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Alteration of rat hippocampal neurogenesis and neuronal nitric oxide synthase expression upon prenatal exposure to tamoxifen

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Abstract

The present study delineates the effect of tamoxifen on neuronal density and expression of neuronal nitric oxide synthase (nNOS) in hippocampal nerve cells during prenatal and postnatal periods in rats. Pregnant rats were administered with tamoxifen one day prior to labor (E21) and on the childbirth day (E22). Hippocampi of embryos at E22 and newborns at postnatal days of 1, 7, and 21 (P1, P7, and P21) were investigated. Density of the neurons in areas of the developing hippocampus including cornu ammonis (CA1, CA3), dentate gyrus, and subiculum were studied. Our findings show that the number of pyramidal neurons was significantly decreased in CA1 and subiculum of tamoxifen-treated rats in E22, P1, and P7. We found that cellular density was lower in early stages of development, however, cellular density and thickness gradually increased during the development particularly in the third week. We found that nNOS expression was decreased in E22, P1, and P7 in animals treated with tamoxifen. The present study shows that tamoxifen affects development and differentiation of postnatal rat hippocampus, CA1 neurons, and nNOS expression.

Keywords: Hippocampus; Tamoxifen; Developing brain; Rat; Nitric oxide

1. Introduction

Nitric oxide (NO) produced by NO synthase (NOS) isozymes plays various prominent functions in the central and peripheral nervous systems. Three NOS isozymes including neuronal-(nNOS), endothelial-(eNOS), and inducible-NOS (iNOS) have been well-characterized [1]. The nNOS is the predominant NOS isoform in the nervous system [2] whereby NO participates in diverse cellular processes including neurogenesis and differentiation [3–5]. Growing evidence suggest that NO is involved in regulating the proliferation and development of neuronal cells. The majority of neurons in the brain are produced during embryonic development. However, certain parts of the germinal zones of the developing brain continue to proliferate into adulthood and generate large numbers of neurons in the adult brain. Nitric oxide and its downstream effectors regulate cell proliferation and differentiation in the developing nervous system and exert important negative regulatory roles on cell proliferation in the adult mammalian brain. The nNOS plays a significant role in the neuronal development of mammalian brain cerebellum, sensory gliaa, and olfactory epitherlium [6]. Nitric oxide also mediates the formation of synaptic connections in developing and regenerating olfactory receptor neurons [7]. Nitric oxide has been suggested as an important modulator of primary inductive and patterning events in the embryo [8]. Nitric oxide negatively regulates cell and tissue differentiation during mammalian adult neurogenesis [9] and plays a neurotrophic role in neuronal cell–cell communication dur-

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ing the development of the brain [10]. NADPH-diaphorase (NADPH-d) has been used extensively as a reliable NOS marker in brain and spinal cord [11]. The nNOS in hippocampus is localized at the sites of neuronal proliferation and migration [5] and responsible for most NO produced in these regions [12]. NADPH-d histochemical technique has been used to study NOS expression in the developing brain and spinal cord and to correlate NOS expression with regions of neurogenesis. These regions include the cerebellum [4], olfactory neurons, the cerebral cortex, and hippocampus [6,13], as well as proliferative zones in the spinal cord [11]. *In situ* evidence also suggests a role for NO in neural proliferation in cultured neonatal rat cerebellum [14,15].

Various hormones regulate differentiation, growth, and development of the nervous system. Estrogens play important roles in the development of nervous system including neuronal and non-neuronal cell growth, neuron differentiation, and survival and death of the brain cells. Antagonism of estradiol receptors potently decreases the neurogenesis and significantly affects neuronal morphometric parameters, suggesting that during early phase of development maternal estrogens are important for proper development of the newborn's hippocampal neurons. Tamoxifen possesses both estrogen agonist and antagonist properties depending on the species, tissue or cell type [16,17]. In central nervous system, tamoxifen antagonizes estrogen receptors and induces cell death in a variety of cells [18,19]. While estrogens enhance the development of hippocampal neurons in CA1 region, [20], tamoxifen inhibits the development and decreases cell number in the hippocampus by altering the estradiol level [16].

The present study examined the ontogeny of nNOS-containing neurons in developing rat brain and investigated the effect of tamoxifen on distribution pattern of NOS-positive neurons in various regions of rat hopocampus. We also tested the hypothesis that tamoxifen suppresses hippocampal NOS expression in the mature rat brain, and studied neurogenesis and the immunohistochemical localization of nNOS in the developing hippocampus of rat brain during the embryonic and postnatal days.

2. Materials and methods

2.1. Animals

Forty adult female and 20 adult male Wistar rats weighing 180–220 g were used. All rats were housed under light–dark cycles of 12 h with access to standard rat food pellets and water *ad libitum*. Vaginal smear from each female rat was examined daily and rats entering estrus were mated with adult males. Vaginal smears of mated females were examined on the following morning. If sperm was observed, this day was designated as Day 1 of pregnancy and the pregnant females were housed in separate cages. One day prior to labor (E21) and on the childbirth day (E22) pregnant rats received tamoxifen (250 μg I.P.) twice daily. Animals at the same gestational age were used as control. Control animals were mated similar to treatment group and received equal amount of the solvent (propylene glycol).

Each group was further divided into the following 4 subgroups: 6 h after the last injection of tamoxifen (E22), 16 h after birth (P1), 1 week after birth (P7), and 3 weeks after birth (P21). Each subgroup contained 6 rats.

2.2. Tissue preparation

Animals were anesthetized with sodium pentobarbital (30 mg/kg I.P.), perfused transcardially with paraformaldehyde (4%) and glutaraldehyde (1%) solution in phosphate buffer (0.1 M, pH 7.4), and hippocampi were removed as described [18,21,22]. A portion of the tissue was immersed in the same fixative and stored overnight at 4 °C in sucrose (30%) in phosphate buffer (0.1 M, pH 7.4). Tissues were embedded in OCT compounds (Tissue Tek, Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen, and sliced 20 μm using a cryostat (Leica 1302). The sections were mounted on poly-Lysine-coated slides and allowed to air dried prior to histochemical and immunohistochemical staining. The other portion of the tissue in paraffin section was dehydrated and stained with Nissl stain (Merck; Germany) for morphological studies using an Olympus photomicroscope (PROVIS AX70, Japan) equipped with a digital camera (DP11, Japan).

2.3. NADPH-diaphorase histochemistry

Cryostat sections were pre-incubated 10 min at room temperature in phosphate buffer saline pH 7.2 (PBS) containing Triton X-100 (0.2%) and incubated with β-NADPH (2 mM; Sigma, St. Louis, MO, USA) and nitro blue tetrazolium (0.3 mM; Sigma, St. Louis, MO, USA) dissolved in PBS containing Triton X-100 (0.2%) for 30 min at 37 °C. Blank samples were prepared similarly except β-NADPH was omitted. After rinsing in PBS, the sections were mounted in glycerol gelatin.

2.4. nNOS immunocytochemistry

The cryostat sections were immersion-fixed at 4 °C and pre-incubated with PBS containing Triton X-100 (0.2%) for 2 h at room temperature. Samples were incubated overnight with one of the following antisera: rabbit antisera against nNOS C-terminal (1:1500; Sigma, St. Louis, MO, USA) or N-terminal (1:1000; Eurodiagnostica, Arnhem, the Netherlands). The primary antisera were diluted in PBS containing bovine serum albumin (BSA, 1%) and Triton X-100 (0.2%). For visualization of the immunoreactive products, the sections were rinsed in PBS and incubated 90 min with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (1:80; Jackson Immunoresearch Laboratories, West Grove, PA, USA), FITC-conjugated donkey anti-sheep IgG (1:80; Sigma, St. Louis, MO, USA), or Texas

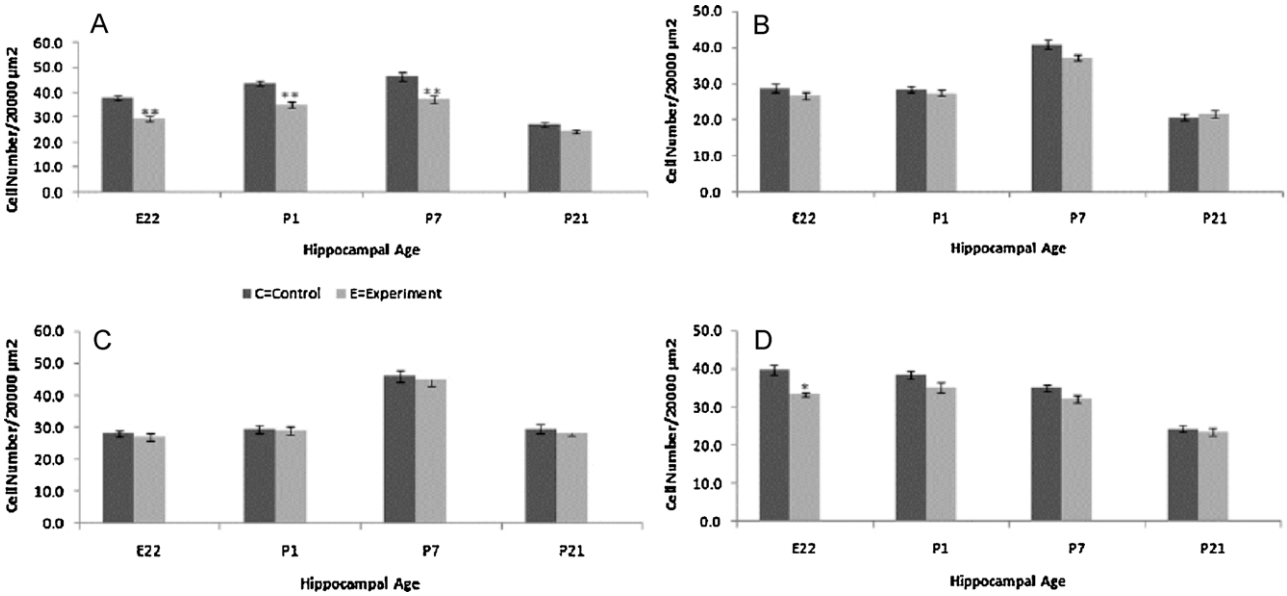

Fig. 1. Quantitative analysis of the effects of developmental stages and tamoxifen treatment on cell number. The number of cells was counted in relation to the subregion and age. (A) Tamoxifen significantly reduced the cell number in CA1 subregion to first week and demonstrated no effects in third week. (B) Tamoxifen did not alter the cell number in CA3 subregion in all ages. (C) Tamoxifen did not alter the cell number in D.G. subregion at either age. (D) Tamoxifen reduced the number of cells in subiculum to first week and demonstrated no effects on in third weeks. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ significantly different from Control.

Red-conjugated affinity purified F (ab')2 fragments of donkey anti-mouse IgG (1:160; Jackson Immunoresearch, West Grove, PA, USA) diluted in PBS containing BSA (1%). Incubations with primary and secondary antisera were performed at room temperature in moisture chambers. Probed samples were rinsed and mounted in PBS/glycerol with *p*-phenylenediamine to prevent fluorescence fading. Micrographs were obtained using an Olympus epifluorescence microscope equipped with filters set for Texas Red and FITC immunofluorescence.

2.5. Western blotting

The hippocampal CA1 regions were homogenized in ice-cold lysis buffer containing Tris HCl (50 mM, pH 8.0), NaCl (150 mM), Nonident P-40 (1%), glycerol (10%), phenylmethyle sulfonyl fluoride (10 (l/ml), sodium deoxycholate (0.5%), and aprotinin (30 (l/ml), subjected to centrifugation at 12,000 × *g* for 20 min, and supernatants were collected. Sixty micrograms from total protein of the supernatant was loaded on each lane and electrophorased on SDS-PAGE gels (12%). Proteins were transferred onto nitrocellulose membranes overnight at 4 °C and blocked with PBS containing non-fat dried milk powder (5%) for 1 h. Membranes were probed with monoclonal anti-nNOS antibody (1:40,000; Sigma, St. Louis, MO, USA) and developed with BCIP/NBT as described [23,24].

2.6. Statistical analysis

Results are expressed as mean ± SE. Non-parametric methods were used to compare the Means. Statistical evaluations were performed using one-way ANOVA followed by Student's *T*-test. Differences were considered significant when *p* value was less than 0.05.

3. Results

3.1. Nissl staining

Fig. 1 shows the number of neuronal cell bodies in the hippocampal subregions CA1, CA3, and DG, and in subiculum at E22, P1, P7, and P21. Fig. 2 shows the variations in chromatin density in neuronal cell bodies in the hippocampal subregions CA1, CA3, DG, and subiculum at E22, P1, P7, P21. As shown, tamoxifen significantly decreased the number of pyramidal neuronal cells in the hippocampal subregion CA1 at E22, P1, and P7. However, no difference was observed at P21 between treated and control samples. Likewise, tamoxifen significantly altered the thickness and the number of pyramidal cells in the CA1 subregion of the hippocampus in all time periods except P21. A minor decrease of neuronal density was observed in subiculum of animals treated with tamoxifen on P21.

3.2. NADPH-diaphorase staining and nNOS immunoreactivity

To examine the expression of nNOS in developing hippocampi, we compared NADPH-d staining and nNOS immunoreactivity in hippocampal subregions CA1 at E22, P1, P7, and P21. Intense NADPH-diaphorase reactivity was

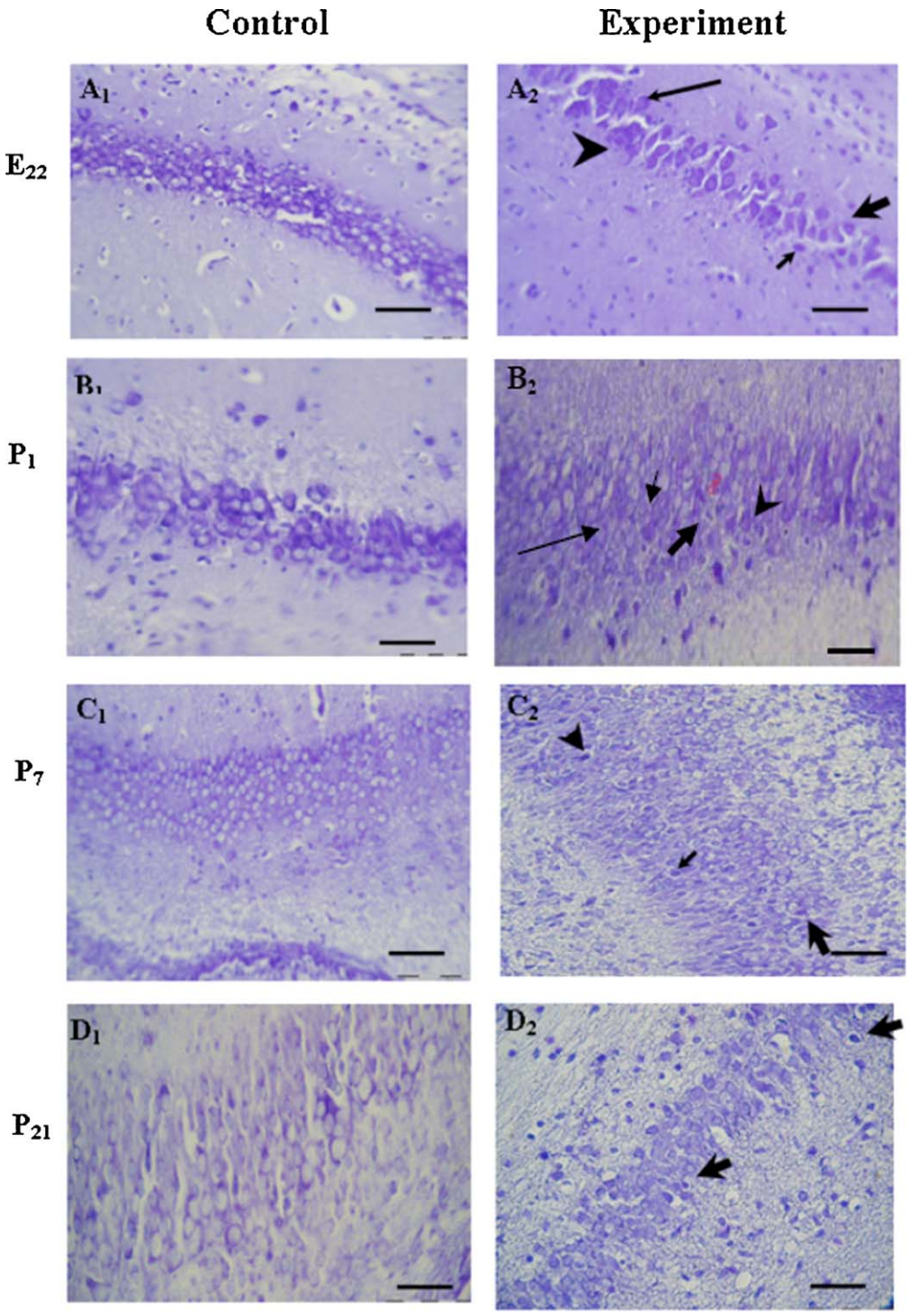

Fig. 2. Photomicrograph of Nissl staining of CA1 neurons of E22 (A1, A2), P1 (B1, B2), P7 (C1, C2), and P21 (D1, D2) of control (Control) and tamoxifen-treated (Experiment) animals. In the control groups the thickness of the layers increases from E22 to P21 and neurons are being matured. In the experimental groups damaged neurons (arrowheads) are heavily stained and dense nuclei with irregular nuclear membrane (short thin arrows), nuclear shrinkage (thick arrows), and chromatin clumping (long thin arrows) are marked. P21 is the least affected area and exhibited shrunken and triangulated neuronal body with a possible lightly reaction in the immediate vicinity. Majority of neurons in P21 area are morphologically unaltered and similar in form and staining to those in the control tissue. Photomicrographs are representative of $n = 8$. Scale bars = 250 μm.

found in neurons in early stage of development (Fig. 3). Our studies demonstrate that tamoxifen-treated rats did not show NADPH-d staining suggesting that NOS expression was inhibited by tamoxifen. In P1 the positive reaction is found without tamoxifen treatment and weak reaction as low density is found without tamoxifen treatment (Fig. 4). Examination of the sections obtained by histochemistry staining at P7 revealed moderate labeling in the CA1 without

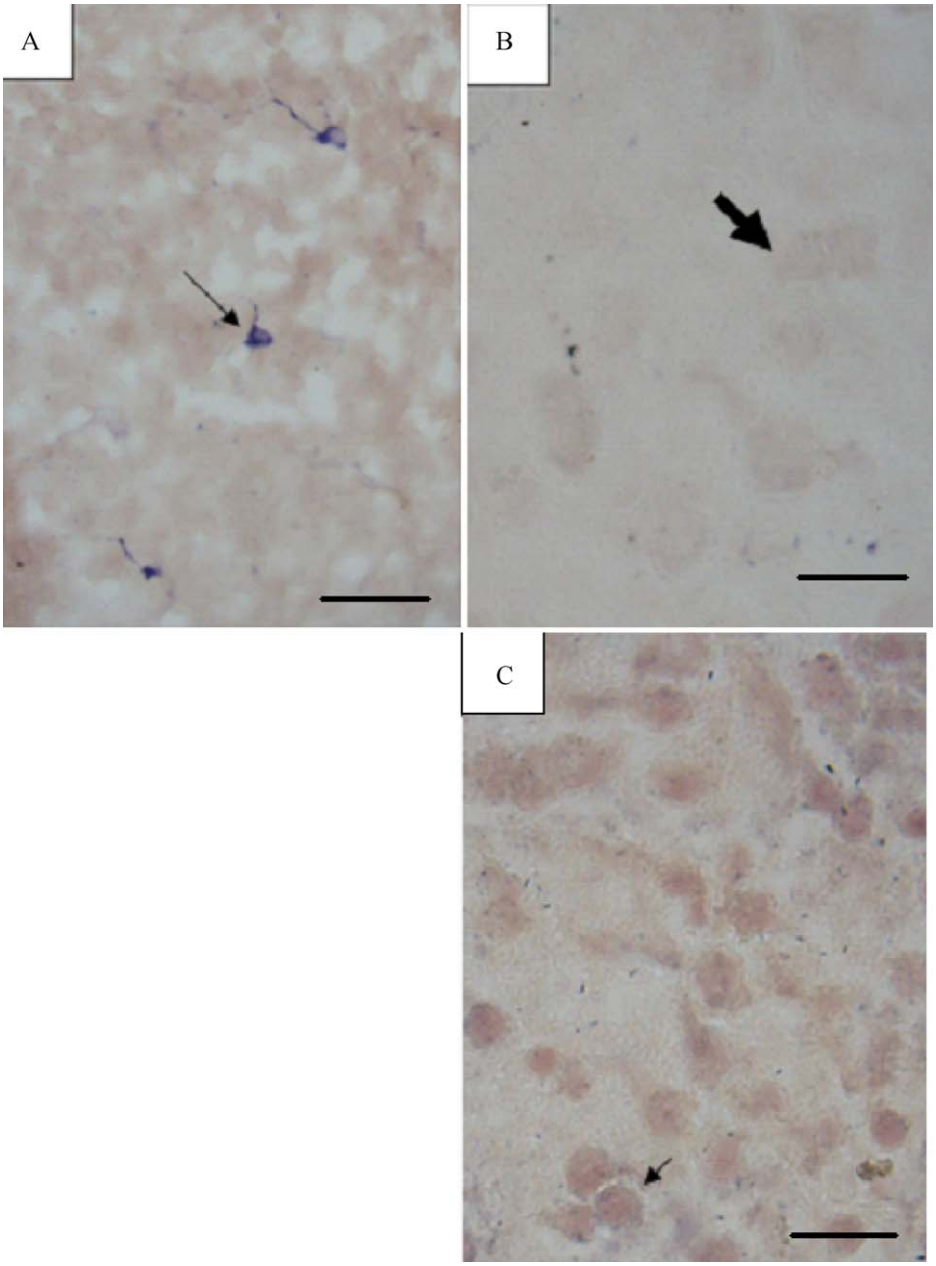

Fig. 3. NADPH-diaphorase staining of CA1 subregion in prenatal E22. Thick arrow shows a neuron. Long thin arrow shows strong NADPH-d reaction. Short thin arrow shows weak NADPH-d reaction. Panel A: staining is shown for NADPH-d reaction in normal group, without treatment, in early development stage. Few neuron show high intense NADPH reaction (long thin arrow). Panel B: blank NADPH-d reaction in treatment group (thick arrow). Panel C: weak NADPH-d reaction in treatment group. (short thin arrow). Photomicrographs are representative of $n = 8$. Scale bar = 100 μm.

tamoxifen treatment (Fig. 5). Very weak NADPH-diaphorase reaction was restricted to small portion of neurons distributed in early developing CA1. A moderate reaction is found in late stage of development (P21) without tamoxifen treatment and strong reaction in early stage of development without tamoxifen treatment (Fig. 6). Considering the biological half-life of 8 h for tamoxifen in rats [18,22], three weeks is sufficient for full clearance of the drug. The highest density of neuronal staining of hippocampus was observed at P21 (Fig. 6C). Results obtained by using immunofluorescent microscopy (Fig. 7) further support the histochemistry data.

3.3. Western blot

Immunoblot was used to further characterize nNOS expression in CA1 samples. Fig. 8 shows nNOS was migrated as a single distinct band at approximately 160 kDa. Consistent with our histological findings, nNOS was present at relatively low level at E22 (Fig. 8). There-

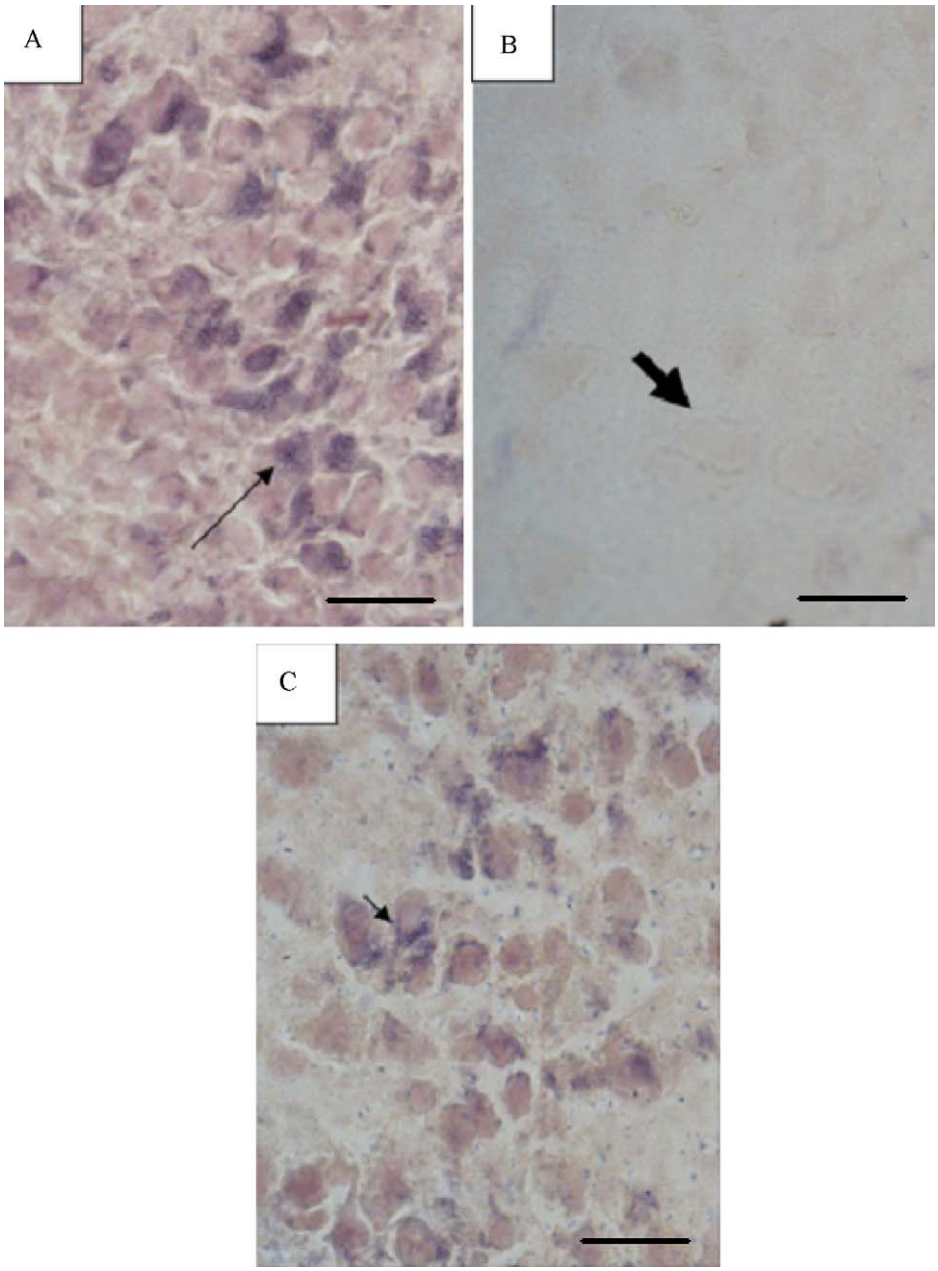

Fig. 4. NADPH-diaphorase staining of CA1 subregion in postnatal P1. Thick arrow shows a neuron. Long thin arrow shows strong NADPH-d reaction. Short thin arrow shows weak NADPH-d reaction. Panel A: NADPH-d reaction is shown in normal group, without treatment, in development stage. We demonstrated intense (weaker than E22) NADPH reaction (long thin arrow). Panel B: blank NADPH-d reaction in treatment group. There is no NADPH-d reaction (thick arrow). Panel C: NADPH-d reaction in treatment group is weak (short thin arrow). Photomicrographs are representative of $n = 8$. Scale bar = 100 μm.

after, nNOS steadily increased to reach maximum level at P21.

4. Discussion

Nitric oxide plays major roles during the development of the central nervous system (CNS). The objective of the present study was to characterize further the profile of NOS expression in developing rat hippocampus. Our findings show that the hippocampal nNOS expression increases during postnatal life and reaches maximum in adult rats. Additionally, the present study sought to test whether administration of tamoxifen decreases hippocampal NOS expression in prenatal rat. The dosage regimen used in our study has shown to produce morphological changes in CNS [21]. Our findings show that tamoxifen caused hippocampal growth restriction and decreased hippocampal NOS expression in E22. These findings indicate that treatment of pregnant rats with tamoxifen affects hippocampus development in prenatal and postnatal

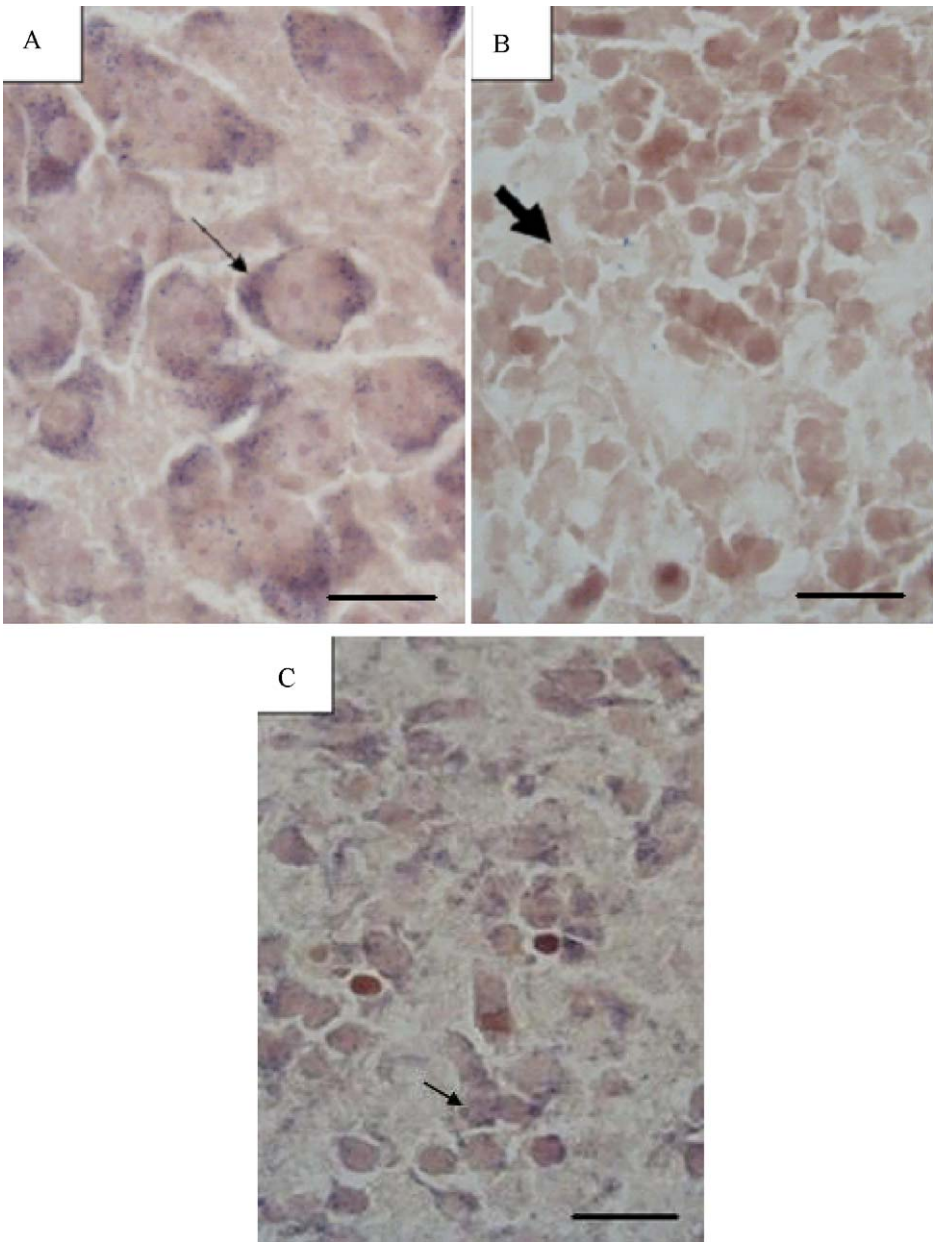

Fig. 5. NADPH-diaphorase staining of CA1 in prenatal P7. Thick arrow shows a neuron. Long thin arrow shows strong NADPH-d reaction. Panel A: reactions are shown for NADPH-d in normal group, without treatment, in more development stage with more neurons. We demonstrated intense but weaker than P1 NADPH reaction (long thin arrow). Panel B: blank NADPH-d in treatment group with no NADPH-d reaction. A neuron is shown by thick arrow. Panel C: NADPH-d reaction in treatment group. We have irregular mild reaction (short thin arrow). Photomicrographs are representative of $n = 8$. Scale bar = 100 μm.

periods. Both estrogen and NOS are involved in developments of the CNS. Tamoxifen exerts estrogen antagonist properties in the CNS. Our findings show lower NOS expression (weak NADPH-d reaction) in the CNS from tamoxifen treated group. Our findings also show that lower NOS expression occurs with lowered neuronal density. These findings indicate involvement of NOS in neuronal development and suggest a neuroprotective role for NOS in developing areas. Our results suggest that NOS plays a critical role as a developmental factor in various areas of the hippocampus. Our results are consistent with those reported by others suggesting that NO regulates the growth and differentiation of hippocampus neurons [25,26]. Studies have indicated that tamoxifen causes significant alterations of the nucleus and mitochondria of certain CNS regions. The nuclei of certain neurons were extremely shrunken and darkened, whereas, in other neurons nuclei were clumping and contained with marginated chromatin [27]. It has been suggested that these alterations are apoptotic changes in CA1 subregion and due to diminished NOS expression [28]. In the early phase of hippocampus development, NOS expression was most abundant in the neural cells. However, treatment with tamoxifen

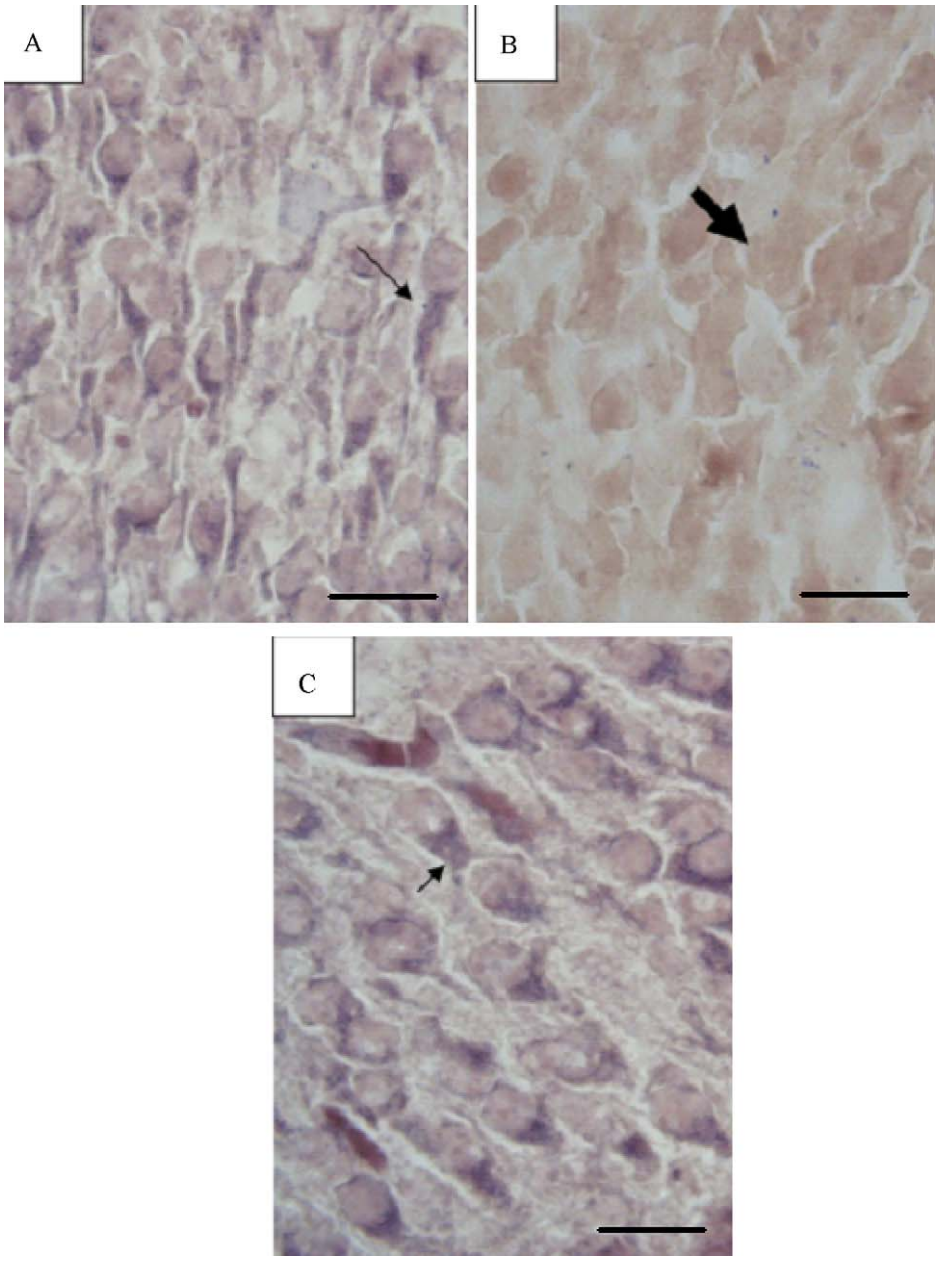

Fig. 6. NADPH-diaphorase staining of CA1 in prenatal P21. Thick arrow shows a neuron. Long thin arrow shows strong NADPH-d reaction. Short thin arrow shows weak NADPH-d reaction. Panel A: shows NADPH-d reaction in normal group, without treatment, in late development stage with much more neurons. We demonstrated low NADPH-d reaction (long thin arrow). Panel B: blank NADPH-d reaction in treatment group. A neuron is marked with think arrow with no NADPH-d reaction (thick arrow). Panel C: NADPH-d reaction in treatment group. We have intense reaction (short thin arrow). Photomicrographs are representative of $n = 8$. Scale bar = 100 μm.

suppressed the enzyme expression. These findings indicate a neuroprotective role for NOS. In addition to apoptotic cells, other neurons showed different morphological patterns of cell death that might reflect different stages of the cell death process [29,30]. It has been reported that decrease in cell number in the hippocampus might be NOS-dependent and changes in hippocampal formation occur following alteration of the estradiol and progesterone levels [16].

Estrogen prevents death of pyramidal neurons in the CA1 subregion and subiculum [20,31]. Developmental changes in the number of the neurons in different regions of rat hippocampus shown in our study are consistent with other studies [32–35]. It has been shown that ageing could decline the number of neurons across all regions of the hippocampus [12,32,36,37]. There are also reports suggesting that the number of the neurons within CA3 region remains constant throughout the life [18,28,38]. Our study demonstrates that tamoxifen treatment also caused nuclear abnormalities. These findings are consistent with the results reported by others [39]. Similar results have been reported for the mito-

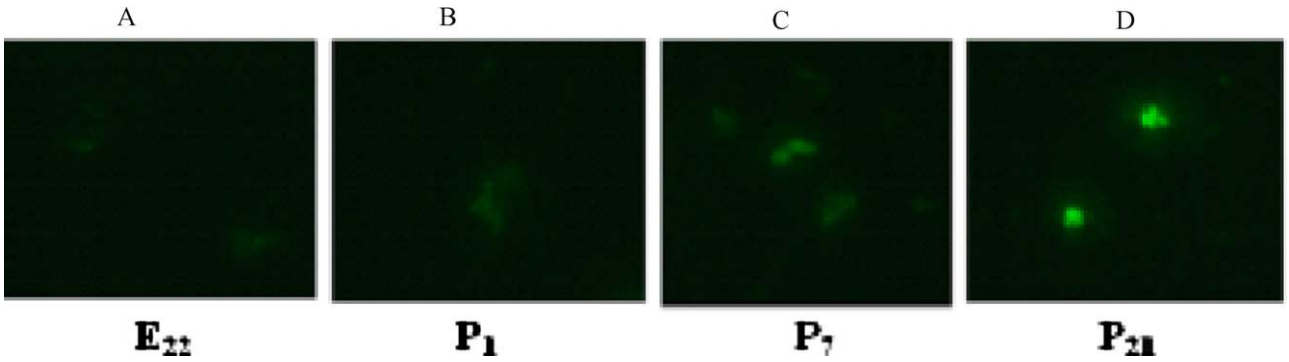

Fig. 7. Immunofluoresent microscopy of nNOS expression in developing hippocampus. Expression of nNOS is shown for prenatal period of E22 (Panel A), postnatal P1 (Panel B), postnatal period P7 (Panel C), and postnatal period P21 (Panel D). Very low nNOS reaction in prenatal E22 is slightly increased in postnatal P1, further increased in postnatal P7, and highly increased 21 days after birth (P21).

chondria of CA1 neurons [40]. Studies in tamoxifen-treated animals have also reported enlargement of mitochondria along with disruptions of mitochondria cristae [41–43]. These changes might occur with the death of the neurons [42,44,45]. Studies have suggested that estrogen acts, in part, by increasing NO in the hippocampal neuronal cell [19,46]. There are also reports on the protective effects of estrogen [20,47] on hippocampus [19] and NOS expression on neurogenesis in developing hippocampus [4,48] that are antagonized by tamoxifen [17,19].

Taken together, our findings indicate that tamoxifen alters the expression of nNOS in certain regions of developing hippocampi and provide new insight into possible mechanisms underlying regulation of NOS expression by estrogen in neuronal cells.

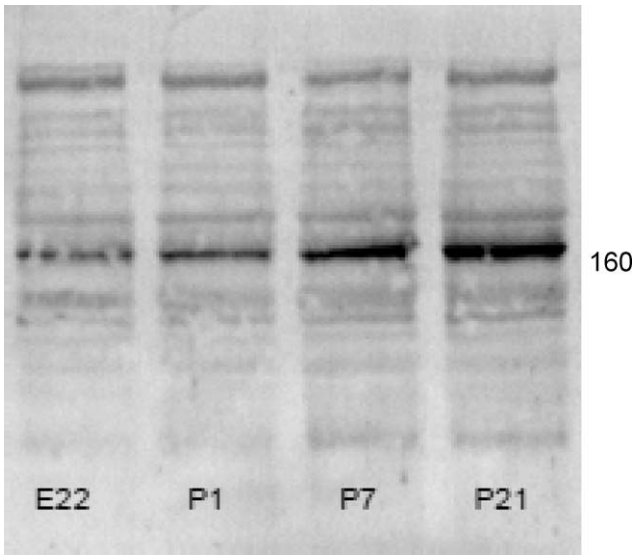

Fig. 8. Western blot analysis of hippocampal nNOS during developing hippocampus. Expression of nNOS in hippocampal CA1 region is shown for prenatal period of E22 (E22), postnatal P1 (P1), postnatal period P7 (P7), and postnatal period P21 (P21). Low nNOS expression at E22 increased during development and peaks at P21.

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