Treatment by Low-Dose Brain Radiation Therapy Improves Memory Performances without Changes of the Amyloid Load in the TgF344-AD Rat Model.

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Research

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Abstract

**Background.** Alzheimer’s disease (AD) is a neurodegenerative condition affecting memory performance. This pathology is characterized by intracerebral amyloid plaques and tau tangles coupled with neuroinflammation. During the last century, numerous therapeutic trials, targeting amyloid, tau or neuroinflammation, unfortunately failed. Development of new therapeutic approaches for AD is eagerly awaited. In this study, we propose to test the use of low-dose brain radiotherapy (LD-RT). LD-RT showed efficacy to reduce amyloid load in patients with peripheral pathologies and also presents anti-inflammatory effects in patients with systemic inflammatory diseases. Furthermore, previous evidence showed an effect on amyloid load, memory performance and modulation of the immune response in AD mice.

**Methods.** The hemi-brain of 14-15 months old TgF344-AD rats was irradiated with two LD-RT schedules (5 fractions of 2 Gy, delivered weekly or daily). Rats underwent behavioral tests to assess memory and general locomotion, before and/or 4 months post treatment. LD-RT impact on amyloid and neuroinflammation was measured using immunohistology.

**Results.** An improvement of spatial working memory was observed in the daily treated group but not in animals treated weekly. On the other hand, both LD-RT schedules were unable to impact amyloid load. An increase of astrocyte reactivity was observed in TgAD groups compared to wild-type animals but LD-RT did not impact it. The weekly treatment increased CD68\(^+\) microglial density in the molecular layer of the hippocampus but no effect was observed with the daily treatment. One major limitation of this study was the already well-established amyloid pathology, which was probably too advanced to observe a potential decrease. Nevertheless, we observed a memory improvement, the main cognitive symptoms of AD, after daily treatment, offering encouraging data to further evaluate the LD-RT therapeutic potential.

**Conclusions.** This study showed a biological effect of LD-RT in an AD rat model on memory performance (after daily treatment) and microglial density (after weekly treatment). The lack of effect of both regimens on amyloid pathology, unexpected, was possibly due to the advanced pathology of our animals. The positive effect on cognition encourages to further evaluate the LD-RT therapeutic potential at an earlier AD stage.

Background

Alzheimer’s disease (AD) is the most common form of dementia and thus represents a major societal challenge. The pathological hallmarks of the disease are amyloid and tau deposits, accompanied by neuroinflammation. Amyloid pathology takes place after abnormal accumulation of amyloid beta (A\(\beta\)) peptides which aggregates to form fibrils, oligomers and finally plaques and progress throughout the disease [1, 2]. The plaque morphology evolves with the pathology, with a shift in favor of fibrillar plaques vs. diffuse plaques at end-stage of the disease [3], while the proportion of dense-core plaques (\(~ 20\%\)) appears to be stable. The amyloid hypothesis, formulated over 25 years ago, suggested that the A\(\beta\)
plaques were at the origin of the neurodegeneration [4]. Nevertheless, the presence of amyloid deposits in persons without cognitive impairments [5] and the absence of direct correlation between amyloid load and cognitive decline [1] shows that amyloid plaques are not, or at least not alone, at the origin of cognitive deficits associated with AD. The tauopathy is due mainly to intraneuronal aggregation of hyperphosphorylated forms of the tau protein involved in the destabilization of microtubules and neuronal dysfunction [6]. The more advanced form of aggregation of tau are the neurofibrillary tangles (NFT). It is well established that the course of the tauopathy is associated with symptom severity [1, 7].

The third component, the neuroinflammation, involves reactive astrocytes and reactive microglia, the immune cells of the brain. Astrocyte reactivity is classically characterized by an increase in intermediate filament protein expressions such as glial fibrillary acidic protein (GFAP) and Vimentin, and a hypertrophy of soma and processes. These structural changes are accompanied by many transcriptomic and functional modifications which may positively or negatively influence the progression of the pathology [8, 9]. Microglial cells also undergo important morphological, transcriptomic and functional changes when they become activated. Indeed, overexpression of proteins such as ionized calcium binding adaptor molecule 1 (IBA1) or Cluster of Differentiation 68 (CD68) is observed and the most extreme reactive state of microglial cells is characterized by an ameboid form. Functionally, reactive glial cells produce higher rate of pro- and anti-inflammatory molecules such as cytokines, chemokines and reactive oxygen species [10]. The involvement of glial cells in Aβ phagocytosis and debris degradation is also well described [11]. However, it is often postulated that neuroinflammation is firstly a mechanism of defense for the brain, but with disease progression, this mechanism is overload. It generates itself pro-inflammatory molecules and reactive oxygen species, harmful for neuronal functioning and survival, consequently also participating to disease damage [12].

Despite multiple clinical trials, no disease-modifying treatment against AD has been validated yet [13]. It is consequently important to develop and test alternative strategies. Radiation therapy (RT) at low-dose (LD) offers promising applications as it has the potential to act on both amyloidosis and neuroinflammation. The ability to disaggregate amyloid pathology, possibly interacting directly with the beta-sheet structure, has been shown in multiple forms of systemic amyloidosis [14]. LD-RT has also shown remarkable anti-inflammatory effects in benign and chronic degenerative inflammatory diseases [see ref 14 for a detailed review]. Only four studies have evaluated the LD-RT effect in the context of AD using AD mouse models [15–18]. In a first report, authors observed a decrease of amyloid plaques, and accompanied by an improvement of memory performances in a pure amyloid model, more important with a fractionated protocol of LD-RT (5 fractions of 2 Grays (Gy), delivered daily) [15]. They hypothesized that pro-inflammatory mechanisms of RT could play a role in Aβ clearance, but a global downregulation of microglial activation through an increase of anti-inflammatory cytokines could also be postulated [19]. The same group confirmed their results on amyloid load in the triple transgenic model of AD (3xTg-AD mice) and described a slight decrease of NFT [16]. In a third model, no effect on amyloid was observed 4 days post RT but LD-RT did reduce microglial activation and improved synaptic protein levels, suggesting that LD-RT protected neurons from cellular death [18]. Eight weeks post treatment, a significant decrease of amyloid plaques was measured, with a shift from a pro-inflammatory to an anti-inflammatory cytokine
production, mainly characterized in vitro [17]. Given the discrepant results reported, the mechanisms involved in amyloid load reduction and the effects of LD-RT on the neuroinflammation in the AD brain need further investigation.

The major aim of this study is to confirm the effectiveness of LD-RT when applied at an advanced stage of the disease and its maintenance at long-term. For that, we investigated the impact of LD-RT on memory performances, amyloid load and neuroinflammation using a TgF344-AD rat model [20]. As the majority of the RT schedules commonly used to treat benign degenerative inflammatory pathologies deliver total doses in the range of 5 to 10 Gy in weekly or daily fractions ≤ 2 Gy [21], in the present study we tested two different brain RT regimens: 5 fractions of 2 Gy, delivered weekly and 5 fractions of 2 Gy, delivered daily.

**Methods**

Animals. The TgF344-AD (TgAD) rats present human APP Swedish and PS1 delta E9 transgenes in the Fisher 344 background [20]. The hemi-brain of TgAD female rats was treated by two LD-RT schedules (described below) and analyzed 4 months after the end of LD-RT. Sham treated TgAD and non-transgenic littermates (WT) were used as controls. Animals were housed in a 12-hours light-dark cycle, with food and water ad-libitum. All the experimentation procedures on our animals were conducted with respect of the ethical approval by the Ethics Committee for Animal Experimentation of the Canton of Geneva, Switzerland (GE9917).

Radiation therapy. TgAD rats were anesthetized (2% isoflurane) and treated with half brain irradiation (right hemisphere) using a Truebeam® Linear Accelerator (Varian Medical Systems, Palo Alto, CA, US) with the technique previously described [22]. Two fractionation schedules were used: a) 10 Gy in 5 weekly fractions of 2 Gy (5 weeks overall treatment time, OTT) or b) 10 Gy in 5 daily fractions of 2 Gy (5 days OTT). Sham-treated groups underwent only anesthesia to induce the same stress due to handling and repeated anesthesia.

Experimental groups. The study design included two cohorts: a) Fourteen-month-old TgAD rats treated with 2 Gy x 5 fractions once a week (n = 10), sham-treated TgAD (n = 10), and WT (n = 10) animals. b) Fifteen-month-old TgAD rats treated with 2 Gy x 5 fractions daily (n = 10) and sham-treated TgAD rats (n = 11). At these ages, the amyloid pathology and neuroinflammation is well-advanced. Animals with the appropriate genotype were randomly assigned to experimental groups.

Behavior. Treated animals of the first cohort (2 Gy x 5 fractions weekly) underwent behavioral experiments before RT and 4 months post treatment at 19-month-old. Treated animals and sham RT rats of the second cohort (2 Gy x 5 fractions daily) performed experiments only 4 months after RT. Locomotor activity was assessed using an Open Field (90 × 90 × 40 cm). Rats were placed at the center and video-track for 30 min using the EthoVision® software (Noldus). The alternative Y maze test was used to evaluate the spatial working memory. Animals were placed at the extremity of the start arm of the device
(50 × 50 × 10 cm) and video-tracked for 5 min. The alternations were measured and analyzed using the EthoVision® software. Rats performing less than 4 total entries were excluded from the analysis. All devices were washed with water and carefully dried between each rat.

Histology. Animals were killed under anesthesia (2% isoflurane) by intracardiac perfusion with a saline solution. Their brain was fixed in a 4% paraformaldehyde solution for 24 h and cryoprotect with a sucrose gradient (5 to 20%). Serial brain sections (35 µm) were realized with a cryostat and stored in an anti-freeze solution at -20 °C until used for immunostaining. Left and right hemispheres were identified by a cut in the left thalamus.

**Immunofluorescence.** For methoxy-XO4 (MXO4; Tocris) plaque labelling, slices were rinsed in PBS0.1M for 3 × 5 min and incubated with 20 µg/ml of MXO4 in PBS0.1M for 30 min at room temperature. After 3 washes, slices were incubated O/N at 4 °C with the following antibodies: anti-GFAP-Cy3 (1/1,000, Sigma), anti-IBA1 (1/600, Rabbit; Wako), anti-Vimentin (1/1,000, Chicken; Abcam), anti-CD68 (1/1,000, Rabbit; Invitrogen), anti-AT8 (1/500, Mouse; ThermoScientific), diluted in 1% BSA/PBS0.1M/0.3% Triton X-100. After 3 × 5 min of washes, slices were incubated for 1 h at room temperature in 1% BSA/PBS0.1M/0.3% Triton X-100 with the appropriate secondary Alexa-fluor conjugated antibodies (1/200, Invitrogen). Slices not stained with MXO4 were stained with DAPI for 10 min at room temperature. Slices were rinsed before being mounted on gelatin slides and coverslipped with Fluorosave™ (Calbiochem).

**Immunohistochemistry.** Slices were rinsed in PBS0.1M for 3 × 5 min and incubated overnight (O/N) at 4 °C with an anti-A4G8 antibody (1/500, Mouse; Biolegend) in 1% BSA/PBS0.1M/0.3% Triton X-100. After 3 washes in PBS0.1M, slices were incubated for 1 h at room temperature in the secondary antibody goat anti-mouse-HRP (1/200, Dako) in 1% BSA/PBS0.1M/0.3% Triton X-100. Slices were rinsed and revealed with a solution of 0.2 mg/ml DAB (Sigma-Aldrich) in 1x PBS, containing 100 µl/l H2O2 for 5 min at room temperature. Slices were mounted and let dried O/N. A coloration with cresyl violet was performed to stain nucleus. After dehydration, the coverslip was added using a mounting medium.

Image analysis. All images were acquired using an Axioscan.Z1® (Zeiss) at 10x and analyzed using ImageJ software. For the A4G8 staining only performed on the cohort treated daily, a manual segmentation of the cortex and the hippocampus was realized to measure the potential dose effect due to laterality from the region targeted by radiations [22]. The number of amyloid plaques and their individual area were measured using a semi-automatic detection of amyloid plaques with size and intensity threshold. The same technic was used to quantify MXO4+ plaques in the hippocampus. A distribution analysis of plaque area was realized using STATISTICA software: for small plaques (< 50 µm²) a pitch of 10 µm² was used and a pitch of 200–250 µm² was used for larger plaques (> 50 µm²). For GFAP and IBA1 staining, the mean grey value was measured in the entire hippocampus (3 fields per slices, 4 slices per animal). For Vimentin and CD68 staining, the percentage of the area positively stained was measured using an intensity threshold in the molecular layer and the hilus of the hippocampus, two separated regions of interest (ROI) manually delimited (2–5 slices per animal). The cellular layer of the
subgranular zone of the dentate gyrus was quantified using an intensity threshold in a defined field (3–4 slices per animal) to measure the percentage of the ROI positively stained with DAPI or Cresyl violet.

Statistical analysis. A sample size analysis with the graphical Douglas Altman's nomogram approach [23] was performed and significant data were reported if $p \leq 0.05$ and $\beta < 0.2$. Analysis was performed in blind conditions and normality of residues was assessed with the Shapiro-Wilks test. For data presenting left and right comparison, Two-way ANOVA (Group and Hemisphere as between factors) and LSD post hoc test were used to compare the groups. Paired two-tailed student's $t$-test or unpaired two-tailed student's $t$-test were used to compare two groups. Chi-2 test was performed to analyze plaque distribution data. All analyses were performed on Prism 8 (GraphPad). Results are presented as Mean ± SEM.

**Results**

All TgAD rats analyzed were unilaterally treated with the two different radiation regimens as planned (Fig. 1a). As the hippocampus, one of the main source of neural stem cells, is known to be particularly sensitive to radiations even at low doses [24], we validated that both LD-RT regimens do not induced a neuronal toxicity, as shown by the absence of hippocampal atrophy and modulation of the dentate gyrus thickness (Additional file 1).

Daily LD-RT improves memory performances and restores locomotion.

The spatial working memory of animals was evaluated using the alternative Y maze test before and after LD-RT. Animals treated with the weekly schedule did not perform better the test ($t(14) = 0.878$, $p = 0.395$, Fig. 1b), however, we observed a significant decrease of locomotion ($t(9) = 2.328$, $p = 0.045$; Fig. 1c). Interestingly, animals treated with the daily schedule greatly improved their memory performances (+ 42% of success to the test, $t(16) = 2.348$, $p = 0.032$; Fig. 1d), accompanied with a reduction of the total distance traveled in the open field after treatment compared to sham-RT animals ($t(19) = 2.163$, $p = 0.044$; Fig. 1e).

LD-RT does not impact amyloid plaques in the hippocampus of aged TgAD rats.

The $\text{MXO4}^+$ amyloid plaques, labelling fibrillar dense-core plaques [25], were counted in the hippocampus of treated and sham-treated TgAD rats (Fig. 2a). LD-RT applied weekly did not impact the density of amyloid plaques (number of plaques per $\mu$m$^2$; Two-way-ANOVA: $F_{(1,15)} = 1.384$, $p = 0.258$ for the main effect of group, $F_{(1,15)} = 1.324$, $p = 0.268$ for the main effect of hemisphere, $F_{(1,15)} = 2.594$, $p = 0.128$ for group x hemisphere interaction; Fig. 2b). We also analyzed the intensity of the staining in order to evaluate if plaques present a more diffuse morphology after treatment but no difference was obtained (Two-way-ANOVA: $F_{(1,13)} = 0.002$, $p = 0.967$ for the main effect of group, $F_{(1,13)} = 0.092$, $p = 0.767$ for the main effect of hemisphere, $F_{(1,13)} = 0.714$, $p = 0.413$ for group x hemisphere interaction; Fig. 2d). The mean plaque area (Two-way ANOVA: $F_{(1,15)} = 0.422$, $p = 0.526$ for the main effect of group, $F_{(1,15)} = 2.662$, $p = 0.124$ for the main effect of hemisphere, $F_{(1,15)} = 0.153$, $p = 0.701$ group x hemisphere interaction;
Fig. 2f) and the distribution of the number of plaques in different categories of plaque size were also unchanged by LD-RT, suggesting that LD-RT did not impact neither small (< 50 µm² corresponding to ~79.2 ± 0.9% of the plaques) or larger (> 50µm²) plaques (Chi-2 test, p > 0.999; Figure 2h). The same results were obtained with the daily schedule (number of plaques per µm²; Two-way-ANOVA: F(1,9) = 0.690, p = 0.428 for the main effect of group, F(1,9) = 2.886, p = 0.124 for the main effect of hemisphere, F(1,9) = 0.967, p = 0.351 for group x hemisphere interaction, Fig. 2c; MXO4 intensity: Two-way-ANOVA: F(1,9) = 1.400, p = 0.267 for the main effect of group, F(1,9) = 2.294, p = 0.164 for the main effect of hemisphere, F(1,9) = 0.164, p = 0.695 for group x hemisphere interaction, Fig. 2e; mean plaque area: Two-way-ANOVA: F(1,9) = 0.011, p = 0.917 for the main effect of group, F(1,9) = 1.894, p = 0.202 for the main effect of hemisphere, F(1,9) = 0.500, p = 0.497 for group x hemisphere interaction, Fig. 2g; plaque area distribution: Chi-2 test, p = 0.524, Fig. 2i).

No lateral effect of LD-RT on amyloid plaques.

To go further, we evaluated the impact of LD-RT on both diffuse and dense-core plaques, using the A4G8 antibody, in the daily treated group which presented significant memory improvements (Fig. 3a). The absence of effect of the daily treatment in the hippocampus was confirmed for all parameters studied: plaque density per µm² (Two-way-ANOVA: F(1,10) = 0.027, p = 0.872 for the main effect of group, F(1,10) = 13.67, p = 0.004 for the main effect of hemisphere, F(1,10) = 0.000, p = 0.987 for group x hemisphere interaction), A4G8 staining intensity (Two-way-ANOVA: F(1,10) = 1.000, p = 0.341 for the main effect of group, F(1,10) = 0.004, p = 0.950 for the main effect of hemisphere, F(1,10) = 0.558, p = 0.472 for group x hemisphere interaction), mean plaque area (Two-way-ANOVA: F(1,10) = 0.012, p = 0.731 for the main effect of group, F(1,10) = 3.164, p = 0.106 for the main effect of hemisphere, F(1,10) = 0.618, p = 0.450 for group x hemisphere interaction); (Fig. 3b). Thus, LD-RT did not impact either diffuse or dense-core plaques. As the radiation dose is less homogenous at the midline in the hemi-brain irradiation [22], we quantified, in addition to the hippocampus, the amyloid plaques in the cortex. No effect was observed in the cortex either: plaque density per µm² (Two-way-ANOVA: F(1,8) = 0.081, p = 0.783 for the main effect of group, F(1,8) = 15.22, p = 0.005 for the main effect of hemisphere, F(1,8) = 0.003, p = 0.955 for group x hemisphere interaction), A4G8 staining intensity (Two-way-ANOVA: F(1,8) = 0.204, p = 0.664 for the main effect of group, F(1,8) = 3.157, p = 0.114 for the main effect of hemisphere, F(1,8) = 0.073, p = 0.794 for group x hemisphere interaction), mean plaque area (Two-way-ANOVA: F(1,8) = 0.078, p = 0.788 for the main effect of group, F(1,8) = 0.034, p = 0.858 for the main effect of hemisphere, F(1,8) = 0.746, p = 0.413 for group x hemisphere interaction); (Fig. 3c). The plaque distribution in different area categories was also unchanged in both regions (Additional file 2). Consequently, the absence of impact on amyloid plaques in our rat model did not differ across the irradiated volume.

Astrocyte reactivity is unchanged by LD-RT treatments in TgAD rats.

As expected in 19-month-old animals, we observed a significant overexpression of GFAP in TgAD groups compared to WT rats, characterizing an astrocyte reactivity in this model (Two-way-ANOVA: F(2,24) =
21.67, $p < 0.0001$ for the main effect of group, $F_{(1,24)} = 0.120$, $p = 0.732$ for the main effect of hemisphere, $F_{(2,24)} = 1.269$, $p = 0.299$ for group x hemisphere interaction; LSD post hoc test: $p < 0.0001$; Fig. 4a, b). No difference was observed after LD-RT compared to the sham-treated group, whatever the regimen studied (for weekly treatment: LSD post hoc test: $p = 0.913$, Fig. 4b; for daily treatment: Two-way-ANOVA: $F_{(1,13)} = 0.102$, $p = 0.754$ for the main effect if group, $F_{(1,13)} = 0.541$, $p = 0.475$ for the main effect of hemisphere, $F_{(2,13)} = 0.102$, $p = 0.801$ for group x hemisphere interaction; Fig. 4c).

To go further, we quantified separately the molecular layer and the hilus of the hippocampus, the second region containing a clear larger number of amyloid plaques. The quantification of the percentage of the ROI positively stained with an anti-Vimentin antibody, reflecting the Vimentin$^+$ astrocyte density in the hippocampus, tended also to be increased in the molecular layer of TgAD rats (Two-way-ANOVA: $F_{(2,22)} = 3.221$, $p = 0.059$ for the main effect of group, $F_{(1,22)} = 1.824$, $p = 0.191$ for the main effect of hemisphere, $F_{(2,22)} = 2.055$, $p = 0.152$ for group x hemisphere interaction; Fig. 4d). The Vimentin$^+$ astrocyte density tended to increase or reached the significance in the hilus of sham-treated and weekly treated TgAD rats respectively, compared to WT, showing that astrocyte reactivity was mainly localized around amyloid plaques, highly present in the hilus (Two-way-ANOVA: $F_{(2,22)} = 4.634$, $p = 0.021$ for the main effect of group, $F_{(1,22)} = 3.423$, $p = 0.078$ for the main effect of hemisphere, $F_{(2,22)} = 1.561$, $p = 0.232$ for group x hemisphere interaction; LSD post hoc test: $p = 0.125$ WT vs sham-treated rats, $p = 0.006$ WT vs RT rats, $p = 0.288$ sham-treated vs RT rats; Fig. 4f). However, neither a weekly or a daily treatment influenced the Vimentin$^+$ astrocyte density compared to sham-treated animals (for weekly treatment: Two-way-ANOVA: $p = 0.059$ for the main effect of group, $p = 0.191$ for the main effect of hemisphere, $p = 0.152$ for group x hemisphere interaction in the molecular layer; Fig. 4d; LSD post hoc test: $p = 0.288$ sham-treated vs RT rats in the hilus; Fig. 4f; for daily treatment: Two-way-ANOVA: $F_{(1,12)} = 2.249$, $p = 0.160$ for the main effect if group, $F_{(1,12)} = 8.896$, $p = 0.011$ for the main effect of hemisphere, $F_{(1,12)} = 0.306$, $p = 0.590$ for group x hemisphere interaction in the molecular layer; Fig. 4e; Two-way-ANOVA: $F_{(1,13)} = 0.376$, $p = 0.550$ for the main effect if group, $F_{(1,13)} = 3.581$, $p = 0.081$ for the main effect of hemisphere, $F_{(1,13)} = 1.332$, $p = 0.270$ for group x hemisphere interaction in the hilus; Fig. 4g).

Microglial reactivity is increased by weekly LD-RT treatment in TgAD rats.

We observed a differential expression of IBA1 between groups (Two-way-ANOVA: $F_{(2,23)} = 3.455$, $p = 0.049$ for the main effect of group, $F_{(1,23)} = 3.703$, $p = 0.067$ for the main effect of hemisphere, $F_{(2,23)} = 0.897$, $p = 0.422$ for group x hemisphere interaction). The overexpression of IBA1 almost reached the significance in sham-treated TgAD groups compared to WT rats, suggesting a microglial reactivity in this model (LSD post hoc test: $p = 0.056$ WT vs sham-treated rats; Fig. 5a, b). However, no treatment effect was observed on IBA1 levels (for weekly treatment: LSD post hoc test: $p = 0.233$ sham-treated vs RT rats, Fig. 5b; for daily treatment: Two-way-ANOVA: $F_{(1,15)} = 0.288$, $p = 0.599$ for the main effect of group, $F_{(1,15)} = 1.210$, $p = 0.289$ for the main effect of hemisphere, $F_{(1,15)} = 0.619$, $p = 0.444$ for group x hemisphere interaction; Fig. 5c).
A second marker, the CD68, more specific of microglial reactivity than IBA1 expression was quantified in the molecular layer and in the hilus of the hippocampus. A clear overexpression of CD68 was observed in microglial cells associated with amyloid plaques (Fig. 5a). The quantification of the percentage of the ROI positively stained with an anti-CD68 antibody, reflecting the CD68+ microglia density in the hippocampus, revealed significant differences in the molecular layer (Two-way-ANOVA: $F_{(2,23)} = 6.245$, $p = 0.007$ for the main effect of group, $F_{(1,23)} = 1.319$, $p = 0.263$ for the main effect of hemisphere, $F_{(2,23)} = 5.859$, $p = 0.009$ for group x hemisphere interaction; Fig. 5d) and in the hilus (Two-way-ANOVA: $F_{(2,26)} = 7.806$, $p = 0.002$ for the main effect of group, $F_{(1,26)} = 1.950$, $p = 0.174$ for the main effect of hemisphere, $F_{(2,26)} = 1.114$, $p = 0.344$ for group x hemisphere interaction; Fig. 5f). Indeed, the CD68+ microglia density significantly increased in both regions in TgAD rats compared to WT rats (LSD post hoc test: $p = 0.018$ WT vs sham-treated rats, $p = 0.003$ WT vs RT rats in the molecular layer, Fig. 5d; $p = 0.068$ WT vs sham-treated rats, $p = 0.002$ WT vs RT rats in the hilus, Fig. 5f). Moreover, a significant increase of CD68+ microglial density was observed in the weekly treated hemisphere compared to the contralateral side in the molecular layer (LSD post hoc test: $p = 0.001$). These results showed a slight increase of microglial reactivity in the hippocampus after a weekly but not daily exposure to LD-RT (Two-way-ANOVA: $F_{(1,12)} = 0.830$, $p = 0.380$ for the main effect of group, $F_{(1,12)} = 0.062$, $p = 0.807$ for the main effect of hemisphere, $F_{(1,12)} = 0.135$, $p = 0.720$ for group x hemisphere interaction, in the molecular layer, Fig. 5e; Two-way-ANOVA: $F_{(1,12)} = 0.128$, $p = 0.727$ for the main effect of group, $F_{(1,12)} = 0.006$, $p = 0.938$ for the main effect of hemisphere, $F_{(1,12)} = 0.081$, $p = 0.781$ for group x hemisphere interaction, in the hilus, Fig. 5g).

**Discussion**

Our study shows that the same LD-RT dose delivered weekly or daily does not have the same therapeutic potential. Indeed, only the dose of 10 Gy delivered in 5 fractions daily presented interesting therapeutic effects at an advanced stage of the pathology as it improved efficiently the memory of TgAD rats (+ 42%) and restored their locomotor activity. However, this regimen did not impact amyloid plaques or neuroinflammation markers. A slight increase of astrocyte and microglia reactivity was instead observed in the treated hemisphere with the weekly schedule, without any influence on amyloid load.

The toxic side effects of high-dose RT for brain tumors are well described [26, 27]. The doses used in this protocol are more than three times lower compared to those used in clinical practice for brain metastases or focal glioblastoma for example and the long-term impact of low-doses irradiation are not fully understood. The toxicity of RT, even at low doses, could be related to hippocampus exposure [24]. Indeed, the two key niches of neural stem cells in the adult brain are present in the subventricular zone and the subgranular layer of the dentate gyrus in the hippocampus [28], making the hippocampus a particularly sensitive region to irradiation [29]. We verified that both regimens did not induce hippocampal atrophy by measuring the total volume of the hippocampus in sham-treated and irradiated animals and also did not induce neuronal loss in the subgranular zone of the dentate gyrus. These results validate the safety of regimens delivered weekly and daily in the TgAD rat model.
The primary aim of this study was to evaluate the therapeutic potential of both radiation regimens when applied at a stage presenting a severe amyloid pathology. The main cognitive alteration in AD being memory deficits, we pay attention to the effect of both treatments on the memory alteration displayed by TgAD rats at 14-month-old. LD-RT delivered weekly did not ameliorate the spatial working memory of treated rats in contrary to the regimen delivered daily which improved it significantly. Interestingly, both regimens reduced the locomotor activity of rats. As a hyperactivity is described in this model [20], this reduction suggests a restoration of the normal behavior of TgAD rats. Consequently, the regimen 2 Gy x 5 fractions delivered daily presents more therapeutic potential.

A positive effect of LD-RT on amyloid load was previously reported in patients presenting peripheral amyloidosis [30–36] and recently in three AD mouse models [15–17]. Nevertheless, this reduction of the number of amyloid plaques was not observed in our rat model. We went further by analyzing plaques with two different methods: Methoxy-XO4 (MXO4), a fluorescent derivative of Congo Red, which labels fibrillar/dense-core plaques [25] and the A4G8 antibody which stains both diffuse and fibrillar/dense-core plaques [37]. No difference was observed for either method, showing that neither diffuse nor fibrillar/dense-core plaques are affected by LD-RT, whatever the regimen. MXO4 and A4G8 staining intensities, which could also reflect the plaque morphology (diffuse vs fibrillar/dense-core plaques), and the size of each individual plaques were also analyzed. LD-RT did not impact plaque morphology, without any differential effect for small or larger plaques, as shown by size distribution analyses. The major difference between this study and the published results in AD mice, in addition to the species studied, is that we applied the treatment at an advanced amyloid stage, whereas studies in mice applied LD-RT at the beginning of amyloid deposit formation. Indeed, for example, our model presented 8326 ± 1562 plaques only in the hippocampus of sham-treated rats compared to 63 ± 6.64 plaques in the whole brain of sham-treated APP/PS1 mice used in the study of Marples and collaborators [15]. Consequently, it is possible that the pathology in our TgAD rats was already too severe to observe a reduction of amyloid plaques in the hippocampus or in the cortex. A recent publication supports this hypothesis as the decrease of amyloid plaques was not observed after the same LD-RT regimen in 5XFAD mice, treated when an aggressive amyloid pathology was already present [18]. Moreover, we cannot exclude that LD-RT did not impact amyloid plaques but decreased the soluble forms of the Aβ such as Aβ40, Aβ42 and/or Aβ oligomers, known to be the most toxic forms [38]. Further analyses are consequently necessary to understand the impact of LD-RT on those soluble forms. Another hypothesis is the importance of the delay post treatment. Indeed, in the publications mentioned above, the maximal delay post RT analyzed is 8 weeks [15–17]. Thus, it is possible that 4 months post RT, as in our study, the beneficial effect of LD-RT on the amyloid is caught up by the disease progression.

Furthermore, it is important to keep in mind that amyloid load is not directly related to cognitive deficits. In addition to amyloid, the neuroinflammation now appears as a key player in AD pathology. For example, it has been recently demonstrated a functional link between neuroinflammation and functional network/cognitive impairment using PET imaging and functional magnetic resonance imaging in AD patients [39]. An anti-inflammatory effect of LD-RT, as shown in benign and degenerative inflammatory diseases in periphery [40–42], could obviously be an interesting aspect for AD. Neuroinflammation
partners in the brain are astrocyte and microglial cells. Both are known to react to alteration in the brain tissue homeostasis. This reaction is characterized by a reactive state, classically identified through the overexpression of markers such as GFAP and Vimentin for astrocytes and IBA1 and CD68 for microglia, as examples. These morphological changes are accompanied by transcriptomic and functional modifications, which clearly influence AD pathology progression [8, 9, 12]. The anti-inflammatory effect of LD-RT, associated with the decrease of amyloid plaques, was also observed in AD mice [15, 18].

Unexpectedly, our study showed that a weekly treatment by LD-RT did not reduce astrocyte reactivity and microglial activation but on the contrary, induced an increase of CD68 overexpression in the molecular layer of the hippocampus. Nevertheless, this increase seems to be slight and is not accompanied by cognitive benefits. Thus, complementary analyses on functional changes induced by LD-RT are necessary. The daily treatment did not influence the expression of the markers studied, suggesting that the delay between each irradiation may be an important parameter in the neuroinflammatory response. Besides, as reactive astrocytes and microglial activation are mainly observed around amyloid plaques, it is possible that the amyloid pathology of our animals was too important to expect a reduction of neuroinflammation at this stage.

On the contrary, we may also hypothesize that daily LD-RT did not reduce amyloid load because it is not able to modulate neuroinflammation partners. The mechanisms by which LD-RT reduces efficiently the amyloid load in peripheral pathologies are not understood. One hypothesis suggests a direct effect of LD-RT on the β-sheet structure of amyloid [43]. But it is also possible that this decrease of amyloid is due to a modulation of inflammation. Indeed, peripheral macrophages can internalize Aβ [44] but this ability could be altered in presence of chronic inflammation, as it is the case for microglial cells at an advanced stage of AD [45]. In our model, LD-RT does not reduce inflammation and therefore does not enhance Aβ clearance capacities of microglia and astrocytes. Consequently, it would be interesting to perform the same experiments in younger animals to validate this hypothesis.

Finally, protective effects of LD-RT have been described against neurodegeneration. Indeed, in 5XFAD mice, a restoration of the immunoreactivity of the neuronal marker NeuN and the pre-synaptic marker synaptophysin was described in the hippocampus (CA1, CA3 and in the molecular layer) after LD-RT [18]. An increase of the microtubule-associated protein 2 (MAP2) and the postsynaptic density protein 95 (PSD95) was also observed in the hippocampus of AD mice after chronic irradiation [46]. These results supposed a restoration of synaptic and neuronal integrity. At the transcriptomic level [46], a modulation of pathways related to synaptic plasticity and a significant decrease of synaptic neurodegenerative processes was pointed out in the hippocampus after a cumulative dose of 6 Gy but not at lower dose (0.3 Gy). Interestingly, a reduction of phosphorylation pathways of microtubule-associated protein tau (Mapt) is also observed in the GO term analysis, suggesting a reduction of the hyperphosphorylation of the tau protein, known to be related with its disassembly with microtubules, their disintegration and the aggregation of tau in NFT [6, 47, 48]. Consequently, the restoration of synaptic and neuronal integrity, in addition to a modulation of hyperphosphorylated forms of tau could explain the memory improvement observed in our model.
One major limitation of this study is the well-advanced amyloid pathology. Indeed, as discussed, it is highly possible that LD-RT treatments were applied too late in the pathology to expect to have a reduction of amyloid plaques and neuroinflammatory markers. Nevertheless, it could be interesting to measure the soluble forms of amyloid peptides, known to be the more toxic forms in AD. As our tissue samples were prepared for immunohistology, it was not possible to process the biochemical experiments to realize these types of measurements. Consequently, further analyses are necessary to better understand the impacts of LD-RT in the brain at early and late stages.

**Conclusions**

In our study, we validated that LD-RT with a schedule delivering 5 daily fractions of 2 Gy improves memory abilities of TgAD rats unlike the same RT dose using 2 Gy delivered once a week. With this regimen, we were unable to replicate previously reported effects on amyloid load or neuroinflammatory mechanisms. A slight increase of microglial activation markers was observed only in the treated hemisphere of weekly treated rats, however without associated memory or behavioral changes. These results contribute to the evidence of a biological effect of LD-RT in AD, in favor of a daily scheme. The lack of effect on amyloid or neuroinflammation at an advanced stage of pathology highlights the importance to study the mechanisms of action of LD-RT at an earlier disease stage.

**Abbreviations**

AD: Alzheimer’s disease  
Aβ: Amyloid beta  
CD68: Cluster of Differentiation 68  
GFAP: Glial fibrillary acidic protein  
Gy: Grays  
IBA1: Ionized calcium binding adaptor molecule 1  
LD-RT: Low-dose radiation therapy  
MAP2: Microtubule-associated protein 2  
MXO4: Methoxy-XO4  
NFT: Neurofibrillary tangles  
OTT: Overall treatment time  
PSD95: Postsynaptic density protein 95
Declarations

Ethics approval and consent to participate

All the experimentation proceeds on animals were conducted with respect of the ethical approval by the Ethics Committee for Animal Experimentation of the Canton of Geneva, Switzerland (GE9917).

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KC, ABF and BBT performed the experiments and analyses. KC, TZ, BBT and VG interpreted the data and write the article. KC, TZ, ABF, PM, NK, GD, GBF, BBT and VG revised the manuscript.

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References
1. Masters CL, Bateman R, Blennow K, Rowe CC, Sperling RA, Cummings JL. Alzheimer's disease. Nat Rev Dis Primers. 2015;1:15056.

2. Thal DR, Rüb U, Orantes M, Braak H. Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology. 2002;58:1791–800.

3. Dickson TC, Vickers JC. The morphological phenotype of beta-amyloid plaques and associated neuritic changes in Alzheimer's disease. Neuroscience. 2001;105:99–107.

4. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. Science. 1992;256:184–5.

5. Mormino EC, Papp KV. Amyloid Accumulation and Cognitive Decline in Clinically Normal Older Individuals: Implications for Aging and Early Alzheimer's Disease. J Alzheimers Dis. 2018;64:S633–46.

6. Barbier P, Zejneli O, Martinho M, Lasorsa A, Belle V, Smet-Nocca C, et al. Role of Tau as a Microtubule-Associated Protein: Structural and Functional Aspects. Front Aging Neurosci. 2019;11:204.

7. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 1991;82:239–59.

8. Ben Haim L, Carrillo-de Sauvage M-A, Ceyzériat K, Escartin C. Elusive roles for reactive astrocytes in neurodegenerative diseases. Front Cell Neurosci. 2015;9:278.

9. Escartin C, Guillemaud O, Carrillo-de Sauvage M-A. Questions and (some) answers on reactive astrocytes. Glia. 2019;67:2221–47.

10. Bagyinszky E, Giau VV, Shim K, Suk K, An SSA, Kim S. Role of inflammatory molecules in the Alzheimer's disease progression and diagnosis. J Neurol Sci. 2017;376:242–54.

11. Ries M, Sastre M. Mechanisms of Abeta Clearance and Degradation by Glial Cells. Frontiers in aging neuroscience. 2016;8:160.

12. De Strooper B, Karran E. The Cellular Phase of Alzheimer's Disease. Cell. 2016;164:603–15.

13. Ceyzériat K, Zilli T, Millet P, Frisoni GB, Garibotto V, Tournier BB. Learning from the Past: a Review of Clinical Trials Targeting Amyloid, Tau and Neuroinflammation in Alzheimer's Disease. Curr Alzheimer Res. 2020;

14. Ceyzériat K, Tournier BB, Millet P, Frisoni GB, Garibotto V, Zilli T. Low-Dose Radiation Therapy: A New Treatment Strategy for Alzheimer's Disease? J Alzheimers Dis. 2020;

15. Marples B, McGee M, Callan S, Bowen SE, Thibodeau BJ, Michael DB, et al. Cranial irradiation significantly reduces beta amyloid plaques in the brain and improves cognition in a murine model of Alzheimer's Disease (AD). Radiotherapy and Oncology. 2016;118:43–51.

16. Wilson GD, Wilson TG, Hanna A, Kulchycki J, Buelow K, Pruetz BL, et al. Low Dose Brain Irradiation Reduces Amyloid-β and Tau in 3xTg-AD Mice. J Alzheimers Dis. 2020;

17. Kim S, Chung H, Ngoc Mai H, Nam Y, Shin SJ, Park YH, et al. Low-Dose Ionizing Radiation Modulates Microglia Phenotypes in the Models of Alzheimer's Disease. Int J Mol Sci [Internet]. 2020 [cited 2020 Jul 21];21. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7353052/
18. Kim S, Nam Y, Kim C, Lee H, Hong S, Kim HS, et al. Neuroprotective and Anti-Inflammatory Effects of Low-Moderate Dose Ionizing Radiation in Models of Alzheimer’s Disease. Int J Mol Sci. 2020;21.

19. Garibotto V, Frisoni GB, Zilli T. Re: Cranial irradiation significantly reduces beta amyloid plaques in the brain and improves cognition in a murine model of Alzheimer’s Disease (AD). Radiother Oncol. 2016;118:577–8.

20. Cohen RM, Rezai-Zadeh K, Weitz TM, Rentsendorj A, Gate D, Spivak I, et al. A Transgenic Alzheimer Rat with Plaques, Tau Pathology, Behavioral Impairment, Oligomeric Aβ, and Frank Neuronal Loss. J Neurosci. 2013;33:6245–56.

21. Kriz J, Seegenschmiedt HM, Bartels A, Micke O, Muecke R, Schaefer U, et al. Updated strategies in the treatment of benign diseases—a patterns of care study of the german cooperative group on benign diseases. Adv Radiat Oncol. 2018;3:240–4.

22. Koutsouvelis N, Rouzaud M, Dubouloz A, Nouet P, Jaccard M, Garibotto V, et al. 3D printing for dosimetric optimization and quality assurance in small animal irradiations using megavoltage X-rays. Zeitschrift Fur Medizinische Physik. 2020;30:227–35.

23. Ashby D. Practical statistics for medical research. Douglas G. Altman, Chapman and Hall, London, 1991. No. of pages: 611. Price: £32.00. Statistics in Medicine. 1991;10:1635–6.

24. Gondi V, Hermann BP, Mehta MP, Tomé WA. Hippocampal dosimetry predicts neurocognitive function impairment after fractionated stereotactic radiotherapy for benign or low-grade adult brain tumors. Int J Radiat Oncol Biol Phys. 2012;83:e487-493.

25. Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT. Neuropathological alterations in Alzheimer disease. Cold Spring Harb Perspect Med. 2011;1:a006189.

26. Makale MT, McDonald CR, Hattangadi-Gluth JA, Kesari S. Mechanisms of radiotherapy-associated cognitive disability in patients with brain tumours. Nat Rev Neurol. 2017;13:52–64.

27. Monje ML, Mizumatsu S, Fike JR, Palmer TD. Irradiation induces neural precursor-cell dysfunction. Nat Med. 2002;8:955–62.

28. Bacigaluppi M, Sferruzza G, Butti E, Ottoboni L, Martino G. Endogenous neural precursor cells in health and disease. Brain Research. 2020;1730:146619.

29. Kazda T, Jancalek R, Pospisil P, Sevela O, Prochazka T, Vrzal M, et al. Why and how to spare the hippocampus during brain radiotherapy: the developing role of hippocampal avoidance in cranial radiotherapy. Radiat Oncol. 2014;9:139.

30. Neuner GA, Badros AA, Meyer TK, Nanaji NM, Regine WF. Complete resolution of laryngeal amyloidosis with radiation treatment. Head Neck. 2012;34:748–52.

31. Luo M, Peng G, Shi L, Ming X, Li Z, Fei S, et al. Intensity-modulated radiotherapy for localized nasopharyngeal amyloidosis: Case report and literature review. Strahlenther Onkol. 2016;192:944–50.

32. Cooper CT, Greene BD, Fegan JE, Rovira D, Gertz MA, Marcus DM. External beam radiation therapy for amyloidosis of the urinary bladder. Pract Radiat Oncol. 2018;8:25–7.
33. Leibovitch I, Selva D, Goldberg RA, Sullivan TJ, Saeed P, Davis G, et al. Periocular and orbital amyloidosis: clinical characteristics, management, and outcome. Ophthalmology. 2006;113:1657–64.

34. Khaira M, Mutamba A, Meligonis G, Rose GE, Plowman PN, O’Donnell H. The use of radiotherapy for the treatment of localized orbital amyloidosis. Orbit. 2008;27:432–7.

35. Ren S, Ren G. External beam radiation therapy is safe and effective in treating primary pulmonary amyloidosis. Respir Med. 2012;106:1063–9.

36. Copperman TS, Truong MT, Berk JL, Sobel RK. External beam radiation for localized periocular amyloidosis: a case series. Orbit. 2019;38:210–6.

37. Alafuzoff I, Pikkarainen M, Arzberger T, Thal DR, Al-Sarraj S, Bell J, et al. Inter-laboratory comparison of neuropathological assessments of beta-amyloid protein: a study of the BrainNet Europe consortium. Acta Neuropathol. 2008;115:533–46.

38. Walsh DM, Selkoe DJ. A beta oligomers - a decade of discovery. J Neurochem. 2007;101:1172–84.

39. Passamonti L, Tsvetanov KA, Jones PS, Bevan-Jones WR, Arnold R, Borchert RJ, et al. Neuroinflammation and Functional Connectivity in Alzheimer’s Disease: Interactive Influences on Cognitive Performance. J Neurosci. 2019;39:7218–26.

40. Rödel F, Frey B, Gaipl U, Keilholz L, Fournier C, Manda K, et al. Modulation of inflammatory immune reactions by low-dose ionizing radiation: molecular mechanisms and clinical application. Curr Med Chem. 2012;19:1741–50.

41. Rödel F, Keilholz L, Herrmann M, Sauer R, Hildebrandt G. Radiobiological mechanisms in inflammatory diseases of low-dose radiation therapy. Int J Radiat Biol. 2007;83:357–66.

42. Arenas M, Sabater S, Hernández V, Rovirosa A, Lara PC, Biete A, et al. Anti-inflammatory effects of low-dose radiotherapy. Indications, dose, and radiobiological mechanisms involved. Strahlenther Onkol. 2012;188:975–81.

43. Bistolfi F. Localized amyloidosis and Alzheimer’s disease: the rationale for weekly long-term low dose amyloid-based fractionated radiotherapy. Neuroradiol J. 2008;21:683–92.

44. Lai AY, McLaurin J. Clearance of amyloid-β peptides by microglia and macrophages: the issue of what, when and where. Future Neurol. 2012;7:165–76.

45. Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer’s disease. Lancet Neurol. 2015;14:388–405.

46. Kempf SJ, Janik D, Barjaktarovic Z, Braga-Tanaka I, Tanaka S, Neff F, et al. Chronic low-dose-rate ionising radiation affects the hippocampal phosphoproteome in the ApoE-/- Alzheimer’s mouse model. Oncotarget. 2016;7:71817–32.

47. Derisbourg M, Leghay C, Chiappetta G, Fernandez-Gomez F-J, Laurent C, Demeyer D, et al. Role of the Tau N-terminal region in microtubule stabilization revealed by new endogenous truncated forms. Sci Rep. 2015;5:9659.
48. Chang E, Kim S, Yin H, Nagaraja HN, Kuret J. Pathogenic missense MAPT mutations differentially modulate tau aggregation propensity at nucleation and extension steps. Journal of neurochemistry. 2008;107:1113.