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High serum androstenedione levels correlate with impaired memory in the surgically menopausal rat: a replication and new findings

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Abstract

After natural menopause in women, androstenedione becomes the primary hormone secreted by the residual follicle-depleted ovaries. In two independent studies, in rodents that had undergone ovarian follicular depletion, we found that higher endogenous serum androstenedione levels correlated with increased working memory errors. This led to the hypothesis that higher androstenedione levels impair memory. The current study directly tested this hypothesis, examining the cognitive effects of exogenous androstenedione administration in rodents. Middle-aged ovariectomized rats received vehicle or one of two doses of androstenedione. Rats were tested on a spatial working and reference memory maze battery including the water-radial arm maze, Morris water maze (MM) and delay match-to-sample task. Androstenedione at the highest dose impaired reference memory as well as the ability to maintain performance as memory demand was elevated. This was true for both high temporal demand memory retention of one item of spatial information, as well as the ability to handle multiple items of spatial working memory information. We measured glutamic acid decarboxylase (GAD) protein in multiple brain regions to determine whether the gamma-aminobutyric acid (GABA) system relates to androstenedione-induced memory impairments. Results showed that higher entorhinal cortex GAD levels were correlated with worse MM performance, irrespective of androstenedione treatment. These findings suggest that androstenedione, the main hormone produced by the follicle-depleted ovary, is detrimental to working memory, reference memory and memory retention. Furthermore, while spatial reference memory performance might be related to the GABAergic system, it does not appear to be altered with androstenedione administration.

Keywords

aging; animal model; GABA; ovariectomy; working memory

Introduction

Clinical evidence suggests that the cognitive outcome following ovarian hormone loss differs depending on the type of menopause experienced, i.e. surgical versus transitional ovarian hormone loss (Nappi *et al.*, 1999; Farrag *et al.*, 2002). We recently showed that, in middle-aged female rats, retention of the follicle-depleted ovary after transitional menopause

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negatively impacted memory (Acosta *et al.*, 2009a). Specifically, using rats treated with 4-vinylcyclohexene-diepoxy (VCD) to experimentally induce follicular depletion, removal of residual ovaries after transitional follicular depletion improved cognition relative to retention of residual ovaries (Acosta *et al.*, 2009a). Rats that underwent VCD-induced transitional menopause followed by ovariectomy (OVX) showed better cognitive performance compared with transitionally menopausal rats that remained ovary-intact. The only difference between these groups was the presence of follicle-depleted ovaries. In Acosta *et al.* (2009a), circulating androstenedione levels were found to be related to cognitive scores, with more working memory errors correlating with higher serum androstenedione levels. These findings are noteworthy given that androstenedione is the main hormone released by the post-menopausal ovary, and it becomes the primary source of plasma estrogens after menopause due to aromatization (Grodin *et al.*, 1973).

Here, we take the next step in this line of research, under the tenet that if androstenedione is related to poorer memory, we should see impairments after administration to a rodent without circulating ovarian-derived hormones. Two doses of exogenous androstenedione were administered to middle-aged surgically menopausal (OVX) rats, with doses based on previous studies (Lea & Flanagan, 1998; Sprando *et al.*, 2004) showing physiological levels of androstenedione comparable with those correlating with poor cognitive performance in Acosta (2009a). We tested androstenedione's impact on cognition by testing animals on a battery of tasks tapping into several memory domains. Mazes included the water radial-arm maze (WRAM) which assessed working and reference memory and incorporated memory load increase across trials, the Morris water maze (MM) which tested spatial reference memory, the delayed-match-to-sample three-choice task (DMS) which examined working and recent memory, and the elevated plus maze (EPM) which assessed anxiety-like behavior. The WRAM and DMS included delays spanning hours, to evaluate high-demand memory retention. Additionally, androstenedione was measured in serum and correlated with memory measures, as done previously (Acosta *et al.*, 2009a, 2010).

We also evaluated whether the GABAergic system plays a role in the relationship between androstenedione and memory. Research suggests the GABA_A receptor (GABA_AR) modulates cognition. GABA_A-R antagonists enhance memory, and GABA_A agonists impair memory (Yonkov & Georgiev, 1985; Yonkov *et al.*, 1987; Brioni *et al.*, 1989; Castellano & McGaugh, 1990; Castellano *et al.*, 1993; Zarrindast *et al.*, 2004). Furthermore, several androgens have effects on GABA_A receptors. For example, dehydroepiandrosterone can act as a GABA_AR negative allosteric modulator (Birzniece *et al.*, 2006), and it improved memory in middle-aged and aged mice (Flood & Roberts, 1988; Farr *et al.*, 2004). Also, androstanediol has been shown to be a positive allosteric modulator of GABA_AR (Reddy & Jian, 2010). To assess androstenedione's impact on the GABAergic system, in the tested animals we measured levels of glutamic acid decarboxylase (GAD), the synthesizing enzyme and rate-limiting step of GABA production, in multiple cognitive brain regions after maze testing concluded.

Materials and methods

Subjects

Twenty-six 14-month-old Fischer-344 female rats born and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN, USA) were used. Upon arrival, rats were pair housed, acclimated for 2 weeks before surgery, had access to food and water *ad-libitum* and were maintained on a 12-h light/dark cycle (lights on at 07:00 and off at 19:00 h) at the Arizona State University animal facility. All procedures were approved by the local IACUC committee and adhered to NIH standards.

OVX and hormone treatment

Approximately 2 weeks before behavioral testing, all rats received ovariectomy under isoflurane inhalation. Ovariectomy consisted of bilateral dorsolateral incisions in the skin and peritoneum, the ovaries and tips of the uterine horns were ligatured and removed, and muscle and skin were sutured. Immediately after surgery, rats received Rimadyl (5 mg/mL/kg) for pain and saline (2 mL) to prevent dehydration.

Rats were randomly assigned to one of three treatment groups: Vehicle ($n = 10$), Androstenedione Low ($n = 8$) and Androstenedione High ($n = 8$) (Steraloids, Newport, RI, USA). Vehicle-treated animals received 0.5 mL of polyethylene glycol (PEG) (Sigma-Aldrich, St. Louis, MO, USA) only. The Androstenedione Low group was given 4 mg/kg daily, and the Androstenedione High group was given 8 mg/kg daily. Doses were based on previous literature (Lea & Flanagan, 1998; Sprando *et al.*, 2004), showing desired serum ranges of androstenedione (2.19 ± 0.25 to 4.71 ± 0.41 ng/mL) comparable with the levels found in Acosta (2009a) that were correlated with greater errors on a working memory task. Hormone or vehicle treatment began 2 days after surgery (12 days before behavioral testing ensued) and continued until the animals were killed (see Fig. 1 for experimental timeline). The assigned substrate was administered daily between 07:30 and 08:30 h via subcutaneous injection into the scruff of the neck. All hormone doses were dissolved in PEG so that each dose was given in 0.5 mL. Injections occurred at least 1 h prior to behavioral testing, 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 was concordant with our prior studies showing correlations between androstenedione and memory (Acosta *et al.*, 2009a, 2010).

Vaginal smears and uterine weights

Vaginal smears were taken 8, 9, 10, 11, 28 and 44 days after OVX. Hormone administration began 2 days after OVX, and thus was being administered during the time smears were performed. Smears were classified as proestrus, estrus, metestrus or diestrus, per prior protocols (Goldman *et al.*, 2007; Acosta *et al.*, 2009b). Prior studies have shown that androgens stimulate the uterus and lead to increased uterine weight (Ruizeveld de Winter *et al.*, 1991; Horie *et al.*, 1992). To further verify androstenedione's effects on uterine tissues, the uteri of all subjects were removed, trimmed of visible fat and immediately weighed (wet weight).

Water radial-arm maze (WRAM)

Subjects were tested for 13 days on the eight-arm win-shift WRAM (Fig. 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 water surface in the ends of four of eight arms (each arm was 38.1×12.7 cm). Each subject was assigned different platform locations that remained constant throughout the 13 days of testing. The subjects were released from the start arm and had 3 min to locate a platform. Once a platform was found, the subject remained on it for 15 s, and was then returned to its heated cage for a 30-s 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. An arm entry was 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 (11 cm into the arm). Orthogonal measures of working 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 that session. Reference memory (RM) errors were the number of first entries into an arm that never contained a platform. Working memory incorrect (WMI) errors were repeat entries into reference memory arms. 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. Each subject was given one session a day for 12 consecutive days. After 12 days at a 30-s ITI, a 6-h ITI was interpolated between trials 2 and 3.

For WRAM analyses, for the three orthogonal measures of WMC, WMI and RM, performance across all testing days were evaluated in six 2-day blocks (Block 1 = days 1–2, Block 2 = days 3–4, Block 3 = days 5–6, Block 4 = days 7–8, Block 5 = days 9–10 and Block 6 = days 11–12). Performance on the final 2 days of regular testing (Block 6) was also assessed at the highest working memory load (Trial 4), as this has revealed steroid hormone-induced benefits and decrements in aged animals (Bimonte-Nelson *et al.*, 2003a; Braden *et al.*, 2010). On Day 13, a 6-h delay was imposed between trials 2 and 3 to assess memory retention for numerous items of information (i.e. the found platform locations on trials 1 and 2). We analysed post-delay trials (Trials 3 and 4) to detect potential delay-induced impairments. Errors committed on the post-delay trials (Day 13, Trials 3 and 4) were compared with errors at baseline (the last day of regular testing, Day 12, Trials 3 and 4).

Delay match to sample (DMS) three-choice task

Subjects were tested for 7 days on the four-arm (each arm was 38.1×12.7 cm) win-stay DMS to evaluate spatial working memory and short-term memory retention (Fig. 2b). The black plexiglass maze was filled with water made opaque with black non-toxic paint. Animals received six consecutive trials a day for 6 days. The platform was located in a new arm each day, but remained in the same arm within a day, for each subject. The drop off location varied for each trial, semi-randomly alternating between the three available arms within a set of trials. Trial 1 was the information trial, informing the animal where the platform was for that day's session, trial 2 was the working memory test trial, and trials 3–6 were recent memory test trials (Frick *et al.*, 1995). Rats were given 90 s to find the platform. Once on the platform, the rat remained on it for 15 s, followed by placement into a heated cage for a 30-s ITI. An arm entry was 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 (11 cm into the arm). First errors were first entries into an arm without a platform, and repeat errors were repeat entries into an arm without a platform within the same trial. The dependent variables were the number of Total (First + Repeat errors) errors, First errors and Repeat errors across testing (Days 1–6 Trials 2–6; Trial 1 was the information trial). After 6 days of testing with a 30-s ITI, an increased time delay was given. On day 7, a 6-h delay was interpolated between Trials 1 and 2 to assess memory retention. Errors committed on the post-delay trials were compared with errors on the last day of regular testing, Day 6 (baseline), to detect impairing effects of a 6-h delay vs. the 30-s ITI.

Morris water maze (MM)

Subjects were assessed on the MM maze (Fig. 2c) for six trials a day for 3 days using a tub (188 cm diameter) filled with black water made opaque using non-toxic paint. A hidden platform (10 cm wide) remained in a fixed location (north-east quadrant) throughout testing, thereby testing spatial reference memory (Morris *et al.*, 1982; Bimonte-Nelson *et al.*, 2006). The subject was placed in the maze from the north, south, east or west location (semi-randomly determined), and had 60 s to locate the hidden platform. Once the rat found the platform the trial was terminated. After 15 s of platform time, the subject was placed into its heated cage until its next trial. The approximate ITI was 15 min. To evaluate whether

subjects localized the platform to the spatial location, after all test trials were completed on day 3, a 60-s probe trial was given whereby the platform was removed. For each trial, a camera suspended above the maze tracked each rat's path and a tracking system (Ethovision, Noldus Instruments, Leesburg, VA, USA) analysed each rat's tracing.

MM performance was assessed by swim path distance (cm) to the platform. For probe trial data, total percentage distance in the previously platformed (target, NE) quadrant was compared with 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).

Elevated plus maze (EPM)

Animals were tested on the EPM (Fig. 2d) for 1 day, with a 5-min trial to evaluate anxiety, based on previously published protocols (Bellani *et al.*, 2006; Huynh *et al.*, 2011). The EPM consisted of four (50.8 cm) contralateral arms (49.5 cm in length, 10.2 cm in width) positioned in accordance to face north, south, east and west. The closed arms (east and west arms) had walls surrounding the perimeters (50.8 cm in height), while the open arms had no walls surrounding the arm floors. Rats were placed into the center of the maze facing a randomly assigned closed arm and given 5 min to explore the environment. For each trial, a camera suspended above the maze recorded the animal. Time spent in the open arms, frequency of open arm entrances, frequency of closed arm entries and total fecal boli were quantified. Time spent in an arm and arm entrances were defined as both front paws crossing into the respective arm quadrant. At the end of the trial, the subject was placed back into its home cage, fecal boli in the maze were counted and the maze was wiped clean with odor eliminator to remove any scents prior to the next trial. A measure of anxiety was calculated for the EPM using the following equation, which unified all EPM parameters into one ratio (Huynh *et al.*, 2011). Anxiety index values ranged from 0 to 1, with a higher value indicating increased anxiety.

$$Anxiety\ Index = 1 - \left[\frac{(open\ arm\ \frac{time}{5}\ min) + (open\ arm\ entry / total\ entry)}{2} \right]$$

Visible platform maze

Since the MM, 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 (39 × 23 inches) was filled with clear water and a black platform (10 cm wide) was elevated above the water surface. Opaque curtains covered obvious extramaze cues. The drop-off location remained the same across trials, and the platform location for each trial varied in space semi-randomly. Animals had to locate the platform protruding from the water. They were given six trials for 1 day. Performance was assessed by latency (s) to the platform.

Brain dissections

Two days after the conclusion of behavior testing, animals were anesthetized with isoflurane, decapitated, brains rapidly dissected and tissues frozen. Dissected tissues were stored in preweighed microcentrifuge tubes at -70°C until analysis. Dissections were performed according to plate designations in Paxinos & Watson (1998) and were as follows: frontal cortex (plates 5–14), cingulate cortex (plates 5–14), basal forebrain (plates 14–16),

perirhinal cortex (plates 39–42), entorhinal cortex (plates 39–42) and CA1/CA2 region of the dorsal hippocampus (plates 33–35). For each brain the frontal cortex was taken first from the dorsal aspect of the intact brain. Next, the cingulate cortex was taken with the longitudinal fissure as the medial border, and the medial border of the frontal cortex cut as the lateral border. The brain was then cut in the coronal plane to obtain access to the basal forebrain. For the basal forebrain, both medial septum and ventral diagonal band were included with the posterior landmark being the crossing point of the anterior commissure. The brains were then cut in the coronal plane to obtain access to the last three brain regions. For the CA1/CA2 region of the hippocampus, dentate gyrus and the alveus were excluded. For the entorhinal cortex, the tissue was dissected from the same slice as the hippocampal sample, taking a 2- to 3-mm sample ventral to the hippocampus. The perirhinal cortex was also collected from this same slice, taking a 2- to 3- mm sample around the perirhinal fissure.

Western blot analysis of GAD 65 + 67

GAD 65 + 67 protein expression levels were analysed in frontal, cingulate, perirhinal and entorhinal cortices, and hippocampus (each from the right hemisphere), as well as the basal forebrain. Samples were sonicated in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, 50 mM Tris) equivalent to ten times the weight of the sample and centrifuged at 10 000 r.p.m. for 10 m at 4°C. Protein concentrations were determined using BCA protein assays (ThermoFischer Scientific, Pittsburgh, PA, USA) and 10 µg of protein from each sample was run on a Run Blue 4–12% SDS gel, using the SureLock minicell (Invitrogen, Carlsbad, CA, USA). Gels were counterbalanced by group and each region was run in two gels. Half of each group was loaded onto a single gel, with two gels in total for each brain region. Proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA, USA) and immunoblotting was performed with working dilutions of rabbit anti-GAD 65 + 67 (ab11070) and rabbit anti-beta Actin (ab25894) primary antibodies (Abcam Inc., Cambridge, MA, USA). Antibody dilutions were 1:10 000 for GAD 65 + 67 and 1:1000 for beta Actin primary antibodies. Membranes were then exposed to a peroxidase-conjugated goat anti-rabbit secondary antibody, 1:20 000 dilution (111-035-003; Jackson Immuno Research, West Grove, PA, USA) and visualized using Pierce ECL (ThermoScientific, Rockford, IL, USA) on a Biospectrum Biochemi 500 Imaging System (UVP, Upland, CA, USA). Bands were identified as the protein of interest, based on molecular weight, using Precision Plus Protein WesternC Standards (Bio Rad, Hercules, CA, USA). The density of GAD 65 + 67 and beta Actin (control protein) was then quantified using ImageJ software (Rasband, 1997–2004). The dependent measure was a proportion of each subject's GAD 65 + 67 density to their beta Actin density, brought to percentage control of Vehicle subjects run on the same gel, as per prior protocols (Braden *et al.*, 2010, 2011; Pandey *et al.*, 1999).

Blood serum analysis

At the time of brain dissection, blood was collected via cardiocentesis (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ, USA). Blood was allowed to clot at 4°C and serum was collected after centrifugation (3220 r.p.m., 20 min). Serum was stored at –20°C until androstenedione assays were performed by the Core Endocrinology Laboratory at Pennsylvania State University College of Medicine. Serum levels of androstenedione were determined by iodinated radioimmunoassay using reagents obtained from Siemens (Los Angeles, CA, USA) as done previously (Acosta *et al.*, 2010). The low-end sensitivity for this androstenedione method is 0.1 ng/ml.

Statistical analyses

Unless otherwise noted, two-tailed tests were used, and alpha was set at 0.05. Data were analysed separately for each maze. WRAM, DMS, MM and Visible Platform were first analysed with a repeated-measures ANOVA with Treatment as the between-subject variable and Days/Blocks and/or Trials as the within-subject variable, as appropriate for the specific maze test. EPM, GAD Western blot, uterine weights and serum analyses were analysed using *t*-tests, with Treatment as the between-subject variable. As our interest was to determine whether each dose of androstenedione enhanced or impaired performance relative to the Vehicle group, all of our comparisons were planned. Any comparisons done between the Androstenedione Low and Androstenedione High groups were done post hoc and such analyses are specifically stated in the Results; in these cases, the Bonferroni correction was applied.

Correlations were used to examine relationships between androstenedione serum levels and behavioral results found to have significance using the ANOVA tests described above. Circulating androstenedione levels were correlated with the following outcome variables: MM distance collapsed across all trials/days; DMS Total Errors postdelay; and WRAM WMI errors Block 6 Trial 4. Correlations were also used to examine relationships between GAD levels in multiple brain regions with the same behavioral results assessed in the androstenedione serum correlation analysis stated above.

Results

Vaginal smears and uterine weights

During hormone treatment, all Vehicle and Androstenedione Low animals showed continuous diestrus smears, while Androstenedione High animals showed vaginal smears with an increased number of cornified cells, indicating uterine stimulation. Uteri of Androstenedione High animals weighed more than those of Vehicle animals (Androstenedione High vs. Vehicle: $t_{16} = 2.47$; $P = 0.025$), as shown in Fig. 3.

Water radial-arm maze

There were no Treatment main effects or interactions for WMC or RM for all days (collapsed across the six blocks of testing). For WMI, there was a significant Treatment $\times$ Block $\times$ Day $\times$ Trial interaction for the Vehicle versus Androstenedione High comparison ($F_{1,16} = 1.951$, $P = 0.0195$). Further analyses showed that on the trial testing the highest memory load (Trial 4) on Block 6 (the last block of testing), the Androstenedione High group committed more WMI errors than both the Vehicle group ($F_{1,16} = 6.673$, $P = 0.018$) and the Androstenedione Low group ($F_{1,14} = 7.862$, $P = 0.014$) (Fig. 4c). There were no treatment main effects or interactions for WMC, WMI or RM for the 6-h delay.

Delay match to sample three-choice task

There were no Treatment effects for Total Errors, First Errors or Repeat Errors during the acquisition phase of testing (Days 1–6). The high-dose androstenedione treatment impaired retention on the 6-h delay manipulation given between trials 1 and 2. Specifically, for the 6-h delay given on Day 7, Androstenedione High was the only group to commit significantly more Total Errors (Fig. 5) ($t_7 = 3.862$, $P = 0.006$) and First Errors ($t_7 = 4.245$, $P = 0.004$) compared with their baseline performance (Trial 2 on Day 6). Vehicle and Androstenedione Low groups were not impaired by the 6-h delay, as they did not differ from their respective baseline scores to their delay scores.

Morris water maze

Figure 6 shows the mean distance to platform scores for each treatment group across the 3 days of MM testing, collapsed across trials. Androstenedione treatment at the high dose impaired spatial reference memory; Androstenedione High rats swam a greater distance to find the platform compared with Vehicle rats ($F_{1,16} = 4.871$, $P = 0.042$). There was also a significant linear trend for dose of androstenedione ($F_{1,2} = 182.905$, $P = 0.047$), with swim distance increasing as dose of androstenedione increased. For the probe trial, each treatment group swam a higher percentage swim distance in the target quadrant versus the opposite quadrant [within-group evaluation: Androstenedione High ($t_7 = 5.693$, $P = 0.001$), Androstenedione Low ($t_7 = 5.565$, $P = 0.001$), Vehicle ($t_9 = 10.336$, $P < 0.0001$)], indicating that all groups, regardless of hormone status, localized the platform quadrant by the end of testing. There were no group differences for platform crossings.

Elevated plus maze

There were no Treatment main effects for number of open arm entries (Fig. 7a), number of closed arm entries (data not shown), amount of time spent in open arms (Fig. 7b) and number of fecal boli (data not shown). In addition, there were no Treatment main effects for the Anxiety Index (data not shown).

Visible platform

There were no Treatment main effects or interactions for latency on the visible platform task (Fig. 8). By the last trial of testing, all subjects found the visible platform within 10 s. These data confirm visual and motor competence to perform swim maze tasks.

Serum androstenedione levels

Each Vehicle rat had undetectable levels of serum androstenedione. The Androstenedione High group had higher androstenedione serum levels than the Vehicle group ($t_{16} = 4.282$, $P = 0.0006$), and the Androstenedione Low group had higher serum levels than the Vehicle group ($t_{16} = 3.846$, $P = 0.0014$) (Fig. 9a).

As shown in Fig. 9b, replicating our prior correlations (Acosta *et al.*, 2009a, 2010), there was a significant correlation between androstenedione serum levels and WRAM WMI errors on Block 6, Trial 4 ($r_{25} = 0.609$, $P = 0.0007$), with higher androstenedione levels correlating with worse performance. There were no significant correlations for androstenedione serum levels and MM distance collapsed across all days or DMS Total errors for the post delay measure.

Western blots

There were no Treatment effects for GAD levels for the frontal cortex, cingulate cortex, basal forebrain, hippocampus, entorhinal cortex or perirhinal cortex. There were also no Treatment effects for the loading control, beta Actin. For correlation analyses, all data points were centered to control for group differences, as done previously (Talboom *et al.*, 2010); for more detailed methods and rationale of this procedure see Hallahan & Rosenthal (2000) and Enders & Tofighi (2007). There was a significant correlation between GAD levels in the entorhinal cortex and MM distance, collapsed across all days and trials. Animals that had higher GAD levels in the entorhinal cortex tended to swim a greater distance on the MM ($r_{23} = 0.431$, $P = 0.036$), as seen in Fig. 10, suggesting that higher GAD levels in the entorhinal cortex are associated with poorer spatial reference memory performance.

Discussion

The current study is the first to methodically evaluate the effects of androstenedione administration on learning and memory in the middle-aged female rodent. We have previously shown endogenous androstenedione serum levels to correlate with poorer working memory scores in a rat model of transitional menopause (VCD). If androstenedione is related to poorer working and reference memory, we should see impairment after we administer it to a rodent without circulating ovarian-derived hormones. Here, we demonstrate that, in fact, androstenedione administration does impair performance within multiple domains of cognitive function. Specifically, in OVX middle-aged rats, androstenedione impaired spatial reference memory as well as the ability to maintain performance with increasing memory demand. This was true for both high-demand memory retention of one item of spatial information (as seen on the DMS task) as well as the ability to handle multiple items of spatial working memory information (as seen on the WRAM task). The lack of group differences on the visible platform task and EPM suggest that the cognitive deficits observed on spatial working and reference memory were probably not attributed to visual impairment or anxiety. We confirmed that androstenedione injections administered daily increased androstenedione serum blood levels and uterine weights. Indeed, consistent with previous findings showing uterine weight increases after treatment with other androgens (Nantermet *et al.*, 2005), androstenedione treatment at the highest dose elevated uterine weights, relative to vehicle treatment, in the current study. Androgens can increase uterine weight via direct action on androgen receptors located in the uterus (Ruizeveld de Winter *et al.*, 1991; Horie *et al.*, 1992), an effect not reversed by estrogen receptor antagonists (Schmidt & Katzenellenbogen, 1979), thereby suggesting that androstenedione effects on the uterus are due to its androgenic actions rather than aromatization to estrogens. Serum evaluation showed that androstenedione levels were higher in both the androstenedione high- and low-dosed groups, relative to vehicle, and every rat in the vehicle group produced serum androstenedione levels that were undetectable. Collectively, these results confirm that only the rats in the androstenedione high- and low-dosed groups had the hormone circulating at levels detectable by the assay used here. Importantly, both doses of androstenedione given here produced blood serum levels similar to the levels achieved in the Acosta (2009a) study. In fact, the androstenedione levels in the current study are concordant with the those seen in follicle-depleted middle-aged rats showing the greatest memory impairment in that study (Acosta *et al.*, 2009a). The study here allowed for a controlled analysis of androstenedione at the levels produced in the transitional model, while also controlling for any interaction that may occur with the presence of the residual ovary and any other hormones secreted from this residual ovary.

Androstenedione dose-dependently negatively impacted spatial reference memory on the MM, with a greater swim distance to the platform as dose increased. This was shown via linear trend analysis. Note that this impairment pervaded across all days of traditional testing, yet there were no group differences in the probe trial for quadrant specificity or platform location crossings. Thus, despite high-dose androstenedione-induced impairments on the MM across the three test days, rats caught up by the end of testing and eventually learned the platform location.

The current study also found that androstenedione impaired the ability to maintain performance as memory demand was increased. This was true for both high-demand memory retention of one item of spatial information (DMS testing), as well as for the ability to handle multiple items of spatial working memory information (WRAM testing). On the final block of testing for WRAM, the rats receiving the high dose of androstenedione committed more WMI errors on Trial 4, when the working memory load was at its highest, compared with those receiving the low dose of androstenedione or vehicle. This

androstenedione-induced impairment was also supported by the significant correlation between WRAM WMI errors on Trial 4 on the last block of testing and androstenedione serum levels. This is consistent with the results from Acosta *et al.* (2009a), who found a significant correlation between WMI errors and androstenedione serum level, with more errors correlating with higher circulating androstenedione levels. In the current study, for the 6-h delay on the DMS task, where one item of information had to be retained for an extended time period, we found that the rats receiving the high-dose androstenedione were the only group to commit significantly more errors after this 6-h delay, versus their baseline error scores. Notably, performance on the DMS task requires accurate spatial navigation and retention of spatial information, as the use of kinesthetic or intramaze cues would not result in successful performance. Steroid hormones such as estrogens have been shown to impact spatial versus response strategy utilization on tests similar to our DMS task, and these effects have been localized to specific brain regions (Korol, 2004; Davis *et al.*, 2005). An alternative interpretation to the androstenedione-induced memory retention deficits, as noted on the test trial after the 6-h delay, should be acknowledged. Indeed, it is possible that androgen milieu impacts learning or application of the task rules, and that the finding we interpret here as an androstenedione-induced memory retention deficit incorporates a lack of rule learning or appropriate task rule application. For example, the androstenedione high-dose animals could have interpreted the test trial after the delay as a new day, and not the continuation of that day's task. Thus, in this case the animal could consider this test trial a new information trial in which the location of the correct arm is random. Future work systematically evaluating androgen by strategy interactions, as well as rule learning, could yield novel insight into the mechanism and specificity of the functional effects of androgens.

In the present study animals were tested on a battery of behavioral assays. It is therefore possible that the experience of being tested on multiple behavior tests influenced performance outcomes and effects of androstenedione, especially on the tests that were given toward the end of the battery, including the EPM. Systematic evaluation of potential behavior testing order effects when assessing androgens is an important future direction in characterizing the cognitive impact of this class of hormones.

The current study suggests that androstenedione's impact on cognition is not due to changes in GABA synthesis, at least as evaluated by changes in GAD, the synthesizing enzyme for GABA. Indeed, we failed to detect group differences in GAD levels in multiple cognitive brain regions. There is a strong positive correlation between GAD mRNA levels and GABA neuronal activity (Erlander & Tobin, 1991), making GAD a good neuronal marker for GABAergic function (Bauer *et al.*, 2000; Milbrandt *et al.*, 2000). Nonetheless, whether androstenedione alters GABAergic receptor activity or other markers of GABA activity remains to be investigated in order to develop a better understanding of androstenedione's impact on the GABAergic system. We did, however, find a correlation between GAD levels in the entorhinal cortex and swim distance to the platform in the MM when data were collapsed across all treatment groups. Specifically, animals that had higher GAD levels in the entorhinal cortex tended to swim a greater distance on the MM, thereby suggesting that higher GAD levels in the entorhinal cortex are associated with poorer spatial reference memory performance. This is a replication of our previous work showing an increase in GAD levels in the entorhinal cortex and impairment in spatial reference memory (Braden *et al.*, 2010). The entorhinal cortex and the hippocampus are associated with, and play crucial roles in, memory processing (Knowles, 1992), which increasing evidence suggests is also related to the GABAergic system (Izquierdo *et al.*, 1993). However, the direct mechanism of androstenedione-induced memory effects remain to be elucidated.

Although exogenous androstenedione administration has not been previously evaluated for rodent spatial cognition, other androgens have been assessed. The most commonly tested

androgens have been dihydrotestosterone, testosterone and dehydroepiandrosterone. Research suggests that dihydrotestosterone has no impact on spatial working or reference memory (Raber *et al.*, 2002; Bimonte-Nelson *et al.*, 2003b; Benice & Raber, 2009). By contrast, testosterone treatment improves working memory (Bimonte-Nelson *et al.*, 2003b), spatial reference memory (Benice & Raber, 2009), and avoidance learning and memory (Flood *et al.*, 1995; Edinger *et al.*, 2004). The difference between androstenedione's negative cognitive impact and testosterone's positive cognitive impact may be related to the type of estrogen directly synthesized from these two androgens. Testosterone is directly aromatized to 17 β -estradiol, while androstenedione is directly aromatized to estrone. Androstenedione's impairment on cognition may be through its conversion to estrone, as it has been shown that androstenedione administration significantly increases estrone serum levels (Jasuja *et al.*, 2005). The exogenous administration of estrone appears to negatively affect cognition; however, this has only been examined in two studies to date. Work done thus far has found that administration of estrone impaired contextual fear conditioning in young adult female rats (Barha *et al.*, 2010) as well as working memory on a DMS win-stay task in middle-aged female rats (Engler-Chiurazzi, 2012).

Future research aimed at discovering the mechanism(s) responsible for androstenedione-induced cognitive impairment could yield valuable insight into the hormonal mechanisms relating to the sometimes reported negative cognitive consequences of menopause (Weber & Mapstone, 2009), whereby follicular depletion occurs along with retention of the residual ovary and androstenedione release. Future studies include methodically evaluating the specific contributions of androstenedione to cognition in female rodent models, including pharmaceutical manipulations of androgenic stimulation by receptor or enzyme blockade.

In conclusion, the current study is, to our knowledge, the first to methodically evaluate the effects of androstenedione administration on learning and memory in the middle-aged female rodent model. Here, we demonstrate that exogenous androstenedione administration impaired spatial reference memory, as well as the ability to maintain performance as memory demand was elevated. The latter was shown on two different tasks. These results, taken with prior findings that higher endogenous androstenedione levels correlate with poorer working memory in the follicle-depleted ovary-intact rat (Acosta *et al.*, 2009a, 2010), indicate that circulating androstenedione could impact memory abilities as aging and menopause ensues. Understanding the mechanisms and parameters through which androstenedione is impairing memory may reveal valuable information toward optimization of factors during menopause that could influence healthy cognitive aging.

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Abbreviations

DMS	delayed-match-to-sample three-choice task
EPM	elevated plus maze
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase

ITI	inter trial interval
MM	Morris water maze
OVX	ovariectomy
PEG	polyethylene glycol
R	receptor
RM	reference memory
VCD	4-vinylcyclohexene-diepoxide
WMC	working memory correct
WMI	working memory incorrect
WRAM	water radial-arm maze

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Fig. 1.
Schematic representation of the experiment.

Maze Schematics

Fig. 2. Diagrams of the behavioral mazes used in the experiment. (a) Water radial arm maze. (b) Delay-match-to-sample three-choice task. (c) Morris water maze. (d) Elevated plus maze.

Fig. 3. Mean ($\pm$ SE) uterine weights for each group. Uterine weights of the Androstenedione High group were greater than those in the Vehicle group ($t_{16} = 2.526$, $P = 0.024$).

Fig. 4.

Mean ($\pm$ SE) working memory incorrect errors on the WRAM. (a) Errors (s) across all days of testing, shown in six 2-day blocks, (b) across all trials on Block 6 (Days 11–12) and (c) on Trial 4 for Block 6, where the Androstenedione High group committed significantly more errors than both the Androstenedione Low group ($F_{1,14} = 7.862$, $P = 0.014$) and the Vehicle group ($F_{1,16} = 6.673$, $P = 0.02$).

Fig. 5. Mean ($\pm$ SE) total errors for each group for post-delay trials on the DMS. The Androstenedione High group was the only group to make significantly more post-delay errors following the 6-h delay between trials 1 and 2 ($t_7 = 3.862$, $P = 0.006$).

Fig. 6.

Mean ($\pm$ SEM) swim distance (cm) during MM testing. (a) Swim distance across all days and all trials of testing, and (b) swim distance across days of testing. The Androstenedione High group swam a significantly greater distance to find the platform across all three days and all six trials of testing compared with Vehicle ($F_{1,16} = 4.871$, $P = 0.042$). There was also a significant linear trend with swim distance increasing as dose of androstenedione increases ($F_{1,2} = 182.905$, $P = 0.047$).

Fig. 7.

(a) Mean ($\pm$ SE) total open arm entries on the elevated plus maze. There were no group differences. (b) Mean ($\pm$ SE) total time spent in open arms (s) on the elevated plus maze. There were no group differences.

Fig. 8.
Mean ($\pm$ SE) swim latency (s) on the visible platform. There were no group differences in swim latency.

Fig. 9.

(a) Mean ($\pm$ SE) androstenedione serum levels (ng/ml). Both the Androstenedione High ($t_{16} = 4.282$, $P = 0.0006$) and Androstenedione Low ($t_{16} = 3.846$, $P = 0.0014$) groups had higher androstenedione serum levels than the Vehicle group. No vehicle animal showed any detectable amounts of androstenedione. (b) Correlation analysis indicated that higher androstenedione levels were associated with more WRAM working memory incorrect (WMI) errors on the last 2 days of testing when the working memory load was at its greatest (Trial 4) ($r_{25} = 0.609$, $P = 0.0007$).

Fig. 10.

Correlation analysis indicated that higher GAD levels in the right entorhinal cortex were associated with swimming a greater distance to find the platform on the MM, collapsed across all days and all trials ($r_{23} = 0.431$, $P = 0.036$).