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研究課題名（和文）The underlying mechanisms of synaptic dysfunction after the irradiation

研究課題名（英文）The underlying mechanisms of synaptic dysfunction after the irradiation

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研究成果の概要（和文）：Pretreatment of N-methyl-D-aspartate (NMDA) receptor antagonist before irradiation on the brain indicates that NMDA receptor mediates X-irradiation-induced drebrin decrease, which may be useful for the prevention of radiation-induced synaptic dysfunction.

研究成果の学術的意義や社会的意義
The effects of radiation on the CNS are of significant interest. In this study suggests that NMDAR-mediated drebrin reduction underlies X-irradiation-induced acute transient cognitive impairment, which may be a basis of further research for radiation protection on the brain.

研究成果の概要（英文）：Previously we have shown that radiation may produce acute temporary cognitive impairment via decreases of postsynaptic protein drebrin from the synapse within 24 hours. In order to reveal the mechanisms radiation-induced synaptic dysfunction and its prevention, the ten-weeks-old C57BL/6N male mice were pretreated by injection of saline or N-methyl-D-aspartate (NMDA) receptor antagonist MK801 ten minutes before of a whole brain of irradiation, and then the immunoreactivity of postsynaptic protein drebrin was analysed on molecular layer of DG of Hippocampus 8 hours following X irradiation. Our results show there was a decrease in the immunoreactivity of drebrin and the N-methyl-D-aspartate (NMDA) receptor antagonist MK801 prevented it. These results indicate that the NMDA receptor mediates X-irradiation-induced drebrin decrease possibly via drebrin exodus.

研究分野：放射線神経生物

キーワード：X-irradiation　cognitive impairment　synaptic dysfunction　drebrin　NMDA receptors

様　式　C－１９、F－１９－１、Z－１９、CK－１９（共通）

１．研究開始当初の背景

[Irradiation and synaptic dysfunction]

Radiation is the emission of energy in the form of waves or particles through space or material medium. The effect of radiation has been a great interest since it has been widely used in the field of medicine as diagnostic and therapeutic modalities. Radiation may affect the brain homeostasis and may disrupt the brain function, including cognitive function. Although mature neurons in the brain are known to be radioresistant, several studies showed that irradiation might alter the function of neurons, such as synaptic dysfunction. Synaptopathy or synaptic dysfunction is known to underlie cognitive impairment. The previous study showed that a single high dose of 10 Gy radiation could cause acute transient synaptic dysfunction [1 and 2]. While a single lower dose of 1 Gy of irradiation causes the late effect on the synaptic function [1]. Previously, we have shown that a single dose of 10 Gy of radiation to the whole brain causes a transient acute decrease in fear memory formation. This transient decrease is thought to be underlying by the temporary reduction of postsynaptic protein, drebrin, *in vivo* [2]. Newly generated neurons known to have a major role in cognitive function and it's radiosensitive. Interestingly, the number of positive newly generated neurons were decreased while the fear memory formation is returned to the normal level. These results suggest that the synaptic dysfunction underlies the acute transient deficit in the fear memory formation after the irradiation and not by cell death [2].

[The involvement of astrocytes in the synaptic dysfunction after the radiation exposure]

There is an acute transient decrease in fear memory formation within 24 hours after the radiation exposure. And this acute transient decrease in parallel with the temporary decrease of postsynaptic protein, drebrin [2]. The temporary elevation of calcium (Ca^{2+}), which could be increased by radiation-induced brain edema has been thought to underlie of transient synaptic dysfunction. Drebrin is an actin-binding protein which is localized in the dendritic spines of mature neurons [3]. The activation of NMDA receptors is causing the Ca^{2+} influx into the cells, and it induced the distribution changes of drebrin from dendritic spines to the dendritic shaft [4]. Shortly after, it was proposed that Ca^{2+} entry through NMDARs was particularly effective at killing neurons compared to entry through other channels, and the dependent rate-limiting processes may trigger early neuronal degeneration [5]. The NMDA receptors (NMDARs) could be activated by the glutamates that release by presynaptic sites. Glutamate-triggered excitotoxicity is a major cause of neurotoxic many acute incidents of brain injuries, and it is believed to play a role in chronic neurodegenerative disorders. This dynamic release and reuptake between astrocytes-neurons lead to the dynamic changes of neuronal potential and synaptic function, which is essential for cognitive function and higher brain function. The balance in release and reuptake by astrocytes is necessary for neuroprotection effects.

２．研究の目的

The purpose of this study is to investigate the underlying mechanisms of synaptic dysfunction, focusing not only on neurons but also on astrocytes after exposure to the radiation.

３．研究の方法

(1) High-throughput primary hippocampal culture method.

The hippocampal neurons from embryonic 18-day-old Wistar rats were dispersed with trypsin and dispensed into serum tubes then stored in liquid nitrogen before seeding. The hippocampal neurons were thawed in 37 ℃ and seeded with density from 5000-10.000/well in a Poly-L-lysine coated 96 well plates (Greiner Bio-One, Germany). Neurobasal Medium (Gibco, Grand Island, NY) with B27 supplement (Gibco, Grand Island, NY), penicillin/streptomycin (Gibco, Grand Island, NY) and L-alanyl-l-glutamine (Gibco, Grand Island, NY) was used as culture medium. Ara-C (0.2 μM final concentration) was added into medium to inhibit glial proliferation at 4 DIV.

(2) Banker's methods with slight modification.

Primary hippocampal cultured neurons were prepared as previously described [6] using Banker's methods with slight modification. The 21 DIV neurons or Glial cells were irradiated and also pretreated with the NMDA receptor antagonist APV with concentration 50 μM an hour before 10 Gy X-irradiation (1.3 Gy/min, Shimadzu X-TITAN 225S X-ray generator, Shimadzu Inc., Kyoto, Japan). The cells were fixed at 8 hours after X-irradiation.

(3) Immunocytochemistry

The cultured neurons were fixed in 4% paraformaldehyde in phosphate buffer for 20 minutes at room temperature. Fixed cells were permeabilised with 0.1% Triton X-100 and blocked with 3% bovine serum albumin. Mouse monoclonal antibody against drebrin (clone M2F6, 1:1) [3] was used as a primary antibody and were incubated overnight at 4 °C. After washing with PBS, neurons were incubated for 1 hour at room temperature with secondary antibody. Alexa 647-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR) was used as a secondary antibody. F-actin was labelled with rhodamine-conjugated phalloidin.

(4) Animal studies

To observe the effects of irradiation and the involvement of NMDA receptors after irradiation on the brain. Ten-week-old male mice (C57BL/6N, Japan SLC Inc., Shizuoka, Japan) were injected with NMDA receptors antagonist MK801 (2 mg/kg, i.p, Sigma-Aldrich, St. Louis, MO) or the same volume of saline 10 minutes before whole brain X-irradiation. Mice were anaesthetized using 2.5% tribromoethanol (250 mg/kg, Sigma-Aldrich, St. Louis, MO) and irradiated with 10 Gy X-irradiation (1.3 Gy/min, Shimadzu X-TITAN 225S X-ray generator, Shimadzu Inc., Kyoto, Japan).

(5) Immunohistochemistry

Eight hours after irradiation, mice were anaesthetized then perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brain tissue was removed and transferred into 30% sucrose in phosphate buffered saline (PBS) until equilibration the brain stored at -80°C in Tissue-Tek® O.C.T.™ Compound. Frozen tissue was cut into 20 µm thick coronal sections using Leica CM3050 S Research Cryostat (Leica Microsystems, Wetzlar, Germany). The brain sections were permeabilised with 0.1% Triton X-100 and incubated with 3% bovine serum albumin in PBS. Immunolabelled with a rabbit polyclonal antibody against drebrin A (DAS2, 1:200; Immuno-Biological Laboratories Co., Maebashi, Japan) in 4 °C for overnight. Then labelled with Alexa 488-conjugated anti-rabbit IgG antibody (Molecular Probes, Eugene, OR). Nuclei were labeled using 4',6-diamidino-2-phenylindole (DAPI). The brain sections were mounted with PermaFluor (Lab Vision Co., Fremont, CA).

(6) Quantification and statistical analysis

To analysed drebrin cluster *in vitro*, we used Drebrin Imaging-Based Evaluation of mature Synapse method [7]. The clusters of drebrin defined as a region with a fluorescent intensity that was two-fold higher than the average fluorescent intensity of the dendritic shaft. Images of a 96-well plate were acquired using IN Cell Analyzer 2200 (GE Healthcare Life Sciences, Pittsburgh, PA) automatically.

To analysed drebrin immunointensity *in vivo,* four regions of interest in the molecular layer of the dentate gyrus of the hippocampus were randomly chosen, and the immunostaining intensity of the regions was automatically obtained with MetaMorph analysis software.

4. 研究成果

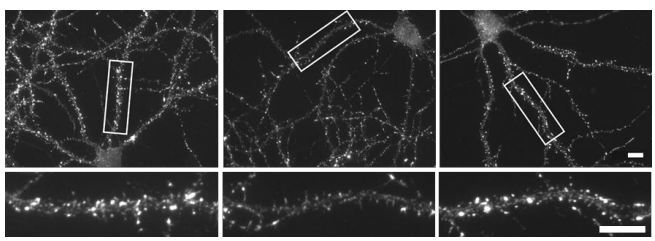
(1) Drebrin cluster decreased after 10 Gy X-irradiation on High-throughput put analysis.

The cultured neurons on 96 wells plated were irradiated at 21 DIV and fixed at 6 hours after. The number of drebrin cluster in the irradiated group decreased significantly comparing sham group, and there is no change in the number of neurons and dendrite length. Using this method allowed us to observe the direct effects of irradiation on neurons with minimum effects of the presence of glial cells.

(2) N-Methyl-D-aspartate receptor mediates X-irradiation-induced decrease of drebrin clusters *in vitro*.

To examine whether the decrease in X-irradiation-induced drebrin immunostaining intensity is due to the activation of NMDARs and presence of glial cells, the primary hippocampal neuronal culture which prepared by Banker`s method was used and analysed the direct effect of X-irradiation on drebrin accumulation at dendritic spines. The neurons at 21 DIV were pretreated with APV 1 hour before 10 Gy X-irradiation then fixed at 8 hours after the

Figure 1. N-Methyl-D-aspartate receptor mediates X-irradiation-induced decrease of drebrin *in vitro*

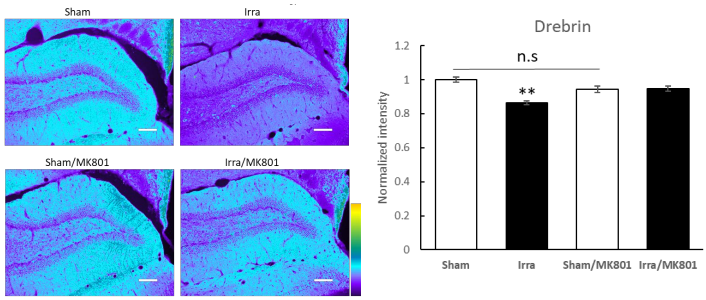


Sham Irra Irra/APV

irradiation. The number of drebrin clusters measured, as described previously [7]. The number of drebrin clusters showing that X-irradiation significantly decreased the drebrin cluster number after 8 hours. On the other hands, the number of drebrin clusters in the irradiated neurons was preventing the decreases of drebrin after irradiation, and it is similar to that in sham neurons (Figure 1). Scale bars 10 μm.

(3) N-Methyl-D-aspartate receptor mediates X-irradiation-induced drebrin decrease in the hippocampus. A whole brain of mice was irradiated with 10 Gy X-rays and analyzed the intensity of drebrin in the molecular layer of the dentate gyrus (Figure 2). The drebrin intensity of each group was normalized using the average intensity of sections from sham mice without MK801, as described previously [2]. As shown in Figure 2 The intensity of drebrin after 10 Gy X-irradiation without MK801 decreased significantly compared to the sham group (Sham, n = 32 slices from 6 mice, Irradiation, n = 28 slices from 6 mice, $p < 0.01$), which was consistent with our previous result [2]. The pretreated mice with MK801, an NMDA receptor antagonist, 10 minutes before X-irradiation showing no difference of drebrin intensity between sham and X-irradiated groups. These results suggest that MK801 has a protective effect on X-irradiation-induced decrease of drebrin immunostaining intensity. Scale bars 100 μm.

Figure 2. N-Methyl-D-aspartate receptor mediates X-irradiation-induced decrease of drebrin *in vivo*



Our study suggests that X-irradiation on neurons cause to decrease of drebrin clusters, and it did not affect by the presence of glial cells. Pretreatment with NMDA receptors antagonists before the irradiation might be a potential therapy for X-irradiation-induced cognitive impairment. Before the potential therapy application, further study of pathological activation of NMDA receptors after X-irradiation and physiological activation are needed.

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〔その他〕

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６．研究組織

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