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Coexistence of vasopressin, neurophysin and noradrenaline immunoreactivity in medium-sized cells of the locus coeruleus and subcoeruleus in the rat

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Vasopressin- and neurophysin-immunoreactive cells have recently been demonstrated in the rat locus coeruleus (A6) and subcoeruleus (A7). Using consecutive 5 μ m thick frozen sections, medium-sized cells throughout the locus coeruleus area, but predominantly in the posterior parts of the A6 displayed coexistence for vasopressin and noradrenaline or neurophysin and noradrenaline immunoreactivity. The putative projection areas of putative fibers from vasopressin-containing cells in the locus coeruleus still remain to be elucidated.

In addition to the previously known hypothalamic paraventricular (PVN), supraoptic (SON) and supra-chiasmatic (SCN)^{2,18} nuclei, vasopressin (VP)- and neurophysin-immunoreactive cells have recently been demonstrated in the bed nucleus of the stria terminalis (BST), medial amygdala (AME), dorsomedial hypothalamus (DMH) and locus coeruleus (LC; A6 and A7) of the rat pretreated with colchicine^{3,17,21}. Since the LC is believed to consist primarily of noradrenergic cells^{1,19} it was investigated whether VP and its associated neurophysin coexist with noradrenaline (NA) in this brain structure. Therefore, consecutive cryostat sections of male Wistar rats, weighing 180–200 g, were stained with either anti-VP, anti-neurophysin or anti-NA. As a control, sections of male Brattleboro rats, weighing 250–300 g, homozygous for diabetes insipidus, which lack the ability to express VP and neurophysin immunoreactivity, were stained in an identical manner.

In animals, obtained from Centraal Proefdielenbedrijf (Zeist, The Netherlands), anesthetized with Hypnorm (Philips Duphar, Amsterdam, The Netherlands), 35 μ g colchicine dissolved in 20 μ l saline was injected into the lateral ventricle. After a survival time of 48 h, each animal was perfused at

high speed (200 ml/min) with 500 ml 5% glutaraldehyde in 0.05 M sodium cacodylate containing 1% Na₂S₂O₅, pH 4.0. Brains were dissected, sliced and immersed overnight in 0.05 M Tris-NaCl buffer pH 7.0, with 1% Na₂S₂O₅ containing 15% sucrose. Na₂S₂O₅ was added in order to inhibit oxidation of noradrenaline. Subsequently, specimens were frozen in liquid nitrogen and 5 μ m cryostat transversal sections were made and collected on chrome-alum gelatin-coated glass slides. Consecutive sections were incubated overnight with the following antisera: (a) Anti-arginine VP (batch W1; diluted 1:1000). This antiserum has characteristics similar to the previously used batches 125 or 126. (b) Anti-rat neurophysin (batch R4, 7/19/73, diluted 1:3000). (c) Anti-NA (batch W3/30-9, diluted 1:750). The NA antiserum was raised according to the procedure of Geffard et al.¹¹ i.e., NA-free base was conjugated by means of glutaraldehyde to bovine serum albumin and subsequently injected into rabbits. This resulted in an antibody sharing slight cross-reactivity with dopamine (unpublished results). Prior to incubation with VP or neurophysin antiserum, sections were treated with Tris-NaCl buffer pH 7.8 containing 0.025% trypsin (Sigma; batch 113F-8130; 12,400 BAEE units/mg protein; BAEE = Sodium Benzoyl 1-Arginine Ethyl

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Ester) at room temperature for 10 min⁹. Prior to incubation with NA antiserum, sections were treated with 0.05 M Tris-NaCl buffer pH 7.6 containing 1% sodiumborohydride for 5 min to saturate the double bonds in the conjugated noradrenaline molecule formed during fixation with glutaraldehyde. Immunocytochemical staining was performed in accordance with the peroxidase-antiperoxidase (PAP) method using 3, 3'-diaminobenzidine as a chromogen. Subsequently slides were rinsed, dehydrated in a graded ethanol series and embedded in Entellan.

In sections of the Wistar rat brain, cells in the LC displaying VP- or neurophysin immunoreactivity in addition to NA immunoreactivity were observed (Figs. 1A, 2A). The trypsin treatment was essential for a clear peptide immunoreactivity in the cryostat sections. VP- or neurophysin-immunoreactive cells also exhibiting NA immunoreactivity were 20–25 μ m in diameter, had multi- or bipolar processes, possessed large nuclei relative to the cytoplasm

and appeared primarily in the caudal part of this nucleus. Because of the diffuse pattern of the NA staining it is unclear whether different cell types were stained. The cells showing coexistence of VP or neurophysin and noradrenaline appeared mostly in the compact A6 region (Fig. 1A, B), but also in the more anterior LC (Fig. 2A, B) and in the subcoerulear area (A7 region). They were first visible approximately at the level of -9.3 mm relative to bregma according to Paxinos and Watson¹⁶ and were traceable to the most caudal part of the LC. In the double-stained cell region of the LC all VP- or neurophysin-immunoreactive neurons also appeared to display NA immunoreactivity (Fig. 1A), while some NA cells lacked peptide immunoreactivity (Fig. 2B). In identically treated sections of the homozygous Brattleboro rat no VP or neurophysin immunoreactivity was visible.

Coexistence of peptides and monoamines has now widely been demonstrated for both the central and

Figs. 1, 2. Pairs of consecutive cryostat sections of Wistar rat LC incubated with antisera raised against vasopressin and noradrenaline (1A, B) or against neurophysin and noradrenaline (2A, B). IV, fourth ventricle; CE, cerebellum; BV, blood vessel.
Fig. 1A, B: vasopressin and noradrenaline immunoreactivity in posterior LC. Note the extent to which vasopressin stains at this level of the LC. Arrowheads indicate a group of cells with both vasopressin and noradrenaline immunoreactivity. Note also that in the case of peptidergic staining the cell nuclei remain unstained.

Fig. 2A, B: neurophysin and noradrenaline immunoreactivity in anterior LC. Note the small portion of neurophysin cells stained at this level. Arrows indicate a cell displaying both neurophysin and noradrenaline immunoreactivity. Arrowheads indicate a noradrenaline-immunoreactive cell which is not stained for neurophysin in the adjacent section.

the peripheral nervous system¹⁴. In the present study 3 experimental procedures enabled the visualization of coexistence of VP and neurophysin with NA in LC cells. First, the use of colchicine which resulted in a concentration of secretory material in the soma by blocking axonal transport¹³. Secondly, the use of trypsin improved staining in neuropeptide immunocytochemistry⁹. The way trypsin enhances staining is unclear. At present, the notion prevails that trypsin treatment reveals epitopes which are masked by fixation-induced cross-linking of various proteins⁹. Also, precursor polypeptides might be cleaved into shorter fragments which could be recognized by the antibodies. Therefore, the possibility exists that trypsin creates new antigenic sites causing false-positive results²⁰. Since in identically treated cryostat sections of the homozygous Brattleboro rat both VP and neurophysin staining were absent, and VP- and neurophysin-immunoreactive cells were present in the LC of the Wistar rat³ also without trypsin treatment, the present results are probably not due to artefactual staining induced by trypsin treatment. The colchici-

ne-trypsin procedure thus enhanced the sensitivity of the immunocytochemical staining and enabled the visualization of hitherto unknown peptidergic neurons⁹. Indeed, in two different experiments we also found a few VP- or neurophysin-immunoreactive Purkinje cells in the Wistar rat, which were undetectable in sections of the homozygous Brattleboro rat (unpublished results). It is possible that these Purkinje cells partly account for the VP found in the cerebellum in the radioimmunoassay system⁷. Thirdly, the use of a NA antibody which allowed the use of a highly concentrated glutaraldehyde fixative resulting in a minimal loss of antigen combined with an optimal preservation of the cell morphology. The fact that this NA antibody slightly cross-reacts with dopamine is not crucial in this study, since the LC is known to contain dopamine- β -hydroxylase and, therefore, noradrenergic neurons¹⁹.

The results demonstrate that numerous cells in the LC contain VP and neurophysin immunoreactivity in addition to NA. The LC noradrenergic cells are small, medium or large in size and have been classi-

fied morphologically into 4 categories¹. In this classification the double-stained cells correspond to type II cells, being medium-sized and having a high nuclear to somal cytoplasm ratio. VP- and neurophysin-immunoreactive cells constitute a substantial part of LC noradrenergic neurons.

The present results add further details to the nature and subdivision of catecholaminergic cells within the A6 and A7 regions. In addition to VP, neuropeptide Y (NPY)⁸, somatostatin¹² and enkephalin⁴ immunoreactivity were also reported present in the rat and cat LC. It is therefore possible that multiple peptides coexist in one neuron in this brain structure. Coexistence of neuropeptides and NA in the LC needs further investigation, since this phenomenon might help to distinguish subpopulations of LC neurons which may project to selective brain areas or perform specific functions¹⁵.

Projection areas from LC vasopressinergic cells yet remain to be elucidated. However, one could speculate that putative LC vasopressinergic fibers account for the following phenomena: first, the known visible VP fibers in the rat central nervous system of which the origin is still unclear, e.g. in the medial amygdaloid nucleus, the ventral hippocampus, the ventral tegmental area and the substantia nigra⁶. However, most of these fiber systems seem to disappear after castration and thus are likely to have their origin in gonadal steroid-sensitive VP nuclei (e.g. bed nucleus of the stria terminalis, medial amygdala)^{6,22}. In addition, preliminary studies performing VP immunocytochemical staining 3 weeks after LC lesion displayed no loss of VP fibers in the rat brain rostral to the LC. Extensive lesion and retrograde transport studies in combination with immunocyto-

chemistry may help to provide a conclusive answer. Secondly, the LC could be the source of the relatively high radioimmunoassayable values of VP that have been reported for some areas in the rat brain containing no or very few visible VP fibers e.g., dorsal hippocampus⁷. In this instance LC lesion studies followed by radioimmunological determination of regional VP and NA levels can provide an answer. Thirdly, it is possible that VP is only present in the cell body exerting some metabolic function.

In some brain areas, corresponding to terminal fields of the dorsal noradrenergic bundle, VP is able to affect NA turnover²³. It has been hypothesized that VP exerts this effect through a presynaptic modulatory mechanism^{10,23}. The present results, however, provide the possibility that coexistence of both transmitters in the presynaptic ending, rather than a presynaptic modulating principle, might be the basis of the above-mentioned effects and must therefore be considered when the interaction of both substances in brain function is studied.

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