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1-[(4-Nitrophenyl)sulfonyl]-4-phenylpiperazine treatment after brain irradiation preserves cognitive function in mice

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Abstract

Background. Normal tissue toxicity is an inevitable consequence of primary or secondary brain tumor radiotherapy. Cranial irradiation commonly leads to neurocognitive deficits that manifest months or years after treatment. Mechanistically, radiation-induced loss of neural stem/progenitor cells, neuroinflammation, and demyelination are contributing factors that lead to progressive cognitive decline.

Methods. The effects of 1-[(4-nitrophenyl)sulfonyl]-4-phenylpiperazine (NSPP) on irradiated murine neurospheres, microglia cells, and patient-derived gliomaspheres were assessed by sphere-formation assays, flow cytometry, and interleukin (IL)-6 enzyme-linked immunosorbent assay. Activation of the hedgehog pathway was studied by quantitative reverse transcription PCR. The in vivo effects of NSPP were analyzed using flow cytometry, sphere-formation assays, immunohistochemistry, behavioral testing, and an intracranial mouse model of glioblastoma.

Results. We report that NSPP mitigates radiation-induced normal tissue toxicity in the brains of mice. NSPP treatment significantly increased the number of neural stem/progenitor cells after brain irradiation in female animals, and inhibited radiation-induced microglia activation and expression of the pro-inflammatory cytokine IL-6. Behavioral testing revealed that treatment with NSPP after radiotherapy was able to successfully mitigate radiation-induced decline in memory function of the brain. In mouse models of glioblastoma, NSPP showed no toxicity and did not interfere with the growth-delaying effects of radiation.

Conclusions. We conclude that NSPP has the potential to mitigate cognitive decline in patients undergoing partial or whole brain irradiation without promoting tumor growth and that the use of this compound as a radiation mitigator of radiation late effects on the central nervous system warrants further investigation.

Key Points

1. Patients undergoing radiotherapy for brain cancer experience cognitive decline over time after treatment.
2. NSPP targets hedgehog pathway to expand neural stem cells and progenitor cells without affecting the tumor cells in the brain.
2. In an animal model NSPP mitigates radiation-induced neuro-inflamation and cognitive impairment.

Importance of the Study

Successful radiotherapy of CNS malignancies inevitably leads to cognitive decline in cancer survivors, and treatment options to mitigate this side effect are limited. We present evidence that a piperazine compound can prevent cognitive decline in mice after total brain irradiation without compromising the antitumor effect of radiation, suggesting that this compound could be used to mitigate radiation side effects in brain tumor patients undergoing radiotherapy.

Exposure of the CNS to ionizing radiation results in normal tissue toxicity.[1] With survival times for cancer patients steadily increasing over the past decades,[2] more and more patients are now at risk of experiencing late effects of radiotherapy. Patients receiving cranial irradiation—and among those in particular pediatric patients—are facing a decline in cognitive function later in life.[3–5] The underlying mechanisms include neuroinflammation, diminished neuronal connectivity, and demyelination.[1] Earlier studies by Limoli and colleagues demonstrated that the functional consequences of brain irradiation can be mitigated by injection of neural stem cells into the brain and that newly derived neurons from this stem cell population integrate into the circuitry of the adult brain.[6] Furthermore, activation of microglia is a critical factor for radiation-induced neuroinflammation, which ultimately leads to cognitive decline. These data indicate that mitigating radiation effects in the intrinsic neural stem/progenitor cell population and microglia cells could be exploited in the radiotherapy setting to prevent radiation-induced cognitive decline.

We previously reported that 1-[(4-nitrophenyl)sulfonyl]-4-phenylpiperazine (NSPP, formerly termed "Compound #5"[7,8]) prevents the acute radiation syndrome in mice by activating the hedgehog signaling pathway.[7,8] In this study we tested if NSPP has an effect on the neural stem/progenitor cell population. Our data show that NSPP when given after cranial irradiation preserves the neural stem/progenitor cell population, inhibits microglia activation, mitigates radiation-induced neuroinflammation, and prevents radiation-induced cognitive impairment in mice.

Materials and Methods

Animals

Nestin–enhanced green fluorescent protein (GFP) mice were a kind gift from Dr Grigori Enikolopov, Cold Spring Harbor Laboratory.[9] C3Hf/Sed/Kam were originally obtained from The MD Anderson Cancer Center. All experiments were performed in accordance with all local and national guidelines for the care of animals.

For orthotopic tumor grafting, 2×10^5 GL261-luciferase cells were implanted into the right striatum of the brains of female C57BL/6 mice using a stereotactic frame (Kopf Instruments) and a nano-injector pump (Stoelting). Injection coordinates were 0.5 mm anterior and 2.25 mm lateral to the bregma, at a depth of 3.5 mm from the surface of the brain. Tumors were grown for 7 days, after which successful grafting was confirmed by bioluminescence imaging.

Cell Culture

A detailed description of the culture conditions for murine neural stem/progenitor cells, microglia cells, glioblastoma (GBM) cells, and human patient-derived lines is provided in Supplementary methods.

Quantitative Reverse Transcription PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). Synthesis of cDNA was carried out using SuperScript Reverse Transcription IV (Invitrogen). Quantitative PCR was performed in the QuantStudio 3 (Applied Biosystems, Thermo Fisher) using the PowerUp SYBR Green Master mix (Applied Biosystems, #A25742). Cycle threshold (C_t) for each gene was determined after normalization to hypoxanthine-guanine phosphoribosyltransferase (HPRT; mouse) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; human), and delta-delta ($\Delta\Delta$)C_t was calculated relative to the designated reference sample. Gene expression values were then set equal to $2^{-\Delta\Delta Ct}$ as described by the manufacturer of the kit (Applied Biosystems). All PCR primers were synthesized by Invitrogen and designed for the murine and human sequences of Ptch1, Ptch2, Gli1, Gli2, and the housekeeping genes HPRT and GAPDH (Supplementary table).

IL-6 Enzyme-Linked Immunosorbent Assays

Enzyme-linked immunosorbent assays (ELISAs) were performed by following the manufacturer's instructions (Mouse IL-6 Quantikine ELISA Kit, Fisher Scientific, #M6000B). The absorbance was read at 450 nm (Spectramax M5, Molecular Devices). A wavelength correction was performed by subtracting readings at 600 nm from those at 450 nm.

Irradiation

Neurosphere cultures were irradiated with 0, 2, or 4 Gy at room temperature using an experimental X-ray irradiator (Gulmay Medical) at a dose rate of 5.519 Gy/min. Control samples were sham irradiated. The X-ray beam was operated at 300 kV and hardened using a 4 mm Be, a 3 mm Al, and a 1.5 mm Cu filter.

Eight-week-old mice were anesthetized with isoflurane, cone beam CT images were acquired, and individual treatment plans were calculated for each mouse using the SmART Plan software package. Subsequently, for the in

vivo experiments the right hemisphere of the brain was irradiated with 4 Gy or 10 Gy using a single beam. For behavioral studies, the whole brain was irradiated with 10 Gy using 2 opposing beams. The X-ray beam was operated at 225 kV.

Dosimetry traceable by NIST (National Institute of Standards and Technology) on both X-ray machines was performed using a micro-ionization chamber.

In Vitro Drug Treatment

NSPP (Vitascreen) was solubilized in dimethyl sulfoxide (DMSO). Three hours after irradiation, neurosphere cultures were treated with NSPP (10 μM) or DMSO.

In Vivo Drug Administration

In vivo neural stem/progenitor experiments: 0.55 mg of NSPP was dissolved in 15 μL DMSO and then suspended in 1 mL of 1% Cremophor EL (CrEL; Sigma-Aldrich). Starting 24 hours after irradiation, mice received 5 daily subcutaneous injections of 5 mg/kg of NSPP or DMSO/CrEL.

For behavioral studies, starting immediately after 10 Gy whole brain irradiation, mice received 5 daily subcutaneous injections of 5 mg/kg NSPP or DMSO/CrEL.

In Vitro Assays with Patient-Derived GBM Specimens

For the assessment of self-renewal in vitro, HK-374, HK-157, and HK-382 cells were irradiated with 0 or 4 Gy. Three hours after irradiation, cells were treated with either DMSO or NSPP. The medium was supplemented with DMSO or NSPP every other day for 2 weeks. The number of spheres formed in each treatment group was normalized against the non-irradiated control.

Brain Dissociation

Five days after drug treatment, the brains of the mice were harvested and placed on the Acrylic Mouse Brain Slicer Matrix (Zivic Instruments, #BSMAA001-1). Coronal sections starting from 2 mm anterior to 2 mm posterior of the bregma were cut and the left hemisphere was separated from the right. The brain tissue was minced into very small pieces using a scalpel and the cells were isolated as mentioned in Supplementary methods (neural stem cell culture). The cells were then used for either flow cytometric analysis to assess the percentage of Nestin-GFP+ cells in different treatment groups or to quantify self-renewal capacity in neurosphere formation assays.

Behavioral Testing

All of the behavioral experiments (Novel Object Recognition [NOR], Object in Place [OIP], Fear Conditioning [FC]) were conducted in the Behavioral Testing Core at UCLA. A detailed description is provided in Supplementary methods.

Flow Cytometry

Passage #2 neurospheres established from the brains of Nestin-GFP mice were harvested and dissociated into single cell suspension as described in Supplementary methods. Single cell suspensions were either subjected to fluorescence activated cell sorting (FACS) (Flow Cytometry Core, Terasaki, BD FACS ARIA) for GFP-high, -medium, and -low cell populations or analyzed for GFP expression using a MACSQuant Analyzer (Miltenyi Biosciences) and the FlowJo software package v10.

Neurosphere-Formation Assay

In order to assess self-renewal capacity, passage #2 neurospheres from Nestin-GFP mice were trypsinized and plated into 96-well nontreated plates containing 1x complete NeuroCult media, at a range from 1 to 1000 cells/well. Growth factors (epidermal and basic fibroblast), were added every 3 days, and the cells were allowed to form neurospheres for 14 days. The number of spheres formed per well was then counted and expressed as a percentage of the initial number of cells plated.

Immunohistochemistry

Immunohistochemistry was performed using standard protocols. A detailed description is included in Supplementary methods.

Statistical Analysis

All statistical analyses were performed using the GraphPad Prism software package. Unless stated otherwise, results were derived from 3 biological replicates or at least 3 animals per group. A P-value ≤0.05 in a Student's t-test or one-way ANOVA was considered statistically significant. Kaplan–Meier estimates were calculated using GraphPad Prism, and a P-value of 0.05 in log-rank test indicated a statistically significant difference.

Results

Radiation Mitigation in Neural Stem/Progenitor Cells In Vitro

Passage #2 neurospheres from Nestin-GFP mice, in which most cells were Nestin-GFP+, were used for all in vitro experiments (Figure 1A). In order to test the self-renewing capacity of the sorted GFP-high, -medium, and -low cells from neurospheres we performed in vitro limiting dilution assays. Nestin-GFP^{high} cells showed 4.3-fold higher sphere formation than Nestin-GFP^{med} cells and 13.5-fold higher sphere formation than Nestin-GFP^{low} cells (Figure 1B), thus supporting the neural stem/progenitor phenotype of Nestin-GFP^{high} cells. Irradiation of the neurospheres with 0, 2, or 4 Gy preferentially reduced the size of the Nestin-GFP^{high} cell population (Figure 1C). This was in line with

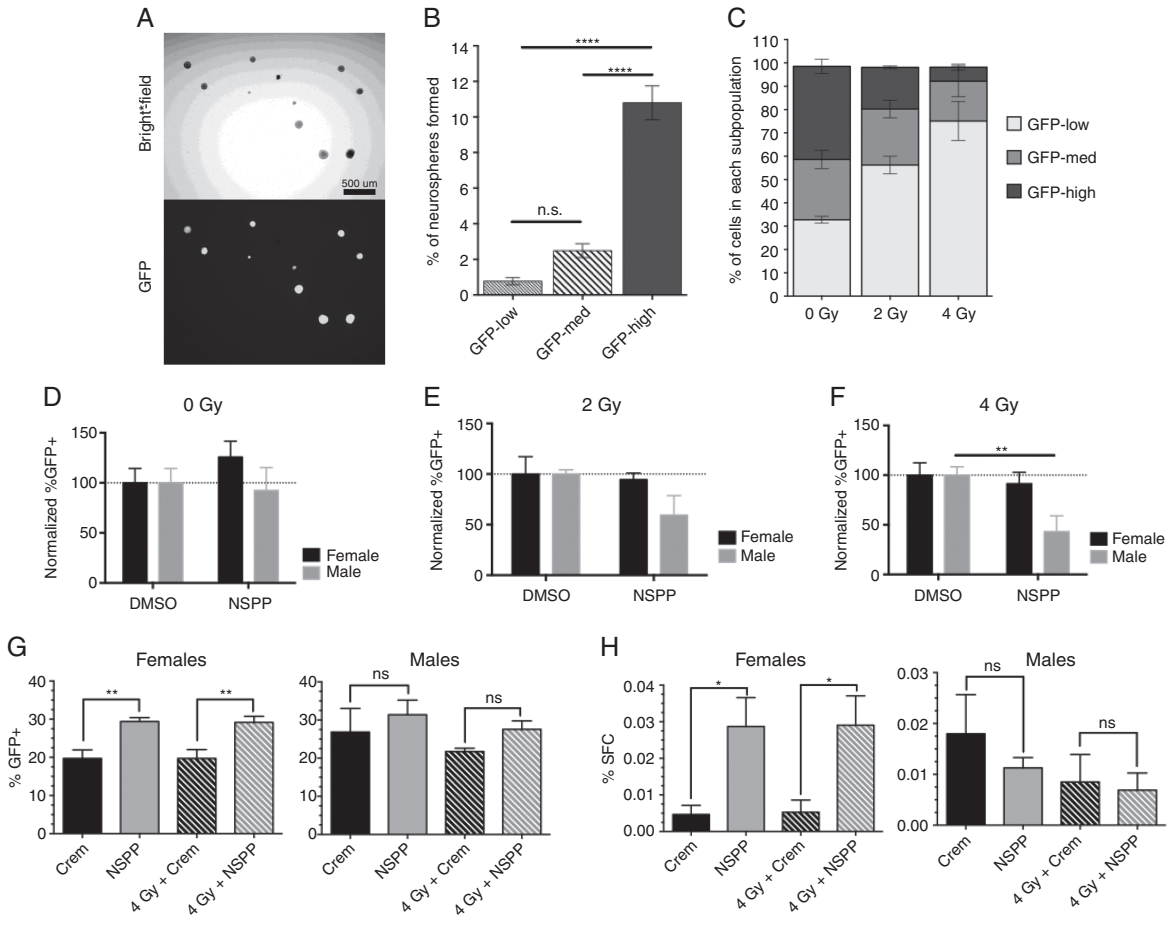

Figure 1. Radiation mitigation in neural/progenitor cells in vitro and in vivo. (A) Representative images of neurosphere cultures established from the brains of Nestin-GFP mice. Bright-field and GFP image (4x). (B) Sorted GFP^{high}, -^{medium}, and -^{low} cells were subjected to an in vitro limiting dilution assay. (C) Effect of radiation (0, 2, or 4 Gy) on 3 different subpopulations of Nestin-GFP neurospheres. Neurospheres (passage #2) from male or female newborn pups were subjected to 0 (D), 2 (E), or 4 Gy (F) followed by a single treatment with either DMSO or NSPP (10 μM) 3 hours post irradiation. Eight-week-old Nestin-GFP male and female mice were sham irradiated or irradiated with 4 Gy. After 24 hours, mice were treated with DMSO/CrEL or NSPP (5 mg/kg) subcutaneously for 5 days. The brains of the mice were harvested, dissociated, and analyzed by FACS (G) or sphere forming assays (H). All experiments in this figure have been performed with at least 3 independent biological repeats. (Unpaired *t*-test. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.)

a previous report on radiation-induced differentiation of neural stem/progenitor cells.[10]

Next, we irradiated neurospheres with 0, 2, or 4 Gy and treated the cells with NSPP. NSPP failed to increase the size of the population of Nestin-GFP^{high} neural stem/progenitor cells (Figure 1D–F). While NSPP did not show any effect on the neurospheres derived from the female newborn pups, it significantly reduced the Nestin-GFP^{high} population of cells derived from the male newborn pups, especially in the 4 Gy treated groups.

Radiation Mitigation in Neural Stem/Progenitor Cells In Vivo

We next considered the possibility that our lead compound targets neural stem/progenitor cells indirectly, which, however, cannot be easily tested in the absence of the correct microenvironment in vitro. To investigate this, 8-week-old male and female Nestin-GFP mice were irradiated with a dose of 4 Gy to the right brain hemisphere. The radiation treatment plan ensured sparing of the contralateral hemisphere from irradiation, thus allowing for an internal unirradiated control for each individual mouse. 24 hours later the animals began treatment with 5 daily injections of CrEL/DMSO or NSPP. The brains were harvested, digested, and analyzed for the number of Nestin-GFP^{high} stem/progenitor cells, and the self-renewing capacity of the isolated cells was evaluated.

NSPP significantly increased the number of Nestin-GFP^{high} stem/progenitor cells in female mice (Figure 1G, left panel). In male mice we observed a similar trend but the effect was not significant (Figure 1H, left panel). In in vitro limiting dilution assays we observed a significant increase in sphere-forming capacity in cells obtained from female mice treated with NSPP but not in cells obtained

from male mice (Figure 1G, H, right panel). Therefore, all remaining studies were conducted in female mice.

NSPP Mitigates Radiation-Induced Neuroinflammation

Six-week-old female C3H mice were treated with a single fraction of 4 or 10 Gy to the right brain hemisphere (Figure 2A). Starting 24 hours after irradiation, the mice were treated with either DMSO/CrEL or NSPP for 5 days. The brains were harvested, fixed in formalin, and embedded in paraffin, and 4 μm sections were subjected to immunohistochemistry. Sections were either stained with hematoxylin and eosin (H&E) or stained against glial fibrillary acidic protein (GFAP; marker for reactive astrocytes marker), Iba1 (ionized calcium binding adaptor molecule 1; an activated microglia marker), and Ki67 (proliferation marker) and subjected to an automated image analysis (Figure 2B–E).

The slides were scored for positively stained cells in the cortex, corpus callosum, *cornu ammonis* 1 stratum pyramidale (CA1sp), and the dentate gyrus. In the non-irradiated group, NSPP did not show significant changes in GFAP, Iba1, or Ki67 expression. When NSPP was given on 5 consecutive days starting 24 hours after irradiation, it led to a significant reduction in GFAP and Iba1 expression, thus indicating mitigation of radiation-induced neuroinflammation (Figure 3A).

To further confirm the anti-inflammatory effect of NSPP, we collected conditioned media from EOC20 microglia cells 24 hours after exposure to irradiation with 0 or 10 Gy and treatment with DMSO or NSPP in vitro. IL-6 secretion levels were assessed using ELISA. In line with the well-known pro-inflammatory effect of radiation, we observed a significant

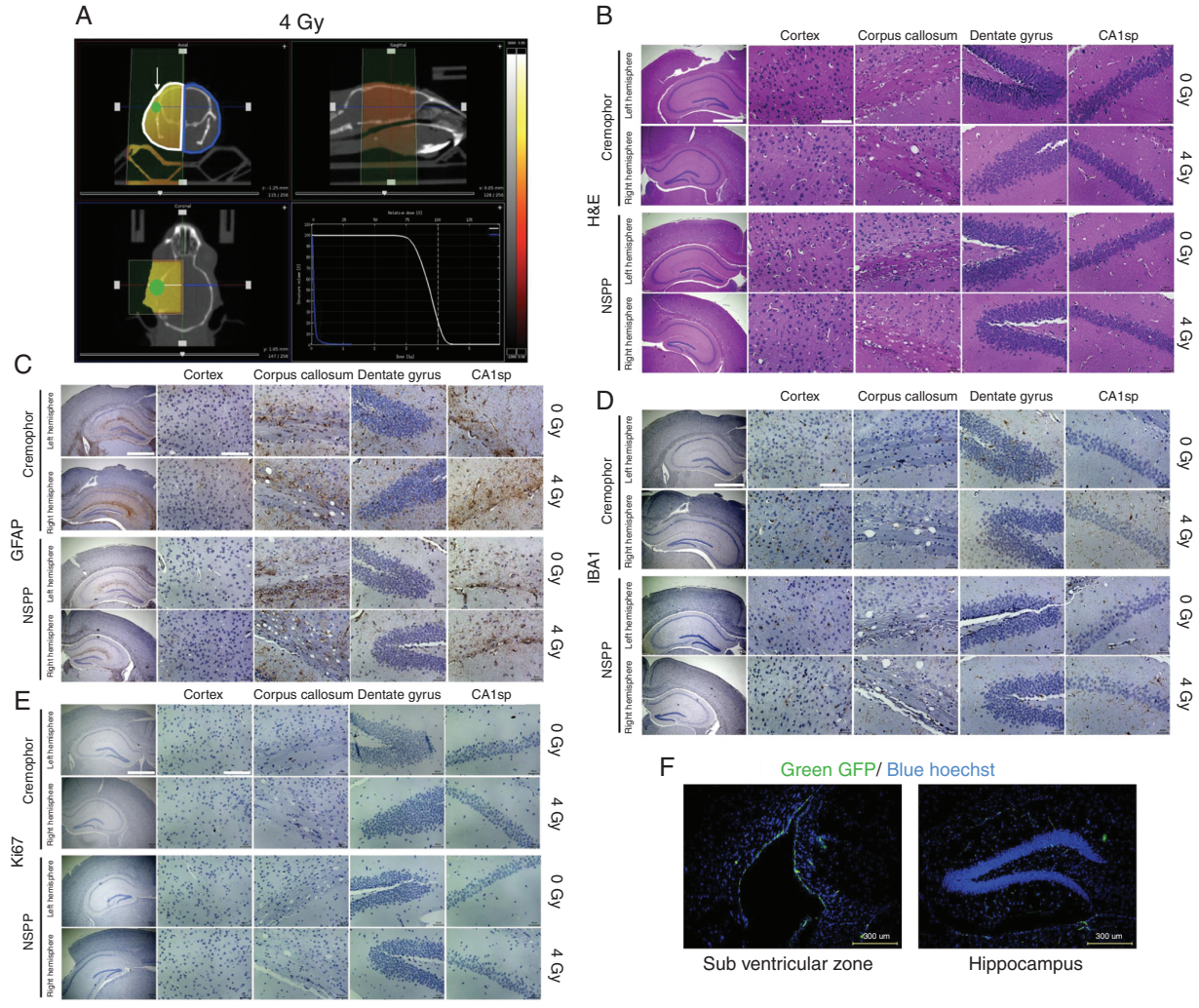

Figure 2. Radiation treatment plans and immunohistochemistry images. (A) Six-week-old C3H female mice were irradiated with 0 or 4 Gy and treated with DMSO/CrEL or NSPP (5 mg/kg, s.c.) for 5 days. Coronal sections were stained with H&E (B) or labeled with antibodies against GFAP (C), Iba1 (D), or Ki67 (E). Scale bars in low power images (4x, left columns): 1000 μm. Scale bars in high power images (40x): 100 μm. (F) Representative images (10x) of the subventricular zone and hippocampus region of 8-week-old Nestin-GFP mice labeled for GFP.

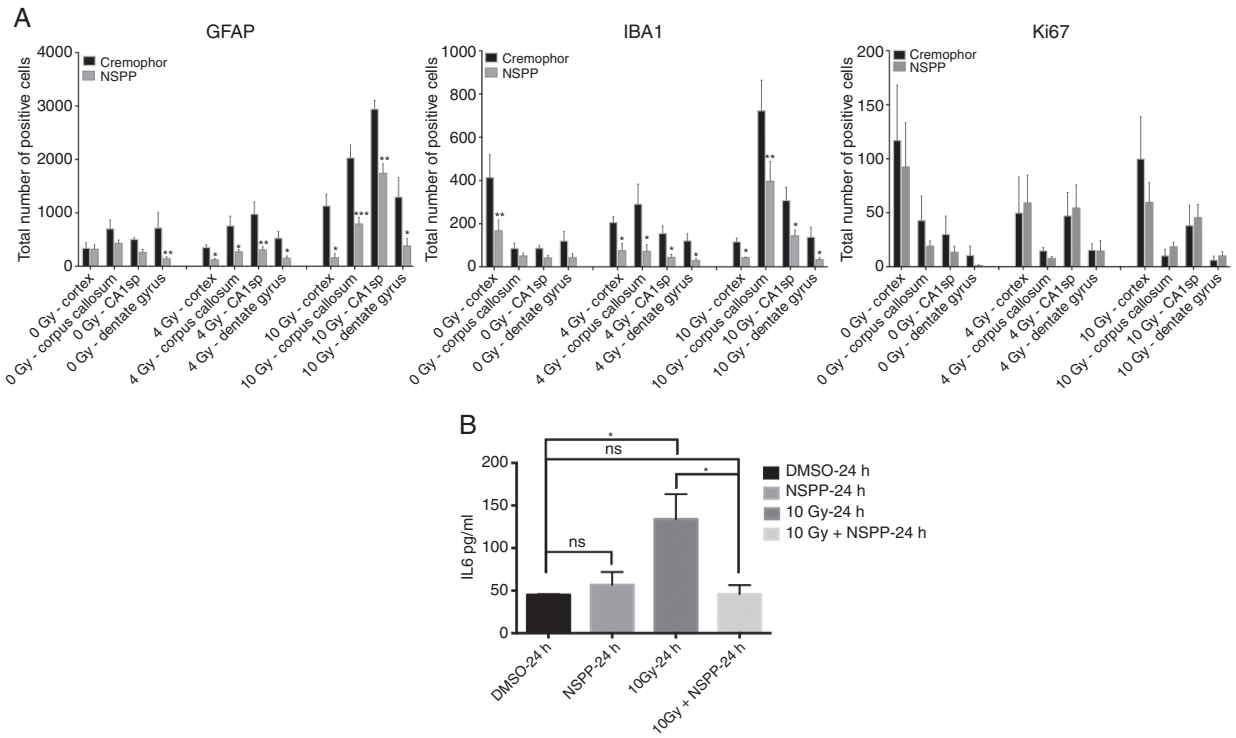

Figure 3. NSPP mitigates radiation-induced neuroinflammation. (A) Coronal sections stained against GFAP (left panel), Iba1 (center panel), and Ki67 (right panel) were quantified for the total number of positive cells in the cortex, corpus callosum, CA1sp, and dentate gyrus regions of the brain in the unirradiated and irradiated mice. (B) ELISA for IL-6 using conditioned media from normal EOC20 microglia cells 24 hours after exposing them to 0 or 10 Gy irradiation and treatment with either DMSO or NSPP. (Unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.)

increase in the secretion of IL-6 in cells treated with 10 Gy. Consistent with an anti-inflammatory effect, NSPP significantly reduced IL-6 secretion levels (10 Gy: 2-fold, $P = 0.0425$) compared with the DMSO control group (Figure 3B).

Preservation of Cognitive Function in Irradiated Mice

Ionizing radiation to the brain has long been known to cause neuroinflammation, which ultimately leads to a decline in cognitive function.[11,12] Our short-term experiments indicated that NSPP mitigates neuroinflammation. Next, we sought to test if treatment with NSPP also translated into improved cognitive function. After total brain irradiation with 10 Gy (Figure 4A), animals were treated with either DMSO/CrEL or NSPP (5 mg/kg) for 5 days. One month after irradiation the animals were subjected to unbiased cognitive testing (Figure 4B). NOR and OIP tests were performed to evaluate impairments in the prefrontal and perirhinal cortices, as well as hippocampus regions. This was followed by FC tasks for studying deficits in memory function dependent on the hippocampal regions.

Mice receiving DMSO/CrEL after cranial irradiation of 10 Gy demonstrated a significant behavioral deficit on both NOR and OIP tasks compared with unirradiated controls, as indicated by their impaired preference for novel object (Figure 4C) or place (Figure 4D). However, in the NSPP-treated group, mice showed significantly improved performance in identifying the novel object (Figure 4C) or place (Figure 4D). Furthermore, the discrimination index (DI) between the unirradiated and the combined treatment groups (10 Gy + DMSO/CrEL or 10 Gy + NSPP) were statistically insignificant, indicating that NSPP had successfully mitigated the radiation effects. In the FC task, the baseline freezing levels were comparable among the 3 treatment groups. All groups also showed an increased freezing behavior post 3 tone-shock pairings (context fear bars). Baseline freezing levels 48 hours post-training were significantly decreased in irradiated mice compared with the unirradiated control mice. Administration of NSPP to the irradiated mice reduced the cognitive deficits (Figure 4E). Treatment of the irradiated mice with NSPP led to an increased freezing behavior compared with the DMSO/CrEL-treated irradiated group, indicating preservation of hippocampal function (Figure 4E).

Effects of NSPP on GBM Cells In Vitro and In Vivo

Radiation mitigators or protectors always bear the risk of radiation protection or mitigation not only in normal tissues but also in tumors. To test the effect of NSPP on GBM cells in vitro we performed sphere-forming capacity assays using 3 different patient-derived GBM cell lines: HK-374, HK-157, and HK-382 in the presence (10 μM) or absence of NSPP in combination with irradiation at 0 or 4 Gy. The

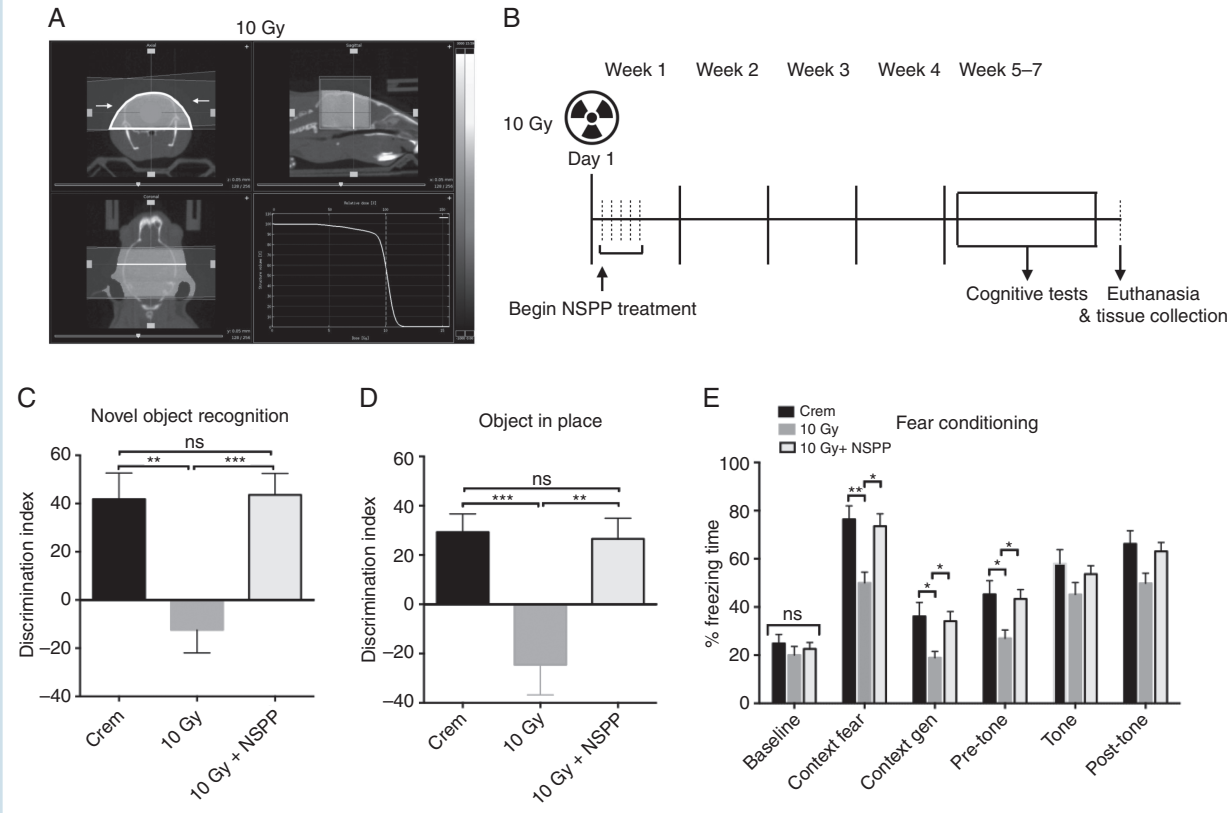

Figure 4. Preservation of cognitive function in irradiated mice. Groups ($n = 10$) of 6-week-old female C3H mice were irradiated with 0 or 10 Gy (A) and treated with NSPP (5 mg/kg) or DMSO/CrEL for 5 days. Schematic representation of the behavioral testing schedule (B). Starting week 5 the mice were tested for cognitive functions by performing NOR (C), OIP (D), and FC (E) tasks. (Unpaired t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.)

gliomaspheres were treated with DMSO or NSPP every other day for 2 weeks, at the end of which the number of spheres formed was counted and presented as the percentage of spheres formed. In HK-374 GBM cells NSPP significantly reduced the cells' self-renewing capacity with or without irradiation (Figure 5A, left panel), while in the HK-157 and HK-382 cell lines NSPP had no effect (Figure 5A, center and right panels).

To test if NSPP interferes with the effects of tumor irradiation in vivo, 2×10^5 GL261-luciferase mouse glioma cells were intracranially injected in C57BL/6 mice. Seven days after implantation, tumor grafting was confirmed by bioluminescence imaging (BLI), and the tumors were either sham irradiated or irradiated with 10 Gy. Immediately after irradiation, the mice were treated with either DMSO/CrEL or NSPP (5 mg/kg) subcutaneously. The treatment was given on a 5-days-on/2-days-off schedule for 3 weeks. Weights of the mice were recorded every day until the study endpoint. Kaplan–Meier survival estimates showed no effects of NSPP alone or in combination with radiation (Figure 5B). Importantly, NSPP did not show any toxicity and did not lead to weight loss (Figure 5C). Tumor growth was monitored by BLI of the tumors at day 7 (pretreatment) and at day 23 (days post implantation; Figure 5D) and indicated no tumor-promoting effects of NSPP.

Effects of NSPP on the hedgehog Pathway in Microglia and GBM Cells

Recent publications have shown that deregulated developmental pathways play a key role in GBM progression and tumorigenesis by conferring drug resistance to the tumor cells and that inhibition of the hedgehog pathway induces apoptosis in GBM cells.[13–15] We had previously demonstrated that NSPP activates the hedgehog pathway by binding to the transmembrane domain of Smoothened.[16] Therefore, we sought to test whether the different sensitivities of microglia and GBM to NSPP would explain its differential effect in normal tissues and tumors. Quantitative RT-PCR for hedgehog target genes was performed in normal microglia cells (EOC20) and HK-374 patient-derived GBM tumor cells 24 hours after treatment with different concentrations of NSPP. The results obtained are presented as a ratio of fold changes of the genes in EOC20 over HK-374 cells. Low doses of NSPP (500 nM to 1 μM) induced the expression of the hedgehog pathway target genes Ptch1, Ptch2, Gli1, and Gli2 in microglia cells more efficiently compared with HK-374 GBM cells, both alone and in combination with radiation (Figure 6A–D), suggesting that microglia cells are more sensitive to NSPP than HK-374 glioma cells.

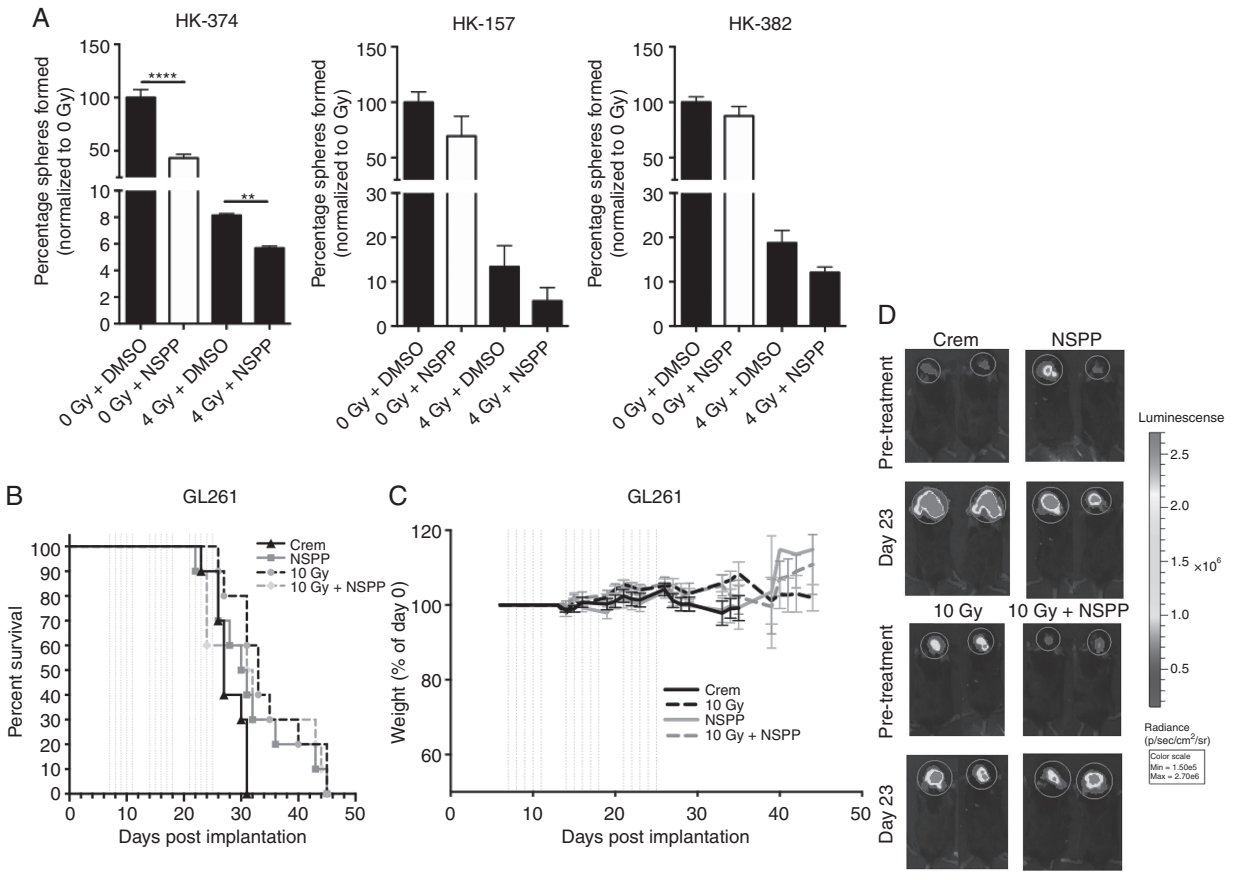

Figure 5. Effects of NSPP on GBM cells in vitro and in vivo. (A) Patient-derived HK-374 (left panel), HK-157 (center panel), and HK-382 (right panel) GBM cells were used to perform sphere-forming assays with sham-irradiated or irradiated cells in the presence or absence of NSPP (10 μM). The cells were treated every other day for 2 weeks. The number of spheres formed under each condition was counted and presented as percentage spheres formed. (Unpaired *t*-test. $^{**}P < 0.01$, $^{****}P < 0.0001$.) (B) 2×10^5 GL261-Luciferase mouse glioma cells were implanted intracranially into six-week-old female C57BL/6 mice. Animals were irradiated with 0 or 10 Gy and treated with DMSO/CrEL or NSPP (5 mg/kg, s.c.) for 3 weeks. The effect of NSPP on survival in tumor-bearing mice was assessed using Kaplan–Meier estimates. (C) Weight curves for the mice in the different treatment groups. (D) Bioluminescence images of mice bearing tumors obtained at day 7 (pretreatment) and at day 23 (days post implantation). Each group had $n = 10$ mice.

Discussion

Aside from surgery, radiotherapy is one of the most effective cancer treatments for patients suffering from brain cancer or cancer metastases to the brain. However, with 5-year survival rates steadily increasing, more and more patients experience long-term treatment side effects, which in the case of cranial irradiation manifest in impaired cognitive functions. Symptoms arise months and years after completion of radiotherapy and are particularly detrimental in childhood cancer survivors where despite tumor control rates often being excellent, the cognitive decline can amount to a loss of 1–2 IQ points per year.[17]

Some experimental approaches, while difficult to translate into the clinic, have shown promising results.[18–20] Approved clinical treatment options for preventing the late sequelae of cerebral radiotherapy are few and are mostly limited to radiation treatment volume reduction[21] or sparing of critical brain structures from irradiation.[22] Previous pharmacological radioprotection studies using, for example, amifostine have been hindered by the lack of blood–brain barrier penetration of the drugs and the general concern of simultaneous protection of tumor cells.[23]

Few pharmacological treatment attempts have been made to mitigate radiation effects to the CNS after completion of treatment, and those are mostly limited to corticosteroids, which are routinely used to acutely reduce edema but are not sustainable as a long-term treatment option. So far experimental approaches have had limited[24] or no success.[25]

We had previously reported that NSPP mitigates the acute intestinal radiation syndrome when given 24 hours or later after a lethal dose of radiation through activation of the hedgehog pathway.[7,16] Motivated by reports in the literature that hedgehog signaling also affects neural stem cells,[26–28] we sought to test if NSPP would mitigate radiation injury in brain tissues.

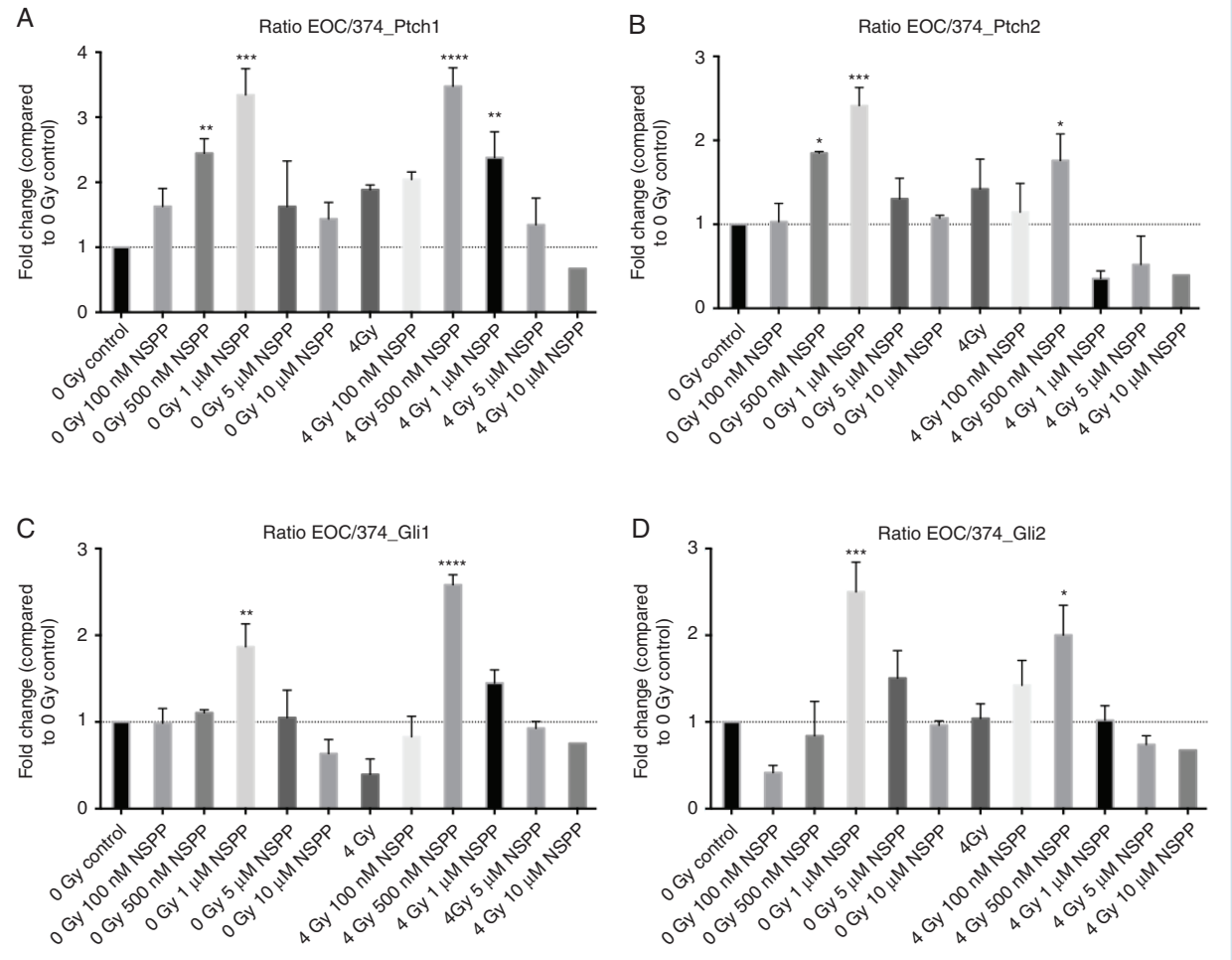

Figure 6. Effects of NSPP on hedgehog signaling in microglia and GBM cells. QRT-PCR for hedgehog target genes Ptch1 (A), Ptch2 (B), Gli1 (C), and Gli2 (D) in EOC20 microglia and HK-374 glioma cells 24 hours after irradiation and treatment with DMSO or NSPP. Fold changes are presented as a ratio of EOC20 microglia cells over HK-374 cells. (Unpaired one-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.)

NSPP is not soluble in water and it was therefore solubilized in DMSO/CrEL. Clinically, CrEL is known for having side effects when drugs like paclitaxel are dissolved in 50% CrEL, and large amounts of CrEL are co-injected with the drug.[29] In our study, CrEL was only used in in vivo experiments as a solvent for NSPP at a concentration of 1%. The total volume injected per animal was on average 225 µL, which amounted to a small total amount of CrEL per animal and did not cause adverse effects.

When given after total brain irradiation, NSPP increased the number of Nestin-GFP+ cells and their self-renewal capacity of the cells in the brains of female mice while it had no effect on male mice. It is noteworthy that the self-renewal of Nestin-GFP+ cells from male mice exceeded that of female mice, both at baseline and after 4 Gy, and that the number and self-renewal capacity of Nestin-GFP+ cells in female mice were not affected by a single dose of 4 Gy. Estrogen dependency of embryonic but not adult neural stem/progenitor cell proliferation and differentiation has been previously described.[30] At the age of 8 weeks, one can assume that the young-adult animals in our study still had active neurogenesis. We speculate that the reported interplay of hedgehog and estrogen signaling[31] could explain the striking sex differences in efficacy for the Smoothened activator NSPP.[8]

Attempts to show the effect of NSPP on passage #2 neural stem/progenitor cells in vitro failed irrespective of sex, indicating that NSPP does not have a direct effect on neural stem/progenitor cells but that it rather affects the microenvironment. The possibility of indirect effects was further supported by data showing a reduction of radiation-induced IL-6 production by microglia cells in vitro and reduction of radiation-induced astrogliosis (GFAP) and microglia activation (Iba1) in vivo.

It is important to point out that NSPP showed efficacy when given 24 hours after exposure of the animals to radiation and when repair of radiation-induced DNA damage has long been completed.[32] Previous attempts to preserve cognitive function have mostly relied upon radioprotectors like amifostine that have to be given before treatment to limit radiation toxicity to the normal tissue and always bear the risk of tumor tissue protection. In our study, NSPP did

not attenuate the effects of radiation on glioma cells in vitro or in vivo, even when given 3 hours after or concurrently with radiation, respectively.

An explanation for the differential effects of NSPP on normal and malignant cells could be that the hedgehog signaling pathway is utilized at a different threshold in gliomas, resulting in differential responses to activators of this pathway. Furthermore, Smoothened agonists are known to inhibit hedgehog signaling at higher concentrations,[33] and the bioavailability of NSPP in normal brain tissues and GBM is likely to differ based on differences between the blood–brain barrier and the blood–tumor barrier.[34] Taken together, the data suggest the possibility of a therapeutic window for NSPP and indicates that NSPP could be safely administered during or after the completion of radiotherapy in patients suffering from GBM, where the presence of residual tumor cells after completion of surgery and radiotherapy is almost always inevitable.

Cranial irradiation is known to disrupt hippocampal neurogenesis in rodents as well as in humans.[35] The resulting decline in cognitive function manifests in memory loss. Using 3 different cognitive tests, we demonstrated that a dose of 10 Gy had profound effects on hippocampal-dependent memory function. In line with the observed effects of NSPP on neuroinflammation and neural stem/progenitor cell populations, NSPP treatment translated into preservation of cognitive function in the animals, with results in irradiated, NSPP-treated animals being statistically indistinguishable from non-irradiated animals.

Despite the promising nature of NSPP as an agent that allows for radiation mitigation and preservation of cognitive function following radiotherapy, there are still some questions that need to be addressed. First, we demonstrated inhibition of radiation-induced neuroinflammation by NSPP after a single dose of 4 or 10 Gy. It remains to be seen if NSPP when given daily during the typical course of fractionated radiotherapy with 30 fractions of 2 Gy still preserves cognitive function. Second, our studies on cognitive function used a single dose of 10 Gy. Based on an alpha/beta ratio of 2 for the CNS,[36] this dose amounts to a biologically effective dose (BED) of only 60 Gy. Although falling short of the BED of 120 Gy calculated for 60 Gy in 2 Gy fractions given in GBM patients, our dose more closely resembled the BED for cranial irradiation in children with leukemia, a patient population most vulnerable to the normal tissue effects of radiation.[37] However, the dose of 10 Gy given in our study accounted for a substantial normal tissue toxicity. Finally, radiation-induced toxicity to the CNS is multifactorial and affects multiple tissue compartments, including demyelination of neurons, activation of microglia, and microvascular damage that all contribute to a decline in cognitive function over time. We demonstrated a reduction in early onset of neuroinflammation and preservation of cognitive function 6 weeks after irradiation but did not test whether NSPP will mitigate cognitive decline at later time points. Future studies will be needed to evaluate if continued application of NSPP over an extended period of time will continue to mitigate radiation-induced cognitive impairment.

In summary, we conclude that NSPP has the potential to mitigate radiation effects to the normal brain when given during or after radiotherapy and warrants further investigation.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

cognitive function | neural stem cells | radiation | radiation mitigation

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Authorship statement. KB, PM, LH, LZ, MS, AI, NTN, SSS, DS performed the experiments and collected the data. CEM performed the immunohistochemical analysis, LML and HIK provided materials. KB and FP analyzed the data and wrote the manuscript. FP conceived of the study. All authors edited and approved of the final version of the manuscript.

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