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EFFECTS OF GONADECTOMY AND AVOIDANCE LEARNING ON THE $GABA_{A\alpha1}$ RECEPTOR DENSITY IN THE PREFRONTAL CORTEX OF MALE AND FEMALE RATS

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We evaluated the effects of gonadectomy and passive avoidance learning on the density of $GABA_{A\alpha1}$ receptors in the prefrontal cortex of male and female rats. Twenty adult males weighing 200 ± 30 g and 20 adult females weighing 150 ± 20 g were divided into four groups: (i) Sham, intact rats with no learning session, (ii) Sham-L, intact rats subjected to the avoidance learning session, (iii) GE, gonadectomized rats without learning, and (iv) GE-L, gonadectomized rats with learning. A shuttle box was used for the induction of passive avoidance learning. The density of $GABA_{A\alpha1}$ receptors was investigated with an immunohistochemical technique; Image Analyzer software was used. Ovariectomy without learning led to significant reduction of the density of $GABA_{A\alpha1}$ receptors in different regions of the prefrontal cortex relative to the control intact group; at the same time, ovariectomized females with learning demonstrated a significantly higher density of $GABA_{A\alpha1}$ receptors in the prefrontal cortex as compared to the Sham-l group. No significant differences in the density of $GABA_{A\alpha1}$ receptors were observed in both castrated male rat groups. The comparison of male and female rats showed that the density of $GABA_{A\alpha1}$ receptors in castrated rats with learning was significantly lower than that in ovariectomized females with learning. Thus, ovariectomy exerts a more potent effect than castration on the $GABA_{A\alpha1}$ receptor density in different regions of the prefrontal cortex. Learning provides increases in the $GABA_{A\alpha1}$ receptor density in different regions of the prefrontal cortex in female rats, while castration of male rats exerts no significant effect from this aspect.

Keywords: gonadectomy, passive avoidance learning, prefrontal cortex, $GABA_{A\alpha1}$, receptors.

INTRODUCTION

During the ontogenesis, gonadal hormones play important roles in the differentiation of CNS cells and formation of neuronal networks. In several brain regions, exposures to testosterone or estrogen within a certain critical period permanently cause significant organizational differences between males and females [1]. There is increasing evidence suggesting that gonadal steroids exert powerful effects on the CNS. Although it is clear that there is a relationship between gonadal steroids and cognitive functions of the CNS, there are conflicting data about the effect of gonadal steroids on learning and memory. Sex steroid hormones act directly and indirectly via different neurotransmitter systems in the CNS. One of these systems is the gamma amino butyric acid A ($GABA_A$) receptor complex that is the main inhibitory system in the mammalian CNS [2].

Inhibitory effects of GABA are realized through two major types of receptors, ionotropic receptors with ligand-gated ion channels ($GABA_A$ and $GABA_C$ receptors) and metabotropic receptors that are G-protein-coupled $GABA_B$ receptors acting via second messengers [3]. $GABA_A$ receptors are pentamers composed of subunits derived from the α, β, γ, δ, θ, ε, and π gene families. There are several different ligands that bind to $GABA_A$ receptors; many of them have distinct binding sites. One of these sites is a binding site for neurosteroids. Steroids capable of influencing receptors in the brain via non-genomic mechanisms are termed neuroactive steroids, or neurosteroids; those

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can be positive and negative endogenous modulators of the $GABA_A$ receptor function [4].

In the brain, testosterone is converted into a more potent androgen, dihydrotestosterone (DHT), by 5α-reductase; it can also be converted into estradiol (E2) by cytochrome P450 aromatase (P450 arom). Estrogens are able to affect both allopregnanolone levels and $GABA_A$ receptor expression [5]. An increasing number of reports during the past two decades have described numerous effects induced by steroids via the $GABA_A$ receptor complex in the brain. For instance, allopregnanolones exert biphasic effects; at low concentrations, they potentiate GABA currents, while at higher concentrations they activate the receptors directly, indicating the presence of two distinct binding sites [5].

Our understanding of the function of GABAergic interneurons is challenged by their startling heterogeneity; indeed, different subtypes of interneurons display distinct morphology, physiological properties, connectivity patterns, and biochemical constituents. It has been reported that $GABA_A$ receptors are responsible for mediating a wide range of activities, including anticonvulsant, sedative, and hypnotic effects. It is also well known that the $GABA_A$ receptors affect learning and memory processes. The α_1 subunit-containing $GABA_A$ receptor is the major subtype contributing to about 60% of all $GABA_A$ receptors in the brain. Behavioral examination of genetically modified animals has shown that the α_1 subunit-containing $GABA_A$ receptors play an important role in modulation of memory acquisition. $GABA_A$ receptor agonists impair memory, while their antagonists facilitate retrieval in different tasks. There is a profound difference in the affinity of $GABA_A$ receptors in different brain regions. Furthermore, recent studies have suggested that an altered cortical GABA transmission could be involved in the prefrontal dysfunction in psychiatric disorders, such as schizophrenia. The GABA system is very essential for the prefrontal cortex function [6].

The prefrontal cortex is crucially involved in many higher cognitive functions, such as planning, goal-directed behavior, and working memory. Evidence from animal studies indicates that the anterior cingulate cortex is involved in the memory formation in training on aversive tasks [7]. Most information has been derived from studies on conditioned behavior, in particular avoidance behavior in rats. In these tasks, an aversive situation was used as a stimulus for learning. Passive avoidance learning refers to the learned inhibition of behavior in order to avoid a punishment. It is a kind of emotional memory measurement. Research on passive avoidance learning has been useful in clarifying the association of personality and temperament characteristics with disinhibited behavior [8].

As was mentioned above, many earlier studies described the effects of the GABAergic system and neurosteroids in the prefrontal cortex on learning and memory, but the effect of learning and also actions of sex hormones on the $GABA_A$ receptor density in the prefrontal cortex have not been compared. So, the aims of our investigation were (i) to compare the $GABA_{A\alpha1}$ receptor density in the cingulate cortex area 1 (Cg1), primary motor cortex (M1), and secondary motor cortex (M2) in male and female rats using an immunohistochemistry technique, and (ii) to estimate the effect of learning in a passive avoidance procedure and of gonadectomy on the $GABA_{A\alpha1}$ receptor density in the Cg1, M1, and M2 areas of the prefrontal cortex in male and female rats.

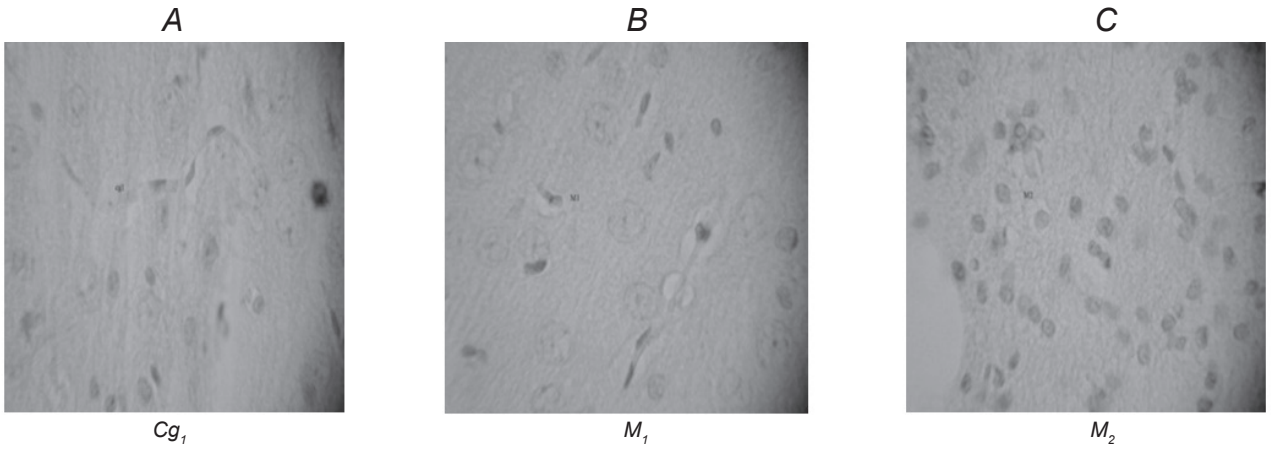
METHODS

Forty adult Sprague–Dawley rats, 20 males weighing, on average, 200 ± 30 g and 20 females weighing 150 ± ± 20 g, were used.

Male and female rats were randomly divided into four equal groups: (i) Sham, intact rats without learning (incision was made without removal of the gonads), (ii) Sham-L, animals without gonad removal learned for passive avoidance, (iii) GE, gonadectomized rats without learning, and (iv) gonadectomized rats with learning (GE-L). Animals in the non-learned groups did not have any learning experience. In the learned groups, animals were handled for 10 days before the learning procedure for reduction of the influence of shuttle-box shock.

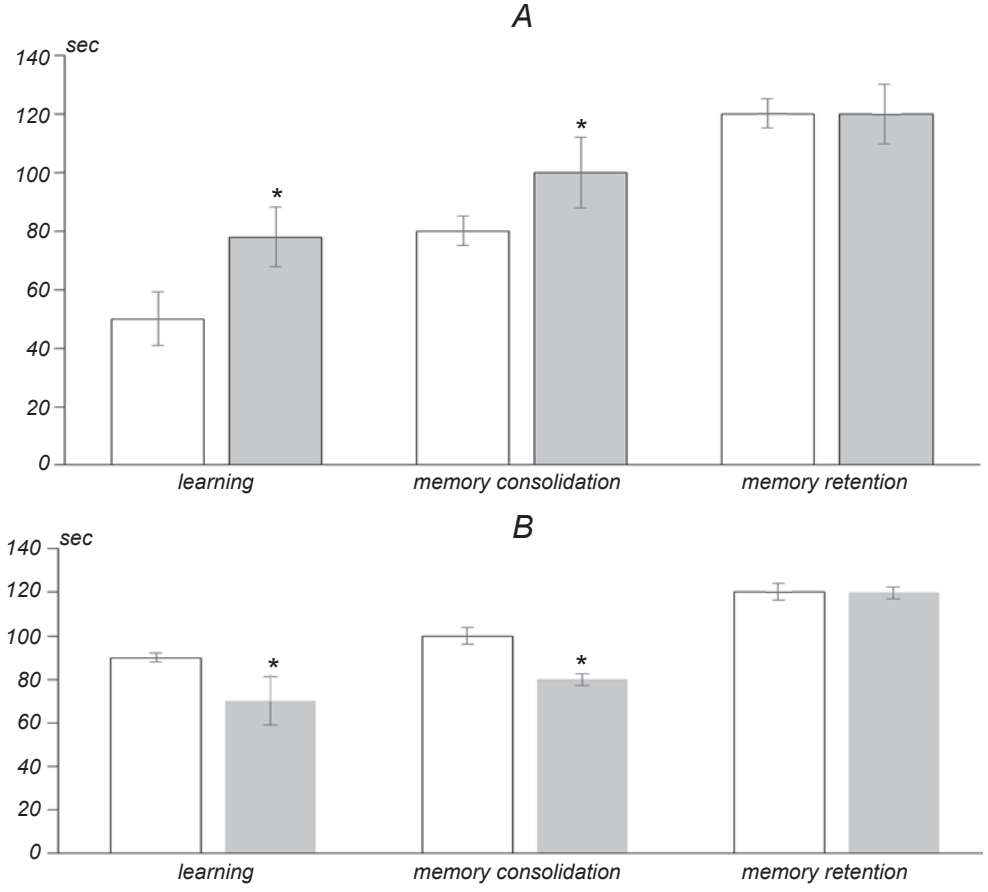
Gonadectomy Procedures. For castration, male rats were anesthetized by thiopental sodium. A horizontal incision was made in the scrotum, and the testes were tied off and removed with a cut distal to the ligature; then the incision was sutured. For ovariectomy, female rats were anesthetized by the same dose of thiopental sodium and ovariectomized through a midline laparotomy under sterile conditions.

Behavioral Procedure. Twenty days after gonadectomy in the respective groups, the learning



F i g. 1. Photomicrographs of immunohistochemical detection of negative control in the Cg1, M1, and M2 areas (A–C, respectively). Magnification ×400.

Р и с. 1. Мікрофотографії результатів імуногістохімічного виявлення в зонах *Cg1, M1* та *M2* щурів групи негативного контролю.



F i g. 2. Effects of gonadectomy on passive avoidance learning (time spent in the light compartment, sec). A and B) Males and females, respectively. Open and dashed columns show data for Sham-L and castrated/ovariectomized rats, respectively. Asterisks show cases of significant intergroup differences ($P < 0.05$).

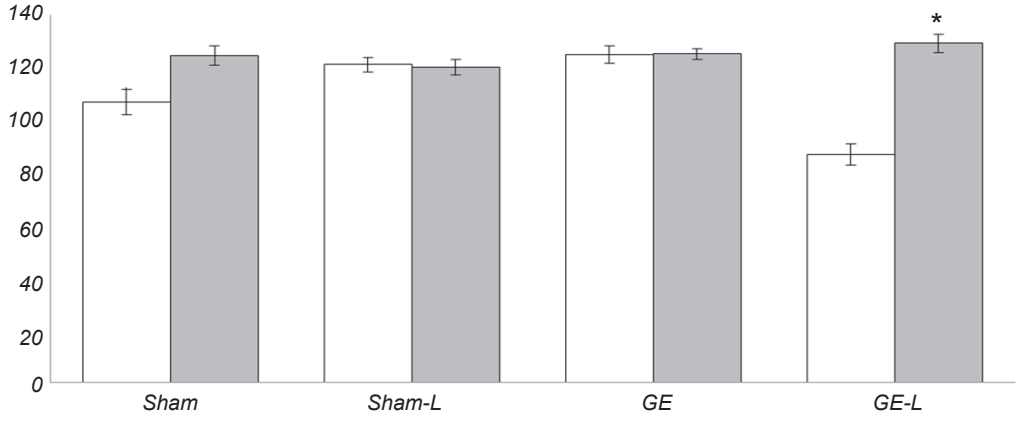
Р и с. 2. Вплив гонадектомії на результати навчання пасивному униканню.

procedure was performed in the Sham-L and GE-L groups. A two-way shuttle-box (Aryo Azma Co, Iran) with acrylic walls and steel floor bars was used for learning. The box, 44×20×19 cm, was bisected by a vertical partition with an opening in the middle allowing the animal to move freely from one compartment to another (including light and dark ones). In the light compartment, the animal was safe, while in the dark compartment it received foot electrical shocks of 0.6 mA for 1 sec, with a latent period of 1 sec.

On the first day, all animals were individually subjected to 2 min of adaptation to the shuttle box, within which the rat could explore the light compartment and move about freely. Within this stage (since rats like the dark compartment), if the rat did not move to the dark compartment in 120 sec, it was removed from the study. This adaptation was repeated 30 min later. On the second day, the rats were placed in the light compartment of the box and, 1 sec after entering the dark compartment, the animal received a 0.6 mA foot shock for 1 sec. On the third day, the procedure was similar to the initial latency day; the third day was considered learning one. On the fourth day, the procedure of memory consolidation was like that within learning days, but without foot shocks. On the fifth day (memory retention), the procedure was similar to that on the fourth day. The rats were considered completely learned if they did not move to the dark compartment in 120 sec during the third, fourth, and fifth sessions.

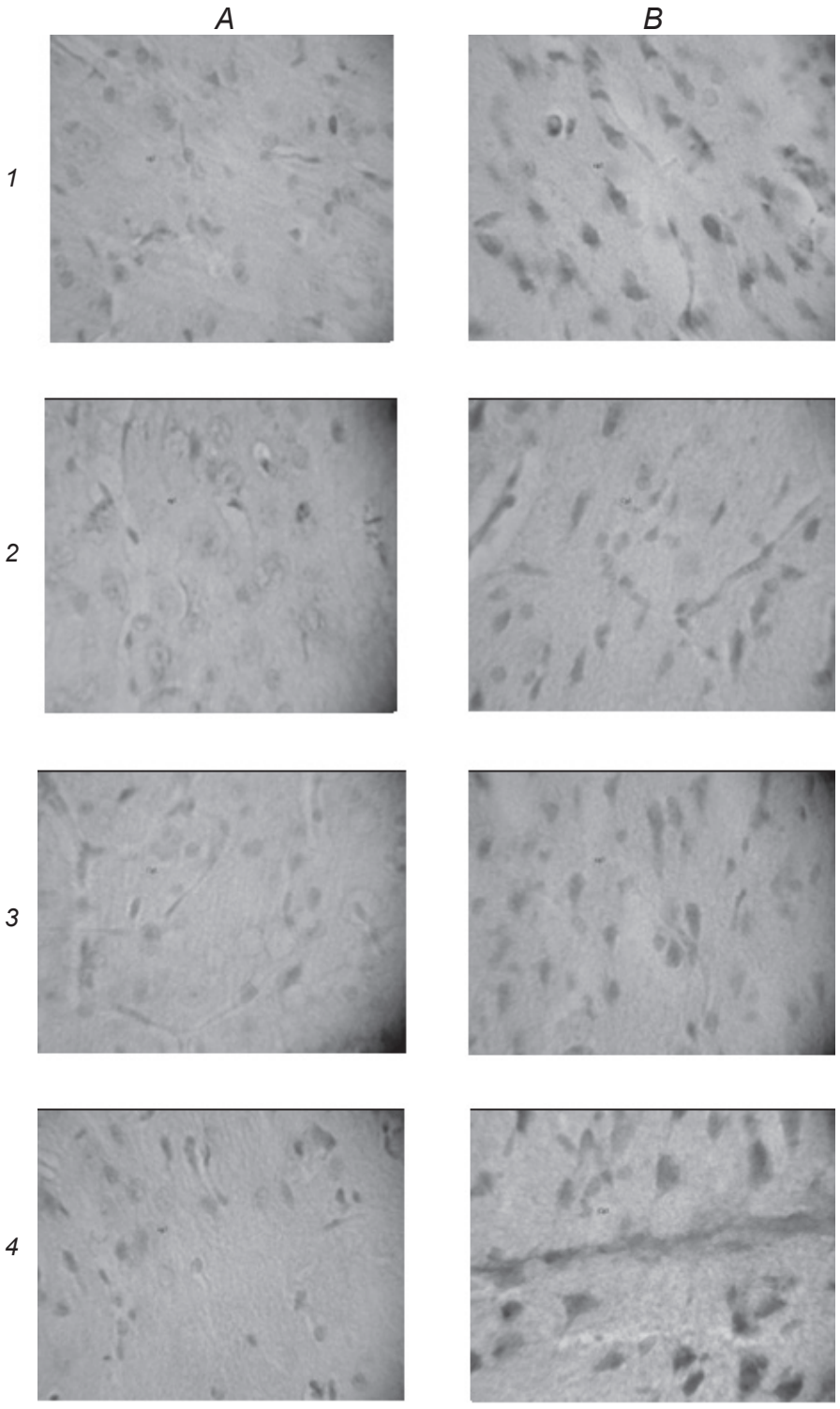
Tissue Preparation. In all groups, after the last learning session, the rats were given with a sodium thiopental overdose (120 mg/kg). After heart perfusion with 10% formaldehyde, the brains were removed, washed with normal saline, and fixed for 72 h in 10% formaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Then the brains were post-fixed in 4% formaldehyde in 0.1 M PB (pH 7.4). Paraffin embedding was done using standard techniques, and 5-μm-thick sections were prepared and mounted on 25% L-lysine-coated glass slides.

Immunohistochemical Study. The mounted slides were dried and stored in a –20°C freezer until used for antibody labeling. For this, the slides were brought to room temperature, washed, and the slides outlined with a liquid-repellent slide marker pen (to retain reagents on the slides during the immunostaining procedure). Slides were incubated overnight with primary antibodies against $GABA_{A\alpha1}$ receptor (Abcam, Great Britain; $4 \cdot 10^{-3}$ dilution). On the next day, slides were rinsed in PBS and incubated in secondary antibody (DAKO Co., Denmark; envision) and next time washed in PBS. Finally, the slides were rinsed in PBS three times for 10 min and reacted with a cromogen (Dab; DAKO Co., Denmark). After rinsing in PBS, the slides were stained with hematoxylin (staining of the nuclei). Negative-control sections were incubated with PBS in the absence of primary antibody, and no immunoreactivity was detected. All procedures in all groups and in all individuals were the same and parallel, and the procedures in groups were



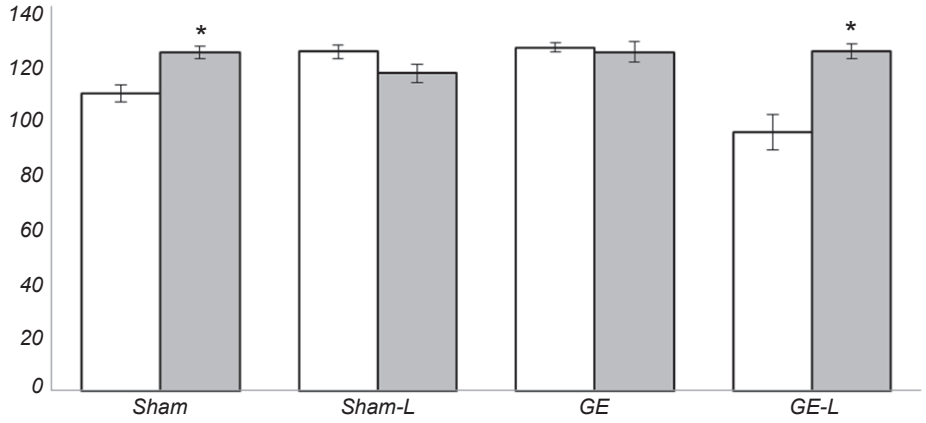
F i g. 3. Comparison of the $GABA_{A\alpha1}$ receptor density in the Cg1 area of male and female rats (open and dashed column, respectively). Results are expressed as means ± s.e.m. Vertical scale) Color intensity, pixels. Asterisks in this and other figures show cases of significant differences ($P < 0.05$).

Р и с. 3. Порівняння щільності $GABA_{A\alpha1}$-рецепторів у зоні *Cg1* самців та самиць щурів.



F i g. 4. Photomicrographs representing comparison of the GABA$_{Aα1}$ receptor density in the Cg1 of male (A) and female (B) rats. 1–4) Experimental groups, Sham, Sham-L, GE, and GE-L (see Methods), respectively. ×400.

Р и с. 4. Мікрофотографії результатів виявлення GABA$_{Aα1}$-рецепторів у зоні *Cg1* самців та самиць щурів.



F i g. 5. Comparison of the $GABA_{A\alpha1}$ receptor density in the M1 area of male and female rats. Designations are similar to those in Fig. 3.

Р и с. 5. Щільність $GABA_{A\alpha1}$-рецепторів у зоні *M1* щурів різних груп.

done randomly. Figure 1 shows the negative control. In these slides, there was no brown color due to the reaction of primary antibody.

After preparing digital images of the slides, the density of α1 subunits of $GABA_A$ receptors was analyzed using Image Analyzer program (version 1.33) for immunohistochemistry procedures. The density of receptors was estimated according to three characters, hue, saturation, and intensity. Then, a number reverse with respect to the receptor density was calculated, meaning that a higher receptor density is represented by a smaller number in the program. Color staining of the neurons was measured in different regions of the prefrontal cortex.

Statistical Analysis. Numerical data are shown below as means ± s.e.m. Statistical analysis was performed using SPSS (version 18) software. One-way ANOVA and the Tukey's *post hoc* test were used to determine the differences between groups. $P < 0.05$ was considered as the level of significance.

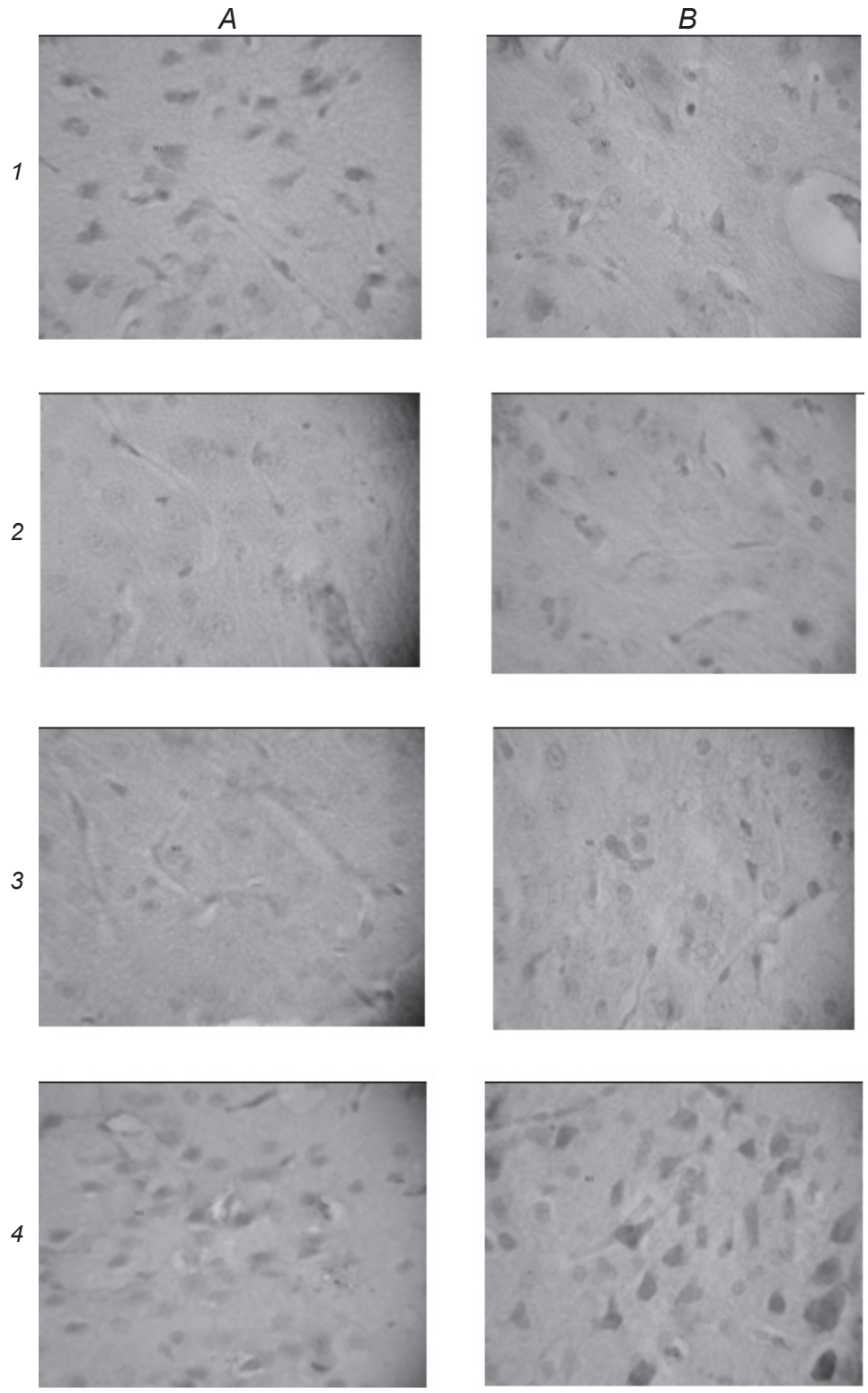
RESULTS

Effects of Gonadectomy on Passive Avoidance Learning. According to the obtained data, castration of male rats was accompanied by noticeable increases in the indices of learning and memory consolidation in the passive avoidance learning procedure ($P < 0.05$; Fig. 2). On the other hand, ovariectomy in female rats significantly ($P < 0.05$) suppressed learning and memory consolidation in the above learning procedure (Fig. 2).

Effects of Castration and Passive Avoidance Learning on the Density of $GABA_{A\alpha1}$ Receptors in the Areas Cg1, M1, and M2 of the Prefrontal Cortex. To determine whether a combination of castration and passive avoidance learning can alter the $GABA_{A\alpha1}$ density in the Cg1, M1, and M2 zones, the immunohistochemistry method was used; this procedure was demonstrated to be a sensitive test for assay of $GABA_{A\alpha1}$ [9, 10]. Our data showed that there was no significant differences ($P > 0.05$) between the densities of $GABA_{A\alpha1}$ in the Cg1, M1, and M2 of male rats (Figs. 3 to 8). Passive avoidance exerted no significant effect on the density of $GABA_{A\alpha1}$ in the Cg1 and M2, but significantly increased the respective index in the M1 region of the prefrontal cortex.

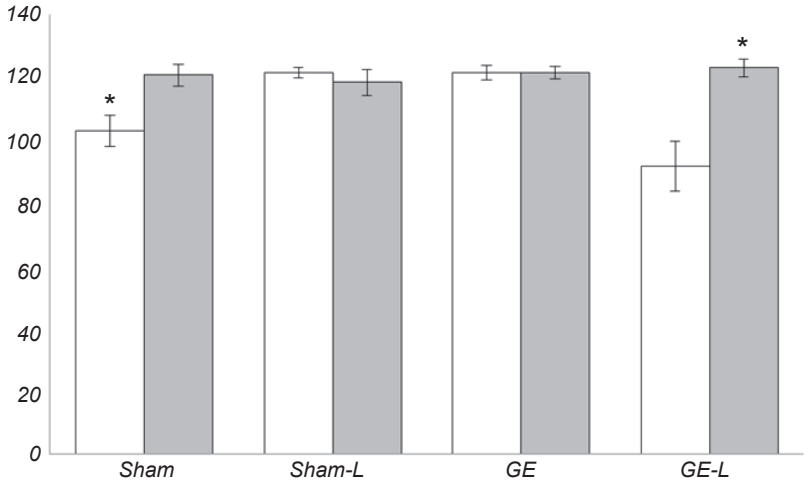
Effects of Ovariectomy and Passive Avoidance Learning on the Density of $GABA_{A\alpha1}$ in the Regions Cg1, M1, and M2 of the Prefrontal Cortex. In female rats, passive avoidance learning induced a significant ($P < 0.05$) decrease in the density of $GABA_{A\alpha1}$ receptors in the Cg1, M1, and M2 of female rats. The $GABA_{A\alpha1}$ receptor density significantly increased in ovariectomized rats with learning, while ovariectomy without learning significantly decreased this index relative to that in control female rats (Figs. 3 to 8).

Comparison of $GABA_{A\alpha1}$ Receptor Density in the Areas Cg1, M1, and M2 of Male and Female Rats. Figures 3 to 8 show that the densities of $GABA_{A\alpha1}$ receptors in the Cg1, M1, and M2 of control females were significantly higher than those in control males. In the Sham group, this density only in the M1 region was significantly lower in female rats than in males. In ovariectomized rats subjected to learning, the density



F i g. 6. Photomicrographs representing comparison of the $GABA_{Aα1}$ receptor density in the M1. Designations are similar to those in Fig. 4.

Р и с. 6. Мікрофотографії результатів виявлення $GABA_{Aα1}$-рецепторів у зоні *M1* самців та самиць щурів.



F i g. 7. Comparison of the $GABA_{A\alpha1}$ receptor density in the M2 area of male and female rats. Designations are similar to those in Fig. 3.

Р и с. 7. Щільність $GABA_{A\alpha1}$-рецепторів у зоні *M2* щурів різних груп.

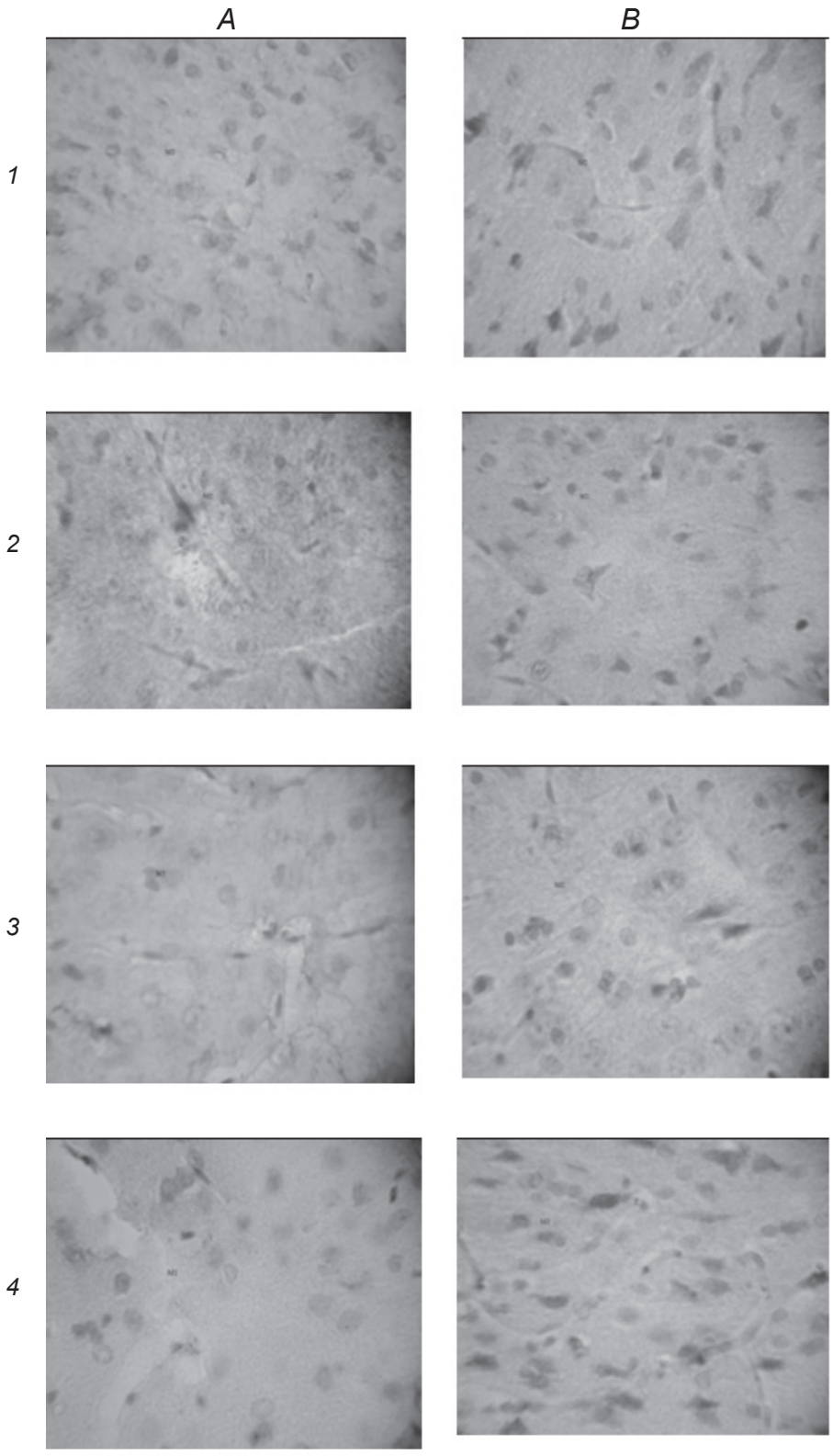
of $GABA_{A\alpha1}$ receptors in the Cg1, M1, and M2 significantly ($P < 0.05$) exceeded the respective index in castrated male rats with learning.

DISCUSSION

We have found that castration of male rats led to some increases in the indices characterizing learning and memory in the passive avoidance task, while ovariectomy decreased these indices. The literature shows that androgen-related effects on learning and memory in adult animals and humans are complex and contradictory. Androgens can improve the cognitive performance; e.g., testosterone replacement in gonadectomized rodents increases acquisition in the T-maze [11]. In contrast, other studies suggested that high levels of androgens might adversely affect memory in humans [12] and laboratory animals [13]. On the other hand, many investigators have reported that castration does not affect learning [14, 15]. In many reports, it was shown that ovariectomy impaired spatial memory and led to morphological changes in cognitive centers of the rat brain [16–18].

Our results indicated that castration exerts no significant effect on the $GABA_{A\alpha1}$ receptor density in the Cg1 and M1 areas of the cortex. Neurosteroids are a class of endogenous steroids that are synthetized in the brain, adrenals, and gonads. These agents exert potent effects on the $GABA_A$ receptor; so, the effect of adrenalectomy/castration is mediated, at least partly, by changes in $GABA_A$ receptor function [19, 20]. Castration resulted in changes in [^{3}H] muscimol binding in the brain areas containing steroid receptors, while certain bain areas are deprived of these receptors. Castration was shown to lead to high binding levels in the preoptic anterior nucleus and in the anterior neostriatum area, i.e., in those brain areas that are known to contain gonadal steroid receptors [21]. Majewska et al. [22] demonstrated that the sulfated form of DHEA (DHEAS) binds to the $GABA_A$ receptors on rat neurosynaptosomes. Further, the authors showed that DHEAS binding decreases GABA-mediated current (a whole-cell voltage-clamp technique was used). Yoo et al. [23] reported that castration can lead to down-regulation of GABAergic neurons, which precedes or coincides with increased post-castration LH secretion, and the duration of the decrease is consistent with the less robust post-castration LH response. Ago et al. [24] showed that castration decreases pentobarbital-induced sleeping time in mice, suggesting that it reduces the $GABA_A$ receptor function. In our study, it may be that testosterone released from the adrenal glands compensated the deficiency of gonadal testosterone; therefore, we did not observe a significant change in the $GABA_{A\alpha1}$ receptor density in castrated male rats.

Our results showed that ovariectomy leads to reduction in the $GABA_{A\alpha1}$ receptor density in the Cg1 and M1 cortex areas. Wu et al. [25] have shown that



F i g. 8. Photomicrographs representing comparison of the $GABA_{A\alpha1}$ receptor density in the M2. Designations are similar to those in Fig. 4.

Р и с. 8. Мікрофотографії результатів виявлення $GABA_{A\alpha1}$-рецепторів у зоні *M2* самців та самиць щурів.

cyclic elevations in the progesterone levels in diestrus are accompanied by increased extrasynaptic $GABA_A$ receptor subunit expression in the hippocampus. There is evidence that cyclic fluctuations in steroid hormones within the estrous cycle regulate some $GABA_A$ receptor subunits. Juptner et al. [26] reported that $GABA_A$ receptors in the CNS are under a modulatory control of ovarian sex steroid hormones. The authors showed that ovariectomy enhances specific binding of [3H] muscimol to GABA receptor sites. It seems that these changes are due to cessation of ovarian steroid production, as they can be reversed by substitution of estradiol and progesterone. Scott and Clarke [27] suggested that GnRH secretion is regulated by GABAergic neurons at the level of the GnRH cell bodies in the preoptic area and that, during the breeding season, this is affected by $GABA_A$ and not by $GABA_B$ receptors. Decrease in the $GABA_{A\alpha1}$ receptor density in our study may result from the fact that estrogen enhances GAD activity in neurons [28]. Furthermore, stress is also associated in some way with changes in learning and memory; neuroactive steroids and neurosteroids are released in response to stress and, potently, can modulate $GABA_A$ receptors [5].

In our experiments $GABA_{A\alpha1}$ receptor density in the Cg1, M1, and M2 of ovariectomized learned female rats was significantly higher than that in castrated rat mails with learning. The effect of progesterone on $GABA_A$ receptors also depends on the metabolizing enzymes. Furthermore, the respective metabolites display both agonistic and antagonistic actions on $GABA_A$ receptors. The differences may be due to the choice of a ligand, neurosteroid concentrations, and animals [29] (rats compared to mice).

Therefore, we found that: (i) ovariectomy leads to suppression of learning and memory for passive avoidance, while castration of males enhances the respective indices somewhat; (ii) ovariectomy of female rats leads to significant decreases in the $GABA_{A\alpha1}$ receptor density in the Cg1, M1, and M2 areas of the prefrontal cortex, while castration exert no significant effect on the above index in the mentioned areas. Some studies in both human and animal models suggest that a balance between the serum testosterone and estradiol levels may be critical for the performance of memory tasks [30]; (iii) passive avoidance learning affects the $GABA_A$ receptor density in areas Cg1, M1, and M2 of the prefrontal cortex of females but not of gonadectomized male rats; (iv) the $GABA_{A\alpha1}$ receptor density increases in female rats following ovariectomy and learning while there is no significant change in castrated male rats. This density in the Cg1, M1, and M2 of ovariectomized rats after learning is significantly higher than that in castrated male rats. These results are in agreement with the statement [29] that the gonads are not the only source of modulators of $GABA_A$ receptors; another likely source of modulators is the adrenal cortex. It was found that intact or gonadectomized female rats displayed a markedly greater corticosterone response and more pronounced increase in GABA receptor binding than intact or castrated males after stress [29].

All procedures involving animal subjects were reviewed and approved by the Institutional Research Ethics Committee of the School of Veterinary Medicine of the Shiraz University.

The authors of this communication, A. Shojaee and M. Taherianfard, confirm the absence of any conflict related to commercial or financial interests, to interrelations with organizations or persons in any way involved in the research, and to interrelations of the co-authors.

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ВПЛИВИ ГОНАДЕКТОМІЇ ТА НАВЧАННЯ УНИКАННЮ НА ЩІЛЬНІСТЬ $GABA_{A\alpha1}$-РЕЦЕПТОРІВ У ПРЕФРОНТАЛЬНІЙ КОРІ САМЦІВ ТА САМИЦЬ ЩУРІВ

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Р е з ю м е

Ми оцінювали впливи гонадектомії та навчання пасивному униканню на щільність $GABA_{A\alpha1}$-рецепторів у префронтальній корі самців і самиць щурів. 20 дорослих самців (маса тіла 200 ± 30 г) та 20 дорослих самиць (150 ± 20 г) були поділені на чотири групи: Sham (інтактні щури, не піддані навчанню), Sham-L (інтактні щури, піддані навчанню), GE (гонадектомовані щури, не піддані навчанню) та GE-L (гонадектомовані щури, що пройшли навчання). Для навчання пасивному униканню використовували човникову камеру. Щільність $GABA_{A\alpha1}$-рецепторів оцінювалася з використанням імуногістохімічної методики (програмне забезпечення «ImageAnalyzer»). Оваріоектомія без навчання зумов-

лювала істотне зменшення щільності $GABA_{A\alpha1}$-рецепторів у регіонах префронтальної кори порівняно з відповідним показником у контрольній інтактній групі; в той же час оваріоектомовані самиці демонстрували значно вищу щільність рецепторів у префронтальній корі, ніж така в Sham-групі. Не спостерігалось істотних відмінностей щільності рецепторів в обох групах кастрованих самців. Результати порівняння показників у самців та самиць засвідчили, що щільність $GABA_{A\alpha1}$-рецепторів у кастрованих щурів, підданих навчанню, була істотно нижчою, ніж така в оваріоектомованих самиць після навчання. Отже, оваріоектомія зумовлює потужніші впливи, ніж кастрація, на щільність $GABA_{A\alpha1}$-рецепторів у різних регіонах префронтальної кори. Навчання униканню призводить до збільшення щільності вказаних рецепторів у різних регіонах префронтальної кори самиць, тоді як кастрація самців не зумовлює істотних впливів у даному аспекті.

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