Progestogens Impact Cognition During the Transition to Menopause in the Rat:

Dissociation of Progestogen- and Memory- Type

by

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ABSTRACT

Progestogens, such as progesterone (P4), medroxyprogesterone acetate (MPA), and micronized progesterone (mP4), are given to ovary-intact women during the transition to menopause to attenuate heavy uterine bleeding and other symptoms. Both progesterone and MPA administration have been shown to impair cognition in ovariectomized (Ovx) rats compared to vehicle-treated controls. mP4, however, has yet to be investigated for cognitive effects in a preclinical setting. Further, progestogens affect the GABA (γ-aminobutyric acid) ergic system, specifically glutamic acid decarboxylase (GAD) the rate limiting enzyme necessary for synthesizing GABA. The goal of this experiment was to investigate the cognitive impact of P4, MPA, and mP4, in an ovary-intact transitional menopause model using 4-vinylcyclohexene diepoxide (VCD) and assess whether these potential changes were related to the GABAergic system. One group of rats received vehicle injections, and the remainder of the groups received VCD to induce follicular depletion, modeling transitional menopause in women. Vehicle or hormone administration began during perimenopause to model the time period when women often take progestogens alone. Rats then underwent testing to assess spatial working and reference memory in the water radial-arm maze (WRAM) and spatial reference memory in the Morris water maze (MWM). Results indicate that P4 and MPA improved learning for working memory measure, but only MPA impaired memory retention in the WRAM. For the WRAM reference memory measure, VCD only treated rats showed impaired learning and memory retention compared to vehicle controls; progestogens did not impact this impairment. Although GAD expression did not differ between treatment groups, in general, there was a relationship between GAD expression

and WRAM performance such that rats that tended to have higher GAD levels also tended to make more WRAM working memory errors. Thus, while P4 and MPA have been previously shown to impair cognition in an Ovx model, giving these hormones early in an ovary-intact perimenopause model elicits divergent effects, such that these progestogens can improve cognition. Additionally, these findings suggest that the cognitive changes seen herein are related to the interaction between progestogens and the GABAergic system. Further investigation into progestogens is warranted to fully understand their impact on cognition given the importance of utilizing progestogens in the clinic.

DEDICATION

I dedicate this research to my wonderful parents Gale and Louis, who have always appreciated and nurtured my love of science. Their support has been unyielding and for that I am forever grateful. To my brother Elliot, for always being able to make me laugh and for providing me with long-lasting friendship. To my aunts and uncle for loving me from afar and for everything that makes them so wonderfully weird. To my mentor Dr. Heather Bimonte-Nelson for giving me the opportunity to be a part of a such a wonderful laboratory and for providing the most amazing mentorship I could have ever hoped for. To Dr. Conrad and Dr. Gipson-Reichardt for providing novel insight into this thesis. To my colleagues in the laboratory for always lending a helping hand and their positive attitudes. Finally, to all my colleagues in the Behavioral Neuroscience department whose collaborative natures and easy friendships I truly treasure.

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INTRODUCTION

Menopause occurs at the average age of 52, and is clinically defined as one year of missed periods (NAMS, 2015). The transition to menopause involves the dysregulation of ovarian hormones as ovarian follicle reserves become deplete. This process is not an abrupt phenomenon; indeed, the transition to menopause can last as long as 10 years (Burger, Hale, Dennerstein, & Robertson, 2008; Harlow & Paramsothy, 2011). During menopause, women can undergo a variety of physical changes that result in unwanted symptoms, including hot flashes, vaginal dryness, sleep disturbances, as well as cognitive changes (NAMS, 2017). In order to combat these undesired effects, hormone therapy containing an estrogen component can be prescribed, which have been utilized for decades to relieve the unwelcome effects of menopause. Unopposed estrogen administration for a woman that has an intact uterus, however, is not advisable as estrogens can increase the risk for endometrial hyperplasia and cancer (NAMS, 2012). In order to attenuate this risk, progestogens, known as both the endogenous progesterone and its synthetic analogs, known as progestins, are prescribed along with estrogens for a combination hormone therapy.

One avenue of research pertinent to understanding women's health during aging concerns hormone therapy and the transition to menopause. During this perimenopausal time period, progestogens can be administered to combat heavy uterine bleeding as well as other symptoms. Endogenously derived progesterone is not often prescribed clinically via the oral route as it has a low oral bioavailability (Kincl, Ciaccio, & Benagiano, 1978). Synthetic progestins that are more bioavailable than natural progesterone have been

produced for oral administration. We aimed to investigate the effects of different progestogens on cognition in a preclinical transitional model of menopause in the rat.

Type of Progestogen Administered for Neuroprotection and Cognition

Progesterone, the endogenous circulating hormone found in women, has been methodically assessed in preclinical models for effects on the brain and cognition. Progesterone has long been known to be neuroprotective in counteracting damage due to ischemic stroke and traumatic brain injury in animal models (Shear, Galani, Hoffman, & Stein, 2002; Wali, Ishrat, Won, Stein, & Sayeed, 2014). Indeed, progesterone and its metabolites, such as allopregnanolone, have various neuroprotective effects. For example, allopregnanolone and 5α-dihydroprogesterone, another metabolite of progesterone, reduced neuronal loss induced via kainic acid excitotoxicity in hippocampal rat neurons (Ciriza, Azcoitia, & Garcia-Segura, 2004). In adult male rats with injury to the prefrontal cortex, vehicle-treated controls demonstrated cell loss at the injury site that was attenuated by progesterone or allopregnanolone treatments (Djebaili et al., 2004). Further, progesterone- or allopregnanolone- treated rats had improved spatial reference memory as assessed on the MWM compared to the vehicle-treated rats with injury at the prefrontal cortex. Progesterone- or allopregnanlone- treated rats did not, however, differ in spatial reference memory from the non-injured vehicle-treated controls, indicating that the neuroprotective effects of progesterone and allopregnanolone on spatial reference memory were limited to injured rats (Djebaili et al., 2004).

Although there is evidence suggesting that progesterone can be neuroprotective, there is also preclinical research suggesting that progesterone administration can have mixed effects on cognition in healthy animals. Variances in outcomes have been shown

to stem from differences in study design such as dose of progesterone, duration of administration, route of administration, cognitive assays assessed, and preclinical model utilized. Some research has suggested that progesterone administration can be beneficial for cognition. For example, in the ovariectomy model (Ovx), where the ovaries have been surgically removed, treatment with a single bolus of a medium (10 mg/kg) or a high dose (20 mg/kg) of progesterone in young Ovx mice improved performance on an object recognition task, a non-spatial task, compared to vehicle controls (Harburger, Pechenino, Saadi, & Frick, 2008). Similarly, in middle-aged and aged Ovx mice, acute progesterone treatment improved memory consolidation in a dose dependent manner during an object recognition task (Lewis, Orr, & Frick, 2008). Additionally, an acute high dose of progesterone (20 mg/kg) improved spatial reference memory on the Morris Water Maze (MWM) task for the aged Ovx mice but not for the middle-aged Ovx mice, indicating that age at treatment plays a role in the potential beneficial cognitive effects of progesterone (Lewis et al., 2008). In contrast, our laboratory has demonstrated that chronic progesterone administration (0.7 mg/day) given to middle-aged Ovx rats impairs spatial working memory (Braden et al., 2015). Further, chronic progesterone (200 mg sustained release over 60 days via pellet) given in combination with 17β-estradiol (the most potent, endogenous, circulating estrogen in mammals) attenuated the beneficial effects of 17β-estradiol alone on spatial reference memory as assessed by the MWM task (H. A. Bimonte-Nelson, Francis, Umphlet, & Granholm, 2006). Of note, the differences in cognitive functioning between these studies could be due to other differences in study design such as the fact that the acute progestogen treatments were given to mice instead of rats. Further, acute treatments of progesterone were administered at a higher dose than

those in the chronic conditions which could have differentially influenced performance on cognitive tasks. These data indicate that a myriad of factors could influence the impact of progestogen treatment on cognitive outcomes.

Synthetic progestins are separated into two distinct classes based on chemical structure, those that resemble progesterone, and those that resemble testosterone and as such they can have differential methods of action in the brain and peripheral tissues (Stanczyk, 2003). The progesterone-like progestins can be further categorized into pregnane derivatives and 19 norpregnane derivatives. One such progestin of interest is medroxyprogesterone acetate (MPA) an acetylated pregnane derivative (Stanczyk, 2003). Currently, perimenopausal women are often administered MPA to alleviate undesired physiological symptoms, such as heavy uterine bleeding; MPA is also available in the clinic as a common form of birth control (brand name DepoProvera) (Depo Provera Prescribing Information, 2006). Although progestogens can have beneficial effects on the uterus to reduce heavy bleeding, their effects on cognition can be varied and should be carefully considered during clinical administration. The Women's Health Initiative Memory Study (WHIMS) called into question the effectiveness of hormone therapy for cognition and for effects on dementia risk in menopausal women 65 years old and over. Data from the WHIMS indicate that postmenopausal women administered conjugated equine estrogens (CEE) with MPA as a combination hormone therapy had an increased risk of dementia that was clinically meaningful as well as a doubled risk of dementia compared to controls (Rapp et al., 2003; Shumaker et al., 2003). MPA alone has been shown by our and other laboratories to have negative effects on cognition in the rat (Braden et al., 2017; Okojie & Oyekunle, 2014). Specifically, MPA has been shown to

have impairing effects on spatial memory in Ovx rats compared to sham controls (Braden et al., 2017, 2011, 2010). It is possible that this cognitive impairment of MPA is non-reversible as rats tested four months after the cessation of MPA treatment (which occurred for four months) still demonstrated cognitive impairment compared to age matched vehicle controls (Braden, Garcia, Mennenga, Lazlo, et al., 2011). Furthermore, *in vitro* MPA treatment has not demonstrated neuroprotection against glutamate-induced excitotoxicity, and when combined with 17β-estradiol, MPA attenuated the neuroprotective effects of 17β-estradiol alone (Nilsen & Brinton, 2002). These findings demonstrate the potential for MPA to impair cognition, as well as brain functioning, in both clinical and preclinical populations.

Women can also be administered micronized progesterone (brand name Prometrium) to combat physiological symptoms associated with perimenopause, specifically endometrial hyperplasia and secondary amenorrhea (Prometrium Prescribing Information). Secondary amenorrhea is the cessation of menstruation for three or more months without concurrent pregnancy, birth control regimen, or menopause transition. Micronized progesterone is the more orally active form of progesterone and is synthesized from yams (De Lignières, 1999; Maxson & Hargrove, 1985). Micronization reduces particle size, increasing the relative surface area and solubility of the particle thereby making it more orally bioavailable (Chakmakjian & Zachariah, 1987; Hargrove, Maxson, & Wentz, 1989; Maxson & Hargrove, 1985; Williams et al., 2013). Micronized progesterone is identical in chemical structure to progesterone, yet smaller in particle size and presumably acts upon the same neurobiological pathways as progesterone. Micronized progesterone has multiple clinical applications; it is prescribed to prevent

preterm labor and subsequent birth (Choudhary, Suneja, Vaid, Guleria, & Faridi, 2014; Glover et al., 2011; Rai et al., 2009) and has recently been investigated for its potential alleviation of symptoms associated with polycystic ovarian syndrome and cocaine abuse although results have been varied (Livadas et al., 2010; Reed, Evans, Bedi, Rubin, & Foltin, 2011). Previous research investigating the effect of MPA and micronized progesterone in combination with conjugated equine estrogens (CEE) in non-hysterectomized, postmenopausal women found that women given micronized progesterone and CEE showed improved quality of life perception over a nine-month period since the beginning of treatment (Ryan & Rosner, 2001). Recently, an ancillary study of the Kronos Early Estrogen Prevention Study (KEEPS), the Cognitive and Affective Study (KEEPS-Cog), investigated the effects of micronized progesterone in combination with CEE or 17ß-estradiol, in ovary-intact women who underwent the natural transition to menopause (Gleason et al., 2015; Miller et al., 2009). No cognitive effects were found, however, with any of the hormone regimens tested (Gleason et al., 2015). This is contradicted by another study which found better performance in working memory as well as improved delayed verbal memory in menopausal women administered CEE with micronized progesterone when compared to their scores before taking this hormone combination therapy (Sherwin & Grigorova, 2011). To date, there has been no research conducted on the effects of micronized progesterone on cognition in a rodent model. It stands to reason that micronized progesterone, with its higher oral bioavailability, might exacerbate effects on cognition and should be investigated.

Transitional Model of Menopause

Preclinical menopause-related progestogen investigations have been limited thus far to models of surgical menopause via Ovx. Ovx models surgical menopause in animals by abruptly removing the primary source for ovarian hormones, resulting in a drastic decrease in circulating ovarian hormone levels. However, the majority of women in the U.S. do not generally undergo surgical menopause. Indeed, roughly 600,000 women a year undergo hysterectomy, the surgical removal of the uterus, half of which occur with concurrent oophorectomy, the surgical removal of the ovaries in women (Whiteman et al., 2008). Instead of surgical menopause, most women undergo a form of naturally occurring menopause during which the finite follicular ovarian pool depletes via accelerated atresia which results in a transition to menopause rather than an abrupt loss of ovaries (NAMS, 2015). The Ovx model provides a blank circulating ovarian-derived hormone profile which makes it an ideal model to assess gonadal hormone deprivation and exogenous hormone administration in rodents. The natural transition to menopause, however, is much more complicated and also requires systematic evaluation.

A newer rodent model of menopause has been created that can be utilized to address the cognitive effects of hormones in a translationally relevant transitional form of menopause. By administering 4-vinylcyclohexene diepoxide (VCD), a metabolite of 4-vinylcyclohexene, researchers can induce ovarian follicular depletion of primary and primordial follicles via accelerated atresia that more closely models a woman's natural menopause transition (Hoyer, Devine, Xiaoming, Thompson, & Sipes, 2001; Kao, Sipes, & Hoyer, 1999; Springer, Flaws, Sipes, & Hoyer, 1996). The VCD model not only more accurately reflects the transitional form of menopause compared to the Ovx model, by

inducing follicular depletion, but also produces a circulating ovarian hormone profile which is more similar to a woman undergoing transitional menopause compared to the Ovx model. Specifically, the intact VCD-treated ovaries produce less progesterone while androgen levels, most predominantly androstenedione, decrease at a much slower rate, creating an androgen-rich hormone milieu (Acosta et al., 2010; Koebele et al., 2017; Mayer, Devine, Dyer, & Hoyer, 2004). These physiological differences between the Ovx and the VCD model, perhaps, lead to distinct behavioral differences between the two models when hormone therapy is additionally administered. For instance, one study found that compared to vehicle in middle-aged controls, CEE administration in middle-aged Ovx rats enhanced cognition while CEE given to middle-aged rats with VCD-induced follicular depletion impaired cognition compared to vehicle controls. These results indicate that transitionally menopausal VCD-treated rats yielding a high androgen, low estrogen, and low progesterone environment respond differently to CEE than Ovx rats with a hormonal environment that is void of endogenous, circulating ovarian hormones (Acosta et al., 2010). Moreover, a negative correlation was found between androstenedione levels in the VCD-treated rats and memory performance on a spatial working and reference memory task (Acosta et al., 2010). It is possible that the androstenedione-dominant milieu in the VCD model could lead to cognitive impairment and thus, the two models could have opposing effects of CEE. Further research suggests that it is the conversion of androstenedione to estrone that underlies the impairment in cognition with transitional menopause (Mennenga, Koebele, et al., 2015). Specifically, our laboratory found that when the activity of the aromatase enzyme, which synthesizes androstenedione to estrone, was blocked, there was a reversal of spatial working and

reference memory impairments induced by androstenedione in young Ovx rats (Mennenga, Koebele, et al., 2015). There was no reversal of these androstenedione-induced memory deficits when the androgen receptor was blocked, indicating that the androstenedione-induced impairments were due its conversion to estrone rather than its activity at the androgen receptor (Mennenga, Koebele, et al., 2015). Another difference between the VCD model compared to the Ovx model is that the VCD model allows for targeted timing of the menopause transition. Ovx is an all or nothing abrupt phenomenon, while follicular depletion with VCD occurs over time. As such, with the VCD model, hormones can be administered during the transition to menopause and scientists can now target perimenopausal time points as avenues of research which had been difficult to investigate prior to this model as the Ovx model lacks a transition stage. There are caveats, however, to the VCD model. VCD is a caustic chemical used in the creation of epoxy and rubber tires (Chhabra, 1989) and recent research has revealed that VCD might lead to oxidative stress and heightened inflammatory factors in young-adult female rats (Abolaji et al., 2016). Assessing exogenous progestogen administration in the VCD model of follicular depletion will further our understanding of hormone interactions given during the perimenopausal transition.

Progestogens and the GABAergic System

Progesterone and MPA have long been implicated in affecting the GABA (γ-aminobutyric acid) ergic system. GABA is the most potent endogenous inhibitory neurotransmitter found in the central nervous system (CNS) (Sieghart & Sperk, 2002). The primary function of the GABAergic system is to inhibit neuronal activity. As such, effects of GABA are primarily anxiolytic and anesthetic in nature. GABA most

commonly acts through the $GABA_A$ receptor, and several metabolites of progesterone have a high affinity for the $GABA_A$ receptor and are considered positive allosteric modulators (Paul & Purdy, 1992). Our laboratory has previously reported that progesterone and MPA treatment can impact the GABAergic system by influencing glutamic acid decarboxylase (GAD), the rate limiting enzyme necessary for synthesizing GABA. Specifically, progesterone and MPA treatment significantly increased GAD 65+67 levels in the entorhinal cortex, and decreased GAD 65+67 levels in the dorsal hippocampus, in aged Ovx female rats, although this effect was tempered for progesterone with a marginal effect for this hormone (Braden et al., 2010). Further, higher levels of serum MPA correlated with decreased expression of GAD 65+67 in the dorsal hippocampus in rats treated with MPA throughout their adult lifespan (Braden, Garcia, Mennenga, Prokai, et al., 2011). MPA has also been shown to increase inhibitory tone in the dentate gyrus (Belelli & Herd, 2003). Additionally, administering bicuculline, a $GABA_A$ receptor antagonist to progesterone-treated middle-aged Ovx rats, attenuated the memory impairments seen in progesterone only-treated rats indicating a role for the GABAergic system in modulating the effects of progesterone on cognition (Braden et al., 2015). As all of these studies have been conducted in the Ovx model, it is necessary to evaluate whether these findings are similar in an ovary-intact model, such as with the VCD model of follicular depletion, to assess if variants in menopause type can affect cognitive outcomes with progestogen treatment. Indeed, utilizing the Ovx model does not allow for evaluation of hormones given during the transition to menopause, as the Ovx model yields an abrupt cessation of endogenous circulating ovarian hormones. Understanding the role of progestogens on the GABAergic system when given during the

transition to menopause will better our understanding of one potential mechanism of the change in cognitive performance associated with progestogen based hormone therapy.

Research Aims and Hypotheses

This study aimed to investigate the effects of three different progestogens, MPA, micronized progesterone, and progesterone, administered alone (without estrogen) in an ovary-intact transitional model of menopause, on spatial working and reference memory as well as the GABAergic system in the rat. Progesterone was added as a treatment group to directly compare the effects of micronization on the two bioidentical hormones. We hypothesized that MPA and progesterone would impair cognition, as has been shown in numerous Ovx rodent studies (Braden et al., 2010, 2011, 2015, 2017). We also hypothesized that micronized progesterone would have impairing effects on cognition, as it the micronized form of the naturally occurring progesterone (De Lignières, 1999; Maxson & Hargrove, 1985). Neither MPA nor micronized progesterone have been investigated in a transitional rodent model of menopause.

MATERIALS AND METHODS

Subjects

49 six month-old, female, virgin, Fischer-344-CDF rats were obtained from the National Institute on Aging (NIA). Animals were pair-housed and fed *ad libitum* for two weeks before hormone administration began which continued until the end of the study. Animals were placed on a 12-hour on/off light-dark cycle, and were treated in compliance with the Arizona State University IACUC protocol.

VCD Administration

Rats were administered either VCD (160mg/kg/day, in 47% dimethyl sulfoxide (DMSO)/saline vehicle, 6% VCD, n=39) (SenesTech Inc., Flagstaff, AZ) or vehicle solution (47% DMSO/saline, n=10) (Sigma-Aldritch, St. Louis, MO) via intraperitoneal (i.p.) injection in accordance with past research and published protocols (Acosta et al., 2009, 2010; Flaws, Doerr, Sipes, & Hoyer, 1994; Koebele et al., 2017; Springer et al., 1996). VCD injections began two weeks after arrival and occurred four times a week (every Monday, Tuesday, Thursday, Friday) for six weeks. One animal died during VCD injections due to a medical condition not related to VCD treatment. The first day of VCD injections was considered day 1 of the study; the rest of the timeline will be referred to as days from first injection of VCD.

Progestogen Administration

Daily subcutaneous injections began on day 52 (from the start of VCD injections). Day 52 was chosen as the beginning point for progestogen injections as previous research had indicated that at 52 days from the beginning of VCD injections, progesterone levels were similar to vehicle controls for young or middle-aged rats administered VCD

(Koebele et al., 2017). Rats received either progesterone (P4) (0.7mg/rat/day in 0.3 ml sesame oil, n=10), micronized progesterone (mP4) (0.7mg/rat/day in 0.3 ml sesame oil, n=9), medroxyprogesterone acetate (MPA) (0.7mg/rat/day in 0.3 ml sesame oil, n=10), or vehicle (0.3 ml sesame oil, n=20). Rats were separated by treatment groups such that all progestogens were administered to rats who had received VCD injections (VCD-P4, VCD-mP4, VCD-MPA). Two groups were given sesame oil vehicle injections (VCD-Vehicle, Vehicle-Vehicle) and were used as controls for this study. The doses of MPA and P4 used herein have been shown by our laboratory to impair spatial working memory (Braden et al., 2015, 2017). The dose for mP4 was chosen to match that of the P4 dose so direct comparisons could be made. Daily injections continued until sacrifice at day 132. See Fig. 1 for study timeline.

Vaginal Smears

Vaginal smears occurred for 10 consecutive days two weeks before behavioral testing began. Smears were determined to be metestrus, diestrus, proestrus or estrus as indicated in a previous protocol (Goldman, Murr, & I, 2007).

Water Radial Arm Maze (WRAM)

Rats began behavioral testing 111 days after receiving the first injection of VCD, and 59 days after the start of vehicle or hormone injections. The WRAM was used to assess spatial memory, specifically working and reference memory paradigms. The maze consisted of eight arms radiating outwardly from a circular center. Each arm was 38.1cm x 12.7cm. Robust visual cues were placed on the walls adjacent to the maze such that they could be seen by the rats. The maze contained water that was kept at 18-20°C and non-toxic black paint was used to create an opaque surface that the animals could not see

below. Four of the eight arms contained hidden platforms (10 cm diameter) 2 cm below the surface of the water. These platforms were kept in consistent locations at the beginning of each testing day for each rat but were semi-randomized between rats. All location patterns used were balanced across the maze such that no quadrant of the maze was underrepresented.

Rats were tested for 12 consecutive days, with four trials each day. The start arm remained constant for all rats and trials. At the start of the first trial, rats were taken out of their cage and placed in the start arm. Rats were allowed to swim freely in the maze until they found a platform or until 3 minutes had elapsed after which rats were led to the closest platform. After rats found a platform they were allowed to stay there for 15 seconds and then they were removed and placed in their warm testing cage for an inter-trial interval (ITI) of 30 seconds. During this ITI, the just-found platform was removed and the maze was cleaned to remove debris and distribute olfactory cues. After the ITI, rats were again placed in the start arm and allowed to follow the same protocol as the first trial. This continued until the end of the fourth trial after which animals were returned to their testing cage for the rest of the day. Performance was assessed via three separate error types: working memory correct (WMC) errors, reference memory (RM) errors, and working memory incorrect (WMI) errors. WMC errors were defined as an entry into an arm which previously contained a platform for that day. RM errors were defined as the first entry into an arm which never contained a platform. WMI errors were defined as all subsequent entries, other than the first, into an arm that never contained a platform. As the trials increased, working memory load increased since rats had to keep more items in

their working memory. On the last day of testing a six-hour delay was implemented between trial 2 and trial 3 to assess delayed memory retention in these rats.

Morris Water Maze (MWM)

The MWM is a water escape task that is used to assess spatial reference memory. The maze was a circular pool that was 188 cm in diameter. The water was kept at 18-20°C and non-toxic black paint was used to create an opaque surface. A platform (10 cm diameter) was hidden 2 cm below the surface of the water in the north-east quadrant of the maze. Robust visual cues were situated on adjacent walls to aid spatial navigation. A camera was mounted above the maze and the computer program Ethovision 10 (Noldus Instruments) was used to capture video and track the rat's path through the maze. Distance to platform (cm) from the drop-off point was recorded and used to assess spatial localization of the platform over a time period of five days.

Rats were tested for five consecutive days, with four trials each day. At the start of the first trial, animals were dropped off at one of four drop off points (North, South, East or West) that were semi-randomly assigned to each trial throughout each of the days. Once in the maze, rats were allowed to swim freely until they found the platform or until one minute had elapsed after which rats were then led to the platform. Rats were allowed to stay on the platform for 15 seconds, after which time the rat was returned to their testing cage for a 5-8 minute ITI. On Day 5, the last day of testing, after all test trials, a probe trial was introduced to assess whether the animals had spatially localized the position of the platform. Before the probe trial began the platform was removed and rats were allowed to swim freely in the maze for one minute after which they were returned to their testing cages.

Visible Platform

The visible platform test was used to assess motor and visual acuity for solving a water escape task. The maze was a rectangular pool (39 X 23 cm) filled with clear water kept at 18-20°C. The maze was encircled by a curtain so that there were no salient visual cues other than those found inside the maze. There was a platform situated inside the maze (10 cm diameter) protruding two cm above the water.

Rats were tested for one day with six trials. At the start of trial 1, animals were placed in the starting position that remained constant throughout trials. The rats were allowed to swim freely until they found the platform. After the rats found the platform they were allowed to stay on it for 15 seconds after which the tester returned rats to their testing cage for an ITI of 5-8 minutes. All rats were tested for trial 1 before moving on to trial 2. Platform locations were assigned semi-randomly across trials. Latency to the platform was measured.

Sacrifice

Rats were sacrificed over two days, at the end of behavioral testing. Final body weights (g) were obtained earlier in the day directly before daily injections on the first day of sacrifices. Rats were anesthetized via isoflurane, and cardiocentesis was performed for blood collection. Rats were then decapitated and brains removed for dissection. Seventeen separate brain regions were raw brain dissected (left and right cingulate gyrus, left and right frontal cortex, basal forebrain, left and right striatum, left and right dorsal hippocampus, left and right perirhinal cortex, left and right entorhinal cortex, left and right temporal association area, and left and right CA1/CA2 region of the

hippocampus) directly after removal. Tissue was weighed immediately afterwards, frozen and stored at -70°C until further analysis.

Uterine horn and ovarian weights, and intestinal removal

Uterine horns and ovaries were dissected out of the body cavity and trimmed of excess fat. Ovaries were cut from the tips of the uterine horns and separately weighed as left and right wet weights (g). Ovaries were then placed in 10% formalin for two days, and then switched to 70% ethanol until future analysis. Uterine horn wet weights (g) were also collected. Intestines were also removed, trimmed of fat, and flash frozen and stored at -70°C for further analysis.

Western Blot

GAD 65 and GAD 67 expression levels were assessed from the right hemisphere in the frontal cortex, dorsal hippocampus, basal forebrain, ventral hippocampus, perirhinal cortex and entorhinal cortex via Western Blot analysis. Samples were homogenized via probe sonicator (Ultrasonic Processor, Cole Parmer, IL, USA) in a 1:50 RIPA buffer (1% Triton X-100, 150mM NaCl, 0.5% Na Deoxycholate, 0.1% SDS, 50mM Tris, phosphatase inhibitor [Cat#: 524625, Millipore-Sigma], and protease inhibitor [Cat#: 5892791001, Millipore-Sigma]). Samples were then centrifuged at 10,000 rpm for 10 minutes at 4°C. A BCA (bicinchoninic acid) protein assay (ThermoFisher Scientific) was utilized to measure protein concentration. Samples were run on a 4-12% Bis-Tris NuPAGE gel using a SureLock Mini-Cell system (Invitrogen, Carlsbad, CA, USA) and transferred to a polyvinylidene difluoride membrane (Immobulin-P). Samples were loaded at 10μg of protein per brain region. Treatment groups were counterbalanced across gels and four gels were run per brain region. After

transfer the membrane was blocked in 5% milk for one hour after which it was incubated overnight in primary antibodies anti-GAD 65 (1:5000, Abcam) and anti-GAD 67 (1:10,000, Abcam) as well as primary antibody anti-beta actin (1:20,000, Cell Signaling) as a control measure. The membrane was then incubated in secondary antibodies anti-mouse horseradish peroxidase (1:2000, Cell Signaling) and anti-rabbit horseradish peroxidase (1:2000, Cell Signaling) for one hour. Chemoluminescence (LumiGlo and Peroxide, Cell Signaling) was used to visualize protein expression using a film developer. ImageJ software was used to perform densitometry. GAD 65+67 levels were normalized to Vehicle-Vehicle samples for analysis.

Statistical Analyses

WRAM data were analyzed across days 2-12 using an omnibus repeated measures analysis of variance (ANOVA) for each of the memory types (WMC, WMI, and RM errors). The repeated measures were Trials nested within Days while the independent variable was Treatment. Data were further separated into three distinct blocks for Days 2-5 (the learning phase), Days 6-9, and Days 10-12 (the asymptotic phase) and each block was individually assessed via an omnibus repeated measures ANOVA for each of the error types. Effects of treatment on Day 12 alone were also assessed as previous research in our laboratory has shown impairments after MPA administration in Ovx rats on the last day of WRAM testing (Braden et al. 2017, 2010). Additionally, we compared performance amongst groups for the highest (Trial 4) working memory load as our laboratory has previously shown that effects of several hormone-altering treatments become apparent when working memory load is highly taxed (Bimonte & Denenberg, 1999; Braden et al., 2010; Koebele et al., 2017). For the delay, for each treatment group

separately, baseline trials from day 12, trials 3 and 4, were averaged and compared to the averaged postdelay trials from day 13, trials 3 and 4, as has been previously reported (Camp et al., 2012; Engler-Chiurazzi et al., 2011; Mennenga, Gerson, et al., 2015; Mennenga, Koebele, et al., 2015; Prakapenka et al., 2018).

MWM total swim distance data were analyzed using an omnibus repeated measures ANOVA for all days of testing. The repeated measures were Trials nested with Days, and the independent variable was Treatment. Additionally, the probe trial was analyzed by assessing percent distance swim distance in the target quadrant (NE quadrant) where the platform had been previously located and in the opposite quadrant (SE quadrant) in order to determine if the rats had correctly spatially localized the platform location.

Visible platform data were assessed using a repeated measures ANOVA where time to platform was the dependent variable. The repeated measure was Trials and the independent variable was Treatment.

Uterine horn data were assessed using an omnibus ANOVA where wet weight (g) adjusted to final body weight (g) was the dependent variable and Treatment was the independent variable.

One way ANOVAs were utilized to assess GAD 65+67 protein levels in the frontal cortex, dorsal hippocampus, basal forebrain, ventral hippocampus, perirhinal cortex, and entorhinal cortex. GAD 65+67 levels were normalized to the Vehicle-Vehicle treatment data. The independent variable was Treatment. To further assess the relationship between GAD expression and cognition, Pearson r correlations were

analyzed for GAD 65+67 in each brain region for WMC and WMI errors for all blocks of WRAM data.

RESULTS

WRAM

The omnibus ANOVA for Days 2-12, including all trials, revealed a main effect of Day for WMC, WMI, RM, and Total errors [WMC, $F(10, 880) = 6.283$, $p < 0.001$; WMI, $F(10, 1320) = 24.652$, $p < 0.001$; RM, $F(10, 1320) = 12.430$, $p < 0.001$; Total, $F(10, 1290) = 15.238$, $p < 0.001$], indicating that all animals learned the task across Days 2-12. There was also a main effect of Trial for Days 2-12 for all days for WMC, WMI, RM, and Total errors [WMC, $F(2, 880) = 610.278$, $p < 0.001$; WMI $F(3, 1320) = 154.272$, $p < 0.001$; RM $F(3, 1320) = 25.594$, $p < 0.001$; Total, $F(3, 1290) = 249.703$, $p < 0.001$], indicating that as trials progressed errors increased and performance deteriorated.

For Days 2-5, there was a main effect of Treatment for WMI errors [$F(4, 396) = 2.740$, $p < .05$] as well as RM errors [$F(4, 396) = 3.393$, $p = < 0.05$] (See Fig. 2 & 3). Fischer post-hoc analyses revealed that for WMI errors, VCD only-treated rats made more errors than Vehicle-treated rats [$p < 0.05$], and that MPA- and P4- treated rats made fewer errors than VCD only-treated rats [VCD-Vehicle *vs.* VCD-MPA, $p < 0.001$, VCD-Vehicle *vs.* VCD-P4, $p < 0.05$], indicating that MPA and P4 treatment attenuated the impairment in working memory seen with VCD treatment alone. For RM errors, Fischer post-hoc analyses revealed that mP4-, P4-, and VCD only- treated rats each made more errors than Vehicle-treated rats [Vehicle-Vehicle *vs.* VCD-mP4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-P4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-Vehicle, $p < 0.01$], indicating

an impairment with VCD-induced transitional menopause, an effect not impacted by the progestogen treatments. There were no effects of Day, Trial, or Treatment for Days 6-9.

For Days 10-12 there was a similar main effect of treatment for RM errors [$F(4, 396) = 2.889$, $p < 0.05$] (See Fig. 4). Fischer post-hoc analyses revealed that all progestogen groups as well as VCD only-treated rats made more errors, indicating worse performance, than Vehicle-treated rats [Vehicle-Vehicle *vs.* VCD-mP4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-MPA, $p < 0.01$, Vehicle-Vehicle *vs.* VCD-P4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-Vehicle, $p < 0.01$]; these results demonstrate an impairment of VCD-induced transitional menopause compared to Vehicle-treatment alone that was not altered by any of the progestogen treatments.

For Day 12 only, for all trials, there was a Trial x Treatment interaction for WMI errors [$F(12, 132) = 8.714$, $p < 0.05$] (See Fig. 5). Analysis of trial 4 alone, the highest working memory load trial, revealed a main effect of Treatment [$F(4, 44) = 2.638$, $p < 0.05$]. Fischer post-hoc analyses revealed MPA-treated rats made more errors than all other treatment groups on trial 4 during the last day of testing [Vehicle-Vehicle *vs.* VCD-MPA, $p < 0.01$, VCD-Vehicle *vs.* VCD-MPA, $p < 0.05$, VCD-P4 *vs.* VCD-MPA, $p < 0.05$, VCD-mP4 *vs.* VCD-MPA, $p < 0.05$], indicating an impairment induced by MPA administration relative to all other progestogen treatments.

For Day 13, after the six-hour delay occurred, there were no main effects of Day for any error type between postdelay trials and baseline trials (data not shown) suggesting that all groups were able to maintain optimal performance after a six-hour delay period.

MWM

There was a main effect of day for all days of testing such that swim distance decreased across days [$F(4, 44) = 75.750$, $p < 0.0001$] (See Fig. 6). There was no main effect of Treatment nor any significant Treatment x Day interaction indicating that there were no differences between treatment groups for learning across days. For the probe trial, there was a main effect of Quadrant such that there was a higher percent swim distance in the target quadrant (NE quadrant), which had previously contained the platform, than the opposite quadrant (SW quadrant), indicating correct spatial localization of the platform [$F(1, 44) = 361.118$, $p < 0.0001$] (See Fig. 6). There were no Treatment x Quadrant interactions, indicating that treatment groups did not differ in their spatial localization of the platform.

Visible Platform

The visible platform test was used to assess the animals' ability to perform the procedural components of a water escape task. There was a main effect of Trial [$F(5, 44) = 8.479$, $p < 0.0001$] such that as trials progressed latency to the platform decreased, indicating better performance as trials progressed with an average latency of 7.55 seconds to reach the platform across all trials (See Fig. 7). There was no main effect of Treatment nor a significant Trial x Treatment interaction, indicating that the groups did not differ in their ability to perform the procedural components of a water-escape task.

Uterine Horn Weights

For uterine horn weights there was a main effect of Treatment when adjusted for final body weight [$F(4, 44) = 7.811$, $p < 0.001$] (See Fig. 8). Fischer post-hoc analyses revealed that the VCD only-treated rats had uterine horns that weighed significantly less

than Vehicle-treated rats [$p < 0.05$]. Additionally, all three progestogens groups had uterine horns that weighed significantly less than Vehicle-treated rats [VCD-MPA *vs.* Vehicle-Vehicle, $p < 0.01$, VCD-P4 *vs.* Vehicle-Vehicle, $p < 0.001$, VCD-mP4 *vs.* Vehicle-Vehicle, $p < 0.01$]. P4-treated rats had uterine horns that weighed significantly less than VCD only-treated rats, $p < 0.05$.

Western Blot Analysis

Western blots were performed to assess GAD 65 and 67 protein expression in the frontal cortex, dorsal hippocampus, basal forebrain, ventral hippocampus, perirhinal cortex and entorhinal cortex. There were no significant effects of treatment for GAD 65+67 for any region (data not shown). After placing a stringent alpha value of $p < 0.01$ to account for multiple correlations, a significant relationship was revealed for cognitive performance in the VCD only-treated group as well as for cognitive performance in the P4-treated group whereby higher levels of GAD 65+67 were associated with worse performance on the WRAM. Specifically, in the perirhinal cortex there was a significant correlation between GAD 65+67 expression and Days 2-5 WMI errors for the VCD-Vehicle group [$r(9) = 0.765$, $p < 0.01$, Fig. 9A] suggesting that the VCD only-treated rats that tended to have higher GAD 65+67 levels in the perirhinal cortex tended to make more WMI errors during the learning phase of WRAM testing. Similarly, in the frontal cortex there was a significant correlation between GAD 65+67 expression and Days 10-12 WMI errors for the VCD-Vehicle group [$r(9) = 0.773$, $p < 0.01$, Fig. 9B] suggesting that the VCD only-treated rats that tended to have higher GAD 65+67 levels in the frontal cortex tended to make more WMI errors during the asymptotic phase of WRAM testing. Additionally, in the ventral hippocampus there was a significant correlation between

GAD 65+67 expression and Days 2-5 WMC errors for the P4-treated group [$r(9) = 0.804$, $p < 0.01$, Fig. 9C] suggesting that the P4-treated rats that tended to have higher GAD 65+67 levels in the ventral hippocampus tended to make more WMC errors during the learning phase of WRAM testing. There were no significant correlations for cognitive performance and GAD 65+67 expression for any other treatment groups for any brain region assessed.

DISCUSSION

The current study is the first to investigate the effects of micronized progesterone, the micronized form of natural progesterone, and MPA, a progestin, on cognition in a transitional model of menopause. We found that, overall, VCD only-treated animals made more errors than Vehicle-treated animals, indicating that follicular depletion impaired spatial working and reference memory on the WRAM, replicating recent findings from our laboratory (Koebele et al., 2017). We demonstrated that MPA impaired spatial working memory relative to vehicle controls on the last day of WRAM testing, similar to our prior findings in the rat Ovx model (Braden et al., 2010, 2017). We found that VCD only-treated rats exhibited impaired spatial reference memory on the WRAM compared to Vehicle-treated rats in both the learning phase and the asymptotic phase, and that none of the progestogen treatments impacted this relationship. Interestingly, we found beneficial effects of MPA and progesterone on spatial working memory during the learning phase of the WRAM. These findings are divergent from previous research in Ovx models which demonstrate that MPA and progesterone can impair spatial working memory on the WRAM (Braden et al., 2010, 2011, 2015, 2017). We also demonstrated that there were no group differences in spatial reference memory on the MWM indicating that the effects of progestogen administration are spatial working memory specific.

Our noted progestogen-induced enhancement could be due, in part, to the fact that these hormones were assessed in a follicle deplete, ovary-intact, VCD rat model. With the VCD rat model, there is a marked difference in hormonal milieu as compared to Ovx, such that there is an androgen-rich hormone profile with VCD treatment, while with the Ovx model there is an abrupt cessation of all ovary-derived hormones, including

androgens. Higher androgen levels that are present in an ovary-intact model of rodent menopause could be interacting with these progestogens to beneficially influence acquisition of the WRAM. Additionally, timing of hormone administration could also play a role in these beneficial effects. Prior research evaluating hormone therapies in rat menopausal models has focused either on the pre-menopausal normally cycling rat, or post-menopausal Ovx rat. With the Ovx procedure, the surgical removal of the ovaries results in an abrupt cessation of hormones. As such, there is no transitional menopausal period during which hormones can be administered. In most VCD studies performed previously, hormone administration usually occurred after animals had become fully follicle deplete. To our knowledge, no other laboratory has studied the effects of hormone administration during the transition to menopause in a follicle depleting model. We have shown that by 52 days after the first injection of VCD, middle-aged rats still had detectable levels of progesterone that did not differ from vehicle treated rats (Koebele et al., 2017). The data collected from Koebele et al (2017) were only a snapshot of hormone levels and information about fluctuations in ovarian hormones at this time point were not obtained. Administration of progestogens on the background of this perimenopausal milieu in the rat could account for the differential impacts to cognition between the VCD and Ovx models.

These findings suggest that there are differential effects of progestogens on spatial working and reference memory when administered during the transition to menopause in the rat. Specifically, VCD-Vehicle animals showed impaired cognition compared to Vehicle-Vehicle animals. MPA attenuated the VCD-induced spatial working memory deficit during learning, but impaired compared to all other treatments at the lattermost

portion of testing during memory retention. Progesterone also attenuated the VCD-induced spatial working memory deficit during learning, but had neutral effects during memory retention. For the reference memory component of the WRAM, all progestogens as well as VCD-Vehicle animals performed worse than Vehicle-Vehicle animals indicating that all three progestogens were unable to alleviate the cognitive impairments imposed by VCD. These findings suggest that differential impacts of progestogens with transitional menopause are dependent upon the type of memory assessed. Spatial working and reference memory are inherently different processes. Spatial working memory is a form of short term memory that needs to be updated within a session while spatial reference memory is form of long term memory that remains constant over time (Bimonte-Nelson et al., 2015). As these two memory types are connected yet distinct in function, it is understandable that progestogens could have differential effects between memory types.

Progestogens and the GABAergic System

In general, there was a trend whereby rats that tended to have higher expression of GAD 65+67 also tended to make more working memory errors on the WRAM for the frontal cortex, perirhinal cortex, and ventral hippocampus. This is consistent with previous research indicating that increased activation in the GABAergic system could lead to memory impairments (Curran, 1986). Since we found similar trends in relationships between memory and several different brain regions, it is probable that the effects of progestogen treatment on the GABAergic system are global in nature. Additionally, given that progestogen treatment influenced spatial working memory rather than spatial reference memory, it is interesting to note that we found correlations between

GAD expression and performance on the WRAM specific to brain regions involved in working memory processes (e.g. frontal cortex and ventral hippocampus) (D'Esposito & Postle, 2015; Kim & Levin, 1996). Also of note, we did not observe group differences for performance on the MWM which assesses spatial reference memory, nor did we observe significant correlations between GAD expression and performance on the WRAM for the dorsal hippocampus, a region of the brain necessary for spatial reference memory (Moser, Moser, & Andersen, 1993). Taken together, these data indicate that the spatial working memory changes induced by progestogen treatment interact with the GABAergic system in a region-specific manner.

It is also possible that progesterone could be influencing the GABAergic system through the progesterone metabolite, allopregnanolone, which has been known to positively regulate the effects of GABA on $GABA_A$ receptors regardless of subunit type (Backstrom et al., 2014). Some research postulates that the cognitive and brain changes seen with progesterone treatment are led by its conversion to allopregnanolone via the synthesizing enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase. Like progesterone, allopregnanolone has been investigated for its effects on cognition in preclinical models. Some studies suggest that allopregnanolone treatment inhibits spatial reference memory on the MWM (Johansson, Birzniece, Lindblad, Olsson, & Bäckström, 2002; Türkmen, Löfgren, Birzniece, Bäckström, & Johansson, 2006). Additionally, partial tolerance to acute allopregnanolone treatment on the MWM was demonstrated and researchers posited that chronic exposure of allopregnanolone in women due to menstrual cycle, pregnancy, and hormone therapy after menopause might affect cognitive outcomes in women that could be mediated by the GABAergic system (Türkmen et al., 2006).

MPA has also been known to inhibit metabolites of progesterone via reduction of 3α-hydroxysteroid dehydrogenase, which is necessary for the synthesis of allopregnanolone, and to increase inhibitory tone of neurons in the dentate gyrus (Belelli & Herd, 2003). Investigation into the interactions between the interplay of these progestogens and the GABAergic system will be imperative to understand the mechanism by which these progestogens influence cognitive outcomes.

Further research should be conducted to replicate these findings, as this is the first study to investigate the effects of progestogen administration during the transition to menopause in a rodent model. Different time points of administration during the transition to menopause should also be evaluated to optimize potential beneficial effects of each progestogen. In order to begin to understand the potential mechanisms of action of progestogen treatment in the brain, additional analysis of brain regions important for spatial working memory, such as the frontal cortex, should be systematically assessed in the follicle-deplete VCD model given varied hormone therapy preparation. Furthermore, micronized progesterone is typically administered via the oral route (De Lignières, 1999; Maxson & Hargrove, 1985). As this study administered micronized progesterone via subcutaneous injection, future studies should assess oral administration of micronized progesterone and other progestogens to model the route of administration utilized by women in the clinic.

Concluding Remarks

Hormone therapy, in which progestogens play a vital role, has been utilized for decades to combat the unwelcome symptoms that occur during the transition to menopause. It is crucial to understand the effects that these hormones can have on

cognition and the brain to discover the optimal hormone therapy for both peripheral and cognitive effects. This study demonstrates that a common hormone therapy component, MPA, can have differential effects on spatial working memory depending upon stage of learning and memory. The current work also suggests that MPA and progesterone are beneficial to cognition in specific circumstances, while prior to this study most work identified MPA and progesterone as cognitively-impairing. Additionally, it is likely that these progestogens play a role in the relationship between GABA-mediated signaling and cognition. Further research should be conducted utilizing the VCD model to assess timing of administration of progestogens, and how progestogens could be interacting with the different hormonal profiles as menopause progresses, for impacts on cognition as well as the GABAergic system.

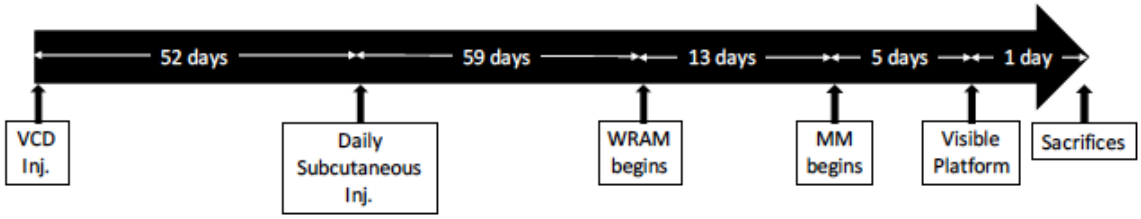

Figure 1. Study Timeline. 52 days after the first VCD injection, animals began receiving daily subcutaneous injections of progestogen or vehicle treatments. 111 days after the first injection of VCD animals began behavior testing which consisted of 13 days of WRAM, 5 days of MWM, 1 day of visible platform, and 2 days of sacrifices. In total the study lasted 132 days since the first injection of VCD.

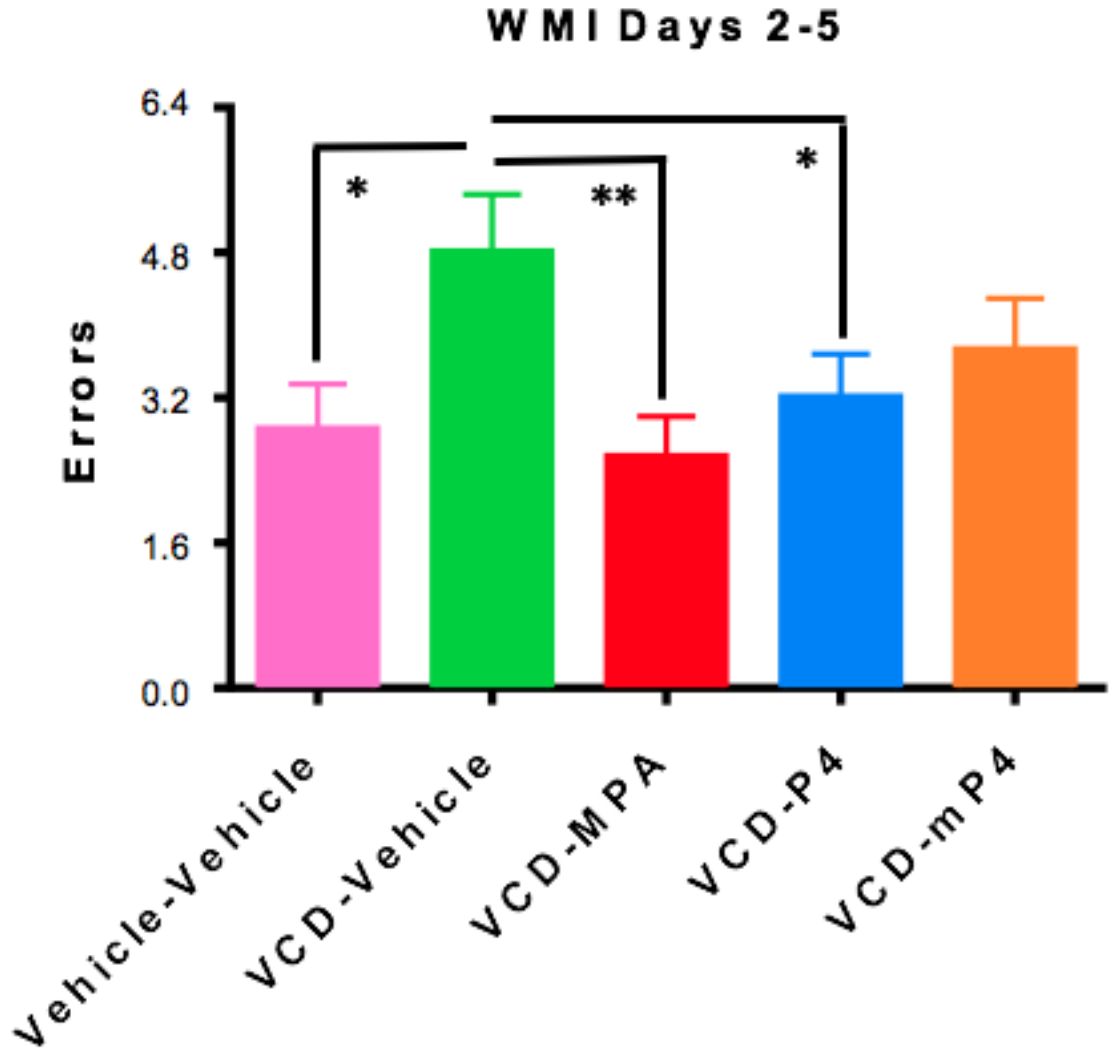

Figure 2. WRAM Days 2-5 WMI errors across treatment groups. Mean error scores (+SE) averaged across trials. Main effect of treatment [$F(4, 396) = 2.740$, $p < .05$]. The MPA- and P4-treated groups performed better than the VCD-Vehicle group [VCD-Vehicle *vs.* VCD-MPA, $p < .001$, VCD-Vehicle *vs.* VCD-P4, $p < .05$]. The VCD-Vehicle group performed worse than Vehicle-Vehicle group [$p < .05$].

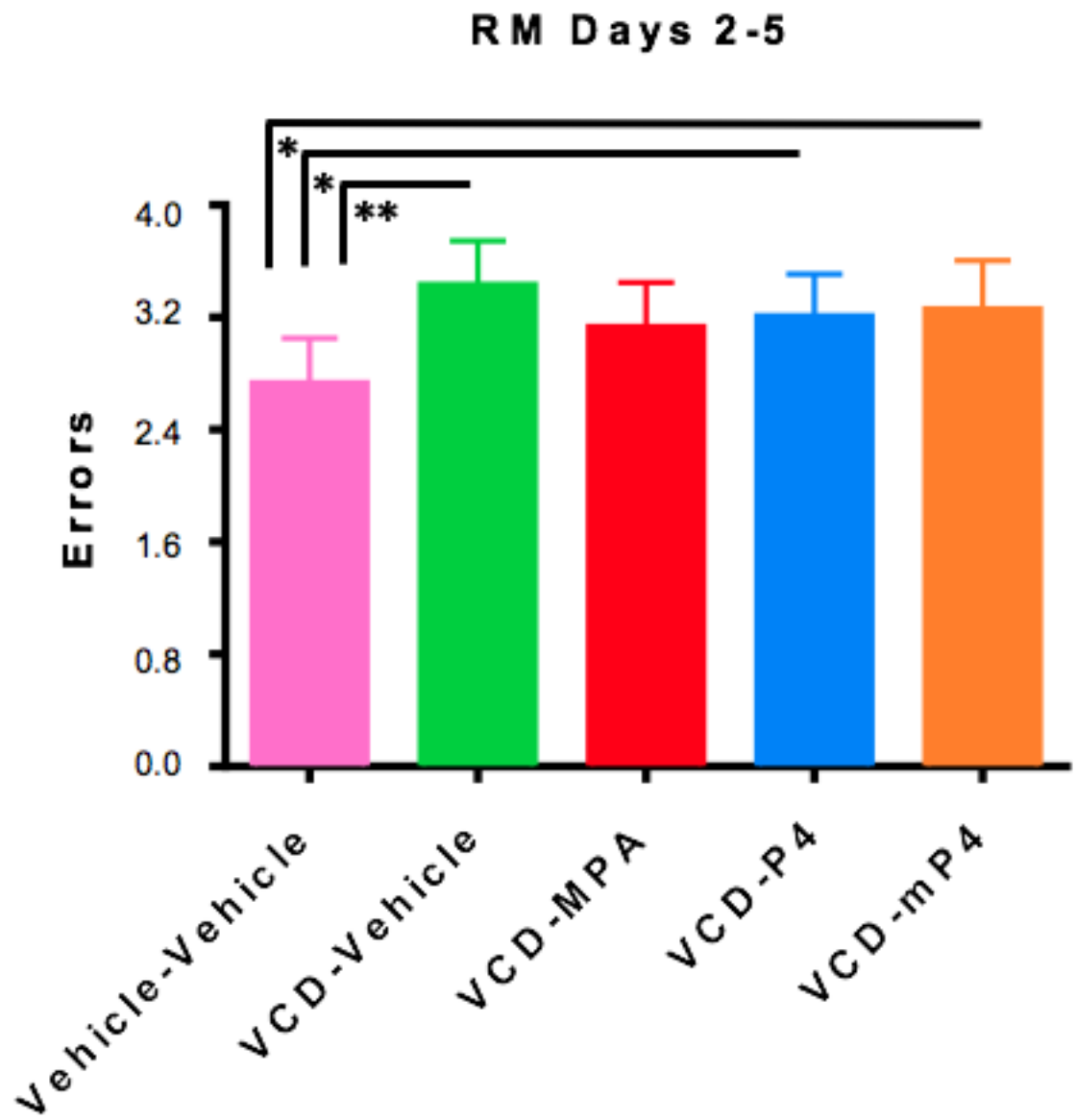

Figure 3. WRAM Days 2-5 RM errors across treatment groups. Mean error scores (+SE) averaged across trials. Main effect of treatment [$F(4, 396) = 3.393$, $p = < 0.05$]. All progestogen groups, except MPA, as well as the VCD-Vehicle group, performed worse than the Vehicle-Vehicle group [Vehicle-Vehicle *vs.* VCD-mP4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-P4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-Vehicle, $p < 0.01$].

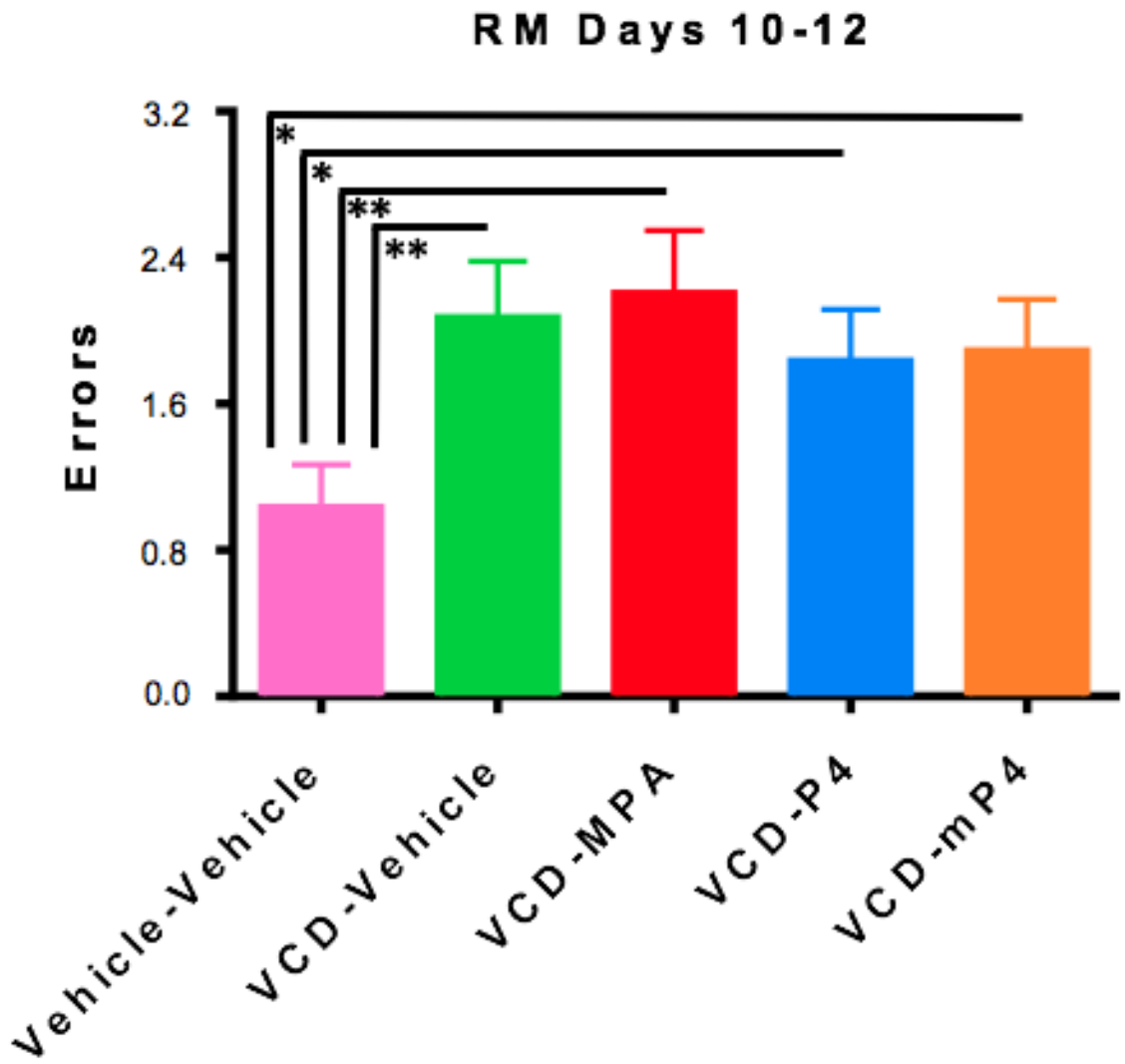

Figure 4. WRAM Days 10-12 RM errors across treatment groups. Mean error scores (+SE) averaged across. Main effect of treatment [$F(4, 396) = 2.889$, $p < 0.05$]. All progestogen groups as well as VCD-Vehicle performed worse than Vehicle-Vehicle [Vehicle-Vehicle *vs.* VCD-mP4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-MPA, $p < 0.01$, Vehicle-Vehicle *vs.* VCD-P4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-Vehicle, $p < 0.01$].

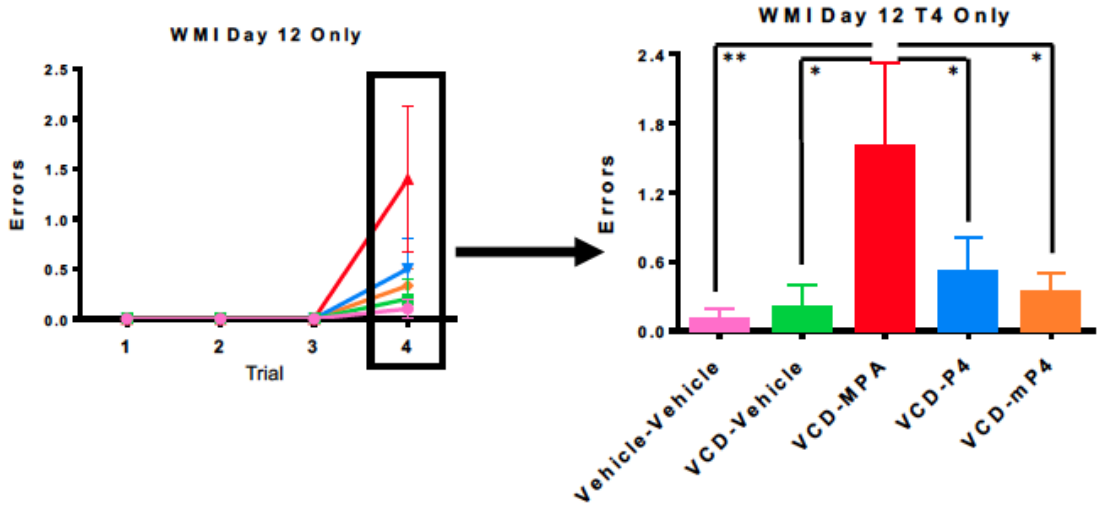

Figure 5. WRAM Day 12 only, WMI errors across treatment groups. Mean error scores (+SE) for all treatment groups. There was a significant Trial x Treatment interaction [$F(12, 132) = 8.714$, $p < 0.05$]. When trial 4 alone was assessed there was a main effect of treatment [$F(4, 44) = 2.638$, $p < 0.05$]. VCD-MPA treated rats performed worse than all other treatment groups on trial 4 [VCD-Vehicle *vs.* VCD-MPA, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-MPA, $p < 0.01$, VCD-P4 *vs.* VCD-MPA, $p < 0.05$, VCD-mP4 *vs.* VCD-MPA, $p < 0.05$].

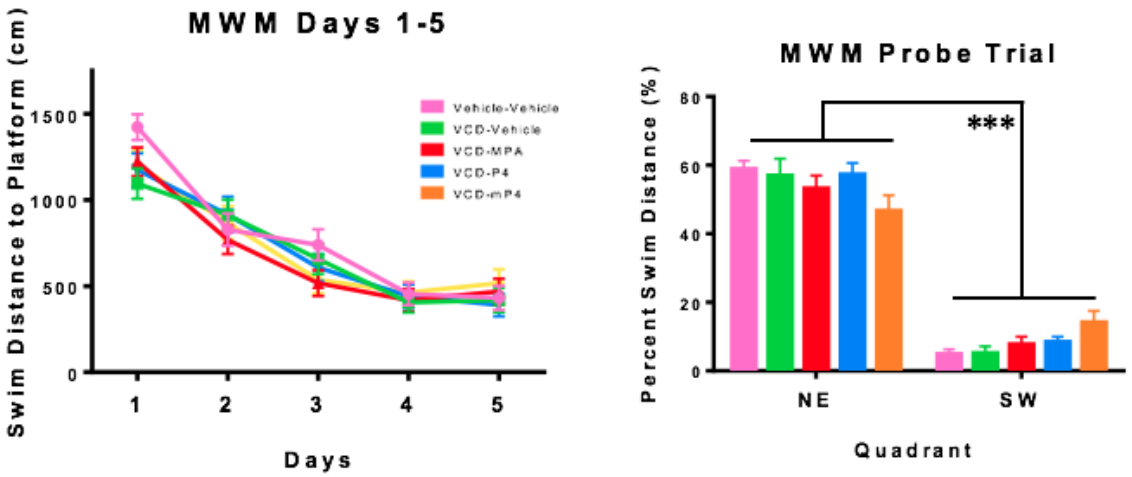

Figure 6. MWM. Mean error scores (+SE) for all treatment groups. There was a significant effect of Day [$F(4, 44) = 75.750$, $p < 0.0001$]. There were no differences between treatment groups. When the probe trial was assessed, there was a significant effect of quadrant [$F(1, 44) = 361.118$, $p < 0.0001$]. Rats had a higher percent swim distance in the target NE quadrant compared to the opposite SW quadrant [NE *vs.* SW, $p < 0.0001$].

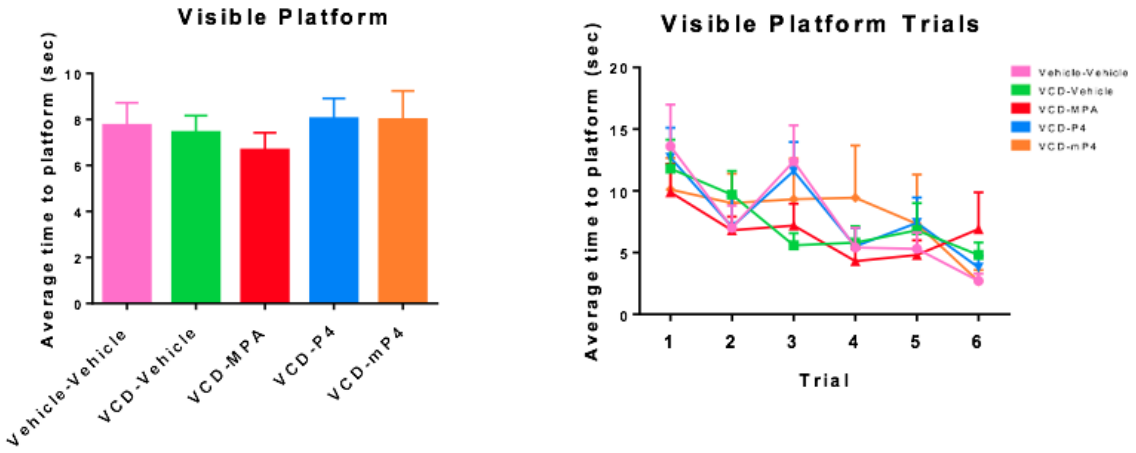

Figure 7. Visible Platform Task. Mean error scores (+SE) for all treatment groups. There was a significant effect of Trial [$F(5, 44) = 8.479$, $p < 0.0001$]. There were no differences between treatment groups.

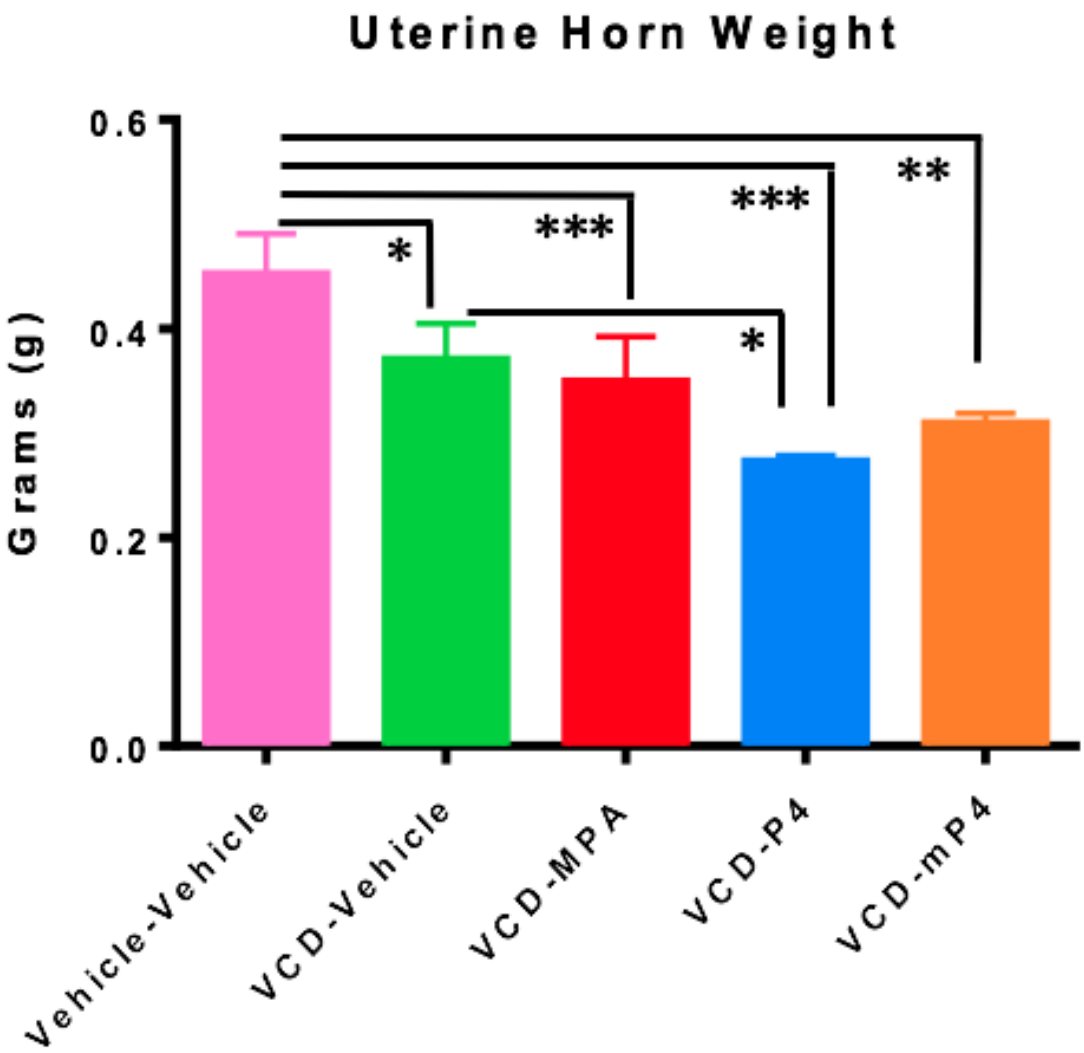

Figure 8. Uterine Horn (g) weight adjusted for final body weight. Main effect of treatment [$F(4, 44) = 7.811$, $p < 0.001$]. All three progestogens groups and the VCD-Vehicle group had uterine horns that weighed significantly less than Vehicle-Vehicle [VCD-Vehicle *vs.* Vehicle-Vehicle, $p < 0.05$, VCD-MPA *vs.* Vehicle-Vehicle, $p < 0.001$, VCD-P4 *vs.* Vehicle-Vehicle, $p < 0.001$, VCD-mP4 *vs.* Vehicle-Vehicle, $p < 0.01$]. VCD-P4 had uterine horns that weighed significantly less than VCD-Vehicle, $p < .05$.

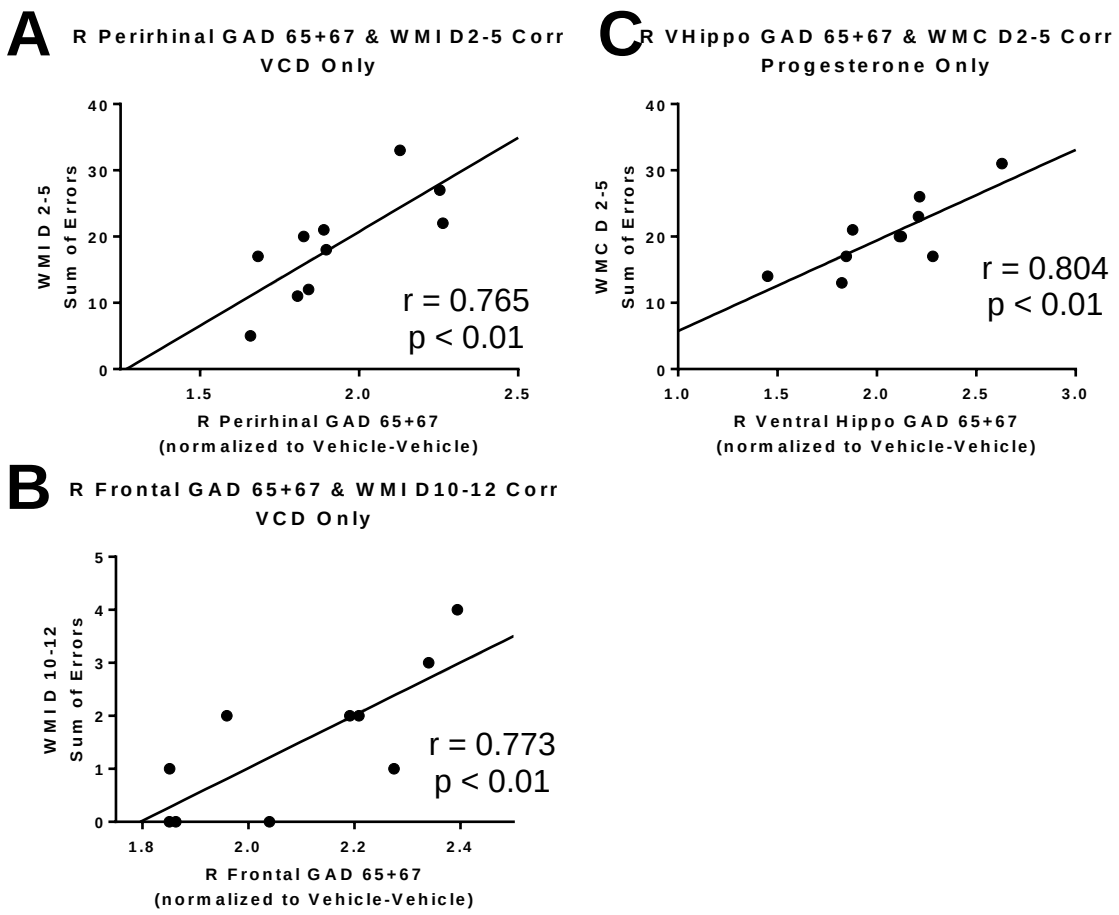

Figure 9. Pearson *r* correlations between WRAM performance and GAD levels for Days 2-5 and Days 10-12 of testing. (A) GAD 65+67 expression in the perirhinal cortex was positively correlated with Days 2-5 WMI errors for the VCD-Vehicle group. (B) GAD 65+67 expression in the frontal cortex was positively correlated with Days 10-12 WMI errors for the VCD-Vehicle group. (C) GAD 65+67 expression in the ventral hippocampus was positively correlated with Days 2-5 WMC errors for the VCD-P4 group.

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