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Distribution of nitric oxide synthase and neuropeptide Y neurones during the development of the hippocampal formation in the rat

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Nitric oxide (NO) is a short-lived radical, which modulates synaptic plasticity, neuronal oscillations and cerebral blood flow. NOS-containing neurones can be detected anatomically by nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry or by NOS immunohistochemistry. Neuropeptide Y (NPY) is the most abundant peptide in the brain. NPY is connected with several vital functions, such as a feeding behaviour, sexual maturation, regulation of circadian rhythms, body temperature, blood pressure and neuroendocrine secretions. Neuropeptide Y also modulates anxiety-related disorders, limbic epileptic seizures as well as learning and memory processes. The study was performed on 45 Wistar rats of various ages (P0, P4, P7, P10, P14, P21, P30, P60, and P120; P — postnatal day). The free-floating sections were stained with standard immunohistochemistry methods. Thereafter the histological sections were studied using the confocal laser microscope equipped. For 3D reconstruction the image analysis program LaserSharp 2000v. 2.0 (Bio-Rad, UK) was used. We found that in the newborn rat both NOS- and NPY-immunoreactivity was weak. It had been increasing gradually until the 7th day of postnatal life, after that until P14 it was maintained on the similar level, and then the number of immunolabelled cells deceased. The developmental changes concerned cell morphology as well — until the 10th day of life the immunoreactive cells were immature, with round or oval bodies and had only a few fibres. From P14 the cells' morphology became similar to that in adult.

key words: nitric oxide, neuropeptide Y, hippocampus, development, rat

INTRODUCTION

Nitric oxide (NO) is a short-lived radical, which modulates synaptic plasticity, neuronal oscillations and cerebral blood flow [23]. It acts as a messenger molecule in neurotoxicity as well [23]. NO is formed by conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) [10]. NOS-containing neurones can be detected anatomically in two ways: by nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry or by NOS immunohistochemistry. Neuronal NOS has been shown to be the same as NADPH-d [19].

In the hippocampus NO is probably involved in the induction of long-term potentation and memo-

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ry formation [13, 24, 27]. Recent studies have also suggested that one of the possible roles of NO released from γ-aminobutyric acid (GABA) ergic neurones is to control hippocampal blood flow [38]. Most non-principal hippocampal neurones are considered to be GABA ergic, and probably play a crucial role in the hippocampal functions [14, 28].

Neuropeptide Y (NPY) is the most abundant peptide in the brain. It acts as a neuromodulator in the central and peripheral nervous system [16, 17, 52]. NPY is present in large quantities in the majority of cortical areas, including the hippocampus [1–3, 30] where it is involved in several biological processes [25]. Physiological studies suggest that NPY is connected with several vital functions, such as feeding behaviour [34], sexual maturation [52], regulation of circadian rhythms, body temperature, blood pressure and neuroendocrine secretions [53]. Neuropeptide Y also modulates anxiety-related disorders, limbic epileptic seizures as well as learning and memory processes [15, 21, 40].

The hippocampal formation is a prominent component of the rat nervous system. It is divided into four cytoarchitectonically distinct regions, including the dentate gyrus, hippocampus proper (which is subdivided into CA1, CA2 and CA3), subicular complex and entorhinal cortex [8].

Both in the dentate gyrus (DG) and hippocampus proper there is one layer of primary cell type (granular layer in DG and stratum pyramidale in the hippocampus) and two in DG or three layers in the hippocampus with non-principal cells (Fig. 1). These non-principal cells are morphologically diverse and they take part in the information circulation within the hippocampal formation. In the dentate gyrus the primary cell type is the granule cell, whose axon crosses the polymorphic layer of DG and enters CA3 sector of the hippocampus proper. The second type represented is the dentate basket cells; their fibres form basket plexuses around the bodies of granule cells. The 3rd type comprises small cells localised within the molecular layer; these cells participate in the forming of basket plexuses. Within the polymorphic layer a few different types of cells are observed. The mossy cells type has large and numerous "thorny excrescences" that are sites of termination of the mossy fibre axons. There is also a variety of interneurones; some of them are fusiform in shape and their fibres extend to the granular and molecular layers. Another group of cells type is localised deeper in the polymorphic layer and give rise to locally ramifying axons [8].

The principal neurone cell type of the hippocampus is the pyramidal cell. It has a basal dendritic tree that extends into the stratum oriens and an apical dendritic tree that extends to the hippocampal fissure. There is a variety of differences in the dendritic organisation of pyramidal cells in CA1 and CA3. In CA3, pyramidal cells are larger and their dendritic total length is larger than pyramidal cells in CA1.

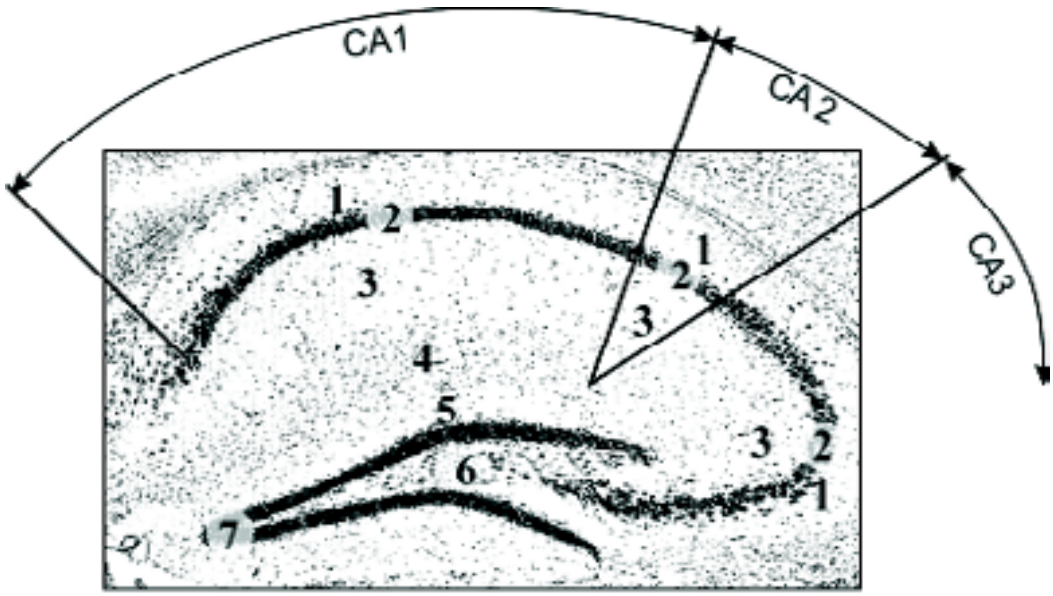

Figure 1. The scheme of hippocampal and dentate gyrus layers. The hippocampal CA1–CA3 sectors consist of four layers 1 — stratum oriens, 2 — stratum pyramidale, 3 — stratum radiatum, 4 — stratum lacunosum-moleculare. The dentate gyrus consists of three layers: 5 — molecular layer, 6 — polymorphic layer and 7 — granular layer.

Besides the pyramidal cells in this layer there is also a population of basket cells, which have various sizes and shapes. They also have apical and basal dendritic trees, but their dendrites have at least a few dendritic spines. These cells' axon extends transversely from the cell body of origin and forms a basket plexus that innervates the cell bodies of the hippocampal pyramidal cells.

The morphologically diverse interneurones are localised in the sparse cell layers. All of them are immunoreactive for GABA, which is the major inhibitory transmitter in the hippocampus [18, 22]. Interneurones of the hippocampus can be classified according to various criteria: 1) morphological characteristics and location [6, 11]; 2) types of postsynaptic target structures [36, 47]; 3) content of neuropeptides [32, 33, 45] and calcium-binding proteins [31, 41, 43, 46] as well as 4) physiological characteristics [50].

It has been found earlier that GABAergic interneurones are generated very early, before birth and earlier than principal cells [7, 48]. On the other hand many previous studies using nitric oxide synthase-immunohistochemistry (NOS-ir) or nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry reported the distribution of neuronal NOS containing GABAergic non-principal neurones scattered throughout the rat hippocampus [28, 42, 51].

In this article we described the morphology and distribution NOS- and NPY-immunoreactive cells in the particular layers of the hippocampus proper and the dentate gyrus from the day of birth until the 4th month of life.

MATERIAL AND METHODS

The study was performed on 45 Wistar rats of various ages (P0, P4, P7, P10, P14, P21, P30, P60, and P120; P — postnatal day). In each group 5 animals were studied. Care and treatment of animals were in accordance with the guidelines for laboratory animals established by the National Institutes of Health as well as by the Local Ethical Committee of the Medical University of Gdańsk. All the animals were deeply anaesthetised with Thiopental (60 mg/kg i.p.) and perfused transcardially with 0.9% solution of saline containing 10,000 units of heparin, followed by 4% solution of paraformaldehyde, and 10% of sucrose in phosphate buffer. The brains were postfixed in 4% paraformaldehyde fixative for 2–4 hours, and then kept in 0.1 M phosphate buffer containing 10% sucrose (overnight at 4°C) and 30% (until sunk). After that the brains were cut into 40-μm-thick coronal sections with a cryostat Jung CM1800 (Leica, Germany).

The free-floating sections were preincubated for 1 hour in 3% normal goat serum (NGS) with 0.3% solution of Triton X-100. Then they were incubated for 24 hrs at 4°C with the monoclonal antibodies against NOS (diluted 1:2000, Sigma, USA) or Neuropeptide Y (1:3000, Affinity, USA) (Table 1). After multiple rises in PBS, sections were incubated (for 2 hrs at room temperature) with appropriate secondary antibody conjugated with Cy3 (Table 1) Controls were incubated with 3% normal goat serum in PBS without the primary antibody. The specificity of staining was checked according to the procedure described by Wouterlood et al. [54].

The histological sections were studied using the MicroRadiance AR-2 (Bio-Rad, UK) confocal laser microscope equipped with an Argon laser producing dichromatic light at 514 nm. The 514-nm line of this laser was applied to excite Cy3, using an excitatory filter 514 and an emission long-pass filter E570LP. For 3D reconstruction the image analysis program LaserSharp 2000 v. 2.0 (Bio-Rad, UK) was used.

RESULTS

Nitric oxide synthase — immunoreactivity

In the newborn rat NOS is expressed mainly in the fibres; the number of immunoreactive cells in all hippocampal parts is very small. Characteristically few NOS-ir cells are localised almost exclusively in the stratum pyramidale of CA3 sector. Their morphology resembles immature cells — they are round with clear nucleus and single, short, thick fibre (Fig. 2A).

Table 1. Specifications and dilutions of the primary and secondary antibodies

Primary antibodies dilution	Manufactures	Secondary antibodies dilution	Manufactures
Mouse anti — NOS 1: 2000	Sigma	Goat anti mouse — CY3 1:800	Jackson Immuno Research
Rabbit anti — NPY 1: 3000	Affinity	Goat anti rabbit — CY3 1:800	

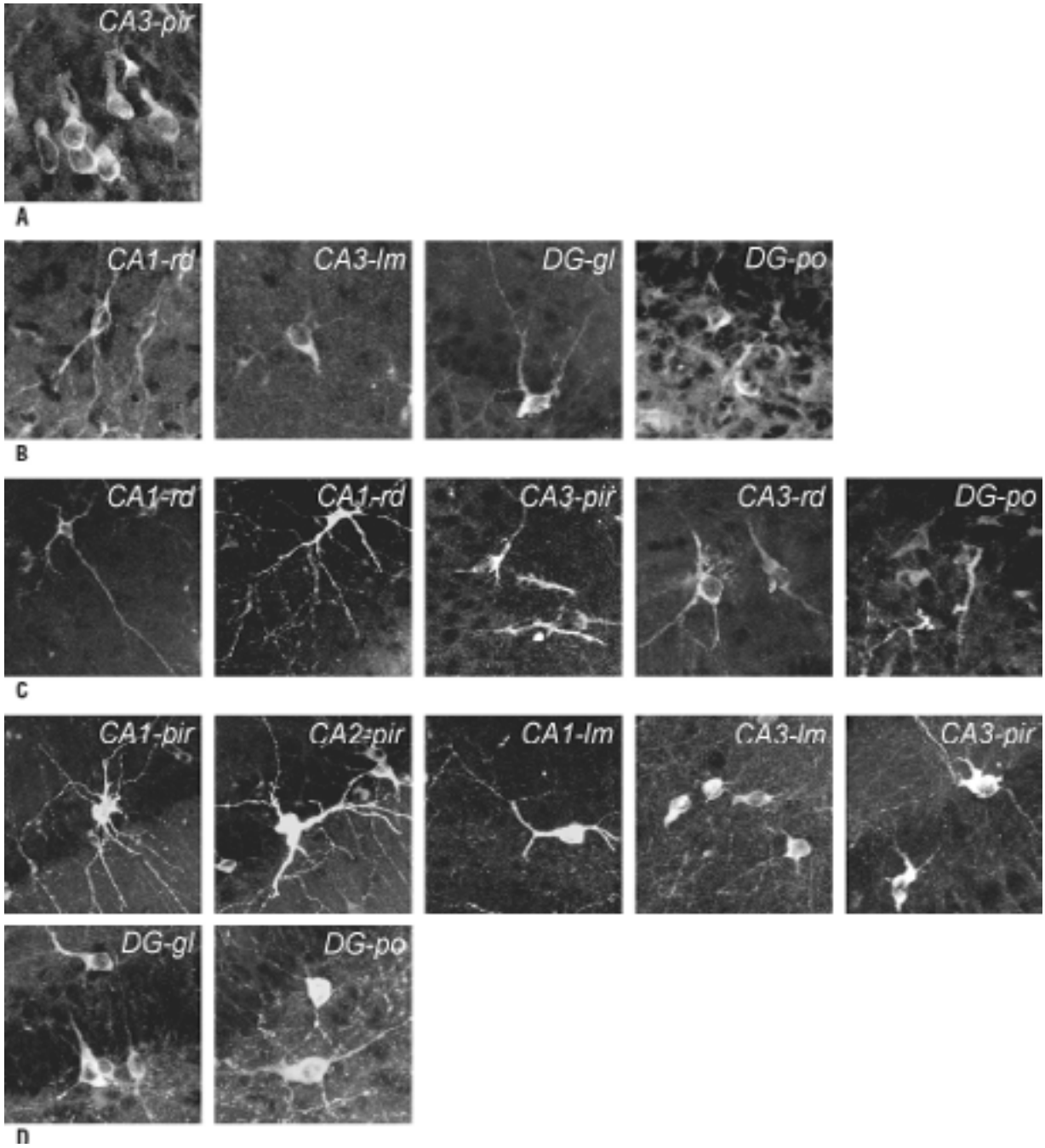

Figure 2. Different types of NOS-immunoreactive cells in particular layer of hippocampus proper and the dentate gyrus in P0 (**A**), P4 (**B**), P7 (**C**) and P10 (**D**).

On the 4th day of postnatal life the number of immunoreactive cells is larger; they are distributed in all hippocampal sectors. In CA1 NOS-ir cells were found at the border between stratum oriens and stratum pyramidale. They have at least two fibres reaching stratum oriens and one long fibre crossing the pyramidal cell layer and reaching stratum radiatum (Fig. 2B). These cells' morphology corresponds to a basket cell type. Characteristically NOS-ir cells in CA3 are similar to those in the stage P0 — they are round with single, short, thick fibre (Fig. 2B). In DG there are some stained cells at the border between the granular and polymorphic layer, giving rise to the molecular layer (Fig. 2B). The morphology of these cells suggests that they are probably dentate basket cells. Within the polymorphic layer there are some immunolabelled cells, they are round with clear nucleus and single, short, thick fibre (Fig. 2B). They are morphologically similar to that in CA3.

On the 7th day of life NOS-immunoreactive cells are the most intense in all stages. NOS-ir cells were observed especially in CA1; the majority of them are multipolar with a lot of varicosities along the fibres (Fig. 2C). Most cells are situated at the border between pyramidal cell layers and stratum radiatum and their fibres are lying perpendicularly to the stratum pyramidale. The cells in the stratum radiatum are multipolar with one long fibre crossing the pyramidal cell layer (Fig. 2C). The NOS-ir cells in CA3 and DG are stained less intensively and they possess no more than three dendrites (Fig. 2C).

In two-week-old rats the number of NOS-ir cells is still large, but smaller than at stage P7. Most NOS-ir cells of CA1 are intensively stained and localised at the border between sparse-cell layers (both oriens and radiatum ones) and stratum pyramidale. They are multipolar and have some long fibres oriented perpendicularly to the pyramidal cell layer. In the pyramidal cell layer of CA1 and CA2 we found some very intense staining cells, which are multipolar in shape and have some long dendrites with varicosities on them (Fig. 2D). At the border between stratum lacunosum-molecure and radiatum we found some large intense stained cells whose fibres run parallel to stratum radiatum (Fig. 2D). In contrast the cells in the CA3 are much less stained and have a few hardly stained dendrites. They were found in the stratum lacunosum-moleculare with fibres running parallel to the pyramidal cell layer (Fig. 2D) and at the border between the stratum pyramidale and radiatum. Cells in the dentate gyrus, localised mostly in the granular and polymorphic layers, are triangular or multipolar with two or three fibres crossing the granular layer or running parallel to this layer (Fig. 2D).

On the 30th day of life the number of immunoreactive cells decreases considerably. There are quite a lot of NOS-ir cells, but their distribution within the hippocampal subdivisions changes. Only a few of them are observed in CA3 now. They are round or oval in shape and have a single, thick dendrite (Fig. 3A). Remaining cells are localised mainly in CA1 and they are characterised by differentiated morphology (triangular or multipolar in shape with no more than three dendrites) (Fig. 3A). NOS-ir cells of DG are of similar morphology to those of CA1 (Fig. 3A).

In two-month-old rats NOS-ir is much weaker than in the previous stages. The number of cells is low, and most of them are fairly stained but they are distributed within all the hippocampal sectors. Most cells, especially in CA1 sector, are oval or multipolar with hardly labelled dendrites, but some immunostained cells within the stratum radiatum have long intense stained fibres (Fig. 3B). In the CA3 immunostained cells are oval in shape with hardly stained fibres (Fig. 3B). Morphologically similar calls were found in the dentate gyrus (Fig. 3B).

On the 120th day of life NOS-ir is very weak; there are only a few fairly stained cells. These cells are oval, multipolar or triangular in shape (Fig. 3C).

Neuropeptide Y-immunoreactivity

In the newborn rat NPY-immunoreactivity concerns mostly fibres and there are only a few immunoreactive cells found in all hippocampal parts (Fig. 4A). The NPY-ir cells are distributed homogeneously within sparse-cell layers in all hippocampal subdivisions. Most of these fairly stained cells are triangular in shape; they have thin fibres. (Fig. 4A)

On the fourth postnatal day the number of immunoreactive cells is larger, but the number was much smaller than that of NOS-ir cells observed in this stage. These cells were localised mostly within the sparse-cell layers both hippocampal sectors and the dentate gyrus. Cells observed in the CA1 are multipolar in shape and they have a few well-stained, long dendrites (Fig. 4B). NPY-ir cells in CA3 are localised mainly at the border between stratum pyramidale and stratum radiatum. They give rise to the stratum oriens and radiatum. Most of them are elongated or oval and its dendrites are hardly stained (Fig. 4B). In the dentate gyrus immunoreactivity concerns neuropil: both fibres and dots (Fig. 4B).

The first postnatal week is characterised by the most intense cell immunoreactivity of NPY populations. NPY-ir cells of all hippocampal subdivisions are triangular or multipolar in shape. In CA1 immunoreactive cells are much more intense than cells in CA3 and the dentate gyrus. In CA1 NPY-ir cells are triangular, with clear unstained nucleus and well-stained dendrites (Fig. 4C). Immunopositive cells in CA3 are less stained, mostly multipolar or fusiform in shape, and its dendrites are hardly stained (Fig. 4C). NPY-ir cells in the dentate gyrus are localised at the border between the granular and polymorphic layers and they are oval and hardly stained (Fig. 4C).

On the 14th postnatal day the number of NPY-ir cells is still large, but smaller than at stage P7. Nevertheless in this stage the widest variety of cells' morphology is observed. The shape of NPY-ir cells depends on their localisation. We observed that NPY-ir cells are oval in the pyramidal layer of CA1 (Fig. 4D) and oval or multipolar in the pyramidal layer of CA3 (Fig. 4D);

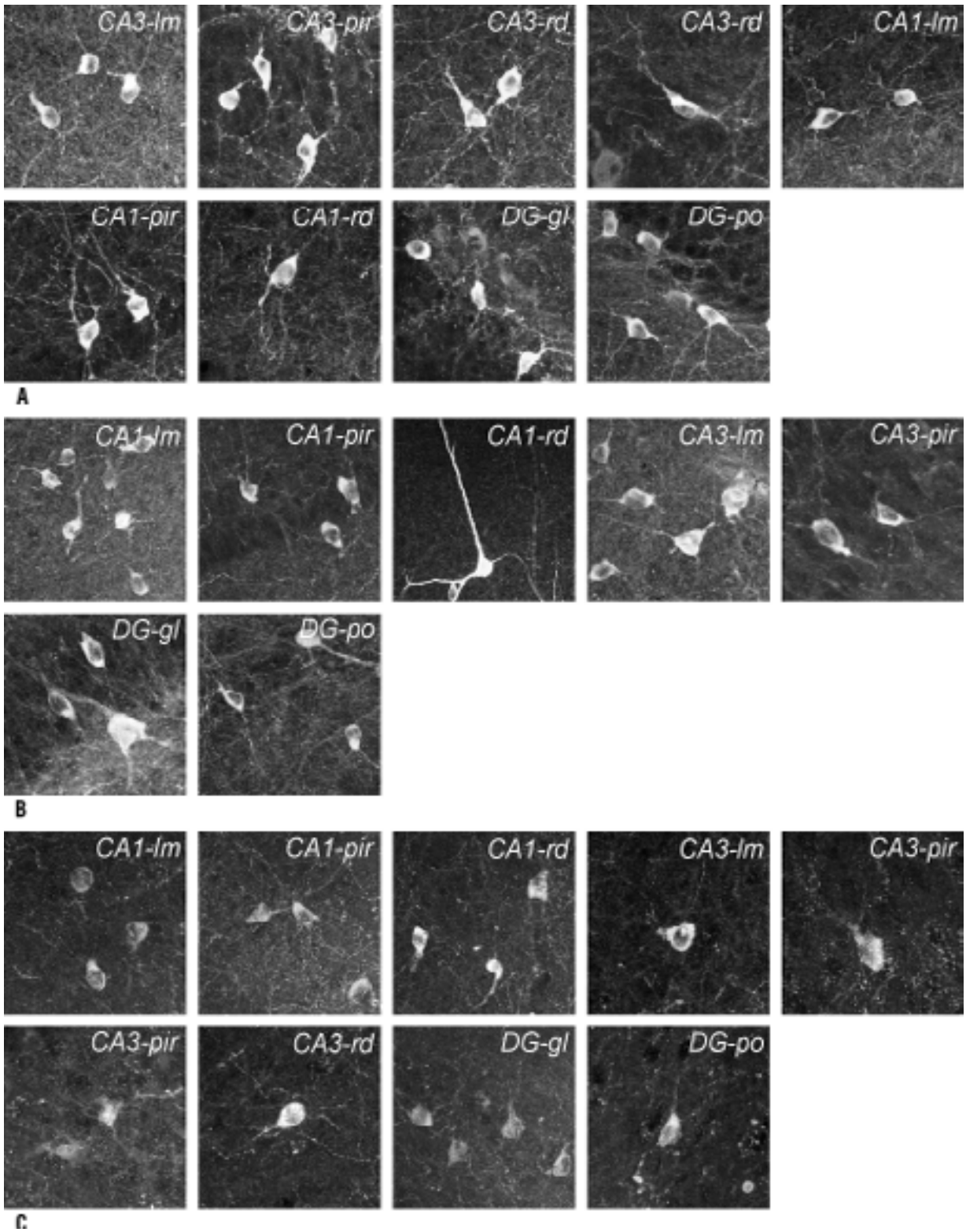

Figure 3. Different types of NOS-immunoreactive cells in particular layer of hippocampus proper and the dentate gyrus in P14 (**A**), P60 (**B**) and P120 (**C**).

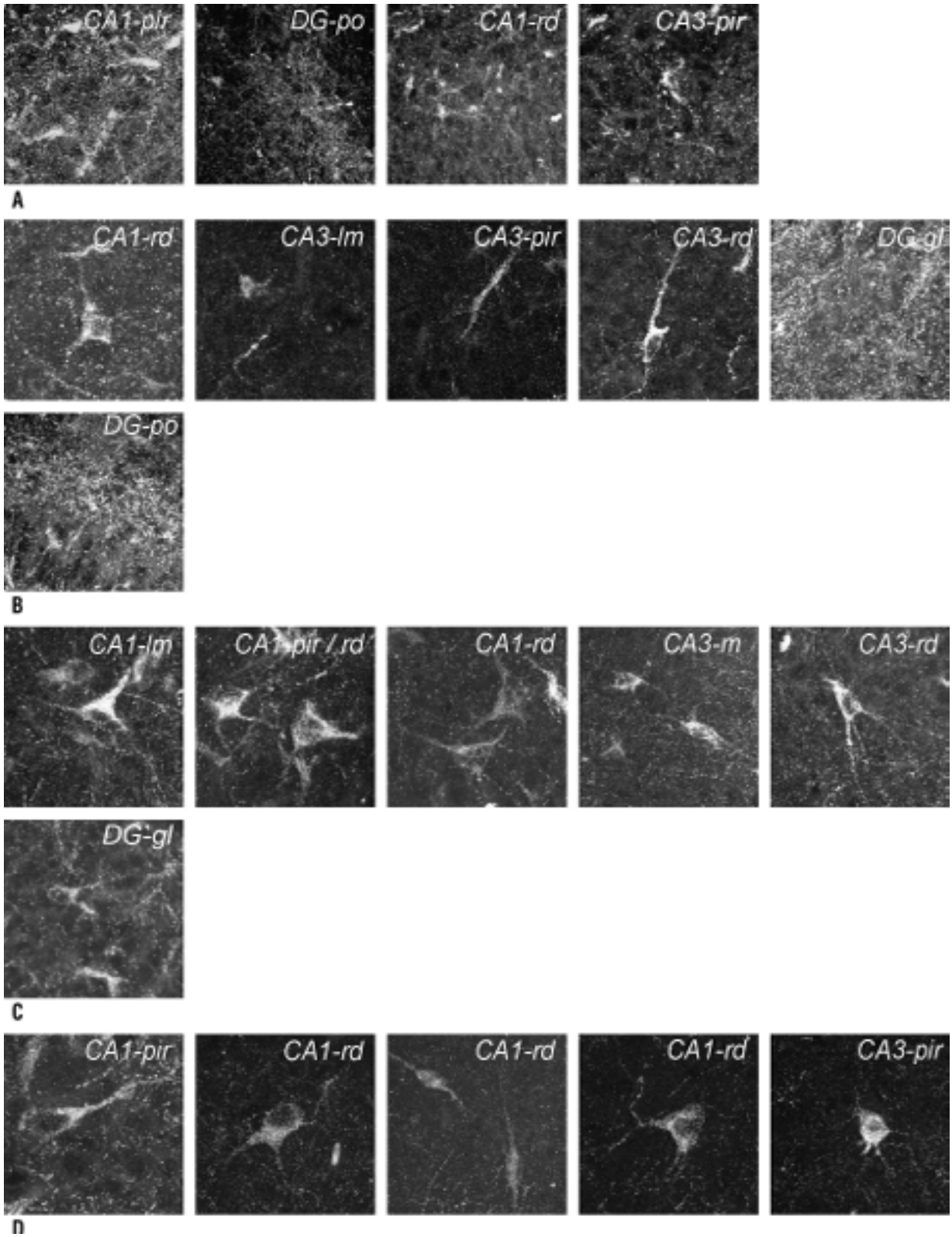

Figure 4. Different types of NPY-immunoreactive cells in particular layer of hippocampus proper and the dentate gyrus in P0 (**A**), P4 (**B**), P7 (**C**) and P10 (**D**).

triangular, fusiform or multipolar with quite long, thick fibres in the radiatum layer of both sectors (Fig. 4D). In the dentate gyrus most cells are localised at the border between the granular and polymorphic layers. Most of them are triangular or oval and have one main fibre that crosses the granular layer and reaches the polymorphic layer (Fig. 4D).

In the first postnatal month the number of immunoreactive cells of both types decreases considerably. In CA1 most cells are oval in shape (Fig. 5A); in CA3 they are oval or multipolar (Fig. 5A). NPY–ir cells in the polymorphic layer are localised at the border between the granular and polymorphic layers; they are oval with a faintly labelled single fibre (Fig. 5A).

In the second postnatal month NPY-ir is weak. The number of cells is low, and most of them are fairly stained. NPY-ir cells of almost all hippocampal subdivisions are mainly multipolar or bipolar (Fig. 5B); only within DG are there some fusiform neurones (Fig. 5B). A similar pattern was observed on the 120th postnatal day (Fig. 5C).

DISCUSSION

The majority of neurones in the mammalian brain are formed before birth. However the period of formation of some neuronal populations extends to postnatal ages [12]. ^{3}H-thymidine autoradiography has been used to study the time of origin of various neurones in the mammalian brain. Within the hippocampus, a precursor of the pyramidal cell population can be labelled during prenatal stages [9, 26], but cannot be labelled when ^{3}H-thymidine is injected postnatally [9], which hardly suggests that they are of prenatal origin. In contrast a large proportion of the granule cells of the dentate gyrus become labelled when injections are made either prenatally or postnatally [4, 5, 9], so these cells are formed either before birth or after it. The studies concerning the developmental pattern of non-pyramidal neurones in CA1 and CA3 [35] shows that on the day of birth the shape of the cell body is irregular, round, ovoid or elongated. They give rise to several shorts dendritic processes. In P5 the dendrites were much longer, and in this time there was observed a difference between the non-pyramidal cells in CA1 and CA3. The cells in CA3 seem to be more mature than cells in CA1. They have longer dendrites with regular varicosities and their dendritic tree traversed large parts of the hippocampal layers while dendrites of non-pyramidal cells in CA1 were restricted to the layer

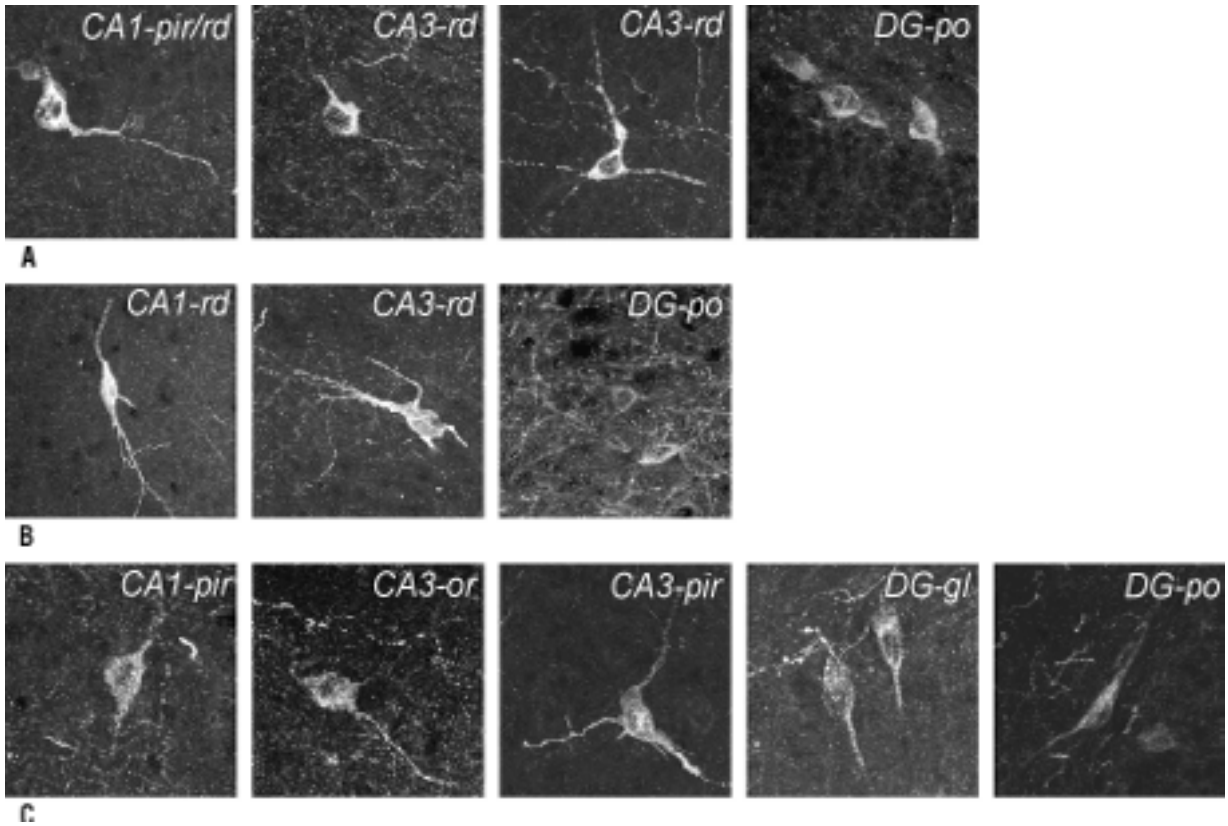

Figure 5. Different types of NPY-immunoreactive cells in particular layer of hippocampus proper and the dentate gyrus in P14 (**A**), P60 (**B**) and P120 (**C**).

of cell bodies' localisation [35]. On the 10th and 20th postnatal day the length of the non-pyramidal cells' dendrites increased, and the differences between cells in CA1 and CA3 became less obvious [35]. The maturation of non-pyramidal neurones is complete by the 3rd postnatal week [35].

Some studies show that developmental patterns of NOS immunoreactivity in the hippocampus are in some points similar to developmental patterns of non-pyramidal cells [39, 49]. NOS immunoreactivity appears in the developing brain on the 13th embryonic day, firstly in the hypothalamus and pons [49]. At the E15 NOS immunoreactivity appears in the thalamus, and at the E19 within the cerebral cortex (including hippocampus) and in the stratum. From that time the number of immunolabelled cells increased gradually and in the second postnatal week positive neurones with the large somata with many processes were distributed widely in the cerebral cortex and hippocampus. Other studies concerning the postnatal development of NADPH-d — positive cells show that NADPH-d reactive somata and processes are present from the day of birth until adulthood in the Ammon's horn [39]. Authors also found that the dentate gyrus has a more delayed period of nitric oxide synthase expression, with the staining appearing only by the end of the first postnatal week.

This is confirmed indirectly by our study. From the 30th postnatal day the pattern of NOS- and NPY-immunoreactivity becomes stabilised. This stabilisation concerned both cells' morphology and the number of immunopositive cells.

The studies [28] concerning analysis of GABAergic containing calretinin (CR) and NOS in the mouse hippocampus show that non-pyramidal cells containing both NOS and GABA were scattered throughout layers in all hippocampal areas, but especially concentrated in the stratum pyramidale and at the border area between stratum radiatum and stratum lacunosum-moleculare. Morphologically, both in Ammon's horn and in the dentate gyrus medium-size multipolar NOS-positive cells were dominant. In other hands the cells, both NOS — and calretinin immunopositive, were small to medium-size, with vertical fusiform somata. Some of them were small to medium-sized with multipolar somata and radially orientated dendrites [28]. In a more precise study Jinno et al. [27] found that there were two groups of NOS-ir neurones — non-principal and principal neurones. The NOS-ir non-principal neurones were scattered throughout layers in all hippocampal areas, especially numerous in the subgranular zone of DG and the stratum pyramidale of Ammon's horn [27, 29]. They were concentrated at the border area between the stratum lacunosum-moleculare and stratum radiatum. On the basis of the studies of NOS-ir in the hippocampus the authors found that in the hilus at the dorsal level, large multipolar neurones were dominant, and some small multipolar neurones were observed [27]. In the molecular layer of DG small-size multipolar neurones were evenly distributed. In the stratum oriens of Ammon's horn, a few small multipolar neurones were observed. In the stratum pyramidale and stratum radiatum of Ammon's horn, small to medium multipolar neurones and relatively few small bipolar neurones were distributed. In the stratum lacunosum-moleculare small multipolar neurones were concentrated at the border area on the stratum radiatum [27]. They found that principal neurones in the mouse hippocampus were NOS-ir, but their immunoreactivities were weaker than NOS-ir non-principal neurones, and that they have noticeable dorsoventral gradients. At the ventral level, CA1 pyramidal neurones adjacent to the subiculum showed NOS-ir of moderate intensity, and CA3 pyramidal neurones adjacent to the hilus showed weak NOS-ir. At the dorsal level, CA1 pyramidal neurones lacked NOS-ir, whereas CA3 pyramidal neurones adjacent to the hilus showed NOS-ir of moderate intensity. Granule cells in the DG were weakly stained for NOS throughout dorsoventral levels [27].

In our study we found that in the adult brain NOS-ir cells were scattered in all hippocampal parts, but most of them are situated in CA1 and DG. NOS-ir cells were round or oval in shape in CA3 and triangular or multipolar in shape in CA1 and DG.

Dun at al. [20] showed that NOS-ir neurones were moderately to strongly labelled. The majority of them had a small diameter (< 10 μm) and several processes (from 3 to >10) and were located in the pyramidal layer. A few NOS-ir neurones were scattered in the stratum oriens, stratum radiatum and subiculum, they were also infrequently detected in the stratum lacunosum. Some large (> 20 μm), multipolar strongly labelled cells were observed in various parts of the hippocampus. In the dentate gyrus, small-diameter NOS-ir cells were seen at the hilar border with granule layer of the suprapyramidal blade. The hilus contains the highest density of NOS-ir neurones compared with other regions of the hippocampus [20].

In our studies we found that the number of NPY--immunopositive cells is much smaller than NOS-ir cells in all studied stages. In each hippocampal part and all developmental stages there were many more

immunostained fibres than cells. Sloviter et al. [44] found that in the adult rat hippocampus NPY-ir neurones were present in the greatest number in the stratum oriens of areas CA1 and CA3, and in the stratum radiatum of area CA3. Significant numbers of NPY-positive cells were present in the granular cells and pyramidal cell layers, but relatively few NPY--positive somata were present in the dentate molecular layer or the stratum radiatum and stratum lacunosum-moleculare of CA1.

In our study NPY-ir cells were situated in all layers of the hippocampus proper and at the border of the polymorphic and granular layers. Most of them were oval or multipolar.

Köhler et al. [30] found a relatively large number of NPY-ir cells throughout all subfields of Ammon's horn with small variations in their total number from dorsal to ventral levels. However, a predominant number are present in the deeper than the superficial layers. In CA1 and CA3 most NPY-ir cells are situated within or adjacent to the pyramidal layer — in the stratum oriens and stratum radiatum. Very few NPY-ir cells are present within the strata lacunosum-moleculare. The authors found also that NPY-ir cells show large variations in soma size and morphology. In strata oriens and pyramidale many medium-size (around 18 μm) bipolar and polymorphic cells are present as well as medium to large size (29–25 μm) cells of multipolar shape. In the stratum radiatum the authors [30] found several small cells with ovoid or round perikarya, and in the deeper parts of this stratum several bitufted NPY-ir cells. The same authors [30] estimating immunoreactive fibres show that Ammon's horn and the dentate gyrus are rich in thin, varicose stained axons present in all layers of the hippocampus. Most of these fibres run in the molecular layer of Ammon's horn and in the outer one-third of the molecular layer of the dentate gyrus, where they form distinct bands.

There are not many studies concerning the developmental pattern of NPY-ir in the hippocampal formation. Most of them concern development of the human foetus or infant hippocampus. Yu et al. [55] found that immunopositive cells and fibres were identified in the hippocampus at 15th week of gestation. These cells were localised in the polymorphic and pyramidal layers and their number increased with the age of the foetuses. Other authors [37] found that high concentrations and widespread distribution of NPY-ir neurones were present in each of the hippocampal formations from birth to 42 years. Authors found that NPY interneurones are particularly numerous in the stratum oriens of CA1; they are multipolar, round, ovoid or triangular, bipolar or fusiform. They also found that in the postnatal brain spurt, which corresponds to the phase of rapid myelination, there is no decline in total number of NPY-ir neurones, but there is a decrease in density [37].

The developmental pattern of NOS- and NPY-immunoreactivity reflects the developmental pattern of non-principal cells population in the hippocampal formation. Until the third postnatal day both NOS- and NPY-ir cells seem to be immature. Most of them were round or oval in shape and have at least two fibres. From the third postnatal day the number of immunopositive cells becomes stabilised and their morphology became typical of mature neurones.

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