Young Adult and Middle-Age Rats Display Unique Working Memory Impairment and Differential Neurobiological Profiles following Hysterectomy

by

Victoria Elaine Woner

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Heather A. Bimonte-Nelson, Chair
Cheryl D. Conrad
Benjamin C. Trumble

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ABSTRACT

Hysterectomy is the second most common gynecological surgery performed in women. Half of these surgeries involve removal of the uterus alone, and half involve concomitant removal of the ovaries. While the field has retained the notion that the nonpregnant uterus is dormant, more recent findings suggest that hysterectomy is associated with cognitive detriment. Of note, the clinical literature suggests that an earlier age at hysterectomy, with or without concomitant ovarian removal, increases dementia risk, implicating age at surgery as a variable of interest. While preclinical work in a rodent model of hysterectomy has demonstrated spatial working memory impairments, the role of age at surgery has yet to be addressed. The current experiment utilized a rodent model of hysterectomy to investigate the importance of age at surgery in post-surgical cognitive outcomes and to evaluate relative protein expression related to brain activity, FosB and ΔFosB, in regions critical to spatial learning processes. Young adult and middle-aged female rats underwent sham surgery, hysterectomy, or hysterectomy with ovariectomy, and were tested on a behavioral battery that evaluated spatial working and reference memory. Following the behavioral battery, animals were sacrificed and brain tissues from the Dorsal Hippocampus and Entorhinal Cortex were processed via Western Blot for relative FosB and ΔFosB expression. Behavioral analyses demonstrated that animals receiving hysterectomy, regardless of age or ovarian status, were generally impaired in learning a complex spatial working memory task. However, rats that received hysterectomy in middle-age uniquely demonstrated persistent working memory impairment, particularly with a high working memory demand. Subsequent

neurobiological analyses revealed young rats that underwent hysterectomy had reduced relative FosB expression in the Entorhinal Cortex compared to sham controls, where no significant effects were observed for rats that received surgery in middle-age. Finally, unique relationships between neurobiological and behavioral outcomes were observed largely for sham rats, suggesting that such surgical manipulations might modulate these relationships. Taken together, these findings suggest that age at surgery plays an important role in learning and memory outcomes following hysterectomy, and demonstrate the need for further research into the role of the uterus in communications between the reproductive tract and brain.

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Young Adult and Middle-Age Rats Display Unique Working Memory Impairment and Differential Neurobiological Profiles following Hysterectomy

Introduction

Hysterectomy, or removal of the uterus, is the second most common gynecological surgery, with the first being cesarean section (Carlson, Nichols, & Schiff, 1993). As such, up to one third of women in the United States experience this gynecological surgery by the age of 60 (Carlson et al., 1993; Whiteman et al., 2008). Hysterectomy can be performed at any point during a woman's lifetime for a variety of reasons, but is most commonly performed for benign conditions before natural menopause onset, which occurs, on average, at the age of 52 (Shifren et al., 2014). Hysterectomy may be performed alone, or with concurrent oophorectomy, which is the surgical removal of the ovaries in women. Because many women who undergo hysterectomy are of reproductive age and have not yet experienced menopause, the ovaries are conserved in approximately half of all hysterectomy cases to prevent an abrupt surgical menopause and its associated negative health outcomes (Bhattacharya & Jha, 2010; Wright et al., 2013). That is, ovarian conservation allows for women to maintain circulating ovarian hormone levels until the natural transition into menopause in midlife. However, if a woman is at high-risk for certain gynecological cancers or other complications, the ovaries are removed at the time of hysterectomy as a prophylactic measure and results in an abrupt loss of circulating ovarian hormones (Lowder et al., 2010).

Although the ovaries are generally thought to function normally following hysterectomy (Chalmers et al., 2002), there is also evidence to suggest that hysterectomy

surgery impacts ovarian outcomes during aging. Specifically, research has indicated that women receiving hysterectomy with ovarian conservation can experience disrupted ovarian function, potentially due to modulation of ovarian blood flow, leading to an earlier point of ovarian failure than women who do not receive such gynecological surgery (Chan, Ng, & Ho, 2005; Farquhar, Sadler, Harvey, & Stewart, 2005; Moorman et al., 2011). However, this can be difficult to evaluate, as the postmenopausal stage in women receiving hysterectomy surgery cannot be identified in the traditional clinical sense, which would be one year of amenorrhea (Shifren et al., 2014). Menopause in these women is primarily indicated by the presence of associated symptoms, which include hot flashes, sleep disturbances, and mood and cognitive changes (Koebele & Bimonte-Nelson, 2016; The Practice Committee of the American Society of Reproductive Medicine, 2004). Reliance on this definition of menopause is problematic, as these symptoms can vary greatly between women, as well as within a particular woman across the menopause transition; some symptoms extend many years into the postmenopausal life stage. Therefore, evaluation of menopause status for hysterectomized women can also involve the measurement of elevated serum follicle-stimulating hormone (FSH) levels in blood. In later reproductive years, FSH levels begin to increase, whereby these increases continue through the menopause transition and remain elevated in postmenopause (Burger, 2006; Burger et al., 1999; Harlow et al., 2012; Koebele & Bimonte-Nelson, 2016; Soules et al., 2001). Levels of serum FSH exceeding 40 IU/liter have been adopted as a marker of the late menopausal transition, particularly in the presence of other indications, such as vasomotor symptoms (Burger et al., 1999;

Randolph et al., 2006). While these trends are generally evident across the menopause transition for women, because FSH levels are not consistent for an individual woman or across women, serum FSH level as a stand-alone measurement is too unreliable to consistently identify the initiation of reproductive senescence (Su & Freeman, 2009). Most literature indicates that levels of ovarian hormones, such as 17β-estradiol or progesterone, largely remain stable long-term following hysterectomy (Beavis, Brown, & Smith, 1969; Chalmers et al., 2002; Kaiser, Kusche, & Würz, 1989; Souza, Fonseca, Izzo, Clauzet, & Salvatore, 1986), where some evaluations report at least short-term changes in serum estradiol and progesterone levels (Vuorento, Maenpaa, & Huhtaniemi, 1992; Xiangying, Lili, & Yifu, 2006). Collectively, such changes in gonadotropin and ovarian hormone levels following hysterectomy suggest that the uterus might play a more active role in regulation of the hypothalamic-pituitary-ovarian (HPO) axis.

These findings implicating the uterus in participating in a network of communication between the ovaries, pituitary, and brain are in contrast to the long-standing characterization of the uterus as a reproductive organ that is responsive to stimulation via the ovaries, but otherwise plays a minimal role in HPO axis feedback. Indeed, the uterus has been described as a "useless organ" outside of its role in maintaining a pregnancy (Navot & Williams, 1991). Given the aforementioned findings concerning ovarian changes following hysterectomy, along with evidence that the uterus contains both gonadotropin- and steroid hormone- receptors (Lessey et al., 1988; Reshef et al., 1990; Stilley et al., 2014; Toft & Gorski, 1966) and is innervated along the central and peripheral nervous systems (Brauer & Smith, 2015; Gnanamanickam & Llewellyn-

Smith, 2011), it seems that the role of the non-pregnant uterus in HPO axis functioning has largely been underestimated. Rather, the uterus is likely involved in this ovarian-brain feedback loop, receiving and sending inputs to neural and peripheral structures to maintain healthy functioning throughout both pregnant and nonpregnant states.

There is some clinical literature indicating that the uterus may indeed send and receive neural inputs important for maintaining cognitive health, and that hysterectomy may disrupt healthy cognitive functioning with aging. Hysterectomy, both alone and with concomitant oophorectomy, has been associated with an increased risk of dementia (Phung et al., 2010; Rocca et al., 2007). Interestingly, an earlier age at hysterectomy alone is related to an exacerbation of this increased risk of dementia and cognitive decline, paralleling findings associated with premenopausal oophorectomy (Rocca, Grossardt, Shuster, & Stewart, 2012). In contrast, one study found a small reduction in risk of Alzheimer's disease associated with hysterectomy (Imtiaz et al., 2014); however, the majority of the women who underwent hysterectomy were postmenopausal at the time of surgery (Imtiaz et al., 2014), implicating age at surgery as a key variable in cognitive outcomes. It is possible that these observed ovarian and brain outcomes following hysterectomy are directly related. If hysterectomy results in ovarian dysfunction and earlier initiation of the menopause transition, then this could contribute towards the cognitive impairments observed in young adult females in the decades following surgery, where the young adult brain has not yet undergone the reorganizational processes that occur across the menopause transition (Koebele & Bimonte-Nelson, 2015), and might be ill-poised to respond to such ovarian dysfunction.

Given these indications concerning the role of the uterus as a potential bi-directional participant in feedback between the brain and ovaries, the utilization of a preclinical model is warranted to systematically investigate these potential relationships. Preclinical rodent models of surgical menopause variants can provide valuable insight into the behavioral and neurobiological mechanisms behind such endocrine-related phenomena as observed in the clinical literature. Indeed, rodents have well-studied reproductive and neurological structures that are comparable in many ways to those of humans. While female rodents experience different patterns of gonadal hormone change with age compared to women, the overarching commonalities between rodent and human HPO tract communication make them an excellent model for preclinical assessments of menopause (Koebele & Bimonte-Nelson, 2016), particularly in the context of surgical manipulations (Acosta et al., 2009; Bimonte-Nelson et al., 2003; Koebele & Bimonte-Nelson, 2016). Whereas observational research in women cannot dissociate between the impact of female aging and the trajectory of reproductive senescence on cognitive outcomes, rodent models allow for the separation of such variables of interest to investigate their unique roles in subsequent cognitive outcomes (Koebele & Bimonte-Nelson, 2016). For example, the preclinical model of surgical removal of the ovaries in the rodent, known as Ovariectomy (Ovx) (Bimonte & Denenberg, 1999), has provided significant contributions to the knowledge of the cognitive effects of the abrupt cessation of circulating ovarian hormones (Bimonte-Nelson, Singleton, Williams, & Granholm, 2004; Bimonte & Denenberg, 1999; Camp et al., 2012). Ovx has been demonstrated to be detrimental to cognition when animals undergo surgery in adulthood (Bimonte &

Denenberg, 1999; Daniel, Roberts, & Dohanich, 1999), but is beneficial when surgery occurs when animals are aged and are evaluated at an extended timepoint post-surgery (Bimonte-Nelson et al., 2003). These preclinical findings correspond with the clinical literature, showing cognitive impairment following surgical menopause as compared to the natural menopause transition (Nappi et al., 1999) and an increased risk of dementia for those who undergo oophorectomy before menopause onset (Rocca et al., 2007; Rocca, Grossardt, & Shuster, 2011).

Until recently, there had been no systematic evaluations of reproductive senescence that included the presence or absence of the uterus as a variable of interest. The Bimonte-Nelson laboratory recently sought to fill this gap in the literature by developing a rodent model of hysterectomy to evaluate whether the surgical removal of the uterus impacted cognitive outcomes in a similar manner to other variations of gynecological surgery, including Ovx (Koebele et al., 2019). In this experiment, young adult female rats received hysterectomy, both alone and with concomitant Ovx, bilateral Ovx, or sham surgery, before undergoing a cognitive battery to assess spatial working and reference memory six weeks following surgery (Koebele et al., 2019). This study found a unique spatial working memory impairment with hysterectomy surgery as compared to the other variations in gynecological surgery, as well as compared to sham controls. Upon inspection of circulating ovarian hormone levels and ovarian cytology at sacrifice, there was some evidence of hormonal changes following hysterectomy, but such changes were not present for measures of ovarian follicle morphology (Koebele et al., 2019). The model has provided significant evidence concerning the role of the uterus

in cognitive functioning, with indications that this relationship is indeed not secondary to ovarian changes as a result of uterine removal (Koebele et al., 2019). While this work is integral to further understanding the relationship between the uterus and the brain, several questions remain concerning the cognitive outcomes associated with this form of gynecological surgery. Collectively, these clinical and preclinical observations concerning the cognitive outcomes following hysterectomy suggest that a methodical evaluation of the role of age at gynecological surgery is necessary to determine how hysterectomy affects cognitive outcomes and impacts potential feedback mechanisms between the brain, pituitary, and reproductive tract.

While there is preliminary evidence of learning and memory changes following hysterectomy in this rodent model, the neurobiological underpinnings behind such cognitive impairment remain elusive. In order to develop effective therapies to postpone or prevent this cognitive impairment in women following gynecological surgery, it is critical to understand these neural mechanisms. One potential mediator of such brain changes associated with learning is Delta (Δ) FosB. ΔFosB and its full-length complement, FosB, are transcription factors of the immediate early gene *fosB*. Generally speaking, these immediate early genes are activated following stimulation or activity, triggering the release of these transcription factors. FosB and ΔFosB can heterodimerize with transcription factor Jun from the immediate early gene *jun* to form activator protein - 1 (AP-1) complexes, which in turn may regulate gene expression (Carle et al., 2007; Ruffle, 2014). Whereas FosB is a characteristically unstable transcription factor with a half-life of approximately an hour (Carle et al., 2007; Zerial et al., 1989), ΔFosB is

unique in its relative stability with a half-life of days to weeks (Carle et al., 2007; Ruffle, 2014), making the latter a useful tool for assessing long-term change within the brain. ΔFosB has largely been studied in the addiction literature, where ΔFosB expression becomes upregulated in the nucleus accumbens (a brain region important for addiction learning) with repeated exposure to drugs of addiction, such as cocaine, and remains persistently elevated after drug cessation (Nestler, Barrot, & Self, 2001). Of note, ΔFosB has also recently been implicated in spatial learning, where the number of FosB/ΔFosB-immunoreactive cells was increased in the CA1 region of the hippocampus following learning of the Morris Water Maze (MWM) task for rats compared to naïve controls, as well as compared to rats that learned the non-spatial version of the MWM, where these cells were demonstrated to increase for all tested animals in the dentate gyrus (Eagle et al., 2015). Given the prolonged nature of ΔFosB, evaluation of the expression of FosB and ΔFosB in brain regions important for learning and memory following hysterectomy surgery might provide insight into the neurobiological mechanisms underlying these observed cognitive changes.

The current experiment sought to characterize the role of age at surgery for hysterectomy, both alone and in conjunction with ovary removal, in altering post-surgical learning and memory outcomes using a rodent model. Young and Middle-aged female rats underwent either Sham, Hysterectomy, or Hysterectomy with concomitant Ovx. Spatial learning and memory were evaluated via a behavioral battery six weeks post-surgery. At the end of the experiment, relative protein expression of FosB and ΔFosB in the Entorhinal Cortex and Dorsal Hippocampus were analyzed via Western Blot in these

animals to evaluate potential brain changes in regions associated with spatial learning and memory as a result of age and surgery. Finally, correlations were run between spatial working memory and relative expression of FosB and ΔFosB in these brain regions to characterize the relationships between neurobiological and cognitive outcomes across each treatment group, and how these factors might be altered across age and by surgery.

Methods

Subjects

Thirty-two 5 month-old (Young) and thirty 11 month-old (Middle-Age) virgin, reproductively-intact female Fischer-344 (CDF) rats were obtained from the National Institute on Aging colony at Charles Rivers Laboratories (Raleigh, NC). Rats were pair housed randomly upon arrival, and were provide food and water ab *libitum* while kept on a 12-hour light/12-hour dark cycle daily for the duration of the study. Rats were given one week to acclimate before surgeries and initiation of the experiment. All experimental procedures were performed with approval from the Arizona State University (ASU) Institutional Animal Care and Use Committee, and followed the standards of the National Institutes of Health.

Experimental Design

A detailed overview of the experimental timeline can be found in Figure 1.

Surgical procedure. Rats within each age group (5 months and 11 months) were randomly assigned to one of three surgical groups: Sham, Hysterectomy, or Hysterectomy with concurrent Ovx (Ovx+Hysterectomy); this resulted in a total of six surgical groups: Young-Sham, Young-Hysterectomy, Young-Ovx+Hysterectomy,

Middle-Age-Sham, Middle-Age-Hysterectomy, Middle-Age-Ovx+Hysterectomy. Surgeries were performed one week after the rats arrived at ASU, and were conducted using a protocol previously established within this laboratory (Koebele et al., 2019). For all surgical procedures, rats were anesthetized via inhaled isoflurane and received 1.0mg/kg meloxicam and 1.2mg/kg buprenorphine for pain management, along with 5.0ml of an isotonic solution to ensure postsurgical hydration. Each surgery involved a ventral midline incision through the skin and peritoneum; groups receiving sham surgery received only this surgical manipulation. Groups receiving hysterectomy surgery had each uterine horn ligated and cut below the ovary, preserving the oviduct, before being separated from attached abdominal fat. This fat was separated down the length of the uterine horn until the utero-cervical junction was reached, wherein it was ligated and cut above the cervix at the base of the uterine body. Rats receiving Ovx+Hysterectomy surgery had ovaries and uterus removed by separating the surrounding abdominal fat until the utero-cervical junction was reached, where it was ligated and cut at the base of the uterine body, preserving the cervix. For all surgical groups, muscle incisions were sutured using dissolvable Vicryl suture, with bupivacaine applied to the muscle surface for further pain management prior to skin closure. The skin incision was closed via surgical staples for all subjects. Rats recovered under a heat lamp and were single-housed for seven days following surgery, before being re-pair housed with their original cage-mate for the duration of the study. Two animals in the Young-Hysterectomy group died following surgery, resulting in a total of 10 rats per treatment group.

Body weights & vaginal cytology. Baseline weights were collected before surgery, and weights were recorded weekly throughout the duration of the study. Three weeks after surgery, vaginal smears were conducted daily for eight consecutive days to monitor estrous cyclicity following surgery, as well as to confirm successful Ovx surgery for the Ovx+Hysterectomy groups. Vaginal smears were classified according to established protocols (Goldman, Murr, & Cooper, 2007) as proestrus, estrus, metestrus, or diestrus. Proestrus was classified by the presence of clustered round epithelial cells with some cornified cells. Estrus was classified by the predominant presence of cornified cells. Metestrus was characterized by the presence of cornified cells, round cells, leukocytes, and some needle-like cells. Diestrus was characterized by the predominant presence of leukocytes, sometimes with the presence of cornified cells or round epithelial cells. Vaginal smears were conducted again on the day prior to sacrifice and on the day of sacrifice for all subjects to confirm estrous cyclicity in ovary-intact subjects at this timepoint, as well as to confirm Ovx status for Ovx+Hysterectomy rats for both ages.

Behavioral battery. Six weeks following surgery, all rats underwent behavioral testing to evaluate spatial working and reference memory performance.

Water radial-arm maze. All rats were first evaluated via the win-shift version of the water radial-arm maze (WRAM), which was used to examine spatial working and reference memory (Bimonte-Nelson, Daniel, & Koebele, 2015). The WRAM is an eight-arm apparatus that is situated in a testing room surrounded by salient visual cues to aid in spatial navigation. The maze is filled with room temperature water (18-20°C), where the surface of the water is made opaque with non-toxic black tempera paint. Four out of the

eight arms contain hidden platforms, which animals must locate to successfully complete the task. Each rat was randomly assigned to a set of platform locations, which were kept fixed for each individual subject across all days of testing.

At the start of each trial, the rat was released from the starting arm and given three minutes to successfully locate a hidden platform; if the rat did not find a platform within the allotted time, it was gently guided to the nearest platform by the experimenter. Once on the platforms, the rats was required to remain there for a total of 15 seconds before being removed by the experimenter and placed into a heated testing cage for a 30 second inter-trial-interval (ITI), wherein the found platform was removed and the maze was swept to distribute any odor cues. After completion of the ITI, each rat was once again dropped off at the starting arm to begin another trial; this continued for a total of four trials per day, such that all platforms were located for each individual animal. In this manner, working memory load increased across each trial for a given subject, as each rat needed to remember not to visit arms where platforms had previously been located for that day. Testing continued in this fashion for a total of 12 baseline days, where on Day 13, a six-hour delay was implemented between Trials 2 and 3 to evaluate delayed memory retention.

Performance on the WRAM was quantified by totaling the number of entries into non-platformed arms, here referred to as errors, for each trial. An arm entry was recorded as an error when the tip of the rat's snout passed 11cm from the opening of the arm, which was clearly marked on the outside of the maze arms. These errors were further split into orthogonal error types that represent spatial working and reference memory

performance, as has been done previously in this laboratory (Bimonte-Nelson, 2015c; Bimonte, Nelson, & Granholm, 2003; Braden et al., 2017; Koebele et al., 2019). These error types were defined by the following criteria: an entry into a previously platformed arm within a testing day was noted as a working memory correct (WMI) error; the first entry into a non-platformed arm for a given day of testing was noted as a reference memory (RM) error; and subsequent entries into non-platformed arms for a given testing day was noted as a working memory incorrect (WMI) error.

Morris water maze. One day following the completion of WRAM testing, all subjects were evaluated on the Morris water maze (MWM), which measures spatial reference memory performance (Bimonte-Nelson, 2015a; Bimonte-Nelson et al., 2015; Morris, Garrud, Rawlins, & O'Keefe, 1982). The MWM consists of a large circular tub, 188 cm in diameter, filled with cool water (18-20°C) which is made opaque with non-toxic black tempera paint. A platform (10 cm diameter) is placed in the northeast (NE) quadrant of the maze and sits hidden below the surface of the water, where it remains across all testing trials. Robust visual cues are placed around the room to aid in spatial navigation.

In this task, each rat was dropped off at a series of designated starting locations (North, West, South, East) that are semi-randomized across testing days. Each subject was given 60 seconds to locate the hidden platform for each trial; if the platform was not located in the allotted time, the animal was gently guided by the experimenter onto the platform. Once located, the rat remained on the platform for a total of 15 seconds before being removed and placed into its heated testing cage for an approximate 5-10 minute

ITI. Each rat performed four testing trials per day across 5 days; an additional 5th trial, referred to as the probe trial, was added at the end of the 5th day of MWM testing, where the platform was removed and each animal was observed swimming for 60 seconds to evaluate spatial localization to the platform. Performance on the MWM was quantified by calculating the distance traveled by each subject to reach the platform for all testing trials, as well as swim latency to the platform, using the Ethovision tracking system (Noldus Instruments, Wageningen, The Netherlands).

Visible platform task. Following the conclusion of MWM testing, each rat was evaluated on the visible platform (VP) task to assess ability to perform the procedural components of a water-escape task (Bimonte-Nelson, 2015b). The VP apparatus is a rectangular tub filled with clear cool water (18-20°C) that contains a black platform, sitting 4cm above the surface of the water. A curtain that surrounded the tub was used to attenuate the use of spatial cues while performing the task. Each rat was dropped off at the designated starting location, and was given 90 seconds to locate the platform. Once located, the animal remained there for 15 seconds before being removed and placed into its heated testing cage, with an inter-trial interval of approximately 5-10 minutes. Each animal completed a total of 6 trials, wherein the platform location was moved semi-randomly across trials between three distinct locations. Performance on this task was quantified by latency (in seconds) to locate the platform for each trial.

Sacrifices

Ten days following the completion of the behavioral battery, all animals were sacrificed at approximately 7 months of age (Young) or 13 months of age (Middle-Age).

Rats were deeply anesthetized with inhaled isoflurane before blood was collected via cardiocentesis to allow for blood serum analysis of various ovarian hormone levels. After decapitation, brains were rapidly removed, where the left hemisphere was post-fixed in 4% paraformaldehyde for 48 hours before being transferred into a 0.1M phosphate buffered solution for subsequent analysis. The right hemisphere was raw dissected to collect frontal cortex, cingulate cortex, dorsal hippocampus, entorhinal cortex, perirhinal cortex, temporal cortex, and ventral CA1/CA2 brain regions. These dissected tissues were individually weighed and flash frozen at -70°C for further analysis.

Ovaries were collected from the ovary-intact animals (Sham and Hysterectomy groups), trimmed of excess fat, individually weighed, and placed into vials filled with 10% formalin for 48 hours before being transferred into a 70% ethanol solution for further analysis. Uteri from the Sham subjects were collected, trimmed of excess fat, and the wet weight was collected.

Western Blot Protein Analysis

Dorsal hippocampus and entorhinal cortex, collected from the right hemisphere of each subject at sacrifice, were evaluated for FosB expression and ΔFosB expression via western blot protein analysis. Raw tissue samples that were flash frozen were suspended in a 1:25 weight-to-volume ratio of RIPA buffer solution (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris HCl), protease inhibitor (Millipore-Sigma, CAT#5892791001) and phosphatase inhibitor (Millipore-Sigma, CAT#524625), and thereafter kept on ice. Tissue samples were then homogenized via probe sonication (Ultrasonic Processor, Cole Parmer, IL, USA), before being centrifuged

at 10,000 rpm for 10 minutes at 4°C. The resulting supernatant was then collected, aliquoted, and frozen at -70°C for further analysis. Protein concentration for each sample was determined using a bicinchoninic acid protein assay (Thermo-Fisher Scientific, Pittsburgh, PA, USA), with samples run in duplicate; a consistent measure of protein concentration was obtained when the coefficient of variation was less then 10% across duplicates.

Treatment groups were counterbalanced and evenly distributed across each gel. Tissue processing was completed using the NuPAGE PowerEase electrophoresis system. Samples for a given region were loaded at an equal protein concentration and run with MES running buffer on a 4-12% NuPAGE Bis-Tris gel in an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA, USA). After protein separation, an Immobilon polyvinylidene difluoride membrane was utilized for protein transfer. The membrane was then blocked in 5% nonfat milk for 1 hour at room temperature before being washed with 1XTBST. The membrane was then incubated overnight in 5% milk with the primary antibody for anti-FosB (1:1000; Abcam, ab184938) at 5°C. The following day, the membrane was washed with 1XTBST before incubation in 5% milk with the secondary antibody anti-rabbit HRP (1:2000; Cell Signaling #7074) for 1 hour at room temperature. The membrane was then washed again before being developed using chemiluminescence (Lumiglo and peroxide solutions, Cell Signaling #7003S) with a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). Resulting films were scanned as JPEG files at 600dpi for subsequent densitometry analyses, which were completed using ImageJ software (Gallo-Oller, Ordoñez, & Dotor, 2018). FosB and ΔFosB expression were

normalized to beta-tubulin expression for each gel. A total of 10 gels were run across the two brain regions.

Statistical Analyses

All statistical analyses were completed using StatView software. For the behavioral and brain data, except those from the WRAM delay, a series of planned comparisons in the form of 2 x 2 analyses were designed between Surgery groups with Age included to address the following questions: How might removal of the uterus affect cognition differentially with age as compared to intact female rats? (Sham vs. Hysterectomy, Young and Middle-Age); How might removal of the uterus in conjunction with ovary removal impact cognition differentially with age as compared to intact females? (Sham vs. Ovx+Hysterectomy, Young and Middle-Age); How might removal of the uterus alone impact cognition differentially with age as compared to removal of both the uterus and ovaries? (Hysterectomy vs. Ovx+Hysterectomy, Young and Middle-Age). All analyses were two-tailed, wherein the alpha level was set to 0.05. Results were designated as marginal if the p value was between 0.05 and 0.10.

Behavioral analyses.

Water radial-arm maze. A repeated measures ANOVA was used to analyze data collected from the WRAM, with Age and Surgery as the independent variables, and Days and Trials as the repeated measures. The dependent variable for these analyses was Errors, whereby separate analyses were conducted for WMC, WMI, and RM errors. Data were first analyzed as an omnibus repeated measures ANOVA across all baseline days of testing (Days 2-12) to determine the general learning patterns for this task for each of the

error types (WMC, WMI, and RM errors). Based upon prior publications demonstrating behavioral differences across days of WRAM testing corresponding to task acquisition and maintenance (Bimonte-Nelson et al., 2015; Koebele et al., 2019; Mennenga et al., 2015; Prakapenka et al., 2018), the WRAM data were then analyzed for each planned comparison and each error type after being separated into three blocks: Days 2-4, the Early Acquisition Phase; Days 5-9, the Late Acquisition Phase; and Days 10-12, the Asymptotic Phase. Furthermore, given that prior research from this laboratory has shown hormone-mediated outcomes specifically when working memory load is highly taxed (Bimonte & Denenberg, 1999; Koebele et al., 2017, 2019; Prakapenka et al., 2018), planned analyses of Trial 3 alone and Trial 4 alone were completed for each error measure within each block of WRAM testing, following the same structure as the planned comparisons outlined above.

For data collected during the WRAM delay, each treatment group was evaluated separately for WMC and WMI errors using a repeated measures ANOVA, whereby Errors served as the dependent variable, and Trial 3 performance on the last baseline day of testing (Day 12) versus Trial 3 performance post-delay (Day 13) served as the repeated measures.

Morris water maze. One rat in the Middle-Age-Hysterectomy group was unable to complete MWM testing due to a benign skin condition unrelated to surgical assignment, and this animal was excluded from these analyses. MWM data were analyzed using a repeated measures ANOVA, with Age and Surgery as the independent variables and Trials within Days as the repeated measures, as there were four testing

trials per day over the course of five days. Swim distance to the platform (cm) served as the dependent variable for each planned comparison. An omnibus repeated measures ANOVA was first analyzed to determine the overall learning patterns for animals across days of testing; subsequent analyses of Surgery effects were performed using the planned comparisons outlined above. The probe trial was analyzed using an ANOVA, with the percent of swim distance in the Target (NE) vs. Opposite (SW) Quadrant served as the dependent measure; this probe analysis was performed separately for each treatment group.

Visible platform task. Data collected from the VP task were analyzed using a repeated measures ANOVA, with the same set of planned comparisons as used above, where Age and Surgery served as the independent variables, Trials as the repeated measure, and Latency to the platform as the dependent measure.

Body weight analysis. Body weight data were analyzed using ANOVA, using the same planned comparisons used for behavioral analyses, where Surgery and Age served as the independent variables and Body Weight at sacrifice served as the dependent variable.

Western blot protein analysis. Western blot analyses were completed using ANOVA, with Age and Surgery as the independent variables, and normalized FosB and ΔFosB expression as the dependent variable for each planned comparison. One sample from the entorhinal cortex produced data for FosB and loading control expression that were found to be outliers using the Grubbs test; data for this subject were therefore excluded from analyses in the entorhinal cortex.

Correlations between WRAM performance & normalized FosB/ΔFosB expression. Pearson r correlations were completed with Fischer's tests of significance between densitometry and WRAM performance to assess relationships between brain and behavioral outcomes for each age and surgical group. Specifically, relative normalized FosB and ΔFosB expression in the R Dorsal Hippocampus and R Entorhinal Cortex were correlated with WMI and WMC errors averaged across trials from each block of WRAM testing to determine the relationship between proteins that govern neurological changes associated with learning and working memory performance within each experimental group (i.e. intraclass correlations). As these correlations served the purpose of exploring potential associations between these outcome variables, corrections for multiple comparisons were not performed for these analyses.

Results

Water Radial-Arm Maze

The WRAM evaluated spatial working and reference memory performance. With the initial omnibus analysis, there was a main effect of Day for each memory measure, with errors decreasing across testing days (Days 2-12) for WMC ($F_{(10,540)} = 9.543$, $p < 0.0001$), WMI ($F_{(10,540)} = 22.268$, $p < 0.0001$), and RM errors ($F_{(10,540)} = 18.283$, $p < 0.0001$), demonstrating learning of the WRAM task (data not shown). For each memory measure, there was no significant Day x Age x Surgery interaction, demonstrating that groups did not differ in WRAM learning across all baseline testing days. All subsequent analyses of WRAM data during baseline testing involved the use of planned comparisons

and blocking of testing days to further characterize differences in acquisition and memory retention phases of WRAM testing.

Early acquisition phase.

For the Early Acquisition Phase (Days 2-4), planned comparisons for the Sham and Ovx+Hysterectomy groups revealed a significant Trial x Surgery interaction for WMC errors ($F_{(2,72)} = 3.286$, $p < 0.05$), where post hoc analyses revealed a main effect of Surgery for Trial 3 ($F_{(1,36)} = 4.535$, $p < 0.05$) such that rats that received Ovx+Hysterectomy surgery made more WMC errors than those in the Sham group, regardless of age at surgery (Figure 2A). Additionally, planned comparisons between the Hysterectomy and Ovx+Hysterectomy groups revealed that, for the highest working memory load trial, Trial 4, there was a marginal Age x Surgery interaction ($F_{(1,36)} = 3.763$, $p < 0.10$; Figure 2B). When each age was analyzed separately, for young animals, there was a main effect of Surgery ($F_{(1,18)} = 3.714$, $p < 0.10$), where the removal of the ovaries in addition to removal of the uterus was facilitative compared to removal of the uterus alone (Figure 2B). However, this effect of Surgery was not present for middle-aged animals. The planned comparison of Sham and Hysterectomy groups revealed no significant effects for WMC errors during the Early Acquisition Phase.

For WMI errors in the Early Acquisition Phase, planned comparisons between Sham and Ovx+Hysterectomy groups revealed a marginal effect of Surgery ($F_{(1,36)} = 3.660$, $p < 0.10$), whereby animals that received Ovx+Hysterectomy surgery tended to make more WMI errors than their sham counterparts, regardless of age. Furthermore, for the moderate working memory load trial alone, there was a main effect of Surgery for the

Sham vs. Ovx+Hysterectomy planned comparison ($F_{(1,36)} = 6.533$, $p < 0.05$); animals that received Ovx+Hysterectomy surgery made more WMI errors than animals that received Sham surgery, independent of age at surgery (Figure 3A). There was also a main effect of Surgery for the Sham vs. Hysterectomy comparison for WMI errors ($F_{(1,36)} = 4.697$, $p < 0.05$) for the moderate working memory load trial during the Early Acquisition Phase, where animals that received Hysterectomy surgery made more WMI errors than Sham counterparts, regardless of age at surgery (Figure 3A). Planned comparisons for Hysterectomy vs. Ovx+Hysterectomy revealed no significant effects for WMI errors during the Early Acquisition Phase, for all trials together as well as for the moderate and high load trials. Overall, this indicates working memory impairment for a moderate working memory load as a result of removal of the uterus, regardless of ovarian status or age at surgery (Figure 3B).

There were no statistically significant effects for RM errors for any of the planned comparisons during the Early Acquisition Phase.

Late acquisition phase.

For the Late Acquisition Phase (Days 5-9), there were no statistically significant effects across any of the planned comparisons for WMC errors.

Analyses of WMI errors in the Late Acquisition Phase for planned comparisons between Sham and Hysterectomy groups revealed a Trial x Age x Surgery interaction for WMI errors ($F_{(3,108)} = 3.971$, $p < 0.05$; Figure 4). Subsequent analyses revealed that at the highest working memory load trial, Trial 4, there was a marginal Age x Surgery interaction ($F_{(1,36)} = 3.231$, $p < 0.10$), such that, for young animals, there was a marginal

effect of Surgery ($F_{(1,18)} = 3.947$, $p < 0.10$), with Hysterectomized rats tending to make more WMI errors than their Sham counterparts; however, for middle-aged animals, there was no effect of Surgery (Figure 4). Likewise, planned comparisons between Hysterectomy and Ovx+Hysterectomy groups revealed a marginal Trial x Age x Surgery interaction ($F_{(3,108)} = 2.583$, $p < 0.10$); subsequent analyses of trial-specific effects of Surgery or Age x Surgery interactions were not statistically significant.

For the Late Acquisition Phase, there were no significant effects for RM errors across any of the planned comparisons.

Asymptotic phase.

During the Asymptotic Phase of WRAM testing (Days 10-12), planned comparisons between Sham and Hysterectomy groups revealed a marginal Age x Surgery interaction ($F_{(1,36)} = 4.103$, $p < 0.10$); follow-up analyses showed that when surgery occurred in middle-age, hysterectomized animals tended to make more WMC errors than their sham counterparts ($F_{(1,18)} = 3.425$, $p < 0.10$), an effect that was not present for young animals. Additionally, this planned comparison revealed a significant Trial x Surgery interaction ($F_{(2,72)} = 4.585$, $p < 0.05$; Figure 5A). Post hoc analyses revealed a marginal Age x Surgery interaction for Trial 3, the moderate working memory load trial ($F_{(1,36)} = 3.648$, $p < 0.10$), but subsequent analyses for middle-aged and young animals did not find significant effects of Surgery on this trial (Figure 5B). Conversely, at the highest working memory load, there was a significant effect of Surgery ($F_{(1,36)} = 4.233$, $p < 0.05$), where Hysterectomized animals made more errors than their Sham counterparts (Figure 5A); however, a marginal Age x Surgery interaction ($F_{(1,36)} = 3.429$, $p < 0.10$) led to further

probing of these relationships (Figure 5B). It was discovered that at the highest working memory load, for middle aged animals, there was a marginal effect of Surgery ($F_{(1,18)}$ = 4.367, $p < 0.10$), where animals that were Hysterectomized in middle-age tended to make more WMC errors at the highest working memory load than their Sham counterparts; for young animals, again, there was no effect of Surgery (Figure 5B). This indicates that, uniquely at the end of testing, animals receiving hysterectomy surgery in middle-age were impaired on a working memory task, with greatest impairments seen at the highest working memory load, and that no impairment is seen in animals receiving surgery when young.

For planned comparisons between Sham and Ovx+Hysterectomy animals during the Asymptotic Phase, there were no significant effects across all testing trials. However, on the moderate working memory load trial, a marginal effect of Surgery was revealed ($F_{(1,36)}$ = 3.344, $p < 0.10$), with animals receiving Ovx concurrent with Hysterectomy tending to make more WMC errors than those receiving Sham surgery; this pattern occurred regardless of age at surgery (Figure 5A).

Planned comparisons between Hysterectomy and Ovx+Hysterectomy groups during the Asymptotic Phase revealed a marginal Age x Surgery interaction ($F_{(1,36)}$ = 3.547, $p < 0.10$), as well as a Trial x Surgery interaction ($F_{(2,72)}$ = 6.213, $p < 0.01$), and a marginal Trial x Age x Surgery interaction ($F_{(2,72)}$ = 2.802, $p < 0.10$) (Figures 5A&B). For the moderate working memory load trial, analyses revealed an Age x Surgery interaction ($F_{(1,36)}$ = 4.934, $p < 0.05$). For young animals, those that received Ovx plus Hysterectomy surgery made more WMC errors on this trial than those that received

Hysterectomy surgery alone ($F_{(1,36)} = 4.688$, $p < 0.05$; Figure 5B). In contrast, no effect of Surgery was seen for animals that received Hysterectomy vs. Ovx+Hysterectomy surgery in middle-age for this trial. On the highest working memory load trial, there was a marginal effect of Surgery ($F_{(1,36)} = 4.073$, $p < 0.05$), where animals receiving Hysterectomy tended to make more WMC errors than those receiving Ovx+Hysterectomy surgery (Figure 5A). Upon inspection of patterns of WMC errors made across testing trials for each age (Figure 5B), a post hoc decision was made to evaluate Surgery effects within each age. Intriguingly, for animals receiving surgery in middle-age, there was an effect of Surgery ($F_{(1,36)} = 4.682$, $p < 0.05$), such that Hysterectomized animals in middle-age made more WMC errors than those receiving concurrent Ovx plus Hysterectomy concurrently, where there was no effect of age for this comparison for young animals (Figure 5B).

When analyzing WMI errors made during the Asymptotic Phase of WRAM testing, planned comparisons between Sham and Hysterectomy groups revealed a marginal effect of Surgery ($F_{(1,36)} = 4.071$, $p < 0.10$), with Hysterectomized animals tending to make more WMI errors than Sham animals. There was also a Trial x Surgery interaction ($F_{(3,108)} = 3.651$, $p < 0.05$) for WMI (Figure 6A). At the highest working memory load trial, there was a marginal effect of Surgery ($F_{(1,36)} = 3.947$, $p < 0.10$), where animals that received Hysterectomy surgery tended to make more WMI errors than those that received Sham surgery (Figure 6A). Like that of WMC errors in the Asymptotic Phase, upon inspection of patterns of WMI errors made across testing trials for each age (Figure 6B), a post hoc decision was made to evaluate Surgery effects within

each age. Subsequent analyses revealed that for animals receiving surgery in middle-age, there was a marginal effect of Surgery ($F_{(1,18)} = 4.281$, $p < 0.10$) such that Hysterectomized animals made more WMI errors at the highest working memory load than their Sham counterparts (Figure 6B). No effect of Surgery was seen for this planned comparison in young animals.

Planned comparisons between Sham and Ovx+Hysterectomy groups revealed no initial effects of Surgery, nor any Age x Surgery interactions, either across all trials, or for Trials 3 or 4 alone. However, given the differing effects of Surgery by Age seen at the highest working memory load for Sham and Hysterectomy groups, the posthoc decision was made to analyze the effects of Surgery separated by Age for Trial 4 alone. Subsequent analyses revealed that, for animals receiving surgery in middle-age, there was a marginal effect of Surgery ($F_{(1,18)} = 4.245$, $p < 0.10$) for Trial 4, where animals that received concurrent Ovx and hysterectomy surgery tended to make more WMI errors than their sham counterparts (Figure 6B). Notably, there was no effect of Surgery for young animals in this planned comparison.

For planned comparisons between Hysterectomy and Ovx+Hysterectomy groups during the Asymptotic Phase, there was no effect of Surgery, nor an Age x Surgery interaction for the analysis across all trials. However, when Trial 3, the moderate working memory load trial, was analyzed alone, a marginal Age x Surgery interaction was revealed ($F_{(1,36)} = 2.880$, $p < 0.10$); of note, subsequent statistical analyses could not be performed, due to the limited variance in these groups (Figure 6B).

For the Asymptotic Phase of WRAM testing, there were no effects of Surgery or Age x Surgery interactions for any of the planned comparisons.

Delayed memory retention.

To evaluate delayed memory retention on the WRAM with the implementation of a 6-hour delay between Trials 2 and 3, performance was evaluated on Trial 3, the immediate post-delay trial. Specifically, Trial 3 for the last baseline day of testing, Day 12 (Baseline) was compared to performance on Trial 3 on the day of the delay, Day 13 (Delay). For WMC errors, the Young-Sham ($F_{(1,9)} = 22.500$, $p < 0.01$), Young-Hysterectomy ($F_{(1,9)} = 16.000$, $p < 0.01$), Young-Ovx+Hysterectomy ($F_{(1,9)} = 7.309$, $p < 0.05$), Middle-Age-Sham ($F_{(1,9)} = 11.250$, $p < 0.001$), and Middle-Age-Ovx+Hysterectomy ($F_{(1,9)} = 5.651$, $p < 0.05$) made significantly more errors on the post-delay trial as compared to baseline indicating delay-induced impairment (Figure 7). Notably, no effect was seen for the Middle-Age-Hysterectomy group. There were no delay-induced effects for WMI errors.

Morris Water Maze

In the omnibus analysis of MWM performance across Days 1-5, there was a main effect of Day ($F_{(4,212)} = 80.190$, $p < 0.0001$) for distance to the platform, with distance decreasing across testing days, indicating learning of the task (Figure 8A). In addition, the omnibus analysis of MWM performance revealed an effect of Trial ($F_{(3,159)} = 12.674$, $p < 0.0001$), where distance to the platform decreased across trials within a day of testing. Finally, there was a significant Day x Trial interaction ($F_{(12,636)} = 2.739$, $p < 0.01$),

indicating that performance within a testing day was more consistently improved for latter days of testing.

When planned comparisons were performed as outlined above to analyze MWM performance across Days 1-5, there was no effect of Surgery nor an Age x Surgery interaction for any two-group comparisons.

Finally, analyses of probe trial performance revealed an effect of Quadrant for each treatment group [Young-Sham ($F_{(1,9)} = 60.851$, $p < 0.0001$); Young-Hysterectomy ($F_{(1,9)} = 73.493$, $p < 0.0001$); Young-Ovx+Hysterectomy ($F_{(1,9)} = 120.132$, $p < 0.0001$); Middle-Age-Sham ($F_{(1,9)} = 18.126$, $p < 0.01$); Middle-Age-Hysterectomy ($F_{(1,8)} = 98.046$, $p < 0.0001$); Middle-Age-Ovx+Hysterectomy ($F_{(1,9)} = 58.383$, $p < 0.0001$)] (Figure 8B). For each group, a greater percentage of total swim distance was spent in the Target Quadrant (NE) as opposed to the Opposite Quadrant (SW), indicating spatial localization of the platform.

Visible Platform Task

For the VP task, for each set of planned comparisons, there was a main effect of Trial (Sham vs. Hysterectomy: $F_{(5,180)} = 5.275$, $p < 0.001$; Sham vs. Ovx+Hysterectomy: $F_{(5,180)} = 6.116$, $p < 0.0001$; Hysterectomy vs. Ovx+Hysterectomy: $F_{(5,180)} = 4.444$, $p < 0.001$), with latency to the platform decreasing across trials (Figure 9). For each two-group planned comparison, there was no effect of Surgery or Age x Surgery interaction. For the final testing trial, no animal had greater than 15 seconds latency to platform, indicating all subjects were capable of performing the procedural components necessary to solve a water-escape task.

Vaginal Cytology

Vaginal smears were performed for eight consecutive days, beginning three weeks after surgery. Rats that underwent Ovx+Hysterectomy surgery showed blank or diestrus-like smears, demonstrating successful surgical removal of the ovaries. Rats in the Sham and Hysterectomy groups showed normal estrous cycles of 4-5 days in length, demonstrating continued post-operative ovarian function. Vaginal smears were again collected on the day prior to sacrifice and the day of sacrifice. Ovx+Hysterectomy groups continued to display blank or diestrus-like smears, whereby Sham and Hysterectomy groups displayed normal estrous cycle activity. There were no perceptible differences in estrous cyclicity across Young and Middle-Age groups within a given surgery type.

Body Weights at Sacrifice

At sacrifice, body weights were collected for each subject. Analyses showed that there there was a main effect of Surgery for each planned comparison (Sham vs. Hysterectomy: $F_{(1,36)} = 4.464$, $p < 0.05$; Sham vs. Ovx+Hysterectomy: $F_{(1,36)} = 85.853$, $p < 0.0001$; Hysterectomy vs. Ovx+Hysterectomy: $F_{(1,36)} = 120.582$, $p < 0.0001$), where Sham rats weighed more than Hysterectomy rats, and Ovx+Hysterectomy rats had the greatest body weight, regardless of age at surgery (Figure 10). For the Sham vs. Ovx+Hysterectomy planned comparison, there was a significant Age x Surgery interaction; upon further analysis of these Surgery effects across the Young and Middle-Age groups, it was found that the effect of Surgery was present for the Sham vs. Ovx+Hysterectomy planned comparison for both ages (Young: $F_{(1,18)} = 73.118$, $p < 0.0001$; Middle-Age: $F_{(1,18)} = 22.836$, $p < 0.001$; data not shown).

Western Blot Protein Analysis

Dorsal hippocampus. Across all sets of planned comparisons, there were no effects of Surgery or Age x Surgery interactions for normalized FosB expression or normalized ΔFosB expression in the Dorsal Hippocampus (Figure 11).

Entorhinal cortex. Planned comparisons between Sham and Hysterectomy animals for normalized FosB expression in the Entorhinal Cortex revealed a marginal Age x Surgery interaction ($F_{(1,35)} = 3.105$, $p < 0.10$). Subsequent analyses of each age separately showed that, for young animals, there was an effect of Surgery ($F_{(1,17)} = 5.950$, $p < 0.05$), where animals that were Hysterectomized in young adulthood had reduced relative FosB expression as compared to their Sham counterparts; there was no effect of Surgery for this planned comparison in middle-aged animals (Figure 12). There were no effects of Surgery or Age x Surgery interactions for relative FosB expression for either the planned comparisons between Sham and Ovx+Hysterectomy groups, or between Hysterectomy and Ovx+Hysterectomy groups, in the Entorhinal Cortex.

Across all planned comparisons, there were no effects of Surgery or Age x Surgery interactions for relative ΔFosB expression in the Entorhinal Cortex.

Correlations

Correlations between relative FosB and ΔFosB expression in the Dorsal Hippocampus or Entorhinal Cortex and WRAM performance (WMC and WMI errors averaged across Days 2-4, Days 5-9, or Days 10-12) were analyzed to further investigate the relationships between these variables, as well as to assess the extent to which these relationships may vary by surgery type or age at surgery. For animals in the Middle-Age-

Sham group, there was a marginal positive correlation between relative ΔFosB expression in the Dorsal Hippocampus and WMI errors made during the Late Acquisition Phase ($r(8) = 0.561$, $p < 0.10$), whereby, for animals receiving sham surgery in middle-age, more working memory errors were somewhat related to greater hippocampal ΔFosB (Figure 13A). Similarly, for animals in the Young-Ovx+Hysterectomy group, there was a positive correlation between relative ΔFosB expression in the R Dorsal Hippocampus and WMI errors made during the Asymptotic Phase of WRAM ($r(8) = 0.753$, $p < 0.01$); for animals that underwent surgical removal of the uterus plus ovaries in young adulthood, more WMI errors during this final phase of WRAM testing marginally correlated with greater hippocampal ΔFosB (Figure 13B). There were no significant correlations for any treatment group for relative FosB expression in the Dorsal Hippocampus.

In the Entorhinal Cortex, all significant correlations with relative FosB/ΔFosB expression were found during the Late Acquisition Phase of WRAM testing. Specifically, for animals in the Middle-Age-Sham group, there was a negative correlation between relative FosB expression in the Entorhinal Cortex and WMC errors made during the Late Acquisition Phase ($r(8) = -0.643$, $p < 0.05$; Figure 14B). For Sham animals that received surgery in middle-age, more WMC errors were associated with reduced FosB expression in Entorhinal Cortex. Additionally, there was a marginal negative correlation between ΔFosB expression in the Entorhinal Cortex and WMC errors made during the Late Acquisition Phase for animals in the Young-Sham group ($r(8) = -0.575$, $p < 0.10$), where greater WMC errors marginally associated with reduced ΔFosB expression for young animals that received Sham surgery (Figure 14C). Finally, there was a marginal negative

correlation for animals in the Middle-Age-Sham group between relative FosB expression in the Entorhinal Cortex and WMI errors made during the Late Acquisition Phase ($r(8) = -0.613$, $p < 0.10$), where greater WMI errors tended to correlate with a reduction in ΔFosB expression for animals receiving Sham surgery in middle-age (Figure 14A).

Discussion

Using a novel rodent model, the current study demonstrated that the cognitive effects of hysterectomy, with and without concurrent Ovx, differ depending upon age at surgery. Hysterectomy-induced working memory deficits were present for young animals in the initial learning phase of testing, while the impairing effects of hysterectomy in middle-age persisted, and were present in both the learning and latter phases of testing, particularly for the highest working memory load trial. Additionally, age-specific alterations in levels of proteins that govern brain changes associated with learning were found for the surgical menopause variants evaluated here. Furthermore, unique relationships between behavioral and neurobiological outcomes were discovered for specific age- and surgical- groups.

The working memory deficits seen in the Young-Hysterectomy group relative to the Sham control group were a replication of the initial work using the hysterectomy model (Koebele et al., 2019). Alternatively, the continued spatial working memory impairment throughout WRAM testing seen in animals that received hysterectomy in middle-age was unexpected, given previous clinical work in women demonstrating increased risk of cognitive decline associated with earlier age at hysterectomy (Phung et al., 2010; Rocca et al., 2012). However, it should first be noted that the current

experiment evaluated spatial learning and memory outcomes associated with hysterectomy only weeks after surgery, where the clinical literature has mainly evaluated dementia risk, which involves examination of cognitive status decades after the initial surgery; future work should characterize the long-term learning and memory outcomes following such surgical manipulation using this preclinical model. It is also possible that this discrepancy reflects a question that must be further probed specifically within the clinical literature, where women that undergo hysterectomy surgery at different ages might have different health conditions that prompt such gynecological surgery, which could be driving this differential risk of dementia. Alternatively, given the differences between female reproductive senescence in rats and in humans (Koebele & Bimonte-Nelson, 2016; Lu, Hopper, Vargo, & Yen, 1979; The Practice Committee of the American Society of Reproductive Medicine, 2004), it is possible that differential hormonal and neurobiological changes may be driving these divergent cognitive outcomes with hysterectomy at later timepoints.

Spatial Memory Performance

Overall, both young and middle-aged animals were able to learn the procedural components of the WRAM, as a main effect of Day was found across Days 2-12, where errors made decreased across days of testing for all error types across all subjects. During the VP task, animals also demonstrated the ability to successfully complete a water-escape task, as main effects of Trial were found across each planned comparison; this would suggest that differences in errors made on the WRAM were neither due to

particular groups being unable to complete such a task due to procedural demand reasons, nor due to a lack of motivation to find the escape platform.

Within the Early Acquisition Phase of testing, for WMC errors, animals that received hysterectomy with concomitant Ovx, regardless of age, were impaired relative to Sham animals at a moderate working memory load. At the highest working memory load, however, there was a unique interaction between Age and Surgery such that, for young animals, rats in the Hysterectomy group tended to make more errors than those in the Ovx+Hysterectomy group. Overall, this would suggest an earlier point of working memory failure for those that undergo hysterectomy with Ovx, regardless of age; this complements the preclinical literature, which states that Ovx generally is detrimental to cognition (Daniel et al., 1999; Rashidy-Pour, Bavarsad, Miladi-Gorji, Seraj, & Vafaei, 2019), as well as the clinical literature, where memory deficits are seen following oophorectomy in women (Nappi et al., 1999; Schaafsma, Homewood, & Taylor, 2010). However, effects shown at the highest working memory load highlight a unique impairment for rats that underwent hysterectomy, where this impairment is not seen for middle-aged animals that underwent hysterectomy. This suggests that animals that received hysterectomy surgery in young adulthood had unique learning impairments, which fits with recent preclinical work from this laboratory that utilized this model, where hysterectomy surgery in young animals was likewise found to be detrimental during learning on the WRAM (unpublished findings).

For WMI errors made during the Early Acquisition Phase, animals that received hysterectomy, regardless of age or of ovarian status, were impaired with a moderate

working memory load demand. It is fitting that effects for WMI errors parallel those of WMC errors, given that both error types concern working memory failures. However, it is noteworthy that all hysterectomized rats would demonstrate such impairment during learning for this error type, as it is a failure of working memory that necessitates a previous failure in reference memory within a given testing day. These data demonstrate that for a moderate working memory load, manipulation of the reproductive tract in general might result in some degree of working memory impairment during learning.

In the Late Acquisition Phase, there is yet again a unique hysterectomy-induced impairment observed for young animals alone, where animals in the Young-Hysterectomy group tended to make more WMI errors at the highest working memory load as compared to those in the Young-Sham group; such impairment was not observed for those that received surgery in middle-age. This again would indicate that animals receiving hysterectomy surgery when young are at a unique disadvantage in learning a spatial working memory task, particularly when working memory is highly taxed.

When analyzing the Asymptotic Phase, the resulting effects largely contrasted those found during Acquisition on the WRAM. For WMC errors, animals receiving hysterectomy with concurrent Ovx were found to be marginally impaired at the moderate working memory load trial as compared to Sham animals. When this impairment was further probed by age, however, it was found that animals in the Young-Ovx+Hysterectomy group were impaired relative to Young-Hysterectomy animals at this timepoint. This suggests that animals receiving hysterectomy plus Ovx, particularly when young, are uniquely impaired on a spatial working memory task at a moderate cognitive

load. Given that a similar impairment was found for both young and middle-aged animals receiving Ovx in the Early Acquisition Phase at the moderate working memory load, the data suggest that young animals are particularly vulnerable to working memory impairment from such gynecological surgery, potentially as a result of the cessation of circulating ovarian hormones (Bimonte & Denenberg, 1999; Daniel et al., 1999; Gibbs, 2000). Furthermore, when the highest working memory load trial was analyzed, a unique effect of hysterectomy was found for middle-aged animals, where the Middle-Age-Hysterectomy group demonstrated impaired working memory performance relative to Middle-Age-Ovx+Hysterectomy and Middle-Age-Sham groups. These findings show that, when working memory is taxed at its highest on the WRAM, animals receiving hysterectomy surgery in middle-age demonstrate unique working memory impairment, making them more vulnerable to cognitive detriments as a result of such surgical manipulation.

Unique working memory impairment as a result of hysterectomy surgery when performed in middle-age was also apparent for WMI errors made during the Asymptotic Phase of WRAM testing. At the highest working memory load trial, animals receiving hysterectomy in middle-age, with or without concomitant Ovx, tended to make more errors than their sham counterparts. With WMC and WMI errors demonstrating the same pattern of cognitive impairment with hysterectomy in middle-age at a high working memory load, it seems evident that the effects of hysterectomy on working memory performance, as evaluated on the WRAM, indeed do differ depending upon age at surgery.

Evaluation of delay-induced impairments on the WRAM revealed that all groups except for the Middle-Age-Hysterectomy group were impaired by the implementation of a delay between Trials 2 and 3. Impairment with WRAM delay has previously been demonstrated in such groups receiving gynecological surgery (Koebele et al., 2019). While it may seem unexpected that the group demonstrating greatest impairment during the final baseline days of testing would not be impaired with the implementation of a delay between trials, it is possible that this group was already sufficiently taxed during baseline testing, such that the delay did not appear to present any additional challenge. This laboratory has previously found that animals that are sufficiently burdened during baseline days of testing demonstrate no further impairment following implementation of a delay on a spatial working memory task (Engler-Chiurazzi et al., 2011).

Finally, evaluation of MWM did not demonstrate significant differences in spatial reference memory performance due to surgery; this was true for every planned comparison analysis. Each group was able to spatially localize the platform, as demonstrated by the significantly greater amount of time spent in the Target quadrant as opposed to the Opposite quadrant during the probe trial for each group. This indicates that all groups were able to learn the task. These findings concur with those demonstrated by previous work using this model (Koebele et al., 2019), where no differences in reference memory performance were detected.

Body Weights & Vaginal Cytology

At sacrifice, there were significant differences in body weight based upon surgery, regardless of age, where Sham animals weighed more than Hysterectomy

animals, and Ovx+Hysterectomy animals had the greatest body weight overall. Weight gain with Ovx has been frequently reported in the literature (Koebele & Bimonte-Nelson, 2016; Koebele et al., 2019; Prakapenka et al., 2018), but the reduction in weight for Hysterectomy animals relative to Sham animals is fairly novel. This weight loss could suggest that removal of the uterus alone may result in dysregulation of the metabolism or the gut microbiome, potentially via modulation of ovarian hormone levels, such as 17β-estradiol (Baker, Al-Nakkash, & Herbst-Kralovetz, 2017). Recent work has revealed that the gut microbiome potentially plays a role in brain functioning, and can impact cognitive outcomes via the 'gut-brain axis' (Hasan Mohajeri, La Fata, Steinert, & Weber, 2018). Future research should explore the extent to which removal of the uterus might disrupt other organ systems and endocrine axes, particularly in middle-age.

Characterization of vaginal cytology both before behavioral testing and at sacrifice indicate that all ovary-intact groups were normally cycling by the conclusion of the study, where Ovx+Hysterectomy animals demonstrated characteristically blank or diestrus-like smears. While vaginal cytology findings indicate that Sham and Hysterectomy groups had normal ovarian function regardless of age at surgery, future characterization of ovarian tissues and serum ovarian hormone levels from these animals would give a more comprehensive depiction of any potential changes in ovarian function as a result of hysterectomy surgery, given some clinical indications of earlier ovarian failure if present (Farquhar et al., 2005; Moorman et al., 2011).

Relative FosB/ΔFosB Expression

In evaluating relative expression of FosB and ΔFosB in both the Dorsal Hippocampus and Entorhinal Cortex, there were no differences across any of the planned comparisons within the Dorsal Hippocampus. However, differences amongst animals that received gynecological surgery were apparent for FosB within the Entorhinal Cortex. Specifically, animals that received hysterectomy in young adulthood had reduced relative FosB expression in the Entorhinal Cortex as compared to sham controls; no effect of surgery was apparent for animals receiving surgery in middle-age. Given that the Entorhinal Cortex serves as a bidirectional pathway for information between the hippocampus and other areas within the cortex, a decrease in expression of FosB for animals that received hysterectomy in young adulthood indicates a potential downregulation in neuronal activity in a brain area critical to spatial learning and memory. This modulation of activity in the Entorhinal Cortex could ultimately negatively impact learning and memory outcomes, which fits well with the behavioral outcomes observed here, wherein animals in the Young-Hysterectomy group demonstrated learning impairments on the WRAM. Although ΔFosB is expressed for longer periods of time (on the order of weeks to months after initial activation) (Eagle et al., 2015; Nestler et al., 2001), it is important to note that basal FosB levels are also maintained within the brain by the *fosB* gene (Ruffle, 2014). Despite the fact that FosB degrades more quickly than ΔFosB, such basal maintenance of FosB within the brain indicates that alternative splicing mechanisms may occur within specific brain regions, which in turn could be associated with hormonal alterations or with aging.

In young animals, it is possible that this reduction in full-length FosB could be due to re-organizational factors motivated by other endocrine or neurological changes as a result of hysterectomy surgery. Future research should work to decipher the effects of full-length FosB within brain regions specific to learning and memory, as well as to further characterize these modulations in FosB expression as a result of such gynecological surgery.

Additionally, it would be remiss not to acknowledge that other neurobiological factors might contribute to these cognitive outcomes as a result of such reproductive tract manipulation. One avenue for further research that appears to hold much promise is the evaluation of how the immune system, peripheral and within the brain, responds to uterine removal. It has been established that the uterus plays an integral role in immune signaling in both the pregnant and nonpregnant states (Lee, Kim, Kim, & Kang, 2015; Mor & Cardenas, 2010). Additionally, some work has been done demonstrating that peripheral immune challenges can impact neuroinflammation, which can subsequently affect spatial working memory outcomes, particularly in older animals (Chen et al., 2008). Furthermore, ovarian hormones like 17β-estradiol have been demonstrated to be protective against neuroinflammation (Suzuki et al., 2007), where increases in inflammation have been demonstrated following removal of the ovaries (Pacifici et al., 1991; Pfeilschifter, Köditz, Pfohl, & Schatz, 2002). Together, these findings warrant further research into whether hysterectomy might alter long-term immune functioning, peripherally and in brain, and how cognition might subsequently be impacted.

Correlations between Behavioral and Neurobiological Outcomes

Correlations between WRAM performance and relative FosB/ΔFosB expression for each treatment group revealed interesting relationships between brain and behavioral outcomes. In the Dorsal Hippocampus, there was a marginal positive correlation for animals receiving sham surgery in middle-age between WMI errors made during the Late Acquisition Phase and normalized ΔFosB expression, where greater expression correlated with increasing working memory errors. Likewise, a significant positive correlation was present for animals in the Young-Ovx+Hysterectomy group between WMI errors during the Asymptotic Phase of testing and normalized ΔFosB expression. Overall, this indicates that, within the Dorsal Hippocampus, aging and hormonal alterations contribute to positive relationships between expression of proteins governing brain changes related to learning and subsequent spatial working memory performance. Further research should explore whether direct manipulation of ΔFosB expression within the Dorsal Hippocampus contributes to spatial learning and memory impairment or enhancement, and how this relationship might be modulated by reproductive tract manipulation as well as across aging.

Where correlations between these neurobiological and behavioral outcomes were all positive within the Dorsal Hippocampus, all subsequent significant or marginal correlations within the Entorhinal Cortex were negative in direction. The difference in direction of these relationships between brain and behavior suggests that FosB and ΔFosB might be playing differential roles within the Dorsal Hippocampus and Entorhinal Cortex. There was a marginal negative correlation for animals in the Middle-Age-Sham group between WMI errors made during the Late Acquisition Phase and normalized FosB

expression, where greater FosB expression correlated with a reduction in WMI errors. Likewise, there was a significant negative correlation for Middle-Age-Sham animals between WMC errors made during this phase of testing and normalized FosB expression; the similar pattern of correlations across WMI and WMC errors for Middle-Age-Sham animals suggests that, in middle-age, greater FosB expression seems to be related to improved cognitive outcomes. This is noteworthy, as Middle-Age-Hysterectomy animals, which demonstrated working memory deficits on the WRAM, has significant reductions in FosB expression in the Entorhinal Cortex. These correlations further demonstrate the need for further research to better characterize the role of full-length FosB in learning and memory. Finally, there was a marginal negative correlation between WMC errors made during the Late Acquisition Phase of WRAM testing and normalized ΔFosB expression for rats in the Young-Sham group, where increased ΔFosB expression was related to a reduction in working memory errors. Of note, for animals in middle-age, relationships were present between cognitive outcomes and FosB expression in the Entorhinal Cortex, whereas in young animals, these cognitive outcomes were demonstrated to have a relationship with ΔFosB expression in the Entorhinal Cortex. As previous studies have demonstrated that alternative gene splicing mechanisms can be modulated by aging (Tollervey et al., 2011) and by 17β-estradiol administration following Ovx (Shults, Pinceti, Rao, & Pak, 2015), further research should explore whether alternative splicing mechanisms of the *fosB* gene might change across aging, and how such surgical manipulations of the reproductive tract might further modulate these outcomes.

Conclusions and Future Directions

The current experiment demonstrates that age at hysterectomy surgery, alone and with concomitant ovarian removal, modulates spatial learning and memory outcomes. Hysterectomy in young animals negatively impacted spatial working memory during learning, whereas surgery in middle-age showed impairments both in the learning and later phases of WRAM testing. These findings sit in contrast to the aforementioned experimental hypothesis that younger age at hysterectomy surgery would demonstrate exacerbated cognitive impairment as compared to those receiving surgery in middle-age, based upon findings from the clinical literature (Phung et al., 2010; Rocca et al., 2012). However, there are some differences between such clinical findings and the current experimental paradigm, namely that the current experiment evaluated cognition at a fairly short interval post-surgery, whereas the majority of clinical research has evaluated dementia risk associated with hysterectomy, which involves evaluation that spans decades after surgery. Some work in the Bimonte-Nelson laboratory using the hysterectomy model has begun to evaluate spatial learning and memory at longer post-surgical timepoints (unpublished findings), but only in animals that receive surgery in young adulthood. Indications from this work so far point to a sustained cognitive impairment for young animals receiving hysterectomy surgery, even 12 months after initial surgical intervention (unpublished findings). Such research should be extended into evaluating longer post-surgical timepoints for animals that receive hysterectomy in middle-age, where impairment could be more severe at short-term evaluations, but could dissipate more quickly with aging than with animals that receive hysterectomy in young adulthood.

Additionally, the exacerbated working memory impairments demonstrated here as a result of hysterectomy in middle-age might be related specifically to the rodent form of female reproductive senescence, which in some ways does differ from that of humans. While young adult rodents have circulating hormone levels that in many ways parallel that of women during their reproductive years, with cyclic fluctuations in 17β-estradiol and progesterone (Koebele & Bimonte-Nelson, 2016), rodents in late middle-age undergo a reproductive transition known as estropause, characterized by a persistent estrus cycle phase with moderate to high circulating levels of 17β-estradiol and moderate gonadotropin levels (Koebele & Bimonte-Nelson, 2016; Lu et al., 1979). Notably, during this time, ovarian follicular reserve declines in the rodent, but is not fully depleted. This sits in stark contrast to the experience of reproductive senescence in women, known as menopause, where the ovarian follicular reserve becomes deplete, and gonadotropins are elevated with declining levels of 17β-estradiol (Burger, 2006; Koebele & Bimonte-Nelson, 2016). It is possible that, with hysterectomy in middle-age, rats are experiencing a disruption in HPO-axis functioning, but that this dysregulation leads to differential cognitive outcomes due to the differential experiences of reproductive senescence. Further work evaluating ovarian tissues and serum ovarian hormone levels from the current experiment could provide insight into whether hysterectomy surgery leads to such disruption.

While previous work has demonstrated no significant changes in ovarian follicle counts for rodents following hysterectomy in young adulthood (Koebele et al., 2019), potential changes with hysterectomy in middle-age remain to be explored. It is possible

that at this middle-age timepoint, where the brain is undergoing reorganization in this sensitive estropausal window (Koebele & Bimonte-Nelson, 2015), surgical removal of the uterus uniquely disrupts HPO axis functioning wherein ovarian changes may occur. In this case, potential ovarian dysfunction following hysterectomy in middle-age might contribute to the cognitive impairments observed with the current experiment that extended beyond that of animals receiving hysterectomy surgery in young adulthood. Future work could also assess a model of the menopause transition in the rodent, namely the 4-vinylcyclohexene diepoxide (VCD) model of transitional menopause. VCD, when administered gradually over a series of 15 injections, has been demonstrated to selectively deplete the ovarian follicular pool, leading to eventual ovarian failure via accelerated atresia (Hoyer, Devine, Hu, Thompson, & Sipes, 2001; Hu, Christian, Thompson, Glenn Sipes, & Hoyer, 2001; Mayer et al., 2002), similar to that of menopausal women. Additionally, this laboratory has demonstrated that serum ovarian hormone levels in VCD-treated rats are similar to those seen in menopausal women (Acosta et al., 2010, 2009). Given the translational value of the VCD model, future work could expand the use of the hysterectomy model to better characterize the spatial learning and memory outcomes associated with uterine removal at the start of the menopause transition via VCD administration, with a rapidly depleting ovarian follicular reserve and fluctuating ovarian hormone levels.

This investigation into the role of age at hysterectomy surgery, both alone and with concomitant ovarian removal, in modulating cognitive and neurobiological outcomes in a rodent model has provided significant insight into the importance of the

nonpregnant uterus across the female reproductive lifespan, where the uterus might participate in communications between the reproductive tract and brain. Indeed, this work contributes to the larger body of literature providing evidence opposite the notion that the nonpregnant uterus is a dormant, useless organ, thereby supporting the tenet that the uterus plays a larger role in HPO axis functioning and modulates cognitive outcomes. The current experiment has expanded the questions that future work should now address concerning the role of reproductive and cognitive aging in surgical variations of menopause, with rodent models providing valuable insight into the experiences that many women face across the aging trajectory. Once these effects are more clearly delineated, options for clinical management can be more directly pursued to yield promise toward a healthy trajectory during aging in women, even with variances in menopause etiology.

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APPENDIX A

FIGURES

Figure 1

Experimental Timeline

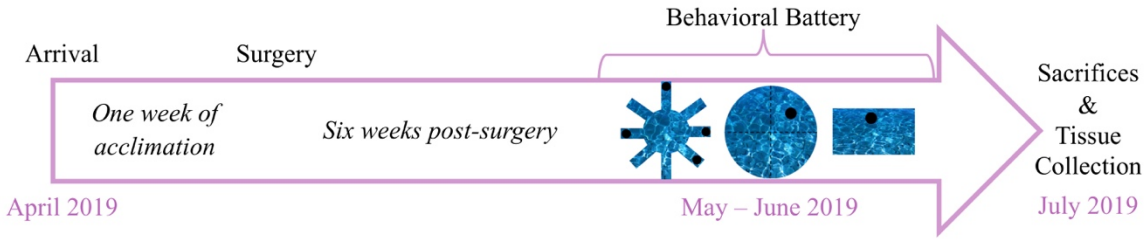


Figure 1: Experimental Timeline. Surgery occurred for the young and middle-aged animals one week after arrival. Six weeks after surgery, animals underwent a behavioral battery that evaluated spatial working and reference memory and included the Water Radial-Arm Maze, the Morris Water Maze, and the Visible Platform Task. Following the conclusion of the behavioral battery, animals were sacrificed, where tissues were collected for further processing.

Figure 2

WRAM: Early Acquisition Phase

WMC Errors

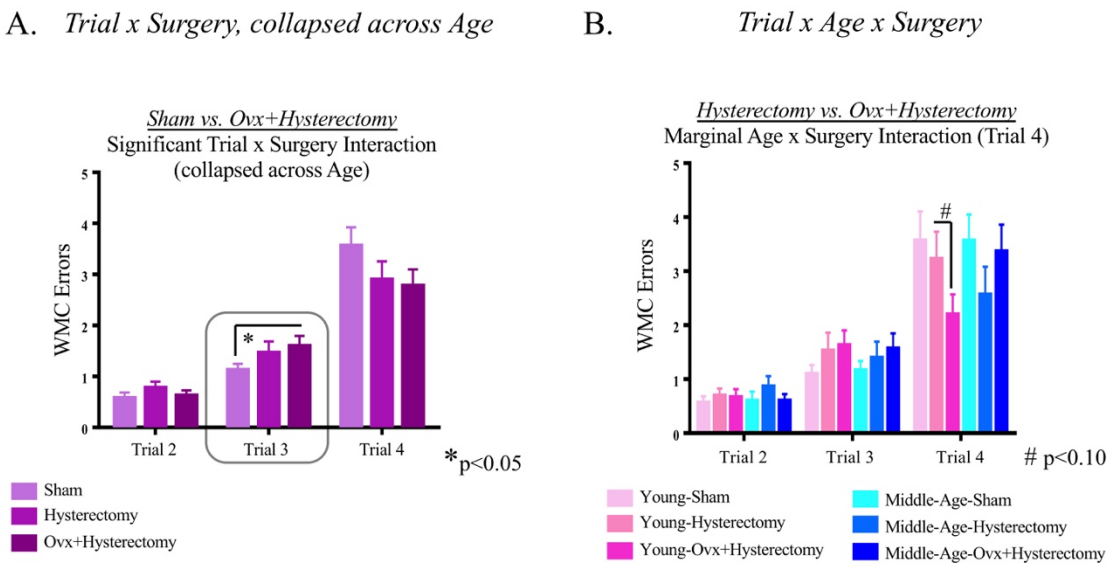


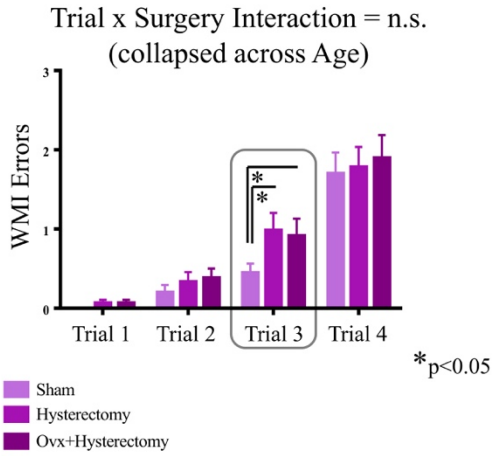
Figure 2: WRAM: Early Acquisition Phase WMC Errors. (A) Following a significant Trial x Surgery interaction for the Sham vs. Ovx+Hysterectomy planned comparison, it was found that, with a moderate working memory load, rats that had their uterus and ovaries removed were impaired relative to Sham rats during learning on the WRAM. (B) There was a marginal Age x Surgery interaction for the Hysterectomy vs. Ovx+Hysterectomy planned comparison on the highest working memory load trial. Subsequent analyses revealed that rats in the Young-Hysterectomy group were marginally impaired relative to rats in the Young-Ovx+Hysterectomy group, but this effect of Surgery was not present for animals that received surgery in middle-age. Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

Figure 3

WRAM: Early Acquisition Phase

WMI Errors

A. *Trial x Surgery, collapsed across Age*



B. *Trial x Age x Surgery*

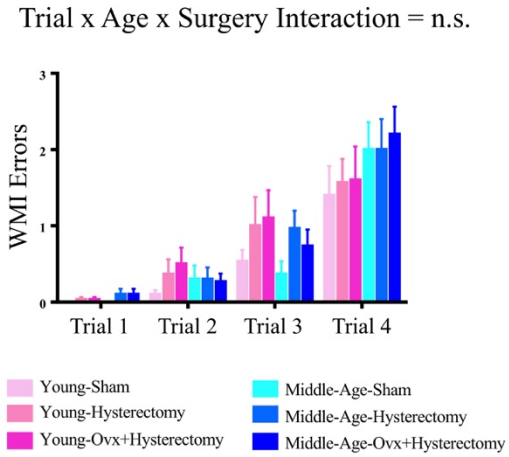


Figure 3: WRAM: Early Acquisition Phase WMI Errors. (A) At the moderate working memory load trial, rats that had their uterus removed, regardless of ovarian status, demonstrated working memory impairment relative to Sham rats during learning on the WRAM when collapsed across age. (B) There was not a significant Trial x Age x Surgery interaction for any of the planned comparisons, demonstrating that the effects of Surgery followed the same pattern across trials, whether animals received surgery in young adulthood or in middle-age. Data are presented as the mean ± SEM. * $p < 0.05$.

Figure 4

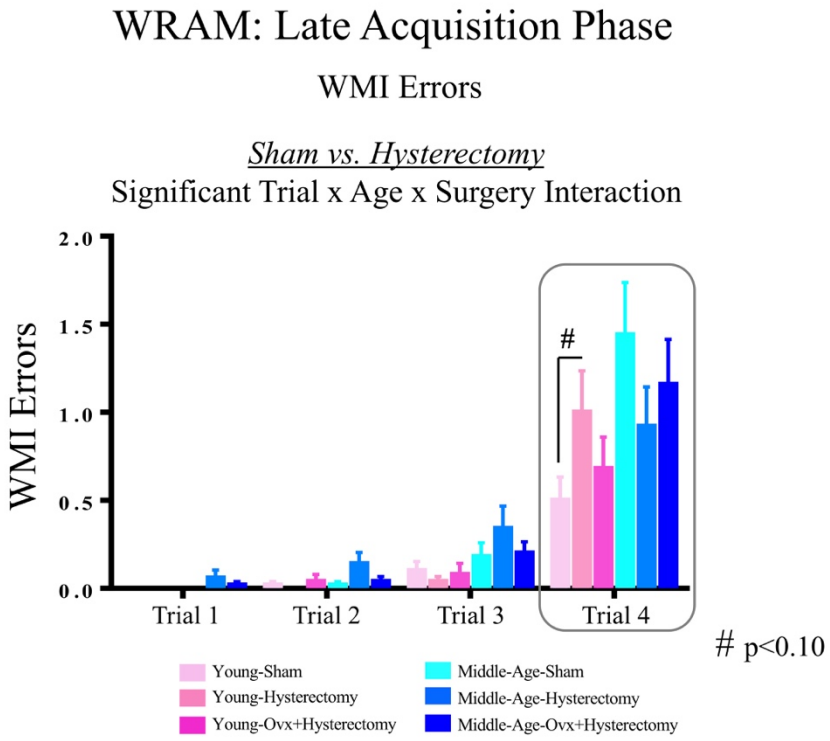


Figure 4: WRAM: Late Acquisition Phase. For the Sham vs. Hysterectomy planned comparison, there was a significant Trial x Age x Surgery interaction. Subsequent analyses showed that, at the highest working memory load trial, rats that had their uterus alone removed in young adulthood tended to demonstrate working memory impairment relative to Sham rats that received surgery in young adulthood; this marginal effect of Surgery was not present for animals that received surgery in middle-age. Data are presented as the mean ± SEM. # $p < 0.10$.

Figure 5

WRAM: Asymptotic Phase

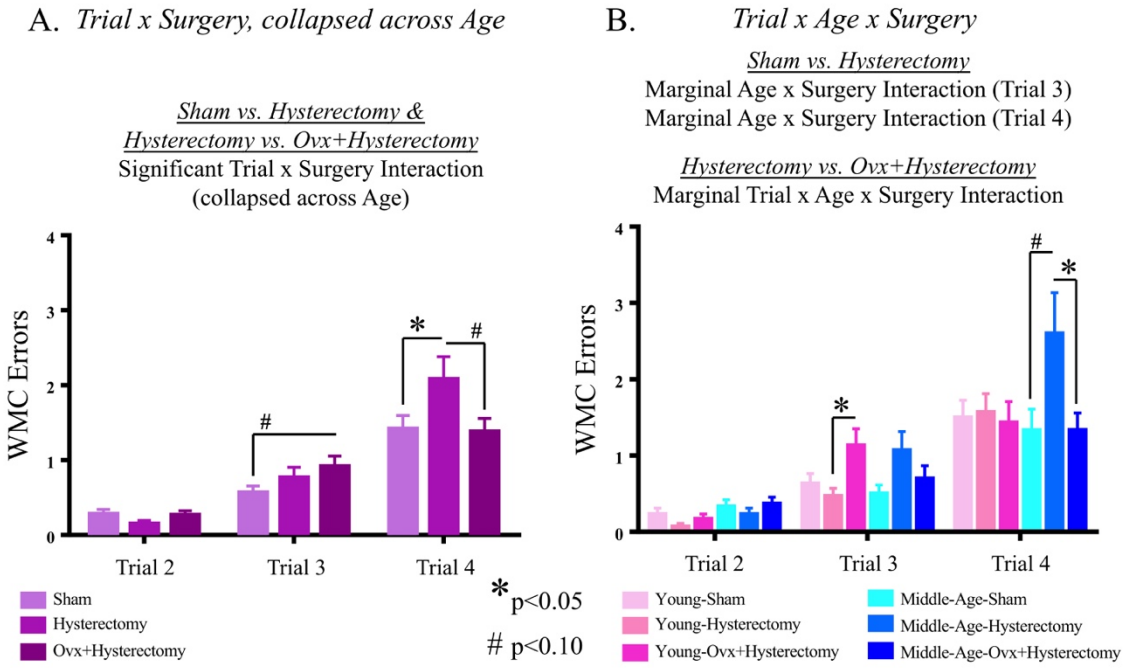


Figure 5: WRAM: Asymptotic Phase WMC Errors. (A) For the Sham vs. Hysterectomy planned comparison, as well as for the Hysterectomy vs. Ovx+Hysterectomy planned comparison, there was a significant Trial x Surgery interaction. Subsequent analyses found that for the moderate working memory load trial, rats that received Hysterectomy plus Ovx surgery were marginally impaired relative to Sham animals when collapsed across age. At the highest working memory load, rats that received Hysterectomy surgery made more WMC errors than Sham rats, and tended to make more WMC errors than Ovx+Hysterectomy rats when collapsed across age. (B) Given marginal Age x Surgery interactions for Trial 3 and Trial 4 with the Sham vs. Hysterectomy planned comparison, as well as a marginal Trial x Age x Surgery interaction for the Hysterectomy vs. Ovx+Hysterectomy planned comparison, the effects of Surgery were further probed across each age for each trial. For the moderate working memory load trial, rats in the Young-Ovx+Hysterectomy group were impaired relative to rats in the Young-Hysterectomy group; no such impairment was found for animals in middle-age. At the highest working memory load, middle-aged rats that received Hysterectomy surgery were impaired relative to those that received Sham or Ovx+Hysterectomy surgery, where no such impairment was found for animals that received surgery in young adulthood. Data are presented as the mean ± SEM. * $p < 0.05$, # $p < 0.10$.

Figure 6

WRAM: Asymptotic Phase

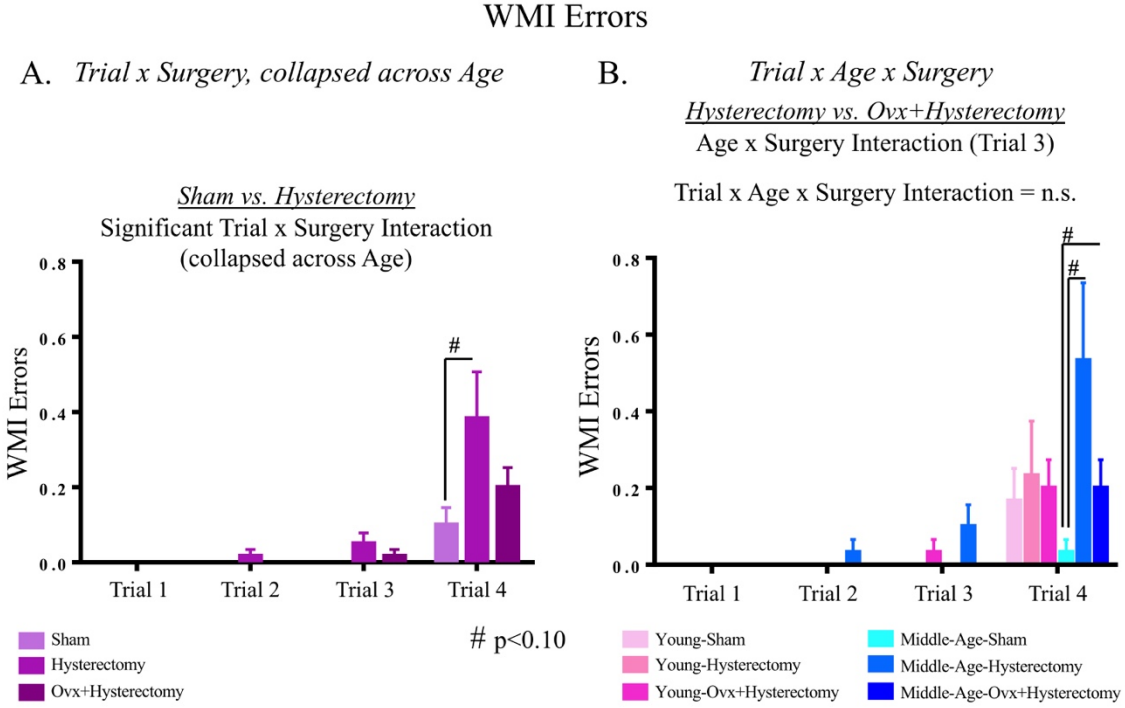


Figure 6: WRAM: Asymptotic Phase WMI Errors. (A) For the Sham vs. Hysterectomy planned comparison, there was a significant Trial x Surgery interaction. Subsequent analyses found that for the highest working memory load trial, rats that received Hysterectomy surgery were marginally impaired relative to Sham animals when collapsed across age. (B) Given Age x Surgery interaction for Trial 3 with the Hysterectomy vs. Ovx+Hysterectomy planned comparison, as well as the general distribution of errors across trials for each treatment group, it was evident that effects of Surgery would need to be further probed across each age for each individual trial. At the highest working memory load, rats that received Hysterectomy surgery in middle-age, regardless of ovarian status, marginally demonstrated working memory impairment relative to rats that received Sham surgery in middle-age; no such Surgery effects were found for animals that received surgery in young adulthood. Data are presented as the mean ± SEM. # $p < 0.10$.

Figure 7

WRAM: Delayed Memory Retention

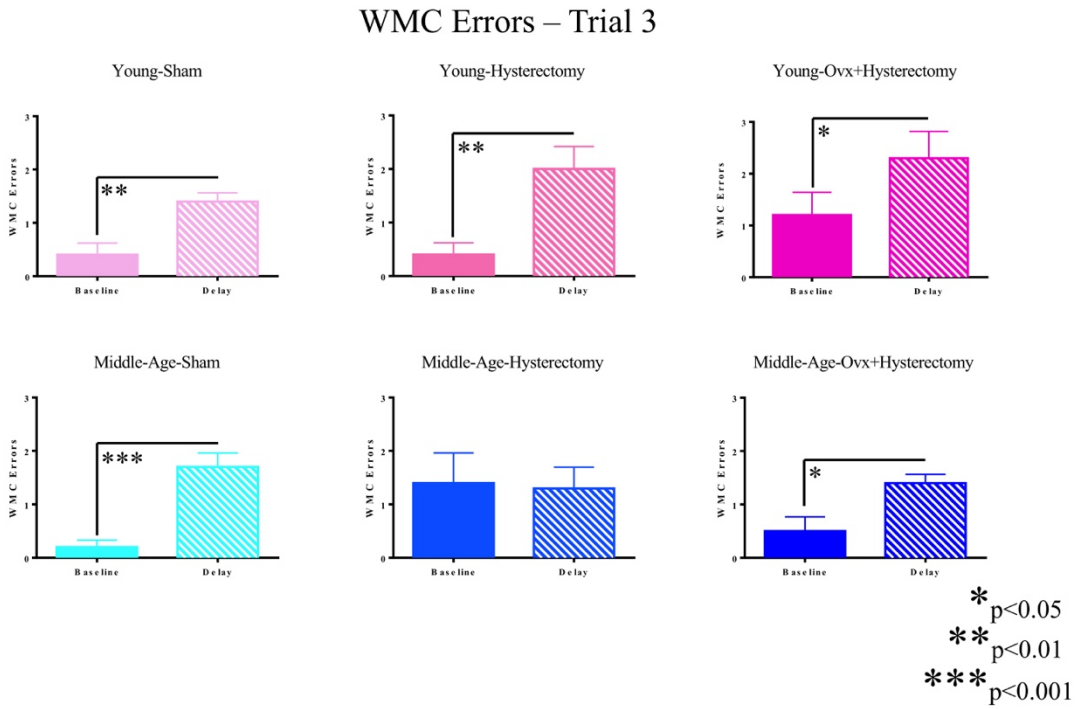


Figure 7: WRAM: Delayed Memory Retention. WMC errors for each treatment group on Trial 3 of the last day of baseline testing, Day 12 (Baseline) compared with the WMC errors made on Trial 3 of the delay day, Day 13 (Delay). All groups but the Middle-Age-Hysterectomy group demonstrated delay-induced impairments in working memory performance. Data are presented as the mean ± SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Figure 8
Morris Water Maze

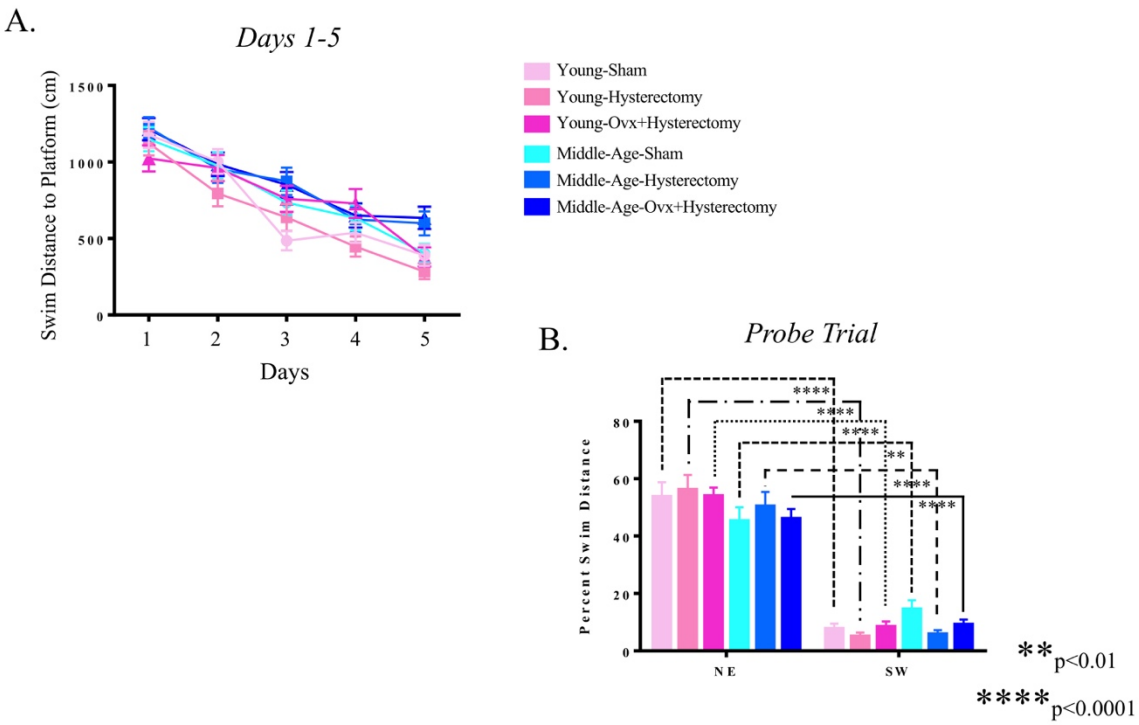


Figure 8: Morris Water Maze. (A) There were no effects of Surgery or Age x Surgery interactions for any of the planned comparisons across MWM Days 1-5. (B) All treatment groups spatially localized to the target quadrant during the probe trial. Data are presented as the mean ± SEM. **** $p < 0.0001$, ** $p < 0.01$.

Figure 9

Visible Platform Task

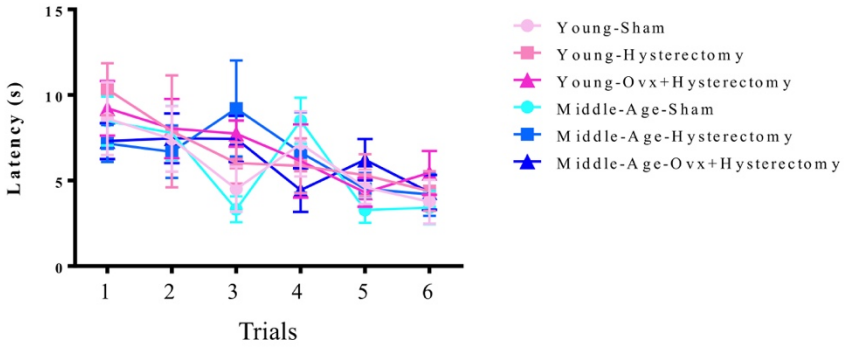


Figure 9: Visible Platform Task. There was a significant effect of Trial for each planned comparison, where latency decreased across trials. No animal had a swim latency that exceeded 15 seconds for the final trial, demonstrating that all animals were capable of completing the procedural components necessary to solve a water-escape task. Data are presented as the mean ± SEM.

Figure 10

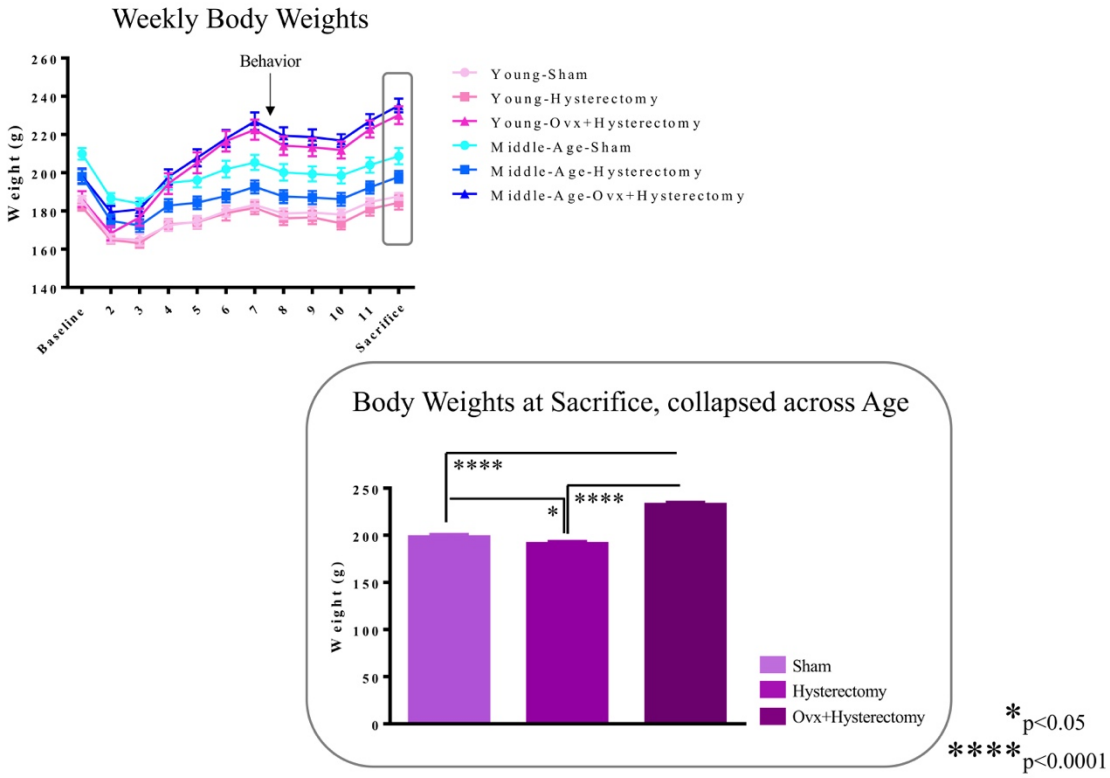


Figure 10: Body Weights. Weekly body weights collected across the duration of the experiment. At sacrifice, final body weights were collected for each subject, where Hysterectomy animals were found to weight significantly less than Sham animals, and Ovx+Hysterectomy animals were found to have greater body weight than Sham animals and Hysterectomy animals. Data are presented as the mean ± SEM. **** $p < 0.0001$, * $p < 0.05$.

Figure 11

Western Blots: Dorsal Hippocampus

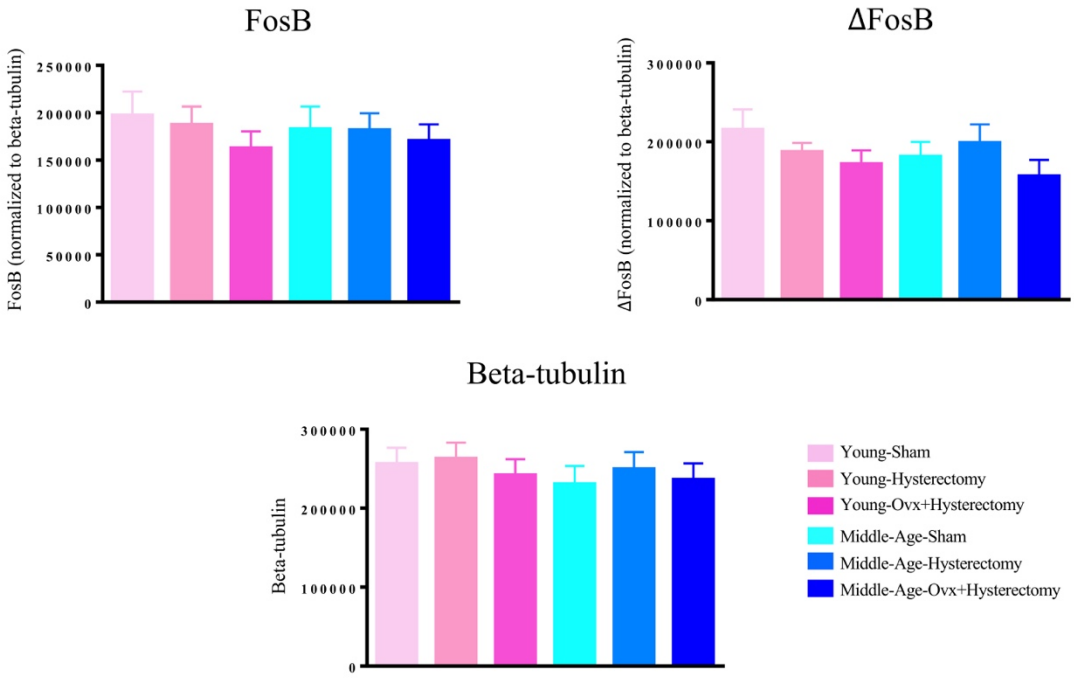


Figure 11: Western Blots: Dorsal Hippocampus. FosB, ΔFosB, and beta-tubulin expression in the Dorsal Hippocampus. There were no significant effects of Surgery or Age x Surgery interactions for any of the planned comparisons in FosB, ΔFosB, or beta-tubulin expression in the Dorsal Hippocampus. Data are presented as the mean ± SEM.

Figure 12

Western Blots: Entorhinal Cortex

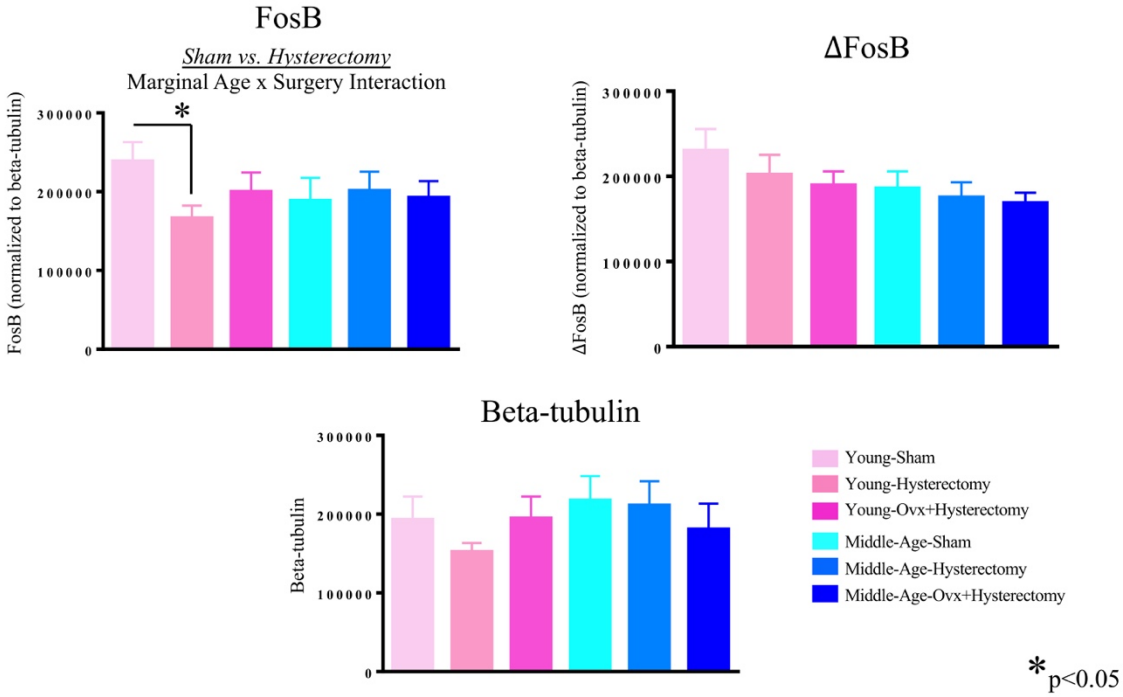


Figure 12: Western Blots: Entorhinal Cortex. FosB, ΔFosB, and beta-tubulin expression in the Entorhinal Cortex. There was a significant Age x Surgery interaction for the Sham vs. Hysterectomy planned comparison for FosB expression, where animals that received Hysterectomy in young adulthood had reduced FosB expression in the Entorhinal Cortex as compared to animals that received Sham surgery in young adulthood; no such effects of Surgery were present for animals that received surgery in middle-age. There were no effects of Surgery or Age x Surgery interactions for ΔFosB or beta-tubulin expression in the Entorhinal Cortex. Data are presented as the mean ± SEM. * $p < 0.05$.

Figure 13

Correlations between WRAM performance & FosB/ΔFosB expression in Dorsal Hippocampus

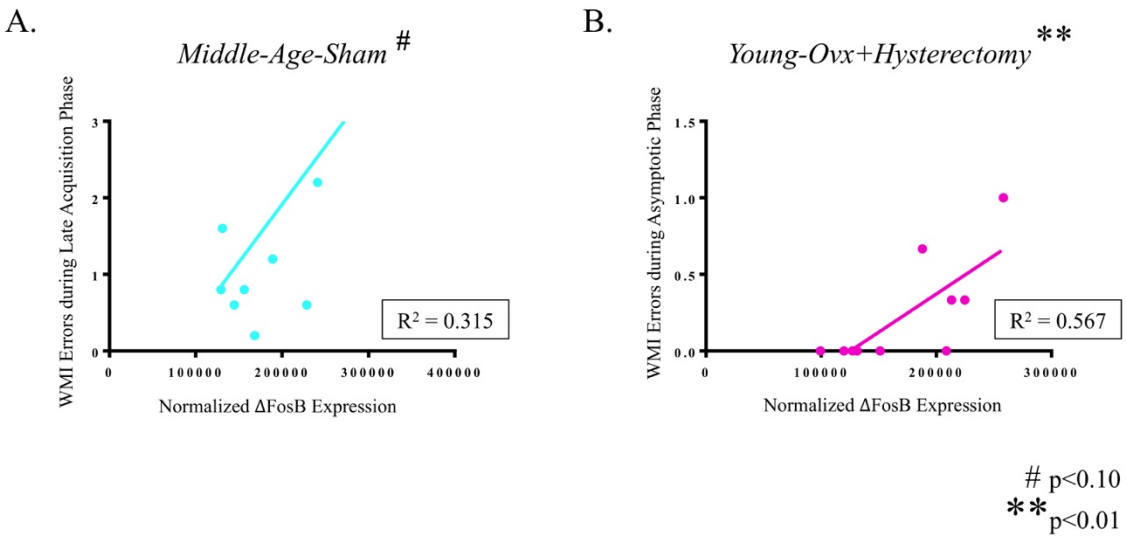


Figure 13: Correlations between WRAM Performance & FosB/ΔFosB Expression in Dorsal Hippocampus. (A) For Middle-Age-Sham animals, a higher number of WMI errors made during the Late Acquisition Phase of WRAM testing was marginally associated with increased ΔFosB expression in the Dorsal Hippocampus. (B) For rats in the Young-Ovx+Hysterectomy group, making more WMI errors made during the Asymptotic Phase of WRAM testing was associated with greater ΔFosB expression in the Dorsal Hippocampus. Data are presented as the mean ± SEM. # $p < 0.10$, ** $p < 0.01$.

Figure 14

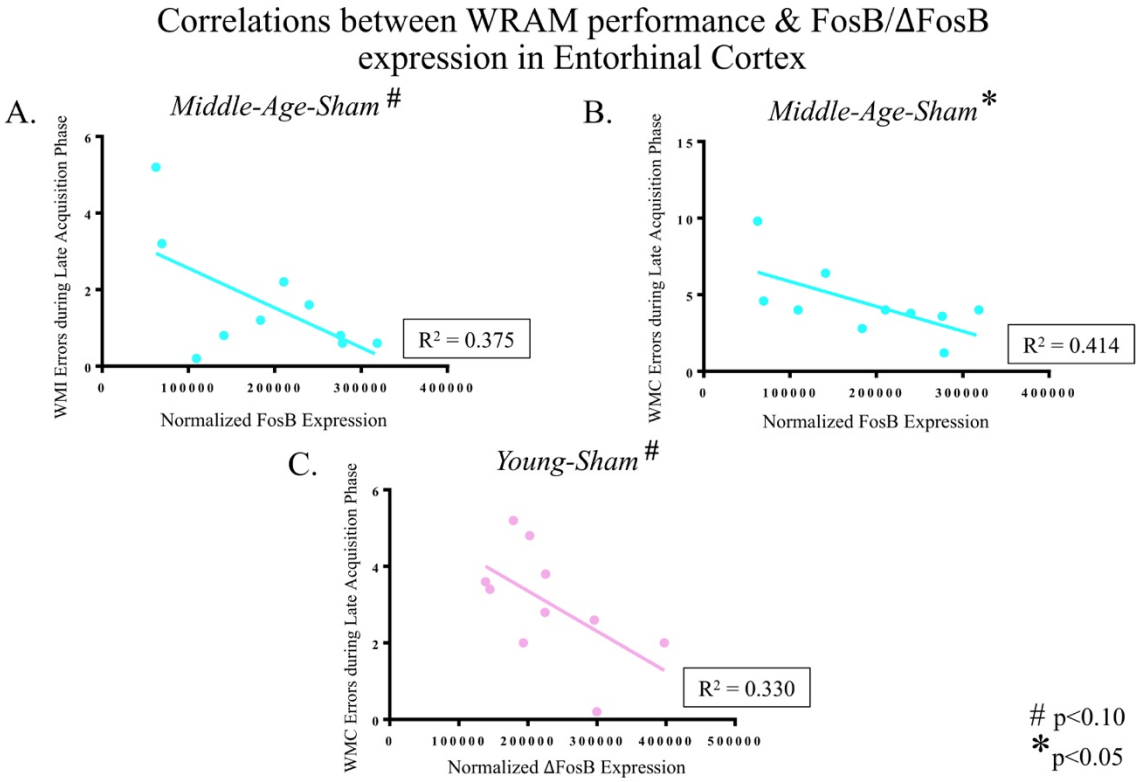


Figure 14: Correlations between WRAM Performance & FosB/ΔFosB Expression in Entorhinal Cortex. (A) For Middle-Age-Sham animals, an increase in number of WMI errors made during the Late Acquisition Phase of WRAM testing was marginally associated with reduced FosB expression in the Entorhinal Cortex. (B) For rats in the Middle-Age-Sham group, a greater number of WMC errors made during the Late Acquisition Phase of WRAM testing was associated with reduced FosB expression in the Entorhinal Cortex. (C) For rats in the Young-Sham group, a higher number of WMC errors made during the Late Acquisition Phase of WRAM testing was marginally associated with reduced ΔFosB expression in the Entorhinal Cortex. Data are presented as the mean ± SEM. # $p < 0.10$, * $p < 0.05$.