ORIGINAL RESEARCH ARTICLE

Use of steroids and their effects on neuronal density in hypothalamus and amygdala

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ABSTRACT

Anabolic-androgenic steroids (AAS) are natural, semi synthetic, or synthetic substances with testosterone-like activity often used in body building gyms and physical training centers without any control or criteria. Previous studies revealed that AAS can cause hypomanic or manic symptoms including aggressive or violent behavior in some individuals. The objective of this experimental study was to evaluate the effects of supraphysiological doses injections of testosterone cypionate and stanozolol on neuronal density in mice lateral hypothalamus and central amygdala. For this experiment 60 Swiss mice were used (30 males and 30 females) divided in three groups of 20 animals with 10 male and 10 female each: group I – control (1,8 mg/kg/day saline solution); group II - Deposteron® (0,8 mg/kg/day testosterone cypionate); and group III - Winstrol Depot® (1,8 mg/kg/day stanozolol). The treatment lasted 30 days (twice-a-week AAS administration) and the animals was submitted to swimming activity (three times a week). Experiment animals were euthanized and their brains removed for histological procedures. Neuronal density estimation was performed according to random simple counting. The use of supraphysiological doses of both anabolic-androgenic steroids (AAS) proved to be statistically significant for decreasing the number of neuron cell bodies in lateral hypothalamus of male and female mice, and in central amygdala of female mice. The use of AAS such as Deposteron® and Winstrol Depot® can induce neuron cell death, compromises lateral hypothalamus and central amygdala functions, and may lead to behavioral changes.

Keywords: neuron; steroids; hypothalamus; amygdala

Introduction

Anabolic-androgenic steroids (AAS) are natural, semi synthetic, or synthetic substances with testosterone-like activity that are used for therapeutic purposes or in sports field due to their anabolic and androgenic properties which increase muscular mass and body weight [1,2]. Steroids hormones synthesized by suprarenal cortex and gonads represent the main source of male sexual hormones that maintains sexual characteristics associated to manliness [3].

These drugs are often used in body building gyms and physical training centers without any control or criteria, thus representing a high risk to their users' health[4-5]. Due to this abusive pattern of AAS intake, they become a type of drugs very important from the toxicological point of view[6].

Athletes use supraphysiological doses of AAS aiming muscular mass and strength increase, in addition to decreasing post-workout muscle recovery time[7,8]. The dosage is usually from 10 to 100 times higher than therapeutic one, and 2/3 of the abusive use occur among non-athletes[9].

The indiscriminate use of AAS began in the middle of 1950s, increased by the 1970s and is still going on to this day, bringing harmful effects to people's health[10,11]. For these reasons AAS were prohibited by the International Olympic Committee since Montreal Olympiads (1976) when AAS control

was performed by the first time[12]. In USA, from 1991 on, AAS were classified as special controlled drugs[13,14]. In Brazil these drugs are also put into consuming-controlled substances class[15].

Supraphysiological doses of AAS lead to several harmful effects in the organism with adverse consequences concerning metabolic, endocrine, cardiovascular, hepatic, neurologic, aesthetic, behavioral and psychiatric aspects. Until now it is not precisely explained how AAS act in human brain; however, there are records of changes in aggressive behavior, anxiety and depression[18].

Corticolimbic neural circuits mediate emotional behavior and they are implicated in psychiatric disturbances pathophysiology such as aggressiveness, anxiety, depression, alcoholism, and schizophrenia, among others[19]. AAS can cause hypomanic or manic symptoms including aggressive or violent behavior in some individuals[20].

Therefore, this study tries to clarify what possible damages are caused by supraphysiological doses of AAS in mice lateral hypothalamus and central amygdala through quantitative and behavioral analyses.

Materials and Methods

Animals

For this experiment 60 Swiss mice were used (30 males and 30 females), with 90 days old (young-adults), weight from 40 to 50 grams, housed in boxes with 5 animals in each one. They received commercial feed and water "ad libitum" and were kept in 12 dark/12 light hour cycle.

The treatment consisted in intraperitoneal (IP) injection of Deposteron® (testosterone cypionate) and Winstrol Depot® (stanozolol). Mice were divided into three groups of 20 animals (10 male and 10 female each): group I - control, received 1,8mg/kg/day saline solution; group II - Deposteron®, received 0,8mg/kg/day testosterone cypionate; and group III - Winstrol Depot®, received 1,8mg/kg/day stanozolol.

Treatment

The treatment lasted 30 days, with twice-a-week AAS administration (on Tuesdays and Thursdays). Treatment doses were calculated to be similar to those used by body building gym customers of the city of Alfenas-MG, according to the allometric extrapolation method.

Training sessions (swimming) happened at the same time three times a week (non-injection days). The animals were put to swimming for 15 minutes in a 43x34x26 cm plastic container with water at 24-26ºC. During swimming, the animals were monitored so they do not float or lean on the edge of the tank and stop moving. After the treatment, the animals undergo euthanasia with deepening of anesthesia (halothane), for the removal of the brains.

Sample Collection

Experiment animals were euthanized by halothane (Alotano®) inhalation and had their brains removed. The specimens were stored by 24 hours in glass containers filled with formaldehyde 4% solution, pH 7.4, and 0.1M[21]. Frontal section samples from each brain's homotypic medium region were taken in order to evaluate the areas selected for this study[22].

Fragments were processed following the standard sequence for conventional histological procedure: alcohol dehydration, xylol diaphanization and paraffin bath. Each sample was blocked and cut into 7m-width slices using a Lupe® microtome and stained with cresyl violet in order to ease Nissl corpuscles' visualization and to mark strongly each cell for counting.

Neuronal density estimation

Neuronal density estimation was carried out using random simple counting. Two random microscopic fields taken from 3 serial cuts of the selected areas (lateral hypothalamus and central amygdala) were obtained, totalizing 6 analyzed fields for each one.

Through the counting frame, one marked neuron cell bodies within the area and inclusion line and excluded those outside the area and on continuous red lines. Thus, only neuron cell bodies for each counted area were quantified but not the overall number of them for selected areas. For each group an identification code was labeled in the slices so making analyses blinded in order to avoid any kind of researcher's influence in relation to a given sample[23,24,25,26].

The analyses were performed by a Carl Zeiss® Axiovision 4 Module Interactive Measurement image analyzing system coupled to a Carl Zeiss® Axio Scope A1 microscope and to a computer.

Statistical Analyses

The variance test performed by Bioestat 5.3

software was used to evaluate neuronal density quantitative analyses and to verify the presence of significant interactions among areas and studied groups. Statistical analyses for aggressive behavior tests were calculated by analysis of variance (One-Way ANOVA) and Tukey's test. Values of $p < 0.05$ were considered indicative of significance for both statistical analyses and analyzed parameters.

Ethics Statement

The present experiment has the approval of the Committee of Ethics in Animal Experimentation (nº 505/2013).

Results

Neuronal density estimation in male mice lateral hypothalamus and central amygdala

Outcomes taken from male mice lateral hypothalamus showed a quite significant decrease in neuronal density estimation in Deposteron® group (31%) as well as in Winstrol Depot® group (33%) when compared to control group (Figure 1A).

Quantitative results related to neuronal density estimation of central amygdala of Deposteron® and Winstrol Depot®-treated male mice indicated no significant difference (Figure 1B).

Neuronal density estimation in female mice lateral hypothalamus and central amygdala

Outcomes taken from AAS-treated female mice showed that neuronal density in lateral hypothalamus was reduced in 38% for Deposteron® group and in 25% for Winstrol Depot® one. This means that the use of Deposteron® supraphysiological doses induced a greater reduction in the number of neuron cell on female mice ($p < 0.01$) when compared to Winstrol Depot® group ($p < 0.05$) (Figure 2A).

One could observe a significant decrease in the number of neuron cell of central amygdala for Deposteron® (36%) and Winstrol Depot® (23%) treated female mice ($p < 0.01$), showing that both treatments promoted a similar reduction in the number of neuron cell bodies when compared with each other (Figure 2B).

General comparison between male and female mice

The results still allow a general comparison of neuronal density quantification between male and female mice for each treatment (Deposteron® and Winstrol Depot®) and between the analyzed structures (lateral hypothalamus and central amygdala) (Figure 3, 4 and 5).

Deposteron® treatment caused a significant decrease in the number of neuron cell bodies in male (31%) and female (38%) lateral hypothalamus while Winstrol Depot® treatment reduced it in 33% for
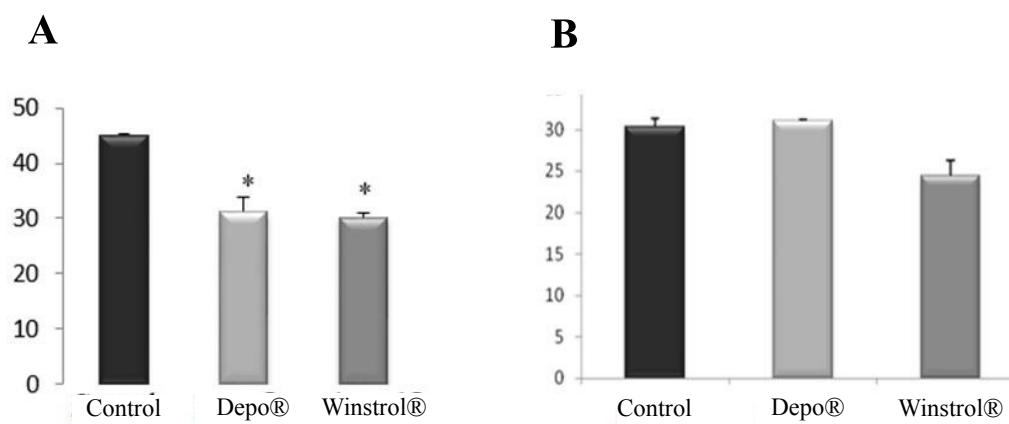

Figure 1. In A: Neuronal density estimation in lateral hypothalamus comparison between the male groups, showing significant decrease in the groups treated with AAS (Deposteron® and Winstrol Depot®). $p < 0.01$ (1A). In B: Neuronal density estimation in central amygdala comparison between the male groups. $p > 0,05$.
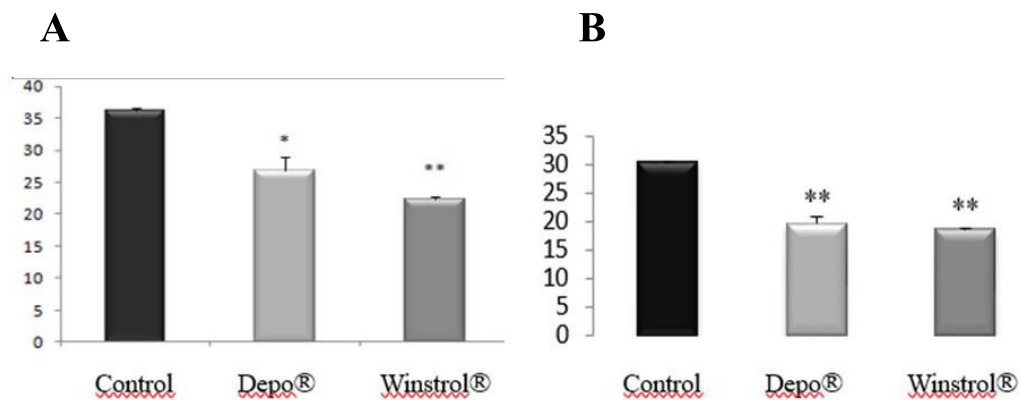
A **B**

Figure 2. – In 1A: Neuronal density estimation in lateral hypothalamus comparison between the female groups showing significant decrease in the groups treated with AAS Deposteron® (*p<0.01) and Winstrol Depot® (**p<0.05). In B: Neuronal density estimation in central amygdala comparison between the female groups showing significant decrease in the groups treated with AAS (Deposteron® and Winstrol Depot®) (**p<0,01).

Lateral hypothalamus neuron cell density
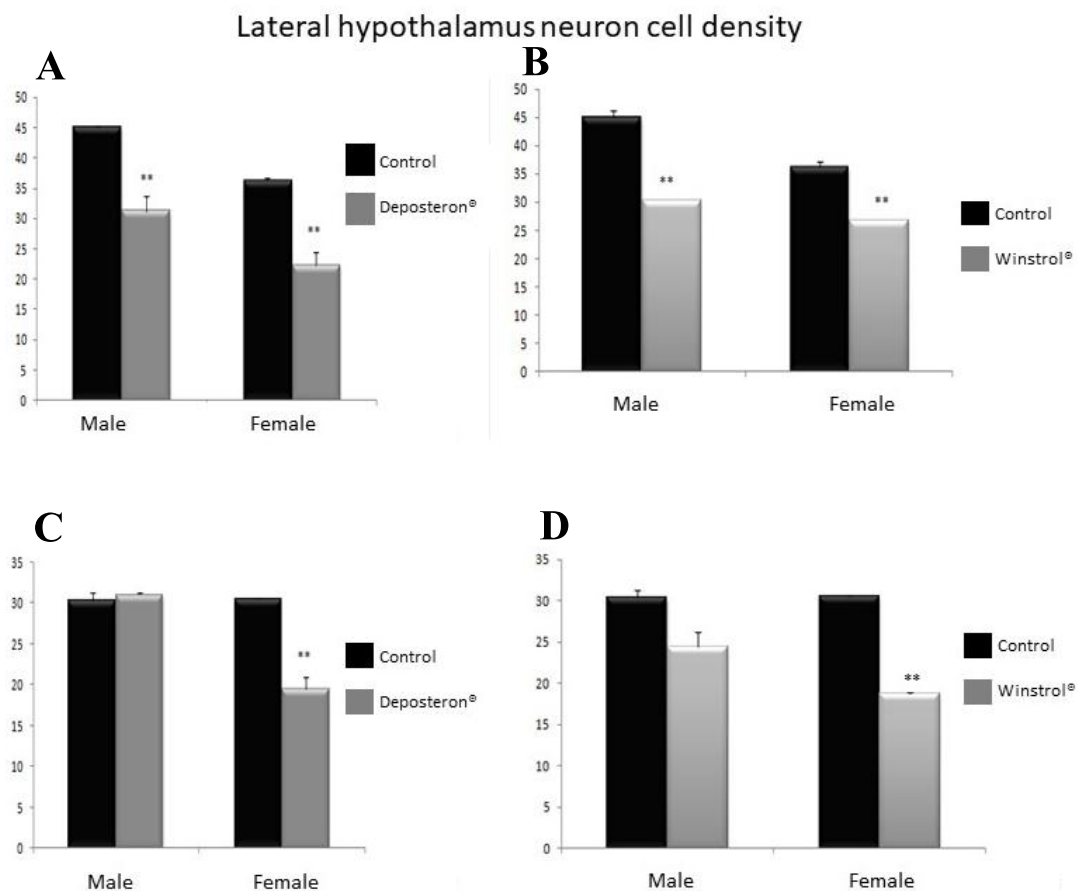
A **B**

C **D**

Figure 3. – In A and B: Lateral hypothalamus neuronal density estimation for Deposteron® (3A) and Winstrol® (3B). Observe the treated groups of male and female mice, showing significant decrease in the number of neuron cell bodies, were Deposteron®-male and female (p<0.01) and Winstrol Depot®-male (p<0.01) and female (p<0.05). In C and D: Central amygdala neuronal density estimation for Deposteron® (3C) and Winstrol Depot® (3D). The female treated groups showed significant decrease, were p<0.01.
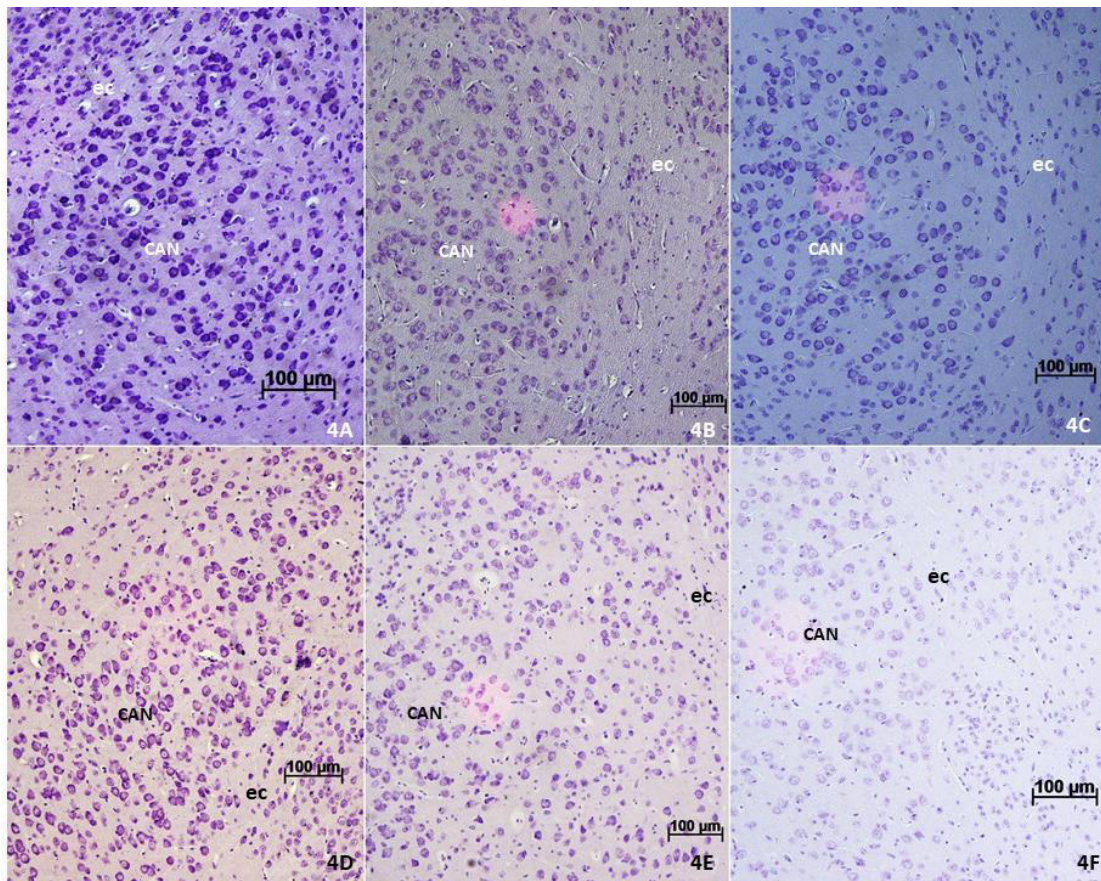
Figure 4. - Photomicrography of central amygdala nucleus (CAN). In 4A, 4B and 4C comparison between the female groups and 4D, 4E and 4F comparison between the male groups. 10x. Cresyl violet. *ec=external capsule.
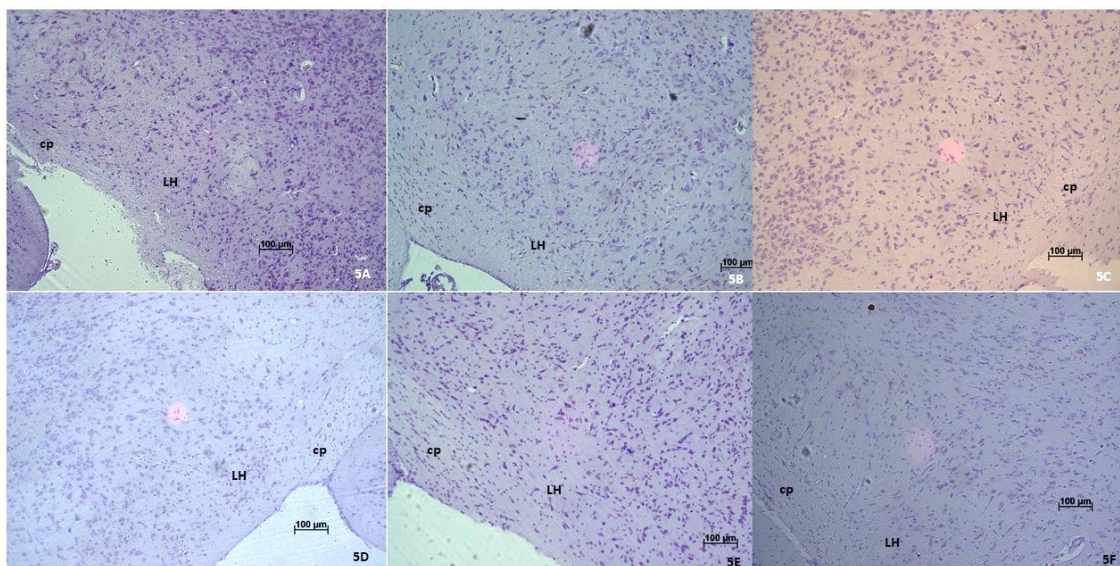

Figure 5. - Photomicrography of lateral hyphotalamus (LH). In 5A, 5B and 5C comparison between the female groups and 5D, 5E and 5F comparison between the male groups. 10x. Cresyl violet. *cp=cerebral peduncle.

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male and 26% for female mice (Figures 3A and 3B).

When one compares neuronal density estimation in central amygdala, only female mice treated with Deposteron® and Winstrol Depot® presented a significant decrease in neuronal density, respectively 36% and 23%. Meanwhile, data showed no significant decrease in the number of neuron cell bodies in male mice central amygdala (Figures 3C and 3D).

Studies in progress will allow us to clarify what are the probable mechanisms that have led to these differences of neuronal loss in the structures then analysed between genders.

Discussion

According to data found in neuronal density analyses for male mice lateral hypothalamus one can assert that Deposteron® and Winstrol Depot® injections in supraphysiological doses can reduce it. This indicates that the use of these drugs can cause harmful consequences to male mice brains (neuron cells death) which can damage the selected areas and impair their functions. These results corroborate the data presented by a paper in which AAS-treated mice had a significant reduction in the number of cortical neuronal density when compared to control group (treated with saline solution)[27,28].

Another experiment also confirmed the outcomes presented in this present study. In this, the authors made a comparison among testosterone, nandrolone, stanozolol and gestrinone effects over excitotoxic cell death induced by N-methyl-D-aspartate (NMDA) in cortical neuron cell rat primary cultures and their results suggested that high doses of these AAS increased neural vulnerability, raised cellular cytotoxicity and eased neuron cells death[29].

One of the selected areas for the present experiment was lateral hypothalamus which is a limbic structure related to angry and aggressive behavior[30]. Therefore, a decrease in the number of neuron cell bodies can alter these functions.

Winstrol Depot® treated male mice had a decrease in neuronal density of central amygdala but this was statistically non-significant. Central amygdala is related to fear and aggressive behavior[31]. Once an emotional stimulus is detected, amygdala can process this stimulus by means of its large cortical areas connections involved in attention, perception and explicit memory cognitive functions[32,33,34]. Therefore, a decrease in neuronal

density in this structure may damage its functions.

AAS and exercise were without effect on BDNF mRNA in females. In sum, we find that AAS elicit sex-specific differences in anxiety and that voluntary exercise accentuates these differences. In addition, our data suggest that these behavioral outcomes may reflect convergent actions of AAS and exercise on a sexually differentiated CRF signaling system within the extended amygdala[35].

Supraphysiological doses of AAS may lead to a variety of neuroendocrine problems. Precisely, the hypothalamic-pituitary-gonadal (HPG) axis is one of the body systems that is mainly influenced by steroidal hormones. Two-dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry analyses identified a total of 17 different proteins that were significantly affected by supraphysiological levels of AAS this results suggest that steroids have the capacity to directly affect the neuroendocrine system by modulating key cellular processes for the control of reproductive function[36]. The use of AAS in female athletes has been associated with adverse effects that include acne, hirsutism, deepening of the voice and menstrual disturbances; life-threatening adverse effects such as cardiac arrhythmias and sudden death have also been reported[37].

Therefore, the previously mentioned articles [35,36,37] may be directly related to the neuronal loss evidenced in this work that triggers a cascade of harmful effects on neuronal mechanisms, both in the amygdala and in the hypothalamus, which may be related to the observed endocrine and behavioral effects in AAS users.

Conclusion

This article pointed toward that Deposteron® as well as Winstrol Depot® treatments induced significant decrease in male and female neuronal density. On the other hand, only female mice central amygdala. This outcome indicates that Deposteron® (testosterone cypionate) and Winstrol Depot® (stanozolol) use may induce a quite significant decrease in the neuronal density of central amygdala in women taking these drugs. Although the results do not point towards the same decrease in male mice, one cannot affirm that AAS use would be safe for men. According to data obtained in the present study as well as other data available scientific literature, there are several harmful and collateral effects for AAS-customers' physical and mental health.

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