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The Interplay of Progestins, Matrix Metalloproteinases, and the Aging Brain

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The Interplay of Progestins, Matrix Metalloproteinases, and the Aging Brain

Keyana N. Porter

Dissertation submitted to the School of Pharmacy
at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Pharmaceutical and Pharmacological Sciences

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amyloid beta degradation, Alzheimer's disease, hormone therapy, glucocorticoid receptor

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Abstract

The Interplay of Progestins, Matrix Metalloproteinases, and the Aging Brain

Keyana N. Porter

Progestins are synthetic hormones that are designed to mimic the biological actions of progesterone. They, however, possess other pharmacological actions and properties, in addition to their progestational activities. Medroxyprogesterone Acetate (MPA) is a progestin used globally in the hormonal contraceptive, Depo Provera®, by women in their reproductive prime and is a major compound found in hormone therapy (HT) formulations used by menopausal women. MPA is used by approximately 1 in 5 adolescents and adult women in the United States who are sexually active. Globally, nearly 48 million women utilize injectable contraceptives to prevent pregnancy, with most users utilizing MPA as their hormone of choice. Despite the extensive use of hormonal methods as either contraception or menopausal HT, there is very little known about the potential effects of these compounds on the cellular processes of the brain. Additionally, MPA promotes changes in the circulating levels of matrix metalloproteinases (MMPs), such as MMP-9, in the endometrium, yet limited literature studying the effects of MPA on neurons and astroglia cells has been conducted. Furthermore, the dysregulation of MMPs has been implicated in the pathology of Alzheimer's disease (AD), where inhibiting the secretion of MMP-9 from astroglia reduces the proteolytic degradation of amyloid beta.

The key objective for my dissertation work was to investigate the effects of acute and chronic administration of MPA on MMP-9 production and secretion, as well as AD-related pathology, by utilizing hormonal modulation of A β -degrading enzymes. Two sets of studies were designed to begin addressing some of the knowledge gaps associated with MPA and its potential effects on the brain. The hypothesis of these studies was that MPA alters the levels of MMP-9 secretion and enzymatic activity, in turn, negatively impacting the degradation of A β and cognitive function.

The first set of studies examined the outcomes of MPA on MMP-9 secretory, proteolytic, and A β -degrading activities. We found that MPA treatment inhibited transcription of MMP-9, which resulted in a subsequent decrease in the production and secretion of MMP-9 protein, in part through the glucocorticoid receptor. Additionally, we investigated the consequences on amyloid beta-degrading activity and found that MPA treatment decreased proteolytic degradation of amyloid beta. Our results suggest MPA suppresses amyloid beta degradation in an MMP-9-dependent manner, *in vitro*, and potentially compromises the clearance of amyloid beta *in vivo*.

The second set of studies was an *in vivo* assessment, evaluating the effects of chronic administration of exogenous progestins on cognitive function and MMP-9 expression. Our findings suggest that long-term, subcutaneous administration of MPA negatively impacts cognitive function, specifically memory consolidation, in wild-type (WT) mice, and enhances cognitive function, in a triple transgenic mouse model of AD (3xTg-AD), but causes a net increase MMP-9 expression in both mouse models.

Collectively, these two studies demonstrated that MPA's actions on the brain need to be further investigated and more inclusive of non-menopausal and AD models. MPA elicits a differential effect in WT and AD animals, however, increases cortical MMP-9 expression in both phenotypes. Overall, these findings suggest that MPA has the potential to elicit differential effects on women in their reproductive prime and women with predispositions for AD and should encourage more elaborate investigations of its effects.

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Black lives matter.

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List of symbols, abbreviations, and nomenclature

17 β -estradiol (E2)
activator protein 1 (AP1)
Alpha-secretase (α -secretase)
Alzheimer's Disease (AD)
Amyloid beta ($A\beta$)
Amyloid beta 1-40 ($A\beta_{1-40}$)
Amyloid beta 1-42 ($A\beta_{1-42}$)
Amyloid precursor protein (APP)
Amyotrophic lateral sclerosis (ALS)
androgen receptor (AR)
angiotensin-converting enzyme (ACE)
apolipoprotein (*APOE4*)
Beta-secretase (β -secretase)
beta-site APP Cleaving Enzyme 1 (BACE1)
Blood-brain barrier (BBB)
brain-derived neutrophic factor (BDNF)
Central nervous system (CNS)
cerebrospinal fluid (CSF)
conditioned media (CM)
conjugated equine estrones (CEE)
dimethylsulfoxide (DMSO)
Early onset Alzheimer's (EOAD)
endothelin-converting enzyme (ECE)
Enzyme-linked immunosorbent assay (ELISA)
estrogen receptor (ER)
extracellular matrices (ECM)
Familial Alzheimer's disease (FAD)
follicle-stimulating hormone (FSH)
Frontotemporal Lobar Degeneration (FTLD)
Gamma-secretase (γ -secretase)
glucocorticoid receptor (GR)
glucocorticoid response element (GRE)
gonadotropin releasing hormone (GnRH)
Hidden platform (HP)
Hormone therapy (HT)
human T-lymphotropic virus type 1 (HTLV-1)
insulin-degrading enzyme (IDE)
interleukin 1 α (IL-1 α)
lactate dehydrogenase (LDH)
Late onset AD (LOAD)
Lewy Body dementia (LBD)

long term potentiation (LTP)
luteinizing hormone (LH)
Matrix Metalloproteinase-2 (MMP-2)
Matrix Metalloproteinase-3 (MMP-3)
Matrix Metalloproteinase-9 (MMP-9)
Matrix Metalloproteinases (MMPs)
medroxyprogesterone acetate (MPA)
membrane progesterone receptors (mPR)
messenger RNA (mRNA)
microRNA (miR)
mineralocorticoid receptor (MR)
multiple sclerosis (MS)
neprilysin (NEP)
Neurofibrillary tangles (NFT)
nuclear factor kappa B (NFκB)
nuclear factor kappa B (NF-κB)
ovariectomized (OVX)
perineuronal net (PNN)
peripheral nervous system (PNS)
prefrontal cortex (PFC)
Presenilin 2 (PSEN2)
Presenilin1 (PSEN1)
Progesterone (P4)
progesterone receptors (PR)
progesterone response element (PRE)
Reversal (Rev)
serum-free media (SFM)
soluble APP α (sAPP α)
Sporadic Alzheimer's disease (SAD)
tissue inhibitors of MMPs (TIMP)
tissue plasminogen activator (tPA)
Traumatic brain injury (TBI)
triple transgenic mouse model of Alzheimer's disease (3xTg-AD)
tumor necrosis factor α (TNF- α)
Vascular Dementia (VaD)
Visible platform (VP)
Wild-type (WT)
Women's Health Initiative Memory Study (WHIMS)

Chapter 1

Introduction & Literature Review

1.1 Introduction to Alzheimer's Disease

1.1.1 Epidemiology and Impact

Dementia is an “umbrella” term used to describe a range of symptoms associated with cognitive impairment. The four most common types of dementia are Alzheimer's disease (AD), Vascular Dementia (VaD), Frontotemporal Lobar Degeneration (FTLD), and Lewy Body dementia (LBD). Within these four types of dementia, AD is the most prevalent. AD is an age-related, progressive dementia and the sixth leading cause of death in the United States (U.S.). AD is also the only disease in the top 10 causes of death in the U.S. that currently has no way of being prevented, cured, or delayed. It is also the only disease in the U.S. top 10 causes of death whose mortality rate consistently and substantially increases, showing a 145% increase since the year 2000. There are 5.8 million Americans that are estimated to be currently living with AD, with 5.6 million over the age of 65. With current estimates, the number of people, age 65 and older with, AD may triple to nearly 14 million by 2050, which emphasizes the need for developments and advances towards slowing or curing this disease. Overall, AD is progressive in nature, responsible for increased deaths, and contributes to poor health outcomes and disabilities in the U.S., which consequently is a huge burden on patients, caregivers, the healthcare system, and the economy.

1.1.2 Progression of AD

The progression of AD can be divided into three stages: mild, moderate, and severe (Burns and Iliffe, 2009). A patient can experience their first symptoms of mild memory loss before the age of 65 (early onset AD, EOAD) and over the age of 65 (late onset AD, LOAD). Morphologically, the cerebral cortex and subcortical regions are atrophied in AD brains. The most important neuropathological phenomena relevant to AD is the progressive

accumulation of extracellular deposits of amyloid beta (amyloid plaques; A β); aggregates of intracellular tau protein (neurofibrillary tangles); and loss of neurons and synapses (reviewed by Haass et al., 2007). These changes are also accompanied by glial activation, neurite dystrophy, and neuronal death (Jović et al., 2019).

Extracellular amyloid plaques are mostly composed of A β peptide, which is generated by proteolytic cleavage of amyloid precursor protein (APP) (De Strooper, 2010). APP is recognized by alpha-secretase (α -secretase), which cleaves the precursor protein, promoting the non-amyloidogenic cleavage of APP, which has been shown to promote neuroprotection and memory enhancement (Ghisso and Frangione, 2002). However, as reviewed by Chen et al., (2015), with aging, α -secretase becomes progressively inefficient. This causes APP to be truncated by non-specific proteases such as beta- and gamma-secretase (β -secretase and γ -secretase), promoting the amyloidogenic cleavage of APP (Holsinger et al., 2002; Yang et al., 2003; Zhang et al., 2014; Carroll and Li, 2016). Cleavage of APP at the β - and γ -secretase sites produces the 40 or 42 amino acid fragments of A β (A β ₁₋₄₀ and A β ₁₋₄₂) and subsequently, A β peptide is released into the extracellular space (Chen, 2015; Xu et al., 2016). Extracellular A β assumes several conformational states ranging from monomers to soluble oligomers and fibrils. These polymers of A β quickly aggregate and form the amyloid plaques which are characteristic of the disease (Pryor et al., 2012). Aggregates of A β , colocalized with activated microglia, is additionally observed early in the development of AD symptoms (Meda et al., 1995; Cagnin et al., 2001). The leading hypothesis regarding the causation of AD-related neurodegeneration attributes abnormal A β accumulation as a key initiating event (Hardy and Selkoe et al., 2002; Hardy et al., 2009).

Tau is a microtubule-associated protein encoded by the *MAPT* gene. Mutations in *MAPT* have been reported to impact the age of onset of AD. Stabilization of cytoskeletal microtubules of axons occurs through the interaction of tubulin and tau protein. Under pathological conditions, tau is abnormally phosphorylated at multiple residues on the protein, destabilizes cytoskeletal microtubules, and in turn, weakens the cytoskeleton to a degree that increases neuronal vulnerability. Hyperphosphorylation of tau eventually aggregates and forms pathological intracellular deposits (neurofibrillary tangles, NFT). The severity of clinical symptoms has been shown to be highly correlated with the progressive accumulation of NFTs, and there is no correlation between clinical symptoms and deposition of A β (Kauwe et al., 2008). However, aberrant tau phosphorylation is reported to be stimulated by A β 's modulation of protein kinases and phosphatases that regulate the phosphorylation of tau (Binder et al., 1985; Grundke-Iqbal et al., 1986; Arriagada et al., 1992; Götz et al., 2001; Bloom et al., 2014; Wilkaniec et al., 2016). Alternatively, Leroy et al. (2012) demonstrated that the absence of tau decreased amyloid plaque burden by nearly 50%. Collectively, the literature suggests there is a strong relationship between the amyloidogenic processing of APP and tau protein.

1.2 Risk Factors for AD

1.2.1 Genetic and environmental risk factors

Multifactorial in nature, AD is caused by heredity and environmental factors. The rate of progression of AD is increased by the existence of AD in a family's history (familial AD, FAD), promoted by mutations in the amyloid precursor protein (APP) and presenilin 1 (PSEN1) and 2 (PSEN2) genes. Mutations in these genes severely impacts the metabolism of

A β (production, aggregation, and clearance) (Murakami et al., 2003; Tsubuki et al., 2003). Notably, the majority of these mutations are linked to PSEN1/2 mutations (Sherrington et al., 1993). Of the mutations in the APP gene, the majority were identified near the binding sites of the secretases responsible for cleaving APP and generating A β , thus affecting APP processing and leading to increased A β deposition. Familial AD is dominantly inherited, with clinical symptoms surfacing by the age of 45 (Scheltens et al., 2016).

The majority of AD cases are sporadic (sporadic AD, SAD) in nature. Although the primary causes of SAD are not extensively known, there are several social and environmental factors that have been identified to interact with genetic risk factors, which influence the onset and progression of the disease. Aging is the highest risk factor for AD, but lifestyle differences such as smoking, diet, and physical exercise, and engagement in cognitive activities, influenced by education and occupation, also contribute to the risk for AD (Mielke et al. 2014). Level of education, for example, has been shown to promote cognitive reserve, or cognitive resilience, which is thought to serve as a protective mechanism against cognitive decline and influence susceptibility to AD-related pathology (Stern et al., 2009).

Stroke has also been shown to be a major risk factor for dementias, such as AD and Vascular dementia (VaD). Post-mortem brain analyses of AD brains have revealed a strong correlation between a history of stroke and more severe dementia (Snowdon et al., 1997). Furthermore, a history of stroke has also been shown to be strongly associated with an earlier onset of dementia (Honig et al., 2003). It is currently proposed that stroke worsens AD-related symptoms, but also, AD increases the risk for stroke. The inflammatory cascade

initiated by stroke-related insults more than likely results in brain damage that potentiates, and maybe even exacerbates, the progression of AD (Honig et al., 2003). Alternatively, AD potentially increases the risk for stroke, by predisposing the brain to pathological and molecular changes, that substantiate the risk for stroke. In fact, stroke, VaD, and AD share common risk factors including aging, hypertension, insulin resistance, diabetes, obesity, and hyperlipidemia (Craft et al., 2009). Thus, stroke and dementia possess an additive or synergistic relationship.

Traumatic brain injury (TBI) is also closely related to neurodegeneration and dementia, both pathologically and metabolically. Cerebrovascular injury has been shown to induce A β and tau deposition, ultimately causing cognitive impairments and AD-related pathology (Iadecola, 2013; Pluta et al., 2013; De Silva and Faraci, 2016; Wolters et al., 2017; Tarasoff-Conway et al., 2015). Herein, TBI promotes further damage to endothelial cells, alterations in the levels of tight junction proteins and MMP, and a disruption in blood-brain barrier (BBB) permeability (Wen et al., 2014; Salehi et al., 2017; Abdul-Muneer et al., 2013). Mitochondrial dysfunction is a consequence of TBI and has also been suggested to be a contributing and potentiating factor in AD (Silva et al., 2013; Fossati et al., 2016; Swerdlow et al., 2018; Szarka et al., 2018). However, the link between mitochondrial dysfunction and TBI have not been extensively investigated.

In addition to metabolic risk factors for SAD, there are genetic risk factors that have been identified to be highly associated with SAD. For example, beta-site APP Cleaving Enzyme 1 (BACE1), a unit component of β -secretase is also positively associated with the risk for SAD. Genomic studies have revealed additional molecular factors that contribute to

increased risk for SAD through their regulatory functions of immunological responses to neuroinflammation (TREM2, CLU, CR1, ABCA7, CD33, EPHA1, and the MS4A family), synaptic function (PICALM, BIN1, CD33, CD2AP, and EPHA1), and transport and metabolism of cholesterol (APOE, SORL1, CLU and ABCA7) (Seshadri et al., 2010; Hollingworth et al., 2011; Jiang et al., 2013; Totso and Reitz, 2013; Griciuc et al., 2013; Hickman et al., 2014; Rosenthal et al., 2015; Ising et al., 2015).

There is an increased risk and accelerated onset of AD, with the presence of the $\epsilon 4$ allele of the apolipoprotein (*APOE4*) gene, which is the greatest known genetic risk factor for SAD (Corder et al., 1993; Saunders et al., 1993). Outside of cholesterol transport, APOE plays an important role in neuroplasticity and APP processing. It binds $A\beta$, negatively impacting clearance mechanisms and thus, promotes aggregation. Genes involved in the regulation of inflammatory responses and cholesterol metabolism were highly correlated with clinical and neuropathological symptoms of AD (Koldamova et al., 2005; Kim et al., 2009; Castellano et al., 2011; Goedeke et al., 2014). Carriers of *APOE4* have also been shown to develop AD at an earlier age, and simply carrying a single allele of this gene has been found to nearly triple the risk of AD (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993).

In general, age-related changes in gene expression are due to epigenetic regulation. This includes DNA methylation, histone modifications, and changes in the expression of microRNA (miR). miR are small, non-coding single-stranded RNA molecules that regulate the expression of genes that are involved in AD. Several miR have been shown to be heavily involved in direct regulation of APP expression in FAD (Delay et al., 2001; Patel et al., 2008;

Hébert et al., 2009; Vilardo et al., 2010; Long et al., 2011; Long et al., 2012; Schonrock et al., 2012). Similarly, genes attributed to SAD have been shown to be regulated by miR. The gene which encodes the active unit of α -secretase (ADAM10), for example, is regulated by miR that are altered in peripheral blood of AD patients (Postina et al., 2008; Augustin et al., 2012; Leidinger et al., 2013). In theory, utilizing miRNA as biomarkers can aid in the early diagnosis and therapeutic approaches for both FAD and SAD (Kiko et al., 2014). Further understanding of these molecular factors at the RNA and protein levels will ultimately lead to an improved understanding in the interactions of these genes and their close association to the symptoms in AD.

1.2.2 Gender- and sex-specific risk factors

The Institute of Medicine (2001) defines sex as the biological and physiological differences between men and women, attributed to the sex chromosomes (X and Y). Gender-specific differences refer to environmental, social, and cultural influences men and women, adding more of an experiential effect to the biological effects. There is a clear gender disparity that exists in the incidence of AD. Collectively, the risk for AD is influenced by factors with the same frequency for both men and women, but a stronger effect in one particular sex, factors that have similar effects in men and women, but different frequencies, and factors that are different both in strength and frequency. Women are disproportionately affected with AD, when compared to men. Of the 5.6 million people age 65 and older who are impacted by AD, 3.5 million are women and 2.1 million are men (Herbert et al. 2013). AD also progresses faster in woman than men (Tschanz et al. 2011), and women experience significantly more severe pathology than men (Ruitenberg et al.,

2001; Corder et al., 2004; Barnes et al., 2005; Schmidt et al., 2008; Sinforiani et al., 2010; Chapman et al., 2011; Hall et al., 2012; Irvine et al., 2012). Several explanations have been proposed to explain the sex difference in AD. Women are twice as likely to develop depression and live more sedentary lifestyles, which are known risk factors for AD (Kessler et al., 1993; Dotson et al., 2010; Blondell et al., 2014; Livingston et al., 2017; Singh-Manoux et al., 2017; Horder et al., 2018). Additionally, *APOE4*, the strongest risk factor for AD, impacts women and men differently, as women with *APOE4* have been found to be more likely to develop dementia than men with *APOE4* (Farrer et al., 1997; Fleisher et al., 2008; Altmann et al., 2014; Sampedro et al., 2015). However, with age being a major risk factor for AD, the most simplistic theory is that women live longer than men, making it appear there are more women living with AD than there are men. This concept of “survival bias” was shown in the Framingham Heart Study, which reported an increased mortality rate in men due to poor cardiovascular health and decreased risk for dementia in men who reached the age of 65 and older, when compared to women (Petrea et al., 2009). Epidemiologists considered the data was impacted by the fact that men with positive cardiovascular health generally living healthier lives, lowering their overall risk factors for dementia.

Sex differences are also observed in several transgenic mouse models of AD, where female AD animals have been reported to experience an earlier on-set of neuropathology, as well as more severe AD-related pathology. Accumulation of A β , for example, is higher in female Tg2576, APP_{swe}XPS1, APP23, APP_{swe}/PSEN1E9, and 3xTg-AD mice (Sturchler-Pierrat and Staufienbiel, 2000; Callahan et al., 2001; Lee et al., 2002; Anderson et al., 2003; Hirata-

Fukae et al., 2008; Halford and Russell, 2009; Carroll et al., 2010). Additionally, performances in behavior assays of cognitive function suggest female transgenic AD mice are more cognitively impaired when compared to male mice. The data from these studies suggest there are sex-specific risk factors that exist.

1.3 Matrix Metalloproteinase-9

1.3.1 Introduction to Matrix Metalloproteinases (MMPs)

The integrity of the central nervous system (CNS) is maintained by the extracellular matrix, which consists of structural and signaling molecules that are essential for cellular differentiation and proliferation. Homeostatic mechanisms that regulate cell migration, extension of neurites, and synaptic plasticity are heavily dependent on proteins secreted into the extracellular matrix, and to maintain homeostasis, these proteins are degraded by proteolytic enzymes. Just as extracellular matrix proteins are regulated, these proteolytic enzymes are also regulated at various levels. Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that have many extracellular substrates. MMPs are expressed in many cell types, including endothelial cells, muscle cells, and, in the central nervous system (CNS), neurons and glia (Genersch et al., 2000; Roomi et al., 2009). To date, there are over 20 MMPs that have been identified (Ethell and Ethell, 2007), with a few residing in the CNS, in normal and pathological conditions (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MT1-MMP, MT5-MMP, and their inhibitors, TIMP-1,-2,-3, and -4) (Tanney et al., 1998; Vaillant et al., 1999; Oh et al., 1999; Llano et al., 1999; Sekine-Aizawa et al., 2001; Agapova et al., 2001; Rosenberg et al., 2001; Schutz et al., 2002; Costa et al., 2002).

1.3.2 Structure, Expression, and Activation

The majority of MMPs share similar structural domains, which includes an N-terminal signal peptide, a propeptide region, a catalytic domain containing a Zn^{2+} binding site, a short hinge region, fibronectin repeats and C-terminal hemopexin-like repeats (Hayashita-Kinoh et al., 2001; Sekine-Aizawa et al., 2001). MMPs are differentiated by the components within their hemopexin-like and fibronectin repeats. Uniquely, MMP-9 and MMP-2 are closely related but distinguished from other MMPs by their fibronectin type II inserts, in their catalytic domains. The fibronectin type II inserts allow for MMP-9 and MMP-2 to bind elastin, laminin, and collagen, as well as cleave their substrates (Van den Steen et al., 2002). The N-terminal signal peptide targets the enzyme for secretion and promotes the cleavage of the MMPs, for activation. The propeptide region contains a “cysteine switch” sequence, and a disruption between the cysteine and Zn^{2+} interaction initiates activation of the MMP (Vallee and Auld, 1990). MMPs are regulated by the propeptide region, which maintains the enzymes in an inactive state by binding tissue inhibitors of MMPs (TIMP) (Cao et al., 1998; Pavlaki et al., 2002).

Intracellularly, MMP-9 is distributed along microtubules and microfilaments, via vesicular transport. Secretion of MMP-9 occurs via the ER-Golgi secretory pathway, in 200 – 1000 nm vesicles. Initially, MMP-9 is secreted extracellularly as a catalytically inactive pro-enzyme (proMMP-9). The pro-form of MMP-9 is incapable of cleaving its substrates until the proteolysis, or protein unfolding, of the active site occurs. ProMMP-9 is bound to TIMP-1 at the C-terminal domain of MMP-9. Once the “cysteine switch” occurs, the proMMP-9•TIMP complex dissociates with the assistance of other MMPs, tissue plasminogen

activator (tPA)-plasmin system, and/or serine proteases, allowing proMMP-9 to become fully activated (MMP-9) (Stawarski et al., 2014). Some MMPs require assistance from membrane type MMPs (MT-MMPs). MMP-2, for example, has a secretory mechanism which involves a tertiary complex with TIMP-2 and MT1-MMP.

1.3.3 Regulation

The expression, secretion, and proteolytic activities of MMPs are tightly regulated, during early development and adulthood, at three levels: transcription, activation, and inhibition by physiological inhibitors. There are also post-translational modifications, compartmentalization, and storage mechanisms that additionally regulate MMP activity. MMP-9 mRNA, protein, and enzymatic levels have been detected in several regions of the CNS, such as the hippocampus, brainstem, cortex, and cerebellum (Oliveira-Silva et al., 2007; Gawlak et al., 2009; Aujla and Huntley, 2014; Bednarek et al., 2014). MMP-9 is mainly expressed in glia and along oligodendrocyte processes (Dzwonek et al., 2004; Gawlak et al., 2009). The expression of MMPs also appears to be function-specific more than cell-specific, for example, protein expression and enzymatic activity of MMP-9 appears localized to cell bodies, dendrites, and excitatory synapses that are found on dendritic spines.

In the adult brain, MMP-9 levels are ubiquitously low, and altered in response to various stimuli, including growth factors, cytokines, metal ions, oncogenes, and hormones (reviewed by Van den Steen et al., 2002). The signaling pathways shown to be involved in the activation of MMPs are ERK-MAP kinase, phosphoinositide 3 (PI-3) kinase, protein kinase C (PKC), and p38 pathways (White et al., 2000; Wang et al., 2002; Hussain et al.,

2002). There are also binding sites for activator protein 1 (AP1), nuclear factor kappa B (NFκB), Smad, and Ets in the promoter sequence of most MMPs.

Importantly, TIMP expression is critical for preventing excessive MMP proteolytic activity. TIMPs appear to be regulated in a similar manner, through the binding of the same nuclear factors that bind MMPs. Due to similar release kinetics, TIMP expression is also inversely related to the expression of MMPs (Sato et al., 2002; Zechel et al., 2002). The literature suggests MMP-9 is specifically inhibited by TIMP-1, via direct binding of TIMP-1 to the catalytic site of MMP-9 or direct binding of MMP-9's hemopexin domain to the C-terminus of TIMP-1. Ultimately, the tight regulation of MMP-9's proteolytic activity is essential to its regulatory functions in the CNS.

1.4 Matrix Metalloproteinase-9 (MMP-9) and Brain Function

MMPs possess both beneficial and detrimental impacts on brain development, the adult brain, and the spinal cord. Collectively, MMPs have been implicated in BBB dysfunction, demyelination, neuroinflammation, neurotoxicity, and CNS diseases such as multiple sclerosis (MS) and stroke. The literature shows MMP-9 holds a unique and essential role in the physiology and pathology of many brain functions and dysfunctions.

1.4.1 MMP-9 and Inflammation

Neuroinflammation typically induces immune response mediators to be released from microglia, the resident macrophages in the brain, as well as neurons and astrocytes. A dysfunctional BBB promotes increased leukocyte infiltration, as well as increased MMP-9 production. Conversely, MMP-9, has been shown to induce proinflammatory cytokines (Candelario-Jalil et al., 2009), which also influences BBB permeability, through the cleavage

of the vascular basal lamina and/or tight junctions between cells belonging to the neurovascular unit (Reijerkerk et al., 2006; Candelario-Jalil et al., 2009; Barkus et al., 2010; Verslegers et al., 2013). This MMP-9-induced BBB disruption serves as the foundation for the pathogenesis of MS, where increased MMP-9 expression and activity have been consistently detected in the serum, cerebrospinal fluid (CSF), and demyelinating lesions of multiple sclerosis (MS) patients (Yong et al., 2007). The literature also suggests MMP-9 plays a role in bacterial and viral infections. During human T-lymphotropic virus type 1 (HTLV-1) infection in glial cells, for example, tumor necrosis factor α (TNF- α) and interleukin 1 α (IL-1 α) yielded increased MMP-9 expression and secretion in glial cells and in CSF (Giraudon et al., 1996).

1.4.2 MMP-9 and Stroke

There is also an abundance of literature, demonstrating MMP-9's role in stroke (Chaturvedi and Kaczmarek, 2014; Yang and Rosenberg, 2015). Within the multiple roles MMP-9 plays in stroke, it mainly influences infarct volume and post-stroke recovery. Genetic ablation of MMP-9 reportedly protects against ischemic stroke (Jin et al., 2010; Rivera et al., 2010). Currently, tPA is the only FDA-approved treatment for ischemic stroke. The limitation of this treatment is its success depends on a highly specific 4.5-hour time window. If administered more than 4.5 hours post-onset of the stroke, MMP-9 is activated, which, in turn, causes further disruption of the BBB (Murata et al., 2008). This ultimately worsens post-stroke outcomes, highlighting the important role of MMP-9 in stroke outcome.

1.4.3 MMP-9 and Synaptic Plasticity

MMP-9's beneficial role in synaptic plasticity is well documented. Dendritic spine morphology is highly dependent on MMP-9 expression and activity (Bilousova et al., 2009; Benson and Huntley, 2012), where overexpression of MMP-9 yields thinner dendritic spines (Michaluk et al., 2011). Additionally, there are several *ex vivo* and *in vivo* studies that show MMP-9 maintains long term potentiation (LTP), and inhibition of MMP-9 leads to LTP impairment, within multiple pathways (Nagy et al., 2006; Meighan et al., 2006; Bozdagi et al., 2007; Okulski et al., 2007; Conant et al., 2010; Wojtowicz and Mozrymas, 2010; Dziembowska et al., 2012; Wiera et al., 2012; Gorkiewicz et al., 2015). MMP-9 has also been shown to be important, during postnatal periods, for the development of sensory circuits and the regulation of synaptogenesis and myelination (Oh et al., 1999; Michaluk et al., 2009; Peixoto et al., 2011). Pharmacological inhibition or genetic deletion of MMP-9 also negatively impacts learning and memory. Localization of MMP-9 expression and activity has been shown to be behavior task-dependent. For example, expression and activity were found to be increased in the hippocampus, prefrontal cortex, piriform cortex, and cerebellum, following the performance of the head-shake response task (Wright et al., 2006), while increased MMP-9 expression was reported in the hippocampus, frontal cortex, and amygdala after the completion of contextual fear conditioning and passive avoidance (Nagy et al., 2007; Ganguly et al., 2013). Collectively, MMP-9 expression is crucial for healthy synaptic function and learning and memory.

1.4.4 MMP-9 and AD

MMP-9 is also heavily implicated in neurogenerative disease, including Parkinson's disease, Huntington's disease, Down syndrome, Amyotrophic lateral sclerosis (ALS), and AD

(Yong, 2005). Regarding AD, there is a clear link between MMP-9 and AD pathogenesis. MMP-9 expression is stimulated by the presence of A β peptide (Deb and Gottschall et al., 1996), and may serve to maintain a homeostatic balance between the production and clearance of A β . There are several zinc-metalloproteinases that mediate the clearance of A β , including endothelin-converting enzyme (ECE), angiotensin-converting enzyme (ACE), insulin-degrading enzyme (IDE), neprilysin (NEP) and matrix metalloproteinases (MMPs) (Saido and Leissring et al., 2012). Among these A β -degrading enzymes, MMP-9 is the most distinguished, as it has been shown to be capable of degrading a range of A β species. Furthermore, deposition of A β has been shown to be inversely associated with MMP activity (Lim et al., 1997; Ridnour et al., 2012). Not only has a genetic association been shown (Helbecque et al., 2003), but postmortem analyses of AD brain tissues show increased MMP-9 levels in neurons, neurofibrillary tangles, and senile plaques (Backstrom et al., 1996; Asahina et al., 2001; Helbecque et al., 2003; Lorenzl et al., 2003). Additionally, MMP-9 levels in the CSF of AD patients were shown to be lower than levels in control patients (Mroczko et al., 2014). TIMP-1 has also been shown to be increased in the CSF of AD patients, most likely due to increased MMP-3 levels in the CSF and plasma (Reitz, et al., 2010; Horstmann et al., 2010). An imbalance between MMP-9 and its regulatory enzyme, TIMP-1, is also implicated in AD. AD patients tend to have high MMP-9/TIMP-1 ratios, along with high levels of tau in their CSF (Talamagas et al., 2007).

Along with A β , tau is also a substrate for MMP-9, as well as MMP-3, which has been shown to be the activator of proMMP-9 (Hernández-Guillamon et al., 2009). Ironically, while MMP-3 has been shown to degrade tau in regions that facilitate fibril formation, MMP-9 has

been shown to promote aggregation via its tau cleavage sites (Nübling et al., 2012). This is attributed to the differences in their affinities for cleaving the protein. However, as previously mentioned, MMP-3 is a known activator of proMMP-9, which would suggest an increase in brain MMP-3 yields an indirect proaggregatory effect on tau via increased MMP-9 activity (Nübling et al., 2012). Moreover, APOE4, a major risk factor for AD, significantly reduces A β -induced MMP-9 levels. Thus, reduction of astrocytic MMP-9 would most likely impact clearance of A β and encourage deposition in AD (Guo et al., 2006; Bell et al., 2012).

1.5 Female Reproductive Aging

1.5.1 Introduction to the female reproductive system

The female reproductive system is controlled by communication between the hypothalamus, anterior pituitary gland, and ovaries, forming the hypothalamic-pituitary-adrenal (HPA) axis (reviewed by Hiller-Sturmhöfel and Bartke, 1998; reviewed by Del Rio et al., 2018). From the hypothalamus, a small peptide, gonadotropin releasing hormone (GnRH), is secreted and travels to the anterior pituitary gland. Secretion of this peptide regulates the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from cells in the anterior pituitary gland, to the ovaries. FSH stimulates the development of ovarian follicles in the ovaries and stimulates the secretion of estrogen by follicle cells, of the ovaries. LH stimulates ovulation and the production of progesterone by the corpus luteum of the ovaries. Once LH reaches a peak/surges, there is a rapid decline in estrogen levels which increases cholesterol receptor levels in the ovaries, more specifically in theca and granulosa cells. This free cholesterol is then converted to progesterone and progesterone exits the cells, binds albumin and corticosteroid-binding albumin and enters

into circulation. To regulate the release of gonadotropins, there is a negative feedback loop exerted on the hypothalamus established, where high levels of circulating estrogen suppresses further release of FSH and high levels of circulating progesterone suppresses further release of LH.

1.6 Adolescence & Adulthood

Once a woman reaches the menarche, or first menses, gonadotropin levels undergo cyclic changes (reviewed by Boswell, 2014; reviewed by Galvez-Peralta, 2015). The menstrual cycle can be divided into two phases. The first 14 days is known as the follicular phase and the last 14 days is known as the luteal phase. In the follicular phase, there is pulsatile release of GnRH from the hypothalamus, which stimulates the synthesis and release of FSH and LH from the pituitary gland. FSH will then stimulate the maturation of ovarian follicles, as well as stimulate inhibin production and the conversion of androgens to estrogens (E2) by granulosa cells. Negative feedback on the pituitary and hypothalamus is exerted by E2 and inhibin, which is only expressed when circulating estrogen levels are low. After about 10 days, once high estrogen levels are achieved, there is positive feedback exerted on the pituitary gland, stimulating the release of LH and triggering ovulation. After ovulation, LH, FSH, and GnRH levels decrease, and the follicle turns into a corpus luteum. The corpus luteum begins to secrete inhibin and progesterone. Inhibin has a negative feedback and inhibits the secretion of FSH, while progesterone has a negative feedback on the hypothalamus, inhibiting the secretion of GnRH, affecting the release of FSH and LH. Progesterone also stimulates the growth of the endometrial lining. Under the assumption there is no fertilization, the corpus luteum degrades to allow new follicles to undergo

maturation and progesterone is no longer inhibiting GnRH release. This causes the endometrial lining to shed (menstruation) and allows for the initiation of the next cycle. The combination of estrogen and progesterone suppresses gonadotropin synthesis and release through their effects on the HPA axis. Estrogen inhibits the release of FSH from the pituitary, in turn inhibiting follicular development. Without the development of ovarian follicles, there is no LH and thus, ovulation will not occur. Progestins in the combination formulation and even in the progestin-only formulations prevent ovulation by inhibiting LH release. This will prevent the LH surge and subsequently prevent ovulation. Additionally, progestins causes thickening of cervical mucus and endometrial atrophy. The thickening of the cervical mucus prevents sperm penetration and endometrial atrophy interferes with implantation, helping to prevent pregnancy.

1.6.1 Senescence

Perimenopause is often referred to as “Menopausal Transition”. Ovarian follicles produce estrogen and inhibin, in response to FSH, allowing inhibin to prevent further synthesis and secretion of FSH. The inevitable decrease of ovarian follicles causes a gradual decline in circulating estrogen levels and inhibin production. Decreased inhibin production leads to subsequent loss of the negative feedback loop to the hypothalamus, increasing GnRH secretion. Women begin to experience irregular menstruation at this stage and these changes in hormonal regulation ultimately leads to a woman’s last menstrual period. At the menopausal stage, the ovaries become depleted of ovarian follicles, massively impacting estrogen and inhibin levels to the point a woman no longer ovulates or menstruates. Post-menopause is characterized by the loss of ovarian sensitivity to FSH and LH, as well as the

lack of the negative feedback that is typically exerted on the HPA axis, by E2 and inhibin. These changes lead to increased production and release of GnRH, FSH, and LH, as well as extremely low levels of estrogen. The combination of progesterone and estrogen is used to “normalize” the menstrual cycle, which during the menopausal periods of a woman’s life is irregular. Therefore, treatment with the combination of progesterone and estrogen will help with peri-menopausal symptoms. Furthermore, the cyclic use of progesterone, two weeks on, two weeks off, will often keep the normal menstrual cycle going. Daily therapy with 0.625 mg of conjugated equine estrones (CEE) + 2.5-5mg of medroxyprogesterone acetate (MPA) will eliminate cyclic bleeding, control vasomotor symptoms, prevent genital atrophy, maintain bone density, and promote a favorable lipid profile. Progesterone's effects on the pituitary apparently contribute to its protective effect against osteoporosis, hypertension, hirsutism, etc. Thus, the complementation of progestin treatment is used to treat estrogen excess or deficiency to help alleviate vasomotor symptoms.

1.7 Progestogens

The term “progestogen” is used to describe both natural progesterone and synthetic derivations of progesterone, or progestins (Stanczyk et al., 2003). MPA is an example of a progestin, found in both birth control formulations and hormone therapies (HT).

1.7.1 Progesterone

Progesterone is a well-characterized female reproductive hormone that plays a major role in ovarian functions, such as implantation and menstruation. It is synthesized in the ovary (corpus luteum and granulosa cells), the testis, and the adrenal cortex from circulating cholesterol, as well as the placenta during pregnancy (Hu et al., 2010). The

reproductive synthesis of progesterone is controlled by communication between the hypothalamus, anterior pituitary gland, and ovaries, forming the hypothalamic-pituitary-ovarian axis. A surge in luteinizing hormone (LH) increases free cholesterol, which is ultimately converted to progesterone (Kuo et al., 2012). Once synthesized, progesterone targets the female genital tract, the pituitary gland, mammary glands, osteoblasts, and the CNS (Taraborrelli, 2015). There is overwhelming evidence that the brain synthesizes steroids, like progesterone, de novo from cholesterol by neural cells of both the CNS and peripheral nervous system (PNS) (Reddy, 2010). Almost all brain cholesterol is found in oligodendrocytes and plasma membranes. Thus, progesterone synthesis occurs in oligodendrocytes, astrocytes, glial cells, Purkinje neurons, and in the peripheral nervous system, progesterone synthesis occurs in Schwann cells (Zwain and Yen, 1999). After the initial findings by Zwain and Yen et al., (1999) demonstrating neural cells have the capacity to synthesize steroids, several lines of strong evidence have followed, in both animal and human studies, reporting progesterone is synthesized in these neural cells.

Synthesis of progesterone in the brain modulates intracellular signaling pathways, channels, and transcription, in turn impacting neuronal activity. In neural cells, progesterone binds to intracellular progesterone receptors. These progesterone receptors are either in the nucleus where they regulate the transcription of progesterone-sensitive genes, or they directly interact with membrane-associated kinases (reviewed by Jacobsen and Horwitz, 2013). Progesterone enters the cell and binds to progesterone receptors (PR) that are distributed between the nucleus and the cytoplasm. To activate gene transcription, the ligand-receptor complex binds to a progesterone response element (PRE) in target

promoters. This leads to an up-regulation or down-regulation of gene transcription and subsequently leads to tissue responses. Progesterone may also act via non-genomic signaling, in which it activates membrane progesterone receptors (mPR), which are usually directly linked to intracellular signal transduction or second messenger systems, mediating rapid tissue responses.

1.7.2 Synthetic Progestins

Synthetic progestins are commonly used in place of natural progesterone due to their ability to inhibit ovulation and their greater bioavailability, half-life, and activity post-administration (Pasqualini et al., 1996; Sitruk-Ware et al., 2000). Since 1938, the year the very first progestin was synthesized, many generations of progestins have been synthesized, differing in their chemical structures and biological effects (Inhoffen and Hohlweg, 1938). Structurally, synthetic progestins either relate to progesterone or testosterone and these structural differences influence their relative binding affinity (RBA) to sex steroid receptors, which in turn impacts their transcriptional activity. In addition to the PR, progestins interact with androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). Like progesterone, progestins enter the cell and bind their cognate receptors that are distributed between the nucleus and the cytoplasm. To activate gene transcription, the ligand-receptor complex binds to a hormone response element (PRE) in target promoters, or bind receptors in the cytoplasm, yielding an up-regulation or down-regulation of gene transcription. Furthermore, although natural progesterone holds a strong RBA for mPRs, it is currently inconclusive whether progestins mediate their actions via mPRs. Within a list of 30 steroidal compounds, the RBAs for mPRs failed to exceed 50%

(Thomas et al., 2007). The non-genomic membrane bound signaling mechanisms of synthetic progestins remain to be elucidated.

1.8 Hormones and Cognitive Decline

Age-related hormonal changes have been found to negatively impact cognitive function and impact risk for AD. There is an abundance of literature which focuses on the effects of estrogen-related compounds, androgens, and progesterone on the brain.

1.8.1 Estrogens

The neuroprotective effects of estrogen-related compounds are well-documented in the literature. Estrogens have been reported to protect against neuronal death (Simpkins et al., 2009, 2010, 2012; Suzuki et al., 2009), antagonize AD-related pathology (Pike et al., 2009), and regulate spine density, LTP, and neurotransmitters, in turn, protecting cognitive function (Cooke and Woolley, 2005). Estrogens were initially linked to A β once it was reported that E2 modifies APP processing, by either increasing enzymes that reduce levels of A β or decreasing enzymes that increase levels of A β , ultimately promoting the nonamyloidogenic pathway (Desdouits-Magnen et al., 1998; Jaffe et al., 1994; Greenfield et al., 2002; Gandy et al., 2003; Thakur and Mani et al., 2005; Bernstein et al., 2009; Amtul et al., 2010; Nord et al., 2010). Estrogen-related compounds have also been shown to promote A β clearance by inducing NEP (Liang et al., 2010; Grimm et al., 2013), IDE (Amtul et al., 2010; Zhao et al., 2011), and transthyretin expression, which sequesters A β to prevent aggregation (Schwarzmann et al., 1994; Quintela et al., 2009; Amtul et al., 2010; Oliveira et al., 2011).

Clinically, there are lower circulating levels of E2 in the blood and the brain, in women with AD, when compared to age-matched controls (Yue et al., 2005; Rosario et al., 2011). Surgical removal of the ovaries from wild-type (WT) female animals, which significantly reduces the levels of circulating E2 and progesterone, has been shown to yield increased levels of A β (Petanceska et al., 2000). Many studies have further demonstrated how E2 can prevent and rescue pathology in AD mouse models (Levin-Allerhand et al., 2002; Xu et al., 2002; Carroll et al., 2007; Carroll and Pike et al., 2008;). Hogervorst et al., (2002) sought out to investigate the effect of ET (estrogens alone) versus HT (CEE+ progestogen) on cognitive function in postmenopausal women. From this double-blinded randomized controlled trial, they reported modest positive cognitive outcomes from HT. The following year, the Women's Health Initiative Memory Study (WHIMS) evaluated the cognitive outcomes of post-menopausal HT consisting of CEE with or without MPA. The findings from this large-scale clinical trial suggests HT fails to delay the progression of cognitive decline in women, and revealed HT increases the risk for dementia (Rapp et al., 2003; Shumaker et al., 2003). These findings have encouraged further investigation and discussion of why the discrepancy between basic research and clinical research data exists (Gleason et al., 2015; Henderson et al., 2016). The age in which HT is initiated, the hormonal components within the formulation, and the treatment regimen are suggested to be the most important influential factors on cognitive outcomes (Carroll et al., 2010; Craig and Murphy, 2010; Whitmer et al., 2011).

1.8.2 Androgens

Androgenic compounds have also been shown to hold neuroprotective roles by promoting the growth of neurons, regeneration of axons, and function of synapses (MacLusky et al., 2004; Huppenbauer et al., 2005; Marron et al., 2005; Brannvall et al., 2005; Hajszan et al., 2008; Hatanaka et al., 2009). Additionally, androgens protect against age-related insults and AD-related pathology, such as A β toxicity and production, by targeting APP processing and metabolizing enzymes (Gouras et al., 2000; Pike et al., 2001; Nguyen et al., 2005; Park et al., 2007; Zhang et al., 2014; McAllister et al., 2010). Clearance mechanisms of A β are also affected by androgenic compounds, where androgens have been shown to increase the expression of NEP, which holds an androgen response element in the promoter region of the *NEP* gene (Shen et al., 2000; Yao et al., 2008; McAllister et al., 2010). The literature also indicates testosterone regulates circulating A β in the CSF and plasma of castrated male animals (Wahjoepramono et al., 2008) and anti-androgen therapy causes an accumulation of circulating A β (Almeida et al., 2004). Moreover, age-related depletion of hormones, in the brain, leads to increased levels of A β , in rat brains (Rosario et al., 2009). In a comparative study, where the effects of various sex steroid hormones on A β levels were assessed, testosterone was shown to reduced levels of A β , to a higher degree, when compared to E2, in castrated 3xTg-AD mice (Rosario et al., 2010). Overall, low levels of testosterone have been linked to increased levels of A β , worsened cognitive outcomes, impaired brain metabolism, and increased risk of AD (Moffat et al., 2004; Verdile et al., 2012; Tan et al., 2013; Butchart et al., 2013).

1.9 Knowledge Gap: Progestogens and Cognitive Function

As previously mentioned, the term “progestogen” is used to describe both natural progesterone and synthetic derivations of progesterone, or progestins (Stanczyk et al., 2003). Progesterone has been shown to be neuroprotective in stroke, TBI, and neurodegenerative diseases such as AD (Goodman et al., 1996; Jiang et al., 1996; Roof et al., 1996; Chen et al., 1999; Roof et al., 2000; Kumon et al., 2000; Nilsen et al., 2002a,b; Nilsen et al., 2003; Morali et al., 2005; Pettus et al., 2005; Kaur et al., 2007; Singh et al., 2013). Synthetic progestins are utilized by healthy women to control ovarian function earlier in life, and to limit menopausal symptoms later in life. The brain is a major target for synthetic progestins. Due to their lipophilicity, they have been shown to easily cross the blood-brain-barrier and rapidly diffuse throughout the CNS, into brain regions such as the hypothalamus, hippocampus, amygdala, and cortex, as well as the spinal cord (Sohrabji, 2007; Wilson et al., 2008; Banks et al., 2012; Diotel et al., 2018). Despite the multiple generations of progestins that have been synthesized, there needs to be further investigation of their effects on brain functions. Notably, the cognitive effects of progestogens, when administered alone, is currently understudied, where majority of investigations study the effects of progestogens when administered with an estrogen.

MPA is an example of a progestin, found in both birth control formulations and HT. MPA, which is used to reduce the risk of cancers resulting from unopposed estrogen therapy, is the most commonly used progestogen in HT regimens (Gambrell et al., 1986; Hirvonen et al., 1996). Despite the fact MPA structurally resembles progesterone more than any other progestin, there are vast differences in their pharmacological and

pharmacokinetic profiles (Hackenberg et al., 1990; Schindler et al., 2003; Winneker et al., 2003; Koubovec et al., 2005; Jodhka et al., 2009). It has also become increasingly clear that although progesterone is considered neuroprotective, MPA is not. MPA has been shown not only to inhibit brain-derived neurotrophic factor (BDNF), which negatively impacts neurogenesis, but also fails to alter glutamate-induced intracellular Ca^{2+} influx, while progesterone's neuroprotective effects have been shown to be dependent on both neurotrophin signaling and attenuation of glutamate-induced increases in Ca^{2+} (Kaur et al., 2007; Jodhka et al., 2009). The neuroprotective effects of E2 are also antagonized by MPA but substantiated by progesterone (Miyagawa et al., 1997; Rosano et al., 2000; Nilsen et al., 2002b; Littleton-Kearney et al., 2005).

Allopregnanolone, a metabolite of progesterone, is a neuroactive steroid. Dysregulation of allopregnanolone has been associated with the pathophysiology of neurodegenerative disorders, including Alzheimer's disease. Allopregnanolone levels are significantly decreased, both in women and in rats, after long-term administration of hormonal contraceptives, with levels being reduced by almost 80% (Follesa et al., 2002; Rapkin et al., 2006; Porcu et al. 2012; Santoru et al., 2014). When administered allopregnanolone, 3xTg-AD mice were shown to have improved performances on cognitive tasks through the regeneration of hippocampal nerve cells (Wang et al., 2010). *In vitro*, allopregnanolone was found to increase the number of neural progenitor cells from the hippocampus of rats and human cerebral cortex (Wang et al., 2005). Additionally, there are several studies which demonstrate restoration of allopregnanolone levels is essential for a therapeutic benefit in AD. These findings suggest that the reduction of allopregnanolone

may play a significant role in the pathogenesis of AD. It is currently unclear if MPA is a substrate for the progesterone metabolizing enzymes, 5-alpha-reductase and 3-alpha-hydroxysteroid dehydrogenase. In lieu of MPA's inability to be converted to neuroactive steroid metabolites, coupled with its reduced potential to convert brain progesterone/neuroprogesterone to allopregnanolone, it is evident MPA has limited neuroprotective efficacy.

Clinically relevant exogenous hormones are globally used, but their long-term effects aren't well understood. While limited research with MPA suggests a negative impact on the brain, these studies need to be extended to model systems relevant to women in their reproductive prime, as majority of efforts have been in menopausal animal models and menopausal women (Shumaker et al., 2003, 2004; Braden et al., 2010, 2011, 2017; Lowry et al., 2010; Olanrewaju et al., 2013). Thus, there is an unmet need for studies focusing on the effects of chronically using MPA, earlier in life, and before menopause. Furthermore, as previously stated, estrogens can modulate the levels of A β -degrading enzymes, such as NEP and IDE. Merlo and Sortino (2012) utilized estrogen to induce the A β -degrading activity of MMP-9, establishing MMP-2 and MMP-9's contribution to the neuroprotective effect of estrogen, *in vitro*, and demonstrating estrogen can also modulate the levels of additional A β -degrading enzymes. There is accumulating evidence, in systems other than the CNS, that MPA can modulate the levels of MMP-9 (Di Nezza et al., 2003; Hwang-Levine et al., 2011; Allen et al., 2019). Thus, investigation of the effects on MPA on MMP-9 secreted from glia remains to be pursued.

The population of AD patients is progressively increasing, while simultaneously advancing the burden on caregivers, healthcare providers, and the healthcare system. Due to the multifactorial and heterogenous nature of AD, the identification of risk factors for AD is essential for understanding how to potentially prevent and delay the progression of this disease. A major issue in the field of AD is nearly two-thirds of AD patients are female. To attribute the gender disparity in AD to survival bias would be logical, however AD-related changes in the brain are initiated nearly 20 years before an official diagnosis, thus the survival bias cannot fully explain the sex- and gender-specific differences (Hof et al., 1996; Perl et al., 2010; Bateman et al., 2012). Collectively, the precise explanation as to why women currently bear the greatest AD burden remains yet to be clearly determined.

The overarching goal of this dissertation work was to characterize the effects of exogenous progestin treatment on AD-related pathology, utilizing hormonal modulation of A β -degrading enzymes. The first strategy was an *in vitro* evaluation of the effects of the commonly used progestin, MPA, on MMP-9. Chapter 2 examined the outcomes of MPA on MMP-9 secretory, proteolytic, and A β -degrading activities. The second strategy was an *in vivo* assessment, evaluating the effects of chronic administration of exogenous progestins on AD-related cognitive function. Chapter 3 further evaluated the *in vivo* effects of long-term, subcutaneous administration of MPA on cognitive functions in WT and 3xTg-AD mice. Chapter 4 summarized the findings from the studies evaluating the acute and chronic effects of MPA on AD-related pathology, *in vitro* and *in vivo*, and outlines the limitations, important takeaways, and future directions of these studies.

2 Chapter 2

Medroxyprogesterone Acetate Impairs Amyloid Beta Degradation in a Matrix Metalloproteinase-9 Dependent Manner

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2.1 Abstract

Despite the extensive use of hormonal methods as either contraception or menopausal hormone therapy (HT), there is very little known about the potential effects of these compounds on the cellular processes of the brain. Medroxyprogesterone Acetate (MPA) is a progestogen used globally in the hormonal contraceptive, Depo Provera, by women in their reproductive prime and is a major compound found in HT formulations used by menopausal women. MPA promotes changes in the circulating levels of matrix metalloproteinases (MMPs), such as MMP-9, in the endometrium, yet limited literature studying the effects of MPA on neurons and astroglia cells has been conducted. Additionally, the dysregulation of MMPs has been implicated in the pathology of Alzheimer's disease (AD), where inhibiting the secretion of MMP-9 from astroglia reduces the proteolytic degradation of amyloid beta. Thus, our hypothesis is that exposure to MPA disrupts proteolytic degradation of amyloid beta through the downregulation of MMP-9 expression and subsequent secretion. To assess the effect of progestins on MMP-9 and amyloid beta, *in vitro*, C6 rat glial cells were exposed to MPA for 48 hours and then the enzymatic, secretory, and amyloid beta degrading capacity of MMP-9 was assessed from the conditioned culture medium. We found that MPA treatment inhibited transcription of MMP-9, which resulted in a subsequent decrease in the production and secretion of MMP-9 protein, in part through the glucocorticoid receptor. Additionally, we investigated the consequences on amyloid beta-degrading activity and found that MPA treatment decreased proteolytic degradation of amyloid beta. Our results suggest MPA suppresses amyloid beta degradation in an MMP-9-dependent manner, *in vitro*, and potentially compromises the clearance of amyloid beta *in vivo*.

Keywords: matrix metalloproteinases, medroxyprogesterone acetate, amyloid beta (A β), amyloid beta (A β) degradation, Alzheimer's disease, hormone therapy, glucocorticoid receptor

2.2 Introduction

Alzheimer's disease (AD) is a neurodegenerative disease with three key pathological hallmarks: the progressive accumulation of extracellular deposits (amyloid plaques); aggregates of intracellular protein (neurofibrillary tangles); and loss of neurons and synapses (reviewed by Haas et al., 2007). Extracellular amyloid plaques are mostly composed of amyloid beta peptide ($A\beta$), which is generated by proteolytic cleavage of amyloid precursor protein (APP) (De Strooper, 2010). APP is recognized by alpha-secretase (α -secretase), which cleaves the precursor protein, promoting the non-amyloidogenic cleavage of APP, which has been shown to promote neuroprotection and memory enhancement (Ghisso and Frangione, 2002). However, as reviewed by Chen et al., (2015), with aging, α -secretase becomes progressively inefficient. This causes APP to be truncated by non-specific proteases such as β -secretase and γ -secretase, promoting the amyloidogenic cleavage of APP (Holsinger et al., 2002; Yang et al., 2003; Zhang et al., 2014; Carroll and Li, 2016). Cleavage of APP at the β - and γ -secretase sites produces the 40 or 42 amino acid fragments of $A\beta$ ($A\beta_{1-40}$ and $A\beta_{1-42}$) and subsequently, $A\beta$ peptide is released into the extracellular space (Chen, 2015; Xu et al., 2016). Extracellular $A\beta$ assumes several conformational states ranging from monomers to soluble oligomers and fibrils. These polymers of $A\beta$ quickly aggregate and form the amyloid plaques which are characteristic of the disease (Pryor et al., 2012).

The clearance of $A\beta$ is mediated by several $A\beta$ -degrading zinc-metalloproteinases, including endothelin-converting enzyme (ECE), angiotensin-converting enzyme (ACE), insulin-degrading enzyme (IDE), neprilysin (NEP) and matrix metalloproteinases (MMPs) (Saido and

Leissring, 2012). MMPs are a family of highly regulated, zinc-dependent enzymes that are produced by neurons and astroglia (Dzwonek et al., 2004). These enzymes are initially secreted as catalytically inactive pro-enzymes (proMMP-9), which are bound to endogenous inhibitors, named tissue inhibitors of metalloproteinases (TIMPs), through the C-terminal domain of each molecule. A disturbance of this complex (proMMP-9•TIMP), by a proMMP activator (MMP-3), causes the pro-enzyme (proMMP-9) to dissociate, which becomes fully activated, and proceeds with the degradation of its substrates (Ogata et al., 1995; Rosenblum et al., 2007). Functionally, MMPs are extremely diverse, with regulatory roles in many important cellular processes including synaptic plasticity and cognition, neuroinflammation, blood-brain barrier (BBB) integrity, cell migration, survival and apoptosis, as reviewed by Vafadari et al., (2016). MMPs have been found to be overexpressed during various pathological conditions such as stroke, epilepsy, schizophrenia, and neurodegeneration (Vafadari et al., 2016). Their expression can be induced by a large variety of factors, including cytokines, growth factors, metal ions, antibiotics, and hormones (Van Den Steen et al., 2002; Vandooren et al., 2017). In the context of AD, A β exposure induces the expression and secretion of MMPs from cultured neurons, reactive astroglia, and neuroblastoma cells (Deb and Gottschall, 1996; Deb et al., 2003; Talamagas et al., 2007; Mizoguchi et al., 2009). When secreted from astroglia, proMMP-9 is immediately cleaved into an active form (MMP-9) in the extracellular compartment, thus contributing to the maintenance of the balance between A β production and clearance (Ogata et al., 1995; Akiyama et al., 2000; Wegiel et al., 2000). In contrast to other A β -degrading proteases, MMP-9 is known to degrade A β fibrils *in vitro*, A β plaques *in*

situ, and A β *in vivo* (Backstrom et al., 1996; Yan et al., 2006; Yin et al., 2006; Hernandez-Guillamon et al., 2015). Consequently, modulation of MMP-9 levels can therefore impact the clearance of A β and promote its deposition.

Medroxyprogesterone Acetate (MPA) is a widely used, synthetic progestin that is the primarily found in the contraceptive, Depo Provera[®], and in HT, Prempro[®] and Premphase[®]. Merlo et al., (2012) utilized estrogen to induce the A β -degrading activity of MMP-9, establishing MMP-2 and MMP-9's contribution to the neuroprotective effect of the hormone *in vitro* (Merlo and Sortino, 2012). Our work focused on MPA, and its ability to modulate the levels of MMP-9, and by extension the degradation of A β . MPA exerts adverse effects on cognition, substantiating the risk of dementia in menopausal women (Shumaker et al., 2003, 2004), promoting memory impairments in menopausal animal models (Braden et al., 2010, 2011, 2017), and inducing amnesia in case study reports of premenopausal women (Gabriel and Fahim et al., 2005). MMP-9 has been shown to be critical for cellular processes involved in learning and memory, as it regulates dendritic spine morphology, maintains late phase long term potentiation (LTP), and controls postnatal brain development (Nagy et al., 2006; Michaluk, 2010; Kamat et al., 2014; Gorkiewicz et al., 2015; Reinhard et al., 2015; Kaczmarek, 2016). MPA has also been shown to alter MMP-9 activity and production in BV2 microglial, cancer, and epithelial cells (Di Nezza et al., 2003; Hwang-Levine et al., 2011; Allen et al., 2019). Additionally, the secretion of MMP-9 was found to be negatively impacted by MPA in the aforementioned cell types (Deb and Gottschall, 1996; Deb et al., 2003; Hwang-Levine et al., 2011; Allen et al., 2019). It is currently unknown if hormonal modulation of glial-secreted MMP-9, using MPA, impacts degradation of A β . The

pharmacological inhibition of MMP-2 and MMP-9, using both specific and non-specific enzymatic inhibitors, has also been shown to attenuate astroglia cell-mediated A β degradation (Yin et al., 2006). Therefore, MPA-mediated reduction of glial-secreted of MMP-9 would likely result in dysregulated APP processing, fostering conditions which would disrupt clearance of A β . On the premises that: 1) this commonly used progestin negatively impacts the secretion of this A β -degrading enzyme, in the aforementioned cell types and 2) inhibition of glial secretion of MMP-9 reduces the proteolytic degradation of amyloid beta, our central hypothesis is that MPA diminishes the secretion of MMP-9, thereby reducing the degradation of amyloid beta.

Here, we provide evidence that illustrates the connection of MPA, MMP-9, and A β . We found that MPA influences A β degradation by modulating the expression and/or activity of A β -degrading enzymes in a glial cell line (C6). C6 rat glial cells are a commonly used glial cell line and have also been identified as a useful cell line to study hormone action in glia (Kumar et al., 1986; Buchanan et al., 2000; Su et al., 2012b). We demonstrate that MPA significantly reduces enzymatic activity and secretion of MMP-9, and that MPA significantly reduces the degradation of A β . Together, these data implicate MPA in a negative effect on the A β -degrading enzyme, MMP-9. These data support a potential role where MPA perturbs A β clearance mechanisms, indicating the necessity for *in vivo* investigations pertaining to MPA's influence on AD-related pathology.

2.3 Materials and Methods

Cell Culture. The C6 rat glial cell line was obtained from the American Type Culture Collection (ATCC #CCL 107). C6 cells were maintained in Hyclone Dulbecco's modified Eagle's medium (DMEM)/high glucose (Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 1% penicillin/streptomycin (Fisher Scientific). Once cells reached 80% confluency, they were trypsinized and spun down at 325.5xg for 3 min. Cells were counted with a Nexcelom Bioscience Cellometer AutoT4 (Lawrence) and seeded at a density of 1×10^6 cell/well in 6-well cluster plates, incubated overnight at 37°C in 5% CO₂.

Treatments. Serum-supplemented media was removed and the cell monolayer was washed once with phosphate buffer saline (1X PBS) and fresh-serum free media (OptiMEM; Fisher Scientific), containing either 0.1% dimethyl sulfoxide (DMSO; Millipore Sigma), various concentrations of Medroxyprogesterone Acetate (MPA; 10 nM, 100 nM, 1 μM, and 10 μM) (Millipore Sigma), GM6001 (100 μM; Calbiochem, La Jolla, CA), or RU486 (2 μM; Millipore Sigma). All treatments were diluted in serum-free medium to a final DMSO concentration of 0.1%. Cell viability was always above 90% as assessed by Calcein AM (Fisher Scientific). C6 cells were incubated at 37°C in 5% CO₂ for 48 hours (48-h) or 72-h. The conditioned media was collected and used to assess MMP-9 enzymatic activity (by gelatinase zymography), quantify extracellular MMP-9 (by ELISA), and assess proteolytic degradation of amyloid beta (by Western blot). Supernatants were stored at -80°C and thawed on ice during experimental analyses. Repeated freeze thaw cycles were avoided by aliquoting the

samples. The cells were collected and used to quantify intracellular MMP-9 protein levels (by ELISA) and assess MMP-9 mRNA expression (by qRT-PCR).

Cell viability. Cell viability was assessed using Calcein AM (Life Technologies) and reconstituted at 2 mM in dimethylsulfoxide (DMSO). About 50,000 C6 cells were seeded in a black-walled clear bottom 96 well plate (Corning; #3601). C6 cells were treated with 10 nM–10 μ M of MPA. After exposure to MPA (10 nM–10 μ M) for 72-h, the plate was washed three times with PBS 1X. A total of 100 μ L of 1 μ M Calcein AM was added to the wells. The plate was incubated at 22°C in the dark for 30 min. The plate was read using a BioTek Synergy H1 Hybrid reader (Winooski, VT, USA).

Lactate Dehydrogenase Assay. Cell death after MPA treatment was assessed using the PierceTM lactate dehydrogenase (LDH) Cytotoxicity Assay (Fisher Scientific) kit. Reaction substrates were prepared as per the manufacturer's instructions. LDH assay was performed with the media from the black-walled clear bottom 96 well plate. Forty-five minutes prior to the end of the 72-h exposure period, 10 μ L 10 $\times$ lysis buffer was added to one control well, and the plate was placed back in the incubator. At the conclusion of the exposure period, 50 μ L of media was carefully removed from each well and transferred to a new 96 well clear bottom assay plate. Next, 50 μ L of the LDH reaction mixture was added to each well, and the plate was incubated for 30 min at room temperature, protected from light. The reaction was stopped by adding 50 μ L of LDH stop solution to each sample. The plate was read using the BioTek Synergy H1 Hybrid reader (BioTek) at absorbance of 490 nm and 680 nm.

Assay of MMP-9 activity by gelatin zymography. The gelatinolytic/proteolytic activity of MMP-9 secreted into the culture medium was determined with gelatin zymography by electrophoresis of serum-free conditioned media (CM) collected from confluent C6 cells, in accordance with Frankowski et al., (2012). Briefly, CM freed of cell debris by centrifugation were mixed with Laemmli sample buffer (Bio-Rad; #1610747) lacking reducing agents. Electrophoresis in precast Novex polyacrylamide zymogram gels (Invitrogen) was performed at a constant voltage of 125V for 90 min in SDS Tris-Glycine Buffer, followed by a series of three 15 minutes washes in 1X renaturation buffer (Invitrogen). The gels were transferred to 1X Developing buffer (Life Technologies) for 30 minutes with gentle shaking and then placed at 37°C for an 18 h incubation. The following day, the gels were stained in a solution with 45% ethanol, 54% sterile diH₂O, 1% acetic acid, and 0.125 g Coomassie brilliant blue R-250 (Sigma) between 45 minutes to 1-h. The gels were then transferred to de-staining solution #1, containing 25% ethanol, 10% acetic acid, and 65% diH₂O for 45 minutes. De-staining solution #1 was decanted and then replaced by a de-staining solution #2, containing 5% ethanol, 7.5% acetic acid, and 87.5% diH₂O for 1-3 h. Areas of gelatinolytic degradation appeared as transparent bands on the blue background. Gels were imaged using ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA). Images were acquired using Bio-Rad Quantity One™ software. The Novex Sharp Pre-stained Protein Standard (Invitrogen) was used to identify MMP species or the MMP-9 Active, Human, Recombinant (Millipore Sigma; #PF024-5UG) was used as a reference standard, showing MMP-9 gelatinolytic activity at 67 kDa. The bands in the gel are quantified using ImageJ 1.38X (NIH).

Intracellular and extracellular MMP-9 levels. CM samples and cell lysates were analyzed with a matrix metalloproteinase-9 (MMP-9) ELISA kit following the product manuals (R&D Systems Quantikine™; #RMP900). Results were calculated from the standard calibration curves on internal standards. After adding stop solution, optical density (OD) was measured at 450-nm with correction wavelength at 550-nm immediately using BioTek Synergy H1 Hybrid plate reader (BioTek). The final readings were obtained by subtracting 450-nm from 550-nm OD reading to correct for optical imperfections of the microplate reader. A standard curve was generated with reagents provided in the kit and the sample values were read against the standard to determine MMP-9 concentrations in each treatment.

RNA isolation and Quantitative Real-Time PCR. Cells were seeded at 1×10^6 cells/well in a 6-well plate and left to reach ~80% confluency over the course of 24-h. Media was gently aspirated from cells and replaced by 2 mL of OptiMEM Reduced Serum Media (Fisher Scientific), supplemented with 1% penicillin/streptomycin (Fisher Scientific), or the various concentrations of MPA. After a 12-h exposure period, cells were collected using a Cell Lifter (Corning, Corning, NY, USA), washed with 1X PBS, and pelleted by centrifugation at $325.5 \times G$ for 3 min, followed by the addition of 1mL QIAzol Lysis Reagent for RNA purification (Qiagen).

Total RNA was purified by using the miRNeasy Mini Kit (Qiagen) as per the manufacturer's instructions. For all lysate samples, 200 μ L chloroform was added, and vortexed for 1 min

followed by incubation on Ice for 5-10 min. Next, the samples were centrifuged for 15 min at $12,000 \times g$ at 4°C . The upper aqueous phase ($\sim 300 \mu\text{L}$) was transferred to a new collection tube and mixed with $600 \mu\text{L}$ 100% ethanol. The solution was then placed on a RNeasy MinElute spin column and centrifuged at $8,000 \times G$ for 15 s. The flow through was discarded and $700 \mu\text{L}$ buffer RW1 (20% Ethanol, 900 mM guanidinium isothiocyanate (GITC), 10 mM Tris-HCl pH 7.5) was added to the spin column and centrifuged at $8,000 \times G$ for 15 s. Two washes of $500 \mu\text{L}$ buffer RPE (80% Ethanol, 100 mM NaCl, 10 mM Tris-HCl pH 7.5) were then performed, with the first lasting 15 s and the second lasting 2 min. The RNeasy MinElute spin column was then placed in a new collection tube and spun at $8,000 \times G$ for 5 min to dry the column membrane. Lastly, the column was placed in another collection tube and $40 \mu\text{L}$ RNase-free water was added to the center of the membrane and incubated for 1 min, and then centrifuged at $8,000 \times G$ for 1 min ending with the purified RNA eluted in the collection tube.

RNA concentrations for each sample were measured using Nano drop 2,000 spectrophotometer (Thermo Scientific). For cDNA synthesis, $0.75 \mu\text{g}$ total RNA was reverse transcribed using the miScript II RT kit (Qiagen). A reaction mix ($20 \mu\text{L}$ total volume) was made using $4 \mu\text{L}$ 5 $\times$ miScript HiFlexBuffer, $2 \mu\text{L}$ 10 $\times$ miScript Nucleics Mix, $2 \mu\text{L}$ miScript Reverse Transcriptase Mix, and $12 \mu\text{L}$ Template RNA/nuclease-free water. Prior to use for quantitative real-time PCR (qRT-PCR), cDNA was diluted in nuclease-free water, at a ratio of 1:10.

Expression of MMP-9 mRNA was determined using target specific RT2 primer assays and the RT2 SYBR® Green PCR kit (Qiagen; 5 µL SYBR® Green; 0.5 µL Target Primer; 4.5 µL diluted cDNA). qRT-PCR reactions were performed in duplicate for each sample, using the CFX384 Touch™ RT PCR Detection System (Bio-Rad) for 45 cycles as follows: 15 s at 94°C, 30 s at 55°C, 30 s at 70°C. Negative control reactions were included as wells containing only master mix and nuclease-free water (no template cDNA). The expression levels of target genes in cell lysates was standardized against Adenylyl cyclase-associated protein 1 (CAP-1) (IDT). Quantification of PCR amplified mRNA specific cDNA was done by comparative cycle threshold CT method ($2^{-\Delta\Delta CT}$). Ct values of mRNA were subtracted from the average Ct of the internal controls, and the resulting ΔCT was used in the equation: relative copy numbers = $(2^{-\Delta\Delta CT})$.

Amyloid Beta preparation and amyloid beta degradation in astroglia-conditioned medium. Synthetic A β 1-42 (Invitrogen; # 30112) was prepared from lyophilized A β 1-42 monomers that were suspended in 167 µL of HPLC grade water (Fisher Scientific) and incubated at room temperature for 5 min. The dissolved A β 1-42 was then diluted to 230 µM by adding 833 µL of Ca²⁺-free phosphate buffered saline (PBS) and incubated for 48-h at 37°C for polymerization. After polymerization, synthetic A β 1-42 (230 µM) at a final concentration of 23 µM was freshly prepared and added to either serum-free media (SFM), untreated CM, MPA-treated CM, or GM6001-treated CM of C6 glial cells. The mixtures were then

incubated at 37°C (Yin et al., 2006). After 24 h, samples were collected and residual A β 1-42 was analyzed by Tris-Glycine—Western blotting.

Western blot was performed using denaturing 4-20% Novex Wedgewell Tris-Glycine SDS gels (Invitrogen) with 90 min electrophoresis at 125V, 30 mA and iBlot Gel Transfer Gel Transfer Stacks (Invitrogen) with 7 min of electrical blotting. The polyvinylidene difluoride (PVDF) membrane (Invitrogen) with proteins transferred was blocked by Odyssey Blocking Buffer (LI-COR Biosciences; #927-40000) for 1-h and was incubated with primary 6E10 (1:1000, Biolegend; #803016) overnight and then with secondary (anti-mouse conjugated with fluorescence) (LI-COR) at room temperature for 90 min. Bands were visualized using LI-COR Odyssey IR Imager (LI-COR). Quantification using densitometric analysis was performed using Odyssey imaging systems (LI-COR biosystems, Lincoln, NE). Densitometry signal for each range of oligomers (low molecular weight aggregates (<15 kDa), intermediate-sized oligomers (~15-55 kDa), high molecular weight oligomers (>56 kDa), or the entire lane for total A β 1-42) was normalized relative to the signal of the control lane (SFM) and fold change over untreated cells was plotted.

Statistical Analyses. All biological experiments were repeated at least three times with n = 3–16 plates/wells per treatment. Results from the experiments are reported as means $\pm$ SEM. All quantitative data were assessed for significance using a one-way ANOVA with

Dunnett's post hoc test. All results were analyzed by GraphPad Prism 8.0 software (GraphPad Software). A p value < 0.05 was used to establish significance.

2.4 Results

Medroxyprogesterone Acetate reduces MMP-9 enzymatic activity

To study the effect of Medroxyprogesterone Acetate (MPA) on matrix metalloproteinase-9 (MMP-9) activity, we incubated C6 cells for 48 and 72-h with increasing concentrations of MPA (10 nM—10 μ M). The conditioned media (CM) were then collected and analyzed by gelatin zymography. This technique allows for the visualization of both active and proenzyme (inactive) forms of gelatinases (MMP-2 and MMP-9) (Frankowski et al., 2012). Untreated C6 cells show constitutive expression of active MMP-9 denoted by the 92 kDa gelatinase band (Figure 1 A, B), with no detections of bands representative of MMP-2 activity. Densitometric analysis of zymograms obtained in 5 different experiments indicated that 48-h incubation of C6 cells with MPA inhibited MMP-9 activity in a dose-dependent manner, with significant reductions occurring at the three highest concentrations (100 nM, 1 μ M, and 10 μ M) compared to untreated control cells (Figure 1 C, D). As expected, GM6001, an MMP inhibitor, suppressed MMP-9 enzymatic activity by 80%. This dose-dependent, inhibitory effect of both MPA and GM6001 on enzymatic activity persisted at 72-h.

To determine whether the inhibitory effect of MPA on MMP-9 activity was due to interference with the activation process of MMP-9, post-secretion, we incubated C6 cells with medium alone. After 48-h, the CM were collected, divided in aliquots, and treated with increasing concentrations of MPA. Media was then incubated for 24 more hours at 37°C and analyzed by gelatin gel zymography. The inhibitory effect of MPA was lost, suggesting that MPA does not directly interact with MMP-9 and confirming the interference with the activity of the protease is cell-mediated (data not shown).

To exclude the possibility of drug toxicity as the reason of its inhibitory effect, C6 cells were incubated with increasing concentrations of MPA (10 nM, 100 nM, 1 µM, and 10 µM) or 1 µM GM6001, then cell viability using Calcein AM and LDH release were assessed. MPA did not cause any appreciable cellular toxicity, even at the highest concentration used (10 µM) (Supplementary Figure 1).

Medroxyprogesterone Acetate reduces MMP-9 production

Based on our finding that MPA caused decreased enzymatic activity of MMP-9, we next assessed whether the inhibitory effect of MPA on MMP-9 enzymatic activity was due to a reduction in overall secretion of MMP-9 protein or TIMP-1 inhibition. To measure extracellular and intracellular MMP-9 protein levels, C6 cells were incubated for 48-h with increasing concentrations of the drug. Both the media and the cell lysates were collected and analyzed by ELISA for MMP-9 (pro-MMP-9, TIMP bound MMP-9, and latent MMP-9)

(Figure 2A, B). The analysis of three experimental replicates showed that MPA significantly inhibited extracellular MMP-9 protein levels (Figure 2A). MMP-9 inhibition ranged from 30% to 60% relative to control (Figure 2A). Intracellular MMP-9 protein expression was also significantly decreased after MPA treatment, in a dose-dependent manner (Control: 0.85548 ng/mL; 10 nM MPA: 0.88669 ng/mL; 100 nM MPA: 0.568602 ng/mL; 1 μ M MPA: 0.468092 ng/mL; 10 μ M MPA: 0.369965 ng/mL) (Figure 2B). Because intracellular MMP-9 protein levels were negatively impacted by our MPA treatment, we evaluated the expression level of MMP-9 in cells treated with MPA by quantitative RT-PCR. Additionally, our zymography experiments revealed no detection of MMP-2 enzymatic activity, thus we performed an analysis of MMP-2 mRNA expression simply to confirm the lack of MMP-2 expression in the C6 cells (Figure 3A). Analysis of MMP-9 mRNA expression shows the experimental groups were significantly lower when compared with the control group (Figure 3A). Such repression appears after 12-h of treatment but is not detectable at later time points (24-h) (Figure 3B).

Medroxyprogesterone Acetate's effects are mediated through the Glucocorticoid Receptor

To investigate a potential mechanism by which MPA causes repression of MMP-9 transcription, we first considered a receptor-mediated approach. At the molecular level, MPA elicits its biological effects through multiple receptors, including the progesterone receptor (PR), androgen receptor (AR), and glucocorticoid receptor (GR) (Africander et al.,

2011). Notably, these cells are devoid of the PR and AR (Su et al., 2012b). The GR, however, is present in C6 cells and MPA binds to the GR with a high affinity, acting as a partial to full agonist for the GR (Koubovec et al., 2004, 2005; Su et al., 2012b, 2012a; Louw-du Toit et al., 2014). We tested the hypothesis that MPA's effects on enzymatic activity and production of MMP-9 are mediated by the GR (Figure 4). C6 cells were pretreated with mifepristone, RU486 (2 μ M), for 30 min, followed by MPA treatment for another 48-h, using the lowest and highest concentrations of MPA at which significant effects were seen on enzymatic activity, intracellular protein, and mRNA levels (100 nM and 10 μ M). Densitometric analysis of the zymogram (Figure 4A) shows pharmacological inhibition of the GR with RU486 attenuated MPA's effect on MMP-9 activity (100 nM MPA: 51.61%; 100 nM MPA/RU486: 82.23%; 10 μ M: 41.99%; 10 μ M MPA/RU486: 64.79% (Figure 4B). This suggests our findings are potentially due, in part, to a GR-mediated mechanism.

Medroxyprogesterone Acetate antagonizes the degradation of A β

Previous reports have suggested that MMP-9 is capable of degrading amyloid beta, *in vitro* (Backstrom et al., 1996). To explore the possibility that inhibition of MMP-9 would interfere with amyloid beta degradation, C6 wells were treated with MPA for 48-h and the CM were incubated with freshly prepared synthetic human A β ₁₋₄₂ for 24-h at 37°C. The doses selected for this experiment were the lowest and the highest dose at which we observed significant effects on enzymatic activity, intracellular protein, and mRNA levels (100 nM and 10 μ M). Using the anti-A β 6E10 antibody, A β levels were then measured by Tris-Glycine Gel-Western

blotting (Figure 5A). This approach yielded the resolution of groups of oligomers as indicated in Figure 5A, consistent with the literature (Prangkio et al., 2012). Incubation of A β with CM resulted in a significant reduction in A β levels, causing the appearance of several A β fragments, which are indicative of MMP cleavage (Backstrom et al., 1996). This A β -degrading activity was antagonized by MPA (100 nM and 10 μ M). Densitometric analysis (Figure 5B-E) established that CM modestly altered low molecular weight aggregates (<15 kDa), while MPA-treated CM samples showed no effect (Figure 5B). However, MPA-treated CM samples overall mitigated proteolytic cleavage of intermediate-sized oligomers (~15-55 kDa) (Figure 5C), high molecular weight oligomers (>56 kDa) (Figure 5D), and total (Figure 5E) A β species. Incubation of A β ₁₋₄₂ with CM significantly abated A β levels and MPA treatment weakened this effect. To verify the contribution of MMPs to A β degradation in CM, we incubated CM with freshly prepared synthetic human A β ₁₋₄₂ for 24-h at 37°C in the presence or absence of the broad-spectrum, MMP inhibitor, GM6001, and found that the A β -degrading activity in CM was attenuated (Supplemental Figure 2). Additionally, we incubated SFM with freshly prepare synthetic human A β ₁₋₄₂ for 24-h at 37°C in the presence or absence of active recombinant MMP-9 protein (rMMP-9). These data confirmed findings in the literature that MMP-9 possesses A β -degrading activity (data not shown) (Backstrom et al., 1996; Yan et al., 2006; Hernandez-Guillamon et al., 2015). Next, we assessed the possibility of a direct action of MPA on A β . We treated SFM with MPA for 48-h. incubated the supernatant with 23 μ M A β ₁₋₄₂, for an additional 24-h at 37°C, and we found MPA-treated SFM failed to digest A β (data not shown). We determined our observed impairment of proteolytic degradation occurred by an indirect, cell-mediated mechanism.

2.5 Discussion

Ninety percent of the cells in the central nervous system (CNS) are glia (Haydon, 2001). Glial cells (astrocytes, oligodendrocytes and microglia) communicate with neurons to regulate synaptic plasticity and neurotransmission (Fields et al., 2014). Accumulating evidence suggests MMP secretion from astroglia contribute to the degradation and clearance of amyloid plaques (Yan et al., 2006; Yin et al., 2006; Wang et al., 2014), emphasizing the critical role of induction and secretion of MMPs in the brain. There are several reports showing astroglia conditioned media (CM) possesses A β -degrading activity, in part, through the secretion of MMPs (Backstrom et al., 1996; Yin et al., 2006; Fragkouli et al., 2014). Moreover, MMP-9 possesses α -secretase-like activity and cleaves APP at several sites, promoting the non-amyloidogenic processing of the precursor protein and clearance of A β (Backstrom et al., 1996; Yin et al., 2006; Talamagas et al., 2007; Filippov and Dityatev, 2012).

The present study investigated the expression of MMP-9 in C6 glial cells treated with MPA. Several reports have demonstrated that MPA can alter MMP-9 levels, where levels are increased in macrophages and, alternatively, reduced in BV2 microglial, endometrial cancer, and primary amnion epithelial cells (Di Nezza et al., 2003; Hwang-Levine et al., 2011; Allen et al., 2019). Consistent with these reports, we found that MMP-9 protein expression and enzymatic activity is lowered by treatment with MPA. However, majority of the previous *in vitro* studies have used non-CNS cell lines to observe the effect of MPA on MMP-9, with

little to no investigation of the effects of MPA on glial secretion of MMP-9. Additionally, due to the lack of focus in a CNS-related system, previous work failed to investigate the potential for MPA to influence AD-related pathology via the hormonal modulation of MMP-9.

To our knowledge, this is the first *in vitro* analysis of the effect of MPA on MMP-9's enzymatic activity and production, in a C6 glial cell line. Our results show that MMP-9 is expressed in C6 glial cells in control conditions and is suppressed by exposure to MPA at both the mRNA and the protein level. We found that MPA suppresses MMP-9 activity in a dose-dependent manner (Figure 1). Decreased MMP-9 activity was reflected by impaired synthesis of the MMP-9 protein (Figure 3A, B) and was further supported with data showing a suppression of MMP-9 mRNA (Figure 3A). We observed a spurious increase at the highest concentration of MPA (10 μ M), which may be indicative of off-target effects because of such a high dose of the hormone. In agreement with our hypothesis, experiments on CM confirmed the inhibitory effects of MPA on the enzymatic activity of MMP-9. Conversely, we found MPA did not cause secretory inhibition of MMP-9, but rather suppressed the transcription of MMP-9 at the mRNA level.

The glucocorticoid receptor (GR) is typically found, in an inactive state, in the cytoplasm, and, upon ligand binding, it becomes activated and transrepresses pro-inflammatory genes. This is thought to be substantiated either through direct DNA-binding, by binding a

glucocorticoid response element (GRE) or the nuclear factor kappa B (NF- κ B) response element (κ BRE) (Meijsing et al., 2009; Surjit et al., 2011; Watson et al., 2013; Weikum et al., 2017; Sacta et al., 2018; Hudson et al., 2018) or 2), or through a DNA independent, direct protein-protein interaction/crosstalk with other transcription factors, including NF- κ B and AP-1 (McEwan et al., 1997; Webster and Cidlowski, 1999; De Bosscher et al., 2003; Liu and Xu, 2012; Trevor and Deshane, 2014). The initial finding of MPA's capability of interfering with the activities of NF- κ B or AP-1, at the promoter level, suggested MPA represses cytokine-induced, AP-1 driven genes, as well as NF- κ B-driven genes, without impacting the DNA-binding activity of NF- κ B, in a GR-dependent manner (Koubovec et al., 2004). Conversely, Simoncini et al., (2004) demonstrated MPA's anti-inflammatory effects and MPA's ability to reduce hydrocortisone-dependent nuclear translocation of NF- κ B in human endothelial cells. C6 cells have spontaneous NF- κ B nuclear activity, suggesting the presence of constitutive NF- κ B activity, which explains the constitutive basal expression of MMP-9 observed in our experiments (Robe et al., 2004). We treated C6 cells with a GR antagonist, which attenuated MPA's effects on MMP-9 enzymatic activity (Figure 4). In this case, we suspect there may be a GR-dependent transrepression of MMP-9, via interaction with either NF- κ B or AP-1, which are generally accepted as regulators of MMP-9 expression (Jonat et al., 1990; Paliogianni et al., 1993; Yokoo and Kitamura, 1996; Barnes, 1998; Bond et al., 2001; Ronacher et al., 2009; Africander et al., 2011; Li et al., 2012; Mittelstadt and Patel, 2012). The literature also extensively supports the notion that glucocorticoids alter MMP-9 expression (Rosenberg et al., 1996; Park et al., 1999; Eberhardt et al., 2002; de Paiva et al., 2006), as well as MMP-3 (Richardson and Dodge et al., 2003; Koyama et al., 2017),

which is the enzyme responsible for the conversion of MMP-9 from an inactive to an active state. Therefore, it's plausible the progestin binds to the cytosolic GR, activates the GR, and then activated GR binds to NF- κ B, in turn, negatively interfering with the transcriptional enhancer and, in turn, promoting transrepression of MMP-9. Notably, C6 cells have also been shown to express mRNA for the membrane progesterone receptors (mPR) (Su et al., 2012a). Salazar et al., (2016) demonstrated MPA elicits progestin-induced intracellular signaling in PR-negative breast epithelial cells, suggesting a potential mode of action via membrane progesterone receptors. It is possible that our observations may be partially due to non-genomic signaling through membrane progesterone receptors, however, there is currently a lack of information regarding MPA's relative binding affinity to membrane progesterone receptors and a lack of evidence relating to MPA's propensity to elicit its effects via non-genomic membrane bound signaling. Future studies should aim to further delineate the precise mechanisms by which our observations in the present study occur.

To our knowledge, this is the first investigation of the effects of MPA treatment on MMP-9's A β -degrading activity. Our objective was to relate progestin-induced reductions of active MMP-9 levels with a loss of A β ₁₋₄₂ degradation. We were interested in observing the effects after exposure to high concentrations of A β (23 μ M) and, using Western blot analysis, we were able to confirm the diminished ability of MPA-treated C6 cells to degrade A β ₁₋₄₂ (Figure 5). A key finding of our current study was that MPA-treated CM samples did not significantly alter low molecular weight aggregates (Figure 5B). However, MPA treatment impaired the degradation of intermediate-sized and high molecular weight oligomeric A β

species.. Of interest is the observed effect on oligomeric A β , which is suggested to be the culprit of the neurodegeneration seen in AD (Lesné et al., 2008, 2013; Lublin and Gandy, 2010). Our data suggests MPA treatment potentially promotes AD-related pathology. Our inclusion of the non-specific MMP inhibitor, GM6001, yielded a greater effect on degrading activity. This affirmed our observations were, in part, MMP-9 dependent, as GM6001 was shown to inhibit the enzymatic activity of MMP-9 by 80% (Figure 1). Enzymatic activity of MMP-9 was inhibited to the same extent by GM6001 and 10 μ M MPA, which was reflected in their hindrance of A β degradation. Degradation of A β was not fully thwarted with GM6001 treatment, implicating the potential for additional proteases that exist in CM. As previously discussed, in addition to MMPs, A β can be degraded by other proteases, including ECE, IDE, and NEP (Saido and Leissring, 2012). Prior literature supports a minimal contribution of IDE, NEP, and ECE as extracellular, secreted amyloid beta scavengers from astrocytes. According to Yin et al., (2006), in addition to NEP, ECE, and IDE, there may be proteases that have yet to be experimentally identified, contributing to astrocyte-mediated degradation of A β , due to the fact that NEP, ECE, and IDE were undetected with Western blot analysis of conditioned media of neonatal mouse astrocytes. Moreover, it has been reported that cultured cells may be incapable of secreting IDE, and it is still undetermined whether NEP is capable of degrading oligomeric amyloid beta (Saido et al., 2012; Song et al., 2018). There is evidence that estrogen promotes amyloid beta degradation through the induction of NEP (Liang et al. 2010), and there is also evidence that IDE is induced by 17 β -estradiol, reducing amyloid beta load *in vivo* (Zhao et al. 2011). Thus, the literature generally supports the idea that hormones are capable of regulating these specific amyloid

beta degrading enzymes and suggests MPA could also potentially regulate additional amyloid beta degrading enzymes. However, we focused on MMP-9, because MMP-9 is the only amyloid beta scavenger shown to possess the ability to degrade A β fibrils *in vitro*, A β plaques *in situ*, and A β *in vivo*, making it the most unique and distinguished of the known scavengers (Backstrom et al, 1996; Yan et al, 2006; Yin et al., 2006; Hernandez-Guillamon et al., 2015). Overall, our findings suggest MMPs are involved in the degradation of A β in CM and MPA impedes on the degradation, through the downregulation of MMP-9 production, in a GR-dependent manner.

We noted several limitations in the present study. First, our synthetic A β formulation and its aggregated forms may not fully represent the *in vivo* phenomena, due to variability in oligomer generation (refer to Supplementary Figure 8). Additionally, the effective concentrations of MPA in our study ranged from 10 nM to 10 μ M. Although our working concentrations are relatively high, peak serum concentrations of MPA fall between 10 nM and 100 nM (Tomasicchio et al., 2013), after women receive an intramuscular injection of 150 mg, every three months. These are concentrations at which our observed effects on MMP-9 and A β -degrading activity occurred. Furthermore, our *in vitro* model utilizes a transformed glial cell line. The utilization of primary cells are more reliable than cell lines, however, this would require pharmacological induction of MMP-9 expression or transfection of an MMP-9 expression vector. As previously mentioned, C6 cells have spontaneous NF- κ B nuclear activity, suggesting the presence of constitutive NF- κ B activity, which explains the constitutive basal expression of MMP-9 observed in our experiments

(Robe et al., 2004), and circumvented the necessity for induction or transfection of MMP-9 expression. Although *in vitro* experiments using cell culture are useful, providing pertinent information, ultimately *in vivo* experiments are needed to confirm these effects.

Based on our findings, progestin-induced downregulation of MMPs is partially responsible for hampering the proteolytic cleavage of $A\beta_{1-42}$ and supports a possible link between MPA administration and AD-related pathology. The importance of MMP-mediated degradation of toxic $A\beta_{1-42}$ species and its potential neuroprotective effect is abolished with MPA-induced reduction in MMP-9 expression and production. Most importantly, this commonly used progestin suppresses both the transcription and the activation of glial MMP-9, which is responsible for reduced $A\beta$ degradation. Taken together, our study confirmed $A\beta$ -degrading activity was reduced, in part, through the impairment of MMP-9 production.

In summary, our work demonstrates the necessity for a further delineation of MPA's effects on MMP-9 production. We also raise awareness for the unmet need of *in vivo* investigation for the potential cognitive and pathological outcomes of MPA. There is currently an abundance of literature which focuses on the effects of estrogen, estradiol, testosterone and progesterone on the brain, and particularly amyloid beta production and clearance (Vest et al., 2013; Li et al., 2014; Giatti et al., 2016; Uchoa et al., 2016). There is still a paucity of published research addressing the potential effects of progestins, more specifically MPA, on the brain. While limited research with MPA suggests a negative impact

on the brain, these studies need to be extended to model systems relevant to women in their reproductive prime, as majority of efforts have been in menopausal animal models and menopausal woman (Shumaker et al., 2003, 2004; Braden et al., 2010, 2011, 2017; Lowry et al., 2010; Olanrewaju et al., 2013). Our finding that MPA limits MMP-9 production could ultimately negatively impact synaptic plasticity *in vivo*, as MMP-9 has been shown to be considered “indispensable” for neuronal plasticity (Nagy et al., 2006; Michaluk et al., 2011; Kamat et al., 2014; Gorkiewicz et al., 2015; Lepeta and Kaczmarek, 2015; Kaczmarek, 2016). Thus, independent of AD-related pathology, MPA’s suppression of MMP-9 production could still prove harmful for learning and memory. Herein, we encourage additional investigations related to MPA’s effects on the brain. Future efforts should be extended to model systems relevant to AD-related pathology. It is conceivable that the prolonged use of MPA will progressively subdue the proteolytic degradation of A β by MMP-9, *in vivo*, promoting AD-related pathology. This hypothesis is currently being tested in our laboratories.

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Author Contributions

KNP designed studies, conducted studies, and composed manuscript. SNS, DAD, MEV, and DDQ aided with studies and revised the manuscript. JWS designed studies and revised the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.6 Figures

Figure 1. MPA reduced the gelatinolytic activity of MMP-9 in C6 glial cells. (A, B) Representative zymograms showing MMP-9 activity of MPA-treated conditioned media (CM) determined by gelatin zymography. After a 48-h (left) or 72-h (right) incubation period in serum-free media, supernatants obtained from 1×10^6 cells were analyzed by gelatin zymography. DMSO at 0.1% was used as the vehicle control and constitutively showed MMP-9 gelatinolytic activity (lane 1). Upon MPA treatment, MMP-9 gelatinolytic activity was significantly decreased (lanes 2–5). GM6001 (lane 6) was used as a negative control for active MMP-9. (C, D) Densitometric analysis of conditioned media from astroglia determined by gelatin zymography. C6 glial cells were incubated for 48-h (left) or 72-h (right) with increasing concentrations of MPA and GM6001, a non-specific MMP inhibitor. Results are expressed as percentage of activity of treated to untreated cells (mean $\pm$ SEM). MMP-9 gelatinolytic activity of untreated cells is expressed as 100%. MPA significantly decreased MMP-9 enzymatic activity in a dose-dependent manner ($P < 0.001$) compared with that found in untreated cells. Results are representative of five independent experiments. *** $p < 0.001$.

Figure 2. The effect of 48-h MPA treatment on MMP-9 protein in C6 glial cells. (A) Extracellular MMP-9 secretion was detected using ELISA kit. MPA inhibited the expression of extracellular MMP-9 in treated C6 glial cells. Results are representative of three independent experiments. (B) The effect of 48-h MPA treatment on intracellular MMP-9 in C6 glial cells. MMP-9 secretion was detected using ELISA kit. Results are expressed as means $\pm$ SEM. MPA inhibited the expression of intracellular MMP-9 in treated C6 glial cells. Results are representative of five independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 3. The effect of MPA treatment on MMP-2/-9 mRNA in C6 glial cells. (A) The mRNA levels of MMP-2 and MMP-9 were measured by qRT-PCR in C6 glial cells treated with MPA for 12-h, using MMP-2 and MMP-9 specific primers. (B) At 24-h, the suppression of MMP-9 mRNA levels is no longer detected. Data are represented by mean $\pm$ SEM of 3 independent qRT-PCR experiments performed in duplicates. The expression levels are represented relative to GAPDH reference gene. Results are representative of five independent experiments. * $p < 0.05$ and ** $p < 0.01$.

Figure 4. RU486 pretreatment antagonized the effects of MPA on MMP-9 activity. (A) Representative zymogram showing MMP-9 activity of MPA-treated conditioned media (CM). DMSO at 0.1% was used as the vehicle control and constitutively showed MMP-9 gelatinolytic activity (lane 1). C6 cells were pretreated with mifepristone, RU486 (2 μ M), for 30 min, followed by MPA treatment for another 48-h. Treatment with RU486, alone, did not alter MMP-9 enzymatic activity (lane 2). Upon MPA treatment, MMP-9 gelatinolytic activity was significantly decreased (100 nM, lane 3; 10 μ M, lane 5). Pretreatment with RU486 (2 μ M) antagonized MPA's effect on enzymatic activity (100 nM, lane 4; 10 μ M, lane 6). (B) Densitometric analysis of conditioned media from astroglia determined by gelatin zymography. Results are expressed as percentage of activity of treated to untreated cells (mean $\pm$ SEM). MMP-9 gelatinolytic activity of untreated cells is expressed as 100%. RU486 treatment is represented by the white bar, 100 nM MPA treatments are represented by the light grey bars (solid and striped), 10 μ M MPA treatments are represented by the dark grey bars (solid and striped). Results are representative of three independent experiments. ** $p < 0.01$.

A

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Figure 5. Western blot detection and densitometric analysis of Aβ using 6E10 antibody (A) Representative Western blot of Aβ using 6E10 antibody. Freshly prepared synthetic human Aβ₁₋₄₂ (23 μM) was added to serum-free media (SFM) (lane 1), SFM that had been conditioned by incubation with C6 glial cells (CM) (lane 2), or CM that had been treated with MPA (100 nM or 10 μM) for 48-h (lanes 3 and 4). The mixture was then incubated for 24 h at 37 °C, and residual Aβ was analyzed by Tris-Glycine—Western blotting. Incubation of Aβ₁₋₄₂ with CM significantly decreased Aβ levels. MPA treatment attenuated this effect. (B-E) Densitometric analysis shows that MPA-treated CM samples induced significant differences in Aβ species. Incubation of Aβ₁₋₄₂ with CM significantly decreased Aβ levels. MPA treatment attenuated this effect. Results are representative of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.

A **B**

Supplementary Figure 1. Effect of MPA on cell viability and LDH release. (A) Calcein AM and (B) Lactate dehydrogenase (LDH) data indicate that significant cell death did not occur at any concentration of MPA treatment.

A **B**

Supplementary Figure 2. Western blot detection and densitometric analysis of Aβ using 6E10 antibody. Representative Western blot of Aβ using 6E10 antibody. Freshly prepared synthetic human Aβ1-42 was added to serum-free media (SFM) (lane 1), SFM that had been conditioned by incubation with C6 glial cells (CM) (lane 2), or CM that had been treated with 1 μM GM6001 (lane 3) for 48-h. The mixture was then incubated for 24 h at 37 °C, and residual Aβ was analyzed by (A), Tris-Glycine—Western blotting. (B) Densitometric analysis of overall total Aβ1-42 shows that incubation of Aβ1-42 with CM significantly decreased Aβ levels and GM6001 treatment attenuated this effect. Results are representative of two independent experiments. *p < 0.05, **p < 0.01.

Supplementary Figure 3. Full Zymogram Gel of Cropped Image as Shown in Figure 1A. Using a 10-well gel, MMP-9 Active, Human, Recombinant (Millipore Sigma; #PF024-5UG) was used as a reference standard showed MMP-9 gelatinolytic activity at 67 kDa (lane 1). DMSO at 0.1% was used as the vehicle control and constitutively showed MMP-9 gelatinolytic activity (lane 3). Upon MPA treatment, MMP-9 gelatinolytic activity was significantly decreased (lanes 4–7). GM6001 (lane 8) was used as a negative control for active MMP-9.

Supplementary Figure 4. Full Zymogram Gel of Cropped Image as Shown in Figure 1B. Using a 12-well gel, DMSO at 0.1% was used as the vehicle control and constitutively showed MMP-9 gelatinolytic activity (lanes 1 and 7). Upon MPA treatment, MMP-9 gelatinolytic activity was significantly decreased (lanes 2–5 and 8–11). GM6001 (lanes 6 and 12) was used as a negative control for active MMP-9.

Supplementary Figure 5. Full Zymogram Gel of Cropped Image as Shown in Figure 4. Using a 10-well gel, DMSO at 0.1% was used as the vehicle control and constitutively showed MMP-9 gelatinolytic activity (lane 1). C6 cells were pretreated with mifepristone, RU486 (2 μ M), for 30 min, followed by MPA treatment for another 48-h. Treatment with RU486, alone, did not alter MMP-9 enzymatic activity (lane 2). Upon MPA treatment, MMP-9 gelatinolytic activity was significantly decreased (100 nM, lane 3; 10 μ M, lane 5). Pretreatment with RU486 (2 μ M) antagonized MPA's effect on enzymatic activity (100 nM, lane 4; 10 μ M, lane 6).

Supplementary Figure 6. Full Western Blot Gel of Cropped Image as Shown in Figure 5A. Li-Cor Pre-Stained Protein Ladder (ChameleonTM Duo; #928-60000) (lane 1). Freshly prepared synthetic human A β 1-42 (23 μ M) was added to serum-free media (SFM) (lane 3), 10% fetal bovine serum (FBS) media (lane 4), SFM that had been conditioned by incubation with C6 glial cells (CM) (lane 5), or to 100 nM MPA-treated CM (lane 6) or 10 μ M MPA-treated CM (lane 7).

Supplementary Figure 7. Full Western Blot Gel of Cropped Image as Shown in Supplementary Figure 2. Li-Cor Pre-Stained Protein Ladder (ChameleonTM Duo; #928-60000) (lane 1). Freshly prepared synthetic human Aβ1-42 (23 μM) was added to serum-free media (SFM) (lane 2), SFM that had been conditioned by incubation with C6 glial cells (CM) (lane 3), CM that had been treated with 1 μM GM6001 (lane 5), or to CM that had been treated with MPA (10 nM, 100 nM, 1μM, and 10 μM) for 48-h (lanes 7-10). As noted in our Discussion, our synthetic Aβ preparation exhibited variability in oligomer generation. The variability in aggregates stems from using HPLC grade water as our solvent, instead of alcohols, such as hexafluoroisopropanol (HFIP), which removes preexisting aggregates and beta sheet secondary structures from Aβ1-42, yielding the peptide in one specific form of Aβ1-42 species. The solvent used to dissolve the lyophilized peptides determines the initial conformation of amyloid beta and also the aggregation kinetics (Wei and Shea, 2006). Additionally, while performing replicates of our studies, our amyloid preparation was stored at -80. Amyloid peptides are shown to be sensitive to temperature and freezing even at low concentrations, where freezing the peptide allows for uncontrolled oligomerization, causing the variation in aggregates once it goes through a freeze/thaw cycle (Filippov et al. 2008). We observed changes in the preparation as an effect of time and temperature. During our experiments and replicates, we found that if the preparation was not freshly made with each replicate, our Western blot detection would then lack the sensitivity required to detect MPA-induced effects on degradation. The effects of CM and GM600, however, could still be detected, using a synthetic preparation that was not freshly made.

3 Chapter 3

The *In Vivo* Effects of Long-Term Administration of Medroxyprogesterone Acetate on Matrix Metalloproteinases in Cognitive Function

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W. Simpkins

3.1 Abstract

Progestins, outside of their action on the endometrium, have multiple nonreproductive functions. Currently, the literature provides only a superficial understanding of the interplay between synthetic progestins and the central nervous system (CNS). While the central effects of progesterone are well-characterized, the effects of synthetic progestins on the brain are understudied. Recently, we have demonstrated that *in vitro* treatment of glial cells with medroxyprogesterone acetate (MPA), a widely used synthetic progestin, resulted in repression of matrix metalloproteinase-9 (MMP-9) expression and activity, as measured by their capabilities to degrade amyloid beta. Matrix metalloproteinases (MMPs) are expressed by neurons and glial cells, in the adult brain, and released in response to enhanced neuronal activity under physiological and pathological conditions. Moreover, synaptic connections rely heavily on the function of MMP-9. We reasoned that MPA's suppression of MMP-9 expression would also negatively impact cognitive and synaptic function, through the downregulation of MMP-9. The present investigation evaluated the long-term effects of MPA on MMP-2/-9 and cognitive function in intact, wild-type (WT) mice and the triple transgenic (3xTg-AD) mouse model of AD. We hypothesized that long-term administration of MPA accelerates the onset of cognitive dysfunctions in 3xTg-AD mice. Additionally, we hypothesized MPA would induce cognitive impairments in the WT mice, through negative impacts on synaptic function via action on MMP-9. Our results indicate that MPA treatment promotes cognitive dysfunction and increases cortical MMP-9, *in vivo*. These results raise the possibilities of yielding similar effects in women who exercise the chronic use of MPA to prevent pregnancy or in HT formulations.

3.2 Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia, characterized by progressive cognitive decline. The pathological hallmarks of AD are extracellular plaques stemming from the accumulation of amyloid beta, neurofibrillary tangles as a result of aggregated hyperphosphorylated tau, neurodegeneration, and the loss of synapses. As the main constituent of amyloid plaques, A β plays a key role in the pathogenesis of AD (Selkoe et al., 2001). Accumulation of A β occurs nearly a decade prior to the onset of clinical symptoms and originates from the proteolytic cleavage of amyloid precursor protein (APP), by beta- and gamma-secretases (Scheltens et al., 2016), which promotes the amyloidogenic cleavage of APP. This produces 40 or 42 amino acid fragments of A β (A β ₁₋₄₀ and A β ₁₋₄₂) and subsequently, A β peptide is released into the extracellular space (Chen, 2015; Xu et al., 2016). Extracellular A β assumes several conformational states ranging from monomers to soluble oligomers and fibrils, which rapidly form aggregates and ultimately amyloid plaques which are characteristic of the disease (Pryor et al., 2012). In a series of experimental models of AD and in AD patients, there is a growing body of evidence that neuronal dysfunction stems from soluble oligomeric A β (Mucke et al., 2000; Gong et al., 2003; Cheng et al., 2007). APP is also recognized by alpha-secretase (α -secretase), which cleaves the precursor protein, promoting the non-amyloidogenic cleavage of APP and producing the N-terminal fragment soluble APP α (sAPP α). sAPP α has been reported to promote neuroprotection and cognitive resilience (Ghisso and Frangione, 2002). Decreased expression of sAPP α in the cerebrospinal fluid (CSF) of familial (FAD) and sporadic AD (SAD) patients (Lannfelt et al., 1995; Almkvist et al., 1997; Colciaghi et al., 2002; Fellgiebel et al., 2009), due

to reduced α -secretase activity and increased β -secretase activity (Tyler et al., 2002), is highly correlated to memory impairments in aged rodents and AD patients (Almkvist et al., 1997; Anderson et al., 1999; Fellgiebel et al., 2009).

MMPs are a family of zinc-dependent enzymes responsible for the degradation and remodeling of extracellular matrices (ECM). In AD, MMP-2 and MMP-9 facilitate the clearance and degradation of A β , influence the aggregation kinetics of tau protein, and additionally regulate synaptic connections (Deb and Gottschall, 1996; Deb et al., 2003; Talamagas et al., 2007; Mizoguchi et al., 2009). In contrast to other A β -degrading proteases, MMP-9 is known to degrade A β fibrils *in vitro*, A β plaques *in situ*, and A β *in vivo* (Backstrom et al., 1996; Yan et al., 2006; Yin et al., 2006; Hernandez-Guillamon et al., 2015). Overexpression of MMP-9 in a transgenic mouse model of AD (5xFAD) resulted in reduced levels of oligomeric A β and cognitive resilience, in specifically female animals when compared to their male counterparts. Additionally, MMP-9 overexpression caused increased levels of sAPP α , via enhanced cleavage of APP at α -secretase cleaving sites. In theory, modulation of MMP-9 levels potentially impacts the progression of AD pathogenesis. Conversely, MMP-9 has been shown to be increased in AD brains (Backstrom et al., 1996). Moreover, there are reports that treatment with a non-specific MMP inhibitor, GM6001, improves A β -induced cognitive impairment and neurotoxicity, *in vivo*. The authors hypothesize MMP-9 likely performs nonselective cleavage of the ECM and neural membranes, leading to neuronal dysfunction and cognitive impairment and suggesting a causal role of MMP-9 in A β -induced cognitive deficits and neurotoxicity (Mizoguchi et al.,

2009). Despite the discrepancies within the literature, taken together, modulating the levels of MMP-9 impacts AD pathogenesis.

Medroxyprogesterone Acetate (MPA) is globally used as the progestin component of hormonal therapy used by menopausal women, Prempro® and Premphase®, as well as a hormonal contraceptive, Depo Provera®. There is accumulating evidence that MPA increases the risk of dementia for menopausal women (Shumaker et al., 2003, 2004), induce cognitive impairment in ovariectomized animal models (Braden et al., 2010, 2011, 2017), and exert amnestic effects in case studies of premenopausal women (Gabriel and Fahim et al., 2005). MPA's cognitive-impairing effects may be mediated via its action on modulating the levels of MMPs. *In vitro* evidence suggests that estrogen-related compounds induce MMP-2 and MMP-9 expression, promoting the degradation of A β and produce neuroprotective effects (Merlo et al., 2012). We have previously reported that hormonal modulation of MMP-9, utilizing MPA, decreases the production and proteolytic activity of MMP-9, in turn, impairing the A β -degrading activity of MMP-9 (Porter et al., 2020). Alarming, the long-term consequences of using MPA during a woman's reproductive prime is understudied, as a majority of the previous investigations utilized menopausal animal models and women during the stages of menopause. In this study, we used a transgenic mouse model of AD to investigate the effects of chronic MPA treatment, in intact animals, on cognitive function. Our central hypothesis is that MPA-treated animals, both the WT and AD groups, will show cognitive impairments. Here, we demonstrate MPA promotes differential effects on memory consolidation. MPA treatment induced a modest memory impairment in WT animals, while yielding a beneficial effect in 3xTg-AD animals.

3.3 Materials and Methods

Animals and drug treatments

Triple APP_{swe}, PS1_{M146V} and Tau_{P301L} transgenic mice (3xTg-AD) and wild-type (WT; B61129SF2/J) mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA). The mice were maintained at constant temperature with an alternating 12 hours light-dark cycle. Food and water were available *ad libitum*. Twenty mice were assessed in this study; female 3xTg-AD (n = 10) and WT (n = 10). Mice (5 animals/group) were randomly assigned and injected subcutaneously (s.c.) with either saline or 4 mg depot medroxyprogesterone acetate (MPA; Pfizer, New York, NY) once every 4 weeks, from the age of 3-months to 6-months. This dose was selected to approximate serum MPA levels achieved by Depo-Provera injection for prevention of undesired pregnancy. The 4 mg dose also produces mean serum concentration of 21.37 ng/ml, respectively (the level that approximates the peak serum concentrations of MPA in women using Depo-Provera for prevention of undesired pregnancy) (Miguel et al., 2012). Control mice received the same volume of vehicle. To confirm the effectiveness of MPA injections on ovulation, vaginal smears were taken for the seven days post-injection 1. Smears were classified as proestrus, estrous, metestrus, or diestrus per prior protocol (Goldman et al., 2007; Acosta et al., 2009). Animals treated with MPA were confirmed to be in a constant diestrus phase, while vehicle-treated animals showed a normal cyclicity. All animal experiments were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals. The animal studies were approved by the Institutional Animal Care and Use Committee of West Virginia University.

Morris Water Maze

After the last injection, mice were tested in the Morris water-maze (MWM) task. The apparatus and the protocol used a circular pool (140 cm in diameter) situated in a room with discrete extramaze cues and filled with water ($23 \pm 1^\circ\text{C}$), that was rendered opaque by the addition of white paint. A clear movable platform (10 cm in diameter) was submerged 1 cm below the water surface. The protocol we followed consisted of a 1-day visible phase (four 60-second trials per day in the presence of a movable visible platform), a 4-day acquisition phase (four 60-second trials per day in the presence of a hidden platform, located in a fixed position (NE quadrant) relative to visible extramaze cues) and a 2-day reversal phase (opposite to the target quadrant i.e. the quadrant where the platform was located during acquisition). Each daily session consisted of four trials and four different starting positions were used in a random order. Each trial had a maximum duration of 60 s and mice who failed to locate the platform within 60 s were gently placed on the platform. At the end of each trial, mice remained on the platform for 15 s. The behavior of animals during the water-maze was analyzed by the behavioral analysis system AnyMaze (Stoelting, Wood. Dale, IL, USA). In order to assess learning in the visible and the acquisition phase of the task, two parameters were analyzed; latency (time in seconds to reach the platform) and swim distance (in meters). The values obtained were averaged per mouse within each daily session. To ensure that behavior in the water-maze did not simply reflect changes in activity, swim speed was also scored; these values were also averaged per mouse within each of the phases of the task (visible, acquisition and reversal).

Enzyme-linked immunosorbent assay (ELISA)

Hemispheric tissue samples were weighed and frozen immediately in liquid nitrogen and stored at -80°C . Using a ratio of $\sim 1\text{g}$ of tissue to 20mL reagent, total proteins were extracted using T-PER tissue protein extraction reagent (Thermo Scientific) added with Halt Protease Inhibitor Cocktail (Thermo Scientific) and 1 mM EDTA (Invitrogen). Supernatant was collected after centrifugation at $10,000\text{ g}$ for 15 min at 4°C , the total protein concentrations were measured using a BCA protein assay kit (Thermo Scientific), and samples were normalized to $65\text{ }\mu\text{g/mL}$ of total protein. Levels of MMP-2 and MMP-9 in mouse hippocampal and cortical homogenates were analyzed using commercially available high-sensitivity ELISA kits (Quantikine, R&D Systems), according to the manufacturer's instructions. The optical density in each well was read at 450 nm (with 540 nm correction) using a microplate reader.

Blood samples were drawn via cardiac puncture into heparinized before perfusion. Plasma was isolated from blood in heparinized tubes, on ice. Samples were then centrifuged for 20 minutes at $2000\times\text{g}$ within 30 minutes of collection. An additional centrifugation step of the plasma at $10,000\times\text{g}$ for 10 minutes at $2-8^{\circ}\text{C}$ is recommended for complete platelet removal. Plasma samples were aliquoted and stored at -80°C until analysis. All measurements were done in duplicate. Similar standard curves for each of the proteins were required in each plot. The immunoassays were carried out following the manufacturer's instructions. All assays were done in duplicate.

3.4 Results

***In Vivo* Assessment of Medroxyprogesterone Acetate-Induced Effects on Memory and Learning**

To test the effects of MPA on memory and spatial learning impairments, we performed the Morris Water Maze test (Figure 1). The acquisition trials (hidden platform: HP) of the MWM task is used to assess learning capacity, which analyzes the escape latency (time required to reach the platform) (Figure 2 and 3) and swimming distance to the platform (Figure 4). Our latency data reveals a significant MPA-induced learning deficit in the WT animals on Day 1 of reversal training (Figure 2A), and this is controlled by similar rates of swim speed (Figure 2B). Additionally, on the final day of acquisition training, MPA enhanced learning in AD animals (Figure 3A) and this is controlled for with no significant differences in swim speed (Figure 3B). On the specific days where we observed significant differences in swim speed (Figure 2B and 3B), we were unable to interpret escape latency data without existing confounding factors and solely relied on the analysis of swim distance across all trials. Swim distance was averaged across all 5 trials, for Day 1 and Day 4, the initial and final days of acquisition training. The WT animals treated and untreated, both successfully improved in performance over the course of training, as indicated by the significant reductions in their swim distances (Figure 4A). However, treatment with MPA revealed a modest enhancement in spatial memory/learning, in AD animals (Figure 4B). We compared performances on trial 1 only, of the first and fourth day (final) of acquisition training (Figure 4C, D), to reflect memory retention and memory consolidation. In the WT animals, the swim distance of vehicle-treated animals significantly decreased from Trial 1 of Day 1 to Trial 1 of Day 4

(Figure 4C). However, this effect was not seen in the WT- MPA animals. Although statistically insignificant, MPA-treated WT animals had increased swim distance on Trial 1 of Day 4 of training, relative to vehicle-treated animals. However, in AD animals, MPA-treated animals show improved memory consolidation, as their swim distance is significantly reduced, from Trial 1 of Day 1 to Trial 1 of Day 4 (Figure 4D). Trial 5 of Day 1 and Day 4 is indicative of short-term or working memory, and all our animals show no effect of treatment in those trials (data not shown).

There were no statistically significant differences in swim distance between groups in the reversal training (Figure 4E, F). Noticeably, average swim distance, on Day 1 of Reversal training, shows WT mice treated with MPA appear to not entirely abandon their initial learning, though the differences in swim speed are not statistically significant (Figure 2A and 4E). There were no major differences in swim distance observed in the AD animals (Figure 4F). By Day 2 of Reversal training, all four animal groups had relatively low swim distances, with no significant effects of treatment (data not shown).

The entries into the former location of the platform (NE) were quantified in order to assess cognitive flexibility. The untreated WT animals show a significant reduction in the number of entries they made into the NE quadrant, while the target quadrant was now the South, but the statistical significance of this effect is not observed in the MPA-treated WT animals (Figure 5A). Additionally, although statistically insignificant, MPA-treated WT animals had higher number of entries into the previous platform zone (NE quadrant), Day 2, Trial 5 (Figure 5C). There was no effect of treatment on the number of entries, in the AD animals

(Figure 5B, D). Collectively, these data imply MPA positively impacts memory consolidation in AD animals, while only potentially impairing memory consolidation in the acquisition training and cognitive flexibility during the reversal training, in WT animals.

The Effect of MPA on MMP-2/-9 Expression

The levels of MMPs are modulated by a variety of stimuli, including hormones that are utilized as either contraception or menopausal hormone therapy (HT), both natural and synthetic. To study the effect of Medroxyprogesterone Acetate (MPA) on matrix metalloproteinase-2 and -9 (MMP-2/-9) expression, we administered MPA subcutaneously to WT and 3xTg-AD mice, once a month, for 3 consecutive months. MMP-2 and MMP-9 have been strongly associated with learning and memory in past studies. To measure hippocampal and cortical MMP-2/-9 protein levels, brain homogenates were analyzed by ELISA for MMP-2/-9 (pro-MMP-2/-9, TIMP bound MMP-2/-9, and latent MMP-2/-9; Figures 6 and 7). Our analysis showed that MPA significantly reduced protein expression of MMP-2 in the frontal cortex (Figure 6C) and the hippocampus (Figure 6D) of AD animals but show no effect of treatment on MMP-2 in WT animals (Figure 6A, B). MPA treatment significantly increased protein expression of MMP-9 in the frontal cortices of both WT and AD animals, with a more robust effect in WT animals. A 2-fold increase in WT MPA animals was shown, relative to control (Figure 7A), while MMP-9 expression in AD animals was also increased, to a lesser extent (Figure 7C). There were no effects of treatment observed in the hippocampal samples of either genotype of animals (Figure 7B, D).

3.5 Discussion

The present study investigated the effects of MPA administration on WT and 3xTg-AD mice. Several reports have demonstrated that MPA can alter MMP-9 levels, where levels are increased in macrophages and, conversely, decreased in several other cell lines (Di Nezza et al., 2003; Hwang-Levine et al., 2011; Allen et al., 2019). We have previously shown that 48 and 72-hour exposure of C6 glial cells to MPA decreased MMP-9 protein expression and enzymatic activity (Porter et al., 2020). In our previous study, we used a relatively acute treatment paradigm, while in our current study we investigated the chronic effects of MPA treatment *in vivo*. Furthermore, most of the previous *in vivo* studies have used ovariectomized female animals to observe the effect of MPA on the brain, with little to no investigation of the effects of MPA on brain MMP-9. Additionally, due to the lack of focus in a CNS-related system, previous work failed to investigate the potential for MPA to influence cognitive function and AD-related pathology via the hormonal modulation of MMP-9. Herein, we provide data showing the effects on brain MMP profiles and cognitive function in intact female mice.

To our knowledge, this is the first *in vivo* investigation of the effect of chronic MPA administration on brain MMP-2/-9's expression, *in vivo*. Our results show that MMP-9 is expressed in the brains of WT and AD animals in the hippocampus and cortex of vehicle-treated animals and is increased by exposure to MPA at the protein level. Interestingly, the observed effects go against our original hypothesis, as we confirmed stimulatory effects of MPA on the expression of MMP-9, whereas in the glial cell line, we observed inhibitory effects of MPA, as shown by the suppression of transcription of MMP-9 at the mRNA level.

Conversely, we found MPA significantly reduced hippocampal and cortical MMP-2 protein expression, in the AD animals, however the biological significance of our observations is minimal despite their statistical significance.

In the cytoplasm, the glucocorticoid receptor (GR) is considered inactive, until a ligand for the receptor binds and activates the receptor. In turn, this typically promotes an anti-inflammatory response through the induction of anti-inflammatory gene expression or transrepression of proinflammatory genes. Recent work from our group revealed MPA's effects on MMP-9 expression are modulated in a GR-dependent manner, where the pretreatment of C6 glial cells with a GR antagonist (RU486), prior to MPA exposure, attenuates MPA's effects on MMP-9 expression (Porter et al., 2020). Moreover, glucocorticoids have been shown to alter MMP-9 expression (Rosenberg et al., 1996; Park et al., 1999; Eberhardt et al., 2002; de Paiva et al., 2006), as well as MMP-3 (Richardson and Dodge, 2003; Koyama et al., 2017), which is the enzyme responsible for the conversion of MMP-9 from an inactive to an active state. The literature extensively supports the notion that MPA binds to the GR with a high affinity, acting as a partial to a full agonist for the GR (Koubovec et al., 2004, 2005; Su et al., 2012a, b; Louw-du Toit et al., 2014). Alternatively, Zhao et al. indicates MPA's agonistic behavior is highly dependent on the expression levels of the GR. Decreasing the expression levels of the GR converts MPA from an agonist to an antagonist of GR-mediated transrepression (Zhao et al., 2003). Therefore, it is plausible that the discrepancy between the effects seen in our *in vitro* study and our *in vivo* study are attributed to alterations in the expression of the GR, as an effect of our chronic MPA

treatment, in both the WT and AD animals. Future studies should aim to further delineate the precise mechanisms by which our observations in the present study occur.

To our knowledge, this is the first investigation of the effects of MPA treatment on cognitive function, and AD-related pathology, in intact female animals. Our objective was to relate progestin-induced elevations of active MMP-9 levels with cognitive impairments. We were interested in observing the effects of monthly subcutaneous injections and, using the Morris Water Maze task, we were able to confirm the differential effects on memory consolidation on WT and 3xTg-AD animals.

Our most significant behavior data suggests MPA-treated AD animals were more capable of retaining memory of the platform's location overnight. We sought out to investigate the effect of chronic MPA treatment on cognitive function in the 3xTg-AD mouse model and found a significant effect on memory consolidation and cortical MMP-9 expression. In the transgenic mouse model of AD which has five familial AD-related mutations (5xFAD), overexpression of MMP-9 reduced oligomeric A β , increased synaptic markers, and prevented cognitive deficits (Fragkouli et al., 2014; Kaminari et al., 2017). Our finding that MMP-9 expression is increased in AD animals, after chronic treatment of MPA, is in line with findings in the literature, where increased MMP-9 expression yields beneficial effects in AD animal models. Although we did not statistically compare the two genotypes used in this study, we noticed the performances between the two vehicle treated groups were not vastly different. The literature states 6-months of age in this specific animal model should have sufficed to induce obvious impairments in AD animals performing the MWM

task (Oddo et al., 2003). It is possible that our AD animals may have been selected at a time before an apparent learning and/or memory deficit.

Within our vehicle groups, we assessed the effect of MPA treatment on performance between the first and final day of acquisition training. When swim distance is averaged across all 5 trials, for Day 1 and Day 4, both the vehicle and MPA-treated groups improved over the course of training. However, if we average the performances for Trial 1 of Day 1 and Day 4, the WT vehicle animals show they are progressively retaining information, and this significant change is not observed in the MPA-treated animals. This suggests MPA treatment impairs memory retention in WT animals, but modestly enhances memory consolidation and working memory in AD animals.

Cognitive flexibility is consistently associated with damage to the prefrontal cortex (PFC) (Ragozzino et al., 1999; Birrell and Brown, 2000; Block et al., 2007; Bissonette et al., 2008). Additionally, the frontal cortex is involved in working memory, cognitive flexibility, attention and other cognitive and executive functions that are affected both in patients and animal models with AD (Baddeley et al., 1991; Brugger et al., 1996; Perry and Hodges, 1999). Cognitive flexibility is the ability to update a strategy or adjust a previously learned behavior in response to alterations in a present task (Bissonette et al., 2013). In the MWM, evidence of loss of cognitive flexibility is shown when treated animals failed to adapt their behavior to learn the new location of the platform, after switching it from the target quadrant (NE) to the opposite quadrant. Reversal learning reveals whether animals can extinguish their initial learning of the platform's location and possess cognitive flexibility to

acquire the new location of the platform. After multiple trials on Day 1 of reversal training, WT mice treated with MPA appear not to entirely abandon their initial learning, though this difference is also not statistically significant. There were no major differences observed in the AD animals. By Day 2 of reversal training, all four animal groups were shown to be capable of uncovering the new location, as shown by lower values of swim distance. Again, although statistically insignificant because of variability within the treatment group, on the last trial of the last day of Reversal training, a portion of the MPA-treated animals continued to enter the previously platformed zone (NE). At this point in training, animals should have grasped the concept that there is no longer any reason to enter the NE quadrant. Figure 4 shows all the other treatment groups have relatively lower number of entries, with less variability within the group.

Our finding that chronic treatment of MPA elevates MMP-9 expression, in WT animals, could ultimately negatively impact the perineuronal net (PNN), as MMP-9 is considered a degrading enzyme for proteins within the PNN. Increased disruption of the PNN in the PFC leads to deficits in cortical GABAergic signaling (Slaker et al., 2015; Sorg et al., 2016; Bozzelli et al., 2018; Paylor et al., 2018; Wen et al., 2018; Holter et al., 2019). Parvalbumin (PV) GABAergic neurons in the PFC are also highly associated with cognitive flexibility (Murray et al., 2015). Thus, decreased GABA levels could play a role in the modest differences observed in the WT animals' MWM performances, correlating their behaviors to the 2-fold increased MMP-9 expression in their cortices. Herein, we encourage additional investigations related to MPA's effects on the PNN. It is conceivable that the chronic use of MPA will progressively increase the proteolytic degradation of the PNN by MMP-9, *in vivo*,

which could potentially promote cognitive dysfunctions in memory consolidation and cognitive flexibility.

Our findings imply MPA positively impacts memory consolidation in AD animals. Additionally, because of our lack of statistical power and low sample size, our data are only able to suggest MPA potentially impairs memory consolidation in the acquisition training and cognitive flexibility during the reversal training, in WT animals. The major limitation of our study is the sample size. Although we had sufficient statistical power for our biochemical analyses, unfortunately we did not for our behavior studies. The inclusion of a behavior task more sensitive or tailored towards assessing cognitive flexibility and/or deficits within the frontal cortex is needed.

Although we were able to measure protein, we were unable to make the correlation of protein with enzymatic activity, due to our inability to detect it via zymography. Future studies could assess MMP-9 activity in histological sections via in situ zymography, where frozen sections are coated with fluorescently labeled matrix proteins, incubated, and observed for black patches in the fluorescent background, indicating proteolysis of the matrix protein (George and Johnson, 2010). Alternatively, frozen sections could also be incubated with dye-quenched (DQ) gelatin, which would become fluorescent upon proteolytic cleavage by MMP-9. In this method, MMP-9 activity would be denoted as bright fluorescent spots (Gawlak et al., 2009). We suggest future studies be performed, with a higher sample size, a more in-depth analysis of how MPA alters MMP-9 protein levels and what the consequences are.

There is currently an unmet need for extensive *in vivo* investigation for the potential cognitive and pathological outcomes of MPA, in animals that are not ovariectomized. The majority of the literature focuses on the effects of estrogen, estradiol, testosterone, and progesterone on the brain, and particularly amyloid-beta production and clearance (Vest and Pike, 2013; Li and Singh, 2014; Giatti et al., 2016; Uchoa et al., 2016). There is still a paucity of published research addressing the potential effects of progestins, more specifically MPA, on the brain. While limited research with MPA suggests a negative impact on the brain, these studies need to be extended to model systems relevant to women in their reproductive prime, as a majority of efforts have been in menopausal animal models and menopausal woman (Shumaker et al., 2003, 2004; Braden et al., 2010, 2011, 2017; Lowry et al., 2010; Akinloye Olanrewaju et al., 2013). In summary, our work demonstrates the necessity for a further delineation of the consequences of chronic MPA administration's effects on brain MMP-9 and cognitive function, in WT and AD mice. Herein, we encourage additional, elaborate investigations related to MPA's effects on the brain. Future efforts should remain extended to model systems relevant to AD-related pathology, with a stronger sample size, and investigate the role of the PNN.

3.6 Figures

Phase	Visible Platform	Acquisition- Hidden Platform				Reversal Training	
Day	1	2	3	4	5	6	7

Figure 1. Outline of Morris Water Maze (MWM) task. (A) Acquisition training platform location: NE
(B) Reversal training platform location: S

A **B**

Figure 2. Swim speed and latency to platform in MWM task of WT mice. Mice (n=5 per experimental group) were tested in the MWM. Swim speeds across 5 trials per day were averaged. HP: Hidden Platform, Rev: Reversal.

Figure 3. Swim speed and latency to platform in MWM task of AD mice. Mice (n=5 per experimental group) were tested in the MWM. Swim speeds across 5 trials per day were averaged.

Figure 4. Swim distance in MWM task. Mice (n=5 per experimental group) were tested in the MWM. Mice were trained on the task until optimal performance was maintained for 2 consecutive days, measured by distance swam, before 2 days of reversal training. (A) WT and (B) AD animals' performance the first day of training versus the last day of training (Day

1 v Day 4). (C) WT and (D) AD animals' performance in Trial 1 of the first day of training versus the last day of training (Trial 1 Day 1 v Trial 1 Day 4). (E) WT and (F) AD animals swim distance in reversal training, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, unpaired one-tailed t-test.

A **B** **C** **D**

Figure 5. NE quadrant entries during reversal training in MWM task. Mice ($n=5$ per experimental group) were tested in the MWM. In reversal training, the platform location was changed and the number of entries in the platform's former location (NE) were measured. (A) WT and (B) AD animals' performance the first trial of training versus the last trial of training, on Day 1 (Trial 1 Day 1 v Trial 5 Day 1). (C) WT and (D) AD animals' number of entries into NE on the last trial of all phases of training (last trial of the last day of training; Day 2 Trial 5), $*p < 0.05$, unpaired one-tailed t-test.

Figure 6. The effect of chronic MPA treatment on MMP-2 expression in the frontal cortex and hippocampus in WT and 3xTg-AD female animals. MMP-2 (ng/mL) in the specified brain regions was detected using the ELISA kit in WT animals (A,B) and AD animals (C,D) MPA-treated and untreated. MPA treatment had no effects on MMP-2 expression in the frontal cortex of WT animals. MPA treatment significantly decreased MMP-2 in the frontal cortex of 3xTg-AD mice. Results are expressed as means $\pm$ SEM. Results are representative of five animals in each treatment group. * p < 0.05, ** p < 0.01, unpaired one-tailed t-test.

Figure 7. The effect of chronic MPA treatment on MMP-9 expression in the frontal cortex and hippocampus in WT and 3xTg-AD female animals. MMP-9 (ng/mL) in the specified brain regions was detected using the ELISA kit in WT animals (A, B) and AD animals (C, D) MPA-treated and untreated. MPA treatment had significantly increased MMP-9 expression in the frontal cortex of both WT and AD animals. Results are expressed as means $\pm$ SEM. Results are representative of five animals in each treatment group. * $p < 0.05$, ** $p < 0.01$, unpaired one-tailed t-test.

Chapter 4

General Discussion

Until the 1980s, women were intentionally excluded from participating in clinical trials. This longtime bias yielded a striking gap in knowledge about women's health and the significant role that sex- and gender-specific differences play in health and disease. The 1993 NIH Revitalization Act required that women be included in all clinical studies and that trials be designed to permit analysis of different effects in women, and in 2016, NIH finally made it an experimental necessity for researchers to factor in sex as a biological variable in animal and human studies, and otherwise required an experimental justification (Mazure et al., 2015). Even though there have been many advances made in women's health research, Alzheimer's disease (AD) is a prime example of a condition for which there is currently limited and superficial understanding of sex differences. Even considering the last decade, sex- and gender-specific differences in AD have been severely understudied. Our ability to address the risks for AD is impeded by the paucity of knowledge regarding how and why the disease differs between men and women. Sex- and gender-specific risk factors are just one of the many areas in which more research into the differences between the sexes in AD is needed. Previous research has often overlooked sex differences in diagnosis, clinical trial design, and treatment outcomes, while also failing to consider the influence of social and environmental factors, in turn, delaying progress in detection and care. In order to optimize the development of current and future interventions in AD, these differences cannot be ignored.

The interplay between biological factors (genetic and hormonal influences) and environmental factors is essential to understanding how the risk for AD can be reduced, and how new strategies for prevention and treatment should be developed and implemented.

Hormonal influences are of interest because there is an extensive amount of literature that suggests menopause and ovariectomy lead to negative cognitive changes and may represent sex-specific risk factors for AD (Pike et al., 2017; Gurvich et al., 2018; Nebel et al., 2018; Rahman et al., 2019). Collectively, sex steroids have been shown to play an important role in the sex differences in the brain and the role of endogenous and exogenous estrogen-related compounds is well-characterized, yet relatively little is known about the long-term effects of exogenous synthetic progestins on the brain and their role in the pathogenesis of AD. Medroxyprogesterone acetate (MPA) is used by approximately 1 in 5 adolescents and adult women in the United State who are sexually active (Mosher et al., 2010). Globally, nearly 48 million women utilize injectable contraceptives to prevent pregnancy, with the majority of users utilizing MPA as their hormone of choice (United Nations, 2013). Therefore, studies that elucidate the mechanisms and long-term effects of exogenous administration of MPA have the potential to impact millions of women, and further the current mission to promote research on the biological sex differences in disease and improving women's health through science, policy, and education.

Two major studies were designed to begin addressing increasingly relevant knowledge gaps associated with the effects of exogenous progestin treatment. The overall goal of my dissertation project was to investigate the acute and chronic effects of MPA administration on AD-related pathology, *in vitro* and *in vivo*, utilizing hormonal modulation of A β -degrading enzymes, matrix metalloproteinases (MMPs). This chapter includes a discussion of the major findings as related to the literature on MPA, MMPs, and cognitive function, and what implications may be valuable for future epidemiological investigations.

The chapter concludes with a brief summary and discussion of the limitations of the study, as well as suggestions for future experiments.

This chapter contains discussion and future research possibilities to help answer the research questions:

(R1): What are the outcomes of MPA on MMP-9 secretory, proteolytic, and A β -degrading activities, *in vitro*?

(R2): What are the effects of long-term, subcutaneous administration of MPA on cognitive functions and AD-related neuropathology in WT and 3xTg-AD mice?

The central hypothesis of this dissertation was that MPA treatment downregulates MMP-9 expression and subsequent secretion, in turn, disrupting the proteolytic degradation of amyloid beta. Further, the effects on MMP-9 would also negatively impact cognitive function and AD-related neuropathology. The proposed research was based upon the notion that the current literature extensively focuses on the effects of other classes of hormones, with very few studies evaluating the effects of MPA on cognition of women in their reproductive prime, despite evidence that MPA negatively impacts cognitive function in menopausal women and animal models (Braden et al., 2010, 2011, 2017; Gabriel and Fahim et al., 2005; Epperson et al., 2013; Greendale et al., 2009; Espeland et al., 2013; Gleason et al., 2015; Henderson et al., 2016; Manson et al., 2017; Shumaker et al., 2003, 2004).

To address this, I first conducted a survey of what is known about progestins based on the current literature, and how the literature on MPA fell short of characterizing its long-

term effects in an intact animal model and in younger women. We designed an *in vitro* exposure model, to determine the impact of MPA on MMP production and activity, and how that may affect amyloid beta degradation. Through this, I was able to show MPA could suppress MMP-9 expression and proteolytic activity, which fell in line with the previously literature, however my experiment was novel in its use of a CNS-related cell line (glial cells). For this study, I used the C6 glial cell line to demonstrate how changes in MMP-9 expression and secretion from glial cells may impact degradation of amyloid beta. We then investigated the consequential effects on amyloid beta degradation and show that MPA impairs the degradation of amyloid beta, in an MMP-9 dependent manner. Furthermore, we revealed MPA's effect on MMP-9 is mediated by the glucocorticoid receptor (GR). The direct impact of this study was to show the potential for MPA to negatively affects cellular processes that are regulated by MMP-9, *in vivo*, due to the negative impact MPA showed, *in vitro*. Our ability to easily perform dose-response and time-course studies, in a homogeneous population of cells, presented a highly controlled environment. However, primary cells are more reliable than transformed cell lines. C6 cells have spontaneous NF- κ B nuclear activity, suggesting the presence of constitutive NF- κ B activity, which explains the constitutive basal expression of MMP-9 observed in our experiments (Robe et al., 2004), and circumvented the necessity for induction or transfection. Considering that we would be required to either induce MMP-9's expression pharmacologically or transfect an MMP-9 expression vector into the primary cells, we decided to perform an *in vivo* experiment using wild-type and triple transgenic Alzheimer's disease mice, to confirm our observed effects.

At the molecular level, MPA elicits its biological effects through multiple receptors, including the progesterone receptor (PR), androgen receptor (AR), and glucocorticoid receptor (GR). The hormone binds to the GR with a high affinity, acting as a partial to full agonist for the GR (Koubovec et al., 2004, 2005; Africander et al., 2011; Su et al., 2012b, 2012a; Louw-du Toit et al., 2014). The initial finding of MPA's capability of interfering with the activities of NF- κ B or AP-1, at the promoter level, suggested MPA represses cytokine-induced, AP-1 driven genes, as well as NF- κ B-driven genes, without impacting the DNA-binding activity of NF- κ B, in a GR-dependent manner (Koubovec et al., 2004). We treated C6 cells with a GR antagonist, which attenuated MPA's effects on MMP-9 enzymatic activity (Chapter 2; Figure 4). In this case, our data implicate a GR-dependent transrepression of MMP-9, via interaction with either NF- κ B or AP-1, which are generally accepted as regulators of MMP-9 expression (Jonat et al., 1990; Paliogianni et al., 1993; Yokoo and Kitamura, 1996; Barnes, 1998; Bond et al., 2001; Ronacher et al., 2009; Africander et al., 2011; Li et al., 2012; Mittelstadt and Patel, 2012). Therefore, it's plausible the progestin binds to the cytosolic GR, activates the GR, and then activated GR binds to NF- κ B/AP-1, in turn, negatively interfering with the transcriptional enhancer and, in turn, promoting transrepression of MMP-9. Future studies should assess MPA's effects on nuclear translocation, utilizing western blot analysis and confocal imaging. Under control conditions, *in vitro*, AP-1 and NF κ B translocate to the nucleus, to bind the promoter region of MMP-9 and, in turn, induce its expression. MPA treatment, *in vitro*, would hypothetically interfere with this translocation, thus the expression of AP-1 and NF κ B subunits, in the nuclear fraction, would decrease, while phosphorylation of proteins in the cytosolic fraction

would also decrease. This can also be shown with immunofluorescent staining, using antibodies for p65 (transactivator of NFkB), phospho-c-Jun (subunit of AP-1), and c-Fos (subunit of AP-1). Under control conditions, *in vitro*, staining would be shown in the nucleus, and upon MPA treatment, the staining will be in the cytoplasm. Future studies should aim to further delineate the precise mechanisms by which our observations in the present study occur.

Next, I demonstrated the effects of MPA on cognitive function and brain MMP-9 expression, *in vivo*. Through this study, I established the correlation between MMP-9 and cognitive function, in intact, female WT and 3x-TgAD mice. The initial goal was to associate reduced MMP-9 expression with cognitive impairments. However, MPA treatment unexpectedly induced MMP-9 expression, in the frontal cortices, of both the WT and AD groups. Additionally, MPA treatment modestly impaired memory consolidation and cognitive flexibility in WT animals, but slightly enhanced memory consolidation in AD animals. The impairment of memory consolidation in the WT animals may be attributed to increased disruption of the perineuronal net (PNN), via the 2-fold increase in MMP-9 expression in the frontal cortex. Notably, with an n of 5 in each group, our behavior study did not have strong statistical power. Thus, our results serve as preliminary/pilot data and should prompt further elaborate investigation of the effects of chronic MPA on cognitive flexibility and memory consolidation, with an evaluation of the role that the PNN plays in the differential effects we observed between WT and AD animals.

Synthetic glucocorticoids are widely used for treatment of many inflammatory conditions. Generally, hormones which act through the GR have been assumed to be anti-

inflammatory, however, several studies have shown that there are several factors that allow hormones to exert pro-inflammatory effects. Short-term exposure to glucocorticoids may suppress neuroinflammation, while chronic exposure may promote inflammation in the frontal cortex and hippocampus (Hu et al., 2016). Chapter 2 demonstrated MPA's effects on MMP-9 expression are mediated in a GR-dependent manner. *In vitro*, MPA caused a dose-dependent reduction in MMP-9's expression and enzymatic activity, in turn negatively impacting the degradation of A β . *In vivo*, MPA increased MMP-9 protein expression, specifically in the frontal cortex. Depending on dose/concentration, treatment paradigm, the tissue being studied, and the steroid hormone being used, glucocorticoids can promote either anti-inflammatory or pro-inflammatory responses (Dinkel et al., 2002; Cruz-Topete and Cidlowski, 2015; Desmet and De Bosscher, 2017). Additionally, whether pro-inflammatory GC effects occur in the brain can depend upon the use of synthetic versus endogenous glucocorticoids and the timing of exposure with respect to the inflammatory challenge, but unfortunately the precise mechanism(s) by which the same hormone can promote two opposing responses are not well understood. The discrepancy between our *in vitro* and *in vivo* studies are most likely attributed to the differences in treatment paradigms, where our *in vitro* treatments were relatively acute treatments and our *in vivo* treatment was a chronic, slow-release depot of the hormone. We expected there would be a cognitive impairment that would be exacerbated with MPA treatment, but in fact, we observed improvement. Moderate levels of glucocorticoids have been shown to enhance both long-term potentiation (LTP) and memory consolidation, while very low or very high levels inhibit these processes (Joels and Krugers, 2007; Wirth et al., 2015). Additionally, too

little or too much receptor occupancy leads to an impairment as well (de Kloet et al., 1999). Therefore, it is plausible that our chronic MPA treatment alters GR expression. In theory, with a lower number of receptors, the receptor can become saturated at lower circulating levels of hormone. We suspect that as a result of our treatment, the AD animals possessed a higher GR expression when compared to the WT-treated animals. Given the clinical implications of the pro-inflammatory effects of MPA, it is crucial for future investigations to focus on the capacity of MPA to increase CNS inflammation and alter GR expression.

Our *in vivo* study allowed for the flexibility to assess the effects of MPA, in two genotypes of mice that are genetically homogenous. The dosage of MPA used was standard dosage form that produced a pseudo pregnancy state and was administered at a controlled dosing interval. Additionally, we were able to perform behavior assessments of the animals and follow these assessments with biochemical analyses. Our data suggests aging the animals to 6-months of age did not show a significant cognitive impairment. The effects of MPA on the GR in brain structures involved in the stress response should also be studied. As previously mentioned, hormones exert negative feedback on the HPA axis, through their actions on the endometrium. Studying the effects of MPA on the HPA axis could determine how the GR adapts after chronic MPA treatment, as well as determine if the MWM behavior task itself promotes any alterations in GR expression. This would determine if MPA impairs or suppressed the ability of the animals to respond to stress, including stress that may have been a byproduct of performing in the MWM task. Future studies should further investigate the effects of chronic MPA treatment on GR expression.

Obviously, mice are not human beings, thus we cannot be aware of the extent that the data would translate into the clinic. We also were forced to make an informed guess as to when cognitive decline would present itself and we seemingly did not observe the mutation-induced cognitive decline in the AD animals. We also selected a single dose and a single dosing regimen. A major limitation of our *in vivo* study was the number of mice per group, which limited our ability to interpret the behavior data. Our protein measurements show increased MMP-9 expression after MPA treatment. However, the specific cell type(s) this effect is attributed to remains to be determined. Immunofluorescence could be used to identify the cell types where increased MMP-9 expression is located. Although we were able to measure protein, we were unable to make the correlation of protein with enzymatic activity, due to our inability to detect it via zymography. Future studies could assess MMP-9 activity in histological sections via *in situ* zymography, where frozen sections are coated with fluorescently labeled matrix proteins, incubated, and observed for black patches in the fluorescent background, indicating proteolysis of the matrix protein (George and Johnson, 2010). Alternatively, frozen sections could also be incubated with dye-quenched (DQ) gelatin, which would become fluorescent upon proteolytic cleavage by MMP-9. In this method, MMP-9 activity would be denoted as bright fluorescent spots (Gawlak et al., 2009). Additionally, Oddo et al. reported that at the 6-month timepoint, in 3xTg-AD mice, synaptic dysfunction and LTP deficits are observed, but extracellular A β deposits are not in the hippocampus, even though intracellular A β immunoreactivity is apparent at this age (2003). We were also unable to achieve detectable levels of amyloid beta in our detergent-soluble samples, of both WT and AD mice. Once we revisited the literature, we found that A β ₄₂ is

significantly increased at 6 months of age, compared to animals at 2 months, in the detergent-insoluble fraction, the form of A β that readily forms aggregates (Billings et al., 2005). Therefore, future studies should prioritize the insoluble fraction of the cortex, and assay for A β_{42} . Inconsistencies within the literature are most likely due to variability in the method chosen to quantify A β , which ultimately influences the species you're able to analyze. Additionally, it is plausible that the behavior study itself altered endogenous glucocorticoid release and receptor expression, influencing the responses to MPA treatment. Future studies should aim to assess the effects of chronic MPA treatment on MMPs in Morris Water Maze-trained and -untrained mice. Such studies could provide insight into the role of education (training that is sometimes stressful in humans) and its association with decreased risk for Alzheimer's disease. We suggest a continuation of the experiments we performed, where there are multiple age groups (3-, 6-, 9-, 12-month), multiple hormone treatment regimens (acute and chronic), an assessment of detergent-insoluble A β_{40} and A β_{42} , and a battery of behavior assessments, in order to fully assess the effects of MPA on learning and memory.

My studies on the acute and chronic effects of MPA have confirmed this progestin can modulate the levels of MMP-9, in a CNS-related system, both *in vitro* and *in vivo*, as well as differentially impact cognitive function in intact, female WT and 3xTg-AD mice. In summary, our *in vitro* data suggests MPA: (a) downregulates MMP-9 expression and production, (b) impairs the neuroprotective effect of the proteolytic cleavage of toxic A β_{1-42} species, and (c) suppresses the transcription of glial MMP-9, through the glucocorticoid receptor. *In vivo*, MPA: (a) enhances memory consolidation and working memory in AD mice, (b) impairs memory consolidation in WT mice, and (c) increases MMP-9 levels in the

frontal cortices of both WT and AD mice. Collectively, these findings indicate MPA's differential effects should be further investigated, along with the underlying mechanisms regarding the signaling mechanisms behind our *in vitro* and *in vivo* observations.

Future Directions & Conclusions

There are specific areas within the sex- and gender- specific risk factors in AD that are currently understudied and must be of priority, due to their direct impact on the future progress of the field. Prior to our work, there weren't many studies that assessed how synthetic progestins influences cognition and AD risk (Braden et al., 2011; Olanrewaju et al., 2013). My work provides indications of acute versus chronic administration of MPA, the effects on MMP-9 expression, and the impacts on cognition and AD-related pathology. Additional investigations must follow these initial studies, as well as studies addressing how synthetic progestins might influence AD biomarkers, in addition to their effects on MMP-9. The field also accepts that AD presents itself more severely and progresses faster in woman more than it does in men, thus the reasoning behind why women are more vulnerable should also be investigated (Barnes et al., 2005; Hua et al., 2010; Holland et al., 2013; Lin et al., 2015; Filon et al., 2016; Koran et al., 2017). Moving forward, when evaluating the effects of exogenous hormones, particularly hormonal contraception, researchers should improve the details they include, regarding the specific type of contraception evaluated (estrogen-containing versus progestin-only). The lack of information regarding the long-term effects of contraceptives, in general, is one of the main rationales behind the Nurses' Health Studies (I and II). In both cases, and even in a follow-up study that occurred in 2014, whether the oral contraceptive was a combination pill versus a progestin-only pill was not recorded; they

were either “Never” or “Ever” users (Charlton et al., 2014). Due to the neuroprotective actions of estrogen-related compounds, this convolutes how we interpret those data. They also recorded HT use and noted there were missing data points, demonstrating not only are their gaps in knowledge, but also gaps in data.

Menopause is associated with fluctuation of endogenous hormone levels, as well as increased risk of AD in women (Fisher et al., 2018). Thus, we understand why majority of the literature has focused on menopausal influence on AD and the effects of exogenous hormones on AD risk. However, moving forward, researchers should be more inclusive of younger, intact (non-menopausal) pre-clinical and clinical models, when evaluating the long-term effects of exogenous hormones. Pike et al., performed 3 individual experiments, evaluating the effects of estrogen and progesterone on AD-related pathology (2007). In their first experiment, they used intact female 3xTg-AD mice to study the development of AD neuropathology as an effect of age. However, in the remainder of their experiments, they used OVX animals and ovariectomy exacerbated A β accumulation and tau hyperphosphorylation. Estrogen treatment provided neuroprotection from these pathologies, but combined estrogen and progesterone treatment attenuated this effect. Interestingly, progesterone alone treatment yielded no effect on the accumulation of A β , but successfully reduced tau hyperphosphorylation, unlike estrogen. It is unclear why their hormone treated groups only included OVX female 3xTg-AD animals and failed to include gonadally intact animals. Oftentimes, researchers use OVX animal models, to reduce variability in their outcome measures, however, the estrous cycle is essentially suspended once the subjects are chronically treated with hormonal contraceptives, thus variability

should not be much of a concern. Nonetheless, our findings in our AD animals are, in part, supported by the conflicting literature, although they utilized postmenopausal models (Paganini-Hill and Henderson, 1996; Tang et al., 1996; Kawas et al., 1997; Hogervorst et al., 2000; Zandi et al., 2002; Maki et al., 2005; Pike et al., 2017). Our contribution to the field is one of the first investigations of the effects of this specific progestin, in an intact WT and AD model.

Not only are women victims to an increased AD burden, but they also have an increased stroke burden, thus there is an additional, urgent need to understand the risk factors, in stroke, that are unique to women. The vast majority of strokes occur in men and women over the age of 65, however nearly 15% of all strokes affect individuals age 45 and younger. Additionally, the narrow focus on the effects of estrogen-related compounds, and paucity of research addressing the potential effects of synthetic progestin compounds, in non-menopausal models, transcends beyond the AD field. The neuroprotective efficacy of estrogen-related compounds is well-established in the stroke field. Thus, the findings from this project can also be applied to acute neurological disease conditions, like stroke, and promote further investigation of the effects of synthetic progestins on post-stroke outcomes.

Many studies have demonstrated that the upregulation of MMPs, specifically MMP-9 levels post-stroke, is strongly correlated with blood-brain barrier (BBB) disruption, increased infarct volumes, and poor post-stroke outcomes (Asahi et al., 2000; Asahi et al., 2001; Rosell et al., 2005; Sotgiu et al., 2006; Vukasovic et al., 2006; Moldes et al., 2008; Sandoval and Witt et al., 2008; Hu et al., 2009; Wang et al., 2009; Li et al., 2013).

Additionally, the literature suggests MMP-9 inhibition is neuroprotective against changes in BBB permeability (Svedin et al., 2007) and highlights the prominent role MMP-9 plays at the BBB post-stroke (Dejonckheere et al., 2011). MMP-9 knockout animals have been shown to have improved post-stroke outcomes (reductions in infarct volume, cerebral edema, and functional deficits). Clinical data also reveals that MMP-9 is increased following stroke in humans and is strongly correlated with worse post-stroke outcomes (Montaner et al., 2001; Copin et al., 2005; Ning et al., 2006; Lucivero et al., 2007; Rosell et al., 2008). Regarding the mechanism by which these effects occurs, the field debates over whether resident brain cells, circulating immune cells, or cells within the vasculature are responsible for the increased expression of MMP-9 post-stroke. Turner et al., (2016) hypothesizes that neutrophils are primary source of MMP-9 and once ischemic injury occurs, MMP-9 transmigrates into the tissue and, in turn, degrades the ECM and disrupts the BBB, consequently promoting the secretion of MMP-9 from neurons and microglia. The precise mechanism must be further elucidated.

Of interest, is the disparity between men and women in disability and recovery following a stroke. According to the Framingham Heart Study, women were more vulnerable to more severe post-stroke outcomes (Petrea et al., 2009). Even though women are known to experience more debilitating strokes and suffer from a lower quality of life post-stroke (Gargano et al., 2007; Martínez-Sánchez et al., 2011a,b), previous research falls short of addressing post-stroke outcomes prior to menopause and fail to specifically evaluate the effects of MPA on post-stroke outcomes, during a woman's reproductive prime. For example, the Women's Health Initiative (WHI) suggests that oral therapy with

combined equine estrogen (CEE) alone or in combination with MPA (CEE/MPA) are risk factors for ischemic stroke in healthy postmenopausal women (Wassertheil-Smoller et al., 2003; Anderson et al., 2004). Littleton-Kearney et al., evaluated the consequences of long-term oral administration of CEE alone or in combination with MPA on infarct volumes and observed reduced cortical lesion volume after 2-months of treating OVX rats with either CEE or CEE/MPA. In Chapter 3, we found that MPA promoted nearly a 2-fold increase of MMP-9 protein levels in the cortex of our WT animals. Theoretically, if the baseline level of MMP-9 is already elevated prior to an ischemic insult, relative to control, this would exacerbate the deleterious effects of MMP-9, post-stroke. The literature states baseline differences between men and women (age, comorbidities, severity, and pre-stroke disability) explains the increased mortality rate in women, thus hormone use could additionally impact post-stroke outcomes. Future research should aim to determine whether MPA worsens post-stroke outcomes, via the upregulation of MMP-9.

Overall, my dissertation project compliments the existing literature which highlights MPA's limited neuroprotective efficacy. Additionally, my dissertation project suggests there may be additional exogenous hormone treatments that, when chronically used, have adverse effects on the brain. The more we understand the long-term effects of progestins on the brain, the more researchers can address the critical knowledge gaps in the literature, and further elucidate if exogenous hormones pose a sex-specific risk factor for cognitive dysfunctions in women. Due to the discrepancies and knowledge gaps within the pre-clinical and clinical literature, further work on the influence of synthetic progestins on brain function and AD risk is warranted as a potential sex-specific risk factor until a consensus is

reached. However, we are hoping our work serves as a pilot study to increase awareness and merit further exploration. Until we elaborately examine the influence of exogenous hormones on the sex and gender differences within AD, the field will never be as productive as it has the potential to be. Addressing the differences between women and men benefits everyone. Proper diagnosis becomes difficult due to differences in symptoms, while management of the disease become challenging due to differences in the responses to treatment. By focusing on sex- and gender-specific differences and developing appropriate strategies for clinical studies, we can continue to fill the current knowledge gaps in the field, ultimately improving health outcomes for both women and men.

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