

## Peripheral inflammatory activation after hippocampus irradiation in the rat

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### Abstract

**Purpose:** To detect the possible biochemical signs of inflammatory activation in the peripheral circulation in a rodent model of hippocampus irradiation, and to examine the effects of L-alpha-glycerylphosphorylcholine (GPC) in this experimental protocol.

**Materials and methods:** Anesthetized Sprague-Dawley rats were subjected to 40 Gy cobalt irradiation of both hemispheres of the hippocampus, with or without GPC treatment (50 mg/kg intravenously (i.v.), 5 min before the irradiation,  $n = 6$ , each). A third group ( $n = 6$ ) served as saline-treated control. Blood samples were obtained 3 h after the end of irradiation in order to examine the changes in plasma histamine, tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin 1-beta, interleukin 6 (IL-6) and interleukin 10 (IL-10); liver tissue samples were taken to determine adenosine triphosphate (ATP) concentrations.

**Results:** The hepatic ATP levels were significantly declined, while plasma concentrations of circulating TNF- $\alpha$ , IL-6, IL-10 and histamine were significantly increased after hippocampus irradiation. GPC treatment significantly reduced the irradiation-induced release of cytokines and histamine, and the liver ATP level was maintained at the control value.

**Conclusions:** Targeted brain irradiation produced measurable pro- and anti-inflammatory cytokine changes in the systemic circulation. GPC supplementation provides significant protection against irradiation-induced peripheral pro-inflammatory activation and ATP depletion.

**Keywords:** Brain irradiation, radioprotection, cytokines, TNF-alpha, ATP, L-alpha-glycerylphosphorylcholine

### Introduction

Brain radiotherapy is used successfully in patients with various primary brain tumors and tumors metastatic to the brain (Kalifa and Grill 2005, Khuntia et al. 2009); however, patients often experience potentially harmful side-effects, such as

interstitial edema with elevated intracranial pressure (Kirste et al. 2011, Liu et al. 2010).

There are numerous potential mechanisms of irradiation-induced adverse reactions in the central nervous system (CNS), but it has been established that a coordinated pro-inflammatory response, including the release of preformed and de novo synthesized mediators may play key roles in radiotherapy-associated tissue injury (Denham and Hauer-Jensen 2002). It has been shown that the expressions of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) genes are rapidly induced after brain irradiation, and these cytokines have also been implicated in edema formation (Mohanty et al. 1989, Hong et al. 1995, Botchkina et al. 1997, McBride et al. 1997, Meistrell et al. 1997, Daigle et al. 2001, Gaber et al. 2003, Han et al. 2006, Shimada et al. 2012).

The spread of pro-inflammatory events is balanced by the release of anti-inflammatory cytokines such as interleukin-10 (IL-10), which downregulates TNF- $\alpha$  activity and inhibits long-term interleukin-6 (IL-6) production (Marshall et al. 1996, Huaux et al. 1999). Indeed, it has been demonstrated that the TNF- $\alpha$  output peaks after 2–8 h and has usually returned to the baseline by 24 h after radiation (Daigle et al. 2001). The sizes and structures of the cytokines are also limiting factors, which exclude their passive diffusion across the blood-brain barrier (BBB).

Nevertheless, the entry of peripherally-produced cytokines into the brain tissue after total-body irradiation or inflammatory syndromes is rather well-documented and this implies that the mechanism that controls the passage of such substances from the blood into the cerebrospinal fluid may be temporarily disturbed. It also follows that the unwanted consequences of brain irradiation might include a release of substances that may have peripheral effects if the pathophysiological opening of the barrier mechanisms is bidirectional. On this basis, we hypothesized that radiation therapy may lead to peripheral pro-inflammatory conse-

quences through the production of mediators that originate from the irradiated brain.

Our primary aim was to investigate the immediate changes in major pro- and anti-inflammatory cytokines in the peripheral circulation after irradiation of the hippocampus with therapeutic doses. An additional aim was to influence the peripheral cytokine response with a potentially anti-inflammatory intervention. Here, we took into consideration the previous finding that pretreatment with phosphatidylcholine (PC) prevented the decrease in hippocampal neurogenesis after a lipopolysaccharide-induced peripheral inflammatory challenge (Tőkés et al. 2011). L-alpha-glycerolphosphorylcholine (GPC) is a water-soluble, deacylated PC derivative, which has proved effective against the loss of the membrane function in CNS injuries (Amenta et al. 1994, Onishchenko et al. 2008). Against this background, experiments were undertaken to characterize the preventive potential of GPC treatment on the brain irradiation-induced cytokine production in the peripheral circulation.

## Materials and methods

### Animals

Experiments were performed on 18 adult male Sprague-Dawley rats (180–250 g, purchased from the Animal House of the University of Szeged) housed in plastic cages in a thermoneutral environment ( $21 \pm 2^\circ\text{C}$ ) under a 12-h dark-light cycle. Food and water were provided *ad libitum*. The experimental protocol was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged and followed the National Institutes of Health (Bethesda, MD, USA) guidelines on the care and use of laboratory animals. The animals were randomly allocated into the study groups.

### Experimental protocol

The animals were anesthetized with 5% chloral hydrate solution intraperitoneally (i.p.) and placed in a supine position on a heating pad. The right jugular vein was cannulated with polyethylene (PE50) tubing for the maintenance of anesthesia (5% chloral hydrate, Fluka Analytical, Buchs, Switzerland) and for treatment. Group 1 ( $n = 6$ ), which served as non-treated controls, received 0.5 ml sterile saline intravenously (i.v.). Computed tomography (CT)-based (Emotion 6-Siemens AG, Erlangen, Germany) three-dimensional conformal treatment planning was performed with the XIO™ (CMS, Elekta, Stockholm, Sweden) treatment planning system. The hippocampus was delineated on each slice on CT images acquired in the treatment position. Two opposed isocentric lateral circle fields 1 cm of diameter were planned, resulting in a homogeneous dose distribution in the target. The field profile and output factor of the custom-made collimator were measured by using film dosimetry and a pinpoint ionization chamber. For the irradiation, the animals were laid on a special positioning scaffold (resembling a bunk-bed, 3 rats at a time). Group 2 ( $n = 6$ ) and group 3 ( $n = 6$ ) were subjected to cobalt 60 teletherapy (Teragam K01, SKODA UJP, Prague,

Czech Republic) of the hippocampus in both hemispheres: 40 Gy (1 Gy/2.25 min), from two opposed lateral fields. The dosage level selected for the study protocol was based on the data of previously published investigations (Münter et al. 1999, Karger et al. 2002, Hideghéty et al. 2013); biological responses to different single doses were defined in pilot experiments as well (see Supplementary Data, to be found online at <http://informahealthcare.com/abs/doi/10.3109/09553002.2013.836617>). It should be added that the radiotolerance of the rat brain is different from human brains, and structural changes, including decreases in cell number and demyelination can be expected in the 50–100 Gy dose range (Münter et al. 1999).

Prior to the start of radiation portal imaging with the gamma ray of the Cobalt unit was performed for field verification. Additionally, group 3 received GPC (Lipoid GmbH, Ludwigshafen, Germany; 50 mg/kg bw, dissolved in 0.5 ml sterile saline, i.v.) 5 min before the start of irradiation. The effects of GPC *per se* were characterized in accompanying studies; the GPC treatment alone did not induce measurable changes in the observed inflammatory biochemical parameters.

Three hours after the completion of irradiation, blood samples were obtained from the inferior vena cava to examine the plasma histamine, TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-10 changes. The animals were then killed by decapitation and additional liver samples were immediately taken to determine tissue adenosine triphosphate (ATP) changes.

### ATP measurements

The liver samples were snap frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$  until assays analysis. The tissue was weighed, placed into a 3-fold volume of trichloroacetic acid (6% w/v), homogenized for 1 min, and centrifuged at 5,000 g. After adjustment of the pH to 6.0 with saturated  $\text{K}_2\text{CO}_3$  solution, the reaction mixtures were prepared by the addition of 100  $\mu\text{l}$  of ATP assay mix (containing firefly luciferase, luciferin,  $\text{MgSO}_4$ , ethylenediaminetetraacetic acid (EDTA), DL-Dithiothreitol (DTT) and Bovine Serum Albumin (BSA) in a Tricine buffer; Sigma-Aldrich GmbH, Munich, Germany) to 100  $\mu\text{l}$  of 5-fold-diluted sample. The ATP determinations were based on the measurement of luciferase chemiluminescence, using a luminometer (LUMAT LB 9507, Berthold Technologies, GmbH, Bad Wilbad, Austria). The ATP levels were calculated with the aid of a standard ATP calibration curve (Sigma-Aldrich GmbH) and the data were referred to the sample weights.

### Measurement of plasma TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10

Blood samples (0.5 ml) were taken from the inferior vena cava into precooled EDTA-containing polypropylene tubes, centrifuged at 1000 g for 30 min at  $4^\circ\text{C}$ , and then stored at  $-70^\circ\text{C}$  until assay. Plasma TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10 concentrations were determined by means of commercially available enzyme-linked immunosorbent assays ((ELISA), Quantikine ultrasensitive ELISA kit for rat TNF- $\alpha$  IL-1 $\beta$ , IL-6 and IL-10; Biomedica Hungaria Kft, Budapest, Hungary). The minimum detectable levels of rat TNF- $\alpha$  and IL-1 $\beta$  were  $< 5$  pg/ml, that of rat IL-10 was  $< 10$  pg/ml and the mean detectable dose of rat IL-6 was 21 pg/ml.

**Measurement of plasma histamine**

Blood samples (0.5 ml) were taken from the inferior vena cava into precooled EDTA-containing polypropylene tubes, centrifuged at 1,000 g for 30 min at 4°C, and then stored at -70°C until assay. Plasma histamine concentrations were determined by means of a commercially available enzyme-linked immunoassay (Quantikine ultrasensitive EIA kit for rat histamine; Biomedica Hungaria Kft).

**Statistical analysis**

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Non-parametric methods were used. Differences between groups were subjected to Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures, median values (M) and 75th percentiles (p75) and 25th percentiles (p25) are given. P values < 0.05 were considered significant: \*p < 0.05 relative to the saline-treated control group, and #p < 0.05 relative to the irradiated group.

**Results**

**Liver ATP levels**

Figure 1 reveals that brain irradiation with 40 Gy resulted in a significant reduction in hepatic ATP level as compared with the saline-treated group (M: 71.9; p25: 57.7; p75: 100.9 vs. M: 120.4; p25: 117.5; p75: 126.6). In the GPC-treated group, the level of liver ATP was significantly higher and did not differ significantly from that observed in the control group (M: 119.4; p25: 113.1; p75: 123.2).

**Plasma TNF-α, IL-1β, IL-6 and IL-10 concentrations**

The irradiation of the rat hippocampus was accompanied by a significant plasma TNF-α level elevation (M: 20.7; p25: 18.7; p75: 23.2) as compared with the control group (M: 9.7; p25: 9.3; p75: 10.06). The i.v. GPC treatment protocol reduced the increase in TNF-α level (M: 12.8; p25: 12.4; p75: 13.6) significantly (Figure 2).

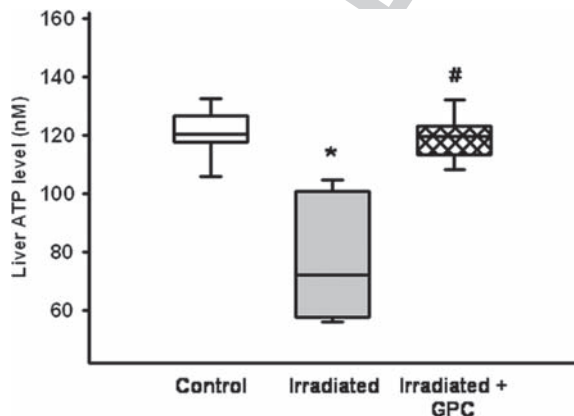


Figure 1. Liver ATP levels 3 h after 40 Gy hippocampus irradiation. The white box plot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the grey box plot to the glycerylphosphorylcholine (GPC)-treated group. Median values and 75th and 25th percentiles are given. \*p < 0.05 relative to the saline-treated control group. #p < 0.05 relative to the irradiated group.

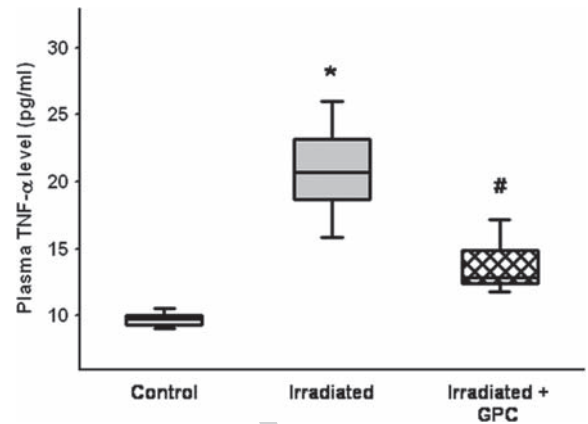


Figure 2. Plasma TNF-α changes after hippocampus irradiation. The white box plot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the grey box plot to the glycerylphosphorylcholine (GPC)-treated group. The plasma TNF-α level was significantly increased 3 h after irradiation as compared with the saline-treated group, and the GPC administration significantly reduced the irradiation-induced inflammatory reaction. Median values and 75th and 25th percentiles are given. \*p < 0.05 relative to the saline-treated control group. #p < 0.05 relative to the irradiated group.

The IL-6 concentration was also significantly higher at 3 h after radiation exposure (M: 347.2; p25: 297.4; p75: 422.3 vs. saline treatment: M: 289.6; p25: 264.7; p75: 323.9); administration of GPC decreased this tendency (M: 333.2; p25: 298.2; p75: 345.5), the plasma level then not differing significantly from that for the control group (Figure 3).

In the case of the plasma IL-1β, no between-group differences were observed (control: M: 126.5; p25: 119.8; p75: 129.9; irradiated: M: 122.3; p25: 116.7; p75: 143.8; GPC-treated: M: 132.7; p25: 129.5; p75: 137.8; Figure 4).

The IL-10 plasma level was significantly higher 3 h after the irradiation (M: 90.7; p25: 82.6; p75: 102.1; Figure 5) than in the saline-treated control group (M: 4.1; p25: 1.2; p75: 5.04); GPC treatment likewise significantly reduced the irradiation-induced IL-10 reaction (M: 19.5; p25: 16.3; p75: 22).

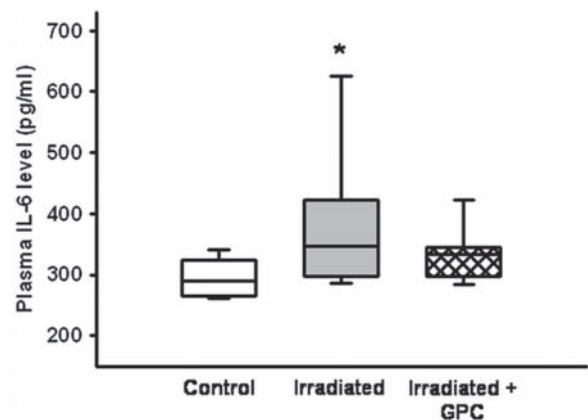


Figure 3. Plasma IL-6 level 3 h after 40 Gy hippocampus irradiation. The white box plot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the grey box plot to the glycerylphosphorylcholine (GPC)-treated group. The IL-6 concentration was significantly higher at 3 h after radiation exposure than after the administration of saline alone. The GPC treatment led to a decreasing tendency, and the result did not differ significantly from that in the control group. Median values and 75th and 25th percentiles are given. \*p < 0.05 relative to the saline-treated control group.

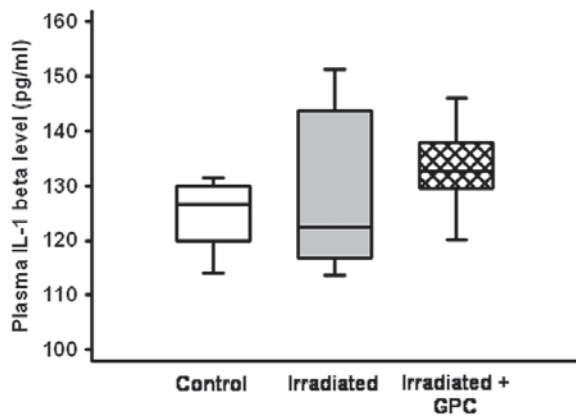


Figure 4. Plasma IL-1 $\beta$  level 3 h after 40 Gy hippocampus irradiation. The white box plot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the grey box plot to the glycerylphosphorylcholine (GPC)-treated group. There was no statistical difference between the groups. Median values and 75th and 25th percentiles are given.

### Plasma histamine changes

The hippocampus irradiation resulted in a significant elevation (M: 49.6; p25: 44.3; p75: 63.9; Figure 6) in plasma histamine level as compared with the non-irradiated control group (M: 23.9; p25: 16; p75: 33.1). Again, after the GPC treatment, the histamine concentration remained at the control level (M: 25.3; p25: 23.7; p75: 28.7).

### Discussion

In the present study irradiation of the rat hippocampus with 40 Gy transiently elevated the concentrations of circulating acute-phase cytokines, and significantly decreased the hepatic ATP content. The results also demonstrated that a single dose of GPC can influence the changes in TNF- $\alpha$ , IL-6, IL-10 and histamine plasma levels and prevents the ATP depletion in the rat liver.

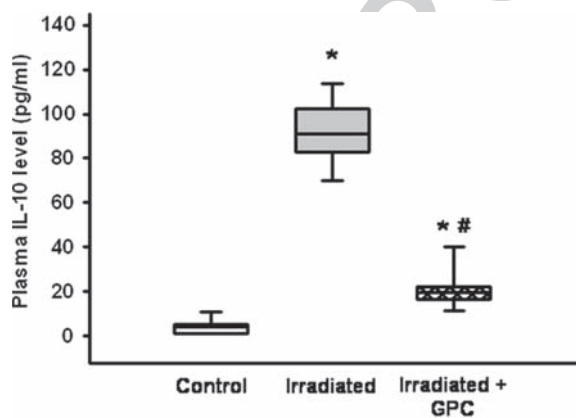


Figure 5. Plasma IL-10 level changes. The white box plot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the grey box plot to the glycerylphosphorylcholine (GPC)-treated group. The plasma IL-10 level at 3 h after the irradiation was significantly higher than that in the saline-treated control group. The GPC treatment significantly reduced the irradiation-induced inflammatory reaction. Median values and 75th and 25th percentiles are given. \* $p < 0.05$  relative to the saline-treated control group. # $p < 0.05$  relative to the irradiated group.

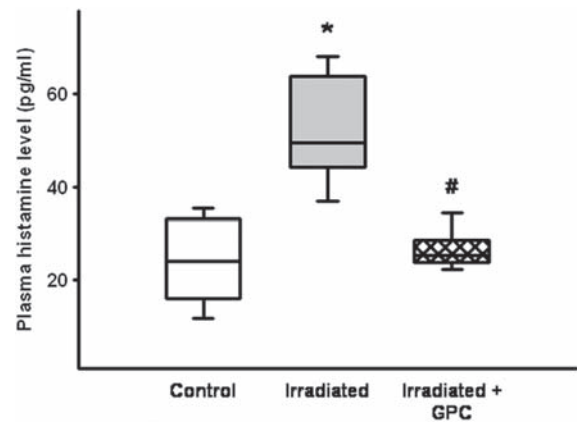


Figure 6. Plasma histamine level in the peripheral circulation 3 h after hippocampus irradiation. The white box plot relates to the saline-treated group, the dark-grey box plot to the irradiated group and the grey box plot to the glycerylphosphorylcholine (GPC)-treated group. The GPC treatment prevented the increase of the plasma histamine and resulted in a significantly lower level as compared with the irradiated group. Median values and 75th and 25th percentiles are given. \* $p < 0.05$  relative to the saline-treated control group. # $p < 0.05$  relative to the irradiated group.

In this experimental set-up the selection of pro-inflammatory cytokines was based on their known key roles in the mediation of signals in a wide spectrum of CNS cell types that exert central roles in acute inflammation (Dinarello 1996, Locksley et al. 2001). There have been several reports demonstrating that the overexpression of TNF- $\alpha$  and IL-1 $\beta$  genes may be associated with the molecular responses of the brain to irradiation (Hong et al. 1995, Gaber et al. 2003, Marquette et al. 2003). *Vice versa*, it has been shown that peripheral TNF- $\alpha$  production plays a detrimental role in neural survival or differentiation in the hippocampus (Vezzani et al. 2002, Monje et al. 2003, Liu et al. 2005). However, the peripheral biochemical consequences of hippocampus irradiation have not been characterized previously.

IL-6 is a multifunctional pro-inflammatory cytokine that plays a role in the mediation of the inflammatory responses after total-body irradiation (Kishimoto 2005), and recent studies have suggested that elevated levels of IL-6 protein expression may be responsible for the radiation-induced inflammation in the brain (Linard et al. 2003, 2004, Marquette et al. 2003). Furthermore, it has also been reported that the exposure of rodents to total-body irradiation selectively activated nuclear factor- $\kappa$ B (NF- $\kappa$ B) and subsequently increased the mRNA expression of TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 in lymphoid tissues (Zhou et al. 2001).

In this line, histamine, mainly released by neurons and mast cells (Ruat et al. 1990) can play additional, roles in the formation of edema in the rat brain. Although an increased histamine release is associated with hypoxia in ischemic and injured brain (Mohanty et al. 1989), the exact interactive roles of the compound in radiation-induced CNS lesion are still largely unknown.

The pro-inflammatory mediator release may be counteracted by increased IL-10 production, which downregulates TNF- $\alpha$  activity, inhibits long-term IL-6 production (Marshall et al. 1996, Huaux et al. 1999), blocks NF- $\kappa$ B activity, and is involved in the regulation of the Janus kinase/signal

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transducers and activators of transcription (JAK-STAT) signaling pathway; thus, it can be considered to be an anti-inflammatory cytokine after irradiation-induced brain damage (Ward et al. 2011).

The study design allowed us to detect distant, peripheral effects of brain irradiation. In this line, we observed for the first time that, shortly after brain irradiation, the inflammatory cytokine levels are significantly elevated at the periphery. It is our working hypothesis that, after irradiation, a significant, local, pro-inflammatory response is activated, and the BBB is temporarily opened. The functional, distant or long-term consequences of this phenomenon are still unknown, but the hippocampus irradiation-induced pro-inflammatory stimuli not only affected the circulating cytokine concentrations, but in parallel, the hepatic ATP production was significantly reduced.

It emerged that the peripheral plasma levels of these key mediators were successfully modulated by GPC administration. GPC is a precursor molecule of the neurotransmitter acetylcholine, and was previously tested as a centrally acting parasympathomimetic drug in dementia disorders and acute cerebrovascular diseases (Barbagallo Sangiorgi et al. 1994, De Jesus Moreno Moreno 2003). GPC acts as a PC precursor (Gallazzini and Burg 2009), and the increased uptake of membrane-forming phospholipids, including PC, proved to exert an anti-inflammatory influence in other experimental studies (Chao et al. 1995, El-Hariri et al. 1992, Erős et al. 2009). Previous investigation revealed that PC treatment prevented microglia accumulation in the hippocampus (Tórkés et al. 2011), and further evidence for the mechanism of action is provided by recent *in vitro* findings of an anti-TNF- $\alpha$  effect and specific inhibition of the Toll-like receptor 4-dependent inflammatory pathway (Ishikado et al. 2009, Treede et al. 2009).

The deacylated, water-soluble PC analogue GPC rapidly delivers choline to the brain across the BBB (Parnetti et al. 2007), and it may be present in the irradiated area where the cytokine-mediated actions are expected. Indeed, the *i.v.* GPC administration prior to the irradiation challenge was associated with enhanced anti-inflammatory protection, and in this respect a central mediatory role of TNF- $\alpha$  is proposed in the transmission of the intracranial inflammatory response to the periphery. However, another possibility whereby signals from the irradiated brain could be influenced through nerves communicating with the periphery. It has been demonstrated that the IL-1 $\beta$  levels in the hypothalamus, thalamus and hippocampus, and the TNF- $\alpha$  and IL-6 levels in the hypothalamus, were increased 6 h after partial-body irradiation, and vagotomy before irradiation prevented these responses (Marquette et al. 2003). Along these lines, it could be hypothesized that the vagus nerve and the cholinergic anti-inflammatory system may also be one of the descending pathways for rapid signaling with respect to irradiation.

## Conclusions

Our data provide strong evidence for the possibility of peripheral inflammatory activation after hippocampus irradiation through the production of mediators leaking from the irradiated brain. Moreover, we show that pre-treatment with

GPC is protective against CNS irradiation-induced peripheral effects. The study has limitations too, because a certain degree of leakage in the cobalt irradiator and the possibility of an internal scatter effect cannot be excluded with certainty, and therefore, theoretically it is possible that the body may have received 2–4 Gy scatter irradiation. However, all animals were exposed to identical doses of irradiation, thus between-group differences were determined unambiguously. Further studies should clarify specific interactions between CNS and peripheral inflammation and protection. However, the inhibition of TNF- $\alpha$  mediation by GPC, leading to a decreased pro-inflammatory cytokine production and an elevated ATP level in the periphery, could be of considerable therapeutic significance if reproduced in clinical practice.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## Supplementary material available online

Supplementary Data.

1 *Supplementary material for Tökés T, et al. Peripheral inflammatory activation after hippocampus irradiation in the rat, Interna-* 60  
2 *tional Journal of Radiation Biology 2013;doi: 10.3109/09553002.2013.836617.* 61

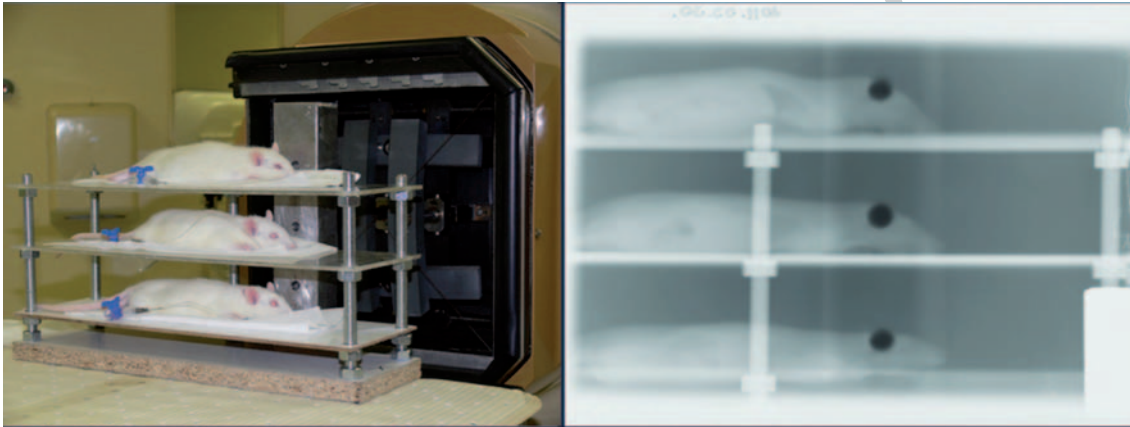
### 3 4 5 **Supplementary Data** 63

6 Biological responses to different single doses of irradiation 64  
7 were defined in pilot rat experiments (Figure 1). 65

8 Brain irradiation-induced biological effects were tested 66  
9 in the 0-120 Gy range in 10 Gy steps. Histopathological 67  
10 changes were evaluated using conventional light micros- 68  
11 copy (OM 50x-400x) by two experienced histopathologists, 69  
12 70

independently (see results in Table 1). Histological analysis 64  
was performed in coded sections, tissue injury was graded 65  
on a 1-4 damage scale with the following criteria: presence 66  
of necrosis (see results in Figure 2), macrophage density 67  
(see Figure 3), hemorrhage (see Figure 4) and calcification 68  
(see Figure 5), respectively, where „1” represented normal 69  
structure. 70

For further details see relevant parts of the manuscript. 71



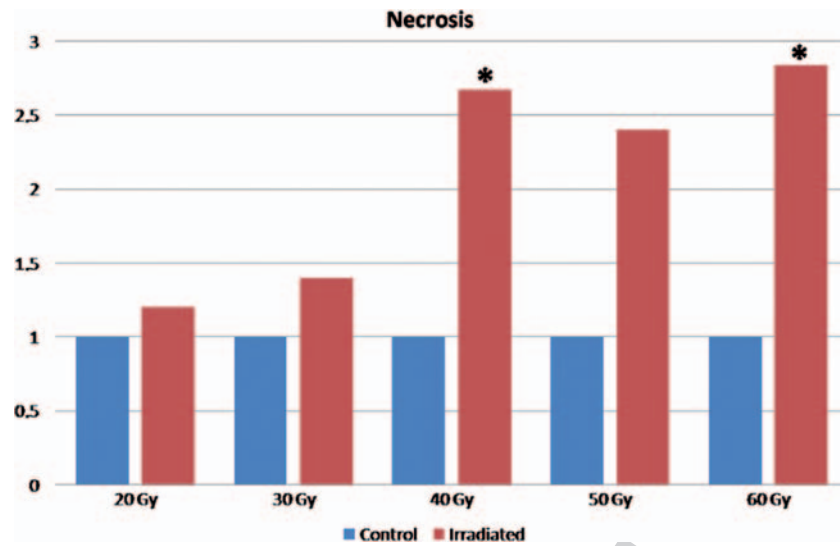
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Supplementary Figure 1. Photograph showing the animals positioned for the irradiation protocol and the exact area of irradiation (black circles). 87  
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Supplementary Table I. Histopathological scores after brain irradiation with 20–60 Gy.

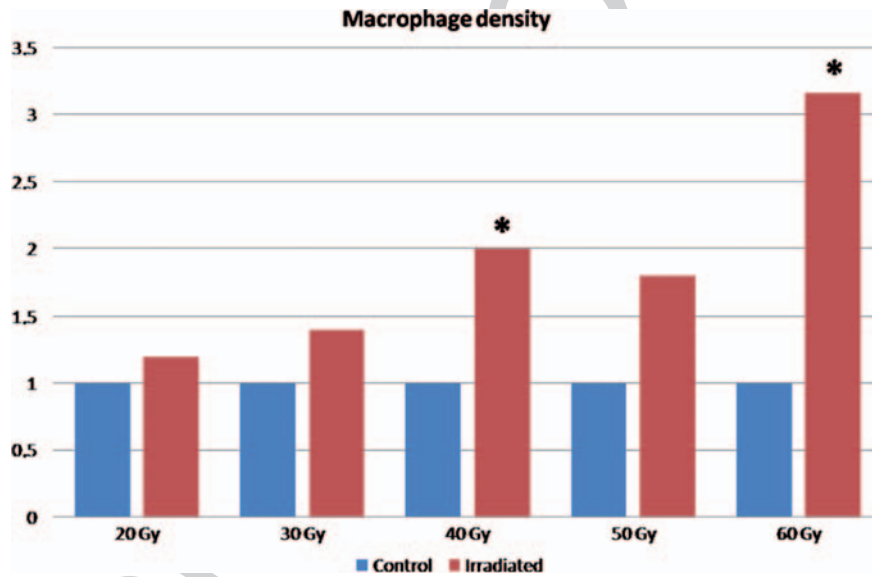
Dose (Gy)	Exp. number	Group	Necrosis 1-4	Macrophage 1-4	Haemorrhage 1-4	Calcification 1-4	Valid
20 Gy	7. ONKO 1	Irradiated	1	1	1	1	1
20 Gy	7. ONKO 2	Irradiated	1	1	1	1	1
20 Gy	7. ONKO 3	Irradiated	1	1	1	1	1
20 Gy	7. ONKO 4	Irradiated	1	1	1	1	1
20 Gy	7. ONKO 5	Irradiated	2	2	1	2	1
20 Gy	7. ONKO 6	Control	1	1	1	1	1
20 Gy	7. ONKO 7	Control	1	1	1	1	1
20 Gy	7. ONKO 8	Control	1	1	1	1	1
20 Gy	7. ONKO 9	Control	1	1	1	1	1
20 Gy	7. ONKO 10	Control	1	1	1	1	1
30 Gy	8. ONKO 1	Irradiated	1	1	2	2	2
30 Gy	8. ONKO 2	Irradiated	2	2	2	2	2
30 Gy	8. ONKO 3	Irradiated	1	1	1	1	2
30 Gy	8. ONKO 4	Irradiated	2	2	1	2	2
30 Gy	8. ONKO 5	Irradiated	1	1	1	1	2
30 Gy	8. ONKO 6	Control	1	1	1	1	2
30 Gy	8. ONKO 7	Control	1	1	1	1	2
30 Gy	8. ONKO 8	Control	1	1	1	1	2
30 Gy	8. ONKO 9	Control	1	1	1	1	2
30 Gy	8. ONKO 10	Control	1	1	1	1	2
40 Gy	9. ONKO 14	Control	1	1	1	1	3
40 Gy	9. ONKO 15	Control	1	1	1	1	3
40 Gy	9. ONKO 16	Control	1	1	1	1	3
40 Gy	9. ONKO 17	Control	1	1	1	1	3
40 Gy	9. ONKO 18	Control	1	1	1	1	3
40 Gy	9. ONKO 19	Control	1	1	1	1	3
40 Gy	9. ONKO 20	Irradiated	3	3	2	3	3
40 Gy	9. ONKO 21	Irradiated	3	2	2	2	3
40 Gy	9. ONKO 22	Irradiated	3	2	2	2	3
40 Gy	9. ONKO 23	Irradiated	2	1	1	1	3
40 Gy	9. ONKO 24	Irradiated	4	3	2	3	3
40 Gy	9. ONKO 25	Irradiated	1	1	1	1	3
50 Gy	10. ONKO 1	Control	1	1	1	1	4
50 Gy	10. ONKO 2	Control	1	1	1	1	4
50 Gy	10. ONKO 3	Control	1	1	1	1	4
50 Gy	10. ONKO 4	Control	1	1	1	1	4
50 Gy	10. ONKO 5	Control	1	1	1	1	4
50 Gy	10. ONKO 6	Irradiated	1	1	1	1	4
50 Gy	10. ONKO 7	Irradiated	1	1	1	1	4
50 Gy	10. ONKO 8	Irradiated	2	2	2	1	4
50 Gy	10. ONKO 9	Irradiated	4	2	3	3	4
50 Gy	10. ONKO 10	Irradiated	4	3	2	3	4
60 Gy	11. ONKO 1	Control	1	1	1	1	5
60 Gy	11. ONKO 2	Control	1	1	1	1	5
60 Gy	11. ONKO 3	Control	1	1	1	1	5
60 Gy	11. ONKO 4	Control	1	1	1	1	5
60 Gy	11. ONKO 5	Control	1	1	1	1	5
60 Gy	11. ONKO 6	Control	1	1	1	1	5
60 Gy	11. ONKO 7	Irradiated	1	1	1	1	5
60 Gy	11. ONKO 8	Irradiated	3	4	2	3	5
60 Gy	11. ONKO 9	Irradiated	4	4	2	3	5
60 Gy	11. ONKO 10	Irradiated	2	3	2	2	5
60 Gy	11. ONKO 11	Irradiated	4	3	3	4	5
60 Gy	11. ONKO 12	Irradiated	3	4	2	1	5

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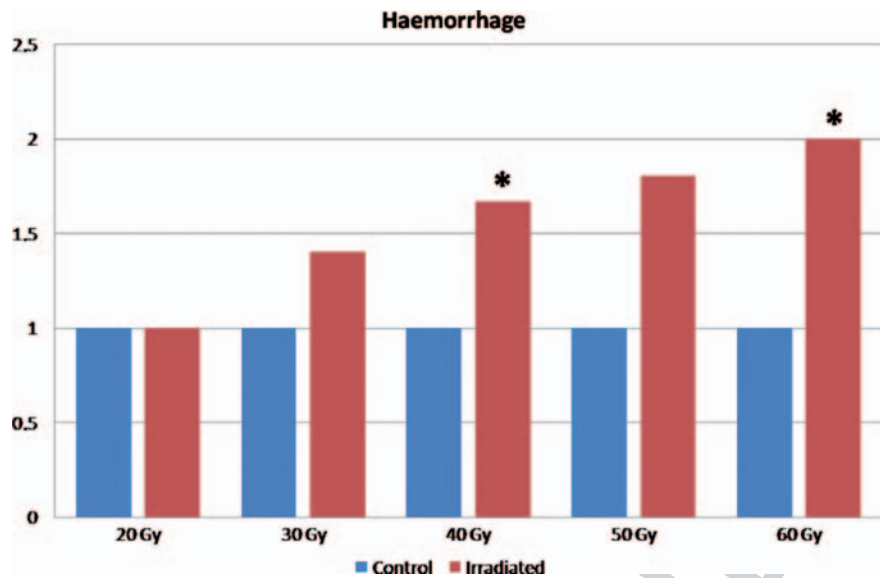




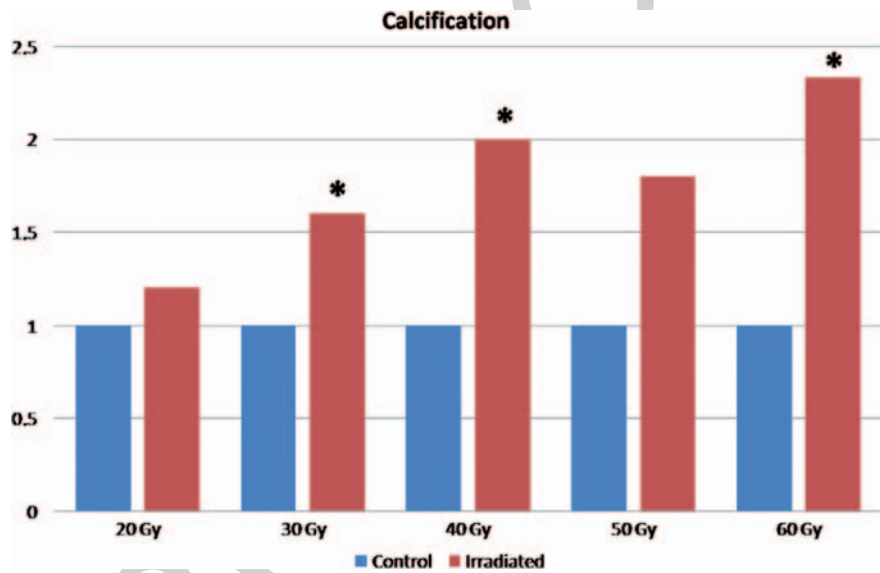
Supplementary Figure 2. Levels of necrosis after different doses of brain irradiation. The ratio of necrotic cells increased significantly after 40 Gy brain irradiation. Data analysis was performed with a statistical software package (StatView 4.54, Abacus Concepts Inc., Berkeley, CA, USA), using analysis of variance (ANOVA) and the Fisher PLSD *post hoc* test. \* $p < 0.05$ .



Supplementary Figure 3. Macrophage density after different doses of brain irradiation. \* $p < 0.05$ .



Supplementary Figure 4. The extent of haemorrhage after different doses of brain irradiation. \* $p < 0.05$ .



Supplementary Figure 5. Signs of calcification after different doses of brain irradiation. \* $p < 0.05$ .