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Genotype differences in anxiety and fear learning and memory of WT and ApoE4 mice associated with enhanced generation of hippocampal reactive oxygen species

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Abstract

Apolipoprotein E (apoE), involved in cholesterol and lipid metabolism, also influences cognitive function and injury repair. In humans, apoE is expressed in three isoforms. E4 is a risk factor for age-related cognitive decline and Alzheimer's disease, particularly in women. E4 might also be a risk factor for developing behavioral and cognitive changes following ⁵⁶Fe irradiation, a component of the space environment astronauts are exposed to during missions. These changes might be related to enhanced generation of reactive oxygen species (ROS). In this study, we compared the behavioral and cognitive performance of sham-irradiated and irradiated wild-type (WT) mice and mice expressing the human E3 or E4 isoforms, and assessed the generation of ROS in hippocampal slices from these mice. E4 mice had greater anxiety-like and conditioned fear behaviors than WT mice, and these genotype differences were associated with greater levels of ROS in E4 than WT mice. The greater generation of ROS in the hippocampus of E4 than WT mice might contribute to their higher anxiety levels and enhanced fear conditioning. In E4, but not wild-type, mice, PMA-treated hippocampal slices showed more DHE oxidation in sham-irradiated than irradiated mice and hippocampal HO-1 levels were higher in irradiated than sham-irradiated E4 mice.

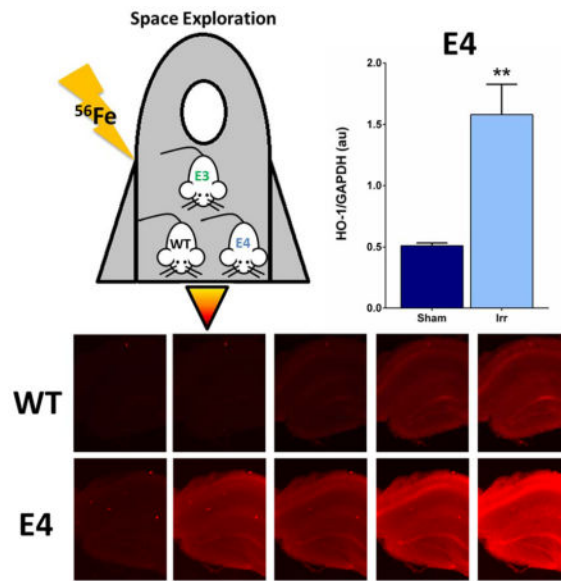
Graphical Abstract

Mice with apolipoprotein E4 (E4), a risk factor for Alzheimer's disease, have greater anxiety-like and conditioned fear behaviors than wild-type (WT) mice. Generation of reactive oxygen species (ROS, in red) three months following ⁵⁶Fe irradiation, a component of the space environment astronauts are exposed to, is more pronounced in the hippocampus of E4 than WT mice. In E4, but not WT, mice, hippocampal levels of the oxidative stress-relevant marker heme oxygenase-1 are higher in irradiated than sham-irradiated E4 mice.

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There are no conflicts of interest to declare.



Keywords

anxiety; cognition; superoxide; DHE; hippocampus; irradiation

Introduction

Among the major human isoforms of apolipoprotein E (apoE) involved in cholesterol and lipid metabolism, apoE4 (E4) is a risk factor to develop age-related cognitive decline and Alzheimer's disease, particularly in women (Farrer *et al.* 1997, Raber *et al.* 2004, Spinney 2014). Effects of cranial ^{56}Fe irradiation (3 Gy, 600 MeV) on hippocampus-dependent cognition in C57BL/6J WT mice three months following irradiation (Villasana *et al.* 2010) and in human apoE mice thirteen months following irradiation (Villasana *et al.* 2011a) are sex-dependent, with female mice being more susceptible to radiation-induced cognitive impairments than male mice. The increased risk of cognitive decline in E4 carriers might be related to a reduced ability to protect against reactive oxygen species (ROS)-related lipid peroxidation (Pedersen *et al.* 2000) and may involve genotype differences in brain levels of ROS. Although acute increases in ROS may be important for learning and memory (Kishida *et al.* 2005a, Kishida & Klann 2007, Hidalgo *et al.* 2007a), a prolonged increase in ROS can lead to injury through oxidation of cellular components such as lipids, proteins and DNA (Knapp & Klann 2002a). The brain is particularly vulnerable to ROS damage, as it has a high oxygen consumption but relatively low levels of antioxidants.

Space radiation exposure, including ^{56}Fe exposure, poses a significant risk to the central nervous system. In rodents, ^{56}Fe irradiation might affect hippocampus-dependent cognitive function (Haley *et al.* 2012a, Villasana *et al.* 2010, Rola *et al.* 2004, Rabin *et al.* 2004, Rabin *et al.* 2002, Rabin *et al.* 2009, Haley *et al.* 2013, Yeiser *et al.* 2013). ROS are altered by irradiation (Manda *et al.* 2008, Mizumatsu *et al.* 2003, Monje *et al.* 2003, Limoli *et al.* 2007, Tseng *et al.* 2013) and, in this way, affect brain function as well.

Most *in vivo* irradiation rodent studies have focused on oxidative damage (Raber *et al.* 2011, Suman *et al.* 2013), levels of antioxidant enzymes, mice lacking or overexpressing a particular antioxidant enzyme (Rola *et al.* 2007, Raber *et al.* 2011), or compounds scavenging ROS (Manda *et al.* 2007, Manda *et al.* 2008, Villasana *et al.* 2013). Although ROS levels have been studied in both the context of aging (Driver *et al.* 2000) and of brain function thirty days after radiation exposure that occurred between 24 and 48 hours after birth (Caceres *et al.* 2010), less is known about the levels of ROS generated in the tissues of mice long after irradiation. Dihydroethidium is a fluorescent dye that is oxidized by superoxide into the stable dihydroxy ethidium (DHE). Based on its ability to detect intracellular and extracellular superoxide, DHE is used for *in vitro* and *in vivo* analysis of superoxide (Peshavariya *et al.* 2007, Hall *et al.* 2012), including hippocampal slices of mice (Haley *et al.* 2012a) and rats (D'agostino *et al.* 2007).

Heme oxygenase-1 (HO-1) catalyzes the degradation of heme to generate carbon monoxide, biliverdin and free iron (Dwyer *et al.* 1992, Vincent *et al.* 1994). Increased HO-1 levels are seen in many neurological conditions associated with increased oxidative stress and inflammation, as well as following X-Ray irradiation (Rugo & Schiestl 2004). While elevated HO-1 levels can restore redox homeostasis and reduce inflammation (Bergeron *et al.* 1997, Ewing *et al.* 1992, Jernigan *et al.* 2001, Lee *et al.* 2011, Ndisang & Jadhav 2009, Zhang *et al.* 2004), excessive heme degradation may result in toxic levels of CO, bilirubin and iron (Cuadrado & Rojo 2008). Based on these results, alterations in DHE oxidation might be related to changes in HO-1 levels.

In the current study, we compared behavioral and cognitive performance of sham-irradiated and ⁵⁶Fe-irradiated wild-type (WT), E3, and E4 mice and analyzed whether behavioral and cognitive changes are associated with changes in hippocampal superoxide and HO-1 levels.

Material and Methods

Animals and irradiation

Human E3 and E4 targeted replacement mice, generated by Dr. Patrick Sullivan and bred in our colony, were used for the present study. C57BL/6J (WT) mice were purchased from Jackson Laboratories and also bred in our colony for the present study. Two-month-old E4 (sham-irradiated: $n = 10$ mice; irradiated: $n = 14$ mice), E3 (sham-irradiated: $n = 4$; irradiated: $n = 6$), and WT (sham-irradiated: $n = 16$ mice; irradiated: $n = 16$ mice) female mice bred in our mouse colony at OHSU were shipped to Brookhaven National Laboratories (BNL) in Upton, NY for cranial ⁵⁶Fe irradiation (3 Gy, 600 MeV/n) or sham-irradiation. Following acclimatization for 1 week at the animal facility, the mice were transferred to the NASA Space Radiation Laboratory (NSRL) on the day of the sham-irradiation or ⁵⁶Fe-irradiation and weighed. All mice received i.p. anesthesia, (ketamine (Sigma), 80 mg/kg and xylazine (Sigma), 20 mg/kg), and ophthalmic solution was placed on the eyes for protection. The mice designated for irradiation were placed in positional cradles to stabilize the head during irradiation. Sham-irradiated mice received the same procedure except that they were not irradiated. Upon recovery from anesthesia, the mice were returned to the animal facility. One week after irradiation, the mice were shipped to Oregon Health and Science University (OHSU) for behavioral and cognitive testing starting three months after irradiation. Four

months after irradiation, the mice were weighed again. At BNL and prior to behavioral testing at OHSU, the mice were housed under a constant 12 hr light: 12 hr dark cycle. Food (PicoLab Rodent Diet 20, no. 5053; PMI Nutrition International, St. Louis, MO), and water were provided *ad libitum*. All procedures were approved by Institutional Animal Care and Use Committee at OHSU and BNL.

Behavioral and cognitive testing

Three months following sham- or ^{56}Fe irradiation, mice were behaviorally and cognitively tested. They were first tested in the open field, light-dark, elevated zero maze, and elevated plus maze (on successive days in week 1); subsequently tested for hippocampus-dependent spatial learning and memory in the water maze (week 2); and finally tested for hippocampus-dependent contextual fear memory and hippocampus-independent cued fear memory (week 3) (detailed methods are described below). The experimenter testing the mice was blinded to the treatment of the mice. All tests with the exception of water maze were conducted in the morning. Water maze test sessions were conducted in the morning and early afternoon (beginning at approximately 8:00 am and 1 pm, respectively).

Open field

The open field was used to evaluate measures of anxiety and locomotor behavior. Mice were placed in a 40.64cm \times 40.64 cm brightly lit (luminescence: 200 lux) open arena equipped with infrared photocells interfaced with a computer (Kinder Scientific, Poway, CA). Active times (single beam breaks within 1 second) and distance moved were recorded for a single 10-minute session. In the open-field, the center zone (20.3 \times 20.3 cm) is more anxiety-provoking than the peripheral zone; therefore, mice that are more anxious in the open field spend less time in the center (Choleris *et al.* 2001, Clement *et al.* 2002). Total distance moved was used as an index of exploratory behavior and percent of time spent in the center of the open field as a measure of anxiety.

Light-dark

The light-dark test was also used to assess anxiety levels. In the light-dark test, mice were placed in the open field enclosure (described above) containing black plastic inserts that covered the sides and the top fifty percent of the open field (Hamilton-Kinder, Poway, CA). A single opening in the wall of the insert adjacent to the open area allowed the mice to enter or exit the more anxiety-provoking light area of the maze (luminescence: 200 lux). Active times and distance moved were recorded for a single 10-minute session. Breaks in the photo beams were used to calculate path length, active times, and rest time in the open and closed compartments of the enclosure. Mice with increased measure of anxiety spend less time in the light side of the enclosure (Bhatnagar *et al.* 2004).

Elevated zero maze

The elevated zero maze was also used to assess measures of anxiety and exploratory behavior. The custom built elevated zero maze (Kinder Scientific) consisted of two enclosed areas with two adjacent open areas. Mice were placed in the closed part of the maze and allowed free access for 10 minutes (luminescence: 200 lux). Mice could spend their time

either in the closed or open area of the maze. A video tracking system (Noldus Information Technology, Sterling, VA, set at six samples/second) was used to calculate the time spent in the open areas and distance moved throughout the maze. Mice that are more anxious in the elevated zero maze spend less time in the open areas (Shepherd *et al.* 1994). Outcome measures included the percent of time spent in the open areas and distance moved.

Water maze

The water maze test was used to assess spatial learning and memory (Morris 1984). A circular pool (140 cm diameter) was filled with water ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$). The water was made opaque with white chalk in order to hide the platform. The platform (20 cm wide) was located approximately 1 cm below the water level. On the first two days of water maze testing, the mice were trained to locate a visible platform (flagged with a visible beacon). There were three trials per session (5-minute inter-trial interval (ITI) and two sessions (two hours apart) per day. The platform was moved to a new quadrant for each of the four visible platform sessions. Trials ended when the mice reached the platform and remained on it for 3 seconds or when 60 seconds elapsed. In trials in which the mice did not find the platform, they were guided to the platform and allowed to remain on it for 3 seconds. Upon removal from the maze, the mice were dried with absorbent towels and returned to their home cages.

After visible platform training, the mice were trained to locate a hidden platform in three trials per session (5-minute ITI) and two sessions (two hours apart) per day. The location of the hidden platform remained constant although the drop location varied for each trial. Mice were allowed to remain on the platform for 3 seconds before they were removed from the pool. Performance measures for visible and hidden platform training included swim speeds and cumulative distance to the platform. Cumulative distance to the platform measures how far the mice are located from the platform over the duration of the trial. The lower the cumulative distance, the better the performance. Thigmotaxis, defined as the percent of time spent in the outer 20 cm perimeter of the pool, was analyzed as an anxiety measure in the water maze.

Probe trials (platform removed) were conducted exactly 1 hour after the last trial of each day of hidden platform training in order to assess spatial memory retention. Percent of time spent in the target quadrant (learned location of the platform during prior hidden platform trials) and cumulative distance to the platform location were used as measures of spatial memory retention. The swimming patterns of the mice were analyzed using the Ethovision video tracking system set at 6 samples/sec.

Contextual and cued conditioned fear

Conditioned fear was used to assess hippocampus- and amygdala-dependent associative memory. In this task, mice learn to associate the environmental context (fear conditioning chamber) or cue (tone) with a mild foot shock (unconditioned stimulus, US). When mice are re-exposed to the context or the tone (conditioned stimuli, CS), conditioned fear results in freezing behavior which is characterized by cessation of all movement except for respiration. Contextual fear conditioning is thought to be hippocampal- and amygdala-dependent, whereas cued fear conditioning is amygdala-dependent, but not hippocampal-dependent.

(Phillips & LeDoux 1992). On the first day of the conditioned fear test, each mouse was placed in a fear conditioning chamber (Med Associates, Inc, St. Albans, VT) and allowed to explore it for 2 minutes before the delivery of a 30 second tone (80 dB) which was immediately followed by a 2 second foot shock (0.35 mA). Two minutes later, a second tone-shock pair was delivered. Mice were removed from the testing chambers 10 seconds after the second shock and were returned to their home cages. Chambers were cleaned with 0.5% acetic acid between animals. The pre-tone time, which was the first 2 minutes of the trial, was used as the baseline measure for freezing behavior. Mice were removed from the testing chambers 10 seconds after the end of the second shock and returned to their home cages. On day 2, each mouse was first placed in the fear conditioning chamber containing the exact same context but without delivery of a tone or foot shock. Freezing was analyzed for 3 minutes. The context of the chambers was changed by adding a smooth floor texture over the grid floor, inserting the shape of a triangle, adding a new scent (hidden vanilla soaked nestlets), and by cleaning the chamber with 70% ethanol rather than acetic acid. One hour after the last contextual test for each mouse, the mice were assessed for cued fear conditioning. Mice were placed in the chambers containing the modified context and were allowed to explore for 3 minutes before they were re-exposed to the fear conditioning tone for 3 minutes. Freezing behavior was analyzed for the first and last 3 minutes of the cued fear conditioning test. Eight mice (WT mice: three sham-irradiated and two irradiated mice; E4 mice: one sham-irradiated and two irradiated mice) were excluded from the complete fear conditioning tests due to experimental error. An additional eighteen mice (WT mice: five sham-irradiated and four irradiated mice; E4 mice: four sham-irradiated and five irradiated mice) were excluded from the cued fear conditioning test due to a technical error.

Freezing was measured using a motion index, calculated based on a proprietary motion analysis algorithm in the Med Associates Video Freeze Software (Med Associates Inc). Briefly, the software analyzes and acquires videos of the trials at a frequency of 30 frames/second. The motion index is based on the sum of the pixel changes in a frame compared to those of a reference frame and to those of successive frames. The reference frame is based on a video capture when the mouse is not in the chamber. The motion index threshold used in the current study was 18. This means that the motion index had to remain below 18 pixel changes to be considered freezing.

Analysis of group differences in freezing before delivery of the first tone during fear conditioning training on day 1 were analyzed as measure of baseline freezing. This allowed us to determine whether there were potential pre-conditioning group differences in behaviors such as immobility, which could contribute to freezing scores. Potential group differences in the motion index during the two shocks on day 1 were also analyzed. This allowed us to determine whether there were possible group differences in sensory response to the shocks. Finally, the percent time freezing during contextual and cued testing on day 2 were analyzed.

DHE oxidation analysis

DHE oxidation levels were assessed approximately one week after fear conditioning in slices from E4 (sham-irradiated: $n = 5$ sections; irradiated: $n = 9$ sections), E3 (sham-irradiated: $n = 2$ sections; irradiated: $n = 4$ sections) and WT (sham-irradiated: $n = 6$ sections;

irradiated: $n = 5$ sections) mice. Hemibrains were placed in 4°C oxygenated (95% oxygen 5% carbon dioxide) cutting solution (in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 5 glucose). Acute brain coronal sections (150 μm) were generated using a Vibratome (Leica Microsystems, St. Louis MO) containing an oxygenated bath. Sections from each mouse were collected and kept separate in a 12-well plate bath containing ice cold and oxygenated cutting solution. Once all sections were collected, the bath solution was replaced with half cutting and half artificial cerebrospinal fluid (ACSF) solution (in mM: 125 NaCl, 2.4 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂ and 25 glucose). Thirty minutes later, the bath solution was changed to a 100% ACSF solution and the bath temperature was gradually increased to 34°C. Sections were transferred and allowed to equilibrate for one hour in a multi-bath chamber placed on an Olympus spinning disk confocal microscope. The multi-bath chamber was situated on the confocal stage and received 36°C oxygenated ACSF using a gravity fed perfusion system and a multiple in-line heater (Warner Instruments, Hamden, CT). The rate of perfusion was 1ml/minute. A superfusion pump was used to perfuse out solution from the chambers, making this an open perfusion system. Each separate chamber contained representative sections from each group of mice. This allowed all the sections from the different treatment groups and two genotypes to be examined under the exact same conditions. Images of the hippocampus (crux, enclosed blade and free blade of the dentate gyrus; areas CA1 and CA3) were acquired for background reference (Ex λ 488 nm; Em λ > 590 nm) before the addition of DHE (10 μM, Molecular Probes, Eugene, OR). Phorbol-12-Myristate-13-Acetate (PMA) induces generation of superoxide via stimulation of the NADPH-oxidase complex (Tejada-Simon *et al.* 2005). Because induction of superoxide is important for learning, memory, and synaptic plasticity (Kishida *et al.* 2006, Kishida *et al.* 2005b, Knapp & Klann 2002b, Thiels *et al.* 2000), we used PMA as a functional assay to assess whether irradiation alters the ability to generate superoxide in E4, E3, and WT mice. PMA (1 μM) or DMSO was added to separate ACSF solution reservoirs approximately 5 min following the addition of DHE. Images were acquired every 2 minutes for up to 20 minutes at a 4× magnification. The optimal x, y, and z coordinates for each slice and each region were selected and programmed before the DHE experiment began. This allowed us to determine the best focal plane before the experiment began, and it also allowed us to label the sections with their corresponding mouse ID number. Images were acquired after addition of DHE using a 4× water objective. As there were slight variations in the focal plane within each slice, several images (3–6) were acquired for optimal image quality. An Olympus confocal microscope and Slidebook 6 Digital Microscopy Software (Intelligent Imaging Innovations Inc.) were used to collect the images and analyze the intensity of oxidized DHE. The experimenter that prepared and analyzed the DHE-oxidation of the slices was blind to the treatment and genotype. There were a total of 3 experiments. Each experiment consisted of 2–3 replicate slices for each mouse from each treatment group and for each drug treatment. The mean temperature was 35.9°C with a range of 34.3 – 37.3°C between individual experiments. The mean location of the hippocampal slices from Bregma was –2.0 with a range of –1.58mm–2.4mm. This anatomical range was selected because previous data suggest that the dorsal hippocampus is more involved in hippocampus-dependent spatial memory compared to the ventral hippocampus (Moser *et al.* 1993).

Western Blot Analysis

Hippocampal tissues were homogenized in RIPA lysis buffer [0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris pH 8.0] containing the phosphatase inhibitor sodium vanadate [NaV, 1mM]. Protein lysates were extracted by centrifugation and protein concentrations were calculated using the MicroBCA protein assay. Equal amounts of protein were separated on sodium dodecyl sulfate polyacrylamide (10% SDS-PAGE) gels and transferred to a polyvinylidene difluoride (PVDF) membrane. Membrane blots were then blocked in tris-buffered saline and tween 20 (TBST) [1 × TBS, 0.1% Tween 20] containing 5% bovine serum albumin (BSA). The membranes were then incubated with primary antibody diluted (1:1,000) in TBST containing 1% bovine serum albumin (BSA) overnight at 4°C. Membranes were then washed in TBST [3×10min] before being incubated in secondary antibody [1:10,000 dilution] for one hour. Membranes were incubated in enhanced chemiluminescence (ECL) reagent before being exposed to CL-Xposure Film to detect protein changes. A representative blot is shown in Fig 1.

Statistical analyses

All statistical tests were conducted using SPSS 22.0 (SPSS Inc, Chicago, IL, USA) or GraphPad Prism software (GraphPad Software, La Jolla, CA) and were considered significant at $P < 0.05$. All figures were generated using GraphPad Prism software. Where relevant, post-hoc corrections for multiple comparisons were applied. Data are reported as averages \pm the standard error of the mean. For all statistical analyses, data were first assessed for normality and homogeneity of variance to determine whether to use parametric or non-parametric statistical analyses. The data distribution was considered normal at a significance of $p > 0.01$ (Shapiro-Wilk test).

RESULTS

General health and body weights

There were no signs of illness or sick-like behaviors as a result of ^{56}Fe irradiation in either genotype. There were no effects of ^{56}Fe irradiation or genotype on activity or measures of anxiety in the light-dark test (not shown).

Activity and anxiety measures

Effects of genotype on activity and anxiety measures were seen in the elevated zero maze, elevated plus maze, and open field. Genotype differences were seen for the activity measures of the open field ($F_{2,58} = 9.172$, $p < 0.001$; Fig. 2A), the elevated zero maze ($F_{2,59} = 15.44$, $p < 0.0001$; Fig. 2B), and the elevated plus maze ($F_{2,59} = 18.76$, $p < 0.0001$; Fig. 2C). Compared to WT and E3 mice, E4 mice explored less in the open field ($p < 0.001$; $p < 0.01$, respectively; Sidak's post-hoc), the elevated zero maze ($p < 0.0001$, both; Tukey's post-hoc), and the elevated plus maze ($p < 0.05$; $p < 0.0001$, respectively; Tukey's post-hoc). E3 mice explored more than WT mice in the elevated plus maze ($p < 0.001$; Tukey's post-hoc). Similarly, genotype differences were found in anxiety measures of the open field ($F_{2,58} = 3.197$, $p < 0.05$; Fig. 2D) and the elevated plus maze ($F_{2,59} = 5.177$, $p < 0.01$; Fig. 2F). WT mice spent more time in the center of the open field ($p < 0.05$; Tukey's post-hoc) and in the

open arms of the elevated plus maze ($p < 0.05$; Tukey's post-hoc) compared to E4 mice. Finally, there was a trend towards a main effect of irradiation in anxiety measures in the elevated zero maze ($F_{2,57} = 3.972$, $p = 0.0511$, Fig. 2E) and in the activity measures in the elevated zero maze ($F_{1,59} = 3.526$, $p = 0.065$), with irradiated mice showing lower anxiety levels and spending more time in the open areas of the maze, yet moving less than their sham counterparts.

Cognitive performance

Next spatial learning and memory were assessed in the water maze. There were no significant effects of genotype or radiation on ability of the mice to locate the visible or hidden platform locations (Table 1). However, irradiation affected spatial memory retention in the water maze probe trials (Fig. 3). To compare performance across probe trials, we analyzed performance as percent time in the target quadrant in the three probe trials using a repeated measures ANOVA. There were main effects of irradiation ($F_{2,58} = 4.693$, $p < 0.05$) and genotype ($F_{2,58} = 4.831$, $p < 0.05$) (Fig. 3A). Independent of genotype, sham-irradiated mice spent more time in the target quadrant than irradiated mice. E3 mice spent more time in the target quadrant than WT mice ($p < 0.05$; Bonferroni's post-hoc). Genotype differences were also observed when cumulative distance to the target was calculated (Fig. 3B). There was a main effect of genotype ($F_{2,58} = 6.85$, $p < 0.01$); E3 mice swam closer to the platform location than WT mice (Bonferroni's post-hoc).

Finally, mice were tested for contextual and cued fear conditioning. There were no effects of genotype or irradiation on average baseline motion (prior to the first tone) (Fig. 4A). There were also no effects of irradiation in response to the shocks. However, there was a main effect of genotype ($F_{2,45} = 6.194$, $p < 0.01$) in response to the two shocks, with E3 and E4 mice showing a greater response than WT mice ($p < 0.01$, $p < 0.05$, respectively; Tukey's post-hoc; Fig. 4B). Similarly, there was an effect of genotype in response to the two tones ($F_{2,45} = 9.573$, $p < 0.001$) (Fig. 4C) and in freezing during the interval between the two tone-shock pairings ($F_{2,45} = 7.366$, $p < 0.01$) (Fig. 4D). E4 mice had higher immobility during the tones than WT mice ($p < 0.001$; Bonferroni's post-hoc) and higher immobility during the interval than WT ($p < 0.01$) and E3 mice ($p < 0.05$; Tukey's post-hoc). Twenty four hours after training, genotype differences were also seen in freezing during the hippocampus-dependent contextual memory test ($F_{2,49} = 7.401$, $p < 0.01$); freezing was higher in E4 than WT mice ($p < 0.001$; Tukey's post-hoc, Fig. 4E). This genotype difference was not limited to the contextual memory test. Freezing during the hippocampus-independent cued memory test was also influenced by genotype ($F_{2,28} = 15.035$, $p < 0.001$, Fig. 3F). E4 mice showed higher freezing levels both before and during the tone than WT mice ($p < 0.000$; Bonferroni's post-hoc). In contrast to these genotype differences and the effects of ^{56}Fe irradiation on spatial memory retention in the water maze, there were no effects of irradiation on freezing levels during the contextual or cued memory tests.

Hippocampal DHE oxidation levels

Representative images showing the time course of DHE oxidation in hippocampal slices of WT and E4 mice are displayed in Fig. 5. The quantification of all the images showed that there were significant time x genotype ($F_{3,016,1092} = 22.348$, $p < 0.000$), time x irradiation

($F_{1,530,1092} = 4.019$, $p < 0.05$), time x drug (PMA or vehicle) ($F_{1,530,1092} = 15.171$, $p < 0.000$), time x genotype x irradiation ($F_{3,016,1092} = 4.816$, $p < 0.01$), time x genotype x drug ($F_{3,016,1092} = 5.304$, $p < 0.001$), and time x irradiation x drug ($F_{1,530,1092} = 5.304$, $p < 0.01$) interactions for DHE oxidation levels, and a trend towards a time x genotype x irradiation x drug interaction ($F_{3,016,1092} = 2.30$, $p = 0.075$). Because there were no interactions with hippocampal region, the average of the five different regions was used for further analyses. A significant main effect of genotype ($F_{2,273} = 42.692$, $p < 0.000$) was found, as well as significant genotype x irradiation ($F_{2,273} = 4.197$, $p < 0.05$) and irradiation x drug ($F_{1,273} = 4.504$, $p < 0.05$) interactions, and a trend toward an interaction of genotype x drug ($F_{2,273} = 2.737$, $p = 0.067$). Bonferroni's post hoc test revealed significant genotype differences in DHE oxidation between WT and E4 ($p < 0.001$) and WT and E3 mice ($p < 0.001$). There was no difference in DHE oxidation between E3 and E4 mice ($p = 0.877$). Based on these results and the significant behavioral and cognitive differences between E4 and WT mice, we proceeded to investigate in more detail the differences in DHE oxidation between WT and E4 mice. A repeated measures ANOVA of vehicle-treated sections revealed a significant time x genotype interaction ($F_{1,380,428} = 52.492$, $p < 0.001$) and a significant main effect of genotype ($F_{1,115} = 105.233$, $p < 0.001$). Sidak's pairwise comparison revealed that the hippocampal slices of E4 mice showed more DHE oxidation than those of WT mice (Fig. 6A).

Finally, we analyzed the response of hippocampal slices of E4 and WT mice to PMA, an inducer of ROS. PMA-treated hippocampal slices showed a significant time x genotype interaction ($F_{1,30,428} = 7.008$, $p < 0.01$) and a significant main effect of genotype ($F_{1,107} = 18.062$, $p < 0.001$; Fig. 6B). Additionally, PMA-treated sections revealed a significant time x genotype x irradiation interaction ($F_{1,30,428} = 6.041$, $p < 0.01$) and a genotype x irradiation interaction ($F_{1,107} = 8.441$, $p < 0.01$). Therefore, we ran a repeated-measures ANOVA in E4 and WT mice separately to examine the differing irradiation effects. The effect of irradiation was significant in both E4 ($F_{1,57} = 5.613$, $p < 0.05$) and WT mice ($F_{1,50} = 5.275$, $p < 0.05$). However, irradiation affected the genotypes differently. Hippocampal slices of irradiated WT mice showed more DHE oxidation than those of sham-irradiated WT mice. In contrast, hippocampal slices of E4 irradiated mice showed less DHE oxidation levels than those of E4 sham-irradiated mice (Bonferroni's pairwise comparison).

Hippocampal HO-1 levels

Hippocampal HO-1 levels were analyzed in sham-irradiated and irradiated WT, E4, and E3 mice. There was an effect of genotype ($F_{2,12} = 5.184$, $p < 0.05$; Fig. 7) with higher hippocampal HO-1 levels in E4 than WT mice ($p = 0.023$). There was also a genotype x radiation interaction ($F_{2,12} = 12.31$, $p = 0.0012$). Hippocampal HO-1 levels were higher in irradiated than sham-irradiated E4 mice ($p = 0.0018$, Sidak's post-hoc). No effect of irradiation on HO-1 levels was seen in WT mice ($p = 0.92$). Finally, there was a trend towards lower HO-1 levels in irradiated than sham-irradiated E3 mice ($p = 0.08$).

DISCUSSION

We found that compared to WT mice, E4 mice explored less in the open field, the elevated zero maze, and the elevated plus maze. Similarly, E4 mice showed higher measures of anxiety in the open field and elevated plus maze than WT mice. Together with the isoform-dependent effects of apoE on measures of anxiety in mice (Raber *et al.* 1998, Robertson *et al.* 2005) and humans (Robertson *et al.* 2005), increased measures of anxiety in apoE-deficient mice (Raber *et al.* 2000, Bongers *et al.* 2004), and isoform-dependent effects of apoE on PTSD-like behaviors (Johnson *et al.* 2015, Kimbrel *et al.* 2015), these data highlight the importance of these effects in the neurobiological effects of apoE (for a review, see Raber 2007, Raber 2008). Genotype differences were also observed in fear conditioning. E4 mice showed a greater response to the shocks and enhanced freezing during training, and enhanced hippocampus-dependent contextual and cued fear memory compared to WT mice. With the increased shock response, it is conceivable that this genotype difference might have contributed to the higher subsequent freezing responses and contextual and cued fear memories in E4 than WT mice. In all genotypes, irradiation induced hippocampus-dependent impairments in spatial memory retention in the water maze. The increased anxiety levels and enhanced fear learning and memory of E4 and WT were associated with increased DHE oxidation.

In the current study, E4 mice did not show impairments in acquisition or retention of spatial memory. Consistent with these data, E4 mice did not show impairments in spatial learning and memory in previous studies (Siegel *et al.* 2010, Haley *et al.* 2012b, Johnson *et al.* 2014, Villasana *et al.* 2011b). However, the experimental conditions might modulate these results as impaired acquisition and retention of spatial memory was reported in E4 mice. (Reverte *et al.* 2013).

Both contextual fear memory and spatial memory retention in the water maze are hippocampus-dependent. However, only spatial memory retention in the water maze was affected three months following ⁵⁶Fe irradiation. A dissociation between spatial memory in the water maze and contextual learning in fear conditioning has been previously reported (Logue *et al.* 1997, Silva *et al.* 1998). Consistent with the current study, whole body ⁵⁶Fe irradiation affected water maze performance but not contextual fear conditioning within one month following radiation exposure in human apoE mice (Haley *et al.* 2012a) or 13 months following ⁵⁶Fe irradiation (Villasana *et al.* 2011a). These data indicate the importance of considering different hippocampus-dependent cognitive tests in assessing effects of irradiation on the brain.

ROS is required for normal synaptic plasticity, learning and memory (Kishida *et al.* 2006, Kishida *et al.* 2005b, Knapp & Klann 2002b, Thiels *et al.* 2000, Hidalgo *et al.* 2007b). Therefore, enhanced DHE oxidation levels in hippocampal slices of E4 mice might have been associated with enhanced fear conditioning. E4 has reduced antioxidant capacity (Colton *et al.* 2005) and it is conceivable that this contributed to the higher basal levels of ROS and enhanced fear conditioning in E4 mice. The lower DHE oxidation in PMA-treated slices of irradiated versus sham-irradiated E4 mice and higher HO-1 levels in irradiated than sham-irradiated E4 mice suggest that the enhanced antioxidant capacity of E4 mice

following ^{56}Fe irradiation might protect them against increases in oxidative stress and ROS. A similar scenario was seen in mice lacking the extracellular form of superoxide dismutase that showed higher basal levels of oxidative stress. Hippocampal oxidative injury, assessed as 3-nitrotyrosine levels, showed less profound increases in mice lacking the extracellular form of superoxide dismutase than in WT mice (Raber *et al.* 2011). In contrast to E4 mice, WT mice had lower basal levels of ROS. DHE oxidation was higher in PMA-treated slices of irradiated than sham-irradiated WT mice. Importantly, the effect of irradiation on ROS levels was only observed upon pharmacological stimulation, which may be functionally relevant to synaptic plasticity, learning and memory (Hidalgo *et al.* 2007b, Kishida *et al.* 2005b, Tejada-Simon *et al.* 2005).

These paradoxical effects seen in pharmacologically stimulated slices of irradiated E4 and WT mice, suggest that the level of ROS required for behavioral and cognitive performance is determined by background levels. Thus, the role of ROS in behavioral and cognitive performance may depend on genetic differences in background levels. Further studies, possibly with antioxidants, should help elucidate the role that background levels of ROS play in the ability to induce ROS following irradiation.

In this study, there was a trend towards reduced measures of anxiety in the elevated zero maze three months following ^{56}Fe irradiation. These results are consistent with reduced measures of anxiety in E4 mice thirteen month following cranial ^{56}Fe irradiation (Villasana *et al.* 2011a). As anxiety, within reasonable limits, serves as a warning signal (Perkins *et al.* 2013), it is hard to determine whether the trend towards reduced measures of anxiety following ^{56}Fe irradiation is beneficial or detrimental. The analysis of performance in the open field, elevated plus maze, and light-dark tests indicates that this trend is task specific. These data are consistent with the notion that distinct anxiety tests differ in detecting anxiety differences in mutant mice (Rizk *et al.* 2004). In E4 mice, these data are consistent with the studies showing that ROS increase anxiety levels and that attenuation of ROS can ameliorate anxiety levels (Novio *et al.* 2011, Xu *et al.* 2014, Bouayed *et al.* 2009). Although the hippocampus is involved in the regulation of anxiety, the amygdala and other brain regions are involved as well. It is conceivable that DHE oxidation levels are differentially affected in brain regions other than the hippocampus. Future studies are warranted to assess this possibility.

In the current study, the mean cumulative distance to the target during the hidden and visible platform conditions was similar. It is conceivable that the groups' similar mean cumulative distance to the target in the two days of visible and three days of hidden sessions is due to the fact that they were trained for task learning prior to the hidden sessions, as well as receiving an extra day of training for the hidden location.

In summary, higher hippocampal ROS levels in E4 mice are associated with increased measures of anxiety and enhanced freezing levels during fear learning and memory. The opposite effects of PMA on DHE oxidation in slices of irradiated and sham-irradiated E4 and WT mice highlight the complex role of ROS in behavioral and cognitive performance. Nevertheless, future efforts are warranted to determine whether reducing ROS levels in E4 carriers is able to reduce anxiety levels

Acknowledgments

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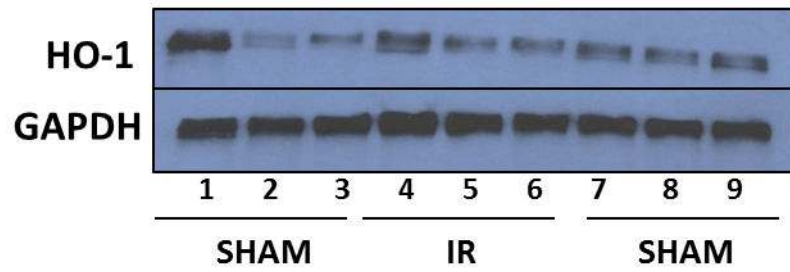


Figure 1.

Representative Western blot of hippocampal HO-1 levels. Western blot analysis showing protein levels of HO-1 (higher band) and GAPDH (lower band, to ensure equal loading) in hippocampal tissues of WT and E4 mice. Lanes 1–3: sham-irradiated WT; lanes 4–6: irradiated WT; lanes 7–9: sham-irradiated E4.

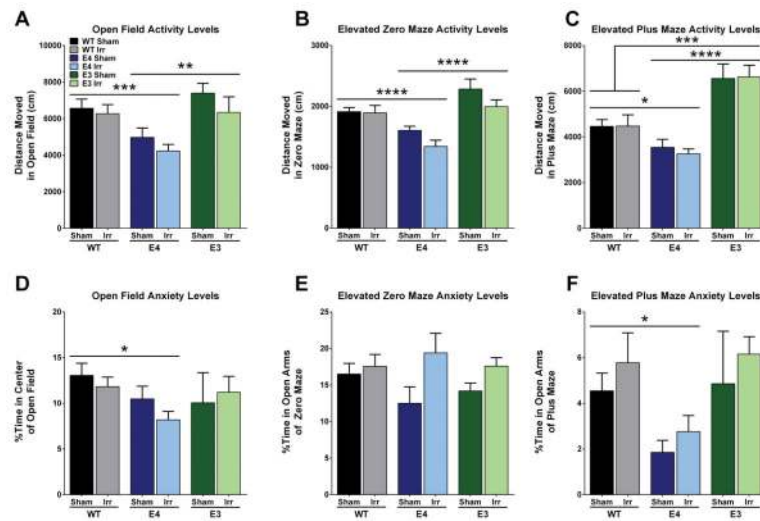


Figure 2.

Anxiety and activity measures in sham- and ⁵⁶Fe-irradiated WT, E3, and E4 female mice. E4 mice moved less in **A.** the open field (** $p < 0.001$; * $p < 0.01$), **B.** the elevated zero maze (***) $p < 0.0001$), and **C.** the elevated plus maze (* $p < 0.05$) than WT and E3 mice. E4 mice also spent less time in **D.** the center of the open field (* $p < 0.05$), and **F.** the open arms of the elevated plus maze ($p < 0.05$) but not the elevated zero maze (**E**). Bars represent the group means \pm SEM. $N = 4-16$ mice per group.

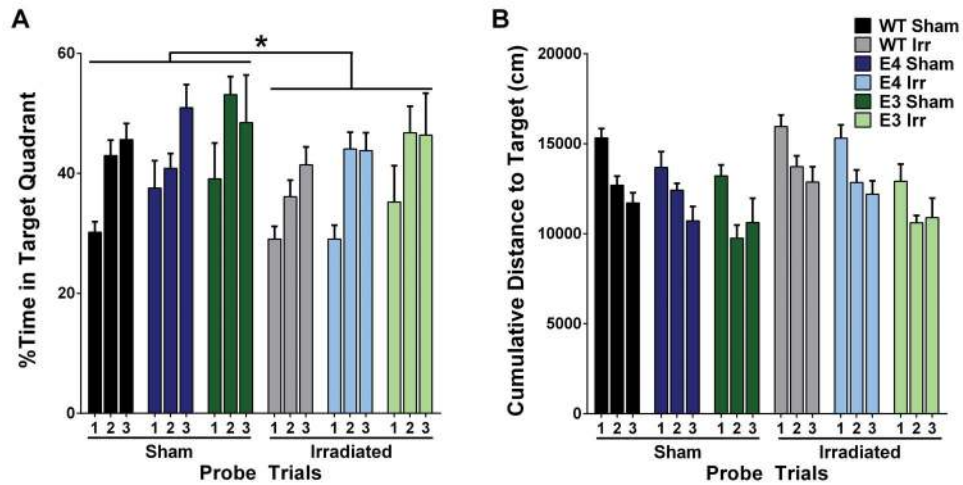
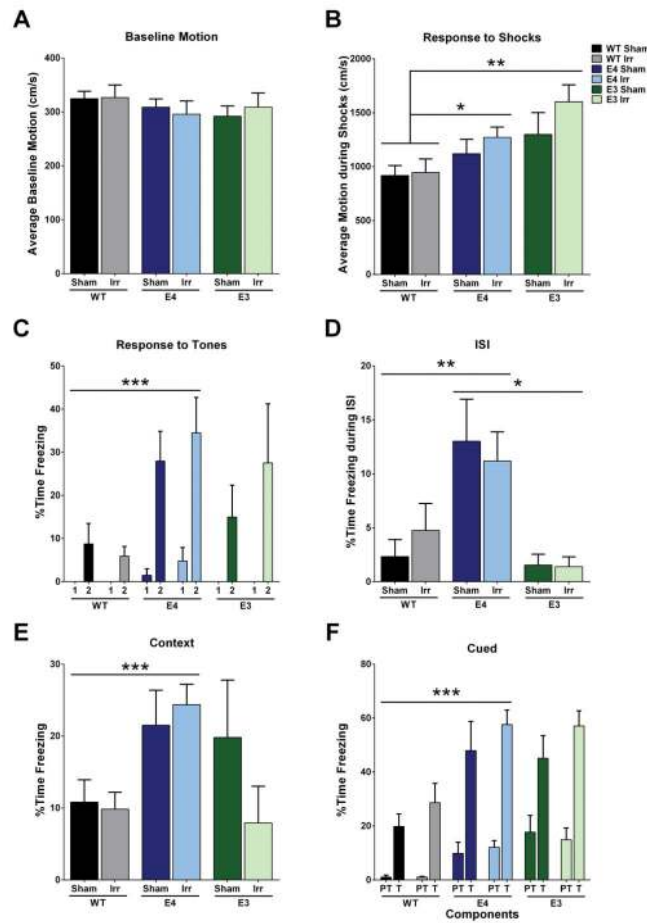


Figure 3. Spatial memory retention of sham and ^{56}Fe -irradiated WT, E3, and E4 mice in the Morris Water Maze. **A.** Percent of time spent in target quadrant in the three probe trials of the water maze. The percent of time spent in target quadrant differed across probe trials and between genotypes and treatment. Sham-irradiated mice spent more time in the target quadrant than irradiated mice ($p < 0.05$) and E3 mice spent more time in the target quadrant than WT mice ($p < 0.05$). **B.** Cumulative distance to the target location in the three probe trials of the water maze. E3 mice swam closer to the platform than WT mice ($p < 0.01$). Bars represent group average \pm SEM. $N = 4\text{--}16$ mice per group. * $p < 0.05$; ^ $p < 0.01$; # $p < 0.001$; + $p < 0.0001$.

**Figure 4.**

Acquisition and memory of conditioned fear in sham and ^{56}Fe -irradiated WT, E3, and E4 mice **A.** Average baseline motion, prior to the first tone on the training day. There were no effects of radiation or genotype. **B.** Response to the average of the two shocks on the training day. WT mice showed lower response levels than E3 ($p < 0.01$) and E4 ($p < 0.05$) mice. **C.** Freezing levels during the two tone-shock pairings on the training day. E4 mice showed higher freezing levels than WT mice ($p < 0.001$). **D.** Immobility during the interval between the two tone-shock pairings. E4 mice showed higher freezing levels than WT ($p < 0.01$) and E3 ($p < 0.05$) mice. **E.** Freezing levels during the contextual fear memory test. E4 mice showed higher freezing levels than WT mice ($p < 0.001$). **F.** Freezing levels prior to (PT) and during the tone (T) in the cued fear memory test. E4 mice showed higher freezing levels than WT mice prior to and during the tone ($p < 0.001$). Bars and line graphs represent group average \pm SEM. Training day and context test day 2: $N = 4 - 12$ mice; cued test day 2: $N = 3 - 10$ mice.

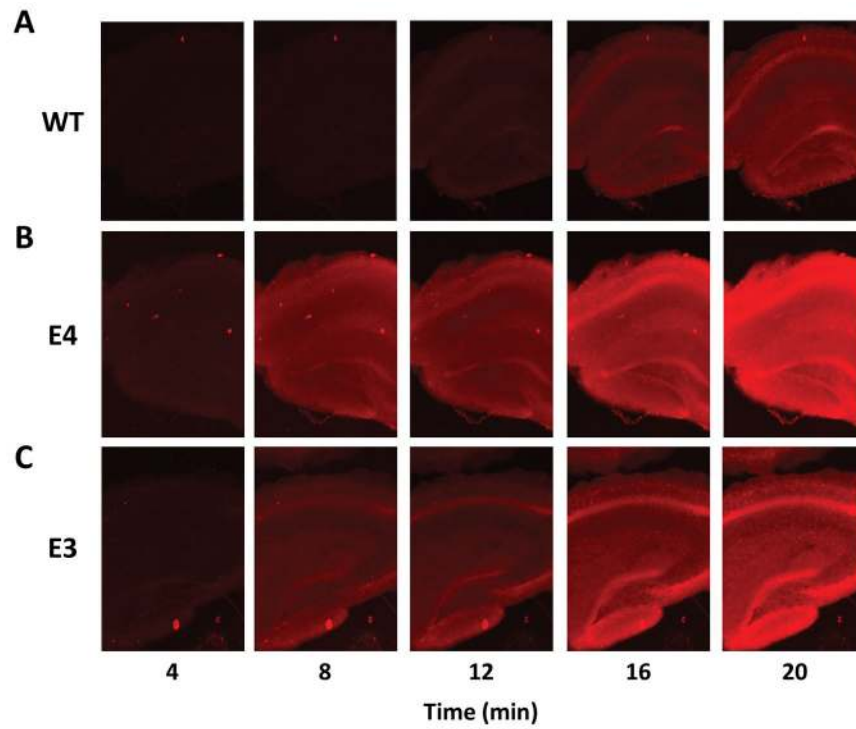


Figure 5. Representative images of time courses of DHE-incubated hippocampal slices from WT (top), E4 (middle), and E3 (bottom) mice demonstrating higher background levels of ROS in E4 than WT mice. Vehicle-treated slices from WT mice had lower levels of DHE-oxidation compared to slices from E4 mice.

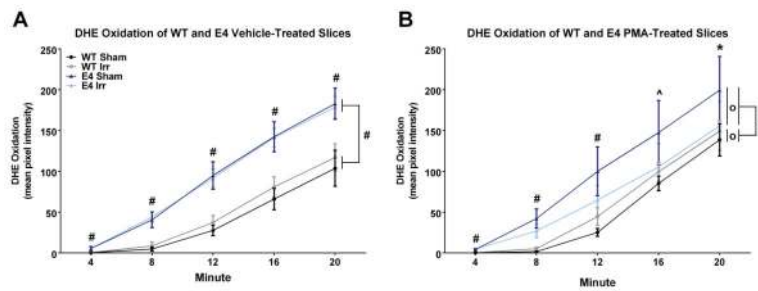


Figure 6.

Baseline and pharmacologically-induced levels of ROS in acute hippocampal slices from sham-irradiated and ^{56}Fe -irradiated WT and E4 female mice. **A.** Baseline levels of DHE oxidation in vehicle-treated hippocampi from E4 mice were higher than those from WT mice at each time point ($\#p < 0.001$ each) and across the average of the time points ($\#p < 0.001$). However, there were no differences in DHE oxidation in vehicle-treated slices from sham and irradiated mice at any time point in either genotype **B.** Following pharmacological induction of ROS with PMA, hippocampal slices from E4 mice showed greater DHE oxidation than those from WT mice at each time point and across the average of the time points ($\#p < 0.001$; $\wedge p < 0.01$; $*p < 0.05$). In E4 mice, DHE oxidation levels were attenuated in PMA-treated hippocampal slices from irradiated mice compared to those from sham-irradiated E4 mice across the average of the time points ($^0p < 0.05$). In contrast, DHE oxidation in slices from PMA-treated irradiated WT mice was higher than those from sham-irradiated WT mice across the average of the time points ($^0p < 0.05$). Data are displayed as the estimated marginal means for each group \pm SEM. $N = 5 - 7$ sections per group.

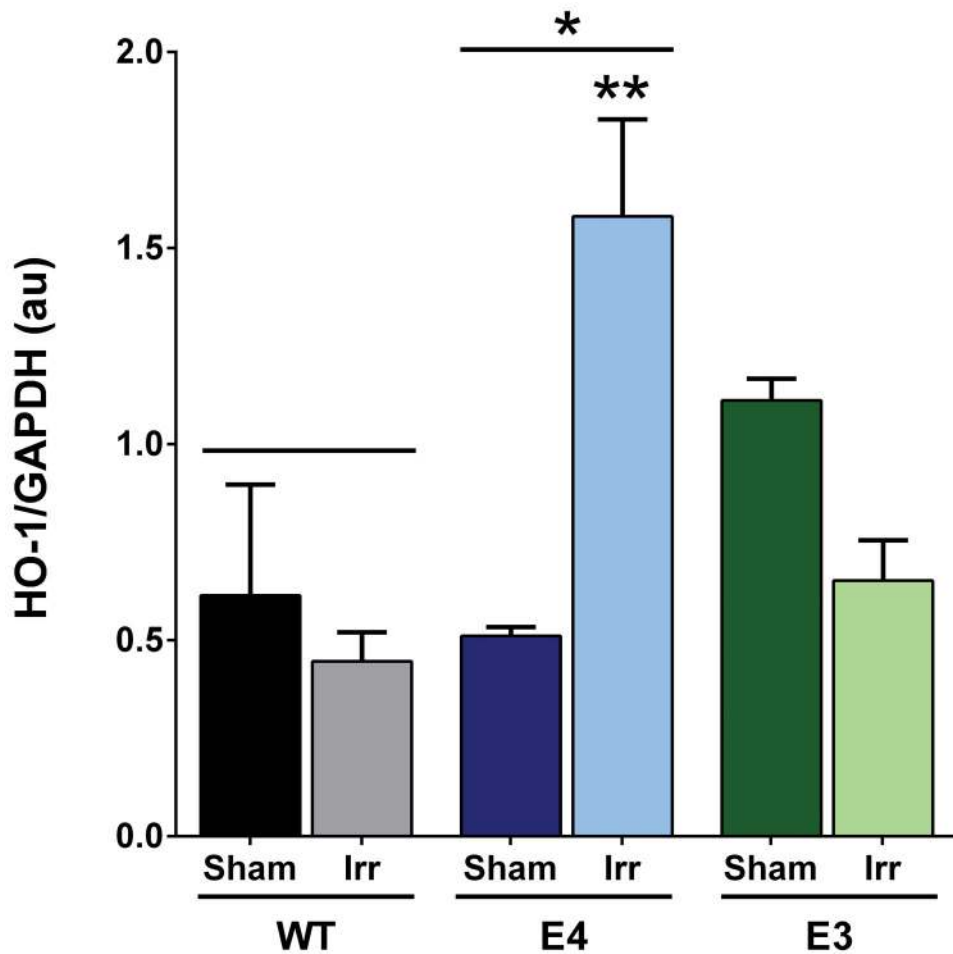


Figure 7.

Hippocampal HO-1 levels in sham-irradiated and irradiated Wt, E4, and E3 mice. There was a genotype x radiation interaction ($F_{2,12} = 12.31$, $p = 0.0012$) and an effect of genotype ($F_{2,12} = 5.184$, $p < 0.05$) (Fig. 7). Hippocampal HO-1 levels were higher irradiated than sham-irradiated E4 mice ($p = 0.0018$, Sidak's multiple comparisons test). No effect of irradiation on HO-1 levels was seen in WT ($p = 0.92$) mice and there was a trend towards lower HO-1 levels in irradiated than sham-irradiated E3 mice ($p = 0.08$). Hippocampal HO-1 levels were also higher in E4 than WT mice. $N = 3$ mice/genotype/treatment. * $p < 0.05$; ** $p < 0.01$ versus sham-irradiated E4.

Cognitive and anxiety measures of sham-irradiated and ^{56}Fe irradiated E4, E3, and WT female mice in the water maze test.¹

Table 1

| Measure | Visible | | | | | | Hidden | | | | | |
|---|------------------|------------------|-------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | Sham-Irradiated | | Irradiated | | WT | | Sham-Irradiated | | Irradiated | | WT | |
| | WT | E4 | E3 | WT | E4 | E3 | WT | E4 | E3 | WT | E4 | E3 |
| Mean Cumulative Distance to target (cm) | 6648.89 ± 790.08 | 5973.12 ± 734.88 | 5514.02 ± 1080.81 | 7572.34 ± 892.13 | 6935.45 ± 732.78 | 6205.69 ± 1214.5 | 6465.46 ± 421.39 | 5963.24 ± 517.24 | 5482.66 ± 698.72 | 7479.77 ± 530.61 | 6951.99 ± 532.69 | 4447.47 ± 546.21 |
| Thigmotaxis (%) | 15.45 ± 2.05 | 13.56 ± 1.82 | 14.66 ± 3.27 | 17.16 ± 2.25 | 15.46 ± 1.99 | 9.87 ± 2.32 | 4.82 ± 0.46 | 4.86 ± 0.91 | 2.23 ± 0.42 | 6.84 ± 0.75 | 9.46 ± 1.66 | 2.96 ± 0.85 |
| Swim Speeds (cm/s) | 17.49 ± 0.36 | 17.97 ± 0.50 | 18.16 ± 0.58 | 17.78 ± 0.26 | 16.40 ± 0.27 | 17.71 ± 0.45 | 18.44 ± 0.31 | 17.51 ± 0.35 | 18.55 ± 0.33 | 18.34 ± 0.28 | 17.16 ± 0.34 | 17.89 ± 0.35 |

¹Data are shown as group averages ± SEM. N= 10–16 per group. All data were analyzed by ANOVA, using genotype and treatment as between-group factors. There were no significant effects in any outcome measure.