Institute of Experimental Morphology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 13
Sofia • 2008

Diaphorase Activity Expression in Dissociated Cortical Cultures from Mouse Embryos

A. Dandov, N. Lazarov

Department of Anatomy, Medical Faculty, Thracian University, Stara Zagora

The free radical nitric oxide (NO) is involved in neuromodulation and present in many species. NO has a short life and a high diffusion coefficient, and is generated by NO synthase (NOS). It is interesting when in the ontogenesis NO first appears. We set it as a goal to investigate NO in embryonic neurons in vitro. We established cortical cultures from seventeen-day-old (E-17) mouse brains. On the sixth day the cultures were processed for investigating the NOS marker, nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d). Our results demonstrated that NADPH-d reactivity is present in many embryonic cortical neurons and is distributed in the perikarya and neurites. A background reaction in the neuroglial cells is also observed. It can be inferred that NO begins its action early in the ontogenesis by its own synthesis. Further investigation is to find out when NO appears in the mouse cortex and what factors activate NOS.

Key words: cortical culture, diaphorase, histochemistry, mouse embryo, nitric oxide synthase.

Introduction

The free radical nitric oxide (NO) is a neural messenger involved in neuromodulation, and present across a large number of animal phyla. Its significance as an important chemical mediator has become apparent only since 1980, when it was first described by Furchgott, and demands a considerable readjustment of our views about neurotransmission and neuromodulation [5]. NO has an important modulatory role on the processing of sensory signals in vertebrates and invertebrates. It has been implicated in the control of neuronal development and of synaptic plasticity in the central nervous system, as well as in appetite and nociception [1, 3, 4].

NO is a molecule with a short life span and a very high diffusion coefficient that readily traverses cell membranes, and thus spreads rapidly around its site of origin. It is generated by the enzyme NO synthase (NOS) from L-arginine, and neurons containing NOS have been found to exist in both vertebrates and invertebrates.

It is still a matter of considerable interest when and where in the ontogenesis NO comes into the group of an already large cast of neurotransmitters and/or modulators. Therefore, in order to answer at least partly this question, we set it as a goal of

this study to investigate the presence of NO in embryonic neurons in a nerve cell culture. To do so, we applied NADPH-diaphorase (NADPH-d) histochemistry, with NADPH-d acting as a marker for NOS. It is very well known now that NADPH-d activity is often used as a marker of NOS localization and this activity has been shown to correspond with the distribution of NOS in nervous systems of both vertebrates and invertebrates.

Materials and Methods

For the present study we used eight seventeen-day-old embryonic (E17) mouse brains, from which were established primary dissociated cortical neuronal cultures. All the procedures described were performed in a laminar flow hood. After a routine dissection of the cortexes, they were thoroughly minced and trypsinized in a 10 ml solution of 2x trypsin-EDTA (Gibco BRL) for exactly 15 min at 37°C in a water bath. The trypsinization process was discontinued by adding 3 ml fetal calf serum (FCS) (Gibco BRL) in 17 ml PBS. The cell preparation was then passed through a fine meshwork (50 μm large pores) to obtain mostly separate cells and further centrifuged at 1.500 rt. for 9 min. The pellets were then gathered in Fischer's medium (for reference cf. 6) containing 5% FCS, re-stirred, and the cell numbers and viability prior to establishing the cultures were assessed using 0.4% Trypan Blue stain (Gibco BRL) and standard hemocytometer counting [6]. The cultures were seeded in ten four-well manufacturer's dishes, preliminarily coated with poly-L-lysine (Sigma Chemicals Co, St. Louis, Mo., USA) and after a starvation period of 24 h, the whole medium was changed and the cultures were then fed on Fischer's modified medium containing 5% FCS on an "every other day" routine. All the cultures were grown in an incubator supplied with 95% air and 5% CO_2.

On the sixth day the cultures were processed for investigating the NOS histochemical marker, NADPH-d. In brief, the cultures were fixed in refrigerated 4% paraformaldehyde, permeabilized in TBS containing 0.3% Triton X-100, and then incubated in a staining solution containing β-NADPH, nitroblue tetrazolium (NBT) and 0.3% Triton X-100 in TBS for 30-60 min at 37°C until the reaction had developed satisfactorily. The cultures were observed under an inverted microscope, photographed with a digital camera, and the images were further processed with the PhotoShop software computer program

Results

Our results clearly demonstrated that NADPH-d reactivity is present in a large population of embryonic cortical neurons, with no intracellular preference. NADPH-d reactivity is well distributed in both the perikarya and the neuritis (Fig. 1). We also observed a steady background histochemical reaction in the neuroglial cells, although in a somewhat weaker manner (Fig. 2).

Discussion

This study focuses on the expression of NOS and its histochemical marker, NADPH-d, in primary dissociated cell cultures from mouse embryonic brains. Our results comply with these obtained by other authors, who also investigated diaphorase ac-

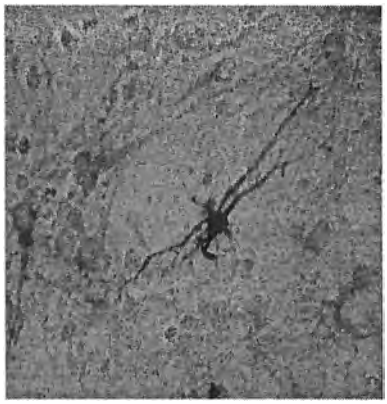

Fig. 1. NADPH-d histochemical reaction in a solitary cortical neuron. The perikaryon and the neuritis are well marked. × 100

Fig. 2. In the photomicrograph, a steady backdround reaction can be observed against a definitely marked neuron. × 100

tivity in cell culture models [2]. First and foremost, we were able adequately to apply the method of diaphorase histochemistry to neuronal culture models to demonstrate such an expression. From the obtained results it can be inferred that NO begins exerting its neuromodulatory action early in the ontogenesis, at that not through diaplacentar diffusion but via its own proper synthesis in the investigated cells in an *in vitro* situation. NO has been implicated in the control of neuronal development and of synaptic plasticity in the central nervous system, as well as in appetite and nociception, and it is very much possible that it starts exerting its functional impacts rather early in the ontogenesis and facilitates the synaptic development and intercellular communication both in the living embryo and the *in vitro* models. It is still a matter of further investigation to find out at what point precisely NO and its synthesizing enzymes first appear in the mouse cortex *in utero* and what factor activates the genes encoding the expression of NOS.

References

1. Bombardi, C, A. Grandis, R. Chiocchetti, M. L. Lucchi. Distribution of calbindin-D28k, neuronal nitric oxide synthase, and nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) in the lateral nucleus of the sheep amygdaloid complex. — Anat. Embryol. (Berl), **211**, 2006, 707-720.
2. Hon, W M, V. J. Chhatwal, H. E. Khoo, S. M. Moochhala. Histochemical method for detecting nitric oxide synthase activity in cell cultures. — Biotech. Histochem., **72**, 1997, 29-32.
3. Fallah, Z. Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity and CB-28kDa immunoreactivity in spinal neurons of neonatal rats after a peripheral nerve lesion. — Iranian Biomed. J., **9**, 2005, 103-110.
4. Chang, H. M., U. I. Wu, T. B. Lin, C. T. Lan, W. C. Chien, W. L. Huang, J. Y. Shieh. Total sleep deprivation inhibits the neuronal nitric oxide synthase and cytochrome oxidase reactivities in the nodose ganglion of adult rats. — J. Anat., **209**, 2006, 239-250.
5. Furchgott, R.F. Endothelium-derived relaxing factor: discovery, early studies, and identification as nitric oxide. — Biosci. Rep., **19**, 1999, 235-251.
6. Schilling, K, M. H. Dickinson, J. A. Connor, J. I. Morgan. Electrical activity in cerebellar cultures determines Purkinjie cell dendritic growth patterns. — Neuron, 7, 1991, 891-902.