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Distribution of NADPH-Diaphorase and Neuronal NO Synthase in the Nuclei of the Medulla Oblongata in Rats

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The distribution of nitroxidergic neurons in the medulla oblongata nuclei in Wistar rats (n = 8) was studied using histochemical (NADPH-diaphorase) and immunohistochemical methods with antisera to the neuronal form of nitric oxide synthase (nNOS). NADPH-diaphorase was found in large and small neurons in the sensory, autonomic, and motor nuclei. The latter were found to contain particularly large numbers of neurons expression this enzyme activity. In contrast to NADPH-diaphorase, nNOS in the corresponding nuclei was always present in smaller quantities of mainly smaller neurons. The sensory nuclei (solitary tract nucleus, reticular parvocellular and lateral nuclei, spinal nucleus of the trigeminal nerve) showed 1.5–3 times more nNOS neurons than the motor nuclei. In some nuclei (the nucleus ambiguus, the nucleus of the hypoglossal nerve) containing large numbers of NADPH-diaphorase-positive neurons, immunoreactive cells were particularly rare.

Keywords: medulla oblongata nuclei, nitroxidergic neurons, NADPH-diaphorase, neuronal NO synthase, rat.

Extensive information has now accumulated on the neuronal organization of the brain's nitroxidergic systems. Increased interest from researchers in nitric oxide (NO) is largely due to its role in supporting a multitude of neuronal functions [2, 5, 6, 8, 12, 17, 21]. Knowledge of the distribution of NO-ergic neurons in the brain thus acquires particular importance. However, there is still no unified view as to the topography or the numbers of neurons expressing the enzyme NO synthase, especially the neuronal form of NO synthase (nNOS), which in physiological conditions has a role in NO synthesis in nerve cells [8, 9, 12].

The locations of nNOS in the structural formations of the brain are often assessed in terms of the fact that they containing NADPH-diaphorase, as this is colocalized with nNOS in some neurons [11, 14–16]. Although results obtained by histochemical detection of NADPH-diaphorase and immunohistochemical detection of nNOS quite frequently do not agree [13, 19, 21], studies of the brain's NO-ergic systems have thus far made wide use of the histochemical reaction for NADPH-diaphorase.

The aim of the present work was to undertake a comparative study of the topography and numbers of NADPH-diaphorase- and nNOS-positive neurons in several medulla oblongata nuclei in rats.

MATERIALS AND METHODS

Experiments were performed on Wistar rats (n = 8) weighing 240–280 g, kept in laboratory animal-house conditions on a standard diet. Experimental manipulations were performed in compliance with the requires of the "Regulations for Studies Using Experimental Animals" (USSR Ministry of Health Decree No. 755 of August 12, 1977). Animals were sacrificed by overdosage with 3% thiopental sodium. Brains were extracted from skull cavities, the medulla oblongata was separated and fixed for 1 h in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C, followed by preparation of sections of thickness 30 ∝m on a cryostat.

NADPH-diaphorase was detected in neurons using the histochemical (HC) method of Hope and Vincent [16]. Reaction specificity was verified by incubating several sec-

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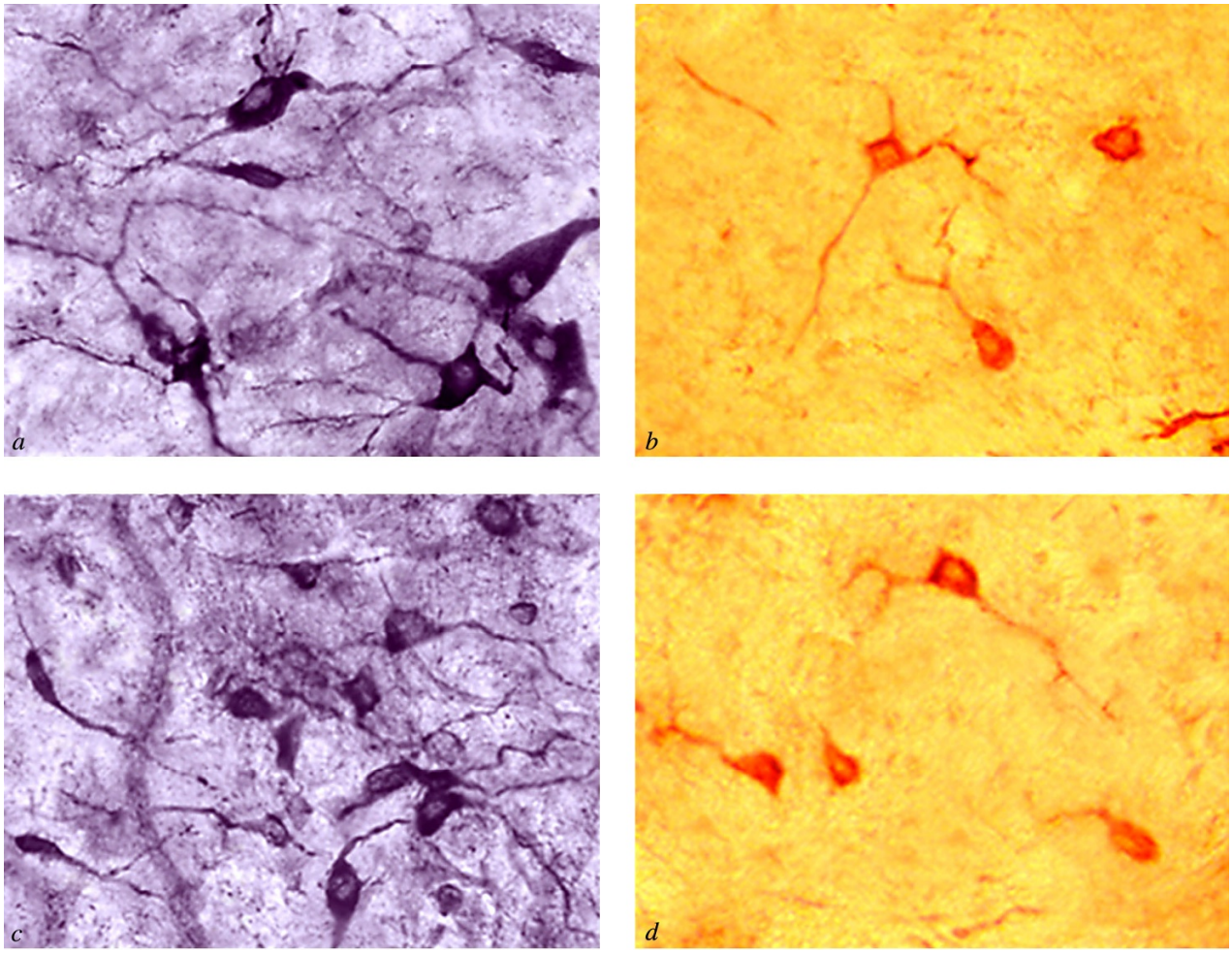

Fig. 1. Nitroxidergic neurons in the nucleus of the hypoglossal nerve (*a*, *b*) and the spinal nucleus of the trigeminal nerve (*c*, *d*) in rats. *a*, *c*) Histochemical reaction for NADPH-diaphorase; *b*, *d*) immunohistochemical reaction for nNOS. Objective · 20, ocular · 15.

tions in solution not containing NADPH or containing NADP instead of NADPH. Immunohistochemical (IHC) studies used polyclonal rabbit antibodies against nNOS (ICN Biomedicals, USA, 1:5000). Reaction product was detected by incubating sections with streptavidin-peroxidase complex (Biomedicals, Germany) using standard avidin-biotin ABC-complex visualization system (Vectastain Elite ABC kit, Vector Labs, Burlingame, USA). Reaction specificity was evaluated by staining sections without primary or secondary antibodies.

Studies addressed the solitary tract nucleus (STN), reticular magnocellular (RMCN), paramagnocellular (RPMCN), parvocellular (RMN), and lateral (RLN) nuclei, the dorsal nucleus of the vagus nerve (DNVN), the nucleus ambiguus (NA), the nucleus of the hypoglossal nerve (NHN), and the spinal nucleus of the trigeminal nerve (SNTN). Series of sequential brain sections were made, one stained with methylene blue, the second used for HC, and the third for IHC. Neuron profile areas ($\propto m^2$) were measured on projections of the section of each nucleus, along with the total number of cells identified by methylene blue staining, the proportion detected by staining for NADPH-diaphorase and the proportion of nNOS-positive neurons, as well as the numbers of these cells per 0.01 mm^3 (relative density), as described previously [6]. All neurons were divided into three size groups on the basis of body area: small (50–250 $\propto m^2$), intermediate (251–680 $\propto m^2$), and large (greater than 681 $\propto m^2$). Data were analyzed quantitatively using the Allegro-MC computer program for automated image analysis [1]. Quantitative data are presented as mean and standard error of the mean obtained by processing at least 12 sections for each nucleus. The significance of numerical data was assessed using Student's *t* test. Differences were regarded as significant at $p < 0.05$.

RESULTS

NO-positive neurons were detected in all the brain nuclei studied, and these differed in terms of shape, size, distribution density, and intensity of reactions (Fig. 1). Most

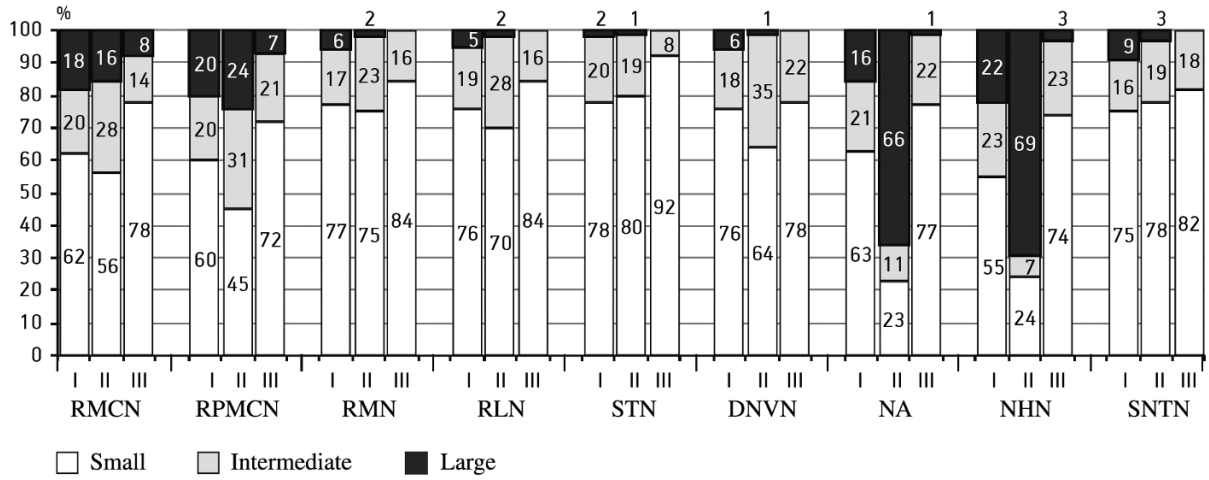

Fig. 2. Percentage ratios of small, intermediate, and large neurons in the medulla oblongata nuclei on staining of cells with methylene blue (I) and using histochemical (II) and immunohistochemical (III) detection of nitroxidergic neurons in rats. RMCN – reticular magnocellular nucleus; RPMCN – reticular paramagnocellular nucleus; RMN – reticular parvocellular nucleus; RLN – reticular lateral nucleus; STN – solitary tract nucleus; DNVN – dorsal nucleus of the vagus nerve; NA – nucleus ambiguus; NHN – nucleus of the hypoglossal nerve; SNTN – spinal nucleus of the trigeminal nerve.

neuron bodies had round, fusiform, or triangular shapes. Polygonal cells were seen more rarely, and occasional stellate cells were also seen. Limited numbers (about 1%) of giant pyramidal neurons with bodies with cross-sectional areas of greater than 2000–2500 per $\propto m^2$ were found in some nuclei (RMCN, RPMCN) or between them; these cells had NADPH-diaphorase activity but were not seen using the reaction for nNOS. Many neuron processes and capillaries were immunonegative, as were larger vessels, which were quite often seen in large numbers in the projections of the nuclei (see Fig. 1, *a*, *c*).

Comparison of data obtained from HC and IHC studies of the corresponding nuclei showed significant differences in the proportions of small, intermediate, and large neurons (Fig. 2). This shows that NOS expression was seen mainly in small neurons. The location of NADPH-diaphorase was less associated with neuron size: along with small neurons, neuron activity was often present in intermediate and large cells. The proportion of large NADPH-diaphorase-positive neurons was greater in the motor nucleus, in which methylene blue also showed the largest numbers of these cells (NA, NHN, RMCN, RPMCN). In the NA and NHN, the proportions of small cells containing this enzyme were barely above 20%, though more than 70% of neurons were immunopositive. In nuclei known to have sensory or associative functions (STN, RMN, RLN, SNTN), large neurons were relatively rare using HC and, in some cases, using IHC (see Fig. 2).

The numbers of NO-ergic neurons were not directly related to the numbers of cells staining with methylene blue (Fig. 3, *a*). The HC method always identified more NO-positive neurons than IHC reactions. For example, of 696 neurons identified on staining with methylene blue in the projection of the STN, 170 cells (24.4%) had NADPH-diaphorase activity and only 86 neurons (12.6%) were immunopositive. While the proportion of NADPH diaphorase-positive neurons in the SNTN approached 18.9%, IHC demonstrated only 9.7% (of 812 cells stained with methylene blue, the HC method identified 154 neurons, IHC 79). In the RMN, of 704 cells staining with methylene blue, 287 (40.8%) were detected by the HC method and 201 (28.6%) by the IHC method. Similar results were obtained from studies of the quantitative distributions of NADPH-diaphorase and immunopositive neurons in the other nuclei studied (see Fig. 3, *a*).

The most significant differences in the numbers of neurons detected by different methods were seen in the NA and NHN. In the former, the proportion of NADPH-diaphorase-positive cells approached 75.8%, of which about 8% were labeled by the nNOS reaction, compared with 87.9% and 5.8% for the HC and IHC methods respectively in the latter.

In those cases in which NO-ergic neurons were numerous and were distributed quite densely, the cell concentrations in the nuclei were higher (see Fig. 3, *b*). Values in IHC studies of the corresponding nuclei were always lower than values obtained with the HC method. However, as the value of this indicator was independent not only of the number of neurons, but also of their sizes, not even the largest number of small cells led to any significant increase in their relative density. Thus, differences in the values of the indicator calculated using the HC and IHC methods were smaller in the SNTN and DNVN than in the other nuclei (RMCN, RPMCN, NA) in which large but numerous neurons with NADPH-diaphorase activity were detected, though the number of immunopositive cells was very limited.

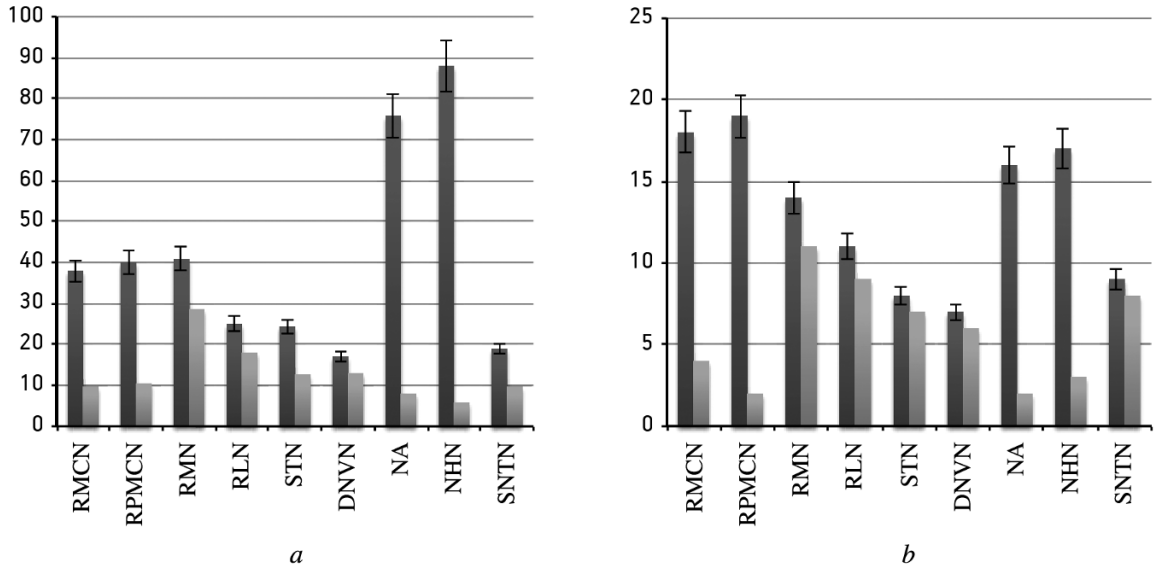

Fig. 3. Proportions (*a*) and relative densities (*b*) of nitroxidergic neurons in the rat medulla oblongata nuclei. Dark columns show results of the histochemical reaction for NADPH-diaphorase; light columns show results of the immunohistochemical reaction for nNOS. The horizontal axes how medulla oblongata nuclei (abbreviations as in Fig. 2); the ordinates show values (%). Values in the corresponding nuclei on staining with methylene blue were taken as 100%. Vertical bars show standard errors.

DISCUSSION

At the beginning of the 1960s, Thomas and Pearse [18] used the then new histochemical method in their studies of the brain to detect NADPH-diaphorase activity, which reduces the oxidized form NADP. These authors identified small numbers (about 2%) of sparsely distributed neurons with intense enzyme reactions in different parts of the brain, and these cells were termed "solitary active cells." However, interest in this enzyme arose again after publication of reports (especially those of Hope and Vincent – the authors of an improved version of the previously used method) that high levels of NADPH-diaphorase activity in particular neurons are accompanied by the expression of constitutive forms of NOS involved in the synthesis of one of the most important members of a new class of nerve spike transmitters – NO [11, 15, 16]. An explanation for this phenomenon was found. In some case, constitutive NOS was found not only to catalyze NO formation, but also to have NADPH-diaphorase activity, using the reduced form NADPH as a cofactor donating electrons on NO synthesis. It was then forgotten that the discussion was about "solitary cells" and "high NADPH-diaphorase activity" and these phenomena were applied to the whole population of NO-ergic neurons. NADPH-diaphorase came to be identified with constitutive NOS, especially nNOS, despite the fact that these are discriminated not only functionally and biochemically [8, 12, 17], but also in terms of their distribution at the cellular and subcellular levels [9, 10, 13, 20, 21]. It is not by chance that many cells with NADPH-diaphorase activity have been shown by double labeling to remain immunonegative with antiserum to nNOS, which was supported by data obtained from various animal species in studies of several parts of the brain and spinal cord and various other organs [10, 13, 17, 19, 21]. However, these undoubtedly interesting observations remained in doubt, as in most cased they were not supported by quantitative data.

With the aim of filling this gap, we undertook a comparative study of the topography and quantitative distribution of neurons detected by each of the methods described in the medulla oblongata nuclei in rats. Our data show that NO-ergic neurons in the brainstem are distributed extremely non-uniformly, though all nuclei showed, along with NADPH-diaphorase-positive neurons, neurons with positive reactions for nNOS. Significant differences in the locations and numbers of each of these two types of neuron were found in the nuclei studied here. Thus, the proportions of neurons with NADPH-diaphorase activity in the motor nuclei were always higher than those in the sensory and associative nuclei. In some nuclei (NA, NHN) containing numerous NADPH-diaphorase-positive neurons, nNOS-expressing cells were particularly rare. Use of IHC showed the reverse relationship: the contents of such neurons in sensory and associative nuclei (STN, RLN, SNTN, RMN) were higher than in the motor nuclei. In all cases, the HC method identified 1.5–3 times more cells in the corresponding nuclei than the IHC method. The cause of these differences remain to be explained, though it should be noted that the specificity of the IHC method for identifying the locations of nNOS has been demonstrated in many studies and is unchallengeable, such that it has undoubted advantages

over the HC method, for which the number of cells depends on more factors, for identification of NO-ergic neurons [3, 14–16].

Our comparative studies of the cellular composition of nuclei using parallel sections stained with methylene blue, as well as the HC and IHC studies, allowed a further difference to be identified in the distributions of the two neuron types. nNOS was found mainly in small cells, while NADPH-diaphorase was also seen in the quite numerous group of intermediate and large cells. In both cases, the ratios of small, intermediate, and large cells differed from the values determined using preparations stained with methylene blue. There is, however, another view on this point: that the ratio of small and large NADPH-diaphorase-positive neurons in the brain nuclei is entirely comparable with the numbers of these cells identified with methylene blue [4].

Another question is that of the processes of NO-positive neurons. HC studies consistently identify cells with numerous long or short processes. The existence of a well-developed system of sensory and efferent conductors with different levels of NADPH-diaphorase activity has repeatedly been noted in the brain and spinal cord and in vascular and intramural nerve plexuses by light and electron microscopy [2, 3, 6, 7, 12]. In contrast, the IHC method allows a limited number of processes to be detected, these being felt to be exclusively neuron dendrites [9, 10, 17].

Thus, despite some common features in the cellular localization of NADPH-diaphorase and nNOS in the medulla oblongata nuclei, there are clear differences in the topography and quantitative distribution of neurons expressing these two enzyme, generating the need for caution in evaluating results of studies of NO-ergic neurons using the HC method for NADPH-diaphorase.

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