Premarin has opposing effects on spatial learning, neural activation, and serum cytokine levels in middle-aged female rats depending on reproductive history

Liisa AM Galea*1,2,3, Meighen M. Roes2, Christina J. Dimech2, Carmen Chow2, Rand Mahmoud1,3, Stephanie E. Lieblich1,2, Paula Duarte-Guterman1,2

Djavad Mowafaghian Centre for Brain Health1, Department of Psychology2, Graduate Program in Neuroscience3, University of British Columbia, Vancouver, BC, CANADA

*Address correspondence to:
Liisa Galea, Ph.D.
Professor,
Dept Psychology
Djavad Mowafaghian Centre for Brain Health
University of British Columbia
2215 Wesbrook Mall
Vancouver, BC
V6T1Z3
phone: 604-822-6536
webpage: galealab.psych.ubc.ca
Abstract

Menopause is associated with cognitive decline, and hormone therapies (HT) may improve cognition depending on type, and timing of HT. Previous parity may influence cognition in later life. We investigated how primiparity and long-term ovariectomy influence cognition, neurogenesis, hormones, cytokines, and neuronal activation in middle-aged rats in response to Premarin, an HT. Nulliparous and primiparous rats were sham-ovariectomized or ovariectomized, administered vehicle or Premarin six months later, and all rats were trained in the Morris water maze. Premarin improved spatial learning and memory in nulliparous rats, but impaired early learning in primiparous rats. With training, primiparity increased hippocampal neurogenesis, and Premarin decreased immature neurons, regardless of parity. Moreover, Premarin increased serum tumor necrosis factor (TNF) α and the CXC chemokine ligand 1 (CXCL1) in nulliparous, but not primiparous, trained rats. However, Premarin decreased the expression of the immediate early gene zif268 in the dorsal CA3 region in primiparous rats after training. Thus, primiparity alters how Premarin affects spatial learning, neuronal activation, and serum cytokines. These findings have implications for the treatment of age-associated cognitive decline in women.

Keywords: Parity; Aging; Memory; Estrone; Premarin; Neurogenesis; Hippocampus; cytokines
Maternal adaptation is the process by which a women’s body has to adapt to allow for a host of physiological changes that must occur to allow for appropriate survival of the fetus. For example, cardiac output, and pulmonary function are increased or decreased, respectively, by as much as 50% in gestating women (Grindheim et al., 2012; Savu et al., 2012). Furthermore, the endocrine system is modified, as the placenta releases a variety of hormones in high concentrations including estradiol, progesterone, and corticotropin releasing hormone (CRH; Brett and Baxendale, 2001; Holl et al., 2009). The mother’s immune system also undergoes modifications during pregnancy in part to foster tolerance to the forming fetus (Ghaebi et al., 2017). Although it has been widely assumed that many aspects of maternal physiology normalize after the expulsion of the placenta, certain physiological changes outlast the pregnancy and early postpartum and even emerge later in life. For example, as parous women age, mid-luteal phase estradiol levels are decreased compared to nulliparous women (Dorgan et al., 1995). In addition, parity alters the immune profile of aged animals (Barrat et al., 1997; Mahmoud et al., submitted). Parity also has lasting consequences on the brain, as long-lasting changes in hippocampal volume and cognition are seen in women and rodents (for review see Roes & Galea, 2015; Hoekzema et al., 2017; Galea et al., 2000; Galea et al., 2014; Barha and Galea, 2015). Two months after parturition, women exhibit reductions of hippocampal volume, and in other brain regions, and this reduction is still evident 2 years post parturition (Hoekzema et al., 2017). In addition, primiparous rats show reduced hippocampal volume and neurogenesis during lactation and throughout the postpartum period (Galea et al., 2000; Pawluski and Galea, 2007, Leuner and Gould, 2007; Workman et al., 2015). Thus, studies suggest that reproductive experience has lasting effects on the maternal physiology and hippocampal structure and plasticity.

Neurogenesis in the hippocampus is reduced in the early and late postpartum in primiparous female rats (Galea et al., 2000; Pawluski and Galea, 2007, Leuner and Gould, 2007; Workman et al., 2015) but curiously in middle age, increased hippocampal neurogenesis is seen in multiparous rats relative to nulliparous rats (Barha et al., 2015). Multiparous rodents have increased levels of brain derived neurotrophic factor (BDNF), synaptophysin, and spinophilin, as well as more immature neurons, compared to nulliparous rats in middle age (Barha et al., 2015; Cui et al. 2014; Macbeth et al., 2008; Rossetti et al, 2016). Furthermore, in response to estrogens, multiparous middle-aged rats show an enhancement in cell proliferation whereas nulliparous middle-aged rats do not (Barha and Galea, 2011). In the hippocampus and amygdala,
neuropathology (neuritic plaques and neurofibrillary tangles) was positively correlated with
parity in older women, but not in men, with the majority of subjects having dementia of probable
AD (Beeri et al., 2009), suggesting some neurological consequences of increased parity in
women. These findings suggest that increased parity is associated with both increased
hippocampal plasticity and pathology well into middle age, long after offspring have been reared.

Previous parity may also influence cognitive ability in middle- and older-aged rodents
and perhaps women. Primiparous and multiparous middle-aged rats display better performance
compared to nulliparous rats in the dry land maze (Gatewood et al., 2005; Love et al., 2005),
water maze (Lemaire et al., 2006; Barha et al., 2015) and in reversal learning tasks (Gatewood et
al., 2005). Importantly, previous reproductive experience may affect various aspects of
hippocampal cognition differently. In middle age, multiparous rats have enhanced early
acquisition in the spatial working memory version of the Morris water maze, but impaired
reference memory acquisition, compared to nulliparous rats (Barha et al, 2015). Studies in
women are more equivocal. However, parity is associated with better memory in older women
with factors such as age of first pregnancy, genotype, and amount of parity playing a moderating
role (Fox et al., 2013; Karim et al., 2016; Roes and Galea, 2015). Taken together these studies
suggest that previous parity may be associated with improvements in learning and memory in
middle-age.

Menopause is associated with a decline in circulating estrogens as well as declines in
certain cognitive domains (Weber, Rubin & Maki, 2013). Conversely, hormone therapy (HT) in
postmenopausal women may improve cognition depending on age at administration and HT
composition (Espeland et al., 2017; Hogeverst et al., 2000; Ryan et al., 2008), but other studies do
not find a beneficial effect on cognition (Gleason et al., 2015; Henderson et al., 2017). Premarin is
a common HT of conjugated equine estrogens (CEE), composed 50% of estrone sulphate and 0.1%
estradiol sulphate. Meta-analyses indicate that fewer studies report positive effects on cognition
with Premarin compared to estradiol-based therapies (Hogevorst et al., 2000; Ryan et al., 2008).
In addition, the ability of HT to improve cognition in middle-age depends on the specific
formulation of HT in animals (Baxter et al., 2013; Prakapenka et al., 2018). Furthermore, the
effects of HT on cognition in women may be dependent on the timing of when HTs were
prescribed (early or late in menopause), with a greater proportion of studies finding detrimental
outcomes, or fewer positive outcomes, when HT is initiated 10 or more years after menopause (Espeland et al., 2017; Hogevoorst et al., 2000; Ryan et al., 2008), although other studies find no such effect (Henderson et al., 2016). These inconsistencies in the literature may have to do with reproductive history, which is often not included in the analyses, and in the present study, we were interested in whether Premarin’s effects on cognition were dependent on past reproductive history.

In a number of studies, a ‘critical window’ exists during which estradiol administration early, but not late, after menopause or ovariectomy improves hippocampus-dependent cognition in middle age (Walf, Paris & Frye, 2009; Gibbs 2010; Vedder et al., 2014; Daniel and Bohacek, 2010). However, the duration of ovariectomy itself, without HT, can influence cognition and may depend on parity, with long-term (6 months) ovariectomy improving spatial memory in nulliparous rats (Bimonte-Nelson et al 2003) or showing no cognitive benefit in multiparous rats (Walf, Paris & Frye, 2009), suggesting that parity modulates the ovariectomy-induced effects on cognition in later life. Thus, in the present study we investigated the effects of long-term ovariectomy on cognition with previous parity.

Reproductive experience can alter the cognitive and neuroplastic effects of estrogens. Estrone and estradiol administration upregulated cell proliferation in the hippocampus of middle-aged multiparous but not nulliparous rats (Barha & Galea, 2011), suggesting that parity preserves the sensitivity of the hippocampus to estradiol and estrone later in life. Furthermore, ovariectomy in middle age has opposing effects on memory in nulliparous versus multiparous rats, improving spatial memory retrieval in nulliparous rats but impairing it in multiparous rats (Barha et al., 2015). Thus, reproductive experience alters hippocampus-dependent memory and neuroplastic response to estrogens in middle age.

Few studies have examined the potential mechanisms of long-lasting alterations in learning and neuroplasticity across aging that depend on parity. Given that steroid and peptide hormones are hijacked during pregnancy it is likely that there may be long-term impacts to these endocrine systems (Barha and Galea, 2017). While it is not known what mechanisms contribute to these long-term changes, current knowledge suggests a role of estrogens, adrenal hormones, and inflammatory signalling to drive long-term changes with parity. Studies in women point to reductions in estrogens across menstrual cycle after parity (Barrett et al., 2014) and estrogens are related to both neuroplasticity and cognition (for review see: Duarte-Guterman
et al., 2015). Cortisol and corticosterone can influence hippocampus-dependent cognition and neuroplasticity (for review see Wingenfield and Wolf, 2014) but studies are mixed as to whether long-lasting alterations to the hypothalamic pituitary adrenal (HPA) axis are seen with parity (Federenko et al., 2006; Lankarani-Fard et al., Tu et al., 2006). Furthermore, immune signalling is challenged with the placenta and fetus (Bonney, 2016), and long-lasting changes in immune signalling may be present (Asztalos et al., 2010; Clendenen et al., 2011; Mahmoud et al., submitted), which also influence cognition (Donzis and Tronson, 2014). Thus, the mechanisms by which parity can alter the aging trajectory are unclear, but may involve changes in inflammatory signatures (Mahmoud et al., submitted; Barrat et al., 1997; Cramer et al., 2017), levels of estrogens (Dorgan et al., 1995; Bridges and Byrnes, 2006; Barrett et al., 2014), and HPA axis changes with parity. Given these findings, levels of estrogens, adrenal mass, and inflammatory signatures were examined in the present study.

The aim of the present study was to determine the effects of primiparity, Premarin, and long-term ovariectomy on spatial memory, and how the experimental manipulations affect hippocampal neurogenesis and neuronal activation in behaviourally trained middle-aged rats. We also examined possible mediating factors such as adrenal mass, serum levels of cytokines, and estrogens. We hypothesized that Premarin treatment in middle-aged rats would affect spatial reference and reversal learning (a measure of cognitive flexibility), neurogenesis, and neuron activation in the hippocampus in a manner that is dependent on reproductive experience and hormone status.

Method

Animals

Ninety-seven female and 16 male Sprague-Dawley rats (Charles River, Quebec) were 2 months old at arrival at the University of British Columbia. Rats were housed in opaque polyurethane bins (24 x 16 x 46 cm) with aspen chip bedding, and were given standard laboratory chow (Harlan, Canada) and tap water ad libitum. Rats were maintained under a 12h:12h light/dark cycle (lights on at 07:00h). Rats were randomly assigned to parity (primiparous (Prim) or nulliparous (Null)), surgery (ovariectomy (OVX) or sham surgery), and treatment (oil or Premarin) conditions (n’s = 12-13 before attrition). Thus, this resulted in 8 groups: Null-Sham-Oil; Null- Sham-Prem; Null-OVX-Oil; Null-OVX-Prem; Prim- Sham-Oil;
Prim-Sham-Prem; Prim-OVX-Oil; Prim-OVX-Prem. Nulliparous refer to females that have never been pregnant or exposed to pups, while primiparous refers to females that were pregnant and mothered once.

Rats were double-housed except during pregnancy and gestation (nulliparous females were single-housed for an equivalent period of time) and recuperation from surgery. To prevent nulliparous rats from being exposed to odours and noises from males and/or pre-weaning pups, nulliparous rats were housed in a separate colony room until breeding and weaning were complete. All testing was conducted in accordance with ethical guidelines set by the Canada Council for Animal Care and all procedures were approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and suffering of animals.

**Apparatus**

The Morris Water Maze was a black circular pool, 180cm in diameter, filled with room temperature water mixed with black tempura paint (non-toxic) to render it opaque. Large and distinct distal cues were placed on all four walls of the room surrounding the pool and remained constant throughout behavioural testing. In the water maze task, the animal must use the distal cues to navigate to a hidden platform (15cm diameter), submerged roughly 2 cm beneath the pool surface. Cameras installed above the center of the pool were connected to ANY-maze 4.98 software (Stoelting, Wood Dale, IL, USA) in order to record measures.

**Breeding and procedures**

See Figure 1 for experimental timeline. During breeding, two females and one male were paired overnight beginning at approximately 5pm. Females were vaginally lavaged each morning between 7:30 and 9:00 am and samples were assessed for the presence of sperm. Upon identification of sperm, females were considered pregnant, weighed, and single housed into clean cages. One day after birth, all litters were culled to 5 males and 5 females. If there were not enough males or females in one litter, pups were cross-fostered from a dam that gave birth the same day, when possible. Cross-fostering occurred twice (OVX-Prem, Sham-Prem), and sex-skewed litters were maintained twice (Sham-Oil, OVX-Prem). Rats were left undisturbed except for cage changing and weighing which occurred weekly during gestation.
Maternal behaviour observations

Observations of maternal behaviours were done three times per day from postpartum days 2 through 8. Observations were taken between 9:00-10:30am, 1:00-2:30pm, and 5:00-6:30pm daily, during which each dam was observed for 10 continuous minutes. The amount of time spent engaging in the following behaviours was recorded: licking and grooming, nursing (arched-back, blanket, passive), and off nest behaviours (including self-grooming and sleeping).

Ovariectomy and Sham Surgery

At 8 months of age, rats underwent bilateral ovariectomy or sham surgery through bilateral flank incisions. Rats were induced and anesthetized with isoflurane, which was delivered at an induction flow rate of 5% in 1.5% oxygen. Rats were maintained at a surgical plane of anesthesia on a warming blanket using an isoflurane flow rate of 1.5-2.5% and were given an injection of Lactated Ringer's Solution (10 mL) to maintain fluid balance, and Ketoprophnen (0.5 mL/kg; Anafen, Merial Canada Inc., Baie d'Urfe', Quebec, Canada) and Marcaine (0.10 mL; Bupivacaine, Hospira Inc., Lake Forest, Illinois, Canada) as analgesics. Sham surgery consisted of skin and muscle incisions that were subsequently sutured without damage to or manipulation of the ovaries. Rats were single-housed for 7 days or until their sutures completely healed, at which point they were re-paired with cage mates.

Between the time of parturition and behavioural testing (from 5 months of age to approximately 14 months of age), 17 animals reached humane endpoint due to various factors common with aging (e.g., respiratory distress, malocclusion, urogenital or mammary tumor), or developed ulcerated foot sores that precluded participation in behavioural testing (Null-Sham-Oil: 2; Null-Sham-Prem: 2; Null-OVX- Oil: 2; Null-OVX-Prem: 1; Prim- Sham-Oil: 4; Prim- OVX-Oil: 1; Prim-Sham-Prem: 3; Prim- OVX-Prem: 2).

Hormone Administration

At 14-15 months of age, rats began a daily subcutaneous injection of either Premarin (20µg Conjugated equine estrogens (CEE)/0.1 ml sesame oil; Wyeth Pharmaceuticals, Markham, ON, Canada, obtained from a veterinarian prescription) or sesame oil (equivalent volume) and continued for 22 days. Injections were given between 7:30 and 9:30 am, at least 2 hours before any behavioural testing. The dose of Premarin was based on Acosta et al (2009) to correspond to
the most common daily dose taken by women (0.625mg; 0.00893 mg drug/kg body weight for a woman of average weight). The equivalent injected dose in rats has previously been found to influence memory (Acosta et al., 2009).

5-bromo-2-deoxyuridine (BrdU) Administration

BrdU is a thymidine analogue that incorporates into the DNA of dividing cells during the synthesis phase of the cell cycle within two hours after administration (Packard, et al., 1973). Depending on the amount of time elapsed between injection and perfusion of the animal, BrdU can be used to assess cell proliferation or cell survival in the dentate gyrus (Taupin, 2007). In the current study, rats were perfused 21 days after BrdU administration and 22 days after first hormone or oil injection. Therefore, the number of BrdU-labelled cells measures the survival of adult-generated hippocampal cells over a 21 day period after being produced and maintained under Premarin or oil administration. On the second day of hormone injections, rats received a single i.p. injection of 200mg/kg 5-bromo-2-deoxyuridine (BrdU: Sigma, St. Louis, MO). The BrdU solution was prepared to a concentration of 20mg/ml just prior to injection by dissolving BrdU in freshly prepared warm (< 40 °C) 0.9% saline containing 0.7% 1N NaOH.

Estrous Cycle

Phase of the estrous cycle can influence both hippocampal plasticity (Tanapat et al., 1999; Woolley et al., 1990; Rummel et al., 2010), spatial learning and strategy use (Warren & Juraska, 1997; Korol et al., 2004). Furthermore, the length, and regularity of the estrous cycle changes with aging in rats (LeFevre & McClintock, 1988). Therefore, estrous cycles were monitored in our study by vaginal lavage across all behavioural testing days. Lavage samples were transferred onto microscope slides, stained with Cresyl Violet, and left to dry. A rat was determined to be in the proestrous stage if at least 70% of cells were nucleated epithelial cells (Byers et al., 2012). Vaginal lavage samples were qualitatively categorized for evidence of normal cycling, abnormal cycling (consecutive lavage-cycles varying in length or order), persistent diestrus (consecutive lavage-cycles consisting primarily of leukocyte-dense cell samples), or persistent estrus (consecutive lavage-cycles consisting primarily of cornified cells; modified from Levefre & McClintock, 1988 to reflect the shorter sampling period in this study). Of the 32 sham rats that completed the study, 11 had irregular cycling, 11 were in persistent
estrus and 10 were cycling normally (See Table 1). Estrous cycle monitoring was conducted throughout behavioural testing.

**Morris Water Maze Training**

Morris Water Maze training is used to assess spatial reference learning (Vorhees and Williams, 2006) and was conducted on injection days 12-17 during 9am - 2 pm each day, with animals counterbalanced. There were four trials each day of 60s in duration or until the rat located the hidden platform. If the rat did not find the platform within 60s, the experimenter guided it to the platform, where it stayed for 10s. The inter-trial interval was approximately 7 minutes. Within each session, trials were randomly started from four different points within the pool, which were never repeated within a training day. The first day of water maze training was a visible platform test of four trials, in order to determine whether there were any visual deficits among the animals. A visible platform (white, 2 cm above the water) was placed in the SE quadrant of the maze. Days 2-6 of training used the standard reference memory version of the water maze; the platform was submerged below the water surface and remained in the NE quadrant throughout training. Five days after the last training trial and 21 days after BrdU injection, rats received a 60-second probe trial, during which the platform was removed from the pool. Percentage of time spent in the platform zone was recorded and is considered a measure of spatial memory retention of the original hidden platform training (Vorhees and Williams, 2006; Morris, 1984). We chose to examine probe performance five days after training to have a measure of long-term memory (Vorhees and Williams, 2006) and also so that the BrdU-labelled cells would be more mature in order to examine activation of new neurons using zif268 (Epp et al., 2011). We also examined a more sensitive proximity measure as an index of memory and search strategy (Pereira and Burwell, 2015), the platform zone. The platform zone was defined as a circular region centered on the hidden platform encompassing 5% of the area of the water maze. Approximately 10 minutes after the probe trial, rats underwent four reversal trials in which the hidden platform was moved to the SW quadrant (Wagner et al 2013). Reversal training was conducted after the probe trial to allow for a measure of memory that was not contaminated by reversal learning, but it should be noted that the probe trial will also serve as an extinction trial as the rats did not find the hidden platform (Vorhees and Williams, 2006). Reversal learning is used as a proxy for cognitive flexibility (Vorhees and Williams, 2006). Intriguingly, reversal learning is altered after parity and in response to estrous cycle phase early after the postpartum period.
(Albin-Brooks et al., 2017; Workman et al., 2013). Total distance (m), swim speed (m/s) and latency (s) to reach the hidden platform were calculated for each day across the four trials using ANY-maze software. Early versus late acquisition can shed light on when treatments may be influencing behaviour and may represent different structural or neural changes with learning (Gholizadeh et al., 2013; Kleim et al., 2004; Packard and Goodman, 2013; Packard, 1999). Thus, we examined the acquisition data by bundling the data into early (Days 2-4) and late (Days 5-6) phases of testing (Engler-Chiurazzi et al., 2011).

Perfusion

Ninety minutes after the probe trial, rats were weighed and administered an overdose of sodium pentobarbitol and were perfused transcardially with 60 ml 0.9% saline followed by 120 ml 4% paraformaldehyde (Sigma-Aldrich). Just prior to perfusion, ovaries (sham rats only), uterine and adrenals were extracted and weighed (organ mass was divided by body mass to calculate a ratio and used in analyses). Due to an error, fewer animals had adrenals collected (n=4-6 per group). Brains were extracted and post-fixed in 4% paraformaldehyde overnight, transferred to 30% sucrose (Fisher Scientific) solution 24h later, and remained in sucrose solution at 4°C until sectioning. Brains were sliced using a Leica SM2000R microtome (Richmond Hill, Ontario, Canada) into 40μm coronal sections. Sections were collected in series of every 10th section throughout the entire rostral-caudal extent of the hippocampus and stored at -20°C in a cryoprotective solution consisting of 30% ethylene glycol (Sigma-Aldrich, St. Louis, MO, USA) and 20% glycerol (Sigma-Aldrich) in 0.1 M phosphate-buffer (PB, pH 7.4).

Hormone Assays

Premarin is a CEE that consists estrone (50%) and estradiol (0.1%), as both of these estrogens have been implicated in neurogenesis (Barha et al., 2009) and hippocampus-dependent learning (Barha et al., 2010), and are often different in parous groups (Bartlett et al., 2014), and thus, these estrogens were also assessed in the present study. Blood was taken at the time of perfusion from the right atrium. Blood samples were stored overnight at 4°C and centrifuged at 10,000g for 15 minutes. Serum was collected and stored at -20°C. To determine the concentration of estradiol and estrone in circulation, radioimmunoassay (RIA) was conducted in duplicate on serum according to manufacturer’s instructions (Beckman Coulter, Mississauga, Ontario, Canada).
sensitivity for the ultrasensitive 17β-estradiol kit is 2.2 pg/mL and the antibody is highly specific for 17β-estradiol, with 2.40 % cross-reactivity with estrone. The sensitivity for the estrone kit is 1.2 pg/mL and has 1.25 % cross-reactivity with 17β-estradiol. Average intra-assay coefficients of variation were 10.66% and 10.73% for the estrone and 17β-estradiol kit, respectively.

**Serum cytokine quantification**

Parity-related alterations in cytokine profiles have been detected in older women (Clendenen et al., 2011) and mice (Barrat et al. 1997). A large body of evidence implicates cytokines in cognitive function in aging (see review McAfoose and Baune, 2009) and cytokines are associated with synaptic plasticity, neurotrophic factors, and neurogenesis in rats (Borsini et al., 2015). Furthermore, cytokine profiles change over the course of the menopausal transition, and with estrogens in postmenopausal women (Abdi et al., 2016; Yasui et al., 2007). These findings point to inflammatory cytokines as an avenue for understanding the impact of parity and HT on cognitive aging. Serum cytokines were quantified using a multiplex electrochemiluminescence immunoassay kit (V-PLEX Proinflammatory Panel 2, Rat) from Meso Scale Discovery (Rockville, MD), used according to manufacturer instructions. Samples were run in duplicates and the following cytokines were quantified simultaneously in each sample: Interferon gamma (IFN-γ), Interleukin-1beta (IL-1β), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), CXCL1, Interleukin-10 (IL-10), Interleukin-13 (IL-13), and tumor necrosis factor alpha (TNF-α). A Sector Imager 2400 (Meso Scale Discovery) was used for plate reading, and the Discovery Workbench 4.0 software (Meso Scale Discovery) was used for data analyses. Lower limits of detection (LLODs) were as follows (pg/ml): IFN-γ: 0.163; IL-1β: 1.48; IL-4: 0.179; IL-5: 7.64; IL-6: 2.4; IL-10: 0.233; IL-13: 0.78; TNF-α: 0.156; and CXCL1: 0.085.

**Immunohistochemistry**

For each of the antibodies used in this study, we included a negative control using a subset of sections incubated in PBS instead of the primary antibodies (omitting one antibody at a time in the case of double-labelled immunofluorescence). All the negative controls resulted in the absence of immunoreactivity.

**DCX** One series of brain tissue was labelled for the immature neuronal marker doublecortin (DCX). DCX is expressed in all immature neurons ranging from hours to 21-30 days of age.
(Brown et al., 2003). Tissue was pretreated with 0.6% hydrogen peroxide for 30 minutes at room temperature after rinsing in 0.1 M PBS. It was then incubated at 4°C for 24 h in a primary antibody solution consisting of 1:1000 polyclonal goat anti-doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat# sc-8066, RRID: AB_2088494), 0.04% Triton-X, and 3% normal rabbit serum, dissolved in 0.1 M PBS. The tissue was washed in 0.1 M PBS and then incubated for 24 h at 4°C in a secondary antibody solution containing 1:500 biotinylated rabbit anti-goat (Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS. Tissue was then incubated for 4 h at room temperature in an avidin-biotin solution containing 1:1000 avidin and 1:1000 biotin in 0.1 M PBS (ABC kit; Vector Laboratories), rinsed in PBS, and washed in 0.175 M sodium acetate buffer. Doublecortin-expressing cells were visualized by developing tissue for approximately 10 minutes in a diaminobenzidine (DAB; Sigma Aldrich) solution. Once staining was complete, tissue was mounted on glass slides, dehydrated, cleared with xylene, and coverslipped using Permount (Fisher Scientific).

Zif268 A series of hippocampal sections was stained for zif268, an immediate early gene, that is required for long-term potentiation and memory consolidation (Bozon et al., 2003). Tissue was rinsed in 0.1M PBS overnight and 3 x 10 minutes the following day. The tissue was incubated in 0.6% H₂O₂ for 30 minutes at room temperature and re-rinsed. The tissue was then incubated in the primary antibody, rabbit anti-Erg-1 (Santa Cruz Biotechnology Cat# sc-189, RRID: AB_2231020) at 4°C for 18 h with 0.04% Triton-X and 3% normal goat serum (NGS; Vector Laboratories) in 0.1M PBS. Next, the tissue was rinsed in PBS and incubated in the second antibody solution (goat anti-rabbit Biotinylated IgG in 0.1M PBS; 1:1000; Vector Laboratories, Burlington, ON, Canada) for 18 h at 4°C. Tissue was then rinsed in 0.1M PBS and incubated for 1 h at room temperature in an avidin-biotin complex dissolved in 0.1M PBS, as per instructions in the ABC kit (Vector Laboratories). The tissue was rinsed first in 0.1M PBS followed by a rinse in sodium acetate. Brain sections were then transferred to a DAB solution and incubated for 5 minutes in a dark room, then rinsed with sodium acetate followed by 0.1M PBS. The tissue was mounted onto glass microscope slides, dried overnight, dehydrated in ethanol, cleared with xylene, and coverslipped with Permount.

BrdU/zif268 Immunofluorescent double-labelling of BrdU and zif268 was conducted to quantify the percentage of 21-day old neurons activated during spatial memory retrieval and
reversal learning. Staining began with three rinses for 10 minutes each in 0.1M PBS. The tissue was then incubated for 24 h in the zif268 primary antibody solution containing rabbit anti-zif268 (1:1000; Egr-1 SC-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA), with 4% normal donkey serum, 0.03% Triton X, and 0.1M PBS. The slices were then rinsed three times for 10 minutes in 0.1M PBS. Tissue was then incubated in the secondary antibody donkey anti-rabbit Alexa 488 (1:500; Invitrogen Molecular Probes, Oregon, USA) for 18 h at 4°C. The tissue was then fixed in 4% paraformaldehyde for 10 minutes, washed twice in 0.9%NaCl for 10 minutes each followed by 2N HCl at 37°C for 30 minutes. Next tissue was rinsed three times in 0.1M PBS and incubated in mouse anti-BrdU (1:500; Roche Diagnostics GmbH, Mannheim, Germany, Roche Cat# 11170376001, RRID: AB_514483) with 4% normal donkey serum, and 0.03% Triton X in PBS for 24 h at 4°C. The tissue was then rinsed three times for 10 minutes in 0.1M PBS after which it was incubated in donkey anti-mouse Cy3 (1:250: Jackson Immuno Research Laboratories Inc., Philadelphia, USA) for 18 h. To conclude, the sections were rinsed three times for five minutes each, mounted on glass slides, and coverslipped using PVA DABCO.

**BrdU/NeuN** A final series of hippocampal sections was double labelled for BrdU and NeuN, a mature neuronal protein, to quantify the proportion of adult-generated hippocampal cells with neuronal morphology. Sections were incubated in 0.1M TBS containing the primary antibody, 1:250 mouse anti-NeuN (EMD Millipore, Cat# MAB377, RRID: AB_2298772) at 4°C for 48 h. The tissue was rinsed for 10 minutes three times in TBS. Tissue was then incubated in the secondary antibody, donkey anti-mouse Alexa 488 (1:200; Invitrogen Molecular Probe, Burlington, ON, Canada), for 18 h. The tissue was then fixed using 4% paraformaldehyde for 10 minutes, rinsed two times for 10 minutes in 0.9% NaCl, and was incubated in 2N HCl at 37°C for 30 minutes. Tissue was then incubated in rat anti-BrdU (1:500; Bio-Rad / AbD Serotec Cat# OBT0030S, RRID: AB_609570) for 48 h at 4°C, and then incubated for 24 h in donkey anti-rat Cy3 (1:500; Jackson ImmunoResearch; PA, USA). The tissue was then rinsed three times for 10 minutes each in TBS, mounted on glass slides, and coverslipped using PVA DABCO.

**Cell counting**

Cells were considered to be BrdU-labelled if they were intensely stained and exhibited medium-sized round or oval cell body (Cameron, et al., 1993). All cell counting was performed
by an experimenter blind to treatment groups. BrdU-labelled cells were counted excepting those in the uppermost focal plane in every 10th section using an Olympus CX22 microscope with a 100x objective. DCX-expressing cells were counted in every 10th section of the hippocampus using a Nikon E600 light microscope under a 40x objective lens as described elsewhere (Barha and Galea, 2013, Barha et al., 2011b, Brummelte and Galea, 2010 and Epp et al., 2011). Total number of BrdU-labelled and DCX-expressing cells in each region (dorsal or ventral) were determined using a modified stereological optical fractionator method to account for low numbers as done previously (Epp et al., 2011; McClure et al., 2013; Tanapat et al., 1999). We noted whether immunoreactive cells were located in the dorsal or ventral GCL using the criterion defined by Banasr et al. (2006), as spatial memory is more strongly associated with the dorsal hippocampus and anxiety/stress behaviour with the ventral hippocampus (See Fanselow & Dong, 2010 for review). BrdU-labelled cells were three-week old cells that had been synthesizing DNA for a 2 h period 21 days prior to termination.

The percentages of BrdU labelled cells co-expressing zif268 or NeuN were calculated by identifying, exhaustively, whether BrdU-labelled cells were co-labelled with zif268 or NeuN. Area measurements for the GCL were obtained with digitized images and the software ImageJ (NIH). Volume estimates of the dentate gyrus were calculated using Cavalieri’s principle (Gundersen & Jensen, 1987).

DCX-expressing cells were classified into stages of maturity based on Plümpe et al. (2006). Type 1 cells (proliferative stage cells) included DCX-expressing cells with no processes or short, plump processes no longer than a cell width; Type 2 cells (intermediate stage cells) had unbranched processes of intermediate length reaching no farther than the moleculayer; Type 3 cells (postmitotic stage cells) had a more mature appearance, with denritic branching in the molecular and/or granule cell layer.

Zif268 Optical Density

We used quantitative densiometric analysis of DAB-stained sections to assess the expression of zif268 to allow us to examine differential expression between groups in specific sub-regions of the hippocampus (DG, CA1, and CA3). For each animal, eight sections of the hippocampus (four ventral, four dorsal) were examined for zif268 expression. Images of these sections were taken at 40X magnification using a Nikon E600. These images were converted to
8-bit images (grey scale) and the optical density of zif268-expressing cells was quantified using Image J (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA). From each segment, an ellipse of 50 μm area was sampled from the dentate gyrus, the CA1 hippocampal field, and the CA3 hippocampal field. To accommodate differences in staining between tissue samples, staining intensity was assessed by quantifying zif268 staining in the tissue background (using the corpus callosum). Therefore, a fourth ellipse was sampled from the background of the image. The optical density (OD) of each region of interest was expressed as the mean gray value per μm³. The background measure was subtracted from the OD in the regions of interest (DG, CA1, CA3) to obtain an OD measure that compensates for differences in intensity of staining.

**Data analyses**

All analyses were conducted using Statistica (Statsoft Tulsa, OK). Analysis of variance (ANOVA) were used on most dependent variables of interest with ovarian status (OVX, sham), hormone treatment (Premarin, oil) and parity (nulliparous, primiparous) as between-subjects variables. For some analyses, repeated measures ANOVA were used with training day (1 to 5), DCX cell maturity stage, region (dorsal, ventral), or hippocampal field (CA1, CA3, DG) as the within-subjects variables. Learning stage (early or late) was also used as a factor in some analyses. We used estrous cycle phase as a covariate in all analyses, and unless otherwise specified, estrous phase did not significantly influence the outcome. Post-hoc tests used Newman-Keuls. Any a priori comparisons were subjected to a Bonferroni correction. Pearson product-moment correlations were performed on dependent variables of interest. Effect sizes are given as partial η² or Cohen’s d where appropriate. The significance level was set at α=0.05

**Results**

Among primiparous rats, surgery and treatment groups were similar in their previous maternal behaviour towards offspring

Treatment and ovarian status groups did not differ significantly in maternal behaviour (See Table 2). There were no significant main effects or interactions between ovarian status or treatment on active (nursing, licking) versus passive/off-nest behaviours recorded on post-natal days 2-8 (p’s > 0.11).
Distance to reach the visible platform did not differ across groups

Two rats (Null-Sham-Oil and Null-Sham-Prem) were unable to find the visible platform during this phase of testing and were excluded from further testing. Rats improved significantly across visible platform training trials (main effect of trial, $F(3, 204) = 6.92, p < 0.01$; partial $\eta^2 = 0.09$ Figure 2A), but there were no other significant main or interaction effects ($p$’s $> 0.31$).

Premarin treatment impaired spatial reference acquisition in primiparous rats and enhanced spatial reference acquisition in nulliparous rats, regardless of ovarian hormone status.

As expected, distance travelled during reference memory training decreased significantly across days, $F(4, 272) = 71.39, p < 0.01$, partial $\eta^2 = 0.77$. There was a significant parity by treatment interaction in distance to reach the hidden platform, regardless of ovarian hormone status, $F(1, 68) = 4.96, p < 0.05$, partial $\eta^2 = 0.068$ (see Figure 2B and C). However, post-hoc tests did not reveal any significant differences between groups (all $p$’s $> 0.19$). Latency to reach hidden platform decreased across days, $F(4, 272) = 77.47, p < 0.01$, partial $\eta^2 = 0.53$, but there were no group differences in latency to reach the hidden platform ($p$’s $> 0.13$). Groups did not differ in swim speed across days ($p$’s $> 0.19$).

Other researchers have found effects of hormone administration and/or parity differ between early and late stages or blocks of learning (Frye, 1995; Barha et al., 2015; Barha & Galea, 2013; Acosta et al., 2009; 2010). To examine whether there were significant differences in total distance travelled during early versus late learning, we averaged the total distance across trials for days 2-4 and 5-6. Premarin-treated primiparous rats travelled longer distances to reach the platform than Premarin-treated nulliparous rats across days 2-4 but not 5-6 ($p$’s $< 0.01$ (Cohen’s $d=0.52$ and 0.73, respectively; Figure 2B and C; learning stage by parity by treatment $F(1,68) = 3.70, p < 0.05$; partial $\eta^2= 0.05$). Furthermore, Premarin treatment increased distance to reach the hidden platform on days 2-4 compared to oil treatment in primiparous rats ($p = 0.03$, Cohen’s $d= 0.41$) but decreased distances to reach the hidden platform in nulliparous rats ($p = 0.01$, Cohen’s $d= 0.51$; see Figure 2B and 2C).

Premarin improved spatial memory retrieval in sham-operated nulliparous rats.
Ovariectomy improved spatial memory retrieval in primiparous rats
Only two groups performed above chance in the percentage of time spent in the target quadrant (p’s < 0.01; both parity groups that were ovariectomized and treated with oil; see Table 3). The repeated measures ANOVA indicated a main effect of quadrant (F(3, 204)=27.00, p <0.01. partial η²= 0.28), indicating that all rats spent more time in the hidden platform quadrant but no other significant main effects (p’s > 0.07; Table 3). In order to determine whether there were any differences in memory retention in a more limited area we also examined platform zone. We expected a priori that reproductive experience would alter the effects of Premarin and perhaps ovarian hormone status on spatial memory retrieval. A priori analyses indicated that under sham surgery, nulliparous but not primiparous had improved probe trial performance with Premarin relative to oil (p’ s = 0.05 (Cohen’s d=1.0) and 0.37, respectively; Figure 3). Under ovariectomy, neither parity group was affected by Premarin (p’ s > 0.44), but Premarin-treated primiparous females spent a greater percentage of time in the platform zone than Premarin-treated nulliparous rats p = 0.03; Cohen’s d=1.0; Figure 3D). No other pairwise comparisons between parity groups or treatment groups were significant, p’s > 0.26. There were no other significant effects (all p’s ≥ 0.19). Only one group performed above chance, under ovariectomy the primiparous group given Premarin (t(12)=2.66, p < 0.05, all other p’s > 0.12).

**Under Premarin treatment, sham-operated primiparous rats have impaired performance on the first reversal trial compared to sham-operated nulliparous rats.**

Under Premarin treatment, primiparous sham-operated rats had greater distance to find the new platform location on the first reversal trial compared to all other groups (p’s < 0.01; Cohen’s d = 0.84 to 1.75; parity by ovarian hormone status by treatment by trial interaction: F(3,201) = 3.93, p < 0.01; partial η²= 0.06; see Figure 3A-C). There were also significant main effects of trials [F(3,201) = 27.19, p < 0.001; partial η²=0.29], surgery [F(1,67) = 5.62, p < 0.05, partial η²=0.077] and interactions (trial by parity and trial by ovarian hormone status by parity, both p’s < 0.05 partial η²=0.04, 0.07, respectively). Under ovariectomy, there were no group differences in distance to reach the hidden platform over trial (see Figure 3C).

**Premarin impairs early acquisition in primiparous but not nulliparous rats**

Due to the differences seen between parity groups in response to Premarin in early acquisition, we also created a variable to examine the contribution of early acquisition. As
expected, Premarin impaired early acquisition in primiparous rats ($p = 0.04$, Cohen’s $d = 0.63$) and compared to nulliparous rats given Premarin ($p=0.02$, Cohen’s $d = 0.71$), while Premarin tended to facilitate acquisition in nulliparous rats ($p> 0.10$; parity by treatment interaction $F (1, 68)=7.12$, $p < 0.01$, partial $\eta^2=0.094$; see Figure 3 E).

**Primiparous rats had greater number of BrdU-labelled cells than nulliparous rats.**
**Premarin decreased the survival BrdU-labelled cells in the ventral dentate gyrus but increased it in the dorsal dentate gyrus in sham-operated females.**

There were no significant differences in volume for the dorsal or ventral dentate gyrus (all $p$’s $>0.09$; Table 3) and thus, estimated total counts are used. Primiparous rats had more BrdU-labelled cells than nulliparous rats, regardless of treatment or ovarian hormone status (main effect of parity: $F (1,66)=4.71$, $p < 0.05$, partial $\eta^2=0.07$; see Figure 4A). In sham-operated females, Premarin increased the number of BrdU-labelled cells in the dorsal GCL ($p = 0.02$; Cohen’s $d=0.99$) but decreased the number of BrdU-labelled cells in the ventral GCL (Cohen’s $d=1.51$; Region by ovarian hormone status by Treatment interaction: $F(1,66) = 19.34$, $p < 0.001$, partial $\eta^2=0.22$), but there was no significant effect of Premarin in OVX groups; Figure 4B). Ovariectomized oil-treated rats had higher levels of BrdU in the dorsal dentate gyrus compared to Sham-oil treated females ($p=0.004$, Cohen’s $d=0.90$) but lower levels of BrdU-labelled cells in the ventral dentate gyrus compared to Sham-oil treated females ($p=0.01$; Cohen’s $d=0.94$). The majority of BrdU-labelled cells co-expressed NeuN (>83%), indicating they were new neurons, and there were no significant differences as a function of parity, treatment, or surgery (all $p$’s $>0.20$, see Table 3). The percentage of BrdU-labelled cells that co-expressing zif268 was not significantly different among groups (all other $p$’s $\geq 0.09$; Table 3).

**Primiparity increased DCX expression in the ventral dentate gyrus, and Premarin treatment reduced DCX expression dependent on region and parity.**

Premarin reduced DCX expression in the dorsal region in nulliparous rats compared to oil ($p=0.034$, Cohen’s $d=0.67$) and in the ventral dentate gyrus in primiparous rats ($p < 0.001$, Cohen’s $d=1.06$; region by parity by treatment interaction $F(1,62) = 12.59$, $p < 0.001$; partial $\eta^2=0.17$; See Figure 5A). Primiparous oil-treated rats had higher expression of DCX in the ventral region than nulliparous oil-treated rats ($p=0.014$, Cohen’s $d=0.61$). There were also main
effects of treatment and region (both $p$’s <0.05; partial $\eta^2$=0.07, 0.32, respectively) but no other significant effects on DCX-expressing cells ($p > 0.06$).

**Primiparity increased the proportion of proliferative immature neurons**

Primiparity increased the proportion of Type 1 (proliferative) DCX-expressing cells compared to nulliparous rats ($p = 0.04$) but parity did not significantly affect the proportion of Type 3 ($p = 0.10$) or Type 2 cells ($p = 0.67$; See Figure 5B; stage by parity interaction, $F(2,108) = 3.11$, $p < 0.05$, partial $\eta^2$=0.05). There were no other significant main or interaction effects on DCX morphology ($p$’s > 0.07).

**Primiparous rats have increased zif268 expression in the CA3 region after spatial memory retrieval and reversal learning compared to nulliparous rats. Premarin decreased dorsal CA3 zif268 expression in primiparous but not nulliparous rats.**

Primiparous rats had greater zif268 expression in the CA3 region than nulliparous rats (main effect of parity ($F(1, 52)= 5.51$, $p<0.05$ partial $\eta^2$=0.10), regardless of ovarian status. Ovariectomy reduced zif268 expression in the ventral CA3 region, regardless of parity ($F (1, 52) =5.57$, $p<0.05$, partial $\eta^2$=0.10). As expected there was greater zif268 expression in the dorsal compared to ventral CA3 region (main effect of region: $F(1,52)=53.39$, $p<0.001$). A priori we expected differences with treatment and parity, and Premarin decreased zif268 expression in the dorsal CA3 in primiparous rats ($p<0.01$ Cohen’s $d$=0.51) but not in nulliparous rats ($p$=0.64; see Figure 6).

In the CA1 region, there was greater zif268 expression in the dorsal versus ventral region, regardless of parity or treatment (main effect of region: $F (1,57)=6.72$, $p< 0.01$, partial $\eta^2$=0.11). Again, ovarian hormone status reduced zif268 expression in the ventral CA1 region, regardless of parity or treatment ($F(1, 57)=3.84$, $p=0.054$, partial $\eta^2$=0.06; Figure 6D). There were no other significant differences in the CA1 region between groups (all $p$’s > 0.17). There were no significant main or interaction effects on zif268 expression in the dentate gyrus (all $p$’s > 0.12; Table 3).

**Premarin increased TNF-α and CXCL1 in sham nulliparous, but not primiparous, rats**
Premarin increased serum levels of TNF-α and CXCL1 in sham nulliparous rats compared to oil-treated nulliparous rats (p=0.006, p=0.048, Cohen’s d=0.61, 1.72, respectively; see Figure 7). Premarin did not increase CXCL1 serum levels in primiparous rats (p’s > 0.7) but there was a significant effect of Premarin to decrease TNFα levels in primiparous rats (p=0.05; Cohen’s d=1.57). For TNF-α there was a significant interaction of parity by treatment by surgery (p=0.02, partial η²=0.13) while for CXCL1 this interaction was a trend only (p=0.07, partial η²=0.10). Sham rats (250.0 ± 35.6) had higher serum levels of CXCL1 than OVX (167.7 ±18.5) rats (main effect of surgery: F (1, 32) = 4.59, p <0.05, partial η²=0.13). There were no other significant effects for TNF-α or CXCL1. However, there was a trend for primiparous rats (8.34±1.87 pg/ml) to have higher serum levels of IL-6 compared to nulliparous rats (4.5±0.68 pg/ml; p=0.08, Cohen’s d=0.6). There were no other significant effects of surgery, Premarin or parity on any of the other cytokines: IL5, IL4, ILβ, IL13, IL10, IFNγ (all p’s > 0.16).

Premarin prevents ovariectomy-induced decrease in relative adrenal mass in primiparous rats only. Premarin treatment reduced relative ovary mass regardless of parity.

Ovariectomy significantly reduced relative adrenal mass (main effect of surgery: F(1,31) = 37.82, p < 0.0001, partial η²=0.22). However a priori results indicated that ovariectomy reduced relative adrenal mass in all groups (p’s < 0.002) except in Premarin-treated primiparous rats (p = 0.47; parity by surgery by treatment interaction, F (1,31) = 3.88, p < 0.05, partial η²=0.09; see Table 4).

Premarin treatment reduced relative ovary mass compared to oil treatment (main effect of treatment: F(1,28) = 6.86, p < 0.01, partial η²=0.20), regardless of parity (all other p’s > 0.36). As expected, ovariectomy significantly decreased relative uterine mass (main effect of surgery F(1,68) = 44.09, p < 0.001, partial η²=0.39). There were no other significant main or interaction effects (p’s > 0.06; see Table 4). As expected, ovariectomized rats were heavier than sham rats (main effect of surgery: F(1,68)=13.044, p<0.001; partial η²= 0.16; see Table 4), but there were no other significant effects on body mass at the time of perfusion.

Premarin treatment increased estrone levels regardless of parity or surgery, and increased 17β-estradiol levels to a lesser degree. Primiparous rats were more likely to be irregularly cycling.
As expected, Premarin increased both serum estradiol and estrone levels (treatment by estrogens interaction, $F(1,56) = 40.42, p < 0.001$; both $p's < 0.001$; partial $\eta^2= 0.42$) compared to oil treatment. Indeed, overall the increase in estrone levels was 8.6x greater with Premarin (Oil: 25.06±1.48; Premarin: 216.77±19.76 pg/ml) while estradiol increased by 14x than control levels with Premarin (Oil: 7.41±0.90; Premarin: 106.20 ± 8.78 pg/ml). In addition, estrone levels were non-significantly greater than estradiol serum levels under oil treatment ($p = 0.09$) but were significantly greater than estradiol concentrations under Premarin treatment ($p < 0.001$). There were no significant effects of parity or surgery on estrone or estradiol levels; however, this was likely due to the overwhelming treatment effects of Premarin. Importantly there were no significant differences between estrone or estradiol levels between OVX and sham groups ($p > 0.30$; See Table 5). Nulliparous rats were more likely to be regularly cycling than primiparous rats ($\chi^2=7.89, p <0.05$; Table 1).

Serum cytokine levels (IL4, IL6, IL10 and IL13) were positively correlated with neurogenesis in the Premarin-treated Primiparous group only, IL-13 was positively correlated with learning in Premarin-treated nulliparous rats only.

Premarin was associated with positive correlations between cytokines and BrdU-labelled cells in primiparous rats only (IL-4: $r=0.88, p=0.002$; IL-6: $r=0.92, p<0.001$; IL10: $r=0.81, p=0.008$; IL-13: $r=0.81, p=0.01$, see Figure 8); and while there were positive correlations in other cytokines, they did not survive Bonferroni correction including: IL-β: $r=0.75, p=0.02$; IFNγ: $r=0.76, p=0.02$; IL-5: $r=0.75, p=0.02$) but no significant correlations with CXCL1 or TNFα (p’s > 0.27). There were two positive correlations in nulliparous rats given Premarin but these did not survive Bonferroni correction (CXCL1: $r=0.77, p=0.02$ and IL-13: $r=0.68, p=0.04$). Primiparous rats given oil had two positive correlations with IL-10 and IL-5 and BrdU-labelled cells ($r=0.83, p=0.01$; $r=0.83, p=0.01$). There were no significant correlations among cytokines and neurogenesis for nulliparous oil-treated rats (all p’s > 0.36).

Significant correlations with cytokines and early learning were seen in nulliparous rats but not primiparous rats (all p’s > 0.10). Nulliparous rats treated with Premarin, regardless of ovarian hormone status, showed a negative correlation with IL-13 and early learning (days 2-4) (IL-13: $r=-0.82, p<0.01$), while there was a positive correlation with IL-5 and early learning that would not survive a Bonferroni correction (IL-5: $r=0.68, p=0.03$). There was a positive correlation
between ventral DCX-expression and distance travelled to reach the hidden platform on days 2 and 3 of training in the Premarin-treated primiparous rats only (r=0.74, p<0.0001) but a non-significant negative correlation in nulliparous rats.

Discussion

Parity influenced learning, neurogenesis and response to the hormone therapy, Premarin, in middle-aged female rodents. In middle age, primiparity was associated with more new neurons in the dentate gyrus, a greater proportion of proliferative immature neurons in the dentate gyrus, and a greater zif268 expression in the CA3 region compared to nulliparity. Furthermore, previously parous rats exhibited more irregular estrous cycling compared to nulliparous controls in middle age. Premarin impaired early spatial learning, early reversal learning and decreased immature neurons in the ventral dentate gyrus in primiparous rats, while Premarin had a beneficial effect on early learning in nulliparous rats. Furthermore, Premarin decreased zif268 expression in the dorsal CA3 region and increased adrenal to body mass ratio in primiparous, but not nulliparous rats. However, nulliparous rats had higher serum levels of the cytokines TNFα and CXCL1 with Premarin compared to primiparous rats. Ovarian hormone status altered fewer outcomes, as long-term ovariectomy enhanced spatial memory (as measured by probe trial performance) in nulliparous, but not primiparous rats, and reduced zif268 expression in the CA1 and CA3 region, regardless of parity. These findings indicate that parity has long-lasting effects on brain, immune signalling and behaviour in middle-aged females, and that a single reproductive experience alters the ability of the hormone therapy, Premarin, to exert its effects on neural and cognitive effects in mid-life. These reproductively experienced and Premarin treated animals all had spatial training and as such, we cannot eliminate the possibility that our neuroplastic findings are influenced by the learning paradigm. As such, the directionality of reproductive experience-dependent effects of Premarin cannot be determined. That is to say, we cannot determine whether Premarin treatment affected behaviour differently in nulliparous versus primiparous rats, which in turn altered physiology (neuroplasticity, adrenal mass, immune milieu), or if the behavioural effects were driven by the observed alterations in physiology. Regardless, our findings provide compelling evidence to suggest that female reproductive history should be considered in aging research.
**Premarin inhibited early spatial learning and reversal learning in primiparous rats but enhanced early learning and memory in nulliparous rats.**

In the present study, Premarin impaired early spatial training and reversal learning in primiparous but enhanced early spatial training in nulliparous animals, regardless of ovarian status. Furthermore, we found that Premarin enhanced spatial memory (probe trial) in sham nulliparous, but not primiparous, rats. These findings are consistent with other studies that show that the same dose of Premarin enhanced working and reference memory in nulliparous middle-aged rats (Acosta et al. 2009; Acosta et al., 2010; Engler-Chiurazzi et al., 2011). In contrast, Premarin impaired both reference and working memory in the radial arm maze in young adult nulliparous rats (Barha and Galea, 2013). Collectively, these results suggest that higher doses of Premarin facilitate learning and memory in middle-aged nulliparous rats, but intriguingly, our novel data suggests Premarin impairs learning and memory in middle-aged primiparous rats. These findings may also shed light on the findings that indicate little cognitive benefit from Premarin in postmenopausal women (Espeland et al., 2004; Gleason et al., 2015). Because the majority of women were parous in the women’s health initiative study (Shadyab et al., 2017), the impairments (or lack of benefit) may be due to the effects of parity on Premarin’s ability to influence spatial and reversal learning.

In the present study, long-term ovariectomy improved spatial memory (measured in the probe trial) in primiparous, but not nulliparous, rats. However, other studies have found short-term ovariectomy impaired spatial memory in middle-aged multiparous rats (Barha et al., 2015) and long-term ovariectomy improved spatial memory in older nulliparous rats (~22 months; Bimonte-Nelson et al., 2003). Collectively, these findings suggest that the duration of ovarian hormone deprivation can influence spatial memory, with short-term ovariectomy impairing, and long-term ovariectomy improving, spatial memory dependent on parity.

In the present study, reversal learning quickly reached asymptotic performance, which likely reflects the preceding probe trial, which would have acted as an extinction trial. Intriguingly, Premarin impaired the first trial of reversal learning only in sham-treated primiparous rats, which is consistent with previous findings indicating that higher levels of ovarian hormones were associated with greater number of perseverative errors in biparous rats (Workman et al., 2013). Collectively, Premarin, slightly but significantly, impaired early
performance in both the spatial and reversal learning in primparous but not nulliparous middle-aged rats.

**Primiparity is associated with greater neurogenesis levels in the dentate gyrus, while Premarin reduces neurogenesis in both nulliparous and primiparous rats dependent on region and age of new neuron following spatial training.**

In middle-aged females, primiparity was associated with increased neurogenesis (BrdU-labelled cells) and a greater number of immature neurons (DCX) in the ventral dentate gyrus compared to nulliparity in rats that underwent a behavioural test, consistent with a previous study examining immature neurons in multiparous middle-aged rats (Barha et al., 2015). While in the present study, we cannot determine whether parity would result in increased neurogenesis in cage controls, in the previous study, Barha et al. (2015) found that multiparity did increase neurogenesis (DCX-expressing cells) compared to nulliparity, regardless of behavioural training. In the present study, primiparous rats also had greater proportion of type 1 proliferative cells, which indicates that the enhanced neurogenesis with parity is associated with increased cell proliferation. Intriguingly, a previous study showed that multiparous rats which did not experience behavioural testing had a greater percentage of the postmitotic type 3 cells (Barha et al., 2015), which in combination with the current findings suggests that behavioural experience can alter the maturational trajectory of new hippocampal neurons dependent on parity. We have previously shown that the amount of parity affects neurogenesis in the early postpartum, with primiparous, but not biparous rats, showing a reduction in the survival of new neurons (Pawluski and Galea, 2007). Amount of parity also affects verbal memory deficits during pregnancy, with multigravid women having worse scores than primigravid women (Glynn, 2012). Taken together, these findings suggest that parity influences neurogenesis in the early postpartum and well into middle-age.

Premarin treatment in behaviourally tested rats increased the survival of BrdU-labelled cells in the dorsal dentate gyrus but decreased it in the ventral dentate gyrus in sham-operated females, regardless of parity. Premarin also increased neurogenesis in young adult nulliparous ovariectomized rats, but that study did not distinguish between dorsal or ventral regions (Barha and Galea, 2013). The finding that there were regional differences with Premarin may be related to the different functions attributed to these regions with the dorsal associated with reference...
memory and the ventral region associated with stress/anxiety. Given that Premarin increased adrenal mass in primiparous animals this may have contributed to the decrease in neurogenesis seen in the present study and may be related to the ability of Premarin to show some alleviation of negative mood symptoms in postmenopausal women (Gleason et al., 2015).

The Premarin-induced reduction in neurogenesis in spatially trained rats is also evident when examining immature neurons (DCX-expressing cells), but with regional differences as the reduction is seen in the ventral dentate gyrus of primiparous rats and the dorsal dentate gyrus of nulliparous rats. However, as described earlier Premarin reduced more mature neurons (the BrdU-labelled cells) in the ventral region, regardless of parity. Although it may seem puzzling that we see regional differences between DCX-expressing cells versus BrdU-labelled cells with parity and Premarin, this is likely due to the broad spread of ages of immature neurons in DCX-expressing cells, given that DCX is expressed for 21 days in rats (Brown et al., 2003). It is also important to keep in mind that new neurons may not function similarly in nulliparous versus primiparous animals. Indeed, we found more immature neurons in the ventral dentate gyrus were associated with poorer performance in the Premarin-treated primiparous, but not nulliparous, rats. Importantly, our findings cannot determine whether the effects of parity and Premarin to influence neurogenesis underlie the observed changes in cognition. Nonetheless, our findings suggest that the relationship between neurogenesis and acquisition depends on previous parity when under the influence of Premarin.

**Primiparous rats had greater activation (zif268 expression) in the CA3 region, while treatment with Premarin decreased expression in the dorsal CA3 in primiparous rats only**

Primiparous rats exhibited increased zif268 expression in the CA3 region after spatial memory retrieval and reversal training. This suggests that neuronal activation levels were higher in primiparous compared to nulliparous rats after spatial memory retrieval and reversal learning. This is partially consistent with findings of greater LTP, BDNF and enhanced pCREB in older multiparous mice compared to nulliparous mice (Tomizawa et al., 2003). Intriguingly, the same increase in zif268 expression was also seen in young adult nulliparous rats after different types of hippocampus-dependent learning compared to males (Yagi et al., 2015, 2017), suggesting that activation of the CA3 region is particularly important for cognition in female rats. Given that we saw increased CA3 activation in the primiparous rats only this may suggest that primiparous rats
have activation patterns similar to young adult female rats after spatial learning. This is reminiscent of the finding that estrogens upregulated cell proliferation in multiparous middle-aged and nulliparous young adult rats, but not nulliparous middle-aged rats (Barha and Galea, 2011). Collectively these studies indicate that parity may protect the hippocampal response to aging. However, future studies need to determine whether these cell-signalling and neurotrophic factors are responsible for the differences in response to hormone therapy in older females that are dependent on reproductive history.

In the present study, there was greater activation (zif268 expression) in the dorsal CA3 region compared to the ventral region, regardless of parity, which is consistent with another study in females (Yagi et al., 2015). We speculate that this increased dorsal relative to ventral expression is related to spatial memory and reversal learning. Indeed, zif268 is required for memory consolidation (Bozon et al., 2003) and dorsal hippocampus zif268 expression is important for reconsolidation (Lee et al., 2010). Further, the dorsal hippocampus is important for spatial reference memory (Pothuizen et al., 2004) and our observations of greater activation in the dorsal region are consistent with the idea that this area is important for spatial memory. However, neither Premarin nor parity altered activation of new neurons in response to spatial memory retrieval and reversal learning, in contrast to findings in young nulliparous rats given a lower dose of Premarin that saw decreased activation (Barha and Galea, 2013). The co-expression of BrdU/zif268 was very low in the present study, likely due to the older age of the rats compared to the previous study in young adult rats. Intriguingly, however, Premarin decreased activation of neurons in the CA3 region of the hippocampus after spatial memory retrieval and reversal learning but only in the primiparous rats. Further research should examine the relationship between immediate early genes and functioning of new neurons under different treatments with differing reproductive history and perhaps examine shorter and longer timelines of memory retrieval in older rats.

Possible mechanisms of long-lasting influence of reproductive experience on neurogenesis and spatial learning: immune signalling, estrogens, and adrenal steroids

TNF-α and CXCL1 were increased by Premarin in nulliparous rats but not in primiparous rats following behavioural testing. Interestingly, TNF-α has beneficial effects on synaptic strengthening (Beattie et al., 2002) and learning and memory (Baune et al., 2008) in the healthy
adult brain. Thus, the effect of Premarin to enhance learning in nulliparous rats and reduce learning in primiparous rats may be related to TNF-α. Because in nulliparous rats Premarin increased TNF-α and facilitated early learning in the same rats, but in primiparous rats Premarin decreased TNF-α and impaired early learning, these findings point to TNF-α as a possible mediator. Further, the same relationship of enhanced CXCL1 with aging and improved cognition is seen in aged male rats, as CXCL1 is also upregulated in the hippocampus after running (Speisman et al., 2013). This may suggest that the enhancement in cognition seen in Premarin-treated nulliparous rats may be related to increased CXCL1 in the present study. Although the direction of these relationships between cognitive function and cytokine levels cannot be determined from the current study, previous work indicates that even in behaviourally-naïve animals, parity alters the immune profile of aged animals (Barrat et al., 1997; Mahmoud et al., submitted). Intriguingly, we also found that IL-13 was positively related to acquisition in the nulliparous groups treated with Premarin only, this same relationship between increased IL-13 and better water maze performance has been shown in male mice (Brombacher et al., 2017). These results suggest differing relationships of circulating cytokine levels to learning dependent on previous parity.

Intriguingly, several cytokines (Il-6, IL-4, IL-10, IL-13) were positively associated with neurogenesis in primiparous rats treated with Premarin, but not in nulliparous rats, after behavioural testing. These data are somewhat consistent with the findings of Urza et al. 2017, showing that only after a challenge (ovarian tumor) did cytokines respond differentially in previous parous versus nulliparous mice. In our study, while we did not have an immune challenge, the rats did undergo challenges with cognitive testing, surgeries, and injections. These findings suggest that previous parity interacts with aging changing the relationship of neurogenesis with cytokine levels after hormone treatment and behavioural testing.

Premarin enhanced adrenal to body mass ratio in primiparous rats only which suggests that Premarin along with behavioural testing increases CORT in primiparous but not nulliparous, rats, as adrenal mass has been linked to serum CORT (Chan et al., 2014). As high CORT can interfere with learning and memory (Conrad, 2010) it is possible that Premarin worked via increased adrenal steroids to negatively affect learning and zif268 expression in the dorsal hippocampus in primiparous compared to nulliparous rats. The question of why this occurred is
an open one, although primiparous rats were more likely to cycle irregularly. Estrogens modulate hippocampus-dependent learning in a dose and estrogen type specific manner (Barha et al., 2010) and this may have played a role. Collectively these data suggest that levels of adrenal mass and specific cytokines are altered with previous parity and with exposure to Premarin. More studies are needed to identify neuroimmune interactions on cognition with parity and aging.

Conclusions

The present study shows that previous reproductive experience can increase neurogenesis, spatial acquisition, and disrupt cycling in middle-aged rats. Intriguingly, treatment with Premarin, the CEE hormone therapy, differentially affected middle-aged rats, dependent on previous reproductive experience. Premarin negatively influenced spatial acquisition and decreased activation of neurons (zif268) in the dorsal CA3 region in primiparous rats only, suggesting that previous parity also interacts with hormone therapy to exert effects on cognitive and neural measures. Premarin also increased serum cytokines TNFα and CXCL1 in nulliparous but not primiparous rats. In this study, all animals engaged in cognitive training and as such, we cannot determine whether the neuroplastic changes we saw are directly the result of Premarin or parity alone but may have been an interaction with training. Indeed, it is important to be aware that often neuroplastic changes with estrogens are either not seen or only emerge in cage controls (Frick et al.2004, Barha and Galea, 2013) but we argue that for translational value it is important to test whether a treatment influences neuroplasticity in conjunction with behavioural testing. As many studies that have examined the influence of age in females have used retired breeders, we caution that the effects seen in studies with retired breeders may be different from those obtained in nulliparous animals. These findings add to the growing field of how reproductive experience can have long-lasting influences on the physiology of the middle-aged female, but also suggest that parity may need to be taken into account when considering the influence of hormone therapies in older age. The fact that previous parity influences the efficacy of hormone treatment contributes to a growing research indicating that other treatments such as selective serotonin reuptake inhibitors influence coping behaviour and neurogenesis differently depending on parity (Workman et al., 2016; Gobinath et al., 2017; Overgaard et al., in press). Clearly, we are in the infancy of this field of determining how maternal experience influence the aging brain. In particular, there are clinical implications that women may need to consider reproductive history.
in their selection of HT and assessment of risk versus benefit of HT use to combat cognitive menopausal symptoms. In summary, our current data suggests that Premarin resulted in different, and sometimes opposing, outcomes on brain and behaviour in middle-aged rats dependent on parity. These findings underscore the necessity of considering reproductive history in individualized treatments in ageing women.

Acknowledgements

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References


Bridges RS, Byrnes EM. Reproductive experience reduces circulating 17beta-estradiol and prolactin levels during proestrus and alters estrogen sensitivity in female rats. Endocrinology. 2006;147:2575-82.


Epp JR, Haack AK, Galea LA. Activation and survival of immature neurons in the dentate gyrus with spatial memory is dependent on time of exposure to spatial learning and age of cells at examination. Neurobiol Learn Mem. 2011 Mar;95(3):316-25.


Rummel J, Epp JR, Galea LA. Estradiol does not influence strategy choice but place strategy choice is associated with increased cell proliferation in the hippocampus of female rats. Horm Behav. 2010;58(4):582-90.


Table 1. Percentage of sham-operated rats exhibiting regular cycling or irregular cycling (sample size in brackets). A chi-square on regular vs irregular cycling was significant $\chi^2=7.89$, $p=0.048$.

<table>
<thead>
<tr>
<th></th>
<th>% regular cycling</th>
<th>% irregular cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nulliparous - oil</td>
<td>66.67 (6)</td>
<td>33.33 (3)</td>
</tr>
<tr>
<td>Nulliparous - Premarin</td>
<td>12.50 (1)</td>
<td>87.50 (7)</td>
</tr>
<tr>
<td>Primiparous - oil</td>
<td>12.50 (1)</td>
<td>87.50 (7)</td>
</tr>
<tr>
<td>Primiparous - Premarin</td>
<td>28.57 (2)</td>
<td>71.43 (5)</td>
</tr>
</tbody>
</table>
Table 2. Mean (± SEM) percentage of time spent in active maternal behaviors versus passive or off-nest behaviors during postnatal days 2-8.

<table>
<thead>
<tr>
<th></th>
<th>Nursing/Licking</th>
<th>Passive/Off-Nest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham - oil</td>
<td>45.31 ± 5.64</td>
<td>54.69 ± 5.64</td>
</tr>
<tr>
<td>Sham - Premarin</td>
<td>58.33 ± 3.31</td>
<td>41.67 ± 3.31</td>
</tr>
<tr>
<td>OVX - oil</td>
<td>53.16 ± 4.97</td>
<td>46.84 ± 4.97</td>
</tr>
<tr>
<td>OVX - Premarin</td>
<td>51.45 ± 3.40</td>
<td>48.55 ± 3.40</td>
</tr>
</tbody>
</table>
Table 3. Mean(± SEM) percentage of BrdU-labelled cells co-expressing NeuN or zif268 in response to spatial memory retrieval and reversal training, dentate gyrus volume and total zif268 expression in the dentate gyrus (DG). There were no significant differences among groups.

<table>
<thead>
<tr>
<th></th>
<th>BrdU/NeuN</th>
<th>BrdU/zif268 %)</th>
<th>%Quadrant (Hidden)</th>
<th>DG Volume (mm³)</th>
<th>Zif268 Expression DG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oil</td>
<td>Premarin</td>
<td>Oil</td>
<td>Premarin</td>
<td>Oil</td>
</tr>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>84.47 ± 3.71</td>
<td>87.85 ± 3.60</td>
<td>0.57 ± 0.06</td>
<td>4.67 ± 2.70</td>
<td>29.4±2.5</td>
</tr>
<tr>
<td>Primiparous</td>
<td>83.99 ± 3.71</td>
<td>89.82 ± 3.76</td>
<td>1.68 ± 0.78</td>
<td>2.38 ± 2.38</td>
<td>28.5±4.6</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>83.07 ± 3.26</td>
<td>88.07 ± 3.07</td>
<td>2.7 ± 1.1</td>
<td>2.2 ± 0.75</td>
<td>31.7±2.2</td>
</tr>
<tr>
<td>Primiparous</td>
<td>90.47 ± 3.42</td>
<td>85.50 ± 2.67</td>
<td>1.9 ± 1.0</td>
<td>2.1 ± 0.9</td>
<td>34.3±2.9</td>
</tr>
</tbody>
</table>
Table 4. Mean (± SEM) body mass (g) and organ mass to body mass ratio for adrenals, uterus, and ovaries.

<table>
<thead>
<tr>
<th></th>
<th>Adrenals</th>
<th></th>
<th>Uterus</th>
<th></th>
<th>Ovaries</th>
<th></th>
<th>Body Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Ovariectomy</td>
<td>Sham</td>
<td>Ovariectomy</td>
<td>Sham</td>
<td>Ovariectomy</td>
<td>Sham</td>
</tr>
<tr>
<td><strong>Oil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>0.0165</td>
<td>0.0107</td>
<td>±0.001</td>
<td>0.0004*</td>
<td>1.207±0.14</td>
<td>0.536±0.15</td>
<td>0.0215±0.007</td>
</tr>
<tr>
<td>Primiparous</td>
<td>0.0170</td>
<td>0.0100</td>
<td>±0.001</td>
<td>0.001*</td>
<td>1.352±0.23</td>
<td>0.469±0.12</td>
<td>0.0216±0.006</td>
</tr>
<tr>
<td><strong>Premarin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>0.0174</td>
<td>0.0108</td>
<td>±0.003</td>
<td>0.001*</td>
<td>0.920±0.21</td>
<td>0.620±0.06</td>
<td>0.0178±0.003</td>
</tr>
<tr>
<td>Primiparous</td>
<td>0.0147</td>
<td>0.0135</td>
<td>±0.002</td>
<td>0.001</td>
<td>1.497±0.22</td>
<td>0.621±0.06</td>
<td>0.0194±0.005</td>
</tr>
</tbody>
</table>

* Indicates significant difference from sham-operated rats
Table 5. Mean(± SEM) serum estrone and estradiol levels (pg/ml) in sham-operated and ovariectomized nulliparous and primiparous rats receiving Premarin or oil treatment.

<table>
<thead>
<tr>
<th></th>
<th>Serum Estrone pg/ml</th>
<th>Serum Estradiol pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nulliparous</td>
<td>Primiparous</td>
</tr>
<tr>
<td>Sham</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oil</td>
<td>26.50 ± 2.52</td>
<td>24.88 ± 3.27</td>
</tr>
<tr>
<td>Premarin</td>
<td>198.05 ± 57.23*</td>
<td>235.25 ± 52.79*</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oil</td>
<td>23.27 ± 2.81</td>
<td>22.27 ± 2.81</td>
</tr>
<tr>
<td>Premarin</td>
<td>227.30 ± 44.74*</td>
<td>204.24 ± 26.34*</td>
</tr>
</tbody>
</table>

* Indicates significant difference from oil-treated group, p < 0.01
Figure Captions

**Figure 1.** Experimental outline. MWM = Morris Water Maze, BrdU= bromodeoxyuridine.

**Figure 2.** Group means ± SEM (standard error of the mean) of the distance travelled to reach the platform in the visible (A) and hidden platform trials (B-E). A. Means and SEM of visible platform learning curves for nulliparous and primiparous rats given oil or Premarin, regardless of ovarian hormone status. There were no significant differences between groups. B. Spatial learning as measured by distance to reach the hidden platform (m) in the Morris Water Maze across all 5 days of training for the nulliparous or primiparous sham or ovariectomized (OVX) rats treated with oil or Premarin. Premarin reduced the distance to reach the hidden platform in nulliparous rats but increased the distance to reach the hidden platform in primiparous rats (an effect more clearly seen in C and D). For C and D: Trials were also split into early and late training trials (Days 2-4 and Days 5-6 and averaged across the days included) to show the differences in Premarin treatment by parity were seen in the early but not late training trials, Nulliparous (C) and Primiparous (D). In E, the graph depicts nulliparous vs primiparous in the early and late training trials treated with Premarin. * denotes $p < 0.05$ indicate different from oil controls (C,D) and between parity groups (E).

**Figure 3.** Mean±SEM (standard error of the mean) of the percent time spent in the platform zone and during the four trials of reversal learning. Reversal Learning: Under sham surgery, Premarin treatment increased distance to reach the hidden platform on the first trial compared to oil in primiparous rats (B) but there were no significant differences with Premarin in nulliparous rats (A). Under ovariectomy, there were no group differences in distance to reach the hidden platform over trials (C). Time spent (%) in the platform zone during probe trial (D). Under sham surgery, nulliparous rats spent significantly more time in the platform zone under Premarin treatment compared to oil treatment. Under ovariectomy, Premarin-treated primiparous females spent significantly more time in the platform zone than Premarin-treated nulliparous rats. Dashed line indicates chance level. * denotes $p < 0.05$ and indicates difference on day 1 between oil and Premarin treated Primiparous rats (B) and between oil and Premarin groups in (D) or between parity groups treated with Premarin in D. (E) An early learning measure was created by aggregating early learning in the initial hidden platform (days 2-4) and in the first trial of reversal training to create an early learning measure. Primiparous rats treated with Premarin had worse performance than primiparous rats treated with oil or nulliparous rats treated with Premarin. *denotes $p <0.05$.

**Figure 4.** Mean ± SEM (standard error of the mean) of the density of BrdU-labelled cells. (A) Representative photomicrograph of a BrdU-labelled cell in a primiparous female, scale bar = 10µm. (B) Confocal image of a BrdU/NeuN co-labelled cell, green=NeuN, red=BrdU. (C) Primiparous rats had more BrdU-labelled cells than nulliparous rats surviving 21 days (+ SEM) in the dorsal and ventral GCL. * denotes $p < 0.05$ (D) Premarin treatment decreased the survival BrdU-labelled cells in the ventral dentate gyrus, but increased survival in the dorsal dentate
gyrus in sham-operated females* denotes $p < 0.05$ indicating difference from oil-treated group.
OVX: ovariectomy, BrdU=bromodeoxyuridine.

**Figure 5.** Mean+SEM (standard error of the mean) of the density of doublecortin (DCX)-expressing cells and proportion of DCX-expressing cells in different stages of development. (A) Premarin treatment decreased the density of doublecortin (DCX)-expressing cells in the ventral dentate gyrus of primiparous rats and in the dorsal dentate gyrus of nulliparous rats compared to oil-controls. Asterisks denote $p < 0.05$ for these comparisons (B) Primiparity increased the proportion of Type 1 DCX-expressing cells compared to nulliparous rats ($p = 0.044$ denoted by *) but parity did not significantly affect the proportion of Type 3 cells ($p = 0.10$) or Type 2 cells ($p = 0.69$). (C) Photomicrographs of representative Type 1 (proliferative), 2 (intermediate) and 3 (postmitotic) DCX-expressing cells.

**Figure 6.** Mean+SEM (standard error of the mean) of optical density of zif268 expression in the CA3 and CA1 region. (A) Primiparity increased zif268 expression in the dorsal and ventral CA3 region in response to spatial memory regardless of treatment, ovarian hormone status, or region (main effect of parity). (B) Ovariectomy decreased zif268 expression in the ventral CA3 region (main effect of ovarian hormone status), regardless of parity or treatment. (C) Premarin decreased zif268 expression in the dorsal CA3 region in response to spatial memory in primiparous but not nulliparous rats. (D) Ovariectomy decreased zif268 expression in the ventral CA1 region, regardless of parity or treatment (main effect of ovarian hormone status) similar to what was seen in the CA3 region. E. Photomicrograph of zif268 expression. Asterisks denote $p < 0.05$.

**Figure 7.** Mean+SEM (standard error of the mean) of serum cytokine levels CXCL1 and tumor necrosis factor (TNF)-α. Premarin increased serum CXCL1 (A) and TNF-α (B) levels in sham nulliparous but not primiparous rats, regardless of ovarian hormone status. Asterisks denote $p < 0.05$ from oil-treated groups.

**Figure 8.** Scatterplots of Pearson product-moment correlations between serum cytokines and BrdU-labelled cells in the hippocampus between nulliparous and primiparous middle-aged rodents treated with Premarin, regardless of ovarian hormone status (A-D). Premarin was associated with positive correlations between cytokines and BrdU-labelled cells in primiparous rats only (IL-4: $r=0.88$, $p=0.002$ (A); IL-6: $r=0.92$, $p<0.001$ (B); IL10: $r=0.81$, $p=0.008$ (C); IL-13: $r=0.81$, $p=0.01$ (D)). In nulliparous rats given Premarin there was one significant correlation with BrdU-labelled cells and serum IL-13 but this did not survive Bonferroni correction (IL-13: $r=0.68$, $p=0.04$ (D)). E and F. Significant correlations with cytokines and early learning were seen in nulliparous rats but not primiparous rats (all p’s $> 0.10$). Scatterplots of early learning distance travelled in the Morris Water Maze in Nulliparous (E) or Primiparous (F) with IL-13 with rats treated with Premarin or oil. Nulliparous rats treated with Premarin, regardless of ovarian hormone status, showed a negative correlation with IL-13 and early learning (days 1-3) (IL-13: $r=-0.82$, $p<0.01$ (E) that was not seen in Primiparous rats treated with Premarin(F).
**Figure 1**

- **Parturition**
- **Ovariection or sham surgery**

Timeline:
- 5 months
- 8 months
- 14 months

**Procedure:**
- **d2**: BrdU 200 mg/kg i.p.
- **d12-17**: MWM training
- **d22**: Probe & Reversal Perfusion

**Procedure details:**
- Daily oil or Premarin injections 22 days (d1-22)
Figure 2

A. Visible Platform Training

B. Hidden Platform Training

C. Comparing Premarin with Parity
Figure 4

A. GCL
Hilus
10 µm

B.

C. Number of BrdU-labelled cells for NULLIPAROUS and PRIMIPAROUS groups in Dorsal and Ventral regions.

D. Number of BrdU-labelled cells for Sham-Oil, Sham-Premarin, OVX-oil, and OVX-Premarin groups in Dorsal and Ventral regions.

* indicates significant difference.
Figure 5

A

Number of DCX-expressing cells

Dorsal Ventral

Nulliparous-Oil Nulliparous-Premarin Primiparous-oil Primiparous-Premarin

* * *

B

Proportion of DCX-expressing cell type

Type 1 Type 2 Type 3

NULLIPAROUS PRIMIPAROUS

C

1 2 3

Scale bar: 10µm
Figure 7

A

![Graph A](null)

Nulliparous Primiparous

Serum CXCL1 (pg/ml)

Nulliparous - Oil

Oil: 269

Nulliparous - Premarin

Premarin: 245

B

![Graph B](null)

Nulliparous Primiparous

Serum TNF-a (pg/ml)

NULLIPAROUS-OIL

PRIMIPAROUS - OIL

NULLIPAROUS-PREMARIN

PRIMIPAROUS-PREMARIN

*