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Effect of Spatial Learning on Hippocampal Testosterone in Intact and Castrated Male Rats

Gisou Mohaddes[1, 2], Nasser Naghdi[*1], Saeed Khamnei[2], Shohreh Khatami[3] and Ali Haeri[4]

[1]Dept. of Physiology and Pharmacology and [3]Dept. of Biochemistry, Pasteur Institute of Iran, Tehran; [2]Applied Drug Research Centre of Tabriz University of Medical Sciences, Tabriz; [4]Dept. of Pharmacology, Shaheed Beheshti University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background: Sex steroids and their receptors exist in hippocampus and affect spatial learning and memory. This study was designed to measure testosterone level of CA1 and to assess the effect of spatial learning on its amount in left and right hippocampus of adult male rats. **Methods:** Sixteen rats were divided into two intact and castrated groups, and then trained in Morris water maze (MWM). Another 40 animals were divided into four groups and their right or left hippocampus cannulated. Half of the animals in each group were castrated simultaneously. All the animals were trained in MWM. Microdialysis was performed and steroid contents of hippocampal dialysate were analyzed through HPLC/ultraviolet detection device method. **Results:** Results showed no significant differences between control and castrated animals in spatial learning after four days of training. Gonadectomy did not change testosterone level in CA1 region of hippocampus. Spatial learning decreased testosterone levels in CA1 region of hippocampus in right hippocampus of the non-castrated group. Significant differences were indicated in testosterone level between left and right hippocampus, in favor of left side in all groups. **Conclusion:** Castration does not affect learning. Testosterone, as a neuromodulator, exists in CA1 region of hippocampus and training can decrease its level only in right hippocampus significantly. Lesser testosterone content of right hippocampus may show the conversion of it to other metabolites. *Iran. Biomed. J. 13 (1): 49-58, 2009*

Keywords: Testosterone, Hippocampus, Spatial learning, Adult rat

INTRODUCTION

The hippocampus, which is involved essentially in learning and memory processes, is known to be a target for the neuromodulatory actions of the steroid hormones. Extensive studies have been performed on the role of steroids in modulating hippocampal plasticity and functions [1, 2].

Although steroid hormones are mainly synthesized in the adrenal glands, the gonads and the feto-placental unit [3], a variety of steroids, can be synthesized in the rodents' brain independently of peripheral glandular sources. Such steroids formed within the brain from cholesterol are defined as neurosteroids. Among them, neuroactive neurosteroids are allosteric modulators of the neurotransmitter receptor activities; hence, regulating different aspects of animal behavior. Particularly, aging process and Alzheimer disease are the beneficial effects of neurosteroids on memory [4].

Estrogens are locally synthesized in the adult hippocampal neurons. In the pathway of steroidogenesis, cholesterol is converted to pregnenolone (by P450scc), dehydroepiandrosterone [by P450 (17α)], androstenediol (by 17β-hydroxysteroid dehydrogenase, 17β-HSD), testosterone (by 3β-HSD) and finally to estradiol (by P450arom) and dihydrotestosterone (by 5α-reductase) [5].

Although changes in neurosteroid levels in adult rodents have been observed in several situations such as circadian and infradian rhythms and heterosexual exposure or stress, their persistence after removal of the adrenals and gonads indicates that the nervous system is permanently exposed to

*Corresponding Author; Tel. & Fax: (+98-21) 6696 8854; E-mail: naghdi@pasteur.ac.ir

neurosteroids. It is amazing that in rodents, the concentrations of neurosteroids are definitely larger in the brain than in plasma [6, 7].

The literature of androgen effects on spatial memory in adult animals and humans is complex and paradoxical. Some evidence suggests a positive correlation between testosterone and spatial cognition [8]. In contrast, several reports indicate that chronic treatment with androgenic compounds impairs spatial learning and retention of spatial information in young and middle-aged animals [9, 10] and humans [11].

A large number of animal studies also support the beneficial effects of estradiol on the functioning and viability of neurons and on learning and memory processes [12].

According to testosterone-mediated effects on learning, this study was designed at first to asses the peripheral steroids effects on spatial learning and memory. Secondly, to measure basal level of testosterone in CA1 region of hippocampus and thirdly, to investigate the effect of spatial learning on hippocampal CA1 region testosterone concentration in adult male rats and fourthly, to compare testosterone level in right and left hippocampus.

MATERIALS AND METHODS

Animals. Male Wistar rats (purchased from Pasteur Institute of Iran, Tehran) weighing about 220-250 g at the time of surgery, were used throughout this study. Before intracerebral cannulation, the animals were housed in groups of five in polyvinyl chloride cages, placed in a well-ventilated environment under controlled temperature 25 ± 2 and humidity 50 ± 5%. The rats were maintained on a 12-h light- dark schedule (lights on at 0700 h) and had free access to food and water. After surgery, rats were placed in individual cages under the same environmental conditions. All experimental procedures were in accordance with the Pasteur Institute Guide for the Care and Use of Laboratory Animals.

Surgery:

Castration surgery. All animals were anesthetized with inhalation of Diethyl ether (Merck, Germany). A horizontal incision was performed in scrotum and the testes were tied off and removed with a cut distal to the ligature, then the incision was sutured.

Stereotaxic surgery. The rats were anesthetized with a mixture of ketamine hydrochloride (Sigma, Germany) and xylazine (ketamine 60 mg/kg, xylazine 12 mg/kg) and mounted in a stereotaxic frame (Stoelting, USA). A midsaggital incision of the skin was made to expose the skull. The bone was cleared with damp cotton buds and a 1-mm diameter hole was drilled in order to insert a guide cannula (Microbiotech 4 Cuprophan, MBA, Sweden). The guide cannula was implanted into the right or left CA1 with the following coordinates: 3.8 mm posterior to the bregma, ± 2.2 laterals to midline and 2.7 ventral to the skull surface [13]. The guide cannula was anchored to the skull with a screw and dental cement. At least a seven-day recovery period was supposed before any other intervention.

Behavioral assessment:

Apparatus. The water maze was a black circular pool with a diameter of 136 cm and a height of 60 cm, filled with water (20 ± 1°C) to a depth of 20 cm. The maze was divided geographically into four equal quadrants: northeast, northwest, southeast, and southwest and release points that were equally designed at each quadrant as north, east, south, and west around the perimeter of the tank. A hidden circular platform (10 cm in diameter), made by Plexiglas, was located in the center of the southwest quadrant, submerged 1.5 cm beneath the surface of the water. Fixed, extra maze visual cues were presented at various locations around the maze (i.e. computer, Morris water maze (MWM) hard wares, posters). An infrared camera (Canon, Japan) was mounted above the center of the maze. An infrared LED was attached to each rat as a probe so that the animal motion can be recorded and sent to the computer. A tracking system was used to measure the escape latency, traveled distance and swimming speed.

Procedure. The animals were placed in the water facing the wall of the pool at one of the four designated starting points (north, east, south, and west) randomly and allowed to swim and find the hidden platform. Each of four starting positions was used once in a series of four trials. The rats were given a maximum of 90 s to find the platform and if it failed to find the platform in 90 s, it was placed on the platform and allowed to rest on it for 30 s. During the first 4 days, the platform position remained constant, in the south west quadrant. On day 5, the platform was elevated above water

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surface, marked by aluminum foil and placed in the south east quadrant [14].

Microdialysis. A MAB/4 probe (MAB 4.15.1.Cu, <6000 kDa, Stockholm, Sweden) was inserted into the guide cannula. A 30-cm piece of PE-50 tubing was attached to a swivel (Eicom, Japan). On the day before training, the animals were placed in a Plexiglas cage to move freely for 30 minutes and first dialysate sample was collected. The second sample was collected on the fourth day immediately after MWM test. All experiments were performed at the same time of the day. Microdialysis probes were perfused with a constant flow rate at 5 µl/min [15] of artificial cerebrospinal fluid (aCSF) (102 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.67 mM NaH_2PO_4, and 0.3 mM Na_2HPO_4, Glucose, pH 7.4) by using a microinfusion pump (WPI, sp 210 iw, USA). Sample was collected for 90 min. At the end of the sampling, microdialysate was capped and stored at -20°C until analysis (30 days later). All brains were examined to ensure that there was no extensive bleeding around the brain probe.

Testosterone measurement:

HPLC/ultraviolet detection device (UVD). A HPLC method with ultraviolet spectrometric detection (UVD) was developed for the rapid and simultaneous measurement of CA1 region of hippocampus testosterone in dialysate. The HPLC set up consisted of a HPLC pump (Waters 510, USA), an injector (Waters, USA), C18 (ODS) column (a 5-µm particle, size 4.6 × 250 mm, and guard column (Teknokroma, Spain), UVD (Waters 486, Tunable Absorbance Detector, USA) and an integrator (Waters 746, USA). Testosterone chromatography was performed on a C18-column with an eluent of water-methanol-tetrahydrofuran (volume ratio 55:35:10, pH 4.0, Merck, Germany) and was detected by UVD at 245 nm [16]. Testosterone standard and corticosterone (as internal standard) were purchased from Sigma, Germany.

Histology. Following behavioral and microdialysis testings, animals were deeply anesthetized with diethyl ether and sacrificed by decapitation, then the brains were removed. For histological examination of guide canula and microdialysis probe placement in CA1 area, a 100-µm thick section was taken and mounted on slides. Slides were stained with cresyl violet and the guide canula and probe track were examined for each rat (Fig. 1). Only those animals whose canulae were exactly placed in CA1 region were used for data analysis.

Experiments:

Experiment 1. A) Sixteen animals were divided into intact and castrated groups and tested behaviorally. All animals were tested for 5 days and all runs began at 9:00 a.m. Each animal received four trials during four daily acquisition sessions. B) Behavioral tests were performed in another 40 rats and they were divided into four groups and nominated as following: Right hippocampus cannulated and non-castrated (RNC), Right hippocampus cannulated and castrated (RC), Left hippocampus cannulated and non-castrated (LNC), Left hippocampus cannulated and castrated (LC).

Experiment 2. Microdialysis tests were performed two times in the same groups of behavioral tests: 1) on the first day of training before procedure and 2) on the fourth day after training in MWM. Microdialysis samples were collected in four animals in each group.

Statistical analysis. Statistical analysis was performed using Student's *t*-test for comparing serum and hippocampal testosterone level between groups. All spatial learning data over training days from hidden and visible platform tests were analyzed by one-way ANOVA, followed by post-hoc analysis using Tukey's honestly significant difference for assessing differences between specific groups. All results were shown as means ± S.E.M. In all comparisons, $P<0.05$ was considered significant.

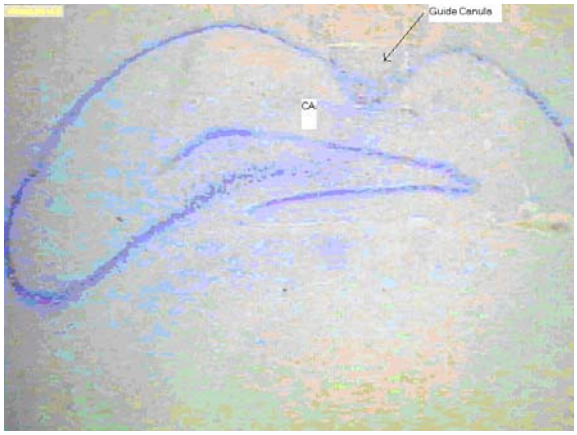

Fig. 1. Nissl-stained coronal brain section from cannulated and microdialysed rats. Cannula position is shown (magnified in Photoshop).

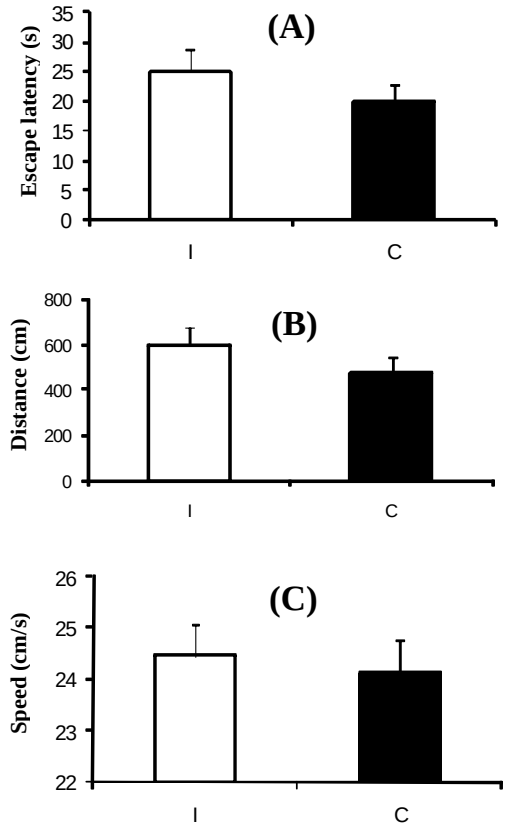

Fig. 2. Mean of escape latency **(A)**, distance **(B)** and swimming speed **(C)** in intact (I) and castrated (C) rats to find hidden platform. There is no significant difference between groups.

RESULTS

Experiment 1:

A) Spatial learning (days 1-4). The groups mean escape latency, traveled distance and swim speed to reach the hidden platform in the water maze by intact and castrated groups (Fig. 2) and RNC, RC, LNC and LC groups (Fig. 3) are shown. There were no significant differences among groups during first four days of training. Figure 4 shows the traveled distance (A) and escape latency (B) were significantly decreased in all groups at the 4th day of training as compared to the first day (*P<0.05) but there are no significant differences in swimming speed (C).

B) Visible platform trials (day 5). There was no significant difference in performance among the groups for latency, distance and speed on 5th day of study.

Experiment 2:

A) Hippocampal testosterone measurement after castration. Castration did not change testosterone level in CA1 region of hippocampus as compared to the non-castrated (control) group. Figure 5 illustrates the mean testosterone level of right and left CA1 in castrated and non-castrated rats before and after training. There were no significant differences between groups.

B)Relationship between hippocampal testosterone and spatial learning. According to the results, testosterone level decreased in all experimental groups after training, but it was significant just in right hippocampus of NC group. As indicated in Figure 6, testosterone level of right CA1 in non-castrated rats decreased significantly after training (P<0.01).

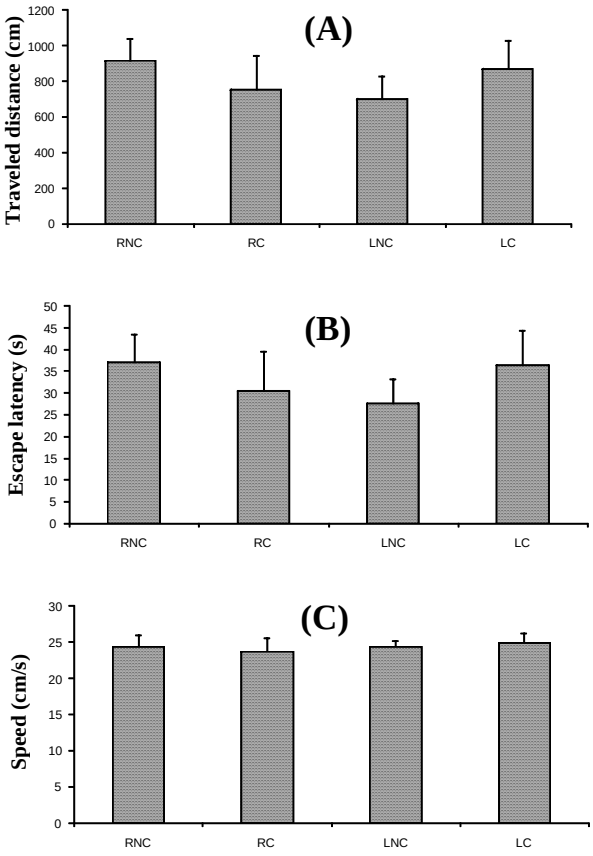

Fig. 3. Average traveled distance **(A)**, escape latency **(B)** and swimming speed **(C)** across all training days. There are no significant differences between groups. Right hippocampus cannulated and non-castrated (RNC), Right hippocampus cannulated and castrated (RC), Left hippocampus cannulated and non-castrated (LNC) and Left hippocampus cannulated and castrated (LC).

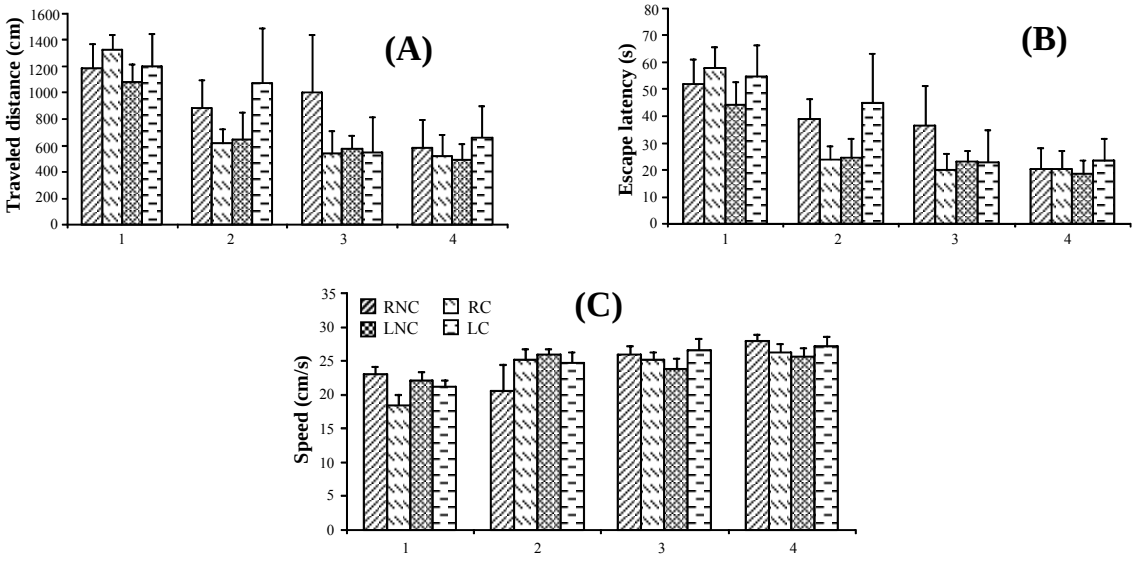

Fig. 4. Comparison of traveled distance **(A)**, escape latency **(B)** and swimming speed **(C)** during same training days within different groups. The figures show the escape latency and traveled distance significantly decreased in all groups at the 4th day of training compared to the first day (**P*<0.05). Right hippocampus cannulated and non-castrated (RNC), Right hippocampus cannulated and castrated (RC), Left hippocampus cannulated and non-castrated (LNC) and Left hippocampus cannulated and castrated (LC).

C) Comparison of right and left testosterone content. Figure 6 shows that mean testosterone level of the left CA1 was significantly higher than that of right side in non-castrated rats before training (*P*<0.001). This preference in testosterone level of left hippocampus was also seen in this group after training (*P*<0.05, Fig. 6). The mean testosterone level of right and left CA1 before training in castrated rats is shown in Figure 6. The levels of testosterone were significantly higher in the left side (*P*<0.05). As Figure 6 illustrates, the preference was also seen in this group after training (*P*<0.001). Therefore, the mean testosterone level of left hippocampus was higher in all experimental groups.

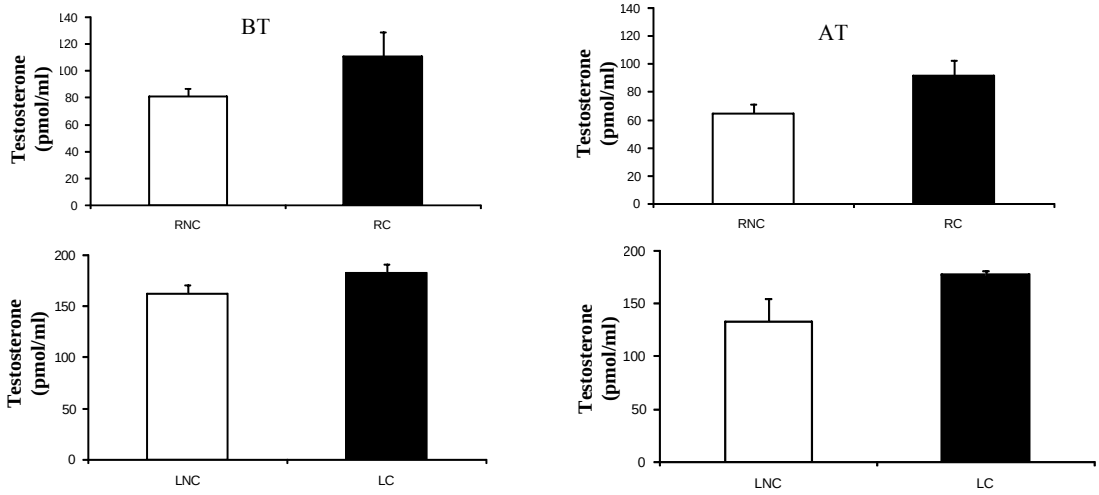

Fig. 5. The Figure illustrates the mean testosterone level of right and left CA1 in castrated and noncastrated rats before (BT) and after (AT) training. Data are shown as mean ± SEM. There is no significant difference between groups. Right hippocampus cannulated and non-castrated (RNC), Right hippocampus cannulated and castrated (RC), Left hippocampus cannulated and non-castrated (LNC), Left hippocampus cannulated and castrated (LC).

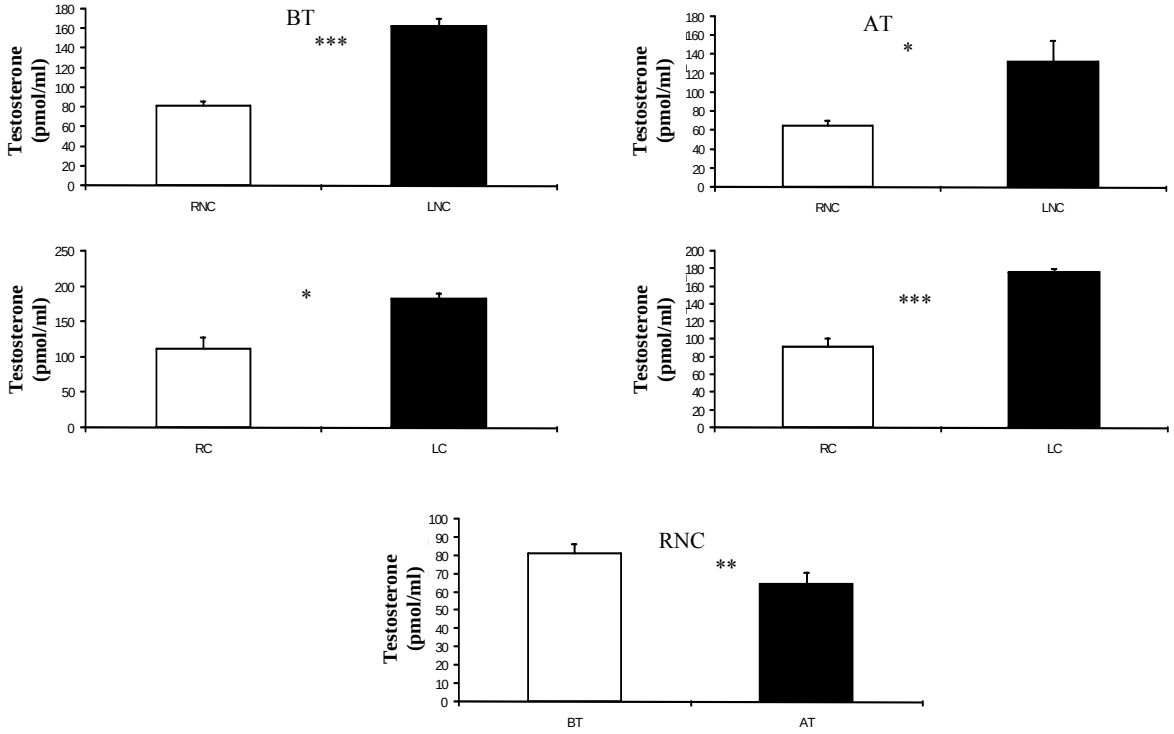

Fig. 6. The Figure represents the mean testosterone level of right CA1 in noncastrated rats before (BT) and After (AT) training (left upper panel), mean testosterone level of right and left CA1 in noncastrated rats before (BT) (right upper panel), mean testosterone level of right and left CA1 in noncastrated rats after (AT) (left middle panel), mean testosterone level of right and left CA1 in castrated rats before (BT) (right middle panel), mean testosterone level of right and left CA1 of castrated rats after (AT) (lower panel). Right hippocampus cannulated and non-castrated (RNC), Right hippocampus cannulated and castrated (RC), Left hippocampus cannulated and non-castrated (LNC), Left hippocampus cannulated and castrated (LC). Data are shown as mean ± SEM. *significant difference (P<0.05), ** significant difference (P<0.01), *** significant difference (P<0.001).

DISCUSSION

Spatial learning and memory. Findings showed that adult castration did not cause alterations in the spatial learning although castration omits the endogenously derived testosterone from sexual gonads [17]; hence, adult circulating levels of androgens do not seem to critically affect escape training. These findings are in agreement with those from previous studies where adult castration of males was shown to have no significant effect on spatial ability as compared to intact controls [18-20]. These findings suggest that gonadal steroids could not be critical in spatial localization of adult male rats.

The literature reflecting effects of systemic androgens on learning and memory are controversial. Numerous studies have examined sex differences and/or the effects of perturbing the gonadal hormonal environment on different aspects and types of learning and memory. These studies report contradictory results.

Destructive effects of androgens on MWM tasks in rats treated with testosterone have been shown. For example, one study showed the effect of testosterone on spatial memory acquisition in intact adult rats and reported that both young and middle age rats display impaired memory retention after testosterone administration [10]. On the contrary, some investigators have been unable to induce a deficit in either the acquisition or retention of the water maze task after chronic treatment with either testosterone propionate or administration of the anabolic-androgenic-steroid cocktail containing testosterone cypionate for 10 weeks [21]. Interestingly, it has been shown that supplementary testosterone administration in the older men had a facilitative effect on spatial memory [8]. Recently, Cherrier *et al.* [22] showed that short-term testosterone administration enhances cognitive function in healthy older men. While there are a growing number of studies addressing roles of gonadal

steroids in mediating and/or modulating spatial learning and memory and also other types of learning and memory processes, the use of different animal strains, behavioral tasks, type of apparatus, and hormone treatment across studies may account for some of the current inconsistencies in findings.

Since there were no statistical differences between the control and experimental groups on the 5th day of training in which the platform was visible; it can be inferred that there were no alterations in non-mnemonic factors such as motivational, motor, and sensory processes.

Hippocampal testosterone quantification. In this work, the first finding is that hippocampal concentration of testosterone in castrated and non-castrated rats was not significantly different and it is also shown that testosterone content of hippocampus was more than plasma. We suppose that testosterone as a neurosteroid, exists in CA1 region of hippocampus, it can be produced there or it may be transported to hippocampus from other brain regions.

Findings of the present study support the findings of Hojo *et al.* [2] and Shibuya *et al.* [23] and that hippocampal pyramidal neurons and granule neurons are equipped with complete machinery for the synthesis of testosterone and 17β-estradiol.

In rodents, concentrations of neurosteroids are definitely larger in the brain than in plasma [7]. For instance, the basal concentration of estradiol in hippocampus is approximately 1 n*M*, which was greater than that in blood plasma [5]. Changes in neurosteroid levels in adult rodents have been observed in several situations, such as circadian and infradian rhythms and heterosexual exposure or stress as well as their persistence after removal of the adrenal glands and gonads indicates that the nervous system is permanently exposed to neurosteroids [7].

The hippocampus has been shown to be critically involved in learning and memory processes [18, 24]. Given the utmost importance of the hippocampus in spatial learning and the concentration of androgen binding sites in CA1 area [25], it would be interesting to question whether testosterone concentration changes after spatial learning through this brain region.

In order to answer this question, concentration of testosterone was measured in both the right and left hippocampal CA1 regions before and after the spatial learning in castrated and non-castrated male rats. According to the results, testosterone level decreased significantly in right CA1 region after spatial learning in RNC group.

In a previous report of our laboratory, it has shown that in hormonally intact adult rats, intrahippocampal injection of testosterone impaired spatial performance. Intriguingly, when flutamide, an androgen receptor antagonist, was injected into the CA1 region, it also resulted in an impaired acquisition of the MWM task [24].

Taken together the decrease of testosterone level after spatial learning and deteriorative effect of testosterone administration, some possibilities were speculated to explain mechanisms underlying testosterone decrement after spatial learning.

In addition to direct steroidal actions, the metabolism of steroids in brain tissues can result in biotransformation and the production of biologically active metabolites [26]. Hippocampal testosterone might have been metabolized to estradiol. Then, the metabolite affects spatial performance since the blockade of androgen receptors in the hippocampus resulted in deficit of the memory task [24].

Neural aromatization of testosterone to estrogen is responsible for effects on the structure and function of the songbird hippocampus. It may accelerate the acquisition of a spatial memory task and increase the size of neurons in the rostral hippocampus of songbird. Neural aromatization of testosterone to estrogen is responsible for effects on the structure and function of songbird hippocampus [27].

In addition, the hippocampus, which is involved in spatial abilities and declarative memory, contains both testosterone receptors and estrogen receptors; therefore, testosterone may have direct effects on the hippocampus through androgen receptors as well as indirect effects from aromatization to estradiol interacting with estradiol receptors [22].

Indeed, androgenic action in the vertebrate brain is often mediated by the enzymatic activity of cytochrome P450arom that catalyzes the conversion of androgen to estrogen [26]. Both P450arom [2] and estrogen [28] are expressed in hippocampus.

Although steroids are classically thought to act via intracellular steroid hormone receptors that bind to DNA to modify gene expression, neurosteroids have been also shown to have a direct effect on ligand-gated ion channels of neuronal membranes. These receptor-active neurosteroids may represent an important class of neuromodulators, which act via novel non-genomic mechanisms. In particular, their action on GABAA receptors has often been involved in numerous responses, including memory performances [7, 29].

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Estradiol also rapidly modulates neuronal signal transduction and the induction of long-term potentiation via N-methyl D-aspartate (NMDA) receptors and putative neuron communication, thereby mediating in learning and memory [23].

Some electrophysiological and binding studies have shown androgen-mediated changes in NMDA sensitivity and NMDA receptor number in hippocampal CA1 pyramidal cells [14]. Estrogen also enhances NMDA receptor-mediated currents and promotes an enhancement of long term potentiation magnitude [30].

Finally, this study demonstrated that testosterone level in the left hippocampus is significantly greater than right side. It is accepted that the two halves of human brain are structurally, functionally and behaviorally are different [31]. Studies on postmortem brains have revealed chemical asymmetries in thalamus and temporal lobes. Asymmetrical distribution has been observed for glutamic acid carboxylase, gamma-amino butyric acid, choline acetyltransferase (ChAT) and dopamine. There is also strong evidence for asymmetrical distribution of ChAT in the temporal lobes. Acetylcholine is an important neuro-transmitter which is considered to play a key role in intellectual activity including memory [32].

In functional view, selective (but not exclusive) spatial memory impairment is associated with right temporal lobe damage that is related to the integrity of the hippocampal functioning [33]. While left temporal lobe contributes to performance on tasks requiring verbal learning and memory, the right temporal lobe is implicated in tasks requiring memory for items that are difficult to verbalize, such as visuospatial materials [34].

In addition, Wolf and Kirschbaum [35] have shown there is a negative correlation between testosterone and verbal fluency in men but in women, higher estradiol levels as well as testosterone levels were associated with better verbal memory.

It seems that enhanced enzymatic activity might be responsible for more conversion of testosterone to its metabolites in right hippocampus that results in less testosterone concentration.

In summary: 1) Elimination of systemic testosterone by castration did not affect spatial performance. 2) Testosterone exists in CA1 region of adult male rats' hippocampus. 3) Castration has no effect on testosterone level in CA1 region of adult male rats' hippocampus. 4) The spatial learning and memory has no effect on testosterone level in CA1 region of rat's hippocampus while in right side they cause the decrease of testosterone. 5) According to our knowledge, this is the first report that testosterone content of left CA1 is more than that of the side in intact and castrated rat's, before and after spatial learning.

These findings suggest that testosterone may affect spatial learning and memory indirectly through conversion to its metabolites such as estradiol. The lower amount of right hippocampus' testosterone content may be the result of higher conversion of testosterone to its metabolites in comparison to that happening in the left hippocampus.

To reveal the type of testosterone's metabolites and its conversion rate in CA1 region of hippocampus, further investigations must be done.

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