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Developmental changes in nitric oxide synthase protein expression in the rat striatum and cerebral cortex

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We examined the expression of brain nitric oxide synthase (bNOS) in two developing rat brain structures, the striatum and the cerebral cortex. For this purpose, we quantified the relative protein concentration level using the Western blotting method and densitometric scanning. 32 Wistar rats, divided according to survival period (P0-P120-postnatal days) were used in this study. Our results demonstrate that bNOS expression rises in these structures during the first week of postnatal life, reaching a maximum in the striatum on the 10th day and in the cerebral cortex on the 7th day of postnatal life. After the period of increase the expression declines and after the 14th day a stabilisation of bone protein concentration is observed, both in the striatum and the cerebral cortex.
These changes in bone protein expression might be related to the important role of nitric oxide in the developing rat brain, especially in synaptogenesis, apoptosis and neurotransmission.

key words: nitric oxide, protein expression, Western blotting, development, cerebral cortex, striatum, rat

INTRODUCTION

Nitric oxide (NO) is a diffusible free-radical gas that plays an important role in the regulation of vascular tone, macrophage-mediated cytotoxity, and neurotransmission in the nervous system [7]. Nitric oxide takes part in the development of the rat central nervous system and in the processing of neuronal cell differentiation and is crucial to synaptogenesis as well as synaptic plasticity and apoptosis [1, 2].

Nitric oxide synthase (NOS, 155 kDa M. W.), the enzyme that synthesises NO, is, given its role in neural development, expressed in the developing rat brain [2]. NO, as well as NOS inhibition, induces behavioural changes such as that of food and water intake [4], alteration in the electrical activity of the cortex and stimulation of renal sympathetic nerve activity [3]. NOS-immunoreactive cell bodies and bNOS proteins have been detected during embryonic development in rats [9].

Nitric oxide is synthesised in a metabolic pathway from L-arginine by nitric oxide synthase in the presence of molecular oxygen and NADPH [5]. Three basic isoforms of NOS have been identified: the calcium/calmodulin-dependent constitutive forms, which are present predominantly in neurons (bNOS) and in endothelial cells (eNOS) and the inducible calcium-independent enzyme (iNOS), which is found in macrophages and in activated microglial and astroglial cells [5].

The aim of this study was to investigate bNOS expression in two developing rat brain areas, the striatum and the cerebral cortex, at the protein concentration level using a Western blotting method.

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MATERIAL AND METHODS

The material consisted of 32 Wistar rats divided according to survival period (P0, P2, P7, P10, P14, P28, P60 and P120-postnatal days). Care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institute of Health as well as by the local ethical committee. All animals were deeply anaesthetised with lethal doses of Nembutal and decapitated. The brains were removed and the samples of the striatum and the cerebral cortex were rapidly microdissected under surgical microscope control.

The tissues were homogenised with buffer, including protease inhibitors, and an assessment of protein concentration was carried out using colorimetric Lowry assay. Samples containing equivalent amounts of proteins were electrophoretically separated and subsequently transferred onto a nitrocellulose membrane by semi-dry blotting. The reliability of sample loading and protein transfer was evaluated by staining the nitrocellulose membrane with Ponceau S solution before immunoblotting. Non-specific binding sites on the membrane were blocked in non-fat dry milk. The membrane was incubated overnight with primary polyclonal antibody — rabbit anti-brain nitric oxide synthase (bNOS, Sigma, USA) diluted 1:10000. After primary incubation the blots were incubated with biotinylated goat anti-rabbit IgG (BioRad, UK) diluted 1:3000. Following incubation with alkaline phosphates — streptavidin complex, colour development reagents (BioRad, UK) were used for visualisation. The results were documented and the relative amount of the proteins in each sample was quantified by densitometric scanning (Laser Pix, Biorad, UK).

In this investigation β-actin was used as a reference protein. The antibodies used for identification and visualisation of β-actin were: mouse monoclonal anti-β-actin (Sigma, USA) diluted 1:5000 as primary IgG and biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, USA) diluted 1:10000 as the secondary antibody. The remaining stage of the examination procedure was carried out with the same protocol and under the same conditions.

RESULTS

When studying the expression of bNOS in the rat striatum during the postnatal period (P0-P120) we observed an increase in band colour intensity between the P0 and P10 groups. The maximum level of relative protein concentration in the striatum was recorded in the P10 group. The intensity of protein staining in older groups of animals (from P14 to P120) stabilised at a similar level but this was lower than in younger groups (Fig. 1).

An analysis of band colour intensity carried out on the cerebral cortex revealed an increase between the P0 and P7 groups, reaching a maximum in the P7 group. After the 7th day of postnatal life a lower concentration of protein was observed in the P10 group. The relative amount of bNOS protein calculated in older groups of animals (P14–P120) stabilised at a lower level (Fig. 2).

To summarise, the results of this investigation show differences in the expression of bNOS between these two parts of the brain, especially during the first 10 postnatal days. In the cerebral cortex the maximum concentration of bNOS is observed on the 7th day and in the striatum on the 10th day of postnatal life.

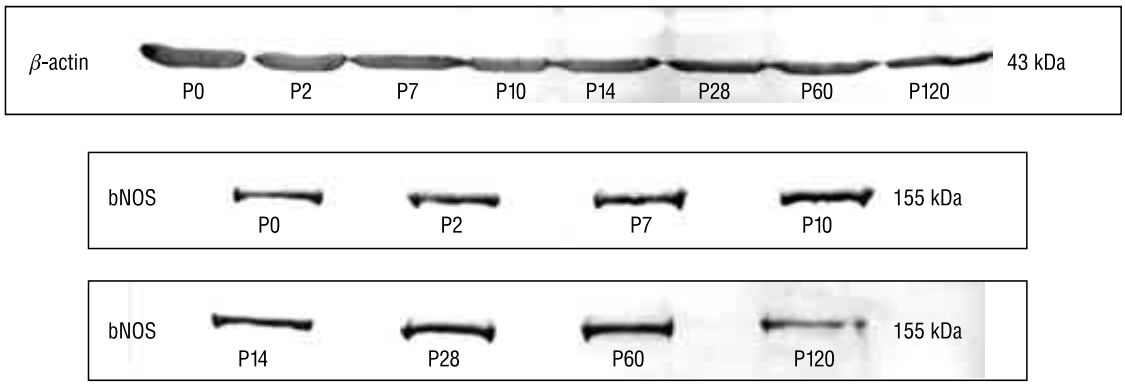

Figure 1. Age-related comparative analysis of bNOS expression in the striatum. Upper panel: Western blots of β-actin carried out in 8 groups (P0–P120 days of postnatal life). Bottom panels: Western blots of bNOS at different ages. Equal amounts of proteins were used in both β-actin and bNOS detection.

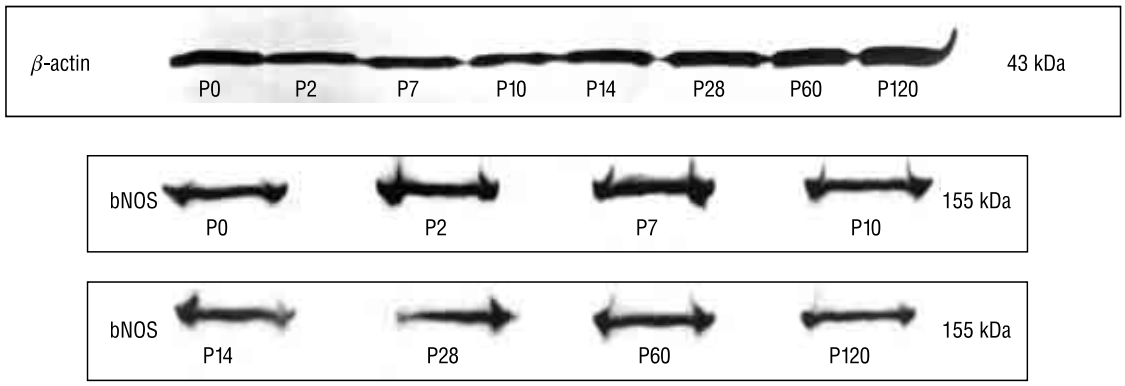

Figure 2. Age-related comparative analysis of bNOS expression in the cerebral cortex. Upper panel: Western blots of β-actin carried out in 8 groups (P0–P120 days of postnatal life). Bottom panels: Western blots of bNOS at different ages. Equal amounts of proteins were used in both β-actin and bNOS detection.

DISCUSSION

The expression of bNOS protein has been detected at the embryonic stage of developing brain. Terada et al. [9] affirmed that both in the striatum and cerebral cortex, bNOS-positive cells were immunohistochemically detected at E19. An increase in bNOS expression was also observed during the first week of postnatal life. Western blotting analysis carried out in our studies revealed an increase in bNOS band colour intensity during the first days of postnatal life, both in the cerebral cortex and in the striatum. This might be connected to the role of nitric oxide in neuronal cell differentiation and synaptogenesis.

The results of the present study demonstrate a decline in bNOS expression after the 7th day, as observed in the cerebral cortex, or the 10th day of postnatal life, as in the striatum. It is suspected that this might be linked to a rapid decrease in apoptosis expression at this time. The role of NO in programmed cell death, which is a normal feature of neurogenesis, has been also postulated [1, 2]. Maciejewska et al. [6] found the greatest number of apoptotic cells in the striatum during the first postnatal week with a rapid drop in number from the 7th day of postnatal life. A similar conclusion concerning neuronal cell development in the cerebral cortex was presented by Spreafico et al. [8] in their study. The authors observed a maximal expression of apoptosis between the 5th and 8th day of postnatal life and a subsequent decrease in apoptotic cell concentration.

In adult animals nitric oxide still plays an important role as a gas neurotransmitter in the central nervous system, so the stabilisation of bNOS expression observed in the present study, both in the cerebral cortex and in the striatum, might be connected with this role.

Differences between bNOS expression in the cerebral cortex and that in the striatum may also suggest that the development dynamic is different in each structure. In the cerebral cortex the expression of bNOS decreases after the first week, whereas in the striatum it decreases after the 10th day of postnatal life. In view of the role of NO in the development of the central nervous system, our results suggest that in some aspects the cerebral cortex might mature earlier than the striatum.

In conclusion, changes in brain nitric oxide synthase expression in the cerebral cortex and striatum are connected with the function of NO in brain development, synaptogenesis, apoptosis and neurotransmission. This investigation proved that the expression of bNOS proceeds differently in these two parts of the brain during the early postnatal period.

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