

GABA_b Receptor Antagonist (CGP₃₅₃₄₈) Improves Testosterone Induced Spatial Acquisition Impairment in Adult Male Rat

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Abstract

The high density of the androgen receptors in fundamental centers of learning and memory, such as hippocampus, shows that there must be some relationship between the androgen receptors and cognitive aspects. On the other hand, Gama Amino Butric Acid (GABA) plays a controlling role in the balance of excitability and inhibitory states in the cortex and hippocampus; a number of reports suggest that removal of the influence of inhibitory GABA receptors lead to memory enhancement and conversely the activation lead to memory inhibition. Sex steroids can rapidly influence neural activity by increasing the binding affinity of neurotransmitters such as GABAergic. To determine the effect of Testosterone on learning and memory in CA₁ region of hippocampus, male albino Wistar rats (200 - 250 g) are bilaterally cannulated into CA₁ of hippocampus then different doses of Testosterone enanthate or CGP₃₅₃₄₈ are injected through the cannulae for assessing of acquisition, consolidation and retrieval in a single-day testing protocol of Morris water maze task. After hippocampal microinjection with Testosterone (T), acquisition is significantly impaired, while after treatment with CGP₃₅₃₄₈, acquisition impairment caused by T can be significantly improved. Also T and CGP₃₅₃₄₈ have no significant effect on consolidation and retrieval stages of spatial memory. These results suggest that CGP₃₅₃₄₈ may have therapeutic value in the treatment of Testosterone-induced acquisition impairment.

Keywords

Spatial Learning and Memory, Hippocampus, Testosterone, CGP₃₅₃₄₈, Morris Water Maze

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1. Introduction

The hippocampus has been shown to be necessary for several types of learning and memory formation in rats and other mammals [1]. The hippocampus may serve several different functions; a collective body of data from maze navigation and electrophysiological lesion studies repeatedly indicates that the hippocampus significantly contributes to the processing of spatial information [2]. Spatial learning means the ability of the animal to locate a particular place by using spatial cues. Researchers have suggested that high levels of androgens may adversely affect memory in laboratory animals and humans [3]. The literature of androgen effects on spatial memory in adult animals and humans is complex and contradictory. Some evidence suggests a positive correlation between Testosterone and spatial ability [4] [5]. In contrast, several reports indicate that this compound has impaired spatial learning and retention of spatial information in adult animals [6] [7]. Previous studies reveal relatively high levels of androgen receptors (AR) in the rat's hippocampus which shows there may be some relationships between androgen receptors and cognitive aspects of brain, however, the action of androgenic remains unclear [8]. Androgenic initiate many of these effects by specifically binding to AR in the cytoplasm.

The androgen-induced reduction of CAILTP found in previous study [9] was consistent with earlier reports that Testosterone, possibly via the AR, down regulated the NMDA receptor in CA₁ in the rat [10]. An organizational role for androgens in the reduction of NMDA-mediated synaptic plasticity in another species had been demonstrated [11].

Since GABA plays a controlling role in the balance of excitability and inhibitory states in hippocampus, a number of reports suggest that removal of the influence of inhibitory GABA receptors lead to memory enhancement and conversely the activation lead to memory inhibition [10] [12]. However, other results have reported the opposite, where GABAergic antagonists inject into the striatum or substantianigra produced amnesia [12]. GABA_B receptor agonists can inhibit the release of various neurotransmitters by inhibiting Ca⁺ channels or activating K⁺ channels, while GABA receptor antagonists can increase the release of neurotransmitters[13] [14].

There are important reciprocal relationships between brain steroid hormone and GABAergic system. Sex steroids can rapidly influence neural activity by increasing the binding affinity of neurotransmitters or by directly altering cell membrane ion conductance in the hippocampus. Neurosteroids can be positive and negative endogenous modulators of GABA receptors [15] [16]. Exogenous Testosterone depresses plasma levels of both gonadotropins and androgen precursors such as dehydroepiandrosteron (DHEA) and its sulfate (DHEAS). DHEAS can activate an allosteric site on the GABA receptor that inhibits the chloride channel opening and thus increases neuronal excitability.

Testosterone appears to exert little regulatory control over GABA receptor subunit. Considering the data given above, we conduct a series of experiments to investigate the role of GABA_b receptor antagonist in CA₁ region on spatial learning and memory and find relation between Testosterone and GABA receptors on effect of T on spatial learning.

2. Materials and Methods

2.1. Animals

Male albino Wistar rats (200 - 250 g, aged 10 - 12 week) were obtained from Pasteur institute of Iran. They were housed in a temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and humidity-controlled room. The animals were maintained under a 12:12h light/dark cycles with lights off at 7:00 p.m. Food and water were available ad libitum. These animal experiments were carried out in accordance with recommendations from the declaration of Helsinki and internationally accepted principles for the use of experimental animals.

2.2. Surgical Procedure

Approximately 7 - 8 days prior to initiation of the behavioral experiments, the rats were anesthetized with intraperitoneal (i.p) injection of a mixture of ketamine and xylazine (100 and 25 mg/kg) [17] [18] and then operated on to implant guide cannulas bilaterally at a site immediately above the CA₁ (AP: -3.8 mm from bregma; ML: ± 2.2 mm from midline; and DV: -2.7 mm from the skull surface) on the Paxinos and Watson's atlas of the rat brain. Two screws were inserted into the skull and cannulae fixed to them with dental cement.

2.3. Microinjection Procedure

Intra hippocampal injection was made via guide cannulae with injection needles (27-gauge) that were connected by polyethylene tubing to 10- μl Hamilton microsyringe. The injection needle was inserted 0.5 mm beyond the tip of the cannula and 0.5 μl vehicle different doses of Testosterone or CGP₃₅₃₄₈ were injected 3 - 4 min. The needle was left in place for another 60 s before it was slowly withdrawn.

2.4. Behavioral Testing

2.4.1. Morris Water Maze Apparatus

The water maze used has been described extensively. Briefly, it consisted of a dark circular pool (140 cm in diameter and 55 cm high) filled with water ($20^\circ\text{C} \pm 1^\circ\text{C}$) to a depth of 25 cm. A transparent Plexiglas platform (11 cm diameter) was located 1 cm below the water surface in the center of the arbitrarily designed north-east (NE), south-east (SE), south-west (SW) or north-west (NW) orthogonal quadrant. The platform provided the only escape from the water. Many extra-maze cues such as racks, a window, a door, bookshelves and picture on the walls surrounded the room where the water maze was housed. These were kept in fixed positions with respect to the swimming pool to allow the rat locate the escape platform hidden below the water surface. The position of the animal was monitored by a camera that was mounted above the center of the pool. The rats marked by a LED display in a ping-pong ball that was held on the rats back by a rubber jacket. The power supply of the LED display was inserted to thin counterbalanced cable. The camera signal was digitized and fed to a computerized tracking system that monitored and stored the position of the rat every 100 ms, thus the time required reaching the platform (latency) and the swimming speed were recorded as were as the time spent in the target quadrant [19] [20].

2.4.2. Training Protocol

The single training session consisted of eight trials with four different starting positions that were equally distributed around the perimeter of the maze. Each rat was placed in the water facing the wall of the tank at one of the four designated starting points (north, east, south, and west) and allowed to swim and find the hidden platform located in the SW quadrant (target quadrant) of the maze. Each of four starting positions was used twice in eight training sessions; their order was randomized. During each trial, each rat was given 90 s to find the hidden platform. After mounting the platform, the animals allowed to remain there for 20 s, then start next trial. After completion of training, the animals returned to their home cages until the retention testing (probe trial) 24 h later. In the probe trial the hidden platform was removed and the animal was released from the north location and allowed to swim freely for 60 s. After the probe trial, the platform was elevated above the water surface and placed in the different position (SE quadrant).

The water maze performance is subject to the influences of other non-spatial learning factors such as the sensory, motivational, emotional, or motor functions of the tested subjects. We took the following measures to ensure that the effects of T and CGP₃₅₃₄₈ were not produced by non-spatial learning factors. First, we examined the recorded swimming speed of the rats. Intra-hippocampal applications of the drugs failed to alter the swimming speed of the rats. Second, we tested the performance of the rats' microinjected with Saline, DMSO, CGP₃₅₃₄₈ and T in a visible platform water maze in which no learning is involved. There were no significant differences for the latencies to find the visible platform among control, CGP₃₅₃₄₈ and T-treated rats.

2.5. Histology

Following behavioral testing, animals were sacrificed and brains were removed and stored in 10% formalin. For histological examination of cannulae and needle placement in the CA₁ region, 100 μm thick sections were taken. Only those animals whose cannulae were exactly placed in the CA₁ region were used for data analysis (Figure 1) [21].

2.6. Experimental Protocol

2.6.1. CGP₃₅₃₄₈ or Testosterone Microinjection

1) Acquisition assessment

The aim of this experiment was to determine the effect of bilateral pre-training injection of Testosterone and

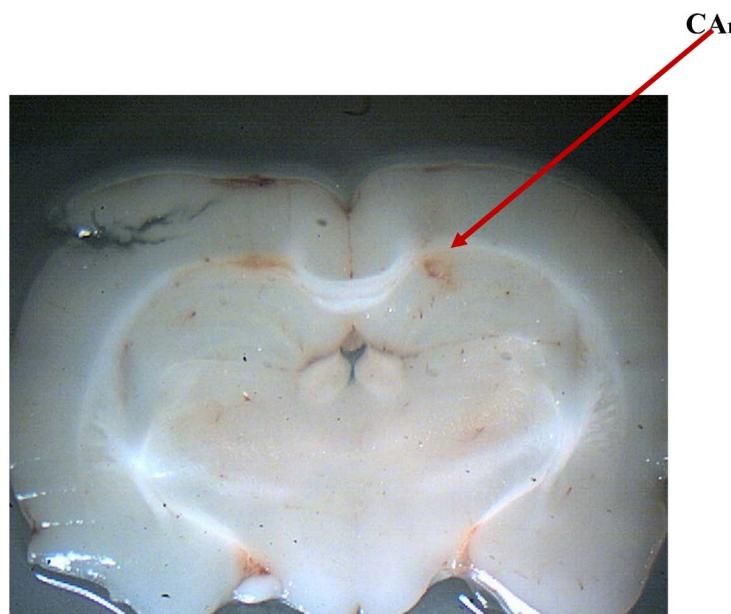


Figure 1. Representative photomicrograph of an infusion site into the rathippocampal CA₁ area. The figure shows the site of infusion in the dorsal hippocampus with the arrowhead pointing to the infusion cannula tract.

CGP₃₅₃₄₈ into CA₁ region on acquisition of spatial memory. 49 rats were divided into seven groups ($n = 7$), three groups received bilateral injection of T into CA₁, 30 min before training with doses 20,40, 80 µg/0.5µl/side [22], and the control animals were injected with 0.5 µl DMSO. In three groups (each group 7 rats) received vehicle (Saline) or CGP₃₅₃₄₈ (2.5 and 5 µg/0.05µl/side) [23], 15 min before training in MWM.

2) Consolidation assessment

The aim of this experiment was to determine the effect of bilateral post-training administration of Testosterone and CGP₃₅₃₄₈ into CA₁ region on consolidation of spatial memory. 32 rats were randomly divided into two control ($n = 8$) and three drug groups ($n = 8$). The rats were trained in two blocks of eight trials within 1 day. Immediately after the final trial, three groups received bilateral injection of T (20, 40 and 80 µg/0.05µl/side) and the control group was injected with a similar volume of DMSO. 24 rats were divided in to three groups ($n = 8$) and received vehicle (Saline) and doses of CGP (2.5 and 5 µg/0.5µl/side) immediately after the last trial. Memory retrieval for the location of the platform was tested 24 h later using a 60 s free swims probe trial.

3) Retrieval assessment

In this experiment the effect of pre-probe injection of Testosterone and CGP₃₅₃₄₈ on retrieval of spatial memory was assessed. On day 2, 56 rats were divided to DMSO group as control ($n = 8$), three drug groups received T (20, 40, 80 µg/0.05µl/side) ($n = 8$) 30 min before probe trial and three groups received CGP₃₅₃₄₈ (2.5 and 5 µg/0.05µl/side) ($n = 8$) and one group as control received Saline (0.05 µl) ($n = 8$) 15min before test on retrieval of spatial memory.

2.6.2. CGP₃₅₃₄₈ + Testosterone

1) Acquisition assessment

The aim of this experiment was to determine the effect of bilateral pre-training injections CGP₃₅₃₄₈ plus Testosterone into CA₁ region of hippocampus. A total of 16 rats were divided into two groups and received 5 µg/0.5µl/side CGP₃₅₃₄₈ + 80 µg/0.5µl/side T respectively or Saline + DMSO as a vehicle with a similar volume with 15 min gap between them, then training test began after a delay 30 min.

2) Consolidation assessment

The aim of this experiment was to determine the effect of bilateral post-training administration of CGP₃₅₃₄₈ plus Testosterone into CA₁ region. 18 rats with cannulas aimed at the bilateral CA₁ areas were trained in two blocks of four trials in one day. Immediately after the final trial, the rats were randomly divided into two groups and received bilateral injections of 5 µg/0.5µl/side CGP₃₅₃₄₈ + 80 µg/0.5µl/side T respectively or Saline + DMSO as a vehicle with a similar volume. Memory retrieval for the location of the platform was tested 24 h lat-

er using 60 s free swim probe trial.

3) Retrieval assessment

The aim of this experiment was to examine the effect of bilateral pre-probe trial administration CGP₃₅₃₄₈ lus T into CA₁ region. 18 rats trained in two blocks of four trials in training day. On day 5, rats were randomly divided into two groups. Then 25 min before the probe trial they received intra-CA₁ injection of 5 µg/0.5µl/side CGP + 80 µg/0.5µl/side T with 15 min gap between them, respectively Saline + DMSO as a vehicle with a similar volume.

2.7. Data Analysis

Data obtained over training from hidden platform tests, probe trials and visible platform tests were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparison. All results have been shown as means ± S.E.M. In statistical comparisons, $P < 0.05$ considered as significant difference.

3. Results

3.1. The Effect of Testosterone

3.1.1. Acquisition

Figures 2(a)-(c) showed that T in dose 80 µg could significantly increase the escape latency ($F = 7, P < 0.005$), traveled distance ($F = 3.2, P < 0.03$), But no significant differences in swimming speed ($F = 2.08, P < 0.06$) compared with sham operated group. Also there was no significant difference of performance among the groups on visible platform day for escape latency ($F = 1.6, P < 0.19$) and travelled distance ($F = 0.22, P < 0.8$).

3.1.2. Consolidation of Spatial Memory

In this experiment results obtained from the injection of T or DMSO immediately after training session. There was no significant differences in number entrance of animal to target quadrant ($F = 0.27, P < 0.8$). There was no significant difference in time spent in target quadrant ($F = 2.2, P < 0.1$), traveled distance in target quadrant ($F = 1.6, P < 0.1$) in probe test, also no significant difference of performance among the groups on the visible platform day in escape latency ($F = 0.6, P < 0.5$) and travelled distance ($F = 0.36, P < 0.7$).

3.1.3. Retrieval

In this experiment results obtained from the injection of T or DMSO were injected 30 min before probe trial. There was no significant difference in number entrance animal to target quadrant ($F = 0.6, P < 0.7$), time spent in target quadrant ($F = 0.8, P < 0.5$) and traveled distance in this quadrant ($F = 0.6, P < 0.7$) comparing with sham operated group. Also there was no significant difference of performance among the groups on the visible platform in escape latency ($F = 2.6, P < 0.06$) and travelled distance ($F = 2.6, P < 0.6$).

3.2. The Effect of CGP₃₅₃₄₈

3.2.1. Acquisition

Figure 3 showed results obtained from the injection CGP₃₅₃₄₈ and Saline as a vehicle on acquisition of spatial learning and memory. A significant difference was found in escape latency ($F = 3.04, P < 0.001$) but no had seen in traveled distance ($F = 0.56, P < 0.5$) and swimming speed ($F = 0.2, P < 0.9$) (**Figures 3(a)-(c)**). Also results obtained from injection of CGP₃₅₃₄₈ on visuo-motor coordination in visible platform showed no significant difference of performance among the groups for escape latency ($F = 0.1, P < 0.8$) and traveled distance ($F = 0.2, P < 0.8$).

3.2.2. Consolidation

The results obtained from injection of CGP₃₅₃₄₈ or Saline immediately after training session was shown that CGP₃₅₃₄₈ in dose 5 µg had no significantly difference the number entrance animal to target quadrant ($F = 0.7, P < 0.4$) comparing with sham operated group. Time spent in target quadrant ($F = 0.4, P < 0.6$), traveled distance in this quadrant ($F = 0.9, P < 0.3$). Results obtained on visuo-motor coordination in visible platform test showed no significant difference of performance among the groups for escape latency ($F = 0.6, P < 0.5$) and traveled distance ($F = 1.2, P < 0.2$).

3.2.3. Retrieval

The results obtained from the injection of CGP₃₅₃₄₈ 15 min before probe trial was generally found no significant

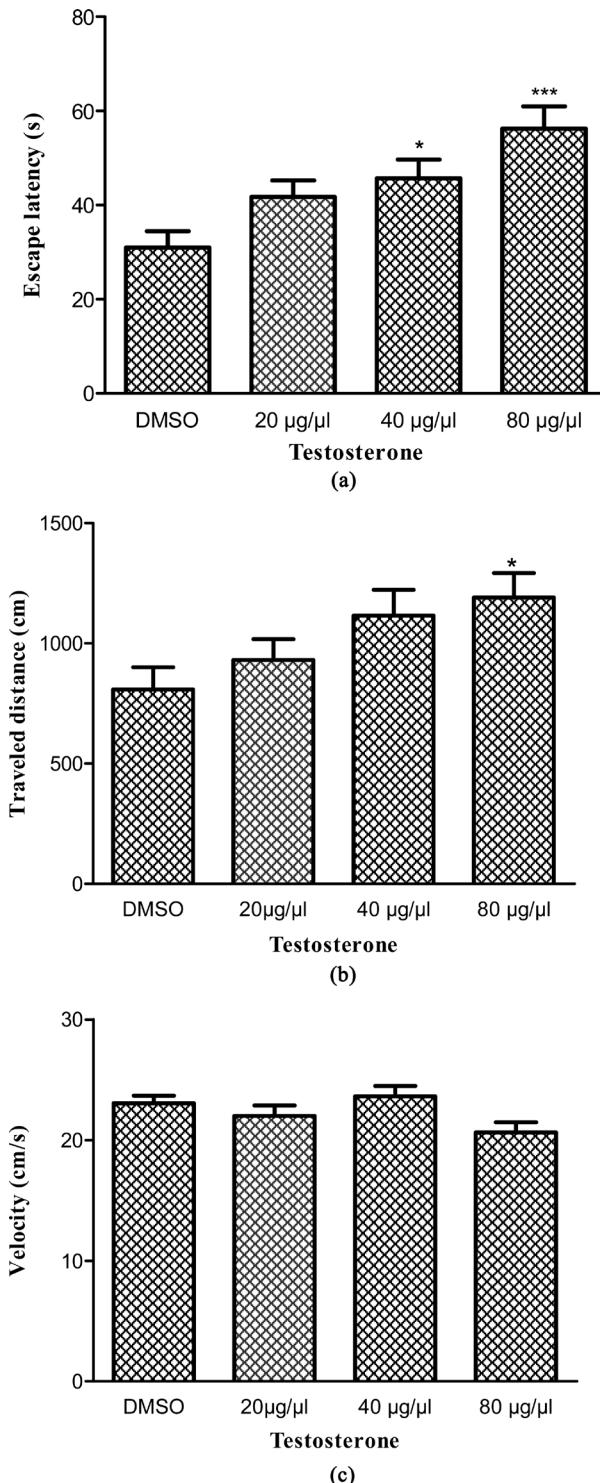


Figure 2. Average escape latency (a), traveled distance (b) and swimming speed (c) in training day. There are significant difference ($^{**}P < 0.005$ and $^{*}P < 0.03$) in escape latency (a) and traveled distance (b) of Testosterone treated groups in compare to control (DMSO) group, but there is no significant difference on swimming speed (c) between groups in compare to control group. Data are presented as the mean (\pm S.E.M.).

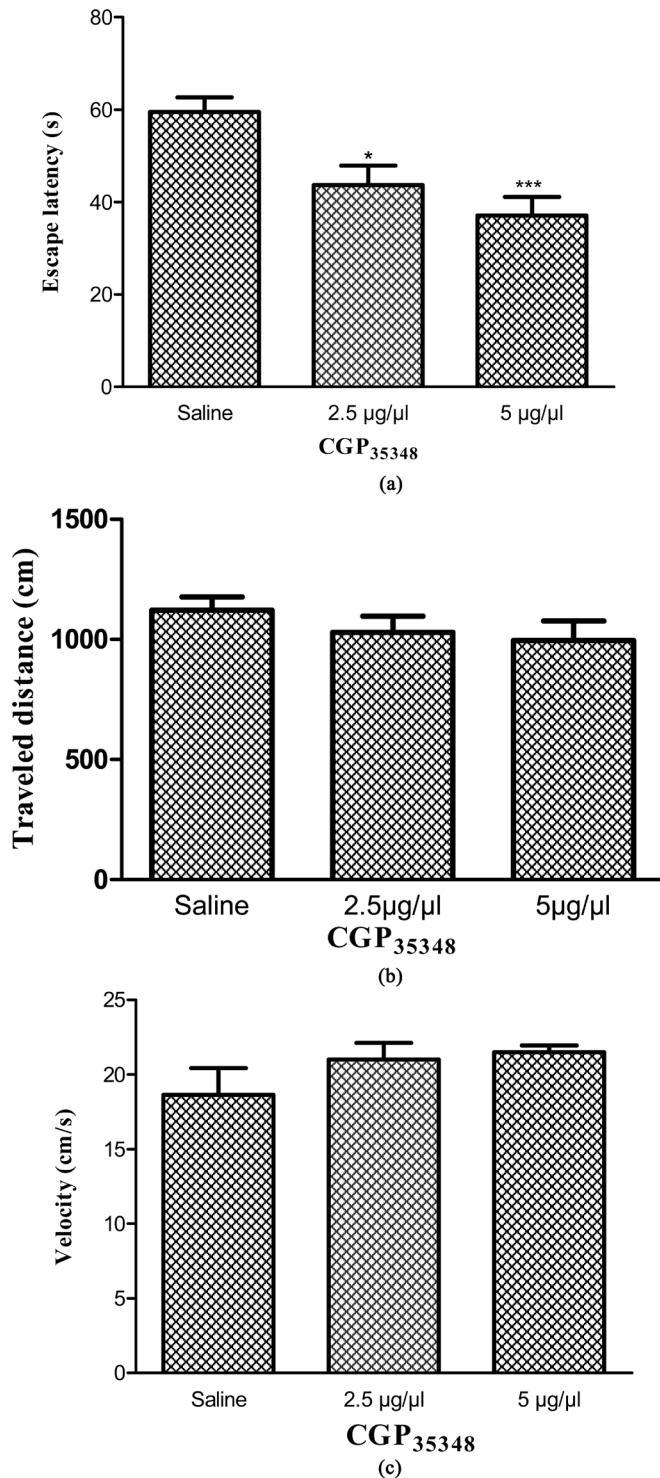


Figure 3. Average escape latency (a), traveled distance (b) and swimming speed (c). Figures show a significant difference in escape latency **P < 0.001 between CGP₃₅₃₄₈ (5 µg/0.5µl) as compare the control group.

difference in number entrance animal to target quadrant ($F = 0.9, P < 0.4$) , time spend in target quadrant ($F = 1.2, P < 0.2$), traveled distance in this quadrant ($F = 1.8, P < 0.1$) comparing with sham operated group. Results obtained from injection of CGP₃₅₃₄₈ on visuo-motor coordination showed no significant difference of performance among the groups for escape latency ($F = 1.1, P < 0.9$) and traveled distance ($F = 2.1, P < 0.14$).

3.3. The Effect of CGP 35348 + Testosterone

3.3.1. Acquisition

Figure 4 show results obtained from the injection CGP₃₅₃₄₈ 5 µg/0.5µl/side + Testosterone 80 µg/0.5µl/side or Saline + DMSO on acquisition in spatial learning and memory. A significant difference was generally found in escape latency ($F = 3$, $P < 0.006$) but not seen in traveled distance ($F = 3.2$, $P < 0.08$) between groups in hidden

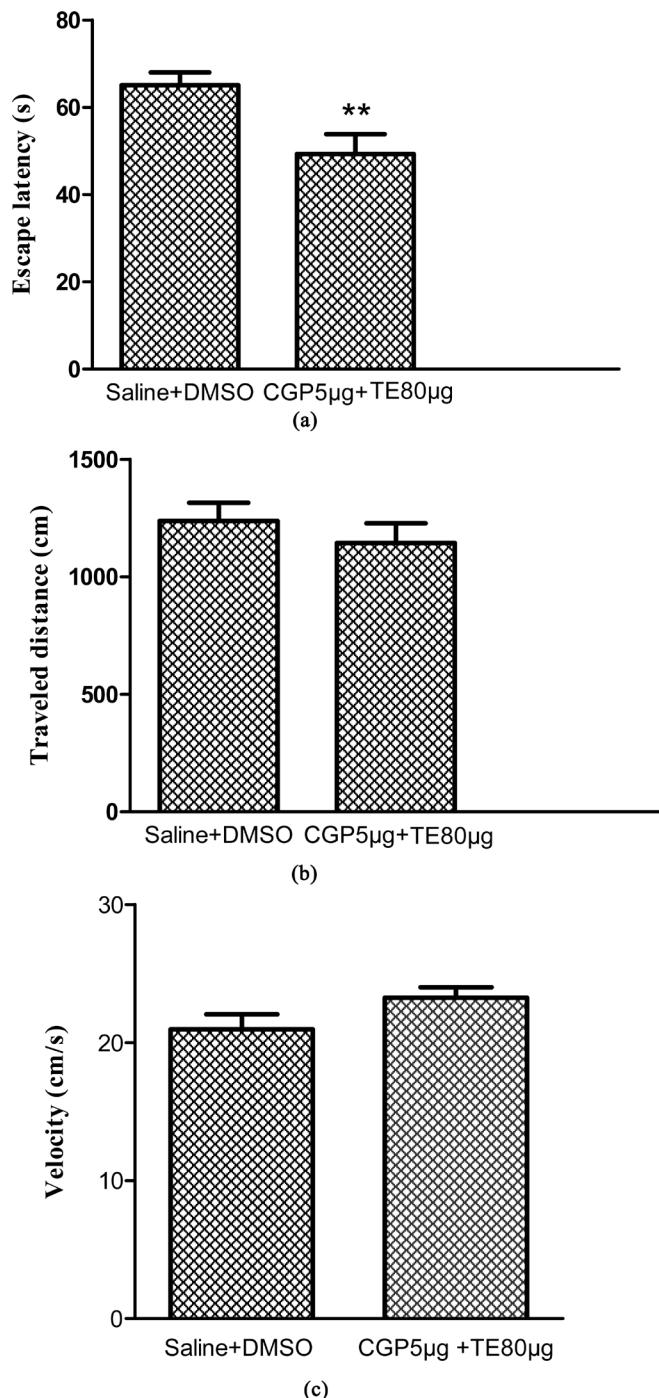


Figure 4. Average escape latency (a), traveled distance (b) and swimming speed (c) in Saline + DMSO (vehicle), CGP₃₅₃₄₈ + Testosterone (5 µg/0.5µl + 80µg/0.5µl/side) groups. * $p < 0.05$ shows a significant difference between treated groups with vehicle in the escape latency. Data are presented as the mean (\pm S.E.M).

platform trials there was no significant differences in swimming speed ($F = 4.8, P < 0.2$) (**Figures 3(a)-(c)**).

Results obtained from the injection of CGP₃₅₃₄₈ + T on visuo-motor coordination on visible platform showed no significant difference among the groups for escape latency ($F = 0.9, P < 0.1$) and traveled distance ($F = 0.7, P < 0.7$).

3.3.2. Consolidation

There was no significant differences in number entrance animal to target quadrant ($F = 0.01, P < 0.9$), time spend in target quadrant ($F = 0.17, P < 0.3$), traveled distance in target quadrant ($F = 0.1, P < 0.6$) between treated groups and sham operated group. There was no significant difference of performance among the groups on visible platform day for escape latency ($F = 0.8, P < 0.1$) traveled distance ($F = 0.24, P < 0.7$).

3.3.3. Retrieval Test

The results obtained from the injection of CGP₃₅₃₄₈ + Testosterone on retrieval in spatial learning and memory was shown that there was no significant difference in number entrance animal to target quadrant ($F = 0.4, P < 0.48$), time spent in target quadrant ($F = 0.8, P < 0.5$), traveled distance in target quadrant ($F = 0.6, P < 0.7$). Also there was no significant difference of performance among the groups on visible platform trial for escape latency ($F = 0.21, P < 0.8$) and traveled distance ($F = 0.7, P < 0.1$).

4. Discussion

The present findings of this study showed that there was no significant difference between the vehicle groups (Saline, DMSO, DMSO and Saline) in acquisition, consolidation and retrieval. Intra-hippocampal injection of Testosterone impaired acquisition of spatial learning but there were no effect on consolidation and retrieval in MWM. Also Intra-hippocampal injection of CGP₃₅₃₄₈ (5 µg/0.5µl) and CGP₃₅₃₄₈ (5 µg/0.5µl) + T (80 µg/0.5µl) could improve spatial acquisition but they had no effect on consolidation and retrieval stage.

There were no statistical differences between the control and experimental groups on the non-spatial visual discrimination task in which the platform was elevated above the water level. It could be inferred that the observed changes could not be attributed to the alternations of non-mnemonic factors such as motivation, motor, or sensory processes induced by the treatment.

The literature of androgen effects on spatial memory in adult animals and humans was complex and contradictory. Some evidence suggested a positive correlation between Testosterone and spatial ability [4] [24]. In contrast, several reports indicated that chronic treatment with androgenic compounds had impaired spatial learning and retention of spatial information in young and middle-aged animals [25] [26] and humans [27] [28]. At the same time, some investigators had reported no association between visuospatial ability and either endogenous or exogenous Testosterone in adult male mammals [7] [25].

Our finding based on Testosterone role in impaired memory processing were compatible with study of Hoorani *et al.* that indicated the administration of Testosterone pre-training, in passive avoidance task impaired acquisition, consolidation and retrieval [29]. Also in other investigation reported that injection of TE into the basolateral nucleus of the amygdale resulted in significant difference escape latency and traveled distance compared to the DMSO receiving group [30]. Different results obtained from Testosterone on spatial learning and memory could be related to diversity in animal race, different doses, different methods, the place of injection (IP, IM, Sc and Local injection) and etc.

There could be several possible explanations for our findings. First, exogenous Testosterone depressed levels of both gonadotropins and androgen precursors such as dehydroepiandrosterone (DHEA) and its sulfate (DHEAS) [31]. DHEAS could activate an allosteric site on the GABA receptor that inhibited the chloride channel opening and thus increasing neuronal excitability [32]-[35]. At the same time, the administration of DHEAS, a negative allosteric modulator of the GABA receptors, could enhance the release of acetylcholine, a neurotransmitter closely associated with learning and memory function from neurons in the hippocampus [36].

Second, there was a possibility that Testosterone could be converted to estrogen by aromatized enzyme [37], hence impairing spatial memory. Third, Testosterone by acting as a nonselective sigma antagonist, might produce a tonic dampening of the function of sigma receptors and consequently cause a decrease in NMDA receptor function [38]. In general, sex steroids could rapidly influence neural activity by increasing binding affinity of neurotransmitters or directly altering cell membrane ion conductance in brain structures including the

hippocampus [8] [39] [40]. Progesterone, and rostenedione, and Testosterone retained some modulator activity on the GABA receptors.

Our results showed that CGP₃₅₃₄₈ (5 µg/0.5µl) could improve acquisition in the MWM as compared to vehicle-treated rats. Since there were no significant differences between the vehicle and experimental groups in visible platform, and there were no significant differences in swimming speed, indicating that it could not be attributed to sensory or motivational processes.

Some of the researchers showed that GABA_b receptor antagonists could elevate brain cAMP levels and elevation of cAMP could promote early response gene expression such as c-fos gene expression, which promoted memory [35] [41]. They had shown that GABA_b receptor antagonists could enhance cognitive performance in primates as well as rodents suggested that GABA_b receptor antagonists might enhance cognitive function by facilitating cholinergic transmission.

In our study, administration of Testosterone + CGP₃₅₃₄₈ decreased escape latency and travel distance as compared to the Saline + DMSO Group. It has been shown that there are important reciprocal relationships between brain androgenic system and GABAergic system.

The swimming speed of animals to find the target platform was not significantly affected in different experimental animals compared to the control group. It appears reasonable to claim that none of the drugs had any effect on the dynamic sensory activities. Following the animals to the training day it was clear that the visual scent of animals was unaffected, since the escape latency and distance traveled did not show significant differences between control and drug receiving groups.

The principal observation of this study is that the Testosterone-derived neurosteroid androstanediol has GABA_a receptor-modulating activity in the adult hippocampus. Androstanediol is an endogenous neurosteroid produced from Testosterone. It has been demonstrated that androstanediol exhibits functional actions on GABA receptors. They found that the neurosteroid markedly potentiates responses to GABA in acutely dissociated CA₁ pyramidal neurons. Overall, these studies strongly support that androstanediol is a positive modulator of GABA_a receptors [42].

In conclusion, our results indicate that Testosterone impair acquisition while CGP₃₅₃₄₈ could exterminate the effect of Testosterone on spatial acquisition. So it is concluded that Testosterone may have action via GABA_b receptor.

Acknowledgements

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