**Title** Complex long-term effects of radiation on adult mouse behaviour

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**Running Title:** Complex long-term effects on mouse behaviour

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**Garrett, L. et al. Complex long-term effects of radiation on adult mouse behaviour. *Radiat. Res.***


## Abstract

We have shown previously that a single radiation event (0.063, 0.125 or 0.5 Gy, 0.063 Gy/min) in adult mice (age: 10 weeks) can have delayed dose-dependent effects on locomotor behaviour 18 months post irradiation (p.i.). The highest dose (0.5 Gy) reduced, whereas the lowest dose (0.063 Gy) increased locomotor activity at older age independent of sex or genotype. The present study explored if higher doses administered at a higher dose rate (0.5, 1 or 2 Gy, 0.3 Gy/min) at the same age (10 weeks) cause stronger or earlier effects on a range of behaviours, including locomotion, anxiety, sensorimotor and cognitive behaviour.

There were clear dose-dependent effects on spontaneous locomotor and exploratory activity, anxiety-related behaviour, body weight and affiliative social behaviour independent of sex or genotype of wildtype and *Ercc2*$^{S737P}$ heterozygous mice on a mixed C57BL/6JG and C3HeB/FeJ background. In addition, smaller genotype- and dose-dependent radiation effects on working memory were evident in males, but not in females. The strongest dose-dependent radiation effects were present 4 months p.i., but only effects on affiliative social behaviours persisted until 12 months p.i. The observed radiation-induced behavioural changes were not related to alterations in the eye lens, as 4 months p.i. anterior and posterior parts of the lens were still normal (see Pawliczek et al., this issue). Overall, we did not find any sensitizing effect of the mutation towards radiation effects *in vivo*.

## Introduction

Incidence and severity of radiation effects increase with absorbed dose but radiation effects also depend on the quality or type of radiation, dose rate, tissue and other factors such as the age or sex of the individual[1-4]. For many medical purposes γ-radiation is used[5], and electromagnetic radiation such as X-rays or γ-rays induces sparse ionization events, where energy is exponentially absorbed by tissues, which can induce primary ionization events with low-energy scattered electrons[3]. The brain and in particular adult neurogenesis that still occurs in the subventricular zone and in the subgranular zone of the hippocampus are highly sensitive towards ionizing radiation as shown for doses of 2-10 Gy[6]. Convergent data from animal and human studies indicate that irradiation of the brain with high doses as used in radiotherapy can result in a dynamic and multiphasic process of brain injury occurring over time, and in cognitive and psychiatric impairments that can be present many years after completion of treatment[7]. Thus, high doses of ionizing radiation can affect mood, learning, memory and olfaction, which in turn are biomarkers for early stages of neurodegenerative diseases. Oxidative stress and inflammatory processes are considered the relevant factors for delayed radiation effects, and they also play a role in neurodegenerative diseases[2,8,9].

However, it is less clear whether low to moderate radiation dose exposure e.g. due to occupation or occasional diagnostic or therapeutic interventions may also have long-term negative effects on brain and behaviour, especially when applied in adulthood and not at young age when developmental processes are not finished yet and mitotic activity is still high. There is no epidemiological evidence for detrimental health effects below 100 mSv and several studies instead suggest beneficial effects of very low doses[8,10-12]. But above this dose exposure could, in principle, increase the risk of adverse effects in the long term. A recent

study in Russian Mayak workers found a linear association between cumulative γ-dose and the incidence of Parkinson's disease (PD), indicating that occupational exposure increased the risk to develop PD later in life[13]. Molecular and cellular mechanisms in the low-dose range are often different from those in the high-dose range, and there is emerging evidence that even doses used for diagnostic purposes can potentially contribute to late cognitive impairments especially when applied to the hippocampus and at young age[9]. While adults are the least radiosensitive age group as it has been shown that radiosensitivity is higher in infants, juveniles and the elderly than in young adults[2,14,15], other factors like genetic predisposition[16,17] or sex[18,19] may also influence radiosensitivity and thus the risk for adverse effects. Many studies have been conducted using animal models, but most of them used either young animals before adulthood or radiation doses above 2 Gy, and the period of observation for delayed radiation effects on brain or behaviour was at maximum 6 or 12 months[20].

To increase the available data on long-term effects of low to moderate γ-radiation doses applied in adulthood, we previously explored the effects of a single dose of either 0.063, 0.125 or 0.5 Gy ($^{60}$Co, dose rate 0.063 Gy/min) administered at 10 weeks of age in wildtype (wt) and phenotypically healthy heterozygous *Ercc2*$^{S737P}$ (het) mice of both sexes up to 24 months p.i.[21-23]. Heterozygous *Ercc2*$^{S737P}$ mice were used to address genetic predisposition to radiosensitivity *in vivo* because their lymphocytes demonstrated an increased sensitivity to radiation-induced DNA damage *in vitro*[16]. We found dose-dependent effects, with the dose below 100 mGy (0.063 Gy) having delayed protective effects and the 0.5 Gy dose inducing late-onset negative effects on locomotor behaviour and microglia irrespective of sex or genotype[23]. Although visual abilities of mice can affect their locomotor behaviour[24], those findings were unrelated to vision since the animals did not develop radiation-induced

cataracts at theses doses[21]. Here we present the results of a new study investigating if higher doses of 0.5, 1 and 2 Gy administered with a higher dose rate (0.3 Gy/min) at the same age and to the same kind of mice induced stronger or earlier effects on a range of behaviours that are known to be affected by alterations in adult neurogenesis or hippocampal function. Spontaneous locomotor, exploratory and anxiety-related behaviour (Open Field Test), Sensorimotor function (Acoustic startle response and its prepulse inhibition), spatial working memory (Spontaneous alternation in the Y-Maze) and olfactory, social memory (Social Discrimination Test) were assessed 4, 12 and 18 months p.i. This study was conducted as part of the interdisciplinary LDLensRad project[25] (see also Pawliczek et al., this issue).

## Materials and Methods

### Animals

In total 323 mice were used for this study, bred and kept under specific pathogen-free conditions at the Helmholtz Center Munich. Mice of the same sex were group-housed and kept on a 12/12-hour dark-light cycle (lights on at 6 am) in a temperature (22 - 24°C) and humidity controlled (50 – 60%) environment and provided ad libitum standard chow and water. Wildtype (wt) mice were F1 hybrids of a C57BL/6JG female and a C3HeB/FeJ male, and heterozygous mutants (het) were F1 hybrids of a wild-type C57BL/6JG mother and a homozygous *Ercc2*$^{S737P/S737P}$ father on a C3HeB/FeJ background. This mixed genetic background overcomes the recessive retinal degeneration caused by a mutation in the *Pde6b* gene[26] present in the C3H strain background that the recessive *Ercc2*$^{S737P}$ mutation was bred on[16], resulting in healthy het *Ercc2*$^{S737P/+}$ mutants with normal vision (see also Pawliczek et al, this issue). At the age of 10 weeks (± 10 days), groups of 20 mice per sex and genotype were

whole-body irradiated by doses of either 0.5, 1 or 2 Gy (dose rate 0.3 Gy/min; $^{60}$Co source in Eldorado 78 tele-therapy irradiator, AECL, Canada). This $^{60}$Co source is one the most widely used sources γ-radiation[27]. Concurrently processed control animals (in total 21 mice per sex and genotype, 7 accompanying each radiation dose group), had the same type of handling and other conditions of exposure, but without dose (sham radiation, 0 Gy group).

**Ethical standards**

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. In particular, the study was approved by the relevant body of the government of Upper Bavaria (ROB-55.2-2532.Vet_02-16-167).

**Behaviour**

Four behavioural tests were performed at 4, 12 and 18 months after radiation exposure: the Open Field, Acoustic Startle, Social Discrimination and spontaneous alternation in the Y-Maze (Fig. 1A). These four tests were performed successively over a timespan of four weeks, always in this order. Tests were mainly carried out during the first half of the light phase, starting 1 hour after lights on. Experimental groups were either tested concurrently in multiple devices or in a counterbalanced design to control for circadian rhythm influences. After the last behavioural test was performed the experiment was terminated.

**Open Field (OF)**

This test evaluates spontaneous locomotor and exploratory activity in a novel environment. Testing is carried out in a square arena (45.5 cm x 45.5 cm x 39.5 cm) surrounded by transparent plastic walls and a metal frame equipped with infrared beam detectors to automatically monitor motor activity and its location (ActiMot, TSE Systems, Bad Homburg,

Germany). Among the recorded parameters are the total distance travelled, average speed, rearing (as measure of exploratory behaviour) and time spent in the exposed centre of the arena (as a measure of anxiety). The test was performed as previously described[28-30].

**Acoustic Startle/Prepulse Inhibition Test (ASR/PPI)**

This test assesses sensorimotor function by prepulse inhibition of the acoustic startle reflex and it was performed as previously described[31]. Deficits could be related to several neuropsychiatric disorders[32]. In brief, testing chambers (Med Associates Inc, St. Albans, VT, USA) are located within soundproof cubicles that isolate the animals from the rest of the lab environment. Two loudspeakers are located in the upper part of the chamber, one of them presenting background noise (65 decibels, dB) throughout the session. A cylinder encloses the animal on top of a piezoelectric motion sensor platform, transducing animal movements into recorded electrical signals. A session starts with a stimulus-free acclimation period of 5 min (except for background noise), followed by 5 startle stimulus alone (110 dB) trials. Then trial types for ASR and PPI are each presented 10 times, arranged in a pseudo-random order and organized in 10 blocks. ASR trial types consist of acoustic stimulus levels of 70, 80, 85, 90, 100, 110, and 120 dB. PPI is assessed for a startle stimulus level of 110 dB with prepulse levels of 67, 69, 73, and 81 dB preceding the startle pulse at an inter-stimulus interval of 50 milliseconds.

**Social Discrimination (SD)**

This test evaluates olfaction and social recognition memory and was performed as previously described[30,31,33]. After a 2 h habituation period to a fresh cage, a test animal was exposed to a stimulus animal during the sample session for 4 min. After a retention interval of 2 h, the test animal was re-exposed to the first stimulus animal (now a familiar subject) together with

158 a second, previously not encountered stimulus animal (unfamiliar subject) for 4 min (test
159 session). Ovariectomized 129Sv females were used as stimulus animals. In both sessions the
160 duration of investigatory behaviour of the test animal towards the stimulus mice was
161 recorded by a trained observer to calculate a recognition index (time spent investigating the
162 unfamiliar subject/ sum of time spent investigating both subjects), which is used as an
163 indicator of social memory. The time spent investigating the stimulus mouse during the
164 sample session was termed "social interest" and the total time spent investigating both
165 stimulus mice during the test session was termed "social approach". Since rodents rely heavily
166 on olfaction in social interactions and have a strong olfactory bulb neurogenesis, alterations
167 in social investigation behaviour can also indicate altered olfactory capacities[30,33].

168 **Y-Maze**

169 This test evaluates spatial working memory and was performed as previously described[30,31,33].
170 In brief, the Y-Maze test apparatus consists of three identical arms (30 x 5 x 15 cm) placed at
171 a 120° angle from each other, made of opaque light grey PVC. To assess spontaneous
172 alternation, defined as consecutive entries into all three maze arms, mice are placed
173 individually at the end of one arm and allowed to freely explore the Y-Maze for 5 min. Arm
174 entries are monitored by a trained observer. The ratio of actual to possible arm alternations
175 (total number of triplets) multiplied by 100 is calculated as % spontaneous alternations (SPAs),
176 and a ratio of alternate arm returns (AARs) and same arm returns (SARs) is calculated likewise.

177 **Statistical Analyses**

178 Behavioural data was analysed using a linear model with random intercept (PASW Statistics
179 18, IBM) to assess the influence of dose, sex, genotype and time p.i. and their interactions on
180 the different measured behavioural parameters. This analysis was deemed appropriate

because of the high number of animals and the repeated measurements. In the absence of interaction effects of dose, sex and genotype (see Table 2) over the course of the study, data of both sexes and gentoypes were subsequently pooled per dose and time p.i. and analysed by a two-way ANOVA with mixed effects using GraphPad Prism 8 (version 8.1.1 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com) and presented in the figures. For social interest and social approach behaviour where significant sex x dose interactions were found, data was only pooled per genotype and analysed separately per sex (see Fig. 3). For Y-Maze parameters (%SPA, %AAR) with significant sex x dose x genotype interactions these subsequent analyses were performed separately for each sex and genotype (see Fig. 4). Reported P-values were adjusted for multiple comparisons using Tukey's test. For all tests, a P value $<0.05$ was used as a level of significance and data are presented as means ± SEM.

## Results

### Radiation dose effects independent of sex or genotype

As shown in Table 2, with the exception of the social recognition index, all behavioural parameters changed over time and thus with ageing, as expected. Radiation dose had a significant effect on all behavioural parameters except for the acoustic startle response and the social recognition index. In addition, we also observed the normal sex differences in OF, ASR/PPI and SD parameters, but a significant sex and radiation dose interaction was only present in the affiliative social behaviours termed social interest (SD sample session) and social approach (SD test session). Significant genotype effects were observed in OF and Y-Maze locomotor parameters, ASR and social approach behaviour, but no dose and genotype

or sex and genotype interactions were detectable. Significant sex, dose and genotype interactions only occurred in working memory parameters %SPAs and %AARs in the Y-Maze.

For all parameters without any interactions, data of all experimental groups were pooled per dose and time p.i. for further analyses of radiation dose effects. Body weight was significantly increased by all doses 4 months p.i., but this radiation effect was less prominent at later time points when body weight had naturally increased in all groups (Fig. 1B). Of note, the 2 Gy group was the only group that did not further increase body weight from 12 to 18 months p.i. and where most animals were lost before the end of the study (see Table 1).

Significant dose-dependent effects were observed on OF parameters 4 months p.i. (Fig. 2A-D). Spontaneous locomotor activity as measured by total distance travelled (Fig. 2A) and average speed of movement (Fig. 2C) as well as anxiety-related behaviour as measured by the percentage of the time spent in the anxiogenic centre of the OF (Fig. 2D) were increased by 0.5 Gy and decreased by 2 Gy. Vertical exploratory rearing activity (Fig. 2B) was decreased by 2 Gy while the lower doses had no effect on this parameter. There were no significant differences at later time points. No significant radiation dose effect on ASR was detected (Fig. 2E), and while there was a small significant overall dose effect on PPI in the linear model ($p<0.05$, see also Table 2), after correction for multiple testing during post-hoc analysis no individual significant differences were detectable (Fig. 2F). The number of entries into the arms of the Y-Maze was significantly reduced by 2 Gy 4 months p.i. and, interestingly, also 18 months p.i. (Fig. 3F).

**Effects of radiation on social behaviour and social recognition memory**

Because of the significant sex x dose interactions in affiliative behaviour parameters in the SD test shown in Table 2, these parameters were only pooled by genotype and analysed and

presented separately per sex (Fig. 3A-D). As shown in this figure, male mice (Fig. 3A,C) spent approximately double the time of female mice (Fig. 3B,D) with social interest and social approach behaviour. Apart from this difference in absolute levels, the dose-dependent radiation effects on these behaviours in males mirror those in females. 0.5 Gy increased affiliative behaviour 4 and 12 months p.i. (Fig. 3A-D), whereas 1 Gy reduced it at 4 months p.i., and also at 12 months p.i. in males. Interestingly, the 2 Gy group did not differ from the sham-irradiated 0 Gy control group 4 months p.i., but 12 months p.i. 2 Gy significantly reduced affiliative behaviour. There were no radiation dose effects detectable 18 months p.i. on social behaviour (Fig. 3A-D). But these strong and persistent radiation effects on social interest and social approach did not affect the formation of social memory, as we did not detect a significant dose effect on the social recognition index (Fig. 3E).

**Sex- and genotype-dependent radiation effects on working memory**

As there were complex radiation x sex x genotype interactions on the working memory parameters %SPA and %AAR in the Y-Maze (see Table 2), these data were not pooled but analysed and presented per sex and genotype (Fig. 4). These analyses revealed genotype-dependent radiation effects on working memory in male mice (Fig. 4A,C,E,G), and no significant radiation effects at all on these working memory parameters in female mice (Fig. 4B,D,F,H). In wt males, 1 Gy increased the %AAR (Fig. 4E) and concomitantly reduced %SPA 4 months p.i. (Fig. 4A), whereas in male *Ercc2*$^{S737P}$ het mice 0.5 Gy increased %SPA (Fig. 4C) and concomitantly reduced %AAR (Fig. 4G) at this time point. There were no significant radiation dose effects at later time points. These results indicate sex- and genotype-specific radiation dose effects on spatial working memory as measured by spontaneous alternation in the Y-Maze 4 months after irradiation.

## Discussion

This study revealed dose-dependent long-term radiation effects of a single radiation exposure in young adulthood to 0.5, 1 or 2 Gy on locomotor, anxiety-related, social and cognitive behaviours as well as on body weight. In line with previous reports[20], dose-response relationships for behavioural outcomes were not simple. The pattern of effects was also heterogenous both in terms of their time course and concerning the influence of sex and the *Ercc2*$^{S737P}$ het genotype. All of these effects were evident 4 months p.i., but only effects on social behaviour were still present 12 months p.i. The only significant radiation effect detected 18 months p.i. was a reduction of arm entries into the Y-Maze by 2 Gy. Most of these effects affected both sexes and genotypes similarly, only radiation effects on working memory were sex- and genotype-specific. Of note, behavioural alterations 4 months p.i. cannot be attributed to radiation effects on the eye lens as there were none detectable at this time point. Only reduced Y-Maze exploration 18 months p.i. could potentially be related to visual impairments that occurred in the 2 Gy group at this stage (see Pawliczek, this issue).

Our results provide further evidence that dose rate influences radiation effects, given that in our previous study[23] 0.5 Gy applied at a lower dose rate (0.063 Gy/min vs. 0.3 Gy/min here) in an otherwise identical experimental design produced different behavioural outcomes. At a lower dose rate 0.5 Gy did not affect locomotor activity 4 months p.i., but induced a delayed long-term reduction of locomotor and rearing activity present 12 and 18 months p.i.[23]. In contrast, at the higher dose rate in this study, 0.5 Gy increased locomotor activity measures 4 months p.i. without detectable differences from the control group at later time points. This finding is of potential clinical relevance since 0.5 Gy is a dose that can be experienced during

cerebral embolisation treatment[34]. Previously it was shown that 0.5 and 2 Gy irradiation at a dose rate of 0.445 Gy/min transiently reduced locomotor activity 6 hours p.i., but did not have any effect during the first 24 hours at a lower dose rate of 0.005 Gy/min, and later time points were not investigated[35]. It is tempting to speculate that in this dose range higher dose rates more effectively activate defence and repair mechanisms, which then prevents late-onset adverse effects, for which there is some evidence from intestinal stem cell turnover[36].

It is unclear if dose rate plays a role for radiation effects on hearing/ASR as well. At the lower dose rate 0.5 Gy induced a small, but detectable and persistent reduction of ASR 4 and 12 months p.i. that was no longer distinguishable from the natural age-related decline in ASR[37] in the sham-irradiated control group 18 months p.i.[23], in contrast to no significant dose-dependent effects on ASR at any time point at the higher dose rate in the present study. At very high radiation doses used in radiotherapy, ionizing radiation can induce sensorineural hearing loss[38] by damaging cochlear structures through the production of reactive oxygen species (ROS), precipitating inflammation and cell death[39]. But this is usually not observed in the dose range used in our study, although during the first 8 days after acute exposure a subtle reduction of ASR by 2 Gy with a maximum 2 days p.i. has been reported[39].

In line with our previous study including the *Ercc2*$^{S737P}$ heterozygous mutation, we did not find any evidence that this genotype increases radiation sensitivity *in vivo* as hypothesized based on their increased sensitivity to radiation-induced DNA damage *in vitro*[16]. In spite of overall genotype differences that were mainly present in activity-related behaviours, the effect of radiation on these behaviours did not differ between genotypes (see Table 2). The only genotype-specific radiation effect, which was also sex-specific, was observed on working memory, which was only affected by radiation in males, but not in females. There was no

linear dose-response relationship in either genotype, and the fact that working memory was improved by 0.5 Gy in het males while it was impaired by 1 Gy in wt males 4 months p.i. is consistent with other findings that this mutation only marginally affects radiosensitivity *in vivo,* and then it is rather protective (see also Pawliczek, this issue).

The literature on sex-specific radiation effects on cognition is inconclusive, also because most studies have only used males. While some studies suggested a higher sensitivity to radiation-induced cognitive impairments in females[40-42], others found sex-specific differences in anxiety and improved fear memory recall in females[43], or even higher adverse neurocognitive effects in males[44]. All of those studies used higher doses, dose rates or higher charged, higher energy radiation sources. Of note, the other measure of cognition used in the present study, the social recognition index, was completely unaffected by radiation in spite of strong dose-dependent effects on social/olfactory investigation. Overall, our results are consistent with previous findings that – in contrast to neonatal exposure - a single radiation dose up to 2 Gy administered in young adulthood does not induce obvious cognitive impairments 6 months p.i., in spite of significant reductions in measures of adult hippocampal neurogenesis up to this time point[45].

The strongest radiation effects observed in our study were those on social/olfactory investigation, which are most likely due to alterations in adult neurogenesis in the SVZ[30]. In adult rodents, neurogenesis in the SVZ, rostral migratory stream and olfactory bulb is much more pronounced than SGZ neurogenesis in the hippocampus, which makes it easier to detect effects of experimental manipulations on SVZ neurogenesis, e.g. early delayed impairments of olfactory memory 3 months after 5 Gy irradiation at the age of 4 weeks[46]. SVZ neurogenesis also recovers better from radiation effects than SGZ neurogenesis in the hippocampus[47]. While the lower doses of 0.5-2 Gy used in our study did not induce social memory

impairments, 0.5 Gy increased and 1 Gy decreased social/olfactory investigation activity 4 and 12 months p.i., while 2 Gy only reduced it 12 months p.i. in both sexes. The underlying mechanisms for this dose-response relationship are unclear. They are likely related to the combined effects of these doses on SVZ neurogenesis, i.e. the stem cell pool, neurogenic niche microenvironment, microvasculature, as well as on neuroinflammatory processes and repair mechanisms, which may unfold with different time courses[45,48].

A significant effect of radiation on body weight gain in female mice was previously reported for doses of 3-12.5 Gy[49,50], and our results extend them to lower doses and to male mice. Similar to our findings, the dose-response curve became inverse 3-4 months p.i.[49], probably due to more adverse or cancerogenic effects of the higher doses. Radiation-induced body weight gain is also experienced by a substantial portion of craniopharyngioma patients, but the mechanisms of radiation effects on the hypothalamus and of hypothalamic obesity are still poorly understood[51-53]. While hypothalamic POMC neurons exert region-specific control over SVZ neurogenesis via long-range projections[54], radiation effects on the ability of hypothalamic leptin to function as the "saturation hormone" may also play a role (see [9] for review). But in the present study an influence on body weight due to peripheral effects can not be excluded.

Taken together, the present results provide further evidence that also early in adult life a single radiation event can have long-term, dose-dependent effects on locomotor, anxiety-related, social and cognitive behaviour, and on body weight. In future experiments, it would be interesting to investigate the molecular neurobiological mechanisms of the time course of changes in neurogenic niches, i.e. the SVZ and the hippocampus.

## Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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## Acknowledgements

The authors thank Erika Bürkle and Monika Stadler for expert technical assistance. The work was supported by the Euratom research and training programme 2014-2018 in the framework of CONCERT under grant agreement No 662287 (LDLensRad), by the German Federal Ministry of Education and Research (grant no. 02NUK045A) and by INFRAFRONTIER (grant no. 01KX1012).

## Authors' contributions

CD, LG, SMH and JG designed the experiments; MCU, JE, DP and FF collected data and tissue; LG, AZ and SMH analysed and interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

## Additional information

## Competing interests' statement

The authors declare that they have no conflict of interest.

## Figure legends

**Fig.1 Experimental design. A** Male and female wild type (wt) and *Ercc2*$^{S737P}$ heterozygous (het) mice were whole-body irradiated with $^{60}$Co source at the age of 10 weeks (2.5 months) with a single dose of radiation. Final dose values were either 0 Gy (sham-irradiated control groups), 0.5 Gy, 1 or 2 Gy, with a dose rate of 0.3 Gy/min. Mice were repeatedly tested at 4,

12 and 18 months post-irradiation (p.i.) with behavioural tests such as the Open field (OF), Acoustic startle/Prepulse inhibition (ASR/PPI), Social Discrimination (SD) and Y-Maze test. **B** Body weight was measured at each timepoint p.i. There was increased body weight (g) in each of the radiation groups compared to sham control at 4 months p.i. that was not evident at the other timepoints p.i. **p < 0.01, ***p < 0.001 vs. 0 Gy control group.

**Fig.2 Effects of radiation on spontaneous locomotor and sensorimotor behaviour over time.** Dose effects after 4, 12 and 18 months p.i. on locomotor and anxiety-related behaviour in the Open Field (OF) **(A-D)** and on the acoustic startle response and its prepulse inhibition **(E, F)**. Because of the absence of interaction effects of dose, sex and genotype (see Table 1), data of both sexes and gentoypes were pooled per dose and time p.i. and analysed by a two-way ANOVA with mixed effects. Data presented are the means +/- SEM per time point, asterisks represent adjusted p-values (Tukey's test) of post-hoc analyses: *p<0.05, ** p<0.01, **** p<0.0001 vs. 0 Gy control group per time point. **A** Distance travelled in the OF was significantly increased by 0.5 Gy and reduced by 2 Gy 4 months p.i. **B** Rearing activity was significantly reduced by 2 Gy 4 months p.i. **C** Average speed in the OF was significantly increased by 0.5 Gy and reduced by 2 Gy 4 months p.i. **D** The time spent in the anxiogenic centre of the OF was significantly increased by 0.5 Gy and reduced by 2 Gy 4 months p.i. **E** There were no significant radiation dose effects on the acoustic startle response at any time point p.i. **F** There were no significant radiation dose effects on prepulse inhibition at any time point p.i. that survived post-hoc correction for multiple comparisons.

**Fig.3 Effects of radiation on social behaviour, recognition and Y-Maze entries over time.** Dose effects after 4, 12 and 18 months p.i. during the Social Discrimination test **(A-E)** and on the total number of arm entries during the Y-Maze test **(F)**. **A,B** Social interest during the

sample session was increased by 0.5 Gy and reduced by 1 Gy 4 and 12 months p.i., whereas it was significantly reduced by 2 Gy 12 months p.i. both in males **(A)** and in females **(B)**. **C, D** Total social approach behaviour towards both subjects during the test session was increased by 0.5 Gy and reduced by 1 Gy 4 and 12 months p.i., whereas it was reduced by 2 Gy 12 months p.i. both in males **(C)** and in females **(D)**. **E** There were no significant radiation dose effects on the social recognition index. **F** Arm entries during the Y-Maze test were significantly reduced by 2 Gy 4 and 18 months p.i. Data presented are the means +/- SEM per time point of data of pooled per genotype **(A-D)** and of data pooled per genotype and sex **(E,F)**. P-values are results of post-hoc analyses adjusted for multiple comparisons (Tukey's test) indicated by asterisks: *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs. 0 Gy control group per time point.

**Fig.4 Effects of radiation on spontaneous alternation in the Y-Maze over time.** Dose effects after 4, 12 and 18 months p.i. on spontaneous alternations (%SPA, **A-D**) and alternate arm returns (%AAR, **E-H**) in the Y-Maze were sex- and genotype-specific (see Table 1). Data presented are the means +/- SEM per time point, asterisks represent adjusted p-values (Tukey's test) of post-hoc analyses: *p<0.05, ** p<0.01 vs. 0 Gy control group per time point. **A** %SPA was significantly reduced by 1 Gy 4 months p.i. in male wildtypes. **B** There were no significant radiation dose effects on %SPA at any time point p.i. in female wildtypes. **C** %SPA was significantly increased by 0.5 Gy 4 months p.i. in male heterozygotes. **D** There were no significant radiation dose effects on %SPA at any time point p.i. in female heterozygotes. **E** %AAR was significantly increased by 1 Gy 4 months p.i. in male wildtypes. **F** There were no significant radiation dose effects on %AAR at any time point p.i. in female wildtypes. **G** %AAR was significantly reduced by 0.5 Gy 4 months p.i. in male heterozygotes. **H** There were no significant radiation dose effects on %AAR at any time point p.i. in female heterozygotes.

**Table 1. Animal numbers per testing time point.** The number of animals per experimental group available for testing at each time point and used in the statistical analyses are shown.

**Table 2. Effects of dose, sex and genotype and their interactions over the course of the study.** Data of key behavioural parameters were analysed with a linear model with random intercept to assess the influence of dose, sex, genotype and time p.i. and their interactions over time. Presented are the p-values of the fixed effects and their interactions; values reaching the level of significance ($p<0.05$) are shaded in grey.

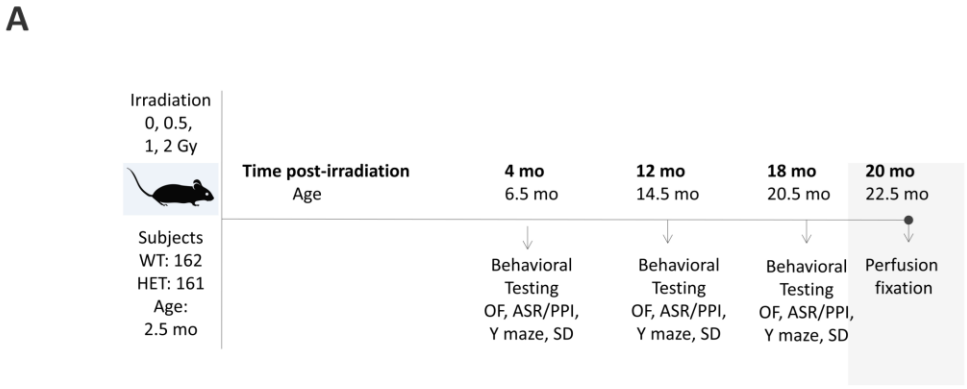
Table 1. Animal numbers

| **Irradiation dose (Gy)** | **Genotype** | **Sex** | **4 months p.i.** | **12 months p.i.** | **18 months p.i.** |
|---|---|---|---|---|---|
| 0 | Wildtype | Male | 21 | 18 | 15 |
| | | Female | 21 | 21 | 19 |
| | *Ercc2*$^{S737P}$ | Male | 21 | 20 | 19 |
| | | Female | 21 | 20 | 20 |
| 0.5 | Wildtype | Male | 20 | 20 | 17 |
| | | Female | 20 | 20 | 18 |
| | *Ercc2*$^{S737P}$ | Male | 20 | 19 | 19 |
| | | Female | 20 | 19 | 17 |
| 1 | Wildtype | Male | 20 | 18 | 17 |
| | | Female | 20 | 19 | 19 |
| | *Ercc2*$^{S737P}$ | Male | 20 | 20 | 18 |
| | | Female | 20 | 17 | 17 |
| 2 | Wildtype | Male | 20 | 20 | 16 |
| | | Female | 20 | 18 | 9 |
| | *Ercc2*$^{S737P}$ | Male | 20 | 20 | 17 |
| | | Female | 19 | 19 | 14 |
| **total** | | | **323** | **308** | **271** |

Table 2. P-values of main variables radiation dose, sex, genotype (geno), time p.i. and of their interactions for key experimental parameters. Soc Int = Social Interest; Soc Appr = Social Approach; Rec Ind = Recognition Index.

| | dose | sex | geno | time p.i. | dose x geno | sex x dose | geno x sex | sex x dose x geno |
|---|---|---|---|---|---|---|---|---|
| BW | .021 | <0.001 | .050 | <0.001 | .440 | .260 | .911 | .340 |
| Distance | <0.001 | <0.001 | <0.001 | <0.001 | .143 | .554 | .730 | .451 |
| Speed | <0.001 | <0.001 | <0.001 | <0.001 | .150 | .571 | .863 | .515 |
| Rearings | .019 | .047 | .004 | <0.001 | .684 | .919 | .307 | .602 |
| %Centre | <0.001 | <0.001 | .639 | <0.001 | .171 | .409 | .536 | .714 |
| Startle | .382 | <0.001 | .036 | <0.001 | .595 | .153 | .102 | .912 |
| PPI | .043 | <0.001 | .168 | <0.001 | .057 | .587 | .694 | .737 |
| Soc Int | <0.001 | <0.001 | .265 | <0.001 | .312 | <0.001 | .178 | .155 |
| Soc Appr | <0.001 | <0.001 | .011 | <0.001 | .286 | <0.001 | .379 | .581 |
| Rec Index | .116 | <0.001 | .193 | .134 | .141 | .596 | .181 | .944 |
| Y Entries | .003 | .556 | <0.001 | <0.001 | .897 | .407 | .318 | .726 |
| %SPA | <0.001 | .530 | .249 | <0.001 | .424 | .429 | .590 | .001 |
| %AAR | .004 | .621 | .062 | .006 | .322 | .453 | .465 | .007 |

573 **Fig. 1**



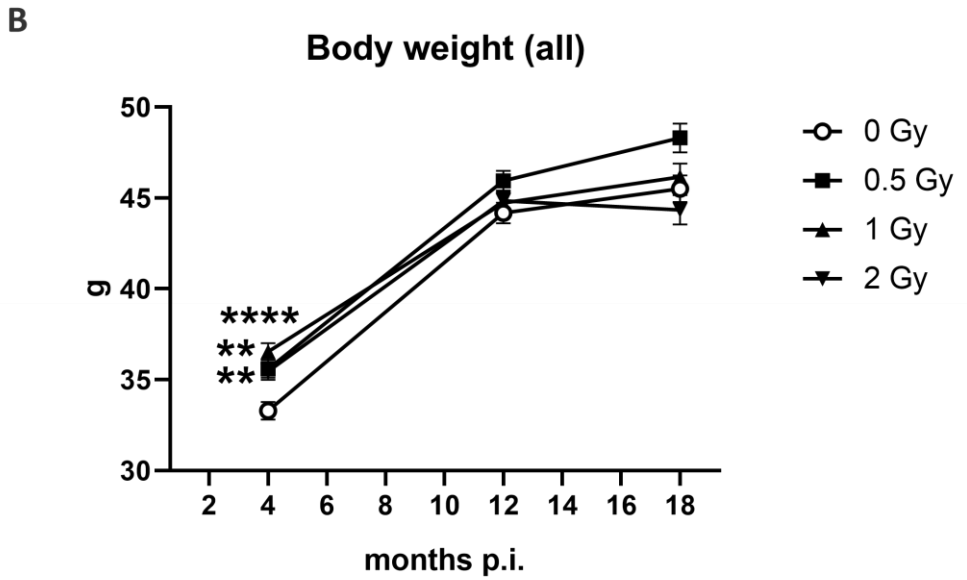




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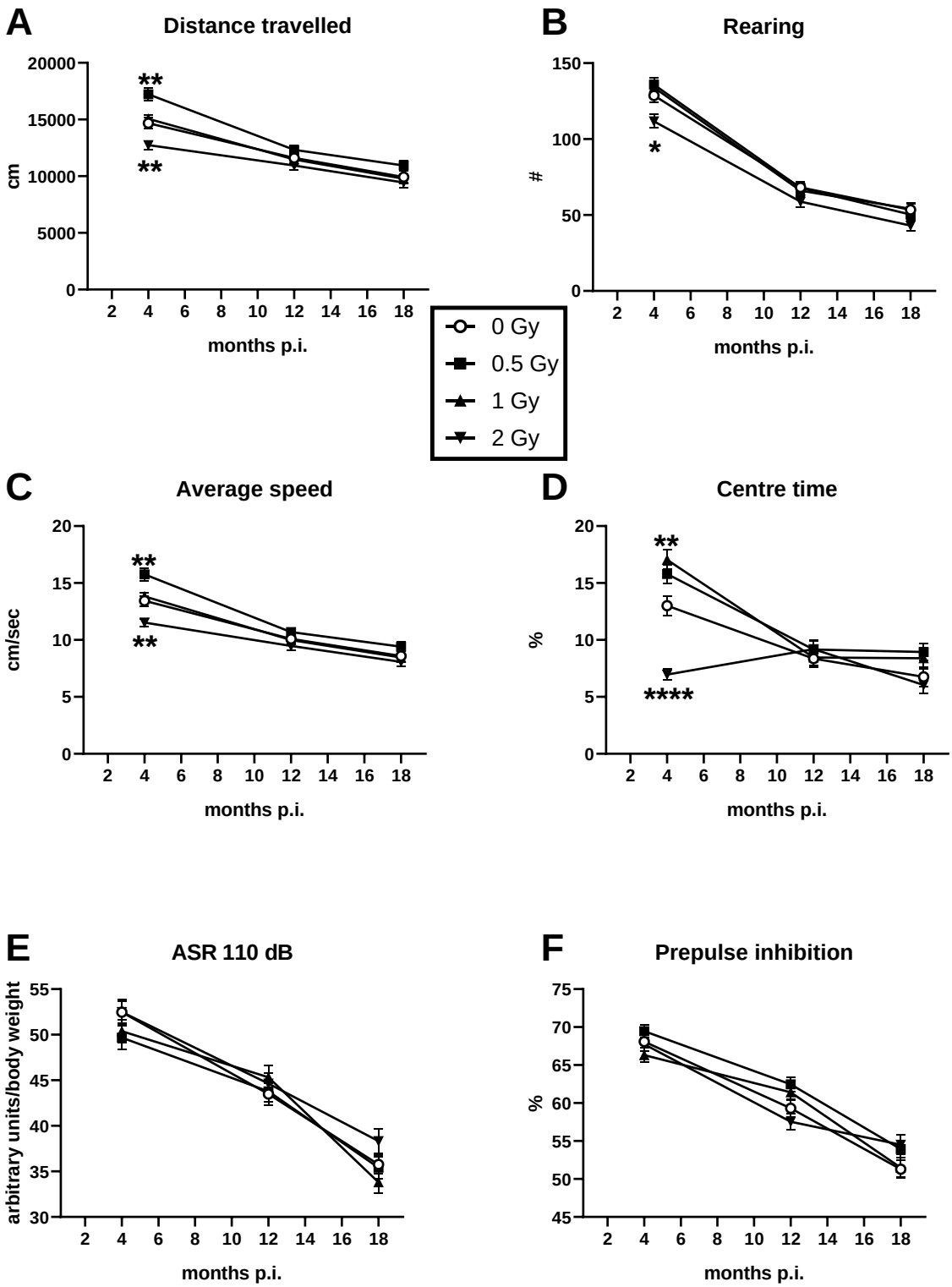
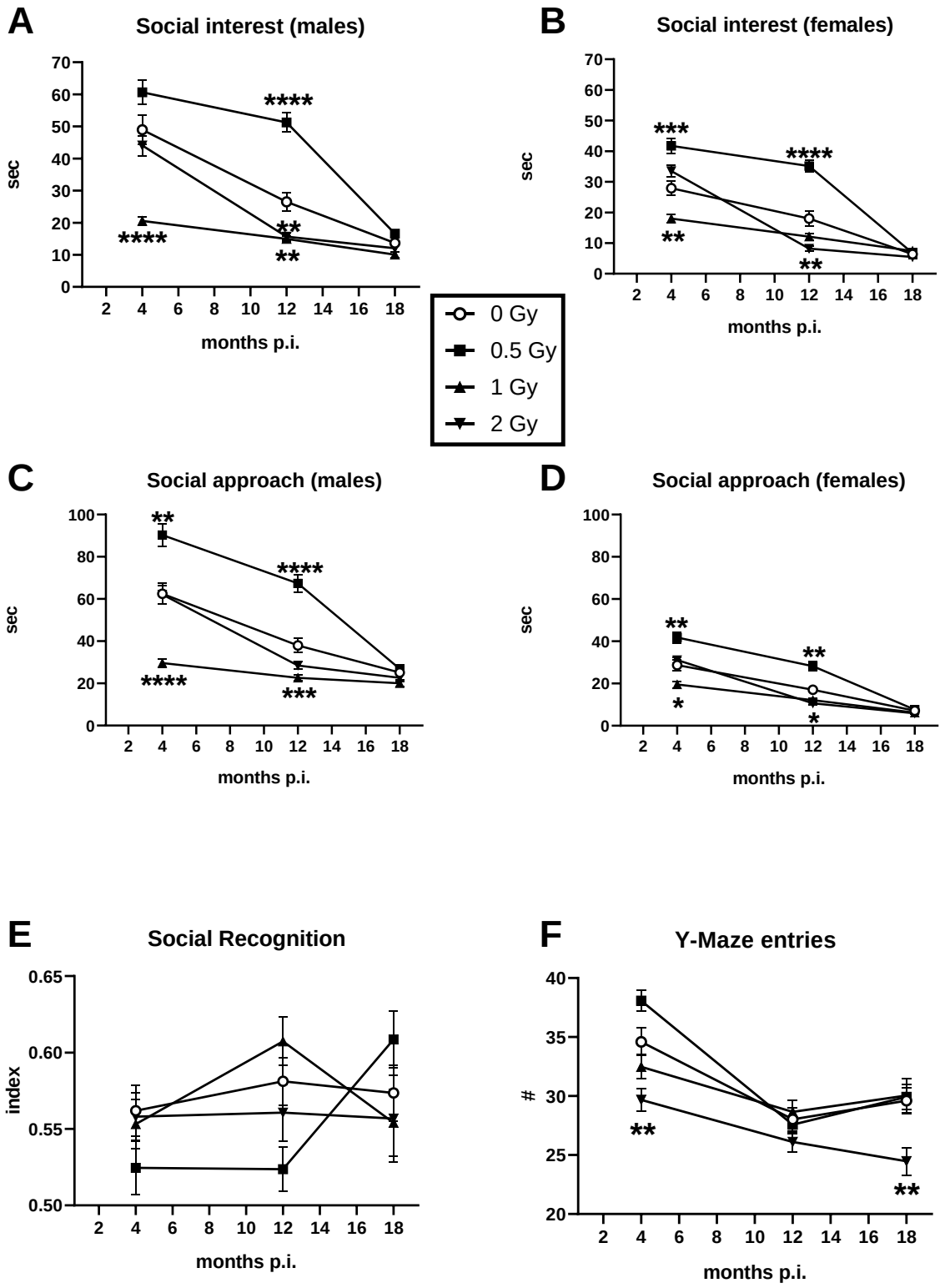
**Fig. 2**


A
Distance travelled
cm
20000
15000
10000
5000
0
**
**
2 4 6 8 10 12 14 16 18
months p.i.
B
Rearing
#
150
100
50
0
*
2 4 6 8 10 12 14 16 18
months p.i.
0 Gy
0.5 Gy
1 Gy
2 Gy
C
Average speed
cm/sec
20
15
10
5
0
**
**
2 4 6 8 10 12 14 16 18
months p.i.
D
Centre time
%
20
15
10
5
0
**
****
2 4 6 8 10 12 14 16 18
months p.i.
E
ASR 110 dB
arbitrary units/body weight
55
50
45
40
35
30
2 4 6 8 10 12 14 16 18
months p.i.
F
Prepulse inhibition
%
75
70
65
60
55
50
45
2 4 6 8 10 12 14 16 18
months p.i.

583 **Fig. 3**

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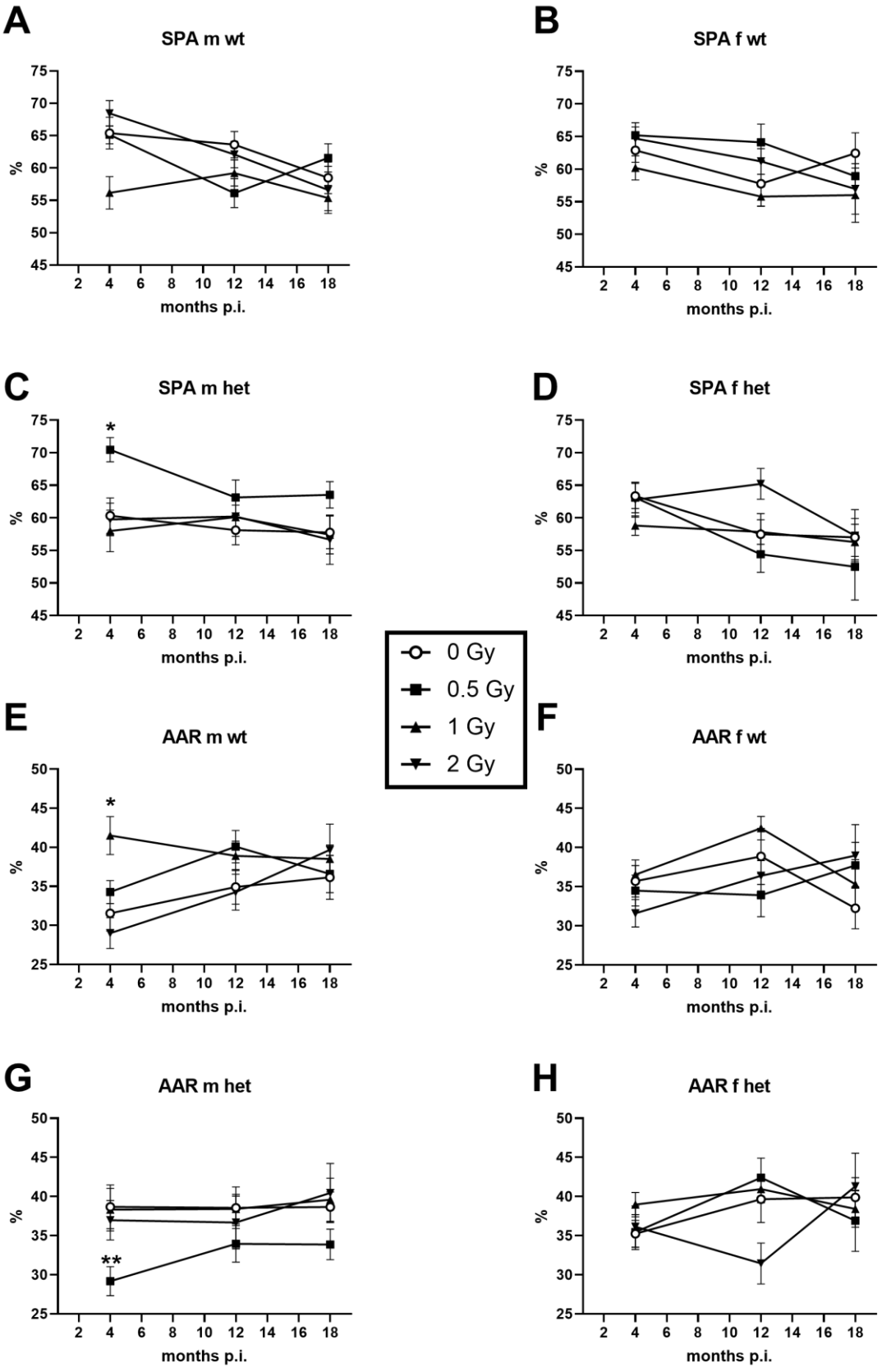
586 **Fig. 4**


A
SPA m wt
B
SPA f wt
C
SPA m het
D
SPA f het
E
AAR m wt
F
AAR f wt
G
AAR m het
H
AAR f het
%
months p.i.
0 Gy
0.5 Gy
1 Gy
2 Gy
*
**

587