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Manipulation of prenatal hormones and dietary phytoestrogens during adulthood alter the sexually dimorphic expression of visual spatial memory

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Abstract

Background: In learning and memory tasks, requiring visual spatial memory (VSM), males exhibit higher performance levels compared to females (a difference attributed to sex steroid hormonal influences). Based upon the results from our companion investigation, this study examined the influence of prenatal sex steroid hormone manipulations on VSM in adulthood, as assessed in the radial arm maze. Additionally, the influence of dietary soy phytoestrogens (i.e., the presence of high or low estrogen-like compounds present in the animal's diet) on VSM was examined in combination with the prenatal hormonal manipulations.

Results: Radial arm maze performance on a phytoestrogen-rich diet: 1) females treated prenatally with testosterone were masculinized and acquired/performed in a manner similar to control or oil-treated males and 2) males treated prenatally with an androgen receptor blocker (flutamide) were feminized and acquired/performed in a fashion typical of control or flutamide-treated females. When a diet change was initiated in adulthood, control phytoestrogen-rich fed females outperformed control females switched to a phytoestrogen-free diet. Whereas, in control males the opposite diet effect was identified. Furthermore, flutamide-treated males fed a phytoestrogen-rich diet outperformed flutamide-treated males switched to a phytoestrogen-free diet.

Conclusions: These results suggest that prenatal hormonal manipulations significantly sex-reverse the normal sexually dimorphic expression of VSM. Specifically, VSM was enhanced in females treated with testosterone and inhibited in males treated with flutamide. Finally, dietary soy phytoestrogens set a bias on learning and memory in these hormonally manipulated animals in a predictable manner and these data confirm and extend the findings in our companion paper.

Background

In learning and memory tasks requiring the use of spatial cues, sex differences in performance have been consistently demonstrated; the noted differences show that males generally acquire spatial tasks more rapidly and exhibit better performance compared to females [1–6]. Gender differences for spatial abilities have been reported in a variety of tests of spatial skill in a number of mammalian species (eg. rats, voles, and humans [7–14]). While general consensus has been reached regarding sex

differences in spatial ability the underlying mechanisms for these differences are not fully understood. However, increasing evidence suggests that gonadal steroids modulate this sexually dimorphic ability.

Support for the hypothesis that gonadal steroids influence memory is found from studies in which castration of male rats inhibit performance, and treatment of intact and ovariectomized female rats with testosterone improves spatial performance [3,11]. Furthermore, neonatal treatment of intact male rats with cyproterone acetate, an androgen antagonist, feminized their performance in a spatial learning task, as adults [10].

Apart from the direct effects, testosterone may play a significant role in the sexual differentiation of spatial ability through its aromatization to estradiol. It is plausible that the "masculinizing" agent may be estrogen through conversion of testosterone to estrogen locally in brain via aromatase [15]. Generally, males exhibit higher levels of brain aromatase compared to females and males have notably stable and abundant levels of substrate (testosterone) for conversion to estradiol [15]. In support of this notion, early postnatal treatment of male rats with an aromatase inhibitor decreased choice accuracy during acquisition of the radial arm maze when compared to controls [4].

Additionally, in spatial and non-spatial learning across the rat estrous cycle, animals in proestrus (the point in the cycle in which estradiol is at its highest) tested on a non-hippocampal cue task, performed significantly better than those in estrus (the point in the estrous cycle in which estradiol drops off rapidly) [5,14,16].

Numerous results, from studies in rats, have demonstrated that spatial performance, in castrated male and female rats, is enhanced when estradiol is administered both in development [2,3,7] and adulthood [1,4–6,8,9,14]. However, neonatal estradiol treatments have been shown to increase errors in intact male rats [11]. Similarly, spatial working memory, evaluated in a water-escape version of the radial-arm maze, is enhanced in intact female rats receiving a physiological dose of estradiol [10]. Taken together these results suggest that testosterone (and estradiol) treatments may have differing effects in intact vs. gonadectomized animals. Furthermore, studies on the organizational effects of gonadal hormones have almost exclusively targeted the first 10 postnatal days as critical for the establishment of sex differences in spatial task performance [2,7,11]. Therefore, prenatal hormonal effects on sexually dimorphic visual spatial ability have not been extensively investigated. However, compelling research assessing prenatal androgen and estrogen exposure on adult spatial learning demonstrated that 70-day old male and female rat's water maze performance was significantly affected by prenatal hormones [3]. Steroid-sensitive sex differences were observed in water maze performance in favor of intact male rats compared to males prenatally-treated with flutamide and castrated at birth. Also, performance was enhanced in testosterone propionate – (TP) and estradiol benzoate-treated (EB) female rats compared to intact female rats [3].

In summary, in learning and memory tasks requiring the use of spatial cues researchers have consistently found sex differences [1–6]. Males exhibit facilitated spatial learning and better spatial memory compared with females [7–14]. This sex difference is partially due to gonadal steroid differences in males and females [1–14]. The presence of testosterone, presumably via its intraneuronal conversion to estradiol [3,11–13], has been shown to enhance visual spatial ability [3,11–13]. However, data collected in intact animals and especially the examination of the effects of the prenatal hormones on visual spatial ability in these animals is scarce. Also, based upon the findings of our companion paper where dietary phytoestrogens significantly influenced visual spatial memory (VSM). Therefore, in this study, we tested the hypothesis that manipulating the prenatal hormonal environment has long-term influences on VSM in adult male and female intact rats (utilizing radial arm maze methods to examine varying aspects of memory). Additionally the influence of phytoestrogens (plant compounds that are structurally and functionally similar to estradiol [17–24]) on VSM was assessed in these animals.

Results

Acquisition – on the Phyto-600 diet (shaping to criterion)

Acquisition of the radial-arm maze as influenced by the prenatal treatment of testosterone or flutamide resulted in significant differences in treatment ($F(3,75) = 13.98$, $p < 0.05$) sex ($F(1,75) = 42.40$, $p < 0.05$) and treatment by sex interaction ($F(3,75) = 8.59$, $p < 0.05$). Post hoc pairwise comparisons confirmed that control males (oil-treatment) acquired the maze significantly earlier than control females ($p < 0.05$). However, the prenatal treatments testosterone or flutamide resulted in no sex difference, see Figure 1 ($p > 0.05$). Furthermore, prenatal testosterone-treated females acquired the maze in significantly fewer days than oil-treated females or flutamide-treated males and females ($p < 0.05$).

Eight-Arm Task – on the Phyto-600 diet

Multivariate analysis of the effects of prenatal treatments on performance in the 8-arm task revealed significant differences among treatments ($p < 0.05$) sex ($p < 0.05$) and treatment by sex interaction ($p < 0.05$). Fur-

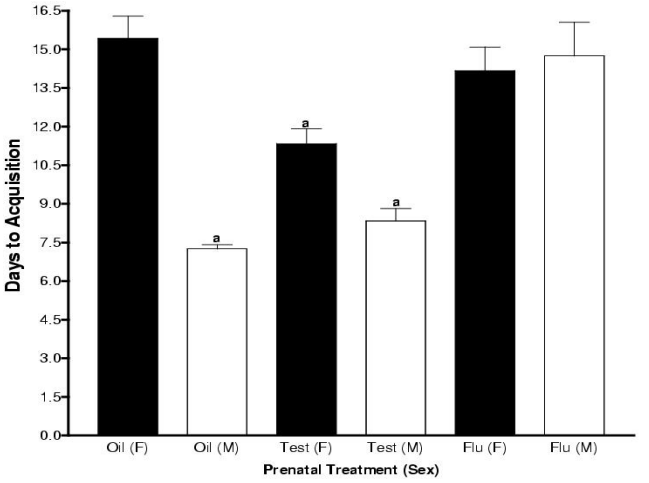

Figure 1
Acquisition: Number of trials (Mean + SEM) required to reach a set criterion level of performance in the eight-arm radial maze by female (F) and male (M) rats prenatally treated with oil, testosterone (test) or flutamide (flu). **a** Reached criterion significantly earlier than oil and flutamide treated females and flutamide treated males ($p < 0.05$) Number of female and male animals ranged from 8 to 12 among the treatment groups.

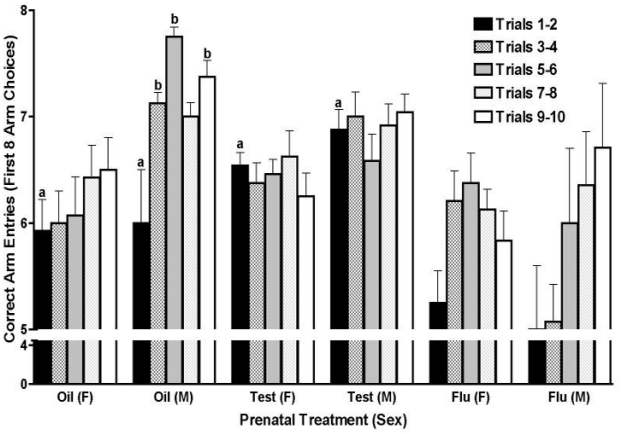

Figure 2
8-Arm Task: Number of correct arm choices, in the first eight arm entries (Mean + SEM), made by female (F) and male (M) rats treated prenatally with oil (control), testosterone (Test) or flutamide (Flu). A correct choice was defined as an entry into a baited arm not yet visited in the trial. **a** Significantly greater than prenatally treated flutamide males and females ($p < 0.05$) **b** Significantly greater than oil and flutamide treated females ($p < 0.05$). Number of female and male animals ranged from 8 to 12 among the treatment groups.

ther analysis revealed that, consistent with previous studies, oil-treated males significantly outperformed oil-treated females on trials 3–4, 5–6, and 9–10 ($p < 0.05$). This sex difference was not however, significant in either the testosterone or flutamide treated animals ($p > 0.05$). Furthermore, prenatally oil- and testosterone-treated animals significantly outperformed flutamide-treated animals (independent of sex) on trials 1–3. These data are represented in Figure 2.

Four-Arm Task – after the diet change (baited/unbaited)

Following the diet change, multivariate analysis of variance identified significant differences among treatments ($p < 0.05$), diets ($p < 0.05$), treatment by diet ($p < 0.05$) and treatment by sex by diet interaction ($p < 0.05$) see Figure 3. Further analysis found the following differences: prenatally oil-treated females fed a Phyto-600 diet made significantly more correct arm choices than oil-treated females fed a Phyto-free diet on trials 1–3, 7–9 and 10–12 ($p < 0.05$). In contrast, oil-treated males fed a Phyto-free diet significantly outperformed oil-treated males fed the Phyto-600 diet on trial 13–15 ($p < 0.05$). Testosterone-treated males and females fed either a Phyto-600 or Phyto-free diet did not differ significantly across the trials ($p > 0.05$). Furthermore, correct arm entries did not differ significantly in prenatally flutamide-treated female fed either diet ($p > 0.05$). However, flutamide-treated males, fed the Phyto-600 diet, had significantly more correct arm entries on trials 4–6, 10–12 and 13–15 compared to oil-treated males fed the Phyto-free diet ($p < 0.05$).

Discussion

The organizational effects of gonadal hormones influencing sex differences in learning and memory tasks requiring the use of spatial cues have almost exclusively targeted the period following birth as critical for the establishment of sexually dimorphic performance, where males outperform females in intact rats [4,7,11]. However, one study demonstrated that prenatal hormone manipulation significantly altered 70-day old male and female rat's (Morris) water maze performance [3], where intact males performance surpassed males prenatally-treated with flutamide, whereas, females prenatally-treated with androgens or estrogens showed enhanced performance compared to intact females. To our knowledge, prenatal hormonal effects on sexually dimorphic VSM, as expressed in the radial-arm maze, have not been investigated to date. Furthermore, we were unable to find any studies that compared the effects of testosterone with an androgen receptor blocker like flutamide in intact male and female rats examining radial-arm performance. Therefore, based upon this information and the results of our companion paper [where we examined the influence of phytoestrogens (estrogen-like plant mol-

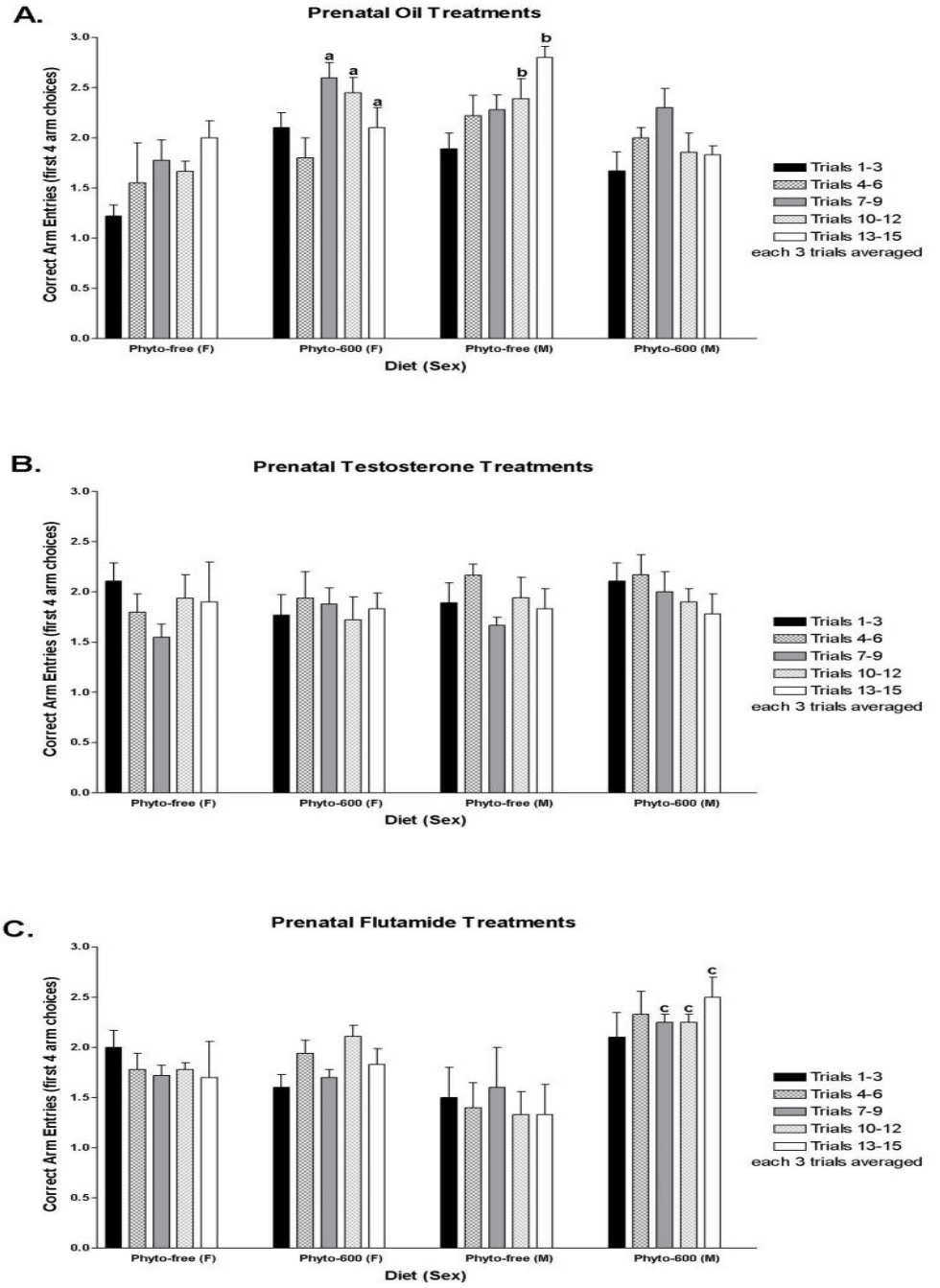

Figure 3
4 Arm-Task (baited/unbaited): Number of correct arm choices, in the first four arm entries (Mean + SEM), made by female (F) and male (M) rats treated prenatally with Oil (control; A), Testosterone (B) or Flutamide (C) and fed either a Life-Long Phytoestrogen-600 diet or the Phyto-600 diet from conception and then switched postnatally to a Phyto-free diet. Phyto-free refers to animals exposed to the Phyto-600 diet from conception until 80 days of age and then placed on the Phyto-free diet from 80 days of age until sacrifice at 120 days of age. A correct choice was defined as an entry into a baited arm not yet visited in the trial. **a** Prenatally oil-treated females fed the Phyto-600 diet outperformed oil-treated females fed the Phyto-free diet ($p < 0.05$) **b** Prenatally oil-treated males fed the Phyto-free diet outperformed oil-treated males fed the Phyto-600 diet ($p < 0.05$) **c** Prenatally flutamide-treated males fed the Phyto-600 outperformed flutamide-treated males fed the Phyto-free diet ($p < 0.05$) Number of female and male animals ranged from 4 to 6 among the treatment and diet groups.

ecules) on VSM, utilizing a radial arm-maze to quantify several aspects of memory], lead us to investigate the influence of dietary soy derived phytoestrogens on VSM in combination with manipulating the prenatal hormonal environment.

In the present study, since it is rare that animals are raised on a phytoestrogen-free diet, all pregnant rats were placed on a phytoestrogen-rich diet at least three to four weeks before insemination. During the last week of pregnancy, the rats received daily subcutaneous injections of flutamide, testosterone or peanut oil (that served as controls). After being exposed to this diet for 60 days, each treatment group by sex was tested to determine the number of trails required to acquire the radial-arm maze and reach a predetermined criterion of performance in the 8-arm task. Control animals (oil-treated) performed as expected (by sex) where males significantly outperformed females in acquisition and 8-arm task maze performance (a finding consistent with our companion paper, where untreated males outperformed untreated females in these tasks when tested in the same experiment). Prenatal testosterone or flutamide treatments significantly altered maze performance, where testosterone-treated females acquired and performed in the maze at a similar level as intact males. These results are in agreement with previous studies demonstrating early postnatal testosterone effects in females in radial-arm maze performance [2,11–13]. Additionally, the prenatal administration of flutamide in the present study complements previous neonatal research where the treatment of intact male rats with an androgen antagonist (cyproterone acetate) feminized male performance in a spatial learning task [10]. Taken together these studies suggest that the critical period for hormonal influence on adult spatial ability span both late pre- and early postnatal development.

Following the 8-arm task, a dietary change was initiated where one-half of the male and female rats were kept on the original phytoestrogen-rich (Phyto-600) diet and the other half was assigned to a phytoestrogen-free (Phyto-free) diet. After the diet change, each rat was tested in the baited/unbaited 4-arm VSM task. Measures of accuracy on the 4-arm task demonstrated that a diet change in young adult control animals (oil-treated) had a positive influence on accuracy in females, but a negative influence on accuracy in males (consistent results with our companion paper). Most striking was the finding that dietary phytoestrogens in combination with the flutamide treatment altered maze performance in the 4-arm task. In fact, prenatal treatment of flutamide in males resulted in diet differences resembling female controls (oil-treated females in this study which can be compared with non-treated females in our companion paper) where flutamide-treated males fed the Phyto-600 diet made significantly fewer errors than flutamide-treated males fed the Phyto-free diet. In other words, feminized males (via the flutamide treatments) fed the Phyto-600 diet outperformed feminized males fed the phytoestrogen-free diet. This result is similar to intact females on the Phyto-600 diet outperforming intact females on the Phyto-free diet, as observed in our companion paper.

Conclusions

Male rats acquire and exhibit superior performance to females in learning and memory task requiring the use of visual spatial cues. This study, examined the influence of the prenatal hormonal milieu on VSM. Within the radial arm maze, prenatal hormones significantly disrupted the normal sexually dimorphic expression of VSM. Specifically, prenatally testosterone-treated females performed in a "masculinized" fashion, more consistent with male performance, while prenatally flutamide-treated males performed in a "feminized" fashion, similar to that of female performance. Furthermore, when a diet change was initiated in adulthood, phytoestrogens, in the animal's diet, improved performance in "feminized" (flutamide-treated) males. Taken together, this research, A) confirms androgen's influence on VSM, B) establishes the prenatal period as a critical developmental interval for visual spatial ability in adulthood and C) confirms and extends the findings in the companion paper of the effects of phytoestrogens, present in animal diets, to alter VSM.

Materials and Methods

Animals

Adult (50 day-old) females, purchased from Charles River Laboratories (Wilmington, MA, USA) for breeding, were caged individually and housed in the Brigham Young University Bio-Ag vivarium and maintained on a 10-hour dark 14-hour light schedule (lights on 1400–0400).

Diets

Upon arrival all animals were allowed *ad libitum* access to a commercially available diet with high phytoestrogen levels (Harlan Teklad Rodent Diet 8604, Madison, WI, USA) containing 600 micrograms of phytoestrogens/gram of diet (in the glycoside form); referred to hereafter as the Phyto-600 diet. This diet contained 420 micrograms/gram of total isoflavones. Later in these experiments, after the 8-arm task a diet change was initiated (see below; similar to that reported previously-see companion paper). Some of the animals remained on the Phyto-600 diet, while some animals were changed to a custom phytoestrogen-free diet; referred to hereafter as the Phyto-free diet, obtained from Ziegler Bros. (Gardner, PA, USA). At 70–80 days of age when all the animals

were on the Phyto-600 diet, females were time-mated, placing them with a sexually active male until two ejaculations occurred. Day of insemination was designated as Gestation Day 0 (GD 0).

Prenatal Treatment

Two weeks following insemination (GD 14) pregnant females were randomly assigned to one of three groups (at least 3 females/treatment) receiving daily subcutaneous injections of: 1) 90 mg/kg body weight/day flutamide (suspended in 0.1 cc ethyl alcohol), 2) 1 mg testosterone (suspended in 0.1 cc peanut oil) or 3) 0.1 cc peanut oil that served as controls, until parturition (GD 22). Thirty days following birth, the animals were weaned and separated by sex into colony cages (4 animals per cage). At 40 days of age, animals were singly caged.

Apparatus

The maze utilized in this research study was an 8-arm radial maze obtained from Columbus Instruments (Columbus, OH, USA). The stainless steel maze consists of eight arms (length 45 cm, width 13 cm, wall height 13 cm) extending outward, at equal angles around a center platform. Five cm from the end of each arm a small receptacle was placed to hold the food out of view from the maze's center. Above the maze a video camera was suspended (at approximately 5 feet) to record each trial for analysis.

Maze Procedure (Acquisition and Eight-Arm Task; working memory)

At 50-days of age rats were put on a limited feeding schedule and maintained at approximately 90% of normal body weight; however, animals were allowed to gain an additional 5 g per week to account for normal growth. One week later, the rats were introduced to the radial arm maze. On 3 consecutive days each rat was placed into the maze for 5 minutes to explore. Following this introduction, rats were placed, alone, in the center of the maze. Three Froot Loops (FL) were placed at the start of each arm. If the rat retrieved FL from at least 5 of the piles within 3 minutes, then on the following day a single FL was placed at the start of each arm. If 5 of the 8 FL were retrieved, the following day a single FL was placed 5 cm further out on the arms. In this manner, the FL were gradually placed farther and farther out on the arms in a systematic manner. Training was completed for a given rat when it retrieved FL from the end of at least 5 of the 8 arms within 3 minutes on 3 consecutive trials. The number of trials required to reach this criterion was recorded for each rat (acquisition). Once this criterion level was reached, for a given rat, it was tested on this task for 10 additional trials, one/day (8-arm task). Each trial was considered complete when all 8 arms were visited, or 3 minutes had passed. An arm choice was scored if the rat traveled three-fourths of the way down the length of an arm. A working memory error was recorded if an animal reentered a previously visited arm.

Diet Change

Following the 8-arm task trials described above, the animals were returned to their home cages and a dietary change was initiated. Approximately one-half the total number of male or female (random cycling) rats was kept on the original Phyto-600 diet (long-term) and the other half was assigned to a Phyto-free diet (short-term; from approximately 80 to 120 days of age). This ad lib feeding continued for 15 days. Following this ad lib period of feeding, rats were again reduced to 90% of normal body weight for 10 days.

Maze Procedure (Four-Arm Task; Reference and Working Memory)

Following reduction, each rat was tested for an additional 15 days, one trial/day, during which only 4 arms were baited (four-arm task). To begin these trial, each animal was placed in a bottomless, opaque box in the center of the maze. The box was removed opening all arms simultaneously to the rat. These trials were considered complete when the 4 baited arms (arms 2,3,6 and 8) had been visited or 3 minutes had passed. During all trials the amount of time required to visit the initial 4 arms and the order in which the arms were entered were recorded. Again an arm entry was noted if a rat traversed at least three-fourths the length of the arm. A working memory error was recorded if a rat reenters a baited arm, a reference memory error was recorded if an animal entered an arm, which was not baited, and a working/reference memory error was recorded if an animal reentered an arm, which was not baited. All testing and analysis was accomplished without knowledge of hormone or diet conditions.

Maze Statistical Analysis (Acquisition and Eight-Arm Task)

Acquisition: A one-way analysis of variance (ANOVA) was used to evaluate acquisition of the radial-arm maze based on the number of trials needed for each rat to meet the specified level of performance.

Accuracy (8 arm task): A multivariate analysis of variance (MANOVA; sex over trials 2×5) was used to determine accuracy of the initial 10 trials (each 2 trials were averaged). Accuracy was defined as the number of correct arm choices (an arm not yet chosen in that trial) in the first eight arm entries.

Maze Statistical Analysis (Four-Arm Task)

Accuracy (4 arm task): Accuracy on the four-arm task was determined by the number of correct arm choices (baited arms not yet chosen in the trial) made in the first

4 arm entries. A MANOVA (sex by diet change over trials $2 \times 2 \times 5$) was used to interpret these findings (each 3 trials were averaged). Also a sex by diet over trials MANOVA was completed to determine the number and types of errors committed in the initial 4 arm entries.

All analyses were performed using SPSS statistical software package and all significant main effects were followed by Bonferroni post hoc comparisons. The alpha level was set at 0.05.

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