

Cranial irradiation induces axon initial segment dysfunction and neuronal injury in the prefrontal cortex and impairs hippocampal coupling

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Abstract

Background. Radiation therapy for brain tumors commonly induces cognitive dysfunction. The prefrontal cortex (PFC) is crucial for a diverse array of cognitive processes, however, its role in radiation-induced cognitive dysfunction is unknown. We previously found that cranial irradiation impairs neuroplasticity along the hippocampal–PFC pathway. Herein, we hypothesized that brain irradiation directly affects the firing properties of PFC neurons, contributing to deficits in neuronal functions.

Methods. In vivo recordings were used to monitor the firing activities of PFC neurons and local field potentials in both PFC and hippocampal CA1/subicular regions after cranial irradiation of Sprague Dawley rats. We further assessed the impacts of irradiation on axon initial segments (AISs) with immunofluorescence assays of PFC slices.

Results. We found that PFC neurons exhibited increased excitation 3 days after radiation and the timing of increased excitation coincided with elongation of the AIS. At 2 weeks, excitation levels returned to nearly normal levels however the population of spontaneously firing neurons decreased. While the number of NeuN-positive neurons in the PFC was not different, persistent neuronal injury, manifested as ATF-3 staining, was present at 2 weeks. Radiation also disrupted communication along the hippocampal–PFC pathway, with elongation of the phase lag between regions. Analysis of paired-pulse ratios suggested that this was secondary to presynaptic dysfunction.

Conclusions. Cranial irradiation excited and injured surviving PFC neurons and was associated with a partial block of PFC's functional coupling to the hippocampus. These deficits in the PFC may contribute to radiation-induced cognitive dysfunction.

Key Points

- Cranial irradiation induced transient excitation of PFC neurons.
- AIS morphological alteration and impaired hippocampal coupling corresponded with abnormal excitation.
- PFC neuronal injury persisted, without a decrease in NeuN-positive cell numbers.

Importance of the Study

Cranial irradiation is an essential treatment for brain tumors but may induce significant cognitive dysfunction. In previous decades, investigations of radiation-induced deficits have largely focused on neurogenesis and the hippocampus. However, the roles of other brain regions, including the prefrontal cortex, which is crucial not only for memory but also for a diverse set of other cognitive processes,

remain unknown. This study found that prefrontal cortex neurons were excited and injured after cranial irradiation. This was accompanied by abnormalities in the axon initial segment as well as impaired coupling with the hippocampus. These results provide evidence for cortical neuronal injury after radiation that may contribute to radiation-induced cognitive dysfunction.

Radiation is a common treatment for primary and metastatic brain tumors. Despite its clinical efficacy, the effects of cranial radiation therapy on cognition and subsequent quality of life can be devastating. Even low-dose radiation treatments can have debilitating cognitive effects, especially in children.^{1–3} Most previous studies on the etiology of radiation-induced cognitive impairments have focused on the loss of neural progenitor cells in the hippocampus.^{4,5} However, interest is growing in other brain regions, including the prefrontal cortex (PFC), a region crucial not only for memory but also for a diverse set of other cognitive processes.^{6,7} The PFC also has close functional connections to the hippocampus. The direct monosynaptic pathway, originating in the hippocampal CA1/subicular region and projecting to the medial orbital and prelimbic areas of the PFC, has been investigated and is highlighted in several theories of cognitive function.^{8–10}

The traditional theory attributes radiation-induced injury to a gradual decline in the number of cells,¹¹ and long-term deficits in cognition after radiation therapy have thus been attributed to the loss of neuronal stem cells.¹² In recent years, new evidence has indicated that surviving neurons also contribute to cognitive dysfunction after brain radiation, and long-term changes in neuronal structure and pathway functions after radiation are being evaluated with relation to radiation-induced cognitive impairment.⁴ We previously found that the plasticity of synapses within hippocampal–PFC pathway was significantly altered after a single cranial radiation dose.¹³ Here, we hypothesized that, in addition to changes in neuronal plasticity, radiation also affects the firing properties and morphologic features of PFC neurons. To test this hypothesis, we recorded the firing activities of PFC neurons and local field potentials in both PFC and the CA1/subicular regions simultaneously at days 3 and 14 after irradiation. Neuronal density, axon initial segment (AIS) length, and neuronal injury were assayed by using immunofluorescent staining to visualize and examine radiation-induced alterations in PFC neurons.

Materials and Methods

Study Approval

The Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center approved all procedures in accordance with federal guidelines.

Animal Care and Preparation

Eight-week-old male Sprague Dawley rats (Harlan Laboratory) were used for all experiments. To avoid the influence of reproductive cycles and hormone fluctuations on neuronal activities, only male animals were used in the current study. Animals were housed with food and water ad libitum in a temperature-controlled room ($23 \pm 0.5^\circ\text{C}$) with a 12-h light/dark cycle. On day 0, rats were anesthetized with isoflurane and either whole-brain radiation to 10 Gy delivered with an XRAD 225Cx (Precision X-Ray) or given sham treatment.

Electrophysiology Recording In Vivo

On day 3 or 14 after irradiation, rats were anesthetized with urethane (Sigma, 1.5 g/kg i.p.) and placed in a stereotaxic frame with body temperature maintained at 37°C by a homeothermic warming blanket. Extracellular recordings, in vivo, were then performed as described in our previous publications.^{13,14} In brief, glass electrodes (2M NaCl) were positioned in the PFC through a small burr hole in the skull by using coordinates based on the atlas of Paxinos and Watson (3.0 mm anterior to bregma, 0.8 mm lateral to the midline, 3.5–4.0 mm deep). Another glass electrode (for recording) or bipolar concentric stainless steel stimulating electrodes (150 μm outer diameter with a 300 μm tip separation, WPI) were placed in the CA1/subicular region of the hippocampus (6.5 mm posterior to bregma, 5.5 mm lateral to the midline, 5.5–6.5 mm deep) ipsilateral to the recording site. A 3-min segment of spontaneous firing activity was recorded from each neuron. Fast Fourier transforms were performed to 3-min segments of local field potential waveforms at frequencies from 0.1 to 100 Hz to check the spectrum power distribution.

Electrical stimulation of the CA1/subicular region evoked a characteristic monosynaptic excitatory postsynaptic field potential (PSP) in the PFC. Test pulses were delivered at an intensity that evoked a response, measured as its amplitude, of 70% of its maximum. At this intensity, the field potential is most likely to reflect summated PSPs. Field potentials were amplified, filtered (band pass 0.1 Hz–3 kHz), and digitized at 20 kHz. No PSP was detected if either electrode was off target. Pulse pairs with varying interpulse intervals (25, 50, 75, 100, and 200 ms) were applied at a repetition rate of 0.25 Hz. Data were acquired and

analyzed with a CED spike2 system (Cambridge Electronic Design Limited). Electrodes were marked with Dil dye on their surfaces to identify the tracks left by extracellular microelectrodes.^{14,15} Data are reported only for those rats in which the electrodes were confirmed to be in the PFC and CA1/subiculum regions.

Immunofluorescence

While still deeply anesthetized, animals were perfused intracardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% formaldehyde/12.5% picric acid solution in 0.1 M PBS. The brains were removed, post-fixed in the perfusion fixative, and cryoprotected in 20% and then 30% sucrose in 0.1 M PBS all at 4°C. Serial frozen sections containing the PFC were cut at 15 μm on a cryostat for immunofluorescence analysis. The sectioned brain slices were incubated for 60 min at room temperature (RT) in a blocking solution of 3% normal donkey serum in PBS with 0.3% Triton-X100. Sections were incubated overnight at 4°C with antibodies against neuronal nuclei antigen (mouse anti-NeuN, 1:150; Chemicon) and Ankyrin-G (rabbit anti-Ank G, 1:100; Santa Cruz Biotechnology) or activating transcription factor 3 (rabbit anti-ATF3, 1:500; Santa Cruz Biotechnology). Sections were washed in PBS and incubated with Cy3- or FITC-conjugated secondary antibodies overnight at 4°C. Finally, the sections were washed 4 \times 10 min in PBS, mounted, and cover-slipped for observation. The length of AIS and the numbers of cells positive for NeuN or ATF3 were measured or counted in at least 3 randomly chosen regions (100 μm \times 100 μm) from each brain section. A minimum of 4 sections from each sham or radiation treated rats were quantified.

Statistics

Statistical analyses were done with Microsoft Excel and SPSS. All data were expressed as means \pm SEM. Potential differences between groups were calculated by analysis of variance, followed by a Tukey post hoc test for multiple comparisons. *P*-values of less than .05 were considered to indicate statistically significant differences.

Results

Cranial Irradiation Transiently Excites Neurons in the PFC

At day 3 after a single 10 Gy cranial radiation dose, PFC neurons (Figure 1A) were found to be highly excited, with a 5-fold increase in their firing rate (Figure 1B). However, this acute radiation-induced excitation was not sustained as the firing rate of PFC neurons returned to control levels at 2 weeks. In association with this initial increase in firing rate, radiation exposure also activated “silent neurons” in the PFC. The number of spontaneously firing neurons increased from 1.71 ± 0.28 to 2.74 ± 0.22 cells/track in the PFC at day 3 after radiation exposure (Figure 1C). However, the population of spontaneously firing neurons dropped

to lower-than-control levels at 2 weeks after radiation (Figure 1C). This evidence of alterations in firing properties suggests that PFC neuron function had been affected by the whole-brain radiation.

Cranial Irradiation Alters Plasticity of the AIS and Injures PFC Neurons

To investigate the possible reasons underlying initial excitation in the PFC, we examined AIS morphology in PFC neurons. The AIS is crucial for regulating neuron excitability. Staining and measurement of the AIS of PFC neurons (Figure 2A) revealed that, as expected, radiation changed not only neuronal firing activities but also the length of the AIS of the PFC neurons. The AIS of PFC neurons was significantly elongated at day 3 after irradiation, but returned to control levels by 2 weeks after radiation exposure (Figure 2B). This radiation-induced change in the plasticity of AIS likely contributed to the transient excitation of PFC neuron firing activity after irradiation.

To explore why the population of spontaneously firing neurons was decreased in the PFC at 2 weeks after radiation, we stained PFC neurons with anti-NeuN antibody and counted the number of neurons on brain slices harvested at day 3 and day 14 after irradiation. No difference was found in neuron density among these time points and sham controls (Figure 3A), suggesting that it was not cell loss that caused the decrease in the number of spontaneous firing neurons. However, staining for ATF-3, a marker of neuronal injury, indicated that PFC neurons were injured (Figure 3B–E), and this prolonged radiation-induced damage could be detected even 2 weeks after radiation (Ctrl: 0.56 ± 0.17 ; RT 3 days: 22.78 ± 1.27 ; RT 2 weeks: 24.78 ± 1.16). Although we do not know these cells' ultimate fate, persistent damage in PFC neurons may contribute to dysfunction following cranial irradiation.

Cranial Irradiation Affects the Functional Connection of the Hippocampal–PFC Pathway

To identify potential effects of radiation on transmission in the hippocampal–PFC pathway, local field potentials at the PFC and CA1/subicular regions were recorded simultaneously in sham and irradiated animals. Cross-correlation analysis, a measure of similarity of 2 series as a function of the displacement of one relative to the other, revealed that the local field potentials at the PFC were synchronized with CA1/subicular activity, and cranial irradiation did not change this synchronization (Figure 4A and B). However, when the data were analyzed by phase analysis, which provides information on the time delay between 2 synchronized signals, irradiation was seen to partially block signal transmission along the hippocampal–PFC pathway. The phase lag between the CA1/subicular and PFC field potentials was increased from 6.32 ± 0.71 ms (sham) to 30.43 ± 3.26 ms at day 3 and to 13.43 ± 2.33 ms at day 14 after irradiation (Figure 4C).

In a further study of the effects of radiation on the hippocampal–PFC pathway, we found the paired-pulse ratio was significantly suppressed at day 3 following radiation. The paired-pulse ratio, the ratio of the amplitude of the

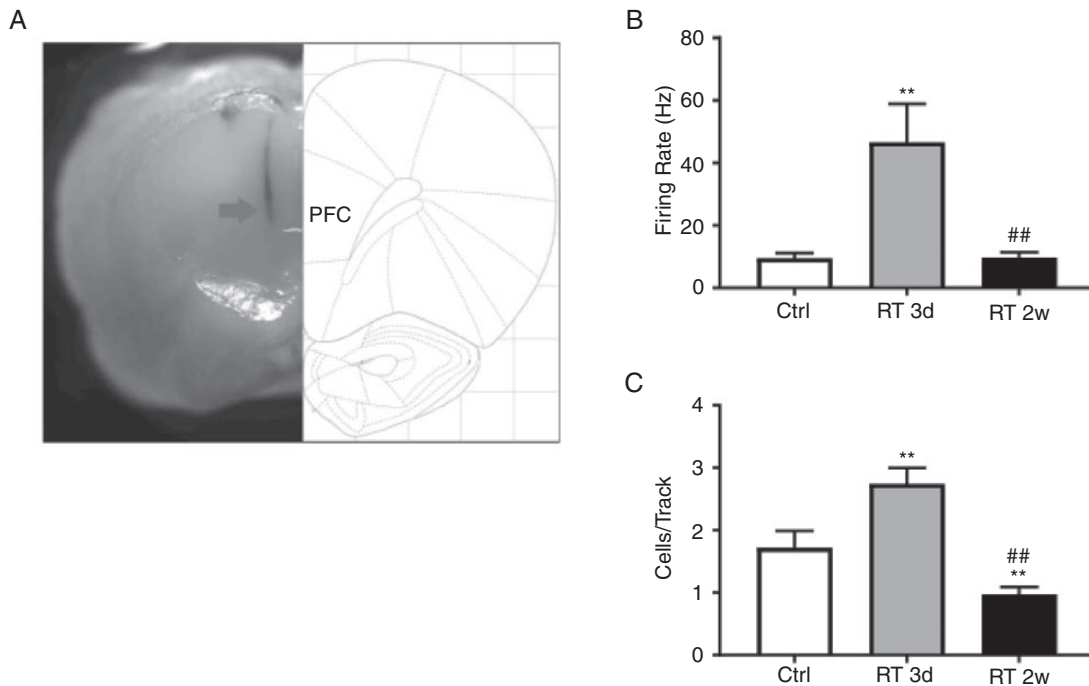


Figure 1. Whole-brain irradiation in a single 10-Gy dose transiently excited neurons in the prefrontal cortex (PFC). (A) A phase-contrast image illustrates that electrode general recording site within the PFC (arrow), (B) firing rates, and (C) numbers of spontaneously firing neurons per recording track were measured in adult rats at 3 and 14 days after radiation (RT). PFC neuron firing rates were 46.41 ± 12.50 Hz ($n = 50$ neurons, from 6 rats) at day 3, a significantly increase over rates in sham controls (Ctrl, 9.41 ± 1.69 Hz, $n = 40$ neurons, from 6 rats), but dropped to 9.64 ± 1.74 Hz ($n = 33$ neurons, from 6 rats) at day 14 after radiation [$F_{(2,120)} = 6.204$, $P < .01$]. The number of spontaneously firing neurons was initially increased (2.74 ± 0.26 , $n = 29$ recording tracks, from 6 rats, day 3 after irradiation) and then reduced (0.97 ± 0.12 , $n = 36$ recording tracks, from 6 rats, day 14 after irradiation), both significantly so relative those of sham controls (1.71 ± 0.28 , $n = 28$ recording tracks, from 6 rats; [$F_{(2,96)} = 15.56$, $P < .0001$]). ** $P < .01$ vs Ctrl; ## $P < .01$ vs RT 3 days.

second response to that of the first, is inversely related to the synaptic release ability and has been widely used as a measure of presynaptic function. The suppressed paired-pulse ratio suggests that presynaptic inhibition was induced by cranial irradiation at CA1/subicular inputs (Figure 5A).

We also observed a change in the patterns of local field potentials recorded in both the CA1/subicular region and the PFC. We next examined the spectrum powers of their local field potentials at frequencies between 0.1 and 100 Hz and found that the spectrum powers at the 59–61 Hz frequency segment were significantly depressed in both areas 2 weeks after radiation (Figure 5B). This frequency segment belongs to the Gamma EEG band (low-amplitude rhythms in the 30–100 Hz range), which is thought to reflect perceptual and cognitive processes.

These findings suggest that the effects of cranial radiation are not limited only to PFC neurons themselves, but that changes the coupling between brain areas, affects the local field potentials patterns, signal transmission, and synaptic plasticity.

Discussion

Radiation-induced cognitive impairment is a common adverse effect of cranial irradiation. Despite the prevalence

and significance of radiation-induced brain dysfunction, the underlying mechanisms are still largely unknown. In non-neurogenic brain regions, changes in the connectivity and function of mature neurons may underlie cognitive deficits. The PFC is one such non-neurogenic brain region that is critical not only for memory but also for diverse cognitive processes including perception, decision making, and emotion.^{7,16,17} In the present study, we investigated how radiation exposure affects PFC functions.

We observed a radiation-induced excitation of neuron firing properties. The PFC neuronal firing rate was remarkably increased at 3 days after a single exposure, which could be explained by the plasticity of the AIS, an excitable neuronal domain between the axonal and somatodendritic compartments that has a pivotal role in initiating action potentials and contributes to the fine regulation of the neuronal output.¹⁸ Previous studies have shown that the structural characteristics of the AIS, such as length and distance from the soma, strongly affect the excitability and firing pattern of neurons.^{19–21} The negative correlation between AIS length and distance from soma has been suggested as favoring maximal neuronal excitability.²² In the present study, the AIS of PFC neurons was greatly elongated within 3 days after irradiation. Furthermore, we found that changes in the length of AIS were also associated with changes in the plasticity of the hippocampal–PFC pathway. A direct monosynaptic pathway that is critical

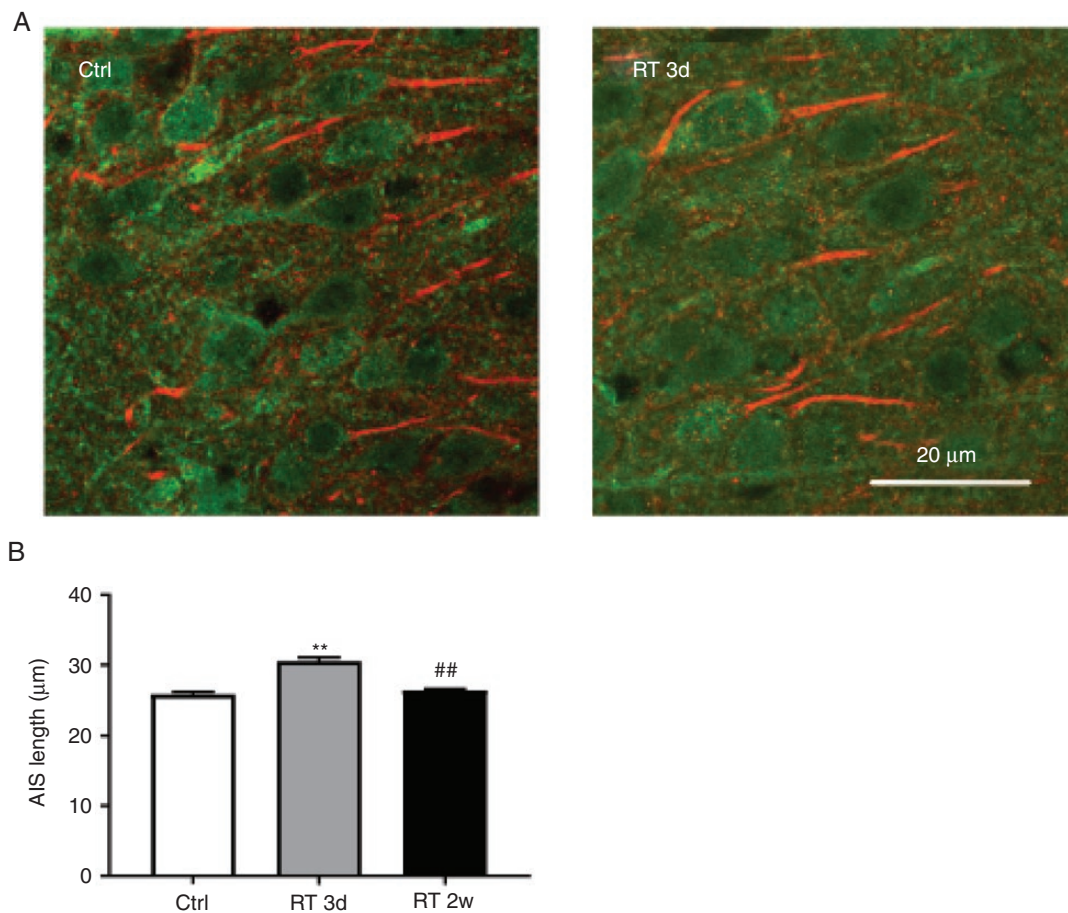


Figure 2. Cranial irradiation altered the plasticity of the axon initial segment (AIS) of neurons in the prefrontal cortex (PFC). (A) Confocal imaging illustrates neuronal soma (green, anti-NeuN) and AIS (red, anti-Ank G) on a PFC slice (bar: 20 μm). (B) Histogram of AIS length showed a similar biphasic response in which the PFC neuron AIS was first elongated from 25.88 ± 0.31 (Ctrl, $n = 110$ from 6 rats) to 30.63 ± 0.45 at day 3 ($n = 100$ from 6 rats) and then reduced to 26.37 ± 0.27 at day 14 ($n = 110$ from 6 rats) after irradiation [$F_{(2,317)} = 55.14, P < .0001$]. ** $P < .01$ vs Ctrl; ## $P < .01$ vs RT 3 days.

for several aspects of cognition and sensitive to insults is known to originate in the hippocampal CA1/subicular region and projects to the medial orbital and prelimbic areas of the PFC.^{9,16,17} Our paired-pulse ratio test suggested that this important excitatory input is affected by radiation exposure. Paired-pulse facilitation is a well-known phenomenon that is thought to be related to an increase in the second postsynaptic response when it is elicited shortly after the first and is regarded as a form of short-term plasticity at many synapses in the central nervous system. It determines the responses of neurons to patterned presynaptic activities, revealing the basic principles of information processing in the nervous system.^{23,24} In a paired-pulse ratio test, the ratio between the amplitude of the second response over the first response is inversely related to the initial release probability, and the depression to synaptic should be dependent on the previous release. Therefore, it has been widely used as an easy measure of the probability of presynaptic release.^{25,26} The decreased paired-pulse ratio found in the PFC neurons suggests the presence of a radiation-induced inhibition of the excitatory

input from the hippocampus, which could be a possible mechanism underlying the elongation of AIS and enhancement of the excitability of PFC neurons.

In addition to changes in firing rates, we observed that “silent” PFC neurons were initially activated by radiation and functionally joined the neuronal network. Similar to our findings on neuron firing rates, the number of spontaneous firing neurons was reduced to a level significantly lower than that of the control group at 2 weeks without a loss of neurons. PFC neurons had sustained radiation-induced damage, as detected via ATF-3 staining. ATF-3 expression has been reported to be associated with several signaling pathways implicated in the cellular stress response in numerous cell types and is regarded as a marker of neuronal injury in the central nervous system.²⁷ We found that 2 weeks after irradiation, most other electrophysiological parameters had returned to near-control levels after acute shifting, but ATF-3 signals could still be distinctly detected in most PFC neurons. Considering the strong excitatory effects to the PFC neurons as addressed above, we believe that these cells were affected

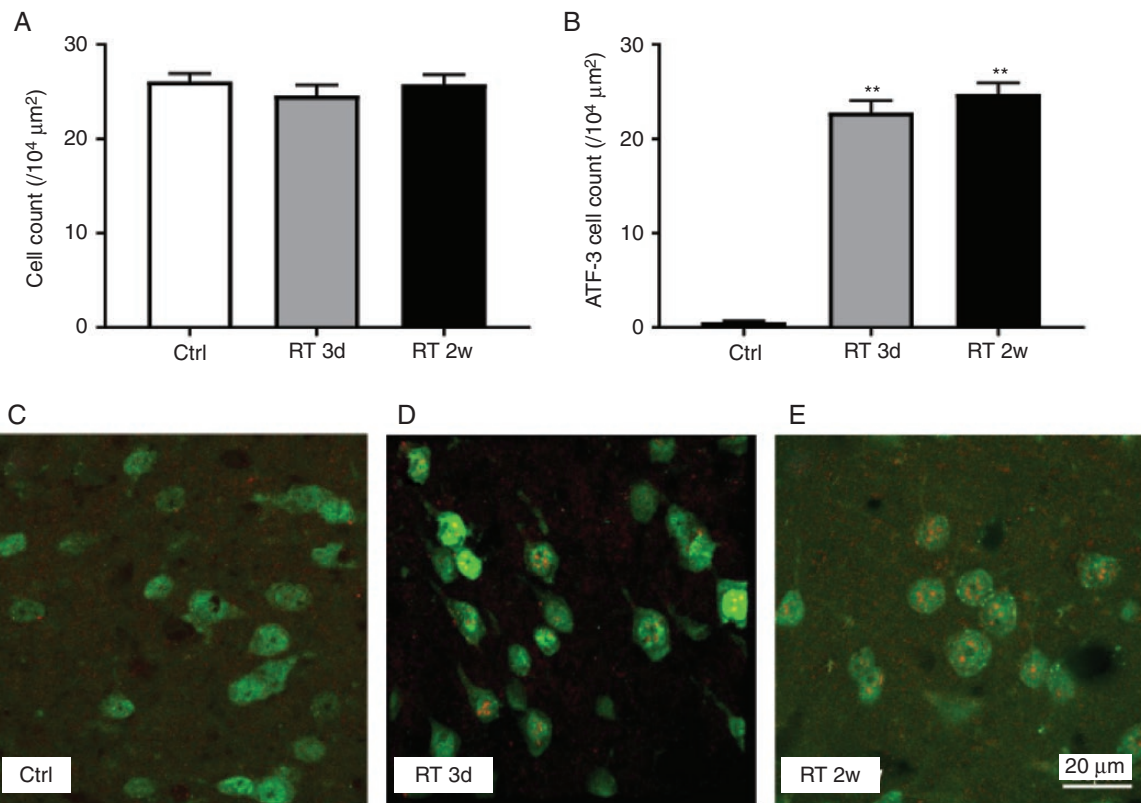


Figure 3. Cranial irradiation damaged, but did not reduce the number of, neurons in the prefrontal cortex (PFC). (A) Radiation did not significantly change the numbers of PFC NeuN-positive neurons (26.11 ± 0.83 , $n = 18$ images from 6 rats, sham control; 24.61 ± 1.11 , $n = 18$ images from 6 rats, day 3 after irradiation; 25.78 ± 1.07 , $n = 18$ images from 6 rats, day 14 after irradiation [$F_{(2,51)} = 0.61$, $P = .55$]). (B) Immunohistochemical staining for ATF-3, a marker of neuronal injury, was detected in PFC neurons at (D) day 3 after irradiation and (E) at day 14 after irradiation, but (C) not in sham controls (0.55 ± 0.17 , $n = 18$ images from 6 rats, sham control; 22.78 ± 1.27 , $n = 18$ images from 6 rats, day 3 after irradiation; 24.78 ± 1.16 , $n = 18$ images from 6 rats, day 14 after irradiation [$F_{(2,51)} = 182.2$, $P < .0001$]). ** $P < .01$ vs Ctrl.

by radiation-induced excitatory toxicity, and that this prolonged neuronal damage induced by radiation could be a reason for the eventual inhibition of spontaneous firing and triggering permanent damage to neuron function.

We also interrogated signal transportation in the hippocampal–PFC pathway. The similarity of local field potentials between hippocampal CA1/subicular and PFC regions was consistent before and after radiation treatment. However, a distinct delay was observed in signal conduction from the CA1/subicular region to the PFC within 3 days of radiation. We also observed a change in the patterns of local field potentials recorded in both the CA1/subicular region and the PFC. The spectrum powers at the Gamma EEG band were found significantly depressed in both areas 2 weeks after radiation. It is well known that the Gamma EEG band is a subject of keen interest in humans^{28–30} after animal models revealed that synchronous discharge bursts within the gamma range were involved in perception and cognition.³¹ Previous animal experiments have demonstrated that the functional significance of synchronous gamma oscillations, which is shown as the synchronicity of gamma discharges, correlates with cognitive function^{32–34} and that disrupted

synchronicity of gamma discharges correlates with diminished cognitive function.^{35,36}

To the best of our knowledge, this is the first study that indicates that radiation-induced changes on PFC neuron firing properties are associated with alterations on AIS plasticity. Limitations of the current study include a lack of behavioral data. However, Tomé's recent review paper indicates that 13 studies have revealed behavioral effects of cognitive deficits in rodents with a single dose of 10 Gy, as was used in our study.³⁷ To better understand the contribution of cortical neuronal dysfunction to cognitive impairments, we are currently investigating the detailed behavioral consequences of radiation-induced actions in PFC, including assays of attention and other cortical domains. Another limitation is that we used whole-brain radiation in the current study which affected PFC functions both directly and indirectly via neuronal inputs. We are planning to perform targeted radiation to specific brain regions to isolate radiation-induced damages in our future work.

In summary, we found that cranial irradiation excited the firing activity of PFC neurons, including AIS elongation, enhanced firing rates, and increased numbers of spontaneously firing neurons. Although this excitatory

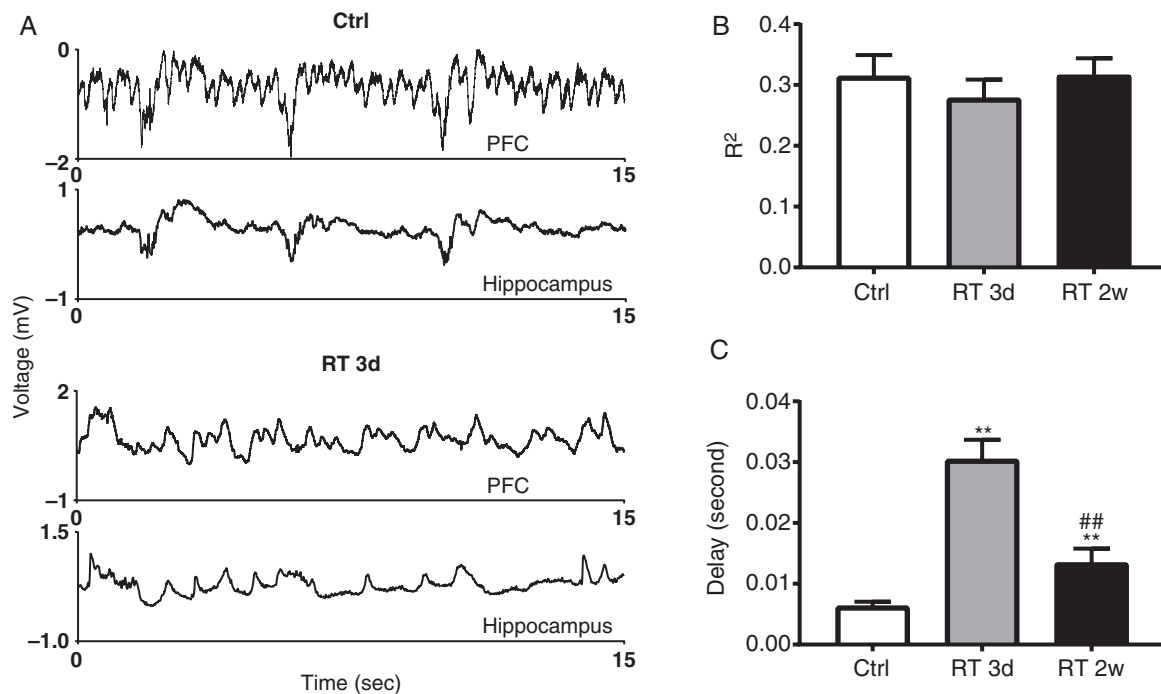


Figure 4. Cranial irradiation partially blocked signal transmission between hippocampus and the prefrontal cortex (PFC). (A) Two 15-s segments of local field potentials recorded simultaneously showed synchronization between PFC and hippocampal activities from control and RT group. (B) Cross-correlations of the activities in the PFC and hippocampus were not significantly affected by cranial irradiation (0.31 ± 0.04 , $n = 40$ neurons from 6 rats, sham control; 0.28 ± 0.03 , $n = 49$ neurons from 6 rats, day 3 after irradiation; 0.32 ± 0.03 , $n = 33$ neurons from 6 rats, day 14 after irradiation [$F_{(2,119)} = 0.47$, $P = .636$]). (C) Phase measurements, however, showed significant delays as early as day 3 that were still detectable at day 14 (6.33 ± 0.72 ms, $n = 40$ neurons from 6 rats, sham control; 30.43 ± 3.26 ms, $n = 49$ neurons from 6 rats, day 3 after irradiation; 13.43 ± 2.34 ms, $n = 33$ neurons from 6 rats, day 14 after irradiation [$F_{(2,119)} = 25.86$, $P < .0001$]), indicating that radiation notably blocked signal transmission between the PFC and hippocampus. ** $P < .01$ vs Ctrl; ## $P < .01$ vs RT 3 days.

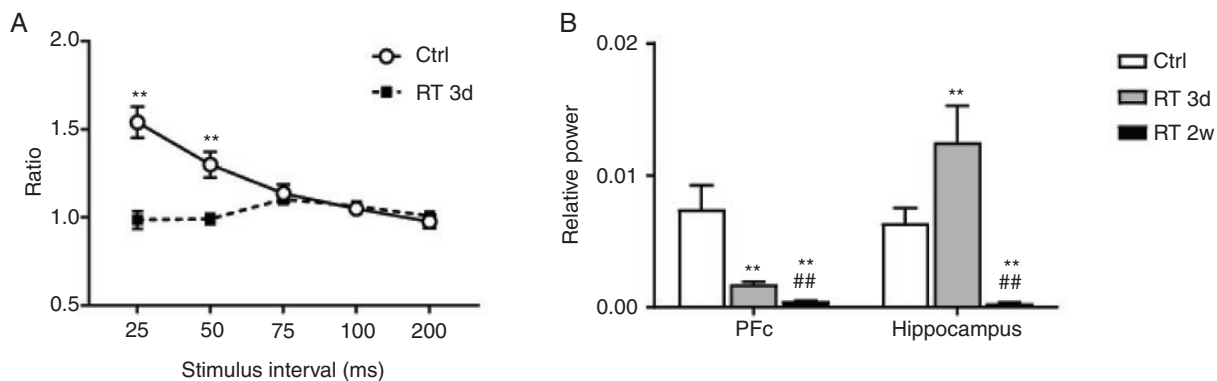


Figure 5. Cranial irradiation reduced the paired-pulse ratio and depressed Gamma EEG. (A) The paired-pulse ratio was significantly decreased at day 3 after irradiation in the prefrontal cortex (PFC) [$F_{(4,190)} = 13.98$, $P < .0001$]. Five animals were used in each group. (B) Spectrum powers at the 59–61 Hz frequency segment were significantly depressed in the PFC and hippocampus after 2 weeks after radiation. (PFC, Ctrl: $7.45E^{-3} \pm 1.81E^{-3}$, $n = 40$ from 6 animals; RT 3 days: $1.74E^{-3} \pm 0.20E^{-3}$, $n = 50$ from 6 animals; RT 2 weeks: $0.45E^{-3} \pm 0.05E^{-3}$, $n = 33$ from 6 animals [$F_{(2,120)} = 12.37$, $P < .0001$]; Hippocampus, Ctrl: $6.38E^{-3} \pm 1.15E^{-3}$, $n = 40$ from 6 animals; RT 3 days: $12.53E^{-3} \pm 2.78E^{-3}$, $n = 50$ from 6 animals; RT 2 weeks: $0.29E^{-3} \pm 0.07E^{-3}$, $n = 33$ from 6 animals [$F_{(2,120)} = 8.64$, $P < .001$]). ** $P < .01$ vs Ctrl; ## $P < .01$ vs RT 3 days.

activity was transient, the negative effects of cranial irradiation on the PFC neurons were prolonged. Radiation-induced neuronal injuries were still detectable when most electrophysiological parameters had returned to near-normal levels at 2 weeks after irradiation. Although radiation did not result in significant cell death, cranial irradiation partially blocked the PFC from functionally receiving signals transmitted from the hippocampus, and persistent PFC neuronal damage was evidenced by ATF-3 staining. We believe that these radiation-induced changes in surviving PFC neurons may contribute to radiation-induced cognitive deficits. The finding of radiation-induced depression of gamma oscillation also has promise for diagnosing and quantifying the cognitive impairments after radiation therapy along with formal neurocognitive testing.

Keywords

axon initial segments | cognitive impairment | cranial radiation | hippocampal-PFC pathway | neuroplasticity

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