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Dose-dependent effects of testosterone on spatial learning strategies and brain-derived neurotrophic factor in male rats

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Abstract

Studies suggest that males outperform females on some spatial tasks. This may be due to the effects of sex steroids on spatial strategy preferences. Past experiments with male rats have demonstrated that low doses of testosterone bias them toward a response strategy, whereas high doses of testosterone bias them toward a place strategy. We investigated the effect of different testosterone doses on the ability of male rats to effectively employ these two spatial learning strategies. Furthermore, we quantified concentrations of brain-derived neurotrophic factor (pro-, mature-, and total BDNF) in the prefrontal cortex, hippocampus, and striatum. All rats were bilaterally castrated and assigned to one of three daily injection doses of testosterone propionate (0.125, 0.250, or 0.500 mg/rat) or a control injection of the drug vehicle. Using a plus-maze protocol, we found that a lower testosterone dose (0.125 mg) significantly improved rats' performance on a response task, whereas a higher testosterone dose (0.500 mg) significantly improved rats' performance on a place task. In addition, we found that a low dose of testosterone (0.125 mg) increased total BDNF in the striatum, while a high dose (0.500 mg) increased total BDNF in the hippocampus. Taken altogether, these results suggest that high and low levels of testosterone enhance performance on place and response spatial tasks, respectively, and this effect is associated with changes in BDNF levels within relevant brain regions.

Keywords

testosterone; spatial memory; rat; response strategy; place strategy; BDNF

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Contributors

K.J. Zhang and M.D. Spritzer made significant contributions to the design of this study and to the writing of the manuscript. All co-authors contributed to data collection, data analysis and interpretation, provided input on revisions of this manuscript, and approved the final version of the submitted manuscript.

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Declarations of interest

None.

1. Introduction

A male advantage on certain spatial tasks is among the most well established sex differences in cognitive ability. Many studies have demonstrated that men significantly outperform women in spatial tasks involving route learning (Postma et al., 2004), maze navigation (Astur et al., 1998), and judgment of line angles (Cherney et al., 2008). A similar sex difference in spatial memory has also been documented in rodents (Gresack and Frick, 2003; Keeley et al., 2013; Saucier, 2002). One reason for this prominent sex difference appears to be that males and females use different spatial learning strategies to navigate (Galea and Kimura, 1993). When examining how one learns a task, a distinction can be made between *place* learning and *response* learning, also commonly referred to as allocentric and egocentric learning, respectively (Packard and McGaugh, 1996). While a place strategy relies on using environmental cues to learn a position in space, a response strategy involves learning to execute a certain motor-response based on sequential cues, such as learning to make a turn in one direction during a specific time point of a task (Schmidt et al., 2009). Studies involving lesioning different brain regions in rodents have demonstrated that while the hippocampus mediates place learning, the dorsolateral striatum is primarily responsible for response learning (Packard and McGaugh, 1996). Moreover, in human studies, it seems that when learning a new route, women preferentially rely on a response strategy by noticing and using landmarks, whereas men preferentially rely on a place strategy by understanding and using the Euclidean geometric properties (i.e. angles of the intersections) of the route to orient themselves (Cherney et al., 2008; Silverman and Choi, 2006). A similar sex difference in strategy use has also been demonstrated for rats. In a Morris water maze task, for example, female rats rely heavily on local landmarks to locate the hidden platform (Jonasson et al., 2004), whereas male rats are biased towards using extra-maze cues (Sava and Markus, 2005).

Unlike adult rats, juveniles do not demonstrate a sex difference in the spatial strategies that they use (Kanit et al., 2000). This suggests that certain sex steroids may play an activational role in the spatial strategy each sex chooses to use. In a study involving female rats, Korol et al. (2004) found that when ovarian steroids were high during proestrus, the rats were biased towards using a place strategy, whereas when ovarian steroids were low during estrus, the rats were biased towards using a response strategy. Similarly, ovariectomized female rats that were systemically injected with either a high or low dose of estradiol showed a significant bias towards using a place strategy or response strategy, respectively (Quinlan et al., 2008). These results suggest that in females, fluctuations in ovarian steroids (estradiol) may result in the use of one spatial strategy over the other. Likewise, testosterone has also been documented to play a role in influencing spatial strategy choices in male rats. Spritzer et al. (2013) found that castrated adult male rats receiving a low dose of testosterone (0.125 mg/rat) were biased towards using a response strategy, while castrated rats receiving a higher dose of testosterone (0.500 mg/rat) were biased towards using a place strategy.

The exact mechanism behind how testosterone influences spatial learning has yet to be fully understood. However, androgen receptors have been found to be distributed throughout regions of the brain critical for spatial learning and memory, including the hippocampus (Beyenburg et al., 2000; Moghadami et al., 2016), striatum (Fernandez-Guasti et al., 2003),

and prefrontal cortex (Nuñez et al., 2003), suggesting that testosterone has a direct effect on these brain regions. Furthermore, since both testosterone and brain-derived neurotrophic factor (BDNF) have been implicated in enhancing spatial memory, it has been suggested that an interaction between the two may affect spatial learning (Egan et al., 2003). First isolated and sequenced from the mammalian brain based on its survival-promoting role in neurons of the dorsal root ganglion, BDNF expression has been observed in a variety of mammalian brain regions, including the hippocampus, striatum, and prefrontal cortex (Murer et al., 2001). BDNF is first synthesized as a 32-kDa proneurotrophin (proBDNF), before being subsequently cleaved by a serine protease to produce a 14-kDa mature protein (mBDNF) (Mowla et al., 2001). While proBDNF preferentially interacts with the pan-neurotrophin receptor p75, mBDNF binds and activates the receptor tyrosine kinase trkB (Chao and Bothwell, 2002; Ibáñez, 2002). Furthermore, by activating two distinct receptor systems, studies have suggested that pro- and mBDNF may lead to opposite biological effects (Lu et al., 2005). It is also important to note that there are inconsistent reports regarding the correlation between BDNF and testosterone levels. While some studies have demonstrated a positive correlation between BDNF and testosterone (Li et al., 2012; Shin et al., 2016), other studies have found no significant correlation (Auer et al., 2016; Bimonte-Nelson et al., 2008).

Given that testosterone seems to influence spatial strategy preferences (Spritzer et al., 2013), the present study examined the effect of testosterone in a dose-dependent manner on both place and response learning in adult male rats. Furthermore, given the inconsistent results regarding the correlation between BDNF and testosterone, we tested whether the different doses of testosterone had an effect on both pro- and mature BDNF levels in the cortex, hippocampus, and striatum.

2. Material and methods

2.1 Subjects

Young adult male Fischer 344 rats (2 months old) were obtained from the National Institute of Aging's Aged Rodent Colony (Bethesda, MD, USA). On the first day of testosterone injections, the rats tested on the place task ($n = 37$) had a mass of 225.65 ± 9.38 g, and the rats tested on the response task ($n = 42$) had a mass of 226.86 ± 7.46 g. All rats were pair-housed in opaque, polypropylene cages ($21 \times 42 \times 21$ cm) supplemented with Tek-Fresh paper bedding (Harlan Laboratories, Indianapolis, IN, USA) and a white polyvinyl chloride (PVC) enrichment tube. Rats had free access to water from glass water bottles and a soy-protein-free rodent diet (Harlan Teklad Diet 2020X), except during the food-restriction period of behavioral testing. The housing and testing rooms were maintained at $24 \pm 5^\circ\text{C}$ with a 12:12 h light-dark cycle (lights on at 0700 h EST). All procedures involving animals were approved by the Middlebury College Animal Care and Use Committee and were carried out in accordance with ethical guidelines of the National Institutes of Health.

2.2 Surgery

All rats were bilaterally castrated 5-7 days after arriving at the animal facility. Surgeries were performed with aseptic technique under isoflurane anesthesia (3.5% in oxygen during

induction, 2.0-3.0% during maintenance). Just before surgery, each subject received an s.c. injection of the analgesic Ketofen (5 mg/kg body mass), and an i.m. injection of the antibiotic Cefazolin (40 mg/kg body mass). Each testis was excised through a single incision at the posterior end of the scrotum and ligated with dissolving (polydioxanone) suture material (Ethicon, Somerville, NJ, USA). The muscular sheath was closed with dissolving sutures, and the skin layer was closed with nylon monofilament sutures (CP Medical, Norcross, GA, USA). Immediately following surgery, topical antibiotic was applied to the incision site and an s.c. injection of lactated Ringer's solution (10 mL/kg) was administered to replace fluids lost during surgery. Rats were single housed for 24 h and then administered another injection of Ketofen (5 mg/kg body mass) before being pair-housed for the remainder of the experiments.

2.3 Treatment

For both experiments, the subjects were divided into four groups each (Table 1). The control group received daily s.c. injections of 0.1 mL sesame oil (Oil group), while the three other groups (T groups) received daily s.c. injections of varying concentrations of testosterone propionate (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 mL sesame oil: 0.125 mg/rat, 0.250 mg/rat, or 0.500 mg/rat. At the start of food restriction (first day of injections), there were no significant differences in the rats' body mass between treatment groups ($p = 0.178$) or between the two experiments ($p = 0.943$). Four days before the start of injections, each rat was handled daily for 4-5 min to become acclimated to the researchers. Injections were conducted daily (0800-0930 h) starting 9 days after surgery and seven days prior to maze habituation. Daily injections continued through the morning of euthanasia (12 days total). In previous work, we found that 7 days of preliminary injections were needed to observe effects of testosterone on spatial memory (Spritzer et al., 2011).

2.4 Apparatus

The rats' place and response learning were quantified using a polycarbonate plus maze that was elevated on a table 91 cm above the floor. The maze was painted semi-gloss black and consisted of four arms (40.5×12.7 cm) with 12.5 cm high walls. Each of the arms was arranged at 90° around a 14×14 cm center. For each trial during the habituation and testing phase, one 45 mg dustless dextrose reward pellet (Bio-Serv, Frenchtown, New Jersey, USA) was placed in a small goal cup located 1 cm from the end of each arm. The testing room was illuminated with dim fluorescent overhead lighting (50 lx) and the maze was in the same configuration relative to the room for all habituation and training trials. The researcher was located in the same position within the room during all trials. For the testing trials, but not habituation trails, two large, high contrast posters were mounted on separate walls of the room as extra-maze cues.

2.5 Habituation and behavioral testing

The behavioral protocol consisted of three days of habituation (beginning seven days after starting daily injections) and one day of either place or response testing. These protocols are based on those used by another lab (Korol and Kolo, 2002), with the main change being the addition of maze habituation days, which we have found to be essential for reducing anxiety-like behavior in male rats (Spritzer et al., 2013). All subjects were placed on food

restriction seven days prior to the first day of habituation, and their weights were maintained at approximately 85% of free-feeding body mass until the end of the experiment. Free-feeding reference rats were castrated at the same time as the subjects for each experiment and were used to calculate the expected daily growth rates. Previous work in our lab has shown that seven consecutive days of testosterone at the same doses used for this study had no significant effect on body mass among rats that were not food restricted (Wagner et al., 2018). In order to reduce neophobia, ten reward pellets were placed daily in each cage during the seven days prior to habituation.

For the first day of habituation, each rat was first placed in a holding cage supplemented with bedding for 1 min. The rat was then placed in the center of the maze and allowed to explore for 5 min. Afterwards, the rat was removed from the maze and placed in the holding cage for 15 s before being returned back to its original home cage stocked with 10 reward pellets. On the second day of habituation, two reward pellets were placed along each arm and one pellet in the goal cup (total of 3 pellets per arm). Each rat was placed in the center of the maze and allowed to explore for a maximum of 10 min or until all of the pellets were eaten. Each rat was then placed in the holding cage for 15 s before being returned to its home cage. Finally, on the third day of habituation, one reward pellet was placed at the end of 3 of the 4 arms (no pellet in the start arm). Each rat was placed in the holding cage for 1 min and then placed at the end of the start arm. Rats were allowed to explore the maze for a maximum of 5 min or until one pellet had been consumed. The rat was then placed in the holding cage for 15 s while the maze was randomly rotated one arm to the left or right. The procedure was repeated a total of 8 times, such that each rat was released twice from each of the four start arms in a random order. After the eighth trial, the rat was removed from the maze and placed in the holding cage for 15 s before being returned to its home cage.

For the rats assigned to the one-day of place testing trials, each rat was trained to locate food in one goal arm that was consistent for all trials involving the rat (Fig. 1a). The goal arm location was randomized for each rat, but counter-balanced across rats within each treatment group. For the rats assigned to the one-day of response testing trials, each rat was trained to locate food in one baited goal arm that was consistently in the same direction (left or right) for all of the trials involving that rat (Fig. 1b). The turn direction (left or right) was randomized for each rat, but counter-balanced across rats within each treatment group.

For each block of 20 trials, the release point was also randomized but counter-balanced across arms for each rat. One reward pellet was placed in the goal arm for each trial. Each rat was placed in the holding cage for 1 min before being placed at the end of the start arm and released. Once the rat's hind legs entered an arm, the choice was scored (left turn, right turn, or straight). The trial ended if the rat turned to leave any arm after entering it, made the correct choice and ate the reward pellet, or made the wrong choice and reached the end of the arm. The rat was prevented from entering more than one arm during each trial. Rats were held in a holding cage for about 15 s between trials. To reduce intra-maze cues, the maze was randomly rotated 90° after every 5 trials. If the rat did not move after 2 min, it was placed back in the holding cage for 1 min before the trial was run again from the same start arm. One hundred testing trials were conducted for each rat.

2.6 Tissue collection and processing

To assay circulating testosterone levels, blood samples were collected from all of the rats one day after the testing day. The rats received their respective dose of testosterone in the morning (0800-0930 h), and euthanasia, blood samples, and brain extractions occurred during the afternoon (1200-1300 h) to correspond with the timing of behavioral testing. Each rat received an i.p. injection of a lethal dose (about 120 mg/kg) of sodium pentobarbital (Vortech Pharmaceuticals, Dearborn, Michigan). Approximately 3.0 mL of blood was collected via cardiac puncture and placed into LoBind microcentrifuge tubes (Eppendorf, Hamburg, Germany) on ice. After the blood was allowed to coagulate overnight at 4°C, the samples were centrifuged at 2,000 g for 15 min. The serum was then transferred to 0.5 mL LoBind tubes and stored at -20°C until testosterone ELISAs were completed.

Following blood collection, the rats were immediately decapitated and their brains were rapidly extracted. The whole brain was placed in 0.1 M PBS (pH=7.4) on ice to rinse off some of the blood and to be cooled down. Brain dissection was then carried out on a cold tray to separate left and right slices of the prefrontal cortex, hippocampus, and striatum (i.e., 6 total samples of brain tissue for each rat). Each sample was weighed and placed in a 1.5 mL LoBind tube and then flash frozen and stored in liquid nitrogen. The brain tissue was stored at -80°C freezer until protein extraction occurred. For protein extraction, samples were thawed and diluted with lysis buffer (100 mM Trizma HCl, 150 mM NaCl, 4 mM EDTA, 2% BSA, 2% Triton X-100, 0.5% sodium deoxycholate, 0.1% NaN₃) and a protease inhibitor cocktail (0.5 mM AEBSF, 0.075 µM Aprotinin, 32.5 µM Bestatin, 3.5 µM E-64, 0.25 µM Leupeptin, 0.25 mM EDTA) to a final concentration of 0.05 g/mL. Samples were homogenized using a hand-held homogenizer with sterile pellet pestles for 1-2 min before being centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was decanted and stored at -80°C until BDNF assays were conducted.

2.7 ELISA

Following manufacturer's instructions, blood serum testosterone levels were quantified using Testosterone ELISA kits (MP Biomedicals, Solon, OH, USA). All samples were run in duplicate. The minimum detection level was 0.050 ng/mL. The manufacturer reported low cross-reactivity with androsterone (1.0%), androstenedione (0.89%), dihydrotestosterone (0.86%), and other steroids (<0.05%). Based upon our own assays, the average intra-assay coefficient of variance was 9.19%, and the average inter-assay coefficient was 3.03%. Absorbance was measured at 450 nm by a microplate reader (Biotek, Synergy HT).

Before adding into the ELISA plate, brain protein samples were thawed and centrifuged again for 30 min at 12,000 rpm at 4°C. Following manufacturer's instructions, mature BDNF Rapid ELISA kits (Biosensis, Thebarton, Australia) and proBDNF Rapid ELISA kits (Biosensis, Thebarton, Australia) were used to assay for mature BDNF and proBDNF, respectively. To be within the kit detection ranges, all brain tissue homogenates were diluted by half with additional lysis buffer before analysis. All samples were run in duplicates. The manufacturer reported that the proBDNF kits were 100% reactive with full length proBDNF and did not detect mature BDNF. The mature BDNF kits were reported to have some cross reactivity with proBDNF (5.3% ± 0.5%), and both kits did not cross react with other

neurotrophins. Based upon our own assays, the proBDNF kits, the average intra-assay coefficient of variance was 6.67%, and the average inter-assay coefficient was 5.03%. For the mature BDNF kits, the average intra-assay coefficient of variance was 6.31%, and the average inter-assay coefficient was 4.38%. Absorbance was measured at 450 nm by a microplate reader (Biotek, Synergy HT).

2.8 Statistical analysis

All data analysis was completed using SPSS 25.0 (SPSS INC., Chicago, IL, USA), and a significance value of $\alpha = 0.05$ was used for all tests. For both experiments, task criterion was reached after the rat made 9 out of 10 correct choices. A one-way ANOVA was used to determine if there were any differences in the number of trials needed to reach criterion among the groups. A repeated measures ANOVA was then used to analyze the learning curves in blocks of 10 trials, with treatment as the between-subject factor and testing block as the within-subject factor. We also analyzed each block of 10 trials separately for a treatment effect using univariate ANOVAs. Testosterone levels were compared among groups within each experiment using univariate ANOVA. Total BDNF concentrations were calculated by adding the proBDNF and mBDNF concentrations for each rat. A repeated measures ANOVA was used to analyze proBDNF, mBDNF, and total BDNF concentrations, with treatment group as the between-subjects factor and brain region (prefrontal cortex, hippocampus, striatum) as the within-subjects factor. After this analysis, each brain region was analyzed separately for a treatment effect using univariate ANOVAs. Fisher's LSD was used for all post-hoc analyses, and values are reported as mean $\pm$ SEM. Additionally, Pearson correlations were calculated to determine the relationship between number of trials to criterion for each task and the concentrations of serum testosterone and brain BDNF levels.

3. Results

3.1 Place task

All of the rats from the Oil group had serum testosterone concentrations below the assay's detection limit (0.050 ng/ml), so this group was excluded from further analysis of serum testosterone. The three treatment groups had significantly different serum testosterone levels (Table 1; $F_{2,25} = 17.832$, $p < 0.0005$), and each treatment group differed significantly from one another (all $p < 0.01$).

On the place task, there was a significant effect of testosterone treatment on the number of trials needed to reach criterion (Fig. 2a; $F_{3,33} = 6.938$, $p = 0.001$). Specifically, post-hoc tests showed that rats in the 0.500 mg T group reached criterion in significantly fewer trials compared to rats in the Oil group and the other two testosterone treatment groups (all $p < 0.010$). None of the other groups differed significantly in the number of trials to reach criterion (all $p > 0.498$).

The learning curves for the place task showed that there was a significant block effect, indicating that rats improved on the task over the course of testing (Fig. 3a; $F_{9,297} = 55.988$, $p < 0.0005$). Furthermore, there was a significant treatment effect ($F_{3,33} = 7.161$, $p = 0.001$) and a significant interaction between block and treatment ($F_{27,297} = 2.421$, $p < 0.0005$).

Subsequent univariate ANOVAs within the 10-day blocks indicated that there was a significant difference between treatment groups during blocks 3, 4, 5, 6, 7, 8, and 10 (all $p < 0.03$). The trend within most of these blocks was similar to that observed for trials to reach criterion, with the 0.500 mg T group generally out-performing the other groups. Specifically, the 0.500 mg T group made significantly more correct choices than the Oil group during blocks 3-7 and 10 (all $p < 0.049$). The 0.500 mg T group also made significantly more correct choices than the 0.125 mg T group during block 3 ($p = 0.049$), significantly more correct choices than the 0.250 mg T group in blocks 5, 6, and 8 (all $p < 0.021$), and made significantly more correct choices than both the 0.125 and 0.250 mg T groups during blocks 4 and 7 (all $p < 0.036$). Finally, the 0.125 mg T group made significantly more correct choices than the 0.250 mg T group during blocks 4 and 8 (both $p < 0.042$).

3.2 Response task

All of the rats from the Oil group had serum testosterone concentrations below the assay's detection limit (0.050 ng/ml) except for one sample that was just above the detection limit (0.051 ng/ml). Due to low variance, this group was excluded from further analysis of serum testosterone. The three treatment groups had significantly different serum testosterone concentrations (Table 1; $F_{2,27} = 18.686$, $p < 0.0005$), and each treatment group significantly differed from one another (all $p < 0.05$). Comparing the two experiments, serum testosterone concentration was significantly higher for the 0.250 mg T group among rats used in the place task compared to the response task ($t_{16} = 3.057$, $p = 0.008$). None of the other groups differed between experiments in their serum testosterone concentrations (all $p > 0.07$).

On the response task, there was also a significant effect of treatment on the number of trials needed to reach criterion (Fig. 2b; $F_{3,38} = 5.127$, $p = 0.004$). Specifically, rats in the 0.125 mg T group reached criterion in significantly fewer trials compared to rats in the Oil group and those in the other two testosterone treatment groups (all $p < 0.038$). No other pairwise comparisons indicated significant differences (all $p > 0.189$).

The response learning curves revealed that there was a significant block effect, indicating that the rats improved on the task over the course of testing (Fig. 3b; $F_{9,342} = 98.236$, $p = 0.0005$). There was also a significant treatment effect ($F_{3,38} = 43.243$, $p = 0.002$), but no significant interaction between block and treatment was observed ($p = 0.242$). Subsequent univariate ANOVAs within blocks showed that there were significant differences between treatment groups during block 1 ($F_{3,38} = 3.188$, $p = 0.034$) and block 3 ($F_{3,38} = 5.631$, $p = 0.003$). During block 1, rats in the 0.125 mg T group made significantly more correct choices compared to rats in either the Oil or the 0.500 mg T group (all $p < 0.018$). During block 3, rats in the 0.125 mg T group made significantly more correct choices compared to rats in either the Oil or the 0.500 mg T group (all $p < 0.015$), and rats in the 0.250 mg T group made significantly more correct choices than rats in the 0.500 mg T group ($p = 0.008$).

3.3 BDNF concentration: Place-task experiment

Concentrations of proBDNF differed significantly among the three brain regions (Fig. 4a; $F_{2,66} = 342.870$, $p < 0.0005$). Post-hoc comparisons showed that the hippocampus had significantly higher concentrations of proBDNF than either the cortex or striatum (both $p <$

0.0005), and the cortex had significantly higher concentrations of proBDNF than the striatum ($p < 0.0005$). There was also a significant interaction between brain region and treatment ($F_{6,66}=7.065$, $p < 0.0005$) and a significant main effect of treatment ($F_{3,33} = 3.668$, $p = 0.022$). Subsequent univariate ANOVAs of proBDNF concentrations within each brain region showed that while there was no significant difference between groups in the cortex ($p = 0.694$), there were significant differences between groups in the hippocampus (Fig. 4a; $F_{3,33} = 7.188$, $p = 0.001$) and striatum (Fig. 4a; $F_{3,33} = 4.546$, $p = 0.009$). Specifically, post-hoc tests indicated that proBDNF concentrations were significantly higher in the hippocampus of the 0.500 mg T group compared to each of the other groups (all $p \leq 0.028$). In the striatum, post-hoc tests showed that proBDNF concentrations in both the 0.125 and 0.250 mg T groups were significantly higher compared to the Oil or the 0.500 mg T group (all $p \leq 0.038$).

Similar to proBDNF, there were significant differences of mBDNF concentrations in the three brain regions (Fig. 4b; $F_{2,66} = 251.573$, $p < 0.0005$). Hippocampal mBDNF concentrations were significantly higher than both cortical and striatal mBDNF concentrations (both $p < 0.0005$) and cortical mBDNF concentrations were higher than striatal concentrations ($p = 0.002$). There was also a significant interaction between brain region and treatment ($F_{6,66} = 10.54$, $p < 0.0005$) but no significant main effect of treatment ($p = 0.219$). Subsequent univariate ANOVAs revealed that there was a significant difference in mBDNF concentrations between groups in the hippocampus (Fig. 4b; $F_{3,33} = 8.816$, $p = 0.001$), with the 0.500 mg T and 0.250 mg T groups having significantly higher mBDNF concentrations than the other two groups (all $p \leq 0.035$). There was no significant difference in mBDNF concentrations between groups in the cortex (both $p = 0.44$) and there was a nearly significant difference between treatment groups in the striatum ($p = 0.054$).

Total BDNF concentrations also differed significantly among the three brain regions (Fig. 4c; $F_{2,66} = 548.695$, $p < 0.0005$), with total BDNF concentrations in the hippocampus significantly higher than in either the cortex or striatum (both $p < 0.0005$). Total BDNF concentrations were also significantly higher in the cortex compared to the striatum ($p < 0.0005$). There was also a significant interaction between brain region and treatment group ($F_{6,66} = 14.770$, $p < 0.0005$) and a significant main effect of treatment ($F_{3,33} = 3.359$, $p = 0.030$). Subsequent univariate ANOVAs showed that although there were no significant differences in total BDNF concentrations between treatment groups in the cortex ($p = 0.954$), there was a significant difference in total BDNF concentrations between groups in the hippocampus (Fig. 4c; $F_{3,33} = 11.691$, $p < 0.0005$) and the striatum (Fig. 4c; $F_{3,33} = 5.427$, $p = 0.004$). In the hippocampus, post-hoc tests showed that total BDNF concentration was significantly higher in 0.500 mg T group than in all of the other groups (all $p \leq 0.012$). Also in the hippocampus, total BDNF concentration was significantly higher in the 0.250 mg T group compared to Oil group ($p = 0.015$). In the striatum, post-hoc tests showed that total BDNF was significantly higher in the 0.125 mg T group than in both the Oil group and 0.500 mg T groups ($p \leq 0.002$). Finally, total striatal BDNF concentration was also significantly higher in the 0.250 mg T group than in both the Oil and 0.500 mg T groups (both $p \leq 0.036$).

3.4 BDNF concentration: Response-task experiment

Concentrations of proBDNF differed significantly among the three brain regions (Fig. 5a; $F_{2,76} = 369.057$, $p < 0.0005$) among the rats tested on the response task. Post-hoc comparisons showed that the hippocampus had significantly higher concentrations of proBDNF than either the cortex or striatum (both $p < 0.005$) and the cortex had significantly higher concentrations of proBDNF than the striatum ($p = 0.005$). There was also a significant interaction between brain region and treatment for proBDNF (Fig. 5a; $F_{6,76} = 2.979$, $p = 0.011$) but no significant main effect of treatment ($p = 0.975$). Subsequent univariate ANOVAs revealed that there were no significant differences in proBDNF concentrations between treatment groups within any of the brain regions (all $p > 0.20$).

There were also significantly different concentrations of mBDNF among the three brain regions (Fig. 5b; $F_{2,76} = 215.144$, $p < 0.0005$). Post-hoc tests showed that the hippocampus had significantly higher concentrations of mBDNF than either the cortex or striatum (both $p < 0.0005$), and the cortex had significantly higher concentrations than the striatum ($p = 0.026$). There was a significant interaction between brain region and treatment ($F_{6,76} = 3.777$, $p = 0.002$) but no significant main effect of treatment ($p = 0.510$). Subsequent univariate ANOVAs showed that while there were no significant differences in mBDNF concentrations between treatment groups in either the cortex ($p = 0.536$) or hippocampus ($p = 0.057$), there was a significant difference in mBDNF concentrations between treatment groups in the striatum ($F_{3,38} = 3.161$, $p = 0.036$). Post-hoc tests revealed that mBDNF concentrations were significantly higher in the striatum of the 0.125 mg T group compared to each of the other groups (all $p < 0.041$).

Lastly, total BDNF differed significantly among the three different brain regions (Fig. 5c; $F_{2,76} = 489.334$, $p < 0.0005$). Post-hoc tests showed that the hippocampus had significantly higher concentrations of total BDNF than either the cortex or striatum (both $p < 0.0005$), and the cortex had significantly higher concentrations of total BDNF than the striatum ($p < 0.0005$). For total BDNF concentrations. There was a significant interaction between brain region and treatment ($F_{6,76} = 5.759$, $p < 0.0005$) but no significant main effect of treatment ($p = 0.457$). Subsequent univariate ANOVAs showed that while there were no significant differences in total BDNF concentrations between treatment groups in the cortex ($p = 0.275$), there were significant differences in total BDNF concentrations between groups in the hippocampus ($F_{3,38} = 5.075$, $p = 0.005$) and the striatum ($F_{3,38} = 3.933$, $p = 0.015$). Post-hoc tests showed that in the hippocampus, total BDNF concentration was significantly higher in 0.500 mg T group compared to all of the other groups (all $p < 0.016$). Furthermore, total BDNF concentrations were significantly higher in the striatum of the 0.125 mg T group compared all of the other groups (all $p < 0.043$). This pattern of the 0.500 mg T group having higher total BDNF concentrations in the hippocampus and the 0.125 mg T group having higher total BDNF concentrations in the striatum was similar for both experiments.

3.5 Correlation analyses

Correlation analyses were used to determine which physiological variables were linearly related to performance on each maze task (Table 2). For the place task, rats with higher testosterone reached criterion quicker ($r = -0.498$; $p = 0.002$). This was not the case for

response task ($p = 0.555$), possibly because the relationship seems to be nonlinear (Fig 1b). Both proBDNF ($r = -0.344$; $p = 0.037$) and total BDNF ($r = -0.372$; $p = 0.023$) were significantly correlated with trials to criterion on the place task. In contrast, mBDNF in the striatum was the only BDNF measurement that was significantly correlated with performance on the response task ($r = -0.306$; $p = 0.049$).

4. Discussion

In support of past experiments with male rats (Gibbs and Johnson, 2008; Gibson and Shettleworth, 2005; Spritzer et al., 2013), our results demonstrated that testosterone had a significant dose-dependent effect on both place and response learning. The higher dose of testosterone (0.500 mg/rat) significantly improved rats' performance on a place task, while a lower dose of testosterone (0.125 mg/rat) significantly improved rats' performance on a response task. Furthermore, we found a pattern in which a low dose of testosterone increased total BDNF in the striatum and a high dose increased total BDNF in the hippocampus.

4.1 Testosterone had dose-dependent effects on spatial strategy performance

Rats treated with the 0.125 mg/rat and 0.500 mg/rat testosterone doses reached criterion in significantly fewer trials compared to the Oil group on the response and place task, respectively. Serum testosterone levels for an intact male rat usually range between 1 and 4 ng/mL, but daily peaks between 6 and 15 ng/mL have also been noted (Bartke et al., 1973; Kalra and Kalra, 1977; Mock et al., 1978). It is likely that our daily injections of testosterone resulted in a surge in testosterone comparable to that observed among intact rats, though natural peaks in testosterone tend to occur near the middle of the light cycle rather than near the start (Keating and Tcholakian, 1979). Based on our serum assays, the rats in the 0.125 mg T group had testosterone levels within the typical physiological range, while rats in the 0.500 mg T group represented high physiological levels (Table 1). Thus, our results suggest that the ability of a male rat to effectively employ one spatial strategy over another may change due to the effects of natural fluctuations in circulating testosterone levels.

This hypothesis parallels similar conclusions from past studies that examined the effects of sex steroids on spatial strategy choices made by male and female rats. Spritzer et al. (2013) used the same doses of testosterone as our experiments and found that male rats injected with the 0.125 mg/rat testosterone dose were biased towards using a response strategy, whereas rats injected with the 0.500 mg/rat dose were biased towards using a place strategy. Castrated control male rats did not show a preference for either strategy (Spritzer et al., 2013). Furthermore, another study found that male rats administered a dose of testosterone similar to our 0.125 mg/rat dose performed worse compared to intact males when they were unable to use a response strategy on a working memory version of the radial-arm maze (RAM) (Gibbs and Johnson, 2008). Low levels of estradiol have been shown to bias female rats toward employing a response strategy, while peaks in estradiol bias them toward employing a place strategy (Korol et al., 2004; McElroy and Korol, 2005; Pleil and Williams, 2010). Furthermore, compared to ovariectomized females administered a relatively high dose of estradiol, control ovariectomized females performed worse on spatial tasks that required the use of a place strategy but performed better on tasks that required the

use of a response strategy (Davis et al., 2005; Korol and Kolo, 2002). However, in our experiments, the Oil group did not perform significantly better than any of the testosterone-treated groups when using either strategy. Therefore, it seems that unlike estradiol and its effect on female rats, a minimum threshold of circulating testosterone must be exceeded to significantly improve the ability of males to use one spatial strategy over the other.

On both tasks, rats treated with the 0.250 mg/rat testosterone dose performed at a level similar to the oil-treated rats. This finding corresponds well with data from a recent study from our laboratory that showed on a RAM task that both the 0.125 mg/rat T and 0.500 mg/rat T doses improved spatial working memory in adult rats, while the 0.250 mg/rat T dose did not (Wagner et al., 2018). We found that the 0.250 mg T group was unable to effectively employ either a place or response strategy. While rats in our experiments were forced to use either a place or response strategy on a plus maze, rats on a RAM are free to use either learning strategy to locate rewarded maze arms. Thus, it would be reasonable to hypothesize that in the study by Wagner et al. (2018), the rats in the 0.125 mg T group could have used a striatum-dependent response strategy, while rats in the 0.500 mg T group could have used a hippocampus-dependent place strategy to successfully complete the task. Additionally, the effects of testosterone are not unique to appetitively motivated tasks, as a similar dose-dependent effect of testosterone was observed when an object-location memory task was used (Wagner et al., 2018). Furthermore, these effects seem to persist into old age, as we have recently obtained comparable results on the RAM using aged (20 months) male rats (Jaeger et al., 2020).

It should be acknowledged that the serum testosterone concentrations were found to be significantly lower for rats in the 0.250 mg T group that was used for the response task compared to the rats used for the place task. Because serum was collected only at the end of the experiment, it is unclear whether this difference persisted throughout the experiment, but this does suggest that improved precision in the dosing would be desirable for future experiments. Namely, rats should be dosed per unit body mass rather than per rat.

One explanation for how testosterone impacts spatial strategy performance could be that testosterone, or its metabolites, may have different effects on the hippocampus and striatum. In particular, it could be that the striatum is more sensitive to testosterone than is the hippocampus, which would help explain why we found that the lower dose of testosterone significantly improved a rats' performance on the response task, while the higher dose of testosterone improved rats' performance on the place task. In support of this idea, Feng et al. (2010) found that the distribution of androgen receptors in the brains of male rats was higher in the cells of the striatum than in the hippocampus. Furthermore, low levels of circulating testosterone have also been shown to downregulate androgen receptor expression in the hippocampus of male rats (Moghadami et al., 2016). These studies suggest that low doses of testosterone, such as the 0.125 mg/rat dose that we used, may preferentially activate the striatum over the hippocampus, and thus improve performance on a response task. The physiological mechanism that caused the 0.250 mg T group to perform relatively poorly on both tasks remains unclear, but it seems to fit into the hypothesis that the hippocampus and the striatum represent memory systems that act in competition with each other (Chang and Gold, 2003; Packard and McGaugh, 1996). Injections of estradiol into the hippocampus of

ovariectomized females improved place learning while having no effect on response learning (Zurkovsky et al., 2007, 2006), and injections of estradiol into the dorsal striatum of ovariectomized females had no effect on place learning while impairing response learning (Zurkovsky et al., 2007). It is possible that similar effects are occurring with testosterone in the brains of male rats, and it may be that the 0.250 mg dose of testosterone was sufficient to inhibit striatal activity but insufficient to enhance hippocampal activity. However, the effects in the striatum must be dose-dependent. It remains unclear how a low dose of testosterone (0.125 mg) would enhance striatal activity but a higher dose would not, but perhaps higher doses induced a down-regulation of androgen and/or estrogen receptors.

4.2 Changes in BDNF concentrations

For all of the rats assigned to either the place or response task, testosterone was observed to significantly alter concentrations of BDNF in the hippocampus and striatum in a dose-dependent manner. A number of studies have shown that castration decreases total BDNF levels in the hippocampus of adult male rats (Ebrahimzadeh et al., 2015; Fainanta et al., 2019; Shin et al., 2016). This effect may, however, vary among the sub-regions of the hippocampus — an effect that our assay was too crude to detect. Specifically, castration increased BDNF levels within the mossy fibers extending from the dentate gyrus to the CA3 layer of the hippocampus (Atwi et al., 2016; Skucas et al., 2013), but castration decreased BDNF production within CA1 layer of the hippocampus (Li et al., 2012). Our results support the conclusion that testosterone enhances total hippocampal BDNF, as in both of our experiments rats administered the relatively high dose of testosterone (0.500 mg/rat) had significantly higher concentrations of total BDNF in the hippocampus compared to the castrated control group. We did not find that a high dose of testosterone changed total BDNF concentrations in the striatum or frontal cortex. In fact, for both the place and response experiments, a relatively low dose of testosterone (0.125 mg/rat) led to significantly higher concentrations of total striatal BDNF compared to the Oil group, an effect that was not observed in the hippocampus or frontal cortex. Thus, a high dose of testosterone led to an increase in hippocampal BDNF and a low dose of testosterone led to an increase in striatal BDNF.

We found that rats with the highest concentrations of total BDNF in the striatum performed significantly better in the response experiment, which is a striatum-dependent task (Packard and McGaugh, 1996). Additionally, rats with the highest concentrations of total BDNF in the hippocampus performed significantly better on the place task, which is a hippocampus-dependent task (Packard and McGaugh, 1996). These results suggest that higher BDNF concentrations in the brain region associated with a particular learning strategy may lead to improved performance using that strategy. Furthermore, despite some variation between the place and response experiments, our results also suggest that elevated BDNF may be a mediating factor between testosterone levels and improved spatial memory. These relationships are correlative, and direct manipulation of BDNF or its receptors would be needed to determine if BDNF acts as a critical link between testosterone and spatial memory. Additionally, the effects of testosterone on BDNF seem to be age-dependent. In another recent experiment from our laboratory, we found that testosterone did not have any effects on total BDNF in aged male rats (Jaeger et al., 2020). This suggests that the signaling

pathway between testosterone and BDNF expression may degrade with increased age, and could explain the weakened effects of testosterone upon spatial memory with increased age (Jaeger et al., 2020; Wagner et al., 2018).

While the pattern of total BDNF concentrations was similar for the place and response tasks, there were some minor differences in proBDNF and mBDNF concentrations between the two experiments. For example, on the place task, rats in the 0.500 mg T group had significantly higher concentrations of hippocampal proBDNF, while rats from the response task in the same testosterone treatment group did not have significantly higher concentrations of hippocampal proBDNF but they did have significantly higher concentrations of total BDNF. By binding to different receptors, proBDNF and mBDNF may have divergent biological effects on the brain. Studies have suggested that proBDNF suppresses neuronal excitability (Gibon and Barker, 2017; Woo et al., 2005), while mBDNF promotes neuronal survival, synaptic transmission, and axonal regeneration (Clatterbuck et al., 1994; Sendtner et al., 1992; Yan et al., 1992). However, studies that did not differentiate between the two forms of BDNF, but rather only examined total BDNF, have reported that total BDNF concentrations are significantly higher in rats that have better learning and memory abilities (Ma et al., 1998). This suggests that despite the opposing effects of proBDNF and mBDNF, an increase in total BDNF is still beneficial for learning and memory. This may simply be because the proBDNF in the brain is rapidly converted to mBDNF, and therefore has minimal negative effects on neural plasticity, but little is known about the conversion rate from proBDNF to mBDNF. Interestingly, correlation analyses showed that proBDNF in the hippocampus was more strongly related to performance on the place task than was mBDNF in the hippocampus, whereas mBDNF in the striatum was more strongly related to performance on the response task than was proBDNF in the striatum. Careful manipulation of trkB and p75 receptors would be needed to determine if these difference has any cognitive significance. Regardless, for both of our experiments total BDNF was significantly increased by the high dose of testosterone (0.500 mg/rat) and low dose (0.125 mg/rat) within the hippocampus and striatum, respectively.

Another explanation for our results could be that instead of BDNF concentrations influencing spatial memory, the use of a spatial strategy itself could upregulate BDNF in different brain regions. One previous study demonstrated that BDNF expression in rats remained relatively elevated for up to a week after using a place strategy to solve a task (Harvey et al., 2008). This could explain the minor differences in BDNF concentrations between our two experiments. However, other studies have also found that BDNF mRNA levels decline back to control levels just 6 h after subjects performed a learning a task (Barrientos et al., 2004). Because the rats in our experiments were sacrificed 24 h after completing the maze task, it would be reasonable to assume that changes in BDNF concentrations between treatment groups were not due to any residual maze-training effects, but rather caused by the testosterone injections, which occurred daily until the morning of brain collection. Moreover, the fact that striatal and hippocampal BDNF concentrations exhibited similar patterns between treatment groups in both the place and response experiments also suggests that the task itself did not lead to differences in BDNF levels.

Testosterone may influence BDNF levels in the brain through an androgen- or estrogen-dependent pathway. An estrogen-response element has been well characterized within the *Bdnf* gene (Sohrabji et al., 1995), whereas androgen receptor binding sites within the *Bdnf* gene remain speculative (Wilson et al., 2016). Thus, BDNF may be upregulated in the male hippocampus by estradiol, comparable to the well-documented effects of estradiol on hippocampal BDNF in female rodents (Cavus and Duman, 2003; Singh et al., 1995). Male rat pups castrated at birth showed a down-regulation of BDNF mRNA levels in the hippocampus that was restored by estradiol treatment (Solum and Handa, 2002). Interestingly, this experiment observed no effect of castration or estradiol treatment on *trkB* receptor mRNA in the hippocampus. Similarly, castration had no effect on hippocampal *trkB* levels in adolescent male rats (Allen et al., 2015), suggesting that regardless of the steroidal pathway, our behavioral results are more likely to be the result of BDNF changes rather than changes in *trkB*.

4.3 Conclusion

We demonstrated that a higher dose of testosterone significantly improved a rat's performance on a place task, while a lower dose of testosterone significantly improved performance on a response task. Moreover, a low dose of testosterone increased BDNF concentration in the striatum, while a high dose increased BDNF concentration in the hippocampus. These results suggest that testosterone can affect performance on a spatial task and that this effect may be due to a testosterone-induced increase in BDNF concentrations in the particular brain region required for performing that task. Future work should focus on how testosterone mechanistically effects BDNF concentrations at the molecular level, and whether the observed effects are estrogen-dependent or androgen-dependent.

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Highlights

- A low testosterone dose significantly improved male rats' performance on a response task.
- A high testosterone dose significantly improved male rats' performance on a place task.
- A low testosterone dose significantly increased BDNF levels in the striatum.
- A high testosterone dose significantly increased BDNF levels in the hippocampus.

Fig 1.

Behavioral testing for plus-maze protocol. Rats were trained to complete 100 trials on either the (A) place task or (B) response task. For the place task, rats were trained to locate food in one baited arm that was consistent for all trials involving the rat. For the response task, rats were trained to locate food in one baited goal arm that was consistently in the same direction (e.g., to right) for all of the trials involving that rat.

Fig 2.

Mean trials to criterion ($\pm$ SEM) for rats receiving daily oil or testosterone injections.

Criterion was reached after the rat made 9 out of 10 choices turning in the correct direction.

(A) Among rats tested on the place task, there was a significant effect of treatment on the number of trials to criterion ($p = 0.001$, with the 0.500 mg T group performing better than all other groups). (B) Among rats tested on the response task, there was also a significant effect of treatment on the number of trials to criterion ($p = 0.004$), with the 0.125 mg T group performing better than all other groups. Letters designate groups that differed significantly from each other ($p < 0.05$).

Fig 3.

Mean number of correct arm choices ($\pm$ SEM) during training over every 10-trial block for rats receiving daily oil or testosterone injections. (A) Among rats tested on the place task, there was a significant block effect, a significant treatment effect, and a significant interaction between block and treatment (all $p < 0.0005$). Analyses within blocks revealed that there were significant differences between treatment groups for most of the blocks (*all $p < 0.05$). (B) Among rats tested on the response task, there was a significant block effect and a significant treatment effect (both $p < 0.002$), but no significant interaction effect. However, analyses within blocks revealed that there were significant differences between treatment groups during only blocks 1 and 3 (*all $p < 0.05$).

Fig 4.

Mean ($\pm$ SEM) cortical, hippocampal, and striatal concentrations of (A) proBDNF, (B) mBDNF, and (C) total BDNF for rats injected with oil or testosterone that completed the place task. For all three cases, hippocampal levels were significantly greater than cortical and striatal levels (all $p < 0.0005$). Letters designate groups that differed significantly from each other within each brain region (all $p < 0.05$).

Fig 5.

Mean ($\pm$ SEM) cortical, hippocampal, and striatal concentrations of (A) proBDNF, (B) mBDNF, and (C) total BDNF for rats injected with oil or testosterone that completed the response task. For all three cases, hippocampal levels were significantly greater than cortical and striatal levels (all $p < 0.0005$). Letters designate groups that differed significantly from each other within each brain region (all $p < 0.05$).

Table 1.

Serum testosterone concentrations (mean $\pm$ SEM) from rats collected 4-6 h after testosterone or oil injections on the day after behavioral testing for each experiment.

Task	Treatment	<i>n</i>	Serum Testosterone (ng/ml) *
Place	Oil	9	0.000 $\pm$ 0.000
	0.125 mg T	8	3.425 $\pm$ 0.262
	0.250 mg T	8	7.557 $\pm$ 0.800
	0.500 mg T	12	11.248 $\pm$ 1.109
Response	Oil	12	0.004 $\pm$ 0.004
	0.125 mg T	10	2.451 $\pm$ 0.384
	0.250 mg T	10	4.810 $\pm$ 0.428
	0.500 mg T	10	9.389 $\pm$ 1.291

* For the testosterone-treated groups, serum testosterone concentrations differed significantly from all other treatment groups within each experiment (all $p < 0.05$).

Table 2.

The relationship (Pearson correlation coefficients) between trials to criterion during each task and concentrations of testosterone and BDNF in tissues collected at the end of each experiment.

	Place Task	Response Task
Testosterone (ng/ml)	$r = -0.498$; $p = 0.002^*$	$r = 0.094$; $p = 0.555$
proBDNF in cortex (ng/g)	$r = -0.061$; $p = 0.721$	$r = 0.204$; $p = 0.195$
mBDNF in cortex (ng/g)	$r = 0.226$; $p = 0.179$	$r = -0.064$; $p = 0.689$
Total BDNF in cortex (ng/g)	$r = 0.104$; $p = 0.542$	$r = 0.125$; $p = 0.429$
proBDNF in hippocampus (ng/g)	$r = -0.344$; $p = 0.037^*$	$r = 0.222$; $p = 0.157$
mBDNF in hippocampus (ng/g)	$r = -0.275$; $p = 0.100$	$r = -0.014$; $p = 0.928$
Total BDNF in hippocampus (ng/g)	$r = -0.372$; $p = 0.023^*$	$r = 0.152$; $p = 0.336$
proBDNF in striatum (ng/g)	$r = -0.067$; $p = 0.694$	$r = -0.118$; $p = 0.457$
mBDNF in striatum (ng/g)	$r = 0.110$; $p = 0.516$	$r = -0.306$; $p = 0.049^*$
Total BDNF in striatum (ng/g)	$r = 0.018$; $p = 0.918$	$r = -0.295$; $p = 0.058$

*
 $p < 0.05$