

Castration and Passive Avoidance Learning Alter the Distribution of N-Methyl D-Aspartate Receptor

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ABSTRACT

In neurophysiological studies on learning and memory, L-glutamate receptors especially N-Methyl, D-Aspartate glutamate (NMDA) receptors have a major role in different aspect of the learning and memory. Two sites of mammalian brain that show high density of these receptors are CA1 region of hippocampus and Purkinje cell layer of cerebellum. Twenty four Sprague-Dawley rats were used in 4 groups: Control 1 (intact without learning); control 2 (intact with learning); experimental 1 (castration without learning) and experimental 2 (castration with learning). A shuttle box apparatus used for passive avoidance learning procedure. Immunohistochemical procedure was used for determination of NR1 subunit of NMDA receptor. Photoshop software was used for determination of color intensity. Data were analyzed by student t-test and one-way ANOVA, Tucky test as post-hoc test was used. The level of significant was considered $p < 0.05$. Immunohistological finding of this experiment indicates that castration has a negative effect on density of NR1 subunit of NMDA receptors in two brain regions. Other finding of this study showed that passive avoidance learning significantly increased density of NR1 subunit of NMDA receptors in two brain regions. These results indicated that the sex hormone can modulate function and expression of the NR1 subunit of NMDA receptor in CA1 region of hippocampus and Purkinje cell layer of cerebellum.

Keywords: Castration, Passive Avoidance Learning, NR1 Subunit of NMDA Receptor, Hippocampus, Cerebellum, Rat

1. INTRODUCTION

New neurons are produced throughout life in the hippocampal granular cell in mice, a brain region important for spatial learning and memory (Kesner, 2007; Raber *et al.*, 2004). In addition, steroid hormones such as estrogens and androgens have recently been suggested to affect hippocampal neurogenesis in rats (Ormerod *et al.*, 2003; Spritzer and Galea, 2007). Androgens alter hippocampus-dependent memory formation (MacLusky *et al.*, 2006). Other studies have shown that sex hormones may alter glutamatergic transmission in the brain regulating expression of glutamate receptors (Diano *et al.*, 1997). Testosterone in

males regulated the ability of MK-801 to block morphine-induced C-fos expression in the caudate-putamen. One mechanism by which this could occur is through steroid regulation of glutamate receptor abundance. Therefore gonadal hormones could modulate levels of glutamate receptor subtypes in the caudate-putamen and medial thalamus, regions of the brain showing gender differences (D'Souza *et al.*, 2002).

Treatment of male rats with high doses of anabolic-androgenic steroids affect the mRNA expression of NMDA receptor subunits in certain areas of the brain, for example after application of high doses of anabolic-androgenic steroids up-regulation of the NR1 subunit of NMDA receptor mRNA in nucleus accumbens was

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occurred (Greves *et al.*, 1997). The glutamate receptor NMDA subtype plays a crucial role in LTP and LTD (Rossbach *et al.*, 2007). Extensive evidence indicates that NMDA glutamate receptors in the hippocampus and the amygdala are involved in the formation of aversive memory (Roesler *et al.*, 2000a; 2000b). Dihydrotestosterone is known to increase glutamate binding to the NMDA receptor in the CA1 region of the male rat hippocampus (Romeo *et al.*, 2005). Most brain regions including the olfactory bulb, cerebral cortex, hippocampus and the putamen showed immunostaining similar to NR1 distribution in rats (Zhao *et al.*, 2006).

There are a few studies on NR1 subunit of NMDA receptor distribution after castration. So the aim of present study was to investigate the effect of castration and learning on distribution of NR1 subunit of NMDA receptor in CA1 region of hippocampus and Purkinje cell layer of cerebellum in rat.

2. MATERIALS AND METHODS

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

2.1. Animals

Twenty four male Sprague Dawley rats weighing 200-220g were used. Food and water were made available ad libitum. The rats were housed under a 12h light/dark (light on at 6 a.m.) and controlled temperature ($20\pm 4^\circ\text{C}$) condition. The rats were randomly divided (random assignment) into 4 equal groups ($n = 6$ each) 1-control group (intact without learning) 2-learning group (intact with learning) 3-Castration and 4-Learning following castration. For castration, rats were anesthetized (120 mg kg^{-1} thiopental sodium). A horizontal incision was performed in scrotum and testes were tied off and removed with a cut distal to the ligature, then the incision was sutured.

A two-way shuttle-box (made by Aryoazma Co) with acrylic walls and steel floor bars was used for learning procedure. The box, $44\times 20\times 19$ cm, was bisected by a vertical partition with an opening in the middle that allows the animal to move freely from one compartment to other, including light and dark compartments. In the light compartment the animal was safe while in the dark compartment it received a foot shock of 0.6 mA for 1 sec; with a latent period of 1 sec.

2.2. Behavioral Procedure

On the first day, all animals were individually subjected to 2 min of adaptation to the shuttle box, in which the rat could explore the light compartment and move about freely; at this stage since the rat likes dark compartment, if the rat did not move to dark compartment after 120 sec it was removed from study. This adaptation was repeated 30 min later. On the second day as initial latency, the rats were placed in the light compartment box and 1 sec after entering to the dark compartment 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 considered as learning. On the fourth day, as memory consolidation, the procedure was like the learning days without foot shock. On the fifth day, as memory retention, the procedure was similar to the fourth day. The rats were considered as completely learned, if they did not move to dark compartment after 120 sec during third, fourth and fifth session of experiments.

2.3. Tissue Preparation

In all groups the rats were euthanized by Na thiopental overdose (120 mg kg^{-1}) and after heart perfusion of 10% formaldehyde the brains were removed and washed by normal saline and fixed for 72 h in 10% formaldehyde in 0.1M Phosphate Buffer (PB, PH 7.4), then the brains were post-fixed in 4% formaldehyde in 0.1 M phosphate buffer (PB, PH 7.4). Paraffin embed were done by Tissue processor and paraffin block were prepared. A microtome was used to cut the brains into $5 \mu\text{m}$ sections and mounted in 25% L-lysine coated glass slides.

2.4. Immunohistochemical Study

The slide-mounted were dried and subsequently stored in a -20°C freezer until used for antibody labeling. After removal from the freezer slide-mounted of rat brain 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 over night with primary antibodies against NR1 subunit of NMDA receptor (1×10^{-3} dilution). The next day, slides were rinsed in PBS and incubated in secondary antibody (envision) and further washed in PBS. Finally the slides were rinsed in PBS three times for 10 min and reacted with cromogen (Dab) and following rinse in PBS, the slides were put in hemotoxilin for nucleus staining.

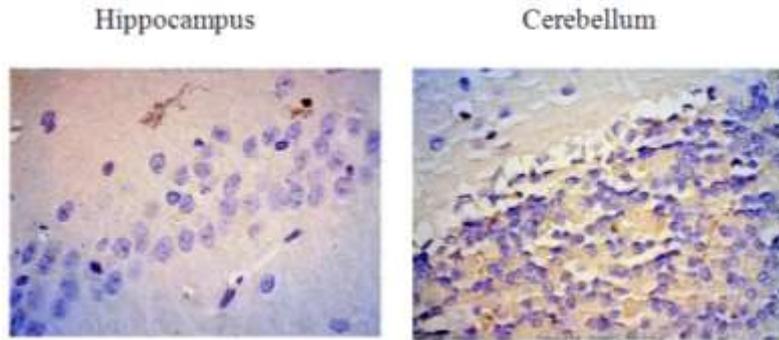


Fig. 1. Micrograph of negative control of CA1 region of hippocampus and Purkinje cell layer of cerebellum

Negative control slides were incubated with PBS in the absence of primary antibody and no immunoreactivity was detected. The **Fig. 1** shows the negative control. In this photograph there was no brown color due to reaction of primary antibody. After preparing of digital image from slides, distribution of NR1 subunit of NMDA receptor of glutamate were analyzed by using Image Analyzer (version 1.33). This program determined the distribution of receptors according to three character, hue, saturation and intensity; and then showed the number that has reverse relation with the receptor distribution; meaning that higher receptor distribution represented by low number in the program.

2.5. Statistical Analysis

Statistical analyses were performed using SPSS (version 18). Student T-Test was used to determine the difference between groups. Data reported as mean±SEM and the level of significant was considered $p < 0.05$.

3. RESULTS

Our data showed that after passive avoidance learning, NR1 subunit of NMDA receptor distribution significantly ($p < 0.05$) increased in CA1 region of hippocampus and Purkinje cell layer of cerebellum (**Fig. 2-4**). Castration significantly ($p < 0.05$) decreased NR1 subunit of NMDA receptor in CA1 region of hippocampus and Purkinje cell layer of cerebellum in comparison to control (**Fig. 2, 3 and 5**). Learning following castration significantly ($p < 0.05$) decreased NR1 subunit of NMDA receptor in comparison to learning group (**Fig. 2, 3 and 6**). The NR1 subunit of NMDA receptor was significantly ($p < 0.05$) higher in learning following castration group than in castration group in CA1 region of hippocampus, but there was no difference between two groups in Purkinje cell layer of cerebellum (**Fig. 2, 3 and 7**).

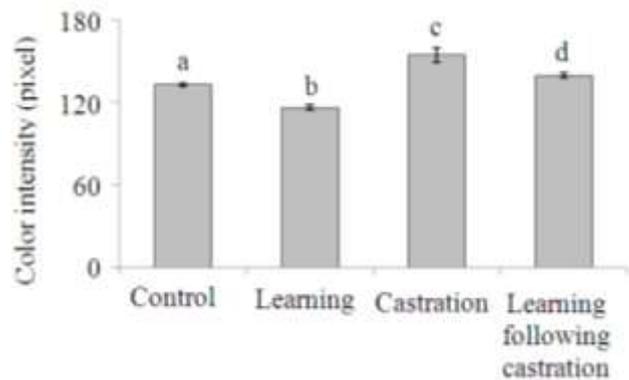


Fig. 2. Effect of castration on NR1 subunit of NMDA receptor distribution in CA1 region of hippocampus. Characters showed significant difference between groups ($p < 0.05$)

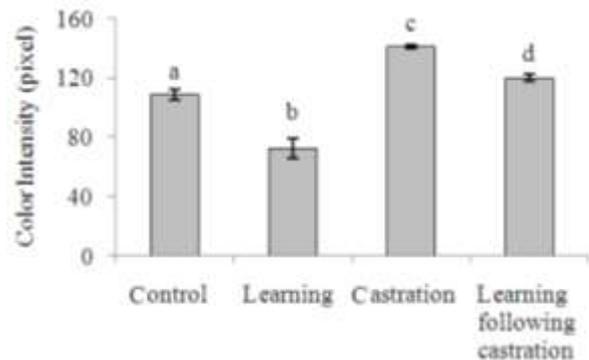


Fig. 3. Effect of castration on NR1 subunit of NMDA receptor distribution in Purkinje cell layer of cerebellum. Characters showed significant difference between groups ($p < 0.05$)

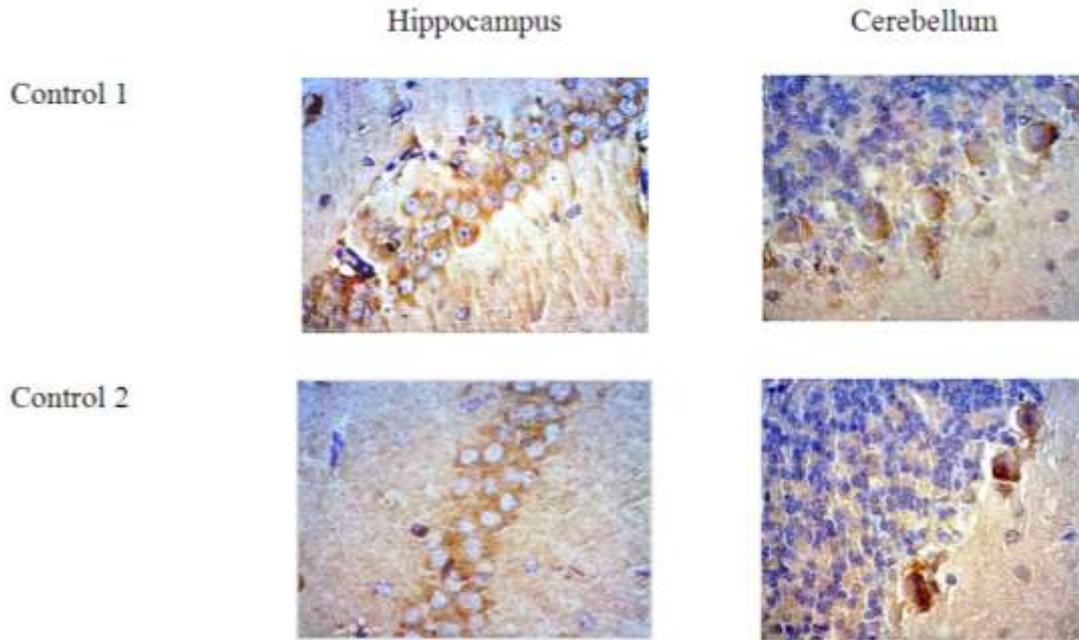


Fig. 4. Micrograph of NR1 subunit of NMDA receptor distribution of CA1 region of hippocampus and Purkinje cell layer of cerebellum in control 1 and control 2 group. $\times 640$

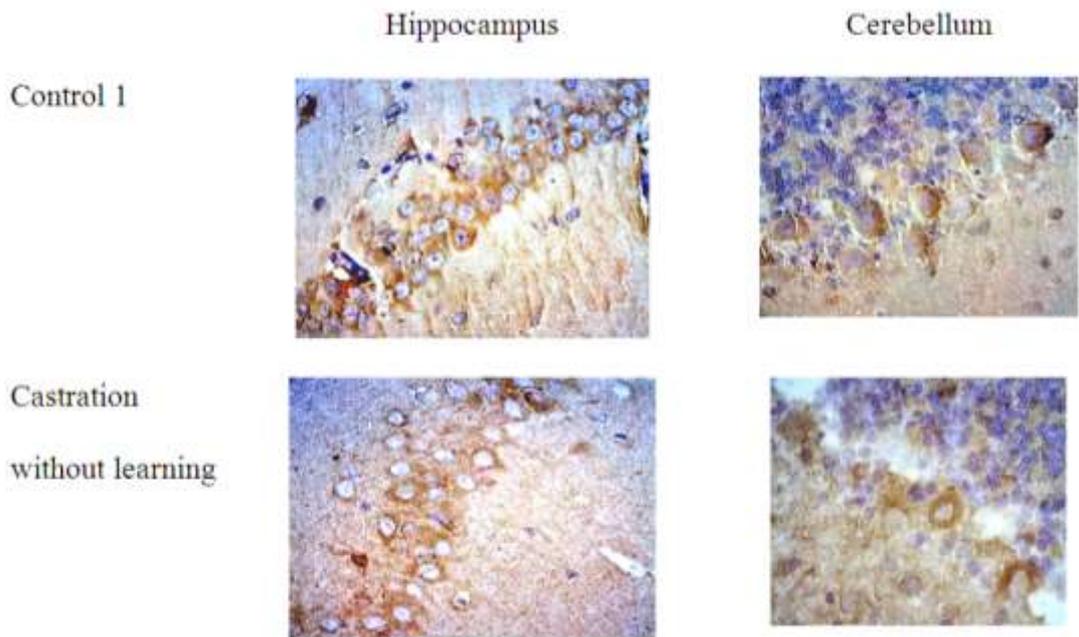


Fig. 5. Micrograph of NR1 subunit of NMDA receptor distribution of CA1 region of hippocampus and Purkinje cell layer of cerebellum in control 1 and Castration without learning group. $\times 640$

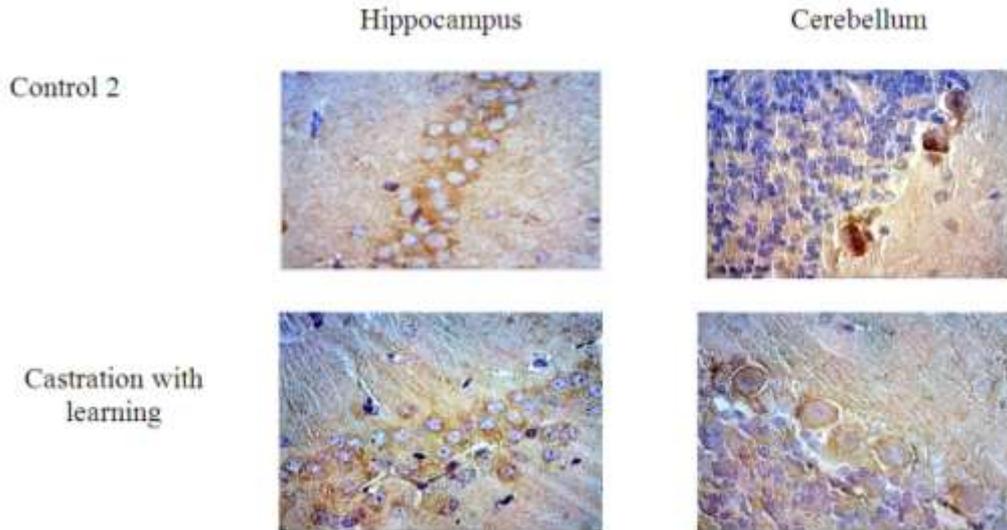


Fig. 6. Micrograph of NR1 subunit of NMDA receptor distribution of CA1 region of hippocampus and Purkinje cell layer of cerebellum in control 2 and Castration with learning. $\times 640$

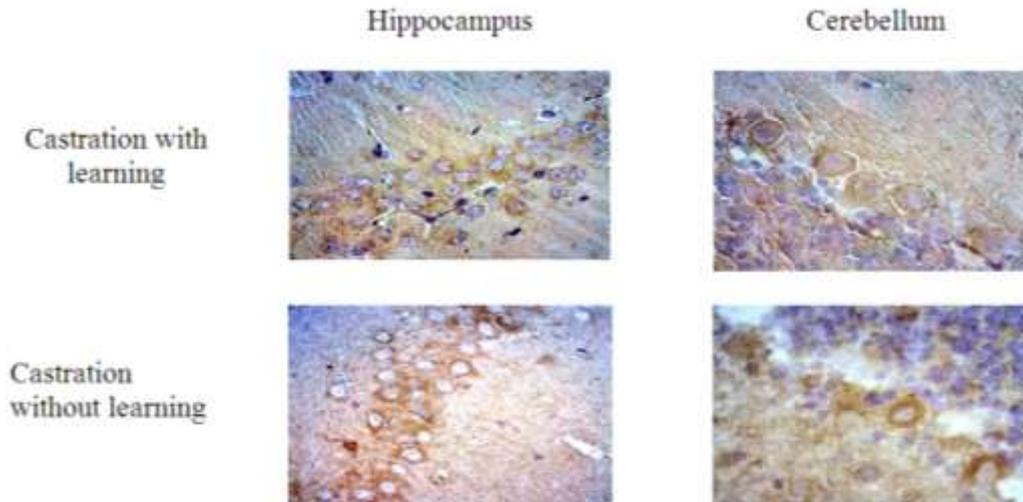


Fig. 7. Micrograph of NR1 subunit of NMDA receptor distribution of CA1 region of hippocampus and Purkinje cell layer of cerebellum in Castration with learning and Castration with learning group. $\times 640$

4. DISCUSSION

Neurosteroids positively and negatively modulate glutamate receptors, which underlie most fast inhibition and excitation in the central nervous system (Mennerick *et al.*, 2001). In the present study NR1 subunit of NMDA receptor distribution increased in response to learning. NMDA receptors are widely

distributed in brain, but the highest concentrations of NMDA binding sites are found in area CA1 of hippocampus, with substantial concentrations also localized within the dentate gyrus. NMDA receptors in hippocampus CA1 area are very important in the regulation of synaptic plasticity and the process of learning and memory, including short-term and long-term memory (Bannerman, 2009; Jafari-Sabet, 2006).

In the present study NR1 subunit of NMDA receptor decreased following castration CA1 region of hippocampus and Purkinje cell layer of cerebellum. In mammals, sex steroid hormones are known to influence spatial learning and memory abilities through their effects on the hippocampus (Frye and Rhodes, 2002). Androgens can improve cognitive performance either by acting directly via the reduced metabolite dihydrotestosterone or by aromatization into estrogens by aromatase (Frye *et al.*, 2008; Hojo *et al.*, 2009). Androgen-dependent modulation of mRNA coding for the NR1 subunit of the NMDA receptor channel in the supraoptic nucleus was investigated. Greves *et al.* (1997) showed that chronic anabolic-androgenic steroids treatment affect the mRNA expression of various NMDA receptor subunits in several brain regions (Greves *et al.*, 1997).

Testosterone in males would regulate abundance of NR1 subunit of NMDA receptor (D'Souza *et al.*, 2003). NR1 subunit of NMDA receptor mRNA in both lateral septum and supraoptic nucleus were decreased in castrated rats when compared to castrate-dihydrotestosterone propionate-treated male rats (Kus *et al.*, 1995b).

In the present study NR1 subunit of NMDA receptor in Learning following castration was higher than castration in CA1 region of hippocampus, but there was no difference between two groups in Purkinje cell layer of cerebellum. Testosterone, as a neuromodulator, exists in CA1 region of hippocampus and training can decrease its level only in right hippocampus significantly (Mohaddes *et al.*, 2009). Benice and Raber (2010) reported that castration lead to reduction in the number of immature neurons in granular cell layer (Benice and Raber, 2010). Kus *et al.* (1995a) observed increase in [125I] MK801 binding in pyramidal cell neurons within CA1 suggests that androgens may potentially affect hippocampal function by modulating pyramidal cell NMDA receptors (Kus *et al.*, 1995a).

5. CONCLUSION

5.1. According to our Results

- Passive avoidance learning lead to increases the NR1 subunit of NMDA receptor distribution in hippocampus and cerebellum
- Castration leads to decreases the NR1 subunit of NMDA receptor distribution in hippocampus and cerebellum
- NR1 subunit of NMDA receptor distribution was higher in castration with learning group than

castration without learning. It means that passive avoidance learning can compensate the down-regulation of NR1 subunit of NMDA receptor in hippocampus but not in cerebellum

5.2. Conflict of Interest

All the authors can confirm that there is no financial or other relationship that would cause a conflict of interest.

6. ACKNOWLEDGMENT

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