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Co-localisation of NOS with calcium-binding proteins during the postnatal development of the rat claustrum

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An immunocytochemical double-staining method was applied in order to study the co-localisation of nitric oxide synthase (NOS) with three calcium-binding proteins, calbindin D28k (CB), calretinin (CR) and parvalbumin (PV) in the claustrum of the rat during the first 4 months of life (postnatal days: P0–P120). The co-localisation of NOS/PV and NOS/CB is reported. These neurons fall into the category of non-pyramidal cells. Double-labelled NOS/CB neurons are observed in the claustrum starting from P4, whereas double-labelled NOS/PV neurons are observed from P14 onwards. The percentages of double-labelled neurons increase in relation to the age. Double-labelled NOS/CB and NOS/PV neurons, although they do not constitute a numerous population, play an important role in the process of maturation of the claustrum. This is confirmed by the occurrence of these types of neurons at definite stages of maturation and by the increase in their number.

key words: calbindin, calretinin, claustrum, development, nitric oxide synthase, parvalbumin

INTRODUCTION

Although questions concerning the development of the rat claustrum (Cl) and the maturation of its neuronal population containing nitric oxide synthase have been the subjects of some publications [5, 7], the co-localisation of NOS with calcium-binding proteins (CaBP) in Cl during the postnatal period has not been studied. Nitric oxide and calcium-binding proteins reveal the important influence on the process of maturation of various structures of the central nervous system. According to some authors the role of NO in the development is concerned with the maturation of the cortical neurons, synaptogenesis and the establishment of functional connections [2, 3]. The role of CaBP in the development is concerned with cellular divisions, development of the dendritic arbor and cellular movements [1]. The protective role of CaBP against cellular death both in physiological and pathological circumstances is discussed [1]. Co-localisation of NOS with CaBP is described in various structures of CNS and reveals a very differentiated character concerning the percentage of co-localising neurons and the morphological types of double-labelled neurons [1, 10].

The aim of the study was morphological and morphometric assessment of the changes of neuronal populations revealing co-localisations of NOS with calbindin D28k, calretinin and parvalbumin in the rat Cl during the 4 months after birth.

MATERIAL AND METHODS

Forty five adult Wistar rats of both sexes were used in this study. Animal care and treatment guidelines outlined by the European Community Council

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Directive (86/609/EEC), as well as those established by the local ethical committee were followed. The animals were divided into 9 groups, according to survival period (postnatal days: P0, P4, P7, P10, P14, P21, P28, P60, P120).

Free-floating sections were processed for fluorescence immunocytochemistry for NOS, CB, CR and PV. The following primary antibodies were used: anti-CB (mouse; 1:100, Sigma Chemical Company, USA), anti-CR (goat; 1:1000, Chemicon, USA), anti-PV (mouse; 1:500, Sigma Chemical Company, USA) and anti-NOS (mouse and rabbit; 1:1000 and 1:1500 respectively, Sigma Chemical Company, USA). After incubation for 1 hour in blocking solution containing 0.1% bovine serum albumin in PBS, 10% normal goat serum, sections were incubated overnight in primary antibodies at room temperature. The sections were incubated for 1 hour at room temperature with the secondary antibodies (goat anti-rabbit, goat anti-mouse or donkey anti-goat, conjugated to indocarbocyanine [Cy-3]; 1:800, or conjugated to fluorescein [FITC]; 1:200, Jackson ImmunoResearch, USA). Sections were rinsed in PBS, mounted on slides, dried and coverslipped with Vectashield (Vector Laboratories, Inc., USA). Double-staining was performed in a mixture of antibodies to assess co-localisation of NOS with CB, CR and PV.

The omission tests of primary or secondary antibodies resulted in a lack of specific labelling, confirming the specificity of the immunocytochemical procedures. The qualitative study was performed with a fluorescence microscope Eclipse E600 (Nikon, Japan) equipped with a confocal imaging system (MicroRadiance, Bio-Rad, UK). The image analysis programs LaserSharp 2000 v. 2.01 (Bio-Rad; UK) as well as LaserPix v. 4.0 (BioRad; UK) were used to prepare the illustrations.

For the quantitative study the double-labelled sections from the anterior (+ 1.6 mm from the bregma), central (–0.26 mm) and posterior (–0.92 mm) parts of Cl were chosen. The borders of Cl were marked as separate inclusion areas under small magnification (4 ×). Neuronal profiles possessing specific immmunocytochemical properties (single labelling or co-localisation within each combination of substances) were counted with the aid of a 20 × objective lens in systematic random test frames of the selected area using the C.A.S.T. Grid system (Computer Assisted Stereological Tool; Olympus, Denmark) working on a microscope BX-51 (Olympus, Japan). The calculations were performed of the percentage values of 1) double-labelled neurons to all labelled neurons in each combination of staining and 2) double-labelled neurons to all NOS-ir neurons observed in each combination of staining. Age-dependent changes in the percentages of co-localisation were tested by the Chi-square test for trends. All experiments with various combinations of substances were conducted on at least three animals.

RESULTS

The co-localisations of NOS with PV and CB were found in Cl. Staining for NOS and CR revealed only single co-localising neurons observed during the whole studied period. NOS/CB double-labelled neurons appeared at P4, whereas NOS/PV double-labelled neurons appeared at P14. The percentages of double-labelled cells in both types of co-localisation increased in the period under study (Fig. 1A, B). In both types of co-localisation the percentage values of co-localising neurons were low and constituted 18% for NOS/CB and 12% for NOS/PV. NOS/PV double-labelled neurons accounted for up to 23% of all NOS neurons, whereas NOS/CB double-labelled neu-

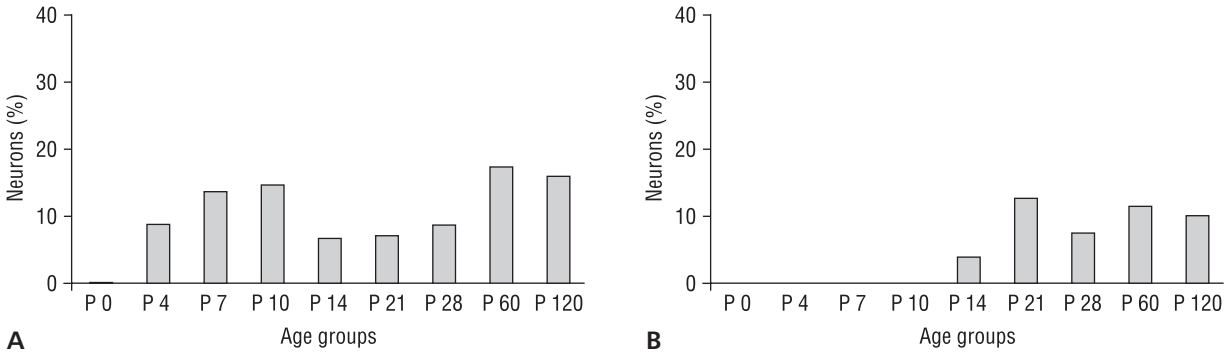

Figure 1. The percentage values of double-labelled NOS/CB-ir (**A**) and NOS/PV-ir (**B**) neurons in relation to all labelled neurons during the period of study.

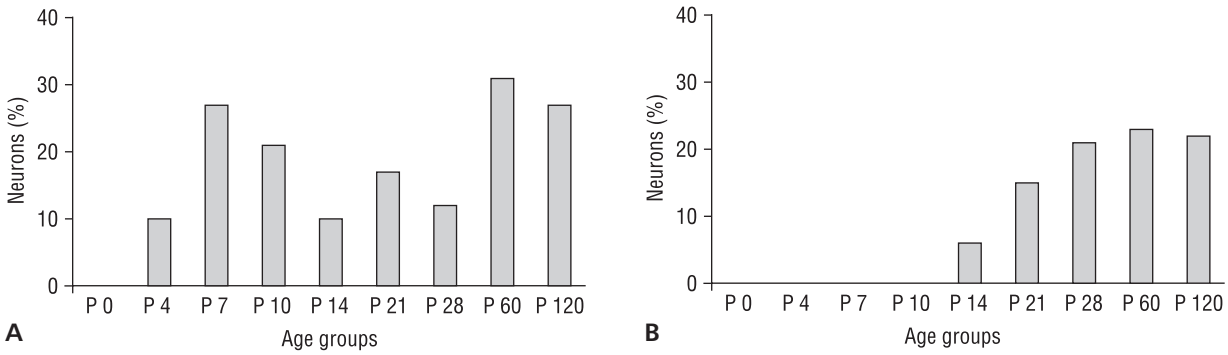

Figure 2. The percentage values of double labelled NOS/CB-ir (**A**) and NOS/PV-ir (**B**) neurons in relation to all NOS-ir neurons observed during the period of study.

rons accounted for up to 31% of all NOS neurons (Fig. 2A, B). Oval and fusiform cells constituted the most numerous morphological types in both types of co-localisation. The differentiation of neuronal shapes increased with age in both types of co-localisation (Fig. 3A, B). The number of bipolar and multipolar neurons of medium and large size became more numerous within the observation period.

DISCUSSION

The differences in the moments of occurrence of co-localising neurons reflect their distinct functions in the process of claustral maturation. According to some authors, occurrence of CB may be related to the moment of accomplished cellular division and formation of the dendritic tree [1]. PV may appear after functional maturation of the neurons and may be related to the process of synaptogenesis [1]. The occurrence of calcium-binding proteins in the neurons of various structures of the central nervous system may be related to their buffering function in relationship to the intracellular Ca^{++} concentration after stimulation of NMDA receptors of NOS-containing neurons [2, 8, 9].

The observed lack of co-localisation of NOS/CR may be explained by the peculiar functional properties of CR which, in many structures of CNS, appears not only in the population of interneurons but also in projecting neurons [4]. Moreover, CR reveals co-localisation with glutamate [4]. Similarly, among NOS-containing neurons two subpopulations can be distinguished. One of these represents a category of

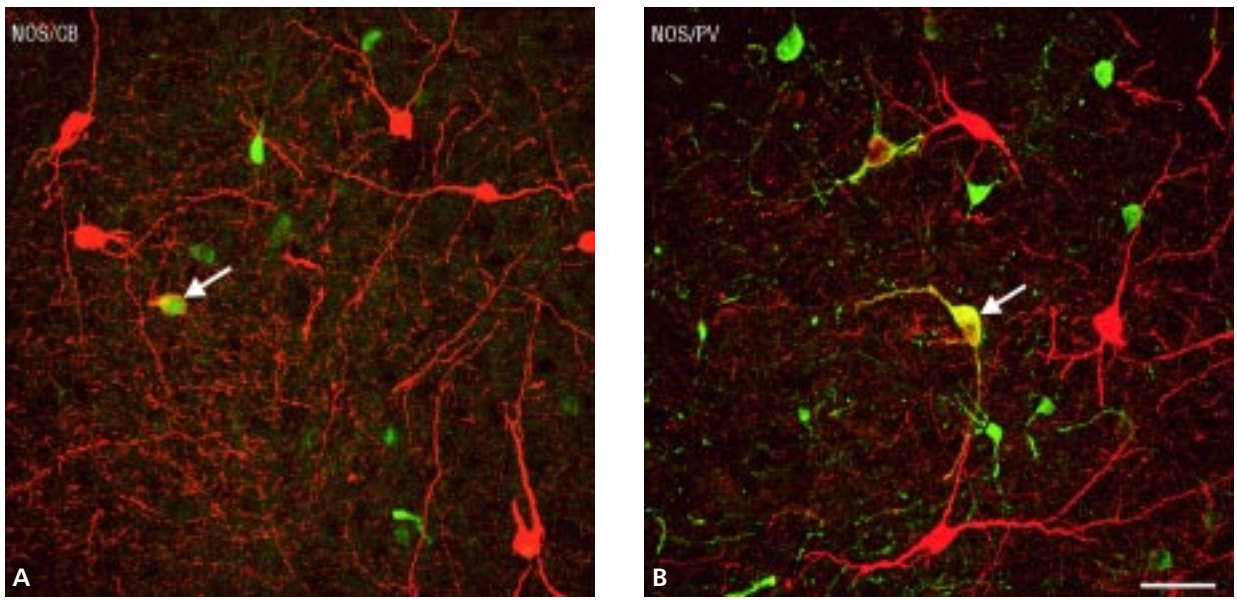

Figure 3. Double-labelled NOS/CB-ir (**A**) and NOS/PV-ir (**B**) neurons (indicated by arrows) of differentiated somatic morphology, observed in the claustrum at P30; double-labelled immunofluorescent staining with Cy3 (for NOS) and FITC (for CaBP); scale bar for A and B = 25 μm.

interneurons co-localising with GABA, whereas the second belongs to the category of projecting neurons, most probably utilising one of the excitatory neurotransmitters [6]. This particular functional differentiation in the populations of NOS and CR neurons may explain the lack of co-localisation in Cl.

Our results indicate that the population of NOS-ir neurons co-localising with CaBP is differentiated with respect to the moment of occurrence in the postnatal life, which corresponds to the functional maturation of the structure studied. Moreover, the co-localising populations of NOS/PV and NOS/CB neurons are most probably engaged in various physiological processes during the period of claustral maturation.

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REFERENCES

1. Andressen C, Blumcke I, Celio MR (1993) proteins: selective markers of nerve cells. Cell Tissue-Res, 271: 181––208.
2. Contestabile A (2000) Role of NMDA receptor activity and nitric oxide production in brain development. Brain Res Rev, 32: 476–509.
3. Cudeiro J, Rivadulla C (1999) Sight and insight — On the physiological role of nitric oxide in the visual system. Trends Neurosci, 22: 109–116.
4. Frassoni C, Radici C, Spreafico R, De Curtis M (1998) protein immunoreactivity in the piriform cortex of the guinea-pig: Selective staining of subsets of non--GABAergic neurons by calretinin. Neuroscience, 83: 229–237.
5. Kowiański P, Moryś JM, Wójcik S, Dziewiątkowski J, Moryś J (2003) Postnatal development of NOS-ir neurons in the rat claustrum. Folia Morphol, 62: 11–17.
6. Kowiański P, Timmermans JP, Moryś J (2001) Differentiation in the immunocytochemical features of intrinsic and cortically projecting neurons in the rat claustrum — combined immunocytochemical and axonal transport study. Brain Res, 905: 63–71.
7. Maciejewska B, Moryś J, Berdel B, Dziewiątkowski J, Narkiewicz O (1997) The development of the rat claustrum — a study using morphometric and in situ DNA end labelling techniques. Eur J Anat, 1: 137–143.
8. Soares-Mota M, Henze I, Mendez-Otero R (2001) Nitric oxide synthase-positive neurons in the rat superior colliculus: Co-localization of NOS with NMDAR1 glutamate receptor, GABA, and parvalbumin. J Neurosci Res, 64: 501–507.
9. Weiss SW, Albers D, Iadarola MJ, Dawson TM, Dawson VL, Standaert DG (1998) NMDAR1 glutamate receptor subunit isoforms in neostriatal, neocortical, and hippocampal nitric oxide synthase neurons. J Neurosci, 18: 1725–1734.
10. Yan XX, Jen LS, Garey LJSO (1996) NADPH-diaphorase--positive neurons in primate cerebral cortex colocalize with GABA and proteins. Cereb Cortex, 6: 524–529.