NADPH-DIAPHORASE ACTIVITY IN THE RAT HYPOTHALAMO-
NEUROHYPOPHYSIAL SYSTEM AFTER SALT LOADING AND REHYDRATION

JUAN L. BLÁZQUEZ, BELÉN PEÑA, FRANCISCO E. PASTOR, ROSA M. LÓPEZ and PEDRO AMAT
Departamento de Anatomía e Histología Humanas, Universidad de Salamanca, 37007 Salamanca, Spain

ABSTRACT
In this work we investigated the histochemical NADPH-diaphorase activity of the rat hypothalamo-magnocellular neurons (with special attention to the nucleus circularis) and of the fibers of hypothalamo-neurohypophysial tract at the level of the median eminence after 5 days of salt loading and salt loading plus rehydration. In all hypothalamo-magnocellular nuclei examined, salt loading increased neuronal NADPH-diaphorase staining, but when the response of the neurons of the nucleus circularis and those of the supraoptic (SON) and paraventricular nuclei (PVN) were compared, nearly all the neurons present in the nucleus circularis were strongly stained whereas in the SON and PVN not all neurons respond to hyperosmotic stimulus, some unresponsive neurons also being observed. In the internal zone of the median eminence some fibers were intensely stained after salt loading but we never observed reactive fibers in the external zone. Rehydration after salt loading reversed the increase in histochemical labelling to the intensity observed in intact animals in all magnocellular neurons. However in the median eminence staining was still stronger after 48 h of rehydration than in untreated rats.

Nitric oxide is rapidly emerging as a major and ubiquitous modulator of a variety of physiological functions (18, 23, 26). The localization of nitric oxide synthase in neurons throughout the brain has been greatly facilitated by the histochemistry of NADPH-diaphorase because, in rat brain, this marker has been identified as largely being due to nitric oxide synthase (3, 9).

In the hypothalamus, NADPH-diaphorase labelling is present in the perikarya of several nuclei, among which are the magnocellular nuclei (1, 2, 20, 22, 30), as well as in the median eminence and the posterior pituitary itself (4, 20). In the rat supraoptic nucleus (SON), NADPH-diaphorase staining is activity-dependent (19) and nitric oxide synthase gene expression in the SON and paraventricular nucleus (PVN) is increased during hyperosmotic stimulation (12). In these nuclei the coexistence of NADPH-diaphorase and vasopressin (5) or oxytocin (17) has been reported. The presence of NADPH-diaphorase neurons has also been demonstrated in several accessory magnocellular nuclei, especially in the nucleus circularis and the nucleus fornicallis (1, 2), and the co-localization in some of these neurons of diaphorase and vasopressin or oxytocin has been shown (21). Nevertheless, in these reports there is no information about experimental modifications of NADPH-diaphorase activity in accessory magnocellular nuclei, particularly in the nucleus circularis, which for years has been considered as a hypothalamic osmoreceptor (8).

In the present work we carried out a histochemical NADPH-diaphorase study of the rat hypothalamic magnocellular neurons (focusing our attention on the response of the nucleus circularis), as well as of the fibers of the hypothalamo-neurohypophysial tract at the level of median eminence, after salt loading and rehydration.

MATERIALS AND METHODS
Eighteen male adult Sprague-Dawley rats (300-350 g) were housed from birth under controlled
Figs. 1 and 2  NADPH-diaphorase histochemistry. Nucleus circularis of salt-loaded rats. Virtually all the magnocellular neurons show a strong labelling. In both cases, note a bundle of fibers emerging dorsally. Bar, 100 μm

Figs. 3 and 4  Nucleus circularis of an intact (Fig. 3) and a rehydrated (Fig. 4) rat. Only some neurons display marker. The intensity of the reaction is variable. Bar, 100 μm

Fig. 5  (a) PVN of a salt-loaded animal. The intensity of reaction and the number of neurons labelled are greatly increased compared to (b), the PVN of a rehydrated rat. Bar, 200 μm
light/dark (12L:12D) and temperature conditions. One group (6 rats) received food and water ad libitum; the other 12 animals received food and a 2% (w/v) NaCl solution as the only source of water over 5 days. Of these animals, 6 were sacrificed after 5 days and the other six were rehydrated for 48 h. The experimental procedures were designed in such a manner that all rats were sacrificed on the same day and histochemically processed at the same time.

Under intraperitoneally-administered sodium thiopental anaesthesia (45 mg/kg body weight), all the animals were perfused through the left ventricle with a fixative solution of 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C, following a wash of the vascular tree with 150 mM NaCl. After perfusion, the hypothalami were dissected out and postfixed at 4°C for 24 h in the same fixative. On a vibratome, 40 μm frontal sections were cut and collected in cold 0.1 M phosphate buffer, pH 7.2. Free-floating sections were incubated to demonstrate NADPH-diaphorase activity in a solution composed of 0.5 ml Triton X-100, 1 mM β-NADPH, 0.8 mM nitroblue tetrazolium, and 8 mM sodium malate in 0.1 M Tris-HCl buffer, pH 8, at 37°C for 45 min under stirring conditions. All reagents were obtained from Sigma. The course of the reaction was controlled under the microscope and terminated by triple washing in phosphate buffer. The sections were then mounted on gelatin-coated slides, air-dried overnight at room temperature, dehydrated through graded alcohols and xylene, and coverslipped with DePeX.

RESULTS

In all hypothalamic-magnocellular nuclei examined in the present study, long-term (5 days) salt loading increased neuronal NADPH-diaphorase staining, whereas 48 h of rehydration after salt loading reversed the histochemical labelling to the intensity observed in intact animals. Thus, in salt-loaded rats, NADPH-diaphorase staining was always very intense in the nucleus circularis and was observed in nearly all the neurons (Figs. 1 and 2). Accordingly, in 40 μm-thick coronal sections, the nucleus circularis appeared as a compact mass in which it was difficult to distinguish the neurons comprising it (Fig. 2). By contrast, in the untreated animals (Fig. 3) and in those rehydrated for 48 h after 5 days of salt overload (Fig. 4), the number of stained neurons and the staining intensity were substantially decreased compared to the dehydrated animals. No differences were seen in the number of stained neurons or in the intensity of the reaction between the untreated animals and those that were rehydrated (Figs. 3 and 4). In salt-loaded rats, a striking observation was the existence of a bundle of fibers emerging dorsally from the nucleus circularis (Figs. 1 and 2).

In salt-loaded animals, the neurons in the PVN and SON also showed a stronger reaction (Figs. 5a and 6a) than that observed in the same nuclei in the rehydrated rats (Figs. 5b and 6b). There were no remarkable differences in the number of labelled neurons or in the staining intensity between rehydrated and intact rats (data not shown). In the PVN, salt loading increased the number of stained neurons considerably compared to the numbers observed in intact animals and after rehydration (Fig. 5, a and b). In the SON of intact and rehydrated rats, the number of weakly stained neurons was high (Fig. 6b) and only some of them seemed to display a more marked degree of labelling after the salt overload (Fig. 6a).

In the internal zone of the median eminence of the dehydrated animals an intense point-like labelling was seen, which must correspond to the fibers of the hypothalamo-neurohypophysial bundle (Fig. 7). The staining intensity clearly decreased by 48 h after rehydration (Fig. 8), but this was still stronger than what was observed in the untreated animals (Fig. 9).

DISCUSSION

In the present work we studied NADPH-diaphorase staining in several structures of the hypothalamo-neurohypophysial system after 5 days of salt loading and after salt loading plus 48 h of rehydration, paying special attention to the response of the nucleus circularis, an accessory magnocellular nucleus. For years, this nucleus has been considered as a hypothalamic osmoreceptor (8) on the basis of its cytoarchitecture and its response to dehydration/rehydration (8, 13), and therefore a differential reactivity of these neurons to hypertonic drinking water would be expected.

Chronic salt loading is a potent stimulus to the hypothalamo-neurohypophysial system and produces a marked depletion of both vasopressin and oxytocin from the rat neurohypophysis (10). At the same time, the synthesis of both hormones is increased; i.e. an increase in the level of both vasopressin and oxytocin mRNA in the hypothalamus.
occurs (7, 14, 16, 28) and an up-regulated synthesis of other neuropeptides present in the hypothalamo-neurohypophysial system is induced; these include cholecystokinin, dynorphin, Leu-enkephalin, corticotropin-releasing factor and galanin (14, 16, 24, 25, 31). More recently, the up-regulation of nitric oxide synthase gene expression in the neurons of the PVN and SON following chronic osmotic stimulation has been reported (12, 29).

With respect to NADPH-diaphorase staining, our results are in agreement with previous works reporting increases in NADPH-diaphorase activity and in the number of stained neurons in the SON (12, 19), PVN (12) together with a stronger degree of staining in the neural lobe (12, 20) after chronic salt loading. Additionally, our observations are consistent with the up-regulation of nitric oxide synthase gene expression observed in the hypothalamo-neurohypophysial system of salt-loaded rats (12, 29). Nevertheless, the present findings are also in partial disagreement with those reported by other authors (6) who, at the light microscopy level, found no noticeable changes in the appearance or distribution of stained cells in the SON of dehydrated rats, although they did observe an increase in stained mitochondria and a more prominent staining of the endoplasmic membranes at the electron microscopy level.

NADPH-diaphorase-stained fibers in the median eminence have not been mentioned generally in the literature. In normal male Long Evans rats, deposits of reaction product in both internal and external palisade zones have been reported (20). The latter results are in disagreement with our observations in that we never observed stained fibers in the external zone of the median eminence, the deposits of reaction apparently being limited to the hypothalamo-neurohypophysial tract in the internal zone. With the data available, it is difficult to offer an explanation for such discrepancy; it could be due to technical differences in the histochemical method used (fixation) or to the difference in reactivity between Long Evans and Sprague Dawley rats of the parvocellular neurons in the PVN and of the axons that, from there, reach the external layer of the median eminence. However, stained fibers were scarce in untreated rats as compared with salt-loaded animals.

Comparison of NADPH-diaphorase staining between the magnocellular neurons in the nucleus circularis and those in the PVN and SON after salt loading revealed that there was strong reactivity in the nucleus circularis, whereas in the SON and PVN not all neurons respond to hyperosmotic stimulus, some unresponsive neurons being also present. This observation may indicate that the neurons of the nucleus circularis would be more sensitive to hyperosmotic stimuli than the neurons in the SON and PVN.

None of the authors mentioned above directed their attention to the recovery process of the hypothalamo-neurohypophysial system after rehydration in respect of NADPH-diaphorase staining. In the magnocellular neurons of the SON, PVN and nucleus circularis, no differences were noted between the untreated and salt-loaded-48 h rehydrated rats, either in the number of neurons stained or in the intensity of the staining. However, in the median eminence, the staining was still stronger after rehydration than in the untreated animals, although less pronounced than in the salt-loaded rats. These observations suggest that the recovery of the hypothalamo-neurohypophysial system after rehydration occurs earlier in the magnocellular nuclei than in the axons of the hypothalamo-neurohypophysial tract at the level of the median eminence; we did not examine the enzymatic activity in the posterior lobe of the hypophysis.

The significance of this enzymatic activity and of its increase in the hypothalamo-neurohypophysial system after hyperosmotic stimuli, remains controversial. Some authors have reported that, in vivo and under conditions of dehydration, nitric oxide attenuates the secretion of oxytocin to promote the preferential release of vasopressin (27) and stimulates drinking behaviour to favour rehydration (11). Additionally, in vitro an inhibitory effect of nitric oxide on the secretion of vasopressin and oxytocin from the neural lobe has been described (15). Moreover, certain authors have suggested that nitric oxide may be released concomitantly with the hormones of the neural lobe and may therefore participate in the increase in blood flow through the neural lobe (see 29).

Fig. 6 (a) SON of a salt-loaded rat. One population of neurons is strongly stained but others show weaker staining. (b) SON of a rehydrated animal. Most of the neurons show only a weak reaction. Bar, 200 μm
Figs. 7–9 Median eminence of salt-loaded (Fig. 7), rehydrated (Fig. 8) and intact (Fig. 9) rats. The intensity of staining decreases after rehydration but is still stronger than in intact animals. Bar, 100 μm

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