Acta Histochemica 116 (2014) 736–739

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Nitrergic neurons during early postnatal development of the prefrontal cortex in the rat: Histochemical study

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ARTICLE INFO

Article history:
Received 24 November 2013
Received in revised form
31 December 2013
Accepted 1 January 2014

Keywords:
Neurons
Dendrites
Prefrontal cortex
NADPH-d
Nitric oxide
Rat

ABSTRACT

The presence of nitrergic cells in the prefrontal cortex has been confirmed, however little is known about the postnatal development of these cells. Nitrergic neurons were studied histochemically by using NADPH-diaphorase staining in the prefrontal cortex of male Wistar rats from postnatal day 7–21 (P7–21). Neuronal NADPH-diaphorase is a nitric oxide synthase that provides a specific histochemical marker for neurons producing nitric oxide (NO). NO acts as a neurotransmitter and intracellular signaling molecule in the nervous system. We observed in 7 day old rats NADPH-d containing neurons that were intensely stained. These neurons were bipolar with a short dendrite with average length of 23 μm. During the second postnatal week, the neurons were mainly bipolar and were rarely multipolar. By P14 the cells were located primarily in cortical layers III–VI. Nitrergic neurons of the 21 day old rats were histochemically identified as multipolar cells with long radial extending dendrites. Dendrites of neurons in 14 and 21 day old rats were a similar length with an average of 57 μm. These results suggest that nitrergic neurons differentiate during a relatively short period of time and reach their structural maturity by the end of the second week of postnatal development.

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Introduction

Nitric oxide (NO) is an important mediator of two pivotal neuronal developmental processes: proliferation and differentiation (Nott and Riccio, 2009). NO represents an endogenous gaseous signaling molecule, synthesized from L-arginine by the enzyme NO synthase. During embryonic and postnatal development neuronal NO synthase (nNOS) is observed in various cell groups of the central nervous system at different times (Li et al., 1997). The neurotransmitter function of nitric oxide is dependent on dynamic regulation of its biosynthetic enzyme. nNOS exists in a particulate form, which is soluble and the various subcellular localization of nNOS may contribute to its diverse functions (Zhou and Zhu, 2009). Nitric oxide regulates biological processes through signaling mechanisms that exploit its unique biochemical properties as a free radical (Hill et al., 2010). Furthermore, NO paradoxically has both neuroprotective and neurotoxic effects on the central nervous system (Calabrese et al., 2007). Recently it has been found that this signaling molecule directly affects processes of adult neurogenesis (Račeková et al., 2009).

The prefrontal association area in front of the motor cortex, which is in front of the hemispheres, has extensive connections with the structures of the limbic system with significant effects on the behavior of the organism. It functions as a planning part of the cortex which develops after birth, and the neural networks are organized through the emotional impulses and learning. It has been well established that the prefrontal cortex (PFC) consists of a large number of distinct areas with varied architecture and connections (Yeterian et al., 2012). There are two main portions of this cortex: the dorsolateral prefrontal cortex (DLPC) and the orbitomedial PFC. The DLPC is primarily involved in executive functions that include working memory, judgment, planning, sequencing of activity, abstract reasoning and dividing attention. The orbitomedial PFC is involved in impulse control, personality, reactivity to the surroundings and mood. This area of the brain is involved in cognitive behavior, personality expression and control of social behavior (Yang and Raine, 2009). Generating purposeful actions is a cardinal aspect of the cognitive functions of the PFC and behavioral planning is central to the organization of action (Tanji and Hoshi, 2001). The PFC of rats has also been implicated in working memory, attention, response initiation and management of autonomic control and emotions (Heibreder and Groenewegen, 2003). PFC has specific connections with the thalamus, basal ganglia, other cortical areas, limbic system structures and monoaminergic structures (Uylings et al., 2003).

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0065-1281/$ – see front matter © 2014 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.acthis.2014.01.001

The aim of the study was to investigate the postnatal development of nitrergic neurons in the rat PFC using a histochemical method of NADPH-diaphorase, which is a simple and selective method for the visualization of neurons containing nitric oxide synthase, the enzyme responsible for nitric oxide synthesis and to determine at which stage of postnatal development the nitrergic neurons are considered as mature.

Materials and methods

Animals

All experiments were performed in accordance with the Committee for Ethics on Animal Experiments at the Faculty of Medicine, Pavol Jozef Safarik University in Kosice, Slovakia. The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocols (No. 1757/10-221/3a).

Male Wistar rats used in the study were from the Laboratory of Research Bio-models, Pavol Jozef Šafárik University in Košice, Faculty of Medicine. The staining pattern in the PFC was studied on postnatal day P7 ($n = 4$), P14 ($n = 4$), P21 ($n = 4$). A total of 12 animals that were reared in undisturbed social conditions, were used for the developmental study of the NADPH-d reactive neurons in the PFC. The animals were exposed to a day/night cycle of 12/12 h at a temperature of $21 \pm 2\,^{\circ}$C and were given free access to food and water.

Perfusion and histological procedures

All animals were anesthetized with ether and perfused through the left ventricle with saline solution (pH 7.4) followed by 4% paraformaldehyde with 0.1 M phosphate buffer. The brains were removed from the skulls and postfixed in the same fixative for 4 h at 4 °C. Brains were then postfixed in same fixative with 15% sucrose for 1 h, and cryoprotected in 30% sucrose overnight at 4 °C. Frontal sections (40 μm) of the cerebral cortex were prepared using a freezing microtome (Microm HM400R Sliding Microtome with Microm Freezing Unit, Microm International GmbH, Walldorf, Germany) and were processed for analysis. 10 sections for each animal were taken from the infralimbic and prelimbic areas of the ventromedial PFC.

NADPH-d histochemistry

NADPH-diaphorase histochemistry was performed according to Vincent and Kimura (1992) and was modified to suit our laboratory conditions as reported in our previous studies (Bolekova et al., 2011, 2012). The free floating sections were incubated for 90 min at 37 °C in a solution containing 5 mg nitroblue tetrazolium (Sigma–Aldrich, St. Louis, MO, USA; N-6876), 200 μm of dimethylformamide, 3.75 mg NADPH (Sigma–Aldrich; N-1630), 8 mg monosodium malate (malic acid, Sigma–Aldrich; M-1125) and 0.06 ml Triton X-100 and 10 ml phosphate buffer. As a negative control for NADPH-d histochemistry the incubation mixture lacked NADPH. This was to test for endogenous reduction activity in the corresponding blue formazan product. No residual staining activity was observed. The histochemical reaction was controlled visually. After staining, sections were rinsed in phosphate buffer then mounted on glass slides, cleared in xylene and coverslipped. The presence of nitrergic neurons was visualized as intensely stained structures with processes. The morphological appearance of the nitrergic neurons in PFC was evaluated histologically on slides using a light microscope (Leica DM500 microscope fitted with Leica ICC50 HD high definition digital microscope camera, Leica Microsystems, Wetzlar, Germany). Leica Application Suite software (LAS EZ, version 2.0.0.) was used.

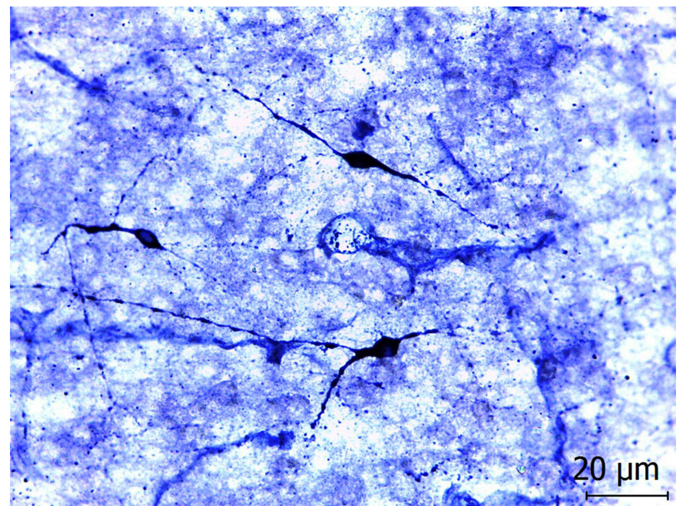

Fig. 1. NADPH-d histochemistry shows bipolar nitrergic neurons with an axon and a dendrite, which are on opposite sides, in PFC of 7 day old rat. Scale bar = 20 μm.

Statistical analysis

All data were analyzed using Friedman test and Repeated Measures ANOVA.

Results

7day old rats

NADPH-d positive neurons (Fig. 1) were visible from layer III to layer VI of the cortical plate with an uneven distribution. They were present mainly in cortical layer III, and occasionally in cortical layer IV, none were seen in cortical layer I. NADPH-d containing cells and processes were found in the infralimbic and prelimbic areas. The neurons were stained dark blue. Cells were represented by mainly bipolar neurons, approximately 10 cells per section, with oval and round soma (perikarya). Neuronal processes were short, unbranched and ran in horizontal and vertical directions. In a microscopy image, fragments of dendrites could be seen.

14 day old rats

During week 2 of postnatal development the NADPH-d positive cells were present from layers III to VI of the cortical plate. Cells were occasionally found in layer I, but the number of cells was low, usually only one or two per section and their cell bodies were located in the lower half of this layer or in the border between cortical layers I and II. They were represented by neurons of varying shape and had the structure of bipolar and multipolar cells with processes running from the cell body (Fig. 2). The number of bipolar neurons was 9 cells per section, whereas the multipolar neurons were 3 cells per section. From P14 the neurons already showed a degree of dendritic development. These dendritic processes were longer in comparison with cells of one week old rats. The microscopic appearance was similar in both the prelimbic and infralimbic areas.

21 day old rats

NADPH-d positive neurons appeared as dark blue stained circular cells with processes located in layers III–VI of the cortical plate.

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