

NADPH-Diaphorase Activity in the Hypothalamic Magnocellular Neurosecretory Nuclei of the Rat

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ARÉVALO, R., F. SÁNCHEZ, J. R. ALONSO, J. CARRETERO, R. VÁZQUEZ, AND J. AIJÓN. *NADPH-diaphorase activity in the hypothalamic magnocellular nuclei of the rat*. BRAIN RES BULL 28(4) 599–603, 1992.—A histochemical study of the distribution of NADPH diaphorase activity in the hypothalamus of normal rats was carried out. Our study demonstrates the presence of NADPH-diaphorase activity in the circularis and anterior and posterior fornical nuclei for the first time. Additionally, we confirm the presence of NADPH-diaphorase-stained neurons in the paraventricular (both magno- and parvicellular neurons) and supraoptic nuclei, as well as a population of isolated positive neurons (not included in any hypothalamic nuclei) located among the different nuclei. Because NADPH diaphorase has recently been shown to be a nitric oxide synthase, our study reveals a wide presence of this enzymatic activity in the hypothalamus of the rat.

NADPH-diaphorase Magnocellular nuclei Hypothalamus Rat

DIAPHORASE (NAD(P)H: dye oxidoreductase, EC 1.6.99) transfers reducing equivalents from NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) to various electron acceptors including tetrazolium dyes (13). It is supposed that diaphorases are widely employed cofactors involved in the electron transfer between NAD(P)H-dependent dehydrogenases and the electron-transport chain (8).

Biochemically, the term *diaphorase* is not used further, because it cannot be applied to a specific enzymatic activity. However, it has been retained in histochemical studies because using NADPH and the dye nitroblue tetrazolium it is possible to stain in fixed brain sections specific populations of neurons known as *NADPH-diaphorase-positive* cells. These neurons reduce nitroblue tetrazolium to an insoluble dark-blue formazan reaction product (6).

The interest of this histochemical technique has been enhanced because it was demonstrated that neuronal NADPH diaphorase is a nitric oxide synthase providing, therefore, a specific histochemical labeling for neurons producing nitric oxide throughout the brain (4).

The possible colocalization of NADPH-diaphorase (ND) activity and different neurotransmitters and neuroactive substances has been studied by several authors (18,23,24,29,34). Thus, it has been shown that ND-active neurons colocalize somatostatin in the cerebral cortex, striatum, and olfactory bulb (18,23,24,32);

neuropeptide Y in the cerebral cortex, striatum, and olfactory bulb (24,32); GABA in a small neuronal population in the retina (29), c-pon in the striatum (30); and acetylcholine in the pontine reticular formation, medial septal nucleus, diagonal band of Broca, and some neurons in the magnocellular preoptic nucleus (23,33). However, the overall distribution of ND activity in the brain with the exception of nitric oxide and citrulline (4) does not match that of any neuroactive substance hitherto described (18,19,22).

Presence of ND activity in the hypothalamic region has been described (14), where it has been proposed as a useful tool for the assessment of neuronal activity in selected neuronal populations. However, these ND-positive regions and populations have not been clearly established, because a detailed morphological study of ND staining in this region is still lacking.

The remarkable selectivity and Golgi-like quality of ND staining enables the morphological identification of neuronal types that have not previously been characterized (29). Additionally, these topographical studies on the distribution of ND-positive neurons are useful for neuropathological studies because these cells are selectively resistant to ischemia and excitatory neurotoxins (1,5) and, in some regions such as the striatum, they are selectively spared in Huntington's disease (2).

Thus, the aim of the present study is to establish clearly the exact location in the hypothalamic magnocellular nuclei of ND

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activity and to describe the characteristics of the ND-positive neurons in this region.

MATERIAL AND METHOD

Five adult female Wistar rats weighting 220–250 g were used for the present study. The animals were deeply anesthetized using ketamine (Ketolar: 50 mg/kg body weight), and perfused through the ascending aorta with 50 ml Ringer's solution followed by a fixative containing 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.3 (PB).

After 2 h, the brains were removed from the skull, the hypothalamic region was dissected out and postfixed at 4°C for a further 2 h in the same fixative. Thereafter, the blocks were rinsed 12 h in PB, transferred overnight to a 30% vol/vol sucrose solution in PB and frozen using liquid nitrogen.

Thirty-micrometer frontal sections were cut on a cryostat and collected in cold (4°C) PB. Free-floating sections were processed for the demonstration of NADPH-diaphorase activity as previously described (22). Briefly, the sections were incubated in a solution made up of 0.08% Triton x-100, 1 mM reduced- β NADPH, and 0.8 mM nitroblue tetrazolium in 0.1 M tris buffer pH 8, at 37°C for 1–3 h. All reagents were obtained from Sigma. The course of the reaction was controlled under the microscope.

After incubation, the sections were rinsed in PB, dehydrated, and mounted on gelatin-coated slides for examination by light microscopy.

Drawings of the distribution of positive neurons were performed by means of a camera lucida-equipped Leitz microscope with the aid of a low power planar lens. The exact location of the positive neurons was determined by means of Nissl-staining of adjacent sections and by phase-contrast microscopy.

RESULTS

Nomenclature

In the present study, the nomenclature and nuclear boundaries proposed by Peterson (11) and Rhodes et al. (12) were used. For the paraventricular nucleus (PVN), the subdivision proposed by Swanson and Kuypers (25) was followed. However, we considered both the anterior and medial magnocellular subdivisions of Swanson and Kuypers as one called commissural subdivision (according to 11 and 12) as described earlier (15–17). Although our study is focused on the magnocellular neurons, in the PVN, both well-known cellular types (magnocellular and parvicellular) were also considered.

Topographical Distribution

Paraventricular nucleus. Both neuronal types of the nucleus (magnocellular and parvicellular) showed positive labeling [Figs. 1 and 2(a–d)].

In the *magnocellular subdivisions*, most of the stained neurons were located in the posterior one, forming a dense cluster of ND-active neurons [Figs. 1 and 2(a, c)]. A few stained cells were seen in the commissural subdivision [Figs. 1 and 2(b)].

In the *parvicellular subdivisions*, the neurons were predominantly located close to the wall of the third ventricle, in the anterior part of the periventricular subdivision (at the level of the commissural magnocellular subdivision) [Figs. 1 and 2(b)]. In the latter, the number of stained neurons was higher in the dorsal part of the ventricle than in the ventral one (Fig. 1).

Positive neurons were also located in the anterior and medial parvicellular subdivisions. However, the number of these neurons was very scarce, specially in the medial subdivision [Figs. 1 and 2(a, d)].

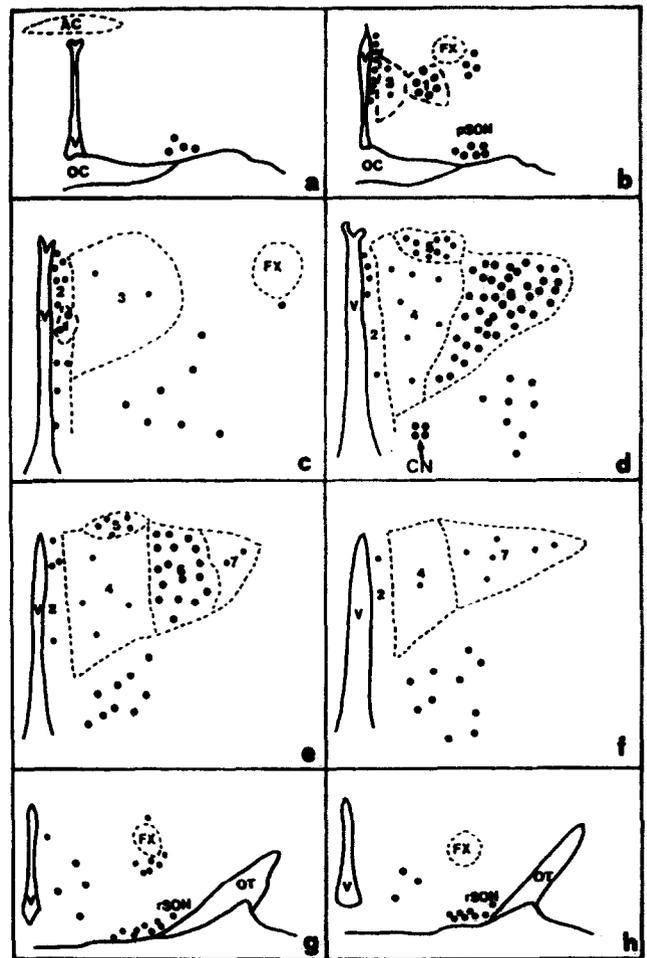


FIG. 1. NADPH-diaphorase activity. Drawings of frontal sections from rostral (a) to caudal (h) planes [(c)–(f) have been exclusively focused on the PVN], showing the distribution of NADPH-diaphorase-stained neurons. AC: anterior commissure; CN: circularis nucleus; FX: fornix; OC: optic chiasma; OT: optic tract; pSON: prechiasmatic supraoptic nucleus; rSON: retrochiasmatic supraoptic nucleus; V: third ventricle; 1: commissural magnocellular subdivision; 2: periventricular parvicellular subdivision; 3: anterior parvicellular subdivision; 4: medial parvicellular subdivision; 5: Dorsal parvicellular subdivision; 6: posterior magnocellular subdivision; 7: lateral parvicellular subdivision.

In the dorsal and lateral subdivisions, some labeled neurons were seen. In the dorsal one, they formed a small cluster located close to the dorsal part of the third ventricle [Figs. 1 and 2(c)]. In the lateral subdivision, only a few labeled neurons were seen [Figs. 1 and 2(d)].

Supraoptic nucleus. In this nucleus, reactive neurons located in both subdivisions were seen [Figs. 1 and 3(a, b)].

In the *prechiasmatic subdivision*, all the extension was occupied by ND-active neurons. However, the number of stained neurons was scarce when compared to those observed in the magnocellular subdivisions of the PVN [Figs. 1 and 3(a)]. It was possible to observe strongly stained neurons intermingled with very weakly stained ones without a specific distribution of both neuronal types into the subdivision [Fig. 3(a)].

In the *retrochiasmatic subdivision*, some well-stained reactive neurons were observed [Figs. 1 and 3(b)]. They were characteristically grouped forming parallel rows of stained cell bodies



FIG. 2. NADPH-diaphorase activity in the paraventricular nucleus. (a) Large population of stained neurons located in the posterior magnocellular subdivision. Some isolated active neurons can be seen in the medial parvicellular subdivision (arrows) and in the dorsal part of the periventricular parvicellular subdivision (arrowheads), $\times 58$. (b) Strongly stained neurons located in the commissural subdivision (arrows) and in the periventricular parvicellular subdivision (arrowheads), $\times 116$. (c) At the level of the posterior magnocellular subdivision (arrows), note the presence of stained neurons located in the dorsal parvicellular subdivision (arrowheads), $\times 58$. (d) In the most posterior part of the PVN, some stained neurons were located in the lateral parvicellular subdivision (arrows). Only a few stained cells were seen in the medial and periventricular parvicellular subdivisions (arrowheads), $\times 58$. V: third ventricle.

(without stained processes) located close to the pial limit of the hypothalamus [Figs. 1 and 3(b)].

Accessory nuclei. In the *circularis nucleus*, stained neurons forming a small cluster of six to seven positive somata were observed [Figs. 1 and 3(c)].

In the anterior and posterior, *fornicals nuclei*, positive neurons were found with the same morphological characteristics as those observed in the magnocellular neurons of the PVN and SON [Figs. 1 and 3(d)].

Finally, some stained neurons were seen in the hypothalamic area between the prechiasmatic subdivision of the SON and the PVN [Figs. 1 and 3(e, f)].

Neuronal Characteristics

In general, the morphological characteristics observed for the reactive neurons located in the magnocellular subdivisions of the PVN, the SON, and the accessory nuclei were quite superimposable.

These neurons showed a strong intensity of reaction (with the exception of some weakly stained neurons located in the prechiasmatic subdivision of the SON) located in the cell body and proximal dendrites. They showed different shapes, mostly rounded. Sometimes it was possible to see one or two well-stained processes (Figs. 2 and 3).

The morphological characteristics observed in the neurons located in parvicellular subdivision of the PVN were very similar to those observed in the previously mentioned neurons [Fig. 2(c, d)].

The isolated neurons located in the hypothalamic area between the SON and the PVN displayed a strong intensity of reaction. The presence of a higher number of long stained processes was noticed. The shapes of these neurons were predominantly pyramidal or oval [Fig. 3(e, f)].

DISCUSSION

NADPH-diaphorase histochemistry stains subpopulations of nerve cells in widely distributed brain regions including, among others, striatum, olfactory bulb, substantia innominata, posterior pituitary, pontine reticular formation, cortex, and several brain-stem nuclei (3,13,14,18,19).

In the hypothalamus, presence of ND activity has been reported (14,35). These authors describe only positive neurons located in the PVN (both in the magno- and parvicellular subdivisions) and in the SON.

In our analysis, we confirmed the presence of those earlier described positive neurons in some magnocellular nuclei such

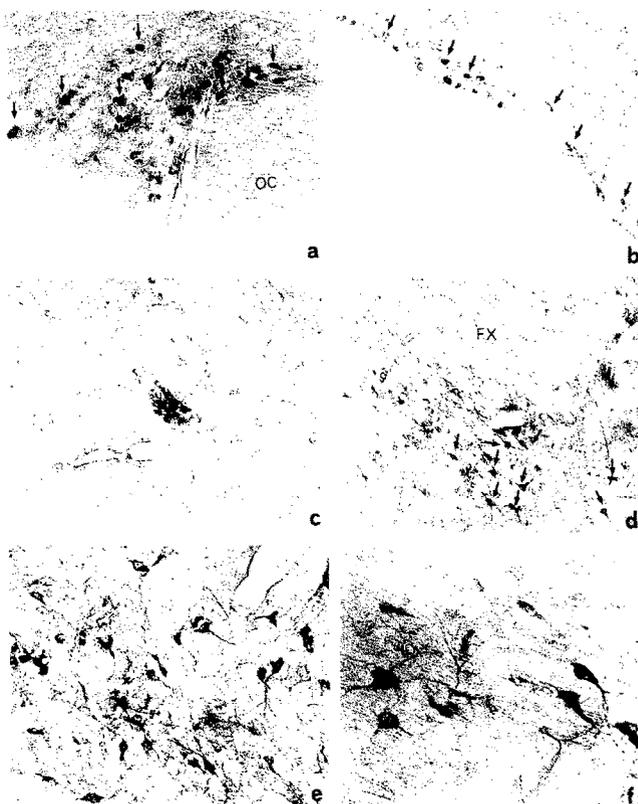


FIG. 3. NADPH-diaphorase activity in the supraoptic nuclei and in the magnocellular accessory nuclei. (a) A few rounded neurons were strongly reactive in the prechiasmatic subdivision of the SON (arrows), $\times 59$. (b) Rounded stained neurons can be seen in the retrochiasmatic subdivision of the SON (arrows), $\times 59$. (c) A small cluster of intensely reactive neurons were located at the level of the circularis nucleus, $\times 59$. (d) In the anterior fornical nucleus, some stained neurons were present (arrows), $\times 59$. (e and f) Throughout the hypothalamic area between the SON and the PVN a lot of stained neurons with various processes were seen: (e) $\times 118$; (f) $\times 236$. OC: optic chiasma. FX: fornix.

as the PVN (both magnocellular and parvocellular cellular types) and the SON (17). Additionally, our study shows ND-positive neurons in some magnocellular accessory nuclei such as the CN and the anterior and posterior fornical nuclei. However, other accessory nuclei such as the medial forebrain bundle nucleus did not display this activity.

Although it is possible to find some isolated positive neurons, especially located in the hypothalamic area between the prechiasmatic subdivision of the SON and the PVN, it is more characteristic of the hypothalamic ND staining that the positive neurons formed very dense clusters, contrary to which it has been observed in other regions. Therefore, the term *solitary active cells* (27,28) frequently used to define the ND-positive cells is not valid in the hypothalamus.

Comparing the distribution of ND staining with that of transmitters and neuroactive substances located in the hypothalamic nuclei, only vasopressin and oxytocin were present in all the nuclei and subdivisions in which ND activity appeared (12,15–17, among others). However, the necessity to use colchicine or adrenalectomy to show the vasopressin-parvocellular reacting neurons is well-known (15–17,21, among others). Thus, this simple histochemical method may be a useful way to visualize specific populations of neurons in this region.

Other peptides and substances described in the rat hypothalamus (CRF, SRIF, VIP, GABA, neuropeptide Y, among

others) (7,9,10,17,21,26, among others) were much more restricted to some nuclei or within the PVN to some subdivisions. Although it has been shown in earlier studies that ND may be a selective marker for neurons containing both somatostatin and neuropeptide Y in other brain areas (31,32), and also based on previous research, including some carried out at our laboratory (17), the more restricted neuronal topography of these two peptides in the hypothalamus does not agree with the wide distribution of ND activity. Detailed studies about the coexistence not only of these two peptides (somatostatin and neuropeptide Y) but also vasopressin and oxytocin with NADPH activity should be carried out to clarify the presence of a partial coexistence.

Finally, it has been recently demonstrated that ND-diaphorase is a neuronal nitric oxide synthase (4). Thus, this histochemical technique allows the cellular location of nitric oxide synthase, showing a wide presence of such oxide in the neurons of the hypothalamic nuclei described in the present study.

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