Brain irradiation leads to persistent neuroinflammation and long-term neurocognitive dysfunction in a region-specific manner

Julie Constanza, Élora Midavaine, Jérémie Fouquet, Martin Lepage, Maxime Descoteaux, Karyn Kirby, Luc Tremblay, Laurence Masson-Côté, Sameh Geha, Jean-Michel Longpré, Benoit Paquette, Philippe Sarret

1. Introduction

Planning of radiation treatment to reach tumor cells infiltrated into the brain parenchyma is complex. Depending on tumor location, brain areas at the periphery of the surgical cavity are irradiated with a significant dose. A therapeutic dose is currently difficult to reach because of the intrinsic sensitivity of healthy brain tissue (Edelstein et al., 2017).

Etiology of the neurocognitive disorders is multi-factorial, including tumor progression, radiotherapy, neurosurgical procedures, chemotherapy and patient lifestyle (van Kessel et al., 2017). Adverse effects can develop and progress months and years after the end of treatment. After brain irradiation, an acute or early late (0–6 months) transient decline of alertness and memory can be followed by a late delay (> 6 months) associated with treatment speed deficits, and loss of...
memory and executive functioning (Correa et al., 2016; Scoccianti et al., 2012). Histopathologically, late delayed injury induced by radiation is characterized by vascular abnormalities, perivascular edema, reactive gliosis, demyelination, and ultimately white matter necrosis, with a dose-volume dependent severity (Lawrence et al., 2010). These long-term sequelae are often progressive and irreversible, and include a broad range of clinical symptoms, such as neuropathy, encephalopathy, seizure, syncope, memory loss and ataxia that severely compromise patients’ quality of life (Diaz and Choi, 2014; Edelstein et al., 2017).

Optimization of radiotherapy requires a better knowledge of the deleterious effects associated with a localized irradiation that may have an impact on interconnected areas. Recent efforts have been made to avoid irradiation of the hippocampus that houses neural stem/progenitor cells for generation of neurons in adult brain (Monje et al., 2003; Monje et al., 2002). Playing a vital role in learning and memory, the hippocampus is particularly vulnerable to radiotherapy (Edelstein et al., 2017). Fortunately, this brain structure is usually free of infiltrating tumor cells despite the extensive invasion of surrounding regions (Mughal et al., 2018). Clinical trials have shown that delayed recall memory and verbal learning can be preserved by avoiding irradiation of the hippocampus (Caine et al., 2016; Gondi et al., 2014).

Although hippocampus-sparing represents a milestone in radiation oncology, analyzing a complex phenomenon by breaking it down into its elementary components is a reductionist view to prevent radiation-induced cognitive impairment. The brain cannot be considered as a series of equivalent functional units since different structures within the brain carry out various functions. The neuroanatomical target theory, as proposed by Peiffer et al. (Peiffer et al., 2013), suggests that some radiation-induced lesions may have similar effects on the functional abilities of other brain lesions. Indeed, a sufficient amount of radiation to the primary motor regions or to the temporal cortex can worsen the overall cognitive function in brain tumor patients, regardless of whether the damage to these two regions occurs by the same mechanism (e.g., vascular injury, inflammation). However, the fact that damage to these cerebral structures leads to injury suggests that selective avoidance of such particular brain sites may also preserve some neurocognitive function (Peiffer et al., 2013).

In this study, we determined whether a specific sensitivity of a brain region to radiation could lead to selective behavioral disturbances. For this purpose, the right primary somatosensory cortex (SIFL) of male Fischer rats was irradiated with a single high dose of radiation using a Gamma Knife (GK). The dose-volume histograms were constructed following delineation of selected anatomical regions of interest (ROI) on post-treatment magnetic resonance images (MRI) (Constanzo et al., 2015). Necrosis, neovascularization and reactive gliosis were determined by $T_2^*$-weighted MRI combined to dynamic contrast-enhanced (DCE) that were then validated by immunohistopathological analyses. The susceptibility of different brain structures to radiation was then monitored over 140 days after Gamma Knife radiosurgery using different behavioral tests assessing learning/memory performances, motor function and coordination, as well as pain and anxiety-like behaviors.

2. Materials and methods

2.1. Subjects and housing

Experiments were performed with adult male Fisher (CDF) rats (250 g, Charles River) housed in a 12 h light/12 h dark cycle and allowed ad libitum access to food and water. Rats were divided in three groups: twenty for behavioral tests (12 irradiated, 8 controls), five for diffusion MRI (5 irradiated, including images before irradiation), six for vascular/permeability MRI (6 irradiated, including images before irradiation), and seven for histology (4 irradiated, 3 controls). The experimental procedures were approved by the Animal Care Committee of the University of Sherbrooke and were in accordance with policies and directives of the Canadian Council on Animal Care.

2.2. Targeted radiation therapy in preclinical animal model and study design

A rat model irradiated with a single isocenter treatment plan deli-
vering 37 Gy at the 30% isodose (corresponding to a dose ≥100 Gy at 100%) in a localized target volume (6 mm3), was used to explore the late delayed effects of radiation. The need for a high radiation dose to induce a necrosis in normal Fischer rat brain was suggested by a previous study in Sprague-Dawley rats, which were irradiated in the right frontal lobe using stereotactic radiosurgery. These animals received a clinically-relevant dose of 60 Gy did not yield histopathological abnormalities. In fact, the loss of endothelial cell integrity occurred only after a dose of 100 Gy, and necrosis was observed in all animals only after receiving 150 Gy (Kondziolka et al., 1992). These results support that, in the rat brain, a radiation dose higher than used clinically is needed to assess corresponding damage inflicted to the human brain by lower doses. The primary somatosensory forelimb (S1FL) of the right hemisphere was thus targeted with a Leksell Gamma Knife Perfusion (Elekta AB) and an appropriate treatment plan was implemented as previously described (Constanzo et al., 2015). The study design including behavioral tests and MRI scanning timepoints following irra-
diation is illustrated in Fig. 1A. After dose-volume histogram calculation, the mean dose of radiation was obtained for different brain regions of interest (Fig. 1B).

2.3. In vivo MRI

Eleven Fisher rats were anesthetized with 5% isoflurane in oxygen and maintained at 2–2.5% isoflurane in oxygen (1.5L/min). They were scanned using a 7 Tesla (T) small animal scanner (Varian Inc) with a dedicated rat head-coil (RAPID MR International, OH) before irradiation, and on days 1, 16, 21, 54, 82 and 110 post-irradiation. During magnetic resonance brain scanning, the temperature of the surrounding air was maintained constant at 30 °C with a warm-air heating system (SA Instruments Inc).

A fast spin echo $T_2^*$ sequence was performed with the following parameters: repetition time $TR = 3000$ ms; effective echo time $T_{Eeff} = 48$ ms; 8 echoes; echo spacing 12 ms; field of view (FOV) = $32 \times 32$ mm$^2$, 5 slices, 0.7 mm axial slice thickness. A dy-
namic contrast-enhanced (DCE) MRI is a gradient echo multi-slice se-
quence, with a protocol consisting of a pre-contrast $T_1$ ($T_{1,0} = 1/(R_1)\alpha$) map calculated from the multiple flip angle approach ($\alpha = 10, 20, 25, 35, 50^\circ$) with TR = 170 ms; TE = 3.5 ms; FOV = $32 \times 32$ mm$^2$; in-plane resolution about 0.125 × 0.125 mm$^2$; 4 averages; 16 slices, 1 mm axial slice thickness. A dynamic acquisition with the same parameters (except for the flip angle which was set to 30°) was repeated for 58 min, with a time resolution of 174 s. A rather low temporal resolution was selected in favor of a high spatial resolution. A solution of Gd-DTPA (142.9 mM Magnevist, Berlex Canada Inc) was administered $320$ s after acquisition initiation (a bolus of 600 $\mu$L of Gd-DTPA [0.143 mol/L] solution was injected over 60 s). The echo time was assumed to be short compared with the $T_2$ relaxation values of the analyzed tissues, and the pre-
jection $T_1$ map, repetition time, equilibrium magnetization, flip angle and granularity of Gd-DTPA at 7 T (3.6 mM$^{-1}$s$^{-1}$) were taken into account (Landis et al., 2000). The $T_2$$^*$ imaging was a 3D gradient echo sequence, which was performed with TR = 90 ms; TE = 25 ms; FOV = $3 \times 3 \times 1.5$ mm$^2$; resolution: 0.117 × 0.117 × 0.156 mm$^3$; flip angle: 15°; 2 averages.

Diffusion-weighted images (DWI) were acquired using a multi-slice spin echo sequence with 15 non-collinear uniform diffusion gradient directions and b = 977 s/mm$^2$. A reference b = 0 image was also acquired. Other imaging parameters were: repetition time (TR)/echo time (TE) = 3500 ms/35 ms, FOV = 38.4 × 38.4 mm$^2$, 25 contiguous coronal slices, 0.3 × 0.3 × 0.35 mm$^3$ resolution, for a 2 h total scan
2.4. Post-processing imaging

The necrosis and neovascularization post-processing, using DCE-MRI and \( T_2^* \) sequences were published in a previous study (Constanzo et al., 2016). To estimate the volume of the necrotic core, the central part of the lesion with no to low contrast enhancement was manually delineated on the first-time frame of the DCE-MRI. The delineation was straightforward because this central part was always surrounded by a shell with high contrast enhancement. The diffusion MRI pre-processing and post-processing steps were described in (Constanzo et al., 2018). Briefly, the methodology used to analyze DWI images was, first a denoising step followed by reconstruction of the diffusion tensor, its associated metrics and computation of the fiber orientation distribution function of spherical harmonics order 6 (Garyfallidis, 2012; Garyfallidis et al., 2014). Tractography algorithms and visualization of streamlines were used to reveal displacements and breakdown in neuronal pathways. In addition, a non-negative least square method was used to compute the diffusion tensors, extracting imaging features such as fractional anisotropy (FA) between 0 and 1 and radial diffusivity (RD) in \( \text{mm}^2 \times \text{s}^{-1} \). The rat brain was divided in 2 main anatomical areas: the cortex and the white matter into the right (irradiated) and the left (non-irradiated) hemispheres. Here, FA and RD were calculated in the corpus callosum (CC).

2.5. Histopathology

Control and irradiated rat brains were analyzed on days 110 and 140. The brain tissue structure (hematoxylin and eosin (H&E)), the Nissl body of neurons (Cresyl violet) and the myelin sheaths (Luxol Fast Blue) staining were assessed to determine the radiation-induced changes, as previously described (Constanzo et al., 2016). These sections were examined with a Nanozoomer slide scanner (2.0-RS, Hamamatsu) combined with the Nanozoomer Digital Pathology software (NDP.view2).

For immune cell staining, brains were frozen and embedded at \(-35 \degree \text{C}\) in O.C.T. compound and then 30 \( \mu \text{m} \) transverse sections were generated using a Leica SM220R sliding microtome (Microtome Leica). Sections were collected in 0.1 M PBS and processed as free-floating sections. Sections were washed in PBS, blocked in 0.2% Triton X-100 supplemented with 5% normal goat serum and 2% BSA in 0.1 M PBS for 1 h at room temperature. After 20 min incubation in 0.1 M glycine solution, sections were labeled with anti-CD3ε antibody (1:500, hamster anti-CD3ε, Santa Cruz, Cat. number sc-1174), anti-CD45 antibody (1:500, mouse anti-CD45, Bio-Rad, Cat. number MCA43R), or anti-Iba1 antibody (1:500, rabbit anti-Iba1, Wako, Cat. number 019–19741), diluted in blocking solution. Sections were then rinsed twice and incubated with the fluorescent secondary antibody (1:500, AlexaFluor® 647 conjugate, goat anti-hamster, Invitrogen, A-21451, ON, CA, 1:500, AlexaFluor® 568 conjugate, goat anti-mouse, Invitrogen, A-11031, ON, CA, 1:500, AlexaFluor® 488 conjugate, goat anti-rabbit, Invitrogen, A-1024116) in blocking solution for 1 h, washed twice in PBS and mounted on SuperFrost Plus slides with Aqua-Poly/Mount (Polysciences). Images of brain slices were acquired using a MicroBrightField/MBF Bioscience (Williston) and a Leica Stimulated Emission Depletion (STED) confocal microscope (Leica Microsystems), using the same acquisition parameters (gain, exposure time).

Brain slices were also stained against the GFAP astrocytes or Iba1 microglial markers. Briefly, sections were treated in 0.3% \( \text{H}_2\text{O}_2 \) solution for 1 h and then blocked in 2% NGS, 0.3% Triton X-100 in 0.1 M PBS for 1 h at room temperature prior to incubation with GFAP primary antibody (mouse anti-GFAP, 1:1000, Sigma, cat#3893) or Iba1 primary antibody (rabbit anti-Iba1, Wako, Cat. number 019–19741). Slices were rinsed and incubated with biotinylated goat anti-mouse antibody (1:200, Goat anti-mouse, Vector Labs, Cat number BA-9200) or anti-
rabbit antibody (1:200, Goat anti-rabbit, Vector Labs, Cat number BA-1000) and then incubated in Elite ABC solution (Vector Laboratories). The product of immune reaction was revealed using 3,3′-diaminobenzidine as a chromogen and 0.015% H₂O₂. The sections were mounted on SuperFrost Plus slides, dehydrated in graded ethanol, de-fatted in xylene and mounted with Permount.

The product of immune reaction was revealed using 3,3′-diaminobenzidine as a chromogen and 0.015% H₂O₂. The sections were mounted on SuperFrost Plus slides, dehydrated in graded ethanol, de-fatted in xylene and mounted with Permount. The specificity of each assay was determined by omitting primary and secondary antibodies on different slices. Images of brain slices were acquired using a Leica DM4000 microscope equipped with a Leica DFC350FX and an InfinityX camera using the same acquisition parameters.

2.6. Actimetry test

The MotorMonitor Version 5.05 (Kinder Scientific Company) was used to assess ambulatory functions of irradiated and controls groups. In the housing room, clear cages were placed in infrared beam-mounted racks for 24 h starting at noon on days 54, 82 and 109 post-irradiation. Parameters analyzed were ambulation, time spent in the center of the cage, basic movements, fine movements and resting time, all of which were determined by the occurrence of X and Y beam breaks. Ambulation occurs when the animal relocates its whole body such as a blocked beam is released while a new beam block occurs. Fine movements represent smaller movement, such as grooming and/or head movements, where the subject changes a beam status but the change does not fit the definition of an ambulation. Ambulation and fine movements thus cannot occur simultaneously.

2.7. Open field (OF) test

The open field test consists of a clear Plexiglas enclosure (40 × 40 cm) placed in the center of a normally lit (400 lx) experimental room. Rats were habituated to experimental conditions, but not to the OF. To initiate testing, rats were placed in the left corner of the enclosure, head facing open space, parallel to one side, and their exploratory behaviors were recorded with the ANY-Maze Tracking software for 5 min across the arena virtually divided into 16 squares. Parameters measured were the number of entries and the time spent in the center of the arena (four virtual central squares), the total distance travelled throughout the arena, and the total time of immobility. An entry in a virtual square occurred when the animal’s midpoint crossed a frontier. Tests were performed on days 54, 109 and 139 post-irradiation.

2.8. Grip strength

To assess the forelimb muscle strength as an indicator of neuromuscular function, the rat was held gently by the base of its tail over the top of the grid so that only its forepaws were able to grip the platform. Rats were then gently pulled backwards until they released their grip. Grip strength performance was evaluated before irradiation, days 33, 54, 82 and 110 post-irradiation, using the rat grip strength meter (Bioseb). The grip strength meter attached to a force transducer automatically records the maximum force applied as the peak tension (in grams) once the grasp is released. Each animal was assayed five times each testing day.

2.9. Rotarod

Motor balance and coordination were determined using a standard rat rotarod (LE 8300, LSI Letica, Panlab Scientific Instruments), which provides a fixed rotational speed: 4 rpm to acclimatize (30 s) rats, and a maximum speed of 12 rpm during the testing period. Five trials per day were performed. Animals were tested before irradiation, days 33, 54, 82 and 110 post-irradiation. The latency to fall off the rotarod was determined automatically by a timer that measures to the nearest second. A cutoff latency of 120 s was used for all rotarod assessments.

2.10. Elevated-plus maze (EPM)

The elevated-plus maze (Stoelting) is a cross-shaped platform constituted of four arms (50 cm long × 10 cm wide) shaped in the form of a cross, and was elevated 40 cm from the floor, as previously described by Walf and Frye (Walf and Frye, 2007). Two opposite arms were enclosed (illuminance: 2 to 4 lx) by side and end walls (40 cm high), and the other two arms were open (illuminance: 15 to 17 lx). The connecting (open) center area measured 10 × 10 cm, allowing rats to move freely into each zone of the maze. Each animal was placed into the center area of the plus maze facing an open arm. 120 days after irradiation, rats were then permitted to explore the maze freely for 5 min. Behaviors were tracked using the ANY-Maze software. The percentage of time spent in the open arms (time in open arms/[time in open arms + time in closed arms] × 100) and the number of entries into the open arms were measured for each group of rats. A zone entry was defined as the presence of 85% of the animal’s body in a specific zone.

2.11. Forced swim test (FST)

The Forced Swimming test (also known as Porsolt’s test) has been extensively used to study the depressive-like behaviors in rodents. Briefly, 120 days after irradiation, rats were placed in a 20-cm diameter cylindrical glass container measuring 50 cm in height and filled with water (24 ± 1 °C), after handling for about 2 min daily, 5 days prior to the beginning of the behavioral procedure. Rats were submitted to a pre-test stage (10 min), 24 h before the test session (5 min). The duration of time spent in active (swimming and climbing) and passive (immobility; i.e. floating with the absence of any movement except for those necessary for keeping the nose above water) behaviors are then scored.

2.12. Morris water maze task (MWM)

The Morris water maze task was used for assessing hippocampal-dependent spatial learning and memory of the rats (130 days after irradiation). Rats were required to find, in a 1.2-m-diameter pool, a submerged platform (14 cm in diameter) located 2 cm below the surface of water (24 °C), rendered opaque by adding white nontoxic paint. Animals were brought into the room 30 min prior to the testing and received four trials per day. Latency was recorded when rats reached the platform. Animals were pseudo-randomly started from a different position at each trial and used distal visuospatial cues located on the room walls to find the hidden escape platform that remained in the center of the same quadrant throughout all the training days (Morris, 1984). Animals were given four trials of 90 s with a 20 min inter-trial interval over 5 consecutive days. Animals were guided to the platform if not located within 90 s, and all rodents remained there for 10 s before removal. After the acquisition phase on day 5, rats were given one probe trial of 60 s in which the platform was removed from the pool to evaluate the number of times the animal crossed the location of the platform and time spent in the target quadrant. This trial was followed by one cued trial of 60 s in which the platform was visible to assess visual and motor deficits and evaluate motivation to escape from water. After each trial, rats were immediately placed under heat lamps to dry and prevent hypothermia. To control for possible effects resulting from circadian cycles, all trials were performed at approximately the same time of day, between 9 and 11 h. Data derived from the MWM task were recorded on computer using a video tracking system (HVS) and calculations were made using the ANY-Maze Tracking software (Stoelting).

2.13. Induction of persistent pain with the formalin test

Fisher rats were placed for 3 consecutive days for 60 min in experimentation room and in the plexiglass formalin cages for habituation. On test day (140 days post-irradiation), the rats were placed in
room for a 30 min habituation period. Thereafter, the rats received a 50 μL subcutaneous injection of diluted 2% formaldehyde into the plantar surface of the right hind paw. Subsequently, rats were placed in clear plastic chambers (40 cm × 30 cm × 30 cm) positioned over a mirror angled at 45° in order to allow an unobstructed view of the paws; and their behaviors were scored for the next 60 min. An intraplantar injection of formalin produced the biphasic nociceptive response typical of this tonic pain model (Tjølsen et al., 1992). The two distinct phases of spontaneous pain behaviors that occur in rodents are proposed to reflect a direct effect of formalin on sensory receptors (phase I) and a longer-lasting pain due to inflammation and central sensitization (phase II). Phase I and II values were calculated between 0 and 9 and 21–60 min, respectively. These two phases are separated by a period of quiescence, the interphase, which is characterized by active inhibition of the formalin-induced nociceptive behaviors.

After injection of formalin into the right hind paw, the experimenter measured the time spent in each of 4 behavioral categories: 0, injected paw is comparable to contralateral paw; 1, injected paw has little or no weight placed on it but is in contact with surface; 2, injected paw is elevated, not in contact with any surface; 3, injected paw is licked, bitten, or flinched. The behaviors believed to represent higher levels of pain intensity were given higher weighted scores (Capone and Aloisi, 2004). Formalin-induced pain-related behaviors were quantified by monitoring the cumulative time spent in behavioral category 3 (flinching/licking/biting) during the three different phases, which involves the recruitment of supraspinal nociceptive circuits. This test was performed by the same experimenter, in a quiet room, between 9 am and 12 pm to avoid any variation related to circadian rhythm (Parent et al., 2016; Roussy et al., 2008).

2.14. Statistical analysis

For in vivo imaging, animals used were their own control (day 0 = first MR scan). Consequently, for quantitative diffusion MRI analyses, data were expressed as mean ± SD, and measurements on irradiated and non-irradiated hemispheres of the corpus callosum (CC) at 4 different time points were performed on every animal. Analyzes were decomposed into 2 nonparametric tests. First, a Friedman test (two-tailed, paired repeated measures) was done to test the effect of time by region and radiation. For this test, the comparison was performed between days 10 and 110 because one rat could not be scanned at baseline. Second, the effect of radiation as a function of time and region was further detected by Wilcoxon test (one-tailed paired test). For the quantitative necrosis volume (DCE-MRI) analysis, a two-tailed paired t-test was used. For the behavioral experiments, data were expressed as mean ± SEM (standard error of mean). Comparison between irradiated and non-irradiated control groups were performed using non-parametric Mann–Whitney tests. Morris water maze data (escape latency) were analyzed using two-way repeated measure ANOVA. A p-value ≤ .05 was considered to be significant. Calculations were performed with GraphPad Prism 7.0.

3. Results

3.1. Vascular and mesostructural changes following brain irradiation

Rats were monitored with four MRI sequences before irradiation until 110 days post-irradiation (Fig. 1a). These MRI sequences were chosen to highlight vascular permeability, neovascularization, necrosis and fiber bundle architectural changes. The mean dose of radiation received by each brain region of interest was obtained by dose-volume histogram, a useful tool in advanced radiotherapy planning (Fig. 1b). Edema was first detected on day 54 using T2w MRI (black arrowheads) (Fig. 2a). The DCE-MRI coupled to T2w MRI demonstrated the presence of small foci suggesting the onset of necrosis (white arrowheads), mainly located in the corpus callosum (CC) and surrounded by possible abnormal neovessels (orange arrowheads) (Fig. 2b, c). These new vessels were leaky, as demonstrated by the uptake of contrast agent (Gd-DTPA) in DCE-MRI (Fig. 2b). As previously discussed (Constanzo et al., 2016), this necrotic region seems to have arisen into the CC on day 54 and to spread throughout M1, S1FL and striatum of the right hemisphere on day 110 (Fig. 2b, c). In addition, hypointense signals surrounding the necrotic zone were observed on the T2w (Fig. 2a) and b0 images (Fig. 2d) and delineated the presence of expected active inflammation (black arrowheads). As observed on b0 images, the fiber bundles were disrupted in the irradiated hemisphere, 110 days after irradiation (Fig. 2d). In addition, the necrotic core volumes, assessed by DCE-MRI image analysis, significantly increased between days 54 and 110, reaching respectively (0.3 ± 0.5) mm3 and (21.7 ± 4.8) mm3 (Fig. 2e). Based on diffusion MR images, it was possible to determine, for each voxel of the image, several metrics, such as fractional anisotropy (FA) and radial diffusivity (RD), which both reflect pathological or age-related processes. For example, during evaluation of white matter infarct, RD is considered to be related to membrane integrity (the Track-HD investigators et al., 2015). In addition, we and others have showed that decrease FA and increased RD are both indirect markers of myelin content (Constanzo et al., 2018; Song et al., 2005). We therefore determined the degree of myelination by measuring the changes in FA and RD parameters in the CC following brain irradiation. As shown on Fig. 2f, we observed reduced FA (*** P < .001) and increased RD (** P < .01) values in CC, when comparing day 10 with day 110. Long-term effects of radiation on the CC was primarily due to the necrosis that led to decreased tissue FA (−25%; * P < .05) and increased RD (+11%; * P < .05) in the irradiated CC on day 110, compared to its contralateral counterpart.

A large volume of necrosis was confirmed on day 110 by H&E histological staining (Bregma = 0.84 mm) (Fig. 3). Neuroplastic changes were further detected by Luxol fast blue staining (Bregma = 0.84 mm), revealing extensive active demyelination spreading along the white matter tracts. On day 110 (Bregma = −2.04 mm), periventricular edema/necrosis was confirmed on H&E stained tissue slices (Fig. 3). This necrosis spread around the hippocampus, leading to fimbria hippocampal demyelination, as pointed by the arrow #1. In addition, Nissl-stained neurons in the irradiated hippocampal formation were disorganized and shrunk. Consistently, a decrease in the number of neurons was observed mainly into the CA2 (Cornu Ammonis) area, which is filled with densely packed pyramidal cells implicated in spatial memory processes (see the contralateral hippocampus for comparison).

3.2. Region-specific immune surveillance and response to radiation

Accumulate evidence indicates that chronic neuroinflammation and subsequent immune responses are inherent complications of brain irradiation, leading to late brain injury and cognitive impairment (Makale et al., 2017). On day 140 post-irradiation, we observed activation of Iba1-positive stained microglial cells and GFAP-positive astrocytes surrounding the necrosis area. Microglia activation followed an increasing gradient, distal to proximal from the lesion (coronal section), and microglia accumulated near newly sprouted blood vessels surrounding the necrosis (Fig. 4a-c). Also, a spatial rostro-caudal activation gradient was observed, with a morphology representative of an increased activation state observed in Bregma 0.84 (Fig. 4b), near the radiation hot spot, as compared to a less active state at −2.04 Bregma level (Fig. 4c). Accordingly, microglial activation was more pronounced proximal to the irradiation level in M1 and S1 brain structures showing a motile amoeboid morphology with a retraction of their long ramifications, an increased cell body volume and an increased cell number compared to Bregma level −2.04 (Fig. 5). Distal to the lesion, and further from the irradiation hot spot, at Bregma −2.04, microglia morphology showed fine and extended processes with a more organized distribution pattern through the tissue whereas at Bregma 0.84 showed a marked microgliosis distal to the region. In addition, this microglial...
activation was accompanied by astrogliosis in the injured brain. Indeed, astrocytic activation was diffuse over the whole ipsilateral hemisphere and, similar to microglia, spread to the contralateral non-irradiated hemisphere (Fig. 6). Qualitatively, the level of astrocytic activation was quite similar between Bregma 0.84 and Bregma −2.04 (compare Fig. 6a, b).

To further characterize the extent of radiation-induced neuroinflammation, the presence of peripheral immune infiltrating cells was examined 140 days following brain irradiation (Fig. 7). Peripheral immune cells are typically CD45<sup>high</sup> with a larger diameter, a round morphology and lack of processes compared to resident microglia. The blood-brain barrier disruption and leaky neovascularization induced by the brain irradiation resulted in the upregulation of activated phagocytic microglia (Iba1<sup>+</sup>/CD68<sup>+</sup> cells) around the lesion site and promoted infiltration of CD45<sup>high</sup> peripheral immune cells. Those CD45<sup>high</sup> immune cells were identified as infiltrating T-cells (CD3<sup>+</sup> cells) and were observed at a greater extent near the lesion border both at Bregma 0.84 and Bregma −2.04.

3.3. Behavioral responses to brain irradiation

Despite exposure to high doses of radiation, irradiated rats exhibited...
similar weight gain throughout the duration of the 140-day behavioral assay compared to non-irradiated control animals (Fig. 8a). A battery of well-established behavioral tests aiming at measuring locomotor activity and muscle function (actimetry, Open Field (OF), Rotarod, Grip strength), anxiety- and depressive-related behaviors (actimetry, OF, Elevated Plus Maze, Forced Swim Test), spatial memory and learning (Morris Water Maze) and persistent pain (formalin test) was then used to assess radiation-induced behavioral alterations. At days 82 and 109 following brain irradiation, freely-moving rats displayed a pronounced hyperactivity, as shown by the increased ambulation in the actimetry box, as compared to day 54 (Fig. 8b). This increased motor activity recorded over 16 h was confirmed by measuring the spontaneous locomotion in the open field arena. Indeed, both the total distance travelled and the average speed were significantly increased 109 and 139 days post-irradiation (Fig. S1a). Likewise, the fine movements representing smaller movements, like grooming or head movements were also significantly increased at days 82 and 109 in rats submitted to gamma knife treatment (Fig. S1b). In order to explore neuromuscular function, all rats were then challenged using the grip strength test. As shown on Fig. 8c, irradiated rats showed no reduced forelimb grip

Fig. 3. Microstructural changes observed 110 days following irradiation. Three different staining were used to highlight necrosis (H&E), demyelination and white matter necrosis (Luxol Fast Blue), as well as neuronal changes (Cresyl violet). A severe necrosis zone was located in M1 and in the corpus callosum of the right hemisphere (Bregma 0.84 mm). A periventricular necrosis was also observed surrounding the hippocampus (Bregma – 2.04 mm) (black arrow on the H&E right panel), leading to demyelination (light blue) in the fimbria of the hippocampus (black arrow #1) and in the external capsule (black arrow #2). Neuronal loss was observed mainly in the CA2 area (Cresyl violet) of the irradiated hippocampus without apparent sign of necrosis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
strength on day 109 compared to control animals. As for the grip test, the performance in the rotarod evaluated at day 110 was not altered in irradiated rats, indicating no movement coordination impairments (Fig. 8d).

We further investigated whether rats submitted to brain irradiation developed anxiety and depressive-related behaviors. We found on days 82 and 109 that irradiated rats were more prone to explore the center of the actimetry box than healthy rats, as shown by their higher number of entries in the center (Fig. 9a). In the Elevated-Plus Maze test, irradiated rats also spent significantly more time in the open arms than control animals at day 120 (Fig. 9b). Similarly, irradiated rats displayed a significant increase in the number of central squares visited in the OF test on day 109 (Fig. 9c). Finally, we used the Forced swimming test to evaluate the depression-like behaviors. The immobility time was significantly reduced in treated rats at day 122, compared to controls (Fig. S1c). Altogether, these behavioral analyses indicated that brain irradiation caused a marked hyperactivity, together with a reduction in anxiety- and depression-like behaviors.

On day 130, we next used the Morris Water Maze in order to evaluate hippocampal-dependent spatial learning and memory in irradiated rats. During training (days 130–135), learning is assessed by the amount of time elapsed before the animal climbs onto the platform to escape the water (escape latency). As shown on Fig. 10a, irradiated rats demonstrated reduced learning abilities by having a longer latency to locate the hidden platform from days 1 to 5 in the spatial component of the test. Accordingly, in the probe trial in which the platform was removed from the pool, irradiated rats spent significantly less percentage of time in the quadrant housing the platform (target quadrant) than sham animals (Fig. 10b). Likewise, irradiated rats displayed fewer platform crossings in the target quadrant than their respective controls (Fig. 10c). This probe trial was finally immediately followed by the visual cue test (visible platform) to assess sensorimotor ability and evaluate motivation to escape from water. Compared to control rats that were able to locate the visible platform relatively quickly, irradiated rats took longer to find the cued platform position (Fig. 10d). This impairment was not attributed to sensorimotor function deficits, since brain-injured rats performed similarly to controls in rotarod and grip strength assays and even displayed a pronounced hyperactivity on ground and in water (increased ambulation and faster swimming speed).

Pain behaviors were also investigated using the formalin test at the end point, 140 days following radiation exposure. Formalin-induced...
pain-related responses were quantified by monitoring specifically the flinching, licking and biting nociceptive behavioral reactions, which are believed to recruit supraspinal nociceptive circuits. Our results revealed that irradiated rats exhibited marked increase in the duration of pain episodes in the inflammatory phase of the formalin test, with no apparent changes in acute and interphase, thus demonstrating the effects of radiation on the development of central sensitization. (Fig. 11).

Fig. 5. Microglia cell morphology at day 140 post-irradiation. Gradual morphological transformations of the Iba1-positive microglial cells surrounding the lesion (distal to proximal) at Bregma 0.84 and −2.04 within the motor cortex (M1); somatosensory cortex (S1) and hippocampus. Morphological states of microglia from the resting state characterized by a ramified (R) morphology to activated amoeboid (A) phenotype, with a range of intermediate activation states: primed (P), hypertrophied (H) and bushy (B). Scale bar = 50 μm.

Fig. 6. Astrocyte morphology at day 140 post-irradiation showing activation of the GFAP-positive reactive cells surrounding the lesion. Scale bars = 1 mm (a, b; left panel) and 100 μm (a, b; right panel).
4. Discussion

Optimization of radiotherapy to treat tumor cells infiltrated into the cerebral parenchyma requires a better knowledge of the specific impact of the irradiated brain regions according to their sensitivity and interconnectivity, as well as evaluating their ability to recover their functions.

In our study, several brain areas were exposed to different doses of radiation: the primary somatosensory cortex (S1FL) involved in pain processing received the highest dose (113 Gy), the motor cortex (M1) implicated in the control of movement was irradiated with 41 Gy, the striatum involved in reward, cognition and motor function was exposed to 35 Gy and finally the hippocampal formation receiving the lowest dose (24 Gy) is mainly involved in learning and memory but can also...
play a key role in emotional regulation. On day 54 post-irradiation, MR images revealed necrotic foci mainly located in the white matter and surrounded by edema (hyperintense signal on T2w, b0 and T2* images). At day 110, the necrotic foci further spread into M1, S1FL, striatum and right ventricle, leading to a loss of integrity of fiber bundles and demyelination in the corpus callosum of the right hemisphere. These structural damages translated into behavioral changes including hyperactivity, disinhibition of the anxiety- and depressive-like behaviors, spatial memory loss, and pain hypersensitivity. Despite the severity of the lesion, our irradiated rats, however, appeared healthy as they gained weight as sham animals, and motor coordination and neuromuscular abilities remained functional over the experimental time course.

In the present study, we found that irradiated rats exhibited less anxiety-like behaviors in the open field, actimetry and elevated-plus maze behavioral assays. Likewise, rats spent less time immobile in the forced swim test than control animals, indicating the absence of depressive-like behaviors. These cognitive deficits observed following radiation exposure are in agreement with previous animal studies. In a single whole brain irradiation of 6 Gy in juvenile rats, Zhou and colleagues indeed reported that the accumulated running distance and the time spent in the open central zone (open field test) were greater for irradiated rats compared to the non-irradiated controls, respectively 74.5% (hyperactivity) and 71.4% (dissinhibition of anxiety-like behaviors) (Zhou et al., 2017). Likewise, the time spent in the central area of the open field increased significantly in six-month old female mice exposed to 2Gy (Kumar et al., 2013). Reduced depressive-like behaviors were also reported within ten days in mice submitted to 20 Gy-ionizing irradiation (Ueno et al., 2019). In contrast, Tome et al. demonstrated that mice exposed to 10 Gy using either whole-brain radiation or hippocampal sparing radiation did not exhibit anxiety-like behaviors (Tomé et al., 2015). The reason for these discrepancies in anxiety-like behaviors between those studies is not clear, however, it might be related to the fact that the radiation-induced brain injury appears in a dose- and time-dependent fashion (Jiang et al., 2015). In spite of the well-known role of the hippocampal neuronal plasticity in anxiety behaviors, depression and post-traumatic stress disorder (PTSD) (Kheirbek et al., 2012), we cannot conclude here that these behavioral changes are directly related to alteration to the hippocampal formation. Indeed, the radiation effects span beyond the irradiated brain regions, and that tissues that are not directly exposed to radiation – ‘bystander effects’, can demonstrate responses that are characteristics of directly irradiated brain structures (Pouget et al., 2018). There are indeed numerous short- and long-range connections in the brain that may lead to these non-targeted effects and then conduct to these phenotypic and behavioral changes. For instance, demyelinating lesions or other adverse effects can be detected in brain structures not directly exposed to radiation. Consequently, although the amygdala was not irradiated in the present study, we cannot exclude for instance that the anxiety-like behaviors observed after radiation exposure are not related to a decline

![Fig. 9. Effects of radiation therapy on subcortical brain function. Brain region-specific sensitivity to irradiation was determined using behavioral tests assessing anxiety-like behaviors (Actimetry, Open Field, Elevated-Plus Maze). The brain damage-related behavioral changes were examined between 54 and 120 days following irradiation. a, The actimetry test revealed that irradiated rats spent significantly more time in the center of the arena, starting at day 82 and increasing at day 109, compared to healthy age paired rats. b, These anxiety-like behaviors were also observed at day 120 in irradiated rats subjected to the Elevated-Plus Maze test, as determined by the increase in the number of entries into open arms and greater percentage of time in open arms. c, Rats also showed anxiety-like behaviors in the Open Field, spending more time in the center of the arena than in the periphery than healthy animals. Sham group n = 8, and irradiated group n = 12. All data represent mean ± S.E.M. Statistical analyses consisted of nonparametric Mann–Whitney tests, where *P < .05 and ***P < .001 versus Sham.](image-url)
of its function, since the amygdala is reciprocally connected to the hippocampus.

Studies on large patient cohorts have documented the significant neurotoxicity after radiotherapy. Children are overrepresented in this literature, as curative treatments for pediatric tumors such as medulloblastoma still require extensive irradiation of the entire craniospinal axis, even if the majority of patients has a localized posterior fossa tumor, due to a significant risk of occult metastases (PDQ Pediatric Treatment Editorial Board, 2002). MRI studies in these young long-term survivors have demonstrated disruption of the white matter integrity, leading to a declining intelligence quotient associated with significant academic struggles, potentially predated by difficulties with attention, memory, and processing speed (Palmer et al., 2007). Cognitive decline is also a known side-effect following irradiation for brain metastases, especially with whole-brain radiotherapy (Chang et al., 2009; McDuff et al., 2013; Tallet et al., 2012). In addition, anxiety and depression have been reported to be two times higher in cancer patients compared to the general population (Hinz et al., 2010). It is nonetheless difficult, if not impossible, to know whether these anxiety behaviors are due to brain irradiation or related to the stressful situation. Stress can indeed precipitate or exacerbate the anxiety-related disorders in brain tumor patients. Interestingly, Cordes et al. demonstrated that the global distress correlated strongly with the Hospital Anxiety score before radiotherapy, but only moderately or weakly with both Hospital Anxiety and Depression Scale (HADS) scores after radiotherapy with the weakest association 6 months after radiotherapy (Cordes et al., 2014). This last finding is consistent with our preclinical behavioral data demonstrating a disinhibition of the anxiety- and depressive-like behaviors. Despite the frequency of these devastating adverse effects, the underlying neuropathological mechanisms remain unclear and the appropriate treatments and, more importantly, prevention are sorely lacking.

Recent studies are refining the global cognitive decline observations by studying the effect of radiation on specific cerebral structures in patients. This consideration has emerged with the neuroanatomical target theory which suggests that a sufficient amount of radiation to the primary motor cortex or to the temporal cortex can lead to the worsening of global cognition, occurring not necessarily by the same mechanism (e.g., vascular injury, demyelination), and avoidance of such structures may preserve their function (Peifer et al., 2013). In 54 patients who underwent fractionated partial brain radiation to treat primary tumors, the cortical regions were vulnerable to dose-dependent radiation atrophy (Seibert et al., 2017). The association between regions of interest in both cortex and their role in memory and attention

![Fig. 10. Effects of radiation therapy on memory performance. The Morris Water Maze test was used to evaluate the effects of brain irradiation on spatial memory and learning performances on days 130 to 135. a, Irradiated rats demonstrated decreased learning abilities with longer latency to locate the hidden platform (escape latency), thus revealing that even though the hippocampus receives “only” 24 Gy (low dose compared to those deposited in S1 and M1), this may indeed result in cognitive impairment (**P < .001 versus Sham, two-way ANOVA). b-d, The rat’s ability to locate the area, where the escape platform previously was, showed a significant difference between groups, as determined by the percentage of time spent in target quadrant and the number of platform crossings during the probe trial as well as during the cued test (visible platform). Sham group n = 8, and irradiated group n = 12. Statistical analyses consisted of nonparametric Mann-Whitney tests, where *P < .05 and ***P < .001 versus Sham.](image-url)

![Fig. 11. Effects of radiation therapy on pain sensitivity. Brain sensitivity to irradiation was determined using the formalin nociceptive test. Radiation-induced pain hypersensitivity was examined at 140 days following radiation exposure. Formalin-induced pain-related behaviors were quantified by monitoring the cumulative time spent in flinching, licking biting during the three different phases (acute, interphase, inflammatory) of this tonic pain model. All data represent mean ± S.E.M. Statistical analyses consisted of nonparametric Mann-Whitney tests, where *P < .05 versus Sham.](image-url)
was selected. Both of these regions showed radiation dose-dependent decrease in cortical thickness approximately one year after radiation therapy, representing the double of the annual cortical atrophy rate observed in patients with Alzheimer’s disease (Seibert et al., 2017). Primary visual and primary somatosensory/motor regions were also assessed, but neither showed cortical atrophy associated with radiation dose, even for patients that received relatively high mean fractionated dose (> 40 Gy, 1.8-2Gy/fraction). It was therefore hypothesized that some regions of higher-order cortex are more radiosensitive (Seibert et al., 2017). Our results seem to support this hypothesis, since behavioral tasks associated to S1FL and M1 (neocortex) that received a dose respectively four times and two times higher than the hippocampus appeared less affected by radiation.

Optimization of radiation treatment should take into account the brain plasticity which refers to the ability of the brain to reorganize itself by forming new connections between neurons, which is an important component of rehabilitation in stroke patients (Hara, 2015). Indeed, the ability of physical exercise to induce neurogenesis in stroke patients represents a therapeutic potential regarding delay or repair of brain damage (Moreno-Gollazos and Orti, 2018). Likewise, in a mouse model of Alzheimer’s disease, physical exercise was associated with increased neurogenesis and decrease in the level of pro-inflammatory cytokines. In the hippocampus, neurogenesis that was induced after physical exercise was associated with prevention of cognitive decline (Ma et al., 2017). The number of astrocytes was also increased in hippocampus and other regions of the brain, which may improve brain homeostasis (Li et al., 2005). Another potential impact of physical exercise is the induction of the expression of glucose transporter1 (GLUT1) in astrocytes (Allen and Messier, 2013). Although physical exercise can thus induce the formation of new neuronal connections and support their activity by improving their glucose supply, its therapeutic potential has never been studied in patients treated by radiotherapy.

In the late 70’s, Burger et al. characterized radiation-induced neuropathologies on patient brain sections. Extensive necrosis of the white matter lateralized to the side of the neoplasm, including coagulative necrosis, telangiectasia and perivascular fibrosis was observed (Burger et al., 1979). In addition, microstructural changes in the ipsilateral hemisphere distant to the tumor bed have occurred and consisted of myelin pallor, reactive gliosis, enlarged microglia, small vessels hyaline- nization and demyelination. The contralateral hemisphere of the patient also contained variable degrees of myelin pallor and glial activation (Burger et al., 1979). In our study, a mixed area characterized by necrosis and neovascularization first occurred in the white matter, leading to long-term demyelination in the irradiated hemisphere which further spread into the surrounding grey matter. The peri-necrotic edema was also associated to a sustained microglial and astrocytic activation. Recent evidences suggest that this chronic inflammation that may also involve infiltrating immune cells contributes to the cognitive decline observed in patients receiving brain irradiation (Greene-Schloesser et al., 2012). Likewise, in rodents this long-lasting inflammation state induces a progressive impairment in neurogenesis as well as hippocampal-dependent spatial learning and memory deficits (Ekdhahl et al., 2003; Lumnizczyk et al., 2017).

Microglia and astrocytes are the primary effectors of neuroinflammation (Sajio et al., 2013; Shrivastava et al., 2017). They are activated by various chemokines and cytokines such as the pro-inflammatory chemokine CCL2, IL-1α and TNFα (Belarbi et al., 2012; Münch et al., 2017), which are overexpressed in brain as soon as 4 h after 15 Gy irradiation (Desmarais et al., 2015). Microglial cells normally participate in CNS development, neuroprotection, synapse connectivity, and the maintenance of hemostasis (Nayak et al., 2014). Over-activated microglia can, however, lead to neuroinflammation, oxidative stress and neuronal dysfunction due to the excess production of a wide range of cytoxic factors, such as TNFα, IL-6, IL-18, reactive oxygen species and nitric oxide (Nayak et al., 2014). Furthermore, irradiated microglial cells have also been shown to induce astrogliosis, which might contribute to radiation-induced edema and astrocytic release of neurotoxic factors inducing neuronal cell death (Hwang et al., 2006; Münch et al., 2017). Astrocytes are also critical for the maintenance of the blood-brain barrier (BBB) integrity. Consequently, re-active astrocytes secreting pro-inflammatory factors promote the BBB breakdown and the infiltration of peripheral immune cells (Wilson et al., 2009). Accordingly, our results reveal the presence of infiltrating T-cells (CD3⁺) at the lesion site of brain-irradiated rats. This is also consistent with previous study showing that CD3⁺ T cells penetrated the brain as early as day 7 after irradiation and persisted even 12 months later (Moravan et al., 2011).

These results suggest that administering an anti-TNFα could prevent early damages induced by radiotherapy. Indeed, a protection of the brain’s microvascular network and lower adhesion of leukocytes were observed in mice treated with anti-TNFα following brain irradiation (Wilson et al., 2009). In another rat model, administration of the TNFα protein synthesis inhibitor 3,6′-dithiothalidomide has restored neuronal function and reverses cognitive deficits that were caused by a neu-roinflammation induced by intracranial injection of lipopolysaccharide (Belarbi et al., 2012). Other studies have focused on the inhibition of microglia-mediated neuroinflammation to mitigate radiation-induced cognitive impairments (Acharya et al., 2016; Jenrow et al., 2013). In irradiated mice, it was shown that depletion of activated microglia through the blockade of the colony-stimulating factor 1 receptor can prevent the radiation-induced cognitive deficits, as assessed with an Open Field test (Acharya et al., 2016). Likewise, inhibition of microglia activation may be beneficial in improving hippocampal-dependent spatial memory (Jang et al., 2010; Wadhwa et al., 2017). Although the most severe effects occur months to years following radiotherapy, it is expected that attenuation of the early damage to the microvascular network and brain parenchyma could likely prevent the propagation of the late-term effects of radiotherapy.

In conclusion, brain irradiation may have significant different impact on the behaviors and cognitive functions according to the areas targeted. Radiotherapy treatment planning could therefore be adapted according to the sensitivity of the targeted brain regions. Therapeutic strategies to block the early events appearing post-radiation should also be explored to prevent the development of long-term cognitive dys-function.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2020.109954.

Ethical statement

The experimental procedures were approved by the Animal Care Committee of the University of Sherbrooke and were in accordance with policies and directives of the Canadian Council on Animal Care.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgements

This work was supported by the Fonds de Recherche Québecois Nature et Technologies awarded to B.P. [grant number 172009] and by the Canadian Institute of Health Research (CIHR) [grant number FDN-148413] awarded to PS. PS is the holder of the Canada Research Chair Tier 1 in the Neurophysiopharmacology of chronic pain. EM was supported by a research fellowship from the Fonds de Recherche en Santé du Québec (FRQ-S). Maxime Descoteaux, Martin Lepage, Laurence Masson-Côté, Benoit Paquette and Philippe Sarret are members of the FRQS-funded Centre de recherche CHUS. The authors thank the Electron Microscopy & Histology Research Core of the FMSS at the
Peiffer, A.M., Leyrer, C.M., Greene-Schlosser, D.M., Shing, E., Kearns, W.T., Hinon, W.H., Tatter, S.B., Ip, E.H., Rapp, S.R., Robbins, M.E., Shaw, E.G., Chan, M.D., 2013. Neuroanatomical target theory as a predictive model for radiation-induced cognitive decline. Neurology 80, 747–753. https://doi.org/10.1212/WNL.0b013e318283bb0a. Pouget, J.-P., Georgakilas, A.G., Ravanan, J.-L., 2018. Targeted and off-target (bystander and abscopal) effects of radiation therapy: redox mechanisms and risk-benefit analysis. Antioxid. Redox Signal. https://doi.org/10.1089/ars.2017.7267.

Roussy, G., Dansereau, M.A., Doré-Savard, L., Belleville, K., Beaudet, N., Richelson, E., Sarret, P., 2008. Spinal NTS1 receptors regulate nociceptive signaling in a rat formalin tonic pain model. J. Neurochem. 105, 1100–1114. https://doi.org/10.1111/j.1471-4159.2007.05205.x.

Saîjo, K., Crott, A., Glans, C.K., 2013. Regulation of microglia activation and deactivation by nuclear receptors. Glia 61, 104–111. https://doi.org/10.1002/glia.22423.

Seibert, T.M., Karunamuni, R., Kafi, S., Burkeen, J., Connor, M., Krishnan, A.P., White, N.S., Farid, N., Bartsch, H., Murzin, V., Nguyen, T.T., Boisvert, A., Brewer, J.B., McDonald, C.R., Dale, A.M., Hattangadi-Gluth, J.A., 2017. Cerebral cortex regions selectively vulnerable to radiation dose-dependent atrophy. Int. J. Radiat. Oncol. 108, 291–308. https://doi.org/10.1016/j.ijrobp.2017.01.005.

Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. 2, 322–328. https://doi.org/10.1038/nprot.2007.44.

Wilson, C.M., Gaber, M.W., Sabek, O.M., Zawrski, J.A., Merchant, T.E., 2009. Radiation-induced astrogliosis and blood-brain barrier damage can be abrogated using anti-TNF treatment. Int. J. Radiat. Oncol. Biol. Phys. 74, 934–941. https://doi.org/10.1016/j.ijrobp.2009.02.035.

Zhou, K., Xie, C., Wickström, M., Dolga, A.M., Zhang, Y., Li, T., Xu, Y., Calmese, C., Boger, M., Zhu, C., Blomgren, K., 2017. Lithium protects hippocampal progenitors, cognitive performance and hypothalamus-pituitary function after irradiation to the juvenile rat brain. Oncotarget 8, 34111–34127. https://doi.org/10.18632/oncotarget.16292.