

# Interaction Between Testosterone and Bicuculline GABA<sub>A</sub> Antagonist in the CA<sub>1</sub> Region of Hippocampus in Spatial Learning in Adult Male Rats

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**T**he hippocampus plays a vital role in spatial learning and memory. Testosterone appears to mediate spatial discrimination and the GABAergic system has also been reported to have a critical role in this effect. In the present study we investigated the interaction between testosterone (androgenic receptor agonist) and bicuculline (GABA<sub>A</sub> receptor antagonist) on spatial learning and memory performance in male Wistar rats.

**Materials and Methods:** Cannulae were implanted into the CA<sub>1</sub> of rats bilaterally and drugs were injected before daily training in the Morris water maze (MWM). In the first experiment, testosterone (0, 20, 40, 80 µg the 0.5 µl DMSO/side) was injected intra-CA<sub>1</sub> before each session. In the second experiment, intra-CA<sub>1</sub> injection of bicuculline (0, 1, 2, 4 µg 0.5 µl saline/side) were given before every session. In the last experiment, testosterone 80 µg, 0.5 µl and bicuculline 2 µg, 0.5 µl were injected into the CA<sub>1</sub>.

**Results:** The results showed that testosterone 80 µg or bicuculline 2 µg, each given separately, and also microinjection of both testosterone + bicuculline increased travel distance and escape latency to find the platform, as compared to their vehicles.

**Conclusion:** It is shown that administration of testosterone and bicuculline separately impaired spatial learning and memory. Microinjection of bicuculline after testosterone treatment did not change spatial learning impairment when com-

pared to testosterone and bicuculline injected separately.

**Key Words:** Hippocampus, Spatial learning and memory, Testosterone, Bicuculline, GABA<sub>A</sub> receptor, Morris Water Maze

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## Introduction

Evidence indicates that the hippocampus is necessary for acquisition and retrieval of spatial information<sup>1</sup> as well as for consolidation/storage.<sup>2,3</sup> Many authors have presented evidence for the specific and disproportional involvement of the hippocampal formation in the spatial aspects of the Morris Water Maze (MWM) learning.<sup>2,4-7</sup>

It has been shown that androgen receptors were found in high density in hippocampal CA<sub>1</sub> pyramidal cells<sup>8</sup> and both androgens and estrogens affect hippocampal synaptic plasticity and learning and memory performance in rats.<sup>9</sup> Testosterone, and its metabolite estradiol, appear to mediate spatial learning and memory performance organizationally and possibly activationally.<sup>10-14</sup> There are conflicting data about the effect of testosterone on learning and memory. Some evidence suggests a positive correlation between tes-

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tosterone and spatial ability,<sup>15-19</sup> e.g. high circulating free testosterone is associated with better performance on tests of memory, executive function, and spatial ability, and with a reduced risk for Alzheimer's disease,<sup>20</sup> adult men with higher testosterone levels showed better cognitive performance on spatial tasks than did women.<sup>11</sup> However several reports indicate that chronic treatment with androgenic compounds has impaired spatial learning and retention of spatial information in young and middle-aged animals<sup>13</sup> and humans.<sup>7,21,22</sup>

Gamma Amino Butyric 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 leads to memory enhancement and conversely the activation leads to memory inhibition. However, other results have reported the opposite, where GABAergic antagonists injected into the striatum or substantia nigra produced amnesia. The majority of GABARs in the CNS are of the GABA<sub>A</sub>-R-type, which is a main target for substances with amnesic properties.<sup>9</sup> It has been shown that the GABA antagonist bicuculline inhibited memory in chicks.<sup>23</sup>

There are important reciprocal relationships between brain steroid hormone and neurotransmitter systems such as cholinergic,<sup>24-26</sup> dopaminergic,<sup>27-28</sup> GABAergic, serotonergic and glutaminergic systems.<sup>22,24,29,30</sup> Sex steroids can rapidly influence neural activity by increasing the binding affinity of neurotransmitters or by directly altering cell membrane ion conductance in brain structures including the hippocampus.<sup>8,29,31</sup> Neurosteroids can be positive and negative endogenous modulators of GABA<sub>A</sub> receptors.<sup>32,33</sup> Exogenous testosterone depresses plasma levels of both gonadotropins<sup>34</sup> and androgen precursors such as dehydroepiandrosterone (DHEA) and its sulfate (DHEAS).<sup>22</sup> DHEAS can activate an allosteric site on the GABA receptor that inhibits the chloride channel opening and thus increases neuronal excitability.<sup>22,35-37</sup> At the same time

it can enhance the release of acetylcholine, from neurons in the hippocampus which is a neurotransmitter closely associated with memory function.<sup>38</sup> Mean levels of DHEAS in human males declines with age,<sup>39</sup> and DHEAS replacement therapy has been reported to result in an improved sense of well-being in aged men and women.<sup>40</sup> In female rats, gonadal steroids regulate hippocampal GABA<sub>A</sub> receptor mRNA levels,<sup>33</sup> but they may have less pronounced effects on GABA<sub>A</sub> receptor subunits in male rats than in female ones.<sup>33</sup> Testosterone appears to exert little regulatory control over GABA<sub>A</sub> receptor subunit mRNA levels.<sup>37</sup> Considering the data given above, we conducted a series of experiments to investigate the association between testosterone and GABA<sub>A</sub> receptors and their interaction on spatial learning.

## Materials and Methods

### Animals

Male albino wistar rats weighing 200–250 g, obtained from the breeding colony of the Pasteur Institute, were housed four per cage in a temperature and light-controlled room under a 12h:12h light:dark cycle, with water and food provided ad libitum. Experiments were carried out in a room where only the water maze setup was placed in standard conditions.

### Surgery

Seven days prior to initiation of the behavioral experiments, the rats were anaesthetized with a mixture of ketamine and xylazine (100 and 3mg/kg, respectively, i.p.) and two guide Cannulae were implanted bilaterally above the CA<sub>1</sub> region of hippocampus at coordinates (in mm): p=-3.72 (posterior to bregma), L= ±2.2 (lateral to the midline) and H=2.7mm (ventral to outer skull surface) based on the Paxinos and Watson's atlas.<sup>41</sup>

### Microinjection procedure

Drugs and vehicles were administered intra- CA<sub>1</sub> region through guide Cannulae (21-gauge) using injection needles (27-gauge) connected by a polyethylene tube to a 1.0μl Hamilton microsyringe; the injection needle

was inserted 0.3mm beyond the tip of the cannula and different doses of testosterone (0, 20, 40, 80  $\mu\text{g}$ , 0.5  $\mu\text{l}$  DMSO/side; 30 min before training) or bicuculline (0, 1, 2, 4  $\mu\text{g}$ , 0.5  $\mu\text{l}$  saline/side; 5min before training) separately or both testosterone (80  $\mu\text{g}$ , 0.5  $\mu\text{l}$  DMSO/side; 25 min before bicuculline injection) and bicuculline (2  $\mu\text{g}$ , 0.5  $\mu\text{l}$  saline/side; 5 min before training) were injected over 3 min; injections (0.5  $\mu\text{l}$  total volume) were delivered over two minutes with a syringe pump, and the needles (extending 0.5 mm from the end of the guide cannulae) were left in place, an additional minute, before they were slowly withdrawn.

### Behavioral assessment

**Apparatus:** The Morris water maze task consisted of a dark circular pool, 140 cm diameter and 55 cm height, filled with water ( $20\pm 1^\circ\text{C}$ ) to a depth of 25 cm. A transparent Plexiglas platform (11 cm in diameter) was located 1cm below the water surface in the center of one of the arbitrarily designed north-east (NE), south-east (SE), south-west (SW), or north-west (NW) orthogonal quadrants. The platform provided the only escape from the water. Extra maze cues such as racks, a door, shelves, and pictures on the walls surrounded the environment; the room was dark during the experiment, where the water maze was placed. These were kept in fixed positions with respect to the swimming pool, to allow the rat to locate the escape platform, hidden below the water surface. A video tracking system (CCD Camera USA) and a PC computer with soft ware (Tivanic Iran & Pasteur Institute of Iran) developed for monitoring and recording the position of the rat in the water maze, was used.<sup>35,36,40-43</sup> Thus, the time required to reach the platform (latency), the swimming path (distance), and the swimming speed were recorded as well as the time and distance spent in each quadrant.

**Procedure:** All rats were given a daily session of four trials for four consecutive days. Each trial involved placing the rat in the pool, close to and facing the wall in one of the four equal quadrants into which the

pool was divided. Animals were allowed to swim freely until they found the escape platform. If a rat failed to find the platform within 90s,<sup>1</sup> the experimenter placed the rat on it. The rat was taken directly from the platform to the new starting point which was changed from trial to trial in aquasi random order; hence each starting point was used once in each session of four trials. To assess visual-motor coordination toward the visible platform, on the fifth day, the platform was elevated above the water surface and placed in the SE quadrant. This assessed visuomotor coordination toward the visible platform.

**Histology:** Following behavioral testing, animals were anesthetized with ether and then sacrificed by decapitation and the brains were removed. For histological examination of cannulae and needle placement in the CA<sub>1</sub> region, 100 $\mu\text{m}$  thick sections were taken, and the cannula track was examined for each rat. We did not use the data from rats, in which cannula tips deviated from the target. In the remaining animals, all cannulae tips were just above the CA<sub>1</sub> region and the injection tracks were exactly spread in a limited area, in the CA<sub>1</sub> region of hippocampus.

**Statistical analysis:** The data were analyzed by one-way analysis of variance (ANOVA) with repeated measures, followed by Tukey's test. All results are shown as mean $\pm$ S.E.M,  $p < 0.05$  was considered statistically significant for all comparisons.

### Experiments

**Experiment 1:** The aim of this experiment was to determine the effect of intrahippocampal injection of DMSO, saline and a group was received saline+ DMSO together, on MWM performance. A total of 24 rats were divided into three groups according to the volume 0.5  $\mu\text{l}$ , and were microinjected 30 min before training every day.

**Experiment 2:** The aim of this experiment was to determine the effect of the intrahippocampal testosterone injection as the agonist for the androgen receptors, on MWM performance. A total of 32 rats divided into four groups according to dose levels, 20, 40 and

80 µg testosterone dissolved in 0.5 µl DMSO, and microinjected 30min before training every day.

*Experiment 3:* The aim of this experiment was to determine the effect on MWM performance of intra- CA<sub>1</sub> injection of bicuculline as an antagonist of GABA<sub>A</sub> receptors; a total of 32 rats were divided into four groups according to dose levels of 1, 2, and 4 µg bicuculline dissolved in 0.5 µl saline and microinjected 5 min before training every day.

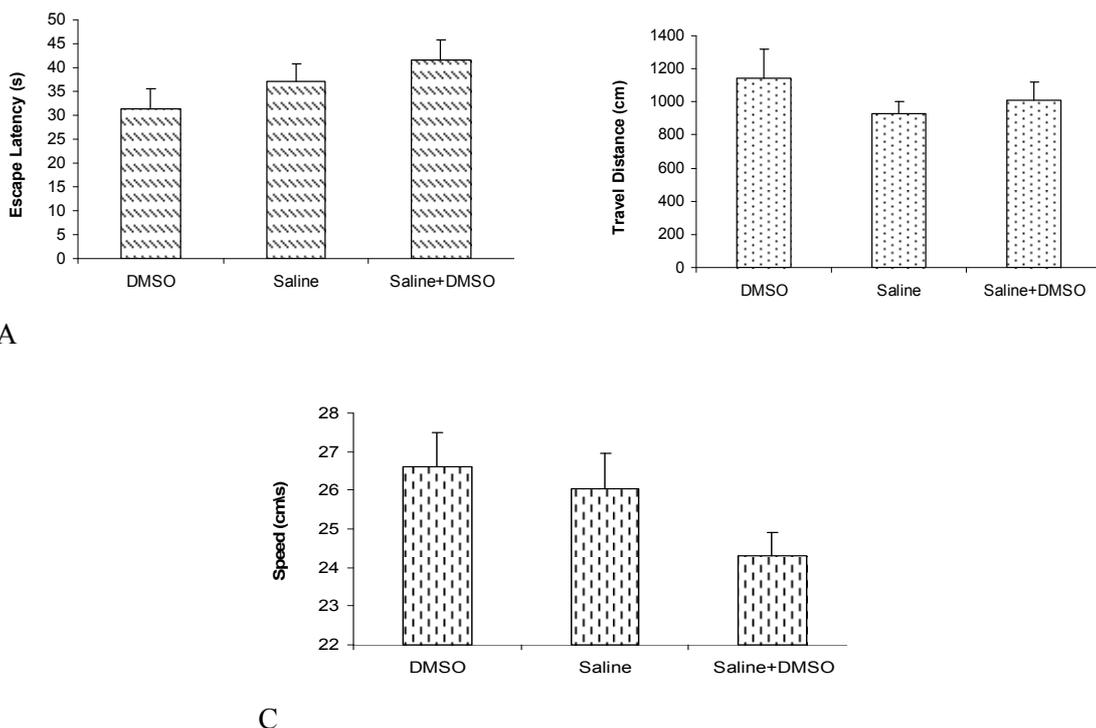
*Experiment 4:* The aim of this experiment was to determine the effect of intra-CA<sub>1</sub> injection of testosterone plus bicuculline on MWM performance. A total of 16 rats were divided two groups according to the effective dose of testosterone (80 µg, 0.5 µl DMSO; 25 min before bicuculline injection) and bicuculline (2 µg, 0.5 µl saline injected 5 min before training) every day.

## Results

Hidden platform trials (days 1– 4) and Visible platform trials (day 5):

### Vehicle effect (Experiment 1)

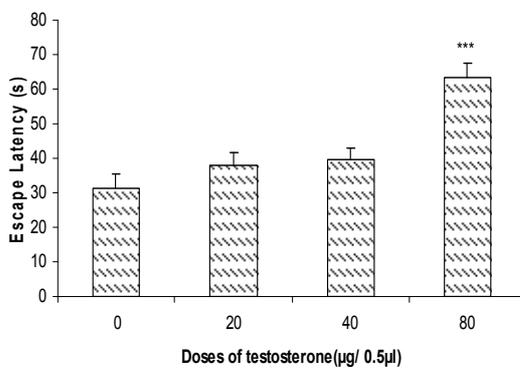
The results obtained from the injection of DMSO or saline, or saline+DMSO combined indicated no significant difference in escape latency ( $F=1.522$ ,  $P=0.225$ ) or traveled distance ( $F=0.796$ ,  $P=0.455$ ) between the groups (Fig. 1). There was no significant difference between the swim speed of the groups ( $F=2.041$ ,  $p=0.1372$ ). These results indicated that injection of DMSO or saline or saline+DMSO had no effect on spatial learning and swimming ability. There was no significant difference in traveled distance ( $F=1.448$ ,  $p=0.2641$ ) or escape latency ( $F=1.094$ ,  $p<0.3587$ ) in the visible test performance between the vehicle groups.



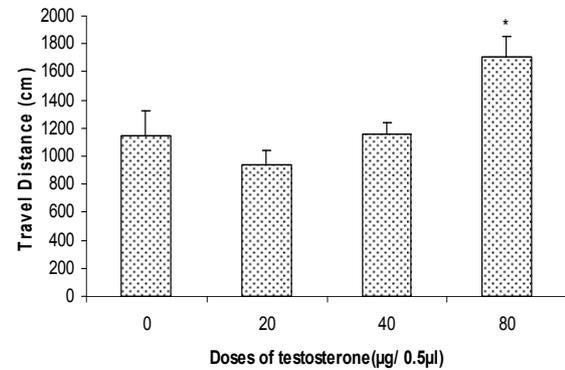
**Figures 1: Average escape latency (A), traveled distance (B), and swimming speed (C) across all training days in vehicles. No significant difference between DMSO, saline and DMSO + saline (n=8 for each group)**

**Testosterone effect (Experiment 2)**

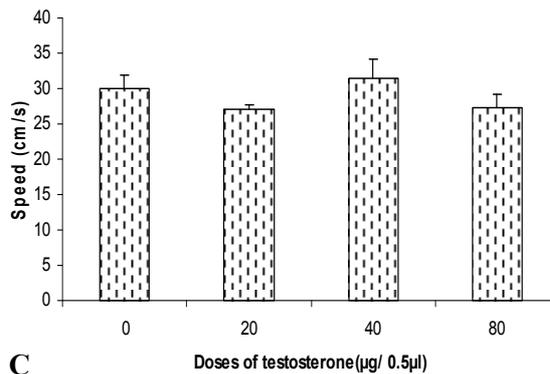
Fig. 2. shows the results obtained from the injection of testosterone and vehicle group (DMSO). A significant increase was generally found in escape latency ( $F=7.97$ ,  $p<0.001$ ) and traveled distance ( $F=6.43$ ,  $p<0.05$ ) between groups (Fig. 2 A and B). There was a significant difference between the 80  $\mu\text{g}$ , 0.5  $\mu\text{l}$  testosterone treated group with the vehicle group. No significant difference found in swimming speed ( $F=1.608$ ,  $p<0.1757$ ) between groups (Fig. 2 C), or in traveled distance ( $F=2.046$ ,  $p=0.1122$ ) or escape latency ( $F=2.374$ ,  $p<0.0737$ ) in the visible test performance between the groups.



A



B



C

**Bicuculline effect (Experiment 3)**

Fig. 3. shows the results obtained from the injection of bicuculline and vehicle group (saline). A significant increase was found in escape latency ( $F=4.41$ ,  $p<0.05$ ) and traveled distances ( $F=0.40$ ,  $p<0.05$ ) between groups (Fig. 3 A and B). There was a significant difference between the 2, 4  $\mu\text{g}$ , 0.5  $\mu\text{l}$  bicuculline treated group and the vehicle group. No significant differences were observed in swimming speed ( $F=0.4043$ ,  $p=0.7501$ ) between groups (Fig. 2 C). There was no significant difference in traveled distance ( $F=2.904$ ,  $p<0.051$ ) or escape latency (2.442,  $p<0.0843$ ) in the visible test performance between the groups.

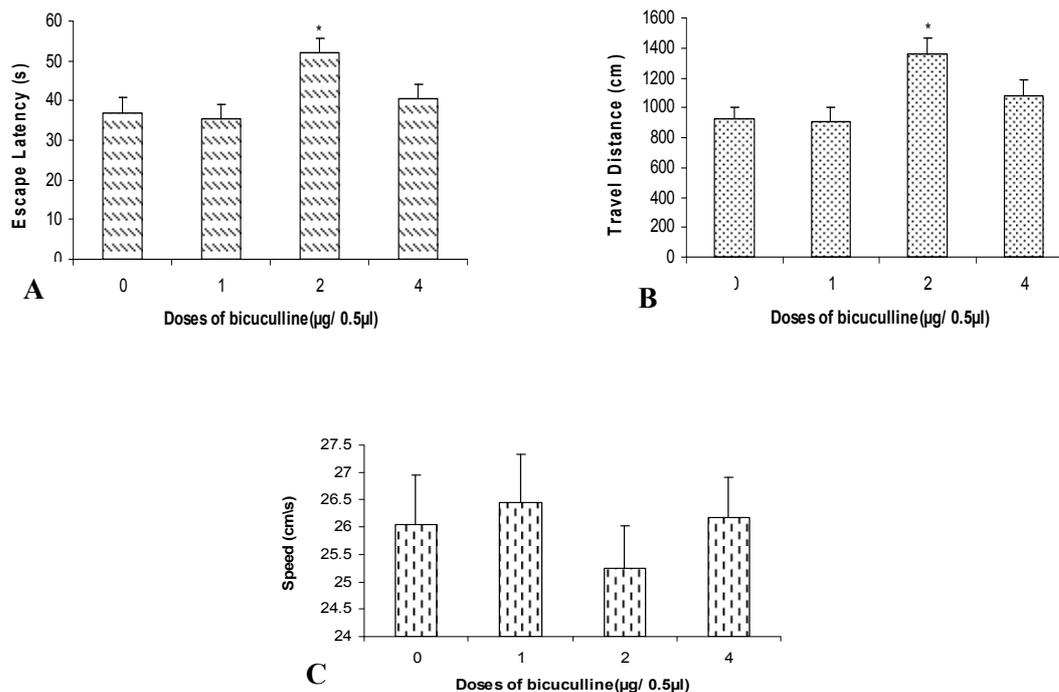
**Figures 2. Average traveled distance (A), escape latency (B), and swimming speed (C) across all training days in testosterone treated groups**

\*\*\* $P < 0.001$ , \* $P < 0.05$  shows a significant difference between 80  $\mu\text{g}/0.5\mu\text{l}$  Testosterone treated

### Bicuculline effect (Experiment 3)

Fig. 3. shows the results obtained from the injection of bicuculline and vehicle group (saline). A significant increase was found in escape latency ( $F=4.41$ ,  $p<0.05$ ) and traveled distances ( $F=0.40$ ,  $p<0.05$ ) between groups (Fig. 3 A and B). There was a significant difference between the 2, 4  $\mu\text{g}$ , 0.5  $\mu\text{l}$  bicucul

line treated group and the vehicle group. No significant differences were observed in swimming speed ( $F=0.4043$ ,  $p=0.7501$ ) between groups (Fig. 2 C). There was no significant difference in traveled distance ( $F=2.904$ ,  $p<0.051$ ) or escape latency ( $2.442$ ,  $p<0.0843$ ) in the visible test performance between the groups.

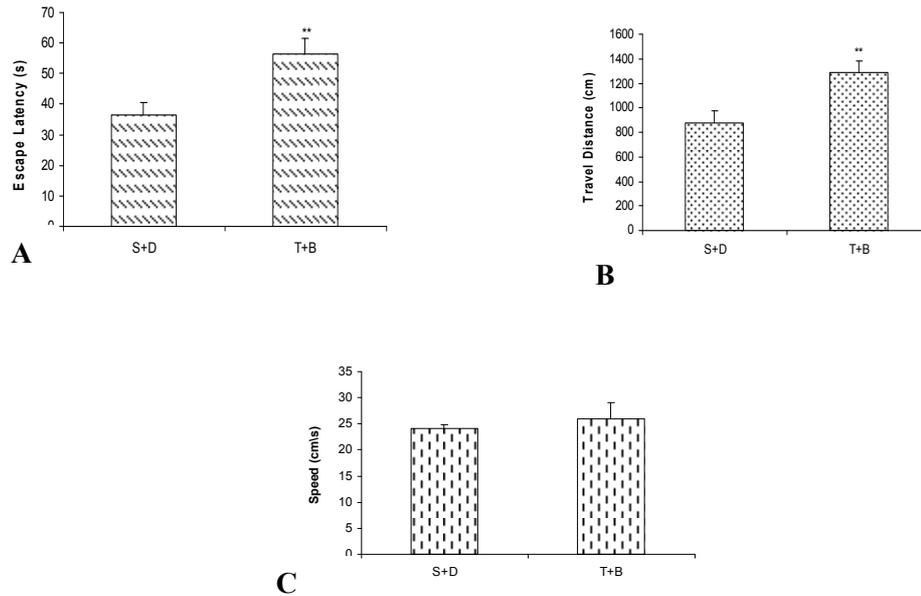


Figures 3. Average escape latency (A), traveled distance (B), and swimming speed (C) across all training days in bicuculline treated group. \* $P < 0.05$  shows a significant difference between 2  $\mu\text{g}/0.5\mu\text{l}$  bicuculline treated group and with the vehicle group ( $n=8$  for each group).

### Testosterone+bicuculline effect (Experiment 4)

Fig. 4. shows the results obtained from the injections of testosterone and bicuculline (T+B) compared to the vehicle group (saline+ DMSO). A significant increase was generally found in escape latency ( $T=3.01$ ,  $p<0.01$ ) and traveled distance ( $T=3.03$ ,

$p<0.01$ ) between groups (Fig. 4 A and B). No significant differences found in swimming speed ( $T=0.5724$ ,  $P=0.5704$ ) between groups (Fig. was 4 C), or in traveled distance ( $T=0.4714$ ,  $p<0.5878$ ) or escape latency ( $T=0.6687$ ,  $p<0.4544$ ) in the visible test performance between the groups.



**Figures 4. Average escape latency (A), traveled distance (B), and swimming speed (C) across all training days in testosterone+ bicuculline treated group (T+B).**

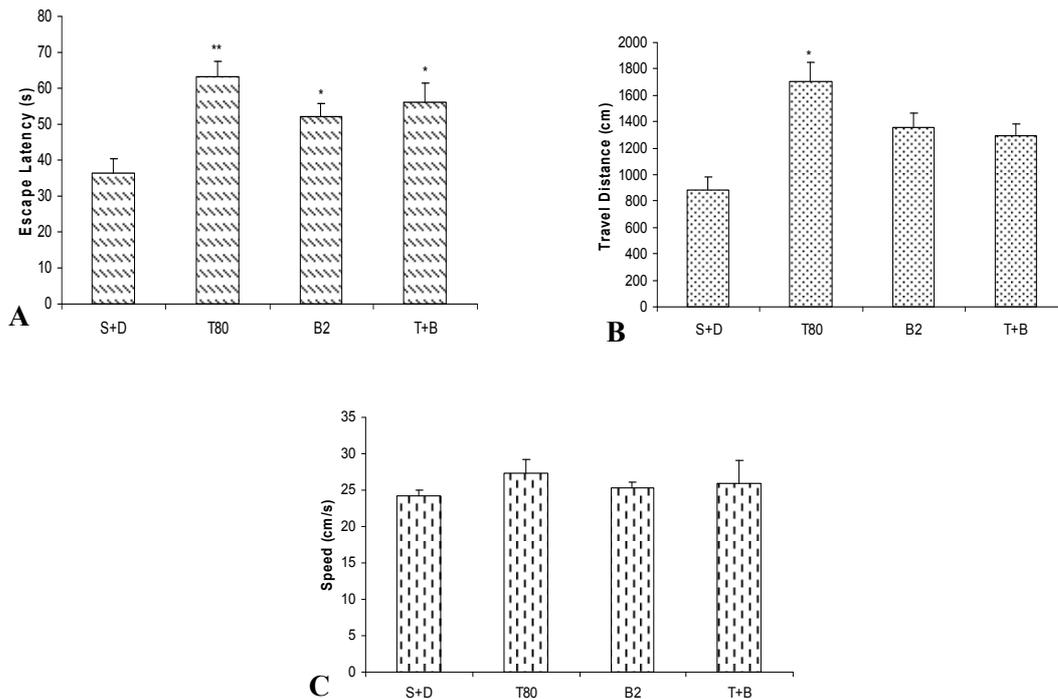
\*\* $P < 0.01$  shows a significant difference between testosterone+ bicuculline treated group and the vehicle group (DMSO+ saline, D+S) ( $n=8$  for each group)

Fig. 5. shows results obtained from the injection T80, B2, T+B and vehicle group (saline+ DMSO). While a significant increase was found in escape latency ( $F=5.890$ ,  $p<0.001$ ) and traveled distance ( $F=3.285$ ,  $p<0.05$ ) between groups (Fig. 5 A and B), no significant differences were observed in swimming speed ( $F=0.5767$ ,  $p=0.6821$ ) between groups (Fig. 5 C), or in traveled distance ( $F=3.670$ ,  $p<0.075$ ) or escape latency ( $F=2.914$ ,  $p<0.0416$ ) in the visible test performance between the groups.

## Discussion

The results indicated that there was no significant difference between the vehicle groups (saline, DMSO, DMSO and saline); DMSO has also been used as a vehicle in other investigations under similar conditions.<sup>39,42,43</sup> We also showed that double injections of DMSO and saline had no significant effect on learning and memory, a finding consistent with some other reports.<sup>19,44</sup>

In our study, testosterone-treated rats displayed impairments of memory acquisition at dose  $80 \mu\text{g}$ ,  $0.5 \mu\text{l}$ . Since there were no significant differences between the vehicle and experimental groups in visible platform performance on the fifth day, it can be inferred that the changes observed could not be attributed to alterations of non-mnemonic factors, such as the motivational or sensory processes induced by the treatments. Since other investigations have shown that chronic treatment with androgenic compounds impaired spatial learning and retention of spatial information in adult animals<sup>39,40,45</sup> and also that there are no significant differences between intact and testosterone depleted or administrated male rats in spatial learning and memory,<sup>46</sup> there could be several possible explanations for our finding. First, exogenous testosterone depresses plasma levels of both gonadotropins<sup>34</sup> and androgen precursors such as dehydroepiandrosterone (DHEA) and its sulfate (DHEAS).<sup>22</sup>



**Figures 5.** Average traveled distance (A), escape latency (B) and swimming speed (C) across all training days in saline+ DMSO (vehicle, S+D), testosterone (T80), bicuculline (B2), testosterone+ bicuculline (T+B) groups. \*\* $P < 0.01$ , \* $P < 0.05$  shows a significant difference between T80, B2 and T+B treated groups with the vehicle group in the escape latency ( $n=8$  for each group).

DHEAS can activate an allosteric site on the GABA receptor that inhibits the chloride channel opening and thus increasing neuronal excitability.<sup>22,35-37</sup> At the same time the administration of DHEAS, a negative allosteric modulator of the GABA<sub>A</sub> receptor, can enhance the release of acetylcholine, a neurotransmitter closely associated with memory function, from neurons in the hippocampus.<sup>38</sup> Second, there is a possibility of aromatization of testosterone to estrogen, which in some nuclei like the hippocampus, can act through estrogen receptors.<sup>22,47</sup> Our previous studies showed that microinjection of both testosterone and flutamid (non-steroidal androgenic receptors antagonist) in the CA<sub>1</sub> impaired spatial learning and memory.<sup>39,40</sup> Third, testosterone, by acting as a non-selective sigma antagonist, may produce a tonic

dampening of the function of sigma receptors and consequently a decrease in NMDA receptor function (low doses of testosterone do not modify the NMDA response).<sup>48</sup>

In the bicuculline-treated group, spatial performance decreased significantly as compared to the vehicle group. Since there were no significant differences between the vehicle and experimental groups on the fifth day of training in visible platform, and there were no significant differences in swimming speed indicating that it could not be attributed to sensory or motivational processes, bicuculline (2, 4  $\mu\text{g}$ , 0.5  $\mu\text{l}$ ) could have impaired acquisition in the MWM as compared to vehicle-treated rats. This is consistent with results of a previous study showing that the selective GABA<sub>A</sub> receptor antagonist, bicu-

culline inhibited reinforced memory in a dose and time dependent manner;<sup>23</sup> also administration of bicuculline and muscimol into the nucleus basalis magnocellularis impaired inhibitory avoidance learning.<sup>50</sup> On the other hand, low doses of GABA to bicuculline-insensitive GABA<sub>A</sub> receptors;<sup>23</sup> in the current data available we observe the inhibitory effect of GABA in the presence of bicuculline.

In our study, administration of both testosterone and bicuculline increased escape latency and travel distance as compared to the DMSO+saline Group. It has been shown that there are important reciprocal relationships between brain steroid hormone systems and neurotransmitter systems such as GABAergic, serotonergic and glutaminergic.<sup>24,29,30</sup> Sex steroids can rapidly influence neural activity by increasing binding affinity of neurotransmitters or directly altering cell membrane ion conductance in brain structures including the hippocampus.<sup>8,29,31</sup> Progesterone, androstenedione, and testosterone retain some modulatory activity on the GABA<sub>A</sub> receptor.<sup>33</sup> Positive modulators of the GABA<sub>A</sub> receptor produce amnesia,<sup>47</sup> suggesting that excessive inhibitory tone could contribute to

cognitive impairments, associated with aging. This hypothesis is consistent with the increased activity of glutamic acid decarboxylase in the brains of aged animals,<sup>19</sup> and the reduced desensitization of GABA<sub>A</sub> receptor in neurons from aged rats.<sup>46</sup> Our present data which indicate the impairment of spatial performance may be explained by the effect of testosterone on the other GABA receptors such as B and C receptors, which are not affected by bicuculline.<sup>23</sup> Since there were no significant differences between the vehicle and experimental group in the swim speed on the fifth day of training in visible platform, performance, it can be inferred that the changes observed could not be attributed to alterations of non-mnemonic factors such as motivational, or sensory processes induced by the treatments.

To conclude, the results showed that administration of testosterone and bicuculline used separately impaired spatial learning and memory. Microinjection of bicuculline after testosterone treatment did not alter the spatial learning impairment when compared to the two used separately.

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