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Organizational effects of testosterone on learning strategy preference and muscarinic receptor binding in prepubertal rats

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ABSTRACT

Prior to puberty, male rats, but not female rats, prefer a striatum-based stimulus-response learning strategy rather than a hippocampus-based place strategy on a water maze task that can be solved using either strategy. Neurochemically, learning strategy preference has been linked to the ratio of cholinergic muscarinic receptor binding in the hippocampus relative to the striatum, with lower ratios displayed by males compared to females and by stimulus-response learners compared to place learners. Sex differences in a variety of different behaviors are established by the organizational influence of testosterone on brain development. Therefore, the current study investigated the potential organizational effects of neonatal testosterone on learning strategy preference and the hippocampus:striatum ratio of muscarinic receptor binding in prepubertal male and female rats. Similar to vehicle-treated control males, prepubertal females treated with testosterone propionate on the first two days of life preferred a stimulus-response strategy on a dual-solution water maze task. Conversely, vehicle-treated prepubertal females were more likely to use a place strategy. Consistent with previous findings, the hippocampus:striatum ratio of muscarinic receptor binding was lower in rats preferring a stimulus-response strategy compared to those using a place strategy and lower in control males compared to control females. However, the hippocampus:striatum ratio was not reversed by neonatal testosterone treatment of females as predicted. The current study is the first to show that sex differences in how a navigational task is learned prior to puberty is impacted by the presence of testosterone during vulnerable periods in brain development.

1. Introduction

Typically, the study of learning focuses on ability and *how well* an individual learns a task or *how much* they have learned. Learning can also be understood in terms of *how* information is acquired, or the strategy used to learn new information. In both humans (Iaria et al., 2003) and rodents (Packard and McGaugh, 1996), navigational learning strategy differs from other measures of learning and memory in that it dissociates how a navigational task is learned into two principal categories, which are linked to two different brain structures. Whereas a hippocampus-based place learning strategy is guided by the relationships between distal cues, striatum-based response and stimulus-response strategies are guided by proprioceptive cues or cues proximal to a goal, respectively (Packard et al., 1989; McDonald and White, 1993; Packard and McGaugh, 1996; White and McDonald, 2002; Compton, 2004; Gold, 2004; Kesner and Rogers, 2004; Squire, 2004; Lee et al., 2008). In contrast to single-solution tasks that determine how well an animal can effectively use a specific strategy, dual-solution tasks are used to examine learning strategy preference in which the location of the goal can be learned equally well by using either strategy. Testing using dual-solution tasks has shown that the hippocampus and striatum process information in parallel and work together to regulate how information is learned. Packard and McGaugh (1996) demonstrated that rats administered saline in either the hippocampus or striatum prior to a probe trial on a dual-solution food-rewarded plus-maze task exhibited a preference for a place strategy early in training (day 8) and a preference for a response strategy with extended training (day 16). However, these same brain regions also compete for control over how information is learned. For example, when the dorsolateral striatum was inactivated by a local anesthetic, adult male rats preferred to use a hippocampus-based place strategy after both eight and 16 days of training. When the dorsal hippocampus was inactivated, there was

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no strategy preference evident after eight days of training; however, a stimulus-response strategy was preferred when training was extended to 16 days (Packard and McGaugh, 1996). Taken together, these findings indicate that the hippocampus and striatum process information in parallel and that dysregulation of function in one brain region may cause the other brain region to assume control over learning.

Both prior to and following puberty, learning strategy preference varies as a function of biological sex. Adult male rats typically prefer a place strategy on dual-solution tasks (Packard and McGaugh, 1996; Chang and Gold, 2003; Elliott and Packard, 2008; Schwabe et al., 2008; Packard and Wingard, 2004; Hawley et al., 2012), although not all studies agree (Martel et al., 2007; Asem and Holland, 2013). Learning strategy preference in adult female rodents is impacted by the activational effects of estradiol, such that females prefer a place strategy when estradiol levels are elevated and a striatum-based strategy when estradiol levels are low (Korol et al., 2004; McElroy and Korol, 2005). However, prior to puberty, the relationship between sex and leaning strategy preference differs (Grissom et al., 2012, 2013). When tested prior to puberty, male rats were more likely to use a striatum-based stimulus-response strategy to learn the location of a visible platform in a water maze. Alternatively, prepubertal females did not demonstrate a strategy preference, using either a stimulus-response strategy or a hippocampus-based place strategy with equal frequency. Notably, testing of prepubertal rats was conducted in the water using a reduced number of training trials massed in one day to capture learning strategy in a very narrow window, a method used in prior research (Akers and Hamilton, 2007; Grissom et al., 2012, 2013).

The effects of gonadal steroids, such as testosterone and its metabolite estradiol, on rodent brain development during early postnatal life are well-established. The presence or absence of these hormones during development influence later expression of a variety of sex-specific behaviors (Phoenix et al., 1959; Dawson et al., 1975; Williams et al., 1990; Roof and Havens, 1992; Roof, 1993a, 1993b; Isgor and Sengelaub, 2003; Foecking et al., 2008; reviews: Arnold, 2009; Becker, 2009; de Vries and Södersten, 2009; McCarthy et al., 2009; Schulz et al., 2009). With regard to learning and memory, adult male rats exhibited better spatial learning and memory relative to females on hippocampus-based spatial tasks (Roof and Havens, 1992; Roof, 1993a, 1993b; Isgor and Sengelaub, 2003). However, female rats treated neonatally with testosterone exhibited spatial learning and memory that was no different than that of adult males (Roof and Havens, 1992; Roof, 1993a, 1993b; Isgor and Sengelaub, 2003). Concomitant with the change in spatial learning and memory, neonatal treatment of females with testosterone increased the width of the granule cell layer of the dentate gyrus (Roof and Havens, 1992; Roof, 1993a), and increased dendritic length and dendritic branching in CA3 of the hippocampus (Isgor and Sengelaub, 2003), when measured in adulthood. Consequently, neonatal treatment of females with testosterone eliminated the differences between males and females in the structure of the hippocampus.

In addition to modulating how well a task is learned, neonatal exposure to gonadal hormones also impacts sex differences in how spatial information is used while learning. Similar to control female rats, adult males that had been castrated neonatally were able to use either room geometry or landmarks to navigate a radial arm maze (Williams et al., 1990). Conversely, adult females that had been treated neonatally with estradiol, a metabolite of testosterone, were only able to navigate by room geometry, which was similar to vehicle-treated males. Taken together, these seminal studies indicate that the development of the hippocampus, spatial learning and memory, and differences in navigation style are sex-specific and organized by exposure to gonadal hormones. Extending prior findings, the current study examined the organizational role of testosterone on the preference for either a hippocampus-based place strategy or striatum-based stimulus-response strategy. Based on earlier reports (Grissom et al., 2012, 2013), it was hypothesized that neonatal treatment with testosterone would result in a stimulus-response strategy preference in prepubertal female rats, similar to that of prepubertal male rats.

Prior reports have linked both muscarinic receptor binding in the hippocampus and striatum, and acetylcholine release in the hippocampus to learning strategy preference. Specifically, rats that used a striatum-based stimulus-response strategy had a significantly lower ratio of muscarinic receptor binding in the hippocampus compared to the striatum than rats that used a place strategy (Grissom et al., 2013). Consistent with these findings, a lower ratio of acetylcholine release in the hippocampus compared to the striatum was detected in rats that used a striatum-based response strategy on a dual-solution task compared to rats that used a hippocampus-based place strategy (Chang and Gold, 2003; McIntyre et al., 2003).

Muscarinic receptor binding in the hippocampus and striatum, as well as acetylcholine release in the hippocampus, also differ as a function of biological sex. Prepubertal male rats, which typically prefer a striatum-based learning strategy, had a significantly lower ratio of muscarinic receptor binding in the hippocampus compared to the striatum than prepubertal female rats, which do not exhibit a preference for either learning strategy (Grissom et al., 2013). Furthermore, adult male rats, which typically prefer a hippocampus-based place learning strategy, exhibited higher levels of acetylcholine in the hippocampus compared to adult females (Mitsushima et al., 2009). However, this sex difference in adulthood is abolished when females are treated neonatally with either testosterone or its aromatase metabolite, estradiol (Mitsushima et al., 2009), suggesting that acetylcholine release is organized by gonadal hormones in early life. Given evidence that the cholinergic system plays a role in learning strategy preference (Chang and Gold, 2003; McIntyre et al., 2003; Grissom et al., 2013), and that sex differences in the cholinergic system of the hippocampus are modulated by neonatal gonadal hormone exposure (Mitsushima et al., 2009), it was also hypothesized that sex differences in the hippocampus:striatum ratio of muscarinic receptor binding would be eliminated by neonatal treatment of female rats with testosterone.

2. Materials & methods

2.1. Animals

Male and female Long-Evans hooded rats were obtained at 65–70 days of age from Harlan, Inc. (Indianapolis, IN) and mated, yielding 6 litters of pups. If birth occurred before 17:00 h, that day was defined as Postnatal Day 0 (PND 0). Within 24 h of birth, litters were culled to 10–12 pups. Breeder rats and their offspring were housed in a climate-controlled facility under a 12:12-h light/dark cycle (lights on at 07:00 h) with free access to food and water. Pups were weaned at PND 21 and group housed by sex and litter. All procedures were approved by the Tulane University Institutional Animal Care and Use Committee in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (1996). The animal care and use program of Tulane University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2. Neonatal hormone treatment

At 24 h and 48 h after birth female pups were injected subcutaneously at the nape of the neck with either 150 μg (300 μg total) testosterone propionate (n = 9; Steraloids, Inc., Newport, RI) suspended in sesame oil vehicle (Sigma-Aldrich, Inc.), or oil vehicle alone (n = 8), based on previously reported methods (Roof, 1993a, 1993b; Roof and Havens, 1992). Males (n = 13) were similarly administered only oil vehicle following the same procedure. The injection site was sealed with tissue glue (Vetbond, 3M, Inc. St Paul, MN) and pups were injected with India ink (American MasterTech Scientific, Lodi, CA) on the right or left paw to indicate treatment group. Pups were then cleaned and rubbed in litter from the home cage before being returned

to the dam. Due to rejection of pups by the dam following injections, one litter was excluded from this experiment. Anogenital distance was measured when rats were killed to confirm efficacy of hormonal treatment.

2.3. Dual-solution water maze task

Learning strategy preference was assessed using a dual-solution water maze task in accordance with previously validated procedures (Hawley et al., 2011; Grissom et al., 2012, 2013). Specifically, a white circular pool, 180 cm in diameter, was filled to a depth of 26 cm with water made opaque by the addition of non-toxic white tempera paint (Crayola, Inc., Easton, PA), and surrounded by three-dimensional extra-maze cues of varying shapes and sizes. The temperature of the water was maintained at approximately 25 °C. A visible black platform, 9.5 cm in diameter, projected 2 cm above the water surface and was located 30 cm from the wall of the pool. One day prior to testing, at PND 27, rats were placed in the pool without a platform present for a 1-min habituation swim.

Twenty-four hours later, on PND 28, rats were trained on the task with the visible escape platform located in the southwest quadrant of the pool. Eight training trials were conducted with the rat entering from each of four cardinal points in a pseudorandomized order. Each rat was allowed 30 s to mount the platform where it remained for an additional 10 s. If a rat failed to escape using the platform within 30 s it was guided to the location.

Training trials were separated by an inter-trial interval of 5 min during which rats were dried and warmed under heat lamps. Learning was indicated by reduced escape latencies as training progressed. Immediately following the completion of training trials, the visible platform was moved to the opposite quadrant of the pool (northeast) and a probe trial was conducted in which rats entered the pool from the south, at a location most distal from the relocated visible platform. During the probe trial, rats that swam directly to the newly relocated visible platform were categorized as stimulus–response learners and rats that initially returned to within 5 cm of the location of the platform during training trials were classified as place learners by an experimenter blind to their condition (McDonald and White, 1994; Akers and Hamilton, 2007; Grissom et al., 2012, 2013). For all trials, the path length to locate the platform was monitored and recorded by an overhead video camera interfaced with tracking software (HVS Image, Ltd., United Kingdom).

2.4. Receptor autoradiography

In vitro autoradiography for muscarinic receptor binding was conducted according to procedures used in our laboratory previously (Dohanich et al., 1985; Wolff et al., 2008; Grissom et al., 2013). Rats were decapitated 1 h after completion of behavioral testing on PND 28. Brains were removed, frozen with powdered dry ice, and stored at −70 °C. Frozen coronal sections (50 μm) were cut using a microtome cryostat, thaw-mounted on positively charged microscope slides, and stored at −70 °C. Sections were collected through the striatum (corresponding to bregma 1.00 mm through bregma 0.20 mm), dorsal hippocampus (corresponding to bregma 23.00 mm through bregma 24.16 mm) and ventral hippocampus (corresponding to bregma 24.80 mm through bregma 25.30 mm). Immediately prior to incubation, sections were thawed and dried at room temperature. Three matched sections from each area (dorsolateral striatum, dorsal hippocampus, and ventral hippocampus) for each subject were incubated in 10 ml of 50 mM sodium-potassium phosphate buffer (pH 7.4) containing the muscarinic receptor antagonist [3H] quinuclidinyl benzilate (QNB, 51.0 Ci/mmol, Perkin-Elmer,) for 1 h. Incubation medium was then poured off, followed by a cold rinse in fresh buffer for 5 min. Nonspecific binding was determined in parallel incubations containing excess atropine sulfate (1 μM). Dry sections were placed in contact with autoradiographic film (GE Healthcare Biosciences, Pittsburg, PA) stored in X-ray cassettes for 14 days. Autoradiographic film was developed with Kodak D-19 Developer for 2 min, rinsed in distilled water for 30 s, and fixed in Kodak Rapid Fixer for 2 min. Autoradiographs were analyzed by computer-assisted densitometry (MCID Imaging Software, 7.0) separately by two different investigators that were blind to conditions. To control for differences in the size of brain areas measured, the same 20 × 20-pixel area was selected for each brain section and the relative optical density (density × area) was recorded. Relative optical densities were converted to nCi/mg protein by reference to plastic tritium standards containing known quantities of radioactivity (Microscales, Amersham, Buckinghamshire, UK) included on each film sheet. Images from sections incubated with atropine were measured to determine the presence of non-specific receptor binding. As levels of non-specific binding were undetectable, indicating a lack of non-specific binding, optical densities from sections without atropine were analyzed and total binding was used for statistical analysis (Wolff et al., 2008; Grissom et al., 2013). Measurements were normalized by determining the density of each film sheet outside the exposure area to obtain a background value.

2.5. Statistical analyses

An analysis of variance (ANOVA) followed by posthoc testing with a Fisher's Least Significant Differences (LSD) test was used to compare measures of anogenital distance and body weight. An independent samples *t*-test was conducted on uterine weight at the time of death, comparing vehicle and testosterone-treated females. A repeated-measures ANOVA with a within-subjects effect of trial block and a between-subjects effect of treatment was conducted to confirm learning of the visible platform maze over learning trials. Learning strategy was categorized as place or stimulus-response and separate chi-square tests were used to compare learning strategy preference. An ANOVA and post-hoc Fisher's LSD tests were conducted comparing the percent of pathlength in the previously trained quadrant as well to confirm differences in strategy preference. Due to a technical malfunction with the tracking software, four animals were excluded from this analysis.

ANOVA were conducted, comparing binding in areas CA1, CA3, and the dentate gyrus of both the dorsal and ventral portions of the hippocampus, as well as the dorsolateral striatum based on condition [Male (vehicle), Female (vehicle), Female (TP)]. Post hoc analysis using Fisher's LSD test were conducted when appropriate. Given the degree of homogeneity in receptor binding throughout the dorsal and ventral regions of the hippocampus, muscarinic receptor binding across areas of the dorsal and ventral aspects were summed to represent total specific muscarinic receptor binding in the hippocampus. Similar to previously published methods (McIntyre et al., 2003; Grissom et al., 2013) we calculated the ratio of muscarinic receptor binding comparing the hippocampus to the dorsolateral striatum. An ANOVA and Fisher's LSD post hoc tests were conducted to examine the ratio of hippocampus to striatum binding based on treatment condition. Additionally, an independent samples *t*-test was conducted on muscarinic receptor binding in CA1, CA3, and the dentate gyrus of the dorsal and ventral aspects of the hippocampus, the dorsolateral striatum, and the ratio of muscarinic receptor binding in the hippocampus compared to the striatum based on learning strategy preference (place, stimulus-response). To determine effect sizes, Eta squared (η^2) was calculated for all ANOVAs and Cohen's *d* was calculated for all *t*-tests.

3. Results

3.1. Anogenital distance, uterine and body weight

There was a significant effect of treatment on anogenital distance [Fig. 1; $F(2,28) = 16.72$, $p < .01$]. Post hoc analysis indicated that males had a significantly greater anogenital distance than both vehicle-

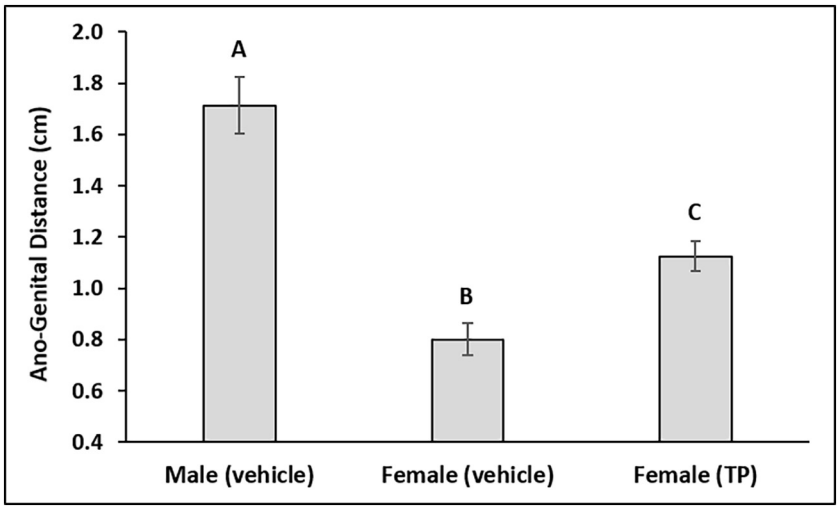

Fig. 1. Anogenital distance of males treated with oil vehicle [Male (vehicle), n = 6], females treated with oil vehicle [Female (vehicle), n = 7] and females treated with testosterone propionate neonatally [Female (TP), n = 8]. Males had the largest anogenital distance, which differed significantly from both groups of females. However, neonatal testosterone treatment significantly increased the anogenital distance in females, compared to vehicle-treated females. (different letters indicate significance at $p < .05$).

treated and TP-treated females [Fig. 1; $p < .05$]. Additionally, the anogenital distance of TP-treated females was significantly greater than in vehicle-treated females [$p < .05$]. Anogenital distance has been demonstrated as a reliable dose-dependent biomarker of testosterone exposure in developing females (Hotchkiss et al., 2007), therefore two females treated with testosterone were excluded from all other analyses as values fell more than two standard deviations from the mean, indicating hormone treatment may not have been fully effective.

There were no differences in measures of uterine weight between vehicle-treated and TP-treated females [data not shown; $t(15) = 0.19$; n.s.]. Body weight was not significantly different between male, vehicle-treated, and TP-treated females [data not shown; $F(2, 28) = 0.37$; n.s.].

3.2. Dual-solution water maze task

Escape latencies decreased significantly across training trial blocks, [Fig. 2; $F(3,78) = 56.67$, $p < .01$], indicating that rats learned the location of the visible platform. There was no main effect of treatment condition [Fig. 2; $F(6,78) = 0.58$, n.s.] indicating that all three treatment groups learned the task equally well. Chi-square tests conducted on learning strategy within each condition indicated that a greater proportion of prepubertal males [Fig. 3B; $\chi^2 = 6.23$, $p < .05$] and TP-treated females [Fig. 3B; $\chi^2 = 5.44$, $p < .05$] used a stimulus-response strategy rather than a place strategy, while a greater proportion of prepubertal vehicle-treated females used a place strategy compared to a stimulus-response strategy [Fig. 3B; $\chi^2 = 4.50$, $p < .05$].

An ANOVA comparing the percent of total pathlength in the previously trained quadrant on the probe trial revealed a significant effect of treatment condition [Fig. 3C; $F(2,16) = 7.22$, $p < .05$]. Post-hoc tests indicated that vehicle-treated females spent a significantly greater portion of their pathlength in the trained quadrant than males [Fig. 3C; $p < .05$] and TP-treated females [Fig. 3C; $p < .05$]. There were no differences in pathlength in the previously trained quadrant between males and TP-treated females [Fig. 3C; n.s.].

3.3. Muscarinic receptor binding

Representative examples of images of the dorsal and ventral hippocampus as well as the striatum can be found in Fig. 4A. Results of the ANOVA based on treatment condition indicated there was a significant difference between groups in the ratio of muscarinic receptor binding in the hippocampus relative to the dorsolateral striatum [Fig. 4B; Table 1; $p < .05$]. Post-hoc analyses indicated that males had lower ratios of muscarinic receptor binding in the total hippocampus relative to the dorsolateral striatum than both vehicle-treated and TP-treated females

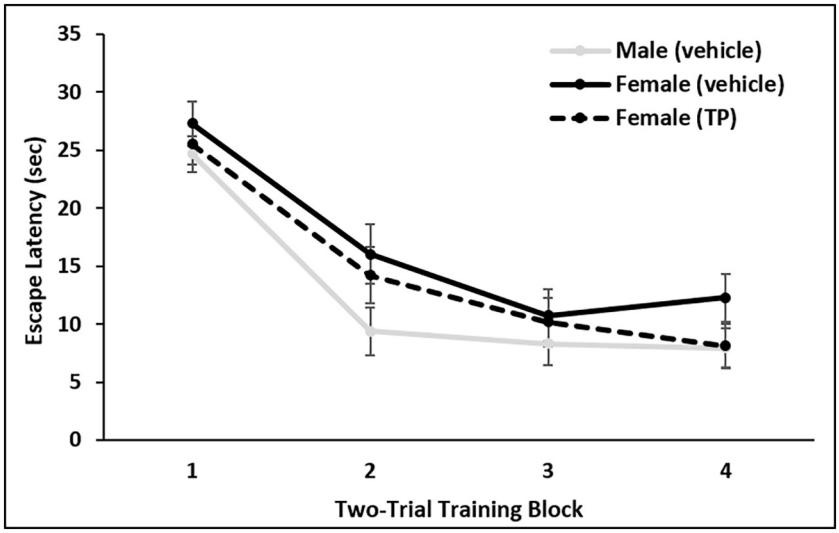

Fig. 2. Regardless treatment condition, prepubertal rats learned to navigate to the visible platform at similar rates as indicated by reductions in escape latencies across training trials.

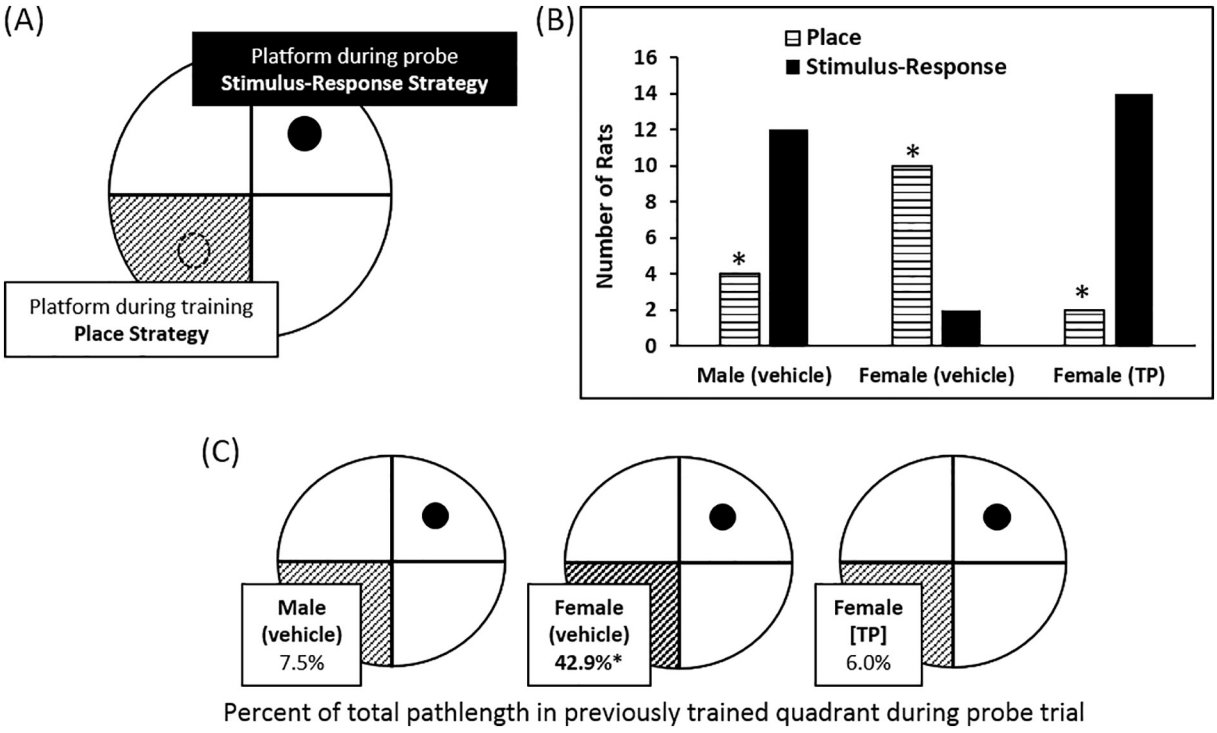

Fig. 3. (A) Rats that swam directly to the newly relocated visible platform during the probe trial were categorized as stimulus-response learners, and those that initially returned to within 5 cm of the location of the platform during training trials were classified as place learners. (B) Males treated neonatally with oil vehicle [Male (vehicle), n = 6], and prepubertal females treated neonatally with testosterone propionate [Female (TP), n = 8] preferred a stimulus-response strategy whereas prepubertal females treated neonatally with oil preferred a place strategy [Female (vehicle), n = 7] (*$p < .05$; stimulus-response versus place). (C) Percentage of escape pathlength on the probe trial spent in the quadrant where the platform was located during training (target quadrant). More time in this quadrant was associated with searching for the platform in the previous location, which was indicative of place strategy. Females treated with oil vehicle [Female (vehicle), n = 7] had a significantly greater percentage of their pathlength in this location than either vehicle-treated males [Male (vehicle), n = 6] or testosterone-treated females [Female (TP), n = 8] (*$p < .05$ vs males and females treated with oil).

[Fig. 4B; $p < .05$]. Table 1 reports the descriptive statistics and results of the ANOVA and post hoc testing conducted on muscarinic receptor binding in specific areas of the dorsal and ventral hippocampus, as well as the dorsolateral striatum.

Results of the independent samples *t*-test comparing muscarinic receptor binding based on learning strategy indicated that stimulus-response learners had lower ratios of muscarinic receptor binding in the total hippocampus relative to the dorsolateral striatum than place learners [Fig. 4C; Table 2; $p < .05$]. Table 2 reports the descriptive statistics and results of the *t*-test for specific areas of the dorsal and ventral hippocampus, as well as the dorsolateral striatum.

4. Discussion

The current results indicate that administration of testosterone to neonatal females impacted the learning strategy preference expressed by females prior to puberty. A significantly higher proportion of prepubertal females treated with testosterone on the first two days of life preferred to use a striatum-based stimulus-response strategy rather than a hippocampus-based place strategy when learning a dual-solution water maze task. The preference for a stimulus-response strategy in testosterone-treated females was identical to that of control males treated with oil vehicle neonatally. Conversely, a significantly higher proportion of prepubertal females treated with oil vehicle neonatally preferred to use a place strategy rather than a stimulus-response strategy when learning the task. Prepubertal rats that preferred a stimulus-response strategy had a lower ratio of muscarinic receptor binding in the hippocampus compared to the striatum than rats that preferred a place strategy, a finding consistent with results reported previously (Grissom et al., 2013). In addition, prepubertal males treated with oil vehicle had a lower ratio of muscarinic receptor binding in the hippocampus compared to the striatum than prepubertal females treated with vehicle, which is also consistent with our previous findings (Grissom et al., 2013). However, in contrast to our prediction, neonatal treatment with testosterone did not abolish this sex difference in muscarinic receptor binding. Specifically, the ratio of muscarinic receptor binding in the hippocampus compared to the striatum was comparable in vehicle and testosterone treated females. Collectively, these results indicate that testosterone treatment in early life modified learning strategy preference without affecting the hippocampus:striatum muscarinic receptor binding ratio.

4.1. Organizational role of gonadal hormones on learning and memory

Only a few reports have documented the effects of early hormone exposure on later cognitive function. Testosterone administered to female rats in early life eliminates sex differences in spatial learning and memory expressed in adulthood (Dawson et al., 1975; Roof and Havens, 1992; Roof, 1993a, 1993b; Isgor and Sengelaub, 2003). The current results in prepubertal rats extends these findings by demonstrating that exposure to testosterone in early life impacts not just *how well* rodents learn, but *how* they learn navigational tasks. Treatment with testosterone shifted learning strategy preference toward a stimulus-response strategy in prepubertal females, completely reversing the learning strategy preference expressed by vehicle-treated females, which preferred to use a place learning strategy. Notably, it is not altogether unexpected that a significant number of females preferred a place strategy given that in two prior experiments (Grissom et al., 2012, 2013) a greater number of prepubertal females used a place strategy rather than a stimulus-response strategy, although this preference failed

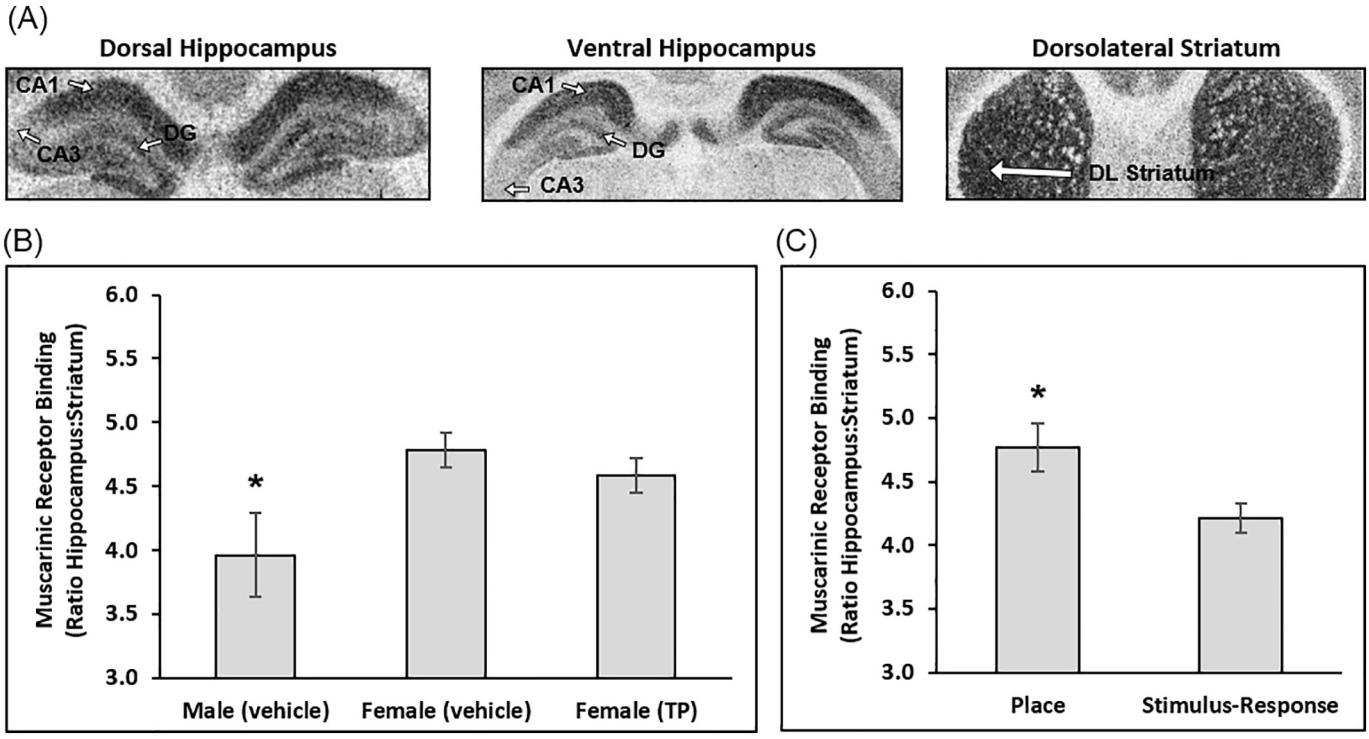

Fig. 4. (A) Representative autoradiographs of muscarinic receptor binding in areas CA1, CA3 and the dentate gyrus (DG) of the dorsal hippocampus and ventral hippocampus as well as the dorsolateral area of the striatum. (B) Prepubertal males treated with oil vehicle [Male (vehicle), n = 6] had a significantly lower ratio of muscarinic receptor binding in total hippocampus relative to the dorsolateral striatum than prepubertal females treated with oil vehicle [Female (vehicle), n = 7], and prepubertal females treated with testosterone propionate [Females (TP), n = 8] (*$p < .05$ vs, female treated with testosterone and female treated with oil). (C) Rats preferring a stimulus-response strategy [n = 12] had a significantly lower ratio of muscarinic receptor binding in total hippocampus relative to the dorsolateral striatum than rats using a place strategy [n = 8] (*$p < .05$).

to reach statistical significance. Taken together, these results indicate that early exposure of prepubertal rats to testosterone results in a greater reliance on a striatum-based stimulus-response learning strategy when learning a dual-solution task that also can be solved by adopting a hippocampus-based place learning strategy.

The training regimen used in the current study, with one day of massed training in the water, has been used in prior studies (Akers and Hamilton, 2007; Grissom et al., 2012, 2013). A shorter training period is necessitated by the small window of time that rats can be tested prior to puberty. Females may enter puberty as early as 32 days of age when levels of estrogens begin to increase, signaling the onset of puberty in rodents (Parker & Parker and Mahesh, 1976). In addition, stimulus-response learning develops earlier than place learning. Therefore, rats cannot be tested using a dual-solution water maze until after 24 days of age (Akers and Hamilton, 2007). With these methodological considerations in mind, it is interesting to consider the possibility that prepubertal male rats in the current study were quicker to switch to a stimulus-response strategy than prepubertal females, an effect that occurs in adult male rats that are tested on dual-solution learning tasks that are motivated by food reward (Packard and McGaugh, 1996).

Whether the organizational effect of early testosterone exposure on learning strategy preference in prepubertal rats is based on the cellular actions of this androgen, or one of its metabolites, is unknown. Previous studies suggest that early treatment with estradiol, a major metabolite

Table 1
ANOVA comparing density of muscarinic receptor binding in the hippocampus (HPC) and dorsolateral striatum (DL striatum) between treatment conditions.

	Male (vehicle) (n = 6)		Female (vehicle) (n = 7)		Female (TP) (n = 8)				
Area	*M*	*SD*	*M*	*SD*	*M*	*SD*	*F*	*p*	η^2
Dorsal hippocampus									
CA1	17.12	1.33	15.72	1.91	15.66	1.95	1.37	0.28	0.13
CA3	10.48	0.83	10.10	1.43	10.71	0.57	0.37	0.69	0.04
Dentate gyrus	15.11	0.91	13.72	1.70	14.40	1.65	1.39	0.28	0.13
Total dorsal HPC	42.71	2.79	39.54	4.75	40.78	4.23	0.98	0.40	0.10
Ventral hippocampus									
CA1	16.86[a]	6.60	22.55 [b]	2.09	22.60 [b]	2.87	4.27*	0.03	0.32
CA3	8.54	3.51	11.55	1.17	11.32	2.02	3.28	0.06	0.27
Dentate gyrus	13.30 [a]	4.97	17.10 [b]	1.95	18.03 [b]	1.74	4.42*	0.03	0.33
Total ventral HPC	38.70 [a]	14.96	51.20 [b]	4.18	51.95 [b]	6.36	4.27*	0.03	0.32
Total HPC	81.41	14.19	90.74	7.35	92.72	9.49	2.19	0.14	0.20
DL striatum	21.12	2.82	19.03	1.51	20.28	1.43	1.94	0.17	0.18
HPC:DL striatum (ratio)	3.91 [a]	0.76	4.78 [b]	0.36	4.57 [b]	0.38	5.06*	0.02	0.36

* $p < .05$; different letters indicate significant differences between groups based on post-hoc tests.

Table 2
Independent *t*-test comparing density of muscarinic receptor binding in the hippocampus (HPC) and dorsolateral striatum (DL striatum) between rats using a stimulus-response or place strategy.

	Stimulus-response (n = 12)		Place (n = 8)				
Area	*M*	*SD*	*M*	*SD*	*t*-Test	*p*	*d*
Dorsal hippocampus							
CA1	16.11	1.80	15.76	1.82	0.42	0.68	0.19
CA3	10.74	1.34	9.99	1.36	1.21	0.24	0.55
Dentate gyrus	14.69	1.46	13.75	1.58	1.37	0.19	0.62
Total dorsal HPC	41.54	3.75	39.51	4.51	1.10	0.29	0.49
Ventral hippocampus							
CA1	20.28	5.77	21.76	2.81	0.67	0.51	0.33
CA3	10.02	3.04	11.36	1.81	1.11	0.28	0.53
Dentate gyrus	15.83	4.11	16.96	2.81	0.68	0.51	0.32
Total ventral HPC	46.14	12.76	50.08	6.96	0.79	0.44	0.38
Total HPC	87.68	12.45	89.59	9.66	0.37	0.72	0.17
DL striatum	21.08	1.56	18.82	2.03	2.82*	0.01	1.25
HPC:DL striatum (ratio)	4.18	0.63	4.77	0.34	2.43*	0.03	1.17

* $p < .05$.

of testosterone, impacted the types of spatial cues used when navigating a radial arm maze (Williams et al., 1990). As adults, males and females treated neonatally with estradiol relied on room geometry to remember the locations of food rewards in the radial arm maze, whereas vehicle-treated females and neonatally castrated males were likely to use both room geometry and landmark cues in the environment. Therefore, in both adult and prepubertal rats, neonatal exposure to gonadal hormones modulates the expression of sex-specific strategies in learning and memory. Given than gonadal hormone levels are low prior to puberty during the time period tested in this study, the activational effects of gonadal hormones on learning strategy preference should be minimal, supporting the hypothesis that sex differences in learning strategy preference are organized by gonadal hormone exposure in early life.

4.2. The cholinergic system and learning strategy

In the current study, rats that used a place strategy had a higher hippocampus:striatum muscarinic receptor binding ratio compared to rats that used a stimulus-response strategy. The lower hippocampus:-striatum ratio in rats using a stimulus-response strategy appears to have been a result of significantly more muscarinic receptor binding in the dorsolateral striatum of rats using a stimulus-response strategy than those using a place strategy, an effect consistent with previous studies (Grissom et al., 2013). These findings are also in agreement with previous reports implicating acetylcholine release in the hippocampus and striatum in the neural regulation of learning strategies (Chang and Gold, 2003; McIntyre et al., 2003), as well as those that have shown that the preference for place learning strategies, relative to response learning strategies, is associated with higher levels of choline acetyltransferase in the hippocampus (Hawley et al. 2015). Although these findings collectively indicate that the cholinergic system is involved in *how* rats learn, other findings have shown that the cholinergic system of the hippocampus and striatum module *how much* information is learned. Specifically, on learning tasks that can only be solved by relying on either the hippocampus-based place strategy or the striatum based response strategy, antagonism of muscarinic receptors in the hippocampus impaired place learning, while antagonism of the receptors in the striatum impaired response learning (Soares et al., 2013). Taken together, these findings support the conclusion that the cholinergic system plays a role in the balance between multiple memory systems that control learning strategy.

In accordance with previous findings, prepubertal females had a significantly higher ratio of muscarinic receptor binding in the hippocampus compared to the striatum than prepubertal males, in part due to significantly more binding in the female ventral hippocampus (Grissom et al., 2013). Although previous research indicated that treating females with testosterone neonatally increased levels of acetylcholine release in the hippocampus of adult females to match that of adult males (Mitsushima et al., 2009), these effects do not appear to extend to the ratio of muscarinic receptors in the hippocampus:striatum prepubertally. Specifically, testosterone treatment shortly after birth did not modify the ratio of muscarinic receptors in the hippocampus relative to the striatum in prepubertal female rat. Though these findings seem to contradict each other, it may be that neonatal hormone treatment in females raises acetylcholine levels without impacting acetylcholine receptors. Additionally, the focus of the current study was to examine muscarinic receptor levels in early life, whereas the sex differences in acetylcholine levels found by Mitsushima et al. (2009) were reported in adulthood.

In adult rats, sex steroids appear to play a role in cholinergic function in both the hippocampus (Shughrue et al., 2000) and striatum (Almey et al., 2012). The cholinergic fibers ascending from the basal forebrain to the hippocampus express estrogen receptors (Gibbs, 1996), though only a small number appear to express androgen receptors (Nakamura et al., 2002). Moreover, in adulthood, testosterone appears to be regulating muscarinic receptor sensitivity given that gonadectomized males are more sensitive to the disruptive effects of the muscarinic antagonist scopolamine than are intact males (Daniel et al., 2003). Whether treatment with testosterone in early life modifies the expression of muscarinic receptors during adulthood in brain areas important for learning and memory remains unclear.

Though sex differences in the ratio of bound muscarinic receptors in the hippocampus:striatum were evident in earlier studies (Grissom et al., 2013), as well as in the current study, it possible that the cholinergic system is acting in concert with other systems that are impacted by exposure to testosterone in early life. The current study examined binding of total muscarinic receptors which included all subtypes (M1-M5). Future studies examining specific muscarinic receptors subtypes, as well as the role of nicotinic receptors, in the hippocampus and striatum would add to the current findings and may provide additional information regarding sex differences in the cholinergic system of the brain during early life as it pertains to learning strategy. Furthermore, other neurotransmitter systems may act in conjunction with, or independently of, the cholinergic system in the hippocampus and striatum to influence learning strategy preference. For example, in addition to acetylcholine, dopamine (Gill and Mizumori, 2006; Lex et al., 2011; Quinlan et al., 2013; Fobbs and Mizumori, 2014), nitric oxide (Kanit et al., 2003), and glutamate (D'Amore et al., 2013) all play a role in learning strategy. Notably, there are sex differences in these systems within the brain (Tanila et al., 1994; Xiang et al., 2011; Hu et al., 2012), which are influenced by changes in levels of gonadal hormones (Davis et al., 2005; Boulware et al., 2013; Khasnavis et al., 2013; Quinlan et al., 2013; Purves-Tyson et al., 2014). Therefore, it is possible that the complete reversal in learning strategy preference as a function of testosterone was due to changes in related neuromodulatory systems.

4.3. Summary

The current study indicates that prior to puberty, learning strategy preference is impacted by exposure to gonadal hormones during a critical organizational period of development. When treated with testosterone shortly after birth, female rats preferred a striatum-based stimulus-response strategy when tested later on a dual-solution water maze prior to puberty. Learning strategy preference in testosterone-treated females mirrored the behavior of prepubertal males and differed from control females that preferred a hippocampus-based place strategy. These results support the hypothesis that sex differences in learning strategy preference are organized by exposure to testosterone

in early life, well before the activational influence of gonadal hormones during puberty and beyond. These findings have important implications for translational research in understanding the development of sex differences in how navigational tasks are learned, and the organizational role of gonadal hormones in learning strategy preference.

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