ORIGINAL ARTICLE

MODAFINIL PREVENTS TESTOSTERONE – PROVOKED SPATIAL LEARNING IMPAIRMENTS IN MORRIS WATER MAZE AND INCREASES THE QUANTITY OF BrdU-POSITIVE CELLS IN DENTATE GYRUS IN MALE RATS

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Abstract

The relationships between the androgen receptors and cognitive processes in some brain regions such as hippocampus had been well documented in previous studies. The aim of this study was to evaluate the effect of bilateral intra-hippocampal infusion of modafinil on testosterone–induced spatial learning alterations in the Morris water maze (MWM) and also the evaluation of the quantity of BrdU-positive cells in dentate gyrus as a marker of cell proliferation. Bilateral intra-hippocampal infusions (1 μL/side) with different doses of testosterone enanthate (10, 50, and 100 μg/side) or modafinil (50, 100, and 250 μM/side) were performed, 30 min prior to training in MWM for four repeated days in male rats. Control groups received dimethyl sulfoxide (DMSO). The behavioural findings of this study, showed considerable raises in time and distance of reaching the hidden platform in testosterone (100 μg/side) – treated animals, and also significant decreases in above mentioned parameters in modafinil (100 μM/side) – treated group compared to their related control groups. We also assessed the effects of modafinil (50, 100, and 250 μM/side) plus testosterone enanthate (100 μg/side), on the spatial learning in MWM and the effect on neurogenesis by recording BrdU-positive in all above mentioned groups. Final results of this study showed that modafinil (100 μM/side) prevented the testosterone (100 μg/side) – induced spatial learning deficits in MWM and also increased the quantity of BrdU-positive cells in dentate gyrus in combination evaluations in comparison with testosterone – treated animals.

Rezumat

Legăatura dintre receptorii androgeni și procesele cognitive în anumite regiuni ale creierului precum hipocampul au fost demonstrate în studii anterioare. Scopul prezentului studiu a fost de a evalua efectul infuziei bilaterale intra-hipocampice a modafinilului asupra afectării învățării spațiale induse de testosteron prin testul Morris al labirintului în cruce (MWM) precum și a cantității de celule BrdU în girusul dentat ca marker al proliferării celulare. Infuzia bilterală intra-hipocampică (1 μL/parte) cu diferite doze de testosteron enantat (10,50 și 100 μg/parte) sau modafinil (50, 100, and 250 μM/parte) s-a realizat la șobolani masculi cu 30 de minute înainte de antrenamentul pentru testul MWM timp de 4 zile consecutive. Grupul de control a primit dimetilsulfoxid (DMSO). Rezultatele studiilor comportamentale au arătat creșteri considerabile a timpului și distanței de atingere a platformei ascunse la animalele tratate cu testosteron (100 μg/parte) și scăderi semnificative a acestora la animalele tratate cu modafinil (100 μg/parte) comparativ cu grupul de control. S-au evaluat totodată și efectele modafinilului (50, 100, and 250 μM/parte) plus testosteron enantat (100 μg/parte) asupra invățării spațiale prin testul Morris al labirintului în cruce (MWM) precum și efectele asupra neurogenezei prin înregistrarea celulelor BrdU-pozitive. Rezultatele finale ale studiului au arătat că modafinilul (100 μg/parte) previne deficitul învățării spațiale induse de testosteron din testul MWM și crește cantitatea de celule BrdU-pozitive în girusul dentat comparativ cu lotul tratat numai cu testosteron.

Keywords: testosterone enanthate, modafinil, spatial learning, dentate gyrus

Introduction

The hippocampus in the limbic system of the brain includes some major sub regions such as CA1, CA3 and dentate gyrus (DG) and acts importantly in different stages of learning and memory formation processes [12, 29, 41, 60].

Production of new neurons (neurogenesis) in some brain regions such as sub-ventricular zone (SVZ) and DG of the hippocampus is a unique form of neuroplasticity [11, 31, 32].

By using BrdU (as a proliferation marker), it has been demonstrated that in the DG, new neurons that arise

from NPCs (neural precursor cells), are finally contributed to cognitive performances [11, 31, 32].
The hippocampus is a key target for androgens and some learning tasks such as inhibitory avoidance and water maze tasks [5, 18, 59].
Densely expression of androgen receptors in rat hippocampal cells indicates the outcome of androgen influences on cognitive processes. Both positive and negative effects of androgens on spatial memory have been founded in previous literatures. These contradictory effects of androgens on memory processes are of interest for researchers [5, 41, 42, 58]. Also, cognitive deficits and long-term memory (LTM) impairments have been reported previously as a result of chronic treatment with androgenic compounds [14, 25, 45].
Prescription of modafinil (2-(diphenylmethyl) sulfinyl) acetamide) for narcolepsy-associated somnolence, shift-work sleep disorder, decrease fatigue and increase a sense of alertness, improvement of concentration and to reduce the signs of attention-deficit hyper-activity disorder (ADHD), and also to treat cocaine/ methamphetamine abuse, has been well documented in previous studies. The role of modafinil for treating cognitive deficits is being investigated. Inhibition of dopamine or norepinephrine transporters, rising dopamine, serotonin, glutamate and orexin or decrease of the extracellular γ-aminobutyric acid (GABA) release, are some probable mechanisms for modafinil actions in documented studies [20, 27, 35, 40, 60, 61].
By considering the multi-functional role of modafinil on several neurotransmitter systems [24, 26, 33, 36, 38, 49, 57, 62], the present research was designed to evaluate the effect of bilateral intra-hippocampal infusion of modafinil on testosterone–induced spatial learning alterations in the Morris water maze (MWM) and also the evaluation of the quantity of BrdU-positive cells in dentate gyrus as a marker of cell proliferation.

Materials and Methods

Animals
Male rats (200 - 250 g) were taken from the animal house of Physiology Department of Tehran University of Medical Sciences, Iran, and kept in controlled conditions in the Plexiglas cages, providing *ad libitum* admittance to food and water. All experimental protocols were in agreement with the Animal Ethics Committee of Tehran University of Medical Sciences.
Drugs
The drugs applied in this research were testosterone enanthate (Sigma Aldrich, U.S.A.), modafinil (Sigma, U.S.A.), ketamine (Alfasan, Holand) and xylazine (Alfasan, Holand). Testosterone and modafinil were dissolved in dimethyl sulfoxide (DMSO) (Sigma, U.S.A.).
Surgery procedure, Behavioural training and Testing
Bilateral intra-hippocampal insertion of guide cannula in CA1 region of the hippocampus was performed using a stereotaxic instrument. All intra-hippocampal infusions were done using a Hamilton syringe, one week after surgery & cannulation and 30 min before training trials, daily for 4 consecutive days. Animal's training in Morris water maze (MWM) was performed for four repeated days as described in previous studies [4, 51, 55, 56].
Each training day consisted of four trials (in each trial, animals were trained to reach the unseen platform in an episode of ninety seconds). The escape latency, travelled distance and the swimming speed for each rat was documented by a video tracking system. The probe test (taking out the hidden platform) was done on day 5 by measuring the time used up in the target quadrant (the quadrant that hidden platform was previously located there) for 90 s (n = 8).
Motivational, motor and sensory processes were assessed by visible trial and open-field motor activity studies (day 5) in both experiments 1 and 2.
Drug treatments
Experiment 1: Rats were divided into four groups (n = 8). In cannulated animals, seven days after recovering from the stereotaxic surgical procedure, testosterone (10, 50 and 100 μg/μL) was infused bilaterally for 4 consecutive days in a volume of 1 μL/side into the CA1 area of the hippocampus 30 min prior to training trials in the MWM task. The control animals are given DMSO in a volume of 1 μL/side for 4 consecutive days. Also, the numbers of BrdU-positive cells were assessed in these groups.
Experiment 2: Rats were divided into four main groups (n = 8). Seven days after recovering from the stereotaxic surgery, modafinil (50, 100 and 250 μM/side) was infused bilaterally for 4 consecutive days in a volume of 1 μL/side into the CA1 area of the hippocampus 30 min prior to training trials in the MWM task. In pilot study, two groups including modafinil (750 μM/side and 1 mM/side) were chosen and modafinil was infused bilaterally for 4 consecutive days as a same manner. The control group received DMSO in a volume of 1 μL/side for 4 consecutive days. Also, the quantity of BrdU-positive cells was assessed in the main mentioned groups.
Experiment 3: The preventive effects of bilateral intra-hippocampal infusion of modafinil (50, 100 and 250 μM/side, 5 min before testosterone infusion) on testosterone (100 μg/side) - induced spatial learning deficits in MWM were investigated in this part of study. The control group is given DMSO/DMSO in a volume of 1 μL/side for 4 consecutive days. Also, the numbers of BrdU-positive cells were assessed.

Neurogenesis assessments

Rats of each group were injected with 5-bromo-2-deoxyuridine (BrdU) as a marker of DNA-synthesis (Sigma, U.S.A., 50 mg/kg/day, i.p.) that was dissolved in 0.9% NaCl during the all training days in MWM.

Tissue sampling

One day after the last i.p. administrations of BrdU, rats were anesthetized with ketamine and xylazine and perfused transcardially with ice cold PBS (0.1 M), followed by 4% paraformaldehyde in PBS. Brains were removed from the skulls and stored at 4% paraformaldehyde at 4°C for two days followed by graded sucrose solution treatment. Coronal sections (30 μm) from the dentate gyrus of the hippocampal area were chosen with an OCT medium using a cryomicrotome and transferred on slides.

BrdU immunohistochemistry

BrdU immunohistochemistry was carried out with the BrdU Immunohistochemistry Kit (ab125306). The brain sections incubated with the quenching solution for 10 min. Two drops of trypsin enzyme were then added to each slide and incubated at 25°C for 10 min, followed by a 3 min rinse in distilled water. Two drops of the denaturing solution were added to each slide and incubated at room warmth for thirty min. The sections were then incubated at room temperature with blocking buffer (10 min), BrdU antibody (ab6362; 1:200) (60 min), and streptavidin-horseradish peroxidase (HRP) conjugate (10 min). The new-born cells were counted under a light microscope (400×) [54].

Quantitation of BrdU - labelled cells

Fifth sections throughout the hippocampus were applied for BrdU immunohistochemistry. All BrdU-labelled cells in dentate gyrus were considered using a light microscope to differentiate single cells within clusters; all counts were performed at 400× magnification.

Statistical Analysis

By using the Graph Pad Prism 5, a p-value of 0.05 or less was considered statistically significant.

Results and Discussion

Findings of the current study showed that all control, testosterone enanthate (TE) and modafinil – treated animals were well trained in MWM task as pointed out by the significant decrease in escape latency and travelled distance parameters by comparison of the first and last day of training in the MWM task (Tables I and II).

Table I

Behavioural parameters for testosterone groups. $p < 0.05$ (a), $p < 0.01$ (b), $p < 0.001$ (c) and $p < 0.0001$ (d)

Treatments Groups	*Escape Latency (sec)*		*Travelled Distance (cm)*		*Swimming Speed (cm/sec)*	
	Day 1	*Day 4*	*Day 1*	*Day 4*	*Day 1*	*Day 4*
Control (DMSO)	64.10 ± 4.95	35.52 ± 5.47[c]	2018 ± 172.4	1209 ± 189.8[b]	31.85 ± 0.78	34.82 ± 1.30
Testosterone 10	57.19 ± 6.08	37.05 ± 4.85[a]	1756 ± 191.5	1150 ± 167.5[a]	30.36 ± 0.60	30.02 ± 0.89
Testosterone 50	73.38 ± 4.72	40.97 ± 4.74[d]	2402 ± 161	1386 ± 167.7[d]	33.33 ± 0.90	34.66 ± 1.55
Testosterone 100	68.28 ± 5.08	36.66 ± 4.60[d]	2272 ± 186.7	1108 ± 132.8[d]	31.81 ± 1.02	30.67 ± 0.93

Table II

Behavioural parameters for modafinil groups. $p < 0.01$ (b) and $p < 0.0001$ (d)

Treatments Groups	*Escape Latency (sec)*		*Travelled Distance (cm)*		*Swimming Speed (cm/sec)*	
	Day 1	*Day 4*	*Day 1*	*Day 4*	*Day 1*	*Day 4*
Control (DMSO)	76.94 ± 5.69	48.00 ± 7.35[b]	2435 ± 199.5	1501 ± 236.5[b]	31.07 ± 0.95	30.43 ± 1.04
Modafinil 50	81.26 ± 3.82	39.40 ± 6.07[d]	2200 ± 100.3	1091 ± 164.9[d]	27.47 ± 0.61	28.91 ± 0.92
Modafinil 100	82.18 ± 3.44	33.65 ± 5.51[d]	2204 ± 92.57	941.1 ± 148.5[d]	27.19 ± 0.63	29.08 ± 0.80
Modafinil 250	80.35 ± 3.92	57.96 ± 7.70[b]	2603 ± 153.1	1857 ± 247.0[b]	32.70 ± 1.01	31.02 ± 1.10

The present data indicate that bilateral intra-hippocampal (i.h.) infusion of testosterone enanthate (50 and 100 μg/side) impaired spatial learning by a significant increase in time and distance to reach the hidden platform during training days (Figures 1A and 1B). One day after completion of training (day 5), by measuring the time spent in target quadrant for 90 s (Probe test), testosterone (100 μg/side) – treated animals showed a significant decrease ($*p < 0.05$) in time spent in target quadrant compared to control animals (Figure 5A). Also, the quantity of BrdU-positive cells in dentate gyrus was decreased in testosterone (100 μg/side) – treated animals in comparison to control group (Figure 2).

It has been shown that TE – induced long term memory deficits may occur *via* both genomic and non-genomic pathways [28, 46]. In addition, previous studies have been suggested that, conversion of TE to oestradiol (E), is one of the probable destructive mechanisms of TE on spatial learning and memory processes. Interestingly, anastrozole (an aromatase inhibitor), reversed TE – induced learning impairments, too [37, 39].

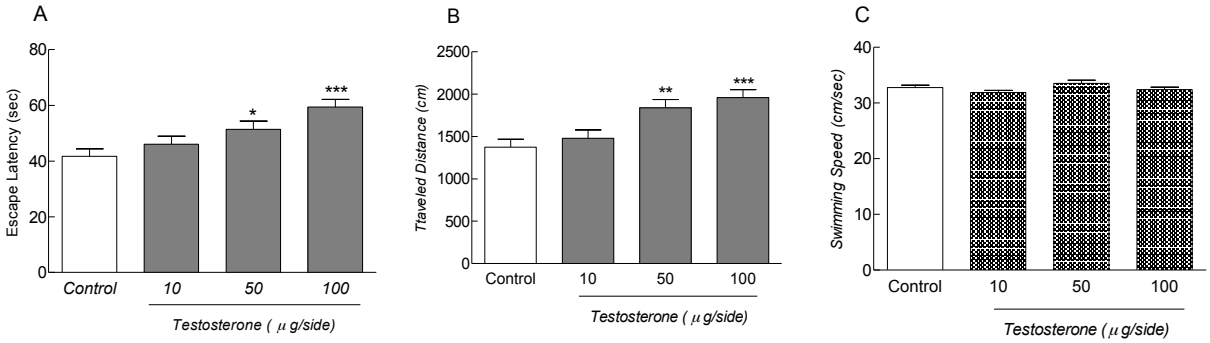

Figure 1.

Average of escape latency (A), travelled distance (B) and swimming speed (C) in MWM at different bilateral intra-hippocampal doses of testosterone enanthate. Each value represents the mean ± S.E.M.
*p < 0.05, **p < 0.01 and ***p < 0.001 significantly different from the control (DMSO) animals (n = 8).

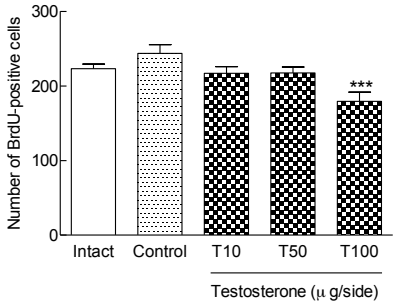

Figure 2.

Quantitative evaluation of the number of BrdU-positive cells in dentate gyrus (DG) in testosterone – treated animals. Each value represents the mean ± S.E.M.
***p < 0.001 significantly different from control (DMSO testosterone – treated) animals (n = 6).

Due to the role of several neurotransmission systems in the hippocampus; an important region for cognitive functions, critical effects of neuro-active steroids may occurs *via* modulatory interactions with these classical transmission pathways such as glutamatergic, GABAergic and cholinergic systems in cognitive processes [47, 48, 65].

In accordance with these data, it has been reported that there is a relationship between TE – induced memory impairments and acetylcholine level decrease [1, 2, 6].

On the other hand, a significant decrease in NMDA receptor's function was observed as a result of decreased function of sigma receptors by testosterone as a non-selective sigma (σ) antagonist [1].

Behavioural and immuno-histochemical results of this research confirmed previous reports about the negative effects of testosterone enanthate on spatial memory.

Four days bilateral infusion of modafinil (100 μM/side, i.h.), caused a significant decrease in distance of reaching the unseen platform in MWM (Figure 3B) and also increased the quantity of BrdU-positive cells in dentate gyrus (Figure 4) in comparison with control animals. Higher doses of modafinil (750 and 1000 μM/side) in pilot study, did not cause any significant alterations in comparison with control and modafinil (250 μM/side) – treated animals (data were not shown).

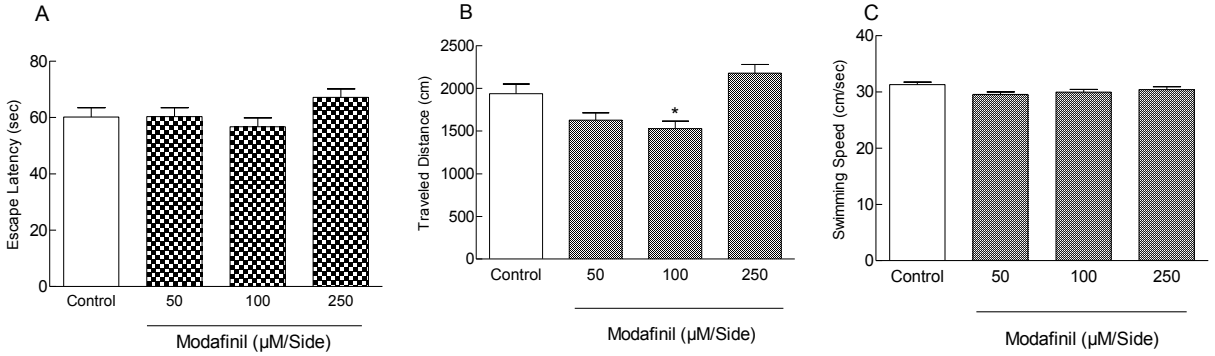

Figure 3.

Average of escape latency (A), traveled distance (B) and swimming speed (C) in MWM at different bilateral intrahippocampal doses of modafinil. Each value represents the mean ± S.E.M.
*p < 0.05 significantly different from the control (DMSO testosterone – treated) animals (n = 8).

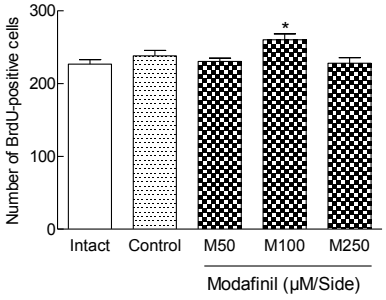

Figure 4.

Quantitative evaluation of the number of BrdU-positive cells in dentate gyrus (DG) in modafinil – treated animals. Each value represents the mean ± S.E.M. *p < 0.05 significantly different from control (DMSO testosterone – treated) animals (n = 6).

On day 5, by measuring the time spent in target quadrant for 90 s (Probe test), modafinil (100 μM/ side) – treated animals showed a significant increase (*p < 0.05) in time spent in target quadrant compared to control (DMSO) animals (Figure 5B).

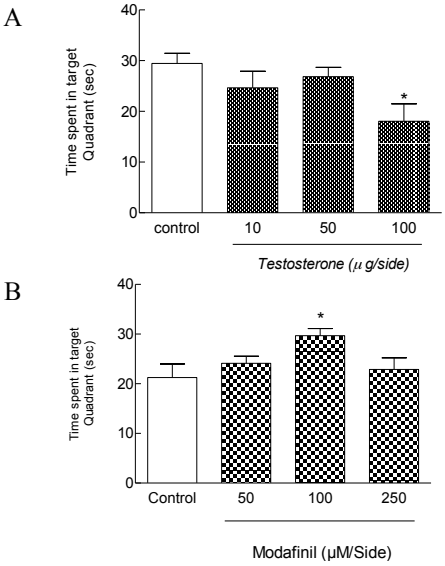

Figure 5.

Time spent in target quadrant one day after completion of training in MWM (Probe test) in testosterone – (A) and modafinil – (B) treated animals. *p < 0.05 significantly different from their related control groups. Values are presented as mean ± S.E.M. (n = 8).

Visible platform trials evaluation showed that there were no considerable differences in performance between the treated groups for latency (day 5) in both experiments 1 (Figure 6A) and 2 (Figure 6B). Open-field locomotor activity assessments showed that there were no considerable differences in open-field parameters (crossing and rearing) in testosterone or modafinil – treated animals in comparison with their related control groups (day 5) in both experiments 1 (Table III) and 2 (Table IV).

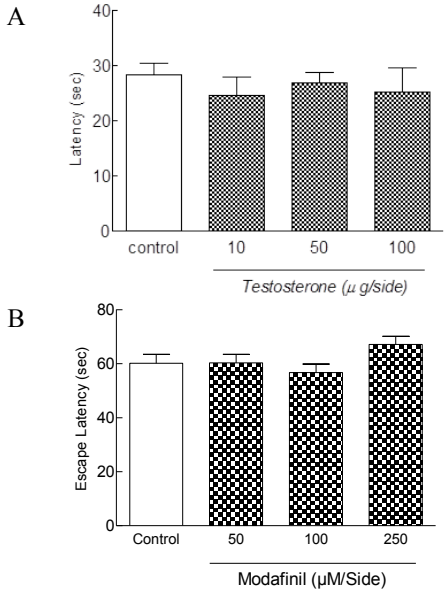

Figure 6.

Evaluation of latency time to find the visible platform in testosterone and modafinil treated animals. It can be inferred that there were no alterations in motivational, motor, and sensory processes.

Table III

Locomotor activity parameters in testosterone groups

Groups	***Open-field parameters***	
	Crossing	***Rearing***
Control (DMSO)	97.78 ± 9.54	16.44 ± 2.10
Testosterone 10	124.9 ± 11.83	14.71 ± 1.63
Testosterone 50	134.3 ± 8.38	13.22 ± 2.15
Testosterone 100	131.4 ± 9.22	16.22 ± 1.33

Table IV

Locomotor activity parameters in modafinil groups

Groups	***Open-field parameters***	
	Crossing	***Rearing***
Control (DMSO)	119.5 ± 12.15	11.17 ± 1.11
Testosterone 10	157.7 ± 11.60	15.50 ± 3.02
Testosterone 50	153.9 ± 12.53	16.86 ± 3.09
Testosterone 100	111.2 ± 13.43	11.83 ± 2.01

Modafinil as a psychostimulant may act *via* distinct neural pathway from other typical psychostimulants. This drug relies on dopamine transporters but does not raise dopamine levels in the *nucleus accumbens* and that is why it has lower abuse potential than mentioned psychostimulants [52].

Studies have been suggested that modafinil modifies the activity of hippocampus and prefrontal cortex [16, 63]. Also, it can advance the performance of tasks that depend on hippocampal functioning [10, 18] and affects many neurotransmitter systems [8, 15-17, 27, 34, 50, 61].

One of the other possible mechanisms of modafinil's action is that modafinil presumably activates D1 receptors that couple to three signalling cascades: (1) the Gs/o/adenylyl cyclase/cAMP signaling; (2) the Gq/phospholipase C/inositol 1,4,5-triphosphate and

(3) the Ras-kinase/ERK kinase signalling [40]. Also, it has been reported that modafinil improves methamphetamine – induced cognitive impairments *via* ERK1/2 signalling pathway modulation, in prefrontal cortex (PFC) [3, 19, 22, 44].

Evidences have been suggested that neurogenesis in the DG is related with cognitive processes [13, 21, 31].

It is notable that disruptions in adult hippocampal neurogenesis lead to reduction in hippocampus-dependent memory, and addition of new hippocampal neurons are related with improved learning and memory functions [9, 21, 30, 53, 64].

Modafinil – induced spatial learning and memory improvements in MWM may be caused as a result of increase in synaptic plasticity in dentate gyrus [8, 10]. The mechanisms underlying modafinil effects on hippocampal neurogenesis remain unknowable [8]. Modafinil has been shown to increase hippocampal activity and probably influencing the development of new-born neurons by this mechanism [7, 43]. Behavioural and immune-histochemical findings of this part of study are in agreement with some previous reports about the improvement effects of modafinil on spatial learning and memory processes.

No considerable differences were found in swimming speed between all treated animals and their related control animals, which is indicative for the non-existence of motor turbulences. Since there were no statistical differences between the control and experimental groups (testosterone or modafinil – treated animals) in probe test (the platform was visible) or in open-field task (assessment of crossing and rearing parameters), it can be inferred that there were no alterations in motivational, motor and sensory processes.

One of the main results of the current research is that modafinil (100 μM/side, i.h.), 5 min before testosterone (100 μg/side, i.h.), significantly decreased the escape latency and travelled distance parameters compared to testosterone – treated animals (Figures 7A and 7B), increased the time spent in target quadrant (day 5, probe test) to a level of control group in MWM task (Figure 8) and also increased the quantity of BrdU-positive cells in dentate gyrus in comparison with testosterone – treated animals (Figure 9).

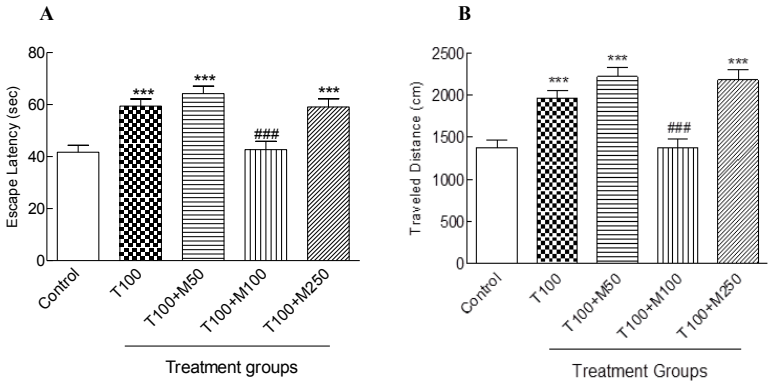

Figure 7.

Average of escape latency (A) and travelled distance (B) in MWM in testosterone/modafinil combination groups Each value represents the mean ± S.E.M. ***p < 0.001 significantly different from control and ###p < 0.001 significantly different from testosterone (100 μg/side) – treated animals (n = 8).

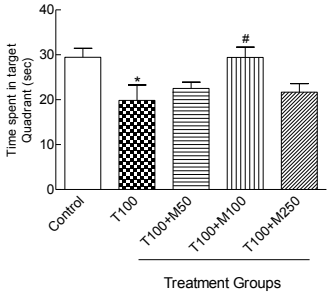

Figure 8.

Time spent in target quadrant one day after completion of training in MWM (Probe test) in testosterone/ modafinil combination group.

*p < 0.05 significantly different from control and #p < 0.05 significantly different from testosterone (100 μg/side) – treated animals (n = 8).

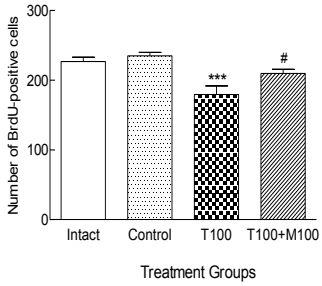

Figure 9.

Quantitative evaluation of the number of BrdU-positive cells in dentate gyrus (DG) in testosterone/ modafinil – treated animals. Each value represents the mean ± S.E.M. ***p < 0.001 significantly different from control and #p < 0.05 significantly different from testosterone (100 μg/side) – treated animals (n = 6).

Conclusions

Thus, due to the interaction of testosterone and modafinil with various neurotransmitter systems, it is reasonable that modafinil probably acts against testosterone-induced learning deficits *via* several possible mechanisms such as modulation of cholinergic, glutamatergic, GABAergic and cAMP/PKA signalling pathways.

Acknowledgement

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