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The role of perinatal testosterone on exploratory behavior, sexual motivation, and spatial working memory in adult rats

Abstract

Aim: Sex differentiation occurs during the perinatal period (four days before and after birth) in the rat brain. The brain remains a female brain unless exposed to testosterone, which gets metabolized to 17β-estradiol and dihydrotestosterone. While aromatase converts testosterone to 17β-estradiol, 5α-reductase converts testosterone to dihydrotestosterone. 17β-estradiol exerts its effects by binding to an estrogen receptor, whereas dihydrotestosterone via an androgen receptor. The role of dihydrotestosterone and 17β-estradiol during the organizational phase of brain differentiation was tested using adult rat behaviors. The study sought to examine the effects of perinatal manipulations on adult rat behaviors.

Materials and methods: Timed-pregnant rats received either the vehicle (5% ethanol and 95% sesame oil), exemestane (4 mg/kg/ml; aromatase inhibitor), or flutamide (20 mg/kg/ml; androgen receptor blocker) subcutaneously during the last four days of pregnancy. Pups from each group continued to receive their treatments during the first four days after birth. From postnatal day 65, these animals were subjected to open field, sexual motivation, and spatial working memory tests.

Results: Male rats receiving flutamide exhibited exploratory behavior significantly more compared to exemestane and control groups. Male rats receiving exemestane had substantially higher plasma testosterone and displayed an increased interest in the estrus rat than the control and flutamide group.

Conclusion: Androgen receptor blockade resulted in increased exploratory behavior in male rats where 17β-estradiol was testosterone's primary metabolite. The blocking of aromatase using exemestane resulted in higher levels of plasma testosterone and enhanced sexual motivation in male rats. Thus, 17β-estradiol and dihydrotestosterone's presence during the organizational phase is essential for normal socio-sexual and exploratory behaviors.

Keywords: Gonadal steroid hormones, Aromatase, Androgen receptors, Sexual dimorphism

Volume 9 Issue 2 - 2021

Chaya Gopalan,[1] Paige Niepoetter,[2] Avery Ogle,[2] Sai Medavaka,[2] Samantha A Olendorff,[3] Kevin R Tucker[3]
[1]Applied Health and Nursing, Southern Illinois University Edwardsville, USA
[2]Biology, Southern Illinois University Edwardsville, USA
[3]Chemistry, Southern Illinois University Edwardsville, USA

Correspondence: Chaya Gopalan, PhD, FAPS, Professor of Physiology, Departments of Applied Health and Nurse Anesthesiology Southern Illinois University Edwardsville , Box 1126, Edwardsville, IL 62026, USA, Tel 618-650-5802, Email cgopala@siue.edu

Received: September 14, 2021 | **Published:** September 28, 2021

Abbreviations: testosterone, T; 17β-estradiol, E2; dihydrotestosterone, DHT; androgen receptors, AR; estrogen receptor, ER; open field, OF; sexual motivation, SM; spatial working memory, SWM

Introduction

An undifferentiated brain remains a female brain unless exposed to testosterone (T).[1,2] Gonadal T, acting via estrogenic and androgenic pathways, is the primary endocrine mediator promoting sexual differentiation of the male brain and sex-specific behaviors.[3] In rodents, the testes produce high levels of T during the perinatal days (four days before and four days after birth), a critical period of brain development, to organize the brain to be masculine.[4–9] Feminization refers to the parallel process of demasculinization where the brain facilitates female sexual behaviors and diminishes the expression of male sex-typical behaviors in rodents.[2] A female rat brain is categorized by having more concentrated axons in the corpus callosum, a lower number of granular cells, a smaller preoptic area, and a smaller cerebellar surface area.[2] Additionally, neonatal females have half as many proliferating cells in the dentate gyrus and hippocampal CA1 regions compared to neonatal male rats.[10]

During the perinatal period, the high levels of circulating T reach the male brain, get converted to 17β-estradiol (E2) and activate the estrogen-receptor-mediated signaling to allow the development of male-typical characteristics.[7] Thus, the perinatal peak of T coupled with steroid receptor activity programs the male brain both phenotypically and neurologically.[10] Such programming or organizing actions of T are linked to several aspects of neuronal development that influence synaptogenesis and neurochemical specifications.[2] For example, the depolarizing action of gamma-aminobutyric acid at the time of birth gradually switches to a hyperpolarizing process in the first six days in both males and females.[11,12]

The male and female sexual behaviors tightly regulated by T occur in two phases: the organizational and the activational phases. The organizational phase occurs during the perinatal period and requires T for both defeminization and masculinization of the brain.[7] On the other hand, the activational phase involves sexual maturation around puberty[13,14] and depends on normal levels of T.[15,6] Any disruption to normal T levels during either phase would affect adult sexual behavior.[16] The action of T, however, is not by itself but through its aromatization to E2[5,9] and dihydrotestosterone (DHT).[7] In rats, the formation of E2 in the brain by aromatization of circulating T appears to be the most important mechanism for the masculinization of the brain.[17] Nevertheless, another source appears to be via local synthesis of E2 in specific parts of the brain, such as the preoptic area.[18]

While DHT brings about its response via androgen receptors (AR),

E2 mediates its effects through two cytoplasmic estrogen receptors, estrogen receptor (ER)-α and ER-β. The masculinizing actions of perinatal T in the male appear to stem from the conversion of T to E2 and its subsequent binding to hypothalamic ER-α.[9] Thus, ER-α appears to be primarily involved in masculinization, and ER-β appears to mediate the defeminization of sexual behaviors.[19–21] Expression levels of ER-α and ER-β in the developing brain reach their peak during the perinatal period.[22] Activation of AR is also known to contribute significantly to masculinization[23] as AR expression continually increases until at least 21 days after birth in the mouse cortex and hippocampus.[24] The expression of AR has been reported to decrease in response to a blockade in AR activity.[7] Thus, a reciprocal interaction between AR and the ER in the brain is suspected.

Exhibiting an interest in a female rat as a marker of a male rat's socio-sexual behavior is often utilized to measure behavioral changes related to abnormal endocrine influences during the perinatal period.[25] However, the role of T and the relative importance of ER and AR on cognitive behaviors in rats are poorly understood.[26] A pharmacological approach was utilized in the present study where the typical actions of T during the perinatal peak of brain sex differentiation were manipulated to understand the organizing effects of perinatal T. More specifically, the roles of DHT and E2 were examined by blocking AR with flutamide or by inhibiting aromatase with exemestane (exemestane) during the perinatal period in both male and female rats. Flutamide acts as a competitive AR antagonist[27] and blocks the effects of androgens by binding to AR both centrally and peripherally.[28] Exemestane is an irreversible steroidal aromatase inhibitor.[29,30] A variety of behavioral tests, including the open field (OF) test, sexual motivation (SM) paradigm, and spatial working memory (SWM) tests, were used in adult rats as a measure of the manipulation of T during the organizational period. In the absence of DHT, male rats were expected to be less attracted to estrus rats. Similarly, decreased SWM in female rats was anticipated due to the absence of E2 in the pups that received exemestane during the perinatal period.[31]

Materials and methods

Animals: Seven timed-pregnant Long Evans rats were purchased from Charles River Laboratories, which arrived on the 14th day of their gestational period. They were housed individually under controlled laboratory conditions (12-hour light/dark cycle with lights on from 7:00 P.M. at a room temperature of 20.0-22.2°C) in solid-bottom, over-sized cages with aspen chip bedding. These animals had access to food and water ad libitum. The cages were checked daily for pups' appearance, and the day of birth was considered postnatal day 0 (PND 0). Newborn pups (N=72; 40 female and 32 male) were housed with their mothers for 21 days in the same laboratory conditions and then separated into their own individual cages, at which time they were able to ingest solid food[32] reliably. Besides, four ovariectomized (OVX) rats were obtained before the start of the SM study. Animal studies were conducted per approved protocol by the Southern Illinois University Edwardsville's Institutional Animal Care and Use Committee. Figure 1 represents the timeline of the study. Day 0 marks the beginning of the study which is the day of the arrival of the timed pregnant rats.

Drugs: Drugs (flutamide or exemestane) were dissolved in 5% ethanol and 95% sesame oil (Sigma-Aldrich) vehicle. The dams were injected subcutaneously with flutamide (Sigma-Aldrich) at 20mg/kg/ml/day, exemestane (exemestane; Cayman Chemical) at 4mg/kg/ml/day, or the vehicle only during the last four days of pregnancy (days 18-21).[30] After birth, the pups from the two flutamide-treated dams were treated with flutamide (N=15). Similarly, pups from two exemestane -treated dams (N=26) were injected with exemestane, and pups from three vehicle-treated dams (N=31) were treated with the vehicle alone for four days (Table 1). These injections ranged from 0.2-0.5ml for the dams and 5-10µl for neonates. Flutamide, exemestane, or the vehicle was injected into the pups via a microsyringe in different subcutaneous locations to avoid bruising. Randomization was accomplished by assigning respective treatments to the dams. Table 1 shows the number of male and female pups in each treatment group (control, exemestane, and flutamide).

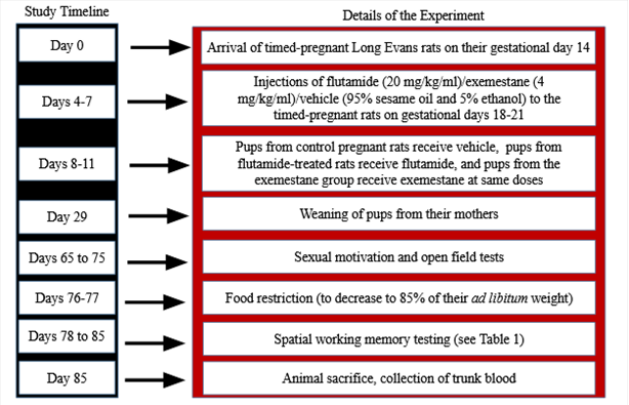

Figure 1 Timeline of the study.

Table 1 Treatment groups and the number of samples

	Males (N)	Females (N)
Group		
Control	10	21
Exemestane	15	11
Flutamide	7	8

Plasma testosterone measurements: Standards of testosterone (1mg/mL in methanol) and 13C3-testosterone (0.1mg/mL in methanol) were purchased from IsoSciences (Amber, PA, USA). Reagent alcohol, ethyl acetate, hexane, ammonium hydroxide, glacial acetic acid, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium fluoride (98%) was purchased from Acros Organics (New Jersey, USA), ammonium acetate (97%) was purchased from RICCA (Arlington, TX, USA), and ammonium bicarbonate was purchased from Avantor (Radnor, PA, USA). T measurements were quantified utilizing gas chromatography-mass spectrometry procedures.

A calibration curve from 0.1ppb to 300ppb with internal standard concentration at 100ppb. All calibration standards were made in 0.25% aqueous ethanol, and initial stocks were made in 20% ethanol. The calibration curve resulted in an R2 value greater than 0.9977.

The extraction procedure was adapted from the CDC published method.[33] Trunk blood was extracted at the time of sacrifice of the rats. The blood serum (200µL) and internal standard (10µL of 13.5 ppb) were combined by mass in a 1.5mL centrifuged tube. Solutions were mixed in an incubator at room temperature for 45 min. Ammonium acetate buffer (100µL, 0.5 M, pH 5.5) was added, and the solution was mixed for 30 min. 40:60 ethyl acetate:hexane (600µL) was added, and the solution was centrifuged (21460×g) for 10 min. The top 500 µL was added to a second 1.5 mL tube, and ammonium bicarbonate buffer (200µL, 0.2 M, pH 8.0) was added. The solution was mixed and allowed to settle, and the top layer (~500µL) was added to a third 1.5 mL tube. The first tube was rewashed with ammonium acetate

buffer and ammonium bicarbonate buffer; the organic layer was added to the third tube. This was dried down in a speed vac (40°C) and reconstituted in 0.2mM ammonium fluoride in 20:80 methanol: water (135µL). All samples were stored at -80°C until analysis.

A Shimadzu Nexera XR LC-20AD coupled to Shimadzu LCMS-8050 with an ESI ion source was used to quantify multiple reaction monitoring (MRM) modes. The column oven was set to 45°C, and a Halo 90 Å, C18, 2.7µm, 2.1 x 100 mm column was used. Mobile phase A was 0.2 mM ammonium fluoride in 20:80 methanol: water, mobile phase B was 100% methanol. The 10-minute gradient had a flow rate of 0.45mL/min, 1µL injection volume, and started with mobile phase B concentration at 30%. The gradient composition of B was as follows: 30% at 0.30 min, 95% at 7.00 min, 95% at 8.00 min, 30% at 8.01 min. The MRM transitions for 13C3-testosterone are 292.00 -> 100.05, 112.20, 256.20m/z with CE -22.0, -24.0, -19.0, respectively. 292.00 -> 100.05m/z was used for quantification. The MRM transitions for testosterone are 289.00 -> 97.05, 109.10, 79.10 m/z with CE -25.0, -26.0, -47.0, respectively. 289 → 97.05 was used for quantification.

Method validation was prepared by gathering several plasma samples and homogenizing to create 3 mL. Standard addition was used to calculate a recovery percentage using 12 replicates. Three replicates were controls, and the other nine were spiked at concentrations in triplicate (25ppb, 50ppb, 100ppb). The samples were masses before and following the addition of the internal standard and the testosterone. The recovery samples were processed identically as described above.

Behavioral testing: Both male and female rats at PND 65 were used in the behavioral studies. At this age, rats are considered young adults and are capable of mating.[32] Behavioral testing included SM, SWM, and OF paradigms. All tests were conducted in the dark with red lights.

A. The OF paradigm is a standard behavioral test that measures locomotor activity, which often is an indirect measure of exploratory behavior.[34] In this test, each subject was placed in the OF apparatus, a 100cm by 100cm opaque plexiglass arena. The animal was allowed to explore the arena for six minutes after being placed in it. The arena was marked as central and peripheral zones where the peripheral zone consisted of 16squares (6400cm) and the central zone occupied nine squares (3600cm). A video-capturing device along with ANY-Maze software was used to collect data. Typical exploratory behavior is when the rat can move freely between the zones versus frozen in one corner for a more extended period. We used the amount of time in the central zone as the criteria in determining exploratory animal behavior. This procedure was repeated eight times over eight consecutive days for each rat being tested.

B. SM paradigm measures interest in a female rat, a marker of male rats' socio-sexual behavior.[25] Tests were conducted in the same arena that was used for the OF study. The arena contained one OVX rat and one estrus rat. The estrus rat was receptive to male during this stage of her estrous cycle. These two female rats were placed in individual wire-mesh cages in the central area of the arena. A male rat was given a choice between approaching the estrus female rat or the OVX rat. Interest in either female was defined by time in proximity. The male rat was allowed to acclimate to the new environment for five minutes. Then movements were tracked for an additional 25 minutes. ANY-Maze software was used to track the male rat's position, movements, and interactions towards either one of the females to test for preference. This procedure was repeated eight times on separate days for each male rat being tested. The total amount of time spent with either of the female rats was used to compare the control and treatment groups.

C. SWM testing assesses cognitive behaviors, such as working memory and reference memory. Behavioral training and testing were performed using a radial arm maze (RAM) that consisted of a platform in the center with eight arms radiating from it. Extra-maze cues were located on walls surrounding the RAM apparatus for animals to use as spatial cues. Six days before testing, the rats were restricted to 12 g of food per day to reduce their body weight to 85% of ad libitum weight at the time of testing. The purpose of restricting food was to motivate the rats to search for food in the maze. A rat was allowed to locate three food pellets (pieces of Fruity Pebbles cereal) placed equidistantly within each arm during the habituation period. However, during the training period, arms 1, 4, 6, and 8 were baited with three food pellets equidistant down each arm, and the rats were allowed to explore the entire maze. On the days of testing, only one food pellet was made available at the farthest point in arms 1, 4, 6, and 8 (Table 2).[35] ANY-Maze software combined with a wall-mounted video camera was used to track the animal. We compared the total amount of time the animal spent in the arms with food between the control and treatment groups.

Table 2 Spatial working memory experimental design

	Number of pellet(s) in Arm	Arms with food pellet(s)	Number of days	Duration of testing (min)
Habituation	3	All 8 arms	3	15
Training	3	1, 4, 6, 8	3	5
Testing	1	1, 4, 6, 8	1	5

Statistics: Seventy-two pups in this study served as dependent variables for the controls and manipulations. We compared the time spent in the middle zone during OF testing between the control and treatment groups by using two-way ANOVA with p-value cutoffs at 0.05; the time spent by the male rat among OVX and estrus rats between the control and the treatment groups, and the averages of the total time spent in radial arms with food between the control and treatment groups for SWM. Plasma T levels were compared between the control and treatment groups using one-way ANOVA. GraphPad Prism (8.0 version) was used to carry out these statistical analyses.

Results

Results from the current study are summarized below:

A. OF testing: As shown in Figure 2, an analysis using two-way ANOVA confirmed an interaction between the male and female rats (rows) and their treatments (columns) ($F=14.46$. DFn=2, DFd=66, $p<0.0001$). Simple main effect analysis row factor was $F=1.90$. DFn=1, DFd=66 whereas the column factor was $F=13.08$. DFn=2, DFd=66; $p<0.0001$. The main effect of the treatment groups using Sidak's multiple comparison test indicated that male rats receiving flutamide (n=7) on average spent significantly more time in the middle zone compared to the male control group (n=10, ****$p<0.0001$) and exemestane-treated males (n=15, ### $p<0.001$). There was no significant treatment effect among the female groups (n=21), exemestane-treated females (n=11), and flutamide-treated females (n=8).

B. SM testing: In general, all male rats, including control animals, spent more time with the estrus rat compared to the OVX rat (Figure 3). An analysis using two-way ANOVA confirmed an interaction between the OVX/estrus (row) and treatment/control (column) factors as significantly different (F=9.53. DFn=2, DFd=58. The p value =0.0003). Treatment had no effect overall (F=0.76. DFn=2, DFd=58. The p value =0.4725) whereas the female rat type (OVX/estrus) variable was significantly different (F=40.61. DFn=1, DFd=58. The P value is <0.0001). Simple effects within rows using Turkey's multiple comparison suggested that male rats receiving exemestane treatment (N=15) spent significantly more time with the estrus rat compared to the control group (N=10; **p<0.01) and the flutamide group (N=7; *p<0.05). Since exemestane-treated rats spent more time with the estrus rat, their time with the OVX rat was significantly reduced p<0.0001).

C. SWM Testing: A two-way ANOVA revealed no interaction between the sex and the treatment variables compared (F=0.35. DFn=2, DFd=66 and the *p* value was 0.7048). Thus, the average total time spent in the arms with food was not significantly different from the total time spent in the arms without food among male and female groups. The treatment (column) variable was F=0.02, DFN=2, DFd=66, and the *p* value was 0.9831 and the sex (row) variable was F=1.12. DFn=1, DFd=66, and the *p* value was 0.2943 (Figure 4). The treatments followed as control male (n=10), control female (n=21), male receiving flutamide (n=7), female receiving flutamide (n=8), male receiving exemestane (n=15), and female receiving exemestane (n=11)

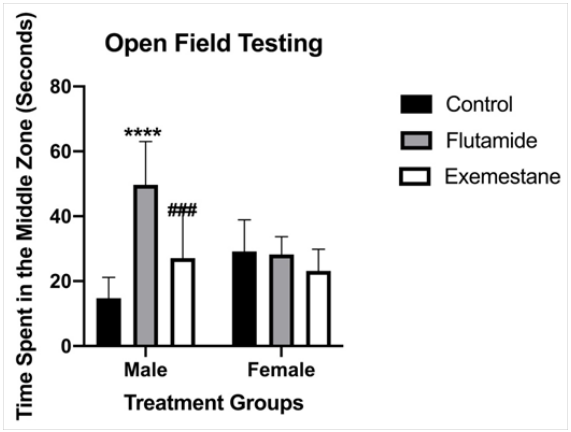

Figure 2 Open field testing results compared between treatment groups.

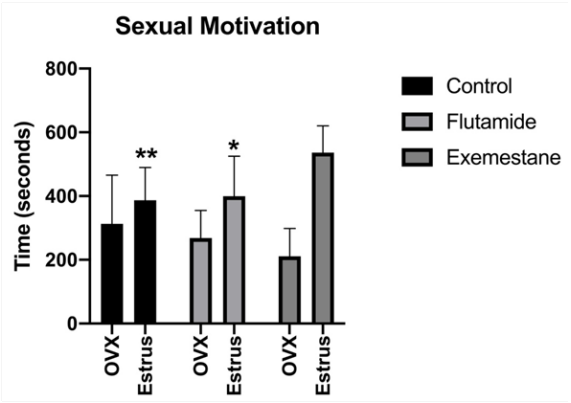

Figure 3 Sexual motivation results.

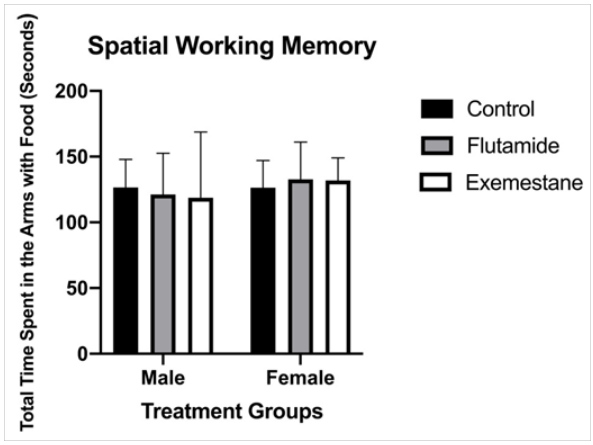

Figure 4 Spatial working memory results.

Plasma T measurement: A one-way ANOVA test was conducted to compare the plasma levels of T between the control and treatment groups. The plasma T levels in the female rats were too low for accurate measurements. On the other hand, there was a significant difference in the plasma concentrations of T between the control and the exemestane-treated male rats [F(2, 26)=4.235; *p*=0.0256; Figure 5). The treatments followed as control male (n=10), male receiving flutamide (n=7), and male receiving exemestane (n=15,* *p*<0.05).

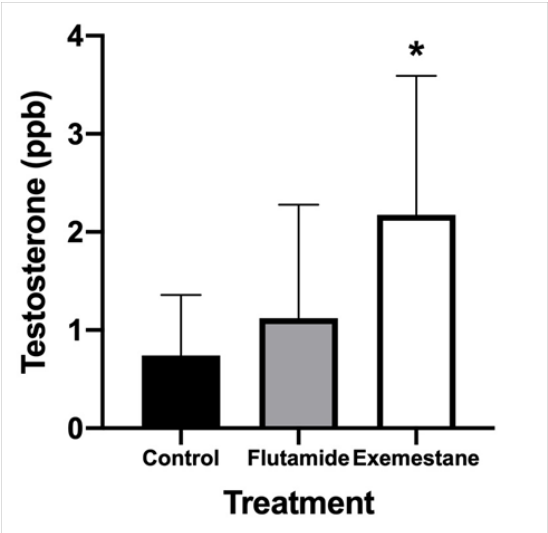

Figure 5 Plasma testosterone levels of treatment groups.

Discussion

We examined the role of T, DHT, and E2 during the window of brain sex differentiation using a pharmacological approach such as blocking ARs to eliminate the effect of DHT and by inhibiting aromatase as a way to remove the effect of E2 and studying the effect on specific behaviors in adult rats. Flutamide served as the perinatal AR blocking agent. On the other hand, exemestane is an inhibitor of aromatase, an enzyme essential in the synthesis of E2.[36] Our study offers advantages by interfering with the animal's normal situation only during the perinatal window using pharmacological manipulations, which is unlike the gene knockout method, for example, where the animal is affected by the manipulation not only during the organizational phase but during the entire lifespan including both the organizational and activational phases.

The absence of AR-mediated effect during the organizational phase in the flutamide-treated animals causes E2 to be the primary gonadal steroid hormone. In the absence of AR activation, the E2-mediated effect in the early stages of development appears to last through adulthood, based on the findings in this study. E2 has been shown to increase exploratory behavior in rats. Djiogue et al.[31] reported that OVX rats exhibited less exploratory behavior. Our study's findings support an E2-mediated increase in exploratory behavior in rats since the male rats receiving flutamide during the perinatal period significantly increased exploratory behavior compared to all other groups tested.

All male rats, irrespective of their treatments, spent more time with the estrus rat than the OVX rat suggesting that the perinatal treatment of flutamide or exemestane was not effective in overcoming their interest in the estrus rat. Since E2 synthesis was interrupted during the organizational phase of exemestane -treated rats, the main gonadal steroid hormones involved in the brain sex differentiation would be DHT and T. Rats receiving exemestane during the perinatal period had higher levels of circulating T as adults suggesting altered levels of steroid hormones in this group (Figure 5).

Although E2 is associated with masculinization and defeminization,[20] the significantly extended time with the estrus rat by the male rats receiving exemestane in our study suggests that DHT and T, in the absence of E2 during the perinatal period, appears to play a role in enhancing socio-sexual behavior during adulthood (Figure 3). Our observation is supported by the findings by Jones et al.,[37] who reported the effects of DHT to differ according to the E2 levels.[38] Using T in combination with E2 demonstrated increased sexually appetitive behaviors in OVX rats, but such effect was lacking in animals treated with E2 alone.[37] The effect of DHT appears to be mediated primarily by the production of hypothalamic reproductive and appetitive regulatory factors.[37] Although the hormonal manipulation in our study occurred during the organizational phase, the effect appears to be lasting into adulthood since greater SM and plasma T levels were shown to be mediated at least in part via T.

The SWM test was not a clear indicator of the hormonal manipulations conducted during the perinatal period. Thus, whether the lack of E2 during the sexual differentiation of the brain has any influence on spatial memory is unclear. Our findings are supported by the recent report by[10] who have shown that the proliferation of cells in the CA1 region of the hippocampus can be elevated to the baseline level using T or E2, inhibition of E2 synthesis or (ER) blockade had no effect. The male rats receiving exemestane spent marginally less time in the arms with food but was not statistically significant.

Conclusion

In conclusion, blocking AR during the perinatal period elevated the exploratory behavior of adult male rats. The absence of AR-mediated effect during the early part of life appears to allow E2 to be a major hormone regulating exploratory behavior during adulthood. Similarly, E2 deprivation during the perinatal period appears to allow DHT and T to be the major gonadal hormones during adulthood and thus an increased interest in the estrus rat. Such long-lasting effects of hormonal manipulation are observed by others.[39]

Acknowledgments

We thank Coral Viernow, Jessica Hammel, and Hannah Ruholl for their assistance with animal behavioral studies.

Funding

None.

Conflicts of interest

The authors declare no conflict of interest.

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Citation: Gopalan C, Niepoetter P, Ogle A, et al. The role of perinatal testosterone on exploratory behavior, sexual motivation, and spatial working memory in adult rats. *Endocrinol Metab Int J.* 2021;9(2):54–59. DOI: 10.15406/emij.2021.09.00309