

**RISK OF GAMMA X-RAYS IRRADIATION AND CYCLOTRON EXPOSURE IN  
THE BRAIN DEVELOPMENT OF RATS.**

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*Abstract*

*X-rays irradiation has been used in the photo thorax and dental panoramic photo, as well cyclotron which has been widely used to treat cancer is also [electromagnetic radiation](#). Both X-rays and cyclotron have the risk of causing damage to the cell and tissue, both in embryonic tissue or adult tissue. Previously we showed that prenatal X-rays gamma irradiation of rats during the late gestation period caused apoptosis of granule cell in the cerebellum. The present report was demonstrate the risk of gamma X-irradiation and cyclotron irradiation kind of gamma irradiation, caused histological abnormal in the cerebellum, especially in in the development of Purkinje cells. Pregnant rats were exposed 2.5 Gy X-irradiation and 1.5 Gy Heavy Ion Medical Accelerator in Chiba (HIMAC) or Cyclotron radiation on gestation day 21 respectively. Pups of rats were observed on postnatal day 5 (P5) and P9. Their cerebella were observed by immunohistochemically to identify Purkinje cells. By postnatal days 5 and 9 of pups, Purkinje cells develop abnormally with shorter and abnormal oriented dendrites and some were failed to migrate. Our research also demonstrate that X-irradiation on GD-21 causes cell death in the EGL resulting in a decreased level of Reelin in the early neonatal period. The lack of Reelin at this stage may contribute to the permanent derangement of Purkinje cells, and possibly also to abnormal foliation of the cerebellum, as we observed before. We suggested effects of cyclotron, also induced decreased level of reelin, so we need to observed level of reelin caused by cyclotron. These results of this report will be necessary to make the security procedure in operating the device that uses gamma radiation.*

**Key Words:** *Abnormal Purkinje cell, Cerebellum, Cyclotron, gamma X-rays.*

**Introduction:**

Exposure of the human to ionizing radiation such as atomic bombing or to X-rays during medical procedures and magnetic resonance imaging has long been a matter of great concern because of the high sensitivity of the fetus. It has been documented the occurrence of tissue abnormalities of the brain after in utero irradiation (Darmanto et al., 1997). It has previously been reported that prenatal exposure to X-radiation on gestation days 19-21 causes heterotopic Purkinje cells in the internal granular layer and white matter of the abnormally foliated cerebellum in the rat (Cowen and Geller, 1960; Inouye M, 1979; Darmanto et al., 1997). Heavy Ion Medical Accelerator in Chiba (HIMAC) or Cyclotron radiation was designed to accelerate beams from 4He to 40Ar. A variety of ion species are, however, required to utilize the full potential of the accelerator for either clinical treatment or irradiation experiments.

Biological effects of radiation on embryos could be responsible for cell death and abnormal neuronal migration during a critical

gestation state of sensitivity to radiation (Sun et al., 1996).

The major histogenetic events of the rat cerebellum take place in the early postnatal days. During this period, precursors of microneurons, e.g., granule cells, form the external granular layer (EGL), extend over the surface of the primordial cerebellum, and actively proliferate. The postmitotic granule cells leave the EGL and migrate to the internal granular layer (IGL). (Altman and Bayer, 1978) On the other hand, young neurons which have been destined to differentiate into Purkinje cells migrate from the ventricular zone toward the surface of the cerebellum (Inouye, 1979) Immature Purkinje cells form a plate underneath the EGL, and then they develop complex dendritic trees (Darmanto et al, 1997).

The cerebellum in this stage of development is particularly sensitive to ionizing radiation. The morphogenesis of abnormal foliation, ectopic Purkinje cells, and ectopic granule cells in the cerebellum of rats and mice following X-irradiation has been described. (Inouye 1978 and Darmanto et al, 1997)

X-irradiation of rat fetuses at 1.7 Gy on gestation day 18 (GD-18), shortly before the major histogenetic period of the cerebellum, arrested the migration of immature neurons destined to differentiate into Purkinje cells. During the post-natal period of rats, an abnormal arrangement of the Purkinje cell layer and malformed folia developed in the anterior region of the cerebellum (Miyata et al, 1996). It has been suggested that the migration of Purkinje cell precursors from the ventricular zone toward the cerebellar cortex may be guided by radial glial fibers.

In addition, the present study aimed to elucidate a possible factor(s) to induce heterotopic Purkinje cells and abnormal cerebellar foliation of the rat following prenatal exposure to X-radiation. For this purpose we examined the expression of morphoregulatory molecules by immunohistochemistry since their functions have been considered essential for the neuronal migration and cellular arrangement of the brain (Crossin, 1990).

However the cellular mechanisms of X-irradiation and cyclotron toxicity, possible involved in the cell death and abnormal caused by X-irradiation and HIMC or Cyclotron are little known.

## Materials and Methods

### Animal Experiments

Pregnant rats from Std:Wistar/st were housed in plastic cages in an air-conditioned room ( $21 \pm 1^\circ\text{C}$ ) with relative humidity of  $50 \pm 10\%$  under an alternating 12 hr light/dark schedule. They were fed a standard food (CE2, CLEA, Japan) and water ad libitum.

Pregnant rats on day 21 of gestation (GD-21, plug day = GD-0) were exposed to whole body X-irradiation at a dose of 2.0 Gy and 2.5 Gy at 7 AM. Radiation factors were 140 kVp, 5 mA, 0.5 mm Cu + 0.5 mm Al added filtration, and  $104.0 \pm 0.5$  m Gy/min exposure rate. Another pregnant rats were exposure with cyclotron dose of 0.75 Gy and 1.5 Gy. To achieve a homogeneous dose distribution, the rats were put in individual plastic cages, and the cages were rotated u 4 rpm on a turntable during exposure. Control pregnant rats were treated in the same manner except for the exposure to X-irradiation. Both groups were allowed b gin birth and rear their litters. Pups of both control ad irradiated rats were anesthetized by diethyl ether, perfused transcardially with 4% formaldehyde, and their brains were fixed with the same solution overnight. Brains rats obtained 12 hr after exposure (after birth), and on postnatal day 5 (PS), P7 and P9. After fixation the cerebella were dehydrated, embedded in paraffin, and serially sectioned in a mid-saggital plane at 5  $\mu\text{m}$  thickness. They were stained with hematoxylin

and eosin, or subjected to a immunohistochemical procedure.

Ca immunohistochemistry against IP3 receptor in Purkinje cells, two or three sections for each cerebellum were selected from the serial sections. Dewaxed sections were sequentially incubated as follows: (1) in 0.6%  $\text{H}_2\text{O}_2$  methanol for 5 min; (2) in 0.1 M phosphate-buffered saline containing 0.3% Triton X-100; (3) in 10% normal goat serum at room temperature for 10 min; (4)' in rat amb-IP3 receptor, diluted 1:100, at  $4^\circ\text{C}$  overnight; (5) in biotinylated universal secondary Ab (Vector) at room temperature for 30 min; (6) in peroxidase-conjugated streptavidin (Vector) at room temperature for 50 min; and (7) reaction in 0.02% diaminobenzidine solution with 0.006%  $\text{H}_2\text{O}$ , at room temperature for 5-10 min.

Sections adjacent to those used for immunohistochemistry of IP3-receptor were immunostained either with anti-NCAM (Chemicon International, Temecula, CA, used at 1:500 in PBS), anti-tenascin (Chemicon, used at 1:500 in PBS), or anti-fibronectin (Becton Dickinson Labware, Bedford, MA, used at 1:100 in PBS) antibodies at  $4^\circ\text{C}$  overnight using the same procedure as immunostaining with anti-IP3 receptor antibody.

These sections were examined light microscopically, and the number of Purkinje cells piling up in the Purkinje cell layer was counted for all lobes of the cerebellum. The data thickness of the Purkinje cell layer was expressed as the median of the cell number, e.g., if the layer thickness was 6 cells at a maximum and 2 cells at a minimum, the median was calculated as 4 cells thick.

### Immunofluorescence of CR-50 and S100 Protein

Pups of both groups were deeply anesthetized with diethyl ether and perfused transcardially with periodate-lysine-paraformaldehyde pH 7.4. Their brains were removed and postfixed with the same solution overnight (McLean et al., 1974). The cerebellum was dissected, rinsed in PBS, and cryoprotected in 10%-20% sucrose in PBS overnight. Specimens were then embedded in OCT compound (Miles, Elkhart, IN), quickly frozen. and serially sectioned in the mid-sagittal plane at 10--12  $\mu\text{m}$  thickness. Serial frozen sections were collected onto poly-L-lysine coated glass slides (Sigma-Aldrich, St. Louis, MO) and air-dried for 1-2 h. The sections were rinsed in PBS, preincubated with 0.3% triton X-100, 3% bovine serum albumin (BSA) and 5% normal goat serum for 1 h, and then incubated with anti-Reelin antibody, CR-50 (Ogawa et al., 1995), diluted 1:10 in blocking solution (normal horse serum diluted 1:50 in PBS) at  $4^\circ\text{C}$  overnight. After incubation with the primary antibody, the sections were washed with PBS and reacted with

biotin conjugated second antibody (anti-mouse IgG) diluted 1:250 in PBS for 1 h, then incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Biomeda, Foster City, CA) diluted 1:50 in PBS for 1 h. Images were taken with a fluorescence microscope (Axiophot 2, Zeiss, Germany). For the negative control, the first antibody was omitted during the labeling procedure.

To detect Bergmann cells and fibers, the sections adjacent to those treated immunohistochemically for Reelin were immunostained with anti-S-100 protein (IBL, Shizuoka, Japan, diluted 1:1,000) at 4°C overnight using the same procedure as immunofluorescence with anti-Reelin antibody

**Northern Blot Analysis**

For Northern blot analysis of *reelin* mRNA, total RNA was extracted from the cerebellum by the method of acid-guanidiniumphenol-chloroform (Chomczynski et al., 1997). The amount of total RNA was determined by the absorbance at 260 nm. Fifteen microgram of total RNA from each sample was subjected to Northern blot analysis under the protocol described previously (Murata et al., 1990). Mouse *reelin* cDNA fragment (a gift from Dr. T Saitoh) was labeled with [P]dCTP (specific activity, 111 TBq/mmol; New England Nuclear, Boston, MA) by the Random Primer Labeling Kit (Boehringer Mannheim, Mannheim, Germany) and used as a probe for hybridization. The density of the bands for *reelin* mRNA was analyzed by the Molecular Imager System (GS-363, Bio-Rad Laboratories, Hercules CA). Autoradiography was performed by exposing the membrane to Kodak X-AR (Eastman Kodak, Rochester, NY) film for 3 days. Membranes were reprobbed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Sato et al., 1996) to monitor the equal delivery and the integrity of RNA.

**Results**

Twelve hr after exposure to X-irradiation, extensive cell death was evident in the EGL of the cerebellum of rats exposed to 2.5 Gy X-irradiation and to 1.5 cyclotron (Fig. 1). There were no distinct differences in the number of cell deaths among animals exposed to the doses

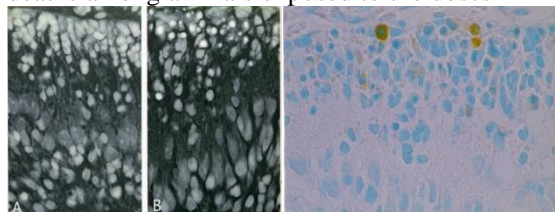


Fig 1. Cell death of EGL after X-irradiation. The vermis of newborn rats prenatally exposed to X-irradiation (right column) and sham-exposed controls (left column) A, B: 12 h

after exposure (P0). By immunohistochemistry of apoptosis method, cells death was confirm as a apoptosis

Figure 1 shows the early histological events after exposure. By 12 h after exposure, extensive cell death and destruction of the EGL became apparent (Fig. 1B), whereas the surface area of the control cerebellum was occupied by 5- to 9-cell-thick EGL (Fig. 1A). There were no findings of Purkinje cell death, suggesting that Purkinje cells are resistant to X-radiation. By 48 h after exposure, all cell debris had disappeared and a small number of surviving granule cells formed clumps together in places, while in other places granule cells were almost missing in the surface (Figure not show). By P4 the EGL started to recover.

**Abnormal Migration of Purkinje Cells in the Irradiated Cerebellum**

The immunoreactivity for IP3 receptor in Purkinje cells was used to identify the morphology and arrangement of Purkinje cells. On P5, Purkinje cells were aligned in the cerebellum of control rats (Fig. 2). Figure 2 shows the development of Purkinje cells after exposure. By P4 the majority of Purkinje cells in the control formed a monolayer with apical cones directing toward the pial surface of the cerebellum (Fig. 2A). In X-irradiated rats, the arrangement of Purkinje cells was irregular, forming a 2- to 7-cell-thick layer with random orientation of the apical cones (Fig. 2B). Purkinje cells in the control on P9 were well aligned with developing dendritic arbors toward the pial surface (Fig. 2E). In contrast, as shown in Figure 2F, Purkinje cells in the irradiated cerebellum remained in a 2- to 3-cell-thick layer. Moreover, many Purkinje cells were heterotopically located in the internal granular layer (IGL). They also exhibited morphological abnormalities: short and thick stem dendrites with few branches and random directions of the stem dendrites or some dendrites extending toward the opposite direction.



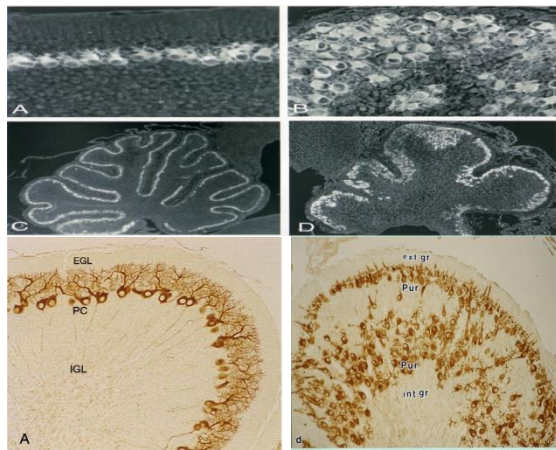


Fig. 2. Arrangement of Purkinje cells in cerebella prenatally exposed to X-irradiation (right column) and sham-exposed control (left column) A,B. Cross section of cerebellum showed the abnormal foliation, D, normal foliation of the control Cerebellum. E: Purkinje cells in the control on postnatal day 4 (P4) aligned with apical cones directing toward the pial surface, F: Purkinje cells were deranged and some apical cones had opposite directions.

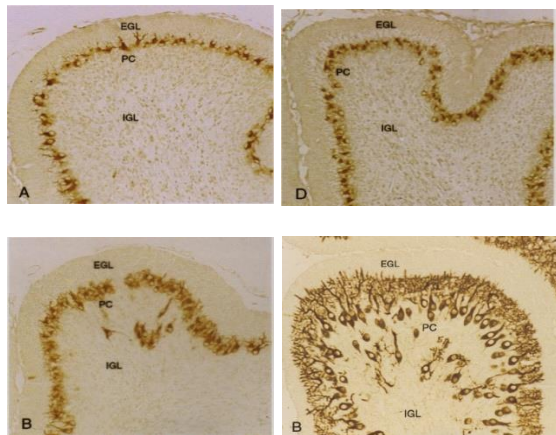


Fig. 3. Arrangement of Purkinje cells in cerebella prenatally exposed to Cyclotron; A; Purkinje cells in the control on postnatal day 4 (P4) aligned with apical cones directing toward the pial surface; B,C.: Purkinje cells were deranged and some heterotopic; D abnormal Purkinje cell on postnatal day 9 (P9).

At the same age, the arrangement of Purkinje cells was irregularly forming a 1-10 cell-thick layer and some scattering in the surface area of the lobules in rats exposed to 2.5 Gy X-irradiation or 1.5 Gy of cyclotron (Fig. 3). The EGL was thinner in rats exposed to 2.5 Gy than that in the control rats. The cerebellar foliation of PS rats following exposure to 2.5 Gy was retarded, resembling the folial pattern of the normal cerebellum on P1 (Fig. not shown). By

P9, the dendritic arborization of Purkinje cells had developed in the control rats (Fig. 2E). In the irradiated pups with 1.5 Gy, some Purkinje cells were seen normally forming one layer (Fig not show), but dendritic arborization did not develop well (Fig. 2F; 3C and 3D). In the irradiated rats with doses of 2.5 Gy, and also cyclotron doses of 1,5 Gy some Purkinje cells remained in the IGL and white matter, showing morphological abnormality with short dendrites and an abnormal dendritic direction (Fig. 3C, D). Some part of the EGL was thinner, resembling an uneven recovery of the EGL, and an abnormal foliation of the cerebellum developed in rats exposed to 2.5 Gy

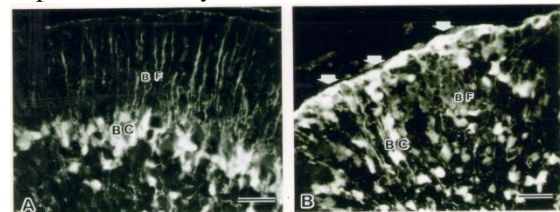


Fig 4. Bergmann cells and their fibers in cerebella prenatally exposed to X-irradiation, B (right column) and sham-exposed controls, A (left column).

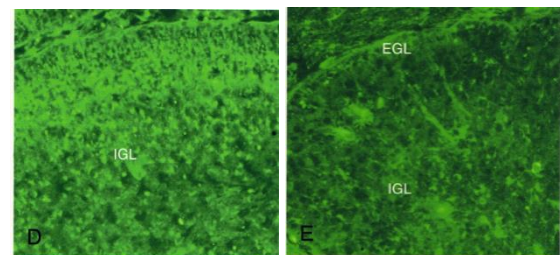
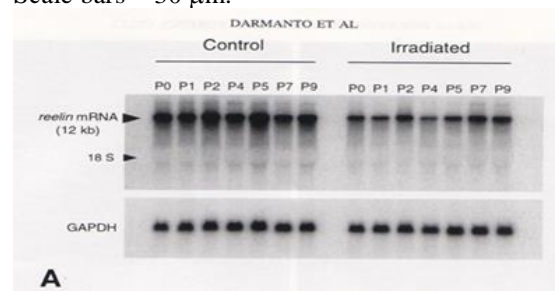


Fig. 5. Immunoreactivity of Reelin in cerebella prenatally exposed to X-irradiation (right column) and sham-exposed controls (left column). A, 9 days after exposure, EGL: external granular layer, ML: molecular layer, IGL: internal granular layer. In controls on P4, cell bodies in the inner half of the EGL and outer part of the IGL were strongly Reelin immunoreactive (A). Reelin decreased on P4 (B). Immunofluorescence stain with antibody CR-50. Scale bars = 30  $\mu$ m.



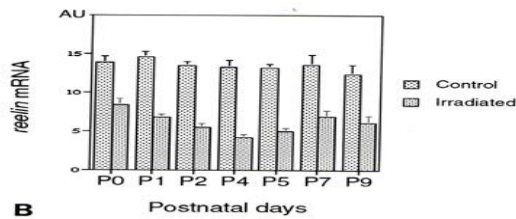


Fig. 6. Northern blot analysis of *reelin* mRNA from P0 through P9. (A) Visualized by autoradiography. A GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe was used for the loading control. (B) Visualized by densitometry. Expression of *reelin* mRNA was decreased in the X-irradiated cerebellum from P0 through P9. Data were presented as mean  $\pm$  SE of 9 X replicate values for *reelin* mRNA expression. Analysis by two-way ANOVA. The difference between control and irradiated groups was statistically highly significant ( $p < 0.0001$ ).

Figure 6A shows *reelin* mRNA as a band at 12 kb. Figure 7B shows the densitometric analysis of the band for *reelin* after correction for the amount of mRNA for the housekeeping gene, GAPDH. The abundance of *reelin* mRNA was almost constant throughout the observation period from P0 to P9 in the control. X-radiation caused a rapid decrease in *reelin* mRNA expression. The decrease was already evident on P0 when Reelin protein still remained comparable with the control. The expression decreased further, being almost one third of the level of the control group on P4. Although the expression tended to recover after P5, the levels were significantly lower than those of controls up to P9 (the end of the observation period).

**DISCUSSION**

In another, the result of teratogenicity of cyclotron or HIMAC is similar effects to the X-irradiation. Observation in the effects of cyclotron and X-irradiation on mice, using immunohistochemistry, the present study demonstrated sequential changes in cellular events in the cerebellum of rats after prenatal X-irradiation. X-radiation caused extensive cell death in the EGL as an acute effect, followed by recovery of the EGL. Although Purkinje cells were resistant to X-ray-induced mortality, they exhibited disturbed alignment with abnormal dendritic arborization. These were partially corrected during the following development, but the incomplete recovery resulted in disorganized Purkinje cell layer and heterotopic Purkinje cells in the IGL with abnormally oriented dendritic arbors. Bergmann cells with abnormal fibers also showed heterotopic locations. These abnormalities occurred before recovery of the EGL.

When the EGL recovered, all Bergmann cells were located beneath the Purkinje cell layer and extended their fibers to the pial surface, being comparable to the control. These results suggest that recovered granule cells push Bergmann cells into the proper area, enabling extension of their fibers.

Abnormal foliation induced by X-irradiation seems to correlate with abnormal cellular events described above. On GD-21, migration of immature Purkinje cells from the ventricular zone to the cortical area of cerebellum was almost complete, however, immature Purkinje cells were still in a multilayer (Altman and Bayer, 1978; Goffinet et al., 1984). X-irradiation at this stage induces abnormal foliation. In contrast, when rats were exposed on P4 to the same dose, almost normal foliation developed even though the majority of granule cells in the EGL were destroyed. Therefore, the critical stage for cerebellar foliation is in the early postnatal period and the alignment of Purkinje cells seems important for a normal pattern of the foliation.

A heterotopic localization of Bergmann cells at the early neonatal days may also be involved in abnormal foliation. When Purkinje cells were in a heterotopic location, Bergmann cells followed the Purkinje cell movement and their fibers were abnormally arranged at this stage. This abnormal arrangement of Bergmann fibers may affect the migrating granule cells and change the direction of fissuration of the cerebellum.

To study the molecular mechanism of the abnormal patterning of Purkinje cells by X-irradiation, we examined the expressions of Reelin as a candidate to mediate the abnormal patterning of Purkinje cells induced by X-irradiation. Reelin is a secretory extracellular protein and has already been suggested to mediate neuronal adhesion and migration at critical stages of development (Ogawa et al., 1995; D'Arcangelo et al., 1995; Sheldon et al., 1997; D'Arcangelo et al., 1988; Howell et al., 1997; Miyata et al., 1997). In reeler mutant mice, in which Reelin is absent, Purkinje cells do not migrate but remain as clusters at deep cerebellar areas (Miyata et al., 1997; Yoneshima et al., 1997; Goldowitz et al., 1997; Tromsdorff et al., 1999; Hirotsune et al., 1995). When normal external granule cells were explanted, reeler Purkinje cells started to migrate around the explanted granule cells (Miyata et al., 1997). These results strongly suggested that Reelin plays a pivotal role in the migration of Purkinje cells. Therefore, it is not possible to specify the critical stage for Reelin to affect the arrangement of Purkinje cells and to discuss the relationship between the Purkinje cell arrangement and foliation. In this study, using X-irradiation, we were able to induce the lack of Reelin at the specific time during cerebellar development. Immunohistochemistry of Reelin in this study clearly demonstrated that X-irradiation

on GD-21 caused the reduction of Reelin 48 h after exposure and the decrease continued up to P9 (data not show), the end of the observation period. During the time when the lack of Reelin occurred in the irradiated pups the alignment of Purkinje cells was completed in the control. Our results thus render evidence that Reelin is important for the arrangement of Purkinje cells at the critical stage.

The decreased *reelin* expression was also demonstrated quantitatively at the level of mRNA. The decrease in *reelin* mRNA was already evident 24 h after exposure, the level was lowest around P4 and started to recover afterward. The decreased *reelin* expression seemed due to cell loss of the EGL. Our results demonstrate that X-irradiation on GD-21 causes cell death in the EGL resulting in a decreased level of Reelin in the early neonatal period. The lack of Reelin at this stage may contribute to the permanent derangement of Purkinje cells, and possibly also to abnormal foliation of the cerebellum, as we observed before. We suggested effects of cyclotron, also induced decreased level of reelin, so we need to observed level of reelin caused by cyclotron.

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