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Estradiol replacement extends the window of opportunity for hippocampal function

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Abstract

We previously reported that treating aged female rats, ovariectomized (OVX) as young adults, with acute proestrous levels of 17 β estradiol (E2) increases CA1 spine density, NMDAR/AMPA ratio, GluN2B-mediated NMDAR current, and LTP at CA3-CA1 synapses if administered by 15, but not at 19, months post-OVX, defining the critical window of opportunity. Importantly, when rats are aged with ovaries intact until OVX at 20 months, hippocampal E2 responsiveness is maintained, indicating the deficit at 19 months post-OVX is a consequence of the duration of hormone deprivation and not chronological age. Here, we find the beneficial effect of E2 on novel object recognition in OVX rats was constrained by the same critical window. Furthermore, chronic low level E2 replacement, commenced by 11 months post-OVX using subcutaneous capsules removed 2 weeks prior to acute proestrous E2 treatment, prevents the loss of hippocampal responsiveness at 19 months post-OVX. These data define the dynamic nature of the critical window showing that chronic replacement with physiological E2 levels within a certain period post-OVX can lengthen the window.

1. Introduction

Estrogen replacement therapy (ERT) is effective in some, but not all women, in the treatment of post-menopausal cognitive decline (Phillips and Sherwin, 1992, Sherwin, 1997, Shumaker, et al., 2004, Shumaker, et al., 2003). Growing evidence suggests this variability in response to ERT may be due in part to a critical period, or window of opportunity, during which time ERT must be initiated to remain beneficial for cognition (Acosta, et al., 2013, B. B. Sherwin, 2007, Daniel and Bohacek, 2010, Davey, 2013, Gibbs, 2000, Lord, et al., 2006,

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Sherwin, 2006, Singh, et al., 2013, Smith, et al., 2010). The ovarian estrogen, 17 β -estradiol (E2), has profound effects on hippocampal structure and function, a brain region critically involved in learning and memory processes. Changes in responsiveness of hippocampal neurons to E2 caused by the duration of hormone deprivation, normal aging processes, or a combination of both, may contribute to the effectiveness, or lack thereof, of ERT in menopause.

In young adult cycling female rats at proestrus or in ovariectomized young adult rats given exogenous E2 to mimic proestrus, dendritic spine density on hippocampal CA1 pyramidal cells, NMDAR transmission mediated by GluN2B-containing receptors, and the magnitude of long-term potentiation (LTP) at CA3-CA1 synapses, a cellular correlate of learning and memory (Malenka and Bear, 2004) are increased (Bi, et al., 2001, Gould, et al., 1990, Smith and McMahon, 2005, Smith and McMahon, 2006, Snyder, et al., 2010, Warren, et al., 1995, Woolley, et al., 1990). Importantly, the increase in GluN2B-mediated transmission is causal to the increase in LTP (Smith and McMahon, 2006) and to enhanced novel object recognition (NOR) in E2-treated OVX rats (Vedder, et al., 2013), mechanistically linking together the heightened plasticity with improved behavioral performance in NOR, a task that requires hippocampal NMDARs (Lewis, 2008; Vedder, et al., 2013).

In aged OVX rats, the duration of ovarian hormone deprivation dictates whether E2 replacement will be beneficial for hippocampal spine density, synaptic physiology, and learning, supporting the critical window hypothesis (Bohacek and Daniel, 2010, Daniel, et al., 2006a, Gibbs, 2000, Gibbs, et al., 2009, Savonenko and Markowska, 2003, Smith, et al., 2010, Wu, et al., 2013). We previously reported that up to 15 months post-OVX, E2 replacement is capable of increasing CA1 dendritic spine density, GluN2B-mediated transmission, NMDAR/AMPA ratio, and LTP similar to that in young adult OVX rats following a 2 week deprivation (Smith and McMahon, 2005, Smith and McMahon, 2006, Smith, et al., 2010). However, E2 replacement was completely ineffective at 19 months post-OVX, defining the window of opportunity for these beneficial effects on hippocampal plasticity following long-term ovarian E2 deprivation. Importantly, the loss of E2 effectiveness in the 19 month post-OVX group is due to the duration of E2 deprivation rather than chronological age because the hippocampus in rats aged with intact ovaries prior to undergoing OVX at the same chronological age as the 19 month-post-OVX group is responsive to subsequent E2 replacement one month post-OVX when the rats are 21 months old (Smith, et al., 2010). This protection of hippocampal responsiveness to E2 replacement is likely a consequence of maintained low plasma levels of ovarian E2 throughout the aging process, since ovarian function enters a phase of constant diestrus or estrus during estropause in rats (Chakraborty and Gore, 2004, Hung, et al., 2003), unlike in women, where ovaries discontinue E2 production in menopause (Klinga, et al., 1982).

In the current study, we first aimed to determine whether E2 enhances NOR within the constraints of the same window of opportunity we previously defined in our experiments of hippocampal structure and synaptic physiology (Smith, et al., 2010). Next, we sought to investigate whether this window can be extended in OVX rats through chronic low level E2 replacement, mimicking the benefits of aging with intact ovaries we previously observed.

2. Methods

All experiments were performed in accordance with the Institutional Care and Use Committee of the University of Alabama at Birmingham.

2.1 Animals

Sprague-Dawley female rats were purchased from Charles River Laboratories and allowed at least one week to acclimate to the animal facility before undergoing ovariectomy (OVX). Rats were kept on a 12hr/12hr light/dark cycle and given unlimited access to food and water.

2.2 Ovariectomy and E2 replacement

All surgeries were performed under isoflurane anesthesia using aseptic conditions. Rats were subcutaneously injected with carprofen (10mg) for analgesia prior to surgery. Removal of the ovaries was performed bilaterally as previously described (Smith and McMahon, 2005). For most experiments, young adult female rats underwent OVX at 7–9 weeks of age and were used in experiments at 9, 15, or 19 months post-OVX to assess the ability of acute proestrous-like E2 to enhance NOR (Fig. 1A–C). The 9 and 15 month post-OVX groups received subsequent chronic E2 or cholesterol replacement, were re-tested for E2 enhanced NOR, then sacrificed for hippocampal synaptic physiology and spine density measurements (Fig. 1A, B and described below). In a separate cohort, rats were aged with ovaries intact and underwent OVX at 20 months of age and tested for the ability of proestrous-like E2 treatment to enhance NOR at the same chronological age as the 19 month post-OVX group.

E2 was replaced using acute and chronic paradigms. To generate acute proestrous-like E2 levels 9, 15, and 19 post-OVX rats, and rats aged with ovaries intact with OVX at 20 months of age were treated with two subcutaneous injections of E2 in cotton seed oil (10 µg/250kg; 24 hr interval) or oil alone, as done previously (Smith and McMahon, 2005, Smith and McMahon, 2006, Smith, et al., 2010, Vedder, et al., 2013, Woolley and McEwen, 1993) and tested for NOR 24 hrs after the second E2 injection (E24).

As mentioned above, following NOR, the 9 and 15 month post-OVX groups were used for the Adult-Replaced and Middle-Age-Replaced groups respectively, in the following chronic replacement experiments. Rats were implanted with silastic capsules (0.058" ID, 0.077" OD, Dow Corning Corp) sealed with type-A medical adhesive (Factor II, Inc) at the nape of the neck to deliver E2 (25% E2 diluted in cholesterol), or cholesterol (Chol) until all rats were 18 months post-OVX (chronological age 20 months). The Adult-Replaced group was implanted with capsules between 9.5 and 11 months post-OVX (chronological age 11.5–13 months) and the Middle-Age-Replaced group at 15 months post-OVX (chronological age 17 months). Therefore the Adult-Replaced group received 7–8.5 months and the Middle-Age-Replaced group received 3 months of E2 (or Chol) replacement. Capsules were replaced every 3 months and were finally removed when rats reached a chronological age of approximately 20 months in order to deplete plasma E2 in those rats with E2-containing capsules; rats were subsequently treated 2 weeks later with proestrous-like levels of E2 using the 2 injection protocol described above (10 µg/250kg; 24 hr interval) and tested for NOR at E24. Two weeks after NOR testing, these rats were treated again with E2 using the

2 injection protocol and sacrificed at E24 for spine density analysis and synaptic physiology experiments.

2.3 Blood collection and serum analysis

To determine the circulating plasma E2 levels produced by the silastic capsules, blood was collected from the saphenous vein at various time points after capsule insertion (Supplemental Fig. 1) using a separate cohort of OVX females (N=3) not undergoing testing. This protocol produced diestrus physiological levels of E2 as well significant weight loss suggestive of E2 exposure (Supplemental Fig. 1A–B) as shown previously (Gibbs, 2000). In experimental rats, blood was collected through the saphenous vein immediately before capsule removal surgery and via cardiac puncture before perfusions prior to decapitation and acute hippocampal slice preparation. Blood samples were kept at room temperature for at least 20 minutes for coagulation to occur and then spun for 20 minute at 3000 rpms for serum collection. Serum samples were stored at -80°C until assayed for E2 levels. E2 serum levels were assayed by Cayman Chemical using an EIA kit (Cayman Chemical).

2.4 Animal body weights and serum E2 levels in chronic replacement studies

Chronic E2 replacement significantly reduced body weight compared to Chol-treated controls in both Adult-Replaced and Middle-Age-Replaced groups (Supplemental Fig. 2A1,B1) (Adult-Replaced, Chol (N=9): $802 \pm 47\text{g}$ vs E2 (N=14): $642 \pm 28\text{g}$, $t(22)=3.14$ $p<0.005$; Middle-Age-Replaced, Chol (N=11): $804 \pm 51\text{g}$ versus E2(N=14): $676 \pm 19\text{g}$, $t(23)=2.58$, $p<0.05$). Serum from replaced rats was collected prior to capsule removal surgery to verify the circulating E2 levels supplied by the capsules. We found pre-capsule removal serum E2 levels were undetectable in all Chol-replaced animals. Average serum E2 levels in E2-replaced groups was $50 \pm 6\text{ pg/mL}$ for Adult-Replaced and $61 \pm 33\text{ pg/mL}$ for Middle-Age-Replaced. These averages did not differ significantly, $t(10)=0.55$ $p=0.60$. Blood was also collected through cardiac puncture just prior to brain slice preparation to further measure the E2 levels following the acute two day injection protocol. These E2 levels were within physiological range for all groups but were significantly higher in Chol treated animals (Adult-Replaced, Chol: $109 \pm 17\text{ pg/mL}$ vs E2: $47 \pm 9\text{ pg/mL}$, $t(23)=3.40$ $p<0.005$; Middle-Age-Replaced, Chol: $80 \pm 12\text{ pg/mL}$ vs E2, $41 \pm 11\text{ pg/mL}$, $t(29)=2.29$, $p<0.05$), although these increased plasma E2 levels did not influence experimental outcome (Figs. 3 and 4).

2.5 Replacement uterine weights

Complete removal of ovaries was verified in each animal after sacrifice for brain slice preparation. In chronic replacement experiments, both Chol and E2-replaced groups were treated acutely with the same two daily injections of E2 ($10\mu\text{g}/250\text{kg}$) prior to the final experiment and therefore all groups had uteri consistent with E2 exposure (Supplemental Fig. 2 A2, B2). E2 replaced rats had significantly higher uterine weights than Chol replaced rats in both the Adult-Replaced and Middle-Aged-Replaced groups likely due to the previous chronic E2 treatment, even though the serum levels after acute E2 injections were higher in Chol replaced rats (Adult-Replaced; Chol (N=10): $0.31 \pm 0.03\text{g}$ vs E2 (N=14): $0.43 \pm 0.03\text{g}$, $t(22)=3.12$, $p<0.005$, Middle-Age-Replaced; Chol (N=11): $0.27 \pm 0.03\text{g}$ vs E2

(N=14): $0.38 \pm 0.02g$, $t(23)=2.91$, $p<0.01$). These results suggest that the replacement protocols also protect the uterine responsiveness to E2.

2.6. Novel object recognition

NOR was performed as done previously (Vedder, et al., 2013). Rats were handled by the experimenter for 1 min and then placed in the empty NOR box (black plexiglas, 40cm $\times$ 40cm $\times$ 80 cm, lxwxh) for 10 min. Twenty-four hrs later, rats were placed in the NOR box with two identical objects (plastic, rubber, or glass objects matched in size). Control experiments were performed to ensure no object preference or side preference. Animals were allowed to investigate the objects for 3 min (training) and were then returned to their home cages. Two hrs later, rats were returned to the NOR box and allowed to investigate one familiar and one novel object for 3 min (testing). Sessions were video-taped and the amount of time spent with the novel versus familiar object was scored by an experimenter blinded to animal treatment and object novelty.

2.7. Electrophysiology

Rats were perfused transcardially with ice cold high sucrose, low calcium, and low sodium artificial cerebral spinal fluid (aCSF) with the following concentrations in mM: 85 NaCl, 2.5 KCl, 4 MgSO₄, 0.5 CaCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 25 glucose and 75 sucrose. The aCSF was saturated with 95% O₂:5% CO₂ (pH 7.4). As described previously (Smith, et al., 2010), acute hippocampal coronal slices were prepared at 400 μ m using a Leica vibratome and stored for 1 hr at room temperature submerged in 95% O₂:5% CO₂ saturated normal aCSF with the following concentrations in mM: 119 NaCl, 26 NaHCO₃, 2.5 KCl, 1 NaH₂PO₄, 2.5 CaCl₂, 1.3 MgSO₄, and 10 glucose. Electrophysiology recordings were performed in a submersion chamber continuously perfused with normal aCSF at 28°C and data were acquired using custom-made Labview software. Extracellular dendritic field potentials (fEPSPs) were generated using a bipolar tungsten electrode placed in stratum radiatum as previously described (Smith and McMahon, 2005, Smith, et al., 2010). After 20 min of stable baseline transmission, high frequency stimulation (HFS, 100 Hz, 0.5 s duration, 4 times, delivered at 20 s intervals, with a current 1.5X that used during baseline stimulation) was applied to induce LTP. Data were excluded if baseline transmission varied by greater than 8%. Recordings were performed using an Axoclamp 2A amplifier (Molecular Devices). Whole-cell patch clamp recordings were obtained using the blind patch technique and an Axopatch 200B amplifier, as done previously (Smith and McMahon, 2005, Smith, et al., 2010). Briefly, electrodes (3–6 M Ω) were filled with 120 mM cesium methanesulfonate, 0.6 mM EGTA, 2.8 mM NaCl, 5 mM MgCl₂, 2 mM ATP, 0.3 mM GTP, 20 mM Hepes, 5 mM QX-314, and 0.4% biocytin. GABA_ARs were blocked with picrotoxin (100 μ M), and L-type Ca²⁺ channels were blocked with nifedipine (10 μ M) to prevent their activation at depolarized potentials. Glutamate currents were evoked using a stimulating intensity that generated a 100–200 pA EPSC amplitude. To maximize the NMDAR contribution to the evoked glutamate current, cells were voltage-clamped at –20 mV. The contribution of GluN2B-containing NMDARs to the total NMDAR current was determined using the selective antagonist Ro 25-6981 (Sigma, RO; 1.0 μ M). Next, the remaining NMDAR current was pharmacologically blocked using DL-APV (Sigma, 100 μ M), and finally, the remaining glutamate current, which should be completely due to activation of

AMPA receptors, was blocked with DNQX (Sigma, 10 μ M). The contribution of each current was calculated by subtraction as done previously (Smith and McMahon, 2006, Smith, et al., 2010) and the NMDAR/AMPA ratio was calculated using the current amplitudes obtained by subtraction from the total glutamate current.

2.8. Histology

During whole-cell recordings, cells were filled with biocytin and at the end of the recording slices were fixed in 4% paraformaldehyde and stored overnight. Next, slices were washed 3 times for 10 min with PBS before 2 hr incubation with streptavidin conjugated Alexa 488 (10 μ M in PBS+1% Triton-X). Finally, slices were washed three times for 30 min each with PBS and mounted for imaging. Cells were imaged on a Leica Confocal microscope using Leica Application Suite Software and Z-stacks (0.2 μ M in thickness) were acquired using a 63X objective and 3X digital zoom. Cells were imaged and analyzed by an experimenter blind to animal treatment. Dendritic spines were counted from sections of dendrites 10 μ m in length by sequentially moving through individual Z-stacks. 5 sections of dendrite were counted per cell with 1–3 cells included per animal.

2.9. Statistics

Significant NOR was defined using a one-sample t-test comparing the percent of time investigating the novel object (time investigating novel object/total time investigating any object) compared to chance (50%). LTP experiments were normalized to the average value of the 20-minute baseline and the percentage of LTP for each slice was obtained by averaging 40 sweeps at 30 minutes post-HFS and comparing to baseline. If more than one slice was recorded from an individual rat, the percent LTP was obtained by averaging the data from all slices. Therefore, the n number represents animal number rather than slice number. For whole-cell data, if more than one cell was recorded from a rat, this data was also averaged together so that numbers represent animal numbers, rather than cell numbers. Statistical comparisons of LTP magnitude, whole-cell recordings and spine density between Chol and E2 replaced groups within either the Adult-Replaced or Middle-Age-Replaced groups were made using student t-tests. For all tests, $p < 0.05$ was considered significant.

3. Results

3.1 Rats with acute E2 treatment at 9 and 15 months post-OVX, but not after 19-months post-OVX, recognize novel objects

We recently reported in young adult OVX rats, that exogenous E2 treatment at proestrous-like levels increases NOR, a behavioral task requiring hippocampal NMDARs (Lewis, 2008, Vedder, et al., 2013). Further, this increase in NOR occurs only at time points at which this same treatment increases the LTP magnitude at CA3-CA1 synapses recorded in slices (Smith and McMahon, 2005, Vedder, et al., 2013). Moreover, both the increase in LTP magnitude and the heightened NOR require GluN2B-containing NMDARs (Smith and McMahon, 2006, Vedder, et al., 2013), linking together enhanced synaptic plasticity and learning. Here we tested whether this association between enhanced LTP and NOR also occurs following prolonged E2 deprivation and aging. We found that vehicle-treated OVX rats at 9, 15, and 19 months post-OVX did not spend more time than chance with the novel

object, consistent with a cognitive impairment (Fig. 2A2-C2, V, 9-month post-OVX (N=11): $55.7 \pm 8.3\%$, $t(11)=0.68$, $p=0.26$; 15 month post-OVX (N=11): $53.5 \pm 7.1\%$, $t(11)=0.49$, 19 month post-OVX (N=7): $57.5 \pm 4.5\%$, $t(6)=1.97$, $p=0.1$). However, acute E2 treatment in both the 9 and 15 month post-OVX, but not in the 19 month post-OVX groups, enables significant NOR (Fig. 2A2-C2, E2, 9-month post-OVX (N=12), $63.4 \pm 5.2\%$, $t(12)=2.56$, $p<0.05$, 15-month post-OVX (N=11): $65.0 \pm 5.3\%$, $t(11)=2.81$, $p<0.005$; E2, 19 month post-OVX (N=11): $55.0 \pm 6.0\%$, $t(10)=0.84$, $p=0.42$). Importantly, these findings of significant NOR only at 9 and 15 months post-OVX parallels precisely our previous findings that E2 increases LTP only at 9 and 15 months post-OVX, but not at 19 months post-OVX (Smith, et al., 2010). No differences in duration of time spent exploring objects during the training or testing sessions were observed in any of the groups (Supplemental Fig. 3A–C). Therefore, the significant NOR enabled by E2 treatment was not due to a difference in motivation to explore, or attention given to objects. These results are in agreement with the concept that the E2-enhanced hippocampal physiology contributes to the ability of E2 to enhance learning and memory.

3.2 Aging with ovaries intact enables NOR

In our previous study (Smith, et al., 2010), we found that the effectiveness of acute E2 treatment on hippocampal structure and function in aged rats was protected when the animals were aged with ovaries intact before undergoing OVX at 20 months of age, with subsequent E2 treatment at the same chronological age as 19 months post-OVX group. Given this, we next tested whether this protection extends to NOR. While the vehicle-treated group was unable to distinguish between the novel versus familiar object (Fig. 2D2, V (N=6) $55.5 \pm 3.0\%$, $t(7)=1.61$, $p=0.08$), the E2-treated group had significant NOR as anticipated (Fig. 1D2, E2 (N=5), $65.2 \pm 4.0\%$, $t(4)=4.29$, $p<0.01$). It is important to note that there was a small, yet significant increase in the total amount of time spent investigating objects during the training session in the E2-treated compared to vehicle-treated rats (supplemental Fig. 3D1, V: 15.6 ± 5.0 s versus E2: 21.3 ± 2.6 s, $t(11)=2.31$, $p<0.05$), which could contribute to the preservation of NOR in E2-treated group.

3.3 Chronic E2 replacement at diestrous concentrations beginning at 9.5–11 months post-OVX prevents the loss of the beneficial effects of acute proestrous-like E2 treatment on synaptic function and NOR at 19 months post-OVX

The protection of hippocampal responsiveness to E2 replacement afforded by aging with intact ovaries is likely due to maintained low E2 plasma levels, since ovarian function in rats enters a phase of constant diestrus or estrus (Chakraborty and Gore, 2004, Hung, et al., 2003), in contrast to women. Therefore, we next wanted to test whether administering chronic low levels of E2 beginning at either 9.5–11 months post-OVX (Adult-Replaced group), or as late as 15 months post-OVX (Middle-Age-Replaced group), would prevent the loss of the beneficial effects of acute proestrous-like E2 treatment on synaptic function and NOR observed at 19 months post-OVX ((Smith, et al., 2010) and Fig. 3C; also see Fig. 1A–B for experimental timeline). To this end, following the NOR studies in Fig. 3, the cohort of 9 month post-OVX rats were surgically implanted between 9.5–11 months post-OVX with silastic capsules to systemically deliver E2 (25% E2 diluted in cholesterol to generate diestrus plasma level) or cholesterol (Chol). At 18 months post-OVX capsules were

removed and 2 weeks later all rats were administered acute E2 to simulate proestrous levels using the 2 day injection protocol and evaluated for NOR at E24 as in Fig. 2. In rats with chronic E2 replacement, acute proestrous-like E2 treatment enabled significant NOR (Fig. 3G, E2 (N=11): $61.25 \pm 5.23\%$, $t(11)=2.15$, $p<0.05$), while acute E2 treatment was ineffective in rats treated with cholesterol (Fig. 3G; Chol (N=8): $54.57 \pm 7.01\%$, $t(6)=0.64$, $p=0.54$), indicating that chronic low level E2 provides protection against the deficit previously observed at 19 months post-OVX (Fig. 2C). No differences occurred between Chol and E2 replaced groups in total time spent investigating objects during the training or testing session (Supplemental Fig. 4A). Importantly, consistent with the NOR results, acute E2 treatment is able to enhance the LTP magnitude at CA3-CA1 synapses (Fig. 3A, Chol (N=6): $114 \pm 4\%$ of baseline fEPSP slope versus E2 (N=7): $130 \pm 4\%$ of baseline fEPSP slope $t(11)=2.60$, $p<0.05$), the NMDAR/AMPA ratio (Fig. 3C, Chol (N=7): 0.36 ± 0.08 versus E2 (N=9): 0.57 ± 0.07 $t(15)=1.74$, $p<0.05$), GluN2B-mediated transmission (Fig. 3D, Chol (N=7): 0.18 ± 0.08 versus E2 (N=9): 0.33 ± 0.04 , $t(15)=2.94$, $p<0.05$) but surprisingly not spine density (Fig. 3E–F, Chol (N=6): 22.3 ± 1.8 versus E2 (N=7): 21.1 ± 1.0 , $t(12)=0.59$, $p=0.56$), only in rats with chronic E2 replacement.

3.4 Chronic E2 replacement at diestrous concentrations beginning at 15 months post-OVX protects the ability of subsequent proestrous-like E2 levels to enable NOR at 19 months post-OVX in the absence of an increase in synaptic function

In Middle-Age-Replaced rats (see Fig. 1B for experimental timeline), acute proestrous-like E2 treatment enabled significant NOR in E2 replaced but not Chol replaced rats (Fig. 4G Chol (N=9): $56.5 \pm 9.0\%$, $t(8)=0.73$, $p=0.25$, E2 (N=16): $59.4 \pm 4.2\%$, $t(14)=2.27$, $p<0.05$), with no difference in total time spent investigating objects (Supplemental Fig. 4B). Surprisingly this preservation of NOR was not accompanied by a protection against the loss of the E2-induced increase in the LTP magnitude previously observed at 19 months post-OVX (Smith, et al., 2010) (Fig. 4A; Chol (N=11): $117 \pm 3\%$ of baseline fEPSP slope vs E2 (N=11): $119 \pm 3\%$ of baseline fEPSP slope, $t(20)=0.32$, $p=0.37$). Along with the lack of LTP, the ability of E2 to enhance the NMDAR/AMPA ratio (Fig. 4C, Chol (N=7): 0.56 ± 0.10 versus E2 (N=16): 0.57 ± 0.08 , $t(18)=0.36$, $p=0.72$), GluN2B-mediated NMDAR current (Fig. 4D, Chol (N=7): 0.39 ± 0.07 versus E2 (N=16): 0.38 ± 0.11 , $t(18)=0.05$, $p=0.96$) and dendritic spine density (Fig. 4E–F, Chol (N=7): 21.4 ± 1.9 versus E2 (N=10): 21.3 ± 1.5 , $t(18)=0.06$, $p=0.95$) are also not protected.

4. Discussion

It has been a decade since the report from the Women's Health Initiative Memory Study citing no benefit or even potential harm of ERT on cognitive function in post-menopausal women (Shumaker, et al., 2004, Shumaker, et al., 2003). Mounting evidence now supports the critical period hypothesis which provides a potential explanation for the disappointing study results (Acosta, et al., 2013, B. B. Sherwin, 2007, Daniel and Bohacek, 2010, Gibbs, 2000, Lord, et al., 2006, Sherwin, 2006, Smith, et al., 2010). Findings presented here provide further support for the critical period hypothesis whereby acute E2 treatment at proestrous-like levels enables significant NOR in female rats 9 and 15 months post-OVX, but not at 19 months post-OVX. E2 treatment enabled NOR in rats aged with intact ovaries

prior to undergoing OVX at the same chronological age as the 19 month post-OVX group, indicating that the period of E2 deprivation causes the lack of responsiveness, rather than chronological age. These behavioral findings perfectly mirror the ability of the same E2 treatment to enhance the LTP magnitude at CA3-CA1 synapses, NMDAR/AMPA ratio, GluN2B-mediated NMDAR current, and CA1 dendritic spine density we reported previously at these post-OVX time points (Smith, et al., 2010). Thus, the shared post-OVX window during which E2 treatment remains beneficial for NOR (current study) and hippocampal synaptic function (Smith, et al., 2010) suggests that the increase in synaptic function and NOR are mechanistically linked (Smith and McMahon, 2006, Vedder, et al., 2013). However, this association does not occur when OVX rats are treated chronically with low E2 levels where in the Middle-Age-Replaced group, significant NOR occurs even in the absence of enhanced hippocampal function.

Our data showing the effectiveness of acute E2 treatment on NOR (current study) and hippocampal synaptic function (Smith, et al., 2010) is preserved when ovaries are left intact for the majority of the lifespan provide an important clue about the critical window. These fortuitous results, we believe, are directly linked to continued ovarian function throughout life in female rats, in contrast to the cessation of ovarian function and hormone production during menopause and aging in women, primates, and mice (Klinga, et al., 1982, Mobbs, et al., 1984, Walker and Herndon, 2008). Because cyclic regulation of ovarian function by the hypothalamus is decreased in aging, most female rats enter a state of constant estrus or diestrus, with some continuing to cycle throughout life (Chakraborty and Gore, 2004, Hung, et al., 2003). Therefore, to mimic menopause using a female rat model, OVX rather than simple chronological aging is required. It is important to also note that in addition to OVX, inducing follicular depletion using 4-vinylcyclohexene-diepoxy in rodents can also model ovarian hormone loss in women (Acosta, et al., 2009). Thus, our findings from ovary-intact aged female rats support the concept that maintained low plasma E2 levels throughout the lifespan will protect hippocampal function in aging. This concept is further supported by our findings that chronic administration of diestrus E2 plasma levels beginning at 9.5–11 months post-OVX (Adult-Replaced group; chronological age 11.5–13 months) prevents loss of hippocampal E2 responsiveness at the 19 month post-OVX time point we observed previously (Smith, et al., 2010) and in the current study. Accordingly, in OVX rats, this chronic exogenous E2 administration mimics the low plasma E2 level provided by ovaries during the aging process, thereby preserving hippocampal E2 responsiveness following late-life OVX. Importantly, the critical period defined in our studies is similar with a previous report where E2 replacement 19 months post-OVX (chronological age 23–24 months) no longer enhances delayed matching to position acquisition (Gibbs, et al., 2009). However, other studies show that E2 replacement only enhances working memory in middle-aged rats when initiated immediately after OVX, but not after 5 months post-OVX (Daniel, et al., 2006b). Thus, the duration of the critical window appears to depend upon the chronological age at which OVX occurs, in addition to the duration of deprivation.

The lack of effect of acute proestrous-like E2 treatment on spine density in the Adult-Replaced group receiving chronic (7–8.5 months) E2 replacement, despite preservation of enhanced synaptic function and NOR, was surprising. This finding challenges the long-held notion that E2-induced increase in spine density is directly related to the heightened

hippocampal dependent learning and memory (Leuner and Shors, 2004), although it must be considered that this association does not seem to occur in aged OVX rats with chronic exposure to low level E2. In young adult OVX rats, the temporal overlap of the increased spine density, selective increase in NMDAR receptor binding, increased transmission mediated by GluN2B-containing NMDARs (immature form of NMDARs), heightened LTP, and elevated NOR (Daniel and Dohanich, 2001, Smith and McMahon, 2005, Smith and McMahon, 2006, Snyder, et al., 2010, Woolley, et al., 1997), suggests a model whereby E2 increases the density of newly developed, highly plastic, silent (NMDAR-only) synapses containing GluN2B NMDARs, similar to early development (Law, et al., 2003, Petralia, et al., 1999, Sans, et al., 2000). Following an LTP inducing stimulus, conversion of these initially silent synapses to active (AMPA and NMDAR containing) synapses causes the heightened LTP magnitude (Smith and McMahon, 2005, Smith and McMahon, 2006), which is also linked to the elevated NOR via a shared requirement for GluN2B-containing NMDARs (Smith and McMahon, 2006, Vedder, et al., 2013). This model is supported by our data showing that the acute E2-induced increase in spine density, GluN2B transmission, NMDAR/AMPA ratio, LTP, and NOR are a “packaged deal”, either occurring together (up to 15 months post-OVX) or not occurring at all (at 19 months post-OVX) (Smith and McMahon, 2005, Smith and McMahon, 2006, Smith, et al., 2010, Vedder, et al., 2013, current report). However, the current results from the Adult-Replaced group suggests that an increase in spine density is not required for either the increase in LTP or NOR, and indicate that the increase in GluN2B-containing NMDARs must be occurring at existing synapses. Fortunately, our results are consistent with previous electron microscopy reports by Morrison and colleagues (Adams, et al., 2004, Adams, et al., 2001) where E2 replacement following acute OVX in 24 month-old female rats increased synaptically located GluN2B-containing NMDARs (Adams, et al., 2004) in the absence of increased spine density (Adams, et al., 2001). In addition, McLaughlin et al 2008 reported that a 5 µg E2 dose administered to adult OVX rats facilitated spatial learning in the Morris Water Maze in the absence of an increase in spine density in area CA1, while a 10 µg E2 dose increased spine density, improved novel object placement, but not spatial learning in the water maze (McLaughlin, et al., 2008). Collectively, these previously published results together with the current study indicate that an increase in spines is not required for E2-induced improvement in cognitive function. In addition, we conclude from the current findings, that in aged OVX rats, an increase in spine density is not required for either heightened synaptic function or NOR. Whether the NOR in this experimental group requires GluN2B NMDARs like in young adult OVX rats remains to be determined.

Perhaps even more surprising is the preservation of NOR in the Middle-Age-Replaced group receiving chronic (3 months) E2 replacement, despite no effect whatsoever on hippocampal synaptic function. Although the E2 induced increase in NOR requires hippocampal GluN2B-containing receptors in young adult OVX rats (Vedder, et al., 2013) and perhaps also in the Adult-Replaced group, clearly some other mechanism stimulated by acute E2 treatment is enabling NOR in the Middle-Age-Replaced group. Because E2 has effects on other brain regions (Gervais, et al., 2013, Hao, et al., 2006, 758, Rasia-Filho, et al., 2012) and NOR is also critically dependent on perirhinal and entorhinal cortices (Barker, et al., 2007, Bellgowan, et al., 2009, Bussey, et al., 1999, Mumby and Pinel, 1994), other regions and/or

mechanisms may be compensating for the lack of responsiveness of hippocampus. It must also be considered that previous exposure to NOR at the 15 months post-OVX time point could facilitate performance in the task when this group was re-tested at 19 months post-OVX following 3 months of E2 replacement. Furthermore, the lack of preservation of hippocampal responsiveness to acute E2 treatment when chronic E2 replacement is delayed to 15 months post-OVX could be a consequence of the duration of deprivation or the duration of the replacement period, or both. However, it is likely the period of deprivation as indicated by our current and previous results as well as those of others (Daniel, et al., 2006a, Gibbs, et al., 2009, Rodgers, et al., 2010). For example, 40 day E2 treatment begun immediately following OVX at 10–11 months of age protects against hormone related cognitive deficits measured up to 8 months later (Rodgers, et al., 2010). Whether E2 is able to protect against the loss of hippocampal synaptic plasticity and morphology at 19 months post-OVX with this shorter 3 month replacement initiated immediately post-OVX when rats are 2 months of age remains to be tested. It is worth noting that the control groups in the Adult-Replaced and Middle-Aged Replaced experiments were implanted with 100% cholesterol-filled capsules rather than with blank capsules. A recent report shows that serum E2 levels are higher when rats are implanted with cholesterol versus blank capsules (McLaughlin, et al., 2010), suggesting that increasing cholesterol levels may increase E2 synthesis despite OVX. This finding however was not replicated in a more recent study (Ortiz, et al., 2013) or in the current study since serum E2 levels are undetectable in all Chol-replaced animals when measured prior to surgical capsule removal.

The mechanisms contributing to the complete loss of NOR in all vehicle-treated long-term post-OVX groups or the improved performance in the Adult-Replaced and Middle-Age-Replaced groups given low E2 levels are unknown, but could be related to decreased expression of ER α in OVX rats that is reversed with E2 treatment (Bohacek and Daniel, 2009). Rodgers et al 2010, (Rodgers, et al., 2010) reported that short-term (40 days) E2 replacement increases expression of ER α in OVX rats for up to 8 months post-treatment, which is associated with improved behavioral performance. In addition, Foster et al., 2008 reported that increasing hippocampal expression of ER α using viral transfection improves spatial learning even in OVX mice, indicating that ovarian E2 is not required (Foster, et al., 2008). Alternatively, in a recent study by Wang et al., 2012, (Wang, et al., 2012) OVX at 9 months of age was associated with an increase in expression of ER β 2 that could be reversed if E2 replacement was initiated within 6 days, but not at 3 months, post-OVX. The decreased ER β 2 expression was associated with improved behavior in the forced swim test. This ER isoform functions as a dominant negative, dimerizing with ER α and ER β , thereby interfering with normal ER function. Because acute E2 treatment remains able to increase spine density, synaptic function, and NOR up to 15 months post-OVX (current report and Smith et al., 2010), it is not clear how or if altered ER β 2 expression is participating in the effects we observe. Degeneration of cholinergic innervation to hippocampus is also obvious candidate (Gibbs, 2010). E2 has been shown to enhance activity of choline acetyl transferase (ChAT) (Luine, 1985), ChAT mRNA levels (McMillan, et al., 1996) as well as hippocampal ACh release (Marriott and Korol, 2003). Removal of ovarian hormones through OVX also decreases ACh release in hippocampus (Marriott and Korol, 2003). The ability of E2 to enhance learning can be prevented when cholinergic projections to hippocampus are

damaged through medial septal lesion (Gibbs, 2002). The amount of degeneration of the cholinergic system during OVX is greater than what occurs during the normal course aging (Marriott and Korol, 2003, Mitsushima, et al., 2009). Also, cholinergic cell loss is a hallmark of Alzheimer's disease (AD) and ERT in women has protective effects against the onset of AD in post-menopausal women (Henderson, 2009, Paganini-Hill and Henderson, 1994, Paganini-Hill and Henderson, 1996). Therefore, periods of long-term ovarian hormone loss may cause cholinergic degeneration to a level that E2 is no longer able to overcome, preventing the beneficial effects of E2 treatment. Much more work in this area is clearly needed.

Here, we further define the critical period during which E2 replacement remains beneficial to hippocampal function in aged OVX rats. At 19-months post-OVX, all beneficial effects of E2 on hippocampal spine density, synaptic function, and NOR are lost (Smith, et al., 2010). However, this lack of responsiveness can be prevented if rats are given chronic E2 replacement before 11-months post-OVX, thereby extending the window of opportunity. Furthermore, despite acute E2 hippocampal responsiveness at 15 months post-OVX, delaying chronic E2 replacement to this time point is unable to interfere with or reverse mechanisms already initiated to preserve hippocampal responsiveness at 19 month post-OVX, despite preserved performance in NOR. Thus, the current findings extend previous knowledge by showing that improved NOR is constrained by the same duration of E2 deprivation as enhanced hippocampal synaptic function and that chronic E2 replacement at physiological levels (diestrus) can extend this window and improve behavior even in the absence of increased spine density (Adult-Replaced) or enhanced hippocampal synaptic function (Middle-Aged-Replaced). Furthermore, the beneficial effects of the low level plasma E2 that occurs as a result of aging with intact ovaries in rats informs the potential use of low level E2 replacement beginning at early menopause to provide hippocampal protection in women during late life. Future studies are needed to examine the effects of more physiological replacement protocols on the critical window, such as cyclic levels of E2 and the addition of progesterone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Timeline and procedures for experimental paradigms

A. 9 month post-OVX NOR and Adult-Replaced experiments. B. 15 month post-OVX NOR and Middle-Age Replaced experiments. C. 19 month post-OVX NOR. D. Aged ovary intact NOR.

Fig. 2. E2-induced NOR is lost after 19-mo post-OVX and this loss is not due to chronological age alone

A1–C1. During training for all groups, the percent time spent on the right object was not different than chance (50%), indicating no side preference. The percent time spent on the novel object during testing was significantly increased compared to chance with acute proestrous-like E2 treatment in 9-month post-OVX (A2) and 15-month post-OVX (B2) but not in 19-month post-OVX (C2) rats. D. Rats aged with ovaries intact and undergo OVX at 20 months of age have significant NOR when treated with acute proestrous-like E2 at the same chronological age as 19-month post-OVX rats. No significant NOR occurred for the Vehicle-treated rats for any of the experimental groups. + $p < 0.05$ compared to chance. Arrows on the right indicate the direction of change in LTP magnitude at CA3-CA1 synapses in E2 versus V-treated OVX rats at these time points previously published in Smith et al, 2010.

Fig. 3. Chronic low-level E2-replacement beginning at 9.5–11 months post-OVX protects against the loss of the beneficial effects of acute proestrous-like E2 treatment on NOR and synaptic function, but not on dendritic spine density

A. Plot showing a significant increase in the average LTP at CA3-CA1 synapses recorded in slices at 19 months post-OVX from rats chronically treated with E2 compared to Chol for 7–8.5 months (Adult-Replaced), then subsequently treated with acute proestrous-like E2 and sacrificed 24 hrs following the second E2 injection. Inset shows average percent LTP with each animal in the dataset presented as an individual closed circle. Representative waveforms for Chol and E2 replaced rats with dotted line representing baseline and solid line representing 30 min post-tetanus. Scale bar represents 0.3 mV on y-axis and 50ms on x-axis. B. Representative whole-cell recording from a Chol-replaced rat. Plot shows change in glutamatergic current with sequential pharmacological blockade of GluN2B-containing NMDARs using Ro 25-6981 (1 μM), NMDARs using D-L, APV (100 μM), and AMPARs using DNQX (10 μM). Waveforms represent total glutamate, AMPAR, NMDAR, and GluN2B currents. Scale bar represents 100pA on y-axis and 300ms on x-axis. C. Bar chart shows a significant increase in the NMDAR/AMPA ratio recorded in CA1 pyramidal cells from E2- versus Chol-replaced rats. D. Bar chart shows a significant increase in the GluN2B/total NMDAR ratio in CA1 pyramidal cells recorded from E2- versus Chol-replaced rats. E. Example images of dendritic spines from Chol and E2-replaced rats. F. Bar chart shows no difference in number of CA1 dendritic spines on tertiary dendrites per 10 μm from Chol and E2-replaced rats. G. Bar chart shows significant NOR only in chronic E2-replaced rats. * indicates $p < 0.05$, + $p < 0.05$ compared to chance.

Fig. 4. Chronic low-level E2-replacement beginning at 15 months post-OVX enables NOR in the absence of preserving the beneficial effects of acute proestrous-like E2 treatment on synaptic function and spine density

A. Plot showing no difference in the average LTP magnitude at CA3-CA1 synapses recorded in slices at 19 months post-OVX from rats chronically treated with Chol or E2 for 3 months (Middle-Age-Replaced), then subsequently treated with acute proestrous-like E2 and sacrificed 24 hrs following the second E2 injection. Inset shows average percent LTP with each animal in the dataset presented as an individual closed circle. Representative waveforms for Chol and E2 replaced rats with dotted line representing baseline and solid line representing 30 min post-tetanus. Scale bar represents 0.3 mV on y-axis and 50ms on x-axis. B. Representative whole-cell recording from a Chol-replaced rat. Plot shows change in glutamatergic current with sequential pharmacological blockade of GluN2B-containing NMDARs using Ro 25-6981 (1 μ M), NMDARs using D-L, APV (100 μ M), and AMPARs using DNQX (10 μ M). Waveforms represent total glutamate, AMPAR, NMDAR, and GluN2B currents. Scale bar represents 100pA on y-axis and 300ms on x-axis. C. Bar chart shows no difference in the NMDAR/AMPA ratio in CA1 pyramidal cells recorded from Chol versus E2-replaced rats. D. Bar chart shows no significant difference in the GluN2B/total NMDAR ratio recorded from CA1 pyramidal cells recorded from Chol versus E2-replaced rats. E. Example images of dendritic spines from Chol and E2-replaced rats. F. Bar chart shows no difference in the number of dendritic spines on tertiary dendrites per 10 μ m between Chol and E2-replaced rats. G. Bar chart shows significant NOR only in chronic E2-replaced rats. * indicates $p < 0.05$, + $p < 0.05$ compared to chance.