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Pharmacological blockade of the aromatase enzyme, but not the androgen receptor, reverses androstenedione-induced cognitive impairments in young surgically menopausal rats

Sarah E. Mennenga^{a,b}, Stephanie V. Koebele^{a,b}, Abeer A. Mousa^{a,b}, Tanya J. Alderete^{a,b}, Candy W.S. Tsang^{a,b}, Jazmin I. Acosta^{a,b}, Bryan W. Camp^{a,b}, Laurence M. Demers^c, and Heather A. Bimonte-Nelson^{a,b}

^aDepartment of Psychology, Arizona State University, Tempe, AZ 85287

^bArizona Alzheimer's Consortium, Phoenix, AZ 85006

^cThe Pennsylvania State University College of Medicine, The M. S. Hershey Medical Center, Hershey, PA 17033

Abstract

Androstenedione, the main circulating ovarian hormone present after menopause, has been shown to positively correlate with poor spatial memory in an ovary-intact rodent model of follicular depletion, and to impair spatial memory when administered exogenously to surgically menopausal ovariectomized rats. Androstenedione can be converted directly to estrone via the aromatase enzyme, or to testosterone. The current study investigated the hormonal mechanism underlying androstenedione-induced cognitive impairments. Young adult ovariectomized rats were given either androstenedione, androstenedione plus the aromatase inhibitor anastrozole to block conversion to estrone, androstenedione+the androgen receptor blocker flutamide to block androgen receptor activity, or vehicle treatment, and were then administered a battery of learning and memory maze tasks. Since we have previously shown that estrone administration to ovariectomized rats impaired cognition, we hypothesized that androstenedione's conversion to estrone underlies, in part, its negative cognitive impact. Here, androstenedione administration impaired spatial reference and working memory. Further, androstenedione did not induce memory deficits when co-administered with the aromatase inhibitor, anastrozole, whereas pharmacological blockade of the androgen receptor failed to block the cognitive impairing effects of androstenedione. Anastrozole alone did not impact performance on any cognitive measure. The current data support the tenet that androstenedione impairs memory through its conversion to estrone, rather than via actions on the androgen receptor. Studying the effects of aromatase and estrogen metabolism is critical to elucidating how hormones impact women's health across the

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Corresponding author: Dr. Heather A. Bimonte-Nelson Arizona State University, Dept. of Psychology Behavioral Neuroscience Division Arizona Alzheimer's Consortium PO Box 871104, Tempe, AZ 85287 Office Phone: 480-727-0766, Fax: 480-965-8544 bimonte.nelson@asu.edu.

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lifespan, and results hold important implications for understanding and optimizing the hormone milieu from the many endogenous and exogenous hormone exposures across the lifetime.

Keywords

Androstenedione; Aromatase; Estrone; Cognition; Memory

Introduction

By the year 2050, the population over the age of 65 in the U.S. is projected to reach 88.5 million people, more than double what it was in the year 2010, and more than half of the population will be female (US Census, 2010). Around the fifth decade of life, most females experience menopause, whereby eggs stop maturing, and eventually ovulation and menstruation cease. With this reproductive senescence, there is a drastic loss of ovarian-derived estrogen and progesterone, and the androgen androstenedione becomes the principal hormone released by the ovaries (Timaras et al., 1995). This androgen-rich hormone milieu is also seen in a rodent model of natural menopause via treatment with 4-vinylcyclohexene diepoxide (VCD), an industrial chemical that induces gradual depletion of primary and primordial follicles in the female rat (Mayer et al., 2002, 2004; Acosta et al., 2009b, 2010).

Accumulating evidence in the female rat suggests that androstenedione has a negative impact on cognition. Our laboratory previously demonstrated that VCD-induced, transitional menopause in middle-aged, female rats elicits inferior cognitive performance across multiple domains, compared to rats that had undergone surgical menopause via ovariectomy (Ovx). Of note, this finding is not apparent in animals that have undergone OvX following their VCD treatment; such that the follicle-deplete ovaries were removed after follicular depletion had ensued (Acosta et al., 2009b). An unexpected finding from this study was that higher serum levels of androstenedione, which is released from the follicle-deplete menopausal ovary (Timaras et al., 1995), correlated with poorer memory scores in follicle-deplete, VCD treated rats (Acosta et al., 2009b). In a follow-up study, we again found that higher androstenedione levels correlated with impaired performance in transitionally menopausal rats demonstrating an androgen-rich serum profile (Acosta et al., 2010). This correlation was evident for multiple types of errors representing several domains of memory, including reference memory, a form of long-term memory that remains constant across all days and trials, as well as two orthogonal measures of working memory, a form of short-term memory that requires updating of information (Acosta et al., 2010). We surmised that if androstenedione was truly related to poorer memory, impairments should be revealed after administration of androstenedione to a “blank” ovarian hormone template. To test this hypothesis, we performed a study in which middle-aged (14 month old) OvX rats were administered either vehicle or one of two doses of androstenedione, and then tested with a battery of mazes that assess learning and memory. Relative to vehicle treatment, androstenedione administration impaired spatial reference memory on the Morris water maze, was detrimental to performance on the water radial-arm maze (WRAM) when the working memory load was most demanding, and impaired memory retention on a win-stay delay match to sample (DMS) task (Camp et al., 2012). Thus, in several different studies we

have shown that androstenedione, released from the follicle-deplete ovary in both women and rats, markedly impairs memory.

Understanding the effects of androstenedione on the brain and its function is critically important to understanding the cognitive impact of natural menopause; ovarian-derived androstenedione is present in menopausal women who maintain their ovaries, an effect observed for at least ten years after menopause ensues (Fogle et al., 2007). Drugs that block the activity of the aromatase enzyme (Santen et al., 2009), which catalyzes the conversion of androstenedione to the estrogen estrone, are some of the tools used to treat metastatic breast cancer prevalent in menopausal women (Glück et al., 2013), as well as manage estrogen-dependent endometrial carcinoma (Gao et al., 2014). Here, we seek to decipher the hormone mechanism(s) underlying the negative cognitive impact of androstenedione using a rat model. Androstenedione could be exerting cognitive effects through a multitude of mechanistic pathways; it is a direct precursor to testosterone via the 17 β -hydroxysteroid dehydrogenase (17 β -HSD) enzyme, and to estrone via the aromatase enzyme, and, further, it binds to androgen receptors (Horton & Tait, 1966; Jasuja et al., 2005). In the rodent model, testosterone administration has been shown to enhance spatial working memory (Bimonte-Nelson et al., 2003b), spatial reference memory (Benice & Raber, 2009), and performance on avoidance tasks (Flood et al., 1995; Edinger et al., 2004). There is also evidence that higher relative levels of testosterone are associated with better spatial ability performance in women, while lower relative levels of salivary testosterone were related to better spatial ability performance in men (Gouchie & Kimura, 1991). We have previously shown that estrone administration in Ovx rats produces cognitive impairments (Engler-Chiurazzi et al., 2012). Given these results, we now hypothesize that androstenedione's conversion to estrone underlies its negative cognitive impact, rather than its actions on the androgen receptor.

The primary purpose of the current study was to systematically evaluate whether androstenedione's conversion to estrone, or its effects on the androgen receptor, are responsible for the negative cognitive effects of androstenedione administration in the surgically menopausal young adult rat. Herein, we tested the hormonal mechanism underlying our previously observed androstenedione-induced cognitive impairments using pharmacological manipulations that either block androstenedione's conversion to estrone, or block androstenedione's androgenic effects by blocking activation of the androgen receptor. Anastrozole, a non-steroidal aromatase inhibitor, or flutamide, a non-steroidal anti-androgen, were co-administered with androstenedione to determine whether androstenedione impairs memory via its conversion to estrone, or via its action on the androgen receptor, respectively. A secondary purpose of this study was to test the effects of anastrozole given alone. Indeed, aromatase inhibitors such as anastrozole are currently used to treat breast cancer and prevent breast cancer recurrence (Santen et al., 2009). Elucidating the impact of aromatase and estrogen metabolism on the brain and its function is critical to our understanding of the systems-level alterations that occur with changes in both endogenous and exogenous steroid hormones.

Materials and Methods

Subjects

Forty-eight four-month-old Fischer-344 virgin female rats born and raised at the National Institute on Aging colony at Harlan Laboratories (Indianapolis, IN) were used. Upon arrival, rats were pair housed, had access to food and water ad-lib, and were maintained on a 12-hour light/dark cycle at the Arizona State University animal facility. All procedures were approved by the local IACUC committee and adhered to NIH standards. Rats arrived two weeks before experiment initiation.

Ovariectomy and hormone treatment

All rats received Ovx 13-14 days before the start of behavioral testing. Animals received bilateral dorsolateral incisions in the skin and peritoneum, the ovaries and tips of the uterine horns were ligatured and removed, and the muscle and skin were then sutured closed. During surgery, rats received an injection of Rimadyl (5mg/ml/kg) for pain and saline (2ml) to prevent dehydration. Hormone or vehicle treatment began 2-3 days after surgery (11 days before behavioral testing ensued) and continued until sacrifice. All assigned treatments were administered daily via subcutaneous injection into the scruff of the neck at an injection volume of 0.5ml. Rats were randomly assigned to one of five treatment groups: Vehicle (n=10), Androstenedione (n=10), Androstenedione+Anastrozole (n=10), Androstenedione+Flutamide (n=10), and Anastrozole (n=10). Vehicle-treated animals received 0.5ml of polyethylene glycol (PEG) (Sigma-Aldrich, St. Louis, MO, USA) only. All rats receiving androstenedione (Steraloids, Newport, RI, USA) were given 2mg daily dissolved in PEG; this dose of androstenedione was based on previous literature (Lea & Flanagan, 1998; Sprando et al., 2004; Camp et al., 2012) and has been shown to produce working memory impairments in middle-aged Ovx rats (Camp et al., 2012). Animals in the Androstenedione+Anastrozole group received 0.025mg/day anastrozole (Tocris, Minneapolis, MN, USA) co-administered with 2mg androstenedione treatment, in order to block activity of the aromatase enzyme, preventing the conversion of androstenedione to estrone. Animals in the Androstenedione+Flutamide treatment group received 27.5mg of flutamide (Sigma-Aldrich, St. Louis, MO, USA) co-administered with 2mg androstenedione treatment, to block the action of testosterone on androgen receptors. The Anastrozole treatment group received 0.025mg/day anastrozole dissolved in PEG.

Twelve days after the initiation of hormone treatment administration, behavioral testing began. Behavioral testing commenced approximately one hour after injections each day, and all treatment groups were counterbalanced across testing squads. All rats were subjected to the complete battery of behavioral evaluations. The order of behavior tests is concordant with our prior studies showing correlations between serum androstenedione levels and memory (Acosta et al., 2009b; Acosta et al., 2010; Camp et al., 2012).

Water radial-arm maze (WRAM)

Subjects were tested for 12 days on the eight-arm win-shift WRAM (Figure 2a) to evaluate spatial working and reference memory, including performance as working memory load increased, as described previously (Bimonte & Denenberg, 1999; Braden et al., 2011). The

black plexiglass maze was filled with water made opaque with black non-toxic paint. The maze contained escape platforms hidden under the surface of the water in the ends of four of eight arms (each arm was 38.1cm × 12.7cm) at the beginning of each day. Each subject was assigned different platform locations that remained constant throughout testing. The subjects were released from the start arm and had 3 minutes to locate a platform. Arm entries were counted when the tip of a rat's snout reached a mark delineated on the outside of the arm and not visible from the inside of the maze (11cm into the arm). Once a platform was found, the subject remained on it for 15s, and was then returned to its heated cage for a 30s inter-trial interval (ITI), until its next trial. During the interval, the just-located platform was removed from the maze. The animal was then placed again into the start alley and allowed to locate another platform. For each animal a daily session consisted of four trials, with the number of platformed arms reduced by one on each subsequent trial. Thus, the working memory system was increasingly taxed as trials progressed, allowing us to assess performance as working memory load increased.

Delay Match to Sample (DMS) Three Choice Task

Following completion of the WRAM, subjects were tested for seven days on the win-stay DMS three-choice task to evaluate spatial working memory and short-term memory retention (Figure 2b). The black plexiglass maze contained four arms (each arm was 38.1cm x 12.7cm), and was filled with water made opaque with black non-toxic paint. There was a single platform hidden beneath the surface of the water; the platform was located in a new arm each day, but remained in the same arm within each day. The animals were released from different arms for each trial, alternating semi-randomly between the three open arms such that animals were dropped off from each arm twice within each testing session. Rats were given 90s to find the platform. Arm entries were counted when the tip of a rat's snout reached a mark delineated on the outside of the arm and not visible from the inside of the maze (11cm into the arm). Once the platform was located, the rat remained on it for 15s, followed by placement into a heated cage for a 30s ITI. Animals received six consecutive trials per day for seven days. Trial one was the information trial, informing the animal where the platform would be located for that day's session, trial two was considered the working memory test trial, and trials three through six were considered recent memory test trials (Frick et al., 1995).

Morris Water Maze

One day after completing DMS testing, subjects were assessed on the Morris water maze (Figure 2c). The apparatus was a tub (188cm diameter) filled with black water made opaque using non-toxic paint. A hidden platform (10cm wide) remained in a fixed location (northeast quadrant) throughout testing, thereby testing spatial reference memory (Morris et al., 1982; Bimonte-Nelson et al., 2006). Animals received six trials per day for three days, and were released into the maze from the north, south, east, or west location varying semi-randomly. Animals were given 60s to locate the hidden platform, and once each subject found the platform, it remained on the platform for 15s, and then was placed into a heated cage until its next trial; the ITI was approximately 15min. Subjects received six trials per day for three days. To evaluate whether subjects localized the platform to the spatial location, after all test trials were completed on Day 3, a 60s probe trial was given where the

platform was removed and each animal was allowed to search for 60s. A camera suspended above the maze and a tracking system (Ethovision, Noldus Instruments, Leesburg, VA, USA) recorded and analyzed each rat's swim pathway.

Visible Platform Maze

Since the Morris water maze, WRAM, and DMS rely on spatial navigation, it was necessary to confirm that all subjects had intact vision and could perform the procedural components of a water escape task without difficulty. A visible platform water escape task was used. A rectangular tub (99 × 58.5cm) was filled with clear water and a black platform (10cm wide) was elevated above the water's surface. Opaque curtains covered obvious extramaze cues. Animals were dropped off from the same location across trials, but the platform location for each trial varied in space semi-randomly across three locations. Animals were given six trials in one day to locate the visible platform; performance was assessed by latency to the platform (s).

Uterine Weights

Prior studies have shown that androgens can stimulate the uterus and lead to increased uterine weight (Ruizeveld de Winter et al., 1991; Horie et al., 1992). To further validate androstenedione's effects on uterine tissues, at sacrifice the uteri of all subjects were removed, trimmed of visible fat, and immediately weighed (wet weight; g).

Blood Serum Analysis

At the time of sacrifice, blood was collected via cardiocentesis. Blood was allowed to clot at 4°C (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ, USA), serum was collected after centrifugation for 20min at 4°C, and serum was stored at -20°C until assays were performed. Serum hormone levels were determined by radioimmunoassay using previously described methods (Acosta et al., 2010; Camp et al., 2012).

Androstenedione was measured in serum using a solid-phase radioimmunoassay (Beckman-Coulter, Webster, TX), based on androstenedione-specific antibodies immobilized to the wall of polypropylene tubes and a ¹²⁵I-labeled androstenedione tracer. Interassay Precision: CV of 7% at mean of 1.1ng/ml (3.8nmol/L), CV of 5% at mean of 3.8 ng/ml (13.3nmol/L). Functional Sensitivity: 1ng/ml.

Testosterone was determined in serum using a competitive solid-phase radioimmunoassay (Beckman-Coulter, Webster, TX) that relies on testosterone-specific antibodies that are immobilized to the wall of polypropylene tubes and compete for testosterone in the sample or purified testosterone standards with ¹²⁵I-labeled testosterone added to the tube as the tracer. Interassay Precision for the assay averages 7% at a mean value of 84ng/dl (2.9nmol/L) and less than 5 % at a mean value of 403ng/dl (13.9nmol/L). Functional sensitivity of the assay is 15ng/dl (0.5nmol/L).

Estrone was determined in serum using a competitive radioimmunoassay (Beckman-Coulter, Webster, TX) with ¹²⁵I-labeled estrone and a highly specific primary antibody. Separation of bound and free antigen was achieved using a double antibody system. Interassay

Precision for the assay averages 11% at a mean value of 35pg/ml. Functional sensitivity of the assay is 5pg/ml.

Statistical Analyses

For WRAM analyses, orthogonal measures of working memory and reference memory errors were quantified as done previously in WRAM studies (Bimonte et al., 2000). Working memory correct (WMC) errors were the number of first and repeat entries into an arm that previously contained a platform within each session. Reference memory (RM) errors were the number of first entries into an arm that never contained a platform within each session. Working memory incorrect (WMI) errors were repeat entries into reference memory arms within each session. Of note, for WMC and WMI errors, the pattern of errors across trials is especially meaningful and critical for interpretation of performance, because working memory demand becomes more elevated as trials progress within each day of testing; the number of WMC or WMI errors an animal can commit is not capped. In contrast, for any given animal four of the eight arms are reference memory arms, and these arms never contain platforms on any trial on any day (these arms never provide reinforcement on any trial); thus, reference memory information needed to solve the task remains constant throughout the entire testing period. It is only possible for animals to make one RM error per arm each day, thereby making this error measure capped at a total of four RM errors per day (with a second or any additional entries into a given reference memory arm counted as WMI). As a result, the later trials for RM errors do not reflect a greater reference memory demand than earlier trials, in contrast to the increasing working memory demand that *does* occur with later trials for WMC and WMI. Testing was divided into three four-day blocks (Block 1=Days 1-4, Block 2=Days 5-8, Block 3=Days 9-12), and the three orthogonal error types (WMC, WMI, and RM) were evaluated separately for each block. Data were analyzed using repeated measures ANOVA with Treatment as the independent variable and number of WMC, WMI, or RM errors across Blocks, Days, and Trials as the repeated measure.

For DMS testing, data were analyzed using repeated measures ANOVA with Treatment as the independent variable and number of total errors across Days and Trials as the repeated measure.

Morris water maze testing was blocked into six three-trial blocks (two Blocks per Day) and analyzed using repeated measures ANOVA with Treatment as the independent variable and swim distance across Blocks and Trials as the repeated measure. For probe trial data, total percent distance in the previously platformed (target, NE) quadrant was compared to that in the quadrant diagonally opposite to the platform (SW). Rats that learned the platform location were expected to swim the greatest distance in the target quadrant (Hyde et al., 2002; Talboom et al., 2008).

Visible platform data were analyzed using repeated measures ANOVA with Treatment as the independent variable, Trials as the repeated measure, and Latency (s) as the dependent variable.

Data were analyzed separately for each maze; two-tailed tests were used throughout, and alpha was set at 0.05. Uterine weights (g), serum androstenedione levels (ng/ml), serum testosterone levels (ng/dl), and serum estrone levels (pg/ml) were analyzed separately using oneway ANOVA, with each respective measure as the dependent variable and Treatment as the independent variable.

Results

Water radial-arm maze

Errors decreased across block for all three memory measures on the WRAM, indicating learning (main effect of Block for WMC [$F_{(2,88)}=52.13$, $p<0.0001$], WMI [$F_{(2,88)}=55.39$, $p<0.0001$], and RM [$F_{(2,88)}=69.25$, $p<0.0001$] errors). There were no Treatment effects for WMC, WMI, and RM for Block 1 (Days 1-4) or Block 2 (Days 5-8) of WRAM testing. We have previously observed effects of exogenous treatment with both androgens and estrogens during the latter portion of testing, so we were particularly interested at effects at the latter testing block (Acosta et al., 2010; Bimonte & Denenberg, 1999; Bimonte-Nelson et al., 2003b; Camp et al., 2012). On Block 3, as predicted, a general pattern emerged, revealing that androstenedione-induced impairments were negated by the addition of the aromatase inhibitor anastrozole, but not by blockade of the androgen receptor through the addition of flutamide. This pattern was observed for all three types of errors evaluated on the WRAM.

For Block 3 of WRAM testing, there was a Treatment $\times$ Trial interaction for WMC errors [$F_{(8,88)}=3.05$, $p<0.01$; figure 2a]. For Trial 4, the trial with the highest working memory load, there was a main effect of Treatment for WMC errors [$F_{(4,44)}=4.31$, $p<0.01$; figure 2a]; post hoc analysis revealed that, on Trial 4, the Androstenedione group committed more WMC errors compared to the Vehicle group (Fisher, $p<0.001$); the addition of aromatase inhibition via anastrozole treatment reversed this androstenedione-induced impairment [Androstenedione vs. Androstenedione+Anastrozole, Fisher, $p<0.01$]. At the highest memory load for WMC, the Androstenedione group also made more errors than the Anastrozole group (Fisher, $p<0.01$) group, and the Androstenedione+Flutamide group committed more errors than the Vehicle group (Fisher, $p<0.05$).

Similar to the effect on Block 3 for WMC, there was also an effect on Block 3 for WMI, with a Treatment $\times$ Trial interaction for WMI errors [$F_{(12,132)}=5.36$, $p<0.0001$; figure 2b]. For Trial 4, there was a main effect of Treatment for WMI errors [$F_{(4,44)}=5.90$, $p<0.001$; figure 2b]; post hoc analysis revealed that, on this trial requiring the highest working memory demand, the Androstenedione group committed more WMI errors compared to Vehicle (Fisher, $p<0.01$). Again, the addition of anastrozole reversed the impairing effect of androstenedione at the highest working memory load [Androstenedione vs Androstenedione +Anastrozole (Fisher, $p<0.01$)], and the Androstenedione group made more errors than the Anastrozole group (Fisher, $p<0.01$). Post hoc analysis also demonstrated that the Androstenedione+Flutamide group committed more WMI errors on Trial 4 than the Vehicle (Fisher, $p<0.01$), Androstenedione+Anastrozole (Fisher, $p<0.01$), and Anastrozole (Fisher, $p<0.01$) groups.

A main effect of Treatment for RM errors was also revealed [$F_{(4,44)}=6.30$, $p<0.001$; figure 2c] for Block 3 of WRAM testing. Post hoc analysis demonstrated that the Androstenedione group committed more RM errors than the Vehicle group (Fisher, $p<0.001$), and, in accordance with effects for both orthogonal working memory error types for the WRAM, the addition of anastrozole reversed reference memory impairments induced by androstenedione [Androstenedione vs. Androstenedione+Anastrozole, Fisher, $p<0.05$]. The Androstenedione group also made more RM errors than the Anastrozole group (Fisher, $p<0.05$), and the Androstenedione+Flutamide group committed more RM errors compared to Vehicle (Fisher, $p<0.001$), Androstenedione+Anastrozole (Fisher, $p<0.01$), and Anastrozole (Fisher, $p<0.05$) groups.

Hormone treatment did not impact performance on the delayed memory retention of multiple platform locations, as there were no treatment effects on the post-delay trials on Day 13 for WMC, WMI, or RM errors on the WRAM.

Delay Match to Sample Three Choice Task

There was a main effect of Day [$F_{(6,264)}=17.17$, $p<0.0001$] with Total Errors decreasing as days progressed. There were no Treatment effects for Total Errors (Days 1-7; figure 3), nor was there a Treatment $\times$ Day interaction.

Morris Water Maze

Analyses revealed a main effect of Block [$F_{(5,220)}=150.06$, $p<0.0001$], with swim distance decreasing across blocks showing learning. There was a Treatment $\times$ Block interaction for Morris water maze testing [$F_{(20,220)}=1.84$; $p<0.05$; figure 4a]. For Block 1, there was a main effect of Treatment [$F_{(4,44)}=2.96$; $p<0.05$; figure 4a]; post hoc analyses revealed that the Androstenedione+Anastrozole group swam a shorter distance to the platform than the Vehicle (Fisher, $p<0.05$), Androstenedione (Fisher, $p<0.05$), and the Androstenedione +Flutamide group (Fisher, $p<0.01$). For the probe trial, there was a main effect of Quadrant [$F_{(1,44)}=982.20$; $p<0.0001$; figure 4b] in the absence of a Quadrant $\times$ Treatment interaction [$F_{(4,44)}=2.30$; $p>0.05$, NS; figure 4b], indicating that all groups equally localized the platform using spatial navigation by the end of Morris water maze testing.

Visible Platform

Figure 5 shows the mean+SEM latency to escape value for each group across all trials for the one day of visible platform testing. There was a main effect of Trial [$F_{(5,220)}=9.32$, $p<0.0001$], with latency decreasing as trials progressed within the day of visible platform testing. There were no Treatment main effects [$F_{(4,44)}=1.49$, $p<0.05$, NS] on latency to escape for the visible platform task. However, there was a Treatment $\times$ Trial interaction [$F_{(20,220)}=2.35$, $p<0.01$], such that there was a main effect of Treatment on Trial 1 [$F_{(4,44)}=3.65$, $p<0.05$]. Further analyses indicated that the Vehicle group took a longer time to reach the platform than the Androstenedione (Fisher, $p<0.05$), Androstenedione+Anastrozole (Fisher, $p<0.01$), and Anastrozole (Fisher, $p<0.001$) groups; no hormone treated group differed from any other hormone treated group. Most importantly, there were no effects of Treatment on any of the remaining trials (Trials 2-6), each animal successfully located the platform on every trial, and by the last trial, all groups found the platform within 16s,

thereby allowing interpretation that animals demonstrated the procedural skills necessary to complete a water maze task.

Uterine Weights

There was a main effect of Treatment for uterine weights [$F_{(4,43)}=13.03$; $p<0.0001$; figure 6]. The Androstenedione group had higher uterine weights than the Vehicle (Fisher, $p<0.0001$), Androstenedione+Anastrozole (Fisher, $p<0.001$), Androstenedione+Flutamide (Fisher, $p<0.0001$), and Anastrozole (Fisher, $p<0.0001$) groups. The Androstenedione +Anastrozole group also had higher uterine weights than the Anastrozole group (Fisher, $p<0.05$).

Serum Levels

There was a main effect of Treatment for serum androstenedione [$F_{(4,31)}=6.04$; $p<0.01$; figure 7a]. Androstenedione treatment increased serum androstenedione levels in all groups receiving this androgen, relative to vehicle treatment [Vehicle vs. Androstenedione (Fisher, $p<0.01$), Vehicle vs. Androstenedione+Flutamide (Fisher, $p<0.05$), Vehicle vs. Androstenedione+Anastrozole (Fisher, $p<0.01$)], and relative to treatment with anastrozole alone [Anastrozole group vs. Androstenedione group (Fisher, $p<0.001$), Anastrozole vs. Androstenedione+Flutamide group (Fisher, $p<0.05$), Anastrozole vs. Androstenedione +Anastrozole group (Fisher, $p<0.01$)].

A main effect of Treatment for serum testosterone was also demonstrated [$F_{(4,28)}=4.60$; $p<0.01$; figure 7b]. The Androstenedione group had higher testosterone serum levels than the Vehicle (Fisher, $p<0.01$) and Anastrozole (Fisher, $p<0.01$) groups, and the Androstenedione+Anastrozole group also had higher serum testosterone levels than the Vehicle (Fisher, $p<0.05$) and Anastrozole (Fisher, $p<0.05$) groups.

The analysis of serum estrone revealed a main effect of Treatment as well [$F_{(4,26)}=96.67$; $p<0.0001$; figure 7c]. The Androstenedione group had higher serum estrone levels than the Vehicle group (Fisher, $p<0.001$), and the addition of anastrozole decreased estrone levels (Androstenedione vs. Androstenedione+Anastrozole Fisher, $p<0.05$), confirming that the anastrozole treatment used herein effectively reduced androstenedione's conversion to estrone. The Androstenedione group also had higher serum levels than the Anastrozole group (Fisher, $p<0.001$), and the Androstenedione+Flutamide group had higher serum estrone levels than the Vehicle (Fisher, $p<0.0001$), Anastrozole (Fisher, $p<0.0001$), Androstenedione (Fisher, $p<0.0001$), and Androstenedione+Anastrozole groups (Fisher, $p<0.0001$). Additionally, the Androstenedione+Anastrozole group tended to have higher serum estrone levels than both the Vehicle ($p=0.05$), and Anastrozole ($p=0.05$) groups, suggesting that the addition of anastrozole did not completely block aromatase activity in this model.

Correlations between serum hormone levels and behavioral tests

Serum estrone levels correlated with average Total Errors on Block 3 of WRAM testing across all four trials ($r=0.39$; $p<0.05$; figure 8a), as well as on Trial 4, the trial with the highest working memory load ($r=0.36$; $p<0.05$; figure 8b). Because we found a clear

bimodal distribution in estrone levels, whereby the Androstenedione+Flutamide group had higher estrone levels than all other groups and therefore held the potential to exert a large amount of influence over these analyses, we also assessed each of these correlations excluding the Androstenedione+Flutamide group. With the Androstenedione+Flutamide group excluded, we found that serum estrone levels still correlated with average Total Errors on Block 3 of WRAM testing across all four trials ($r=0.58$; $p<0.01$; figure 8a), as well as on Trial 4, the trial with the highest working memory load ($r=0.62$; $p<0.01$; figure 8b).

Discussion

Our laboratory has recently reported that androstenedione produces spatial memory impairments in the female rat. Specifically, we have found positive correlations between endogenous androstenedione levels and maze error scores, and subsequently confirmed these relationships by methodically manipulating androstenedione levels in older Ovx rats and showing that exogenous androstenedione treatment impairs memory across multiple domains (Acosta et al., 2009b, 2010; Camp et al., 2012). Our present goals were to extend our previous findings and demonstrate that exogenous androstenedione administration produces memory impairment in young adult animals, and to evaluate the hormonal mechanism(s) underlying these androstenedione-induced cognitive impairments. Because we have previously observed spatial memory impairments following tonic administration of estrone (Engler-Chiurazzi et al., 2012), we hypothesized that the conversion of androstenedione to estrone was, at least in part, responsible for the memory impairments observed when androstenedione is administered to otherwise ovarian-hormone blank (Ovx) animals.

Replicating our previous findings in middle-aged animals, in the current study androstenedione impaired several dimensions of cognition including spatial reference and working memory in young adult Ovx rats. Offering support to our hypothesis regarding the mechanism underlying these effects, androstenedione administration did not induce memory impairments on any measure evaluated here when it was paired with an aromatase inhibitor, anastrozole. Anastrozole blocks the activity of the aromatase enzyme, which is responsible for the conversion of androstenedione to estrone. This treatment still allows the exogenously delivered androstenedione to act both directly as well as indirectly, through its conversion to testosterone, on the androgen receptor. Pharmacological blockade of androgen receptor activation did not block the cognitive impairing effects of androstenedione. Together, these findings offer support to the tenet that androstenedione produces robust memory impairments due to its conversion to estrone, rather than due to its androgenic effects.

Androgens are typically thought of as masculine hormones and are rarely associated with menopause. However, increasing evidence indicates that studying the impact of androgens on cognition is crucial to our understanding of natural transitional menopause and associated cognitive changes. Female rats have been shown to express high concentrations of androgen receptors in cognitive brain areas such as the hippocampus and cerebral cortex (Simerly et al., 1990), which have been shown to be sensitive to both Ovx and androgen administration (Lu et al., 1998), and activation of which could impact cognitive function through gene transcription (McPhaul & Young, 2001). There has been a paucity of research evaluating the

learning and memory effects of endogenous or exogenously administered androstenedione. In fact, as far as we are aware, the current experiment and our prior research findings (Camp et al., 2012) are the only studies testing the effects of androstenedione administration on learning and memory in the rat.

Much of the prior research testing the effects of androgens on rodent cognition has focused on dihydrotestosterone, testosterone, and dehydroepiandrosterone. Interestingly, while reports indicate that dihydrotestosterone has no impact on spatial working or reference memory (Raber et al., 2002; Bimonte-Nelson et al., 2003b; Benice & Raber, 2009), we and others have shown that testosterone administration enhances working memory (Bimonte-Nelson et al., 2003b), spatial reference memory (Benice & Raber, 2009), and performance on avoidance tasks (Flood et al., 1995; Edinger et al., 2004). The metabolism of androstenedione versus testosterone is likely related to the divergence in their respective cognitive impacts; testosterone is directly aromatized to 17 β -estradiol, whereas androstenedione is directly aromatized to estrone. Many studies have demonstrated that 17 β -estradiol can enhance cognition in female rats (e.g. Bimonte & Denenberg 1999; Gibbs, 1999, 2005; Gibbs et al., 2004; Daniel et al., 2006; Talboom, 2008; Rodgers et al., 2010; for review see Acosta et al., 2013). Thus far, the only two studies investigating the cognitive impact of estrone have found that estrone treatment was detrimental to contextual fear conditioning in young adult female rats (Barha et al., 2010), as well as working memory in middle-aged female rats (Engler-Chiurazzi et al., 2012).

The potential clinical implications of the current findings are far-reaching. Indeed, this work could generate new insight into the already immensely complex relationship between the loss of ovarian hormones in menopause and memory changes (Weber & Mapstone, 2009; Weber et al., 2013; Fischer et al., 2014). Cognitive effects likely depend on an individual's menopause status, including whether they have intact ovaries (Nappi et al., 1999), what phase of the menopause transition they are in (Weber et al., 2013), circulating levels of androstenedione, as well as other steroid hormones and gonadotropins (Acosta et al., 2009b), and prior hormone exposure history (Bimonte-Nelson et al., 2010). Knowledge of how these factors interact is particularly salient towards our goal of optimizing hormone therapy for relief of menopausal symptoms. For example, we have demonstrated that conjugated equine estrogen (CEE) hormone therapy benefits cognition following surgical hormone loss, but impairs cognition following transitional menopause in which the residual, androstenedione-producing ovaries remain intact (Acosta et al., 2010). The current results underscore the tenet that CEE is not the optimal hormone therapy for menopausal women. Support for this assertion comes from several intersecting lines of evidence, including the current data indicating that this may be especially relevant for women who retain their ovaries; indeed, CEE is over 50% estrone sulfate (Kuhl, 2005; Gleason et al., 2005). Estrone sulfate is converted to estrone by the liver, further adding to the estrone load derived from ovarian-produced androstenedione. It is possible that a bioidentical estradiol hormone therapy approach may produce more favorable cognitive outcomes, as it would act to bring the hormonal milieu closer to ratios seen in pre-menopausal women (Kuhl, 2005; Gleason et al., 2005).

The study of aromatase and estrogen metabolism is critical to moving the endocrine field forward, and to our understanding of systems-level changes occurring with hormone loss and replacement during menopause. Highlighting the need for a non-estrogenic compound that could safely relieve some of the symptoms of menopause, many women are unable to utilize estrogen-inclusive hormone therapy due to an increased risk of, or history of, breast cancer. The aromatase enzyme is found in breast tissue, and aromatase inhibitors are currently used to treat breast cancer and prevent breast cancer recurrence (Santen et al., 2009). Furthermore, there is a greater degree of androstenedione aromatization to estrogen as the body mass index and obesity increase in postmenopausal women, suggesting that conversion of androstenedione to estrogens can vary across the menopausal population (Santen et al., 2009). Should aromatase inhibitors prove to offset some of the negative cognitive consequences of menopause, this would further add to their value. In fact, it is noteworthy that, in the current study, anastrozole alone did not impair any of our many measures of cognition; indeed, anastrozole is one of the currently prescribed aromatase inhibitors used for breast cancer. Important future directions include developing a better understanding of the downstream hormone and brain mechanism(s) through which androstenedione and estrone produce cognitive impairments. A primary goal of this research is to evaluate alternative hormone therapy options that produce favorable outcomes for improved cognition in the menopausal female, utilizing a systematic approach that acknowledges and accounts for contributions of the many interacting variables that produce cognitive changes throughout aging.

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Figure 1.

Study timeline and depiction of behavioral tasks used. The timeline depicts the relative time frames between ovariectomy, hormone treatment administration, behavioral testing, and sacrifice, along with a pictorial representation of each behavior task utilized.

Figure 2.

Water radial arm maze performance. a) Mean+SEM WMC errors for Block 3 of WRAM testing. There was a Treatment $\times$ Trial interaction for WMC errors. For Trial 4, the trial with the highest working memory load, there was a main effect of Treatment for WMC errors; the Androstenedione group committed more WMC errors than the Vehicle group, and the addition of aromatase inhibition via anastrozole reversed this androstenedione-induced impairment. The Androstenedione group made more errors than the Anastrozole group, and the Androstenedione+Flutamide group committed more errors than the Vehicle group. Because the working memory demand is elevated as trials progress, WMC errors are presented both across trials, and on Trial 4 alone, which is the trial with the highest working memory load. b) Mean+SEM WMI errors for Block 3 of WRAM testing. There was a Treatment $\times$ Trial interaction for WMI errors. For Trial 4, there was a main effect of Treatment for WMI errors; the Androstenedione group committed more WMI errors than the Vehicle group. The addition of anastrozole reversed the impairing effect of androstenedione. The Androstenedione+Flutamide group also committed more WMI errors on Trial 4 than the Vehicle, Androstenedione+Anastrozole, and Anastrozole groups. WMI errors are presented both across trials, and on Trial 4 alone to show performance on the trial with the highest working memory load. c) Mean+SEM RM errors for Block 3 of WRAM testing. There was a main effect of Treatment for RM errors on Block 3 of WRAM testing. The Androstenedione group committed more RM errors than the Vehicle group, and, again, the addition of anastrozole reversed the reference memory impairments induced by androstenedione. The Androstenedione group also made more RM errors than the Anastrozole group, and the Androstenedione+Flutamide group committed more RM errors compared to Vehicle, Androstenedione+Anastrozole, and Anastrozole groups. Shown here are RM errors for each trial in a line graph, and also collapsed across all trials in a bar graph.

Of note, which arms are reference memory for a given animal remain constant across all trials; reference memory arms never contain a platform, and they constitute the first entry in each of the four arms making this error measure capped at four errors per day. Therefore, Trial 4 does not represent performance scores of reference memory information at a higher load as compared to the earlier trials, in contrast to the working memory measures whereby Trial 4 *does* represent performance scores of working memory information at a higher load compared to the earlier trials.

Figure 3. Delay match to sample performance. Mean+SEM errors for DMS testing. There were no Treatment effects for Total Errors during testing (Days 1-7).

Figure 4.

Morris water maze performance. a) Mean+SEM swim distance for Morris water maze.

There was a Treatment $\times$ Block interaction for MM testing. For Block 1, there was a main effect of Treatment; the Androstenedione+Anastrozole group swam a shorter distance to the platform than the Vehicle, Androstenedione, and the Androstenedione+Flutamide groups. b) For the probe trial, there was a main effect of Quadrant in the absence of a Quadrant $\times$ Treatment interaction, indicating that all groups equally localized the platform using spatial navigation by the end of Morris water maze testing.

Figure 5. Visible platform performance. Mean+SEM distance swam for visible platform maze. There were no Treatment main effects on latency to escape for the visible platform task.

*p<0.05
 *** p<0.001
 **** p<0.0001

Figure 6.

Uterine weights. Mean+SEM wet uterine weight (g). There was a main effect of Treatment for uterine weights. The Androstenedione group had higher uterine weights than the Vehicle, Androstenedione+Anastrozole, Androstenedione+Flutamide, and Anastrozole groups. The Androstenedione+Anastrozole group also had higher uterine weights than the Anastrozole group.

#p=0.05
 *p<0.05
 ** p<0.01
 *** p<0.001
 ****p<0.0001

Figure 7.

Serum levels of androstenedione, testosterone, and estrone. a) Mean+SEM serum androstenedione (ng/ml). There was a main effect of Treatment on androstenedione levels. As expected, androstenedione treatment increased androstenedione levels in all groups receiving this androgen, relative to vehicle treatment, and relative to treatment with anastrozole alone. b) Mean+SEM serum testosterone (ng/dl). There was a main effect of Treatment on testosterone levels. The Androstenedione group had higher testosterone levels than the Vehicle and Anastrozole groups, and the Androstenedione+Anastrozole group had higher testosterone levels than the Vehicle and Anastrozole groups. c) Mean+SEM serum estrone (pg/ml). There was a main effect of Treatment on estrone levels. The Androstenedione group had higher estrone levels than the Vehicle group, and the addition of anastrozole decreased estrone levels. The Androstenedione group also had higher estrone levels than the Anastrozole group, and the Androstenedione+Flutamide group had higher estrone levels than the Vehicle, Anastrozole, Androstenedione, and Androstenedione +Anastrozole groups.

a) Correlations between serum estrone & WRAM total errors on all trials

b) Correlations between serum estrone & WRAM total errors on the highest memory load trial

Figure 8.

Correlations between serum estrone levels and cognitive performance. a) Serum estrone levels correlated with average Total Errors on Block 3 of WRAM testing across all four trials including all groups ($r=0.39$; $p<0.05$). Because there was some divergence of the Androstenedione+Flutamide group, to be conservative, we tested this same correlation without this group to confirm it was not carrying the significance of the correlation. With the Androstenedione+Flutamide group excluded, we found that serum estrone levels still correlated with Total Errors on Block 3 of WRAM testing across all four trials ($r=0.58$; $p<0.01$). b) Serum estrone levels correlated with average Total Errors on Block 3, Trial 4, the trial with the highest working memory load ($r=0.36$; $p<0.05$). Again, with the Androstenedione+Flutamide group excluded, we found that serum estrone levels still correlated with Total Errors on Block 3 of WRAM testing on Trial 4 ($r=0.62$; $p<0.01$).