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Changes in Histological Construction and Decrease in ^{3}H-QNB Binding in the Rat Brain after Prenatal X-irradiation

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X-irradiation / Hippocampus / Histological change / MRI / Muscarinic acetylcholine receptor binding

To elucidate the mechanisms involved in deleterious neuronal and behavioral changes after prenatal ionizing irradiation, in vitro muscarinic acetylcholine (mACh) receptor binding and histological construction were investigated in 9-week old rat brains after 1.5 Gy X-ray exposure on embryonic day 15 (E15). A gross anatomical examination with a magnetic-resonance imaging system showed an irregular tissue construction in the hippocampus and cortex of the irradiated rat brain. Histological sections stained with hematoxylin and eosin also indicated that the structures of the hippocampus and cortex were obviously changed. In irradiated rats, the laminar structure of pyramidal cells was selectively deranged in the CA1 region. *In vitro* ^{3}H-Quinuclidinyl benzilate binding in the hippocampus was significantly decreased (about 10%) in prenatal irradiated rats compared to that in sham-treated rats. On the other hand, no significant change in mACh receptor binding was observed in the cerebral cortex. The present study revealed that prenatal exposure to ionizing radiation may induce dysfunction of the cholinergic neuronal systems, especially in the hippocampus, resulting in deleterious changes in memory and behavior.

INTRODUCTION

A number of studies have reported that the developing brain is sensitive to ionizing radiation[1,2]. Histological changes in the brain following prenatal X-irradiation have been reported in experimental animals and humans[3–7]. Impairments in learning and memory functions have also been observed. Prenatal irradiation at about 1 Gy is known to cause a widevariety of deleterious behavioral changes in animals[8–10], and similar exposure to ionizing radiation during weeks 8–15 of gestation in humans has been associated with an increased incidence of severe metal retardation, as well as reductions in intelligence and the level of school performance[11,12]. However, little is known about the neurochemical mechanisms involved in these radiation effects.

Recently, some studies have shown that the hippocampal region is closely related to the learning and memory functions in animals and humans[13,14]. In the hippocampus, cholinergic systems, such as choline acetyltransferase and acetylcholine (ACh) receptor, play important roles in learning and memory functions[15,16].

In the present study, to elucidate the possible neurochemical mechanisms involved, we assessed any histological changes and changes in the function of the cholinergic systems in the rat brains after prenatal exposure to X-rays. Histological and anatomical

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observations were performed not only with conventional hematoxylin and eosin staining, but also with magnetic-resonance imaging systems. The changes in cholinergic functions were measured with an *in vitro* muscarinic acetylcholine (mACh) receptor binding assay using ^{3}H-Quinuclidinyl benzilate (^{3}H-QNB) in the same irradiated rats as that used for the histology.

MATERIALS AND METHODS

Animals and X-ray irradiation

Male and female Wistar rats (Slc; Hamamatsu, Japan) were housed under a 12-h dark-light cycle and had continuous access to food and water. Eight-week old nulliparous females were paired with potent males in cages overnight and checked for vaginal plugs the next morning. When a vaginal plug was present, this day was considered to be day 0 of pregnancy (E0). Pregnant females were exposed to a single whole-body X-ray irradiation at a dose of 1.5 Gy on E15. The physical factors of the X-rays used were 200 kVp, 15 mA, 0.5 mm Cu + 0.5 mm Al filter, 90 cm distance, and 0.45 Gy/minute exposure rate. Control pregnant mice were treated in the same manner, except for X-irradiation.

The irradiated females' offspring at eight or nine weeks after birth were used in the following experiments. All of the animal experiments were carried out with permission and under the regulation of the Insti-

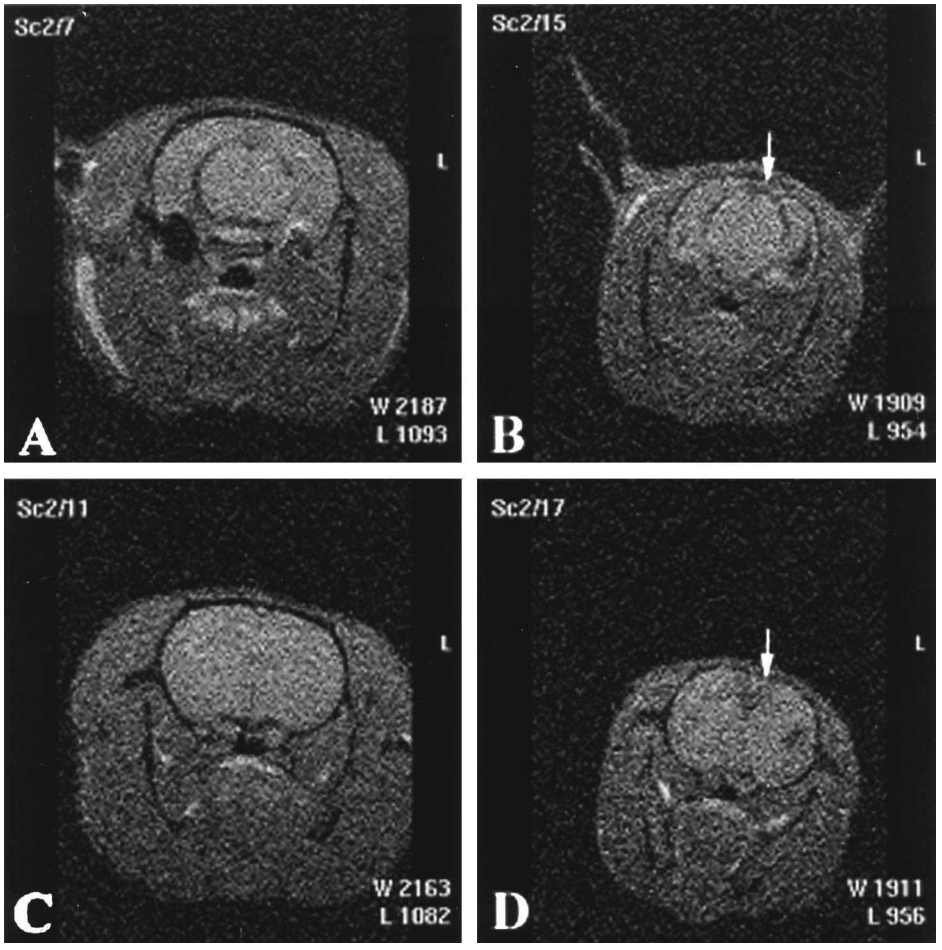

Fig. 1. Typical T1-weighted MRI images at different levels in the brain of 9-week old rats. (A and C). A regular arrangement of brain tissues, such as the cortex, hippocampus and thalamus, is observed in the controls. (B and D). A disarrangement of the tissue structure and a low signal density are recognized in the internal region of the cerebral cortex and the hippocampal area (arrows) in the irradiated animal.
The levels relative to Bregma are 1.5 mm for A and C, and 3.5 mm for B and C, as estimated from the atlas of Swanson[21].

tutional Committee for Animal Safety and Welfare of the National Institute of Radiological Sciences, and in accordance with the Regulations on Appropriate Animal Breeding and Treatment, Ministry Office of Japan.

MRI scan

The gross anatomy of the brain was examined using a clinical MRI system (Gyroscan Intra 1.5T, Philips Medical Systems). Each rat was anesthetized with ketamine (40 mg/kg) and xylazine (10 mg/kg), and coronal T1W images of the head region were obtained with the following settings: factory standard surface coil with an inner diameter of 40 mm; FOV 40.0 mm; multi-slice fast field echo; TE/TR=4.9/50 ms; 22 slices with each thickness of 1.0 mm; slice gap of 0 mm; 3 accumulations; total scan time of 14.5 min.

Histology

Rats were sacrificed by decapitation and the brains were removed. The brains were dissected into two blocks from a position 5 mm away from the Bregma line. One was used for hematoxylin and eosin (H.E.) staining, and the other for in vitro binding. Both of the tissue blocks contained hippocampal regions. The block for the staining was fixed in Bouin's solution, dehydrated, and embedded in paraffin. Cut sections (5 μm) were stained with H.E..

In vitro ^{3}H-QNB binding

The brains for binding were quickly removed and frozen. Coronal sections (30 μm) were prepared on a cryostat at –20°C. The brain sections were preincubated for 10 min at 25°C in 50 mM sodium-potassium (Na-K) phosphate buffer (pH 7.4), and then incubated with 1 nM of ^{3}H-QNB (1.8 GBq/μmol; New England Nuclear, Boston, MA, USA) for 60 min in a corresponding buffer at 25°C. These labeled sections were exposed to a ^{3}H-Imaging Plate (BAS-TR; Fuji Photon Film) together with a ^{3}H-microscale (RPA-507; Amersham, UK). After 3 days of exposure, the images were analyzed. The radioactivity concentrations in the hippocampus and cerebral cortex were determined using a Bio-Imaging Analyzer System (BAS-1500; Fuji Photo Film), and expressed as photo-stimulated lumi-

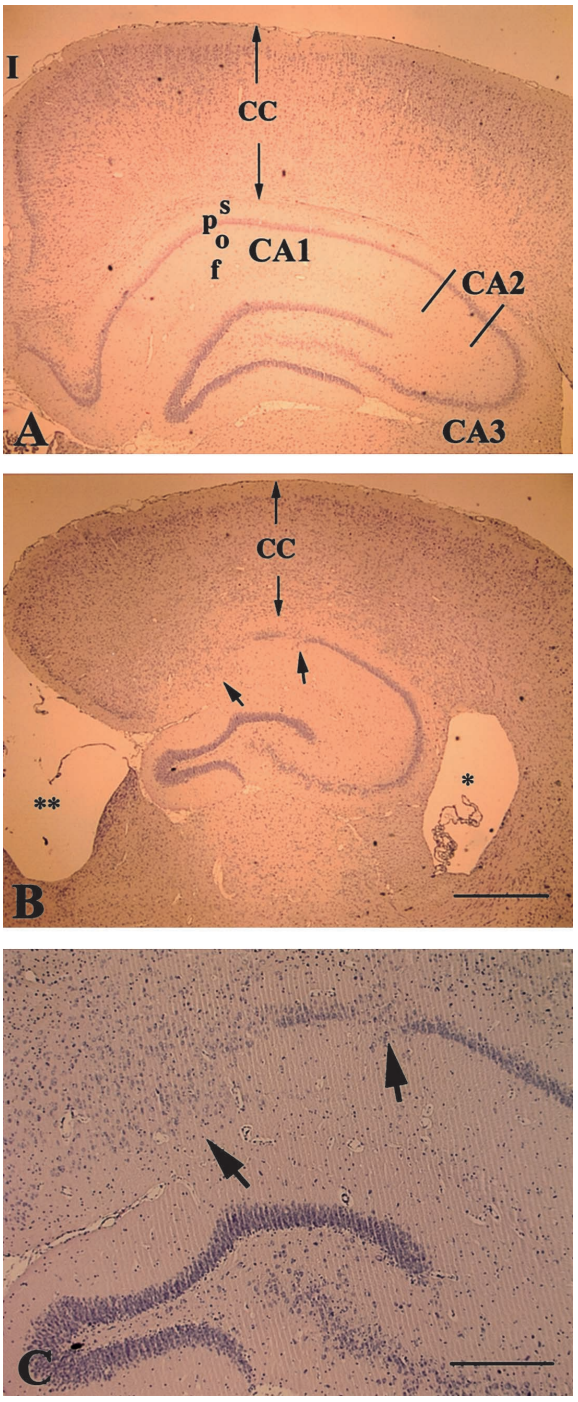

Fig. 2. Micrographs of the hippocampus of 9-week old rats. A shows a normal laminar organization and three fields (CA1, CA2 and CA3) of the hippocampus in the control rat. CC is the cerebral cortex. B shows laminar structure disordered and ectopic cell mass (arrows) in the CA1 area of the hippocampus in the irradiated rat. The asterisks (*) indicate an enlarged ventricle. C shows a higher magnification view of B. s, stratum lacunosum-moleculare; p, pyramidal cell layer; o, stratum oriens; f, fiber-containing alveus; I, internal region of the cerebral cortex; H.E., stain; scale bar, 500 μm for A and B, and 250 μm for C.

nescence values (PSL/mm^2).

RESULTS

The MRI images are shown in Fig. 1. Regular arrangements of brain tissues, such as the cortex, hippocampus and thalamus, were observed in the controls. A disarrangement of the tissue structures and a low signal density were recognized in the internal regions of the cerebral cortex and the hippocampal area (arrows in Fig. 1) in the irradiated animals. The areas with abnormal tissue arrangements corresponded well to the areas with histological changes observed by H.E. staining. The low signal density observed in the cortex and hippocampus region of the irradiated animals may reflect an enlargement of the ventricles (asterisks in Fig. 2) or sinus that was caused by an atrophic reaction of neuronal tissues to the radiation, since a low signal density indicates hard tissue or a space filled with tissue fluid, such as a ventricle under the MRI setting used here.

A histological examination showed a regular laminar organization in the hippocampi of the control rats.

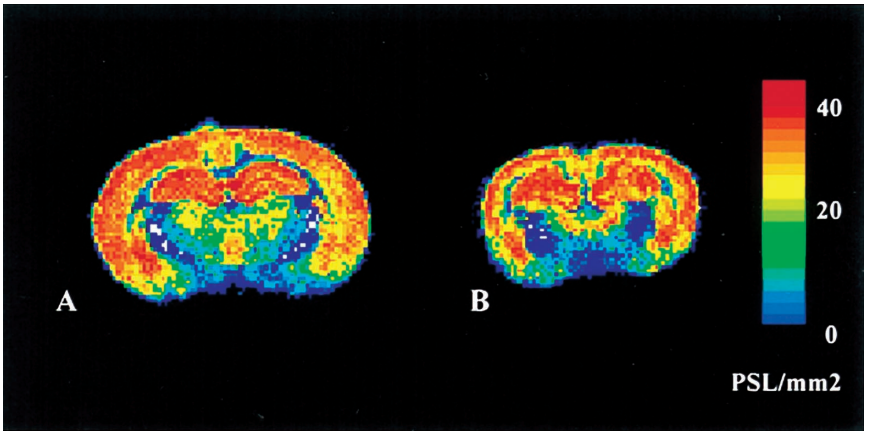

Fig. 3. Typical autoradiograms of *in vitro* ^{3}H-QNB binding in the brain. A shows a sham-treated rat brain and B an irradiated rat brain.

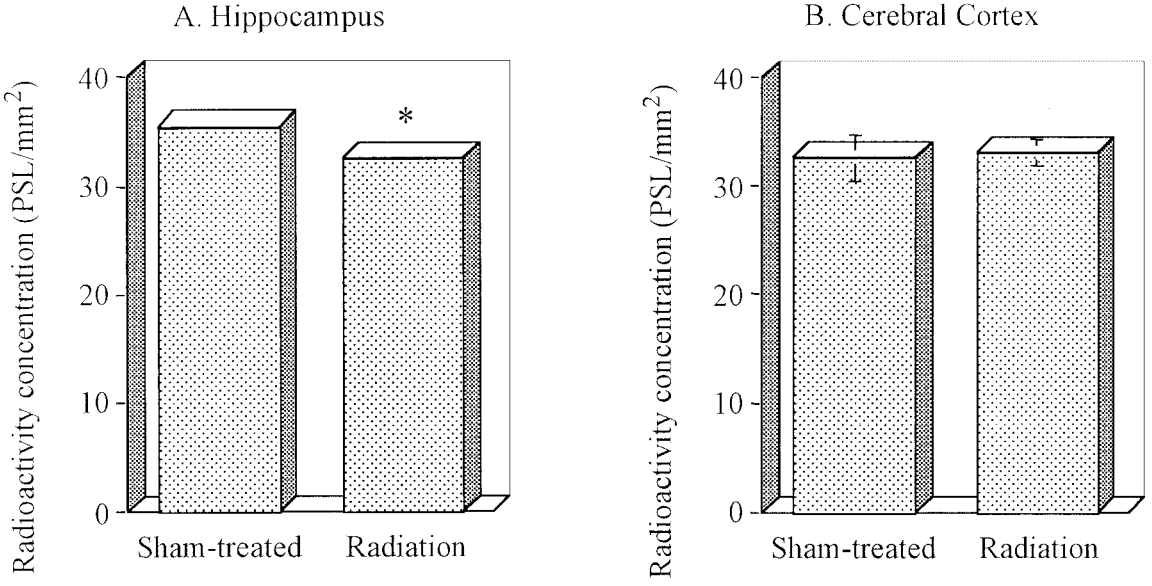

Fig. 4. Result of a quantitative analysis by BAS. The values are expressed as PSL/mm^2 (photo-stimulated luminescense). Each value is the mean ± S.D. of 3 animals. * P<0.01 vs. sham-treated (Student's t-test).

The cellular layers from the stratum lacunosum-moleculare, pyramidal cell layer and stratum oriens to the fiber-containing alveus could be identified in the fields of CA1, and CA2 (Fig. 2A). In the irradiated group, such cell laminar structures were disordered in the CA1 area. Neurons of various types (pyramidal neuron and non-pyramidal neuron), identified on H.E. stained sections based on their cell body size and their shape, were mixed together and formed ectopic neuronal masses located between the internal region of the cerebral cortex and the CA1 area (Fig. 2B, 2C). Pyramidal neurons in the ectopic neuronal masses were distributed randomly.

Autoradiograms of the *in vitro* ^{3}H-QNB binding assay are shown in Fig. 3. The radioactivity concentrations within the region of interest (ROI) covering the entire hippocampus were decreased by about 10% in prenatal irradiated rats (Fig. 4A), suggesting a significant decrease in the number of binding sites or binding affinity. In contrast, no significant change in ^{3}H-QNB binding was observed in the cerebral cortex (Fig. 4B), although histological changes were apparently present at the same position.

DISCUSSION

Histological abnormalities observed in the cortex and hippocampus after prenatal X-irradiation were well consistent with those of previous reports[3–7]. Miki *et al*. (1999) revealed in rats exposed to 1.2 Gy X-ray on E15 that the laminar structure was disarranged, or had partially disappeared, in the CA1 region, and that pyramidal cells in the CA1 region were ectopically located into cortex areas. In the present experiments, a similar derangement was observed in the CA1 region at a dose of 1.5 Gy X-rays on E15. One of the important implications of the present study is that the necessary operation parameters for an MRI analysis could be established for detecting gross-anatomical abnormalities, which had been confirmed by H.E. staining. Because MRI scanning is a non-invasive technique, it may be possible for long-term histological changes after prenatal irradiation in the same subject to be followed by this modality.

As shown in Fig. 4, *in vitro* ^{3}H-QNB binding was significantly decreased in the hippocampus, suggesting a dys- or mal-function of the cholinergic neuronal systems. It has been reported that prenatal irradiation may induce deleterious effects on learning and memory[9]. Therefore, it seems that changes in the cholinergic neuronal systems of the hippocampus, measured here as a decrease in ^{3}H-QNB binding, may, at least in part, be related to impairments of learning and memory functions, since the cholinergic systems, such as choline acetyltransferase and the acetylcholine (ACh) receptor, play important roles in learning and memory functions[15,16].

mACh receptors have been classified into two main subtypes: M_1 and M_2[12,13]. In Alzheimer's disease, the number of M_1 receptor subtypes is decreased[10]. It is very important to examine whether the M_1 receptor subtype is also selectively decreased by prenatal X-irradiation. In addition, B_{max} (the maximum number of binding sites available) and K_D (affinity constant) can be considered as factors in any decrease of *in vitro* ^{3}H-QNB binding. In order to determine whether B_{max} or K_D was decreased by embryonic exposure, a saturation study of ^{3}H-QNB binding will be necessary in a future study.

In the cerebral cortex, Sun *et al*. (1999) reported that neurons in the dorsal regions formed a unique four-layered cortex in irradiated mice compared to controls[20]. Fushiki *et al*. (1997) also demonstrated disarrangement of neuronal cells in the cortex after long-term and short-term premature X-ray exposure[5]. In the present paper, MRI and HE histology clearly indicated decrease in size as well as disarrangement of the tissue structure, including the enlarged ventricles, atrophic tissues, thinner cortex, and ectopic gray matter. In contrast, *in vitro* ^{3}H-QNB binding in the cerebral cortex was not affected by the irradiation. These results demonstrated that the histological changes did not directly correlate to the neuro-chemical functions, such as mACh receptor binding, at least, in the cortical region. However, it should be noted that such a study of *in vitro* binding using brain slices has limitations, since large individual variations of both biochemical

and histological alterations may be supposed. An i*n vivo* binding study by PET (positron emission tomography) or SPECT (single photon emission computed tomography) will be valuable to selectively follow the long-term changes in mACh receptor binding, including the receptor subtype.

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