

## Neurobehavioral Changes in Mice Exposed to Fast Neutrons *in utero*

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### Neutrons/Fetus/Neurobehavioral effects.

Epidemiological studies have revealed that radiation causes brain development abnormalities in atomic bomb survivors exposed *in utero*. Rat and mouse studies have also shown that prenatal exposure to low-linear energy transfer radiation induces developmental brain anomalies. Because the effects of prenatal irradiation on adult behavior patterns remain largely unknown, the present study investigated the effects of neutron exposure *in utero* on postnatal behavior patterns in mice. [C57BL/6J × C3H/He] hybrid (B6C3F1) mice were exposed to cyclotron-derived fast neutrons with peak energy of 10 MeV (0.02–0.2 Gy) or Cs-137 gamma-rays (0.2–1.5 Gy) on embryonic day 13.5. At 5.5–8 months of age, the neurobehavior of male offspring was examined by Rota-rod treadmill and locomotor activity. The accumulation of radio-labeled drug at muscarinic acetylcholine and serotonin receptors in mice from control and neutron-irradiated groups was determined by the tracer method. Locomotor activity during the dark period increased in the 0.02 Gy neutron-irradiated group. Furthermore, at 5.5 months of age, tracer binding *in vivo* to the muscarinic acetylcholine increased and to the serotonin receptors decreased in the 0.02 Gy neutron-irradiated group. In conclusion, the present study reveals that a certain “low-dose window” may exist for radiation-induced changes in neurobehavior and binding to neurotransmitter receptors, because there was correlation in neurobehavior and binding to neurotransmitter receptors in the 0.02 Gy neutron-irradiated group though there was not correlation in the neutron-irradiated groups more than 0.05 Gy.

### INTRODUCTION

On September 30, 1999, a criticality accident at a uranium conversion facility in Tokai-mura, Ibaraki Prefecture, Japan severely exposed three workers to neutrons and gamma rays. The accident generated neutrons identified as potential

human hazards, which prompted research on the biological effects of neutron radiation.<sup>1,2)</sup> Relative biological effectiveness (RBE) values for neutrons inducing prenatal effects in rodents were previously reported as approximately two to four.<sup>3,4)</sup> RBE is dependent on energy and is greater at low doses. Friedberg *et al.*<sup>5)</sup> estimated that RBE for prenatal mortality in mice was larger at 0.2 *kerma* (Gy) than 0.4 Gy suggesting that RBE is altered in a dose-dependent manner. Therefore, we previously examined the effects of RBE of low-dose neutrons on fetal nervous development. Mice were exposed to cyclotron-derived fast neutrons with peak energy of 10 MeV (0.02–1.0 Gy) or 1.0–2.0 Gy of gamma rays on embryonic day 13.5. The dose response curves were best fitted to linear quadratic models and the RBE was estimated to be 9.8. This estimated RBE was considered large for prenatal effects and acute tissue injury induced by low-dose neutrons. These results suggested that more studies were needed to determine the effects of neutron exposure *in utero* to accurately tease out the low-dose radiation effects.<sup>6)</sup>

Epidemiological studies on atomic bomb survivors exposed *in utero* have revealed that radiation causes brain development abnormalities.<sup>7–9)</sup> Otake *et al.*<sup>10)</sup> reported significant effects on the developing brain among those individuals exposed to radiation during weeks 8–25 of gestation.

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Radiation exposure during gestational weeks 8–15 manifests itself through increased frequency of severe mental retardation, decreased intelligence quotient (IQ) scores and school performance, and increased seizure occurrence. Of 30 severe mental retardation cases, 18 (60%) had small heads. Since the effect of prenatal irradiation on behavior patterns during adulthood remains largely unknown, the present study is designed to investigate the effects of neutron exposure *in utero* on the postnatal behavior pattern in mice. The mouse brain of embryonic day 13.5 (E13.5) is highly radiosensitive, and E13.5 in mice correspond to stage when causes abnormality such as severe mental retardation for atomic bomb survivors.<sup>11–13)</sup> The purpose of this study is to evaluate the neurobehavioral effects on mice exposed to fast neutrons *in utero*.

## MATERIALS AND METHODS

### *Animals*

All animals were bred in the animal facility of National Institute of Radiological Sciences. They were housed in an air-conditioned room ( $23 \pm 1^\circ\text{C}$ ) with a relative humidity of  $50 \pm 5\%$  under an alternating 12-hour light/dark cycle lights on at 7:00 a.m.. Mice were fed a standard commercial laboratory diet (Funabashi Farm Co., Tokyo, Japan), and given chlorinated water *ad libitum*. Female C57BL/6JNrs mice were caged with male C3H/HeNrs mice from 5:00 p.m. to 10:00 a.m.. Pregnancies were dated from embryonic day 0 (E0) which commenced the first midnight after mating. Forty-two pregnant mice were divided among nine groups: non-irradiated control group, four groups for fast neutron-irradiation (doses 0.02, 0.05, 0.1 and 0.2 Gy) and four groups for gamma rays-irradiation (doses 0.2, 0.4, 0.8 and 1.5 Gy). Pregnant mice were placed in acrylic irradiation chambers and irradiated on embryonic day 13.5, which corresponds to the radiosensitive stage of cerebrum development.<sup>14,15)</sup> [C57BL/6J  $\times$  C3H/He] hybrid (B6C3F1) male offspring born by natural birth were divided into 9 groups ( $n = 11$ –13/group). Adult mice were tested for behavioral change using the Rota-rod treadmill and locomotor activity beginning at age 5.5 months. Then brains were removed, weighed, and examined histopathologically. Another adult mice ( $n = 3$ /group) were also analyzed for accumulation of radio-labeled tracers to the muscarinic acetylcholine (mACh) and serotonin receptors by the tracer method on the other hand.

All animal experiments were carried out with permission and under regulation of the Institutional Committee for Animal Safety and Welfare at the National Institute of Radiological Sciences (NIRS).

### *Neutrons and Gamma-rays Irradiation*

Neutrons were generated from the NIRS cyclotron, using the deuterium-beryllium reaction. Analyses of the field energy spectrum<sup>16)</sup> and field characteristics<sup>17)</sup> have been reported

elsewhere. Briefly, peak energy of 10 MeV was established for the estimated forward neutron spectra. The dosimetry was conducted using an ion chamber filled with tissue-equivalent gas. The mean dose rate for neutrons was 0.15 Gy/min. The contamination of gamma-rays was estimated to be less than 5% of the neutron dose. Four dose groups (0.02, 0.05, 0.1 and 0.2 Gy) were employed.

Exposure to <sup>137</sup>Cs-generated gamma-rays was conducted with a Gammacell (Nordion, Inc., Ottawa, ON, Canada). The mean dose rate of gamma-rays was 0.65 Gy/min. Four dose groups (0.2, 0.4, 0.8 and 1.5 Gy) were employed.

### *Rota-rod Treadmill Test*

Mice at age 5.5 months were trained to the single lane Rota-rod treadmill (Muromachi Kikai Co., Tokyo, Japan) for five consecutive days prior to the locomotor activity. The Rota-rod treadmill apparatus consisted of a base platform, a 3 cm rotating bar, and a non-slippery surface. The four paws of individual mice were placed approximately 30 cm above the floor on the rotating bar. The number of falls from the bar over a 60 sec (days 1–4) or 300 sec (day 5) period was recorded. The rotating speeds of the bar were 2, 3, 10, 20 or 4–40 rpm. Impaired coordination was detected by removing mice with motility disturbance, i.e., individual mice which fell from the bar by the third day, and conducting comparison examinations with the remaining mice on that particular day.

### *Locomotor Activity*

Spontaneous locomotor activity was examined following Rota-rod treadmill tests in 6 month old mice. During motor activity measures, mice were individually placed in cages and recorded continuously for 4 days. Individual motion scores were calculated and averaged every 3 hours. The behavioral measurement system included the Supermex system, a sound-attenuating chamber, a 64-channel interface, and a personal computer with CompACT AMS software (Muromachi Kikai Co., Tokyo, Japan). Spontaneous motor activity of individual mice was measured by sensors that detect body heat while the mice were housed in their respective home cages of the Supermex system.

### *Histopathological Examination*

Mice were euthanize and sacrificed to investigate neurotransmitter-related factor change by immunohistochemical staining in the brain at 7 days after behavioral tests. Brain were removed from the mice and fixed in 4% paraformaldehyde buffer (PFA) for 24–48 h at 4°C. PFA-fixed brain were immersed and decalcified in 30% sucrose in 0.01 M phosphate buffered saline for 2 days at 4°C. Then tissue were embedded O.C.T compound (4583, Sakura Finetek Japan Co., Tokyo, Japan) and freezed on dry ice. The section were cut at 4–6  $\mu\text{m}$  with a freezing microtome and mounted onto slides. Immunohistochemical staining were performed

according to routine indirect immunohistochemistry method. The primary antibody were used rabbit anti-tyrosine hydroxylase (TH) polyclonal antibody (1:1000, AB152, Chemicon, MA, USA), goat anti-choline acetyltransferase (ChAT) polyclonal antibody (1:100, AB144P, Chemicon) and mouse anti-serotonin (5HT) monoclonal antibody (1:500, ab16007, abcam, Cam, Britain) to observed dopaminergic neuron, cholinergic neuron and serotonergic neuron respectively, and the secondary antibody was used simplestain MAX-PO (Nichirei biosciences, Tokyo, Japan). The specimen were visualized with 3, 3'-diaminobenzidine (DAB) and counterstained with hematoxylin.

#### Accumulation to Receptors by Tracer method

Another adult mice ( $n = 3/\text{group}$ ) were also analyzed for accumulation of radio-labeled tracers to the muscarinic acetylcholine (mACh) and serotonin receptors by the tracer method on the other hand.

In this study, the changes in cholinergic functions were measured with an *in vivo* mACh receptor binding assay using  $^3\text{H}$ -QNB. mACh has the action to excite the nerve. The accumulation of radio-labeled tracer was measured with the corpus striatum, the hippocampus, the cortex and the cerebellum where mACh receptor existed.

Also, measurements were made with an *in vivo* binding assay using  $^3\text{H}$ -*N*-methyl-spiperone ( $^3\text{H}$ -NMSP) to the serotonin receptor. Serotonin is the inhibitory neurotransmitter. The accumulation of radio-labeled tracer to the serotonin receptor was evaluated by measuring them in the cortex and the cerebellum from which the serotonergic neuron was projected.

$^3\text{H}$ -Quinuclidinyl benzilate ( $^3\text{H}$ -QNB: 1.0 TBq/mmol, NEN Research Product) and  $^3\text{H}$ -*N*-methyl-spiperone ( $^3\text{H}$ -NMSP : 2.0 TBq/mmol, NEN Research Product) were used to detect the mACh receptor.  $^3\text{H}$ -QNB was administered to mice 30 min before sacrifices through the tail vein. Mice were sacrificed by decapitation followed by tracer injection. The plasma was obtained from centrifuged whole blood. Brain regions were removed from whole brain. All samples were weighed and completely dissolved in 1 mL of tissue solubilizer (Soluene-350, PerkinElmer Japan, Yokohama, Japan). Ten mL of scintillation cocktail (Hionic-Fluor, PerkinElmer Japan, Yokohama, Japan) was added in each vial. Radioactivity in the tissue sample was measured with a liquid scintillation counter (LS-6000TA, Beckman Coulter, CA, USA). The results were expressed as % injected dose per gram of tissues. These methods were conducted as reported.<sup>18,19)</sup>

#### Statistical Analysis

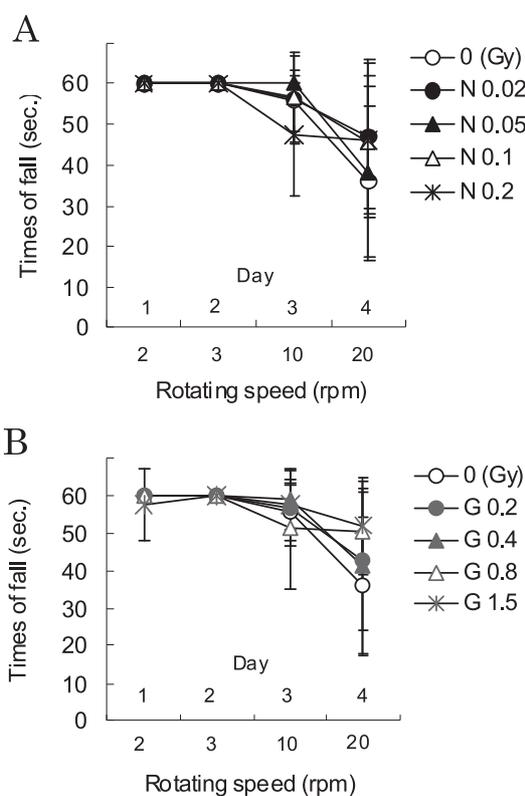
The data are expressed as the mean  $\pm$  standard error. Statistical analysis was performed on body weights data and accumulation of radio-labeled tracer in brain using the Dunnett's test. A value of  $p < 0.05$  or  $p < 0.01$  were considered statistically significant.

## RESULTS AND DISCUSSION

The present study revealed that: (1) locomotor activity during the dark period showed the tendency to increase in the 0.02 Gy neutron-irradiated group, (2) the binding of tracer to mACh and serotonin receptor *in vivo* changed in the 0.02 Gy neutron-irradiated group, especially, the accumulation of  $^3\text{H}$ -QNB to the mACh receptor in the corpus striatum and the hippocampus increased significantly, and the accumulation of  $^3\text{H}$ -NMSP to serotonin receptor in the cortex has decreased significantly.

At 2 and 3 rpm, no muscular force alteration was observed on the Rota-rod treadmill test between the irradiation groups and the control group (Fig. 1). There was also no change at the latter half (10 and 20 rpm) of the constant-speed bar schedule for motility disturbance. At day 5, there was also no observed statistical difference between the number of falls for any dose of the neutron-irradiated group or the 0.8 and 1.5 Gy gamma-rays irradiated groups as compared to control.

The mice were sacrificed in one week following the completed behavior tests, body and brain weights were measured. Both body weights and absolute brain weights were



**Fig. 1.** Times of fall for Rota-Rod treadmill test. Score on mice exposed radiation at days of 1–4. (A) Neutron-irradiated groups, (B) Gamma-rays-irradiated groups. There was no change for the constant-speed bar schedule in motility disturbance or muscular force.

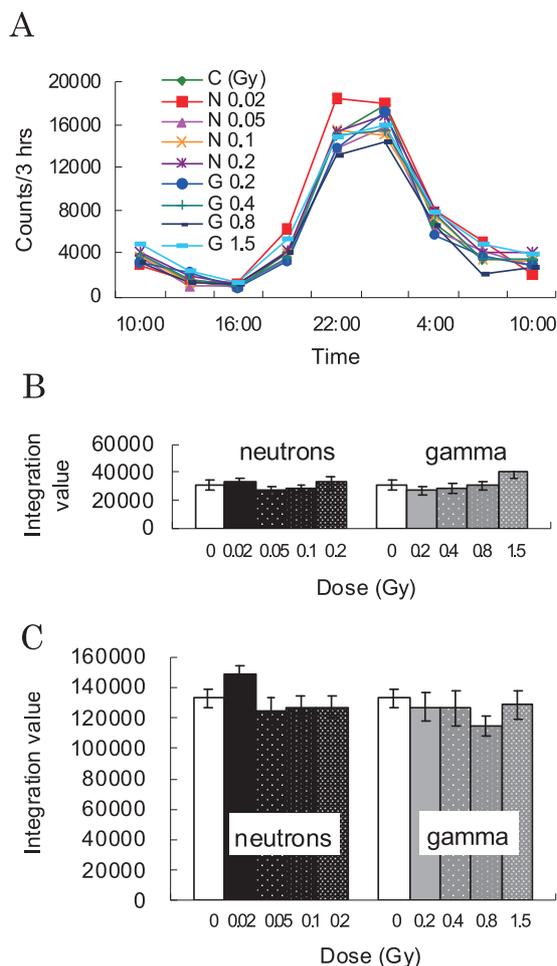
**Table 1.** Body weight and absolute brain weight of mice exposed to neutrons *in utero*.

| Radiation       | Dose (Gy) | Body weight (g) | Brain weight (mg) |
|-----------------|-----------|-----------------|-------------------|
| Non-irradiation | 0         | 30.6 ± 1.7      | 428.0 ± 8.5       |
| Neutron         | 0.02      | 30.5 ± 2.1      | 421.8 ± 11.7      |
|                 | 0.05      | 30.3 ± 1.6      | 422.6 ± 10.6      |
|                 | 0.1       | 30.1 ± 1.4      | 404.4 ± 8.6       |
|                 | 0.2       | 28.1 ± 2.1      | 380.5 ± 4.8*      |

Numerals represents the mean ± S.E..

\*:  $p < 0.01$  vs control (Dunnett's test).

The body weight and brain weight tended to decrease for dose dependence.

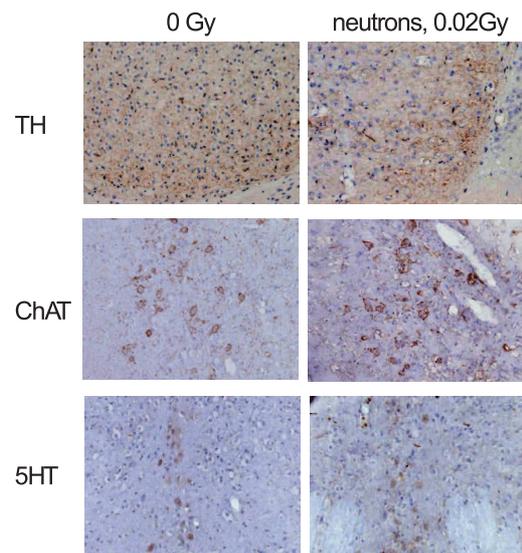


**Fig. 2.** Counts and integration value of locomotor activity about the light period and dark period at the day of 3 in the program. (A) A representative locomotor activity of 24 hrs of mice. Values are average of counts/3 hrs. (B) Integration value of light period, (C) Integration value of dark period. The locomotor activity during dark period increased only in 0.02 Gy neutron-irradiated group though showed no significant differences.

decreased by radiation effects as known well and decreased dose-dependency also in neutron-irradiated groups (Table 1) though the change in muscular force was not confirmed. Especially absolute brain weight decreased significantly in 0.2 Gy.

Many reports suggest that brain weights are reduced in the offspring of pregnant animals exposed to radiation. Antal *et al.*<sup>20)</sup> reported that damage to the central nervous system in the mouse fetus exposed to neutron irradiation at embryonic day 18 is considered to be due to killing and/or inhibition of neuroblasts. Similar results were obtained by Howard and Vogel.<sup>21)</sup> In the present study, both the body and absolute brain weights were decreased in a dose-dependent manner, in spite of no observed change in the radiation quality. In our another study used prenatal mice exposed to neutrons or gamma-rays, thickness of cerebral cortex were measured quantitatively at adult mice. Consequently, the loss of thickness on the cerebral cortex was noted at all of irradiated groups and the body weight decreased for dose dependence. Hypoplasia was observed for the highest dose, especially. It is thought that the decrease in the number of neuronal cells is related to the loss of brain weight. With regard to the neurobehavioral change, the imbalance of the neurotransmitter seems to play more important role than the decreased number of neuroblasts that cause the loss of brain weight or the thickness of cerebral cortex.

The examination of locomotor activity measured the monitored amount of basic activity. Mice are nocturnal creatures,



**Fig. 3.** Histopathological change by immunohistochemical staining. Cyosections were performed immunohistochemical staining in the localization for tyrosine hydroxylase (TH), choline acetyltransferase (ChAT) and the serotonin (5HT) which were a neurotransmitter-related factor. TH; in corpus striatum, ChAT; in tegmental nuclei, 5HT; in raphe nuclei. Histopathological change to three neurotransmitter-related factors was not observed.

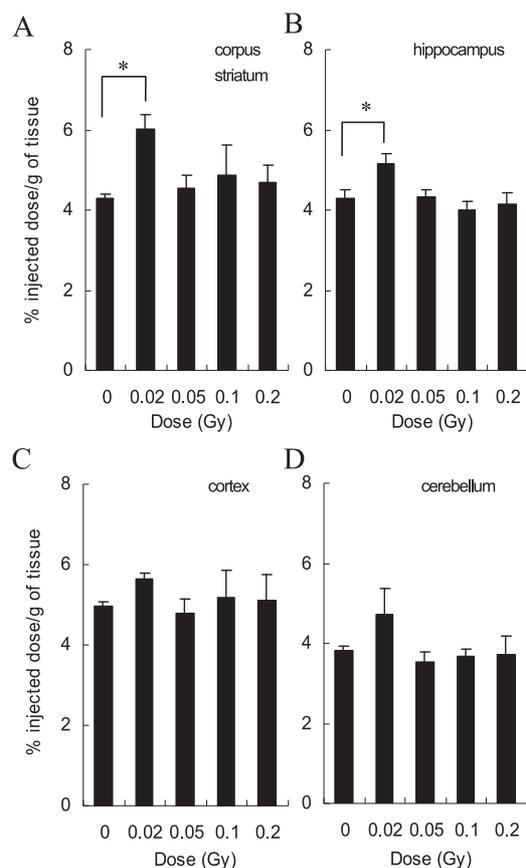
therefore, they remained more active in the dark period as compared to the light period (Fig. 2, A). The amount of motion was totaled during the light and dark period on the third day of the program. Figure 2, B showed integration value of locomotor activity about the light period at the day of 3 in the program. There was slightly increased tendency in the locomotor activity during the light period in the 1.5 Gy gamma-rays-irradiated group compared with control, and the integration value increased dose-response relationship. Figure 2, C showed integration value of locomotor activity about the dark period. There was a trend towards increased the locomotor activity during the dark period despite no significant difference in the 0.02 Gy neutron-irradiated group only. In contrast, there was a trend towards slightly decreased the locomotor activity in all other groups as compared to the control group.

Rota-rod tests, measurement of locomotor activity, and others are typically used to evaluate the effects of chemicals and medicine on behavior by each purpose.<sup>22-25</sup> In the present study, there was no observed muscular force alter-

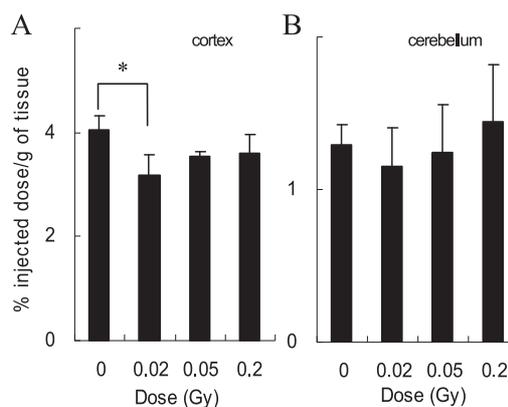
ation or motility disturbance on the Rota-rod test in the irradiation groups as compared to the control group. The locomotor activity during the dark period only increased tendency in the 0.02 Gy neutron-irradiated group with decreases observed among the other groups as compared to the control. Furthermore there was slightly increased tendency in the locomotor activity during the light period only in the 1.5 Gy gamma-rays-irradiated group compared with control, and integration value during same period differed significantly between the four gamma-rays-irradiated groups and increased dose-response relationship. However since such a difference was not shown in gamma-rays groups of other experiments, it is necessary to examine future still in detail. The dose which becomes equivalent biological effects to 0.02 Gy neutron-irradiated group around 1.5 Gy gamma-rays irradiated group may be able to be presumed. These results suggest that behavioral abnormalities may occur with external neutron irradiation at low doses and that these behaviors may differ depending upon the dose range.

There was no change to irradiation in immunohistochemical staining for three neurotransmitter-related factors 0.02 Gy neutron-irradiated group compared with control (Fig. 3). The clear change was observed in high-dose-irradiated groups. Qualitative histological alterations by immunohistochemical staining were not observed any neurotransmitter-related factor.

There was a significant difference in the accumulation of  $^3\text{H}$ -QNB to the mACh receptor in the corpus striatum (Fig. 4, A) and the hippocampus (Fig. 4, B) whereas there was a trend towards increased accumulation despite no significant difference in the cortex (Fig. 4, C) and the cerebellum (Fig. 4, D) by 0.02 Gy in the neutron-irradiated group. The accumulation of  $^3\text{H}$ -NMSP to serotonin receptor which opposes the actions of the mACh receptor was decreased significantly in the cortex (Fig. 5, A) whereas there was a trend towards



**Fig. 4.** Accumulation to receptor in mouse tissue by tracer method. Accumulation of  $^3\text{H}$ -QNB tracer to muscarinic acetylcholine receptor was measured in brain experiment on neutron-irradiated groups (A) corpus striatum, (B) hippocampus, (C) cortex, (D) cerebellum. \*:  $p < 0.01$  (Dunnett's test)



**Fig. 5.** Accumulation to receptor in mouse tissue by tracer method. Accumulation of  $^3\text{H}$ -N-methyl-spiperone tracer to serotonin receptor was measured for neutron-irradiated groups. (A) cortex, (B) cerebellum. \*:  $p < 0.05$  (Dunnett's test)

decreased accumulation despite no significant difference in the cerebellum (Fig. 5, B) by 0.02 Gy in the neutron-irradiated group. Cerebellum is the important organ for motor functions and neuromuscular coordination, and there are the reports that the cerebellum is related to a recognition function recently.<sup>26,27)</sup> In our results, the change of neurotransmitters in the cerebellum, despite not statistically significant, likely contribute to the change of the locomotor activity.

Recently, the hippocampus and cholinergic systems such as choline acetyltransferase and the acetylcholine (ACh) receptor have been shown to play important roles in learning and memory functions. Adult rats which had received 1.5 Gy X-rays at embryonic day 15 showed significant learning disabilities during the water-maze task and were divided into three groups: 1) slightly damaged, 2) mildly damaged, and 3) severely damaged. The severely damaged group demonstrated a remarkable learning disturbance and prominent ectopic neurons present in abnormal locations in the dorsal hippocampus while the slightly damaged group demonstrated no ectopia in the hippocampus. Thus, cognitive dysfunction may at least partly be induced by prenatal exposure to X-ray irradiation attributable to ectopic neurons of the hippocampus.<sup>28)</sup> Momosaki *et al.*<sup>29)</sup> evaluated the effects of prenatal ionizing irradiation on the mechanisms involved in deleterious neuronal and behavioral changes. Brains removed from 9 week old rats who were treated with 1.5 Gy X-ray exposure on embryonic day 15 were evaluated by *in vitro* mACh receptor binding and histological construction. Hippocampus binding of <sup>3</sup>H-QNB was significantly decreased in irradiated rats as compared to sham-treated rats. The mACh receptor binding in the cortex showed no significant change. This study concluded that deleterious alterations in memory and behavior may result from prenatal exposure to ionizing radiation inducing dysfunction of the cholinergic neuronal systems especially in the hippocampus. In our study, accumulation of <sup>3</sup>H-QNB to the mACh receptors are increased in the cortex, striate body, hippocampus, and cerebellum by 0.02 Gy in the neutron-irradiated group. On the other hand, accumulation of <sup>3</sup>H-NMSP to the serotonin receptor, which opposes to the mACh receptor, is decreased in the cortex by 0.02 Gy in the neutron-irradiated group. These results corresponded with the locomotor activity test, however, changes in immunohistochemical staining were not observed. Although there is not as for the significant difference, the change that resembled a change to accumulation of tracer was observed only by locomotor activity, these results indicate that change of locomotor activity might be an indicator for radiation-induced effect more sensitive than histopathological change.

In conclusion, results from the present study suggest that a certain 'low-dose window' may exist for radiation-induced changes in neurobehavior and binding to neurotransmitter receptors. In lower dose than the dose to which the decrease of the brain weight or the thickness of cerebral cortex is

caused, that is, neuronal death is induced, radiation damage seems to appear to the change of neurobehavior or the imbalance of the neurotransmitter by the fetal period exposure. However, the significant changes in the corpus striatum and the hippocampus weren't directly contributed to the change of locomotor activity. Furthermore the cerebellum responsible for motor functions that likely control the locomotor activity wasn't shown significantly change. In neutrons, there may be the dose-range that has a more than expected effects for emotional disturbance on dose that is not yet examined well. Therefore, the biological effect study of the embryonic exposure is still important with the low dose.

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