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Short Communication

The sparing effect of FLASH-RT on synaptic plasticity is maintained in mice with standard fractionation

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ABSTRACT

Long-term potentiation (LTP) was used to gauge the impact of conventional and FLASH dose rates on synaptic transmission. Data collected from the hippocampus and medial prefrontal cortex confirmed significant inhibition of LTP after 10 fractions of 3 Gy (30 Gy total) conventional radiotherapy. Remarkably, 10x3Gy FLASH radiotherapy and unirradiated controls were identical and exhibited normal LTP.

Ultra-high dose rate "FLASH" radiotherapy (FLASH-RT) spares normal tissue toxicities when compared to conventional dose rate radiotherapy (CONV-RT) yet does not compromise anti-tumor efficacy. The combination of these in vivo effects has been coined the FLASH effect and provides opportunities for expanding the therapeutic window to improve patient outcomes and quality of life. Whether this can be realized in the clinic, however, will depend in large part on carefully controlled preclinical studies working within established beam parameters that have been rigorously validated in vivo using selected biological outcomes.

To date, most reports documenting the neurological benefits of FLASH-RT, have implemented single dose or hypofractionated FLASH-RT regimens [1–5]. Whether such benefits could be realized under standard-of-care fractionation protocols used to treat certain brain malignancies such as multiple metastases or other cancers has been a matter of debate. To address this gap in knowledge directly, we implemented a standard-of-care fractionation regimen (10 fractions of 3 Gy, 30 Gy total, BED = 60 Gy using α/β of 3 for normal brain) delivered at FLASH- or CONV dose rates and evaluated mice 4 months later using an electrophysiologic measure of synaptic plasticity, long-term potentiation (LTP). The details of LTP have been studied extensively in laboratory animals for more than 45 years [6]. Naturalistic neuronal firing patterns in the theta frequency firing range that have been observed during learning, and necessary for long-term memory, activate a vast array of membrane receptors, second messenger pathways, and structural proteins (among others) that orchestrate the translocation of synaptic vesicles and re-organization of pre- and post-synaptic mediators that change synaptic structure. The end point leads to the strengthening of synaptic function to enhance communication between synapses. Establishing an LTP-like effect in humans is now on the way and it will be interesting to see if learning-induced stimuli can indeed induce human-LTP in cortical synapses [7]. As such, it has provided a functional readout of CNS functionality that often tracks with changes in cognition in rodents, and our past work under hypofractionated dosing regimens has confirmed that FLASH-RT can spare multiple indices of behavioral performance and theta burst-induced LTP, whereas CONV-RT impairs these endpoints [8,9]. Here we provide the first report demonstrating preservation of LTP after a standard-of-care fractionation regimen suggesting that the sparing effect of FLASH is maintained.

Materials and methods

Animals

Animal procedures were conducted in accordance with the Swiss ethics committee (VD3852) and the University of California, Irvine Institutional Animal Care and Use Committee (IACUC, AUP-

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21–025) for animal experimentation. C57BL/6J female mice (n = 6/treatment) were purchased from Charles River Laboratories (France) and were allowed to acclimate. Mice were 11 weeks of age at the time of irradiation.

Irradiation

Whole-brain irradiations were performed on a prototype Oriatron 6e, 6-MeV electron beam linear accelerator (LINAC) at the Lausanne University Hospital (Lausanne, Switzerland)[10]. Extensive description of dosimetric protocols supporting whole brain irradiations with this LINAC has been previously published, including lateral and depth dose profiles of both FLASH and CONV beams [10,11]. Mice received 10 whole-brain, head only, doses of 3 Gy, from Monday to Friday using a 17-mm diameter graphite applicator at either CONV dose rate (0.09 Gy/s) or ultra-high-dose-rate FLASH delivered in a single 1.8 μs pulse (1.6×10^6 Gy/s). In both cases, prescription dose was defined as surface dose in a 30×30 cm^2 solid water phantom centered behind the applicator, which was shown to correspond (within 2 %) to the dose recorded by thermoluminiscent detectors (TLDs) positioned in the proximal part of the brain between two cerebral hemispheres.

Electrophysiology

Female mice (n = 6/treatment, 18 total) were sampled for electrophysiology and hippocampal slices prepared as described previously [12]. The uteri of female mice were dissected and weighed prior to LTP assessments, confirming that none of the subjects were in estrus. Mice were anesthetized, decapitated, and the brains rapidly removed into ice-cold, oxygenated dissection medium containing (in mM): 124 NaCl, 3 KCl, 1.25 KH2PO4, 5 MgSO4, 0 CaCl2, 26 NaHCO3, and 10 glucose. Hippocampal and prefrontal cortex slices (320 μm, coronal) were cut from a vibratome (Leica, Model:VT1000S) before transfer to an interface recording chamber containing prewarmed (31 ± 10C) artificial cerebrospinal fluid (aCSF) composed of (in mM): 124 NaCl, 3 KCl, 1.25 KH2PO4, 1.5 MgSO4, 2.5 CaCl2, 26 NaHCO3, and 10 glucose. Slices were perfused continuously at a rate of 1.75–2 ml/min while the surface of the slices were exposed to warm, humidified 95% O2/5% CO2. Recordings began following at least 2 hr of incubation.

Field excitatory postsynaptic potentials (fEPSPs) were recorded from CA1b stratum radiatum apical dendrites using a glass pipette filled with 2 M NaCl (2–3 MΩ) in response to orthodromic stimulation (twisted nichrome wire, 65 μm diameter) of Schaffer collateral-commissural projections in CA1 stratum radiatum. Pulses were administered 0.05 Hz using a current that elicited a 50% maximal spike-free response. After maintaining a stable baseline (20 min), long-term potentiation (LTP) was induced by delivering 5 'theta' bursts, with each burst consisting of four pulses at 100 Hz separated by 200 msec (i.e., theta burst stimulation or TBS). The stimulation intensity was not increased during TBS.

Coronal slices (1.70 – 1.98 mm anterior to bregma) from the ventral medial prefrontal cortex were prepared as described above. Field recordings were obtained by placing a bipolar stimulation electrode (FHC, 25 um diameter) within cortical layer IV and a glass recording electrode in layer III (Kirkwood and Bear, 1994; J. Neuroscie; Hebbian Synapses in visual cortex). LTP was induced by delivering 4 trains of 5 theta bursts at 0.05 Hz. The stimulation intensity was not increased during TBS. Data from both brain regions were collected and digitized by NAC 2.0 (Neurodata Acquisition System, Theta Burst Corp., Irvine, CA) and stored on a disk.

Data in the text are presented as means ± SD, while in the figures as mean ± SEM. The fEPSP slope was measured at 10–90% fall of the slope and data in figures on LTP were normalized to the last 20 min of baseline. Electrophysiological measures were analyzed using a 1-way ANOVA. Electrophysiological assessments and analyses were done blinded to the treatment groups.

Results

Long-term potentiation following standard fractionation is preserved after FLASH-RT but not CONV-RT, 4 months after irradiation. A schematic of the experimental set-up for measuing LTP is shown in Fig. 1.

Theta burst stimulation (TBS) applied to the Schaffer collaterals within the hippocampus produced a rapid and robust increase in LTP, quantified as the relative change in the slope of evoked field

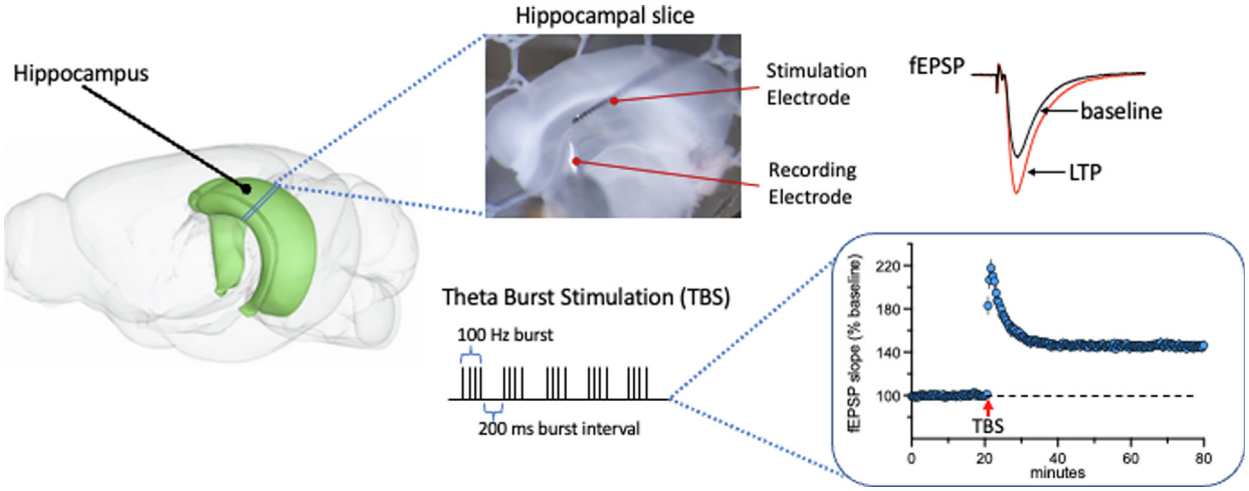

Fig. 1. Schematic representation of long-term potentiation (LTP) recordings in the mouse hippocampus. Hippocampal slices are sectioned and equilibrated in artificial cerebrospinal fluid in preparation for electrophysiological assessments across the selected brain region. Theta bursts stimulation (TBS) delivered by the stimulating electrode is used to evoke field excitatory postsynaptic potentials (fEPSPs) measured by the recording electrode. After the TBS, the slope of the fEPSPs (derived from downstream dendrites) gradually decays to more stable levels of potentiation. This latter measure of sustained potentiation is significantly reduced after CONV-RT when compared to FLASH-RT or unirradiated controls that maintain higher levels of stable potentiation. Procedures performed in the hippocampus are functionally equivalent to those performed in the medial prefrontal cortex.

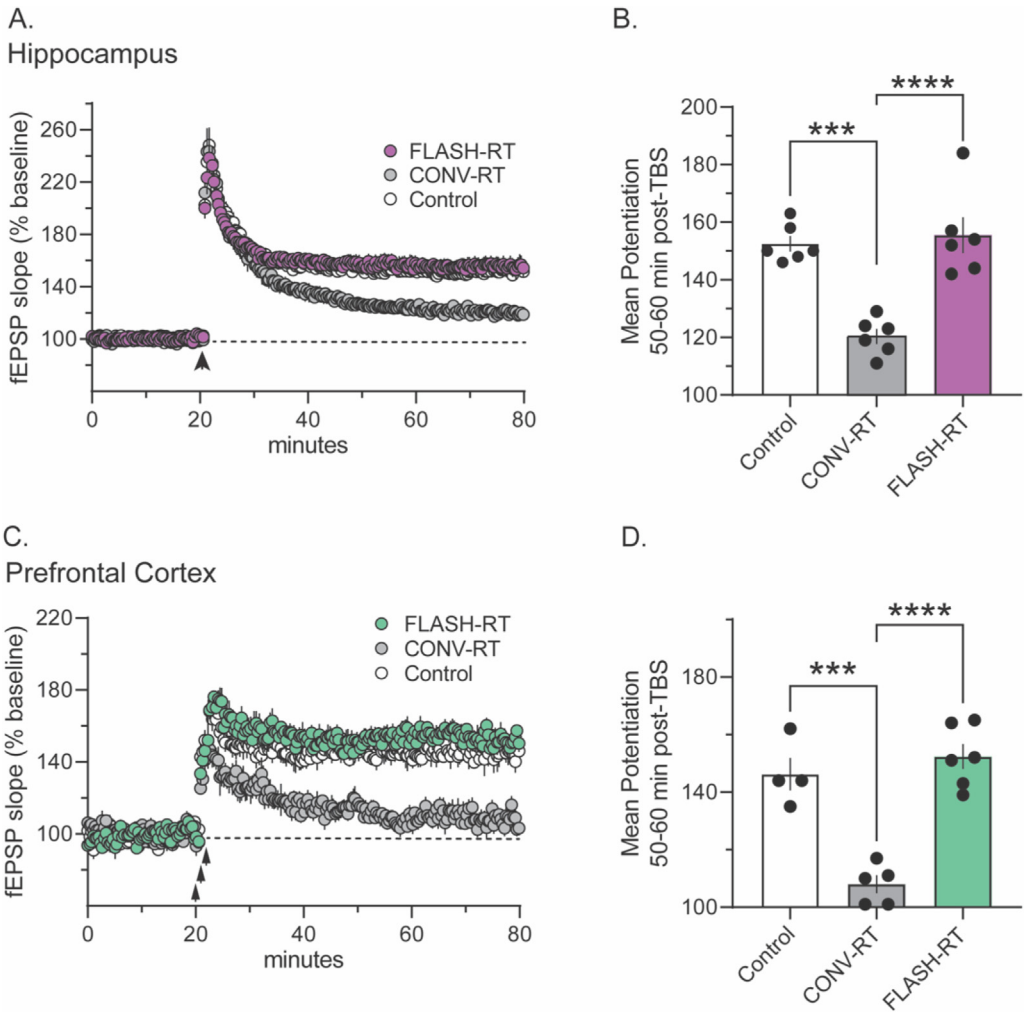

Fig. 2. LTP is maintained following a standard fractionation regimen of FLASH-RT, but not CONV-RT. (**A**) Theta burst stimulation (TBS) applied to the Schaffer collaterals in the hippocampus yields a robust increase in fEPSP slope (as percent of baseline) in unirradiated control and FLASH irradiated female mice but is reduced significantly in CONV mice four months after exposure (upper panels). (**B**) Levels of potentiation in the fEPSP slope maintained 1 h post-TBS were markedly reduced in the hippocampus of CONV-RT mice, but not in control or FLASH irradiated mice. Similarly, TBS in applied to layer IV of the pre/infralimbic subregions of the mPFC layers yielded remarkably similar outcomes, where FLASH-RT was found to maintain the increase in fEPSP slope **(C)** and potentiation 1 h post-TBS **(D)** compared to CONV-RT. For each treatment there were 6 animals/cohort. Scale: 1 mV/5 ms. Data were analyzed using a one-way ANOVA followed by Bonferroni's multiple comparison test. *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

excitatory postsynaptic potentials (fEPSPs) generated by CA1 apical dendrites (Fig. 2**A**). Following the TBS, the fEPSP slope immediately increased and then gradually decayed to more stable levels of potentiation for all cohorts. Notably, mean potentiation levels in the fEPSP slope maintained at 1 h post-TBS were reduced significantly in the hippocampus following CONV-RT, but not in unirradiated control or following FLASH-RT (Fig. 2**B**; one-way ANOVA: $F_{(2,15)} = 21.89$, $P < 0.0001$; Bonferroni *post-hoc*: CONTROL vs CONV: $P = 0.0002$; FLASH vs CONV: $P < 0.0001$).

Using a similar experimental paradigm, TBS applied to layer IV of the pre/infralimbic subregions of the medial prefrontal cortex (mPFC) elicited similar outcomes. The robust increase in fEPSPs 50–60 min post-TBS were statistically similar between Control and FLASH cohorts but were inhibited significantly following CONV-RT (Fig. 2**C**). As in the hippocampus, mean potentiation levels in the mPFC 1 h post-TBS were again reduced after CONV-RT, but spared after FLASH-RT (Fig. 2**D**; one-way ANOVA: $F_{(2,12)} = 31$, $P < 0.0001$; Bonferroni *post-hoc*: Control vs CONV: $P = 0.0002$; FLASH vs CONV: $P < 0.0001$). As these data were collected 4-months post-irradiation, results indicate that radiation-induced changes were relatively permanent and point to the capability of FLASH-RT to preserve a critical readout of synaptic plasticity.

Discussion

Here we report on the capability of FLASH-RT delivered in a clinically used standard fractionation protocol to preserve LTP, in marked contrast to the inhibition of LTP found under CONV-RT. In the clinic, whole brain RT is used under an identical fractionation regimen for the control of multiple brain metastasis [13,14]. Cognitive endpoints have been used in multiple recent clinical trials of whole brain RT [15,16], in which most patients develop measurable cognitive decline by 4 months after irradiation. These studies, which delivered whole brain RT in 10 fractions of 3 Gy, also evaluated the cognitive sparing effects of memantine, a NMDAR antagonist known to attenuate tonic excitation, and intensity-modulated radiation therapy (IMRT) to reduce the dose specifically to the hippocampus [15–17]. Results show that memantine and hippocampal sparing IMRT provide modest relative sparing of cognitive decline. These intermediate findings have now been corroborated in the recent final report by Gondi et.al., [18] that showed whole brain IMRT delivered in 10 fractions of 3 Gy + memantine + hippocampal avoidance sustained preservation of cognitive function and prevention of patient reported neurologic symptoms compared to the same treatment without hippocampal avoidance. No differences in survival or other toxicity were reported for either treatment.

The N107C/CEC.3 trial found that after brain metastasis resection, stereotactic radiosurgery (in which the volume of brain irradiation is limited) was associated with far less loss of early and late cognitive function and quality of life than whole brain RT, but with only about half the 12-month intracranial control rate [19].

A treatment strategy providing both high intracranial control and cognitive sparing remains an important clinical need. The current results demonstrating lack of electrophysiologic change after standard fractionation FLASH compared to unirradiated controls is a first step supporting the applicability of FLASH under an accepted clinical regimen. In recent and ongoing work in mice, we found nearly identical results with hypofractionated regimens [8,9] and single dose (10 Gy, manuscript in preparation) FLASH irradiation, suggesting that there is no dose response for LTP with a possible threshold at or below 10 Gy single fraction or equivalent. Nonetheless, this work indicates that conventional fractionation does not preclude achieving the FLASH sparing effect at least in the brain, although further work confirming the anti-tumor efficacy of FLASH under these conditions is also necessary. As the clinical translation of FLASH-RT awaits further preclinical data, current results suggest that WBRT delivered at FLASH dose rates has the potential to control brain metastases without incurring similar levels of neurologic toxicity as current standard of practice.

Conclusions

To date, single dose and hypo-fractionated regimens of whole brain FLASH-RT have been shown to reduce the adverse cognitive and pathological complications routinely observed after the same fractionation delivered with CONV-RT. In this study, our aim was to evaluate the impact of a clinically used standard fractionation regimen on brain function. Ten fractions of 3 Gy (10x3Gy) were delivered whole brain over two weeks using CONV and FLASH (eRT6/Oriatron) and theta burst-induced LTP was used to provide direct readouts of the strength of synaptic transmission. While behavioral testing remains the gold standard for validating the functional impact of cranial irradiation on the brain, electrophysiological assessments and LTP are direct measurements of synaptic plasticity, a cellular mechanism thought to underlie memory processes. Our previous results in pediatric and adult mouse models showed that consistently with neurocognitive preservation, LTP was preserved after FLASH-RT when delivered in single and hypo-fractionated regimens but was significantly inhibited after the same doses of CONV-RT. In this study, we again found no adverse impact of FLASH-RT on LTP, where responses were statistically indistinguishable from unirradiated controls across both brain regions analyzed. Contrary to these findings, CONV-RT resulted in significant inhibition of LTP. Importantly, and given that the high 30 Gy total dose was delivered in smaller, daily fractions of 3 Gy, radiation-induced oxygen depletion remains an unlikely mechanistic explanation to account for present findings, a topic discussed at length in our recent reviews of FLASH-RT [20,21]. While further work is ongoing to establish the temporal response of the electrophysiologic benefits observed after FLASH-RT, whether LTP can serve as an early biomarker, and the sustained anti-tumor efficacy of conventionally fractionated FLASH, these results provide the first evidence that brain functionality is preserved after standard fractionation with FLASH-RT.

Declaration of competing interest

BWL has received research support from Varian Medical Systems, is a cofounder and board member of TibaRay, and is a consultant on a clinical trial steering committee for BeiGene.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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