Specific Immunoelectronmicroscopic Localization of Vasopressin and Oxytocin in the Neurohypophysis of the Rat*

F.W. van Leeuwen and D.F. Swaab

Netherlands Central Institute for Brain Research, Amsterdam, The Netherlands

Summary. An immunoelectronmicroscopic method for the specific localization of neurohypophyseal hormones was developed in neurohypophyses of Wistar and Brattleboro rats, the latter strain being homozygous for diabetes insipidus. If the proper precautions were omitted, a marked cross reactivity between anti-vasopressin and anti-oxytocin preparations was found. Cross reaction of an anti-vasopressin plasma with oxytocin, at a dilution of less than 1:1600, resulted in electron density of all granules within neurosecretory fibres of the Brattleboro and Wistar neurohypophyses. However, this cross reactivity could be eliminated either by sufficient dilution of the anti-plasma, or by its purification. Purification of the antibodies was performed by absorption to agarose beads coated with the cross reacting component. Upon incubation with anti-vasopressin (diluted unpurified 1:1600 or purified 1:80) and unpurified anti-oxytocin (1:400) plasma, sections of a Wistar neurohypophysis revealed two types of neurosecretory fibres, containing either electron dense or lucent granules. Oxytocin and vasopressin containing neurosecretory fibres were found as clusters in the neurohypophysis. The specificity of both unpurified anti-vasopressin (1:1600) and anti-oxytocin (1:400) plasma was confirmed on serial sections of a Wistar neurohypophysis, alternately incubated with the solutions of the two antibodies.

These data prove that the one-cell-one-hormone hypothesis holds true for the hypothalamic-neurohypophyseal system.

Key words: Vasopressin — Oxytocin — Neurohypophysis — Specificity — Immunoelectronmicroscopy.

Send offprint requests to: F.W. van Leeuwen, Netherlands Central Institute for Brain Research, IJdijk 28, Amsterdam-O., The Netherlands

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Introduction¹

With the use of immunocytochemical techniques, the presence of oxytocin and vasopressin has been established in the hypothalamic-neurohypophyseal system (HNS) of the rat at the light microscopic level (e.g., Swaab et al., 1975a). The simultaneous demonstration within one section of each of the two hormones in different perikarya of the supraoptic (SON) and paraventricular (PVN) nuclei (Vandesande and Dierickx, 1975a), the characteristic distribution in the fronto-caudal (Swaab et al., 1975b) and dorso-ventral (e.g., Van Leeuwen et al., 1976) directions of hormone-containing cell bodies in both the SON and the PVN, and the fact that parts of the same cell, observed in two subsequent sections, only stained with one of the two antibodies (Swaab et al., 1975a) suggest very strongly that the "one-cell-one-hormone hypothesis" (e.g., Sokol and Valtin, 1967) holds true for the HNS. Conclusive evidence for this assumption, however, can be obtained by immunoelectronmicroscopy.

The two immunoelectronmicroscopic investigations performed on the hypothalamic-neurohypophyseal system (HNS) of two different species, the rat (LeClerc and Pelletier, 1974) and the guinea pig (Silverman and Zimmerman, 1975) respectively, showed, in contrast to expectation, only one type of granule in the neurohypophysis. However, these authors used absorption as proof for specificity, a procedure which does not exclude cross reaction with related peptides (Swaab and Pool, 1975).

In the present study both vasopressin and oxytocin were localized specifically with a technique that has been optimized in various aspects (Van Leeuwen, in preparation).

When the present study was completed, the paper of Aspeslagh et al. (1976) appeared. Although the present paper deals with the same subject and confirms Aspeslagh's observations, it differs with respect to fixation and therefore to the absolutely electron lucent neurosecretory granules after control incubation, the use of Brattleboro neurohypophyses as a control for the specificity of anti-vasopressin plasma, and the observation that endings containing one of either hormone are localized in separate clusters. In addition, the present paper contains the new observation that cross reaction is disappearing during dilution, a fact which makes purification of the antibodies redundant.

Materials and Methods

Male Wistar rats and male Brattleboro rats (homozygous for diabetes insipidus), all weighing about 200 g were obtained from TNO, Zeist. The Brattleboros were checked for homozygosity in metabolism cages (Swaab et al., 1973). The animals received tap water and standard chow ad libitum.

The pituitary glands were fixed by immersion in situ with 4% buffered formalin (1965 mOsm/kg H₂O) ('Analar', BDH) for 24 h at -4°C, dissected out and washed for 24 h in buffered sucrose (350 mOsm/kg H₂O; for details, see Swaab et al., 1975a). The neural lobes were then embedded in Epon 812 (Merck) (Luft, 1961). Ultrathin sections were mounted on 300 mesh nickel grids. In order to observe the ultrastructure and the presence of intragranular material, some sections

¹ Part of this study appeared as an abstract (J. Histochem. Cytochem., 24, 618, 1976)

Fig. 1a and b. Immunocytochemically stained-formalin fixed neurohypophyseal section of Wistar rat with: **a** anti-vasopressin plasma (1:800); *n* neurosecretory granules, *p* perivascular cell with nucleus (*nu*) and endoplasmic reticulum (*er*). Note that all nerve fibres are stained; **b** control plasma (1:800). Note absence of osmiophilia in neurosecretory granules (*n*) and rather high background staining. *cf* collagen fibres in perivascular space (*pvs*), *c* capillary

were doubly contrasted for 30 min with 2% uranyl acetate in 50% ethanol and for 5 min with lead citrate (Reynolds, 1963).

For immunocytochemistry, post-embedding staining was performed by the unlabeled antibody enzyme method (see Sternberger, 1974). All incubations lasting 10 min were performed by placing the grids in drops of the immunocytochemical reagents. The first antibody was either anti-arginine-vasopressin (126) or anti-oxytocin (02C) plasma. Only anti-vasopressin plasma was purified once with agarose beads coated with oxytocin (for details, see Swaab and Pool, 1975). The purified and unpurified antiplasmas were diluted from 1:40 up to 1:200 and 1:200 up to 1:3200 respectively in phosphate-buffered saline (PBS, pH 7.4, 258 mOsm/kg H₂O) containing 1% swine serum. As second antibody, swine anti-rabbit IgG (Fc fragment) serum (Nordic) was used in a dilution of 1:10 in PBS. As the next step, peroxidase-anti-peroxidase (PAP) containing 1.49 mg peroxidase and 4.15 mg rabbit anti-peroxidase in one millilitre was used. On the day of use, this PAP was diluted 1:60 in PBS containing 1% of swine serum.

All three steps were preceded by an incubation period with swine serum diluted 1:30 in PBS. After incubation with diaminobenzidine (Sigma) and subsequently with OsO₄ (Merck) no further "staining" was performed. All antisera were stored at -25°C.

Controls, Specificity

For control incubations pre-immune rabbit plasma was used instead of the first antibody. The specificity of arginine-vasopressin was tested on sections of homozygous Brattleboro neurohypophyses. The specificity of anti-oxytocin and of anti-vasopressin plasma was confirmed by staining of the same nerve fibres in serial sections that were alternately incubated either with anti-vasopressin or with anti-oxytocin. In order to minimize masking of these sections by grid bars, sections were collected on 100 mesh nickel grids that were coated with 2% pioloform F (Wacker-Chemie) in chloroform.

Results

The neurosecretory granules (mean diameter 150 nm) of all neurosecretory fibres of the neurohypophyses of Wistar rats showed intensive staining after incubation with either anti-vasopressin or anti-oxytocin plasma, in dilutions of less than 1:1600 (Fig. 1a) and 1:400, respectively. Control incubations with normal rabbit plasma did not reveal any reaction product (Fig. 1b), while conventional staining still showed intragranular material (Fig. 3, inset).

Upon incubation of sections of a Brattleboro neurohypophysis in which no vasopressin is present, with anti-oxytocin plasma in dilutions up to 1:400, a positive reaction was found in all neurosecretory granules of all nerve fibres (Fig. 2a). Also, a positive reaction was apparent after incubation with anti-vasopressin plasma in dilutions less than 1:1600 (Fig. 2b). This pointed to cross reaction of this anti-vasopressin plasma with the heterologous hormone (oxytocin). However, this cross reaction could be eliminated either by dilution or by purification of the antiplasma. With dilutions of 1:1600 for unpurified

Fig. 2a and b. Immunocytochemically stained-formalin fixed neurohypophyseal section of Brattleboro rat homozygous for diabetes insipidus with: **a** anti-oxytocin plasma (1:400); **b** anti-vasopressin plasma (1:400), showing cross reaction with oxytocin. These nerve fibres are situated in separate groups distributed over entire extent of neurohypophysis. *Inset*: anti-vasopressin plasma (1:1600). Note absence of reaction product within neurosecretory granules. *c* capillary; *n* neurosecretory granules; *p* pituicyte; many lipid droplets (*l*) in cytoplasm; *nu* nucleus

Fig. 4a and b. Immunocytochemically stained-formalin fixed serial neurohypophyseal sections of Wistar rat with: **a** anti-vasopressin plasma (1:1600); **b** anti-oxytocin plasma (1:400). *dn* electron dense neurosecretory granules, *ln* electron lucent neurosecretory granules

antiplasma and 1:80 for purified anti-vasopressin plasma, the Brattleboro neurohypophysis did not show any staining of granules (Fig. 2, inset).

When sections of the Wistar neurohypophysis had been treated with these dilutions of anti-vasopressin plasma, two types of neurosecretory fibres were observed, the one containing only *electron dense* (Fig. 3a1), the other only *electron lucent* neurosecretory granules (Fig. 3a2). Equally, after incubation with anti-oxytocin plasma in a dilution of 1:400, these two types of neurosecretory fibres were observed in Wistar rat neurohypophyses (Fig. 3b).

With serial sections of Wistar neurohypophyses the specificity of the antibodies was confirmed. Incubation with either anti-vasopressin (1:1600) or anti-oxytocin (1:400) plasma alternately resulted in a complementary set of nerve fibres, one type which stained after incubation with anti-vasopressin plasma being unstained after incubation with anti-oxytocin plasma, and another type showing the reverse. Each nerve fibre contained only one or the other of the two types of neurosecretory granules (Fig. 4).

Fig. 3a and b. Immunocytochemically stained-formalin fixed neurohypophyseal sections of Wistar rat with: **a** anti-vasopressin plasma (1:1600), showing nerve fibres containing either electron dense (*dn*) a1 or lucent (*ln*) a2 neurosecretory granules. Groups of one of these two types of nerve fibres distributed over sections may occupy considerable areas. Therefore it is practically impossible to show both fibre types in one micrograph. **b** anti-oxytocin plasma (1:400), showing region where these two types are near each other. Between positive granules some granule-like space (arrows). *Inset*: Same spaces in section contrasted conventionally (arrows). *dn* electron dense neurosecretory granules, *nu* nucleus of pituicyte (*p*)

Discussion

With formalin-fixed neurohypophyseal sections of Wistar and Brattleboro rats, a reasonable morphology and a good preservation of the antigenicity of the hormone was obtained. The unlabeled antibody enzyme method resulted in a distinct staining of the neurosecretory granular content while the control incubations did not show any reaction product. In agreement with the report of Silverman and Zimmerman (1975) we could not show any extragranular staining of both hormones.

Cross reactivity of antiplasmas is one of the pitfalls in immunocytochemistry. The commonly used absorption test as proof for specificity of antiplasmas had proven to be invaluable since cross reacting components will be also neutralized during this procedure (Swaab and Pool, 1975). A recent example of cross reactivity problems has been a report on the light microscopic localization of neurohypophyseal hormones (Sokol et al., 1976). Their finding that some cells stained with antibodies against both oxytocin and vasopressin can be fully explained by the presence of cross reacting components that they showed to be present using Brattleboro neurohypophyseal tissue.

At the electron microscopic level the same problem with cross reacting antibodies is present. In former reports (Leclerc and Pelletier, 1974; Silverman and Zimmerman, 1975) concerning the immunoelectronmicroscopic localization of vasopressin, the absorption test was also used as a specificity test. In the present study we used the neurohypophysis of the homozygous Brattleboro rat as a test for specificity of the anti-vasopressin plasma. This strain of rats provides us with a natural model system since vasopressin is not synthesized in this strain (e.g., Miller and Moses, 1969). Since the dilution of anti-vasopressin plasma, which revealed no staining in the Brattleboro neurohypophysis, resulted in staining of a number of fibres in Wistar rat neurohypophysis, one may conclude that in this strain the vasopressin containing granules bind the anti-vasopressin antibodies. From the fact that in the same sections also a number of non-staining fibres is present, in which electron lucent granules readily are observed, the conclusion can be drawn that these latter ones contain oxytocin. In this way the problem of aspecific staining because of cross reactivity is overcome. The specificity of both antibodies was confirmed by showing a complementary pattern of nerve fibres in alternately incubated serial sections of the Wistar neurohypophysis. In contrast to light microscopic immunolocalization (Swaab and Pool, 1975), no improvement of the reaction was obtained with the purified antiplasma in a dilution (1:80) that resulted in specific staining. At the light microscopical level purification of antiplasma was necessary since the staining capacity was lost at high dilutions of the antiplasma. In addition, it appears from our results that the difference between specificity of the same antiplasma in localization techniques (high cross reaction) and radioimmunoassay (low cross reaction) (Swaab et al., 1975b) might, at least partly, be explained by the higher dilutions used in radioimmunoassay.

The present results lead to the conclusion that the one-cell-one-hormone theory (e.g. Sokol and Valtin, 1967) holds true for the HNS and that the two hormones are present in distinct clusters in the neurohypophysis. These

conclusions are in agreement with former light (Vandesande and Dierickx, 1975; Swaab et al., 1975b) and electron microscopic (Aspeslagh et al., 1976) reports. However they are contradictory to other previous light (Sokol et al., 1976) and electron microscopic (Leclerc and Pelletier, 1974) reports. It seems reasonable to assume that those results are due to cross reactivity of the antibodies (see above). From the present study it is obvious that rather large areas of the neurohypophysis are occupied exclusively by either vasopressin or oxytocin fibres. Thus, it seems not impossible that only one type of fibres is observed, if one is not aware of this phenomenon. In the light of this, part of the controversial data of Leclerc and Pelletier (1974) may find an explanation.

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