Extrahypothalamic Vasopressin and Oxytocin Innervation of Fetal and Adult Rat Brain

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INTRODUCTION

After Ernst Scharrer (1951) demonstrated the existence of exohypothalamic pathways in the garter snake by means of the Gomori staining technique, Legait (1958) and Barry (1961), using the same method, convincingly demonstrated that this phenomenon holds true for many other species, ranging from fishes and amphibia to mammalians. Independently of each other, Barry et al. (1958) and Legait and Legait (1958) demonstrated, respectively, oxytocinergic activity in the area of the amygdala, and antidiuretic activity in the habenular region by means of bioassays. Twenty years later, Dogterom et al. (1978) were able to confirm and extend these studies using highly sensitive and specific radioimmunoassays for vasopressin (AVP) and oxytocin (OXT).

As early as 1954, Barry pointed to the possibility that these exohypothalamic fibres terminated by means of "de synapses neurosécrétoires" containing Gomori-positive material. However, the Gomori staining technique did not allow electron microscopic observation and identification of these structures.

In 1974 Sterba demonstrated the existence of such peptidergic synapses in the CNS of the salamander, using electron microscopy and an oxidation technique which was later described by Naumann and Sterba (1976). Due to the limitations of these techniques, particularly with regard to its specificity (Sterba et al., 1980), little or no attention was paid to these excellent studies. The demonstrated exohypothalamic fibres might represent the physiological route of transportation within the CNS for centrally effective vasopressin and oxytocin. An alternative route of transportation, the cerebrospinal fluid, was also proposed (De Wied and Gispen, 1977). We therefore decided to study the localization of vasopressin and oxytocin in the rat central nervous system using immunocytochemical techniques.

LOCALIZATION OF VASOPRESSIN AND OXYTOCIN IN THE RAT BRAIN

Antibodies against these two peptides were raised and purified using agarose beads coupled with the heterologous antigen (Swaab and Pool, 1975). Rat brains were perfused and fixed in 2.5% glutaraldehyde–1% paraformaldehyde in 0.1 M cacodylate buffer, after which the tissue was either embedded in paraffin for an immunolight microscopical procedure, or sectioned on a vibratome to be used both for immunolight- and immunoelectron microscopical observations. Immunolight- and immunoelectron microscopy were performed

using the unlabelled antibody enzyme method of Sternberger (1974). For further details about fixation, staining of the tissue and specificity controls see Buijs et al. (1978) and Buijs and Swaab (1979).

In the adult rat, vasopressin or oxytocin was demonstrated in cell bodies of the magnocellular nuclei and in fibres running towards the neurohypophysis, as well as in cell bodies scattered throughout the hypothalamus. From the paraventricular nucleus (PVN), vasopressin and oxytocin fibre pathways were found to run (Buijs, 1978; Buijs et al., 1978; Fig. 1): (a) via the ventral commissure of the fornix and subiculum, to reach the ventral hippocampus and entorhinal cortex (the ventral hippocampus is also reached by a ventral pathway via the amygdala); (b) via the stria terminalis to the nuclei of the amygdala; and (c) via the central grey and substantia nigra into the medulla oblongata and substantia gelatinosa of the spinal cord. The extent to which the SON contributes to the exohypothalamic system (EHS) is still unclear, but some of the fibres in the amygdala and ventral hippocampus might be derived from this nucleus. In rostal brain regions, including the hippocampus and "central grey", far more vasopressin than oxytocin fibres are present, while oxytocin fibres predominate more caudally. From the parvocellular suprachiasmatic nucleus (SCN), only vasopressin-containing fibres emerge, which run rostrally towards the organum vasculosum of the lamina terminalis and the lateral septum and dorsocaudally towards the lateral habenular nucleus (Buijs, 1978; Fig. 1).

Immunocytochemistry has enabled us to demonstrate vasopressin- and oxytocin-containing cells and/or fibres in the fetal rat hypothalamus as early as day 16. In contrast to the adult, OXT- and/or AVP-containing cells in the fetus come into direct contact with the ventricular system, with their somata lodged between the ependymal cells and their pro-

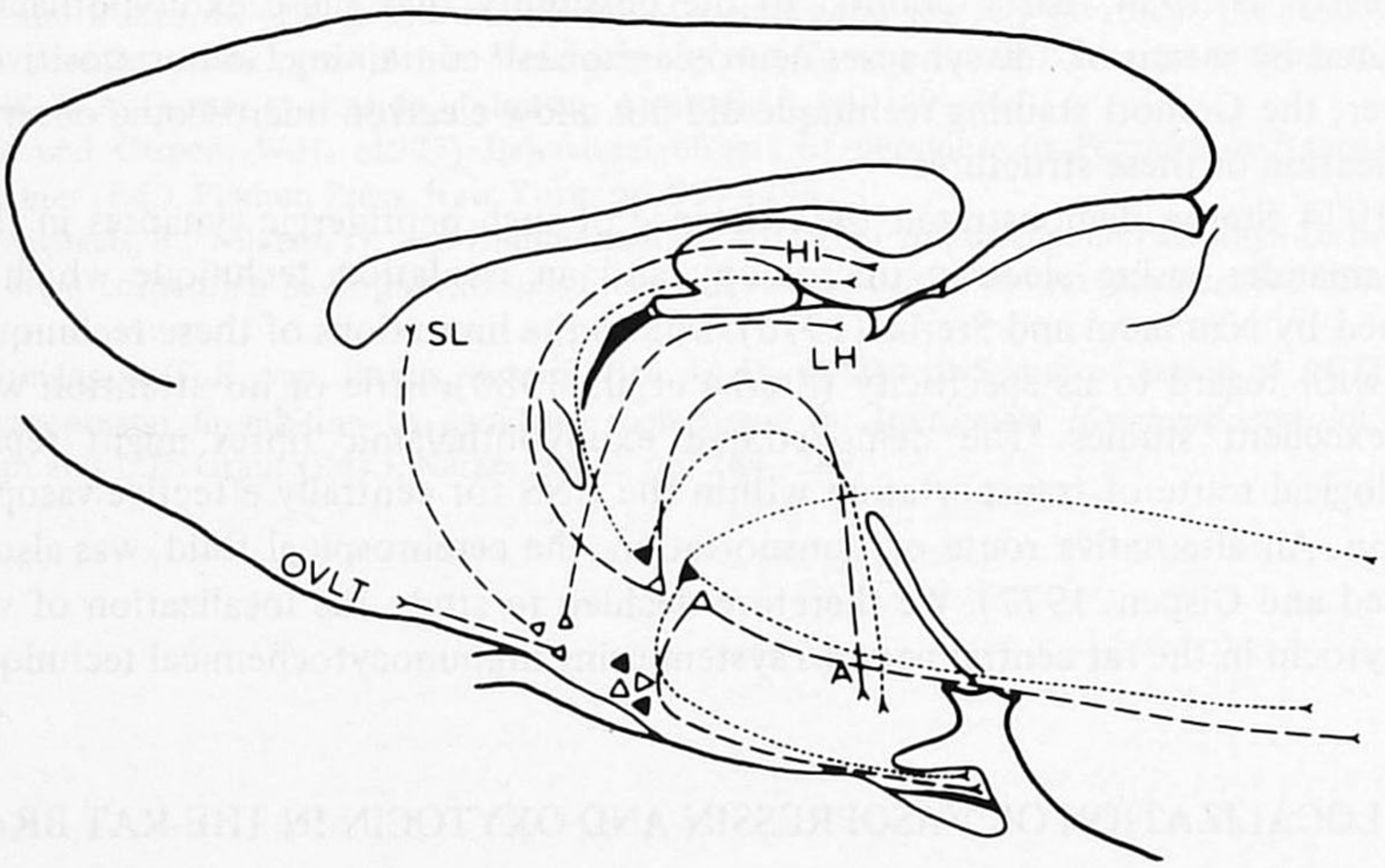
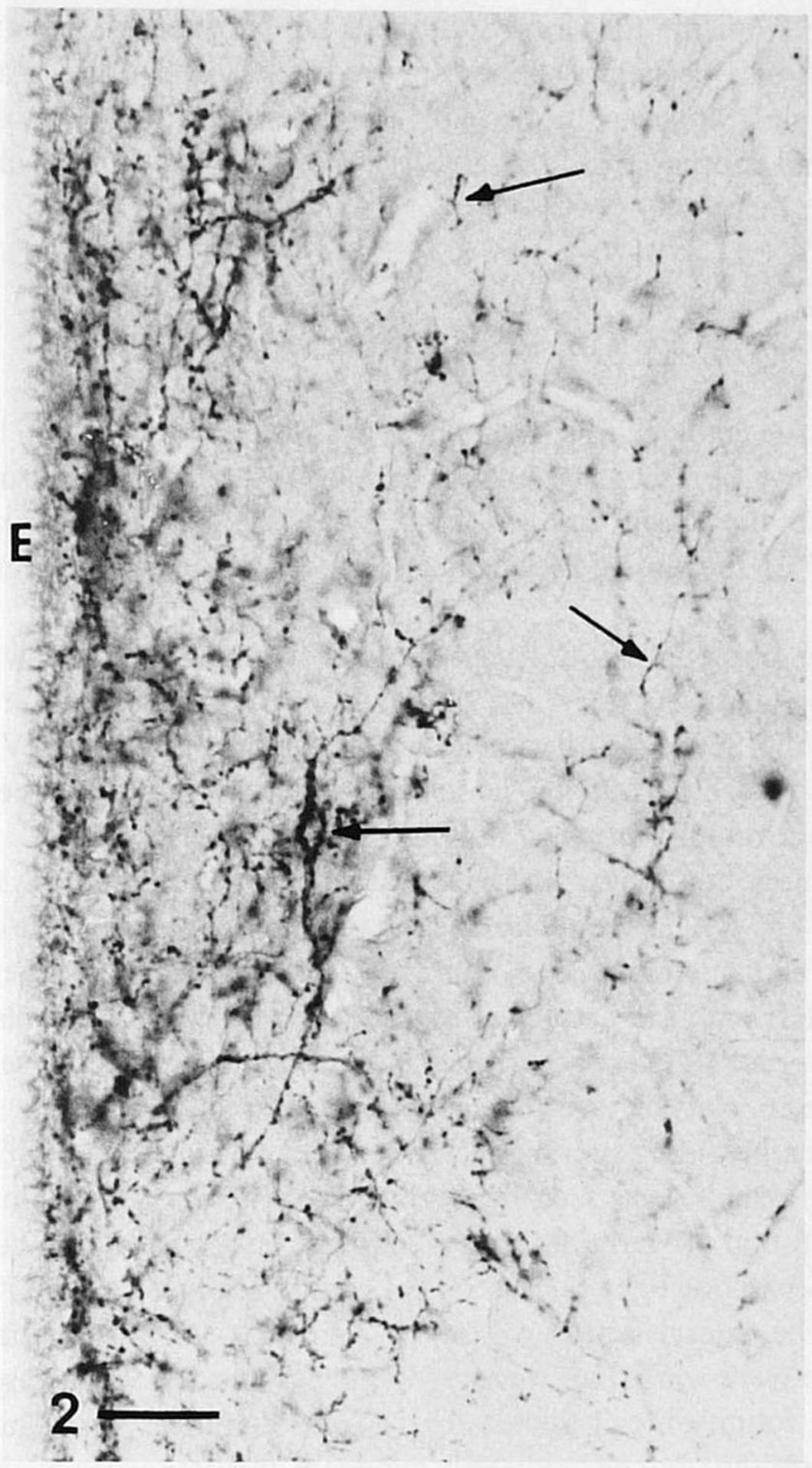
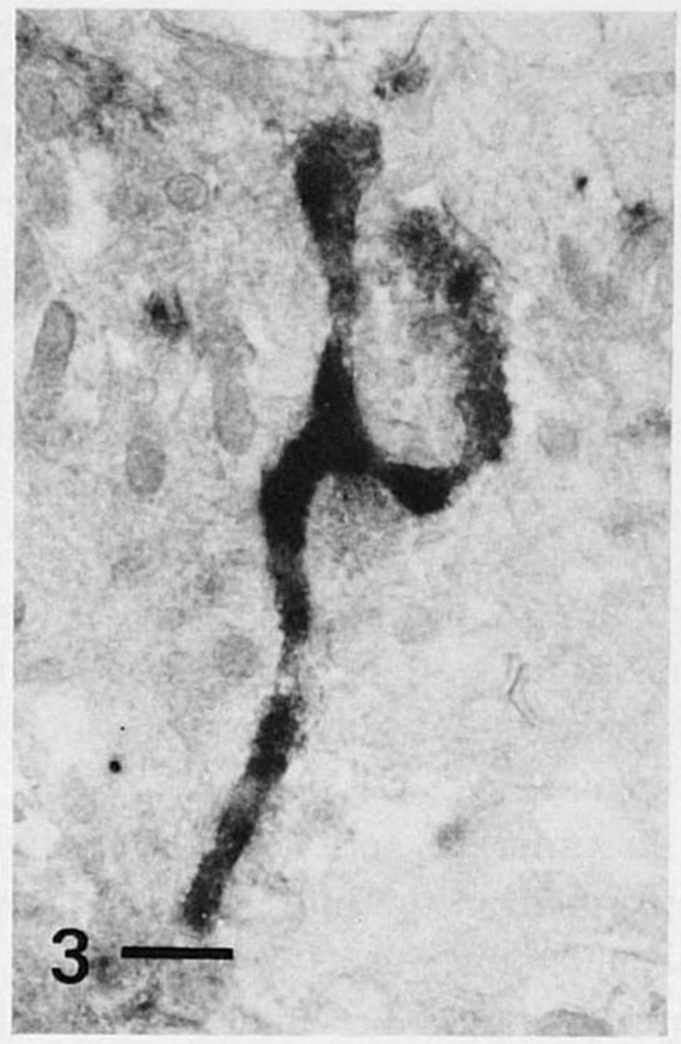


Fig. 1. Diagram illustrating pathways and sites of termination, originating from the suprachiasmatic nucleus (small triangles) and paraventricular nucleus (upper group of large triangles). The open triangles and broken lines indicate vasopressin-containing cell bodies and fibres while the closed triangles and dotted lines represent respectively, cell bodies and fibres containing oxytocin. From the suprachiasmatic nucleus, fibres run to the organum vasculosum lamina terminalis (OVLT), the lateral septum (SL) and the lateral habenular nucleus (LH). Paraventricular nucleus fibres run to the hippocampus (Hi) in which they terminate ventrally, the amygdala (A), and nuclei in the medulla oblongata and spinal cord.





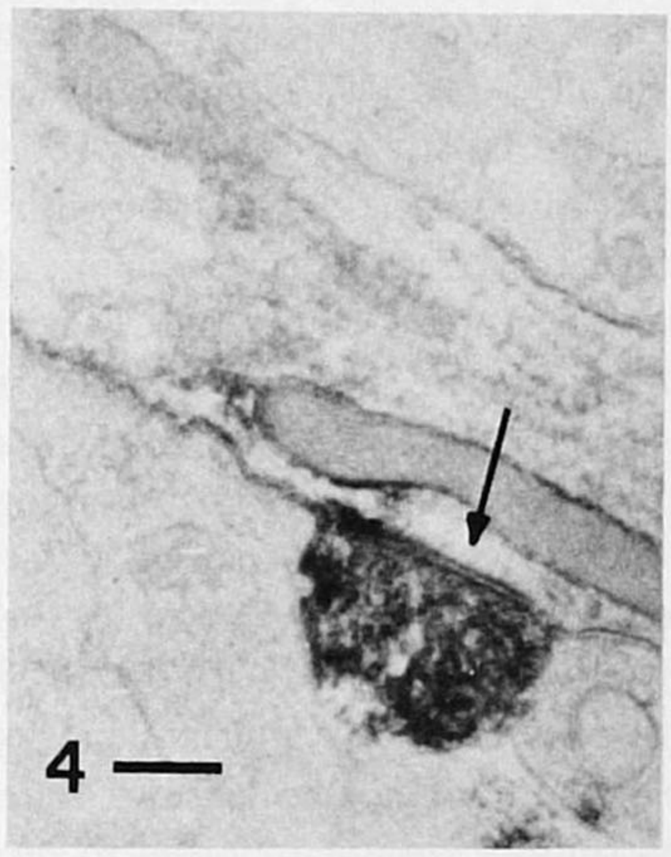


Fig. 2. Vasopressin-containing fibres in a transversal section of the lateral septum. Note the pericellular terminations, fibre branching (arrows) and the high concentration of fibres in the most lateral part of the septum. E, ependyma. Bar, 20 μm.

Fig. 3. Branching of a vasopressin-containing fibre in the lateral septum as seen by means of immunoelectronmicroscopy. Bar, 0.5 μm.

Fig. 4. Vasopressin-positive terminal forming a synapse (arrow) with an unlabelled dendrite in the lateral septum. Bar, 0.25 μm.

cesses pointing into the hypothalamus. The possibility of transport of neurohypophyseal hormones to other brain sites via the cerebrospinal fluid may thus be seriously considered for the developing brain (Boer et al., 1980). A ventromediorostral to dorsolaterocaudal gradient appeared to be present for the development of AVP- and OXT-containing cells, similar to the gradient reported for the general maturation of hypothalamic nuclei (Ifft, 1972; Anderson, 1978). In the fetal brain, large numbers of growth cones containing neuro-

hypophyseal hormones seem to arise from the SON and PVN, and can be traced down into the central grey. Fibres of the EHS can be demonstrated in the nuclei of the amygdala from day 18. AVP in the parvocellular SCN can be demonstrated only from day 3 post-natally while innervation of the lateral septum is still absent (De Vries, unpublished results from The Netherlands Brain Research Institute).

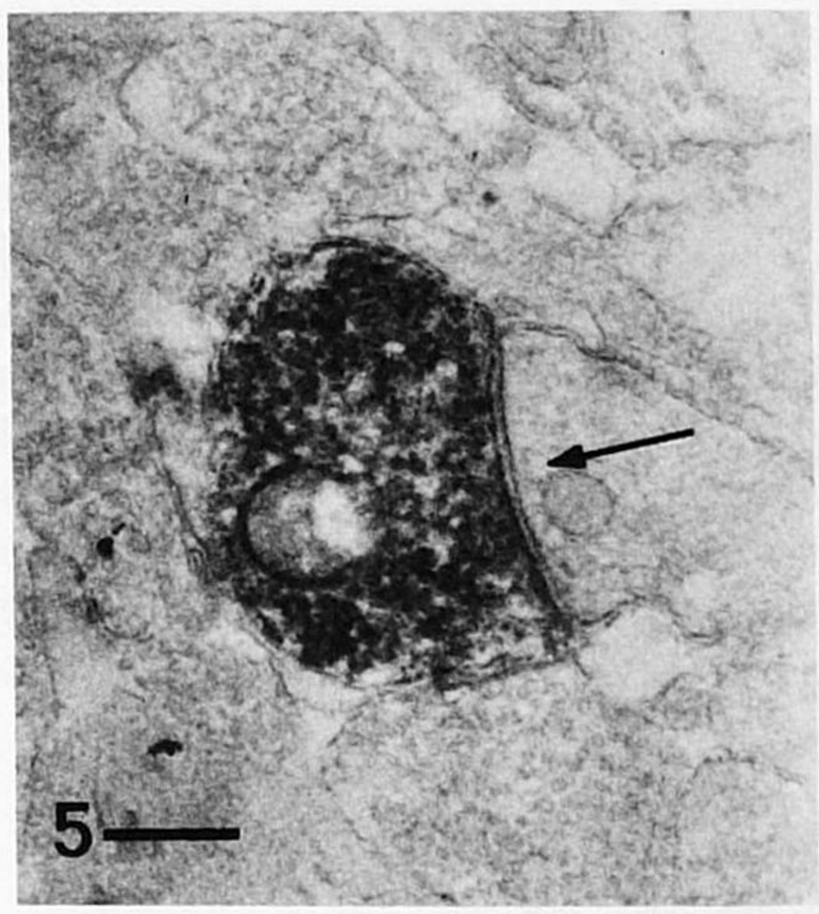
THE PEPTIDERGIC SYNAPSE

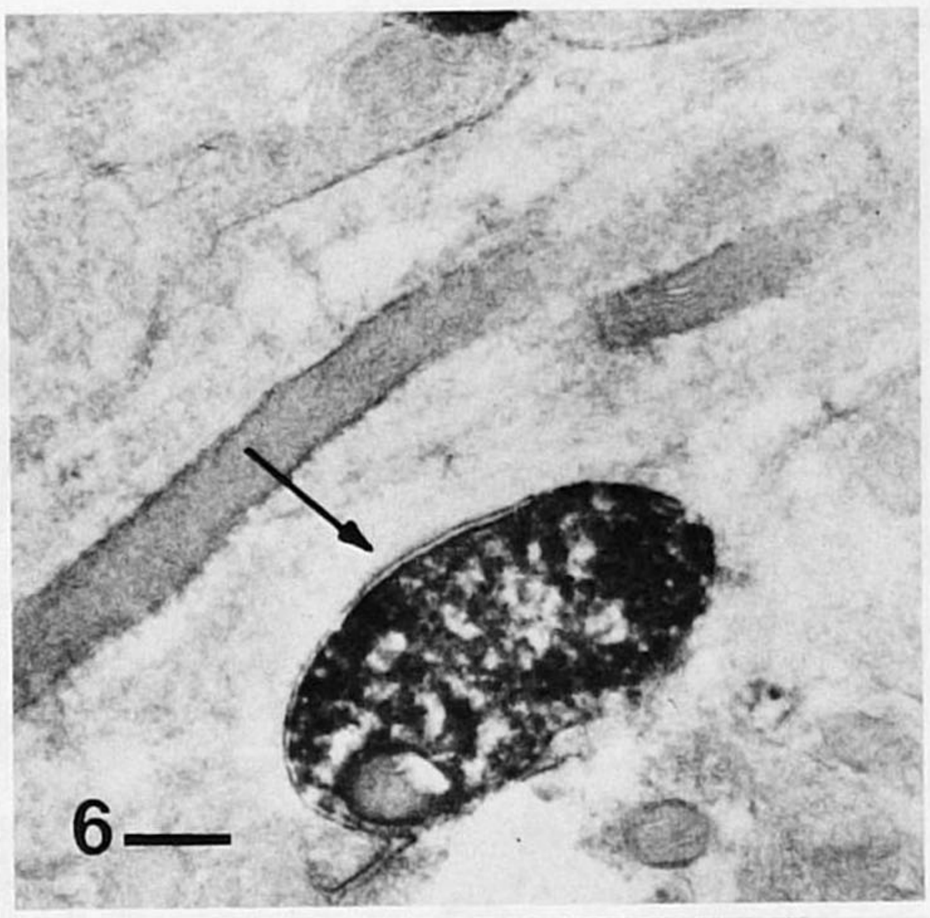
Although no conclusive evidence for actual fibre termination in a given structure can be obtained by means of light microscopy, fibre density and branching and perineuronal structures indicate that this is the case in the lateral septum (Figs. 2 and 3), lateral habenular nucleus, ventral hippocampus, nuclei of the amygdala, nucleus tractus solitarius and nucleus ambiguus.

In order to verify these putative fibre terminations, immunoelectron microscopy was performed on the most densely innervated structures; i.e. the lateral septum and the lateral habenular nucleus as target areas of the SCN, and the nuclei of the amygdala for the PVN fibres. Using the pre-embedding staining technique, a very good correlation was found between the light microscopical results and electron microscopy (Buijs and Swaab, 1979).

In all 3 regions, synaptic structures containing vasopressin were frequently found to terminate mostly on dendrites (Figs. 4–7). These synapses contained clear vesicles and/or dense core vesicles, the latter with a diameter of approximately 100 nm were positively stained for vasopressin. A synaptic cleft with the presence of a postsynaptic density was also sometimes noted (Buijs and Swaab, 1979). These structures containing neurohypophyseal hormones were indistinguishable from classical neurotransmitter-containing synapses, as visualized by means of a similar pre-embeddding staining technique (e.g. Pickel et al., 1976). Infrequently, an oxytocin-containing synapse was seen in the nuclei of the amygdala. Most of these peptidergic synapses were found to terminate on dendrites, and not on the cell bodies as had been supposed on the basis of light microscopical results (Buijs et al., 1978; Sofroniew and Weindl, 1978). In the lateral septum, synapses "en passage" terminating both on a cell body and its dendrite were sometimes observed (Figs. 8 and 9). Since vasopressin-containing terminations were found on cell bodies (Fig. 7) and dendrites, and since monoamines are not reported to be present in cell bodies or dendrites in these parts of the limbic system, it is not plausible that the effect of vasopressin and oxytocin on the monoamine metabolism in these regions (Kovacs et al., 1980) is a result of a direct action of these peptides. In order to enable a functional correlation between the finding of Kovacs et al. and ours, it seems necessary first to establish what type of transmitter the innervated cells produce and to what regions they project.

In the positively stained nerve fibres in the lateral septum and lateral habenular nucleus, vasopressin was present in granules of approximately 100 nm. This is in agreement with the observation that these fibres are derived from the SCN, where the same kind of vesicles are found (van Leeuwen et al., 1978). However, the presence of AVP and OXT in the amygdala and of OXT in the spinal cord, in granules of 100 nm, does not fit in with the observation that the PVN (where these fibres originate), contains granules of a larger size viz. 130 nm (Krisch, 1974). A comparable observation was reported by Dube et al. (1976) who demonstrated vasopressin-positive dense core vesicles, 90 nm in diameter, in fibres within the external zone of the median eminence, which are also thought to come from the PVN (Vandesande et al., 1977). Electrophysiological results suggest, moreover, that these fibres





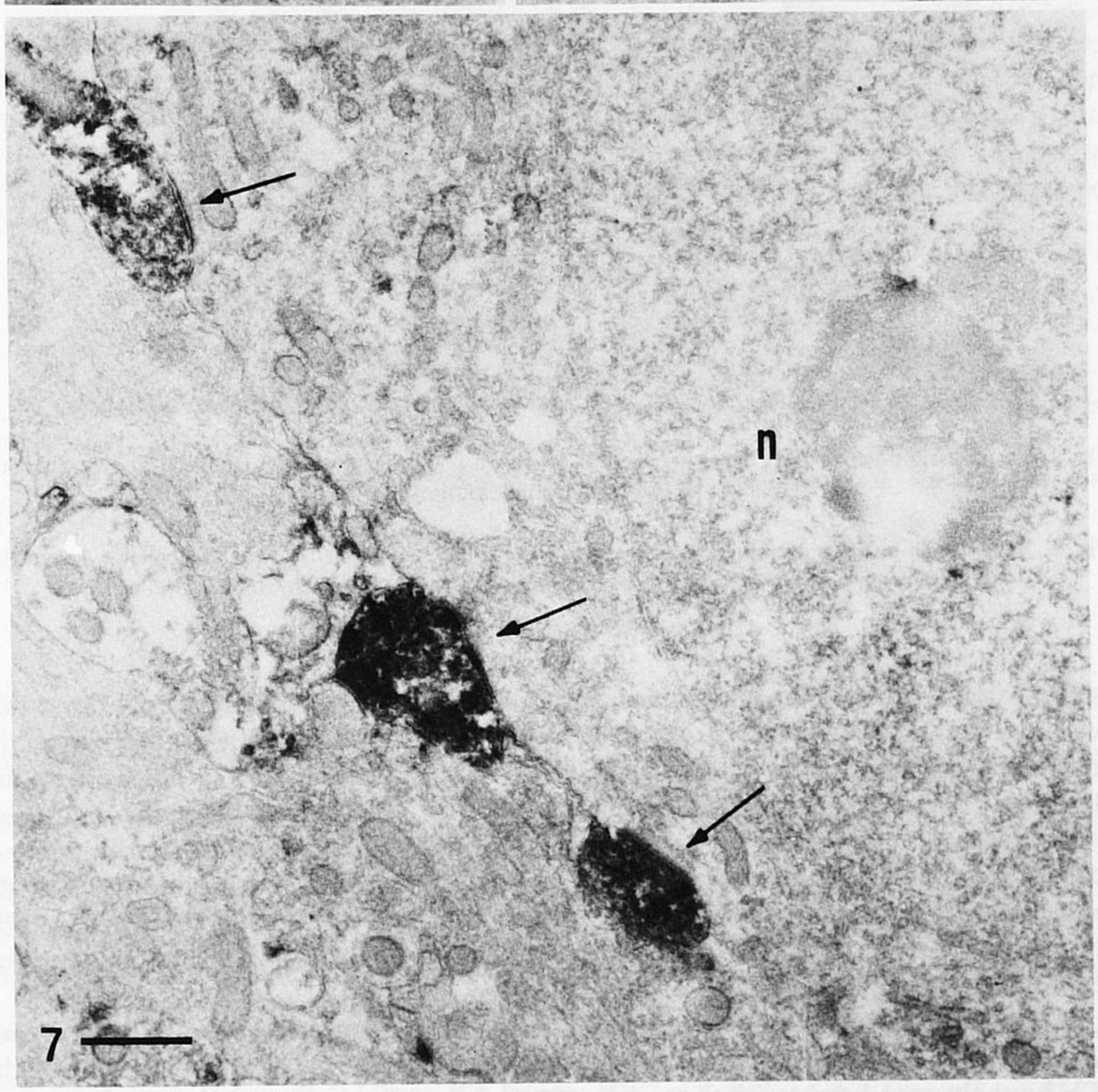


Fig. 5. Vasopressin-positive terminal forming a synapse (arrow) with a dendrite in the medial nucleus of the amygdala. Bar, 0.25 μm.

Fig. 6. Vasopressin-positive synapse (arrow) with an unlabelled dendrite in the lateral septum. Bar, 0.25 μm.

Fig. 7. Three vasopressin-positive synapses (arrows) with a cell body in the lateral septum. n, nucleus. Bar, 0.5 μm.

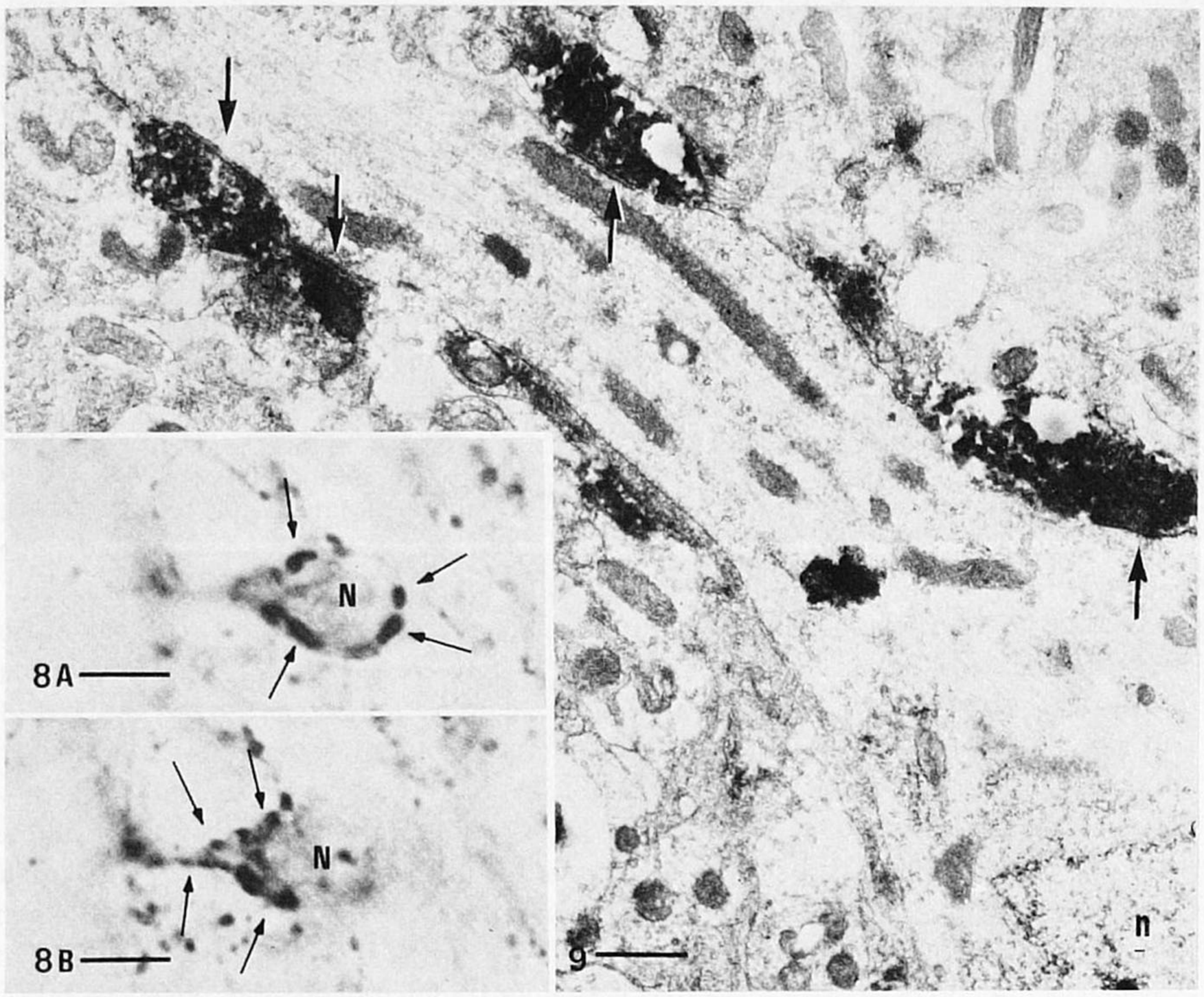


Fig. 8. Vasopressin-positive fibre (arrows) around a neuron (N) and its process, focused at two different levels (A, B) in a 20 μm thick section. Bar, 10 μm.

Fig. 9. Vasopressin-positive terminals (arrows) on a cell body (n, nucleus) and its dendrite, suggesting synapses "en passage". Contrasted with uranyl acetate and lead citrate. Bar, 0.5 μm.

in the external zone of the median eminence might be axon collaterals of PVN fibres that run towards the neurohypophysis (Pittman et al., 1978). Since in the neurohypophysis the granular size is approximately 150 nm (van Leeuwen and Swaab, 1977), this would point either to a selection mechanism for small granules in axon collaterals, or to a different release mechanism in central brain regions and the neurohypophysis. Vasopressin immunoreactivity was frequently found at the surface of clear, vesicle-like structures. This might fit in with the idea that the smooth endoplasmic reticulum serves as an alternative vehicle for intra-axonal transport of non-granular neurosecretory material that gives rise to clear vesicles (Alonso and Assenmacher, 1978).

It is curious to note that neither the SCN nor its massive projections into the limbic system (Fig. 2) had been reported in the older studies by Barry, Legait or Sterba. Since the SCN has only once been reported to stain positively with such methods (Joussen, 1970), it is likely that its neurosecretory product is less readily stained than is that of the magnocellular system. In addition, the SCN fibres in general are thinner than those of the magnocellular PVN or SON, which could make it impossible to detect these fibres, as has also been reported in the case of Sterba's pseudo-isocyanine technique (Sterba et al., 1979).

Since the limbic structures innervated by the SCN, are also found to be important for the AVP effects on avoidance behavior (van Wimersma-Greidanus et al., 1976), the SCN might be necessary in this respect. Another function in which the SCN might be involved is the central regulation of water balance. Thus lesions in the terminal field of the AVP fibres coming from the SCN, or lesions interrupting these fibres, caused a disturbed water balance in rats and goats (Johnson and Buggy, 1978; Andersson et al., 1975). This could mean that the SCN is involved in an indirect feed-back control mechanism upon the peripheral release of AVP. An indication in support of such a possibility is that septal stimulation inhibits PVN spike frequency (Negoro et al., 1973). In addition, direct connections exist between cells of the septum and the SON (Ellendorff et al., 1979).

CONCLUSION

It has been found both by immunocytochemistry and by radioimmunoassay (Dogterom et al., 1978) that AVP and OXT are present in a wide variety of regions in the central nervous system. In addition, it has now been demonstrated that these peptides are localized within synaptic structures which are morphologically indistinguishable from the classical neurotransmitter-containing synapses. It is therefore tempting to speculate that these peptides act as neurotransmitters within the central nervous system.

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REFERENCES

Alonso, G. and Assenmacher, I. (1978) The smooth endoplasmic reticulum in neurosecretory axons of the rat neurohypophysis. *Biol. Cell.*, 32: 203–206.

Anderson, C.H. (1978) Time of neuron origin in the anterior hypothalamus of the rat. *Brain Res.*, 154: 119–122.

Andersson, B., Leksell, L.G. and Lishajko, F. (1975) Perturbation in fluid balance induced by medially placed forebrain lesions. *Brain Res.*, 99: 261–275.

Barry, J. (1954) Neurocrinie et synapses "neurosécrétoires". *Arch. Anat. Mikr.*, 43: 310–320.

Barry, J. (1961) Recherches morphologiques et expérimentales sur la glande diencephalique et l'appareil hypothalamo-hypophysaire. *Ann. Sci. Univ. Besancon Zool. Physiol.*, Ser. 2: 3–133.

Barry, J., Besson, S. and LaMarche, M. (1958) Recherches sur les activités post hypophysomimetiques des extraits de noyaux amygdaliens de cobayes. *Ann. Endocrinol. (Paris)*, 19: 1045.

Boer, G.J., Swaab, D.F., Uylings, H.B.N., Boer, K., Buijs, R.M. and Velis, D.N. (1980) Neuropeptides in rat brain development. In *Adaptive Capabilities of the Nervous System, Progress in Brain Research, Vol. 53*, P. McConnell, G.J. Boer, H.J. Romijn, N.E. van de Poll and M.A. Corner (Eds.), Elsevier, Amsterdam, pp. 207–227.

Buijs, R.M. (1978) Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat; pathways to the limbic system, medulla oblongata and spinal cord. *Cell Tiss. Res.*, 192: 423–435.

Buijs, R.M. and Swaab, D.F. (1980) Immuno-electron microscopical demonstration of vasopressin and oxytocin synapses in the limbic system of the rat. *Cell Tiss. Res.*, 204: 355–365.

Buijs, R.M., Swaab, D.F., Dogterom, J. and Leeuwen, F.W. van (1978) Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. *Cell Tiss. Res.*, 186: 423–433.

Dogterom, J., Snijdewint, F.G.M. and Buijs, R.M. (1978) The distribution of vasopressin and oxytocin in the rat brain. *Neurosci. Lett.*, 9: 341–346.

Dube, D., Leclerc, R. and Pelletier, G. (1976) Electronmicroscopic immunohistochemical localization of vasopressin and neurophysin in the median eminence of normal and adrenalectomized rats. *Amer. J. Anat.*, 146: 103–108.

Ellendorff, F., Poulain, D.A. and Vincent, J.D. (1978) An electrophysiological study of septal input to oxytocin and vasopressin neurones in the supraoptic nucleus of the rat. *J. Physiol. (Lond)*, 284: 124P.

Ifft, J.D. (1972) An autoradiographic study of the time of final division of neurons in rat hypothalamic nuclei. *J. comp. Neurol.*, 144: 193–204.

Johnson, A.K. and Buggy, J. (1978) Periventricular preoptic hypothalamus is vital for thirst and normal water economy. *Amer. J. Physiol.*, 234: R122–R129.

Joussen, F. (1970) Zur morphologie des supraoptico-hypophysären systems beim Kaninchen. *Z. Zellforsch.*, 103: 544–558.

Kovács, G.L., Bohus, B. and Versteeg, D.H.G. (1980) The interaction of posterior pituitary neuropeptides with monoaminergic neurotransmission: significance in learning and memory processes. In *Adaptive Capabilities of the Nervous System. Progress in Brain Research, Vol. 53*, P. McConnell, G.J. Boer, H.J. Romijn, N.E. van de Poll and M.A. Corner (Eds.), Elsevier, Amsterdam, pp. 123–140.

Krisch (1974) Different populations of granules and their distribution in the hypothalamo-neurohypophysical tract of the rat under various experimental conditions. I. Neurohypophysis, nucleus supraopticus and nucleus paraventricularis. *Cell Tiss. Res.*, 151: 117–140.

Leeuwen, F.W. van, Swaab, D.F. and Raay, C. de (1978) Immunoelectronmicroscopic localization of vasopressin in the rat suprachiasmatic nucleus. *Cell Tiss. Res.*, 193: 1–10.

Leeuwen, F.W. van and Swaab, D.F. (1977) Specific immunoelectronmicroscopic localization of vasopressin and oxytocin in the neurohypohysis of the rat. *Cell Tiss. Res.*, 177: 493–501.

Legait, H. (1958) Les voies extra-hypothalamo-neurohypophysaires de la neurosécrétion diencéphalique dans la série des vertébrés. In *Zweites internationales symposium über neurosekretion*, W. Bargman, B. Hanström, B Scharrer and E. Scharrer (Eds.), Springer, Berlin, pp. 42–51.

Legait, H. and Legait, E. (1958) Présence d'une voie neurosécrétoire hypothalamo-habénulaire et mise en évidence d'une activité antidiurétique au niveau des ganglions de l'habénula chez la poule. *Comp. Rend. Soc. Biol.*, 152: 828–830.

Naumann, W. and Sterba, G. (1976) Ultrastructural studies on neurophysine-containing vesicles of the neurosecretory system of vertebrates. *Cell Tiss. Res.*, 165: 545–553.

Negoro, H., Visessuwan, S. and Holland, R.C. (1973) Inhibition and excitation of units in paraventricular nucleus after stimulation of septum amygdala and neurohypophysis. *Brain Res.*, 57: 479–483.

Pickel, V.M., Joh, T.H. and Reis, D.J. (1976) Monoamine synthesizing enzymes in central dopaminergic noradrenergic and serotonergic neurons. Immunocytochemical localization by light- and electronmicroscopy. *J. Histochem. Cytochem.*, 24: 792–806.

Pittman, Q.J., Blume, H.W. and Renaud, L.P. (1978) Electrophysiological indications that individual hypothalamic neurons innervate both median eminence and neurohypophysis. *Brain Res.*, 157: 364–368.

Scharrer, E. (1951) Neurosecretion X. A relationship between the paraphysis and the paraventricular nucleus in the garter snake (Thamnopsis sp.). *Biol. Bull.*, 101: 106–113.

Sofroniew, M.V. and Weindl, A. (1978) Projections from the parvocellular vasopressin and neurophysin containing neurons of the suprachiasmatic nucleus. *Amer. J. Anat.*, 153: 391–430.

Sterba, G. (1974) Ascending neurosecretory pathways of the peptidergic type. In *Neurosecretion – The Final Neuroendocrine Pathway*, F. Knowles, L. Vollrath (Eds.), Springer, Berlin, pp. 38–47.

Sterba, G., Hoffman, E., Solecki, R., Naumann, W., Hoheisel, G. and Schober, F. (1979) The neurosecretory hypothalamo-hindbrain pathway and its possible significance for the regulation of blood pressure and the milk ejection reflex. *Cell Tiss. Res.*, 196: 321–336.

Sterba, G., Naumann, W. and Hoheisel, G. (1980) Exohypothalamic axons of the classic neurosecretory system and their synapses. In *Adaptive Capabilities of the Nervous System, Progress in Brain Research, Vol. 53*, P. McConnell, G.J. Boer, H.J. Romijn, N.E. van de Poll and M.A. Corner (Eds.), Elsevier, Amsterdam, pp. 141–158.

Sternberger, L.A. (1974) *Immunocytochemistry,* New York, Prentice Hall, Englewood Cliffs.

Swaab, D.F. and Pool, C.W. (1975) Specificity of oxytocin and vasopressin immunofluorescence. *J. Endocr.,* 66: 263–272.

Vandesande, F., Dierickx, K. and Mey, J. de (1977) The origin of the vasopressinergic and oxytocinergic fibres of the external region of the median eminence of the rat hypophysis. *Cell Tiss. Res.,* 180: 443–452.

Wied, D. De and Gispen, W.H. (1977) Behavioral effects of peptides. In *Peptides in Neurobiology,* H. Gainer (Ed.), New York, Plenum Press, pp. 397–448.

Wimersma-Greidanus, Tj.B. van, Bohus, B. and Wied, D. De (1976) CNS sites of action of ACTH, MSH and vasopressin in relation to avoidance behavior. In *Anatomical Neuroendocrinology,* W.E. Stumpf and L.D. Grant (Eds.), Karger Basel, pp. 284–289.