The Role of Apolipoprotein E Isoform and Reactive Oxygen Species on the Cognitive Function of Female Mice following Cranial Irradiation

by

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

^{137}Cs-^{137}Cesium

^{56}Fe- ^{56}Iron

ACSF- artificial cerebral spinal fluid

AD-Alzheimer's disease

ALA- alpha-lipoic acid

ANOVA- analysis of variance

apoE- apolipoprotein E

BNL- Brookhaven National Laboratories

CF- conditioned fear task

DG- dentate gyrus

DHE- dihydroethidium

EC-entorhinal cortex

EC-SOD- extracellular superoxide dismutase

EPA- environmental protection agency

EPM- elevated plus maze

EZM- elevated zero maze

Gy- Gray

HNE-4-hydroxynonenal

HZE- high-energy particle radiation

ITI- inter-trial-interval

LD- light-dark task

LDL-low-density lipoproteins

LTP- long-term potentiation

NASA- National Aeronautics and Space Administration

OF- open field task

PMA- phorbol ester myristate

ROS- reactive oxygen species

TBI- total body irradiation

VLDL-very low-density lipoproteins

WM- water maze task

WBI- whole brain irradiation

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Abstract

Exposure to radiation can lead to deficits in cognitive function, including impairments in hippocampal-dependent learning and memory. However, not all individuals exposed to irradiation develop cognitive impairments, suggesting the involvement of genetic risk factors. Apolipoprotein E (apoE), a protein important for neuronal repair, might influence susceptibility to developing radiation-induced cognitive impairments. Humans express three major apoE isoforms, apoE2, apoE3 and apoE4. Compared to apoE3, apoE4 increases the risk to develop Alzheimer's disease while apoE2 decreases this risk. ApoE4 is also associated with cognitive deficits following neurotrauma. Moreover, deficiency of apolipoprotein E (apoE) in mice worsens cognitive impairments following irradiation. There might also be sex differences in the risk for developing radiation-induced cognitive impairments. In both humans and rodents, females are more susceptible to the effects of irradiation on cognition than males. The neurobiological mechanisms underlying the effects of irradiation are not clear but may involve increases in reactive oxygen species (ROS), a major component of oxidative stress.

My dissertation addressed three main questions. First, are the effects of irradiation on the cognitive function of female mice apoE isoform-dependent? Second, does ROS play a role in the effects of irradiation on cognitive function?

Third, are the effects of irradiation on cognition and ROS specific to the type of irradiation? The last question was of interest because humans are exposed to different types of irradiation such as ^{137}Cs gamma and ^{56}Fe high-energy particle radiation,

To address these questions, 2-month-old apoE2 and apoE4 female mice were sham-irradiated or ^{137}Cs-irradiated and behaviorally tested 3 months later to assess hippocampal and non-hippocampal functions. Following testing, group differences in baseline and phorbol-myristate ester (PMA)-induced ROS in *ex vivo* hippocampal slices of the behaviorally tested mice were compared using the fluorescent dye, dihydroethidium (DHE).

Surprisingly, compared to sham-irradiated apoE4 mice, ^{137}Cs-irradiated apoE4 mice showed enhancements in spatial memory retention in water maze probe trials. This was not observed in apoE2 mice. Spatial memory enhancements in apoE4 mice were associated with an enhanced response to PMA induction of ROS. The increase in ROS was specific to PMA-induction as there were no irradiation-induced increases in baseline levels of ROS. When this was followed up with ^{56}Fe irradiation to assess whether the effects of irradiation are specific to ^{137}Cs irradiation, apoE4 female mice no longer showed enhancements in spatial memory retention in the water maze but instead, showed enhanced contextual fear conditioning. Enhancements in contextual memory were not associated with enhancements in PMA-induction of ROS.

While these data are surprising, they also emphasize that effects of irradiation are specific to the type of irradiation, apoE isoform, and type of memory. These studies also show that changes in induction of ROS generation are associated with radiation-induced enhancements in spatial memory retention in the water maze. Moreover, these studies make an important distinction between a general increase in ROS and stimulus-induced generation of ROS as it relates to spatial memory. Potential mechanisms involved in the role of ROS in cognition are proposed.

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General Introduction

General Introduction

Humans are exposed to distinct forms of radiation under different situations. By definition, radiation is the transmission of particles of energy through space or a medium. Most of the radiation humans encounter consists of non-ionizing radiation. Non-ionizing radiation, as found in electronic devices such as microwaves, has a very low frequency and is not considered a health hazard. In contrast, ionizing radiation has enough energy to remove electrons from atoms, thus, creating free radicals. This can cause biological damage such as burns, radiation sickness, and DNA mutations (Environmental Protection Agency, 2010). Ionizing radiation, the type of radiation released following nuclear accidents such as Chernobyl and Fukushima, includes alpha, beta, and gamma particles. Because of industrial uses of radiation and fall out from nuclear accidents, the general public is exposed daily to low levels of ionizing radiation. Airline pilots and crews are exposed to higher levels of radiation than the general public (Lim 2001).

Radiation exposure can lead to life-long and progressive impairments in cognitive function including: deficits in hippocampal-dependent learning and memory, attention, speed of information processing, and executive function (Twijnstra et al. 1987; Lee et al. 1989; Douw et al. 2009). The hippocampus, a brain region important for learning and memory (Scoville and Milner 1957; Squire and Zola-Morgan 1991; Tulving and Markowitsch 1998; Schacter and Wagner 1999) is particularly sensitive to irradiation (Packer et al. 1989; Roman and

Sperduto 1995; Raber et al. 2004; Gutierrez et al. 2007; Monje et al. 2007). In fact, the extent of cognitive impairments is associated with the dose of irradiation delivered to the medial temporal lobes, the site of the hippocampus (Roman and Sperduto 1995). In the U.S., approximately 170,000 patients per year are treated for brain metastasis. It is estimated that 50-90% of patients who survive up to 6 months following whole brain irradiation (WBI) will develop life-long cognitive deficits (Ramanan et al. 2010), and 10% of patients will develop progressive dementia (Shaw et al. 2006). To date, there are no proven strategies for preventing radiation-induced cognitive impairments (Peper et al. 2000; Monje and Palmer 2003; Shaw et al. 2006).

Irradiation and the hippocampus:

A comprehensive understanding of the cognitive domains affected by irradiation can help identify brain areas that are susceptible to the effects of irradiation. As previously noted, the hippocampus is a brain region that has been identified as sensitive to the effects of irradiation. The hippocampus is critical for the formation of new memories such as declarative memories, which are memories for facts and events (Squire and Zola-Morgan 1991; Tulving and Markowitsch 1998; Schacter and Wagner 1999). Historically, damage to the hippocampus was thought to be restricted to anterograde amnesia (inability to form new memories); however, it appears that retrograde amnesia (loss of memories formed before hippocampal damage) can also occur (Gilboa et al. 2006). Non-

declarative memories, such as learned skills and habits, are not dependent on the hippocampus (Squire and Zola-Morgan 1991).

The hippocampus is also important for spatial navigation (O'Keefe and Dostrovsky 1971; Morris 1981). Spatial navigation requires processing of information about the orientation of different environmental components in relation to each other in order to form a "cognitive map" (O'Keefe et al. 1975). Place cells, specialized neurons, are found within the hippocampus and are thought to support spatial navigation. They are distinct from other cells in that their firing rate increases whenever an animal is within a particular place within a cell's field (O'Keefe and Dostrovsky 1971). The dorsal region of the hippocampus has a greater concentration of place cells compared to the ventral region (Moser et al. 2008). Dorsal lesions to the hippocampus result in greater spatial memory impairments than ventral lesions (Moser et al. 1993), suggesting that the dorsal hippocampus might be more involved in spatial memory than the ventral region.

The processing of information within the hippocampus involves distinct regions. The main regions of the hippocampus are the dentate gyrus, the CA1 and the CA3 areas (Lisman 1999; Lavenex and Amaral 2000; Squire et al. 2004). The entorhinal cortex (EC) is a major source of cortical afferent projections for the hippocampus (Zola-Morgan et al. 1994; Squire et al. 2004). Cortical information is received from the EC by the dentate gyrus, which projects to the CA3 region via the mossy fiber tract. The CA3 region then projects to the CA1

region where the information is sent back to the cortex via efferent projections from the subiculum and EC (Squire and Zola-Morgan 1991; Lavenex and Amaral 2000; Adams and Sweatt 2002). Simultaneous processing of various bits of cortical information by the hippocampus is thought to give rise to a memory of a whole event (Squire and Zola-Morgan 1991).

A cellular phenomenon thought to be a neural substrate for learning and memory is long-term potentiation (LTP) (Maren and Baudry 1995; Kim et al. 1996; Shapiro 2001). Hippocampal LTP is a long-lasting increase in synaptic efficacy induced by high-frequency stimulation (Bliss and Collingridge 1993). Similar to long-term memory, expression of LTP is also dependent on protein synthesis (Otani and Abraham 1989; Scharf et al. 2002). Although the exact mechanisms underlying LTP are not fully understood, the general steps giving rise to LTP have been described (Kandel et al. 2000; Abraham and Williams 2008). One of the processes involved in LTP is the activation of extracellular signal regulated kinase (ERK) (English and Sweatt 1997; Winder et al. 1999; Watabe et al. 2000). The importance of ERK has been demonstrated in several different types of memories including spatial memory in the water maze (Blum et al. 1999; Selcher et al. 1999), contextual fear conditioning (Atkins et al. 1998), taste aversion (Berman et al. 1998), and avoidance learning (Walz et al. 2000). There is evidence that irradiation increases ERK phosphorylation (Silasi et al. 2004), suggesting that ERK might play a role on the effects of irradiation on cognitive function. Furthermore, previous data show that ERK phosphorylation is

dependent on generation of superoxide, a free radical belonging to the ROS family (Kishida et al. 2005a). Irradiation results in increases in ROS, suggesting the possibility that increases in ERK activity following irradiation might be mediated through coinciding increases in ROS.

Effects of ^{137}Cs and ^{56}Fe irradiation on learning and memory:

Humans are exposed to various types of irradiation, including different forms of ionizing radiation. In the clinical setting, ^{137}Cs radiation is a common form of gamma radiation used for radiotherapy (Camphausen and Lawrence 2009). Although whole brain irradiation (WBI) for brain metastasis is a life-saving treatment, unfortunately, it is not without serious and life-long side effects (Roman and Sperduto 1995; Abayomi 2002; Byrne 2005; Sarkissian 2005). As previously mentioned, in humans WBI can lead to progressive and long-term deficits in cognitive function including deficits in learning and memory, attention, speed of information processing, and executive function (Twijnstra et al. 1987; Lee et al. 1989; Douw et al. 2009). In children, the effects of irradiation on cognition are also reflected by lower academic achievement and lower IQ score (Lee et al. 1989). Children that were irradiated at a very young age show more severe cognitive impairments (Packer et al. 1989), indicating that age is an important factor in the effects of irradiation on cognition.

Noteworthy, however, is that the literature on the effects of gamma irradiation on the hippocamapal-function of rodents is not completely consistent. For

example, both contextual fear conditioning and water maze are hippocampal-dependent tests (Morris 1984; Brandeis et al. 1989; Kim and Fanselow 1992; Phillips and LeDoux 1992; Young et al. 1994; Maren et al. 1997), yet some studies have found radiation-induced deficits in contextual fear conditioning but not spatial memory retention in the water maze (Saxe et al. 2006; Wojtowicz et al. 2008; Hernandez-Rabaza et al. 2009). In other studies, spatial memory deficits following irradiation are reported in the hippocampal-dependent Barnes maze task but not the water maze test (Raber et al. 2004; Rola et al. 2004). Similarly, radiation-induced deficits have been reported in the water maze test but not the hippocampal-dependent passive avoidance test (Acevedo et al. 2008a). Furthermore, other studies assessing the effects of irradiation on hippocampal-dependent working memory, have reported no deficits (Benice and Raber 2010) or even enhancements (Saxe et al. 2007) in the performance of irradiated mice compared to that of sham-irradiated mice. Enhancements in spatial memory retention of mice and rats following gamma irradiation have also been previously reported (Arnold and Blair 1956; Raber et al. 2011). Collectively, these data indicate that irradiation differentially affects different types of hippocampal-dependent memories. Furthermore, it is also very likely that some of the differences in outcome between studies might be related to differences in task protocols.

Humans are also exposed to other forms of radiation such as high-energy (HZE) particle irradiation, which includes heavy ions from elements such as

carbon and iron (Brookhaven National Laboratories, 2010). Astronauts undergo prolonged exposure to this form of radiation during space missions. The space environment includes ionized atomic nuclei of all stable elements, including hydrogen, helium, oxygen, carbon and ^{56}Fe (Bahadori et al. 2011). HZE irradiation, such as with carbon ions, has had more recent use in the treatment of tumors that are not responsive to other forms of cancer treatments such as gamma irradiation (Normile 1995; Sawajiri et al. 2003). Compared to gamma irradiation, relatively little is known about the effects of HZE irradiation on the cognitive function of humans.

In rodents ^{56}Fe irradiation is associated with impairments in hippocampal-dependent tasks, such as the water maze task (Higuchi et al. 2002; Shukitt-Hale et al. 2007; Manda et al. 2008; Villasana et al. 2011) and contextual fear conditioning (Villasana et al. 2010). Impairments in the operant conditioning of rats have also been observed following ^{56}Fe irradiation (Rabin et al. 2005b).

There are significant energy differences between ^{137}Cs and ^{56}Fe radiation that warrant consideration. The energy released by ^{137}Cs is 662 KeV while that of ^{56}Fe is substantially greater, 600 MeV. In addition, although it is estimated that the radiation energy from galactic cosmic rays, which are mostly comprised of ^{56}Fe particles, can exceed 10 MeV n^{-1} (Bahadori et al. 2011), accurate dosimetry measurements of radiation absorption by astronauts after space missions have not been possible (Testard and Sabatier 1999; Greco et al. 2003). This uncertainty presents a challenge to the radiation research community with

respect to designing experiments to compare the biological effects of HZE radiation with other forms of radiation. The doses of ^{137}Cs radiation used in radiotherapy cover a large range depending on treatment evaluation, but typically are in the 20 – 40 Gy range and are delivered using multiple fractions (Mulhern et al. 2004). Although the energy difference and lack of dose knowledge make it difficult to compare the effects of the two forms of irradiation on cognition, studies indicate that the relative biological effectiveness of HZE radiation is greater than that of gamma radiation (Sawajiri et al. 2003; Guida et al. 2005), which might be due to the size of the particles (^{56}Fe are smaller) and the significant greater energy of HZE radiation (Normile 1995).

The effects of the two forms of irradiation on cognitive function have not been compared side by side within the same study. Indeed, this is a very challenging feat because BNL has the only source of ^{56}Fe radiation in the country and access to its facilities is very limited.

Irradiation, aging and cognitive function:

The effects of gamma irradiation on cognitive function resemble the cognitive decline that occurs with aging. In general, some of the cognitive changes associated with aging in humans include: 1) changes in spatial learning and memory (Erickson and Barnes 2003); 2) reduced processing speed (Salthouse 1996); 3) a decline in fluid intelligence, which is the ability to perform a task based on new information (Salthouse 1996); 4) increased preservative behavior-

the inability to adjust behavior based on task contingencies (Ridderinkhof et al. 2002); and 5) changes in executive function - attention, planning, cognitive flexibility, abstract thinking, rule acquisition, initiating appropriate actions and selecting relevant sensory information (Hedden and Gabrieli 2005). As noted at the beginning of this section, gamma irradiation in children and adults induces impairments in many of these cognitive functions.

In rodents, the effects of irradiation on cognitive function also resemble the cognitive decline that is observed in aged rodents. In fact, it has been proposed that ^{56}Fe irradiation results in accelerated aging (Joseph et al. 2000; Rabin et al. 2005a); however, to the best of my knowledge, the effects of irradiation and aging on cognitive function have not been experimentally compared within the same study to appropriately address the accelerated aging hypothesis. Nevertheless, there are striking resemblances that warrant a close examination of how the effects of irradiation compare to those of aging. Some of the similarities between the effects of irradiation and aging on cognitive function include: deficits in hippocampus-dependent learning and memory (Gallagher and Pelleymounter 1988; Raber et al. 2004; Villasana et al. 2011), increases in anxiety-like behaviors (Boguszewski and Zagrodzka 2002; Rabin et al. 2007), and changes in motor coordination (Joseph et al. 1992). Neurobiological similarities between irradiation and aging include: reductions in adult born hippocampal neurons (Raber et al. 2004; Jinno 2011), reductions in striatal

dopaminergic cells (Joseph et al. 1992), and increases in oxidative stress (Clausen et al. ; Limoli et al. 2007).

If irradiation mimics or accelerates cognitive aging, then another concern is how irradiation might influence the development of age-related neurodegenerative diseases. According to Shaw et al. (2006), 10% of patients that receive WBI or partial brain irradiation develop dementia. Some of the neuropathological characteristics of irradiation are also similar to those observed in Alzheimer's disease (AD). These characteristics include: demyelination of cerebral white-matter, decreases in cerebral perfusion, metabolism and in levels of N-acetyl aspartate (Shaw et al. 2006). There is also a high degree of concordance in pathways associated with cognitive dysfunction between the brains of irradiated mice and those of patients with AD (Lowe et al. 2009). These pathways include genes for ion channels, Ca^{2+} and glutamate signaling, as well as genes associated with amyloid processing.

Risk factors influencing effects of irradiation on cognition:

Not all people exposed to irradiation develop cognitive deficits (Crossen et al. 1994; Mulhern et al. 2004), indicating that individual biological differences influence the effects of irradiation on cognition. The literature on the effects of irradiation on human cognition suggests that sex might be a factor that determines the effects of irradiation on cognition (Butler and Mulhern 2005; Harila-Saari et al. 2007; Lahteenmaki et al. 2007). For instance, girls treated with

radiotherapy for acute lymphoblastic leukemia (ALL) show greater cognitive impairments than boys (Butler and Mulhern 2005). However, not all girls exposed to irradiation develop cognitive deficits, indicating that genetic risk factors might also play a role in the effects of irradiation on cognition.

Apolipoprotein E (apoE) is a 34 kDa protein that is mostly found in the liver but is also present in the brain (Mahley 1988). ApoE is important for neuronal repair (Samatovicz 2000) and for the metabolism and distribution of lipoproteins and cholesterol (Mahley 1988). In non-neuronal cells in the brain, astrocytes and microglia are the main producers of apoE. Neuronal cells, on the other hand, are mainly responsible for the production of low density lipoprotein (LDL) receptors, receptors for apoE (Beffert et al. 2004). Historically, the apoE receptors have been mostly characterized for their role in lipid transport and clearance (Huang et al. 2004). However, they are also involved in signal transduction pathways (Qiu et al. 2006; Korwek et al. 2009).

ApoE might modulate cognitive susceptibility to WBI. The absence of apoE expression in mice (*Apoe*$^{-/-}$) increases the risk to develop cognitive deficits following cranial ^{56}Fe irradiation (Higuchi et al. 2002; Acevedo et al. 2008a). Susceptibility to radiation-induced cognitive impairments might be further influenced by apoE genotype. There are 3 major apoE isoforms expressed in humans, which are encoded by 3 distinct alleles, ε2, ε3 and ε4 (Mahley 1988). The ε3 allele is the most frequently expressed allele (79-80%) followed by ε4 (10 -15%) and ε2 (5-10%) (Lahiri 2004). The idea that apoE isoform might influence

cognitive outcome following irradiation is based on evidence suggesting that apoE has isoform-dependent antioxidant properties (Ihara et al. 2000; Lauderback et al. 2002; Dafnis et al. 2010) and different degrees of protection against oxidative stress (apoE2 > apoE3 > apoE4) (Pedersen et al. 2000; Butterfield et al. 2002; Jofre-Monseny et al. 2008). Irradiation causes long term increases in reactive oxygen species (ROS) (Monje et al. 2003; Limoli et al. 2007; Manda et al. 2007b; Dayal et al. 2008), which can lead to neuronal damage through oxidative stress (Harman 1981). Therefore, the different antioxidant properties of the apoE isoforms might result in different levels of protection against the effects of irradiation on brain function.

Although the exact mechanisms responsible for differences in the antioxidant properties of the apoE isoforms are not clear, they may be related to differences in their ability to bind to 4-hydroxynonenal (HNE) (Pedersen et al. 2000). HNE is a byproduct of lipid peroxidation (Esterbauer et al. 1991). Free HNE can cause cellular damage by oxidizing proteins (Blanc et al. 1997; Mark et al. 1997; Blanc et al. 1998) and cellular death by provoking apoptotic cascades (Kruman et al. 1997). Fortunately, the apoE isoforms can attenuate the harmful effects of HNE by binding to it via their cysteine residues (Pedersen et al. 2000). The 299 amino acid sequence of the apoE isoforms differs only in the number of cysteine residues: apoE2 has two (at position 112 and 158), apoE3 has 1 (at position 112 and an arginine instead at position 58) and apoE4 has none (two arginines at 112 and 158) (Rall et al. 1983; Weisgraber 1990). Therefore, the differences in

cysteine residues are thought to be responsible for the inferior protection of apoE4 against oxidative damage (Pedersen et al. 2000). Still, a different study (Miyata and Smith 1996) suggests that the antioxidant property of apoE involves metal sequestration, but this might be a general property of apoE as no apoE isoform antioxidant differences were observed in the study. Other data suggest that the receptor-binding domain of apoE is responsible for its free radical scavenging property and that apoE3 is more effective at inhibiting LDL oxidation compared to apoE4 (Pham et al. 2005).

The different protective properties of the apoE isoforms in terms of oxidative stress might be related to disease risk and outcome following various brain challenges (Butterfield et al., 2002). Compared to apoE3, apoE4 increases the risk to develop AD, particularly in women, whereas apoE2 reduces this risk (Farrer et al. 1997). Post-mortem brains of AD patients show increased lipid peroxidation (Ramassamy et al. 1999; Ramassamy et al. 2000; Tamaoka et al. 2000) and increased blood levels of the hydroxyl radical (Ihara et al. 2000). These findings support the notion that different antioxidant capacities of the apoE isoforms might be related to the risk of developing AD and/or contribute to its pathology (Butterfield et al. 2002; Jofre-Monseny et al. 2008). In addition, apoE4 is also associated with a risk of developing cognitive impairments following brain trauma (Friedman et al. 1999; Kutner et al. 2000) and cardiac bypass surgery (Tardiff et al. 1997).

Considering the different protective properties of the apoE isoforms in response to environmental challenges, it is possible that the apoE isoforms could also differentially influence the effects of irradiation on cognitive function. Furthermore, because the apoE4 increases the risk of women to develop AD (Farrer et al. 1997), it is possible that apoE4 expression in females also increases the risk to develop radiation-induced cognitive impairments. As previously noted, females are more susceptible to the effects of irradiation and this differences is evident in both humans and rodents (Silasi et al. 2004; Butler and Mulhern 2005; Yazlovitskaya et al. 2006; Acevedo et al. 2008b).

Mice expressing human apoE provide an excellent model for assessing the role of apoE-isoform on the effects of irradiation on cognitive function. Targeted replacement apoE mice were created by replacing the mouse *apoE* gene with human APOE2, APOE3 or APOE4 alleles (Sullivan et al. 1998; Knouff et al. 1999). In these mice, the mouse apoE promoter is used to express human apoE, thereby excluding expression of mouse apoE (Sullivan et al. 1998; Knouff et al. 1999). There is evidence to suggest that the different isoforms express different levels of apoE. For instance, levels of apoE in the hippocampus, cortex and amygdala are higher in female apoE2 mice compared to female apoE4 mice (Siegel et al. 2011). Similarly, within the hippocampus, cortex and cerebellum, male apoE2 mice show a trend towards greater apoE levels compared to apoE4 male mice (Sullivan et al. 2004). ApoE2 male mice also have higher plasma levels of apoE compared to apoE3 and apoE4 mice (Sullivan et al. 2004).

The cognitive function of human apoE transgenic mice has been previously characterized. Compared to apoE3 female mice, apoE4 female mice show impairments in reference and working memory in the water maze tasks and in active and passive avoidance tasks (Bour et al. 2008). Interestingly, 3-4 month-old apoE4 mice show enhancements in LTP compared to apoE2 mice (Korwek et al. 2009). This observation is consistent with previous findings in our lab that show enhanced spatial memory of apoE4 female mice in the water maze task (Siegel et al. 2011). However, in that study it appeared that increased levels of anxiety contributed to the better performance in the water maze. Other studies by our lab have found similar increases in anxiety levels of mice that express apoE4 only in the brain (Robertson et al. 2005).

Potential mechanisms underlying the effects of irradiation on cognition:

The mechanisms responsible for the effects of irradiation on hippocampal function are unclear. Two major responses of the brain to irradiation are reductions in adult born hippocampal cells (Shors et al. 2001; Mizumatsu et al. 2003; Rola et al. 2004; Drew et al. 2010) and increases in inflammatory responses including ROS (Monje et al. 2003; Chan et al. 2004; Rabin et al. 2005c; Limoli et al. 2007; Manda et al. 2007a; Shukitt-Hale et al. 2007; Rola et al. 2008).

To determine whether radiation-induced changes in neurogenesis occur in an apoE isoform-dependent manner, preliminary studies by our lab were conducted

using doublecortin, a marker of immature neurons. These preliminary studies showed that neither ^{137}Cs nor ^{56}Fe irradiation-induced reductions in doublecortin-positive immature neurons occurred in an apoE isoform-dependent manner. These data indicate that neurogenesis is unlikely a primary mechanism to explain potential apoE isoform-dependent effects of irradiation on cognitive function. Therefore, efforts were focused on radiation-induced changes in ROS as a potential mechanism underlying the effects of irradiation on cognitive function. Nevertheless, because only one aspect of neurogenesis (i.e. immature neurons) was assessed by our preliminary study, the role of neurogenesis in learning and memory, and its association with irradiation is still discussed in this dissertation. The aforementioned preliminary data are also presented in the Appendix.

Neurogenesis

The adult brain is capable of producing new neurons. Neurogenesis is most robust in the sub-ventricular zone of the lateral ventricles (SVZ) and the sub-granular zone (SGV) of the granule-cell layer in the dentate gyrus of the hippocampus (Altman and Das 1965; Eriksson et al. 1998). Other brain regions where neurogenesis occurs, albeit at much lower levels, include: the neocortex (Gould et al. 1999), the striatum (Van Kampen et al. 2004), the amygdala (Bernier et al. 2002), and the substantia nigra (Zhao et al. 2003). New cells follow three stages of development: proliferation, migration, and maturation (Mizumatsu et al. 2003; Ming and Song 2005). Cells born in the SVZ migrate to

the olfactory bulb via the rostral migratory stream. Cells born in the sub-granular zone layer that survive become functionally integrated into the hippocampal circuitry (van Praag et al. 2002). These neurons display passive membrane properties, conduct action potentials, and form synaptic inputs with neighboring cells that are similar to those of mature neurons (van Praag et al. 2002).

The discovery of adult born neurons occurred over half a century ago, but interest in their potential role in cognitive function has only recently received much deserved attention. Initial lack of interest was possibly due to the fact that new cells constituted a very low percent of the total hippocampal population, less than 4% (Ming and Song 2005) and because adult neurogenesis in primates and humans was not observed until the late 1990s (Eriksson et al. 1998; Gould et al. 1999). Interest in neurogenesis increased as researchers began to observe an association between levels of neurogenesis and learning and memory in animals such as songbirds (Goldman and Nottebohm 1983). More recent studies show that activities that improve learning and memory such as environmental enrichment and exercise are associated with levels of neurogenesis (van Praag et al. 1999; Kempermann et al. 2002). The latter discoveries in neurogenesis have propelled interest in what is now one of the largest fields in neuroscience.

Although there are several studies showing an association of adult neurogenesis with cognitive function, the exact role of neurogenesis is still debatable. The contributions of adult neurogenesis to hippocampal function may involve: spatial and associative learning and memory (Raber et al. 2004; Zhang

et al. 2008; Drew et al. 2010); timing of events through pattern separation of neuronal activity (Deng et al. 2010); and binding of contextual elements which is thought to be associated with depression (Thomas and Peterson 2008). While some studies report deficits in hippocampal-dependent memory associated with reductions in adult neurogenesis (Shors et al. 2001; Raber et al. 2004; Ming and Song 2005; Winocur et al. 2006; Wojtowicz et al. 2008; Zhang et al. 2008; Drew et al. 2010), other studies report no changes (Meshi et al. 2006; Jaholkowski et al. 2009). Furthermore, some studies report memory enhancements following reductions in adult neurogenesis (Saxe et al. 2007; Raber et al. 2011). Therefore, the exact contributions of neurogenesis to hippocampal function are not yet clear.

Irradiation is commonly used to study adult neurogenesis since proliferating cells are highly sensitive to irradiation (Peissner et al. 1999; Mizumatsu et al. 2003). However, irradiation can also lead to other major changes in the brain, such as inflammatory responses (Limoli et al. 2003; Monje et al. 2003; Limoli et al. 2007). Therefore, it is difficult to determine whether radiation effects on the brain are caused by changes in neurogenesis or by other non-specific effects.

Reactive oxygen species

A well-established biological effect of irradiation is a long-term increase in inflammatory responses (Mizumatsu et al. 2003; Monje et al. 2003; Limoli et al. 2007; Rola et al. 2008) and ROS (Jou et al. 2002; Limoli et al. 2007; Collins-

Underwood et al. 2008; Brown et al. 2010), which can lead to cell damage through oxidative stress. Reactive oxygen species (ROS) are a major component of oxidative stress and include free radicals such as superoxide, nitric oxide, hydrogen peroxide and hydroxyl radicals. High levels of ROS can lead to cell damage or death through oxidation of cellular components such as lipids, proteins and DNA (Bartsch and Nair 2006). Because of its high oxygen consumption and relatively low levels of antioxidants, the brain is particularly vulnerable to oxidative stress (Halliwell 1992). Damage to cellular function as a result of oxidative stress is associated with learning and memory deficits, as well as age-related cognitive decline (Harman 1981). Cellular defense against the damaging effects of ROS is afforded through cell repair mechanisms, as well as by the antioxidant defense system. This includes enzymatic ROS scavengers such as superoxide dismutase, catalase, and glutathione peroxidase (GSH). Oxidative damage occurs when the production of ROS exceeds the ability of the cell to repair itself and is greater than what the antioxidants can scavenge (Harman 1981).

Conventionally, ROS have been considered to mostly have pathological influences on cognitive function; however, ROS such as superoxide and nitric oxide also have critical functions as second messenger molecules in signal transduction pathways involved in learning and memory (Klann 1998; Klann et al. 1998; Kishida et al. 2005b; Kishida et al. 2006a; Hidalgo et al. 2007; Overeem et al. 2010). In line with the positive role of superoxide in learning and memory, we

previously observed radiation-induced enhancements in the hippocampal-function of male mice deficient in extracellular superoxide dismutase (EC-SOD $^{-/-}$) (Raber et al. 2011). Compared to sham-irradiated genotype-matched mice, ^{137}Cs-irradiated EC-SOD $^{-/-}$ mice showed better performance in the water maze probe trials, novel location recognition task, and contextual fear conditioning. Wild-type mice did not show a similar beneficial effect of irradiation. Additionally, hippocampal levels of 3-nitrotyrosine, a marker of oxidized proteins, was elevated in irradiated wild-type mice but not in irradiated EC-SOD $^{-/-}$ mice, suggesting that EC-SOD $^{-/-}$ mice were resistant radiation-induced increases in hippocampal oxidative damage. These findings raise the possibility that irradiation might enhance hippocampal-dependent learning and memory in mice with higher background levels of ROS. Such a possibility could very well apply to apoE4 mice because evidence suggests that the apoE4 isoform is associated with higher levels of ROS (Lauderback et al. 2002; Dafnis et al. 2010). Therefore, evidence supporting a critical and positive role of ROS in learning and memory is discussed below.

Critical role of ROS in learning and memory:

Previous studies have demonstrated that ROS, such as superoxide, are required for LTP and for activation of kinases such as ERK (Kanterewicz et al. 1998; Klann et al. 1998; Klann and Thiels 1999; Kishida et al. 2005b). For instance, removal of superoxide with the addition of superoxide scavengers into the

recording chamber of a hippocamapal slices blocks expression of LTP (Klann 1998). Consistent with this observation, mutant mice that overexpress cytoplasmic superoxide dismutase (SOD1) and extracellular superoxide dismutase (EC-SOD) have abnormal LTP (Gahtan et al. 1998; Thiels et al. 2000). Similarly, mice that lack proteins that make part of the NADPH-oxidase, a superoxide-generating complex, also show LTP impairments that are associated with deficits in hippocampal-dependent task (Kishida et al. 2006a). Memory deficits in humans have also been reported in people who have mutations in the NADPH-oxidase complex (Pao et al. 2004). Adding to the evidence that superoxide is critical for learning and memory, it was previously shown that that superoxide is necessary for NMDA-receptor activation of ERK (Kishida et al. 2006b). As noted above, ERK is an important kinase in LTP and learning and memory. Another target of ROS appears to be the ryanodine receptors, which are responsible for the release of calcium from intracellular stores (Huddleston et al. 2008). ROS-mediated activation of ryanodine receptors can facilitate transcription of genes important for synaptic plasticity (Hidalgo et al. 2007). Finally, the presence and functionality of NADPH-oxidase has been described in neurons (Tejada-Simon et al. 2005). The NADPH-oxidase complex is made up of several membrane bound and cytosolic subunits (Park et al. 1992; Sumimoto et al. 2005). The membrane bound proteins are gp91^{phox} and p22^{phox}; and the cytosolic proteins are p40 ^{phox}, p47 ^{phox,} p67 ^{phox} and a small GTPase protein, rac. Upon phosphorylation of p47 ^{phox,} the cytosolic proteins translocate to the

membrane bound proteins. This causes activation of gp91 ^{phox}, which is responsible for the transfer of electrons from NADPH to molecular oxygen, thereby producing superoxide. The finding that NADPH-oxidase is present and functional at neuronal synapses provided a great contribution to advancing the idea that superoxide plays an important role in learning and memory. This is because NADPH oxidase produces superoxide only upon stimulation (Lambeth 2004; Quinn and Gauss 2004), which is an important feature given that chronic elevations in ROS can lead to oxidative damage.

Dissertation goals, experimental design and rationale

Dissertation goals

The goals of this dissertation were to determine whether apoE isoform influences the effects of irradiation on the cognitive function of female mice and to determine whether changes in ROS following irradiation can explain apoE isoform-dependent effects of irradiation on cognitive function. In order to determine whether the effects of irradiation are specific to radiation source, two forms of irradiation, ^{137}Cs and ^{56}Fe, were assessed. Finally, because the literature suggests that the effects of irradiation might be specific to certain types of learning and memory, performance on various hippocampal and non-hippocampal tasks were assessed.

Chapter 1 addressed whether the effects of ^{137}Cs irradiation on the cognitive function of apoE2 and apoE4 female mice are apoE isoform-dependent and

whether ROS is a potential mechanism that can explain the apoE isoform-dependent effects of irradiation. Chapter 2 describes a follow up study to determine whether the effects of ^{56}Fe irradiation on cognitive function and ROS levels of apoE4 mice are similar to those following ^{137}Cs irradiation.

Experimental design and rationale

Subjects

Human apoE2 and apoE4 mice were chosen because these two genotypes differ the most in terms of ROS modulation. Additionally, our lab previously showed that these mice exhibit spatial memory retention with the water maze paradigm that was used in this dissertation (Siegel et al. 2010a). Female mice were chosen because, in both humans and rodents, females are more susceptible to the effects of irradiation. The estrous cycle of the mice was not monitored. This was decided mainly because of concern that stress related to daily vaginal swabs could interfere with performance on behavioral tests. Moreover, there are no consistent effects of the estrous cycle on hippocampus-dependent learning and memory (Berry et al. 1997; Stackman et al. 1997; Healy et al. 1999). Therefore, it was anticipated that the more profound effects of irradiation would override potential influences of the estrous cycle.

Irradiation

Because both gamma and HZE cranial irradiation are associated with cognitive impairments, one of the goals of this dissertation was to determine whether apoE isoform-dependent effects of irradiation on cognitive function are specific to the type of irradiation. Unfortunately, at the time of the BNL visit, we did not have enough apoE2 mice to address a potential apoE-genotype effect of ^{56}Fe irradiation. However, we did have enough apoE4 mice to determine whether in regular diet fed mice, the effects of ^{56}Fe irradiation are similar to those of ^{137}Cs irradiation.

Head only irradiation was chosen since the main interest of this dissertation was to model the effects of irradiation on the cognitive function of patients who receive whole brain irradiation. However, radiation-induced cognitive deficits are also observed following total body irradiation (TBI) (Peper et al. 2000; Shukitt-Hale et al. 2000). The effects of TBI also include reductions in bone marrow cells, immune system compromises, and damage to vital organs such as the lungs and kidneys (Ina and Sakai 2005; Bogdandi et al. 2010; Down and Yanch 2010; Esteban et al. 2010). Additionally, the doses used for TBI are much lower than what can be used for cranial irradiation. For instance, TBI doses greater than 10 Gy (gamma) can be lethal if not given in multiple fractions (Tsao et al. 2006; Wambi et al. 2008; Brown et al. 2010; Esteban et al. 2010).

For ^{137}Cs cranial irradiation, the dose (10Gy) was based on three reasons previously described (Lee et al. 2010): 1) it is the lowest dose that shows a

biological effect; 2) it is below the threshold for vascular changes, demyelination or mature cell death; and 3) it is a clinically relevant dose in humans. To determine the optimal dose for observing effects of ^{56}Fe irradiation on cognitive function, a ^{56}Fe irradiation dose response study was conducted (Villasana et al. 2010). That study showed that 3 Gy but not 1 or 2 Gy impaired the performance of wild-type female mice on contextual fear conditioning. As previously mentioned, accurate dosimetry measurements for absorption of HZE irradiation by humans have not been possible. However, estimates of the total HZE exposure for a 2-year space mission have been calculated at 2 Gy (Curtis et al. 2000; Higuchi et al. 2002) and were used for basis of the ^{56}Fe irradiation dose curve in our previous study.

3-month period between irradiation and testing

The 3-month time point was chosen because it was originally thought that neurogenesis could explain potential apoE isoform-dependent effects of irradiation on cognitive function: it takes approximately 2 months for adult born cells to have similar synaptic properties as those of mature cells (Deng et al. 2010). Thus, the levels of hippocampal neurogenesis should be minimal 3 months following irradiation.

Animal models to assess effects of irradiation on the cognitive function of mice

There are several different behavioral assays that can be used to assess the cognitive function in mice. Furthermore, specialized paradigms can be used to assess hippocamapal-and non-hippocampal-dependent learning and memory. The behavioral models used in this dissertation to assess the effects of irradiation are described below. Noteworthy comparisons between the hippocampal-dependent tests are made at the end of this section. More descriptive protocol details of the behavioral tests are provided in the subsequent chapters.

Novel location recognition test

The novel location recognition test is a spatial learning and memory task that assesses the ability of animals to recognize a change in the spatial configuration of familiar objects in an environment. In rats, and it is sensitive to hippocampal damage (Save et al. 1992). In mice, this task is sensitive to hippocampal reductions in neurogenesis (Goodman et al. 2010). The test involves 5 10-minute trials, each separated by a 4-minute inter-trial interval (ITI). During the first 3 trials, mice are allowed to explore 3 objects that are each placed in a different corner of the chamber. Prior to the 4th trial, one of the objects is moved to a novel location. The percent time exploring the object in its new location versus its old location (in the 3rd trial), is used as the measure of novel location recognition. Another cognitive test that our lab uses in conjunction with the novel location recognition test is a novel object recognition test. In this test, the

location of objects is not changed. Instead, a novel object is presented and the ability of the mice to detect that novel object in a familiar environment is assessed. Rats with hippocampal lesions show anterograde novel object recognition (Gaskin et al. 2003). The novel object recognition paradigm we use is considered hippocampus-independent.

Water maze

The water maze is a spatial learning and memory task that assesses the ability of mice to acquire and retain information about the configuration of spatial cues outside of the water maze (Morris 1984; Brandeis et al. 1989; Gallagher and Nicolle 1993; Moser et al. 1998; Vorhees and Williams 2006). In other words, this test assesses the ability of mice to form a cognitive map of their environment (O'Keefe and Dostrovsky 1971). In our version of the water maze test, we first train mice to locate a cued platform in order for mice to learn the task rules. This part of the task involves forming an association between reaching the visible platform and removal from the pool. The mice are then trained to locate a hidden platform using spatial cues in the room. Once mice learn the location of the hidden platform, a probe trial is conducted to assess whether mice show preferential searching in the area of the pool where the platform was previously located. Preference for the learned location of the platform is measured by how far the mice swim from where the platform was located (cumulative distance to the target). Performance in the water maze task is very sensitive to hippocampal

damage (Morris 1981; Logue et al. 1997). However, damage to other brain regions such as the nucleus accumbens (Setlow and McGaugh 1998) and striatum (Whishaw et al. 1987) can also affect water maze performance.

Although our water maze paradigm is hippocampal-dependent in that it requires formation of a spatial map, because the probe trials are conducted one hour after training the mice, the memory for the spatial information may not necessarily reflect hippocampal consolidated memory, as this is a protein synthesis-dependent process that requires a longer period of time (Dudai 2002; Scharf et al. 2002; Pfeiffer and Huber 2006).

Contextual Fear Conditioning

Contextual fear conditioning is an emotional learning and memory task that assesses the ability of mice to make an association between an aversive stimuli (unconditioned stimulus, US) and a specific environment (conditioned stimulus, CS) for review see Sander et al. (2003). When mice are reintroduced to the environment in which they received the US, they display a freezing-like behavior, which is used as an index of memory for the CS-US association. Contextual fear conditioning is sensitive to hippocampal and amygdala damage (Kim and Fanselow 1992; Phillips and LeDoux 1992; Young et al. 1994; Maren et al. 1997). In contrast, cued fear conditioning, a test that is often used in conjunction with contextual fear conditioning, is amygdala but not hippocampal-dependent (Phillips and LeDoux 1992; Helmstetter and Bellgowan 1994). Contextual fear

conditioning involves binding of polymodal stimuli (i.e. various contextual elements) with the US. In contrast, in cued fear conditioning, a unimodal stimulus (often a tone) is paired with the US. These differences are thought to explain why cued fear conditioning is amygdala but not hippocampal-dependent (Phillips and LeDoux, 1992). Hence, the combination of the two tests can help determine whether changes in contextual fear conditioning are due to the hippocampus only or whether changes are potentially influenced by both the hippocampus and amygdala.

As noted at the beginning of this section, there are noteworthy differences between the described hippocampal-dependent tasks. For example, in the water maze test, performance is based on the ability to form a spatial map. Yet, it is not clear that acquisition of a spatial configuration is required for contextual fear conditioning as non-hippocampal systems might compensate by using more simple cues (Wiltgen et al. 2006), but see McHugh and Tonegawa (2007). Moreover, although the amygdala modulates spatial memory retention in the water maze, evidence suggests that it is not critical for the formation or retention of spatial memory (McDonald and White 1993; Packard et al. 1994; Brambilla et al. 1997; Packard and Teather 1998), but is for contextual fear conditioning (Phillips and LeDoux 1992; Helmstetter and Bellgowan 1994). Additionally, and as mentioned above, there is evidence to suggest that contextual fear conditioning occurs in the absence of the hippocampus (Wiltgen et al. 2006). Thus, although the hippocampus is the primary region for processing this type of

learning, other brain regions might compensate in its absence. Similar findings have not been reported for the water maze test.

There is also an inherent difference between the water maze and novel location tasks. In the water maze task, a natural aversion to cold water drives the mice to perform the task. However, in the novel location recognition task, the natural tendency to explore novelty is used as the motivational drive to perform the task. Thus, the degree of motivation to perform the task might be different between water maze and novel location recognition. This difference could influence how well mice perform the tasks.

Because the type of memory and brain regions involved in the aforementioned hippocampal-dependent tasks might be differentially susceptible to irradiation, all three of the described tasks were included in my dissertation in order to increase the ability to detect effects of irradiation on learning and memory.

Behaviors potentially contributing to performance in hippocampus-dependent cognitive tests

There are several factors that can influence the performance of mice on cognitive tests such as anxiety, attention, motivation to perform the task, exploratory behavior, and sensorimotor function. These behaviors, with the exception of attention, were assessed to determine their potential influence on performance during the cognitive tests. The tests used to assess these behaviors are briefly described below.

Open field

The open field test is widely used to assess anxiety-like and exploratory behaviors (Crawley 1985; Bolivar et al. 2000; Belzung and Griebel 2001; Choleris et al. 2001). Mice are placed in a brightly-lit open chamber and their activity is measured throughout the duration of the test. Initially, mice tend to spend their time close to the walls of the chamber, although mice will eventually explore their surroundings, including the center of the chamber. Anxiolytic drugs influence the time spent in the center (Prut and Belzung 2003). Thus, the percent time spent in the center of the chamber is a sensitive index for assessing anxiety. Distance moved provides a measure of exploratory behavior and can be used as a general measure of locomotor activity.

Light Dark

In the light-dark test, mice have a choice between exploring a brightly lit open area of the chamber or a dark and enclosed area. The light-dark test is also sensitive to anxiolytic drugs (Crawley 1985; Hascoet et al. 2001; Bourin and Hascoet 2003). Mice typically prefer the dark compartment but they will eventually explore the light compartment. The time spent in the light compartment is used as an index for assessing anxiety and distance moved is used as a measure of exploratory and locomotor behavior.

Elevated zero maze

The elevated zero maze is also used to assess anxiety-like behaviors (Shepherd et al. 1994; Tarantino et al. 2000; Cook et al. 2001). Mice are placed on a circular platform, which has two enclosed walls that are equally separated by two open areas. Initially, mice typically stay within the enclosed walls but eventually explore the open areas of the maze. The time spent in the open areas of the maze is used as an index of anxiety. Distance moved is used as a measure of exploratory and locomotor behavior.

Elevated plus maze

The elevated plus maze is also used to assess anxiety-like behaviors (Pellow and File 1986; Hogg 1996; Crabbe et al. 1999; Menard and Treit 1999; Carobrez and Bertoglio 2005). Similar to the elevated zero maze, the elevated plus maze has two open and two enclosed compartments. However, because it has a plus shape, mice are forced to turn around before returning to the enclosed portions of the maze. Another difference between the zero and elevated plus maze is that the elevated plus maze has a center region that is neither a completely enclosed or an open region.

Assessing the role of ROS on the effects of irradiation on cognitive function:

Alpha lipoic acid

Restoration of the balance between ROS and antioxidants may prevent oxidative damage. Therefore, supplementation of antioxidants might antagonize the effects of irradiation on cognitive function. The antioxidant, alpha-lipoic acid (ALA) is naturally present in cells. It is also found in a variety of foods and in FDA approved over the counter supplements (Biewenga et al. 1997). ALA has diverse pharmacological and antioxidant properties. In addition to its direct ability to scavenge ROS, ALA can also chelate redox active transitional metals, thereby inhibiting production of other ROS such as hydrogen peroxide and the hydroxyl radical (Moini et al. 2002). ALA also appears to restore age-related reductions in the antioxidant glutathione (Suh et al. 2004) and participates in the recycling of vitamin C and vitamin E. In addition to its antioxidant properties, ALA in its reduced form, dihydrolipoic acid (DHLA), increases acetylcholine production by increasing the activity of choline acetyltransferase (Haugaard and Levin 2000). Finally, in insulin-resistant neurons, ALA appears to increase glucose uptake (Bitar et al. 2004). The protective properties of ALA have been shown in the context of a variety of challenges including irradiation (Limoli et al. 2007; Manda et al. 2007b; Manda et al. 2008). Manda et al. (2008) showed that an intraperitoneal injection of ALA (200mg/kg) administered to mice prior to whole body ^{56}Fe irradiation (1.5 Gy) prevents radiation-induced deficits in water maze reference memory and attenuates measures of oxidative stress in the cerebellum. However, in the aforementioned study, the protective effects of ALA were only assessed in irradiated male mice. Because female mice are more

sensitive to the effects of irradiation on cognitive function, it is important to determine whether ALA can prevent radiation-induced deficits in the population that is at a higher risk. Considering that ROS are also required for learning and memory, the potential outcome of ALA treatment is not clear. Therefore, the effects of ALA on the cognitive function of sham-irradiated mice are also of interest. ALA was used in this project to begin addressing the role of ROS in radiation-induced cognitive impairments in apoE female mice. It was administered through diet (food) 2 weeks prior to irradiation using a concentration (0.165%) that was previously shown to delay age-related cognitive decline in mice (Sharman and Bondy 2001). This concentration also attenuated hippocampal-dependent deficits in TG2576 mice, an animal model of AD (Quinn et al. 2007). ROS levels were compared between non-ALA and ALA-supplemented sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice as described below.

Evaluating radiation-induced changes in ROS:

Dihydroethidium is a fluorescent dye that is oxidized by superoxide into the stable byproducts dihydroxyethidium (DHE) and ethidium. These two products have distinct emission peaks corresponding to the production of superoxide and hydrogen peroxide, respectively (Robinson et al. 2006). The ability to produce stable and measurable byproducts makes DHE an attractive probe for measuring ROS (Benov et al. 1998; Quick and Dugan 2001; Peshavariya et al. 2007). This

feature is critical because ROS are highly reactive and quickly form other products. For instance, superoxide dismutase leads to the production of hydrogen peroxide when it reacts with superoxide and catalase produces oxygen and water when it reacts with hydrogen peroxide (Chelikani et al. 2004).

DHE has been used in cell cultures (Benov et al. 1998). Its oxidation has also been assessed in the brains of mice that were IP injected with DHE before they were sacrificed (Hu et al. 2006). To the best of my knowledge, this is the first study to use DHE in live hippocampal slices. The major advantage of incubating slices with DHE is that pharmacological experiments on hippocampal slices can be conducted to determine whether there is a change in the ability to generate ROS, making this a functional assay. In these studies, phrobol-myristate acetate (PMA), a phorbol ester that stimulates generation of ROS (Jofre-Monseny et al. 2007) was used to determine whether irradiation changed the ability of the hippocampus to generate ROS upon a stimulus. The experimental design involved comparison of baseline levels of ROS generation to PMA-induction of ROS in ex vivo hippocampal slices of sham-irradiated and irradiated apoE2 and apoE4 mice.

A potential concern of using DHE is that 2 non-overlapping emission filters are required to distinguish dihydroxyethidium (for superoxide) from ethidium (for hydrogen peroxide). A second potential concern of using DHE is that its oxidation in hippocampal slices can only be stopped by freezing of the slices (Invitrogen Technical support, personal communication). As such, the incubation

time between samples needs to be carefully monitored and recorded to ensure appropriate comparisons of levels of DHE oxidation.

Summary and Hypotheses:

While the majority of the literature suggests that irradiation has negative effects on cognitive function, impairments are rarely consistent across different types of hippocampal-dependent tasks (Raber et al. 2004; Saxe et al. 2006; Acevedo et al. 2008a; Wojtowicz et al. 2008; Hernandez-Rabaza et al. 2009; Villasana et al. 2011). Furthermore, a few studies have even reported enhancements in spatial, contextual and hippocampal-dependent working memory following cranial irradiation (Arnold and Blair 1956; Saxe et al. 2007; Raber et al. 2011). Taken together, these studies demonstrate that the effects of irradiation on learning and memory are not completely predictable and might depend on the type of memory assessed.

In humans, not all individuals exposed to radiation develop cognitive impairments (Roman and Sperduto 1995). Individual differences such as apoE genotype might influence the effects of irradiation on cognitive function. As previously discussed, the apoE isoforms differentially modulate ROS, which might be related to differences in their antioxidant capacity (Ihara et al. 2000; Lauderback et al. 2002; Dafnis et al. 2010) and differences in their ability to protect against oxidative damage (Pedersen et al. 2000; Butterfield et al. 2002; Jofre-Monseny et al. 2008). Conventionally, ROS are thought to have only

negative effects on cognitive function; however, there is a plethora of evidence demonstrating that ROS are also critically involved in learning and memory (Knapp and Klann 2002). Background levels of ROS might determine the effects of irradiation on cognitive function. We previously showed that irradiated EC-SOD $^{-/-}$ mice, performed better on hippocampal-dependent tasks compared to sham-irradiated genotype matched mice (Raber et al. 2011). Similar radiation-induced enhancements were not observed in wild-type mice, suggesting that higher background levels of ROS in EC-SOD $^{-/-}$ mice may have allowed them to benefit from irradiation. Because apoE4 isoform is associated with higher levels of ROS and oxidative damage one might predict that similar to EC-SOD$^{-/-}$ mice, apoE4 mice might also benefit from irradiation. However, such a prediction is complicated by the fact that apoE4 is a risk factor for AD (Farrer et al. 1997) and more importantly is associated with poor recovery following neurotrauma (Friedman et al. 1999; Kutner et al. 2000). Therefore, understanding the influence of apoE genotype on the effects of irradiation on cognitive function is a major interest of this dissertation.

The overarching goals of this dissertation are to determine whether apoE modulates the effects of irradiation in an isoform-dependent manner and, if so, to better understand what neurobiological mechanisms might contribute to these effects. The goals of dissertation were addressed by asking the following questions:

1) Is there an apoE isoform-dependent effect of ^{137}Cs irradiation on the cognitive function of female mice? *Compared to sham-irradiated genotype-matched mice, I hypothesized that the cognitive function of apoE4 mice would be more affected than that of apoE2 mice.* This prediction was based on the association of apoE4 isoform with AD, poor recovery following neuronal injury, and its inferior antioxidant capacity compared to apoE2 isoform.

2) If the effects of irradiation on cognitive function are apoE isoform-dependent, do radiation-induced changes in ROS occur in an apoE isoform-dependent manner? Because chronic elevations in ROS can lead to oxidative damage and because apoE4 isoform is associated with greater oxidative damage compared to apoE2 isoform, *I hypothesized that compared to sham-irradiated genotype-matched mice, irradiated apoE4 mice would show greater radiation-induced increases in ROS generation than irradiated apoE2 mice.* Furthermore, to determine whether irradiation-induced changes in ROS are responsible for the effects of irradiation on cognitive function, half of the mice were placed on an ALA-supplemented diet. *I hypothesized that compared to non-ALA supplemented mice ALA-supplementation would prevent or attenuate irradiation-induced increases in ROS and subsequent impairments in cognitive function.*

3) Are the potential effects of ^{137}Cs irradiation on cognitive function and ROS generation specific to ^{137}Cs irradiation? *I hypothesized that the effects of ^{56}Fe irradiation on the cognitive function and ROS generation of apoE4 mice would be similar to that of ^{137}Cs. That is, compared to sham-irradiated apoE4 mice, I*

hypothesized that ^{56}Fe-irradiated apoE4 would show cognitive impairments and radiation-induced increases in ROS generation.

A diagram of a proposed mechanism to explain the apoE isoform-dependent effects of irradiation on cognitive function is presented in Figure 1.

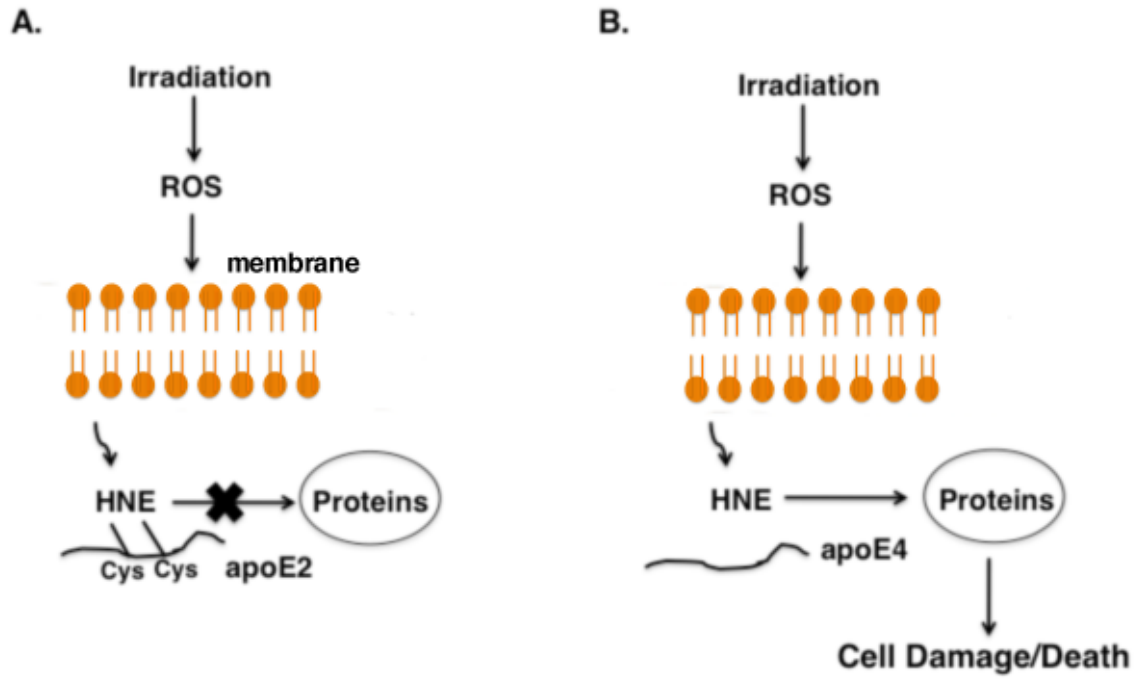

Figure 1. Proposed mechanism for apoE isoform-dependent effects of irradiation on cognition. Because apoE2 contains two cysteines that can bind to HNE, proteins are spared from HNE-induced oxidation. ApoE4, on the other hand, contains no cysteine residues that can bind to HNE and prevent protein oxidation resulting in cell damage or death.

2

Effects of ^{137}Cs irradiation on the cognitive function of apoE2 and apoE4 female mice: the role of ROS

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Abstract

Radiation exposure, such as in the clinical setting, can lead to long-term changes in cognitive function, including impairments in learning and memory. Individual risk factors such as sex and genetic background might determine how irradiation affects cognitive function. For instance, in both humans and rodents, females are more susceptible to developing cognitive impairments following irradiation. Apolipoprotein E (apoE) genotype might also influence the effects of irradiation. ApoE is a protein important for neuronal repair and is expressed in three different isoforms in humans, apoE2, apoE3 and apoE4. Compared to apoE3, apoE4 increases the risk to develop Alzheimer's disease (AD), while apoE2 reduces this risk. ApoE4 is also associated with poor recovery following neurotrauma. The apoE isoforms also have different degrees of antioxidant capacity with apoE2 being the most effective followed by apoE3 and apoE4, respectively. Expression of apoE4 might increase the risk to develop cognitive impairments following irradiation. Although the exact mechanisms underlying the effects of irradiation on cognitive function are not clear, they might involve increases in reactive oxygen species (ROS).

The purpose of the experiments described in this chapter were to determine whether the effects of ^{137}Cs irradiation on the cognitive function of female mice are apoE isoform-dependent and whether changes in generation of ROS are associated with radiation-induced changes in cognitive function. To this end,

female apoE2 and apoe4 mice were sham-irradiated or ^{137}Cs-irradiated at 2-months of age. Prior to irradiation, half of the mice from each genotype were placed on a diet containing the antioxidant, alpha lipoic acid (ALA). Three months after irradiation, mice were behaviorally tested on a battery of tests to assess potential effects of irradiation on cognitive function. One week after the last behavior tests, baseline levels and PMA-induced generation of ROS were assessed in hippocampal slices from the different groups of mice via oxidation of dihydroethidium (DHE), a ROS sensitive dye.

ApoE isoform-dependent effects of ^{137}Cs irradiation were found; however, not in the direction hypothesized. Compared to sham-irradiated mice, ^{137}Cs-irradiated apoE4 mice showed enhancements in spatial memory in the water maze. An enhancement in the ability to respond to PMA-induction of ROS was also observed in hippocampal slices from irradiated apoE4 mice. Similar radiation-induced changes in spatial memory and PMA-induction of ROS were not observed in apoE2 mice. Analysis of DHE oxidation in slices from ALA-supplemented mice showed that ALA did not act as an antioxidant. Accordingly, ALA-supplementation did not attenuate the effects of irradiation on cognitive function.

These data show that the effects of ^{137}Cs irradiation are apoE isoform-dependent and that ROS are associated with these changes. Specifically, data from this study suggest that under specific conditions, namely apoE4 genotype,

radiation-induced changes in PMA-induction of ROS is associated with enhancements in spatial memory in the water maze.

Introduction

Whole brain irradiation (WBI) for radiotherapy is a standard and life-saving treatment for brain metastasis; unfortunately, it is not without serious and life-long side effects (Roman and Sperduto 1995; Abayomi 2002; Byrne 2005; Sarkissian 2005). WBI can lead to progressive and long-term deficits in cognitive function including deficits in learning and memory, attention, speed of information processing, and executive function (Twijnstra et al. 1987; Lee et al. 1989; Douw et al. 2009). In children, the effects of irradiation are also reflected in lower academic achievement and lower IQ scores (Lee et al. 1989). In the U.S., approximately 170,000 patients per year are treated for brain metastasis, and it is estimated that 50% of those patients who survive up to 6 months following WBI will develop life-long cognitive deficits (Ramanan et al. 2010).

The severity of cognitive impairments induced by WBI is associated with the dose delivered to the medial temporal lobes (Roman and Sperduto 1995), the site of the hippocampus, which is a critical structure for learning and memory (O'Keefe and Dostrovsky 1971; Morris 1981; Zola-Morgan et al. 1986; Squire and Zola-Morgan 1991; Nadel and Moscovitch 1997; Tulving and Markowitsch 1998; Schacter and Wagner 1999). There is ample evidence that irradiation changes hippocampal function in both humans (Roman and Sperduto 1995; Abayomi 2002; Butler and Mulhern 2005; Byrne 2005) and rodents (Rabin et al. 2002; Raber et al. 2004; Yazlovitskaya et al. 2006; Acevedo et al. 2008b). However,

not all individuals exposed to radiation develop cognitive impairments. For instance, the effects of irradiation on cognition are greater in girls (Butler and Mulhern 2005). Girls show greater cognitive impairments than boys when treated for acute lymphoblastic leukemia (ALL). Susceptibility of females to the effects of WBI on cognitive function is also observed in mice (Silasi et al. 2004; Yazlovitskaya et al. 2006; Acevedo et al. 2008b).

Individual risk factors for developing radiation-induced cognitive impairments might also be genetic (Correa et al. 2007). Apolipoprotein E (apoE) is a protein important for neuronal repair (Samatovicz 2000) and for the metabolism and distribution of lipoproteins and cholesterol (Mahley 1988). ApoE might modulate cognitive susceptibility to WBI. The absence of apoE expression in mice (*Apoe*$^{-/-}$) increases the risk of developing cognitive deficits following cranial ^{56}Fe irradiation (Higuchi et al. 2002). This susceptibility might be further influenced by apoE genotype. There are 3 major apoE isoforms in the human population, apoE2, apoE3, and apoE4, that are encoded by 3 distinct alleles (Mahley 1988). In humans, ε3 is most frequently expressed allele (79-80%) followed by ε4 (10 - 15%) and ε2 (5-10%) (Lahiri 2004). Compared to apoE3, apoE4 increases the risk of developing AD, particularly in women, whereas apoE2 reduces this risk (Farrer et al. 1997). ApoE4 is also associated with a risk of developing cognitive impairments following brain trauma (Friedman et al. 1999; Kutner et al. 2000) and cardiac bypass surgery (Tardiff et al. 1997). An important distinction between the apoE-isoforms is their antioxidant capacity. ApoE2 is the most effective, followed

by apoE3 and apoE4 respectively (Lauderback et al. 2002). These differences are thought to contribute to AD risk, pathological states of disease and brain injury (Butterfield et al. 2002; Jofre-Monseny et al. 2008). It is possible that the antioxidant differences between the apoE isoforms might also contribute to a higher risk of developing cognitive impairments following irradiation.

The mechanisms responsible for the effects of irradiation on cognition are unclear but may involve long-term increases in inflammatory responses (Monje et al. 2003; Limoli et al. 2007; Manda et al. 2007b; Dayal et al. 2008) and reactive oxygen species (ROS) (Jou et al. 2002; Limoli et al. 2007; Collins-Underwood et al. 2008; Brown et al. 2010). ROS are a major component of oxidative stress. If sustained at high levels, ROS can lead to cell damage and death through oxidation of cellular components (Harman 1981). Because of its high oxygen consumption and relatively low levels of antioxidants, the brain is particularly vulnerable to ROS damage (Halliwell 1992). There is strong evidence implicating ROS in learning and memory deficits, as well as age-related cognitive decline (Serrano and Klann 2004). Moreover, previous studies suggest that attenuation of radiation-induced increases in inflammatory responses such as activated microglia and ROS can prevent radiation-induced neuronal damage and cognitive impairments (Monje et al. 2003; Chan et al. 2004; Rabin et al. 2005c; Manda et al. 2007a; Shukitt-Hale et al. 2007). For instance, Manda and colleagues **(**2008**)** showed that alpha lipoic acid (ALA), a potent antioxidant, prevents radiation-induced cognitive deficits in male wild-type mice and that the preventative effect

of ALA is associated with reduced measures of oxidative stress. The findings from this study provide strong evidence for the role of oxidative damage in the development of cognitive deficits following irradiation. In addition, these findings propose a treatment to prevent radiation-induced long-term cognitive dysfunction.

Several important questions arise from these findings: (1) Can ALA also prevent cognitive deficits in females, the more susceptible sex to radiation-induced cognitive injury? (2) Can ALA prevent cognitive deficits in female mice exposed to ^{137}Cs irradiation, which is the form of irradiation used in the clinical setting and is more commonly encountered by humans? (3) Is the response to ALA apoE isoform-dependent? The latter question is raised because the apoE2 and apoE4 isoforms differentially modulate ROS, and therefore, they might also show a differential response to ALA. To our knowledge, an apoE isoform-dependent response to ALA under the context of irradiation has not been established. This is an important question to address as it can contribute to the success of future research using ALA as a therapeutic strategy for diseases and pathologies involving oxidative damage.

As earlier mentioned, ROS have an important role in learning and memory. Therefore, the effects of ALA on cognitive function are not easily predictable. For instance, it is possible that reductions in baseline levels of ROS with ALA might actually induce cognitive deficits.

To begin addressing the role of apoE isoform and ROS in cognitive function, the diets of apoE2 and apoE4 female mice were supplemented with ALA

(0.165%) 2 weeks prior to irradiation. Half of the other mice from each genotype remained on regular diet. Because these experiments involve a very large number of mice that must be bred at a specific time and shipped to and from Brookhaven National Laboratories in New York, the study was limited to apoE2 and apoE4 female mice. These genotypes were chosen as they differ the most in terms of ROS modulation (Ihara et al. 2000; Lauderback et al. 2002; Dafnis et al. 2010) and oxidative stress (Pedersen et al. 2000; Jofre-Monseny et al. 2008). Female mice were chosen because previous studies indicate that attenuation of ROS prevents radiation-induced impairments in male mice and rats (Rabin et al. 2005c; Manda et al. 2007a; Shukitt-Hale et al. 2007). Yet, similar studies have not been conducted on female mice. In total, there were 4 different treatment groups for each genotype: sham-irradiated regular diet, sham-irradiated ALA - supplemented diet, irradiated regular diet and irradiated ALA-supplemented diet. Following irradiation, hippocampal-dependent and independent functions were assessed by the novel location and novel object recognition tests, the water maze test, and contextual and cued conditioned fear tests. Because anxiety and exploratory behaviors can influence performance on these behavioral tasks, potential group differences in anxiety-like and locomotor behaviors were assessed in the open field, light-dark, elevated zero maze and elevated plus maze tests.

Group differences in ROS were assessed with dihydroethidium (DHE), a fluorescent dye that is oxidized by superoxide into the stable byproducts

dihydroxyethidium and ethidium (Robinson et al. 2006). These byproducts correspond to superoxide and hydrogen peroxide respectively. Baseline levels of ROS generations were compared to stimulus response generation of ROS using the agonist phorbol-myristate acetate (PMA)(Jofre-Monseny et al. 2007). Because ROS are required for learning and memory, we were interested in determining whether irradiation changed not only baseline levels of ROS but also the ability of the hippocampus to generate ROS upon stimulation. This distinction was of interest because in a previous study we found that ^{137}Cs irradiation enhanced the spatial and contextual memory of male mice deficient in extracellular superoxide dismutase (EC-SOD), as assessed by the novel location, water maze, and contextual fear tests. Although EC-SOD $^{-/-}$ mice showed higher levels of oxidative stress compared to wild-type mice, they did not show an increase hippocampal oxidative stress following irradiation whereas wild-type mice did. Because EC-SOD $^{-/-}$ mice lack EC-SOD, the possibility that increases in superoxide following irradiation could be responsible for the enhancements in hippocampal function came to light. The apoE4 isoform is also associated with higher levels of ROS (Ihara et al. 2000; Lauderback et al. 2002; Dafnis et al. 2010) and oxidative stress (Pedersen et al. 2000; Jofre-Monseny et al. 2008). Therefore, there was a recognized possibility that apoE4 mice could also show enhancements in hippocampal function following irradiation. In preparation for such a possibility, a functional assay for superoxide generation was of interest in

order to help determine whether potential enhancements in learning and memory are associated with changes in ROS generation.

Nevertheless, due to the association of apoE4 allele with AD, poor cognitive outcome following brain trauma and inferior antioxidant capacity (Friedman et al. 1999; Kutner et al. 2000; Pedersen et al. 2000; Lauderback et al. 2002), it was hypothesized that apoE4 would be more impaired by ^{137}Cs irradiation compared to apoE2 mice. Because increases in ROS were suspected to mediate changes in cognitive function following irradiation, it was hypothesized that ALA would prevent radiation-induced deficits on hippocampal-dependent tasks. Given the more effective antioxidant properties of apoE2, it was further hypothesized that hippocampal slices from apoE4 mice would show greater chronic ROS levels compared to slices from apoE2 mice following irradiation, and therefore, would be non-responsive to PMA stimulation.

Materials and Methods

Mice

Human apoE targeted replacement mice created on the C57Bl6/J background (Sullivan et al. 1997; Sullivan et al. 1998) were generously provided by Dr Patrick Sullivan. Although our colony is maintained by homozygous matings, every 2-3 years new breeders are used from Dr. Sullivan who maintains his colony by heterozygous and homozygous matings. However, to be clear, mice in our colony were not specifically backcrossed to C57Bl/6J mice to prevent a genetic drift.

The offspring received unique ear identifications at the time of weaning and were only housed with genotype-matched mice. The mice were kept on a 12:12 hour light-dark schedule (lights on at 6AM), with lab chow (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO), and water given *ad libitum*. All procedures were according to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the Oregon Health and Science University (OHSU). OHSU has an Association for the Assessment and Accreditation of Laboratory Animal Care approved animal facility. Figure 3 shows a timeline of experiments conducted on the mice.

ALA Diet

At 6-weeks of age, mice were placed either on an ALA diet (Rodent Pico Chow 20 + 0.165 % ALA, Animal Specialties Inc, Woodburn, OR) or maintained on a regular chow diet (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO). The concentration of ALA used in the current study was based on previous reports showing that this concentration attenuates age-related changes in nitric oxide synthase (NOS) levels in male wild-type mice when given at 3-months of age for a 6-month duration (Bondy 2002). A separate study also showed that this concentration of ALA attenuated memory deficits of TG2576 mice, an animal model of AD (Quinn et al. 2007). In the current study, mice remained on their selected diets throughout testing.

Irradiation

Following i.p. anesthesia (ketamine (Sigma, St. Louis, MO), 80 mg/kg and xylazine (Sigma), 20 mg/kg), mice were sham-irradiated or ^{137}Cs-irradiated (7-9 per genotype per treatment) at 10 Gy in a Mark 1 Cesium Irradiator (Shepherd and Associates, San Fernando, CA) at a dose 1.3Gy/minute. The cerebellum, eyes, and body were shielded with lead (Figure 2). Mice were housed singly starting 3 days prior to the first behavioral test.

Body weight assessment

Although the weight of all the mice were recorded during sham-irradiation or ^{137}Cs irradiation, only the mice that were used for DHE experiments (randomly

selected within each group) were weighed before they were sacrificed. Thus, weight changes were available for 3 sets of mice (one set was not weighed). Initially, we projected that all of the mice would be weighed as we expected to run the DHE oxidation analyses on all of the mice; however, we found that the DHE experiments were too involved to assess all of the mice.

Behavioral testing

The sequence of behavioral testing was such that the tests were administered in the order of increasing stress level. Mice were tested in the open field, light-dark test, elevated zero maze, and elevated plus maze on week 1; novel location and novel object recognition on week 2; water maze on week 3; and conditioned fear tests on week 4. The person testing the mice was blinded to the genotype and 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). Behavioral experiments were conducted 3-months following irradiation to allow for comparison with previous studies using that same time point (Villasana et al. 2010; Raber et al. 2011). The 3-month time point was originally chosen to assess the potential role of neurogenesis on the effects of irradiation on cognition. It takes approximately 2 months for adult born cells to have similar synaptic properties as those of mature cells (Deng et al. 2010). Figure 3 summarizes the sequence of the behavioral experiments.

A. **B.**

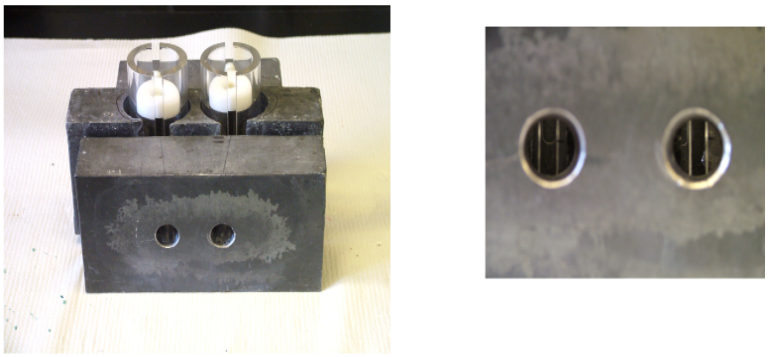

Figure 2. Lead shielding blocks used during ^{137}Cs irradiation. Following i.p. anesthesia, mice were positioned inside plexiglass tubes which contained several ventilation holes. The tubes were placed in a lead block (A). Only the back of the heads of mice was exposed through a small opening in the lead block (B) to prevent irradiation of non-targeted areas

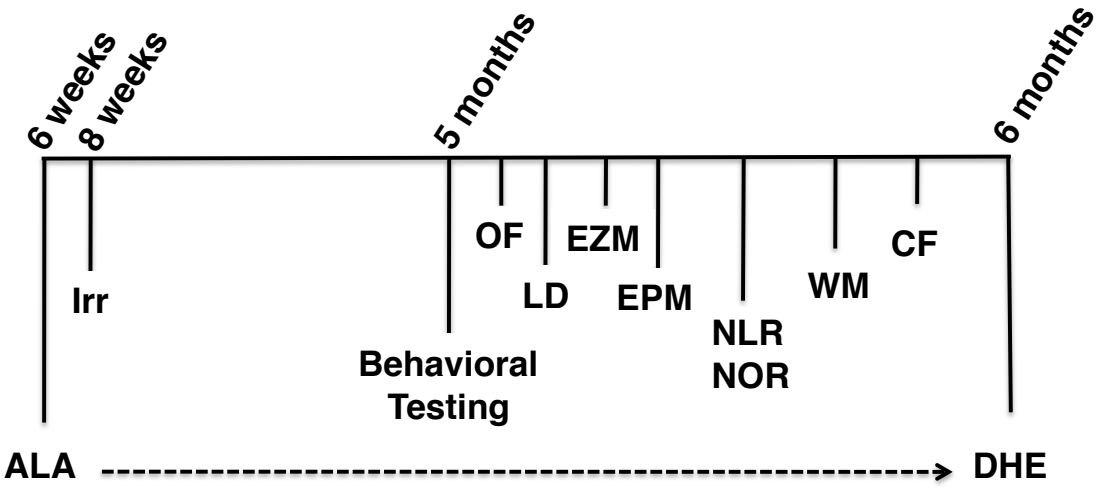

Figure 3. Timeline of experimental procedures conducted on apoE2 and apoE4 female mice. At 6-weeks of age, mice were placed on an alpha-lipoic acid diet (ALA) or were allowed to remain on the regular diet. Mice remained on their selected diets throughout the experiments. At 8-weeks of age, mice were either sham-irradiated or ^{137}Cs-irradiated. Three months later, mice were tested in the open field (OF), light dark test (LD), elevated zero maze (EZM), and elevated plus maze (EPM). The following week, mice were tested on novel location and novel object recognition tests (NLR and NOR). In the third week, mice were tested on the water maze (WM) task followed by conditioned fear tests (CF) in the last week of behavioral testing. Hippocampal levels of ROS in mice were determined by DHE oxidation approximately 1 week after the last behavioral test for each mouse.

Open field

The open field task was used to evaluate measures of anxiety and locomotor behavior (Crawley 1985; Bolivar et al. 2000; Belzung and Griebel 2001; Choleris et al. 2001). Mice were placed in brightly lit (luminescence: 200 lux) open arena (40.64cm X 40.64 cm) 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 x 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). Measures of interest included the percent time in the center of the open field, which was used as an index of anxiety. Total distance moved was used as an index of exploratory and locomotor behavior.

Light-dark

The light-dark test was the second test used to assess anxiety-like and locomotor behaviors (Crawley 1985; Hascoet et al. 2001; Bourin and Hascoet 2003). In the light-dark test, mice were placed in the open field enclosure (described above) containing black plastic inserts which 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). Measures of interest included the percent time in the lighted side of the enclosure and distance moved.

Elevated zero maze

The Elevated zero maze was the third test used to assess anxiety-like and locomotor behaviors (Shepherd et al. 1994; Tarantino et al. 2000; Cook et al. 2001). 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; Carobrez and Bertoglio 2005). Measures of interest included the percent time in the open areas and distance moved.

Elevated Plus-maze

The Elevated plus maze was the last test used to measure anxiety (Pellow and File 1986; Hogg 1996; Crabbe et al. 1999; Menard and Treit 1999; Carobrez and Bertoglio 2005). The elevated plus maze consisted of two open arms and two closed arms equipped with infrared photocells interfaced with a computer (Kinder Scientific Poway). Time in the open arms and distance moved were recorded for a single 10-minute session (luminescence: 200 lux). Recorded beam breaks were used to calculate path lengths and time spent within each arm of the maze. Mice with increased measures of anxiety in this maze spend less time in the open arms (Pellow et al. 1985). Measures of interest included the percent time spent in the open arms and distance moved.

Novel location and novel object recognition tests

The novel location recognition task assesses the ability of mice to recognize a change in the spatial configuration of an environment. In rats, this test is sensitive to hippocampal damage and is therefore considered a hippocampus-dependent task (Poucet 1989; Save et al. 1992; Malleret et al. 2001). The novel object recognition task assesses the ability of mice to recognize a novel object and is a hippocampal-independent task depending on the delay interval used (Save et al. 1992; Ennaceur and Aggleton 1997; Warburton and Aggleton 1999; Hammond et al. 2004).

Figure 28A (Appendix) illustrates the protocol used to assess novel location and novel object recognition. Each mouse was first habituated to an open chamber similar to that used in the open field for 3 consecutive days (10 minutes per day; 200 lux). On the fourth day, mice were tested on the novel location/novel object recognition tasks, which consisted of 5 10-minute trials with 4-minute inter-trial intervals (ITI). Each mouse was placed in the habituated chamber containing 3 different objects (plastic horse, camel and lion, Playmobile, Zirndorf, Germany) which were individually glued to a plexiglass platform in order to help prevent the objects from tipping over if mice climbed on them; however, the platform was not fastened to the arena floor because the objects needed to be replaced and cleaned between trials. All of the objects faced the center of the arena and were located 3 cm away from the wall of the arenas. After 3 familiarization trials, the location of one of the objects was moved to a different corner. Before the 5^{th} trial, one of the familiar objects was replaced with a novel object (cow). A change in the environment, either a spatial reconfiguration or a novel object will typically induce re-exploration of a displaced object and increase exploration of a novel object (Poucet 1989; Benice and Raber 2008). Hence, mice that recognize a change in the environment typically explore a displaced object more during trial 4 compared to trial 3 (before that object was moved). Similarly, mice that recognize a novel object typically show exploratory preference for the novel object compared to familiar objects. Because performance on this task depends on the motivation of mice to explore the objects, total exploration of the objects

was compared between the groups of mice. Additionally, the time spent exploring each object was assessed to determine whether there was an object bias. Exploration of the objects was defined by the approach of the mice to the objects with their noses 2 cm from the object, and scored using a video tracking system as previously described (Benice and Raber 2008) (Ethovision XT, Noldus Information Technology, Wageningen, Netherlands).

Water maze

The water maze test was used to assess spatial learning and memory (Morris 1984; Brandeis et al. 1989; Gallagher and Nicolle 1993; Moser et al. 1998; Vorhees and Williams 2006). A circular pool (140 cm diameter) was filled with water (22°C $\pm$ 2°C). The water was made opaque with white chalk in order to hide the platform. The circular platform was (20 cm wide) and sat approximately 1 cm below water level. On the first 2 days of water maze testing, the mice were trained to locate a visible platform (flagged with a visible beacon). There were 3 trials per session (5-minute ITI) and two sessions (two hours apart) per day. The platform was moved to a new quadrant for each of the 4 visible platform sessions. Trials ended when the mice reached the platform and remained on it for 3 seconds or when 60 seconds elapsed, at which point the mice 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 using 3 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 location training included swim speeds and cumulative distance to the platform. The latter measures how far the mice swim from the platform over the duration of the trial. The lower the cumulative distances, the better the performance. Thigmotaxis behavior, defined by the percent time spent in the outer zone of the pool, which was 20 cm from the wall, was assessed as an anxiety-like measure in the water maze (Saucier et al. 1996; Cain 1998; Barbier and Wang 2009).

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. Cumulative distance to the target (learned location of the platform) was used as the measure of spatial memory retention. The swimming patterns of the mice were analyzed using the Ethovision video tracking system set at 6 samples/sec.

Conditioned Fear

Conditioned fear was used to assess hippocampal-and amygdala-dependent associative memory (Kim et al. 1993; Anagnostaras et al. 2001; Maren 2001; Sanders et al. 2003). In this task, mice learn to associate the environmental context (fear conditioning chamber) and 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 (Kim and Fanselow 1992; Phillips and LeDoux 1992; Young et al. 1994; Maren et al. 1997), whereas cued fear conditioning is amygdala, but not hippocampal-dependent (Phillips and LeDoux 1992; Helmstetter and Bellgowan 1994).

Conditioned fear was conducted 2 days after the last day of the water maze test and was the last behavioral test. 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 for 2 minutes before 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. The pre-tone time, which was the first 2 minutes of the trial was used as the baseline measure for freezing behavior. 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 scored for 3 minutes. The context of the chambers were then changed by adding a smooth floor texture over the grid floor; changing the shape of the chamber to a triangle; adding a new scent (hidden vanilla soaked nestlets); and by cleaning the chamber with 70% ethanol versus 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 was measured by a motion index, which was calculated by a 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 (Anagnostaras et al. 2010). The motion index is based on the sum of the pixel changes in the current frame 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.

Group differences in freezing before delivery of the first tone during fear conditioning on day 1 were analyzed and is referred to baseline freezing. This analysis 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 potential group differences in sensory response to the shocks. There are 2 different measures of foot shock responses that are provided by the software, the average motion index and the maximum motion index. Both measures for each shock were analyzed because they provide slightly different and complementary measures.

Assessment of ROS in *ex vivo* hippocampal slices

The DHE oxidation imaging protocol was designed prior these studies in order to measure ROS generation in response to pharmacological stimulation in live hippocampal slices. DHE oxidation was used in this experiment to compare the generation of superoxide in *ex vivo* hippocampal slices of the behaviorally tested sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice that were on a regular or ALA-supplemented diet.

Superoxide and hydrogen peroxide levels, as assessed by oxidation of DHE, were compared between the groups of mice approximately one week after the last behavioral test. The brains of mice were removed following cervical dislocation and placed in 4°C oxygenated (95% oxygen, 5% carbon dioxide) brain slicing (cutting) solution (in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 5 glucose). Brain coronal sections (150 µm) were made with a Vibratome (Leica Microsystems, St. Louis MO)

equipped with 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 and dissected for isolation of the hippocampus, the bath solution was replaced with a 1:1 cutting solution and ACSF solution (in mM: 125 NaCl, 2.4 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ 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 1 hour in a custom made multi-bath chamber. The multi-bath chamber sat over 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 chamber in the bath contained representative sections from each group of mice. This allowed all of the sections from the different treatment groups, including the 2 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 collected for background reference (Excitation λ 488 nm; Emission $\lambda > 590$ nm) before the addition of DHE (10 μM, Molecular Probes, Eugene, OR). PMA (1μM, Sigma, St. Louis, MO) or DMSO (4μl in 25 ml) were added to separate ACSF solution reservoirs approximately 5 minutes following the addition of DHE.

Images were acquired every 2 minutes for up to 20 minutes at a 4X magnification. The optimal x, y, and z coordinate for each slice and each region was determined 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. 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 4.2 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 completely blind to the different treatments of the slices. There were a total of 4 experiments. Each experiment consisted of 2-3 replicate slices for each mouse from each treatment group and for each drug treatment. Replicates were averaged. N = number of mice. 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 -2.0mm with a range from -1.58mm to -2.4mm. This anatomical range was chosen because previous data suggests that the dorsal hippocampus is more involved in hippocampal-dependent spatial memory compared to the ventral hippocampus (Moser et al. 1993).

Statistical Analyses

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). When appropriate and as indicated, data were normalized by removal of outliers identified as significantly different from the group mean by SPSS software (SPSS Inc, Chicago, IL, USA) or by transformation of the data to satisfy normality.

For the open field, light-dark, elevated zero maze and elevated plus maze tasks, genotype, irradiation treatment, and diet were used as between-subject factors to assess potential group differences using a 3-way ANOVA. Dependent variables included the percent time spent in the center (open field); light side of the enclosure (light-dark test) and open areas of the zero and elevated plus mazes. The distance moved throughout the duration of the trials was also used as a dependent variable for each task.

For the novel location test, a repeated-measures ANOVA with the percent exploration of the displaced object in trials 3 and 4 was used as the within-subject factor. Genotype, irradiation treatment and diet were used as between-subject factors. The outcome of Mauchly's Test of Sphericity was assessed for all analyses involving a repeated-measures ANOVA. Novel object recognition was assessed within each group using the percent exploration time for each object within trial 5 as the dependent variable. The total exploration time of the objects during the familiarization trials was analyzed to determine if there were potential

group differences in motivation to explore the objects (3-way ANOVA). Potential group differences in the percent time spent exploring each object during the familiarization trials were also assessed to determine whether the different groups showed a potential object bias. In order to do so, a repeated measures ANOVA was used with object as the within-subject factor and genotype, irradiation treatment and diet as the between-subject factors.

For the water maze test, potential group differences in swim velocity and thigmotaxis were assessed using the average of these measures across the four visible platform training sessions with genotype, irradiation treatment and diet as the between-subject factors in a 3-way ANOVA. For the platform training sessions, learning curves were analyzed with genotype, irradiation treatment and diet as between-subject factors and sessions as within-subject factors using repeated measures ANOVAs. The outcome of Mauchly's Test of Sphericity was assessed for analyses involving repeated-measures ANOVA. Cumulative distance to the platform was used as the measure of performance. Visible and hidden platform training sessions were analyzed separately. The probe trials were analyzed using repeated-measures ANOVA with probe trial as the within-subject factor and genotype, treatment and diet as between-subject factors. Potential group differences were also assessed using the average cumulative distance to the target of the 3 probe trials.

For conditioned fear analyses, potential group differences in response to the 2 foot shocks during fear conditioning on day 1 were analyzed using a multivariate

analyses. The dependent measures included the average and maximum motion index for both shocks. Baseline freezing (day 1) and freezing during the contextual test (day 2), were analyzed using a 3-way ANOVA with genotype, irradiation treatment and diet as between-subject factors. Potential group differences in cued fear conditioning were analyzed using repeated-measures ANOVA with time as the within-subject factor, which included the first 3 minutes before the tone and the last 3 minutes after the tone.

For the DHE oxidation analyses, group differences in the rate of DHE oxidation (mean pixel intensity) were assessed using a repeated-measures ANOVA for time points 4-20 minutes in increments of 4 minutes (a total of 5 time points). Genotype, irradiation treatment, diet, hippocampal region and drug (+/- PMA) were the between-subject factors. Temperature and bregma location of each slice were used as covariates in the analyses. The individual background (autofluorescence) for each slice was subtracted from the mean pixel intensity for each corresponding slice after DHE-incubation.

When appropriate, main effects were assessed using Fisher's PLSD test as indicated**.** Data are expressed as MEANS $\pm$ SEM or expressed as the estimated marginal means $\pm$ SEM as indicated. The statistical analyses were conducted using SPSS software and were considered significant at $P < 0.05$. All figures were generated using GraphPad Prism Software (GraphPad Software, La Jolla, CA).

Results

Condition of the mice

The mice appeared to tolerate irradiation well, as there were no signs of illness or significant differences in weight changes before and after irradiation (Figure 4). The weights of the mice were recorded just prior to irradiation, which was 2 weeks after ALA supplementation. Therefore, diet was not used in the weight analysis for the repeated measures ANOVA. The 5-month time point was taken immediately before the DHE experiments were conducted.

Open Field

There was an overall genotype effect on the percent time spent in the center of the open field ($F_{1, 55} = 28.82$; $P < 0.001$), apoE4 mice spent less time in the center than apoE2 mice (Figure 5A). Although there was a marginally significant genotype x irradiation treatment x diet interaction on the percent time spent in the center of the open field, the interaction did not reach significance ($F_{1,55} = 3.84$; $P = 0.06$). An effect of diet was observed on the total distance moved in the open field ($F_{1, 55} = 7.14$; $P < 0.01$), ALA-supplemented mice moved more than regular diet fed mice (Figure 5B, inset).

Light-Dark

There was an effect of diet on the percent time spent in the light area of enclosure ($F_{1, 58} = 11.52$; $P < 0.001$), ALA-supplemented mice spent more time in

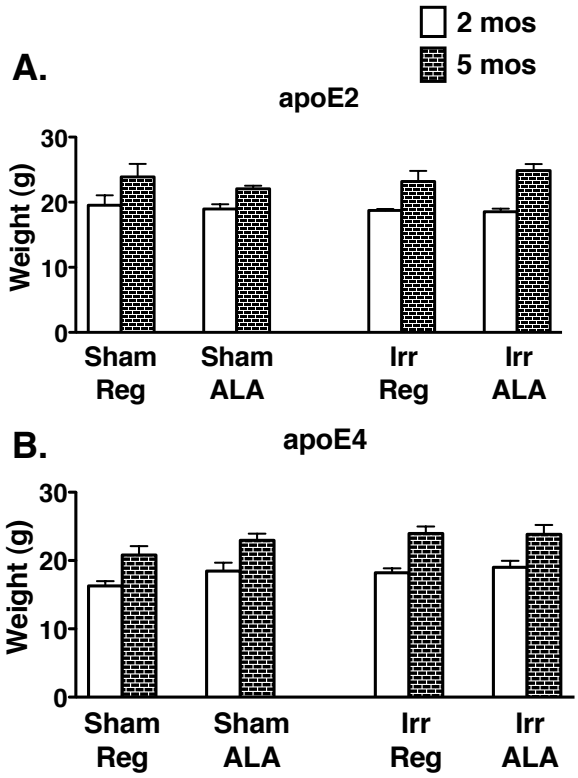

Figure 4. The body weight of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet. There were no effects of genotype or irradiation on weight change (repeated measures ANOVA). N= 3 per genotype/ irradiation treatment/diet. Bars represent the group means and $\pm$ SEM.

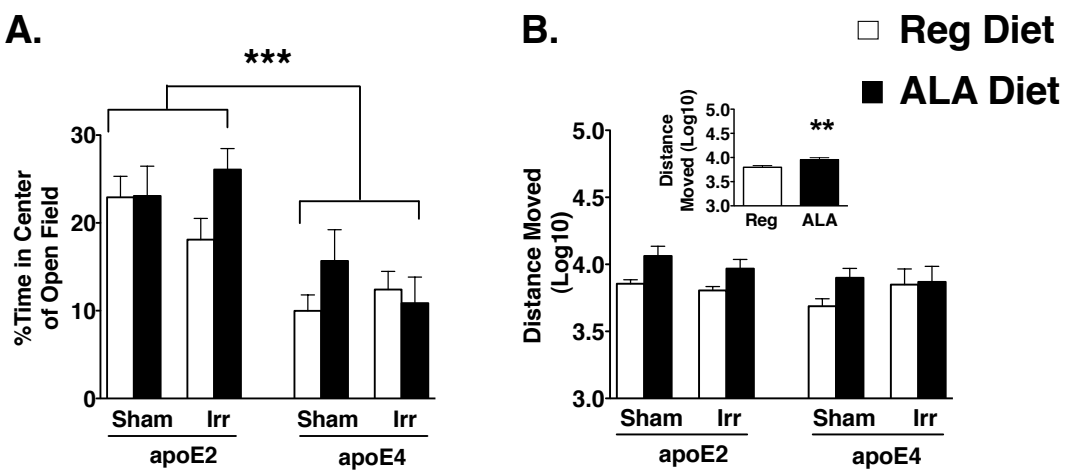

Figure 5. Anxiety and locomotor behaviors in the open field of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet. ApoE4 mice spent less time in the center of the open field compared to apoE2 mice (***$P < 0.001$, A). ALA-supplemented mice moved more than regular diet fed mice (**$P < 0.01$, B inset). The mean and $\pm$ SEM for each group are presented for the percent time spent in the center of the chamber. Group averages for distanced moved were transformed in order to normalize the data. N = 7-9 mice per genotype/irradiation treatment/diet.

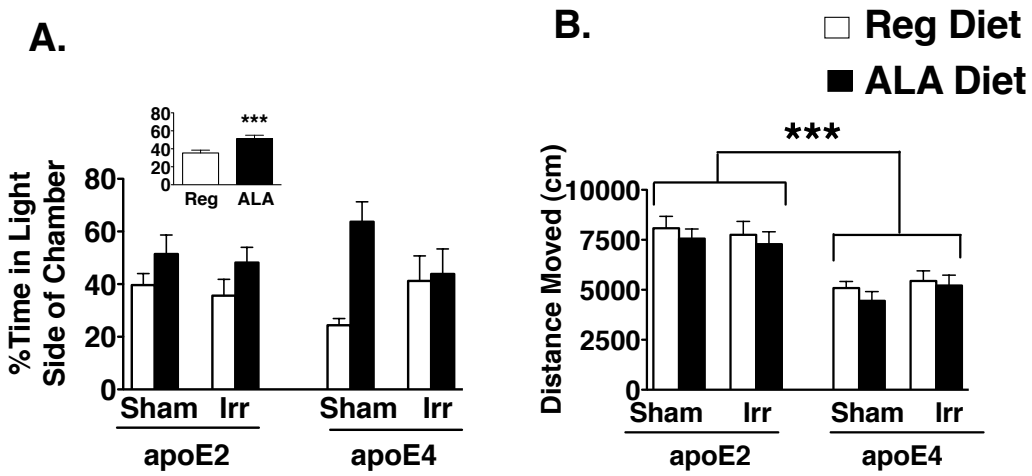

Figure 6. Anxiety and locomotor behaviors in the light-dark test of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet. ALA-supplemented mice spent more time in the light area of the enclosure (***P < 0001, A inset). ApoE4 mice moved less compared to apoE2 mice (***P < 0.001, B). Bars represent the group mean and ± SEM. N = 7-9 mice per genotype/irradiation treatment/diet.

the light area of the enclosure than regular diet fed mice (Figure 6A, inset). An effect of genotype was observed on the distance moved within the enclosure ($F_{1, 58} = 46.76$; $P < 0.001$), apoE4 mice moved less than apoE2 mice (Figure 6B).

Elevated zero maze

A genotype effect was observed on the percent time spent in the open areas of the elevated zero maze ($F_{1, 54} = 62.79$; $P < 0.01$), apoE4 mice spent significantly less time in the open areas than apoE2 mice (Figure 7A). A similar genotype effect was observed on distance moved in the elevated zero maze ($F_{1, 54} = 137.46$; $P < 0.001$), apoE4 mice moved less than apoE2 mice (Figure 7B).

Elevated plus maze

A genotype effect was observed on the percent time spent in the open arms of the elevated plus maze ($F_{1, 57} = 40.38$; $P < 0.001$), apoE4 mice spent less time in the open arms than apoE2 mice (Figure 8A). Additionally, an effect of irradiation treatment was observed on the percent time spent in the open arms ($F_{1, 57} = 6.8$; $P < 0.05$), irradiated mice spent less time in the open arms compared to sham-irradiated mice (Figure 8A, inset). There was an effect of genotype on distance moved ($F_{1, 57} = 22.23$; $P < 0.001$). ApoE4 mice moved less than apoE2 mice (Figure 8B). There was an effect of diet on distance moved ($F_{1, 57} = 10.83$; $P < 0.01$); ALA-supplemented mice moved less than regular diet fed mice (Figure 8B, inset).

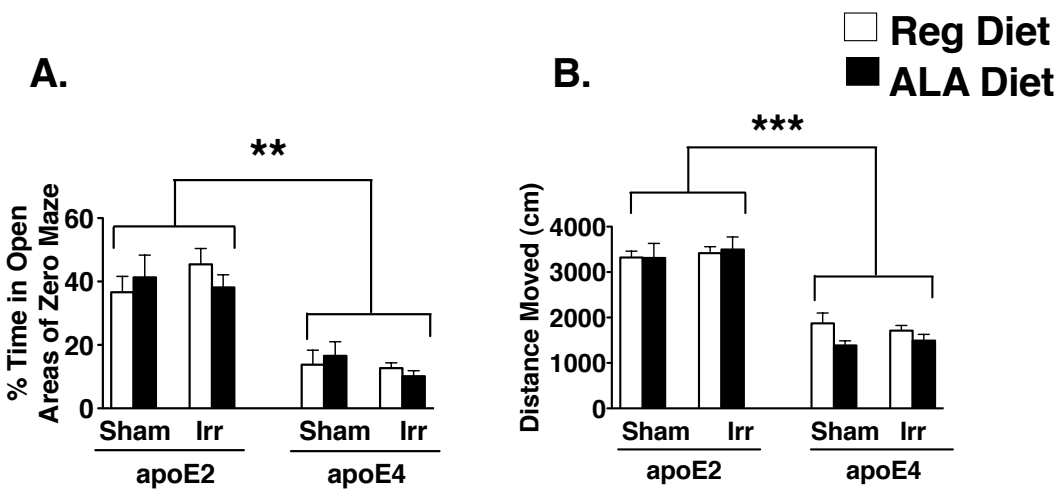

Figure 7. Anxiety and locomotor behaviors in the elevated zero maze of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet. Compared to apoE2 mice, apoE4 mice spent less time in the open areas (**P < 0.01, A) and moved less (***P< 0.001, B). Bars represent the group mean and ± SEM. N = 6-9 mice per genotype/irradiation treatment/diet.

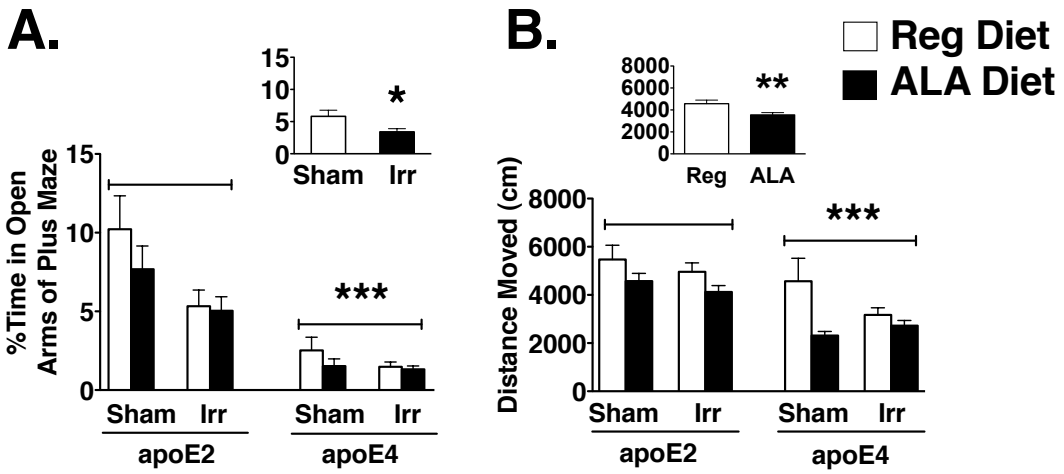

Figure 8. Anxiety and locomotor behaviors in the elevated plus maze of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet. Compared to apoE2 mice, apoE4 mice spent less time in the open arms (***$P < 0.001$, A) and moved less (***$P < 0.001$, B). Irradiated mice spent less time in the open arms compared to sham-irradiated mice (*$P < 0.05$, A inset). ALA-supplemented mice moved less than mice on a regular diet (**$P < 0.01$, B inset). Bars represent the group mean $\pm$ SEM. N = 7-9 mice per genotype/irradiation treatment/diet.

Novel Location Recognition

There were no group differences in the total exploration time with the objects across the familiarization trials (Appendix Figure 27). In contrast, there was an object x genotype x irradiation treatment x diet interaction when the three objects were compared ($F_{2, 114} = 3.13$; $P < 0.05$, Appendix Figure 28B and C). When the data were split up by genotype and irradiation treatment, an object x diet interaction was observed in sham-irradiated apoE2 mice ($F_{2, 32} = 4.44$; $P < 0.05$); the object preference was significant in sham-irradiated ALA-supplemented apoE2 mice ($F_{1.27, 10.14} = 6.75$; $P < 0.05$, Appendix Figure 28B). A multivariate test used to determine the object responsible for the group difference also showed a genotype x irradiation treatment x diet interaction (Wilkes Lambda = 0.88, $F_{2, 56} = 3.86$; $P < 0.05$). An effect of diet was again observed in apoE2 sham-irradiated mice when the data were split by genotype and irradiation treatment (Wilkes Lambda = 0.55, $F_{2, 15} = 6.07$; $P < 0.05$). Finally, a Bonferroni pairwise comparison test within sham-irradiated apoE2 mice revealed that the lion (1 of the 3 objects) was responsible for the object bias in ALA-supplemented mice ($P < 0.01$, Appendix Figure 28B). Noteworthy, the lion was the displaced object for the novel location recognition task.

In the novel location recognition test, a trial x genotype x diet interaction was observed ($F_{1, 56} = 5.02$; $P < 0.05$, Appendix Figure 29). When the data were split by genotype, a trial x diet interaction was observed in apoE2 mice ($F_{1, 32} = 5.33$; $P < 0.05$). However, when the data were further split by diet, the effect of trial

was not significant in either diet. Again, mice that exhibit novel location recognition should show greater exploration for the displaced object during trial 4 compared to trial 3. Thus, the non-significant effect of trial suggests that although the degree of exploration for the lion object in trial 3 versus trial 4 differed between apoE2 mice on regular diet and ALA-supplemented diet, neither group showed novel location recognition. In fact, none of the groups of mice showed much more than chance exploration (33%) for the lion object in trial 4 (Appendix Figure 29). Similarly, none of the groups of mice showed novel object recognition (Appendix Figure 30). This lack of novel location and novel object recognition might be related to the longer ITI used than that of previous studies (Acevedo et al. 2008a; Acevedo et al. 2008c; Siegel et al. 2010b).

Water maze

A diagram of the water maze paradigm is illustrated in Figure 9. There were no effects of genotype, irradiation treatment or diet on swim velocity (Figure 10A) or on the percent time spent swimming in the outer zone of the pool (Figure 10B). There were no effects of genotype, irradiation treatment or diet on cumulative distance to the platform in visible platform training sessions (Figure 11). There was an effect of genotype on the hidden platform sessions ($F_{4, 184} = 2.57$; $P < 0.05$); apoE4 mice had greater cumulative distance to the platform than apoE2 mice (Figure 11).

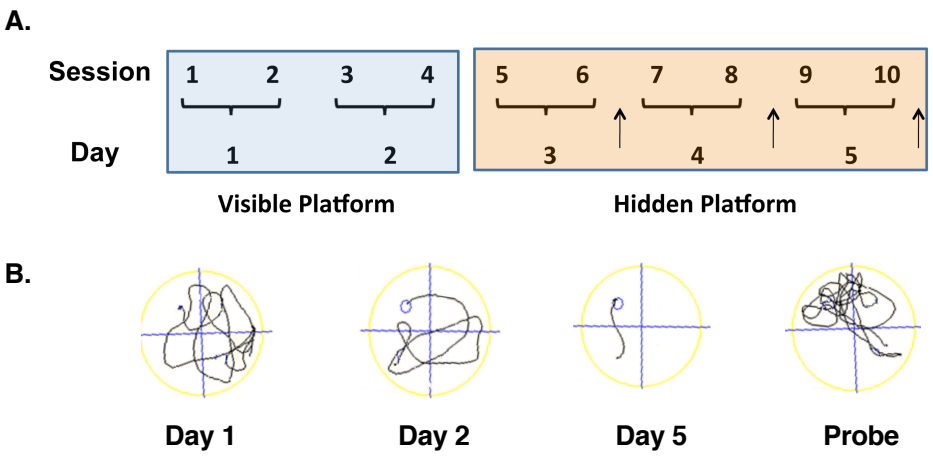

Figure 9. Illustration of the water maze paradigm (A). Mice were trained to locate a visible, then a hidden platform. Probe trials (↑) were conducted 1 hour after the last training session. An example of improved performance (reduced cumulative distance to the target) across training days is shown in panel B. The last example in panel B shows a target bias, as the majority of the search is closer to the target (upper left corner of the pool).

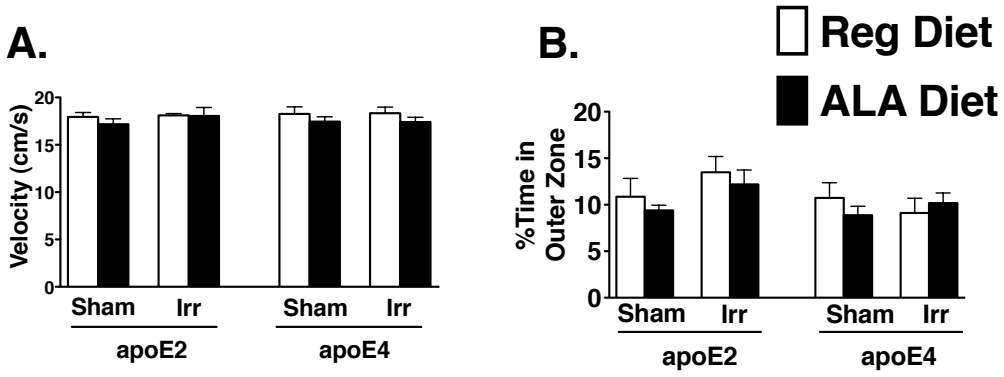

Figure 10. Swim velocity and thigmotaxis behavior during the visible platform training sessions of the water maze of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet. There were no group differences in swim speed or thigmotaxis. Bars represent group mean and $\pm$ SEM. N = 7-9 mice per genotype/irradiation treatment/diet.

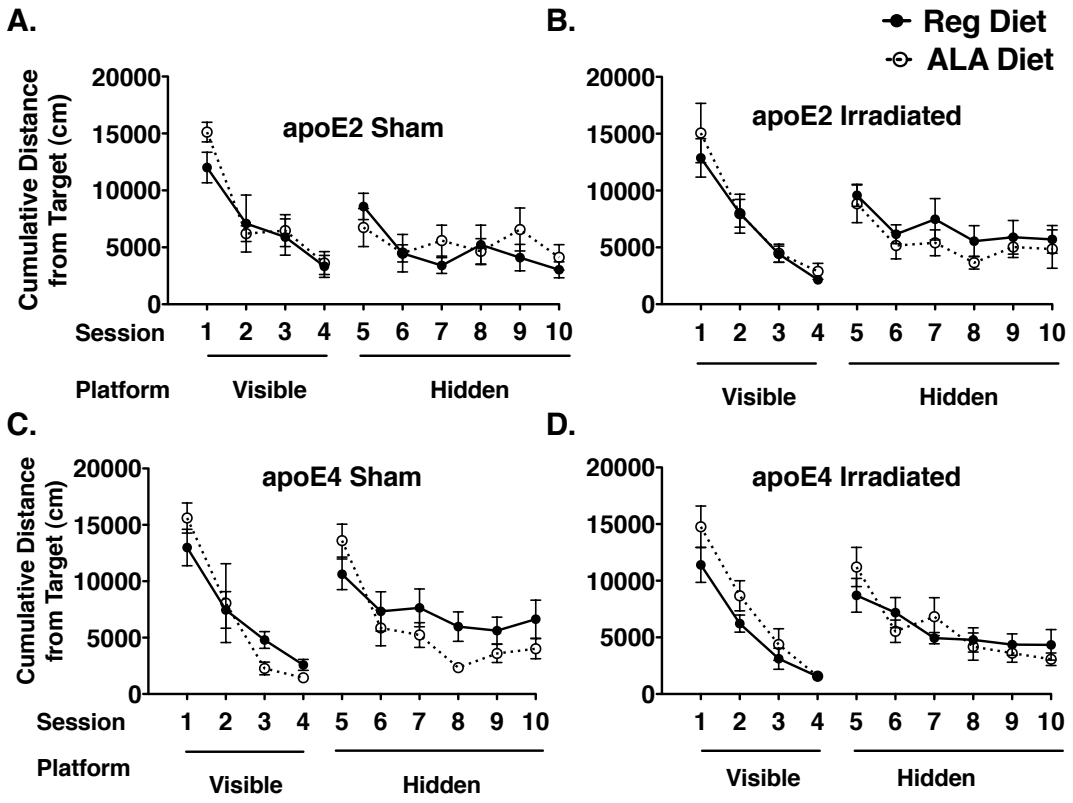

Figure 11. Water maze learning curves of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet. ApoE4 mice (C and D) had greater a cumulative distance to the platform than apoE2 mice (A and B). Data represent mean and ± SEM for each group. N = 7-9 mice per genotype/irradiation treatment/diet.

In the water maze probe trials, there were no significant interactions between probe trial, genotype, irradiation treatment, or diet; however, there was a significant genotype x irradiation treatment interaction across the average of the probe trials ($F_{1, 51} = 7.89$; $P < 0.01$, Figure 12). When the data were split by genotype, the effect of irradiation was significant in apoE4 mice ($F_{1, 28} = 6.24$; $P < 0.05$), irradiated apoE4 mice showed lower cumulative distance to the target compared to sham-irradiated apoE4 mice (Figure 12B). Although irradiated apoE2 mice appeared to have greater cumulative distance to the target than their sham-irradiated counterparts, particularly on the 2nd and 3rd probe trials, the effect of irradiation treatment was not significant ($P = 0.18$, Figure 12A).

When the data were split by irradiation treatment, an effect of genotype was observed ($F_{1, 28} = 5.16$; $P < 0.05$), irradiated apoE4 mice showed lower cumulative distance to the target across the average of the probe trials compared to irradiated apoE2 mice (Figure 12). This genotype difference was not significant in sham-irradiated mice ($P = 0.14$).

A genotype x diet interaction was also observed across the average of the probe trials ($F_{1, 51} = 5.3$; $P < 0.05$). When the data were split by genotype, an effect of diet was observed in apoE2 mice ($F_{1, 27} = 4.21$; $P < 0.05$); ALA-supplemented apoE2 mice showed greater cumulative distance to the target compared to regular diet fed apoE2 mice (Figure 12A).

Because anxiety levels can influence water maze performance, the percent time spent in the open arms of the elevated plus maze was assessed for a

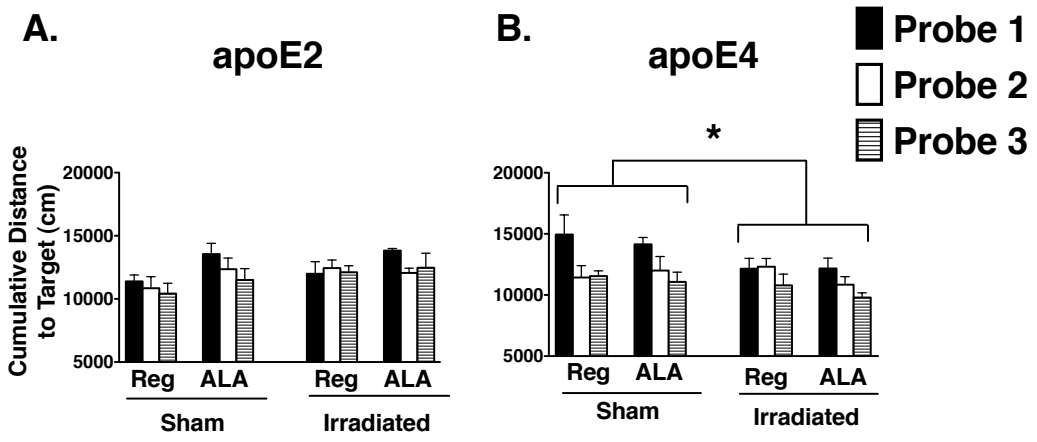

Figure 12. ^{137}Cs irradiation-induced enhancements in spatial memory retention of apoE4 female mice in the water maze probe trials. Across the average of the probe trials, irradiated apoE4 mice performed better than their sham-irradiated counterparts and better than irradiated apoE2 mice (*$P < 0.05$).

potential correlation with the water maze performance across the average of the probe trials. Pearson correlations determined that there was no significant correlation between the percent time spent in the open arms of the elevated plus maze and the probe trial performance in irradiated apoE4 mice on a regular diet ($P = 0.13$) or on a diet containing ALA supplementation ($P = 0.69$).

Conditioned Fear

Data for 2 trials, 1 training trial and 1 cued trial were lost due to technical issues. Because 4 chambers were operated per trial, data for 4 mice were lost. However, the mice still received the experimental stimuli during these trials. The lost foot shock response and baseline freezing data were from mice from each of the different four groups. For the cued trials, the lost data were from 2 apoE2 sham-irradiated mice on regular diet and from 2 apoE2 ^{137}Cs-irradiated mice also on regular diet. Therefore for the cued freezing data, there were 6 apoE2 sham-irradiated regular diet fed mice and 6 ^{137}Cs-irradiated regular diet fed mice.

Figure 13 is a diagram of the fear conditioning protocol used in this study. There were no effects of genotype, irradiation treatment or diet on baseline measures of freezing (Kruskal-Wallis test) (Table 1). Data for the average and maximum motion index for the first and second shock were transformed (square root) in order to meet normality. A multivariate analysis revealed a genotype effect in response to the shocks ($F_{4, 47} = 19.57$; $P < 0.001$, Table 1). ApoE4 mice

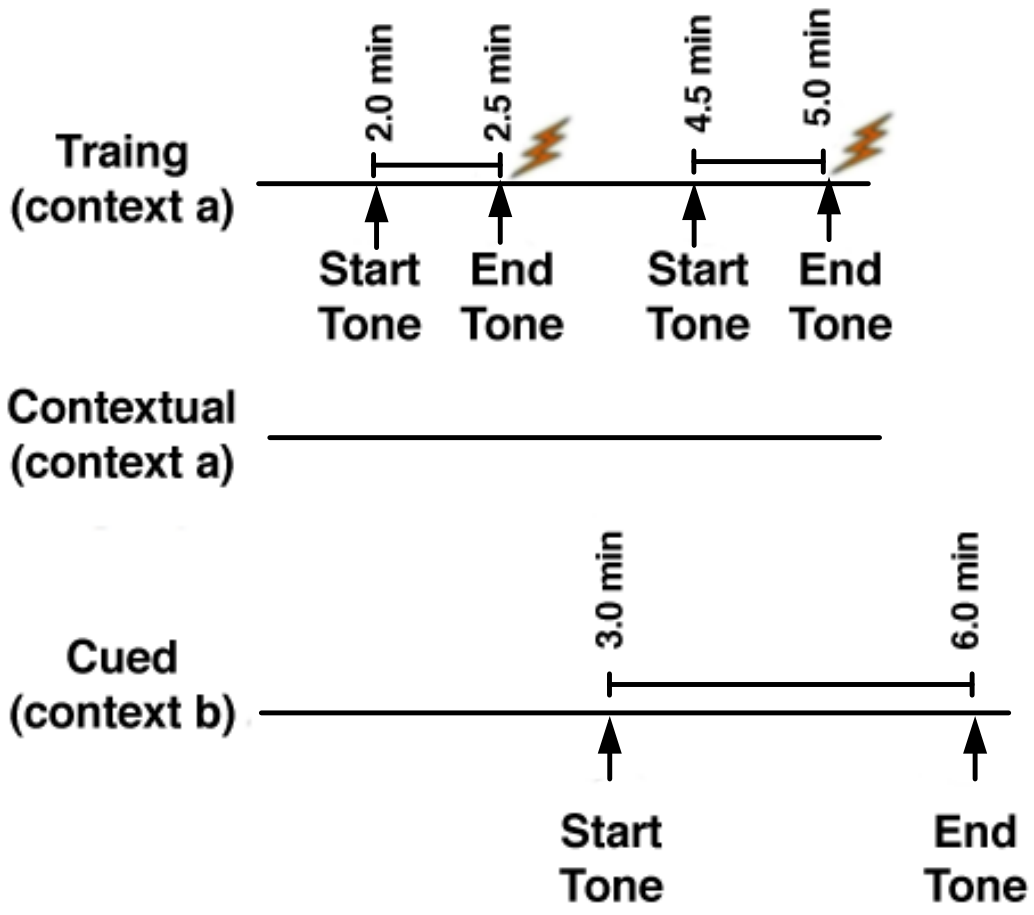

Figure 13. Illustration of the fear conditioning protocol. As described in the methods section, the mice received fear conditioning training on day 1. On the second day context-induced freezing was scored when mice were returned to the same context (chamber) in which they received the tone and shocks. Freezing behavior was assessed for 3 minutes. One hour later, tone-induced freezing was scored when the mice were placed in a different chamber containing a different context than before. Freezing behavior was assessed before the tone and after the tone was initiated.

Table 1. Baseline measures of freezing and motion response to a foot shock[1] of apoE2 and apoE4 mice.

Geno-type	Treat-ment	Diet	Baseline % freezing	Average motion index response		Maximum motion index response	
				1st Foot shock ***	2nd Foot shock*	1st Foot shock**	2nd Foot shock***
apoE2	SHAM	Reg	0.0	17.4 ± 0.7	28.6 ± 1.4	50.0 ± 2.9	45.9 ±2.9
apoE2	SHAM	ALA	0.0	17.0 ± 0.7	29.4 ± 2.3	48.1 ± 3.3	48.1±3.34
apoE2	^{137}Cs	Reg	0.0	17.2 ± 0.7	28.5 ± 1.8	45.1 ± 2.2	44.0 ± 2.4
apoE2	^{137}Cs	ALA	0.0	16.9 ± 0.7	30.0 ± 1.9	49.0 ± 1.6	49.1 ± 3.4
apoE4	SHAM	Reg	0.4 ± 0.3	13.7 ± 1.2	32.0 ±12.0	53.8 ± 3.5	54.5 ± 3.0
apoE4	SHAM	ALA	0.2 ± 0.2	11.5 ± 1.0	29.8 ± 2.2	50.9 ±3.95	49.6 ± 3.3
apoE4	^{137}Cs	Reg	0.0	11.4 ± 0.6	32.3 ± 2.9	56.7 ± 4.1	55.8 ± 3.6
apoE4	^{137}Cs	ALA	0.3 ± 0.3	13.1 ± 0.8	30.3 ± 1.5	53.7 ± 3.6	52.2 ± 1.7

[1]Data for the foot shock response were transformed to the square root of the original data in order to normalize the data. ***$P < 0.001$, **$P < 0.01$ and * $P < 0.05$ effect of genotype.

had greater motion index measures for both shocks in all cases except for the average motion index during shock 1. For that measure, apoE2 mice had a greater response ($F_{1, 50} = 65.95$; $P < 0.001$). The statistics for the other measures where apoE4 had greater responses are as follows: average motion index for shock 2 ($F_{1,50} = 4.23$; P 0.05); maximum motion index for shock 1 ($F_{1, 50} = 8.52$; $P < 0.01$) and shock 2 ($F_{1,50} = 13.48$; $P < 0.001$).

An effect of genotype was also observed on contextual freezing ($F_{1,56} = 52.74$; $P < 0.001$, Figure 14A, inset). ApoE4 mice froze more than apoE2 mice. A tone x genotype x irradiation treatment x diet interaction was observed on cued freezing ($F_{1,51} = 5.2$; $P < 0.05$, Figure 14B). When the data were split by genotype and diet, the effect of irradiation was observed in apoE2 ALA-supplemented mice ($F_{1,14} = 18.66$; $P < 0.001$); irradiated mice exhibited higher freezing levels than sham-irradiated mice (Figure 14B). This was significant for freezing during the tone ($F_{1,14} = 18.74$; $P < 0.001$) but not during the pre-tone.

When the data were then split by irradiation treatment and diet, an effect of genotype on the repeated measure (tone) was observed in every group except for irradiated mice on regular diet. In that group, the effect of genotype was observed during the tone ($F_{1,12} = 52.34$; $P < 0.001$) but not the pre-tone. The statistics for the other measures where apoE4 had greater responses are as follows: sham-regular diet fed mice, ($F_{1,12} = 25.43$; $P < 0.001$) for both the pre-tone ($F_{1,12} = 4.81$; $P < 0.05$) and the tone ($F_{1,12} = 33.28$; $P < 0.001$); sham-irradiated ALA-supplemented mice, ($F_{1,12} = 56.48$; $P < 0.001$) for both pre-tone

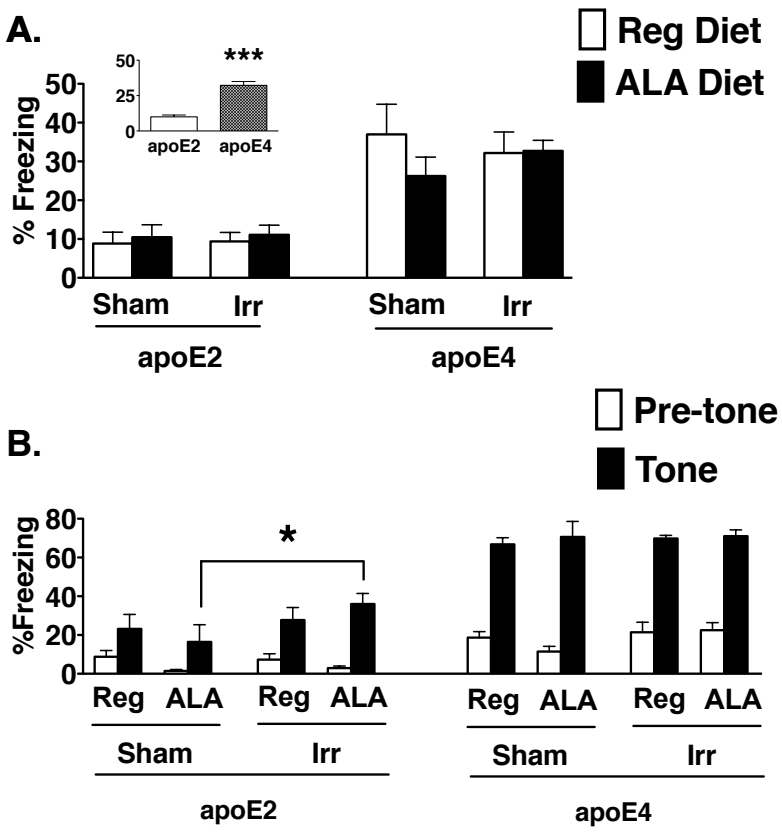

Figure 14. Contextual and cued freezing of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet. ApoE4 mice exhibited higher levels of contextual freezing than apoE2 mice (***P< 0.001, A inset). ^{137}Cs-irradiated apoE2 mice froze more in response to the tone compared to sham-irradiated apoE2 mice during cued fear conditioning test (*P < 0.05, B). Data represent mean and $\pm$ SEM. N = 6-9 mice per genotype/irradiation treatment/diet.

($F_{1,12}$ = 12.50; $P < 0.01$) and tone ($F_{1,12}$ = 57.34; $P < 0.001$); and ^{137}Cs-irradiated ALA-supplemented mice, ($F_{1,15}$ = 4.68; $P < 0.05$) for pre-tone ($F_{1,15}$ = 26.70; $P < 0.001$) and tone ($F_{1,15}$ = 28.50; $P < 0.001$).

DHE oxidation

An example of a DHE-treated hippocampal slice with the regions that were examined is shown in Figure 15. Table 4 (in Appendix) contains data for the 5 hippocampal regions for each genotype, irradiation, diet and drug treatment across the 5 different DHE oxidation time points that were assessed.

The analysis of DHE oxidation revealed a time x genotype x irradiation treatment x drug (+/-PMA) interaction ($F_{1.3, 273.8}$ = 21.05; $P < 0.001$, Figure 16 and 17). As there was no effect of region, the data were averaged across the 5 hippocampal areas and used for further analyses. Similarly, as there was no effect of diet, the data were collapsed for both diets. When the data were split by genotype and irradiation treatment to assess the effect of drug, a time x drug interaction was observed in the hippocampal slices from apoE2 sham-irradiated mice ($F_{1.24, 12.44}$ = 15.99; $P < 0.001$) and apoE4 irradiated mice ($F_{1.34, 13.44}$ = 7.09; $P < 0.05$, Figure 17). In both cases, PMA significantly increased DHE oxidation. Representative images are shown in Figure 18. Although there was a significant interaction between time, genotype, irradiation treatment, and drug, the multivariate analysis that was used to assess the effect of drug within individual time points did not reach significance and therefore pairwise comparisons for the

effect of drug within each individual time point were not analyzed. However, there was a significant effect of drug across the averaged time points for the hippocampal slices from sham-irradiated apoE2 mice ($F_{1,10} = 21.21$; $P < 0.001$, Figure 17A) and irradiated apoE4 mice ($F_{1,10} = 8.3$;$P < 0.05$, Figure 17D). To summarize this interaction, the effect of drug in the hippocampal slices from the mice revealed that PMA significantly increased ROS generation in slices from sham-irradiated apoE2 and irradiated apoE4 mice, but not in slices from irradiated apoE2 or sham-irradiated apoE4 mice.

When the data were then split by genotype and drug to assess the effect of irradiation treatment, a time x irradiation treatment interaction was observed in vehicle-treated hippocampal slices from apoE2 but not apoE4 mice ($F_{1.53,16.85} = 7.77$; $P < 0.01$, Figure 19). Hippocampal slices from irradiated apoE2 mice showed higher DHE oxidation compared to hippocampal slices from sham-irradiated apoE2 mice Figure 19A. There was no difference in DHE oxidation levels among slices from sham-irradiated and irradiated apoE4 mice (Figure 19B).

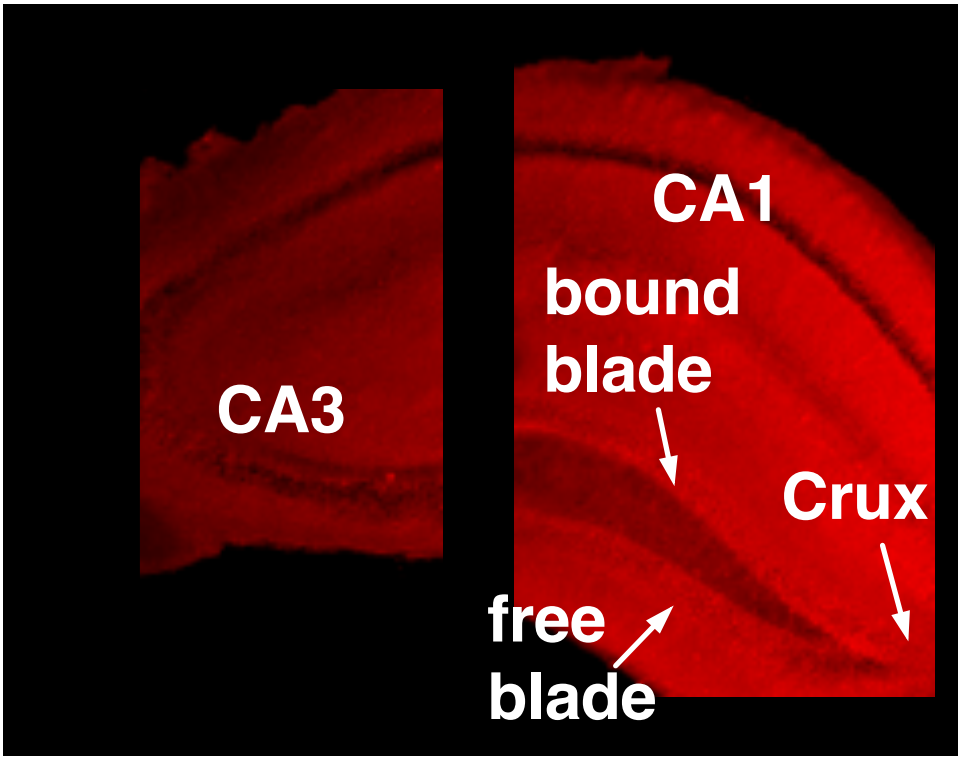

Figure 15. Representative figure of a hippocampal slice with the regions of interest labeled. Regions of interests included CA1, CA3 and 3 dentate regions: the crux, the bound blade and the free blade. These regions were traced and analyzed for pixel intensity across 5 time points, 0 to 20 minutes. The autofluorescence (background) of each region for each hippocampal slice was determined and subtracted from the 5 different DHE oxidation time points assessed.

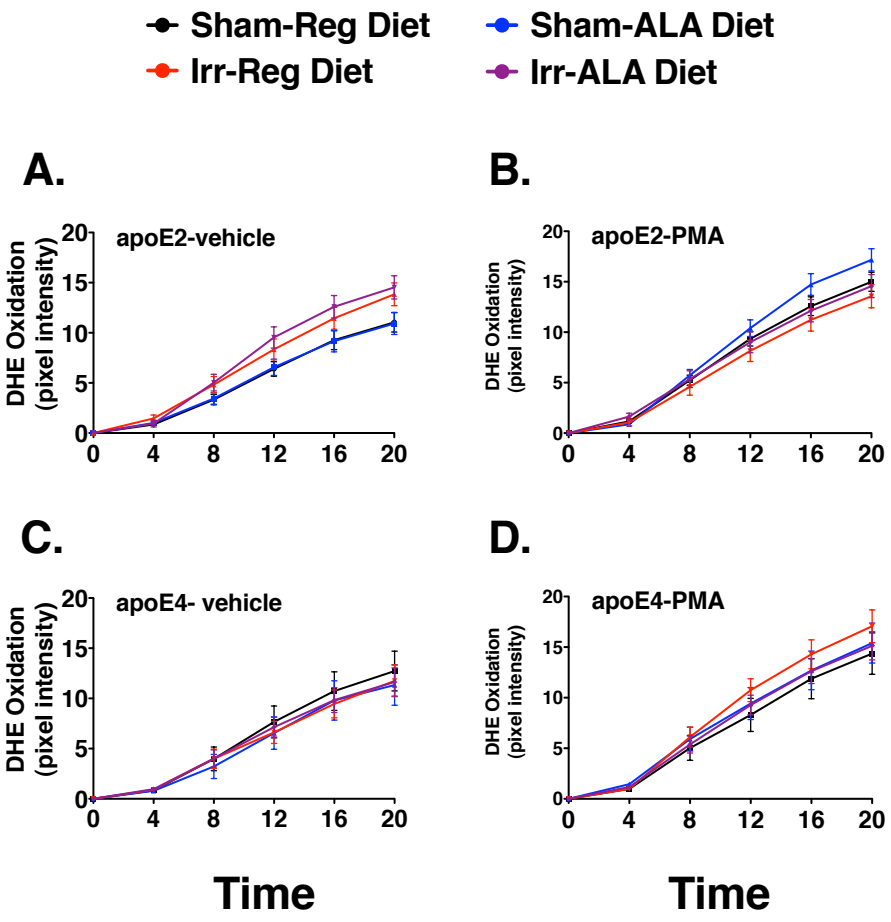

Figure 16. DHE oxidation of hippocampal slices from all of the experimental groups. Graphs show DHE oxidation of hippocampal slices treated with vehicle (left column) or PMA (right column). Four different groups within each genotype are presented together for comparison. ALA-supplementation did not reduce levels of ROS (no effect of diet). Data represent the estimated marginal means ± SEM across the average of the hippocampal regions (no effect of region). N = 3-4 mice from each each genotype/irradiation treatment/diet/drug (+/-PMA).

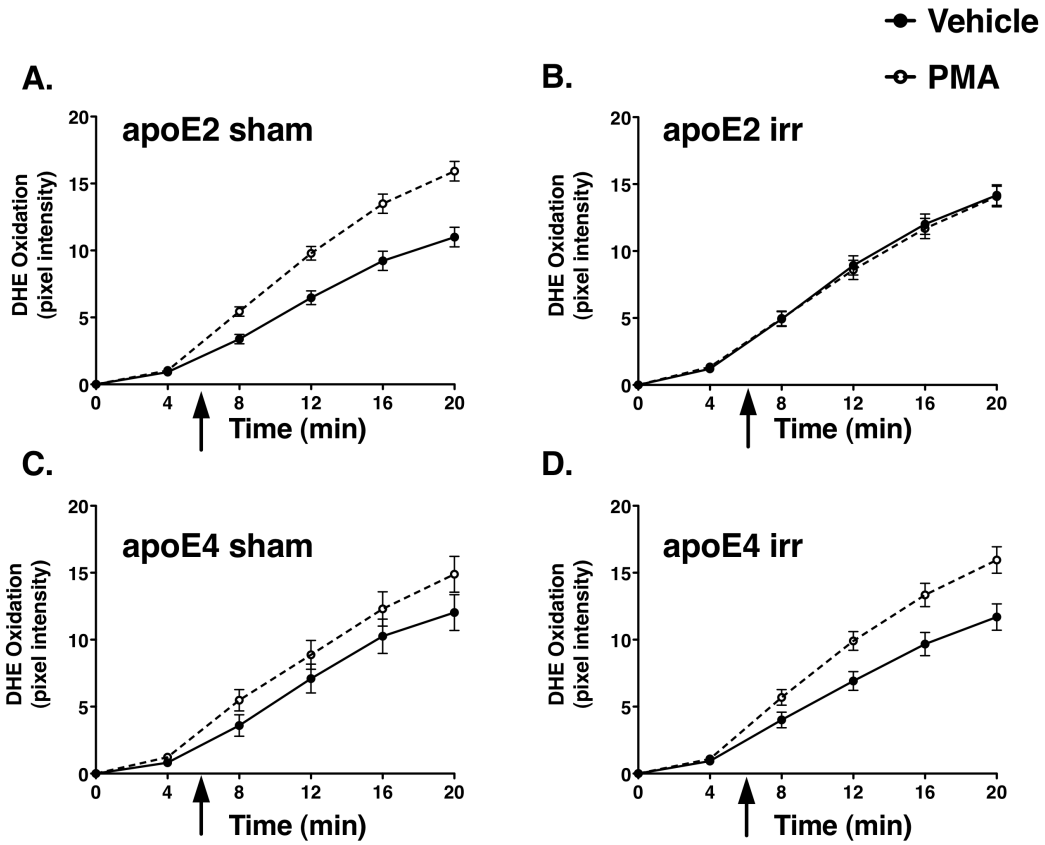

Figure 17. Paradoxical effects of ^{137}Cs irradiation on PMA-induced ROS generation in hippocampal slices from apoE2 and apoE4 mice. Irradiation blunted PMA-induced increases in DHE oxidation in hippocampal slices from apoE2 mice (B compared to A). In contrast, irradiation enhanced PMA-induced increases in DHE oxidation in hippocampal slices from apoE4 mice (D compared to C). PMA was added just before the 6 minute time point (arrow). Data represent the adjusted marginal means and $\pm$ SEM. N = 7-8 mice per genotype/irradiation treatment/drug treatment.

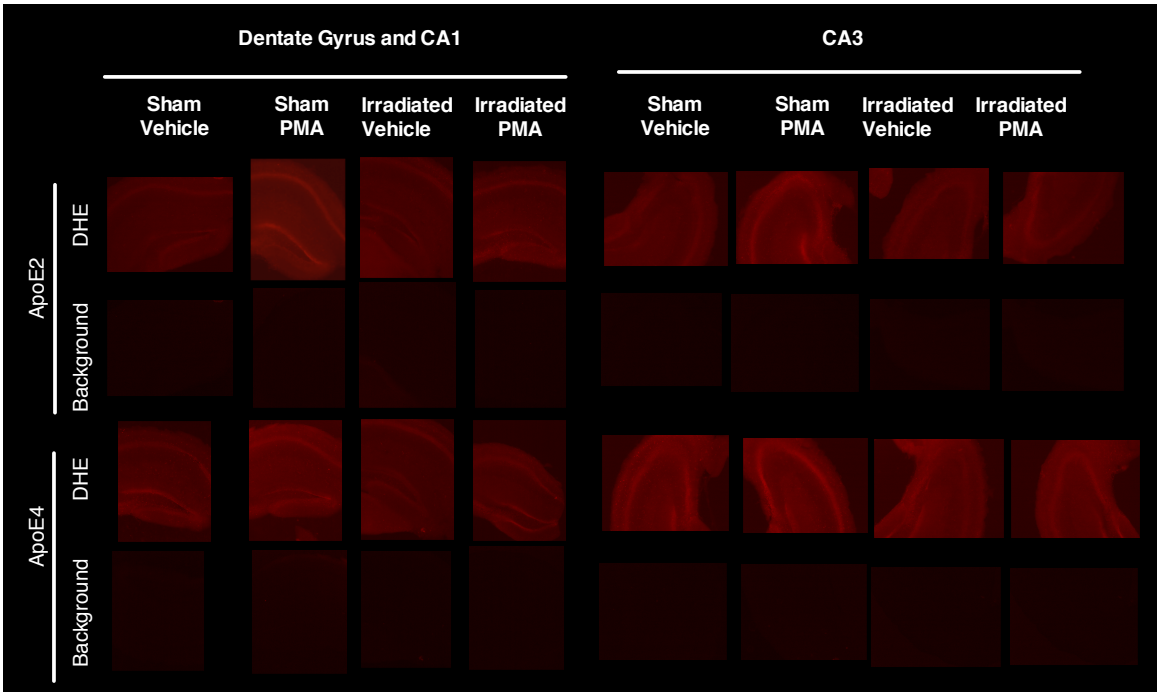

Figure 18. Representative images of DHE incubated hippocampal slices from sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice. Different hippocampal slices from each mouse received vehicle or PMA to assess baseline levels of ROS as well as stimulus-induced levels of ROS. Hippocampal slices from each irradiation treatment group were pharmacologically treated and examined in the same experimental chamber. Thus, all of the slices within a chamber were exposed to the same experimental conditions. The intensity of the background image for each slice was used to subtract its autofluorescence for DHE oxidation. ^{137}Cs-iradiation blunted PMA-induction of ROS in slices from apoE2 mice but enhanced that of slices from apoE4 mice.

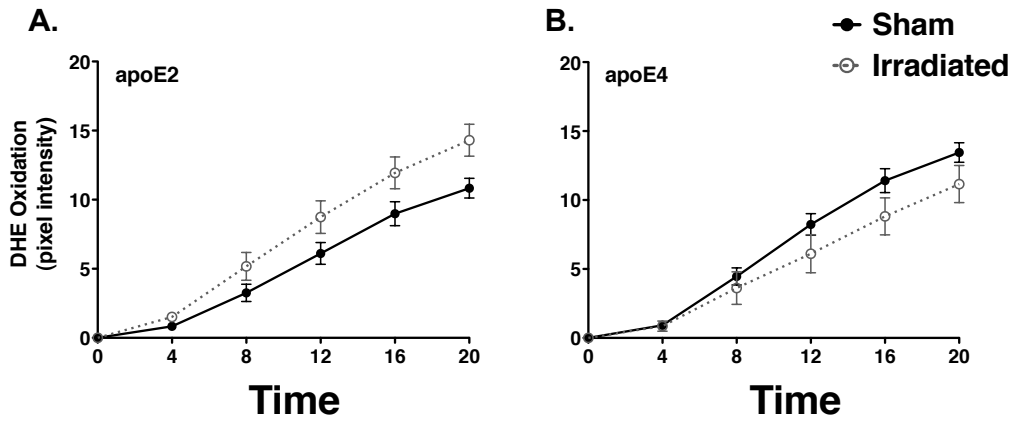

Figure 19. Radiation-induced changes in baseline levels of ROS. Data are from vehicle-treated hippocampal slices. ^{137}Cs irradiation increased levels of DHE oxidation in slices from apoE2 mice (A) but not apoE4 mice (B). Data represent the estimated marginal means and $\pm$ SEM. N = 7-8 mice per genotype/irradiation treatment.

Discussion

This study shows that the effects of ^{137}Cs irradiation on spatial memory retention in the water maze are apoE isoform-dependent in female mice. The results are striking in that they show a beneficial effect of ^{137}Cs irradiation on spatial memory, and even more striking, is that this occurs in apoE4 mice. Specifically, in the water maze probe trials, irradiated apoE4 mice performed better than sham-irradiated apoE4 mice and better than irradiated apoE2 mice (Figure 12). Moreover, we found that the radiation-induced enhancement in spatial memory of apoE4 mice coincided with an enhanced hippocampal response to PMA-induction of ROS generation (Figure 17D).

While several studies show that ROS such as superoxide are critical for learning and memory, to our knowledge, there are no studies suggesting that higher levels can be beneficial. Of significance is that our study makes an essential distinction between higher levels of ROS and the ability to generate ROS upon a stimulus. This is critical because it indicates that it is not simply a matter of having more ROS that is associated with enhanced spatial memory, but rather the ability to generate it upon a specific stimulus. The current study shows this via pharmacological stimulation. However, previous studies on the role of ROS in synaptic plasticity and learning and memory, (Klann et al. 1998; Klann and Thiels 1999; Knapp and Klann 2000; Thiels et al. 2000; Kishida et al. 2005b) imply that learning and memory demands during cognitive tasks could also act as the stimuli for ROS generation.

In contrast to hippocampal slices from irradiated apoE4 mice, hippocampal slices from irradiated apoE2 mice did not show enhanced PMA-induction of ROS, but rather were unresponsive to the PMA-induction (Figure 17B). Irradiated apoE2 mice also did not exhibit radiation-induced enhancements in spatial memory retention in the water maze. This observation strengthens the notion that enhanced hippocampal PMA-induction of ROS following irradiation might be responsible for enhancements in spatial memory retention. Yet, the opposite is not true. That is, that blunted PMA-induction of ROS does not necessarily affect spatial memory. A blunted response to PMA was observed in hippocampal slices form irradiated apoE2 mice, but they did not show changes in spatial memory retention in the water maze. Although it appeared that irradiation reduced spatial memory retention of apoE2 mice compared to their sham-irradiated counterparts, the effect of irradiation did not reach significance (Figure 12A). It is possible that a more sensitive water maze paradigm would reveal differences. Alternatively, it is also possible that a reduction in PMA-induced ROS generation is not necessarily associated with changes in spatial memory even though an enhancement in PMA-response is.

The blunted response to the PMA-induction of ROS observed in slices from irradiated apoE2 mice might be related to changes in baseline levels of ROS. In vehicle-treated hippocampal slices, irradiation increased ROS levels in hippocampal slices from apoE2 but not apoE4 mice (Figure 19). Higher baseline levels might make it more challenging for PMA to induce a response relative to

vehicle-treated hippocampal slices. Therefore, it is possible that the blunted PMA response of slices from irradiated apoE2 might be explained by a general increase in baseline levels of ROS. Ironically, this was the hypothesis made for apoE4 mice. Another possibility for the blunted response to PMA might have to do with potential changes in threshold levels or other factors that can influence induction of ROS. For instance, ROS inducing challenges can change subsequent responses to ROS induction through adaptive mechanisms (Trosko 1998; Williams and Iatropoulos 2002). Radiation hormesis has been described as an adaptive response to low doses of irradiation. Such responses include increases in expression of antioxidants, dose-dependent changes in immune function and reductions in chromosomal damage through induction of DNA repair mechanisms (Olivieri et al. 1984; Wolff 1998; Ina et al. 2005; Otsuka et al. 2006). It is possible that both apoE2 and apoE4 mice in the current study benefited from radiation hormesis.

The effects of irradiation on hippocampal-dependent cognitive function of apoE4 female mice appear to be specific to spatial memory in the water maze. For instance, irradiation had no effect on the contextual fear conditioning of apoE4 mice in (Figure 14A). The conditioned fear paradigm could account for this dissociation. Recent work by Hen and colleagues (2010) showed that in 129S6/SvEvTac male mice, the effects of irradiation on contextual fear conditioning are sensitive to a single CS-US exposure but not to multiple CS-US exposures. However, we previously showed that the paradigm used in the

current study is sensitive to the to the effects of irradiation in C57Bl/6J female mice (Villasana et al. 2010) and in male mice lacking extracellular superoxide dismutase (Raber et al. 2011). Nevertheless, because in the current study, apoE4 female mice in general showed higher anxiety levels in the anxiety tests, it is possible that a more sensitive fear conditioning paradigm might be required in order to observe potential subtle differences between sham-irradiated and irradiated mice.

In contrast to contextual fear conditioning, an effect of irradiation was observed on the cued fear conditioning test (Figure 14B). Specifically, irradiation increased the cued fear conditioning of apoE2 ALA-supplemented mice, but had no effect on the cued fear conditioning of any of the apoE4 groups of mice. It is possible that the high levels of freezing exhibited by apoE4 mice in the cued fear test (approximately 80%) could have masked potential effects of irradiation. Furthermore, the majority of the analyses for the foot shock response indicate that apoE4 mice were more responsive than apoE2 mice (Table 1). As such, it is possible that apoE4 mice may have acquired fear conditioning differently than apoE2 mice. Thus, analyses of the fear conditioning acquisition of apoE2 and apoE4 mice might provide a more accurate description of potential apoE isoform-dependent effects of irradiation on fear conditioning.

Previous data show that higher anxiety levels of apoE4 mice are associated with better performance on the water maze (Siegel et al. 2010a). However, there were several findings in the current study to suggest that the enhancing

effects of irradiation on the spatial memory retention of apoE4 mice in the water maze were not likely attributed to changes in anxiety-like behaviors. First, there were no group differences in thigmotaxis (Figure 10A), a behavior thought be reflective of higher anxiety levels during the water maze task (Venero et al. 2004; Herrero et al. 2006). Second, there was no effect of irradiation in either the visible or hidden platform training sessions (Figure 11). Third, while the effect of irradiation on the elevated plus maze was observed in both genotypes (Figure 8A), only apoE4 mice showed an effect of irradiation in water maze (Figure 12). Fourth, irradiation did not affect the performance of apoE4 female mice in the conditioned fear tasks (Figure 14), which are sensitive to group differences in anxiety levels (Uys et al. 2003; Luyten et al. 2011; Sartori et al. 2011). Finally, in irradiated apoE4 mice, the percent time spent in the open arms of the elevated plus maze was not correlated with probe trial performance in the water maze test. These data suggest that increased anxiety levels were unlikely responsible for the enhanced performance of irradiated apoE4 mice in the water maze probe trials. Be that as it may, the fact remains that irradiation-induced changes in elevated plus maze were observed. Thus, the potential influence of anxiety levels on the cognitive performance of irradiated apoE4 mice should not be easily dismissed. Therefore, we will next discuss the data that support the potential influence of irradiation-induced anxiety on the water probe trial performance.

The effect of irradiation on elevated plus maze appeared very modest (less than 2% differences from sham-irradiated mice) and was not observed in the

other anxiety tests. However, the elevated plus maze also appeared to be more anxiety-provoking than the other tests because mice spent considerably less time in the open arms of the elevated plus maze compared to the center of the open field, light enclosure of the light-dark test, or the open areas of the elevated zero maze. Hence, it may be the case that the changes in anxiety levels following irradiation might only be seen under more anxiety-provoking environments. If so, because the water maze is a stressful task, it is plausible that the effects of irradiation on anxiety could have influenced probe trial performance. However, as outlined in the previous paragraph, the data against this possibility outweighs the data in favor of it.

The effects of irradiation on novel location recognition, another spatial memory test, could not be determined because none of the groups of mice showed novel location recognition (Appendix Figure 29). Because there are three objects, 33% is considered chance exploration and none of the mice showed greater than 33% exploration for the displaced object in trial 4. Similarly, none of the groups of mice showed more than chance exploration for the novel object in trial 5. Previous studies in our lab with the same paradigm used a 3-minute ITI between trials (Acevedo et al. 2008a; Acevedo et al. 2008c; Siegel et al. 2010b). The current study used a 4-minute ITI. The difference in ITI occurred because we are now able to run 4 mice at once (we have switched to automated scoring) versus 1 mouse at a time (manual scoring). The longer ITI could explain why mice in the current study did not show novel location or novel object

recognition (Hammond et al. 2004; Sanderson and Bannerman 2011). In retrospect, fewer mice should have been tested at once in order to reduce the ITI.

In addition to a lack of novel location and novel object recognition, an object preference during the familiarization trials was also observed in our study (Appendix Figure 28B). Although our lab previously showed that there was no object bias for a very similar set of Playmobile objects (Benice and Raber 2008), it is nearly impossible to predict whether a specific genotype or experimentally treated group of mice will show a bias towards a particular object. For example, in the current study, only 1 group out of 8 showed an object bias and it was between groups of similar sex, age, and genotype. For this reason, an analysis for object bias was assessed in the current study. Finally, we cannot dismiss the possibility that the lack of novel location recognition could have been due to a lack of motivation to explore the objects (Kazlauckas et al. ; Darvas and Palmiter 2009). For instance, a lack of sufficient exploration of the objects during the familiarization trials could affect acquisition and, thus, memory. A study from our lab using a similar design but with male and female C57Bl/6J wild-type mice (Siegel et al. 2010b), showed greater exploratory times across the trials compared to those in the current study. Moreover, that study showed novel object recognition, indicating that apoE2 and apoE4 female mice might require a different novel object recognition paradigm in order to perform the task.

In the anxiety tests, apoE4 mice showed greater levels of anxiety compared to apoE2 mice. In 3 of the 4 anxiety tests (Figures 5-8), the light-dark test being the exception, apoE4 mice showed lower percent times in the anxiety-provoking areas of the enclosures or mazes. ApoE4 mice also showed lower distances moved in 3 of the 4 anxiety tests, the open field test being the exception. The percent time and total distance moved in the elevated zero maze and elevated plus maze tests were lower in apoE4 mice compared to apoE2 mice. This indicates that the effect of genotype on anxiety levels may be more reliably seen with these two tasks. Greater anxiety levels of apoE4 mice are consistent with our previous studies (Raber 2007; Siegel et al. 2010a; Villasana et al. 2011).

In contrast to the consistent effects of genotype on anxiety levels observed in the different anxiety tests, similar effects of diet or irradiation on anxiety levels were not observed. For instance, an effect of diet on the time spent in the more anxiety-provoking areas of the tests was only observed in the light-dark test (Figure 6A): ALA-supplemented mice spent more time in the light area of the enclosure compared to mice on regular diet, suggesting that ALA reduced anxiety levels. Furthermore, the effect of diet on distance moved was not consistent. ALA had opposite effects on distance moved in the open field test (Figure 5B) compared to that of the elevated plus maze (Figure 6B). In the open field, ALA-supplemented mice moved more, whereas in the elevated plus maze, they moved less. Previous findings show that ALA diet supplementation (5% w/w dose for 2 weeks) is associated with increased locomotor behavior in male rats

(Hagen et al. 1999). However, a different study in male mice using the same concentration of ALA diet supplementation as used in the current study, reported no increases in locomotor behavior (Bondy et al. 2002).

As previously discussed, irradiated mice spent less time in the open arms of the elevated plus maze compared to sham-irradiated mice. This was the only measure in the anxiety tests that showed an effect of irradiation on anxiety-like behavior. Radiation-induced increases in anxiety-like behavior in the plus maze observed in the current study is not in agreement with previous studies showing no effect of irradiation on the behavior of female androgen receptor transgenic mice on the elevated plus maze test (Acevedo et al. 2008c). Nor is it consistent with reports of reduced anxiety levels of irradiated *Apoe*$^{-/-}$ female mice in the open field following ^{137}Cs irradiation (Acevedo et al. 2008a). Together these data suggest that the effect of irradiation on anxiety might be critically modulated by genotype.

Another important finding in the current study was that ALA did not act as an antioxidant. Analysis of DHE oxidation determined that hippocampal slices from ALA-supplemented mice were no different than hippocampal slices from mice on regular diet. This likely explains why ALA did not antagonize the effects of irradiation on the spatial memory of apoE4 mice in the water maze (Figure 12B). ALA however did have an effect on the spatial memory retention of apoE2 mice in the water maze (Figure 12A). Although there were no interactions between diet and genotype on the levels of DHE oxidation, it appeared that hippocampal

slices from irradiated ALA-supplemented apoE2 mice, showed greater DHE oxidation levels than those of apoE2 mice on regular diet (Figure 16). Importantly, if ALA acted as a prooxidant as suggested by this trend, it could explain the ALA-induced deficits in spatial memory retention of apoE2 mice. Oxidative stress measures in tissue from ALA-supplemented apoE2 mice could help confirm whether ALA was indeed acting as a pro-oxidant.

Because ALA did not act as an antioxidant, the relationship found between ROS and spatial memory is limited to an association at this point. Future studies with an effective antioxidant treatment will allow us to determine whether attenuation of radiation-induced ROS blocks enhancements of the spatial memory retention of apoE4 female mice. There could be several reasons why the expected effects of ALA were not observed. We based our dose and method of administration on a study that showed reductions in NOS when given to wild-type males at 3-months of age for a duration of 6-months (Bondy et al. 2002). However in that study, although ALA was associated with reductions in NOS, it also resulted in deficits in place recall familiarity. This was assessed by reductions in locomotor activity to a previously exposed environment. Mice that were not on ALA-supplementation showed reduced exploration to the previously expose environment whereas ALA-supplemented mice did not. There were no preexisting differences in locomotor behavior to explain the group differences in place recall. Another study also used a similar concentration of ALA in the diet of Tg2576 mice, which is a mouse model of AD that develops cortical and

hippocampal β-amyloid plaque depositions. In that study, ALA reduced memory deficits in contextual fear conditioning of Tg2576 mice compared to Tg2576 mice that were not given ALA (Quinn et al. 2007). However, ALA did not reduce markers of oxidative stress or plaque deposition. More importantly, ALA did not induce deficits in wild-type mice. Thus, the effects of ALA-supplementation on measures related to ROS, oxidative damage or on cognitive function of mice are unclear. The divergent findings may be related to several different factors. For instance, one important difference between the current study and the aforementioned studies is the age at which mice were placed on an ALA-supplemented diet. In our study, mice were placed on an ALA-supplemented diet at 6-weeks of age, whereas mice in the other studies were at least 3-months of age when they were placed on an ALA-supplemented diet. Other potential factors influencing the divergent findings could include the different ROS measures assessed (e.g. NOS versus superoxide/hydrogen peroxide), sex, and the genotype of the mice.

The apoE isoform-dependent effects of ^{137}Cs irradiation on spatial memory retention in the water maze supports the hypothesis that apoE isoform critically modulates the effects of irradiation on cognitive function. However, the original hypothesis that apoE4 mice would be more adversely affected by ^{137}Cs irradiation compared to apoE2 mice was not supported. In contrast to what was predicted, apoE4 mice showed enhancements in spatial memory. While this was unexpected, it was not completely surprising. As discussed in the introduction,

our lab previously showed that compared to sham-irradiated genotype-matched mice, ^{137}Cs irradiation enhanced the spatial and associative memory of male mice deficient in EC-SOD (Raber et al. 2011). This was observed in the novel location recognition task, the water maze test and the contextual fear conditioning test. Of significance is that this cognitive enhancement was associated with a hippocampal resistance to oxidative stress as assessed by 3-nitrotyrosine, a marker of oxidized proteins. This suggests that increased background levels of ROS might provide protection against ROS-inflicted damage. Similar to EC-SOD mice, previous studies suggest that apoE4 mice also have higher levels of ROS (Ihara et al. 2000; Lauderback et al. 2002; Dafnis et al. 2010) and oxidative stress (Pedersen et al. 2000; Butterfield et al. 2002; Jofre-Monseny et al. 2008). Thus, we were prepared for the possibility that apoE4 mice might show enhancements in hippocampal function following irradiation. The idea that higher levels of ROS could provide protection against ROS inflicted damage, could explain protection against radiation-induced cognitive impairments, but how could this explain enhancements in memory? As next discussed, the role of ROS in learning and memory might provide insight into a potential mechanism.

Conventionally, ROS are regarded as deleterious to cognitive function. However, studies have convincingly shown that ROS are also necessary for normal LTP and memory formation (Klann 1998; Knapp and Klann 2002; Kishida et al. 2005b). For instance, Klann and colleagues have shown that superoxide is

required for LTP (Klann 1998; Klann et al. 1998) and NMDA-receptor activation of extracellular regulated kinase (ERK) (Kishida et al. 2005b), a kinase important for learning and memory (Atkins et al. 1998; Schafe et al. 1999; Selcher et al. 1999). In addition, mutations in NADPH-oxidase, a superoxide-generating system, results in performance deficits on hippocampal-dependent tasks in mice (Kishida et al. 2006a). Mutations in the NADPH-oxidase in humans (chronic granulomatous disease) are also associated with cognitive deficits (Pao et al. 2004). Furthermore, Tejada-Simon et al. (2005) characterized NADPH-oxidase as a source of superoxide in neurons, making it an attractive mechanism because it generates superoxide only upon specific stimulation (Lambeth 2004; Quinn and Gauss 2004). There is evidence to suggest that irradiation increases NADPH-oxidase activity in rat brain endothelial cells (Collins-Underwood et al. 2008), which could lead to increases in superoxide production. Furthermore, Silasi et al. (2004) showed that chronic doses of irradiation increase ERK activity in wild-type female mice. Increases in ERK activation are associated with enhanced hippocampal function (Kim-Han and Dugan 2005; Kim et al. 2008). Considering these data, a potential mechanism to explain the enhancements in spatial memory retention of apoE4 mice in the water maze might involve increases in ERK phosphorylation via the specific activation of the NADPH-oxidase complex. Indeed, our study showed that a general increase in ROS was not associated with spatial memory enhancements in the water maze (Figures 12B and 19B). Instead, enhancements were associated with a more specific

generation of ROS, namely PMA-induction of ROS (Figure 17D). We propose that NADPH-oxidase is a good candidate for explaining radiation-induced enhancements in PMA-induction of ROS and its associated enhancements in spatial memory in the water maze test.

In conclusion, this study suggests that radiation-induced increases in ROS could improve hippocampal-dependent spatial memory of individuals with higher background levels of ROS. While it is exciting to find that this benefit was rendered to a genotype that is associated with increased risk to develop neurological diseases and poor recovery following brain injury, our study is too preliminary to suggest a clinical relevance. Additional analyses of the effects of ^{137}Cs cranial irradiation should be conducted to determine potential effects on different forms of cognitive function. Furthermore, because the role of ROS on learning and memory appears to be age-dependent (Serrano and Klann 2004; Hu et al. 2007), the long-term effects of ^{137}Cs irradiation on cognitive function should be determined as well.

3

Effects of ^{56}Fe irradiation on the cognitive function of apoE4 female mice: the role of reactive oxygen species

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Abstract

In the previous chapter, ^{137}Cs irradiation was associated with enhancements in the spatial memory retention of apoE4 mice in the water maze probe trials. These enhancements were also associated with enhancements in PMA-induction of ROS. The purpose of the current study was to determine whether ^{56}Fe irradiation is also associated with enhancements in spatial memory retention and PMA-induction of ROS in apoE4 female mice. To address this, apoE4 female mice were sham-irradiated or ^{56}Fe-irradiated at 2 months of age and were behaviorally tested 3 months later using a battery of tests that included anxiety tests, hippocampal-dependent and non-hippocampal dependent cognitive tests.

Although the data from the previous chapter suggest that irradiation does not cause impairments in hippocampal function of apoE2 or apoE4 mice, previous studies show that ^{56}Fe irradiation is associated with hippocampal-dependent impairments in water maze and contextual fear conditioning in wild-type mice. Therefore, it was hypothesized that apoE4 mice would show impairments in hippocampal-dependent cognitive tests compared to sham-irradiated apoE4 mice. Additionally, it was hypothesized that enhancements in PMA-induction of ROS would no longer be observed because such enhancements were previously associated with enhanced spatial memory in the water maze task.

In contrast to ^{137}Cs irradiation, we found that ^{56}Fe irradiation was not associated with enhancements in the spatial memory of apoE4 mice in the water maze probe trials but, instead, was associated with enhancements in contextual memory in the fear conditioning test. However, enhancements in PMA-induction of ROS were no longer present in hippocampal slices from irradiated apoE4 mice compared to those of sham-irradiated mice. Instead, a reduction in ROS was observed in slices from irradiated mice, including PMA-treated slices.

Taken together, these data suggest that radiation-induced enhancements in memory are dependent on the source of radiation, the type of memory, and apoE isoform. Furthermore, the data suggest that there is a difference in how ROS contributes to spatial memory in the water maze and contextual memory in the conditioned fear test.

Introduction

In our previous study we found that ^{137}Cs irradiation improved the spatial memory retention of apoE4 mice compared to sham-irradiated apoE4 mice. Additionally, we observed that these enhancements were associated with an enhancement in PMA-induced generation of ROS as assessed by dihydroethidium (DHE) oxidation. The purpose of the current study was to determine whether ^{56}Fe irradiation, a form of radiation humans encounter during space missions, has similar effects on spatial memory retention of apoE4 female mice in the water maze test.

In the clinical setting, ^{137}Cs irradiation is a common form of radiation used for radiotherapy (Camphausen and Lawrence 2009). Humans are also exposed to other forms of radiation such as high-energy (HZE) particle irradiation, which includes heavy ions from elements such as carbon and iron (NASA/BNL 2010). Astronauts undergo prolonged exposure to this form of radiation during space missions. The space environment includes ionized atomic nuclei of all stable elements, including hydrogen, helium, oxygen, carbon and ^{56}Fe (Bahadori et al. 2011). Heavy ion irradiation has also had more recent use in the treatment of tumors that are not responsive to other forms of cancer treatments such as gamma irradiation (Normile 1995; Sawajiri et al. 2003). Therefore, increased exposure of this form of irradiation in humans warrants studies to assess how it might affect cognitive function.

Compared to gamma irradiation, the effects of heavy ion irradiation on the cognitive function of humans are relatively unknown. However, studies in rodents suggests that ^{56}Fe irradiation increases anxiety levels (Rabin et al. 2007) and impairs higher order cognitive function including operant conditioning (Rabin et al. 2005b), contextual memory (Villasana et al. 2010), and spatial memory retention in the water maze (Higuchi et al. 2002; Shukitt-Hale et al. 2007; Manda et al. 2008; Villasana et al. 2011).

To begin assessing whether ^{56}Fe irradiation has similar effects as ^{137}Cs irradiation on the cognitive function of apoE4 female mice, mice were sham-irradiated and ^{56}Fe-irradiated at 2 months of age and tested 3 months later (similar to ^{137}Cs irradiation). A 3 Gy (600 MeV/amu iron particles) dose of ^{56}Fe irradiation was used because our previous study determined that contextual fear conditioning in female mice was sensitive to this dose (Villasana et al. 2010). The dose rate was 1.3 Gy/min. Head only irradiation was used, as we wanted to keep the current study similar to the previous study in order to compare irradiation effects on brain function and hippocampal ROS levels. The treatment groups were limited to sham-irradiation and irradiation. The behavioral battery and DHE oxidation experiments were exactly the same as those of the previous experiments.

Statistical comparisons between ^{137}Cs-irradiated and ^{56}Fe-irradiated apoE4 mice were not conducted because ^{56}Fe-irradiated mice were shipped, whereas ^{137}Cs-irradiated mice were not. Previous studies show that shipping of animals

can cause widespread physiological and behavioral effects in rodents. These effects include: increases in corticosterone levels (Tuli et al. 1995), blood pressure (Hoorn et al. 2011), anxiety (Obernier and Baldwin 2006), and changes in reproductive behaviors (Ismail et al. 2001). Moreover, early life stressors in rodents (neonatal up to 8-weeks of age) can cause long-term changes in anxiety-like behaviors (Calvo-Torrent et al. 1999; Adamec et al. 2006; Tsoory et al. 2007; Olesen et al. 2011). Furthermore, although we could assess potential effects of shipping in sham-irradiated mice, we could not determine whether there was a potential interaction between shipping and irradiation. For these reasons, we did not statistically compare irradiated apoE4 mice from the two studies.

Because previous studies in rodents show memory impairments following ^{56}Fe irradiation, we hypothesized that ^{56}Fe-irradiated apoE4 mice would show cognitive impairments compared to sham-irradiated apoE4 mice.

Materials and Methods

Mice

The mice were generated and maintained as described in the previous study except that none of the groups received ALA-supplementation.

Weight assessment

All mice were weighed the day of sham-irradiation or ^{56}Fe irradiation and again 2 months later.

Irradiation

Two-month-old apoE4 female mice bred in our mouse colony at OHSU were shipped to BNL for brain only ^{56}Fe irradiation (n = 6) or sham-irradiation (n = 7). Following acclimatization of 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 ^{56}Fe-irradiaiton. Following i.p anesthesia, (ketamine (Sigma, St. Louis, MO), 80 mg/kg and xylazine (Sigma), 20 mg/kg), ophthalmic solution was placed on the eyes of the mice for protection. The mice designated for irradiation were placed in positional cradles to stabilize the head during irradiation. The cradles were then placed in a chamber designed for the beam line (Rola et al. 2004). The entire head of each mouse was irradiated with 3 Gy

(600 MeV/amu iron particles). Sham-irradiated mice received the same procedure except that they

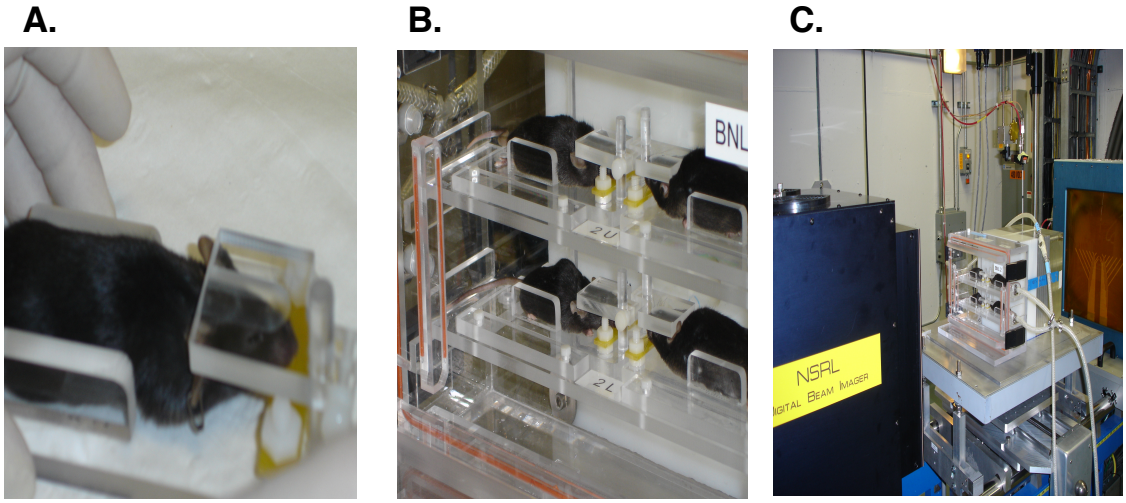

Figure 20. Set up of ^{56}Fe irradiation of mice at Brookhaven National Laboratories. Mice were sedated and placed in positional cradles (A), which were loaded onto holding chambers (B) that had calibrated positions for the beam. The position of the holding chamber was then placed in the beam line (C).

were not treated with ^{56}Fe irradiation. Upon recovery from anesthesia, the mice were returned to the animal facility. All mice were monitored for a few days before they were shipped back to OHSU for cognitive testing and DHE oxidation analyses (3 months later). The chambers used for ^{56}Fe irradiation are shown in Figure 20.

Behavioral Testing

All of the behavioral tests were conducted exactly as described in the previous chapter. Because the data from the previous study had not yet been analyzed at the time of the current study, we were not aware that the novel location and novel object recognition tasks paradigms were not optimal for apoE4 female mice. Therefore, the same protocol was used in the current study.

Assessment of ROS in *ex vivo* hippocampal slices

DHE oxidation measures were conducted as described in the previous chapter.

Statistical Analyses

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). When appropriate and as indicated, the data were normalized by removal of outliers identified as significantly different

from the group mean by SPSS software (SPSS Inc, Chicago, IL, USA) or by transformation of the data to satisfy normality.

Statistical analyses for the behavioral tasks were conducted as described in the previous chapter, except they did not include genotype and diet as between subject factors.

For analysis of DHE oxidation levels, data were standardized to the control, because the data were not normally distributed across the different time points (Shapiro Wilk, $P < 0.01$). Temperature was no longer used as a covariate. This was because slices from sham-irradiated mice (controls) were within the same chamber as the slices from the irradiated mice. Thus, they had the exact same bath temperatures. Distance from bregma was still used as a covariate in the analyses.

Results

Condition of mice

The mice tolerated ^{56}Fe irradiation well as there were no signs of illness or significant differences in weight changes before and after irradiation (Figure 21).

Open field

There were no effects of ^{56}Fe irradiation on the percent time spent in the center of the open field or on distance moved (Figure 22A and B).

Light-dark test

There was no effect of ^{56}Fe irradiation on the percent time spent in the light areas of the enclosure or on distance moved (Mann-Whitney U test, Figure 22C and D).

Elevated zero maze

Compared to sham-irradiated mice, ^{56}Fe-irradiated mice spent more time in the open areas of the elevated zero maze ($F_{1,9} = 7.41; P < 0.05$, Figure 22E) and moved less ($F_{1,9} = 6.26$; $P < 0.05$, Figure 22F).

Elevated plus maze

There was no statistically significant effect of irradiation on the percent time spent in the open arms of the elevated plus maze ($P = 0.07$) or on distance moved (Figure 22G and H).

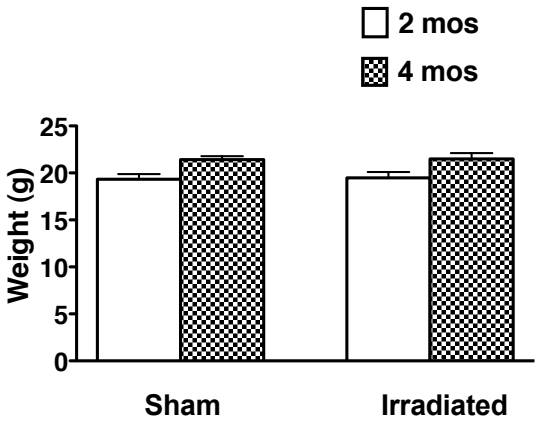

Figure 21. No effect of ^{56}Fe irradiation on the body weight of apoE4 mice. Mice were weighed at 2-months of age (pre-irradiation) and at 4-months of age (post-irradiation). Irradiation did not affect changes in weight (repeated measures ANOVA).

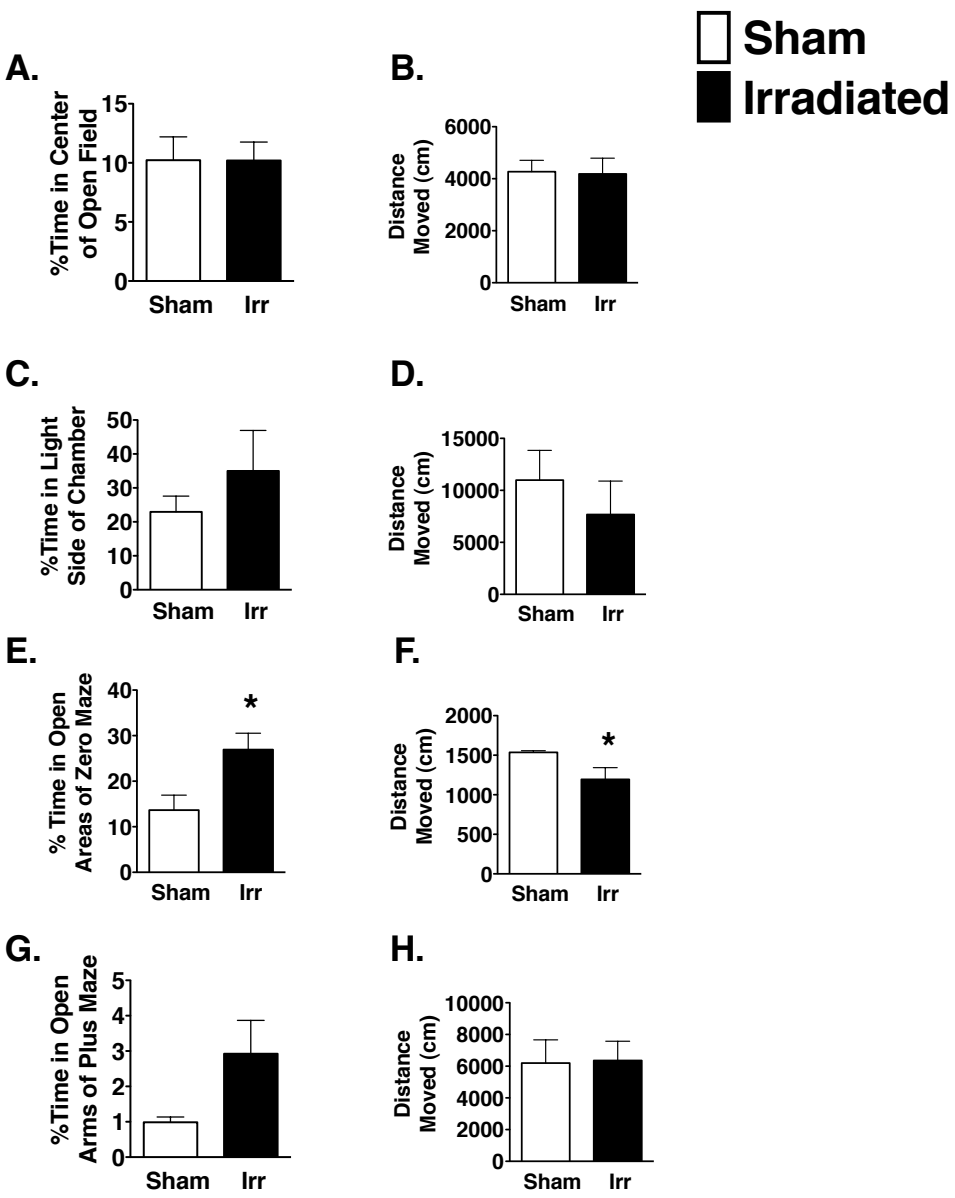

Figure 22. Anxiety and locomotor behavior of sham-irradiated and ^{56}Fe-irradiated apoE4 mice in anxiety tests. Irradiated mice spent more time in the open areas of the elevated zero maze (*$P < 0.05$, E) but also moved less than sham-irradiated mice (F, *$P < 0.05$, F). Bars represent the group mean and $\pm$ SEM. N = 5-7 mice per group.

Novel location and novel object recognition tests

There was no effect of ^{56}Fe irradiation on total exploration time with the objects during the familiarization trials (trials 1-3) (Appendix Figure 31A)**.** During the familiarization trials, neither sham-irradiated nor irradiated mice showed an object bias for any of the objects (Appendix Figure 31B**).** However, none of the groups of mice showed novel location or novel object recognition (Appendix Figure 32**).**

Water Maze

There were no effects of irradiation on swim velocity (Figure 23A), on time spent in the outer zone (Figure 23B), or on cumulative distance to the target during the visible or hidden platform sessions (Figure 23C). The repeated measures analysis of the probe trials showed no significant effect of irradiation (23D). Similarly, there was no effect of irradiation across the average of the probe trials.

Conditioned fear

Two mice (one from each group) were excluded from the fear conditioning tests due to experimental error. There was no effect of ^{56}Fe irradiation on baseline freezing (Mann-Whitney U test, Table 2). The motion index data for the response to shock were transformed (square root) in order to normalize the data (Table 2). There was no effect of irradiation on the average or maximum motion index for either shock. Contextual freezing was increased in ^{56}Fe- irradiated mice compared to sham-irradiated mice ($F_{1,9} = 6.39$; $P < 0.05$, Figure 24A).

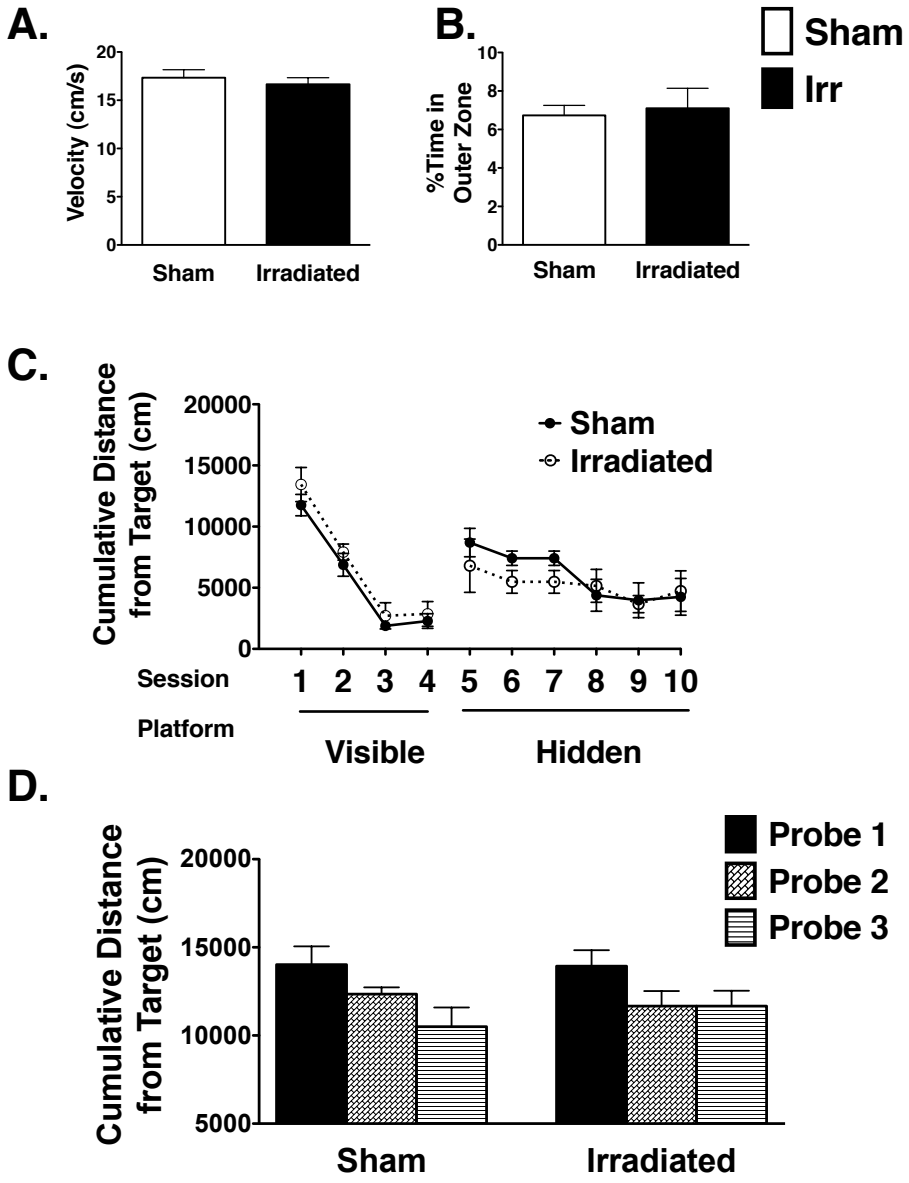

Figure 23. Performance of sham-irradiated and ^{56}Fe-irradiated apoE4 mice in the water maze test. There was no effect of irradiation on swim velocity (A), thigmotaxis (B), or on cumulative distance to the platform in the visible or hidden water maze sessions (C). There was no effect of irradiation on probe trial performance either (D). Bars represent the group mean and $\pm$ SEM. N = 5-7 mice per group.

Table 2. Baseline freezing and foot shock response[1] of apoE4 mice.

Treat-ment	Baseline %Freezing	Foot Shock Response Mean		Foot Shock Response Max	
		1	2	1	2
SHAM	0.3 ± 0.2	13.4 ± 1.2	31.3 ± 3.2	57.1 ± 3.0	54.7 ±2.4
Irr	0.7 ± 0.7	12.73 ± 0.8	33.9 ± 2.6	59.8 ± 1.0	56.0 ± 4.6

[1]Data for the foot shock response were transformed to the square root of the original data.

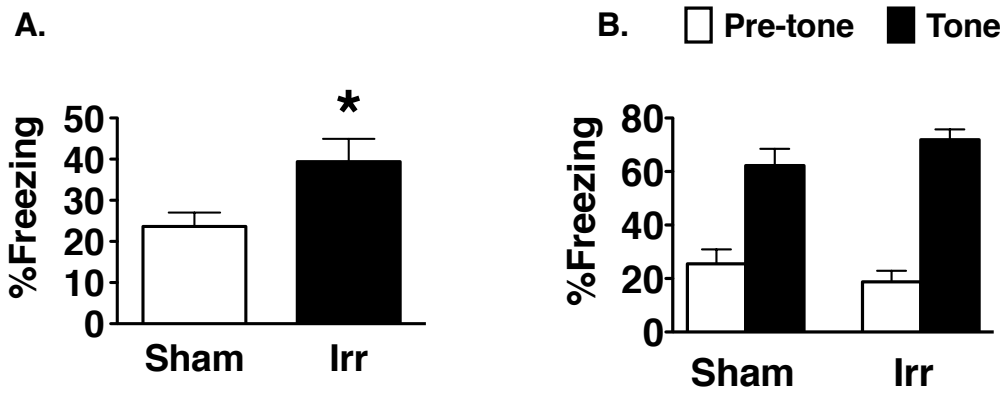

Figure 24. Enhanced contextual memory of ^{56}Fe-irradiated apoE4 mice in the fear conditioning test. Irradiated apoE4 mice exhibited greater freezing levels than sham-irradiated mice during the contextual fear conditioning test (A). Irradiation had no effect on the freezing response to the fear conditioning tone during the cued fear conditioned test (B).

There were no effects of irradiation on freezing when mice were re-exposed to the fear conditioning tone in a different context (Figure 24 B).

DHE oxidation

There was a time x irradiation treatment interaction on DHE oxidation ($F_{1.7, 100.5} = 3.26$; $P < 0.05$), but there was no significant effect or interaction with drug (+/- PMA, Figure 25). Table 5 in the Appendix shows the values for each treatment, drug, hippocampal region, and for each of the 5 different time points. As there was no effect of region, the data were averaged across the 5 hippocampal areas and used for further analyses. Slices from ^{56}Fe-irradiated mice showed lower levels of DHE oxidation (Figure 25). A multivariate analysis for each time point supported the time x irradiation treatment interaction (Wilkes Lambda = .138; $F_{5,9} = 11.27$; $P < 0.001$). Follow up Bonferroni pairwise comparisons showed that the effect of irradiation was significant at the 4-min ($P < 0.001$), 8-min ($P< 0.05$), 12-min ($P < 0.05$), and 20-min ($P < 0.05$) time points (Figure 25). The effect of ^{56}Fe irradiation on DHE oxidation was also significant across the average of the 5 different time points ($F1,59 = 46.12$; $P < 0.001$). Representative images of vehicle-and PMA-treated slices from sham-irradiated and irradiated apoE4 mice are presented in Figure 26.

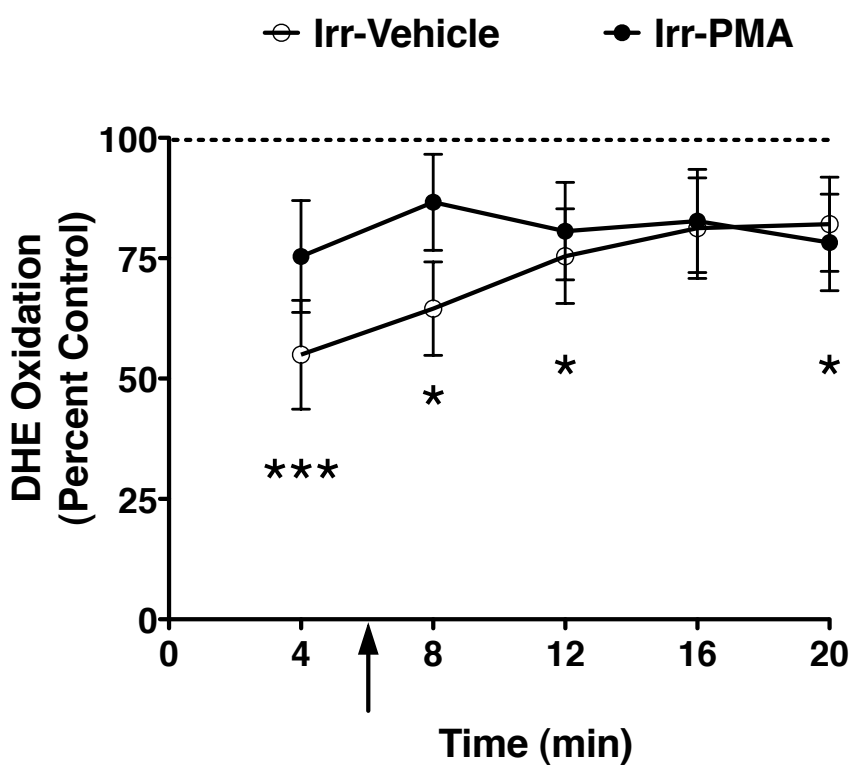

Figure 25. Irradiation-induced reductions in ROS levels in hippocampal slices of apoE4 mice. DHE oxidation was reduced in hippocampal slices from irradiated mice compared to slices from sham-irradiated mice (control, dashed line). The first twelve minutes and last time point show a significant effect of irradiation (***P < 0.001, *P< 0.05, versus the control). Data for both vehicle-and PMA-treated slices from irradiated mice are shown here but there were no significant differences between the two. PMA was added just before the 6-minute time point (arrow). Data represent the estimated marginal means for each group ± SEM. N = 4 mice per irradiation treatment/drug (+/- PMA).

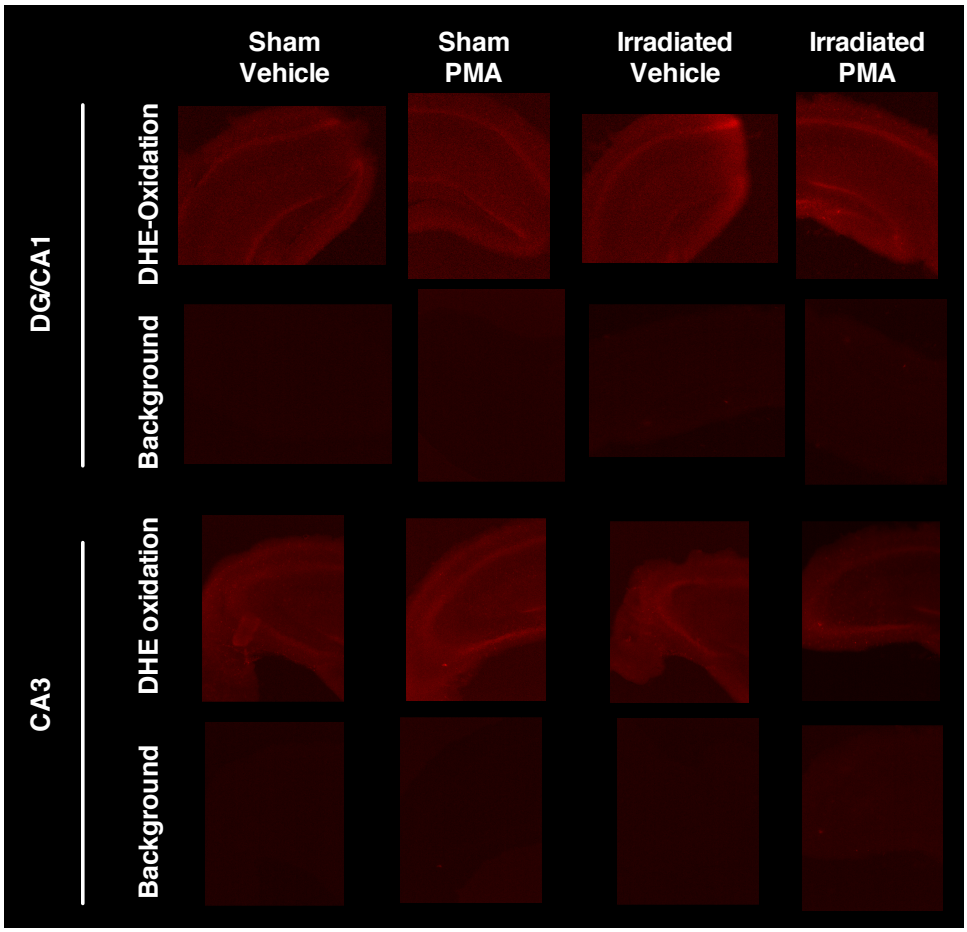

Figure 26. Representative images of DHE-incubated slices from sham-irradiated and ^{56}Fe-irradiated apoE4 mice. Background images were used to subtract the autofluorescence from each corresponding DHE-treated slice. Slices from irradiated mice had lower levels of DHE oxidation compared to slices from sham-irradiated mice.

Table 3. Summarized experimental results of chapters 2 and 3.

				^{137}Cs		^{56}Fe
Assay	**Within Assay**	**Index Measure**	**measure**	**apoE2**	**apoE4**	**apoE**
Weight		Health	g	NE	NE	NE
OF		Anxiety	%Time center	NE	#↑	NE
OF		Exploratory Locomotor Activity	cm	+↑		NE
LD		Anxiety	%Time light	+↑		NE
LD		Exploratory Locomotor Activity	cm	NE	#↓	NE
ZM		Anxiety	%Time open	NE	#↑	*↓
ZM		Exploratory Locomotor Activity	cm	NE	#↓	*↓
PM		Anxiety	%Time open	*↑	#↑	NE
PM		Exploratory Locomotor Activity	cm	+↓	#↑	NE
WM	Thigmo-taxis	Anxiety Task learning	%Time periphery	NE	NE	NE
WM	Velocity	Locomotor	cm/s	NE	NE	NE
WM	Visible LC	Sensory/Motivation/ Task Learning	cumulative distance (cm)	NE	NE	NE
WM	Hidden LC	Spatial Acquisition	cumulative distance (cm)	NE	#↓	NE
WM	Probes (avg)	Spatial memory retention	cumulative distance (cm)	+↓	*↑	NE
CF	Baseline	Anxiety	Immobility	NE	NE	NE
CF	Contextual	Contextual Memory	Immobility	NE	#↑	*↑
CF	Cued	Cued Response Memory	Immobility	*↑[1]	NE	NE
DHE	Baseline	DHE oxidation	Mean pixel intensity	*↑	NE	*↓
DHE	PMA-stimulated	DHE oxidation	Mean pixel intensity	*↓	*↑	*↓

The direction of the arrow indicates an effect of genotype (#), ALA (+) or irradiation (*) on the index measure. NE = no effect. Arrows in the middle of a cell indicate an effect of ALA independent of genotype. [1]Irradiated mice on ALA-supplementation exhibited higher freezing levels than mice on regular diet. Results from the novel location and novel object recognition tests are not included because none of the groups showed novel location or novel object recognition.

Discussion

The purpose of this study was to determine whether the effects of ^{56}Fe irradiation on the cognitive function and hippocampal ROS levels of apoE4 mice are similar to those of ^{137}Cs-irradiation. There were several different effects of ^{56}Fe irradiation on the cognitive function and ROS generation of apoE4 mice compared to ^{137}Cs irradiation. Data from the two studies are summarized in Table 3.

In the current ^{56}Fe irradiation study, we again observed a beneficial effect of irradiation, however it was observed in a different type of memory. In the previous study in chapter 2, ^{137}Cs irradiation enhanced spatial memory retention of apoE4 mice in the water maze test, but not contextual memory in the fear conditioning test. Paradoxically, in the current study, ^{56}Fe irradiation enhanced contextual fear conditioning but not spatial memory retention in the water maze. The fact that similar enhancements in cued fear conditioning were not observed following ^{56}Fe irradiation suggests that the enhancement in contextual fear conditioning was specific to changes in hippocampal function. Thus, ^{137}Cs- and ^{56}Fe irradiation appear to have different effects on spatial memory retention in the water maze and contextual memory in the conditioned fear tests. Nonetheless, both types of memories in the different tasks benefited from the different forms of irradiation.

The dissociation between spatial and contextual memory observed between the ^{137}Cs and ^{56}Fe irradiation studies might be related to the relationship between

the hippocampus and other brain regions that can influence performance on hippocampal-dependent tasks (Silva et al. 1998). White and McDonald provided one of the first examples of how hippocampal-spatial learning might influence associative learning (White and McDonald 1993; McDonald and White 1995). Their studies suggest that the hippocampus suppresses amygdala-dependent associative learning, specifically, cued response learning. This conclusion was based on the observation that pre-exposure to a radial arm maze attenuated conditioned cued preference (CCP) in rats when compared to non pre-exposed rats or rats exposed to a different environment. Because the hippocampus is involved in spatial acquisition of an environment, the authors concluded that the hippocampus inhibited amygdala-dependent acquisition or expression of CCP. Later, other studies showed that post-training but not pre-training hippocampal lesions impaired contextual fear conditioning (Frankland et al. 1998; Wiltgen et al. 2006). The data from these studies suggest that an intact hippocampus inhibits non-hippocampal systems that could support contextual memory.

In agreement with these reports, and particularly germane to the current study, previous studies show a dissociation between spatial memory in the water maze and contextual learning in fear conditioning (Logue et al. 1997; Silva et al. 1997; Cho et al. 1998; Burwell et al. 2004). For instance, in these studies, impairments were observed in spatial memory in the water maze but not contextual memory in fear conditioning and vice versa. Although the findings in this dissertation do not show the exact same dissociation, the trend was similar in

that enhancements were observed for spatial but not contextual memory following ^{137}Cs irradiation and vice versa for ^{56}Fe irradiation. In other words, the two types of memories were always dissociated. Therefore, this observation might speak to the proposed inhibitory role of the hippocampus on non-hippocampal systems that might contribute to contextual memory. It is possible that the enhanced hippocampal function observed in the ^{137}Cs irradiation study could have suppressed underlying enhancements in contextual learning. While this possibility is purely speculative, it is also supported by the fact that when enhancements in contextual memory were observed in the ^{56}Fe irradiation study, enhancements in spatial memory were no longer present. However, there is a caveat to the notion that competitive processes may have been responsible for the lack of enhanced contextual memory in the presence of enhanced spatial memory. The competitive relationship proposed by the findings of the aforementioned studies suggests that non-hippocampal systems do not contribute to contextual memory when the hippocampus is intact (Silva et al. 1998). The question then becomes: if non-hippocampal-systems do not significantly contribute to contextual memory while the hippocampus is fully functional, why would inhibition of such systems influence contextual memory? In other words, if they were not involved in contextual memory, why would inhibiting them affect contextual memory? Hence, it is not clear that the different effects of the two forms of irradiation on spatial and contextual memory are due

to competitive processes between the hippocampus and other regions that can subserve contextual memory.

An alternative and more simplified explanation for the dissociation between the two forms of irradiation on spatial and contextual memory might be related to hippocampal changes in PMA-induction of ROS. In the previous study, ^{137}Cs irradiation-induced enhancements in PMA-induction of ROS were associated with enhancements in spatial memory retention in the water maze. In contrast, the current study shows that reductions in PMA-induced ROS generation following ^{56}Fe irradiation were associated with enhanced contextual memory in fear conditioning. Collectively, these data open up the possibility that some forms of hippocampal-dependent memory might benefit from enhancements in stimulus-induced ROS generation, while other forms of hippocampal-dependent memory might be better served by a blunted stimulus-induction of ROS generation.

Yet a third potential explanation for the different effects of the two forms of irradiation might be related to early life stress associated with shipping. For example, it is possible that early life shipping in ^{56}Fe-irradiated mice could have attenuated radiation-induced enhancements in spatial memory retention in the water maze. Future studies will involve ^{137}Cs irradiations at BNL in order to better compare the effects of the two forms of irradiation on cognition and ROS levels.

A remarkable and consistent finding between the two studies is that enhancements in spatial memory in the water maze were only observed when PMA-induction of ROS was enhanced. In contrast to ^{137}Cs-irradiated apoE4

mice, neither ^{137}Cs-irraidated apoE2 nor ^{56}Fe-irradiated apoE4 mice showed enhancements in PMA-induction of ROS and neither showed enhancements in spatial memory retention in the water maze. Taken together, these data suggest that enhancements in hippocampal-dependent spatial memory retention in the water maze depend on hippocampal enhancements in ROS-induction.

The effects of irradiation on anxiety-like behaviors were different between the two sources of irradiation. In the previous study, ^{137}Cs irradiation-induced increases in anxiety levels of apoE4 mice were observed in the elevated plus maze. In contrast, in this study, ^{56}Fe irradiation induced reductions in measures of anxiety were observed in the elevated zero maze. Although not significant, a trend for ^{56}Fe irradiation induced reductions in anxiety was also observed in the elevated plus maze. The ^{56}Fe irradiation induced reduction of anxiety levels of apoE4 mice in the elevated zero maze is consistent with our previous study of older apoE4 female mice (approximately 15 month-old) (Villasana et al. 2011). However, there are some notable differences between the two studies. In the study involving older mice, the ^{56}Fe irradiation-induced decreases in anxiety measures were in relation to apoE3 mice. Specifically, in sham-irradiated mice, apoE4 mice showed higher levels of anxiety than apoE3 mice. ^{56}Fe irradiation eliminated the genotype difference in anxiety levels by decreasing that of apoE4 while increasing that of apoE3 mice. In the current study we show that the irradiation-induced reduction in anxiety levels of apoE4 mice occur in relation to sham-irradiated apoE4 mice.

In the current study we also show that ^{56}Fe irradiation induced reductions in anxiety levels of apoE4 mice was associated with reductions in ROS levels. This finding is consistent with the idea that ROS are adversely involved in anxiety and that attenuation of ROS can ameliorate anxiety levels (Bouayed et al. 2009; Zafir et al. 2009; Novio et al. 2011). Therefore, the effects of ^{56}Fe irradiation on measures of anxiety described in the current study provide further support for targeting ROS for anxiety-related disorders (Rammal et al. 2008; Bouayed et al. 2009). Whether irradiation-induced reductions in anxiety levels are limited to apoE4 has yet to be comprehensively determined.

In conclusion, the data presented in this study do not support the hypothesis that ^{56}Fe irradiation impairs the cognitive function of apoE4 mice. Instead, it suggests that ^{56}Fe irradiation enhances contextual memory in the fear conditioning task but has no effect on spatial memory retention in the water maze task. As with the ^{137}Cs irradiation study, these data are too preliminary to suggest a clinical relevance for treatment of cognitive impairments.

4

General Discussion

Major findings of the dissertation:

Several effects of irradiation were observed on the behavior and ROS levels of apoE2 and apoE4 female mice. There were 3 major findings that addressed the overarching goals and hypothesis of this dissertation. Potential mechanisms to explain the major findings are described in a subsequent section of this chapter.

The first major finding of this dissertation shows that the effects of cranial ^{137}Cs irradiation on hippocampal-dependent spatial memory retention in the water maze are apoE isoform-dependent. However, these effects were not as hypothesized. ApoE4 mice did not show cognitive impairments following irradiation, but instead showed an enhancement in spatial memory retention in the water maze probe trials. This enhancement was specific to spatial memory retention in the water maze as no changes in contextual memory in the conditioned fear task were observed. In contrast to apoE4 mice, apoE2 mice did not show enhancements in spatial memory retention in the water maze following irradiation. These data reject the hypothesis that ^{137}Cs irradiation impairs the cognitive function of apoE4 female.

The second major finding was that in contrast to ^{137}Cs irradiation, ^{56}Fe irradiation in apoE4 mice did not improve spatial memory retention in the water maze but, instead, improved contextual memory in fear conditioning. Thus, it appeared that the two forms of irradiation enhance different types of hippocampal-dependent memory in apoE4 female mice. These findings reject

the hypothesis that ^{56}Fe irradiation impairs the cognitive function of apoE4 female mice.

The third major finding was that in apoE4 mice, enhancements in PMA-induction of ROS generation were associated with enhancements in spatial memory retention in the water maze but not contextual memory in fear conditioning. Such enhancements were not observed in ^{137}Cs-irradiated apoE2 mice, which also did not show enhancements in spatial memory. Thus, enhancements in spatial memory were only seen in instances where it was accompanied by enhancements in PMA-induction of ROS. In contrast to my hypothesis, irradiation did not increase ROS levels in apoE4 mice.

Contrasts to radiation literature:

The findings reported in this dissertation are in stark contrast to most of the radiation literature on several different levels. First, while not all the literature on the effects of irradiation on the cognitive function of rodents reports irradiation-induced cognitive impairments, the vast majority of it does (Roman and Sperduto 1995; Shukitt-Hale et al. 2000; Abayomi 2002; Shukitt-Hale et al. 2003; Raber et al. 2004; Byrne 2005; Rabin et al. 2005a; Sarkissian 2005; Saxe et al. 2006; Acevedo et al. 2008a; Acevedo et al. 2008c; Wojtowicz et al. 2008; Villasana et al. 2010; Villasana et al. 2011). In the studies described in this dissertation, neither ^{137}Cs-nor ^{56}Fe irradiation induced deficits in spatial memory retention in the water maze or in contextual or cued fear conditioning in either apoE2 or

apoE4 female mice. Previously, we showed that ^{56}Fe irradiation is associated with impairments in contextual fear conditioning in C57Bl/6J female mice (Villasana et al. 2010). A similar contextual fear conditioning paradigm was used in that study, suggesting that the effects of ^{56}Fe irradiation on the contextual fear conditioning of female mice are critically influenced by the expression of human apoE.

Second, not only were irradiation-induced deficits in cognitive function not observed in this dissertation, instead enhancements in spatial memory retention in the water maze and in contextual fear conditioning tasks were observed in apoE4 mice following ^{137}Cs-and ^{56}Fe irradiation, respectively. Although a few studies have shown irradiation-induced enhancements in cognitive function following gamma- (Arnold and Blair 1956; Harlow and Moon 1956; Saxe et al. 2007) and HZE-irradiation (Raber et al. 2011), the majority of radiation studies instead describe irradiation-induced cogntive deficits.

The third finding that is in contrast to the majority of the literature is that an enhancement on a hippocampal-dependent task was associated with an increase in the generation of ROS. Although it is becoming increasingly accepted that ROS play a critical role in learning and memory (Knapp and Klann 2002), to the best of my knowledge, there are no reports suggesting that more ROS such as superoxide results in learning and memory enhancements.

The third and most provocative findings and implications of this dissertation were that apoE4 mice benefited from irradiation. By far, the radiation-induced

enhancements in spatial and contextual memory of apoE4 mice were the most surprising. As previously discussed, apoE4 is associated in neurodegenerative diseases such as AD (Farrer et al. 1997) and with poor recovery following neurotrauma (Nicoll et al. 1995; Kutner et al. 2000). However, the current findings suggest that apoE4 might be protective against the effects of irradiation. Evolutionary advantages of apoE4 have been proposed (Jofre-Monseny et al. 2008). This is based on the observation that apoE4 can provide resistance to the effects of infectious diseases that are commonly present in under-industrialized societies. These diseases include Giardia (Oria et al. 2007), diarrhea (Oria et al. 2005), and liver damage by Hepatitis C (Wozniak et al. 2002). However, there are no reports that apoE4 confers protection against the effects of irradiation. On the contrary, a study assessing the cognitive function of individuals who had received radiotherapy for low grade gliomas, indicates that apoE4 might be a risk factor for developing radiation-induced cognitive impairments (Correa et al. 2007). The study however had a very low number of apoE4 carriers, included individuals who had also received chemotherapy, and was only able to show a trend in lower non-verbal scores of ε4 carriers compared to non-ε4 carriers.

Potential Mechanisms:

The role of ROS

Why apoE2 and apoE4 mice responded differently to irradiation in terms of spatial memory and PMA-induction of ROS requires a more in depth analysis than what is provided in this dissertation. However, some hypotheses about potential mechanisms to explain the different apoE isoform-dependent responses to irradiation can be made based on the findings. First, differences in the PMA-induction of ROS, as shown in (Figure 17), might be related to changes in the baseline (vehicle treated) levels of ROS. Changes in baseline levels of ROS could in turn affect subsequent responses to ROS stimulation via adaptive mechanisms (Trosko 1998; Williams and Iatropoulos 2002). If so, this would imply that increases in baseline ROS levels of irradiated apoE2 mice changed the threshold for PMA-induction. Indeed, this is what is shown in Figures 19A-B. But why irradiation differentially changed baseline levels of ROS in apoE2 and apoE4 mice still remains a question. The answer might have to do with the background levels of ROS prior to irradiation.

As noted in the main introduction, the apoE2-isoform is a more effective antioxidant than the apoE4-isoform. This difference is thought to explain the greater ROS (Ihara et al. 2000; Lauderback et al. 2002; Dafnis et al. 2010) and oxidative stress levels that are associated with the apoE4 isoform (Pedersen et al. 2000; Butterfield et al. 2002; Jofre-Monseny et al. 2008). The higher background ROS levels in apoE4 mice might render them with a higher threshold

level for changes in ROS or oxidative stress induced by irradiation. Resistance to irradiation-induced oxidative damage was previously observed in EC-SOD$^{-/-}$ mice (Raber et al. 2011). It would make sense that the adaptive mechanisms proposed for apoE4 mice would not be recruited because there would be no changes in baseline levels of ROS to evoke adaptive responses. Thus, lack of baseline changes in ROS would leave the threshold for PMA-induction of ROS in apoE4 mice unchanged. This proposed mechanism is based on the DHE oxidation studies and are purely speculative in order to propose a mechanism for the apoE isoform-dependent responses to PMA-induction of ROS. Future studies using PMA dose response curves can begin assessing whether irradiation indeed changes the threshold for ROS induction.

Similar to ^{137}Cs-irradiated apoE2 mice, ^{56}Fe-irradiated apoE4 mice did not show enhancements in spatial memory in the water maze (Figure 23D), nor did they show hippocampal-enhancements in PMA-induction of ROS (Figure 25**)**. Thus, the data from the two studies suggest that enhancements in PMA-induction of ROS might be responsible for spatial memory enhancements in the water maze. However, inhibition of PMA-induction of ROS observed in ^{137}Cs-irradiated apoE2 and ^{56}Fe-irradiated apoE4 mice was not necessarily associated with spatial memory impairments. This suggests that while an increase in PMA-induction of ROS can be beneficial, a decrease does not result in memory impairments. Future studies using an effective antioxidant regimen will provide additional insight into this phenomenon.

Neurogenesis

Previous data from preliminary studies suggest that neurogenesis cannot explain the apoE isoform-dependent effects of ^{137}Cs irradiation on spatial memory (Appendix Figure 33). Although doublecortin-positive cell counts were reduced following irradiation, the reduction occurred in an apoE isoform-independent manner. However, these data present an acute effect of irradiation on neurogenesis, and it is possible that apoE isoform differences might be revealed at later time points. For this reason, we chose to assess the effects of ^{56}Fe irradiation on neurogenesis 3-4 months after irradiation. This was also critical because access to BNL resources is limited and therefore needed to maximize the chances of detecting a genotype effect. However, it appears that even at later time points, apoE isoform does not influence reductions in doublecortin-positive immature neurons following ^{56}Fe irradiation (Appendix Figure 34). More specific to the ^{56}Fe irradiation study in chapter 3, is the observation that irradiation decreased doublecortin-positive cells in apoE4 mice **(**Appendix Figure 34 and 35**)**. These data however do not demonstrate that neurogenesis is not involved in hippocampal function, just that it is not required for the paradigms used in these studies to determine spatial and contextual memory. For instance, it is still possible that increases in ROS could compensate for reductions in neurogenesis. Moreover, the effects of irradiation were focused on immature neurons and it is possible that apoE isoform differences in Ki-67, a marker of precursor cells might be observed following irradiation. Brain tissues from acute

^{56}Fe irradiation studies in apoE2, apoE3, and apoE4 mice have been collected and will be assessed for doublecortin- and Ki-67 positive cells to determine whether earlier time points show apoE isoform-dependent effects.

Limitations and future directions:

As with all studies, some limitations of the experiments should be acknowledged. The rest of this discussion will focus on describing such limitations and suggestions for future experiments.

Because estrogen might modulate fear conditioning in contextual and cued fear conditioning (Jasnow et al. 2006), the potential effects of the estrous cycle on the results presented in this dissertation cannot be ruled out. Although it is highly unlikely that all of the females from each group were within similar phases at the time of testing, this possibility needs to be considered. Future experiments should assess whether radiation exposure changes the estrous cycle in female mice and whether such potential changes are modulated by apoE genotype.

Along the lines of the unexpected effects of ALA, another limitation is that we were unable to conclusively determine whether attenuation of ROS prevents the effects of irradiation on spatial memory retention in the water maze and whether it also attenuates irradiation-induced changes in ROS generation. Future experiments are needed to help determine an optimal dose or a better regimen for ALA-treatment. Because the apoE isoforms have different antioxidant properties, it would be prudent to also determine and confirm an optimal

antioxidant regimen for each genotype. Obviously, given the complexity of the goals of this dissertation, this could not have been easily assessed, as there were many different conditions. Given the findings of ALA however, this will be an important step in future studies.

One limitation of the DHE experiments was that superoxide was not distinguished from hydrogen peroxide. Such a distinction would only be possible using a second emission filter (Robinson et al. 2006). As mentioned in the general introduction, DHE can be oxidized into dihydroxyethidium and ethidium, which correspond to the production of superoxide and hydrogen peroxide, respectively. This is an important distinction because superoxide and hydrogen peroxide might have different effects on cognitive function. For instance, it has been demonstrated that high or chronic levels of hydrogen peroxide can impair synaptic function (Auerbach and Segal 1997; Kamsler and Segal 2003b; Kamsler and Segal 2003a). Future experiments using a specific filter to measure superoxide without a potential contribution of hydrogen peroxide would help determine whether the observed changes in DHE oxidation levels following irradiation are due to changes in superoxide levels.

Another limitation of the current study is that analyses of changes in ROS were focused only on the hippocampus. Because the amygdala is involved in conditioned fear (Phillips and LeDoux 1992; Helmstetter and Bellgowan 1994), and because it can modulate performance in the water maze (Packard et al. 1994; Packard and Teather 1998), initial experiments were conducted to assess

whether the amygdala could be imaged in the DHE experiments. Imaging the amygdala with DHE oxidation was not possible because unlike the distinct architecture of the hippocampus that allows it to be easily identified, the different amygdala regions could not be easily located. Therefore, conclusions about how changes in ROS levels or PMA-induction of ROS within the amygdala might have affected cognition could not be made.

In the water maze, the assessment of memory for the platform location likely involved short-term memory and not long-term memory (Vorhees and Williams 2006). The probe test was assessed one hour after the last hidden platform trial for each mouse. Although the spatial aspect of the trial is hippocampus-dependent, the memory itself is likely independent of consolidation, a hippocampal process typically requiring a longer period of time for protein synthesis (Abraham and Williams 2008). There are data to suggest that short-term and long-term memory processes can be differentially affected by hippocampal lesions (Sanderson et al. 2009). Therefore, it is of high interest to assess whether radiation-induced changes in spatial memory are also observed in water maze when the probe trial is assessed after a longer retention interval (e.g. 24 hours). For instance, we previously showed that ^{56}Fe irradiation does not enhance spatial memory of apoE4 female mice when assessed 24-hours after the last training session (Villasana et al. 2011). However, these were older mice (15 months) that were tested approximately 13 months after irradiation.

Navigating through the water maze involves different brain regions, which can act to complement each other, or in some cases, compete with each other (Lee et al. 2008). For example, the striatum is involved in stimulus-response learning (Packard and Knowlton 2002). Experiments conducted by Lee et al. (2008) demonstrate that lesions to the striatum cause deficits in cued learning and enhancements in spatial learning; whereas, lesions to the dorsal hippocampus cause deficits in spatial learning and enhancements in cued learning. It is of interest to determine whether this relationship can explain radiation-induced enhancements in spatial memory retention in the water maze. The interest for this comes from data suggesting that irradiation reduces potassium-evoked release of dopamine within the striatum (Joseph et al. 1992). If apoE4 mice were to show greater striatal susceptibility to irradiation, it would be reasonable to predict that they might perform better on the spatial component of the task compared to apoE2 mice. To assess whether the striatal-hippocampal relationship plays a role in spatial memory following irradiation, a future experiment could involve comparing cued response to spatial learning in the water maze. Briefly, cued response learning in the water maze involves constantly changing the location of the platform between trials but in such a way that it is always paired with a constant cue. An experiment could consist of testing half of the mice from each genotype (apoE2 and apoE4) and ^{137}Cs irradiation treatment group on a cued version and the other half of the experimental group on a spatial version. Because ^{137}Cs-irradiated apoE4 mice

tested in this dissertation showed enhanced spatial memory retention in the water maze, I would predict enhanced performance in the spatial version of the water maze as compared to genotype-matched sham-irradiated mice. However, I would expect them to perform worse in the cued version. Because in this dissertation, the spatial memory retention of apoE2 was not affected by ^{137}Cs irradiation, I would not expect any changes either the cued or spatial version of the proposed water maze task.

Although our behavioral battery has the advantage of assessing several aspects of cognitive function, we cannot rule out the possibility of potential test order effects. During the behavioral battery, mice were exposed to various experiences such as handling, novel environments and stressful stimuli. These can influence behaviors on subsequent tests (Bouwknecht et al. 2004). Even though all mice were subjected to the same treatment, different types of mice might response differently to such experiences. Future experiments should be conducted to compare performance of irradiated mice given a single test to that of irradiated mice given a battery of tests.

As stated in chapters 2 and 3, while it is exciting to find that irradiation improved hippocampal function of a genotype that is associated with neurological diseases and poor recovery following brain injury, these studies are too preliminary to suggest clinical relevance. First and foremost, the limitations of these studies need to be addressed to assess whether additional experiments corroborate the current findings. Second, additional cognitive measures to

assess different types of learning and memory should be considered. For example, in a previous study (Raber et al. 2011), ^{137}Cs irradiation-induced enhancements in spatial and contextual memory were associated with deficits in novel object recognition. This suggests that while irradiation can enhance some types of memory, it might come at the expense of other forms of memory. Because none of the mice in the current studies showed novel object recognition, it was not possible to determine whether a similar impairment in novel object recognition in apoE4 mice was associated with enhanced spatial memory in the water maze.

Third, the radiation-induced enhancements in memory presented in this dissertation cannot be easily generalized and are present under very specific conditions. As such, it is possible that individual and experimental differences such as age or test paradigm can result in different cognitive outcomes. For example, in a previous study, we did not find that a similar dose of ^{56}Fe irradiation at 2-months of age enhanced contextual memory of apoE4 female mice when tested at an older age (15-months) (Villasana et al. 2011). These findings are in accord with studies suggesting that the dual role of ROS on cognitive function is age-dependent (Serrano and Klann 2004; Hu et al. 2007).

Finally, although there was not enough evidence in the current studies to conclude that the effects of irradiation on anxiety were responsible for the observed enhancements in spatial or contextual memory, the fact remains that ^{137}Cs radiation-induced changes on measures of anxiety levels were observed in

the plus maze. Although these measures were not correlated with the water maze probe trial performance of apoE4 mice, it is interesting that in the second study, ^{56}Fe irradiation was not associated with increased levels of anxiety and enhancements in spatial memory were no longer observed. Thus, one interpretation is that irradiation-induced increases in anxiety, specifically in apoE4 mice, might improve spatial memory retention in the water maze.

Conclusions:

In general, it is accepted that there is a fine balance in ROS required for learning and memory and that deviations from it can lead to memory impairments (Halliwell 1992; Thiels et al. 2000; Knapp and Klann 2002; Kishida et al. 2006a). Conversely, the findings in this dissertation suggest that having more ROS generation can be beneficial. Even though this appears to be the case, the data in this dissertation clearly show that irradiation-induced enhancements in memory occur under very specific conditions. Because this is an important conclusion of this thesis, the next few paragraphs in this section will describe these conditions.

One condition has to do with the type of memory and test. The results suggest that radiation-induced enhancements in PMA-induction of ROS generation are specific to spatial memory retention in the water maze. ApoE2 ALA-supplemented mice also showed ^{137}Cs irradiation-induced enhancements in cued fear conditioning; however, because DHE oxidation was not examined in

the amygdala, a relationship between ROS and amygdala-dependent cued fear conditioning could not be concluded and remains of future interest.

A second specific condition for radiation-induced memory enhancements appear to be related to apoE isoform. ApoE4 but not apoE2 mice benefited from ^{137}Cs irradiation on spatial memory and this was associated with apoE isoform-specific changes in ROS generation. Therefore, the genetic background appears to be a condition for radiation-induced enhancements in memory.

The third condition has to do with generation of ROS. The pharmacological component of the DHE assay shows that it is not a matter of having more ROS in general that is associated with enhancements in spatial memory in the water maze, but rather the ability to generate ROS upon stimulation. Thus, the results from this dissertation suggest that the conditions for which irradiation can result in memory enhancements might depend of the type of memory, genetic or ROS background levels, and changes in induction of ROS generation.

In conclusion, the studies presented in this dissertation suggest that irradiation does not impair hippocampal-dependent spatial or contextual memory but rather can enhance these types of memory. Such enhancements however appear to be very specific to the source of irradiation, apoE isoform, and type of memory. Furthermore, enhancements in the ability of the hippocampus to respond to a ROS-inducing stimulus might explain enhancements in water maze spatial memory.

Finally, the importance of distinguishing between general increases in ROS and stimulus induced increases in ROS cannot be overemphasized. The past two decades are filled with studies that convincingly demonstrate the importance of ROS in learning and memory (Klann 1998; Klann et al. 1998; Thiels et al. 2000; Kishida et al. 2005b; Hu et al. 2006; Kishida et al. 2006a; Hidalgo et al. 2007; Overeem et al. 2010). Yet, ROS are often used synonymously with oxidative stress, and studies often fail to make a distinction between the two. Furthermore, as reviewed by Massaad and Klann (2011), the role of ROS in cognitive function is very complex. The authors describe studies that suggest that differences in the type of reactive oxygen species, the type of antioxidant, and the age of individuals critically influence how ROS affects synaptic plasticity and cognition. Therefore, generalizations between ROS and oxidative stress can result in missed opportunities to better understand the role of ROS under both healthy and pathological conditions. Out of the scientific contributions made in this dissertation, the functional DHE assay might be the most beneficial because it demonstrates that the role of ROS on the effects of irradiation could have easily been dismissed or misinterpreted had the experiments only focused on general increases in ROS or on markers of oxidative stress.

Appendix

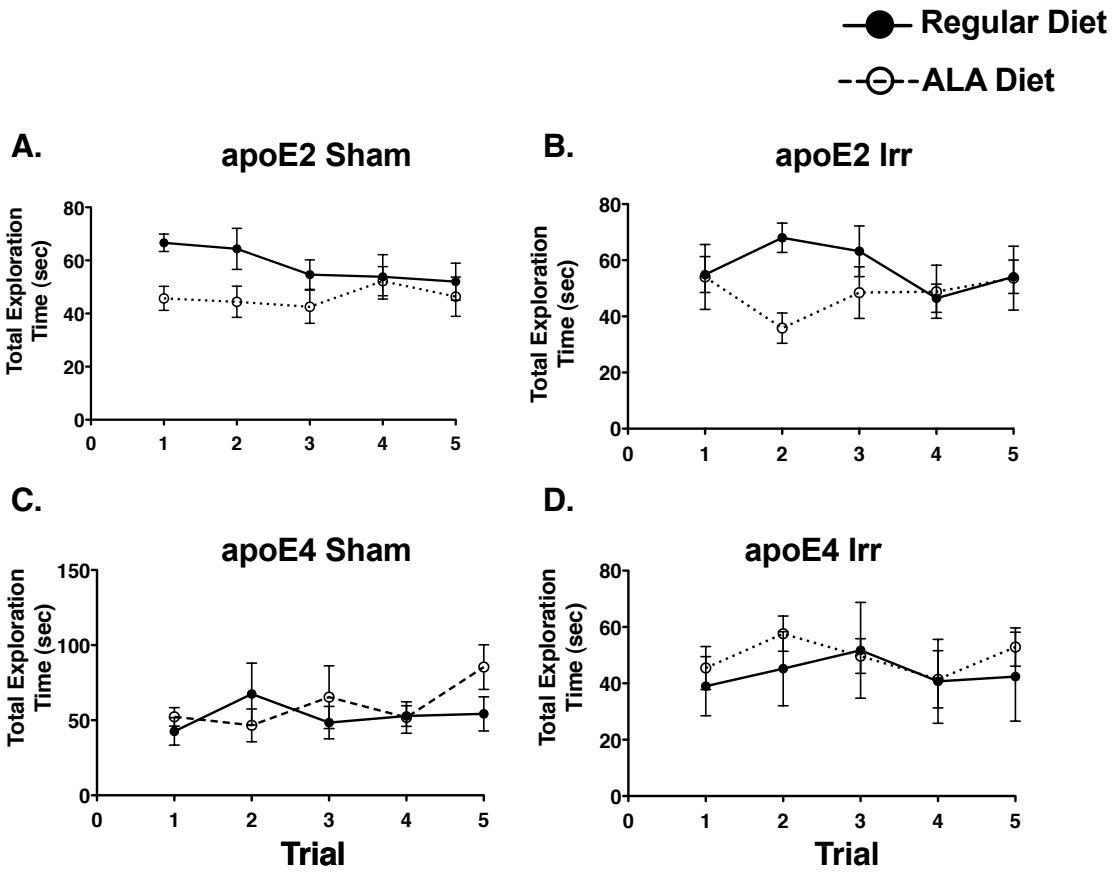

Figure 27. Total exploration time of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet during object familarization trials (1-3), the novel location (4) and novel object recognition (5) trials. All 3 objects were combined for each data point. There were no effects of genotype, irradiation treatment or diet on the total exploration time during the familiarization trails. Bars represent the group mean and $\pm$ SEM. N = 7-9 mice per genotype/irradiation treatment/diet.

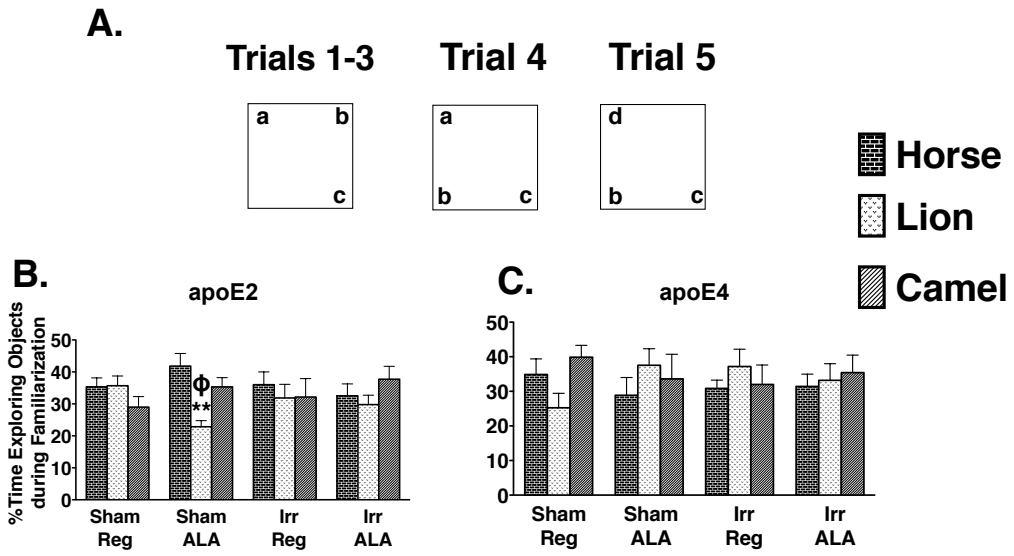

Figure 28. Object bias assessment of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on regular or ALA-supplemented diet. Illustration of the novel location and novel object recognition task paradigm (A). As described in the methods section, mice were allowed to explore 3 objects for 3 10-min consecutive trials. In the 4th trial, one of the objects (lion) was moved. In the 5th trial, a new object (cow) was introduced. Data for a potential object bias during the familiarization trials were assessed in apoE2 (B) and apoE4 (C) mice. ALA-supplemented sham-irradiated apoE2 mice showed an object bias during the familiarization trials ($\phi P < 0.05$ object preference in ALA-supplemented sham-irradiated apoE2 mice, B). There was an exploration bias towards the lion object (**$P < 0.01$, lion versus horse or camel, B). An object preference was not observed in any other group of mice. Bars represent the group mean and $\pm$ SEM. N = 7-9 mice per genotype/irradiation treatment/diet.

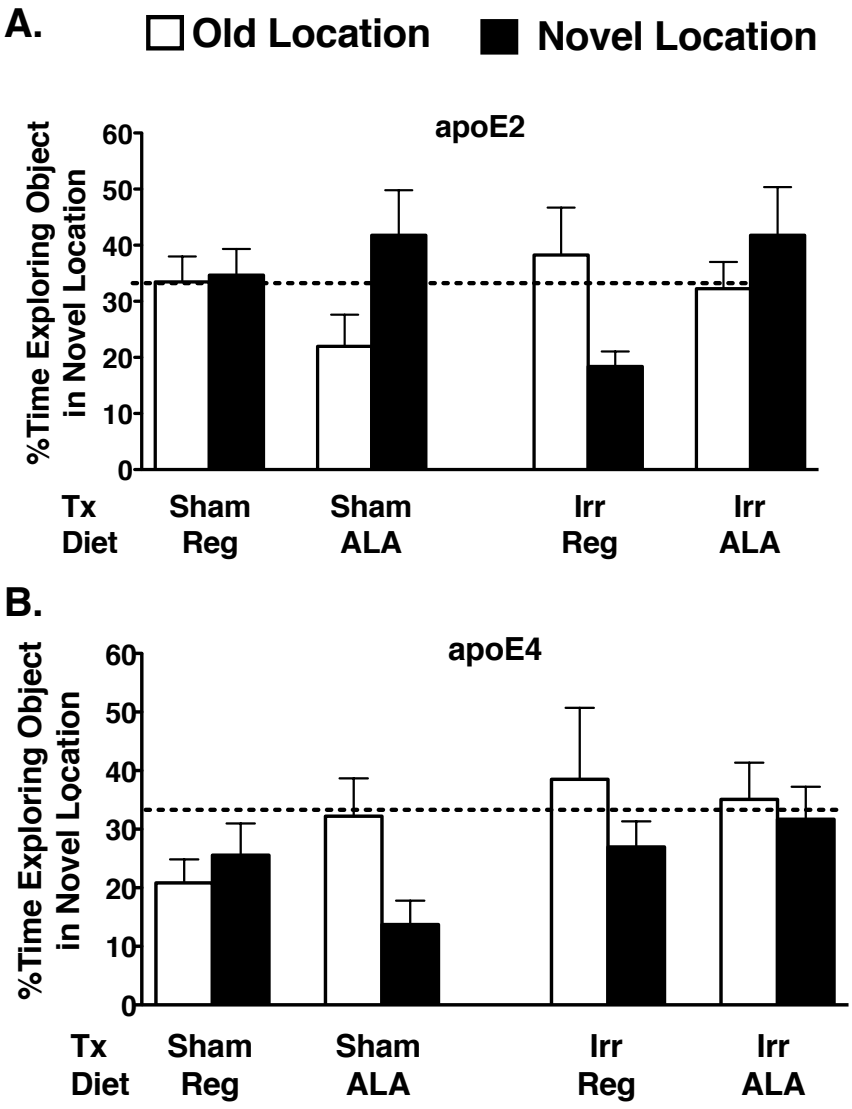

Figure 29. Performance of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet in the novel location recognition task. None of the groups of mice showed novel location recognition (33% is chance exploration as there were 3 objects and is represented by the dashed line). Bars represent the group mean and + SEM. N= 7-9 mice per genotype/irradiation treatment/diet.

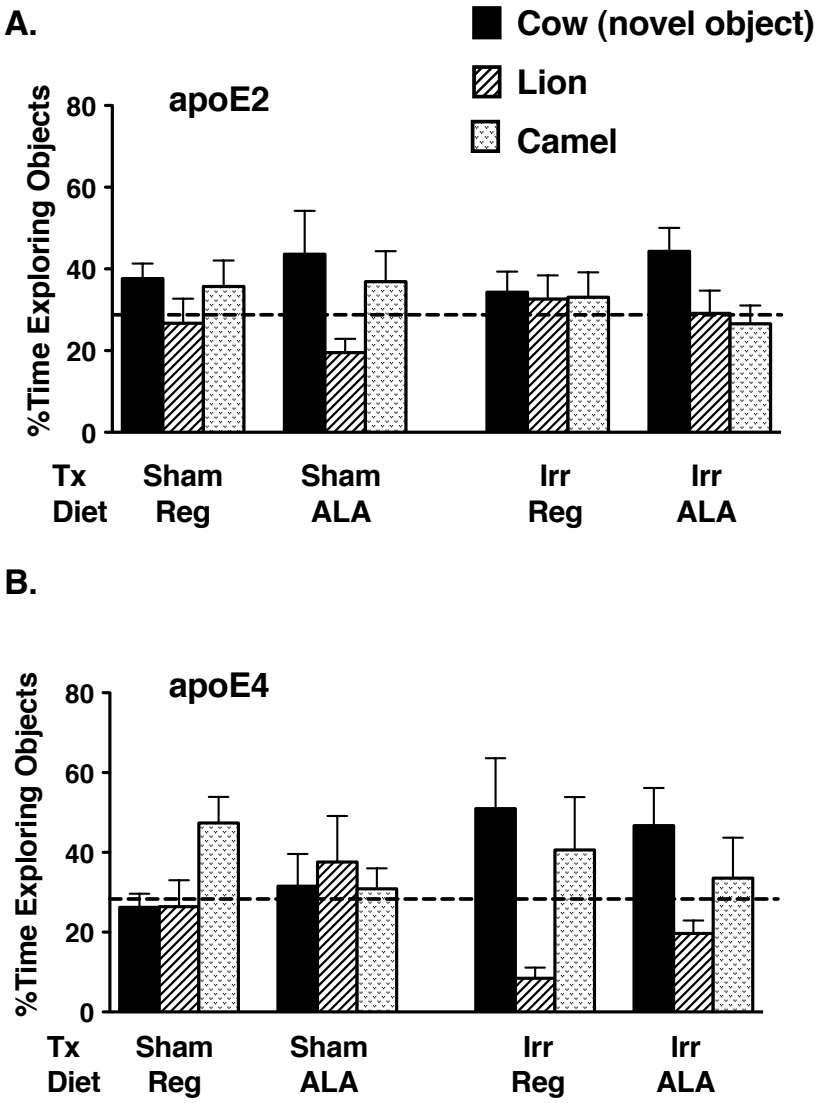

Figure 30. Performance of sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice on a regular or an ALA-supplemented diet in the novel object recognition task. None of the groups of mice showed novel location recognition (33% is chance exploration as there were 3 objects and is represented by the dashed line). Bars represent the group mean and $\pm$ SEM. N = 7-9 mice per genotype/irradiation treatment/diet.

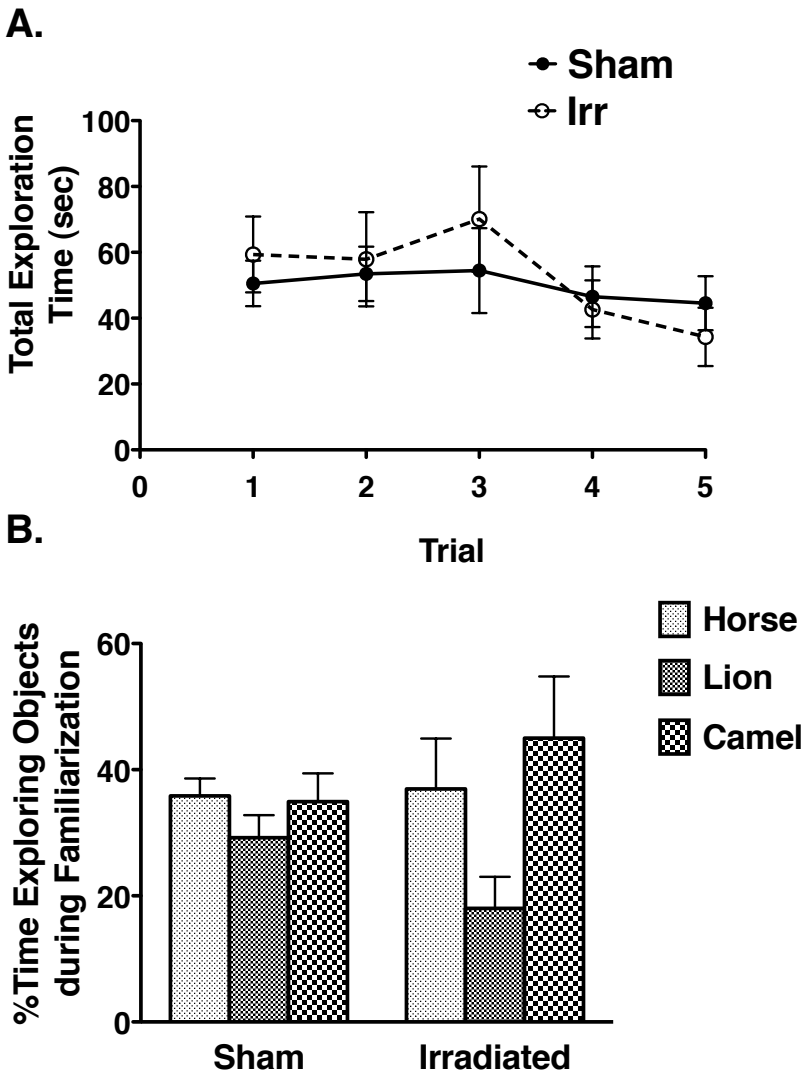

Figure 31. Total exploration and object bias assessment of sham-irradiated and ^{56}Fe-irradiated apoE4 mice during the novel location and novel object recognition tests. Panel A shows the total exploration during the familiarization trials (1-3), the novel location (4) and novel object recognition trials (5). All 3 objects are combined for each trial. Panel B shows the exploration times with each individual object during the familiarization trials (trials 1-3). Bars represent the group mean and $\pm$ SEM. N = 5-7 mice per group.

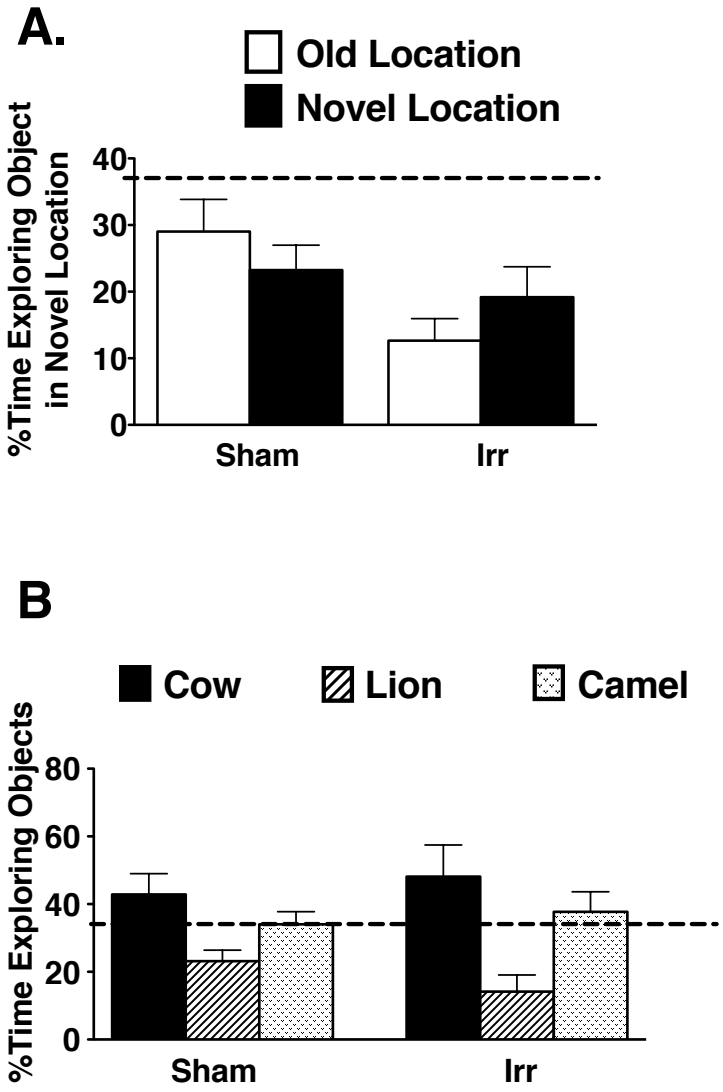

Figure 32. Performance of sham-irradiated and ^{56}Fe-irradiated apoE4 mice in the novel location and novel object recognition tests. Neither groups of mice displayed novel location (A) or novel object recognition (B). Dashed lines represent chance levels. Bars represent the group mean and $\pm$ SEM. N = 5-7 mice per group.

Table 4. DHE oxidation (pixel intensity) of hippocampal slices from sham-irradiated and ^{137}Cs-irradiated apoE2 and apoE4 mice with or without ALA diet supplementation[1].

Genotype	Tx	Diet	Drug	Area	4min	8min	12min	16min	20min
apoE2	Sham	Reg	PMA	1	1.31 ± 0.27	6.26 ± 1.06	10.78 ± 1.51	14.55 ± 1.80	17.34 ± 1.97
apoE2	Sham	Reg	PMA	2	1.28 ± 0.27	6.18 ± 1.06	10.86 ± 1.51	14.32 ± 1.80	16.72 ± 1.97
apoE2	Sham	Reg	PMA	3	1.31 ± 0.27	5.50 ± 1.06	9.51 ± 1.51	12.50 ± 1.80	14.84 ± 1.97
apoE2	Sham	Reg	PMA	4	1.06 ± 0.27	5.28 ± 1.06	8.91 ± 1.51	11.60 ± 1.80	13.49 ± 1.97
apoE2	Sham	Reg	PMA	5	1.28 ± 0.27	6.00 ± 1.06	10.73 ± 1.51	14.52 ± 1.80	17.74 ± 1.97
apoE2	Sham	Reg	Veh	1	0.80 ± 0.27	2.91 ± 1.06	5.62 ± 1.51	8.05 ± 1.80	9.91 ± 1.96
apoE2	Sham	Reg	Veh	2	0.84 ± 0.27	3.08 ± 1.06	5.92 ± 1.51	9.15 ± 1.80	10.96 ± 1.96
apoE2	Sham	Reg	Veh	3	0.84 ± 0.27	2.48 ± 1.06	5.25 ± 1.51	8.07 ± 1.80	9.91 ± 1.96
apoE2	Sham	Reg	Veh	4	0.83 ± 0.27	3.68 ± 1.06	6.75 ± 1.51	9.32 ± 1.80	10.88 ± 1.96
apoE2	Sham	Reg	Veh	5	0.72 ± 0.27	3.13 ± 1.06	6.15 ± 1.51	9.57 ± 1.80	11.48 ± 1.96
apoE2	Sham	ALA	PMA	1	0.93 ± 0.31	6.57 ± 1.23	11.50 ± 1.74	16.14 ± 2.08	18.10 ± 2.27
apoE2	Sham	ALA	PMA	2	0.97 ± 0.31	6.82 ± 1.23	12.19 ± 1.74	16.89 ± 2.08	19.83 ± 2.27
apoE2	Sham	ALA	PMA	3	1.01 ± 0.31	6.27 ± 1.23	10.89 ± 1.74	15.32 ± 2.08	17.86 ± 2.27
apoE2	Sham	ALA	PMA	4	1.00 ± 0.31	6.20 ± 1.23	10.69 ± 1.74	14.52 ± 2.08	16.93 ± 2.27
apoE2	Sham	ALA	PMA	5	1.03 ± 0.31	6.54 ± 1.23	11.89 ± 1.74	16.49 ± 2.08	19.46 ± 2.27
apoE2	Sham	ALA	Veh	1	1.14 ± 0.31	4.12 ± 1.23	6.99 ± 1.74	9.16 ± 2.08	10.54 ± 2.27
apoE2	Sham	ALA	Veh	2	1.07 ± 0.31	4.10 ± 1.23	7.66 ± 1.74	10.69 ± 2.08	12.81 ± 2.27
apoE2	Sham	ALA	Veh	3	1.05 ± 0.31	3.88 ± 1.23	7.25 ± 1.74	9.78 ± 2.08	11.71 ± 2.27
apoE2	Sham	ALA	Veh	4	1.01 ± 0.31	3.86 ± 1.23	7.18 ± 1.74	10.66 ± 2.08	12.70 ± 2.27
apoE2	Sham	ALA	Veh	5	1.14 ± 0.31	3.73 ± 1.23	6.85 ± 1.74	9.02 ± 2.08	10.81 ± 2.27
apoE2	Irr	Reg	PMA	1	1.34 ± 0.27	5.00 ± 1.06	8.72 ± 1.51	11.84 ± 1.80	14.33 ± 1.96
apoE2	Irr	Reg	PMA	2	1.04 ± 0.27	4.45 ± 1.06	8.12 ± 1.51	11.24 ± 1.80	13.38 ± 1.96
apoE2	Irr	Reg	PMA	3	1.04 ± 0.27	4.53 ± 1.06	8.17 ± 1.51	11.37 ± 1.80	13.73 ± 1.96
apoE2	Irr	Reg	PMA	4	1.01 ± 0.27	4.03 ± 1.06	7.12 ± 1.51	9.82 ± 1.80	11.91 ± 1.96
apoE2	Irr	Reg	PMA	5	0.98 ± 0.27	4.65 ± 1.06	8.60 ± 1.51	11.99 ± 1.80	14.68 ± 1.96
apoE2	Irr	Reg	Veh	1	1.25 ± 0.27	5.75 ± 1.06	9.43 ± 1.51	12.86 ± 1.80	15.20 ± 1.96
apoE2	Irr	Reg	Veh	2	1.25 ± 0.27	5.05 ± 1.06	8.55 ± 1.51	11.65 ± 1.80	14.10 ± 1.96
apoE2	Irr	Reg	Veh	3	1.22 ± 0.27	5.30 ± 1.06	8.83 ± 1.51	11.74 ± 1.80	14.35 ± 1.96
apoE2	Irr	Reg	Veh	4	1.15 ± 0.27	4.00 ± 1.06	6.68 ± 1.51	9.14 ± 1.80	11.02 ± 1.96
apoE2	Irr	Reg	Veh	5	2.50 ± 0.27	4.72 ± 1.06	8.93 ± 1.51	12.49 ± 1.80	15.22 ± 1.96
apoE2	Irr	ALA	PMA	1	1.60 ± 0.27	5.27 ± 1.06	8.99 ± 1.51	11.99 ± 1.80	14.43 ± 1.96
apoE2	Irr	ALA	PMA	2	1.47 ± 0.27	5.25 ± 1.06	9.11 ± 1.51	12.22 ± 1.80	14.69 ± 1.96
apoE2	Irr	ALA	PMA	3	1.64 ± 0.27	5.64 ± 1.06	9.91 ± 1.51	13.57 ± 1.80	16.42 ± 1.96
apoE2	Irr	ALA	PMA	4	1.57 ± 0.27	4.73 ± 1.06	7.69 ± 1.51	9.99 ± 1.80	11.94 ± 1.96
apoE2	Irr	ALA	PMA	5	1.91 ± 0.27	5.43 ± 1.06	8.96 ± 1.51	12.67 ± 1.80	15.19 ± 1.96
apoE2	Irr	ALA	Veh	1	0.91 ± 0.27	4.86 ± 1.06	8.01 ± 1.51	10.56 ± 1.80	12.25 ± 1.97
apoE2	Irr	ALA	Veh	2	0.90 ± 0.27	5.78 ± 1.06	10.18 ± 1.51	13.51 ± 1.80	15.57 ± 1.97
apoE2	Irr	ALA	Veh	3	0.89 ± 0.27	4.84 ± 1.06	9.24 ± 1.51	12.17 ± 1.80	13.90 ± 1.97
apoE2	Irr	ALA	Veh	4	0.95 ± 0.27	4.76 ± 1.06	9.18 ± 1.51	12.06 ± 1.80	13.95 ± 1.97
apoE2	Irr	ALA	Veh	5	1.11 ± 0.27	6.08 ± 1.06	12.11 ± 1.51	15.66 ± 1.80	17.97 ± 1.97

apoE4	Sham	Reg	Veh	1	0.73	± 0.27	3.73	± 1.06	7.38	± 1.51	10.44	± 1.80	12.40	± 1.96
apoE4	Sham	Reg	Veh	2	0.88	± 0.27	4.21	± 1.06	7.90	± 1.51	10.84	± 1.80	13.05	± 1.96
apoE4	Sham	Reg	Veh	3	0.72	± 0.27	3.68	± 1.06	6.98	± 1.51	9.82	± 1.80	11.47	± 1.96
apoE4	Sham	Reg	Veh	4	0.78	± 0.27	3.56	± 1.06	6.28	± 1.51	8.62	± 1.80	10.12	± 1.96
apoE4	Sham	Reg	Veh	5	1.20	± 0.27	5.81	± 1.06	11.10	± 1.51	15.52	± 1.80	18.37	± 1.96
apoE4	Sham	Reg	PMA	1	0.89	± 0.27	4.62	± 1.06	7.81	± 1.51	10.73	± 1.80	12.85	± 1.97
apoE4	Sham	Reg	PMA	2	0.94	± 0.27	4.40	± 1.06	7.59	± 1.51	11.16	± 1.80	13.60	± 1.97
apoE4	Sham	Reg	PMA	3	0.94	± 0.27	4.62	± 1.06	7.89	± 1.51	11.06	± 1.80	13.50	± 1.97
apoE4	Sham	Reg	PMA	4	0.99	± 0.27	5.42	± 1.06	8.21	± 1.51	11.56	± 1.80	13.85	± 1.97
apoE4	Sham	Reg	PMA	5	1.16	± 0.27	5.12	± 1.06	9.04	± 1.51	12.98	± 1.80	15.80	± 1.97
apoE4	Sham	ALA	Veh	1	0.85	± 0.27	3.10	± 1.06	6.18	± 1.51	9.41	± 1.80	10.77	± 1.96
apoE4	Sham	ALA	Veh	2	0.86	± 0.27	3.19	± 1.06	6.73	± 1.51	10.48	± 1.80	11.92	± 1.96
apoE4	Sham	ALA	Veh	3	0.71	± 0.27	3.06	± 1.06	5.68	± 1.51	8.66	± 1.80	10.07	± 1.96
apoE4	Sham	ALA	Veh	4	0.81	± 0.27	4.26	± 1.06	7.83	± 1.51	11.73	± 1.80	13.15	± 1.96
apoE4	Sham	ALA	Veh	5	0.83	± 0.27	3.81	± 1.06	7.86	± 1.51	11.11	± 1.80	13.47	± 1.96
apoE4	Sham	ALA	PMA	1	1.54	± 0.27	7.38	± 1.06	10.95	± 1.51	14.64	± 1.80	17.42	± 1.96
apoE4	Sham	ALA	PMA	2	1.47	± 0.27	5.58	± 1.06	9.20	± 1.51	12.24	± 1.80	15.00	± 1.96
apoE4	Sham	ALA	PMA	3	1.18	± 0.27	5.28	± 1.06	8.62	± 1.51	11.54	± 1.80	14.27	± 1.96
apoE4	Sham	ALA	PMA	4	1.47	± 0.27	5.21	± 1.06	8.40	± 1.51	11.59	± 1.80	14.05	± 1.96
apoE4	Sham	ALA	PMA	5	1.57	± 0.27	5.63	± 1.06	9.37	± 1.51	13.04	± 1.80	15.85	± 1.96
apoE4	Irr	Reg	Veh	1	0.77	± 0.31	3.59	± 1.23	6.22	± 1.74	8.74	± 2.08	10.78	± 2.27
apoE4	Irr	Reg	Veh	2	0.75	± 0.31	2.96	± 1.23	5.52	± 1.74	8.28	± 2.08	10.14	± 2.27
apoE4	Irr	Reg	Veh	3	0.76	± 0.31	3.09	± 1.23	5.19	± 1.74	7.38	± 2.08	9.44	± 2.27
apoE4	Irr	Reg	Veh	4	0.93	± 0.31	3.62	± 1.23	6.92	± 1.74	9.74	± 2.08	12.18	± 2.27
apoE4	Irr	Reg	Veh	5	0.90	± 0.31	3.66	± 1.23	5.82	± 1.74	9.71	± 2.08	12.44	± 2.27
apoE4	Irr	Reg	PMA	1	0.85	± 0.31	4.91	± 1.23	9.26	± 1.74	12.56	± 2.08	15.16	± 2.27
apoE4	Irr	Reg	PMA	2	0.92	± 0.31	5.84	± 1.23	10.65	± 1.74	14.18	± 2.08	16.62	± 2.27
apoE4	Irr	Reg	PMA	3	0.85	± 0.31	4.68	± 1.23	8.62	± 1.74	11.59	± 2.08	13.89	± 2.27
apoE4	Irr	Reg	PMA	4	1.02	± 0.31	5.58	± 1.23	8.78	± 1.74	12.08	± 2.08	14.37	± 2.27
apoE4	Irr	Reg	PMA	5	1.22	± 0.31	6.18	± 1.23	11.38	± 1.74	15.35	± 2.08	19.06	± 2.27
apoE4	Irr	ALA	Veh	1	0.88	± 0.27	4.04	± 1.07	7.57	± 1.51	10.50	± 1.81	12.02	± 1.98
apoE4	Irr	ALA	Veh	2	0.81	± 0.27	3.64	± 1.07	6.89	± 1.51	9.67	± 1.81	11.59	± 1.98
apoE4	Irr	ALA	Veh	3	0.75	± 0.27	3.29	± 1.07	6.32	± 1.51	8.52	± 1.81	10.24	± 1.98
apoE4	Irr	ALA	Veh	4	0.91	± 0.27	3.69	± 1.07	6.17	± 1.51	8.57	± 1.81	9.99	± 1.98
apoE4	Irr	ALA	Veh	5	0.79	± 0.27	3.49	± 1.07	7.24	± 1.51	10.32	± 1.81	12.34	± 1.98
apoE4	Irr	ALA	PMA	1	1.14	± 0.27	5.45	± 1.06	9.39	± 1.51	12.84	± 1.81	15.46	± 1.97
apoE4	Irr	ALA	PMA	2	1.18	± 0.27	4.83	± 1.06	8.61	± 1.51	11.82	± 1.81	14.28	± 1.97
apoE4	Irr	ALA	PMA	3	1.03	± 0.27	4.70	± 1.06	8.46	± 1.51	11.62	± 1.81	14.01	± 1.97
apoE4	Irr	ALA	PMA	4	1.28	± 0.27	4.43	± 1.06	7.31	± 1.51	9.84	± 1.81	11.83	± 1.97
apoE4	Irr	ALA	PMA	5	1.05	± 0.27	5.68	± 1.06	9.74	± 1.51	12.89	± 1.81	15.21	± 1.97

[1] Data represent values for hippocampal slices from the 8 different groups of mice, for each drug condition (+/-PMA), each region and the 5 different time points. Values are estimated marginal means ± SEM based on the covariates, temperature and location from Bregma. Areas 1-5 are dentate crux, bound blade, free blade; and areas CA1 and CA3 respectively.

Table 5. DHE oxidation (pixel intensity) of hippocampal slices from sham-irradiated and ^{56}Fe-irradiated apoE4 mice[1].

Tx	Drug	Area	4min	8min	12min	16min	20min
Sham	vehicle	1	100.4 ± 12.7	100.4 ± 12.7	100.4 ± 12.7	100.4 ± 12.7	100.4 ± 12.7
Sham	vehicle	2	101.6 ± 11.0	101.6 ± 11.0	101.6 ± 11.0	101.6 ± 11.0	101.6 ± 11.0
Sham	vehicle	3	101.1 ± 11.7	101.1 ± 11.7	101.1 ± 11.7	101.1 ± 11.7	101.1 ± 11.7
Sham	vehicle	4	101.2 ± 11.1	101.2 ± 11.1	101.2 ± 11.1	101.2 ± 11.1	101.2 ± 11.1
Sham	vehicle	5	100.9 ± 11.3	100.9 ± 11.3	100.9 ± 11.3	100.9 ± 11.3	100.9 ± 11.3
Sham	PMA	1	100.7 ± 12.7	100.7 ± 12.7	100.7 ± 12.7	100.7 ± 12.7	100.7 ± 12.7
Sham	PMA	2	102.5 ± 11.0	102.5 ± 11.0	102.5 ± 11.0	102.5 ± 11.0	102.5 ± 11.0
Sham	PMA	3	101.8 ± 11.7	101.8 ± 11.7	101.8 ± 11.7	101.8 ± 11.7	101.8 ± 11.7
Sham	PMA	4	102.0 ± 11.2	102.0 ± 11.2	102.0 ± 11.2	102.0 ± 11.2	102.0 ± 11.2
Sham	PMA	5	101.4 ± 11.3	101.4 ± 11.3	101.4 ± 11.3	101.4 ± 11.3	101.4 ± 11.3
Irr	vehicle	1	71.9 ± 12.7	72.2 ± 12.7	62.1 ± 12.7	70.4 ± 12.7	84.0 ± 12.7
Irr	vehicle	2	75.4 ± 11.0	75.9 ± 11.0	93.8 ± 11.0	76.7 ± 11.0	74.6 ± 11.0
Irr	vehicle	3	82.0 ± 11.7	85.2 ± 11.7	87.0 ± 11.7	83.9 ± 11.7	81.8 ± 11.7
Irr	vehicle	4	75.0 ± 11.2	80.1 ± 11.2	83.9 ± 11.2	83.8 ± 11.2	81.1 ± 11.2
Irr	vehicle	5	75.3 ± 11.4	79.0 ± 11.4	83.9 ± 11.4	87.8 ± 11.4	80.6 ± 11.4
Irr	PMA	1	45.4 ± 12.6	50.5 ± 12.6	58.8 ± 12.6	46.7 ± 12.6	73.1 ± 12.6
Irr	PMA	2	78.1 ± 10.9	46.1 ± 10.9	67.0 ± 10.9	53.7 ± 10.9	77.4 ± 10.9
Irr	PMA	3	79.7 ± 11.7	58.8 ± 11.7	82.7 ± 11.7	71.4 ± 11.7	84.8 ± 11.7
Irr	PMA	4	76.9 ± 11.1	67.8 ± 11.1	91.6 ± 11.1	81.6 ± 11.1	88.2 ± 11.1
Irr	PMA	5	76.9 ± 11.3	70.1 ± 11.3	92.4 ± 11.3	84.9 ± 11.3	86.1 ± 11.3

[1] Data represent values for hippocampal slices sham-irradiated and ^{56}Fe-irradiated apoE4 mice for each drug condiiton (+/-PMA), each region and the 5 different time points. Values are estimated marginal means ± SEM based on the covariate location from bregma. Areas 1-5 are dentate crux, bound blade, free blade; and areas CA1 and CA3 respectively.

The following data are from two separate studies using different groups of mice as those used in chapters two and three. Assessment of doublecortin-positive immature neurons in apoE2, apoE3, and apoE4 female mice are provided to illustrate the effects of ^{137}Cs and ^{56}Fe irradiation on hippocampal neurogenesis. The methods and results are presented here. Discussion to how the data relates to the dissertation is presented chapter 4.

Effects of ^{137}Cs irradiation on doublecortin-positive immature neurons of apoE2, apoE3, and apoE4 mice

Adapted from:

Villasana L.E., Pfankuch, T., and Raber, J. 2010 "Isoform-dependent effects of apoE on doublecortin and microtubule-associated protein 2 immunoreactivity following 137Cs irradiation" Radiat Environ Biophy 49, 421-26.

Materials and Methods

Mice

Mice were generated and maintained as described in chapter 2 except that they did not receive ALA-supplementation and that they were behaviorally naïve.

^{137}Cs irradiation

Irradiations were conduced as previously described in chapter two. N = 3-4 mice per genotype/hour assessed.

Microscopy and Immunohistochemistry

Mice were anesthetized (100 mg/kg ketamine, 10m/kg xylazine, 2mg/kg acepromazine) and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) 6, 8, 12, 18, or 24 hours following irradiation. The brains were removed and place in 4% PFA in phosphate buffered solution (PBS). The next day, the brains were placed in 30% sucrose in PBS overnight then frozen (-80 °C) until further use.

Coronal sections (30 µm) of the entire dentate gyrus of the hippocampus were collected using a sliding microtome. Sections were serially mounted on Superfrost microscope slides and stored at -80 °C. Upon use, slides were allowed to air dry for 10 minutes at room temperature then placed on a slide warmer for 45 minutes to promote adhesion. A 5% normal donkey serum (Jackson Immunoresearch) was used to block nonspecific binding (20 min RT). Slices were then incubated with anti-doublecortin (1:100, Santa Cruz) overnight at 4°C. Sections were washed 4 times (every 15 min) and incubated with Texas Red coupled anti-mouse secondary antibodies (1:100, Jackson Immunoresearch). Sections were washed 4 times with PBS containing 0.2% Triton X-100 (PBT). Anti-fade (Vectashield) solution was added before cover slipping. Doublecortin-positive cells within the hippocampal dentate gyrus were

quantified bilaterally using a 40x objective lens of an Olympus spinning disk confocal microscope (IX81, Olympus Imaging Corp.) equipped with Slidebook Software (Intelligent Imaging Solutions). DAPI staining was used to confirm whether doublecortin staining corresponded to a true cell. A cell was counted when it appeared on the first Z stack but was not counted if it appeared in the preceding stack. Timothy Pfankuch conducted the immunohistochemistry (IHC) and cell counts for this study.

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). All statistical analysis were conducted using SPSS and were considered significant at $P < 0.05$. All figures were generated using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

A Friedman test showed that there was an effect of irradiation across time ($x^2(40)= 33.23$; $P < 0.001$) but no effect of genotype (Figure 33). A planned comparison (Bonferroni pairwise comparison) was conducted to determine if apoE-isoform differences in the number of doublecortin-positive cells existed at particular time points following irradiation. The planned comparison confirmed a significant effect of genotype at the 8-hour time point; apoE3 mice had lower radiation-induced reductions in doublecortin-positive cells compared to apoE2 ($P< 0.01$) and apoE4 mice ($P < 0.001$). However, as previously noted, there was no overall genotype effect on radiation-induced reductions in doublecortin-positive cell counts.

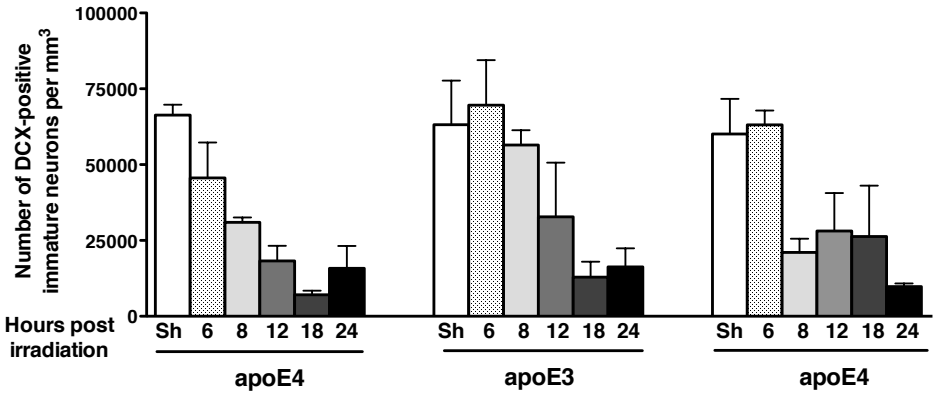

Figure 33. ApoE isoform-independent effects of $^{137}Cs^{-}$irradiation on doublecortin-positive immature neurons of apoE2, apoE3 and apoE4 mice. The number of doublecortin-positive cells was reduced shortly after irradiation ^{137}Cs-irradiation but was similarly reduced in all apoE genotypes. Sh = sham. Bars represent the mean and $\pm$ SEM. N = 3-4 mice per genotype/hour assessed.

Effects of ^{56}Fe irradiation on doublecortin-positive immature neurons of apoE2, apoE3, and apoE4 mice.

Mice

Mice were generated as described in chapter 2 except their food was not ALA-supplemented and they were not behaviorally naïve. The battery of tests they received was similar to that of those in this dissertation with some paradigm modifications.

Materials and Methods

Measures of doublecortin-positive immature neurons of sham-irradiated and ^{56}Fe-irradiated mice were assessed at one time point (4-5 months following irradiation) and were similar to those of the previous study presented above.

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). All statistical analysis were conducted using SPSS and were considered significant at $P < 0.05$. All figures were generated using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

A 2-way ANOVA determined that irradiation reduced the number of doublecortin-positive immature neurons ($1,14 = 59.12$; $P < 0.001$). However, there was no effect of genotype or an irradiation x genotype interaction (Figure 34).

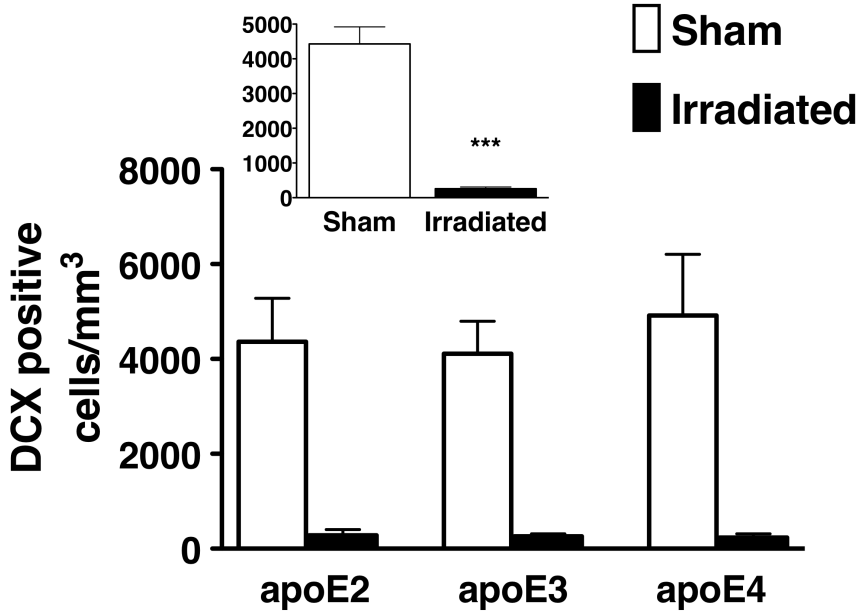

Figure 34. ApoE isoform-independent effects of ^{56}Fe irradiation on doublecortin-positive immature neurons of apoE2, apoE3 and apoE4 mice. Irradiation reduced the number of doublecortin-positive cells (inset) but there was no effect of genotype. ***$P < 0.001$, effect of ^{56}Fe irradiation. Bars represent the group mean $\pm$ SEM. N = 3-4 mice per genotype/irradiation treatment.

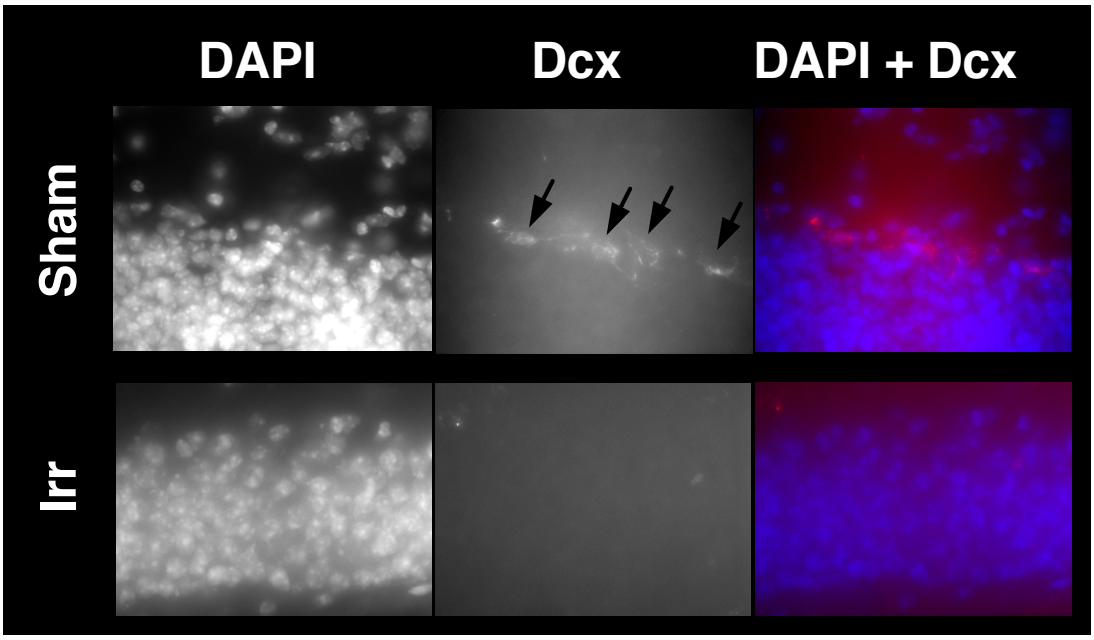

Figure 35. Representative image of doublecortin-positive immature neurons in the dentate gyrus of sham-irradiated and ^{56}Fe-irradiated apoE4 mice. Hippocampal sections were immunolabeled with a doublecortin (Dcx) antibody and counterstained with DAPI. Arrows point to doublecortin-positive immature neurons. The DAPI staining that was projected from a different channel was used to confirm whether the immunolabeling of Dcx corresponded to an actual cell.

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