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Author(s):
Abderrafi Benotmane, Mieke Verslegers, Pier Giorgio Mastroberardino, Simonetta Pazzaglia, Soile Tapio, Géza Sáfrány, Victòria Linares Vidal, Per Eriksson, Dimitry Bazyka, Zacharias Scouras, Mike Hawkins, Florent de Vathaire, Nathalie Impens

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1. Introduction

Up to now, the direct effects of ionizing radiation (IR) on the central nervous system remain elusive and are subject to many debates and uncertainties, especially concerning low doses of irradiation (LD-IR). In the context of the FP7 CEREBRAD (Cognitive and Cerebrovascular Effects Induced by Low Dose Ionizing Radiation, grant agreement n°295552) project, we set the stage to answer these questions by means of two approaches: (1) a direct health assessment through epidemiological studies on exposed individuals and (2) an investigation of dose-dependent and radiation-type dependent biological effects using a mouse model. Furthermore, to correctly inform on the risk estimates, we compared internal and external exposure paradigms and evaluated a possible synergistic effect of radiation with other environmental pollutants. This multidisciplinary approach was achieved by the joint effort of a European consortium including radiobiologists, epidemiologists, neurobiologists, bio-informaticians, paediatricians and dosimetrists. Finally, and importantly, our obtained results were shared with other researchers outside the radiobiology field, with stakeholders and with the general public via workshops, peer-reviewed publications, the creation of a website (http://www.cerebrad-fp7.eu/) and the organization of training courses and student's days.

2. Human data

2.1. Cerebrovascular effects of low doses

We set up a case-control study, including 233 cases of strokes having occurred 5 years or more following a childhood cancer and 233 controls matched on cohort, gender, age and date of childhood cancer, and length of follow-up. We performed very detailed radiation dose estimation in any structure of the brain and in cerebral arteries In a linear model, the EOR of stroke, all types together, per Gy of average radiation dose to the cerebral arteries, was equal to EOR/Gy=0.49 (95%CI: 0.22 to 1.17). To add an exponential or a quadratic term did not improve the fit of the data. The radiation dose received to brain structure other than brain arteries did not play any role.
Our findings strongly differed according to the type, ischemic or hemorrhagic of the cerebrovascular diseases. When considering hemorrhagic strokes, an exponential model fitted better the data. Therefore the risk due to low doses was low (EOR/1GY=0.13 (95%CI : 0.07 to 0.21). At the opposite, when considering ischemic strokes, a linear (negative) exponential model was the best model. In this model the linear for low dose was very high: 2.64 (95%CI: 0.39 ; 17.18) (for more detail see D.2.2).

The EOR/1Gy we evidenced for cerebrovascular diseases, considered together (EOR/Gy=0.49 (95%CI: 0.22 to 1.17)) is coherent with the ones observed in most of the other studies. In Hiroshima Nagasaki survivors, in whom the EOR/1Gy was equal to 0.09

(95%CI: 0.01 to 0.17) when considering stroke as underlying cause of death and EOR/1Gy=0.12 (95%CI: 0.05 to 0.19) when considering stroke as underlying or contributing cause of death (Shimizu, Kodama et al. 2010), but, this cohort the EOR/1GY was equal to 0.36 in survivors who were less than 10 years old at time of atomic bomb (Shimizu, Kodama et al. 2010), what was the age of most of the children at time of radiotherapy in our cohort. In a meta-analysis of several cohort studies, including the international nuclear workers study and the Hiroshima-Nagaski cohort, the EOR/1Gy has been estimated to 0.27 (95%CI:0.20 to 0.34) for stroke (Little, Tawn et al. 2010, Little, Azizova et al. 2012). Lastly, in Mayak workers, the EOR/1GY for external radiation has recently been estimated as being 0.33 (95C%I: 0.19 to 0.50) (Simonetto, Schollnberger et al. 2015).

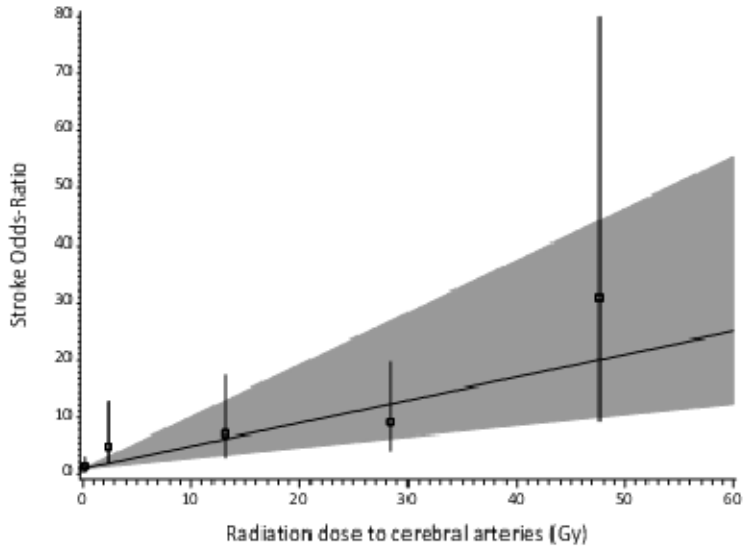


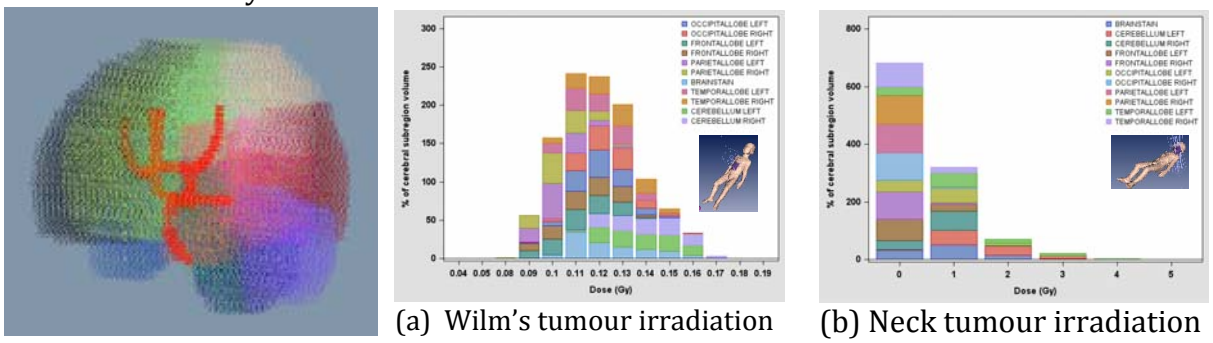
Figure 1: Odds-ratio of stroke (all types together) as a function of the average radiation dose received to the cerebral arteries: observed values and linear model

Our results concerning ischemic strokes have to be considered carefully because of the relatively small number of cases (n=97). Nevertheless, the very high EOR/1Gy we evidenced for low doses, 2.64 (95%CI: 0.39 ; 17.18), could be of importance if verified. At our knowledge, up to now, no other study focused ischemic strokes.

In our study, we only evidence a risk of radiation dose to the cerebral arteries, and not to other brain structures or organs. In particular, we did not evidence a role of the radiation dose received to the kidneys, which is known to induce hypertension. This finding is coherent with the one of the cerebrovascular disease mortality study previously published by IGR/INSERM team (Haddy, Mousannif et al. 2011).

All these results are nevertheless based on average radiation dose to the cerebral arteries. It has to consider that the very strong gradients of dose near to borders of the radiation therapy fields, have as a consequence a very strong heterogeneity of dose within the cerebral arteries, whatever the average radiation dose. Therefore the results of any analysis based on only one parameter of the dose distribution within cerebral arteries (mean, median mode, minimal…) are strongly dependent of the dose distribution, and may not be extrapolated, at all, to uniform irradiation (for more detail see D.2.1). Further analyses will be performed using methods in course of development, able to take into account the distribution of the radiation dose within the cerebral

arteries, rather than only one parameter. These methods will include functional statistics and analyses of isovolumes.



(a) Wilm's tumour irradiation (b) Neck tumour irradiation

Figure 2: Left image: 3D representation of the voxels available at brain level for the calculation of the brain sub- structures doses distributions in the case of a 17 years aged male patient. Right graphs -Dose distribution to cerebrovascular sub regions in the cases of (a) radiotherapy for a Wilm's tumour and (b) radiotherapy for a tumour located at the neck region. In the figure 3.b, the value 0 on the x axis means less than 1 Gy.

2.2. Cognitive effects of low doses

2.2.1. Medical irradiation during childhood

The study was deliberately designed in a similar way to the study conducted by Hall et al. and Blomstrand et al. to enable comparisons (Hall, Adami et al. 2004, Haddy, Mousannif et al. 2011).

The study cohort consisted of the ANGIO cohort. These subjects were treated in the vast majority before the age of one year. This cohort was established between 1985 and 1995 by IGR/INSERM team to study radiation-induced pathologies (Dondon, de Vathaire et al. 2004, Haddy, Andriamboavonjy et al. 2009, Haddy, Dondon et al. 2010, Haddy, Mousannif et al. 2012). Doses of ionizing radiation received by all organs of the body were estimated for all these children, regardless of the site of the haemangioma.

Topics to be included have been selected to people living in the Ile-de-France region. One hundred sixty-seven individuals whose brain radiation received dose estimates are less than 1 Gy to the brain have been identified. A total to 115 subjects were interviewed, the average age at time of questionnaire tests being 50 (from 42 to 63). Neurocognitive assessments of participating subjects undergo and initial interview based on 7 standerdized questionnaires (for more detail see D.2.3).

The RAVLT test and particularly the "delay recall" task is specific to evaluate the episodic memory. Our finding concerning the role of the maximum brachytherapy dose to the temporal lobes in the RAVLT test scores seems relevant since the episodic memory uses neural networks in the hippocampus and more broadly in the inside of the temporal lobes. Indeed, The hippocampus appears to play a central role in the temporary and more durable storage explicit information related to different cortical structures (Hall, Adami et al. 2004).

The MoCA test involved many cognitive domains (executive function, language, memory) and most of patients lost points in memory task. It could explain the relationship between the temporal dose and the MoCA test score but this test is too general and uses several brain structures to conclude a causal relationship.
A higher total radiation dose (Brachytherapy and X-rays) to the cerebral hemispheres was significantly associated to a lower education (p=0.035). Nevertheless the total radiation dose received in cerebral hemispheres, whatever the structure considered was not significantly linked to any of the neurocognitive test used in our study, at the exception of a near from significant result when evaluating depression based on HAD-D score when considering left hemisphere (Table 2 6).
A higher average radiation dose to cerebral hemisphere was also significantly or nearly significantly associated to a degradation in the value of most of neurocognitive tests we used (Table 2 6, Table 2 7): The FactCog (Perceive cognitive impairments) and HAD-D scores were more degraded with higher average radiation dose to the brain hemispheres than with higher radiation dose to other structures, whereas RALVT Decay recall and MOCA scores were more impacted by average radiation doses to the temporal lobes. Among all dosimetric parameters, no one had a significant correlation with the HAD-A and FactCog (perceived cognitive ability).
For the HAD-D test there was a trend for increasing scores with increasing dose to the thyroid and with the maximum brachytherapy dose to the Hemispheres from thresholds equal to 0.12 Gy and 0.054 respectively. Approximately the same threshold (0.059 Gy) of the radiation dose to the left hemisphere lob is obtained to show a significant increase of the FactCog Perceive cognitive impairments scores. RALVT delay recall scores according the years schooling (threshold = 3 years). The maximum brachytherapy dose to the temporal lobes was also significantly associated to this test scores above 0.054 Gy.

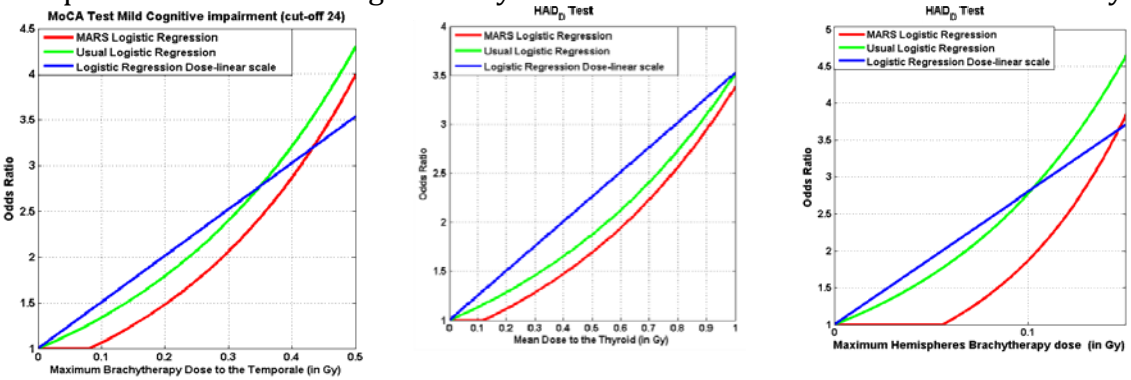


Figure 3: The dose–effect relatioship between the radiation dose to the brain structures and the cognitive test scores via linear (for the Fact-Cog and RAVLT tests) and logistic regression (HAD with cut-off 10 points and MOCA tests with cut-off 24 points).

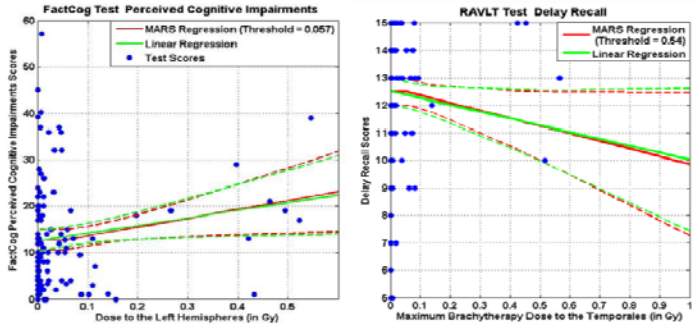


Figure 4: The dose–effect relationship between the radiation dose to the brain structures and the cognitive test scores via linear (for the Fact-Cog and RAVLT tests) and logistic regression (HAD with cut-off 10 points and MOCA tests with cut-off 24 points).

Thyroid tissue is one of the most radiosensitive human tissues and hypothyroidism and hyperthyroidism were previously been associated with depression (Blomstrand, Holmberg et al. 2014) and our results concerning the correlation between the mean thyroid dose and the HAD_D scores could be explained by a possible impact of a low doses on the thyroid hormone regulation functions. Although we found an effect of low-dose radiation on verbal cognitive performance, it should be emphasized that we analysed few subjects (115), warranting caution before making statements about causality. Besides that, children with the largest hemangiomas on the face were likely treated with the highest doses, resulting in the most mutilating scars. This may have influenced their self-esteem or confidence and thereby also their test results and performance in the educational system (for more detail see D.2.3).
More detailed neuropsychological examinations on a larger patient dataset, e.g. analysis of executive functions, would likely have enabled us to draw further conclusions about the nature of the effects of low-dose radiation, if any, on cognitive performance.

2.2.2. Chernobyl studies

Cognitive function is influenced by the radiation dose and age at exposure. The level of subjective distress caused by traumatic event is higher in young adults exposed in utero/ There is some increase of somatoform symptoms and levels of anxiety, insomnia and social dysfunction (for more detail see D.2.4).
Subjects exposed in utero during the check at age of 25–27 years exhibit an excess of the disorders of autonomic nervous system (ICD-10: G90). Neurological microsymptoms as well as neurotic, stress-related and somatoform disorders (F40–F48) dominate.

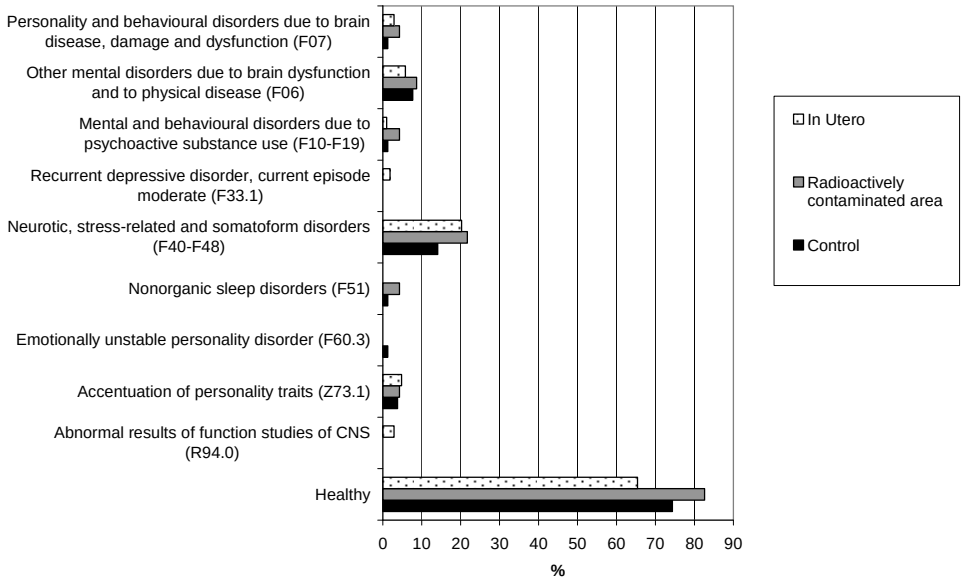


Figure 5: Structure of Mental and behavioural disorders in investigational groups

Subjects exposed to ionizing radiation at adulthood as cleanup workers exhibit symptoms of mild cognitive impairment according to the operational criteria of the

MMSE (mean group scores range =24–27). The cleanup workers have significantly higher level of mental disorders according to the BPRS in dose-related manner, than young adults. This could be the effect of the age and radiation dose. Cleanup workers exposed to doses over 250 mSv and, especially, 500 mSv demonstrate significant cognitive deficit in comparison with exposed below 250 mSv and non-exposed patients. In comparison with previous studies an excess of cognitive dysfunction was significant at doses of 250 mSv and higher (for more detail see D.2.4).

Table 1- Distribution of subjects by group of cognitive dysfunction

COGNITIVE FUNCTION GROUPS	Mini-Mental State Examination (MMSE)	Other criteria	N	%
Normal	28 or more	No cerebrovascular disease, confirmed by neurologist	77	25
Mild Cognitive Impairment (MCI)	24-27	Cerebrovascular disease, confirmed by neurologist	183	60
Dementia (VaD, mainly vascular)	23 or less	Cerebrovascular disease, confirmed by neurologist	46	15
Total			306	100

3. Animal studies

For all animal studies, we used either C57Bl/6 or NMRI mice that were exposed to prenatal irradiation at embryonic day 11 (E11) or to irradiation after birth at postnatal day 10 (PND10) or at postnatal week 10 (W10). Different modes of radiation were used, including whole-body irradiation (pre- and postnatal IR) or local cranial irradiation (postnatal IR). Afterwards, different post-irradiation time points were chosen, ranging from hours to days and months after radiation exposure. Mice were exposed to a range of low to moderate doses of X-rays or gamma-irradiation (Co-60/Cs-137) for external irradiation, and to Cs-137 for internal contamination. Dosimetry simulations using Monte-Carlo codes allowed to evaluate the distribution of the doses in soft tissues and bones to correctly estimate the doses absorbed by the embryonic brain (for more detail see D.3.2)

In the following paragraphs, the effects of LD-IR on the cerebrovascular system and cognition will be discussed, and possible underlying mechanisms are proposed.

3.1 Cerebrovascular diseases

To assess whether prenatal/neonatal radiation exposure exerts an effect on the brain vasculature, we studied the effect of local head irradiation on **blood-brain barrier (BBB) damage and repair**, known to contribute to a proper brain functioning and related to an increased cell ageing. To this end, whole-brain irradiation of animals and

humans has indeed been reported to lead to late delayed vascular damage (Yoshii and Phillips 1982).
Local brain irradiation induced acute endothelial cell activation in the cortex, hippocampus and cerebellum in W10 irradiated mice, and in the cerebellum in PN10 irradiated mice, indicating a higher sensitivity of older mice to radiation induced acute inflammatory reactions compared to young mice. Next, a very important finding was the chronic radiation-induced BBB damage in the hippocampus and cerebellum of W10 IR animals and in the hippocampus of PND10 IR animals, which was induced both by low and high doses. Yet, it should be noted that only a small number of animals could be used for these experiments, leading to a relatively high standard deviation. As such, the trend towards a radiation-induced BBB damage, which is present even 1 month after irradiation, is very promising but will need further confirmation at later time points and with more biological replicates (for more detail see D.3.4).
In any case, our data are of particular importance, since they are corroborated by previous research but also contradict other studies. A radiation-induced blood–brain barrier (BBB) breakdown has been supposed to explain the acute radiation syndrome and the delayed brain radiation injury, but it has been clearly demonstrated only at high doses (Remler, Marcussen et al. 1986). Furthermore, a previous study has shown that 20Gy and 40Gy brain irradiation produced an early permanent increase in BBB permeability in rats, while 10 Gy had no effect at all (Liu, Xiao et al. 2010). Finally, Mao and colleagues demonstrated a time- and dose-dependent loss of the vasculature following gamma and proton radiation exposure in rodents, and decrements in vessel growth were found and could be observed as long as 12 months after a single 8- or 28-Gy exposure (Mao, Archambeau et al. 2003).

3.2 Cognitive defects

3.2.1 Single radiaton exposure

To address persistent effects of external/internal irradiation at the embryonic or early postnatal stage, we subjected animals to a battery of behavioral tests (neuromotor, exploration and learning tests).
In case of ***in utero* radiation** at E11 (external IR: from 0 to 1.0 Gy, or internal IR: from 500 to 50000 Bq/kg), mice externally irradiated with 1 Gy were overall less active when compared to other groups. Further, this group showed an increased sociability and a declined spatial learning. Concerning internal IR, Cesium low/mid activity exposed animals show an improvement in the working memory, spontaneous behavior and less anxiety than control animals (for more detail see D.3.1).

We suggest that in future studies, the range of the activity of internal contamination should be expanded towards a range resulting in doses comparable to the external irradiation doses.

Thus, these data clearly indicate persistent aberrations in cognition and learning as a result of prenatal exposure to irradiation. Even more importantly, as was seen for swim strategies in the Morris water maze, a low dose of external IR (0.1 Gy) already showed difficulties in finding the hidden platform (Figure 1).

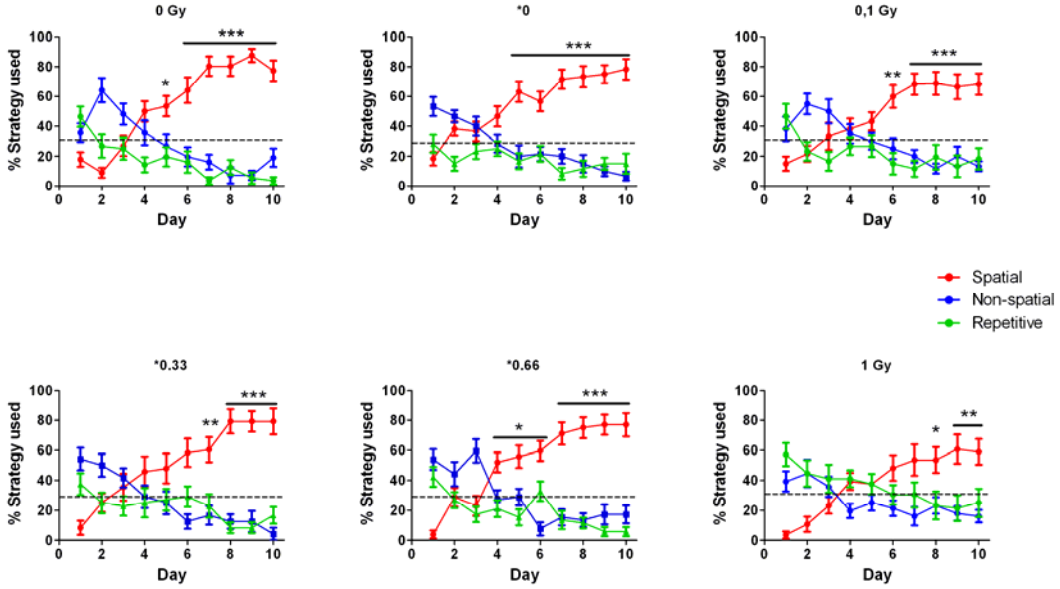


Fig. 6: The use of different strategies during the acquisition phase of the Morris water maze. Swimming patterns were divided into three different strategies: spatial, non-spatial and repetitive. When mice learned to find the hidden platform, they started to use the spatial strategy. Control mice used the spatial strategy predominantly from day 5 on. The other groups showed some delay in this use of the spatial strategy, with exception from the mice irradiated with 0.66 Gy. Data are presented as means ± S.E.M., asterisks indicates significant difference from the chance level (33.33%) (*p < 0.05, **p<0.01, ***p<0.001, two-way RM ANOVA with Bonferroni test for post-hoc comparison).

In contrast, mice **neonatally exposed** to external radiation only displayed differences in behavior starting from 0.5 Gy of gamma-irradiation, while showing a clear dose-response at 1.0 Gy. This discrepancy might be explained by the use of different behavioral paradigms (water maze strategies vs. spontaneous behavior), addressing different aspects of behavior. As such, additional behavioral tests for our groups would be of interest to soundly analyze radiation-induced cognitive aberrations. Importantly, by performing behavioral tests on both males and females, we could rule out a possible gender effect that could be attributable to the observed dose differences. Similarly, differences in behavior between mouse strains (C57Bl6 vs NMRI) could be ruled out.
In case of internal contamination, we again noted a difference in exploratory behavior (less anxiety).

Even though slight differences in dose-responses were observed, we still can conclude that behavior is similarly affected in *in utero* and PND10 exposed animals. Therefore, we need to address this issue of LD-IR induced persistent cognitive effects in our community, to improve health assessment (for more detail see D.3.1).

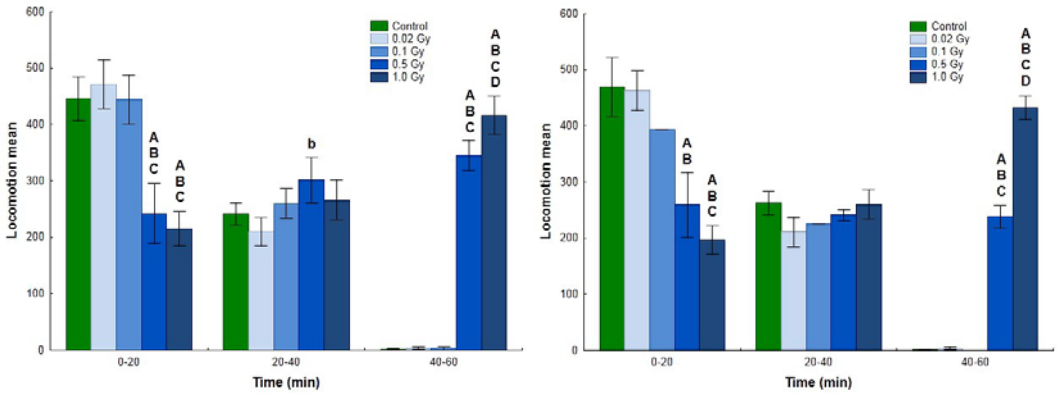


Fig 7: Spontaneous behaviour in 2-month-old C57 bl male mice (up) and 2-month-old C57 bl (Charles River) female mice (down) exposed on PND 10 to a single oral dose of gamma-radiation (0, 0.02, 0.1, 0.5 and 1.0. Statistical analysis, ANOVA with split-plot design and pair-wise testing with Tukey´s test. The height of each bar represents the mean ± SD. A=p≤0.01 vs. control (0 Gy); a=p≤0.05 vs. control; B=p≤0.01 vs. 0.02 Gy; b=p≤0.05 vs. 0.02 Gy; C=p≤0.01 vs. 0.1 Gy; c=p≤0.05 vs. 0.1 Gy; D=p≤0.01 vs. 0.5Gy); d=p≤0.05 vs. 0.5 Gy).

3.2.2 Combined exposure to radiation and toxicants

As a second main aim, based on the high risk for consequences of exposure to IR and toxic agents of the developing nervous system, we characterized the (synergistic) effect of radiation and toxicants such as PBDE, methylmercury, paraquat and nicotine on mouse behavior at the adult age of 2 and 4 months, preceded by irradiation at PND10.

External irradiation

The results obtained within CEREBRAD indicate a synergistically defective spontaneous behavior in IR+PBDE exposed mice, suggestive for an altered cognitive function in adult mice neonatally exposed to gamma irradiation at doses where the sole compounds did not cause any effect. The effects on single exposure are in agreement with earlier published reports on IR (Eriksson, Fischer et al. 2010, Buratovic, Stenerlow et al. 2014) and PBDE99 (Eriksson, Fischer et al. 2006).

In agreement with earlier published work (Fredriksson, Fredriksson et al. 1993), we additionally showed an interaction between IR and 0.4 mg/kg MeHg (Eriksson, Fischer et al. 2010) as well as with paraquat (dose-dependent) and nicotine (for more detail see D.3.5).

Dose-Response curve

In general, we propose a shift in the dose-response curve when such environmental toxicants are combined with IR exposure, resulting in a lowering of the threshold dose of about 300 mGy (Figure 2) (for more detail see D.3.3).

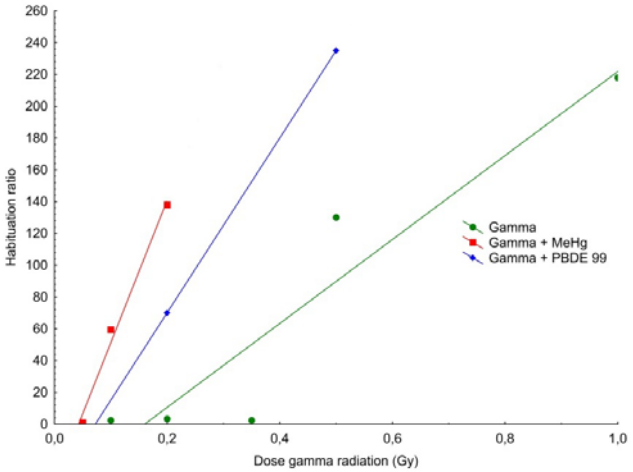


Fig. 8. Habituation capability is the ratio between performance in spontaneous behaviour in a novel home environment taken from period 40-60 min and 0-20 min in 2-month-old NMRI male mice exposed on PND 10 to a single external dose of gamma-radiation (0, 0.02, 0.1, 0.35, 0.5 and 1.0, (green), , a combination dose of gamma radiation and PBDE 99 or a vehicle (20% fat emulsion) , a combination dose of gamma radiation (0, 0.2 and 0.5 Gy) and PBDE 99 (0.8 mg/kg bw) (Blue), a combination dose of gamma radiation (0, 0.05, 0.1 and 0.2 Gy) and MeHg (0.4 mg/kg bw) (Red).

Based on this finding, and since we are living in a mixed environment combining different physical and chemical agents, a threshold theory cannot be adopted. Future research need to focus on combining multiple agents to be more in line with our real life. Additionally, extrapolation of this research to specific diets to investigate life style would emphasize other elements of our modern society that might contribute to radiation risk estimate.

Internal irradiation

A single subcutaneous dose of Cesium-137 was combined with paraquat, bisphenol A, nicotine or MeHg. While paraquat, nicotine or bisphenol A alone increased anxiety, co-exposure of either of those toxicants with lower doses of Cs caused a reversion of this effect. In addition, unexpectedly, Cs and co-exposed groups spent less time searching in the water maze target quadrant, while paraquat alone showed an increased time spent in the target quadrant. Nonetheless, no clear additive or synergistic effect of IR and environmental toxicants could be observed using the current internal contamination paradigm. A more detailed analysis will thus be required to disentangle these remarkable findings, for example by using higher doses of internal contamination (for more detail see D.3.5). For an overview of all cognitive tests performed within CEREBRAD and the main conclusions drawn, we refer to table 1.

Table 2: Overview of all cognitive defects **occurring after external** (EX) and internal (INT) radiation exposure during the prenatal and postnatal period.

		Prenatal exposure			Postnatal exposure		
		Low	***Medium***	***High***	***Low***	***Medium***	***High***
Spatial learning	EX	+	++	+++	ND	ND	ND
	INT	=	=	+	=	=	=
Spontaneous behavior	*EX*	=	=	+	=[b]	+++[abcd]	+++
	INT	ND	ND	ND	ND	ND	ND
Motor function	*EX*	=	=	=	ND	ND	ND
	INT	=	=	=	=	=	=
Exploration/sociability	*EX*	=	+	++	ND	ND	ND
	INT	(+)	(+)	=	+	+	+[bc]
Working memory	*EX*	ND	ND	ND	ND	=[c]	ND
	INT	(+)	(+)	=	=	(+)	=

3.3 Underlying cellular and molecular mechanisms

To explain the observed cerebrovascular and cognitive defects that result from pre/neonatal radiation exposure, we investigated early and late cellular and molecular events that might be at the origin of these anomalies.

3.3.1 Early effects

Neurogenesis & corticogenesis

In the developing neocortex, we noted an impact of prenatal LD-IR on different aspects of brain development as well as on brain cytoarchitecture, as demonstrated by a defective hippocampal neurogenesis and differentiation. Our data also demonstrate that the developing neocortex is, next to the hippocampus, highly susceptible to LD-IR. Further studies will have to be designed to investigate permanent defects in this anatomical region following prenatal irradiation. In any way, these first indications could potentially explain the observed permanent behavioral changes that cannot solely be attributed to hippocampal aberrations.

Early genetic changes after pre- and postnatal radiation that are linked to a deviant neurogenesis and cortical development might be attributed to a p53-mediated DNA damage signaling and apoptosis, which is probably cell-type specific. Besides, we showed a dose-dependent alternative transcription of a shorter isoform for several genes in the irradiated embryonic brain 2 h after X-irradiation, for which p53 was shown to bind the promotor sequence of this short isoform (for more detail see D.4.6). On these premises, we believe that the exact mechanisms explaining LD-IR long-term effects at the organism level can be unraveled only by achieving a better understanding

of the early effects (hours to days) and at the level of the different neuronal populations, of which we provided first important insights.

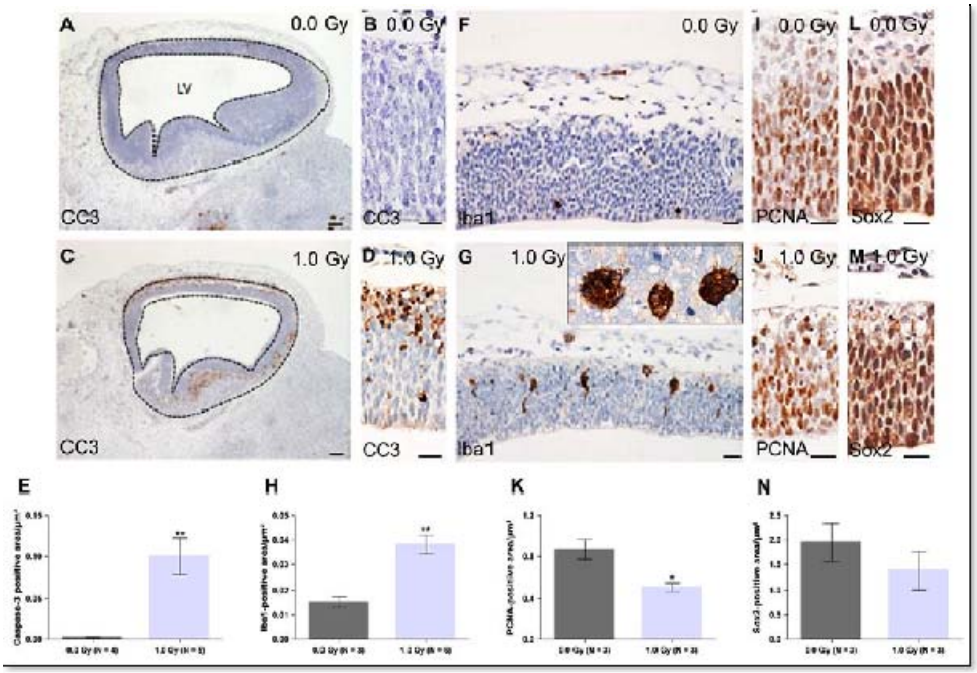


Fig 9: Massive apoptosis, microglial activation and decreased PCNA expression at 24 h postirradiation. Quantification of CC3 (A-D), Iba1 (F,G), PCNA (I,J) and Sox2 (L,M) immunostaining in the embryonic brain of controls (A,B,F,I,L) and 1 Gy exposed mice (C,D,G,J,M) in the region around the lateral ventricle (LV; dashed lines in A and C). B and D are magnifications of the CC3 staining shown in A and C. In the upper right corner of G, an enlargement of Iba1-positive microglial cells which engulf apoptotic nuclei is shown. The quantification indicated a marked increase in apoptosis (E) and in Iba1 expression (H), a decreased PCNA expression (K), and no significant change in Sox2-positive progenitor cells (N) after irradiation. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 for comparison with controls. Scale bar: 20 µm (100 µm in A and C).

In all, multiple developmental processes crucial for the correct wiring and functioning of the brain, such as apoptosis, cell division and cell differentiation, were altered following radiation with low and/or high doses of irradiation.

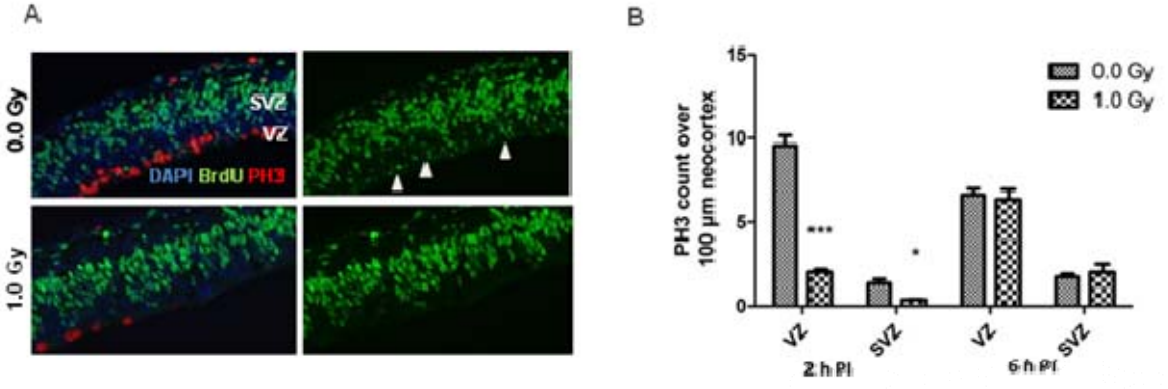


Fig 10: (A) Representative immunostainings for BrdU (green) and PH3 (red), labeling cells in their S-phase and mitotic phase respectively. The neocortex of 0.0 Gy and 1.0 Gy irradiated E11 embryos is shown, 2 h post-irradiation, in which a clear reduction of PH3/BrdU positive cells can be observed in 1.0 Gy irradiated brains at the ventricular lining. (B) Bar graph showing a reduction in mitotically active cells (PH3 positive) in the VZ and SVZ at 2 h post-irradiation, whereas differences were no longer observed at 6 h post-irradiation. VZ = ventricular zone, SVZ = subventricular zone. N = 6. * p<0.05, *** p<0.001.

Yet, more focused research with emphasis on cell-type specific apoptosis and cell cycle arrest, or investigating a possible premature differentiation or a delayed migration will be key to fully comprehend the consequence of LD-IR on brain development.

3.3.2 Late effects

Neurogenesis

Similar as for early effects, we noted an impaired juvenile hippocampal neurogenesis with some defects persisting until 6 months after *in utero* and postnatal radiation exposure. Intriguingly, in animals irradiated at 10 weeks of age and analyzed 6 months after irradiation, the 0.1 Gy dose induced a significant increase in the number of neurons, pointing at potential hormetic mechanisms. Whether the changes elicited by such low doses are potentially detrimental, for instance by synergizing with other environmental or genetic factors, remains to be addressed (for more detail see D.4.2, D.4.3).

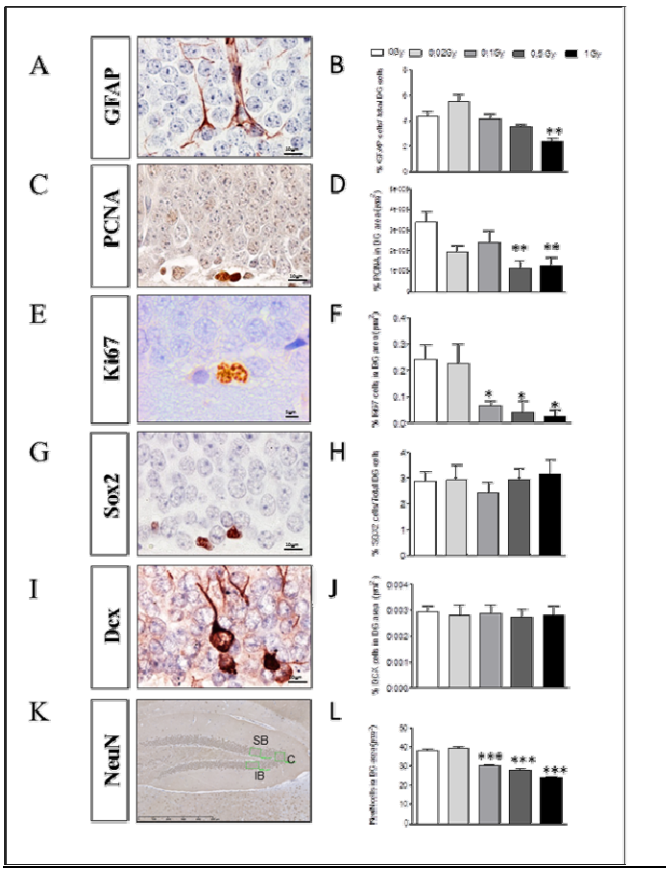


Figure 11: Radiation-induced alterations in the cellular composition of the DG. Expression and quantification of GFAP (A, B), PCNA (C, D), Ki67 (E, F), Sox2 (G, H) Dcx (I, J) and NeuN (K, L).

Chronic inflammation

Nowadays, there is a growing awareness that even low doses of irradiation can cause long-term defects and possibly also a chronic elevation of inflammation. We hypothesize that this neuroinflammatory milieu contributes to the observed neurocognitive defects, including inhibition of hippocampal neurogenesis and synaptic function (see further).
At 6 months after **external irradiation**, we found that apoptosis and microglia activation only occurred following 2 Gy IR and only in PND10 irradiated mice in comparison to W10 irradiated mice, suggesting a higher sensitivity of the young brain to radiation effects. In addition, we could suggest a radiation-induced neuroinflammation based on a persistent astrogliosis, again only after high dose irradiation at PND10 but not at W10. Finally, and in contradiction to the aforementioned, we could note a change in the microvessel length of the hippocampus only in W10 irradiated animals. Furthermore, even though radiation was shown to induce inflammation in irradiated animals, no synergistic effect was noted when animals were predisposed to inflammation (EAE mice). On the contrary, the effects were antagonistic, since irradiation decreased inflammation-mediated cytokine upregulation both after low (0.1 Gy) and high (2 Gy) radiation doses, at least for CD54 (for more detail see D.4.2, D.4.3).
At 2 and 4 months after **internal Cs exposure** at E11, only a few cytokines were shown to be differently expressed in the hippocampus, while BDNF expression showed the strongest increase at 20 weeks post-irradiation. Similarly, for PND10 irradiated groups, a few trends were apparent, especially for BDNF expression at 40 weeks post-irradiation, but a high standard deviation prevented us from making sound conclusions.

Neuronal plasticity and communication

Based on our proteomics studies, we discovered a persistent molecular fingerprint in the brain after neonatal irradiation. This fingerprint included both proteins and miRNAs that were always found to be deregulated in a similar manner in different mouse strains (NMRI and C57Bl/6), in both genders and in both the hippocampus and cortex. This finding was clearly observed after a dose of 0.5 Gy but not after a low dose of 0.1 Gy. The molecular components of this fingerprint included Rac1 (always downregulated) and cofilin (always upregulated), which are key players in the Rac1-Cofilin pathway. The latter is essential for neuronal spine morphology and maturation, hippocampal synaptic plasticity and spatial learning, and thus correlates well with the aberrant learning observed in adult mice irradiated at E11 or PND10 (for more detail see D.4.6).

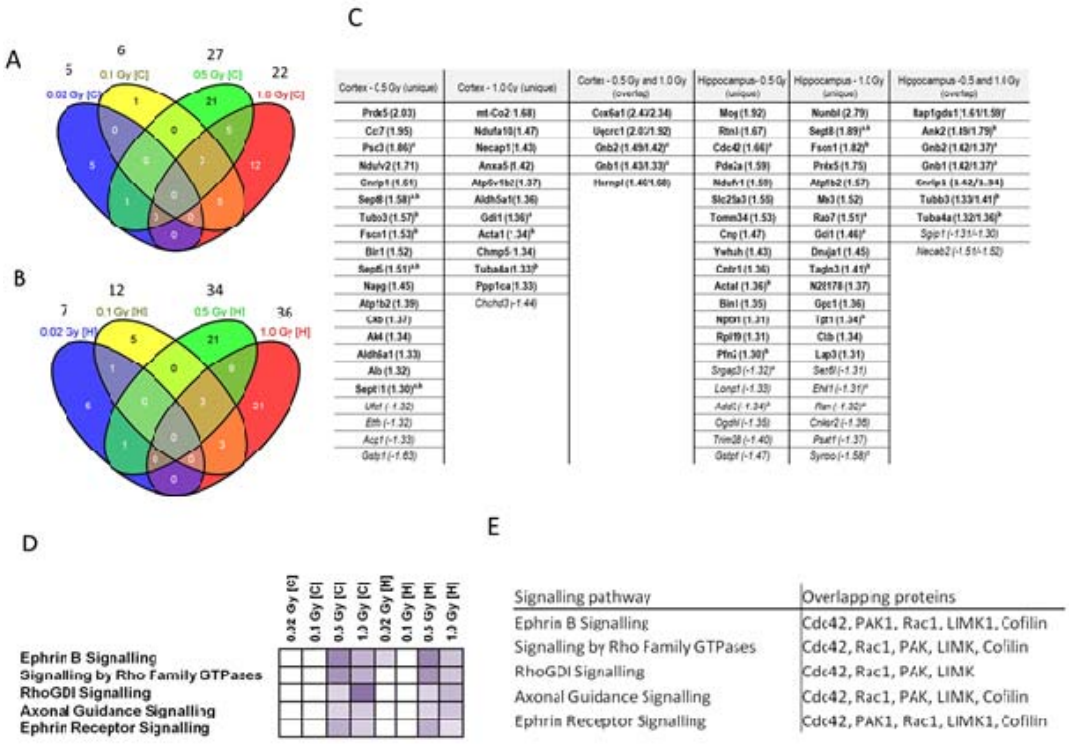


Figure 12. Analysis of signalling pathways from proteomic experiments.
Venn diagrams of deregulated proteins from cortex [C] (A) and hippocampus [H] (B) exposed to 0.02 Gy, 0.1 Gy, 0.5 Gy and 1.0 Gy from global proteomics approach are shown. H: n=4; C: n=5. The number above each dose shows the total number of deregulated proteins at this dose.

Brain morphology

We investigated brain regional differences via a voxel-based MRI morphometric approach. From this, we revealed a clear decline in total brain volume, accompanied by enlarged ventricles and a relative decrease in volume of the prefrontal cortex in 1.0 Gy irradiated animals, which indeed indicates a correlation with the Morris water maze results. Yet, other factors might be in play, since behaviour was also affected at doses below 1.0 Gy. As such, additional analyses need to be performed to unveil all causes leading to an aberrant learning and cognition, e.g. by focusing on other brain regions or on more subtle effects as compared to a reduction in brain size (for more detail see D.4.2).

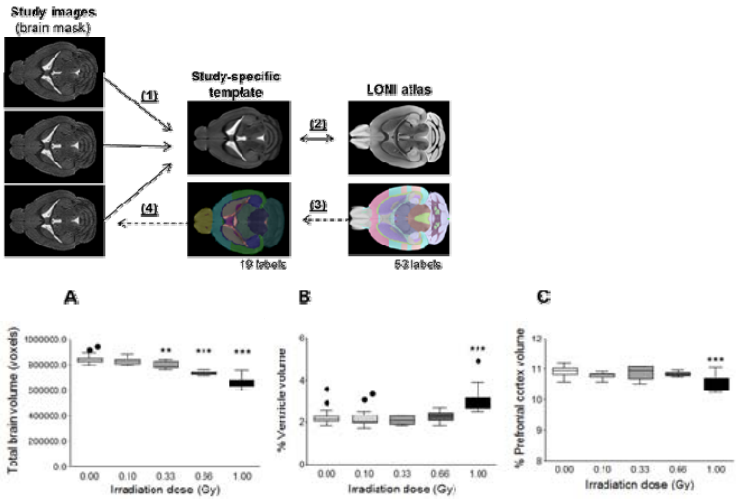


Figure 13: Brain weight changes induced by in utero exposure to radiation. (A) The total brain volume was decreased significantly from a dose of 0.33 Gy onwards. (B) When corrected for total brain volume, the volume of the ventricles was increased in the animals irradiated with the highest dose. (C) The volume in total brain volume was indeed not homogeneously since we also found a decrease in relative frontal cortex volume in 1.00 Gy-exposed mice as compared to controls.

Mitochondrial function

Mitochondrial function and basal respiration in particular, was shown to be altered in whole body irradiated mice, 4 weeks post-IR. At 24 weeks post-IR, however, respiration at higher doses returned to normal levels and alterations were only significant in the 0.1 Gy exposed group. Remarkably, in this low dose irradiated group, respiration was found to be higher than in controls, which is suggestive for hormetic mechanisms activated by very low radiation doses.

Irradiation also altered the neuronal oxido-reductive state and its associated signaling, and effects were again detectable at the lowest 0.1 Gy dose. Interestingly, the dose-response relationship between mitochondrial respiration, oxido-reductive state, or signaling and LD-IR differed between animals analyzed 4 or 24 weeks after irradiation, and was only found to be linear in the former case. These data are consistent with a threshold model in which an adaptive response is activated only when factors signaling the perturbation surpass a certain edge (illustrated in Figure 3). Unambigious validation of this model, however, would require additional and extensive research, for instance monitoring the different parameters (e.g. mitochondrial function, oxido-reductive state, signaling) in real-time with advanced imaging methods (for more detail see D.4.3).

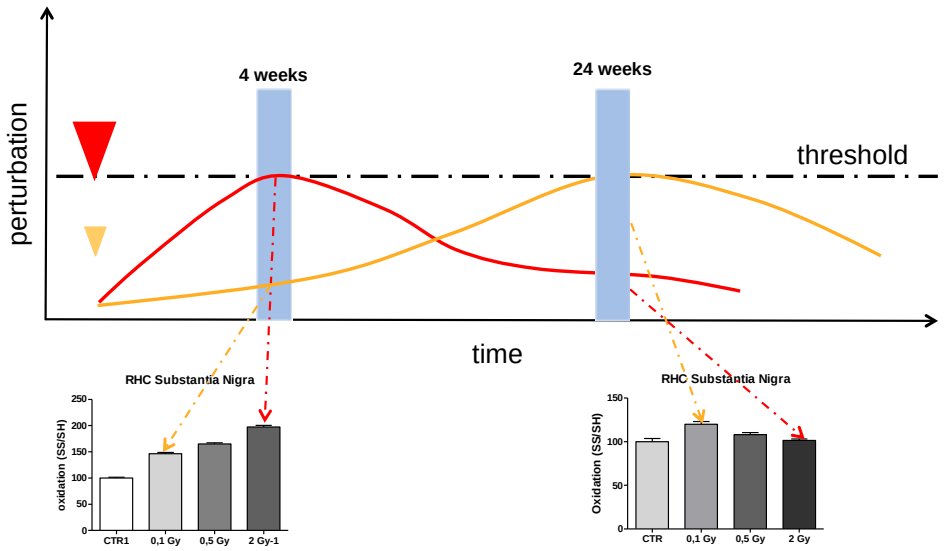


Figure 14. The presented data are consistent with a threshold model, in which an adaptive response is activate only when a perturbation threshold has been surpassed. The kinetic of the perturbation is faster if the insult is stronger (red arrowhead, which might represent a higher LD-IR dose) and will result in swifter achievement of the threshold (blue-shaded area on the left). Once the threshold is reached, the adaptive response is activated and perturbation is consequently reduced. When the insult is weaker (yellow arrowhead), kinetic is slower and longer time will be necessary to activate the adaptive response. This model explains why, at later time points (blue-shaded area on the right), alterations may be more pronounced in samples exposed to lower ionizing radiation.

Experiments to investigate mitochondrial function and oxido-reductive changes in locally irradiated mice confirmed a different susceptibility to LD-IR at the anatomical level, being that the cerebellum is less sensitive than the cortex or the hippocampus. Alterations were often not linear in time and presented biphasic trends. Importantly, also in this case, the 0.1 Gy dose was sufficient to induce radiation-induced defects (for a general overview of changes in the cortex, cerebellum and hippocampus, see table below).

Table 3- Mitochondrial function and oxido-reductive changes in cortex of locally irradiated mice

Table 1: Radiation induced changes in the cortex

	Irradiated at the age of 10 weeks								Irradiated at the age of 10 days							
	day 1		day 7		1 month		6 months		day 1		day 7		1 month		6 months	
	0.1Gy	2Gy	0.1Gy	2Gy	0.1Gy	2Gy	0.1Gy	2Gy	0.1Gy	2Gy	0.1Gy	2Gy	0.1Gy	2Gy	0.1Gy	2Gy
Complex I	=	↓	↓	↑	↓	↓	=	↓	↑↑	↑	=	=	=	↓↓	↓↓	↓↓
Complex II	=	=	↓	=	=	↑	↓	↓	↑	=	=	↑	↑	↓	=	↓
Complex III	=	↓↓	=	↓	↓	↓↓	↓	↓↓	=	=	↓	=	=	=	↓↓	↓↓
Complex IV	=	=	=	↓	=	=	=	=	=	↓	=	=	↓	↓	=	↓
Superoxid anion	↑	↑	↓	↓	↓	↑	=	=	↑	↑	↑	↑	=	=	=	↓
Protein carbonylation	↓	↓	↑	↑	=	↓	=	=	=	↓↓↓	↓	↓↓	=	↓↓	↓	↓↓
Lipid peroxidation					↓	=	↓	=					=	↓	=	↓
Total antioxidant capacity					=	↓	=	↑					↓	↓	↑	↑
SOD	=	=	=	=	=	=	=	=	=	↑↑↑	=	=	=	↑	=	=

Of note, the mode of irradiation seems to influence the outcome, given that the results observed with total body irradiation do not fully overlap with those observed with local cranial irradiation. For instance, 24 weeks after 2 Gy irradiation, protein carbonylation was significantly decreased in animals subjected to local irradiation, while no significant differences could be observed at the same time point in animals that had been exposed to whole-body irradiation. The same applies to mitochondrial analyses, even though local and whole body irradiated samples have been generated and analyzed in different laboratories. Differences observed in total- versus local-irradiation might be attributable to systemic effects generated by LD-ID peripherally and related to processes such as inflammation and/or hormonal changes that might ultimately have consequences to the brain for more detail see D.4.3).

4. General conclusions

The consequences of LD-IR on the brain are dimly understood. CEREBRAD provided extensive information unraveling molecular and functional details of LD-IR effects.
Our results revealed obvious effects of LD-IR on the brain at multiple levels and opened prospective avenues for further exploration. In general, we could observe a clear dose-dependent effect and could unveil different anomalies induced by the lowest X-ray dose studied (0.1 Gy) in terms of cognition, cell death and neurogenesis. Of interest, next to the administered dose, also the age at which exposure occurs strongly influences radiation effects, which are mostly exacerbated when irradiation is induced at earlier stages. Yet, this is sometimes highly dependent on the brain region of interest, for example the cerebellum which is more vulnerable to neonatal radiation as compared to prenatal radiation.

Of interest for our studies, the transcriptional and protein landscape emerged from microarray and proteomics studies confirmed alterations in processes such as inflammation, mitochondrial electron transport, neuron development and axonogenesis. In addition, we could identify several new p53 target genes that are induced in a dose-dependent, transient manner at 2 h after prenatal radiation exposure. However, while the early responses are easy to decipher, the long-term effects are more difficult to interpret. This is most likely related to the nature of the insult (i.e. acute radiation), which results in a strong, yet transient biological effect (DNA damage), and in turn activates a multitude of downstream biological processes. Furthermore, the possibility of radiation-induced epigenetic changes is a challenging field of research, and needs to be thoroughly investigated. Besides, further and dedicated studies eventually involving animal models with mutations in pathways of interest that could synergize with low doses of irradiation will be required to better understand the impact of our findings on human health and disease.

The experimental data obtained within CEREBRAD were organized, integrated and elaborated with an extraordinary bioinformatics approach that, to our best knowledge, is unprecedented in the field of radiation biology (for more detail see D.4.1, D.4.4, D.4.5). One aim was to classify and integrate functional, molecular and behavioral data in a custom made computational platform, which we called Brain Radiation Information Data Exchange (BRIDE). BRIDE is extremely rich as well as quite challenging to explore for pathways inference in the context of particular phenotypes under study, such as cognitive deficits in adult, prenatally exposed mice. Obviously, the BRIDE platform can be further improved to become an established data mining approach among radiation biologists. One potential future plan is to provide a text-mining suite for literature scans, so that the contents can be maintained current, with minimal effort by semi-automated monitoring of the literature. Furthermore, importantly, our systems biology approach confirmed that exposure with 0.1 Gy alters biological processes in the brain. Hereto, it is essential to reiterate that, at present, it is unclear whether these changes are detrimental and further focused studies will be critical to conclusively address this issue (for more detail see D.4.1, D.4.4, D.4.5)..

5. Future perspectives

At this point, numerous questions remain open and more research will be essential in several areas to complement the CEREBRAD findings and answer society's question about cognitive and cerebrovascular risks of LD-IR.

In regard to the animal studies, the following issues need to be addressed for a **future**

approach in LD-IR research

- It is imperative to determine whether the effects elicited by LD-IR are detrimental. This includes anomalies in processes that are fundamental for a proper brain functioning and that might lead to pathology when deranged (e.g. mitochondrial respiration).
- Future studies involving models for neurodegenerative diseases will be indispensable to fully estimate risks of LD-IR during infancy in adulthood and to provide accurate directions to the public.
- The threshold model requires further validation. Addressing the mechanisms activating an adaptive response and its kinetic is extremely important for neuroprotection. An adaptive response, in fact, could be preventively elicited to mitigate undesired off-target effects of medial approaches involving the use of radiation or to protect individuals in the case of a radiation environmental emergency. Studies to identify the specific signaling molecules translating radiation-induced damage into a concerted response are therefore essential.
- Is hormesis really happening? Our data on mitochondrial function and neurogenesis suggest that this might indeed be the case, and focused

experiments to test conclusively this possibility are inescapable. It will also be important to determine the specific doses and conditions eliciting putative hormesis.

- CEREBRAD analyzed molecular and cellular changes up to 24 weeks after irradiation. The presence of alterations at this time point strongly suggest that LD-IR might influence natural ageing and lays foundation for studies in aged mice. However, it is still unclear whether LD-IR could promote senescence and, eventually, in which neuronal cell type.
- The molecular and cellular findings are in high correlation with the observed cognitive deficits in pre- and neonatally irradiated mice. In particular, the defective cortical development that was observed together with a disturbed hippocampal neurogenesis nicely links to the decreased thickness of the prefrontal cortex at the long term. This thus urges for more experiments investigating higher cognitive functions related to the prefrontal cortex in irradiated animals.
- Are the observed early radiation-induced effects the only contributors to the persistent cognitive decline and inflammation? To address this matter, a wider investigation of different brain regions is desired, preferably accompanied by the use of transgenic animals deficient for certain brain developmental factors or cytokines.
- Our systems biology results require experimental validation. Is the identified signature specific and sufficient to reveal LD-IR exposure? The bioinformatics platform should be classified as an infrastructure to be expanded to include more experimental evidence. Additionally, further investment would be necessary to fully develop the usefulness of this tool for the community involved in radiation biology research, and to make it sustainable in the frame of CONCERT.
- Finally, future research need to focus on combining multiple agents to be more in line with our real life. Additionally, extrapolation of this research to specific diets to investigate life style would emphasize other elements of our modern society that might contribute to radiation risk estimate.

In general, our results provide critical information for the EU citizens and highlight the importance of further experiments in the same line of research. Identifying neuropathological signs after LD-IR is particularly relevant when framed in the context of medical imaging, which relies on LD-IR for diagnostic purposes. Ensuring that such diagnostic procedures do not pose substantial neurological risks for the patients at later life stages therefore constitutes a fundamental public health issue. This issue becomes even more compelling when approached from the perspective of ageing, which has become an emergency in the EU and in the advanced economies in general. Indeed, in a society where life expectancy is steadily increasing, addressing the potential consequences of LD-IR exposure in young life on elders has a significant societal and

economic relevance. Moreover, we are obliged to inform the community about the additional risks of IR exposure when predisposed to other environmental toxicants, for example nicotine, which are often underestimated and which need our focused attention.

In conclusion, CEREBRAD generated solid evidence and paved the road for future studies in the field.

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