitizers include: cytotoxics such as actinomycin D, alkylating agents, and antimetabolites (Hall 1978a).

2.2.3 Dose-Response Relationship

The dose-response relationship or cell survival curve expresses the relationship between the dose of radiation absorbed and the proportion of cells which survive (Hall 1978c). One of the consequences of damage to

cells incurred by radiation is cell death. For proliferating tissues, cell death is a loss of reproductive integrity, whereas, for differentiated cells it represents a loss of specific function (Hall 1978c).

In sparsely ionizing radiation which includes X-rays and gamma rays from a radiotherapy unit, the ionizing events are widely separated in space (Hall 1978e). The survival curves are characterized initially by a small shoulder followed by a portion which is almost straight (Hall 1978c). The width of a shoulder in a cell survival curve represents a part of the dose which is wasted. Hence with fractionated doses, there is a greater extent of wastage (Hall 1978e).

Cell survival curves after exposure to X-rays and gamma rays have initial shoulder widths which are large and variable. A large shoulder indicates that cells can accumulate and repair a large amount of sublethal radiation damage (Hall 1978e).

2.2.4 Phases of the Cell Cycle

The cell cycle or mitotic cycle time is the average time interval between successive divisions (Hall 1978d, Howard & Pelc 1951). In 1951, Howard and Pelc first described the events of the cell cycle and shortly after in 1953, they had introduced autoradiography (Hall 1978f). This technique allowed investigators to determine cell cycle times and at what stage of the cycle cells are radiosensitive and radioresistant.

The cell cycle consists of: 1) mitosis; metaphase, anaphase and telophase, 2) G1 phase; post-mitotic pre-DNA synthesis phase, 3) S; DNA synthetic phase and 4) G2; the post-synthesis gap corresponds to the gap between S phase and prophase which is followed by mitosis (Lewis 1979). Prophase corresponds to late G2. In a population of cells, the cell cycle times can vary due to differences in the length of the G1 phase; G2, M (mitosis) and S phases are constant (Hall 1978d).

2.2.5 Effects of Radiation on Cell Turnover

In the mitotic cell cycle, the late G2 and M phase is the most radiosensitive and the S phase is radioresistant (Hall 1978f). Primitive mitotic cells are vulnerable to radiation during late prophase (G2), whereas, post-mitotic primitive cells are radiosensitive at the early stages of differentiation (Yamazaki 1966).

It is known that radiation delays mitosis in late prophase (G2) and produces prior to division chromosomal abnormalities and aberrations. If the X-ray dose is administered close to the critical period in a mitotic cycle, the cell is most radiosensitive just prior to the breakdown of the nuclear membrane, exposing clearly formed chromosomes (Patt & Brues 1954, Carlson 1954). Some of the observed effects include radiation induced inhibition of DNA synthesis, delay in the breakdown of the nuclear membrane and an

increase in chromosomal stickiness and retardation of chromosomal separation and formation of the cleavage furrow (Patt & Brues 1954, Carlson 1954).

Cells which are irradiated early in interphase prior to DNA and chromosomal replication, sustain chromosomal aberrations including ring and dicentric formations, whereas, if irradiation is administered later in interphase (G2), after DNA replication, there are gene mutations, chromosomal breakage or chromatid aberrations, such as the formation of an anaphase bridge (Hall 1978c).

The mechanisms for the age-response function are not fully understood. It has been postulated that with radiation the changes in the form or amount of DNA results in variation in cell sensitivity (Hall 1978f). It has been shown that in small rodents the naturally occurring sulfhydryl compounds in cells provide protection against total body gamma radiation (Hall 1978f, Hall 1978a). Sulfhydryl compounds are efficient protectors for sparsely ionizing radiation, such as X-rays or gamma rays. One explanation suggests that these compounds block the production of free radicals by reacting with them, thus reducing the effects of radiation (Hall 1978f, Hall 1978a).

The damage of cells incurred by radiation during early development results in mitotic cell death. Several possible explanations include: 1) radiation administered during metaphase or anaphase of a cell's

cycle results in mitotic cell death, 2) chromosomes sustain irrepairable damage and the cells ultimately die later during mitosis, 3) the differentiating cells do not establish their synaptic contacts and die, 4) the differentiating cells are altered or destroyed ultimately affecting their inductive influence on each other and other cell populations (Hicks & D'Amato 1966).

At the time of radiation exposure of a population of asynchronous cells, the effect of a single dose will vary due to cells occupying different phases of the cell cycle (Hall 1978f). There will be a lower fraction of surviving cells which are at or close to the sensitive part of the cell cycle (at or close to M), whereas a smaller proportion of cells will be killed which occupy the S phase (Hall 1978f).

Compared to a single dose, with fractionated doses, the proportion of surviving cells increases due to the cell's ability to repair itself from the sublethal radiation damage during the time intervals between exposure (Hall 1978g).

2.2.6 Other Factors Influencing Cell Turnover

2.2.6.1 Nutritional State

In the rat, 97% of the cells in the cerebellum are produced in the first three postnatal weeks. In undernutrition experiments, it was observed that rats exhibited a 15 to 20% permanent reduction in cell number by the weaning stage (Lewis 1979). It was shown in undernourished

brains in the subependymal and external germinal layer of rats up to 22 days old postnatally, the S phase in dividing cells was prolonged with a small effect on the cell cycle time (Lewis 1979, Lewis et. al. 1975, Patel et. al. 1973). It was noted that the S phase was lengthened up to 8 hours resulting in the severe reduction of the G1 phase.

2.2.6.2 Blood Flow

Blood flow to an exposed area also determines the sensitivity to radiation. Radiosensitivity and radiation injury parallels the blood flow to an exposed area (Patt & Brues 1954). Reduced blood flow results in reduced oxygen levels which decreases the effects by radiation. During the 5th to 10th day postnatally in the rat, the cerebral vascularity rises rapidly and decreases markedly at the end of the first month (Craigie 1945).

In assessing the effects of radiation in the brain, the role of vascular changes is not conclusively established. Cowen and Geller (1960) studied the long term effects of prenatal exposure to a single dose of 250 rad at different gestational periods and followed the offspring for several months subsequently, found no acute or chronic vascular changes. Hicks and other investigators (Hicks 1953, Hicks 1953, Deroo 1986) also reported no vascular changes with prenatal exposure to radiation.

Depending on the stage of irradiation, other investigators who irradiated rats prenatally and examined them postnatally reported

vascular changes including perivascular edema, diapedetic hemorrhages, hyaline thrombi in pre-capillaries, small hemorrhages in the brainstem, and retardation in the growth of capillary networks (Aleksandrovskaya 1961, Roizin et. al. 1962, Clemente et. al. 1960, Kosmarskaya & Barashnev 1959). Clemente et. al. (1960) found exposure to 200 to 500 rad from the 5th through the 10th day postnatally results in retardation of capillary growth and cessation of capillary multiplication.

2.2.7 Cell Cycle Times in the Developing Cerebellum

The cell turnover studies by Lewis and his co-workers (Lewis 1979, Lewis et. al. 1975) of the cerebellar external germinal layer in rats from day 1 through day 21 postnatal revealed that the cell cycle time decreases with increasing age. The cell cycle time (Tc), varied from 19 hours at PN day 1 to 17.3 hours at PN day 21 (Lewis 1979). With increasing age the S phase increases to a maximal time of 11.1 hours at PN day 11 and decreases there after to 9.7 hours at PN day 21. This correlates with the maximal proliferation of cells in the external germinal layer at PN day 12 (Altman 1972a). The G2 phase shows a tendency to decrease with increasing age from 2.9 hours at PN day 1 to 1.4 hours at PN days 21 (Lewis 1979). The G1 phase also decreases with increasing age, though a wider variation is apparent from 8.3 hours at PN day 1 to 4.8 hours at PN day 21 (Lewis 1979).

From autoradiographic studies by Schultze et. al. (Schultze et. al 1974) the cycle time for Purkinje cells between the 12th and 15th embryonic day has been calculated to be 9.9 hours. Knowing that there are 500,000 Purkinje cells in the rat cerebellum (Inukei 1928), this information was used to determine at what day proliferation of Purkinje cells begins in order to produce this number of cells by exponential growth. They found that Purkinje cells begin to proliferate on day 8.2 of embryonic development and that the duration of proliferation is 7.8 days (Schultze et. al. 1974).

Thymidine studies have determined the cell cycle time for granule cells to be 19 hours (Fujita 1967).

2.3 Neuroanatomical Nomenclature of the Cerebellum

2.3.1 Lobes, Lobules and Fissures

The cerebellum is located inferior to the occipital lobe of the cerebral cortex and posterior to the fourth ventricle (Addison 1911). It receives sensory input and transmits motor output to other areas of the nervous system by way of its attachment to the brainstem via three cerebellar peduncles; superior, middle and inferior (Larsell 1952). The cerebellum is involved in coordinating voluntary movements including balance, locomotion and fine hand movements (Addison 1911).

Historically, the cerebellum is comprised of the corpus cerebelli and the flocculonodular lobe. These two lobes are delimited by the

posterolateral (dorsolateral) fissure (Larsell 1952). The two lobes of the corpus cerebelli, corresponding to the anterior and posterior lobes, are separated by the primary fissure (Larsell 1952).

As the volume of the cerebellum increases, the fissures deepen and the onset of secondary foliation begins. The broad vermis with its expanded lateral cerebellar hemispheres within the anterior and posterior lobes are transformed into lobules (Larsell 1952). The vermian segments within the anterior lobe are designated lobules I to V, whereas, within the posterior lobe it is lobules VI to IX. The flocculonodular lobe is also represented as lobule X (Larsell 1952). Some lobules are further subdivided by secondary furrows into sublobules.

2.4 Embryonic Development of the Cerebellum

2.4.1 Embryonic Zones

The cerebellar anlage first begins to appear on embryonic day 13 (ED-13) when the neuroepithelium of the paired dorsal metencephalic plates (DMP) begin to collapse (Altman & Bayer 1978b). At the beginning of ED-14, three zones are apparent in the DMP; the superficial cell free fibrous layer or marginal layer, a middle mantle layer consisting of differentiating neurons and neuroglia, and an inner ependymal lining derived from proliferating ventricular cells (Altman & Bayer 1978b, Addison 1911). Between ED 15-16, two more zones appear; a superficial nuclear zone, which

is seen as early as ED-13, gives rise to the deep cerebellar nuclei and the transitory zone, found beneath the intermediate fibrous layer which is composed of pre-migratory Purkinje cells (Altman & Bayer 1978b, Das & Nornes 1972, Schultze et. al. 1974).

On ED 15-16, the midline fusion of the cerebellar plates begins but this is not completed until ED-22 or the day of birth (Altman & Bayer 1978b, Addison 1911). At the same time, on ED-16 in the ventrocaudal metencephalic plates, the germinal trigone appears (once the production of Purkinje cells occurs). On ED-17, the germinal trigone gives rise to the transient external germinal layer which stretches continuously across the fused midline and covers the surface of the cerebellum by ED-19 (Altman & Bayer 1978b, Addison 1911).

2.4.2 Formation of Cortical Layers

2.4.2.1 External Germinal Layer

The appearance of the transient subpial external germinal layer from the superior prong of the germinal trigone on ED-17 occurs after the onset of the production of Purkinje cells. At birth, this layer is comprised of a superficial proliferative zone and an inner pre-migratory zone (Altman & Das 1966, Altman 1972a, Addison 1911). The outer proliferative zone is comprised of actively mitotic round cells (Altman & Das 1966, Altman 1972a, Addison 1911). Despite the rapid proliferation that

occurs in this zone after birth, the depth of the proliferative zone is constantly maintained at 4 to 5 cells deep (Altman 1972a).

The inner pre-migratory zone consists of fusiform spindle shaped cells. These represent the transfer of proliferative cells, which have lost their mitotic ability and begun their differentiation into either granule, basket or stellate cells (Altman 1972a).

Between postnatal days 3 to 10 (PN 3-10), there is rapid cell proliferation in the outer proliferative zone which gradually declines by PN 15-18 and by PN 20-21, the external germinal layer decreases rapidly in cell thickness and disappears altogether, except for vestigial remains in few areas of the cerebellum, namely the flocculus (Altman & Das 1966, Altman 1972a, Addison 1911). The reduction of mitosis and migration of cells from this layer occurs at similar rates in both the vermis and lateral hemisphere (Addison 1911).

2.4.2.2 Molecular Layer

The molecular layer arises from the cell free marginal layer that is not occupied by the outer germinal layer (Addison 1911). At birth, the cell free molecular layer is defined with its occupying Purkinje dendrites (Addison 1911). Initially, this layer is narrow and at PN 8-10 days, the width in the molecular layer increases to accommodate the rapid expansion of the Purkinje cell dendrites (Addison 1911). By PN 21-25 days,

the molecular layer has attained a mature appearance and the increase in the thickness of the molecular layer is concomitant with the decline in the external germinal layer (Altman & Winfree 1977, Altman 1972a, Addison 1911). During this time, cells from the external germinal layer migrate through the molecular layer and some cells, the basket and stellate, occupy a permanent position in the molecular layer (Addison 1911).

2.4.2.3 Purkinje Cell Layer

The Purkinje cells, first identified by Johannes Evangelista Purkinje, begin to arise from the transitory zone during ED 13-16 with peak formation (over 50%) occurring on ED-15 (Altman 1972b, Altman & Bayer 1978b, Das & Nornes 1972, Schultze et. al. 1974). On ED 14-15, the early Purkinje cells begin to differentiate and by ED 16-17, these cells begin to migrate radially from the transitory zone through the differentiating neurons in the nuclear zone and cluster superficially underneath the intermediate fibrous layer (Altman & Bayer 1978b). Depending when the Purkinje cells arise, the migration and dispersion occurs till approximately 4 days after birth (Altman 1972b).

At birth, the Purkinje cells are irregularly distributed, 6-12 cells deep, between the molecular and inner granular layer (Altman 1972b). By the 5th day following birth, the Purkinje cells are dispersed in one continuous row. The decrease in Purkinje cells to a single layer is

concomitant with the expansion and maturation of the cerebellar surface (Altman & Das 1966, Altman & Winfree 1977, Altman 1972b, Addison 1911). With continued growth of the cerebellar cortex, the space between the Purkinje cells increases (Addison 1911).

2.4.2.4 Inner Granular Layer

Although the inner granular layer is recognizable at birth, it is not until the 8th day postnatally that this layer is clearly defined from the medullary layer underneath (Addison 1911). The cells comprising this layer arise from two sources: the outer germinal layer (granule cells) and the mantle layer (Golgi cells and neuroglia) (Altman 1972c, Addison 1911). Initially at birth, this layer increases slowly in width, but from PN 8-21 it increases rapidly as a result of the accumulation of cells from the external germinal layer (Addison 1911). In addition to cells, this layer also houses axons from 1) mossy fibers and climbing fibers from the medullary layer and 2) the efferents of Purkinje cells (Altman 1972c, Addison 1911).

2.4.3 Neuronal Development of Prenatal Macroneurons

2.4.3.1 Origin of Purkinje Cells

The development and origin of Purkinje cells has been described previously.

2.4.3.2 Origin of Deep Cerebellar Nuclei

The deep cerebellar nuclei, namely the medial fastigial, intermediate interpositus and lateral dentate, begin to arise on ED-13 from

the superficial nuclear zone (Altman & Bayer 1978b). By the end of ED-14, 80% of the neurons are already formed.

2.4.4 Neuronal Development of Prenatal Microneurons

2.4.4.1 Origin of Pale Cells

Pale cells begin to arise on ED-19 from the non-collapsing neuroepithelium of the cerebellum and continue production throughout the perinatal period (PN-2) (Altman & Bayer 1978b, Altman & Das 1965, Altman & Bayer 1977). The bulk of these cells (60%) arise on ED-19 and 20 prior to the complete dispersion of the external germinal layer over the surface of the cerebellum (Altman & Bayer 1978b). Pale cells are preferentially distributed in the inner granular layer of the nodulus, ventral uvula, lingula and flocculus (Altman & Bayer 1978b). They are larger than granule cells and smaller than Golgi cells.

2.4.4.2 Origin of Golgi Cells

The Golgi cells begin to arise on ED-19 and continue production till after birth (PN-2) (Altman 1972c, Altman & Bayer 1978b). They are derived from the regressing ventricular neuroepithelia (mantle layer) (Altman & Bayer 1978b). The Golgi cells in the adult cerebellum are interspersed amongst the granule and pale cells of the inner granular layer.

2.4.5 Neuronal Development of Postnatal Microneurons

2.4.5.1 Origin of Granule Cells

Granule cells arise from the external germinal layer between PN 4-21 with the bulk being formed during the 2nd week (PN-7 to PN-15) (Altman & Das 1965, Addison 1911). The precursors of granule cells cease to multiply and towards the end of the 1st week after birth, they enter the pre-migratory zone (Altman & Das 1965). The differentiation and migration of granule cells from the external germinal layer to the inner granular layer is a protracted process. From the vertically oriented granule cells, first a horizontal branch of parallel fiber forms then a vertical branch along which the nucleus of the cell migrates through the molecular layer (Altman & Das 1965). The descending granule cells exhibit no synaptogenic activity in the molecular layer until they reach the inner granular layer. In the inner granular layer, the dendrites of the granule cells form synapses with mossy fiber rosettes in glomeruli which begins at PN-5 and increase in size and number for PN 21-30 (Altman & Das 1965). From PN-15 onward, granule cells are densely packed in the inner granular layer. The migration of granule cells from the external germinal layer to the inner granular layer occurs via the assembly of parallel fibers in the molecular layer in a temporal fashion, from bottom upward (Altman & Das 1965).

2.4.5.2 Origin of Basket Cells

Basket cells arise from the external germinal layer postnatally from day 4 to 11 (Altman 1972a, Altman & Bayer 1978b) and occupy a position in the lower portion of the molecular layer (Altman & Anderson 1972, Altman & Das 1965). The presence of descending basket cell axons are needed to ensure a normal upright growth and orientation of the Purkinje cell stem dendrite (Altman 1976a, Altman 1976b). In the adult, basket cell axons synapse onto the soma of Purkinje cells (Altman 1972b).

2.4.5.3 Origin of Stellate Cells

Stellate cells arise from the external germinal layer postnatally from day 6 to 15 with peak formation between days 8-11 (Altman 1976c, Altman & Bayer 1978b). They occupy a position in the upper one third of the molecular layer (Altman & Anderson 1972, Altman & Das 1965). The presence of descending stellate cells are required for the production of secondary smooth branches on the Purkinje cell stem dendrite (Altman 1976c, Altman 1976b).

2.5 Cerebellar Granule Cell Migration

2.5.1 Mode of Migration

The migrating cerebellar granule cells follow the biphilic mode of migration (Rakic 1990, Rakic 1971). Granule cells arise from the proliferative zone of the external germinal layer. Following the completion

of final mitosis, the proliferative granule cells descend and enter the pre-migratory zone of the external germinal layer and remain there for 28 hours synthesizing the required adhesion molecules necessary for their migration inwards (Chuong 1990). The migration of granule cells from the external germinal layer to the inner granular layer occurs in 4 hours (Chuong 1990).

During migration, the granule cell extends 2 classes of neurites. The two horizontal cytoplasmic processes extending from the granule cell behave as neurophilic. These processes form the parallel fibers of the molecular layer which run parallel to the pia and extend along the surface of previously generated parallel fibers. The single vertical descending neurite from the cell soma is gliophilic; it follows Bergmann glial fibers (Rakic 1990, Rakic 1971). The vertical shaft forms synaptic contacts with Purkinje cell dendrites.

Rakic (1971) observed that during migration the Bergmann glial fibers are closely opposed to the granule cell soma and its leading process and these fibers may serve as guides for inward radial migration. At the level of the Purkinje cell layer, the granule cell reaches the Bergmann cell soma and detaches from the guiding fiber to take up its final position in the inner granular layer (Rakic 1990).

2.5.2 Neuronal-Glial Relationships

In vitro model systems such as microcultures have been developed to study the mechanisms of glial-guided neuronal migration (Hatten & Mason 1990). With the aid of time lapse video microscopy, the movements of migrating neurons along glial fibers have been recorded (Hatten & Mason 1990).

It has been shown that migrating cerebellar granule neurons oppose their cell soma against a Bergmann glial fiber and form a specialized migration or interstitial junction underneath their soma at the site of contact with the glial fiber (Hatten & Mason 1990). This contact site with the glial fiber is maintained as the neuron moves inward.

These junctions are characterized by a wide intercellular space with filamentous material spanning the cleft and binding the membranes of each cell with connections to the cytoskeleton of each cell (Hatten & Mason 1990). As observed by video microscopy, these migration junctions appear to be the force-generating locus during forward movement of the cell (Hatten & Mason 1990).

These migrating neurons express a highly bipolar shape along the glial fiber with the highly motile vertical leading process extended in the direction of migration (Hatten & Mason 1990). Migration is achieved by the neuron pushing its soma against the glial fiber rather than the leading process

pushing or pulling the neuron along the glial guide. Video microscopy has shown as the neuron migrates, the tip of the leading process rapidly extends and retracts its short lamellopodia and filopodia which enfold the glial fiber (Edmondson & Hatten 1987). Neither the motions of the leading process nor the lamellopodia or filopodia are synchronized with the motions of the cell soma (Edmondson & Hatten 1987, Gasser & Hatten 1990). The migrating neuron moves along the glial fiber by forming a "footpad" beneath the cell soma followed by extension of its leading process along the glial fiber (Edmondson & Hatten 1987).

The role of the leading process is to select the glial fiber for migration; it sets the directionality of migration (Hatten & Mason 1990). It has been shown in vitro that prior to reaching their final destination, migrating neurons shift from one glial fiber to another closely apposing glial fiber (Hatten & Mason 1990).

Questions arose whether it was the neuron or the glial cell regulating migration. In the weaver mutant mouse, the granule cells fail to attach to the glial fiber and migrate because of defects in the glial cells and subsequently die in ectopic positions (Caviness & Rakic 1978, Rakic & Sidman 1973, Sotelo & Changeux 1984). The glial fibers in this mutant are deranged, misaligned; they are not in the radial pattern typical of migration, and display abnormal arbors (Rakic & Sidman 1973).

From the homotypic and heterotypic co-culture experiments in the weaver mutant mouse it was shown that normal neurons could migrate along both normal and weaver glia, but weaver neurons could migrate on neither glial fibers. This suggests that a defect in the form and organization of glial fibers results from the neuron failing to bind to the glial process and that the granule cell is the site of action of the expression of the weaver gene (Hatten & Mason 1990).

In the reeler mutant, neurons attach to glial fibers and migrate but migration is arrested prematurely because the neuron fails to detach from its glial guide (Pinto et. al. 1982), supporting further evidence that the neuron regulates migration.

2.5.3 Molecular Mechanisms of Neuronal Migration: Role of Adhesion Molecules and Extracellular Matrix

Adhesion molecules thought to have a role in granule cell migration, have been identified, purified and characterized largely by immunological assays using antibodies which react with the surface components of the cell, inhibiting cell adhesion in vitro (Jessell 1988, Rutishauser et. al. 1988). The adhesion molecules that are thought to be involved in cerebellar granule cell migration include N-CAM, Ng-CAM, cytotactin and astrotactin (Chuong 1990, Jessell 1988, Grumet et. al. 1984, Edmondson et. al. 1988, Stitt & Hatten 1990).

The neural cell adhesion molecule N-CAM, a cell surface glycoprotein, is expressed in all the cerebellar cortical layers but the various isoforms of N-CAM are not all expressed in all layers and Bergmann glial fibers during granule cell migration (Chuong 1990, Chuong et. al. 1987).

Both Ng-CAM and cytotactin are neural-glia adhesion molecules and are not equally expressed throughout the cerebellar cortical layers (Chuong 1990). Ng-CAM begins to be expressed in the pre-migratory zone of the external germinal layer and the molecular layer (Chuong 1990). Cytotactin or tenascin is an extracellular matrix protein produced by oligodendroglia, astrocytes, and some glioma cell lines (Chuong 1990, Grumet et. al. 1984). Cytotactin is expressed throughout but it is abundant in the molecular layer (Chuong 1990).

Astrotactin is a neural-glia adhesion molecule. The cerebellar granule neurons express astrotactin at specific developmental stages. It is abundant in postnatal cells and in migrating neurons but not in proliferative cells in the proliferative zone of the external germinal layer (Hatten & Mason 1990). Astrotactin is expressed in high levels during granule cell migration along glial fibers, and assembly of granule cells into the inner granular layer and expression is reduced after migration and assembly have ceased (Stitt et. al. 1990).

Studies by Edmondson et. al. (1988) and Stitt and Hatten (1990) have shown that the glycoprotein astrotactin functions as a ligand which binds the migrating neuron to the glial fiber (Hatten & Mason 1990). It has been reported that antibodies against astrotactin prevent neuronal membranes from binding to glial cells (Stitt & Hatten 1990), to establish neuron-glia contacts, and to organize neuron positioning by glial processes (Edmondson et. al. 1988). In contrast, they found antibodies against N-CAM did not block in vitro any of these neuron-glia interactions (Edmondson et. al. 1988, Stitt & Hatten 1990).

It has been proposed that granule cell migration along radial glial fibers involves several adhesion molecules, extracellular matrix proteins and proteolytic enzymes (Chuong 1990). Granule cells migrate from the external germinal layer to the inner granular layer through intact tissue. The granule cell adheres to the Bergmann glial fiber through adhesion molecules, moves along the fiber and upon reaching its final destination, the inner granular layer, the neuron detaches from the glia (Chuong 1990). It has been suggested that extracellular proteolysis may be essential during granule cell migration. It has been shown in cerebellar explants that if the proteolytic enzymes plasminogen activator and plasmin (Moonen et. al. 1982) are not present, granule cell migration is inhibited (Verrall & Seeds 1988). These

enzymes could digest surrounding tissues or remodel adhesion attachments allowing cells to migrate inwardly (Chuong 1990).

Neurons migrate along glial fibers via neuron-glia ligands and detach from the glial guide via neuron-neuron interactions (Hatten & Mason 1990). The question arises what arrests migration and diverts the neuron from the glial guide to assemble into the neuronal layer? It is suggested that migration is arrested via 1) neuron-neuron contacts by previously arrived neurons in the target layer, 2) by afferents or 3) cleavage by enzymes (Hatten & Mason 1990).

Firstly, neuron-neuron interactions mediated by cell adhesion molecules would divert the neuron from the glial guide to assemble into the inner granular layer (Hatten & Mason 1990). Once migration is complete, the neuron in the target area would signal the migrating neuron to cease moving and abandon its fiber. A second possibility is neuronal-axon interaction during migration. During migration, when the Purkinje and granule cells enter the target region of the climbing and mossy fibers, there is immediate contact and interaction (Hatten & Mason 1990). The climbing fibers arise from the inferior olivary nucleus and establish contact with Purkinje cells. The mossy fibers enter the inner granular layer of the cerebellum during granule cell migration and the small growing tips on their fine branches contact both Purkinje and the inwardly migrating granule cells (Hatten & Mason 1990,

Mason 1987). The afferent growth cone contacts the migrating cell signalling the cessation of migration with subsequent detachment from the glial fiber (Hatten & Mason 1990). A third suggestion is cleavage by proteolytic enzymes. Granule cell migration involves some molecules for adhesion and some for de-adhesion (Chuong 1990). The de-adhesion molecules would reduce the adhesion forces and allow translocation of the neuron from the glial fiber into the neuronal layer. This de-adhesion can be achieved by cleavage through proteolytic enzymes of the adjacent molecules involved in conformational change of the adhesion molecule (Chuong 1990).

2.6 Effects of Radiation on Cerebellar Development

2.6.1 Sensitive Phases of Development

Radiation can affect three population of cells: proliferating, differentiating and migrating cells (Hicks & D'Amato 1966). The susceptibility to any teratogenic insult, including radiation, is dependent upon the stage of development. In general, during histogenesis, with increasing age, the sensitivity of the developing neural tissue decreases as does its capacity for repair (Patt & Brues 1954, Kameyama & Hoshino 1986).

The CNS has a long lasting sensitive period extending from the beginning of organogenesis to the neonatal period (Kameyama & Hoshino 1986). In rats, histogenesis of the macroneurons of the cerebellum starts from embryonic day 13 to 15 when the Purkinje and the Golgi cells are produced

followed by their migration. The microneurons produced from the external germinal layer; granule, basket and stellate cells, arise postnatally from day 4 to day 21. Since maturation of the cerebellum is largely postnatal, the sensitive phase of its histogenetic components extends from embryonic day 14 to postnatal day 21 (Altman 1972a, Altman 1972b, Altman 1972c, Altman & Bayer 1978b, Altman & Das 1965).

2.6.2 Radiosensitivity of Various Developmental Periods

2.6.2.1 PreImplantation Period

The preimplantation period is the period from fertilization to the time of attachment of the embryo to the uterine wall (Hall 1978k). In the rat which has a gestation period of 21 to 22 days, this corresponds from day 0 to 7 (Russell 1954, Russell & Russell 1954).

Studies by Russell (Russell 1954, Russell & Russell 1954) showed that rats irradiated in utero with 200 rad during the preimplantation period results in a high incidence of prenatal death but there were no abnormalities in the surviving embryos at term. Similar findings were reported by Cowen & Geller (1960), Hicks (1953) and Kosaka (1927).

2.6.2.2 Organogenesis Period

In the rat, organogenesis corresponds from gestational day 8-15 and is a period characterized by major organ formation (Hall 1978k, Russell 1954). In general, exposure to irradiation during this period results

in surviving embryos with major congenital anomalies, overall growth reduction and less prenatal mortality (Hicks 1953, Russell 1954, Russell & Russell 1954, Hall 1978k).

Both Russell (1954) and Hicks (1953 & 1954a) reported that exposure of pregnant rats to 150 to 300 rad irradiation during organogenesis produced a broad spectrum of anomalies in the CNS. Using doses between 150 to 300 rad during various developmental stages, Hicks (1954a) constructed a timetable of various radiation induced anomalies in the nervous system for both rats and mice. His findings are supported by the studies of Job et. al. (1935), Wilson (1954), Warkany and Schraffenberger (1947) and Kaven (1938). During organogenesis, Hicks et. al. (1961) has shown that the mitotic cells which are proliferating in vast numbers are highly radiosensitive.

2.6.2.3 Fetal and Neonatal Period

The fetal period in the rat is from day 16 to term (day 21 or day 22) and it is characterized by further growth and maturation (Russell 1954, Hall 1978k). Irradiation during this period results in no prenatal death, but from embryonic day 14 to about 3 weeks after birth, the cerebellum is progressively more radiosensitive and results in various cerebellar defects (Hicks 1954a).

The neonatal period from birth to 3 weeks postnatally is a period of cerebellar growth and maturation (Russell 1954, Yamazaki 1966). Hicks et. al. (1961) demonstrated that the cells in the fetal and neonatal period which are not undergoing mitosis, but just beginning their differentiation are highly radiosensitive.

2.6.3 Malformation Patterns

The developmental anomalies that occur as a response to radiation are determined by the dose, dose rate, type of radiation, stage of development and species of animal (Hicks & D'Amato 1966). There is a considerable degree of uniformity of radiation induced anomalies observed between offspring of the same litter and different litters of the same species when exposed to radiation at the same stage of development (Hicks & D'Amato 1966). However, there exists some variability in the radiation response amongst genetic strains, different species and amongst members of the same litter (Hicks & D'Amato 1966).

Many radiation studies involving rodents utilize X-ray exposure between 100 and 400 rad. This range secures a spectrum of reproducible anomalies when animals are irradiated between the 7th prenatal day and a week or more postnatally (Hicks & D'Amato 1966).

Extensive experimental studies in rats and mice has shown that the type of anomalies induced by radiation is closely related to the stage of

development upon exposure (Job et. al. 1935, Kaven 1938, Russell 1950, Wilson et. al. 1953, Wilson 1954, Wilson et. al. 1951, Hicks & D'Amato 1966). Russell's work (1950, 1954, 1956, 1957) and that of Russell and Russell (1954) in mice documents the various skeletal, visceral and body anomalies induced by radiation at various stages of development (Russell 1950, 1954, 1956, 1957, Russell & Russell 1954).

2.6.4 Effects on Behavior and Motor Function

Studies on the effects of radiation on the behavioral development and function have been emerging. These studies have attempted to correlate the morphological alterations of the developing nervous system observed in prenatally irradiated animals with their behavior. It has been noted that behavioral changes correlate to the stage of development during which radiation was administered (Hicks & D'Amato 1966).

Furchtgott and Echolls (1958) showed that there is a relationship between the irradiation dose and age of exposure to motor activity. In their studies, they irradiated rats with varying single doses between 50 to 300 rad on gestational days 14 to 18, and examined the rats 27 days postnatally. The offspring exhibited deficits in motor co-ordination, namely decreased motor strength and weakness. Werboff et. al. (1961) found that in rats exposed in utero on gestational days 5, 10, and 15 and examined 27 days PN that a

threshold dose of 25 rad produced deficits in motor co-ordination whereas doses below 25 rad did not.

Offspring of rats irradiated prenatally with 125 rad on gestational days 13, 14, and 15 exhibited hyperactivity, tremors, spasticity, incoordination and a hopping gait in the first postnatal week followed by reduced motor activity 4 weeks postnatally (Hicks & D'Amato 1980b, Jensh et. al. 1986-87, Jensh & Brent 1986-1988, Norton 1986, 1989, Norton et. al. 1976, Semagin 1961). A "hopping gait" is characterized by the hind and forelimbs moving synchronously rather than in the normal opposition pattern. Other behavioral effects that were noted in prenatally irradiated rats included: deficits in maze learning and visual discrimination, defecation, urination and increased emotional behavior (Furchtgott & Echols 1958, Yamazaki 1966, Kaplan 1962).

2.6.5 Human Studies

Information on the effects of ionizing radiation on the developing nervous system in humans is meager and most is derived from the following three sources: 1) the surviving children who were exposed in utero during the atomic bombing of Hiroshima and Nagasaki, 2) the offspring of mothers receiving therapeutic pelvic irradiation and 3) the surveys of the occurrence of neoplasms in children whose mother's pelvis was irradiated during pregnancy (Yamazaki 1966). Human neural tissue and that of animals respond similarly towards ionizing radiation (Yamazaki 1966). Otis and Brent

(1952) have constructed a timetable corresponding to the stages of development in a mouse with the corresponding gestational day in humans.

One characteristic of radiation damage to the developing brain that is commonly exhibited in both experimental animals and humans is microcephaly (Yamazaki 1966). The incidence of microcephaly is related to the dose, stage of development during the time of exposure, and the period of exposure (Yamazaki 1966).

2.6.5.1 Hiroshima and Nagasaki

In assessing the radiation effects of the children exposed in utero during the bombing of Hiroshima and Nagasaki, the type of radiation, the total dose absorbed and the stage of development at the time of exposure must be considered (Yamazaki 1966). The dose of radiation exposure is determined by knowing the distance from the hypocenter; the point on the ground where the bomb exploded.

Compared to Hiroshima, the bomb dropped at Nagasaki released little neutron energy (Yamazaki 1966). In Hiroshima, the bomb dropped released a high percentage of neutrons, whereas at Nagasaki more gamma rays were released. For the same dose, neutrons have a greater carcinogenicity than gamma rays (Hall 1978).

The surviving children of mothers, who were within 2000 meters of the hypocenter, exhibited microcephaly, mental and growth

retardation. The severity decreased as the distance from the hypocenter increased (Miller 1956, Otake & Schull 1984, Yamazaki 1966, Plummer 1952, Sutow 1954). It was shown that the highest degree of mental retardation in the offspring occurred when they were exposed in utero, 8 to 15 weeks of gestation, and the damage was expressed linearly with the dose received (Otake & Schull 1984). In both cities, it was noted that males were more susceptible than females to the effects of radiation.

2.6.5.2 Diagnostic Studies

Depending on the stage of development at the time of exposure in utero, the anomalies manifested in children postnatally included: microcephaly, blindness, microphthalmia, mental deficiency, defects in motor coordination, skull anomalies, spina bifida, retinitis, cataracts and strabismus to name a few (Russell 1954, Yamazaki 1966). As early as 1926, Zappert reported children who were exposed in utero to radiation during the first trimester exhibited microcephaly (Yamazaki 1966, Zappert 1926).

There is much speculation in a correlation between low dose diagnostic radiation in utero and the incidence of increased malignancies; leukemia, neoplasms and tumors of neural cell origin (Heyssel et. al. 1960, Court-Brown 1958). Epidemiologic studies by Stewart et. al. (1958) and MacMahon (1962) revealed that mothers who were exposed to X-rays during their pregnancy via diagnostic abdominal or pelvic irradiation,

showed an increased incidence of tumors of the CNS and cancer mortality in their children. The frequency of tumors of neuroectodermal origin suggests that irradiation during the first trimester of pregnancy, at a time when the embryonic neural cells are highly radiosensitive, renders the neural cells susceptible to malignant tumors (MacMahon 1962, Yamazaki 1966, Court-Brown 1958, Stewart et. al. 1958).

In one study, pregnant women received therapeutic pelvic irradiation, either with X-rays or radium implants at an estimated dose of 30 to 250 rad during the first trimester of pregnancy. The children born exhibited microcephaly, microphthalmia and optic atrophy (Goldstein 1930).

The present investigation is concerned with the effects of maternal exposure to a single dose of whole body irradiation (0.5 Gy) during late gestation on the development of the cerebellum in the rat.

3.0 MATERIALS AND METHODS

3.1 Experimental Animals

3.1.1 Animals

The offspring of 36 pregnant Sprague-Dawley rats, totalling 288 animals, were used in this study.

3.1.2 Animal Care and Environment

The rats were cared for in accordance with the guidelines set by the Canadian Council on Animal Care. The rats were housed in wire mesh cages and maintained under controlled room temperatures (20±2°C) and illumination (12 hour light/dark cycle, 2000-0800 dark). Water and laboratory rat chow were given ad libitum.

3.2 Experimental Protocol

3.2.1 Determination of Pregnancy

Timed pregnancies were obtained by housing two females (between 200-225 g) with a male rat of the same strain overnight. The following day, the females were checked for vaginal plugs and vaginal smears were obtained. If the females showed sperm-positive smears, they were designated as gestational day 1 (GD-1) and randomly assigned to either a control or treatment group.

3.2.2 Assignment of Groups

Pregnant females were housed individually in plastic cages with bedding. Groups of pregnant rats, (N = 3-6/group), were randomly allocated to one of the following control or treatment groups:

#1) Control	Day 17
#2) Irradiation	Day 17
#3) Control	Day 18
#4) Irradiation	Day 18
#5) Control	Day 19
#6) Irradiation	Day 19
#7) Control	Day 20
#8) Irradiation	Day 20

Within each group, several pregnant rats were killed either 21 or 28 days postnatally. Within 36 hours after parturition, all litters in each group (control or treatment) were reduced to 8 neonates.

3.2.3 Procedure for Irradiation

Between 1600-1700 hours, pregnant rats were placed in a specially constructed 20x10x7 cm clear lucite plexiglass cage, divided into two equal compartments. The rats were exposed to 50 cGy or 50 Rad (1 Gray = 100 Rad) of 60 Co gamma radiation using a Theratron F Cobalt Radiotherapy Unit (AECL of Canada). The calibration and performance of the unit is

periodically checked by the Medical Physics Department of the Manitoba Cancer Treatment and Research Foundation.

To ensure uniform dose distribution throughout the irradiated volume, the animals were exposed to parallel opposed radiation fields (antero-posterior and postero-anterior), positioned 75 cm above or below the surface of the plexiglass cage on the treatment table. The time of exposure for both fields was calculated at 16.5 seconds. Total time of confinement was 20-30 minutes. Control animals were treated in the same manner except that they were not irradiated. All irradiation experiments were completed within a 12 month period.

3.2.4 Sacrifice of Animals

At 21 or 28 days PN, the rats were anesthetized with an intraperitoneal injection of Nembutal (50mg/Kg). While under anesthesia, the rats breathed spontaneously, and when they were unresponsive to mechanical stimulation, the chest cavity was opened. The animals were perfused transcardially by means of a #18 gauge catheter with 2.5% glutaraldehyde-2% paraformaldehyde in 0.12M phosphate buffer and 0.02 mM calcium chloride. The 37°C fixative was delivered from a column height of 110-115 cm for a period of 15-20 minutes.

3.2.5 Fixation, Embedding and Staining of Tissue

Following perfusion, the brains were excised, examined for gross anomalies and stored overnight in a fresh solution with the same fixative at 4°C.

For light microscopic examination (LM), a representative number of brains (N=10 or 12/group) were randomly selected for processing. The tissues were rinsed with a rinse solution (0.12 M phosphate buffer with 0.02 mM calcium chloride and 8% dextrose). The tissues not immediately used were placed in this dextrose rinse solution which was changed every two to three weeks to prevent growth of mold.

Initially, the brains were processed with the Dioxane procedure (Appendix II) followed by embedding in paraffin. The dioxane method resulted in brittle tissue during sectioning. For subsequent tissue preparation, the chloroform method was employed (Appendix II) yielding better tissue sectioning. The tissues were serially sectioned at 5 μm from rostral to caudal in the coronal plane with 100 μm intervals between sections. Every 3rd to 5th section was stained with thionin or Bodian (Appendix IV).

3.3 Determination of Cell Analysis

3.3.1 Purkinje Cell Counts

The cerebella were serially sectioned at 5 μm in the coronal plane with 100 μm intervals between sections. Every 3rd to 5th section was stained

with thionin. Using the criteria described by Wetts and Herrup (1982a) the Purkinje cell number in each cerebellum was determined by counting all cells in every 5th section in the pyramis; lobule 8 of the posterior lobe. At 10X, using a 100 μm grid every cell in each section was counted if 1) it was in the Purkinje cell layer 2) had a large-diameter soma, and 3) had a portion of both the nucleus and nucleoli in the plane of section.

The Purkinje cell density in the pyramis and also within the vermis and both lateral hemispheres was obtained by dividing the number of Purkinje cells in the section by the section's length of the Purkinje cell line in millimeters (mm). There were 8-10 cerebellar sections at 25 micrometer intervals that were counted for each animal.

To avoid double counting, sections with questionable Purkinje cell nucleoli were differentiated by examining the sections that preceded and immediately followed the cell in question.

3.3.2 Granule Cell Counts

Granule cells were counted from the same sections used for Purkinje cell counts. Granule cells were counted using the criteria described by Wetts and Herrup (Wetts & Herrup 1982b). Superimposed throughout the granule cell layer, the granule cell density was determined by counting at 40X magnification the number of granule cells within a 100 μm grid. Only the granule cells within the boundaries of the grid were counted. This process

was repeated for 9 areas within the inner granular cell layer in each of the 8-10 cerebellar sections that were counted.

The granule/Purkinje cell ratio was calculated from the linear density in each animal and an average was obtained. The total number of granule cells in the pyramis was calculated by multiplying the average density of granule cells in the granular cell layer by the length of the granule cell layer.

3.4 Determination of Cerebellar Length Measurements

The length of the Purkinje and granule cell layer was measured by tracing the outline of the granular cell layer through a microscope attachment onto a Zeiss digitized X-Y pad. The length of both lateral hemispheres, vermis and the total length was measured in the pyramis. The length was measured in every section where both the Purkinje and granule cells were counted.

3.5 Parameters Analyzed

The effects of maternal exposure to a single dose of whole body irradiation (0.5 Gy) on gestational days 17, 18, 19, and 20 on the development of the cerebella were examined in the offspring at 21 and 28 days postnatally. The gross external morphology of the cerebellar cortex, namely the fissures, sulci and lobule formation were examined in each animal. The internal microscopic structure of all 3 lobes of the cerebellum; anterior, posterior and

flocculonodular, was analyzed for the following: 1) the effect of low dose irradiation on the molecular, Purkinje and inner granular layers and its neurons, 2) the internal cortical layers and its microneurons between the vermis and lateral hemisphere, 3) the arrangement and orientation of the Purkinje cells within the lobes, and 4) if exposure to irradiation during the late gestational days has any effect on the Purkinje and the granule cells, then examine any direct/indirect correlation between the Purkinje and the granule cells.

3.6 Statistical Analysis

Differences between the cerebella of control and irradiated animals were analyzed statistically using Fisher's Exact, chi-square tests and ANOVA.

4.0 RESULTS

4.1 Behavior and Motor Effects.

Rats irradiated with 0.5 Gy on gestational days (GD) 17, 18, 19, or 20 and examined at either 21 or 28 days postnatally exhibited hyperactivity, spasticity and a hopping gait. This motor deficit was noted in all irradiated groups; however, the walking patterns were only observed and not recorded.

4.2 Cerebellar Gross Morphology

4.2.1 Cerebellar Size and Fissures

The size of the cerebella in rats irradiated on GD-17, 18, 19, and 20 and examined at either 21 or 28 days postnatally were comparable to controls. The cerebella in all groups of irradiated rats were normal in size, as well as in folial, sulci and fissure formation.

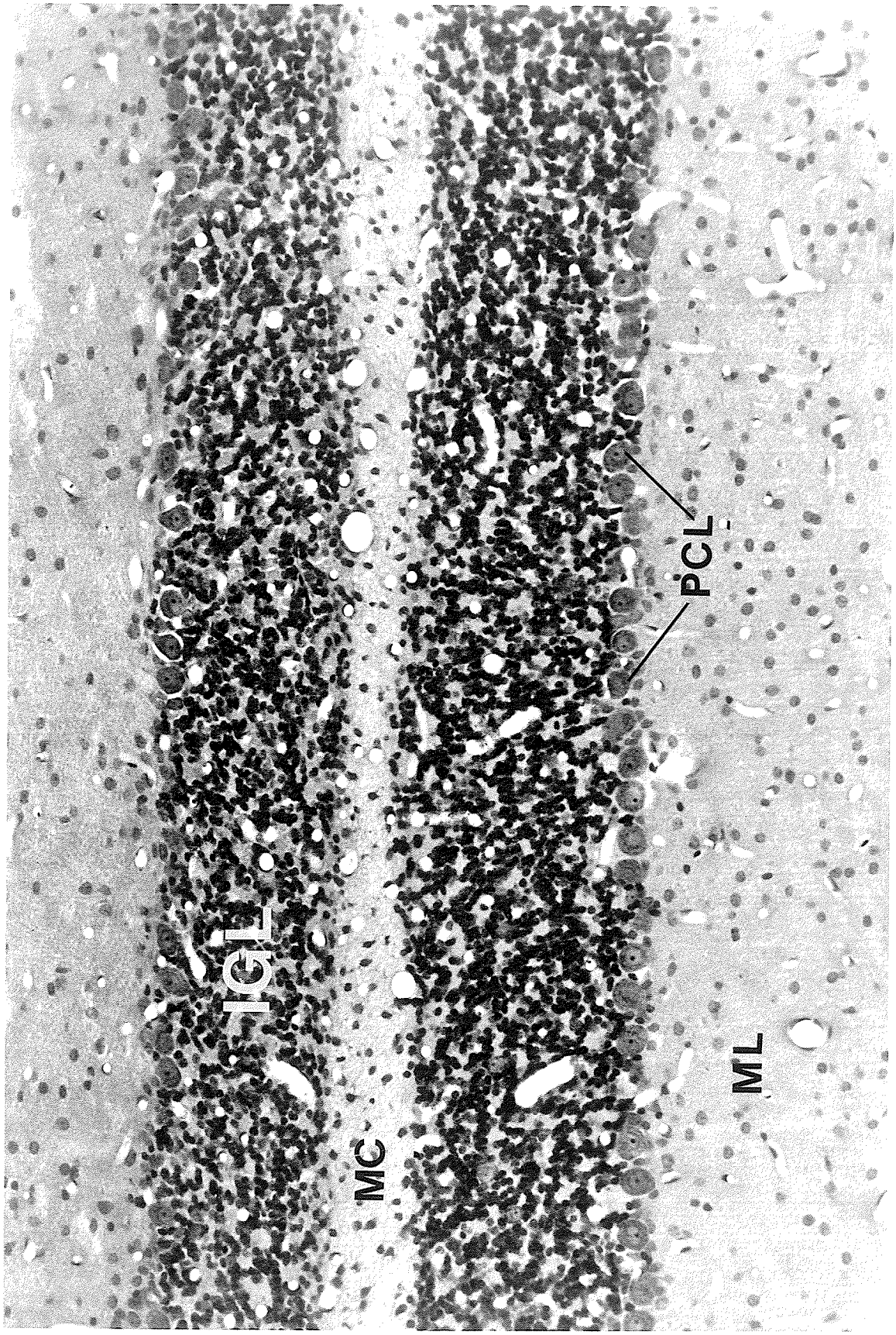
4.2.2 Anterior, Posterior and Flocculonodular Lobes

In all groups of irradiated rats, no gross anomalies were observed in the anterior, posterior and flocculonodular lobes.

4.3 Histological Examination of Cerebellar Cytoarchitecture

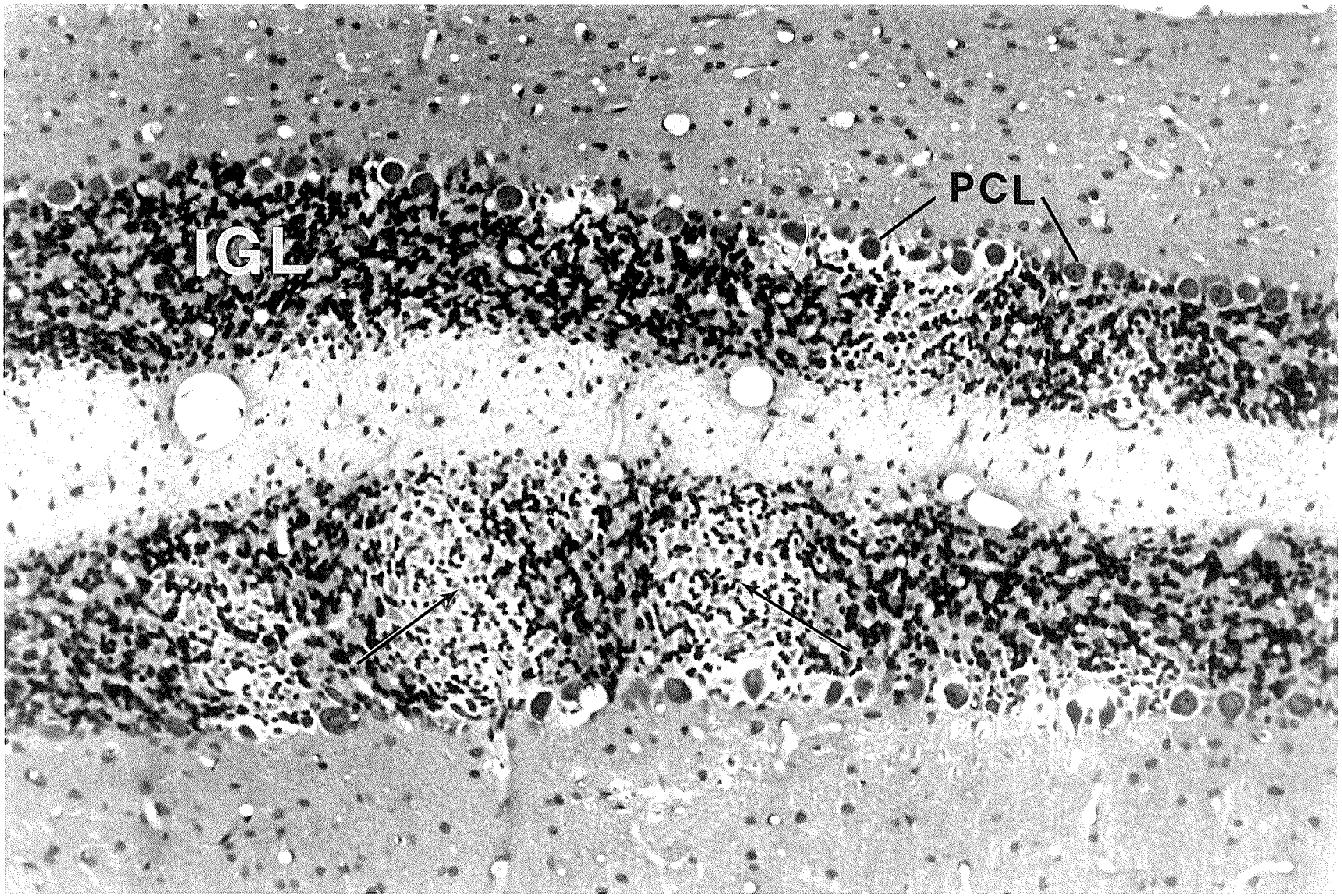
Comparative thionin stained coronal sections of cerebella from rats irradiated on GD-17 and a control are illustrated in (Figures 1 and 2). The trilaminar cortical architecture was maintained and well defined in the cerebella of both control (Figure 1) and all groups of irradiated rats (Figure 2).

FIGURE 1 Coronal section of cerebellar folia from a 28 day old non-irradiated control rat illustrating the normal trilaminar cortical architecture. Thionin stained, 5 μm, 40X. ML, molecular layer; PCL, Purkinje cell layer; IGL, inner granular layer; MC, medullary center.



51a

FIGURE 2 Cerebellum of a 28 day old rat irradiated with 0.5 Gy on GD-17 illustrating the circumscribed lesions (solid arrows) distributed amongst the population of normal appearing granule cells within the inner granular layer. Coronal section, thionin stained, 5 μm, 40X. GC, granule cells; IGL, inner granular layer; PCL, Purkinje cell layer.


PCL
IGL

4.3.1 Molecular Layer

Within the individual lobules there was variation in the size and contour of the molecular layer but this was observed in both control and all irradiated rats. In examining sections stained by thionin and Bodian the molecular layer was not found to be altered.

4.3.2 Purkinje Cell Layer

The most striking changes in the Purkinje cell layer following irradiation was the presence of gaps, variable in length, and atrophied cells. These gaps and/or atrophy of Purkinje cells were present immediately above the lesions in the inner granular layer (Figures 3, 4, 5 and 6). Occasionally, Purkinje cells were found in the inner granular layer scattered amongst the granule cells. The Purkinje cells were readily identified based on their size, flask shape and their dendrites oriented towards the pial surface. The Purkinje cells were aligned in a monolayer throughout the entire cerebella in all lobes examined in all irradiated groups.

4.3.3 Inner Granular Layer

The most conspicuous change in the cortices of rats irradiated between gestational days 17, 18, 19, and 20 was the presence of several circumscribed cerebellar lesions (CL) throughout the inner granular layer (Figure 2). These lesions were characterized by reduced granule cell numbers and condensation and/or atrophy of some of the remaining granule cells

FIGURE 3 Coronal section of the inner granular layer from the cerebellum of a 28 day old non-irradiated rat illustrating the normal population of granule cells clustered beneath the Purkinje cells. Thionin stained, 5 μm, 80X. GC, granule cells; PC, Purkinje cells. A higher magnification of Figure 1.

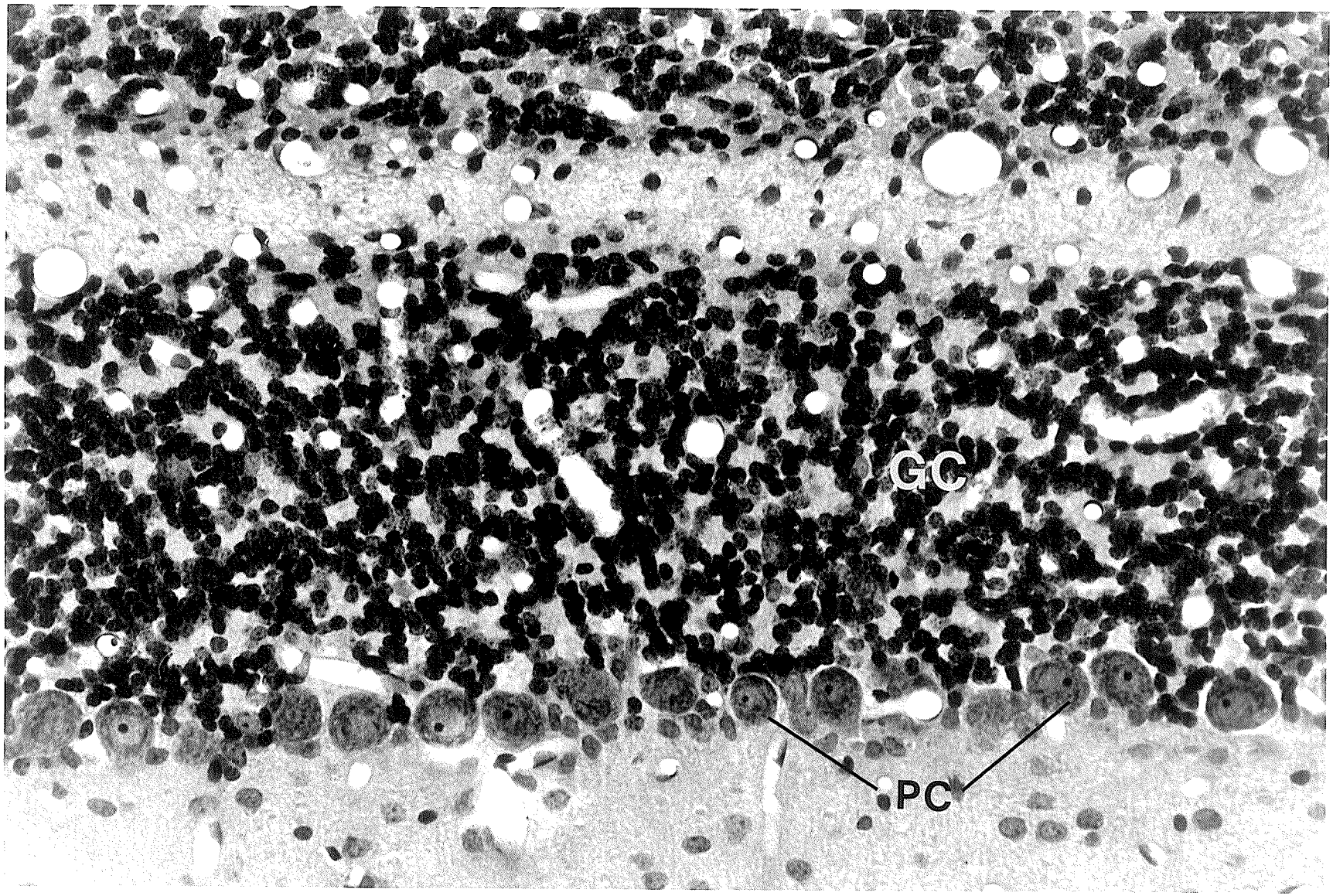

GC
PC

FIGURE 4 Cerebellum of a 28 day old rat irradiated with 0.5 Gy on GD-17 illustrating the clusters of lesions among the normal population of granule cells within the inner granular layer. Note that within these lesioned areas, the granule cells are reduced in number and some appear atrophied. Coronal section, thionin stained, 5 μm, 80X. PC, Purkinje cells; GC, granule cells; CL, circumscribed lesion. A higher magnification of Figure 2.

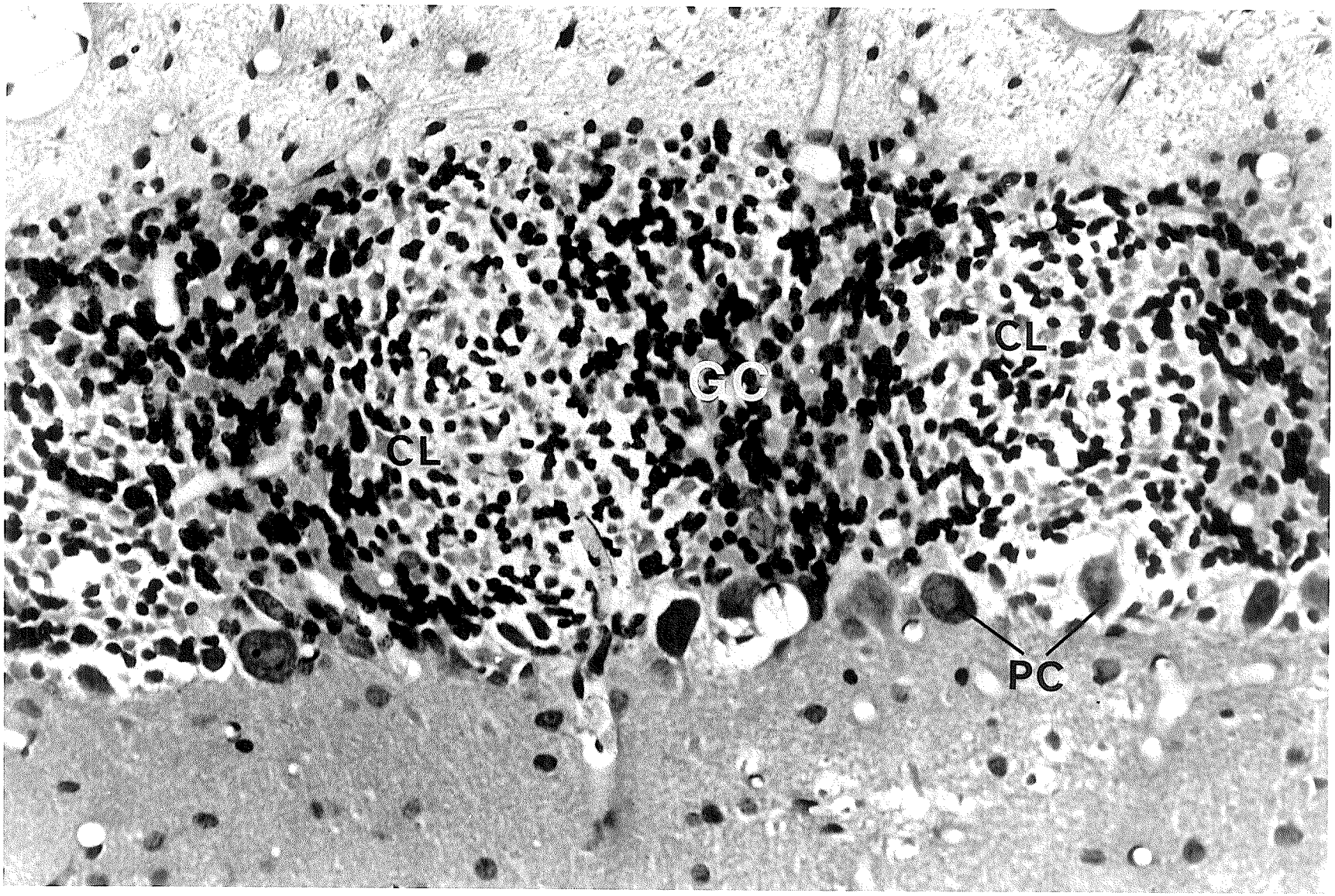

CL
GC
CL
PC

FIGURE 5 Inner granular layer from the cerebellum of a 28 day old non-irradiated rat illustrating the normal population of granule cells clustered beneath the Purkinje cells. Coronal section, thionin stained, 5 μm, 160X. GC, granule cells; PC, Purkinje cells. A higher magnification of Figure 1.

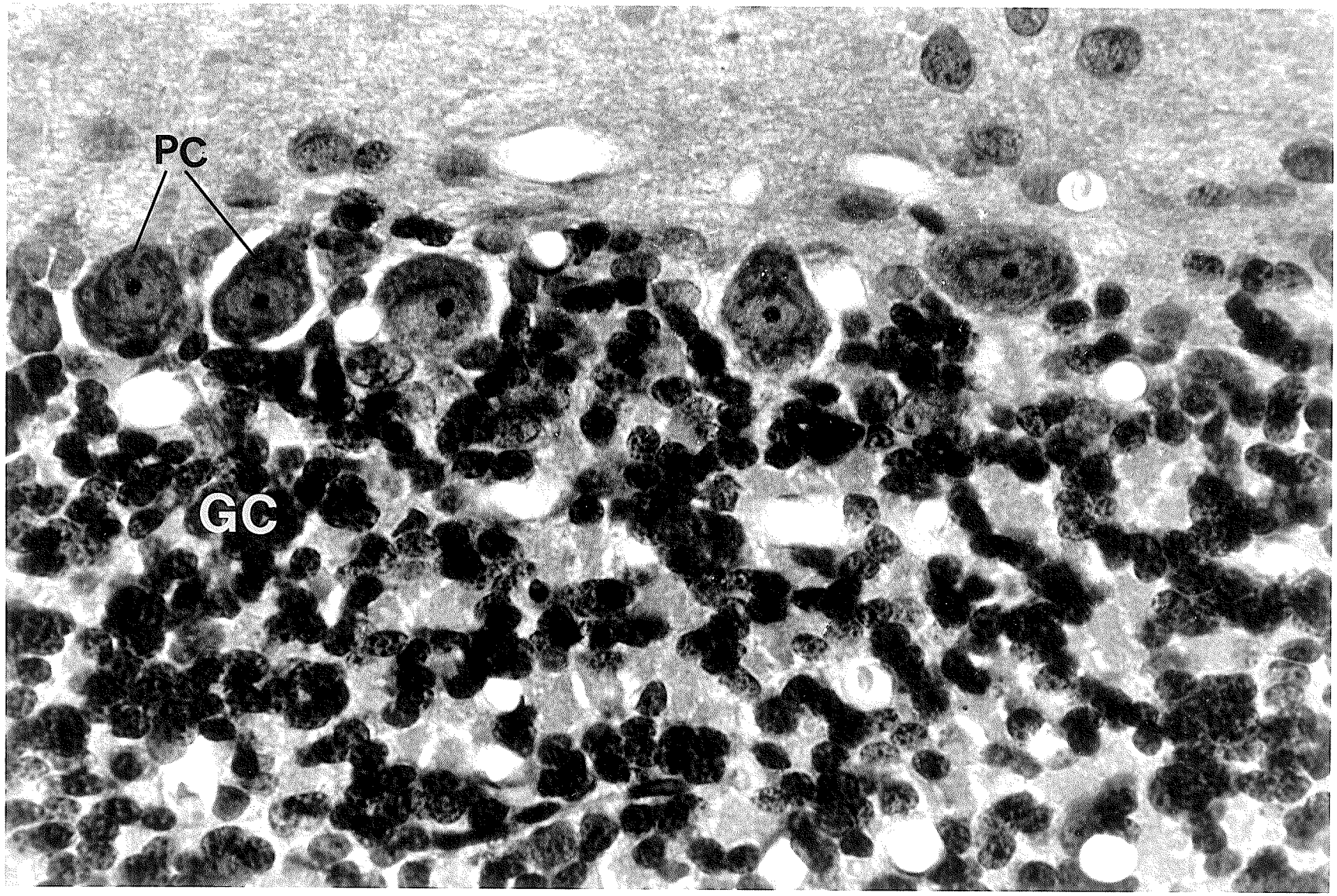
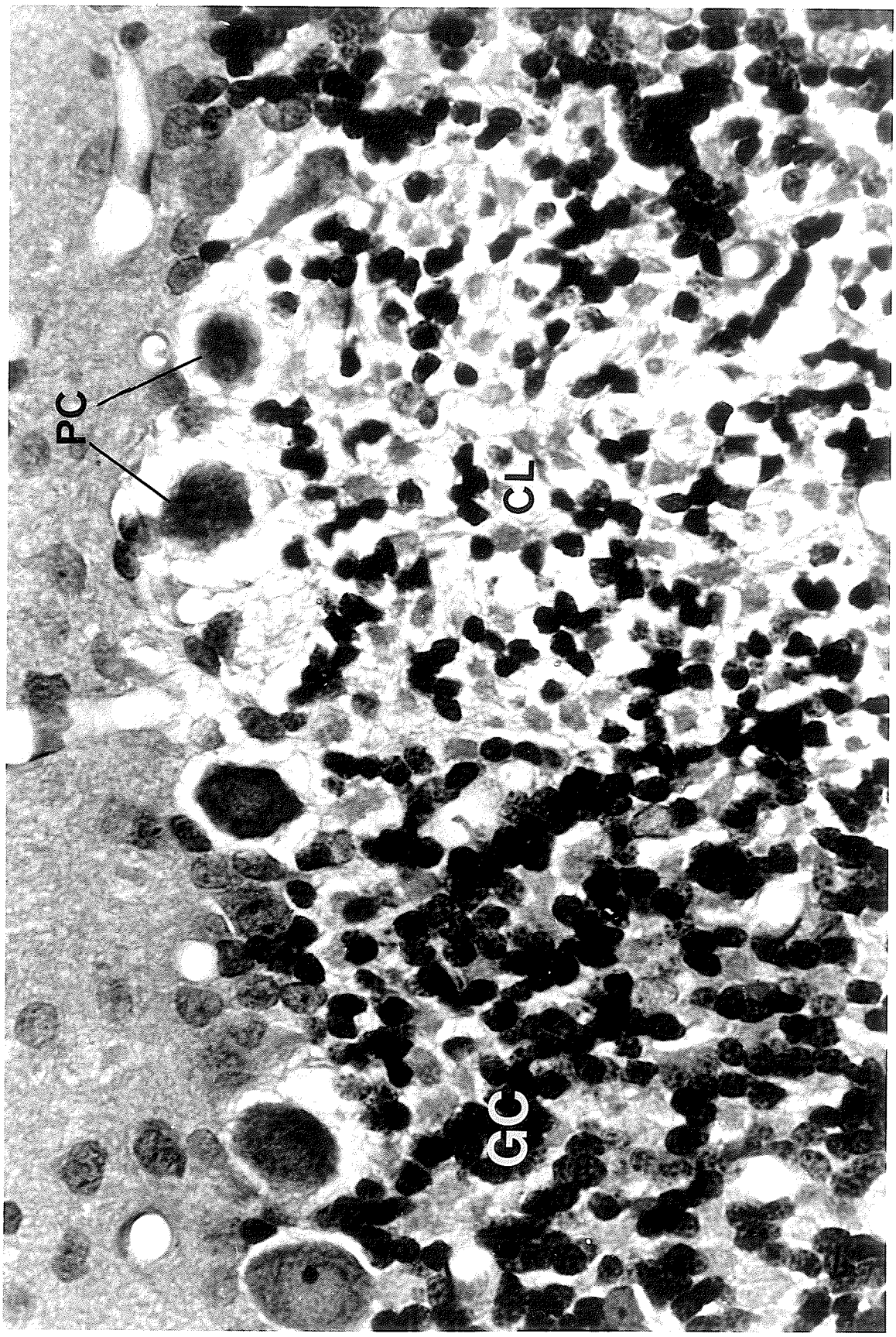

PC
GC

FIGURE 6 Inner granular layer from the cerebellum of a 28 day old rat irradiated with 0.5 Gy on GD-17 illustrating the granule cells in a lesioned area immediately beneath the layer of Purkinje cells. Within these lesioned areas, the granule cells are reduced in number and some appear atrophied. Note the Purkinje cells immediately above the lesion are either absent or pyknotic. Coronal section, thionin stained, 5 μm, 160X. PC, Purkinje cells; GC, granule cells; CL, circumscribed lesion. A higher magnification of Figure 2.



57a

(Figures 4 and 6). These CL distributed within the normal granule cell population varied in size and distribution and in some instances would span across the entire width of the inner granular layer.

4.4 Histological Evaluation of the Inner Granular Layer in Anterior and Posterior Lobes after Irradiation at GD-17, 18, 19, and 20

4.4.1 Incidence of Cerebellar Lesions

These radiation induced lesions were found in all irradiated groups in varying proportions. Some control animals also exhibited these lesions but the proportion was few and compared to the irradiated group statistically not significant.

The incidence of cerebellar lesions within the inner granular layer is summarized in (Tables 1 and 2). The values are compared using Fisher's exact test. In the cerebella of most irradiated rats the occurrence of the lesions were significantly different ($p < 0.05$, Table 1) from controls.

These radiation induced lesions were found in various proportions throughout the irradiated groups. In 21 day old rats, these CL found in 78% on GD-18 and 57% on GD-19 differed significantly ($p < 0.05$) from controls (Table 1). In 28 day old postnatal rats, these CL found in 83% on GD-17, 89% on GD-18, 100% on GD-19 and 80% on GD-20 differed significantly ($p < 0.05$) from controls (Table 1). Irradiation within this four

Table 1. **Incidence of cerebellar lesions within the internal granular layer in 21- and 28 day old control and irradiated rats.**			
Gestational Day of Irradiation	Rat Age Days	Control Rats with Lesions	Number of Irradiated Rats with Lesions
17	21	3/7 (43%)	3/4 (75%)
	28	2/7 (29%)	* 5/6 (83%)
18	21	1/6 (17%)	* 7/9 (78%)
	28	0/7 (0%)	* 8/9 (89%)
19	21	0/7 (0%)	* 4/7 (57%)
	28	4/10 (40%)	* 10/10 (100%)
20	21	4/9 (44%)	3/3 (100%)
	28	3/12 (25%)	* 8/10 (80%)

* $p < 0.05$ Significantly different from controls (Fisher's exact test)

Table 2. Incidence of lesions within the internal granular layer of specific lobes of the cerebellum in 21- and 28 day old irradiated rats.				
		Number of Rats with Lesions in:		
Gestational Day of Irradiation	Rat Age Days	Anterior Lobe	Posterior Lobe	Combined Lesions (Ant. & Post.) Lobes
17	21 (4) 28 (6)	3 (75%) 4 (67%)	0 (0%) 4 (67%)	0 (0%) 3 (50%)
18	21 (9) 28 (9)	7 (78%) 7 (78%)	4 (44%) 7 (78%)	4 (44%) 6 (67%)
19	21 (7) 28 (10)	4 (57%) 9 (90%)	2 (29%) * 9 (90%)	2 (29%) * 8 (80%)
20	21 (3) 28 (10)	2 (67%) 8 (80%)	** 3 (100%) 8 (80%)	2 (67%) 8 (80%)

** $p < 0.05$ Significantly different from GD-17 at 21 days of age. $F = 9.23$ ($df = 1$; $p = 0.0038$)

* $p < 0.05$ Significantly different between 21 days of age for the same day of irradiation (ANOVA). $F = 6.72$ ($df = 1$; $p = 0.0125$)

Number of animals are in parentheses.

day period resulted in a tendency of these lesions to vary on the different days of exposure to irradiation. However, comparing the animals irradiated on GD-17 with those on GD-20, or between 21 and 28 days of age, resulted in no significant difference.

These lesions were distributed in both the anterior and posterior lobes. However, irradiation on GD-17 yielded more rats with lesions within the anterior lobe (75%) than those irradiated on GD-20 (67%). Comparing 21 day old rats irradiated on GD-17 with those on GD-20, there was a trend exhibiting more lesions within the anterior lobe (75%) on GD-17 and (67%) on GD-20. In 28 day old rats, the reverse was true. Irradiation on GD-17 yielded fewer lesions, (67%) within the anterior lobe and more on GD-20, (80%) (Table 2).

In the posterior lobe the only significant difference between treatment and day of irradiation was noted in 21 day old rats. Irradiation on GD-17 yielded no lesions whereas, on GD-20, all rats (100%) exhibited these lesions which differed significantly ($p < 0.05$) from the controls (Table 2).

4.4.2 Total Number of Cerebellar Lesions

The total number of these lesions were counted within the anterior and posterior lobes and the values compared using ANOVA. The results are summarized in (Table 4). It was observed in rats irradiated on GD-17 that the total number of lesions were higher within the anterior lobe

whereas on GD-20 there were more within the posterior lobe. Within the posterior lobe, there was a significant difference ($p < 0.05$) with respect to the total number of lesions and the age of the animals ($p < 0.05$) for each day of irradiation (Table 4).

4.4.3 Comparison between Vermis and Lateral Hemispheres

The incidence and total number of these CL within the vermis and lateral hemispheres were analyzed with ANOVA and the results are summarized in (Tables 3 and 4). Within the anterior lobe, irradiation on both GD-17 and GD-20 yielded more lesions in the vermis in 21 day old rats, whereas in 28 day old rats, more lesions were found in the lateral hemispheres. This difference was not statistically significant. Within the posterior lobe, irradiation on GD-18 and GD-19 produced more lesions in the vermis in 28 day old rats which was significantly different ($p < 0.05$) from lesions in 21 day old rats (Table 3). Irradiation on GD-19 produced more lesions in the lateral hemispheres in 28 day old rats which was significantly different ($p < 0.05$) from lesions in 21 day old rats. Within the posterior lobe, irradiation yielded more CL on GD-20 (100%) which was significantly different ($p < 0.05$) from lesions in GD-17 (0%) (Table 3).

The total number of CL within the anterior and posterior lobes was not statistically significant, and no interaction with day of treatment or age was observed. It was observed in 28 day old rats irradiated on GD-17

Table 3. **Comparison of the incidence of cerebellar lesions in the internal granular layer of the vermis and lateral hemispheres (L.H.) of the anterior and posterior lobes in 21- and 28 day old late prenatally irradiated rats.**

		Anterior Lobe			Posterior Lobe		
Gestational Day of Irradiation	Rat Age Days	Vermis	L.H.	Lesions in both	Vermis	L.H.	Lesions in both
17	21 (4) 28 (6)	2 (50%) 2 (33%)	1 (25%) 4 (67%)	0 (0%) 2 (33%)	0 (0%) 3 (50%)	0 (0%) 2 (33%)	0 (0%) 1 (17%)
18	21 (9) 28 (9)	5 (56%) 5 (56%)	4 (44%) 5 (56%)	2 (22%) 3 (33%)	1 (11%) * 6 (67%)	4 (44%) 7 (78%)	1 (11%) * 6(67%)
19	21 (7) 28 (10)	2 (29%) 6 (60%)	3 (43%) 9 (90%)	1 (14%) 6 (60%)	2 (29%) * 8 (80%)	2 (29%) * 8 (80%)	2 (29%) 7 (70%)
20	21 (3) 28 (10)	2 (67%) 4 (40%)	1 (33%) 7 (70%)	1 (33%) 3 (30%)	1 (33%) 6 (60%)	** 3 (100) 4 (40%)	1(33%) 3 (30%)

** $p < 0.05$ Significantly different between GD-17 at 21 days of age. $F = 4.47$ ($df = 1$; $p = 0.0394$)

* $p < 0.05$ Significantly different between 21 days of age for same day irradiation (ANOVA). $F = 6.72$ ($df = 1$; $p = 0.0125$

Table 4.	**Comparison of the total number of cerebellar lesions in the internal granular layer of the vermis and lateral hemispheres (L.H.) of the anterior and posterior lobes in 21- and 28 day old irradiated rats.**			
Gestational Day of Irradiation	Rat Age Days	Anterior Lobe	Posterior Lobe	Combined Lesions (Ant. & Post) Lobes
17	21 (4) 28 (6)	3 64	0 * 33	3 97
18	21 (9) 28 (9)	24 32	22 * 68	46 100
19	21 (7) 28 (10)	14 131	15 * 144	29 275
20	21 (3) 28 (10)	7 23	6 * 45	13 68

* $p < 0.05$ Significantly different between 21 days for the same day of irradiation (ANOVA).

Number of animals are in parentheses.

that the total number of CL was more in the lateral hemispheres of both anterior and posterior lobes whereas, on GD-20, more were found in the vermis in the posterior lobe and in the lateral hemispheres in the anterior lobe (Table 5).

4.5 Examination of the Inner Granular Layer in Posterior Lobules 7, 8, and 9

4.5.1 Incidence of Cerebellar Lesions

These lesions were counted and analyzed with ANOVA and the results are summarized in (Table 6). In lobules 8 and 9, irradiation on GD-19 yielded more lesions in 28 day old rats which was significantly different ($p < 0.05$) from 21 day old rats. In lobule 8, in 21 day old rats, there were fewer lesions after irradiation on GD-17 (0%) which differed significantly ($p < 0.05$) from GD-20 (100%). No other significant differencs were observed between the lobules.

4.5.2 Total Number of Cerebellar Lesions

The total number of lesions in lobules 7 and 9 was significantly different ($p < 0.05$) between 21 and 28 day old rats for each gestational day of irradiation. In lobule 8, this same difference was almost significant ($p = 0.057$) (Table 8).

Table 5. **Comparison of the total number of cerebellar lesions in the internal granular layer of the vermis and lateral hemispheres (L.H.) of the anterior and posterior lobes in 21- and 28 day old irradiated rats.**

		Anterior Lobe			Posterior Lobe		
Gestational Day of Irradiation	Rat Age Days	Vermis	L.H.	Total	Vermis	L.H.	Total
17	21 (4) 28 (6)	2 17	1 47	3 64	0 14	0 19	0 33
18	21 (9) 21 (9)	13 14	11 18	24 32	2 20	20 48	22 68
19	21 (7) 28 (10)	6 50	8 81	14 131	9 55	6 89	15 144
20	21 (3) 28 (10)	5 6	2 17	7 23	1 32	5 13	6 45

Number of animals are in parentheses (ANOVA).

Table 6. Incidence of cerebellar lesions within the internal granular layer in specified lobules of the posterior cerebellum in 21- and 28 day old irradiated rats.				
Gestational Day of Irradiation	Rat Age Days	7 Folium Tuber and Vermis	8 Pyramis	9 Uvula
17	21 (4) 28 (6)	0 (0%) 2 (33%)	0 (0%) 1 (17%)	0 (0%) 2 (33%)
18	21 (9) 28 (9)	3 (33%) 7 (78%)	2 (22%) 6 (67%)	3 (33%) 5 (56%)
19	21 (7) 28 (10)	2 (29%) 7 (70%)	1 (14%) * 7 (70%)	0 (0%) * 6 (60%)
20	21 (3) 28 (10)	0 (0%) 5 (50%)	** 3 (100%) 4 (40%)	1 (33%) 4 (40%)

** $p < 0.05$ Significantly different from GD-17 at 21 days of age.

* $p < 0.05$ Significantly different between 21 days of age for the same day of irradiation (ANOVA).
$F = 4.27$ ($df = 1$; $p = 0.0440$)

Number of animals are in parentheses.

4.5.3 Comparison between Vermis and Lateral Hemispheres

The incidence and total number of CL within the vermis and lateral hemispheres in lobules 7, 8, and 9 were compared using ANOVA and the results are summarized in (Tables 7, 8 and 9). In lobule 8, there were fewer lesions with irradiation on GD-17 and more on GD-20. This was not statistically significant. No other interaction was observed. With irradiation on GD-18 there were more lesions in the lateral hemispheres and the vermis in the 28 day old rats in lobule 7 ($p < 0.05$) than in 21 day old rats. In lobule 9 the 28 day old rats had more lesions in the vermis with irradiation on GD-19 ($p < 0.05$) than the 21 day old rats. No other interaction was significant. It was observed after irradiation on GD-17 and GD-20 that more CL were found in the lateral hemispheres, in lobules 7 and 8, and in lobule 9, more CL were found in the vermis (Table 7).

Comparing the total number of lesions within the lobules, it was observed in lobules 7 and 8 in 28 day old rats, that irradiation on GD-17 yielded more lesions in the lateral hemispheres whereas, on GD-20, more lesions were found in the vermis (Table 9). In lobule 9, irradiation on both GD-17 and GD-20 yielded more lesions in the vermis. These differences were not statistically significant and no other interactions were noted.

Table 7. Incidence of cerebellar lesions within the internal granular layer of the vermis and lateral hemispheres (L.H.) in specified lobules of the posterior cerebellum in 21- and 28 day old irradiated rats.

		Lobules					
		7 Folium Tuber and Vermis		8 Pyramis		9 Uvula	
Gestational Day of Irradiation	Rat Age Days	Vermis	L.H.	Vermis	L.H.	Vermis	L.H.
17	21 (4) 28 (6)	0 (0%) 1 (17%)	0 (0%) 2 (33%)	0 (0%) 1 (17%)	0 (0%) 1 (17%)	0 (0%) 2 (33%)	0 (0%) 0 (0%)
18	21 (9) 28 (9)	1 (11%) * 6 (67%)	2 (22%) * 6 (67%)	0 (0%) 3 (33%)	2 (22%) 6 (67%)	1 (11%) 1 (11%)	2 (22%) 4 (44%)
19	21 (7) 28 (10)	2 (29%) 7 (70%)	2 (29%) 6 (60%)	1 (14%) 3 (30%)	1 (14%) 5 (50%)	0 (0%) * 6 (60%)	0 (0%) 3 (30%)
20	21 (3) 28 (10)	0 (0%) 2 (20%)	0 (0%) 3 (30%)	1 (33%) 2 (20%)	2 (67%) 2 (20%)	0 (0%) 4 (40%)	1 (33%) 0 (0%)

* $p < 0.05$ Significantly different between 21 days of age for the same day of irradiation (ANOVA). $F = 7.56$ ($df = 1$; $p = 0.0083$)

Number of animals are in parentheses.

Table 8. **Comparison of the total number of lesions within the internal granular layer in specified lobules of the posterior cerebellum in 21- and 28 day old late prenatally irradiated rats.**				
Gestational Day of Irradiation	Rat Age Days	7 Folium Tuber and Vermis	8 Pyramis	9 Uvula
17	21 (4) 28 (6)	0 13	0 16	0 3
18	21 (9) 28 (9)	5 24	7 12	6 5
19	21 (7) 28 (10)	7 49	2 33	0 27
20	21 (3) 28 (10)	0 10	3 12	2 15

Number of animals are in parentheses (ANOVA).

Table 9. Comparison of the total number of lesions within the internal granular layer of the vermis and lateral hemispheres (L.H.) in specified lobules of the posterior cerebellum in 21- and 28 day old irradiated rats.

		7 Folium Tuber and Vermis		8 Pyramis		9 Uvula	
Gestational Day of Irradiation	Rat Age Days	Vermis	L.H.	Vermis	L.H.	Vermis	L.H.
17	21 (4)	0	0	0	0	0	0
	28 (6)	4	9	6	10	3	0
18	21 (9)	1	4	0	7	1	5
	28 (9)	13	11	3	9	1	4
19	21 (7)	3	4	1	1	0	0
	28 (10)	20	29	13	20	12	15
20	21 (3)	0	0	1	2	0	2
	28 (10)	7	3	8	4	15	0

Number of animals are in parentheses (ANOVA).

4.6 Determination of Length along the Laterolateral Axis in Lobule 8

4.6.1 Comparison between GD-17, 18, 19, and 20

The laterolateral length measurements of the pyramis revealed that irradiation throughout this four day period resulted in a significant increase in the length, ($p < 0.001$) compared to the controls (Table 12). There was also a correlation in the length measurements with respect to the age of the animal ($p < 0.001$). The length of the pyramis was greater with irradiation on GD-17 than on GD-20 but this was not statistically significant.

4.6.2 Comparison between Vermis and Lateral Hemispheres

The length measurements revealed that both the lateral hemispheres ($p < 0.05$) and the vermis ($p < 0.001$) in rats irradiated on GD-17 to GD-20 resulted in significant increases from the controls (Table 13). Irradiation on GD-19 in 28 day old rats showed a significant increase ($p < 0.05$) in the length of the lateral hemisphere from 21 day old rats. With irradiation on GD-17, 18, 19, and 20 the length of the vermis differed significantly, ($p < 0.05$) from controls. In the vermis, in 21 day old rats irradiation on GD-17 resulted in a greater increase (17%) ($p < 0.05$) than on GD-20.

Table 10. Distribution of cerebellar lesions within the internal granular layer of the vermis and lateral hemispheres (L.H.) of the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old irradiated rats.

			Number of Rats with Lesions in:	
Gestational Day of Irradiation	Rat Age Days	Total Rats with Lesions	L.H.	Vermis
17	21 (3) 28 (4)	0 (0%) 1 (25%)	0 (0%) 1 (25%)	0 (0%) 1 (25%)
18	21 (6) 28 (7)	1 (17%) 4 (57%)	1 (17%) 4 (57%)	0 (0%) 2 (29%)
19	21 (6) 28 (8)	1 (17%) 6 (75%)	1 (17%) 4 (50%)	1 (17%) 3 (38%)
20	21 (1) 28 (9)	1 (100%) 4 (44%)	0 (0%) 2 (22%)	1 (100%) 2 (22%)

Number of animals are in parentheses (ANOVA).

Table 11. **Comparison of the total number of cerebellar lesions in the internal granular layer of the vermis and lateral hemispheres (L.H.) of the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old irradiated rats.**				
Gestational Day of Irradiation	Rat Age Days	Total Number of Lesions	L.H.	Vermis
17	21 (3) 28 (4)	0 16	0 10	0 6
18	21 (6) 28 (7)	1 8	1 6	0 2
19	21 (6) 28 (8)	2 28	1 15	1 13
20	21 (1) 28 (9)	1 11	0 4	1 7

Number of animals are in parentheses (ANOVA).

Table 12. Total laterolateral length measurements (mm) of the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.			
Gestational Day of Irradiation	Rat Age Days	Control	Irradiated (0.5 Gy)
17	21	17.65 (6)	* 19.58 (3)
	28	19.07 (6)	* 20.39 (4)
18	21	18.53 (5)	* 19.47 (6)
	28	18.26 (6)	* 21.01 (7)
19	21	17.74 (6)	* 18.83 (6)
	28	19.05 (7)	* 20.20 (8)
20	21	17.98 (8)	* 18.46 (1)
	28	19.91 (11)	* 20.60 (9)

* $p < 0.001$ Significantly different from controls and for the same day of irradiation (ANOVA). $F = 14.39$ ($df = 1$; $p = 0.0003$).
* $p < 0.001$ Significantly different between the ages for the same day of irradiation (ANOVA). $F = 14.10$ ($df = 1$; $p = 0.003$).

Number of animals are in parenthesis.
Values are means. SEM for Control: 21 days = 0.30; 28 days = 0.27; Irradiated: 21 days = 0.47; 28 days = 0.29.

Table 13. Total length measurements (mm) of the vermis and both lateral hemispheres (L.H.) of the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.

		Control		Irradiated (0.5 Gy)	
Gestational Day of Irradiation	Rat Age Days	L.H.	Vermis	L.H.	Vermis
17	21	12.28 (6)	5.37 (6)	* 13.30 (3)	** 6.28 (3)
	28	13.47 (6)	5.61 (6)	13.90 (4)	** 6.49 (4)
18	21	13.49 (5)	5.04 (5)	13.78 (6)	** 5.70 (6)
	28	12.94 (6)	5.32 (6)	* 14.95 (7)	** 6.06 (7)
19	21	12.62 (6)	5.11 (6)	* 13.11 (6)	** 5.72 (6)
	28	13.51 (7)	5.54 (7)	* 14.11 (8)	** 6.09 (8)
20	21	12.66 (8)	5.32 (8)	12.44 (1)	** 6.02 (1)
	28	13.91 (11)	6.00 (11)	* 14.18 (9)	** 6.42 (9)

** $p < 0.001$ Significantly different from controls. $F = 33.96$ ($df = 1$; $p = 0.0001$).
** $p < 0.05$ Significantly different between the ages for the same day of irradiation. $F = 10.02$ ($df = 1$; $p = 0.0022$).
* $p < 0.05$ Significantly different from controls (ANOVA). $F = 4.33$ ($df = 1$; $p = 0.04$).
Numbers of animals are in parentheses.
Values are means. SEM for LH Control: 21 days = 0.25 and 28 days = 0.24; LH Irradiated: 21 days = 0.41 and 28 days = 0.25. SEM Vermis Control: 21 days = 0.10 and 28 days = 0.09; Vermis Irradiated: 21 days = 0.16 and 28 days = 0.10.

4.7 Determination of Cell Number along the Laterolateral Axis in Lobule 8

4.7.1 Purkinje Cell Analysis

4.7.1.1 Purkinje Cell Number

The mean number of Purkinje cells were counted in the laterolateral axis in the pyramis and analyzed with ANOVA. The results are summarized in Table 14. The number of Purkinje cells were overall significantly reduced ($p < 0.001$) from controls in all irradiated groups. The number of Purkinje cells was relatively constant with irradiation on GD-17 and GD-20. No other interaction was significant. The loss of Purkinje cells in both the lateral hemispheres ($p < 0.001$) and in the vermis ($p < 0.05$) differed significantly from controls (Table 15). It was observed in all gestational periods following irradiation in both 21 and 28 day old rats that there was a greater reduction in Purkinje cells in the vermis than the lateral hemispheres. The number of Purkinje cells in both the lateral hemispheres and vermis remained relatively constant with irradiation on GD-17 and GD-20 (Table 15).

4.7.1.2 Linear Density

The linear density of PCs of the pyramis was calculated and analyzed with ANOVA. The results are summarized in Tables 16 and 17.

Table 14. Total laterolateral distribution of the Purkinje cell counts in the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.			
Gestational Day of Irradiation	Rat Age Days	Control	Irradiated (0.5 Gy)
17	21	81 (6)	* 56 (3)
	28	89 (6)	* 67 (4)
18	21	93 (5)	* 74 (6)
	28	68 (6)	71 (7)
19	21	80 (6)	* 65 (6)
	28	75 (7)	76 (8)
20	21	76 (8)	* 57 (1)
	28	95 (11)	* 66 (9)

* $p < 0.001$ Significantly different from controls (ANOVA). $F = 14.37$ ($df = 1$; $p = 0.0003$).
Number of animals are in parentheses.
Values are means. SEM for Control for 21 and 28 days is 0.02; Irradiated = 21 days = 0.03 and 28 days 0.02.

Table 15. Distribution of the Purkinje cell counts in the vermis and both lateral hemispheres (L.H.) of the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.

		Control		Irradiated (0.5 Gy)	
Gestational Day of Irradiation	Rat Age Days	L.H.	Vermis	L.H.	Vermis
17	21 28	53 (6) 61 (6)	28 (6) 28 (6)	**40 (3) **45 (4)	* 16 (3) * 22 (4)
18	21 28	63 (5) 49 (6)	30 (5) 19 (6)	**50 (6) 50 (7)	* 23 (6) 21 (7)
19	21 28	54 (6) 55 (7)	26 (6) 20 (7)	**49 (6) 56 (8)	* 17 (6) 20 (8)
20	21 28	56 (8) 67 (11)	21 (8) 28 (11)	**42 (1) **46 (9)	* 15 (1) * 20 (9)

** $p < 0.001$ Significantly different from controls. $F = 11.86$ ($df = 1$; $p = 0.0009$).
* $p < 0.05$ Significantly different from controls (ANOVA). $F = 8.80$ ($df = 1$; $p = 0.0039$).
Number of animals are in parenthesis.
Values are means. SEM for LH Control at 21 and 28 days = 0.02. LH Irradiated at 21 days = 0.03; 28 days = 0.02. Vermis Control at 21 and 28 days = 0.03; Vermis Irradiated at 21 days = 0.05; at 28 days = 0.03.

Table 16. Linear density of PC (number of PCs/mm) in the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.			
Gestational Day of Irradiation	Rat Age Days	Control	Irradiated (0.5 Gy)
17	21 28	4.6 (6) 4.7 (6)	* 2.9 (3) * 3.3 (4)
18	21 28	5.0 (5) 3.7 (6)	* 3.8 (6) 3.4 (7)
19	21 28	4.5 (6) 3.9 (7)	* 3.5 (6) 3.8 (8)
20	21 28	4.2 (8) 4.8 (11)	* 3.1 (1) * 3.2 (9)

* $p < 0.001$ Significantly different from controls (ANOVA). $F = 30.09$ ($df = 1$; $p = 0.0001$).
Number of animals are in parentheses.
Values are means. SEM for Control at 21 days = 0.17; at 28 days = 0.15; Irradiated at 21 days = 0.26; at 28 days = 0.16.

Table 17. Linear density of PC (number of PCs/mm) in the vermis and both lateral hemispheres (L.H.) of the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.

		Control		Irradiated (0.5 Gy)	
Gestational Day of Irradiation	Rat Age Days	L.H.	Vermis	L.H.	Vermis
17	21 28	4.3 (6) 4.5 (6)	5.2 (6) 5.0 (6)	* 3.0 (3) 3.2 (4)	* 2.5 (3) * 3.4 (4)
18	21 28	4.7 (5) 3.8 (6)	6.0 (5) 3.6 (6)	* 3.6 (6) 3.3 (7)	* 4.0 (6) 3.5 (7)
19	21 28	4.3 (6) 4.1 (7)	5.1 (6) 3.6 (7)	* 3.7 (6) 4.0 (8)	* 3.0 (6) 3.3 (8)
20	21 28	4.4 (8) 4.8 (11)	3.9 (8) 4.7 (11)	* 3.4 (1) * 3.2 (9)	* 2.5 (1) * 3.1 (9)

* $p < 0.001$ Significantly different from controls (ANOVA). LH: $F = 22.72$ ($df = 1$; $p = 0.0001$); Vermis: $F = 17.35$ ($df = 1$; $p = 0.0001$).
Number of animals are in parentheses.
Values are means. SEM for LH Control at 21 days = 0.17; at 28 days = 0.15; LH Irradiated at 21 days = 0.26; at 28 days = 0.16. Vermis Control at 21 days = 0.29; at 28 days = 0.27; Vermis Irradiated at 21 days = 0.46; at 28 days = 0.28.

In all irradiated groups, the linear density was significantly reduced ($p < 0.05$) from controls. In 28 day old rats irradiated on GD-17 and GD-19, the linear density did not show a significant reduction from controls. No other significant interaction was noted. In most irradiated groups, there was a significant decrease in the density of Purkinje cells in both the lateral hemispheres and vermis ($p < 0.001$) from controls (Table 17).

4.7.2 Granule Cell Analysis

4.7.2.1 Granule Cell Number

The laterolateral distribution of granule cells was calculated and analyzed with ANOVA. The results are summarized in Tables 18 and 19. The granule cell number was significantly reduced ($p < 0.001$) from controls in all irradiated groups (Table 18). There was also a significant interaction ($p < 0.05$) between day of treatment and between day of treatment and age of animals.

In both 21 and 28 day old rats, irradiation on GD-20 yielded more reduced granule cell number than on GD-17 ($p < 0.05$). Both the lateral hemispheres and vermis showed a significant reduction in the number of granule cells ($p < 0.001$) from controls in all irradiated groups (Table 19). For the lateral hemisphere, there was a relationship between the day of treatment and with the age of the animal ($p < 0.05$). With respect to

Table 18. Total laterolateral distribution of granule cells in the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.			
Gestational Day of Irradiation	Rat Age Days	Control	Irradiated (0.5 Gy)
17	21 28	27,358 (6) 30,893 (6)	* 23,496 (3) * 26,915 (4)
18	21 28	28,722 (5) 32,685 (6)	* 23,948 (6) * 24,792 (7)
19	21 28	30,158 (6) 30,861 (7)	* 20,901 (6) * 22,826 (8)
20	21 28	29,128 (8) 31,856 (11)	* 21,044 (1) * 21,630 (9)

* $p < 0.001$ Significantly different from controls. $F = 309.65$ ($df = 1$; $p = 0.0001$).
* $p < 0.05$ Significantly different for interaction between day of treatment and also between treatment, day and age (ANOVA). $F = 3.00$ ($df = 3$; $p = 0.035$).
Number of animals are in parentheses.
Values are means. SEM Control and Irradiated at 21 and 28 days = 0.01.

Table 19. Distribution of the granule cells in the vermis and both lateral hemispheres (L.H.) of the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.

		Control		Irradiated (0.5 Gy)	
Gestational Day of Irradiation	Rat Age Days	L.H.	Vermis	L.H.	Vermis
17	21 28	12,771 (6) 14,817 (6)	2,739 (6) 2,917 (6)	* 11,305 (3) * 12,927 (4)	* 2,198 (3) * 2,531 (4)
18	21 28	13,760 (5) 15,787 (6)	2,671 (5) 3,086 (6)	* 11,575 (6) * 12,259 (7)	* 2,223 (6) * 2,182 (7)
19	21 28	14,513 (6) 14,726 (7)	2,811 (6) 2,881 (7)	* 10,095 (6) * 11,147 (8)	* 1,945 (6) * 2,071 (8)
20	21 28	13,926 (8) 15,301 (11)	2,766 (8) 3,060 (11)	* 9,952 (1) * 10,635 (9)	* 2,047 (1) * 1,926 (9)

* $p < 0.001$ Significantly different from controls. LH: $F = 216.09$ ($df = 1$; $p = 0.0001$); Vermis: $F = 273.17$ ($df = 1$; $p = 0.0001$).
* $p < 0.05$ Significantly different between treatment/day and treatment/day/age (ANOVA). LH: $F = 2.92$ ($df = 3$; $p = 0.039$); Vermis: $F = 3.36$ ($df = 3$; $p = 0.023$).
Number of animals are is parentheses.
Values are means. SEM for LH and Vermis Control and Irradiated at 21 and 28 days = 0.01.

the vermis, between the different days of irradiation, as well as the age of the animals, there was a significant ($p < 0.05$) reduction in the number of granule cells.

4.7.2.2 Linear Density

The linear density of the granule cells of the pyramis was calculated and analyzed with ANOVA. The results are summarized in Tables 20 and 21. The linear density of granule cells was significantly reduced ($p < 0.001$) from controls in all irradiated groups (Table 20). There was also a correlation between the day of irradiation and age of animals in all groups ($p < 0.05$). There was a tendency for the linear density of granule cells to decrease with irradiation on GD-19 and GD-20.

Both the lateral hemispheres and vermis showed a significant reduction ($p < 0.001$) in the linear density in all irradiated groups from the controls (Table 21). In the lateral hemisphere, there was an interaction with day of irradiation and with the age of the animal ($p < 0.05$). Irradiation on GD-20 yielded a reduced density of granule cells in both the lateral hemispheres ($p < 0.05$) and vermis ($p = 0.057$). It was observed with various days of irradiation that the density of granule cells varied but overall there was a reduction.

Table 20. **Linear density of GC (number of GCs/mm) in the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.**			
Gestational Day of Irradiation	Rat Age Days	Control	Irradiated (0.5 Gy)
17	21 28	1550 (6) 1620 (6)	* 1200 (3) * 1320 (4)
18	21 28	1550 (5) 1790 (6)	* 1230 (6) * 1180 (7)
19	21 28	1700 (6) 1620 (7)	* 1110 (6) * 1130 (8)
20	21 28	1620 (8) 1600 (11)	* 1140 (1) * 1050 (9)

* $p < 0.001$ Significantly different from controls. $F = 175.68$ ($df = 1$; $p = 0.0001$).
* $p < 0.05$ Significantly different for interaction between treatment, day and age (ANOVA). $F = 3.14$ ($df = 3$; $p = 0.0297$).

Number of animals are in parentheses.

SEM for Control at 21 days = 0.19; at 28 days = 0.18; Irradiated at 21 days = 0.31; at 28 days = 0.19.

Table 21. Linear density of GC (number of GCs/mm) in the vermis and both lateral hemispheres (L.H.) of the pyramis (lobe 8) of the posterior cerebellum in 21 and 28 day old control and irradiated rats.

		Control		Irradiated (0.5 Gy)	
Gestational Day of Irradiation	Rat Age Days	L.H.	Vermis	L.H.	Vermis
17	21 28	1040 (6) 1100 (6)	510 (6) 520 (6)	* 850 (3) * 930 (4)	* 350 (3) * 390 (4)
18	21 28	1020 (5) 1220 (6)	530 (5) 580 (6)	* 840 (6) * 820 (7)	* 390 (6) * 360 (7)
19	21 28	1150 (6) 1090 (7)	550 (6) 520 (7)	* 770 (6) * 790 (8)	* 340 (6) * 340 (8)
20	21 28	1100 (8) 1100 (11)	520 (8) 510 (11)	* 800 (1) * 750 (9)	* 340 (1) * 300 (9)

* $p < 0.001$ Significantly different from controls. LH: $F = 104.66$ ($df = 1$; $p = 0.0001$; Vermis: $F = 205.56$ ($df = 1$; $p = 0.0001$).
* $p < 0.05$ Significantly different between treatment, day of irradiation and age (ANOVA). LH: $F = 3.22$ ($df = 3$; $p = 0.0270$); Vermis: $F = 4.30$ ($df = 3$; $p = 0.0072$).

Number of animals are in parentheses.
SEM for LH Control at 21 days = 0.21; at 28 days = 0.20; LH Irradiated at 21 days = 0.34; at 28 days = 0.21. Vermis Control at 21 days = 0.24; at 28 days = 0.23. Vermis Irradiated at 21 days = 0.39; at 28 days = 0.24.

4.7.3 Granule-Purkinje Cell Distribution

4.7.3.1 Comparison between GD-17 18, 19, and 20

The calculated GC/PC ratio of the pyramis is summarized in Table 22. Irradiation on GD-20 yielded a smaller ratio than on GD-17 ($p < 0.05$). Irradiation on GD-17 and GD-18 in 21 day old rats yielded larger GC/PC ratios than controls, whereas on GD-19 and GD-20, the ratios were reduced. In 28 day old rats, GD-17 yielded a higher GC/PC ratio than controls. On GD-18, GD-19 and GD-20 the GC/PC ratio was reduced. These differences were statistically not significant. The GC/PC ratio between the animals irradiated on GD-17, 18, 19 and 20 and their corresponding controls remained relatively constant.

Table 22. The laterolateral GC/PC ratio of the pyramis (lobe 8) of the posterior cerebellum in 21- and 28 day old control and irradiated rats.

		Control			Irradiated (0.5 Gy)		
Gestational Day of Irradiation	Rat Age Days	PC	GC	GC/PC*	PC	GC	GC/PC*
17	21	4.6	1550	337 (6)	2.9	1200	414 (3)
	28	4.7	1620	345 (6)	3.3	1320	400 (4)
18	21	5.0	1550	310 (5)	3.8	1230	324 (6)
	28	3.7	1790	484 (6)	3.4	1180	347 (7)
19	21	4.5	1700	378 (6)	3.5	1110	317 (6)
	28	3.9	1620	415 (7)	3.8	1130	297 (8)
20	21	4.2	1620	386 (8)	3.1	1140	368 (1)
	28	4.8	1600	333 (11)	3.2	1050	328 (9)

Number of animals are in parentheses. * Calculated on an individual basis for each animal. The mean value for the group is given (ANOVA).

5.0 DISCUSSION

5.1 Effect of Irradiation on the Inner Granular Layer

In this study, maternal exposure to a single dose of whole body irradiation (0.5 Gy) on gestational days (GD) 17, 18, 19 and 20 did not result in any gross cerebellar anomalies. The cerebella of all irradiated animals had attained a normal trilaminar architecture consisting of an outer molecular layer, a middle monolayer of Purkinje cells, and an inner granular layer. However, the most pronounced radiation induced change in the cerebellum was observed in the inner granular layer. Regardless of the gestational day of irradiation, all irradiated animals exhibited well defined clusters of cerebellar lesions (CL) distributed throughout the inner granular layer in both the anterior and posterior lobes. These lesions were characterized by a reduced number of granule cells and atrophy of some of the remaining granule cells. The Purkinje cells immediately above the lesion were either missing, atrophied or normal in appearance.

Irradiation on GD-17 resulted in more CL in the anterior lobe and vermis whereas irradiation on GD-20, resulted in more CL in the posterior lobe and in the lateral hemispheres. This coincided with a greater decrease in both Purkinje cells and granule cells in the vermis with irradiation on GD-17, whereas, with irradiation on GD-20, both of these cells were reduced in the lateral hemispheres. It is known that Purkinje cells have a role in the

induction of the development and maturation of granule cells. The number of Purkinje cells and the time these cells spend in the presence of granule cells during their critical period of development determine the number of granule cells. Fewer Purkinje cells and inadequate interaction between Purkinje cells and granule cells result in granule cell death (Wetts & Herrup 1983). The difference in the location of these CL suggests developmental differences. Since the anterior lobe and vermis arises embryologically first, (Larsell 1952), the Purkinje cells and granule cells in these areas would be more sensitive to the effects of irradiation with exposure on GD-17 than on GD-20. This would result in fewer Purkinje cells and ultimately fewer granule cells. The reduction in the number of granule cells would be evident by the presence of more CL within the inner granular layer in the anterior lobe and vermis. The posterior lobe and the lateral hemispheres, which are the lateral extensions of the vermian segments, arise later, and the number of both Purkinje cells and granule cells would be more reduced with irradiation on GD-20 than on GD-17. This would result in more CL in the posterior lobe and within the lateral hemispheres with irradiation on GD-20. The medial to lateral and anterior to posterior gradient in the deficits of both granule cells and Purkinje cells observed in this radiation study has been also reported in mutant mice (Chen & Hillman 1989).

The total length of both lateral hemispheres and the vermis of the pyramis was significantly increased from controls. The increase in length could suggest slight swelling as a result of irradiation.

In a study by Bruni et. al. (1993) rats irradiated with 0.5 Gy on GD-15 and GD-18 also exhibited similar lesions as in the present study. It was reported that these lesions were more profound with irradiation on GD-18 than on GD-15. However, the specific distribution of these lesions was not investigated.

5.2 Effect of Irradiation on the Purkinje Cell Layer

In the cerebella of irradiated rats that exhibited these lesions, the Purkinje cells immediately above the lesion were either missing, atrophied or normal in appearance. Since Purkinje cells have an inductive influence on the development and maturation of granule cells, any alteration in the number of Purkinje cells would result in a reduction in the number of granule cells. The number of Purkinje cells were significantly reduced in all irradiated rats compared to controls. However, the number of Purkinje cells was relatively the same with irradiation on GD-17 and on GD-20, yet more granule cells were lost in the vermis than in the lateral hemispheres, with animals irradiated on GD-20. One possible explanation could be there are more than one class of Purkinje cells that are being affected by irradiation. It has been demonstrated in the mutant lurcher mouse, that the cerebellar cortex contains

at least two classes of Purkinje cells (Tano et. al. 1992). These classes of Purkinje cells are organized into alternating arrays of parasagittal bands. The classification of Purkinje cells into parasagittal bands is evident in the vermis and less in the lateral hemispheres (Hawkes & Gravel 1991). The compartmentation of the cerebellum was demonstrated using the molecular marker zebrin, which is expressed selectively by Purkinje cells (Tano et. al. 1992, Hawkes & Gravel 1991). It has been reported both in the vermis and lateral hemisphere, that zones of Purkinje cells which are zebrin positive, alternate with zones that are zebrin negative (Hawkes & Gravel 1991). In the lurcher mouse zebrin is expressed by Purkinje cells in the pyramis, uvula and flocculonodular (Tano et. al. 1992). Irradiation on GD-20 could have affected one class of Purkinje cells in the vermis that were being expressed in one zone, rendering them more radiosensitive, while the other subset of Purkinje cells were not expressed in that zone, and therefore more radioresistant to irradiation. Fewer or malformed Purkinje cells would subsequently alter the induction of granule cell development resulting in fewer granule cells. The expression of Purkinje cells in parasagittal bands could explain why the presence of the CL within the inner granular layer of the vermis is arranged in alternating clusters.

5.3 Effect of Irradiation on the Number and Interaction of Granule Cells and Purkinje Cells

The numbers of granule and Purkinje cells were determined for rats irradiated on each of GD-17, 18, 19 and 20 and for corresponding controls. The results from this study indicate that both granule cells and Purkinje cells in the pyramis were significantly reduced in rats irradiated on each of GD-17, 18, 19 and 20. Irradiation on GD-20 yielded a greater deficit in granule cells than on GD-17. Compared to controls Purkinje cells were significantly reduced in number with irradiation on both GD-17 and GD-20. However, the decrease did not correspond to the degree of reduction in the number of granule cells compared to controls. The number of Purkinje cells remained relatively constant despite the significant reduction in granule cell number.

These results indicate that there is a direct relationship between the number of Purkinje cells and the number of granule cells. The present results are in agreement with previous studies (Hillman & Chen 1981, Wetts & Herrup 1982, 1983, Chen & Hillman 1989) which have shown that there is a direct correlation between the number of granule cells and the number of Purkinje cells. It is known that the survival of granule cells is dependent upon two factors: the number of granule cells and the length of time the Purkinje cells are present during the critical period of granule cell development (Wetts & Herrup 1983). If granule cells do not establish contact with their major

postsynaptic target, the Purkinje cells, they die (Landis & Sidman 1978, Sotelo & Changeux 1974).

Both the Purkinje cells and the granule cells were significantly reduced in rats irradiated on each of GD-17, 18, 19 and 20 from controls. However, the number of Purkinje cells was not reduced proportionally to the number of granule cells. Rather, the number of granule cells was reduced proportionally to the number of Purkinje cells. This parallel reduction of both cells kept the cell ratios between the granule cells and Purkinje cells relatively constant in rats irradiated on each of GD-17, 18, 19 and 20. This implies that the relationship between Purkinje cells and granule cells is a linear pattern. Chen & Hillman (1989) have shown that there is a linear relationship between the reduction in the number of Purkinje cells and the number of granule cells. As a result of the parallel reduction of both granule cells and Purkinje cells, they found the ratio between these two cells did not significantly change. Since the ratio was relatively the same, this suggests that there is a matching of ratios between the number of granule cells and Purkinje cells (Chen & Hillman 1989).

The present results are in agreement with other studies which further support this direct relationship that exists between the Purkinje cells and the granule cells. Altman & Anderson (1972) have shown that with prolonged X-irradiation started at birth, the proliferating granule cells are killed with no

change in the number of Purkinje cells. Using a malnutrition model, Hillman & Chen (1981) have shown that the granule cell number was reduced while the Purkinje cell number remained constant. In another study, Chen & Hillman (1989) used a Purkinje cell reduction model and a granule cell reduction model to determine the ratios between these two cells. They injected a single dose of MAM (methylazoxymethanol acetate) into pregnant rats on GD-14 and on the first postnatal day. MAM is an antimitotic agent which prevents the replication of chromosomes and ultimately kills cells while it is being detoxified (Nagata & Matsumoto 1969). Injection of MAM on GD-14 reduced the number of Purkinje cells by 35 - 40% (Chen & Hillman 1989). The number of granule cells was proportionally reduced. However, MAM administered on the first postnatal day resulted in an agranular cerebella. Regardless of the granule cell deficit, the reduced number of granule cells did not alter the number of Purkinje cells. The results of the Purkinje and the granule cell reduction model generated different effects on the number of each companion neuron. Chen & Hillman (1989) have shown that the target neurons, the Purkinje cells, determine the maximum number of afferent neurons, the granule cells.

This suggests that there is a unidirectional control over the number of granule cells supported by Purkinje cells and not a bidirectional control over cell number between granule cells and Purkinje cells (Chen & Hillman 1989).

There is evidence that suggests several systems have unidirectional regulation of target to afferent neurons over the number of neurons which are formed or the number which survive overproduction (Altman et. al. 1969, Das 1977). Chen & Hillman (1989) have shown in the rat that the target neurons, the Purkinje cells can support a maximum number of afferent neurons, the granule cells. Using a Purkinje cell reduction model, they have found that one Purkinje cell can support 480 granule cells in the normal adult rat, (Chen & Hillman 1989).

Further support for the relationship between the absence of Purkinje cells resulting in a reduction in granule cell number comes from studies using SV40 transgenic mice and mutant mouse strains. Between embryonic day 14 and postnatal day 6, the SV40 line of transgenic mice exhibit transgene expression resulting in the selective destruction of immature Purkinje cells. This destruction of Purkinje cells alters the postnatal development of granule cells (Feddersen et. al. 1992). Since Purkinje cells are the first neurons arising within the embryonic cerebellum, the immature Purkinje cells have been implicated to have an important role in late embryonic and early postnatal cerebellar development (Miale & Sidman 1961). It has been suggested that the role of Purkinje cells in granule cell neurogenesis is to provide developmental signals or exogenous factors for granule cell differentiation (Gao et. al. 1991). If there is no interaction between the

Purkinje cells and granule cells, the development of granule cells within the external germinal layer is altered. Without the signals or exogenous factors from Purkinje cells, the proliferation and differentiation of granule cells is decreased, followed by subsequent death of the postmigratory granule neurons (Feddersen et. al. 1992).

Additional support for this conclusion comes from prepared homotypic granule cell cultures from postnatal mouse cerebella, PN-5 to PN-10 (Gao et. al. 1991). It was observed with an increased duration of the culture, that there was an increasing number of dying granule cells. From these results it was suggested that the survival of granule cells requires both autocrine mechanisms to stimulate proliferation and developmental signals supplied by the interaction with Purkinje cells for granule cell differentiation (Gao et. al 1991, Feddersen 1992).

Further evidence for the role of Purkinje cells in the development and maintenance of granule cells comes from mutant mouse strains. Mutant mouse strains are characterized by degeneration of a specific population of neurons followed by subsequent degeneration of subsets of mature neurons (Sidman 1983). This same characteristic is also evident in genetic neurological disorders in humans such as hereditary ataxias (Landis et. al. 1974) and Huntington's disease (Bird 1980). The genetic lurcher mutant mice are born with a normal complement of Purkinje cells. Between the second and fifth

weeks postnatally, the lurcherers lose virtually all of their Purkinje cells (Caddy & Biscoe 1979). As a consequence of the degeneration of Purkinje cells, the number of granule cells is reduced by 90% and only a small number do survive (Caddy & Biscoe 1974). Compared to the lurcher mouse, the Purkinje cells in the staggerer mutant mouse are reduced in number, but not all are lost. The remaining Purkinje cells are malformed. These malformed Purkinje cells never establish synaptic contact with the granule cells and the staggerer loses all of its granule cells (Hirano & Dembitzer 1975, Herrup & Mullen 1979a, 1979b). Studies using mutant mouse strains provide further support that the survival of granule cells is dependent upon the number of Purkinje cells and also the length of time the Purkinje cells are present during the critical period of granule cell development. The present radiation studies are in agreement with the conclusion that granule cells during their critical period of development are dependent upon the presence and interaction of Purkinje cells.

5.4. Mechanisms Regulating Neuronal Cell Numbers

Rats irradiated on each of GD-17, 18, 19 and 20 exhibited a significant proportion of circumscribed cerebellar lesions in the inner granular layer. These rats also exhibited a significant reduction in the number of both Purkinje cells and granule cells. The GC/PC ratio in irradiated rats was relatively similar to controls since the number of granule cells was

proportionally reduced to the number of Purkinje cells. These lesions were characterized by a reduction in granule cell number and atrophy of some of the remaining granule cells. The Purkinje cells immediately above the lesion were either missing or atrophied.

Purkinje cells originate prenatally between embryonic days 13 to 16 and mature postnatally (Das 1972, Altman & Bayer 1978). The granule cells arise postnatally from day 4 through day 21 with the bulk being formed during the second week (Altman & Das 1965). The question arises as to why these radiation induced lesions appear in the inner granule layer when the animals were irradiated at a time during which the granule cells are not being formed? Of interest, these same circumscribed lesions were found in some control animals but the proportion was few and compared to the irradiated group statistically not significant.

One explanation could lie in the fact that irradiation does not affect the population of granule cells directly, but rather indirectly. The reduction in the number of granule cells and atrophy of the remaining granule cells could be an indirect consequence of reduced critical interaction with Purkinje cells. The question arises what possible mechanisms are regulating the ratio of neuronal numbers between target cells, the Purkinje cells, and their afferents, the granule cells?

The results from this study can be interpreted by three possible mechanisms. Firstly, irradiation could alter the genetic expression of Purkinje cells which would ultimately alter their inductive control over granule cells resulting in secondary death of granule cells. Genetic control is a major factor in the development and maintenance of mature phenotypes of neurons. Studies of genetic mutant mouse strains and exposure to prolonged X-irradiation have shown that the number and maintenance of Purkinje cells are genetically controlled (Altman & Anderson 1972, Herrup & Mullen 1979b, Rakic & Sidman 1973b, Sotelo 1975, Herrup & Sunter 1986). In the lurcherer and staggerer mutant mice, the genetic induced cell death of Purkinje cells occurs during late development and early adulthood. The genetic induced cell death of Purkinje cells results in an indirect cell death of granule cells. The loss of granule cells matches the number of Purkinje cells lost so that a constant ratio between these two cells is maintained (Caddy & Biscoe 1979, Herrup & Mullen 1979b, Wetts & Herrup 1982, Herrup & Sunter 1986). Although lurcher mice are born with a normal number of Purkinje cells, these cells begin to degenerate during the beginning of synaptogenesis. The death of granule cells follows the degeneration of Purkinje cells. In the staggerer, the Purkinje cells are genetically altered and the death of granule cells correlates with the inability of the granule cells to

establish synaptic contact with the Purkinje cells. Any reduction or alteration in the number of target neurons proportionally reduces their afferents.

In the rat, Purkinje cells complete their final cell division between embryonic days 12 and 16 (Altman 1972, Schultze et. al. 1974). Since there are 500,000 Purkinje cells in the rat cerebellum (Inukei 1928) and the average cell cycle time of Purkinje cells is 9.9 hours (Schultze et. al. 1974), the time of commitment of Purkinje cells can be estimated. In order to produce this number of Purkinje cells by exponential growth, the commitment of Purkinje cells occurs on embryonic day 8.2 and has a duration of 7.8 days (Schultze et al. 1974). In neural development, the commitment of Purkinje cells coincides with the neural plate to neural fold stage of development (Wetts & Herrup 1982).

Since the commitment of Purkinje cells is already established prior to their formation from the transitory zone between embryonic days 13 to 16 (Altman 1972, Das & Nornes 1972, Altman & Bayer 1978, Schultze et. al. 1974), irradiation between GD-17, 18, 19 and 20 could not be affecting the progenitors of Purkinje cells. On embryonic day 14 to 15, the early Purkinje cells begin to differentiate and by embryonic day 16 to 17, these cells begin to migrate radially from the transitory zone through the differentiating neurons in the nuclear zone and cluster superficially underneath the intermediate fibrous layer (Altman & Bayer 1978). Depending on when the

Purkinje cells arise, the migration and dispersion occurs till approximately 4 days after birth (Altman 1972).

Irradiation between GD-17 and GD-20 coincides with the stage of differentiation and migration of Purkinje cells. Irradiation during this time could alter the genetic expression of some but not all of the Purkinje cells. Irradiation could also alter the synthesis of proteins required to produce the exogenous factors or signals for the induction of granule cells by Purkinje cells. Alternately, irradiation between GD-17 and GD-20 could interfere with the synthesis of the neural-glia adhesion molecule astrotactin which is required for the positioning and cell specification of granule cells onto the radial glial fiber.

The glycoprotein astrotactin functions as a ligand which binds the migrating neuron to the glial fiber (Edmondson et. al. 1988, Hatten & Mason 1990, Stitt & Hatten 1990). It has been reported that antibodies against astrotactin prevents the neuron from binding to the glial fibers (Stitt & Hatten 1990). The binding of the neuron to the glial cell is required to establish the neuron-glia contact and to organize the position of the neuron onto the glial fiber (Edmondson et. al. 1988). Astrotactin is expressed in granule neurons at specific stages of development. It is abundant in postnatal cells and in migrating neurons (Hatten & Mason 1990). Astrotactin is expressed in high levels during granule cell migration along radial glial fibers and during

assembly of the granule cells into the inner granular layer (Stitt & Hatten 1990). Any alteration in the expression of astrotactin prior to granule cell migration could alter both the neuron-glial contacts and the organization and positioning of the granule neuron onto the glial fiber. This would ultimately result in fewer granule cells assembling into the inner granular layer and would be manifested by the presence of radiation induced lesions within the inner granular layer.

A second possible mechanism could be that irradiation may be turning on the genetic expression of programmed cell death. This could explain why a certain percentage of control animals also exhibited these CL, although not statistically significant, within the inner granular layer. It is known that natural cell death is a principal mechanism which regulates the overproduction and selective interaction between target cells and their afferents (Cunningham 1982, Cowan et. al. 1984, Oppenheim 1985).

Physiological cell death is a normal developmental process observed in vertebrates and invertebrates (Vaux 1993, Clarke 1990). Cells undergoing natural cell death undergo changes termed PCD - programmed cell death or apoptosis (Vaux 1993). Apoptosis allows for the elimination of damaged, excessive or precancerous cells (Schulte-Hermann 1992). The characteristic changes accompanying cells undergoing apoptosis include rapid DNA fragmentation of isolated cells with no inflammatory infiltrate (Vaux 1993,

Clarke 1990). Multicellular organisms have developed the molecular mechanisms to implement cell death. The occurrence of cell death is widespread and is observed during the development of the gastrointestinal tract (Potten 1992), during insect metamorphosis (Lockshin 1969), during retinal development in humans (Provis & Van Driel 1985), and during regression of Mullerian and Wolffian ducts in sexual development (Ortiz 1945). Naturally occurring cell death has an important role in controlling the size, shape and constitution of compartments in normal developmental processes (Oren 1992, Gavrieli et. al. 1992). Cell death also exerts a homeostatic function in maintaining a balance between cell replication and cell death (Gavrieli et. al. 1992). It is known that cell death exerts a protective role against carcinogenesis and disease (Schulte-Hermann 1992). This is evident in the removal of skin cells during exposure to UV radiation (Danno & Horiot 1982) and in the removal of epithelial cells in the gastrointestinal tract during exposure to carcinogens in the diet (Potten et. al. 1977). Programmed cell death can also be induced by manipulating the hormone levels in hormone dependent tissues such as the prostate and the mammary gland (Tenniswood et. al. 1992) and by radiation (Potten 1992). Potten (1992) had reported that cell death in the crypts of the gastrointestinal mucosa was elevated by small exposure to radiation with doses of 1, 9, and 12

Gy. He had noted in the crypts that there was a strong dose-response dependence from 0 to 1 Gy.

The lesions occurring within the inner granular layer in control animals could be granule cells undergoing the natural process of cell death, to remove the excess granule cells produced during development, as a part of maintaining homeostatic function. In the animals irradiated on each of GD-17, 18, 19 and 20, there was a significant high proportion of these lesions observed in the inner granular layer. These lesions coincided with a loss in the number of granule cells and atrophy of some of the remaining granule cells. Radiation between GD-17 and GD-20 could have induced spontaneous cell death in attempts to eliminate the granule cells that were genetically affected during exposure to radiation. By eliminating the unwanted defective granule cells, the overall homeostatic balance is maintained.

The number of Purkinje cells between animals irradiated on GD-17 and GD-20 was not significantly different. The number of Purkinje cells would remain relatively constant, if during the period of apoptosis, radiation stimulated compensatory proliferation of the remaining Purkinje cells.

In the crypts of the gastrointestinal mucosa, exposure to radiation resulted in cell death but also early compensatory proliferation of the remaining cells (Potten 1992). Programmed cell death does not occur randomly but rather preferentially (Schulte-Hermann 1992). Gavrieli et. al.

(1992) has reported that PCD appears in tissues in clusters. The lesions appearing in the inner granular layer in this study occurred in clusters and is in agreement with Gavrieli et. al. (1992).

A third possible explanation for the appearance of these lesions within the inner granular layer is that irradiation could affect the precursors of granule cells that are replicating simultaneously as the Purkinje cells, thereby, altering the genesis of granule cells.

The results from this study suggest that a direct relationship exists between the proliferation, migration, development and maturation of granule cells and their induction by Purkinje cells. Furthermore, this study supports the view that both cell death and the regulation of granule cells by Purkinje cells play an essential role in the effective development and organization of the cerebellum.

6.0 SUMMARY AND CONCLUSIONS

The effects of late maternal exposure to irradiation on the development of the cerebellum are summarized as follows:

1. Prenatal exposure to 0.5 Gy of ionizing radiation during late gestation produced no gross cerebellar anomalies but resulted in several circumscribed lesions distributed throughout the inner granular layer.
2. These circumscribed lesions found in rats irradiated on each of GD-17, 18, 19, and 20 were characterized by a loss of granule cells which was associated with atrophy or absence of adjoining Purkinje cells.
3. In 21 day old rats, irradiation on GD-17 resulted in more CL anteriorly (75%) and in the vermis, whereas on GD-20, the CL predominated posteriorly (100%) and in the lateral hemispheres.
4. In 28 day old rats, irradiation on both GD-17 and GD-20 resulted in lesions equally distributed in both the anterior and posterior lobes. However, with irradiation on both GD-17 and GD-20, lesions in the anterior lobe predominated in the lateral hemispheres, whereas in the posterior lobe, they predominated in the vermis.

5. The total number of combined lesions in both the anterior and posterior lobes was higher in rats irradiated on GD-17 than on GD-20.

6. The laterolateral length of the pyramis was significantly increased ($p < 0.001$), compared to the controls. Irradiation on both GD-17 and GD-20 resulted in a greater increase in the length of the vermis than in the lateral hemispheres.

7. In 21 day old rats, irradiation on GD-20 produced more lesions in lobule 8 than irradiation on GD-17.

8. The distribution of both granule cells and Purkinje cells of the pyramis in rats irradiated on GD-17, 18, 19, and 20 were significantly reduced ($p << 0.001$), compared to controls.

9. There was a greater deficit in granule cell number with irradiation on GD-20 than on GD-17. Purkinje cells were reduced with irradiation on both GD-17 and GD-20; however, the decrease did not correspond to the degree of reduction in the number of granule cells.

10. Irradiation on GD-17 resulted in a greater reduction of both granule and Purkinje cells in the vermis, whereas on GD-20, both cells were more reduced in the lateral hemispheres.

The results from this study suggest that a direct relationship exists between the proliferation, migration, development and maturation of granule cells and their induction by Purkinje cells. The findings also support the view that both cell death and the regulation of granule cells by Purkinje cells play an essential role in the effective development and organization of the cerebellum.

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8.0 APPENDICES

8.1 APPENDIX I

FIXATIVE

2.5% Glutaraldehyde - 2% Paraformaldehyde in 0.12 M Phosphate Buffer with 0.02 mM CaCl2

Stock Solutions:

5% Paraformaldehyde

Paraformaldehyde	10 gm
Distilled water	200 ml
1 N NaOH	4-6 drops

To dissolve the paraformaldehyde, the water was heated until it steamed slightly, then added to the hot water stirring constantly until dissolved and then the NaOH was added until the solution was clear.

0.4 M Standard Phosphate Buffer

Sodium phosphate monobasic	5.3 gm
Potassium phosphate dibasic (anhydrous)	28.0 gm
Add distilled water to make	500 ml

Final Perfusate

5% Paraformaldehyde	200 ml
50 % Glutaraldehyde (Biological Grade)	25 ml
0.4 M Standard phosphate buffer	150 ml
0.5% CaCl2	2 ml
Add distilled water to make	500 ml

Filter perfusate through #44 filter paper prior to use.

Rinse Solution

8% Dextrose in 0.12 M Phosphate Buffer with 0.02 mM CaCl2

Dextrose	8 gm
Phosphate buffer 0.4 M	30 ml
CaCl2 0.5%	0.4 ml
Distilled water to make	100 ml

8.2 APPENDIX II

Tissue Processing

Chloroform Method for Processing Brains in Paraffin for Light Microscopy

1) Tissues rinsed in the rinse solution.
2) 50% alcohol for 1 hour.
3) 70% alcohol for 1 hour.
4) 95% alcohol for 1 hour.
5) 100% alcohol, 2 changes for 1 hour each.
6) Methanol (anhydrous), 2 changes for 1 hour each.
7) Chloroform, 2 changes for 1 hour each.
8) Paraffin, 2 changes (in tissue processor) for 4 hours.
9) Embed in paraffin.

Dioxane Method for Processing Brains in Paraffin for Light Microscopy

1) Tissues rinsed in running water for 3 hours.
2) 50% water/ 50% Dioxane for overnight.
3) 100% Dioxane, 2 changes for 3 hours each.
4) 100% Dioxane for overnight.
5) Paraffin, 2 changes for 2 hours each.
6) Paraffin, 2 changes for 2 hours each in a vacuum.
7) Embed in paraffin.

8.3 APPENDIX III

Staining

Thionin Stain for Brain Tissue

1) Deparaffinize in xylol, 2 changes for 5 minutes each.
2) 100% alcohol, 2 changes for 2 minutes each.
3) 95% alcohol for 2 minutes.
4) 70% alcohol for 2 minutes.
5) Distilled water for 5 minutes.
6) Thionin stain (4-6 seconds).
7) Distilled water, 2 changes, 2-3 dips.
8) 70% alcohol, 2 changes for 1 minute each.
9) 95% alcohol for 2 minutes.
10) 100% alcohol, 2 changes for 2 minutes each.
11) Xylol, 2 changes for 2 minutes each.
12) Coverslip with permount.

Bodian's Stain for Nerve Fibers and Nerve Endings

Solutions

1% Protargol Solution

Protargol	1.0 gm
Distilled water	100 ml

Sprinkle the protargol on the surface of the water and allow it to remain undisturbed until it dissolves.

Reducing Solution

Hydroquinone	1.0 gm
Formalin, 37 - 40%	5.0 ml
Distilled water	100 ml

1% Gold Chloride Solution

Gold Chloride	1.0 gm
Distilled water	100 ml

2% Oxalic Acid Solution

Oxalic Acid	1.0 gm
Distilled water	100 ml

5% Sodium Thiosulfate (Hypo) Solution

Sodium Thiosulfate	5.0 gm
Distilled water	100 ml

Aniline Blue Solution

Aniline Blue	0.1 gm
Oxalic Acid	2.0 gm
Phosphomolybdic Acid	2.0 gm
Distilled water	300 ml

Staining Procedure

1) Deparaffinize and hydrate to distilled water.
2) Place slides in protargol solution and add 4 to 6 gm of clean copper shot per 100 ml of solution. Let stand at 37° C for 48 hours.
3) Rinse in distilled water, 3 changes.
4) Reducing solution for 10 minutes.
5) Rinse in distilled water, 3 changes.
6) Tone in gold chloride solution for 10 minutes.
7) Rinse in distilled water, 3 changes.
8) Develop in oxalic acid solution under the microscope until background is gray and nerve fibers appear clearly, about 3-5 minutes.
9) Rinse in distilled water, 3 changes.
10) Sodium thiosulfate for 5 minutes.
11) Counterstain with aniline blue solution, 2-3 quick dips.
13) Dehydrate in 95% alcohol, 100% alcohol, and clear in xylol, 2 changes each.
14) Mount with permount.