Immunocytochemically stained vasopressin binding sites on blood vessels in the rat brain

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Following two weeks of cerebroventricular administration of arginine-vasopressin (AVP) by Accurel implants, two types of binding sites for this peptide were immunocytochemically visualized in blood vessels in the brain of Brattleboro (*di/di*) rats: (1) endothelial cells of capillaries were stained with the highest density in hippocampus, striatum, and locus coeruleus (LC), whereas only few such stained cells were present in the septum and cerebral cortex. (2) Bound AVP was also present on endothelial cells and pericyte-like cells in larger blood vessels in striatum and the LC. Both types of vasopressin binding site staining on blood vessels were dose-dependent and could be further enhanced by additional *in vitro* preincubation of the fixed sections with AVP. This staining was not present in rats implanted with either oxytocin or α -melanocyte-stimulating hormone.

An effect of arginine-vasopressin (AVP) on the permeability of the blood–brain barrier (BBB) has been reported for transport of various nutrient substances^{3,4,19,26}. In addition, AVP is known to affect water permeability of brain microvessels^{7,22,27}. Binding assays have shown the presence of binding sites on isolated brain microvessels^{8,13,15,16,20}. Most studies concerning binding sites in the vascular bed of the brain were performed with homogenate binding assays or receptor autoradiography, which has a low resolution. Therefore, morphological localization of these binding sites *in situ* has not been reported so far.

In the present study, a recently introduced immunocytochemical (ICC) procedure has been applied for the localization of AVP binding sites in the vascular bed of the brain. This procedure was developed for visualizing peptidergic binding sites in the rat kidney and brain with a higher resolution than autoradiographic techniques^{23,24,25} and is based upon continuous intracerebroventricular administration of peptides, using Accurel polymer tubing filled with a

ligand^{2,17}. The ligand is subsequently immunocytochemically stained in fixed tissue.

Male Brattleboro rats, 3 months of age and homozygous for diabetes insipidus (*di/di*), were obtained from the Central Animal Supply House, CPB-TNO (Zeist, the Netherlands). These vasopressin-deficient rats were chosen in order to avoid cross-reactive immunostaining of the antisera with endogenous AVP, as we have reported earlier^{23,24}.

Unilateral implants of AVP/Accurel in the lateral ventricle contained either 0.15, 1.5 or 15 μ g of AVP ($n = 7$) or water-filled tubing ($n = 5$). In addition, 6 rats were implanted with 15 μ g oxytocin (OXT) and 5 with α -melanocyte-stimulating hormone (α -MSH) filled Accurel tubing. The following preparations of peptides were used: AVP (Sigma, St. Louis, MO, grade VIII, 358 IU/mg); OXT (Sigma, St. Louis, MO, grade X, 540 IU/mg); α -MSH (WB 1138 K1, kindly donated by Dr. H. Greven, Organon, The Netherlands). Two weeks later, the rats were perfused intracardially with 2.5% glutaraldehyde and 1% paraformaldehyde. Transverse free-floating vibra-

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tome sections, 50 μm thick, were stained with antibody directed against lysine-vasopressin (LVP, No. 121/4, 24.12.72, kindly donated by Prof. J.L. Toubier, AMC, Amsterdam) at a dilution of 1:1000. Pilot experiments showed that the use of anti-LVP resulted in better staining of bound AVP in the brain as compared to anti-AVP. This may be explained by the observation that anti-LVP (No. 121) binds in radioimmunoassay to a different epitope of the AVP molecule than anti-AVP antibodies. For OXT- or α -MSH-implanted brains, a 1:1000 dilution of the homologous antibodies was used: anti-OXT (0-1-V, 4.4.75) and α -MSH (43994, 23.4.75) respectively. Specificity of the staining for in vivo bound AVP on blood vessels was validated by the following control experiments. (1) Method specificity was tested by using a preimmune serum (Truus CTRL, 27.2.85, 1:1000) as first antibody. (2) To exclude possible cross-reaction of anti-LVP with endogenous oxytocin, an ICC staining of AVP-implanted rats was performed with anti-OXT as well (0-1-V, 4.4.75, diluted 1:1000). (3) In order to test for non-specific effects due to the Accurel implantation procedure, staining was, in addition, performed on rats implanted with water-filled tubings ($n = 5$) and in order to check for possible aspecific binding of peptides to blood vessels, rats were implanted with Accurel tubing loaded with other peptides, i.e. (4) 15 μg of OXT ($n = 6$) or (5) α -MSH ($n = 5$), after which staining was applied using the homologous antibodies.

Sites of bound AVP were revealed in two distinct patterns: (1) on endothelial cells in capillaries and (2) on endothelial cells and pericyte-like cells in larger blood vessels. The highest staining density of capillaries was observed in the hippocampus (Fig. 1b). The striatum, septum and the area around the locus coeruleus (LC) were less densely stained, while the cortex contained only very few stained capillaries. The second type of AVP binding sites was found in endothelial and pericyte-like cells of larger blood vessels (diameter $> 50 \mu\text{m}$). This type of staining was present in the striatum and to a lesser extent in the LC area (Fig. 1a,c,d). Electron microscopy is needed for further structural characterization of these binding sites.

Staining of AVP binding sites was dose dependent and could be enhanced by in vitro preincubation of the sections with AVP. The low and medium dose

(0.15 and 1.5 μg) implanted brains, needed higher concentrations of AVP (30 and 6 μM respectively) during the in vitro preincubation compared to the high dose (15 μg) implanted animals (0.6 μM), before an enhancement of staining could be observed.

The various control experiments all provided evidence that AVP was indeed stained in the AVP-Accurel implanted rats: all 5 control procedures resulted in complete absence of binding site staining on blood vessels, indicating that binding was specific for AVP. In rats which had an OXT implant (15 μg) for two weeks followed by subsequent ICC staining of the fixed brains with anti-OXT, no staining of AVP binding sites on blood vessels was observed. Staining of endothelial cells in capillaries was completely absent and the pericyte-like cells in the large blood vessels were not immunoreactive (Fig. 1e).

Enhancement of staining following in vitro incubation of the implanted brains with the ligand (AVP), reinforces the idea that binding sites for AVP are stained. Such a phenomenon is attributed to high affinity binding sites and has been earlier reported for renal VP receptors^{23,29} as well as for receptors for LVP⁶, estrogens¹⁸ and LH-RH³⁰.

Staining of AVP binding sites in capillaries agrees with recent reports of binding assays which indicate AVP binding to isolated microvessels of the rat hippocampus and pig cerebral microvessels, cerebral cortex and hippocampus^{15,16,20}. In previous reports we made use of the same ICC procedure to visualize perineuronal and cytoplasmic AVP binding sites in various regions of rat brain^{24,31}. This means that the earlier reported binding sites using binding assays and receptor autoradiography might be present in at least two compartments: neurons and blood vessels. Vasopressin might reach these compartments by fibers innervating these structures⁵ or by means of the blood stream respectively.

The physiological function of pericytes still has to be elucidated. The strategic placement of pericytes around the blood vessels indicates that they may play an important role within the brain microcirculation¹². Electron microscopical studies support this idea as they reveal the presence of filaments in the cytoplasm of pericytes, resembling the myofilaments of smooth muscle cells, suggesting that pericytes are capable of contraction and may participate in regulating the blood flow in small vessels²⁸. According to

Fig. 1. Immunocytochemical staining of bound AVP in the vascular bed of Brattleboro (*di/di*) rat brain: in all ICC stainings transverse free floating vibratome sections ($50\text{ }\mu\text{m}$) were used. Sections a–d are ICC stained with LVP-antiserum after AVP/Accural implantation for two weeks; (e) is a stained section from a rat after ventricular administration of OXT for two weeks and subsequent ICC staining with OXT-antiserum. a: pericyte-like cell in a blood vessel in the striatum showing immunoreactivity for vasopressin in the cytoplasm. b: bound vasopressin in capillaries of the hippocampus. c: bound vasopressin in endothelial cells of a large blood vessel in the striatum. Note that the cells are aligned to the walls of the blood vessel. d: pericyte-like cells in a large blood vessel in the striatum. e: pericyte-like cell in a large blood vessel (see arrowhead) in the striatum of a rat implanted for two weeks with $15\text{ }\mu\text{g}$ OXT and subsequently immunocytochemically stained with the homologous antibody, 0-1-V; control group IV. Note the negatively stained pericyte-like cell in the vessel and the positively stained OXT fibers around it. Calibration: $20\text{ }\mu\text{m}$ for (a, b, d, e); $50\text{ }\mu\text{m}$ for (c).

ultrastructural ICC studies, neurons and nerve fibers containing AVP are directly connected with brain blood vessels^{14,21}.

Our findings indicate a heterogeneous distribution of two types of specific AVP binding sites in the vascular bed of the brain: (1) endothelial cells in capillaries in various brain regions and (2) endothelial cells and pericyte-like cells in large blood vessels. The presence of AVP binding sites on these cells is a new observation. The two types of binding sites might regulate two separate processes.

(1) Bound AVP on endothelial cells in capillaries may serve as a trigger for the alteration in transport of various compounds across the BBB, although the mechanism linking binding and altered permeability

is yet unclear. Permeability changes are of importance, e.g. in aging and Alzheimer's disease in which functional changes of the BBB have been described¹ and in which many changes of vasopressin systems in the brain have been reported in both rat and human brain^{9,10,11,31}.

(2) Binding of AVP in endothelial cells and pericyte-like cells in large blood vessels may play a role in the control of brain microcirculation.

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