NADPH-diaphorase Positive Neurons of the Rat Hippocampal Formation: Regional Distribution, Total Number and Colocalization with Calcium Binding Proteins

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Received May 24, 2005, Accepted June 20, 2005

Key words: Nitrogen oxide synthase – Interneuron – Dentate gyrus – Ammon's horn

This work was supported by the Hungarian Science Fund, grants #A234 and #TS 040753.

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Abstract: The present study aimed to asses the total number and distribution of the NADPH-diaphorase-positive non-pyramidal neurons in Ammon's horn and dentate gyrus of rat hippocampal formation. Cell bodies were counted according to the "disector" principle. The total numbers varied from 27 000 to 32 400. In all strains, approximately one third of the NADPH-diaphorase-reactive non-principal cells were found in the dentate gyrus and the remaining two thirds were within the Ammon's horn. Analysis of the dorsoventral differences revealed that approximately 70% of NADPH-diaphorase-positive cells were in the dorsal and 30% in the ventral hippocampus. Distribution of NADPH-diaphorase-reactive cells in the different layers of the dentate gyrus and Ammon's horn was similar in all strains. Double-labelling studies revealed colocalization of NADPH-diaphorase with calretinin, but none with calbindin or parvalbumin. NADPH-diaphorase-positive neurons appear to form the largest chemically identified subpopulation of the GABAergic inhibitory cell population of the hippocampal formation.

Introduction

The freely diffusible, reactive free radical gas, nitric oxide (NO), has been proposed to function as a biological intercellular signalling messenger, capable of communicating among neurons, glia and the vasculature [1, 2]. In the hippocampal formation, the endothelial form of the nitric oxide synthase (NOS) enzyme is localized in pyramidal cells [3], whereas nNOS immunoreactivity is characteristic for interneurons [4, 5, 6, 7]. In the hippocampal formation, nNOS and NADPH-diaphorase (NADPHd) positive neuronal populations are very similar, if not identical [7, 8]. Neurons in the hippocampus and cuneate nucleus showing NADPHd or nNOS- reactivity were revealed to be immunoreactive for GABA, thus providing direct evidence that they represent interneurons [7, 9, 10]. NADPH, in the presence of NOS, supplies electrons to the dye nitroblue tetrazolium (NBT). This reaction forms an insoluble dark blue formazan precipitate which deposits in cells thereby staining a NOS-containing cell population [5, 11].

Estimates of neuron numbers have been useful in computational modelling of hippocampal function as well as in the studies of neurodegenerative disorders. The relative and total numbers of the interneurons in the rat hippocampus are only estimates without knowing their regional distribution or the ratio between axosomatic and axodendritic cell populations [12]. There is no data about the total number of any of the chemically identified GABAergic subpopulation in rat strains. It is known however, that the number of granule cells of the hippocampal dentate gyrus differs between rat strains although the number of pyramidal cells of Ammon's horn is similar [13, 14].

In the present study, NADPHd-histochemistry was used to stain a subpopulation of local-circuit GABAergic neurons in the hippocampal

formation to investigate the total number, the areal (dentate gyrus or Ammon's horn) and dorso-ventral distribution in those rat strains that are frequently used in morphological and behavioural studies [15]. However, it was not the purpose of the present study to describe minute differences among the included rat strains, especially because data indicated remarkable similarity in cell counts. We assume that variability inside one strain would be similar as among the different strains.

Colocalization of NADPHd with calcium binding proteins and neuropeptides has extensively been described (for details see the review of Freund and Buzsáki [12]). In this study, we include data to demonstrate that the NADPHd-positive cell population does not overlap with the parvalbumin (PV) containing axo-somatic and axo-axonic cell populations, or with the calbindin (CB) containing local circuit neurons. In addition, the relatively low rate of colocalization with calretinin (CR), that is a marker of axo-dendritic cells, indicates that NADHd-positive cells form a distinct subpopulation of the axo-dendritic GABAergic neurons.

Materials and Methods

All experiments were conducted in accordance with NIH guidelines on the use of laboratory animals (NIH Publications No. 80–23, revised 1996) and in accordance with local statutory regulations. Three month old, male rats from three different strains, Sprague-Dawley, Wistar and CFY were obtained from Charles River Breeding Laboratory (Gödöllő, Hungary). Animals under deep ketamine anaesthesia (0.3 ml/100g) were perfused transcardially first with 100 ml phosphate-buffer of 0.1 M (PB) and then with 500 ml of fixative containing 4% formaldehyde in PB (pH 7.4). The brains were then removed from the skulls, and postfixed for 1 h in fresh fixative solution. Serial coronal 50μm thick sections were collected along the septo-temporal extent of the hippocampal formation with the Vibratome.

Histological procedure for light microscopy

NADPH diaphorase histochemistry

After extensive washing in 0.1 M PB, free floating sections were incubated in a phosphate-buffered (0.1 M) solution containing 0.1% NADPH 0.02% Nitroblue Tetrazolium and 0.3% Triton X-100 (all reagents from Sigma) at 37° C.
The incubations were accompanied by gentle shaking until the appropriate staining pattern, determined by light microscopic examination, was achieved. The best results were obtained with incubation periods between 90–120 min. The reaction was stopped with cold PB. Since NBT turns into a visible product (formazan), a set of sections was promptly removed from the developer solution to assess the reaction pattern of the neurons that rapidly displayed intense reactivity. This was necessary in order to ensure that the product of the subsequent immunohistochemical staining, performed on the same section,

would still be visible. Furthermore, as the brown 3,3'-diaminobenzidine tetrahydrochloride (DAB) insoluble product darkens after histochemistry, the immunocytochemistry was always performed second. This allows histochemical (blue) and immunohistochemical (brown) reaction products to be distinguished.

Immunocytochemistry
Additional sections stained with NADPHd histochemistry were further processed for immunocytochemistry. After extensive washing in 0.1 M PB, sections were preincubated for 1 h in blocking solution containing 10% normal goat serum, 0.5% Triton X-100 in PB at room temperature. Sections were then incubated for different antibodies, namely in rabbit anti-calretinin (1:3000; Swant, Switzerland), mouse anti-parvalbumin (1:5000; Swant, Switzerland), mouse anti-calbindin (1:5000; Swant, Switzerland) at 4° C for three days. The second layer was goat anti-rabbit (1:200; Vector Laboratories, Inc.) or goat anti-mouse (1:200; Vector Laboratories, Inc.). Phosphate-buffer containing 1% normal goat serum was used for washing and for antisera dilutions. Sections were processed according to the ABC method using a Vectastain Elite ABC Kit (Vector Laboratories). After several rinses in PB, bound antibodies were visualized with DAB. The slices were then washed in PB, mounted on gelatine coated glass slides and air-dried. The sections were dehydrated in graded series of ethanol, cleared in xylene and covered with DEPEX (Fluka).

Quantitative analysis
Quantitative analysis was performed using one animal from each strain. Cell count was made unilaterally except for the CFY rat, where cells were counted on both sides to evaluate possible differences between the two hemispheres. From a coronal series every second 50μm section was analyzed using the Neurolucida reconstruction system (Microbrightfield, Colchester VT). First, the contours of the hippocampal layers were traced under the microscope, then cells were marked in each layer using a 100x objective and focusing through the width of the section, but omitting cells in the outermost focal plane (surfaces of the sections). This way we were able to avoid tracing the same cell body in neighbouring sections. The total number of NADPHd-positive cells was estimated by multiplying the number of cells by two. The intensity of neuronal staining for NADPHd varied from dense and complete to very weak and partial. The weakly and partially stained cells were not included in the counts. As an example, there are 3 large and 5 small NADPHd-positive cells on Colour Figure 3 (3 in pyramidal layer, 1 in stratum radiatum and 1 in stratum lacunosum-moleculare), whereas 2 large and 3 small NADPHd-positive cells on Colour Figure 4 that would be included into the counts.

Twenty additional, double labelled sections (NADPHd plus CR or PV or CB), five sections per animal, were examined. The labelled non-pyramidal neurons

were counted at the mid-septotemporal level and the number of double labelled neurons is expressed as a percentage of the neurons labelled with one of the two markers.

Results

Light microscopic morphology, total number and localization of the NADPHd-positive hippocampal neurons

NADPHd-positive cells were found in all layers of the dentate gyrus and Ammon's horn (Figure 1 and Colour Figure 1). They had somata of variable size and shape, spineless dendrites and beaded axons that appeared to avoid the principal cell layers (Colour Figures 1, 2, 3). Axons of the NADPHd-positive cells were less well stained in the hippocampal formation than in the bordering neocortical areas, where strongly stained axons revealed a rich network. Many of the strongly stained cells were in close apposition to capillaries (Colour Figures 2 and 3). Some dendrites even bended around the capillaries (Colour Figure 2), suggesting that nitric oxide plays a role in coupling neuronal activity with regional blood flow in the hippocampal formation, similarly as it is known in other parts of

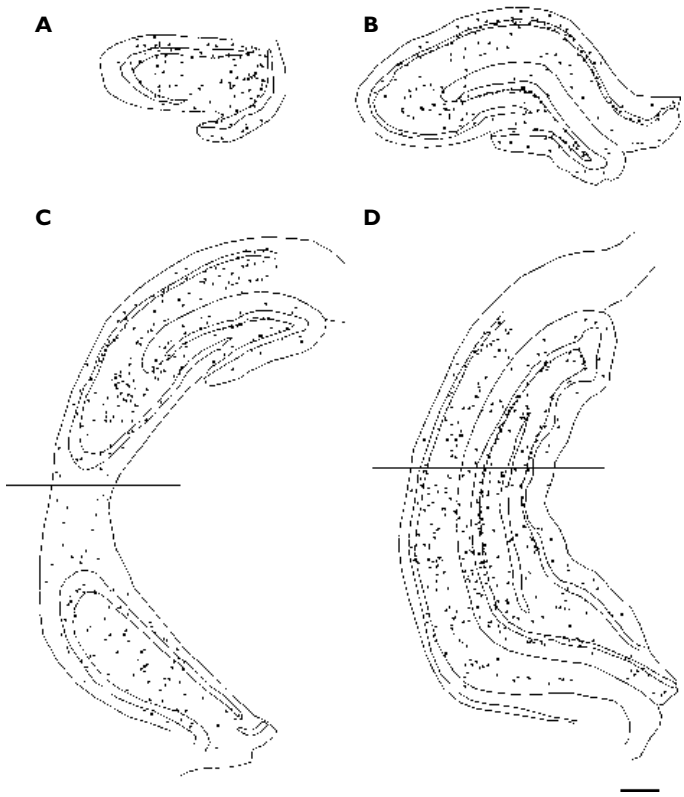

*Figure 1 – Computer aid camera-lucida drawings of the distribution of NADPHd-positive cells in representative individual sections at four different levels (**A** –2; **B** –3.8; **C** – 4.3; **D** – 6.0 from Bregma) [23] along the septo-temporal axis in a CFY rat. The areas of the dentate gyrus and Ammon's horn are outlined and dots represent individual labelled neurons. Suprapyramidal blade (or upper blade) of the dentate gyrus locates closer to the CA1, "above" the CA3 area, infrapyramidal blade (or lower blade) faces the thalamus. Dorsal and ventral hippocampus was divided in a horizontal plane at 5.5 mm distance from the plane of the surface of the skull [23] as indicated by the solid lines on **C** and **D**. Bar = 0.5 mm.*

the central nervous system. However, not all NADPHd-positive cells appeared to contact capillaries (Colour Figure 4). Estimated from the punctate staining intensity, axon terminals were most frequent in the molecular layer of the dentate gyrus just above the granule cells layer (Colour Figures 1A and 2) and at the border between strata radiatum and lacunosum–moleculare of Ammon's horn (Colour Figure 1B).

The morphology and general distribution of NADPHd-positive cells in coronal sections of the hippocampal formation of the three rat strains was similar to previous data from horizontal sections of the hippocampus and no meaningful differences were found in the light microscopy of labelled cells along the septo-temporal axis [7]. Therefore, we limit the light microscopic documentation and description to the regional distribution of the neurons. The total number of the NADPHd-containing cells in the three rat strains slightly varied in the dentate gyrus and in Ammon's horn (Table 1). Most labelled cells were found in the Wistar strain, whereas the hippocampus of CFY rat contained the fewest. There was no major

Table 1 – The number* of NADPHd containing interneurons of the rat hippocampus**

	Sprague Dawley	Wistar	CFY right	CFY left
Dentate gyrus	10 500	12 400	10 800	10 600
Ammon's horn	17 500	20 000	15 700	16 800
Hippocampus (total)	28 000	32 400	26 500	27 300

* numbers are rounded to hundreds

** n = the hippocampus of one side of one animal was analysed from each strain except for the CFY rat where hippocampi of both hemispheres were counted

Table 2 – Distribution of NADPHd positive cells in the hippocampal subregions

Hippocampal regions	Sprague Dawley	Wistar	CFY right	CFY left
dorsal Dentate Gyrus	6 970	10 100	8 540	7 540
dorsal CA1	7 480	8 200	7 400	7 410
dorsal CA2	980	1 850	1 350	1 390
dorsal CA3	3 430	4 000	3 030	2 960
Total number in the Dorsal Hippocampus	**18 860 (67%)**	**24 150 (75%)**	**20 320 (77%)**	**19 300 (70%)**
ventral Dentate Gyrus	3 500	2 350	2 280	2 020
ventral CA1	2 440	2 250	2 300	2 720
ventral CA2	390	550	320	520
ventral CA3	2 750	3 100	1 280	1 740
Total number in the Ventral Hippocampus	**9 080 (33%)**	**8 250 (25%)**	**6 180 (23%)**	**8 000 (30%)**

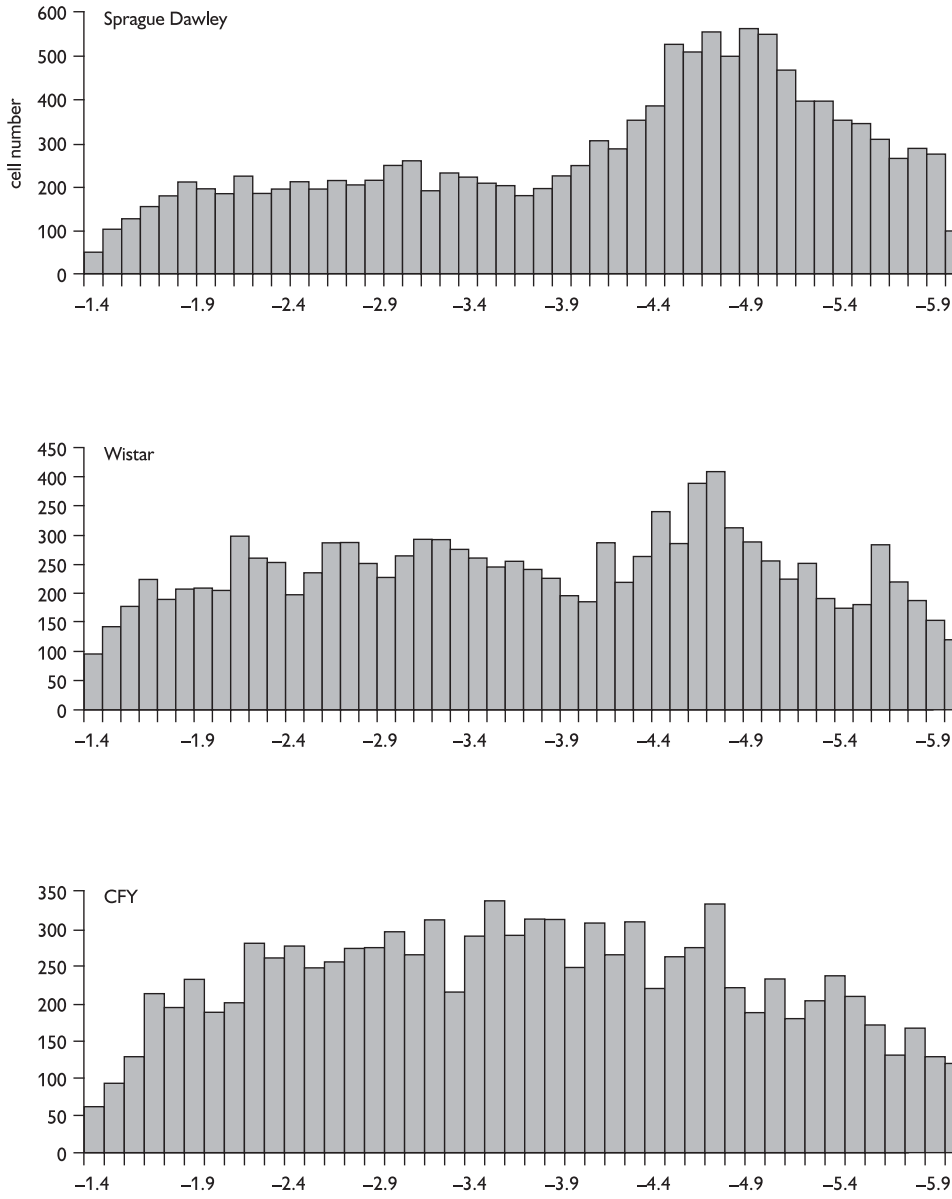

Figure 2 – The septo-temporal distribution of NADPH-diaphorase-reactive cells in the three rat strains. Each column represents the numbers of labelled cells in every second section of the series. Coordinates of the sections are given in relation to Bregma according to the atlas of Paxinos and Watson [23]. Coronal sections that included both the dorsal and ventral hippocampus (cut from the anteroposterior range of –4.2 to –6.0 mm from Bregma) contained more NADPH-diaphorase-reactive cells, than those more septally, because the latter include only the dorsal hippocampus. Depending on the angle of sectioning, more or fewer sections contained the entire hippocampus. The number of NADPHd-positive cells were counted in 44–48 coronal sections along the septo-temporal axis.

difference between the hippocampi of the right and left side of the same brain. Labelled cells were more numerous in Ammon's horn (60–65%) than in the dentate gyrus (35–40%). In addition, the distribution of NADPHd-containing cells was not homogeneous within Ammon's horn, with 30–40% found in the CA1-2 region, and only 20–30% in the CA3 region (Table 2).

The distribution of NADPHd-positive non-pyramidal cells in the dorsal and ventral parts of the hippocampal formation revealed that about 70% of the NADPHd-positive cells were located within the dorsal hippocampus, whereas the ventral part contained approximately 30% of the labelled cells (Table 2). The septo-temporal distribution of the NADPHd-positive neurons was even in all three strains. Most of the individual 50mm thick sections contained 250–300 labelled cells with higher numbers in those regions where the sections included both the ventral and dorsal parts of the hippocampus (Figures 1 and 2).

Regional distribution of NADPHd-positive cells in the sublayers of hippocampal formation. The dentate gyrus (Table 3)

Stratum moleculare. The molecular layer contains 2–7% of the labelled neurons. Most of the labelled cells are medium-sized, radially oriented, fusiform or round cells. It was characteristic in every section that there were more labelled cells in the suprapyramidal (the blade towards the CA1 area) – than in the infrapyramidal blade.

Table 3 – Percentage of NADPHd containing cells in the layers of the hippocampal subregions

Hippocampal region	Sprague Dawley	Wistar	CFY right
Dentate gyrus	**37%**	**35%**	**40%**
stratum moleculare (suprapyramidal)	7%	4%	6%
stratum granulosum (suprapyramidal)	9%	15%	14%
stratum granulosum (infrapyramidal)	5%	9%	8%
stratum moleculare (infrapyramidal)	4%	2%	5%
hilus	12%	5%	7%
CA3	**22%**	**25%**	**23%**
stratum oriens	2%	1%	1%
stratum pyramidale	4%	6%	5%
stratum lucidum	2%	2%	2%
stratum radiatum	11%	14%	12%
stratum lacunosum-moleculare	3%	2%	3%
CA1-2	**41%**	**40%**	**37%**
stratum oriens	4%	2%	2%
stratum pyramidale	11%	13%	13%
stratum radiatum	24%	24%	20%
stratum lacunosum-moleculare	2%	1%	2%
Total	**100%**	**100%**	**100%**

Stratum granulosum. NADPH-diaphorase containing cells are more abundant in the suprapyramidal blade (9–15%) of the dentate gyrus than in the infrapyramidal blade (5–9%). Most of the stained cells were located at the hilar border, although some occurred within the granular cell layer. Subgranular cells were medium-sized to large and of two main types: longitudinally oriented, fusiform neurons, and radially oriented, triangular or bipolar neurons (Colour Figure 1A). The processes of these cells crossed the granule cell layer and branched in the molecular layer, whereas other dendrites branched in the polymorph layer. The granule cell layer contained the largest proportion of labelled cells in the dentate gyrus.

Hilus. Stained neurons (5–12%) were predominantly large, multipolar cells, with their dendritic branches covering the entire extent of the hilar region (Colour Figure 1A). Deep hilar cells were included in this group, because cells at the border between granule cell layer and hilus were counted to the granular layer.

The Ammon's horn (Table 3.)

Stratum oriens. This area contained only a small proportion of the labelled cells, 2–4% in the CA1-2 region, and 1–2% in the CA3 region. Two types of stained cells were found in the stratum oriens. Neurons of the first type, found scattered mainly in the inner half of the layer, were medium to large multipolar cells with richly arborizing dendrites, some of which crossed the pyramidal layer. The neurons of the second type were smaller, longitudinally oriented bipolar cells, found mainly in the outer half of the layer close to the alveus.

Pyramidal layer. This area contained a substantial proportion of the labelled cells, especially in the CA1-2 region (11–13%) and a smaller amount in the CA3 region (4–6%). Stained neurons in the pyramidal layer were mostly radially oriented and bipolar. In CA3 they were of uniform large size, evenly distributed throughout the thickness of the pyramidal layer. Dendrites of these cells enter stratum oriens as well as stratum radiatum, frequently branching in stratum lacunosum-moleculare. Similar but smaller neurons were stained in CA1 (Colour Figure 1B).

Stratum lucidum of CA3 area. Large multipolar neurons, consisting of about 2% of all labelled cells were found in this region. They resembled stained cells of the pyramidal layer.

Stratum radiatum. This area contained the largest proportion of labelled cells, with 20–24% in the CA1-2 region, and 11–14% in the CA3 region. Most were large, radially oriented multipolar or fusiform cells (Colour Figure 4). They had large dendritic trees that covered the whole thickness of the layer and branched in neighbouring layers (Colour Figures 1B and 4). A distinct class of longitudinally oriented fusiform cells was found to possess numerous fine dendritic branches along the border with stratum lacunosum-moleculare.

Stratum lacunosum-moleculare. The stained cells in this layer included large, longitudinally oriented, bipolar cells and small, multipolar neurons with small radial dendritic trees. They represented 1–3% of all labelled cells both in the CA1-2 and CA3 region (Colour Figure 3).

Along the septo-temporal axis the frequency of NADPHd-positive cells did not show any meaningful accumulation. It appeared as if these cells were distributed rather homogenously along the longitudinal axis while differences between layers are obvious (Figure 1 and Table 3). The number of NADPHd-positive cells appeared to correlate with the number of principal cells, such that sections with a larger hippocampal cross-section contained proportionally more labelled cells. Unfortunately, it was not possible in these preparations to estimate the number of principal cells therefore we have no direct data to confirm this.

Colocalization with other markers

CR and NADPHd colocalization. Single and double stained cells could be differentiated, since NADPHd-positive cells displayed a blue cytoplasm with a light, non-stained nucleus, whereas calretinin immunoreactive cells showed brown staining in both the cytoplasm and nucleus (Colour Figure 5C). Thus, the CR/NADPHd neurons presented dark blue stained-cytoplasm and a brown nucleus (Colour Figure 5C and D). NADPHd-activity was detected in 31% of the calretinin-immunoreactive neurons in the dentate gyrus, 47% in CA3, and 34% in CA1-2, whereas calretinin-immunoreactivity occurred in 13% of NADPHd-positive neurons in the dentate gyrus, and 31% in the CA3, 15% in the CA1-2 region. The morphology of the CR/NADPHd neurons varied considerably such that their features could not be used as criteria for classification.

PV and NADPHd colocalization. The distribution of *PV*-containing neurons showed a characteristic pattern: practically all cell bodies were located in strata pyramidale and oriens of the hippocampus and in stratum granulosum and the hilus of the dentate gyrus: no double stained cells were detected in our material (Colour Figure 5A and B).

CB and NADPH-d colocalization. Calbindin was present in both principal cells and in a few interneurons of the hippocampal formation. NADPHd did not co-localize with this calcium binding protein in principal cells or in local circuit neurons.

Discussion

The main result of the present study is that NADPHd-reactive non-pyramidal cells form a substantial subpopulation of the hippocampal GABAergic cells. There are no significant differences in this cell population among the examined rat strains.

The distribution of NADPHd-positive non-pyramidal cells within the different layers of the hippocampal formation was very similar in the various rat strains, suggesting a similar organization of the nitric oxide producing system. Approximately one third of the NADPHd-positive non-principal cells was found in the dentate gyrus and the remaining two thirds were within Ammon's horn in all strains studied. Dorso-ventral differences in the distribution of NADPH-diaphorase-positive cells were large, with 70% of the labelled cells in the dorsal hippocampus and 30% in the ventral hippocampus. Since the ventral part of the hippocampus is much smaller in volume, this distribution suggests a higher NADPHd-positive cell density in the ventral hippocampus, which data is consistent with the previous report of Nomura et al. [16]. There was no appreciable difference in the number of NADPHd-positive neurons between the two hemispheres, which is in agreement with previous results that showed no differences in granule cell numbers between the hippocampi of the two hemispheres [17].

Our previous results concerning the number of GAD_{65}-positive cells together with total numbers of NADPHd-positive cells, suggest that the NADPHd--reactive neurons represent approximately one third of the GAD_{65}-positive neurons in the hippocampal formation of Spraque-Dawley rats [18]. This is in good agreement with previous results in the mouse hippocampus that showed that about 30% of GAD_{67}-immunoreactive neurons were nNOS-positive [10]. In the Sprague-Dawley rat the number of dentate granule cells is slightly higher than the number of CA1-3 pyramidal cells [14, 19, 20]. Therefore, in the Sprague-Dawley strain, the NADPHd-positive cells form 1.05% of the granule cells (1 million) in the dentate gyrus and 2.3% of pyramidal cells (0.75 million) in Ammon's horn.

The generally accepted estimate of 10% for GABAergic neurons in the rat hippocampus [12] is somewhat higher than the ratio indicated by the present results. In Ammon's horn, the ratio can be approximately 7–8%, but in the dentate gyrus it is not higher than 4%. Considering that in different rat strains [14, 19, 20], the total number of granule cells of the dentate gyrus is larger (0,6–1 million) than the number of pyramidal cells of Ammon's horn (0.5–0.8 million), GABAergic neurons may represent about 6 % of neurons of the hippocampal formation including the dentate gyrus and Ammon's horn. In the subiculum and in the entorhinal cortex the ratio of GABAergic neurons is higher than in Ammon's horn [16]. Systematic cell counts of GABAergic neurons along the septo-temporal axis of the entire hippocampus of different rat strains may reveal more accurate numbers. It cannot be ruled out that the percentage of GABAergic neurons varies in the hippocampal formation of different rodent strains.

In agreement with previous reports [19, 21, 22] we found a distinct subpopulation of GABAergic interneurons containing both calretinin and

NADPHd. Megias et al. [22] reported a detailed distribution of co-localization of CR / NADPHd, and our results are similar to those data. They showed that 39% of calretinin positive neurons co-expressed NADPHd and in our samples this ratio was 37%. However, in their study the percentage of NADPHd-positive neurons co-expressing calretinin was 31% [22], whereas in our samples it was 19%. The main difference appears to be in the CA1 region where Megias et al. [22] detected 41% of NADPHd-positive cells co-expressing calretinin, whereas in our samples it was only 15%. At present we do not have an explanation for the difference, but differences in staining intensity of immunoreaction may explain these results.

Parvalbumin positive neurons have also been reported to be immunoreactive for NOS [4]. However, in our material no PV-positive cells were double-labelled with NADPHd. Furthermore, nor was there any NADPHd-reactivity in CB-immunoreactive cells.

Previous observations suggest that nNOS-positive local circuit neurons of the hippocampal formation form exclusively axo-dendritic symmetric synapses [8, 24]. Since nNOS and NADPHd-positive neuronal populations are very similar [7, 8], it can be concluded that NADPHd-positive neurons of the rat hippocampal formation form a large subpopulation of the axo-dendritic GABAergic neurons, controlling the information processing of the dendrites of principal cells. In addition, NADPHd-positive cells have the capacity to influence non-synaptically the neighbour neurons, because nitrogen oxide as a transcellular messenger have a non-synaptic effect on intracellular regulatory enzymes and through the direct connections with capillaries, may couple neuronal activity with regional blood flow [1, 2, 25, 26].

Acknowledgements: Authors wish to thank dr Hajnalka Ábrahám for her help with the Figures. The authors are also grateful to Dr. Susan Totterdell (Department of Pharmacology, University of Oxford) for critically reading and correcting the manuscript.

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