INTRODUCTION

The hippocampus is an important region of the brain that regulates cognitive and emotional functions. In this study, we examined the impact of perinatal administration of testosterone propionate (TP) on the number of pyramidal neurons in the CA1 and CA3 regions of the hippocampi of female rats.

METHODS

Five groups of rats were used in this study. Three groups of female rats were administered TP in either both the prenatal and the postnatal periods (Group 1), only the prenatal period (Group 2) or only the postnatal period (Group 3). The other two groups of rats included control females (Group 4) and control males (Group 5). The rats were sacrificed on postnatal Day 120 and their brains were analysed for hippocampal pyramidal neuron number using stereological methods.

RESULTS

Control male rats (Group 5; p = 0.043) and TP-treated female rats in Groups 1 (p = 0.012) and 2 (p = 0.037), but not Group 3 (p > 0.05), had a significantly higher number of pyramidal neurons than control female rats (Group 4). The rats in Group 1 had the highest number of pyramidal neurons among the female rats.

CONCLUSION

Perinatal TP treatment has an augmenting effect on the number of pyramidal neurons in the hippocampi of female rats. We also found gender-based differences in the hippocampi of male and female rats, with a higher number of pyramidal neurons seen in male rats. Continuous TP administration during the prenatal and postnatal periods is more effective than administration only in the prenatal or postnatal period.

Keywords: androgens, female, perinatal, rat, stereology
postnatal periods. The left brain hemispheres of all rats were selected for the estimation of hippocampal pyramidal neuron numbers, following which the total number of pyramidal neurons in the hippocampal CA1, CA2 and CA3 regions of the rats were estimated using the optical fractionator method.

METHODS

Wistar albino female rats (n = 12) were purchased from the Animal Research Laboratory of Pamukkale University, Turkey. Female rats whose vaginal smears revealed polygonal superficial cells on microscopic inspection were housed together with one male rat overnight, three female rats at a time. Day 0 of gestation was taken to be the day on which a sperm-positive vaginal smear or a vaginal plug presence was observed. Pregnant female rats were then individually housed in different cages. The cages had a plastic base and a stainless steel covering, and were placed in an animal room. The environmental conditions of the animal room were controlled (temperature: 22 ± 2°C; humidity: 50 ± 5%; 12-hour dark/light cycle). Two pregnant rat groups (6 rats in each group) were randomly assigned on gestation Day 16 to receive either intraperitoneal 1 mg TP in 0.5 mL sesame oil or 0.5 mL pure sesame oil every morning (i.e. daily) until parturition. The gender of the pups was determined by measuring the anogenital distance, following which the pups were randomly assigned to five groups after birth (Table I). The day of birth was marked as postnatal Day 0.

The two groups of female pups whose dams had been treated with TP were assigned to receive either 100 μg TP in 0.1 mL sesame oil (Group 1) or 0.1 mL pure sesame oil (Group 2) by subcutaneous injection around the neck on postnatal Days 0–3. Female pups of the dams treated with pure sesame oil were also assigned to receive either 100 μg TP in 0.1 mL sesame oil (Group 3) or 0.1 mL pure sesame oil (Group 4) by subcutaneous injection around the neck on postnatal Days 0–3. Group 5, which consisted of the male pups of dams treated with pure sesame oil, was similarly treated with 0.1 mL sesame oil on postnatal Days 0–3. The study procedure is summarised in Table I. Pups were housed with their dams in the same cage and under the same laboratory conditions until postnatal Day 21. Thereafter, the pups were separated from their dams and kept in different cages until postnatal Day 120, on which they were sacrificed.

On postnatal Day 120, the rats were transcardially perfused with 150 mL saline (0.9% sodium chloride) to rinse the vessels, and with 400 mL of 4% paraformaldehyde solution in phosphate buffer (0.1 M, pH 7.4) for fixation. The brains of the rats were removed and immersed in 30% sucrose solution at 4°C for two days before sectioning. The brains were placed in a cryostat chamber (Leica CM3050; Leica Microsystems Nussloch GmbH, Nussloch, Germany) for sectioning, and coronal sections of 100 μm thickness were cut serially at –15°C to –20°C. Every fourth section was taken, according to systematic uniform random sampling rules, and mounted on gelatin-coated slides for staining. The mounted sections were immersed in xylene for 10 minutes and an ethanol series (consecutive 100%, 100%, 70% and 50% ethanol solutions at 5-minute intervals) for hydration. Subsequently, the sections were stained with cresyl violet for 5 minutes, washed in running tap water, rinsed in distilled water, dehydrated in an ethanol series (consecutive 50%, 70%, 100%, 100% ethanol solutions at 5-minute intervals) and immersed in xylene for 10 minutes. The stained sections were coated with methyl methacrylate (Micromount; Surgipath Medical Industries Inc, Toronto, Canada) and covered with a cover slip.

For microscopic analysis, a microscope (Olympus CX31; Olympus Europa Holding GmbH, Hamburg, Germany) equipped with a microcator (Heidenhain MT-12, ND 221 B; Dr. Johannes Heidenhain GmbH, Traunreut, Germany) and a video camera (SSC-DC88P; Sony, Tokyo, Japan), which was connected to a monitor (LMD-2010; Sony, Tokyo, Japan), was used. A series of ten or twelve brain sections that traversed the hippocampus were used from each brain to count the number of hippocampal pyramidal neurons. Real-time images from the pyramidal cell layers of the hippocampus were obtained using the microscope. The boundaries of the pyramidal cell layer of the hippocampus were first detected using the 4× objective, after which a 100× oil objective was used to measure thicknesses and count cells. Features of the hippocampus proper were identified in the sections, approximately 1.72–6.84 mm behind the bregma, according to Paxinos and Watson’s The Rat Brain in Stereotaxic Coordinates.

![Table I. Study procedure and group features.](image-url)
defined according to the literature,\(^{13,14}\) and the distance between the upper and lower surfaces of the sections was measured using a microcator in every dissector sample.\(^{10,11}\)

The optical fractionator method was used for counting pyramidal neurons in the hippocampus.\(^{10}\) A two-dimensional unbiased counting frame with an area of \(20 \times 20 \mu m^2\) was used to count the cells. The section sampling fraction was 0.25, and the area sampling fraction was 0.016 in all rat groups. The height sampling fraction was calculated by dividing the height of the dissector by the height of the section. The total pyramidal neuron number of the hippocampus was estimated using the following formula: \(N = (\sum Q^-) \times (1/ssf) \times (1/asf) \times (1/hsf)\), where \(N\) = neuron number; \(Q^-\) = counted total pyramidal neuron number; \(ssf\) = section sampling fraction; \(asf\) = area sampling fraction; and \(hsf\) = height sampling fraction.

The estimated intra-animal coefficient of error for the estimated total number of pyramidal neurons and the observed inter-animal coefficient of variation were calculated in accordance with the methodology outlined by West et al.\(^{10}\) and Hosseini-Sharifabad and Nyengaard.\(^{14}\) Volume shrinkage was not taken into consideration for calculating the total number of pyramidal neurons. All data were collected from the left hemispheres as a higher number of androgen receptors and hippocampal CA1 pyramidal cells have been reported to be present in the left hemisphere.\(^{9}\) Variables were analysed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test.

**RESULTS**

The dams’ mean weight on gestation Day 16 was 290 ± 12 g in the TP-treated group (\(n = 6\)) and 284 ± 11 g in the sesame oil-treated group (\(n = 6\)) (p > 0.05). The mean birth weights of the pups whose dams were treated with TP was 7.8 ± 0.2 g, while that of those whose dams were treated with sesame oil was 8.2 ± 0.8 g (p > 0.05). The mean body weights of the rats in Groups 1–5 were measured before sacrifice, and the data are presented in Table II and Fig. 1. There were statistically significant differences among the groups (p = 0.000). The control male rats in Group 5 and the female rats in Group 1 had significantly higher body weights than the control female rats in Group 4.

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The difference between the means of the estimated total number of pyramidal neurons of the various groups was statistically significant (p = 0.011). Compared to the control female rats group (Group 4), a significantly higher total number of pyramidal neurons was found in the control male group (Group 5; p = 0.043) and the TP-treated females in Group 1 (p = 0.012) and Group 2 (p = 0.037), but not Group 3.
Comparative photographs of the coronal sections of the hippocampal regions from the various groups are shown in Fig. 3. There was no demonstrative difference apparent between the various groups.

Treatment with TP improved the body weight and total number of pyramidal neurons in the hippocampi of female pups. Group 1 rats had the highest neuron number among all the female rats. These results suggest that long-term androgen treatment during the perinatal period may be more potent in improving the total number of pyramidal neurons than prenatal or postnatal androgen treatment.

DISCUSSION

Our results indicate that perinatal TP administration caused masculinisation in female rats, as previously reported. Demissie et al.\textsuperscript{15} reported that prenatally androgenised rats exhibited significantly increased body weight, and the findings of our study concur. We found statistically significant differences between the mean body weights of rats on postnatal Day 120, although such differences were not observed at birth. Although the anabolic effect of androgens is well known, it may be worth investigating how TP treatment during the early stages of life affects body weight in the later stages.

Current data indicates that there are significant differences in the morphology, volume and number of pyramidal neurons in the hippocampal CA1, CA2 and CA3 regions in wild-type adult rat brains between different genders, with male rats showing a higher number of pyramidal neuron and neuronal dendrite than females.\textsuperscript{3,8,15,16} It has also been reported that exposure to TP in the perinatal period significantly reverses such gender differences in the pyramidal neuron layer of the hippocampus of female or castrated male rats.\textsuperscript{3,16} In our study, treatment with TP during the prenatal and postnatal periods or only in the prenatal period significantly changed the number of hippocampal pyramidal neurons in female rats. Postnatal TP treatment, however, did not produce statistically significant effects. These findings are in disagreement with the Isgor and Sengelaub study, which suggested that continuous treatment with TP in the late prenatal and early postnatal periods are
ineffective in increasing the number of pyramidal neurons in adult female and castrated male rats.\(^3\)

Studies have reported the effects of gonadal steroids on the proliferation and morphology of pyramidal neurons, as well as the synaptic plasticity of the hippocampus.\(^1\) The brain was shown to be most sensitive to experimental hormonal manipulations during the developmental period.\(^1\) According to Isgor and Sengelaub, who studied the effects of prenatal gonadal steroids on the number and morphology of CA1 and CA3 pyramidal neurons in rats, exposure to androgens in the last week of gestation significantly increased hippocampal CA1 and CA3 pyramidal cell field volumes and neuronal soma size.\(^3\) However, they did not find any significant difference in the estimated total number of pyramidal neurons in the CA1 and CA3 regions between the two genders, and between the androgen-treated female rat groups and control rats.\(^3\) These findings are contrary to the results of our study.

It would be inadequate to attribute the observed discrepancies between the results of the two studies to methodological differences since Isgor and Sengelaub also used stereological methods (optical dissector and Cavalieri estimator), which are unbiased and as effective as the optical fractionator used in our study. However, in the Isgor and Sengelaub study, a total of approximately 50 pyramidal neurons per field per animal were sampled using a systematic random sampling procedure for sections and areas,\(^3\) while in our study, similar sampling procedures were used but 250–300 pyramidal neurons per animal were counted to estimate the total number of pyramidal neurons. The main difference between the two studies appears to be the duration of androgen treatment, and it is possible that the positive effects of androgen on the number of pyramidal neurons might be related to the duration of TP treatment. However, further research is needed to determine the mechanisms involved in the sexual dimorphism seen, with regard to the total number of pyramidal neurons.

Madeira et al found that the total number of CA1 pyramidal neurons displays sexual dimorphism in unmanipulated male and female rats aged 30 and 180 days.\(^17\) The study dealt with the selective vulnerability of hippocampal pyramidal neurons to hypothyroidism in rats, and found that sexual dimorphism in the total number of pyramidal neurons in the CA1 region was not affected by thyroid hormone manipulations.\(^17\) The results of our study had similar findings with Madeira et al’s study regarding sexual dimorphism in the total number of pyramidal neurons in the hippocampus. However, while rats aged 30 and 180 days were used in the study by Madeira et al, rats in the foetal and neonatal period were treated in our study. Further differences existed between these two studies with regard to the kind of hormonal manipulation used and the duration of treatment. A study by Wimer and Wimer showed that there was greater granule cell density in the hippocampi of male mice compared to female mice of the same strain, in six different strains of mice.\(^10\) Notwithstanding differences with respect to the species of rodents used and the type of hippocampal cells assessed, the gender differences described by Wimer and Wimer were also found between the hippocampi of male and female rats in our study.

A study by West et al has suggested that volumetric changes, such as shrinkage and oedema, have an impact on the condensation and counting of neurons.\(^10\) For instance, Madeira et al reported that hypothyroidism induced a reduction in the volume of the pyramidal cell layer and caused a parallel increase in the numerical density of its neurons, although the total number of pyramidal neurons was not affected.\(^17\)

The optical fractionator, which was the preferred method in the current study, allows for the estimation of the total number of pyramidal neurons without taking into consideration the volume of the pyramidal cell layer.\(^15\) Furthermore, in our study, the estimations of total number of pyramidal neurons were made for comparison between the various groups rather than for the exploration of the exact number of total pyramidal neurons. While it is possible to investigate hippocampal pyramidal neuron numbers using various counting and quantification methods – all of which have underlying assumptions and principles\(^19\) – we chose the optical fractionator in our study. However, we do not claim that the optical fractionator is the best option for estimating total cell numbers.

In summary, we found gender-based differences in the total number of pyramidal neurons in the hippocampi of male and female rats, with male rats showing a higher number of pyramidal neurons. This difference was also observed between female rat groups treated with TP and control rats, with perinatal TP treatment having an augmenting effect on the number of pyramidal neurons in the hippocampus. Continuous treatment with TP in the prenatal and early postnatal periods in female rats was more effective than administration in only the prenatal or postnatal periods. More studies will need to be conducted to shed light on the mechanisms by which testosterone produces such effects on hippocampal pyramidal neuron numbers.

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**REFERENCES**


### SMA EVENTS JULY - AUGUST 2013

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<th>EVENT</th>
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