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Postnatal development of NOS-ir neurons in the rat claustrum

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The morphological features of nitric oxide synthase (NOS)-containing neurons in the rat claustrum (Cl) were studied during the period of four months after birth. Forty-five animals divided into nine groups, according to survival period (P0, P4, P7, P10, P14, P21, P28, P60, P120) were used in the study. The immunocytochemical staining to neuronal NOS was performed and the material was studied both qualitatively and quantitatively using unbiased stereological methods. Our observations indicate that the process of maturation of NOS-immunoreactive (ir) neurons in Cl takes place during the early postnatal period. We report the increase of numerical density of immunoreactive neurons, changes in neuronal size, expressed by the decrease of the percentage of small neurons with simultaneous increase of the participation of medium-sized neurons and large neurons. In the whole studied period the prevalence of oval and fusiform neurons is observed. However, the increase of the proportion of multipolar neurons takes place. Round neurons are most characteristic in the youngest groups of animals and later become dominated by the developing subpopulations of ir-neurons of other shapes. In the anterior, central and posterior parts of Cl, a similar pattern of maturation of NOS-ir neurons is observed. No prevalence of characteristically shaped neurons is observed in any part of Cl. The adult-like pattern of morphological features in the NOS-ir neuronal population in Cl is reached in the third postnatal week.

The maturation of NOS-ir neurons in the claustrum is a dynamic process which is not stabilised at the moment of birth. It may be assumed that characteristic changes of the NOS-ir population of neurons may be influential on the physiological processes observed in Cl. These may in particular have some importance for the processes of synaptogenesis and establishing as well as refining of numerous claustral connections with the other structures of the central nervous system.

key words: claustrum, development, interneurons, nitric oxide, nitric oxide synthase, rat

INTRODUCTION

The claustrum is a cortically-dependent structure whose function relies on the integration of the various forms of information coming prevalently from the numerous cortical areas [12, 16, 17, 22]. This main function of the claustrum is realised by the system of internal connections established by numerous non-pyramidal neurons of differentiated immu-

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nocytochemical characteristics. The immunocytochemical studies of the claustral interneurons were performed in various species [5, 7, 13, 20]. Among substances represented in the population of interneurons, nitric oxide (NO) is one of the greatest importance, because of its fundamental role in the processes of neuronal development and maturation. However, studies on NO synthesising neurons in Cl were performed only in the adult representatives of various species [8, 13, 23]. The morphological changes of claustral NOS-ir interneurons during their development and maturation have not been described yet. In the central nervous system (CNS) nitric oxide is produced by three isoforms of nitric oxide synthase: neuronal (nNOS, NOS-I), inducible (iNOS; NOS-II) and endothelial (eNOS, NOS-III) [1, 3, 30]. Two of these — NOS-I (neuronal) and NOS-III (endothelial) — are commonly regarded as Ca^{2+} dependent, constitutively expressed enzymes. A third form — NOS-II (inducible) — is a Ca^{2+} independent enzyme found in activated macrophages and in microglia [1, 3]. During development mRNA of all NOS isoforms is present from the 10^{th} embryonic day onwards, whereas protein amounts of nNOS and iNOS are first detectable at E15 in the brain of the rat [10]. In the cortical plate nNOS is transiently expressed at E15, whereas in the cerebral cortex NOS-positive neurons are present at E19 [2, 24, 25].

NO plays numerous roles during development of the various structures in CNS. It is partly responsible for synaptogenesis and synaptic plasticity [6, 14, 18], regulation of cerebral blood flow [6, 14] establishment and refinement of axonal projections [6, 21, 26, 28] and neuronal death [2–4, 6, 26].

The objective of our study was to disclose the character of both qualitative and quantitative changes occurring in the population of NOS-ir neurons in the rat claustrum during its maturation.

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 Directive of 24 November 1986 (86/609/EEC), as well as those established by the local ethical committee, were followed. The animals were divided into nine groups, according to survival period (P0, P4, P7, P10, P14, P21, P28, P60, P120). The animals were deeply anaesthetised with sodium pentobarbital (100 mg/kg i.p. body weight) and perfused transcardially in a standard manner with physiological saline (at pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (at pH 7.4 and 4°C). After removal from the skull, the brains were immersed in the same fixative for 120 min and stored in 30% sucrose in 0.1 M phosphate buffer (at pH 7.4 and 4°C) for at least 24 hrs. The tissue was frozen and sectioned coronally into 40-mm-thick serial sections on a sliding microtom.

Immunocytochemical procedure

The free-floating sections were processed for nNOS immunocytochemistry. The primary mouse anti-nNOS antibody was used diluted 1:1000 (Sigma Chemical Company, USA). After incubation for 1 h 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. Subsequently, they were incubated in secondary antibodies (goat anti-mouse, conjugated to indocarbocyanine [Cy-3] used at 1:800, or conjugated to fluorescein [FITC] used at 1:200 (Jackson ImmunoResearch, USA), for 1 h at room temperature. Sections were rinsed in PBS, mounted on slides, dried and coverslipped with Vectashield (Vector Laboratories, Inc., USA).

Control procedures

Sections were processed through the identical immunocytochemical procedure, except that primary or secondary antibody was omitted. In all cases the omission of primary or secondary antibodies resulted in lack of specific labelling, confirming the specificity of immunocytochemical procedures.

Qualitative study

The sections were examined with a fluorescence microscope Eclipse E600 (Nikon, Japan) equipped with a confocal imaging system (MicroRadiance, Bio-Rad, UK) supplied with an Argon laser (excitation 488/514 nm). 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.

Quantitative study

The sections coming from the anterior (+1.6 mm from bregma), central (–0.26 mm) and posterior (–0.92 mm) parts of the claustrum were chosen under 10 × objective, which corresponded to the planes described in the atlas of Paxinos and Watson [19]. The numerical density (number of cells per square millimetre) of nNOS-ir neurons was estimated. The borders of Cl were marked as separate inclusion areas under the small magnification (4×).

Neuronal profiles were counted with the aid of a 20× objective lens in systematic random test frames of selected area using C.A.S.T. Grid system (Computer Assisted Stereological Tool; Olympus, Denmark) working on a microscope BX-51 (Olympus, Japan). Along with the counting procedure, the cells were classified as belonging to one of five specified types. The percentage distribution of cell types was calculated. The mean and standard deviations of numerical density estimates were calculated. All calculations were performed in Excel 2000 (Microsoft, USA).

Statistics

Cell distribution was estimated on samples including more than 100 cells in each group. For the analysis of changes of the numerical density and of the proportions of various cell types among age groups, nonaparametric Jonckheere test for trend was used. The significance level was 0.05.

RESULTS

Qualitative study

P0. At this stage only single small and medium-sized, weakly immunostained neurons occur in Cl (Fig. 1A). Their shapes are predominantly oval, fusiform and round. Unipolar neurons of round or oval shape prevail. Practically no immunoreactive neuropil is observed at this stage.

P4. The number of bipolar-ir neurons increases (Fig. 1B). In all immunoreactive neurons, short and thickened fragments of dendrites are clearly visible. The intensity of neuropil staining increases in this studied group.

P7. Apart from the previously described morphological types of neurons, triangle and single multipolar cells are observed (Fig. 1C). Neuropil is even more intensively stained. The borders of Cl are more easily identified at this stage in comparison to the former groups. No significant differences among immunoreactive neurons are reported in the antero-posterior direction.

P10. The morphological heterogeneity of Cl neurons increases at this stage of maturation. Oval, fusiform as well as multipolar, round and triangular ir-neurons of various sizes are observed in all studied parts of Cl (Fig. 1D). The oval and round unipolar neurons are still relatively numerous in all parts of Cl. Neuropil is intensively stained.

P14. Multipolar neurons of various sizes become more numerous in comparison to the previous groups (Fig. 1E). The number of dendrites coming from the cell bodies increases at this stage. The intensity of neuropil staining reaches the adult-like pattern. The proportion of unipolar neurons is smaller in comparison to the younger groups.

P21. NOS-ir neurons are scattered on the cross-sections of Cl. The examples of all studied neuronal shapes are well represented in Cl at this stage (Fig. 1F). The NOS-ir neuronal fibres and granulations are more densely localised. The proximal fragments of dendrites are still thickened as in the younger groups.

P28. Relatively numerous immunoreactive neurons of differentiated morphology are present in Cl. In the majority of these neurons two to four dendrites emerge from the cell somata. In contrast to previously described groups, longer fragments of ir-dendrites and axons can be observed. A dense network of NOS-ir fibres is present in the whole Cl.

P60. The neuronal shapes are differentiated in a way characteristic for adult Cl (Fig. 1G). Generally, the number of ir neurons in Cl is smaller than in the neighbouring structures — insular cortex and endopiriform nucleus.

P120. The population of NOS-ir neurons is morphologically stabilised. In the anterior and central parts of Cl, oval, fusiform and multipolar neurons are localised most frequently. In the posterior part oval and fusiform neurons prevail. In the multipolar neurons four to five immunostained dendrites project from the neuronal bodies (Fig. 1H). The unipolar neurons are almost absent at this stage. The dense network of immunoreactive fibres with varicosities is present in the sections coming from all studied parts of Cl.

Quantitative study

The numerical density of NOS-ir neurons in Cl increases in the studied period (Fig. 2).

The analysis of neuronal size reveals that the proportions of medium- and large-sized NOS-ir neurons increase, whereas in the same time the proportion of small NOS-ir neurons decreases (Fig. 3). In all studied groups with the exception of P0 the percentage of medium-sized neurons is the highest. The large NOS-ir neurons occur from P4 onwards. Their percentage increases in the studied period.

The analysis of neuronal shapes in all studied groups reveals that the relative proportions of oval and fusiform NOS-ir neurons are the highest (Fig. 4). The proportion of multipolar neurons increases in the studied period. In the third postnatal week the proportions of the variously shaped neurons reach the pattern characteristic for the adult Cl.

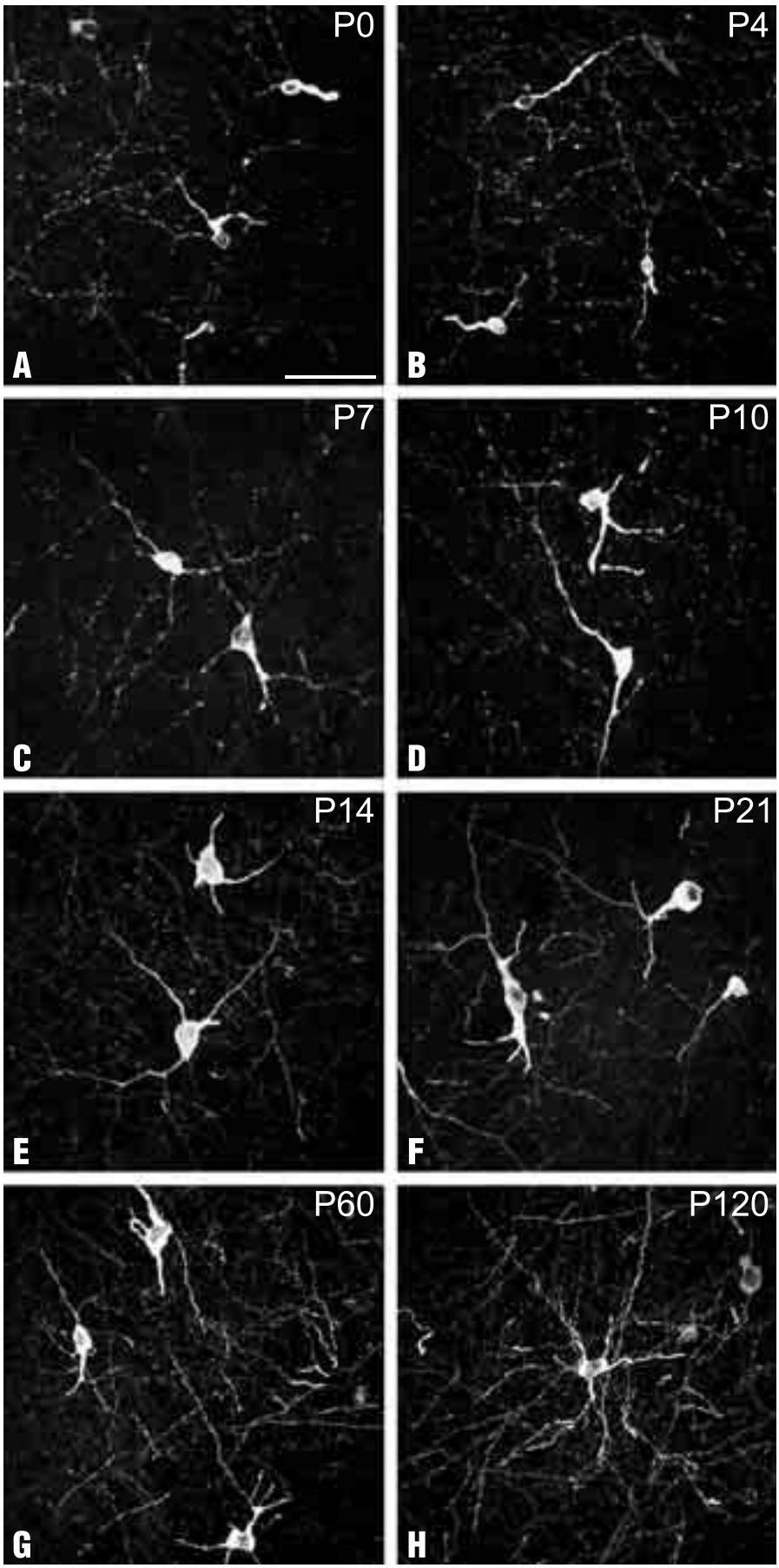

Figure 1. Changes of shapes in the population of claustral NOS--ir neurons in the studied period. Most representative examples of immunoreactive cells for selected age groups are presented in the panel. **A**. Unipolar neurons of oval shape at P0 (scale bar = 50 μm); **B.** More numerous unipolar and single bipolar neurons at P4; **C.** Examples of fusiform and triangular neurons at P7; **D.** Variously shaped immunoreactive neurons at P10; **E.** Multipolar neurons with long proximal fragments of dendrites at P14; **F.** Fusiform, round and oval neurons at P21; **G.** Neuronal morphology at P60 is differentiated and characteristic for adult Cl; **H.** Multipolar immunoreactive neurone at P120.

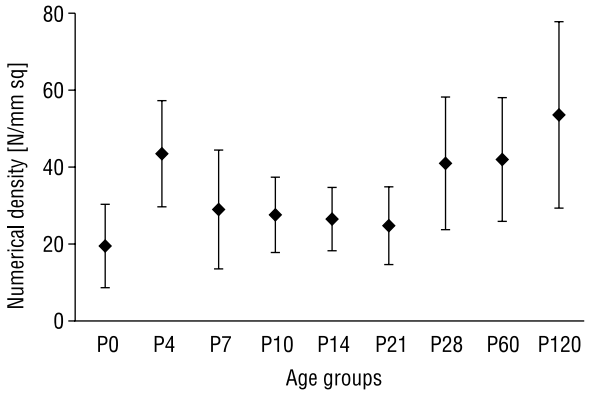

Figure 2. Changes of numerical density of NOS-ir claustral neurons in the studied period.

In three studied parts of Cl the prevalence of oval and fusiform neurons is maintained. The increase of the proportion of multipolar neurons is observed in all three parts of Cl. The morphological heterogeneity of the NOS-ir neuronal population is most clearly expressed in the anterior and central parts of Cl.

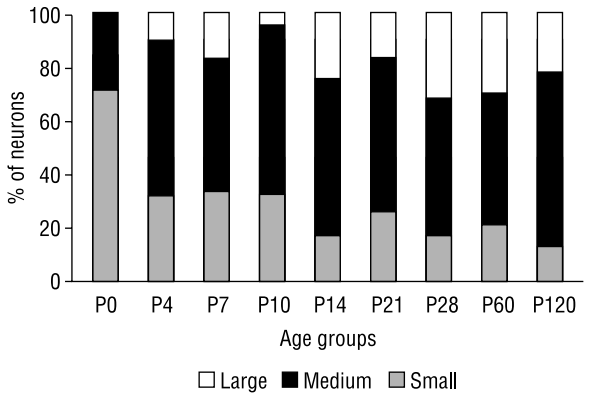

Figure 3. Percentage distribution of small, medium and large NOS-ir claustral neurons in the studied period.

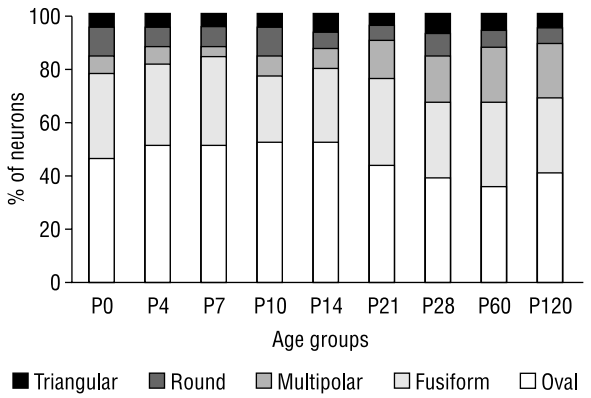

Figure 4. Percentage distribution of variously shaped NOS-ir claustral neurons in the studied period.

DISCUSSION

NOS is present in the claustral neurons in the earliest period of postnatal life. However, the population of the immunoreactive neurons undergoes characteristic changes in the studied period. The expression of NOS increases in the population of claustral neurons, which is reflected in the changes of neuronal density. This result corresponds well to the observations performed in the cerebral cortex by other authors, who also reported the increase of the number of NOS-ir or NADPH-positive neurons in the early post-gestational period. By the end of the second postnatal week in the neocortex, both the number and the laminar distribution of these neurons were adult-like [25, 29].

Our statement that the intensity of neuropil staining increases systematically and reaches the adult-like pattern during the third week may speak in favour of the hypothesis that the NOS-ir population of claustral interneurons is ready to undertake its functions at the stage when the neuronal processes develop their connections with the other claustral neurons and are cytochemically matured. In the cerebral cortex neuropil reaches its adult form during the second postnatal week [29]. The intense synaptogenesis in the rat brain occurs at 2–3 postnatal weeks [14]. This event is preceded by increased activity of nNOS [14, 18].

The increased expression of NOS in the rat Cl may be related to the elimination of a substantial number of projecting neurons. A characteristic decrease of 30% of the total number of Cl neurons was described in the literature [15]. The stabilisation of the morphometric parameters in the rat Cl occurs in the third postnatal week. Similarly, in the rabbit the morphological structure of the claustrum, studied both in a qualitative and a quantitative way, reveals the features of maturation at P21 and does not change significantly in the later period [27].

The character of morphological changes in the population of NOS-ir neurons corresponds to some extent with the character of maturation of the claustrocortical connections. Although the process of development and maturation of these connections in the rat was not studied in the quantitative way, some general tendencies may be distinguished on the basis of the results obtained in the rabbit [11]. In this species the changes of both the total number and numerical density of projecting neurons stabilise during the first month of life. It may be suspected that the decrease of the number of projecting

neurons and their numerical density is the result of the coexistence of numerous processes, among others — cell death, competition for the trophic factors, elimination of the incorrect connections [2, 6, 9, 26, 28]. It may also be the result of the refinement of claustrocortical connections undergone under the influence of nitric oxide [6, 21, 26, 28]. Finally, it is worth mentioning that, apart from the well documented data confirming the occurrence of NOS in the population of the claustral non-projecting neurons, there are some observations coming from the double-staining experiments of retrogradely labelled neurons, that small population of claustral cortically projecting neurons is characterised by the presence of NOS [13]. The physiological role of this population of claustral neurons necessitates further intensive studies.

Summarising, our results indicate that, although development of NOS-ir cells begins in Cl, as in the various structures of CNS, in the prenatal period, dynamic changes of the number of ir-neurons and their shapes occur after birth. The process of maturation of NOS-ir neurons in the claustrum is dynamic and takes place during three weeks after birth. Taking into account observations concerning claustral maturation in various species, it may be suspected that NO is at least partially responsible for physiological processes, such as refinement of the claustrocortical connections, synaptogenesis and programmed cell death. The result of these processes may be the adjustment of the claustrum to its role in the adult brain.

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