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Immunoelectronmicroscopic Localization of Vasopressin in the Rat Suprachiasmatic Nucleus*

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Summary. The classical areas for arginine-vasopressin (AVP) synthesis are the magnocellular supraoptic (SON) and paraventricular nuclei. More recently AVP was also demonstrated in neurons of the parvocellular suprachiasmatic nucleus (SCN) of the rat. This result was substantiated in the present study by means of immunoelectron microscopy, by subjecting sections to anti-vasopressin plasma. Conventional electron microscopy revealed dense-core vesicles in the SCN cell bodies and fibres (mean diameter 94.7± 0.9 nm and 84.0± 1.1 nm respectively). These vesicles were infrequent within the cell bodies and could not be accumulated by ethanol administration. Immunoelectron microscopy showed a positive reaction in the cell bodies and fibres within vesicles of 93.7± 1.1 nm and 98.5± 1.2 nm respectively. By comparison, the cell bodies and fibres of the SON showed immunoreactive granules of 143.0± 1.8 and 147.3± 1.8 nm respectively. The presence in the SCN of AVP in vesicles of different size than those in the SON suggests that synthesis of this substance is indeed occurring within the SCN cells.

Key words: Hypothalamus – Suprachiasmatic nucleus – Supraoptic nucleus – Vasopressin – Immunoelectron microscopy.

Peptidergic (arginine-vasopressin, AVP), luteinizing-hormone-releasing hormone (LHRH) and dopa decarboxylase positive substances have been localized in cell bodies of the suprachiasmatic nucleus (SCN), while the presence of adrenaline, serotonine, AVP, LHRH, thyrotrophin releasing hormone, somatostatin, sub-

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stance P and vasoactive intestinal peptide have been reported in fibres. Histamine has been measured also in this area but was not localized immunocytochemically (for review see Hökfelt et al., 1978).

Paraldehyde fuchsin staining in the rabbit parvocellular SCN (Joussen, 1970) indicated for the first time the possible presence of neurosecretory material comparable to that in the magnocellular cells of the supraoptic (SON) and paraventricular (PVN) nuclei. Indeed immunocytochemical studies at the light microscopic level revealed neurophysin (Vandesande et al., 1974) and vasopressin (e.g., Swaab et al., 1975; Vandesande et al., 1975; Van Leeuwen et al., 1976). This peptide is most probably AVP since it is absent in the SCN cells of homozygous Brattleboro rats (Swaab and Pool, 1975) and is also demonstrable by means of radioimmunoassay (George and Forrest, 1976). Yet, definite proof for the AVP nature, e.g., by a combination of separation and immunocytochemical methods (see Swaab et al., 1977), has still to be given.

Conventional electron microscopy in the SCN of rat, rabbit and mouse has revealed a wide range of dense-core vesicle diameters (50–250 nm) in the cell bodies and processes of this nucleus (Suburo and Pellegrino de Iraldi, 1969; Clattenburg et al., 1972, 1975; Wenisch, 1975). In the present study immunoelectron microscopy was used in order to establish whether vasopressin is present in the dense-core vesicles of the rat's SCN. In addition, the granules of the SON cells were studied in some of the same rats to determine the possible difference between the neurosecretory granules of parvo- and magnocellular origin.

Materials and Methods

Seven male Wistar rats and three male Brattleboro rats (homozygous for diabetes insipidus), weighing 200–300 g, were obtained from TNO (Zeist, The Netherlands) and received tap water and standard chow ad libitum. The Wistar rats were given different treatments in order to search for an accumulation of vesicles in the SCN cell bodies: a) untreated control animals ($n = 2$); b) animals subjected to a regime of 7 days of ethanol (10 ml 50% ethanol/kg body weight) given by gastric tube daily at 9.00 a.m. and 17.00 p.m. ($n = 2$); c) animals that received the same amount of tap water according to an identical scheme ($n = 3$). On the 7th day, 1–2 h after the last treatment the animals were anaesthetized with Nembutal (0.1 ml/100 g body weight i.p.), perfused intracardially with 0.9% saline followed by 2.5% glutaraldehyde – 1% paraformaldehyde in 0.1 M sodium cacodylate/sucrose buffer, pH 7.35. Brain slices of about 1 mm which contained the SCN and SON were subsequently immersed in the same fixative for 2 h at 4° C, dehydrated (van Leeuwen, 1977) and embedded in Epon 812. The Epon mixture was modified in order to obtain harder material and thus large (up to 4 square mm) ultrathin sections. It contained 9.5 ml of Epikote 812, 4.0 ml of dodecenyl succinic anhydride, 5.8 ml of methyl nadic anhydride and 0.3 ml of 2,4,6-Tris (dimethyl aminomethyl) phenol. The capsules were polymerized for 24 h at 45° C followed by 48 h at 60° C. In order to select the vasopressin containing part of the SCN (Fig. 1), semithin (2 µm) Epon sections were etched for 2 min in sodium methanolate according to Mayor et al. (1961), and stained immunocytochemically with anti-vasopressin (# 125 diluted 1 : 400; for details on the procedure see Buijs et al., 1978). Ultrathin sections containing both sides of the SCN were then mounted on nickel grids of 200 mesh. Some sections were doubly contrasted for 60 min with 2% uranyl acetate in 50% ethanol and for 10 min with lead citrate (Reynolds, 1963). The immunocytochemical procedure of Van Leeuwen and Swaab (1977) was followed, except for the incubation with anti-vasopressin (# 126, diluted 1 : 800) for up to 24 h at 4° C.

Diameters of dense core vesicles were measured in micrographs (magnification 26,600) with the use of a dissecting microscope (magnification 3.5×) in which an object micrometer was placed (100 lines/cm). The magnification was controlled with an object micrometer grid ($d = 0.463\,\mu$). For

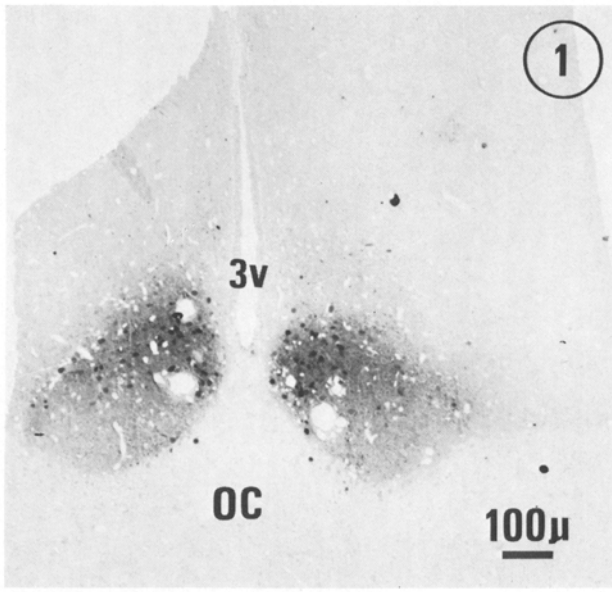

Fig. 1. Semithin section of immunocytochemically stained SCN of Wistar rat hypothalamus, incubated with anti-vasopressin plasma (1:400). Note reaction in cell bodies and fibres. Oblique boundary at left used for orientation of specimen. *OC* optic chiasm; *3V* third ventricle. ×68

comparison, the granules of the SON were measured in three animals (one from each group). In the SCN all granules were measured, in the SON only randomly selected ones.

For control incubations pre-immune rabbit plasma was used instead of the first antibody or anti-vasopressin plasma (# 126) pre-absorbed to AVP containing agarose beads (Swaab and Pool, 1975). In addition, sections of homozygous Brattleboro SCN were incubated with anti-vasopressin plasma (1:800) in order to confirm its specificity.

Results

The majority of the SCN cell bodies had only a small rim of cytoplasm around the nucleus in all three experimental groups. Sometimes the nonmyelinated fibres could be followed for a considerable distance (up to 22 µm). Only few dense core vesicles were seen in the cell bodies and fibres. In the SCN cell bodies and fibres of the homozygous Brattleboro rat that showed no obvious differences from those of the Wistar SCN, dense core vesicles were present. After an incubation time of 24 h with the first antibody a positive reaction was observed in the Wistar SCN and SON cell bodies and fibres (Figs. 2, 3). No difference in the mean diameter of the vesicles in the SCN cell bodies was observed among the three experimental groups; after conventional "staining" they were respectively a) 95.6± 1.7, (SEM), b) 95.3± 1.4, c) 93.3± 1.5 nm. After immunoincubation with anti-vasopressin the values were: a) 94.1± 2.2., b) 93.7± 3.4, c) 93.5± 1.5 nm. In conventionally stained nerve fibres these data were a) 86.4± 1.9, b) 83.0± 1.8 and c) 82.9± 1.8 nm; after the immunoincubation, a) 100.0± 2.3, b) 98.0± 2.5, and c) 97.7± 1.7 nm. Therefore the values of the measurements in these groups were pooled (Table 1).

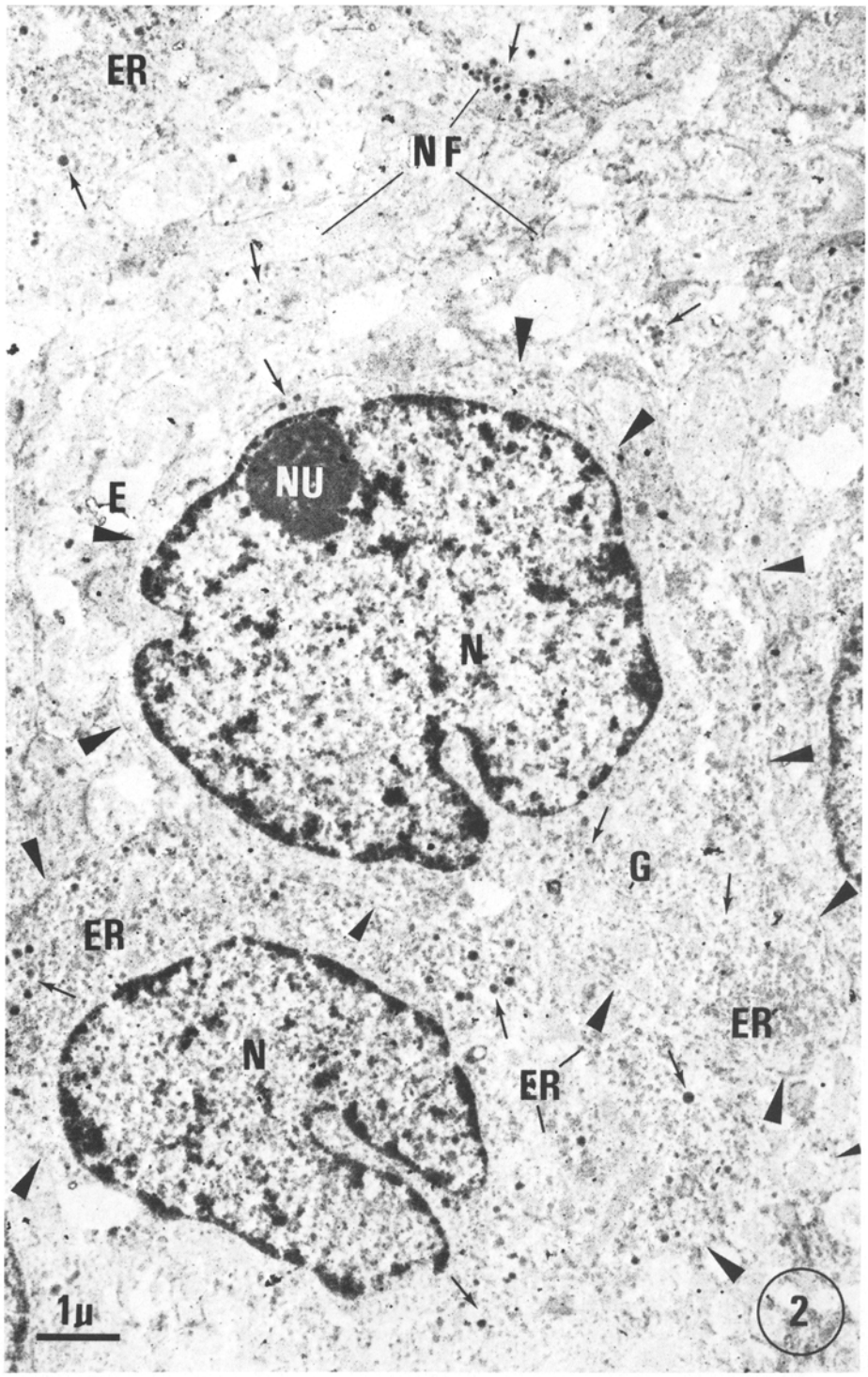

Fig. 2. Ultrathin SCN section of Wistar rat immunoelectron microscopically stained with anti-vasopressin plasma (1 : 800) showing positive reaction of perikaryonal dense-core vesicles (arrows) near endoplasmic reticulum (*ER*) and within nerve fibres (*NF*). Cell boundaries indicated by arrow heads. *E* extracellular space; *G* Golgi area; *N* nucleus; *NU* nucleolus. × 12,000

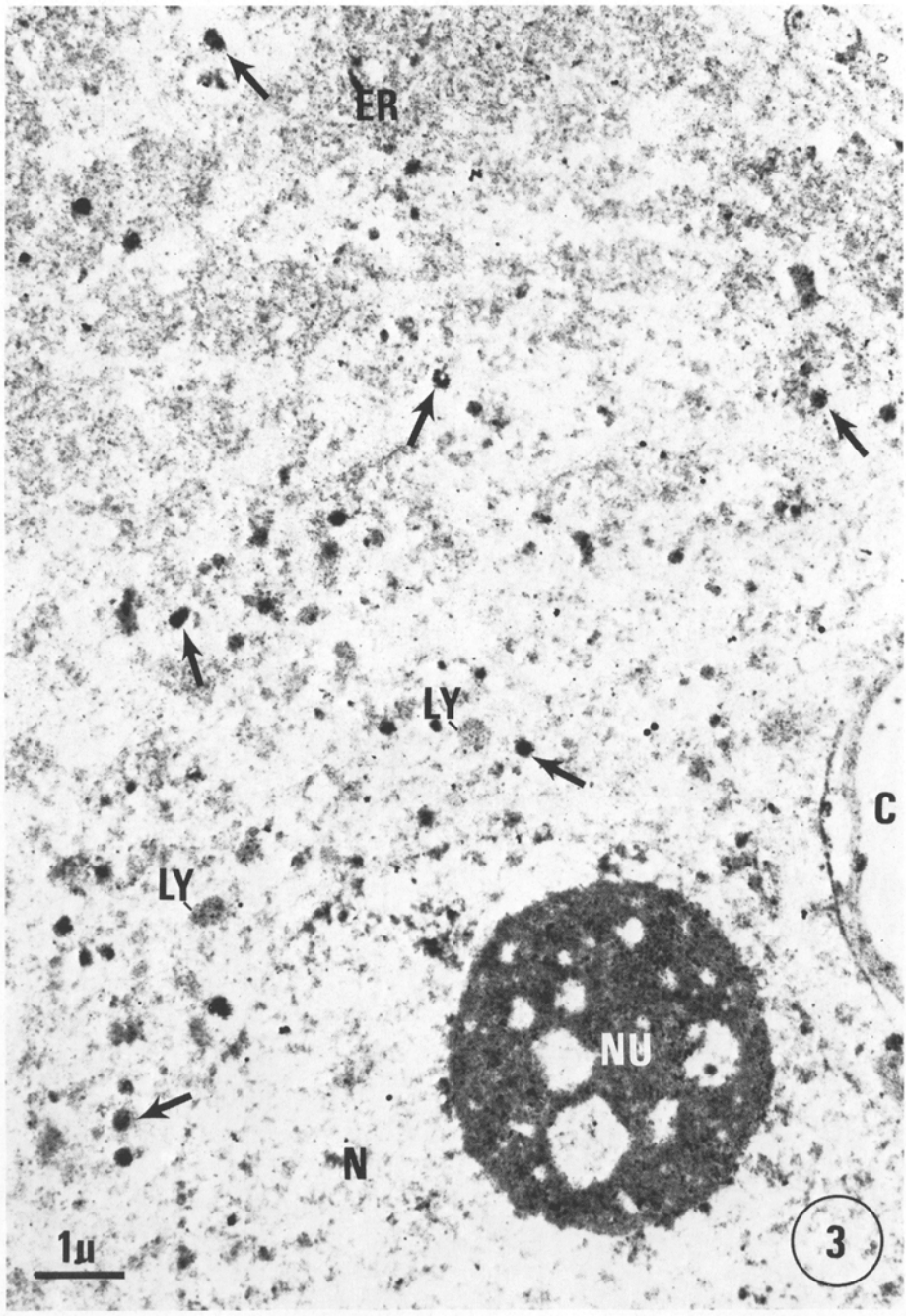

Fig. 3. Ultrathin SON section of same rat at same magnification as Fig. 2, stained immunocytochemically with anti-vasopressin plasma (1 : 800). Note positive reaction in numerous neurosecretory granules (arrows). *C* capillary; *ER* endoplasmic reticulum; *LY* lysosome; *N* nucleus; *NU* nucleolus. ×12,000

Table 1. Measurements of vesicular or granular diameters in rat suprachiasmatic and supraoptic nucleus

		U + Pb					AVP			
		$\bar{x}$ + SEM	*n*	*N*	diameter range (nm)		$\bar{x}$ + SEM	*n*	*N*	diameter range (nm)
SCN										
I	cell bodies	94.7 ± 0.9	246	31	58–141	III	93.7 ± 1.1	165	13	62–134
II	fibres	84.0 ± 1.1	207	46	48–123	IV	98.5 ± 1.2	194	31	65–160
SON										
V	cell bodies	142.3 ± 1.8	150	9	81–208	VII	143.0 ± 1.8	150	9	93–216
VI	fibres	146.3 ± 1.7	150	9	99–206	VIII	147.3 ± 1.8	150	9	99–204

U + Pb: "staining" with uranyl acetate and lead citrate. AVP: "immunostaining" with anti-arginine vasopres plasma. n = number of vesicles or granules. N = number of cell bodies or fibres. The significance of differen between the values was calculated using the Student t-test. A value for $P < 0.05$ was considered to be statistica significant. I–II $P < 0.001$, I–III $0.40 < P < 0.50$, II–IV $P < 0.001$, III–IV $P < 0.005$, V–VI $0.10 < P < 0.20$, V–V $0.70 < P < 0.80$, VI–VIII $0.60 < P < 0.70$, VII–VIII $0.05 < P < 0.10$

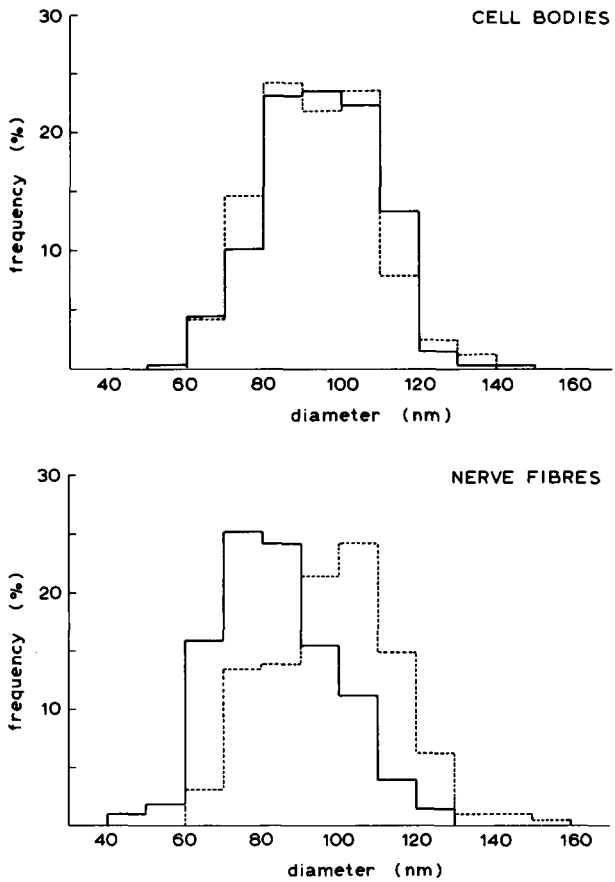

Fig. 4. Histograms showing distribution of dense-core vesicle diameters within cell bodies and nerve fibres of SCN after conventional (solid line) and immunocytochemical (broken line) staining. Note similar distribution in SCN cell bodies and different distribution in fibres

In the cell bodies of the SCN no difference in the diameter of the vesicles was observed after conventional versus immunostaining, whereas the vesicles in the fibres stained by immunoelectron microscopy were larger than those stained conventionally (Fig. 4, Table 1, and Discussion). In the SCN fibres the diameter of the AVP containing vesicles became larger as compared with the vesicles in the cell bodies. No significant difference exists between the diameters of the granules within the SON cell bodies and fibres after both staining procedures.

All control incubations showed no reaction product within the vesicles.

Discussion

The sparse number of vesicles in the cytoplasm of SCN neurons is one of the reasons why it is so difficult to localize vasopressin in the SCN at the electron microscopic level (Krisch, 1977). Selection of the AVP containing part of the SCN by immunocytochemical treatment of semithin sections appeared to be a valuable expedient for orientation. In addition, an attempt was made to accumulate these SCN vesicles by the administration of ethanol as previously reported by Midoux et al. (1975) for the PVN. Unfortunately, no such accumulation of vesicles occurred in the SCN following this treatment.

A second problem is the difficulty in staining the intravesicular content with the incubation procedures for the neurohypophysis (Van Leeuwen and Swaab, 1977). An extension of the incubation time up to 24 h at 4° C (Moriarty, 1973) enhanced the staining of the vesicle content. This also holds true for the granules in the SON cell bodies. The presence of AVP in vesicles suggests that in the SCN cell bodies synthesis is occurring although definite proof for this assumption has to be obtained with the use of pulse-chase studies (see Norström, 1975).

Earlier ultrastructural studies showed that a wide range of dense core vesicles is present in the perikarya of the SCN. Suburo and Pellegrino de Iraldi (1969) described 'granulated vesicles' of 50–250 nm in diameter in the SCN somata of the rat and three types of neuronal processes which frequently contained dense core vesicles of 70–80 nm. Clattenburg et al. (1972) observed dense core vesicles in the rabbit SCN with a diameter never exceeding 110 nm, while in a later paper dense core vesicles were measured with a mean of 104 nm and a diameter range of 70–170 nm. In the mouse SCN two groups of dense core vesicles were described (50–80 nm and 100–130 nm; Wenisch, 1975, 1976). Our measurements in the rat SCN are more in agreement with those in the rabbit (Clattenburg et al., 1972, 1975) and the mouse (Wenisch, 1976) SCN than those reported thus far in the rat (Suburo and Pellegrino de Iraldi, 1969), inasmuch as we were unable to confirm the presence of large vesicles in the SCN cells. Unfortunately, no further details about the frequency distribution of the vesicle diameters were given by the former authors. The difference in the present study between vesicular diameters within the SCN cell bodies and fibres after conventional staining (Table 1) can be explained by the presence of a second type of small dense core vesicles in the nerve fibres (Fig. 4). These non-AVP vesicles that were also described by Suburo and Pellegrino de Iraldi (1969) might contain the substances reported in the Introduction. The higher granular diameter in the nerve fibres than in the SON perikarya (see Zambrano and

De Robertis, 1966) appeared not significant in the present study; the larger size of AVP containing vesicles in the SCN nerve fibres as compared to those in the cell bodies was not reported before. It has to be noted that the moderate ultrastructural preservation (especially of the membranes, which is unavoidable in immuno-electron microscopy; see van Leeuwen, 1977), makes this material less appropriate for highly accurate morphometrical studies than that prepared by the conventional glutaraldehyde – osmium tetroxide techniques.

The function of AVP in the SCN is still unknown. Recent light microscopic immunocytochemical studies show that SCN fibres run to various brain areas: (a) organum vasculosum laminae terminalis, (b) lateral septum, (c) via the nucleus periventricularis and the PVN towards the lateral habenular nuclei, and (d) in the direction of the SON (Buijs et al., 1978; Buijs, 1978, submitted). In some of these areas, i.e., the lateral septum and the lateral habenular nuclei, punctate pericellular profiles containing AVP were observed suggesting a neurotransmitter-like function of AVP in these areas. Whether these fibres play a role in vasopressin-dependent behavioral processes (De Wied et al., 1976) has yet to be established. Formerly the AVP containing SCN fibres that spread also in a caudal direction (Buijs et al., 1978) were thought to run to the zona externa of the median eminence (Vandesande et al., 1974). Also electrophysiological and autoradiographical techniques suggested a pathway of SCN cells to the median eminence (Makara et al., 1972; Swanson and Cowan, 1975), although it cannot be said whether this concerns AVP containing SCN cells or fibres. Vesicles of mainly 90–110 nm containing neurohypophyseal substances were indeed localized in the zona externa of the median eminence (Silverman and Zimmerman, 1975; Dube et al., 1976). Moreover, the presence of small AVP containing vesicles also in the SCN is in agreement with their hypothesis. However, two recent publications offer proof that the neurohormone containing fibres of the zona externa arise mainly from the PVN (Vandesande et al., 1977; Lobo Antunes et al., 1977).

From our own study it appears that the SCN dense-core vesicles are definitely smaller than those in the SON of the same animals. Whether this size difference permits discrimination as to the origin of vasopressinergic nerve fibres in the central nervous system (Buijs et al., 1978) depends on the presence of a class of small immunoreactive granules in the PVN. For comparison, the neurosecretory granules of the SON were, in the present study, also stained with unpurified anti-vasopressin plasma at a low (1 : 800) dilution that might cross react with oxytocin in this nucleus. For further studies of the SON and PVN, purified or diluted antibodies (Van Leeuwen and Swaab, 1977) are needed in order to distinguish between the vasopressin and oxytocin synthetizing cells.

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