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A component of Premarin® enhances multiple cognitive functions and influences nicotinic receptor expression

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Abstract

In women, ovarian hormone loss at menopause has been related to cognitive decline, and some studies suggest that estrogen-containing hormone therapy (HT) can mitigate these effects. Recently, the Women's Health Initiative study found that conjugated equine estrogens, the most commonly prescribed HT, do not benefit cognition. Isolated components of conjugated equine estrogens (tradename Premarin®) have been evaluated in vitro, with $\Delta^{8,9}$-dehydroestrone (Δ^8E1) and equilin showing the strongest neuroprotective profiles. It has not been evaluated whether Δ^8E1 or equilin impact cognition or the cholinergic system, which is affected by other estrogens and known to modulate cognition. Here, in middle-aged, ovariectomized rats, we evaluated the effects of Δ^8E1 and equilin treatments on a cognitive battery and cholinergic nicotinic receptors (nAChR). Specifically, we used ^{125}I-labeled epibatidine binding to assay the neuronal nicotinic receptor containing 4α and 2β subunits (α4β2-nAChR), since this nicotinic receptor subtype has been shown previously to be sensitive to other estrogens. Δ^8E1 enhanced spatial working, recent and reference memory. Δ^8E1 also decreased hippocampal and entorhinal cortex α4β2-nAChR expression, which was related to spatial reference memory performance. Equilin treatment did not affect spatial memory or rat α4β2-nAChR expression. Neither estrogen impacted $^{86}Rb^+$ efflux, indicating lack of direct action on human α4β2 nAChR function. Both estrogens influenced vaginal smear profiles, uterine weights, and serum luteinizing hormone levels, analogous to classic estrogens. The findings indicate that specific isolated Premarin® components differ in their ability to affect cognition and nAChR expression. Taken with the works of others showing Δ^8E1-induced benefits on several dimensions of health-related concerns associated with menopause, this identifies Δ^8E1 as a new avenue to be investigated as a potential component of HT that may benefit brain health and function during aging.

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Keywords

Premarin®; estrogen; $\Delta^{8,9}$-dehydroestrone; equilin; learning; memory; nicotinic acetylcholine receptor; working memory; reference memory

Introduction

By the year 2050, an estimated 45 million postmenopausal women in the United States will have to make the choice of whether to utilize hormone therapy (HT; U.S. Census Bureau, 2007). Ovarian hormone loss due to surgical or natural menopause has been associated with cognitive decline in women (Nappi et al., 1999; Phillips and Sherwin, 1992; Sherwin, 1988); however, the question of how HT impacts cognition is unclear. Premarin®, a complex conjugated equine estrogen preparation synthesized from the urine of pregnant mares, has been given since 1942 and is the most widely used estrogen component of HT in the United States (Hersh et al., 2004; Stefanick, 2005). Some studies in women demonstrate that Premarin®-containing HT improved memory (Campbell and Whitehead, 1977; Kantor et al., 1973; Ohkura et al., 1995). However, the largest placebo-controlled double blind study conducted to date evaluating HT effects on all-cause dementia, the Women's Health Initiative Memory Study (WHIMS), found that conjugated equine estrogen treatment given alone did not reduce incidence of dementia or mild cognitive impairment (Shumaker et al., 2004), and that treatment with conjugated equine estrogens plus the progestin medroxyprogesterone acetate increased the risk of cognitive decline and probable dementia, and did not prevent mild cognitive impairment (Rapp et al., 2003; Shumaker et al., 2003). A recent follow-up study evaluating WHIMS participants found that Premarin® treatment, with or without medroxyprogesterone acetate, was associated with brain atrophy as assessed via MRI scans (Resnick et al., 2009). Thus, while noting many factors likely affected the WHIMS findings, including the global nature of the cognitive measure, older age of participants, and duration of hormone deprivation before treatment initiation (for discussion see Sherwin, 2005), the collected findings suggest that although the absence of ovarian hormones is not optimal for cognition, neither is the most commonly utilized HT.

Premarin® contains the sulfates of more than 10 estrogens, is over 50% estrone, 20–25% equilin, 3.5% $\Delta^{8,9}$-dehydroestrone (Δ^{8}E1), and contains only trace amounts of 17β-estradiol (the most potent naturally circulating estrogen in women and rats); after metabolism, the resulting biologically active circulating hormones are primarily estrone and equilin, and after estrone's conversion, 17β-estradiol (Bhavnani, 2003; Bhavnani et al., 1998; Mayer et al., 2008; Sitruk-Ware, 2002). In the last decade, landmark basic science research from the Brinton laboratory has led to several discoveries regarding the neuroprotective properties of estrogens. This work demonstrated that some components of Premarin® enhanced markers of neuroprotection, while others showed little benefit (Brinton et al., 1997; Zhao and Brinton, 2006). The estrogens Δ^{8}E1 and equilin, found naturally in horses but not in women or rats, were the two primary Premarin® components that showed the most consistent and potent neuroprotective effects in vitro (Zhao and Brinton, 2006). Δ^{8}E1- and equilin-mediated neuroprotection in vitro could potentially translate to improved function of neural networks and brain regions mediating cognitive function, resulting in mnemonic enhancements in vivo.

Basal forebrain cholinergic neurons project to the hippocampus and surrounding cortical areas, and play an important role in learning and memory (Hasselmo, 2006). 17β-estradiol enhances basal forebrain cholinergic function, as evidenced by expression of different cholinergic markers and pharmacological cholinergic challenges (e.g., Gibbs, 2000a; Markowska and Savonenko, 2002; Packard and Teather, 1997). In fact, choline acetyltransferase (ChAT) increases in the basal forebrain months after transient exposure to 17β-estradiol, noting effects

are sensitive to several variables including timing and dose (Bohacek et al., 2008; Gibbs, 1997; Rodgers et al., 2010). Our laboratory has demonstrated Premarin®-induced benefits to the cholinergic system, whereby Premarin® treatment prevented scopolamine-induced amnesia and increased number of ChAT positive neurons in the vertical diagonal band of the basal forebrain in ovariectomized (Ovx) rats (Acosta et al., 2009b). Cholinergic signals are mediated by muscarinic acetylcholine receptors (mAChR) and nicotinic acetylcholine receptors (nAChR), which are present in brain regions mediating memory (Clarke et al., 1985; Court and Clementi, 1995; Tice et al., 1996; Vaucher et al., 2002) and are implicated in mnemonic processing (Hasselmo, 2006; Konopacki et al., 1992; Rouse et al., 1999). Nicotine administration improves memory in humans (Buccafusco et al., 2005), and we and others have noted nicotine-induced memory enhancement in rats across multiple memory domains (Arendash et al., 1995; French et al., 2006; Riekkinen and Riekkinen, 1997; Socci et al., 1995). 17β-estradiol and ethinyl β-estradiol bind to the α4β2-nAChR, the most abundant nAChR subtype in the brain, and directly potentiate the function of human α4β2-nAChRs (hα4β2-nAChR); ethinyl β-estradiol, but not 17β-estradiol, potentiates rat α4β2-nAChRs (rα4β2-nAChR) (Curtis et al., 2002; Paradiso et al., 2001). Recently, in vivo research has demonstrated that nicotine co-administered with estradiol potentiates visual spatial memory in Ovx rats, beyond that of either compound administered alone (Taylor and Maloney, 2010). Thus, there is a link between estrogens, nAChRs, and memory.

Numerous studies have demonstrated that 17β-estradiol benefits spatial working and reference memory in young Ovx rats (Bimonte and Denenberg, 1999; Daniel et al., 1997; Daniel et al., 2005; El-Bakri et al., 2004; Fader et al., 1998; Feng et al., 2004; Gibbs, 2007; Hruska and Dohanich, 2007; Korol and Kolo, 2002; Luine and Rodriguez, 1994), as well as in middle-aged Ovx rats (Bimonte-Nelson et al., 2006; Foster et al., 2003; Markham et al., 2002; Talboom et al., 2008). Premarin® can also benefit cognition in middle-aged Ovx rats, although these effects are dose and task specific (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009; Walf and Frye, 2008). The current study utilized a similar model, the middle-aged Ovx rat, to test the impact of Δ^8E1 and equilin on memory and rα4β2-nAChR expression and function. We used a battery of water-escape mazes previously shown to be influenced by age as well as ovarian hormone loss and estrogen replacement (Acosta et al., 2009a; Acosta et al., 2009b; Bimonte-Nelson et al., 2006; Engler-Chiurazzi et al., 2009; Talboom et al., 2008). These mazes evaluate working memory, which is information that needs to be updated and is pertinent for a short time, and reference memory, which is information that remains constant over time (see Jarrard et al., 1984; Jones, 2002). ^{125}I-labeled epibatidine (I-epi) radioligand binding assays were used to evaluate rα4β2-nAChR expression levels in the hippocampus and entorhinal cortex, and cell culture was used to evaluate whether Δ^8E1 and equilin directly altered hα4β2-nAChR function via $^{86}Rb^+$ efflux experiments. Lastly, several peripheral markers routinely noted to change with 17β-estradiol treatment, including serum luteinizing hormone (LH) levels, vaginal smears, and uterine weights, were assessed.

Materials and Methods

Subjects

Subjects were 50 middle-aged (12–13 month old) Fischer-344 female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN). Animals were acclimated for several weeks to the vivarium at Arizona State University, had exposure to food and water ad-lib, and were maintained on a 12-h light/dark cycle at 23°C. Procedures were approved by Arizona State University IACUC and adhered to the Guide for the Care and Use of Laboratory Animals and NIH standards.

Hormone manipulation

Ovariectomy, group assignment and hormone dosing—Thirty days after arrival, all rats received Ovx under isoflurane anesthesia. Dorsolateral incisions were made in the skin and peritoneum, and ovaries and tips of uterine horns were ligated and removed. Rats were randomly assigned into a control group receiving vehicle only or groups receiving one of three doses of hormone delivered via osmotic minipump. Specifically, the groups were: Ovx plus vehicle (Veh, n=8), Ovx plus 2.6 μg/day of Δ^8E1 (Δ^8E1-Low, n=7), Ovx plus 17.5 μg/day of Δ^8E1 (Δ^8E1-Med, n=7), Ovx plus 35 μg/day of Δ^8E1 (Δ^8E1-High, n=7), Ovx plus 2.6 μg/day of equilin (Equilin-Low, n=7), Ovx plus 6.25 μg/day of equilin (Equilin-Med, n=7), and Ovx plus 12.5 μg/day of equilin (Equilin-High, n=7). All hormones were purchased from Steraloids Inc. (Newport, RI). Low doses of Δ^8E1 and equilin were derived from our studies demonstrating cognitive enhancement using 3.6 μg of Premarin®. Doses were adjusted to account for Δ^8E1 and equilin being in its unconjugated form[1], and to approximate the rat body weight equivalent of the 0.625 mg dose, which was used in the WHIMS and is what women are most commonly prescribed (Acosta et al., 2009b;Engler-Chiurazzi et al., 2009). The high doses for Δ^8E1 and equilin were based on the only other available reports in the rat model using these hormones, wherein Δ^8E1 decreased depolarization-induced cardiac synapse norepinephrine release, and equilin elevated vascular reactivity in the mesenteric vascular bed (Eskin et al., 2003;Mark et al., 2007). The medium doses of Δ^8E1 and equilin were half of the high doses.

Hormone treatment procedure—Hormone treatment began approximately 16 days after Ovx (Bimonte-Nelson et al., 2006), 12 days before behavior testing began. Vehicle only (polyethylene glycol, PEG, average molecular weight of 300, Sigma-Aldrich, St. Louis, Missouri), or Δ^8E1 or equilin in vehicle, was administered via Alzet pumps (Model 2004; Durect Corporation, Cupertino, California). The concentration of Δ^8E1 or equilin, suspended in PEG, in the pumps was calculated to deliver the low, medium or high dose for each hormone per day. Pumps were prepared the day before surgery and allowed to equilibrate in physiological saline at 37 °C overnight. For pump insertion, animals were anesthetized under vaporized isoflurane anesthesia, an incision was made in the dorsal scruff of the neck, the pump was inserted, and the skin was stapled. Pumps remained in the subjects throughout behavioral testing and until sacrifice (approximately 4 weeks), which provided consistent exposure to their assigned treatment for the duration of behavioral testing, through sacrifice.

Behavior testing

Win-stay DMS plus maze (spatial working and recent memory)—We used the DMS plus maze task to test spatial working and recent memory. The protocol was based on studies showing 17β-estradiol- and Premarin®- induced improvements in Ovx rats (Acosta et al., 2009b; Gibbs, 2000b; Korol and Kolo, 2002; Sandstrom and Williams, 2004). The black plexiglass maze (each arm was 38.1cm × 12.7cm) was filled with water made opaque with black nontoxic paint. The submerged platform was hidden at the end of one of the four arms. The start location varied across trials, and the platform location stayed in the same arm within a day, but semi-randomly changed arms across days. This a place learning task, whereby animals must learn to navigate to a hidden escape platform by using distal spatial cues (Restle,

[1]The 20–36 μg of Premarin® powder that we have shown to enhance cognition was actually 2.0–3.6 μg of estrogens, respectively, since it was ≈ 10% hormone and ≈ 90% filler (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009). Premarin® is a mixture of estrogens conjugated to sulfate; the hormones must be deconjugated by the liver to become bioactive (Bhavnani et al., 1998). We used bioactive, unconjugated Δ8E1 and equilin in this study. To attain an approximately equivalent amount of estrogen molecules between our current Δ8E1 and equilin low dose, and our previous Premarin dose, we had to account for the fact that estrogens in Premarin are conjugated, and conjugated estrogens weigh more. Since the molecular weights of Δ8E1 sulfate and equilin sulfate (the conjugated estrogens) are each 370.08 (as monosodium salts), while that of Δ8E1 and equilin (as unconjugated estrogens) are each 268.35, the final concentrations for the low doses were reduced by 28% (i.e., 3.6 μg/day - 28% ≈ 2.6 μg/day).

1957). Since the platform changed position relative to space each day, this task is differentiated from other match-to-sample or match-to-position tasks where animals solve the task using response learning (i.e., making the same turn regardless of the spatial location) (Restle, 1957). Rats received 6 consecutive trials within a daily session, for 7 days, and were given 90 seconds to locate the platform. Once on the platform, the rat remained on it for 15 seconds, followed by placement into a heated cage for a 30 second inter-trial interval. An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Entry into any non-platformed arm was counted as an error, and fewer errors are indicative of better performance. At the start of an animal's daily testing session, it had no information regarding the platform location. Hence, on trial 1, the rat swam until it located the platform. The rat then needed to remember this platform location and return to it on subsequent trials. Working memory performance was evaluated by assessing the ability to navigate to the new platform location on trial 2. Hence, trial 1 was the information trial, trial 2 was the working memory test trial, and trials 3–6 six were recent memory test trials (Acosta et al., 2009b; Engler-Chiurazzi et al., 2009). The dependent variables were the number of initial and repeat errors committed during trial 2 (as a measure of working memory) or trials 3–6 (as a measure of recent memory). Day 1 was considered the training day, as no prior information regarding task demands or platform location was available to the animals. We routinely treat day 1 as a training day on working memory tasks (Acosta et al., 2009a; Bimonte-Nelson et al., 2003a; Bimonte-Nelson et al., 2003b; Bimonte-Nelson et al., 2004; Bimonte and Denenberg, 1999; Bimonte et al., 2003; Huentelman et al., 2009).

Morris maze (spatial reference memory)—Using a previously published protocol (Markham et al., 2002), three days after DMS plus maze testing concluded, we tested spatial reference memory via the Morris maze, as we and others have shown enhancements due to 17β-estradiol treatment in Ovx rats on this task (Bimonte-Nelson et al., 2006; Markham et al., 2002; Talboom et al., 2008). Similar to the DMS plus maze, the Morris maze task assesses place learning; however, in the Morris maze the platform remains in the same spatial location for all days and trials, making it a spatial reference memory task. This can be contrasted with the DMS plus maze, which is a spatial working memory task since the platform is moved to a new spatial location every day. The Morris maze (Morris et al., 1982) consisted of a round tub (188 cm in diameter) filled with water made opaque with black, non-toxic paint. The rat was placed in the maze from any of four locations (North, South, East, or West) and had 60 seconds to locate a submerged hidden escape platform which remained in a fixed location (Northeast quadrant) throughout testing. After 15 seconds on the platform, the rat was placed into its heated cage until the next trial; the inter-trial-interval was 5–8 minutes. For each rat, the testing session consisted of 6 trials/day for 3 days. A video camera recorded each rat, and a tracking system (Ethovision XT 5.1, Noldus Information Technology, Wageningen, Netherlands) analyzed each rat's path. The dependent measure was swim distance (cm), with less swim distance interpreted as better spatial reference memory performance. To assess possible differences in the learning rates among our treatment groups, data were collapsed into 3-trial blocks so that Treatment × Block interactions could be tested. To assess platform localization, a probe trial was given on an additional trial (trial 7) on the last day of testing, whereby the platform was removed from the maze. For the probe trial, percent of total swim distance (cm) in the target Northeast quadrant (i.e., quadrant that contained the platform on the test trials) as compared the opposite Southwest quadrant, was the dependent measure.

Visible platform (motoric and visual competence)—One day after Morris maze testing, we evaluated motoric and visual competence using the visible platform task. A rectangular tub (39 × 23 in) was filled with clear room temperature water. A black platform (10 cm wide) was positioned 1.5" above the water surface following previously published methods (Hunter et al., 2003). Opaque curtains covered extramaze cues. Animals were given

6 trials in 1 day. The drop off location remained the same across trials, and the platform location for each trial varied semi-randomly. Each rat had 90 seconds to locate the platform, where it remained for 15 seconds before being placed back into its heated cage awaiting the next trial. The inter-trial-interval was 5–8 minutes. Latency (seconds) to reach the platform was the dependent measure.

Evaluation of vaginal smears, uteri, and serum LH

We evaluated the effects of Δ^8E1 or equilin treatments on several peripheral markers that are routinely noted to change with 17β-estradiol treatment. To confirm Ovx as well as Δ^8E1 and equilin treatments, vaginal smears were taken prior to Ovx and pump surgery, immediately before behavior testing, and the day before sacrifice. Vaginal cytology via smears was classified as proestrus, estrous, metestrus or diestrus (Goldman et al., 2007). Final vaginal smears were stained with eosin Y and hematoxylin (Sigma) for further brightfield microscopic examinationa. At sacrifice, animals were anesthetized with isoflurane. We confirmed complete Ovx via visual inspection of uterine horns, and we evaluated uterine weights (Acosta et al., 2009b). A ventral incision was made in the abdominal region, and the uterus was cut above the junction with the cervix and on the uterine horn below the ligature remaining from Ovx (Ashby et al., 1997). Uteri were trimmed of all visible fat and were immediately weighed to obtain wet weight, which was the dependent measure. Also, at this time, blood was collected via cardiocentesis. Five subjects per treatment group were randomly chosen to obtain serum LH levels. LH was determined at the Core Endocrine Laboratory at Pennsylvania State University College of Medicine by competitive radioimmunoassay using reagents obtained from ALPCO Diagnostics (Salem, NH), based on prior protocols (Roman et al., 2003). The assay uses ^{125}I-labeled rat LH tracer for binding to a highly specific rabbit polyclonal antibody and separation of bound from free trace with an anti-rabbit IgG precipitating antibody. Results are expressed in ng/ml. Lower limit of quantification for the assay is 0.5 ng/ml and within run and between run accuracy averaged 7% and 9% respectively at a concentration of 4.2 ng/ml.

Brain dissection and nAChR agonist binding

Using radioligand binding assays, we investigated whether treatment with Δ^8E1 or equilin affected rα4β2-nAChR expression levels.I-epi binding was used to estimate number of β2*- and β4*-nAChRs in the hippocampus and entorhinal cortex (Whiteaker et al., 2000; Whiteaker et al., 2008). At sacrifice, brains were rapidly dissected; one subject was excluded due to technical error. Each brain was cut on the coronal plane. Next, the entorhinal cortex (taking a 2- to 3- mm sample ventral to the hippocampus) and the CA1/CA2 region of the hippocampus (dentate gyrus and alveus excluded) were dissected. Dissected tissues were immediately placed in microcentrifuge tubes, frozen on dry ice, and stored at −70 °C until analysis. Hippocampal and entorhinal cortex tissues were separately homogenized in ice-cold 1× Ringer's buffer with sodium azide (in mM : 144 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 50 Tris-base; and 0.02 % (w/v) NaN_3; pH 7.4; binding buffer). Homogenates were washed three times by centrifugation (12,000 × g; 15 minutes; 4 °C) and rehomogenized into 1× binding buffer and stored, pelleted, at 4 °C. I-epi binding was assessed at 1 nM. Non-specific binding was assessed in the presence of 300 mM carbamylcholine. Specific binding was defined as the difference between total and non-specific binding as fmol per mg of protein; this served as the dependent variable for statistical analyses. Since spatial memory processing is dependent on both the hippocampus and entorhinal cortex, and the functioning of these regions is intimately linked (for review see Jarrard, 1993; Zola-Morgan et al., 1994), specific I-epi binding data in the hippocampus and entorhinal cortex were analyzed together creating a single measure; the combined hippocampal and entorhinal cortex data will now be referred to as the hippocampus+entorhinal cortex. In the hippocampus/entorhinal cortex the contribution of β4*-nAChR was minimal (data not shown). Accordingly, I-epi binding populations in the hippocampus/entorhinal cortex are

considered to represent β2 subunit-containing nAChR, likely α4β2-nAChR (Whiteaker et al., 2000), as such, specific I-epi binding will be referred to as rα4β2-nAChR expression.

$^{86}Rb^{+}$ efflux for Δ^{8}E1 and equilin effects on hα4β2-nAChR function

Routine culture of SH-EP1 cells—To ascertain whether hormone effects could be due to direct actions on receptor function, we evaluated whether Δ^{8}E1 or equilin exposure directly affected function of heterologously expressed, hα4β2-nAChR assessed using $^{86}Rb^{+}$ efflux experiments. SH-EP1 cells stably expressing hα4β2-nAChR were maintained as previously described (Eaton et al., 2003). Briefly, cells were grown in Dulbecco's modified Eagle's medium (high glucose, bicarbonate-buffered, with 1 mM sodium pyruvate and 8 mM L-glutamine) supplemented with 10% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Invitrogen, Carlsbad, CA) plus 5% fetal bovine serum (Hyclone, Logan, UT) on 100-mm diameter plates in a humidified atmosphere containing 5% CO2 in air. Positive selection for the human α4 and β2 subunits (in pcDNA3.1/zeo and pcDNA3.1/Hygro, respectively; also from Invitrogen) was maintained by further supplementation of the growth medium with 0.25 mg/ml zeocin (Invitrogen), and 0.4 mg/ml hygromycin B (Calbiochem, San Diego, CA). Cells were maintained at low passage numbers (1–26 from frozen stocks), and passaged weekly by splitting confluent cultures 1/20 – 1/40 to maintain cells in proliferative growth.

$^{86}Rb^{+}$ efflux assays in the presence of acute hormone—Cells were harvested at confluence from 100-mm plates by mild trypsinization before being resuspended in growth medium and evenly seeded at a density of one confluent 100-mm plate per 24-well plate. Cells were allowed to adhere for a minimum of 6 hours. Medium was removed and replaced with 250 μl/well of medium supplemented with approximately 300,000 cpm of $^{86}Rb^{+}$ (PerkinElmer; counted at 40 % efficiency using Cerenkov counting, PerkinElmer Tricarb 1900 LSC). After 4 hours of loading, $^{86}Rb^{+}$ efflux was measured using the flip-plate technique (Lukas et al., 2002). Following aspiration of the bulk of $^{86}Rb^{+}$ loading medium from each well of the cell plate, each well containing cells was rinsed three times with 2 ml of fresh efflux buffer (130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 50 mM HEPES, pH 7.4) to remove extracellular $^{86}Rb^{+}$ After removal of residual rinse buffer by aspiration, the flip-plate technique was used again to simultaneously introduce fresh efflux buffer containing drugs of choice at indicated final concentrations from a 24-well "efflux/drug plate" into the wells of the cell plate. In the case of the acute hormone effect experiments, the drugs used consisted of a test agonist (carbamylcholine at 100 μM) in the presence of Δ^{8}E1, equilin, and PEG (95 fM-1 μM), representing concentrations below, at, or above physiological relevant brain levels of 17β-estradiol (see Woolley, 2007). After a 5-minute incubation, the solution was "flipped" back into the efflux/ drug plate, any remaining medium was removed by aspiration. Suspensions in each well were then subjected to Cerenkov counting (PerkinElmer Wallac Micobeta Trilux 1450; 25% efficiency) after placement of inserts (PerkinElmerWallac 1450-109) into each well to minimize cross-talk between wells. Specific $^{86}Rb^{+}$ efflux was calculated as efflux above the no-agonist control and expressed as a percentage of efflux evoked by a maximally-effective carbamylcholine (1 mM) control stimulation. Increases or decreases in specific agonist-induced efflux (i.e. efflux above non-specific levels) would be interpreted as direct, nAChR-mediated, acute allosteric agonist or antagonist (respectively) effects. Intrinsic agonist activity of Δ^{8}E1, equilin or PEG was calculated in samples containing the same concentrations tested in the presence of agonist. The amount of specific $^{86}Rb^{+}$ efflux, expressed as % of control, in response to log dilutions of Δ^{8}E1, equilin, and PEG served as the dependent measure of hα4β2-nAChR receptor function.

$^{86}Rb^{+}$ efflux assays after chronic hormone treatment—Cell plating for chronic hormone studies were the same as described previously for assessment of acute hormone

effects, with the exception that cells were seeded more thinly into the 24-well plates (one half of a confluent 100-mm plate per six 24-well plates), allowing them to be grown for 48-hours in the presence of either Δ^8E1 or equilin (9 aM - 1 μM) or 0.1% PEG and to reach confluence at the end of this period. Effects of chronic hormone exposure on $^{86}Rb^+$ efflux were assessed as previously described for measurement of acute effects with minor exceptions. Briefly, after the 48-hour hormone pretreatment period, media containing the hormone was removed and replaced fresh media containing $^{86}Rb^+$ but devoid of hormone for the 4 hour isotope loading period. Cells were rinsed free or extracellular isotope, and hormones at desired concentrations or 0.1% PEG (matching conditions used in the preincubation period) were then simultaneously introduced in fresh efflux buffer with or without carbamylcholine from a 24-well "efflux/drug plate" into the wells of the cell plate. After a 5-minute incubation, the solution was "flipped" back into the efflux/drug plate, any remaining medium was removed by aspiration. The dependent measure was identical to the acute experiment (see above).

Statistical Analyses

For behavior assessments, data were analyzed using an omnibus mixed model ANOVA with Treatment as the between variable, and Days, Block, and/or Trials as the within variable(s), as appropriate for the specific maze test. rα4β2-nAChR expression data were analyzed using an omnibus factorial ANOVA with Treatment and Brain region as between variables. Uterine weight and LH levels were analyzed using an omnibus one-way ANOVA with Treatment as between variable. In all ANOVAs conducted, each hormone type was analyzed separately, so each omnibus ANOVA included the Veh group as well as the Low, Med, and High group of either Δ^8E1 or equilin. Since our interest was to determine whether each hormone dose enhanced performance relative to the Veh group, all follow-up comparisons employed Fisher post hoc tests when a significant omnibus ANOVA was found, noting that Type I error correction is not necessary with orthogonal planned comparisons (Keppel and Wickens, 2004). For efflux experiments, multiple regression analyses evaluated if the slope of the "line of best fit" differed from 0, followed by a Runs analysis to determine if the data deviated from linearity.

Results

Win-stay DMS plus maze: spatial working and recent memory

Spatial working memory was assessed using the win-stay DMS plus maze task. Analysis of repeat errors committed on trial 2 alone (the working memory trial) for Δ^8E1 revealed a significant main effect of Treatment ($F(3,25) = 3.02$, $p = 0.0405$). The Δ^8E1-Med and Δ^8E1-High groups made fewer repeat errors on trial 2 as compared to the Veh group (ps < 0.0107; Figure 1a). The analysis of repeat errors for trials 3–6 (recent memory trials) revealed a marginal Treatment main effect for Δ^8E1 ($F(3,25) = 2.45$, $p = 0.0867$). When all DMS test trials were combined (trials 2–6, working and recent memory trials) there was a Treatment main effect for Δ^8E1 ($F(3,25) = 3.61$, $p = 0.0272$). The Δ^8E1 -Med and Δ^8E1-High groups made fewer repeat errors for all test trials combined as compared to the Veh group (ps < 0.0107). There were no main effects or interactions for repeat errors for equilin (Figure 1b), nor were there effects for initial errors for either hormone.

Morris maze: spatial reference memory

Animals were tested on the Morris maze to evaluate spatial reference memory. There was a main effect of Treatment ($F(3,26) = 6.64$, $p = 0.0018$), in the absence of a Block x Treatment interaction ($F(3,26) = 0.21$, $p = 0.8921$). Post-hoc analyses revealed that Δ^8E1 -Low, Δ^8E1 -Med, and Δ^8E1-High groups each swam less distance than the Veh group (ps < 0.0027; Figure 2a). For equilin, there were no significant main effects or interactions (Figure 2b). For the probe trial, each treatment group localized to the Northeast quadrant which previously contained the

hidden escape platform, with a higher percent distance in the Northeast quadrant (Quadrant main effect: Δ^8E1 $F(1,26) = 181.21$, $p < 0.0001$; Figure 2c; Equilin $F(1,26) = 116.19$, $p < 0.0001$; Figure 2d). This was in the absence of a Treatment x Quadrant interaction for either hormone, indicating that all groups localized to the previously platformed quadrant.

Visible platform task: motoric and visual competence

Motor and visual competence was assessed using a visible platform task. There were no main effects of Treatment for Δ^8E1 or equilin, thereby indicating that animals, regardless of hormone status, were able to locate the visible platform (Δ^8E1 $F(3,26) = 0.83$, $p = 0.4908$; Figure 3a; Equilin $F(3,26) = 0.59$, $p < 0.6302$; Figure 3b). All animals found the platform within 10 seconds on the last two trials, thereby indicating that all animals were able to perform the procedural components of a swim maze task.

nAChR agonist binding: rα4β2-nAChR expression levels

We investigated whether treatment with Δ^8E1 or equilin affected nAChR expression levels using a radioligand binding assay. Analysis of Δ^8E1 in the hippocampus+entorhinal cortex revealed a significant main effect of Treatment ($F(3,48) = 4.33$, $p = 0.0089$), with each Δ^8E1 group showing decreased ra4p2-nAChR expression as compared to the Veh group ($ps \leq .05$; Figure 4a). Moreover, when Δ^8E1 -Low, Δ^8E1-Med and Δ^8E1-High groups were combined into one Δ^8E1 group, there was a Treatment main effect ($F(1,54) = 5.25$, $p = 0.0259$; Figure 4a). There were also greater rα4β2-nAChR levels in the entorhinal cortex compared to the hippocampus (Brain Region main effect: ($F(1,48) = 59.81$, $p < 0.0001$; Figure 4a), and there was a null Treatment x Brain Region interaction indicating this pattern was shown for all Δ^8E1 all doses. In the equilin analysis, there was no significant Treatment main effect ($F(3,50) = 1.84$, $p = 0.1517$); however, as seen in the Δ^8E1 omnibus ANOVA, there were greater rα4β2-nAChR levels in the entorhinal cortex compared to the hippocampus (Brain Region main effect: ($F(1,50) = 41.63$, $p < 0.0001$; Figure 4b), in the absence of a Treatment x Brain Region interaction.

Regression: relation between nicotinic receptor agonist binding and memory

Since a decrease in rα4β2-nAChR expression was noted with Δ^8E1 treatment in the hippocampus+entorhinal cortex, we conducted primary regression analyses relating rα4β2-nAChR expression in the hippocampus+entorhinal cortex to maze performance. Specifically, rα4β2-nAChR expression in the hippocampus+entorhinal cortex was the predictor variable in the model, and Morris maze swim distance collapsed across all days, repeat DMS plus maze errors on trial 2 alone (collapsed across days 2–7), or repeat DMS plus maze errors on trials 2–6 (collapsed across days 2–7), was the outcome variable. There was a positive relationship between rα4β2-nAChR expression in the hippocampus+entorhinal cortex and Morris maze swim distance ($b = 0.03$, $r = 0.64$, $z(28) = 3.75$, $p = 0.0002$; Figure 5). Since greater swim distance indicates poorer performance, better spatial reference memory performance was associated with less rα4β2-nAChR expression in the hippocampus+entorhinal cortex. When the data were centered to control for group membership (for more detailed methods and rationale see Enders and Tofighi, 2007;Hallahan and Rosenthal, 2000), the regression analysis remained significant ($b = 7.71$, $r = 0.44$, $z(28) = 2.36$, $p = 0.0182$). There were no significant regression analyses relating rα4β2-nAChR expression in the hippocampus+entorhinal cortex to working or recent memory repeat errors on the win-stay DMS plus maze (trial 2: $b = 0.001$, $r = 0.06$, $z(28) = 0.26$, $p = 0.7829$; trials 2–6: $b = 0.001$, $r = 0.15$, $z(28) = 0.78$, $p = 0.4365$).

Efflux experiments: evaluation of direct estrogenic actions on hα4β2-nAChR function

We evaluated whether Δ^8E1 or equilin exposure directly affected the function of hα4β2-nAChRs assessed using $^{86}Rb^+$ efflux experiments in vitro. For either acute or chronic

administration, regression analyses revealed that slopes of the best fit line did not differ from zero (*p*s > 0.05), and the data did not deviate from linearity (Runs test, all *p*s > 0.05; Figure 6a, 6b and 6c). PEG did not affect or interact with Δ^8E1 or equilin in altering $^{86}Rb^+$ efflux during acute, or after chronic exposure. This indicates that neither acute nor chronic effects of hormone or vehicle on hα4β2-nAChR function occur at the concentrations tested.

Vaginal smears, uterine weights and serum LH: classic estrogenic actions

We tested peripheral markers that are routinely noted to change with 17β-estradiol treatment. Before Ovx surgeries, all animals demonstrated normal cyclicity. After Ovx (but before hormone treatment), all rats consistently exhibited leukocytic, diestrus smears, as expected. Two days prior to behavior testing, after assigned vehicle or hormone had been administered, all Veh-treated rats presented consistent diestrus smears, whereas hormone-treated rats showed constant cornified, estrous smears demonstrating uterine stimulation. These cytological profiles remained stable through sacrifice (Figure 7). Furthermore, each Δ^8E1 group, and each Equilin group, had increased uterine weight compared to the Veh group (Δ^8E1 $F(3,25) = 13.51.$, $p < 0.0001$; Figure 8a; Equilin $F(3,25) = 34.96$, $p < 0.0001$; Figure 8b; Fisher *p*s < 0.0028), and lower LH levels as compared to the Veh group (Δ^8E1 $F(3,15) = 4.95$, $p = 0.0139$; Figure 8c; Equilin $F(3,15) = 8.67$, $p = 0.0014$; Figure 8d; Fisher *p*s < 0.0143).

Discussion

The present study is the first to evaluate whether Δ^8E1 and equilin, two primary components of Premarin® shown to have the most neuroprotective effects in vitro (Brinton et al., 1997; Zemlyak et al., 2002; Zhao and Brinton, 2006), influence cognition. In middle-aged surgically menopausal rats, Δ^8E1 treatment enhanced spatial reference, working, and recent memory. In these same animals, Δ^8E1 treatment decreased rα4β2-nAChR expression (i.e., specific I-epi binding) in the hippocampus and entorhinal cortex, which was related to better spatial reference memory performance on the Morris maze. Equilin treatment did not impact spatial memory nor rα4β2-nAChR expression. There were no direct effects on hα4β2-nAChR function with acute or prolonged exposure to Δ^8E1, equilin or PEG (vehicle).

Δ^8E1 and equilin effects on spatial working and recent memory

The Δ^8E1 medium and high doses enhanced working and recent memory performance on the DMS plus maze task, while equilin did not. These Δ^8E1-induced spatial working memory enhancements corroborate previous findings whereby 17β-estradiol, and higher doses of Premarin®, benefited spatial working memory in Ovx rats (Acosta et al., 2009b; Bimonte and Denenberg, 1999). It is conceivable that the Δ^8E1 medium and high doses facilitated better performance on this task by predisposing these animals to use a place strategy, as this is seen with 17β-estradiol treatment (Korol and Kolo, 2002). In our DMS plus maze task, animals using a response strategy (i.e., turn left or turn right) would have been inefficient at locating the platform, as the start location was varied and the goal arm remained in the same place in space within a day, leading to more errors. We speculate that the use of a place strategy allowed Δ^8E1 medium and high dose treated animals to accumulate fewer errors by creating a more stable cognitive map, in part by having a better ability to hold the spatial location of the platform in working memory (Barnes et al., 1997; Wilson et al., 2004).

Δ^8E1 and equilin effects on spatial reference memory

We found that all doses of Δ^8E1 enhanced spatial reference memory performance. This was evidenced by less overall Morris maze swim distance in the Δ^8E1 treated groups as compared to the Vehicle group. Equilin treatment did not affect Morris maze performance. Tonic subcutaneous Δ^8E1-induced spatial reference memory enhancements are in accordance with Morris maze improvements shown by others after tonic subcutaneous 17β-estradiol treatment

(Bimonte-Nelson et al., 2006; Foster et al., 2003; Talboom et al., 2008), and after cyclic subcutaneous Premarin® treatment (Acosta et al., 2009b), in middle-aged Ovx animals. Interestingly, Δ^8E1's spatial reference memory enhancement did not vary within the dose range studied, and the effect was robust. In fact, Δ^8E1's effects noted here are relatively more pronounced as compared to other reports using 17β-estradiol or Premarin® where effects were specific to aiding overnight retention or performance during the first 6 test trials (Acosta et al., 2009b; Markham et al., 2002; Talboom et al., 2008).

It is noteworthy that the Morris maze learning curves and probe trial data suggest that by the last testing day all groups learned the task and localized to the platform quadrant. Further, all animals readily learned the visible platform task with no group differences. That all animals could learn these two tasks suggests that our observed Treatment effects were not likely due to differences in visual competence or motoric ability.

Δ^8E1 and equilin effects on α4β2-nAChR expression and function

All doses of Δ^8E1 decreased rα4β2-nAChR expression in the hippocampus and entorhinal cortex, while equilin did not alter rα4β2-nAChR expression in any region. Our hippocampus and entorhinal cortex nAChR data are in agreement with other studies demonstrating that 17β-estradiol treatment decreased the number of nAChRs and mAChRs in the central nervous system of Ovx rodents (Cardoso et al., 2004; El-Bakri et al., 2002), although not all studies show this effect (see Centeno et al., 2006; Lapchak et al., 1990). The current results also indicate that hippocampus+entorhinal cortex rα4β2-nAChR expression has implications for cognition. Indeed, a decrease in rα4β2-nAChR expression in the hippocampus+entorhinal cortex was associated with better spatial reference memory performance (i.e., $r = 0.64$), representing a large effect size as defined by Cohen (Cohen, 1988).

We performed a follow-up study to assess possible direct actions of Δ^8E1and equilin on hα4β2-nAChR, as ethinyl β-estradiol has been found to directly potentiate both hα4β2- and rα4β2-nAChR function (i.e., ion efflux) in vitro (Paradiso et al., 2001). We found that exposure to Δ^8E1 or equilin, below, at, or above physiologically relevant brain concentrations of 17β-estradiol (95 fM-1 μM for our acute experiments, and 9 aM-1 μM for our chronic experiments) (see Woolley, 2007), had no detectable effect on function of hα4β2-nAChRs. Previous in vitro studies reporting potentiation of α4β2-nAChRs by estrogens found that in some cases, rα4β2-nAChRs and hα4β2-nAChR do not show comparable responses to the same estrogens, and the concentrations of estrogens used were higher in comparison to the current evaluations (Paradiso et al., 2001). These higher concentrations may not be physiologically relevant in the context of endogenous estrogen levels (Paradiso et al., 2001). This previous report also suggests that the lack of a hydroxyl group at the 17th position on Δ^8E1 may prevent it from potentiating hα4β2-nAChR (Paradiso et al., 2001). Taken together with our data indicating the absence of direct Δ^8E1 effects on hα4β2-nAChR, we hypothesize that Δ^8E1-mediated alterations in rα4β2-nAChRs expression reflect adaptation to Δ^8E1-mediated changes via other aspects of the cholinergic system. It is possible that, similar to 17β-estradiol (Marriott and Korol, 2003), Δ^8E1 treatment leads to enhanced ACh release in the hippocampus and entorhinal cortex which may, in turn, result in reduced expression of α4β2-nAChRs as a compensatory mechanism.

Mediation of Δ^8E1's effects and future directions

Δ^8E1 and equilin bind to, and produce biological activity via, both estrogen receptor (ER) subtypes (Bhavnani et al., 2008). It is likely that Δ^8E1-mediated spatial memory enhancements are primarily, but not solely, mediated by ER activation, as 17β-estradiol's place learning enhancement is influenced by hippocampal ER activation (Zurkovsky et al., 2006). Recent evidence also suggests that estrogenic effects are influenced by the putative G-protein coupled membrane-bound ER (ER-X; reviewed in Toran-Allerand, 2005) and/or GPR30, a G-protein

coupled receptor that responds rapidly to estrogen (reviewed in Prossnitz et al., 2008). It is possible that Δ^8E1's cognitive effects are mediated in part by these non-classic ERs. In fact, recently, Gibbs and colleagues found that the GPR30 agonist G1 enhanced DMP learning (Hammond et al., 2009). It is also noted that Δ^8E1-induced mnemonic enhancements could be related to non-genomic mechanisms as well, as estrogens impart robust non-genomic effects (Prokai and Simpkins, 2007)

Our current experimental design did not allow us to decipher the effects of behavior testing from the effects of hormone treatment on rα4β2-nAChRs expression. Indeed, rα4β2-nAChR expression may have been influenced by behavior testing alone, in a similar manner as that previously noted for hippocampal dendritic spines (see Frick et al., 2004). In addition, the regulation of nicotinic receptor expression by ligands occurs by diverse mechanisms, and these are conserved across different systems (for review see Gaimarri et al., 2007; Govind et al., 2009; Lester et al., 2009). However, the use of a model system allowed us to study hormone effects on a single, well-defined subtype. In our in vitro model system, whole-animal pharmacokinetics and the effects of brain-region differences was an obviated issue. The lack of effect in the model system (where there is no synaptic function), in contrast to the effects in vivo, allows us to conclude that our in vivo effects on 4β2-nAChRs likely reflect a functional adaptation in response to the Δ^8E1 treatment, and not a direct effect of the hormones on the receptors themselves. Further studies are necessary to detail the specific contributions of Δ^8E1 on cholinergic function and rα4β2-nAChR expression, independent of behavioral testing effects, as well as to fully identify the mechanisms of Δ^8E1-mediated mnemonic enhancements both at the cellular and the systems level.

Conclusions

There is increasing evidence that Δ^8E1 demonstrates unique properties compared to more widely-studied estrogens such as 17α- and 17β- estradiol (Baracat et al., 1999; Bhavnani, 1998). Data from other laboratories have demonstrated that Δ^8E1 has an attenuated toxic potential compared to other Premarin® components (Zhang et al., 2001), shows distinct tissue and cell-specific estrogenic properties in relation to estrone (Baracat et al., 1999), and appears to be converted to only one metabolite in women, giving Δ^8E1 a unique pharmacokinetic profile (Bhavnani, 1998). Two studies have evaluated Δ^8E1 in women. These works found that Δ^8E1 exerted an overall beneficial profile for health-related concerns associated with menopause (Baracat et al., 1999; Bhavnani et al., 1998; for review see Utian et al., 2006). Together with the data presented here, the collected evidence suggests that Δ^8E1 is a uniquely beneficial estrogen that warrants further study as a potential nootropic therapy. In summary, Δ^8E1 enhanced multiple domains of learning and memory, with a link to the cholinergic system as shown by changes in nicotinic receptor expression. Equilin did not affect spatial memory and had no effects on nicotinic receptor expression. Select components of Premarin® may offer promising new HT options that positively impact brain health during aging. An exciting new direction might be to further define the individual components of Premarin®. This would allow composition of a novel combined estrogen therapy, with the ultimate goal to optimize the potential benefits, and obviate the risks, of currently used estrogen therapies.

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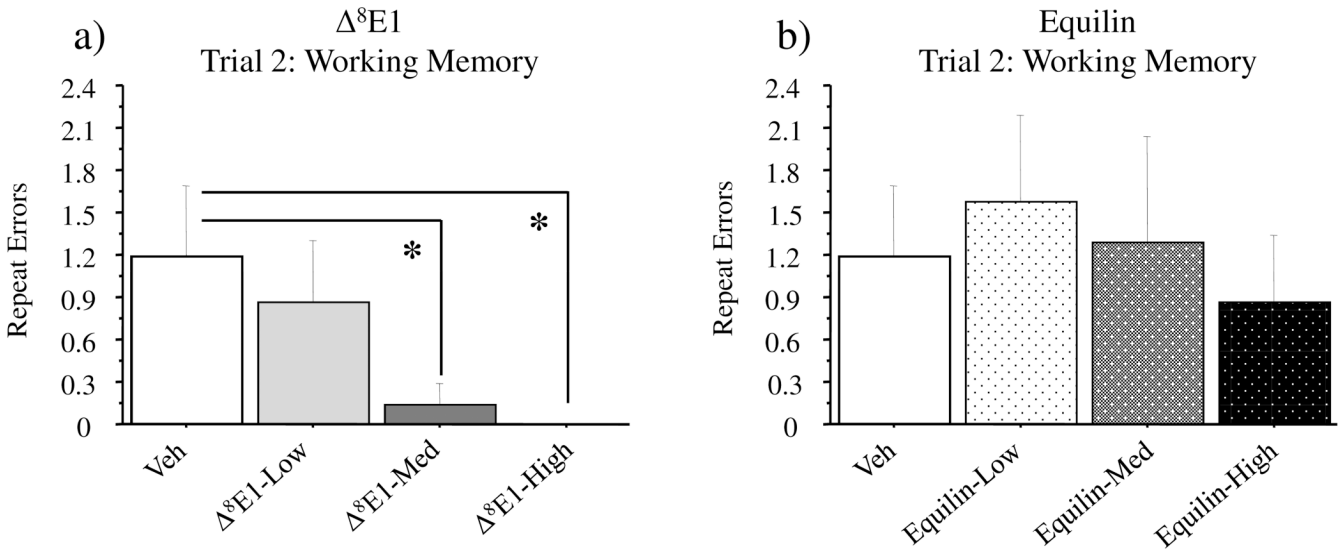

Figure 1.

DMS plus maze. Mean number±*SE* of repeat errors committed on the working memory trial across testing days 2–7 for: a) Veh and each Δ^8E1 group, and b) Veh and each Equilin group. On the working memory trial, the medium and high doses of Δ^8E1 decreased the number of repeat errors committed relative to the Veh group. Equilin treatment did not yield significant effects. * $p < 0.05$.

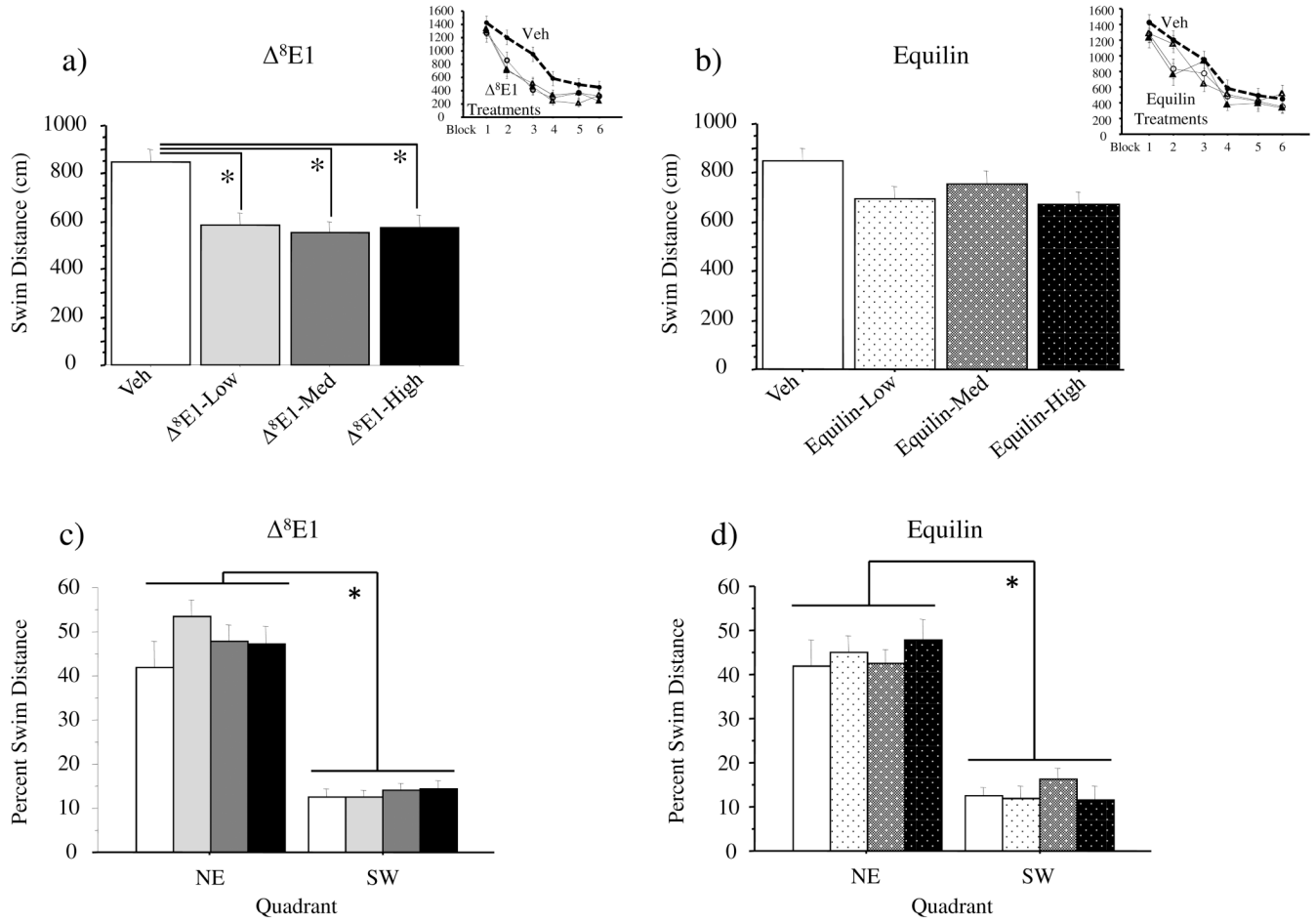

Figure 2.

Morris maze. Mean±*SE* swim distance (cm) collapsed across days for: (a) Veh and each Δ^8E1 group, and b) Veh and each Equilin group; the inset graphs represent swim distance over the 6 blocks of testing. c) and d) depict mean±*SE* percent swim distance in the target and opposite quadrants on the probe trial for the Veh and each Δ^8E1 group, and the Veh and each Equilin group, respectively. Each Δ^8E1 dose enhanced spatial reference memory relative to the Veh group, collapsed across all days. Equilin did not impact spatial reference memory performance. For the probe trial, all groups localized to the quadrant that previously contained the platform (target, NE) as compared to the opposite quadrant (SW). * $p < 0.05$.

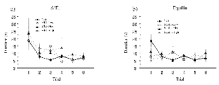

Figure 3.

Visible platform. Mean±*SE* latency (seconds) to reach the platform on the visible platform task for: a) Veh and each Δ^8E1 group, and b) Veh and each Equilin group. There were no Treatment effects, and all groups readily located the visible platform within 10 seconds. These data confirm visual and motor competence by all subjects for platform search and localization.

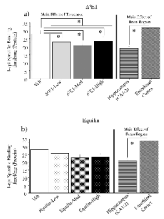

Figure 4.
Mean±*SE* rα4β2-nAChR expression in the hippocampus and entorhinal cortex for: a) Veh and each Δ^8E1 group, and b) Veh and each Equilin group. As represented in the left panel, for the hippocampus+entorhinal cortex (regions combined), all Δ^8E1 groups had decreased α4β2-nAChR expression as compared to the Veh group. Equilin treatment did not alter rα4β2-nAChR expression. As represented in the right panel, both the Δ^8E1 and equilin analyses, more α4β2-nAChRs were present in the entorhinal cortex as compared to the hippocampus. * $p < 0.05$.

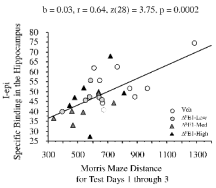

Figure 5.

Scatterplot representing the relation between rα4β2-nAChR expression levels in the hippocampus+entorhinal cortex and Morris maze swim distance across all days and trials for all Δ^8E1 groups and the Veh group. The line represents the linear regression analysis of best fit. Better reference memory performance (lower swim distance) was related to lower rα4β2-nAChR expression.

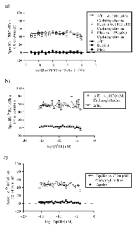

Figure 6.

Concentration-response profiles showing effects on hα4β2-nAChR function (i.e., ion efflux) following: a) acute treatment with Δ^8E1, equilin or vehicle PEG, b) 48-hour treatment with Δ^8E1 and c) 48-hour treatment with equilin. The lines on each graph represent the linear regression analysis of best fit. No systematic effect of acute or 48-hour hormone or vehicle exposure on specific $^{86}Rb^+$ efflux was observed over the range of hormone or vehicle concentrations tested.

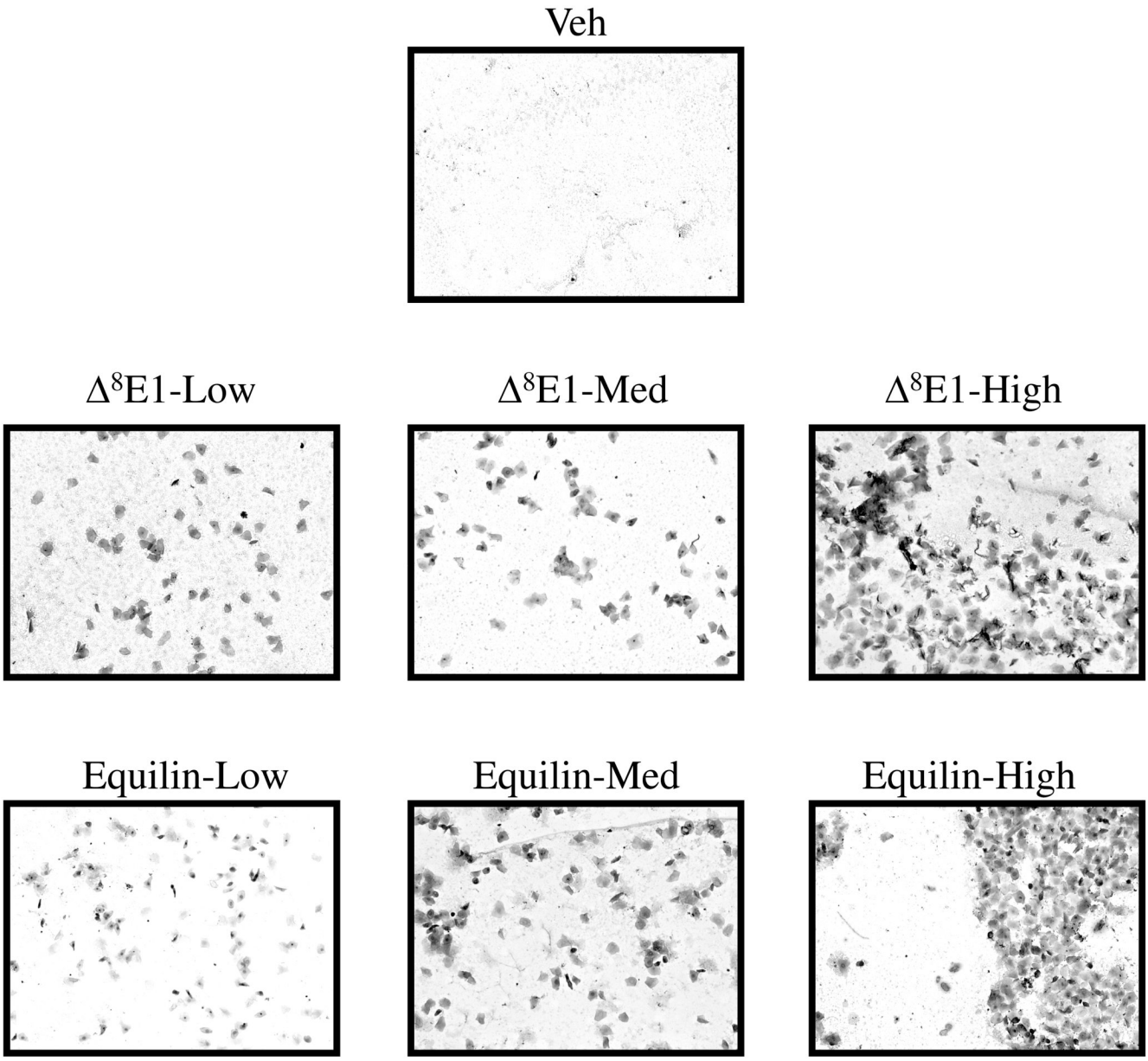

Figure 7.
Representative brightfield eosinY- and hematoxylin- stained vaginal cytology pictures (10X) from Veh, Δ^8E1, and Equilin groups, taken the day before sacrifice. Veh animals displayed few cells, consistent with diestrus and no uterine stimulation. Each group receiving Δ^8E1 or equilin had numerous stained cornified cells, indicative of estrous and uterine stimulation.

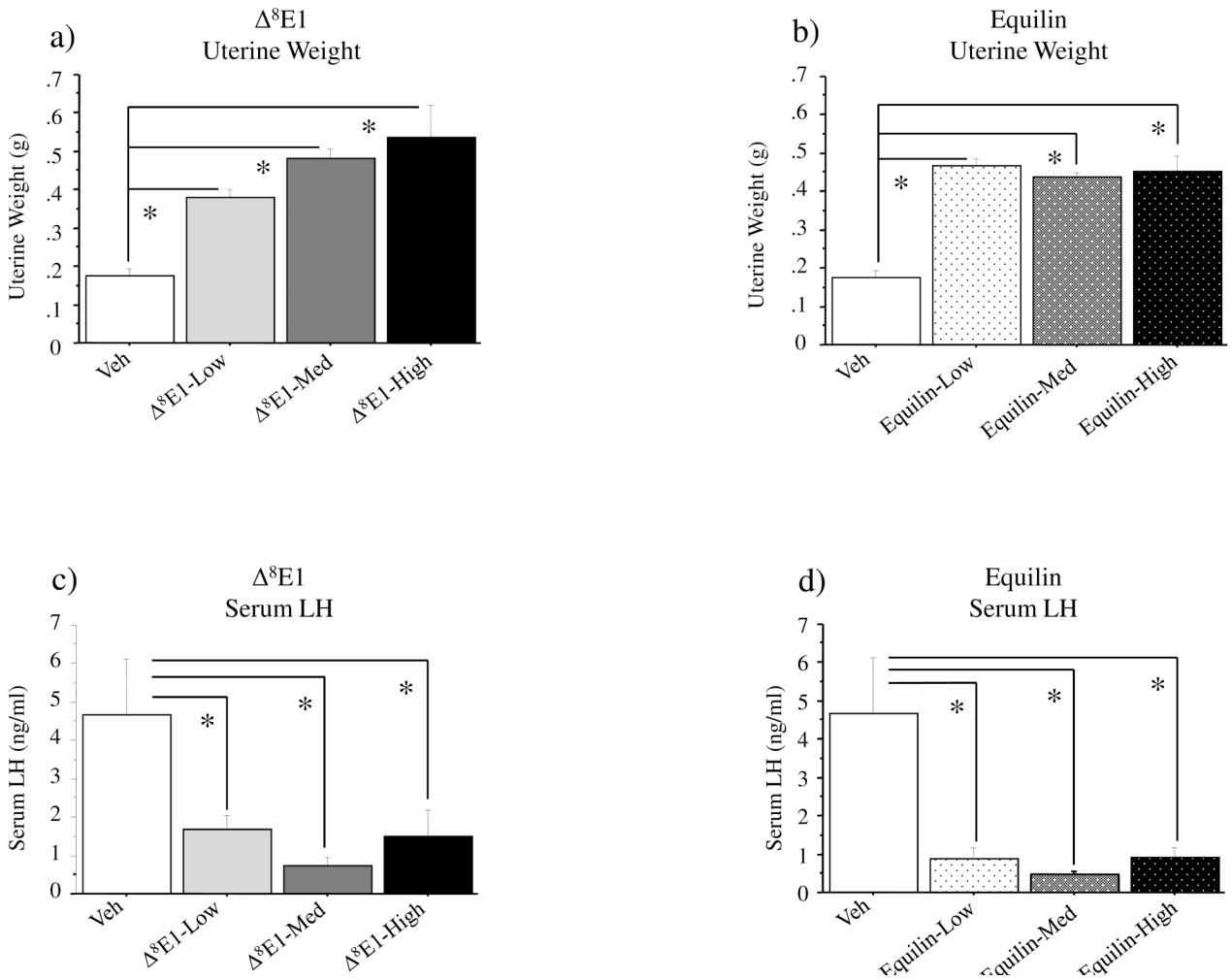

Figure 8.

Mean±*SE* uterine weight (grams) for: a) Veh and each Δ^8E1 group, and b) Veh and each Equilin group. Mean±*SE* serum LH levels (ng/ml) for: c) Veh and each Δ^8E1 group, and d) Veh and each Equilin group. Each dose of Δ^8E1 and equilin increased uterine weights and decreased serum LH levels as compared to the Veh group. * $p < 0.05$.