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ANALYSIS OF ADULT NEUROGENESIS AND HIPPOCAMPUS-DEPENDENT MEMORY IN MOUSE MODELS FOR AGING, ALZHEIMER'S DISEASE AND CRANIAL IRRADIATION-INDUCED NEUROINFLAMMATION

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ABSTRACT

Aging in the brain is a complex process that affects every living being differently. Age-associated memory decline can be mild; however, memory can deteriorate quickly with in patients with mild cognitive impairment or a significant brain injury. Investigating therapeutics that support learning and memory mechanisms in subjects at risk for cognitive impairment is crucial for aging populations. One therapeutic that has drawn interest in Alzheimer's disease (AD) research is the diabetic drug, rosiglitazone which specifically binds to the nuclear receptor, peroxisome proliferating activated receptor gamma (PPARγ). Inhibition of p38α activity has also been shown to be a viable therapeutic target for conserving cognition in neurodegeneration models. Briefly, the hippocampus is responsible for encoding long-term memories associated with spatial and contextual cognitive tasks and is susceptible to AD pathology. The hippocampus is one of the few areas in the adult brain where new neurons are continuously produced throughout life. Furthermore, hippocampal neurogenesis has been shown to support neighboring neurons and correlate with hippocampal learning and memory performance. However, it is unknown how PPARγ and p38α impinge on adult neurogenesis and hippocampal dependent learning and memory in mouse models with risk factors associated with cognitive decline. To empirically answer this question, we chose three models of cognitive decline: aged wild type mice, cranial-irradiated mice and a model of Alzheimer's disease, Tg2576(APP KM670/671NL Swedish). Here we show attenuating p38α activity improves context fear discrimination and survival of adult-born neurons in aged mice. Similarly, the PPARγ agonist RSG rescued cranial irradiated deficits in context discrimination that was independent of adult neurogenesis. In the same model we observed attenuation of Iba-1 expressing microglia in the dorsal hippocampus which has been shown to protect cognition. Lastly, RSG was able to reverse Morris water maze spatial learning and long-term memory but not object recognition or context fear discrimination deficits in the Tg2576 Alzheimer's mouse model. In conclusion the evidence here supports a role for p38α and PPARγ in reversing hippocampal dependent memory deficits.

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LIST OF ABBREVIATIONS

UTMB	University of Texas Medical Branch
GSBS	Graduate School of Biomedical Science
TDC	Thesis and Dissertation Coordinator
RSG	Rosiglitazone
PPARγ	Peroxisome Proliferator Activated Receptor gamma
MAPK	Mitogen Activated Protein Kinase
MWM	Morris Water Maze
NOR	Novel Object Recognition
AD	Alzheimer's disease
Tg2576	Transgenic 2576
APP	Amyloid Precursor Protein
LTP	Long-Term Potentiation
DG	Dentate Gyrus
MEC	Medial Entorhinal Cortex
LEC	Lateral Entorhinal Cortex
CA1/3	Cornus Ammonus 1 and 3
CREB	cAMP Response Element Binding protein
BrdU	BromodeoxyUridine
SGZ	Sub-Granular Zone
CS	Conditioned Stimulus
US	Unconditioned Stimulus

Introduction

Hippocampal learning and memory

Memory formation by the hippocampus has been well studied in the past and shown to require changes in neurochemistry, synapses and gene expression. Briefly, the hippocampus is a subcortical structure in the mammalian brain that is responsible for encoding episodic/spatial memories (Kandel, 2000; R. G. Morris, Garrud, Rawlins, & O'Keefe, 1982; Scoville & Milner, 1957). A component of the limbic system, the hippocampus connects to many structures regulating mood and cognition including the amygdala, thalamus, hypothalamus, cortex, cingulate gyrus, and mammillary bodies(Kandel, 2000). Simply, plasticity in the hippocampus is achieved through activation of glutamate/acetlycholine receptors that promote synaptic connections. Long-term potentiation results from persistent activation of these synaptic connections and leads to memory formation (Bliss & Collingridge, 1993; Winson & Abzug, 1978). Spatial memories are continuously being recorded when sensory information like sight, sound, smell and touch from associative cortices are transmitted to the medial and lateral entorhinal cortices (MEC and LEC, respectively) right outside the hippocampus (Knierim, Neunuebel, & Deshmukh, 2014). Grid cells in the MEC function to establish a spatial map of a new context while the LEC provides specific information about objects in a given environment (Knierim et al., 2014; O'Keefe & Dostrovsky, 1971). Schaffer collateral cells transmit, “where” and “what” information into the hippocampus where pattern separation and completion is accomplished by granule cells in dentate gyrus and pyramidal cells in the CA1, and CA3. Lastly, spatial memory is shunted to cortical regions through the subiculum for long-term storage. This simplistic overview of spatial memory encoding is sufficient for the scope for this study however, several factors depend on how accurately these memories are coded by the

hippocampus. For example, *N*-methyl-D-aspartate receptor (NMDAr) mediated synaptic plasticity in the hippocampus is crucial for facilitating spatial learning (R. G. Morris, Anderson, Lynch, & Baudry, 1986; R. G. Morris & Frey, 1997). Further, knock out of NDMA receptor in CA1 diminishes distal but not proximal navigational memory suggesting NMDA mediated plasticity is necessary for pattern completion (Tsien, Huerta, & Tonegawa, 1996). Another major component of hippocampal plasticity is downstream biochemical signaling; i.e., the cAMP(second messenger)-PKA(Protein Kinase A)-CREB(cAMP Response Element Binding Protein) pathway is necessary to facilitate late-LTP from short-LTP after repeated activation (Frey & Morris, 1997). Lastly, the nuclear transcription factor, CREB is responsible for expressing genes necessary for synaptogenesis and ultimately LTP (Mizuno et al., 2002).

Hippocampal potentiation is heavily dependent on the behavioral task carried out. Dorsal but not ventral hippocampal lesions revealed deficits in spatial acquisition in Morris water maze and Y-maze paradigms(Bannerman et al., 2003; Bannerman et al., 2004; Bannerman et al., 1999). Yet, subjects with ventral hippocampal lesions exhibit less fear in aversive contexts suggesting this part of the hippocampus is strongly associated with the amygdala(B. J. Hock, Jr. & Bunsey, 1998). Together, these studies provide evidence for specific hippocampal dependent learning and memory investigations.

Adult hippocampal neurogenesis

Neurogenesis is the complex process of generating neurons and was thought to only occur during embryogenesis. We now know from historical research that the adult mammalian brain is capable of producing new neurons throughout adulthood. By injecting a marker to track

cell division, tritiated thymidine-H^3, Joseph Altman and Gopal D. Das, 1965, observed proliferating cells in the lateral ventricles and sub-granular region in the hippocampus of adult rodent brains. Moreover, they reported granule cell migration/differentiation positively correlated with time after injection however, proliferation decreased with age. This study was the first to demonstrate adult neurogenesis was present postnatally and that aging significantly affected production of new neurons(Altman & Das, 1965).

Due to the vast research describing adult neurogenesis in the lateral ventricles and sub-granular zone (SGZ), we will focus on adult hippocampal neurogenesis as it relates to spatial and contextual learning and memory. Though still significant in rodents, the neurogenic niche in lateral ventricles far exceeds proliferation in the hippocampus. Further, integration of new born neurons in the olfactory bulbs may function in odor discrimination learning and memory (Alvarez-Buylla & Garcia-Verdugo, 2002; Doetsch & Alvarez-Buylla, 1996; Gheusi et al., 2000).

Since Altman and Das's discovery, several lines of evidence have contributed to describing the biology of adult neurogenesis. The radioactive thymidine analogue Thymidine-H^3 has since been replaced with the safer thymidine analogue, 5-Bromo 2'-deoxyuridine (BrdU) and like thymidine-H^3, it incorporates into the DNA of dividing cells during S-phase of the cell cycle. This new chemical led neuroscientist to discover that adult neurogenesis is not unique to rodents and song birds. By injecting BrdU into terminally ill patients, Peter S. Erickson and colleagues were able to demonstrate neuronal proliferation and differentiation in the SGZ of human hippocampi. These observations were later confirmed in non-human primates. Moreover, adult neurogenesis was recently confirmed in humans by measuring carbon-14 concentrations in neuronal nuclei (Eriksson et al., 1998; Kornack & Rakic, 1999; Spalding et al., 2013). Further,

this compound is now commonly used by neuroscience researchers to visualize and track adult neurogenesis in the adult brain. In fact, by injecting BrdU, it was discovered that adult new born neurons are derived from astrocytes as staining methods showed BrdU and Glial Fibrillary Acidic Protein (GFAP) co-localized in the hippocampal SGZ immediately following intra-peritoneal injections (Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). Using similar techniques, it was demonstrated that mitogen factors like EGF, BDNF, IGF-1 and BMP increase neuronal proliferation/differentiation *in vitro* and *in vivo*, supporting the presence of a neurogenic niche within the hippocampus (B. Y. Chen et al., 2013; Lie, Song, Colamarino, Ming, & Gage, 2004; Ming & Song, 2011). Stages of neurogenesis continues with neural stem cells that arise from proliferating astrocytes and can be visualized with nestin, followed by the immature neural marker doublecortin (DCX) and finally NeuN for mature neurons. The fate of newly divided cells is not certain and can be terminated at any stage with only a small degree of them becoming mature granule cells in the hippocampus (G. Kempermann, Jessberger, Steiner, & Kronenberg, 2004). Survival of new born neurons can vary among species and even between gender suggesting adult neurogenesis can be modulated genetically and by factors like hormones (Gerd Kempermann, H Georg Kuhn, & Fred H Gage, 1997). Further, neural stem cell differentiation is promoted with neural activity and release of neurotransmitters, for example; immature neurons are excited by GABAergic signaling at approximately 4-10 days of age. Moreover, arborization and integration into the neural network depend mainly on glutamatergic signaling but new born neurons can also respond to other neurotransmitters like acetylcholine and dopamine etc… at 2-4 weeks of age (Ming & Song, 2011). The revelation that adult neurogenesis can be modified endogenously prompted research into exogenous factors that mediate production of new neurons. For example, administration of antidepressants like selective

serotonin reuptake inhibitor (SSRI) increased levels of new born neurons in the hippocampus. Conversely, drugs of abuse like alcohol, methamphetamine and opiates decreased adult hippocampal neurogenesis(Duman, Nakagawa, & Malberg, 2001; Eisch & Harburg, 2006).

Behavioral manipulations have also been shown to influence adult hippocampal neurogenesis. Henriette Van Praag and colleagues demonstrated that exercise such as running can increase survival of neurons in the hippocampus. Although exercises like swimming and navigational training in water maze improved hippocampal neurogenesis, it wasn't to the extent voluntary running had on neuronal survival. Moreover, housing rodents in enriched conditions has consistently proven to increase survival of adult born neurons in the hippocampus (Clemenson et al., 2015; David J Creer, Carola Romberg, Lisa M Saksida, Henriette van Praag, & Timothy J Bussey, 2010; G. Kempermann, H. G. Kuhn, & F. H. Gage, 1997; Henriette van Praag, Kempermann, & Gage, 1999; H. van Praag, Shubert, Zhao, & Gage, 2005). In contrast, stressful conditions can negatively alter adult hippocampal neurogenesis (Duman, Malberg, & Nakagawa, 2001).

Since adult neurogenesis resides in an area of the brain that is susceptible to pathological insults, it's no surprise that hippocampal neurogenesis is also affected with brain diseases. As the first report describing adult neurogenesis and others have shown, aging is detrimental to the neurogenic niche and survival of new born neurons(Altman & Das, 1965; Galvan & Jin, 2007). Further, animal models harboring mutations in genes associated with Alzheimer's disease i.e. amyloid precursor protein and gamma secretase, have accelerated loss in proliferation and neuronal survival in mid to late stages of life(Hamilton et al., 2010). Though some conflicting reports observed increased proliferation and differentiation in Alzheimer's mouse models(Q. Chen et al., 2008; K. Jin et al., 2004; Yu et al., 2009). *In vitro* experiments reveal amyloid beta

diminishes proliferation and differentiation in neurosphere cultures though this report doesn't consider growth factors that may be elevated in humans with Alzheimer's disease (Haughey et al., 2002; K. Jin et al., 2004; Mu & Gage, 2011). Mutations in microtubule associated protein tau gene (MAPT) that increase tau phosphorylation decreased adult neurogenesis as early as two months of age (Komuro, Xu, Bhaskar, & Lamb, 2015). Alterations to adult neurogenesis are not limited to neurodegeneration; mental illness like major depression, schizophrenia, and epilepsy also disrupt adult hippocampal neurogenesis (Duan et al., 2007; G. Kempermann, 2002).

Altogether, it is obvious the adult mammalian brain continuously produces new neurons after embryogenesis and this cellular process can be altered with age, drugs and mental illness. The question that remains is what role does new born neurons serve in learning and memory given they reside in area of the brain that is responsible for recording new environments throughout life? Recent reports on this matter suggest new born neurons are necessary for pattern separation/contextual discrimination learning and memory(Aimone, Deng, & Gage, 2011).

We know the hippocampus is necessary for encoding new spatial memories but how does it accomplish this task without interrupting previous memories of the same or similar environments? Theoretical research speculate that the dentate gyrus is crucial for pattern separation while connections from the entorhinal cortex to CA3 is important for pattern completion or recall of these memories. Computational studies support this theory and propose this may be due to the large abundance of granule cells yet sparse activation in the dentate gyrus (Marr, 1971; Treves & Rolls, 1994). This appears to be the case as granule cells in the dentate gyrus were activated in novel contexts where as CA1 neurons were reactivated in familiar contexts (Clelland et al., 2009; Deng, Mayford, & Gage, 2013; Neunuebel & Knierim, 2014). The role of new born neurons in the dentate gyrus may then serve to discriminate between

similar contexts. In fact, ablation of adult neurogenesis prevented mice from discriminating in a spatial navigation and visual discrimination tasks (Clelland et al., 2009). Moreover, adult born neurons role in spatial discrimination was confirmed using optogenetic ablation and *in vivo* recording techniques (Danielson et al., 2016).

Contextual fear discrimination

Contextual fear conditioning is a hippocampus dependent learning task. Based on Pavlovian classical conditioning principle; freezing (reflexive behavior) is measured in response to training in context where a conditioned stimulus (auditory cue) is paired with an unconditioned stimulus (footshock). In a natural setting, the ability for rodents to pair an aversive stimulus like a cat or snake with their environment is crucial for their survival. Researchers have since used derivations of this technique to elucidate the hippocampus's role in learning and memory. Post-training hippocampal lesions at specific time points revealed severe retrograde amnesia for recently acquired but not distant memory recall. This suggests the hippocampus is involved in temporary storage of CS-US association memories (Anagnostaras, Gale, & Fanselow, 2001; Kim & Fanselow, 1992; Maren & Fanselow, 1997). Conversely, animals with pre-training hippocampal lesions performed comparably to non-lesioned subjects. Discrepancies in hippocampal based contextual fear conditioning may due to complete hippocampal lesions and strong amygdala connections to the ventral hippocampus (anterior hippocampus in primates). Thus, lesioning the dorsal (DH) hippocampus only, reveals contextual fear memory remains intact but is disrupted during contextual fear discrimination tasks (Frankland, 1998; Phillips & LeDoux, 1992, 1994).

Contextual fear discrimination is derived from foreground fear conditioning paradigm which excludes an auditory cue during training and challenges subjects to recall discrete information in their environment. Moreover, this technique forces animals to recall specific details in their training context that is otherwise negligible when a tone cue is delivered with a foot shock. The elemental solution postulates that one or two elements in a context is sufficient for hippocampal lesioned animals to infer fear memory(Anagnostaras et al., 2001). Indeed, Frankland et al. 1998 and Phillips R.G and Le doux 1994 demonstrate DH lesions do not disrupt contextual recall until the training context is slightly altered upon testing. The addition of new information in a similar context should elicit exploratory behavior or decreased freezing. Though, animals with DH lesions exhibit comparable freezing in shock and similar but not dissimilar contexts i.e. a context apparatus with salient context elements like walls and shock grid masked with inserts. These studies show contextual fear discrimination is a discrete memory task that requires high cognitive function than contextual testing. Furthermore, these studies demonstrate that the DH is required for detailing environments and severe lesions can elicit heightened fear behavior in contextually overlapping but different environments. This generalized fear suggests animals have lost the ability to adapt to new environments which is not evolutionarily favorable for a species. If contextual discrimination is so important to survival, then what factors contribute to the maintenance of this type of learning and memory and how does it diminish. Recent work on this matter demonstrated conserved or enhanced production of adult born hippocampal neurons may serve to improve discrimination and reduce interference of overlapping environmental cues(Aimone, Deng, & Gage, 2010; Besnard & Sahay, 2016; G. Kempermann, 2008).

Aging and Alzheimer's Disease

Aging is a common risk factor for all leading causes of death in developed countries. In particular, aging in the brain imposes serious problems on human's cognitive reserve, as one common complaint in older adults is memory loss. Today, over 46 million Americans are over the

age of 65 with this population projected to double in the near future (Bureau, 2016). In aging patients, neurocognitive deficits like processing speed, language and executive reasoning are demonstrable with clinical tests. Along with cognitive deficits, neuronal changes are visible with reductions in hippocampal and para-hippocampal cortices (Harada, Natelson Love, & Triebel, 2013). Experimentally, neurocognitive deficits from ageing can be recapitulated in animal models; for example, aged rats (17 months) exhibit deficits compared to younger subjects in hippocampal dependent memory tasks like classical fear conditioning and radial arm maze (Oler & Markus, 1998).

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that accelerates cognitive decline and is characterized post-mortem by the presence of amyloid beta (senile) plaques, shrunken grey/white matter and neurofibrillary tau tangles (Association, 2017). Currently, there are over 5 million Americans living sporadic Alzheimer's diseases and 200,000 that have a genetic link and early onset of the disease. The chances of 65 and older individuals developing dementia due to Alzheimer's is 1 in 10 with their chances increasing every year. Economically, this disease will cost $259 billion in Medicare/Medicaid and without a cure or therapy to curve the progressive nature of this disease, this amount will soar when the remaining baby boomer population is greater than 65 years of age (Association, 2017). Mutations in beta-amyloid precursor protein (APP) and gamma secretase genes are the strongest risk factors for developing AD. These mutated genes accelerate aberrant production of toxic amyloid beta-40, 42 peptides(Walsh & Selkoe, 2007). Transgenic animal models, inoculation with oligomeric Abeta-42 *in vivo* and *in vitro* studies demonstrate this protein causes synaptic dysfunction in cortical and hippocampal regions (Mucke & Selkoe, 2012; Reilly et al., 2003). Further evidence from AD mouse models demonstrated behavioral and Long-Term Potentiation (LTP) deficits as early

as 6 months of age (Jacobsen et al., 2006; Rupp, Wegenast-Braun, Radde, Calhoun, & Jucker, 2011). Therapeutics targeting amyloid beta aggregation have shown promise in similar studies but have yet to pass clinical trials (Comery et al., 2005; C. Hock et al., 2003; Walsh & Selkoe, 2007).

Although transgenic animals serve as excellent models of disease, they do not recapitulate all hallmark AD pathologies like neuronal loss and thus, investigating targets associated with environmental risk factors could prove beneficial for the greater portion of AD patients. Risk factors for patients with sporadic AD include: ageing, metabolic disease like type-2 diabetes, and head trauma. Ageing is the greatest risk factor for all diseases though clinical studies have also shown a strong incidence of type-2 diabetes and AD (Janson et al., 2004; Ott et al., 1999). Research on this matter demonstrated that APP mutant mice crossed with obesity/diabetic mice exhibited increased diabetic and AD pathologies including early cognitive deficits (Takeda et al., 2010). Furthermore, decreased insulin sensitivity in type-2 diabetes disrupts mitogen activated protein kinase (MAPK) which have been shown to be elevated in human AD brains and AD mouse models(Dineley et al., 2001; Hensley et al., 1999). Moreover, Type-2 diabetes and AD increase reactive oxygen species (ROS) and chronic activation of inflammatory pathways in the brain (Sims-Robinson, Kim, Rosko, & Feldman, 2010). Converging evidence suggests, finding therapies that attenuate type-2 diabetes pathology may improve health in a fast-growing population at risk for AD.

Functional role of inflammation

Inflamm-ageing is persistent inflammation concomitant with ageing and is posited to be a risk factor that may contribute to mild cognitive impairment. (Godbout & Johnson, 2009). Proinflammation signals like like IL-beta TNF-alpha trigger resident microglia and astrocyte.

These signals are beneficial for glial cells to clear foreign material in the brain like an infection or Alzheimer's disease pathologies. However, during states of chronic inflammation, the neuroimmune response increase reactive oxygen species that ultimately leads to neuronal cell death. Therapies targeting TNF-alpha effectively reduces neuronal death and conserves cognition in diseases related to chronic inflammation (McCoy, Ruhn, Blesch, & Tansey, 2011; McCoy & Tansey, 2008). Lastly, aging is detrimental to production of new neurons in the hippocampus(Spalding et al., 2013). Some recent work suggests chronic inflammation associated with age or Alzheimer's disease can disrupt production of new neurons (Russo, Barlati, & Bosetti, 2011). Since astrocytes provide several roles in the brain including neuroinflammation, its possible during disease states such as Alzheimer's disease, their neurogenic role is shifted to produce more astrocytes during chronic inflammation (Adamowicz, Mertens, & Gage, 2015). Neurocognitive decline associated with ageing is natural but at what point does it become pathological to cognitive reserve? Investigating therapies that target inflammatory pathways related to risk factors for AD may help promote cognitive reserve throughout life.

PPARγ agonism in brain health

Peroxisome proliferator activated receptor gamma (PPARγ) is a ligand modulate transcription factor and binds to genes responsible for regulating inflammation, and metabolism. A member of the nuclear receptor superfamily, PPARγ contains phosphorylation, sumoylation sites and binding domains for DNA, co-activators, and ligands. PPARγ binds to endogenous eicosanoids and the FDA approved class of drugs; a full agonist shown to improve glucose metabolism in insulin resistant type 2 diabetic patients. Specifically, PPARγ agonism increases expression of glucose transporters and insulin receptors. Knockout of PPARγ in adipose tissue showed reduced adipose accumulation and protection from insulin resistance in mice (Jones et al., 2005). Since the brain requires a high degree of glucose and lipid homeostasis, then PPAR gamma activity should play a big role in maintaining a healthy brain throughout life.

PPARγ is largely expressed in neurons and to a lesser extent astrocytes in the adult mouse and human brain (Warden et al., 2016). In AD mouse models, PPARγ agonism restores hippocampal dependent memory by binding to activated ERK (MAPK); a protein reported to be aberrantly active in AD. Furthermore, neuronal transmission between entorhinal cortex and hippocampus was restored in similar studies. (Jahrling, Hernandez, Denner, & Dineley, 2014; Nenov, Laezza, et al., 2014; Nenov, Tempia, Denner, Dineley, & Laezza, 2014). Further, PPARγ agonism increased breakdown of amyloid beta through microglial and astrocytic activation in double AD mutant mice (Mandrekar-Colucci, Karlo, & Landreth, 2012). Lastly, PPARγ agonism protected hippocampal adult neurogenesis and memory in lipopolysaccharide disease model (Ormerod et al., 2013). These reports suggest PPARγ plays a dynamic role in supporting cognition by: converging onto learning and memory MAPK pathway, preserving adult hippocampal neurogenesis and improving neuronal transmission.

Objective

Adult neurogenesis appears to be crucial for hippocampus dependent context discrimination learning and memory. However, little is known about how to rescue cognition dependent on adult hippocampal neurogenesis in aged models of disease. To study this, we attenuated p38α kinase activity, aged them to 13-17 months and analyzed context discrimination and adult neurogenesis. Further, we studied cognitive effects the PPARγ agonist rosiglitazone had in aged model of Alzheimer's disease and cranial irradiation. This thesis argues cognitive dependent adult hippocampal neurogenesis will be rescued in models of age, neurodegeneration and cranial irradiation.

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Chapter 1

Aged dominant negative p38alpha MAPK mice are resistant to age-dependent decline in adult-neurogenesis and context discrimination fear conditioning

ABSTRACT

A major aspect of mammalian aging is the decline in functional competence of many self-renewing cell types, including adult-born neuronal precursors. Since age-related senescence of self-renewal occurs simultaneously with chronic up-regulation of the p38MAPKalpha (p38α) signaling pathway, we used the dominant negative mouse model for attenuated p38α activity (DN-p38$\alpha^{AF/+}$) in which Thr180 and Tyr182 are mutated (T→A/Y→F) to prevent phosphorylation activation (DN-p38$\alpha^{AF/+}$) and kinase activity. As a result, aged DN-p38$\alpha^{AF/+}$ mice are resistant to age-dependent decline in proliferation and regeneration of several peripheral tissue progenitors when compared to wild-type littermates.

Aging is the major risk factor for non-inherited forms of Alzheimer's disease (AD); environmental and genetic risk factors that accelerate the senescence phenotype are thought to contribute to an individual's relative risk. In the present study, we evaluated aged DN-p38$\alpha^{AF/+}$ and wildtype littermates in a series of behavioral paradigms to test if p38α mutant mice exhibit altered baseline abnormalities in neurological reflexes, locomotion, anxiety-like behavior, and age-dependent cognitive decline. While aged DN-p38$\alpha^{AF/+}$ and wildtype littermates appear equal in all tested baseline neurological and behavioral parameters, DN-p38$\alpha^{AF/+}$ exhibit superior context discrimination fear conditioning. Context discrimination is a cognitive task that is supported by proliferation and differentiation of adult-born neurons in the dentate gyrus of the hippocampus. Consistent with enhanced context discrimination in aged DN-p38$\alpha^{AF/+}$, we discovered enhanced production of adult-born neurons in the dentate gyrus of DN-p38$\alpha^{AF/+}$ mice

compared to wildtype littermates. Our findings support the notion that p38α inhibition has therapeutic utility in aging diseases that affect cognition, such as AD.

INTRODUCTION

Aging is a complex organismal process that is controlled by genetic, environmental, and behavioral factors. A major aspect of mammalian aging is an age-associated decline in functional competence of many self-renewing cell types, including adult stem cells and other proliferative progenitors (Blau, Cosgrove, & Ho, 2015; Janzen et al., 2006; Molofsky et al., 2006). Further, age-related senescence of self-renewal occurs simultaneously with up-regulation of the p38MAPKalpha (p38α) signaling pathway (Brien, Pugazhendhi, Woodhouse, Oxley, & Pell, 2013; Haq et al., 2002; Hsieh & Papaconstantinou, 2002; Hsieh, Rosenblatt, & Papaconstantinou, 2003; Iwasa, Han, & Ishikawa, 2003). One consequence of chronically elevated p38α is a chronic pro-inflammatory state that promotes the expression of a physiologically complex aging phenotype both in the periphery and the CNS leading to susceptibility of age-related diseases including cancer and neurodegenerative diseases (Bachstetter & Van Eldik, 2010; Xu, Li, Xiang, & Sun, 2014). As such, it has been proposed that modulation of p38 MAPK activity may provide new avenues for treating certain age-related degenerative diseases.

Previous work by ourselves and others using mice that express a mutant form of p38α in which Thr180 and Tyr182 are mutated (T→A/Y→F) to prevent phosphorylation activation results in dominant negative mitigation of p38α (DN-p38α$^{AF/+}$) kinase activity. Consistent with the notion that p38α is a significant regulator of cell senescence, aged DN-p38α$^{AF/+}$ mice are resistant to age-dependent decline in the proliferation and regeneration of several peripheral

tissue progenitors when compared to wild-type littermates (Papaconstantinou et al., 2009, 2015; Wong et al., 2009). This has led to the hypothesis that in the periphery, p38 modulates the regenerative capacity of tissues that rely upon a progenitor population (e.g., muscle, liver, and pancreas).

Aging is the major risk factor for non-inherited forms of Alzheimer's disease (AD); environmental and genetic risk factors that enhance chronic inflammation and accelerate the senescence phenotype are thought to significantly contribute to an individual's relative risk for AD. It is not surprising then that p38α MAPK represents an actively pursued therapeutic target for age-dependent cell senescence, neuroinflammation, and AD (Bachstetter & Van Eldik, 2010; Munoz & Ammit, 2010; Pinsetta, Taft, & de Paula da Silva, 2014). In the present study, we evaluated aged DN-p38$\alpha^{AF/+}$ and wildtype littermates in a series of behavioral paradigms to test if p38α mutant mice exhibit altered baseline abnormalities in neurological reflexes, locomotion, anxiety-like behavior, and age-dependent cognitive decline. While DN-p38$\alpha^{AF/+}$ and wildtype littermates appear equal in baseline neurological and behavioral parameters, DN-p38$\alpha^{AF/+}$ exhibit superior context discrimination fear conditioning; a cognitive task that is supported by enhanced production of adult-born neurons in the dentate gyrus of the hippocampus, one of the major CNS progenitor sources (David J Creer et al., 2010; Sahay et al., 2011; Spalding et al., 2013). Quantification of new adult-born neurons revealed that aged DN-p38$\alpha^{AF/+}$ have more BrdU/NeuN-positive neurons in the granule cell layer of the dentate gyrus compared to wildtype littermates. Our findings support the notion that p38α inhibition has therapeutic utility in aging diseases that affect hippocampus-dependent cognitive function, such as AD.

MATERIALS AND METHODS

Materials. Chemicals and reagents were purchased from Sigma.

Animals. Dominant negative-p38$\alpha^{AF/+}$ (DN-p38 $\alpha^{AF/+}$) mutant mice were created on a C57Bl/6j background strain in the laboratory of Dr. Dmitry V. Bulavin (David J Creer et al., 2010; Wong et al., 2009) and provided by Dr. John Papaconstantinou (Papaconstantinou et al., 2009)Wildtype and heterozygous DN-p38$\alpha^{AF/+}$ were generated according to breeding strategies employed in ((Papaconstantinou et al., 2009, 2015; Wong et al., 2009) and housed in the Animal Resource Center Facilities at the University of Texas Medical Branch (UTMB). UTMB Animal Resource Center Facilities operate in compliance with the USDA Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, under OLAW accreditation, and IACUC approved protocols. Male and female mice were housed separately, $n \leq 5$ per cage, with food and water *ad libitum* and aged (10-19 months of age). Median age of wild type and DN-p38 was 10MO, 13MO, respectively. Average age for wild type and DN-p38$\alpha^{AF/+}$ was 12.5MO, 13.5MO, respectively. There was no significant difference in distribution of age as indicated by student t test [t=0.87, df=22.02] p value =0.39. Genotyping was performed as described in (Papaconstantinou et al., 2009, 2015; Wong et al., 2009). Heterozygous DN-p38$\alpha^{AF/+}$ were used as homozygous mutant mice die *in utero*. Experimenters were blinded to genotype during key data acquisition and analysis steps.

To optimize contextual-fear discrimination, 2.5-month old C57Bl/6J were purchased from Jackson Laboratory and aged to 3-months of age in animal care facilities here at UTMB, before testing. For this experiment, mice (n=10; 5 male, 5 female) were exposed to shock context on day 0 where they were allowed to explore for 178s before receiving a 0.75mA footshock for 2-s; the session was terminated 15 seconds after receiving the footshock (**Figure 4A**) and mice

were placed back in their home cages. From day 1 and onward, % freezing was recorded after mice were placed in the shock context A or the safe context B as indicated in **Figure 4B**.

General health, neurological, behavioral and cognitive tests were performed on 29 DN-p38$\alpha^{AF/+}$s and 13 wildtype mice (roughly equal for gender) in UTMB's *Rodent in Vivo Assessment* (*RIVA*) core facility (directed by Dr. Kelly Dineley). In spite of a disparate number of wildtype and DN-p38$\alpha^{AF/+}$ mice, F tests for unequal variance indicated statistically equal variance between the two groups (*see statistics, below*). All mice were tested during 12hr light cycle (06:00 – 18:00hr) then returned to home cages prior to the 12hr dark cycle. Further, mice were acclimated to the testing room at least 1hr before behavioral and cognitive tests were performed. Furthermore our genotypes were analyzed as a single cohort in the order listed below and only one test battery, e.g., SHIRPA, grip strength, etc. was performed each day.

Behavioral Assessment. The *SHIRPA* (Rogers et al., 2001) general health and neurological screen was used to reveal discrete phenotypes in DN-p38$\alpha^{AF/+}$ and wildtype mice. Briefly, weight, coat appearance, and spontaneous activity were recorded using the arbitrary scale 0-4; where 0=none, 1=low, 2=moderate, 3=fair, and 4=good. Next, observations related to cage activity included transfer to testing cage, arousal, gait, etc., were recorded on the same scale. Lastly, provoked activity i.e. trunk curl, wire maneuver and reflex tests were also recorded.

Forelimb Grip Strength. To assess forelimb strength, we utilized the DFIS-2 Digital Force Gauge (Chatillon®). Each mouse was gently but firmly held by the base of the tail and allowed to grab a metal bar with both forelimbs. While grasping the metal bar, grip strength was recorded by gently pulling the mouse away from the metal bar until the grip was lost. Average grip strength in (kg), was calculated for each mouse from three consecutive trials.

Open Field. The open field test measures exploratory behavior and locomotor activity in mice. Briefly, each mouse was placed in the center of a white 38 x 38cm Plexiglas™ box and allowed to explore for 10min. Digital video-based data capture and analysis was achieved with TopScan software (Clever Sys) using central (12.6 cm^2) and peripheral (25.4 cm^2) arenas to track exploratory behavior. Total distance travelled and velocity was calculated for the peripheral and central arenas.

Elevated Plus Maze. A standard elevated plus maze (30 cm long x 6 cm wide x 13 cm tall, closed arms) 50 cm off the ground) was used to measure anxiety-like behavior and exploration patterns. Mouse subjects were placed into the center arena of the maze and allowed to freely explore for 5min. Percent time spent in each arm, total distance travelled, and velocity was calculated using TopScan digital video-based data capture and analysis software (Clever Sys).

Fear Conditioning and Context Discrimination. The contextual fear-discrimination paradigm is a hippocampus-dependent cognitive task that measures an animal's ability to distinguish between two similar yet different environmental contexts (Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998). On training day 0 (**Figure 4**), each subject was placed in a standard mouse fear conditioning chamber (Med Associates) which served as context A, the shock context. Context A training (**Figure 1.3 A**) ensued for 195sec during which time a single 2sec 0.75mA shock was delivered through the electrified grid floor at the 178sec mark; mice then had an additional 15sec to explore the context then were returned to their home cages.

On testing days (day1 onward, **Figure 1.3B**), mice were randomly assigned to experience first either context A, or a similar yet non-identical "safe" context, context B. Context B resembled context A in terms of the grid floor being exposed. Context B contained cardboard inserts, vanilla extract (2 μL pipetted onto a KimWipe®), and the chamber light and fan were

turned off. Like context A, mice were placed in context B for 185s, however, no shock was delivered. A 2hr inter-trial interval between each context was utilized for each subject for each day of testing. Following each session, 70% ethanol was used to thoroughly wipe down Context A and 95% isopropyl alcohol was used to clean Context B.

Digital video-based data capture and analysis with FreezeFrame software (Actimetrics) was used to assess freezing behavior. Discrimination ratios (% freezing in Context B ÷ % freezing in Context A+B) were calculated for each group on each test day.

As additional controls to test for memory of the shock context A and that a completely novel safe context can be recognized, we analyzed %freezing during the training session on day 0 and context discrimination in a completely unique context in which the electrified grid floor was concealed by Plexiglas™ on day 1.

Shock Threshold. Footshock perception was assessed using a protocol previously described (Dineley, Xia, Bui, Sweatt, & Zheng, 2002; Hernandez, Kayed, Zheng, Sweatt, & Dineley, 2010). Using Actimetrics FreezeFrame software, a protocol was created to deliver a 1-s foot shock every 30 seconds while the shock amplitude was manually increased by 1/10 mA until flintching, vocalization, and jumping behaviors were observed, then the protocol was terminated. Upon termination, mice were placed back in their home cages and the fear conditioning box was cleaned with 70% ethanol before the next subject was tested.

BrdU Incorporation. All animals tested received a total of five IP injections of 50 mg/kg BrdU over five days, 30 days prior to behavioral testing; animals were aged for an additional 30 days before harvesting tissue. At 60 days post-injection, animals were transcardially perfused first with 30 ml 0.1 M phosphate buffered saline (PBS) then with 4% paraformaldehyde (PFA) in PBS. Brains were harvested, post-fixed overnight in 4% PFA at 4°C then stored in 30% sucrose-

PBS until sectioned. Left hemispheres were sagittal sectioned (30 μm) on a Leica CM3050-S cryostat between interaural coordinates (0.36-2.4mm; Franklin and Paxinos) that represent the entire dorsal dentate gyrus; right hemispheres were used for an alternative study. Serial sections were placed in cryoprotectant (30% ethylene glycol, 30% glycerol, 0.1 M PBS and distilled water) for storage at -20°C.

For BrdU staining, free floating sections were washed with 0.1 M PBS and then treated with 2N HCL for 20 minutes at 60°C for antigen exposure. Next, sections were blocked with 5% normal donkey serum-0.1MPBS-0.1%tween (Sigma-Aldrich 9663) for 1hr at room temperature and then probed in 3%BSA-0.1MPBS-0.1%tween (Sigma-Aldrich A9647) with rat anti-BrdU (1:1,00; Abcam 6326), mouse anti-NeuN (1:700; Millipore MAB377), goat anti-doublecortin (1:250; Santa Cruz sc-8066) and rabbit anti-Ki67 (1:400; Cellsignaling 9129s) overnight at 4°C. Sections were washed 5X with 0.1 M PBS then probed with secondary antibodies diluted in in 3% BSA-0.1MPBS-0.1%tween: anti-rat or anti-rabbit Alexa-Fluor® 488, anti-goat Alexa Fluor® 568, and anti-mouse Alexa Fluor® 647 (ThermoFisher A-21208, A-31571, A-11057, A-21206) for 1 hr at room temperature.

BrdU-positive cells were counted from five serial sections representing the entirety of the dorsal dentate gyrus. According to the coordinates interaural-Lateral 0.36-2.40mm based on Franklin and Paxinos 3rd edition (2007). The entire granule cell layer of each section was counted for cells co-labeled with BrdU and NeuN. Z-series were captured on a Nikon A1 confocal microscope system using the 20x objective magnification. Images were analyzed using ImageJ Software (NIH) and total cell counts were calculated for each animal. To remain unbiased, genotypes were revealed only after all BrdU, doublecortin, and Ki67 counts were totaled.

Statistics. All statistics were performed using GraphPad Prism and SPSS. Analyses for general health, neurological, behavioral, and cognitive performance were reported as mean +/- SEM with 95% confidence intervals, where appropriate. Genotype differences were tested using an unpaired two-tailed t test. The F-test was used to test for equal variance between groups. In all cases but one (Context C testing, Figure 5B), the F test did not fail. Context discrimination ratios were calculated and tested using a one sample t test against chance, a theoretical mean of 0.5. A repeated measures two-way ANOVA using discrimination ratios was used to determine an effect via genotype vs day design. The Levene's test was used to determine equal variances for within subject's design (days). Further, a within-subject repeated measures two-way ANOVA between subjects using raw freezing data was used (total freezing Context A vs total freezing Context B). BrdU counts were totaled from five mice of each genotype from five sagittal sections (every sixth) that represented the lateral extent of the hippocampus. Statistical significance was determined using a p value ≤ 0.05.

RESULTS

The SHIRPA primary behavioral screen scores a variety of general health measures as well as baseline behaviors and reflexes (Rogers et al., 2001). Across 34 metrics evaluated (**Table 1**), we found no significant differences between aged DN-p38$\alpha^{AF/+}$ and wildtype littermates.

Previous studies found significant delay of age-dependent decline of skeletal muscle myofibers and progenitor cells (Papaconstantinou et al., 2009). Therefore, we gauged muscular performance in aged DN-p38$\alpha^{AF/+}$ and wildtype mice using forelimb grip strength testing.

Average grip strength was not statistically significantly different between groups as indicated by the unpaired t test (**Figure 1.0**).

Baseline locomotion and exploratory behavior was comparable between DN-p38$\alpha^{AF/+}$ and wildtype littermates. Neither distance travelled **(Fig. 1.1A)** in peripheral and central arenas nor velocity **(Fig. 1.1B)** was significantly different. This result suggests that suppression of p38 activity has no influence on baseline exploratory or locomotor behaviors. Open field is also a preliminary screen for anxiety-like behavior (Crawley, 1985) and our results indicate that DN-p38$\alpha^{AF/+}$ mice do not differ from wildtype mice in this parameter. However, to investigate this possibility, we performed additional testing using the elevated plus maze.

To further ensure that motivation to explore and anxiety levels were no different between genotypes, each mouse was evaluated in the elevated plus maze. Percent time in the open, closed, and center arenas of the maze as well as total distance and velocity were compared between wildtype and DN-p38$\alpha^{AF/+}$ groups. The time spent in the open, closed, and center arenas of the EPM were no different between groups (**Figure 1.2A**). Similarly, there was no significant difference in total distance traveled (**Figure 1.2B**) and speed while mice explored the maze (**Figure 1.2C**). In summary, DN-p38$\alpha^{AF/+}$ mice displayed no notable differences in general health measures as well as baseline behaviors and reflexes as determined with SHIRPA and they perform indistinguishably from their wildtype littermates in tests for locomotion, exploration, anxiety-like behavior, and grip strength. Thus, attenuation of p38 MAPK activity does not impinge upon the overall health or behavioral phenotype of these mice compared to wildtype littermates.

Based on the notion that DN-p38$\alpha^{AF/+}$ mice are a model for delayed senescence, we wanted to test our cohort in a cognitive task that has been shown to be highly dependent upon

adult neurogenesis. We therefore adopted the context discrimination fear conditioning paradigm (Frankland et al., 1998; Sahay et al., 2011). To validate the protocol, we subjected young (3 months old) wildtype C57Bl/6j mice to context discrimination (**Figure 1.3B**). Young wildtype mice were able to significantly discriminate between contexts on days 3-5 as determined by analyzing %freezing (**Figure 1.3C**) and discrimination indices (**Figure 1.3D**). The discrimination ratio was calculated as the ratio of total freezing in context A over the sum of total freezing in both context A + B and statistically tested against chance (0.5 or 50%). Young C57Bl/6j mice learn to context discriminate by day 3 onward but not on the first two days of testing.

We next tested our cohort of aged wildtype and DN-p38$\alpha^{AF/+}$ mice in the context discrimination fear conditioning paradigm, a hippocampus-dependent memory task that relies upon intact neurogenesis (Gerd Kempermann, 2015). During training in context A on day 0, both wildtype and DN-p38$\alpha^{AF/+}$ displayed equivalent freezing (**Figure 1.4A**). On testing day 1 (**Figure 1.3B**), mice were tested to discriminate a grossly different context, context C, after testing in both contexts A and B. Both groups of mice exhibited similar %freezing in context C (**Figure 1.3B**), and statistically significant discrimination ratios compared to context A on day 1 ($p<0.05$, one sample t test against a theoretical mean = 0.5), data not shown. Repeated measures ANOVA of discrimination ratios did not show a significant effect for day or genotype [$F(5,200)=0.83$; $p=0.52$]. However, repeated measures ANOVA of discrimination ratios in young wild type mice shows significance compared to aged C57Bl6 mice [$F(1,20) = 11.6$] p 0.002; Sidak multiple comparisons revealed significant discrimination on day 5. Further, a within subjects two-way repeated measures using raw freezing data for each aged group showed a significant discrimination on day 3 for DN-p38$\alpha^{AF/+}$, [$F(5,140)=4.0$; $p=0.001$]; Sidak multiple

comparisons reveal significant discrimination on day 3 but no significant discrimination was observed for aged wild type mice [F(5,60)=0.94; p=0.45]. Upon testing for discrimination between context A and context B on days 1-6, aged wildtype mice were only able to discriminate between context A and B on the last day of testing (**Figure 1.4E**). In contrast, DN-p38$\alpha^{AF/+}$ exhibited significant context discrimination on days 3, 5 and 6 (**Figure 1.4F**), suggesting that attenuated p38 kinase activity is protective against age-dependent decline in context discrimination. At the completion of context discrimination testing, all mice were subjected to shock threshold testing for flinching, vocalization, and jumping with no significant differences noted (**Table 2**).

Since it has been established that hippocampal adult born neurons aid performance in context discrimination (Besnard & Sahay, 2016; Frankland, Kohler, & Josselyn, 2013; Lacar, Parylak, Vadodaria, Sarkar, & Gage, 2014; Wojtowicz, 2012), we evaluated the production of adult born neurons in the dentate gyrus of aged wildtype and DN-p38$\alpha^{AF/+}$. By counting BrdU-positive neurons within the granule cell layer in aged wildtype and DN-p38$\alpha^{AF/+}$ 60 days post injection of BrdU we were able to compare how many dentate gyrus neurons were produced and survived within this timeframe. Aged DN-p38$\alpha^{AF/+}$ exhibited significantly higher number of cells dually positive for BrdU and NeuN compared to wildtype littermates (**Figure 1.5**) suggesting that hippocampal neurogenesis is more pronounced in aged DN-p38$\alpha^{AF/+}$. Moreover, DN-p38$\alpha^{AF/+}$ exhibited numbers of immature (DCX-positive) neurons compared to aged wild type mice as indicated by two-tailed unpaired t test [t=5.31 df=8.0; p<0.05] (Figure 7). However, proliferation in hippocampus between young DN-p38$\alpha^{AF/+}$ and wild type mice was not different for BrdU and Ki67 markers, [t=0.68 df=4; p=0.53], and [t=1.17 df=4; p=0.34], respectfully (Figure 8).

In summary, C57bl/6j mice exhibit an age-dependent decline in pattern separation as measured by the context discrimination fear conditioning paradigm in 3 months old versus aged C57bl/6j; DN-p38$\alpha^{AF/+}$ mice are resistant to this age-dependent phenotype. DN-p38$\alpha^{AF/+}$ and wildtype mice froze to the same extent during fear conditioning training, have similar shock thresholds, and are able to discriminate a wholly unique context (context C), suggesting that fear conditioning deficits in aged wildtype mice are specific to context discrimination fear conditioning rather than an inability to perceive the shock or associate it with a particular context. Enhanced context discrimination in DN-p38$\alpha^{AF/+}$ mice was accompanied by enhanced BrdU-positive neurons and immature doublecortin neruons in the dentate gyrus suggesting that enhanced neurogenesis underlies this behavioral phenotype. Furthermore, DN-p38$\alpha^{AF/+}$ do not present with any alterations in baseline general health, neurological function, locomotor exploration, or anxiety-like behavior. Thus, as has been demonstrated with a variety of p38 MAPK manipulations in a range of model systems as well as reported for the DN-p38$\alpha^{AF/+}$ mouse model, attenuation of p38 MAPK activity delays tissue senescence; in this case, decline of adult hippocampal neurogenesis that underlies context discrimination.

FIGURES

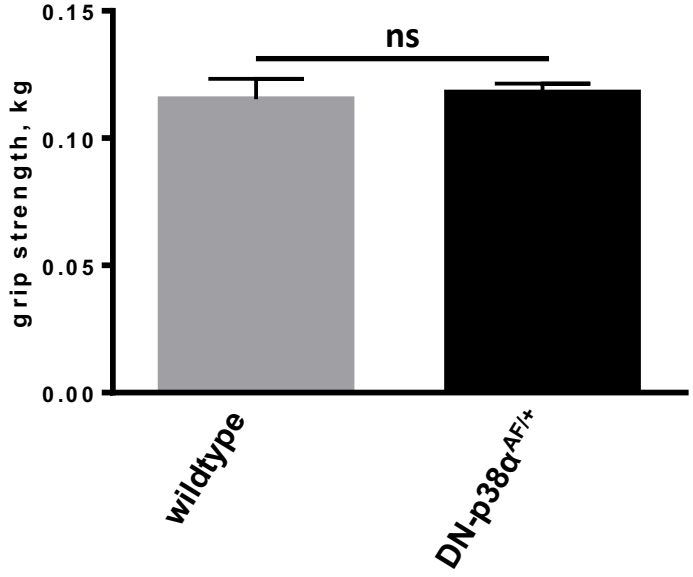

Figure 1.0 ***Equivalent grip strength between aged WT and DN-p38α$^{AF/+}$ mice.*** Two-tailed unpaired t test (p=0.7). ns, not significant.

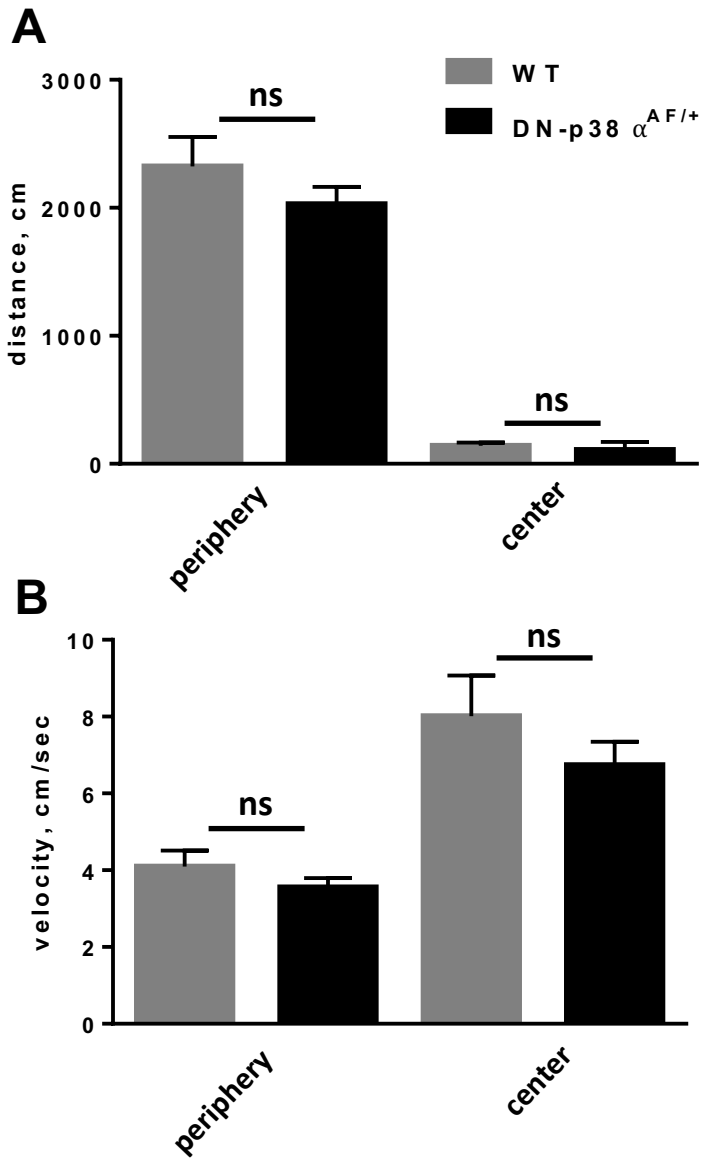

Figure 1.1: ***Equivalent performance in the open field between aged WT and DN-p38α$^{AF/+}$ mice.*** A, Comparison of distance in the periphery and center arenas of the open field are similar between WT and DN-p38α$^{AF/+}$ mice. Two-tailed unpaired t test (p=0.3, 0.2 for the peripheral and center arenas, respectively). Velocity, B, was also comparable. Two-tailed unpaired t test (p=0.2, 0.3 for velocity in the peripheral and center arenas, respectively) ns, not significant.

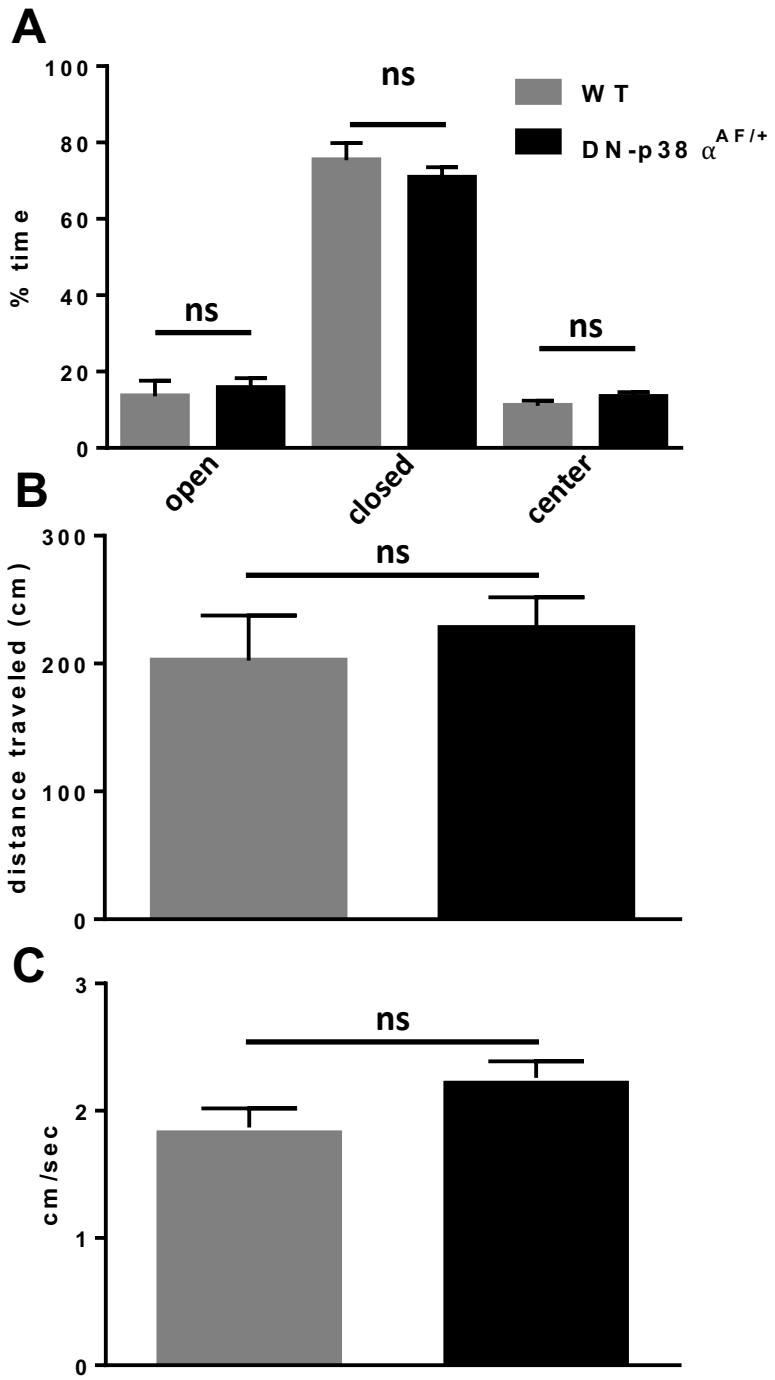

Figure 1.2: ***Equivalent performance in the elevated plus maze between aged WT and DN-p38α$^{AF/+}$ mice.*** A, Comparison of time spent in the open, closed, and center areas of the elevated plus maze are comparable between WT and DN-p38α$^{AF/+}$ mice. Two-tailed unpaired t test (p=0.6, 0.4, 0.2 for open, closed and center arms, respectively). Total distance travelled, B, and velocity, C, are also similar. Two-tailed unpaired t test (p=0.6, 0.07 for distance travelled and velocity, respectively) ns, not significant.

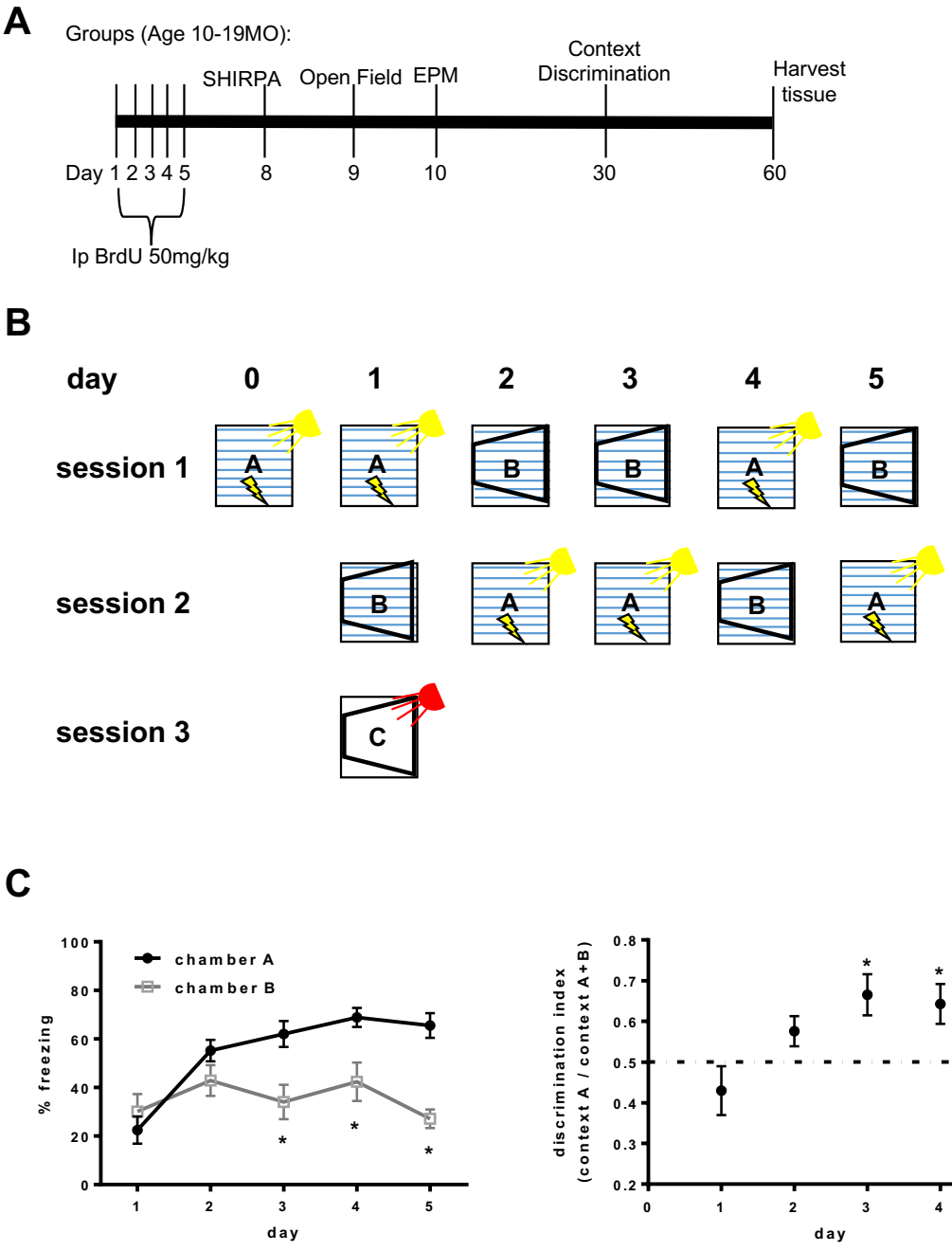

Figure 1.3: ***Context discrimination protocol validation in young C57Bl/6j mice.*** A, Behavioral timeline for aged DN-p38α$^{AF/+}$ and wild type mice. B, For testing, mice are randomly assigned to either context A or context B to measure freezing. On day 1, an additional test is performed in a completely novel context C. C, By day 3, young C57Bl/6j mice freeze significantly less in context B (two-tailed unpaired t test p=0.006, 0.009, <0.0001 for days 3, 4, and 5, respectively) indicative of context discrimination. D, Analysis of discrimination ratios by one sample t test against a theoretical mean of 0.5 revealed significant context discrimination on days 3, 4, and 5 (p=0.01, 0.02, 0.0004, for days 3, 4, and 5, respectively.

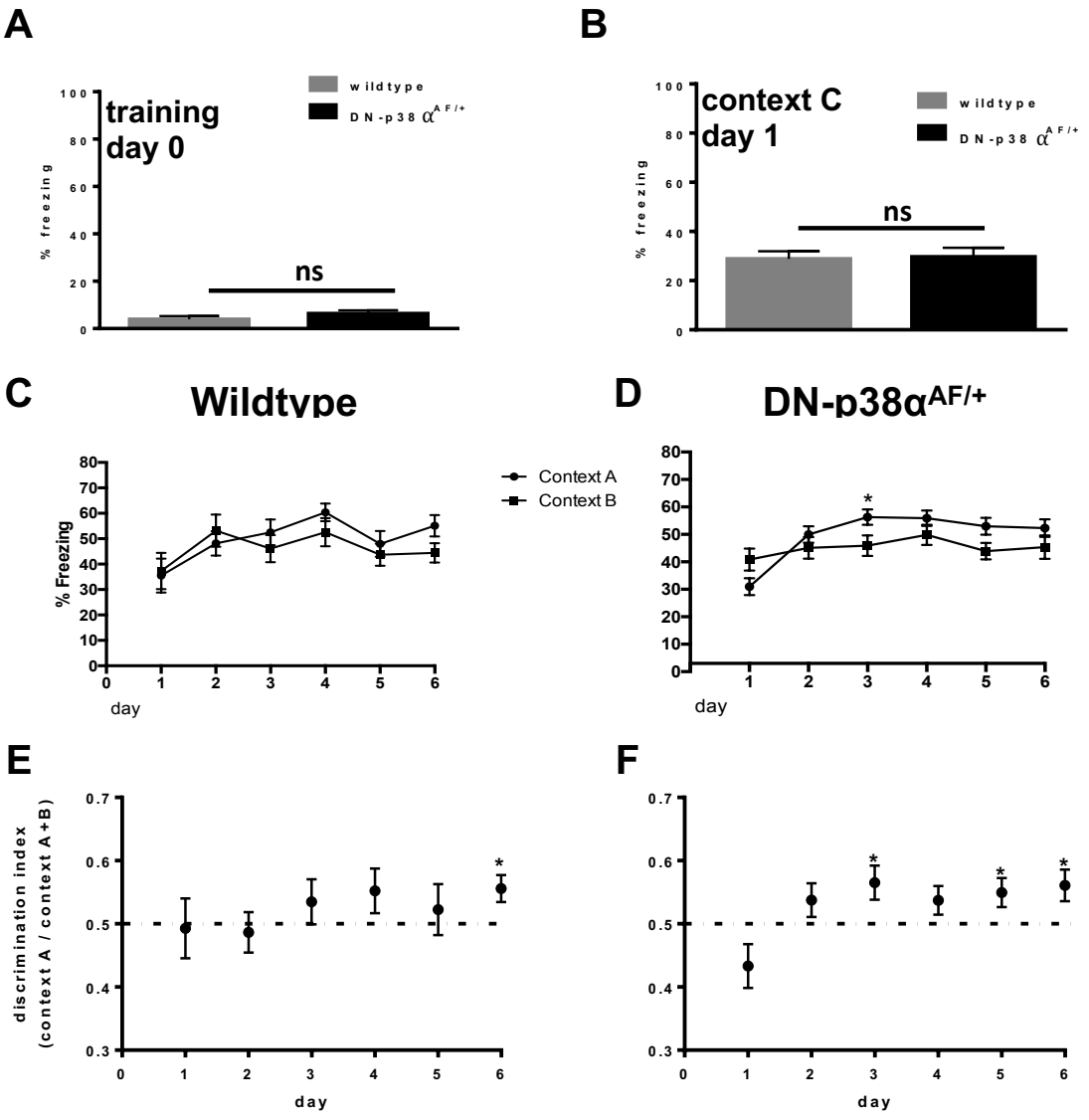

Figure 1.4 ***Enhanced context discrimination in aged DN-p38α$^{AF/+}$ mice.*** **A**, Wildtype and DN-p38α$^{AF/+}$ freeze equivalently during the training session on day 0. Unpaired two-sided t test p=0.3. **B**, Wildtype and DN-p38α$^{AF/+}$ freeze equivalently in a novel context C on day 1 following exposure to contexts A and B. Unpaired two-sided t test with Welch's correction for unequal variance [F(28,12)=3.18], p=0.9. **C** and **D**, two-way RM ANOVA for raw freezing. C, no significant context discrimination in (Context vs. Day) wildtype [F(5,60)=0.9] p=0.4. D, significant discrimination (Context vs Day) Dn-p38α$^{AF/+}$ [F(5,140)=4.0] p=0.001; sidak multiple comparisons reveal significant discrimination on Day3. E, One sample t test against a theoretical mean of 0.5 revealed significant context discrimination in aged wildtype between context A and B only on day 6 (p=0.03). F, DN-p38α$^{AF/+}$ exhibit significant context discrimination on days 3, 5, and 6 (p=0.02, 0.04, and 0.02, respectively). ns, not significant.

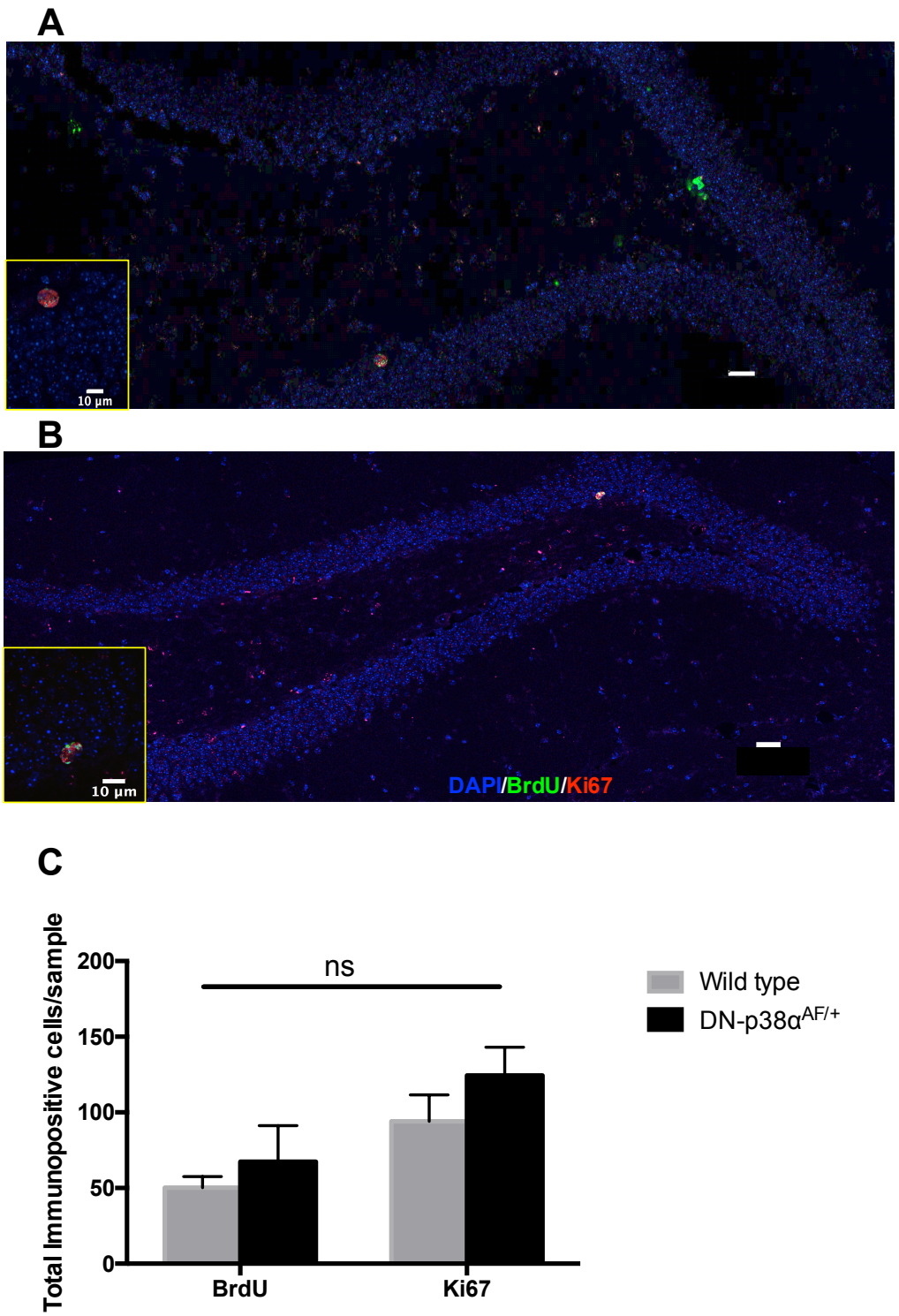

Figure 1.5 ***Dominant negative p38α mutation has no effect on hippocampal adult neuron production in 6MO mice.*** 6MO mice received a single IP BrdU injection 24hrs before brain harvest. **A**, DN-p38α$^{AF/+}$. **B,** WT. sagittal sections were co-labeled with antibodies against BrdU (**green**) and Ki67 (red) then double-labelled cells were counted as described in Methods. **C**, No

significant difference for either BrdU or Ki67 markers between DN-p38α$^{AF/+}$ and wild type mice as indicated by two-tailed unpaired t test [t=0.68, df=4, p=0.53] and [t=1.17 df=4, p=0.3], respectively.

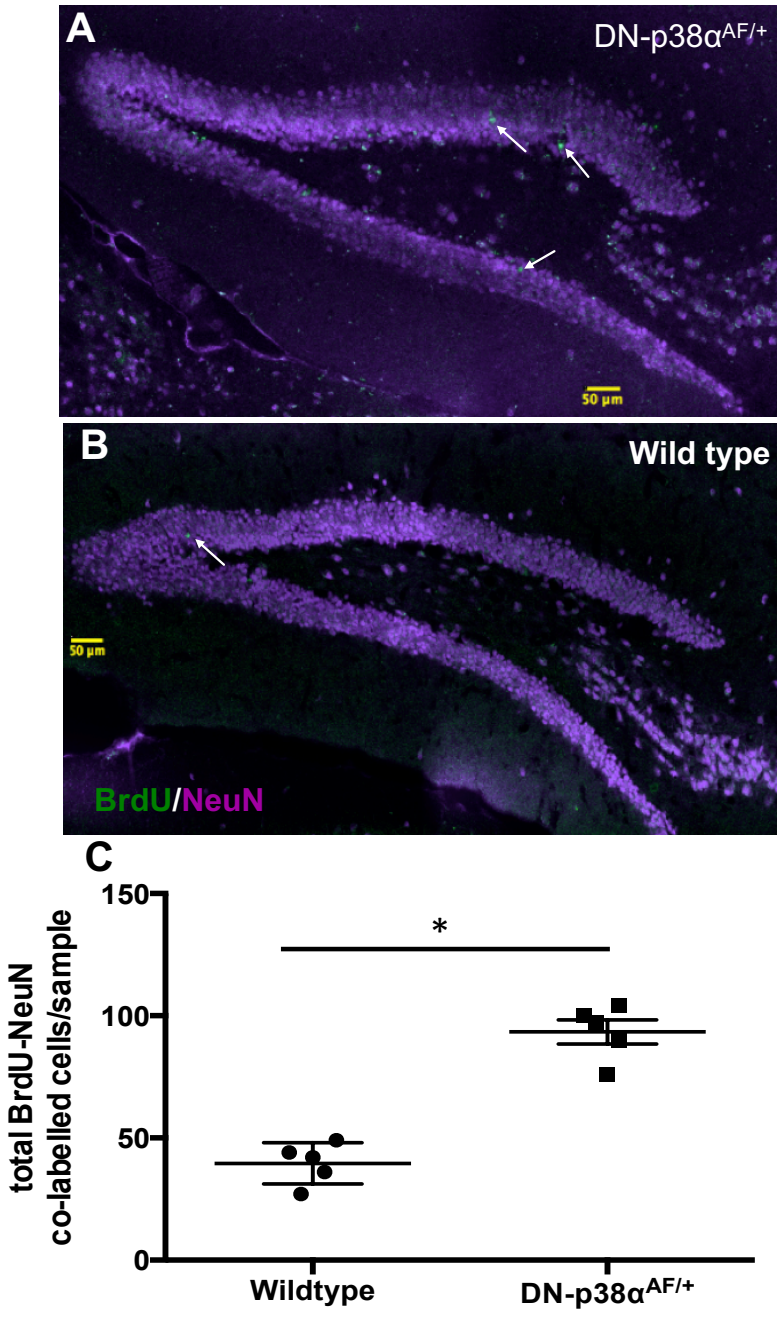

Figure 1.6: ***Enhanced production of BrdU-positive neurons in dentate gyrus of DN-p38$^{\alpha AF/+}$ compared to wildtype mice.*** Sixty days after BrdU injection wildtype, **A**, and DN-p38α$^{AF/+}$, **B**, sagittal sections were co-labeled with antibodies against BrdU (**green**) and NeuN (**purple**) then double-labelled cells were counted. DN-p38α$^{AF/+}$ had a greater number of adult-born neurons in

the granule cell layer of the dentate gyrus than wild type mice, **C**. Two-tailed unpaired t test *p<0.0001).

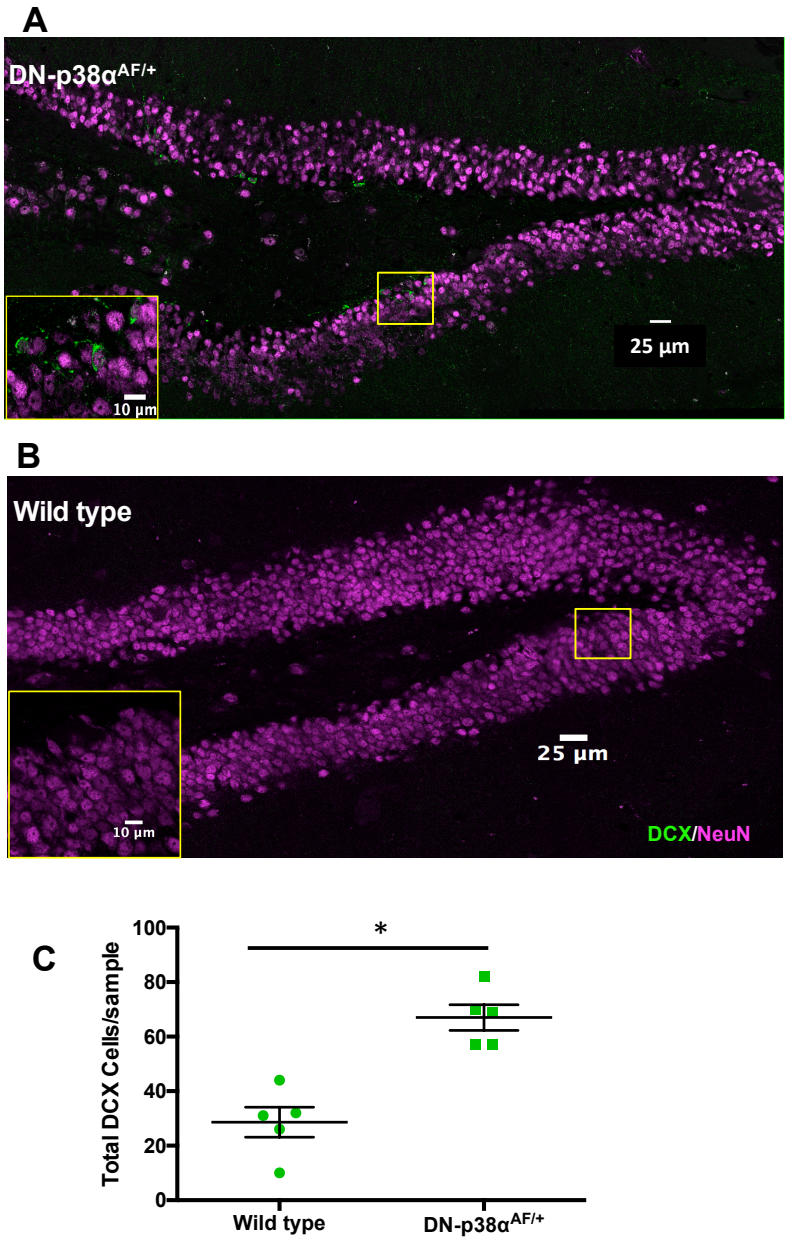

Figure 1.7: Increased *immature neuron pool in aged DN-p38α$^{AF/+}$ mice compared to aged wt mice.* 10-19MO mice received a single IP BrdU injection for five consecutive days, 60 days before brain harvest. Brain sections were co-labeled with antibodies against BrdU (**green**), doublecortin (red), and NeuN (**purple**) then counted. **A**, DN-p38α$^{AF/+}$, and **B**, WT, triple labelling of the dentate gyrus of the hippocampus. **C**, DN-p38α$^{AF/+}$ had a greater number of immature neurons in the dentate gyrus than wild type mice as indicated by a Two-tailed unpaired t test (***p<0.001).

SHIRPA	Wildtype	DN-p38α$^{AF/+}$	Two-tailed
Metric	n=13	n=29	t Test
	Average		p Value
Weight(g)	30.7	32.8	0.07
Body Position	4	4	0.53
Spontaneous Activity	2	1.92	0.5
Respiration Rate	2	2	>0.99
Tremor	2	2	>0.99
Transfer Arousal	5	5	0.21
Palpebral Closure	2	2	>0.99
Piloerection	1	1	>0.99
Gait	3	3	>0.99
Pelvic Elevation	2	2	>0.99

Tail Elevation	1	1	>0.99
Touch Escape	2	2	0.91
Postional Passivity	4	4	>0.99
Trunk Curl	1	1	>0.99
Limb Grasping	1	1	>0.99
Visual Placing	3	3	>0.99
Body Tone	1	1	0.6
Pinna Reflex	1	1	>0.99
Corneal Reflex	1	1	>0.99
Toe Pinch	0	0.96	0.23
Wire Maneuver	4	4	>0.99
Skin Color	2	2	>0.99

Hear Rate	1	1	>0.99
Limb Tone	3	3	>0.99
Abdominal Tone	1	1	>0.99
Lacrimation	1	1	>0.99
Salivation	2	2	>0.99
Provoked Biting	1	1	>0.99
Righting Reflex	3	3	>0.99
Negative Geotaxis	4	4	>0.99
Fear	1	1	>0.99
Irratibility	1	1	>0.99
Aggression	1	1	>0.99
Vocalization	1	1	>0.99

Table 1. ***SHIRPA protocol reveals no phenotypic differences between DN-p38α$^{AF/+}$ and wildtype mice.*** Normal behavior was observed in all mice tested as indicated by the following arbitrary scales for each SHIRPA metric: **Body Position**, 0=Completely flat, 1=Lying on side, 2=Lying prone, 3=Sitting or Standing, 4=Rearing on hind legs, 5=Repeated vertical leaping; **Spontaneous Activity** 0=None,resting, 1=Casual scratch, groom, slow movement, 2=Vigours scratch, groom, moderate movement, 3=Vigorous, rapid/dart movement, 4=Extremely vigorous, rapid/dart movement; **Respiration Rate** 0=Gasping, irregular, 1=Slow, shallow, 2=Normal, 3=Hyperventilation; Tremor 0=Important, 1=Mild, 2=None; Transfer Arousal 0=Coma, 1=Prolonged freeze, then slight movement**,** 2=Extended freeze, then slight movement, 3=Brief freeze (few seconds), then active movement, 4=Momentary freeze, then swift movement, 5=No freeze, immediate, 6=Extremely excited; **Palpebral Closure** 0=Eyes closed, 1=Eyes half closed, 2=Eyes wide open; **Piloerection** 0=Coat stood on end, 1=None; **Gait** 0=Incapacity, 1=Limited

movement only, 2=Fluid but abdnormal, 3=Normal; **Pelvic Elevation** 0=Markedly flattened, 1=Barely touches, 2=Normal, 3=Elevated; **Tail Elecation** 0=Dragging, 1=Horizontally extended, 2=Elevated/straub tail; **Touch Escape** 0=No response, 1=Mild, 2=Moderate, 3=Vigorous; **Positional Passivity** 4=Struggles when held by tail, 3=Struggles when held by neck, 2=Struggles when laid supine, 1=Struggles when held by hind legs, 0=No struggle; **Trunk Curl** 0=Absent, 1=Present; **Limb Grasping** 0=Absent, 1=Present; **Visual Placing** 0=None, 1=Upon nose contact, 2=Upon vibrasse contact, 3=Before vibrasse contact, 4=Early vigorous extension; **Body Tone** 0=Flaccid, no return of cavity to normal, 1=Slight resistance, 2=Extreme resistance, board like; **Pinna Reflex** 0=None, 1=Active retraction, moderately brisk flick, 2=Hyperactive, repetitive flick; **Corneal Reflex** 0=None, 1=Active single eye blink, 2=Multiple eye blink; **Toe Pinch** 0=None, 1=Slight withdrawal, 2-Moderate withdrawal, 3=Brisk, rapid withdrawal, 4=Very brisk repeated extension and flexion; **Wire Maneuver** 0=Falls immediately, 1=Unable to lift hindlegs, falls within seconds, 2=Unable to grasp with hindlegs, 3=Difficulty to grasp with hinlegs 4=Active grip with hindlegs; **Skin Color** 0=Blanched, 1=Pink, 2=Bright, deep red flush; **Heart Rate** 0=Slow, bradycardia, 1=Normal, 2=Fast, tachycardia; **Limb Tone** 0=No resistance, 1=Slight resistance 2=Moderate resistance, 3=Marked resistance, 4=Extreme resistance; **Abdominal Tone** 0=Flaccid, no return of cavity to normal, 1=Slight resistance, 2=Extreme resistance, board like; **Lacrimation** 0=Present, 1=None; **Salivation** 0=Wet zone entire sub-maxillary area, 1=Slight margin of sub-maxillary area, 2=None; **Provoked Biting** 0=Present, 1=Absent; **Righting Reflex** 0=Fails to right when placed on back, 1=Lands on back, 2=Lands on side, 3=No impairment; **Contact Righting Reflex** 0=Absent, 1=Present; **Negative Geotaxis** 0=Falls off, 1=Does not move within 3- seconds, 2=Moves, but fails to turn, 3=Turns but then freezes, 4=Turns and climbs the grid; **Fear** 0=Freezes during transfer arousal, 1=None; **Irritability** 0=Freezes during transfer, 1=Struggle during supine restraint; **Aggression** 0=None, 1=Provoked biting or attack, **Vocalization** 0=None, 1=Provoked during handling.

Response	DN-p38α$^{AF/+}$	Wildtype	Student's t Test
to Footshock	Average Amplitude(mA)		p Value
Jump	0.49	0.47	0.84
Vocalization	0.34	0.33	0.69
Flinch	0.18	0.15	0.15

Table 2. Footshock perception equivalent in aged DN-p38α$^{AF/+}$ and wildtype mice. Footshock threshold was employed to determine shock intensity that provoked jumping, vocalization and flinching behaviors. Average amplitude for all behavior was not statistically different between groups as indicated by Student's t test.

DISCUSSION

Aging is the primary risk factor for all neurodegenerative diseases and other leading causes of death. Based on the early observation of aberrant MAPK signaling (e.g., ERK, p38) in human AD brains and AD mouse models (Dineley et al., 2001; Dineley et al., 2002; Hensley et al., 1999; Hyman, Elvhage, & Reiter, 1994; Savage, Lin, Ciallella, Flood, & Scott, 2002; Zhu et al., 2000), aberrant MAPK activity has long been thought to be a significant contributor to AD. Furthermore, the age-dependent induction of CNS p38α activity that drives stress signals to promote neuroinflammation and cognitive impairment in AD and the converse outcome of acute inhibition of p38α activity that improved spatial memory in AD mouse models (Ashabi, Alamdary, Ramin, & Khodagholi, 2013; Ashabi et al., 2012) has driven recent pursuits of p38α inhibitors as a therapeutic strategy (Bachstetter & Van Eldik, 2010; Munoz & Ammit, 2010; Pinsetta et al., 2014).

DN-p38α$^{AF/+}$ mice express a mutant form of p38α in which Thr180 and Tyr182 are mutated (T→A/Y→F) to prevent phosphorylation activation of p38α and exhibit resistance to age-dependent decline in the regenerative potential of liver, pancreatic islet, and skeletal muscle tissues (Papaconstantinou et al., 2009, 2015; Wong et al., 2009). To determine if reduced p38α MAPK activity affected a cognitive metric associated with adult neurogenesis, we aged heterozygous DNp38α$^{AF/+}$ mutant and wildtype mice then tested our cohorts in the context discrimination fear conditioning paradigm. Control studies were included to determine whether general health, neurological function, locomotor exploration, or anxiety-like behavior were affected. Finally, BrdU injections and immunofluorescence imaging was employed to quantify adult-born neuronal maturation and survival.

Here, we demonstrated that aged DN-p38α$^{AF/+}$ mice appear to have no behavioral or gross neurological abnormalities as they perform comparably to aged wildtype mice in the SHIRPA primary screen for general health, baseline behaviors and reflexes. Although, aged DN-p38α$^{AF/+}$ showed higher hind limb gastrocnemius density (Papaconstantinou et al., 2009), we found no significant difference in forelimb grip strength between aged DN-p38α$^{AF/+}$ and wildtype mice. This is somewhat surprising given that previous studies found delayed loss of age-dependent gastrocnemius muscle mass (Papaconstantinou et al. 2009). Perhaps, forelimb muscle mass is not affected to the same extent as hind limb gastrocnemius.

Further, DN-p38α$^{AF/+}$ mice appeared to have similar motivation to explore open field and elevated plus maze while showing no difference in control metrics like speed and distance traveled. Since aged DN-p38α$^{AF/+}$ mice appear to have no baseline behavioral or neurological deficits, we tested cognition in a context fear-discrimination task. Context discrimination is a hippocampus-dependent task that challenges an animal's ability to distinguish contextual cues in

similar contexts. Several studies have demonstrated that increasing production of adult born neurons in the hippocampus; that is increasing adult neurogenesis levels improves performance in context discrimination while decreasing neurogenesis has the opposite effect (Clemenson et al., 2015; D. J. Creer, C. Romberg, L. M. Saksida, H. van Praag, & T. J. Bussey, 2010; Danielson et al., 2016; Frankland et al., 1998; Merritt & Rhodes, 2015; Sahay et al., 2011). Young neurons are more sensitive to perforant pathway input necessary for driving the encoding of new contexts (Frankland et al., 2013; Lacar et al., 2014; Wojtowicz, 2012). Previous work showed that nearly complete inhibition of p38α activity in CA1 abolished context discrimination in C57Bl6 mice (S. X. Jin, Arai, Tian, Kumar-Singh, & Feig, 2013), whereas our data showed that partial reduction of p38 activity enhanced context discrimination. While this seems contradictory, we would argue that extensive inhibition of CA1 p38 is interfering with the consolidation of memory trace required for context discrimination and that the partial activity remaining in DN-p38$\alpha^{AF/+}$ mice is sufficient to complete the hippocampal tri-synaptic circuit and preserve context discrimination. Analyzing hippocampal proliferation markers, BrdU and Ki67 in young DN-p38<alpha> and wild type mice revealed no significant difference between either group. However, DN-p38<alpha>AF/+ maintained immature neuronal pools while also showing higher retention of new born neurons compared to aged wild type mice. Together, this data reveals attenuation of p38 activity preserves neurogenesis mechanisms with age. Similarly, interventions slowing age dependent decline of adult neurogenesis show benefit in a spatial navigation task ((H. van Praag et al., 2005).

Thus, our finding that aged DN-p38$\alpha^{AF/+}$ mice produce more adult-born neurons than wildtype littermates suggests that p38α negatively contributes to age-dependent decline in neurogenesis that underlies the age-dependent decline in context discrimination.

Our study is the first to demonstrate that *in vivo* attenuation of p38α activity slows age-associated decline in dentate gyrus neurogenesis and associated cognitive function. Overall, our findings suggest that the p38α MAPK pathway plays a role in the decline of cognition with age, and potentially contributing to age-dependent neurodegenerative disease.

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Chapter 2

Profiling hippocampal dependent cognition for Rosiglitazone treated 9-month old Tg2576(Swe-APP) mouse model of Alzheimer's Disease

ABSTRACT

Introduction: Insulin resistance in type 2 diabetic patients has been shown to be a significant risk factor for developing dementia due to Alzheimer' disease. Finding therapies to reverse specific memory deficits in at risk populations for Alzheimer's disease could improve clinical trial success for drug therapies. In this study, we treated the Alzheimer's disease mouse model Tg2576 with at mid-age with the PPARγ agonist rosiglitazone to profile hippocampal memory deficits that are reversible at this age.

Methods: Three cohorts containing Tg2576 mice and wild type littermates were aged to 8-months of age and treated with either control or 30mg/kg rosiglitazone food for 30 days. Behavioral tests Novel Object Recognition, Morris water maze, foreground fear conditioning and context fear discrimination were administered at 9-month of age.

Results: Tg2576 mice treated with RSG show improved spatial learning and reference memory compared to untreated Tg2576 subjects though long-term memory deficits in object recognition were not altered. In a separated cohort, Tg2576 and aged match wild type mice performed comparably in classical fear conditioning. In the last cohort, RSG treatment did not rescue context fear discrimination for Tg2576 and age matched control groups.

Conclusion: Together, the results in this study elaborate on the deficits that arise from over expression of amyloid beta in 9-month old Tg2576 model of Alzheimer's Disease. Moreover, these findings demonstrate a narrow the range of cognitive deficits that are reversed with PPARγ agonism by way of RSG treatment.

INTRODUCTION

Alzheimer's disease, the most common form of dementia, is a progressive neurodegenerative disease that is characterized by progressive memory deficits, brain atrophy and presence of misfolded proteins: amyloid beta plaques and tau fibrillary tangles in the brain(Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). To date, Alzheimer's disease affects over 5 million people will cost $259 billion in health care this year in the United States(Association, 2017). Though, a majority of these patients do not have genetic link to the disease suggesting environmental risk factors play a strong role in Alzheimer's disease. One risk factor in particular, type 2 diabetes, has been shown to exhibit pathologies similar to Alzheimer's disease (Jayaraman & Pike, 2014). Interestingly, the risk of developing Alzheimer's disease significantly increases in patients with type-2 diabetes(Cheng, Huang, Deng, & Wang, 2012; Janson et al., 2004; Luchsinger, Tang, Shea, & Mayeux, 2004; Willette et al., 2015). It has since been revealed that impaired insulin signaling affects mechanisms in the brain such as: synaptic plasticity, spatial memory and neuro-inflammation (Blazquez, Velazquez, Hurtado-Carneiro, & Ruiz-Albusac, 2014; Srodulski et al., 2014). Similarly, Alzheimer's pathologies arise from a disruption in amyloid beta processing that triggers neuro-inflammation, synaptic degradation, and tau phosphorylation that leads to neuronal cell death and ultimately memory impairment (Jack et al., 2010). Further, in the brains of AD patients, insulin receptor and its active state are significantly decreased in the

hippocampus suggesting insulin resistance may coincide with cognitive impairment in Alzheimer's disease (Bedse, Di Domenico, Serviddio, & Cassano, 2015; Watson & Craft, 2003). Diabetic drugs, like thiazolidinediones (TZD) that improve glucose metabolism in patients not responding to insulin therapy, have also been shown to improve memory in preclinical AD models and patients with Mild cognitive impairment (MCI)(Watson et al., 2005). The target, a nuclear transcription factor, (Peroxisome Proliferative-Activated Receptor gamma) PPARγ is modulated by this class of drugs to promote insulin sensitivity. In our hands, the TZD rosiglitazone (RSG) improves hippocampal dependent memory and neuronal transmission between the entorhinal cortex and dentate gyrus(Nenov, Laezza, et al., 2014; Rodriguez-Rivera, Denner, & Dineley, 2011). Transcriptional RNA sequencing data reveals a direct link between PPARγ and MAPK/ERK singling. More recently, (Jahrling et al., 2014), our lab provided support for this notion by demonstrating a relationship between PPARγ and phospho-ERK during memory consolidation *in vivo* and with *in vitro* studies(Denner et al., 2012). Our lab has demonstrated a relationship between PPARγ and phospho-ERK that appears to be important for rescuing hippocampal dependent learning and memory in AD mice however, memory deficits in Alzheimer's disease are diverse and thus further investigation into whether RSG benefits extend to spatial, discrimination and recognition learning and memory is obligatory.

The Tg2576 AD mouse model is ideal to study progressive cognitive impairment as a result of amyloid beta overexpression. The Tg2576 (APP-Swe) Alzheimer's disease mouse model harbors the human Swedish mutation KM670/671NL to Amyloid Precursor Protein. The overexpression of amyloid beta protein leads to progressive accumulation of amyloid beta plaques and memory deficits throughout its life (Hsiao et al., 1996). In fact, at 5 months Tg2576 exhibit synaptic loss, deficits in contextual fear conditioning, object recognition and spatial navigational memories

while amyloid beta plaques are not observable until 11 months of age(Jacobsen et al., 2006). Since we have previously shown that 4 weeks of RSG can rescue contextual associative memory and neural circuitry in the presence of heightened amyloid beta levels at 9 months of age; here, we asked if RSG can rescue spatial navigation, contextual discrimination and object recognition learning and memory at this age. Briefly, these tasks are hippocampal dependent however, we don't know if RSG can affect a wide range of hippocampal dependent learning and memory. To answer this question, we aged Tg2576 male and female mice to 8 months of age at which we supplemented their food with either RSG milled or control chow for 30 days. We later administered object recognition and Morris water maze assays. In similar conditions, a separate cohort of 9-month old Tg2576 mice underwent context discrimination training and testing. Lastly, in a separate cohort, 9-month old Tg2576 mice were subjected to foreground fear conditioning paradigm. Here we show that RSG treatment improved spatial navigation but not object recognition memory. Further, we demonstrate that Tg2576 mice do not appear to have deficits in foreground compared to age-matched wild type controls. This result led us to pursue a similar memory task in context fear discrimination which showed RSG does improve not context discrimination. The research presented here demonstrates that RSG can improve navigational learning and memory at 9 months of age however, RSG treatment did not rescue object recognition memory and context discrimination from overexpression of amyloid beta. Interestingly, at this age, loss of synapses concomitantly with accumulation of amyloid beta does not diminish performance in foreground fear conditioning. This study contributes to the body of research focusing on the cognitive benefits of PPARγ agonism in a transgenic model of Alzheimer's Disease.

MATERIALS AND METHODS

Animals

Animals were bred by mating heterozygous Tg2576 males with C57Bl6/SJL (F1) females (Jackson Laboratory). The University of Texas Medical Branch operates in compliance with the United States Department of Agriculture Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and IACUC approved protocols. Animals were randomly assigned to experimental groups such that each group contained roughly equivalent numbers of males and females, wild type and transgenic. Some animals expired during the study unrelated to treatment. Experimenters were blinded to treatment and genotype during key data acquisition and analysis steps. For Morris water maze, data was collected from two cohorts of animals; for object recognition, three cohorts were used.

Insulin Sensitizer Treatment

Four cohorts containing male and female 8MO Tg2576 and wildtype (WT) littermates were randomly assigned to groups fed control (CTRL) or 30mg/kg RSG diet (Bio-Serve) for 30 days, as previously described (Denner et al., 2012; Rodriguez-Rivera, Denner, & Dineley, 2011).

Water Maze

Two cohorts of mice were tested for spatial navigation learning and memory. Four groups (up to 28 mice/group) consisting of Tg2576 and wildtype littermates either untreated or treated with RSG for one month. Water maze testing was conducted in a circular pool (dimensions: 1 m diameter, 75 cm height; water height = 36 cm) made of white, opaque plastic (Coulbourn Instruments; Holliston, MA). Water was made opaque by the addition of nontoxic white tempera paint (16 oz

per fill) and maintained at 25°C ± 2.0°C. Extramaze cues (high contrast geometric images) were adhered to black non-reflective curtains to hide the experimenter. The pool was divided into 4 quadrants, designated NE, NW, SE AND SW, and 4 release points were centered between quadrants (N, S, E and W). The pool was sanitized and water refreshed daily prior to each testing session. Visible Platform Task: The pool was filled to the surface of a 35 cm high visible platform (square, 14 cm^2). The platform was flagged with a dark plastic block (3x3x9-cm) to ensure visibility. Daily visible platform training blocks (4 trials/day) were conducted for 3 consecutive days for cohort 1 and 1 day for cohort 2 since 1 day of visible platform training proved to be sufficient. For each trial, the platform locations (NE, NW, SE AND SW) and release points (centered between quadrants, N, S, E, W) were pseudo-randomized for each trial. The mouse was allowed 60 sec to locate the platform. If unsuccessful within the allotted time period, the mouse was directed towards the platform using a dark narrow rod and allowed a 30 sec rest period before the next trial. Following the 4^{th} trial, the mouse was retrieved from the platform and placed on a warming blanket until dry then replaced to its home cage. Hidden Platform Task: On the day following visible platform, daily hidden platform training blocks (4 trials/day) were conducted for 9 consecutive days. In contrast to visible platform tests, the platform location was stationary and centered within the SE quadrant and hidden from visibility by submersion ~1 cm below the water line. Mice were released from points centered at quadrant walls, excluding the SE quadrant with the hidden platform. Each mouse had a single training block per day. A training block was defined as 4 consecutive trials and the inter-trial interval was 30 sec. For each trial, the mouse was allowed to swim a maximum of 60 sec to locate the platform. When successful, the mouse was allowed a 30-sec rest period on the platform. If unsuccessful, the mouse was directed towards the platform using a dark narrow rod, allowed a 30-sec rest period.

Probe trials: At the beginning of the 4th and 7th day of hidden platform training and on day 10, a probe trial was conducted to measure spatial memory for the platform location in the target quadrant (SE). The platform was removed and mice were allowed to search for the platform for 60 sec.

Water maze exclusion criteria: An animal was removed from the study if it did not follow the rod to the platform or locate the platform. An animal was also removed if it did not exhibit appropriate search behavior (e.g., no swimming activity) or thygmotaxis.

Water maze data analysis: Swimming activity of each mouse was monitored via a Panasonic BP344 camera mounted directly above the pool. Watermaze3 video-tracking software (Coulbourn Instruments) was utilized to record, analyze and export platform search parameters: latency (sec), distance traveled (cm) and swim speed (cm/sec), as well as the percentage of total time spent in the target quadrant for a probe trial. An assessment of spatial bias was accomplished by measuring the percentage of time spent in the target quadrant and performing one-sided Student's t-test against a theoretical mean of 25%. A one- or two-way analysis of variance (ANOVA), with repeated measures when indicated, was used to compare water maze performance (latency, distance, swim speed and percent time in target quadrant) between genotypes and treatments. Tukey post hoc analysis was used to perform pairwise analyses; $p \leq 0.05$ was considered significant.

Novel object discrimination

Each group consisted of 15-25 mice/group to assess object recognition. Four groups consisting of Tg2576 and wildtype littermates either untreated or treated with RSG for one month. Based on our previously published protocol (Taglialatela, Hogan, Zhang, & Dineley, 2009) we tested whether mice were able to discriminate between a familiar and novel object using a 4-hour inter-

trial interval. Object discrimination data analysis: To assess object recognition memory, a discrimination ratio (novel object interaction time/total interaction time) for each mouse was calculated and analyzed. A one-sided Student's t test against a theoretical mean of 0.5 was used to test for novel object preference; $p \leq 0.05$ was considered significant.

Foreground fear conditioning

In a separate cohort containing 15-16 mice per group, 9MO wild type and Tg2576 subjects were placed in a fear conditioning box (Lafayette Instruments; Lafayette, IN) and allowed to explore for the first 4 minutes of training. At minute 4 and 5, a 2sec 0.5mA foot-shock was delivered from the metal floor grid. Mice were allowed to explore the training context for additional 2 minutes and then placed back in their home cages. Contextual testing began 24 hours later where trained subjects were placed back in aversive environment but not administered a foot shock for the entire 5 minutes of testing. Total freezing was measured using recordings and FreezeFrame software (Actimetrics; Wilmette, IL). Motion indices were set according to individual freezing observed by experimenter blinded to genotypes and sex.

Context Fear Discrimination

A separate cohort that included 15-20mice/group of 9MO Tg2576 and age matched wild type littermates were randomly trained and tested in context fear discrimination adapted from (Frankland, 1998). Refer to (Cortez et al., 2017) for material and method details.

RESULTS

Morris water maze. We previously showed that one-month PPARγ agonism with RSG improves hippocampus-dependent associative learning and memory in 9MO Tg2576 as measured with the contextual fear conditioning paradigm (Denner et al., 2012; Jahrling et al., 2014; Rodriguez-Rivera et al., 2011). We therefore tested two cohorts of Tg2576 and WT littermates in the Morris water maze (R. Morris, 1984; R. G. Morris et al., 1982). After visible platform training, mice were subject to four daily trials to reach the hidden platform over nine consecutive days. During this training, the mice exhibited variable swim speeds (Figure 1A), therefore, distance to the platform was used to test for spatial learning. Two-way repeated measures ANOVA indicated that selective groups improved during the training in that there was a group and day effect but no interaction **(Figure 2.0A).** Untreated Tg2576 had impaired learning to spatially navigate the location of the hidden escape platform as indicated by the significantly longer distance to reach the escape platform on day 3 and days 5-9. Tg2576 treated with RSG performed statistically equivalent to WT groups on days 1-9 indicating that Tg2576 undergoing PPARγ agonism treatment were now able to learn to spatially navigate to the escape platform. This is further supported by visual inspection of escape paths representative of each group (Figure 1B). Whereas untreated Tg2576 display what can be interpreted as a random circular search strategy; all other groups, including RSG-treated Tg2576, appear to triangulate their position relative to the spatial cues placed external to the pool in order to find the hidden platform. Intermingled within the 9 days of hidden platform training, mice were three probe trials (days 4, 7, 10) in which the mice were placed in the pool and allowed to swim for 60 sec; %time in each of four virtual quadrants was quantified. Two-way ANOVA detected a significant interaction and treatment effect **(Figure 2.0C).** One sample t test against a theoretical

mean of 25% did not detect that Tg2576 swam in the target quadrant preferentially during any of the probe trials. However, RSG treated Tg2576 swam in the target quadrant significantly more than chance level for all probe trials. By trial 2, Tukey post hoc analysis detected that RSG treated Tg2576 performed significantly better than untreated Tg2576, indicating enhanced spatial memory. WT groups, untreated and treated, swam preferentially in the target quadrant as well and performed significantly better than untreated Tg2576. Thus, PPARγ agonism not only enhances hippocampus-dependent associative learning and memory (Denner et al., 2012) but also improves spatial learning and memory.

Novel Object Recognition. We previously demonstrated that Tg2576 exhibit object recognition deficits when we tested intermediate and long-term memory by using a 4 and 24-hour retention interval; the interval between exploring two identical objects and when one of them was replaced with a novel object (Taglialatela, Hogan, Zhang, & Dineley, 2009). At 5MO of age, these deficits were ameliorated when the animals were acutely treated with the CaN inhibitor, FK506. FK506 also reversed 5MO Tg2576 hippocampus-dependent associative learning deficits as measured in the contextual fear conditioning task (Taglialatela, Hogan, Zhang, & Dineley, 2009). Since CaN inhibition did not enhance contextual fear conditioning at 9MO (data not shown) but PPARγ agonism did (Denner et al., 2012) we tested the hypothesis that RSG would ameliorate object recognition deficits in 9MO Tg2576. Object discrimination ratios revealed wild type control and RSG treated subjects explored the novel object more than the familiar object, 4 hours after training as expected. However, both untreated and RSG treated Tg2576 mice explored the familiar and novel object equally during testing indicating PPARγ agonism does not rescue deficits in novel object recognition **(Figure 2.1)**.

Foreground Fear Conditioning. We next set out to determine if 9-month old Tg2576 mice develop deficits in foreground fear conditioning paradigm. Previous work in our lab utilized background-delayed fear conditioning to reveal deficits in Tg2576 model of Alzheimer's Disease. Lesions to the dorsal hippocampus produce deficits to background but not foreground fear conditioning(Frankland, 1998; Odling-Smee, 1978). In this study we asked whether 9-month Tg2576 mice exhibit similar phenotype to animals with dorsal hippocampus lesions. Here we used a similar paradigm, though the tone was absent when the two 2-sec foot shocks at 0.5mA were delivered at minutes four and five. Contextual testing was administered 24 hours later, where subjects were placed back in the training environment but not exposed to foot shocks. As expected, Tg2576 and age matched controls explored the training context equally which decreased with each foot shock (**Figure 2.2A)**. In contextual testing, Tg2576 mice explored the same training context equally to age matched controls confirming Tg2576 do not exhibit contextual deficits at this age in foreground fear conditioning (**Figure 2.2B).**

Context Fear Discrimination. Since it appears 9-month old can associate foot-shocks with context in foreground fear conditioning; we next asked if RSG treated and control groups adapt to contextual fear discrimination paradigm. To determine if RSG has an effect in adult hippocampal neurogenesis dependent behavior; we challenged RSG treated and control Tg2576 groups in context fear discrimination paradigm. Recently, research into the role adult hippocampal neurogenesis plays in cognition showed that stimulation with exercise can rescue deficits while genetic knock down of adult hippocampal neurogenesis impairs their ability to discriminate between highly similar contexts (van Praag, Christie, Sejnowski, & Gage, 1999; (Sahay et al., 2011)). In addition, a report from (Frankland, 1998; H. van Praag, Christie, Sejnowski, & Gage, 1999) demonstrated that the dorsal hippocampal lesions impair a rat's ability to discriminate

between an aversive context and highly similar safe context. Since contextual deficits are demonstrable in 9-month old Tg2576 mice and RSG treatment can reverse this pathology, we asked the question: can RSG treatment rescue a similar but more challenging training paradigm that appears to be dependent on adult hippocampal neurogenesis in the dorsal hippocampus. Briefly, Tg2576 mice were placed into context A on training day 0 and allowed to freely explore for 298sec at which point a 2sec 0.75mA footshock was delivered, followed training ended 15sec later. For the next 6 days, subjects were randomly presented with context A and the highly similar context B where cardboard inserts and a vanilla scent were added however, the most salient cue being the metal grid floor remained exposed. Here, control and RSG groups wild type groups exhibited generalized fear in both contexts for the first three days **Figure 2.3A, B**. Though on day 4, both groups explored the safe context B more than the shock context A. Particularly, wild type RSG group continued this trend on day 5 while control group was unable to discriminate on the final day of testing. Further, both control and RSG Tg2576 groups significantly discriminated on days 5 and 6, one day after wild type groups (**Figure 2.3C, D)**. Discrimination ratios revealed there was no significant difference between groups throughout testing (**Figure 2.3E)**. To demonstrate there was no altered baseline exploratory behavior in Tg2576 and RSG treatment groups, we compared total freezing in training context and completely dissimilar context C after discrimination testing on testing day one (**Figure 2.3F, G)**. As expected, there was no difference in total freezing among groups during initial training and when mice were exposed to a context in which the metal grid was masked with a plastic insert.

FIGURES

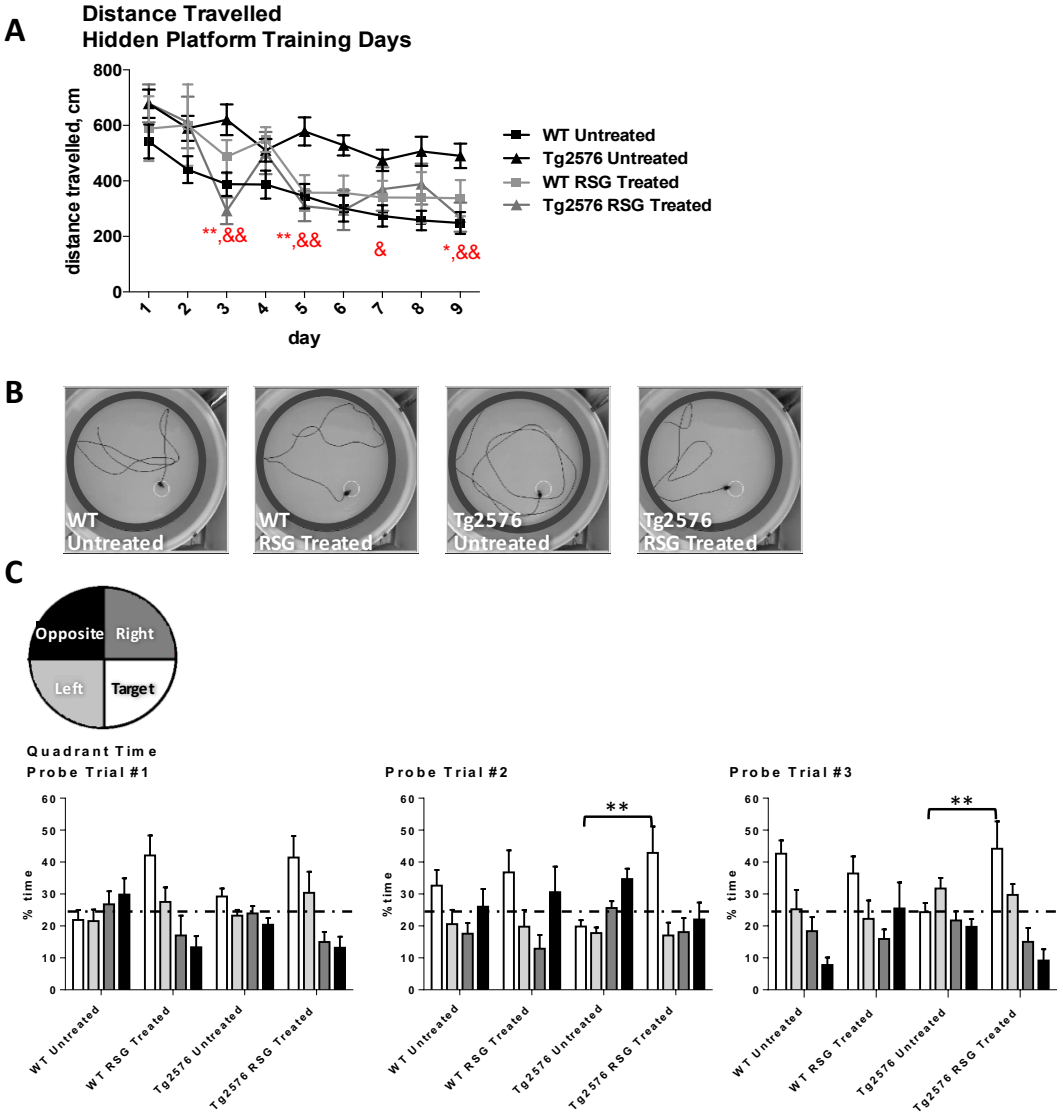


Figure 2.0: PPARγ agonism restores navigational learning and memory in Tg2576. **A.** Spatial navigation memory was assessed using Morris water maze task. **A.** Distance traveled to hidden platform was significantly longer in untreated Tg276 mice whereas RSG treated AD and wild type mice performed comparably during hidden platform trial training (Group F(3, 64)=10.82; p value<0.0001). **B.** Representative images tracing the path taken for each group during probe 2 trial. After 6 days of hidden platform training, untreated Tg2576 mice displayed aimless swimming patterns. **C.** Probe trials 2 and 3 where the platform was removed showed RSG treated Tg2576 mice spent more time exploring target quadrant than untreated Tg2576 mice (probe trial 2, Quadrant (F(3, 228)=9.41 p value< 0.0001)); (Probe trial 3, Quandrant (F(3, 208)=17.67.

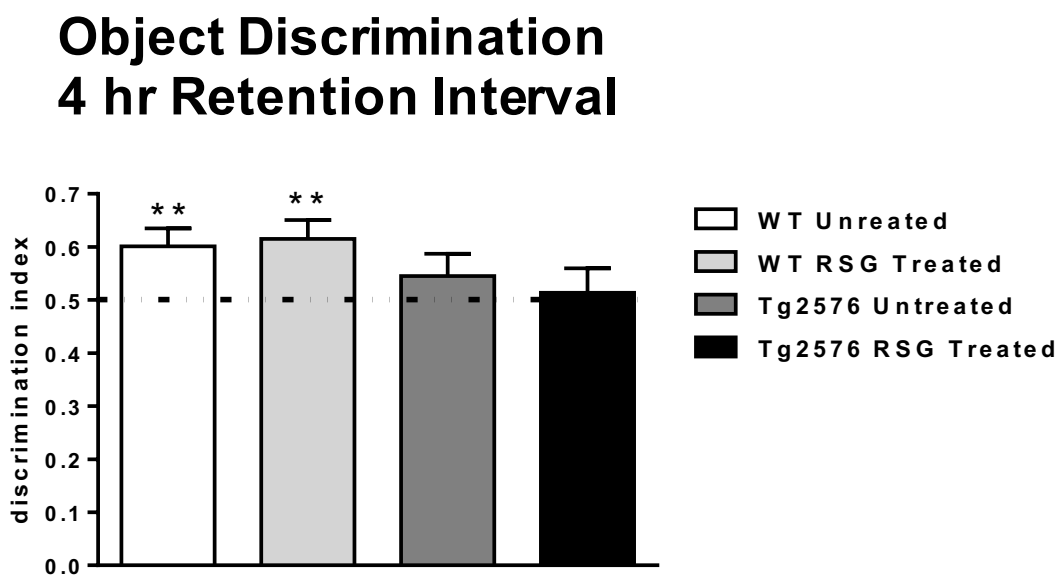


Figure 1.1: Novel object recognition was not rescued with PPARγ agonism four hours after object familiarization. Wild type groups (control, n=17; RSG,n=16) displayed significant preference for novel object introduced during testing that was unchanged with PPARγ agonism treatment. Tg2576 groups (control, n=10; RSG, n=11) exhibited novel object deficits. Two-way ANOVA (Interaction ($F(1, 50)=0.32$ pvalue=0.82).

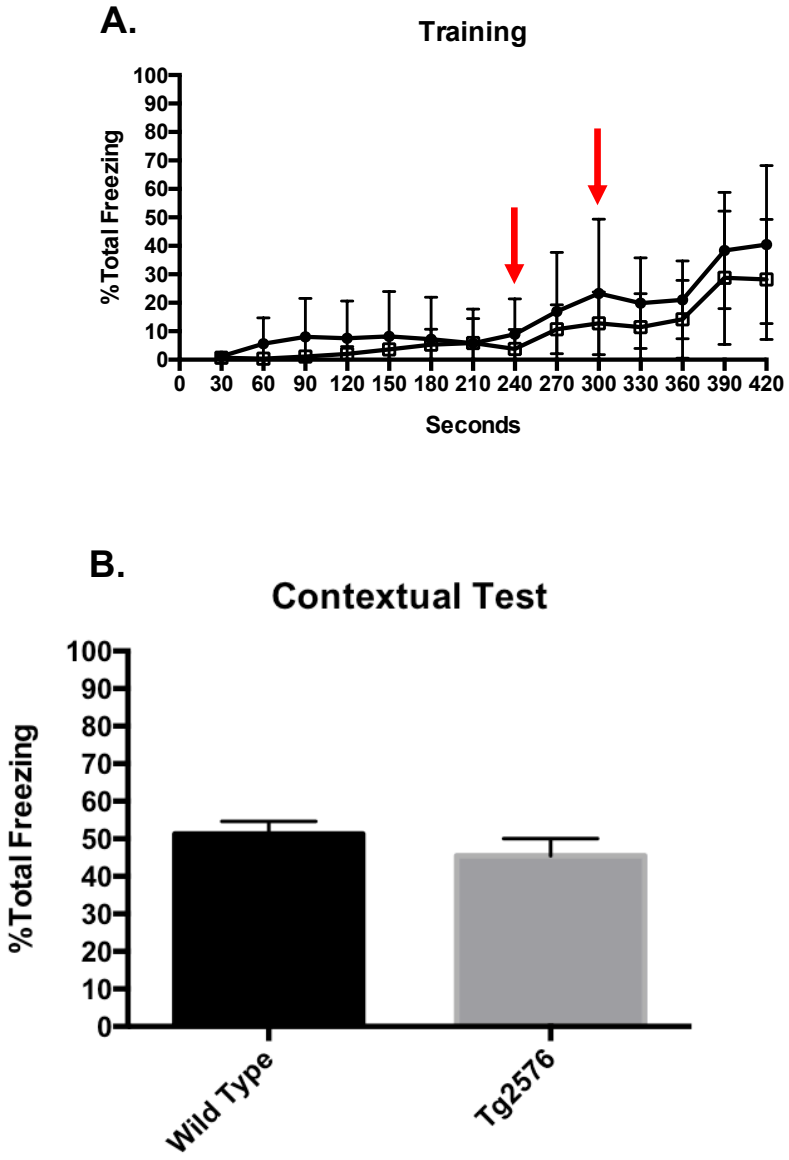
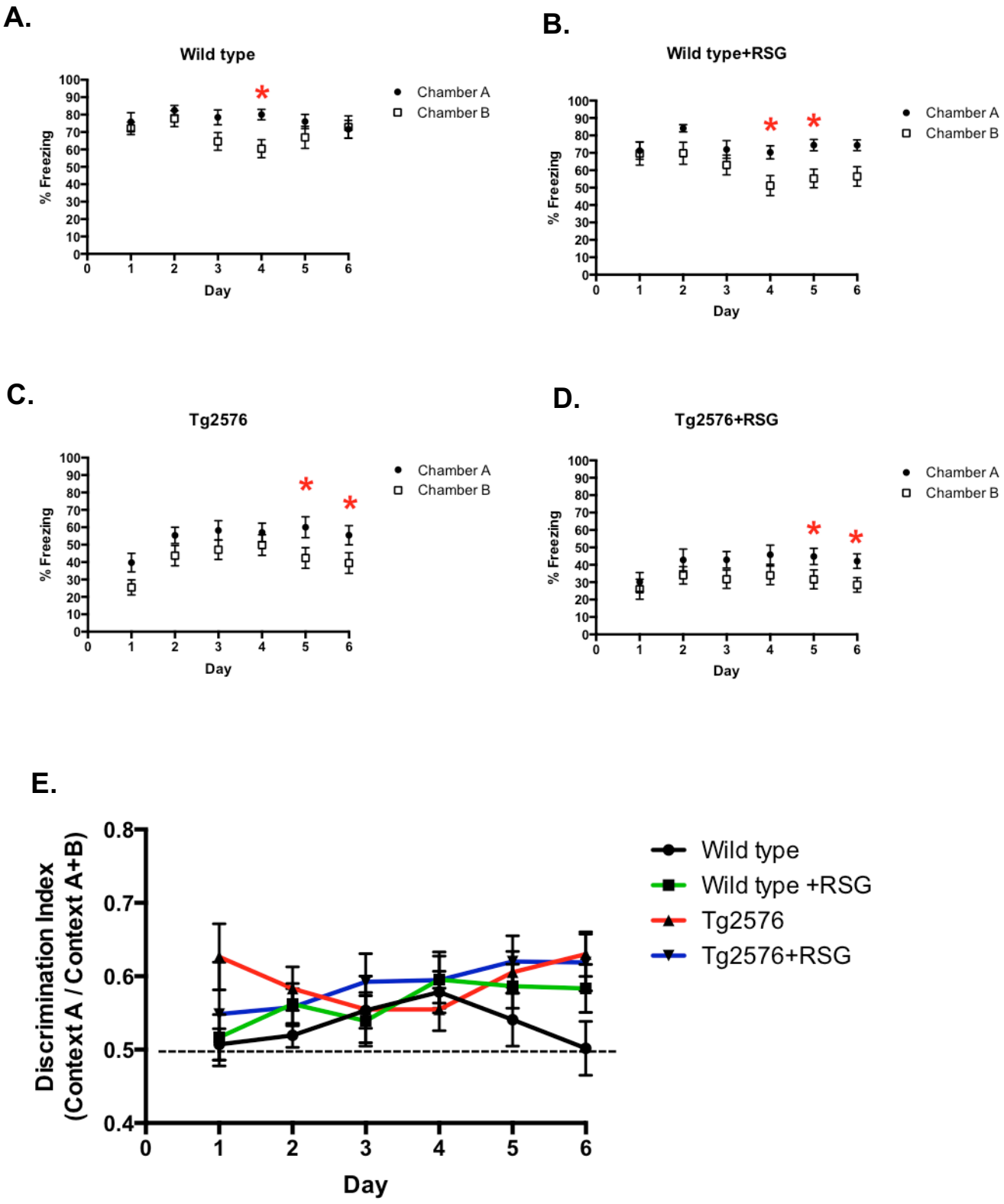
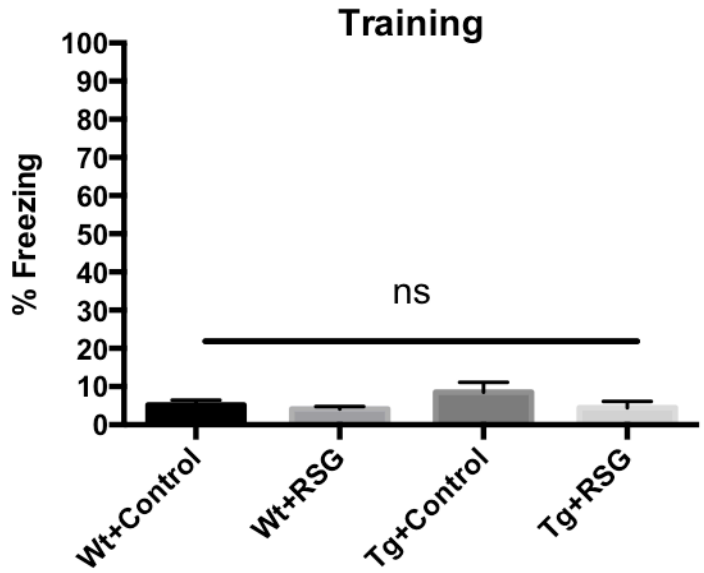


Figure 2.2: Tg2576 AD mice exhibit deficits in unsignaled foreground contextual fear conditioning compared to age matched controls. Performance in foreground training **A.** and contextual test 24 hours later **B.**. During training, both groups explore the novel context equally while freezing begins to increase after aversive stimulus at 240 seconds and 360sec, as indicated by red arrows. 24 hours later, both groups were placed back into training context to measure memory recall. Increased freezing to training context indicates both groups recall aversive context. Throughout contextual testing, Tg2576 mice explored the aversive training context comparably to wild type controls. Student's t test was used to calculate pvalue. Training (pvalue=0.117). Context (pvalue=0.307).


A.
Wild type
% Freezing
Day
Chamber A
Chamber B
B.
Wild type+RSG
% Freezing
Day
Chamber A
Chamber B
C.
Tg2576
% Freezing
Day
Chamber A
Chamber B
D.
Tg2576+RSG
% Freezing
Day
Chamber A
Chamber B
E.
Discrimination Index
(Context A / Context A+B)
Day
Wild type
Wild type +RSG
Tg2576
Tg2576+RSG

F.



G.

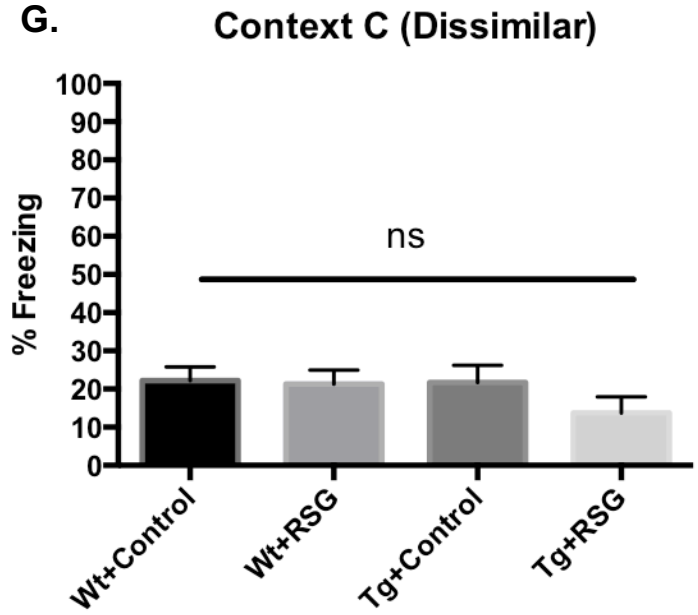


Figure 2.3: RSG does not reverse context discrimination deficits in aged wild type and Tg2576 mice. Figures represent total freezing in context discrimination for wild type **A.,** Wild type+RSG B., Tg2576 **C.,** and Tg2576+RSG **D.**. Raw freezing data reveal that control and RSG treated wild type subjects first distinguish between aversive and highly similar contexts as calculated by within group two-way repeated measures ANOVA; CONTROL: (Context x Day), $F(5,84) = 1.115$, (Pvalue=0.358); Sidak's multiple comparisons Day 4, *pvalue<0.05. RSG: (Context x Day) $F(5, 204)=0.498$, pvalue=0.776; Sidak's multiple comparisons Day 5,6 * pvalue<0.05; Tg2576: (Context x Day) $F(5,95)=0.496$; pvalue=0.778; Sidak's multiple comparisons Day5,6* pvalue<0.05; Tg2576+RSG: (Context x Day) $F(5,85)=0.551$, pvalue=0.730; Sidak's multiple comparisons Day5,6*. Discrimination ratio comparisons as calculated by dividing total freezing in context A by the sum of freezing in context A and B

show similar discrimination between untreated and RSG treated groups over the entire course of the study **E.**; two-way repeated measures ANOVA (Group x day), F(15, 325) =0.779. pvalue=0.70. Exploration in training (**F.**) and context C (dissimilar) (**G.**) context was not altered between untreated and RSG treated groups,Training: one-way ANOVA (Mean, SEM) wild type, n=15 (5.21, 2.84); wild type+RSG, n=16 (4.110, 4.26), Tg2576, n=20 (5.21, 2.73); Tg2576+RSG, n=18 (4.47, 2.77) pvalue=02.96. Context C: one-way ANOVA (mean, SEM); wild type, n=15 (22.17, 6.15); wild type+RSG, n=16 (21.28, 5.84); Tg2576, n=20 (21.73, 5.93); Tg2576+RSG; n=18 (13.68, 5.74), pvalue= 0.40.

DISCUSSION

Patients with Alzheimer's Disease express a dynamic range of cognitive deficits upon diagnosis. For example, patient's chief complaint is having difficulty navigating through and recalling familiar environments. Upon further testing, it is apparent patients suffer from host of cognitive deficits that include working memory impairments(McKhann et al., 2011). Similarly, mouse models of Alzheimer's disease exhibit age dependent decline in hippocampal and working memory dependent cognition. Particularly, the Alzheimer's disease mouse model, Tg2576 show synaptic deficits early in life and subsequently hippocampal deficits arise at 5 months of age. We previously established that RSG recues hippocampal dependent memory and dentate gyrus neuronal transmission for 9-month old Tg2576 mice. In this study we attempted to expand our studies to demonstrate RSG's effects on hippocampal dependent cognitive tasks.

Morris water maze. Our lab and others have previously established RSG treatment preserves associative memory for Alzheimer's disease models in a hippocampal-dependent memory task in delayed fear conditioning (Escribano et al., 2010; Jahrling et al., 2014; Ormerod et al., 2013; Rodriguez-Rivera et al., 2011). Using field recordings and other neuronal physiology techniques in the mouse hippocampus, we demonstrated improved performance may be due to restored neuronal transmission between the entorhinal cortex and dentate gyrus granule cell layer with

our current RSG treatment regimen(Nenov, Tempia, et al., 2014). Since synaptic plasticity plays a strong role in spatial learning and reference memory we asked: Does RSG treatment rescue Morris water maze deficits in Tg2576 mice? Here we show RSG treated Tg2576 mice demonstrated similar navigating strategies to wild type controls to locate a hidden platform based off of distal cues. Further, untreated Tg2576 mice lacked directional navigation patterns during hidden platform trials, suggesting RSG treatment rescues spatial learning at this age. In probe trials 2 and 3, when the platform was absent, RSG treated Tg2576 mice spent more time in the destination quadrant of the pool, indicating RSG improves spatial memory recall. Similarly, (Escribano et al., 2010), demonstrated RSG treatments longer than 4 weeks improves spatial navigation in a 13-month old double APP mutant mouse model (J20). Though it's difficult to generalize information between Alzheimer's mouse models, the amelioration in spatial memory observed with longer treatment but not a duration similar to our methods could be due to heightened amyloid beta burden exhibited in the double mutant compare to the Tg2576 which harbors a single APP mutation. These observations contribute to PPARγ biology in hippocampal dependent spatial learning and memory.

Novel Object Recognition (NOR). Classical object recognition tests an animal's ability to recognize novelty after familiarization of two identical objects and either a short or long retention interval(Antunes & Biala, 2012). Animals with para-hippocampal and hippocampal lesions are unable to recognize novel objects in this task(Aggleton, Albasser, Aggleton, Poirier, & Pearce, 2010). Interestingly, deficits from hippocampal lesions are associated with longer retention intertrials whereas para-hippocampal lesions impair performance with shorter retention trials(Aggleton et al., 2010; Hammond, Tull, & Stackman, 2004). Previously, it's been shown

that 5-month old Tg2576 mice exhibit NOR deficits with 4 and 24 hour retention intertrial, that were ameliorated with calcineurin inhibition (Taglialatela, Hogan, Zhang, & Dineley, 2009). Therefore, using similar methods, we asked whether PPARγ agonism with RSG would restore object recognition after an intermediate term retention intertrial. Here we show 4 weeks of RSG treatment does not improve NOR memory deficits that arise from amyloid beta pathology. Though, it's been demonstrated that longer RSG treatment with RSG in a similar AD mouse model restored NOR memory suggesting; further consideration into longer treatments could elucidate PPARγ effects in our AD model (Escribano et al., 2010).

Foreground fear conditioning. In our lab, RSG reverses deficits for Tg2576 mice in classical fear conditioning which pairs a auditory conditioned stimulus (CS) with an aversive unconditioned stimulus (US) (Rodriguez-Rivera et al., 2011). During training, subjects learn to associate the context with this US-CS pairing. It is widely accepted that hippocampal lesions before training and Tg2576 mice can disrupt memory recall when these subjects are placed back in the training environment (Anagnostaras et al., 2001; Barnes & Good, 2005; H. van Praag et al., 1999). Here, we attempted to increase characterization of 9-month old Tg2576 mice by subjecting them to foreground fear conditioning. Similar to background fear conditioning, though mice are not presented with a tone/conditioned stimulus but instead challenged to associate the novel context with two-foot shocks. It is postulated that the auditory cue masks context features in background fear conditioning and since animals rely on multimodal cues to recall the aversive context. In foreground fear conditioning the animals are not distracted by the auditory cue and can recall multiple cues in the context. Here, we show Tg2576 perform comparably to age matched controls in foreground fear conditioning. With freezing as a metric of learned behavior, it

appears Tg2576 mice have intact contextual fear conditioning but it has been postulated that hippocampal lesions can shunt contextual fear learning to extrahippocampal-amygdala regions (Biedenkapp & Rudy, 2009; Maren, Aharonov, & Fanselow, 1997). This result indicates RSG may restore hippocampal processing of some contextual fear conditioning paradigm as RSG treated Tg2576 subjects increases freezing to wild type level. Previous research demonstrated that dorsal hippocampal lesion can impair performance in background but not foreground fear conditioning suggesting Tg2576 mice at this age have similar impairments (Phillips & LeDoux, 1994).

Context fear discrimination. Our lab previously demonstrated that RSG treatment induces pERK-PPARγ complex formation that appears to be necessary for memory recall in 9-month old Tg2576 AD mice(Jahrling et al., 2014; Rodriguez-Rivera et al., 2011). Further, RSG treatment in this model improves neuronal firing and synaptic vesicle release between entorhinal cortex and hippocampus (Nenov, Laezza, et al., 2014; Nenov, Tempia, et al., 2014). To demonstrate if RSG plays a role in adult hippocampal neurogenesis; we used similar treatment methods but tested cognition 9-month old Tg2576 mice in a context fear discrimination paradigm. Adult hippocampal neurogenesis supports cognition in classical and spatial memory tasks (H. van Praag et al., 1999). Interestingly, enhancing adult neurogenesis improves animals in context fear discrimination learning and memory (Wu, Luna, & Hen, 2015). Conversely, genetic ablation or cranial irradiation depletes adult neurogenesis and diminishes performance in contextual fear conditioning tasks(Danielson et al., 2016; Saxe et al., 2006). In our paradigm, subjects were allowed to explore a novel context for the first few minutes and then delivered a single foot shock. The testing phases began 24 hours later where subjects were randomly exposed to the

training context and a highly similar-safe context for the next 6 days (Cortez et al., 2017). This test is similar to learning in foreground fear conditioning but more challenging since animals are required to adapt and differentiate between overlapping contextual cues in these different contexts. In this study, control and RSG treated groups explore the training context A similarly on day 0. Though, generalized fear was observed for all groups in the first few testing days as freezing levels were similar between context A and B. Though on day 4, both control and RSG treated wild type groups significantly discriminated between aversive and non-aversive contexts. A similar trend was observed in Tg2576 control and RSG treated groups. Discrimination ratios were used as indicator of learning and calculated using total freezing in context A by freezing in context A and B. Again, we see no significant discrimination between these groups indicating RSG has no effect on context fear discrimination learning and memory. Together, this result eliminates the possibility that RSG affects adult hippocampal neurogenesis dependent contextual fear discrimination. It does however, support the notion that RSG treatment protects dorsal hippocampal cognition that is independent of functional adult neurogenesis as we see heightened freezing in context fear discrimination and foreground fear conditioning.

CONCLUSION

Together, the results in this study elaborate on the deficits that arise from over expression of amyloid beta in 9-month old Tg2576 model of Alzheimer's Disease. Moreover, these findings demonstrate a narrow the range of cognitive deficits that are reversed with PPARγ agonism by way of RSG treatment. With this evidence, we postulate specific learning and memory deficits can be rescued with PPARγ using the diabetic drug, rosiglitazone. Lastly, we propose RSG may

protect insulin resistant and diabetic patients suffering from hippocampal dependent associate and spatial memory deficits.

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Chapter 3

Peroxisome Proliferator Activated Receptor-gamma Agonism attenuates age-dependent decline in fear-based context discrimination with unique effects following cranial irradiation: evidence for neurogenesis-independent mechanisms

ABSTRACT

In the brain, radiotherapy can increase neuro-inflammation and interfere with endogenous adult neurogenesis that may ultimately lead to memory impairment in young and adult patients. Investigation into therapies that blunt adverse effects from cranial irradiation could be considered for patients immediately after treatment. PPARγ agonism has been shown to improve cognition in LPS and Alzheimer's disease mouse models. We postulate, RSG treatment will protect adult hippocampal neurogenesis dependent context discrimination in 9MO wild type mice. To achieve our objective, 8-month old wild type mice were left untreated or treated with RSG followed by context discrimination testing and immunohistochemical evaluation of neurogenesis. We found that PPARγ agonism enhanced context discrimination performance in aged wild type mice concomitant with an amelioration of activated microglia in the hippocampus suggesting PPARγ agonism may blocks chronic inflammation that is deleterious to hippocampus-dependent context discrimination. We propose, aged subjects exposed to radiotherapy could benefit from therapies that dampen chronic microglial signaling like PPARγ agonist rosiglitazone.

INTRODUCTION

The Peroxisome Proliferator-activated Receptor gamma (PPARγ) is a ligand-modulated nuclear transcription factor and a therapeutic target for treating insulin resistance in type-2 patients. As such, FDA-approved drugs are highly effective treatments for type-2 diabetes in the form of the thiazolidinediones (TZDs) such as rosiglitazone (RSG).

Much is known regarding the role of PPARγ in peripheral tissues (Desvergne & Wahli, 1999), its role in neuronal function emerged following immunohistological identification of PPARγ expression in brain areas associated with higher cognitive function, including the neurons of the cortex, basal ganglia, hypothalamus, and hippocampus (Inestrosa, Godoy, Quintanilla, Koenig, & Bronfman, 2005; Moreno, Farioli-Vecchioli, & Ceru, 2004; Sarruf et al., 2009; Warden et al., 2016). PPARγ agonism is generally recognized as neuroprotective (Breidert et al., 2002; Feinstein, 2003; Hyong et al., 2008; Victor et al., 2006) via attenuated levels of pro-inflammatory proteins (e.g. iNOS, TNFα, MMP9), reactive oxygen species (ROS), and Aβ. Thus, PPARγ is a therapeutic target in many CNS diseases including Parkinson's disease (Barbiero et al., 2014; Pisanu et al., 2014; Ridder & Schwaninger, 2012), ischemia-reperfusion injury (Gele et al., 2014; Singh, Singh, & Bhatti, 2014), and traumatic brain injury (Sauerbeck et al., 2011; Thal et al., 2011) in addition to Alzheimer's disease (AD).

In rodents and humans, PPARγ is extensively expressed in brain, primarily in neurons and somewhat in astrocytes; PPARγ is essentially absent from microglia (Warden et al., 2016). This strong neuronal signature raises the possibility that PPARγ agonism most likely targets neurons rather than glia to produce neuroprotection or alleviate neurodegenerative disease processes.

We and others have demonstrated that PPARγ agonism improves cognitive performance in AD mouse models, predominantly in tasks that require intact hippocampal ERK signaling (Escribano et al., 2009; O'Reilly & Lynch, 2012; Pedersen et al., 2006; Rodriguez-Rivera et al., 2011). Cognitive enhancement has been shown to be accompanied by improved AD biomarker profiles: alleviation of amyloid and tau pathology (Escribano et al., 2010; Jiang et al., 2012; Kummer et al., 2014; Mandrekar-Colucci et al., 2012; O'Reilly & Lynch, 2012), reduced neuroinflammation (Escribano et al., 2010; Prakash & Kumar, 2014; Yin et al., 2013), increased

antioxidant protection (Nicolakakis et al., 2008; Yin et al., 2013), amelioration of central insulin resistance (Masciopinto et al., 2012; Yin et al., 2013), and normalization of several transcripts and proteins related to ERK and insulin signaling in the hippocampus, including reversal of down-regulated PPARγ (Denner et al., 2012).

Several clinical studies have tested the efficacy of TZDs (RSG and PIO) in AD patients, mostly reporting failure to prevent or improve cognitive and functional decline in those suffering moderate to advanced AD. In contrast, those AD pilot clinical trials assessing RSG or PIO in subjects with early stage disease have found cognitive benefit in subjects comorbid for insulin resistance or those that are APOE4-negative (Craft, 2012; Gold et al., 2010; Hanyu, Sato, Kiuchi, Sakurai, & Iwamoto, 2009; Risner et al., 2006; Sato et al., 2011; Watson et al., 2005).

The lack of success in AD clinical trials using PPARγ agonists to improve cognition is puzzling and has led researchers to investigate PPARγ mechanisms in early stage disease that are amendable to using preclinical animal models. In this context, we and others have demonstrated that PPARγ agonism improves cognitive performance in aged AD mouse models, predominantly in tasks that require intact hippocampal ERK signaling (Escribano et al., 2009; O'Reilly & Lynch, 2012; Pedersen et al., 2006; Rodriguez-Rivera et al., 2011). Cognitive enhancement has been shown to be accompanied by improved AD biomarker profiles: alleviation of amyloid and tau pathology (Escribano et al., 2010; Jiang et al., 2012; Kummer et al., 2014; Mandrekar-Colucci et al., 2012; O'Reilly & Lynch, 2012), reduced neuroinflammation (Escribano et al., 2010; Prakash & Kumar, 2014; Yin et al., 2013), increased antioxidant protection (Nicolakakis et al., 2008; Yin et al., 2013), amelioration of central insulin resistance (Masciopinto et al., 2012; Yin et al., 2013), and normalization of several transcripts

and proteins related to ERK and insulin signaling in the hippocampus, including reversal of down-regulated PPARγ (Denner et al., 2012).

Because the adult hippocampus is capable of continuous production of new-born neurons throughout life and this mechanism has been shown to correlate with performance in hippocampus-dependent learning and memory, we set out to establish whether PPARγ agonism with RSG improved age-dependent decline in context discrimination (Cortez et al.) and determine its effects on context discrimination ablation with cranial irradiation(Aimone et al., 2010; Cortez et al., 2017; Wu et al., 2015).

To achieve our objective, 8-month old wild type mice were left untreated or treated with RSG followed by context discrimination testing and immunohistochemical evaluation of neurogenesis. To evaluate the effect of RSG on recovery of context discrimination following cranial irradiation, mice were subjected to a single dose of X-ray followed by context discrimination testing and immunohistochemical evaluation of neurogenesis. In aged wild type C57Bl6-SJL mice, PPARγ agonism with RSG stimulated hippocampal progenitor production but did not promote neuronal differentiation or survival from these progenitors; although, context discrimination was enhanced in RSG-treated mice. In the absence of adult hippocampal neurogenesis following cranial irradiation, we found that PPARγ agonism enhanced context discrimination performance in aged wild type mice concomitant with an amelioration of activated microglia in the hippocampus suggesting PPARγ agonism may blocks chronic inflammation that is deleterious to hippocampus-dependent context discrimination.

MATERIALS AND METHODS

Animals. C57bl/6 mice were bred and aged in the Animal Resource Center at the University of Texas Medical Branch at (UTMB). UTMB Animal Resource Center Facilities operate in compliance with the USDA Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, under OLAW accreditation, and IACUC approved protocols. Mice were separated by sex, identified upon weaning and allowed food and water *ad libitum* while they aged until 8 months old. (Add cranial coordinates) At 8 months old, mice were exposed to a single dose of 10Gy x-ray irradiation or isoflurane and supplemented with either 50mg/kg rosiglitazone milled chow or control chow for 30 days after x-ray treatment. Body weight was measure once before X-ray treatment and upon first day of behavioral testing.

Behavioral Assays. The open field test was used to measure natural exploration and ambulation in this study 30 days post irradiation treatment (9 months of age. Briefly, mice were placed in the center of a 38cm^3 white plexiglas^{TM} box and allowed to explore for 10 minutes. Digital video-based data capture and analysis was achieved with TopScan software (Clever Sys) using central (12.6 cm^2) and peripheral (25.4 cm^2) arenas to track exploratory behavior. Total distance travelled and velocity was calculated for the peripheral and central arenas. Subsequently, all groups were tested in context fear discrimination paradigm adapted from, (Frankland, 1998; Sahay et al., 2011). Briefly, this cognitive task measures an animal's ability to distinguish between two similar yet different environmental contexts (Frankland et al., 1998). On training day 0, each subject was placed in a standard mouse fear conditioning chamber (Med Associates) which served as context A, the shock context. Context A training ensued for 195sec during which time a single 2sec 0.75mA shock was delivered through the electrified grid floor at the

178sec mark; mice then had an additional 15sec to explore the context then were returned to their home cages.

On testing days mice were randomly assigned to experience first either context A, or a similar yet non-identical "safe" context, context B. Context B resembled context A in terms of the grid floor being exposed. Context B contained cardboard inserts, vanilla extract (2 µL pipetted onto a KimWipe®), and the chamber light and fan were turned off. Like context A, mice were placed in context B for 185s, however, no shock was delivered. A 2hr inter-trial interval between each context was utilized for each subject for each day of testing. Following each session, 70% ethanol was used to thoroughly wipe down Context A and 95% isopropyl alcohol was used to clean Context B.

Digital video-based data capture and analysis with FreezeFrame software (Actimetrics) was used to assess freezing behavior. Discrimination ratios (% freezing in Context B ÷ % freezing in Context A+B) were calculated for each group on each test day.

As additional controls to test for memory of the shock context A and that a completely novel safe context can be recognized, we analyzed %freezing during the training session on day 0 and context discrimination in a completely unique context in which the electrified grid floor was concealed by Plexiglas™ on day 1.

*Immuno-fluorescence and Imaging***.** 6 serial brain sections spanning the dorsal hippocampus, (~180µm between sections) were collected for each animal and washed in 1x PBS for 5 minutes, 3 times. Next, free-floating sections were permeabilized in 0.3% tween-1x PBS for 15 minutes and then blocked in 5% normal donkey serum in 1x PBS for 1 hour at room temperature. After, sections were incubated with goat anti-doublecortin (Santa Cruz), mouse anti-NeuN (Millipore), rabbit anti-Ki67 (Cell signaling) and rabbit anti-Iba (WAKO) in 3%BSA-1xPBS, overnight at

4°C. Next, sections were washed 5 times in 1xPBS and next stained with secondary antibodies: Alexa-fluor® donkey anti-goat 488nm, Alexa-fluor® donkey anti-rabbit 488nm and 568nm and Alexa-fluor® donkey anti-mouse 647nm for 1 hour at room temperature in 3%BSA-1x PBS. Lastly, sections were mounted on SuperFrost® glass slides and sealed in Vecta sheild® mounting media. All representative sections were imaged on a A1 Nikon confocal microscope.

Statistics. All statistics were performed using GraphPad Prism. Data was analyzed using a One-Way ANOVA between treated groups for all metrics in open field test, area under the curve and immunofluorescence. Analyses for cognitive performance were reported as mean +/- SEM with 95% confidence intervals, where appropriate. RSG treatment differences were tested using an unpaired two-tailed t test. The F-test was used to test for equal variance between groups. Context discrimination ratios were calculated and tested using a one sample t test against chance, a theoretical mean of 0.5. A repeated measures two-way ANOVA using discrimination ratios was used to determine an effect via genotype vs day design. The Levene's test was used to determine equal variances for within subject's design (days). Further, a within-subject repeated measures two-way ANOVA between subjects using raw freezing data was used (total freezing Context A vs total freezing Context B).

RESULTS

Behavioral tests

Open field. To confirm that 30-day treatment of RSG and a single low dose cranial irradiation do not alter baseline behavior in mice, we tested natural exploration and anxiety like behavior by exposing all groups to a novel context in open field test for 5 minutes. Thirty days after initial treatments, percent time was similar among groups exploring the periphery and center of open

field box, **Figure 1.0 A,B**. Further, there was no difference in total distance traveled and speed between groups, **Figure 1.0 C, D**. Treated groups displayed similar anxiety like behavior to control group in all metrics of the open field test demonstrating that cranial irradiation and RSG treatments did not change baseline behavior in this study.

Context fear discrimination. To measure cognition, animals were tested in a contextual fear discrimination memory task **Figure 1.2**. Total percent freezing in each context was used to measure learned behavior each day and compared within groups in this study **Figure 1.3**. All groups displayed similar freezing to context A (shock) on day 0. To demonstrate subjects can discriminate between highly dissimilar contexts, we tested groups in context C (novel) on day 1 where we observed less freezing in the novel context than context A and B; though there was no significant difference among groups **Figure1.3 A,B,C,D**.

Discrimination testing between highly similar contexts began day 1 where all groups were able to associate context A with a foot shock however, context A freezing was not different among groups, **Figure 1.4 A**. In the same day, freezing was lower in context B but not different among groups tested on day 1, **Figure 1.4 B**. Assessment of discrimination between highly similar contexts continued for the next the next five days. By day 3, untreated, RSG treated and irradiation+RSG groups froze significantly more in shock context than in similar safe context. However, irradiation treated group were not able to discriminate between shock and safe context over the entire study indicating irradiation disrupts context discrimination, **Figure 1.4 C**. Among groups, discrimination learning was higher for control, RSG, and irradiation+RSG groups compared to irradiation only treated group **Figure 1.4D**. Significantly higher discrimination was observed on day 6 for RSG and irradiation+RSG groups compared to control and irradiation

groups. This was confirmed by measuring cumulative performance where irradiated subjects exhibited lower discrimination among other groups in this study

Figure 1.5 A, B.

Adult Hippocampal Neurogenesis

Proliferation. It has been reported that PPARg agonism improves proliferation in the ventricles and *in vitro* but has yet to be demonstrated in wild type mouse hippocampus (Morales-Garcia et al., 2011). Here we show that RSG significantly increases Ki67 expressing cells in the subgranular zone of the hippocampus compared to untreated **Figure 1.6**. This result was confirmed by treating subjects with mitotic marker 5-bromo-2-deoxyuridine (BrdU 100mg/kg) and harvesting tissue 24 hours later. Immunostaining for BrdU revealed increased abundance of recently divided cells in the subgranular zone of RSG treated subjects compared to untreated subjects.

Differentiation. PPARg agonism influences neurite growth *in vitro* and hippocampal immature neuron abundance (Ormerod et al., 2013) however, we show that the marker for immature neurons, doublecortin immunoreactive cells were not significantly different between untreated and RSG treated groups **Figure 1.7**. Conversely, PPARg agonism restores hippocampal neuroblast species 30 days after initial radiation exposure and chronic RSG treatment **Figure 1.7**.

Newborn Neuron Survival. To determine if RSG affects new neuron survival, we treated 8-month old mice with RSG as previously described. 24 hours after first of day RSG treatment, mice were given 50mg/kg BrdU intraperitoneal injections, for 5 consecutive days. Quantification of BrdU positive cells were restricted to granule cell layer of the dorsal hippocampus. Although

immature neuron abundance is unchanged in aged wild type mice, we see a mild increase in BrdU+NeuN positive cells with RSG treatments.

Iba-1 Immunoreactive Microglia. Iba-1 reactive microglia perform complex processes including synaptic pruning, phagocytosis and inflammatory signaling. Increased expression of Iba-1 has been observed in neurodegenerative diseases and thus we used this marker to identify activated microglia. Here we show RSG diminished microglia abundance induced by cranial irradiation 45 days after initial treatment. Cranial irradiation significantly induced large cell body microglia which RSG restored to control group levels **Figure 1.9**.

FIGURES

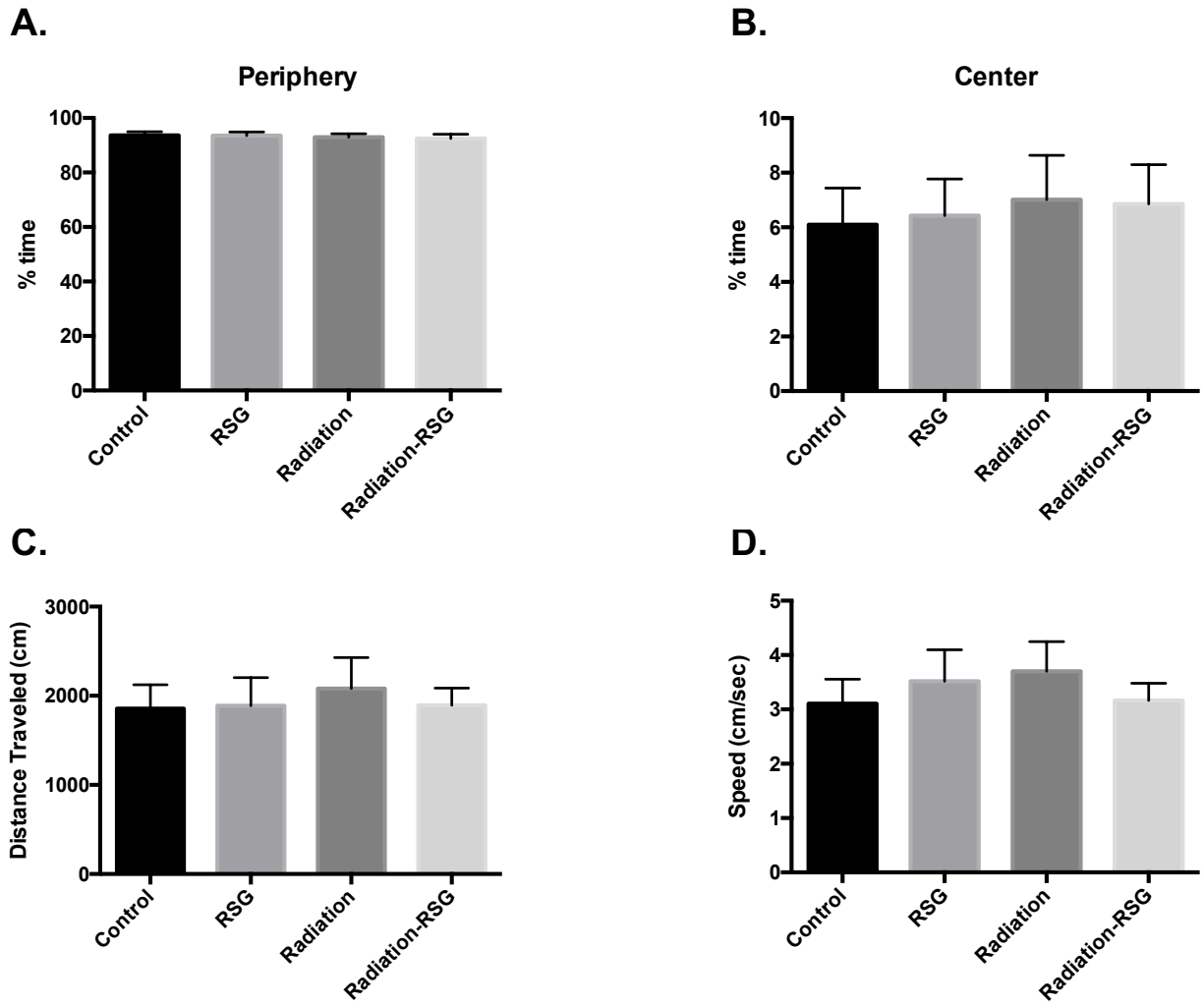

Figure 3.0: Radiation and rosiglitazone do not alter exploratory behavior in aged wild type mice. All subjects were allowed to explore an open field test box for 5 minutes to measure natural exploratory behavior in a novel context. **A.** ANOVA ($F(3, 71)=0.14$) (pvalue=0.92) revealed wild type control (mean=93.63%) animals spent similar percent time exploring the periphery of the open field box as did groups treated with RSG (mean=93.52%), radiation (mean=92.92%) and RSG+Radiation (mean=92.50). **B.** ANOVA ($F(3,71)=0.82$) (pvalue=0.96) test revealed controls (mean=6.09%), RSG (mean=6.24%), radiation (mean=7.1%) and Radiation+RSG (mean=6.85%) time exploring the center of the box was no different this study. **C.** Average total distance traveled for controls(mean=1855cm), RSG(mean=2081cm), radiation(mean=2081cm) and Radiation+RSG(mean=1895cm) traveled similar distance throughout the trial; ANOVA($F(3,71)=0.18$) (pvalue=0.9). **D.** Velocity for controls(mean=3.1cm/sec), RSG(mean=3.51cm/sec), radiation(mean=3.7) and radiation+RSG(3.16) were comparable in open field test; ANOVA ($F(3,71)=0.34$)(pvalue=0.79).

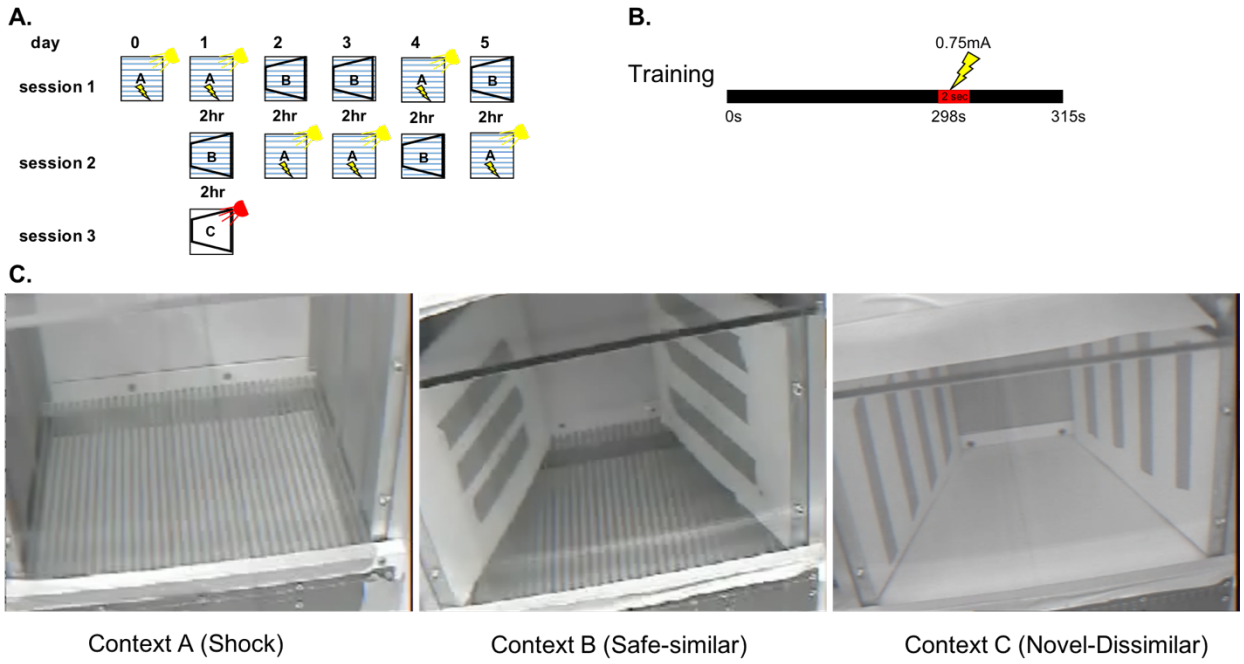

Figure 3.1: ***Context discrimination fear conditioning paradigm.*** **A**. Schematic illustrating random context presentations across 6 six days. **B**. On training day 0, subjects were placed in shock context A and allowed to explore the shock context for 298 seconds at which point a 2 second 0.75mA foot shock was delivered; subjects are kept in context A for an additional 15 seconds, totaling 315 seconds training. On test days, subjects were randomly presented with contexts A and B for the same period of time. Between context presentation, a 2-hour inter-trial interval was inserted during which time mice are returned to their home cage. On test day 1, mice are presented with an additional novel context, C, to control for baseline propensity to freeze. Total percent freezing was measured for each mouse in every context.

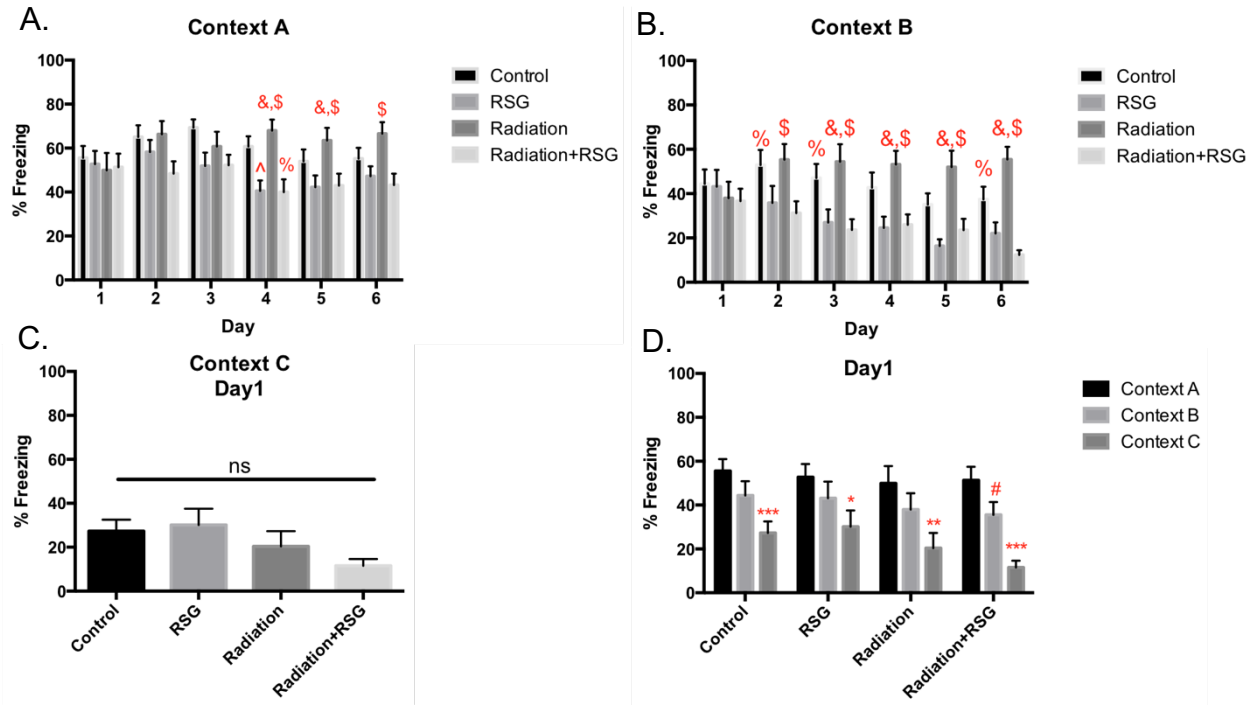

Figure 3.2: RSG treatments reduced conditioned and general fear compared to cranial irradiated and aged matched controls after persistent training. All groups performed similarly on day 1 in each context presentation. Further, all groups displayed lower freezer in context B and even less in context C on day 1. **A.** A significant difference was observed in group ($F(3,59)=5.73$)($pvalue<0.05$) and by day ($F(15,295)=1.374$)($pvalue<0.05$) as measured by repeated measures two-way ANOVA. Tukey's post hoc multiple analysis reveal that RSG and/or cranial irradiation had no effect on groups associating foot shock with context A on the first few days of training. Though on Day 4, RSG and radiation+RSG treated groups froze significantly less than radiation and control groups, with similar trends on day 5 and 6. **B.** Significant interaction ($F(15,295)=1.921$)($pvalue<0.05$) between group ($F(5,295)=3.456$)($pvalue<0.05$) and day($F(3,59)=10.03$)($pvalue<0.05$) was observed in context B (highly similar) as measured by repeated measures two-way ANOVA. Tukeys post hoc multiple analysis revealed radiation+RSG group displayed significantly less general fear than radiation exposed group on day 2 that continued throughout the entire test. Further, RSG treated group froze significantly less than radiation group on days 3-6. Interestingly, radiation+RSG group froze significantly less than control group on days 3 and 6. **C.** On day 1, all groups exhibited similar exploration and freezing in context C (dissimilar-novel); One-way ANOVA ($F(3,59)=2.152$)($pvalue=0.1033$). **D.** Day 1 performance showed each group froze similar when presented with each context. Though In context B, all groups mildly froze in the similar context but significantly froze less to novel context C compared to context A; repeated measures two-way ANOVA, context, ($F(2,118)=24.11$)($pvalue<0.05$). ^Control vs RSG, $^{\&}$RSG vs Radiation, $^{\$}$Radiation+RSG vs Radiation, $^{\%}$Control vs Radiation+RSG, ***ContextA vs ContextC, $^{\#}$ContextB vs ContextC.

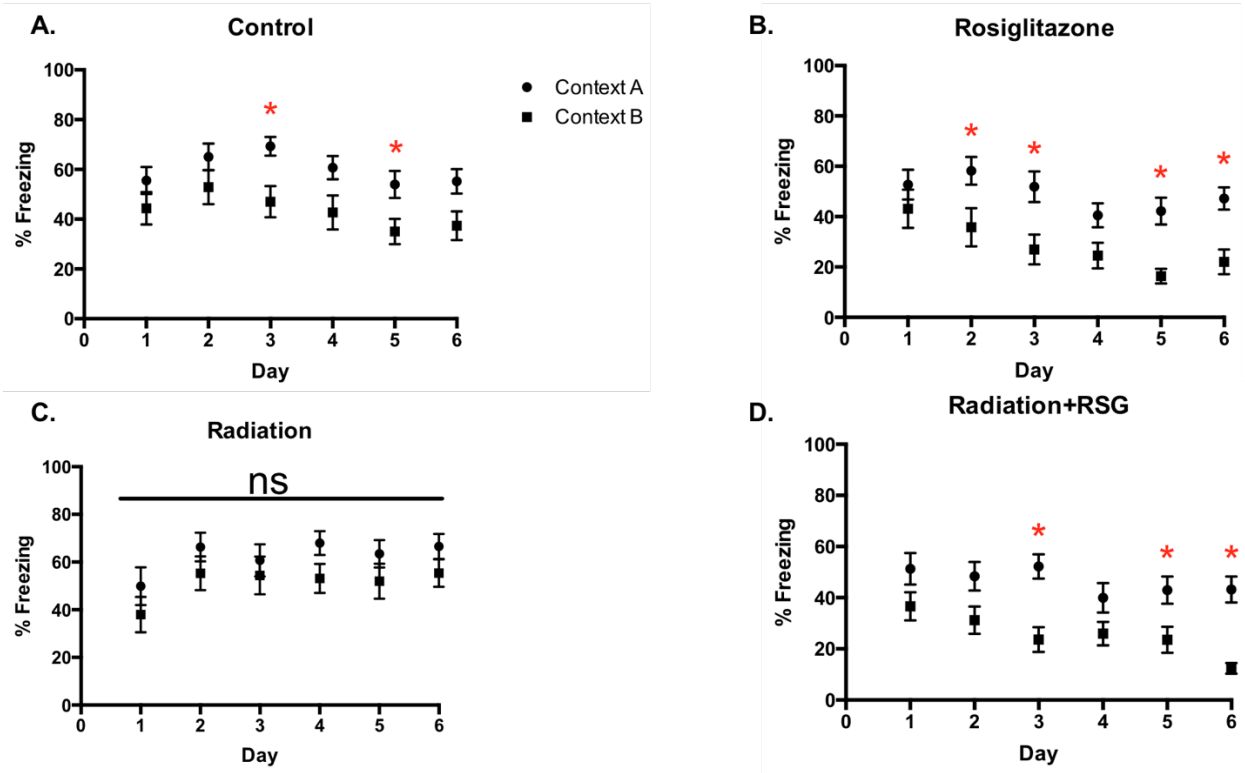

Figure 3.3: ***RSG treatment improved context discrimination in aged wild type and cranial irradiated mice.*** Percent time freezing in context A (circles) and context B (squares) for each treatment group. Data represented as mean ± SEM.. *p <0.05 **A**. *Within* group two way repeated measures ANOVA and Sidak's *post hoc* pairwise comparisons determined that aged wild type mice (n=16), (Context vs Day)(F(5,90)=0.368)(pvalue=0.86); significantly discriminated on day 3 while (**B**.) RSG-treated mice (n= 16), (Context vs Day)(F(5,150)=0.908)(pvalue=0.47) discriminated on day 2, (**C**.) *Within* group two way repeated measures ANOVA and Sidak's *post hoc* pairwise comparisons determined that discrimination was eliminated in cranial irradiated mice (n=14) (Context vs Day) (F(5,30)=0.127)(pvalue=0.98),(**D**.) while RSG-treated cranial irradiated mice (n=17), (Context vs Day)(F(5,160)=1.159)(pvalue=0.33) discriminated on day 3.

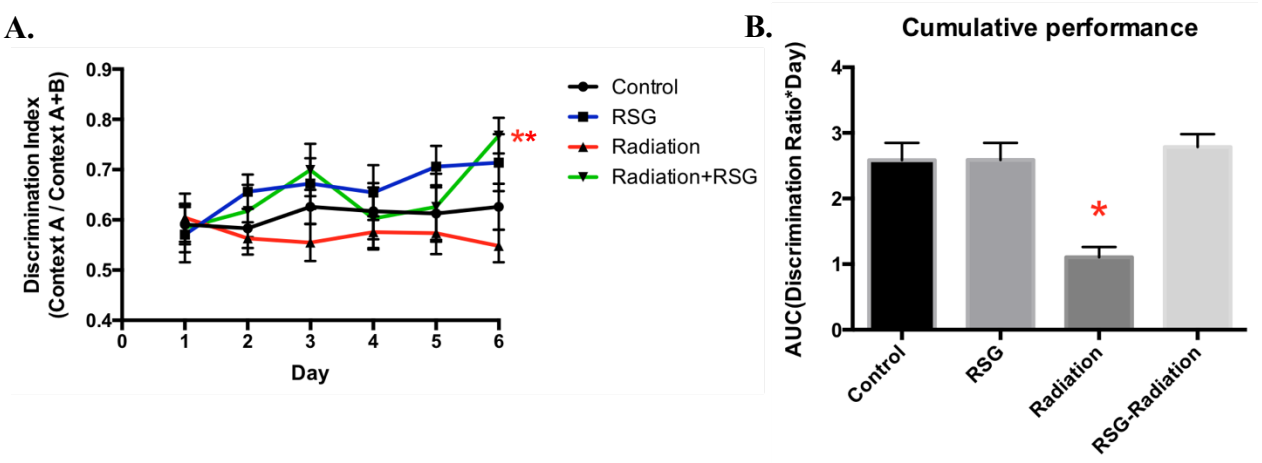

Figure 3.4: ***RSG treatment had no effect on discrimination ratios, except in cranial irradiated mice.*** **A.** Discrimination ratios were calculated by dividing total percent freezing in context A by percent freezing in both context A and B. RSG-treated groups had improved learning by day 6 as measured with the discrimination index. Repeated measures two-way ANOVA group($F(15,295)=0.8817$)(pvalue=0.057); Tukey's post hoc day 6 multiple analysis **Radiation vs Radiation+RSG. **B.** AUC calculations determined that RSG restored context discrimination in cranial irradiated animals. One-way ANOVA ($F(3,20)=12.17$)(pvalue<0.05).

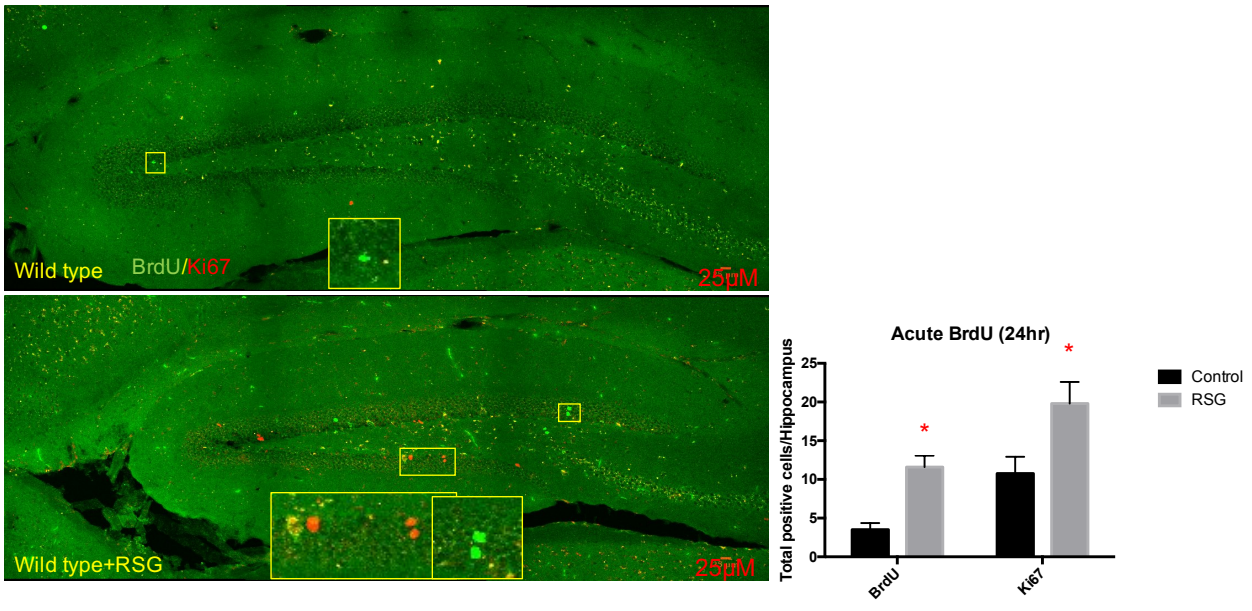

Figure 3.5: ***PPARγ agonism with RSG stimulated progenitor proliferation in hippocampus subgranular zone.*** **A**. Confocal images of sagittal sections of dorsal hippocampus from 9-month old wild type mice treated with RSG (n=3) or untreated (n=3) following BrdU injection 24 hrs

prior to trace dividing cells. Ki67 (Red) and BrdU (Green) show that recently divided cells are more abundant in subjects treated with RSG. **B**. Dividing cells were significantly more abundant in dorsal hippocampi of RSG-treated mice. Data represented as mean ± SEM. Significant effects were established with Student's t test (*p<0.05).

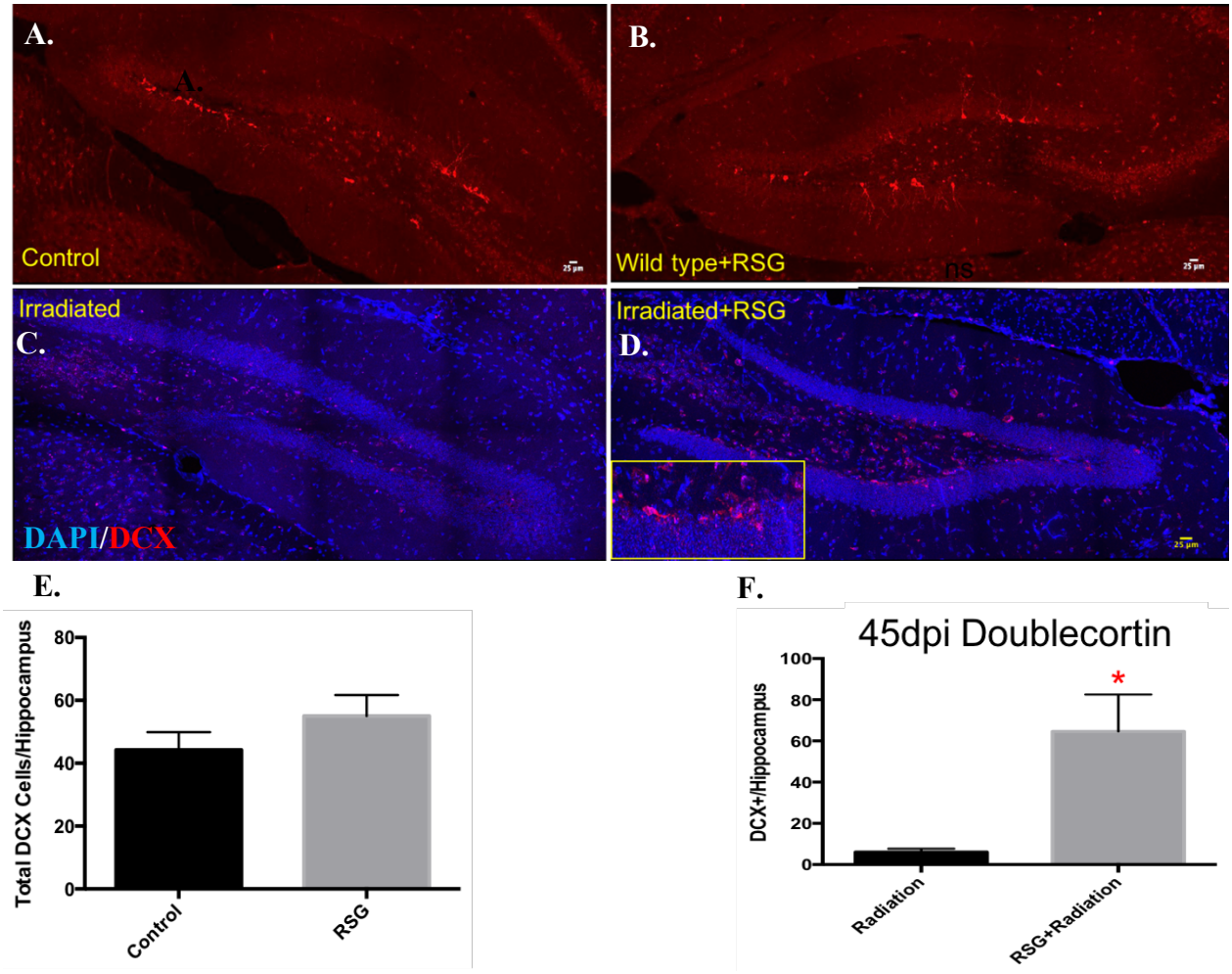

Figure 3.6: ***RSG did not promote new neuron production in dorsal hippocampus, except following cranial irradiation.*** Young neurons were labeled with the new neuron marker, doublecortin (DCX, red). **A**. Dorsal hippocampus section from untreated 9-month old wild type mice. **B**. Dorsal hippocampus from 9-month old wild type mice treated with RSG beginning at 8-months of age. **C**. Abundance of doublecortin-positive neurons in the hippocampus was similar between treatment groups, (n=3mice/group; p=0.26, Student's t test). Data represented as mean ± SEM. **D**. Doublecortin (DCX, red; DAPI, blue) labeling was greatly reduced following

irradiation 45 days prior. **E**. Whereas, RSG-treated mice exhibit substantial doublecortin labeling. **F**. Quantification of doublecortin-posicitve cells in the SGZ were increased in RSG-treated mice 45 days after cranial irradiation (n=3mice/group; $p<0.05$, Student's t test). Data represented as mean ± SEM.

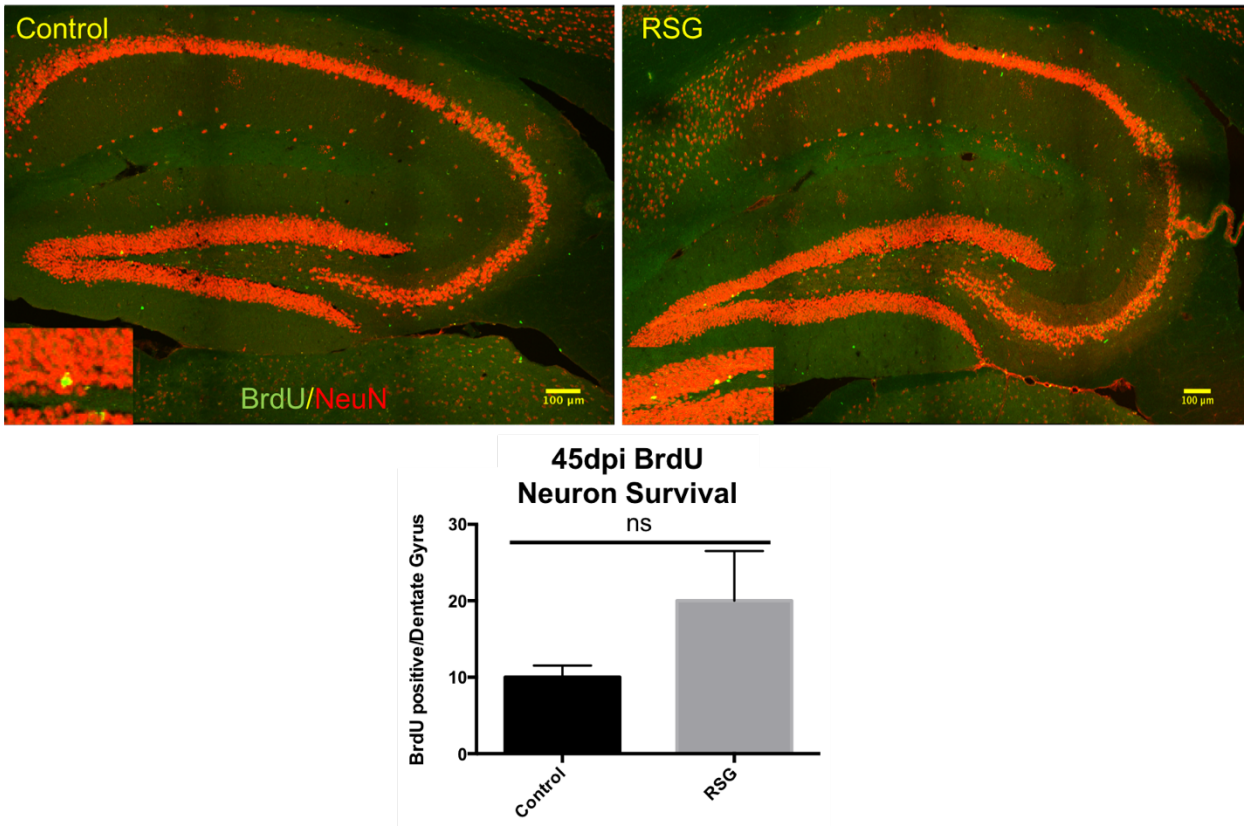

Figure 3.7: ***Long-term neuron survival in the granule cell layer of the dentate gyrus was unchanged with RSG treatment.*** Wild type animals were left untreated or treated with the PPARγ agonist RSG beginning at 8-months of age. BrdU (50mg/kg) injections were delivered for 5 consecutive days, also beginning at 8-months of age. Tissue was harvested 45 days after the last BrdU injection. Immunofluorescent images of dentate gyrus stained for BrdU (green) and NeuN (blue) from untreated, **A**, and RSG-treated mice, **B**. **C**. The number of BrdU-positive cells

were comparable between untreated and RSG-treated groups (n=3mice/group; p>0.05, Student's t test). Data represented as mean ± SEM.

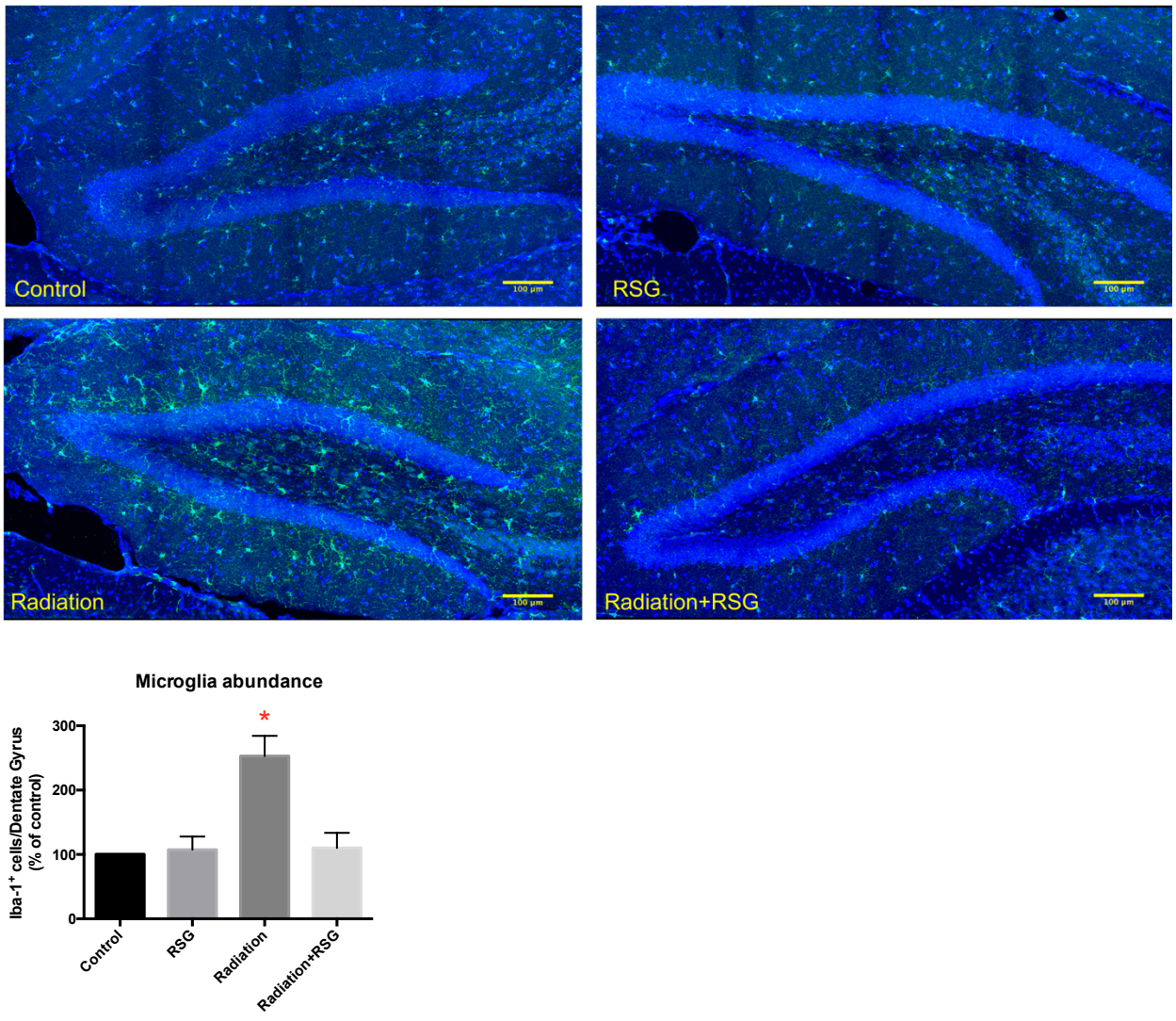

Figure 3.8: ***RSG treatment attenuated cranial irradiation-induced microglia response.*** Dorsal hippocampal sections from 9-months old wild type mice left untreated, **A**, and RSG-treated, **B**, labeled for Iba-1 (green) and DAPI (blue) were captured using confocal microscopy. **C**, Iba-1-positive cells in the dentate gyrus were significantly lower in RSG-treated mice. Data is represented as ± SEM, N= 3mice/group. Significance was established when *p<0.05, ANOVA followed by Tukey's *post hoc* multiple comparisons test.

DISCUSSION/CONCLUSION

Radiotherapy is a common treatment used to abolish metastatic tumors. In the brain though, radiotherapy can interfere with endogenous adult neurogenesis that may ultimately lead to memory impairment in young and adult patients. In fact, children and adults treated with radiotherapy can experience memory impairments months to years after final treatment. Therapeutics that reduce inflammation and restore adult neurogenesis will hasten cognitive recovery from radiotherapy.

PPARγ agonism has been shown to improve memory in models of Alzheimer's disease and humans diagnosed with mild cognitive impairment (Rodriguez-Rivera et al., 2011; Watson et al., 2005). In this regard, Denner L. et al. 2011, proposed through transcriptomic/proteomic analysis that PPARγ agonism induces transcriptional machinery that converges onto MAPK signaling to rescue hippocampal-dependent cognition in AD mice. Indeed, Jahrling et al. 2014, demonstrated PPARγ agonism mediates PPARγ/pERK formation that correlates with cognitive reserve in humans and fear conditioning performance in AD mice. Given that ERK MAPK signaling plays a role in cellular proliferation and differentiation, we set out to demonstrate if PPARγ agonism affects adult hippocampal neurogenesis dependent context discrimination.

Here we demonstrate that PPARγ agonism protects context discrimination learning and memory against cranial irradiation. Baseline activity was not altered by radiation exposure as all groups equally explored a novel open field box **(Figure 3.0)**. Furthermore, freezing in pretraining context A (Day0) and novel context C (Day1) were comparable among groups, which confirms that natural exploration behavior was

unaltered in this study **(Figure 3.1D)**. During training, conditioned fear was comparable in the first few days of testing suggesting all groups were able to recall the foot shock in context A; though mild decay in fear was observed in control, with a significant reduction observed in RSG and RSG+Radiation groups compared to radiation treated subjects, on day 4 and onward **(Figure 3.1A)**. In the innocuous similar context (B), RSG treatment reduced general fear that was heightened and maintained by radiation treated and aged control groups **(Figure 3.1B)**. While RSG seems to dampen fear response with persistent training, we measured learning by calculating discrimination ratios **(Figure 3.4A)**. That is, calculating the proportion of freezing in context A by freezing in contexts A and B. This task requires subjects to associate discrete contextual cues unique to context B to override fear associate with foot-shock grid. Subjects with context discrimination impairment will associate the shock grid to each context and have heightened fear in both context A and B. Here we demonstrate RSG reverses fear discrimination impairments induced by cranial irradiation. It appears that irradiation+RSG and RSG only subjects learn context B is not aversive as freezing declined over the course of the study when exposed to this context, but freezing in context A remain consistent. Context discrimination was comparable among groups for the first five days but on the last day, RSG improved learning in radiation treated group. This result suggests radiation and RSG do not alter initial learning and memory but with extensive training, RSG treatment can improve discrimination impairments. Moreover, area under the curve analysis revealed RSG restored discrimination to control group levels throughout the entire course of the study despite cranial irradiation **(Figure 3.4B)**. Adult hippocampal neurogenesis has been shown to correlate with performance in context discrimination learning and

memory task. In particular, the dorsal hippocampus is crucial for distinguishing between highly similar contexts (Frankland et al., 1998). Thus, we proposed any improvements with RSG treatment would result from enhanced adult hippocampal neurogenesis. Since our method of cranial irradiation obliterated adult hippocampal neurogenesis, we instead compared control and RSG treated groups to determine if RSG had an effect in aged matched controls. Here we show that 45 days of RSG improves endogenous proliferation but not differentiation and new born neuron survival in the hippocampus of 9-month old mice **(Figure 3.7).** However, after a single dose of 10Gy cranial irradiation, PPARγ agonism was only able to restore doublecortin expressing neuroblasts in the SGZ **(Figure 3.6)**. At the moment, our results indicate PPARγ agonism may play a larger role in triggering hippocampal proliferation but not neuronal maturation and survival in adult mice. We note a mild increase in new born neuron survival with RSG treatment but would need more samples to confirm any such result. Previous studies demonstrated exercise and genetic manipulation of adult hippocampal neurogenesis is essential for context discrimination learning and memory (David J Creer et al., 2010; Sahay et al., 2011; Wu et al., 2015). However, the mild presence of neuroblast in the SGZ does not help explain memory improvements by irradiated+RSG subjects, in this study. In future studies we plan to perform acute and chronic BrdU studies at 45 days post irradiation to confirm RSGs effect on adult hippocampal neurogenesis.

Memory impairment from cranial irradiation has been linked to increased microglia abundance that can lead to chronic neuroinflammation after only a single treatment. Since RSG has been shown to reduce inflammation we set out determine if RSG had any effect on the presence of microglia in the hippocampus. Briefly, microglia are resident immune cells in the brain that are

necessary to maintain homeostasis. While microglia perform complex neuro-processes like synaptic pruning and immune reactivity, microglia can become toxic during infections and neurodegenerative diseases. Using Iba-1 as a marker for total microglia, we show that RSG reduces microglia induced by cranial irradiation to control group levels suggesting context discrimination improvements may be due to a reduction in chronic microglia **(Figure 3.8)**. Future studies include performing reactive microglia studies with CD68 marker and employing CSF1R reporter mice to confirm our results.

Together, we show that RSG protects context discrimination in wild type mice exposed to cranial irradiation. In the absence of adult hippocampal neurogenesis to explain this result, we observed that a reduction of microglia by RSG may be responsible for improved discrimination performance. This initial study contributes the body of knowledge regarding adult hippocampal neurogenesis dependent context discrimination and provides evidence of microglia serving a function in learning and memory.

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CONCLUDING REMARKS

The behavioral evidence from Henry Molaison and similar medial temporal lobectomies demonstrated that, resection of the hippocampus induced permanent anterograde amnesia (Scoville & Milner, 1957). Lesion studies in rodents have confirmed these observations and identified several cognitive tasks that require an intact hippocampus such as: spatial navigation, classical fear conditioning and object recognition (Aggleton, Albasser, Aggleton, Poirier, & Pearce, 2010; Anagnostaras, Gale, & Fanselow, 2001; Morris, Garrud, Rawlins, & O'Keefe, 1982; Phillips & LeDoux, 1992, 1994). Synaptic plasticity, synaptic release, and gene expression in the hippocampus are essential mechanisms that are crucial for long term potentiation after training in any of these tasks. Additionally, adult neurogenesis, first reported in the 60s and then widely accepted in the late 90s, is posited to be crucial for pattern separation, temporal learning, and memory resolution (memory acuity) (Aimone et al., 2014; Altman & Das, 1965; van Praag, Kempermann, & Gage, 1999). Since this discovery, researchers have elucidated the biology and functional role adult hippocampal neurogenesis plays in cognitive tasks (Kempermann, 2002; Kempermann, Song, & Gage, 2015; Ming & Song, 2011). From these reports, it's evident that adult hippocampal neurogenesis is sensitive to a wide range of environmental factors from age, neurodegeneration and exercise (Ming & Song, 2011). Moreover, context discrimination appears to be strongly associated with adult hippocampal neurogenesis(Nakashiba et al., 2012; Niibori et al., 2012; Sahay et al., 2011). In fact, cranial irradiation, and genetic ablation of adult neurogenesis diminishes cognitive performance in discrimination tasks(Denny, Burghardt, Schachter, Hen, & Drew, 2012; Sahay et al., 2011). Conversely, exercise, enrichment improved discrimination and adult neurogenesis (Clemenson et al., 2015; Creer, Romberg, Saksida, van Praag, & Bussey, 2010; Denny et al., 2012; Sahay et al., 2011; Wu, Luna, & Hen, 2015).

Since context discrimination is hippocampal neurogenesis dependent, we asked: Can adult neurogenesis and context discrimination be rescued in models affected by senescence, Alzheimer's Disease and cranial irradiation? To answer this question, we aged mice with genetic attenuation of p38/MAPK activity, a protein involved in inflammation. Further, we treated the Tg2576 Alzheimer's mouse model and cranially irradiated mice with the PPARγ agonist and insulin normalizing drug, rosiglitazone.

Context fear discrimination is similar to contextual fear conditioning i.e. it challenges subjects to associate an aversive stimulus with an environment to recall at a later time, but more complex as it exposes subjects to the aversive context and highly similar yet different non-aversive context. This learning and memory task challenges an animal's ability to adapt to new environments with overlapping cues from previous aversive environments. Successful discrimination is observed when the subject significantly freezes more in the aversive context than the highly similar safe context or by observing a discrimination ratio score greater than 0.5. Subjects that are not able discriminate between highly similar contexts exhibit heightened freezing in both contexts indicating the subject is inhibited or distracted by the overlapping contextual cues and thus subjects generalize fear in non-aversive contexts.

Here we show that attenuating p38/MAPK activity in aged mice (mean age=13 months) successfully discriminated between aversive and non-aversive contexts on day 3. Whereas aged wild type littermates were never able successfully discriminate on any day of testing. Without altering baseline exploration and anxiety, partial p38 kinase activity in aged mice conserved

contextual discrimination learning and memory that was otherwise diminished in aged wild type mice. Though, improvements observed in DNp38$\alpha^{AF/+}$ mice were mild compared to 3-month old wild type mice indicating that age plays a strong role in this cognitive task. The role phospho-p38 plays in the hippocampus is dynamic and has been linked to improved performance in passive avoidance (Alonso, Bevilaqua, Izquierdo, Medina, & Cammarota, 2003). Conversely, suppressing p38 activity blocks LTP disruption in the dentate gyrus induced by IL-β injections(Coogan, O'Neill, & O'Connor, 1999). Similarly, LPS induced TNF-α increases p38 activity and is associated with neuronal death (Xing, Bachstetter, & Van Eldik, 2011). Since pro-inflammatory cytokines IL-1β and TNF-α converge on the p38 MAPK signaling then suppressing its activity can possibly be beneficial to the aging brain. To date, we are the first to describe a link between conserved context discrimination and p38α activity in aged animals. Lastly, analysis of adult hippocampal neurogenesis revealed aged mutant mice retain a pool of immature and new born neurons in the dorsal hippocampus compared to wild type mice. This result was only evident in aged but not young mice which correspond to protective effects observed in peripheral tissue from an earlier report using this mouse model (Papaconstantinou et al., 2009). Since context discrimination is correlated with intact hippocampal neurogenesis; we suggest p38α activity plays a role in the preservation of adult hippocampal neurogenesis and cognition in the aging brain.

In a model of Alzheimer's disease, Tg2576 (Swe-APP) mice exhibit accelerated synaptic degradation, LTP deficits and cognitive decline as a result of overexpression of the mutant amyloid precursor protein human gene(Hsiao et al., 1996). Our lab and others have demonstrated PPARγ agonism rescues long term memory recall in Alzheimer's mouse models and patients

diagnosed with mild cognitive impairment(Colucci et al., 2009; Escribano et al., 2010; Pedersen et al., 2006; Rodriguez-Rivera, Denner, & Dineley, 2011; Watson et al., 2005). Here we show PPARγ agonist, RSG rescues spatial learning deficits for Tg2576 in hidden platform trials. This suggests RSG Tg2576 mice learn to navigate and escape from the pool using distal cues whereas untreated Tg2576 group use generalized navigational strategy to find the hidden platform. Improved performance hidden platform testing correlates with enhanced synaptic plasticity(Morris, Davis, & Butcher, 1990). Further, RSG treated Tg2576 spent more time in the platform quadrant than untreated Tg2576 mice. This result confirms RSG treatment rescues reference memory and reverses deficits that arise from overexpression of amyloid beta. However, RSG does not reverse deficits in object recognition nor context fear discrimination learning and memory. It's possible specific memory deficits can exist while others are reversed or unaltered. In fact, dorsal hippocampal lesions impair delayed background but not foreground fear conditioning(Phillips & LeDoux, 1994). Moreover, similar lesions studies induced deficits in context fear discrimination but not foreground fear conditioning (Frankland, 1998). In humans, it's been reported that patients with probable Alzheimer's disease and hippocampal atrophy have higher errors discriminating between similar landscapes/scenes than non-demented and semantic dementia patients with perirhinal cortex atrophy. In the same study, semantic dementia patients performed poorly compared to Alzheimer's patients in word-picture test(A. C. Lee, Levi, Davies, Hodges, & Graham, 2007). Lastly, we report Tg2576 mice do not appear to have deficits in foreground fear conditioning. Together, we propose 9-month Tg2576 mice suffer from dorsal hippocampal and perirhinal deficits and RSG rescues dorsal hippocampal dependent background fear conditioning and spatial learning and memory. The data here and with previous work in our lab suggest RSG may improve spatial and associative learning and memory via

improvement in synaptic transmission and firing properties in the dentate gyrus that is independent of adult neurogenesis (Supplemental data)(Nenov, Laezza, et al., 2014; Nenov, Tempia, Denner, Dineley, & Laezza, 2014).

Cranial irradiation is commonly used as a therapy to treat patients with brain tumors. Although very effective, radiotherapy can have short and long-term effects on patient's cognition(Liyuan Zhang, 2015). Since evidence of adult hippocampal neurogenesis has been established in humans(Eriksson et al., 1998; Spalding et al., 2013), it's likely cognitive deficits can be due to loss of adult born neurons. In fact, pre-clinical studies utilize cranial irradiation as an effective strategy to induce cognitive deficits and abolish adult neurogenesis(Denny et al., 2012; Monje, 2008). Thus, we utilized radiotherapy as tool to study the effects RSG has on an adult neurogenesis dependent cognitive task. Here we show a single 10Gy dose of X-ray irradiation abolishes proliferating cellular pools in the dorsal hippocampus. As expected, loss of adult hippocampal neurogenesis was accompanied with diminished performance in context fear discrimination. Interestingly, RSG treatment: rescued context discrimination deficits induced by cranial irradiation, restored neuroblast pools but did not restore adult neurogenesis as measured by doublecortin counts. Further, RSG treatment quickly reduced generalized fear observed in X-ray irradiated only subjects. This indicates PPARγ agonism can protect discrimination learning strategies that is independent of adult hippocampal neurogenesis. We are not the first to observe improved context discrimination without improving adult hippocampal neurogenesis. For instance, (Wu, Luna, & Hen, 2015) showed running in aged mice improves context discrimination without increasing adult hippocampal neurogenesis which suggests compensatory mechanisms may arise in the absence of functional neurogenesis. Indeed, cranial irradiation has

been reported to induce chronic neuro-inflammation long after final treatment(W. H. Lee, Sonntag, Mitschelen, Yan, & Lee, 2010). Moreover, attenuating neuro-inflammation has been shown to improve cognition after cranial irradiation(Acharya et al., 2016). Here we show RSG treatment attenuated Iba-1 microglia abundance in dorsal hippocampal sections. We note that improved context discrimination may be due to dampening of chronic neuro-inflammation from persistent microglia presence in the dorsal hippocampus. Indeed, PPARγ agonism has been shown to protect hippocampal dependent cognition by blunting neuro-inflammation from LPS and modulating glial activity to clear amyloid beta in a mouse model of Alzheimer's disease(Mandrekar-Colucci, Karlo, & Landreth, 2012; Ormerod et al., 2013). Here, we conclude that PPARγ agonism protects context discrimination learning and memory by way of attenuating microglial abundance/signaling and not through adult hippocampal neurogenesis mechanisms.

Inhibitors of neuroinflammation linked proteins like p38 have developed to rescue cognition from neurodegenerative pathologies but as we demonstrated here, these antagonists should be considered as a therapy to protect adult neurogenesis in the aging brain. Further, cognitive improvement by agonism of the nuclear transcription factor PPARγ has been well documented in ours and other research labs. Although, some labs report PPARγ agonism improves cognition by restoring hippocampal neurogenesis, we demonstrate in a model of Alzheimer's disease and cranial irradiation that this is not the case. Instead, PPARγ's role could be restoring spatial and associative cognition in Alzheimer's disease specific to the dorsal hippocampus and blunting chronic microglial activity after acute insult from cranial irradiation.

Future studies include viral knock down of PPARγ in the dorsal hippocampus at different time point during training in background fear conditioning and Morris water maze to

definitively identify its role in long term memory consolidation. Lastly, long-term treatment with RSG in Tg2576 mice at 5MO and tested at 9MO should be investigated to show PPARγ's effects on parahippocampal dependent object recognition and adult neurogenesis context discrimination.

In conclusion, our results support interventions in p38α signaling can protect declining hippocampal neurogenesis and context discrimination with age. Lastly, our rosiglitazone studies support a role for PPARγ in rescuing AD related cognitive decline and cranial irradiation induced neuroinflammation.

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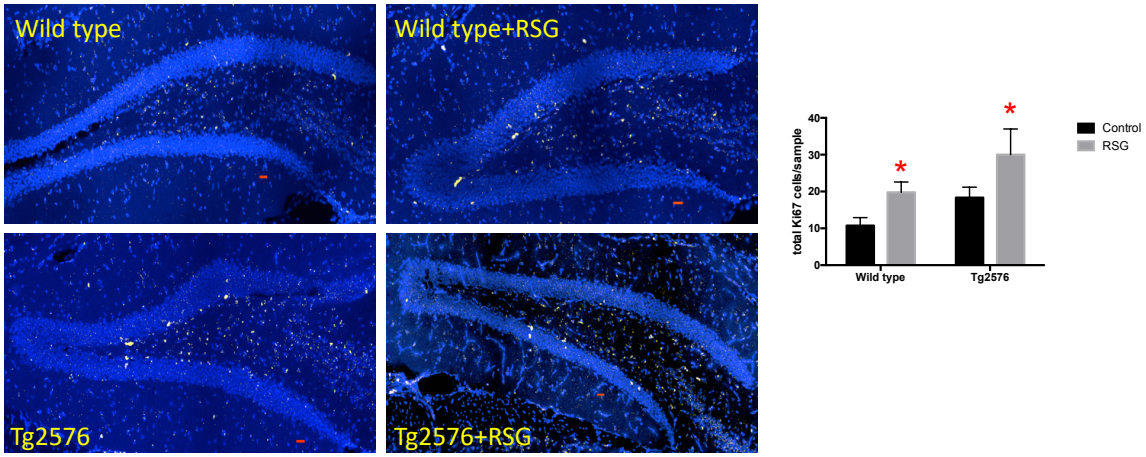
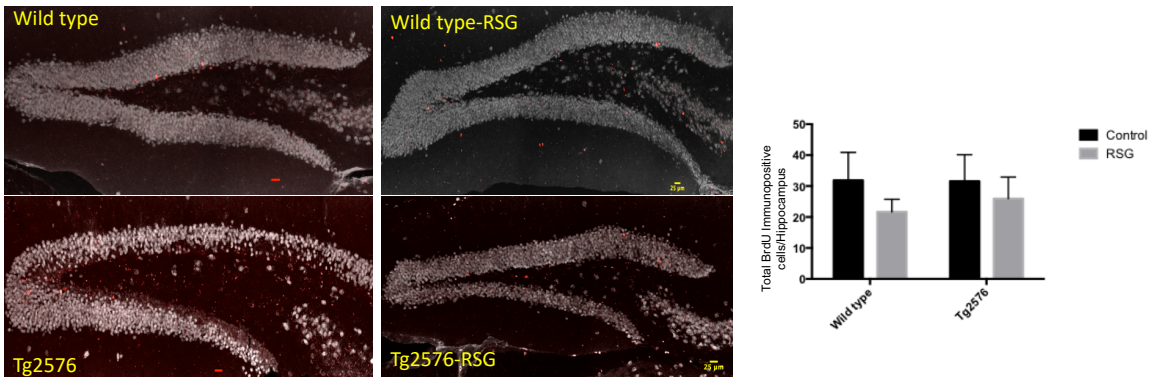
Supplemental Figures



Supplemental Figure 1: Increased mitotic marker Ki67 in Tg2576 and RSG treated mice. Sagittal sections (30μM) immunolabeled with ki67 (yellow). Total proliferating cells were identified and counted from 6 sections/mouse spanning the entire dorsal hippocampus. Red scale bars represent (25μM). N=3/group, Two-way ANOVA *pvalue<0.05.



Supplemental Figure 2: Adult born neuron survival persists equally across all groups. Sagittal sections (30μM) immunolabeled for Bromodeoxyuridine (BrdU) (red). Total new born neurons were counted from 6 sections/mouse in the granule cell layer spanning the entire dorsal hippocampus. N=3/group. Two-way ANOVA, pvalue=0.768.

Vita

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Professional Experience

Engaged in results-driven interdisciplinary research projects including, aging, behavioral neuroscience, hippocampal-learning and memory, neurodegeneration, induced pluripotent stem cells, confocal microscopy, environmental toxicology, molecular biology, and biochemistry with a history of publications in peer-reviewed journals. Routinely designed and executed large-scale behavioral assays with genetic mouse models, processed and sub-dissected brain tissue to measure learning and memory associated proteins, analyzed RNA for pro-inflammatory markers, and probed for hippocampal adult neurogenesis/microglial markers. Whilst, preparing manuscripts and grants, reviewing manuscripts, and mentoring other researchers. Highly experienced with collaborations and oral presentations. U.S. Citizen.

Research Interest

My current interest is in researching adult neurogenesis, neuro-inflammation, and hippocampus dependent learning and memory for age-related neurological diseases. Currently, my research correlates adult hippocampal neurogenesis as a mechanism for contextual discrimination learning and memory. We see that interventions in MAPK and PPARγ signaling can impact adult neurogenesis and hippocampus dependent memory in mouse models of aging, Alzheimer's disease and cranial irradiation. Further, we observed microglia abundance was affected with PPARγ agonism suggesting neuro-inflammation plays a role in hippocampus dependent learning and memory. In the near future, I would like to utilize behavioral, neurophysiological and *in vitro* techniques to reverse neuro-inflammation associated cognitive impairment in aged mouse models.

Publications

Gorgun, MF. Zhuo, M. **Cortez, D.** Dineley, KT. Englander, EW. Acute inhalation of combustion smoke triggers neuroinflammation and persistent anxiety like behavior in the mouse. Journal of Inhalation Toxicology. 2017.

Wsu, J. Wildburger, N. Haidacher, S. Nenov, M. Folorunso, T. Singh, A. Chesson, B. Franklin, W. **Cortez, D.** et al. PPARgamma agonists rescue increased phosphorylation of FGF14 at S226 in the Tg2576 mouse model of Alzheimer's disease. Journal of Experimental Neurology. 2017.

Cortez, D. Bulavin, D. Wu, P. et al. Aged dominant negative p38α MAPK mice are resistant to age-dependent decline in adult neurogenesis and context discrimination fear conditioning. Behavioral Brain Research. 2016.

Cortez, D. Viteck, C. Persans, M. Lowe, K. Seasonal detection of atrazine *atzA* in man-made waterways receiving agricultural runoff in a subtropical, semi-arid environment. World Journal of Microbiology Biotechnology. 2016.

Hernandez, C. **Cortez, D.** Gu, Z. et al. Research tool: Validation of floxed α7 nicotinic acetylcholine receptor conditional knockout mice using *in vitro* and *in vivo* approaches. The Journal of Physiology. 2014.

Education

Master of Science
Major study in Microbiology

The University of Texas-Pan American	Edinburg, Texas
Graduation Date:	August, 2011
Cumulative GPA:	3.9

Bachelor of Science
Major study in Biology; minor study in Chemistry/Psychology

The University of Texas-Pan American	Edinburg, Texas
Graduation Date:	May, 2008
Cumulative GPA:	3.36

Honors

Hispanics in Engineering, Science and Technology Scholarship	2010
UTMB Bridge to PhD fellowship	2009-2011
Excellence Scholarship	2008-2009
STARS Scholarship	2006-2008
Dean's List	2004, 2007
Annual Forum on Aging Excellence in Basic Science Research	2016
Austin's Conference on Learning and Memory Travel Scholarship	2017

Teachings at The University of Texas-Pan American

Teacher's Assistant-Introduction to Biology lab	2008-2009
Teacher's Assistant- Environmental Biology lab	2008-2010

Memberships

Texas Academy of Science	2008-2011
American Society for Microbiology	2010-2011
ISTAART	2012-2013
Society for Neuroscience	2012-Present
Molecular and Cellular Cognition Society	2012-Present

Positions Held

Treasurer Society for Neuroscience-Galveston Chapter	2014-Present
Academic Subcommittee UTMB Diversity Council	2015-2016
Officer Ultimate Frisbee at UTMB	2013-Present

Volunteer

Brain Fair-Education Outreach(Semi-annual)	2014-Present
Community Brain Health Outreach	2015-Present
UTMB United to Serve	2014-Present
Diabetic Support Group outreach	April 4, 2017

Presentations

Poster

Cortez, D. Denner, L. Dineley, K. Context Discrimination in Alzheimer's Disease and Aging Mouse Models: Contributions of PPARγ Agonism on Microglia, Neurogenesis. 2017. Austin's Learning and Memory Conference. Austin, TX.

Cortez, D. Denner, L. Dineley, K. Environmental Risk for Alzheimer's Disease. 2017. Mitchell Center for Neurodegenerative Diseases Symposium. Galveston, TX.

Cortez, D. Denner, L. Dineley, K. Context Discrimination in Alzheimer's Disease and Aging Mouse Models. Effects of PPARγ Agonism. 2017. Mitchell Center for Neurodegenerative Diseases Symposium. Galveston, TX.

Cortez, D. Denner, L. Dineley, K. Aged dominant negative p38α MAPK mice are resistant to age-dependent decline in adult neurogenesis and context discrimination fear conditioning. 2016. The Annual Forum on Aging. Galveston, TX.

Cortez, D. Denner, L. Dineley, K. Aged dominant negative p38α MAPK mice are resistant to age-dependent decline in adult neurogenesis and context discrimination fear conditioning. 2016. Society for Neuroscience. San Diego, CA.

Cortez, D. Denner, L. Dineley, K. Aged dominant negative p38α MAPK mice are resistant to age-dependent decline in adult neurogenesis and context discrimination fear conditioning. 2016. Society for Neuroscience. San Diego, CA.

Cortez, D. Hernandez, C. Dineley, K. Generation of floxed α7 nAChR mice: Validation Through Targeted Cell-specific deletion. 2013. Society for Neuroscience. San Diego. CA.

Cortez D, Lowe K. Characterization of bacteria inhabiting snake venom. 2008.Texas Academy of Science-poster presentation, Corpus Christi, TX.

Cortez D, Lowe K. Isolation and characterization of bacteria tolerant to pesticides atrazine and oxamyl for the potential use in bioremediation techniques. 2010. American Society for Microbiology-poster presentation, San Diego, CA.

Cortez D, Lowe K. Monitoring atrazine degrading and atrazine tolerant bacteria in South Texas agricultural canals using Quantitative-PCR. 2010 HESTEC- poster and oral presentation, Edinburg, TX.

Oral

Cortez, D. Denner, L. Dineley, K. PPARγ agonism attenuates reactive microglia and conserves context discrimination conditioning after cranial irradiation. 2017. Neuroscience Graduate Program Symposium. Galveston, TX.

Cortez, D. Denner, L. Dineley, K. Aged dominant negative p38α MAPK mice are resistant to age-dependent decline in adult neurogenesis and context discrimination fear conditioning. 2016. National Student Research Forum. Galveston, TX.

Cortez, D. Denner, L. Dineley, K. Context Discrimination in Alzheimer's disease model reveals adult neurogenesis mechanism. 2015. Society for Neuroscience. Chicago, IL.

Cortez D, Lowe K. Isolation and characterization of bacteria tolerant to pesticides atrazine and oxamyl for the potential use in bioremediation techniques. 2010. Texas Academy of Science. Junction, TX.

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