Age Related Changes in Cognition and Brain:

A Focus on Progestogens

by

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ABSTRACT

Cognitive function declines with normal age and disease states, such as Alzheimer's disease (AD). Loss of ovarian hormones at menopause has been shown to exacerbate age-related memory decline and may be related to the increased risk of AD in women versus men. Some studies show that hormone therapy (HT) can have beneficial effects on cognition in normal aging and AD, but increasing evidence suggests that the most commonly used HT formulation is not ideal. Work in this dissertation used the surgically menopausal rat to evaluate the cognitive effects and mechanisms of progestogens proscribed to women. I also translated these questions to the clinic, evaluating whether history of HT use impacts hippocampal and entorhinal cortex volumes assessed via imaging, and cognition, in menopausal women. Further, this dissertation investigates how sex impacts responsiveness to dietary interventions in a mouse model of AD. Results indicate that the most commonly used progestogen component of HT, medroxyprogesterone acetate (MPA), impairs cognition in the middle-aged and aged surgically menopausal rat. Further, MPA is the sole hormone component of the contraceptive Depo Provera, and my research indicates that MPA administered to young-adult rats leads to long lasting cognitive impairments, evident at middle age. Natural progesterone has been gaining increasing popularity as an alternate option to MPA for HT; however, my findings suggest that progesterone also impairs cognition in the middle-aged and aged surgically menopausal rat, and that the mechanism may be through increased GABAergic activation. This dissertation identified two less commonly used progestogens,

norethindrone acetate and levonorgestrel, as potential HTs that could improve cognition in the surgically menopausal rat. Parameters guiding divergent effects on cognition were discovered. In women, prior HT use was associated with larger hippocampal and entorhinal cortex volumes, as well as a modest verbal memory enhancement. Finally, in a model of AD, sex impacts responsiveness to a dietary cognitive intervention, with benefits seen in male, but not female, transgenic mice. These findings have clinical implications, especially since women are at higher risk for AD diagnosis. Together, it is my hope that this information adds to the overarching goal of optimizing cognitive aging in women.

DEDICATION

I would like to dedicate my dissertation to three people: my mom, Marian Braden, my dad, Dr. Bill Braden, and Keley Rose Schaefer. My parents have provided me with a lifetime of support, from never failing to answer late-night, stressed-out phone calls to faithfully delivering a rent check every month. For as long as I can remember, there's never been anything that I didn't think I could accomplish if I gave it my all. I owe that faith in myself entirely to them. Finally, the love that I received from Keley Schaefer is the most beautiful gift I could have ever hoped for. It sustained me every day throughout the completion of this dissertation and I have no doubt it will sustain me for the rest of my life.

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CHAPTER 1

GENERAL INTRODUCTION

Cognitive Aging and Alzheimer's Disease

By the year 2050 there will be an estimated 90 million people in the United States who are over the age of 65 (U.S. Census Bureau, 2007). Many of these individuals will experience cognitive decline as aging ensues and some will develop Alzheimer's disease (AD). Indeed, aging is the number one risk factor for developing AD (Alzheimer's Association, 2010). Currently, there are more than 5 million people in the U.S. diagnosed with AD and that number is expected to more than double by the year 2050 (Alzheimers Association, 2010). Despite the rapidly growing prevalence of this disease, there are still no demonstrably effective treatments to reverse or halt the pathology or symptoms.

Alzheimer's Disease: Sex Differences and Dietary Interventions

As women age, they are at a higher risk to develop AD than men (Andersen et al., 1999). Some evidence suggests that loss of ovarian hormones at menopause puts women at a greater risk for AD (Solerte et al., 1999) .Indeed, estrogen-containing HT has been shown to reduce AD risk (Zandi et al., 2002) and enhance cognition in postmenopausal women with AD (Asthana et al., 1999). However, the largest study to date evaluating the ability of HT to reduce risk of AD found that one type of hormone therapy (HT) regimen, Prempro, actually increased the risk of AD in post-menopausal women (Shumaker et al., 2003). Chapters 2 through 5 in this dissertation evaluate the cognitive effects of a primary hormone component in Prempro, medroxyprogesterone acetate (MPA), in

the surgically menopausal aged rodent model, and Chapter 6 assesses the effects of HT on cognition and brain volumes in menopausal women.

Another potential risk factor for AD is diet. Several epidemiological studies have demonstrated that nutrition is an important factor in susceptibility to AD (Morris, 2009), and in animal models of AD, there is support that a multitude of nutritional interventions can modulate disease-related cognitive decline and pathology (Frydman-Marom et al., 2011; Joseph et al., 2003; J. W. Lee et al., 2009; Wang et al., 2008). However, in AD patients, there is only modest evidence that single nutrients can have favorable outcomes on cognition, while others fail to demonstrate any benefits (Kamphuis & Scheltens, 2010). One potential shortcoming of human trials evaluating dietary intervention as a potential AD treatment is the focus on single-nutrient treatments. Chapter 7 in this dissertation addresses whether or not a combination of multiple nutrients can have positive effects on cognition and brain markers in animal models of AD, and if these effects interact with sex.

Menopause, Hormone Therapy, and Cognition

Within our aging population, that is estimated to include 90 million people in the United States who are over the age of 65 by the year 2050, over half will be postmenopausal women (U.S. Census Bureau, 2007). Menopause, occurring typically in the fifth decade of life, is characterized by loss of ovary-derived circulating hormones, including estrogen and progesterone (Timaras, Quay, & vernadakis, 1995). Menopause-induced hormone loss has been linked to many symptoms that affect quality of life in women including hot flashes, urogenital

atrophy, and memory decline (Freedman, 2002; Nappi et al., 1999; Sherwin, 1988). HT is given to women to attenuate menopause-induced symptoms.

Several clinical studies in menopausal and postmenopausal women have demonstrated positive effects of estrogen-containing HT on memory and cognition (Campbell & Whitehead, 1977; Duka, Tasker, & McGowan, 2000; Kantor, Michael, & Shore, 1973; Ohkura et al., 1995; Phillips & Sherwin, 1992; Sherwin, 1988; Wolf et al., 1999). However, recently the cognitive effectiveness of HT has been of much debate, due to the unexpected findings of the large, placebo-controlled, randomized Women's Health Initiative Memory Study (WHIMS) conducted by the National Institute of Health. Post-menopausal women taking Premarin (estrogens) alone did not differ significantly from those taking placebo for dementia diagnoses (Shumaker et al., 2004). In contrast, twice as many women receiving Prempro (estrogens + a progestin) were diagnosed with dementia as compared to the placebo group, a significant effect (Shumaker et al., 2003).

Hormone Therapy and Mesial Temporal Lobe Structure Volumes

The hippocampus is a brain structure intimately involved in learning and memory, and loss of hippocampal volume is a hallmark brain change seen in AD progression (Buckner et al., 2005; Scahill, Schott, Stevens, Rossor, & Fox, 2002) which is related to cognitive functioning (Jack et al., 2000; Jack et al., 1999). Hippocampal volumes have also been shown to be modulated by HT. In the WHIMS trial, a subset of women underwent a follow-up MRI regional volumes study, including evaluation of the hippocampus, and results were consistent with

HT-associated changes in dementia risk. Specifically, Premarin (conjugated equine estrogens) treatment led to no significant changes in hippocampal volumes, compared to placebo (p=.18); however, in women receiving Prempro (conjugated equine estrogens + the progestin MPA), there was a marginally significant effect (p=.09), with HT-treated women having smaller hippocampi than placebo (Resnick et al., 2009). These findings are in disagreement with other trials that have shown estrogen-containing HT to prevent hippocampal volume loss in a mix of healthy and demented women (Eberling et al., 2003) and women at genetic risk for AD (Yue et al., 2007). Multiple factors seem to modulate whether or not HT is associated with a protection or exacerbation of hippocampal volume loss, such as the interval between menopause and HT initiation (e.g., critical window hypothesis) (Erickson, Voss, Prakash, Chaddock, & Kramer, 2010) and current versus past HT use (Boccardi et al., 2006; Lord, Buss, Lupien, & Pruessner, 2008).

The entorhinal cortex is a brain structure heavily implicated in AD progression (Braak & Braak, 1991; Hyman, Van Hoesen, Damasio, & Barnes, 1984; Hyman et al., 1984; Braak and Braak, 1991) and cognitive performance in normal individuals (Fernandez, Brewer, Zhao, Glover, & Gabrieli, 1999), but much less studied for responsiveness to HT-induced atrophy protection. However, one voxel-based morphometry MRI study did evaluate HT effects on entorhinal cortex volumes and found that HT use was associated with larger entorhinal cortex volumes, as compared to non-users (Boccardi et al., 2006). Chapter 6 examined the ability of continuous or discontinuous HT to alter volumes of the

hippocampus and entorhinal cortex, as well as cognitive function, in post-menopausal women.

Progestins and Memory: MPA

Premarin, a complex combination of horse-derived estrogens, is the most widely used estrogenic component of HT (Hersh, Stefanick, & Stafford, 2004). Women with a uterus that are taking estrogens must include a progestin in their regimen because of increased risk of endometrial hyperplasia associated with unopposed estrogen treatment (Smith, Prentice, Thompson, & Herrmann, 1975; Ziel & Finkle, 1975). Prempro is the most widely prescribed progestin-containing HT in the United States, with an estimated 20 million prescriptions written per year within the last decade (Hersh et al., 2004).

The progestin, MPA, may be a key factor in Prempro that induced cognitive impairments in the WHIMS trials, although many variables could be involved in this negative outcome (Sherwin, 2005). Until recently, there had been no study directly testing the hypothesis that MPA is detrimental to cognition in women or an animal model, however, there is indirect evidence that MPA is detrimental to the brain and its function. Indeed, MPA exacerbated neuronal death by glutamate-induced excitotoxicity (Nilsen, Morales, & Brinton, 2006), reduced estrogen-mediated neural protection against excitotoxicity (Nilsen & Brinton, 2002b), and completely blocked the glutamate-stimulated calcium increase produced by 17 β-estradiol, a positive mechanism by which estrogens may modulate cognitive functioning (Nilsen & Brinton, 2002a). Chapter 2 tested the

cognitive effects of MPA in the surgically menopausal aged rat, and Chapters 3, 4b, and 5b tested these effects in the surgically menopausal middle-aged rat.

MPA is also the sole hormone component of the contraceptive Depo Provera. Depo Provera was approved by the Food and Drug Administration in 1992 (Bakry et al., 2008), and has been prescribed to over 10 million women in the United States since this time (Mosher, Martinez, Chandra, Abma, & Willson, 2004). This widely prescribed contraceptive is often the choice for women who cannot take estrogen-containing contraceptives because of medical conditions (Spencer, Bonnema, & McNamara, 2009), for nursing mothers (Rodriguez & Kaunitz, 2009), and for those who prefer the convenience of an intermittent injection once every three months over the daily pill. There have been no methodical clinical evaluations of the cognitive effects of Depo Provera in women; however, there is a documented case study of amnestic effects corresponding with Depo Provera use (Gabriel & Fahim, 2005). Chapter 3 studied the long-term cognitive effects of MPA administration, giving MPA to young ovary-intact rats and then testing at the middle-age time point, with or without MPA given at middle-age as well. This addressed how MPA treatment during young-adulthood could interact with subsequent MPA treatment at middle-age.

Progestins and Memory: NETA and LEVO

With evidence mounting that MPA is not the optimal progestin to be used in HT and birth control taken by women, it is important to determine if there are safer progestins, with respect to cognition. Based on chemical structures, synthetic progestins either structurally resemble progesterone or testosterone,

considering the A-ring and the functional group in the 17 beta position of the steroidal skeleton (Brinton et al., 2008; Stanczyk, 2003). Progesterone-related drugs, including acetylated (e.g., MPA; Figure 12b) and non-acetylated pregnans and 19-norpregnans, respectively, are commonly used for HT and contraception in the USA. The majority of testosterone- and especially 19-nortestosterone-resembling progestins contain an ethynyl group in the 17 alpha position with (e.g., norethindrone acetate, NETA, Figure 12c) or without acetylation of the 17 beta hydroxyl group. A homologous subcategory within this series consists of those progestins that carry a C-13 substitution with an ethyl group (Stanczyk, 2003). These progestins are often referred to as 13-ethyl gonanes, and a typical representative of this subgroup of progestins is levonorgestrel (LEVO, Figure 12d), a popular choice of progestin in Europe (Brinton et al, 2008).

MPA is in the acetylated progesterone derivative category. When exploring progestins not in the acetylated progesterone derivative category, the two most commonly used choices for HT and birth control are NETA from the ethynylated 19-nortestosterone family, and LEVO from the homologous 17 alpha ethynyl nortestosterone ("13-ethyl gonane") series (P. A. Murphy & Brixner, 2008; Curtis, 2006). There have been limited scientific investigations for HT regarding the effects of NETA, paired with ethinyl- or 17β- estradiol, on cognition and brain processes in healthy menopausal women. In these studies the combination HT was associated with greater brain activation during a memory test (Persad et al., 2009; Smith et al., 2006), protection from memory decline after one and two years of treatment (Tierney et al., 2009), or no impact on self-report

of memory and concentration difficulties (Gambacciani et al., 2003). Chapters 4a and 4b tested the cognitive effects of NETA at different doses and treatment parameters in the surgically menopausal middle-aged rat.

There have been no scientific investigations of the effects of LEVO on cognition, neither when included as a part of combination therapy in menopausal women, nor when given alone in rodents, leaving little information to hypothesize the safety of this progestin on the brain and its function. However, differences in chemical structure from the cognitively-impairing progestin, MPA, and its global use, make LEVO a worthy candidate for scientific studies evaluating this endpoint. Chapter 4b tested the cognitive effects of LEVO in the surgically menopausal middle-aged rat.

Progesterone and Memory

In both clinical and preclinical studies, natural progesterone has been associated with detrimental cognitive effects. In healthy women, a large oral progesterone dose is detrimental to memory (Freeman, Weinstock, Rickels, Sondheimer, & Coutifaris, 1992). In aged rats, ovariectomy (Ovx) improves cognition (Bimonte-Nelson, Singleton, et al., 2003), which is likely related to removal of elevated progesterone levels (Lu, Hopper, Vargo, & Yen, 1979), since progesterone administration reverses the beneficial effects of Ovx (Bimonte-Nelson, Singleton, Williams, & Granholm, 2004). However, the literature on progesterone effects on cognition in young Ovx rodents is mixed, with some studies showing benefits, mostly related to non-spatial memory (Frye, Duffy, & Walf, 2007; Frye & Walf, 2008; Harburger, Pechenino, Saadi, & Frick, 2008; Orr,

Lewis, & Frick, 2009). When combined with 17 β-estradiol, progesterone abolishes 17 β-estradiol-induced memory enhancements (Bimonte-Nelson, Francis, Umphlet, & Granholm, 2006; Gibbs, 2000; Harburger, Bennett, & Frick, 2007; but see Gibbs, 2000) and attenuates 17 β-estradiol's neurotrophic effects in vivo (Bimonte-Nelson, Nelson, & Granholm, 2004), and in cell culture (Aguirre & Baudry, 2009; Nilsen & Brinton, 2002a). Further, administration of the progesterone metabolite, allopregnanolone, can impair cognition in healthy women (Kask, Backstrom, Nilsson, & Sundstrom-Poromaa, 2008) and young rats (Frye & Sturgis, 1995; Johansson, Birzniece, Lindblad, Olsson, & Backstrom, 2002). Chapter 2 aimed to replicate the cognitive impairing effects of progesterone in aged Ovx rats, and Chapter 5a tested these effects in the middle-aged Ovx rat.

Progestogens and GABA

Because the ring-A reduced metabolites of progesterone have a high affinity for the $GABA_A$ receptor, and several are potent positive allosteric modulators (Paul & Purdy, 1992), it is a reasonable hypothesis that progesterone-induced memory impairments are related to GABAergic system alterations. Providing support for this hypothesis, experimental manipulation of progesterone alters the GABAergic system. For example, progesterone administration decreases glutamic acid decarboxylase (GAD), the synthesizing enzyme and rate limiting step of GABA production, activity in the rodent dorsal hippocampus (Wallis & Luttge, 1980), and can alter mRNA expression of subunits of the $GABA_A$ receptor in the hippocampus of Ovx rats (Pazol, Northcutt, Patisaul,

Wallen, & Wilson, 2009; Weiland & Orchinik, 1995). Further, MPA has been shown to enhance $GABA_A$ receptor-mediated inhibition (Belelli & Herd, 2003), possibly by alter progesterone's metabolic conversions (Penning, Sharp, & Krieger, 1985). Chapters 2 and 3 tested the effects of progesterone and MPA administration on protein levels of GAD in cognitive brain regions. Chapter 5 directly tested the hypothesis that progesterone- (Chapter 5a) and MPA- (Chapter 5b) induced memory impairments are via activation of the $GABA_A$ receptor. This was done via pharmacologically manipulating the GABAergic system, by treating middle-aged Ovx animals concomitantly with progesterone or MPA plus the $GABA_A$ antagonist, bicuculline. Chapter 5a also tested the effects of progesterone, MPA, and/or bicuculline on number of GABA-producing cells in the hippocampus.

Progestogens and Neuronal Health

While we have shown that progesterone impairs cognition in aged rats (Bimonte-Nelson et al., 2004b) and reverses the cognitive benefits of 17β-estradiol in middle-age rats (Bimonte-Nelson et al., 2006), in young rodent models of cortical contusion, progesterone improves cognitive performance (Roof, Duvdevani, Braswell, & Stein, 1994), and reduces oedema and neuronal degeneration (Roof et al., 1994; Roof, Duvdevani, Heyburn, & Stein, 1996; Roof, Duvdevani, & Stein, 1993). Further, progesterone modulates cyclooxygenase-2 (Cox-2), a key enzyme in the formation of prostaglandins in the inflammatory response that is found in the cortex and other subcortical structures in the rat brain (Breder, Dewitt, & Kraig, 1995), and induced in glutamate excitotoxicity (Tocco

et al., 1997). One study found that progesterone decreased Cox-2 expression in the frontal cortex following cortical contusion (Cutler et al., 2007), while another found that progesterone increased Cox-2 expression in response to lipopolysaccharide (LPS)-induced cerebrovascular inflammation (Sunday, Tran, Krause, & Duckles, 2006). MPA has also been tested in these models of neuroprotection, wherein others have found that MPA reduces cerebral oedema but does not protect against cognitive impairments (Wright, Hoffman, Virmani, & Stein, 2008), and exacerbates Cox-2 expression in response to LPS-induced cerebrovascular inflammation (Sunday et al., 2006). Indeed, on global measures of neuroprotection in cell culture, progesterone protects against glutamate-induced excitotoxicity (Nilsen & Brinton, 2002b). Conversely, MPA does not seem to demonstrate neuroprotective properties in vitro (Nilsen & Brinton, 2002b), but in fact exacerbates glutamate- induced excitotoxicity (Nilsen et al., 2006). Further, MPA results in a greater attenuation of 17 β-estradiol's neurotrophic actions than natural progesterone (Nilsen & Brinton, 2002a). Chapter 5 tested the effects of progesterone (Chapter 5a) and MPA (Chapter 5b) treatment on number of Cox-2-positive cells in the frontal cortex, in middle-aged Ovx rats.

Neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), is another system that is influenced by progesterone, and is involved in neuronal function and age-related memory changes (Backman et al., 1996). Progesterone counteracts estrogen-induced increases in entorhinal and frontal cortex neurotrophin levels in aged Ovx rats (Bimonte-Nelson, Nelson,

et al., 2004). This same effect is seen in hippocampal slice cultures for BDNF (Aguirre & Baudry, 2009); however, in cerebral cortex slice cultures, progesterone treatment alone increases BDNF levels (Kaur et al., 2007). It has yet to be determined whether progesterone or MPA impacts neurotrophin levels in vivo, and if these changes are related to the cognitive impairing effects of progestogens. Chapter 2 tested the effects of progesterone and MPA treatment on BDNF levels in cognitive brain regions of aged Ovx rats that were cognitively characterized.

Work in this dissertation used the surgically menopausal rat to provide insight into the cognitive effects and mechanisms of multiple progestogens clinically used in women. I also translated these questions to the clinic, evaluating whether the history of HT use impacts volumes of cognitive brain structures and memory measures in menopausal women. Further, this dissertation adds insight into how sex impacts responsiveness to dietary interventions in a mouse model of AD. Together, it is my hope that this information adds to the overarching goal of optimizing cognitive aging in women.

CHAPTER 2

MEDROXYPROGESTERONE ACETATE IMPAIRS MEMORY AND ALTERS THE GABAERGIC SYSTEM IN AGED SURGICALLY MENOPAUSAL RATS

By the year 2050 there will be an estimated 90 million people in the United States who are over the age of 65, and over half of these individuals will be postmenopausal women (U.S. Census Bureau, 2007). Menopause, occurring typically in the fifth decade of life, is characterized by loss of ovary-derived circulating hormones, including estrogen and progesterone (Timaras et al., 1995). Menopause-induced hormone loss has been linked to many symptoms that affect quality of life in women including hot flashes, urogenital atrophy, and memory decline (Freedman, 2002; Nappi et al., 1999; Sherwin, 1988). HT is given to women to attenuate menopause-induced symptoms. Premarin, a complex combination of horse-derived estrogens, is the most widely used estrogenic component of HT (Hersh, et al., 2004). Women with a uterus that are taking estrogens must include a progestin in their regimen because of increased risk of endometrial hyperplasia associated with unopposed estrogen treatment (Smith et al., 1975; Ziel and Finkle, 1975). Prempro (Premarin + MPA) is the most widely prescribed progestin-containing HT in the United States, with an estimated 20 million prescriptions written per year within the last decade (Hersh et al., 2004). Further, over 10 million women in the United States have been prescribed MPA as the injectable contraceptive Depo Provera (Mosher et al., 2004). Thus, MPA is widely clinically utilized.

Several clinical studies in menopausal and postmenopausal women have demonstrated positive effects of estrogen-containing HT on memory and cognition (Campbell and Whitehead, 1977; Duka et al., 2000; Kantor et al., 1973; Ohkura et al., 1995; Phillips and Sherwin, 1992; Sherwin, 1988; Wolf et al., 1999). However, recently the cognitive effectiveness of HT has been of much debate, due to the unexpected findings of the large, placebo-controlled, randomized WHIMS conducted by the National Institute of Health. Menopausal women taking Premarin alone did not differ significantly from those taking placebo for dementia diagnoses (Shumaker et al., 2004). In contrast, twice as many women receiving Prempro were diagnosed with dementia as compared to the placebo group, a significant effect (Shumaker et al., 2003).

MPA may be a key factor in Prempro that caused cognitive impairments, although many variables could be involved in this negative outcome (Sherwin, 2005). There has been no study directly testing the hypothesis that MPA is detrimental to cognition in women or an animal model, however, there is indirect evidence that MPA is detrimental to the brain and its function. Indeed, MPA exacerbated neuronal death by glutamate-induced excitotoxicity (Nilsen et al., 2006), reduced estrogen-mediated neural protection against excitotoxicity (Nilsen & Brinton, 2002b), and completely blocked the glutamate-stimulated calcium increase produced by 17 β-estradiol, a positive mechanism by which estrogens may modulate cognitive functioning (Nilsen & Brinton, 2002a). In both clinical and preclinical studies, progesterone has been associated with detrimental cognitive effects. In healthy women, a large oral progesterone dose is detrimental

to memory (Freeman et al., 1992). High circulating progesterone levels are observed in most rats following estropause (reproductive senescence) (Lu et al., 1979). It is noteworthy that Ovx in aged rats improves cognition (Bimonte-Nelson, Singleton, et al., 2003), which is likely related to progesterone removal, since progesterone administration reverses the beneficial effects of Ovx (Bimonte-Nelson et al., 2004b). Additionally, administration of the ring-A reduced progesterone metabolite, allopregnanolone, can impair cognition in healthy women (Kask et al., 2008) and young rats (Frye & Sturgis, 1995; Johansson et al., 2002).

Because the ring-A reduced metabolites of progesterone have a very high affinity for the $GABA_A$ receptor (Paul & Purdy, 1992), we hypothesize that progesterone-induced memory impairments are related to GABAergic system alterations. Providing support for this hypothesis, the hippocampus and related brain regions affected by aging and mediating memory processing are largely controlled by the GABAergic system (Izquierdo et al., 1993; Mora, Segovia, & del Arco, 2007), and experimental manipulation of progesterone alters the GABAergic system. For example, progesterone administration decreases GAD, the synthesizing enzyme and rate limiting step of GABA production, activity in the rodent dorsal hippocampus (Wallis & Luttge, 1980). Additionally, after 17 β-estradiol priming, progesterone but not MPA decreased mRNA expression of the α4 subunit of the $GABA_A$ receptor in the CA1 of Ovx rats (Pazol et al., 2009). Indeed, MPA results in different ring-A reduced metabolites (dihydroMPA and tetrahydroMPA) that do not influence binding at the benzodiazepine site of the

$GABA_A$ receptor as the progesterone ring-A reduced metabolites do, as seen with allopregnanelone (McAuley, Kroboth, Stiff, & Reynolds, 1993).

Neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), is another system that is influenced by progesterone, is involved in neuronal function and in age-related memory changes (Backman et al., 1996). Progesterone counteracts 17β-estradiol-induced increases in entorhinal and frontal cortex neurotrophin levels in aged Ovx rats (Bimonte-Nelson et al., 2004a). This same effect is seen in hippocampal slice cultures for BDNF (Aguirre & Baudry, 2009); however, in cerebral cortex slice cultures, progesterone treatment alone increases BDNF levels (Kaur et al., 2007). Thus, alteration of neurotrophin levels is a neuronal mechanism by which progesterone-induced memory changes may be mediated.

While studies suggest that progesterone has a profound impact on GABAergic and neurotrophic systems, as well as cognition, these findings cannot be extrapolated to effects of the synthetic progesterone, MPA. Given that MPA is the progestin component in Prempro, the most widely used combination HT, the negative in vitro findings regarding this hormone are particularly salient. Methodical investigations are warranted to determine whether these detrimental in vitro effects translate to brain functions such as learning and memory. The goal of the present study was to determine whether MPA exerts detrimental effects on the brain and its function. To do this, we tested the cognitive, GABAergic, and neurotrophic effects of MPA in the aged surgically menopausal rat, comparing effects to natural progesterone and vehicle control Ovx and Sham animals.

Methods

Subjects

Subjects were 37 eighteen month-old Fischer-344 female rats born and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). Rats were acclimated for several weeks before surgery, had access to food and water ad-lib, and were maintained on a 12-hour light/dark cycle at the Arizona State University animal facility. All procedures were approved by the local IACUC committee and adhered to NIH standards.

Ovariectomy and Hormone Treatment

Rats were randomly assigned to one of five treatment groups: Sham (ovary-intact), Ovx, Ovx+Prog, Ovx+Low MPA, and Ovx+High MPA. Approximately two months before behavioral testing, all rats received Ovx or sham surgery. All rats were anesthetized via isofluorene inhalation. Rats receiving Ovx underwent bilateral dorsolateral incisions in the skin and peritoneum, and the ovaries and tips of uterine horns were ligatured and removed. Muscle and skin were then sutured. Rats receiving sham surgery underwent identical skin incision and suture. At the time of surgery, Alzet osmotic pumps (2ML4; Durect Co., Cupertine, CA) containing either proplyene glycol (vehicle, Sigma-Aldrich, St. Louis, MO, USA), progesterone (PROG; 21mg dissolved in 2mL propylene glycol, Sigma-Aldrich, St. Louis, MO, USA), or MPA (low dose: 14mg; high dose: 21mg, dissolved in 2mL propylene glycol (Sigma-Aldrich, St. Louis, MO, USA)) were implanted in the neck scruff. Hormone administration continued throughout behavior testing and sacrifice. Doses were based on prior research

(Zhang, Fishman, & Huang, 1999), multiplied by a factor of 10 to account for the increased weight from the mouse to the rat. After surgery, rats received Rimadyl (5mg/mL/kg) for pain and saline (2 mL) to prevent dehydration. Animals underwent pump reinsertion surgery every 31-32 days; behavioral testing began 66 days after the first pump insertion. Thus, hormone administration continued throughout behavior testing and sacrifice.

Vaginal Smears and Uterine Weights

Vaginal smears were taken 16 days after Ovx and pump insertion. Smears were classified as proestrus, estrous, metestrus or diestrus, per prior protocols (Acosta, Mayer, Talboom, Zay, et al., 2009; Goldman, Murr, & Cooper, 2007). As expected, all Ovx animals, regardless of treatment, showed leukocytic smears, while sham animals showed one of the four phases of the estrous cycle. To examine drug effects on uterine tissues, at sacrifice the uteri of all subjects were removed, trimmed of visible fat and immediately weighed (wet weight).

Water Radial-Arm Maze (WRAM)

Subjects were tested for 13 days on the eight-arm win-shift WRAM to evaluate spatial working and reference memory, including performance as working memory load increased, as described previously (Bimonte & Denenberg, 1999, 2000; Bimonte, Granholm, Seo, & Isacson, 2002; Bimonte, Hyde, Hoplight, & Denenberg, 2000). The maze contained escape platforms hidden under the water surface in the ends of 4 of the 8 arms. Each subject had different platform locations that remained fixed throughout the experiment. A subject was released from the start arm and had 3 minutes (m) to locate a platform. Once a platform

was found, the animal remained on it for 15 seconds (s), and was then returned to its heated cage for a 30s inter-trial interval (ITI) until its next trial. During the interval, the just-chosen platform was removed from the maze. The animal was then placed again into the start alley and allowed to locate another platform. For each animal a daily session consisted of four trials, with the number of platformed arms reduced by one on each subsequent trial. Thus, the working memory system was increasingly taxed as trials progressed, allowing us to access working memory load. Each subject was given one session a day for 12 consecutive days.

Quantification and blocking were based on prior studies (Bimonte & Denenberg, 2000; Bimonte et al., 2002; Bimonte et al., 2000; Hyde, Hoplight, & Denenberg, 1998; Hyde, Sherman, & Denenberg, 2000). An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Errors were quantified using the orthogonal measures of working and reference memory errors (Jarrard, Okaichi, Steward, and Goldschmidt, 1984), as done previously in WRAM studies (Bimonte et al., 2002; Bimonte et al., 2000; Hyde et al., 2000). Working Memory Correct (WMC) errors were the number of first and repeat entries into any arm from which a platform had been removed during that session. Trial 1 is not analyzed for WMC errors because no platform has yet been removed. Reference memory (RM) errors were the number of first entries into any arm that never contained a platform. Working Memory Incorrect (WMI) errors were repeat entries into a RM arm.

Morris Maze (MM)

The MM was tested for 6 trials/day for 5 days using a tub (188 cm diameter) filled with black water made opaque using non-toxic paint. A hidden platform (10 cm wide) remained in a fixed location, thereby testing spatial reference memory (Bimonte-Nelson et al., 2006; R. G. Morris, Garrud, Rawlins, & O'Keefe, 1982). The rat was placed in the maze from the North, South, East, or West location, and had 60s to locate the hidden platform (10 cm wide), which remained in a fixed location (Northeast quadrant) throughout testing. Once the rat found the platform the trial was terminated. After 15s platform time, the rat was placed into its heated cage until its next trial. The approximate ITI was 15m. To evaluate whether rats localized the platform to the spatial location, after all test trials on day 5, a 60s probe trial was given whereby the platform was removed. For each trial, a camera suspended above the maze tracked each rat's path and a tracking system (Ethovision 3.1, Noldus Instruments) analyzed each rat's tracing.

MM performance was assessed by swim path distance (cm) and latency (s) to the platform. For probe trial data, percent of total distance in the previously platformed (target) quadrant was compared to the quadrant diagonally opposite the platform. Rats that learned the platform location were expected to spend the greatest percent distance in the target quadrant (Stavnezer, Hyde, Bimonte, Armstrong, & Denenberg, 2002).

Visible Platform Maze

Since the MM and WRAM rely on spatial navigation, it was necessary to confirm that all subjects had intact vision and could perform the procedural task

components without difficulty. A visible platform (VP) water-escape task was used in this regard. A rectangular tub (39 x 23 in) was filled with clear water and a black platform (10 cm wide) with a white flag (2 x 3 in) was elevated above the water surface. Opaque curtains covered extramaze cues. The drop off location remained the same across trials, and the platform location for each trial varied in space semi-randomly. Animals had to locate the flagged platform protruding from the water, and were given 8 trials/day for 2 days. Performance was assessed by latency (s) to the platform.

Brain Dissections

Two days after conclusion of behavior testing, animals were anesthetized, decapitated and brains rapidly dissected and frozen. Dissected tissues were stored in preweighed microcentrifuge tubes at -70°C until analysis. Dissections were according to plate designations in Paxinos and Watson (1998) and were as follows: frontal cortex (plates 5-14), cingulate cortex (plates 5-14), basal forebrain (plates 14-16), perirhinal cortex (plates 39-42), entorhinal cortex (plates 39-42), and CA1/CA2 region of the dorsal hippocampus (plates 33-35). For each brain the frontal cortex was taken first from the dorsal aspect of the intact brain. Next, the cingulate cortex was taken with the longitudinal fissure as the medial border, and the medial border of the frontal cortex cut as the lateral border. Next, the brain was cut in the coronal plane to obtain access to the basal forebrain. For the basal forebrain, both medial septum and ventral diagonal band were included with the posterior landmark being the crossing point of the anterior commissure. The brain was then cut in the coronal plane to obtain access to the last three brain regions.

For the CA1/CA2 region of the hippocampus, the dentate gyrus and the alveus were excluded. For the entorhinal cortex, the tissue was dissected from the same slice as the hippocampal sample, taking a 2- to 3-mm sample ventral to the hippocampus. The perirhinal cortex was also collected from this same slice, taking a 2- to 3-mm sample around the perirhinal fissure.

Western Blot Analyses of GAD 65+67

GAD 65+67 protein expression levels were analyzed in frontal, cingulate, perirhinal, and entorhinal cortices, and hippocampus, from the right hemisphere, and the basal forebrain. Samples were sonicated in RIPA buffer (150mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na Deoxycholate, 50mM Tris) equivalent to 10 times the weight of the sample and centrifuged at 10,000 RPM for 10m at 4°C. Protein concentrations were determined using BCA protein assays (ThermoFisher Scientific, Pittsburgh, PA, USA) and 10 μg of protein from each sample was run on a NuPAGE Bis-Tris gel, using the SureLock mini-cell (Invitrogen, Carlsbad, CA, USA). Gels were counterbalanced by group and each region was run on two gels. Approximately half of each group was loaded onto a single gel, corresponding to 2-4 subjects from each treatment group. Proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA, USA) and immunoblotting was performed with working dilutions of a rabbit anti-GAD 65+67 (ab11070) and rabbit anti-beta Actin (ab25894) primary antibodies (Abcam Inc., Cambridge, MA, USA). Antibody dilutions were 1:10,000 for GAD 65+67 and 1:2,000 for beta Actin primary antibodies. Membranes were then exposed to a peroxidase-conjugated goat anti-rabbit secondary antibody, 1:10,000

dilution (111-035-003; Jackson Immuno Research, West Grove, PA, USA) and visualized using Pierce ECL Western Blotting Substrate (ThermoScientific, Rockford, IL, USA) on a Biospectrum Biochemi 500 Imaging System (UVP, Upland, CA, USA). Bands were identified as the protein of interest, based on molecular weight, using Precision Plus Protein WesternC Standards (Bio Rad, Hercules, CA, USA). The density of GAD 65+67 and beta Actin (control protein) were then quantified using ImageJ software (Rasband, 1997-2004). The dependent measure was a proportion of each subject's GAD 65+67 density to their beta Actin density, brought to percent control of Ovx subjects run on the same gel, per prior protocols (Pandey, Zhang, Mittal, & Nayyar, 1999).

Growth Factor ELISAs

NGF and BDNF levels were assessed in frontal, cingulate, and entorhinal cortex, and hippocampus, from the left hemisphere. Using Promega kits (Madison, WI), NGF and BDNF can be quantified in the range of 7.8–500 pg/mL, and cross-reactivity with other trophic proteins is < 2–3%. This procedure has been utilized routinely in our laboratory with excellent replicability (Bimonte-Nelson et al., 2004a). In brief, flat-bottom plates were coated with the corresponding capture antibody, which binds the neurotrophin of interest. The captured neurotrophin was bound by a second specific antibody, which was detected using a species-specific antibody conjugated to horseradish peroxidase as a tertiary reactant. All unbound conjugates were removed by subsequent wash steps according to the Promega protocol. After incubation with chromagenic

substrate, color change was measured in an ELISA plate reader at 450nm. The dependent measure was pg of BDNF or NGF/mg tissue.

Blood Serum Analyses for MPA

Liquid-liquid extraction of aliquots (1 mL) from serum samples was performed according to (Kim & Kim, 2001) after adding 10 ng/mL of d9-progesterone (C/D/N Isotopes, Pointe-Claire, Quebec, Canada) as an internal reference compound. MPA concentrations were determined by liquid chromatography–atmospheric-pressure ionization tandem mass spectrometry. A reversed-phase column and isocratic elution were used for chromatographic separation of MPA and internal reference. Measurements were performed on a Surveyor – LTQ-FT system from Thermo Fisher (San Jose, CA). In 13x100 mm glass tubes, aliquots (1 mL) of serum were diluted with identical volumes of potassium phosphate buffer (100 mM, pH 7.0) followed by addition of an internal reference compound (d9-progesterone, 10 µL from an 1-µg/mL solution in ethanol). Pentane (4 mL) was then added and the mixture was vortexed for 1 min. After centrifugation at 1000g for 10 min, the organic layer was removed and the extraction was repeated. The pooled organic extracts were dried under nitrogen stream at room temperature and, then, reconstituted in 100 µL acetonitrile before analysis. MPA concentrations were determined by liquid chromatography–atmospheric-pressure ionization tandem mass spectrometry (LC–APCI-MS/MS). Analytes were separated on a 50 mm x 2.1 mm i.d. Supelco (Bellefonte, PA) Discovery HS C18 reversed-phase column. Isocratic elution was used with a mobile phase of water (22.5% v/v), acetonitrile (77% v/v) and acetic acid (0.5%

v/v) delivered at 0.25 mL/min flow rate by a Surveyor MS pump (Thermo Fisher, San Jose, CA). Injections (6 µL each) were made by an autosampler (Leap Technologies, Carrboro, NC). The entire column effluent was introduced to the APCI source of an LTQ-FT instrument (Thermo Fisher) whose linear ion trap was used to perform MS/MS data acquisitions. Selected-reaction monitoring (SRM) was employed to record MPA and d9-progesterone signals. MPA and d9-progesterone were measured through SRM chromatograms obtained by monitoring the fragments m/z 327 and m/z 306, respectively, which represented fragments of the protonated compounds (m/z 387 for MPA and m/z 324 for d9-progesterone). MPA concentrations were determined by performing calibrations from the analysis of serum samples spiked with 1, 2, 5 and 10 ng/mL of analyte, respectively, and using the added d9-progesterone as an internal reference to compensate for slight changes in extraction efficiency and LC–APCI-MS/MS conditions among samples. The dependent measure was ng/mL of MPA in serum.

Statistical Analyses

For behavior assessments, data were analyzed separately for each maze, first using an omnibus repeated measures ANOVA with Treatment as the between variable and Days and/or Trials as the within variable/s, as appropriate for the specific maze test. This was done to allow interpretation of Days and/or Trials repeated measures effects in the context of potentially complex Treatment interactions, and for these omnibus analyses all Treatment groups were included. Since our interest was to evaluate effects of Ovx, progesterone, and dose-specific MPA administration, two-group planned comparisons were run on the specific

measures of the last WRAM testing day, the delay WRAM testing day, and Morris maze overnight forgetting (described below), noting that Type I error correction is not necessary with orthogonal planned comparisons (Keppel & Wickens, 2004).

For WRAM analyses, for all three orthogonal measures of WMC, RM and WMI, learning across all testing days was evaluated in order to detect Day x Treatment interactions. Performance on the final day of regular testing, Day 12, was evaluated at the highest working memory load (Trial 4), as this has revealed Ovx-induced benefits and progesterone-induced impairments in aged animals in our laboratory (Bimonte-Nelson et al., 2003b; Bimonte-Nelson et al., 2004b). On Day 13, a two hour delay was imposed between trials 2 and 3 to assess memory retention for numerous items of information (i.e., the found platform locations on trials 1 and 2). Treatment effects and Trial x Treatment interactions were analyzed for the post-delay trials (Trials 3 and 4) in order to detect impairing effects of the delay within that day. Errors committed on the post-day trials were also compared to errors on the last day of regular testing, Day 12 (baseline) in order to detect impairing effects of a two hour delay versus the 30s ITI.

For MM, in addition to the learning evaluation across all days, in order to test overnight forgetting of the platform location on the MM, we compared distance scores from the last trial of each day (Trial 6) to the first trial of the following day (Trial 1), collapsed across all testing days, as done previously (Acosta, Mayer, Talboom, Zay, et al., 2009; Bimonte-Nelson et al., 2006; Markham, Pych, & Juraska, 2002). Using a priori two-group planned comparisons we examined

Treatment x Trial interactions for effects of Ovx, and progesterone and MPA administration across the overnight interval (Trial 6 to Trial 1). We also examined within group trial comparisons of the overnight interval (Trial 6 to Trial 1) in order to determine which treatment groups increased their distance swum across the overnight interval, thus showing overnight forgetting of the platform location.

Uterine weight, neurotrophins, and GAD protein levels were assessed via a priori planned comparisons using t-tests. Alpha was set at 0.05, two-tailed, for all analyses except for the Sham vs. Ovx working memory load effect, as we have previously established that Ovx decreases errors on this measure (Bimonte-Nelson et al., 2003).

Results

Water Radial-Arm Maze

There were no Treatment main effects or interactions across the 12 testing days for WMC, WMI, or RM errors.

Working memory load effects

On the final testing day with a 30s ITI (Day 12), for the omnibus ANOVA there was a Treatment x Trial interaction for WMC errors [$F(8, 64) = 2.078$, $p = .05$]; both progesterone and high MPA impaired ability to handle numerous items of information as working memory load increased [Treatment x Trial interaction: Ovx vs. Ovx+Prog: $F(2, 24) = 11.01$; $p < .0005$; Ovx vs. Ovx+High MPA: $F(2, 26) = 8.671$; $p < .005$]. At the highest memory load on Trial 4 there was a Treatment main effect for the omnibus ANOVA [$F(4, 32) = 2.77$; $p < .05$]. As we have shown previously in aged rats, Ovx improved

performance [Sham vs. Ovx: $t(14) = 1.77$; $p < .05$], and Ovx+Prog impaired performance [Ovx vs. Ovx+Prog: $t(12) = 4.483$; $p < .001$] (Figure 1a). High MPA impaired memory on the trial with the highest working memory load as well [Ovx vs. Ovx+High MPA: $t(13) = 3.357$; $p < .01$] (Figure 1a). There was not a significant difference between Ovx+Prog and OVX+High MPA treated animals. Also, there were no group differences for RM or WMI errors.

Delayed memory retention on the WRAM

On Day 13 a two hour delay was given between Trials 2 and 3. There was a Treatment x Trial interaction on Day 13 for WMC errors for the omnibus ANOVA with Trials 2-4 included [$F(8, 64) = 2.540$; $p < .05$]. High MPA impaired performance on Day 13, as compared to OVX [Treatment x Trial interaction including trials 2-4: $F(2, 26) = 6.448$; $p < .01$] (Figure 1b). Further, there was a Treatment x Trial interaction for WMC errors across Trials 3 and 4 for Day 13 [$F(4, 32) = 2.752$; $p < .05$; Ovx vs. Ovx+High MPA $F(1, 13) = 7.626$; $p < .05$], where Ovx+High MPA animals showed an increase in WMC errors across these post-delay trials [$t(9) = 3.109$; $p < .05$], while errors committed by OVX animals remained stable across these post-delay trials (ns) (Figure 1b). There was no effect of low MPA, progesterone or Ovx on Day 13, nor were there treatment group differences for WMI or RM errors.

It is important to note that Ovx+High MPA animals did not commit any more errors after the delay of two hours as they did on the previous baseline day (Day 12) with an ITI of 30 s. Indeed, scores on Day 12 did not differ from scores on Day 13 for Ovx+High MPA animals. Ovx+Prog animals improved

performance from baseline Day 12 to the delay Day 13 [$t(6) = 3.158$; $p < .05$], and were no longer different from Ovx animals (ns), showing no delay induced impairment but instead a continuation of their learning curve. There were also no longer differences between Sham and Ovx groups (ns), again showing no delay-induced impairment.

Morris Maze

There were no Treatment effects for MM performance as measured by Distance or Latency to reach the platform (data not shown). There was a Day main effect for each measure for the omnibus ANOVA [Distance: $F(4, 24) = 12.153$; $p < .0001$ (Figure 2a); Latency: $F(4, 24) = 10.526$; $p < .0001$ (Figure 2b)], with Distance and Latency scores decreasing across days, and no Treatment x Day interaction for either variable. Ovx+High MPA animals showed overnight forgetting, while Ovx animals did not, as evidenced by a Treatment x Trial interaction for the Ovx vs. Ovx+High MPA comparison [$F(1, 12) = 8.526$; $p < .05$]. Distance scores remained stable after the overnight interval in the Ovx group [$t(6) = .307$; $p = .77$], and increased after the overnight interval in the Ovx+High MPA group [$t(6) = 4.059$; $p < .01$] (Figure 2c). There were no interactions between Ovx and Ovx+Prog [$F(1, 10) = 3.166$; $p = .11$], Ovx+Low MPA [$F(1, 9) = 2.417$; $p = .15$], or Sham [$F(1, 11) = 1.01$; $p = .34$] animals, nor was there a significant increase across the overnight interval for any of these groups [Sham: $t(5) = .96$; $p = .38$; Ovx+Prog: $t(4) = 2.02$; $p = .11$; Ovx+Low MPA: $t(3) = 1.32$; $p = .28$], indicating that only the Ovx+High MPA group showed an impaired ability to remember the location of the platform overnight.

Traditional probe analyses comparing distance swum in the target quadrant (NE) to the opposite quadrant (SW) showed no overall Quadrant preference (Quadrant main effect, ns) indicating animals did not localize to the previously platformed spatial location. There was a null Treatment x Quadrant interaction suggesting groups did not differ in this pattern (Figure 2d). Because the probe trial indicated that animals did not localize to the target quadrant, we further assessed probe trial data by analyzing the annuli. Indeed, we and others have shown that animals can use a motoric pattern of circling the middle annulus to locate the platform (Bimonte-Nelson et al., 2006; Stavnezer et al., 2002). This was not the case in the current study, however. Analysis of the probe trial showed an Annulus main effect [$F(2, 24) = 112.785$; $p < .0001$], and no Treatment main effect nor Treatment x Annulus interaction (ns), suggesting that treatments did not differ in annuli preference. Animals showed a preference for the outer annulus when compared to both the inner [$t(24) = 12.766$; $p < .0001$] and middle [$t(24) = 8.898$; $p < .0001$] annuli (figure 2e). This was further confirmed by visual, qualitative inspection of the swim paths during regular testing (figure 2f) and the probe trial (figure 2g). It appeared that animals circled in the outer annulus until they made a direct trajectory to the platform.

The decrease in Distance scores across days described above, suggests that animals swam a lesser distance within the allotted maximum search time, or until the platform was located. To confirm that animals were not floating or showing a decrease in movement within the maximum search time, and that this was not why distance scores decreased across days, we quantified the number of trials

within a day that each animal successfully found the platform (and thus escaped) within the 60s time limit. The number of trials escaped increased across days [Day main effect: $F(4, 24) = 22.92$; $p < .0001$] (Figure 2h inset), with a null Treatment x Day interaction. Every group showed an increase in the number of Escapes across days, suggesting learning by every group [Sham: $F(4, 5) = 7.899$; $p < .0001$; Ovx: $F(4, 6) = 2.984$; $p < .05$; Ovx+Prog: $F(4, 4) = 3$; $p = .05$; Ovx+Low MPA: $F(4, 3) = 4.123$; $p < .05$; Ovx+High MPA: $F(4, 6) = 13.552$; $p < .0001$] . There was no effect of Treatment on Escapes across all testing days, and no group significantly differed from any other group. Please see Figure 2H for Escapes.

Visible Platform

There were no Treatment main effects or interactions. There was a Day x Trial interaction [$F(7, 168) = 3.483$; $p < .0016$] , with Time decreasing across all trials of testing (Figure 3). By the second day of testing, all subjects found the visible platform within 6 s. Together, these data confirm visual and motor competence to perform the maze tasks.

GAD 65 & 67

Differences in GAD levels across groups were present in the hippocampus and entorhinal cortex. Because GAD protein levels did not differ between the Ovx+Low MPA and Ovx+High MPA groups, they were combined for statistical analyses. In the hippocampus, MPA treatment (doses combined) significantly [$t(15) = 2.251$; $p < .05$], and Prog treatment marginally [$t(10) = 1.96$; $p < .10$], decreased GAD levels as compared to Ovx (Figure 4a). However, in the

entorhinal cortex, both progesterone and MPA (doses combined) treatment increased GAD levels as compared to Ovx [Ovx vs. Ovx+MPA: $t(15) = 2.542$; $p < .05$; Ovx vs. Ovx + Prog: $t(9) = 2.428$; $p < .05$] (Figure 4b). There was no Treatment effect or any group differences for the loading control, beta Actin (ns).

Neurotrophins

There were no group differences in BDNF or NGF levels in any brain region analyzed (Table 1).

Blood Serum Levels of MPA

Low MPA (mean = 4.9 ng/mL) and High MPA (mean = 6.67 ng/mL) - treated groups were the only groups to show MPA concentrations in serum, as expected, since this is not a naturally occurring hormone. Therefore, this confirms presence of the drug (Table 2).

Uterine Weights

Uteri of Sham animals weighed more than those of all other treatment groups [Sham vs. Ovx: $t(11) = 9.239$; $p < .0001$; Sham vs. Ovx+Prog: $t(9) = 6.057$; $p < .0005$; Sham vs. Ovx+Low MPA: $t(8) = 5.028$; $p < .001$; Sham vs. Ovx+High MPA: $t(11) = 7.208$; $p < .0001$]. MPA uterine weights were greater than those of Ovx [Ovx vs. Ovx+Low MPA: $t(9) = 4.079$; $p < .005$; Ovx vs. Ovx+High MPA: $t(12) = 3.523$; $p < .005$] (Table 2).

Discussion

The current study is the first to test the synthetic progestin MPA, the most commonly utilized progestin component of HT given to women in the United States (Hersh et al., 2004), for learning and memory in the female rodent. We

found that MPA impairs cognition and alters the GABAergic system in the aged surgically menopausal rat. Specifically, MPA impaired the ability to handle an increasing working memory load on the WRAM across 30 second and 2 hour delays, and exacerbated overnight forgetting on the MM spatial reference memory task. MPA effects were dose specific, with only the highest dose showing detrimental effects. Natural progesterone also impaired performance on the WRAM at the highest working memory load with a 30 second delay, which is in agreement with our prior findings of progesterone administration to aged Ovx rats (Bimonte-Nelson et al., 2004b). We also replicated our previous findings, the herein being our third report, that Ovx is beneficial in aged rats, by improving performance on the WRAM at the highest working memory load at the lattermost portion of testing (Bimonte-Nelson et al., 2003b; Bimonte-Nelson et al., 2004b), likely mediated in part by the removal of high endogenous progesterone levels (Bimonte-Nelson et al., 2004b). It is noteworthy that the current working memory load findings in aged female rats correspond with prior findings that decrements at the highest working memory load on the WRAM are exacerbated with aging (Bimonte, Nelson, & Granholm, 2003). Additionally, working memory deficits associated with an elevated demand on the system via increasing the number of items to be remembered (Bimonte & Denenberg, 1999) or extended temporal delays (Engler-Chiurazzi et al., 2009) can be attenuated by 17 β-estradiol or conjugated equine estrogen administration, further demonstrating that different types of working memory demand increases are sensitive to steroid hormones.

Our data also concur with findings that the progesterone metabolite, allopregnanolone, impairs reference memory in young male rats (Johansson et al., 2002) and can impair reference memory and working memory in female rats (Frye & Sturgis, 1995). Progesterone's detrimental effects on cognition have also been seen in women. Data suggest that in pregnant women the "maternal amnesia" phenomenon is due to high circulating progesterone levels (Brett & Baxendale, 2001; Freeman et al., 1992). Moreover, as demonstrated by the WHIMS collective findings, there is evidence that the combination of estrogen and MPA has a greater negative impact on cognition (Shumaker et al., 2003), than estrogen alone (Shumaker et al., 2004).

The current findings demonstrate that both natural progesterone and the synthetic MPA either significantly or marginally alter GAD levels in the hippocampus and entorhinal cortex. GAD is the synthesizing enzyme and rate limiting step of GABA synthesis. There is a strong positive correlation between GAD mRNA levels and GABA neuronal activity (Erlander & Tobin, 1991), making GAD a good neuronal marker for GABAergic function (Bauer, Brozoski, Holder, & Caspary, 2000; Milbrandt, Holder, Wilson, Salvi, & Caspary, 2000; Raol, Zhang, Budreck, & Brooks-Kayal, 2005). Our findings that progesterone marginally, and MPA significantly, decreases GAD in the hippocampus are consistent with the literature for progesterone. Others have shown that progesterone decreased GAD activity in the dorsal hippocampus (Wallis & Luttge, 1980), and reversed 17 β-estradiol-induced increases in GAD mRNA in hippocampal CA1 and hilus (Weiland, 1992). This same decrease of GAD in the

hippocampus is observed after treatment with the $GABA_A$ agonist diazepam (Raol et al., 2005), suggesting that progesterone- and MPA-induced GAD decreases may result from increased $GABA_A$ receptor activation. Recent work testing both progesterone and MPA found that after 17 β-estradiol priming, progesterone but not MPA decreased mRNA expression of the α4 subunit of the $GABA_A$ receptor in the CA1 of young Ovx rats (Pazol et al., 2009). Our effects somewhat conflict with these findings in that both progesterone and MPA impacted the GABAergic system; however, our animals were not estradiol primed and were much older.

To our knowledge, the current report is the first to measure the impact of progesterone or MPA on the GABAergic system in the entorhinal cortex. In contrast to the progesterone- and MPA- mediated decreases seen in the hippocampus, both progesterone and MPA treatment increased GAD levels in the entorhinal cortex. Although no study has measured progesterone or MPA-related changes in the GABAergic system in the entorhinal cortex, it has been shown that alteration of the GABAergic system in this region affects memory function. Post-training infusion of the $GABA_A$ agonist muscimol into the entorhinal cortex blocked memory formation (Ferreira, Da Silva, Medina, & Izquierdo, 1992), and lesions of the GABAergic system in entorhinal cortex layer III disrupted hippocampal CA1 place fields, causing impaired spatial representation (Brun et al., 2008). The entorhinal cortex and the hippocampus are intimately associated (Knowles, 1992), and both play crucial roles in memory processing, which increasing evidence suggests is facilitated in part through the GABAergic system (Izquierdo et al., 1993).

Given the strong links between progesterone and memory, and the GABAergic system and memory, it is tempting to speculate that the progesterone- and MPA-induced alterations of the GABAergic system begin to explain the cognitively-impairing effects of progestins. However, our data do not allow clear interpretations of these links. Indeed, in the current study there was a dissociation of progestin-induced memory changes and progestin-induced GAD changes. Specifically, while GAD levels were altered with the low-dose and high-dose MPA treatments, low-dose MPA had no impact on behavior, while high-dose MPA did. Thus, it is not clear whether the alterations in GAD levels underlie the observed differences in cognitive performance. Progesterone and MPA both bind to the progesterone receptor, and progesterone is known to interact with several other steroid receptors (Pluchino et al., 2006; Pridjian, Schmit, & Schreiber, 1987), presumably all of which may lead to altered synaptic function through changes in gene transcription. Several of progesterone's ring-A reduced metabolites are potent agonists of the $GABA_A$ receptor (Paul & Purdy, 1992). While MPA's ring-A reduced metabolites do not seem to influence the $GABA_A$ receptor (McAuley et al., 1993), MPA has been shown to alter progesterone's metabolic conversions (Penning et al., 1985), wherein these alterations might lead to enhanced synaptic and extrasynaptic $GABA_A$ receptor-mediated inhibition (Belelli & Herd, 2003). However, at supraphysiological levels, MPA moderately inhibits benzodiazepine binding at the $GABA_A$ receptor (McAuly et al., 1993). Additionally, MPA pretreatment exacerbates neuronal death induced by glutamate excitotoxicity in cultured hippocampal neurons (Nilsen et al., 2006). Therefore,

while the specific mechanism(s) by which progestins alter GAD levels in the hippocampus and entorhinal cortex is unknown, several neuronal actions of these compounds are documented which could independently or interactively contribute to altered GAD levels. Further studies manipulating the GABAergic system after progestin administration will be integral to this mechanistic understanding.

Although previous literature shows that progesterone increases neurotrophins in cerebral cortex slice cultures (Kaur et al., 2007) and counteracts estrogen-induced increases in neurotrophins in the entorhinal and frontal cortices (Bimonte-Nelson et al., 2004a) and hippocampal slice cultures (Aguirre & Baudry, 2009), we found no effects of progesterone or MPA treatment on neurotrophin levels in several cognitive brain regions. Perhaps in vivo, progestins do not have independent, but only regulatory actions, of estrogen's effects on neurotrophins. More research is necessary to investigate this hypothesis.

We were able to confirm presence of MPA in serum with mass spectrometry. Not only was this verification of hormone administration, but it also allowed us to compare resulting hormone levels in our animals to known serum concentrations of hormones in women taking HT that includes MPA. According to the prescribing information of Prempro (Prempro Prescribing Information, 2009), women administered the most common dose of MPA given as HT (5mg), achieved peak plasma concentrations of 4.8 ng/mL. This corresponds almost exactly to our low-dose MPA serum blood levels (4.9 ng/ml) and is still strikingly similar to our high-dose MPA (6.67 ng/ml). These data confirm the clinical relevance of the MPA doses used in this study.

Several lines of evidence converge to suggest that progestins such as MPA have a negative consequence on neuronal health yielding detrimental effects on brain functions such as learning and memory. This is exemplified across multiple realms of the literature, from clinical to basic science. Clinical evidence shows detrimental cognitive effects of combined HT (Shumaker et al., 2003). Basic science evidence shows that both MPA (current report) and progesterone (Bimonte-Nelson et al., 2004b) impair cognition, and that progesterone abolishes 17 β-estradiol-induced memory enhancements (Bimonte-Nelson et al., 2006; Harburger et al., 2007; but see Gibbs, 2000) and attenuates 17 β-estradiol's neurotrophic effects in vivo (Bimonte-Nelson et al., 2004a) and in cell culture (Aguirre & Baudry, 2009). Moreover, when comparing MPA and progesterone, it is noteworthy that MPA does not demonstrate neuroprotective properties, while progesterone does (Nilsen & Brinton, 2002b), and that MPA causes a greater attenuation of 17 β-estradiol's neurotrophic actions (Nilsen & Brinton, 2002a). This in vitro work is in accordance with our current findings that MPA has broader detrimental effects for learning and memory, as compared to progesterone. In fact, progesterone's detrimental effect on learning of the WRAM seemed to be more of a transient nature, as effects were only seen on the last testing day with a 30 second ITI, and not after a two-hour delay to challenge extended memory retention. Furthermore, while no study has directly evaluated the impact of MPA on cognitive health in younger women using this pharmaceutical as a contraceptive, it should be noted that these effects could translate to cognitive impairments in these younger women as well, and account

for some of the negative effects reported with use of this drug. Indeed, a documented case study reports amnesic effects corresponding with use of Depo Provera, a contraceptive wherein MPA is the sole hormone component (Gabriel & Fahim, 2005).

In conclusion, the current study is the first to show that not only natural progesterone, but also the synthetic progestin MPA, the most common progestin included in HT, impairs memory and alters the GABAergic system in cognitive brain regions in the aged surgically menopausal rat. To our knowledge, there has been no study directly evaluating MPA-mediated cognitive effects in younger, or older, women. Increasing evidence suggests that this is clearly warranted. The current study has significant implications for the components of HT that might impact cognitive functioning, and suggest that MPA is detrimental within this domain.

CHAPTER 3

COGNITIVE-IMPAIRING EFFECTS OF MEDROXYPROGESTERONE ACETATE IN THE RAT: INDEPENDENT AND INTERACTIVE EFFECTS ACROSS TIME

Nearly every woman will face the decision of whether to take exogenous hormones, either for contraception or for HT. MPA, a synthetic progestin, is the sole hormone component of the contraceptive Depo Provera, and the most commonly prescribed progestin component of HT (Prempro and Premphase: MPA + conjugated equine estrogen). Depo Provera was approved by the Food and Drug Administration in 1992 (Bakry et al.,2008), and has been prescribed to over 10 million women in the United States since this time (Mosher et al., 2004). This widely prescribed contraceptive is often the choice for women who cannot take estrogen-containing contraceptives because of medical conditions (Spencer et al., 2009), for nursing mothers (Rodriguez & Kaunitz, 2009), and for those who prefer the convenience of an intermittent injection once every three months over the daily pill. Prempro has been prescribed since 1995 (Stefanick, 2005) with as many as 19.7 million prescriptions written per year (Hersh et al., 2004). At menopause, many women choose to take HT to attenuate menopause-induced symptoms, including memory decline (Freedman, 2002). Women with a uterus must include a progestin in their HT regimen to offset the increased risk of endometrial hyperplasia related to unopposed estrogen treatment (Smith et al. 1975; Ziel & Finkle 1975). While there have been no methodical clinical evaluations of the cognitive effects of MPA alone in any population of women,

there is evidence implicating that MPA is detrimental to cognition. For Depo Provera, there is a documented case study of amnestic effects corresponding with its use (Gabriel & Fahim, 2005). Moreover, the large, placebo-controlled, randomized WHIMS reported that twice as many women receiving Prempro were diagnosed with dementia as compared to the placebo group, a significant finding (Shumaker et al., 2003). Those women taking Premarin (conjugated equine estrogen only) showed a nonsignificant increase in risk of dementia compared to those taking placebo ($p = .18$) (Shumaker et al., 2004).

Recently, we showed that MPA impaired learning and memory in aged ovariectomized (Ovx) rodents (Braden et al., 2010; Chapter 2). In that study we also replicated previous findings (Bimonte-Nelson et al., 2004b) that natural progesterone impairs cognition in aged Ovx rodents. Others have shown that infusions of the progesterone metabolite, allopregnanolone, into the lateral ventricles of young Ovx rats can lead to impaired spatial reference and working memory performance (Frye & Sturgis, 1995). Progesterone and allopregnanolone have also been shown to impair working and reference memory, respectively, in young intact male and female rats (Johansson et al., 2002; Sun et al., 2010). However, the literature on progesterone and allopregnanolone cognitive effects in young rodents reports mixed results, with task-specific benefits noted sometimes, but not always (Frye et al., 2007; Frye et al., 2010; Frye & Walf, 2008; Harburger et al., 2008; Lewis et al., 2008; Orr et al., 2009). The animal study findings of progestin-induced cognitive impairments are corroborated by clinical reports of the detrimental effects of progesterone and allopregnanolone on memory in

women (Freeman et al., 1992; Kask et al., 2008). Yet, progesterone can enhance other cognitive processes such as attention, response/processing speed, cognitive flexibility and visuomotor coordination (Sofuoglu, Mouratidis, & Mooney, 2011). These clinical findings highlight the need for more scientific investigations, both clinical and preclinical, of the negative impact progestins could have on cognition. A progestin is included in every contraceptive, and in HT when given to women with a uterus; thus, safe progestin use is a critical medical issue affecting every woman who chooses to take exogenous hormones.

MPA's cognitive-impairing effects may be mediated via the GABAergic system. Indeed, we have shown that both progesterone and MPA alter the GABAergic system in cognitive brain regions of behaviorally tested aged Ovx animals; in this study MPA decreased GAD, the synthesizing enzyme for GABA, in the dorsal hippocampus and increased GAD in the entorhinal cortex (Braden et al. 2010). MPA has also been shown to enhance synaptic and extrasynaptic $GABA_A$ receptor-mediated inhibition (Belelli & Herd, 2003). Further, in vitro evidence implicates that MPA has a negative effect on neuronal function by exacerbating glutamate-induced excitotoxicity (Nilsen et al., 2006). Taken together, in vitro, preclinical, and clinical evidence of MPA's negative effects on the brain and its function are particularly salient. Moreover, there have been no evaluations of the possible long-term effects of MPA administration on cognition or interactions with subsequent MPA use as HT, nor whether there is a long-term impact on the GABAergic system in vivo. These issues are especially relevant as we embark on a new generation of menopausal women who were much more

likely to have been prescribed contraceptives, as compared to generations before (Diczfalusy, 1991). We hypothesize that MPA treatment during young adulthood and middle-age impairs cognition in the rat model, by enhancing $GABA_A$ receptor-mediated inhibition in the hippocampus, as evident by a compensatory decrease in GAD. Thus, the current study investigated the long-lasting effects of MPA given in young adulthood (to model contraception) on cognition and the GABAergic system in middle-age, and how such early treatment interacts with later MPA use as HT, in the rat model.

Methods

Subjects

Subjects were 44 Fischer-344 female rats born and reared at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). Rats were four months old at the beginning of the study, had unrestricted access to food and water, and were maintained on a 12-hour light/dark cycle at the Arizona State University animal facility. All procedures were in accordance with the local IACUC committee and follow NIH standards.

Hormone Treatment and Ovariectomy

Rats were randomly assigned to one of four treatment groups: Control, Early-MPA, Late-MPA, and Early+Late-MPA. The study was divided into two phases of injections (Figure 5). Phase 1 of weekly injections of either vehicle (0.4ml sesame oil + 0.02ml dimethyl sulfoxide [DMSO], both from Sigma-Aldrich, St. Louis, MO, USA) or MPA (3.5mg, Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.4ml sesame oil + 0.02ml DMSO, began at 4 months of age

and continued until 8 months of age. This regimen of MPA treatment was selected based on previously established anti-ovulatory effects (Bhowmik & Mukherjea, 1988), in order to mimic the biological action of Depo Provera taken by women (Depo Provera Prescribing Information, 2006). We chose to keep animals ovary-intact to most closely model the hormone milieu of fertile women prescribed Depo Provera.

At 10 months of age, all rats received Ovx surgery. Rats were anesthetized via isofluorane inhalation, bilateral dorsolateral incisions were made in the skin and peritoneum, and the ovaries and tips of uterine horns were ligatured and removed. Finally, muscle and skin were sutured. At time of surgery, rats received a single injection of Rimadyl (carprofen; 5mg/ml/kg) for pain and saline (2ml) to prevent dehydration. Phase 2 of weekly injections (either vehicle or MPA) began 2-3 days after Ovx surgery and continued throughout behavior testing until sacrifice. Testing began at 12 months of age, approximately 2 months after the beginning of phase 2 injections.

Animals in the Control group received vehicle injections during both injection phases. Early-MPA animals received MPA injections during phase 1 and vehicle injections during phase 2, Late-MPA animals received vehicle injections during phase 1 and MPA injections during phase 2, and Early+Late-MPA animals received MPA injections during both phases (Figure 1). During both injection phases, body weights were recorded weekly.

Vaginal Smears and Uterine Weights

To assess the long-term effects of 4 months of MPA injections on ovulation, vaginal smears were taken at 8 months of age for three days in the behaviorally tested animals, starting 9 days after the last injection (Figure 1). Smears were classified as proestrus, estrous, metestrus or diestrous per prior protocol (Acosta et al., 2009; Goldman et al., 2007). At sacrifice, the uteri of all subjects were removed, trimmed of visible fat, and immediately weighed (wet weight), in order to examine drug effects on uterine tissues (Braden et al. 2010, Chapter 2),

Water Radial-Arm Maze

Two months after initiation of phase 2 of injections, subjects were tested for 11 days on the 8-arm win-shift water radial-arm maze (WRAM) to evaluate spatial working and reference memory, including performance as working memory load increased, as described previously (e.g., Bimonte & Denenberg, 1999). There were escape platforms hidden under the water surface in the ends of 4 of the 8 arms. The temperature of the water was 18° Celsius (C). Platform locations varied across subjects, but remained fixed for each individual subject, throughout the experiment. Once released from the start arm, a subject had 3 minutes (m) to locate a platform. After a platform was found, the animal remained on it for 15 seconds (s), and was then placed in its heated cage for a 30s inter-trial-interval (ITI) until its next trial. During the interval, the just-chosen platform was removed from the maze. This process was repeated until each platform was found. This totaled to four trials in each animal's daily session, with the number

of platformed arms reduced by one on each subsequent trial. The working memory system was increasingly challenged as trials progressed, allowing us to access working memory load. Each subject was given one session a day for 11 consecutive days.

Quantification and blocking into acquisition and asymptotic phases were based on prior studies (e.g., Bimonte & Denenberg, 200). An arm entry was counted when the tip of a rat's snout reached a mark 11cm into the arm. Orthogonal measures of working and reference memory errors were quantified as done previously in WRAM studies (Bimonte et al., 2000; Braden et al., 2010, Chapter 2). Working Memory Correct (WMC) errors were the number of first and repeat entries into an arm that previously contained a platform during that session. Reference memory (RM) errors were the number of first entries into an arm that never contained a platform. Working Memory Incorrect (WMI) errors were repeat entries into a reference memory arm. Errors were analyzed across all Days (1-11) of testing and on the latter portion of regular testing at the highest working memory load, as this has revealed progesterone- and MPA- induced impairments (Bimonte-Nelson et al., 2003b; Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2).

Morris Maze

Two days after WRAM completion, subjects were tested on the Morris maze (MM) for 4 trials/day for 4 days. To test spatial reference memory (Bimonte-Nelson et al., 2006; Morris et al., 1982), a tub (188cm diameter) filled with black water made opaque using non-toxic paint was used, with a hidden

platform (10cm wide) remaining in a fixed location. The temperature of the water was 18° C. During initial testing (Days 1-4) the rat was started at the North, South, East, or West location in the maze, and had 60s to locate the hidden platform in the Northeast (NE) quadrant. The trial was terminated once the rat found the platform. After 15s on the platform, the rat was placed into its heated cage until its next trial. The ITI was approximately 10m. Swim path distance (cm) and latency (s) to the platform across all days of initial testing (Days 1-4) were used to asses MM performance. Distance scores from the last trial of each day (Trial 6) were compared to the first trial on the following day (Trial 1) in order to test overnight forgetting of the platform location (Bimonte-Nelson et al., 2006; Markham et al., 2002), as this measure has revealed MPA-induced impairments (Braden et al., 2010, Chapter 2). The first overnight interval was omitted from this analysis, as we noted that all animals were extending their learning curve without forgetting during this time interval (Figure 7a). In order to determine which treatment groups increased their swim distance across the overnight interval, thus showing overnight forgetting of the platform location we examined within-group trial comparisons of the overnight interval (Trial 6 to Trial 1).

After all test trials on day 4, a 60s probe trial was initiated whereby the platform was removed. This was used to evaluate whether rats localized the platform to the spatial location. Percent of total distance in the previously platformed (target) quadrant was compared to the quadrant diagonally opposite the platform. Rats that spent the greatest percent distance in the target quadrant were interpreted as localizing the platform to the spatial location. To attenuate the

possibility of extinction due to the probe trial, rats were placed back into the maze immediately following the probe trial with the platform replaced in the NE quadrant. Next, to test relearning of a novel platform location, on Day 5 the platform was switched from the NE quadrant to the Southwest (SW) quadrant, and rats were given 8 trials with the new platform location. Distance and latency were analyzed across all trials for the platform switch (Day 5).To evaluate whether rats localized the new platform to the spatial location, after the eighth trial, a 60s probe trial was given whereby the platform was again removed. For each trial, a rat's path was recorded from a camera suspended above the maze, and a tracking system (Ethovision 3.1, Noldus Instruments) analyzed each rat's tracing.

Visible Platform Maze

To confirm that all subjects had intact vision and could perform the procedural task components of MM and WRAM spatial navigation without difficulty, subjects were tested on a visible platform water-escape task, as previously described (Braden et al., 2010, Chapter 2). Briefly, animals were placed into a rectangular tub (39 x 23in) where they had to locate a flagged platform protruding from the water. The temperature of the water was 18° C. Extramaze cues were covered by opaque curtains. Animals were given 6 trials, with the drop off location the same across trials, and the platform location for each trial varied in space semi-randomly. Performance was assessed by latency (s) to the platform across all trials.

Brain Dissections

One day after the conclusion of behavior testing, animals were anesthetized with isofluorane and brains were rapidly dissected and frozen. Dissected tissues were stored at -70°C in preweighed microcentrifuge tubes until analysis. Plate designations (Paxinos & Watson, 1998) for dissections were as follows: frontal cortex (plates 5-14), anterior cingulate cortex (plates 5-14), posterior cingulate cortex (plates 15-35), entorhinal cortex (plates 39-42), and the CA1/CA2 region of the dorsal (plates 33-35) and ventral (plates 39-42) hippocampus. For each brain the frontal cortex was taken first from the dorsal aspect of the intact brain. The medial border of the frontal cortex cut served as the lateral border for the cingulate cortex dissection, with the longitudinal fissure as the medial border. Designation between anterior and posterior cingulate cortex was made according to Paxinos & Watson (1998) plate 14. Next, the brain was cut in the coronal plane at plate 33 to obtain access to the dorsal CA1/CA2 region of the hippocampus. Finally, the brain was cut again in the coronal plane at plate 39 to obtain access to the last two brain regions (the hippocampus and entorhinal cortex). It was here that the ventral CA1/CA2 region of the hippocampus was taken. For the hippocampus, the dentate gyrus and the alveus were excluded. For the entorhinal cortex, the tissue was dissected from the same slice as the ventral hippocampus sample, taking a 2- to 3- mm sample ventral to the hippocampus.

Western Blot Analyses of GAD 65+67

GAD 65+67 protein expression levels were analyzed in frontal, anterior cingulate, posterior cingulate and entorhinal cortices, and the dorsal and ventral

hippocampus, from the right hemisphere. Each sample was sonicated in RIPA buffer (150mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na Deoxycholate, 50mM Tris) equivalent to 10 times its weight and centrifuged at 10,000 rpm for 10m at 4°C. BCA protein assays determined protein concentration (ThermoFisher Scientific, Pittsburgh, PA, USA) and 10µg of protein from each sample were run on a NuPAGE Bis-Tris gel, using the SureLock mini-cell (Invitrogen, Carlsbad, CA, USA). PVDF membranes were used for transfer (Millipore, Bedford, MA, USA) and immunoblotting was performed with working dilutions of a rabbit anti-GAD 65+67 (ab11070) and rabbit anti-beta Actin (ab25894 or ab8227) primary antibodies (Abcam Inc., Cambridge, MA, USA). Antibody dilutions were 1:10,000 for GAD 65+67 and a range of 1:400 - 1:20,000 for beta Actin primary antibodies. Membranes were then incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody, 1:10,000 dilution (111-035-003; Jackson Immuno Research, West Grove, PA, USA) and visualized using Pierce ECL Western Blotting Substrate (ThermoScientific, Rockford, IL, USA) in a film developer. Precision Plus Protein WesternC Standards (Bio Rad, Hercules, CA, USA) were used to identify bands as the protein of interest, based on molecular weight. ImageJ software (Rasband, 1997-2004) was used to quantify the density of GAD 65+67 and beta Actin (loading control protein). The dependent measure was a ratio of each subject's GAD 65+67 density to their beta Actin density, brought to percent control of Control subjects run on the same gel, per prior protocol (Braden et al., 2010, Chapter 2; Pandey et al., 1999).

Blood Serum Analyses for MPA, Progesterone, and Allopregnanolone

Serum MPA determinations were performed according to the method reported earlier (Braden et al., 2010, Chapter 2). In addition, the assay was extended to measure progesterone and allopregnanolone, similar to an earlier procedure (Prokai, Frycak, Stevens, & Nguyen, 2008). Briefly, levels were measured by the isotope dilution method through the addition of deuterium-labeled internal standards d_9-progesterone and d_4-allopregnanolone (C/D/N Isotopes, Pointe-Claire, Quebec, Canada); 10µL each from an 1µg/mL solution in ethanol to 1ml of serum. Progesterone, d_9-progesterone, allopregnanolone and d_4-allopregnanolone were measured through selected-reaction monitoring (SRM) signals of m/z 315 $\rightarrow$ 297, 324 $\rightarrow$ 306, 301 $\rightarrow$ 287 and 305 $\rightarrow$ 287, respectively, obtained by liquid chromatography–atmospheric-pressure chemical ionization tandem mass spectrometry (LC–APCI-MS/MS). Progesterone and allopregnanolone concentrations were calculated by dividing the areas under their corresponding SRM peaks in the LC–APCI-MS/MS chromatograms with those of d_9-progesterone and d_4-allopregnanolone, respectively, multiplied with the known concentrations (10ng/mL each) of these added internal standards. Calibrations from the analysis of serum samples spiked with 1, 2, 5 and 10ng/mL of analyte, respectively, were used to determine MPA concentrations. Additionally, d_9-progesterone was used as an internal reference to compensate for slight changes in extraction efficiency and LC–APCI-MS/MS conditions among samples. The dependent measures of the assay were ng/mL of MPA, progesterone, and allopregnanolone in serum, respectively.

Statistical Analyses

For behavior assessments, data were analyzed separately for each maze. Since our interest was to evaluate Treatment effects regarding timing of MPA administration, two-group planned comparisons were run on specific measures. To evaluate potentially complex higher order Treatment interactions with Days or Trials, we utilized an omnibus repeated measures ANOVA including all groups, with Treatment as the between variable and Days and/or Trials as the within variable/s. For GAD protein levels, serum levels of MPA, progesterone, and allopregnanolone, uterine weights, and body weights, data were assessed via a priori planned comparisons using t-tests. For all of these analyses, alpha was two-tailed and set at 0.05, with two exceptions where analyses were one-tailed: for the Trial 4 WMC effects for the latter portion of testing, as we have previously established that MPA impairs performance on this measure (Braden et al., 2010, Chapter 2), and uterine weights comparisons (Control vs. Late-MPA and Control vs. Early+Late-MPA), as we have previously shown that MPA administration after Ovx increases uterine weight (Braden et al., 2010, Chapter 2). For all planned comparisons, it was noted that Type I error correction is not necessary (Keppel & Wickens, 2004).

Correlations

Pearson r correlations were run between GAD levels in each brain region and 1) serum MPA levels, 2) serum progesterone levels, 3) serum allopregnanolone levels, and, 4) memory scores from each of the mazes tested. Additionally, correlations were run between each serum hormone and memory

scores. The memory scores used for correlations were, for WRAM: WMC, WMI, and RM errors across all days of regular testing; for MM: distance across all days of testing, and overnight forgetting on the last two overnight intervals. Alpha was adjusted to 0.01 to account for multiple correlations.

Results

Table 3 summarizes the significant behavior effects.

Water Radial-Arm Maze

Two group planned comparisons revealed that animals treated with MPA at any time point made more WMC errors than Control animals, across all days of testing [Control vs. Early-MPA: $t(20) = 2.17$; $p < .05$; Control vs. Late-MPA: $t(18) = 2.817$; $p < .05$; Control vs. Early+Late-MPA: $t(21) = 2.285$; $p < .05$] (Figure 6a). For WMI errors, only animals that received MPA at both time points (Early+Late-MPA) made more errors than Control animals [$t(21) = 2.431$; $p < .05$] (Figure 6b). There were no differences between Control and Early-MPA, or Control and Late-MPA, groups for WMI errors, and no Treatment group differences for RM. Across all testing days (Days 1-11), there was a Day main effect for each error type for the omnibus ANOVA [WMC: $F(10, 39) = 5.627$; $p < .0001$; WMI: $F(10, 39) = 13.009$; $p < .0001$; RM: $F(10, 39) = 10.288$; $p < .0001$], with errors decreasing across days demonstrating learning. There was no Day x Treatment interaction.

During the asymptotic phase of testing (Days 5-11), we investigated group differences at the highest working memory load, Trial 4, as we have previously shown memory load impairments at the end of testing after progesterone

treatment (Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2), and MPA treatment given to Ovx animals in old age (Braden et al., 2010, Chapter 2). For WMC errors on Trial 4, animals treated with MPA at any time point made more errors than Control animals [Control vs. Early-MPA: $t(20) = 1.973$; $p < .05$; Control vs. Late-MPA $t(18) = 2.681$; $p < .05$; Control vs. Early+Late-MPA: $t(21) = 1.990$; $p < .05$] (Figure 6c). For WMI errors, only animals that received MPA at both time points (Early+Late-MPA) made more errors on Trial 4 than Control animals [$t(21) = 2.270$; $p < .05$] (Figure 6d). There were no differences between Control and Early-MPA, or Control and Late-MPA for Trial 4 WMI errors, and no Treatment group differences for Trial 4 RM errors.

Morris Maze

Initial Testing (Days 1-4, platform in the NE quadrant)

Planned comparisons revealed no effect of MPA treatment on initial MM performance, as measured by distance and latency. There was a Day main effect for Distance and Latency for the omnibus ANOVA [Distance: $F(3, 40) = 114.966$; $p < .0001$ (Figure 7a) ; Latency: $F(3, 40) = 107.947$; $p < .0001$ (data not shown)], with scores decreasing across days, demonstrating learning of the task. There was no Day x Treatment interaction for initial MM testing (NE platform location) performance as measured by Distance or Latency.

As we have shown previously (Braden et al., 2010, Chapter 2), animals who received MPA after Ovx only (Late-MPA) showed overnight forgetting. Distance scores increased across the overnight interval in the Late-MPA group [$t(8) = 2.538$; $p < .05$], while they remained stable across the overnight interval in

the Control group [t(10) = 1.717; p = .12] (Figure 7b). There were no significant increases across the overnight interval for Early-MPA or Early+Late-MPA animals.

The first probe trial, assessing learning of the initial platform location (NE quadrant), revealed a main effect of Quadrant [F(1, 40) = 221.798; p < .0001]. We confirmed that each group spent more percent Distance in the target NE quadrant as compared to the opposite SW quadrant, showing localization of the platform location [Control: t(10) = 9.931; p < .0001; Early-MPA: t(11) = 6.134; p < .0001 ; Late-MPA: t(8) = 6.202; p < .0005; Early+Late-MPA: t(11) = 9.628; p < .0001 (Figure 7c)].

Platform Switch (Day 5, platform in the SW quadrant)

For the MM testing where the platform was moved from the NE to the SW quadrant (platform switch), planned comparisons revealed no effect of MPA treatment on distance or latency. There was a Trial main effect for both Distance and Latency for the omnibus ANOVA [Distance: F(7, 40) = 20.789; p < .0001 (Figure 8a) ; Latency: F(7, 40) = 30.257; p < .0001 (data not shown)], with both decreasing across Trials. It is noteworthy that, when comparing distance for the first eight trials of the original location to the distance for the eight trials of the platform switch, there was an Original vs. Platform Switch main effect [F(1, 40) = 44.171; p < .0001], and an Original vs. Platform Switch x Trial interaction [F(7, 280) = 4.325; p < .0001], for the omnibus ANOVA. Collapsed across all groups, animals learned the platform switch location at a much faster rate than the original platform location [Trial 2: F(1, 40) = 55.572; p < .0001; Trial 3: F(1, 40) =

11.133; $p < .005$; Trial 4: $F(1, 40) = 28.501$; $p < .0001$; Trial 5: $F(1, 40) = 6.783$; $p < .05$; Trial 6: $F(1, 40) = 6.857$; $p < .05$; Trial 7: $F(1, 40) = 4.607$; $p < .05$ (Figure 8a)], demonstrating understanding of the rules of the task. There were no interactions with Treatment.

The second probe trial, assessing learning of the new platform location (SW quadrant), revealed a main effect of Quadrant [$F(1, 40) = 33.950$; $p < .0001$ (Figure 8b)] with a preference for the SW quadrant, and no interactions with Treatment or Treatment main effect.

Visible Platform

Planned comparisons revealed no Treatment effects for latency to the visible platform. There was a Trial main effect [$F(5, 40) = 13.259$; $p < .0001$(Figure 9)], with Latency decreasing across all trials, demonstrating learning of the task. By the fourth trial, all subjects found the visible platform within 6s, confirming visual and motor competence to perform swim maze tasks for all groups.

Blood Serum Levels of MPA, Progesterone, and Allopregnanolone, Vaginal Smears, Uterine Weights and Body Weights

Groups with MPA administered in middle-age were the only groups to show MPA concentrations in serum at sacrifice. Indeed, Late-MPA (mean = 9.538 ng/mL) and Early+Late-MPA (mean = 11.126 ng/mL) -treated groups demonstrated detectable MPA concentrations, and these groups did not significantly differ from each other. Control and Early-MPA animals had no detectable levels of MPA. This confirms presence of MPA in animals receiving

the drug at sacrifice, and successful clearing of the drug in animals that were not receiving MPA at time of sacrifice but had received it as earlier treatment (Figure 10a). For progesterone, Control animals had higher levels than all MPA-treated groups [Control vs. Early-MPA: $t(21) = 2.553$; $p < .05$; Control vs. Late-MPA: $t(18) = 3.734$; $p < .005$; Control vs. Early+Late-MPA: $t(21) = 3.92$; $p < .001$] and animals that received MPA only during young adulthood (Early-MPA) had greater progesterone levels than animals treated with MPA during middle-age [Early-MPA vs. Late-MPA: $t(19) = 3.288$; $p < .005$; Early-MPA vs. Early+Late-MPA: $t(22) = 2.642$; $p < .05$] (Figure 10b). Only Control animals had detectable allopregnanolone levels (Figure 10c). There were no significant correlations between serum hormone concentrations and memory scores.

Although it has previously been shown by Bhowmik & Mukherjea (1988) that weekly injections of 3.5mg of MPA are sufficient to halt ovulation for at least 7 days, we confirmed this in a pilot study with animals, not included in behavioral testing, at 3 months of age and of the same strain as the behaviorally tested animals. Animals were given one injection of 3.5mg MPA, and smears were then performed, classified as proestrus, estrous, metestrus or diestrous per prior protocol (Acosta et al., 2009; Goldman et al., 2007). This one injection proved to be the minimum dose sufficient to inhibited ovulation for 9±2 days in these animals, as evidenced by continually diestrous smears, and no cornified estrous smears, which would indicate ovulation (Goldman et al., 2007). Smears were taken at 8 months of age for three days in the behaviorally tested animals, starting 9 days after the last injection (Figure 5). Eighty-eight % of the MPA-treated

animals (Early-MPA and Early+Late-MPA groups) showed the diestrous phase while 12% had begun cycling again; all animals given vehicle injections showed indication of cycling as evident by at least one day of cornified estrous smears.

Similar to our prior findings (Braden et al., 2010, Chapter 2), animals treated with MPA during middle age had heavier uteri than Control animals [Control vs. Late-MPA: $t(18) = 1.914$; $p < .05$; Control vs. Early+Late-MPA: $t(21) = 1.787$; $p < .05$]. Control and Early-MPA uteri weights did not differ (Table 4).

During phase 1 of injections, both groups receiving MPA had greater body weights than the Control group [Control vs. Early-MPA: $t(21) = 7.724$; $p < .0001$; Control vs. Early+Late-MPA: $t(21) = 4.147$; $p < .001$] (Table 4), and the group not receiving MPA (Late-MPA) did not differ from the Control group. During phase 2, animals that had received MPA during phase 1 (Early-MPA and Early+Late-MPA) still had greater body weights than the Control group [Control vs. Early-MPA: $t(21) = 5.227$; $p < .0001$; Control vs. Early+Late-MPA: $t(21) = 2.353$; $p < .05$] (Table 4), but the Late-MPA animals did not weigh more than the Control group, even though they were receiving MPA at the time [Control vs. Late-MPA: $t(18) = .217$; p .83], which is in accordance with other reports of MPA treatment to middle-aged rodents (Isserow et al., 1995).

GAD 65 & 67

There were no Treatment effects for any brain region analyzed (Table 5). Pearson r correlations revealed that in animals that had detectable levels of serum MPA (Late-MPA and Early+Late-MPA), there was a negative correlation

between MPA levels and GAD protein in the dorsal hippocampus [r (19) = -.571, p = .0059] (Figure 11) indicating that higher MPA serum levels were associated with decreased GAD protein levels in the dorsal hippocampus. There were no correlations between GAD protein and the memory scores evaluated.

Discussion

The synthetic progestin MPA is widely prescribed as the contraceptive Depo Provera, and is in the HTs Prempro and Premphase. The current study supports the hypothesis that MPA has detrimental effects on cognition in the female rodent. We previously found that MPA impaired learning and memory in aged Ovx rats, when given in old age (Braden et al., 2010, Chapter 2). Here, we show that MPA administered during young adulthood, middle-age, or both, impaired working memory in middle-aged Ovx rats. Specifically, MPA given at any time point tested herein increased WMC errors on the WRAM across all days of testing and at the highest working memory load on the latter portion of testing, relative to vehicle treatment. MPA given at both time points was particularly detrimental to WRAM performance, as evidenced by a concomitant increase in errors on two orthogonal working memory measures. Notably, the detrimental effects of MPA were especially prominent when the task was most taxing and the load on working memory was highest. Compared to working memory, MPA seems to have less of an impact on reference memory. In fact, the only finding on a reference memory measure tested here was the replication of our prior work showing that MPA induced overnight forgetting, as shown when administered after Ovx only (Braden et al., 2010, Chapter 2). Indeed, the current results show

that the Late-MPA group, who only received MPA post-Ovx, had poorer retention of the platform location overnight. Table 3 summarizes the significant behavior effects.

The current findings are in agreement with clinical evidence implicating MPA as detrimental to cognition in young women when given as a contraceptive, as reported in one case study (Gabriel & Fahim, 2005), and may be related to the increased risk of dementia seen in the WHIMS MPA + conjugated equine estrogen trial (Shumaker et al., 2003). Further, administration of progesterone or its metabolite, allopregnanolone, is detrimental to memory in healthy pre-menopausal women (Freeman et al., 1992; Kask et al., 2008). In rodents, we and others have shown progesterone-related decrements in working memory (Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2; Frye & Sturgis, 1995; Sun et al., 2010) and reference memory (Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2; Johansson et al., 2002). However, the literature on progesterone and allopregnanolone's effects on cognition in young rodents is mixed, with some studies showing benefits and others not showing benefits (Frye & Walf, 2008; Frye et al., 2007; Frye et al., 2010; Harburger et al., 2008; Orr et al., 2009). Likewise, in ovary-intact middle-aged rodents, for some memory tasks higher levels of progesterone or allopregnanolone in cognitive brain regions were associated with better performance, while for others, lower levels were associated with better performance (Paris, Walf, & Frye, 2010). In similar age populations, post-training injections of progesterone had no effect on MM performance in middle-age rodents, but attenuated overnight forgetting in aged Ovx rodents, as

well as differentially facilitated object recognition across different age groups (Lewis, Orr, & Frick, 2008).

It must be considered that the water maze tasks used in the present study could involve a stress response, exacerbated by colder water temperatures which can facilitate memory formation (Sandi, Loscertales, & Guaza, 1997)(Sandi et al. 1997). Although MPA does bind to the glucocorticoid receptor at one-tenth of the potency as the synthetic glucocorticoid, dexamethasone (Pridjian et al., 1987), and at high doses has been shown to curb the stress response seen in cancer patients (Lang, Zielinski, Templ, Spona, & Geyer, 1990), in the current report the working memory load effect lends support to the hypothesis that MPA modulates memory, specifically. The nature of the working memory load in the radial-arm maze is such that the only aspect that changes across trials is the memory demand and not the motivator involved. Indeed, in the earlier trials when memory load was lowest but the non-cognitive stressors of the task were the same, MPA had no effect on performance.

MPA, progesterone, and allopregnanolone have each been shown to affect the GABAergic system (Belelli & Herd, 2003; Braden et al., 2010, Chapter 2; Paul & Purdy, 1992; Pazol et al., 2009; Wallis & Luttge, 1980). In the current report, there was a negative correlation between serum MPA levels and GAD in the dorsal hippocampus. In animals receiving MPA treatment at test (Late-MPA and Early+Late-MPA), higher MPA levels correlated with less GAD in the dorsal hippocampus, which is in agreement with our previous findings of MPA-induced decreases in GAD in the dorsal hippocampus in aged Ovx animals (Braden et al.,

2010, Chapter 2). There is an increasing focus on research, including both in vitro and in vivo studies, evaluating how MPA could impact the GABAergic system. Findings have indicated that although MPA's ring-A reduced metabolites do not directly bind to the $GABA_A$ receptor (McAuley et al., 1993) like natural progesterone's ring-A reduced metabolites (Paul & Purdy, 1992). MPA alters progesterone's metabolic conversions (Penning et al., 1985) which may be responsible for MPA-induced enhanced $GABA_A$ receptor-mediated inhibition (Belelli & Herd, 2003) as well as changes in GAD (Braden et al., 2010, Chapter 2). Similar to the relation between reduced GAD levels in dorsal hippocampus and serum MPA levels we show here, Raol et al (2005) demonstrated a decrease in GAD levels in the dorsal hippocampus after administration of the $GABA_A$ agonist diazepam in rodents, in turn suggesting that MPA-induced GAD decreases may be a consequence of increased $GABA_A$ receptor activation. In fact, increased $GABA_A$ receptor activation impaired learning as well as immediate and delayed recall in humans (Curran, 1986). There is also evidence that MPA has a negative effect on neuronal function via exacerbation of glutamate-induced excitotoxicity when tested in vitro (Nilsen et al., 2006) and lacks neuroprotective effects seen with natural progesterone (Nilsen & Brinton, 2002b). However, further research is needed to understand the complete mechanism behind MPA's detrimental effects on neuronal function and cognition in order to determine safe progestin use.

In aged Ovx rats, we previously found a main effect whereby MPA treatment decreased GAD levels in the dorsal hippocampus (Braden et al., 2010,

Chapter 2). Here, we show a similar relationship via correlation, in the absence of a main effect, in middle-aged Ovx rats. We hypothesize it is a difference in age of the animals in the current study (12 months old) versus our previous report (20 months old; Braden et al. 2010, Chapter 2). This may represent that some animals receiving MPA when middle-aged still showed resilience to MPA-induced GABAergic changes while others did not, thereby enriching variability conducive to significant correlations among individual subjects rather than main effects via ANOVA. While the relation between progestins (including MPA, progesterone and allopregnanolone) and the GABAergic system has been evaluated using in vitro techniques with tissues from young adult animals (Paul & Purdy, 1992; Pazol et al., 2010; McAuley et al., 1993; Belelli & Herd, 2003), whether these findings translate to similar effects in vivo, or in aged animals, is an exciting and clinically relevant question that remains to be explored. Specifically, how brain aging alters the trajectory of progestin-induced GABAergic changes has yet to be methodically detailed.

There were MPA-induced changes in serum levels of MPA, progesterone, and allopregnanolone. For serum MPA, animals receiving MPA at test (Late-MPA and E+Late-MPA) were the only groups to show MPA concentrations in serum at sacrifice, confirming presence of the drug after administration, as well as successful clearing of the drug in blood for the group that received MPA treatment in young adulthood only. Further, serum MPA levels are within the range of MPA used clinically (Depo Provera Prescribing Information 2006; Prempro Prescribing Information 2009). The prescribing information for Depo

Provera and Prempro states that MPA is absorbed, distributed, metabolized, and excreted in an identical manner for both formulations. Presence of progesterone is assumed to be from the adrenal glands which have been shown to significantly contribute to total circulating progesterone levels in rodents (Fajer, Holzbauer, & Newport, 1971). MPA administration at any time inhibited serum levels of both progesterone and allopregnanolone, as compared to Controls. For progesterone, our data suggest that this inhibition was partially recovered over time as the Early-MPA animals had greater levels than animals treated with MPA during middle-age. The long-lasting effects of the MPA-induced decrease in endogenous progesterone and allopregnanolone levels represent another possible mechanism by which MPA may impair cognition. This outcome of MPA treatment is particularly striking because it is the only biological effect that is present in all groups that showed cognitive impairments. Indeed, ovarian hormone removal via Ovx (thereby removing endogenous progesterone as well as estrogen) impairs cognition in young rats (Bimonte & Denenberg, 1999; Talboom, Williams, Baxley, West, & Bimonte-Nelson, 2008), but not in middle-aged or aged rats (Savonenko & Markowska, 2003; Talboom et al., 2008), under normal test conditions without pharmacological challenge. Removal of elevated progesterone levels may be one mechanism by which Ovx improves cognitive performance in aged rats (Bimonte-Nelson et al., 2003b; Bimonte-Nelson et al., 2004b). Indeed, while in some cases progesterone treatment can improve cognitive performance in Ovx rats (Frye et al., 2007; Walf, Rhodes, & Frye, 2006), and in stroke and cortical contusion rodent models (Roof et al., 1993; Roof & Hall, 2000), these

improvements were seen in young animals. The collected findings suggest that age may be a factor in these effects since progesterone treatment impairs cognition in aged Ovx rats (Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2), and hormone status at middle-age impacts cognition (Markowska, 1999) with estropause status characterized with high progesterone levels related to poorer cognitive performance (Warren & Juraska, 2000). Additionally, in one study, progesterone treatment improved memory in young Ovx mice, but progesterone plus MPA did not (Frye, Koonce, & Walf, 2010), further demonstrating the lack of efficacy of MPA on improving cognition in the rodent model, even when tested in young adulthood. Recently, there have been investigations of combined estrogens and MPA treatment in middle-aged rodents. In Ovx animals, long-term treatment of 17β-estradiol plus MPA led to impaired spatial memory performance as compared to chronic or cyclic 17β-estradiol alone or 17β-estradiol plus progesterone, suggesting that the addition of MPA was detrimental to cogntiion (Lowry, Pardon, Yates, & Juraska, 2010).

Another possible mechanism of MPA-related memory impairment is halting ovulation. It is tempting to speculate that the negative effects on cognition in Early-MPA and Early+Late-MPA could be due to prolonged acyclicity in young adulthood, as opposed to direct effects of MPA on the brain. Inherent to the complexities associated with administration of a contraceptive to evaluate cognition, is that we are stopping ovulation. While we cannot rule out the possibility that the cascade of events related to a lack of ovulation might have impacted our findings, a recent publication shows no effect of MPA treatment on

plasma 17β-estradiol levels in middle-aged ovary intact rodents (Frye et al., 2010). Further, we observed memory impairment in the Late-MPA group that had no evidence of acyclicity in young adulthood, because they were ovary-intact and not exposed to MPA at that time.

MPA impacted uterine and body weights. For uterine weight, we replicated our previous findings that animals treated with MPA after Ovx had heavier uterine weights than Control animals (Braden et al., 2010, Chapter 2), while Early-MPA animals did not differ from Control animals. Finally, for body weight, MPA treatment in young adulthood but not middle-age (Isserow et al., 1995) caused an increase in body weight as compared to Control. The body weights of animals treated with MPA during young adulthood only (Early-MPA) did not return to Control-like weights after the cessation of treatment suggesting that early MPA treatment causes long lasting weight gain, in the rodent model. There is some evidence of this in clinical research as well (Bigrigg et al., 1999; Hani, Imthurn, & Merki-Feld, 2009).

In conclusion, the current report indicates that in the rodent model: 1) MPA administration during young adulthood results in long lasting working memory impairments evident later in life (middle-age), even in the absence of circulating MPA levels at the time of test; 2) MPA administered during middle-age, extending through time of test, impairs working memory and overnight retention; 3) MPA administered during young adulthood and again at middle-age impairs working memory, an effect that is robust as it is seen on two orthogonal working memory measures. Based on the latter result, our study suggests there is

added risk to working memory when MPA is given both in young adulthood and middle-age. These detrimental effects may, in part, be mediated by changes to the GABAergic system in the dorsal hippocampus. Overall, the current study builds on earlier in vivo and vitro research indicating that MPA is detrimental to brain health and function. Given the large number of prescriptions that are written every year containing MPA, as it is contained in both contraceptives and HTs, more investigation into this synthetic hormone's effect on cognition and brain is warranted.

CHAPTER 4

PROGESTINS DIFFERENTIALLY IMPACT LEARNING AND MEMORY DEPENDING ON HORMONE CLASS AND REGIMEN

A progestin, the synthetic form of progesterone, is included in every hormone therapy prescribed to women with a uterus, as well as every birth control formulation. The need to develop this class of steroids was due to the poor bioavailability and short biological half-life of the natural hormone, although micronized progesterone somewhat overcomes these caveats (Sitruk-Ware, 2002). The necessity to include a progestin in hormone therapies and contraceptives is well documented, as it offsets the increased risk of endometrial hyperplasia related to unopposed estrogen treatment (Smith et al., 1975; Ziel & Finkle, 1975). However, the potential risks to cognition and other crucial processes that progestins pose have been largely unexplored. There is indirect evidence via the large, placebo-controlled, randomized WHIMS that the most commonly prescribed progestin in the USA, MPA, may adversely affect cognition. Indeed, a series of studies found that the combination hormone therapy Prempro (conjugated equine estrogens; CEE, plus MPA) was associated with a significant increased risk of dementia (Schumaker et al., 2003), while CEE-only hormone therapy produced a nonsignificant increase in risk of dementia ($p = 0.18$), compared to those taking placebo (Shumaker et al., 2004). Further, MPA is the only active ingredient in the contraceptive Depo Provera, and a documented case study revealed amnestic effects associated with its use (Gabriel & Fahim, 2005). Recently we were the first to methodically investigate the cognitive effects of

MPA in a rat model. We found that MPA impairs cognition in aged (Braden et al., 2010, Chapter 2) and middle-aged (Braden et al., 2011, Chapter 3) ovariectomized (Ovx) rats, as well as initiates long-lasting cognitive-impairing effects when given to young adult, ovary-intact rodents, evident when these animals reach middle-age (Braden et al., 2011, Chapter 3). With evidence mounting that MPA is not the optimal progestin to be used in hormone therapy and birth control taken by women, it is imperative to determine if there are safer progestins, with respect to cognition.

Based on their chemical structures, synthetic progestins either structurally resemble progesterone or testosterone considering the A-ring and the functional group in the 17 beta position of the steroidal skeleton (Stanczyk, 2003; Brinton et al., 2008). Progesterone-related drugs include acetylated (e.g., MPA, Figure 12b) and non-acetylated pregnans and 19-norpregnans, respectively, commonly used for hormone therapy and contraception in the USA. The majority of testosterone- and especially 19-nortestosterone-resembling progestins contain an ethynyl group in the 17 alpha position with (e.g., norethindrone acetate, NETA, Figure 12c) or without acetylation of the 17 beta hydroxyl group. A homologous subcategory within this series consists of those progestins that carry C-13 substitution with an ethyl group (Stanczyk, 2003). These progestins are often referred to as 13-ethyl gonanes, although the use of gonane as the parent name for these progestins only is misleading as a broad range of steroids are “gonane” per se (Edgren & Stanczyk, 1999; Moss, 1989). The term gonane refers to the parent tetracyclic hydrocarbon without C-10 and C-13 methyl groups and C-17 substitution (Moss,

1989). A typical representative of this subgroup of progestins is levonorgestrel (LEVO, Figure 12d), a popular choice of progestin in Europe (Brinton et al, 2008).

Having already established an acetylated progesterone derivative (MPA) as being cognitively impairing under certain parameters, it is reasonable to explore the safety of other frequently used progestins, as well as MPA when given under different treatment regimen .We selected NETA from the ethynylated 19-nortestosterone family of progestins for investigating its impact on cognition, because NETA is commonly used in both hormone therapy and contraceptive formulations (Curtis, 2006; Murphy & Brixner, 2008) in the USA and Europe. There have been limited scientific investigations of the effects of NETA, paired with ethinyl- or 17β-estradiol as hormone therapy, on cognition and brain processes in healthy menopausal women. In these studies the combination hormone therapy was associated with greater brain activation during a memory test (Smith et al., 2006; Persad et al., 2009), protection from memory decline after one and two years of treatment (Tierny et al., 2009), or no impact on self-report of memory and concentration difficulties (Gambacciani et al., 2003). While these investigations are encouraging in that they indicate potential beneficial cognitive effects of combination therapies, it still remains to be methodologically seen how NETA alone impacts the brain and its function. Another type of frequently used progestin, within the homologous 17 alpha ethynyl nortestosterone ("13-ethyl gonane") series is LEVO which is commonly prescribed in contraceptive formulations (Murphy and Brixner, 2008) but is a part of only one hormone

therapy regiment (Curtis et al., 2006). Unfortunately there have been no scientific investigations of the effects of LEVO on cognition neither in combination therapy in menopausal women nor alone in rodents, leaving little information to hypothesize the safety of this progestin on the brain and its function. However, differences in chemical structure from the cognitively-impairing progestin, MPA, and its global use make LEVO a worthy candidate for scientific studies evaluating this endpoint. In two separate studies, the current investigation aims to evaluate both NETA and LEVO's effects on cognition in the middle-age Ovx rat when given tonically, via injections, or both as compared to vehicle. We will also be exploring the cognitive effects of MPA, previously shown to impair cognition, under a new (daily injections) schedule of administration.

Study 3a

This study was designed to investigate whether the progestin NETA, given tonically, alters cognitive performance in the Ovx rat.

Methods

Subjects

Subjects were 33 twelve month old, Fisher-344 female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). Animals were acclimated for several weeks and pair housed with an identical treatment assigned cage-mate in the Arizona State University animal facility. All animals had exposure to food and water ad-lib, and were maintained on a 12-h light/dark cycle at 23°C. Procedures were approved by the ASU IACUC committee and adhered to NIH standards.

Ovariectomy and Hormone Treatment

Rats were Ovx at 12 months of age and divided into three treatment groups. Each rat was randomly assigned to either vehicle (propylene glycol, Sigma-Aldrich, St. Louise, MO) or one of two doses of NETA (Steraloids, Inc., Newport, RI), administered tonically via a Alzet osmotic pump (2006; Durect Co., Cupertine, CA). Treatment groups included: Ovx+Vehicle (n=8), Ovx+Tonic NETA-Low (4µg/day, n=9), and Ovx+Tonic NETA-Moderate (20µg/day, n=5). The low and moderate doses of NETA were based on doses given to women taking NETA hormone therapy; 1mg in FemHRT and 5mg in Aygestin (Curtis et al., 2006), respectively, adapted for body weight of the rat[1]. Ovx was performed on all rats while under isoflurane inhalation. Bilateral dorsolateral incisions were made in the skin and peritoneum, followed by ligature and removal of the ovaries and tips of the uterine horns. Muscle and skin were then sutured. At the time of surgery, Alzet osmotic pumps containing propylene glycol alone for vehicle animals, or propylene glycol plus the appropriate dose of NETA for drug-assigned animals, were inserted under the skin at the scruff of the neck. This pump model released hormone for a 6 week duration. After surgery, rats received Rimadyl (5 mg/mL/kg) for pain and saline (2 mL) to prevent dehydration. Animals underwent pump re-insertion surgery 35 days after Ovx. Behavioral testing began 42 days after the first pump insertion (7 days after the second pump re-insertion; Figure 13a). Thus, hormone administration continued throughout behavior testing and sacrifice.

[1] mg/kg calculations were based on a 70kg woman.

Initially, we included an Ovx+Tonic NETA-High (65µg/day) dose because therapeutic efficacy of higher NETA doses have not been determined, and we wanted to capture a dose response curve for NETA effects. We chose this higher dose to be one-tenth of the dose of MPA we have shown to alter cognition (Braden et al., 2010, Chapter 2), since NETA is 10 times more potent than MPA (King & Whitehead, 1986). However, upon inspection of the Alzet pumps at sacrifice, we found pump failure (pumps had cracked) for all animals in the Ovx+Tonic NETA-high group and for three animals in the Ovx+Tonic NETA-Moderate group. These animals were not used for analyses as the company informed us drug release is halted with pump cracking, thereby leaving analyses of only Ovx+Tonic NETA-Low and Ovx+Tonic NETA-Moderate animals with confirmed intact pumps.

Vaginal Smears and Uterine Weights

Vaginal smears were performed 36 days after Ovx. Each smear was identified as proestrus, estrous, metestrus or diestrus, per prior protocols (Acosta et al., 2009; Goldman et al., 2007). Smears were repeated four days later. At sacrifice, uteri from all subjects were removed, trimmed of fat and weighed immediately (wet weight) in order to examine drug effects.

General Considerations for Statistics on Behavioral Data

For behavior assessments, data were analyzed separately for each maze. Specific days, trials and blocking details are given below for each maze. First, in order to determine learning on each maze, an omnibus ANOVA including all groups was run to investigate a significant main effect of all Days and/or Trials.

For Treatment effects, since our interest was to determine whether each dose enhanced or impaired performance relative to the Ovx-Vehicle group, two-tailed, two-group planned comparisons were conducted and alpha was set at 0.05.

Water Radial-arm Maze

Subjects were tested for 13 days on the eight-arm, win-shift, WRAM to evaluate spatial working and reference memory, including performance as working memory load increased, as described previously (e.g., Bimonte & Denenberg, 1999, 2000; Bimonte et al., 2000, 2002). The maze contained escape platforms hidden under the water surface in the ends of four of the eight arms. Each subject had different platform locations that remained fixed throughout the experiment. A subject was released from the start arm and had three minutes (m) to locate a platform. Once a platform was found, the animal remained on it for 15 seconds (s), and was then returned to its heated cage for a 30s inter-trial interval (ITI) until its next trial. During the interval, the just-chosen platform was removed from the maze. The animal was then placed again into the start alley and allowed to locate another platform. For each animal a daily session consisted of four trials, with the number of platformed arms reduced by one on each subsequent trial. Thus, the working memory system was increasingly taxed as trials progressed, allowing us to access working memory load. Each subject was given one session a day for 12 consecutive days. On day 13 a six hour delay was instilled between trials 2 and 3.

Quantification and blocking were based on prior studies (e.g., Bimonte & Denenberg, 2000; Bimonte et al., 2000, 2002; Hyde et al., 1998, 2000). An arm

entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Errors were quantified using the orthogonal measures of working and reference memory errors (Jarrard, Okaichi, Steward, & Goldschmidt, 1984), as done previously in WRAM studies (Bimonte et al., 2000, 2002; Hyde et al., 2000). Working memory correct (WMC) errors were the number of first and repeat entries into any arm from which a platform had been removed during that session. Trial 1 is not analyzed for WMC errors because no platform has yet been removed. Reference memory (RM) errors were the number of first entries into any arm that never contained a platform. Working memory incorrect (WMI) errors were repeat entries into a reference memory arm.

Data were blocked into days 2-6, which is considered the acquisition phase, and 7-12, which is considered the asymptotic phase. Blocking data into these two phases is the traditional protocol for analyzing WRAM data (Bimonte & Denenberg, 1999, 2000; Bimonte et al., 2000; Hyde et al., 1998, 2000).

For WMC, WMI, and RM errors, the Ovx-Vehicle group was compared to each progestin group using planned comparison two-group repeated measures ANOVAs. To analyze effects of the 6 hour delay, Day 12 Trial 3 was considered the baseline day/trial (the last day of regular testing) and this was compared to the post delay trial given on Day 13, Trial 3. This was done to examine memory retention after a 6 hour delay. Each group was analyzed separately using a 2-within (baseline trial and post-delay trial) repeated measures ANOVA.

Morris Maze

The MM consisted of testing for 6 trials/day for three days using a tub (188 cm diameter) filled with black water made opaque with non-toxic paint. A hidden platform (10 cm wide) remained in a fixed location, thereby testing spatial reference memory (Bimonte-Nelson et al., 2006; Morris et al., 1982). The rat was placed in the maze from the North, South, East, or West location, and had 60 s to locate the hidden platform (10 cm wide), which remained in a fixed location (Northeast quadrant) throughout testing. Once the rat found the platform the trial was terminated. After the 15s platform time, the rat was placed into its heated cage until its next trial. The approximate ITI was 10m. To evaluate whether rats localized the platform to the spatial location, after all test trials on day 3, a 60s probe trial was given whereby the platform was removed. For each trial, a camera suspended above the maze tracked each rat's path and a tracking system (Ethovision 6, Noldus Instruments) analyzed each rat's tracing.

MM analyses tested distance (cm) to the platform by comparing Ovx-Vehicle to each progestin group using a planned comparison two-group repeated measures ANOVAs. For probe trial data, percent distance in the previously platformed (target) quadrant was compared to the diagonally opposite quadrant using an omnibus ANOVA (Talboom et al., 2008). Rats that learned the platform location should spend the greatest percent distance in the target vs. opposite quadrant (Stavnezer et al., 2002). For Treatment effects, an omnibus ANOVA with Treatment as the between variable, and Quadrant as repeated-measures, was used.

Visible Platform Maze

Since the WRAM and MM rely on spatial navigation, it was necessary to confirm that all subjects had intact vision and could perform the procedural swim task components without difficulty. A visible platform water-escape task was used. A rectangular tub (39 x 23 in) was filled with clear water and a black platform (10 cm wide) was elevated above the water surface. Opaque curtains covered extramaze cues. The drop off location remained the same across trials, and the platform location for each trial varied in space semi-randomly. Animals had to locate the platform protruding from the water, and were given 8 trials. Performance was assessed by latency (s) to the platform.

Results

Vaginal Smears and Uterine Weights

All animals, regardless of treatment group, exhibited diestrous vaginal smears with primarily leukocytic cells. Uterine weights were analyzed using ANOVA with Treatment as the between variable. Mean ± SE uterine weights were: Ovx+Vehicle (0.163 ± 0.038), Ovx+Tonic NETA-Low (0.114 ± 0.006), and Ovx+Tonic NETA-Moderate (0.152 ± .038). Uterine weights did not differ between treatment groups [$F(2, 19) = 0.869$; $p = 0.44$]. These results are in agreement with the known non-estrogenic activity of NETA (e.g., Sitruk-Ware, 2006).

Water Radial-arm Maze

Acquisition and Asymptotic Testing Phases

Across Days 2-12 (combined acquisition and asymptotic testing phases) there was a Day main effect for the omnibus ANOVA for each type of error [WMC: $F(10, 190) = 3.564$; $p < 0.0005$; WMI: $F(10, 190) = 2.913$; $p < 0.005$; RM: $F(10, 190) = 3.661$; $p < 0.0005$], with errors decreasing across days, demonstrating learning of the task.

For the acquisition phase, there was a main effect of Treatment for the Ovx+Vehicle vs. Ovx+Tonic NETA-Moderate comparison for WMC [$t(11) = 2.68$; $p < 0.05$ (Figure 14a)], WMI [$t(11) = 2.96$; $p < 0.05$ (Figure 14b)], and RM [$t(11) = 2.20$; $p < 0.05$ (Figure 14c)], with the moderate NETA dosed group committing more of all three error types and therefore, showing impaired performance. There were no main effects or interactions of Treatment for Ovx+Vehicle vs. Ovx+Tonic NETA-Low on the acquisition phase or either NETA dose for the asymptotic phase.

Delay Testing

To evaluate which groups were impaired by the 6 hour delay, we compared performance on Day 12 Trial 3 (baseline), to Day 13 Trial 3 (post-delay). For WMC errors, on Trial 3, the trial immediately following the delay, the Ovx+Tonic NETA-Low and the Ovx+Tonic NETA-Moderate groups each made more errors as compared to baseline [Ovx+Tonic NETA-Low Day effect for baseline day vs. delay day: $t(8) = 4.00$; $p < 0.05$; Ovx+Tonic NETA-Moderate Day effect for baseline day vs. delay day: $t(4) = 3.5$; $p < 0.05$ (Figure 15)],

indicating that the delay impaired performance in both NETA groups. This delay-induced decrement was not observed in Vehicle treated rats (Ovx+Vehicle Day effect for baseline day vs. delay day: $p = 0.57$). There were no delay-induced effects for WMI or RM errors.

Morris Maze

There was a Day main effect for distance for the omnibus ANOVA [Distance: $F(2, 58) = 237.316$; $p < 0.0001$], with scores decreasing across days, demonstrating learning of the task. For the Ovx+Vehicle vs. Ovx+Tonic NETA-Moderate comparison, there was a Day x Treatment interaction [$F(2, 22) = 3.44$; $p < 0.05$], with Ovx+Tonic NETA-Moderate animals exhibiting higher distance scores as compared to Ovx+Vehicle animals on Days 2 and 3 [Treatment effect for Days 2 and 3 combined: t (11)=2.16; $p = 0.05$ (Figure 16a)]. The Day x Treatment interaction was not significant for the Ovx+Vehicle vs. Ovx+Tonic NETA-Low comparison ($p = 0.73$), and there were no Treatment main effects for either dose.

For the probe trial, a higher percent distance was spent in the previously platformed (NE) versus the opposite (SW) quadrant [Quadrant main effect: F (1, 19) = 77.77; $p < 0.0001$ (Figure 16b)]. This pattern did not differ by Treatment [null Quadrant x Treatment interaction], suggesting that all groups localized the platform location by the end of testing.

Visible Platform

There was a main effect of Trial [$F(5, 95) = 7.985$; $p < 0.0001$ (Figure 17)], with latency decreasing across trials. There were no Treatment effects for

either Vehicle vs. NETA dose group for the visible platform task. The average time to find the platform was 7 s by the last trial of testing, confirming visual and motor competence for solving a water-escape swim task.

Study 3b

In Study 3a, the progestin NETA impaired cognition in the middle-age Ovx rat when given tonically via osmotic pump. Study 3b was designed to investigate whether it is the tonic schedule of administration of NETA, in Study 1, and MPA, in a previous study (Braden et al., 2010, Chapter 2), that led to the observed impairing effects. Study 3b also examines the cognitive effects of a progestin novel to cognitive testing, LEVO, which is of a different chemical structure category than NETA and MPA. Thus, in Study 3a, the effects of MPA and NETA, given via injections, on cognition were investigated and compared to the progestin novel to cognitive testing, LEVO. Additionally, in keeping with the Food and Drug Administrations' recommendation of limiting the length of hormone therapy use (Food and Drug Administration, 2009), we reduced the duration of progestin treatment before test.

Methods

Subjects

Subjects were 42 thirteen month old, Fisher-344 female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). Acclimation, housing, food and water, and light/dark procedures were identical to study 3a.

Ovariectomy and Hormone Treatment

All rats were ovariectomized (Ovx) at 13 months of age, 15 days prior to behavioral testing, under isoflurane inhalation. The Ovx procedure was identical to Study 3a. Rats were randomly assigned to one of five treatment groups, with vehicle (0.4mL sesame oil + 0.02mL dimethyl sulfoxide [DMSO], both from Sigma-Aldrich, St. Louis, MO, USA) or drug given via injection (inj): Ovx+Vehicle (n=8), Ovx+Inj MPA (700 µg, n=9; Sigma-Aldrich, St. Louise, MO), Ovx+Inj NETA-Moderate (20 µg, n=8; Steraloids, Inc., Newport, RI), Ovx+Inj NETA-High (70 µg, n=4), Ovx+Inj LEVO-Low (0.06 µg, n=9; Sigma-Aldrich, St. Louise, MO), and Ovx+Inj LEVO-High (0.6 µg, n=9). The dose of MPA was based on that of a previous study (Braden et al., 2010, Chapter 2). The NETA-Moderate dose was based on a dose given to women in HT (5mg in Aygestin; Curtis et al., 2006) adapted for the body weight of a rat, and the NETA-High dose was one-tenth of the dose of MPA since NETA is 10 times more potent than MPA (King and Whitehead, 1986). The low and high doses of LEVO were based on doses given to women taking LEVO clinically, 0.015mg in Climara Pro and 0.15mg in Seasonalle (Climara Pro Prescribing Information; Seasonique Prescribing Information), respectively, adapted for body weight of the rat[2]. Behavioral testing began 13 days after hormone administration was initiated and continued throughout the study (4 weeks; Figure 13b). The last treatment injection was administered 1 hour before sacrifice.

[2] mg/kg calculations were based on a 70kg woman.

Vaginal Smears

Vaginal smears were performed 2 weeks after Ovx, one day prior to behavioral testing. Each smear was identified as either proestrus, estrous, metestrus or diestrus, per prior protocols (Acosta et al., 2009; Goldman et al., 2007).

Uterine Weights, Statistical, Water Radial-arm Maze and Morris Water Maze procedures

Procedures were identical to study 3a.

Results

Water Radial-arm Maze

Acquisition and Asymptotic Testing Phases

Across Days 2-12 (combined acquisition and asymptotic testing phases) there was a Day main effect for the omnibus ANOVA for each type of error [WMC: $F(10, 390) = 13.567$; $p < 0.0001$; WMI: $F(10, 390) = 6.565$; $p < 0.0001$; RM: $F(10, 390) = 7.912$; $p < 0.0001$], with errors decreasing across days, demonstrating learning of the task.

For the asymptotic phase, there was a main effect of Treatment for the Ovx+Vehicle vs. Ovx+Inj NETA-High comparison for WMC [$t(10) = 2.31$; $p < 0.05$ (Figure 18a)], with the high NETA dose group committing fewer errors and therefore, showing enhanced performance. This difference was also evident at Trial 4, at the highest working memory load [$t(10) = 2.57$; $p < 0.05$ (Figure 18b)]. There were no effects of the high NETA dose on WMI or RM errors for the asymptotic phase, or any of the three error types for the acquisition phase. For

each of three error types, the moderate NETA dose, MPA, and both doses of LEVO had no effect on the acquisition or asymptotic phases yielding no main effect or interactions with Treatment.

End of Testing: Days 11-12

In order to examine a potential replication of our previously published MPA- (Braden et al., 2010, Chapter 2) and progesterone- (Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2) induced impairments, we investigated group differences at the end of testing. For Days 11-12, there was a main effect of Treatment for the Ovx+Vehicle vs. Ovx+Inj MPA comparison for WMI [t (15) = 1.86; $p < 0.05$ (Figure 19a)] and RM [t (15) = 1.86; $p < 0.05$ (Figure 19b)], with the MPA group committing more errors and therefore, showing impaired performance. There were no differences between Ovx+Vehicle and Ovx+Inj MPA groups for WMC Errors. For each of the three error types, both doses of NETA and both doses of LEVO had no effect at the end of testing.

Delay Testing

To evaluate which groups were impaired by the 6 hour delay, we compared performance on Day 12 Trial 3 (baseline), to Day 13 Trial 3 (post-delay). For WMC errors, on Trial 3, the trial immediately following the delay, all groups made significantly more errors as compared to baseline [effect for baseline day vs. delay day: Ovx+Vehicle: t (7) = 8.28; $p < 0.0001$; Ovx+Inj MPA: t (8) = 5.5; $p < 0.001$; Ovx+Inj NETA-Moderate: t (7) = 4.96; $p < 0.005$; Ovx+Inj LEVO-Low: t (8) = 3.46; $p < 0.01$; (Figure 20)], except for Ovx-Inj NETA-High who made marginally more errors [t (3) = 3; $p = 0.06$] and Ovx+Inj LEVO-High,

who were not impaired [t (6) = 1; p = 0.36]. These findings indicate that the delay impaired performance in Vehicle, MPA, NETA-Moderate, and LEVO-Low groups. This delay-induced decrement was marginally observed in NETA-High treated rats (p =0.06) and not seen at all in LEVO-High treated rats. There were no delay-induced effects for WMI or RM.

Morris Water Maze

There was a Day main effect for distance for the omnibus ANOVA [Distance: F (2, 78) = 241.328; p < 0.0001 (Figure 21a)], with scores decreasing across days, demonstrating learning of the task. There were no Treatment main effects or Day x Treatment interactions between Ovx-Vehicle versus any progestin-treated group.

For the probe trial, a higher percent distance was spent in the previously platformed (NE) versus the opposite (SW) quadrant [Quadrant main effect: t (40) = 20.1; p < 0.0001 (Figure 21b)]. This pattern did not differ by Treatment [null Quadrant x Treatment interaction], suggesting that all groups localized the platform location by the end of testing.

Discussion

The effectiveness of clinically used progestins in offsetting the increased risk of endometrial hyperplasia associated with unopposed estrogen treatment is well documented (Smith et al., 1975; Ziel and Finkle, 1975); however, little is known about the potential risks to cognition due to clinically-used progestins. With a progestin included in every HT given to women with a uterus, and in every birth control formulation, this is an essential line of research both clinically and

preclinically. The current study corroborates previous evidence from our laboratory that the most commonly used progestin in the USA for HT, MPA, impairs learning and memory in the Ovx middle-aged rodent. The study also present for the first time conditions under which two other types of frequently used progestins for hormone therapy and contraception, via NETA and LEVO, improve and/or impair cognition in the Ovx female rat.

In Study 3a, both the low and moderate doses of NETA, given tonically, impaired working memory retention of a six hour delay on the WRAM, as compared to vehicle treated rats. While this was the only impairment seen in the tonic low dose NETA rats, the tonic moderate dose NETA rats were impaired in working and reference memory performance during the acquisition of the WRAM and on reference memory performance on the MM, as compared to vehicle. In Study 3b this same moderate dose of NETA, as well as a high NETA dose, was investigated using a treatment regimen of daily injections. Under the method of daily injections, the moderate NETA dose that previously impaired cognition when given tonically had no effect as compared to vehicle-treated rats. Further, the high NETA dose, when given via injections, improved working memory performance during the asymptotic phase of testing on the WRAM and partially protected against a six hour delay-induced impairment on the same task, both effects as compared to vehicle. It was at the six hour delay on the WRAM that we also saw cognitive enhancements as a result of daily injections with the high dose of LEVO, as compared to vehicle. Finally, in Study 3b we examined the effects of daily injections of MPA at a dose that we have previously shown to impair

cognition when given tonically (Braden et al., 2010, Chapter 2) or via weekly injections (Braden et al., 2011, Chapter 3). Indeed, daily injections of MPA resulted in impaired working and reference memory performance during the latter part of testing on the WRAM, as compared to vehicle. Taken together, our past (Braden et al., 2010 & 2011, Chapter 2 & 3) and present results with MPA treatment clearly show the detrimental effect of this 17α-acetoxy-6α-methyl progesterone on cognition independently from the treatment regimen. Figure 22 summarizes the significant behavior effects.

Most notable in our results are the divergent effects of NETA depending on the parameters associated with its treatment. In Study 3a, NETA animals were treated tonically for six weeks prior to test at doses of 4 and 20 µg/day, both of which led to cognitive impairments. In Study 3b, NETA animals were treated via daily injections for two weeks prior to test at doses of 20 and 70 µg/day, with the 70µg/day dose leading to cognitive enhancements. Thus, we conclude that longer durations of lower doses of NETA given tonically are associated with impaired cognition, while short durations of higher doses of NETA given cyclically improve cognition, in the middle-aged Ovx rodent. Further research needs to be done to determine exactly which of these three parameters (dose, schedule of treatment, and treatment duration) are critical in modulating the cognitive decrements or enhancements associated with NETA treatment. The cognitive enhancing effects of NETA are in line with clinical studies examining the effects of combined HT using this progestin (Smith et al., 2006; Persad et al., 2009; Tierny et al., 2009). It is noteworthy that in our studies NETA only enhanced

cognition in Study 3b when the route of administration was daily injections, presumably inducing pharmacokinetics and pharmacodynamics more similar to a woman taking a daily oral pill, as in the clinical investigations. Metabolism and bioavailability under various route of administration may also be a significant parameter influencing NETA's, or in general progestins', impact on brain functions.

The current study is the first to investigate the cognitive effects of LEVO in any basic science or clinical setting. Here we found that while LEVO did not have a profound effect on cognition, it did prevent a delay induced impairment on the WRAM, suggesting that LEVO may enhance memory retention. Taken together, our hypothesis was supported in that progestins with a different chemical structure (Figure 12c, d) than the cognitively impairing MPA (an acetylated pregnane derivative, Figure 12b) can enhance cognition in the middle-age Ovx rat, given the right parameters.

This is now our third report showing that MPA when given alone impairs cognition in the rodent model. Other rodent studies have shown that long-term treatment of 17β-estradiol plus MPA led to impaired spatial memory performance, as compared to 17β-estradiol, in Ovx rats (Lowry et al., 2010). Additionally, in young Ovx mice, the addition of MPA to natural progesterone treatment reversed the cognitive benefits seen with progesterone alone (Frye et al., 2010). In other basic science work, MPA has attenuated or blocked the beneficial effects of 17β-estradiol on neuronal health in hippocampal cell cultures (Nilsen & Brinton, 2002a, 2002b), and exacerbated glutamate-induced toxicity

when given alone (Nilsen et al., 2006). Further, our findings are corroborated by a case study of amnesia corresponding with the use of MPA in the contraceptive depo provera (Gabriel & Fahim, 2003) and indirect clinical evidence, via the combined estrogen alone and estrogen+progestin trials, that the addition of MPA to hormone therapy increases risk of dementia (Shumaker et al., 2003, 2004).

It is critical to find a progestin option for women taking hormone therapy and birth control that will protect against estrogen induced endometrial hyperplasia but will not have adverse effects on cognition, such as those seen with MPA. Here we present two progestins, NETA and LEVO, as alternative options to be evaluated for cognition in women. Future research must be done to better understand how these two progestins interact with estrogens for cognition both in the clinic and in animal models, as well as the mechanisms behind divergent effects of progestins on cognition.

CHAPTER 5

THE $GABA_A$ ANTAGONIST, BICUCULLINE, ATTENUATES PROGESTERONE, BUT NOT MPA, INDUCED MEMORY-IMPAIRMENTS

Chapter 5a

The $GABA_A$ antagonist bicuculline attenuates progesterone induced memory-impairments

Nearly every woman will face the decision of whether to take exogenous hormones, either for contraception or for HT, and those with a uterus must include a progestogen in their regimen (Smith et al., 1975). The most commonly prescribed progestin component of HT, and the sole hormone component of the contraceptive Depo Provera, MPA, has been associated with memory impairments in rodent models (Braden et al., 2010, 2011, Chapters 2 & 3) and adverse in vitro cellular effects (Nilsen et al., 2006; Nilsen & Brinton, 2002a, 2002b). In postmenopausal women, combination HT treatment including MPA lead to an increase in risk of dementia (Shumaker et al., 2003). There is evidence in women that, like MPA, natural progesterone is associated with detrimental effects on cognition in both the phenomenon of "maternal amnesia" (Brett & Baxendale, 2001) and in a controlled study of healthy women receiving a large dose of oral progesterone (Freeman et al., 1992). Progesterone has also been shown to impair memory in rodent models (Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2; Sun et al., 210) and reverse the beneficial effects of 17β-estradiol in vivo (Bimonte-Nelson et al., 2006; Harburger et al., 2007) and in vitro (Nilsen & Brinton, 2002a; Aguirre & Baudry, 2009). These findings are

particularly salient since the use of progesterone (specifically, natural progesterone in its micronized form to be orally bioavailable) as the progestogen component of HT has only become an option for women in the United States as recently as 1998 (Langer, 1999), and is a promising new candidate to replace MPA as the progestogen component in HT (Langer, 1999; Sherwin & Grigorova, 2011).

A potential mechanism for progesterone-induced memory impairments in the rodent model is the GABAergic system. Indeed, we have shown that progesterone impacts the GABAergic system of cognitive brain regions in the Ovx rat (Braden et al., 2010, Chapter 2). However, it remains to be determined, through direct pharmacological manipulation, whether the GABAergic system is a mechanism of progesterone's effects on cognition during aging in the Ovx rat. Evidence suggests that progesterone impairs memory by increasing $GABA_A$ stimulation in the hippocampus, in turn resulting in greater inhibition of the hippocampus, and thereby detrimentally affecting cognition. We propose to address the question of whether progesterone's effects on memory are, in part, due to $GABA_A$ modulation by administering the $GABA_A$ receptor antagonist, bicuculline, to progesterone-treated animals. We hypothesize that bicuculline will prevent progesterone's negative effects on cognition. In this study, we also aim to determine if in vivo treatment with progesterone and/or bicuculline alters the number GABA-producing cells in the hippocampus. We predict that the number of GABA-producing cells will be down regulated by progesterone treatment, similarly to the protein changes observed in Braden et al. (2010, Chapter 2), and

that this will be reversed by concomitant treatment with bicuculline, mirroring the expected behavioral outcomes.

Contrary to the negative effects of long-term progesterone treatment on cognition in normal aging (Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2), short-term progesterone treatment after traumatic brain injury has been associated with favorable outcomes in both humans and animal models (Stein, 2012). Specifically, in cortical contusion models, progesterone improves cognitive performance and reduces oedema and neuronal degeneration (Roof et al., 1993, 1994, 1996). The mechanism of these effects may be modulation of Cox-2, a key enzyme in the formation of prostaglandins in the inflammatory response (Breder et al., 1995) that is induced in glutamate excitotoxicity (Tocco et al., 1997). Previous research indicates progesterone can result in upregulation or downregulation on this system; indeed, one study found a progesterone-induced decrease of Cox-2 expression following cortical contusion (Cutler et al., 2007) and another an increase after LPS-induced inflammation (Sunday et al., 2006). In the current study, we aimed to determine if changes in Cox-2 expression in the frontal cortex, a brain area intimately involved in working memory (Jones, 2002), are related to memory-impairing effects of progesterone in the middle-aged Ovx rat. Investigating the GABAergic and neuroinflammatory system's relationship to the cognitive impact of progesterone in an animal model is an early step in answering questions of the mechanisms by which natural progesterone impairs cognition. With this knowledge, we can move forward in the field of hormone

treatment with prosgetogens that do not share these pharmacological characteristics, and are thus potentially safer alternatives for inclusion in HTs.

Methods

Subjects

Subjects were 37, 13 month old Fischer-344 female rats born and reared at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). Rats were acclimated for several weeks before surgery, had access to food and water ad-lib, and were maintained on a 12-hour light/dark cycle at the Arizona State University animal facility. All procedures were approved by the local IACUC committee and adhered to NIH standards.

Hormone Treatment and Ovariectomy

Rats were randomly assigned to one of four treatment groups: Ovx+Vehicle, Ovx+Progesterone (Ovx+Prog), Ovx+Bicuculline (Ovx+Bic), or Ovx+Prog+Bic. Identical to Study 3b (Chapter 4), at 13 months of age all rats were anesthetized via isofluorane inhalation, underwent bilateral dorsolateral incisions in the skin and peritoneum, and the ovaries and tips of uterine horns were ligatured and removed. Muscle and skin were then sutured. Two days after surgery all drugs were administered via two daily subcutaneous injections in the scruff of the neck. For the first injection, animals received either a control vehicle solution (0.4mL sesame oil + 0.02mL dimethyl sulfoxide [DMSO], both from Sigma-Aldrich, St. Louis, MO, USA) or progesterone (0.7 mg, Sigma-Aldrich St. Louis, MO, USA; dissolved in 0.4mL sesame oil + 0.02mL DMSO) and for the second injection, animals received either a control vehicle solution (sesame oil +

10% DMSO) or bicuculline (3.5mg/kg; dissolved in sesame oil + 10% DMSO). Injections continued throughout behavior testing until sacrifice.

Vaginal Smears and Uterine Weights

Vaginal smears were taken 12 and 13 days after Ovx and classified as proestrus, estrous, metestrus or diestrous per prior protocol (Acosta et al. 2009; Goldman et al. 2007). At sacrifice, the uteri of each subject was removed, trimmed of visible fat, and immediately weighed (wet weight), in order to examine hormone and drug effects on uterine tissues (Braden et al., 2010, 2011; Chapter 2 & 3).

Water Radial-Arm Maze

Thirteen days after initiation of injections, subjects were tested on the WRAM for 15 days to evaluate spatial working and reference memory, including performance as working memory load increased, as described previously (Bimonte & Denenberg, 2000; Bimonte et al., 2000, 2002). The maze contained escape platforms hidden under the water surface in the ends of 4 of the 8 arms. Each subject had different platform locations that remained fixed throughout the experiment. A subject was released from the start arm and had three minutes (m) to locate a platform. Once a platform was found, the animal remained on it for 15 seconds (s), and was then returned to its heated cage for a 30s inter-trial interval (ITI) until its next trial. During the interval, the just-chosen platform was removed from the maze. The animal was then placed again into the start alley and allowed to locate another platform. For each animal a daily session consisted of four trials, with the number of platformed arms reduced by one on each subsequent trial.

Thus, the working memory system was increasingly taxed as trials progressed, allowing us to access working memory load. Each subject was given one session a day for 14 consecutive days. On Day 15, a 6 hour delay was administered between Trials 2 and 3 as in Studies 3a and 3b (Chapter 4).

Quantification and blocking were based on prior studies (Bimonte & Denenberg, 2000; Bimonte et al., 2000, 2002; Hyde et al., 1998, 2000). An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Errors were quantified using the orthogonal measures of working and reference memory errors (Jarrard, Okaichi, Steward, & Goldschmidt, 1984), as done previously in WRAM studies (Bimonte et al., 2000, 2002; Hyde et al., 2000). Working Memory Correct (WMC) errors were the number of first and repeat entries into any arm from which a platform had been removed during that session. Trial 1 is not analyzed for WMC errors because no platform has yet been removed. Reference memory (RM) errors were the number of first entries into any arm that never contained a platform. Working Memory Incorrect (WMI) errors were repeat entries into a RM arm.

Morris Maze

The day after WRAM completion, subjects were tested on the MM for 6 trials/day for 3 days using a tub (188 cm diameter) filled with black water made opaque using non-toxic paint. A hidden platform (10 cm wide) remained in a fixed location, thereby testing spatial reference memory (Bimonte-Nelson et al., 2006; Morris et al., 1982). The rat was placed in the maze from the North, South, East, or West location, and had 60s to locate the hidden platform, which remained

in the Northeast quadrant throughout testing. Once the rat found the platform the trial was terminated. After 15s platform time, the rat was placed into its heated cage until its next trial. The approximate ITI was 10-15m. To evaluate whether rats localized the platform to the spatial location, after all test trials on day 3, a 60s probe trial was given whereby the platform was removed. For each trial, a camera suspended above the maze tracked each rat's path and a tracking system (Ethovision 3.1, Noldus Instruments) analyzed each rat's tracing.

MM performance was assessed by swim path distance (cm) and latency (s) to the platform. For probe trial data, percent of total distance in the previously platformed (target) quadrant was compared to the quadrant diagonally opposite the platform. Rats that learned the platform location were expected to spend the greatest percent distance in the target quadrant (Stavnezer et al., 2002).

Visible Platform Maze

To confirm that all subjects had intact vision and could perform the procedural task components of MM and WRAM spatial navigation without difficulty, subjects were tested on a VP water-escape task. A rectangular tub (39 x 23 in) was filled with clear water and a black platform (10 cm wide) was elevated above the water surface. Opaque curtains covered extramaze cues. The drop off location remained the same across trials, and the platform location for each trial varied in space semi-randomly. Animals had to locate the protruding platform, and were given 6 trials in one day. Performance was assessed by latency (s) to the platform.

Tissue Preparation

After the conclusion of behavioral testing, rats were anesthetized with isofluorane. Brains were removed, blocked anterior to the hippocampus, and post-fixed in PFA for 48 hours. After 48 hours the brains were moved to PB and stored at 4 °C. Two days prior to sectioning, brains were placed in a 30% sucrose solution for 48 hours. Brains were coronally sectioned at 40 μm on a cryostat (Microtome HM 500 OM Cryostat) at -20 °C, and free floating sections were collected for immunohistochemistry.

Immunohistochemistry and Quantification of GAD 67

Dorsal hippocampal sections in the posterior block of brains (Plates 33 – 37; Paxinos and Watson, 1998) were washed three times for 5m each in PBS, and then incubated in 1% H2O2 diluted in PBS for 30m at room temperature (RT). Next, sections were washed three times (all GAD 67 washes = 10m for the remainder of staining) and placed in blocking buffer (5% normal goat serum, 2.5% BSA, 0.2% Triton-X, in 1x PBS) for 30m at RT. Incubation in a 1:500 dilution of mouse anti-GAD 67 primary antibody (MAB5406; Millipore, Billerica, MA, USA) diluted in blocking buffer, for 48 hours at 4°C, followed. Next, sections were washed four times in PBS and incubated in a 1:500 dilution of Alexa Fluor 568 goat anti-mouse IgG (H+L) (A-21206; Invitrogen, Grand Island, NY), diluted in blocking buffer, for two hours at RT, followed by a second round of four washes. Finally, sections were mounted on slides, left to air dry, and coverslipped using anti-fading mounting medium (Vectashield, Vector Labs, Burlingame, CA, USA). Control procedures were run excluding primary and

secondary antibodies. Exclusion of the primary antibody resulted in no cell staining, and exclusion of secondary antibody resulted in a lack of fluorescent development.

One experimenter, blind to treatment group status of the animals, performed all image analysis procedures. Images were acquired using Olympus DP70 digital camera fitted on an Olympus BX-50 microscope using DP-BSW Software (version 2.02, Olympus America Inc., Center Valley, CA). A 10X objective was used to capture representative images, bilaterally, from the CA1, CA3, and DG of the hippocampus. Captured images were manually counted using the Cell Counter plugin under NIH ImageJ software (Rasband, 1997–2004) in three sections of the dorsal hippocampus of five animals per treatment group, similarly to procedures used previously in our laboratory (Acosta et al., 2009). A cell was included as positive for GAD labeling if the interior was greater than 75% filled with a pixel intensity darker than the background.

Immunohistochemistry and Quantification of Cox-2

Frontal cortex sections in the anterior block of brains (Plates 9 – 17; Paxinos and Watson, 1998) were washed twice (all Cox-2 washes = 5m) in PBS then incubated in 0.2% H2O2 diluted in PBS for 15 m at RT. Next, sections were washed twice and placed in blocking buffer (1% BSA, 0.2% Triton-X in 1x PBS) for 30m at RT. Incubation in a 1:250 dilution of rabbit anti-Cox-2 primary antibody (ab15191; Abcam Inc., Cambridge, MA, USA) diluted in blocking buffer, for two hours at RT, followed. Next, sections were washed three times for in PBS and incubated in a 1:500 dilution of biotin-conjugated goat anti-rabbit

(111-065-003; Jackson Immuno Research, West Grove), diluted in blocking buffer, for one hour at RT, followed by a second round of three washes. Finally, sections were treated with ABC reagent (Vector Laboratories, Burlingame, CA) and incubated for 45m at RT. Sections were washed three times in PBS, and then incubated with DAB Peroxidase Substrate (Vector). After the desired color was achieved (dark purple) brain sections were washed three times in PBS, mounted on slides, dehydrated, and coverslipped. Control procedures were run excluding primary and secondary antibodies. Exclusion of the primary antibody resulted in no cell staining, and exclusion of secondary antibody resulted in a lack of DAB Peroxidase Substrate color development.

One experimenter, blind to treatment group status of the animals, performed all image analysis procedures. Images were acquired using an Olympus DP70 digital camera fitted on an Olympus BX-50 microscope using DP-BSW Software (version 2.02, Olympus America Inc., Center Valley, CA). A 4X objective was used to capture images. Captured images were manually counted using the Cell Counter plugin under NIH ImageJ software (Rasband, 1997–2004), bilaterally, in 12 sections of the frontal cortex of 4-5 animals per treatment group, similarly to procedures used previously in our laboratory (Acosta et al., 2009). A cell was included as positive for Cox-2 labeling if the interior of the cell had a pixel intensity of four standard deviations greater than the mean pixel intensity of the region of interest, as other protocols have done (Parent, Vexler, Gong, Derugin, & Ferriero, 2002).

Statistical Analyses

For behavior assessments, data were analyzed separately for each maze. To evaluate learning and potentially complex higher order Treatment interactions with Days or Trials, we utilized an omnibus repeated measures ANOVA including all groups, with Treatment as the between variable and blocks of Days and/or Trials as the within variable/s. For uterine weights, GAD cell counts, and Cox-2 cell counts, Treatment effects were assessed via ANOVA. When ANOVA yielded a significant effect of Treatment, two group comparisons using t-tests were used to investigate group differences. For all analyses, alpha was two-tailed and set at 0.05.

Results

Water Radial-Arm Maze

Learning

Across all testing days (Days 1-14), and including all treatment groups, there was a day main effect for each error type for the omnibus ANOVA [WMC: $F(13, 429) = 5.828$; $p < .0001$ (Figure 23a); WMI: $F(13, 429) = 11.638$; $p < .0001$ (Figure 23c); RM: $F(13, 429) = 12.839$; $p < .0001$ (data not shown)], with errors decreasing across days, demonstrating learning.

Treatment Effects

To evaluate Treatment effects and interactions, days 1-14 were blocked into 2-3 day blocks. We traditionally block data into two large blocks (e.g. Bimonte & Denenberg, 1999); however, for the current study we further divided blocks to provide a finer tuning of when effects occur, based on our prior

progesterone data showing effects on learning (Bimonte-Nelson et al., 2004b; Braden et al., 2010, Chapter 2). For WMC errors, there was a significant omnibus ANOVA main effect of Treatment [$F(3, 33) = 2.955$; $p < .05$], and a Treatment x Trial interaction [$F(6, 66) = 4.092$; $p < .005$] for Block 3 (Days 6-8). For Block 3, the Ovx+Prog+Bic group committed fewer errors than all other treatment groups [Ovx+Vehicle vs. Ovx+Prog+Bic : $t(17) = 2.222$; $p < .05$; Ovx+Prog vs. Ovx+Prog+Bic: $t(16) = 3.061$; $p < .01$; Ovx+Bic vs. Ovx+Prog+Bic: $t(16) = 2.125$; $p < .05$ (Figure 23a)], demonstrating a working memory enhancement of the combination of progesterone and bicuculline. On Trial 4 alone, at the highest working memory load, there was a main effect of Treatment [$F(3, 33) = 5.367$; $p < .005$] with Ovx+Prog animals committing more errors than Ovx+Vehicle [$t(17) = 2.186$; $p < .05$ (Figure 23b)]. Interestingly, Ovx+Prog animals also committed more errors than Ovx+Prog+Bic [$t(16) = 3.146$; $p < .01$] animals, demonstrating that the progesterone-induced working memory impairment is reversed by the addition of bicuculline (Figure 23b). Ovx+Bic animals also committed more errors than Ovx+Vehicle [$t(17) = 2.399$; $p < .05$] and Ovx+Prog+Bic [$t(16) = 3.528$; $p < .005$] animals on Trial 4 during Block 3, demonstrating a bicuculline-induced working memory impairment that is reversed by progesterone (Figure 23b). For WMI errors, there was also a significant omnibus ANOVA main effect of Treatment [$F(3, 33) = 4.007$; $p < .05$] and Treatment x Trial interaction [$F(9, 99) = 3.096$; $p < .005$] for Block 3. For Block 3, the Ovx+Prog group committed more errors than all other treatment groups [Ovx+Prog vs. Ovx+Vehicle: $t(17) = 2.364$; $p < .05$; Ovx+Prog vs. Ovx+Prog+Bic: $t(16) = 2.21$; $p < .05$; Ovx+Prog vs.

Ovx+Bic: $t(16) = 2.878$; $p < .05$ (Figure 23c)]. In Block 3, there was a main effect of treatment at the highest working memory load (Trial 4) [$F(3, 33) = 4.117$; $p < .05$], where the same pattern was evident, with the Ovx+Prog group committing more errors than all other treatment groups [Ovx+Prog vs. Ovx+Vehicle: $t(17) = 2.386$; $p < .05$; Ovx+Prog vs. Ovx+Prog+Bic: $t(16) = 2.734$; $p < .01$; Ovx+Prog vs. Ovx+Bic: $t(16) = 2.561$; $p < .05$ (Figure 23d)]. These WMI effects further demonstrate a progesterone-induced working memory impairment that is reversed by the addition of bicuculline. There were no significant effects for RM errors during any block.

Delay

After regular testing, a 6 hour delay was administered between trials 2 and 3. For WMC errors, we compared baseline performance on Trial 3 (Day 14, last day of regular testing) to Trial 3 immediately after the delay on Day 15, within each group. The Ovx+Prog group was the only group to significantly increase errors after the delay [Ovx+Prog: $t(8) = 3.162$; $p < .05$], demonstrating a susceptibility to delay-induced working memory impairments in progesterone-treated animals (Figure 24). The progesterone-induced impairment was obviated with the addition of bicuculline [Ovx+Prog+Bic: $t(8) = .634$; $p = .54$]. No group displayed a delay-induced increase in WMI or RM errors.

Morris Maze

Learning

For the omnibus ANOVA including all treatment groups, there was a Day main effect for Distance and Latency [Distance: $F(2, 66) = 259.666$; $p < .0001$

(Figure 25a); Latency: $F(2, 66) = 138.796$; $p < .0001$ (data not shown)], with scores decreasing across days, demonstrating learning of the task.

Treatment Effects

Trials were blocked into three trial blocks, as done previously (Talboom et al., 2010). For Block 5, there was a main effect of Treatment [$F(3, 33) = 2.903$; $p < .05$]. Ovx+Prog+Bic animals swam a shorter distance to the platform than Ovx+Vehicle animals for block 5 [$t(17) = 2.448$; $p < .05$], demonstrating a reference memory enhancement induced by the combination of progesterone and bicuculline (Figure 25a).

Probe

The probe trial revealed a main effect of Quadrant [$F(1, 33) = 182.603$; $p < .0001$] and no main effect of, or interactions with, Treatment. Each group spent a greater percent Distance in the target NE quadrant as compared to the opposite SW quadrant, showing localization of the platform location for all groups [Ovx+Vehicle: $t(9) = 4.861$; $p < .001$; Ovx+Prog: $t(8) = 7.997$; $p < .0001$; Ovx+Bic: $t(8) = 10.783$; $p < .0001$; Ovx+Prog+Bic: $t(8) = 5.782$; $p < .0005$ (Figure 25b)].

Visible Platform

There was a Trial main effect [$F(5, 165) = 16.642$; $p < .0001$ (Figure 26)], with Latency decreasing across all trials, demonstrating learning of the task. The omnibus ANOVA effect for Treatment was not significant for latency to the visible platform. By the fourth trial, all groups found the VP within 9s, confirming visual and motor competence to perform swim maze tasks for all groups.

Vaginal Smears

Twelve and 13 days after Ovx, vaginal smears were taken and classified as proestrus, estrous, metestrus or diestrous, per prior protocol (Acosta et al., 2009; Goldman et al., 2007). As expected, all animals, regardless of treatment, showed leukocytic smears, indicative of the diestrous phase.

Uterine Weights

The omnibus ANOVA effect of treatment was not significant for uterine weight, and there were no significant uterine weight effects for each prog and/or bic group as compared to Vehicle. (Table 6).

Frontal Cortex Cox-2 Cell Counts

The omnibus ANOVA effect of treatment was not significant for frontal cortex Cox-2 cell counts, and there were no significant Cox-2 cell count effects for each prog and/or bic group as compared to Vehicle. (Table 7).

Hippocampal GAD Cell Counts

The Treatment effect for the omnibus ANOVA for GAD cells counts was not significant for CA1, CA3, or DG. Within each region there were no significant effects for each prog and/or bic treated group compared to Vehicle (Table 7).

Discussion

Natural progesterone is a clinically used component of HT, and a promising new candidate to replace MPA as the most commonly used progestogen component in HT (Sherwin & Grigorova, 2011; Langer, 1999). We have previously shown that both progesterone and MPA impair memory in the

menopausal rat model (Bimonte-Nelson et al., 2004b; Braden et al., 2010, 2011, Chapters 2 & 3), and the results from this study further support our previous findings. Herein, progesterone treatment impaired working memory performance and exacerbated a delay-induced impairment on the WRAM. This study intended to evaluate the mechanism by which progesterone impairs memory, in the menopausal rat model, building on previous research that progesterone's metabolites interact with the GABAergic system acting as positive allosteric modulators (Lan & Gee, 1994). Thus, we hypothesized that progesterone-mediated cognitive impairments may be initiated via pro-GABAergic actions, and that concomitant treatment with the $GABA_A$ receptor antagonist, bicuculline, would block these impairments. We found that both the progesterone impairment on working memory performance and delay retention, as evaluated by the WRAM, were reversed by the addition of bicuculline. Further, the combination of Prog+Bic enhanced working and reference memory performance on the WRAM and MM, with respect to vehicle, indicating a synergistic effect of these two compounds for memory enhancement.

We have previously shown that both long-term (two months) progesterone and MPA treatment in aged Ovx rats decreased protein levels of CA1/2 hippocampal GAD (Braden et al., 2010, Chapter 2); in the current study, we aimed to determine if short-term (2 weeks) progesterone treatment affects number of GABA-producing cells in the hippocampus. Neither progesterone nor bicuculline treatment alone, nor the combination together, had an effect on any of the hippocampal regions that were quantified (CA1, CA3, DG). In accordance

with our findings, others have also found progesterone to alter GAD mRNA levels in the CA1, but not number of GABA-producing cells (Weiland et al., 1992). Moreover, progesterone has been shown to decrease mRNA levels of the α4 subunit of the $GABA_A$ receptor in the CA1 and increase γ2 mRNA (Pazol et al., 2009; Weiland & Orchinik, 1995). Thus, the mechanism of progesterone-induced memory impairments, and bicuculline reversal, may simply be changes in GAD protein levels (Braden et al., 2010, Chapter 2) or mRNA (Wallis & Luttge, 1980), or alterations in $GABA_A$ receptor subunit expression.

It is noteworthy that, behaviorally, bicuculline completely reversed the memory-impairing effects of progesterone and synergized with this hormone to enhance memory. Presumably, progesterone and bicuculline have opposing actions on the GABAergic system, with progesterone metabolites acting as positive allosteric modulators (Lan & Gee, 1994) and bicuculline acting as an antagonist (Brickley, Cull-Candy, & Farrant, 1996). Other steroid hormones that can exert memory enhancing effects, specifically 17β-estradiol, have been shown to act on the GABAergic system in the hippocampus in order to increase the dynamic range of the hippocampus, as a mechanism of cognitive enhancement (Rudick & Woolley, 2001). Increasing the dynamic range of the hippocampus, and thus expanding the potential of this systems response to synaptic input and memory formation, may also be the mechanism by which the combination of progesterone and bicuculline enhances cognition. Future investigations are warranted to further elucidate this mechanism.

Based on prior research in cerebrovascular insult models, we hypothesized that another potential mechanism of progesterone-induced memory impairments is the modulation of Cox-2, a key enzyme in the formation of prostaglandins in the inflammatory response (Breder et al., 1995). However, in our study, progesterone or bicuculline, alone or together, had no effect on number of Cox-2 positive cells in the frontal cortex. Previous research testing progesterone after cortical contusion or LPS insult shows mixed results, with one study finding that progesterone decreased Cox-2 expression in the frontal cortex after cortical contusion (Cutler et al., 2007), while another found a progesterone-induced increased of Cox-2 in cerebral blood vessels following LPS insult (Sunday et al., 2006). These findings support the possibility that progesterone does not exacerbate neuronal inflammation in an animal without an experimental traumatic incident. Indeed, to our knowledge this is first examination of the effects of progesterone on a marker of neuronal inflammation in an animal that did not undergo a model of physiological trauma, unlike Cutler et al. (2007) and Sunday et al. (2006).

In conclusion, we determined that, 1) short-term progesterone treatment impairs working memory performance and exacerbates a delay-induced impairment on the WRAM, 2) progesterone impairments are reversed by concomitant treatment with the $GABA_A$ receptor antagonist, bicuculline, 3) compared to vehicle treated animals, the combination of progesterone and bicuculline enhances working and reference memory performance on the WRAM and MM. Future investigations should aim to elucidate the mechanism of short-

term progesterone-induced memory impairments, with initial investigations looking at changes in the subunits of the $GABA_A$ receptor. This work will be integral to the mechanistic understanding of progestogen-induced cognitive impairments in order to one day determine safe progestogen use for combination birth control and HT prescribed to women.

Chapter 5b

The $GABA_A$ antagonist bicuculline does not attenuate medroxyprogesterone acetate-induced memory-impairments but reverses changes in number of GABA-producing cells in the hippocampus and Cox-2 positive cells in the frontal cortex.

Nearly every woman will face the decision of whether to take exogenous hormones, either for contraception or for HT, and those with a uterus must include a progestogen to their regimen (Smith et al., 1975). Medroxyprogesterone acetate (MPA) is the most commonly used progestogen component of HT, and it is the sole hormone component of the contraceptive Depo Provera. In postmenopausal women, MPA may be a key factor that lead to an increased risk of dementia in the WHIMS trials, as a significant increase was observed after combination HT treatment including MPA, but not with conjugated equine estrogen-only HT treatment (Shumaker et al., 2003; 2004). Further, a documented case study linked use of MPA in Depo Provera with amnesia (Gabriel & Fahim, 2005). In the rat model, we and others have shown that MPA alone (Braden et al., 2010; 2011, Chapters 2 & 3), or in combination with 17β-estradiol (Lowry et al., 2010), induces cognitive impairments. Finally, in vitro MPA exacerbates neuronal

death by glutamate-induced excitotoxicity (Nilsen, Morales, & Brinton, 2006), reduces 17β-estradiol-mediated neural protection against excitotoxicity (Nilsen & Brinton, 2002b), and completely blocks the glutamate-stimulated calcium increase produced by 17 β-estradiol, a positive mechanism by which estrogens may modulate cognitive functioning (Nilsen & Brinton, 2002a).

A potential mechanism for MPA-induced memory impairments in the rodent model is the GABAergic system. Indeed, we have shown that in Ovx rats who had demonstrated MPA-induced cognitive impairments, MPA also impacted the GABAergic system in cognitive brain regions (Braden et al., 2010, Chapter 2). However, it remains to be determined, through direct pharmacological manipulation, whether the GABAergic system is indeed a mechanism underlying MPA's effects on cognition during aging in the Ovx rat. Evidence suggests that MPA increases $GABA_A$ stimulation in the hippocampus, in turn resulting in an increase of inhibition of the hippocampus, compared to control treatment (Belelli & Herd, 2003), which may be the mechanism of MPA's detrimental effects on cognition. We propose to address the question of whether MPA's effects on memory are, in part, due to $GABA_A$ modulation, by administering the $GABA_A$ receptor antagonist bicuculline to MPA-treated animals. We hypothesize that bicuculline administration will prevent MPA's negative effects on cognition. In this study, we also aim to determine if in vivo treatment with MPA and/or bicuculline alters the number GABA-producing cells in the hippocampus. We predict that the number of GABA-producing cells will be down regulated by MPA treatment, similarly to the protein changes observed in Braden et al. (2010,

Chapter 2), and that this will be reversed by concomitant treatment with bicuculline, mirroring the expected behavioral outcomes.

Additionally, MPA has been shown to modulate Cox-2, a key enzyme in the formation of prostaglandins in the inflammatory response (Breder et al., 1995) that is induced in glutamate excitotoxicity (Tocco et al., 1997). We aim to determine in this study if in vivo treatment with MPA and/or bicuculline alters Cox-2 positive cells in the frontal cortex. Only one study has examined the link between MPA and Cox-2; findings in Ovx rats showed an MPA-induced exacerbation of Cox-2 expression in cerebral blood vessels following LPS-induced inflammation (Sunday et al., 2006). This suggests that MPA may have neuroinflammatory-producing properties that may be related to cognitive impairments. Indeed, MPA does not improve cognitive performance in young rodent models of cortical contusion (Wright et al., 2008), while natural progesterone does (Roof et al., 1994). Thus, in the current experiment, we predict that MPA treatment will increase the number of Cox-2-positive cells, relative to vehicle treatment, in the frontal cortex, a brain region important for working memory (Jones, 2002). The frontal cortex is also a brain area where natural progesterone has been shown to modulate Cox-2 protein levels after cortical contusion (Cutler et al., 2007). By answering the question of the mechanism by which MPA impairs cognition in the female rodent, we can move forward to identify progestogens that do not share these pharmacological characteristics, and may represent safer alternatives for inclusion in birth control and HTs.

Methods

Subjects

Subjects were 38, 13 month old Fischer-344 female rats born and reared at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). Rats were acclimated for several weeks before surgery, had access to food and water ad-lib, and were maintained on a 12-hour light/dark cycle at the Arizona State University animal facility. All procedures were approved by the local IACUC committee and adhered to NIH standards.

Hormone Treatment and Ovariectomy

Rats were randomly assigned to one of four treatment groups: Ovx+Vehicle, Ovx+MPA, Ovx+Bicuculline (Ovx+Bic), or Ovx+MPA+Bic. These are the same Ovx+Vehicle and Ovx+Bic animals used in Study 4a. Identical to Study 3b (Chapter 4), at 13 months of age all rats were anesthetized via isofluorane inhalation, underwent bilateral dorsolateral incisions in the skin and peritoneum, and the ovaries and tips of uterine horns were ligatured and removed. Muscle and skin were then sutured. Two days after surgery all drugs were administered via two daily subcutaneous injections in the scruff of the neck. In the first injection, animals received either a control vehicle solution (0.4mL sesame oil + 0.02mL dimethyl sulfoxide [DMSO], both from Sigma-Aldrich, St. Louis, MO, USA) or MPA (0.7 mg, Sigma-Aldrich, St. Louis, MO, USA; dissolved in 0.4mL sesame oil + 0.02mL DMSO), and for the second injection, animals received either a control vehicle solution (sesame oil + 10% DMSO) or

bicuculline (3.5mg/kg; dissolved in sesame oil + 10% DMSO). Injections continued throughout behavior testing until sacrifice.

Vaginal Smears and Uterine Weights

Vaginal smears were taken 12 and 13 days after Ovx, and classified as proestrus, estrous, metestrus or diestrous per prior protocol (Acosta et al. 2009; Goldman et al. 2007). At sacrifice, the uteri of all subjects was removed, trimmed of visible fat, and immediately weighed (wet weight), in order to examine drug effects on uterine tissues (Braden et al., 2010, 2011, Chapters 2 & 3).

Water Radial-Arm Maze

Thirteen days after initiation of injections, subjects began testing on the WRAM for 15 days to evaluate spatial working and reference memory, including performance as working memory load increased, as described previously (Bimonte & Denenberg, 2000; Bimonte et al., 2000, 2002). The maze contained escape platforms hidden under the water surface in the ends of 4 of the 8 arms. Each subject had different platform locations that remained fixed throughout the experiment. A subject was released from the start arm and had three minutes (m) to locate a platform. Once a platform was found, the animal remained on it for 15 seconds (s), and was then returned to its heated cage for a 30s inter-trial interval (ITI) until its next trial. During the interval, the just-chosen platform was removed from the maze. The animal was then placed again into the start alley and allowed to locate another platform. For each animal a daily session consisted of four trials, with the number of platformed arms reduced by one on each subsequent trial. Thus, the working memory system was increasingly taxed as trials progressed,

allowing us to access working memory load. Each subject was given one session a day for 14 consecutive days. On Day 15, a 6 hour delay was administered between Trials 2 and 3 as in Experiments 3a and 3b (Chapter 4).

Quantification and blocking for analyses were based on prior studies (Bimonte & Denenberg, 2000; Bimonte et al., 2000, 2002;Hyde et al., 1998, 2000). An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Errors were quantified using the orthogonal measures of working and reference memory errors (Jarrard et al., 1984), as done previously in WRAM studies (Bimonte et al., 2000, 2002; Hyde et al., 2000). Working Memory Correct (WMC) errors were the number of first and repeat entries into any arm from which a platform had been removed during that session. Trial 1 is not analyzed for WMC errors because no platform has yet been removed. Reference memory (RM) errors were the number of first entries into any arm that never contained a platform. Working Memory Incorrect (WMI) errors were repeat entries into a RM arm.

Morris Maze

The day after WRAM completion, subjects began testing on the MM for 6 trials/day for 3 days using a tub (188 cm diameter) filled with black water made opaque using non-toxic paint. A hidden platform (10 cm wide) remained in a fixed location, thereby testing spatial reference memory (Bimonte-Nelson et al., 2006; Morris et al., 1982). The rat was placed in the maze from the North, South, East, or West location, and had 60s to locate the hidden platform, which remained in the Northeast quadrant throughout testing. Once the rat found the platform the

trial was terminated. After 15s platform time, the rat was placed into its heated cage until its next trial. The approximate ITI was 10-15m. To evaluate whether rats localized the platform to the spatial location, after all test trials on day 3, a 60s probe trial was given whereby the platform was removed. For each trial, a camera suspended above the maze tracked each rat's path and a tracking system (Ethovision 3.1, Noldus Instruments) analyzed each rat's tracing.

MM performance was assessed by swim path distance (cm) and latency (s) to the platform. For probe trial data, percent of total distance in the previously platformed (target) quadrant was compared to the quadrant diagonally opposite the platform. Rats that learned the platform location were expected to spend the greatest percent distance in the target quadrant (Stavnezer et al., 2002).

Visible Platform Maze

To confirm that all subjects had intact vision and could perform the procedural task components of MM and WRAM spatial navigation without difficulty, subjects were tested on a VP water-escape task. A rectangular tub (39 x 23 in) was filled with clear water and a black platform (10 cm wide) was elevated above the water surface. Opaque curtains covered extramaze cues. The drop off location remained the same across trials, and the platform location for each trial varied in space semi-randomly. Animals had to locate the protruding platform, and were given 6 trials in one day. Performance was assessed by latency (s) to the platform.

Tissue Preparation

After the conclusion of behavioral testing, rats were anesthetized with isofluorane. Brains were removed, blocked anterior to the hippocampus, and post-fixed in PFA for 48 hours. After 48 hours the brains were moved to PB and stored at 4 °C. Two days prior to sectioning, brains were placed in a 30% sucrose solution for 48 hours. Brains were coronally sectioned at 40 μm on a cryostat (Microtome HM 500 OM Cryostat) at -20 °C, and free floating sections were collected for immunohistochemistry.

Immunohistochemistry and Quantification of GAD 67

Dorsal hippocampal sections in the posterior block of brains (Plates 33 – 37; Paxinos & Watson, 1998) were washed three times for 5m each in PBS, then incubated in 1% H2O2 diluted in PBS for 30m at room temperature (RT). Next, sections were washed three times (all GAD 67 washes = 10m for the remainder of staining) and placed in blocking buffer (5% normal goat serum, 2.5% BSA, 0.2% Triton-X, in 1x PBS) for 30m at RT. Incubation in a 1:500 dilution of mouse anti-GAD 67 primary antibody (MAB5406; Millipore, Billerica, MA, USA) diluted in blocking buffer, for 48 hours at 4°C, followed. Next, sections were washed four times in PBS and incubated in a 1:500 dilution of Alexa Fluor 568 goat anti-mouse IgG (H+L) (A-21206; Invitrogen, Grand Island, NY), diluted in blocking buffer, for two hours at RT, followed by a second round of four washes. Finally, sections were mounted on slides, left to air dry, and coverslipped using anti-fading mounting medium (Vectashield, Vector Labs, Burlingame, CA, USA). Control procedures were run excluding primary and secondary antibodies.

Exclusion of the primary antibody resulted in no cell staining, and exclusion of secondary antibody resulted in a lack of fluorescent development.

One experimenter, blind to treatment group status of the animals, performed all image analysis procedures. Images were acquired using Olympus DP70 digital camera fitted on an Olympus BX-50 microscope using DP-BSW Software (version 2.02, Olympus America Inc., Center Valley, CA). A 10X objective was used to capture representative images bilaterally from the CA1, CA3, and DG of the hippocampus. Captured images were manually counted using the Cell Counter plugin under NIH ImageJ software (Rasband, 1997–2004) in three sections of the dorsal hippocampus of five animals per treatment group, similarly to procedures used previously in our laboratory (Acosta et al., 2009). A cell was included as positive for GAD labeling if the interior was greater than 75% filled with a pixel intensity darker than the background.

Immunohistochemistry and Quantification of Cox-2

Frontal cortex sections in the anterior block of brains (Plates 9 – 17; Paxinos and Watson, 1998) were washed twice (all Cox-2 washes = 5m) in PBS then incubated in 0.2% H2O2 diluted in PBS for 15m at RT. Next, sections were washed twice and placed in blocking buffer (1% BSA, 0.2% Triton-X in 1x PBS) for 30m at RT. Incubation in a 1:250 dilution of rabbit anti-Cox-2 primary antibody (ab15191; Abcam Inc., Cambridge, MA, USA) diluted in blocking buffer, for two hours at RT, followed. Next, sections were washed three times for in PBS and incubated in a 1:500 dilution of biotin-conjugated goat anti-rabbit (111-065-003; Jackson Immuno Research, West Grove), diluted in blocking

buffer, for one hour at RT, followed by a second round of three washes. Finally, sections were treated with ABC reagent (Vector Laboratories, Burlingame, CA) and incubated for 45m at RT. Sections were washed three times in PBS, and then incubated with DAB Peroxidase Substrate (Vector). After the desired color was achieved (dark purple) brain sections were washed three times in PBS, mounted on slides, dehydrated, and coverslipped. Control procedures were run excluding primary and secondary antibodies. Exclusion of the primary antibody resulted in no cell staining, and exclusion of secondary antibody resulted in a lack of DAB Peroxidase Substrate color development.

One experimenter, blind to treatment group status of the animals, performed all image analysis procedures. Images were acquired using an Olympus DP70 digital camera fitted on an Olympus BX-50 microscope using DP-BSW Software (version 2.02, Olympus America Inc., Center Valley, CA). A 4X objective was used to capture images. Captured images were manually counted using the Cell Counter plugin under NIH ImageJ software (Rasband, 1997–2004) bilaterally in 12 sections of the frontal cortex of 4-5 animals per treatment group, similar to procedures used previously in our laboratory (Acosta et al., 2009). A cell was included as positive for Cox-2 labeling if the interior of the cell had a pixel intensity of four standard deviations greater than the mean pixel intensity of the region of interest, as other protocols have done (e.g. Parent et al., 2002).

Statistical Analyses

For behavior assessments, data were analyzed separately for each maze. To evaluate learning and potentially complex higher order Treatment interactions

with Days or Trials, we utilized an omnibus repeated measures ANOVA including all groups, with Treatment as the between variable and blocks of Days and/or Trials as the within variable/s. For GAD cell counts, Cox-2 cell counts, and uterine weights, Treatment effects were assessed via ANOVA. When ANOVA yielded a significant effect of Treatment, two group comparisons using t-tests were used to investigate group differences. For all analyses, alpha was two-tailed and set at 0.05.

Results

Water Radial-Arm Maze

Learning

Across all testing days (Days 1-14), and including all treatment groups, there was a day main effect for each error type for the omnibus ANOVA [WMC: $F(13, 442) = 7.909$; $p < .0001$ (Figure 27a); WMI: $F(13, 442) = 11.502$; $p < .0001$ (Figure 27b); RM: $F(13, 442) = 12.432$; $p < .0001$ (Figure 27c)], with errors decreasing across days, demonstrating learning.

Treatment Effects

For RM errors, there was a significant omnibus ANOVA main effect of Treatment [$F(3, 34) = 3.717$; $p < .05$] for Block 2 (Days 3-5), with Ovx+MPA+Bic animals committing more errors than Ovx+Vehicle [$t(17) = 2.947$; $p < .01$] and Ovx+MPA [$t(17) = 2.64$; $p < .05$] animals, demonstrating a reference memory impairment induced by the combination of MPA and bicuculline (Figure 27c). There were no significant effects for WMC or WMI errors during any block.

Delay

After regular testing, a 6 hour delay was administered between trials 2 and 3. We compared baseline performance on Trial 3 (Day 14, last day of regular testing) to Trial 3 immediately after the delay on Day 15, within each group. The Ovx+MPA and Ovx+MPA+Bic groups were the only groups to significantly increase WMC errors after the delay [Ovx+MPA: $t(9) = 3$; $p < .05$: Ovx+MPA+Bic: $t(8) = 2.828$; $p < .05$], demonstrating a susceptibility to delay-induced working memory impairments in MPA-treated animals that is not reversed by bicuculline (Figure 28). Ovx+Vehicle and Ovx+Bic animals were not impaired on WMC errors with the delay, and no group displayed a delay-induced increase in WMI or RM errors.

Morris Maze

Learning

For the omnibus ANOVA including all treatment groups, there was a Day main effect for Distance and Latency [Distance: $F(2, 68) = 143.064$; $p < .0001$ (Figure 29a); Latency: $F(2, 68) = 184.2$; $p < .0001$ (data not shown)], with scores decreasing across days, demonstrating learning of the task.

Treatment Effects

For Block 6, there was a main effect of Treatment [$F(3, 34) = 3.062$; $p < .05$]. Ovx+MPA+Bic animals swam a longer distance to the platform as compared to Ovx+Vehicle [$t(17) = 2.345$; $p < .05$] and Ovx+Bic [$t(16) = 2.737$; $p < .05$] animals on Block 6, demonstrating a reference memory impairment induced by the combination of MPA and bicuculline (Figure 29a).

Probe

The probe trial revealed a main effect of Quadrant [$F(1, 34) = 108.254$; $p < .0001$] and no main effect of, or interactions with, Treatment. Each group spent a greater percent Distance in the target NE quadrant as compared to the opposite SW quadrant, showing localization of the platform for all groups [Ovx+Vehicle: $t(9) = 4.861$; $p < .001$; Ovx+MPA: $t(9) = 3.462$; $p < .01$; Ovx+Bic: $t(8) = 10.783$; $p < .0001$; Ovx+MPA+Bic: $t(8) = 4.984$; $p < .005$ (Figure 29b)].

Visible Platform

There was a Trial main effect [$F(5, 170) = 19.951$; $p < .0001$ (Figure 30)], with Latency decreasing across all trials, demonstrating learning of the task. The omnibus ANOVA for Treatment was not significant for latency to the visible platform. By the fourth trial, all groups found the VP within 9s, confirming visual and motor competence to perform swim maze tasks for all groups.

Vaginal Smears

Twelve and 13 days after Ovx, vaginal smears were taken and classified as proestrus, estrous, metestrus or diestrous per prior protocol (Acosta et al., 2009; Goldman et al., 2007). As expected, all animals, regardless of treatment, showed leukocytic smears, indicative of the diestrous phase.

Uterine Weights

There was a significant omnibus ANOVA effect of Treatment [$F(3, 34) = 15.398$; $p < .0001$]. Similar to our prior findings (Braden et al. 2010; 2011, Chapters 2 & 3), animals treated with MPA had heavier uteri than vehicle animals [Ovx+Vehicle vs. Ovx+MPA: $t(18) = 5.529$; $p < .0001$], and this was not reversed

by the addition of bicuculline [Ovx+Vehicle vs. Ovx+MPA+Bic: $t(17) = 6.486$; $p < .0001$] (Table 8).

Frontal Cortex Cox-2 Cell Counts

There was a significant omnibus ANOVA main effect of Treatment [$F(3, 15) = 22.311$; $p < .0001$]. Ovx+MPA animals had more cells labeled positive for Cox-2 in the frontal cortex than every other treatment group [Ovx+MPA vs. Ovx+Vehicle: $t(8) = 23.717$; $p < .005$; Ovx+MPA vs. Ovx+MPA+Bic: $t(8) = 25.35$; $p < .005$; Ovx+MPA vs. Ovx+Bic: $t(7) = 20.815$; $p < .005$ (Figure 31a)], indicating that MPA administration induced an increase in number of Cox-2 cells in the frontal cortex, and that this was reversed by concomitant bicuculline treatment. There were no significant differences between any other groups.

Hippocampal GAD Cell Counts

There was a significant omnibus ANOVA main effect of Treatment in the CA1 for number of cells labeled positive for GAD [$F(3, 16) = 3.602$; $p < .05$]. Ovx+MPA animals had more cells labeled positive for GAD in the CA1 region of the hippocampus than Vehicle [$t(8) = 2.416$; $p < .05$] and Ovx+MPA+Bic [$t(8) = 2.595$; $p < .05$ (Figure 32a)] animals, indicating that this MPA-induced increase in CA1 GAD is reversed by bicuculline. Ovx+Bic animals did not differ significantly from any group, and there were no significant effects of Treatment for CA3 or DG (Table 9).

Discussion

The synthetic progestin, MPA, is the most commonly used progestogen component of HT and the sole hormone component in the contraceptive Depo

Provera. We have previously shown that MPA impairs memory in the surgically menopausal rat model (Braden et al., 2010, 2011, Chapters 2 & 3). The results from this study support our previous behavioral findings; however, it is noteworthy that the impairing effects of MPA in the current report were observed on only one memory measure, where previously we have seen impairments on multiple memory measures, after MPA treatment (Braden et al., 2010, 2011, Chapters 2 & 3). In the current study, MPA treatment exacerbated a delay-induced memory-retention impairment on the WRAM. The goal of this study was to evaluate a plausible mechanism by which MPA impairs memory, using the rationale that previous research has shown that MPA interacts with the GABAergic system, enhancing $GABA_A$ receptor-mediated inhibition (Belelli & Herd, 2003). We hypothesized that MPA-mediated cognitive impairments were associated with pro-GABAergic actions, and that concomitant treatment with the $GABA_A$ receptor antagonist, bicuculline, would block these impairments. Our hypothesis was not supported; we found that the MPA delay-induced retention impairment on the WRAM was not reversed by the addition of bicuculline. Further, the combination of MPA and bicuculline impaired reference memory performance on the WRAM and MM, indicating an unexpected synergistic effect of these two compounds for memory impairment.

We have previously shown that long-term MPA treatment (two months) in aged Ovx rats decreased protein levels of GAD in the CA1/2 hippocampal region (Braden et al., 2010, Chapter 2). In the current study, we aimed to determine if MPA treatment affects the number of GABA-producing cells in the hippocampus.

Results showed that MPA treatment increased number of GAD-positive cells, and thus the number of GABA-producing cells, in the CA1 of the hippocampus, compared to vehicle treatment, an effect reversed by the addition of the $GABA_A$ antagonist, bicuculline. The MPA effect observed herein is in the opposite direction of our previous finding, showing that MPA treatment resulted in a decrease of GAD protein levels in the CA1/2 hippocampal region (Braden et al., 2010, Chapter 2). In cultured hippocampal neurons, others have found that a change in number of GAD-positive cells is mirrored by a change in GAD protein levels, demonstrating a direct correlation in the two GABAergic measures (Murphy, Cole, Greenberger, & Segal, 1998). A critical difference between our current and past studies that could have resulted in the different effects observed on the GABAergic system, is the amount of time MPA was given. In our first investigation, MPA treatment of a longer duration (two months), resulted in a decrease of hippocampal GABAergic markers (Braden et al., 2010, Chapter 2), and in our current investigation, a shorter duration of MPA treatment (two weeks) resulted in an increase of hippocampal GABAergic markers. Interestingly, 17β-estradiol treatment has also been associated with divergent effects on hippocampal GABAergic markers at different time points after treatment (Rudick & Woolley, 2001). Specifically, in young Ovx rats, 17β-estradiol initially (24 hours after treatment) decreases number of hippocampal GABA-producing cells; and 48 hours after treatment, 17β-estradiol increases number of hippocampal GABA-producing cells, compared to vehicle (Rudick & Woolley, 2001). Although our MPA investigations are measuring GABAergic makers after longer

treatment durations (two weeks versus two months) than the work with 17β-estradiol (24 hours versus 48 hours), it seems that as time progresses, MPA treatment may induce an inverse of GABAergic effects on the hippocampus compared to the effects induced by 17β-estradiol treatment. Explicitly, 17β-estradiol initially decreases and later increases hippocampal GABAergic markers (Rudick & Woolley, 2001), while MPA initially increases and later decreases hippocampal GABAergic markers (current data and Braden et al., 2010, Chapter 2). MPA has been shown to have opposite effects to 17β-estradiol on the hippocampal glutamatergic system as well. Specifically, MPA treatment exacerbates NMDA-induced excitotoxic cell death while 17β-estradiol protects against it (Nilsen et al., 2006). Thus, MPA may exert inverse effects on the hippocampal GABAergic and glutamatergic systems as compared to 17β-estradiol, and this may be related to the cognitive enhancing properties of 17β-estradiol in rodents and women (Frick, 2009; Maki & Sundermann, 2009) and the cognitive impairing properties of MPA, alone or in combination with estrogens, in rodents and women (Braden et al., 2010, 2011, Chapters 2 & 3; Lowery et al., 2010; Maki & Sundermann, 209).

We hypothesized that another potential mechanism of MPA-induced memory impairments is the modulation of Cox-2, a key enzyme in the formation of prostaglandins in the inflammatory response (Breder et al., 1995) that is induced in glutamate excitotoxicity (Tocco et al., 1997). This hypothesis was supported, as we found that MPA treatment increased number of Cox-2 positive cells in the frontal cortex, compared to vehicle. Notably, bicuculline reversed this

MPA-induced increase. MPA's effects on Cox-2 have only been investigated in one previous study, and consistent with our findings, MPA-induced an exacerbation of Cox-2 expression in cerebral blood vessels of Ovx rats, following LPS inflammatory insult (Sunday et al., 2006). Our results are also consistent with in vitro work, showing that MPA exacerbates glutamate-induced excitotoxic cell death (Nilsen et al., 2006), and are the first evidence that, in vivo, MPA may be damaging to neurons independent of experimentally-induced insult.

In reconciling behavioral outcomes with our previous research, as well as changes in neurochemical markers observed in this study, we encountered some unexpected findings that will require follow-up investigations. First, in the current study MPA treatment did not impair cognition on multiple measures of learning and memory, as we have previously shown (Braden et al., 2010, 2011, Chapters 2 & 3). This is most likely due to differences in treatment duration. For this study, MPA was given approximately two weeks prior to test, versus two months before test in Braden et al., (2010, 2011, Chapters 2 & 3) where we showed potent MPA-induced cognitive detriments on multiple maze tasks. This suggests that longer MPA treatment durations are associated with greater cognitive impairments, and future investigations are warranted to methodically test this hypothesis. Results from this study also suggests that an MPA-induced increase in GABA-producing cells in the CA1 of the hippocampus, or Cox-2 positive cells in the frontal cortex, as we found herein, does not necessarily lead to robust memory changes. Further, bicuculline did not attenuate MPA-induced memory impairments, but completely blocked GAD and Cox-2 changes, suggesting that reversal of an MPA-induced

increase in GABA-producing cells in the CA1 of the hippocampus or Cox-2 positive cells in the frontal cortex may not be the mechanism of bicuculline's reversal of the MPA-induced memory retention impairment. Finally, the combination of MPA and bicuculline, two compounds presumable having opposing actions on the GABAergic system (Belelli & Herd, 2003; Brickley et al., 1996) synergized to impair memory in middle-aged Ovx animals, with respect to vehicle. Future investigations are warranted to further elucidate the memory-impairing mechanisms of MPA, and MPA in combination with bicuculline, in the surgically menopausal rodent model.

The results from the current study indicate that, in middle-aged Ovx rats: 1) short-term MPA treatment exacerbates a delay-induced impairment on the WRAM, increases number of GABA-producing cells in the CA1 of the hippocampus, and increases Cox-2 positive cells in the frontal cortex, 2) bicuculline treatment reverses MPA-induced increases in GAD and Cox-2-positve cells, but does not prevent delay-induced memory impairments, 3) the combination of MPA and bicuculline impairs reference memory performance on the WRAM and MM. Future investigations should aim to elucidate the timeline of MPA changes on cognition and the GABAergic system. We now have evidence showing that 1) two weeks of MPA treatment in middle-aged Ovx rats induces a modest memory impairments and an increase in the number of GABA-producing cells in the hippocampus (current report) and 2) two months of MPA treatment in middle-aged or aged rats induces robust memory impairments and decreases levels of GAD protein in the hippocampus (Braden et al., 2010, 2011, Chapters 2

& 3). Future studies are needed to investigate the effects of MPA treatment at multiple time points in between two weeks and two months (i.e. four weeks and six weeks) on cognition and GABAergic markers. Further, measuring these GABAergic markers (number of GABA-producing cells and GAD protein levels) in the hippocampi of the same rats, will allow direct evaluation of the correlation between these two measures, in the surgically menopausal rat model. This information will add to the understanding of whether or not it is possible to have an MPA-induced up-regulation in number of GABA-producing cells concomitant with a down-regulation of GAD protein levels. Continuing this work in behaviorally tested animals will allow researchers to relate MPA-induced GABAergic changes with changes in cognition. Specifically, we need to understand the MPA-induced GABAergic milieu (number of GABA-producing cells and GAD protein levels) of the hippocampus that is present at the onset of robust memory impairments, in the aging Ovx rat model. This work will be integral to the mechanistic understanding of MPA-induced cognitive impairments in order to one day determine safe progestin use for combination birth control and HT prescribed to women.

CHAPTER 6

HIPPOCAMPAL AND ENTORHINAL CORTEX VOLUMES OF POST-MENOPAUSAL WOMEN ARE MODULATED BY HORMONE THERAPY STATUS: CORRELATIONS WITH AGE AND COGNITIVE MEASURES

Menopause-induced hormone loss has been linked to many symptoms that affect quality of life in women including hot flashes, urogenital atrophy, and memory decline (Freedman, 2002; Nappi et al., 1999; Sherwin, 1988). HT is given to menopausal and post-menopausal women to attenuate menopause-induced symptoms and clinical studies have demonstrated that estrogen-containing HT can prevent (Sherwin, 1988; Phillips & Sherwin, 1992) or improve memory decline in healthy women (Campbell & Whitehead, 1977; Duka et al., 2000; Joffe et al., 2006; Linzmayer et al., 2001; Maki & Sundermann, 2009; Phillips & Sherwin, 1992; Wolf et al., 1999), Further, AD is more prevalent in women than men (Anderson et al., 1999), and there is some evidence to suggest that a loss of ovarian hormones at menopause puts women at a greater risk (Solerte et al., 1999). Indeed, a history of estrogen-containing HT can reduce AD risk (Zandi et al., 2002), and as a treatment, estrogen can enhance cognition in post-menopausal women with AD (Asthana et al., 1997)

Over the last decade, the cognitive effectiveness of HT has been of much debate, due to the unexpected findings of the large, placebo-controlled, randomized WHIMS conducted by the National Institute of Health. Post-menopausal women taking Conjugated Equine Estrogens (CEE) alone did not differ significantly from those taking placebo for dementia diagnoses (Shumaker

et al., 2004). In contrast, twice as many women receiving CEE + MPA were diagnosed with dementia as compared to the placebo group, a significant effect (Shumaker et al., 2003). Recently, Maki and Sundermann (2009) conducted a systematic review of randomized clinical trials of hormone therapy's effects on verbal memory, decline in which is the earliest predictor of AD. Findings indicated that there is a beneficial effect associated with estrogen-only HT on verbal memory in younger menopausal women and a detrimental effect of CEE+MPA on verbal memory in menopausal women (Maki and Sundermann, 2009). This review brings back into question the complex role estrogen-containing HT plays in normal cognitive aging, and risk of AD, in women.

Loss of hippocampal volume is another hallmark brain change seen in AD progression (Buckner et al., 2005; Scahill et al., 2002) and is related to cognitive functioning in AD patients (Jack et al., 1999; 2000). A subset of women who participated in the WHIMS underwent a follow-up MRI regional volume study, including evaluation of the hippocampus, 1.4 - 3 years after the completion of the WHIMS trial (Resnick et al., 2009). Results were consistent with findings showing HT-associated changes in dementia risk, and specifically CEE treatment alone, lead to no significant changes in hippocampal volumes compared to placebo ($p=.18$); however, in women receiving CEE+MPA, there was a marginally significant effect ($p=.09$), with HT-treated women having smaller hippocampi than placebo (Resnick et al., 2009). Just as findings from the WHIMS dementia assessment are in disagreement with many other trials measuring the cognitive impact of estrogen-containing HT, so are findings from the follow-up

volumetric study. Eberling et al. (2003) reported that estrogen-containing HT was associated with no hippocampal volume loss in a mix of healthy and demented women. Further, Yue et al. (2007) found that in women at genetic risk for developing AD, a history of HT was associated with larger hippocampal volumes, as compared to women at the same genetic risk that had not used HT.

Much effort has been put into reconciling the negative WHIMS findings of HT-associated dementia risk and hippocampal volume loss, with positive effects on both measures associated with HT use demonstrated in other studies. Several hypotheses have emerged, including the critical window hypothesis, that HT initiation at or near the time of menopause is related to cognitive benefits, while delayed initiation is not (Khoo et al., 2009; Maki, 2006; Sherwin, 2005). The critical window hypothesis seems to also be important for determining whether HT is associated with a protection of hippocampal volume. A longer interval between menopause and HT initiation is associated with smaller hippocampal volumes, while participants initiating HT at time of menopause seem to have larger hippocampal volumes than never users (Erickson et al., 2010). Further, current versus past HT use seems to differentially affect hippocampal volume size, but the literature is mixed, with some showing largest volumes of middle temporal gyrus structures in current users (Lord et a., 2008) and others in past users (Boccardi et al., 2006). Still other research has shown no differences in hippocampal volumes correlated with current or past HT use (Low et al., 2006), or a decrease in overall gray matter associated with HT (Greenberg et al., 2006), in line with findings from the WHIMS.

The entorhinal cortex is a brain structure heavily implicated in AD (Hyman et al., 1984; Braak and Braak, 1991) and cognitive performance, including memory, (Fernandez et al., 1999), but much less studied for responsiveness to HT-induced atrophy protection. The entorhinal cortex was evaluated in a voxel based morphometry study by Boccardi et al. (2006), where HT use was associated with larger entorhinal cortex volumes, as compared to non-users. More research evaluation of the effects of HT on preserving entorhinal cortex volumes, in addition to hippocampal volumes, may add to the understanding of divergent findings within the field of HT and cognitive aging.

The overarching aim of this study was to further investigate the relationship between HT, mesial temporal lobe structure volumes (hippocampus and entorhinal cortex), and cognition. Post-menopausal women were recruited, divided into three HT groups based on continuous, discontinuous, and never use, and underwent a cognitive battery and MRI scans to determine regional volumes of the hippocampus and entorhinal cortex. We hypothesized that HT use would be associated with favorable outcomes for cognitive measures and hippocampal and entorhinal cortex volumes, as opposed to never using HT. Further, based on the findings of Boccardi et al., (2006) we hypothesized that within HT use, the greatest benefits for hippocampal and entorhinal cortex volumes would be seen with discontinuous use, as compared to continuously or never using HT. We hypothesized that this distinction would transfer into cognition as well, with discontinuous users outperforming continuous and never users. This prediction was based on the relationship between hippocampal volumes and memory

performance (Jack et al., 2000) and findings relating past, but not current, HT use with a decrease in dementia risk (Zandi et al., 2002). Finally, it is well known that mesial temporal lobe volumes and cognitive performance undergo age-related decline. We aimed to indirectly determine if prior HT use prevents age-related decline in these variables, by investigating correlations between age and volumes and/or cognitive scores, within HT groups. We hypothesized that in continuous or discontinuous HT groups age would not correlate with mesial temporal lobe volumes and cognitive performance

Methods

Participants

Participants were recruited from three participating institutions of the Arizona Alzheimer's Consortium; Barrow Neurological Institute, Sun Health Research Institute, and Mayo Clinic-Arizona. All participants provided written consent which was approved by the individual institutions' Internal Review Boards. The data included participants from several separate imaging studies on aging performed at Barrow Neurological Institute from 2000-2007, all of which include cognitive assessments and MRI structural scans. There were 50 postmenopausal women. General inclusion criteria for study participants were: 1) Age 50 or greater; 2) No clinical diagnosis of Mild Cognitive Impairment or dementia from the treating neurologist and Mini Mental State Exam (MMSE) score of 26 or greater, in order to exclude those participants with significant cognitive impairment; and 3) No evidence of depression as measured by either the Geriatric Depression Scale (GDS) or Beck Depression Inventory-II (BDI).

Post-menopausal was defined as one year or more since the final menstrual period. Detailed histories of endogenous and exogenous estrogen exposure were gathered from all participants and included age at menopause, history of partial or complete hysterectomy, years on and type of HT (estrogen and/or progesterone use), and age at which HT was initiated and terminated. Participants were divided into three groups based on lifetime estrogen exposure; continuous users (CU), discontinuous users (DU), and never-users (NU). CU (n = 16) were women who began HT at menopause and had consistently taken HT since therapy initiation. DU (n = 22) had used HT for varying periods of time during their postmenopausal years (minimum of 1 year of use), but their use was not continuous. NU (n = 12) had never taken HT, or if they did, the period of time did not exceed six-months (n = 2).

Image Acquisition

All participants underwent imaging on a 1.5- or 3-tesla GE scanner at Barrow Neurological Institute. Images of the whole brain were collected using an axial SPGR (spoiled gradient) T1-weighted, 3-dimensional acquisition with the following parameters: TR (repetition time) = 24 ms, TE (echo time) = 6 ms, flip angle= 40 degrees, NEX (number of excitations) = 1, slice thickness = 1.9 mm, 0 skip between slices, Field of view = 240 mm, in plane resolution =.9375 square mm voxels.

Volumetric Data

Hippocampal and entorhinal cortex volumes were manually traced. The outlines of the hippocampus and entorhinal cortex were traced from the three-

dimensional SPGR images using ANALYZE (version 7.5). Both structures were visualized in all three planes, landmarked in the sagittal plane, and drawn in the coronal plane. We employed the guidelines of Machulda et al. (2001) to define the hippocampal boundaries. The anterior boundary was defined by observing the white matter band and/or the cerebrospinal fluid space between the amygdala and hippocampus in the sagittal plane. The posterior aspect of the posterior region was initially landmarked in the sagittal plane by locating the posterior edge of the hippocampus and then checking in the coronal plane to ensure that the fornices were completely visualized.

We employed the guidelines of Insausti et al (1998) to define the entorhinal cortex boundaries. In the sagittal plane, the most anterior portion of the entorhinal cortex was marked where the uncus transformed into the gyrus ambiens. At this area in the coronal plane, the superior border of the entorhinal cortex was the gyrus ambiens and the inferior border was the collateral sulcus. In the posterior portion of the entorhinal cortex the hippocampal fissure was used as the superior border. Finally, the crus cerebri was landmarked in the sagittal plane and served as the most posterior area of the entorhinal cortex. For hippocampal and entorhinal cortex size, values are presented as % of total intracranial volume (TIV). Total intracranial volume was generated using Statistical Parametric Mapping (SPM8; http://www.fil.ion.ucl.ac.uk/spm/)

Cognitive Assessment

Participants were administered either the California Verbal Learning Test (CVLT-II) or the Rey Auditory Verbal Learning Test (RAVLT), which are both

auditory learning and memory tasks consisting of a supraspan word list that is repeated 5 times for a total word recall learning measure as well as a free recall trial 20-30 minutes later to assess delayed, or retained memory (Delis, Freeland, Kramer, & Kaplan, 1988; Rey, A., 1964). Participants were also administered the Trail Making Test (TMT) parts A and B ('TrailsA and TrailsB"), which are measures of psychomotor speed and executive functioning, respectively (Reitan and Wolfson, 1985).

Statistical Analyses

Memory performance was expressed as percentage of the maximum possible (Total Words (words learned over 5 trials): 80 for the CVLT and 75 for the RAVLT; Delayed Memory: 16 for the CVLT and 15 for the RAVLT). ANOVA was run to assess group differences on control demographic variables (age, years of education, years since menopause, and MMSE) and TIV. Two group planned comparisons using t-test were run to evaluate group differences on measures of interest. Specifically, planned comparisons included: 1) CU vs. NU, 2) DU vs. NU, and 3) CU vs. DU on the dependent measures: 1) Left hippocampal volume, 2) Right hippocampal volume, 3) Left entorhinal cortex volume, 4) Right entorhinal cortex volume, 5) Total Words, 6) Delayed memory, 7) TrailsA and, 8) TrailsB. Significance was defined as a p value less than 0.05; a p value between 0.05 and 0.10 was deemed a marginal effect. For all planned comparisons, it was noted that type I error correction is not necessary (Keppel & Wickens, 2004).

Intragroup correlations using Pearson's r coefficient were run between age and 1) Left hippocampal volume, 2) Right hippocampal volume, 3) Left entorhinal cortex volume, 4) Right entorhinal cortex volume, 5) Total Words, 6) Delayed memory, 7) TrailsA and, 8) TrailsB. Correlations were also run between each volume (1. Left hippocampal volume, 2. Right hippocampal volume, 3. Left entorhinal cortex volume, 4. Right entorhinal cortex volume) and cognitive scores (1. Total Words, 2. Delayed memory, 3. TrailsA and, 4. TrailsB). Due to multiple comparisons, significance for correlations was defined as a p value less than 0.01, similar to Braden et al., (2011, Chapter 3) and a p value less than 0.05 was deemed a trend.

Results

Demographics

There were no group differences on age [F(2, 47)=0.852; p=0.433], years of education [F(2, 47)=0.117; p=0.890], or postmenopause length of time [F(2, 47)=0.647; p=0.528]. The age range of participants was 53 – 91 years. Groups also did not differ on MMSE scores [F(2, 47)=1.263; p=0.292]. Participants were either Caucasian (90%) or Latino (10%) and 70% had undergone hysterectomy (Table 10). Of the women who had used HT, 76.3% had a known history of taking unopposed estrogens only (CU: 81.3%; DU: 72.7; Table 10).

Volumetric Data

Total Intracranial Volume (TIV)

There was no difference between groups for TIV [F(2, 47)=0.509; p=0.604 (Table 11)].

Hippocampal Volumes

For both hippocampi (left and right), DU had significantly larger volumes than NU [Left: t(32)=2.74; p=0.01 (Figure 33a); Right: t(32)=2.39; p=0.023 (Figure 33b)]. For the right hippocampus only, CU had marginally larger volumes than NU [t(26)=1.80; p=0.082 (Figure 33b)]. CU did not differ from NU on left hippocampal volumes [t(26)=1.01; ns] and DU did not differ from CU on either hippocampal volume [Left: t(36)=1.34; ns; Right: t(36)=0.25; ns] (Table 11).

Entorhinal Cortex Volumes

For the right entorhinal cortex, CU had significantly larger volumes than NU [t(26)=2.12; p=0.044 (Figure 33d)] and DU had marginally larger volumes than NU [t(32)=1.95; p=0.06 (Figure 33d)]. For the left entorhinal cortex, CU had marginally larger volumes than NU [t(26)=2.02; p=0.054 (Figure 33c)] and DU did not differ from NU [t(32)=1.23; ns]. DU did not differ from CU on either entorhinal cortex volume [Left: t(36)=1.20; ns; Right: t(36)=0.54; ns] (Table II).

Cognitive Assessment

For Delayed Memory, DU remembered a marginally higher percentage of words than NU [t(32)=1.95; p=0.06 (Figure 34)] and CU did not differ from either group[CU vs. DU: t(36)=1.10; ns; CU vs. NU: t(26)=1.02; ns]. No group differences were observed for Total Words or for Trails A or B tests (Table 12).

Correlations

Continuous Users

Figure 35 depicts the relationships between brain volume measures, cognitive performance, and age for the CU group. Both the left and right

hippocampi were negatively correlated with age [Left: r(16)=−0.623, p=0.0084; Right: r(16)=−0.621, p=0.0088 (Figure 35)], indicating a decrease in bilateral hippocampal volume as age increases. There were no significant correlations between hippocampal volume and cognitive scores for CU. Further, age did not significantly correlate with cognitive scores or entorhinal cortex volumes, and entorhinal cortex volumes did not correlate with cognitive scores (data not shown).

Discontinuous Users

Figure 36 depicts the relationships between brain volume measures, cognitive performance, and age for the DU group. Age negatively correlated with Total Words [r(20)=−0.542, p=0.0082 (Figure 36)] and there was a trend for a negative correlation between age and Delayed Memory [r(20)=−0.499, p=0.017 (Figure 36)], indicating lower cognitive scores as age increases. Right hippocampal volumes also showed a trend towards positively correlating with Delayed Memory [r(20)=0.479, p=0.023 (Figure 36)], indicating an association between larger right hippocampi and better scores on delayed memory performance. Age and hippocampal volumes did not significantly correlate and Trails A and B and entorhinal cortex volumes did not correlate with each other or any other measure (data not shown).

Never Users

Figure 37 depicts the relationships between brain volume measures, cognitive performance, and age for the NU group. Age negatively correlated with Total Words [r(10)=−0.749, p=0.0036 (Figure 37)], Delayed Memory

[r(10)=−0.731, p=0.0052 (Figure 37)], and right hippocampal volume [r(10)=−0.714, p=0.0073 (Figure 37)], indicating an association between an increase in age with a decline in memory scores and hippocampal volumes. Age positively correlated with Trails A [r(10)=0.763, p=0.0046 (Figure 37)] and Trails B [r(10)=0.793, p=0.0023 (Figure 37)] speed, indicating an association between an increase in age and an increase in processing speed and executive functioning. Hippocampal volumes did not correlate with cognitive scores, and entorhinal cortex volumes did not correlate with any other measure (data not shown).

Discussion

This study investigated the effects of prior HT use on mesial temporal lobe structure volumes (hippocampus and entorhinal cortex), and cognition. HT status as continuous, discontinuous, or never use differentially impacted hippocampal and entorhinal cortical volumes. For hippocampal volumes, discontinuous HT use was associated with the most favorable outcome. Indeed, discontinuous HT users demonstrated a bilateral preservation of hippocampal volumes as compared to women who never used HT. Continuous HT appeared to be less protective than discontinuous HT, with continuous HT users showing only a marginal hippocampal volume protection as compared to never users for the right hippocampus only. These findings are in accordance with others who have shown positive effects of estrogen-containing HT on hippocampal volumes in menopausal women (Eberling et al., 2003; Yue et al., 2007; Erickson et al., 2010; Lord et al., 2008; Boccardi et al., 2006). Further, our disparate findings between continuous and discontinuous users align with a voxel-based morphometry study

by Boccardi et al. (2006), detailing that past HT use is associated with greater volumes of gray matter, including the hippocampal complex, than current HT use.

Conversely to hippocampal results, for entorhinal cortex volumes, continuous HT use was associated with the most favorable outcome. Continuous users had significantly larger right and marginally larger left entorhinal cortex volumes, as compared to never users. Discontinuous HT use demonstrated no protection of entorhinal cortex volume loss, as compared to never users, but also did not differ from continuous users. Again, our findings are in accordance with Boccardi et al. (2006) who found that when grouping past and current HT users together, HT protected against entorhinal cortex volume loss as compared to non-users, but was not a region that showed greater protection in past users as compared to current users. To our knowledge, only one study has previously evaluated the ability of estrogen-containing HT to protect against age-related reduction in entorhinal cortex volume through manual tracings. Results from this longitudinal study are difficult to interpret because at the five year follow-up, neither the HT group nor the control group exhibited a significant decrease in entorhinal cortex volume (Raz, Rodrigue, Kennedy, & Acker, 2004).

The volumetric results reported are corrected for TIV as is convention in most reports of regional volumetric analyses. Since total intracranial volume is an estimate of general brain size, it is interesting that no significant group differences were observed on this measure, suggesting that HT did not have a generalized effect on brain size in our study.

We found HT status to have a modest impact on verbal memory performance. There was a marginal benefit to discontinuous HT use compared to never using HT, as demonstrated by a higher percentage of words remembered after a delay. Continuous HT use did not demonstrate the same modest delayed memory performance enhancement, as compared to never use, but also did not differ from discontinuous HT user's performance. These findings are in accordance with others showing that past, but not current, HT use protects against a longitudinal MMSE decline (Matthews, Cauley, Yaffe, & Zmuda, 1999) and an increased risk of dementia (Zandi et al., 2002). In our study, psychomotor speed and executive functioning, as measured by the TrailsA and TrailsB, did not seem to be affected by HT status, which is in accordance with other findings as well (Etnier & Sibley, 2004; Pefanco et al., 2007).

Our sample size precluded formal analyses of complex interactions of HT, cognition, and brain volumes; however, we performed separate correlation analyses by group to visualize possible differences in the expected age-related declines in both brain structure volumes and cognition. These correlations revealed that HT status appears to modulate the relationship between age, hippocampal volumes, and cognition. Specifically, women who had never used HT showed the expected age-related decline in hippocampal volume and cognition as measured by verbal memory, psychomotor speed (TrailsA), and executive function (TrailsB). HT use on any timeframe (continuous or discontinuous) showed differential effects on the relationship between age and either hippocampal volumes or cognitive measures. In continuous users, age was

negatively correlated with bilateral hippocampal volume, but there was no significant age-related decline on any cognitive measure. This suggests that continuously using HT alters the age-related decline in cognitive performance, but, remarkably, not hippocampal volume loss. In discontinuous users, age-related decline was observed for verbal memory performance, but did not show a significant relationship with hippocampal volumes. This suggests that a history of HT use produces some protection against the expected age-related declines in hippocampal volume but did not protect against a decline in verbal memory performance with age. Interestingly, the discontinuous HT users were also the only group to demonstrate a relationship, albeit modest, between hippocampal volume and memory performance, which may indicate that discontinuous HT use could have a biological effect to more strongly relate hippocampal volume with verbal memory performance. This is particularly noteworthy since discontinuous HT use was associated with the most favorable outcomes on both hippocampal volume and verbal memory performance. Future studies are warranted to elucidate the differential roles, and potentially mechanisms by which, continuous and discontinuous HT use ameliorates age-related declines in hippocampal volumes and cognitive performance.

Entorhinal cortex volumes showed no correlation in any group with age or cognitive measures. The lack of correlation with age is not surprising since others have also demonstrated a decline in hippocampal volumes with age, but not in entorhinal cortex volumes (Knoops, Gerritsen, van der Graaf, Mali, & Geerlings, 2012). Entorhinal cortex volumes did not correlate with cognitive measures in any

group. Further, although continuous HT use was associated with the largest benefit in entorhinal cortex volume, this group did not demonstrate a cognitive enhancement, relative to never users, on any measure. Instead, the only cognitive benefits seen herein were related to discontinuous HT use, the group that also showed the most benefit in hippocampal volumes. Studies in humans (Fell, Klaver, Elger, & Fernandez, 2002) and animals (Ferreira et al., 1992) suggest that the hippocampus and entorhinal cortex have different roles in memory formation based on encoding phase. Perhaps the tasks used in this study are not sensitive to the cognitive enhancements associated with larger entorhinal cortex volumes. Future research is warranted to elucidate the functional consequences of larger entorhinal cortex volumes in continuous versus never HT users.

Our sample size precluded us from obtaining statistical power to evaluate two other factors that are important to consider, which are content of HT and hysterectomy. Collective findings from the WHIMS suggests that women who had undergone hysterectomy and received HT containing CEE only did not show the HT-associated detriments in dementia risk and brain volumes (Shumaker et al., 2003; Resnick et al., 2009). Women who had not undergone hysterectomy, and were thus assigned to receive CEE+MPA, did exhibit HT-associated detriments in dementia risk and brain volumes (Shumaker et al., 2004; Resnick et al., 2009). It is possible that the less favorable outcomes on delayed memory and hippocampal and entorhinal cortex volumes seen in the never users in our study, are a result of fewer incidents of hysterectomy in this group, versus both continuous and discontinuous users. Indeed, animal models of transitional

menopause demonstrate detrimental effects associated with the retention of the follicular deplete ovary (Acosta, Mayer, Talboom, Tsang, et al., 2009). Additionally, our HT population in this study primarily consisted of women who had a history of using estrogens without progestogens. This may partially explain why our study showed HT-related benefits for brain volumes and cognition contrary to the WHIMS CEE+MPA trial (Shumaker et al. 2003; Resnick et al., 2009). Moreover, the review by Maki and Sundermann (2009), showing beneficial effects associated with estrogen-only HT, but detrimental effects of CEE+MPA, on verbal memory in menopausal women, further support this hypothesis. Future studies with larger sample sizes are warranted to determine the contributions hysterectomy and content of HT have on brain volumes and cognition in postmenopausal women.

In conclusion, HT use is associated with larger volumes of mesial temporal structures, with greater benefits observed for hippocampal volume with discontinuous HT use and greater benefits for entorhinal cortex with continuous HT use, both as compared to no HT use. Discontinuous HT use was associated with a modest verbal memory advantage that was also positively correlated with hippocampal volume. Finally, continuous and discontinuous HT use differentially modulated age-related decline in hippocampal volumes and cognitive performance. Thus, here, we provide further evidence that HT treatment in menopausal women can have favorable outcomes on the preservation of mesial temporal structure volumes and cognitive performance. Future investigations are

warranted to further elucidate the parameters under which HT can ameliorate the symptoms of menopause and benefit cognitive aging in women.

CHAPTER 7

BROAD-BASED NUTRITIONAL SUPPLEMENTATION IN 3XTG MICE CORRECTS MITOCHONDRIAL FUNCTION AND INDICATES SEX-SPECIFICITY IN RESPONSE TO ALZHEIMER'S INTERVENTION

Alzheimer’s disease (AD) is a neurodegenerative condition that is the most prevalent cause of dementia in the elderly. Due to shifting demographics, it is projected that the number of AD cases worldwide will increase dramatically during the first half of the 21st century (Ferri et al., 2005). While study of early-onset AD has uncovered genetic mutations that modulate production of the betaamyloid (Aβ) protein thought to be key in the disease, the vast majority (>98%) of AD cases are late-onset and idiopathic. Because of the limited knowledge of the underlying cellular and molecular causes of late-onset AD, detailed examination of risk factors is essential fordeveloping a stronger understanding of the pathogenic process. Based on the existence of dozens of epidemiological studies demonstrating that nutrition is an important factor in susceptibility to AD (Morris et al., 2009), several ongoing or recently completed human trials have investigated the neuroprotective capacities of many different nutrients (Kamphuis et al., 2010). In addition to these human trials, the wide-availability of AD rodent models has allowed for multiple experimental studies, with a significant body of work supporting the role of nutrients in modulating disease-related cognitive decline and pathology: blueberry supplementation can avert both cognitive decline and deficits in signal transduction (Joseph et al., 2003), curcumin demonstrates an ability to prevent cognitive decline (Yang et al.,

2005), green tea can slow cognitive decline through multiple mechanisms (Lee et al., 2009), and both cinnamon extracts (Frydman-Marom et al., 2011) and grape polyphenol extracts (Wang et al., 2008), can inhibit Aβ oligomerization and cognitive decline. Additionally, omega-3 fatty acids can reduce Aβ burden (Green et al., 2007; Grimm et al., 2011; Lim et al., 2005; Oksman et al., 2006), improve cognition (Arsenault, Julien, Tremblay, & Calon, 2011), and protect from dendritic pathology (Calon et al., 2004; Green et al., 2007). Medical food "cocktails" can reverse cognitive decline and attenuate soluble Aβ pathology (Parachikova, Green, Hendrix, & LaFerla, 2010), and the nutritional supplement S-adenosyl methionine has recently been shown to be effective in delaying tau or Aβ pathology in 3xTG mice (Lee, Lemere, Frost, & Shea, 2012) and may have wide-ranging effects on amyloid levels, tau phosphorylation, and DNA methylation (Fuso & Scarpa, 2011).

Whereas, until recently, most studies of dietary intervention in animal models largely have been focused on examining the effects of a single nutrient, the Memory Preservation Nutrition Supplement Program (MPNSP) used here combines three separate broad-based supplements: a phyto-nutrient powder, high-quality fish oil, and an amalgam of herbs and spices based on an evidence-based integrated whole foods program, Memory Preservation Nutrition (Emmerson Lombardo et al., 2005). The MPNSP was designed to delay the onset and slow the progression of AD in humans by providing broad-based nutritional supplementation targeting multiple organs and pathways. In this study, we administered this supplementation regimen to the 3xTG mouse, which is the first

transgenic mouse harboring the PS1M146V, APPSwe, and tauP301L transgenes, and therefore recapitulates several hallmarks of AD, including concomitant plaque and tangle formation in AD vulnerable regions (Oddo et al., 2003). The dietary cocktail was chosen for the possibility that multiple nutrients could extend and enhance the established successes shown in single nutrient studies due to additive and synergistic effects across the multiple nutrients. As a further benefit, these products have been demonstrated to be well-tolerated (Capodice et al., 2009; Freund-Levi et al., 2006) and are considerably less expensive than most conventional pharmaceutical treatments. We hypothesized that the MPNSP would prevent or reverse the brain regional glucose uptake declines we have shown in a variety of murine models of AD (Nicholson et al., 2010; Reiman et al., 2000; Valla, Gonzalez-Lima, & Reiman, 2008; Valla, Schneider, & Reiman, 2006).

Human fluorodeoxyglucose positron emission tomography (FDG PET) studies show that regionspecific alterations in the cerebral metabolic rate for glucose (CMRgl) are found in AD, with hypometabolism present in the posterior cingulate-precuneus (PCC), parieto-temporal, and prefrontal regions (Minoshima, Foster, & Kuhl, 1994; Reiman et al., 1996). Interestingly, decrements in CMRgl are localized to these same regions in aged pre-symptomatic homozygotes (Reiman et al., 1996) and young-adult carriers (Reiman et al., 2004) of the APOE 4 allele, the primary genetic risk factor for late-onset AD. We extended these findings to demonstrate reduced mitochondrial function, as measured by cytochrome c oxidase activity (Complex IV of the electron transport chain), in postmortem PCC as well as isolated platelets from living AD patients (Valla,

Berndt, & Gonzalez-Lima, 2001; Valla, Schneider, Niedzielko, et al., 2006). Further, the same deficiency was measured in platelets from patients with mild cognitive impairment (MCI) (Valla et al., 2006), and in the PCC of young-adult 18-40 yearold) carriers of the APOE 4 allele in the absence of AD histopathology (Valla et al., 2010), the latter thus indicating that, along with the CMRgl changes, energy metabolism decline may be among the earliest detectable brain changes associated with AD risk. This indicates that mitochondrial functional decline, measured via cytochrome oxidase (Complex IV), is effective at detecting some of the earliest cellular-level changes in AD pathogenesis or vulnerability and is therefore an attractive biomarker for use in evaluation of interventions and therapeutics, particularly those aimed at preclinical and early-stage AD. Thus, in addition to behavioral cognition and brain regional fluorodeoxyglucose uptake, we analyzed mitochondrial cytochrome oxidase activity, as well as fibrillar and soluble AD pathology and neuroinflammatory markers.

Methods

This study was performed under protocols approved by the respective Institutional Animal Care and Use Committees at the Barrow Neurological Institute, St. Joseph's Hospital and Medical Center and Arizona State University (ASU). 3xTG and wildtype (WT) controls (129/sv x C57BL6) were bred in our colony at the Barrow Neurological Institute from founders received from the University of California, Irvine. Transgene and diet status were blinded until analyses were complete. 3xTG (N=28, 14/condition) and wildtype (N=28, 14/condition) mice were placed in age- and sex-matched 4-mouse cohorts, with

one mouse of each genotype fed supplementation and the other standard diet (below). Mice were group housed in Plexiglas shoebox-style cages prior to study start and housed in singly or in pairs once supplementation began, with access to food (supplemented or standard) and water ad libitum.

Supplementation

Dietary supplementation was initiated at an average age of 38 weeks (range 29-46 weeks) and continued for 25 weeks, until the mice were euthanized. Mice on the supplemented condition were fed LabDiet 5001 (Purina, St. Louis, MO) containing the supplemental phyto-nutrient powder, herbal/spice amalgam, and cod liver oil integrated into the chow (for respective compositions see Table 13). Dose calculations were based on the MPNSP-recommended dose to an 80 kg human scaled to a 35 g mouse consuming 4 g/day. Mice on the nonsupplemented condition were fed LabDiet 9F 5020 (Purina, St. Louis, MO). Daily food consumption was monitored for the first three weeks, and mice were weighed every third week for the duration of the study. After approximately 18 weeks, all mice were transferred from the Barrow Neurological Institute to ASU to undergo behavioral testing; they remained at ASU until euthanized. Figure 38 provides a flowchart of the experimental protocols.

Delayed Match to Position Three Choice Task

Mice were tested on the Delayed Match to Position (DMP) Three Choice Task for 6 trials/day for 10 days, evaluating spatial working and short-term memory retention. A black Plexiglas maze (each arm was 38.1 cm x 12.7 cm) with four open arms, was filled with water made opaque using non-toxic paint,

average temperature 17.5°C, and contained one escape platform. The platform was moved to a new arm each day, but remained in the same arm within a day. Trial 1 was the information trial informing the animal where the platform was for that day's session, trial 2 was the working memory test trial, and trials three 3-6 were recent memory test trials (Frick, Baxter, Markowska, Olton, & Price, 1995). Mice were given 90 s to find the platform. Once located, the animal remained on the platform for 15 s. The inter-trial interval (ITI) was 30 s. An arm entry was counted when the tip of a mouse's snout reached a mark 11 cm into the arm. First errors were first entries into an arm without a platform, and Repeat errors were repeat entries into an arm without a platform. The dependent variables were the number of Total errors (First + Repeat errors), First errors, and Repeat errors across testing (i.e., days 1-10, trials 2-6) for each treatment group. To investigate the effects of learning vs. asymptotic phases of testing, errors were blocked into days 1-5 vs. days 6-10. For all measures of regular testing, an omnibus ANOVA was run to investigate main effects and interactions. When significant, analyses were followed by post hoc t-tests, with the a priori hypothesis that diet condition would influence cognitive performance. On day 11, mice were given a delay challenge of a 30 min ITI between trials 5 and 6. Planned comparisons to analyze effects of the delay were run comparing errors on the baseline day/trial of day 10, trial 6 (the last day of regular testing) versus the post delay trial given on day 11, trial 6. Effects of $p \leq 0.05$ were considered significant. Effects of $p < 0.05$ were considered significant.

Morris Maze

Two days after DMP concluded, each mouse received 4 trials/day for 6 days using a tub (188 cm diameter) filled with opaque water using non-toxic paint (average temperature 17.5°C). The hidden platform (10 cm wide) remained in a fixed location, thereby testing spatial reference memory (Bimonte-Nelson et al., 2006; Morris et al., 1982). Mice were placed in the maze from the North, South, East, or West location, and had 60 s to locate the platform in the Northeast quadrant. Once the mouse found the platform the trial was terminated and the mouse remained there for 15 s. The mouse was then placed into its heated cage until its next trial with an approximate ITI of 10m. Performance was assessed by swim path distance (cm) and latency (s) to the platform. To evaluate whether mice localized the platform to the spatial location, after all test trials on day 6, a 60s probe trial was given with the platform removed. Percent of total distance in the previously platformed (target) quadrant was compared to the quadrant diagonally opposite the platform. For each trial, a camera suspended above the maze tracked each mouse's path and a tracking system (Ethovision 5.1, Noldus Instruments) analyzed each mouse's tracing. Distance and latency across all days of testing and percent distance in the target and opposite quadrants during the probe trial were analyzed via an omnibus ANOVA to investigate main effects and interactions. When significant, analyses were followed by post hoc t-tests. Effects of $p < 0.05$ were considered significant.

Tissue Processing

Each animal was given an i.p. injection of 18 μCi/100 g body weight [14C]-fluorodeoxyglucose (FDG; American Radiolabeled Chemicals, St. Louis, MO) in sterile saline. During the subsequent 45 min uptake period each animal was placed into an empty individual cage in a dark and quiet cabinet. Mice were then decapitated, and the brain rapidly extracted and frozen. Brains were stored at -20°C until sectioned. 40 μm coronal sections were taken in four series, creating three matched slide sets and an aliquot of tissue divided into 3 pools (anterior to hippocampal formation, containing hippocampal formation, and posterior to hippocampal formation) for each subject. At each level of the series, 4 sections were also cut at 20 μm and dried for later immunohistochemistry. FDG autoradiography and CO histochemistry and subsequent densitometric imaging proceeded as performed previously (Nicholson et al., 2010).

Image Analysis

Defined regions-of-interest (ROIs) corresponded to those presented in Paxinos & Franklin, (2001), with the exception that the retrosplenial gyrus was divided into three defined anteroposterior ROIs to localize any reductions in PCC, based on previous work (Nicholson et al., 2010): posterior cingulate (approximately bregma –1.4), posterior cingulate level 2 (bregma –2.1), and retrosplenial (bregma –2.6). Autoradiographic and histochemical data were analyzed independently; based on the behavioral outcomes, sex was included as a variable in omnibus 2x2x2 (genotype by supplementation by sex) analyses of variance, with $\alpha = 0.05$. Significant effects were followed by post hoc Student's t-

tests at $\alpha = 0.05$: we did not correct for multiple comparisons but report the calculated p values; while this does not minimize Type I errors, the results are consistent with our previous analyses. Individual ROI scores showing a Studentized residual >3.0 in the ANOVA were deemed to be outliers and removed from the final analysis.

Fibrillar Amyloid & Tau Pathology

For Thioflavin S staining for amyloid plaques, slides were fixed with 4% buffered paraformaldehyde (PFA), rinsed with tap water, rinsed in distilled H2O, immersed in 4% thioflavin S in distilled H2O for 5 min, differentiated in 70% ethanol, rinsed twice with distilled H2O, and coverslipped with aqueous mounting media. Immunohistochemistry for hyperphosphorylated tau (clone AT8) was performed on frozen coronal sections. Frozen sections were fixed with 4% PFA, blocked with hydrogen peroxide (3% for 5 min) and 3% bovine serum albumin (BSA) and 2% goat serum (1 hour), and probed on-slide with an antibody for phosphorylated tau (AT8; Pierce) and visualized with diaminobenzidine-hydrochloride (DAB; Dako LSAB+ System). Assessments of both were performed by a single rater, blinded to conditions, on an Olympus BX61 microscope using a 3-point scale (heavy – moderate - light) in the areas of highest deposition/tangle density (hippocampal subiculum, CA1, CA3).

Brain tissue extract for ELISA and Western blotting

Ten of the cryostat-cut coronal sections, from each of the 3 levels, were homogenized in 200 µl of 50 mM Tris-HCl at pH 8.0, with 1mM EDTA and a cocktail of protease and phosphatase inhibitors (Pierce); these were aliquoted and

stored until the ELISA. For Aβ ELISA, samples were further extracted with guanidine HCl, whereas for glial fibrillar acidic protein (GFAP) and synaptophysin ELISA, samples were further extracted in 4 volumes of 5x RIPA buffer.

Aβ ELISA

Thirty μL of Tris buffer-extracted brain homogenates were extracted further with 7M guanidine- HCl to obtain a final concentration of 5M guanidine-HCl. Extraction was performed at 4oC for 17 hours on shaker. Protein concentrations of these samples were determined by MicroBCA assay. ELISA kits for human Aβ42 and Aβ40 (Invitrogen) were used according to manufacturer's instructions. For both Aβ42 and Aβ40 ELISA, 1200 ng of protein were loaded to each well, and duplicate wells from the same samples were assayed and averaged.

GFAP ELISA

The GFAP ELISA was developed in-house, using a capture antibody of purified anti-GFAP cocktail (BD Biosciences) used at 0.25 μg in 100 μL per well, and a detection antibody of rabbit polyclonal anti-GFAP (DAKO) used at 1:10,000 dilution. Anti-rabbit IgG-conjugated horseradish peroxidase (rabbit IgG-HRP; Pierce) was used at 1:20,000 dilution. Purified GFAP (Calbiochem) was used to manufacture standards to cover a range from 0.156 to 10 ng/mL. Capture antibody coating buffer was sterile PBS, pH 7.4. Wash buffer was composed of PBS with 0.05% Tween 20 (PBST), pH 7.4. Sample dilution buffer was PBST with 1% skim milk while blocking buffer contained 3% skim milk powder. Plates

were coated with capture antibody for 18 hours at 4oC, followed by washing 5 times with wash buffer installed in a plate washer. Plates were blocked for 1 hour using blocking buffer at room temperature, followed by washing 5 times. RIPA bufferextracted brain samples were loaded at 100 µg per 100 µL per well for 2 hours, and detection antibody incubation was carried out for 2 hours after washing 5 times. Anti-rabbit IgG-HRP diluted in wash buffer was applied to the wells for 2 hours followed by 5x wash. Colorimetric enzymatic reaction was achieved by incubating 30 m with substrate for anti-rabbit HRP (R&D Systems). Reaction was stopped by 1M sulfuric acid solution. Optical absorbance for the color products in each well was read by a spectrophotometer at 450 nm. Data was calculated according to the linear regression of the protein standards and absorbance.

Synaptophysin ELISA

Synaptophysin ELISA was also developed in-house, using a capture antibody of mouse antisynaptophysin IgM antibody (Covance, clone SP17) used at 1:4000; detection antibody was rabbit anti-synaptophysin IgG (Chemicon) used at 1:2000. Synaptophysin protein standard (Abnova) was used in a range from 0.156 to 5 ng/mL. Samples were loaded to each well at 1 µg protein per 100 µL per well. Anti-rabbit IgG-HRP (Pierce) was used at 1:3000. Wash buffer was 0.1 M Tris buffer, pH 7.4, containing 0.05% Tween 20 (TBT). Sodium carbonate buffer, pH 9.6, was used as coating buffer for the capture antibody. Blocking buffer was TBT containing 5% skim milk, while sample diluent was TBT containing 2% skim milk powder. Incubation schedule was performed as described above.

Western blot detection of microglial markers

Protein (10 μg/lane) from brain samples prepared for ELISA was prepared for electrophoresis with LDS loading buffer and reducing reagent DTT (1 μg/μL) and run on NuPage 10-14% Bis- Tris mini gels with MES running buffer (Invitrogen). Separated proteins were transferred to nitrocellulose membranes at 30 volts for 60 m. Membranes were air-dried and blocked with 50 mM Tris-buffered saline, pH 8.0, containing 0.05% Tween 20 (TBS-T) and 5% nonfat dried milk powder. Microglial specific marker Iba-1 (ionized calcium binding adapter protein 1) was detected with a rabbit polyclonal antibody (Wako Chemicals U.S.A., Richmond, VA). Microglial alternative activation markers Ym1 (chitinase 3-like 3), MMR1 (macrophage mannose receptor 1), and ARG1 (arginase 1) were detected with polyclonal antibodies raised in either goat or sheep (R&D Systems, Minnesota), followed by secondary antibodies conjugated with horseradish peroxidase (HRP) of appropriate species. Blots were then reacted with Dura chemiluminescent substrate for HRP (Thermo Scientific) and imaged. Specific bands were identified by appropriate molecular weight and the density of immunoreactive bands was measured with Alpha View software (Cell Biosciences) and normalized to β-actin.

Results

MPNSP supplementation was generally well-tolerated. Mortality during the study appeared unrelated to supplementation: 1 WT mouse and 3 3xTG mice died or were euthanized prematurely due to illness while on supplementation, 2 WT and 4 3xTG mice died or were euthanized while on the standard diet. Data

from these animals are included when possible and when not obviously impacted by illness preceding death. Final sample sizes under supplementation were N=12 3xTG (6M, 6F) and N=10 WT (4M, 6F), and nonsupplemented were N=13 3xTG (5M,8F) and N=13 WT (5M, 8F). Consumption of the two diets did not differ during the initial 3-week monitoring period; overall, each supplemented mouse consumed 3.8±0.5 g/day and each mouse on standard chow 3.8±0.6 g/day ($p=0.89$, 2-tailed t-test; Figure 39a). Mean body weight in the two groups diverged early in the feeding (3-6 weeks) and then stabilized, with supplemented mice weighing less in comparison at each point (Figure 39b), but there was no indication of increasing divergence between the groups over time. Given the relatively broad range in age of mice at initiation of supplementation, we first explored the possibility of age-related effects by analyzing behavioral, FDG, and CO data by similarly-aged cohorts (approximately 29-35 weeks, 36-41 weeks, and 42-46 weeks at initiation), and via ANCOVA with age as covariate; no significant effects of age were found (data not shown). In contrast, analysis of the behavioral results demonstrated that sex accounted for more measured variability than age; thus, all subsequent analyses included sex as a variable.

Delayed Match to Position Three Choice Task

Across all days of regular testing, there was a main effect of genotype for each type of error [Total: $F(1, 45) = 6.103$, $p<0.05$ (Figure 40); First: $F(1, 45) = 8.359$, $p <0.01$; Repeat: $F(1, 45) = 4.694$ $p<0.05$] with 3xTG mice making more errors than WT mice. To investigate the effects of learning vs. asymptotic phases of testing, errors were blocked by days 1-5 vs. days 6-10. Here there was a

significant interaction between block, genotype, and sex [$F(1, 41) = 7.390$, $p<0.01$]. Because our a priori hypothesis was that diet would impact cognitive performance, we further investigated diet effects within the significant block, genotype, and sex interaction. During the asymptotic phase, the supplemented male 3xTG mice made fewer total errors than the nonsupplemented male 3xTG mice [$t(8) = 5.267$, $p=0.05$ (Figure 40)]. The female 3xTG mice did not differ by supplement status.

On Day 11 there was a 30 min delay given between trials 5 and 6. No group significantly increased the number of errors committed between Trial 6 of the last day of regular testing (Day 10) and Trial 6 after the delay (Day 11).

Morris Maze

Across all days of testing, there was a main effect of Day for Distance and Latency in the WT and 3xTG mice [WT Distance: $F(5, 130) = 117.887$; $p<0.0001$; WT Latency: $F(5, 130) = 104.124$; $p<0.0001$; 3xTG Distance: $F(5, 90) = 21.396$; $p<0.0001$; 3xTG Latency: $F(5, 90) = 15.428$; $p<0.0001$], with scores decreasing across days, demonstrating learning of the task for both genotypes. There was a main effect of genotype for each measure [Distance: $F(1, 44) = 9.671$, $p<0.005$; Latency: $F(1, 44) = 7.995$, $p<0.01$] with 3xTG mice swimming a longer distance and exhibiting a greater latency to locate the platform, than WT mice (Figure 41). There were no supplementation effects, sex effects, day x supplementation interactions, nor day x sex interactions for performance as measured by either measure. The probe trial, assessing learning of the platform location (NE quadrant), revealed a main effect of Quadrant [t (36) = 15.5;

$p<0.0001$] and a Quadrant x Genotype interaction [t (36) = 3.13; $p<0.005$]. Each group spent more percent distance in the target NE quadrant as compared to the opposite SW quadrant, showing localization of the platform location [WT: F(1, 22) = 392.851; $p<0.0001$; 3xTG: F(1, 18) = 40.375; $p<0.0001$]; however, WT mice spent significantly more percent distance in the target NE quadrant [t(40) = 14.747; $p<0.0005$] and significantly less percent distance in the opposite quadrant SW [t(36) = 4.747; $p<0.05$] than 3xTG mice, suggesting that WT mice showed better localization to the platform location than 3xTG mice.

FDG Autoradiography

The omnibus 2x2x2 ANOVA revealed a significant main effect ($p<0.05$) of genotype in 44 of 55 ROIs, consistent with our previous results in these mice at a slightly older age (18 months; Nicholson et al., 2010). Also consistent with previous results, our usual data normalization to average whole brain activity was precluded by the widespread gene-related changes in glucose uptake, so analysis proceeded with nonnormalized FDG uptake data. Modestly significant main effects of supplement status were found (reticular thalamus, nucleus basalis, and medial geniculate), and within these, significant interactions between genotype and supplementation (medial geniculate) and genotype, supplementation, and sex (nucleus basalis). Completion of post hoc 2-tailed ttests revealed that significant effects in medial geniculate were mediated by a supplementdriven increase in WT mice (supplemented vs. nonsupplemented: 871±210 vs. 657±122, mean±standard deviation, p=0.009), particularly the males (supplemented vs. nonsupplemented: 921±176 vs. 605±40, p=0.025). A similar trend was apparent in male WT nucleus

basalis (supplemented vs. nonsupplemented: 826±167 vs. 498±68, p=0.020). Four other ROIs showed the identical relationship: middle cingulate gyrus (p=0.012), anteroventral thalamus (p=0.020), reticular thalamus (p=0.033), and mediodorsal thalamus (p=0.041). Only one ROI showed a similar effect in WT females, the mammillary bodies (1226±148 vs. 869±249, p=0.011). Of note, significant effects of the supplementation were seen across the expanse of the WT cingulate gyrus: anterior (p=0.047), middle (p=0.039), posterior (p=0.039), retrosplenial (p=0.034), except posterior level 2 (p=0.094), and also in the heavily interconnected hippocampal subiculum (p=0.042). WT somatosensory barrel fields, lateral posterior thalamus, and pontine nuclei also showed significant effects of supplementation ($p<0.05$). All significant changes were increases, and collectively, all WT ROI values were increased in the supplemented group. No significant post hoc effects were seen across the 3xTG groups or between comparisons of sex within the 3xTG; all apparent regional glucose uptake effects were mediated by changes in WT mice.

Cytochrome Oxidase Histochemistry

Previous analyses of 3xTG mice from our colony have demonstrated significant CO activity declines in several brain regions (unpublished data), similar to our findings in other AD mouse models (Valla et al., 2007). The omnibus 2x2x2 ANOVA revealed many regional significant main effects (Table 14). Several strong interactions with sex were identified, prominently including the entorhinal cortex and hippocampal formation subregions. Significant differences between subgroups of 3xTG mice were again confirmed by post hoc

2-tailed t-tests. In contrast to FDG uptake, the CO changes under supplementation were mediated almost exclusively by 3xTG mice: supplementation induced significant increases in several brain regions, to approximately WT levels (Table 15), most significantly in posterior cingulate (3xTG supplemented vs. nonsupplemented: $p<0.001$), primary somatosensory ($p=0.001$) and barrel field ($p=0.009$) cortex, mediodorsal thalamus ($p=0.003$), lateral hypothalamus ($p=0.007$), and lateral posterior thalamus ($p=0.013$). A similar trend was apparent in basolateral amygdala. The directionality and effect size of these changes was roughly the same in 3xTG males and females. However, an alternate pattern emerged when comparing across sex in areas showing other significant ANOVA effects (Table 15): in entorhinal cortex, male and female 3xTG showed a significant difference after supplementation (201 ± 16 and 166 ± 20, $p=0.039$), but 3xTG males increased significantly to WT levels, while 3xTG females decreased nonsignificantly from WT levels, indicating a profound difference in regional function. Such a trend was also apparent in CA1 and the medial mammillary nuclei. Similarly, in CA3 and dentate gyrus, supplementation elicited little change in 3xTG males but caused the females to significantly decline from WT levels (Table 15).

Fibrillar Amyloid & Tau Pathology

Thioflavin staining for amyloid deposition revealed a consistent involvement of the hippocampal formation, particularly the anterodorsal subiculum and CA1, but often extending through ventroposterior aspects and to the amygdala. Lesser deposition was also seen in the basal forebrain and frontal

cortices. This pattern is consistent with our previous report (Nicholson et al., 2010). All wildtype mice were negative, and no apparent differences were observed between supplementation conditions in the 3xTG mice. AT8-positive structures resembling neurofibrillary tangles were consistently located in 3xTG hippocampal CA1 and CA3; in images captured from these areas, no apparent difference in the density or composition of these tangles was seen between supplementation conditions. In the case of both Aβ and tau, deposition tended to worsen with increasing age across the range studied here.

Biochemical measurements of Aβ, synaptophysin, and glial activation markers

ELISA measurement of Aβ40 and Aβ42 in 3xTG mouse brain tissues at the level containing hippocampus showed a trend toward lower levels in supplemented mice. Analyzing by gender, it was apparent that the trend was driven by female mice, which showed significantly higher levels compared to the males. Further analysis of the 3xTG female subgroup (age at sacrifice [weeks]: supplemented: 64.1±2.5; nonsupplemented: 67.0±2.0, N=5/group) showed a significant reduction in Aβ42 (Table 16; $p<0.038$). This phenomenon was not observed in Aβ40 or male mice, although the trend in Aβ40 was similar in 3xTG females. The concentration of synaptophysin was not affected by supplementation (Table 16). Activated astroglial marker, GFAP, was used as an initial indicator of neuroinflammation. Supplementation did not lead to differential GFAP levels in either WT or 3xTG mice (Table 16). To determine the effects of diet treatment on microglial activation, we measured whether there were changes in the levels of

Iba1, Ym1, MMR1, and ARG1 expressions. Iba1 has been used widely as a marker for brain microglia across a spectrum of activation states, whereas Ym1, MMR 1, and ARG1 have been used specifically to identify alternatively-activated microglia in AD and APP transgenic mouse brains (Colton et al., 2006). Western blotting results showed a lack of effect of supplementation on most of the microglial markers except on ARG1 in female 3xTG mice ($p<0.05$, Table 16).

Discussion

We have confirmed that MPNSP supplementation can modulate markers of brain energy metabolism and alter other indicators of cognition and pathophysiology in a mouse model of AD, depending on sex. MPNSP supplementation prevented or reversed cognitive decline, with the modality affected dependent upon sex in this model. In post hoc analyses, MPNSP supplementation increased WT FDG uptake in several regions, including retrosplenial cingulate, posterior cingulate, and subiculum, but showed few effects in the 3xTG mice, who maintained significant FDG decreases in most ROIs. In contrast, analysis of regional CO activity in the 3xTG demonstrated several key regions in which the enzyme activity was corrected to WT levels. Following on the behavioral analysis, a breakdown by sex revealed striking differences in brain regional effects, particularly in regions in and closely connected to the hippocampal formation (e.g., entorhinal cortex).

Deposition of fibrillar plaques and AT8-positive tau did not appear to be ameliorated by the supplementation, possibly a function of the timing of intervention: evidence of intracellular and extracellular Aβ in the 3xTG mice has

been reported at 4 and 6 months, respectively (Billings, Oddo, Green, McGaugh, & LaFerla, 2005). Memory impairment has been reported at 4 months (Billings et al., 2005) and synaptic dysfunction at 6 months (Oddo et al., 2005). Our earliest intervention began at approximately 7 months, after these processes should be underway; that said, biochemical measurements of the levels of human Aβ40 and Aβ42 in 3xTG mice showed supplementation-induced changes, with a strong trend toward differences dependent upon sex. A significant Aβ42-lowering effect was observed in the female subgroup, which had much higher Aβ levels overall as reported in this model previously (Hirata-Fukae et al., 2008). Our analyses indicate that our sample may have been underpowered for detection of several of the tested effects; alternatively, an earlier start time or higher dosing may be required to maximize the benefit of broad-based nutritional supplementation.

Our earlier regional FDG analyses of mouse models of AD, including this model (Nicholson et al., 2010), indicated consistent declines in brain regional FDG uptake, some similar to those seen in human AD (Reiman et al., 1996; Minoshima et al., 2004). We were surprised to find that the supplementation did not correct this aberrant FDG uptake, which is an extremely plastic marker of brain activity, while it did correct mitochondrial CO function, also a plastic marker but much less so, in many of the same regions. One possible explanation for this dichotomy may be that aspects of the transgene-induced changes have direct and perhaps irreversible effects on mechanisms of FDG uptake, while the mitochondrial changes may be more amenable to our treatment, which includes compounds thought to be mitochondrial enhancers (Lagouge et al., 2006).

Concerning cognition, we were able to detect robust differences between the WT and 3xTG groups for both spatial working and short-term memory on the DMP task, and spatial reference memory on the MM, establishing an excellent paradigm for the evaluation of interventions in the 3xTG AD model. Importantly, MPNSP supplementation generated significant changes in short-term spatial memory as demonstrated in the DMP task, but not for spatial reference memory on the MM. These divergent effects may represent a specificity of memory type enhancement through MPNSP supplementation, aiding more in short-term working memory functional recovery rather than reference memory. Indeed, these effects are in line with the brain region-specific recovery of CO activity in supplemented male 3xTG entorhinal cortex, an area that may be more important for short-term working memory processing than long-term reference memory, as shown in patients with MCI using MRI voxel-based morphometry (Schmidt-Wilcke, Poljansky, Hierlmeier, Hausner, & Ibach, 2009). Although behavioral analysis often revealed only marginal effects of the supplementation, we could determine that subject sex contributed substantially to variability and, when analyzed accordingly, differences between the sexes were indeed significant. Further, in the subsequent imaging and pathology analyses, including sex as a covariate illuminated other sex-specific changes. Specifically, in entorhinal cortex, medial mammillary nuclei, and in the CA3 and dentate gyrus of the hippocampus, a pattern emerged whereby in these regions supplementation corrected CO activity to WT levels in 3xTG males only, and decreased levels in supplemented 3xTG females only. Given the prominent role of these nuclei in the

behavioral tasks tested, these regional effects may reflect the sex-specific performance differences we observed. It follows then, that these network differences may reflect differences between male and female correction and/or compensation of functional deficits; i.e., 3xTG females activate different pathways than males in order to perform the task, or suffer enhanced regional vulnerability (or differences in recovery) which forces them to do so. That the entorhinal cortex and hippocampus appeared central to this sex-specific network change in mitochondrial activity was no surprise; previous research from our group has indicated that these regions are particularly sensitive to sex steroid-induced alterations in female rodents (Braden et al., 2010, Chapter 2). In fact, estrogen and progesterone have been shown to modulate AD pathology and hippocampal-dependent behavioral function in 3xTG mice (Braden et al., 2010, Chapter 2), and female 3xTG mice display larger deficits in hippocampal-dependent behavior than matched males (Carroll et al., 2010). The CO biomarker identified changes in areas (including the hippocampal formation) relevant to the apparent preservation of cognitive function that resulted from supplementation in 3xTG males. This lends support to further examination of CO as an endpoint in preclinical interventional studies, and our concomitant work in humans (Valla et al., 2001, 2006, 2010) indicates that CO may be a useful biomarker early in the disease process and a quantifiable endpoint for the effects of consequential intervention.

As many components of the supplement diet used in this study may possess anti-inflammatory properties, we also examined whether the diet could

alter the activation of glial cells. We found that in female 3xTG mice the supplementation significantly increased the expression levels of ARG1, a marker for alternative activation of microglia. This result suggested that the supplementation might have potential to shift a subpopulation of microglia from classical to alternative activation phenotype. Alternatively-activated microglia have an enhanced ability to phagocytize Aβ (Colton et al., 2006). However, ARG1 expression could also be increased with severity of AD as indicated by previous data obtained in AD brains and in 70 week-old Tg2576 mice, an animal model for amyloid pathology in AD (Valla et al., 2007). Increases in ARG1 have also been observed in the lipopolysaccharide-induced inflammatory response in the rTG2510 transgenic mice, an animal model overexpressing P301L tau (D. C. Lee et al., 2010)(Lee et al., 2010).

This study has demonstrated that broad-based nutritional supplementation can positively impact certain parameters of cognition, mitochondrial function, and pathophysiology in a transgenic mouse model of AD. There were no indications that the supplementation carried unwanted side effects; indeed, these supplements have a long history of safe use in humans, including in formal clinical trials (Kamphuis et al., 2010; Capodice et al., 2009). Further research is needed to establish the optimal time for such supplementation to begin and its optimal dose (in both mice and humans). The youngest mice in this study at initiation were approx 7 months old, and cognitive deficits, Aβ accumulation, and bioenergetic deficits have been previously reported in this model prior to that age; nonetheless, MPNSP supplementation induced several potentially beneficial effects. And,

while not directly addressed in this study, it is possible that supplementation may be more effective as a preventative therapy, possibly via mechanisms bolstering brain reserve (i.e., increasing metabolic capacity via effects on mitochondrial function) or keeping levels of pathological species of tau or Aβ below critical thresholds. Given the relative safety and low cost of such intervention relative to current traditional pharmaceutical treatments with similar efficacy, broadbased supplementation such as the MPNSP or comprehensive diet plans of similar design hold great promise in the prevention and potential treatment of AD.

CHAPTER 8

GENERAL DISCUSSION

The work in this dissertation used the surgically menopausal rat to evaluate the cognitive effects and mechanisms of multiple progestogens clinically used in women. Findings suggest that MPA and progesterone may not be the best candidates for the progestogen component of HT and birth control, and point towards alternatives, such as NETA and LEVO. Moreover, in a clinical translation, this work suggests that a history of HT use is associated with positive impacts in volumes of mesial temporal lobe structures and memory measures in menopausal women. Finally, data herein suggest that sex is a variable that can alter outcome when evaluating the impact of a dietary intervention in a mouse model of AD.

Cognitive Effects

MPA

This series of experiments was the first to test the synthetic progestin, MPA, for learning and memory in the female rodent. MPA is the most commonly utilized progestin component of HT given to women in the United States (Hersh et al., 2004) and the only hormone found in the injectable contraceptive Depo Provera. In Chapter 2, we found that MPA impaired cognition in the aged surgically menopausal rat. Next, in Chapter 3, we investigated the possible long-term effects of MPA administration during young adulthood, and/or interactions with subsequent MPA administration in post-Ovx middle-age, on cognition. Specifically, MPA given in either young adulthood, middle-age, or both, impaired

cognition, and MPA given at both time points was particularly detrimental to cognitive performance, as evidenced by an impairment on multiple memory measures. Further, we were able to confirm through blood serum analyses that, even in the absence of circulating MPA levels at the time of test, MPA administration during young adulthood resulted in long lasting working memory impairments evident later in life (middle-age). Finally, in Chapters 4b and 5b, we found that a shorter duration of MPA treatment impaired performance during the latter part of testing (Chapter 4b), or exacerbated a delay-induced memory retention impairment (Chapter 5b) on the WRAM. The current findings are in agreement with clinical and basic science evidence implicating MPA as detrimental to the brain and its function in women (Gabriel & Fahim, 2005; Shumaker et al., 2003), animals (Lowry et al., 2010), and cell culture (Nilsen et al., 2006).

NETA

With evidence mounting that MPA is not the optimal progestin choice for women, we turned our efforts to evaluating the cognitive effects of other popular progestins for HT and contraception that are of a different progestin class than MPA. First, in Chapter 3a we tested two doses of tonically administered NETA in the middle-age Ovx rat, and found that both doses impaired cognitive performance, with greater impairments observed in the higher dosed group. However, in Chapter 3b, when we administered NETA via daily injections for a shorter duration, this same moderate dose of NETA that previously impaired cognition when given tonically, had no effect. Moreover, a high NETA dose

improved cognitive performance under these treatment parameters. Collectively, our findings suggest that the cognitive impact of NETA, in the middle-aged Ovx rat, depends on the parameters involved in treatment (dose, schedule of treatment, and treatment duration). Divergent NETA results have also been observed clinically, with studies suggesting that NETA-containing HT can have positive effects (Tierney et al., 2009), or no impact (Gambacciani et al., 2003) on memory function.

LEVO

In Chapter 4b, we were the first to evaluate the cognitive effects of LEVO in any basic science or clinical setting. Here, we found that while LEVO did not have a robust impact on cognition, at the high dose, via daily injections, it did prevent a delay-induced impairment on the WRAM, suggesting that LEVO may enhance memory retention. While there has been no other research on the effects of this hormone for memory or any marker of brain function, these findings do support our hypothesis that progestins with a different chemical structure than the cognitively-impairing MPA can enhance cognition in the middle-age Ovx rat.

Progesterone

In Chapter 2, we replicated previous findings that natural progesterone impairs performance on the WRAM in the aged Ovx rodent (Bimonte-Nelson, Singleton et al., 2004). Further, we replicated our previous findings, the herein being our third report, that Ovx is beneficial in aged rats, by improving performance on the WRAM (Bimonte-Nelson et al., 2006), likely mediated in part by the removal of high endogenous progesterone levels (Bimonte-Nelson et al.,

2004b). In Chapter 5a, we extended these findings to the middle-aged Ovx animal, showing that daily injections of progesterone, beginning two weeks before test, impaired performance on the WRAM. Our data concur with findings in humans, that administration of progesterone or its metabolite, allopregnanolone, is detrimental to memory in healthy women (Freeman et al., 1992; Kask et al., 2008).

Mechanism

GABA

The findings from Chapter 2 demonstrate that in aged Ovx animals both natural progesterone and the synthetic MPA either significantly or marginally alter GAD levels in the hippocampus and entorhinal cortex. GAD is the synthesizing enzyme and rate limiting step of GABA synthesis, and is routinely used as a neuronal marker for GABAergic function (Bauer et al., 2000; Milbrandt et al., 2000). Our progesterone findings are in line with others who have shown that progesterone decreased GAD activity in the dorsal hippocampus (Wallis & Luttge, 1980). In middle-aged Ovx animals, while there was no main effect of MPA treatment on GAD levels in any of the brain regions analyzed, we found a negative correlation between serum MPA levels and GAD in the dorsal hippocampus (Chapter 3). In animals receiving MPA treatment at test, higher MPA levels correlated with less GAD in the dorsal hippocampus, which is in agreement with our findings of MPA-induced decreases in GAD in the dorsal hippocampus in aged Ovx animals (Chapter 2).

Previous research demonstrating that progesterone metabolites (Lan & Gee, 1994) and MPA (Belelli & Herd, 2003) can increase GABA-mediated inhibition in the hippocampus, as well as progestogen-induced changes that we have observed in GABA markers (Chapter 2), led us to pharmacologically investigate the role of the GABAergic system in progestogen-induced memory impairments. In Chapter 5a, we found that in middle-aged Ovx animals, concomitant treatment with the $GABA_A$ antagonist bicuculline, completely reversed progesterone-induced memory impairments. This suggests that progesterone-induced memory impairments may be via activation of the GABAergic system. In this study we also measured changes in number of GABA-producing cells in the hippocampus of behaviorally tested animals. Progesterone and/or bicuculline had no effect, suggesting that a change in number of GABA-producing cells in the hippocampus may not be the mechanism of progesterone-induced memory impairments or the bicuculline reversal.

In contrast to effects with progesterone, in Chapter 5b, we found that concomitant treatment with bicuculline had no effect on MPA-induced memory impairments in middle-aged Ovx animals. This suggests that MPA-induced memory impairments may not be via activation of the GABAergic system. However, when we examined MPA and/or bicuculline-induced changes in number of GABA-producing cells in the hippocampus, we found that MPA treatment increased the number of GABA-producing cells, an effect reversed by bicuculline. Here, we see a dissociation between bicuculline's ability to block MPA-induced changes in hippocampal GABAergic markers and cognitive

impairments, further suggesting other mechanisms of MPA-induced memory changes. Moreover, this increase in a hippocampal GABAergic marker seen after short-term MPA treatment (two weeks) is in the opposite direction of our previously established MPA-induced decrease in GAD protein levels in the hippocampus after long-term treatment (two months; Chapter 2). This suggests that MPA may exert differential effects on the hippocampal GABAergic system depending on duration of treatment.

Neuronal Health

Cox-2

Progesterone and MPA have both been shown to modulate Cox-2 expression after insult (Cutler et al., 2007; Sunday et al., 2006). Previous research of progesterone's effects on Cox-2 is mixed, with one study finding a progesterone-induced decrease in Cox-2 expression (Cutler et al., 2007), and another a progesterone-induced exacerbation of Cox-2 (Sunday et al., 2006), both after insult. In Chapter 5a, we found no effect of progesterone on number of Cox-2 positive cells in the frontal cortex, suggesting that inflammation in the frontal cortex is likely not the mechanism of progesterone-induced memory impairments. MPA, however, has only been associated with an exacerbation of Cox-2 expression after experimental procedures to increase inflammation (Sunday et al., 2006), and indeed we found in Chapter 5b, that MPA treatment increased number of Cox-2 positive cells in the frontal cortex. Interestingly, although the $GABA_A$ antagonist bicuculline did not reverse the memory-impairing effects of MPA in this study, it did completely block the MPA-induced increase in frontal cortex

Cox-2-positive cells, suggesting that this may not be the mechanism of MPA-induced memory impairments in the Ovx middle-aged rat, at least as tested on the WRAM and MM. However, an important research question should be to determine if an MPA-induced increase in Cox-2 positive cells in the frontal cortex has functional consequences on tasks that tap memory types that were not evaluated in the tasks we used to test cognition herein. For example, the frontal cortex is thought to mediate behavioral flexibility, among other cognitive functions, and damage to this area can lead to perserverative behaviors (Kesner & Churchwell, 2011).MPA-induced increases in frontal cortex Cox-2 positive cells may be related to a deficit in reversal learning and strategy switching that could be measured by the plus maze (Young & Shapiro, 2009). Future investigations elucidating the functional consequences of MPA-induced increases in frontal cortex Cox-2 positive cells on cognition are warranted.

BDNF

Although previous literature shows that progesterone increases neurotrophins in cerebral cortex slice cultures (Kaur et al., 2007) and counteracts 17β-estradiol-induced increases in neurotrophins in the entorhinal and frontal cortices (Bimonte-Nelson et al., 2004a), and hippocampal slice cultures (Aguirre & Baudry, 2009), in Chapter 2 we found no effects of progesterone or MPA treatment on neurotrophin levels in several cognitive brain regions. Perhaps in vivo, progestogens do not have independent, but only regulatory actions, of estrogen's effects on neurotrophins. This could be methodologically evaluated by cognitively testing progesterone and 17β-estradiol alone, and together, in a single

study, with an endpoint of evaluating neurotrophic markers. Results would add further mechanistic understanding of the role neurotrophins have in the cognitive effects of these two clinically used hormones.

Hormone Therapy, Cognition, and Mesial Temporal Lobe Volumes

In Chapter 6, we found that HT use is associated with larger volumes of mesial temporal structures, and a modest verbal memory enhancement, in post-menopausal women. Effects were dependent on HT status, with discontinuous users demonstrating larger hippocampal volumes bilaterally, and continuous users only a marginally larger right hippocampal volume, both as compared to women who never used hormone therapy. Continuous users, however, had significantly larger right and marginally larger left entorhinal cortex volumes, as compared to never users. Discontinuous HT use was not related to a change in entorhinal cortex volume, as compared to continuous or never use. Our effects are consistent with previous research showing that HT use is associated with larger hippocampal (Boccardi et al., 2006; Eberling et al., 2003; Erickson et al., 2010; Lord et al., 2008; Yue et al., 2007) and entorhinal cortex (Boccardi et al., 2006) volumes, with deferential effects depending on previous or current HT status (Boccardi et al., 2006). We showed that discontinuous HT users were the only HT group to show a verbal memory enhancement, as compared to never users, and these results are also in line with findings from others on HT effects on dementia risk (Zandi et al., 2002) and MMSE (Mathews et al., 1999).

Alzheimer's Disease, Sex, and Dietary Intervention

In Chapter 7, we investigated the ability of a broad-based nutritional supplement to reverse the cognitive deficits seen in transgenic AD model mice, as compared to WT mice. Interestingly, we found that effects of the diet depended on sex, with cognitive benefits seen in male transgenic mice only, as compared to non-supplemented male transgenic mice. Multiple mechanisms of AD-associated cognitive impairments and dietary intervention correction were investigated in subsequent imaging and pathology analyses. One variable revealed sex-specific changes. Across both sexes, AD mice exhibited altered CO activity, a marker for mitochondrial function, in several brain areas, as compared to WT mice. However, in the entorhinal cortex, medial mammillary nuclei, and in the CA3 and dentate gyrus of the hippocampus, supplementation corrected CO activity to WT levels in AD male mice only, and decreased levels in supplemented AD female mice. Changes in mitochondrial function of cognitive brain regions thus represent a potential mechanism by which dietary intervention enhances cognition in male AD mice, and creates a vulnerability in female AD mice. Whether these effects in an animal model translate to AD patients is an important question, especially because incidence of AD is greater in women, as aging ensues (Anderson et al., 1999).

Final conclusions

Several lines of evidence converge to suggest that naturally circulating and synthetic hormones affect cognition and related brain mechanisms in women and animal models. Specifically, 1) sex can modulate responsiveness to dietary

intervention in an animal model of AD, 2) HT use is related to increased volumes of mesial temporal lobe structures and a verbal memory enhancement in post-menopausal women, 3) progesterone has negative effects on cognition in the menopausal rodent model, which may be related to the GABAergic system, 4) MPA has negative effects on cognition in the menopausal rodent model, and alters the GABAergic system, which increasing evidence suggests may not be the mechanism of MPA-induced cognitive impairments, 5) NETA can impair or improve cognition, depending on parameters used, in the menopausal rat model, and 6) LEVO can improve cognition in the menopausal rat model. Optimizing cognitive aging in women has been the overarching goal of this dissertation, and more specifically, I hope this research aids in the determining of a progestogen component, for birth control and HT, that is safe for brain health and function.

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Table 1

Mean neurotrophin levels in pg/mg in the Cingulate Cortex, Hippocampus, Entorhinal Cortex, and Frontal Cortex.

Neurotrophin Levels (pg/mg)

	Cingulate Cortex		Hippocampus		Entorhinal Cortex		Frontal Cortex	
	BDNF	NGF	BDNF	NGF	BDNF	NGF	BDNF	NGF
Sham	1.36 ±.38	4.71 ±.97	6.30 ±1.21	3.56 ±.47	1.44 ±.44	1.49 ±.34	.77 ±.10	1.39 ±.24
OVX	1.10 ±.12	4.47 ±.64	6.03 ±1.32	3.05 ±.47	1.34 ±.16	1.49 ±.20	.79 ±.15	1.18 ±.25
OVX+PROG	1.58 ±.17	4.78 ±.97	5.90 ±1.45	3.21 ±.50	1.31 ±.30	1.38 ±.20	.90 ±.15	1.07 ±.11
OVX+Low MPA	1.58 ±.56	5.55 ±1.24	3.95 ±.30	2.07 ±.16	1.34 ±.18	1.68 ±.33	.79 ±.19	1.16 ±.24
OVX+High MPA	1.19 ±.30	4.05 ±.80	5.03 ±.53	3.28 ±.62	1.69 ±.24	1.56 ±.15	1.13 ±.27	1.56 ±.47

Table 2

Mean blood serum concentrations of MPA in ng/mL (±SE) and mean uterine weights in g (±SE).

MPA Serum Levels (ng/ml) and Uterine Weights (g)

	Sham	OVX	OVX+ PROG	OVX+ Low MPA	OVX+ High MPA
MPA Serum Levels	Not Detected	Not Detected	Not Detected	4.90 ± .56	6.67 ± 1.08
Uterine Weights	.47 ± .03	.16 ± .01	.20 ± .03	.25 ± .02	.22 ± .01

Table 3

Summary of significant behavior effects.

Summary of Effects

Treatment	WRAM				MM
	WMC D1-11	WMC D5-11 Trial 4	WMI D1-11	WMI D5-11 Trial 4	Overnight Forgetting
Early-MPA	Impaired	Impaired			
Late-MPA	Impaired	Impaired			Impaired
Early+Late-MPA	Impaired	Impaired	Impaired	Impaired	

Table 4

*Mean uterine and body weights in g (±SE). * p < .05 vs. Control*

Uterine and Body Weights (g)

	Control	Early-MPA	Late-MPA	Early+Late-MPA
Uterine Weight	.37 ± .10	.20 ± .06	.66 ± .12*	.62 ± .10*
Body Weight Phase 1	189.8 ± .9	215.4 ± 1.2*	191.6 ± .1	209.3 ± 1.4*
Body Weight Phase 2	232.0 ± 1.3	256.2 ± 1.2*	233.2 ± 1.6	243.9 ± 1.3*

*$p < .05$ vs. Ctrl

Table 5

Mean GAD protein levels in % control (±SE).

GAD Protein Levels (% Ctrl)

	Frontal Cortex	Anterior Cingulate Cortex	Posterior Cingulate Cortex	Entorhinal Cortex	Dorsal Hippocampus	Ventral Hippocampus
Control	100 ± 6	100 ± 20	100 ± 14	100 ± 7	100 ± 7	100 ± 5
Early-MPA	100 ± 6	84 ± 18	79 ± 8	104 ± 11	96 ± 6	99 ± 12
Late-MPA	89 ± 3	71 ± 15	103 ± 18	97 ± 5	98 ± 8	107 ± 10
Early+Late-MPA	98 ± 4	92 ± 20	87 ± 11	93 ± 5	96 ± 9	96 ± 9

Table 6

Mean uterine weights in g (±SE).

Mean uterine weights in grams (±SE)

	Ovx+Vehicle	Ovx+Prog	Ovx+Bic	Ovx+Prog+Bic
Uterine Weight (g)	.169 ± .04	.205 ± .02	.194 ± .03	.189 ± (.01)

Table 7

Mean frontal cortex cox-2 cell counts and hippocampal GAD cell counts (±SE).

		Mean cell counts per section (SE)			
		Ovx+Vehicle	Ovx+Prog	Ovx+Bic	Ovx+Prog+Bic
Frontal Cortex Cox-2		2.242 ± .27	4.469 ± .45	1.500 ± .20	3.375 ± .25
Hippocampus	CA1 GAD	11.133 ± .70	13.067 ± .60	12.767 ± .56	14.467 ± .94
	CA3 GAD	11.633 ± .56	13.967 ± .78	14.567 ± .90	13.433 ± .85
	DG GAD	18.933 ± .89	18.167 ± .93	18.700 ± 1.28	18.300 ± 1.13

Table 8

*Mean uterine weights in g (±SE). *p < .05 vs. Ovx+Vehicle*

Mean uterine weights in grams (±SE)

	Ovx+Vehicle	Ovx+MPA	Ovx+Bic	Ovx+MPA+Bic
Uterine Weight (g)	.169 ± .04	.301 ± .02*	.194 ± .03	.329 ± .02*

*$p < .05$ compared to Ovx+Vehicle

Table 9

Mean hippocampal GAD cell counts (±SE).

Hippocampal GAD mean cell counts per section (±SE)

	Ovx+Vehicle	Ovx+MPA	Ovx+Bic	Ovx+MPA+Bic
CA3	11.633 ± .56	13.633 ± .75	14.567 ± .90	12.713 ± .89
DG	18.933 ± .89	18.700 ± .95	18.700 ± 1.28	16.167 ± .96

Table 10
Demographic information by group.

Demographics

	Total (n =50) Mean (S.D.) Range	CU (n =16) Mean (S.D.) Range	DU (n =22) Mean (S.D.) Range	NU (n =12) Mean (S.D.) Range
Age	75.2 (8.2) 53 – 91	76.3 (6.7) 59 - 86	73.5 (8.2) 58 - 87	76.8 (10.2) 53 - 91
Education	14.1 (2.3) 10 - 20	14.3 (2.4) 10 - 20	14.0 (2.4) 11 - 19	14.0 (2.3) 12 - 18
Years since menopause	28.3 (9.8) 3 - 46	28.5 (10.4) 8 - 46	29.6 (9.1) 15 - 44	25.3 (10.4) 3 - 41
Hysterectomy (% yes)	70	87.5	72.7	41.7
MMSE	28.3 (1.3) 26 - 30	28.2 (1.4) 26 - 30	28.5 (1.1) 26 - 30	27.8 (1.3) 26 - 30
Estrogen only (% yes)	--	81.3	72.7	--

Table 11

*Brain volumes by group. *p < .05 vs. NU; # p < .10 vs. NU*

Brain Volumes

	CU Mean (S.E.)	DU Mean (S.E.)	NU Mean (S.E.)
TIV (ml)	1376.1 (60.0)	1376.8 (33.2)	1444.3 (65.2)
Left Hippocampus (%TIV)	.231 (.013)	.25 (.007)*	.213 (.013)
Right Hippocampus (%TIV)	.227 (.011)#	.231 (.008)*	.199 (.012)
Left Entorhinal Cortex (%TIV)	.504 (.035)#	.454 (.025)	.400 (.037)
Right Entorhinal Cortex (%TIV)	.447 (.040)*	.421 (.029)#	.335 (.029)

*$p < .05$ versus NU

# $p < .10$ versus NU

Table 12

Cognitive scores by group. # $p < .10$ vs. NU

Cognitive Scores

	CU Mean (S.E.)	**DU** Mean (S.E.)	**NU** Mean (S.E.)
Total Words (% remembered)	.578 (.027)	.603 (.031)	.545 (.050)
Delayed Memory (% remembered)	.643 (.044)	.705 (.035)#	.553 (.083)
Trails A (sec.)	39.17 (4.68)	39.58 (2.50)	38.10 (3.53)
Trails B (sec.)	99.83 (7.69)	103.79 (10.50)	110.10 (18.600)

#$p < .05$ versus NU

Table 13.

Summary of the 3 MPNSP supplements integrated into the mouse chow. Each was purchased over-the-counter at a local grocery.

Freeze-dried phyto-nutrient powder (target: 5.25 mg/day/mouse)

blueberry	cranberry	parsley	
brown rice*	ginger*	pomegranate*	
brussels sprout*	green cabbage*	red cabbage*	
chicory*	kale	rose hip*	
cinnamon*	okra*	spinach	
Concord grape*	papaya*	turmeric*	

*cultured with following probiotic species, 1 billion per serving at the time of manufacture:
L. casei, L. plantarum, L. salivarius, L. acidophilus, L. rhamnosus, S. thermophilus, B.bifidum, B. infantis, B. longum, & B. breve

Herbal/spice liquid amalgam (target: 1.94 µl/day/mouse)
Rosemary (leaf) - supercritical & ethanolic extracts (23% total phenolic antioxidants)
Turmeric (rhizome) - supercritical (45% turmerones) & ethanolic extracts (7% curcuminoids)
Ginger (rhizome) - supercritical (30% pungent compounds) & ethanolic extracts (3% pungent compounds)
Holy Basil (leaf) - ethanolic extract (2% ursolic acid)
Green Tea (leaf) - hydroethanolic extract (45% polyphenols)
Hu Zhang (root and rhizome) - hydroethanolic extract (8% resveratrols)
Chinese Goldthread (root) - hydroethanolic extract (6% berberine)
Barberry (root) - hydroethanolic extract (6% berberine)
Oregano (leaf) - supercritical extract (4% total phenolic antioxidants)
Baikal skullcap (root) - hydroethanolic extract (17-26% baicalein complex, 0.4-0.9% wogonin)
Other: extra virgin olive oil, maltodextrin, silica, yellow beeswax, palm kernel oil

Cod liver oil (target: 6.56 µl/day/mouse)

DHA	EPA	Vitamin D	
20.40	16.10	15.00	mg / kg / day

Table 14.

Comprehensive list of all regions-of-interest assessed and the calculated probability values for each main effect and interaction from the 2x2x2 ANOVA on the cytochrome oxidase imaging data. Regions showing significant ($p<0.05$) main effects and interactions were assessed with post hoc 2-tailed t-tests.*

ROI	**Genotype (G)**	**Diet (D)**	**Sex (S)**
Optic tract (opt)	0.751	0.280	0.172
Cingulate gyrus, retrosplenial (RSG	0.028*	0.539	0.376
Cingulate gyrus, posterior 2 (CGp2)	0.418	0.661	0.086
Cingulate gyrus, posterior (CGp)	0.105	0.001*	0.380
Cingulate gyrus, middle (CGm)	0.063	0.225	0.211
Cingulate gyrus, anterior (CGa)	0.102	0.375	0.074
Posterior parietal cortex (PPtA)	0.139	0.951	0.058
Lateral entorhinal cortex (LEnt)	0.026*	0.316	0.560
Primary somatosensory cortex (S1)	0.062	0.022*	0.389
Primary somatosensory, BF (S1BF)	0.046*	0.066	0.225
Secondary somatosensory cortex (S2)	0.090	0.064	0.149
Primary auditory cortex (Au1)	0.020*	0.522	0.100
Primary visual, monocular (V1)	0.002*	0.737	0.302
Piriform cortex (Pir)	0.841	0.862	0.350
CA1 (CA1)	0.002*	0.542	0.571
CA3 (CA3)	0.005*	0.119	0.327
Dentate gyrus (DG)	0.170	0.348	0.526
Subiculum (S)	0.001*	0.606	0.061
Medial mammillary nucleus (M)	0.869	0.359	0.563
Substantia nigra (SNR)	0.131	0.964	0.273
Basolateral amygdala (BLA)	0.372	0.056	0.262
Anteroventral thalamus (AV)	0.958	0.828	0.779
Reticular thalamus (Rt)	<0.001*	0.149	0.367
Reuniens nucleus (Re)	0.639	0.461	0.278
Ventromedial thalamus (VM)	0.296	0.127	0.137
Ventrolateral thalamus (VL)	0.023*	0.021*	0.090
Mediodorsal thalamus (MD)	0.116	0.009*	0.438
Laterodorsal thalamus (LD)	0.051	0.391	0.267
Lateral posterior thalamus (LP)	0.599	0.010*	0.620
Parafascicular thalamus (PF)	0.112	0.792	0.062
Ventroposterolateral thalamus (VPL)	0.230	0.183	0.609
Ventroposteromedial thalamus (VPM)	0.168	0.677	0.057
Posterior thalamus (Po)	0.155	0.310	0.227
Lateral habenula (LHb)	0.806	0.683	0.450
Rostral caudoputamen (rCPu)	0.181	0.018*	0.269
Caudal caudoputamen (cCPu)	0.021*	0.790	0.224
Lateral globus pallidus (LGP)	0.228	0.706	0.301
Subthalamus (STh)	0.269	0.366	0.099
Nucleus accumbens (Acb)	0.742	0.216	0.318
Nuc. of vertical diagonal band (VDB)	0.409	0.205	0.303
Medial septal nucleus (MS)	0.936	0.246	0.170

Lateral septal nucleus (LSI)	0.100	0.096	0.183
Nucleus basalis (B)	0.043*	0.099	0.333
Anterior hypothalamus (AH)	0.337	0.634	0.063
Lateral hypothalamus (LH)	0.387	0.004*	0.478
Dorsolateral geniculate (DLG)	0.196	0.291	0.102
Medial geniculate (MG)	<0.001*	0.354	0.008*
Superior colliculus (SC)	0.001*	0.281	0.052
Pontine nuclei (PnC)	0.111	0.394	0.598
Inferior colliculus, central (CIC)	0.267	0.051	0.500
Periaqueductal gray (PAG)	0.114	0.226	0.975
Vestibular nuclei (Ve)	0.956	0.048*	0.950
Reticular nucleus, giganto (Gi)	0.605	0.095	0.633
Cerebellar lobules 1-5 (Cb)	0.008*	0.763	0.133
Simple lobule (Sim)	0.081	0.936	0.042*
Crus 1 lobule (Crus1)	0.043*	0.349	0.191

ROI	**GxD**	**SxG**	**SxD**	**SxGxD**
opt	0.916	0.105	0.649	0.611
RSG	0.648	0.909	0.350	0.058
CGp2	0.690	0.269	0.505	0.655
CGp	0.006*	0.298	0.829	0.635
CGm	0.606	0.571	0.433	0.947
CGa	0.321	0.772	0.654	0.927
PPtA	0.754	0.562	0.691	0.554
LEnt	0.394	0.689	0.008*	0.620
S1	.018*	0.443	0.789	0.785
S1BF	0.289	0.909	0.857	0.512
S2	0.173	0.780	0.767	0.913
Au1	0.821	0.453	0.508	0.916
V1	0.986	0.299	0.989	0.025*
Pir	0.764	0.648	0.805	0.171
CA1	0.742	0.321	0.617	0.009*
CA3	0.676	0.003*	0.989	0.018*
DG	0.125	0.743	0.242	0.008*
S	0.451	0.673	0.491	0.279
M	0.505	0.251	0.275	0.008*
SNR	0.600	0.457	0.019*	0.730
BLA	0.168	0.924	0.938	0.882
AV	0.517	0.485	0.398	0.623
Rt	.402	0.471	0.553	0.013*
Re	0.778	0.479	0.897	0.436
VM	0.188	0.965	0.688	0.693
VL	0.175	0.965	0.570	0.600
MD	0.053	0.362	0.148	0.585
LD	0.614	0.311	0.150	0.073
LP	0.183	0.375	0.088	0.593
PF	0.823	0.462	0.695	0.268
VPL	0.400	0.710	0.919	0.153
VPM	0.629	0.368	0.763	0.346
Po	0.539	0.777	0.731	0.372
LHb	0.380	0.935	0.454	0.448
rCPu	0.837	0.539	0.755	0.735
cCPu	0.360	0.643	0.991	0.242

LGP	0.928	0.797	0.963	0.999
STh	0.689	0.276	0.909	0.727
Acb	0.869	0.384	0.668	0.592
VDB	0.277	0.875	0.514	0.749
MS	0.376	0.803	0.840	0.947
LSI	0.893	0.872	0.990	0.883
B	0.389	0.325	0.235	0.162
AH	0.708	0.150	0.602	0.935
LH	0.436	0.935	0.737	0.699
DLG	0.839	0.443	0.366	0.360
MG	0.080	0.236	0.022*	0.499
SC	0.120	0.516	0.055	0.968
PnC	0.362	0.744	0.826	0.132
CIC	0.420	0.278	0.365	0.003*
PAG	0.938	0.523	0.405	0.091
Ve	0.614	0.299	0.936	0.222
Gi	.930	0.958	0.258	0.865
Cb	0.858	0.870	0.244	0.592
Sim	0.370	0.994	0.160	0.393
Crus1	0.256	0.884	0.100	0.958

Table 15.

*Mean (±SD) from selected regions showing significant main effects and/or interactions in the cytochrome oxidase imaging analysis. WT=wildtype; TG=3xTG; Supp=on supplemented diet; NS=on standard diet; M=male; F=female. * Significantly different from same group nonsupplemented, 2-tailed t-test, p<0.05. ** Significantly different from males of the same group, p<0.05.*

ROI	WT-Supp	WT-NS	TG-Supp	TG-NS
Cingulate gyrus, posterior (CGp)	271±24	275±35	288±25 *	234±14
Lateral entorhinal cortex (LEnt)	208±51	216±63	185±29	183±34
Primary somatosensory cortex (S1)	242±28	242±27	247±25 *	207±13
Primary somatosensory, BF (S1BF)	252±24	246±22	245±30 *	212±12
CA1 (CA1)	208±16	212±27	190±31	186±28
CA3 (CA3)	250±23	260±47	221±25	243±35
Dentate gyrus (DG)	282±29	273±33	253±41	280±43
Medial mammillary nuclei (M)	297±54	311±75	285±66	331±101
Basolateral amygdala (BLA)	243±40	236±29	248±30 *	213±30
Mediodorsal thalamus (MD)	262±45	255±33	268±35 *	219±20
Lateral posterior thalamus (LP)	272±50	258±34	284±38 *	239±29
Lateral hypothalamus (LH)	216±34	198±23	216±23 *	183±25

ROI	TGM-Supp	TGM-NS	TGF-Supp	TGF-NS
CGp	286±30 *	234±15	290±21 *	233±15
LEnt	201±16 *	166±20	166±31 **	196±38
S1	245±27	210±10	249±26 *	205±16
S1BF	247±35	229±41	244±28	214±13
CA1	195±26	173±38	174±28	200±23
CA3	223±28	219±36	220±25 *	260±25
DG	273±34	258±46	229±39 *	295±37
M	316±61	263±57	240±49	366±104
BLA	255±31	220±45	242±30 *	208±13
MD	279±42 *	204±14	258±29	228±18
LP	297±47 *	219±28	274±30	252±23
LH	219±24	187±37	213±23 *	180±15

Table 16.

*Results from ELISA and Western blot analyses by sex (mean and SE). WT=wildtype; TG=3xTG; Supp=on supplemented diet; NS=on standard diet; M=male; F=female. *Significantly different between supplemented and nonsupplemented females, 2-tailed t-test, p<0.05. **Significantly different between supplemented and nonsupplemented, p<0.05.*

ELISA:	**TGM-Supp**	**TGM-NS**	**TGF-Supp**	**TGF-NS**
Human Abeta 42 (ng/mg)	10.0±5.55	3.20±1.13	33.47±8.70 *	60.50±2.19*
Human Abeta 40 (ng/mg)	0.12±0.05	0.06±0.021	44.55±10.68	70.82±1.28
Synaptophysin (pg/µg)	418.61±25.19	416.97±46.07	429.55±39.54	394.54±11.71
GFAP (ng/µg)	10.56± 2.31	9.11±2.51	11.79±2.26	13.91±0.95
Western Blot:				
IBA1 (17kD)	2.52±0.21	2.13±0.11	3.15±0.67	3.09±0.31
ARG1	0.56±0.20	0.67±0.32	0.42±0.06**	0.36±0.08**
YM1	1.35±0.27	1.69±0.14	0.67±0.08	0.59±0.07
MMR	0.15±0.03	0.15±0.02	0.13±0.02	0.14±0.02

ELISA:	**WTM-Supp**	**WTM-NS**	**WTF-Supp**	**WTF-NS**
Human Abeta 42 (ng/mg)	n/a	n/a	n/a	n/a
Human Abeta 40 (ng/mg)	n/a	n/a	n/a	n/a
Synaptophysin (pg/µg)	301.79±13.84	361.10±29.74	388.86±22.92	340.80±14.61
GFAP (ng/µg)	12.61±0.75	10.33±2.02	9.64±1.79	12.65±1.28
Western Blot:				
IBA1 (17kD)	2.71±0.26	2.34±0.11	2.83±0.15	2.91±0.19
ARG1	0.56±0.11	0.67±0.15	0.55±0.08	0.48±0.10
YM1	1.55±0.13	2.18±0.51	0.84±0.10	0.80±0.12

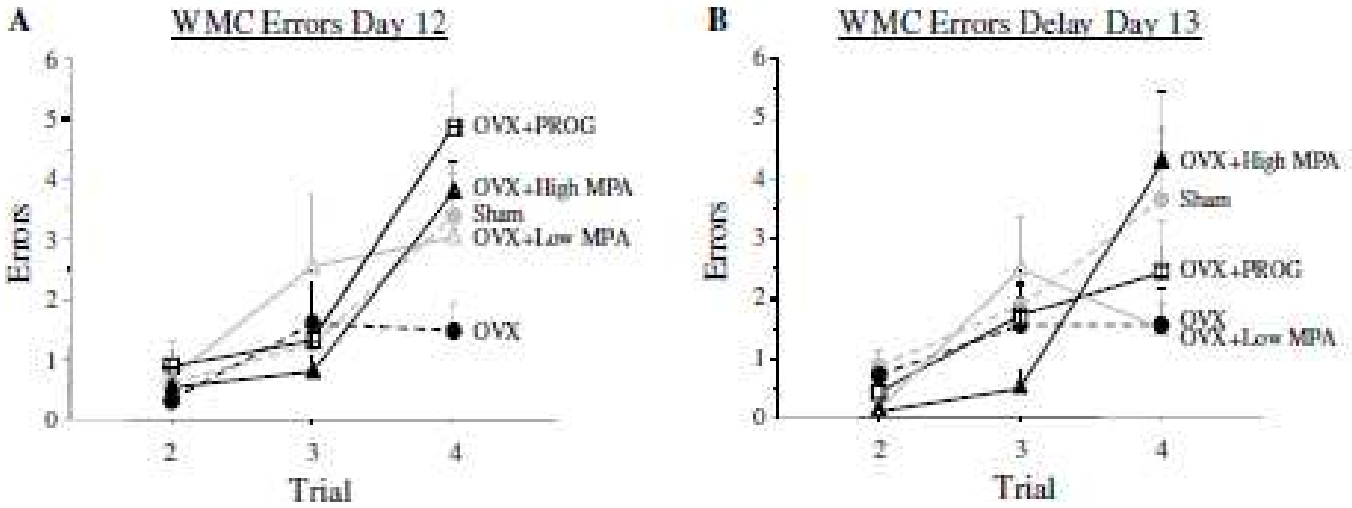

A
WMC Errors Day 12
Errors
Trial
OVX+PROG
OVX+High MPA
Sham
OVX+Low MPA
OVX
B
WMC Errors Delay Day 13
Errors
Trial
OVX+High MPA
Sham
OVX+PROG
OVX
OVX+Low MPA

Figure 1. Mean error scores for WMC (+SE) on the water radial-arm maze for (a) day 12 and (b) delay day 13. *p < .05

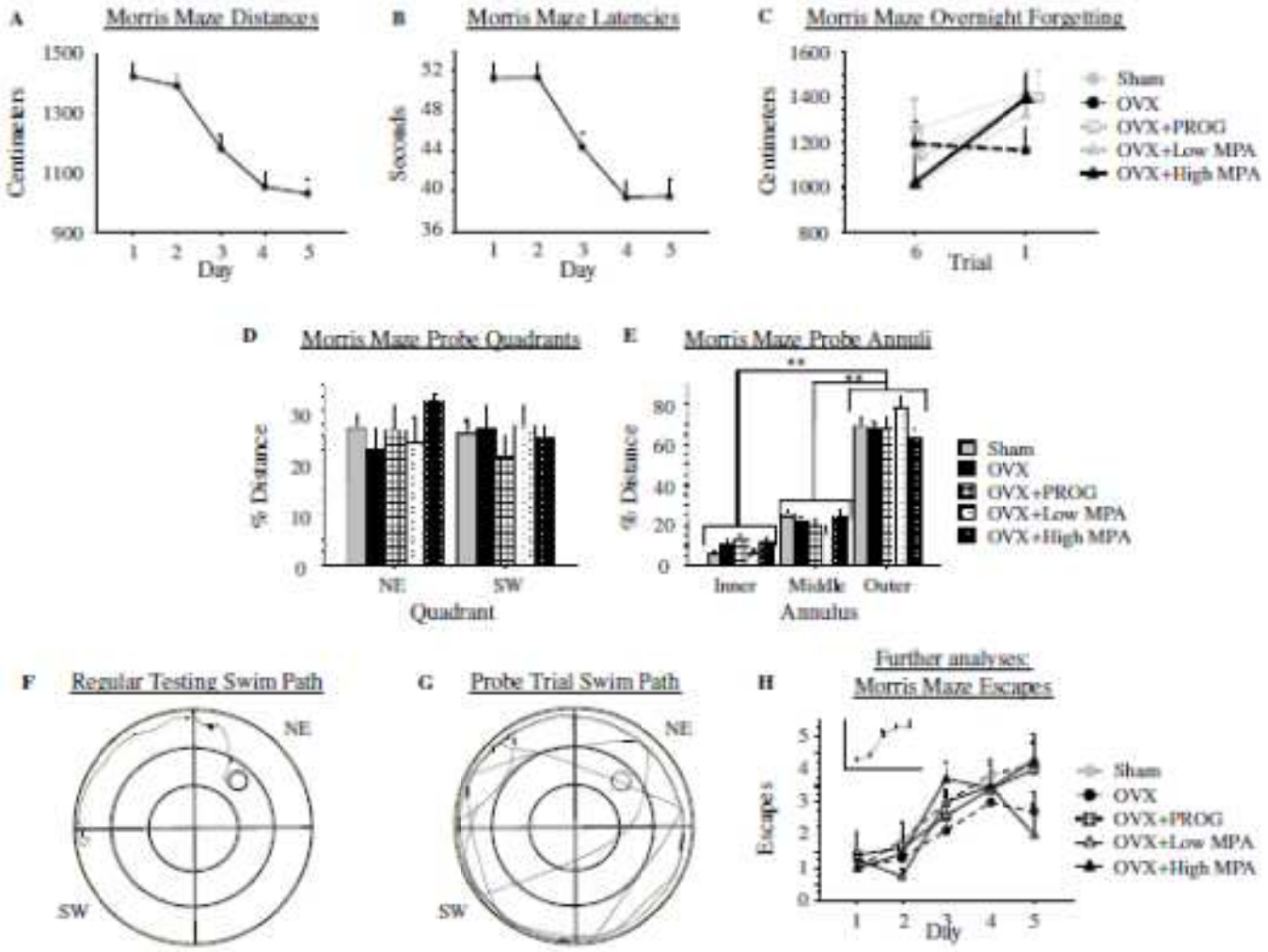

A
Morris Maze Distances
Centimeters
1500
1300
1100
900
1 2 3 4 5
Day
B
Morris Maze Latencies
Seconds
52
48
44
40
36
1 2 3 4 5
Day
C
Morris Maze Overnight Forgetting
Centimeters
1600
1400
1200
1000
800
6
Trial
1
Sham
OVX
OVX+PROG
OVX+Low MPA
OVX+High MPA
D
Morris Maze Probe Quadrants
% Distance
30
20
10
0
NE
SW
Quadrant
E
Morris Maze Probe Annuli
% Distance
80
60
40
20
0
Inner
Middle
Outer
Annulus
Sham
OVX
OVX+PROG
OVX+Low MPA
OVX+High MPA
F
Regular Testing Swim Path
NE
SW
G
Probe Trial Swim Path
NE
SW
H
Further analyses:
Morris Maze Escapes
Escapes
5
4
3
2
1
0
1 2 3 4 5
Day
Sham
OVX
OVX+PROG
OVX+Low MPA
OVX+High MPA

Figure 2. (a) Mean distance scores in centimeters (+SE) on Morris maze days 1-5. (b) Mean latency scores in seconds (+SE) on Morris maze days 1-5. (c) Mean Distance scores in centimeters (+SE) on Morris maze representing overnight forgetting. (d) Mean percent Distance (+SE) in the target NE quadrant as compared to the opposite SW quadrant during the probe trial (NW) over the opposite quadrant (SE) by any group. (e) Mean percent distance scores (+SE) spent in the annuli of the Morris maze during the probe trial. (f) A swim path of an animal during regular testing. (g) A swim path of an animal during the probe trial. (h) Mean escape scores (+SE) on Morris maze days 1-5. **$p < .0001$

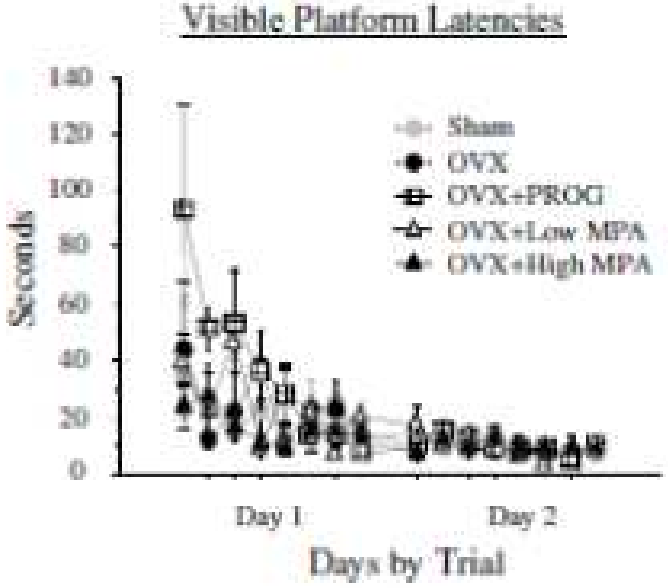

Visible Platform Latencies
140
120
100
80
60
40
20
0
Seconds
Sham
OVX
OVX+PROG
OVX+Low MPA
OVX+High MPA
Day 1
Day 2
Days by Trial

Figure 3. Mean latency scores in seconds (±SE) on the visible platform maze.

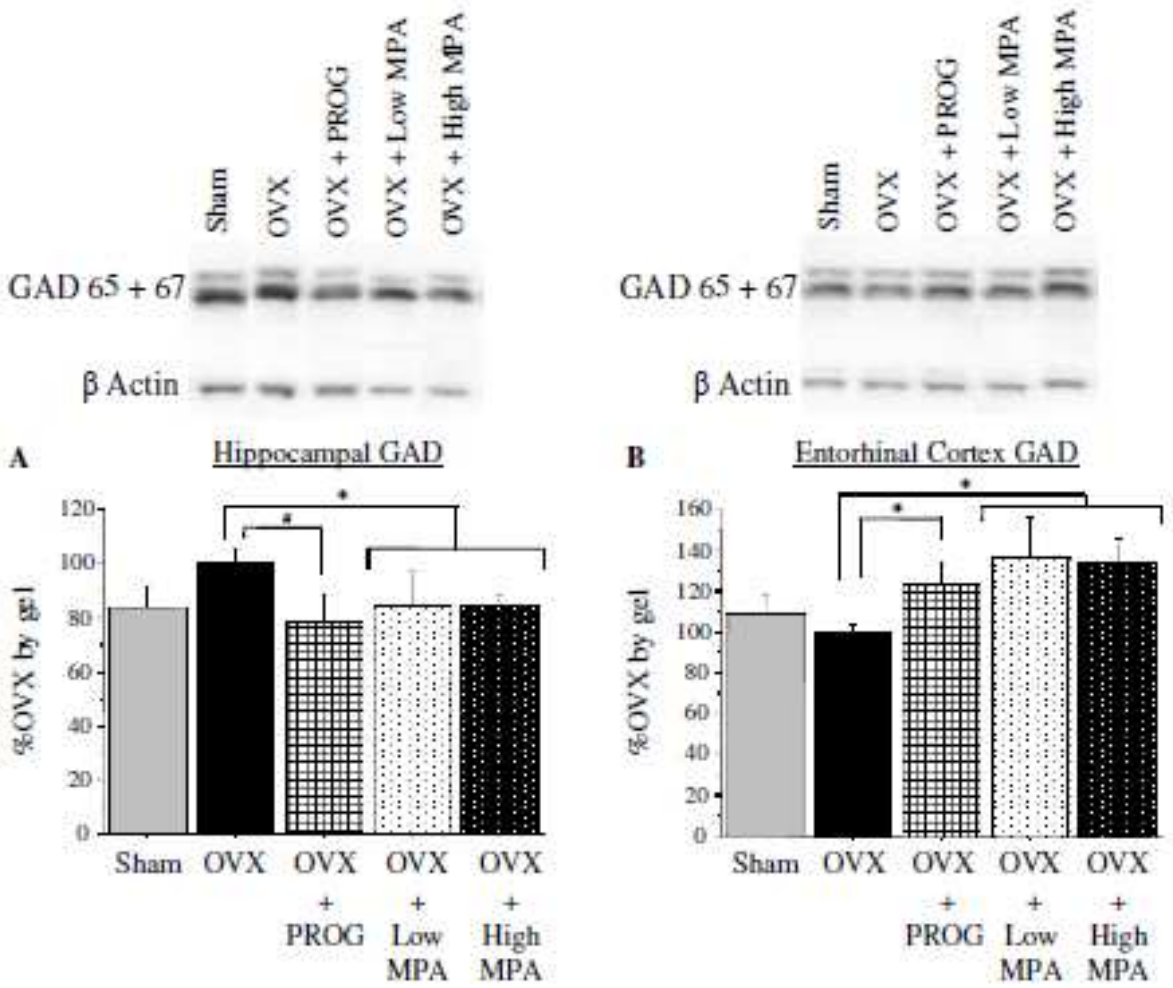

Sham
OVX
OVX + PROG
OVX + Low MPA
OVX + High MPA
GAD 65 + 67
β Actin
A
Hippocampal GAD
%OVX by gel
0
20
40
60
80
100
120
Sham
OVX
OVX + PROG
OVX + Low MPA
OVX + High MPA
B
Entorhinal Cortex GAD
%OVX by gel
0
20
40
60
80
100
120
140
160
Sham
OVX
OVX + PROG
OVX + Low MPA
OVX + High MPA

Figure 4. (a) Mean % OVX by gel (+SE) of GAD protein expression and representative bands, as determined by Western blot luminescence, in the hippocampus. (b) Mean % OVX by gel (+SE) of GAD luminescence and representative bands, in the entorhinal cortex. *$p < .05$; #$p < .10$

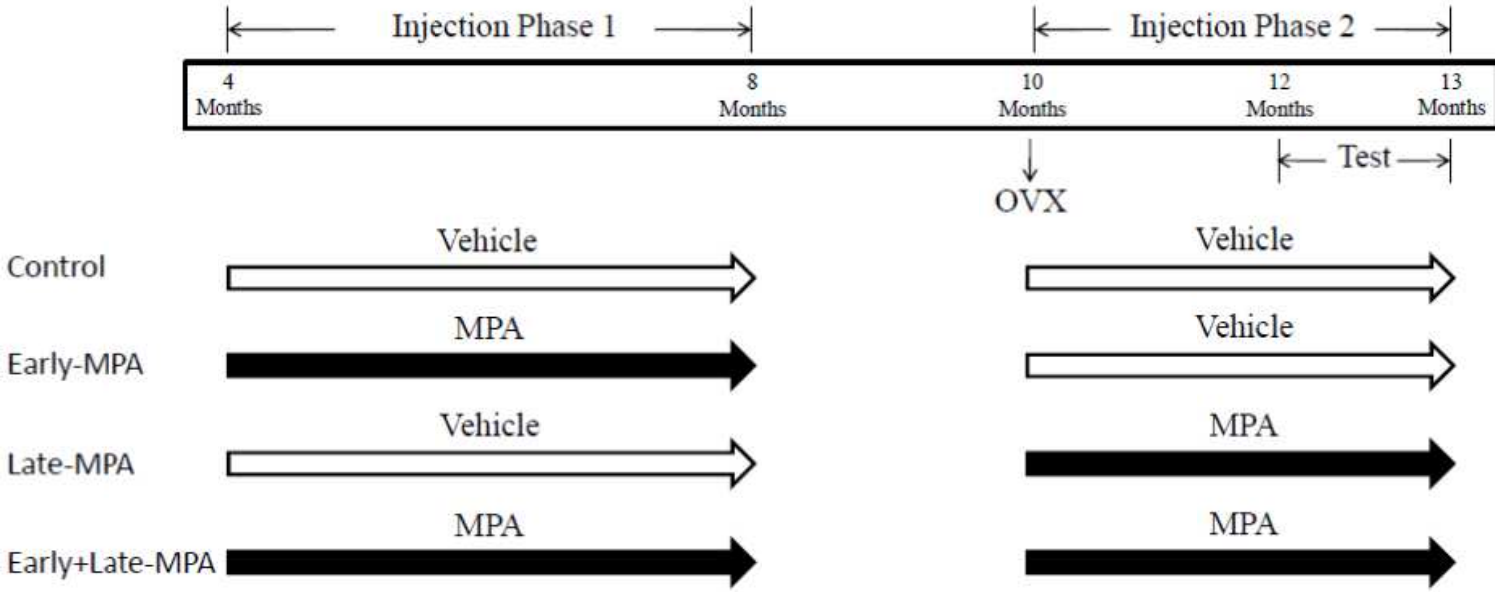

Injection Phase 1
Injection Phase 2
4 Months
8 Months
10 Months
12 Months
13 Months
OVX
Test
Control
Vehicle
Vehicle
Early-MPA
MPA
Vehicle
Late-MPA
Vehicle
MPA
Early+Late-MPA
MPA
MPA

Figure 5. A time line summarizing injections, ovariectomy, and behavioral testing for each group.

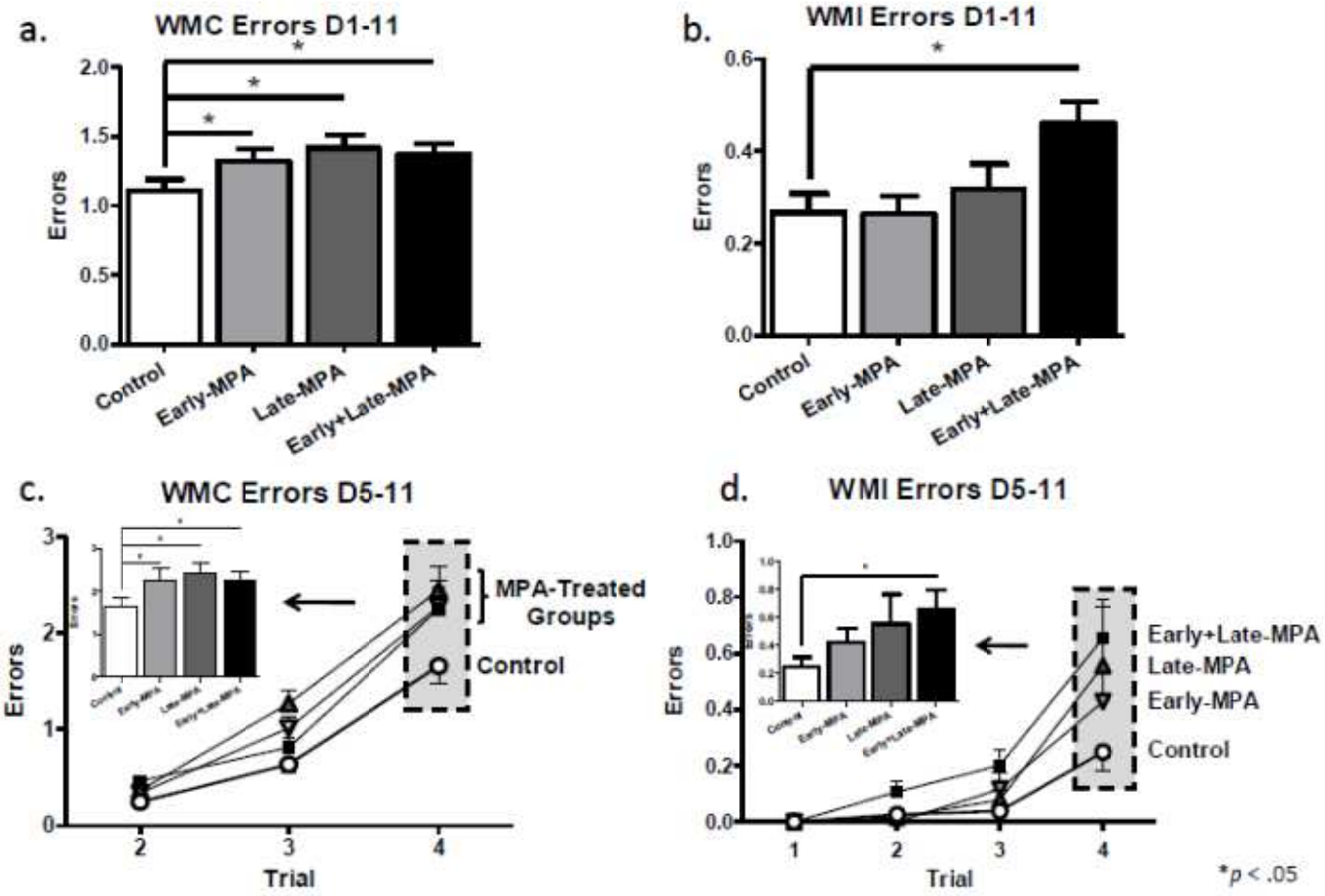

a.
WMC Errors D1-11
Errors
2.0
1.5
1.0
0.5
0.0
Control
Early-MPA
Late-MPA
Early+Late-MPA
b.
WMI Errors D1-11
Errors
0.6
0.4
0.2
0.0
Control
Early-MPA
Late-MPA
Early+Late-MPA
c.
WMC Errors D5-11
Errors
Trial
MPA-Treated Groups
Control
d.
WMI Errors D5-11
Errors
Trial
Early+Late-MPA
Late-MPA
Early-MPA
Control
*p < .05

Figure 6. Mean error scores (+SE) on the water radial-arm maze. (a) WMC errors across all days of regular testing (days 1-11). (b) WMI errors across all days of testing. (c) WMC errors at the highest working memory load during the latter portion of testing (days 5-11). (d) WMI errors at the highest working memory load during the latter portion of testing (days 5-11). *$p < .05$

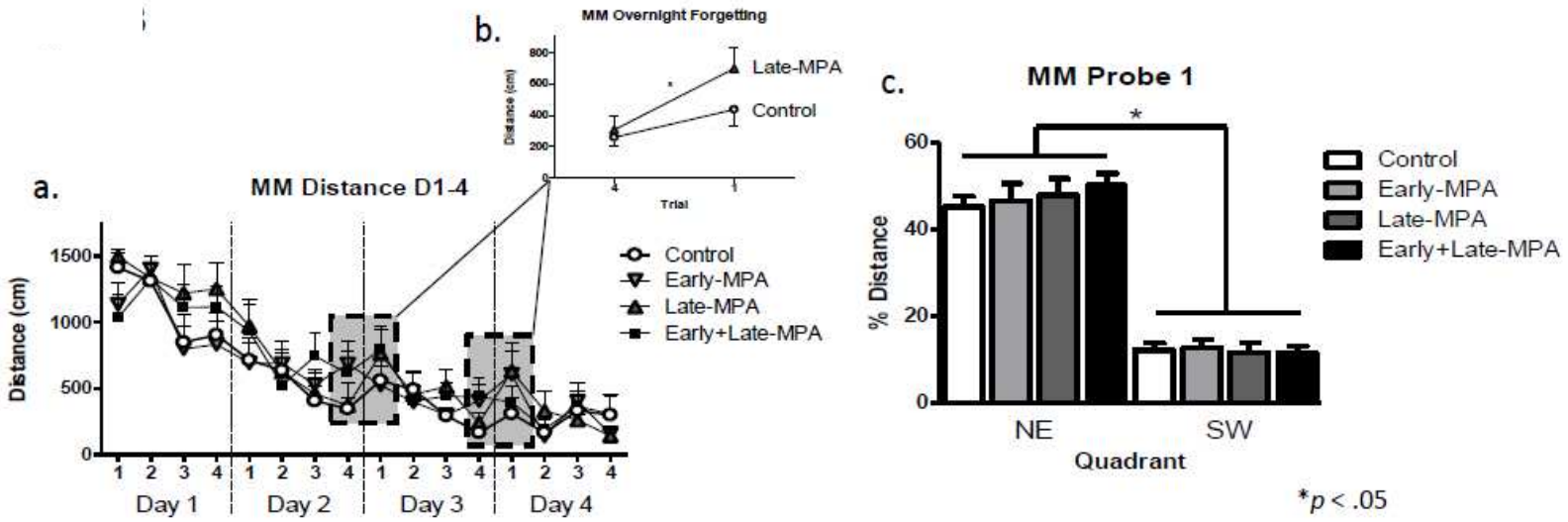

a.
MM Distance D1-4
Distance (cm)
1500
1000
500
0
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Day 1
Day 2
Day 3
Day 4
Control
Early-MPA
Late-MPA
Early+Late-MPA
b.
MM Overnight Forgetting
Distance (cm)
800
600
400
200
0
*
4
1
Trial
Late-MPA
Control
c.
MM Probe 1
*
% Distance
60
40
20
0
NE
SW
Quadrant
Control
Early-MPA
Late-MPA
Early+Late-MPA
*p < .05

Figure 7. Mean distance scores in centimeters (+SE) on Morris maze initial testing days 1-4. (a) Distance scores across all days of initial testing (days 1-4). (b) Distance scores across the overnight interval. (c) Mean percent Distance in the target NE quadrant as compared to the opposite SW quadrant during the probe trial day 4. *$p < .05$

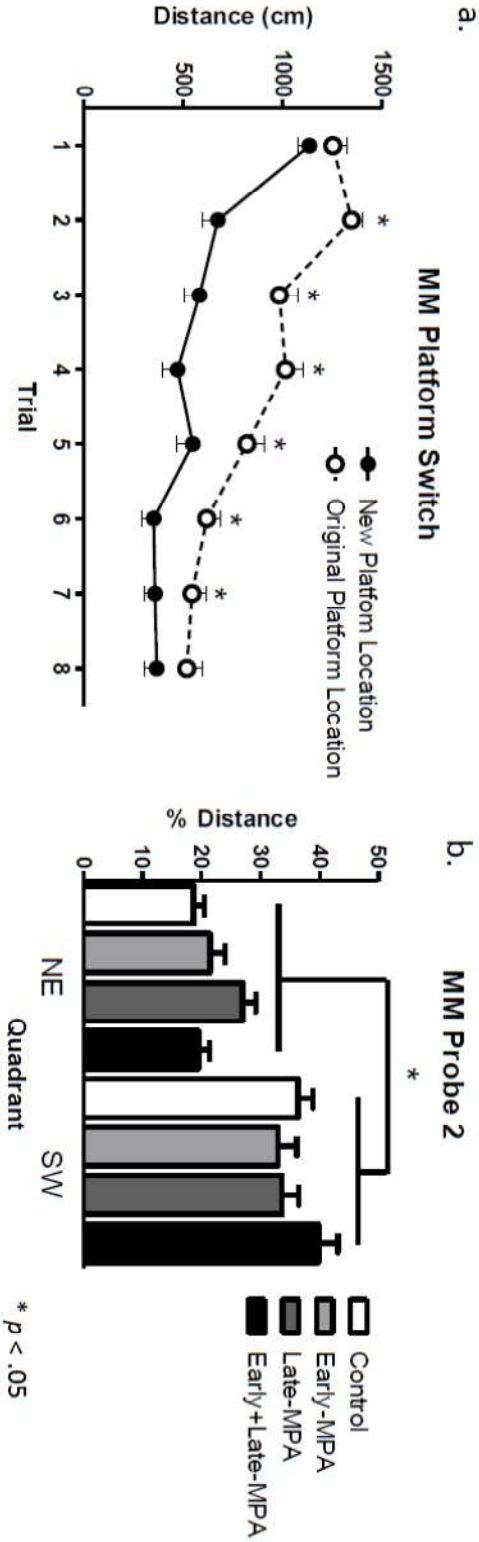

a.
MM Platform Switch
Distance (cm)
1500
1000
500
0
1
2
3
4
5
6
7
8
Trial
*
New Platform Location
Original Platform Location
b.
MM Probe 2
% Distance
50
40
30
20
10
0
NE
SW
Quadrant
*
Control
Early-MPA
Late-MPA
Early+Late-MPA
* p < .05

Figure 8. Mean Distance scores in centimeters (+SE) on Morris maze Platform Switch testing day 5. (a) Distance across all eight trials by new vs. original platform location. (b.) Percent Distance in the target SW quadrant as compared to the opposite NE quadrant during the probe trial day 5. *$p < .05$

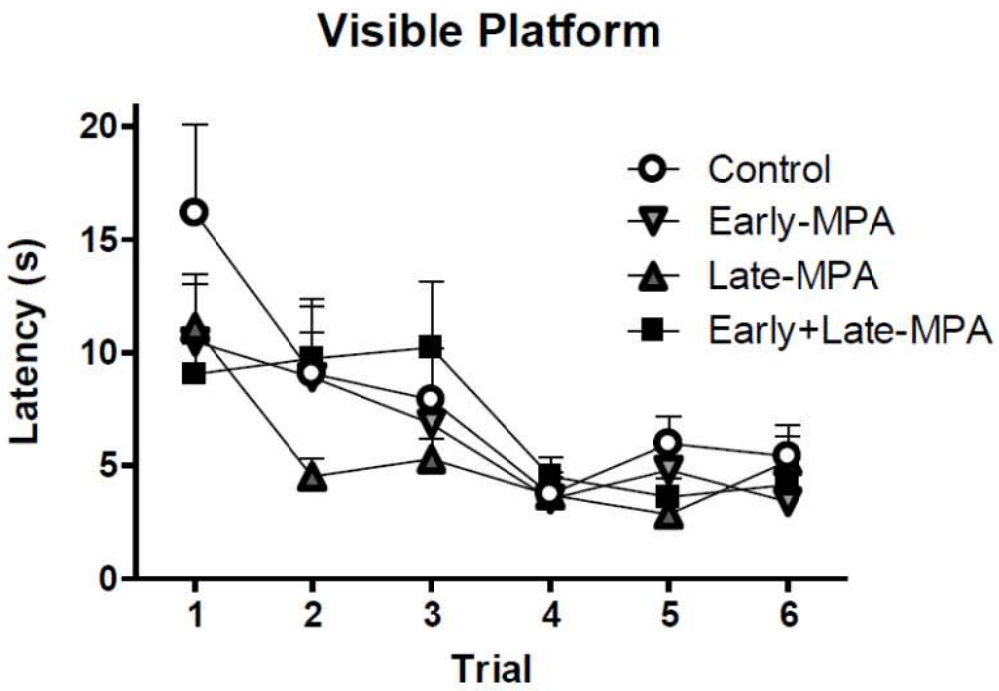
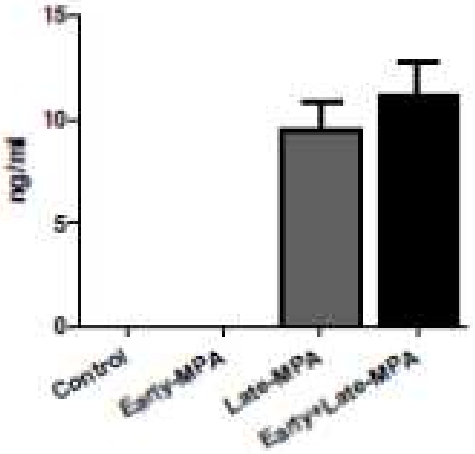
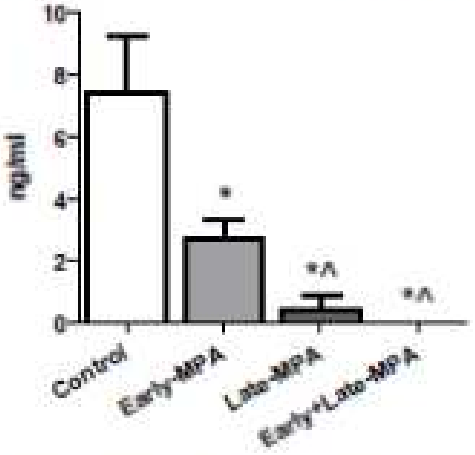

Visible Platform
Latency (s)
20
15
10
5
0
1
2
3
4
5
6
Trial
Control
Early-MPA
Late-MPA
Early+Late-MPA

Figure 9. Mean latency scores in seconds (+SE) on the visible platform maze.

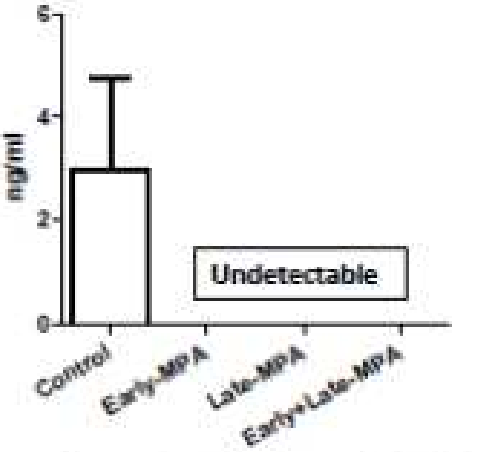
a. MPA Serum Levels



b. Progesterone Serum Levels



c. Allopregnanolone Serum Levels



* Sig. vs. Control; ^ Sig. vs. Early-MPA

Figure 10. Mean serum concentrations (+SE). (a) MPA concentrations. (b) Progesterone concentrations. (c) Allopregnanolone concentrations. *p < .05 vs. Control; ^p < .05 vs. Early-MPA

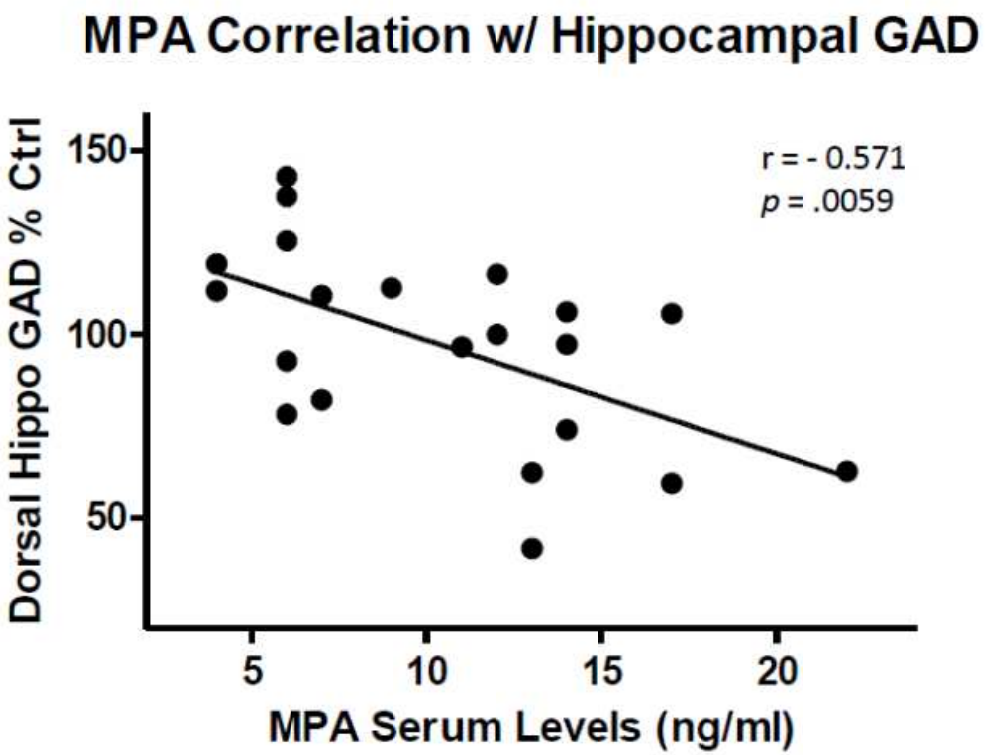
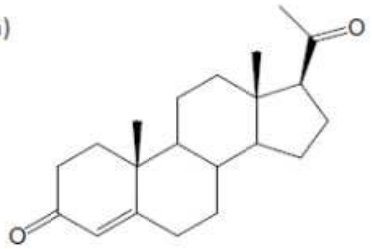

MPA Correlation w/ Hippocampal GAD
Dorsal Hippo GAD % Ctrl
150
100
50
r = - 0.571
p = .0059
5
10
15
20
MPA Serum Levels (ng/ml)

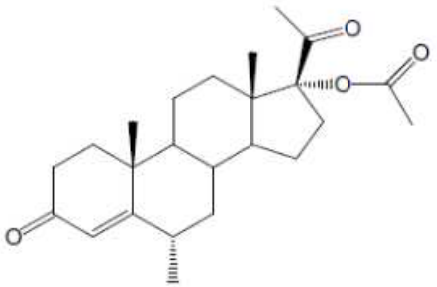
Figure 11. Relationship between MPA serum concentrations and GAD protein in the dorsal hippocampus in animals that had detectable levels of MPA at sacrifice (Late-MPA and Early+Late-MPA).

a)



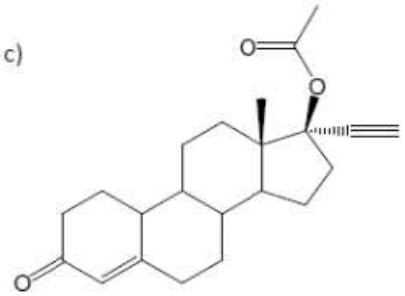
Progesterone

b)



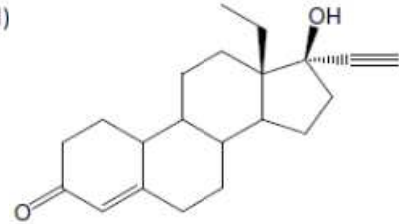
Medroxyprogesterone Acetate (MPA)

c)



Norethindrone Acetate (NETA)

d)



Levonorgestrel (LEVO)

Figure 12. Chemical structures of (a) progesterone (b) medroxyprogesterone acetate (MPA) (c) norethindrone acetate (NETA) (d) levonorgesterel (LEVO).

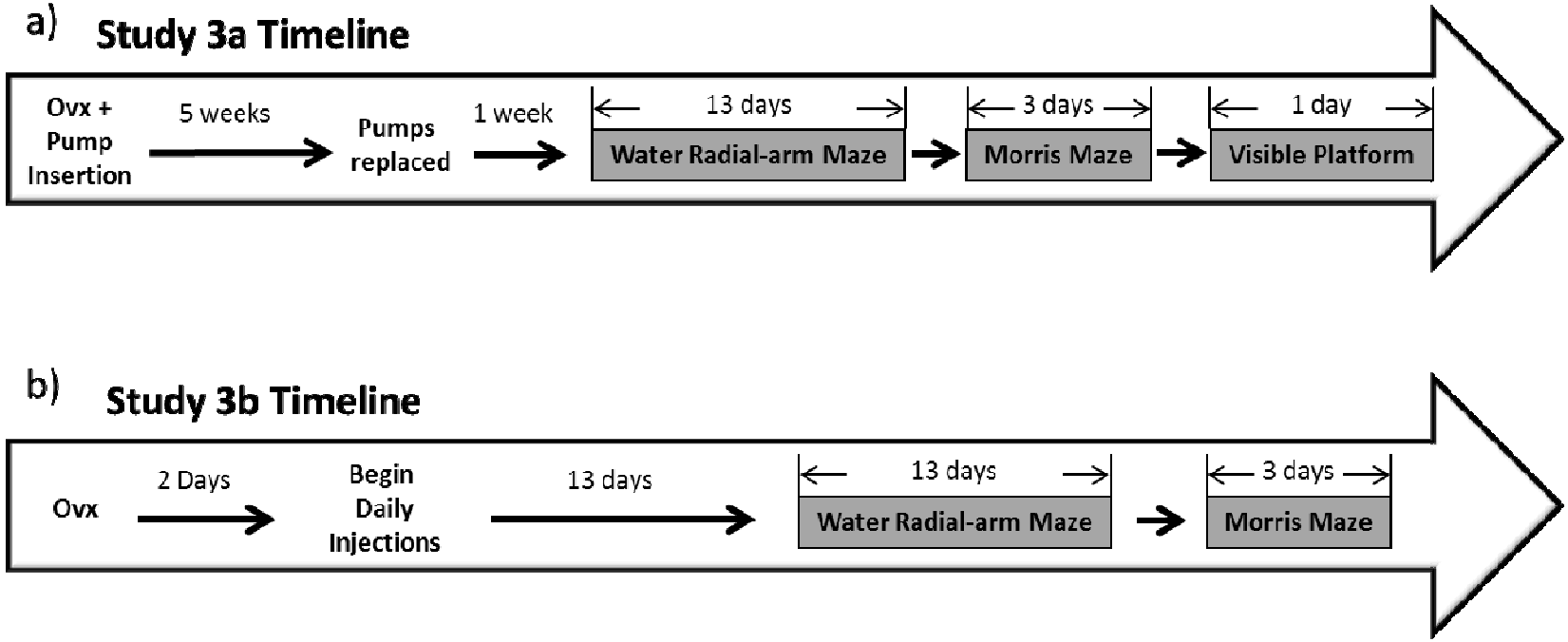

a)
Study 3a Timeline
Ovx + Pump Insertion
5 weeks
Pumps replaced
1 week
13 days
Water Radial-arm Maze
3 days
Morris Maze
1 day
Visible Platform
b)
Study 3b Timeline
Ovx
2 Days
Begin Daily Injections
13 days
13 days
Water Radial-arm Maze
3 days
Morris Maze

Figure 13. A time line for (a) Study 3a and (b) Study 3b summarizing injections, ovariectomy, and behavioral testing for each group.

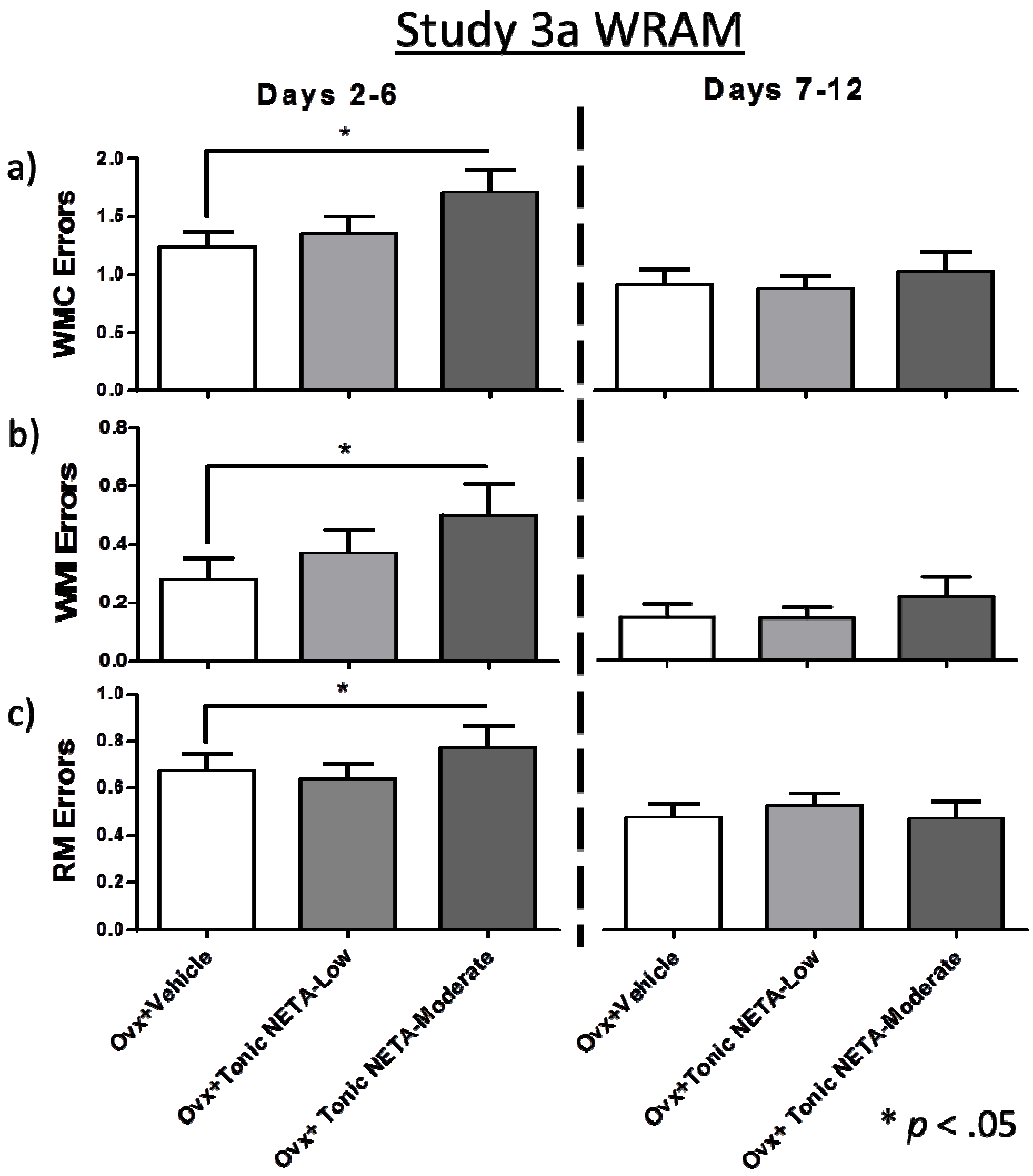

Study 3a WRAM
Days 2-6
Days 7-12
a)
WMC Errors
b)
WM Errors
c)
RM Errors
*
Ovx+Vehicle
Ovx+Tonic NETA-Low
Ovx+ Tonic NETA-Moderate
* p < .05

Figure 14. Mean error scores (+SE) on the water radial-arm maze during the learning (days 2-6) and asymptotic (days 7-12) testing phases. (a) WMC errors. (b) WMI errors. (c) RM errors. *$p < .05$

Study 3a WRAM: 6 Hour Delay

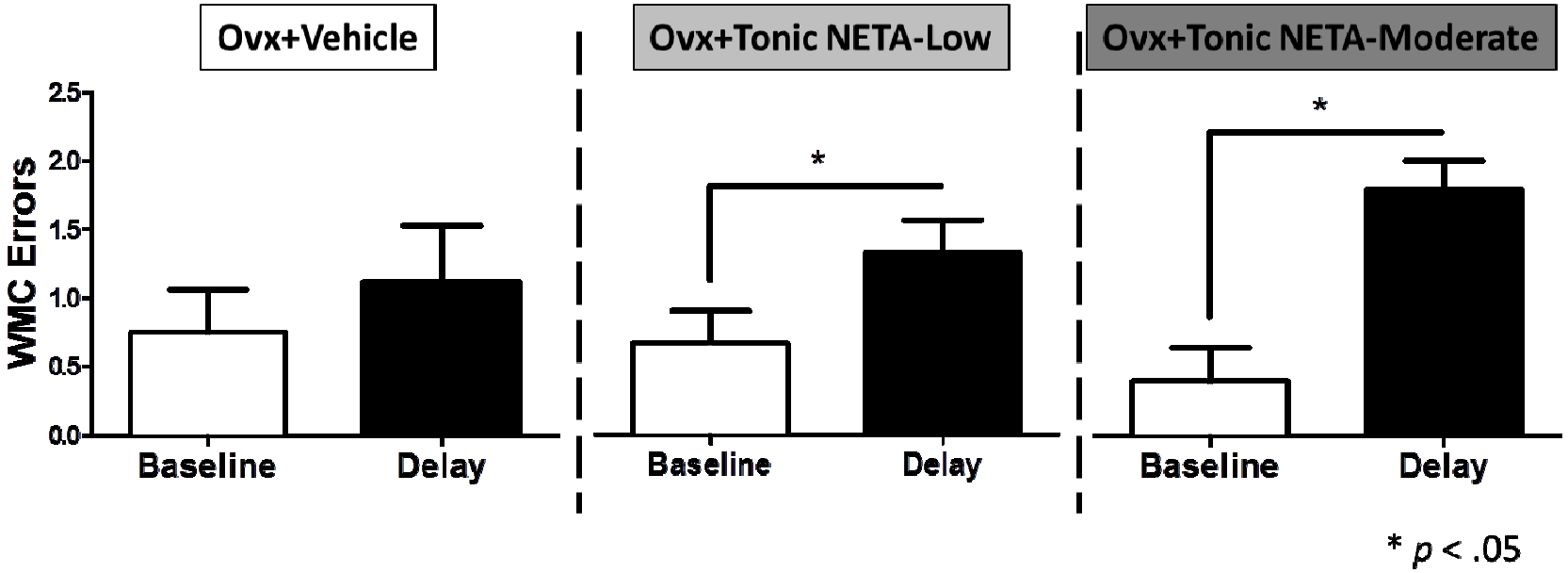


Figure 15. Mean WMC error scores (+SE) on the water radial-arm maze for baseline (last day of regular testing trial 3) vs. delay (trial 3 immediately following a 6 hour delay). *$p < .05$

Study 3a MM

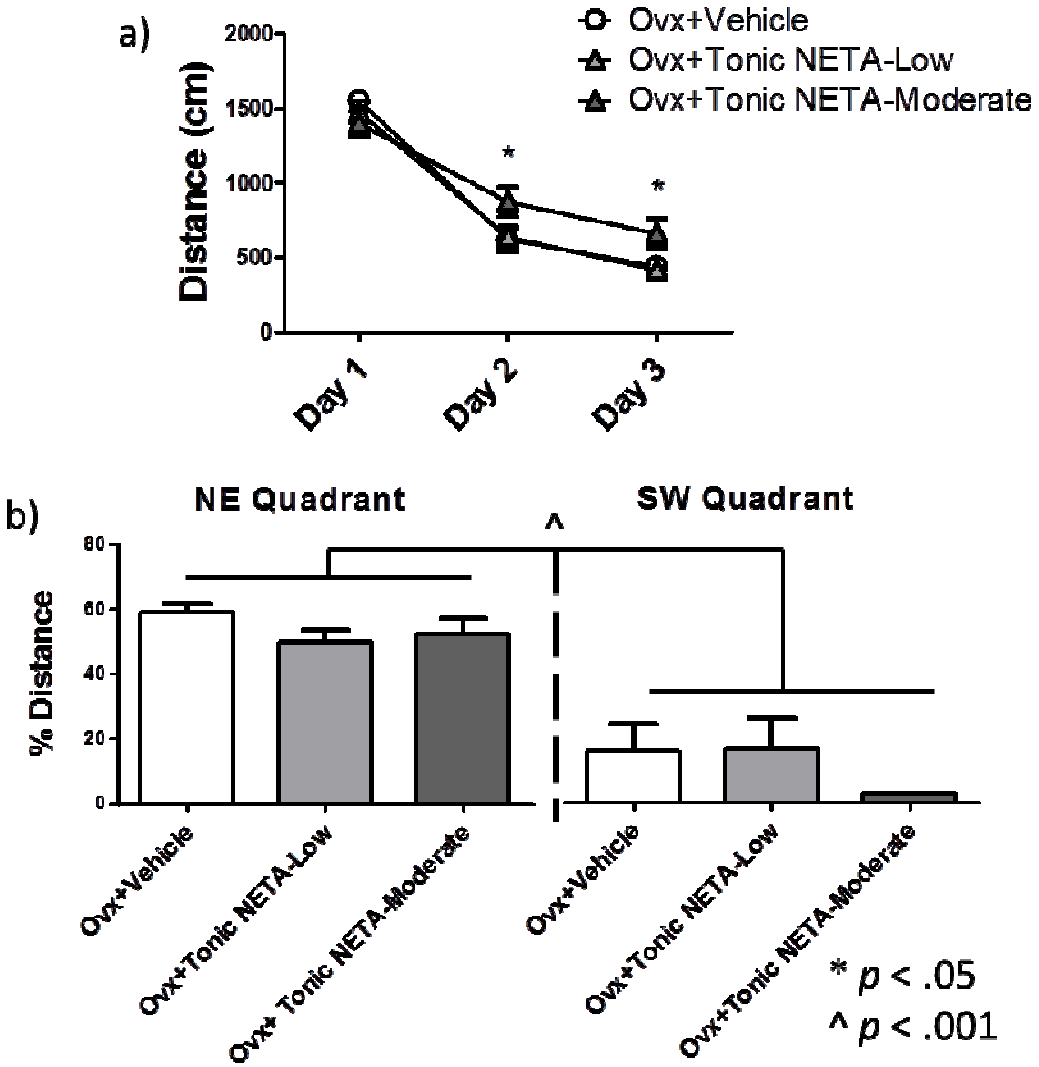

a)
Ovx+Vehicle
Ovx+Tonic NETA-Low
Ovx+Tonic NETA-Moderate
Distance (cm)
2000
1500
1000
500
0
*
*
Day 1
Day 2
Day 3
b)
NE Quadrant
SW Quadrant
^
% Distance
80
60
40
20
0
Ovx+Vehicle
Ovx+Tonic NETA-Low
Ovx+ Tonic NETA-Moderate
Ovx+Vehicle
Ovx+Tonic NETA-Low
Ovx+Tonic NETA-Moderate
* p < .05
^ p < .001

Figure 16. Mean Distance scores in centimeters (+SE) on Morris maze. (a) Distance scores across all days of testing (days 1-3). (b) Mean percent Distance in the target NE quadrant as compared to the opposite SW quadrant during the probe trial. *$p < .05$; ^$p < .0001$

Study 3a Visible Platform

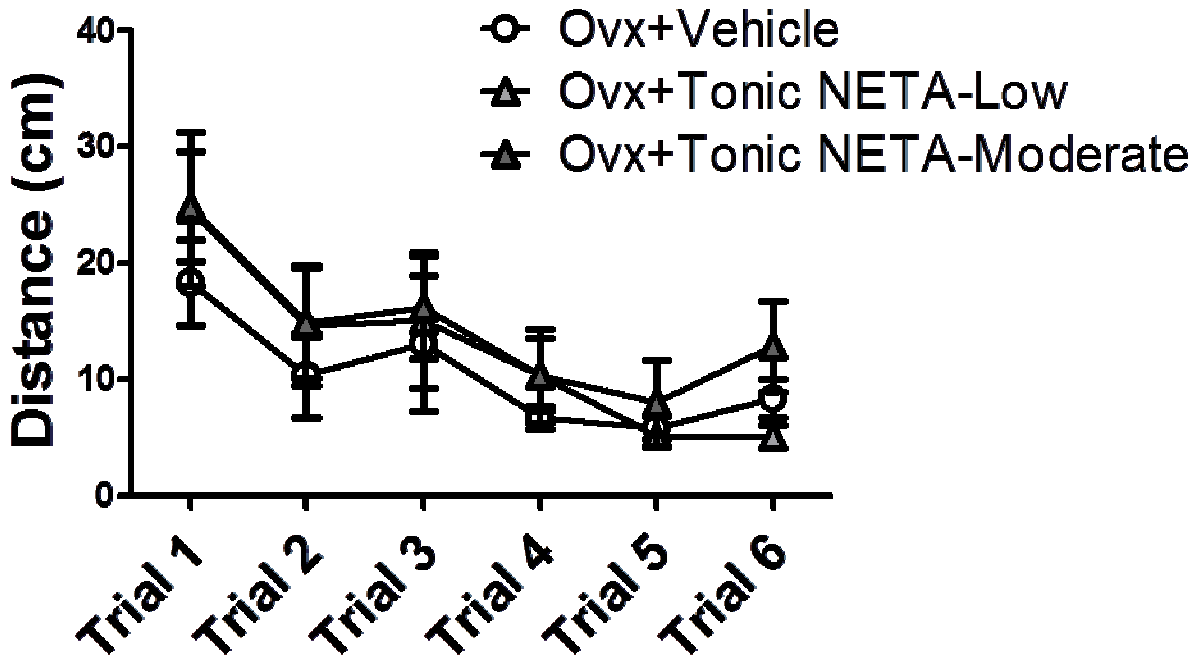


Figure 17. Mean latency scores in seconds (+SE) on the visible platform maze.

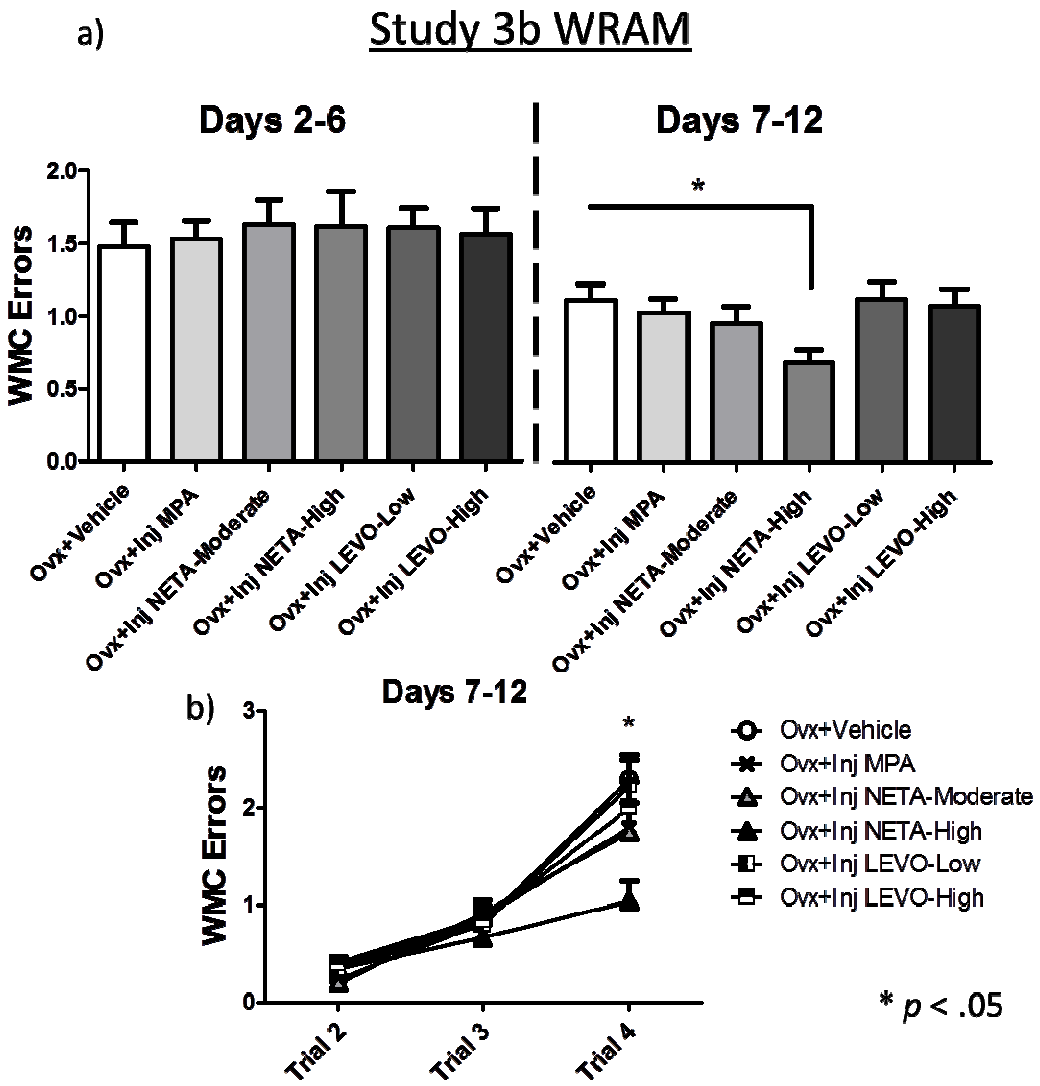

a)
Study 3b WRAM
Days 2-6
Days 7-12
WMC Errors
2.0
1.5
1.0
0.5
0.0
*
Ovx+Vehicle
Ovx+Inj MPA
Ovx+Inj NETA-Moderate
Ovx+Inj NETA-High
Ovx+Inj LEVO-Low
Ovx+Inj LEVO-High
b)
Days 7-12
WMC Errors
3
2
1
0
*
Trial 2
Trial 3
Trial 4
Ovx+Vehicle
Ovx+Inj MPA
Ovx+Inj NETA-Moderate
Ovx+Inj NETA-High
Ovx+Inj LEVO-Low
Ovx+Inj LEVO-High
* p < .05

Figure 18. Mean WMC error scores (+SE) on the water radial-arm maze. (a) WMC error scores during the learning (days 2-6) and asymptotic (days 7-12) testing phases. (b) Working memory load effect during the asymptotic testing phase. *$p < .05$

Study 3b WRAM: Latter Testing

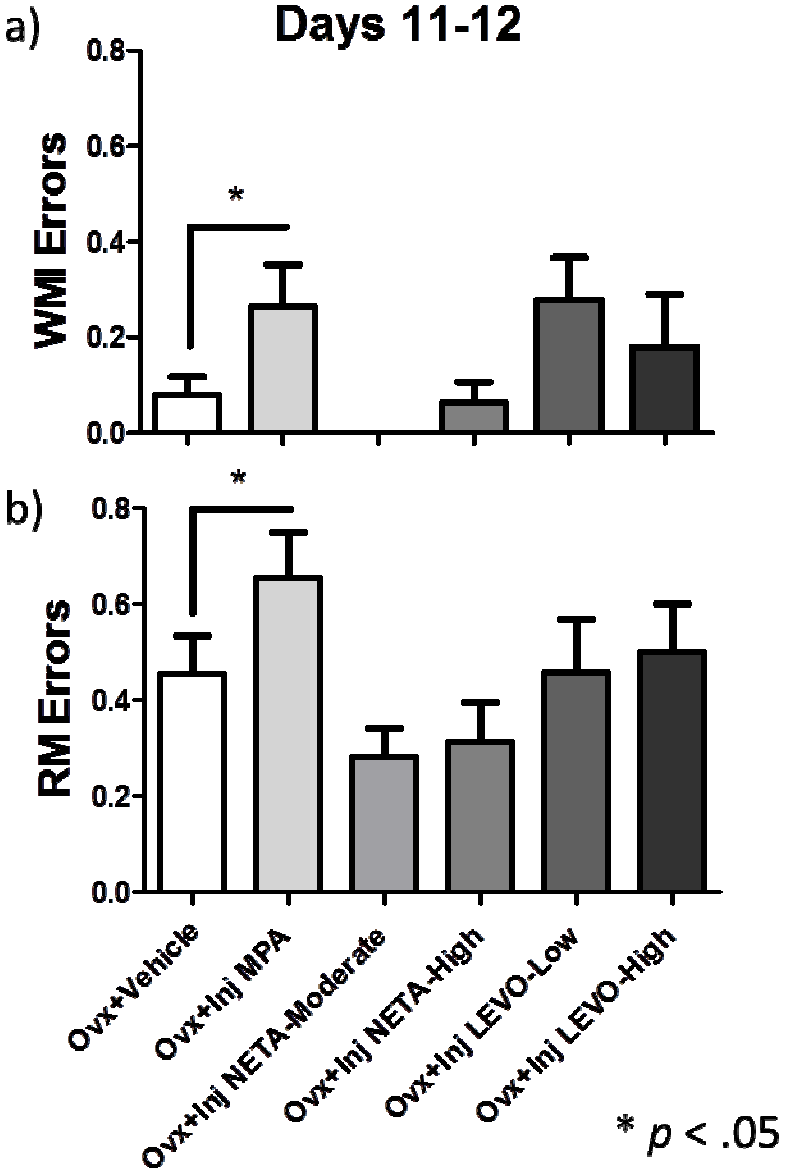
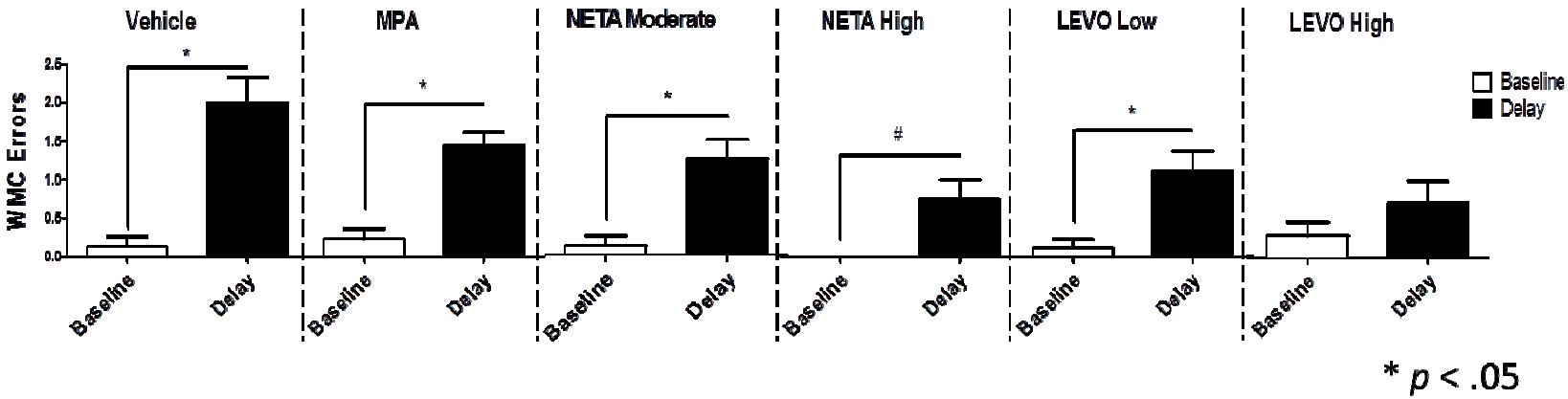


Figure 19. Mean error scores (+SE) on the water radial-arm maze during the latter portion of testing (days 11-12). (a) WMI error scores (b) RM error scores. *p < .05

Study 3b WRAM: 6 Hour Delay



* $p < .05$

Figure 20. Mean WMC error scores (±SE) on the water radial-arm maze for baseline (last day of regular testing trial 3) vs. delay (trial 3 immediately following a 6 hour delay). *$p < .05$

Study 3b MM

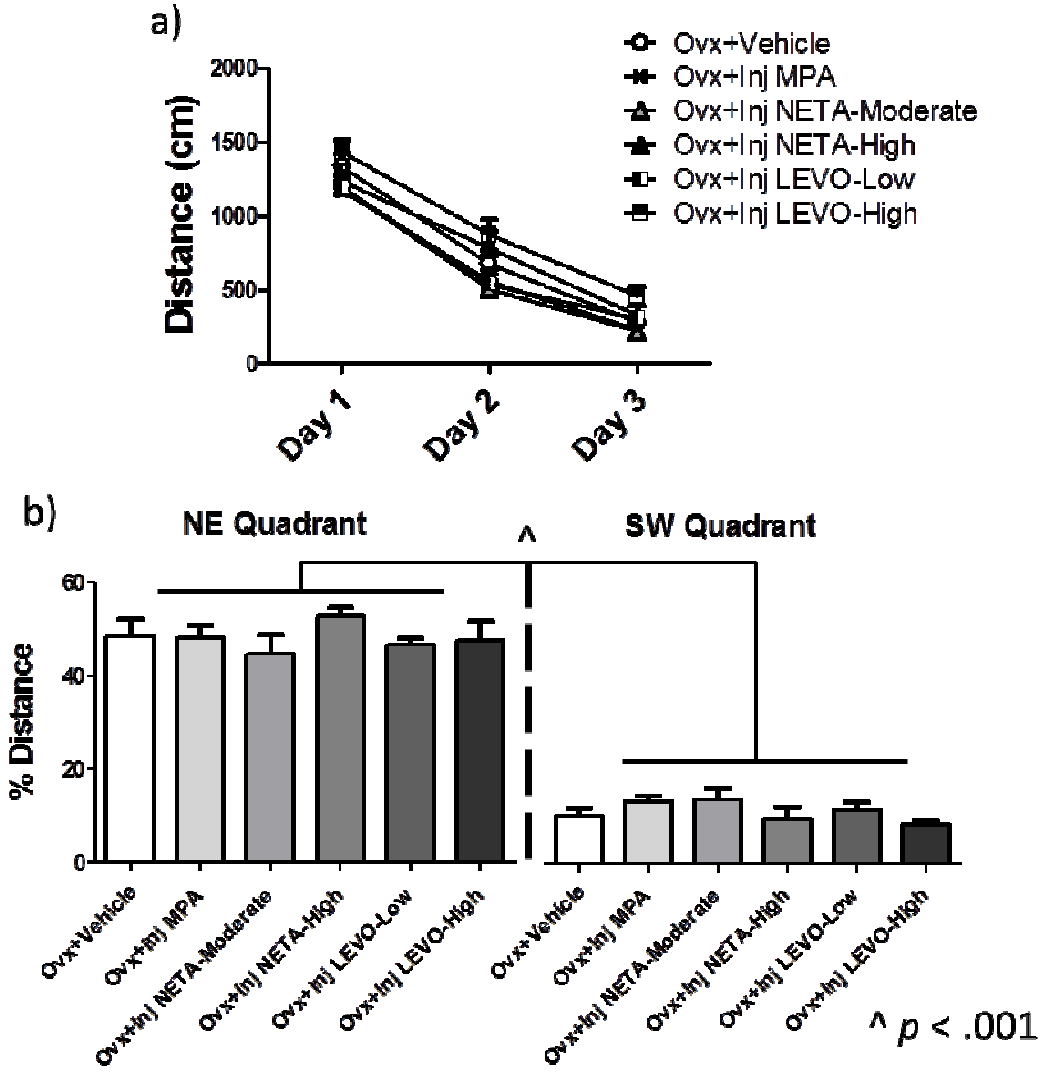

a)
Distance (cm)
2000
1500
1000
500
0
Day 1
Day 2
Day 3
Ovx+Vehicle
Ovx+Inj MPA
Ovx+Inj NETA-Moderate
Ovx+Inj NETA-High
Ovx+Inj LEVO-Low
Ovx+Inj LEVO-High
b)
NE Quadrant
^
SW Quadrant
% Distance
60
40
20
0
Ovx+Vehicle
Ovx+Inj MPA
Ovx+Inj NETA-Moderate
Ovx+Inj NETA-High
Ovx+Inj LEVO-Low
Ovx+Inj LEVO-High
^ p < .001

Figure 21. Mean Distance scores in centimeters (+SE) on Morris maze. (a) Distance scores across all days of testing (days 1-3). (b) Mean percent Distance in the target NE quadrant as compared to the opposite SW quadrant during the probe trial. *$p < .05$; ^$p < .0001$

Conclusions Summary

Study 3a

Treatment	Outcome
NETA Low	Impaired
NETA Moderate	Impaired
NETA High	Data not included (pump failure)

Study 3b

Treatment	Outcome
NETA Moderate	Null
NETA High	Improved
LEVO Low	Null
LEVO High	Improved
MPA	Impaired

Figure 22. Conclusions summary for Study 3a (tonic treatment) and 3b (injection treatment).

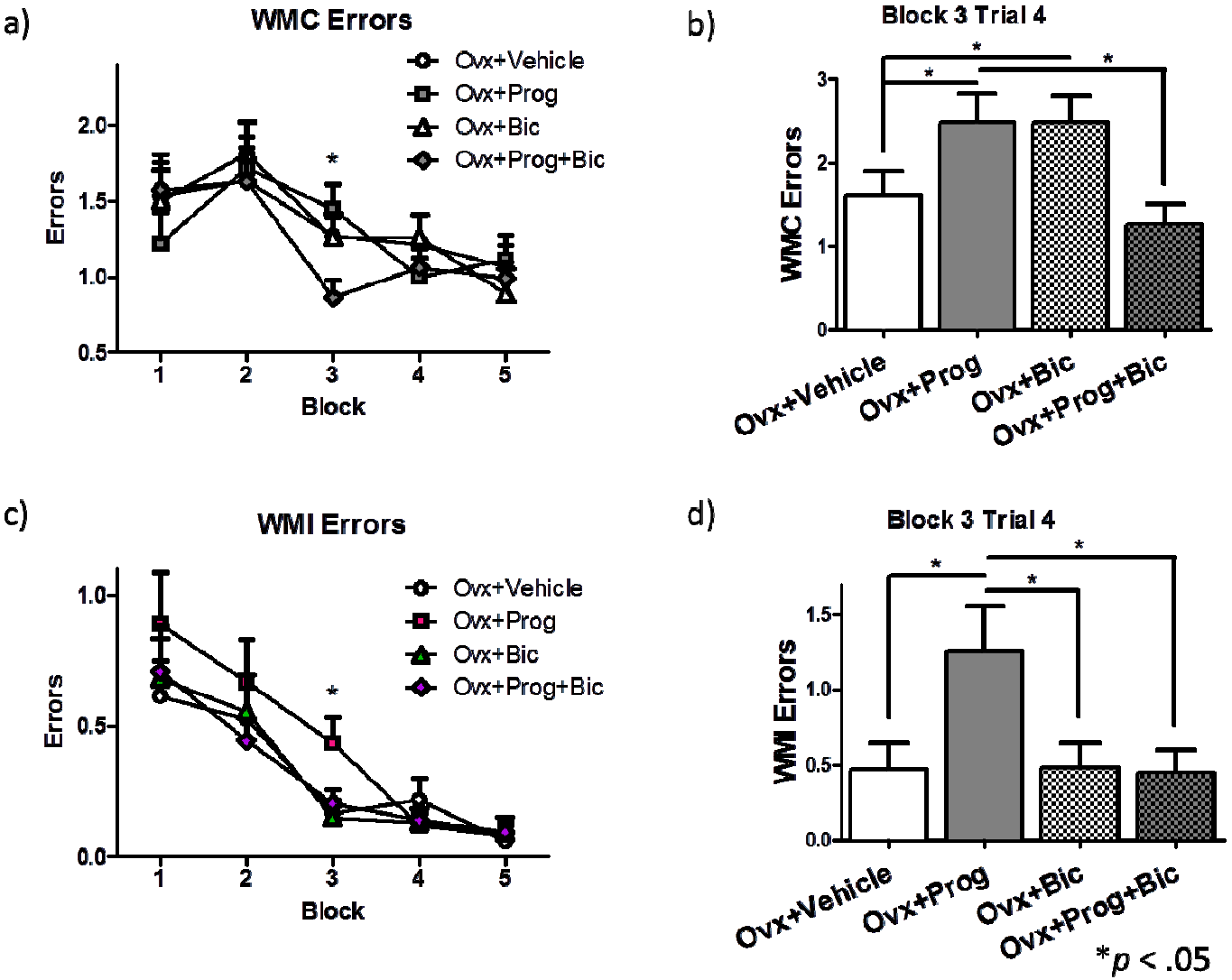

a)
WMC Errors
Ovx+Vehicle
Ovx+Prog
Ovx+Bic
Ovx+Prog+Bic
Errors
Block
b)
Block 3 Trial 4
WMC Errors
Ovx+Vehicle
Ovx+Prog
Ovx+Bic
Ovx+Prog+Bic
c)
WMI Errors
Errors
Block
d)
Block 3 Trial 4
WMI Errors
*p < .05

Figure 23. Mean error scores (+SE) on the water-radial arm maze. (a) WMC error scores across all days of regular testing in 2-3 day blocks. (b) WMC error scores on block 3 trial 4. (c). WMI error scores across all days of testing in 2-3 day blocks. (d) WMI error scores on block 3 trial 4. *$p < .05$

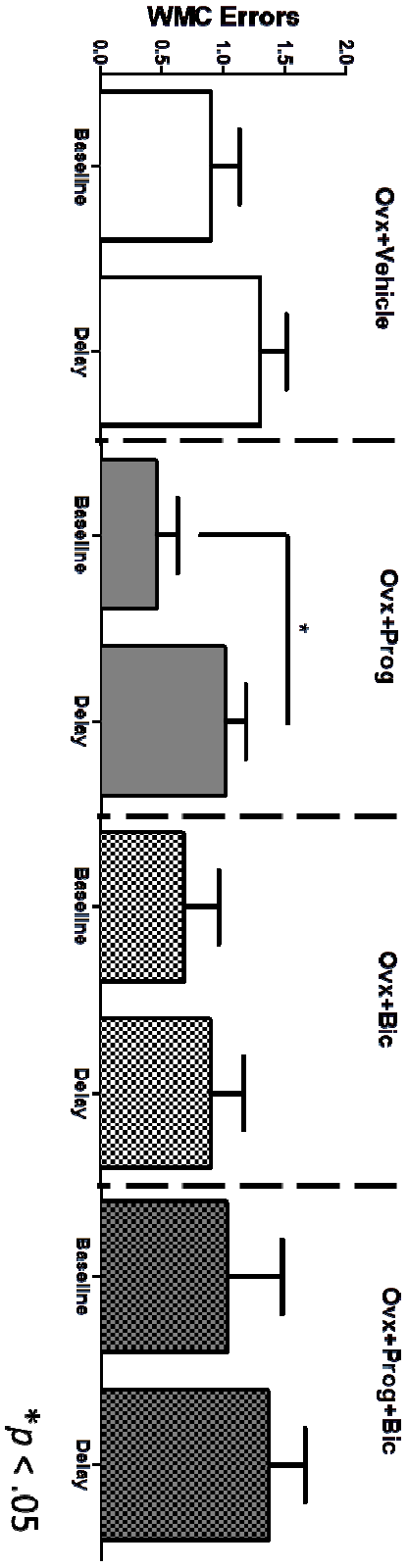

WMC Errors
0.0
0.5
1.0
1.5
2.0
Baseline
Delay
Ovx+Vehicle
Ovx+Prog
Ovx+Bic
Ovx+Prog+Bic
*
*p < .05

Figure 24. Mean WMC error scores (+SE) on the water radial-arm maze for baseline (last day of regular testing trial 3) vs. delay (trial 3 immediately following a 6 hour delay). *$p < .05$

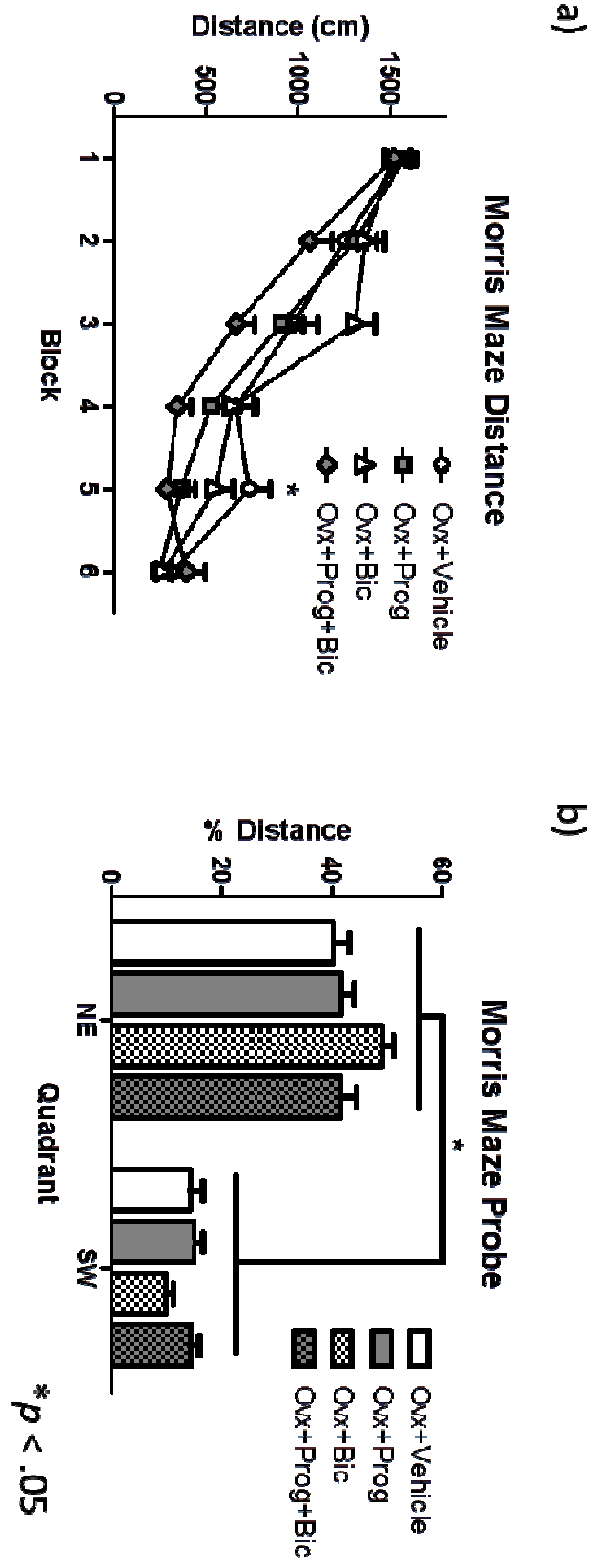

a)
Morris Maze Distance
Distance (cm)
0
500
1000
1500
Block
1
2
3
4
5
6
*
Ovx+Vehicle
Ovx+Prog
Ovx+Bic
Ovx+Prog+Bic
b)
Morris Maze Probe
% Distance
0
20
40
60
NE
SW
Quadrant
*
Ovx+Vehicle
Ovx+Prog
Ovx+Bic
Ovx+Prog+Bic
*p < .05

Figure 25. Mean Distance scores in centimeters (+SE) on Morris maze. (a) Distance scores across all days of testing in 3 trial blocks. (b) Mean percent Distance in the target NE quadrant as compared to the opposite SW quadrant during the probe trial. *$p < .05$

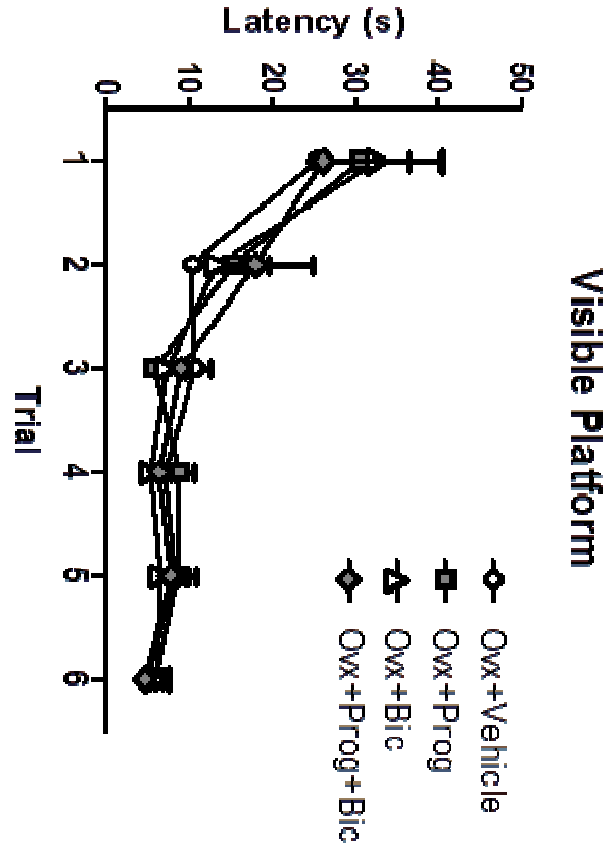
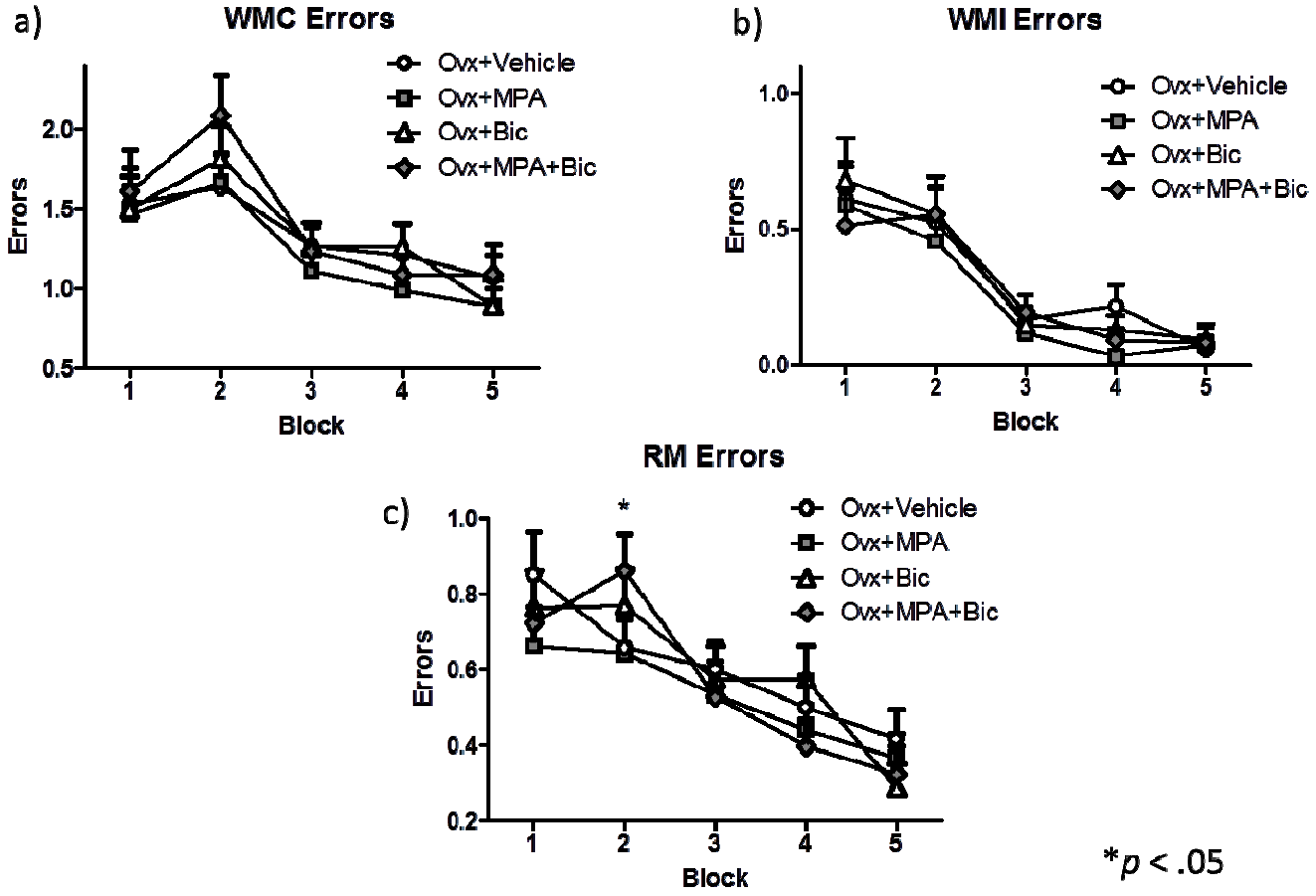

Visible Platform
Latency (s)
0
10
20
30
40
50
Trial
1
2
3
4
5
6
Ovx+Vehicle
Ovx+Prog
Ovx+Bic
Ovx+Prog+Bic

Figure 26. Mean latency scores in seconds (+SE) on the visible platform maze.


a)
WMC Errors
Ovx+Vehicle
Ovx+MPA
Ovx+Bic
Ovx+MPA+Bic
Errors
2.0
1.5
1.0
0.5
1
2
3
4
5
Block
b)
WMI Errors
Ovx+Vehicle
Ovx+MPA
Ovx+Bic
Ovx+MPA+Bic
Errors
1.0
0.5
0.0
1
2
3
4
5
Block
RM Errors
c)
*
Ovx+Vehicle
Ovx+MPA
Ovx+Bic
Ovx+MPA+Bic
Errors
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
Block

*$p < .05$

Figure 27. Mean error scores (+SE) on the water-radial arm maze across all days of regular testing in 2-3 day blocks for (a) WMC error scores (b) WMI error scores and (c) RM error scores. *$p < .05$

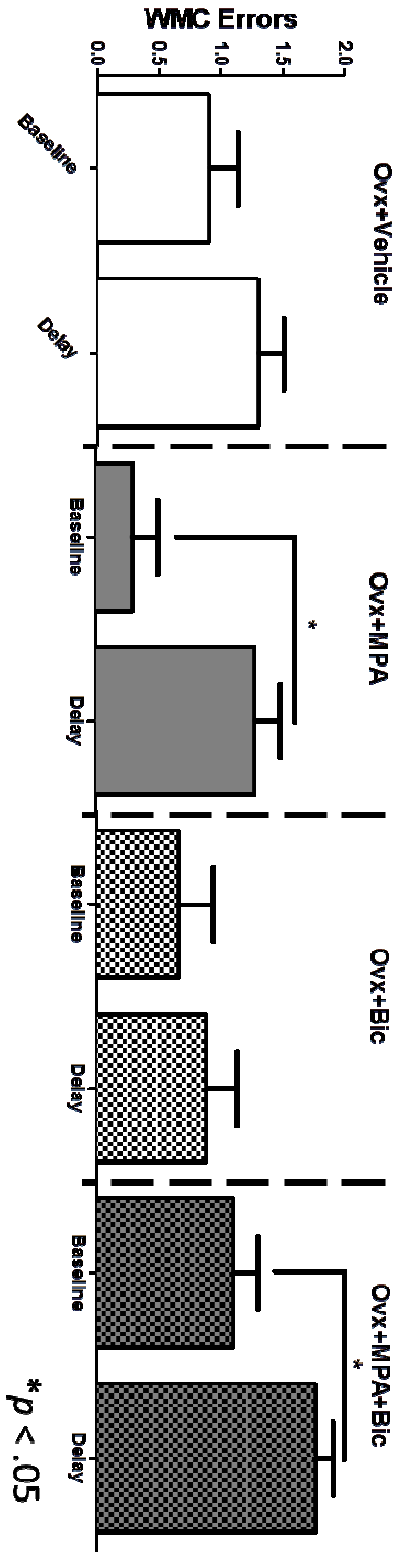
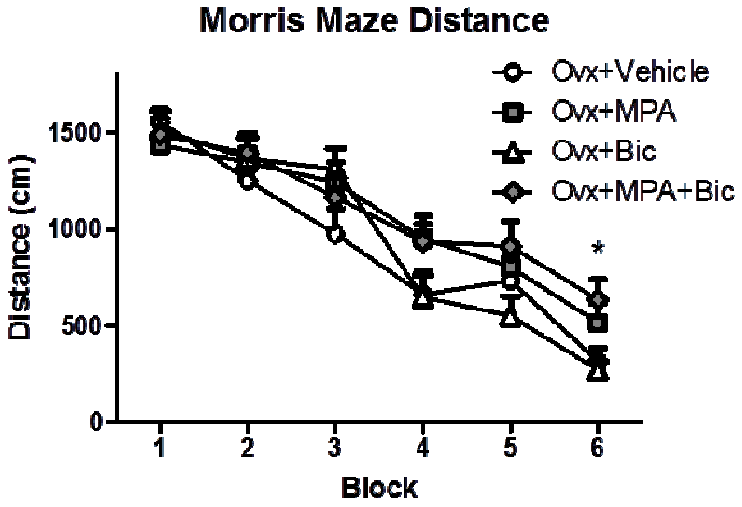

WMC Errors
0.0
0.5
1.0
1.5
2.0
Ovx+Vehicle
Baseline
Delay
Ovx+MPA
Baseline
Delay
*
Ovx+Bic
Baseline
Delay
Ovx+MPA+Bic
Baseline
Delay
*
*p < .05

Figure 28. Mean WMC error scores (+SE) on the water radial-arm maze for baseline (last day of regular testing trial 3) vs. delay (trial 3 immediately following a 6 hour delay). *$p < .05$

a)


Morris Maze Distance
Ovx+Vehicle
Ovx+MPA
Ovx+Bic
Ovx+MPA+Bic
Distance (cm)
1500
1000
500
0
1
2
3
4
5
6
Block
*

b)

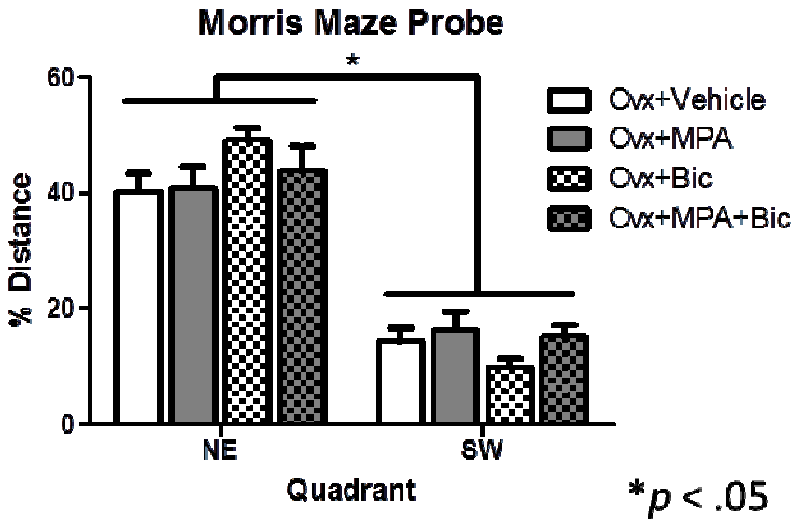

Morris Maze Probe
*
60
40
20
0
% Distance
Ovx+Vehicle
Ovx+MPA
Ovx+Bic
Ovx+MPA+Bic
NE
SW
Quadrant
*p < .05

Figure 29. Mean Distance scores in centimeters (+SE) on Morris maze. (a) Distance scores across all days of testing in 3 trial blocks. (b) Mean percent Distance in the target NE quadrant as compared to the opposite SW quadrant during the probe trial. *$p < .05$

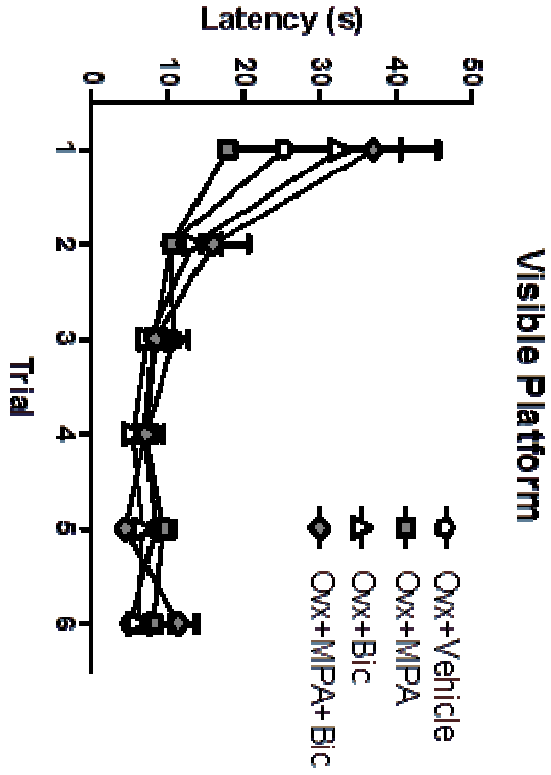
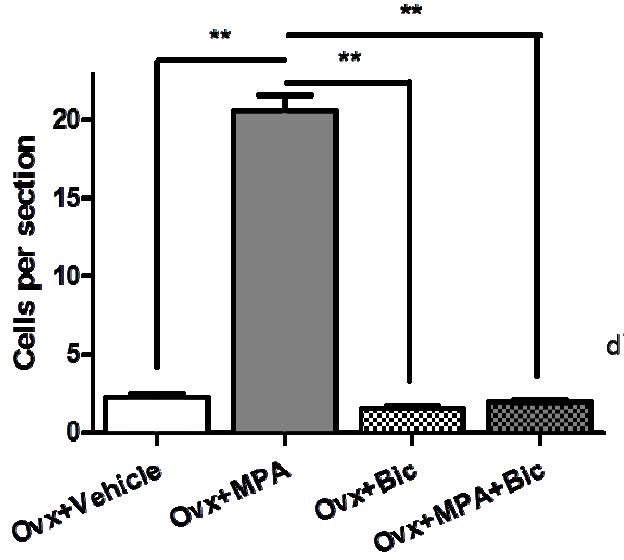

Visible Platform
Latency (s)
0
10
20
30
40
50
Trial
1
2
3
4
5
6
Ovx+Vehicle
Ovx+MPA
Ovx+Bic
Ovx+MPA+Bic

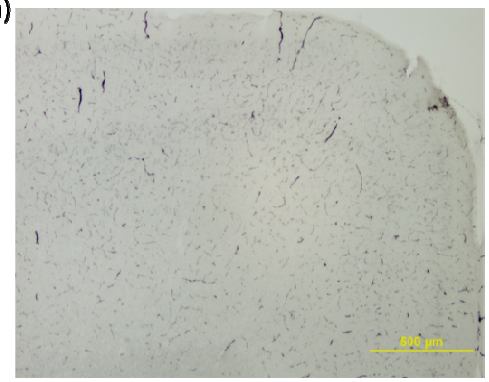
Figure 30. Mean latency scores in seconds (+SE) on the visible platform maze.

a) **Frontal Cortex Cox-2 Cell Counts**

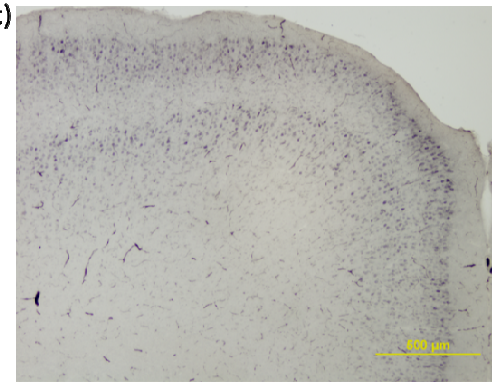


$^{**}p < .005$

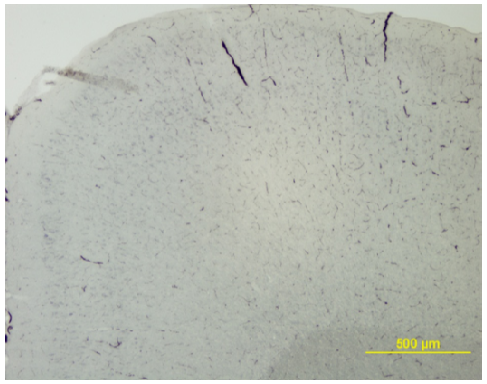
b) Vehicle



c) MPA



d) Bic



e) MPA+Bic

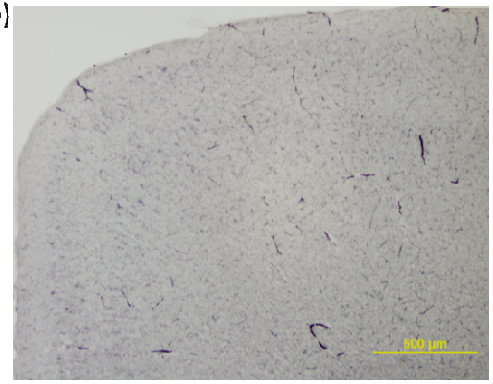


Figure 31. (a) Mean frontal cortex Cox-2 Cell Counts per section (+SE). Representative images from (b) Ovx+Vehicle, (c) Ovx+MPA, (d) Ovx+Bic, and (e) Ovx+MPA+Bic. *p < .05

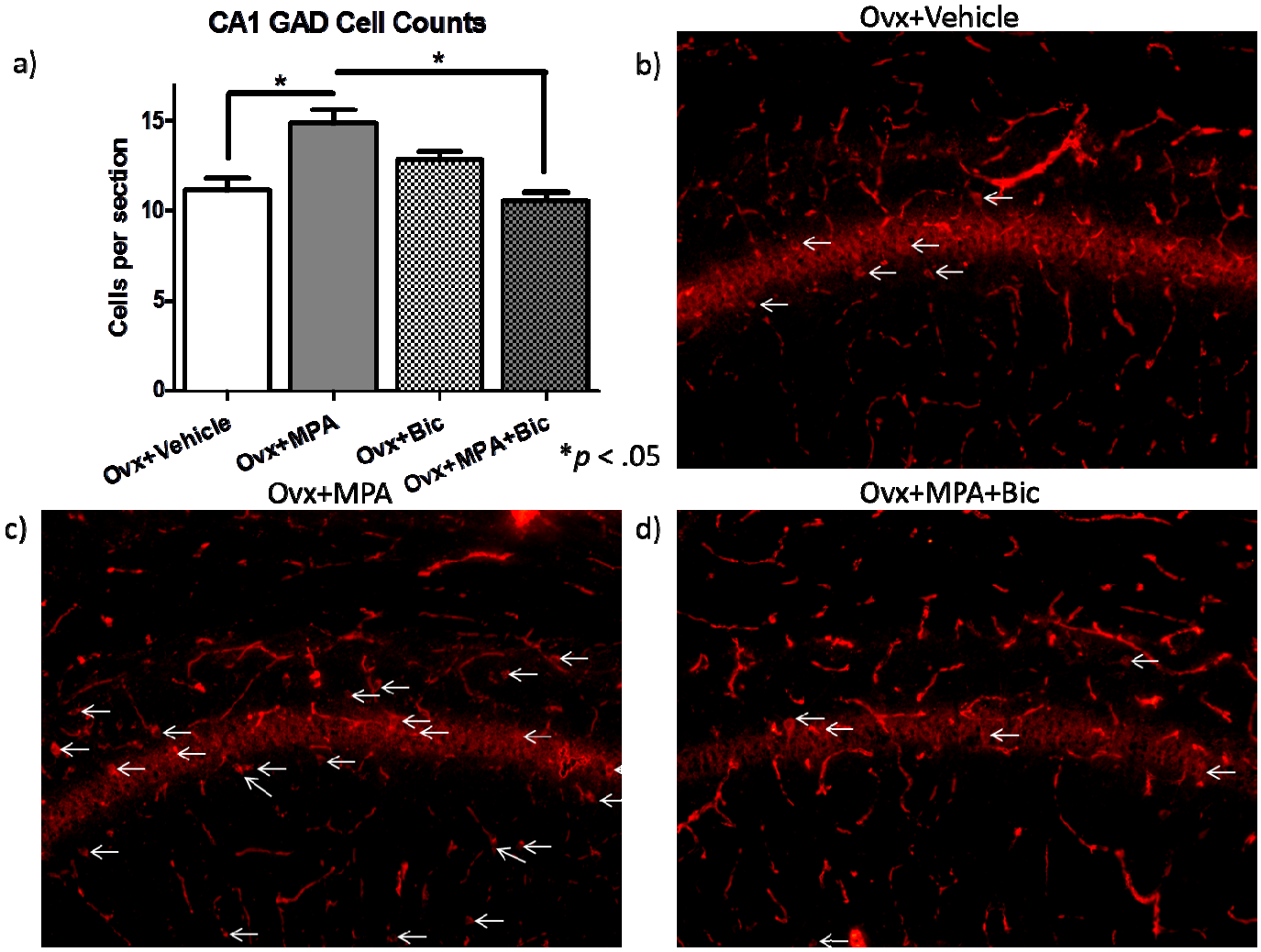
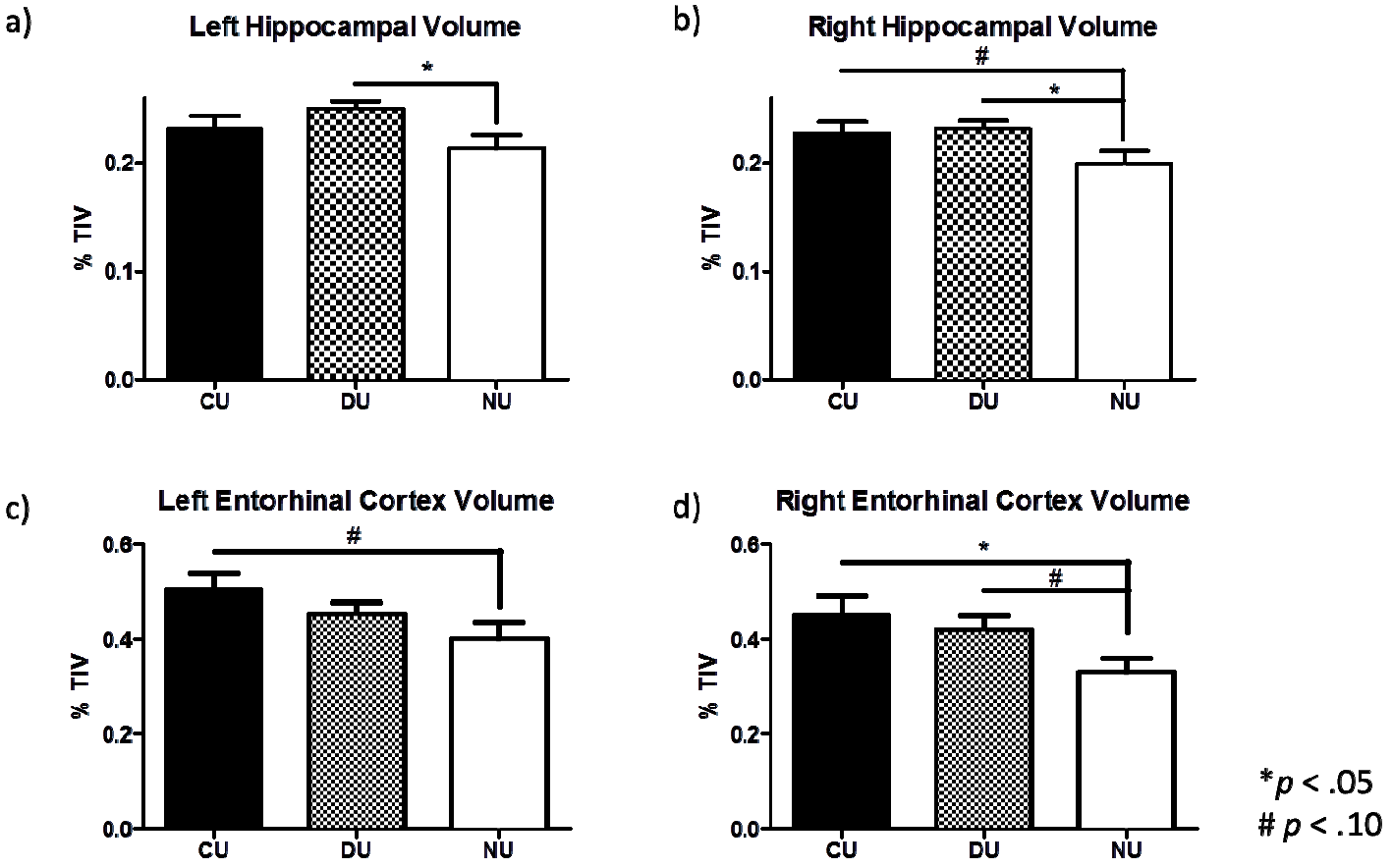

a)
CA1 GAD Cell Counts
Cells per section
0
5
10
15
*
*
Ovx+Vehicle
Ovx+MPA
Ovx+Bic
Ovx+MPA+Bic
*p < .05
b)
Ovx+Vehicle
c)
Ovx+MPA
d)
Ovx+MPA+Bic

Figure 32. (a) Mean CA1 GAD Cell Counts per section (+SE). Representative images from (b) Ovx+Vehicle, (c) Ovx+MPA, and (d), Ovx+MPA+Bic. White arrows indicate positive GAD cells. *$p < .05$


a)
Left Hippocampal Volume
% TIV
0.0
0.1
0.2
CU
DU
NU
*
b)
Right Hippocampal Volume
% TIV
0.0
0.1
0.2
CU
DU
NU
#
*
c)
Left Entorhinal Cortex Volume
% TIV
0.0
0.2
0.4
0.6
CU
DU
NU
#
d)
Right Entorhinal Cortex Volume
% TIV
0.0
0.2
0.4
0.6
CU
DU
NU
*
#

*$p < .05$
# $p < .10$

Figure 33. Mean %TIV (+SE) for (a) left hippocampal volumes, (b) right hippocampal volumes, (c) left entorhinal cortex, and (d) right entorhinal cortex. *p < .05, #p < .10

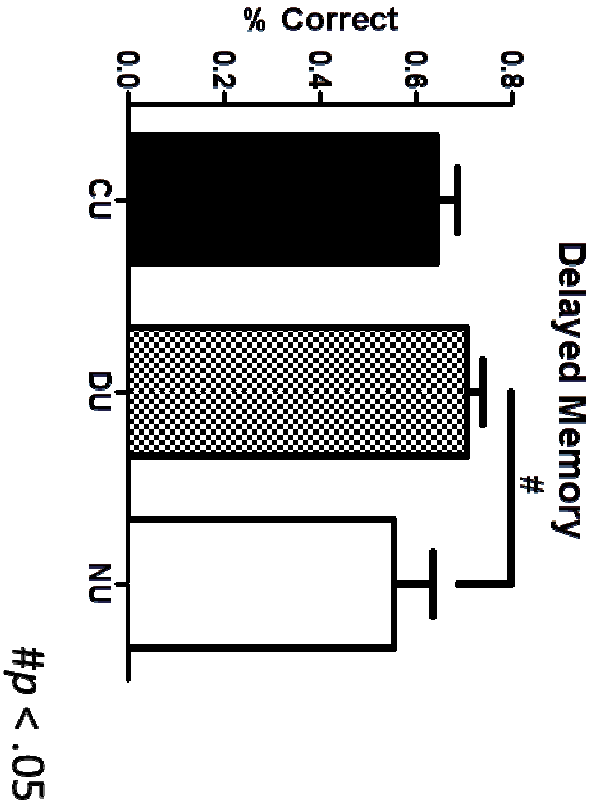

% Correct
0.0
0.2
0.4
0.6
0.8
CU
DU
NU
#
Delayed Memory
#p < .05

Figure 34. Mean % correct words (+SE) for delayed memory. #p < .10

Continuous Users Correlations

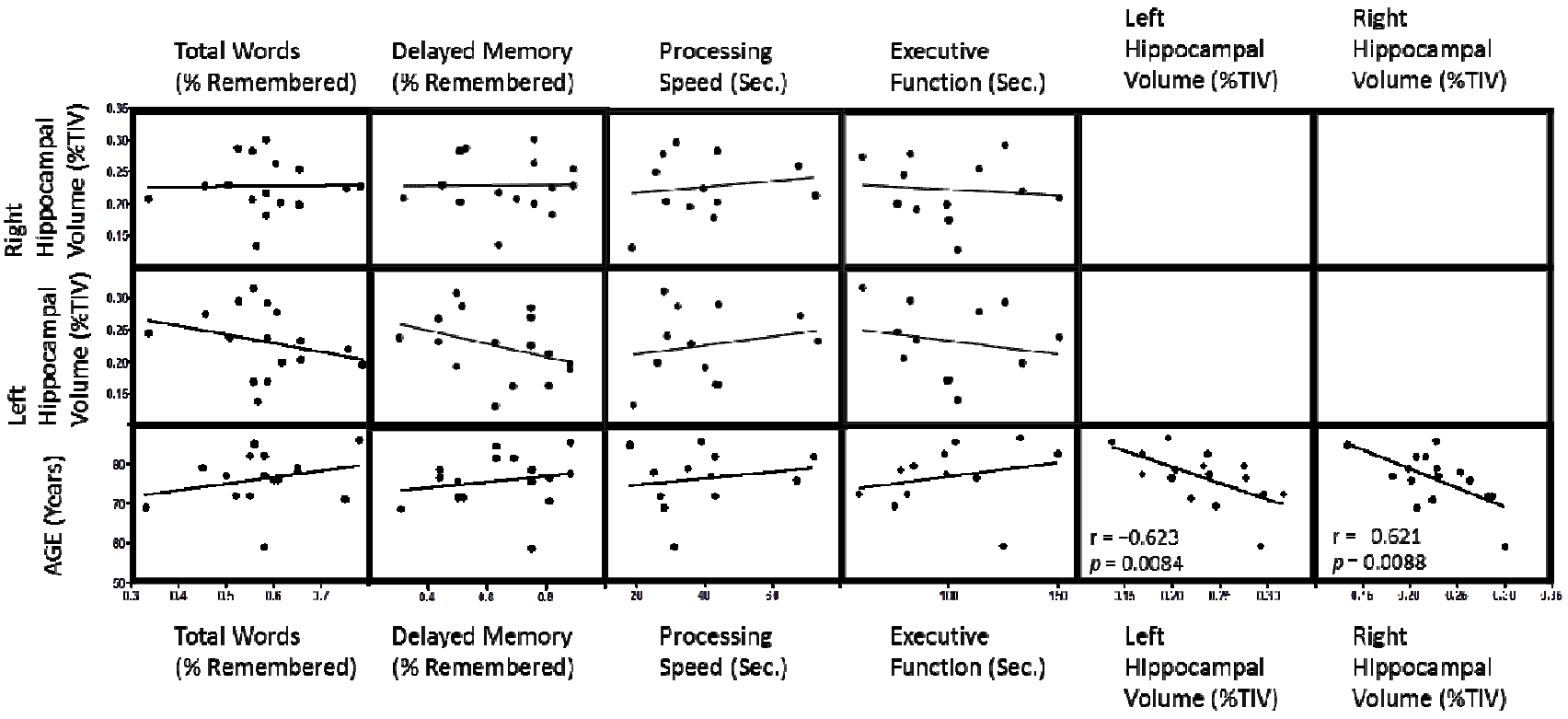


Figure 35. Correlation matrix for continuous users showing correlations between age, brain volumes (hippocampus and entorhinal cortex), and cognitive scores (Total words, Delayed memory, TrailsA, and TrailsB). p values and Pearson r values listed for significant ($p < .01$) and marginal ($p < .05$) correlations.

Discontinuous Users Correlations

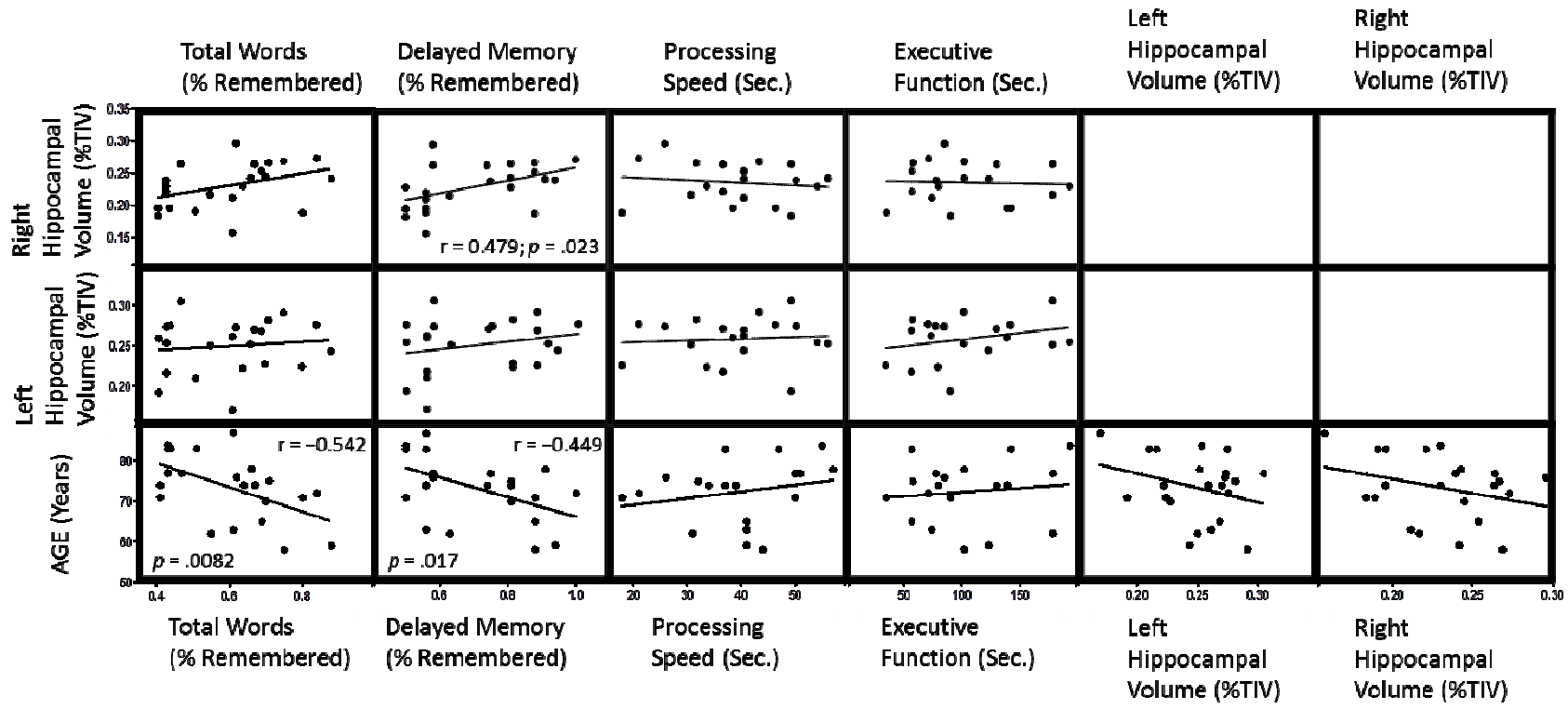


Figure 36. Correlation matrix for discontinuous users showing correlations between age, brain volumes (hippocampus and entorhinal cortex), and cognitive scores (Total words, Delayed memory, TrailsA, and TrailsB). p values and Pearson r values listed for significant ($p < .01$) and marginal ($p < .05$) correlations.

Never Users Correlations

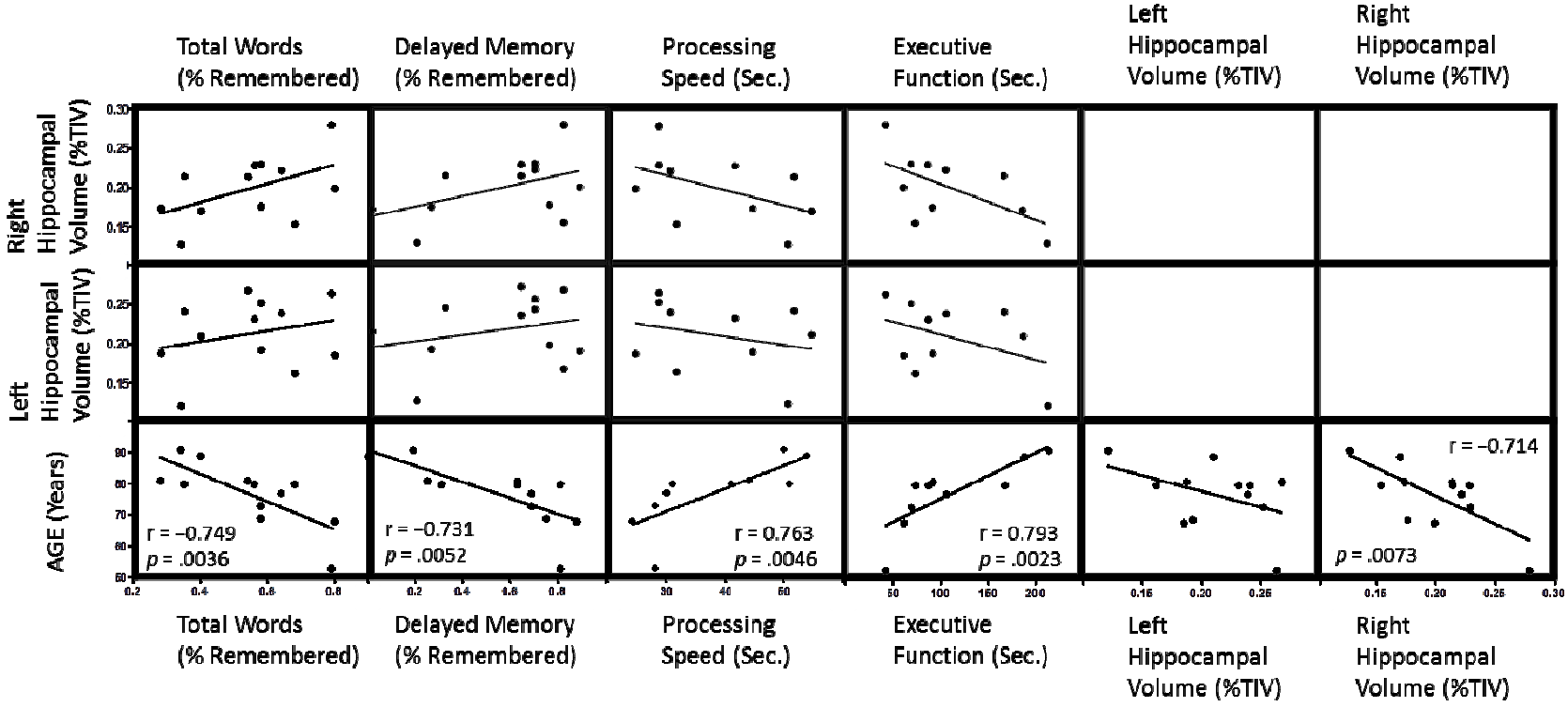


Figure 37. Correlation matrix for never users showing correlations between age, brain volumes (hippocampus and entorhinal cortex), and cognitive scores (Total words, Delayed memory, TrailsA, and TrailsB). p values and Pearson r values listed for significant ($p < .01$) and marginal ($p < .05$) correlations.

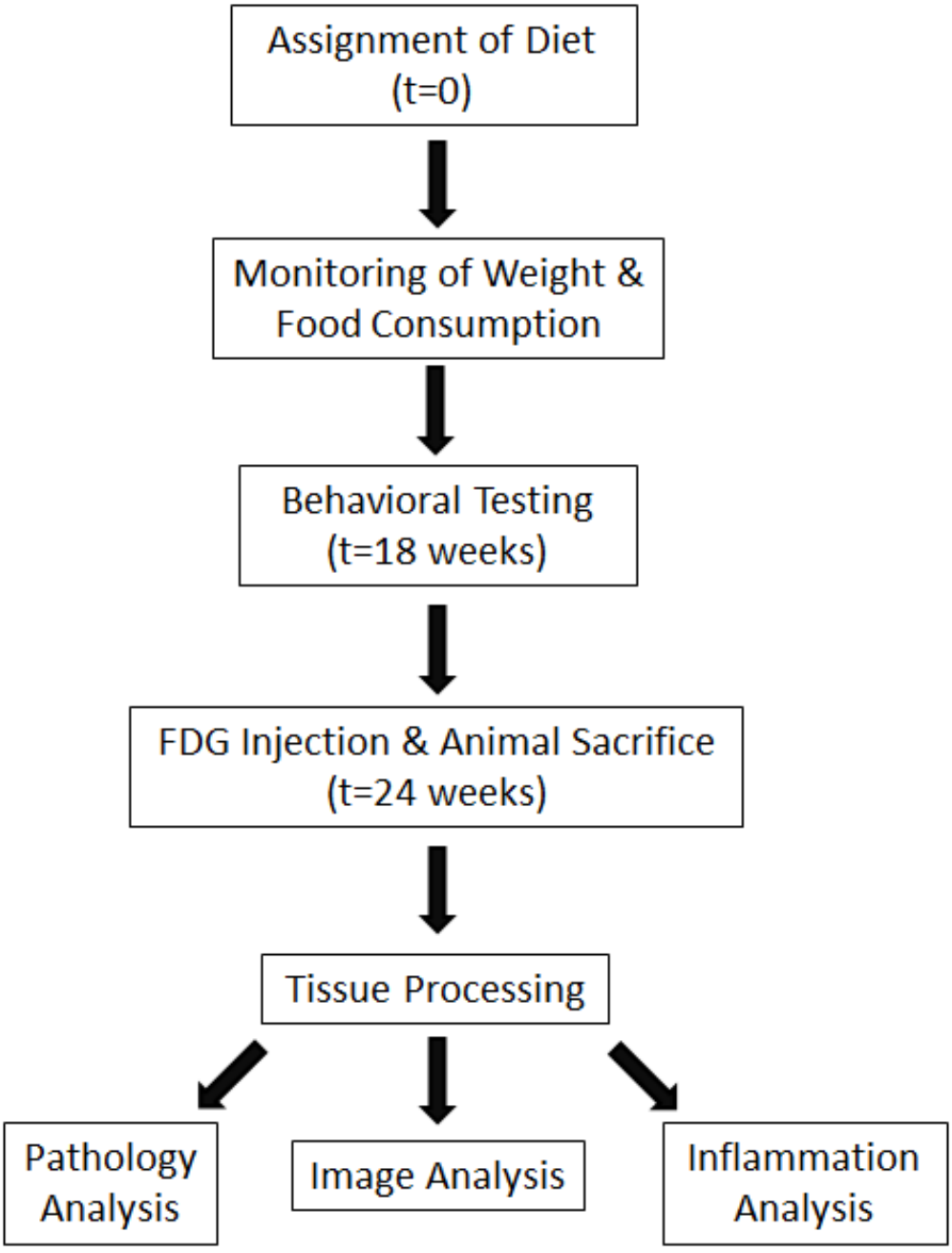

Assignment of Diet
(t=0)
Monitoring of Weight &
Food Consumption
Behavioral Testing
(t=18 weeks)
FDG Injection & Animal Sacrifice
(t=24 weeks)
Tissue Processing
Pathology
Analysis
Image Analysis
Inflammation
Analysis

Figure 38. Flowchart summarizing each stage of the study. The same animals were used for each analysis throughout the study, with tissues distributed from the behaviorally-tested animals.

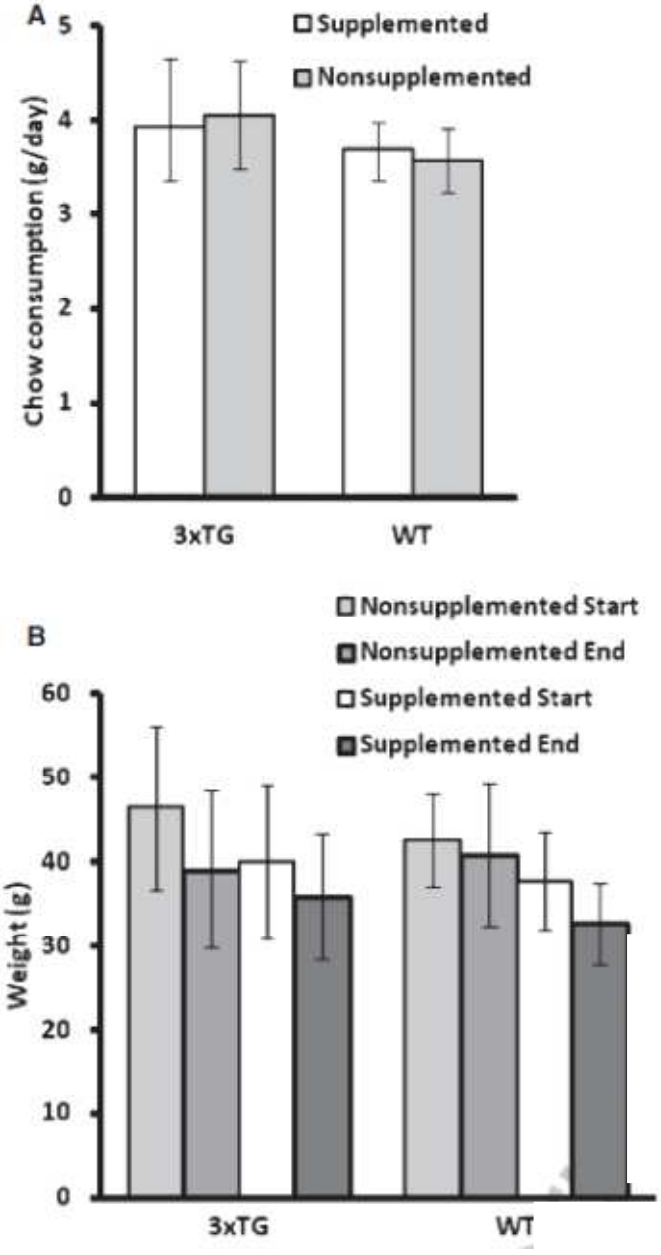

A
Supplemented
Nonsupplemented
Chow consumption (g/day)
0
1
2
3
4
5
3xTG
WT
B
Nonsupplemented Start
Nonsupplemented End
Supplemented Start
Supplemented End
Weight (g)
0
10
20
30
40
50
60
3xTG
WT

Figure 39. (a) Mean (±SD) chow consumption (g/day) for each group during the first three weeks of supplementation. (b) Mean (±SD) starting and ending body weights (g) for each group.

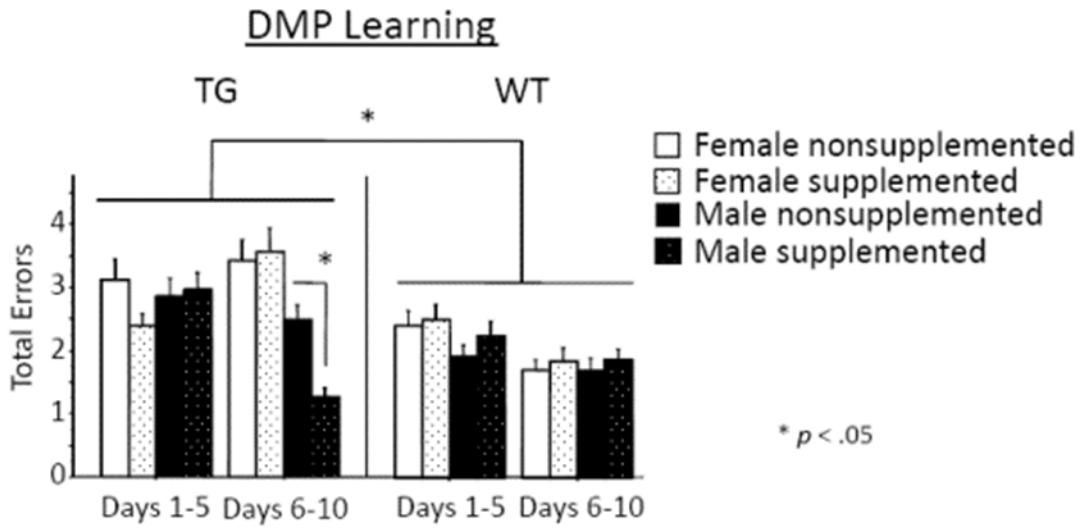
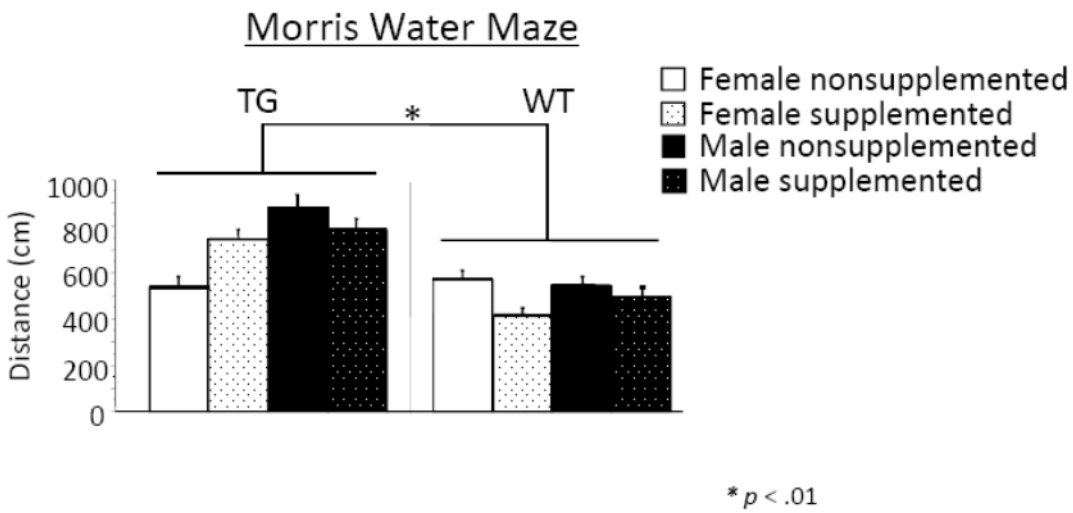

DMP Learning
TG
WT
*
*
Total Errors
0
1
2
3
4
Days 1-5
Days 6-10
Days 1-5
Days 6-10
Female nonsupplemented
Female supplemented
Male nonsupplemented
Male supplemented
* p < .05

Figure 40. Mean error scores for total errors (±SE) on the delayed match to position for regular testing days 1-10, separated into learning (days 1-5) and asymptotic (days 6-10) phases.


Morris Water Maze
TG
*
WT
Female nonsupplemented
Female supplemented
Male nonsupplemented
Male supplemented
Distance (cm)
1000
800
600
400
200
0

* $p < .01$

Figure 41. Mean distance scores in centimeters (±SE) on Morris maze days 1-6.

APPENDIX A

SECURED PERMISSION TO INCLUDE PUBLISHED RESEARCH

I have secured permission from all authors to include published research in the current dissertation.

APPENDIX B

MINT JULEP

Ingredients

10 mint leaves, stems removed

1 tablespoon of powdered sugar

1 1/2 ounce clean fresh spring water

3 ounces premium Bourbon

Crushed ice

1 sprig of mint for garnish

Directions

Put the leaves in a 12 ounce cocktail glass and pour the sugar on top. Muddle them together gently with a muddler or the handle of a wooden spoon. When the leaves and wet sugar begin to turn to a little mushy, add the water and the bourbon, and stir. Top with crushed ice and garnish with the sprig of mint.