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## Physical Activity Attenuates Brain Irradiation-Associated Skeletal Muscle Damage in the Rat

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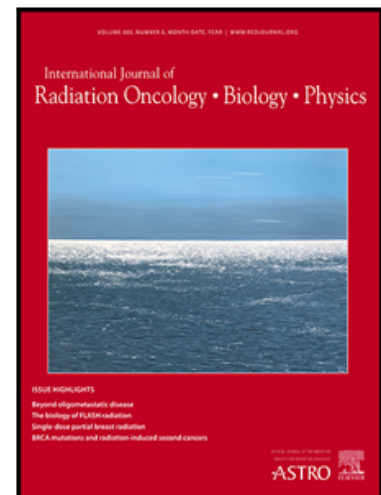
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Physical activity attenuates brain irradiation-associated skeletal muscle damage in the rat

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#### **[Data Availability Statement for this Work]**

Research data are stored in an institutional repository and will be shared upon reasonable request to the corresponding author.

#### **[Ethical Statement]**

All authors certify to comply with the ethical guidelines for authorship and publishing in the International Journal of Radiation Oncology - Biology - Physics. This animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Animal investigations performed in this study is in accordance with the current European directive (2010/63/EU). Ethical approval was obtained from the regional committee (CENOMEXA) and the French Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation (APAFIS#12496).

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## **ABSTRACT**

**Background:** Radiotherapy for brain tumors increases patient survival. Nonetheless, side effects are increasingly reported such as cognitive deficits and fatigue. The etiology of fatigue remains poorly described. Our hypothesis is that the abscopal effects of radiotherapy on skeletal muscle may be involved in fatigue. The present study aims to assess the impact of brain irradiation on skeletal muscles, its

relationships with fatigue and to analyze whether physical activity could counteract brain radiation-induced side effects.

**Methods:** Adult Wistar rats were randomly distributed between 4 groups: control (CTL), irradiated (IR), non-irradiated with physical activity (PA) and irradiated with physical activity (IR+PA). Irradiated rats were exposed to a whole-brain irradiation (WBI) of 30 Gy (3x10 Gy). Rats subjected to PA underwent sessions of running on a treadmill, 3 times/week during 6 months. The effects of WBI on muscles were evaluated by complementary approaches: behavioral tests (fatigue, locomotion activity), magnetic resonance imaging (MRI) and histological analyses.

**Results:** Irradiated rats displayed a significant fatigue and a reduced locomotor activity at short term compared to the CTL group which were attenuated with PA at 6 months after whole-brain irradiation. The IR rat's gastrocnemius mass decreased compared to CTL rats which was reversed by physical activity at 14 days after WBI. Multiparametric MRI imaging of the skeletal muscle highlighted an alteration of the fiber organization in IR rats as demonstrated by a significant decrease of the mean diffusivity in the gastrocnemius at short term. Alteration of fibers was confirmed by histological analyses: the number of type I fibers was decreased while that of type IIa fibers was increased in irradiated animals but not in IR+PA group.

**Conclusions:** The data show that whole-brain irradiation induces skeletal muscle damage which are attenuated by PA. This muscle damage may explain, at least in part, the fatigue of patients treated with radiotherapy.

**KEYWORDS:** Radiotherapy – Brain – Skeletal muscle – Fatigue – Sarcopenia – Abscopal effects - Rat

## INTRODUCTION

With advances in anticancer therapies, patients are surviving longer but adverse effects of treatments are increasingly described <sup>1</sup>. In the world, brain radiation therapy (RT) is performed on hundreds of thousands of patients with brain cancer each year <sup>2</sup>. Although RT has a toxic effect on tumor cells, it inevitably results in dose deposition in the healthy tissue surrounding the tumor leading to harmful consequences on the patient's post-treatment recovery and quality of life. Indeed, most patients report extreme fatigue in few weeks after the brain RT and cognitive impairments that appear during months after RT <sup>1,3</sup>.

While the mechanisms underlying RT-induced cognitive decline have been extensively discussed <sup>1</sup>, the etiology of fatigue remains poorly understood <sup>4</sup>. In some clinical studies based on different cancer types, skeletal muscle loss was described after cancer treatment <sup>5</sup> and recently, it has been evidenced that sarcopenia, defined as a decrease of muscle mass and physical performances, is a factor of bad prognosis after RT <sup>6</sup>. A prospective clinical study is underway to evaluate the muscle atrophy after RT in woman with breast cancer and the link with RT-induced fatigue (NCT02567669). However, the relationship between fatigue and skeletal muscle damage following RT was not analyzed in primary and metastatic brain tumors.

The skeletal muscle and the brain are intimately linked by peripheral nerves as well as by the blood circulation. This relationship may explain the abscopal effects, defined as non-target actions in non-irradiated cells, distant from the irradiated target <sup>7</sup>. These remote actions of irradiation have been highlighted by Kovalchuk and colleagues who showed that irradiation of the liver in the rat induced alterations in the dendritic organization in the brain as well as cognitive deficits <sup>8</sup>. Similarly, it has been reported that irradiation of hindlimb in the mouse led to significant brain injury as

reflected by multifocal hypometabolism and persistent neuroinflammation <sup>9</sup>. These findings suggest that peripheral irradiation is able to alter the function and the structure of brain tissue. Nonetheless, consequences of brain irradiation on other organs of the body have not been studied to date, especially on the skeletal muscle that could be involved in RT-induced fatigue.

A major axis of the current cancer research is to understand the origin of adverse effects of cancer treatments and to propose therapeutic strategies to prevent or counteract them. The therapeutic management of the harmful effects of RT on fatigue and cognition remains difficult since most of the drugs evaluated in clinical trials have not shown conclusive results with mixed and short-lasting patient's responses <sup>3,10</sup>. The physical activity (PA) is increasingly considered as a relevant non-drug therapy for rehabilitation in patients during and after cancer treatments because it prevents several diseases and improves cognitive abilities. In fact, the PA can reduce cancer-induced mortality and also, improves cognitive functions in animal models of many neurological disorders and in aged patients with cognitive decline <sup>11</sup>. In 2010, Brown and colleagues demonstrated that moderate-intensity resistance exercise decreases the fatigue of patients with cancer <sup>12</sup>. Patients with brain tumors experience weakness and physical function loss which can be attenuated by physical activity <sup>13</sup>. Moreover, the resistance exercise increases lean mass and reduces muscle wasting <sup>14</sup>. However, the origin of fatigue is still unknown and its management is difficult for clinicians.

One of the current challenges in the management of brain tumor patients is to elucidate the etiology of fatigue and propose relevant therapies to reduce the radiation side effects of brain RT. The aim of this study, performed in the adult rat is double: 1/ to assess the impact of brain irradiation on skeletal muscles and its

relationship with fatigue and 2/ to define the impact of physical exercise on these radiation-induced side effects.

## METHODS

### Animals

Six-month-old male WISTAR rats were maintained on a 12-hour light:dark cycle at 21-22°C with water and food *ad libitum*. Animal investigations were performed under the current European directive (2010/63/EU). Ethical approval was obtained from XXX (XXX) and XXX (XXX - anonymized for review)). In accordance with animal ethics, the rats were euthanized when they had reached the humane endpoints defined by excessive weight loss during consecutive days that cannot be improved by animal care, disheveled hair, lack of grooming, an animal with a hunched or prostrate back. Rats were distributed randomly in 4 groups: non-irradiated-rats (CTL group), irradiated-rats group (IR group), non-irradiated-rats subjected to physical activity (PA group) and irradiated-rats subjected to physical activity (IR+PA group). The whole experimental protocol is detailed in **Supplementary Figure 1**.

### Whole-brain irradiation

During the brain irradiation procedure, anesthesia was induced with 5 % of isoflurane and then maintained at 2 % with a mix of gas (30 % O<sub>2</sub> / 70 % N<sub>2</sub>O). The whole brain, except cerebellum, was exposed to X-rays with an irradiator dedicated to small animals and coupled with a scanner (X-RAD 225Cx, Precision X-ray). To ensure spatial accuracy of the deposited dose and homogenous irradiation in the brain, whole-brain irradiation (WBI) was preceded by a treatment planning system (TPS) through the use of SmART-Plan software (**Supplementary Figure 2**). To confirm the precision of TPS, the actual doses absorbed by the brain tissue were



measured by an *in vivo* dosimetry based on a scintillating fiber dosimeter <sup>15</sup>. WBI consisted to a fractionated irradiation with a total dose of 30 Gy distributed equally over 3 consecutive days as described in Yang and colleagues <sup>16</sup>. Although 30 Gy is a relatively high radiation dose since the median lethal dose (LD50) to brain irradiation was set at 33 Gy in rodents <sup>17,18</sup>, we retained this dose to have translational relevance by ensuring that the pathophysiology induced after cerebral irradiation is relatively similar between rats and humans. Indeed, a minimum dose of 25 or 30 Gy is required in rats to cause vascular damage and induce demyelination without white matter necrosis <sup>16,19,20</sup>, which are the main radiation-induced brain injuries associated to cognitive deficits in brain tumors patients treated by radiotherapy <sup>21,22</sup>. The details of WBI protocol were: voltage=225 kV, intensity=13 mA, energy=80 keV, dose rate=3.3 Gy/min, 25 mm diameter circular collimator. Copper filtration (thickness=1 mm) was used to remove lower energy X-ray photons, which ensures good penetration of the X-rays into the depth of the tissue and contribute to better radiation dose. After brain irradiation, rats were allowed to recover from anesthesia and returned to their home cages. The animals were then monitored daily until the end of the study (up to 6 months post-brain irradiation).

### **Physical activity**

The physical activity (PA) was carried out through the use of a treadmill according to a published standard protocol <sup>23</sup>. Briefly, all rats for all experimental groups (CTL, PA, IR, IR+PA) were daily familiarized with the treadmill 15 days before the irradiation. Rats ran at 6 cm/s for 10 min at the beginning and until 12 cm/s for 30 min at the end of the habituation period. The PA sessions were applied from the third day after WBI until the end of the study only for animals of PA and IR+PA groups. The PA sessions were performed 3 times a week with one day of break between the exercises. A

session of PA lasted 30 min per rat with 3 progressive speeds: the warm-up phase (13 cm/s for 5 min), the fast-walking phase (18 cm/s for 5 min) and the run phase (36 cm/s for 20 min).

## **Behavioral tests**

### ***Fatigue test***

Fat-test is a test which was developed in our laboratory to assess the fatigue in the rats. The experimental system is made of an inclined board of 2 m long and 25 cm wide with an inclination angle of 30° to the ground which allows animals to go to a darker corner at the end of the board (located at a height of 1 m relative to the ground). The time to reach the corner was quantified.

### ***Open-field test***

The open-field test was performed in a square white box (1m x 1m x 0,6m). A videotracking system on the ceiling detects animals in the open-field and analyses their movements (Viewpoint software). For all trials, rats were placed at the lower left corner of the open-field and during 10 min, the distance traveled and movement velocity were quantified. The velocity is quantified on long animal movements and greater than 15 cm/s. The apparatus was cleaned between each rat with 70% ethanol.

## **MRI examinations**

MRI (7 Tesla PharmaScan, Bruker BioSpin) of the whole brain and muscles of both hindlimbs were performed from 14 days up to 6 months after WBI. During MRI sessions, rats were anesthetized with isoflurane (2 %) in 30 % O<sub>2</sub>/70 % N<sub>2</sub>O. Breathing rate and rectal temperature were monitored throughout the MRI acquisitions.

### ***MRI of the brain***

For each animal, the following sequences were performed during 30 min using a crossed coiled configuration: (1) T2-weighted acquisition (T2w) (Turbo-RARE (Rapid Acquisition with Relaxation Enhancement), TR/TE = 5000/30 ms, factor RARE = 4, 64 slices of 0.5 mm, , repetitions = 4, FOV = 256x256 pixels and spatial resolution = 0.125 x 0.125mm: acq time = 16 min) and (2) EPI T2\* acquisition before and after P904 (Chematech) injection (200  $\mu$ mol/kg in the tail vein of the rat) with a waiting time of 4 minutes between the acquisitions (TE/TR = 13/20000 ms, 32 slices of 1 mm, mean = 1, repetition = 6, matrix = 110x55 pixels, spatial resolution = 0.290 x 0.290 mm). The cerebral blood volume (CBV) maps were generated thanks to homemade macro in ImageJ software (version 1.52k) (NIH, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>)<sup>24</sup>. The CBV is determined by the Steady-State method (SS- $\Delta R2^*$ ), according to a previous work<sup>25</sup>.

### ***MRI of the muscles***

Since the T2 signal in MRI is very sensitive to the water content in the tissues, T2 mapping is relevant to quantify T2 relaxation time (T2rt). Diseases affecting the properties of the muscle fibers or leading to changes of muscle fiber type are known to modify T2rt values<sup>26–28</sup>. Indeed, T2rt values increase because of edema or inflammation in skeletal muscles, but decrease in the presence of muscle fibrosis.

Diffusion tensor imaging (DTI) is of interest to assess the skeletal muscle architecture. Different diffusion parameters could be quantified by MRI such as the mean diffusivity (MD) that represents the average speed of water molecules moving through the tissue and is sensitive to typology of muscle fibers and their sizes. Moreover, the axial diffusivity (AD parameter) and radial diffusivity (RD parameter) reflect the rate of diffusion of water molecules in a particular direction, which allows to

appreciate the changes of the length and the diameter of muscle fibers respectively  
26,28,29

For each animal, the following sequences were performed during 15 min: (1) T2w MSME (Multi-slice Multi-Echo, TR=3000 ms, 15 echoes of 8-120 ms, 20 consecutive slices of 2mm, 2 averages, FOV=150x100 pixels, spatial resolution = 0.4x0.4 mm) and (2) DTI (Diffusion Tensor Imaging, TE/TR = 19.2/3000 ms, 64 directions, 5 A0 images, b-value = 600 s/mm<sup>2</sup>, 20 slices of 2mm, average = 1, repetition = 1, FOV = 75x50 pixels, spatial resolution = 0.8x0.8 mm). T2 maps were generated from Paravision 6.0 software. The diffusion images were treated using Paravision 6.0 software to generate maps of the following DTI-derived parameters: mean diffusivity MD=  $(\lambda_1+\lambda_2+\lambda_3)/3$ , axial diffusivity (AD=  $\lambda_1$ ) and the radial diffusivity (RD=  $(\lambda_2+\lambda_3)/2$ ). Through the use of ImageJ software, circular regions of interest (ROI) were positioned on the gastrocnemius (GNM) along 3 consecutive slices to quantify T2, MD, AD and RD (**Supplementary Figure 3**).

## ***Ex vivo analyses***

### ***Tissue sampling***

At 14 days, 1 and 6 months after WBI, transcatheter perfusion with saline was performed on deeply anesthetized rats. Then, gastrocnemius (GNM) and extensor digitorum longus (EDL) were harvested and weighed and thereafter frozen in isopentane and stored at -80 °C until use. The right GNM and EDL were used for histological analyses. The epididymal white adipose tissue was weighed to get insight on the evolution of white adipose tissue.

### ***Histological analyses***

Transverse sections of the GNM of 10  $\mu\text{m}$  thickness were subjected to Myosin ATPase staining (at pH 4.53) as described elsewhere<sup>30</sup>. For each staining, 2 pictures per animal were acquired in white light with a microscope (DMi8, Leica) at 10X objective.

### **Statistical analyses**

All data are presented as mean  $\pm$  standard deviation (SD). Statistical analyses were performed with GraphPad Prism® (GraphPad Prism 9.3.1.471 software). One-way or two-way ANOVA followed with LSD Fisher's post-hoc test was performed. Correlation tests were made to determine Pearson's correlation coefficient ( $r$ ) and the  $p$ -value was obtained with Z test. The number of animals for each experiment is detailed in each figure legend.

## **RESULTS**

### **Physical exercise reduces brain irradiation-induced mortality**

The follow-up of the animals during 6 months revealed that the survival was lower in irradiated animals in comparison to the other groups (**Figure 1a**). At 6 months, the mortality rate was of 24 % in IR rats ( $p < 0.01$  compared to CTL group) and 4 % in IR-PA rats. Of note, the mortality of the irradiated rats subjected to PA was not different from the non-irradiated control group (CTL). In the group of irradiated rats, 5 were euthanized between D10 and D14 because they had reached the humane endpoints, and 4 died suddenly, between 3 and 6 months following the irradiation, without a known reason. In these animals, MRI of the brain at 1 month failed to show any lesion of the of structures responsible of vital life functions (e.g. brain stem) which

could explain the cause of death. The animals that died during the protocol due to a technical problem such as anesthesia during MRI acquisitions (2 CTL, 1 IR and 1 IR+PA) were not included in the survival study.

### **Physical exercise counteracts brain irradiation-induced cerebral alterations**

As expected with a total dose of 30 Gy in the rat, brain irradiation did not induce any visible edema nor necrosis at all the time points analyzed (**Figure 1b**). Nonetheless, the quantification of MRI revealed that irradiation resulted in a significant reduction of the brain tissue volume at 6 months (-5.3 %,  $p < 0.05$ ) compared to CTL group. This brain atrophy was not attenuated by physical exercise (**Figure 1c**). At that time, the irradiated rats had a decreased cerebral blood volume (CBV) in the cortex (**Figure 1d**) compared to CTL group (-11.7%,  $p < 0.05$ ). Interestingly, PA was able to counteract these alterations of CBV (**Figure 1d**).

### **Physical exercise attenuates brain irradiation-induced fatigue and locomotor deficits**

The Fat-test demonstrated that the irradiated rats spent more time to climb the tilted board (**Figure 2a**). At 6 months following WBI, this deficit was still persistent ( $10.9 \pm 4.4$  s for IR group against  $6.3 \pm 1.7$  s for CTL group,  $p < 0.05$ ). In rats submitted to PA only, the time made to ascent the slanted board and reach the dark corner was similar to the time of the control rats during all the analyzed time points. Nonetheless, while PA was not able to prevent irradiation-induced fatigue at 14 days, it significantly restored the ability of animals at 6 months post-WBI as the irradiated rats spent the same time to climb the tilted board as those submitted to PA alone (**Figure 2a**).

These data show that PA was able to mitigate at long-term the brain irradiation-induced fatigue.

In the open-field test, the locomotor activity, as evidenced by the distance travelled (**Figure 2b**), was reduced in irradiated rats throughout the time period analyzed relative to non-irradiated rats (although the statistical significance was not reached at 6 months for the distance travelled;  $p=0.0001$ ,  $p=0.0009$  and  $p=0.291$  respectively at 14 days, 1 month and 6 months after the irradiation). In rats subjected to PA only, the distance travelled in the open-field was not modified during all the studied time points in comparison to control rats. However, physical exercise improved the locomotor activity at 6 months after WBI since the irradiated rats subjected to PA travelled the same distance as the respective controls (**Figure 2b**).

The velocity of locomotion measured in the open-field was decreased at 14 days ( $p<0.0001$ ), 1 month ( $p<0.0001$ ) and 6 months ( $p<0.0001$ ) after irradiation compared to CTL group (**Figure 2c**). However, PA improved this parameter both at the acute and chronic phases after WBI as highlighted by non-significant difference between velocity values for PA and IR+ PA groups at 14 days and 6 months after brain irradiation (**Figure 2c**). Altogether, the results indicate that brain irradiation induces deficits in locomotor activity which are attenuated at long term period by physical exercise.

### **Physical activity counteracts brain irradiation-induced muscle wasting**

Before brain irradiation, the mean body mass was homogenous between the 4 groups (**Figure 3a**). The CTL and PA groups displayed a significant increase of body mass until the sixth month of the experimental protocol ( $p<0.0001$ ). However, both IR and IR+PA groups displayed a persistent reduced body mass compared to control

groups (**Figure 3a**). Nonetheless, the comparison between IR+PA and IR groups showed that the trained rats had a significant improved body mass at 6 months after irradiation ( $p < 0.05$ ) (**Figure 3a**).

To quantify the effects of brain irradiation on skeletal muscles, the muscle mass of gastrocnemius (GNM) and extensor digitorum longus (EDL) were weighed in some animals euthanized at 14 days and 6 months following brain irradiation. For the GNM, the muscle mass of irradiated rats was significantly reduced compared to the control group at 14 days (-18%,  $p < 0.05$ ) and at 6 months (-21%,  $p < 0.0001$ ), respectively (**Figure 3b**). PA alone had no significant impact on the mass of the GNM (**Figure 3b**). However, PA prevented the wasting of the GNM associated to brain irradiation at 14 days after the irradiation as the mass of the GNM was not different between IR+PA and PA groups (**Figure 3b**). Similarly, brain irradiation induced a reduction in the mass of the EDL compared to CTL group at 14 days and 6 months after irradiation which was counteracted by PA only at 6 months (**Figure 3c**).

In order to examine if the reduction of the muscle mass observed in irradiated animals is merely related to the reduction of body mass, we correlated the mass of the GNM and the adipose tissue to the body weight of the animals. As presented in **Figure 3d**, the GNM mass is not correlated with the body weight of the animals, except in the only irradiated rats. Interestingly, the strong correlation between GNM and body mass in irradiated rats ( $r = 0.901$ ) no longer exists when the animals are subjected to physical activity ( $r = 0.169$ ). However, there is a significant correlation between and body mass in most animals (**Figure 3d**). These correlations may indicate that the irradiation-induced body weight could not be only attributed to a loss of muscle weight but rather mainly to adipose tissue.



### **MRI reveals muscle alterations at the acute phase following brain irradiation**

To examine brain irradiation-associated muscle alterations, T2 relaxation time (T2rt) and diffusion MRI-derived parameters (mean (MD), axial (AD) and radial (RD) diffusivities) were measured on GNM through the use of MRI for all groups at 14 days, 1 month and 6 months (**Supplementary Figure 3**).

At 14 days after the irradiation, the GNM displayed reductions in T2rt in IR and IR+PA groups compared to CTL group ( $p < 0.05$ ), but T2rt values normalized between groups and there was no major difference whatever the group considered (**Figure 4a**). Furthermore, MD, AD and RD values quantified 14 days after WBI were significantly decreased in IR group compared to CTL group (**Figures 4b-4d**). Interestingly, PA prevented the reduction of these diffusion-derived parameters at this time (**Figures 4b-4d**) as evidenced by non-significant difference between IR+PA and PA groups. At 1 and 6 months, the MRI-derived parameters had mainly similar values between both animal groups, although irradiated rats tended to have mean values of MD, AD and RD that differed from control animals (**Figures 4b, 4c and 4d**).

To analyze the significance of MRI-derived parameters, we correlate T2rt, MD, AD and RD with GNM mass, fatigue index and locomotor activity. The fatigue index was negatively correlated with MD ( $p < 0.05$ ) (**Figure 5a**). The mass of GNM and the locomotor activity of rats were positively correlated with MD ( $p < 0.05$ ,  $p < 0.001$  respectively) (**Figures 5b-5d**). T2rt, AD and RD were not, or inconsistently correlated with the mass of the muscle, the fatigue and the locomotor activity (**Supplementary Table 1**). These data suggest that MD parameter derived to diffusion MRI could be considered as the best sensitive parameter to detect brain irradiation-associated muscle modifications.

## Physical activity attenuates brain-irradiation-induced changes in muscle fiber typology

The muscle fiber typology in the GNM was analyzed by myosin ATPase staining to discriminate the skeletal muscle fibers of types I, IIa and IIb. As presented in **Figure 6a**, GNM of irradiated rats had few type I fibers (black) and enlarged type II fibers (light and dark brown) at 6 months after WBI. However, irradiated rats subjected to PA displayed a typology similar to control rats (**Figure 6a**). These observations were confirmed by quantification of the proportion of each muscle fiber types in the GNM. At 14 days after brain irradiation, the irradiated rats did not show any difference in the proportion of the type I, IIa and IIb fibers relative to non-irradiated rats (**Figure 6b**). Nonetheless, at 6 months, irradiated rats showed a significant decrease in type I fibers (-63 %) compared to control rats ( $p < 0.05$ ), and this was associated to an increase of the proportion of type IIa fibers: 25 % for IR group and 36 % for CTL group ( $p = 0.0591$ ) (**Figure 6c**). Interestingly, the proportions of fiber I (21 %) and fiber IIa (25 %) in GNM of irradiated exercise-trained rats were equivalent to values obtained for CTL and PA groups. These data highlighted that PA prevented the typology fiber changes in skeletal muscle observed in long-term effects of brain irradiation.

## DISCUSSION

One of major challenges in cancer management is to reduce the non-targeted effects of radiotherapy, in particular, the fatigue which is widely expressed by patients that underwent brain radiotherapy<sup>31,32</sup>. The goal of this study was to assess the impact of brain irradiation on skeletal muscles and its relationship with fatigue. Moreover, it also aimed to define the impact of physical exercise on these radiation-induced side

effects. Through the use of an animal model of controlled WBI and multiple approaches of analyses both in the acute and the chronic phases in tumor-free rats, we report that brain irradiation was associated to a marked muscle damage and a persistent fatigue. Interestingly, physical exercise attenuated these alterations.

Radiation-induced cognitive deficits have been broadly described both in animal models and patients <sup>1</sup>. However, the etiology of fatigue and its related mechanisms are poorly understood <sup>4</sup>. In some clinical studies based on different cancer types, skeletal muscle loss was described after cancer treatment <sup>5,33</sup> and could be involved in cancer-induced fatigue <sup>34-36</sup>. Recently it has been evidenced that sarcopenia, defined as a decrease of skeletal muscle mass and function, may be a poor prognostic factor after RT <sup>37,38</sup>.

In our model, fractionated WBI resulted in increased mortality rate, a significant loss of cerebral tissue and reduced cortical cerebral blood volume at 6 months following irradiation. In these irradiated rats, fatigue, as quantified by an original test developed in our laboratory, was evidenced as soon as 2 weeks after irradiation and lasted up to 6 months. Nonetheless, the motivation of the animals to perform the test and to reach the dark platform may modify their abilities. If this is the case, the contribution of motivation would not be significant as we have been able to show, using the splash test <sup>39</sup>, no difference between the irradiated rats and the control rats (data not shown). Moreover, irradiated animals displayed a decreased locomotor activity along the entire duration of the experimental protocol. These data are in agreement with clinical investigations in which weakness of legs and reduced locomotor performances were observed in patients subjected cerebral radiotherapy <sup>32,35</sup>. In order to further understand the origin of the fatigue and the alteration of motor activity associated to brain irradiation, we hypothesized that the skeletal muscle may

be altered remotely through the abscopal phenomenon defined as distant actions of irradiation<sup>8,9,40</sup>. To analyze this hypothesis, we examined the muscles and found that WBI was associated with significant reduced GNM weight as well as changes in the typology as exemplified by a decrease of type I fibers and an increase of type IIA fibers associated to enlargement of these fibers at 6 months after irradiation. These changes in muscle fiber typology induced by brain RT could be related to immobilization studies, following muscle denervation in rats, showing a switch from type I to type II fibers, which is due to a progressive atrophy of type I fibers associated with a hypertrophy of type II fibers<sup>41</sup>. Similarly, switches from type I to type II fibers are observed in many neuromuscular disorders<sup>42</sup>.

Interestingly, this preclinical study highlights that diffusion MRI, especially quantification to mean diffusivity (MD) parameter could be promising to assess to radiation-induced sarcopenia. From 2 weeks after WBI, MD, AD and RD values measured in GNM were significantly decreased in irradiated rats compared to controls animals. Diffusion tensor imaging (DTI) is interesting to assess the skeletal muscle architecture based to probes the motion of water molecules in tissue. The MD represents the average speed of movement of water molecules in the tissue and is sensitive to cell density. AD and RD, which reflect the speed of diffusion of water molecules in a particular direction, make it possible to assess the effects on the length and diameter of muscle fibers respectively. The fact that these parameters decrease reflects that the speed of water molecules is lower and that organelles or cellular debris are blocking their diffusion<sup>26,28,29</sup>. Furthermore, our results show that MD parameter is negatively correlated with fatigue level and positively correlated with locomotor activity of rats. Thus, MD parameter could be a sensitive imaging biomarker to detect brain irradiation-induced muscle modifications as previously

suggested in clinical studies conducted on sarcopenia<sup>43</sup>. Diffusion tensor imaging (DTI) has been successfully applied for muscle diseases, including sarcopenia, and injuries<sup>44</sup>. For instance, it has been shown, in healthy volunteers, that DTI is sensitive to age-related changes affecting the muscles of the lower limb<sup>45</sup>. Furthermore, Ponrartana and collaborators found a significant correlation of MD with age and muscle strength in pediatric patients affected by Duchenne dystrophy<sup>46</sup>.

The abscopal effects of brain irradiation could be explained by the production of oxidative stress and inflammation in the cerebral tissue that could reach skeletal muscle via the blood circulation. It is well known that irradiation produces water radiolysis that is a major source of reactive oxygen species (ROS). ROS can be released in the brain or in the blood, leading to mitochondrial dysfunctions, which would reduce the energy sources available to the cells and would be involved in the fatigue felt by the patients<sup>47</sup>. On the other hand, Wilke and collaborators demonstrated that brain irradiation produces neuroinflammation by oxidative damage<sup>48</sup>. Inflammatory cells-produced ROS and pro-inflammatory cytokines (TNF- $\beta$  and IL-1 $\beta$ ) could be released in blood flow and alter muscle which known to be sensible to oxidative stress and inflammation<sup>49</sup>.

Non-targeted effects of brain irradiation could also be related to dysfunction of cholinergic transmission in the neuromuscular junction that may take place following radiation-induced damage to the corticospinal bundle. Currently, no study has investigated the impact of the brain irradiation on corticospinal bundles. However, data exist in the literature to show that brain RT induces axon demyelination which is involved in cognitive deficits<sup>10</sup>. Thus, white matter demyelination after RT could affect the corticospinal bundle, and so the nervous conduction, which limit the release of acetylcholine in the neuromuscular junction. This may reduce muscle contractions

and cause immobilization of skeletal muscle and muscle wasting. The brain irradiation-induced muscle damage evidenced in our study are similar to those found in rodent models of sarcopenia. In these models, muscle atrophy can be induced by different methods like aging, denervation, hindlimb unloading and immobilization. In the cast, velcro or spiral wire immobilization models, a minimum of 1 to 2 weeks of complete immobilization is necessary to induce muscle damage<sup>50</sup>. In our model, although the irradiated rats displayed a reduced locomotor activity, they were not totally immobilized (Figures 2b and 2c). The reduction in the locomotor activity of the rats after irradiation may partly explain the muscle damage, but with regard to muscle alterations observed from 14 days post-irradiation by measurement of muscle mass (Figure 3) or by MRI (Figure 4), other biological phenomena than the partial immobilization of the rats are probably involved.

Whatever the underlying mechanisms, our data show that brain irradiation induces muscle damage that is associated with fatigue. These results suggest that brain irradiation is associated with a form of sarcopenia.

All the studied parameters (behavioral, MRI and histology) assessed in non-irradiated rats subjected to PA are similar with the control animals. The lack of PA effects in non-irradiated animals can be explained by the protocol of PA used. Indeed, the protocol of forced PA with the treadmill<sup>23</sup> was not gradual so the trained animals had the habit to run and at the end of the protocol, the race did not require an additional effort, inducing an effect only at short term. Moreover, this PA corresponds to an endurance exercise, opposed to the aerobic exercise. But, other parameters such as the intensity, the duration and the frequency of exercise can exert different actions on skeletal muscle<sup>51</sup>. However, PA has a real impact on animals submitted to WBI. We showed that PA reduced the mortality related to brain irradiation. In fact, it

reported that the PA reduces the risk of cancer, increase the overall survival <sup>52</sup>. Our MRI and histological data show that PA attenuated the muscle wasting and muscle fiber disorganization induced by brain irradiation in rats. So, PA counterbalances brain irradiation-induced muscle damage. The beneficial impact of PA is found in clinical studies. In 2001, Al-Majid and colleagues demonstrated that PA can reduce the muscle wasting with resistance exercise <sup>14</sup>. As well as its direct effects on the skeletal muscle, moderate-intensity resistance exercise decreases the fatigue of patients with cancer <sup>12</sup> and attenuated weakness and physical function loss of patients with brain tumor <sup>13</sup>.

For pragmatic reasons, this preclinical study was conducted only male animals to avoid any heterogeneity that could be linked to variations in the hormonal cycle of females. These variations may complicate the interpretation of the results of behavioral tests, especially those performed in a longitudinal manner. However, gender is an important factor in the effects of irradiation and physical activity. Indeed, several studies have reported that the sex of animals influences cranial irradiation-induced behavioral impairments and brain damage <sup>53–57</sup>. Similarly, it is also described that the gender affects the efficiency of physical activity <sup>58–60</sup>. Therefore, for translational relevance in brain and non-brain cancer patients treated by radiation therapy, it is important to confirm, in future experiments, the results found in female animals.

In conclusion, our data showed that brain irradiation induces a fatigue, a diminution of locomotor activity, a decrease of muscle mass and alterations of organization and typology of fibers. These muscle damage observed after cerebral irradiation are associated with the fatigue. Interestingly, muscle alterations and its consequences can be attenuated by physical exercise. These results emphasized that physical

activity is very pertinent intervention to manage the quality of life of patients treated by RT.

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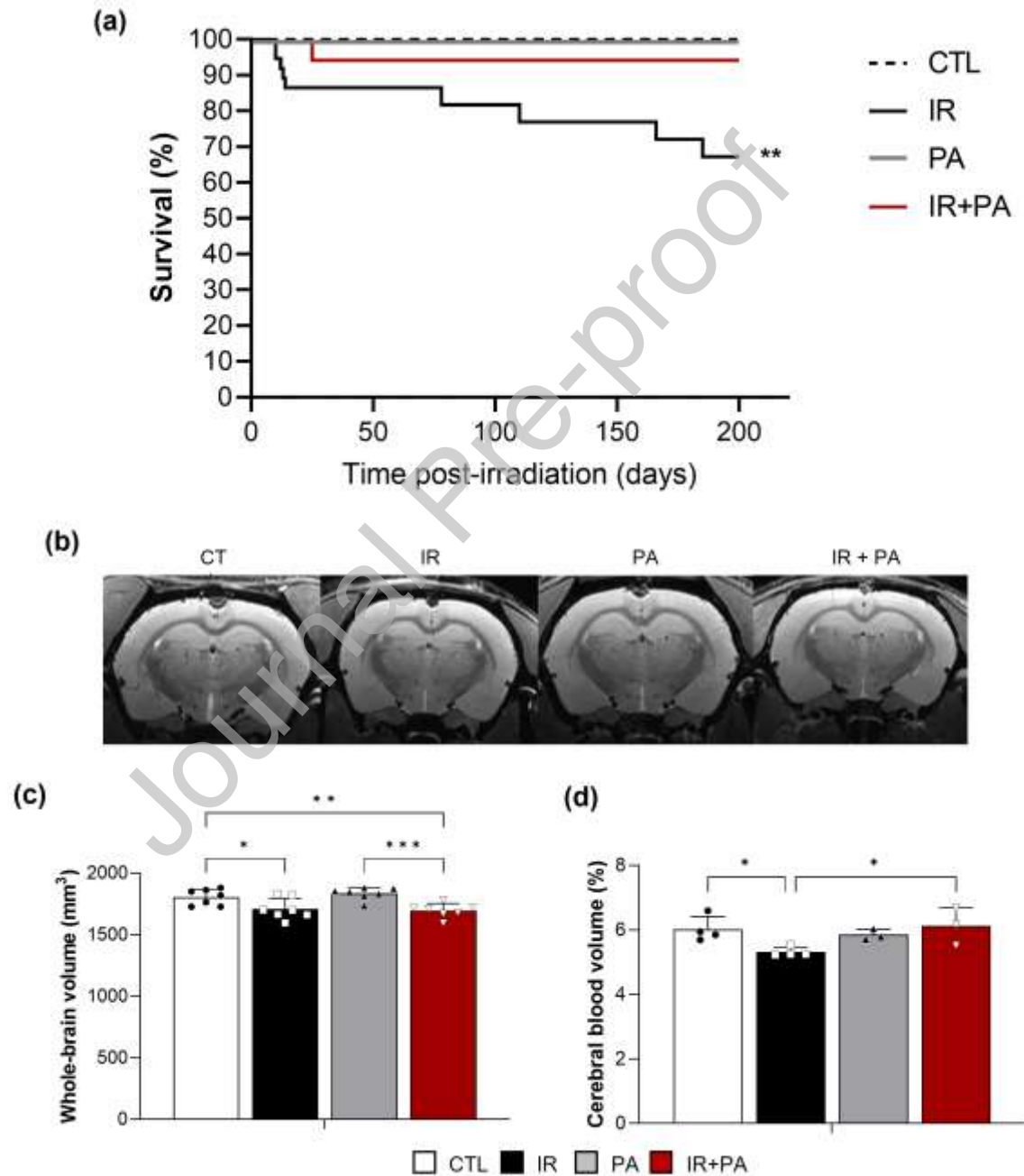
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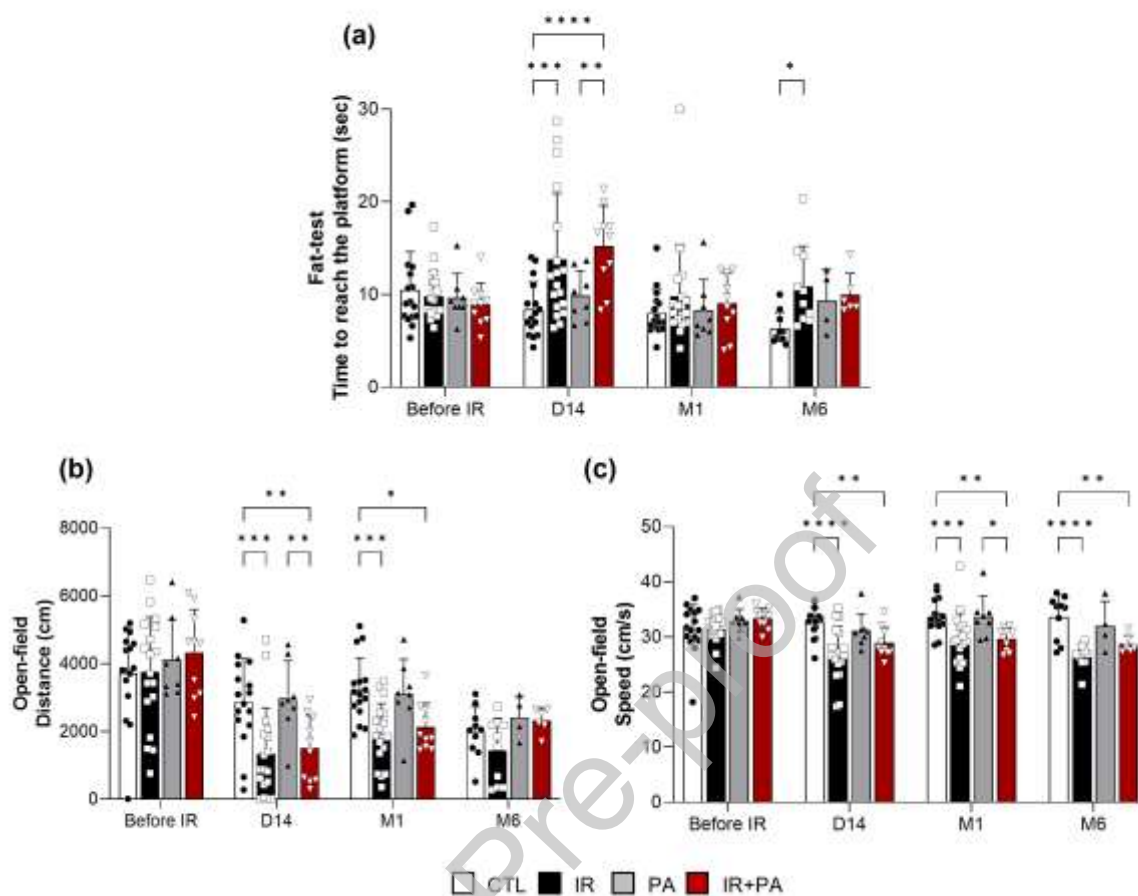
FIGURE 1



**Figure 1**

**Physical activity reduces brain irradiation-induced mortality and cerebral alterations.** **(a)** The survival probability was determined from the number of dead rats in each experimental group: unirradiated rats (CTL group = none dead), irradiated rats (IR group = 9 dead), non-irradiated rats subjected to physical activity (PA group = none dead) and irradiated rats subjected to physical activity (IR+PA group = 1 dead). Animal number for each experimental group was  $N_{CTL}=28$ ,  $N_{IR}=37$ ,  $N_{PA}=20$ ,  $N_{IR+PA}=23$ . The statistical analyses were based on a log-rank test; \*\*  $p < 0.01$  vs CTL. **(b)** Representative T2-weighted images of unirradiated rats (CTL), irradiated rats (IR), non-irradiated rats subjected to physical activity (PA) and irradiated rats subjected to physical activity (IR+PA). At 6 months after whole-brain irradiation, no edema or radionecrosis was observed. **(c)** Quantification of whole-brain volume based to T2-weighted images obtained 6 months after irradiation. Animal number for each experimental group was  $N=7$ . **(d)** Quantification of cerebral blood volume (CBV) in the cortex. CBV was measured from T2 star MRI before and after intravenous injection of a contrast agent (P904®). Animal number for CTL and IR experimental group were  $N=4$  and for PA and IR+PA experimental group were  $N=3$ . All data were presented as mean + SD. Statistical analyses were performed with on one-way ANOVA followed by Fisher's LSD test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

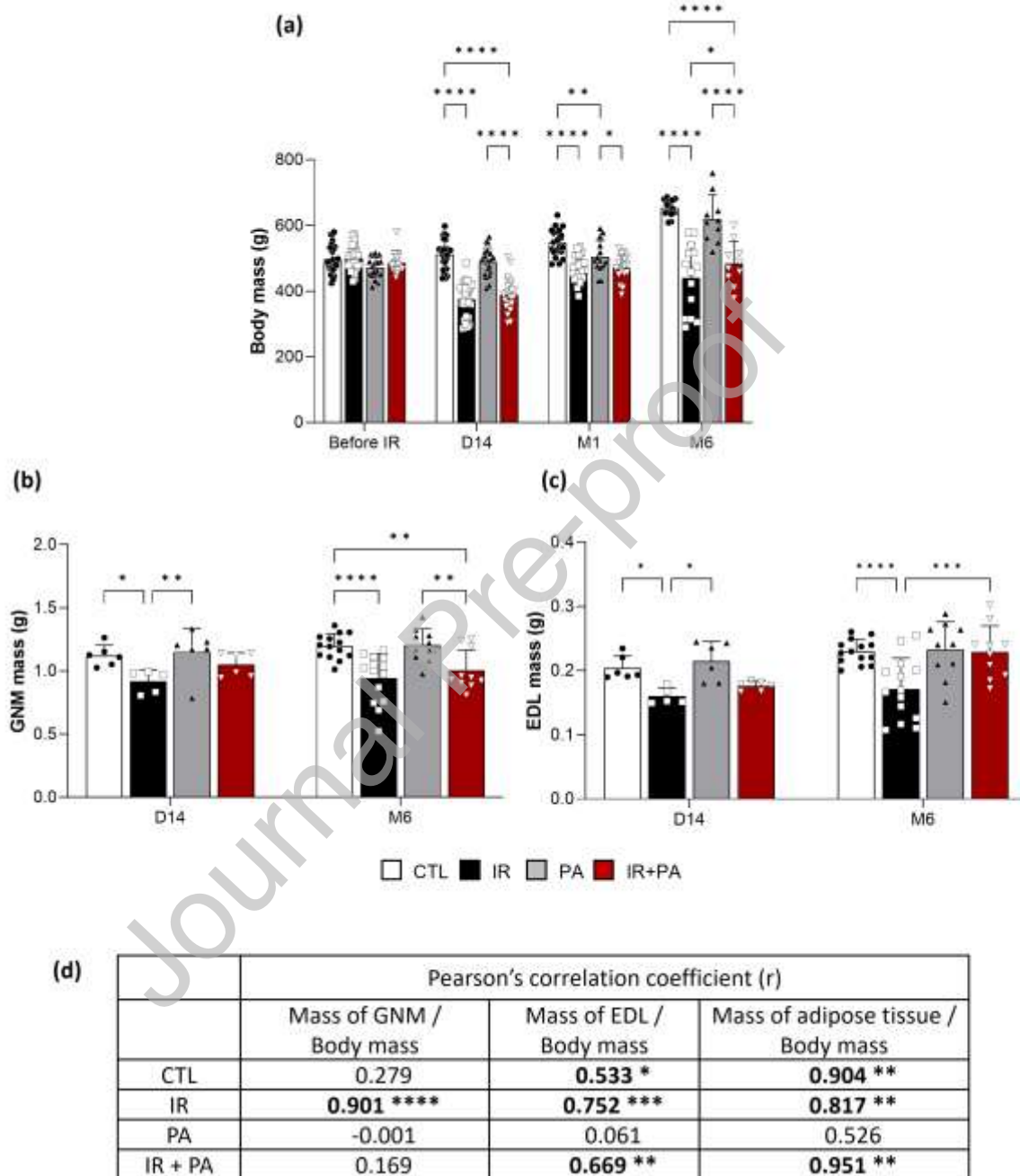
FIGURE 2

**Figure 2**

**Brain irradiation induces fatigue and a decreased locomotor activity which were attenuated by physical activity.** The unirradiated rats (CTL), irradiated rats (IR), unirradiated rats with physical activity (PA) and irradiated rats subjected to physical activity (IR+PA) were submitted to fatigue (Fat-test) and open-field tests **(a)** Assessment of fatigue with the Fat-test. The time taken by the animals to climb an inclined plane in order to reach a platform was quantified. **(b-c)** Quantification of the distance traveled by the rats in the open-field during 10 minutes as well as the speed of movement of the animals. All data were presented as mean + SD. The number of animals for each experimental group was respectively  $N_{CTL}=10-16$ ,  $N_{IR}=10-18$ ,

$N_{PA}=4-8$  and  $N_{IR+PA}=6-10$ . Statistical analyses were performed with two-way ANOVA followed by Fisher's LSD test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$

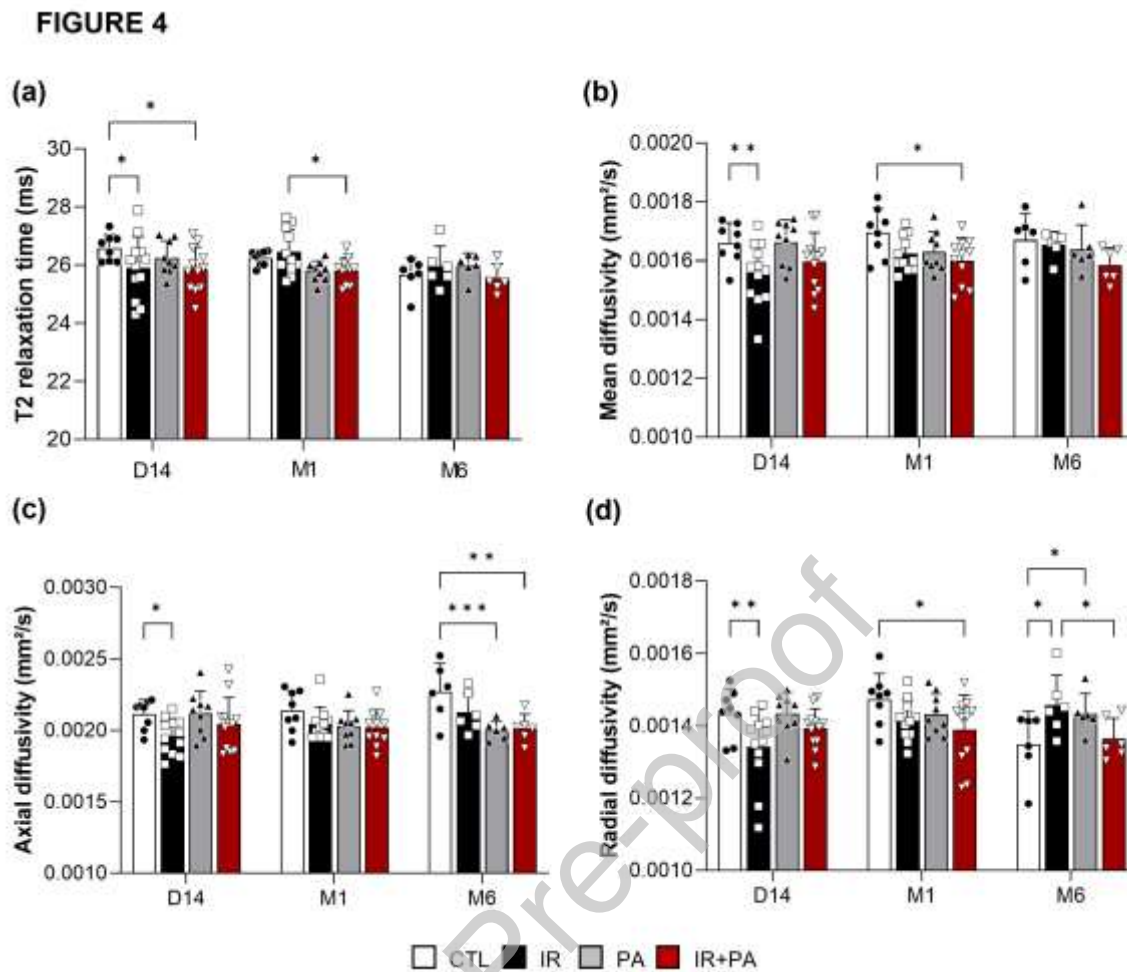
FIGURE 3

**Figure 3**

**Brain irradiation is associated to skeletal muscle mass reduction which was attenuated by physical activity. (a) Evolution of body weight in non-irradiated rats**



(CTL group), irradiated rats (IR group), non-irradiated rats subjected to physical activity (PA group) and irradiated rats subjected to physical activity (IR+PA group). The number of animals for each group was respectively,  $N_{CTL}=14-28$ ,  $N_{IR}=14-28$ ,  $N_{PA}=10-20$  and  $N_{IR+PA}=10-22$ . **(b-c)** Masses of gastrocnemius (GNM) and extensor digitorum longus (EDL) at early 14 days and 6 months after cerebral irradiation. The number of animals for each group was respectively,  $N_{CTL}=6-14$ ,  $N_{IR}=5-14$ ,  $N_{PA}=6-10$  and  $N_{IR+PA}=6-10$ . All data were presented as mean + SD. Statistical analyses were performed with two-way ANOVA followed by Fisher's LSD test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . **(d)** Correlation analyses were performed between body weight, skeletal muscles (GNM and EDL) and gonadal white adipose tissue. The number of animals was 16-20 for GNM/body masses, 16-20 for EDL/body masses and 6-12 for adipose tissue/body masses. For each correlation, the Pearson's correlation coefficient ( $r$ ) is shown. The statistical significance was achieved with the z correlation test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$

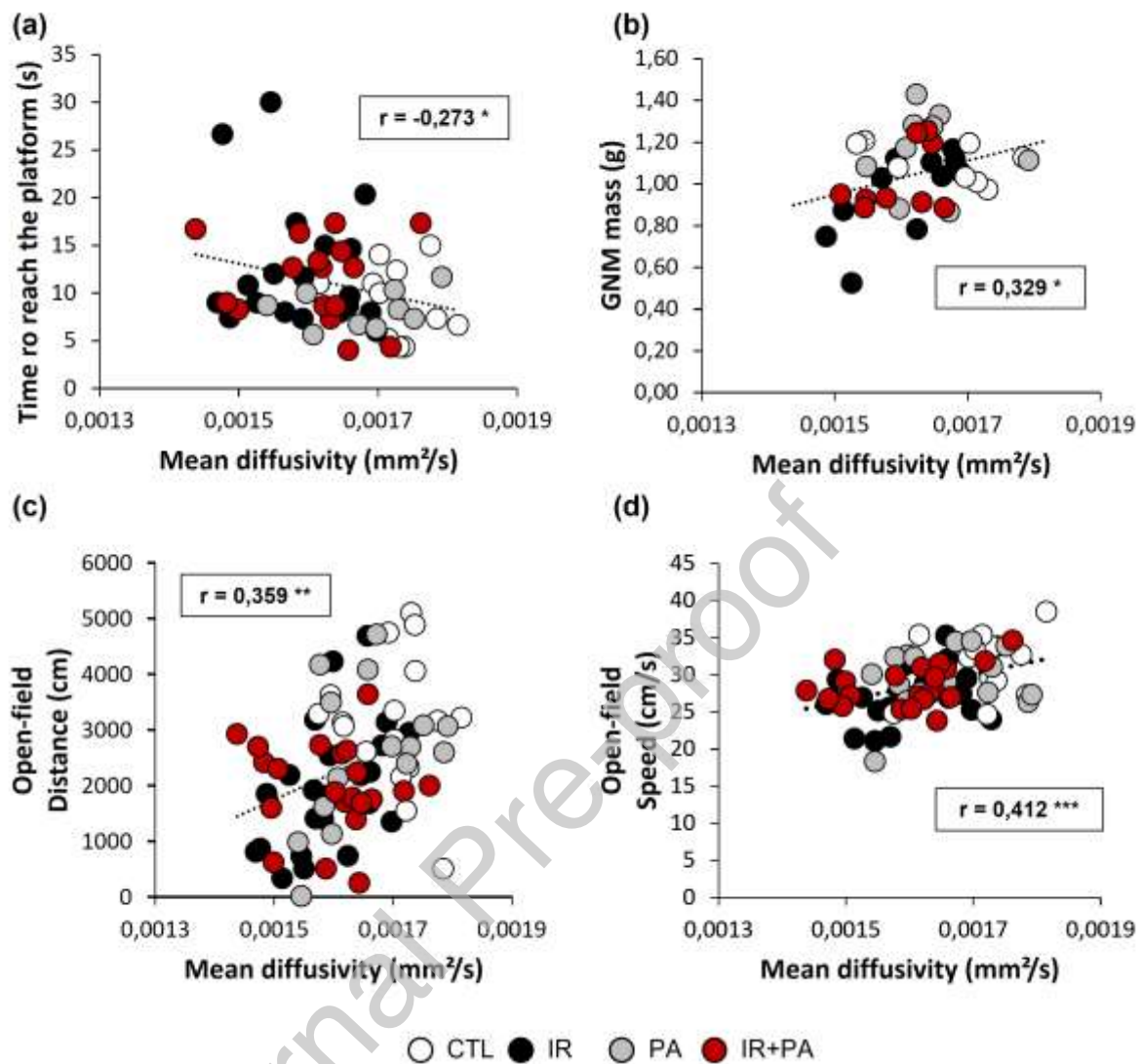


**Figure 4**

**MRI examinations reveal brain irradiation-associated muscle alterations.**

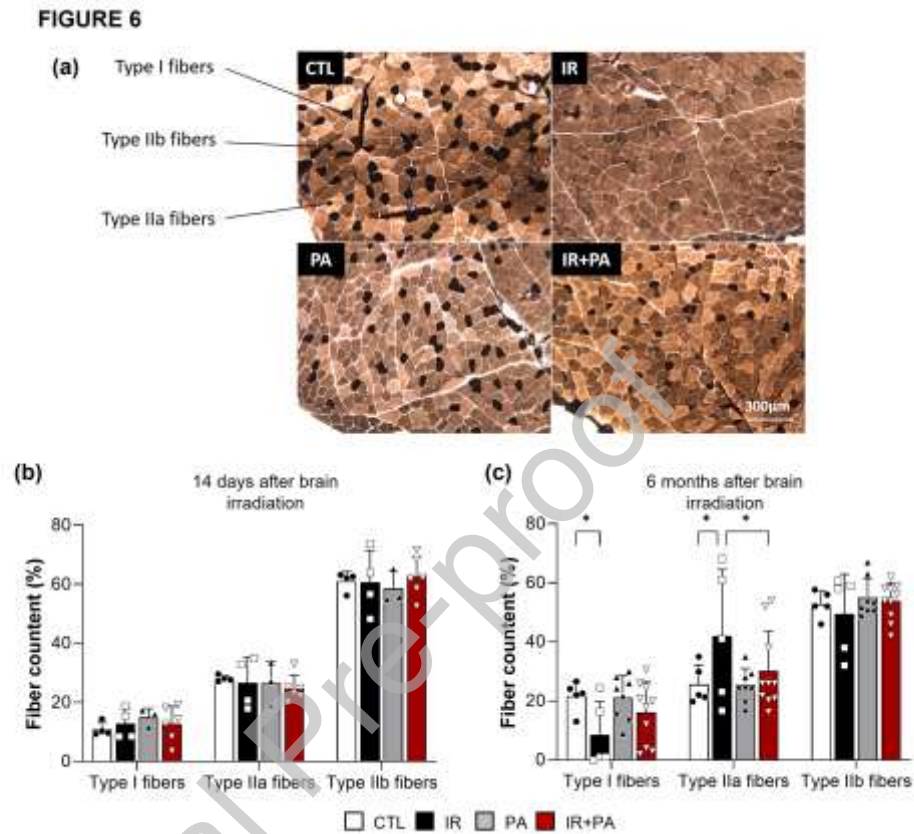
Multiparametric MRI of the lower part of the hind leg were acquired at 14 days (D14), 1 month (M1) and 6 months (M6) in non-irradiated rats (CTL), irradiated rats (IR), non-irradiated rats subjected to physical activity (PA) and irradiated rats subjected to physical activity (IR+PA). **(a)** T2 relaxation time (T2rt) **(b)** mean diffusivity (MD), **(c)** axial diffusivity (AD) and **(d)** radial diffusivity (RD) were quantified in the gastrocnemius. The number of rats in each group was respectively  $N_{CTL}= 6-8$ ,  $N_{IR}= 6-11$ ,  $N_{PA}= 6-9$ ,  $N_{IR+PA}= 6-11$ . All data were presented as mean + SD. Statistical analyses were performed with two-way ANOVA followed by Fisher's LSD test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$

FIGURE 5

**Figure 5**

**Correlations between MRI parameters of the muscle and fatigue, locomotion and skeletal muscle mass following brain irradiation. (a)** Correlation between the mean diffusivity (MD) values and the time taken by the animals to climb the inclined plane in order to reach the dark corner located on the platform, a quantitative indicator of fatigue level. Animals number for each group was  $N_{CTL}=11$ ,  $N_{IR}=19$ ,  $N_{PA}=9$ ,  $N_{IR+PA}=16$ . **(b)** Correlation between the mean diffusivity (MD) values and the gastrocnemius (GNM) mass ( $N_{CTL}=7$ ,  $N_{IR}=12$ ,  $N_{PA}=9$ ,  $N_{IR+PA}=9$ ). **(c-d)** Correlation between the mean diffusivity (MD) values and the locomotor activity reflected by the

distance traveled by the rats as well as the speed of movement of the animals during open-field test ( $N_{CTL}=11$ ,  $N_{IR}=19$ ,  $N_{PA}=9$ ,  $N_{IR+PA}=16$ ). Correlation tests were made with the Pearson's correlation test; \*  $p < 0.05$ , \*\*\*  $p < 0.001$



**Figure 6**

**Physical activity prevents the modifications of the typology of muscle fibers observed after brain irradiation.** Histochemical analysis of muscle fibers type was performed by myosin ATPase staining on the gastrocnemius (GNM) sections at early (14 days) or late (6 months) times after whole-brain irradiation. (a) Representative images of myosin ATPase staining in unirradiated rats (CTL), irradiated rats (IR), non-irradiated rats subjected to physical activity (PA) and irradiated rats subjected to physical activity (IR+PA). Myosin ATPase staining performed to pH 4.53 allows to discriminate the skeletal muscle fibers of types I (black), IIa (light brown) and IIb (dark brown) fibers. Quantification of the proportion of type I, IIa and IIb fibers in GNM at 14

days **(b)** or 6 months **(c)** after brain irradiation. The number of animals for each group was respectively:  $N_{CTL}=4-5$ ,  $N_{IR}=4-5$ ,  $N_{PA}=3-8$ ,  $N_{IR+PA}=6-10$ . All data were presented as mean + SD. Statistical analyses were performed with two-way ANOVA followed by Fisher's LSD test; \*  $p < 0.05$

## **SUPPLEMENTARY DATA**

### **Supplementary Figure 1**

**Timeline of the experimental design.** Irradiated rats received a fractionated whole-brain irradiation (WBI) on 3 consecutive days for a total dose of 30 Gy. Rats of all experimental groups were daily familiarized with the treadmill during 15 days before irradiation. Then physical exercise sessions were applied 3 times per week from 3 days after irradiation until the end of protocol (6 months) only for non-irradiated with physical activity rats (PA group) and irradiated with physical activity rats (IR+PA group). Behavioral tests and magnetic resonance imaging (MRI) were performed at 14 days, 1 month and 6 months after the brain irradiation. At these time points, some rats were euthanized for tissue sampling

### **Supplementary Figure 2**

**Validation of the homogeneity of dose deposition of x-rays in the whole-brain.**

The dose deposition maps, generated by Monte Carlo simulations from the treatment planning system (TPS), showed that all the brain structures received a dose of 10 Gy at each irradiation fraction. The dose-volume histogram (DVH) curves confirmed that

whole-brain irradiation procedure delivered uniform dose of x-rays in cortical and sub-cortical structures

### **Supplementary Figure 3**

**Analyses of muscle MRI.** (a) circular regions of interest (ROI) were positioned on the center of gastrocnemius (GNM) along 3 consecutive slices of T2-weighted images. (b) From T2 and DTI images, several MRI parameters such as T2rt (T2 relaxation time), MD (mean diffusivity), AD (axial diffusivity) and RD (radial diffusivity) were quantified using ImageJ software. In this figure, representative maps of SI (signal intensity derived from T2 imaging), T2rt, MD, AD, RD were shown for one representative rat for each experimental group at 14 days after whole-brain irradiation: unirradiated rats (CTL group), irradiated rats (IR group), unirradiated rats subjected to physical activity (PA group) and irradiated rats subjected to physical activity (IR+PA group)

### **Supplementary Table 1**

**Correlations between MRI parameters and GNM mass, fatigue index and locomotor activity.** MRI parameters (T2rt, MD, AD and RD) were correlated to the time during the Fat-test, the GNM mass and to the distance and speed during the open-field test. The number of animals was 45-80 for T2rt's correlation, 37-76 for MD's correlation, 36-76 for AD's correlation and 38-78 for RD's correlation. For each correlation, the Pearson's correlation coefficient ( $r$ ) is shown. The statistical significance was achieved with the z correlation test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$