

Exploring DNA double strand breaks as an underlying mechanism of learning and the influence of apolipoprotein E

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Abbreviations

53BP1	p53 Binding Protein 1
ACD	Age-associated Cognitive Decline
AD	Alzheimer's Disease
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of Variance
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
Arc	Activity-regulated cytoskeletal-associated gene
ATM	Ataxia- telangiectasia serine/threonine kinase
BAPTA	1,2-bis(<i>o</i> -aminophenoxy)ethane- <i>N,N,N',N'</i> -tetraacetic acid
BOLD	Blood-oxygen level dependent
CaMKII	Calcium-calmodulin-dependent kinase II
ChIP	Chromatin Immunoprecipitation
ddPCR	Digital droplet PCR
DG	Dentate gyrus
DSBs	Double Stranded Breaks
HR	Homologous Recombination
i.p.	intraperitoneal
i.v.	intravenous
IEGs	Immediate Early Genes
IR	Ionizing Radiation
LOAD	Late Onset Alzheimer's Disease
LTP	Long term potentiation
MANOVA	Multivariate Analysis of Variance
MCI	Mild Cognitive Impairment
MRI	Magnetic Resonance Imaging
NADPH	Nicotinamide adenine dinucleotide phosphate
NHEJ	Non-Homologous End Joining
NMDAR	N-methyl-D-aspartate Receptor
Npas4	Neuronal PAS domain protein 4
PFA	Paraformaldehyde
PS1	Presenilin 1
PTSD	Post-traumatic Stress Disorder
ROS	Reactive Oxygen Species
RT	Radiation Therapy
RTFs	Regulatory Transcription Factors
SSBs	Single stranded breaks
TSS	Transcriptional Start Sites
WT	C57BL6/J (Wild-Type) mice

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Abstract

DNA double strand breaks (DSBs) lead to apoptosis or tumorigenesis. The complexity of their repair has led to DSBs being classified as the most harmful type of DNA damage. In a sudden shift, a novel role for DSBs in immediate early gene (IEG) expression, learning, and memory has been suggested. Inducing neuronal activity *in vitro* and learning events *in vivo* lead to increases in DSBs and upregulation of IEGs. Additionally, inhibiting DSB repair and increasing DSBs impair long-term memory and alter IEG expression. DNA damage increases and DNA repair decreases with aging; post-mortem tissue from patients diagnosed with mild cognitive impairment or Alzheimer's disease (AD) in life show increases in DSBs. In rodent carrying dominant mutations that cause AD, increased baseline DSBs and impaired DSB repair were observed. Together, the data suggest that there may be an adaptive role for DSBs in the central nervous system and that dysregulation is a driving factor in age-related cognitive decline (ACD) and age-related diseases. Using two common cancer treatments—amifostine and etoposide—I altered DSBs in WT mice and assessed learning, memory and IEG expression. Systemic administration of these two compounds in male and female C57Bl6/J (WT) mice led to changes in long-term contextual fear memory in a sex-dependent fashion: amifostine increased contextual fear memory in males, and etoposide impaired long-term contextual fear memory in females. Additionally, infusions of etoposide into the CA1 region of the hippocampus before fear conditioning impaired long-term contextual fear memory, but not cued fear memory, in both males and females. cFos and Arc, two IEGs, were decreased in the CA1 following etoposide infusions. These data support the hypothesis that DSBs contribute to learning and memory. To further investigate how DSBs and IEGs may be modulated based on known risk factors for ACD and AD, I used targeted replacement apolipoprotein E (apoE) male and female mice. The 3

human apoE isoforms (E2, E3, or E4) confer differential risk for diseases: E4 increases risk for AD, while E2 increases risk and severity of post-traumatic stress disorder (PTSD). Women are also at higher risk to develop both AD and PTSD. The neural mechanisms that lead to apoE-dependent risk for different diseases remain unclear. Using whole-brain cFos imaging, I assessed brain-wide changes in neural activation and connectivity in young, female apoE mice following contextual fear conditioning. E4 mice had lower cFos immunoreactivity across regions important for somatosensation and contextual encoding compared to E2 mice, and showed altered connectivity compared to E3 mice. I also assessed the interaction of age, sex, and apoE using male and female young (3 month) and middle aged (12 month) mice. Using immunofluorescence and γ H2Ax-chromatin immunoprecipitation (ChIP) followed by digital droplet PCR (ddPCR), I found that all these factors influence the formation of DSBs near *Fos* and *Npas4* in the hippocampus. Middle-aged E4 males and females showed increased DSB-associated IEGs at baseline. These results add to the evidence that dysregulation of adaptive DSBs could be a driving mechanism in aging and disease development. Altogether, this dissertation adds to the evidence that DSBs have an adaptive function in the brain, and that minor alterations in typical DSB formation and repair can alter IEGs, learning, and memory.

Chapter 1: Introduction

Learning and memory: A brief history

The goal to understand the mechanisms that lead to effective learning and memory bridge a wide range of domains: psychological, educational, social, political, and biological, to name a few. Research into methods to effectively encode, store, and recall information has identified important distinctions across sub-types of learning and memory. Notably, Dr. Brenda Milner's work with Henry Gustav Moliason in the late 1950s was key in identifying the distinction between explicit (requiring conscious effort) and implicit (not requiring conscious effort) learning and memory [1]. Mr. Moliason developed life-long, explicit anterograde amnesia following a bilateral temporal lobectomy to address his severe epilepsy, yet his implicit memory remained intact. Experiments examining Mr. Moliason's differing learning abilities started to clarify what behavioral neuroscientists (or "physiological psychologists," as they were called at the time) had been attempting to identify for a while: the location of the engram.

Dr. Karl Lashley had been searching for the engram—the physical location of memory—for most of his career, systematically lesioning large areas of the brain and using subsequent mazes to assess learning abilities [2]. In the 1940s, his frustration at failing to find the engram led to him reflecting on the difficulty of studying learning, saying "I sometimes feel, in reviewing the evidence of the localization of the memory trace, that the necessary conclusion is that learning is just not possible. It is difficult to conceive of a mechanism that can satisfy the conditions set for it. Nevertheless, in spite of such evidence against it, learning sometimes does occur" [3].

Prior to both Dr. Lashley and Dr. Milner, Dr. Ivan Pavlov described what he called the "conditioned reflex" in dogs while conducting experiments looking at mechanisms of digestion

[4]. This gave birth to the concept now known as classical conditioning: the association of a neutral stimulus with an unconditioned stimulus, resulting in novel, conditioned responses. Since then—and combined with the additional knowledge of various types of learning dependent on different brain regions—classical conditioning has been widely used to study an individual's ability to learn and remember. A range of species quickly and effectively acquire fear memories associated with contexts and cues, known as fear conditioning.

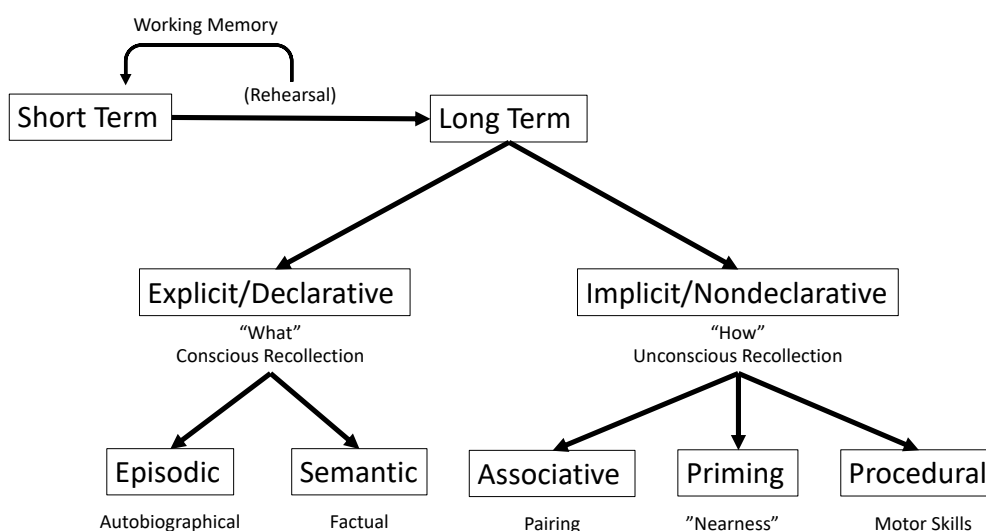


Figure 1.1. Schematic of types of learning and memory. Over decades, the distinction between explicit and implicit, as well as the sub-types, have arisen from hundreds of researchers' work.

The extensive use of fear conditioning specifically has created a wealth of understanding related to the encoding, storage, and recall stages of classical conditioning. Notably, the neuroanatomy of various types of fear conditioning has been described in great detail [5, 6]. Both the amygdala and hippocampus are essential for the learning and memory phases of different paradigms: specifically, the amygdala is a crucial region for cued fear memory [7, 8], while the hippocampus is essential for contextual fear memory [5].

Tremendous effort on the part of the broader neuroscience community has led to groundbreaking discoveries about the underlying cellular mechanisms that drive learning and

memory. Several notable mechanisms have changed perceptions of the central nervous system. Here, I will briefly summarize two: adult neurogenesis and long-term potentiation.

Adult neurogenesis. Scientists for decades believed that the brain existed in a fixed state, unable to regenerate or produce new neurons. However, in the 1960s, Dr. Joseph Altman began publishing findings of neurogenesis in adult mammal brains [9-11]. These findings were not widely accepted until the 1980s when several researchers started reporting on additional evidence for adult neurogenesis in rodents and birds [12, 13]. Notably, findings pointed towards adult neurogenesis primarily occurring in the subventricular zone and dentate gyrus sub-region of the hippocampus [14], leading to hypotheses that learning and memory could be traced to new-born neurons [15].

Indeed, there are a plethora of reports on the importance of adult neurogenesis for hippocampus-dependent memory. Yet, it quickly became clear that hippocampal neurogenesis could not entirely explain learning and memory. Ablation of adult neurogenesis via inducible knock-out in adult mice was sufficient to impair spatial learning and memory in the Morris water maze test [16]. However, in this same study, contextual fear conditioning (known to be hippocampus-dependent) was not impaired. Another series of experiments showed the opposite: ablation of hippocampal neurogenesis via X-ray irradiation in mice impaired contextual fear conditioning, but did not affect water maze performance [17]. Previous reports from our lab have found that decreasing neurogenesis in the subventricular zone by X-ray irradiation led to impaired spatial learning and memory in the Barnes Maze, but not the Morris Water Maze [18]. Moreover, radiation with X-rays did not impair rotarod performance or open field activity, even with significant decreases in neurogenesis [19]. Adding to the nuanced complexity, toxin-

induced ablation of hippocampal neurogenesis in rats impaired learning of trace fear conditioning, but not contextual fear conditioning or water maze learning [20].

Together, the evidence points to both a role for adult neurogenesis in some forms of learning and memory—such as pattern separation [21]—and suggests that there are other mechanisms necessary for encoding and maintenance.

Long term potentiation. Around the same time as Dr. Altman’s first descriptions of adult neurogenesis, Dr. Terje Lømo discovered long-term potentiation (LTP) in the rabbit hippocampus [22, 23]. This discovery—that repeated stimulation of the same neural connections leads to more efficient signal transmission—provided evidence to support hypotheses originally put forth (independently) by Drs. Santiago Ramón y Cajal and Donald Hebb [24, 25].

Techniques to draw a causal link between LTP and learning and memory were delayed, but highly pursued. After further characterization of the mechanisms of LTP (namely, the dependence on glutamate, NMDA receptors, and the calcium-calmodulin-dependent kinase II (CaMKII) pathway), researchers were able to target LTP during learning and memory testing. Early experiments showed that inhibition of NMDA receptors impaired learning and memory in the water maze test [26]. Subsequent studies showed that LTP “saturation” (stimulation to the point of maximal LTP expression) in the dorsal hippocampus impaired spatial reversal learning in the Barnes maze [27]. Targeting LTP with chemical agents, such as inhibiting CaMKII-mediated phosphorylation, impaired hippocampal LTP and prevented learning in the water maze [28]. Moreover, others have reported an increase in amygdalar LTP following training in cued fear conditioning [29], and that inhibiting hippocampal LTP via saturation was sufficient to occlude learning and memory in the water maze [30].

Moving beyond early LTP, though, late LTP (which lasts for hours) is characterized by induction of protein synthesis and is known to be necessary for more long-term memory formation and maintenance [31-33].

The role of immediate early genes in synaptic plasticity, learning, and memory

One family of proteins linked to synaptic plasticity, learning, and memory is the family of immediate early genes (IEGs) [33, 34], induced following neuronal electrical activity by numerous stimuli—from behavioral experiences to drugs of abuse. Likely hundreds of genes fall into this IEG category. IEGs are expressed at very low baseline levels and are then transiently increased within minutes of stimulation. Notably, IEGs do not require *de novo* protein synthesis for their transcription to be induced, as inhibition of known protein synthesis pathways do not impair IEG expression [35-38]. The mechanisms behind this rapid induction of IEG expression is still unclear. Expression of IEGs is then cleared relatively quickly, returning to baseline levels within 2-3 hours following stimulation [39]. Because of this, IEGs are widely used as markers of neuronal activation in the central nervous system [34, 35, 39-41].

The exact functions of IEGs range from initiating transcription to direct effects on synaptic maintenance. This has led to their sub-classification into “effector” IEGs—genes that directly affect cellular function, or “regulatory transcription factors” (RTFs)—genes that affect cellular function indirectly by regulating expression of other genes [42-45]. Of these, it is estimated that the majority of IEGs are effectors, while ~15 are RTFs [42, 44]. A select few relevant to my dissertation are discussed in this section (**Table 1.1**).

Fos & Jun. The first description of the proto-oncogene *c-fos* in the brain was following induction of seizures [46]. mRNA levels of c-Fos were seen to peak around 60 minutes after seizure induction, while protein levels peaked around 90 minutes. Levels of mRNA transiently went below baseline 3 hours post-stimulus and then returned to baseline levels at 16 hours. Around the same time, *c-Jun* was identified as a component of the activator protein 1 (AP-1) transcription factor [47]. It was quickly identified that *c-Fos* and *c-Jun* form a heterodimer to create AP-1 and subsequently act as a transcription factor, indirectly contributing to synaptic plasticity [48].

There is a wealth of literature from the last 40 years about the role of *fos*, *jun*, and the AP-1 complex in learning and memory. Focusing on fear conditioning, *c-fos* has been repeatedly shown to be upregulated in anatomically relevant regions following fear conditioning. For example, c-Fos increased in the mammillary bodies and anterior thalamus of rats after fear conditioning [49] and in the dorsal CA1 in mice after contextual fear conditioning [50]. Moreover, inhibition of c-Fos in the CA1 impairs contextual recall [51].

Over the years, other members of the *fos* and *jun* families have been identified, such as FosB, JunB, and JunD [52, 53]. Less is known about the function of these other variants, though FosB and a splice variant, Δ FosB, appear to persist as a more long-term molecular switch that plays a role in development of addiction [52, 54-56]. JunB was also found to be up-regulated in the hippocampus after contextual fear conditioning in mice [50], suggesting a role in contextual encoding. Additionally, the binding of JunD to the AP-1 complex is associated with fear extinction and decreased c-Fos in the dorsal hippocampus [57].

Arc/Agr3.1. In contrast to *fos* and *jun*, the activity-regulated cytoskeletal-associated gene (*Arc*, otherwise known as *Agr3.1*) is an example of an “effector” IEG [44, 58, 59]. It is a highly regulated protein primarily expressed in neurons. Following activation, it is rapidly targeted to activated synapses, where it increases the number of thin spines and decreases the density of AMPA receptors [60]. Interestingly, *Arc* knock out mice display intact short-term memory [61]. However, inhibition or lack of *Arc* impairs late-LTP [62] and long-term memory consolidation [61]. Inhibiting *Arc* after fear conditioning is also sufficient to impair contextual fear memory [63], highlighting the importance of *Arc* in memory maintenance.

Npas4. Neuronal PAS domain protein 4 (*Npas4*) is another RTF. *Npas4* functions to maintain the balance between excitation and inhibition: one of its major downstream effects is increasing inhibitory synapses on cell bodies while decreasing inhibitory synapses on apical dendrites in the CA1 [64]. This appears to be modulated by the downstream activation of brain derived neurotrophic factor (BDNF) [41]. The accurate maintenance of inhibitory signaling is especially crucial during adolescence and development. *Npas4* is important for inhibitory synapse development in the prefrontal cortex of mice specifically during adolescence [65], implicating *Npas4* dysregulation in disorders such as schizophrenia [66]. Moreover, increased *Npas4* expression suppresses fear memory in a post-traumatic stress disorder (PTSD) mouse model [67], further highlighting the importance of inhibitory signaling related to psychological disorders.

Other IEGs. This is not an exhaustive review of all known IEGs and their functions. Other IEGs of note include *Egr1/zif268*, *Homer1*, and *Cyr61*. *Egr1*—also known as *zif268*—is

expressed following contextual fear conditioning [68] and induced by LTP [69]. *Egr1* appears to be both an effector and an RTF: its exact transcriptional targets are unknown, but evidence suggests that it binds to DNA and promotes transcription, as well as works directly in response to cellular damage [70]. *Homer1* is an effector IEG that is known to mediate the binding between mGluRs and IP3 receptors, thereby negatively regulating excitatory synapses [41]. *Homer1a* is upregulated in the CA1, CA3, and dentate gyrus following exposure to a novel environment, contextual fear conditioning, and contextual memory test [71]. Lastly, *Cyr61* plays an essential role in dendritic growth in the hippocampus [72].

Table 1.1. Selection of IEGs and their known primary function in synaptic plasticity.

Name	Classification	Primary Function	References
cFos	RTF	Binds with cJun to create the AP1 complex, thereby promoting transcription	[48]
cJun	RTF	Binds with cFos to create the AP1 complex, thereby promoting transcription	[48]
Arc/Arg3.1	Effector	Involved in endocytosis of AMPA receptors and increasing thin spines	[41]
Npas4	RTF	Mediates the balance between inhibitory and excitatory signals, notably by controlling inhibitory synapse growth	[41]
Egr1/zif268	RTF & Effector	Transcription factor; important in cell survival, differentiation, and death especially after injury	[68, 69]
Homer1a	Effector	Negative regulation of excitatory synapses via mediating the binding between mGluRs and IP3 receptors	[41]
Cyr61	Effector	Promotes adhesion of endothelial cells and aids in DNA synthesis; regulates dendritic growth	[72]

DNA double strand breaks: A dangerous and complex history

The importance of maintaining DNA integrity is critical for faithful gene expression. Yet, DNA is susceptible to many forms of damage caused during normal cellular functioning: it is

estimated that up to tens of thousands DNA-damaging events happen daily per cell [73]. These include base-pair mismatch, which can occur during the DNA replication process; oxidative base damage; single stranded breaks (SSBs), often induced by ionizing radiation or reactive oxygen species; and double stranded breaks (DSBs) [74]. All types of DNA damage have the potential to lead to a host of negative consequences, such as apoptosis or tumorigenesis. Of the types of DNA damage, though, DSBs are considered the most cytotoxic, [75] since when unrepaired, DSBs serve as a signal for apoptosis [76-78]. This has led to the evolution of highly complex DSB repair processes.

DSBs can result from a range of factors, including chemical toxins (such as cisplatin, bleomycin, and etoposide) and ionizing radiation (IR) [77]. They can also be harnessed for therapeutic effects in disease states, as chemotherapy and radiation therapy (RT) are commonly used to treat cancers. Chemical- and RT-induced DSBs have been an effective way to kill tumorigenic cells and eliminate tumors; however, side effects, including those involving injury to healthy bystander cells, are common and debilitating [79]. Particularly, IR often leads to formation of many SSBs; when SSBs are close together and present in opposite DNA strands they produce DSBs, often with “dirty ends” (single-stranded overhangs) that lead deletion of nucleotides during the DSB repair process [80]. Yet, DSBs are not always pathological. DSBs play an important, adaptive role in the immune system, with the best understood examples being involved in generation of antibody diversity during VDJ recombination and class-switching of immunoglobulins [81]. DSBs are also seen in typical cell division during the S/G2 phases, when a sister chromatid is available as a template [82]. The fine line between damage-induced/pathological versus physiologically relevant DSBs has spurred renewed interest in possibly related behavioral changes.

In the clinic, amifostine (also known as Ethyol) protects non-cancerous cells during RT and is commonly co-administered during treatment [83]. Amifostine, which is metabolized into the active agent WR-1065, scavenges free radicals, increases the speed of DSB repair, and decreases the total number of DSBs that form, preferentially protecting non-tumor tissue [83, 84]. Reported side effects range from gastrointestinal disturbances to severe skin conditions, with a high rate of adverse side effects leading to discontinuation of use, especially in head and neck cancers [85]. Conversely, etoposide (also known as Vepesid, Etopophos, or Toposar) induces DSBs and is used during chemotherapy to increase apoptosis in tumors [86]. Etoposide increases DSBs by interfering with the complex of covalently-bound topoisomerase-II β to cleaved DNA, thereby preventing DSB repair from occurring [87]. As with amifostine, major side effects include a host of gastrointestinal disturbances, as well as extreme fatigue and weakness [88]. Yet, research into sex-dependent effects of amifostine and etoposide and at doses much lower than typically used as part of cancer therapy is lacking. In cancer patients, women clear plasma amifostine faster than men [89]. Etoposide also has a shorter half-life in females, though clearance is similar between the sexes [90]. The major side effects of both drugs suggests that amifostine and etoposide may be more toxic than thought for unknown reasons, which could be related to central nervous system (CNS) injury (related to so-called “chemobrain”) [91].

As mentioned above, faithful DSB repair is essential for a healthy, functional cell. DSBs that form during mitosis are repaired via homologous recombination (HR), where breaks are mended using a sister chromatid strand to faithfully restore genetic information that may have been lost when the break formed [77]. DSBs induced by damage, or DSBs in non-dividing cells, are repaired by non-homologous end joining (NHEJ), whereby a series of complex signaling cascades lead to the processing and ligation of the two DNA ends. This process is highly error-

prone, offering the opportunity for insertions and deletions to form, but is independent of the cell cycle [75, 76]. Rapidly upon DNA breaking, histone variant H2Ax is phosphorylated at serine-139, forming γ H2Ax, which occurs near the site of the break [92]. γ H2Ax appears to play an important role in initiating DSB repair, both by increasing accessibility to the broken DNA and by recruiting other proteins important for the repair process [92]. One of these proteins—p53 binding protein 1 (53BP1)—is a marker for repair by the NHEJ pathway [93]. Due to the rapid focal accumulation of these two proteins at DSB sites, they are often used to identify the anatomic and genomic location of breaks.

Notably, the adaptive roles of DSBs have been primarily describe in peripheral, dividing cells. Yet, the central nervous system (CNS) is comprised of post-mitotic cells. DSBs in the CNS have been characterized as harmful as a result. DSBs in the hippocampus specifically have been identified throughout the lifespan as harmful for learning, memory, and general survival. Mice irradiated at prenatal days 3, 10, or 21 had persisting γ H2Ax in the dentate gyrus of the hippocampus until 15 months of age and decreased survival [94]. Repeated exposure to fractionated low-dose radiation (0.1 Gy) in juvenile and adult mice increased 53BP1 in the dentate gyrus and decreased neurogenesis [95].

Double strand breaks, synaptic plasticity, and aging

In a sudden shift, recent reports suggest that DSBs may also have an adaptive role in healthy brain function. As mentioned above (see section on IEGs), the mechanisms leading to the rapid expression of IEGs are unknown. More and more evidence has been emerging showing a connection between DSBs and IEGs, potentially offering an explanation for this rapid expression upon stimulation (**Table 1.2**).

Notably, an early study identified that sub-toxic stimulation of ionotropic glutamate receptors in cultured rat neurons resulted in γ H2Ax formation and cell survival [96]. Transient increases of γ H2Ax and MRE11 were observed following activation of NMDARs (15 μ M) or AMPARs (25 μ M). NMDAR activation led to peak γ H2Ax signal 10 minutes after bath application, while AMPAR activation led to peak γ H2Ax signal 30 minutes after application. Both vitamin E and the calcium-chelator BAPTA attenuated this increase, though not to baseline levels, indicating that, while calcium plays a role, DSBs formed following subtoxic glutamatergic stimulation are not entirely calcium-dependent.

Several years later, a transient increase in γ H2Ax was observed in the brains of C57BL/6J wild-type (WT) mice following exposure to a novel environment [97]. Also noteworthy were the observations that γ H2Ax existed at low baseline levels, returned to these baseline levels in 24 hours, and were seen exclusively in brain regions important for contextual encoding. Similarly, visual stimulation resulted in an increase of γ H2Ax in the corresponding hemisphere of the visual cortex, but not the opposite hemisphere [97]. This region-specific change in γ H2Ax levels suggest that typical, external stimuli trigger DSBs.

A subsequent study showed that DSBs could be induced by stimulating cultured hippocampal neurons and that these breaks occurred at promoter regions for IEGs, such as *Fos*, *Npas4*, and *Egr1* [98]. The breaks were associated with increased gene expression, suggesting DSBs promote IEG expression. DSBs induced by neuronal activation were shown in this study to be dependent on topoisomerase-II β (topoII β), as reducing topoII β decreased formation of γ H2Ax and IEG expression, and to occur near promoter regions. These data suggest that DSBs may remove constraints preventing promoter and enhancer regions from interacting [99].

Moreover, WT mice trained in a contextual fear conditioning test showed an increase in γ H2Ax foci in the hippocampus, though IEG expression was not assessed from *in vivo* samples [98].

Around the same time, another report showed evidence that DNA breaks are involved in transcriptional elongation [100]. Using HEK293 cells, DSBs induced by serum were found downstream of transcriptional start sites (TSS) of the IEGs *Egr1*, *Jun*, *Fos*, and *Myc*. Similar to the findings from Madabhushi et al. [98], these IEGs were upregulated, again supporting that the DSBs induced by physiological activation were involved in gene expression. Data from this study indicated that ATM serine/threonine kinase (ATM) and DNA-dependent protein kinases (DNA-PKcs)—both known responders to DNA damage—phosphorylate Trim28, which holds RNA polymerase II (PolII) in pause. Once phosphorylated, Trim28 releases the pause on PolII, allowing transcription to begin. Similar to Madabhushi et al [98], topoII β was found to be important in stimulus related DSBs [100]. These two studies indicate that neuronal activation induces topoII β -mediated DSBs and results in transcription of a subset of genes (**Fig. 1.2A**).

Inhibiting gene transcription causes long-term memory disruptions [31], thus, interfering with typical DSB formation and/or repair might affect learning and memory. The Gadd45-family of proteins is involved in DNA repair and de-methylation [101], and thus serves as a target to interfere with typical DSB repair. In one study, inhibition of Gadd45 γ in the prelimbic area of mice during trace fear conditioning altered IEG expression and decreased long-term memory [102]. Notably, the first wave of IEG expression was not altered with the Gadd45 γ inhibitor; rather, a second peak of IEG signaling (specifically for *Arc*, *cFos*, *Npas4*, and *Cyr61*) at 5 hours post-test was identified to be affected following Gadd45 γ inhibition. In this same study, use of etoposide in the prelimbic area also impaired expression of *Arc*, *cFos*, *Npas4*, and *Cyr61* at 5 hours post-test; mice that received etoposide infusions also displayed decreased freezing in

response to the conditioned stimulus. This direct manipulation of the DSB repair timeline added to the evidence that tight regulation of the formation and repair of DSBs may be critical for learning and memory.

Subsequent to these studies looking at the role of DSBs in the initial encoding and storage stages of learning, researchers began exploring the possible role of DSBs in other stages of the learning and memory process. Indeed, increased DSBs were seen in the CA1 region of the hippocampus in Sprague-Dawley rats during fear reconsolidation [103]. The genomic location was highly specific: these DSBs were found in the promoter region for *Npas4*, but not *cFos*. Moreover, inhibition of topoII β in the CA1 using siRNA during the retrieval trial impaired memory and decreased DSBs on the *Npas4* promoter. Relatedly, exposing mice to whole-body gamma radiation (which induces widespread DSBs with overhangs) after training in contextual fear conditioning increased fear memory on the subsequent day and decreased the number of cFos immunoreactive positive cells in the infralimbic cortex [104]. Thus, timing and specificity of DSBs appear to influence the effects on learning and memory. The number of parvalbumin-positive cells was also decreased in mice exposed to post-training gamma radiation [104], suggesting that cellular-subtype is another important factor to consider in DSB location.

Others have also pursued identifying if DSBs occur in a variety of cells in the brain. There is evidence that DSBs induced by contextual fear conditioning occur in both neuronal and non-neuronal cells in the hippocampus and medial prefrontal cortex [105]. In neurons, genes related to synaptic function were upregulated, including IEGs like *Arc*, *Npas4*, *Fos*, *Nr4a1*, *Actb*, *Ntrk2*, *Egr1*, and *Plk2*. *Arc* was also upregulated in non-neuronal cells. Glial cells—including astrocytes, oligodendrocytes, and microglia—uniquely showed increased DSBs and gene expression following exposure to corticosterone. These upregulated genes primarily related to

cellular homeostasis, cell motility and adhesion, and proliferation and cell death, again indicating specificity of DSBs for type of stimulus [105]. Moreover, these data suggest that DSBs contribute to gene expression across all major cell-types of the brain.

The importance of timely DSB repair is another important factor to consider when exploring and adaptive role of DSBs in the brain. It was shown that DSB repair markers remained high in *drosophila* and mice deprived of sleep after exposure to a novel environment or whole-body gamma radiation [106]. Conversely, flies or mice allowed to sleep following stimulation displayed significantly lower levels of DSBs. Males were notably more affected by sleep deprivation than females: γ H2Ax markers were higher in the frontal cortex of sleep-deprived male mice 3 and 7 hours after whole-body irradiation, but not in females. Sleep is important for learning and memory processes, and there is a known circadian rhythm in IEG expression [107-109]. Sleep deprivation is well-known to impair learning and memory, with hippocampus-dependent learning and memory being particularly susceptible to the negative effects of sleep deprivation [110]. The importance of sleep, then, for DSBs is logical, though more research is needed to understand the driving mechanisms underlying this.

DSB repair declines steadily with age, while DNA damage increases with age [111, 112]. Age is the highest predictive risk factor for developing age-associated cognitive decline (ACD) and AD [113]. Age and disease synergistically increase the amount of pathological DSBs (*i.e.*, increased reactive oxygen species (ROS) and decreased repair, causing more DSBs), potentially creating more “noise” vs the “signal” of physiological DSBs when trying to detect the latter [111, 114-116]. Several dominant familial mutations that cause AD have been identified in amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2). Transgenic mice expressing mutated human APP were reported to have higher levels of DSBs at baseline

compared to WT mice and did not return to baseline levels 24 h after exploring a novel environment [97]. Moreover, APP/PS1 mice showed higher DSBs than WT mice and a decrease in DSB repair signals in the hippocampus [117]. Consistent with these mouse studies, levels of γ H2Ax and 53BP1 were recently shown to be higher in cortical areas of patients with mild cognitive impairment and AD compared to controls [118]. These studies suggest that elevated levels of pathological DSBs could be a driving factor in development of dementia or other learning- and memory-related disorders (**Fig. 1.2B**).

Table 1.2. Summary of findings related to an adaptive role of DSBs in IEG expression.

Reference	Year	System	Sex	Age	Stimulation	Main Findings	IEGs Upregulated	IEGs Unchanged
Crowe et al	2006	Primary cortical rat neurons	Not reported	Not reported	AMPA, NMDA, Electrical pulse	Sub-toxic stimulation of ionotropic glutamate receptors resulted in γ H2Ax formation	n/a	n/a
Suberbielle et al	2013	Wildtype/APP-PS1 mice	Males and Females	4-7 months	Exposure to novel environment Visual stimulation	Transient increase in γ H2Ax foci in relevant brain regions; high baseline levels of γ H2Ax in APP/PS1 mice and elevated levels at 24 h compared to WT mice.	n/a	n/a
Madabhushi et al	2015	Primary hippocampal mouse neurons Wildtype mice	Not reported	Not reported	Potassium chloride, bicucullin, NMDA, etoposide Fear conditioning	Physiological stimulation induces DSBs on transcriptional start sites that leads to up-regulation of a subset of genes, mostly IEGs	FosB, Fos, Nr4a1, Npas4	n/a
Bunch et al	2015	HEK239 Cells	n/a	n/a	Heat Shock Serum	DSBs occur downstream of TSS, leading to transcriptional elongation.	Egr1, Jun, Fos, Myc	n/a
Bellesi et al	2016	Drosophila Wildtype mice	Males and Females	3 months	Exposure to novel environment Whole-body gamma irradiation	Increased markers of DSB repair during sleep; impaired DSB repair when sleep is prevented	n/a	n/a
Kugelman et al	2016	Wildtype mice	Males	1.5 months	Whole-body gamma irradiation	Whole-body gamma irradiation after fear training led to increased fear expression but decreased cFos in GABA cells in the infralimbic cortex	cFos	n/a
Li et al	2019	Wildtype mice	Males	2 months	Trace fear conditioning	Inducing DSBs with etoposide led to prolonged increase of IEG expression and impaired memory	Arc, cFos, Npas4, Cyr61	n/a
Navabpour et al	2020	Sprague Dawley rats	Males	2 months	Fear reconsolidation	Increased DSBs in promoter region of Npas4 following fear memory test; impaired fear retention following inhibition of topoisomerase II β	Npas4	cFos
Stott et al	2021	Wildtype mice	Males	4 months	Contextual fear conditioning	Increased DSBs in neurons and glial following contextual fear conditioning Glial cells have an increase DSBs in response to corticosterone	Egr3, Junb, Egr1, Npas4, Nr4a1	n/a

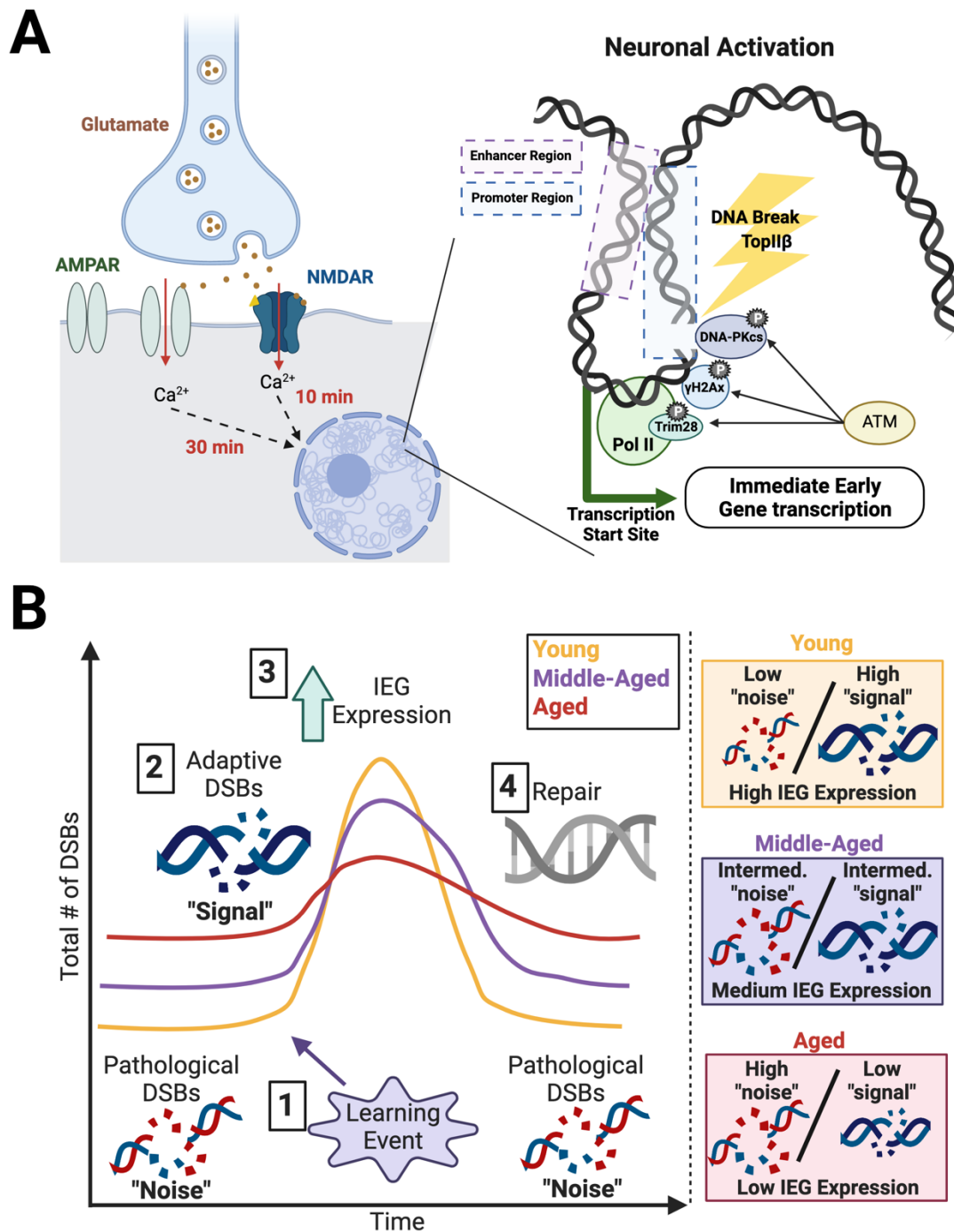


Figure 1.2. Suggested method of adaptive DSBs in youth and aging. **A)** Neuronal activation occurs with the binding of glutamate to NMDAR and AMPARs, allowing an influx of calcium that initiates a signal cascade into the nucleus. Following this cascade, topoisomerase II β initiates a DSB downstream of the transcription start site. The DSB activates ATM, which induces phosphorylation of DNA-PKcs, Trim28, and H2Ax. Trim28 phosphorylation releases RNA polymerase II (Pol II), which then becomes active to induce transcription of IEGs. Additionally, the DSB releases topological constraints, allowing the enhancer and promoter regions to interact. **B)** At baseline, DSBs are low. Once stimulation occurs, such as a learning event, there is a rapid increase in DSBs leading to rapid IEG expression. DSBs are repaired and return to baseline levels, as do IEGs. In aging, increases in baseline DNA damage lead to more aberrant, pathological DSBs, increasing “noise.” Upon stimulation, there is less of an increase in adaptive DSBs, or less “signal,” and a failure to repair DSBs as quickly. Made with biorender.com.

Apolipoprotein E, sex, and age: Differential risk for neurological disorders

The highest predictive risk factors for ACD and AD are age, biological sex, and apolipoprotein E (apoE) isoform [113, 119-121]. In this section, I will summarize the data exploring the risks related to *APOE* genotype, sex, and their interactions.

ApoE is a protein widely expressed throughout the body—most highly in the liver, brain, and kidneys—that has a wide range of functions. Primarily, apoE is a major component of lipid transport, metabolism, and injury response. ApoE's role in the brain as a cholesterol transporter is known to contribute to synaptogenesis and synapse maintenance [122, 123]. Yet, apoE knock-out mice are relatively normal [124] and no cognitive impairments were seen in an apoE-deficient person [125]. In the brain, astrocytes are the main producers of apoE, though other cell types (*i.e.* neurons, microglia, and oligodendrocytes) have been shown to express apoE under stress or following injury [126-132]. ApoE was also recently discovered to directly function as a transcription factor with high affinity for binding double-stranded DNA [133]. Taken together, the variety of functions of apoE indicate its importance in the central nervous system under physiological and pathological conditions.

There are three human isoforms of apoE: E2, E3, and E4. In the general population, allele frequencies are around 8.4% for $\epsilon 2$, 77.9% for $\epsilon 3$, and 13.7% for $\epsilon 4$ [119, 134]. People with one or two $\epsilon 4$ alleles have a relatively higher risk of developing late onset AD (LOAD) compared to E3 carriers; conversely, E2 carriers are relatively protected from LOAD [119]. Notably, this relative protection/risk for LOAD is ethnicity-dependent: the E4-LOAD link is seen in White and Japanese populations but absent in Black, Native American, and non-white Hispanic [119, 135, 136]. Carrying E4 is also highly predictive of developing frontotemporal dementia, vascular dementia, and dementia with Lewy bodies, suggesting a common underlying mechanism [137-

139]. While E2 is protective against LOAD compared to E3, it is associated with higher diagnoses and greater severity of post-traumatic stress disorder (PTSD) [140-142]. E2 is also associated with poor outcomes in melanoma, whereas E4 conferred better outcomes (reduced tumor size, metastasis, and increased survival) in mice [143]. How apoE isoforms lead to differential—and specific—disease risk or protection is still unclear, though. Specifically studying the E2 isoform in human populations has been difficult due to its rarity. E2 is often excluded from being analyzed [144, 145], but often E2 carriers are included and classified as “E4-” carriers.

Women are at a greater risk to develop AD than men, with roughly 2/3 of all cases diagnosed in women [146]. Women also show greater and faster cognitive deterioration than men despite no differences in amyloid-beta levels [120, 147]. The exact reason for this sex-specific risk is unknown. Some hypotheses include changes in hormone levels (specifically estradiol) during menopause [120, 148], sex differences in mitochondrial function and oxidative stress [149, 150], and increased pro-inflammatory markers in aging women [151]. Moreover, females with the $\epsilon 4$ allele have an even greater risk to develop AD (4.37 odds ratio) than males with the $\epsilon 4$ allele (3.14 odds ratio) [152]. Women are also more likely to be diagnosed with PTSD at a rate two times more than men [153, 154]. Differences in PTSD prevalence are hypothesized to be influenced by a combination of biological and environmental factors [155]. Estradiol is known to affect the hypothalamus-pituitary-adrenal axis response to stress as well as cognitive processing of fear- and trauma-related memories [156]. The interaction of sex and *APOE* genotype in PTSD is relatively unexplored, though a recent study from our lab found sex-specific changes in trauma response in human apoE targeted replacement mice where E2 females

had higher plasma corticosterone, adrenal gland weight, and impaired spatial memory following trauma [141].

The distinct risks associated with sex and apoE isoform are especially intriguing when looking at the bi-directional link between PTSD and AD. A meta-analysis recently reported that diagnosis of PTSD in mid-life was specifically associated with diagnosis of LOAD and vascular dementia [157]. Other reports have found connections between chronic stress, amyloidgenesis, and amyloid-beta deposition [158, 159]. Patients diagnosed with PTSD were also found to have higher levels of DNA breaks in blood (measured by Fluorometric Detection of Alkaline DNA Unwinding) that were decreased following psychotherapy, which could be a possible avenue for accelerated aging and cognitive decline [160].

Notably, apoE isoform- and sex-dependent differences in behavioral and cognitive performance and brain activity are seen throughout life. School-aged girls with the $\epsilon 4$ allele had impaired visual and spatial memory compared to E3-carrying girls, but no differences were detected between E3- and E4-carrying boys [161]. In cognitively intact middle-aged people, E4 carriers (both male and female) showed increased blood-oxygen level dependent (BOLD) signal during a learning task despite no differences in cognitive performance [162]. Young adult E4-carriers also had lower rates of glucose metabolism in regions important for cognition, though no differences were seen in neuropsychological scores [163]. Similar differences were seen in H₂¹⁵O PET imaging in college-age women and men, where E4 carriers had greater temporal lobe activation during a non-verbal memory task [164]. Thus, distinctions in the mechanisms of learning and memory are likely present throughout life with and without overt, observable behavioral changes in the different apoE isoforms, or between the sexes.

Dissertation studies

In my review of the literature, I included investigations looking at an adaptive role of DSBs in IEG expression as it relates to apoE isoform. Data from these studies suggest that DSBs are formed in specific regional and genomic locations that lead to upregulation of specific genes, that these are dependent on the stimulus, and that this process is controlled by topoII β . Moreover, the data indicate that disruptions in the typical DSB and DNA damage response process—namely, increasing baseline levels or interfering with DSB repair—impairs learning and memory. However, it is still unclear if there is a causal link between DSBs, IEGs, and learning and memory. Moreover, it is unclear if DSBs differ based on risk factors for ACD and AD, such as sex, age, and apoE isoform.

My dissertation research is split into two major aims: 1) to determine if manipulating DSBs alters learning, memory, and IEG expression, and 2) to determine if age and apoE isoform differentially affect DSBs and IEGs. With these two aims, I set out to address 4 main questions: 1) does manipulating DSBs with common cancer treatments alter learning, memory, and/or IEG expression; 2) do males and females respond differently to these drugs; 3) are there changes in DSBs and/or IEG expression in the different apoE genotypes; and 4) do age and sex play a role?

In both aims, I used fear conditioning, which has clearly defined neurocircuitry, to be able to focus my attention on specific brain regions. I refer to baseline—and potentially harmful—DSBs as “pathological” DSBs, while DSBs that form following a stimulus “adaptive” DSBs. Brief descriptions of each of the experiments are indicated below, with more in-depth background, methodology, and results within each chapter.

Aim 1. I hypothesized that interfering with hippocampal DSBs during a learning task would impair hippocampus-dependent memory and change IEG expression. Turning to the cancer literature, we chose to use amifostine (WR-2721) to decrease DSBs and etoposide to increase DSBs before or after training C57Bl/6J (WT) mice in fear conditioning. Importantly, our first experiments involved a single, systemic (*i.p.*) administration of these compounds. We tested memory 24 h and 2 weeks later, and analyzed the IEGs cFos and Δ FosB, γ H2Ax, and NADPH levels in the hippocampus and amygdala. We predicted that amifostine would increase fear memory and that etoposide would impair it. We also predicted that cFos and Δ FosB levels would decrease following etoposide (**Fig. 1.3**). In addition to advancing our understanding of the role of DSBs in learning and memory, learning and memory-related effects of these drugs at doses much lower than used in the context of cancer therapy have not been described. Moreover, any possible sex-dependent differences in responses to these drugs have not been described.

Experiments with direct infusions provide more evidence for the specificity of timing and region-specific effects of interfering with DSBs. Therefore, we tested the effect of direct infusion of etoposide into the hippocampus of WT mice before being trained in fear conditioning. Again, we tested memory 24 h and 2 weeks later, and assessed the IEGs cFos and Arc, and γ H2Ax by immunohistochemistry in the hippocampus. We predicted that contextual fear memory (hippocampus-dependent) would be impaired after etoposide, but that cued fear memory (hippocampus-independent) would not. We also predicted that the IEG timeline would be interrupted, with cFos and Arc levels remaining high 5+ h after infusion based on previous data.

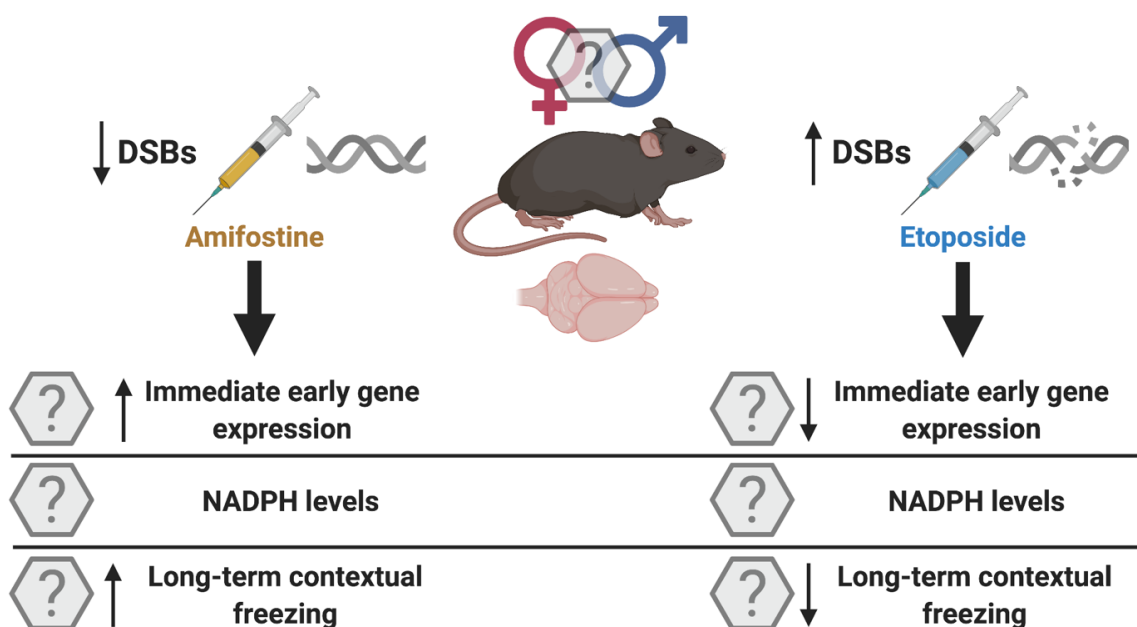


Fig. 1.3. Prediction of the effects of amifostine and etoposide on IEG expression and long-term memory. *Left:* Amifostine, which decreases DSBs, would lead to an increase in IEG expression, potentially affect NADPH levels, and increase long-term contextual freezing. *Right:* Etoposide, which increases DSBs, would lead to a decrease in IEG expression, potentially affect NADPH levels, and decrease long-term contextual freezing. *Middle:* Possible sex-dependent effects of these two compounds is unknown. Made with biorender.com.

Aim 2. I hypothesized that apoE isoforms differentially affect DSBs and IEG expression under baseline conditions and following a learning experience. Based on the known sex- and age-interactions, I further hypothesized that age and sex influence DSB and IEG expression. Using human apoE targeted replacement mice, I trained mice in a contextual fear conditioning paradigm known to show differences in extinction based on apoE isoform [165]. Using immunohistochemistry, whole-brain imaging, and chromatin immunoprecipitation followed by droplet digital polymerase chain reaction (ddPCR), we assessed the anatomic and genomic location of modulation of the IEGs cFos and Npas4, and the DSB repair protein 53BP1. We compared the signals at “peak” time (1.5 h after training) to those in behaviorally naïve and “recovery” (24 h after training) in genotype-, sex- and age-matched mice. We predicted that middle-aged E4 mice would show higher pathological levels of DSBs, and that they would be spread randomly through the genome. Further, we predicted to see decreased IEG expression following a learning task and impaired DSB repair at the 24 h time point in E4 compared to age- and sex-matched E2 and E3 mice (**Fig 1.4**). To the best of our knowledge, these are the first experiments to investigate possible differences in DSBs and IEGs based on apoE isoform.

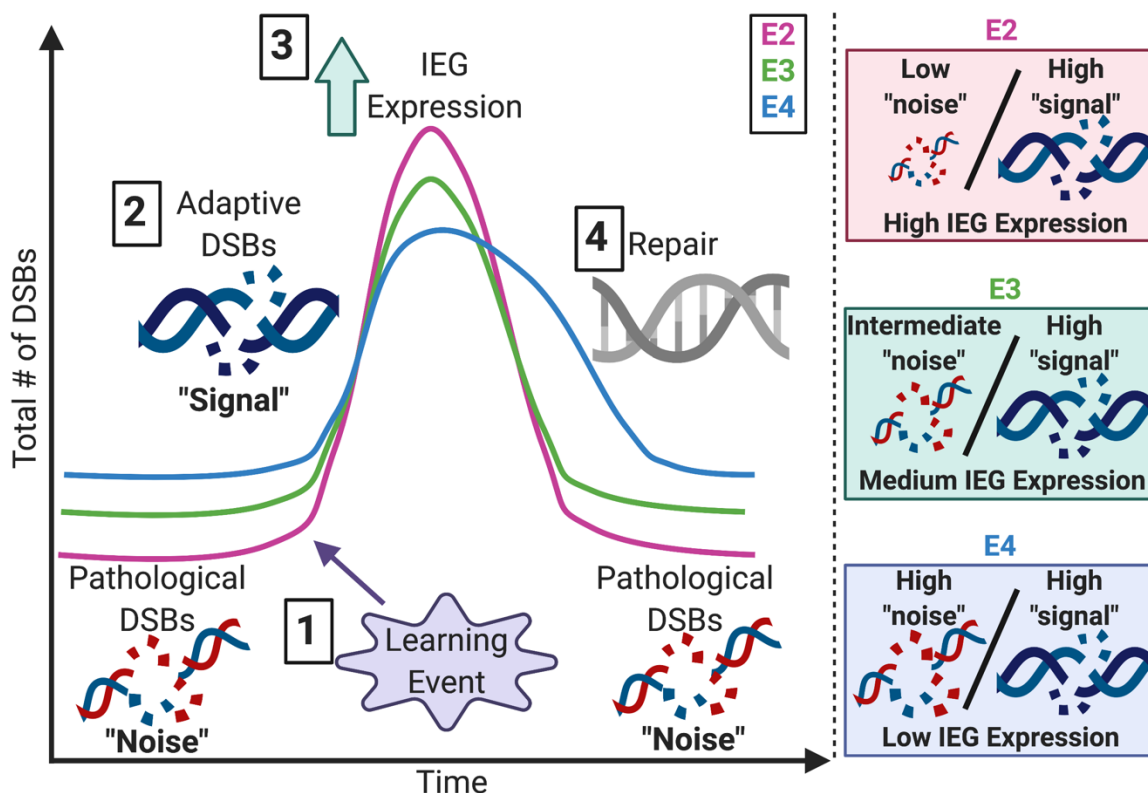


Fig 1.4. Predicted model of DSB and IEG expression in the different apoE genotypes. *Left:* Pathological (“noise”) DSBs exist at a low rate. After a stimulus, adaptive DSBs form that lead to expression of IEGs. DSBs are repaired within 24 h, possibly contributing to long-term memory. *Right:* Summarized hypotheses. ApoE isoforms have different levels of pathological DSBs, impeding the repair of adaptive DSBs, and different levels of peak IEG expression. Made with biorender.com.

These studies have several key innovative aspects. To the best of my knowledge, these experiments were the first investigation to explore 1) the direct effects of common cancer treatments on learning and memory in both sexes, 2) changes in IEG expression due to these drugs, and 3) the role of DSBs and IEGs in the apoE isoform-specific risks to develop AD and/or PTSD.

Chapter 2: Common cancer treatments targeting DNA double strand breaks affect long-term memory and relate to immediate early gene expression in a sex-dependent manner

Abstract

DNA double strand breaks (DSBs) have been highly studied in the context of cancers, as DSBs can lead to apoptosis or tumorigenesis. Several pharmaceuticals are widely used to target DSBs during cancer therapy. Amifostine (WR-2721) and etoposide are two commonly used drugs: amifostine reduces DSBs, whereas etoposide increases DSBs. Recently, a novel role for DSBs in immediate early gene expression, learning, and memory has been suggested. Neither amifostine nor etoposide have been assessed for their effects on learning and memory without confounding factors. Moreover, sex-dependent effects of these drugs have not been reported. We administered amifostine or etoposide to 3-4-month-old male and female C57Bl/6J mice before or after training in fear conditioning and assessed learning, memory, and immediate early genes. We observed sex-dependent baseline and drug-induced differences, with females expressing higher cFos and FosB levels than males. These were affected by both amifostine and etoposide. Post-training injections of amifostine affected long-term contextual fear memory; etoposide affected contextual and cued fear memory. These data support the hypothesis that DSBs contribute to learning and memory, and that these could play a part in cognitive side effects during common treatment regimens. The sex-dependent effects also highlight an important factor when considering treatment plans.

Introduction

Unrepaired DNA double strand breaks (DSBs) are signals for apoptosis [76] and drugs to induce DSBs or protect non-tumorous tissue from DSBs have been developed to treat cancers. Amifostine (WR-2721) is metabolized into the active agent WR-1065, and protects cells from DSBs by scavenging free radicals, inducing cellular anoxia, and condensing DNA [84, 166, 167]. Conversely, etoposide induces DSBs by stabilizing the complex of covalently-bound topoisomerase-II β to cleaved DNA [87]. Recent evidence suggests that DSBs may induce immediate-early gene (IEG) expression [97, 98].

The precisely timed expression of IEGs is essential for learning and memory [40]. The *fos* family of genes, including *fos* proto-oncogene (cFos) and FosB, are IEGs important in fear learning and memory [168]. cFos is transiently expressed, with low basal levels that rapidly increase upon stimulation, and return to baseline levels within hours [169]. FosB has similarly low basal levels, though the truncated form (Δ FosB) accumulates over time and persists for weeks following a stimulus [56]. Hippocampal cFos and Δ FosB are essential for contextual learning and hippocampal synaptic plasticity [50, 170]. However, the mechanism underlying the rapid expression of IEGs is unclear.

Following physiological stimulation, DSBs were detected on promoter regions of IEGs, including *fos* and *fosB*, leading to their up-regulation [98]. Transient increases in the DSB repair marker γ H2Ax were seen in relevant brain regions in mice following contextual learning [97, 98], and inhibition of DSB repair in the mouse prelimbic area prolonged IEG expression and impaired long-term memory [171]. Thus, DSB induction and timely repair may facilitate IEG transcription and learning. Relatedly, physiological DNA-damaging agents, such as reactive oxygen species (ROS) and superoxide, might contribute to learning and memory. Increasing and

inhibiting activity of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which creates ROS and superoxide, impairs learning and memory [172]. NADPH is a target for cancer treatments, as NADPH oxidases contribute to chronic gastrointestinal inflammation and cancer [173], and targeting NADPH homeostasis might reduce tumors [174].

Despite their wide clinical use, there is little information about how amifostine and etoposide affect learning and memory. Etoposide may modulate learning and memory by inducing IEG expression [98], though prolonged IEG expression and impaired long-term memory were seen following microinjections of etoposide in the mouse prelimbic area [171]. Assessment of hippocampal volume in pediatric brain cancer survivors indicated smaller hippocampal volume following chemotherapy treatment, which included etoposide and amifostine, that corresponded to verbal memory performance [175]. As etoposide and amifostine were used in combination with other chemotherapy agents, it is impossible to conclude which effects were caused by these specific drugs. To the best of our knowledge, investigations into the effects of amifostine alone have not been reported, nor have there been reports on timing of administration of these drugs related to learning and memory. Timing is important to consider, as irradiation with gamma rays or X-rays (known to induce DSBs) after fear learning increased long-term fear memory in mice [104], which directly contrasts to impaired memory when mice are irradiated prior to fear learning [176]. Preclinical research into sex-dependent effects of amifostine and etoposide is also lacking. In cancer patients, women clear plasma amifostine faster than men [89]. Etoposide also has a lower half-life in females, though clearance is similar between the sexes [90].

In this study, we investigated the effects of amifostine and etoposide on hippocampus-dependent and -independent fear conditioning [5] and IEG expression in male and female C57Bl/6J mice.

Methods:

Mice

Male and female C57Bl/6J (WT) mice ($n = 299$) purchased from Jackson Labs (Sacramento, CA) were used. Mice were 3-4 months old at the time of behavioral testing. All mice were group housed to 5/cage; starting 5 days before behavioral testing, mice were singly housed and provided with extra enrichment. Standard chow and water were provided *ad libitum*. Lights were on a 12-hour cycle; testing took place during the light cycle. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the AAALAC-certified Oregon Health and Science University (OHSU).

Compounds

Amifostine trihydrate (WR-2721) was purchased from Sigma Aldrich (Cat #1019406-150 MG) and diluted in saline solution the day prior to injection. Mice received a single intraperitoneal (*i.p.*) injection of one of six doses of amifostine: 0 (saline), 53.5, 80.25, 107, 160.5, or 214 mg/kg. These doses were chosen based on a range of typical doses tested in the context of radioprotection [167, 177].

Etoposide phosphate (etopophos) powder was purchased through the OHSU research pharmacy (E.R. Squibb & Sons, LLC, New Brunswick, NJ). It was diluted in saline to a 20 mg/mL stock solution and stored at -80°C until the day before use. Mice received a single *i.p.*

injection of etoposide (0, 35, or 70 mg/kg). These doses were chosen based on the reported range commonly used for acute dosing [178].

Behavioral Experiments

Experiment 1: Effects of pre-training injections on long-term memory

Amifostine was delivered *i.p.* (6 doses, $n = 8-12$ mice/sex/dose) 10 minutes before being placed into a sound-attenuating chamber for fear conditioning (Med Associates, Fairfax, VT). The timing between injection and training was chosen as amifostine peaks in brain tissue 7-15 min after a systemic injection [179]. A separate series of experiments were conducted to test the effects of etoposide. Etoposide was administered *i.p.* 90 min before fear training (3 doses, $n = 8-9$ mice/sex/dose). This route and time of injection were chosen after we assessed concentration of etoposide in the brain following *i.p.* or *i.v.* injection: as both routes showed similar brain levels 2 hours after injection, we proceeded with *i.p.* injections (data not shown).

We followed a similar fear conditioning paradigm as previously described [180]. Mice explored the box for a 2-minute baseline period, after which a 30-second tone (80 dB, 2800 Hz) played that co-terminated with a 2-s foot shock (0.5 mA); this was followed by a 90-s inter-stimulus interval (ISI). The tone-shock pairings were repeated a total of 4 times. All chambers were cleaned with 0.5% acetic acid between trials. The researcher doing the injections and the researcher handling the mice for behavior were distinct, and both were blinded to treatment groups during testing.

To assess long-term hippocampus-dependent and hippocampus-independent memory, mice underwent contextual and cued fear recall tests 24 h and 2 weeks after training as described [5, 104, 180, 181]. For the contextual recall test (hippocampus-dependent), mice were placed

into the same chamber for a period of 5 minutes; no tones and no shocks were administered. Chambers were cleaned with 0.5% acetic acid between contextual trials [104, 180]. For the cued recall test (hippocampus-independent), a distinct floor, ceiling, and smell were placed in the chambers. After a baseline period of 90 s, the tone was played for a 3-min period. Chambers were cleaned with 10% isopropanol between cued trials [181].

Twenty-four hours after the last recall test, mice were euthanized by rapid cervical dislocation. Brain tissue was collected: the amygdala and cortex were dissected from half of the animals, while the hippocampus and cortex were dissected from the other half to assess tissue for long-term changes in Δ FosB levels. Tissues were flash frozen in liquid nitrogen and stored at -80° C until use. An experimental timeline is illustrated in **Figure 2.1A** (made with Biorender.com).

Experiment 2: Effects of post-training injections on long-term memory

To ensure that differences in long-term memory were not a result of differences induced by either drug during acquisition, we injected animals immediately after fear training. Based on the results of experiment 1, we chose a single dose of amifostine (107 mg/kg) and a single dose of etoposide (35 mg/kg) to compare to post-training saline injections for these experiments ($n = 7/\text{dose/sex}$). Fear training, contextual, and cued recall tests were all performed in the same way as described above (**Fig. 2.1B**).

Experiment 3: Effects of pre-training injections on cFos and NADPH

To assess the effects of amifostine and etoposide on IEG expression, mice were euthanized by cervical dislocation 2 hours after systemic injection followed by fear training to

capture peak cFos signal [169]. For this experiment, we used 4 doses of amifostine ($n = 5$ mice/sex/dose: 0 (saline), 53.5, 107, or 214 mg/kg) or 3 doses of etoposide ($n = 5$ mice/sex/dose: 0 (saline), 35, or 70 mg/kg). Hippocampus and cortex were dissected from all animals and flash frozen in liquid nitrogen. Tissue was stored at -80°C until use (**Fig. 2.1C**).

Experiment 4: Effects of pre-training injections on DSBs

To assess the effects of amifostine on DSB formation in the hippocampus, mice were injected with saline, 107 mg/kg of amifostine, or 35 mg/kg of etoposide and euthanized immediately upon completion of fear conditioning via perfusion (**Fig. 2.1D**).

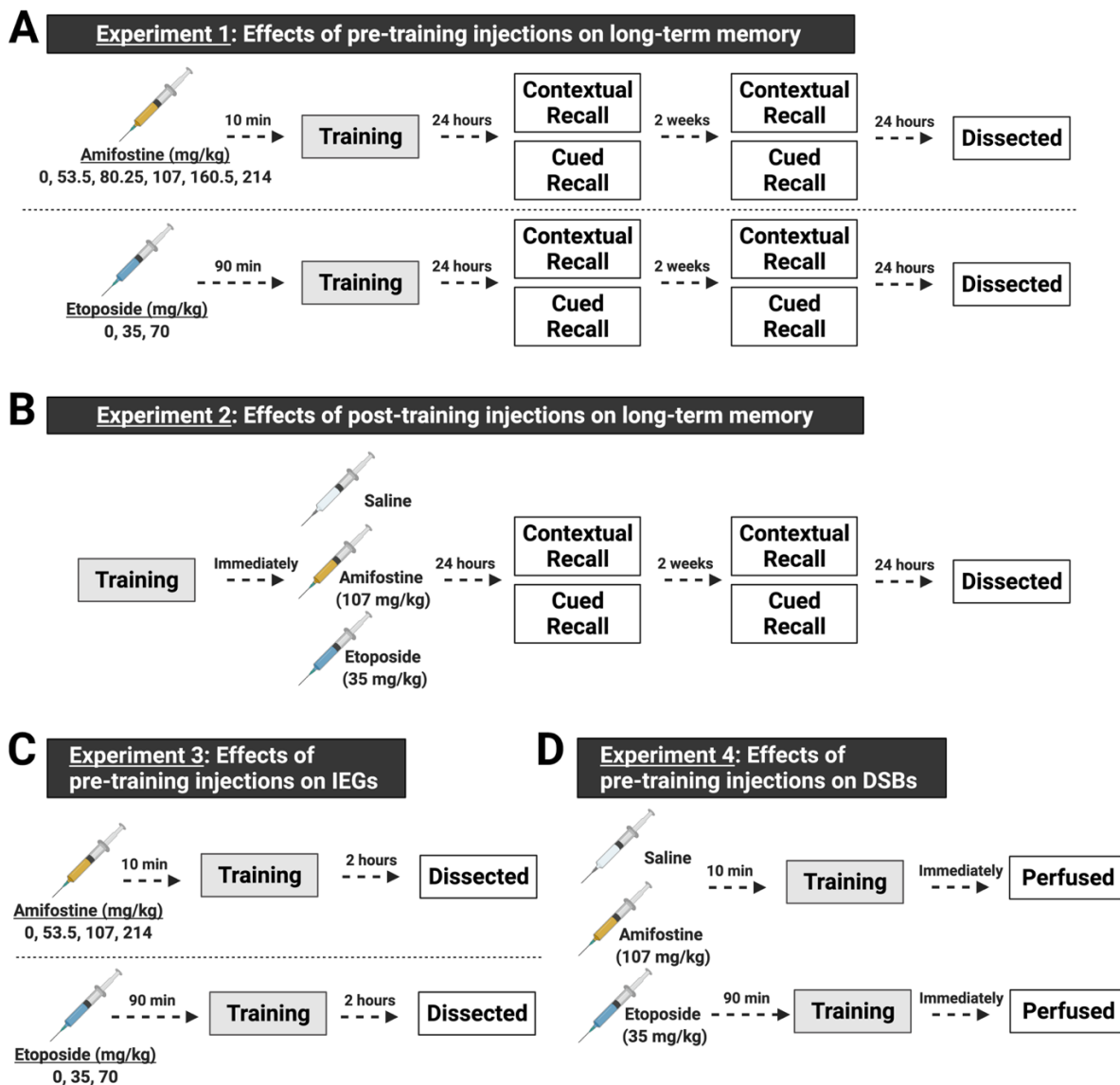


Figure 2.1. Schematic of the experimental design, made with Biorender.com. Both male and female C57Bl/6J mice were used in all experiments. **A)** Experiments for understanding the effects of pre-training injections on learning and memory. In experiment 1, mice received a single *i.p.* injection prior to being trained in fear conditioning. We tested 6 doses (including saline, $n = 8-12$ mice/sex/dose) of amifostine administered 10 min before fear training, or 3 doses (including saline, $n = 8-9$ mice/sex/dose) of etoposide administered 90 min before fear training. Twenty-four hours and 2 weeks later, mice underwent contextual and cued recall tests. One day after the last recall test, mice were euthanized, and brain tissue dissected. **B)** Experiments for understanding the effects of post-training injections on learning and memory. In experiment 2, mice were trained in fear conditioning and immediately received an *i.p.* injection of saline, 107 mg/kg amifostine, or 35 mg/kg of etoposide upon completion ($n = 7$ mice/sex/dose). Doses were picked based on the results of experiment 1. Mice underwent the same timeline for recall tests as in experiment 1. **C)** Experiments for understanding the effects of pre-training injections on immediate early genes. We tested 4 doses (including saline, $n = 5$ mice/sex/dose) of amifostine and 3 doses (including saline, $n = 5$ mice/sex/dose) of etoposide following the same timing as in experiment 1. Mice were euthanized 2 hours later to assess cFos expression. **D)** Experiments for understanding the effects of pre-training injections on DSB formation. Based on results from the previous experiments, we injected mice with saline, 107 mg/kg amifostine, or 35 mg/kg of etoposide before training in fear conditioning ($n = 3$ mice/sex/dose). Immediately upon completion, mice were perfused to assess DSB formation.

Western blots

Brain tissue was prepared as described [182]. Tissue was homogenized and sonicated in lysate buffer (1M Tris-Cl, pH 7.5; 6M NaCl; 10% SDS; 0.5M EDTA; Triton-X 100; Phosphatase Inhibitor #3, Roche, #05-892-970-001; Protease Inhibitor, Sigma-Aldrich, #P0044) and total protein concentrations determined with a Pierce BCA Kit (Thermo Fisher, #23227).

Samples were boiled in SDS buffer for 10 min and 10 μ g of protein loaded into wells of 10-20% Tris-Glycine gels. Gels were run for 75 min at 125 V to separate samples. Proteins were transferred to Immobilon-FL PVDF membranes (Millipore, #IPF00010) for 75 min at 30 V on ice. Total protein for quantification was assessed using REVERT™ Total Protein Stain (Li-Cor Biosciences, Lincoln, NE) and imaged on a Li-Cor Odyssey CLx. Blots were then blocked in Odyssey Blocking Buffer (Li-Cor Biosciences) for 1 h at room temperature and incubated overnight at 4° C with primary antibodies against Δ FosB (Cell Signaling, Cat# 14695S, rabbit, 1:1000), cFos (Santa Cruz, Cat #2119, mouse, 1:500), or γ H2Ax (Cell Signaling, Cat #9718, rabbit, 1:1000,). The following day, blots were washed and incubated in appropriate secondary antibodies (goat anti-rabbit IR800 CW, Li-Cor, #926-32211, 1:10,000; goat anti-mouse IR700 IR680 LT, Licor, #926-68050, 1:10,000) for 1 h at room temperature.

Images were analyzed with ImageJ software and normalized to total protein detected by the REVERT stain for comparison. More details can be found in Supplemental Materials.

ELISAs

cFos, FosB, and NADPH levels were analyzed using ELISA kits from MyBioSource (San Diego, CA) according to manufacturer directions. Hippocampus and cortical samples from experiment 3 were assessed for cFos (Cat# MBS2887418; HIP 1:10, CTX 1:100) and NADPH (Cat# MBS2605848; AMY 1:10, HIP 1:20, CTX 1:40); hippocampus samples were also assessed

for FosB (Cat# MBS9718096; HIP 1:10). Hippocampus and amygdala samples from experiment 1 were analyzed for FosB and NADPH levels. Standards were run in duplicate on all plates; samples were run singly. For analysis, all optical density readings were normalized to total protein determined by a Pierce BCA Kit (Thermo Fisher, #23227).

Immunofluorescence & Microscopy

Perfused brains from experiment 4 were cut into 40 μm sections using a Cryostat to assess the formation of γH2Ax foci, a common marker for DSB repair [97, 98]. We followed our standard immunofluorescent protocol [182]. Briefly, sections were rinsed in 1x PBS and blocked in 4% normal goat serum before overnight incubation in primary antibody targeting γH2Ax (rabbit-anti- γH2Ax , Cell Signaling, #9718, 1:500). On day 2, sections were rinsed and incubated in secondary antibody (goat-anti-rabbit, AlexaFluor594, Invitrogen, #A-11012, 1:250) for 2 hours. Following washes, 2.5 $\mu\text{g/mL}$ DAPI (Sigma D9542) in PBS was applied for 20 min. Sections were rinsed one more time, then slide mounted with CitiFluor CFMR2 Antifadent Solution, and sealed with Biotium CoverGrip Coverslip Sealant. Z-stack images at 0.5 μm steps were taken using a Zeiss LSM 980 with Airyscan 2 at 63x zoom.

ImageJ (NIH) was used to analyze γH2Ax foci. ROIs were created for each nucleus in the DAPI channel based on a single image in the center of the Z-stack. Within the mask, the number and size of γH2Ax foci were quantified.

Statistical Analysis

Researchers were blinded throughout all experiments. Data were assessed for normality of variance. Statistical analyses were performed using SPSS v.25 software (IBM, Armonk, NY)

or GraphPad v.7 software (Prism, San Diego, CA). Analyses for effects of amifostine and etoposide were performed separately. Sex and dose were used as between-group variables; when sex was not found to be significant, it was dropped from the statistical model. When sex was found significant, analysis of the sexes was done separately. Dunnett's *post hoc* test was used to compare doses against saline following all ANOVAs, as statisticians indicate that running *post hoc* tests in the absence of significant ANOVAs is informative and valid [183].

Fear conditioning: For analysis of training, data from all experiments with pre-training injections of amifostine were combined, and data from experiments with pre-training injections of etoposide were combined. Average motion and percent time freezing were analyzed using Analysis of Variance (ANOVAs). Total percent time freezing during the 24 h and 2 week contextual tests was analyzed with a repeated measures ANOVA; additionally, time freezing over the 5 minutes within each trial was analyzed with a repeated measures ANOVA. For the cued recall tests, percent time freezing during the tone at 24 h and 2 weeks was analyzed with a repeated measures ANOVA. A repeated measures ANOVA was also used to analyze percent time freezing during the baseline and tone periods for each recall test.

To assess the effects of pre- vs. post-training injections on contextual and cued recall, the corresponding doses of amifostine and etoposide from experiment 1 were analyzed against the 24 h and 2 week contextual and cued recall tests from experiment 2 with ANOVAs.

Molecular measures: For analysis of Western blots, bands of interest were normalized to total protein transferred to the blot and analyzed with a 2-way ANOVA. For analysis of ELISAs, optical density readings were interpolated based on the standard curve in each plate using GraphPad v. 7 and normalized to total protein determined with the BCA kit. For analysis of γ H2Ax foci, the size and count of foci were normalized to total DAPI area. A 2-way ANOVA

was used to analyze differences based on dose and sex. In the case of experiments 2 and 4, the same saline control group was used for comparison against amifostine and etoposide respectively, with corrections for type I statistical errors applied.

Results

Pre- and post-training injections of 107 mg/kg of amifostine increase long-term contextual, but not cued, freezing in male mice

Fig. 2.1 shows a timeline of all experiments. Mice received an intraperitoneal (*i.p.*) injection of saline, 53.5, 80.25, 107, 160.5, or 214 mg/kg amifostine 10 min prior to being trained in fear conditioning. Contextual and cued memory were tested 24 h and 2 weeks later (**Fig. 2.1A**).

Amifostine had no effects on average motion or percent time freezing during fear training (**Table 2.1**). All groups increased freezing over the four tones ($p < 0.001$) and four inter-stimulus intervals (ISI; $p < 0.001$, **Fig. 2.2B**), indicating similar fear learning. Females froze more than males during the ISIs ($p = 0.046$). Similarly, we detected no effects of amifostine on motion during the ISIs or tones.

Analysis of total percent time freezing during the contextual recall tests revealed an effect of dose ($p < 0.001$) and sex ($p = 0.046$). Dunnett's post hoc test indicated that mice injected with 107 mg/kg froze more than controls ($p = 0.014$). When we analyzed sexes separately, we found an effect of dose in both males ($p = 0.004$; **Fig. 2.2C, left**) and females ($p = 0.036$; **Fig. 2.3C, right**). Dunnett's post hoc test indicated that males injected with 107 mg/kg froze more than saline-injected controls ($p = 0.017$), but this did not reach significance in females.

Conversely, percent time freezing during the tone in the cued recall tests was not different between groups. All mice showed the expected increase in freezing when the tone was played at both 24 h ($p < 0.001$; **Supplemental Fig. 2.1A**) and 2 weeks ($p < 0.001$; **Supplemental Fig. 2.1B**). There was a time-by-sex interaction at 2 weeks ($p < 0.001$), where females froze less during the baseline period than males. When males and females were analyzed separately, there was a time-by-dose interaction in males only ($p = 0.043$), which appeared to be driven by the saline and 53.5 mg/kg groups showing similar freezing levels during the baseline and tone. No effects were found in females at the 2-week time point.

Hippocampus and amygdala from these animals were assessed for Δ FosB levels by Western blot (**Fig. 2.2D**). Analysis of hippocampal Δ FosB normalized to total protein revealed that females had higher levels than males ($p = 0.0029$; **Fig. 2.2E, left**). There was also a trend towards an effect of dose ($p = 0.0521$). When sexes were analyzed separately, no effect of dose was seen in males ($p = 0.6366$). Conversely, an effect of dose was seen in females ($p = 0.0244$), with Dunnett's post hoc test indicating a trend towards a decrease in females that received 107 mg/kg ($p = 0.0569$). Amygdala Δ FosB levels were also higher in females than males ($p < 0.0001$), but were not affected by dose (**Fig. 2.2E, right**).

We next trained a cohort of animals in the same fear conditioning task and immediately delivered an *i.p.* injection of saline or 107 mg/kg of amifostine upon completion (**Fig. 2.2F**). Injection time (pre- vs. post-training) affected baseline motion, where animals that received pre-training injections moved more at baseline than animals that received post-training injections regardless of drug ($p < 0.001$, **Fig. 2.2G**). No differences were detected in shock response or percent time freezing during training (**Table 2.1**).

When we compared contextual freezing between pre- and post-training injections at 24 h and 2 weeks, a time by sex interaction ($p = 0.004$) and dose by sex interaction ($p = 0.033$) led us to split males and females for analysis. Amifostine increased time freezing in male mice ($p = 0.015$) regardless of injection time. Conversely, there were no effects or interactions in females.

Analysis of time freezing during the tone in the cued recall tests at 24 h and 2 weeks revealed that females froze more than males ($p = 0.029$) and a trend towards an effect of dose ($p = 0.088$). There were no significant differences based on injection time ($p = 0.736$), and no significant differences were found when we analyzed the sexes separately. All mice showed the expected freezing increase in response to the tone at 24 h ($p < 0.001$; **Supplemental Fig. 2.1C**) and 2 weeks ($p < 0.001$; **Supplemental Fig. 2.1D**), though this was distinct between males and females, shown by a time by sex interaction at both time points ($p = 0.010$, $p < 0.001$, respectively). When we split males and females for analysis, we found an effect of injection time in females only at 24 h ($p = 0.046$), where post-training injections led to higher freezing. There were no effects detected during the 2 week recall test.

We also assessed hippocampal Δ FosB in animals that received post-training injections (**Fig. 2.2I**). When we compared pre- and post-training injections of saline or 107 mg/kg of amifostine in males and females, we found an effect of injection time ($p = 0.001$), dose ($p = 0.049$), and sex ($p = 0.011$), and a dose by time interaction ($p = 0.045$). To clarify these effects, we split males and females. We detected an effect of injection time in males ($p = 0.041$, **Fig. 2.2I left**) and an effect of dose in females ($p = 0.044$, **Fig. 2.2I right**). Post hoc tests did not indicate any differences between specific groups, though these results indicate intriguing sex- and time-dependent effects of amifostine.

Table 2.1. Fear conditioning motion and time freezing during training for mice in Experiment 1 and 2 investigating the effects of amifostine. No differences were detected between sexes or doses.

Pre-Training							Post-Training	
Injections							Injections	
Dose (mg/kg)	0	53.5	80.25	107	160.5	214	0	107
Baseline	247.06 ±	254.44 ±	242.39 ±	253.51 ±	211.07 ±	235.21 ±	<i>See</i>	<i>See</i>
Average Motion (au)	7.78	7.74	6.24	11.26	11.62	8.88	<i>Fig. 2</i>	<i>Fig. 2</i>
Shocks	774.76 ±	837.52 ±	717.34 ±	826.64 ±	804.86 ±	801.35 ±	734.39 ±	735.37 ±
Average Motion (au)	33.18	42.79	43.3	52.10	32.83	36.08	42.17	36.30
Last Tone	29.57 ±	25.00 ±	30.34 ±	34.10 ±	31.71 ±	35.20 ±	35.94 ±	32.38 ±
% Time Freezing	4.02	2.90	3.75	4.74	6.25	4.67	6.69	4.29
Last ISI	<i>See</i>	<i>See</i>	<i>See</i>	<i>See</i>	<i>See</i>	<i>See</i>	24.37 ±	26.87 ±
% Time Freezing	<i>Fig. 2</i>	<i>Fig. 2</i>	<i>Fig. 2</i>	<i>Fig. 2</i>	<i>Fig. 2</i>	<i>Fig. 2</i>	4.28	4.97

All data are presented as means ± SEM.

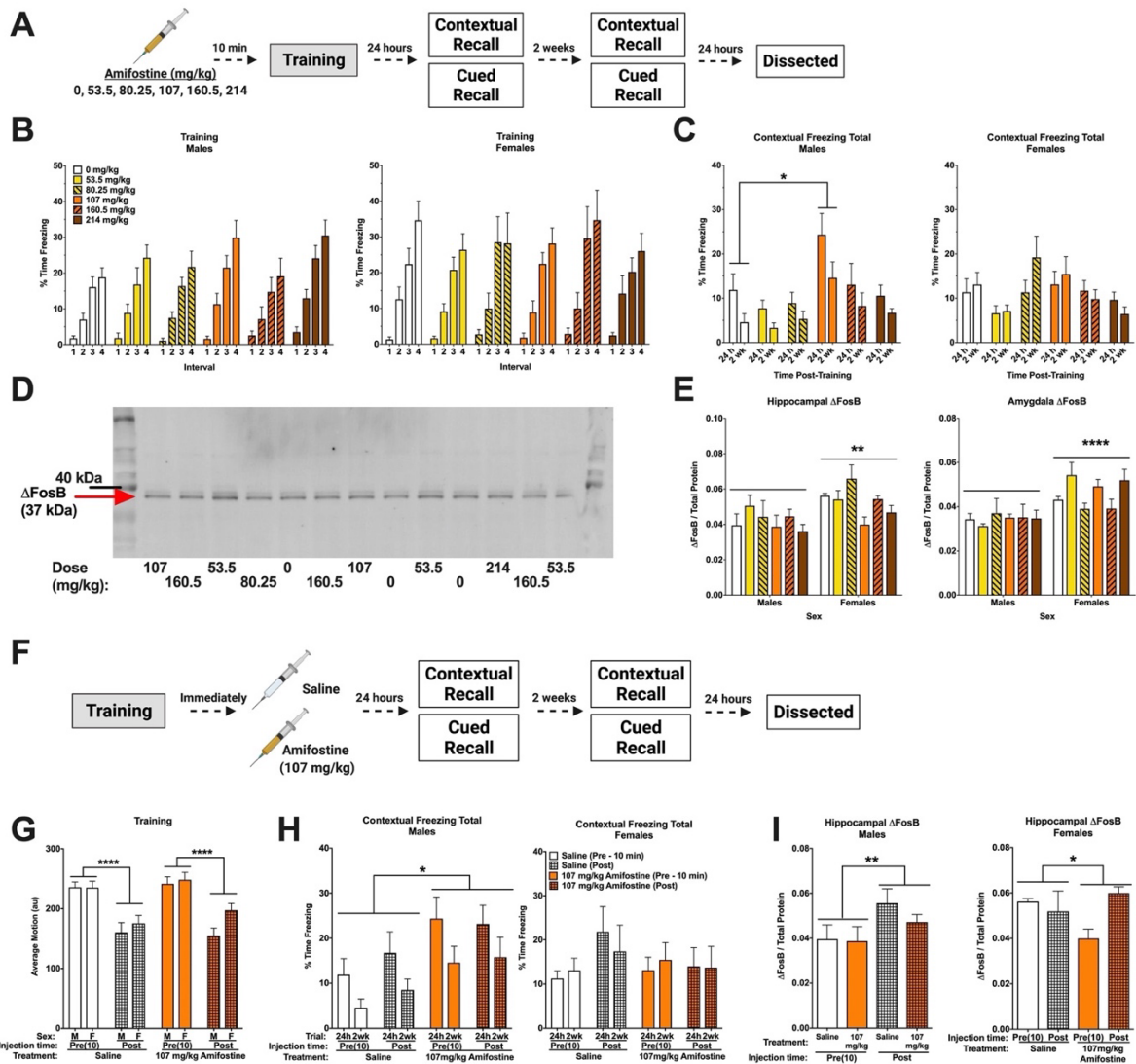


Figure 2.2. Pre- and post-training injections of amifostine lead to long-term increases in contextual freezing in males. **A**) Experiment 1 design. **B**) Percent time freezing during the inter-stimulus intervals on training day. All mice showed similar increases in time freezing, indicating fear learning. **C**) Percent time freezing during the 5-min contextual recall tests done at 24 h and 2 weeks in males (left) and females (right). In males, a significant main effect of dose was detected ($F(5,54) = 3.999, p = 0.004$) with males injected with 107 mg/kg freezing more than 0 mg/kg ($p = 0.017$, Dunnett's post hoc). In females, there was a significant main effect of dose detected ($F(5,54) = 2.588, p = 0.036$). Dunnett's post hoc test did not indicate any significant differences compared to the 0 mg/kg group. **D**) Representative Western blot for Δ FosB. **E**) Quantification of hippocampal (left) and amygdala (right) Δ FosB levels. A 2-way ANOVA for hippocampal Δ FosB showed a significant main effect of sex ($F(1,47) = 9.908, p = 0.0029$) and a trend towards a main effect of dose ($F(5,47) = 2.387, p = 0.0521$). Sexes were split for analysis, and revealed a main effect of dose in females ($F(5,23) = 3.202, p = 0.0244$), with Dunnett's post hoc test indicating a trend towards lower Δ FosB in the 107 mg/kg group compared to the 0 mg/kg group ($p = 0.0569$). There was no effect detected in males ($p = 0.6366$). A 2-way ANOVA for amygdala levels also indicated a significant main effect of sex ($F(1,47) = 21.6, p < 0.0001$), but no differences based on dose were detected. **F**) Experiment 2 design. **G**) Average baseline motion during the training session. Regardless of dose, pre-training injections increased average baseline motion ($F(1,84) = 50.11, p < 0.001$). **H**) Percent time freezing during the 24 h and 2-week contextual recall tests in males (left) and females (right). Both pre- and post-training injections of 107 mg/kg of amifostine increased percent time freezing in male mice ($F(1,29) = 6.741, p = 0.015$). 107 mg/kg of amifostine did not affect percent time spent freezing in female mice ($p = 0.611$). **I**) Hippocampal Δ FosB levels following pre- and post-training injections of saline or 107 mg/kg in males (left) and females (right). A 3-way ANOVA revealed a main effect of injection time ($F(1,42) = 7.132, p = 0.001$), dose ($F(1,42) = 4.108, p = 0.049$), and sex ($F(1,42) = 8.265, p = 0.011$), and a dose by time interaction ($F(1,42) = 4.284, p = 0.045$). Sexes were split for further analysis; an effect of time was found in males ($F(1,21) = 4.724, p = 0.0413$). In females, an effect of dose was found ($F(1,21) = 4.601, p = 0.0438$). Data show averages $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Amifostine decreases hippocampal DSBs but does not change sex-dependent cFos levels

We used a separate cohort of mice to assess the immediate effects of pre-training injections of amifostine on DSBs and IEGs. Hippocampal tissue from animals euthanized 2 hours after training was assessed for cFos, FosB, and NADPH (**Fig. 2.3A**). Analysis of hippocampal cFos by ELISA indicated that females had higher levels than males ($p = 0.0068$; **Fig. 2.3C**), which was confirmed with Western blot ($p = 0.0180$, **Table 2.2**). No sex or dose differences were detected in hippocampal FosB or NADPH, nor in γ H2Ax when assessed with Western blot (**Table 2.2**).

No differences based on dose or sex were detected when cortical tissue was analyzed for cFos and NADPH levels; however, Dunnett's post hoc test indicated a trend towards a difference between 214 mg/kg and 0 mg/kg in females ($p = 0.084$, **Fig. 2.3D**).

To determine the effects of systemically administered amifostine on DSBs, a cohort of animals received injections of saline or 107 mg/kg of amifostine, were trained in fear conditioning, and euthanized immediately by perfusion for immunohistochemistry (**Fig. 2.3B**). Analysis of γ H2Ax foci in the CA1 region of the hippocampus indicated a decrease in both males and females that received amifostine compared to saline ($p = 0.0342$; **Fig. 2.3E**). Representative images can be seen in **Fig. 2.3F**.

Table 2.2. Molecular measures from mice in Experiments 1 and 3. In the amifostine cohorts, there were no differences detected based on dose or sex for hippocampal FosB, NADPH, or γ H2Ax, nor in cortical NADPH. In the etoposide cohorts, there were no differences detected based on dose or sex for hippocampal FosB, cFos, or γ H2Ax, nor in cortical NADPH.

Amifostine				
Dose (mg/kg)	0	53.5	107	214
Hippocampal FosB (pg/ug)	0.33 $\pm$ 0.006	0.48 $\pm$ 0.014	0.40 $\pm$ 0.007	0.41 $\pm$ 0.007
Hippocampal NADPH (ug/mg)	40.99 $\pm$ 0.25	46.06 $\pm$ 0.21	41.48 $\pm$ 0.23	46.97 $\pm$ 0.17
Hippocampal γH2Ax (yH2Ax / Total Protein)	0.087 $\pm$ 0.004	0.0971 $\pm$ 0.006	0.0859 $\pm$ 0.004	0.091 $\pm$ 0.005
Cortical NADPH (ug/mg)	35.88 $\pm$ 0.33	40.66 $\pm$ 0.30	38.11 $\pm$ 0.33	31.50 $\pm$ 0.25
Etoposide				
Dose (mg/kg)	0	35	70	
Hippocampal FosB (pg/ug)	0.302 $\pm$ 0.003	0.394 $\pm$ 0.006	0.288 $\pm$ 0.004	
Hippocampal cFos (ug/mg)	4.71 $\pm$ 0.29	5.06 $\pm$ 0.15	5.03 $\pm$ 0.37	
Hippocampal γH2Ax (yH2Ax / Total Protein)	0.116 $\pm$ 0.017	0.093 $\pm$ 0.010	0.10 $\pm$ 0.012	
Cortical NADPH (ng/ug)	2.92 $\pm$ 0.16	2.87 $\pm$ 0.13	2.62 $\pm$ 0.17	

All data are presented as means $\pm$ SEM.

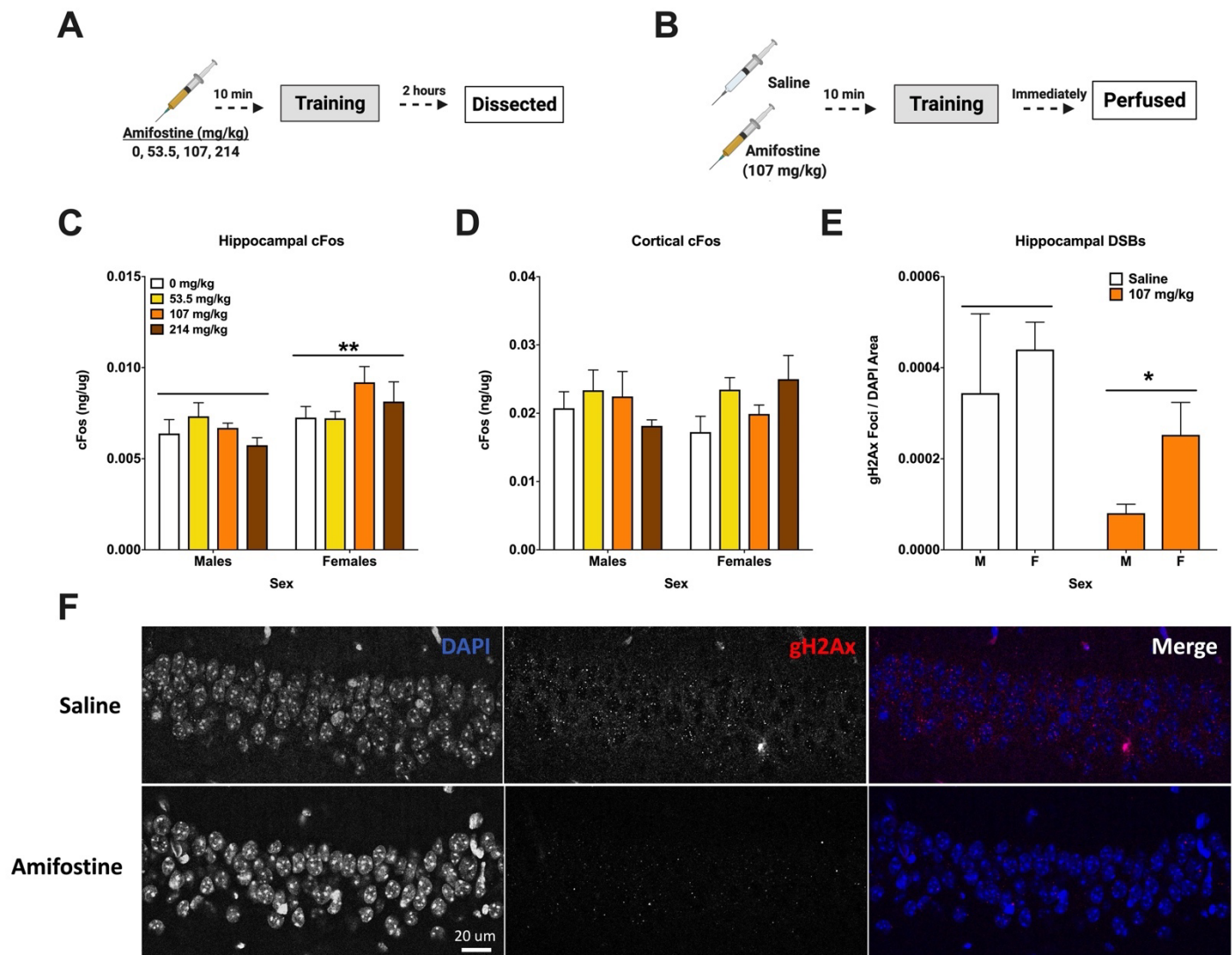


Figure 2.3. Amifostine decreases hippocampal DSBs but not cFos. **A)** Experiment 3 design. **B)** Experiment 4 design. **C)** Hippocampal cFos levels were measured by ELISA. Females showed a higher levels of hippocampal cFos than males ($F(1,32) = 8.387$, $p = 0.0068$), which was confirmed by Western blot analysis ($F(1,32) = 6.222$, $p = 0.0180$). No effect of dose was detected. **D)** Cortical cFos levels were measured by ELISA. There were no overall main effects detected, however Dunnett's post hoc test revealed a trend towards higher cFos in females injected with 214 mg/kg than saline controls ($p = 0.084$). **E)** Quantification of γ H2Ax foci per DAPI area. An injection of 107 mg/kg of amifostine prior to fear training decreased γ H2Ax in the CA1 ($F(1,20) = 5.167$, $p = 0.0342$). **F)** Representative images for γ H2Ax IHC of the CA1. Data show averages $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$.

Post-training injections of etoposide decrease cued freezing in females

We next assessed how pre-training injections of etoposide affect learning, memory, and IEG expression. Mice received *i.p.* injections of saline, 35, or 70 mg/kg of etoposide 90 min before being trained in fear conditioning. Contextual and cued memory were assessed 24 h and 2 weeks later (**Fig. 2.4A**).

Analysis of the effects of etoposide on learning revealed that females moved more than males during the initial baseline period ($p = 0.044$), though there was no effect of dose. There were no differences during shocks or in percent time freezing during the tones (**Table 2.3**). However, percent time freezing during the inter-stimulus intervals was higher in females ($p = 0.000$), and etoposide dose affected freezing based on sex ($p = 0.010$). Males showed an effect of dose ($p = 0.020$), with Dunnett's post hoc test revealing that mice injected with 70 mg/kg froze more during the intervals than controls ($p = 0.0106$; **Fig. 2.4B, left**). This effect was not detected in females ($p = 0.216$; **Fig. 2.4B, right**), suggesting that pre-training injections of etoposide had sex-dependent effects on learning. Moreover, there were no dose-dependent differences on average motion during the intervals ($p = 0.872$), indicating that etoposide did not affect motion.

There were no differences detected when time freezing during the contextual recall tests was analyzed (**Fig. 2.4C**). However, minute-by-minute analysis of the 2 week trial revealed a significant effect of dose in females only ($p = 0.0149$; **Supplemental Fig. 2.2A**), suggesting a possible subtle, female-specific increase. No differences were detected when time freezing during the tone in the cued recall tests at 24 h and 2 weeks was analyzed (**Supplemental Fig. 2.2B**). All mice showed the expected increase in freezing between baseline and the tone at 24 h ($p < 0.001$; **Supplemental Fig. 2.2C**) and 2 weeks ($p < 0.001$; **Supplemental Fig. 2.2D**), with no differences detected based on dose or sex at either time point.

Western blot was used to assess Δ FosB levels in hippocampus and amygdala from these animals (**Fig. 2.4D**). Analysis of hippocampal Δ FosB revealed an effect of dose ($p = 0.0331$), but no effect of sex and no sex by dose interaction. When we removed sex as a factor from the statistical model, this effect of dose still held ($p = 0.040$), though Dunnett's post hoc testing did not indicate any differences compared to the saline-injected group (**Fig. 2.4E, left**). Analysis of amygdala Δ FosB by Western blot indicated a trend towards a difference based on sex ($p = 0.0660$), but not dose (**Fig. 2.4E, right**).

We next tested the effects of 35 mg/kg of etoposide administered after training on memory (**Fig. 2.4F**). As observed before, pre-training injections led to an overall increase in baseline motion compared to post-training injections regardless of drug ($p < 0.05$; **Fig. 2.4G**). No other differences were detected during training, with all animals showing the expected increase in freezing during training (**Table 2.3**). Analysis of percent time freezing during the 24 h and 2 week contextual trials revealed that females froze more than males ($p = 0.033$). When sexes were analyzed separately, we detected a trend towards a drug by injection time interaction ($p = 0.055$), and no effects of drug ($p = 0.409$) or injection time ($p = 0.445$) in male mice (**Fig. 2.4H, left**). In females, we again observed a trend toward a drug by injection time interaction ($p = 0.070$) and a trend towards an effect of injection time ($p = 0.089$). Subsequent pairwise comparison of injection time revealed that females injected with 35mg/kg of etoposide after training froze significantly less than those that received pre-training etoposide injections ($p = 0.003$; **Fig 2.4H, right**).

Analysis of freezing during the tone at 24 h and 2 weeks also indicated an effect of sex ($p = 0.009$), an effect of injection time ($p = 0.002$) and an injection time by dose interaction ($p = 0.010$). When we analyzed the sexes separately, we found a trend toward an effect of injection

time ($p = 0.051$) and a significant drug by injection time interaction ($p = 0.048$) in males. Post hoc analysis showed that post-training injections in males decreased freezing ($p = 0.035$; **Fig. 2.4I, left**). In females, we found a significant effect of injection time ($p = 0.016$), as well as a trend toward an injection by drug interaction ($p = 0.087$). Subsequent analysis indicated that the effect of injection time was driven by the females that received post-training etoposide ($p = 0.007$; **Fig. 2.4I, right**). Assessment of freezing at baseline and during the tone showed that females injected with 35 mg/kg of etoposide after training had a blunted response to the tone at both 24 h ($p = 0.035$) and 2 wks ($p = 0.004$), which was not seen in males (**Supplemental Fig. 2.3A-D**).

Hippocampal Δ FosB was also analyzed in these animals compared to pre-training injections at the same doses. We found a significant effect of injection time ($p < 0.0001$) and trends towards a sex by dose interaction ($p = 0.064$) and a sex by time interaction ($p = 0.059$). Analysis in males only showed this same effect of injection time ($p = 0.0061$) but no effect of dose ($p = 0.199$). *Post hoc* comparisons to identify the time differences showed that mice that received saline pre-training had lower hippocampal Δ FosB than 35 mg/kg post-training ($p = 0.036$; **Fig. 2.4J left**). Analysis in females only also showed an effect of time ($p < 0.0001$) and a trend towards an effect of dose ($p = 0.059$); post hoc analysis showed that mice injected with 35 mg/kg etoposide pre-training injections had lower levels than post-training saline ($p = 0.0005$) and post-training 35 mg/kg mice ($p = 0.002$; **Fig. 2.4J right**). Again, these data highlight the importance of sex and timing of administration when assessing effects of etoposide.

Table 2.3. Fear conditioning motion and time freezing during training for mice in experiment 1 and 2 investigating the effects of etoposide. No differences were detected between sexes or doses.

Dose (mg/kg)	Pre-Training Injections			Post-Training Injections
	0	35	70	35
Baseline				
Average Motion (au)	214.16 ± 9.65	210.26 ± 10.52	202.60 ± 10.07	<i>See Fig. 4</i>
Shocks				
Average Motion (au)	899.24 ± 45.42	797.34 ± 38.86	841.68 ± 39.77	845.82 ± 48.51
Last Tone % Time Freezing	28.17 ± 4.59	32.95 ± 5.25	30.02 ± 6.38	30.44 ± 5.15

All data are presented as means ± SEM.

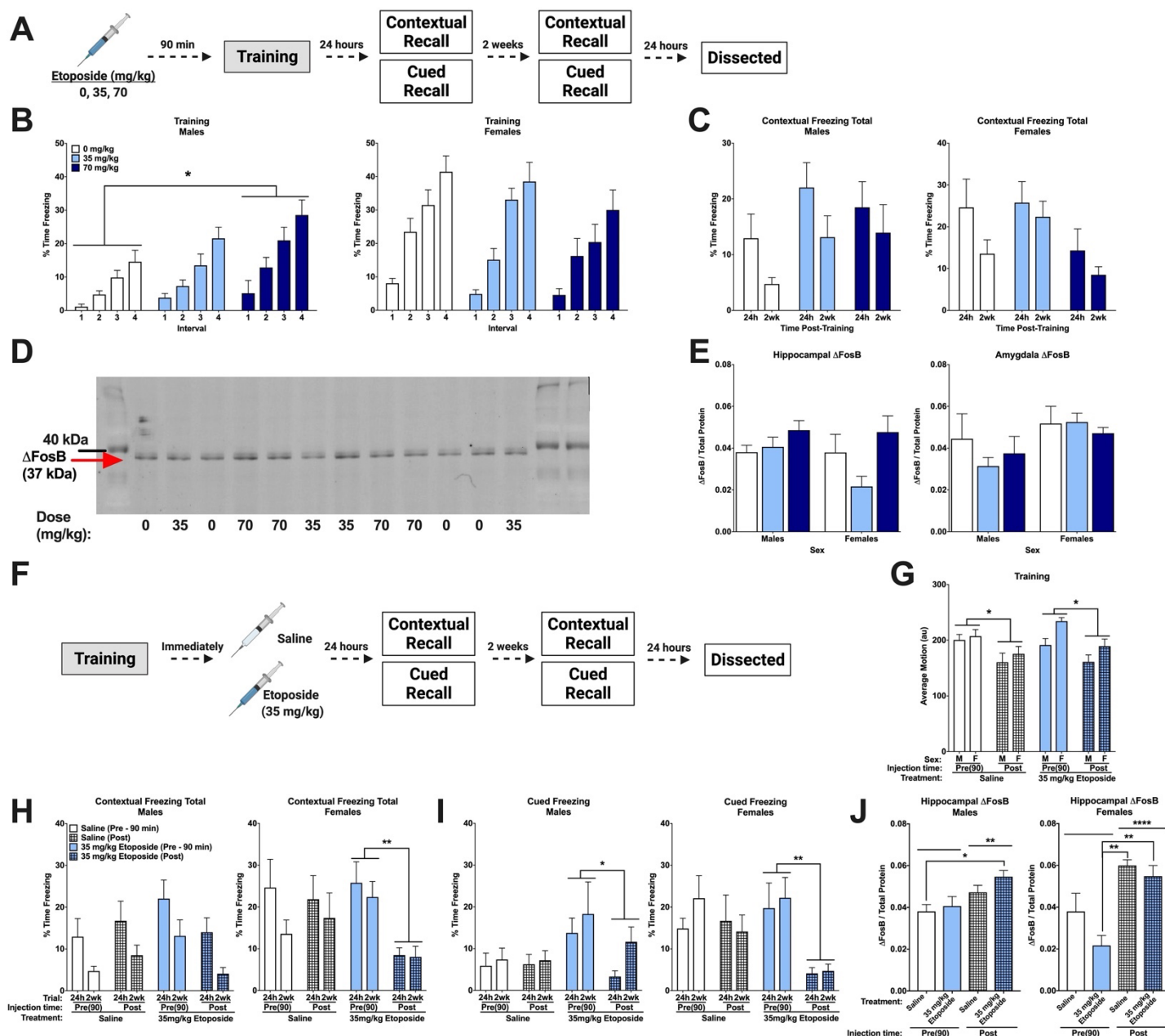


Figure 2.4. Post-training injections of etoposide lead to long-term decreases in contextual and cued freezing in females. **A)** Experiment 1 design. **B)** Percent time freezing during the inter-stimulus intervals on training day. A repeated measures ANOVA in males (*left*) showed a main effect of dose ($F(2,37) = 4.358, p = 0.020$) with Dunnett's post hoc test indicating that males injected with 70 mg/kg froze more than 0 mg/kg ($p = 0.0106$). There was no main effect of dose detected in female mice ($p = 0.216$, *right*). **C)** Total percent time freezing in males (*left*) and females (*right*) during the 24 h and 2 week contextual trials. No significant differences were detected. **D)** Representative Western blot for Δ FosB. **E)** Quantification of hippocampal (*left*) and amygdala (*right*) Δ FosB. A 2-way ANOVA of hippocampal levels revealed a significant main effect of dose ($F(2,18) = 4.143, p = 0.0331$), though no differences compared to the saline injections were detected with Dunnett's post hoc test. Analysis of amygdala levels indicated a trend towards females having higher levels than males ($F(1,19) = 3.806, p = 0.0660$). **F)** Experiment 2 design. **G)** Average baseline motion during the training session. Regardless of dose, pre-training injections increased average baseline motion ($F(1,73) = 17.919, p < 0.001$). Males also moved less than females ($F(1,73) = 7.239, p = 0.009$). **H)** Percent time freezing during the 24 h and 2-week contextual recall tests in males (*left*) and females (*right*). In males, we found a trend towards a significant dose by injection time interaction ($F(1,27) = 4.024, p = 0.055$), with a trend towards a difference between pre- and post-training injections of 35 mg/kg etoposide ($p = 0.087$). In females, we saw a trend toward a main effect of injection time ($F(1,25) = 3.317, p = 0.089$) and a trend towards a dose by injection time interaction ($F(1,25) = 3.573, p = 0.070$). Comparison of injection time revealed a difference between pre- and post-injections of 35 mg/kg etoposide ($p = 0.003$). **I)** Percent time freezing during the tones at 24 h and 2-weeks after training in males (*left*) and females (*right*). Repeated measures analysis in males revealed a significant dose by injection time interaction ($F(1,27) = 4.292, p = 0.048$) and a trend towards a main effect of dose ($F(1,27) = 4.185, p = 0.051$). Pre- and post-training injections of 35 mg/kg etoposide were significantly different ($p = 0.035$). In females, a significant main effect of injection time ($F(1,26) = 6.631, p = 0.016$) and trend toward a dose by injection time interaction ($F(1,26) = 3.156, p = 0.087$) were found. A significant difference between pre- and post-training injections of 35 mg/kg etoposide ($p = 0.007$) was found. **J)** Hippocampal Δ FosB quantification in males (*left*) and females (*right*) that received pre- or post-training injections of saline or 35 mg/kg of etoposide. A 3-way ANOVA showed a significant effect of injection time ($F(1,37) = 27.412, p < 0.0001$) and a trend towards a sex by dose ($F(1,37) = 3.641, p = 0.064$) and sex by injection time interaction ($F(1,37) = 3.788, p = 0.059$). When split, males showed a significant effect of injection time ($F(1,19) = 9.526, p = 0.0061$) with post hoc tests indicating a difference between mice injected with saline pre-training compared to mice injected with etoposide post-training ($p = 0.0359$). Females also showed a significant effect of injection time ($F(1,18) = 26.95, p < 0.0001$) and a trend towards a dose difference ($F(1,18) = 4.065, p = 0.059$). Post hoc tests indicated that pre-training injections of etoposide had lower Δ FosB than post-training saline ($p = 0.0005$) and post-training etoposide ($p = 0.0020$). Data show averages $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$.

Etoposide decreases hippocampal NADPH in females and hippocampal DSBs in both sexes

We used a separate cohort of animals to assess the immediate effects of pre-training injections of etoposide on DSBs and IEGs. Hippocampal tissue from animals euthanized 2 hours after training was assessed for cFos, FosB, and NADPH (**Fig. 2.5A**). Males had higher NADPH levels than females ($p = 0.0002$), and NADPH levels were affected by dose ($p = 0.0285$). When we split sexes for analysis, it was clear that this was driven by the females, as an effect of dose was detected in females ($p = 0.0368$), but not males ($p = 0.526$; **Fig. 2.5C**). Dunnett's post hoc test indicated that both the 35 mg/kg and 70 mg/kg groups had lower NADPH levels than 0 mg/kg ($p = 0.041$ and $p = 0.033$, respectively). There were no differences detected for cFos, FosB, or γ H2Ax when measured by Western blot or ELISAs (**Table 2.2**).

Cortical tissue was also analyzed for cFos and NADPH levels. Analysis of cortical cFos levels did not indicate a dose difference in females ($p = 0.100$), but Dunnett's post hoc test revealed a trend towards a difference between 0 and 70 mg/kg ($p = 0.0648$). This was not seen in males (**Fig. 2.5D**). Unlike the hippocampus, no differences in cortical NADPH were detected (**Table 2.2**).

Another cohort received either saline or 35 mg/kg of etoposide prior to training and were euthanized immediately upon completion (**Fig. 2.5B**). Analysis of hippocampal DSBs indicated that 35 mg/kg of etoposide decreased γ H2Ax foci per DAPI area compared to saline controls in both male and female mice ($p = 0.0285$; **Fig. 2.5E**). Representative images can be seen in **Fig. 2.5F**.

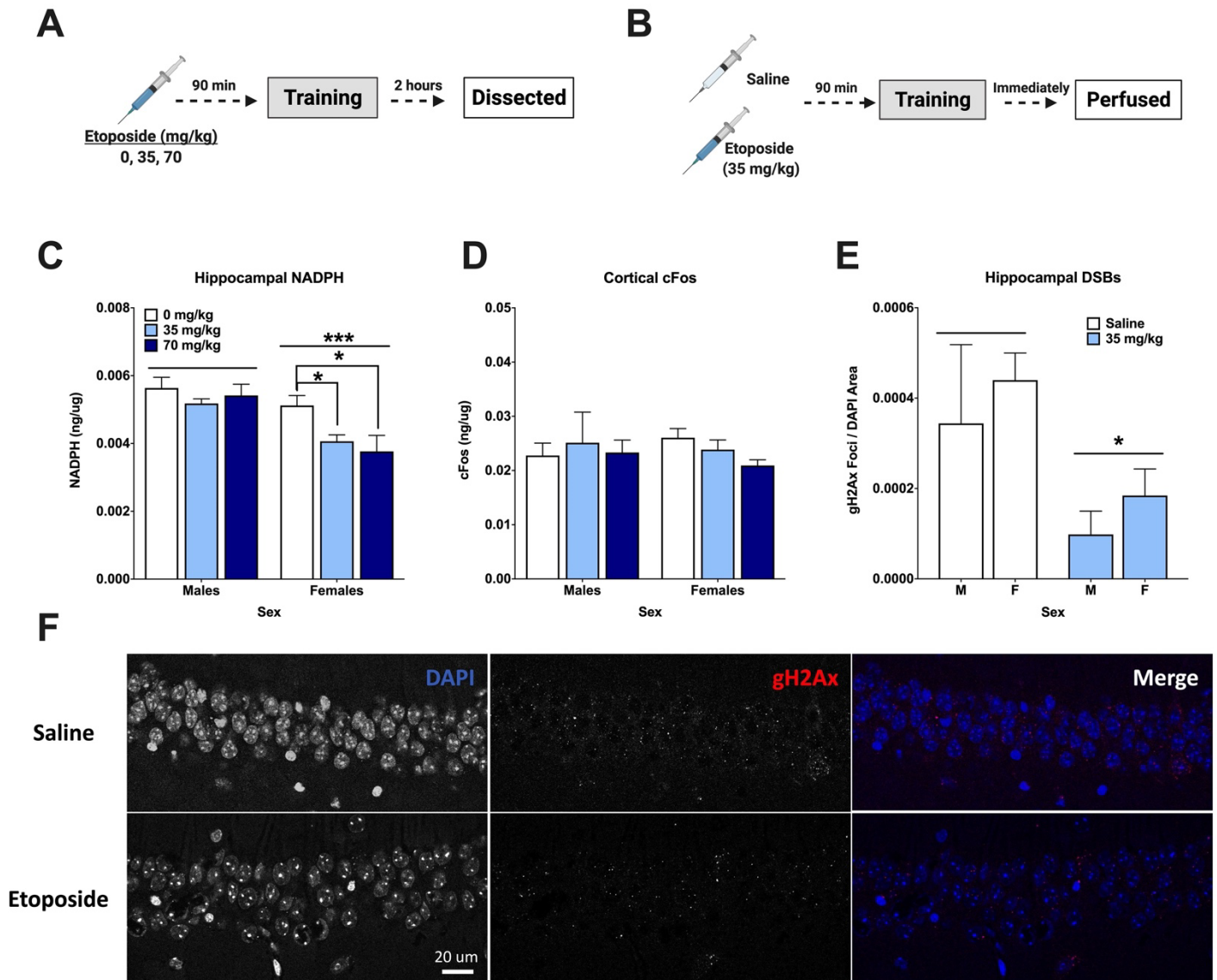


Figure 2.5. Etoposide decreases hippocampal gH2Ax, hippocampal NADPH, and cortical cFos in a sex-dependent manner. **A)** Experiment 3 design. **B)** Experiment 4 design. **C)** Hippocampal NADPH levels. A 2-way ANOVA showed a main effect of dose ($F(2,24) = 4.141$, $p = 0.0285$) and that males had higher levels than females ($F(1,24) = 18.68$, $p = 0.0002$). A 1-way ANOVA indicated a significant effect in females ($F(2,12) = 4.404$, $p = 0.0368$), with Dunnett's post hoc test indicating a difference between 0 and 35 mg/kg ($p = 0.041$) and between 0 and 70 mg/kg ($p = 0.033$). There was no effect of dose detected in males ($p = 0.216$). **D)** Cortical cFos levels. No significant differences were detected by a 2-way ANOVA. In females, a 1-way ANOVA did not reveal an overall difference based on dose ($F(2,12) = 2.796$, $p = 0.100$), but Dunnett's post hoc test indicated a trend towards a difference between 0 mg/kg and 70 mg/kg ($p = 0.0648$). There were no differences detected in males by 1-way ANOVA ($p = 0.8993$). **E)** Quantification of γ H2Ax foci per DAPI area. An injection of 35 mg/kg of etoposide prior to fear training decreased γ H2Ax in the CA1 ($F(1,20) = 6.271$, $p = 0.0210$). **F)** Representative images of γ H2Ax in the CA1. Data show averages $\pm$ SEM. * $p < 0.05$; *** $p < 0.001$.

Discussion

To the best of our knowledge, this is the first study to examine the effects of systemic injections of DSB-altering agents commonly used during cancer treatment on learning, memory, and IEG expression in both sexes. Despite their clinical use for decades [83, 86], research into their specific effects is confounded by additional treatments and disease state [175]. Impairments in cognitive function are widely reported among patients undergoing cancer treatment, which is often termed “chemo-brain” [184-187]. Our results suggest that amifostine and etoposide have distinct effects on learning and memory dependent on sex and timing of administration. Both reduce hippocampal γ H2Ax signal after fear training, suggesting that interrupting typical DSB formation and repair may lead to disruptions in learning and memory. The middle doses used (107 and 35 mg/kg, respectively), which reflect clinical levels, also had the most pronounced effects on memory.

Amifostine did not impair animals' ability to learn, but etoposide (70 mg/kg) increased fear learning in males that was not due to effects on motion. Across experiments, we observed sex differences in freezing levels, with females often showing higher freezing than males. This is consistent with previous work showing that female mice froze more during contextual fear conditioning training than males, and showed greater fear generalization to other contexts [188]. This is also reflective of human conditions, as women diagnosed with post-traumatic stress disorder have greater fear acquisition than men [189]. Reports of emotional distress in cancer patients has indicated that women have increased clinically significant emotional distress [190], anxiety, and depression [191] compared to men. While the complexity of cancer makes it nearly impossible to identify a single driving factor, our observation of the sex-dependent effects of

these common drugs on fear learning and memory suggest they could be contributing to emotional distress measured in patients.

These compounds also affected long-term memory. We detected an increase in hippocampus-dependent contextual fear memory in male mice injected with 107 mg/kg of amifostine. The similar effects of pre- and post-training amifostine injections is likely due to the relatively fast pharmacokinetics, as amifostine peaks in the brain 7-15 min after systemic administration and we conducted fear training and pre/post injections within a 20 min time window [166]. Conversely, we did not detect any differences in hippocampus-independent cued fear memory, suggesting region-dependent susceptibility. We similarly saw an effect of etoposide on long-term contextual fear memory, though in females and only following post-training injections. On top of that, we also observed a decrease in cued freezing in both sexes after post-training etoposide injections.

Other research on hippocampus-dependent memory following amifostine has suggested similar results. 214 mg/kg of amifostine administered prior to gamma radiation rescued novel object recognition and restored hippocampal neurogenesis in mice [176]. Specific effects of etoposide on hippocampus-dependent and -independent function have not previously been described, even in conjunction with radiation. One study indicated that etoposide may be useful for hippocampal tumors due to reduction in hippocampal polyamines [192], but function was not assessed. Investigations into the effects of combined cancer treatment have identified the hippocampus as particularly susceptible. For example, ovariectomized female rats treated with cyclophosphamide and doxorubicin displayed impaired contextual, but not cued, fear memory 1 week after completing treatment [193]. In patients, hippocampal connectivity was shown to be

altered after chemotherapy [185] and reductions in hippocampal volume were correlated with impaired cognition [194].

A possible mechanism contributing to changes in cognition may be IEG regulation. While our data are inconclusive, patterns indicate that these drugs may affect synaptic plasticity via disrupting IEGs which should be further explored. Here, cortical cFos was mildly affected by both drugs in a sex-dependent manner. Females treated with the highest dose of amifostine trended toward higher cortical cFos levels, whereas females treated with the highest dose of etoposide trended toward lower levels. Additionally, our data suggested that 107 mg/kg lowered hippocampal Δ FosB levels in females, and that etoposide had an overall effect on hippocampal Δ FosB, where 35 mg/kg etoposide group appears to have slightly lower levels.

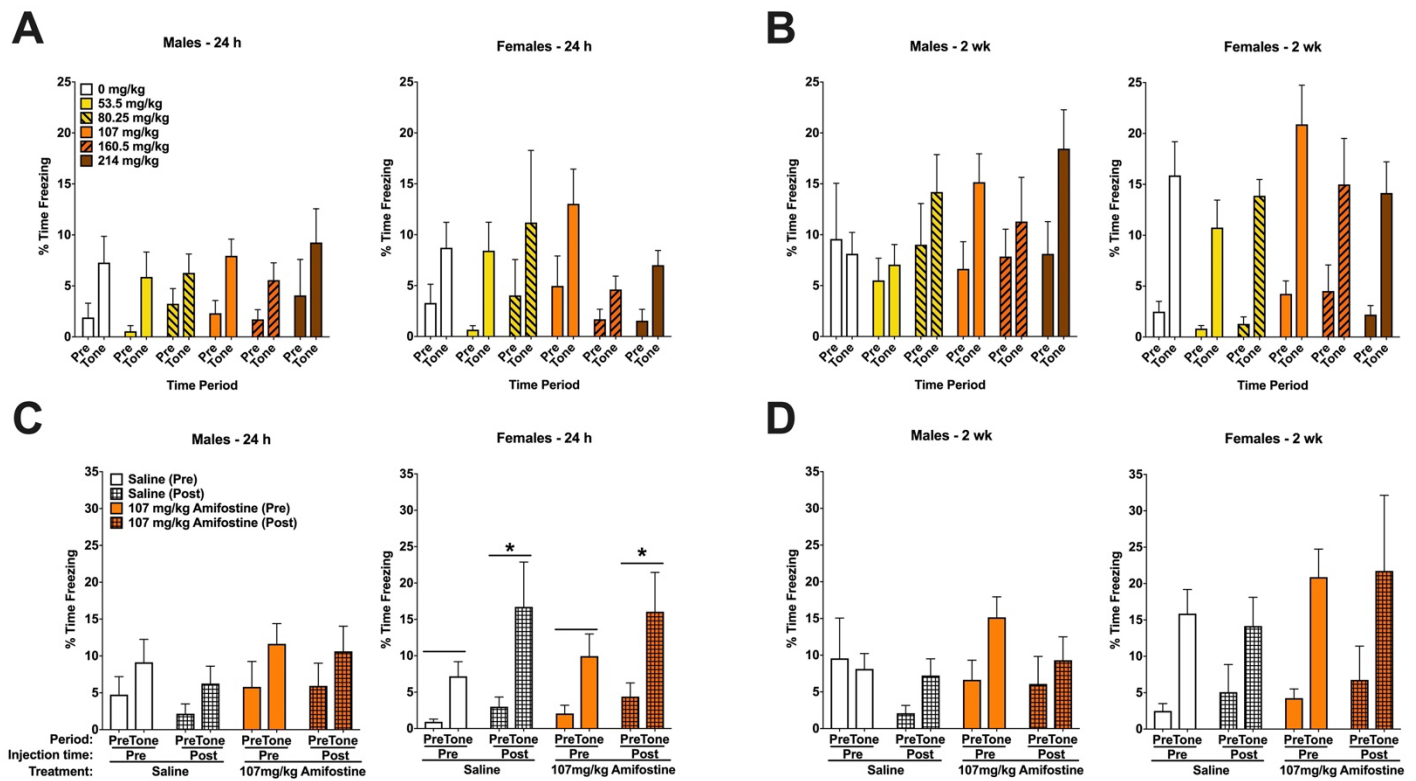
The complexity of measuring IEG induction and expression is well-known, and observed here in the stark differences in hippocampal Δ FosB levels based on timing of injection (pre-training or post-training). We observed higher levels in most animals that received post-training injections, with the exception of females that received amifostine. The complexity of the IEG expression timeline has been a recent interest in development of cancer treatments [195]. Our results indicate that more work needs to be done for a better understanding of the role of IEGs in cancers and cancer treatments.

Notably, we observed sex differences in our molecular measures. Levels of hippocampal cFos, Δ FosB, and amygdala Δ FosB were higher in females than males, though this was only seen in the amifostine cohorts. Timing of the *i.p.* injections (10 vs. 90 min pre-training) likely has an influence on stress response and IEG levels [196-198]. Other studies suggest that sex differences in cFos activation might be dependent on brain region: male mice had greater dorsal hippocampal cFos induction following contextual fear retrieval, whereas females showed greater

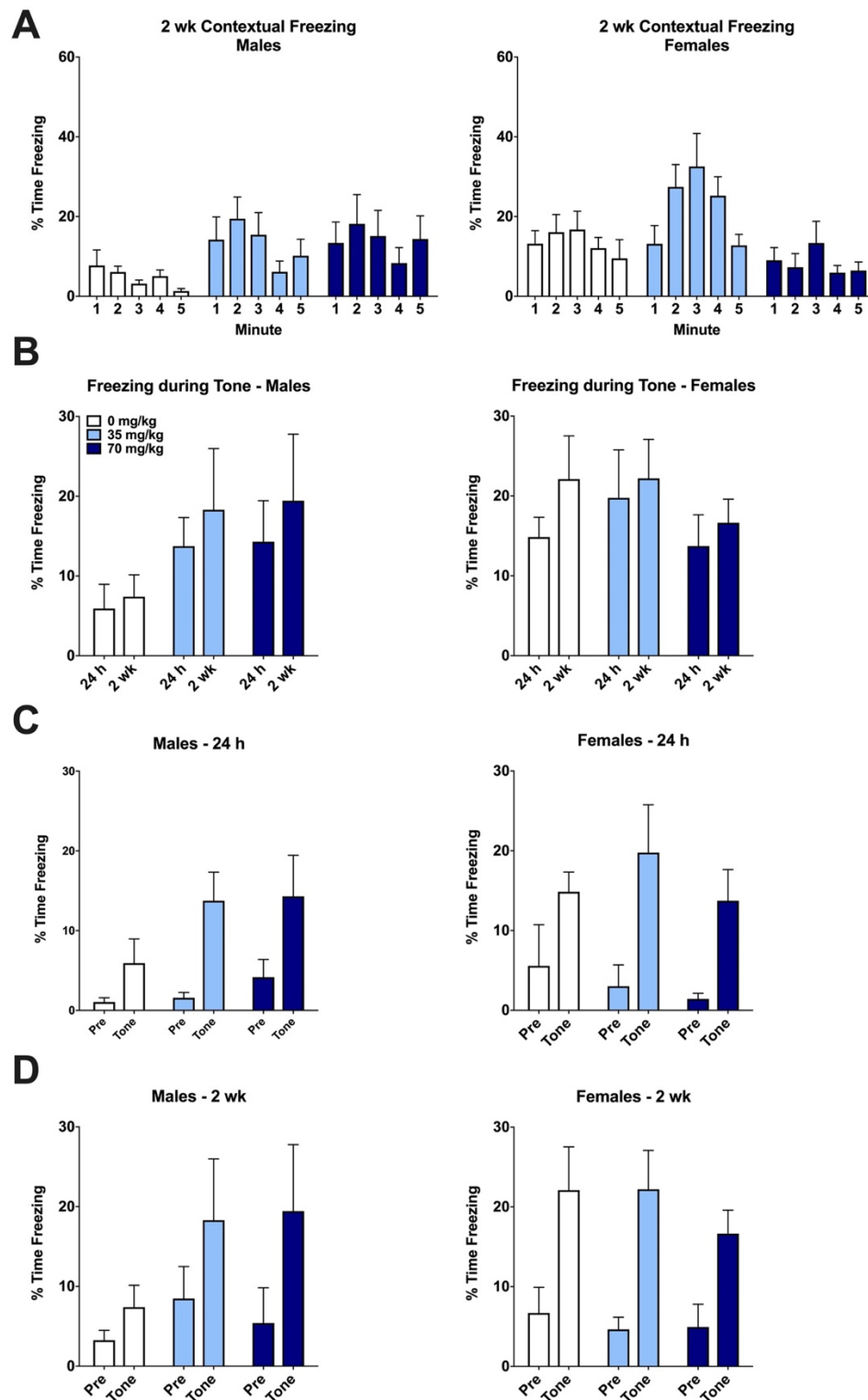
basal amygdala induction [188]. Assessment of estrous cycle in rats suggested that sex differences in cFos mRNA were dependent on stage of the cycle [199], which could account for discrepancies in our results and the literature.

Hippocampal NADPH levels were different based on sex in the etoposide cohorts, with higher levels seen in males. Females generally appear to have less oxidative damage than males, resulting in less NADPH oxidase activity [200]. We also detected a dose-dependent decrease in hippocampal NADPH levels in females. NADPH has previously been shown to counteract the effects of etoposide [201], which is in line with our results, as NADPH levels would decrease as it is oxidized to NADP⁺. Conversely, NADPH levels were not different based on amifostine dose, which is itself a reactive oxygen species scavenger. These sex-dependent changes in cFos, Δ FosB, and NADPH did not directly mirror the observed behavioral changes. However, our observations are important to consider for future efforts looking at learning, memory, and response to drugs in males and females, as highlighted by Shansky and Murphy [202].

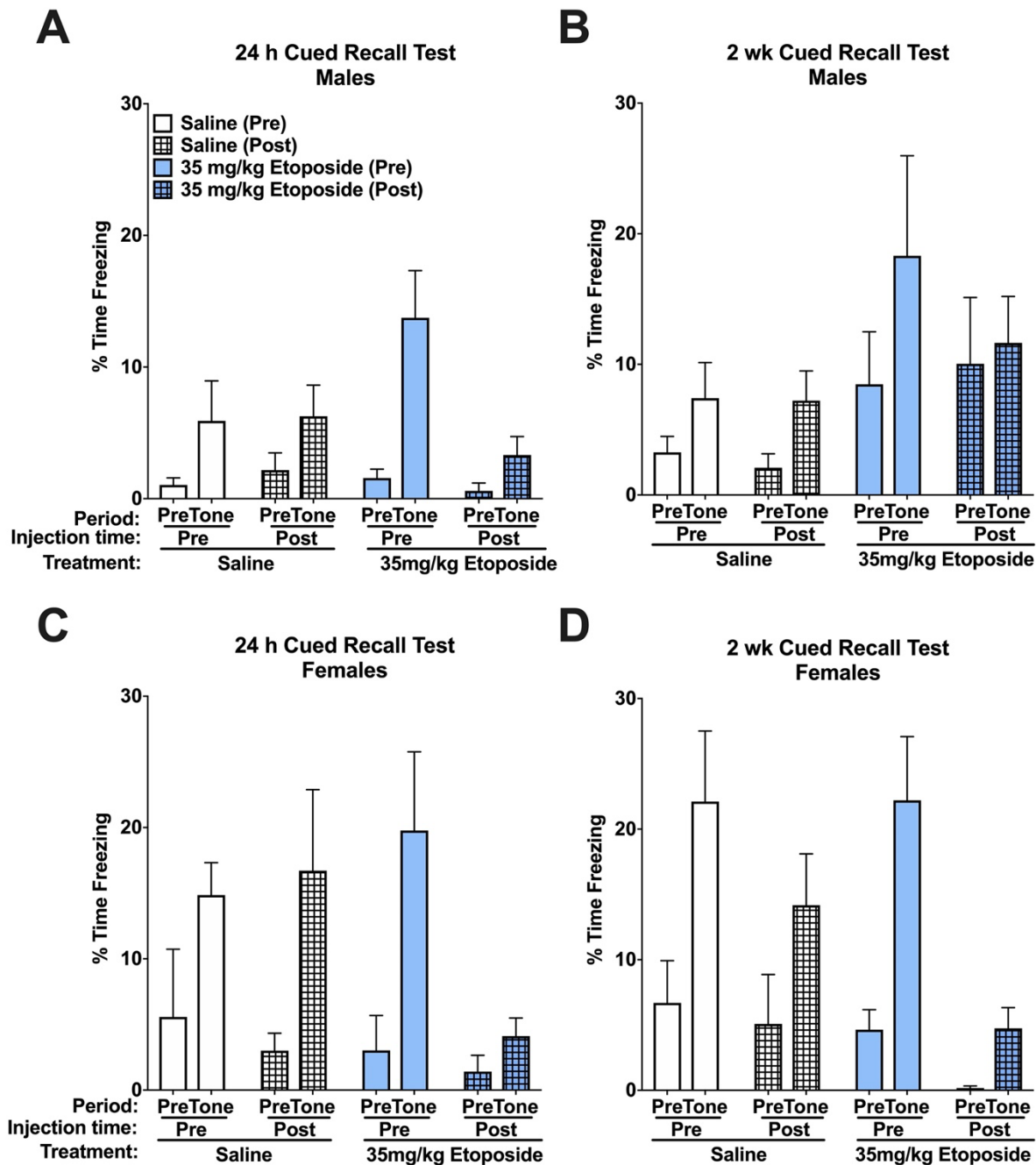
In summary, our results reveal important information about the effects of amifostine and etoposide on learning, memory, and IEGs that can optimize treatment strategies. Newer analogs of these drugs, such as PrC-210 [203], might reduce these side effects and improve patients' quality of life. Additionally, assessing the direct effects of these drugs on specific brain regions (via central injections) will be useful to clarify the underlying mechanisms driving learning and memory changes, which we pursued as described in the subsequent chapter.



Supplemental Figure 2.1. Cued recall tests at 24 h and 2 wks after pre- and post-training injections of amifostine. **A)** Percent time freezing at baseline (“pre”) and in response to the tone 24 h after pre-training injections in males (*left*) and females (*right*). All groups froze more in response to the tone ($F(1,108) = 77.189$, $p < 0.001$). **B)** Percent time freezing at baseline and during the tone 2 wks after pre-training injections in males (*left*) and females (*right*). All groups froze more when the tone played ($F(1,108) = 99.639$, $p < 0.001$). We also found a significant time by sex interaction ($F(1,108) = 21.281$, $p < 0.001$), with females freezing less during the baseline period. Analysis in males showed a significant time by dose interaction ($F(5,54) = 2.472$, $p = 0.043$). **C)** Percent time freezing at baseline and during the tone 24 h after post-training injections in males (*left*) and females (*right*). All groups showed an increase in response to the tone ($p < 0.001$), though we found a time by sex interaction ($F(1,64) = 6.961$, $p = 0.010$). Analysis in females revealed a significant effect of injection time ($F(1,34) = 4.270$, $p = 0.046$). **D)** Percent time freezing at baseline and during the tone 2 wks after post-training injections in males (*left*) and females (*right*). All groups showed increased freezing in response to the tone ($F(1,64) = 42.424$, $p < 0.001$). We also found a time by sex interaction ($F(1,34) = 14.952$, $p = 0.000$). Data show averages $\pm$ SEM. * $p < 0.05$.



Supplemental Figure 2.2. Cued recall tests at 24 h and 2 wks after pre-training injections of etoposide. **A)** Percent time freezing over the 5 minutes of the 2 wk contextual recall test in males (*left*) and females (*right*). Minute-by-minute analysis indicated a main effect of dose in females ($F(2,21) = 5.173, p = 0.0149$). **B)** Percent time freezing during the tone in the 24 h and 2 wk recall tests in males (*left*) and females (*right*). No differences based on dose or sex were detected. **C)** Percent time freezing at baseline (“pre”) and during the tone in the 24 h recall tests in males (*left*) and females (*right*). All mice showed the expected increase in freezing in response to the tone ($F(1,43) = 54.258, p < 0.001$). **D)** Percent time freezing at baseline and during the tone in the 2 wk recall tests in males (*left*) and females (*right*). All mice showed the expected increase in freezing in response to the tone ($F(1,43) = 66.264, p < 0.001$). Data show averages $\pm$ SEM. * $p < 0.05$.



Supplemental Figure 2.3. Cued recall tests at 24 h and 2 wks after post-training injections of etoposide. **A)** Percent time freezing at baseline (“pre”) and during the tone in the 24 h recall tests in males. All mice increased freezing during the tone ($F(1,27) = 20.733, p < 0.001$). **B)** Percent time freezing at baseline and during the tone in the 2 wk recall tests in males. All mice showed the expected increase in response to the tone ($F(1,27) = 18.359, p < 0.001$). **C)** Percent time freezing at baseline and during the tone in the 24 h recall tests in females. All mice increased freezing in response to the tone ($F(1,26) = 26.036, p < 0.001$), but a time by injection time by dose interaction indicated a blunted response in females that received post-training injections of etoposide ($F(1,26) = 4.935, p = 0.035$). **D)** Percent time freezing at baseline and during the tone in the 2 wk recall tests in females. All mice showed an increase in response to the tone ($F(1,26) = 60.079, p < 0.001$), though a time by injection time interaction indicated a blunted response in females that received post-training injections of 35 mg/kg etoposide ($F(1,26) = 10.307, p = 0.004$).

Chapter 3: Infusion of etoposide in the CA1 disrupts hippocampal immediate early gene expression and hippocampus-dependent learning

Abstract

Tight regulation of immediate early gene (IEG) expression is important for synaptic plasticity, learning, and memory. Recent work has suggested that DNA double strand breaks (DSBs) may have an adaptive role in post-mitotic cells to induce IEG expression. Physiological activity in cultured neurons as well as behavioral training leads to increased DSBs and subsequent IEG expression. Additionally, infusion of etoposide – a common cancer treatment that induces DSBs – impairs trace fear memory, and systemic injections impair long-term contextual fear memory. Here, we assessed the effects of hippocampal infusion of 60 ng of etoposide on IEG expression, learning, and memory in 3-4-month-old C57Bl/6J mice. Etoposide altered expression of the immediate early genes *cFos* and *Arc* in the hippocampus and impaired hippocampus-dependent contextual fear memory. These data add to the growing evidence that DSBs play an important role in IEG expression, learning, and memory, opening avenues for developing novel treatment strategies for memory-related disorders.

Introduction

Immediate early genes (IEGs) – including the proto-oncogene *cFos* and activity-regulated cytoskeleton-associated protein (*Arc*) – are important for synaptic plasticity and learning and memory [40, 62, 204]. At baseline, these genes are expressed at low basal levels. Activity within neurons leads to a rapid increase in their expression and a subsequent return to baseline levels within hours [168]. This precise timing is important for accurate encoding and recall, especially related to hippocampus-dependent contextual learning and memory [50, 205, 206]. Yet, what exactly leads to the rapid expression of these genes is still unclear.

Recently, evidence has emerged that DNA double strand breaks (DSBs) may contribute to IEG expression [97, 98]. DSBs are observed after physiological neuronal activation [97], and are associated with an increase in mRNA levels of a select sub-set of genes, many of which are part of the IEG family [98]. Contextual fear conditioning also induces DSBs in neuronal and non-neuronal cells that leads to transcriptional changes in neurons and glia [105]. Notably, the timing of DSB induction and their proper repair are important components of this process. Mice carrying mutations that cause Alzheimer's disease show higher basal levels of DSBs and impaired DSB repair compared to wild-type mice, measured by a common DSB repair marker, γ H2Ax [97]. Post-mortem analysis in people reflects this as well: patients with Alzheimer's disease have higher γ H2Ax than those with mild cognitive impairment or cognitively unimpaired age-matched controls [118].

In attempts to identify causal links, the chemotherapy agent etoposide, which induces DSBs by interfering with the topoisomerase-II beta complex, is being used [87]. We have recently shown that systemic administration of etoposide impairs long-term contextual and cued fear memory and changes hippocampal FosB protein levels [207]. In the clinic, etoposide's

effects on learning and memory are confounded by the presence of many other variables but do generally point towards impaired hippocampus-dependent cognitive performance [175].

Yet, etoposide's penetration past the blood brain barrier is poor (<3%) [208], and toxicity is seen in cell cultures at doses around 10 $\mu\text{g/mL}$ [209]. As such, direct infusions into the central nervous system are used in patients being treated for brain tumors. Cerebral spinal fluid levels were around 3 $\mu\text{g/mL}$ in patients 2 hours after receiving 0.5 mg intraventricular infusions [210]; while headaches were the main side effects reported, there was no assessment of learning or memory. In mice, direct infusion of 0.1 $\mu\text{g}/\mu\text{L}$ (but not 1 $\text{ng}/\mu\text{L}$) of etoposide in the prelimbic area impairs long-term fear memory and alters the timeline of *cFos*, *Arc*, *Npas4*, and *Cyr61* RNA expression [171]. More research is needed to identify the extent and specificity of DSBs in learning and memory as well as characterize the molecular changes induced by lower doses. Namely, testing the effects of etoposide in brain regions known to be important for learning and memory—such as the hippocampus—is an important next-step in understanding the role of DSBs in the learning and memory process, as well as testing lower doses similar to those observed in the clinic. Further clarifying possible mechanisms, like disruption to IEG expression, is also needed for a better understanding of this system.

Here, we used 60 ng of etoposide to induce DSBs selectively in the CA1 region of the hippocampus during fear acquisition. We hypothesized that CA1 infusions of etoposide would interfere with hippocampal IEG expression and impair hippocampus-dependent long-term contextual (but not hippocampus-independent cued) fear memory. Etoposide increased γH2Ax in the CA1, but not in other regions of the hippocampus. Etoposide also impaired *cFos* expression throughout the hippocampus and decreased long-term contextual-dependent freezing. These data point to a nuanced disruption of intra-hippocampal signaling by etoposide and provide more

direct causal evidence that DSBs are involved in hippocampus-dependent learning and memory via regulation of IEGs.

Methods

Animals

A total of 84 male ($n = 57$) and female ($n = 27$) C57Bl/6J mice from Jackson Labs (Bar Harbor, ME) were delivered to OHSU at 5 weeks of age. Surgical cannula implantation occurred at 8-11 weeks of age. Infusion and behavioral testing occurred at 10-13 weeks of age. A total of 9 animals were euthanized due to surgical complications.

All animals were group housed at 5 mice/cage until the day of surgery. Animals were singly housed for 1-week after surgery to allow for recovery; after 1 week, animals were group-housed 3 mice/cage for the remaining duration of experiments. Body weights were recorded weekly starting the day of surgery.

All animal procedures were reviewed and approved by the Oregon Health & Science University IACUC and in accordance with AAALAC and ARRIVE guidelines. Researchers were blinded to the treatment groups throughout all experiments.

Cannula Surgery

Surgeries were performed according to our standard protocol [211]. Briefly, mice were treated with oral meloxicam (5 mg/kg) prior to being induced with 5% isoflurane and maintained at 1.5–2% isoflurane throughout surgeries. A water heating pad was used to maintain body temperature; depth of anesthesia and breathing were monitored. Mice were secured in a stereotaxic frame (World Precision Instruments, Sarasota, FL) with fixation bars. Ophthalmic

ointment was applied to the eyes, and their heads were shaved. A local subcutaneous injection of lidocaine was given, and heads sterilized by alternating 3 swabs of betadine and 2 swabs of 70% isopropanol. An incision was made on the midline of the scalp to expose the skull and holes drilled bilaterally above the hippocampus (Bregma coordinates: AP: -1.7mm, ML: ± 1.0). A bilateral guide cannula (DV projection: 1.2mm) was then lowered and secured in place with dental cement (Lang Dental Mfg. Co., Wheeling, IL). Once secured, a dummy cannula was inserted and mice removed from the stereotaxic frame. Mice received two days of post-op treatment with oral meloxicam (5 mg/kg).

Infusions & Fear Conditioning

Etoposide phosphate (etopophos) powder was purchased through the OHSU research pharmacy (E.R. Squibb & Sons, LLC, New Brunswick, NJ). It was diluted in saline to a 20 mg/mL stock solution and stored at -80° C until the day before use. The working solution was diluted to a final concentration of 0.1 $\mu\text{g}/\mu\text{L}$. This dose was chosen based on the previous use of direct infusions of etoposide in the prelimbic area of mice [171], as well as clinical data for common doses used in patients [210] and accounting for toxicity levels [208].

For infusions, mice were lightly anesthetized with 5% isoflurane. Mice were removed from anesthesia and gently held by the researcher. Internal cannula were then inserted; 0.3 μL of saline or etoposide were infused in each hemisphere at a rate of 0.3 $\mu\text{L}/\text{minute}$. Internal cannula were left *in situ* for 60 seconds before mice were placed back into the home cage.

Fear conditioning was conducted 2 hours after infusions. We implemented a common protocol previously used by our lab [207]. Briefly, mice were placed in to fear conditioning chambers (MedAssociates, Fairfax, VT) for a baseline period of two minutes before being

exposed to 4 tones that co-terminated with a 2 s, 0.5 mA shock. There were 90 s inter-stimulus intervals between each tone-shock pairing. Upon completion, mice were returned to their home cages and fear chambers cleaned with 0.5% acetic acid.

For assessment of long-term hippocampus-dependent and -independent memory, mice were tested in contextual and cued fear conditioning 24 h after training. The contextual trial (hippocampus-dependent) was a 5-min period in the same chambers as training; no tones were presented, and chambers were cleaned with 0.5% acetic acid. For the cued trial (hippocampus-independent), contextual clues were eliminated by inserting a new floor, walls, and roof, as well as cleaning with 10% isopropanol. The test was comprised of a 90 s baseline period followed by a 3-min presentation of the same tone played during training. There was a 3-hour period between the contextual and cued recall tests. Lastly, we assessed contextual memory again at 2 weeks to explore the lasting effects of hippocampal etoposide infusions [207].

Tissue Collection

We randomly split our animals into two experimental categories: the “immediate” cohorts and the “long-term” cohorts.

To assess effects of infusions on γ H2Ax foci and IEGs, a cohort of male mice received infusions, were trained in fear conditioning, and euthanized immediately post-training (2 h after infusion, $n = 4/\text{treatment}$) or 5 h post-training (7 h after infusion, $n = 7-8/\text{treatment}$; **Fig. 3.1A, 3.2A**). These times were chosen as peak IEG signal ranges from 15 min – 120 min after stimulation and typically return to baseline levels around 4 hours later [33, 69, 195]. Additionally, direct infusions of etoposide were shown to lead to increased IEG expression 5 h after infusion [171]. For analysis of long-term effects of infusions on γ H2Ax, separate cohorts of

male and female mice were euthanized 15 minutes after completion of the 2-week contextual recall test ($n = 13-15/\text{sex/treatment}$; **Fig. 3.3A**).

All animals were euthanized by perfusion. Mice received an *i.p.* injection of a ketamine-xyline cocktail. Once animals were non-responsive, they were perfused with ice-cold 1X PBS followed by 4% paraformaldehyde (PFA). Brains were removed and soaked in 4% PFA overnight, then sunk in 30% sucrose solution. Brains were then sectioned at 40 μm thickness using a cryostat; the lower left hemisphere was notched to indicate side when sectioning. Sections were stored in cryopreserve until use.

Immunohistochemistry

cFos and Arc Immunohistochemistry

Free floating sections ($n = 4/\text{animal}$) were stained for cFos and Arc using a standard 3,3'-diaminobenzidine tetrahydrochloride (DAB) protocol [212, 213]. Briefly, tissue was rinsed 3 x 5 minutes in 1x PBS and activated with 0.3% hydrogen peroxide for 15 minutes. Tissue was again rinsed 3 x 5 minutes in 1x PBS and blocked in 4% normal goat serum (NGS) in 0.3% PBS-Triton X. Sections were then incubated in primary antibody diluted in 4% NGS for approximately 48 hours at 4°C (rabbit anti-cFos: 1:5000, Santa Cruz, sc-52, lot #G2612; mouse anti-Arc: 1:300, Santa Cruz, sc-17839, lot #K1609). Tissue was washed 3 x 5 minutes before incubating in secondary antibody diluted in 4% NGS for 1 hour (goat anti-rabbit biotinylated: 1:200, Vector Labs, BA-100, lot #ZH0615; goat anti-mouse biotinylated: 1:200, Vector Labs, BA-9200, lot #X0623). After 3 x 5 minute washes, avidin-biotin complex was applied for 1 hour (Vector labs), and washed 3 x 5 minutes again. Chromagen was applied for exactly 10 minutes and the reaction stopped with deionized water, washed 2 x 5 min in 1x PBS, and slide mounted.

Slides were coverslipped with Cytoseal-60 after going through an ethanol-xylene dehydration series.

γ H2Ax Immunofluorescence

Free floating sections ($n = 4$ sections/animal) were stained for γ H2Ax foci using our standard 3-day fluorescence protocol [182]. Briefly, tissue was rinsed 3 x 5 min in 1x PBS and blocked for 1 h in 4% NGS. Sections were incubated overnight at room temperature in rabbit-anti- γ H2Ax (1:1000, Cell Signaling, 97185, lot #17), rinsed 3 x 5 min in 1x PBS, then incubated overnight in goat-anti-rabbit Alexa 594 secondary (1:1000, Invitrogen, A11012, lot #1892265). Tissue was again rinsed 3 x 5 min in 1x PBS, then incubated for 20 min in a DAPI counterstain (1:200, Sigma Aldrich, St. Louis, MO). After another 20 min rinse in 1x PBS, tissue was mounted and coverslipped with Citifluor CFMR2 antifade mounting media (Electron Microscopy Sciences, Hatfield, PA, #17979-10).

Fluoro-Jade C

To confirm that infusions did not cause widespread cell death, we stained sections for Fluoro-Jade C as previously described [214, 215]. Four hippocampal sections per animal were slide mounted and dried for 2 hours on a slide warmer at 55° C. Slides were immersed for 5 minutes in 80% EtOH with 1% NaOH, then 2 minutes in 70% EtOH with 1% NaOH. Following a 2 minutes rinse in ddH₂O, slides were incubated for 15 minutes in 0.06% KMnO₄, then rinsed again for 2 minutes in ddH₂O, and incubated for 15 minutes in 0.0001% Fluoro-Jade C (16A, Histo-Chem, Inc., AR) dissolved in 0.1% acetic acid. Slides were rinsed 3 x 1 minute in ddH₂O, and allowed to air dry overnight in the dark. Slides were cleaned in xylene, then cover slipped with Permount™ mounting medium (Fisher Scientific, MA).

Microscopy and quantification

For all DAB-stained sections, images were captured with an upright Olympus IX81 microscope (Olympus, Center Valley, PA, USA) equipped with CellSens imaging software. Images of each sub-region of the hippocampus were taken at 10x magnification with 100 ms exposure time. cFos- and Arc-positive cells were identified by the dark nuclear label and counted using ImageJ software (NIH, Bethesda, MD). Fluoro-Jade C sections were also captured using the Olympus IX81 upright microscope at 10x magnification with 5 s exposure time using a FITC filter.

For γ H2Ax-stained fluorescent sections, images were captured with a Zeiss LSM 980 FastAiry microscope (Zeiss, Thornwood, NY, USA) equipped with Zen imaging software. Images of each sub-region of the hippocampus were taken at 63x magnification in oil immersion. Analysis of γ H2Ax foci was performed using ImageJ software. The number of foci was normalized to the area occupied by DAPI for analysis [182].

Statistics & Reproducibility

All data were assessed for normality prior to proceeding with parametric tests. Statistical analyses were conducted using Prism v. 7 (GraphPad, San Diego, CA) and SPSS v 27 (IBM, Armonk, NY) software.

For fear conditioning, the average motion (au) during the initial baseline period was analyzed with a 2-way ANOVA. The average motion (au) during the shocks, percent time freezing during the tones, and percent time freezing during the inter-stimulus intervals were

analyzed using a repeated measures ANOVA with sex and treatment as between-group variables and time as the within-group variable.

For all immunohistochemical analyses, hemisphere was initially included as a variable to confirm there were no hemisphere-dependent differences. In all cases, there were no significant effects or interactions with hemisphere, so it was dropped from the statistical model.

For γ H2Ax foci analysis, the number of γ H2Ax foci normalized to the DAPI area (mm) was averaged in each section. We then used a repeated measures ANOVA with treatment as the between group variable and section as the within-group variable for analysis.

For analysis of the number of *Arc* and *cFos* positive cells, we analyzed the average number of immunopositive cells per section using a repeated measures ANOVA with treatment and time post-training as between-group variables and section as the within-group variables. Sidak's post hoc test was used following these ANOVAs.

Results

Fear conditioning and hippocampal injection of etoposide increases γ H2Ax foci in the CA1

Using male C57Bl/6J (WT) mice, we bilaterally implanted cannula targeting the CA1 region of the hippocampus. Body weights were used to track health and recovery of mice throughout all experiments (**Table 3.1**). To determine the effects of etoposide on hippocampal IEGs and hippocampus-dependent learning and memory, we bilaterally infused 0.3 μ L of etoposide (0.1 μ g/ μ L) or saline into the CA1 region of the hippocampus in male WT mice. Two hours later, mice were euthanized (behaviorally naïve, n = 4-5/treatment) or trained in a fear conditioning paradigm and immediately euthanized (n = 4/treatment).

We first assessed the effects of etoposide infusions on γ H2Ax formation by immunofluorescence in the CA1, CA3, and dentate gyrus (DG) regions of the hippocampus (**Fig. 3.1A**). Mice trained in fear conditioning had significantly more γ H2Ax foci in the CA1 than behaviorally naïve animals ($p = 0.001$; **Fig. 1B**). Additionally, etoposide infusions increased the number of γ H2Ax foci in the CA1 region ($p < 0.001$; **Fig. 3.1B**). Sidak's post hoc testing indicated that mice that received fear conditioning and etoposide infusions had significantly more γ H2Ax than fear conditioned, saline-infused mice ($p < 0.05$). Representative images of γ H2Ax in the CA1 can be seen in **Fig. 3.1C**.

Similarly, γ H2Ax foci in the CA3 were significantly increased in animals that underwent fear conditioning ($p < 0.001$; **Fig. 3.1D**). There was no difference between saline- and etoposide-infused animals in the CA3 region ($p = 0.995$), though we found a significant condition by treatment interaction ($p = 0.038$; **Fig 3.1D**), where etoposide increased γ H2Ax in the behaviorally naïve mice but decreased it in the fear conditioned mice. Representative images of γ H2Ax in the CA3 can be seen in **Fig. 3.1E**.

Analysis of γ H2Ax foci in the DG also revealed a significant effect of behavioral condition ($p < 0.001$; **Fig. 3.1F**), as well as a significant condition by treatment interaction ($p = 0.005$). Sidak's post hoc testing indicated that fear conditioned, etoposide-infused mice had less γ H2Ax than conditioned-matched, saline-infused mice ($p < 0.05$). There was no main effect of treatment ($p = 0.140$). Representative images of γ H2Ax in the DG can be seen in **Fig. 3.1G**. These region- and condition-dependent change in γ H2Ax focus formation suggests that CA1-targeted infusion of etoposide disrupt DSBs in the hippocampus at baseline and during a learning event.

To confirm that our infusions were not causing cell death, we assessed Fluoro-Jade C staining in the hippocampus. There was no visible sign of Fluoro-Jade C staining in any hippocampal regions, indicating that neither saline nor etoposide infusions caused cell death (Supplemental Fig. 3.1A-B).

Table 3.1. Body weights before cannula implantation (“Pre-Surgery”) and at the end of the study (“Post-Infusion”) in male and female mice.

Sex	Time & Treatment			
	Pre-Surgery		Post-Infusion	
	Saline	Etoposide	Saline	Etoposide
Male	24.33 ± 0.39	24.13 ± 0.38	28.00 ± 0.43	26.35 ± 0.63
Female	19.28 ± 0.46	18.86 ± 0.46	19.88 ± 0.60	20.35 ± 0.29

Data presented as averages ± SEMs.

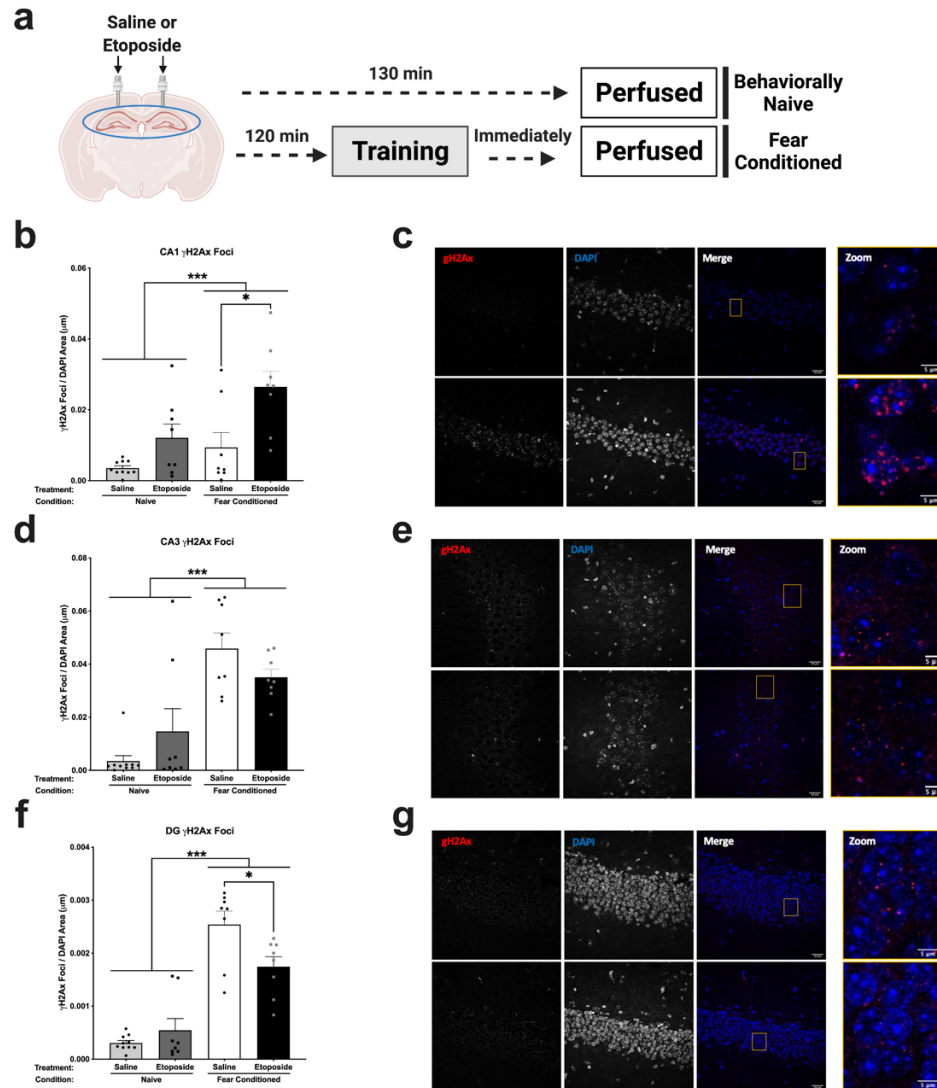


Figure 3.1. Analysis of γ H2Ax in the hippocampus following infusions of saline or etoposide and fear conditioning. **A**) Schematic of the experimental timeline ($n = 4$ -5/behavioral group/treatment). **B**) γ H2Ax foci in CA1. Fear conditioning significantly increased the number of γ H2Ax foci ($F(1,30) = 12.599, p = 0.001$). Etoposide infusions significantly increased the number of γ H2Ax foci ($F(1,30) = 20.587, p < 0.001$). **C**) Representative images of γ H2Ax staining in the CA1 from fear conditioned mice. The far-right panel is zoomed in on the yellow box in the "merge" image. **D**) γ H2Ax foci in CA3. Fear conditioning significantly increased γ H2Ax foci ($F(1,30) = 53.260, p < 0.001$). Etoposide infusions did not change the number of γ H2Ax foci ($F(1,30) = 0.003, p = 0.995$). There was a significant interaction between behavior and treatment groups ($F(1,30) = 4.689, p = 0.038$). **E**) Representative images of γ H2Ax staining in the CA3. **F**) γ H2Ax foci in the DG. Fear conditioning significantly increased γ H2Ax foci ($F(1,30) = 128.782, p < 0.001$). There was a significant interaction between behavior condition and treatment ($F(1,30) = 9.203, p = 0.005$), where etoposide infusions significantly decreased the number of γ H2Ax foci in the fear conditioned group ($p < 0.05$) but increased it in the naïve group. **G**) Representative images of γ H2Ax staining in the DG. Data are presented as total number of foci normalized to area of DAPI (mm) $\pm$ SEM. $*p < 0.05$.

Etoposide disrupts timeline of Arc and cFos expression in the hippocampus

Induction of DSBs with etoposide in the prelimbic area of mice interfere with the timeline of IEG expression, increasing expression 5 h after stimulation [171]. Thus, we looked at two IEGs – *Arc* and *cFos* – using immunohistochemistry immediately following fear training (2 hours after infusion, $n = 4/\text{treatment}$) or 5 hours after training (7 hours after infusion, $n = 7-8/\text{treatment}$; **Fig. 3.2A**) in male WT mice. Additionally, we analyzed *cFos* in behaviorally naïve animals that received infusions of saline or etoposide ($n = 4-5/\text{treatment}$). A representative image of the cannula location and hippocampal regions is illustrated in **Fig. 3.2B**. Representative images of cFos and Arc staining in the CA1, CA3, and DG can be seen in **Supplementary Fig. 3.2A-F**.

In the CA1, mice that underwent fear conditioning had significantly more cFos⁺ cells than behaviorally naïve mice ($p < 0.001$), and mice euthanized immediately after fear conditioning had more than mice euthanized 5 h later ($p = 0.001$; **Fig. 3.2C**). We also found a significant time post-training by behavior interaction ($p < 0.001$). Sidak's post-hoc comparison indicated that fear conditioned, saline-infused mice euthanized 5 h post-training had significantly fewer cFos⁺ cells compared to the immediately-euthanized, fear conditioned saline group ($p < 0.0001$). However, this was blunted in the fear-conditioned etoposide-infused groups: mice euthanized 5 h post-training were not different than mice euthanized immediately ($p = 0.2607$; **Fig. 3.2C**).

Similarly, fear conditioning significantly increased the amount of cFos in the CA3 ($p < 0.001$; **Fig. 3.2D**). We also found a time post-training by behavior interaction in the CA3 ($p < 0.001$). Sidak's post-hoc comparison showed that both saline- and etoposide-infused animals

euthanized immediately after fear conditioning had more cFos+ cells than mice euthanized 5 h after fear conditioning ($p < 0.0001$, $p = 0.0037$, respectively; **Fig. 3.2D**).

There were also more cFos+ cells in the DG in fear conditioned animals than behavioral naïve animals ($p < 0.001$; **Fig. 3.2E**). We found significant time by treatment ($p = 0.041$), time by behavior condition ($p = 0.034$), and treatment by time by behavior condition ($p = 0.011$) interactions, as well as a trend towards a time by treatment interaction ($p = 0.052$). Sidak's post hoc test indicated that only the fear conditioned, saline-infused groups showed a drop in the number of cFos+ cells ($p < 0.0001$); this pattern was absent in the fear conditioned, etoposide-infused groups ($p = 0.7132$; **Fig. 3.2E**).

We next analyzed the number of Arc+ cells in the CA1, CA3, and DG in mice that underwent fear conditioning with saline or etoposide infusions. In the CA1, we found a significant effect of time ($p = 0.030$), though in the opposite direction compared to the number of cFos+ cells (**Fig. 3.2F**). Both saline- and etoposide-infused mice euthanized 5 h after training had more Arc+ cells than the immediate groups. In the CA3, the number of Arc+ cells was affected by treatment ($p = 0.023$), but not time ($p = 0.414$), with the etoposide-infused groups showing more Arc+ cells than the saline-infused groups (**Fig. 3.2G**). Lastly, the number of Arc+ cells in the DG was affected by time ($p < 0.001$), treatment ($p = 0.006$), and a time x treatment interaction ($p = 0.006$; **Fig. 3.2H**). Sidak's post-hoc testing indicated that the number of Arc+ cells dropped over time in the saline-infused groups ($p < 0.001$) but not the etoposide-infused groups ($p = 0.539$). Overall, the etoposide groups had fewer Arc+ cells (**Fig. 3.2H**).

To assess if communication between hippocampal regions was disrupted by etoposide, we ran correlations of the number of cFos+ cells between the CA1, CA3, and DG. We found significant positive correlations for both saline- and etoposide-treated animals between the CA1

and DG ($p < 0.0001$; **Fig. 3.2I**), the CA1 and CA3 ($p < 0.0001$; **Fig. 3.2J**), and the CA3 and DG ($p < 0.0001$; **Fig. 3.2K**). Similar analysis of intra-hippocampal Arc signal did not reveal any significant correlations between regions in either treatment group (**Supplemental Fig. 3.3A-C**).

To assess if there was a relationship between cFos and Arc, we correlated signals in each region. There was no relationship between the number of cFos⁺ and Arc⁺ cells in the CA1 or CA3 regions. However, there was a positive correlation between the number of immunopositive cFos and Arc cells in the DG in saline-infused mice only ($p < 0.001$; **Fig. 3.2L**); there was no correlation in etoposide-infused mice ($p = 0.772$). We also correlated the number of γ H2Ax foci with the number of cFos⁺ cells in each region. There were no significant correlations in the CA3 or DG, though we found significant positive correlations in the CA1 of both saline- ($p = 0.0176$) and etoposide-infused ($p = 0.0239$) mice that underwent fear conditioning (**Fig. 3.2M**). There was no significant correlation in the CA1 of behaviorally naïve animals.

Altogether, these data suggest that hippocampal infusion of etoposide blunts cFos expression, and blunts Arc in the DG but increases it in the CA3 region.

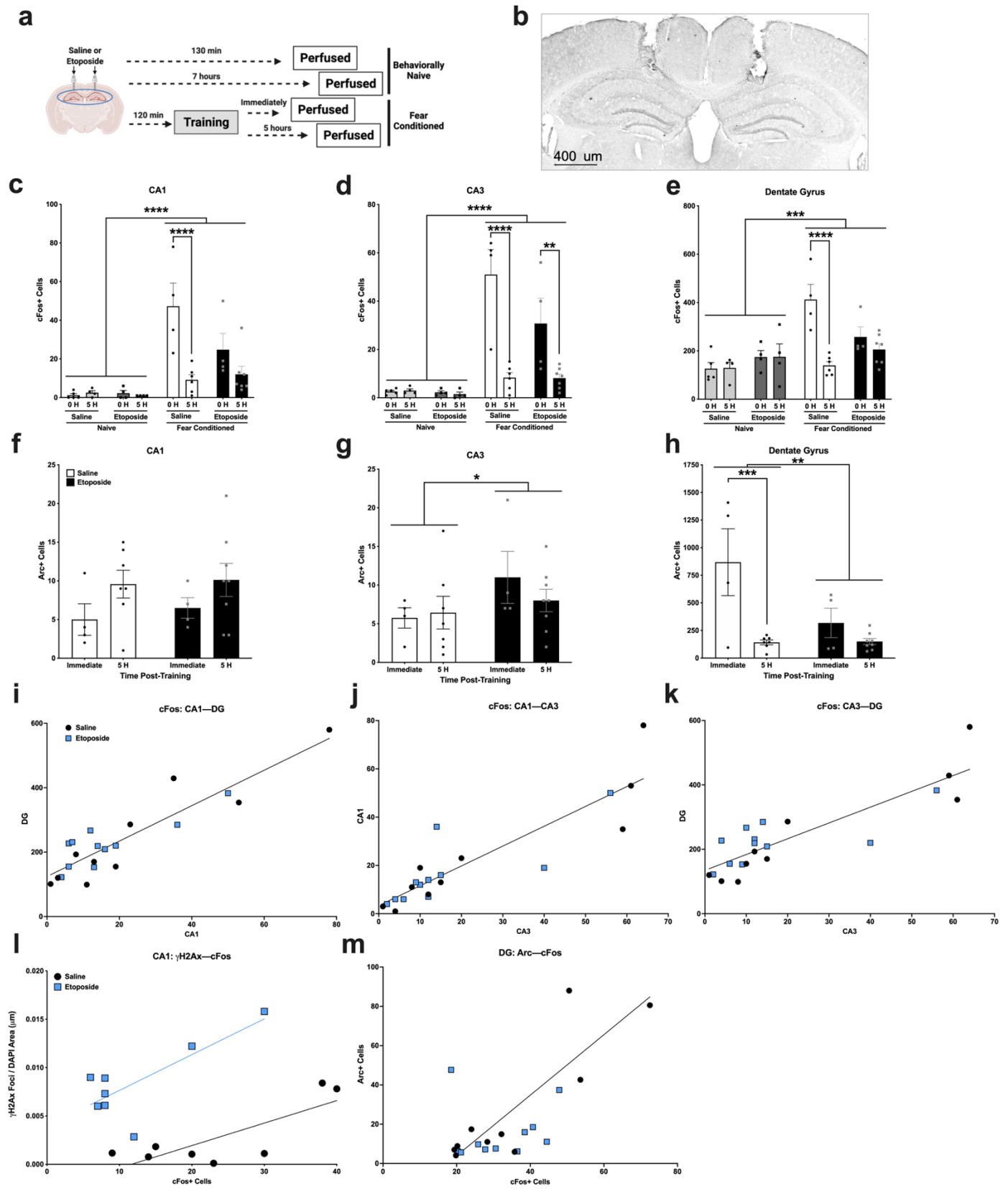


Figure 3.2. Analysis of cFos and Arc by immunohistochemistry in the hippocampus at two time points after fear conditioning. **A)** Schematic of the experimental timeline ($n = 4-7/\text{behavior group/treatment/time point}$). **B)** Representative overview image to show cannula placement. **C)** Total number of cFos+ cells in the CA1. There were more cFos+ cells in animals that underwent fear conditioning ($F(1,30) = 43.165, p < 0.001$), and in the group euthanized immediately post-training ($F(1,30) = 12.608, p = 0.001$). There also was a significant time post-training by behavior interaction ($F(1,30) = 13.769, p < 0.001$). Sidak's post-hoc comparison indicated that the fear conditioned saline group euthanized 5 h post-training had significantly fewer cFos+ cells compared to the immediately euthanized group ($p < 0.0001$), but the 5 h post-training etoposide group was not different than the immediate group ($p = 0.2607$). **D)** Total number of cFos+ cells in the CA3. There were more cFos+ cells in the animals that underwent fear conditioning ($F(1,30) = 43.071, p < 0.001$), and in the the group euthanized immediately post-training ($F(1,30) = 23.265, p < 0.001$). There also was a significant time post-training by behavior interaction ($F(1,30) = 23.747, p < 0.001$). Sidak's post hoc comparison indicated that the fear conditioned saline group euthanized 5 h post-training had significantly fewer cFos+ cells compared to the immediately euthanized group ($p < 0.0001$), and that the fear conditioning etoposide group euthanized 5 h post-training had fewer cFos+ cells compared to the immediately euthanized group ($p = 0.0037$). **E)** Total number of cFos+ cells in the DG. There were more cFos+ cells in the animals that underwent fear conditioning ($F(1,30) = 22.965, p < 0.001$). There was also a significant time by treatment interaction ($F(1,30) = 4.583, p = 0.041$), time by behavior interaction ($F(1,30) = 4.940, p = 0.034$), treatment by time by behavior interaction ($F(1,30) = 7.441, p = 0.011$), and a trend towards a time by treatment interaction ($F(1,30) = 4.583, p = 0.052$). Sidak's post hoc testing revealed a significant decrease in the fear conditioned saline-infused group 5 h post-training compared to immediately post-training ($p < 0.0001$), but not in the fear conditioned etoposide-infused groups ($p = 0.7132$). **F)** Total number of Arc+ cells in the CA1. There was a significant increase in Arc+ cells in animals euthanized 5 h post-training compared to immediately post-training ($F(1,42) = 5.054, p = 0.030$). **G)** Total number of Arc+ cells in the CA3. Mice infused with etoposide had overall more Arc+ cells ($F(1,42) = 5.067, p = 0.023$). **H)** Total number of Arc+ cells in the DG. There was a significant effect of time post-training ($F(1,42) = 22.104, p < 0.001$), treatment ($F(1,42) = 8.362, p = 0.006$), and a time by treatment interaction ($F(1,42) = 8.222, p = 0.006$). Sidak's post hoc test showed that the number of Arc+ cells dropped over time in the saline-infused groups ($p < 0.001$), but not the etoposide-infused groups ($p = 0.539$). **I)** A significant positive correlation between the total number of cFos+ cells in the CA1 and DG after fear conditioning ($F(1,19) = 83.13, p < 0.0001$). There was no difference between the saline- and etoposide-infused mice. **J)** A significant positive correlation between the total number of cFos+ cells in the CA1 and CA3 ($F(1,19) = 67.62, p < 0.0001$). There was no difference between the saline- and etoposide-infused mice. **K)** A significant positive correlation between the total number of cFos+ cells in the CA3 and DG ($F(1,19) = 56.16, p < 0.0001$). There was no difference between the saline- and etoposide-infused mice. **L)** Correlation between average number of γH2Ax normalized to DAPI area in the CA1 and the total number of cFos+ cells in the CA1. There was a significant positive correlation in both the fear conditioned saline-infused animals ($F(1,6) = 10.53, p = 0.0176$) and the fear conditioned etoposide-infused animals ($F(1,6) = 9.012, p = 0.0239$). **M)** Correlation between cFos and Arc cells in the DG. There was a significant positive correlation in the fear conditioned saline-infused animals ($F(1,8) = 24.23, p = 0.0012$), but not in the fear conditioned etoposide-infused animals ($F(1,8) = 0.089, p = 0.7724$). Data are presented as total number of immunopositive cells $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Hippocampal injection of etoposide impairs long-term contextual, but not cued, fear memory in both males and females

Based on the observed molecular changes in the hippocampus, we subsequently assessed long-term hippocampus-dependent and -independent memory. Our previous work indicated that systemic injections of etoposide affected males and females differently [207], thus we assessed long-term memory in both males and females here ($n = 13-15/\text{sex}/\text{treatment}$). All animals went through the same infusion and fear training timeline and were subsequently tested for contextual (hippocampus-dependent) and cued (hippocampus-independent) memory at 24 h and contextual fear memory at 2 weeks (**Fig. 3.3A**). No differences were detected in body weight based on drug treatment ($p = 0.828$). Males weighed more than females throughout the study ($p < 0.001$; **Table 3.1**).

There were no differences in performance during fear training based on treatment. All animals showed similar activity levels at baseline ($p = 0.653$; **Fig. 3.3B**), motion in response to the shocks ($p = 0.252$; **Fig. 3.3C**), percent time freezing during the tones ($p = 0.643$; **Fig. 3.3D**), and percent time freezing during the inter-stimulus intervals ($p = 0.127$; **Fig. 3.3E**). Additionally, there were no differences between males and females except for the response to shock, where males moved more in response to the shock than females ($p = 0.027$; **Fig. 3.3C**). These data indicate that infusions of etoposide prior to fear conditioning did not alter acquisition (*i.e.*, fear learning).

Twenty-four hours later, we tested the mice for recall of cued memory, 3 hours after the first contextual recall test (described below). Analysis of the response to the tone during the cued recall test indicated that all animals recognized the tone, shown by the increase in percent time freezing in all groups when the tone was played ($p < 0.001$; **Fig. 3.3F**). There were no effects of

treatment ($p = 0.823$), sex ($p = 0.905$), or interaction between dose and sex ($p = 0.771$), suggesting that non-hippocampus-dependent learning and memory was unaffected by our etoposide infusions into the CA1 region.

We also tested the animals for recall of contextual memory at 24 h, and again 2 weeks after training to include assessments of long-term effects of etoposide on hippocampus-dependent memory. There was an effect of treatment, with the animals that received etoposide freezing less at both time points compared to those that received saline ($p = 0.029$; **Fig. 3.3G**). There were no differences between the sexes ($p = 0.522$), and no interaction between dose and sex ($p = 0.659$).

Lastly, we euthanized these animals 15 minutes after the last contextual test to analyze γ H2Ax foci in the CA1 region. There were no long-term differences in this DSB signal based on treatment ($p = 0.534$) or sex ($p = 0.347$; **Table 3.2**).

Altogether, these data indicate that CA1 infusions of etoposide selectively impair long-term, hippocampus-dependent memory.

Table 3.2. γ H2Ax foci normalized to DAPI area (mm) in the CA1 region of animals that were euthanized 15 minutes after the last contextual recall test.

Sex	Treatment	
	Saline	Etoposide
Male	0.34 ± 0.18	0.33 ± 0.18
Female	0.77 ± 0.29	0.52 ± 0.19

Data presented as averages $\pm$ SEMs.

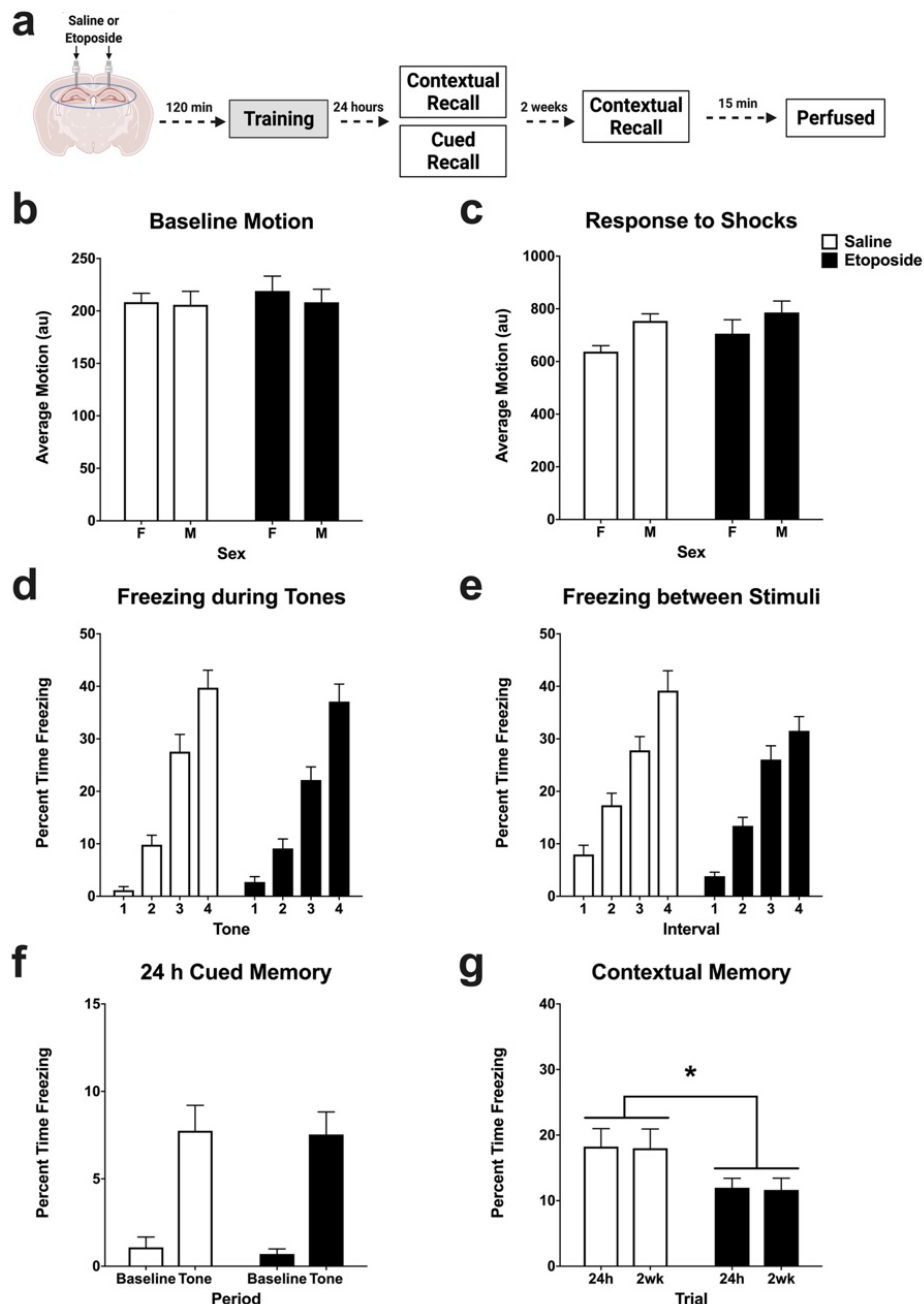


Figure 3.3. Behavioral performance in fear conditioning, cued, and contextual tests. **A)** Schematic of the experimental design. **B)** Average motion (au) during the 2-min baseline period in fear training. No differences were detected based on treatment ($p = 0.653$) or sex ($p = 0.649$). **C)** Average motion during the shocks (au). Males moved more during the shocks than females ($F(1,66) = 5.125$, $p = 0.027$), but no differences were detected based on treatment ($p = 0.252$). **D)** Percent time freezing during the 4 tones. All animals increased over the course of training ($F(2.382,161.992) = 131.083$, $p < 0.001$), with no differences found between treatment ($p = 0.455$) or sex ($p = 0.816$). **E)** Percent time freezing during the 4 inter-stimulus intervals. All animals increased over the course of training ($F(2.260,153.674) = 113.594$, $p < 0.001$), with no differences found between treatment ($p = 0.108$) or sex ($p = 0.708$). **F)** Percent time freezing at baseline and during the tone in the 24 h cued recall test. All animals showed increased freezing in response to the tone ($F(1,43) = 54.312$, $p < 0.001$); no differences were detected based on treatment ($p = 0.823$) or sex ($p = 0.771$). **G)** Percent time freezing during the contextual recall tests at 24 h and 2 weeks post-training. Mice that received etoposide infusions froze less at both time points than saline-infused mice ($F(1,43) = 5.112$, $p = 0.029$). No differences were seen based on sex ($p = 0.522$). All data are presented as mean $\pm$ SEM. * $p < 0.05$.

Discussion

The results of this study provide evidence that DSBs are involved in regulating IEG expression and long-term memory. Etoposide infusions in the hippocampus impaired long-term contextual memory, which is hippocampus-dependent, but not cued memory, which is hippocampus-independent. Mice infused with etoposide did not have higher levels of cFos⁺ cells 2 hours after infusion compared to 7 hours after infusion. All mice that underwent fear conditioning had more γ H2Ax foci in the CA1, CA3, and DG than behaviorally naïve mice, though we observed interactions between behavioral condition and treatment in the CA3 and DG. Additionally, the number of Arc⁺ cells was decreased in the DG and increased in the CA3 region following etoposide infusions.

DSBs play an important role in transcriptional elongation during cell division [100, 216] and generating molecular diversity in the immune system [217], but in non-dividing cells (such as neurons) DSBs have been thought of as the result of damaging factors and signals for apoptosis [218]. However, our data add to a growing literature that suggest an adaptive function of DSBs in non-dividing cells via regulating gene expression related to learning and memory. Previous studies have shown that stimulation of electrical activity in cultured hippocampal neurons led to DSBs forming on promoter regions of a small subset of genes, which led to their subsequent up-regulation [98]. These genes – including *fos*, *Npas4*, and *Egr1* – are known to regulate synaptic plasticity and related protein synthesis pathways. In mice, increased levels of γ H2Ax foci were observed in relevant brain regions following introduction to a novel environment [97]. Importantly, the increase in this DSB marker was transient: levels returned to baseline after 24 hours. This same study also showed that mice carrying dominant Alzheimer's disease genetic mutations had higher baseline levels of γ H2Ax foci and those levels failed to

return to baseline levels 24 h after introduction to the novel environment. These initial studies indicated that the precise timing and location of physiological DSBs may have a role in learning and memory and could be dysregulated in neurodegenerative disease.

Subsequent studies explored the role of DSBs across cell types in the mouse brain and in different fear conditioning paradigms. Mice tested in contextual fear conditioning showed increased DSBs in the hippocampus and medial prefrontal cortex, many of which occurred on or near genes important for regulating synaptic plasticity [105]. Both neuronal and non-neuronal cells displayed an increase in DSBs in that study; we did not assess cellular sub-types in this current study. Adding to the role of DSBs in fear learning and memory, direct infusion of 0.1 $\mu\text{g}/\mu\text{L}$ etoposide into the mouse prelimbic area impaired trace fear conditioning and disrupted IEG expression [171]. However, 1 $\text{ng}/\mu\text{L}$ of etoposide did not alter behavior. Our results extend these findings, demonstrating that an intermediate dose is sufficient to alter DSBs and IEGs in the hippocampus. While our infusions were directly into the CA1 region, we also observed alterations in the CA3 and DG. One possibility is that etoposide itself spread to these regions and directly affected DSB formation and IEG expression. Another is that the intra-hippocampal signaling was altered due to changes in the CA1, thereby indirectly altering DSBs and IEGs in the CA3 and DG [219]. Intriguingly, we discovered an interaction between behavioral condition (naïve or fear conditioned) and treatment (saline or etoposide), where etoposide infusions decreased γH2Ax foci in the DG and CA3 in fear conditioned mice but increased foci in naïve mice. This relationship likely reflects the sensitivity of interfering with the proper timing for adaptive DSBs during periods of learning.

Notably, our results indicate that inducing DSBs in the CA1 region led to blunted IEG expression immediately following fear conditioning. Previous research has shown that etoposide

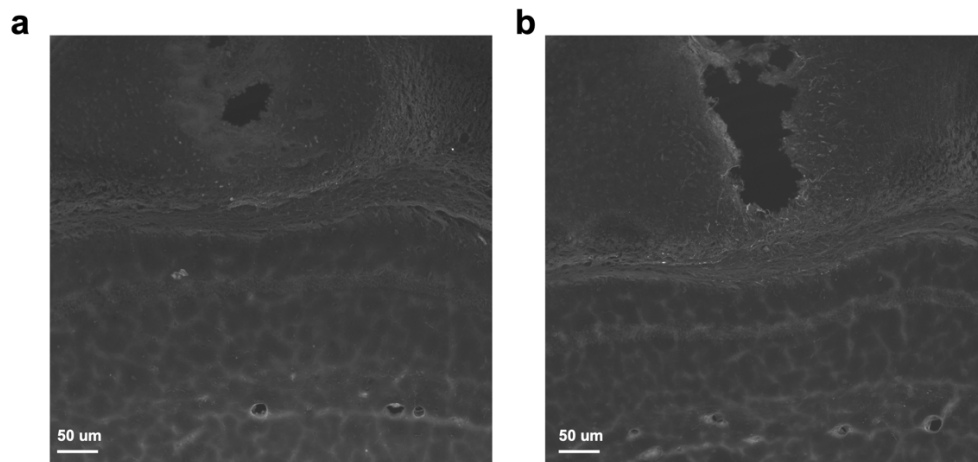
blunted the second wave of IEG mRNA levels (5 h post-training) in the prelimbic area [102]. Ionizing radiation—which induces single and double-strand breaks—has been shown to both up- and down-regulate IEG expression depending on dose, tissue analyzed, and timing [220-222]. For example, 2 Gy of X-ray radiation in hippocampal cultured neurons decreased cFos and Egr1 mRNA compared to sham controls at 60 min, but 2 Gy + NMDA activation led to increased Egr1 mRNA [223]. The 2 Gy alone and the 2 Gy + NDMA activation also resulted in increased γ H2Ax to similar levels. These data indicate that while DSBs may contribute to IEG expression, alterations to precise regulation and timing can both increase and decrease these genes. Our data here suggest that this dose of etoposide combined with fear conditioning led to a down-regulation, essentially preventing a “peak” like the one observed in the saline-infused, fear conditioned group. This lack of an increase is likely the driving factor in the statistical interactions. However, it is possible that other doses or timing could result in up-regulation, warranting further studies to explore this.

Moreover, our results increase understanding of the central effects of etoposide, which is commonly used in cancer treatment regimens [86, 87, 192, 210]. Very little data exist on the possible side effects of etoposide, as cancer and treatment status confound direct ties to etoposide alone. Etoposide does cross the blood brain barrier, though not well, with only ~3% entering the brain [209]. Even in small amounts, the hippocampus may be particularly susceptible to etoposide, as etoposide reduced hippocampal polyamines [192]. However, hippocampus-dependent function was not assessed in that study. Research into combined cancer treatment suggest that the hippocampus is sensitive to chemotherapy: transient treatment with cyclophosphamide and doxorubicin in ovariectomized female rats impaired contextual, but not cued, fear memory [193]. Additionally, following chemotherapy in patients, hippocampal

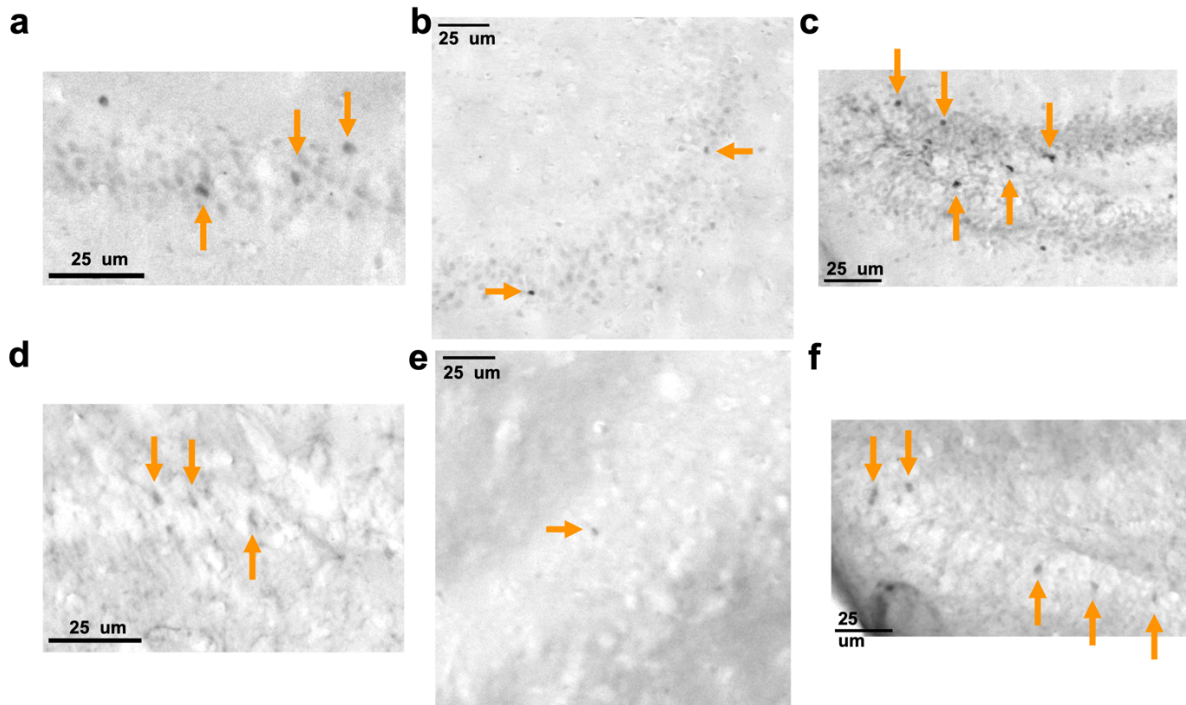
volume and connectivity are altered and correlate with impaired cognition [185, 194]. Direct delivery of 0.5 mg of etoposide in the ventricles of patients (with peak CSF levels reaching 9 $\mu\text{g/mL}$) elicited side effects of headaches and meningitis; learning and memory were not assessed, though [210]. Our previous experiment showing that systemic injections of a medium dose of etoposide impaired contextual and cued fear memory tied etoposide specifically to learning and memory impairments [207].

While IEGs generally tend to increase upon stimulation, there is task- and region-dependent specificity [44]. Our Arc and cFos cell count data were correlated only in the DG, but not in the CA1 or CA3 regions of the hippocampus. We also observed an increase in Arc in the CA3, in contrast to Arc in the DG and cFos in all sub-regions analyzed. cFos immunoreactivity was correlated in the hippocampal sub-regions analyzed, but Arc was not. Others have shown similar results, with region-, task- and time-specific increases in different IEGs [206, 224, 225]. Within the hippocampus, the CA1 is shown to be required for both acquisition and recall of contextual memory, while the CA3 is only required for acquisition [226], and timing of context related IEG expression in the CA1, CA3, and DG are distinct [225]. Notably, *Arc* and *cFos* fall into two subcategories of IEGs: *Arc* is classified as an “effector” IEG, while *cFos* is classified as regulatory transcription factor (RTF) [42-44]. Thus, while related, others have found that Arc and cFos RNA levels in rats are correlated in some regions (such as the entorhinal cortex) but not others (like the hippocampus) following spatial learning [44]. This sub-categorization may account for the distinct effects we observed in the hippocampus. Even with this subtle difference, our results suggest that etoposide affects both sub-types of IEGs. Further research should continue to identify specific IEG families that are regulated by DSB-induced gene expression, as well as explore more brain regions and task-dependency.

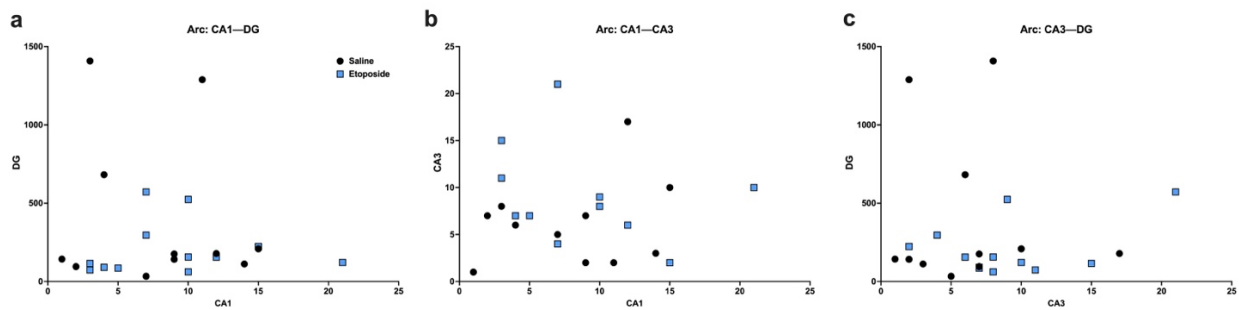
In summary, these experiments indicate that induction of DSBs in the hippocampus interferes with learning and memory and that proper regulation of DSB timing and location is related to intact learning and memory. Future efforts are warranted to identify the role of DSBs in other forms of learning and other brain regions, such as the amygdala. Additionally, characterizing the effects of a wider range of doses will be important, especially when considering the translational relevance.



Supplemental Figure 1. No Fluoro-Jade C staining in the CA1 region of the hippocampus in **A)** a saline-infused animals and **B)** an etoposide-infused animal. There was no observable indication of cell death in any animals.



Supplemental Figure 2. Representative images of cFos staining in **A)** the CA1, **B)** the CA3, and **C)** the DG. Representative images of Arc staining in **D)** the CA1, **E)** the CA3, and **F)** the DG. Orange arrows point to examples of cells that were counted as immuno-positive.



Supplemental Figure 3. No correlation of total number of Arc+ cells between the **A)** CA1 and DG, **B)** CA1 and CA3, and **C)** CA3 and DG.

Chapter 4: Apolipoprotein E isoform-dependent effects on neuronal ensembles as measured by cFos activation patterns across fear-related brain regions

Abstract

Apolipoprotein E (apoE) isoforms confer differential risk for diseases. Of the 3 isoforms (E2, E3, or E4), E4 is associated with increased risk for Alzheimer's disease (AD), while E2 is associated with increased risk and severity of post-traumatic stress disorder (PTSD). Women are also at higher risk to develop both AD and PTSD. In cognitively normal adults, MRI and PET scans show that E4 carriers have greater brain activation during learning and memory tasks in the absence of behavioral differences. Mice expressing the human apoE isoforms display differences in fear extinction that parallel human data, such that E2 mice fail to extinguish, mirroring heightened PTSD symptoms in E2 combat veterans. Yet, the neural mechanisms that lead to apoE-dependent risk for different diseases remain unclear. We used young female apoE targeted replacement mice to assess brain-wide changes in neural activation following contextual fear conditioning with whole-brain cFos imaging. We analyzed the magnitude of cFos activation in regions important for contextual fear conditioning, as well as co-activation across regions similar to functional MRI analyses. E4 mice froze more during contextual fear conditioning and had lower cFos immunoreactivity across regions important for somatosensation and contextual encoding compared to E2 mice. E4 mice also showed altered co-activation compared to E3 mice. Our data in mice correspond to human MRI and behavioral data examining the apoE isoforms, indicating that there are differences in brain activity at young ages independent of outwardly observable behavior. This information is important to consider in the clinic for enhanced precision treatment and prevention strategies.

Introduction

Sporadic Alzheimer's disease (AD) cases—those with no known cause—comprise more than 90% of AD diagnoses [227]. Age and female sex are the two highest risk factors for development of late onset AD (LOAD) [113, 228]. Additionally, risk to develop post-traumatic stress disorder (PTSD) is higher in women than men [229]. Behind these, apolipoprotein E (apoE) isoform is the next greatest predictor for LOAD [119, 230]. ApoE exists in 3 human isoforms: E2, E3, and E4. Of the 3 human isoforms, people with one or two E4 alleles have a relatively higher risk of developing LOAD compared to E3 carriers; conversely, E2 carriers are relatively protected from LOAD [119]. Notably, this relative protection/risk for LOAD is ethnicity-dependent: the E4-LOAD link is seen in White and Japanese populations but absent in Black, Native American, and non-white Hispanic populations [119, 135, 136]. While compared to E3, E2 is protective against LOAD, it is associated with higher rates and greater severity of post-traumatic stress disorder (PTSD) [140-142]. It was also recently shown that people and mice with the E2 isoform had poorer outcomes in melanoma, whereas E4 conferred better outcomes (reduced tumor size, metastasis, and increased survival) [143]. Exactly how the apoE isoforms lead to differential and specific disease risk or protection is still unclear.

Investigations looking at brain activation at rest and during learning and memory tasks have shown apoE-dependent differences in cognitively healthy adults. Cognitively intact middle-aged E4-carriers display greater blood oxygen levels during learning trials compared to age- and sex-matched E3 controls in the absence of behavioral differences [162]. In young adults, E4 carriers show increased brain glucose metabolism [163]. These studies together suggest that E4 carriers have increased energy usage even at young ages. There are also sex- and apoE-

dependent differences in 7-10-year-olds [161]: female E4 carriers perform worse on visual and spatial recognition tasks than female E3 carriers. However, this effect is not seen in males.

In rodents, immediate early genes (IEGs)—such as cFos—are used as markers of neuronal activation. IEGs are important for synaptic plasticity, learning, and memory [33, 34, 44, 69, 195, 231]. Interfering with typical IEG expression impairs memories retrieval [63] and induced IEG expression can create false memories [232] in rodents. ApoE-dependent differences in expression of IEGs are relatively unexplored, though some studies have shown apoE-dependent differences in IEG expression. On a high fat diet, E4 mice show less IEG expression in the hippocampus than E3 mice [233], and apoE-dependent changes in cFos phosphorylation are observed in induced pluripotent stem cells (iPSCs) [234].

Contextual fear conditioning is a well-studied learning test with clearly defined neuroanatomy [5, 6, 235]. cFos expression increases in specific brain regions rodents after fear conditioning dependent on the paradigm used [49, 50]. Increases are seen in the hippocampus following contextual fear conditioning, for example [50], and inhibition of c-Fos in the CA1 impairs contextual recall [51]. Beyond the hippocampus, many regions have been defined that contribute to different aspects of the fear encoding process, including somatosensation (such as the thalamus and locus coeruleus), contextual encoding (such as the entorhinal cortex and hippocampus), fear integration (amygdala nuclei), and fear expression (such as periaqueductal gray and medulla) [5]. These regions identified in rodents mirror those observed in human functional magnetic resonance imaging (fMRI) during Pavlovian fear conditioning [236].

There are differences in fear learning and memory in mice expressing human apoE isoforms under control of the murine apoE promoter. E2 mice display impaired fear extinction [165], and E4 mice show increased anxiety-like behavior [237]. Moreover, E2 mice show other

alterations in the endocrine system and behavior following exposure to trauma, which are potentially related to the greater diagnosis and higher PTSD clinical scores and blunted cortisol levels seen in E2-carriers from combat veteran and refugee populations [140, 141]. To the best of our knowledge, neuroanatomical or fMRI changes in humans in response to fear based on apoE status have not been studied.

Early-life differences in IEG signaling might contribute to the apoE-specific risks for LOAD or PTSD. Recent advances in whole-brain imaging have opened up the ability to probe for brain-wide alterations in neuronal activation [238] as well as analyses of co-activation across regions similar to functional MRI analyses [212, 239]. Due to increased risk for females to develop AD and PTSD, we chose to investigate possible early changes in female mice [228]. Here, we analyzed cFos across the brain in young female apoE targeted replacement mice trained for contextual fear conditioning. We hypothesized that each human apoE isoform would show distinct patterns of activation across the highly defined fear circuitry, and thus provide insight into apoE-dependent differences that may contribute to relative risk for neurological conditions.

Materials & Methods

Mice

Two- to three-month old female targeted replacement human apoE mice generated by Sullivan et al. [240-242] and bred in our colony were used for the experiments. For behavioral experiments, $n = 60$ mice in total ($n = 19-22$ mice/genotype) were used. For the whole-brain experiments, $n = 30$ mice in total ($n = 10$ mice/genotype) were used. All mice were singly housed starting 4 days prior to testing, at which point extra nesting material was provided. Food and water were provided *ad libitum* and lights were on a standard 12 h light: dark cycle.

All animal procedures were reviewed and approved by the OHSU IACUC and in accordance with AAALAC standards. All procedures followed the ARRIVE guidelines. Researchers were blinded to genotype groups throughout the duration of experiments.

Contextual Fear Conditioning

We used a standard 2-shock contextual fear conditioning paradigm, as described previously [165]. Briefly, mice were placed into plexiglass chambers with a shock grid (Med Associates, St. Albans, Vermont) and allowed to explore for a baseline period of 148 s. A 2 s, 0.5 mA shock was administered, followed by a 148 s inter-stimulus interval (ISI), a second 2 s shock, and a final 60 s ISI. After these 6 minutes, mice were removed from the fear conditioning chambers. Chambers were thoroughly cleaned with 0.5% acetic acid after each trial. Trials were recorded with Video Freeze software (Med Associates). Time freezing, defined as the cessation of all movement except breathing, and average indices of motion, measured by changes in video-pixel composition in arbitrary units (au), were analyzed.

Tissue collection & whole-brain imaging

Two hours after fear conditioning, mice were injected with 50 mg/ml lethal ketamine-xylazine cocktail to capture peak cFos levels [196, 198]. Mice were intracardially perfused with 15 ml of ice-cold saline followed by 15 ml of 4% paraformaldehyde (PFA). Brains were removed and stored overnight in 4% PFA. After 24 h, brains were switched to phosphate-buffered saline (PBS). The brains were analyzed by Certerra, Inc. for whole-brain clearing, cFos staining and imaging, as previously described [238].

Statistical analysis

All data were first analyzed for normality; we were able to proceed with standard parametric tests for all measures. Considering the uniqueness of this type of data set, we implemented Bonferroni corrections to reduce likelihood of Type I errors.

For fear conditioning, average motion and percent time freezing were analyzed as outcome measures. We used a 1-way ANOVA for average motion during the baseline period and average motion in response to the shocks. For learning, we analyzed the percent time spent freezing during the ISIs with a repeated-measures ANOVA. Bonferroni's post hoc comparisons were used to compare the behavioral data of mice expressing distinct apoE isoforms.

For whole-brain analysis, we first analyzed the raw number of cFos⁺ cells throughout the brain. We discovered significant differences between cohorts, likely due to the sensitive nature of IEGs. As such, we transformed the cFos counts into Z-scores, normalized to the E3 animals in each cohort.

We then proceeded to analyze differences in cFos activation across the brain using the Z-scores. We first identified brain regions known to be important for contextual fear learning [5, 6] and organized them into 4 distinct subcategories: somatosensation, contextual encoding, fear integration, and fear expression. **Table 4.1** identifies these regions, the acronyms, and their primary function related to contextual fear learning. We then used repeated-measures ANOVAs in these sub-categories to identify regional and isoform-dependent differences, with Bonferroni's post hoc corrections to compare the isoforms. To localize regional differences, we subsequently used MANOVAs with Bonferroni corrections to identify isoform differences in specific brain regions.

We have previously used this type of data as an indirect measure of connectivity [212, 239]. Here, we calculated the Pearson's coefficient for all fear-related brain regions. We then built correlation matrices and compared the matrices to identify apoE isoform-specific changes in co-activation of cFos using a method previous described [243]. The statistic defined by Chang and colleagues [243] tests whether two covariance matrices are identical. Critically, this method is sensitive to small numbers of pairwise differences in covariance matrices by testing whether the maximum element-wise difference between the two covariance matrices is 0, making the test appropriate for examining differences in covariance matrices in cases where small numbers of pairwise differences may drive overall differences in covariance patterns, such as in gene expression [244] or neuronal connectivity [239]. The test statistic, $\hat{T}$, is defined as the maximum of the squared, element-wise differences between the two covariance matrices, normalized by its variance. The p -value is determined by creating an empirical null distribution of $\hat{T}$ using a multiplier bootstrap [245] and counting the proportion of bootstrap statistics that are larger than the test statistic. This was shown to be appropriate even in cases where the number of dimensions is greater than the number of subjects [243].

Results

ApoE isoform-dependent differences in fear learning and whole-brain cFos expression

We first assessed if apoE isoform affects contextual fear learning in young female mice (**Fig. 4.1A**). Prior to any shocks, we detected a difference in baseline motion between the mice with distinct apoE isoforms ($p = 0.002$; **Fig. 4.1B**). Bonferroni's post hoc testing indicate that E4 mice moved less than E3 mice ($p = 0.0001$) and trended towards moving less than E2 mice ($p = 0.070$). We did not find any differences in average motion during the shocks ($p = 0.979$), suggesting that all mice responded similarly to the aversive stimulus.

Table 4.1. List of brain regions involved in contextual fear learning. Brain regions were organized into 4 functional classifications for analysis purposes.

ROIs	Full Name	Functional Classification	Detailed Function
ILM	Intralaminar area of the dorsal thalamus	Somatosensation	Somatic sensation
DORsm	Thalamus, sensory-motor cortex related	Somatosensation	Sensation of aversive unconditioned stimulus
PO	Posterior complex of the thalamus	Somatosensation	Sensation of aversive unconditioned stimulus
PB	Parabrachial Nucleus	Somatosensation	Sensation of aversive unconditioned stimulus
LC	Locus coeruleus	Somatosensation	Sensation of aversive unconditioned stimulus
AI	Agranular insular area	Somatosensation	Somatic sensation
PIR	Piriform area	Contextual Encoding	Olfaction
SSp	Primary somatosensory area	Contextual Encoding	Integration of sensation of aversive unconditioned stimulus
PERI	Perirhinal cortex	Contextual Encoding	Temporal encoding
SUBv	Subiculum, ventral part	Contextual Encoding	Contextual encoding
ENT	Entorhinal cortex	Contextual Encoding	Contextual encoding
HIP	Hippocampus	Contextual Encoding	Contextual encoding
LA	Lateral Amygdala	Fear Integration	Integration of sensation of aversive unconditioned stimulus
BLA	Basolateral amygdala	Fear Integration	Integration of associative learning
BMA	Basomedial amygdala	Fear Integration	Integration of associative learning
CEA	Central amygdala	Fear Integration	Output of autonomic components of emotion
PAG	Periaqueductal gray	Fear Expression	Freezing
PRNc	Pontine reticular nucleus, caudal part	Fear Expression	Potentiated startle
BST	Bed nuclei of the stria terminalis	Fear Expression	Corticosterone release
VMH	Ventromedial hypothalamic nucleus	Fear Expression	Corticosterone release
LZ	Hypothalamic lateral zone	Fear Expression	Cardiovascular response
DMX	Dorsal motor nucleus of the vagus nerve	Fear Expression	Cardiovascular response
VTA	Ventral tegmental area	Fear Expression	Corticosterone release
PVT	Paraventricular nucleus of the thalamus	Fear Expression	Corticosterone release
MY	Medulla	Fear Expression	Cardiovascular response
SI	Substantia innominate	Fear Expression	Arousal

The percent time freezing during the inter-stimulus intervals (ISIs), a measure of fear learning, was also dependent on apoE isoform ($p = 0.0008$; **Fig. 4.1C**). E4 mice froze significantly more during the ISIs than either E2 ($p = 0.0319$) or E3 mice ($p = 0.0006$). Additionally, while all mice displayed an increase in freezing from ISI 1 to ISI 2 ($p < 0.0001$), this pattern was distinct in the isoforms, shown by a significant time-by-isoform interaction ($p = 0.0285$).

Following fear conditioning, we assessed brain activation via cFos across the whole brain and in regions known to contribute to contextual fear learning (**Table 4.1**). There were apoE isoform differences in the global magnitude of cFos activation ($p = 0.020$, **Fig. 4.1D**), with Bonferroni's post hoc test showing that E4 mice had less cFos immunoreactivity than E2 mice ($p = 0.0319$) and trended towards less cFos immunoreactivity than E3 mice ($p = 0.0664$). We next focused our analysis on regions important for contextual fear learning. Again, there was an overall difference dependent on apoE isoform ($p = 0.0172$; **Fig. 4.1E**). Post hoc testing revealed that E4 mice had less cFos immunoreactivity in all regions involved in fear learning compared to both E2 ($p = 0.0270$) and E3 mice ($p = 0.0434$). A representative image of the whole-brain stained for cFos immunoreactivity can be seen in **Fig. 4.1G**.

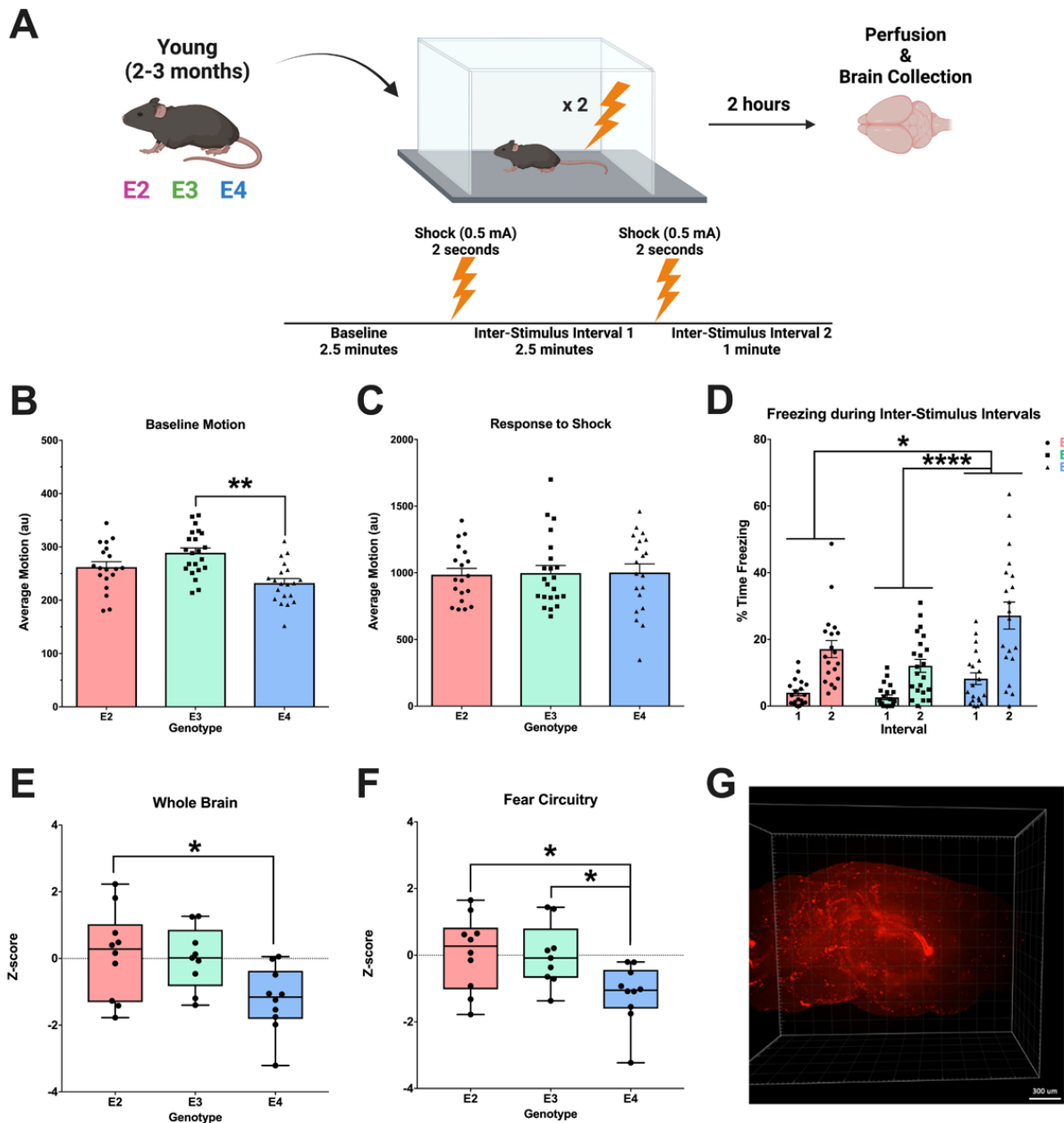


Figure 4.1. Effects of apoE isoform on fear learning. **A)** Schematic of the experimental design. **B)** Average baseline motion in the initial 2 minutes in the fear conditioning test. There was a main effect of apoE isoform ($p = 0.002$, $F(2,58) = 9.896$). The Bonferroni's post hoc test revealed a difference in E3 vs. E4 ($p = 0.001$) and a trend towards a difference in E2 vs. E4 ($p = 0.070$). **C)** Average motion during the two shocks. There were no significant apoE isoform differences ($p = 0.979$). **D)** Percent time freezing during the ISIs. There was an effect of time ($p < 0.0001$, $F(1,58) = 92.43$), isoform ($p = 0.0008$, $F(2,58) = 8.134$), and a time-by-isoform interaction ($p = 0.0023$, $F(2,58) = 3.785$). The Bonferroni's post hoc test revealed a difference in E2 vs. E4 ($p = 0.0319$) and E3 vs. E4 ($p = 0.0006$) mice. **E)** Z-scores of cFos-positive cells in the entire mouse brain. There was a main effect of apoE isoform ($p = 0.020$, $F(2,26) = 4.573$). The Bonferroni's post hoc test revealed differences in E2 vs. E4 ($p = 0.0319$) mice and a trend towards a difference in E3 vs. E4 mice ($p = 0.0664$). **F)** Z-scores of cFos-positive cells in all regions involved in contextual fear learning. There was a main effect of apoE isoform ($p = 0.0172$, $F(2,26) = 4.773$). Post hoc testing revealed differences between E2 vs. E4 ($p = 0.0270$) and E3 vs. E4 ($p = 0.0434$) mice. **G)** Representative 3-D image of a hemisphere illustrating whole-brain cFos immunoreactivity. Scale bar is 300 μ m. Behavioral data are presented as mean $\pm$ SEM. cFos immunoreactivity data are presented as minimum to maximum boxplots. Each point represents an individual animal. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Schematic made with biorender.com

ApoE isoform-dependent differences in magnitude of cFos activation in fear circuitry

To clarify differences between the apoE isoforms in brain activation, we organized brain regions involved in contextual fear learning into distinct categories: somatosensation, contextual encoding, fear integration, and fear expression (**Table 4.1**).

We found a significant difference between isoforms in regions important for somatosensation ($p = 0.031$; **Fig 4.2A-B**). Bonferroni's post hoc showed that this isoform effect was driven by the difference between E2 and E4 mice ($p = 0.028$) throughout. Subsequent MANOVA identified that the thalamus, sensory-motor cortex-related area was significantly different based on apoE ($p = 0.048$; **Fig. 4.2D**), with post-hoc testing indicating a trend that this was due to differences in E2 compared to E4 ($p = 0.058$). We also detected a trend towards an isoform difference in the posterior complex of the thalamus ($p = 0.085$, **Fig. 4.2E**). The intralaminar area (**Fig. 4.2C**), parabrachial nucleus (**Fig. 4.2F**), locus coeruleus (**Fig. 4.2G**), and agranular insular area (**Fig. 4.2H**) did not reach significance after correction for multiple testing.

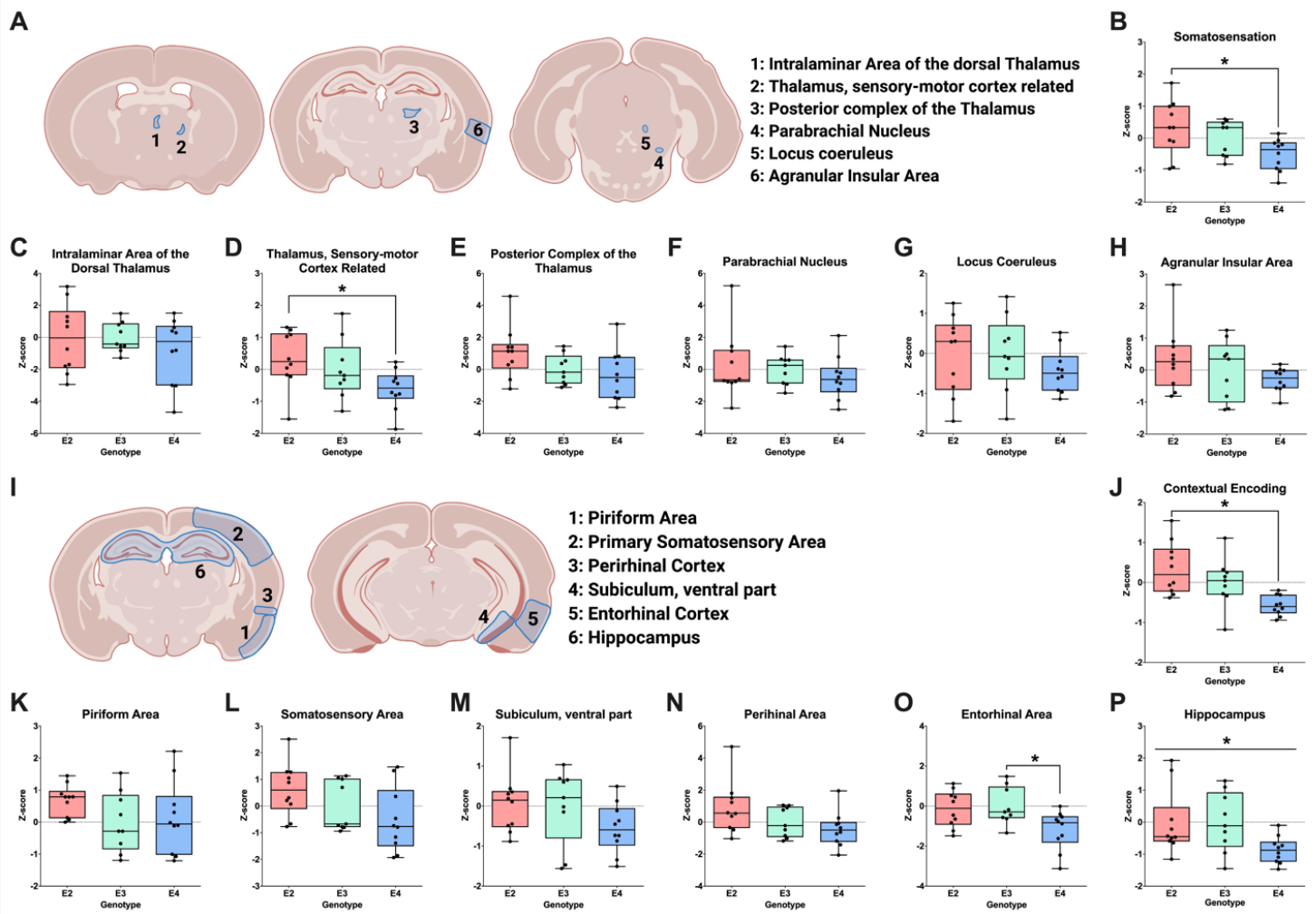
When we analyzed regions important for contextual encoding, we detected a significant effect of region ($p = 0.040$, Greenhouse-Geisser corrected; **Fig. 4.2I-J**), indicating that these sub-regions had distinct patterns of cFos activation. Additionally, we found a main effect of isoform ($p = 0.002$). Again, E4 mice had significantly lower cFos levels than E2 mice ($p = 0.002$) and a trend towards lower levels than E3 mice ($p = 0.078$). There were no differences between E2 and E3 mice ($p = 0.506$). Follow-up analyses to explore specific sub-regions revealed that cFos immunoreactivity was different based on apoE isoform in the entorhinal cortex ($p = 0.018$; **Fig. 4.2O**) and hippocampus ($p = 0.045$; **Fig. 4.2P**), though post-hoc analysis only revealed a difference between E3 and E4 ($p = 0.031$) and a trend towards a difference between E2 and E4 ($p = 0.057$) in the entorhinal area. There were also trends towards an apoE isoform difference in the

primary somatosensory area ($p = 0.074$; **Fig. 4.2L**) and the perirhinal area ($p = 0.074$; **Fig. 4.2N**). No apoE isoform differences were detected in the piriform area (**Fig. 4.2K**) or the subiculum, ventral part (**Fig. 4.2M**).

Next, we analyzed cFos immunoreactivity in the amygdalar nuclei (**Fig. 4.3A**). We did not find any overall apoE isoform-dependent differences in cFos immunoreactivity levels ($p = 0.272$; **Fig. 4.3B**). However, MANOVA identified a significant apoE isoform difference in the basolateral amygdala ($p = 0.035$; **Fig. 4.3D**), with E2 mice having higher levels of cFos immunoreactivity than E4 mice ($p = 0.035$). There was also a trend towards an apoE isoform-dependent difference in the lateral amygdala ($p = 0.078$; **Fig. 4.3C**). No differences were found in the basomedial (**Fig. 4.3E**) or central amygdala (**Fig. 4.3F**).

Lastly, we explored apoE isoform differences in brain regions important for expression of fear (**Fig. 4.3F**). There was a trend toward a main effect of apoE isoform ($p = 0.055$; **Fig. 4.3G**), again with E4 mice overall showing a trend toward lower cFos immunoreactivity levels than E2 mice ($p = 0.052$). A MANOVA of these regions revealed that cFos immunoreactivity in the periaqueductal gray was different based on apoE isoform ($p = 0.029$). Here, the difference was between E3 and E4 mice ($p = 0.025$; **Fig. 4.3I**). No differences were found in the pontine reticular nucleus, caudal part (**Fig. 4.3J**); bed nuclei of the stria terminalis (**Fig. 4.3K**); ventromedial hypothalamic nucleus (**Fig. 4.3L**); hypothalamic lateral zone (**Fig. 4.3M**); dorsal motor nucleus of the vagus nerve (**Fig. 4.3N**); ventral tegmental area (**Fig. 4.3O**); paraventricular nucleus of the thalamus (**Fig. 4.3P**); medulla (**Fig. 4.3Q**); or substantia innominata (**Fig. 4.3R**).

Taken together, these analyses suggest that apoE isoform-dependent differences in contextual fear circuitry are localized to areas important for somatosensation and contextual encoding, with lower cFos immunoreactivity levels in E4 mice.



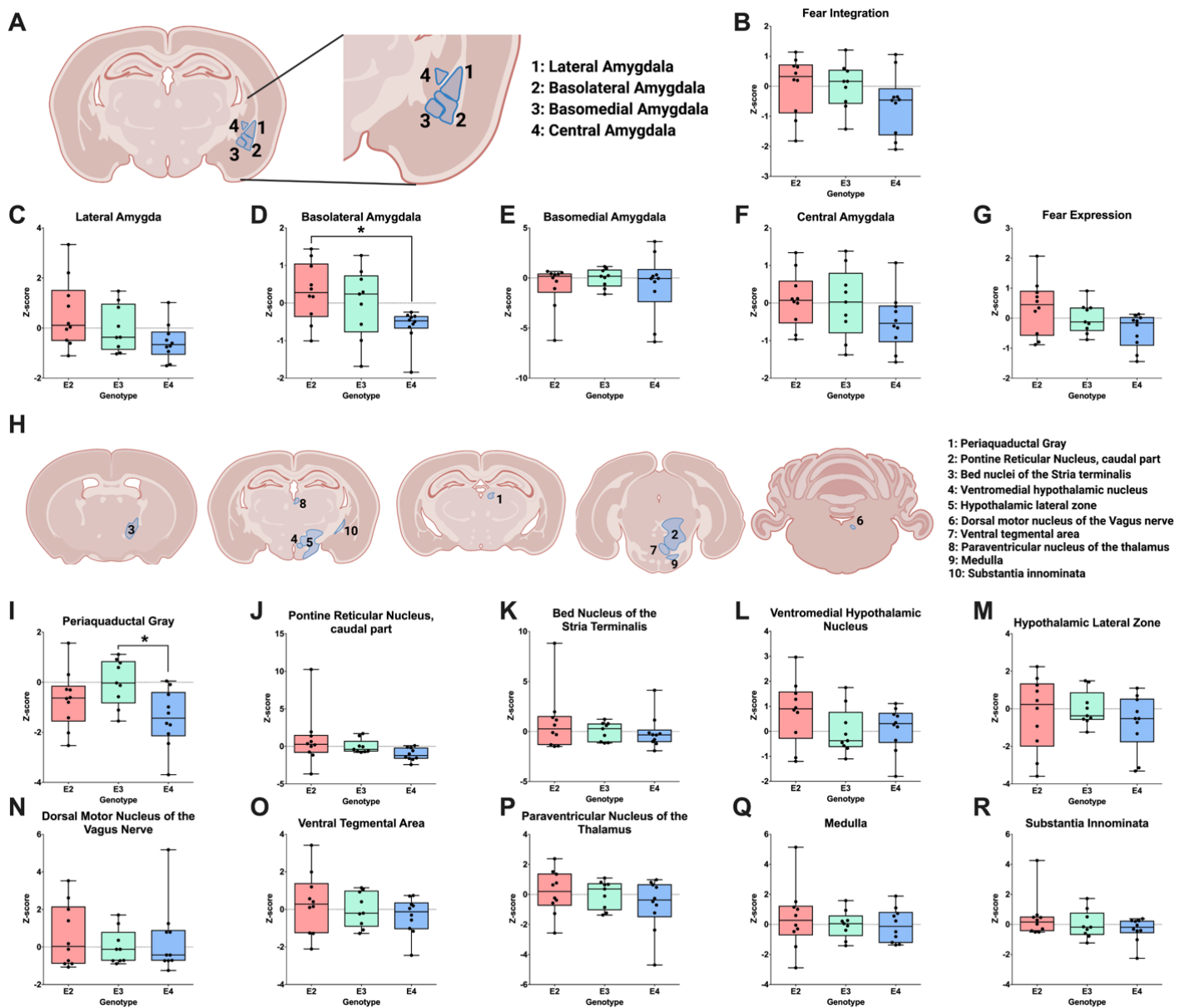


Figure 4.3. cFos immunoreactivity in brain regions important for integration of contextual fear learning and fear expression. **A)** Schematic of brain regions involved in fear integration. **B)** Average of regions involved in fear integration. A repeated measures ANOVA in these regions did not reveal any overall differences between apoE isoforms ($p = 0.272$). Z-scores of cFos immunoreactivity levels were seen in **(C)** the lateral amygdala, **(D)** the basolateral amygdala, **(E)** the basomedial amygdala, and **(F)** the central amygdala. Subsequent analysis identified apoE isoform-specific differences in the basolateral amygdala ($p = 0.035$, $F(2,26) = 3.810$) and a trend towards apoE isoform differences in the lateral amygdala ($p = 0.078$). The Bonferroni's post hoc test revealed that the difference in the basolateral amygdala was driven by E2 vs. E4 mice ($p = 0.035$). **G)** Average of regions involved in fear expression. **H)** Schematic of brain regions involved in fear expression. A repeated-measures ANOVA in these regions revealed a trend towards an overall differences between apoE isoforms ($p = 0.055$, $F(2,26) = 3.239$). Z-scores of cFos immunoreactivity levels were seen in **(I)** the periaqueductal gray; **(J)** the pontine reticular nucleus, caudal part; **(K)** the bed nucleus of the stria terminalis; **(L)** the ventromedial hypothalamic nucleus; **(M)** the hypothalamic lateral zone; **(N)** the dorsal motor nucleus of the vagus nerve; **(O)** the ventral tegmental area; **(P)** the paraventricular nucleus of the thalamus; **(Q)** the medulla; and **(R)** the substantia innominata. Subsequent analysis identified apoE isoform-specific differences in the periaqueductal gray ($p = 0.029$, $F(2,26) = 4.092$). The Bonferroni's post hoc test revealed that the difference was driven by E3 vs. E4 mice ($p = 0.025$). Data are presented in minimum to maximum boxplots. Each point represents an individual animal. * $p < 0.005$. Schematic made with biorender.com

ApoE isoform-dependent differences in co-activation of cFos immunoreactivity across regions important for contextual fear learning

The unique benefits of collecting data from the entire brain allowed us to analyze the co-activation of cFos cells within the identified fear circuitry. We calculated the Pearson's coefficient for these regions and built covariance matrices categorized into the 4 sub-categories for each apoE isoform (**Fig. 4.4A-C**). When we analyzed these matrices, we found that the co-activation of cFos immunoreactivity in E4 mice was significantly different than that in E3 mice ($p = 0.0174$, $\hat{T} = 3.9302$; **Fig. 4.4B & 4.4C**). There was no difference detected between co-activation of cFos immunoreactivity in E2 and E3 mice ($p = 0.208$, $\hat{T} = 3.0344$; **Fig. 4.4A & 4.4B**) or between E2 and E4 mice ($p = 0.2348$, $\hat{T} = 2.9955$). These results suggest that communication between fear-related brain regions may be preferentially altered in young E4 mice.

To identify what correlations were driving these results, we examined all brain regions that had r -values above 0.7 or below -0.7. **Fig. 4.4D-F** show these analyses, which revealed a few major patterns between the apoE isoforms (**Fig. 4.4G**). First, there was only one overlap in regional correlations across the three apoE isoforms (between the agranular insular area and the substantia innominata). Second, E2 and E3 mice showed high correlations between contextual encoding regions, while E4 mice did not have any strong correlations between these regions. Third, E4 mice showed more strong negative correlations (5 total) than either E2 or E3 (2 each). Finally, E4 mice showed the least overlap with either of the other two apoE isoforms.

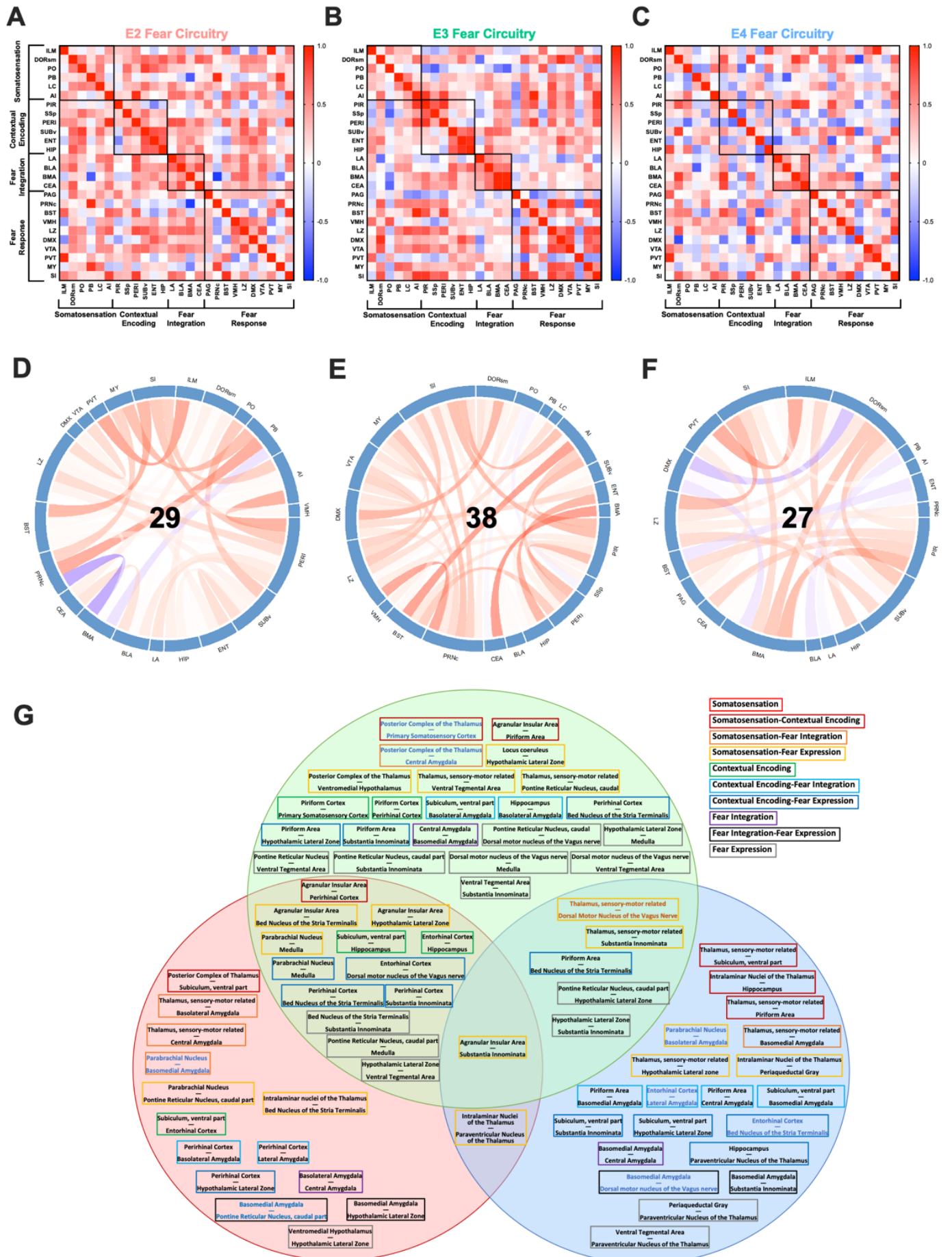


Figure 4.4. Correlation matrices of brain regions involved in contextual fear learning. Pearson's correlations were run for **(A)** E2, **(B)** E3, and **(C)** E4 mice to identify co-activation of cFos immunoreactivity across regions. Comparison of the correlation matrices revealed that E4 mice were different from E3 mice ($p = 0.0174$, $\hat{T} = 3.9302$). To identify the drivers of this difference, we mapped the regions that had r values >0.7 or <-0.7 and generated chord diagrams for **(D)** E2, **(E)** E3, and **(F)** E4 animals. **(G)** A Venn diagram of the regions that were identified as having the strongest correlations. Black text indicates a positive correlation; blue text indicates a negative correlation; orange text indicates an overlap but a mismatch in direction. The outline color of the box indicates the categories the two regions fall into.

Discussion

Our results provide insight into early life distinctions between the human apoE isoforms in female mice. Notably, young E4 female mice repeatedly displayed lower cFos immunoreactivity levels across regions important for somatosensation of aversive stimuli and contextual encoding. Specific regions that had lower cFos immunoreactivity levels in E4 mice include thalamus, sensory-motor cortex-related; entorhinal area; hippocampus; basolateral amygdala; and periaqueductal gray. E4 mice also showed increased fear learning (*i.e.*, enhanced time freezing during ISIs). Lastly, E4 mice showed altered co-activation of brain regions, with more negative correlations of cFos immunoreactivity across regions, compared to E3 mice. These data indicate that early-life alterations in IEG expression in brain may play a role in the apoE isoform-specific risks for developing neurological disorders.

The observed apoE differences in time freezing during the ISIs are in line with our earlier, independent studies [237, 246]. Beyond training day, we have also previously and consistently found apoE-dependent differences in fear extinction. Notably, both male and female E2 mice show impaired fear extinction compared to E3 and E4 [140, 165]. The E2 isoform has been relatively less-studied due to its rarity in the general population [119]. However, it is clear that E2 carriers show a decreased risk to develop LOAD and carrier status is associated with longevity [247-250]. E2 is not without neurological disease risk, though: apoE2 is associated with higher rates of PTSD, PTSD symptom severity measured by the Clinician-Administered PTSD-scale, and problematic alcohol use [140, 142, 251]. Impaired fear extinction in mice closely matches one of the primary symptoms of PTSD—the perseveration of traumatic memories. Moreover, there is a lack of data on fear acquisition related to apoE isoform; our

results add to knowledge about distinctions in relation to a highly salient learning event, though the after-effects of exposure a single traumatic event are complex.

E4 has also been linked to PTSD symptoms. E4-homozygous combat veterans with high levels of combat exposure have a higher lifetime prevalence of PTSD and increased PTSD symptoms severity, as measured by the Davidson Trauma Scale [252]. The Davidson Trauma Scale (in contrast to the Clinician-Administered PTSD-scale) is a self-report measure used as a screening for PTSD diagnosis [253, 254]. While highly reliable [255], the different rating scales used could partially account for the distinct apoE isoform-related conclusions. Moreover, there was no increased risk for PTSD in E4-carriers who had low combat exposure. The neuropathological impact of traumatic brain injury (TBI) has been shown to be worse in E4 carrying patients [256, 257] and E4 targeted replacement mice [258-260]. This includes increased tau phosphorylation, accumulation of amyloid beta 40, and inflammation, as well as decreased levels of brain derived neurotrophic factor (BDNF). Thus, the interaction between combat exposure (and thus likelihood of repeated TBIs) with apoE isoform may play a role in the patterns of PTSD presentation seen the different apoE isoforms and contribute to the divergent findings. PTSD also might have a bi-directional relationship with dementia: PTSD increases the risk for late-onset dementia, and dementia increases the risk for delayed-onset PTSD in those who experienced a significant trauma earlier in life [157].

Here, we assessed cFos as a possible contribution to isoform-specific risks immediately following a traumatic event (fear conditioning). The importance of IEGs for synaptic plasticity is well-known [34]. IEG expression can be induced by a range of stimuli, including exposure to novelty or stress [40, 44, 231, 261]. Dysregulation in IEGs has been implicated in a range of neurological diseases, including increased circulating *Egr1* in patients with schizophrenia [262,

263], increased Arc in the amygdala of patients with major depressive disorder [264, 265], and increased cFos and cJun in the hippocampus and amygdala in patients with AD [266-268]. Rodent research has highlighted dysregulation of IEGs in both AD and PTSD models. Young (4-6-month-old) mice carrying mutations in the amyloid precursor protein (APP) gene—a autosomal dominant cause of AD—were shown to have increased hippocampal delta-FosB, which led to subsequent downregulation of cFos and was associated with cognitive impairments [269]. APP knock out mice displayed high baseline levels of Egr1, cFos, and BDNF in the prefrontal cortex [270]. These high baseline levels interfered with the expected increase in expression following exposure to novelty compared to WT mice, suggesting that functional APP is important for proper IEG regulation. *In vitro*, E4 has been shown to stimulate amyloid beta production by increasing APP transcription more than E3 or E2 [234], indicating a possible modulating role of apoE in IEG signaling.

In a PTSD model, elevated levels of cFos immunoreactivity were seen in the bed nucleus of the stria terminalis (BNST), paraventricular hypothalamus (PVH), basolateral amygdala (BLA), and central amygdala (CEA) [271]. In another PTSD model, elevated cFos immunoreactivity levels in BNST, PVH, CEA, medial amygdala, paraventricular hypothalamus, paraventricular thalamic nuclei, and periaqueductal gray (PAG) were identified [272]. Notably, these PTSD-like brain maps highlight regions that were different between the apoE isoforms here, such as the BLA and PAG. E2 and E3 had higher cFos immunoreactivity than E4 in these regions, respectively, which are important in fear integration and expression. It is possible, then, that the higher cFos levels in E2 and/or lower levels in E4 could be contributing to different fear expression—namely, more freezing in E4 mice during training and previously reported impaired extinction in E2 mice [140].

IEG expression in the context of apoE isoforms has only recently been explored. As mentioned above, evidence suggests a relationship between APP and IEG expression. Upstream, apoE influences the transcription of APP via phosphorylation of cFos [234]. Using iPSCs derived from males, it was shown that E4 stimulates phosphorylation of ERK1, stimulates phosphorylation of cFos, increases APP levels, and increases amyloid beta secretion more than E3, and E3 does so more than E2 [234]. Moreover, inhibiting cFos with an AAV-delivered RNAi decreased overall APP levels [234]. A subsequent study also identified a stepwise decrease from E4 to E3 to E2 in activation of synapse-building genes via phosphorylation of CREB [273].

Initially, these results appear paradoxical to our current findings, where the previous experiments identify E4 as inducing more cFos phosphorylation. However, the iPSC experiments used exogenous apoE as stimulation and—as stated by the authors—explored transcription and synapse growth in reduced systems. E4 carriers have lower apoE levels than non-E4 carriers throughout the body and independently of AD diagnosis [274-276]. E4 mice on a high fat diet (HFD) did not show increases in cFos and Arc hippocampus, cortex, and hypothalamus, while E3 mice on a HFD did [233]. Thus, it is conceivable that lower apoE levels in E4 carriers lead to less overall IEG expression, though this hypothesis requires further investigation. Notably, we observed higher freezing in E4 mice despite generally lower cFos expression. We did not detect any correlations between cFos immunoreactivity and behavior, though alterations in brain activation have been reported in E4 carriers in the absence of overt behavioral differences [162, 163]. In human populations, E2 is relatively understudied due to the relative rarity of this isoform. Yet, recent studies have focused more on E2: in one study, young adult E2 carriers had larger hippocampal gray matter and relied more on hippocampus-dependent spatial strategies to solve a navigation task [277]. We detected an apoE-dependent difference cFos⁺ cells in the

hippocampus of mice, which could be reflective of increased hippocampal activation during the contextual fear conditioning trial, complementing these findings in the young human population.

The data from human imaging studies are also in line with the distinctions we observed when we analyzed regional correlations. There was a difference between the correlation matrices of E3 and E4 animals, but no difference between E2 and E3 or E2 and E4 mice. When we examined the strongest correlations ($r > 0.7$ or $r < -0.7$), there was a single overlapping correlation between all 3 isoforms. This finding—that the 3 isoforms are more separate than similar in this measure—is an important contribution to understanding the differential risks associated with E2 and E4. Moreover, E4 mice did not show any strong positive correlations within the contextual encoding regions and had more strong negative correlations than E2 or E3. This was clear when looking at the correlation between the entorhinal cortex and hippocampus: E4 mice displayed a negative correlation (-0.621), while E2 and E3 mice displayed positive correlations (0.71 and 0.906, respectively). Analysis of functional connectivity to the entorhinal cortex in an older human population also identified distinctions in each apoE isoform: E2 carriers showed the strongest functional connectivity of the entorhinal cortex with the frontal and middle temporal gyrus; E3 with the postcentral gyrus, inferior parietal lobule, thalamus, and insula (among others); and E4 with the precuneus, angular gyrus, superior parietal lobule, and posterior cingulate (among others) [278]. In this population—which included both healthy controls and patients with mild cognitive impairment—the E4 alterations in functional connectivity was associated with increased speed of disease progression, while the alterations seen in E2 carriers were associated with protection against cognitive decline.

Altogether, these data suggest that IEGs are differentially regulated by apoE isoform at a young time point. Alterations in IEG signaling, specifically in E4 mice, could be a driving factor in E4-specific risk for diseases.

Chapter 5: Age, sex and apoE isoform affect baseline and learning-induced DSBs and IEG expression in the hippocampus

Abstract:

Age, sex, and apolipoprotein E (apoE) isoform are the three greatest risk factors to develop Alzheimer's disease (AD). Of the 3 human isoforms, E4 confers greater relative risk to develop AD compared to E3, while E2 is relatively protective against AD and associated with longevity. Women are more likely than men to develop AD and show faster disease progression. Age and disease synergistically increase the amount of DNA damage and decrease DNA repair mechanism; women are also shown to have increased oxidative stress and inflammatory markers in aging. Yet, the mechanisms underlying the relative risks of apoE, sex, and their interactions in aging is unclear. Recently, an adaptive role of DNA double strand breaks (DSBs) in immediate early gene expression (IEG) has been described. IEGs are known to be important for synaptic plasticity, learning and memory, as interfering with typical IEG expression impairs various types of long-term memory. Here, we used young (3 month) and middle-aged (12 month) male and female targeted replacement apoE mice to investigate the influence of age, sex, and apoE on DSBs and IEGs at baseline and following fear conditioning. We found that all of these factors influence the formation of DSBs near *Fos* and *Npas4* in the hippocampus, with middle-aged animals showing increased expression at baseline such that $E4 > E3 > E2$. Middle-aged E4 mice also froze more than age- and sex-matched E3 and E2 mice. These results add to the evidence that dysregulation of adaptive DSBs could be a driving mechanism in aging and disease development.

Introduction

Apolipoprotein E (apoE) is a protein widely expressed throughout the body involved in cholesterol metabolism. In the brain, its role as a cholesterol transporter is important for synaptic maintenance [122, 123], stress response [126-132], and transcription [133]. There are 3 major human apoE isoforms: E2, E3, and E4. Of these, E4 is known to be a relative risk factor for late-onset Alzheimer's disease (AD) compared to E3 in White and Japanese populations [119, 134]. E4 is also associated with increased risk for many forms of dementia, including frontotemporal dementia, vascular dementia, and dementia with Lewy bodies [137-139]. Conversely, E2 is relatively protective against AD, and associated with greater longevity [247-250]. However, the E2 isoform increases risk for developing post-traumatic stress disorder (PTSD) and PTSD severity [140, 142, 251]. The reasons underlying the isoform-specific differences of apoE for disease risk are still unclear.

Females are also at a higher risk to develop AD and PTSD [113, 228, 229]. Women account for the majority of AD diagnoses and show faster cognitive decline than men despite similar amyloid-beta levels [120, 147]. This sex difference in cognitive decline is possibly due to increased oxidative stress and pro-inflammatory markers in aging women [149-151]. Moreover, there is an interaction of apoE isoform and sex, where E4 females have even higher risk to develop AD than males [152]. Even at young ages, there are distinct effects of apoE isoform dependent on sex: grade-school girls with the $\epsilon 4$ allele had impaired visual and spatial memory compared to girls without the $\epsilon 4$ allele [161]. This difference between isoforms was not seen in school-aged boys. Increased BOLD signal was also seen in cognitively healthy adults with the $\epsilon 4$ allele despite no differences in cognitive performance [162].

The family of genes known as immediately early genes (IEGs) are important for synaptic plasticity, learning and memory. These genes—such as *cFos*—are used as markers for neuronal activity, as they exist at low baseline levels, are rapidly expressed upon stimulation, and return to baseline levels within a few hours [33, 34]. *cFos* is upregulated in relevant brain regions following fear conditioning [5, 44, 49, 196], such as the dorsal CA1 in mice after contextual fear conditioning [50]. Inhibition of *cFos* in the hippocampus during fear recall is sufficient to impair contextual memory [51]. The precise timing and expression of these genes is essential: even within the hippocampus, expression of *cFos* in the CA1 and CA3 contribute to different stages of encoding and extinction [226].

The mechanisms that lead to immediacy of IEG expression are unclear. Recently, evidence has emerged for a role for DNA double strand breaks (DSBs) in IEG expression. Stimulation of hippocampal neurons *in vitro* led to DSBs on the transcription start sites of IEGs [98]. Exposure of 4-6 month old C57Bl/6J (WT) mice to a novel environment led to a transient increase γ H2Ax—a DSB repair marker—in relevant brain regions [97]; WT mice trained in fear conditioning also showed increased DSBs in the hippocampus [98]. Yet, the tight regulation of DSBs and their proper repair is likely important for this mechanism. Young mice carrying familial dominant mutations for AD had higher baseline levels of γ H2Ax at baseline and failed to return to baseline levels 24 hours after stimulation [97]. Increased γ H2Ax and 53BP1 (another DSB repair marker) were also found in post-mortem tissue of patients with mild cognitive impairment (MCI) and AD [118], suggesting that dysregulation of DSBs is an early, driving factor in AD progression.

Considering the relative risk associated with the apoE isoforms, female sex, and age, it is likely that these factors all influence DSBs and IEG expression. Here, we used young (3 month)

and middle-aged (12 month) male and female apoE targeted replacement mice to assess DSBs and IEG expression at baseline and following contextual fear conditioning. We hypothesized that these 3 factors (age, sex, and apoE) would interact to influence pathological (baseline) and adaptive DSBs.

Methods

Mice

Human apoE targeted replacement mice generated by Sullivan et al. [240-242] and bred in our colony were used for these experiments. We used both males and females at two ages: young (3 months old) and middle-aged (12 months old). These experiments required a total of $n = 249$ mice. Experiment 1 (immunofluorescence) required $n = 126$. Experiment 2 (chromatin immunoprecipitation) required $n = 123$. Mice were singly housed until 4 days before behavioral testing; they were provided with extra nest material for enrichment. Food and water were provided *ad libitum*, and lights were on a standard 12 h light:dark cycle.

All animal procedures were reviewed and approved by the OHSU IACUC and in accordance with AAALAC standards. All procedures followed the ARRIVE guidelines. Researchers were blinded to genotype groups throughout the duration of all experiments.

Experimental Design: Contextual Fear Conditioning & Tissue Collection

A schematic of the experimental design can be seen in **Fig. 5.1**.

In experiment 1, mice were randomly assigned to one of three groups: behaviorally naïve, fear training, or fear training + recovery ($n = 3-5$ mice per genotype per age per sex; **Table 5.1**).

Mice in the fear training group were trained in contextual fear conditioning (described in Chapter 4), then returned to their home cages and perfused with ice-cold saline and 4% paraformaldehyde 1.5 h later. This time point was chosen as to find the peak overlap between cFos expression and 53BP1 [93, 196]. Mice in the fear training + recovery group were trained in the same contextual fear conditioning, then returned to their home cages and perfused 24 h later. Behaviorally naïve mice were never exposed to fear conditioning; these animals were perfused on the same day as the other groups.

In experiment 2, mice were randomly assigned to one of two groups: behaviorally naïve, or fear training ($n = 4-6$ mice per genotype per sex per age; **Table 5.1**). Mice in the fear training group were trained in the same contextual fear conditioning paradigm; 5 min after completing the trial, they were euthanized by rapid cervical dislocation and decapitation. This time point was chosen based on data indicating that γ H2Ax formation is highest within 5 min after stimulation [103]. Behaviorally naïve mice were never exposed to fear conditioning and euthanized in the same way. The hippocampus and cortex were dissected and flash frozen in liquid nitrogen, then stored at -80°C until use.

Table 5.1. Break down of number of animals in each group for Experiment 1: Immunofluorescence and Experiment 2: ChIP-ddPCR.

Experiment 1		Young			Middle-Aged		
	Genotype	Naïve	1.5 h	24 h	Naïve	1.5 h	24 h
Females	E2	3	5	3	3	3	3
	E3	3	3	3	3	3	3
	E4	3	4	5	4	4	3
Males	E2	3	4	3	3	3	3
	E3	3	4	3	4	4	4
	E4	3	3	3	4	4	4

Experiment 2		Young		Middle-Aged	
	Genotype	Naïve	5 min	Naïve	5 min
Females	E2	4	4	4	6
	E3	4	4	6	6
	E4	4	4	5	5
Males	E2	4	4	6	6
	E3	4	4	5	5
	E4	4	4	4	4

Dual-label immunofluorescence & microscopy

Brains were sectioned at 40 μm thickness using a cryostat to assess 53BP1, a DSB-repair protein, and c-Fos following standard immunofluorescence protocols [104, 182]. Briefly, sections were rinsed in 1x phosphate buffer saline (PBS) and blocked in 4% normal goat serum (NGS) before overnight incubation in primary antibody targeting 53BP1 (rabbit, 1:3000, Bethyl Labs, # A300-272A). On day 2, sections were rinsed and incubated in secondary antibody (goat-anti-rabbit, AlexaFluor647, Invitrogen, # A-21245, 1:250) for 2.5 h. Sections were then thoroughly rinsed in 1x PBS, blocked again in 4% NGS, and incubated in mouse-anti-c-Fos overnight (mouse, 1:500, PhosphoSolutions, # 309-cFOS). On day 3, sections were rinsed and incubated in secondary antibody (goat-anti-mouse, AlexaFluor488, Invitrogen, #A-11001, 1:250) for 2.5 h, then washed in 1x PBS. Following washes, 2.5 $\mu\text{g/mL}$ DAPI (Sigma D9542) in PBS was applied for 20 min. Sections were rinsed one more time, then slide mounted with CitiFluor CFMR2 Antifadent Solution, and sealed with Biotium CoverGrip Coverslip Sealant.

Three sections were imaged per animal. Z-stack images at 0.25 μm steps were taken using a Zeiss LSM 980 with Airyscan 2 at 63x zoom. Images were taken of the CA3 and CA1. The number of cells with 53BP1 foci, cFos, and co-localized signal were manually counted using ImageJ (NIH, Bethesda, MD).

Chromatin Immunoprecipitation & digital droplet PCR

Chromatin immunoprecipitation (ChIP) was done with a ThermoFisher MAGnify Kit (ThermoFisher, #492024) as previously described [279]. Hippocampi were prepared according to instructions: tissue was chopped finely in ice-cold PBS, homogenized using 18g followed by 21g needles, fixed with 1% formaldehyde for 10 min at room temperature, quenched with glycine for

5 min at room temperature, washed in cold PBS 3 x 10 min, incubated in MAGnify lysis buffer with proteinase inhibitor cocktail for 5 min, and sonicated for 5 min using a Covaris s220 sonicator. Samples were then spun down to remove cell debris. 1% of the sample was taken for chromatin input, while the rest was incubated with 2 μ g of γ H2Ax antibody (Cell Signal, #9718) overnight at 4°C. DNA reverse-crosslinking and purification were done according to the MAGnify protocol. Concentrations for ChIP and input samples were measured using the Qubit dsDNA High Sensitivity kit (ThermoFisher). Samples were stored at -20°C until use in digital droplet PCR (ddPCR).

ddPCR was used to assess apoE- and age-dependent effects on cFos and Npas4 expression [280]. Samples were prepared according to the standard BioRad QX200™ Droplet Digital™ PCR System manual (BioRad, Hercules, CA). Briefly, 10 μ L of ChIP sample were prepared per 22 μ L reaction with EvaGreen QX200 ddPCR SuperMix and BioRad primers designed for Fos (#10031258) or Npas4 (#10031258). Samples were converted into droplets with the QX200™ system, then PCR done using a T1000 thermal cycler (BioRad) with the following protocol: 95°C for 5 min, then 34 cycles of 96°C for 30 s then 58°C for 1 min, stabilization at 4°C for 5 min, then 90°C for 5 min. Fluorescence was measured on a QX200™ droplet reader on the EvaGreen channel and analyzed using QuantaSoft™ software. The number of positive droplets normalized to total droplets generated and copies/ μ L were analyzed.

Statistics

All data were first analyzed for normalcy and homogeneity. Statistics were run using SPSS vs. 28 (Armonk, NY) and Prism v. 7 (Graphpad, XXX). For experiment 1, only data from young animals has been analyzed for cFos+53BP1 dual labeling due to time restrictions.

Percent time spent freezing and average motion (in arbitrary units (au)) were obtained from Med Associates Video Freeze software. Average baseline motion and average motion during the shocks were analyzed with an ANOVA including sex, genotype, and age as between-group variables. Percent time freezing during the inter-stimulus intervals (ISIs) was analyzed with a repeated measures ANOVA with sex, genotype, and age as between-group variables. Due to the repeated significant main effects of sex and interactions with sex, we subsequently split males and females for all analyses and repeated the above statistics with genotype and age as between-group variables.

The number of cFos⁺ cells, 53BP1⁺ cells, and cFos-53BP1⁺ cells in the CA1 and CA3 were analyzed with a multi-way ANOVA including time post-training, sex, and genotype as between group variables. Again, repeated significant differences based on sex led us to split sexes for analysis.

The concentration of cFos and Npas4 (measured in copies/ μ L) was analyzed with an ANOVA with sex, genotype, age, and time post-training as between group variables. Sexes were again split for analysis for clarification.

For all analyses, Tukey's or Bonferroni's post-hoc corrections were applied to compare apoE isoforms and time post-training.

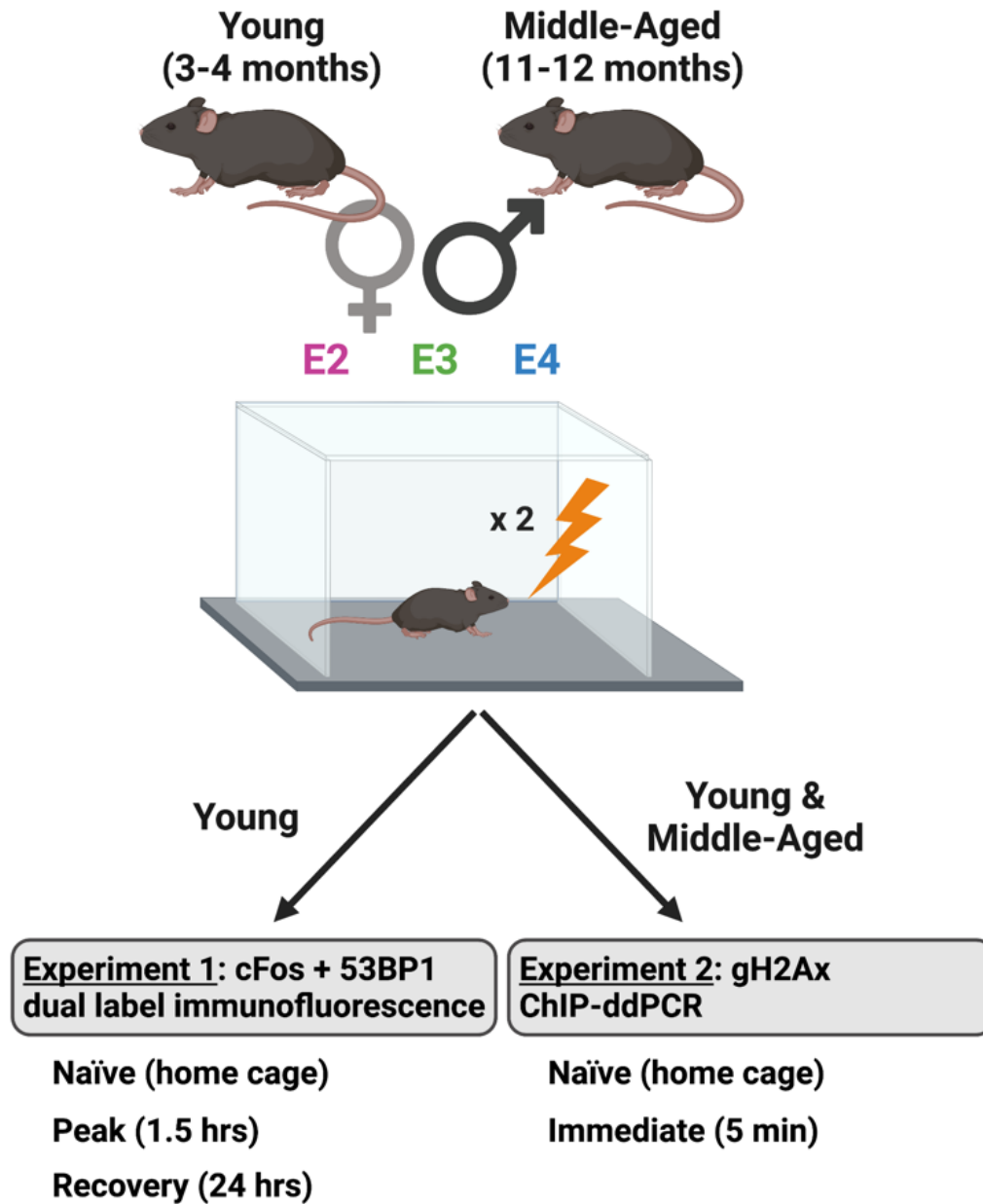


Figure 5.1. Experimental design. Young (3 month) and middle aged (12 month) male and female E2, E3, and E4 targeted replacement mice were trained in contextual fear conditioning. In experiment 1, young mice were perfused 1.5 h or 24 h after completing training. Behaviorally naïve age- and sex-matched controls were also perfused. In experiment 2, fresh hippocampus was dissected from young and middle-aged mice 5 min after completing fear training. Hippocampi were also dissected from behaviorally naïve age- and sex-matched controls. Schematic made with biorender.com

Results

ApoE isoform, sex, and age affect contextual fear training

A schematic of the experimental design can be seen in **Fig. 5.1**. All data from mice trained in fear conditioning were analyzed together. Average motion during the initial 2.5-minute baseline period was analyzed in females and males separately. We found a significant main effect of age in females ($p < 0.001$), where middle-aged females moved more than young females (**Fig. 5.2A**). In males, we detected a main effect of genotype ($p = 0.031$; **Fig. 5.2B**). Post-hoc testing identified that E2 males moved less than E3 males ($p = 0.042$). The average motion in response to the shocks was also analyzed separately in females and males. There was a main effect of age in both females ($p < 0.001$; **Fig. 5.2C**) and males ($p = 0.006$; **Fig. 5.2D**), as well as a trend towards an effect of genotype in females ($p = 0.070$) and males ($p = 0.068$).

We next analyzed percent time freezing during the ISIs. Analysis in females revealed a main effect of genotype ($p = 0.024$; **Fig. 5.2E**) and a trend towards an age by genotype interaction ($p = 0.055$); Bonferroni's post hoc indicated this was driven by a difference in E2 v. E4 overall ($p = 0.041$), and E2 vs E4 ($p = 0.0052$) and E3 vs E4 ($p = 0.0392$) in middle-aged females. Analysis in males also revealed a significant effect of genotype ($p = 0.005$; **Fig. 5.2F**), age ($p < 0.001$) and trend towards a genotype by age interaction ($p = 0.060$). Overall, these effects were driven by higher freezing in middle-aged E4 males compared to E2 ($p = 0.008$) and E3 ($p = 0.014$) middle-aged males. Additionally, we found a time by genotype interaction ($p = 0.043$), driven by a lack of increase in freezing between ISI1 and ISI2 in E2 males.

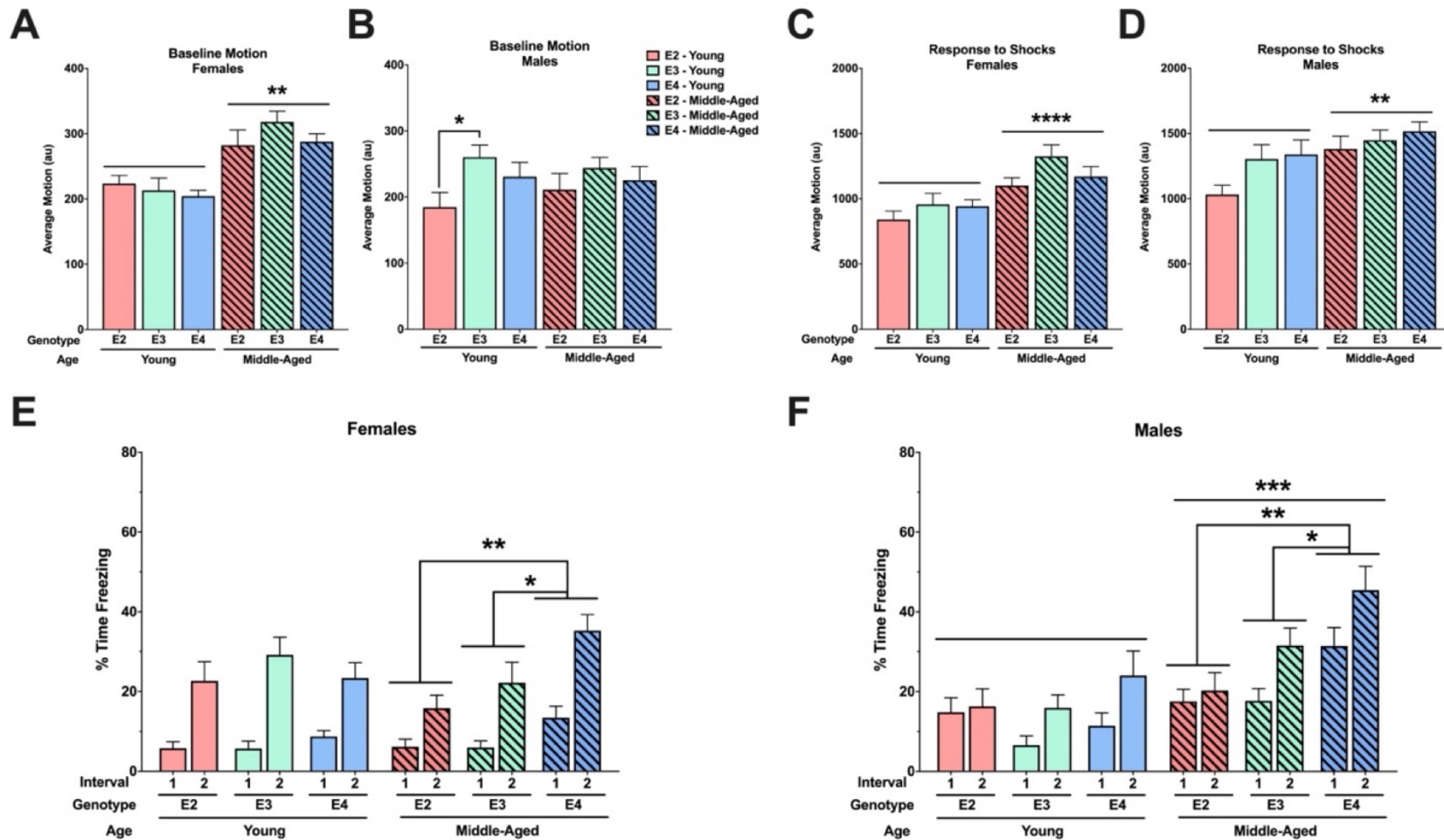


Figure 5.2. Contextual fear conditioning data. Average baseline motion was analyzed in (A) females and (B) males separately. There was a main effect of age in females ($p < 0.001$, $F(1,69) = 42.131$) and a main effect of genotype in males ($p = 0.031$, $F(2,60) = 3.480$). E2 males moved less than E3 males ($p = 0.042$). Response to shock in (C) females and (D) males. There was a main effect of age in both females ($p < 0.001$, $F(1,69) = 25.064$) and males ($p = 0.006$, $F(1,60) = 8.246$), as well as a trend towards an effect of genotype in females ($p = 0.070$) and males ($p = 0.068$). Percent time freezing during the ISIs in (E) females and (F) males. There was a main effect of genotype ($p = 0.024$, $F(2,69) = 3.927$) and a trend towards an age by genotype interaction ($p = 0.055$) in females; Bonferroni's post hoc indicated this was driven by a difference in E2 v. E4 overall ($p = 0.041$), and E2 vs E4 ($p = 0.0052$) and E3 vs E4 ($p = 0.0392$) in middle-aged females. A time by genotype interaction ($p = 0.043$, $F(1,60) = 3.318$), a significant effect of genotype ($p = 0.005$, $F(2,60) = 5.900$), age ($p < 0.001$, $F(1,60) = 19.087$) and trend towards a genotype by age interaction ($p = 0.060$) were seen in males. Bonferroni's post hoc showed higher freezing in E4 males compared to E2 ($p = 0.008$) and E3 ($p = 0.014$) in middle-aged males. Data are averages $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Sex and apoE isoform differentially affect hippocampal cFos and cFos-53BP1 colocalization

Following contextual fear conditioning, mice in Experiment 1 were intracardially perfused 1.5 hours or 24 hours later to assess cFos and 53BP1 in the hippocampus. Behaviorally naïve mice were also perfused for comparison to “baseline.”

Analysis of cFos, 53BP1, and colocalization in the CA1 region of the hippocampus revealed many significant differences based on sex; thus, we proceeded to analyze the sexes separately. Representative images can be seen in **Fig. 5.3A**. An ANOVA for the number of cFos⁺ cells in females revealed a main effect of time post-training ($p < 0.0001$; **Fig. 5.3B**), but no differences between genotypes. Sidak’s post-hoc testing indicated that all mice perfused 1.5 h post-training had higher levels of cFos than naïve mice and mice perfused 24 h post-training. In males, there was also a significant effect of time post-training ($p = 0.0165$), as well as a trend towards an effect of genotype ($p = 0.055$; **Fig. 5.3C**). However, differences between time points post-training failed to reach significance following post-hoc corrections.

We also assessed 53BP1 in the CA1. There was a significant main effect of time post-training ($p = 0.050$) in females (**Fig. 5.3D**), though no effect of genotype. Post-hoc testing only reached significance when comparing naïve to 1.5 h post-training in E3 females. In males, we did not detect any significant effect of genotype ($p = 0.400$) or time post-training ($p = 0.2101$; **Fig. 5.3E**).

We next analyzed colocalization of cFos and 53BP1 in the CA1. Again, we detected a main effect of time post-training in females ($p < 0.0001$) and a trend towards an interaction between time and genotype ($p = 0.073$; **Fig. 5.3F**). Sidak’s post-hoc testing revealed that all animals perfused 1.5 h after training had higher colocalization than animals at 24 h; yet, E2 females perfused 1.5 h after training did not have significantly higher colocalization levels than

E2 females at baseline ($p = 0.0670$). Additionally, E3 females 1.5 h post-training had more colocalization than E2 females 1.5 h post-training ($p = 0.0451$). Colocalization analysis in males similarly revealed a significant effect of time post-training ($p = 0.0052$) and trend towards a genotype-by-time interaction ($p = 0.0894$; **Fig. 5.3G**). Post-hoc testing again identified a difference between E2 and E3 peak colocalization ($p = 0.0122$). However, only E3 males at 1.5 h post-training had more colocalization than naïve or 24 h E3 males; this peak failed to occur in E2 or E4 male mice.

As in the CA1, we analyzed cFos, 53BP1, and colocalization in the CA3 (**Fig. 5.4A**). We found a main effect of time post-training in cFos levels in females ($p < 0.0001$; **Fig. 5.4B**). E2 females failed to show a significantly higher peak of cFos at 1.5 h post-training, while E3 mice and E4 mice both significantly increased compared to baseline; E4 females were the only ones to have significantly lower cFos levels at 24 h compared to 1.5 h. cFos analysis in the CA3 of males failed to show any significance: there was a trend towards an effect of time post-training ($p = 0.0567$; **Fig. 5.4C**), though cFos did not peak at 1.5 h in any genotype. 53BP1 was not different based on genotype or time-post training in either females (**Fig. 5.4D**) or males (**Fig. 5.4E**).

Colocalization of cFos and 53BP1 in the CA3 of females was significantly different between time points post-training ($p = 0.0005$; **Fig. 5.4F**). Sidak's post-hoc testing identified E2 females had higher colocalization levels at 1.5 h compared to 24 h, while E4 mice had higher levels at 1.5 h compared to behaviorally naïve E4 animals; E3 females at 1.5 h were not different from baseline or 24 h following post-hoc corrections. Similarly, colocalization was different in the CA3 of males based on time post-training ($p = 0.0027$) as well as genotype ($p = 0.0277$; **Fig. 5.4G**). Post-hoc testing identified that the genotype difference was driven by a difference

between E2 and E3 mice at 1.5 h ($p = 0.0058$). Of the genotypes, E3 males were the only to show a significant difference between naïve and 1.5 h, and 1.5 h and 24 h post-training.

Altogether, these data indicate that cFos levels and colocalization of cFos and 53BP1 in the hippocampus are dependent on sex and apoE-isoform in a region-specific manner.

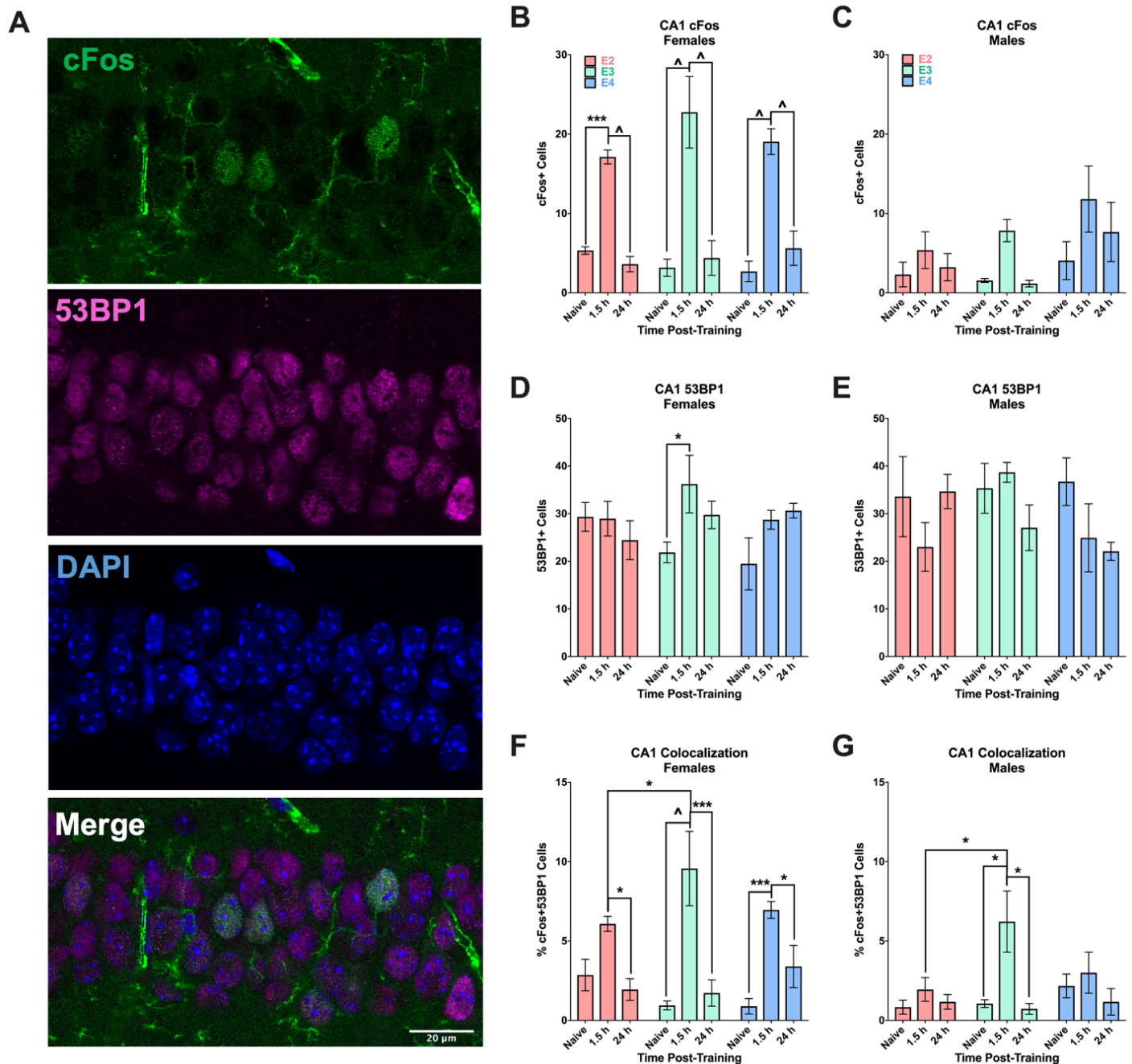


Figure 5.3. cFos, 53BP1, and colocalization analysis in the CA1. **A)** Representative images of the CA1. There was a significant effect of time post-training in **(B)** females ($p < 0.0001$, $F(2,22) = 67.89$) and **(C)** males ($p = 0.0165$; $F(2,19) = 5.136$). 53BP1 in the CA1 was different based on time post-training in **(D)** females ($p = 0.050$, $F(2,22) = 3.44$) but not in **(E)** males ($p = 0.2006$). Colocalization of cFos+53BP1 was significant based on time-post training in **(F)** females ($p < 0.0001$, $F(2,22) = 32.15$) and **(G)** males ($p = 0.0052$, $F(2,17) = 7.298$). Data are averages $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; [^] $p < 0.0001$.

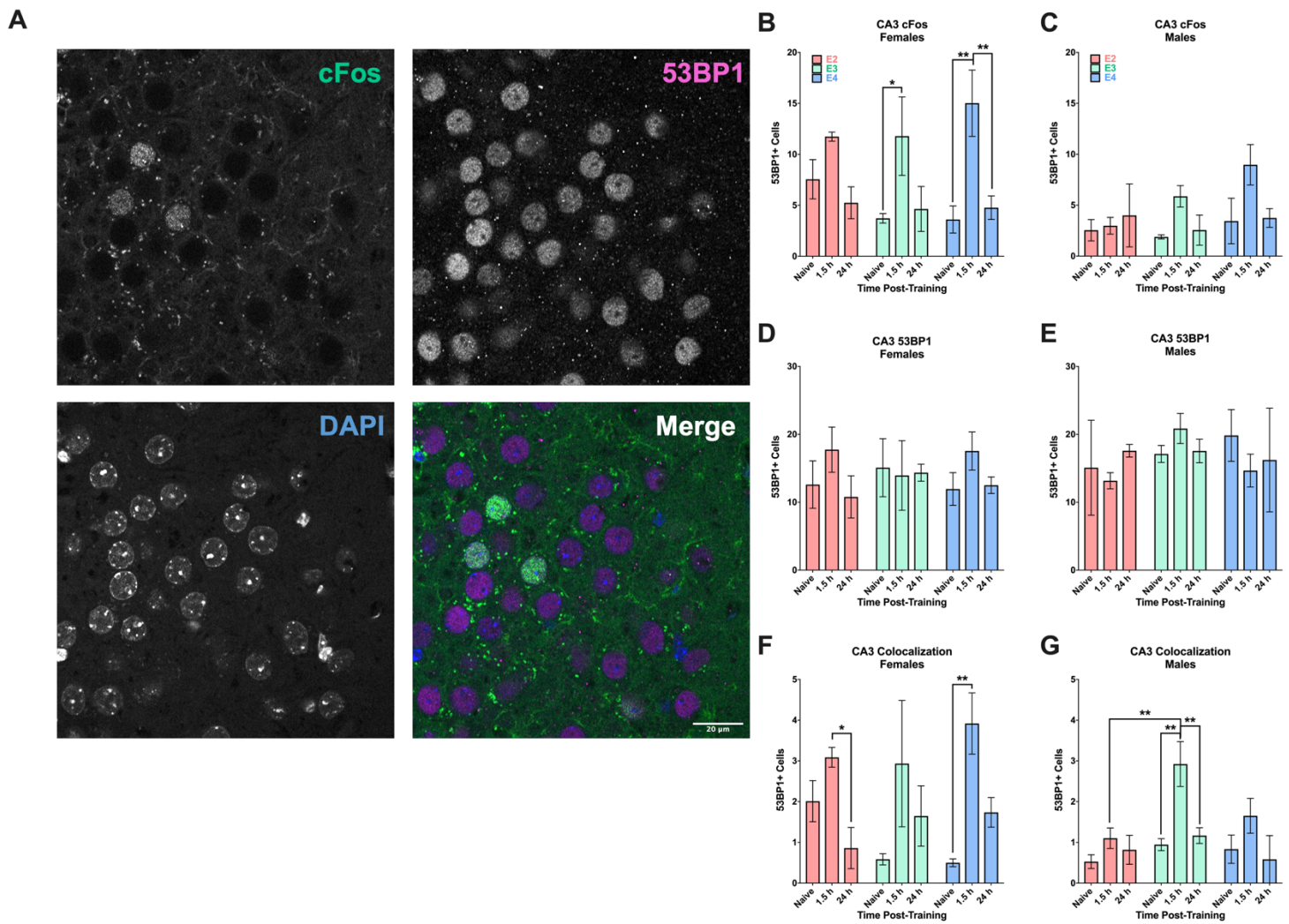


Figure 5.4. cFos, 53BP1, and colocalization analysis in the CA3. **A)** Representative images of the CA3. There was a significant effect of time post-training in **(B)** females ($p < 0.0001$, $F(2,21) = 15.11$) and a trend in **(C)** males ($p = 0.0567$; $F(2,18) = 3.379$). 53BP1 in the CA1 was not different based on time post-training in **(D)** females ($p = 0.3137$) or **(E)** males ($p = 0.9305$). Colocalization of cFos+53BP1 was significant based on time-post training in **(F)** females ($p = 0.0005$, $F(2,21) = 11.04$) and **(G)** males ($p = 0.0027$, $F(2,19) = 8.182$). There was also a difference based on genotype in males ($p = 0.0277$, $F(2,19) = 4.255$). Data are averages $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ^ $p < 0.0001$.

Age, sex, and apoE isoform affect Fos and Npas4 expression at baseline & after fear conditioning

Analysis of the amount of *Fos* and *Npas4* near the site of γ H2Ax binding similarly revealed sex-, age- and apoE-dependent effects. In females, an ANOVA for concentration of Fos (copies/ μ L) indicated a main effect of genotype ($p = 0.022$) and behavior group ($p = 0.007$) and significant genotype-by-behavior group ($p = 0.005$) and behavior group-by-age ($p = 0.004$) interactions (**Fig. 5.5A**). Tukey's post-hoc tests indicated that middle-aged, naïve E3 and E4 mice had higher Fos concentration than middle-aged, naïve E2 mice ($p = 0.022$, $p = 0.0009$, respectively). Middle-aged, naïve E4 mice also had significantly higher levels than young, naïve E4 mice ($p = 0.0095$). We also found a significant decrease in Fos concentration in middle-aged E4 females in the fear conditioning group compared to middle-aged, naïve E4 mice ($p = 0.0001$) and a trend towards a decrease in middle-aged, fear conditioned E3 females compared to middle-aged, naïve E3 females ($p = 0.054$).

In males, we did not detect any significant main effects when we assessed Fos concentration. However, we did find significant genotype-by-age ($p = 0.026$), behavior group-by-age ($p = 0.014$), and genotype-by-behavior group-by-age ($p = 0.002$) interactions (**Fig. 5.5B**). Post-hoc testing identified that middle-aged, naïve E2 and E4 males had higher Fos concentrations than middle-aged, naïve E3 males ($p = 0.0098$, $p = 0.0049$, respectively). Additionally, we found that young, fear conditioned E2 males had a higher Fos concentration than age- and group-matched E3 ($p = 0.010$) and E4 ($p = 0.0173$) males. Lastly, young, fear conditioned E2 males had higher Fos levels than young, naïve E2 males ($p = 0.047$) in contrast to middle-aged, fear conditioned E2 males having higher Fos levels than age matched, naïve E2 males ($p = 0.0294$).

Analysis of *Npas4* concentration revealed similar results in both females and males. In females, there was a significant effect of age ($p = 0.048$) and a trend towards an effect of behavioral group ($p = 0.065$). We also found significant genotype-by-behavior group ($p = 0.002$) and behavior group-by-age ($p = 0.037$) interactions (**Fig. 5.5C**). Like with the *Fos* levels, *Npas4* concentration was higher in middle-aged, naïve E3 ($p = 0.034$) and E4 ($p = 0.0081$) females compared to age- and group-matched E2 females. Middle-aged, fear conditioned E2 females also had higher *Npas4* levels than middle-aged, fear conditioned E4 females ($p = 0.010$). Lastly, middle-aged, fear conditioned E4 females had lower *Npas4* concentration than middle-aged, naïve E4 females ($p = 0.0002$).

For *Npas4* analysis in males, we found a main effect of genotype ($p = 0.011$) and age ($p = 0.008$), as well as genotype-by-age ($p = 0.009$) and genotype-by-behavior group-by-age ($p = 0.024$) interactions (**Fig. 5.5D**). There was a trend towards a behavior group-by-age interaction, as well ($p = 0.056$). Post-hoc testing identified that middle-aged, naïve E2 and E4 males had higher *Npas4* concentrations than middle-aged, naïve E3 males ($p = 0.0130$, $p = 0.0043$, respectively). Young, fear conditioned E2 males also had higher *Npas4* concentrations than group- and age-matched E3 ($p = 0.0019$) and E4 ($p = 0.0026$) males. Young E2 mice in the fear conditioning group also had higher *Npas4* than young, naïve E2 males ($p = 0.0446$).

To summarize, these data indicate that DSBs occur more near *Npas4* in aging; that there is a step-wise increase from E2 to E3 to E4 in DSBs near *Fos* and *Npas4* in aging females; and that fear conditioning induces DSBs near *Fos* and *Npas4* in young E2 males. Together, these results suggest that age, sex, and apoE all affect pathological and adaptive DSBs.

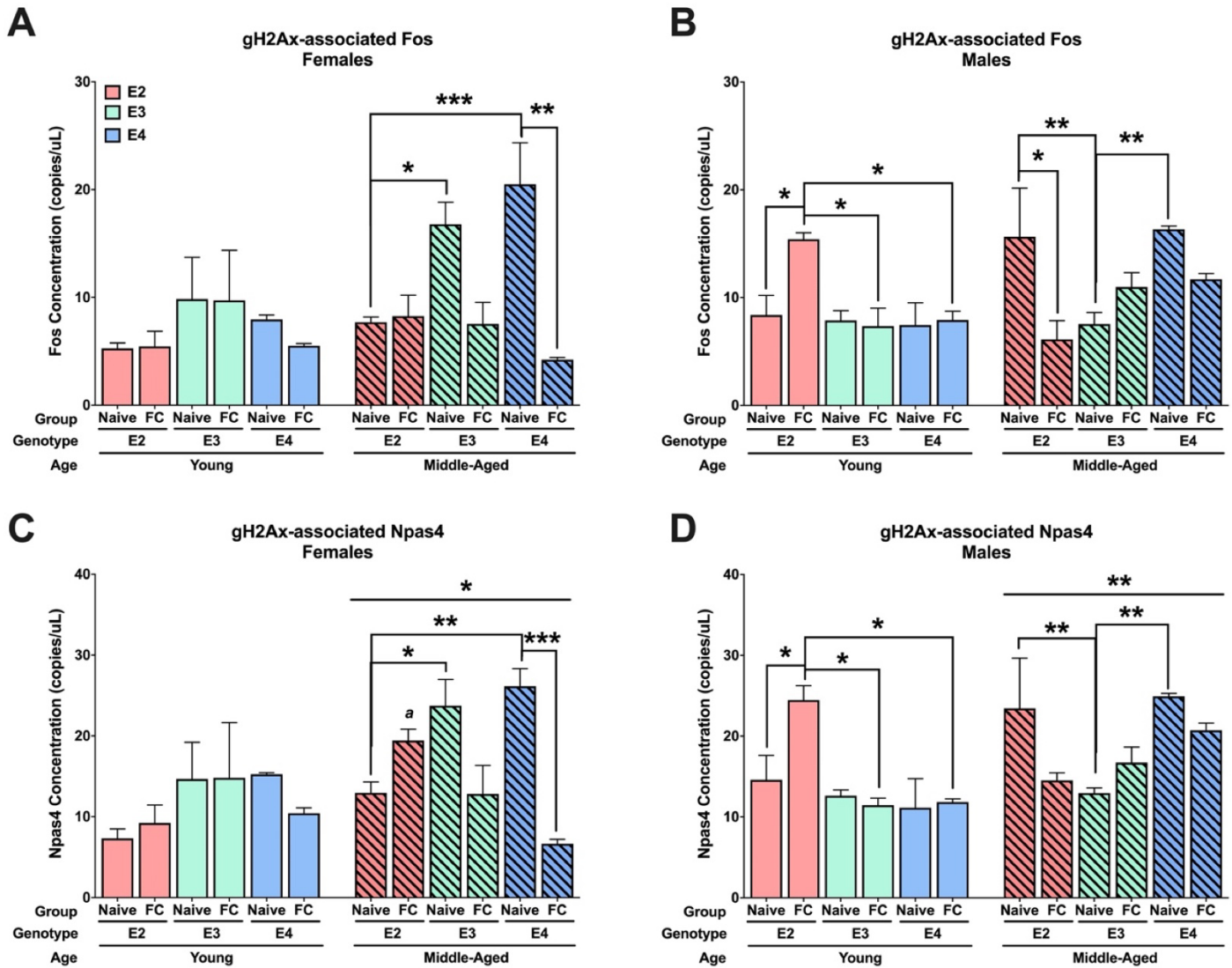


Figure 5.5. ddPCR results from hippocampi following ChIP with γ H2Ax. Analysis of cFos expression in (A) females and (B) males. In females, there were main effects of genotype ($p = 0.022$, $F(2,37) = 4.217$) and group ($p = 0.007$, $F(1,37) = 8.125$) and genotype-by-group ($p = 0.005$, $F(2,37) = 6.115$) and group-by-age ($p = 0.004$, $F(1,37) = 9.507$). In males, there were genotype-by-age ($p = 0.026$, $F(2,31) = 4.131$), group-by-age ($p = 0.014$, $F(1,31) = 6.843$), and genotype-by-group-by-age ($p = 0.002$, $F(2,31) = 7.411$) interactions. Analysis of Npas4 expression in (C) females and (D) males. In females, there was a main effects of age ($p = 0.048$, $F(2,33) = 4.236$) and significant genotype-by-group ($p = 0.002$, $F(2,33) = 7.636$) and group-by-age ($p = 0.037$, $F(1,33) = 4.750$) interactions. In males, there were main effects of genotype ($p = 0.011$, $F(2,34) = 5.151$) and age ($p = 0.008$, $F(1,34) = 7.898$), and genotype-by-age ($p = 0.009$, $F(2,34) = 5.443$) and genotype-by-group-by-age ($p = 0.024$, $F(2,34) = 4.178$) interactions. Data are averages $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ^a $p < 0.05$, comparison middle-aged FC female E2 to middle-aged, FC female E4.

Discussion

We show that fear learning is different in male and female apoE mice dependent on age; namely, middle-aged male mice freeze more than young males, and middle-aged E4 mice freeze more than age- and sex-matched E2 and E3 mice. We also discovered sex-dependent effects on hippocampal cFos expression and colocalization of cFos and 53BP1. Young females showed higher cFos levels and more robust, transient peaks in cFos signal than males in the CA1 and CA3. Genotype differences in colocalization of these signals was more pronounced in males, with E2 and E4 males failing to show significant increases in the colocalization in the CA1 or CA3. Lastly, baseline levels of DSBs near *Fos* and *Npas4* increased with age in females in a step-wise fashion such that $E4 > E3 > E2$; in males, middle-aged E3 mice had lower levels of baseline DSB-associated *Fos* and *Npas4* than E2 or E4. Young E2 males trained in fear conditioning also had higher *Fos* near DSBs than young, fear conditioned E3 or E4 males. Altogether, these data highlight that apoE, and sex interact to have distinct effects on DSBs and IEG expression at different points in life.

Our results add to other evidence that DSBs are involved in both physiological and pathological aging. Transient increases in γ H2Ax were observed in both WT mice and mice carrying mutations in the amyloid precursor protein (APP) gene following exposure to a novel environment [97]. We did not observe clear increases in DSBs with our immunofluorescence, likely due to differences in γ H2Ax and 53BP1 function and timing, nor increases in DSB-associated IEGs after fear conditioning as reported by others [103, 105]. This could be due to slight differences in methodology and/or mechanisms of distinct IEGs, as both Stott et al. [105] and Navabpour et al. [103] observed fear conditioning-induced increases in *Npas4* but not *Fos*.

we did detect changes in hippocampal *Fos* and *Npas4* dependent on apoE genotype at baseline levels.

However, we did detect an increase in γ H2Ax-associated IEG expression at baseline in middle-aged E4 female and male mice. This mirrors the previously reported data showing baseline levels of γ H2Ax were higher in the APP mutant mice, and γ H2Ax failed to return to baseline levels after 24 h [97]. Notably, the previous study used mice 4-7 months of age and collapsed sexes for analysis, while we assessed males and females mice at young (3 month) and middle-aged (12 months) periods. Another study found evidence that both young (5 month) and aged (16 month) APP/PS1 mice had increased γ H2Ax signal in the hippocampus, but detected no change in hippocampal 53BP, which is also in line with our results [117].

In humans, recent research has identified an increase in neuronal γ H2Ax and 53BP1 in brains from patients that had MCI or AD compared to healthy, age-matched controls [118]. Increased oxidative stress, which is known to induce DSBs, is seen in typical and diseased aging [111, 112, 114-116]. 8-Hydroxyguanine (8-OHdG) was found to be higher in post-mortem hippocampal tissue from clinically diagnosed AD patients compared to cognitively and pathologically normal, age-matched controls [115]. Additionally, 53BP1 and PTEN (another DNA repair protein) were decreased in this clinical AD population, suggesting impaired DSB repair [115]. Another study found that age was associated increased DNA damage in the frontal cortex, leading to down-regulation of genes important for synaptic plasticity [111]. Decreased DNA repair—specifically decreases in accurate 53BP1 recruitment—were also observed in aged human cell cultures [112]. [111, 112, 114-116]. While we did not detect differences in 53BP1 levels in our young animals, our results showing increased DSB-associated *Fos* and *Npas4* in middle-aged animals is indicative of increased aberrant signaling (or “pathological” DSBs) with

aging. Additionally, our observation that this was more pronounced in middle-aged E4 females and males parallels others' findings that age and AD synergistically increase DNA damage.

Previous research exploring the role of apoE in cFos expression identified that apoE influences transcription of APP by mediating the phosphorylation of cFos [234]. This study indicated that E4 stimulated amyloid beta production in cell culture by increasing cFos more than E3 and E2. The link from E4 to increased cFos expression to increased amyloid beta begins to clarify a possible mechanism underlying the E4-specific risk for dementias that is supported by our results. Importantly, the effects of sex on the effects of age and apoE observed are important when considering the translational relevance of these data.

In summary, our results are the first to show that apoE isoform affects DSBs and IEG expression. These data indicate that aging E4 males and females may have more aberrant IEG expression, which could contribute to their increased risk for developing age-related cognitive decline, mild cognitive impairments, and dementias.

Chapter 6: Discussion & Future Directions

General Summary of Results

I set out to address 4 main questions: 1) does manipulating DSBs with common cancer treatments alter learning, memory, and/or IEG expression; 2) do males and females respond differently to these drugs; 3) are there changes in DSBs and/or IEG expression in the different apoE genotypes; and 4) do age and sex play a role? I addressed the first two questions in chapters 2 and 3 using WT mice, and the latter 2 in chapters 4 and 5 using human targeted replacement apoE mice.

Results from experiments in Chapters 2 and 3 suggest that the systemic administration of common chemotherapy agents amifostine and etoposide alter long-term memory in a sex-dependent manner (**Fig. 6.1**). Male mice injected with 107 mg/kg of amifostine before and after fear conditioning showed increased long-term contextual memory; this was absent in females. This dose of amifostine also decreased hippocampal γ H2Ax, though no dose-dependent differences were observed in IEG expression. Conversely, female mice injected with 35 mg/kg of etoposide after fear conditioning showed impaired long-term contextual memory, and both males and females injected with this dose after training showed impaired long-term cued memory. Post-training injections of etoposide also increased hippocampal Δ FosB in both sexes, decreased hippocampal γ H2Ax, and decreased hippocampal NADPH in females.

Following up on these systemic injection experiments, we microinjected 60 ng of etoposide directly into the hippocampus of male and female WT mice before fear conditioning. Both sexes showed impaired long-term contextual fear memory and no change in cued fear memory, indicating specificity of our infusions to the hippocampus. γ H2Ax was increased in the CA1 of animals infused with etoposide, suggesting an increase in DSBs. Intriguingly γ H2Ax was

decreased in the dentate gyrus. Analysis of the IEGs cFos and Arc revealed that animals infused with etoposide had decreased IEG expression in the CA1 and dentate gyrus, though notably only cFos was decreased in the CA1 while both cFos and Arc were decreased in the DG. Together, these experiments utilizing drugs known to alter DSBs suggest that even slight interference with typical DSB regulation is sufficient to alter behavior and IEG expression.

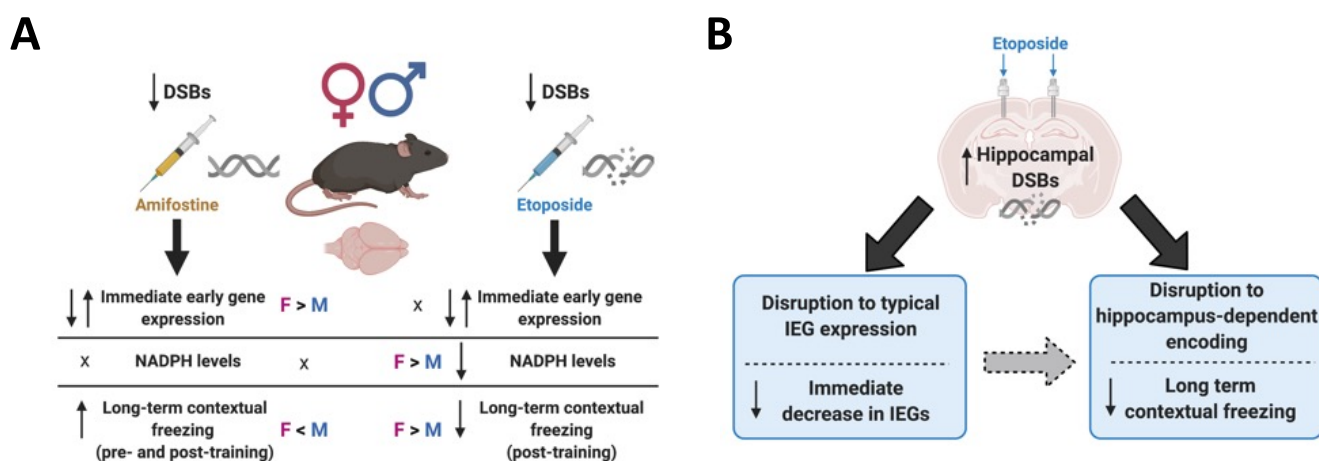


Figure 6.1. Schematic of the results from experiments in Chapter 2 and 3. A) Systemic injections of amifostine decreased hippocampal DSBs, altered immediate early gene expression, and increased long-term contextual freezing in males. Systemic injections of etoposide decreased hippocampal DSBs, altered immediate early gene expression, decreased NADPH levels in females, and decreased long-term contextual freezing in females. B) Central injections of etoposide increased DSBs and decreased cFos and Arc in the CA1 and impaired long-term contextual memory in both males and females.

The evidence that slight dysregulation can interrupt typical learning and memory opens a novel path to explore in disease, leading into the experiments conducted as part of Aim 2. Using whole brain cFos imaging, we found that young (3 month) E4 female mice generally show lower cFos expression in the brain and in relevant regions following fear conditioning compared to age- and sex-matched E2 and E3 mice. E4 mice also showed altered co-activation of brain regions in the fear circuitry compared to E3 mice. Notably, E4 mice failed to show strong positive correlations between regions important for contextual encoding. This global decrease in

cFos was independent of behavior, as E4 mice also froze more during contextual fear training than E2 or E3 mice.

We also incorporated age and sex into our experiments exploring the role of apoE in DSBs and IEG expression. Middle-aged (12 month) E4 male and female mice spent more time freezing during contextual fear training than sex- and age-matched E2 or E3 males. Middle-aged males also froze more than young males, and E2 males at both ages failed to show an increase in freezing between ISI 1 and ISI 2, suggesting impaired fear acquisition. These behavioral data suggest that age increases the distinctions between apoE genotypes, as we expected it would. Additionally, analysis of cFos, 53BP1, and their co-occurrence by immunofluorescence revealed sex- and apoE-dependent patterns in young animals. Females had higher cFos levels and colocalization of cFos and 53BP1 in the CA1 and CA3 than males, but no differences in 53BP1 alone. Colocalization of cFos and 53BP1 were different between genotypes in males, with E3 males displaying a transient increase in signal that was absent in E2 and E4 males. These sex-specific effects in young mice indicate that sex and apoE likely interact throughout life to modulate brain activation.

We also found that age, sex, and apoE isoform interact to affect DSBs near *Fos* and *Npas4*. In behaviorally naïve females, middle-aged E4 mice had more DSBs near *Fos* and *Npas4* than young E4 mice, and more than middle-aged E2 mice. Similarly, middle-aged, behaviorally naïve E4 males had higher baseline DSBs near *Fos* and *Npas4* than E3, though middle-aged E2 mice also showed elevated *Fos* and *Npas4* compared to E3. In mice that underwent fear conditioning, young E2 males had higher DSB-associated IEGs than age matched E3 and E4 male mice. These data indicate that there are more baseline DSBs in middle-aged E4 female and

E2 and E4 male mice, suggesting a possible accelerated aging timeline and partially explaining the relative risk and protection for ACD and AD.

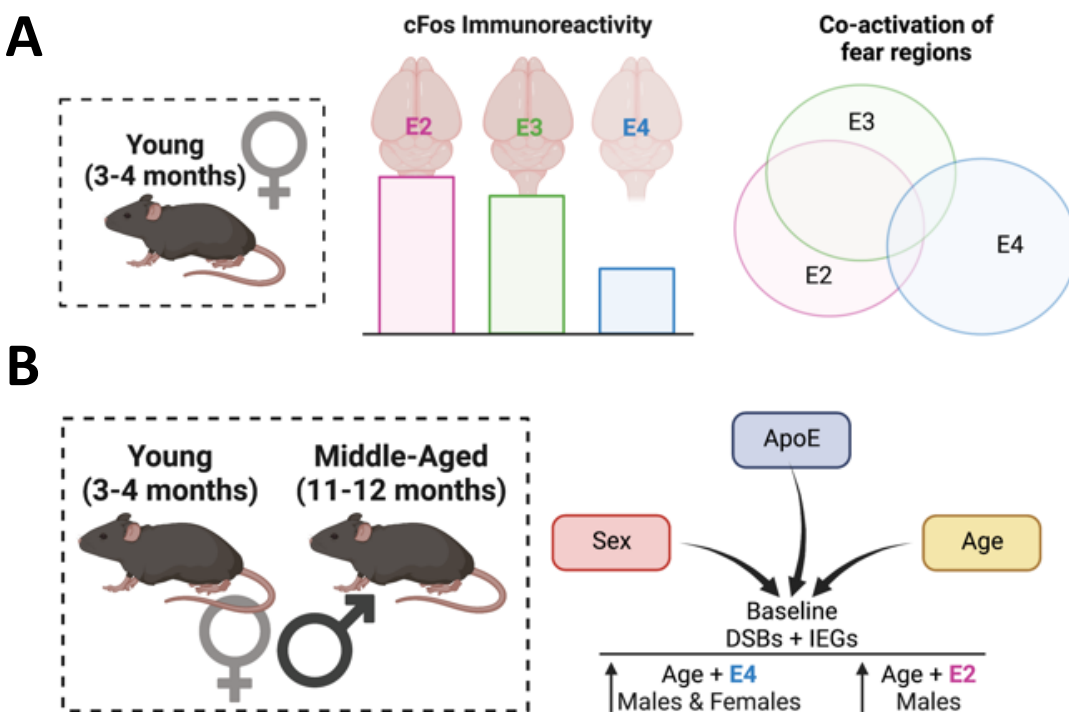


Figure 6.2. Schematic of results from experiments in Chapters 4 and 5. **A)** Whole brain cFos data indicated that young, E4 females generally have lower cFos immunoreactivity and have less overlap in connectivity with young female E3 mice. **B)** ApoE, sex, and age all affected baseline levels of DSB-related IEGs.

In summary, the data from my dissertation studies supports the hypothesis that DSBs are involved in synaptic plasticity, learning and memory, and that the precise timing and regulation of their repair is essential. These results add to growing evidence that adaptive DSBs are a mechanism for rapid IEG expression, opening a novel path to pursue for early diagnosis and intervention for age-related diseases. The subsequent sections incorporate data from others and address specifically how the results from my research complement others' results, as well as identify important future directions. More detailed discussions related to each experiment can be found in each chapter.

Manipulation of DSBs with amifostine and etoposide

Despite their wide use for decades, these studies are the first to specifically look at the effects of amifostine and etoposide on learning and memory in both male and female WT mice. Previous reports have been confounded by other chemotherapy agents or radiation exposure. Even with confounding variables, amifostine does appear to have long-term learning- and memory-enhancing effects. A high dose (214 mg/kg) rescued hippocampus-dependent memory and neurogenesis after exposure to gamma radiation in male mice 24 h later [176]. We also showed that a medium (107 mg/kg) and high (214 mg/kg) dose of amifostine were able to rescue cognitive impairments induced by simulated space radiation in male mice 3 months later [239]. Notably, amifostine and radiation combined impaired novel object recognition in female mice, suggesting that the sexes may respond differently to DSB manipulation, and/or have distinct DSB response mechanisms. For example, sex differences in microglia number have been observed, where females have more than males [281]. However, analysis of the transcriptome of microglia in males revealed more inflammation-related transcription, while the female microglia transcriptome was more related cellular regulation and neuroprotection [281]. These data are in line with our findings that amifostine increased long-term memory in males, but not females.

Conversely, systemic etoposide had a greater effect on long-term memory in females than in males. These results are an important contribution to the field of cancer biology, as the effects of systemic etoposide on learning and memory have not previously been described, even with radiation. Other chemotherapy agents—such as doxorubicin, which has a similar mechanism of action to etoposide [282]—impaired contextual fear memory in ovariectomized females [193].

Thus, while we found that etoposide's effects were more pronounced in females, it is unclear if this is due to interactions with circulating hormones.

Both compounds are useful in generally manipulating DSBs, but they are both non-specific. Our experiments looking at systemic administration revealed important information about the sensitivity of DSBs in learning and memory, but more specificity is needed. To reduce the effects of circulating factors, as well as by-pass the blood-brain barrier (as penetration of both drugs is measurable, but poor [208, 283]), we directly infused etoposide into the hippocampus of male and female WT mice. Previous reports indicated that infusing 0.1 $\mu\text{g}/\mu\text{L}$ etoposide directly into the prelimbic area of mice 2 h before trace fear conditioning was sufficient to impair fear memory 24 h later. Our dose of 60 $\text{ng}/\mu\text{L}$ infused into the hippocampus 2 h before fear training also impaired hippocampus-dependent fear memory and is closer to typical clinical levels of administration: 0.5 mg of etoposide delivered intraventricularly leads to peak cerebral spinal fluid levels of 9 $\mu\text{g}/\text{mL}$ in patients [210]. Notably, we did not see any differences in hippocampus-independent cued fear memory, indicating the specificity of increasing DSBs to a relevant behavior. Combined with the data from Li et al [171], our results indicate the region-specific importance of tight DSB regulation, and opens a possible novel path to explore in learning- and memory-related diseases.

The influence of apoE on DSBs and IEG expression

Differences in global brain activation based on apoE isoform

Early-life differences in brain activation and behavior based on apoE isoform have been reported repeatedly, though sometimes in contrast. We found that young female E4 mice overall had lower cFos immunoreactivity in the brain, and lower cFos immunoreactivity in regions

important for contextual fear conditioning. Additionally, we found that E4 mice had less co-activation of brain regions in the fear circuitry. This is in line with data from humans, showing that young E4/E4 children showed thinning of the entorhinal cortex and lower fractional anisotropy in the hippocampus, as well as poorer working memory [284]. In college-aged people, E4 carriers had hypometabolism of glucose in the posterior cingulate, parietal, temporal and prefrontal cortices [163]. We specifically saw that E4 mice had lower cFos immunoreactivity in the entorhinal cortex, mirroring this human data.

Information on brain activation in E2 individuals is scarce due to the rarity of the isoform in the general population [119]. One study indicated that E2 individuals relied more heavily on hippocampus-dependent spatial strategies during a navigation task and had larger hippocampal gray matter [277]. Considering the lack of data on brain activation in E2 carriers, our data on brain-wide cFos activation is widely beneficial.

Relationship between DSBs and IEGs: The interplay of sex, age, and apoE isoform

ApoE, sex, and age have been referred to as “the Triad” for risk of AD [121]. Our findings from the dual-label immunofluorescence and ChIP-ddPCR experiments are the first to indicate that apoE, sex, and age influence the relationship between adaptive DSBs and IEG expression. Previous research exploring DSBs in aging and AD have used relatively rare dominant familial mutations, such as mutations in the amyloid precursor protein (APP) [97] and presenilin 1 (PS1) [117] genes. Moreover, these studies did not analyze IEGs, only DSB repair markers such as γ H2Ax, 53BP1, and RAD51. APP has been shown to modulate IEG expression, namely by down-regulating *Egr1*, *cFos*, *BDNF*, and *Arc* [270]. Five-month-old APP knock-out mice expressed high baseline levels of these IEGs and failed to show an increase following

exposure to novelty [270]. ApoE has recently been shown to modulate APP via phosphorylation of cFos *in vitro* in an isoform-specific way, where E4 increased APP production more than E3, and E4 and E3 more than E2 [234]. Our experiments are the first to directly assess the role of apoE on DSBs and IEGs *in vivo*, connecting these experiments together to create a fuller picture of differences in regulation of neuronal activation.

Additionally, we incorporated sex as a variable due to the discrepancies in neurological diagnoses between males and [113, 120, 147, 228, 229]. The previous research mentioned above either did not distinguish between males and females [97, 117] or only used males or male-derived cells [234]. There are very few reports on sex differences in DSB repair in the brain. Circulating estrogen and androgens were identified to promote the non-homologous end-joining (NHEJ) repair pathway in breast and prostate cancer, indicating the potential for sex-specific modulation of DSB repair [285, 286]. However, there are known interactions between apoE and sex, ranging from behavioral differences in E4 girls [161] to increased late onset AD rates in E4 women [121, 287]. Our data that there are distinct apoE patterns in DSBs and cFos expression dependent on sex further emphasize the importance of studying both males and females and informs prevention and treatment approaches in the clinic.

To round out the triad, we chose to assess two ages: “young” and “middle-aged.” Importantly, the middle-aged time point is a critical period, as AD pathology and neurodegeneration can occur for decades prior to diagnosis [288]. Analysis of brain activation between young and middle-aged people identified that E4 carriers show less recruitment of the parietal lobe during learning tasks and more diffuse baseline activation at middle age despite equal cognitive performance [289]. The middle-age, E4-specific increase in baseline cFos expression complement these human findings and add to the hypothesis of accelerated aging in

E4 carriers. Our analysis of DSB and IEG regulation offers insight into prodromal neural changes that could be addressed in the clinic.

Methodological Considerations and Future Directions

Stages of learning & memory

I focused my attention on assessing DSBs and their effect on IEG expression during learning. In Chapter 2, I addressed two distinct stages of learning: acquisition (pre-training injections) and consolidation (post-training injections). Notably, etoposide affected long-term memory when injected during the consolidation phase, but not during the acquisition phase. Amifostine affected long-term memory when injected during both acquisition and consolidation. These results suggest that manipulating DSBs at distinct stages in the learning and memory process could lead to distinct outcomes.

Others have shown that DSBs occur in the brain during fear reconsolidation [103]. DSBs led to increased expression of *Npas4*, but not *cFos*. γ H2Ax was also correlated with histone H3 lysine-4 trimethylation (H3K4me3) levels, and inhibition of DSBs before retrieval impaired long-term memory [103]. These results suggest that DSBs are likely involved at many stages of the learning and memory process, though future experiments are needed to further characterize the importance of DSBs at various points.

Thus, building on the experiments I have conducted and reported here, there are a few routes to pursue for future experiments. The first that stands out is using direct hippocampal infusions of etoposide before contextual fear extinction. I would train WT mice in a similar contextual fear conditioning paradigm, and 24 h later I would infuse etoposide or saline into the hippocampus 2 h prior to contextual fear recall (with no shocks). Another 24 h later, I would re-expose mice to the same context. I predict that mice infused with etoposide before the extinction

trial would show higher levels of freezing than saline-infused animals, as the encoding of new information (that the context is “safe”) would be impaired. These would build on the previously mentioned results that impairing DSBs with RNA interference (RNAi) during contextual retrieval inhibits contextual fear recall [103]. An experiment such as this would add to our knowledge about the importance of DSBs at different stages in the learning and memory process. This line of investigation would also have potential implications for treatment strategies; similar to histone deacetylase (HDAC) inhibitors, targeting DSBs during cognitive behavioral therapy may be able to promote fear extinction [290]. **Fig. 6.3** demonstrates several stages of learning that future experiments could target.

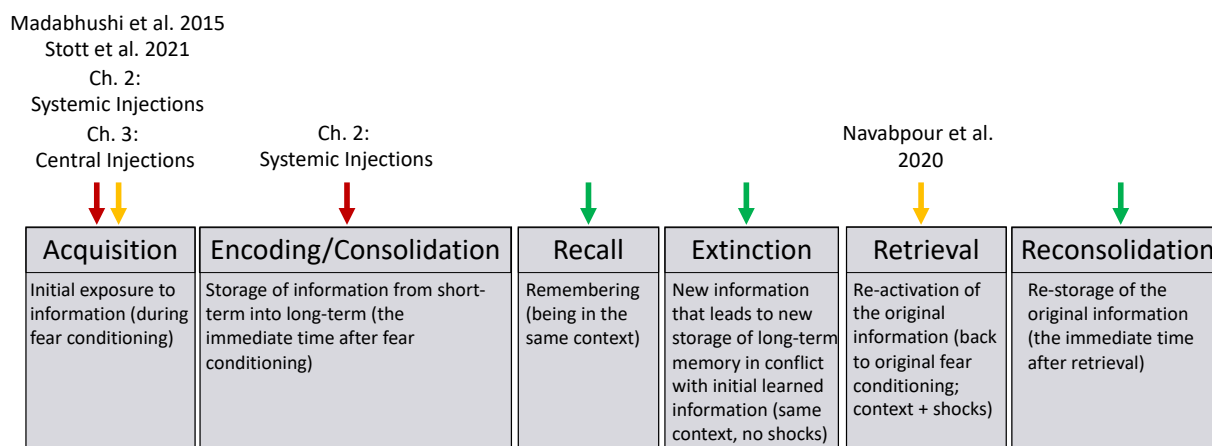


Figure 6.3. Outline of the possible stages of learning related to fear conditioning that have been or could be targeted. Red arrows indicate stages that I have targeted, orange arrows indicate stages that others have targeted, and green arrows indicate stages that could be targeted in the future.

Increased specificity

As mentioned, amifostine and etoposide are “dirty” drugs, meaning they have widespread, untargeted effects. For my purposes, these compounds worked well to assess how even

untargeted alteration of DSBs before and after a learning task affected long-term memory. The next step, though, is to increase specificity, both regionally and in genomic targeting.

A first next-step I would take would be to directly infuse amifostine into the hippocampus before fear conditioning. As amifostine also has poor (though measurable) blood-brain barrier penetration [283], direct hippocampal infusions would add to the evidence that amifostine enhances contextual acquisition, leading to stronger long-term contextual memory. I would design this experiment in a very similar way to the methods described in chapter 3, though one major difference would be timing of injections. Due to the fast pharmacokinetics of amifostine [89, 166, 283], I would infuse amifostine or saline 15 minutes before fear conditioning. Besides this minor adjustment, I would conduct the rest of the experiments (including the testing and tissue collection timelines) as described. By removing systemic considerations, this line of investigation would more precisely address the role of DSBs in the learning process; additionally, our results indicating that males and females responded similarly to direct infusions of etoposide suggest that direct infusions of amifostine might also affect the sexes similarly. Using both males and females will elucidate the mechanisms of amifostine in both sexes.

Others have used RNAi to precisely target players in the DSB repair process. For example, interfering with Gadd45 γ (a protein in the DSB repair pathway) using RNAi prevented DSB repair and impaired long-term trace fear memory [171]. Inhibition of topoisomerase-II β in the hippocampus by RNAi before contextual retrieval also impaired contextual memory [103]. These indicate that new technology is making it possible to increase specificity to target DSBs at distinct time points, opening more lines of investigation into the role of DSBs in synaptic plasticity, learning and memory. Drugs are also being developed to increase specificity and

targets of amifostine, such as PrC-210 [203]. Thus, future experiments will benefit from advanced technology for specifically altering stages of the DSB repair cascade. If I were to pursue this, I could conduct experiments similar to those described in chapter 3 using an RNAi against topoisomerase-II β instead of etoposide, which also affects topoisomerase-II α [87, 291]. Or, using an RNAi to inhibit 53BP1, which would thereby impair the NHEJ repair pathway [93]. By increasing specificity of the target while maintaining the same infusion, behavior, and tissue collection timelines, the molecular dependency of adaptive DSBs will be better understood.

Anatomically, future endeavors could also include infusing DSB-altering agents into the amygdala (**Fig. 6.4**). In the future, I would want to compare amygdalar infusions directly to the results from my hippocampal infusions. Thus, I would keep all methods the same: I would infuse 60 ng of etoposide bilaterally into the lateral amygdala (LA), as lesions of the LA have been shown to impair auditory fear memory [292]. Two hours later, I would train male and female WT mice in fear conditioning and test their contextual and cued fear memory 24 h and 2 weeks later. As there are contradicting findings on the role of the LA in contextual fear memory [292, 293], I predict that etoposide infusions in the LA would impair cued fear memory but may or may not affect contextual fear memory.

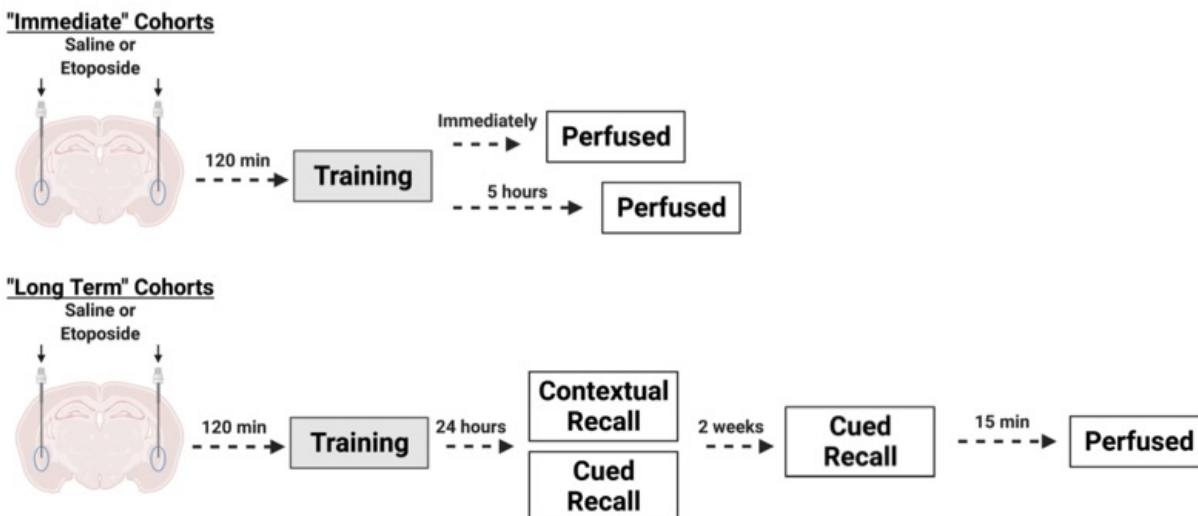


Figure 6.4. Proposed future experiment to compare infusions of etoposide in the lateral amygdala to the experiments described in Chapter 3 using male and female WT mice.

Cellular sub-types

Assessing the cell types that contained DSBs and/or IEG expression was beyond the scope of my dissertation. While IEGs are primarily expressed in neurons, non-neuronal cells do show IEG expression rapidly upon stimulation, mostly in response to insult or disease [294-296]. Others have reported that adaptive DSBs do occur in both neuronal and non-neuronal cell populations [105], with glucocorticoids specifically increasing DSBs in glial cells but not neuronal cells. This suggests that adaptive DSBs form in specific cell-types in response to corresponding stimuli.

Even within neurons, sub-type is an important factor in accurate modulation of learning and memory. IEGs are expressed in all neuronal subtypes: for example, cFos is found in both excitatory and inhibitory neurons in the barrel cortex following exposure to a novel environment [297], and in the medial prefrontal cortex of mice following exploration and ingestion of palatable foods [298]. To the best of my knowledge, no one has investigated DSBs in neuronal

sub-types. This is an important next step in understanding the extent that adaptive DSBs are involved in synaptic plasticity and modulation of neuronal activity.

If I were to address this, I would start by using immunohistochemistry to triple-label cells with γ H2Ax, markers of specific cell-types—such as parvalbumin for GABA-ergic cells [104, 299] or vGlut1 for glutamatergic cells [300]—and cFos. Antibodies exist and are widely used for these cell-types, such as a mouse-anti-parvalbumin from Sigma (#P3088), which we have previously used [104], and a mouse-anti-vGlut1 from Abcam (#134283). Similar to my methods in Chapter 5, quantifying the number of GABA- or glutamatergic cells containing γ H2Ax, cFos, and both would reveal important functions of DSBs in specific cell-types. Moreover, performing these experiments in targeted replacement apoE mice would further clarify the distinctions in cellular activation. It is possible, for example, that the decrease in global cFos in young E4 females we observed in Chapter 4 is in glutamatergic neurons, while the increase in *Fos* near DSBs seen in middle-aged E4 females could be in GABA-ergic neurons, or vice versa. This possible future experiment would advance our understanding of the role of apoE isoform in IEG expression.

Genomic location of DSBs

We chose to pursue a biased approach (PCR) in identifying the location of DSBs on the genome in Chapter 5. Based on previous research from others, we chose candidate genes (namely, *Fos* and *Npas4*) that would likely be near the site of DSBs to assess the effects of apoE, sex, and age. We initially ran a pilot study to establish ChIP using a γ H2Ax antibody followed by genome sequencing. The pilot study did not identify any notable peaks in gene expression, though, and due to time and COVID-19 restraints, we decided to take a biased approach. In future experiments, I would return to pursue an unbiased approach in identifying the role of

apoE, sex, and age on genomic location of DSBs using ChIP-Seq, as described by others [98, 100, 103, 105]. I would maintain a similar experimental design to the one described in Chapter 5. Notably, I would add a 3rd age in targeted replacement apoE mice: young (3 month), middle-aged (12 month), and old (16 month). This third, aged group would further identify the trajectory of DSBs in the apoE isoforms. As in my previous experiments, I would use both male and female mice to fully investigate “the Triad” of risk for AD. By taking an unbiased approach in genome sequencing, these experiments would provide a fuller picture of the distinction between the hypothesized pathological and adaptive DSBs.

While common, use of γ H2Ax to identify the location of DSBs is indirect and dependent on the recruitment of γ H2Ax to the site of a break. Recent interest has spurred the development of several methods for direct tagging of DSBs without relying on the recruitment of repair proteins, such as End-Seq [301], Breaks Labeling *In Situ* and Sequencing (BLISS) [302], and i-BLESS [303]. These various methods are novel ways to directly localize DSBs, enhancing precision and specificity. Thus, subsequent experiments can and should utilize these novel techniques to address specifically DSBs. This would be particularly interesting in the experiment I described just above—in young, middle-aged, and old apoE targeted replacement mice. Comparing End-Seq and ChIP-Seq results would be insightful into the amount of DSBs compared to the amount of γ H2Ax recruitment.

Final Remarks

To briefly summarize, these dissertation studies have added to growing evidence for an adaptive role of DSBs, and furthered our understanding of apoE-, sex-, and age-specific modulation of cellular mechanisms related to learning and memory. There are new avenues of

investigation that have been spurred by this research (described above). There is no doubt that the pursuit of understanding the role of DSBs in physiological function will continue and provide insight into the mysteries of the brain.

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