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Chronic irradiation with low-dose-rate ^{137}Cs-γ rays inhibits NGF-induced neurite extension of PC12 cells via Ca^{2+}/calmodulin-dependent kinase II activation

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ABSTRACT

Chronic irradiation with low-dose-rate ^{137}Cs-γ rays inhibits the differentiation of human neural progenitor cells and influences the expression of proteins associated with several cellular functions. We aimed to determine whether such chronic irradiation influences the expression of proteins associated with PC12 cells. Chronic irradiation at 0.027 mGy/min resulted in inhibition of NGF-induced neurite extension. Furthermore, irradiation enhanced the nerve growth factor (NGF)-induced increase in the phosphorylation of extracellular signal–regulated kinase (ERK), but did not affect the phosphorylation of NGF receptors, suggesting that irradiation influences pathways unassociated with the activation of ERK. We then examined whether irradiation influenced the Akt—Rac1 pathway, which is unaffected by ERK activation. Chronic irradiation also enhanced the NGF-induced increase in Akt phosphorylation, but markedly inhibited the NGF-induced increase in Rac1 activity that is associated with neurite extension. These results suggest that the inhibitory effect of irradiation on neurite extension influences pathways unassociated with Akt activation. As Ca^{2+}/calmodulin-dependent kinase II (CaMKII) is known to inhibit the NGF-induced neurite extension in PC12 cells, independent of ERK and Akt activation, we next examined the effects of irradiation on CaMKII activation. Chronic irradiation induced CaMKII activation, while application of KN-62 (a specific inhibitor of CaMKII), attenuated increases in CaMKII activation and recovered neurite extension and NGF-induced increases in Rac1 activity that was inhibited by irradiation. Our results suggest that chronic irradiation with low-dose-rate γ-rays inhibits Rac1 activity via CaMKII activation, thereby inhibiting NGF-induced neurite extension.

KEYWORDS: ^{137}Cs-γ rays, Ca^{2+}/calmodulin-dependent kinase II, low-dose irradiation, nerve growth factor, neurite extension

INTRODUCTION

The effects of low-dose or low-dose-rate irradiation on neuronal cells have drawn increasing attention in recent years. Research has indicated that chronic irradiation with low-dose-rate ^{137}Cs-γ rays inhibits the differentiation of human neural progenitor cells in a dose-dependent manner [1]. Furthermore, such chronic irradiation has been shown to influence the expression of proteins associated with cell adhesion, apoptosis, and metabolism, among other functions [1]. Similarly, additional studies have reported that low-dose or low-dose-rate ^{137}Cs-γ radiation inhibits neuronal differentiation

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of neuroblastoma and neural stem–like cells by inhibiting the expression of proteins associated with cell cycle and cell proliferation [2]. In contrast, we have previously reported that transient irradiation with X-rays promotes NGF-induced neurite extension in PC12 cells [3]. Similarly, low-dose-rate ^{137}Cs-γ radiation has been reported to facilitate neurite extension in mouse neural stem–like cells [4]. These findings suggest that low-dose or low-dose-rate radiation have substantial impacts on the differentiation of neural cells and may produce completely opposite effects, according to dose amounts or dose rates. Despite these findings, however, the precise mechanisms by which low-dose or low-dose-rate irradiation modifies intracellular signals to affect neuronal differentiation remain unclear.

Previous studies have elucidated the differentiation signal induced by nerve growth factor (NGF), which causes PC12 cells to differentiate into neural cell–like cells, and this has been widely utilized in developing models of neuronal differentiation [5–7]. NGF-induced activation of specific receptors is followed by activation of extracellular signal–regulated protein kinase (ERK) via Raf and Ras, which in turn induces the expression of a variety of proteins that together constitute the function of a neural cell [5–8]. In addition, PI3 kinase–induced activation of Akt is followed by activation of Rac1 and Cdc42, which in turn induces neurite extension and helps to shape the morphology of neural cell–like cells [5–8]. Thus, models of neural differentiation with known patterns of intracellular signaling serve as a highly effective tool for analyzing intracellular signals associated with the modification of neuronal differentiation by means of low-dose or low-dose-rate irradiation. In the present study, we aimed to elucidate the effects of chronic, low-dose-rate ^{137}Cs-γ radiation on NGF-induced neurite extension in PC12 cells, and to examine fluctuations in intracellular signaling associated with this process.

MATERIALS AND METHODS

Reagents

We utilized the following reagents to investigate the effects of chronic, low-dose-rate ^{137}Cs-γ radiation on NGF-induced neurite extension in PC12 cells: NGF 2.5S (N-100, Alomone Labs, Jerusalem, Israel); anti-ERK1/ERK2 mouse-monoclonal antibody (MAB1576) and anti-phosphorylated ERK1/ERK2 rabbit-polyclonal antibody (MAB1018, both R & D systems, Minneapolis, MN, USA); anti-Trk A rabbit polyclonal antibody (sc-118) and anti-phosphorylated Trk A mouse-monoclonal antibody (sc-8058, both Santa Cruz Biotechnology, Dallas, TX, USA)—Trk A is a high-affinity nerve growth factor receptor; anti-tyrosine hydroxylase rabbit-monoclonal antibody (ab137869); anti-Akt rabbit polyclonal antibody (ab8805) and anti-phosphorylated Akt rabbit monoclonal antibody (ab81283); anti-Ca^{2+}/calmodulin-dependent kinase II (CaMKII) rabbit polyclonal antibody (ab131468) and anti-phosphorylated CaMKII rabbit polyclonal antibody (ab5683, all Abcam plc, Cambridge, UK); KN-62 (I2142, Sigma-Aldrich, St Louis, MO, USA).

Neuronal extension assay

Rat pheochromocytoma cells (PC12) were obtained from the RIKEN Cell Bank (Ibaraki, Japan) and were maintained as previously described [9]. The cells were cultured in a 75-cm^2 flask at 37°C in a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DF medium 1:1, vol/vol) containing 15 mM HEPES buffer (pH 7.4), 50 units/ml penicillin G, 0.1 mg/ml streptomycin sulfate, 5% horse serum and 5% fetal calf serum in a 5% CO_2 humidified atmosphere.

For the neuronal extension assay, cells were cultured in serum-containing medium in 75-cm^2 collagen-coated flasks for 3 h. Cells were treated with 50 ng/ml NGF in serum-free medium (DF medium containing 15 mM HEPES buffer [pH 7.4], 50 units/ml penicillin G, 0.1 mg/ml streptomycin sulfate, and ITS Premix [Becton Dickinson, Franklin Lakes, NJ, USA]), for 5 days. Lengths or numbers of neurites were quantified using nerve axon image analysis software Ver 1.50 (Kurabo, Osaka, Japan) [3, 10]. The average length (μm) per single cell and number of neurites per single cell were calculated and expressed as a ratio relative to the non-irradiated group [3].

^{137}Cs γ irradiation

Irradiation with ^{137}Cs γ-rays was performed using an irradiation system at the Radiation Biology Center of Kyoto University, in conjunction with NGF stimulus onset, for 5 days. The radiation dose-rate was 0.027 mGy/min (total dose: 194 mGy).

Western blot analysis

Proteins were extracted using the CytoBuster™ Protein Extraction Reagent (Merck Millipore, Billerica, MA, USA) and concentrated or diafiltered with an Amicon Ultra device (Merck Millipore). Cell lysates were subjected to SDS-PAGE (12.5%), followed by western blotting. The target proteins were visualized using a WesternBreeze® Chemiluminescent Kit (Thermo Fisher Scientific, Waltham, MA, USA) and were detected using a Molecular Imager VersaDoc™ MP 5000 (Bio-Rad, Hercules, CA, USA). The band intensity of western blots was determined by densitometry with Image J and the mean value was obtained. The degrees of phosphorylation of signal proteins were expressed as the relative ratio of the amount of phosphorylated signal protein compared with the amount of the intact signal protein, i.e. phosphorylated NGF receptor/NGF receptor (P-NGFR/NGFR), phosphorylated Erk/Erk (P-Erk/Erk), phosphorylated Akt/Akt (P-Akt/Akt) or phosphorylated CaMKII/CaMKII (P-CaMKII/CaMKII).

Measurement of Rac1 activity

Rac1 activity was determined using an absorbance-based G-LISA Rac1 Activation Assay Biochemistry Kit (Cytoskeleton, Denver, CO, USA), according to the manufacturer's instructions. Activity was expressed as a relative ratio to the non-irradiated group.

Inhibition of CaMKII

CaMKII activity was inhibited using KN-62, a specific inhibitor of CaMKII that suppresses the autophosphorylation of Ca^{2+}/CaM kinase in PC12 D cells [11], a subclone of PC12 cells that are characterized by a very rapid extension of neurites in response to NGF [12]. Based on this previous report, PC12 cells in this study were

simultaneously treated with 10 μM KN-62 under conditions of NGF stimulation.

Statistical analysis

Statistical analyses were performed using Excel 2010 software (Microsoft Corp., Redmond, WA, USA). Data were expressed as mean ± standard error. Statistical significance between the control and irradiated group was determined by Student's *t* test. Differences with a *P* value < 0.05 were considered to be significant.

RESULTS

NGF stimulation clearly induced neurite extension in PC12 cells, as well as their subsequent alteration into neural cell–like cells (Fig. 1a). The proportion of cells that showed neuronal extension was 11.3 ± 0.39% in NGF-untreated cells and 91.8 ± 1.87% in NGF-treated cells. The average length and number of neurites after 5 days of NGF-treatment were 96.7 ± 13.0 μm/cell and 3.07 ± 0.46/cell, respectively. In contrast, chronic irradiation with low-dose-rate γ-rays resulted in substantial inhibition of this NGF-induced neurite extension (Fig. 1a–c). The average length and number of neurites after 5 days of irradiation were 34.8 ± 2.77 μm/cell and 1.58 ± 0.10/cell, decreasing by approximately 35% and 50%, respectively, when compared with those of the non-irradiated group (Fig. 1b and c). In contrast, the neurite extension of NGF-untreated cells was unaffected by irradiation (Fig. 1a–c), suggesting that irradiation suppressed specific signaling steps involved in NGF-induced neurite extension.

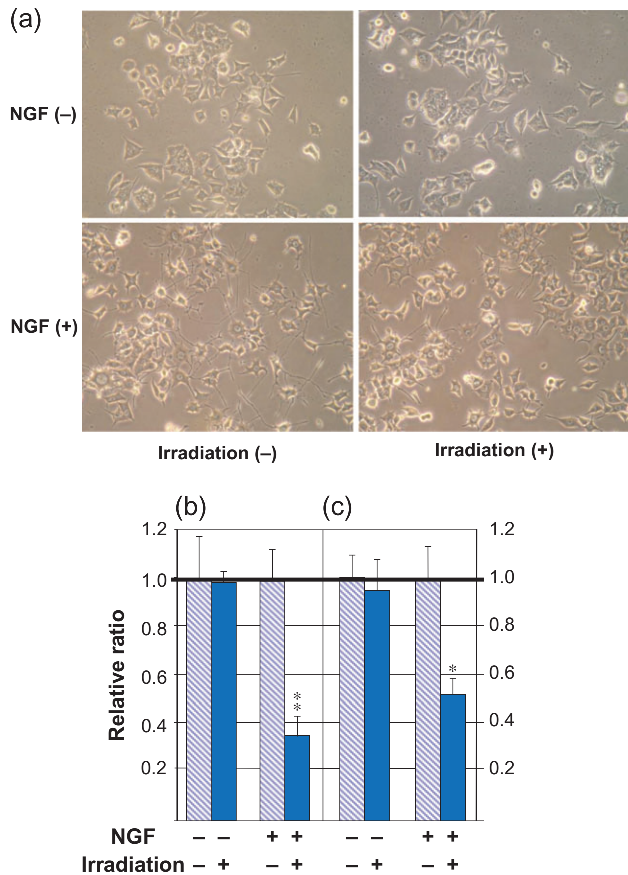

Fig. 1. Irradiation with ^{137}Cs γ-rays depresses NGF-induced neurite extension in PC12 cells. (a) Phase-contrast micrographs, (b) lengths of neurites and (c) numbers of neurites in PC12 cells after 5 days of NGF stimulation or non-stimulation, with or without ^{137}Csγ-ray irradiation. Lengths or numbers of neurites are expressed as the relative ratio to the non-irradiated group. Data are presented as the mean ± standard error of triplicate samples. *$P < 0.05$, **$P < 0.01$ vs non-irradiated group. NGF = nerve growth factor.

We subsequently examined whether chronic irradiation with low-dose-rate γ-rays affects the phosphorylation of NGF-induced NGF receptors. Even though NGF clearly enhanced phosphorylation of receptors (Fig. 2a: the relative ratios of Lane 1 and Lane 3 were 0.51 ± 0.02 and 0.87 ± 0.05, respectively), irradiation scarcely affected the degree of phosphorylation (Fig. 2a: the relative ratios of Lane 3 and Lane 4 were 0.87 ± 0.05 and 0.85 ± 0.08, respectively). In contrast, phosphorylation of NGF receptors was weak in NGF-untreated cells, regardless of whether irradiation was applied (Fig. 2a: the ratios of Lane 1 and Lane 2 were 0.51 ± 0.02 and 0.62 ± 0.06). We then assessed the activation status of ERK as a downstream target of NGF receptors. NGF markedly increased ERK phosphorylation, which was further increased by irradiation

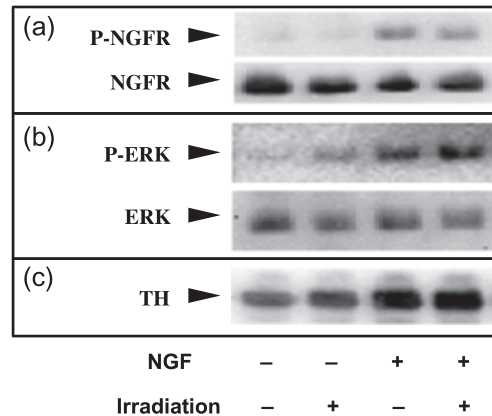

Fig. 2. Western blot analysis of NGF stimulation–related proteins in PC12 cells. Immunoblot showing varying levels of (a) phosphorylated NGF receptor (P-NGFR, upper) and NGF receptor (NGFR, lower), (b) phosphorylated ERK (P-ERK, upper) and ERK (lower), (c) tyrosine hydroxylase (TH) observed in PC12 cells after 5 days of NGF stimulation or non-stimulation, with or without ^{137}Csγ-ray irradiation. Similar results were obtained in three separate experiments. NGF = nerve growth factor, ERK = extracellular signal–regulated kinase.

(Fig. 2b: the ratios of Lane 1, Lane 2 and Lane 3 were 0.58 ± 0.05, 0.82 ± 0.07 and 0.93 ± 0.03, respectively). Furthermore, irradiation also increased ERK phosphorylation in NGF-untreated cells (Fig. 2b: the ratios of Lane 1 and Lane 2 were 0.58 ± 0.05 and 0.72 ± 0.04, respectively). These results suggest that, although irradiation does not affect the stimulation of NGF receptors, increased signaling is observed. Furthermore, because ERK activation occurred following irradiation, these results suggest that the irradiation-induced suppression of neurite extension is not due to the suppression of ERK activation, and that irradiation influences pathways involved in morphological change that are not affected by the activation of ERK.

NGF stimulation causes PC12 cells to undergo functional changes, such as enhancement in the expression of tyrosine hydroxylase (TH), an enzyme involved in dopamine synthesis, as well as morphological changes, such as increased neurite extension [13]. Increased expression of TH is induced via ERK activation of a variety of proteins [13]. Our results indicate that NGF-induced TH expression exhibits further increases following irradiation (Fig. 2c).

On the other hand, studies have indicated that morphological changes, such as neurite extension are associated with the activation of Rac1, a small-molecular-weight, GTP-binding protein, via the phosphorylation of Akt, a signal protein unaffected by ERK activation [14]. Thus, we examined whether chronic low-dose irradiation affected this Akt–Rac1 pathway. We observed that NGF stimulation increased Rac1 activation (Fig. 3a), whereas this increase in Rac1 activation was markedly inhibited by irradiation (Fig. 3a). In contrast, although Rac1 activation in NGF-untreated cells was also inhibited by irradiation, this effect was marginal (Fig. 3a). These findings correspond to the morphological changes observed in Fig. 1. On the other hand, NGF stimulation markedly enhanced Akt phosphorylation, which was not changed by irradiation (Fig. 3b: the ratios of Lane 1, Lane 3 and Lane 4 were 0.62 ± 0.06, 1.01 ± 0.15 and 1.13 ± 0.14, respectively). In addition, Akt phosphorylation was enhanced slightly in NGF-untreated cells after irradiation (Fig. 3b: the ratios of Lane 1 and Lane 2 were 0.62 ± 0.06 and 0.79 ± 0.05, respectively). These results suggest that the irradiation-induced suppression of neurite extension is not due to the suppression of Akt activation, and that a separate mechanism associated with Rac1 activation is involved this process.

Previous reports have indicated that NGF-induced neurite extension in PC12 cells is inhibited by increased levels of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) expression [15]. This phenomenon is triggered independently of ERK and Akt activation [16]. An additional report has indicated that γ-ray irradiation increases CaMKII activation within a cell [17]. In the light of these findings, we hypothesized that irradiation-induced CaMKII activation may be involved in the inhibition of neurite extension observed in the present study. Our findings revealed that irradiation produced CaMKII activation, regardless of the presence or absence of NGF stimulation (Fig. 4a: the ratios of Lane 1, Lane 2, Lane 3 and Lane 4 were 0.75 ± 0.03, 1.13 ± 0.07, 0.83 ± 0.03 and 1.19 ± 0.03, respectively). Subsequently, we examined the effects of KN-62, a CaMKII-specific inhibitor that interacts with the calmodulin binding site of CaMKII and then inhibits autophosphorylation of CaMKII [11], to determine whether this activation is related to the inhibition of NGF-induced neurite extension. We observed that KN-62 inhibited CaMKII activation (Fig. 4a: the ratios of Lane 4 and Lane 5 were 1.19 ± 0.03 and 0.86 ± 0.05, respectively), and that neurite extension previously inhibited by irradiation had been recovered (Fig. 4b and c). Similarly, KN-62 recovered Rac1 activation that had been inhibited by irradiation, although it did not increase NGF-induced Rac1 activation in non-irradiated cells (Fig. 4d). These results suggest that chronic γ-ray irradiation inhibits Rac1 activation via CaMKII activation, thereby inhibiting NGF-induced neurite extension.

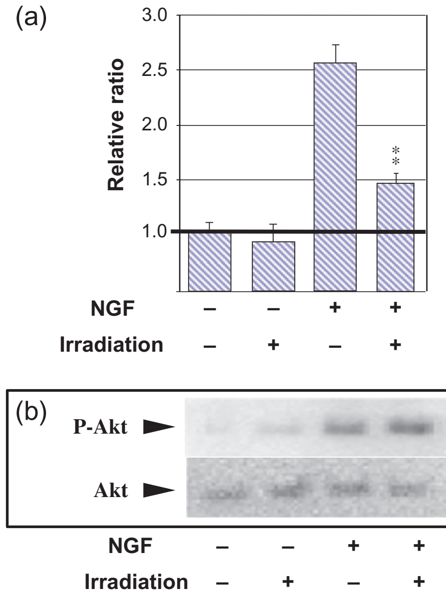

Fig. 3. Irradiation with ^{137}Cs γ-rays attenuates NGF-induced Rac1 activation without increasing phosphorylation of Akt in PC12 cells. (a) The activity of Rac1 in PC12 cells after 5 days of NGF stimulation or non-stimulation, with or without ^{137}Csγ-ray irradiation. Rac1 activity is expressed as the relative ratio to the NGF non-stimulated group without irradiation. Data are presented as the mean ± standard error of triplicate samples. **$P < 0.01$ vs non-irradiated group. (b) Immunoblot showing varying levels of phosphorylated Akt (P-Akt, upper) and Akt (lower) observed in PC12 cells after 5 days of NGF stimulation or non-stimulation, with or without ^{137}Csγ-ray irradiation. Similar results were obtained in three separate experiments. NGF = nerve growth factor.

DISCUSSION

In this study, during the period when neurite outgrowth could be sufficiently induced, the cells were cultured at a dose rate

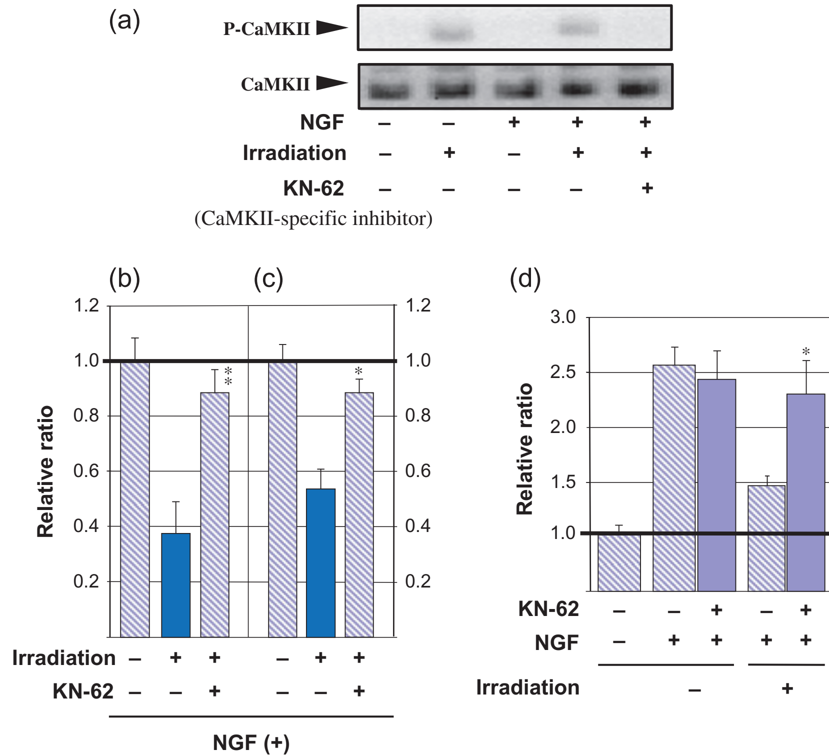

Fig. 4. Inhibition of CaMKII activity results in ^{137}Csγ-ray irradiation-induced depression of NGF-induced neurite extension in PC12 cells. (a) Immunoblot showing varying levels of phosphorylated CaMKII (P-CaMKII, upper) and CaMKII (lower) observed in PC12 cells after 5 days of NGF stimulation or non-stimulation, with or without ^{137}Csγ-ray irradiation in the presence or absence of KN-62. Similar results were obtained in three separate experiments. (b) Lengths and (c) numbers of neurites in PC12 cells after 5 days of NGF stimulation or non-stimulation, with or without ^{137}Csγ-ray irradiation in the presence or absence of KN-62. Lengths or numbers of neurites are expressed as the relative ratio to the non-irradiated group. Data are presented as the mean ± standard error of triplicate samples. *$P < 0.05$, **$P < 0.01$ vs non-irradiated group. (d) The activity of Rac1 in PC12 cells after 5 days of NGF stimulation or non-stimulation, with or without ^{137}Csγ-ray irradiation in the presence or absence of KN-62. Rac1 activity is expressed as the relative ratio to the NGF non-stimulated group without irradiation in the absence of KN-62. Data are presented as the mean ± standard error of triplicate samples. *$P < 0.05$ vs non-irradiated group. NGF = nerve growth factor, CaMKII = Ca^{2+}/calmodulin-dependent protein kinase II.

of <0.027 mGy/min. This dose rate is sufficiently lower than the dose recommended as a low dose rate by the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) [18, 19].

The total dose was less than ~200 mSv, which UNSCEAR initially proposed as a low dose. Although this proposal value was changed to 100 mSv or less later [18, 19], we believe that the biological effects of 200 mSv makes it worth considering as a low dose. This is because the difference in biological radiation effects between 100 mSv and 200 mSv has not been sufficiently elucidated.

The findings of the present study indicated that low-dose-rate ^{137}Cs-γ irradiation results in clear inhibition of NGF-induced neurite extension, consistent with the findings of previous reports [1, 2]. Furthermore, our results revealed that activation of Rac 1 (a protein that induces neurite extension) is influenced by the irradiation-induced activation of CaMKII, independent of the ERK and Akt pathways. Previous studies have reported similar activation of CaMKII following irradiation of neural cells in neonatal mice, inhibiting neuronal differentiation and leading to cognitive impairment [17], consistent with the findings of the present study.

NGF increases intracellular levels of Ca^{2+} and induces neuronal differentiation [20]. Research has indicated that low-dose-rate ^{137}Cs-γ irradiation inhibits this increase in a dose-dependent manner [21]. These findings suggest that the irradiation-induced activation of CaMKII can be attributed, not to modification of the Ca^{2+} concentration, but to that of a different pathway. Previous reports have indicated that irradiation induces reactive oxygen species (ROS), which activate CaMKII in a variety of cells [22–24]. Such findings suggest that ROS are produced following irradiation activate CaMKII (Fig. 5).

CaMKII activation and the associated inhibition of Rac1 seem to suggest that CaMKII directly controls the activation of Rac1. However, some research indicates that the mechanisms underlying CaMKII and Rac1 differ. For example, even though both CaMKII and Rac1 act as a regulatory factor for the neurites of neuronal cells, Rac1 exerts a stimulatory control on neurite formation, while

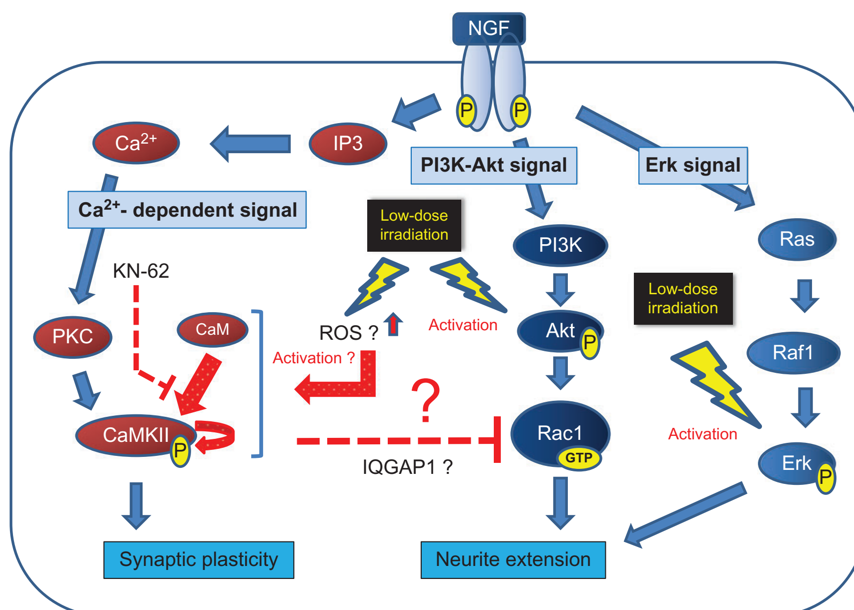

Fig. 5. Hypothesis of inhibition mechanism of the NGF-induced neurite extension by chronic irradiation with low-dose-rate ^{137}Cs-γ rays via Ca^{2+}/calmodulin-dependent kinase II activation. Chronic irradiation with low-dose-rate γ rays activates CaMKII in the downstream of the Ca-dependent signal induced by NGF. The production of reactive oxygen species generated by irradiation may be involved in this activation of CaMKII. The CaMK II suppresses NGF-induced neuronal extension by inhibition of the activation of Rac1 downstream of the PI3K-Akt signal. IQGAP1, which is considered to contribute to neurite extension, may be involved in this inhibition of Rac1 activity. NGF = nerve growth factor, ERK = extracellular signal–regulated kinase, PI3K = phosphoinositide 3-kinase, IP3 = inositol trisphosphate, CaM = calmodulin, CaMKII = Ca^{2+}/calmodulin-dependent protein kinase II, ROS = reactive oxygen species, IQGAP1 = Ras GTPase-activating-like protein.

CaMKII is involved in the dynamic control of neurite movement [25]. In addition, one study reported that activation of Ras GTPase-activating-like protein (IQGAP1), which is considered to contribute to neurite extension, is enhanced by Rac1 and suppressed by CaMKII [26]. Such findings suggest that CaMKII does not directly affect Rac1 activation, but rather suppresses neurite extension via a separate mechanism, and that Rac1 activation instead occurs as a result of this suppression (Fig. 5). However, the precise mechanism underlying the irradiation-induced activation of CaMKII activation and inhibition of Rac1 remains unknown, necessitating closer examination in future studies.

Previously, it was reported that a transient irradiation with X-rays (dose rate: 0.1 Gy/min, total dose: 500 mGy) promoted NGF-induced neurite extension in PC12 cells [3]. Similarly, low-dose-rate (0.95 Gy/min, total dose: 6 Gy) ^{137}Cs-γ radiation facilitated neurite extension in neural stem–like cells [4]. The cause of the difference between the results of promoting neuronal differentiation in the above-mentioned reports and the results of suppression of neuronal differentiation obtained in reports included in this study is not quite clear. The total irradiation doses used in studies on irradiation-induced inhibition of neurite extension, including the present study, range from 0.01 to 2.1 Gy [1, 2], overlapping with those used in studies on the promotion of neurite extension, which range from 0.1 to 6 Gy [3, 4]. In contrast, irradiation dose rates used in reports on the irradiation-induced inhibition of neurite extension range from 0.4 to 15 mGy/h [1, 2], whereas those used in reports on the promotion of neurite extension range from 5 to 57 Gy/h [3, 4]. Given this large discrepancy, we suspect that the irradiation dose rate, rather than the total irradiation dose, determines the effect on neurite extension. However, further studies are required in order to clarify this point.

The results of the present study revealed that chronic irradiation with low-dose-rate ^{137}Cs γ-rays inhibits NGF-induced neurite extension, and further suggested for the first time that CaMKII activation may be involved in this phenomenon. Our results may therefore provide valuable information for studying the effects of chronic, low-dose-rate irradiation on tissues of the nervous system.

CONFLICT OF INTEREST

The authors state that there are no conflicts of interest.

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