THE ROLE OF 5-LIPOXYGENASE IN THE STRESS-MEDIATED EXACERBATION OF THE ALZHEIMER'S DISEASE PHENOTYPE

A Dissertation
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
DOCTOR OF PHILOSOPHY

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May 2015

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ABSTRACT

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Doctor of Philosophy

Temple University, 2015

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BACKGROUND: Alzheimer's disease (AD) is the most common aging-associated neurodegenerative dementia. Current epidemiological trends indicate that a rapidly aging population, in conjunction with the economic impact of AD and lack of disease-modifying agents for AD, make AD an enormous public health challenge. AD pathology has been well characterized: it consists of extracellular plaques composed of Aβ protein and intraneuronal tangles of hyperphosphorylated tau protein. Genetic analyses of AD cases have identified causative mutations in the pathways of Aβ protein production but these mutations are rare. Therefore environmental factors that modify AD risk are of increasing importance. One such environmental factor that has received attention recently is stress. Biomarkers of stress (i.e., plasma and urinary cortisol) are associated with increased AD risk and more precipitous AD decline. Animal models have also largely recapitulated these results: stress exacerbates the AD phenotype in several studies. One of the actions of stress hormones such as glucocorticoids, is to upregulate the activity of the 5-lipoxygenase protein (5LO). 5LO is widely expressed in the central nervous system and is responsible for producing leukotrienes from arachidonic acid. 5LO has been previously shown to positively modulate Aβ production as well the phosphorylation of tau protein. Therefore,

while stress is associated with increased AD vulnerability, stress hormones modulate the 5LO protein, and the 5LO protein has been shown to modulate AD pathology, but the importance of 5LO in the stress-mediated exacerbation of the AD phenotype has not yet been explored.

HYPOTHESIS: The central hypothesis of this thesis is that 5LO plays a central role in the stress-mediated exacerbation of the AD phenotype.

METHODS: We used the 3xTg animal system, an AD transgenic mouse model which expresses both plaques and tangles and crossed 3xTg animals with 5LO knockout mice to create 3xTg animals without 5LO (3xTg/5LO-/-). We challenged both 3xTg and 3xTg/5LO-/- animals with dexamethasone (7 d, 5mg/kg i.p.) and restraint/isolation stress (28 d, 60 min/d) in separate studies to interrogate how the stress-response to Aβ, tau and fear-conditioned memory were altered by lack of 5LO in the AD context.

RESULTS: In our study with dexamethasone, we found that no memory insults occurred in either 3xTg or 3xTg/5LO-/- animals as a result of a 7 d 5mg/kg dexamethasone i.p. injection challenge. We also found no elevation in brain levels of Aβ after dexamethasone exposure, although 3xTg/5LO-/- animals had less Aβ than 3xTg animals, a finding our group has previously published. However we found that 3xTg animals had greater phosphorylation of tau and generation of insoluble tau following dexamethasone treatment. This tau pathology was

associated with elevation in GSK3β activity. 3xTg/5LO-/- animals lacked any dexamethasone-associated advancement of tau pathology or elevation in GSK3β activity.

In our study with restraint/isolation stress, we found that 3xTg/5LO-/- animals were protected against fear-conditioned contextual and cued insult recall caused by stress found in 3xTg animals. No change in Aβ was found as a function of either genotype or stress condition. As with our study with dexamethasone, we found that 3xTg animals had greater phosphorylation of tau and generation of insoluble tau following restraint/isolation stress. This tau pathology was associated with elevation in GSK3β activity. 3xTg/5LO-/- animals lacked any restraint/isolation-associated tau pathology or GSK3β activity. We additionally found that knockout of 5LO exerted a protective effect against restraint/isolation-mediated impairment in long-term potentiation.

CONCLUSION: Our work reveals, for the first time, the importance of the 5LO protein in stress-mediated exacerbation of the AD phenotype. These data indicate that 5LO-targeted interventions could be of use in individuals vulnerable to this environmental risk factor, and more broadly, in a preventative strategy against AD.

DEDICATION

This thesis is dedicated to my family—to my grandparents who raised me, my parents who propelled me, and my younger sister, who simultaneously vexes and energizes me (as all little sisters ought!).

ACKNOWLEDGMENTS

We see farther and more than our predecessors not because we have keener vision or see from greater heights but because we are picked up and borne aloft on the shoulders of giants.

If I have had any success, it is because I have been lucky enough to be in the right situation at the right time, taking advantage of the patience of those who are much wiser. I would like to thank Dr. Domenico Praticò for his role as a mentor both professionally and personally. I am unable to imagine another individual who would have trained me to think scientifically about clinical problems as well and with such incredible vigor and excitement. The bar has been set high, perhaps unreasonably so.

I also deeply appreciate and value the time and thoughtfulness of my committee members as they have guided me: Drs. Barrie Ashby, Nae Dun, Lynn Kirby, and Ellen Unterwald. The process of becoming a scientist is an active one and my committee has helped me in that process in wonderfully supportive and incredibly constructive ways. Their efforts have ensured I have a fighting shot at negotiating the vast marketplace of science that is the modern biomedical enterprise. In that spirit, I also thank Dr. Vinay Parikh for agreeing to be an external reader and devoting his time and energy to that goal.

I am also indebted to all former and current members of the lab, but especially Jin Chu, Jian-Guo Li, Phillip Giannopoulos, Antonio Di Meco, Elisabetta Lauretti,

Ni Meng, Jiamin Zhou, and Simone Puccio for their help technically, but also in making the lab a great place to work.

A special acknowledgement also must go out to Dr. Margaret Sperow for aiding with electrophysiology studies that improved our understanding of 5LO and the work presented in thesis. Her time and commitment yielded incredible dividends.

The greater Temple community requires mention. In particular, I could not have asked for a better MD/PhD program director than Dr. Dianne Soprano—her constant watchful eye was much appreciated in negotiating the nuanced aspects of medical and graduate school. Her advice has always been on-target, something I have heavily relied upon over the past seven years. To Tracy and Tracey: I would be spinning my wheels without your excellent and much-needed direction. I also very much appreciate the back-and-forths and insights provided by many faculty, but especially Drs. Scott Shore and Bassel Sawaya over the years. To the clinical mentors over the past two years too numerous to mention: I have learned much from your examples—time to put it into practice.

To my friends –a veritable rogue's gallery— currently or formerly in medical school, graduate school, MD/PhD program, or otherwise: thank you for preserving my sanity and commiserating with me when it did me some good (and also at other times when it did not). And also for some exceptional fun and havoc that we shared/caused (specific individuals, of course, shall necessarily remain nameless).

And of course, Rina—for everything.

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LIST OF ABBREVIATIONS

5LO	5-lipoxygenase protein
ACTH	adrenocorticotrophic hormone
AD	Alzheimer's disease
ADAM	a disintegrin and metalloproteinase
ALOX5	5-lipoxygenase gene
APH-1	anterior-pharynx defective protein 1
APP	amyloid β precursor protein
Aβ	amyloid beta
BACE1	β-APP cleavage enzyme 1
C83	carboxy-terminal fragment 83
C99	carboxy-terminal fragment 99
cdk5	cyclin-dependent kinase 5
CRF	corticotrophin-releasing factor
CRH	corticotrophin-releasing hormone
fEPSP	Field excitatory postsynaptic potential
FDA	Food and Drug Administration
FLAP	5-lipoxygenase activating protein
GR	glucocorticoid receptor
GSK3β	glycogen synthase kinase 3 β
HPA	hypothalamus-pituitary-adrenal
i.p.	intraperitoneal
LT	leukotriene
LTP	long-term potentiation
MAPT	microtubule associated protein tau
NFT	neurofibrillary tangle
NMDA	N-methyl-d-aspartate
Pen2	presenillin enhancer 2
PET	positron emission tomography
PS1	presenillin 1
sAPPα	soluble APP fragment α
sAPPβ	soluble APP fragment β

CHAPTER 1

INTRODUCTION

Alzheimer's disease: background

Alzheimer's disease (AD) is the most common aging-associated neurodegenerative dementia. The condition is eponymously named after the German psychiatrist and neuropathologist Dr. Aloysius "Alois" Alzheimer, who described the case of one Auguste D. in a 1906 lecture at the 37th Conference of South-West German Psychiatrists in Tublingen (Hippius 2003). He described the 51-year old Auguste D. as having significant cognitive impairments, hallucinations, delusions and erratic behavior, with near total impairment in her daily activities of living.

Clinically, AD is characterized by progressive, irreversible memory loss and global cognitive decline. In the vast majority of cases, symptoms typically manifest late in life, usually after the age of 65. Current estimates place the prevalence of AD at over 5 million in the United States alone, with approximately 11% of 65 year and older, and 32% of 85 years and older thought to have symptomatic AD (Alzheimer's Association, 2013). Given improvements in the past century in biomedical research, healthcare technology, and therapeutic development in multiple arenas, median lifespan has increased, with larger numbers of the aged surviving into their 80s, 90s and beyond. In parallel, the "baby boomer" generation, a cohort born between the 1940s-1960s thought to

comprise ~20% of the population, has begun to reach the age of 65 beginning in 2010 (Alzheimer's Association 2013). These trends of increased longevity as well as a rapidly aging of the population have important implications for the future burden AD. For example, current projections indicate that incidence of AD would increase from 2010 estimate of ~450,000/year to over 900,000/year in 2050 (Alzheimer's Association, 2015). Similarly, by 2025, prevalence of those 65 and older with AD will reach over 7 million, from 5 million currently, reaching over 13 million by 2015.

While AD clearly affects patients and their families, its toll on the healthcare system is staggering with 2014 estimates placing the cost of AD at over $200 billion USD—at current projections, this cost is believed to eclipse $1 trillion in 2050. While the public health challenge from AD is immense, the contemporary clinical armamentarium is woefully inadequate as no FDA-approved therapies exist to modify disease course, and are limited to cholinesterase inhibitors and N-methyl-d-aspartate (NMDA) receptor antagonists (Farlow and Cummings, 2007).

Alzheimer's disease: molecular pathology of amyloid β and tau

Over the last several decades, the brain pathology described by Dr. Alzheimer has been well-characterized. In the AD brain, two classical lesions have been discovered: an accumulation of extracellular plaque composed of aggregated amyloid β (Aβ) peptides and intraneuronal aggregation of neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein (Querfurth and LaFerla, 2010; Holtzman et al., 2011; Iqbal et al., 2010). Investigation of these two

molecules in the past forty years has revealed that their metabolism and dysfunction are crucial in the development of not only the molecular brain pathology of AD but also its behavioral sequelae.

Aβ is formed by the sequential cleavage of the Aβ precursor protein, APP, through a number of cleavage steps as shown in Figure 1. APP belongs to an evolutionarily conserved family of type 1 transmembrane glycoproteins (Goldgaber et al., 1987, Wasco et al., 1993a; Wasco et al., 1993b). While its physiological function is unclear, APP is thought to play a role in several processes, including in neural development and neuronal transport (Nicolas and Hassan, 2014). Nevertheless, APP is expressed in neurons and may be acted on by a number of cleavage enzymes leading to peptides of different lengths (Haass et al., 2012; Brunholz et al., 2011). Classically, two different cleavage fates of APP are thought to be relevant in the AD context: the non-amyloidogenic pathway, and the amyloidogenic pathway.

In the non-amyloidogenic pathway, APP is cleaved by the α-secretase, resulting in a soluble APPα fragment (sAPPα) and an 83kd transmembrane protein (C83) whose products are not involved in AD pathology (Anderson et al., 1992). Proteins in the ADAM family (a disintegrin and metalloprotease domain) are thought to mediate this cleavage action, with ADAM 9, 10, 17 and 19 showing evidence of possessing this activity (Allinson et al., 2003; Tanabe et al., 2007).

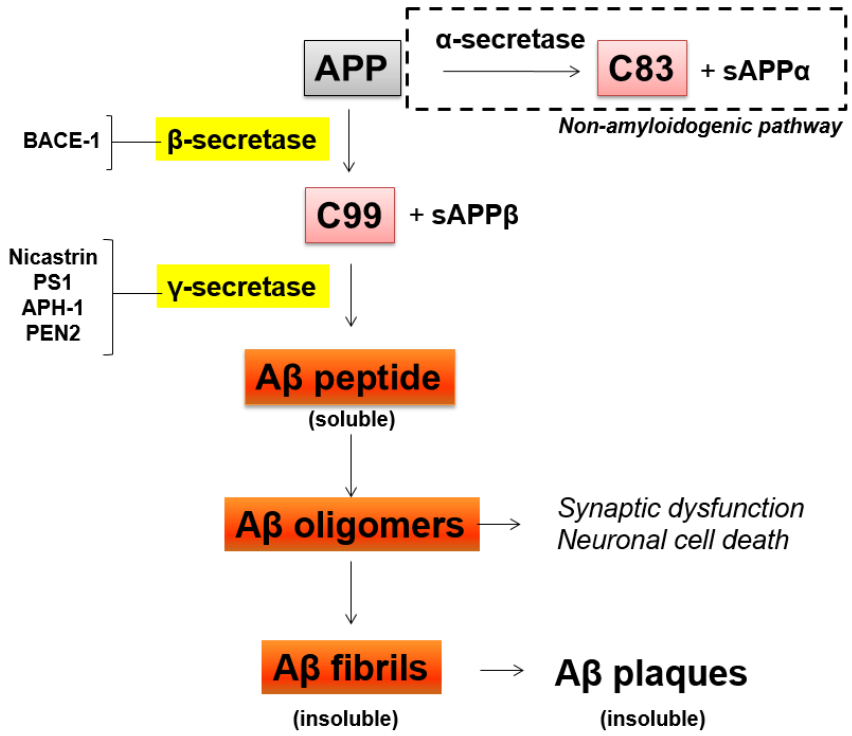

Figure 1 Production of Aβ from the Aβ precursor protein

Aβ precursor protein (APP) is either cleaved by α-secretase to yield non-amyloidogenic products, or sequentially cleaved by β-secretase and then γ-secretase to yield Aβ peptides. Aβ peptides aggregate, forming oligomers eventually forming Aβ plaques. Current evidence indicates Aβ oligomers may directly cause synaptic dysfunction and neuronal cell death seen in Alzheimer's disease.

In the pro-amyloidogenic pathway, APP is cleaved first by the β secretase, resulting in a soluble APPβ fragment (sAPPβ) and a 99kd transmembrane protein (C99) (Palmert et al., 1989). β-secretase activity is thought to be carried out by the β-site APP cleaving enzyme 1 (BACE-1) (Schonlein et al., 1993). The C99 product produced by BACE-1 is then free to be acted on by the γ secretase (Citron et al., 1995; De Strooper et al., 1993). Unlike α and β secretases, the γ secretase is a four-protein enzyme composed of the following proteins: presenillin, nicastrin, anterior pharynx defective 1 protein (APH1), and presenillin enhancer 2 (PEN-2) (Lu et al., 2014; Fraering et al, 2004). These proteins compose the γ secretase in a 1:1:1:1 stoichiometry. Cleavage of C99 by γ secretase occurs within the lipid bilayer (i.e., intramembranous cleavage) and is thought to be promiscuous leading to the production amyloid β peptide of different lengths, with the majority of products being between 39 and 43 amino acids long (Murphy et al., 1999). Aβ is normally found in the brains of both healthy controls as well as AD patients, but its physiological function has not been clarified (Puzzo and Arancio, 2013). However, it is thought that in AD, the pro-amyloidogenic pathway is privileged.

Amyloid β proteins have the ability to self-aggregate, and in human brains, $A\beta_{42}$ peptides have been found to be the most prone to aggregative behavior (Tabaton and Gambetti, 2006; Yang et al., 1995). As Aβ peptides aggregate they form low-*n* oligomers (i.e, dimers, trimers, etc.), continuing to aggregate until they form fibrils, β-pleated sheets, and, ultimately, aggregates that form histopathologically-

significant plaques in brain parenchyma (Serrano-Pozo et al., 2011). Aβ pathology localizes to cerebral cortex, entorhinal cortex and hippocampus and basal ganglia but Aβ progression is somewhat variable and not robustly correlated with behavioral decline (Serrano-Pozo et al., 2011; Bennet et al., 2006; Knopman et al. 2003). Typically, significant amyloid deposition in subcortical nuclei and cerebellum does not occur unless the disease stage is advanced (Braak and Braak, 1997).

While it was originally believed that plaques cause the majority of cellular damage in AD, current data suggest that low-*n* oligomers cause AD-associated neuronal dysfunction and death (Ono and Yamada, 2011). Although future work on the biophysical properties of Aβ peptide conformation, length, and concentration are needed to clarify specific pathophysiological mechanisms of Aβ neurotoxicity, some themes have emerged.

The Aβ peptide itself is thought to possess oxidative properties. The sulfur atom of methionine 35 in Aβ peptide has been shown to produce free radicals (Butterfield et al., 2013; Yatin et al., 1999). This observation seems to support significant oxidative damaged observed in AD brains post-mortem wherein oxidatively damaged proteins, lipids and nucleic acids are abundantly found (Pratico 2008). Replacing methionine with cysteine greatly reduces oxidative damage in model organisms such as *C. elegans*, and substitution of sulfur with a methylene moiety reduces oxidation *in vitro* of the Aβ peptide (Dai et al. 2007).

Additionally, Aβ species have been shown to bind to acetylcholine and N-methyl-d-aspartate (NMDA) receptors, both critical for learning and memory, causing not only disruptions in synaptic plasticity and long-term potentiation (LTP) but also excitotoxicity, leading to cell death (Nalbantoglu et al., 1997; Kim et al. 2001; Hynd et al., 2004; Ikonomovic et al 2009; Lombardo and Maskos, 2014; Medeiros et al., 2011). Amyloid β monomer injection into the brains of adult rats leads to inhibition of hippocampal LTP, which supports the idea that memory insults may be tied to amyloid β concentration (Walsh et al. 2002). Many other pathologic mechanisms have been described, including Aβ-associated disruption of appropriate calcium signaling, interaction with transition metals (i.e., Cu, Fe, Zn), reduction of neuron-supportive trophic factors and pathologic modulation of non-neuronal cells including astrocytes and glia (Kawahara 2010; Savelieff et al., 2013; Zhang et al., 2012). Based on such evidence, Aβ peptide is likely a multifaceted molecular target in AD-associated neurodegeneration.

In addition to amyloid β, tau also plays a critical role in AD. Tau protein, the major pathologic component of NFTs, is the predominant microtubule-associated protein found in neurons in the adult brain. In humans, six tau isoforms are generated from alternatively splicing of the mRNA transcript of the *MAPT* gene found on chromosome 17 (Binder et al., 1985, Andreadis et al., 1992; Neve et al. 1986) . The isoforms of tau are classified based on the presence of either of 3 or 4 microtubule binding domains (3R, or 4R), or 0, 1 or 2 aminoterminal inserts of 29 amino acids (Goedert and Jakes, 1990). Tau promotes assembly and

stabilization of microtubules which maintains appropriate axonal cargo trafficking and ultrastructure (Mandelkow et al., 1995). Tau function and its microtuble-associating and –stabilizing functions are heavily influenced by its phosphorylation. The protein sequence of tau contains over 80 serine/threonine-proline phosphorylation motifs, regulated by a balance of tau-associated kinases as well as phosphatases, as shown in Figure 2 (Buee et al. 2000).

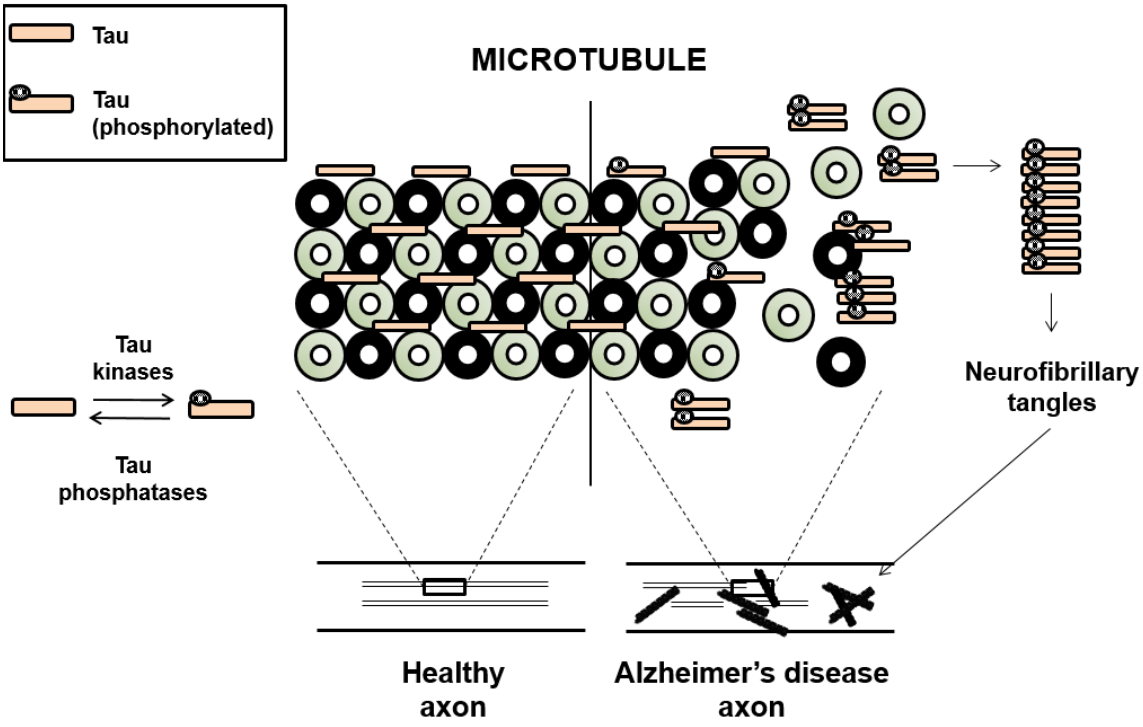

Figure 2 Microtubule associated tau protein in healthy neurons and in Alzheimer's disease. In healthy axons, tau is associated closely with microtubules, stabilizing them and allowing for appropriate cargo trafficking. In Alzheimer's disease, hyperphosphorylated tau dissociates from microtubules, destabilizing them, and aggregating such that neuronal ultrastructure and appropriate cargo trafficking is disrupted.

In AD, tau is hyperphosphorylated, and tau-associated kinases, such as the glycogen synthase kinase 3 (GSK3) and cyclin dependent kinase 5 (cdk5) have been found to be abnormally regulated in the brains of AD patients (Hanger et al., 1992; Baumann et al., 1993). Upon hyperphosphorylation and dissociated with microtubules, tau begins to oligomerize, forming paired helical filaments (Badiola et al., 2010). As aggregation proceeds, tau species become insoluble, evolving into intraneuronal tangles distinguishable in stained gross brain specimen. The distribution of neurofibrillary tangles stereotypically begins in the entorhinal cortex and hippocampus, spreading to limbic structures, and with advanced disease, various areas of the cerebral cortex (Serrano-Pozo et al., 2011). In contrast to amyloid plaque burden, tau tangle burden correlates with severity and duration of dementia, and corresponds to temporal profile of cognitive and behavioral insults seen in patients (Nelson et al., 2007). Tau-associated neurotoxicity in AD is thought to be mediated due to microtubule disruption which results in abnormal trafficking of cell cargo such as mitochondria, which lead to abnormal cell energetics and generation of reactive oxygen species (Iikima-Ando et al., 2012). In addition tau-associated synaptic disruption and loss has been described in several animal models, and is thought to be closely linked to tau-associated disruption of calcium homeostasis (Reynolds et al., 2008; Usardi et al. 2011; Liao et al., 2014; Sul et al., 2009; Pierrot et al., 2006).

While both amyloid β and tau pathologies are found in AD, amyloid β has historically been considered the cardinal pathology due to a number of genetic data. First, rare familial forms of AD with mutations in APP and secretase proteins are associated with early development of AD and accelerated decline (Bagyinszky et al., 2014). Recent data have also revealed protective APP mutations reducing risk of AD (Jonsson et al., 2012; Jonsson et al., 2013). Additionally, the presence of an extra chromosome 21, as seen in trisomy 21 (Down's syndrome), which increases brain levels of Aβ, dramatically increases risk of AD in those patients (de la Monte, 1999). Based on these observations, the amyloid hypothesis of AD has been posited, which dictates that Aβ is central to the pathology of AD (Hardy and Selkoe, 2002). In part due to the amyloid hypothesis of AD, significant effort has been made to devise clinical interventions aimed at either reducing brain levels of amyloid β peptide. Although a number of amyloid-targeted therapeutics have been attempted in clinical trials, they either suffered from unacceptable adverse events, or have failed to achieve therapeutics goals (Mullane and Williams, 2013). While controversial, these results have brought to light potential weakness in the amyloid hypothesis of AD. Critics of the amyloid hypothesis maintain that significant and repeated failures of direct amyloid β targeted therapy imply that amyloid β does not play an important enough role in AD to be crucial to therapeutic development (Giacobini and Gold, 2013; Teich and Arancio, 2012). On the other hand, amyloid hypothesis apologists point out that amyloid β therapy is carried out when AD symptoms are manifest, at a point where the molecular damage of AD may be unsalvageable

(Toyn and Ahlijanian, 2014). Resolution of these issues will require large clinical trials aimed at addressing pathology at a pre-symptomatic state in patients.

Stress and the HPA axis

The feedback pathways by which stressful stimuli alter an organism's biology have been characterized, and revolve around a feedback system involving the hypothalamus, pituitary, and adrenal glands (HPA; HPA axis) (for a full review, see Aguilera, 2011). In response to psychological or physiological stress, corticotrophin-releasing factor (CRF; also called corticotrophin-releasing hormone) is secreted from the paraventricular nucleus of the hypothalamus, as seen in Figure 3. CRF acts on the neuroendocrine cells of the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), which enters circulation induces secretion of stress hormones, such as glucocorticoids, from the adrenal glands**.** The primary glucocorticoid in humans is cortisol which binds to mineralocorticoid as well as glucocorticoid receptors (GRs), forming complexes that translocate to the nucleus to modulate patterns of gene expression. Emerging evidence also indicates that in the brain there exist membrane-associated receptor sensitive to corticosteroids which may explain rapid changes in cell physiology too fast for genomic modulation (Dorey et al., 2011). The level of circulating glucocorticoids is kept in homeostatic balance through negative feedback mediated by several brain regions. Application of synthetic glucocorticoids reduces the excitability of paraventricular nucleus cells and lowers their secretion of CRF through an endocannabinoid-mediated reduction in

presynaptic glutamate release (Di et al., 2003). Cortisol, corticosterone, and dexamethasone exposure to the anterior pituitary leads to reduction of ACTH production, reducing circulating CRF and glucocorticoid levels, providing another locus for glucocorticoid level regulation (Widmaier and Dallman, 1984). Others brain regions such as prefrontal cortex, amygdala and hippocampus have also been implicated in glucocorticoid and HPA axis homeostasis (McEwen et al., 1968; Sapolsky et al., 1990).

The vast majority of circulating endogenous glucocorticoids is bound by corticosteroid binding globulin while a small portion is bound to serum albumin, with only the free steroids acting on tissues (Moisan et al., 2014). Under basal physiological conditions, glucocorticoid levels follow patterns of discrete pulsatile release, approximately hourly, punctuated by low levels between release, with the highest circulating levels occurring in the early morning and the lowest levels occurring in the evening (Nicolaides et al. 2014). However, this periodicity and circadian cycle of glucocorticoid release is plastic and can be greatly influenced by different physiological contexts or disease states.

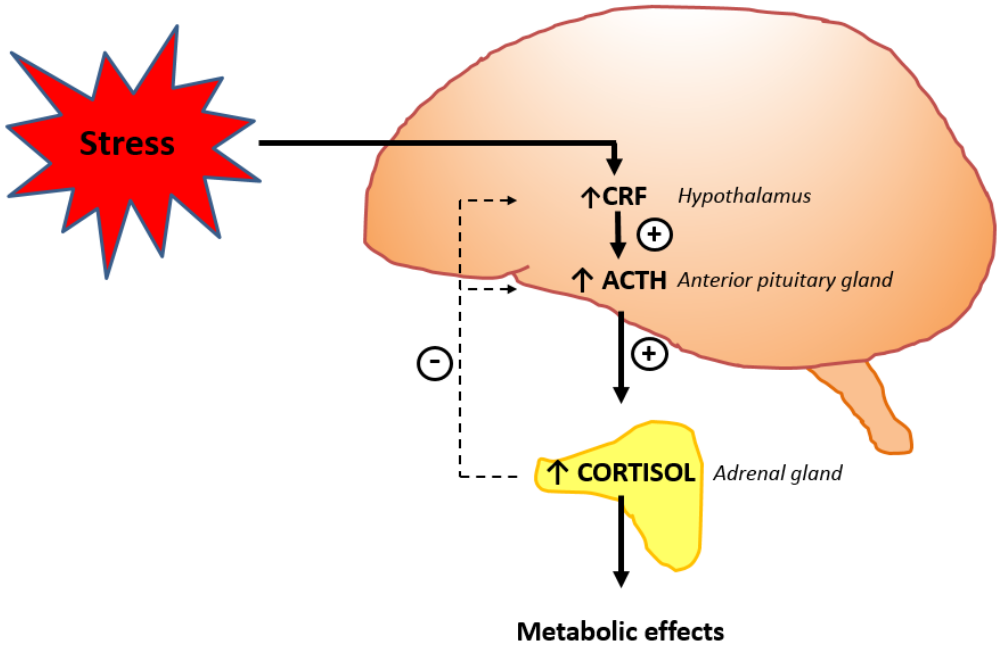

Figure 3 Stress and Hypothalamus-Pituitary-Adrenal (HPA) axis. Stressful stimuli activate the HPA axis. In response to stress, CRF is released from the hypothalamus, which in turns stimulates ACTH release anterior pituitary, which acts on the adrenal gland to release cortisol, the principle corticosteroid in humans. With healthy HPA axis functioning, cortisol negatively feedbacks at multiple levels of the HPA axis.

Glucocorticoid theory of brain aging

Decades of investigation have revealed that glucocorticoids and stress are critical modulators of memory, with significant attention being paid to the effects of glucocorticoid on the hippocampus, a limbic system structure essential to memory (Sapolsky, 2000; Scoville and Milner, 1957).

The hippocampus is a medial temporal lobe structure which consists of densely-packed layers of neurons that has been compared in appearance to a ram's horn. The hippocampus is part of a larger hippocampal formation which includes the dentate gyrus, subiculum, and entorhinal cortex (Li et al., 2009). Work using electrophysiological and anatomical techniques has revealed that informational flow through the hippocampus is generally unidirectional, receiving input from multiple brain regions (Jacobson and Sapolsky, 1991). In addition to the formation of new memories, the hippocampus is thought to be particularly important for spatial navigation.

Because aging is the strongest non-modifiable non-genomic risk factor for AD, Landfield and colleagues originally posited the hypothesis that glucocorticoids promote brain aging based on studies in rodents which indicated corticosteroids produce aging-associated neurodegenerative changes in hippocampus, a brain locus where GRs are richly expressed (Landfield et al., 1991; Landfield et al., 2007; Sapolsky et al, 1986)**.** Moreover, several experimental data supported the importance of the hippocampus in regulating the HPA axis. For example, complete or partial destruction of the hippocampus increases plama

corticosteroid levels in response to stressors, while electrical stimulation of the hippocampus decreases corticosteroids elvels in a number of species (Jacobson and Sapolsky 1991). Hippocampal efferents modulate the activity of CRF-containing neurons at multiple sites, although there is data to support the notion that input from the hippocampus is an important modulator of paraventricular nucleus neurons (Herman et al. 1992).

Since hippocampal neurons are important negative feedback regulators of the HPA axis, it was conjectured that extended stress or glucocorticoid exposure and aging acted cooperatively to produce cognitive decline (Conrad and Bimonte-Nelson, 2010; Jameison and Dinan, 2001; Jacobson and Sapolsky, 1991). Animal and human studies revealed elevations in circulating stress hormones correlate with cognitive decline (Montaron et al., 2006;), and longitudinal studies link cortisol with loss of hippocampal volume (Knoops et al., 2010). Extended activation of the HPA axis and elevation of stress hormones also structurally change the hippocampus, resulting in atrophy and altered metabolism (de Quervain et al., 2003). Functionally, positron emission tomography (PET) and functional magnetic resonance imaging have revealed that stress/glucocorticoid administration reduces blood flow and decreases hippocampal activation during memory retrieval tasks (Symonds et al., 2012; Oei et al., 2007). Based on these data, stress, and stress hormones have been understood to be a risk factor for cognitive decline associated with brain aging in general.

Stress and Alzheimer's disease

While inherited mutations in APP and γ-secretase proteins have been discovered that lead to early-onset AD in several families, these AD cases only account for ~5% of all disease incidence. Since purely genetic factors are unable to account for the majority of AD incidence, environmental factors are thought to play a significant role in AD vulnerability, progression and severity. Of those environmental factors, recent attention has been placed on the role of stress and dysfunction of the hypothalamus-pituitary-adrenal axis in AD. Because stress is an unavoidable aspect of life, understanding of how stress modulates AD vulnerability is useful both for the development of clinical therapeutic strategies as well as investigation of AD disease biology.

Several human studies have been carried out in humans that suggest stress and stress hormones are important in AD. AD patients display higher basal salivary cortisol levels than controls and higher HPA activity, as measured by plasma cortisol, correlates with more severe disease progression in mild and moderate cases of AD (Arsenault-Lapierre et al., 2010; Rasmuson et al., 2002; Csernansky et al., 2006). Post-mortem analyses of cerebrospinal fluid (CSF) cortisol levels also show a similar trend between AD and age-matched controls (Hoogendijk et al., 2006). In elderly patients without detectable dementia, higher levels of chronic distress are associated with greater risk for development of mild cognitive impairment, which some have argued to be a prodrome for AD, as well as AD itself (Wilson et al., 2006). Higher urinary cortisol excretion is also associated

with greater incidence of cognitive impairment (Karlamangla et al., 2005). In AD patients, hyperactivity of the HPA axis also negatively correlates to hippocampal volume, with lower volume being associated with lower scores on neuropsychological batteries of episodic and visuospatial memory (Elgh et al., 2006; Huang et al., 2009). Administration of exogenous glucocorticoids, such as prednisone has also been reported to cause behavioral decline in AD patients (Aisen PS, et al., 2000).

Many of these observations have been recapitulated in several animal studies. In rodents, suppression of glucocorticoids from mid- to late-life increases neurogenesis in the hippocampi of aged animals while chronic long-term activation of the HPA axis results in cognitive dysfunction and reduction in neurogenesis (Hu et al., 2012; Montaron et al., 2006). Work by multiple investigators has shown that behavioral stress, across a variety of paradigms (including restraint, isolation and/or immobilization stress), worsens AD-like pathology and exacerbates memory impairments in various rodent models of AD (Jeong et al., 2006; Carroll et al., 2011). Administration of synthetic glucocorticoids as well as endogenous corticosteroids also exacerbates the AD-like phenotype, while corticosteroid antagonists are protective (Dong et al., 2008; Dong et al., 2004; Green et al., 2006). In wild-type animals, stress also dramatically augments cognitive insults of Aβ peptide infusions in the brain (Lee et al., 2009).

In addition to stress and glucocorticoids, a role for CRF has been implicated in the pathogenesis of AD. CRF exposure acutely elevates brain Aβ levels and phosphorylated tau in transgenic AD animals which is prevented by using CRF antagonists (Kang et al., 2007). Overexpression of CRF in an AD mouse also results in faster progression of the AD phenotype, while disruption of the CRF receptor results in normalization of pathology (Dong et al., 2012; Rissman et al., 2012). These early results seem to indicate that stress, in addition to elevating glucocorticoids, facilitates neurodegeneration through CRF. However, there is discrepancy between those studies and other data derived from both animal as well cell culture, in which CRF is shown to protect against AD (Facci et al., 2003; Scullion et al., 2013)

Other important aspects of the HPA axis, including the neuroactive properties of the mineralocorticoid receptor and ACTH, are less well studied and may also play a role in AD. Additionally, since the HPA axis can also be modulated by circulating catecholamines such as epinephrine and norepinephrine, further investigation of these in the context of stress may prove fruitful, but have not yet been systematically carried out.

These data, taken as a whole, support the idea that stress is an environmental factor actively involved in the neurobiology of AD.

5-lipoxygenase

Lipoxygenases (LOs) are a family of evolutionarily conserved, non-heme iron-containing enzymes that are present across plant and animal kingdoms. LOs are so named because of their enzymatic action, inserting molecular oxygen into free and esterified polyunsaturated fatty acids. LO nomenclature is such that individual enzymes are named by their positional specificity, i.e., 12-lipoxygenase inserts molecular oxygen into carbon-12 of fatty acids. Thus, the protein 5-lipoxygenase (5LO), coded by the arachidonate 5-lipoxygenase gene (*ALOX5)* inserts molecular oxygen into the carbon-5 of free or esterified fatty acids, most notably arachidonic acid as shown in Figure 4 (Radmark and Samuelsson, 2010). In order to carry out this reaction, 5LO also requires the 5LO activating protein, or FLAP, which presents substrate for enzymatic action (Sampson, 2009). Immediate products of 5LO include unstable 5-hydroperoxyeicosatetraenoic acid which is either reduced to 5-hydroxyeicosatetraenoic acid or leukotriene A_4 (LTA_4) (Radmark et al., 2014). Depending on the cellular milieu, LTA_4 can be metabolized either to leukotriene B_4 or C_4 (LTB_4 or LTC_4), with LTC_4 further being metabolized to LTD_4 and LTE_4. Both LTB_4 and cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) have G-protein coupled receptors (respectively: BLTR1 and BLTR2; and cysLTR1 and cysLTR2) which have been linked to immune cell chemotaxis and are critical molecular players in the inflammatory pathophysiology of asthma and allergy (Tantisira and Drazen, 2009).

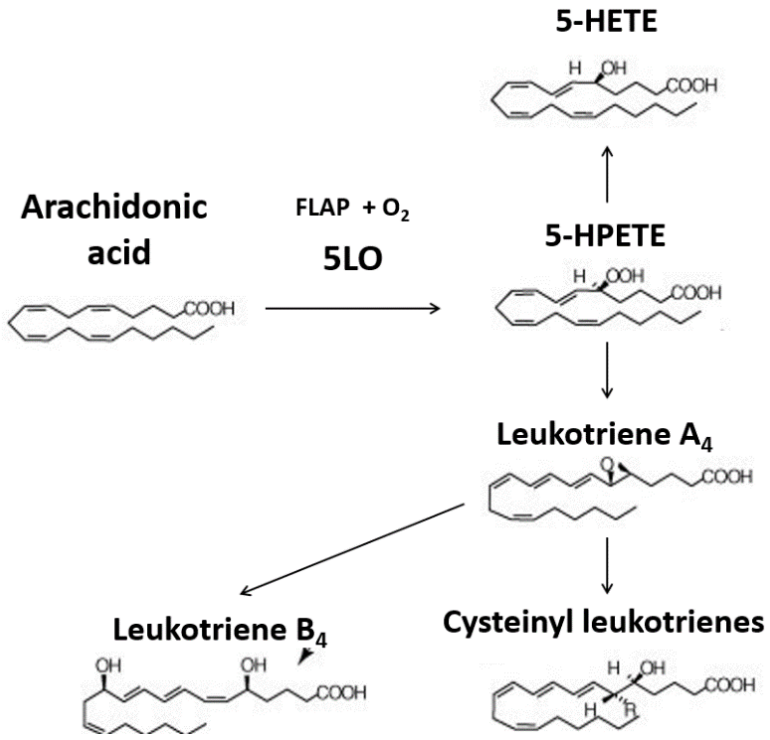

Figure 4 Metabolism of arachidonic acid by 5-lipoxygenase 5LO uses arachidonic acid as substrate to produce 5-HPETE, which is metabolized to LTA_4 which can be metabolized to either LTB_4 or the cysteinyl leukotrienes (i.e., LTC_4,D_4 and E_4) depending on the cellular milieu. 5LO carries out this reaction with the FLAP protein, requiring molecular oxygen. Adapted from Giannopoulos et al., 2014.

Although the vast majority of published work investigating 5LO focuses on its role in health and disease outside the brain, its role in neurobiology is just now being fully appreciated. 5LO is found in vasculature, in endothelial cells, as well as throughout the central nervous system, in both neurons and glia (Lindgren et al., 1984; Miyamoto et al., 1987; Pergola and Werz, 2010; Mamot et al., 1995; Matsuo et al., 1995). Over the last 30 years, 5LO and its metabolites have shown to be important molecular players in traumatic brain injury (Corser-Jensen et al., 2014), neuroendocrine hormone release (Dan-Cohen et al., 1992), ischemic stroke (Shi et al., 2013), cerebral edema (Di Gennaro et al., 2004), brain tumor-associated vascular permeability (Nathoo et al., 2006; Soderstrom et al., 2003; Baba et al., 1994), brain inflammation (Kellom et al. 2012), cerebellar granule neuron proliferation (Uz et al., 2001), prion protein neurotoxicity (Stewart et al., 2001), spinal cord injury (Ersahin et al., 2012), cocaine sensitivity (Chen and Manev, 2011), neurotrophic hormone modulation (Dzitoyeva et al., 2008), central modulation of hypertension (Waki et al., 2013), and Parkinson's disease (Kang et al., 2013). Interestingly, 5LO expression and its metabolites increase with age in both animal models as well as human subjects (Uz et al., 1998; Manev et al., 2000). 5LO protein levels are particularly enriched in both cortex as well as hippocampus, areas known to be particularly vulnerable in neurodegeneration, and in AD in particular (Chinnici et al. 2007; Lammers et al., 1996).

Glucocorticoids and 5-lipoxygenase

While glucocorticoids are known to possess anti-inflammatory effects in the periphery, their effects in brain tissue is more nuanced. Recent evidence has indicated that glucocorticoids positively modulate 5LO in the brain.

For example, male Sprague-Dawley rats administered exogenous corticosterone or dexamethasone for 10 d displayed increased 5LO protein expression in hippocampus as well as cerebellum (Uz et al., 1999). In primary neuronal cultures of rat cerebellar granular neurons, dexamethasone increased 5LO protein and mRNA, which was inhibited by both glucocorticoid receptor antagonism and application of glucocorticoid receptor-specific antisense oligonucleotides (Uz et al., 2001).

5-lipoxygenase and Alzheimer's disease

A small pilot study in humans has linked 5LO gene polymorphisms to early- and late-onset AD (Qu et al., 2001), although large-scale population studies have yet to confirm these findings. On immunohistochemical analyses in human AD brain, 5LO colocalizes with amyloid β plaques and NFTs (Ikonomovic et al., 2008). In neuronal cells harboring the Swedish APP mutation, a well-accepted *in vitro* model of AD, knockout of 5LO reduces the production and release soluble Aβ (Chu and Pratico, 2011a). This effect is due to reduced activity of γ-secretase, with 5LO knockout lowering steady-state expression of nicastrin, PS1, APH--1 and Pen-2 proteins without modulating either APP, the β-secretase or α-secretase expression (Chu and Pratico, 2011a). This effect is also seen when

5LO is disrupted in an AD mouse model of amyloidosis, the Tg2576 mouse (Firuzi et al., 2008). On the other hand, overexpression of 5LO produces the opposite effect *in vitro*: Aβ levels are elevated, and associated with increased protein and mRNA levels of all four γ-secretase proteins (Chu et al., 2012). Importantly, pharmacological inhibition of 5LO also reproduces 5LO knockout effects (Chu and Pratico, 2011b).

Although γ-secretase action produces Aβ peptides, which are pathologic in AD, it is also involved in the processing of other substrates, including Notch, a protein critical for appropriate neuronal functioning and differentiation. Clinical trials investigating γ-secretase inhibitors for use in AD have to date been largely unsuccessful because these agents are hypothesized to have altered Notch production as well as Aβ production (Imbimbo et al., 2011). However, with 5LO modulation, γ secretase-dependent Notch production is unperturbed, making any future and potential use of this class of drug a feasible alternative to γ-secretase inhibitors (Chu and Pratico, 2011a).

By directly controlling the substrate availability for 5LO, FLAP modulation similarly affects Aβ production with FLAP knockout and inhibition reducing Aβ through reduction in γ-secretase expression. *In vitro* data in neural cells have been reproduced in mouse models of AD-like amyloidosis with similar effects of 5LO and FLAP on Aβ, which includes immunohistochemical evidence of plaque reduction upon 5LO or FLAP knockout (Giannopoulos et al., 2013). Recently,

these data have also been reproduced in TgCRND8 animals, with reduction found in amyloid-associated angiopathy upon administration of MK886, an inhibitor of FLAP (Hawkes et al., 2013)

In addition to Aβ, 5LO also modulate tau phosphorylation. In cells overexpressing 5LO, endogenous tau is hyperphosphorylated, generating both early-stage, as well as advanced-stage tau phosphoepitopes (Chu et al., 2013; Chu and Pratico 2013). Beyond tau phosphorylation, 5LO overexpression results in production of paired helical filaments of tau, precursors to insoluble tau deposition and neurofibrillary tangle formation. Knockout or inhibition of 5LO or FLAP results in amelioration of tau hyperphosphorylation. Both *in vivo* in murine models and *in vitro*, 5LO pathway changes are mediated by 5LO and FLAP influence on cyclin-dependent kinase 5 activity (Chu et al., 2013; Chu and Pratico, 2013).

As previously discussed, abnormalities in Aβ metabolism are presumed to be upstream of tau phosphorylation but 5LO effect on tau phosphorylation seems to be independent of Aβ. In a series of *in vitro* experiments, γ-secretase blockade in the presence of 5LO overexpression did not prevent 5LO-mediated hyperphosphorylation of tau (Chu et al., 2012). These *in vitro* results have also been corroborated in mouse models of amyloidosis such as the Tg2576 mouse (Chu and Pratico, 2011).

Beyond neuropathology, knockout of 5LO or FLAP mitigates age-dependent AD learning and memory impairments seen in two different models of AD. In both Tg2576 and 3xTg mice, 5LO knockout or inhibition is associated with improvements in learning and memory over baseline in fear conditioning paradigms (Giannopoulos et al., 2014, Chu et al., 2012) while overexpression of 5LO is associated with greater cognitive deficits (Chu et al., 2011; Giannopoulos et al., 2013; Giannopoulos et al. 2013). Knockout or inhibition of 5LO and FLAP also ameliorates reducation of synaptic protein levels synaptophysin and post-synaptic density protein 95 (Giannopoulos et al., 2013, Giannopoulos et al., 2014), in AD animals and restores hippocampal long-term potentiation to wild-type levels. Even in aged transgenic mice, when significant AD pathology has been deposited, targeting the 5LO pathway improves the overall AD phenotype (Di Meco et al. 2014)

Taken together these data support the idea that 5LO plays a central role in the development of the AD phenotype.

Summary: Alzheimer's disease, stress and 5LO

AD is an aging-associated neurodegenerative condition which is costly and poised to be a tremendous health challenge given a rapidly aging population. This dilemma is further underscored by the fact that our current therapeutic armamentarium is limited and does not modify AD course. While causative genetic mutations of AD have been identified, this knowledge is of limited utility in

over 95% of AD cases, which do not possess such mutations. Moreover pharmacolopical intervention using currently available FDA-approved medications does not change disease course, and is especially of limited utility when AD symptoms are apparent. These factors have led to significant interest in modifying environmental risk factors that advance AD. One such risk factor is stress. Both pharmacological and behavioral stress are positively associated with increased AD vulnerability and precipitous decline in humans and have been shown to accelerate the molecular pathology of AD in animal models and cell culture.

One of the pleotropic actions of stress hormones, glucocorticoids, is to upregulate the 5LO protein in the brain. Glucocorticoid administration of corticosteroids *in vitro* increase both the mRNA and transcription of 5LO in multiple brain regions. Intriguingly, 5LO modulates AD-associated amyloid β metabolism, tau phosphorylation and memory impairment in animal models of AD. In a preliminary study, our group has reported *in vitro* evidence supporting a link between stress hormones, glucocorticoids and AD (Puccio et al. 2011). Our group has found in neuroblastoma cells, dexamethasone administration result in increased 5LO activity, which leads to a dexamethasone-dependent increase in AD pathology. In mouse embryonic fibroblasts in which 5LO is knocked out, dexamethasone fails to achieve this effect.

Thus, while evidence suggests that stress and 5LO, separately, are involved in AD pathogenesis, and stress hormones modulate 5LO, the question of whether 5LO is relevant to the stress-induced pathology and cognitive decline of AD is still unanswered.

Hypothesis

Based on the literature presented above, our central hypothesis is that 5LO plays a functional role in the stress-associated exacerbation of the AD phenotype.

Approach and Aims

In order to test our hypothesis we employed an *in vivo* approach using the 3xTg mouse model of Alzheimer's disease. 3xTg animals evolve both amyloid plaques and neurofibrillary tau tangles as a function of aging, exhibiting worsening memory impairments and learning deficits. We crossed 3xTg mice with mice null for 5LO to yield 3xTg/5LO-/- animals and subjected both 3xTg and 3xTg/5LO mice to a short course of dexamethasone, as well as a longer course of restraint/isolation stress, a significant behavioral stressor. Using this general strategy to test our central hypothesis, we sought to address the following Aims:

Aim 1: Does 5LO modulate dexamethasone-mediated acceleration of the AD phenotype?

1.1 Does 5LO knockout protect against dexamethasone-mediated memory impairment?

1.2 Does 5LO knockout protect against dexamethasone-mediated amyloid β production?

1.3 Does 5LO knockout protect against dexamethasone-mediated tau phosphorylation?

Aim 2: Does 5LO modulate the behavioral stress-mediated acceleration of the AD phenotype?

2.1 Does 5LO knockout modulate behavioral stress-mediated memory impairment?

2.2 Does 5LO knockout protect against behavioral stress-mediated amyloid β production?

2.3 Does 5LO knockout protect against behavioral stress-mediated tau phosphorylation?

CHAPTER 2

EFFECT OF 5LO KNOCKOUT ON THE DEXAMETHASONE-INDUCED ALZHEIMER'S DISEASE PHENOTYPE

Introduction

While Alzheimer's disease (AD) is a neurodegenerative disease of late life, non-heritable environmental factors are thought to accelerate accumulation of AD pathology, Aβ plaques and tau tangles, well before cognitive decline is detected. Recent evidence has suggested that high circulating levels of glucocorticoids could be one such environmental factor. AD patients display higher levels of circulating glucocorticoids than age-matched controls and higher levels of glucocorticoids confer greater risk of developing dementia in cognitively intact aged persons (Rasmuson et al., 2002; Csernansky et al., 2006). In animal models of AD, dexamethasone and corticosterone administration has been linked to acceleration of the AD phenotype, while administration of glucocorticoid receptor antagonists such as mifepristone have been linked to reduced Aβ and tau pathology (Green et al., 2006; Carroll et al., 2011; Jeong et al., 2006)

Stress hormones such as glucocorticoids have multiple pleotropic effects depending on tissue type. One such effect is increasing expression of the 5-lipxoygenase protein, a protein expressed in the central nervous system whose expression increases with aging, whose functioning is critical to the production of leukotrienes (Uz et al., 1998; Uz et al., 1999; Uz et al., 2001; Chinnici et al.,

2007). *In vitro*, pharmacological inhibition of 5LO blunts dexamethasone-mediated AD pathology, while knockout of 5LO reduces amyloid burden in a mouse model of AD amyloidosis (Chu and Pratico, 2011). Therefore, while glucocorticoids accelerate AD neuropathology and upregulate 5LO, and, separately, knockout of 5LO reduces AD neuropathology, it is still an unanswered question whether 5LO contributes to the *in vivo* glucocorticoid-mediated exacerbation of the full spectrum of the AD--like phenotype, which includes amyloid and tau pathology, as well as behavioral deficits.

In the present study, we investigated how knockout of 5LO modulated the effect of dexamethasone treatment in the triple transgenic (3xTg) mouse model of AD, a system in which both the signature pathologies, Aβ plaques and neurofibrillary tau tangles are expressed. In the below study, 3xTg and 3xTg genetically deficient for 5LO (3xTg/5LO-/-) were challenged with a 7 d course of dexamethasone and its effect on memory, Aβ and tau were investigated in both animal strains.

Materials and Method

Animals

All animal procedures were approved by the Institutional Animal Care and Usage Committee, in accordance with the National Institutes of Health Guidelines. All animals were housed on a 12-h light/dark cycle in the Medical Research Building at the Temple University Health Sciences Campus, which is fully accredited by

the Association for the Assessment and Accreditation of Laboratory Animal Care International. Standard chow and water were provided *ad libitum*.

Naïve female 3xTg and 3xTg/5LO-/- mice aged 3.5 months were used for this study. The 3 x Tg mouse harbors a mutant APP (KM670/671NL), a human mutant PS1 (M146V) knock-in, and tau (P301L) transgenes (Oddo et al., 2003). To generate the 3xTg/5LO-/- mouse, mice lacking 5LO (5LO-/-) were cross-bred with 3xTg mice to generate founder 3xTg/5LO-/- animals (Goulet et al., 1991). These 3xTg/5LO-/- animals were backcrossed at least 10 generations, with the resulting progeny used for all studies. Mice were euthanized by CO_2 administration and brains were removed 24h after behavioral studies. Cortices were dissected from one hemibrain and stored -80º C while the other hemisection was fixed in paraformaldehyde overnight, processed and paraffin-embedded, and used for immunohistochemistry analyses.

Dexamethasone administration

Animals were either given phosphate-buffered saline (PBS) as control, or dexamethasone intraperitoneally (DEX; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 5mg/kg for 7 d, n=5 3xTg (PBS), n = 7 3xTg (DEX), n = 4 3xTg/5LO-/- (PBS), n = 5 3xTg/5LO-/- (DEX). Intraperitoneal injections of dexamethasone were always given between 0800 and 1000 hours.

Behavioral paradigms

For behavioral assessment of working memory the Y-maze paradigm was used, and to assess hippocampal- and amygdala-associated learning and memory recall the fear conditioning paradigm was used. Y-maze behavior was carried out one day after last dexamethasone injection, and fear conditioning was carried out one ay after Y-maze behavior was assessed. For all test on both apparatuses, 70% ethanol was used to thoroughly clean all surfaces after every trial.

The Y-maze apparatus consists of three arms 32 cm long x 10 cm wide with 26 cm walls (San Diego Instruments, San Diego, CA, USA). Animals were placed in the central portion of the maze and allowed to freely explore for 5 min. Entries into each arm were recorded using a video camera. An entry was counted if all four paws entered an arm. An exit from an arm was counted if all four paws left an arm. In addition to total entries, total alternation percentage was also recorded. An alternation was defined as three consecutive arm entries into different arms. Total alternation percentage was derived using the formula: total number of alternations /(total entries -2).

For fear conditioning, tests were conducted in a fear conditioning chamber with black methacrylate walls, a speaker, and grid floor (Start Fear Systems, Harvard Apparatus, Holliston, MA, USA) and was carried out over 2 days. On day 1, mice were placed in the conditioning chamber for 2 min to freely explore before the onset of a 2000Hz neutral tone of 90dB which lasted 30 sec. The last 2 sec was

paired with a 0.6 mA scrambled foot shock. Mice were removed from the chamber 1 min after the shock. A total of 3 pairs of foots shocks and tones were delivered with 30 s intervals without footshocks or tones between trials. On day 2, mice were assessed for contextual and cued recall. Freezing behavior was defined as movement recorded by the system associated with animal breathing in the absence of any ambulation. For contextual fear recall, mice were put back into the same conditioning chamber for 5 min and their freezing behavior was recorded. In this phase, no neutral tones were sounded and no shocks were delivered. Cued fear recall was assessed ~3 h after contextual fear recall was assessed. For cued fear recall, mice were placed back into the chamber with altered floor, odor, color, and lighting for 5 min. The same tone used for training was sounded at the 2 min mark, with 3 tones delivered, exactly as with the training phase with 30 seconds between tones. The percentage of time spent freezing was used for calculation for the training, contextual and cued recall phase.

Biochemical analyses

Mouse brain cortical homogenates were sequentially extracted first radioimmunoprecipitation assay buffer (RIPA), containing EDTA-free protease-inhibitor (Roche, Indianapolis, IN, USA) and phosphatase inhibitor (ThermoFisher) for the amyloid β soluble fractions and then in formic acid (FA) for the amyloid β insoluble fractions as previously described (Giannopoulos et al. 2014). Amyloid β1-40 and amyloid β1-42 levels were assayed by a sensitive sandwich enzyme-linked immunosorbent assay kit in accordance to the

manufacturer's protocols (Wako Chemicals, USA). All biochemical assays were performed in triplicate per animal sample.

Immunoblotting

Brain homogenate RIPA or pH-neutralized FA samples were electrophoretically separated using 10% Bis-Tris gels of 3-8% Tris-acetate gel (Bio-Rad, Philadelphia, PA, USA), according to the molecular weight of the target molecular and then transferred into nitrocellulose membranes (Bio-Rad). Membranes were blocked with Odyssey-blocking buffer and then incubated with primary antibodies overnight at 4ºC. After three washing cycles in TBS-T, membranes were incubated with IRDye secondary antibodies (LI-COR; Lincoln, NE, USA) at 22 ºC for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR, Lincoln, NE, USA). Actin was always used as the loading control. Antibodies and dilutions were as follows: HT7 (1:200, Cell Signal, Danvers, MA, USA), AT180 (1:200, Pierce, Morris Plains, NJ, USA) PHF13 (1:200; Pierce), PHF1 (1:200, a generous gift from Dr. Peter Davies), GSK3α/β (1:200; Santa Cruz, Dallas TX, USA), cdk5 (1:200; Santa Cruz), p35/p25 (1:200, Santa Cruz), and β-actin (1:1000, Santa Cruz)

Immunohistochemistry

Immunohistochemistry analysis was performed as previously described (Chu and Pratico, 2011). Briefly, serial 6 µM thick coronal sections were mounted on 3-aminopropyl triethoxysilane (APES)-coated slides. Brain sections were deparaffinized, hydrated, bathed in 3%H_2O_2 in methanol and treated with 10mM

citric acid to retrieve antigen. Sections were blocked in 2% fetal bovine serum before incubated with primary antibody against PHF13 (1:20) or PHF1 (1:200) overnight at 4ºC. Sections were washed, incubated with appropriate secondary antibody and developed using the avidin-biotin complex method (Vector Laboratories, Burlingame, CA, USA) with 30, 30-diaminobenzidine as chromogen.

Kinase activity

Kinase activities of GSK3β and cdk5 were carried out as previously described (Joshi et al. 2012). Briefly, GSK3β and cdk5 were coimmunoprecipitated from 75 µg cortical lysate using 1.5 µg respective antibodies. The immunoprecipitates were washed with lysis buffer three times followed by once by HBS buffer (10-mM HEPES, pH 7.4, 150 mM NaCl). Activity of GSK3β was determined using phosphor-glycogen synthase peptides 2 (Millipore, Billerica, MA, USA) as substrate while cdk5 activity was determined using histone H1 (Santa Cruz Biotechnology, Dallas, TX, USA) as substrate. In both experiments immunoprecipitates were incubated in HBS containing 15 mM $MgCl_2$, 50 mM ATP, 1 mM dithiothreitol, and 1 µCi of [32P] ATP. After 30 min incubation at 37* C, the reaction products were determined by a liquid scintillation counter. Kinase activity assays were carried out in triplicate for each animal sample.

Statistical analysis

Data are presented as the mean ± standard error of the mean. One-way analysis of variance (ANOVA), with Bonferroni multiple comparison tests were performed

using Prism 5.0 (GraphPad Software, La Jolla, CA). All data are presented as mean +/- standard error of the mean. Significance was set at $p < 0.05$. While the studies contained in this thesis are published, during the course of this candidate's dissertation defense, it was pointed out the two-way ANOVA may have been a better choice for statistical analyses, and therefore could have led to slightly different conclusions than stated.

Results

Dexamethasone does not impair fear-conditioned recall in 3xTg or 3xTg/5LO-/- animals

It has been previously shown that dexamethasone administration impairs learning and memory in transgenic AD animals (Caroll et al., 2011). In order to see to see how this stress system behaved in our hands, as well as observing the effect of dexamethasone administration in animals lacking 5LO, we administered a 7 d course of dexamethasone at a dose of 5mg/kg to 3xTg and 3xTg/5LO-/- animals as has been previously reported (Green et al., 2006). We first assessed animals in the fear conditioning paradigm. In the training phase, no differences in genotype or treatment were observed as shown in Figure 5A, with freezing percentage $\pm$ S.E.M. as follows: 3xTg, 80.83 $\pm$ 6.96%; 3xTg/DEX, 73.45 $\pm$ 7.16%; 3xTg/5LO-/-, 72.93 $\pm$ 12.45%; 3xTg/5LO-/- DEX, 75.5 $\pm$ 13.46%, $p<0.94$. After ensuring that animal had similar freezing behavior in the training phase, we then assessed memory in the contextual recall portion of the fear conditioning paradigm. Once again no differences were found, as shown in

Figure 5B with percentage of freezing behavior ± S.E.M. for each condition as follows: 3xTg, 91.52 ± 2.1%; 3xTg/DEX, 86.51 ± 3.15%; 3xTg/5LO-/-, 82.68 ± 6.13%; 3xTg/5LO-/- DEX, 86.84 ± 2.9%, p<0.43. Following assessment of contextual recall, we assessed cued recall in all animals and conditions as shown in Fig 5C. In this phase too, we were unable to detect any changes in freezing behavior, with percentage freezing behavior ± S.E.M. as follows: 3xTg, 50.17 ± 4.87%; 3xTg/DEX, 53. ± 7.57%; 3xTg/5LO-/-, 60.25 ± 4.90%; 3xTg/5LO-/- DEX, 40.86 ± 2.9%, p<0.317

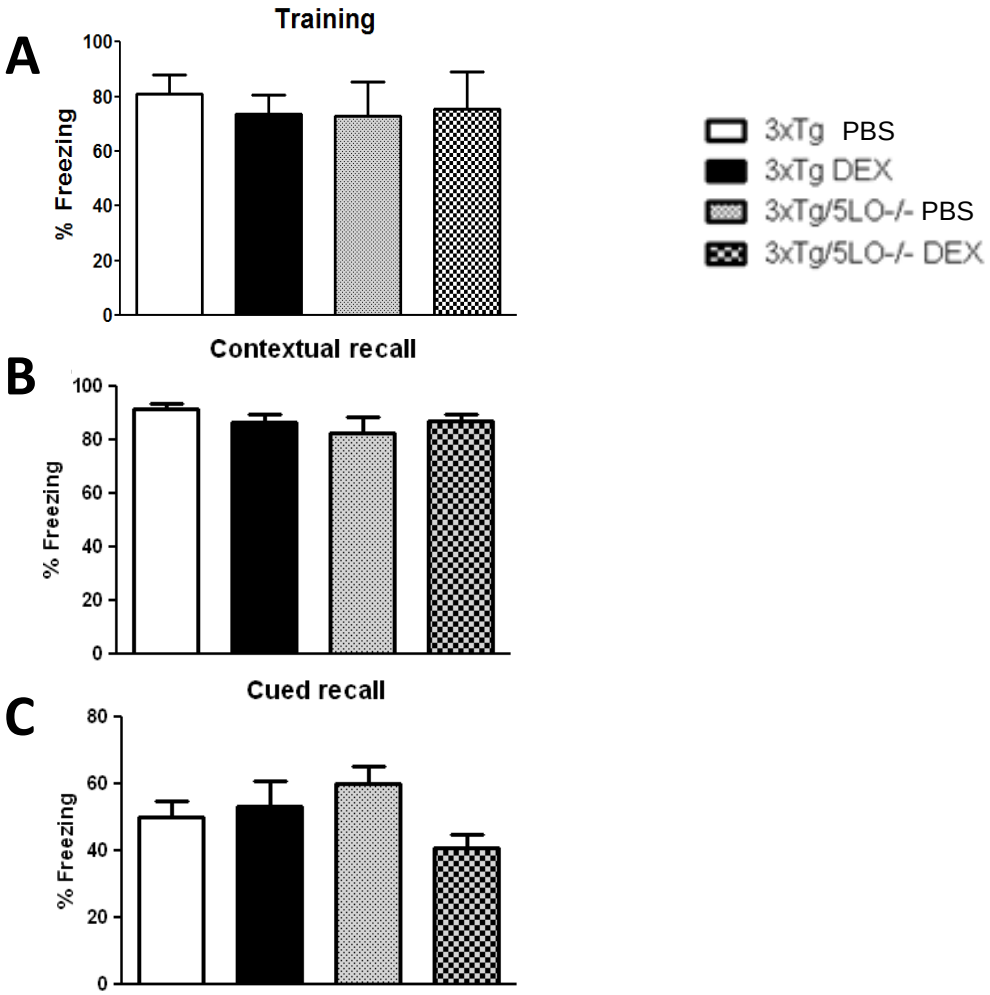

Figure 5 Fear conditioned memory in 3xTg and 3xTg/5LO-/- animals given PBS or dexamethasone. Freezing behavior in the (A) training phase, (B) contextual recall phase, and (C) cued recall phase of the fear conditioning paradigm. 3xTg animals treated with PBS, i.p. (n=5), 3xTg animals treated with dexamethasone (DEX), 5mg/kg i.p. for 7d (n= 7), 3xTg/5LO-/- animals treated with PBS (n=4), i.p., and 3xTg/5LO-/- animals treated with dexamethasone (DEX), 5mg/kg i.p. for 7d (n=5) were used for this study.

Dexamethasone does not change Y maze behavior in 3xTg and 3xTg/5LO-/- animals

Although we did not observe any changes in fear conditioned behavior in dexamethasone-treated animals, dexamethasone has been described to affect short-term memory in addition to long-term memory. It is possible that insults to short-term memory may have not been adequately assessed through the fear-conditioned memory paradigm. To assess short term memory in our animals, we utilized the Y-maze paradigm. As shown in Figure 6A, we found no difference between overall arm entries between all groups (3xTg, 18.25 $\pm$ 4.78; 3xTg/DEX, 22.00 $\pm$ 4.39; 3xTg/5LO-/-, 15.25 $\pm$ 3.12; 3xTg/5LO-/- DEX, 11.8 $\pm$ 2.07, $p<0.23$). Similarly, we did not find any differences in alternation percentage as shown in Figure 6B (3xTg, 59.69 $\pm$ 7.851%; 3xTg/DEX, 70.82 $\pm$ 12.19%; 3xTg/5LO-/-, 62.55 $\pm$ 10.10%; 3xTg/5LO-/- DEX, 50.98 $\pm$ 13.46%, $p<0.50$)

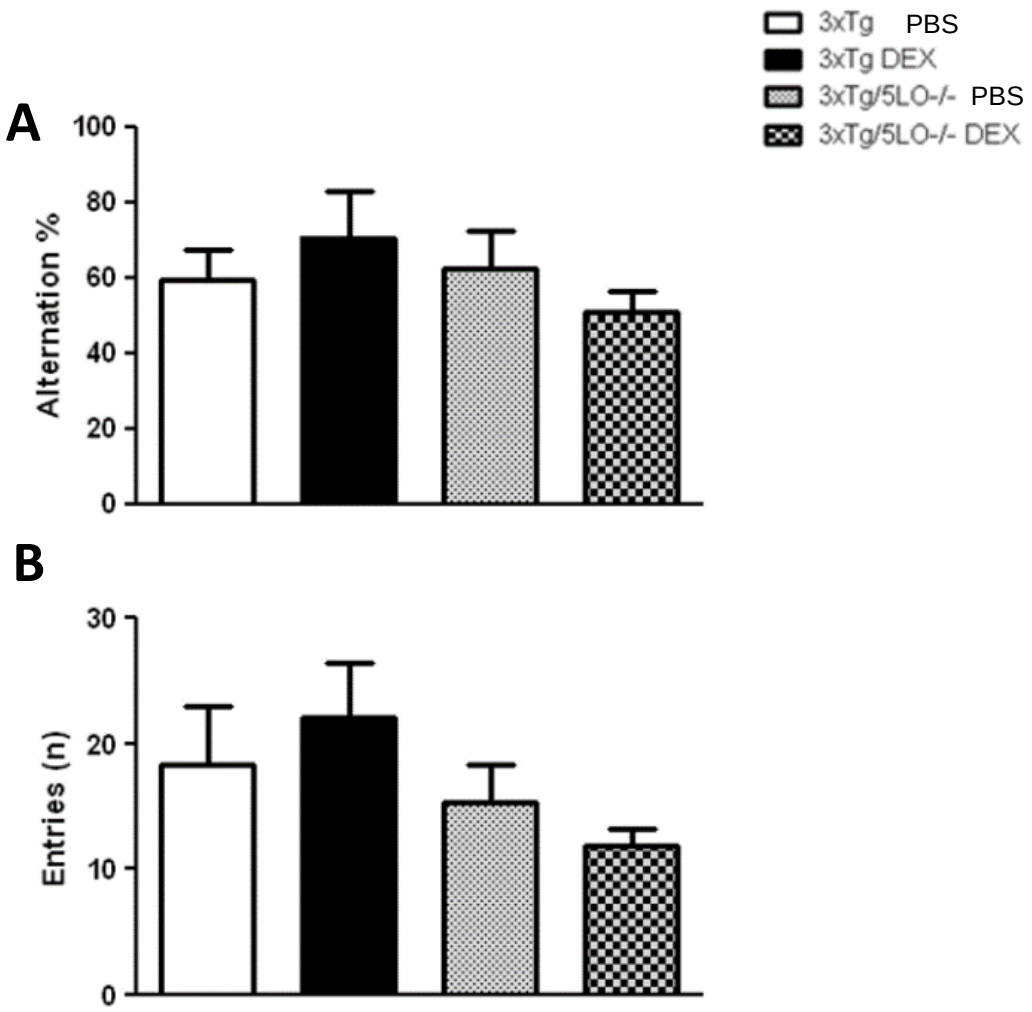

Figure 6 Y maze behavior in PBS or dexamethasone-treated 3xTg and 3xTg/5LO-/- animals 3xTg animals treated with PBS, i.p. (n=5), 3xTg animals treated with dexamethasone (DEX), 5mg/kg i.p. for 7d (n= 7), 3xTg/5LO-/- animals treated with PBS (n=4), i.p., and 3xTg/5LO-/- animals treated with dexamethasone (DEX), 5mg/kg i.p. for 7d (n=5) were assessed in the Y maze paradigm for both alternation percentage as well as total entries.

Dexamethasone does not increase amyloid levels in 3xTg or 3xTg/5LO-/-animals

Because there have been several reports that dexamethasone administration increases soluble Aβ species (Green et al., 2006; Carroll et al., 2011), we next assayed the cortical homogenates for soluble $A\beta_{40}$ and $A\beta_{42}$ (Joeng et al., 2006). We found that a 7 d treatment of dexamethasone was not sufficient to significantly alter $A\beta_{40}$ or $A\beta_{42}$ as shown in in Figure 7. However, we found, as we have previously reported, that overall amyloid β levels were significantly lower in 3xTg/5LO animals than in 3xTg animals (Giannopoulos et al., 2013; Giannopoulos et al., 2014). Using protein-corrected values with 3xTg animals as baseline we found that soluble $A\beta_{40}$ levels $\pm$ S.E.M. were as follows: 3xTg, 100.0 $\pm$ 3.55%; 3xTg/DEX, 82.91 $\pm$ 6.98%; 3xTg/5LO-/-, 51.71 $\pm$ 5.65%; 3xTg/5LO-/- DEX, 45.13 $\pm$ 14.03%, *$p<0.03$. Similarly corrected soluble $A\beta_{42}$ levels were found to be as follows: 3xTg, 100.0 $\pm$ 3.94%; 3xTg/DEX, 84.81 $\pm$ 6.31%; 3xTg/5LO-/-, 44.2 $\pm$ 6.65%; 3xTg/5LO-/- DEX, 38.73 $\pm$ 9.33%, *$p<0.01$

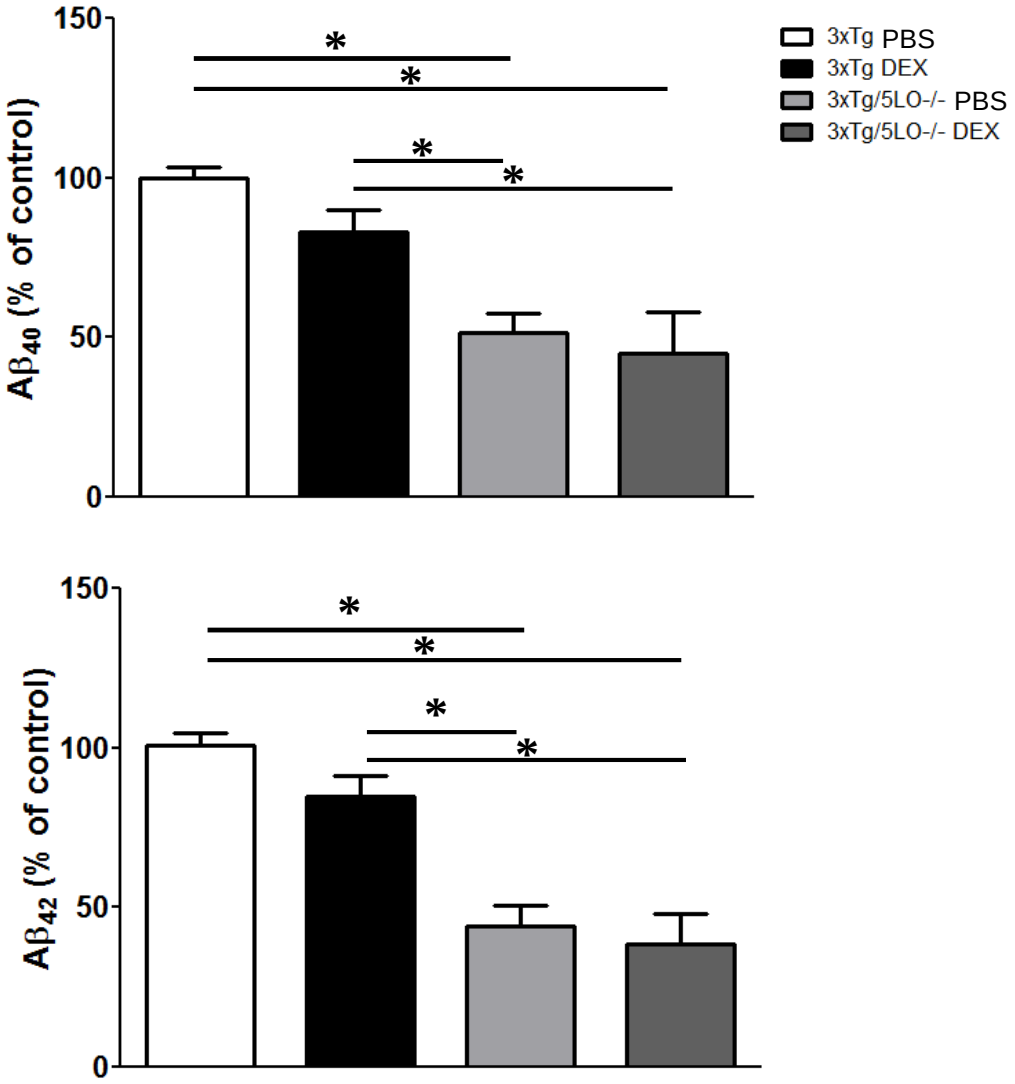

Figure 7 Soluble $A\beta_{40}$ and $A\beta_{42}$ levels in the brains of PBS or dexamethasone-treated 3xTg and 3xTg/5LO-/- animals Levels of soluble $A\beta_{40}$ and $A\beta_{42}$ in cortical RIPA lysate as detected by EIA in brains of 3xTg animals treated with PBS, i.p. (n=5), 3xTg animals treated with dexamethasone (DEX), 5mg/kg i.p. for 7d (n= 7), 3xTg/5LO-/- animals treated with PBS (n=4), i.p., and 3xTg/5LO-/- animals treated with dexamethasone (DEX), 5mg/kg i.p. for 7d (n=5). These data represent triplicate samples from all animals.

Knockout of 5LO prevents dexamethasone-induced soluble PHF13 tau phosphorylation

Since we did not observe any changes in behavior or amyloid β as a function of dexamethasone, we investigated how tau changed in our animals and treatment conditions. Using cortical brain lysates, we performed immunoblotting of total tau (HT7 tau) as well as phosphorylated tau species thought to represent advanced phosphorylation and precursors of insoluble tau deposition (PHF13 tau and PHF1 tau) in all animals and conditions. As shown in Figure 8A, B we found that dexamethasone increases PHF13 tau in 3xTg animal but not in 3xTg/5LO-/- animals (protein corrected and HT7 normalized values $\pm$ S.E.M: 3xTg, 100.0 $\pm$ 3.55%; 3xTg/DEX, 172.8 $\pm$ 5.73%; 3xTg/5LO-/-, 117.6 $\pm$ 5.65%; 3xTg/5LO-/- DEX, 86.67 $\pm$ 6.95, *$p<0.001$). We found no difference in HT7 tau or PHF1 tau between 3xTg, 3xTg/5LO-/- and dexamethasone-treated 3xTg/5LO-/- animals. To further assess phosphorylation, we performed immunohistochemistry analysis to support our immunoblotting data. In agreement with our immunoblotting results, as shown in Figure 9, we found that immunostaining of PHF13 was increased in dexamethasone-treated 3xTg animals but not in 3xTg/5LO-/- animals. Immunostaining of PHF1 tau was similar regardless of genotype or treatment.

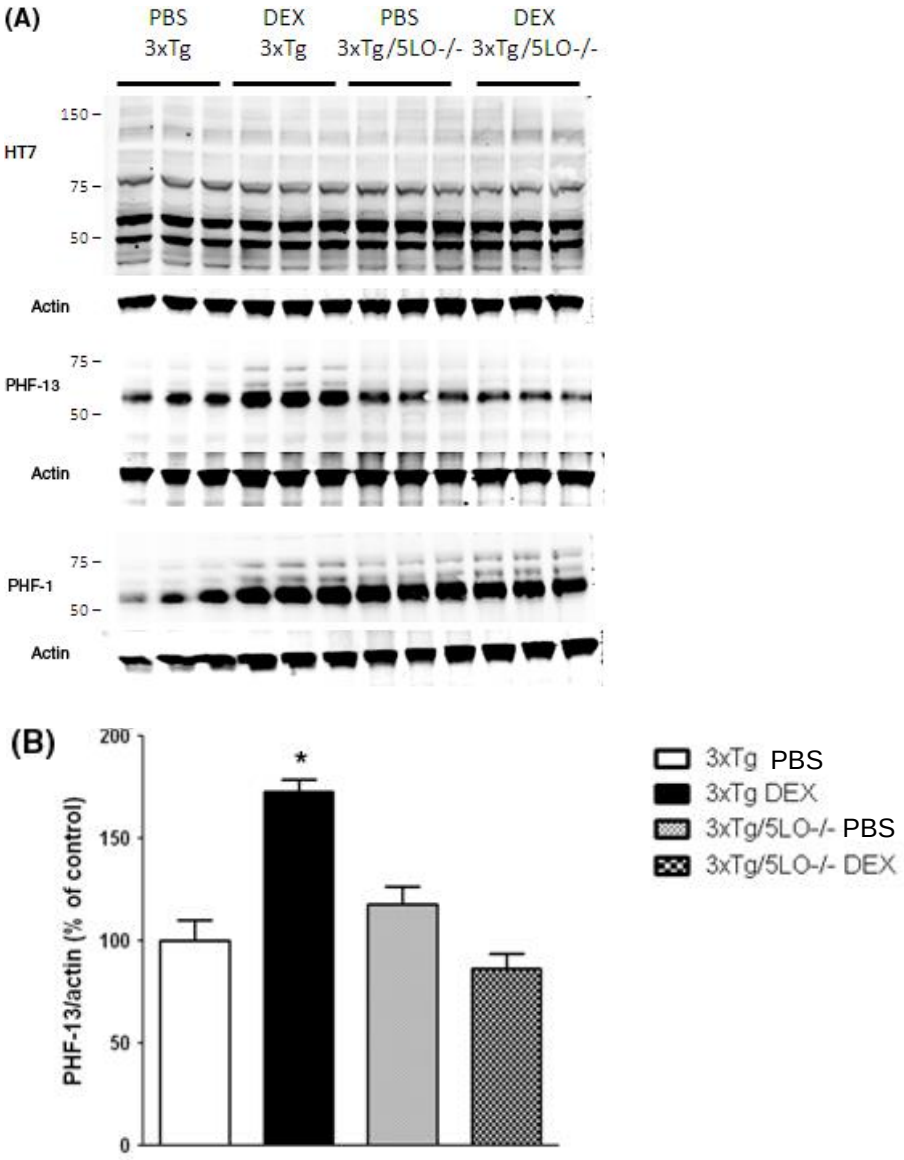

Figure 8 Tau phosphorylation in PBS or dexamethasone-treated 3xTg and 3xTg/5LO-/- animals (A) Representative immunoblots of cortical RIPA lysates from 3xTg animals treated with PBS, i.p. (n=5), 3xTg animals treated with dexamethasone, 5mg/kg i.p. for 7d (n= 7), 3xTg/5LO-/- animals treated with PBS (n=4), i.p., and 3xTg/5LO-/- animals treated with dexamethasone, 5mg/kg i.p. for 7d (n=5). (B) Densitometric analysis of PHF13 tau immunoreactivity in (A), with 3xTg/DEX group showing significance compared to all other groups *p<0.001.

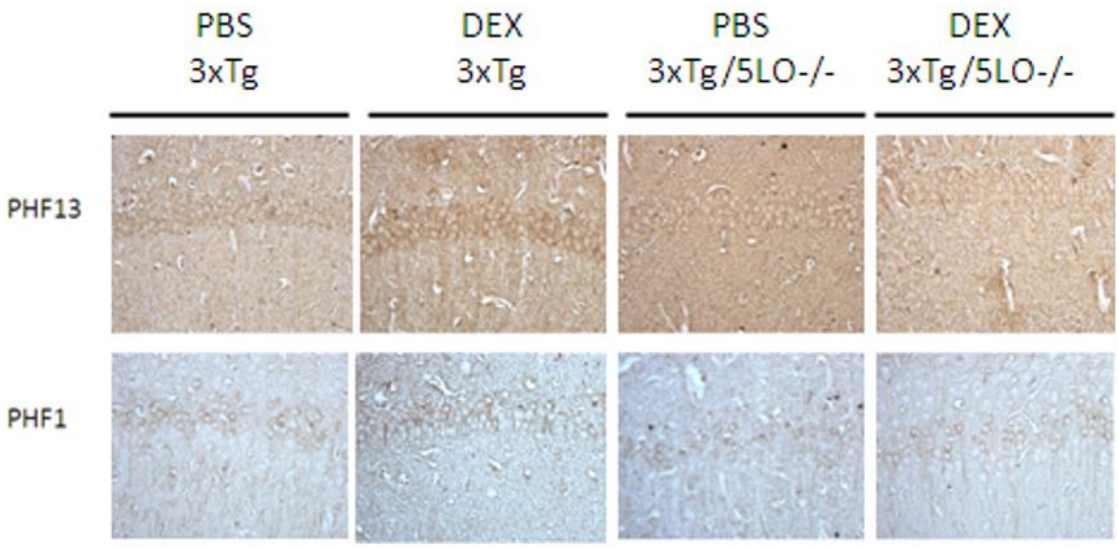

Figure 9 Tau immunostaining in the brains of PBS or dexamethasone-treated 3xTg and 3xTg/5LO-/- animals Representative PHF13 and PHF1 immunostaining in brain sections from 3xTg animals treated with PBS, i.p. (n=5), 3xTg animals treated with dexamethasone (DEX), 5mg/kg i.p. for 7d (n= 7), 3xTg/5LO-/- animals treated with PBS (n=4), i.p., and 3xTg/5LO-/- animals treated with dexamethasone (DEX), 5mg/kg i.p. for 7d (n=5).

Dexamethasone increases insoluble tau deposition in 3xTg animals but not in 3xTg/5LO-/- animals

Since elevation of soluble species of phosphorylated tau is thought to occur before deposition of insoluble tau, we next investigated whether dexamethasone accelerates generation of insoluble tau species in our animal model system. As shown in Figure 10, immunoblotting for total tau using HT7 antibody in formic acid-solublized pellets of RIPA cortical homogenates, dexamethasone administration results in the generation of insoluble tau in 3xTg animals. Dexamethasone-injected 3xTg/5LO-/- animals did not exhibit acceleration of insoluble tau pathology.

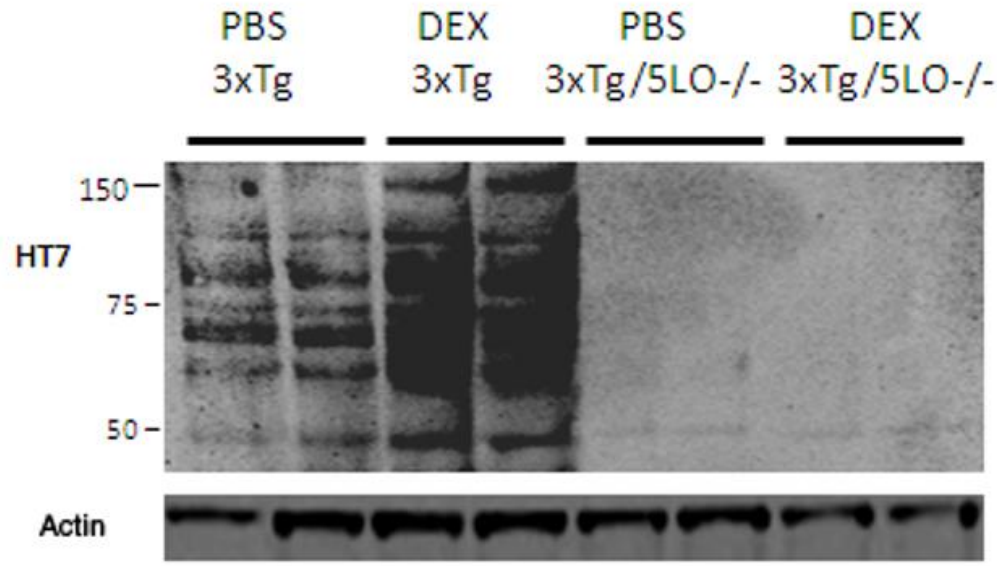

Figure 10 Insoluble total tau in cortical brain homogenates of PBS- and dexamethasone-treated 3xTg and 3xTg/5LO-/- animals Representative immunoblot of insoluble tau, as probed by the HT7 antibody in 3xTg animals (n=5), 3xTg animals treated with dexamethasone (DEX) (n=7), 3xTg/5LO-/- (n=4), and 3xTg/5LO-/- animals treated with dexamethasone (DEX) (n=5).

Dexamethasone-induced tau phosphorylation is associated with elevated GSK3β activity

To investigate the mechanism whereby 5LO modulates dexamethasone-induced tau phosphorylation, we assayed for two kinases that phosphorylate tau and have been shown to be altered in the brains of AD patients in cortex and hippocampus: glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (cdk5) (Hanger et al., 1992; Baumann et al., 1993). As shown in Figure 11, we found no changes in the steady state levels of either total GSK3α and GSK3β. Additionally, we found no changes in levels of cdk5, or its p35 and p25 coactivators. Although our immunoblotting results could imply that activity of these kinases were unperturbed in our experimental conditions, we nevertheless further investigated kinase activity directly.

When assessing GSK3β and cdk5 using kinase activity assays with their respective substrates, as shown in Figure 12, we found that GSK3β activity was increased in the cortical lysate of dexamethasone-treated 3xTg animals while no such increase was noted in dexamethasone-treated 3xTg/5LO-/- animals (radioactivity measured in arbitrary units, A.U. $\pm$ S.E.M.: 3xTg, 1.00 $\pm$ 0.1328; 3xTg/DEX, 2.326 $\pm$ 0.3567; 3xTg/5LO-/-, 0.9832 $\pm$ 0.0869; 3xTg/5LO-/- DEX, 1.346 $\pm$ 0.1675, *$p<0.001$). We did not detect significant differences in cdk5 activity in any condition.

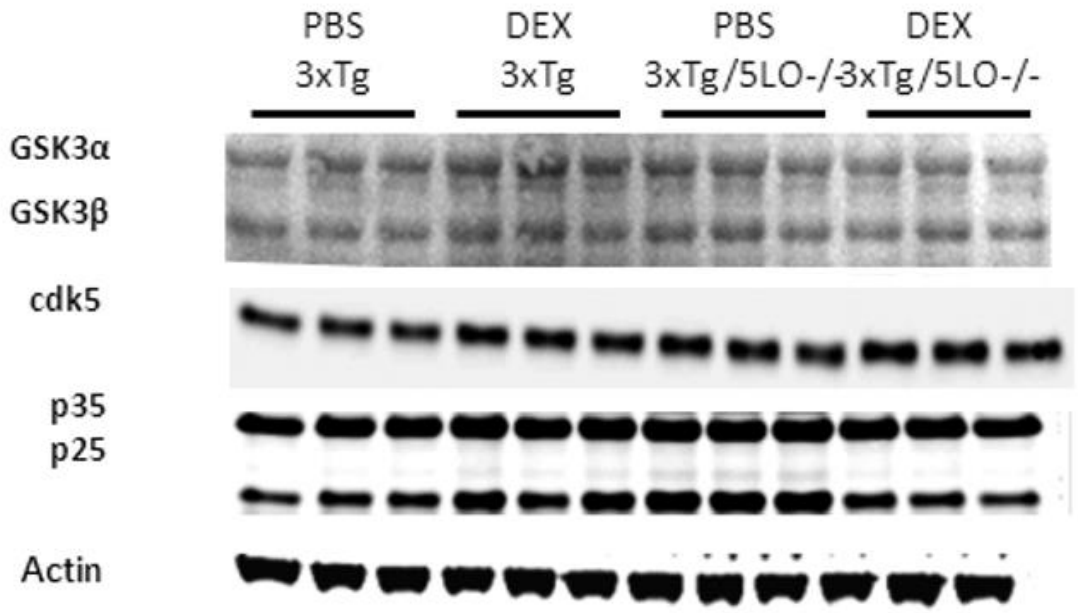

Figure 11 Levels of GSK3β and cdk5 in cortex of PBS and dexamethasone-treated 3xTg and 3xTg/5LO-/- animals. Representative immunoblots from RIPA cortical lysates of phosphorylated and total α and β subunits, as well as csdk5, and its coactivators, p35 and p25 in 3xTg animals treated with PBS (n=5), 3xTg animals treated with dexamethasone (DEX) (n=7), 3xTg/5LO-/- treated with PBS (n=4), and 3xTg/5LO-/- animals treated with dexamethasone (DEX) (n=5).

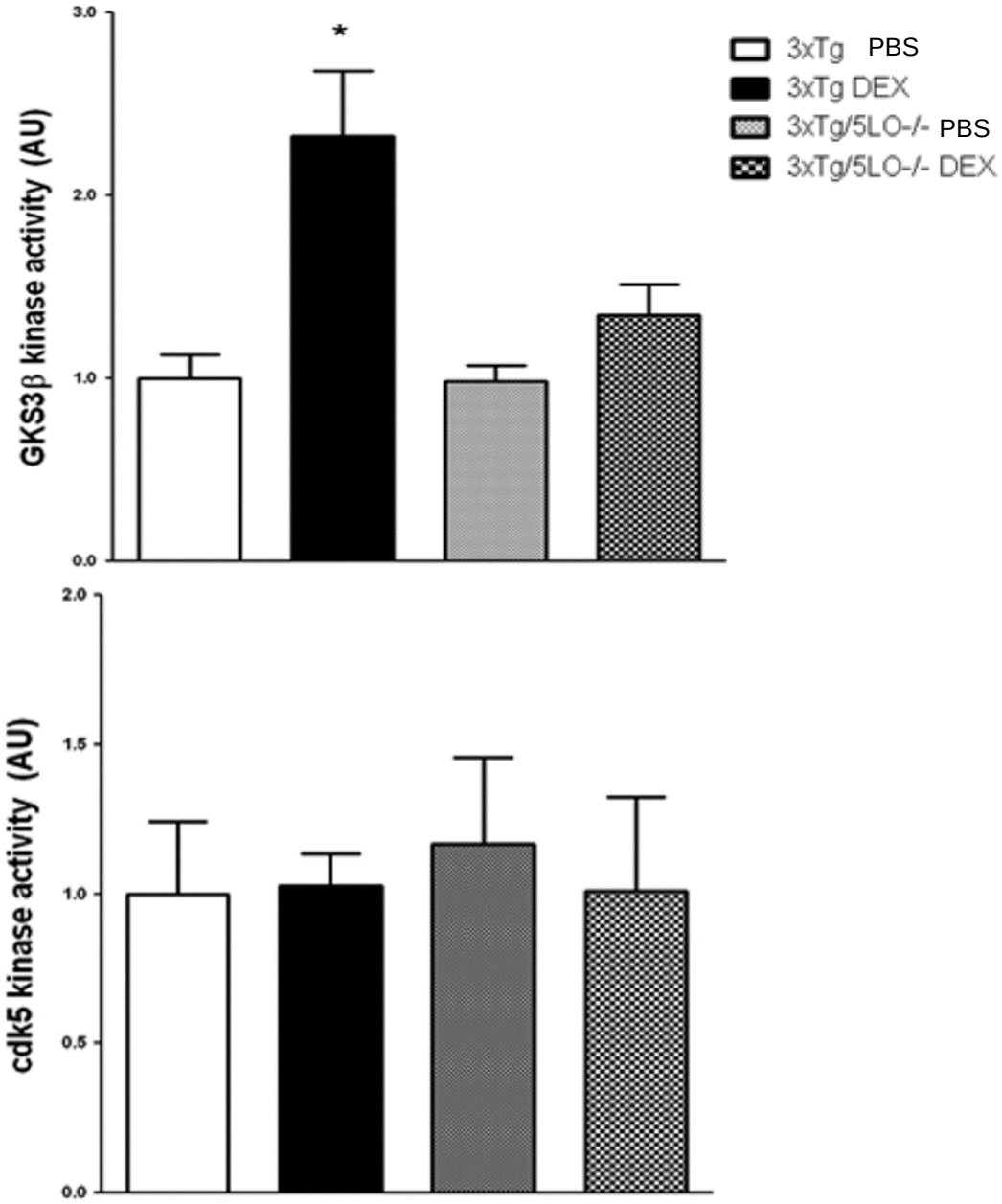

Figure 12 Tau-associated kinase activity in PBS or dexamethasone-exposed in 3xTg and 3xTg/5LO-/- animals Cortical brain homogenates from 3xTg, dexamethasone-treated 3xTg, 3xTg/5LO-/- and dexamethasone-treated 3xTg/5LO-/- animals, assayed for GSK3β and cdk5 kinase activity. Values are expressed in arbitrary units (A.U), with each/genotype condition representing 3 triplicate assay from 3 different animals per condition (n=3 mice/condition).

Discussion

In the current study, we investigated the importance of 5LO as it relates to the dexamethasone-induced exacerbation of the AD phenotype. We found that a 7 day course of dexamethasone 5mg/kg i.p. did not cause deficits in fear-conditioned memory recall, nor in Y-maze behavior in 3xTg or 3x/5LO-/- animals. While we observed that amyloid β pathology was reduced in 3xTg/5LO-/- animals compared to 3xTg animals, a finding previously reported in older animals, we did not observe any changes in amyloid β pathology as a function of dexamethasone exposure (Giannopoulos et al., 2013; Giannopoulos et al., 2014). By contrast, we found that tau phosphorylation and insoluble tau generation was accelerated in 3xTg animals after dexamethasone administration, which was not observed in 3xTg/5LO-/- animals. Associated with this increase in tau phosphorylation, we found that dexamethasone treatment was associated with an increase in GSK3β activity, but not cdk5 activity.

In summary, we show for first time that the 5LO enzymatic pathway is involved in the dexamethasone-dependent increase of tau phosphorylation by modulating the activity of the GSK3β kinase in a rodent model of AD with plaques and tangle, the 3xTg mouse.

Given human data on stress and AD vulnerability, it is intriguing that we did not observe any changes in Aβ levels as a function of dexamethasone administration. In fact, in the 3xTg mouse model of disease, an identical dose and length of dexamethasone administration has been reported by Green and colleagues to modestly increase brain levels of Aβ, which is at odds with our

currently presented results (Green et al., 2006). However, in studies performed by Green and colleagues, not only did they used a different extraction buffer for solubilization of brain homogenates to assay for Aβ levels, they homogenized whole-hemibrains, which could certainly lead to our reported differences. However, differences in methodology do not explain larger discrepancies in Aβ production that have been reported among different mouse models of AD exposed to stress hormones. In the 3xTg mouse model of AD, stress hormone-driven elevation of Aβ is associated with increased steady state levels of APP and BACE1, which imply that stress hormone increase Aβ load by increasing in starting substrate (Green et al., 2006). However, in the Tg2576 mouse model of AD, which has the identical human APP transgene (but not PS1 or tau transgenes found in the 3xTg mouse model), stress-related elevation of Aβ is completely unrelated to APP machinery, utilizing a mechanism that has yet to be elucidated clearly (Caroll et al., 2011). One evident explanation of this discrepancy is that the APP transgene in the 3xTg animal model is driven by the Thy1 promoter while the hamster prior promoter drives APP expression in the Tg2576 model—it is possible that glucocorticoid receptor-mediated activation modulates these promoters differently, and therefore, APP expression is increased in one but not both strains. Further study in both animal strains about stress-hormone induced modulation of Aβ production is required, and would be informative.

While our data and the work of Green and colleagues differ on Aβ, they are in concordance regarding tau pathology. In their study, they found dexamethasone exposure increased somatodendritic mislocalization of tau. In agreement with their work, we found stress hormone-associated elevation of the paired helical filament phosphoepitopes of tau, which represents tau that poorly associates with microtubules and has an increased tendency to aggregate and become insoluble. This was also supported by our finding that insoluble tau was generated by stress-hormone exposure in 3xTg animals. While tau is one of the cardinal pathologies in AD, it has generally been thought to play a secondary role in disease etiology and progression. However recent data seems to challenge this notion. For example, tau pathology and its progression in the brains of human AD cases appears to correlate closely to neurocognitive decline (Nelson et al., 2007). Moreover, purified tau protein oligomers isolated from the cortices of human AD cases are sufficient to propagate abnormal tau conformations of endogenous tau in non-transgenic mice (Lasagna-Reeves et al., 2012). Based on these data, dexamethasone-mediated tau phosphorylation, and its disruption by 5LO knockout, present important events in AD progression and therapeutic intervention, respectively.

Glucocorticoids have been shown to have variable effects on the activity of GSK3β, which depend heavily on the cellular milieu being analyzed. In some contexts, such as in osteoblasts, glucocorticoids cause increased GSK3β kinase activity while in others, GSK3β activity is reduced by glucocorticoids (Smith et al.,

2002). In our hands, we were able to observe the glucocorticoid-associated elevation of GSK3β activity in the brains of transgenic AD animals, supporting its role as a functional part of the stress hormone response in AD. Precisely how glucocorticoid modulate GSK3β, and the precise mechanism of how 5LO modulates GSK3β remains important topics of future work.

Since stress is linked to AD-associated memory insult in humans, our finding that a 7 d 5 mg/kg dexamethasone paradigm does not alter fear-conditioned recall does not allow us to interpret whether 5LO knockout would have a beneficial effect on stress hormone-associated memory impairment. A parsimonious explanation may be that 3xTg animals may have memory deficits better revealed by other paradigms (i.e., the Morris Water Maze, a paradigm commonly utilized in interrogating visuospatial memory impairment). Alternatively, our results argue that even before behavioral decline is evident in the 3xTg animal model, stress accelerates tau pathological burden. This interpretation reflects human data which suggest that AD pathology is present well before symptoms manifest (Villemagne et al., 2011; Chakroborty et al., 2012). For example, cerebrospinal fluid analysis in those individuals who have mild cognitive impairment (MCI) with amnesia, an AD prodrome, reflect high burden of both amyloid and tau pathology (Vos et al., 2013). This cerebrospinal burden of AD pathology also correlate with imaging studies in humans that predict that those individuals with MCI with higher amyloid load progress to AD (Westman et al., 2012; de Leon et al., 2007). Viewed in this frame, 5LO

knockout-mediated protection from glucocorticoid-induced memory impairment would most likely be evident at a later age in the same animals.

With regard to aging, disruption of 5LO, either by pharmacological inhibition or knockout, has been previously shown to protect 3xTg animals from AD-associated pathological burden and memory impairment in AD animals between 8 and 16 months, where pathological burden is significantly appreciable (Giannopoulos et al., 2013; Giannopoulos et al. 2014). An important assumption underlying those studies with 5LO is that since 5LO brain expression increases in an aging-associated fashion, the impact of 5LO modulation in the AD context would be best observed at an advanced age. However, our finding that 5LO knockout reduces Aβ pathology (irrespective of dexamethasone administration) as well as tau phosphorylation at an early age suggests that 5LO is an active modulator independent of age *in vivo*. Further studies are needed understand the development importance of 5LO to not only the AD phenotype, but also as it relates to amyloid neurobiology in general.

CHAPTER 3

EFFECT OF 5LO KNOCKOUT ON THE RESTRAINT-ISOLATION STRESS-INDUCED ALZHEIMER'S DISEASE PHENOTYPE

Introduction

Recent evidence has implicated stress as an environmental factor involved in the advancement the AD phenotype. Higher plasma or urinary cortisol in late life confers greater risk for AD development and a positive correlation exists between stress hormone levels and rate of AD progression (Elgh et al., 2006; Karlmangla et al., 2005; Rasmuson et al., 2002; Csernansky et al., 2006). The mechanisms of stress-associated worsening of the AD phenotype remain elusive but our previous study investigating the role of 5LO in the dexamethasone-associated worsening of the AD phenotype suggests that 5LO is required to mediate dexamethasone-associated advancement of tau phosphorylation, insoluble tau generation and dexamethasone-associated increase in GSK3β activity (Joshi et al., 2013). Despite these data, stress leading to AD progression in human is likely due to chronic, rather than acute exposure to stress. Moreover, while dexamethasone is very specific to the glucocorticoid receptor, it may fail to mimic the fuller repertoire of the effects of corticosterone, the endogenous glucocorticoid in mice. To address this issue, we subjected both 3xTg animals and 3xTg animals genetically deficient for 5LO (3xTg/5LO-/-) to a chronic stressor, 28 days of chronic restraint/isolation stress, and observed its effects on memory, Aβ and tau.

Materials and Method

Animals

All animal procedures we-re approved by the Institutional Animal Care and Usage Committee, in accordance with the National Institutes of Health Guidelines. All animals were housed on a 12-h light/dark cycle in the Medical Research Building at the Temple University Health Sciences Campus, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Standard chow and water were provided *ad libitum*.

Naïve female 3xTg and 3xTg/5LO-/- animals aged 4-4.5 months were used for our studies. The 3 x Tg mouse harbors a mutant APP (KM670/671NL), a human mutant PS1 (M146V) knock-in, and tau (P301L) transgenes (Oddo et al., 2003). To generate the 3xTg/5LO-/- mouse, mice lacking 5LO (5LO-/-) were cross-bred with 3xTg mice to generate founder 3xTg/5LO-/- animals (Goulet et al., 1991). These 3xTg/5LO-/- animals were backcrossed at least 10 generations, with the resulting progeny used for all studies. Mice were euthanized by CO_2 administration and brains were removed 24h after behavioral studies. Whole blood was collected at the time of sacrifice using EDTA as anticoagulant with final concentration of EDTA being 4.5 mM for each sample. EDTA-treated whole blood was centrifuged at 14,000 rpm for 10 minutes to yield plasma. This plasma was collected, alliquoted, and stored at -80º C for later analysis.

Cortices were dissected from one hemibrain and stored -80º C while the other hemisection was fixed in paraformaldehyde overnight, processed and paraffin-embedded, and used for immunohistochemistry analyses.

Restraint/isolation stress

Restraint/isolation (R/I) stress was administered as described by Carroll and colleagues by placing mice in a well-ventilated chamber (12 cm length x 3 cm diameter), which prevented locomotion (3xTg, n=6; 3xTg R/I stress, n=7; 3xTg/5LO-/-, n=4; 3xTg/5LO-/- n=5) (Carroll et al., 2011). Animals were taken from their group-housed cages, gently put into the R/I chamber and then placed in a clean cage with clean bedding and housed 1 mouse/cage. Animals were subjected to R/I stress for 60 min/day for 28 days. R/I stress was carried out between 0800 to 1000 hours daily. Control animals were briefly handled and then returned to their home cages. After R/I stress, animals were removed from R/I chamber and placed back into their original group-housed cages. Body weights of animals monitored at weekly intervals during our studies and was not found to deviate from the weight of unstressed controls.

Behavioral paradigms

For behavioral assessment of working memory the Y-maze paradigm was used, and to assess hippocampal- and amygdala-associated learning and memory recall the fear conditioning paradigm was used. Y-maze behavior was carried out one day after last dexamethasone injection, and fear conditioning was carried out

one ay after Y-maze behavior was assessed. For all test on both apparatuses, 70% ethanol was used to thoroughly clean all surfaces after every trial.

The Y-maze paradigm consists of three arms 32 cm long x 10 cm wide with 26 cm walls (San Diego Instruments, San Diego, CA, USA). Animals were placed in the central portion of the maze and allowed to freely explore for 5 min. Entries into each arm were recorded using a video camera. An entry was counted if all four paws entered an arm. An exit from an arm was counted if all four paws left an arm. In addition to total entries, total alternation percentage was also recorded. An alternation was defined as three consecutive arm entries into different arms. Total alternation percentage was derived using the formula: total number of alternations /(total entries -2).

For fear conditioning, tests were conducted in a fear conditioning chamber with black methacrylate walls, a speaker, and grid floor (Start Fear Systems, Harvard Apparatus, Holliston, MA, USA) and was carried out over 2 days. On day 1, mice were placed in the conditioning chamber for 2 min to freely explore before the onset of a 2000Hz neutral tone of 90dB which lasted 30 sec. The last 2 sec was paired with a 0.6 mA scrambled foot shock. Mice were removed from the chamber 1 min after the shock. A total of 3 pairs of foots shocks and tones were delivered with 30 s intervals without footshocks or tones between trials. On day 2, mice were assessed for contextual and cued recall. Freezing behavior was defined as movement recorded by the system associated with animal breathing

in the absence of any ambulation. For contextual fear recall, mice were put back into the same conditioning chamber for 5 min and their freezing behavior was recorded. In this phase, no neutral tones were sounded and no shocks were delivered. Cued fear recall was assessed ~3 h after contextual fear recall was assessed. For cued fear recall, mice were placed back into the chamber with altered floor, odor, color, and lighting for 5 min. The same tone used for training was sounded at the 2 min mark, with 3 tones delivered, exactly as with the training phase with 30 seconds between tones. The percentage of time spent freezing was used for calculation for the training, contextual and cued recall phase.

Biochemical analyses

Mouse brain cortical homogenates were sequentially extracted first radioimmunoprecipitation assay buffer (RIPA), containing EDTA-free protease-inhibitor (Roche, Indianapolis, IN, USA) and phosphatase inhibitor (ThermoFisher) for the amyloid β soluble fractions and then in formic acid (FA) for the amyloid β insoluble fractions as previously described. Amyloid β1-40 and amyloid β1-42 levels were assayed by a sensitive sandwich enzyme-linked immunosorbent assay kit in accordance to the manufacturer's protocols (Wako Chemicals, USA). All biochemical assays were performed in triplicate per animal sample. Plasma levels of corticosterone and brain levels of leukotriene B_4 were assayed using enzyme-linked immunosorbent assay kits in accordance with the manufacturer's protocols (Enzo Life Science, Farmingdale, NY, USA) in

accordance with the manufacturer's protocols.For assessment of leukotriene B_4, cortical lysate was used.

Immunoblotting

Brain homogenate RIPA or pH-neutralized FA samples were electrophoretically separated using 10% Bis-Tris gels of 3-8% Tris-acetate gel (Bio-Rad, Philadelphia, PA, USA), according to the molecular weight of the target molecular and then transferred into nitrocellulose membranes (Bio-Rad). Membranes were blocked with Odyssey-blocking buffer and then incubated with primary antibodies overnight at 4ºC. After three washing cycles in TBS-T, membranes were incubated with IRDye secondary antibodies (LI-COR; Lincoln, NE, USA) at 22 ºC for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR, Lincoln, NE, USA). Actin was always used as the internal loading control. Antibodies and dilutions were as follows: HT7 (1:200, Cell Signal, Danvers, MA, USA), AT180 (1:200, Pierce, Morris Plains, NJ, USA) PHF13 (1:200; Pierce), PHF1 (1:200, a generous gift from Dr. Peter Davies), GSK3α/β (1:200; Santa Cruz, Dallas TX, USA), cdk5 (1:200; Santa Cruz), p35/p25 (1:200, Santa Cruz), synaptophysin (SYP, 1:200, Santa Cruz), PSD95 (1:200, Cell Signal) and β-actin (1:1000, Santa Cruz)

Kinase activity

Kinase activities of GSK3β and cdk5 were carried out as previously described (Joshi et al., 2013). Briefly, GSK3β and cdk5 were coimmunoprecipitated from 75 µg cortical lysate using 1.5 µg respective antibodies. The immunoprecipitates

were washed with lysis buffer three times followed by once by HBS buffer (10-mM HEPES, pH 7.4, 150 mM NaCl). Activity of GSK3β was determined using phosphor-glycogen synthase peptides 2 (Millipore, Billerica, MA, USA) as substrate while cdk5 activity was determined using histone H1 (Santa Cruz, Biotechnology, Dallas, TX, USA) as substrate. In both experiments immunoprecipitates were incubated in HBS containing 15 mM $MgCl_2$, 50 mM ATP, 1 mM dithiothreitol, and 1 µCi of [32P] ATP. After 30 min incubation at 37* C, the reaction products were determined by a liquid scintillation counter. Kinase activity assays were carried out in triplicate for each animal sample.

Electrophysiology

Electrophysiological experiments were performed as previously described (Giannopoulos et al. 2014). Mice were sacrificed by rapid decapitation 24 h after assessment on Y-maze and fear conditioning paradigms, and brains placed in ice-cold artificial cerebrospinal fluid (ACSF) in which sucrose (248 mM) was substituted for NaCl. Hippocampal slices (400 µm) were cut using a vibratome and placed in ACSF with bubbled 95% O2/5%CO2 at room temperature to recover for 1 hour. Slices were transferred to a recording chamber and continuously perfused with ACSF maintained at 32-34oC. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum using an extracellular glass pipette (3-5 MΩ) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200-300 µm from the recording pipette.

Stimulation intensities were chosen to produce a fEPSP at 1/3 of the maximum amplitude based on an input/output (I/O) curve using stimulations of 0-300 μA. Paired-pulse facilitation (PPF) experiments were performed using a pair of stimuli of the same intensity delivered 20, 50, 100, 200 and 1000 ms apart. Baseline was recorded for 20 min before tetanization with pulses every 30 s. Long-term potentiation (LTP) at CA3-CA1 synapses was induced by four trains of 100 Hz stimulation delivered in 20 s intervals. Recordings were made 30 s for 90 min following tetanization. The fEPSP rise/slope between 30% and 90% was measured offline using Clampfit 10.3 (Molecular Devices, Sunnyvale, CA, USA) and normalized to the mean rise/slope of the baseline. All tests were performed by an experimenter who was unaware of genotype or treatment.

Statistical analysis

Data are presented as the mean ± standard error of the mean. One-way analysis of variance (ANOVA), with Bonferroni multiple comparison tests were performed using Prism 5.0 (GraphPad Software, La Jolla, CA). All data are presented as mean +/- standard error of the mean. Significance was set at $p < 0.05$. While the studies contained in this thesis are published, during the course of this candidate's dissertation defense, it was pointed out the two-way ANOVA may have been a better choice for statistical analyses, and therefore could have led to slightly different conclusions than stated.

Results

Restraint/isolation stress increases corticosterone and leukotriene levels in 3xTg but not 3xTg/5LO-/- animals

As shown in Figure 13, the R/I stress paradigm was sufficient in inducing an elevation of plasma corticosterone in 3xTg but not in 3xTg/5LO-/- animals (protein corrected using 3xTg baseline: 3xTg, 100.0±5.32%; 3xTg/R/I, 136.6±15.2%, 3xTg/5LO-/-, 85.35±10.94; 3xTg/5LO-/- R/I, 94.14±7.15%, *p<0.01). Since abnormalities in circulating corticosterone can lead to changes in glucocorticoid receptor expression in the brain, we also assessed glucocorticoid receptor levels in homogenized brain lysates. We found no difference in glucocorticoid receptor levels between either genotype or experimental condition as shown in Figure 14. Finally, to support the association between stress and 5LO function, we measured the levels of the downstream 5LO metabolite, leukotriene B_4, in the brains of all our animals. We found that restraint/isolation-stressed 3xTg animals had significantly elevated LTB_4 levels compared to unstressed 3xTg animals as shown in Figure 15. As expected, no detectable levels of LTB4 were found in the brains of 3xTg/5LO-/- animals regardless of treatment (3xTg, 100.0±6.3%; 3xTg/R/I, 135.0±12.6%, 3xTg/5LO-/-, not detectable; 3xTg/5LO-/- R/I, not detectable, *p<0.01).

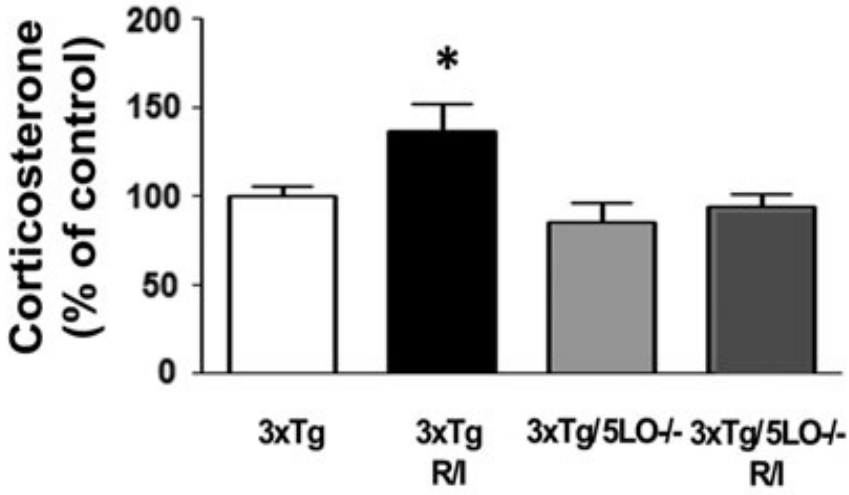

Figure 13 Plasma corticosterone levels in 3xTg and 3xTg/5LO-/- animals after restraint/isolation stress Levels of plasma corticosterone were assayed from 3xTg (n=6), R/I stressed 3xTg (n=7), 3xTg/5LO-/- (n=4), R/I stressed 3xTg/5LO-/- (n=5) animals.

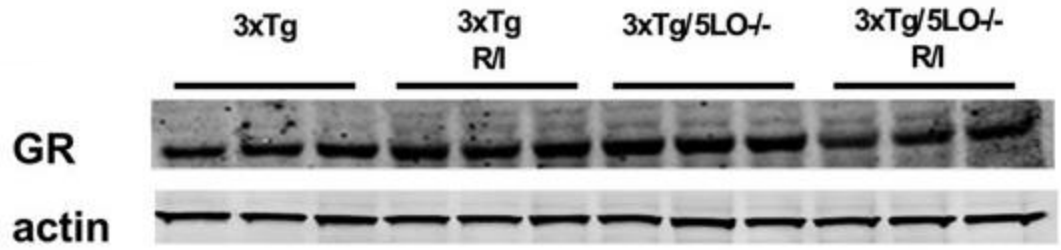

Figure 14 Glucocorticoid receptor expression in brains of 3xTg and 3xTg/5LO-/- animals after restraint/isolation stress Representative immunoblot of glucocorticoid receptor (GR) assayed from brain lysates of 3xTg (n=6), R/I stressed 3xTg (n=7), 3xTg/5LO-/- (n=4), R/I stressed 3xTg/5LO-/- (n=5) animals.

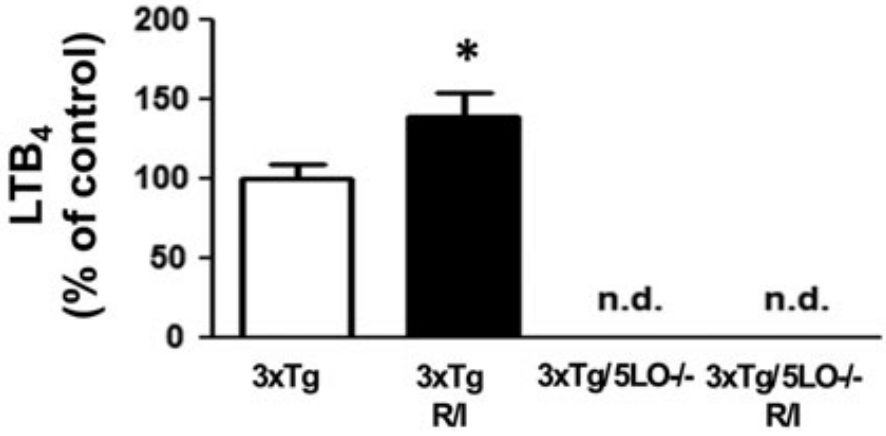

Figure 15 Leukotriene B_4 levels in cortical lysates of restraint/isolation-stressed 3xTg and 3xTg/5LO-/- animals LTB_4 EIA were carried out on cortical brain homogenates obtained from 3xTg (n=6), R/I stressed 3xTg (n=7), 3xTg/5LO-/- (n=4), R/I stressed 3xTg/5LO-/- (n=5) animals. *p<0.01

Restraint/isolation stress impairs fear-conditioned recall in 3xTg but not 3xTg/5LO-/- animals

Since we found that R/I stress increased plasma cortisol levels, we next investigated whether this was linked to impairments in learning and memory. As with our studies with dexamethasone, we assessed our animals in the fear-conditioning paradigm. As shown in Figure 16, we found no differences between animals or treatment conditions in the training phase of the fear-conditioning paradigm (3xTg, 74.65 $\pm$ 3.31%; 3xTg/R/I, 69.13 $\pm$ 9.47%; 3xTg/5LO-/-, 76.83 $\pm$ 9.46%; 3xTg/5LO-/- R/I, 72.73 $\pm$ 9.06%, p<0.92). However when contextual memory was assessed, we found that restrain/isolation-stressed 3xTg animals

had significant impairment when compared to 3xTg/5LO-/-. No differences were found in contextual recall between 3xTg, unstressed 3xTg/5LO-/- and restraint/isolation stressed 3xTg/5LO-/- mice (3xTg, 78.65 $\pm$ 4.11%; 3xTg/R/I, 56.48 $\pm$ 4.47%; 3xTg/5LO-/-, 80.53 $\pm$ 3.82%; 3xTg/5LO-/- R/I, 75.5 $\pm$ 4.6%, *p<0.01) Assessment of cued recall also showed trends similar to contextual conditioning data. Restraint/isolation-stressed 3xTg animals showed impaired cued recall while 3xTg/5LO-/- showed no such insult. No difference was observed between unstressed 3xTg, unstressed 3xTg/5LO-/- or stressed 3xTg/5LO-/- animals (3xTg, 40.0 $\pm$ 7.3%; 3xTg/R/I, 18.9 $\pm$ 3.61%; 3xTg/5LO-/-, 48.0 $\pm$4.34%; 3xTg/5LO-/- R/I, 44.2 $\pm$ 1.72%, *p<0.01.

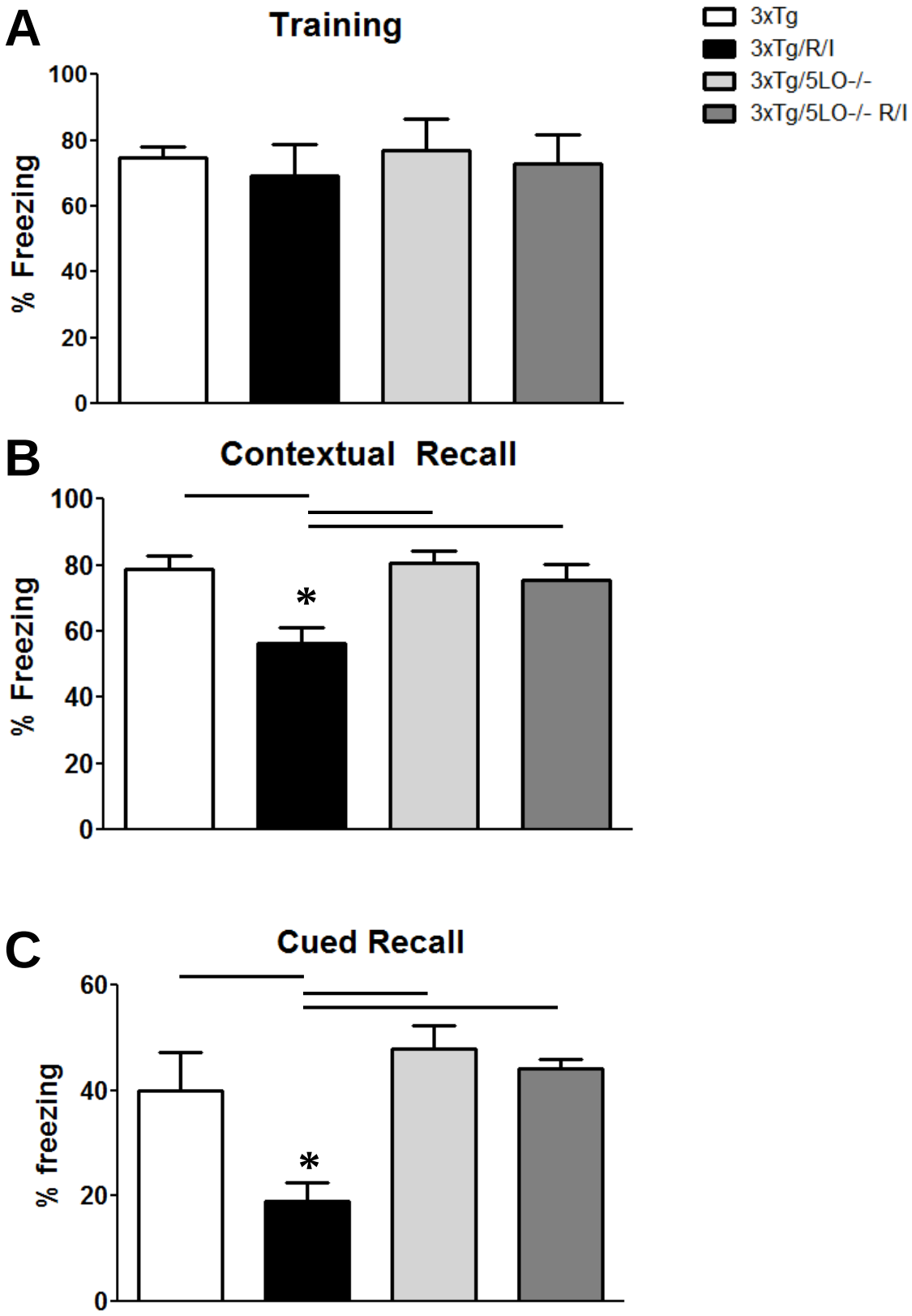
A
Training
3xTg
3xTg/R/I
3xTg/5LO-/-
3xTg/5LO-/- R/I
% Freezing
100
80
60
40
20
0
B
Contextual Recall
% Freezing
100
80
60
40
20
0
*
C
Cued Recall
% freezing
60
40
20
0
*

Figure 16 Fear conditioned memory in 3xTg and 3xTg/5LO-/- animals after restraint/isolation stress Freezing behavior in the (A) training phase, (B) contextual recall phase, and (C) cued recall phase of the fear conditioning paradigm. 3xTg (n=6), R/I stressed 3xTg (n=7), 3xTg/5LO-/- (n=4), R/I stressed 3xTg/5LO-/- (n=5) animals were used for this study. *p<0.01

Restraint/isolation stress does not change Y maze behavior in 3xTg and 3xTg/5LO-/- animals

Since we found changes in fear-conditioned behavior in 3xTg animal in the restraint-isolation stress condition, we next assessed Y-maze behavior to assess working memory. As shown in Figure 17, we found no difference in overall arm entries between all groups (3xTg, 21 $\pm$ 2.45; 3xTg/R/I, 24.75 $\pm$ 2.78; 3xTg/5LO-/-, 21.67$\pm$ 2.028; 3xTg/5LO-/- R/I, 20.25 $\pm$ 2.07, p<0.56). Similarly, we did not find any differences in alternation percentage (3xTg, 58.35 $\pm$ 10.49%; 3xTg/R/I, 60.43 $\pm$ 4.18%; 3xTg/5LO-/-, 62.17 $\pm$ 6.428%; 3xTg/5LO-/- R/I, 60.89 $\pm$ 13.46%, p<0.98

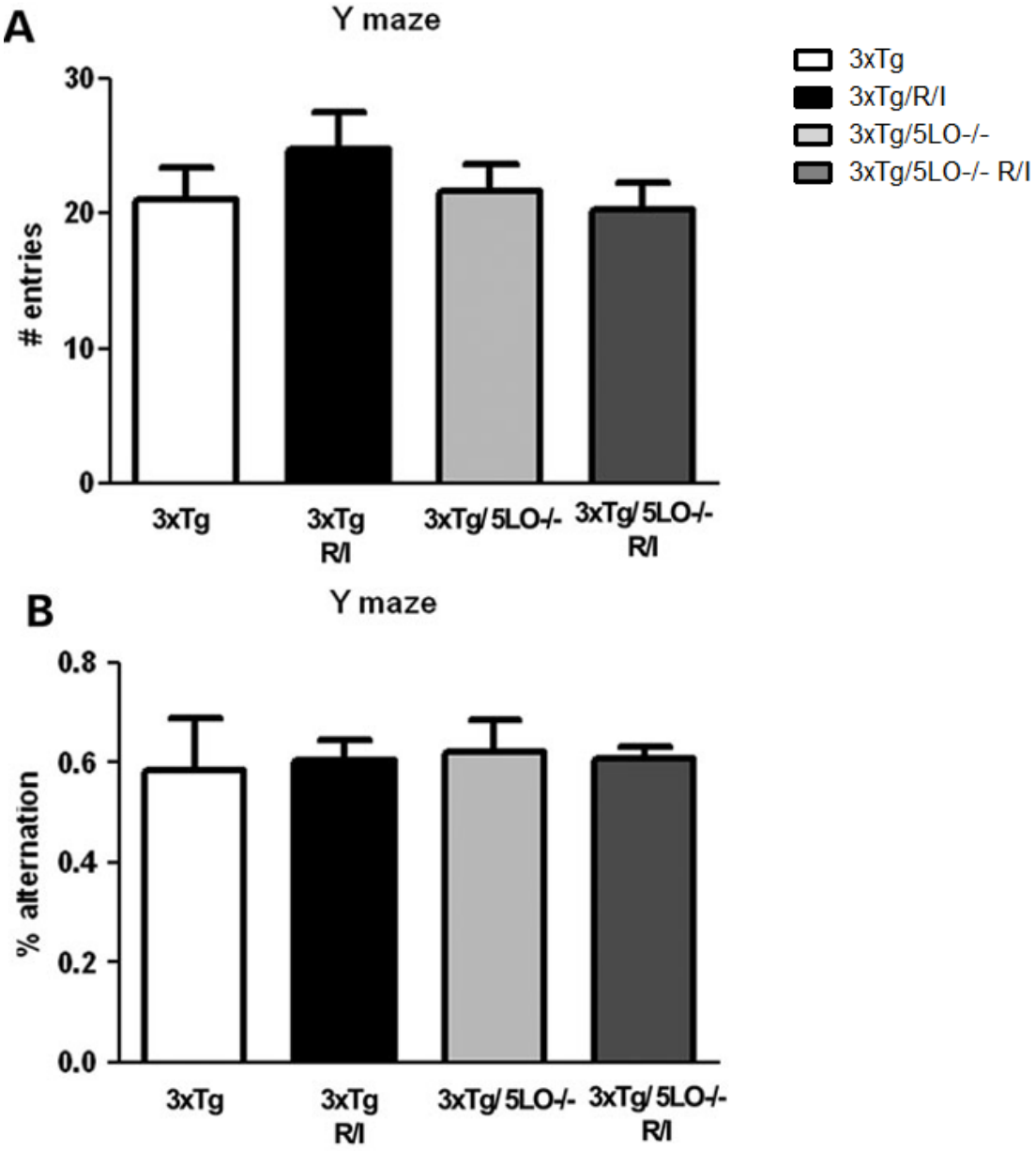

Figure 17 Y maze behavior in 3xTg and 3xTg/5LO-/- animals undergoing restraint/isolation stress 3xTg animals. (n=6), 3xTg animals exposed to restraint/isolation stress (R/I), (n= 7), 3xTg/5LO-/- animals (n=4), i.p., and 3xTg/5LO-/- exposed to R/I stress were assessed in the Y maze paradigm for both total number of entries (A) and alternation percentage (B).

Restraint/isolation stress does not increase amyloid levels in 3xTg or 3xTg/5LO-/- animals

Since we found behavioral changes in restraint-isolation stressed animals, we next asked the question of whether, if at all, this observation was associated with changes in amyloid beta levels. We carried out EIAs of cortical brain lysate restraint/isolation-stressed 3xTg and 3xTg/5LO-/- animals, and as shown in Figure 18, we found no effect of restraint-isolation stress on $A\beta_{40}$ or $A\beta_{42}$ levels in either 3xTg or 3xTg/5LO-/- animals. However, we found that knockout of 5LO-/- reduced Aβ levels independent of experimental condition, similar to our dexamethasone studies. ($A\beta_{40}$ levels, expressed as % of protein-correct 3xTg: 3xTg, 100.0 $\pm$ 12.03%; 3xTg/R/I, 85.55 $\pm$ 7.79%; 3xTg/5LO-/-, 39.65 $\pm$ 9.46%; 3xTg/5LO-/- R/I, 25.72 $\pm$ 8.17%, *$p<0.001$) ($A\beta_{42}$ levels, expressed as % of protein-correct 3xTg: 3xTg, 100.0 $\pm$ 8.38%; 3xTg/R/I, 95.04 $\pm$ 5.07%; 3xTg/5LO-/-, 56.90 $\pm$ 3.6%; 3xTg/5LO-/- R/I, 39.14 $\pm$ 4.58%, *$p<0.001$)

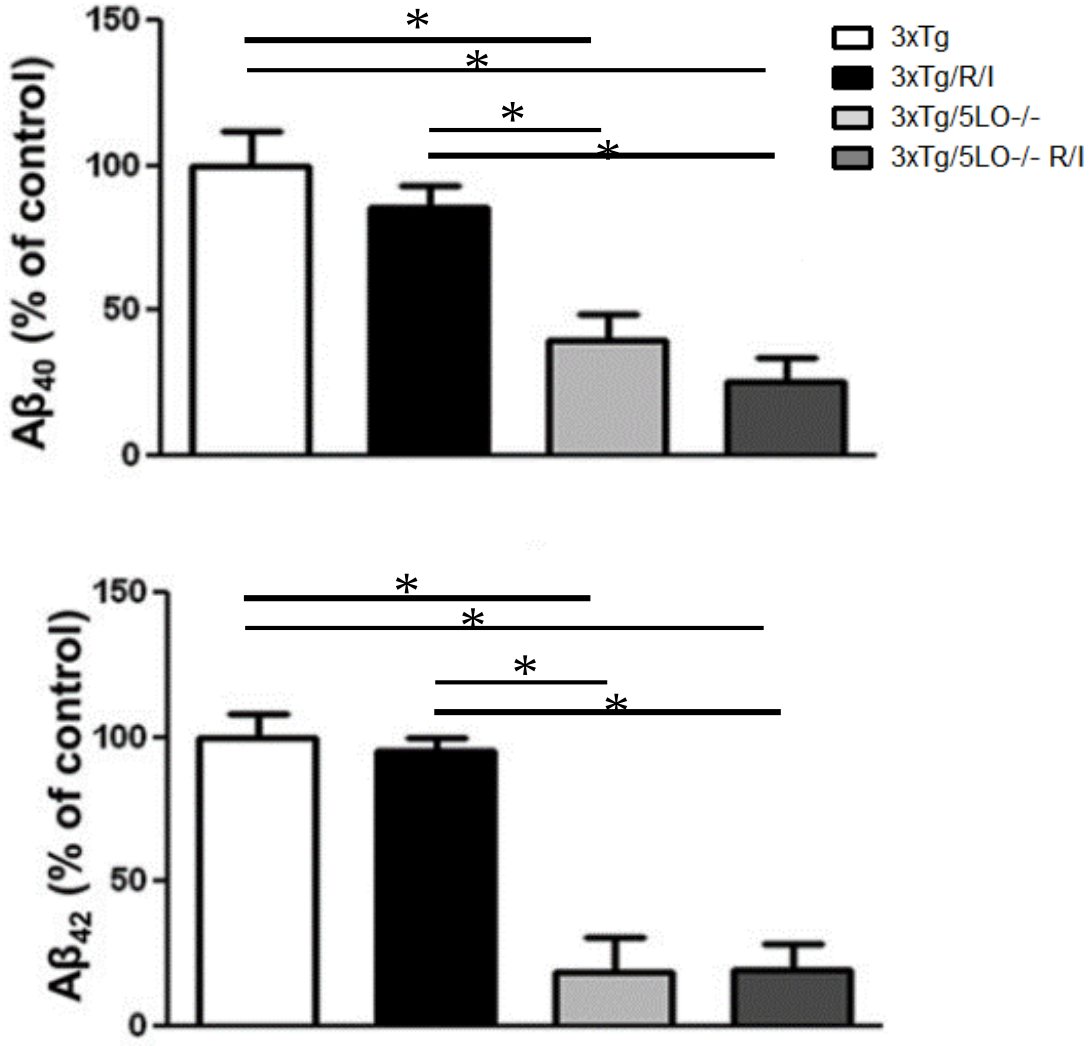

Figure 18 Soluble $A\beta_{40}$ and $A\beta_{42}$ levels in the brains of restraint/isolation stressed 3xTg and 3xTg/5LO-/- animals. Sensitive and specific EIAs targeting $A\beta_{40}$ and $A\beta_{42}$ were used to probe amyloid levels in cortical RIPA lysates in 3xTg (n=6), restraint/isolated 3xTg (n=7), 3xTg/5LO-/- (n=4) and restraint/isolated 3xTg/5LO-/- (n=5) animals. These data represent triplicate samples from all animals.

Tau phosphorylation is promoted by restraint/isolation stress in 3xTg mice but not 3xTg/5LO-/- mice

Upon finding no changes in Aβ levels as a function of stress condition, we next investigated if phosphorylated tau species changed in this model of stress. We chose to assay for HT7, PHF13, PHF1 and AT180. While PHF13 and PHF 1 tau assess advanced tau phosphorylation, AT180 is a phosphorylated tau epitope that is thought to occur earlier in disease and signify an early event in neurofibrillary tau accumulation. As shown in Figure 19, we found that restraint/isolation stress increases PHF13 and PHF1 tau levels but not AT180 in 3xTg animals. This effect was not observed in 3xTg/5LO-/- animals. (protein-corrected PHF1/HT7 tau immunoreactivity expressed as percent of 3xTg baseline: 3xTg, 100.0 $\pm$ 1.89%; 3xTg/R/I, 140.0 $\pm$ 11.06%; 3xTg/5LO-/-, 101.0 $\pm$ 3.2%; 3xTg/5LO-/- R/I, 109.3 $\pm$ 9.18%; protein-corrected PHF13/HT7 tau immunoreactivity expressed as percentage of 3xTg baseline: 3xTg, 100.0 $\pm$ 11.25%; 3xTg/R/I, 187.4 $\pm$ 35.23%; 3xTg/5LO-/-, 101.1 $\pm$ 10.81%; 3xTg/5LO-/- R/I, 98.49 $\pm$ 4.75%; *$p<0.05$)

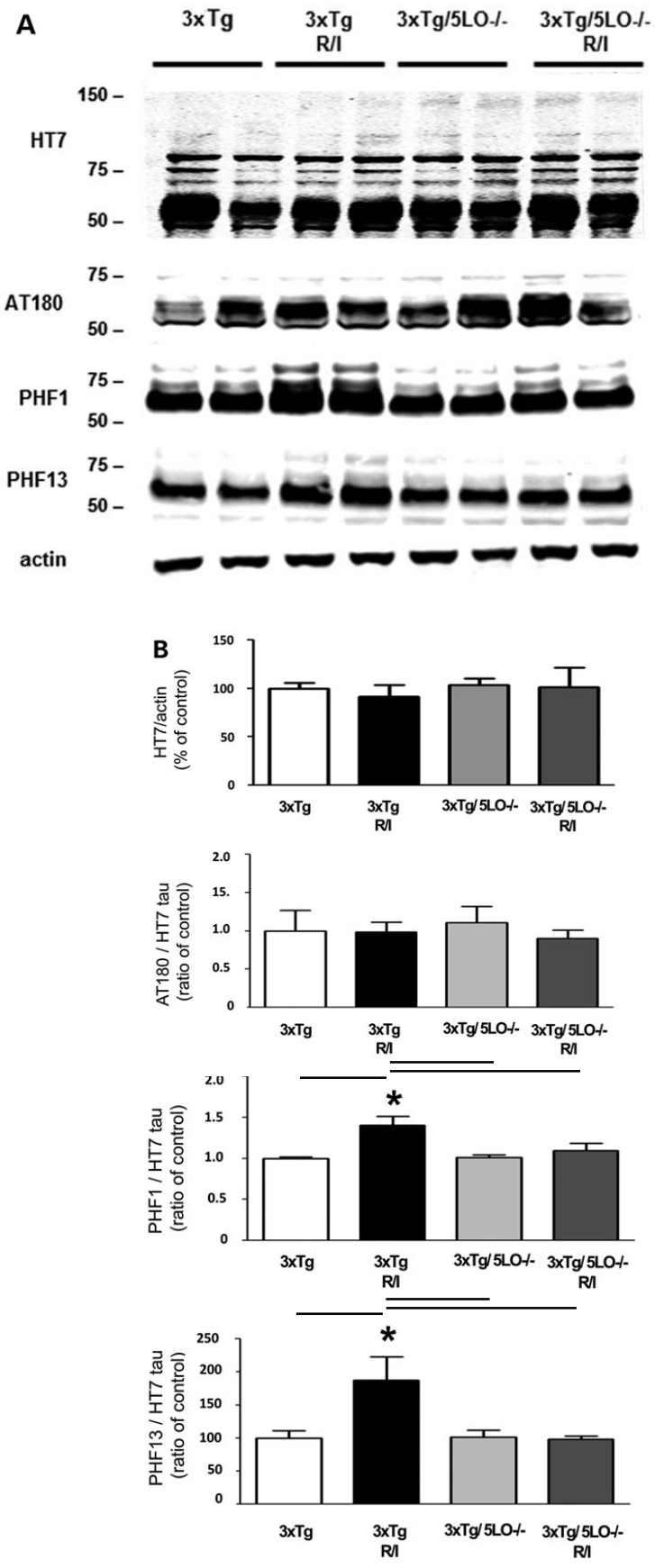
A
3xTg
3xTg R/I
3xTg/5LO-/-
3xTg/5LO-/- R/I
150 –
HT7
75 –
50 –
75 –
AT180
50 –
75 –
PHF1
50 –
75 –
PHF13
50 –
actin
B
HT7/actin (% of control)
150
100
50
0
3xTg
3xTg R/I
3xTg/5LO-/-
3xTg/5LO-/- R/I
AT180 / HT7 tau (ratio of control)
2.0
15.
1.0
0.5
0
PHF1 / HT7 tau (ratio of control)
2.0
1.5
1.0
0.5
0
*
PHF13 / HT7 tau (ratio of control)
250
200
150
100
50
0
*

Figure 19 Soluble tau species in the brains of 3xTg and 3xTg/5LO-/- animals following restraint/isolation stress Representative immunoblot (A) utilizing cortical lysates of 3xTg (n=6), restraint/isolated 3xTg (n=7), 3xTg/5LO-/- (n=4) and restraint/isolated 3xTg/5LO-/- (n=5) animals to probe for HT7, AT180, PHF1 and PHF13 phosphorylated tau species. (B) Densitometric analysis of immunoblot data presented in panel (A) with *$p < 0.05$.

Insoluble tau is deposited in stressed 3xTg animals, but not 3xTg/5LO-/- animals In order to understand whether elevation in both PHF13 and PHF1 also led to the development of insoluble tau, we used neutralized formic-acid cortical lysates to immunoblot for insoluble tau. Using HT7 antibody as probe, we found that restraint-isolation stress led to the development of insoluble tau species in stressed 3xTg animals as shown in Figure 20. By contrast, we did not find detectable levels of insoluble tau in stressed 3xTg/5LO-/- animals.

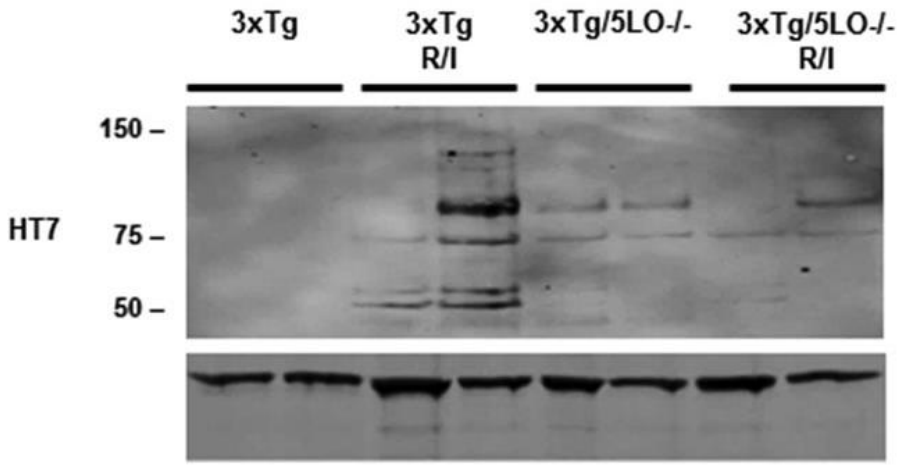

Figure 20 Insoluble tau in cortical brain homogenates of restraint/isolation stressed 3xTg and 3xTg/5LO-/- animals Representative immunoblot of cortical lysates from 3xTg (n=6), restraint/isolated 3xTg (n=7), 3xTg/5LO-/- (n=4) and restraint/isolated 3xTg/5LO-/- (n=5) animals probed for insoluble tau, as probed by the HT7 antibody in 3xTg and 3xTg/5LO-/- animals exposed to restraint/isolation (R/I) stress.

GSK3β activity is associated with restraint-isolation stress induced tau phosphorylation

Our previous studies with dexamethasone revealed that increased GSK3 activity was associated with tau phosphorylation. To see this was also true with restraint-isolation stress-induced tau phosphorylation and insoluble tau generation, we first carried out immunoblotting of tau kinases using cortical lysates as shown in Figure 21. We found no changes in the levels of GSK3α. GSK3β, phosphorylated GSK3α, phosphorylated GSK3β, cdk5, p35 or p25 in 3xTg or 3xTg/5LO-/-

animals regardless of stress condition. However, when assessing for kinase activity, we found that GSK3β activity was elevated in 3xTg mice that were restraint/isolation stressed as shown in Figure 22. No such increase was found in 3xTg/5LO-/- animals, with similar GSK3β activity unstressed 3xTg, unstressed 3xTg/5LO-/- and stressed 3xTg/5LO-/- animals (radioactivity measured in arbitrary units, A.U. ± S.E.M: 3xTg, 14,350 ± 1,120; 3xTg/R/I, 28,185 ± 1347; 3xTg/5LO-/-, 15,142 ± 1,434, 3xTg/5LO-/- R/I, 13,714 ± 1,754, *p<0.0001). No changes in cdk5 activity was found, regardless of genotype or stress condition.

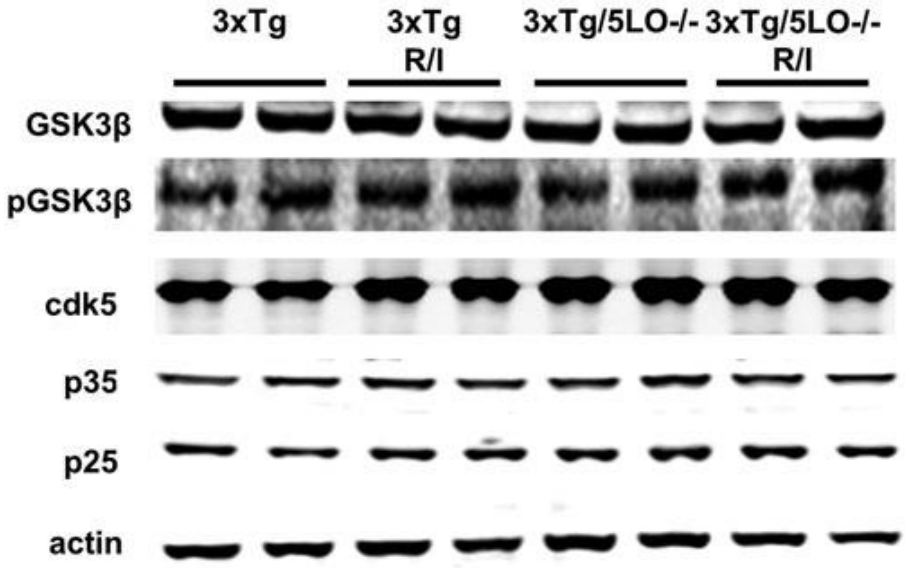

Figure 21. GSK3β and cdk5 steady-state expression in cortical lysates in restraint/isolation stressed 3xTg and 3xTg/5LO-/- animals

Representative immunoblots from RIPA cortical lysates of phosphorylated and total GSK3α and β subunits, as well as csdk5, and its coactivators, p35 and p25.

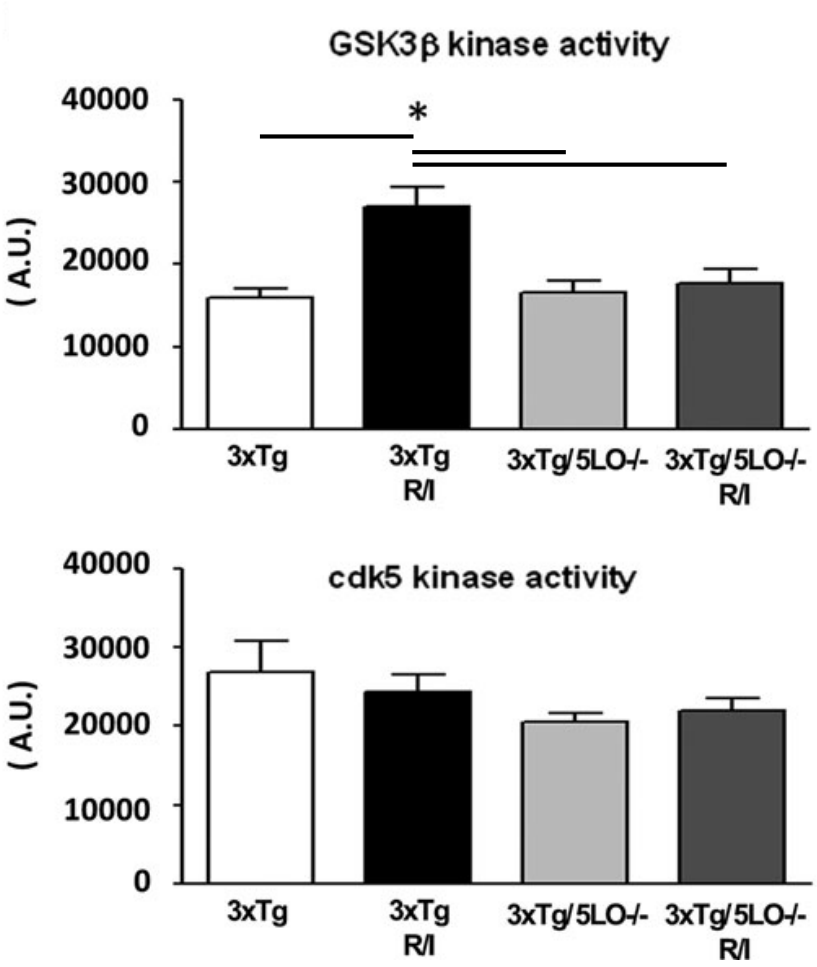

Figure 22. Tau-associated kinase activity in restraint/isolation stress-exposed in 3xTg and 3xTg/5LO-/- animals Cortical brain homogenates from 3xTg, R/I stressed 3xTg, 3xTg/5LO-/- and R/I stressed 3xTg/5LO-/- animals, assayed for GSK3β and cdk5 kinase activity. Values are expressed in arbitrary units (A.U), with each/genotype condition representing at least 3 triplicate assay from 3 different animals per condition (n=3 animals/condition).

Knockout of 5LO prevents R/I stress-mediated synaptic dysfunction

Since emerging evidence indicates synaptic disruption underlying Aβ and tau-associated neurotoxicity underlie insults to learning and memory seen in AD, we studied synaptic function by electrophysiological studies in both stressed and unstressed genotypes. These electrophysiological studies were performed exclusively by, and in collaboration with, Margaret Sperow, PhD under the guidance of Lynn Kirby, PhD. Using hippocampal slices, we first established that basal synaptic transmission was similar between animals and treatment groups by generating input/output curves and measuring fEPSPs elicited in the CA1 by stimulating Schaffer collaterals as shown in Figure 23A. We also examined paired-pulse facilitation to assess for short-term plasticity as shown in Figure 23B. We found no differences amongst groups in either parameter. We then assessed long-term potentiation (LTP) over 90 min as shown in Figure 23C-E, and found that compared to 3xTg baseline, R/I-stressed 3xTg animals displayed a reduced LTP response. By contrast, 3xTg/5LO-/- animals had an augmented LTP response but this was reduced to 3xTg baseline when subjected to R/I stress.

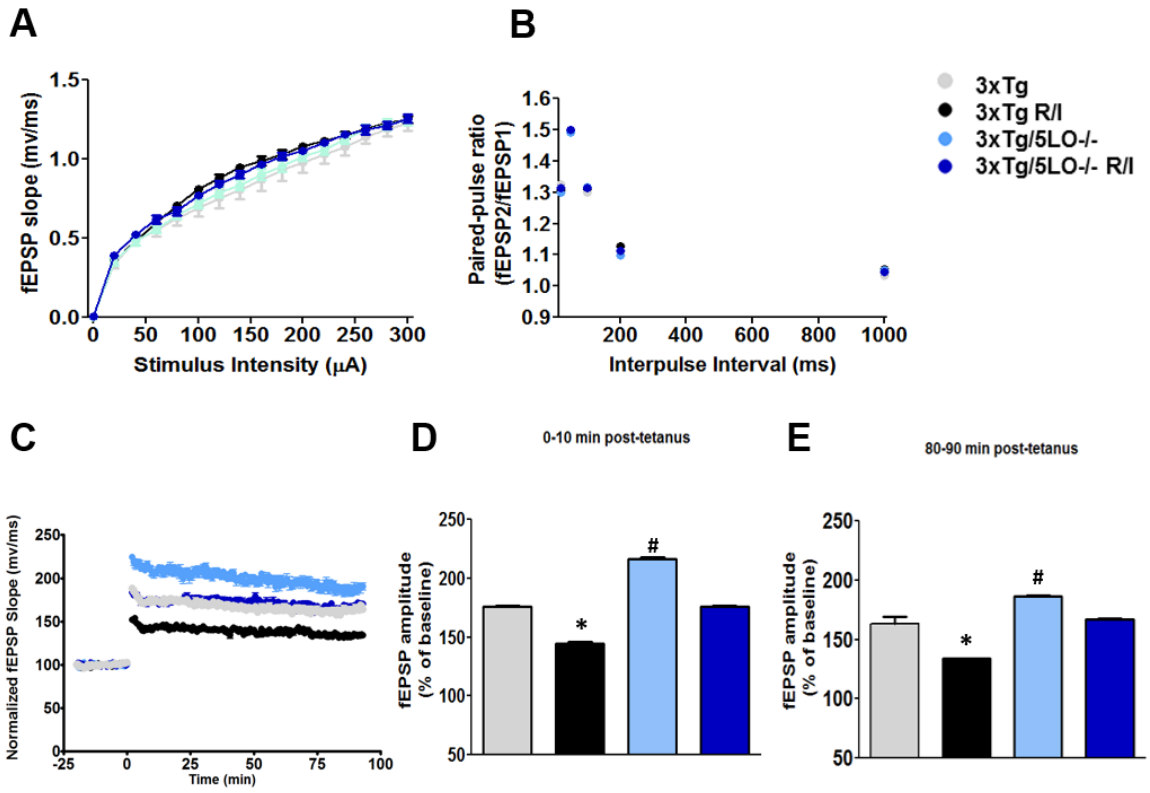

Figure 23 Absence of 5LO rescues synaptic dysfunction in 3xTg mice undergoing chronic restraint/isolation stress. For electrophysiology studies hippocampal slices were used (n= number of slices/number of animals; 3xTg, n = 13/3; 3xTg R/I stress, n= 13/3; 3xTg/5LO-/-, n = 14/3; 3xTg/5LO-/- R/I stress, n = 14/3). (A) Input/output curves and representative field excitatory post-synaptic potentials (fEPSPs) at increasing stimulus strengths. (B) Mean fEPSPs slopes as a function of interpulse interval between first and second fEPSPs evoked at CA3-

CA1 synapses in slices from the same mice at 20, 50, 100, 200 and 1000 ms in the same animals. (C). 90 min recordings of fEPSP expressed as percentage of 3xTg pre-tetanus baseline. (D). LTP magnitudes at 0-10 min post-tetanus and at 80-90 min (E) showing reduced fEPSPs in 3xTg R/I stressed animals compared to all animal groups (*), and elevated fEPSPs in 3xTg/5LO-/- animals compared to all groups (#), with similar levels of fEPSPs in unstressed 3xTg and stressed 3xTg/5LO-/- animals. Results are mean ± sem (*$p < 0.05$).

Discussion

In this study, we found that 3xTg AD mice exposed to 28 days R/I stress paradigm manifest significant memory impairments and that genetic absence of 5LO protected them from this effect. In addition, R/I stress significantly increased tau phosphorylation, increased the amount of insoluble tau, increased activity of GSK3β, and that none of these effects were seen in stressed 3xTg animals genetically deficient for 5LO. Finally, we found that R/I stress significantly impaired LTP in 3xTg mice, but 3xTg/5LO-/- animals undergoing R/I had a LTP response indistinguishable from 3xTg LTP without stress. Taken together these results highlight the novel functional role that 5LO plays in the development of the biochemical, electrophysiological and behavioral sequelae of stress in the AD context.

In summary, we show for the first time that the 5LO enzymatic pathway is involved in the behavioral impairment, tauopathy and synaptic insult following a

behavioral stressor in a rodent model of AD with plaques and tangles, the 3xTg mouse.

To the best of our knowledge, our report is the first to interrogate the effects of restraint/isolation stress in young 3xTg animals, and the second study in the literature to use restraint/isolation stress in an AD mouse model. Restraint/isolation stress has been used previously in a mouse model of amyloidosis, the Tg2576 mouse, which only expresses the human transgenic APP, evolving only the amyloid β plaques found in AD (Carroll et al., 2011). In their study, Carroll and colleagues found that both young (~4 month) and old (~14 month) Tg2576 animals exposed to restraint/isolation stress had evidence of elevated amyloid levels in the brain. However, restraint/isolation paradigm used in their studies was for 6 hours/day which represents a significantly greater magnitude of stress exposure than the AD animals used in our study experienced (60min/day). Nevertheless, even with an increased amyloid load, Carroll and colleagues were unable to find any changes in APP, α-, or β-secretase to account for their results and speculated that such an increase was the result of reduced Aβ clearance from the brain, and not from alteration from Aβ-producing machinery per se. Indeed, in the same study, an increase in brain Aβ was not found when the same investigators used a chronic variable stress model which randomized mice to experience alternating stressors of a long swim in 30ºC bath, short swim in 15ºC bath, restraint for 15 min, housing with soiled bedding, and housing under constant light for 24 hours. On the other hand

Cuadrado-Tejedor and colleagues found that six weeks of a combination of low intensity stroboscopic illumination in darkness for 8h, intermittent bell ringing (10db, 1/10s), white noise from an untuned radio (4h), 45 degree cage tilt, rat odor, 3h daytime darkness, transfer of cages to another room for 4h, overnight water and food deprivation, illumination and removal of nesting material (12h), and swimming in cold water 18ºC, 5min), was sufficient to lead to modest elevation in soluble brain amyloid levels (Cuadrado-Tejedor et al., 2012). Taken as a whole, these data indicate that it is likely that specific stressor type and exposure strength, and not just behavioral stress in general, leads to modulation of amyloid pathology. Our data support the idea that restraint/isolation stress, at least for the duration and time we selected, may not be enough to modulate Aβ pathology in 3xTg.

In line with the our previous work describing the importance of 5LO to the evolution of dexamethasone-associated tauopathy in 3xTg mice, the data in this study reveal that 5LO is required for restraint/isolation stress-associated tauopathy. Several studies have shown that the phosphorylation of tau occurs following multiple different stressors, including cold stress, hypoglycemia, starvation and hibernation (Korneyev et al., 1995; Feng et al., 2005; Papasozomenos 1996; Yanagisawa et al., 1999; Arendt et al., 2003). In rats, Yan and colleagues have shown that 14 d of 1 hour restraint stress produces hyperphosphorylation of tau, but does not produces any insoluble tau species (Yan et al., 2010). In a similar vein, Zhang and colleagues have found that

neonatal isolation for 4 hours daily between the first day of life to post-natal day 20 produces phosphorylation of tau which resembles the phosphorylation found in early stage AD tau pathology (Zhang et al., 2011). Given these data in non-transgenic AD animals, it is unsurprising that restraint/isolation stress accelerated tau pathology in the 3xTg model. However, our results are the first to report that the 5LO protein is required for tau phosphorylation produced by behavioral stress.

While we have showed that genetic absence of 5LO prevents a short course of dexamethasone from advancing tau pathology, in that study we were unable to detect any memory impairment caused by our treatment and thus were unable to establish that 5LO-associated tau phosphorylation had any behavioral relevance (Joshi et al, 2013). In the current study, we have shown that 5LO is required for stress-related cognitive impairment and LTP deficits. Two previous reports have found that in older 3xTg animals, recall and LTP insults are mitigated by 5LO pathway disruption but ours is the first to report an LTP change in young 3xTg animals (Giannopoulos et al., 2013; Giannopoulos et al., 2014). Interestingly, stressed 3xTg/5LO-/- animals, though lacking any overt differences in fear-conditioning and Y-maze when compared to unstressed 3xTg or unstressed 3xTg/5LO-/- animals, nevertheless had similar LTP responses to 3xTg animals. Since unstressed 3xTg/5LO-/- animals had the best LTP response, these results suggests that 5LO-/- confers augmentation to the LTP response found in 3xTg animals, which is modulated by stressors. *In vitro* data have shown that 5LO may

modulate glutamate receptor internalization, but studies linking electrophysiological changes cause by 5LO to brain circuitry leading to behavior have not yet been systematically carried out (Imbesi et al., 2008). Why and how this 5LO-mediated modulation of LTP affects behavior will clearly need further inquiry to clarify its role in not only AD, but also in neuronal long-term potentiation, in general.

CHAPTER 4

DISCUSSION

General Discussion

The goal of our studies was to investigate the role of the 5LO protein in the stress-mediated exacerbation of the AD phenotype. While some genetic mutations have been linked to the early development of AD in several families, the vast majority of AD patients lack these mutations (Querfurth and Laferla, 2010). Therefore environmental factors such as stress exposure have been conjectured to play a disease-accelerating role in AD. Stress, and stress hormones, such as glucocorticoids, have a variety of pleotropic cellular effects. Among them, glucocorticoids have been shown to increase the expression of the 5LO protein (Uz et al., 1999; Uz et al., 2001). 5LO is a widely expressed protein in the central nervous system, and produces leukotriene metabolites from arachidonic acid. 5LO is a positive modulator of amyloid β and tau phosphorylation, both critical molecular players in AD pathophysiology. In light of this data, we reasoned that disruption of 5LO via a knockout strategy could blunt the detrimental effects of stress in transgenic AD animals. We utilized the 3xTg animal model of AD, one which develops both amyloid plaques and neurofibrillary tau tangles. In our approach, we used both pharmacologic stress, dexamethasone, as well as behavioral stress, using the restraint/isolation paradigm, in both 3xTg and 3xTg/5LO-/- animals. In our studies, we investigated the effects of stress on the AD phenotype both biochemically and behaviorally.

In Aim 1, in Chapter 3, we investigated the importance of 5LO as it relates to the dexamethasone-induced exacerbation of the AD phenotype. In those studies we found that a 7 day course of dexamethasone 5mg/kg i.p. did not produce any deficits in 3xTg or 3x/5LO-/- animals as measured by the Y-maze and fear conditioning paradigm. While amyloid β pathology was reduced in 3xTg/5LO-/- animals compared to 3xTg animals no changes in amyloid β pathology as a function of dexamethasone exposure was found. However, tau phosphorylation and insoluble tau generation was accelerated in 3xTg animals after dexamethasone administration, and this was not observed in 3xTg/5LO-/- animals. Associated with this increase in tau phosphorylation, we found that dexamethasone treatment was associated with an increase in GSK3β activity, but not cdk5 activity.

In Aim 2, in Chapter 4, we investigated the importance of 5LO to the behavioral-stress-induced exacerbation of the AD phenotype. We found that a 28 day period of restraint/isolation stress was sufficient in increasing plasma cortisol and brain levels of leukotriene B_4 in 3xTg animals but not in 3xTg/5LO-/- animals. Additionally, we found that knockout of 5LO did not alter globally alter expression of glucocorticoid receptor. While restraint/isolation stress was sufficient to cause contextual and cued-related fear conditioning deficits in 3xTg animals, 3xTg/5LO-/- animals were spared from such insults. By contrast, Y-maze behavior was unaltered by restraint/isolation stress or genotype. Spontaneous alternations recorded in the Y-maze reflect appropriate functionining of not just hippocampus,

but also thalamus, prefrontal cortex, striatum and cerebellum (Lalonde, 2002), while the fear-conditioning paradigm reveals out deficits localizable to the hippocampus and amygdala (Maren 2008). This discrepancy between performance on these two tasks suggests that R/I stress may spare insult to thalamus, prefrontal cortex, striatum and cerebellum to the extent that such insults were not detectable through our Y-mase assay.

In concordance with our studies with dexamethasone, we did not to find any changes in Aβ in the brains of 3xTg or 3xTg/5LO-/- animals caused by R/I stress, although we were able to confirmed that 3xTg/5LO-/- animals indeed had lower brain levels of Aβ than their 3xTg counterparts. R/I stress significantly worsened tau phosphorylation, increased the amount of insoluble tau, which was associated with an increased activity of GSK3β kinase in 3xTg animals but not 3xTg/5LO-/- animals. Finally, while R/I stress significantly impaired LTP in 3xTg mice, 3xTg/5LO-/- animals undergoing R/I had a LTP response indistinguishable from unstressed 3xTg.

Taken together these results highlight the novel functional role that 5LO plays in the development of the biochemical, electrophysiological and behavioral sequelae of stress in the AD context. Our work extends what is known about the mechanism of stress-associated insult in AD, as well as further advances knowledge of the importance of the 5LO protein in aging-associated neurodegenerative diseases.

Our studies bring to light the pleotropic nature of the action of stress and stress hormones in the brain. Classically, glucocorticoids have been known to be potent anti-inflammatory agents, which includes reducing expression and downstream metabolic signaling of 5LO. For example, in rheumatoid arthritis synovium, intraarticular glucocorticoids such as triamcinolone reduces 5LO expression (Gheorghe et al., 2009). However, recent evidence suggests this is tissue-specific. In placental tissue, dexamethasone increases 5LO activation and LTB_4 production (Zhang et al., 2014). Recent work from the laboratory of Robert Sapolsky sheds light on this disconnect: in the work by Munhoz and colleagues, corticosteroid administration potentiated the proinflammatory effects of lipopolysaccharide by activation of nuclear factor κB (NFκB) in a dose-dependent fashion especially in the frontal cortex, and to a lesser effect, the hippocampus (Munhoz et al., 2010). In a separate study by the Sapolsky group, the same NFκB-mediated potentiation by lipopolysaccharide has been described to result from chronic unpredictable stress in rats, which consisted of: 60 min restraint on day 1, 15 min forced swim on day 2, 90 min cold isolation on day 3, overnight lights-on period on day 4, 5 min forced swim on day 5, overnight food/water deprivation day on 6, 120 min restraint on day 7, lights off on day 8, 5 min forced swim on day 9, overnight lights-on day 10, 90 min cold isolation on day 11, 60 min restraint on day 12, overnight food/water deprivation on day 13, 60 min restraint on day 14 (Munhoz et al. 2006). While our work did not feature administration of pro-inflammatory agents, amyloid peptides have pro-

inflammatory properties themselves (Carrero et al. 2012). It is likely then, that on a background of a pro-amyloidotic state (as in the 3xTg mouse), that dexamethasone administration and restraint/isolation stress advanced a brain inflammation, which includes 5LO action. Speculation aside, how stress hormones and glucocorticoids control 5LO remains unresolved. The 5LO promotor region includes glucocorticoid response elements (Hoshiko et al., 1990), which would suggest that stress and stress hormones increase its steady state expression by GR binding to the 5LO promoter. This appears to be supported by both *in vitro* and *in vivo* studies in which downstream metabolites of 5LO are elevated in response to stress or stress hormones but this assumption has not been directly tested (for example, with genetic manipulation of the 5LO promoter region) in the AD context.

Our studies also bring a particular focus to the relative vulnerability of tau to stress hormones and stress in general. While debate regarding the specific importance and priority of amyloid β and tau in AD is likely to remain unresolved for some time, the evolution of insoluble tau pathology early in life, combined with recent data suggesting pathological tau species may propagate in a prion-like fashion (Lasagna-Reeves et al., 2012), imply that targeting tau early in disease course would be key for prevention. Conversely, if pathological insoluble tau is already present, it is unclear how effective interventions targeting tau phosphorylation would be. Since, as discussed above in Chapter 4, tau appears to be vulnerable to hyperphosphorylation from a number of different stressors,

5LO-targeted therapeutics may hold particular promise preventatives for tauopathies.

Clinical Relevance

Since currently-approved therapeutics for AD are limited, our current work adds to previously published work on 5LO in the AD context, providing further preclinical evidence of using the 5LO pathway for drug targeting. As the pathological burden of AD appears years before symptoms arise, our work is particularly relevant for a preventative strategy for AD, especially since exposure to stress is an unavoidable risk factor for AD.

Future Directions

Although the work presented in this thesis is novel and timely, it serves only as platform for many additional avenues of investigation that promise to be fruitful and relevant to AD, especially as they relate to stress and 5LO neurobiology.

First and foremost, analysis of AD pathology using specific cortical and subcortical brain regions in AD mice in response to stressors would be instructive. This is particularly important with regards to 5LO biology, since a comprehensive analyses of its expression in the brain (aside from its presence in whole cortex and hippocampus) has not yet been carried out to the best of our knowledge.

Second, although we have shown that 5LO is required for stress and stress hormones to promote their AD-advancing effects, it is not known whether

this is due to the presence of 5LO, its metabolites or both. Additionally, it is not yet clear, based on the work presented in this thesis or currently in the literature, how interactions between neurons, astrocytes and microglia are modulated by knockout of 5LO. Would knockout of 5LO be functionally equivalent to genetic knockout of all leukotriene receptor types in the brain? Is knockout of 5LO in neurons *in vivo* sufficient to prevent AD pathology, or do astrocytic and microglial 5LOs contribute to AD? How would leukotriene receptor knockout, either specifically (i.e., only one BLTR or cysLTR), or in totality, affect the stress response in AD? These questions must be resolved for a nuanced understanding of 5LO neurobiology.

Third, while we have used dexamethasone and restraint/isolation stress as proxy stressors, it would be important to see if 5LO knockout protects AD animals from other dysregulations of the HPA axis. Specifically, the role of CRF in the 5LO-mediated protection from stress would be useful as other investigators have linked it to stress-mediated pathology in AD (Rissman et al., 2012). While we did not specifically investigate this aspect of the HPA axis, dexamethasone administration would likely lead to reduced CRF production (based on known feedback loops), while restraint/isolation stress would likely lead to an increase in CRF to initiate the stress response. To that end, conditional AD model systems exist in which CRF is overproduced driven by an inducible tetracycline promotor (Guo et al. 2012)—it would be intriguing to see if 5LO knockout or pharmacological modulation would confer protection against the development of the AD phenotype in such a system (Dong et al., 2012). Similar understandings

of how stress-induced catecholamine release and mineralocorticoid receptor activation would be modulated by 5LO would be important.

Fourth, while stress hormones have been shown to modulate 5LO, they have many other effects. What stress hormone-associated metabolic effects are altered by the absence of 5LO in AD transgenic models? For example, glucocorticoid exposure leads to altered insulin signaling and insulin resistance, and insulin-associated signaling pathways, and their dysregulation, have been found recently to relevant to the AD brain (De Felice FG, 2013). The importance of 5LO in this aspect of AD pathophysiology would be novel and important.

Finally, since we found that tau, not Aβ, is the critical pathology that is modulated by both stress and 5LO knockout, it would be important to investigate stress in animal models of tauopathy to observe whether this effect exists outside the AD context. This investigation should not only be extended to genetic and neurodegenerative disease in which tau is abnormally phosphorylated, but also in cases where tau pathology is acquired, for example, as in traumatic brain injury.

Conclusions

In conclusion, our work reveals the importance of the 5LO protein in stress-mediated exacerbation of the AD phenotype. Since stress is unavoidable, our data indicate that 5LO-targeted interventions could be of use in individuals particularly vulnerable to this environmental risk factor, and. more broadly, in a preventative strategy against AD.

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