Brain Research Bulletin 76 (2008) 565–571

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

NPY-, SOM- and VIP-containing interneurons in postnatal development of the rat claustrum

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ARTICLE INFO

Article history:
Received 18 September 2007
Received in revised form 3 March 2008
Accepted 16 April 2008
Available online 13 May 2008

Keywords:
Amygdaloid body
Claustrum
Development
Interneurons
Neuropeptides

ABSTRACT

A growing body of evidence indicates the common origin of the claustrum, endopiriform nucleus, and the basolateral nuclear complex of amygdala from the lateral and ventral parts of the pallium, as the claustroamygdaloid complex. It seems very probable that at least some of the claustral interneurons derive from subcortical sources.

The postnatal development of neuropeptide Y-, somatostatin- and vasoactive intestinal polypeptide-containing interneurons was studied during the 4 postnatal months (P0–P120; P, postnatal day). The study was conducted on 45 Wistar rats of both sexes.

Our results indicate that neuropeptide-containing interneurons are not morphologically mature at the moment of birth. The characteristic features of neuronal bodies and the relatively long period of postnatal development may indicate their migration from the subcortical neurogenetic centers. Morphological changes in the neuropil are also reported.

Although developmental patterns differ between various neuropeptide-containing neuronal subpopulations, two phases of development can be distinguished in each of them: the early phase (P0–P4) during which undifferentiated neurons and neuropil dominate, and the late phase (P7–P28) during which the characteristic features of an adult-like structure gradually appear. Later these observed developmental changes are terminated.

The postnatal development of neuropeptide-containing interneurons is completed after 4 weeks of life. This period, which is important for the structural and functional development of the claustrum, must be taken into account in future studies on this structure.

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1. Introduction

The claustrum (Cl; dorsal claustrum or claustrum proper) is a telencephalic structure characterized by extensive, reciprocal connections with numerous neocortical areas [14]. A growing body of evidence indicates that it plays a role in processes of cross-modal sensory integration [3,7,12,23], memory [54,55] and epileptogenesis [47]. Because of its numerous cortical connections, the Cl is able to play a particular role enabling the transfer of sensory information of various modalities between cortical areas. The special function of the Cl is also concerned with kindling propagation, as well as with the generalization of seizures [34,35].

The Cl consists of two morphologically distinguished neuronal subpopulations [31]. The prevalent group consists of glutamatergic projecting neurons with spiny dendrites. The second group of GABA-ergic interneurons, represented by variously shaped somata and aspiny dendrites, constitute, in various species, between 7 and 12% of all claustral neurons [5,17,25,50]. Apart from GABA, the occurrence of neuropeptides such as neuropeptide Y (NPY), somatostatin (SOM), vasoactive intestinal polypeptide (VIP) and cholecystokinin (CCK) has been described in the Cl [14,15,27,48,49].

The expression patterns of developmental regulatory genes in mice indicate the origin of the Cl to be from the lateral and ventral pallial divisions [33,43,45]. Neurogenesis of the claustral neurons in the cortical neuroepithelium of the rat was estimated to be between E15 and E16 [4]. However, there is evidence that at least some of the claustral interneurons may originate in the subpallium and migrate tangentially during development [29,43].

According to previously published results, the development of the Cl is not complete at the moment of birth. The increase of its total volume and the loss of nearly 30% of neurons, including apoptotic death, take place after birth [30]. Moreover, characteristic

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0361-9230/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2008.04.004

morphological changes among interneurons containing calcium-binding proteins (CaBP) and nitric oxide synthase (NOS) have been described [13,22,26].

Neuropeptide-containing interneurons may be very important for the initiation of integrative function and memory processes coordinated by the Cl [12]. The stabilizing influence of peptide neuromodulators on the activity of claustral projecting neurons may be crucial for the development of claustro-cortical connections.

Our studies focused on the morphological changes of claustral interneurons, immunoreactive to three neuropeptides (NPY, SOM and VIP), during the first 4 postnatal months.

2. Materials and methods

Forty-five Wistar rats of both sexes were used in this study. Animal care and treatment guidelines established by the local Ethical Committee, as well as standards defined by the European Communities Council Directive of 24 November 1986 (86/609/EEC), were followed. The animals were divided into nine age groups: P0, P4, P7, P14, P21, P28, P60, P90, and P120, with five animals in each group.

The animals were deeply anaesthetized with sodium pentobarbital (thiopental sodium, Biochemie GmBH, Germany; 80 mg/kg of body weight, i.p.) and transcardially perfused with 200 ml of physiological saline (at pH 7.4) followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, 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 PBS (at pH 7.4 and 4 °C) for at least 24 h. Subsequently, the brains were frozen and sectioned coronally into 40-μm-thick serial sections on a sliding microtome.

2.1. Immunocytochemical procedure

The free-floating sections were processed for fluorescence immunocytochemistry. Three primary antibodies were used in the study: anti-NPY (rabbit; 1:400, Affiniti, UK), anti-SOM (rabbit; 1:300, Euro-Diagnostica AB, Sweden), and anti-VIP (rabbit; 1:100, Cappel ICN Pharmaceuticals Inc., USA). After incubation for 1 h in a blocking solution containing 0.1% bovine serum albumin in PBS and 10% normal goat serum, the sections were incubated overnight in primary antibodies at room temperature. Subsequently, they were rinsed in PBS and incubated in the secondary antibody (goat anti-rabbit; conjugated to indocarbocyanine [Cy-3] used at 1:800, Jackson ImmunoResearch, USA) for 1 h at room temperature. The sections were rinsed in PBS, mounted on slides, dried, and coverslipped with Vectashield (Vector Laboratories Inc., USA).

In order to confirm the specificity of staining, the sections were processed by immunocytochemical procedure with the omission of primary or secondary antibody. In all cases the omission tests resulted in a lack of specific labeling.

2.2. Qualitative study

In each immunocytochemically distinguished neuronal subpopulation, the shapes of the perikarya and their polarity were studied. Additionally, the morphological features of the neuropil were evaluated. These included the density of neuronal fibers, the presence of immunolabeled ramifications, the presence of immunolabeled points and varicosities, and the intensity of staining.

The morphological features of mature neurons were defined based on the increased amount of cytoplasm in relationship to the nucleus and the decreased proportion of unipolar neurons in relationship to all immunolabeled neurons.

The morphological features of neuropil maturation were assumed to be: increased neuronal fiber density, the occurrence of immunolabeled ramifications, varicosities and immunoreactive points, and increased intensity of staining.

In order to compare the selected parameters of immunoreactive neuronal populations, the following criteria of classification were established. Immunoreactive neurons were classified, according to the shapes of perikarya, as belonging to one of five categories (round, oval, fusiform, triangular, or multiangular). Additionally, three morphological types of neuronal polarity were distinguished (unipolar, bipolar, and multipolar).

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). Image analysis programs (LaserSharp 2000 v. 2.01 and LaserPix v. 4.0, Bio-Rad, UK) were used to prepare the illustrations.

2.3. Quantitative study

The quantitative study involved the assessment of the neuronal density in each of the immunoreactive populations. Sections of the anterior (+1.6 mm from bregma), central (−0.26 mm), and posterior (−0.92 mm) parts of the Cl were chosen under 10× magnification. The borders of Cl were marked as separate inclusion areas under low magnification (4×).

The neuronal profiles were counted and the numerical density (number of cell profiles per mm^2) was calculated with the aid of a 20× objective lens in systematic random test frames of the selected area using a C.A.S.T. grid system (Computer Assisted Stereological Tool, Olympus, Denmark) and microscope (BX-51, Olympus, Japan).

2.4. Statistics

All the calculations were performed using Excel 2000 (Microsoft, USA). Raw data concerning the number of cells per mm^2 were collected. Mean values and standard deviations were calculated for each group. The comparisons of neuronal densities between various immunoreactive neuronal populations were performed using a nonparametric multiple comparison test (Steel–Dwas test, Tukey equivalent test).

3. Results

3.1. Neuropeptide-containing neurons in the Cl

Neuronal bodies and neuropil containing NPY, SOM, and VIP were present in the Cl during the entire study period. These neurons were randomly distributed in all parts of the structure. Morphological changes of immunoreactive cell bodies and neuropil were observed, which could be divided into two phases of development. In the first phase (P0–P4), immature neurons were observed. These were characterized by small somata, very often of unipolar or bipolar type, with nuclei surrounded by a narrow rim of cytoplasm and short processes. These morphological features may be attributed to migrating neurons. In the second phase (P7–P28), differentiating neurons were observed, with a greater amount of cytoplasm and longer, prevalently ramified dendrites. The intensity of staining increased inhomogenously. The last period of observation (after P28) was characterized by the occurrence of differentiated neurons of various shapes and clearly differentiated dendritic arbors, as well as visible proximal fragments of the axons.

3.2. NPY immunoreactivity in the Cl

3.2.1. Neurons

In the first phase (P0–P4), NPY-ir bipolar and unipolar neurons containing a small volume of cytoplasm were present (Fig. 1A). Neuronal somata of oval and fusiform shapes were most numerous. Only short and dilated fragments of one to three neuronal processes were visible.

In the second phase (P7–P28), the neuronal types became more differentiated (Fig. 1D). As well as those previously observed, multipolar neurons occurred for the first time. Oval and fusiform shapes of neuronal somata were most numerous in this phase. The amount of cytoplasm increased gradually. Neuronal processes were narrower and longer than before, and their morphological differentiation into dendrites and axons could be traced more easily.

After P28, neurons of multipolar and bipolar types and of variously shaped neuronal somata were observed (Fig. 1G). The initial fragments of axon and two to five dendrites originated from neuronal somata, dividing dichotomically into consecutive branches.

The numerical density of NPY-ir neurons increased during the first 2 weeks of life (Fig. 2A).

3.2.2. Neuropil

In the first phase (P0–P4), NPY-ir neuropil was poorly developed (Fig. 1A). Short fragments of fibers with varicosities and scattered immunoreactive points were present. In the second phase (P7–P28), an increase in the number of immunoreactive elements and the length of immunoreactive fibers was observed (Fig. 1D). After 4 weeks the morphological structure of NPY-ir neuropil reached its mature form (Fig. 1G). The intensity of staining did

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