

# ***Median Raphe Nucleus of adult rat: sexual dimorphism and effects of female gonadal steroid deprivation***

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## **Abstract**

**Introduction:** During the last decades it is shown that the gonadal steroids affects the nervous system. Gonadal steroids easily cross the brain blood barrier and insert their effects on morphometric parameters of the certain areas of the mammalian brain despite their direct or indirect roles in sexual behavior, the effects that generally called sexual dimorphism (SD). Among the different brain neurotransmitter system, serotonergic system is of more interest to study for SD due to its extensive projections and the role of its neurotransmitter, serotonin (5HT), in different physiological and pathological conditions including neuronal firing, human emotional control, and affective disorders. Although the fact that 5HT is affected by the sexual gonadal steroids, it is not known whether the nuclei related to this system also influenced by gonadal hormones. Median raphe nucleus (MRN) is the largest one among the other nuclei in human brain and involves in many important functional roles. There are not enough evidences regarding the SD in median raphe nucleus of mammalian brain and also the influence of gonadal steroids on its morphometric parameters. The present study was supposed to answer the mentioned doubtfully questions.

**Material & Methods:** Sixty adult male and female Sprague-Dawley rats (200-230g) were used in this study. The animals randomly were divided to four groups including normal female group, normal male group, ovariectomized group (OVX) and sham surgery group. For SD the animals of normal male and female groups were compared and for the study of the effects of female gonadal steroids the normal female group compared with OVX group. The animals perfused and fixed transcardially, brain stem was removed, coronal sections were obtained and processed for light microscopic study. Nissl and Golgi staining used for study morphometric and neuronal morphologic parameters of MRN. Data analyzed and the results presented by means± SD.

**Results:** Based on our findings MRN showed sexual dimorphism and gonadal steroid deprivation via ovariectomy significantly influenced certain morphometric parameters of MRN.

**Conclusion:** According to the results of this study, sexual dimorphism of MRN and the influence of female gonadal steroids on serotonergic nuclei should consider in normal and pathological conditions.

**Key words:** Median raphe nucleus, female gonadal steroids, ovariectomy, sexual dimorphism

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## Introduction

In living organisms ranging from fungi to humans many structural and functional biological properties are determined by sexual and non-sexual steroid hormones.<sup>(1)</sup> As a manner of fact when the behavior of the sexes differs markedly, there must be sex differences in brain structure mediating those behavioral differences. Historically it was believed that there is a unidirectional relation between brain and reproductive system, accordingly only certain areas of the nervous system that directly involves in control of reproductive behavior are different between male and female. During the last decades it has been shown that the sex steroid hormones are small molecules that easily cross blood brain barrier and influence certain parts of the mammalian nervous system at different anatomical and functional levels.<sup>(1)</sup> The effects of gonadal steroids on the nervous system leads to differences between male and female brain and spinal cord areas, which known as SD or sexual dimorphism in nervous system.<sup>(2)</sup> It is now known that levels of circulating sex steroid hormones during development and in adult hood play a critical role in determining physiology and behavior.<sup>(3,4)</sup> The neural mechanisms underlying hormonal effects on brain function and morphology have been the subject of numerous studies. Traditionally, steroid hormone effects

have been divided into 2 categories including organizational or genomic and activational or non-genomic effects.<sup>(5)</sup> Whereas organizational effects have been defined as permanent structural changes induced by hormones during development, activational effects have been defined as the turning on or off of previously established neuronal circuits in adulthood.<sup>(5,6)</sup> For the regions of the CNS which directly involve in reproductive behavior SD varies from differences in the size of individual neurons, dendritic branching patterns, and synaptic organization, to dramatic dimorphisms in the length and volumes of the nuclei. Numerous findings show that these morphometric parameters are not limited to these areas and have been reported in regions not necessarily involved in reproductive behavior.<sup>(7,8)</sup> Since the morphologic characteristics of neurons have been shown to influence the functional properties of the neurons, it is likely that these hormone induced structural changes contribute significantly to the activation of neural circuits necessary for certain behaviors.<sup>(9)</sup> Regarding to the critical periods for sex steroidal effects on structure and function of NS, recent findings emphasize that these effects are not limited to the prenatal and before puberty period but also the level of circulatory sexual hormones in all developmental stages even in adult animal has an important role in

physiology and behavior by affecting the cellular, molecular and microscopic parameters of the neurons nuclei and neural circuits activity.<sup>(10, 11)</sup> Some of these findings mostly obtained from sex steroidal deprivation studies in adult animals that demonstrated any manipulation of sex steroid hormone levels in adulthood can induce reversible or non-reversible dramatic macroscopic and microscopic changes in certain regions of the central nervous system,<sup>(12,13,14)</sup> such as neurons of adult avian song system,<sup>(15,16)</sup> corpus callosum and anterior commissure,<sup>(17,18,19)</sup> bulbocavernous spinal nucleus,<sup>(19,20)</sup> spinal motoneurons,<sup>(21)</sup> rat purkinje cell,<sup>(22)</sup> SDN-POA of hypothalamus,<sup>(23,24,25)</sup> hippocampal pyramidal cells,<sup>(26)</sup> bed nucleus of human stria terminalis,<sup>(27)</sup> nigrostriatal dopamine neurons,<sup>(28)</sup> rat arcuate nucleus,<sup>(29,30,31)</sup> human median raphe nucleus,<sup>(32)</sup> substantia nigra<sup>(33)</sup> and dendritic spine density of the rat dorsal raphe nucleus/DRN.<sup>(34)</sup> The serotonergic system is the most expansive and complex anatomic and neurochemical system in mammalian brain, its projections receive to many areas of the brain, the nuclei and its neurotransmitter are involved in numbers of important physiological and pathological functions.<sup>(35,36)</sup> It is reported that the serotonergic system is particularly sensitive to female gonadal steroids as any alterations in level of female sex hormones dramatically changes

serotonergic system physiology.<sup>(37,38)</sup> Among the serotonergic nuclei, DRN and MRN received the most attention, SD has been reported for DRN but not for MRN, it is also not known whether the female sex steroid deprivation could influence on MRN or not. In the present study MRN as the largest raphe nucleus in human is evaluated firstly for SD and in second for the effects of female gonadal steroid deprivation.

### Materials and Methods

Sixty female and male adult Sprague\_Dawley rats (200-250 g) were used in this study. The animals prepared from Razi Institute (Karj, Hesark Institute,) and were maintained in standardized environment with respect to both photoperiod cycle (10 h light, light at 06.00) and temperature (22° C). Food and water were provided ad libitum throughout the experiments. All the procedures were approved by local committee of Ethic and Research of IUMS. The animals randomly divided to four groups (n=15) including intact female, intact male, ovariectomized rats (OVX), sham surgery. In order to study SD in MRN the normal male and female groups were compared and for the study of the effects of female gonadal steroids deprivation the normal female group compared with OVX group. The morphometric parameters including length, volume, total neuronal number, density and morphology of the neurons of MRN were considered in this study.

For ovariectomy female rats were anesthetized with IP injection of mixture of Ketamine (100mg/kg) and Xylazine(1/8), bilateral ovariectomy was done by midline ventral incision, the ovaries removed, remaining uterus ligated. For sham surgery group the animals under went same surgical procedure without removing the ovaries. One month of interval time considered for both groups. For Nissl staining the animals were perfused and fixed transcardially with aldehyde solutions (1.25% Glutaraldehyde, 4% Paraformaldehyde in Phosphate Buffer 0.1M pH 7.4) followed by 10% sucrose solution in Phosphate Buffer for cryo-protection. The brain stem was removed and maintained in graded 10%, 20% and 30% sucrose solution in Phosphate Buffer 0.1M pH 7.4 in 4°C for 3 days. By using freezing microtome, coronal sections of 30µm sections were obtained and processed for Nissl staining. Golgi-impregnation method was done for study the morphology of the neurons of MRN as follows, all the rats were deeply anesthetized with intraperitoneal injection of same anesthetic mixture and transcardially perfused with 300 ml 1% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate-buffered saline (PBS; pH 7.6) followed by 450 ml of second fixative containing 6% potassium dichromate, 6% chloral hydrate and 10% formaldehyde in 0.1M phosphate-buffered saline (PBS; pH 7.6). Brain stems were removed, blocks

of 3 mm thick containing DRN prepared and postfixed in the second fixative for 24 h. Then, the blocks put in the solution of 3.5% potassium dichromate in distilled water for 48h, after that stored in 1.5% silver nitrate for 72 h in dark room. Dehydration and paraffin embedding were done and by using a rotatory microtome coronal sections of 80µm thickness were cut, mounted and coverslipped. Light microscopic study was done and by using Olysia Bioreport software morphometric parameters of MRN including length, volume, total neuronal count and neuronal density was studied. The morphology of the neurons of MRN also studied by light microscopic. All the slides containing Golgi-impregnated and Nissl staining brain stems were coded prior to quantitative analysis. The code was not broken until after the analysis was completed. By using SPSS 15, the means of variable were determined and these data were subjected to 1-way ANOVA analysis, the results were presented in mean± SD. The  $P_v < 0.05$  was considered as significant.

## Results

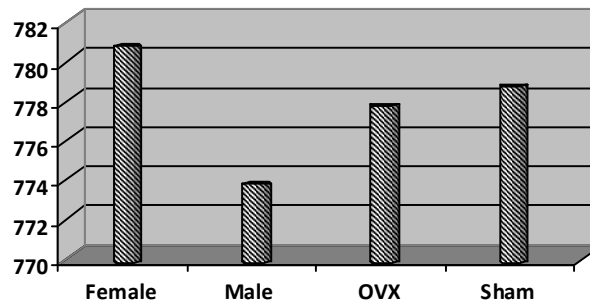
In the present study the location of MRN in coronal sections of brain stems was defined based on Paxinos and Watson atlas of Rat Brain, according to the atlas the MRN is located ventral to decussation of superior cerebellar peduncles (xscp) and medial longitudinal fasciculus, the nucleus extended

rostromedially from xscp appears to pontine reticular nucleus (PnC) appears in sections. MRN also is surrounded by a very well defined vascular ring that helps us to define the border of the nucleus; the mentioned ring was recognizable in whole length of MRN that separates MRN from paramedian raphe area (Fig 1 A & B). Two types of neural staining including Nissl and Golgi were used in this study; the results respectively are as follows.

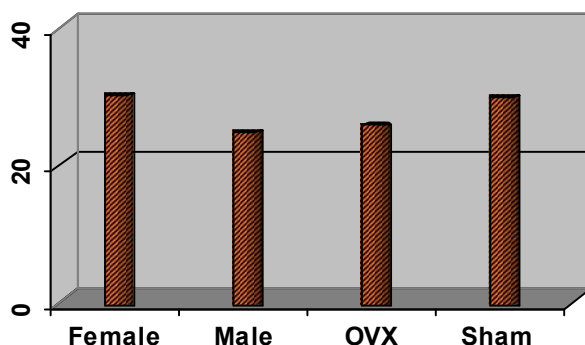
***I- Results of Nissl staining***

Nissl staining was used to study and compare the morphometric parameters of MRN including

rostromedial length, total volume, number and density of the neurons among male, female, OVX and sham groups. For SD we compared the mentioned parameters between male and female rats. Our findings showed SD in length, volume and number of the neurons of MRN Total volume of MRN (fig.1 and tables 1-3). According to our data the length, volume and number of the neurons of MRN of female rats was larger and higher than male rats (graph 1-3).



**Graph1: MRN volume**



**Graph 3: MRN neuronal number**

For the volume and number of the neurons of MRN the sex differences was considered significant with  $P_v$  less than 0.05 ( $P_v = 0.0001$ ,  $P_v = 0.02$  respectively). Regarding the rostrocaudal length of the MRN, our data showed non-significant longer length in MRN of female rats than male ( $P_v=0.65$ ). To study of the effects of female gonadal steroid deprivation the results obtained from female rats considered as control group and

compared with OVX and sham groups. Our findings showed significant decrease of the numbers of the neurons of MRN in OVX rats comparing to normal and sham animals (fig. 3 A-F). For the volume of MRN there was also significant decrease of volume of MRN between OVX rats with sham and normal female ( $P_v < 0.05$ ). Length of MRN of OVX rats non-significantly is less than normal female and sham animals (table 1-3, graph 1-3).

<b>Group</b>	<b>Mean</b>	<b>SEM</b>
<b>Female</b>	<b>176.2</b>	<b>3.955</b>
<b>Male</b>	<b>150</b>	<b>3.317</b>
<b>OVX</b>	<b>164.8</b>	<b>2.154</b>
<b>Sham surgery</b>	<b>180.8</b>	<b>2.557</b>

**Table1: MRN volume ( $10^{-3} \text{ mm}^3$ )**

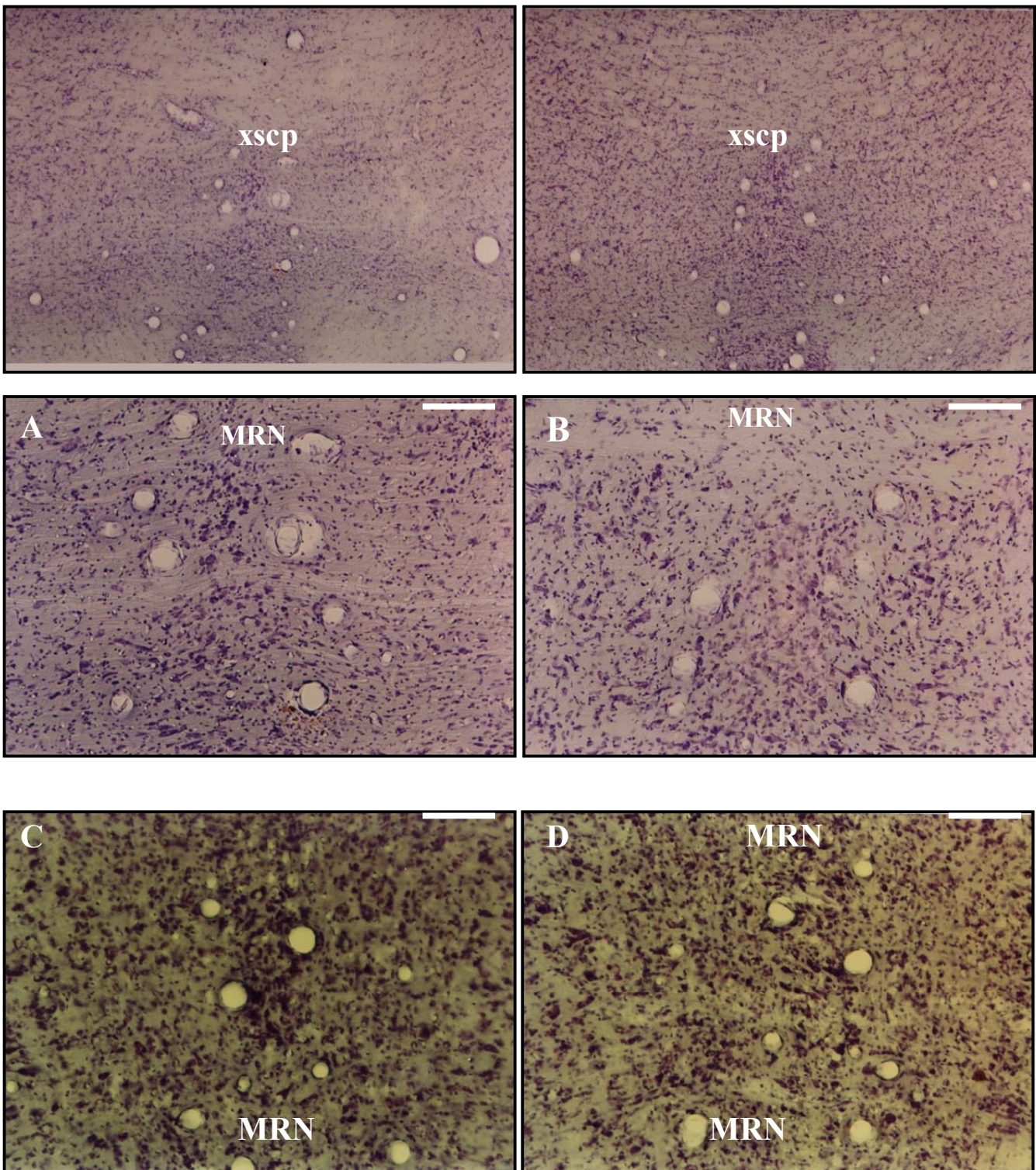
<b>Group</b>	<b>Mean</b>	<b>SEM</b>
<b>Female</b>	<b>30.86</b>	<b>0.9011</b>
<b>Male</b>	<b>25.46</b>	<b>0.5454</b>
<b>OVX</b>	<b>26.50</b>	<b>1.0302</b>
<b>Sham surgery</b>	<b>30.52</b>	<b>0.8438</b>

**Table3: Total cell number**

## **II- Result of Golgi staining**

We used Golgi staining only to study and compare the morphology of the neurons of MRN among the male, female and OVX groups. As MRN is surrounded by a recognizable vascular ring so light microscopic examination of Golgi-impregnated tissue revealed reliable and consistent neuronal staining throughout the MRN of all brain stems. In particular, three neuronal populations

of MRN including polygonal, fusiform and round neurons were always well represented and easily identifiable in all parts of MRN of the Golgi stained sections of all groups (Fig. 3 A-F). Same population from morphological point of view was seen in male, female and OVX animals. We did not compare the number, density and fine structure of the neurons among the groups.



**Fig 3: Photomicrographs of MRN. Intact female (A-C-E) , OVX (B-D-E) Scale bar for A & B 50 μm, C & D 30 μm, E & F 10 μm**



## Discussion

Results of the present study exactly showed SD in MRN, it is also showed that short-term female sex steroid deprivation through ovariectomy resulted in significant changes of morphometric parameters of MRN compared with that of intact females. SD has been reported for certain various nuclei of the brain in rodent, mammalinas, primates and humans based on morphometric parameters including volume, length, number and density of the neurons,<sup>(13,28,39)</sup> In most of the cases the SD parameters in male nuclei such as DRN,<sup>(34)</sup> SDN-POA,<sup>(25)</sup> bed nucleus of human stria terminalis,<sup>(27)</sup> and rat arcuate nucleus,<sup>(31)</sup> are more dominant than female. To our knowledge the only exception among these sexually dimorphic nuclei with dominancy in female than male reported in 1996 by Gorski et al is AVPN-SDN.<sup>(24)</sup> There are few reports regarding SD in MRN, Cordero et al in 2000 reported SD in number and proportion of neurons in the human MRN via postmortem study, they showed male dominancy in MRN. No reports exist for SD in rodents, our findings not only showed SD in rat MRN but also female dominancy, which is different from our previous study for rat DRN that showed SD with male dominancy.<sup>(34)</sup> To explain our findings for SD of MRN attention to cellular and molecular mechanisms of sex steroidal effects on nervous system is needed. Numerous researches over the

last decades has provided evidences for extensive steroid-dependent plasticity in the adult brain. Neuroactive steroid compounds apply to those steroids that have biological effects or activities in nervous system which may originate from various sources: a) the endocrine system mainly adrenal and sexual glands,<sup>(40, 41)</sup> b) the nervous system itself where steroids can be synthesized de novo,<sup>(28,39,42)</sup> c) exogenous from various environmental sources.<sup>(2,43)</sup> Sex steroids regulate neuronal function by influencing neurons via steroid receptors which have been defined in many parts of the brain, either involving in reproductive behavior or not.<sup>(28,44)</sup> Neurons responsive to gonadal steroids at least may have three types of steroids receptors acting throughout different mechanisms: 1) the well known intracellular steroid receptors responsible for steroidal genomic action,<sup>(45,46,47)</sup> that once activated act as transcription factors and may trigger gene expression and are typically responsible for late and long lasting neuronal response<sup>(2,9,48,49)</sup> 2) effects mediated by membrane receptors for non-genomic actions, 3) the recently described membrane steroidal receptors<sup>(50,51)</sup> which may be coupled directly to membrane ion channels or second messengers system that elicit rapid and transitory changes on neural structure.<sup>(52,53,54)</sup> In the case of steroidal effects on the serotonergic nuclei, it seem to be linked to the presence of estrogen and progesterin-sensitive

neurons in the midbrain raphe nuclei and/or as well as possibly nongenomic actions in brain area to which serotonin neurons project their axons.<sup>(6)</sup> In our beliefs these considerations are not enough to explain different findings for SD of DRN and MRN in rats. There might be other mechanisms are still unknown and needs more reseach. For the results of the effects of sex steroid deprivation, there are numerous evidences that show female sex steroid deprivation leads to dramatical changes of morphometric parameters of certain nuclei in rodents and primates brain. Gorski et al (1996) demonstrated that OVX in rats significantly decreases the length and volume of sexual dimorphic nucleus of AVPN-POA.<sup>(24)</sup> Sexual dimorphic of dopaminergic neurons of substantia nigra have been frequently studied. Leranath et al in 2000 reported that OVX and gonadal steroids deprivation in female monkeys after 30 days will cause a 30% decrease in these cells and steroid treatment will not compensate it, but it is compensating with steroid prescription after 10 days.<sup>(28)</sup> Jameie et al in 2004 reported same results for DRN of OVX rats, that followed OVX the number, density of denderitic spines and length of denderits significantly reduced.<sup>(34)</sup> Regarding these data neuroprotective properties suggested for female sex steriods. Growing evideces show that there are potent mechanisms to explain neuroprotective function of estrogens

and preventing of apoptosis is one of these mechanisms<sup>(2,11,28)</sup> Low level of circulatory female sex steroidal hormones following OVX might induces apoptosis that in turn leads to morphometric changes of the nuclei. The exact mechanisms of this phenomenon is still unknow and needs more reseach.

### **Conclusion**

In summary, the cross talk between sex steroidal hormones and central nervous system play important roles in regulating behaviors either physiologic or pathologic. This interaction should consider in diagnosis, clinical and therapeutical plan.

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