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Aging-dependent changes in the radiation response of the adult rat brain

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Abstract

Purpose—To assess the impact of aging on the radiation response in the adult rat brain.

Methods and Materials—Male rats 8, 18, or 28 months of age received a single 10Gy dose of whole brain irradiation (WBI). The hippocampal dentate gyrus (DG) was analyzed one and ten weeks later for sensitive neurobiological markers associated with radiation-induced damage: changes in the density of proliferating cells, immature neurons, total microglia, and activated microglia.

Results—A significant reduction in basal levels of proliferating cells and immature neurons and increased microglial activation occurred with normal aging. WBI induced a transient increase in proliferation that was greater in older animals. This proliferation response did not increase the number of immature neurons, which decreased following WBI in young rats but not in old rats. Total microglial number decreased following WBI at all ages but microglial activation increased markedly, particularly in older animals.

Conclusions—Age is an important factor to consider when investigating the radiation response of the brain. In contrast to young adults, older rats show no sustained decrease in the number of immature neurons following WBI but have a greater inflammatory response. The latter may play an enhanced role in the development of radiation-induced cognitive dysfunction in older individuals.

Keywords

WBI; aging; microglia; neurogenesis; hippocampus

Introduction

Each year over 200,000 patients are treated with large field or WBI, the front line treatment for primary and metastatic brain tumors. Some 50% of long-term survivors develop late-delayed cognitive deficits due to radiation-induced injury of normal brain tissue (1,2). Age is an important risk factor for the development and severity of cognitive dysfunction following

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WBI, with pediatric and elderly patients appearing to be more vulnerable to WBI-induced brain injury than young adults (3-6). There is a paucity of experimental data regarding the effects of old age on the radiation response in the brain despite evidence that the average age for developing the cancers that require treatment with WBI is > 50 years old, making middle-aged and older adults a commonly treated patient population (7).

As a region of ongoing neurogenesis, the subgranular zone (SGZ) of the hippocampal DG is particularly sensitive to therapeutic levels of radiation. Following WBI, many neural progenitor cells undergo apoptosis within a few hours, followed by a transient increase in proliferation one week later (8,9). Surviving progenitor cells then are chronically suppressed, possibly by increased inflammation (10). These changes have been associated with cognitive deficits in young rodents, suggesting that in the young brain neurogenesis is important in some learning and memory pathways that are vulnerable to WBI (11,12).

The inflammatory system and microglia, the intrinsic immune cells of the brain, may be central in mediating many cellular interactions that contribute to dysfunction after WBI. A chronic inflammatory response, identified by increases in ED1⁺ activated microglia, has been demonstrated repeatedly in the CNS of young animals months after irradiation (8,10,13,14). To date, however, experimental studies of radiation-induced inflammation, brain injury and cognitive dysfunction have been conducted almost exclusively in animals a few weeks to a few months old, young ages that do not reflect important neurobiological changes that occur with normal aging, such as decreased proliferation and neurogenesis (15-18), increased microglial activation (19,20) and expression of pro-inflammatory cytokines (20-22). Experimental studies of stroke, traumatic brain injury, exogenous cytokine administration, and axotomy support the hypothesis that aging impacts the intensity and duration of brain inflammation and glial activation following challenges (23-28). Moreover, evidence that old age impacts the duration of some radiation-induced cognitive deficits in rodents (29,30) suggests greater radiation-induced injury in older rats. These basal and injury-induced differences between young adult and older brains indicate that radiation-induced brain injury may be both quantitatively greater and mechanistically different in middle-age and elderly patients. Therefore, it is important for experimental and translational models of radiation-induced brain injury to represent the primary patient population that undergoes treatment and often develops side effects.

In this study, we irradiated rats 8 (young adult), 18 (middle-age), and 28 (old) months of age to test whether aging alters radiation-induced changes in proliferation and the production of immature neurons in the SGZ of the hippocampus, neurobiological changes thought to contribute to the development of cognitive dysfunction (11,12). In addition, we tested whether old animals show a greater inflammatory response to a single 10Gy dose of WBI by assessing the total number and activation state of the microglia at one and ten weeks post-irradiation.

Methods and Materials

Animals and irradiation procedures

Eighty male Fischer 344 × Brown Norway (F344×BN) F₁ hybrid rats were purchased from the colony maintained by Harlan Sprague Dawley, Inc. for the National Institute on Aging. Rats were obtained at 7, 17, and 27 months of age, housed on a 12:12 light:dark schedule with food and water available ad libitum, and acclimated for one month. The animal facility at WFUSM is accredited by the American Association for Accreditation of Laboratory Animal Care and complies with Public Health Service-National Institutes of Health and institutional policies and standards for laboratory animal care. The Institutional Animal Care and Use Committee approved all protocols described herein. Animals in each age group were divided randomly into irradiated and sham control groups. All rats were anesthetized with a ketamine/xylazine

mixture (80/4 mg/kg body weight). Irradiated rats received treatment in a ^{137}Cs irradiator with collimating devices for delivery to the whole brain and lead shielding to protect the body and eyes. Irradiated rats received 10Gy at an average dose rate of 4.23Gy/min; half the dose (5Gy) was delivered to each side of the head to ensure equal midline radiation. Irradiated and sham rats were divided into two survival groups ($N=5-7/\text{group}$), euthanized at one and ten weeks post irradiation/anesthesia. One and ten weeks represent times where previous studies in young rodents have shown a transient increase in proliferation (one week) and a sustained decrease in neurogenesis (ten weeks) and increase in inflammatory markers (ten weeks) after WBI (8, 13). Non-anesthetized, non-irradiated controls also were included to assess the influence of anesthesia; these rats did not differ significantly from sham rats on any measure (data not shown) so statistical comparisons were limited to irradiated and sham rats.

Tissue processing

For tissue collection, rats were deeply anesthetized with ketamine/xylazine (80/12 mg/kg body weight) and decapitated. The brains were removed rapidly and hemisected at the midline. The right hemisphere was immersion fixed in phosphate buffered 4% paraformaldehyde for 48 h. Brains were cryoprotected in 10%, 20%, and 30% sucrose and frozen in embedding medium. Cryosectioned 40 μm thick coronal sections were collected in an antifreeze solution (1:1:2 ethylene glycol, glycerol, 0.1M PBS).

Immunohistochemistry and immunofluorescence

For each antibody (see below), a 1-in-24 series of sections representing the entire anterior to posterior extent of the hippocampus DG was washed in tris-buffered saline (TBS, pH 7.4). For immunohistochemical (IHC) staining, endogenous peroxidase activity was blocked in 0.6% H_2O_2 in TBS. Sections were first incubated in blocking solution (5% normal serum and 0.2% Tx-100 in TBS) and then overnight at 4°C in primary antibody diluted in the blocking solution. The primary antibodies were: rabbit monoclonal anti-Ki67 (labels mitotic cells (31), Abcam, 1:300), mouse monoclonal anti-CD68 (ED1 clone; labels activated macrophages/microglia (32), AbD Serotec, 0.83 $\mu\text{g}/\text{mL}$), rabbit polyclonal anti-Iba1 (labels all macrophages/microglia (33), WAKO, 0.083 $\mu\text{g}/\text{mL}$), and goat polyclonal anti-doublecortin (DCX; labels dividing neuroblasts and post-mitotic immature neurons (34), Santa Cruz, 1 $\mu\text{g}/\text{mL}$). Ki67, ED1 and Iba1 labeling were detected using biotinylated secondary antibodies (1:300) and visualized using peroxidase conjugated avidin-biotin complex (ABC Elite kit) with nickel enhanced diaminobenzidine (DAB) as substrate (Vector Laboratories). DCX labeling was visualized with a Cy5-conjugated secondary antibody (Jackson 1:250). Sections were counterstained with the nuclear binding dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma Aldrich, IHC labeling) or Sytox Green (Molecular Probes/Invitrogen, IF labeling) to facilitate recognition of anatomical landmarks.

Quantitative analyses

All analyses were performed blindly using coded sections. For sections labeled by IHC, a modification of the optical dissector method (35,36) was used for quantification of immunolabeled cells in the SGZ. As described previously (16), we determined the area of the SGZ (defined as the region extending 25 μm on either side of the border between the hilus and the granule cell layer, GCL, of the DG) and then counted immunolabeled cells within the SGZ using the NeuroLucida system (Microbrightfield, Inc., Colchester, VT). Immunolabeled cells were counted exhaustively in the SGZ, excluding cells in the top focal plane to avoid overestimation. The clustering and cytoplasm staining of DCX⁺ cells made it difficult to reliably count using brightfield microscopy, so DCX⁺ cells were counted (in the combined SGZ and GCL) in stacks of optical sections obtained using a Leica TCS SP2 confocal

microscope. The length of the SGZ at the GCL-hilar border was used to standardize the counts. All counts are expressed as # of cells/mm of SGZ.

Statistical analysis

For each dependent variable at each time point after WBI the mean density of immunolabeled cells in the region of interest was calculated for each group and compared by two-way analysis of variance (ANOVA) using Sigmapstat (SYSTAT), testing for main effects of age (8, 18 and 28 months old when irradiated), condition (irradiated versus sham) and interactions. Where indicated, post hoc analyses (Holm-Sidak test) were performed. The threshold of significance was $p \leq 0.05$.

Results

1. Effects of WBI on proliferation and neurogenesis vary with age and time post-irradiation

The density of proliferating Ki67⁺ cells was quantified in the SGZ of irradiated and age-matched sham rats one and ten weeks after single dose WBI (Figure 1). One week after WBI significant effects of age ($F=16.829$ $p<0.01$) and radiation ($F=54.262$ $p<0.01$), as well as an age $\times$ radiation interaction ($F=9.101$ $p<0.01$), were demonstrated by ANOVA. Post hoc analyses revealed age-related differences in basal proliferation between young adult and middle-aged sham rats ($p<0.01$) and between young adult and old sham rats ($p<0.01$), but not between middle-aged and old sham rats ($p=0.413$). The density of proliferating cells in the SGZ was greater in irradiated than in age-matched shams for middle-aged and old rats. The greatest radiation-induced increase was seen in old animals (343%, $p<0.01$); the increase was less in middle-age animals (156%, $p<0.01$) and was not statistically significant in young adults (14%, $p=0.263$). Ten weeks after WBI, regardless of age, the density of proliferating cells in the SGZ in irradiated rats declined compared to the levels one week post-WBI (Figure 1E, 1F). In middle-aged and old rats proliferation in irradiated animals returned to age-matched sham levels; in the young adult rats proliferation was lower than in age matched shams ($p<0.05$).

In addition to analyzing proliferation, we quantified immature neurons using the DCX antibody (Figure 2). An ANOVA revealed significant effects of age ($F=29.869$, $p<0.01$), radiation ($F=31.694$, $p<0.01$), and an age $\times$ irradiation interaction ($F=13.225$, $p<0.01$) at one week after WBI. Post-hoc analyses revealed that the density of immature neurons in sham animals was significantly greater in young adults than in middle-aged (52% reduction, $p<0.01$) and old animals (58% reduction, $p<0.01$), but did not differ between middle-aged and old rats ($p=0.205$). One week after WBI, the density of immature neurons was significantly reduced in young adult rats (49% reduction, $p<0.01$) and appeared slightly decreased in middle-aged rats (25% reduction, $p=0.07$), but did not differ between irradiated and sham animals in the old group ($p=0.693$). Ten weeks after WBI, there were significant effects of age ($F=10.187$, $p<0.01$), radiation ($F=5.150$, $p<0.05$) and an age $\times$ radiation interaction ($F=3.976$, $p<0.05$) on the density of DCX⁺ cells. Post hoc comparisons of irradiated and sham rats indicated that the number of immature neurons remained suppressed in irradiated young adult rats ($p<0.01$) but was not affected by irradiation in middle-aged ($p=0.106$) or old ($p=0.396$) rats (Figure 2F).

2. Effects of WBI on the density of microglia are independent of age and time post-irradiation

Given evidence of increased proliferation in the absence of an increase in DCX⁺ cells one week after WBI and the expectation that a WBI-induced inflammatory response might include microglial proliferation, we analyzed the density of microglia in the SGZ (Figures 3 and 4). The basal density of Iba1⁺ microglia did not change with age but there was a significant overall effect of radiation at one week after WBI ($F=98.606$, $p<0.01$), with similar and significant WBI-induced decreases in the density of microglia in young adult (35%), middle-aged (29%), and old (23%) rats (all $p<0.01$, Figure 4A). The density of microglia remained reduced ten

weeks after WBI ($F=24.377$, $p<0.01$). As at one week, post hoc comparisons revealed decreases in the irradiated animals compared to age-matched sham controls at all three ages (young adult 23%, middle-aged 20%, old 18%, all $p<0.05$, Figure 4D).

3. Effects of WBI on the density and fraction of activated microglia are dependent on age and time post-irradiation

We analyzed another component of a brain inflammatory response, activation of microglia visualized using the ED1 anti-CD68 antibody (Figures 3 and 4). Significant overall effects of age ($F=68.420$, $p<0.01$) and radiation ($F=32.782$, $p<0.01$) on the density of ED1⁺ cells in the SGZ were observed. The density of ED1⁺ cells increased with age in sham animals: in middle aged rats the density was 279% greater than in young adults ($p<0.01$), and in old rats the density was 519% greater than in young adults ($p<0.01$) and 63% greater than in middle-aged rats ($p<0.01$). One week following WBI the density of activated microglia was increased in irradiated rats compared to age-matched shams (young adult 178%, $p<0.05$, middle-aged 61%, $p<0.01$, old 54% elevation, $p<0.01$). Although a greater relative increase from baseline was seen in the young adult age group, in which the basal level of activation was very low, the density of activated microglia in the old irradiated rats was almost four-fold higher than in irradiated young adult rats (Figure 4B). At ten weeks after WBI, there was a significant effect of age, but not irradiation, on the density of ED1⁺ cells ($F=68.084$, $p<0.01$ and $F=2.739$, $p=0.11$, respectively). Regardless of age, the density of activated microglia in the SGZ in irradiated animals returned to baseline levels, with no significant differences between sham and irradiated animals (Figure 4E).

Since the neuronal microenvironment may be influenced both by the total number of activated microglia and by the fraction of the microglial population that is activated, we estimated the percentage of microglia that were activated using the counts of Iba1⁺ and ED1⁺ cells (Figure 4C, F). At the one week time point significant effects of age ($F=35.681$, $p<0.01$) and radiation ($F=51.561$, $p<0.01$) were evident. In young adult sham rats only 6% of microglia in the SGZ were activated, significantly lower than the fraction found in middle-aged (24%, $p<0.05$) and old (38%, $p<0.01$) rats. WBI increased the percentage of activated microglia to 25%, 50% and 75% of the total population in young adult, middle-aged and old rats, respectively (Figure 4C). Ten weeks after WBI, both age ($F=40.239$, $p<0.01$) and radiation ($F=12.348$, $p<0.01$) had significant effects on the percentage of activated microglia, which remained elevated in irradiated rats compared to age matched shams (Figure 4F). In the oldest rats 60% of all microglia remained activated 10 weeks after a single dose of WBI, whereas only 20% were activated in young adult rats.

Discussion

This study provides the first quantitative assessment of radiation-induced changes in the neurogenic and microglial cell populations in experimental animals at ages corresponding to the adult patient population commonly treated with WBI. Basal and WBI-induced changes in proliferation were dependent on age, as were basal and WBI-induced changes in the density of immature neurons. Neither the basal microglial density nor the WBI-induced decrease in microglial density changed with age, but the density and proportion of activated microglia increased with age and following WBI, such that the level of activated microglia was much greater in irradiated old rats than in irradiated young adult rats. The results of this study are the first to indicate that the neurobiological response to WBI is qualitatively and quantitatively different in old rodents compared to young adults.

In order to model life stage specific responses in humans (37), one can approximately map rodent ages to humans based on postnatal brain development (38-40), vulnerability to injury (23,41), and other life-stage specific changes. In F344xBN rats, a strain widely used in studies

of normal aging, 18 and 28 months represent the 95% and 75% points on the normal survival curve, approximately equivalent to 50 and 65 years of age in the US population (42), ages that better represent the middle age and older patients that commonly undergo WBI. Radiation-induced changes in these middle-aged and old rats provide new insight into the mechanisms of radiation-induced injury in an important clinical population.

WBI-induced deficits in hippocampal function have been associated with decreased neurogenesis (11,12,43), which potentially could arise from changes in cell proliferation, commitment, and/or survival. We examined both the density of proliferating, Ki67⁺ cells in the SGZ of the rodent hippocampus and, more specifically, the density of immature, DCX⁺ neurons. The density of Ki67⁺ and DCX⁺ cells was lower in middle aged and older rats than in young adults, as shown previously (15-18). Recent evidence indicates that the aging-related decrease in neurogenesis results from decreased proliferation, rather than changes in survival or cell cycle length (17,44). Regardless of the mechanism(s) of changes in normal aging, our results reveal that advanced age is an important modifier of the effects of radiation on the neurogenic population. WBI decreased the density of DCX⁺ neurons in young adults, as previously reported (8,11,13), but had no effect on the density of DCX⁺ cells in old rats (Figure 2). One must be circumspect when interpreting the effects of a change or lack of change in the number of DCX⁺ cells on neuronal replacement (34,45-47); DCX is expressed in dividing neuroblasts and for approximately two weeks in differentiating, post-mitotic immature neurons (34,45) but not all DCX⁺ neurons survive to maturity. Where examined in previous studies, however, WBI-induced decreases in neurogenesis in younger animals demonstrated by S-phase labeling with bromodeoxyuridine (BrdU) combined with neuron specific markers have been paralleled by decreases in DCX labeling (8,11). Additional studies will be required to assess whether WBI in old rats decreases neurogenesis by some mechanism that does not change the number of differentiating neurons, but the present findings suggest that in old rats WBI-induced changes in neurogenesis contribute very differently, if at all, to the development of radiation-induced cognitive dysfunction.

Clearly altered neurogenesis is only one mechanism that may lead to cognitive dysfunction. In the clinic, cognitive deficits following radiation therapy in adult patients include functions mediated by neural regions in which there is no adult neurogenesis, including non-hippocampal regions of the medial temporal lobe and the frontal cortex (1,48-51). In the laboratory, rats irradiated at four months of age show deficits in a non-hippocampal dependent task, novel object recognition, as well as in hippocampal-dependent maze performance (52,53). To date, only one laboratory has compared the development of cognitive deficits in irradiated old rodents versus irradiated young rodents (29,30), assessing a limited array of cognitive functions. These earlier studies demonstrated that WBI induced progressive and irreversible memory dysfunction in 18 month old rats, whereas the deficit was reversible in rats irradiated at 45 days or four months of age. In contrast to the memory effects, radiation-induced learning dysfunction was not age dependent, but occurred more rapidly in younger rats than in 18 month old rats. The experiments provided no insight into mechanisms but clearly indicate that radiation-induced cognitive deficits may differ in nature and extent depending on the subject's age at irradiation, and possibly on the neural regions that contribute to the affected cognitive functions. One important and common contributor to radiation-induced brain injury appears to be changes in inflammation, which may be particularly important in the development of cognitive deficits at later ages.

Microglia play a central role in the brain inflammatory response (54-56) and are affected both by aging and WBI (Figures 3 and 4). Our finding that the basal density of microglia in the SGZ did not change with age is consistent with previous studies of the murine hippocampus (57). We suspected that the transient increase in proliferation observed one week after WBI was due to increased microgliogenesis, since others have shown that the production of new cells by

proliferating cells in the SGZ is altered following WBI and results in increased genesis of oligodendrocytes and microglia (10,13): BrdU labeling of three week old and two month old mice several weeks after WBI revealed an increased percentage of BrdU⁺ cells labeled for CD68 (8,12). We did not quantify microglial proliferation in the current study but qualitative assessment of sections double-labeled for Ki-67 and Iba1 indicated that the majority of Ki-67⁺ cells in the SGZ at one week after WBI were Iba1⁺ microglia and that the percentage of Iba1⁺ cells in the DG that were Ki-67⁺ increased from less than 1% in control animals to approximately 20% in irradiated rats (data not shown). Increased microglial proliferation in irradiated animals did not, however, produce an increase in the total number of microglia in the SGZ; microglial density decreased at one and ten weeks following WBI (Figure 4A,D). A decrease in total microglial number may appear inconsistent with evidence of increased proliferation but it must be recognized that microglial number represents a balance of birth, death, and migration. Careful stereological analyses of microglial number throughout the hippocampus and in other brain regions will be required to clarify the dynamic regulation of the microglial population following WBI.

Microglial proliferation is just one component of the inflammatory response following WBI. The change in microglia from resting to the activated state results in many physiologic changes, including up-regulation of the lysosomal antigen CD68 recognized by the ED1 antibody, (32) and increased production of pro-inflammatory proteins (58). In the present study, total microglial density did not change with age (Figures 3A,C and 4A,D) but basal levels of activated microglia were elevated in older animals (Figure 3B,D), with only a few percent of ED1⁺ microglia in young adult animals (Figure 4C) versus 28% in the oldest animals (Figure 4F). Similar age-dependent increases in markers of activated microglia have been reported in non-human primates (59) and humans (60). The decrease in overall microglial density concomitant with the increase in the number of activated microglia after WBI demonstrates a striking pro-inflammatory microenvironment in the SGZ across ages; in old rats approximately 75% of all microglia are activated (Figure 4E,F). Such chronic brain inflammation has been shown to be deleterious for function in many models of aging-related neurodegenerative diseases (55,56), and may contribute to more severe and prolonged WBI-induced cognitive dysfunction in older animals (29,30). Moreover, although the role of microglia in a pro-inflammatory response has been investigated more extensively, microglia also serve trophic functions by producing and secreting neurotrophic peptides (61,62). Thus, following WBI, both loss of trophic support from resting microglia and heightened pro-inflammatory responses from activated microglia may contribute to a feed-forward cycle of chronic inflammation and damage that is significantly exacerbated in older animals.

This study demonstrates that radiation-induced changes in the adult brain are significantly influenced by age. The radiation-induced decrease in density of immature neurons in the hippocampus that appears to contribute to cognitive deficits in young rodents does not occur in old rats. In contrast, the radiation-induced inflammatory state is exacerbated in older rats and may play a greater role in the development of neural dysfunction following WBI. In translational efforts to develop new treatments to prevent or reduce the deleterious effects of brain irradiation on cognitive function, it is important to include animal models that represent the age of the primary clinical population, since the aged brain appears to differ significantly from the young adult brain in its response to challenge, and also may differ in its response to treatments.

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Conflicts of Interest: None

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Figure 1.

Cell proliferation was demonstrated by the distribution of Ki67⁺ cells in the GCL, Hilus, and SGZ in young (A, C) and old (B, D) rats under sham conditions (A, B) and at one week after WBI (C, D). Scale bar = 100μm, 25μm (inset). E. The mean density of Ki67⁺ cells in the SGZ of sham animals (open bars) decreased with age and increased one week after irradiation (closed bars) in middle-aged and old animals. F. At ten weeks after WBI the density of Ki-67⁺ cells decreased in irradiated young adult rats but did not differ between irradiated and sham rats at middle and old age. *p<0.01 vs young adult sham (E, F); #p<0.01 vs age-matched sham (E), #p<0.05 (F).

Figure 2.

Neurogenesis was evident in the DG from young (A,C) and old (B,D) sham rats in sections immunolabeled for DCX (red) and counterstained with SYTOX green. In old rats DCX⁺ cells were rarer and less likely to occur in clusters. Scale bar = 15 μ m. E-F. Quantitative analysis demonstrated an aging-related decline in the density of immature neurons in shams (open bars) and demonstrated that irradiated (closed bars) young rats had an acute (1 wk, E) and sustained (10 wk, F) decline in the density of immature neurons that was not apparent in middle-aged and old rats. * $p < 0.01$ vs young adult sham; # $p < 0.01$ vs age-matched sham.

Figure 3. Representative sections labeled with anti-Iba1 (A,C) and anti-CD68 (ED1) (B,D) revealed labeled microglia throughout the DG of young adult (A,B) and old (C,D) sham rats. An increased density of ED1⁺ (activated) microglia is evident in old sham rats (D) compared to young adults (C). Scale bar 25 μ m.

Figure 4.

Counts of Iba1 (A, D) and ED1 labeled cells (B, E) and the percent of Iba1⁺ cells that were ED1⁺ (C, F) in sham animals (open bars) and at one (A-C) and ten (D-F) weeks after WBI (closed bars) quantified the effects of aging and irradiation on microglia. The density of Iba1⁺ microglia was stable across ages but decreased at one (A) and ten (D) weeks after WBI, regardless of age. The density of activated, ED1⁺ microglia (B,E) increased with age and following WBI. The extent of microglial activation at each age and under each condition is indicated by the fraction of all microglia that were ED1⁺ (C, F). *p<0.01 vs young adult sham, ** p<0.05 vs middle-aged sham, #p<0.01 vs age-matched sham (A, C, E), #p<0.05 (B, D, F).