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Sex and Age Differences of Neurons Expressing NOS Immunoreactivity in the Pag of Male and Female Rats

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Expression of the enzyme nitric oxide synthase (NOS) was studied in the periaqueductal gray matter (PAG) of male and female rats during postnatal development at 30, 60 and 90 days of age. NOS-immunoreactive neurons were located in the dorsolateral (dlPAG), lateral and ventrolatelal (vlPAG) longitudinal subdivisions. Morphometric analysis revealed sexual dimorphism in the density of NOS-immunopositive neurons in the vlPAG of 30 days old prepubertal, 60 days old pubertal and 90 days old rats. Females showed numerous NOS-immunopositive neurons than males. The present results suggest that sex differences in the number of NOS-immunopositive neurons in the vlPAG may be related to epigenetic effects of gonadal hormones in the postnatal development.

Key words: PAG – NOS, postnatal development, sex differences, rat

Introduction

The midbrain periaqueductal gray (PAG) plays a modulatory role in a variety of behaviors including antinociception, reproduction, fear and anxiety, aggression and vocalization and sex differences are modulated by both the organizational and activational effects of gonadal steroids [5]. It is described to possess four longitudinal cell-rich columns - dorsomedial (dmPAG), dlPAG, lateral and vlPAG subdivisions, which serve as distinct anatomical modules for the specific functions [see 10]. The PAG integrates input from the limbic forebrain (including the amygdala) and the diencephalon with ascending input from the dorsal horn [2] and projects to the rostral ventromedial medulla (RVM). The RVN in turn projects to the dorsal horn of the spinal cord and elicits the antinociceptive effects of opiates, as well as sex differences in opioid analgesia are modulated by effects of gonadal steroids [5]. Despite the critical role played by the PAG–RVM system in the spinal response to noxious stimulation, very little is known about the control exerted by brain stem descending fibres during postnatal development [2]. One set of primary factors that contribute to brain sexual differentiation are steroid hormones that are produced of the gonads and act directly in the developing brain. There are several ways to categorize the molecular mechanisms that drive brain deve-

lopment with or without sexual differentiation. One class of molecules that control gene expression is transcription factors, second class is effector molecules, which control and contribute to signaling from one cell to another. The potential molecular effector is nitric oxide (NO), which is a product of the enzymatic conversion of L – arginine to citrulline and is produced by three forms of NOS – neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). NO plays many roles in development as well as adulthood. NO helps cell migration, cell proliferationand survival, which are all important factors for sexual differentiation [see 3].

In the light of these issues, the arm of the present study was to determine the density of NOS -immunopositive neurons in the PAG during postnatal development of the brain in male and female rats.

Material and methods

Nine female and 9 male Sprague-Dawley rats were used to study the localization of NOS immunoreactivity in the developing PAG. Intact animals were classified into 3 age groups: 30 days old, prepubertal rats, 60 days old, pubertal rat and 90 days old, young postpubertal rats. Animals were anaesthetized with thiopental (40 mg/kg body weight). Transcardial perfusion was performed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brains were removed from the skulls and postfixed for 1 h in the same fixative. Afterwards, brains were washed in 0.1 M phosphate buffered saline (PBS) overnight at 4°C. Coronal sections (40 μm thick) were cut on a freezing microtom (Reichert-Jung, Germany). Sections were made at three PAG levels: cranial PAG - between bregma - 5.3 and bregma - 6.3, middle PAG - between bregma - 6.3 and bregma - 7.3 and caudal PAG - between bregma - 7.3 and bregma -8.3 [6]. Free-floating sections were preincubated for 1 h in 5% normal goat serum in PBS. Afterwards, incubation of the sections was performed in a solution of the primary antibody for 48 h at room temperature. We used a monoclonal anti-nNOS antibody (Sigma, St. Louis MO, USA) in a dilution of 1:1000 according to instructions of the manufacturers. After rinsing in PBS, sections were incubated with biotinylated anti-mouse IgG (Vector Labs. Inc. Burlingame, Calif., USA, dilution, 1:500) for 2 h. Sections were washed in PBS and incubated in a solution of avidin-biotin-peroxidase complex (Vectastain Elite ABC reagent; Vector Labs., Burlingame Calif., USA; dilution 1:250 in PBS) for 1 h. This step was followed by washing in PBS and then in 0.05 M Tris-HCl buffer, pH 7.6, which preceded incubation of sections in a solution of 0.05% 3,3¢-diaminobenzidine (DAB, Sigma) containing 0.01% H_2O_2 for 10 min at room temperature for the visualization. Sections were collected in Tris-HCl buffer 0.05 M, pH 7.6. In control sections, no significant staining was observed under the control conditions. Morphometric analysis was performed by capturing images of PAG through a 40 objective using a microanalysis system Nikon photomicroscope ECLIPSE 80i (digital camera DXM 1200C and the measured area of 0.360185 mm^2). Data the entire drawings were entered.

Results and discussion

Areal staining patterns on coronal sections of across the rostrocaudal axis in PAG subdivisions at levels of +5.3 to + 8.3 mm from bregma [6] were analyzed (Fig. 1).

The principal findings were as follows. First, immunostaining of the NOS immunoreactivity showed a striking specific pattern of neuronal profiles in dlPAG, vlPAG

and around aqueductus cerebri (AC) in male and female rats (Fig. 2). The distribution of the NOS-immunoreactive neurons in the PAG generally coincided with that observed in previous studies [1, 8]. Most of NOS-immunoreactive neurons are medium size ovoid, fusiform to multipolar or small rounded neurons arrenged in dlPAG, vlPAG and around AC (Fig. 2), a phenomenon reported [1, 4].

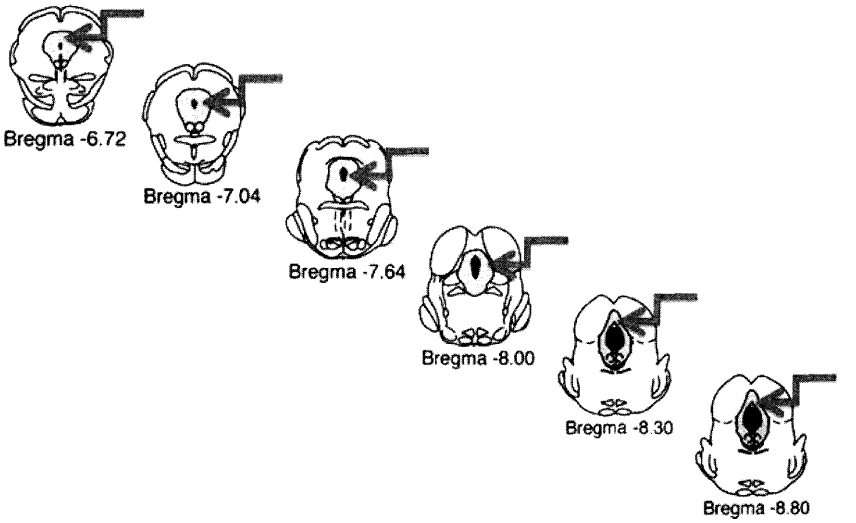

Fig. 1. Sections were made at three PAG levels: cranial PAG – between bregma – 5.3 and bregma – 6.3. middle PAG – between bregma – 6.3 and bregma – 7.3, caudal PAG – between bregma – 7.3 and bregma – 8.3

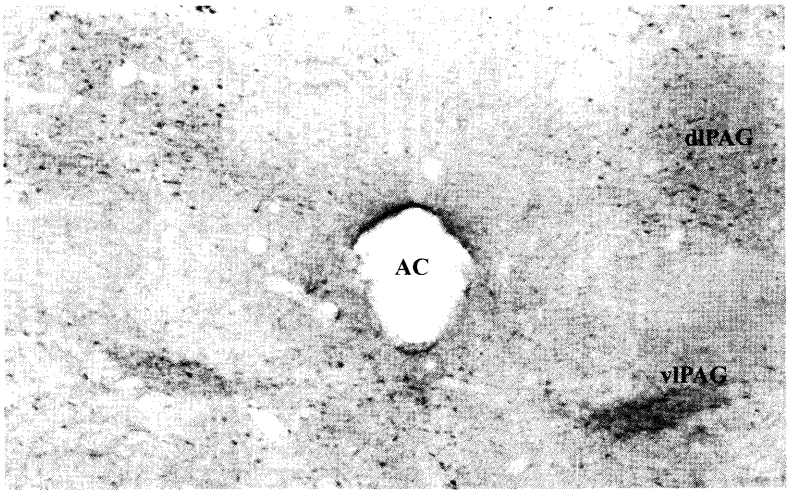

Fig. 2. NOS – immunopositive neurons are located in dl PAG, vlPAG and around aqueductus cerebri (AC). × 4

Second, the average density of NOS-immunoreactive neurons per μm^2 in the vl-PAG of female rats was greater than in males of the tested age groups (Fig. 3. Females showed a greater density of NOS-immunoreactive neurons than males and increased with age in both sexes. The average density of NOS-immunoreactive neurons in the dlPAG of male and female rats were similar in all age groups ($P>0.1$; Fig. 4). However, NOS-immunoreactive neurons showed a increase in number per μm^2 during aging in both sexes (Figs. 3, 4).

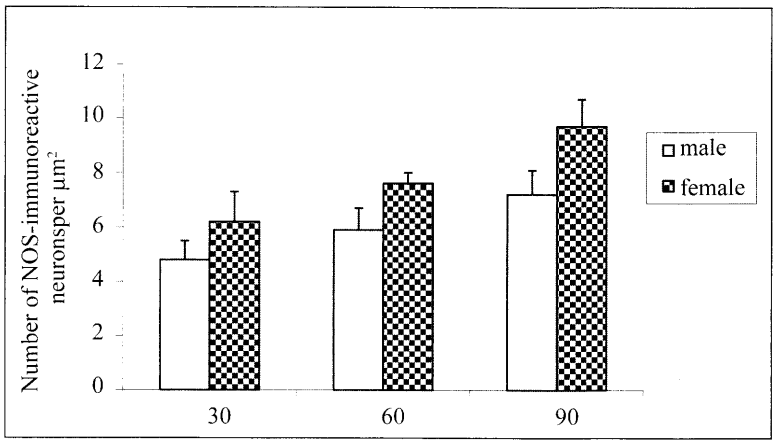

Fig. 3. The average density of the NOS-immunoreactive neurons in the vlPAG of female irats is greater than in male rats at 30 days, 60 days and 90 days male rats. There is a statistically significant increase in the neuronal density from female to male rats ($P < 0.05$).

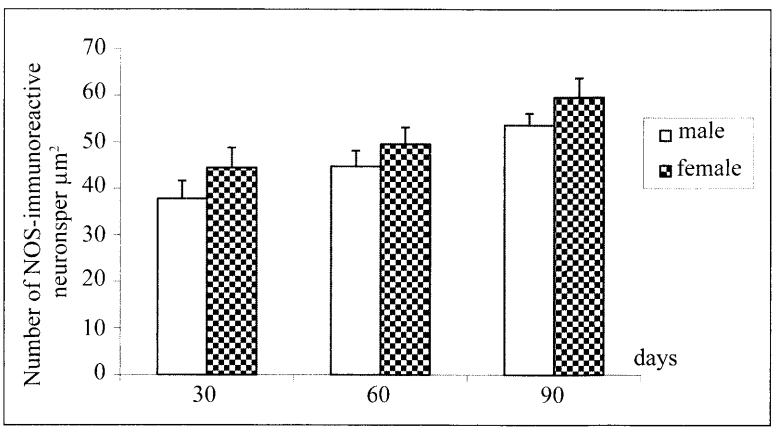

Fig. 4. The average density of the NOS-immunoreactive neurons in the dlPAG of female is greater than in male rats at 30 days, 60 days and 90 days male rats. There is not a statistically significant increase in the neuronal density from female to male rats ($P < 0.1$).

Third, these results suggest that sex differences in the density of NOS-immunoreactive neurons in the rat vlPAG is related to epigenetic effects of gonadal hormones during early stages of development and undergo additional modifications in later stages.

This conclusion corresponds to results that showed such a correlation between androgens and expression of different neuroactive substances in various brain regions [7, 9].

In summary, our morphometric study reveals that sex-dependent differences in the density of NOS-immunoreactive neurons of the postnatal vlPAG is established in all postnatal ages. These new data emphasize the need to examine NOS immunoreactivity in neurons in postnatal PAG after experimental manipulation of the hormonal balance.

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