Neuroscience Letters, 77 (1987) 137–142
Elsevier Scientific Publishers Ireland Ltd.

NSL 04626

En bloc immunohistochemistry reveals extensive distribution of histidine decarboxylase-immunoreactive neurons on the ventral surface of the rat hypothalamus

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(Received 27 January 1987; Revised version received and accepted 23 February 1987)

Key words: Histamine; Histidine decarboxylase; Tuberomammillary nucleus; Immunohistochemistry; Cerebrospinal fluid; Rat

En bloc immunohistochemistry was used to examine the distribution of L-histidine decarboxylase (HDC)-immunoreactive neurons on the ventral surface of the rat hypothalamus. Following standard paraformaldehyde fixation, the ventral hypothalamus containing the tuber cinereum was carefully dissected free and incubated en bloc with antisera to HDC followed by standard avidin–biotin complex immunohistochemistry. Microscopic examination of these en block preparations revealed the existence of an extensive plexus of HDC-immunoreactive neurons located directly upon the ventral hypothalamic surface. HDC-immunoreactive neurons were largely restricted to the caudal half of the hypothalamic surface, and were multipolar, with 2–5 dendrites radiating in all directions from the soma. The proximity of these neurons to the ventral surface of the brain suggests that histaminergic neurons might be capable of responding to cerebrospinal fluid borne substances. Thus, one form of input to hypothalamic histaminergic neurons may be humoral rather than synaptic.

The tuberomammillary (TM) nuclei of the hypothalamus contain the cells of origin of the central histaminergic system as demonstrated by antibodies against both histamine [10, 15] and its synthetic enzyme L-histidine decarboxylase (HDC) [11, 21]. The intimate relationship between these putative histaminergic neurons and the cerebrospinal fluid (CSF)-containing spaces of the brain is suggested by several observations. The cell bodies themselves are clustered near CSF-containing spaces including both the mammillary recess and the ventral surface of the hypothalamus. Furthermore, using a newly developed coupled intracellular horseradish peroxidase–monoamine oxidase technique, we have recently demonstrated that TM neurons located well

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within the parenchyma of the brain extend their dendrites to the ependymal surface [17].

While these data are suggestive, a more direct approach is afforded by the use of en bloc immunohistochemistry. This technique, which involves incubation of large blocks of tissue in antibody followed by standard immunohistochemical procedures, has recently been used to visualize serotonin-immunoreactive neurons on the ventral surface of the medulla [3]. In the present study, a similar en bloc immunohistochemical approach has been used to demonstrate the existence of an extensive plexus of HDC-immunoreactive neurons on the ventral surface of the hypothalamus.

Ten adult male Wistar rats (200–300 g) were deeply anesthetized with chloral hydrate (600 mg/kg, i.p.) and perfused transcardially with 100 ml of room temperature phosphate-buffered saline (10 mM, pH 7.4) followed by 300 ml of chilled 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed from the skull and the ventral hypothalamus containing the tuber cinereum dissected free. This en bloc preparation was then washed for several hours in Tris-buffered saline (TBS, 50 mM, pH 7.4) at 4°C. The pia and associated vasculature were then carefully stripped from the underlying tissue using a dissecting microscope, taking care to keep the brain surface moist with TBS.

The en bloc preparation was then subjected to standard HDC immunohistochemistry using a polyclonal antibody whose specificity has been previously documented [20]. The primary incubation was made at a dilution of 1:10,000 in TBS with 0.3% Triton-X and 2% normal goat serum for 48 h at 4°C. This incubation was followed by a standard immunohistochemical protocol using the avidin–biotinylated horseradish peroxidase technique of Hsu et al. [5]. The tissue was washed 3×20 min in TBS, incubated in 0.5% biotinylated goat anti-rabbit (Vector Labs) with 0.3% Triton-X and 2% normal goat serum in TBS for 1 h at room temperature. Following another 3×20 min wash TBS, the block of tisue was incubated in 1% avidin–biotinylated horseradish peroxidase complex (Vector) in TBS with 2% normal goat serum. Following a final 3×20 min in wash in TBS, the tissue was pre-incubated in 50 mM Tris-buffer containing 0.025% 3-3′-diaminobenzidine and 1 mM imidazole for 10 min, followed by addition of 0.0075% H_2O_2. The reaction was permitted to proceed for 10 min and terminated by transfer to TBS. Clearing of the tissue was accomplished by immersion in 100% dimethyl sulfoxide [3a] for 4 h, followed by 1 h each in 100% ethanol and xylene. The en bloc preparation was then placed in a custom made well on a microscope slide, embedded in permount, and dried for several days in a warm (75°C) oven. Immunopositive neurons were observed and photographed on an Olympus BH-2 microscope.

In order to assess the degree of penetration of antiserum into the tissue, in selected cases the tissue was placed overnight in cryoprotectant (25% sucrose, 10% glycerol in 5 mM Tris-buffer) at 4°C, and then cut coronally on a freezing microtome at 30 μm thickness, mounted on slides, dehydrated and coverslipped with permount.

Examination of en bloc preparations under low power revealed an extensive plexus of HDC-immunoreactive neurons on the caudal half of the ventral hypothalamic surface (Fig. 1A). HDC-immunoreactive neurons covered much of the caudal tuber

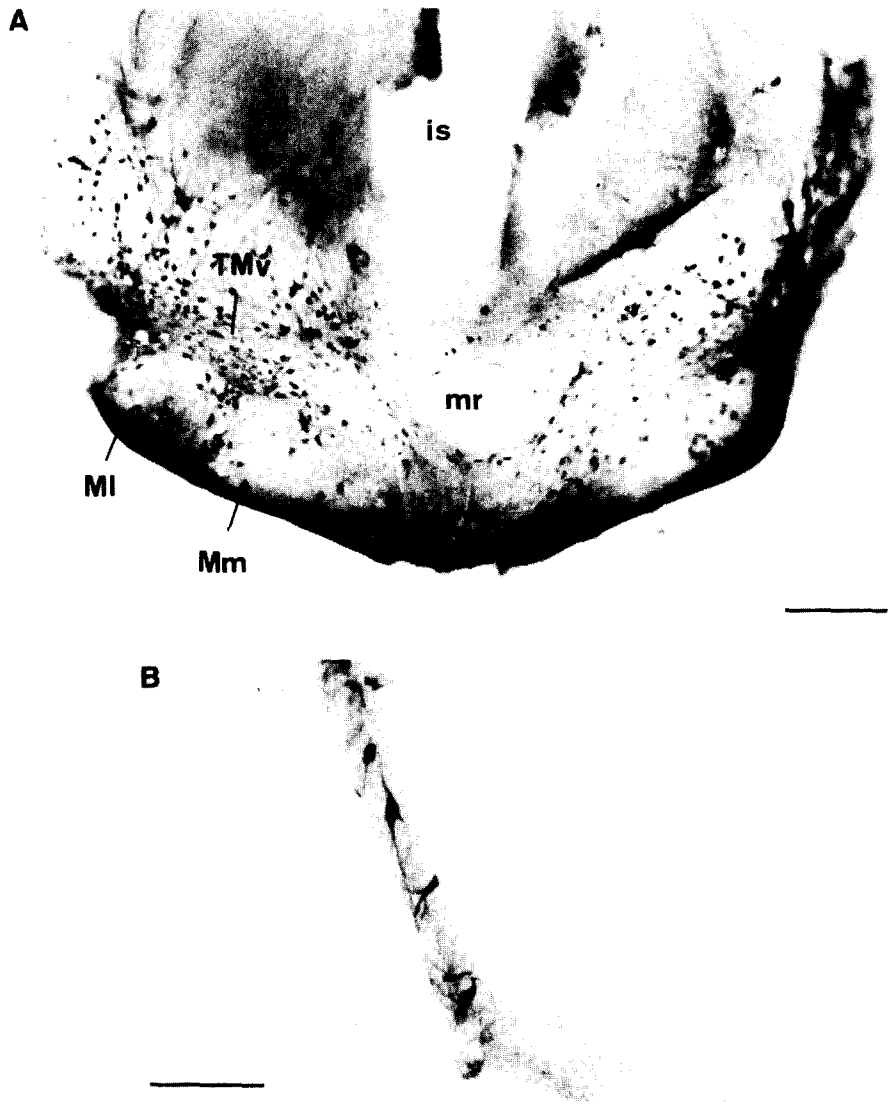

Fig. 1. A: view of the ventral hypothalamus stained for en bloc HDC immunohistochemistry. HDC-immunoreactive neurons can be seen distributed over much of the caudal tuber cinereum, with a particularly dense cluster evident overlying the ventral division of the tuberomammillary nucleus (TMv). Note the absence of HDC-immunoreactive neurons over the medial (Mm) and lateral (Ml) subdivisions of the lateral mammillary nucleus. mr, mammillary recess; is, infundibular stalk. Bar = 600 μm. B: coronal section (30 μm thickness) of the tuberomammillary nucleus previously stained using the en bloc HDC immunohistochemical protocol. Immunopositive neurons are seen only at the extreme edge of the tissue section. Bar = 100 μm.

cinereum and, especially laterally, extended rostrally along the ventral surface of the hypothalamus. A particularly dense cluster could be seen at the rostral edge of the confluence of the medial and lateral subdivisions of the lateral mammillary nucleus,

apparently representing the ventral division of the TM nucleus as defined by Köhler et al. [6]. Close examination of the surface of the tuber cinereum revealed that there were areas in which HDC-immunoreactive neurons were conspicuously absent, in particular overlying the lateral and medial subdivisions of the lateral mammillary nucleus, as well as the posterior division of the medial mammillary nucleus. However, cell bridges were evident connecting the cluster of HDC-immunoreactive neurons in the ventral TM with those located in the postmammillary caudal magnocellular nucleus of Bleier et al. [1]. The distribution of these latter neurons is not shown because of the extreme difficulty of photographing neurons on such an oblique surface. However, here too HDC immunoreactive neurons were extensively distributed on the surface of the brain.

HDC-immunoreactive neurons as seen in en bloc preparations were multipolar, with 2–5 dendrites radiating in all directions from the soma. Indeed, over much of the caudal tuber cinereum, the dendrites of HDC-immunoreactive neurons formed a lattice work, with the dendrites of adjacent neurons frequently overlapping each other. The morphology of HDC-immunoreactive neurons in these en bloc preparations was strikingly similar to that seen in standard immunohistochemical material sectioned in either coronal, sagittal or horizontal planes [4, 6, 14, 22].

The protocol employed herein stained only neurons located within approximately 40–50 μm of the ventral hypothalamic surface as evidenced by staining tissue according to the en bloc protocol followed by coronal sectioning at 30 μm (Fig. 1B). This value must be taken as an approximation only, since a gradient of staining existed, with neurons at the edge of the hypothalamus staining more intensely than those located some distance within the hypothalamic parenchyma, even when heavy metal intensification of the diaminobenzidine reaction product was employed. It is not known if the very lightly staining cells furthest from the ventral surface can be seen when viewed from the surface of the en bloc preparation.

These data extend previous studies using tissue sections in which the intimate relationship of TM neurons to the ventral surface of the hypothalamus has been noted [6, 8]. In their ultrastructural studies, Maeda et al. [8] reported that processes of TM neurons invade the glia limitans, just below the pial surface. The present results not only reveal that HDC-immunoreactive neurons on the ventral surface of the hypothalamus are far more extensively distributed than had previously been suspected, but also provide details of their topography in relation to major hypothalamic landmarks.

Anatomical [6, 16, 18, 19], biochemical [2] and physiological [12] studies have repeatedly shown that TM neurons send their axons throughout the central nervous sytem, including direct projection to the cerebral cortex. It is not known if HDC-immunoreactive neurons located in the extreme ventral positions described herein project widely through the brain as well, but examination of the data of Köhler et al. [6] suggests that this may indeed be the case.

The functional significance of these findings relates largely to the potential for HDC-immunoreactive neurons to extrude substances into the CSF as well as to be responsive to neuroactive substances carried by the CSF. The location of the tuber

cinereum at the rostral end of the interpeduncular fossa suggests that the latter possibility may be particularly relevant. Since CSF flows out of the fourth ventricle through the foramina of Luschka, down around the convexities of the brainstem and from there rostrally along the ventral surface of the brain towards the interpeducular fossa, any CSF-sensing function of these neurons is likely to be an end-stage rather than en passant phenomenon.

The notion that TM histaminergic neurons might be capable of sensing CSF-borne substances dovetails nicely with their purported role in control of behavioral state [7, 9, 13, 19, 21]. It has long been suspected that states of consciousness, which wax and wane over exceedingly long periods of time, might be controlled by humoral factors of unknown origin. The present data suggest that TM histaminergic neurons, with their proximity to the CSF-containing spaces of the brain and their extensive projections throughout the central nervous system, might be the anatomical substrate of such a phenomenon.

We thank Dr. E.G. McGeer for her generous provision of laboratory facilities, and for a critical reading of the manuscript. Supported by the Medical Research Council. P.B.R. is a Fellow of the MRC.

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