Immuno-Electron Microscopical Demonstration of Vasopressin and Oxytocin Synapses in the Limbic System of the Rat*

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Summary. The extensive distribution of exohypothalamic vasopressin or oxytocin containing nerve fibres is thought to be the anatomical basis for the involvement of these neuropeptides in central processes. Following light microscopic observations suggesting that these fibres terminate on other neurons, the present study was undertaken to demonstrate the existence of such endings in the limbic system, which is one of the main target areas for these peptides. For immunoelectron microscopy glutaraldehyde-paraformaldehyde perfused brains of male Wistar rats and Brattleboro rats, homozygous for diabetes insipidus, with and without postfixation in OsO_4 , were used. Post-embedding staining revealed false positive reaction product on all dense core vesicles, e.g., in the lateral septum. With pre-embedding staining, however, intense and specific reactions were observed for both vasopressin and oxytocin at their sites of production, as well as the neurohypophysis and in the extrahypothalamic limbic brain regions.

In the lateral septum and habenular nucleus only vasopressin-containing synapses could be demonstrated, while in the medial nucleus of the amygdala synapses containing either vasopressin or oxytocin were observed. These peptide containing synapses do not seem to differ in any fundamental way from the classical transmitter-containing synapses in the brain.

Key words: Vasopressin – Oxytocin – Peptide synapse – Neuropeptide.

Vasopressin (AVP) and oxytocin (OXT), the two neurohypophyseal hormones, are synthesized in the paraventricular (PVN) and supraoptic nuclei (SON), while the suprachiasmatic nucleus (SCN) contains AVP only (Swaab and Pool, 1975). The

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exact mode of action of these neuropeptides in controlling behavior (Bohus, et al., 1978; De Wied and Bohus, 1979) is not yet known, although effects on transmitter metabolism and electrical properties have been found. Intracerebro-ventricular administration of vasopressin has pronounced effects upon monoamine metabolism (Tanaka et al., 1977; Versteeg et al., 1979) and hippocampal theta rhythm in the rat (Bohus et al., 1978). Moreover, LVP (Barker, 1977) and recently also AVP (Termaat, pers. communication) have been shown to have long lasting facilitating effects on spike frequency of snail neurons.

The anatomical basis of these effects is thought to be the extensive AVP and OXT system of fibre projections found in many parts of the brain, well beyond the hypothalamus (Buijs et al., 1978). As a result of studies involving light microscopic immunocytochemistry, the existence of three types of peptidergic fibre terminations has been suggested (Buijs, 1978a, b): (1) the "classical" neurosecretory endings in the neurohaemal organs, i.e., the neurohypophysis, the median eminence, and the organon vasculosum laminae terminalis, (2) endings on dendrites, i.e., on the dendritic tree of the ventral hippocampus, and (3) punctate perineuronal structures, e.g., in the lateral septum and amygdala. However, due to the limitations of light microscopy it was impossible to demonstrate conclusively that such fibres indeed terminate on neuronal structures in the CNS. AVP or OXT synapses have not been previously described. The aim of the present immuno-electron microscopical study was, thus, to establish the shape and the mode of termination of such peptidergic fibres in the brain. Three different brain regions were selected: (1) the lateral septum, (2) the lateral habenular nucleus, and (3) the medial nucleus of the amygdala. The first two receive vasopressin containing fibres, from the SCN, while the third region is reached by both vasopressin and oxytocin fibres from the PVN (Buijs, 1978a).

Materials and Methods

Twenty-seven male Wistar rats and 8 male rats of the Brattleboro strain, homozygous for diabetes insipidus (Ho-DI) (TNO, Zeist), weighing 180–200 g, were used in the present study. The animals were anaesthetized with Nembutal (0.1 mg/100 g body weight i.p.), perfused intracardially with saline followed by 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M cacodylate buffer pH 7.25 (all chemicals from Merck). The desired brain regions were isolated free-hand in slices of approximately 2 mm and subsequently immersed in the same fixative for 2–3 h at 4° C.

Tissue blocks for *post-embedding* staining were postfixed in 2% OsO₄ in the cacodylate buffer, dehydrated in a graded ethanol and epoxypropane series (for details see Van Leeuwen and Swaab, 1977).

For *pre-embedding* staining 20–80 µm sections were made from tissue blocks with a vibratome (Oxford Instruments) into 0.05 M TRIS/HCl, buffer pH 7.6 in 0.9% NaCl. Sections were handled with a paint brush and placed into glass vials. The immunocytochemical procedures were essentially the same as previously described (Buijs et al., 1978), and consisted of subsequent incubations at 4° C in (a) 10% swine serum for 30 min, (b) rabbit vasopressin #125 or oxytocin #02C antiserum purified (1:400) or non-purified (1:1000) for 16 h, (c) washed in TRIS/saline for 1 h, (d) swine anti-rabbit IgG (Fc fragment) serum (1:40) (Nordic) for 2 h, (e) washed in TRIS/saline for 1 h, (f) peroxidase anti-peroxidase (PAP) (1:200) for 2 h, (g) washed in TRIS/saline for 4 h, (h) 0.5 mg/ml 3,3-diaminobenzidine (DAB) (Sigma) 0.01 H₂O₂ (Merck) in TRIS/saline for 3–8 min, (i) washed in TRIS/saline for 30 min, (j) 2% OsO₄ in TRIS/saline for 30 min, (k) washed in TRIS/saline for 60 min, (l) dehydrated in a graded ethanol series, and (m) flat embedded in Epon 812. Ultrathin sections were examined in a Philips EM 200 electron microscope both with and without staining in uranyl acetate and lead citrate. The influence of the

addition of Triton X-100 to the 10 % swine serum and the first antiserum was checked in concentrations ranging from 0.1 to 0.4 %. Specificity controls used were: (1) replacement of the first antiserum by normal rabbit serum, (2) adsorption of vasopressin antiserum and oxytocin antiserum to vasopressin and oxytocin containing agarose beads respectively, (3) Ho-DI rat brain sections of the lateral septum as a control for the specificity of the vasopressin antiserum, and (4) SCN sections for the specificity of the oxytocin antiserum. For details of the method involved in the production and purification of antisera the reader is referred to Buijs et al. (1978) and Swaab and Pool (1975)

Results

Post-embedding Staining

Initially, an attempt was made to localize vasopressin in ultrathin OsO_4 fixed brain sections by means of post-embedding staining. However, as no reaction was obtained in the lateral septum with the normal procedure (Van Leeuwen and Swaab, 1977), it was necessary to use longer incubation times in the first antiserum (48 h, 4° C) and in the DAB mixture (20 min). This resulted in the staining of dense core vesicles in nearly each synapse and fibre of the lateral septum. Since no staining was observed in controls with AVP antiserum exhausted with AVP-containing agarose beads, or with normal rabbit serum instead of the first antiserum, specificity was suggested. However, when sections from the Ho-DI rats were treated with the purified AVP antiserum, staining was again observed on all sizes of dense core vesicles in the lateral septum, indicating that the staining produced by the longer incubation times was non-specific. This experience forced us to seek for a different technique that might permit specific staining.

Pre-embedding Staining

The light microscopic data obtained by pre-embedding staining of vibratome sections of the lateral septum, habenula, and amygdala, are the same as those found in previous studies (Buijs, 1978a; Buijs et al., 1978) with 6 μm paraffin sections. In contrast to the result of post-embedding staining, however, no staining was observed in the lateral septum of the Ho-DI rat if purified vasopressin antiserum was used. In addition, all other controls, described in the methods section, remained negative in light- as well as in electron-microscopy. The use of 0.1 % Triton improved the penetration of the antisera, resulting in a stained zone reaching approximately 20 μm into either side of the section. Without Triton this penetration was only 5 μm . The use of concentrations higher than 0.1 % Triton resulted in considerably more damage to the ultrastructure of the membranes than if no Triton was used. Also the rest of the procedure caused some damage of membranes. An increase in glutaraldehyde concentration up to 5 % did not result in any perceptible improvement of membrane preservation. In contrast, the use of OsO_4 , for postfixation, resulted in good ultrastructural preservation, but in the loss of all immunoreactivity.

Pre-embedding staining of the neurohypophyses resulted in a positive reaction of granula, while extragranular DAB deposits were also observed. In slices of

Fig. 1. Vasopressin positive terminal forming synapse (*black arrow*) with unlabelled dendrite in lateral habenular nucleus. Note DAB deposit around clear vesiclelike structures (*arrows*). $\times 60,000$. Bar = $0.25\ \mu\text{m}$

Fig. 2. Vasopressin positive terminals (*arrows*) around cell body (*n* nucleus) in lateral septum.

Fig. 3. Vasopressin positive terminal forming synapse (*arrow*) with unlabelled dendrite in medial nucleus of amygdala. $\times 84,000$. Bar = $0.25 \mu\text{m}$

Fig. 4. Vasopressin positive terminal forming two synapses (*arrows*) with two different unlabelled dendrites in lateral septum. $\times 60,000$. Bar = $0.25 \mu\text{m}$

Figs. 5-7. Light- and electron-micrographs of different animals showing vasopressin positive reaction in fibres and terminals around cell body and its process in lateral septum

Fig. 5. Vasopressin positive terminals (*arrows*) on cell body (*n* nucleus) and its dendrite. Contrasted with uranyl acetate and lead citrate. $\times 21,000$. Bar = $0.50\mu\text{m}$

Figs. 6 and 7. Vasopressin positive fibre around neuron and its process (*arrows*), focussed at two different levels in $20\mu\text{m}$ thick section. $\times 1600$. Bar = $10\mu\text{m}$

Figs. 9–11. Electron micrographs demonstrated DAB deposit on vasopressin or oxytocin containing dense core vesicles, all figures of the same magnification: note difference in granule size

Fig. 8. Vasopressin containing granules (*arrows*) in lateral septum; **Fig. 9** in medial nucleus of amygdala, and **Fig. 11** in neurohypophysis

Fig. 10. Oxytocin containing granules in spinal cord of Ho-DI Brattleboro rat. $\times 38,000$. Bar = $0.25 \mu\text{m}$

hypothalamic tissue containing the SCN and PVN processed in the same way, poor preservation of dense core vesicles occurred. Electron dense reaction product was present not merely on the vesicles, only a few of which remained intact, but also around smaller structures with a clear vesicle-like appearance (Fig. 1).

Synaptic structures positively stained by means of this procedure were frequently found in sections of the lateral septum, lateral habenular nucleus and

nuclei of the amygdala. In the septum and habenular nucleus only vasopressin fibres could be demonstrated while in the medial amygdala both vasopressin and oxytocin synapses were observed. Besides the difference in peptide content no morphological distinction could be made between amygdala sections incubated with either purified vasopressin or oxytocin antisera. Although some endings were found on cell somata (Fig. 2), the majority of the terminations were seen on dendrites (Figs. 1, 3, 4). In addition, in the amygdala AVP or OXT synapses were sometimes seen on glial cells. In the lateral septum structures resembling synapses "en passage" were observed (Figs. 5-7). Most synapses appeared to have a widened synaptic cleft, while infrequently a postsynaptic density was present. In the "positive" nerve fibres in the lateral septum, lateral habenular nucleus, and amygdala, clusters of reaction product were, moreover, seen upon dense core vesicles that had a diameter of approximately 100 nm (Figs. 8-10). In the neurohypophysis, however, the positively reacting dense core vesicles had a diameter of approximately 180 nm (Fig. 11). No terminations could be seen on these peptide-containing fibres.

Discussion

Post-embedding Staining

The false positive reaction in the procedure for post-embedding staining demonstrates that it is hardly possible to claim that an immunocytochemical technique is "specific" for a certain compound if a useful control such as an antigen-deprived mutant is lacking (Swaab et al., 1977). The fact that dense core vesicles of all possible diameters were "positive" and that increase of the incubation times produced an increase in background staining as well as staining of dense core vesicles, was sufficient to indicate nonspecific staining. The false positive reaction occurred not only in OsO_4 postfixed material, but also in glutaraldehyde-paraformaldehyde fixed material. Moreover, from this result it can be concluded, that the presence of unstained dense core vesicles in the same section, is an additional criterion for evaluating the specificity of post-embedding staining.

Pre-embedding Staining

That preservation of optimal ultrastructure is in general incompatible with maintenance of immunoreactivity (Sternberger, 1974), was confirmed by the results of pre-embedding staining. Until now an intense immuno-reactivity has been accompanied by some unavoidable loss of ultrastructure. The small number of "positive" dense core vesicles observed in the PVN, SCN and their processes with this staining procedure is in contrast with the prevalence of such granules in optimally OsO_4 fixed material. Many of the dense core vesicles seem to be ruptured, possibly as a result of the penetration of the antisera and of DAB polymerisation during this procedure. For the same reason the presence of dense core vesicles could often not be established in the neurosecretory terminations in extrahypothalamic

areas. In the neurohypophysis, however, the vast majority of the dense core vesicles remained intact throughout the procedure, pointing to the possibility that the extrahypothalamic area contains neurosecretory vesicles that are different from those in the neural lobe. This possibility is reinforced by the difference in granular size found in the extrahypothalamic area and the neurohypophysis. In the amygdala AVP and OXT are present in 100 nm vesicles, while in the SON, PVN and neurohypophysis generally larger, 140–160 nm neurohypophysial hormone containing vesicles are found (Zambrano and De Robertis, 1966; Flament-Durand, 1971; Livingston, 1971). In this connection, it is notable that the AVP-containing fibres, seen in the external zone of the median eminence and assumed to come from the PVN (Vandesande et al., 1977), also contain small 90 nm dense core vesicles (Dube et al., 1976). As the possibility exists that the extrahypothalamic fibres from the PVN are largely derived from different cells to those which project towards the neurohypophysis (Ono et al., 1978; Schober, 1978), these different cells may contain granules of a different size. The DAB deposits found around clear, vesicle-like structures might also fit in with the idea that the smooth endoplasmatic reticulum is a transport vehicle for nongranular, intra-axonal transport of neurosecretory material (Droz et al., 1973, 1975), and thus gives rise to clear vesicles (Alonso and Assenmacher, 1978).

Another question in relation to the exohypothalamic neurosecretory fibres is whether they are axonal or dendritic in character (Buijs, 1978a). The absence of synapses on the AVP/OXT fibres and terminals and that of dendrite markers, e.g., ribosomes, argues for the axonal nature of the exohypothalamic fibres. The bi- or multipolarity generally observed in neurosecretory cells suggests that they may have more than one axon, allowing simultaneous release in the neurohypophysis and in extrahypothalamic brain regions. The synchronous elevation of AVP in blood and CSF after vagal stimulation (Heller et al., 1968) or hemorrhage (Vorherr, 1968) supports this possibility. However, electrophysiology has so far failed to confirm that the neurohypophysial and extrahypothalamic fibres are derived from the same cell (Pittman et al., 1978). On the other hand, some arguments support the possibility that different cells project towards the neurohypophysis and the extrahypothalamic area respectively. HRP injections placed into the neurohypophysis or the spinal cord seem to label largely different populations of cells (Ono et al., 1978).

Besides its influence on behavior, AVP in the lateral septum may play a role in the regulation of the release of AVP in the neurohypophysis. Septal lesions, or lesions interrupting the AVP pathways from the SCN, disturb the water balance in rat (Johnson and Buggy, 1978) and goat (Andersson et al., 1975), while stimulation of the septal region results in a change of spike frequency in PVN cells (Negoro et al., 1973).

The present results demonstrate synapses containing vasopressin in the lateral septum and lateral habenular nucleus and AVP or OXT containing synapses in the medial nucleus of the amygdala. Surprisingly, most synapses are found on dendrites and not on cell bodies as had been supposed from light microscopic observations (Buijs, 1978a; Sofroniew and Weindl, 1978). An explanation for this discrepancy could be that the light microscopic staining technique makes the fibre structures around cell bodies more easily recognizable. The possibility that AVP and OXT

may influence neurotransmitter metabolism is demonstrated by reports that these peptides, when injected centrally, influence monoamine metabolism in specific brain regions (Tanaka et al., 1977; Versteeg et al., 1979). However, these transmitters are not found in the neurons of the lateral septum, lateral habenular nucleus, or amygdala. For a functional interpretation of the peptide synapses described it would be necessary, to know what kind of transmitter the postsynaptic neurons produce and the regions to which they project.

The presence of these peptide synapses in the limbic system seems to be the morphological basis for the effects of AVP and OXT on avoidance behavior, for which this structure is of considerable importance (Van Wimersma Greidanus et al., 1979). In the hippocampus, where both AVP and OXT terminals are present, locally injected OXT reduced, while AVP facilitated, passive avoidance behavior. Injection of each peptide into the dorsal septal nucleus, however, resulted only in facilitation (Kovacs et al., in press). This suggests that in the lateral septum, where no OXT containing synapses occur, only AVP receptors are present, and that these are sensitive to OXT as well.

It has been found both by radioimmunoassay and immunocytochemistry that AVP and OXT are present in a wide variety of regions in the CNS (Dogterom et al., 1978). In addition, it is known that AVP has a long lasting effect on membrane properties (Barker, 1977; Termaat, pers. communication). The present study demonstrated their localization in synaptic structures. With these findings several "criteria for establishing the identity of a neuroregulator in the central nervous system" (Barchas et al., 1978) as a neurotransmitter are fulfilled. Since it is still unknown whether the criteria developed for "classical" neurotransmitters also apply to the category of peptide transmitters, it cannot be decided how far we are from "proving" the transmitter nature of these compounds.

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