Rapid changes on nitrinergic system in female mouse hippocampus during the ovarian cycle

RAPID CHANGES ON NITRINERGIC SYSTEM IN FEMALE MOUSE HIPPOCAMPUS DURING THE OVARIAN CYCLE

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Abstract

Fluctuating levels of estradiol and progesterone during the estrous cycle may induce structural changes in several brain nuclei including the hippocampus, where some neurons express estrogens receptors. Nitric oxide plays a wide range of functions in the nervous system generally by acting as a neurotransmitter-like molecule. It has been demonstrated that long-term treatments with estradiol in ovariectomized females and with testosterone in castrated males induce neuronal nitric oxide synthase (nNOS) expression in rat hypothalamus, whereas changes in nNOS immunoreactivity or in associated NADPH-diaphorase activity were observed both in hypothalamus and in amygdala during different phases of estrous cycle. Estradiol could induce nNOS expression in several brain regions in rodents. Therefore, to clarify if the hippocampal NO producing system is a target for gonadal hormones in physiological conditions, we have investigated the effects of estrous cycle in the expression of nNOS immunoreactivity on two months old intact female mice.

Immunoreactive cells were observed in all hippocampal subregions: the higher number was detected in the pyramidal layer of CA1 region and in polymorph layer of dentate gyrus. The number of nNOS positive neurons fluctuates during the estrous cycle, reaching its peak during proestrus and metaestrus, and these variations were statistically significant in CA1, CA2 and CA3 regions. These results suggest that the nitrinergic system is a target for estrogen action in the hyppocampus, and that this action may take place in physiological conditions according to the short-term variations of gonadal hormones during the estrous cycle.

Keywords: nitric oxide synthase, gonadal hormones, estrous cycle, neuronal plasticity, mice

Introduction

Gonadal steroid hormones are known to regulate brain function by producing long-term and/or short-term structural changes in the organization of several brain nuclei (Melcangi and Panzica, 2006), including the hippocampus (for reviews see McEwen, 2001; Prange-Kiel et al., 2006; Ogiue-Ikeda et al., 2008). The density and the number of dendritic spines on hippocampal CA1 pyramidal neurons are sensitive to fluctuating levels of estradiol and progesterone during the estrous cycle in the adult female rat (Gould et al., 1990; Woolley and McEwen, 1992), in addition, recent studies demonstrated the importance of estradiol local synthesis in *in vitro* models (Prange-Kiel et al., 2006) and the neuroprotective effects of estrogens as well as phytoestrogens on hippocampal neurons (Azcoitia et al., 2006). In rat, pyramidal neurons of CA1 and CA3 areas and granule cells of the dentate gyrus express estrogens receptors alpha and beta (Shughrue et al., 1997) and estradiol induced rapid modulation of synaptic transmission and spine morphology (Mukai et al., 2007; Hojo et al., 2008; Ogiue-Ikeda et al., 2008). Generally, estrogens exert stimulating effects and this positive induction requires activation of N-methyl-D-aspartate (NMDA) receptor (Woolley and McEwen, 1994; Gazzaley et al., 1996). Moreover, several brain proteins has been shown to fluctuate over the estrous cycle in rodents (Chen et al., 2007; Diao et al., 2007; Diao et al., 2008a; Diao et al., 2008b; Spencer et al., 2008).

Nitric oxide (NO) has been largely accepted to play a wide range of functions in the nervous system (Bredt and Snyder, 1992; Garthwaite and Boulton, 1995; Ohkuma and Katsura, 2001) generally by acting as a neurotransmitter-like molecule that readily

diffuses across cellular membranes. NO can contribute to both anterograde and retrograde signalling at the synapse. The classic NO target is the soluble guanylyl cyclase (Ignarro, 1990; 2002) and the given effect is to increase the intracellular levels of cyclic GMP (cGMP), followed by the cGMP-dependent protein phosphorylation cascades. Recent findings have shown that higher concentrations of NO can modify proteins through direct chemical reactions like S-nitrosylation without stimulating production of cGMP (Ahern et al., 2002).

Since the gaseous properties of NO make it near impossible to localize, major insights about its production site came from the study of the three nitric oxide synthase (NOS) enzymes involved in its synthesis: endothelial (eNOS) and neuronal (nNOS) forms, Ca^{2+} dependents, and macrophagical NOS (mNOS), Ca^{2+} independent (Moncada et al., 1991; Moodley, 2002). All these types of NOS are present within the nervous system, though the nNOS type is more frequently observed (for a review see Panzica et al., 2006).

Gonadal hormones can trigger nNOS expression (for a review see Panzica et al., 2006): it has been demonstrated that long-term treatments with estradiol in ovariectomized females and with testosterone in castrated males induce nNOS expression in rat hypothalamus and hippocampus (Weiner et al., 1994; Ceccatelli et al., 1996; Du and Hull, 1999; Grohe et al., 2004) whereas changes in nNOS immunoreactivity or in associated NADPH-diaphorase (ND) activity during different phases of estrous cycle were observed both in hypothalamus (Martini et al., 2004; Sica et al., 2009) and in the amygdala (Collado et al., 2003; Carrillo et al., 2007). Biochemical data show that nNOS

can bind to the postsynaptic scaffoid molecule PSD-95, forming a complex with the NMDA receptor (Christopherson et al., 1999) and this effect depends on estrogens (d'Anglemont de Tassigny et al., 2007, 2009); moreover, in female mice, during the proestrus, there is an increase in the expression of PSD-95 (Spencer et al., 2008).

Previous studies have described the presence of NO producing neurons in the hippocampus of rodents using both the ND histochemical technique or antibodies against nNOS. ND positive cells have been described in rat (Vincent and Kimura, 1992; Valtschanoff et al., 1993) and in mouse (Cork et al., 1998), whereas nNOS-immunoreactive (nNOS-ir) neurons were observed in rat (Chung et al., 2004) and in male mice (Gotti et al., 2005).

Western-blot studies demonstrated changes in nNOS expression after exposure to estrogens of ovariectomized female rats (Grohe et al., 2004). To clarify if the hippocampal NO producing system is a target for gonadal hormones also in physiological conditions, we have here investigated the effects of estrous cycle in the expression of nNOS immunoreactivity on two month-old intact female mice.

Materials and methods

Animals

A total of 24 female mice were used for this study. The mouse strain employed in this study was previously obtained in our laboratory by crossing two lines of mice: C57BL/6J and DBA2. At the age of 4 weeks the animals were housed into groups of five per cage and were maintained in LD 12:12 photoperiods (lights on 0700 h EST) at 20 ± 2°C with relative humidity of 50 ± 5% until the sacrifice. Food and tap water were available *ad libitum*. Determination of the stage of the estrous cycle was assessed by examining vaginal cytology (for details see Becker et al., 2005). The animals were inspected immediately before the sacrifice, and females at different points in the estrous cycle were randomly selected (e.g., one in proestrus, one in diestrus, one in metestrus, and one in estrus) for tissue collection.

Animal care was in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and the animal investigation committee of Italian MIUR approved the experimental protocol.

Fixation and tissue preservation

All animals were sacrificed at the age of 8 weeks. Mice were deeply anaesthetized with an intraperitoneal injection of xilazine-ketamine (50mg/kg body weight) and perfused through the heart with saline solution (0.9%) until vessels were completely blood-free, and then with 400 ml of fixative (4% paraformaldehyde in 0,1 M phosphate buffer, pH 7,3). Brains were dissected out of the skull, post-fixed for 2 hours

in the same fixative and rinsed in 0.01 M phosphate buffer saline (PBS). They were then placed overnight in a 30% sucrose solution in PBS, frozen in precooled liquid isopentane at -35°C, and stored in a deep freezer at –80°C until sectioning.

Brains were serially cut in the coronal plane at 25 µm thickness using a cryostat. The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the mouse brain atlas (Paxinos and Franklin, 2001). Sections were collected in a cryoprotectant solution (Watson et al., 1986) at -20°C. Every forth section (one section every 100 µm) was stained for nNOS immunohistochemistry. The use of alternate sections is a routine technique in our laboratory and has two advantages, the first is the possibility of comparing adjacent sections to understand the spatial relationships among the investigated systems, and the second is to reduce drastically the number of animals for the research. Adjacent sections were Nissl-stained with toluidine blue, or used for controls. Brain sections were always stained in groups containing females from all estrous cycle points, so that between assays variance could not cause systematic group differences.

nNOS immunocytochemistry

The free-floating sections collected in the cryoprotectant solution were washed overnight in PBS at pH 7.3. The following day, sections were first washed in PBS containing 0.2 % Triton X-100 for 30 min and then treated to inhibit endogenous peroxidase activity with a solution of PBS containing methanol/hydrogen peroxide for 20 min (Streefkerk, 1972). Sections were incubated for 30 min with normal goat serum

(Vector Laboratories, Burlingame, CA, USA) and incubated overnight at room temperature with anti-nNOS rabbit antibody (ImmunoStar, Hudson, WI, USA) diluted 1:12,000 in PBS, pH 7.3-7.4, containing 0.1-0.2% Triton X-100. A biotinilated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) was then employed at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by 60 min incubation with avidin-peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA). The peroxidase activity was visualized with a solution containing 0.187 mg/ml 3,3-diamino-benzidine (Sigma, Milan, Italy) and 0.003% hydrogen peroxide in 0.05M Tris-HCl buffer pH 7.6. Sections were mounted on chromallum coated slides, air dried, cleared in xylene and cover slipped with Entellan (Merck, Milan, Italy).

The commercial antibody against nNOS was generated in rabbit against a C-terminal synthetic peptide sequence (1419-1433) of human nNOS. The manufacturer (dr. Jeffrey Spangenberg, IncStar, Stillwater, MN) tested the specificity of the antibody by Western blot analysis and pre-adsorption with synthetic human nNOS (5 mg per ml of antibody at working dilution). No cross reactivity with other isoforms of NOS was reported (Dawson et al., 1991; Eliasson et al., 1997). The nNOS antiserum has been successfully used in human, rat, mouse, cat and monkey tissue. In particular, the specificity of this antibody for mouse central nervous tissue was tested in nNOS-knock out mice where the staining was totally abolished (Kriegsfeld et al., 1999). We have performed the following controls in our material: a) the primary antibody was omitted or replaced with

an equivalent concentration of normal serum (negative controls); b) the secondary antibody was omitted. In these conditions, cells and fibers were totally unstained.

Sections were examined and photographed using a Nikon Eclipse 80i microscope connected to a Nikon DS-Fi digital video camera. All images were processed using Adobe Photoshop 7 on a Power PC G4 Macintosh.

Quantitative analysis

To identify the neuroanatomical structures we followed the mouse brain atlas (Paxinos and Franklin, 2001). We selected three corresponding sections of anterior hippocampus from each animal (Bregma -1.58, -1.70, -1.82). Acquired images were analysed using ImageJ 1.36b software (Wayne Rasband, NIH, Bethesda, MD, USA) running on a Power PC G4 Macintosh; measurements were performed on just one side (right).

During the various steps a few sections were lost and consequently some animals were excluded from the quantitative analysis. Therefore, we have finally analyzed 5 proestrus, 5 estrus, 5 metaestrus, and 5 diestrus female mice.

For each animal and section we quantified the area of the hippocampus as well as that of CA1, CA2, CA3 and Dentate Gyrus (DG) subregions by manually tracing the corresponding boundaries: this measure was utilized to compute, based on the thickness of the section (25µm), the relative volume. Total sampled volumes of both hippocampus and each subregions were calculated summing the volumes of the three examined levels.

Cells were counted using the particle counting protocol of ImageJ. We counted all the positive neurons (identified for the presence of a clearly labelled cell body), in the CA1, CA2, CA3 and Dentate Gyrus (DG) subregions of the hippocampus for the three selected levels. The absolute number of cells was determined using the Abercrombie method (1946) and, finally, we obtained the cell density (reported as number of positivite cells per mm^3) for each animal and region by dividing the absolute cell number for the total volume.

Volume values were compared by one-way analysis of variance (ANOVA) followed, when appropriate, by a post-hoc comparison test (Fisher PLSD).

Cell density (mean value for the three sections) was compared by repeated measures ANOVA (considering the different subregions of the hippocampus as repeated measures, while the independent variable was the estrous cycle). This first analysis reported a significant effect (see results), therefore we subsequently performed a one-way ANOVA for each subregion followed, when appropriate, by a post-hoc comparison test (Fisher PLSD).

The analysis were performed using the software StatView 5 (Abacus Concepts, Inc., Berkeley, CA, USA). Data are presented as means ± standard error (S.E.) and differences were considered statistically significant for values of $p < 0.05$.

Results

General distribution of nNOS neurons

The immunostaining for nNOS provided an easy characterization of the hippocampus, of its boundaries and of its different layers. The region between the CA3 field and the DG was the more intensely stained zone (Fig. 1A, 2).

nNOS immunoreactive neurons are distributed in the various layers in a homogeneous manner, though it is possible to note generally a larger number of cells in the pyramidal layer of CA1 region and in polymorph layer of dentate gyrus in comparison with the other regions and layers; a consistent number of neurons was also present in the radiatus layer, while CA2 region shown only a few positive neurons.

Ammon's horn

Stratum oriens. Only few cells, prevalently located in CA1, were observed. Medium-sized, multipolar with a round-shaped cell body neurons were located in the middle of the layer; these cells were weakly-stained if compared to pyramidal cells and have weakly stained dendrites (Fig.1B). A few, bipolar and weakly stained neurons were present near the alveus.

Pyramidal layer. It's the layer with the higher number of neurons. Based on cell location and morphology these cells are likely to be local interneurons. In particular, large-sized, intensely stained cells, with a well-defined unstained nucleus, were present in CA1 region. These neurons have a round-shaped cell body, with long dendrites reaching both stratum radiatum and stratum oriens (Fig.1C). A small number of cells were bipolar, with processes reaching the adjacent layers (Fig.1D). In CA3 region there are nNOS-medium-sized immunopositive cells with less-stained processes.

Stratum radiatum. Medium-sized neurons, prevalently located in CA1 region, were present. These cells have long and branched dendritic trees that reached the adjacent layers. A larger part of these neurons was unipolar cells (Fig.1E).

Stratum lacunosum-moleculare. The smallest neurons of all layers were present in this stratum. These cells were less stained and had short dendrites.

Dentate gyrus

In the granular cell layer, nNOS-immunopositive cells were present in limited number; these neurons were prevalently localized near the polymorph layer (Fig.1F). In the molecular layer, positive neurons were localized in the inner portion of the layer.

The polymorph layer had the major number of cells; these neurons were multipolar, with a round-shaped cell body. It is possible to note a large number of processes (Fig.1G).

Quantitative analysis

The volume of the hippocampus did not change (data not shown) and the ANOVA analysis reports not significant effect of the cycle ($F(3,16) = 0.355$, $p = 0.7862$). On the contrary, the density of nNOS positive neurons fluctuates during the estrous cycle, reaching its peak during proestrus and metaestrus; the diestrus, instead, is characterized by the lower number of nNOS positive cells (Fig.2). The repeated measures ANOVA

performed on the cell density shows an overall statistically significant effect of the estrous cycle in all subregions ($F(3,16) = 3.638$, $p = 0.0357$).

We have therefore performed a one-way ANOVA for each subregion, followed by the post-hoc comparison test (Fisher PLSD) (Fig. 3). More in details:

- CA1 region: Metaestrus mice have the highest density of positive cells (1354.200 ± 130.738/mm^3); the one-way ANOVA analysis reports not significant effect of the cycle ($F(3,16) = 2.094$, $p = 0.1413$), however, the post-hoc comparison with Fisher PLSD test show a statically significant differences between metaestrus and diestrus $p = 0.0327$.
- CA2 region: Proestrus mice have the highest density of positive cells (996.000 ± 82.013/mm^3), and the one-way ANOVA reported a significant effect of estrous cycle ($F(3,16) = 3.931$, $p = 0.0281$). The post-hoc Fisher PLSD test reveals significative differences between proestrus and diestrus ($p=0.0052$) and between metaestrus and diestrus ($p = 0.0195$).
- CA3 region: In this region metaestrus mice show the highest density of positive cells (871.600 ± 127.933/mm^3). One-way ANOVA reported a significant effect of estrous cycle ($F(3,16) = 3.840$, $p = 0.0302$). The post-hoc Fisher PLSD test reveals significative differences between proestrus and diestrus ($p = 0.0166$) and between metaestrus and diestrus ($p = 0.0062$).
- DG region: Proestrus mice have the highest density of positive cells (1128.600 ± 97.344/mm^3), and the one-way ANOVA reported no significant effect of estrous

cycle (F (3,16) = 0.628, p = 0.6073). The post-hoc Fisher PLSD test reported no statistically significant differences for all the comparisons.

Discussion

In the present study we report the first detailed anatomical description of the distribution of nNOS-ir neurons in the hippocampus of female mouse. Previous studies performed in rat show difference among the different strains: in male Wistar rats the nitrinergic neurons are prevalently located in pyramidal neurons in the CA1 pyramidal layer (Rodrigo et al., 1994); instead, in male Sprague-Dawley rats nNOS-immunopositive cells are prevalently located in interneurons in the pyramidal layer, in the granular layer of the dentate gyrus and in the subiculum. A discrete number of cells were also noted in the CA3 pyramidal layer and in the molecular layer of the dentate gyrus (Valtschanoff et al., 1993). No detailed data are available for female rat.

Based on previous studies performed in rat (Vincent and Kimura, 1992; Valtschanoff et al., 1993; Chung et al., 2004), our data show that the hippocampal distribution of mouse nNOS-ir neurons is largely different. In fact, in the present study we show that the nitrinergic system of female mouse has a more homogeneous distribution among the layers and an overall less cell density compared to rat, with the highest cell density (interneurons) in the pyramidal layer of CA1 and in the polymorph layer of dentate gyrus.

In addition, our data indicate that the density of nNOS-ir neurons significantly fluctuates in several hippocampal subregions of female mice during the estrous cycle. This finding

is in agreement with our previous studies in mouse hypothalamus (Martini et al., 2004; Sica et al., 2009), rat medial amygdala (Carrillo et al., 2007) and rat basal nucleus of the accessory olfactory tract (Collado et al., 2003). As reported for the mouse hypothalamic nuclei, the effect of estrous cycle phase on nNOS-ir cell density is not similar for all the investigated regions and reach a statistically significant value in CA2, CA3 and partially in CA1. In these regions the increase of nNOS positive cells happens during the proestrus (which correspond to a peak of estradiol in the cycle) and the metaestrus (which correspond to a peak of progesterone in the cycle), in all regions the lowest cell density has been observed during the diestrus, when both hormones are at low levels.

As reported in the introduction, a direct relation among estradiol and nNOS expression has been demonstrated in experimental conditions in female rat ventromedial nucleus of hypothalamus (Ceccatelli et al., 1996) and hippocampus (Grohe et al., 2004). However, in these experiments ovariectomized females were treated for several days with high levels of estrogens, and there is no demonstration that this effect is also present in physiological conditions.

Who regulates whom? It is known that pyramidal neurons in hippocampal CA1 and CA3 area and granule cells in the dentate gyrus expresses ER alpha and beta (Shughrue et al., 1997; Shughrue et al., 2000) and that progestin target cells were seen in the pyramidal layer of the CAl, CA2, and CA3 and in the dentate gyrus of hippocampus (Shughrue et al., 1992).

The genes encoding both eNOS and iNOS contain estrogens response elements (EREs) in their 5'-flanking promoter region (Grohe et al., 2004). However, these EREs are not

present in the nNOS gene (Forstermann et al., 1998). Thus, it is highly probable that the estrogenic effect on nNOS may be due to an indirect action, involving the intervention of regulation factors able to regulate nNOS promoter, as, for example, the estrogen-dependent transcription factor AP-2 (Hall et al., 1994). AP-2 is able to induce the activation of several genes such the receptor of estrogens (McPherson and Weigel, 1999), its expression may vary during the estrous cycle (Patrizi et al., 2005) and AP-2 expressing cells are present in mice hippocampus (Coelho et al., 2005).

The other player during estrous cycle is progesterone. Previous studies showed that in the preoptic area and in the hypothalamus of ovarectomized rat the contemporary administration of estrogens and progesterone greatly enhances the NO/cGMP signalling pathway, increasing levels of nNOS (Chu et al., 2004); a similar regulation might take place also in the hippocampus of female mice. In addition, recent data show that a putative progesterone response element is present in the 5'-flanking region of the mouse iNOS gene (Coughlan et al., 2005). However, no data are available for nNOS gene and no significant changes in progesterone receptor isoform content in the hippocampus were observed in the normal fluctuation of gonadal hormones during the estrous cycle (Guerra-Araiza et al., 2003).

Finally, the expression of nNOS could be modulated through changes in signalling proteins that are expressed at different levels during the estrous cycle (Diao et al., 2007). Among them, some nitric oxide associated signalling proteins as the NG-proteins (NG, NG-dimethylarginine dimethylaminohydrolase type 1 and 2) that hydrolyze the arginine

residues to citrulline and increase NO generation, may induce modulation of nNOS in vivo.

In conclusion, the data reported in this study illustrate the presence of a nitrinergic population of neurons in the female mice hippocampus that may vary, in a region-specific way, according to different physiological conditions linked to the variations of gonadal hormones during the estrous cycle. The relationships among physiological variations of estrogen levels and in vivo modulation of nNOS expression remain unknown, however, it seems highly probable that they depend by a multifactorial series of events that may include, gonadal hormone receptors distribution and co-existence, estrogen-regulated transcription factors and signalling proteins.

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Figure Legends:

Figure 1

A. Coronal sections illustrating the CA1, CA2, CA3 and DG regions of the hippocampus in female mice and the distribution of nNOS immunoreactive neurons in the various layers. **B**. High magnification of nNOS immunopositive multipolar cells in oriens layer. **C**. Multipolar cell in the pyramidal layer. **D**. Bipolar cell in the pyramidal layer. **E**. Pseudounipolar cell in the radiatum layer. **F**. Multipolar cells in the granular cell layer. **G**. Multipolar cells in the polymorph layer. Or, oriens layer; Py, pyramidal cell layer; Rad, stratum radiatum; Lmol, lacunosum moleculare layer; Mol, molecular layer of dentate gyrus; Gr, granule layer of dentate gyrus; Pol, polymorph layer of dentate gyrus. Magnification bars: A = 300 μm; B-G = 20 μm.

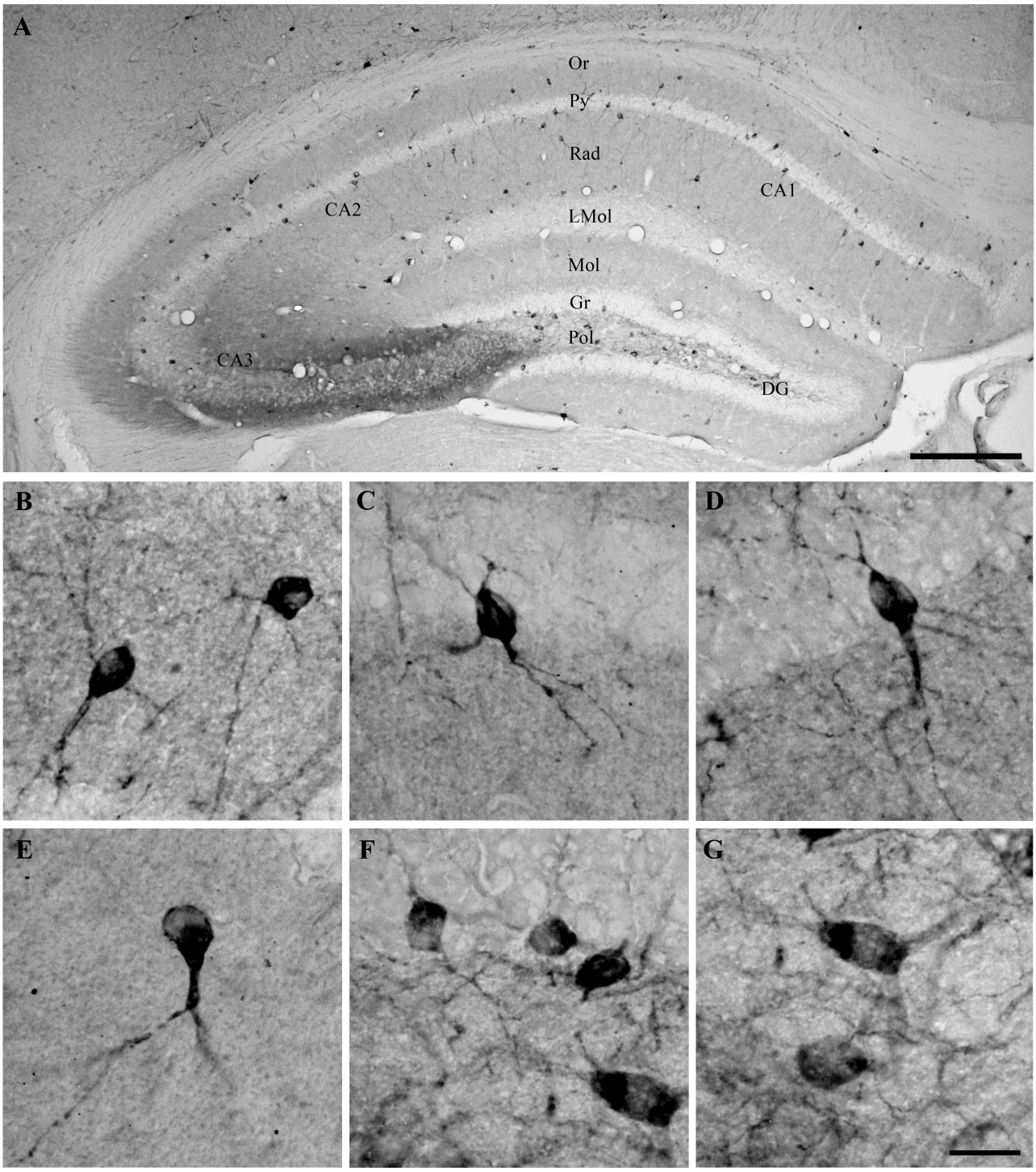
Figure 2

Coronal sections illustrating the CA2, and CA3 regions of the hippocampus. Comparison between among proestrus, estrus, metaestrus, and diestrus female mice in the distribution of nNOS immunopositive cells. It is possible to note that in proestrus and metaestrus female mice there are a major number of cells and fibers. Magnification bar = 200 μm.

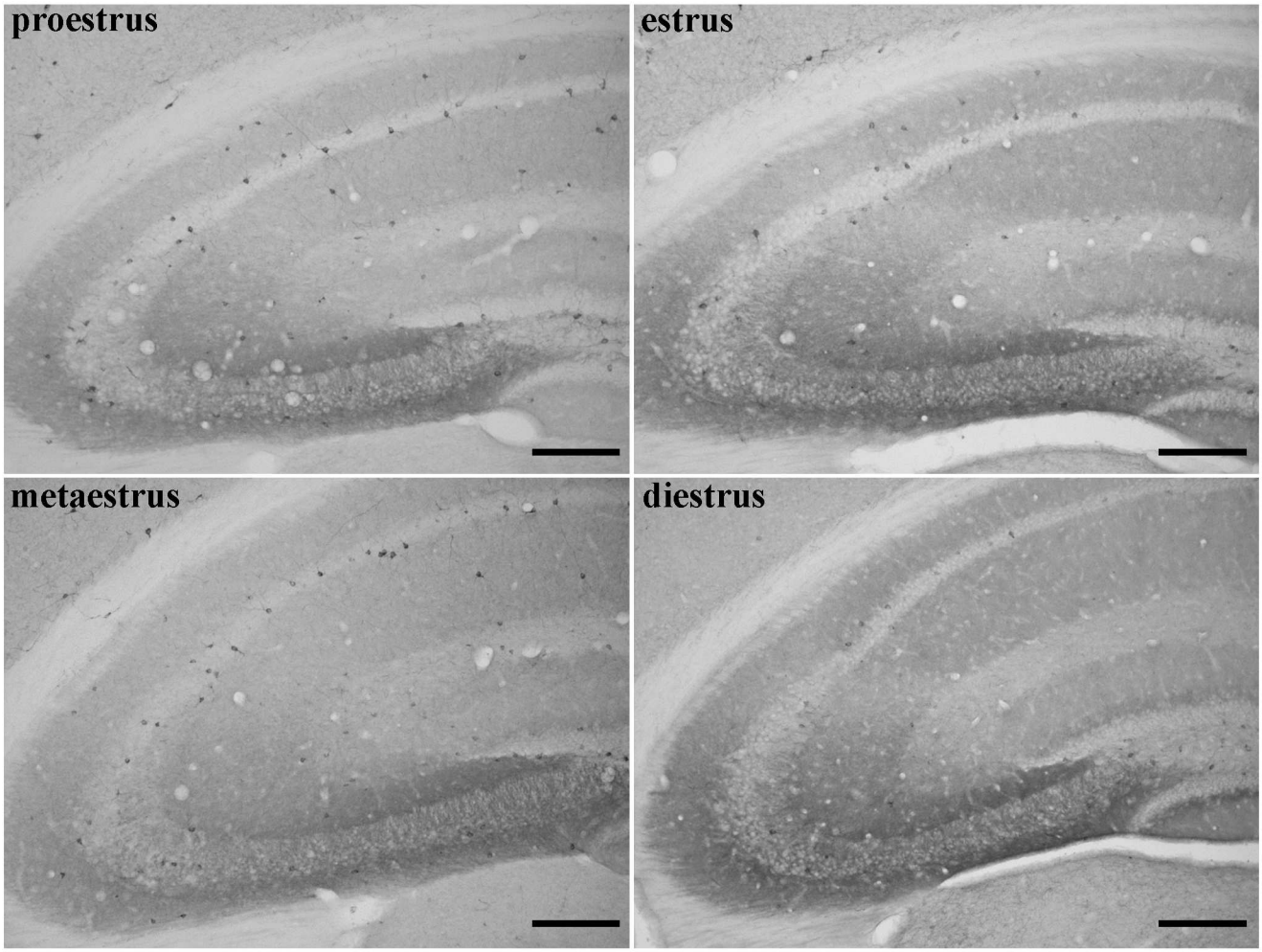
Figure 3

Histograms illustrating the variations of nNOS-positive cell density (expressed as mean ± S.E.) of in CA1, CA2, CA3 and DG region of the hippocampus during female estrous cycle. Cell Density (* $p \leq 0.05$ metaestrus compared with diestrus mice in CA2 and proestrus compared with diestrus mice in CA3; ** $p < 0.01$ proestrus compared with diestrus mice in CA2 and metaestrus compared with diestrus mice in CA3.

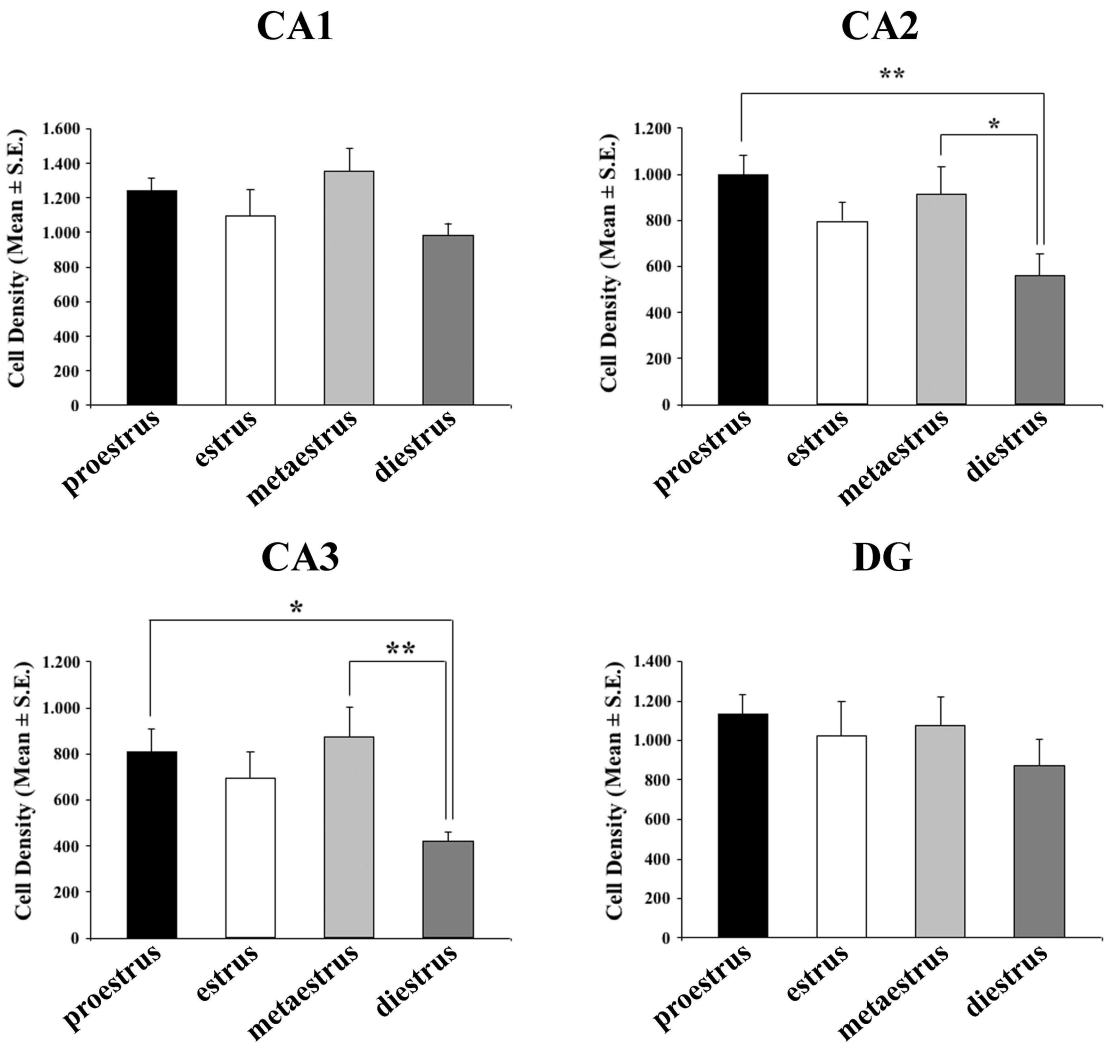
Figure(1)



Figure(2)



Figure(3)


CA1
Cell Density (Mean ± S.E.)
1.600
1.400
1.200
1.000
800
600
400
200
0
proestrus
estrus
metaestrus
diestrus
CA2
**
*
Cell Density (Mean ± S.E.)
1.200
1.000
800
600
400
200
0
proestrus
estrus
metaestrus
diestrus
CA3
*
**
Cell Density (Mean ± S.E.)
1.200
1.000
800
600
400
200
0
proestrus
estrus
metaestrus
diestrus
DG
Cell Density (Mean ± S.E.)
1.400
1.200
1.000
800
600
400
200
0
proestrus
estrus
metaestrus
diestrus