T H E M E D I A N E M I N E N C E

AN ELECTRON MICROSCOPIC STUDY WITH SPECIAL REFERENCE TO GONADOTROPIN RELEASE IN THE RAT

PROEFSCHRIFT

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We dance round in a ring and suppose,
But the Secret sits in the middle and knows.
(Frost)

CONTENTS

Abbreviations

ACTH	adrenocorticotropic hormone, an anterior pituitary hormone
AV[1)]	agranular vesicle(s) or microvesicle(s), neuronal vesicle(s) about 50 nm in diameter which after fixation with aldehyde-osmium tetroxide are electron-lucent and which resemble synaptic vesicles. After incubation with 5-OHDA and fixation with potassium permanganate, microvesicles in certain axonal swellings have an electron-dense content. These vesicles are then called small granular vesicles
CRF	corticotropin-releasing factor, one of the hypophysiotropic substances
m.e.	median eminence, part of the neurohypophysis
FSH	follicle-stimulating hormone, an anterior pituitary gonadotropic hormone
Gn-RH	gonadotropin-releasing hormones, one of the hypophysiotropic substances
GV	granular vesicle(s), neuronal vesicles of about 60-220 nm in diameter which after fixation by aldehyde-osmium tetroxide have an electron-dense content. Such vesicles are after fixation with potassium permanganate generally electron-lucent. Then, they are indicated with 'GV'.
HRP	horseradish peroxidase, an enzyme used here as extracellular tracer
5-OHDA	5-hydroxydopamine, a 'false' transmitter which accumulates in monoaminergic varicosities
IF[1)]	those hypophysiotropic substances which inhibit release of a particular anterior pituitary hormone
LH	luteinizing hormone, an anterior pituitary gonadotropic hormone
LRF[1)]	luteinizing hormone-releasing factor, one of the hypophysiotropic substances (here used as synonym for Gn-RH)
P	probability
RF[1)]	those hypophysiotropic substances which stimulate release of a particular anterior pituitary hormone
SEM	standard error of the mean
TRH	thyrotropin-releasing hormone, one of the hypohysiotropic substances

1) only used in Appendix papers

I. INTRODUCTION

1. *Neuroendocrine relationships*

Adaptation of an organism to changes in the external and internal environment is in vertebrates brought about by two more or less separate integrative systems: the nervous system and the endocrine system. The nervous system is primarily equipped for rapid and short lived responses, the endocrine system for slower but longer lasting ones.

The cells of nervous system and endocrine system have many features in common but they differ, apart from rapidity and duration of the effects exerted, in the way they achieve "privacy" (Wurtman, 1970) in their intercellular communication. In the nervous system "privacy" is attained primarily by anatomical means whereas chemical messengers, operating over a long distance, are particularly used in the endocrine system.

In the nervous system, neurons are the cells adapted for reception, integration and rapid transmission of information. Transmission occurs along dendrites and axons, which are elongated parts of the neurons themselves, and from one cell to another at morphologically identifiable sites of contact, the synapses. Transmission is mediated by a limited number of substances, neurotransmitters, which are released extremely close to receptors of the affected cells.

The endocrine system is more loosely organized. The system mainly consists of a number of glands of which the anterior pituitary gland occupies, to a certain extent, a key position. The cells of the anterior pituitary gland and peripheral endocrine glands are adapted for reception and transmission of information. The information is carried by specific substances, hormones, which are released into the extracellular space and which are generally transported by the circulation. In principle, every cell of an organism can be reached, but only certain cells are able to decode the information

enclosed in the hormonal signal. For this purpose, the so-called target cells are equipped with specific (hormone) receptors. For example, receptors of certain cells in testis and ovary are able to recognize and bind specific hormones, the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH), secreted by the anterior pituitary gland. This may result in a biological response of the cells.
It is not surprising that nervous system and endocrine system are not operating independently. Cells particularly involved in the interrelation of these systems are the neuroendocrine cells, cells by which neural signals are converted into hormonal signals. The present study deals with several aspects of the ultrastructure of one group of these neuroendocrine cells, the hypophysiotropic cells. These cells are located in the brain (fig. a) and regulate the secretion of the endocrine cells of the anterior pituitary gland by releasing hypophysiotropic substances from their endings into a special vascular system, the pituitary portal system. The site of release is the median eminence.
Present concepts about these neuroendocrine cells have emerged gradually and a concise historical survey may therefore be appropriate.

1.1. history in short

The assumption that relations exist between nervous system and pituitary was expressed already by Galen (200), albeit that waste products of the brain were though to pass to the pituitary via a funnel-shaped infundibulum, an area roughly corresponding to the structure called later the median eminence (m.e.; for terminology, see p. 4). From the pituitary, pituita, or nasal mucus, was believed to pass into the nasal cavity[1)].
Later, the view arose that the function of the anterior pituitary gland is under the control of the central nervous system. This was initially suggested by clinical observations. Experimental evidence for this view came from studies on the rabbit (Harris, 1937; Haterius and Derbyshire, 1937), since electrical stimulation of the hypothalamus, a part of the

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1) In this respect it is interesting that the reverse route appears to be a rapid and effective way to deliver substances into the brain (Anand Kumar et al, 1976)

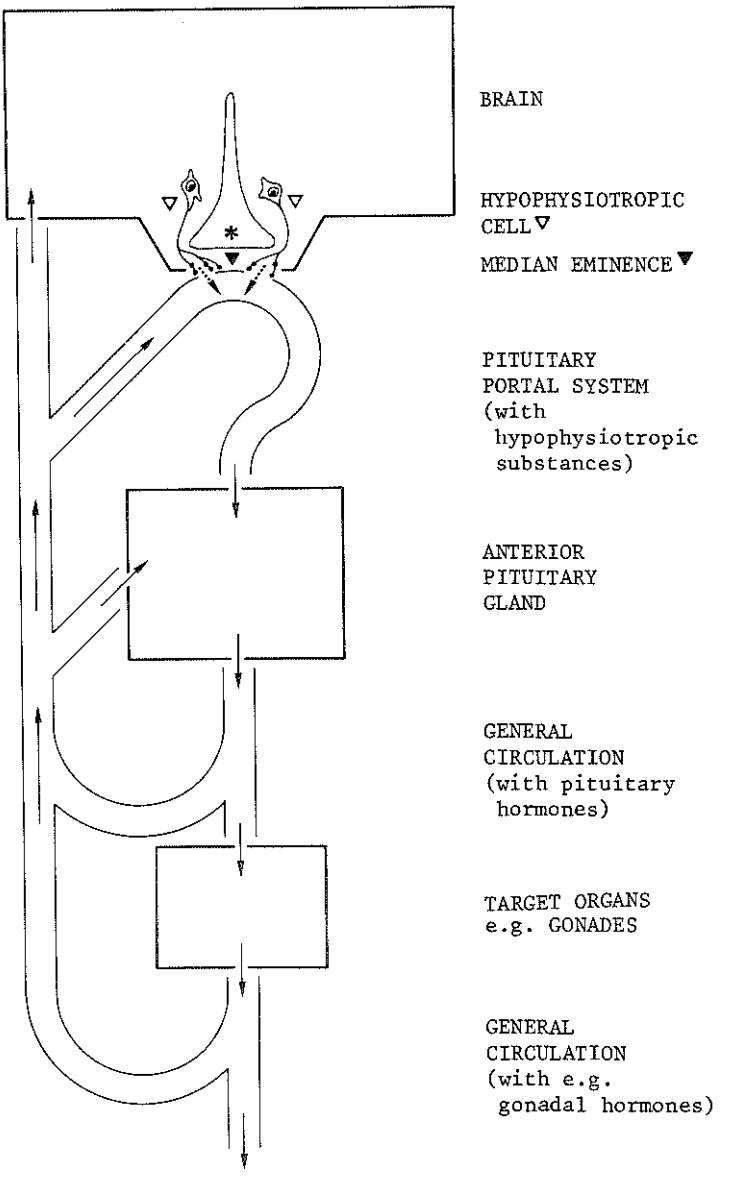


Fig. a. Relations between brain, median eminence, anterior pituitary gland and target organs. The drawing of the median eminence represents a coronal section showing above the median eminence the widened basal part of the third ventricle, viz. the infundibular recess (*).

The pituitary gland - terminology

The pituitary gland or hypophysis consists of two parts. One part arises during ontogeny as a (neural) downgrowth from the hypothalamus, which is the most basal region of the diencephalic part of the brain and largely surrounds the third ventricle (fig. b). This neural part develops into the neurohypophysis.

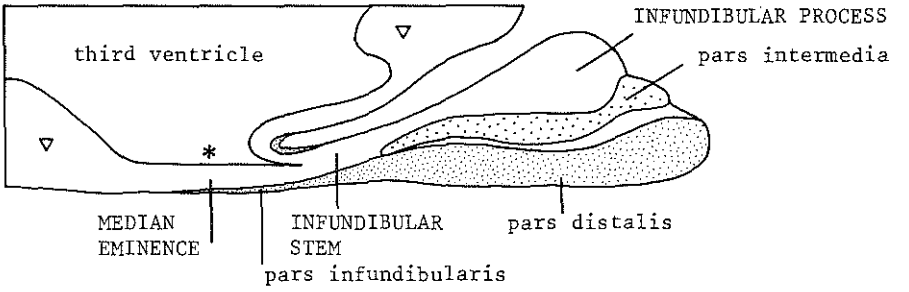


Fig. b. Diagram of a sagittal section of the neurohypophysis (MEDIAN EMINENCE, INFUNDIBULAR STEM and INFUNDIBULAR PROCESS) and of the anterior pituitary gland (pars infundibularis, pars distalis and pars intermedia). Hypothalamus (▽) and third ventricle are , in part, visible. * infundibular recess

The other part, the future anterior pituitary gland or adenohypophysis, is said to arise as a dorsal evagination (Rathke's pouch) from the roof of the oral ectoderm, although recent data are suggestive of a neuroectodermal origin (Pearse and Takor Takor, 1976).

The neurohypophysis comprises

1. the median eminence (m.e.), the part continuous with the hypothalamus and connected to
2. the infundibular stem, the part which connects the m.e. and
3. the infundibular process (neural lobe, posterior pituitary gland), the distal enlargement of the neurohypophysis.

In the literature, infundibulum generally refers to m.e. plus infundibular stem. Terminologies in use are rather confusing, mainly because of species differences. In some species, e.g. man, the infundibular stem is elongated, in which case neural stalk is occasionally used instead of infundibular stem. Some authors (Daniel and Prichard, 1975) regard the m.e. as a part of the infundibular stem (see I 2.1.)

The anterior pituitary gland is divided into

1. the pars distalis, the bulk of the anterior pituitary gland,
2. the pars infundibularis (pars tuberalis), which lines m.e. and infundibular stem and
3. the pars intermedia (intermediate lobe) which is located between pars distalis and infundibular process.

Thus, the pituitary gland comprises infundibular process, pars distalis and pars intermedia.
In addition to these terms, pituitary stalk is occasionally used, which refers to both infundibular process and surrounding pars infundibularis.

The pituitary portal system

In most mammals, the arterial supply of the anterior pituitary gland is at best scanty (see Moll, 1958). The main supply is generally derived from a primary capillary plexus in m.e. and infundibular stem (fig. c)

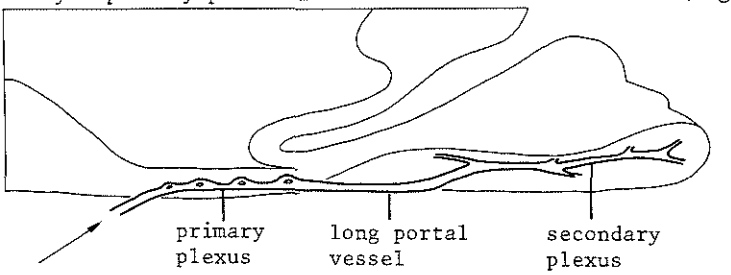


Fig. c. Drawing of the main elements of the pituitary portal system (sagittal section, cf. fig. b).

The major component of the primary plexus is the surface network (Duvernoy, 1972), while an additional deep network exist in many vertebrates. The surface network is located at the periphery of m.e. and infundibular stem and is covered by pars infundibularis and pial membranes. According to Duvernoy (1972), in mammals the deep network consists of long capillary loops (Green and Harris, 1947) and subependymal network, which parts seem somewhat separate (Moll, 1957). The capillaries of the primary plexus are mainly drained by the long portal vessels toward the secondary plexus in the pars distalis.
In addition, numerous anastomoses exist between primary plexus and capillaries in infundibular process on the one hand and tuber cinereum, the nearby part of the hypothalamus, on the other (Török, 1964; Szentágothai et al, 1968; Akmayev, 1971; Duvernoy, 1972; Page et al, 1976; Page and Bergland, 1976). According to Porter et al (1977) the anastomoses are especially located subependymally and the blood flow is from infundibular process to m.e. and tuber cinereum (see also Oliver et al, 1977).
In addition to the long portal vessels, short portal vessels exist between anterior pituitary gland and infundibular process. Although it was generally assumed that the anterior pituitary received blood by these short portal vessels (Landsmeer, 1951; Porter et al, 1973), recently the reverse direction has been proposed (Oliver et al, 1977; Porter et al, 1977) whereas Bergland and Page (1976) emphasize the possibility of flow reversal.

brain, induced ovulation, a phenomenon caused by increased release of LH. Stimulation of the pituitary appeared to be less effective (Markee et al, 1946; Harris, 1948b). Furthermore, lesions involving the basal part of the hypothalamus, or more specifically the m.e. caused disturbances in the reproductive functions (Dey et al, 1940; Dey 1941, 1943; Taleisnik and McCann, 1961), as was the case after section of the

pituitary stalk. Two important features were established: 1. the ocurrence of portal vessels (see p. 5) between a capillary plexus in the m.e. and one in the anterior pituitary gland (Popa and Fielding, 1930a,b, 1933; Wislocki and King, 1936; Wislocki, 1938; Xavier Morato, 1939; Green, 1951) and 2. the scarcity, possibly absence, of an innervation of the pars distalis (Green and Harris, 1947; Green, 1951; Unsicker, 1971). These observations suggested a neurovascular link between hypothalamus and anterior pituitary gland, viz. a vascular transport of information originating in the hypothalamus. This possibility was especially advocated by Harris (1948a, Green and Harris, 1949).

Subsequent studies (Harris, 1950; Harris and Jacobsohn, 1952; Harris, 1955; Nikitovitch-Winer and Everett, 1958), which included hypothalamic stimulation and lesions, section of the pituitary stalk and transplantation of the pituitary, gave strong support to the neurohumoral hypothesis of Green and Harris, implying that hypophysiotropic hormones are synthetized by hypothalamic neurons, transported through axons to m.e. and infundibular stem, released from nerve endings located near the primary plexus and distributed to the pituitary by the portal vessels.

Later, direct evidence for the existence of hypothalamic hormones stimulating release of anterior pituitary hormones became available. Data on the discovery of the hypothalamic hormone(s) stimulating release of LH and FSH follow here (see further Table I). McCann et al (1960), McCann (1962) and Campbell et al (1961) infused extracts of stalk- m.e. tissue intravenously or directly into the anterior pituitary gland and concluded that release of LH was increased. The substance held responsible for the LH-releasing activity was called LH-releasing factor or LRF. Such extracts were found to cause in addition release of FSH (Mittler and Meites, 1964; Igarashi and McCann, 1964) but whether the effects on LH and FSH are caused by two different substances or, more likely, by one substance (Schally et al, 1976a) is still disputed. Since the structure is known yet of a substance causing release of both LH and FSH (Schwartz and McCormack, 1972; Schally et al, 1973a; Ondo et al, 1973a), the general term gonadotropin-releasing hormone (Gn-RH) seems more appropriate and will be used in this thesis. In the Appendix papers, the term LRF is still used. In addition to substances causing release of pituitary hormones, the hypothalamus appeared to contain substances causing inhibition of release. Such a substance was called an inhibiting

Table I Observations in favour of the neurohumoral view on control of gonadotropin secretion

1. extraction and synthesis of Gn-RH	Geiger et al, 1971; Matsuo et al, 1971; Schally et al, 1971, 1973b
2. high concentration of Gn-RH in the m.e., especially near the primary plexus, as shown by bioassay (a), radioimmunoassay (b) and immunohistochemistry (c)	a. Watanabe and McCann, 1968; Crighton et al, 1970; Krulich et al, 1971, 1977; Quijada et al, 1971 b. Palkovits et al, 1974a; Morris, 1975; Wheaton et al, 1975; Kizer et al, 1976 a,b; Estes et al, 1977; Knigge et al, 1977; Krulich et al, 1977 c. Barry et al, 1973; King et al, 1974; Kordon et al, 1974; Pelletier et al, 1974a; Barry and Carette, 1975; Barry and Dubois, 1975; Sétáló et al, 1975b; Barry, 1976; Fuxe et al, 1976a; Kozlowski et al, 1976a; Silverman, 1976
3. high concentration of Gn-RH in pituitary portal blood	Kamberi et al, 1969; Porter et al, 1970, 1973; Ben-Jonathan et al, 1973; Fink et al, 1977; Neill et al, 1977
4. increased amounts of Gn-RH in portal blood during pro-oestrus (a), after castration (b) and after preoptic stimulation (c),	a. Sarkar et al, 1976; Fink et al, 1977 b. Eskay et al, 1974; Porter et al, 1976 c. Harris and Ruf, 1970; Ben-Jonathan et al, 1974; Fink and Jamieson, 1974, 1976; Chiappa et al, 1975; Eskay et al, 1977; Porter et al, 1977
5. effects of antiserum against Gn-RH on gonadotropins, gonads and sex steroids (a), preovulatory surge of gonadotropins (b) and LH surge after preoptic stimulation (c)	a. Fraser et al, 1974; Fraser, 1975; Arimura, 1976; Bercu et al, 1976; Kerdelhue et al, 1976; Hauger et al, 1977; Koch, 1977; b. Koch et al, 1973; Arimura et al, 1974; Fraser, 1975; Shiina et al, 1976; Koch, 1977 c. Porter et al, 1977
6. deficiency of Gn-RH and abnormal gonadal development	Cattanach et al, 1977

factor or, in case its structure was elucidated, an inhibiting hormone. In Table II different (putative) hypophysiotropic substances are listed.

1.2. neurosecretion

Neurovascular control of the anterior pituitary gland presupposes that cells located in the brain release biologically active substances into capillaries draining towards the glands. It was, and generally is, taken for granted (Harris, 1955; Szentágothai et al, 1968) that these substances are synthetized in specialized neurons (see Knowles, 1974). Morphological evidence for the existence of neurons with endocrine properties dates from 1919 (Knowles, 1974). Such specialized neurons formed the morphological basis for the concept that so-called neurosecretory cells, originally cells characterized by the presence of histologically stainable (neurosecretory) material, secrete their - hormonal - product into the blood stream (Scharrer and Scharrer, 1940, 1954; Bargmann, 1966, 1969; Knowles and Bern, 1966).
It is emphasized that terms like neurosecretion and neurosecretory cells are somewhat confusing since their criteria gradually changed (Knowles and Bern, 1966) and since a generally accepted definition is lacking (Bargmann, 1974; Knowles, 1974). In the terminolgy of Bargmann (1966), a neurosecretory cells is a neuron that also possesses glandular activity. It has indeed been demonstrated that the so-called classical neurosecretory cells of the hypothalamo-neurohypophyseal system (I 4.1.2.) have fundamental electrical properties in common with other central neurons (Cross and Green, 1959; Kandel, 1964; Kelly and Dreifuss, 1970; Cross et al, 1975; Hayward, 1977). The glandular properties of these cells are also beyond dispute (Dean et al, 1968; Picard, 1969; Pilgrim, 1969; Thorn, 1970; Douglas et al, 1971; Dreifuss et al, 1973; Norström, 1975). However, ordinary neurons possess glandular features as well, since 'the prime function of a neuron is to produce and apply to other tissues a chemical activator' (Bishop, 1965; see also Scott, 1905). Thus, differences between neurosecretory cells producing neurohormones and other neurons producing neurotransmitters (Scharrer, 1969) do not seem to be fundamental (Bargmann, 1969). A main difference seems to be the distance between site of release and location of receptors. The difference in distance of transport and therefore in distribution volume, has consequences for the levels of synthetic activity, for the amounts of intraneuronal secretory products, for the rapidity of degradation of bio-

TABLE II Hypophysiotropic substances[1)]

hypophysiotropic substance	abbreviation	structure
gonadotropin-releasing hormone (luteinizing hormone-releasing hormone)[2)]	Gn-RH (LRF)	decapeptide
corticotropin-releasing factor	CRF[3)]	?
thyrotropin-releasing hormone	TRH	tripeptide
growth hormone (somatotropin)-releasing factor	GH-RF	?
growth hormone (somatotropin)-release-inhibiting hormone or somatostatin	GH-RIH	tetradecapeptide
prolactin-releasing factor	PRF	?
prolactin release-inhibiting factor (=dopamine?)	PIF	?[4)] (catecholamine)
melanocyte-stimulating hormone-releasing factor	MRF	?
melanocyte-stimulating hormone-release-inhibiting factor (=dopamine?)	MIF	tripeptide? (catecholamine)

1) for general references, see McCann and Porter, 1969; Guillemin and Burgus, 1972; Schally et al, 1973b; Reichlin et al, 1976

2) the existence of a FSH-RF separate from Gn-RH is not very likely (Shahmanesh and Jeffcoate, 1976; Schally et al, 1976a)

3) "My eyes told my mind about him. My mind sent a message to my hypothalamus, told it to release the hormone CRF into the short vessels connecting my hypothalamus and my pituitary gland. The CRF inspired my pituitary gland to dump the hormone ACTH into my bloodstream. My pituitary had been making and storing ACTH for just such an occasion. ...And some of the ACTH in my bloodstream reached the outer shell of my adrenal gland, which had been making and storing glucocorticoids for emergencies. My adrenal gland added the glucocorticoids to my bloodstream. They went all over my body..." (K. Vonnegut, in Breakfast of champions, 1973)

4) McCann: "... But this is going to take a great deal of work, would you not agree, Dr. Schally, to isolate and determine the structure of PIF?"
Schally: "No, I think that is a question of only 6 to 12 months ..."

- J. Reprod. Fert. Suppl. 20, 164 (1973) -

logically active substances and for the duration of the effects exerted at the target cells.

If differences between neurosecretory cells and ordinary neurons are primarily quantitative in nature, many intermediate 'types' of cells will exist. Such a continuum of cell 'types' may well be extended to the chromaffin cells of the adrenal medulla and possibly also to the peptide secreting cells of the anterior pituitary gland and the gastro-intestinal tract (cf. Guillemin, 1977a; Pearse, 1977).

In view of the complications mentioned, the neuronal systems dealt with in I 4. are not classified in terms of neurosecretory or non-neurosecretory. There is, however, no urgent reason to dismiss the term neurosecretory completely. Certain neurons are simply more neurosecretory than others.

2. *The median eminence*

2.1. location

The m.e. is, according to Tilney (1936), an elevation in the midline on the basal surface of the brain caudal to the optic chiasm. This elevation consists of the pars infundibularis, a part of the anterior pituitary gland (p. 4), and a neural part. In recent literature, only this neural part is denoted as m.e. It is roughly located between optic chiasm and mammillary body. At the lateral side the infundibular sulcus separates the m.e. from the tuber cinereum, a poorly myelinated part of the hypothalamus. Although the m.e. was, and sometimes is, called the m.e. of the tuber cinereum, the m.e. is considered as part of the neurohypophysis (p. 4).

The main part of the m.e., the m.e. pars oralis, is in the rat and most other vertebrates (primates excluded) located rostral to the infundibular stem. Since the basal part of the third ventricle is widened into the infundibular recess (fig. a), the m.e. constitutes the relatively large but thin layered bottom of the third ventricle. The remaining part of the m.e., the m.e. pars caudalis, which is located dorso-caudal to the infundibular stem is, in the rat, relatively small and poorly vascularized (Landsmeer, 1947; Rinne, 1960). If not specified, data on the m.e. refer to the m.e. pars oralis.

It may be added that according to Daniel and Prichard (1975), in man

an area corresponding to the m.e. of lower mammals cannot be accurately defined. Instead of m.e., they use "upper infundibular stem". In this respect, the main difference between man and lower mammals seems to be the difference in relative size of m.e. pars oralis and pars caudalis.

2.2. general light microscopic features

At the light microscopical level, the m.e. has many peculiar features if compared with most other areas of the central nervous system (Christ, 1951; Green, 1951; Nowakowski, 1951; Spatz, 1951; Dellman, 1962; Szentágothai, 1964; Akmayev, 1969):

1. the ependymal cells are unusual in shape; certain cells, the tanycytes, have processes to the base of the brain,
2. a subependymal layer of glial fibres is absent,
3. only a few neuronal cell bodies are present,
4. fibres of the neurosecretory cells, located in supraoptic and paraventricular nuclei, pass through the m.e. to the infundibular process,
5. near the primary plexus of the pituitary portal system, there is an abundance of small unmyelinated axons and aberrant terminals,
6. an outer glial layer is absent,
7. a prominent vascular plexus occurs at the periphery of the m.e.; from this plexus capillary loops penetrate into the m.e.,
8. a part of the anterior pituitary gland, the pars infundibularis, is located between m.e. and pial membranes.

2.3. general electron microscopic features

In all vertebrate species studied, the m.e. has several features in common, like a particular topographical succession of structural elements from the infundibular recess (fig. a,b) to the base of the brain. Some general observations on the ultrastructure of the rat m.e. will be summarized in this sequence (for terminology see fig. d). Data refer to material fixed by aldehyde-osmium tetroxide and are in many respects comparable to the descriptions of Kobayashi et al (1970) and of Raisman (1971).

INFUNDIBULAR RECESS

Small numbers of intraventricular cells, axons and axonal swellings are present near the ependymal cells. In these axonal swellings, vesicles with an electron-dense core, so-called granular vesicles (GV) are frequently present. Sizes of GV vary over a wide range, including GV larger than 160 nm in diameter (see fibre layer).

INTERNAL ZONE

ependymal and subependymal layer

Non-neuronal elements - specialized ependymal cells form the floor of the infundibular recess. Two types may be distinguished: 1. brick-like cells which form the largest part of the floor, 2. tanycytes, mostly located at the lateral side. In addition to the ependymal cells, one or more layers of subependymal (or hypendymal) cells may be observed, which cells occasionally show an apical process contacting the cerebrospinal fluid. Certain ependymal and subependymal cells are in contact with the single basement membrane of subependymal capillaries. Fenestrations are practically absent in these capillaries, which are only exceptionally contacted by neuronal endfeet.

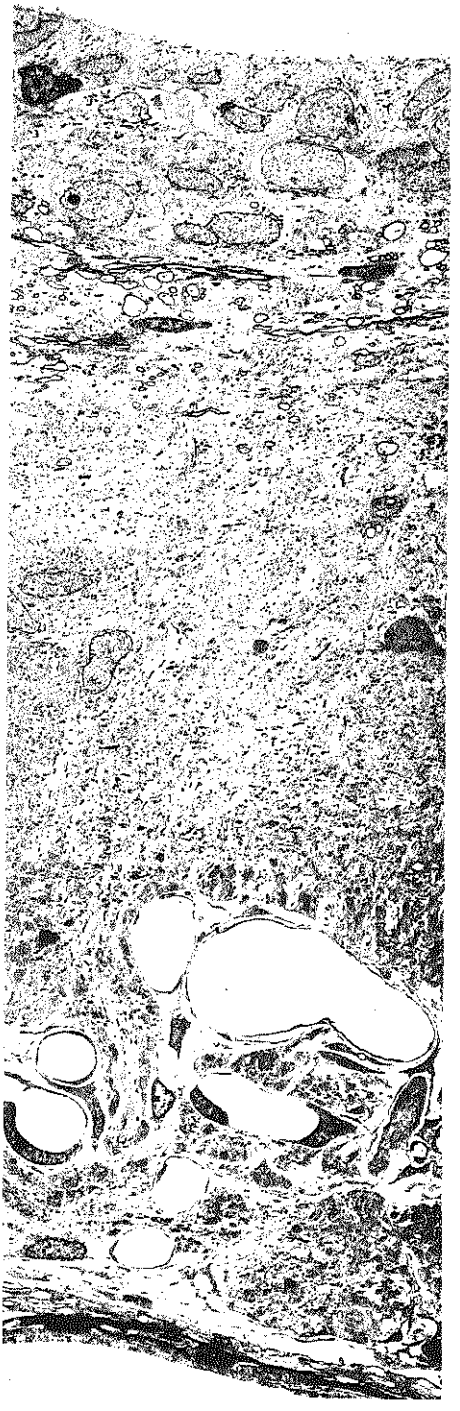
Neuronal elements - axons, axonal swellings and occasionally dendrites are present between ependymal and subependyal cells. Frequently axonal swellings contain GV about 100 nm in diameter. These swellings presumably belong to monoaminergic, especially noradrenergic neurons (see I 4.2.; Kobayashi et al, 1970; Ajika and Hökfelt, 1973; Löfström et al, 1976a). Certain axonal swellings contain much larger GV (see fibre layer). Axonal swellings showing a synaptic contact with a neuronal cell body or a dendrite or a synaptoid contact with a non-neuronal cell contain mainly electron-lucent microvesicles about 50 nm in diameter.

fibre layer

Non-neuronal elements - in addition to processes of ependymal and subependymal cells, mainly oligodendrocytes occur, as well as occasionally loops of the primary plexus (p.5).

Neuronal elements - the fibre layer of the internal zone is characterized by the presence of axons which mainly belong to the hypothalamo-neurohypophyseal system (I 4.1.2.). These axons contain GV about 150-220 nm in diameter (App. 3), assumed to contain oxytocin or vasopressin and neurophysins. In certain axonal swellings, large accumulations of such vesicles are present. Most axons are unmyelinated but such large GV are also present in myelinated axons and swellings, in contrast of the opinion of e.g. Cross (1971), Constantinides (1974), Clattenburg (1974) and Hayward (1977). Unmyelinated axons and axonal swellings with smaller GV are also present.

Fig. d. Coronal section of the m.e. The capillaries of the primary plexus in the external zone are empty, due to perfusion fixation. A-D indicate sub-zones of the palisade zone (App. 3). x 1100


infundibular recess
of third ventricle
ependymal and
subependymal
layer
fibre layer
INTERNAL
ZONE
D
C
palisade
zone
B
A
EXTERNAL
ZONE
zona granulosa
pial membrane

EXTERNAL ZONE

palisade zone

Non-neuronal elements - cells containing lipid droplets and resembling the pituicytes of the infundibular process are frequently seen. Such lipid droplets are also present in ependymal and subependymal cells. The processes of these non-neuronal cells give this area its palisade-like appearance. Endfeet of tanycytes and pituicyte-like cells are in contact with the outer basement membrane (or more accurately basal lamina) of the primary plexus. In certain non-neuronal endfeet, membrane-bound material of variable size, shape and electron-density is present.

Neuronal elements - unmyelinated axons and swellings are present in large numbers. The axons are of small diameter (mainly 0.1-0.6 µm) and contain predominantly neurotubules. The varicosities are characterized by the presence of GV of varying sizes, mainly 60-140 nm in diameter. They are assumed to contain predominantly hypophysiotropic substances. Some swellings show synaptoid contacts with non-neuronal elements. Microvesicles are numerous in axonal swellings which contact the outer basement membrane.

capillaries and perivascular space

A wide, irregular perivascular space is present between the inner basement membrane lining the fenestrated capillaries of the primary plexus and the outer basement membrane lining the neuronal and non-neuronal endfeet of the m.e. The perivascular space contains collagen fibres and connective tissue cells, especially fibroblasts and occasionally a mast cell.

zona granulosa (see App. 3)

The part of the external zone located peripherally to the palisade zone is not characterized by a palisade appearance. Preterminal and terminal parts of neuronal and non-neuronal cells are grouped in 'islands'. Most profiles are in direct contact with the outer basement membrane. In this area large configurations of loose membranous whorls are present.

The area between m.e. and pial membranes contains cells of the pars infundibularis and axon bundles probably derived from the superior cervical ganglia.

2.4. function of the median eminence

As visualized in fig. e, there are two main views with respect to the function of the m.e.,

1. the m.e. is the area where nerve endings release hypophysiotropic substances into the capillaries of the primary plexus. Thus, the m.e. serves as a neuroendocrine transducer, translating neural information derived from higher integrative centres into hormonal signals (I 1.1.),

2. the m.e. is the interface between the cerebrospinal fluid in the infundibular recess and the blood in the primary plexus, where specialized

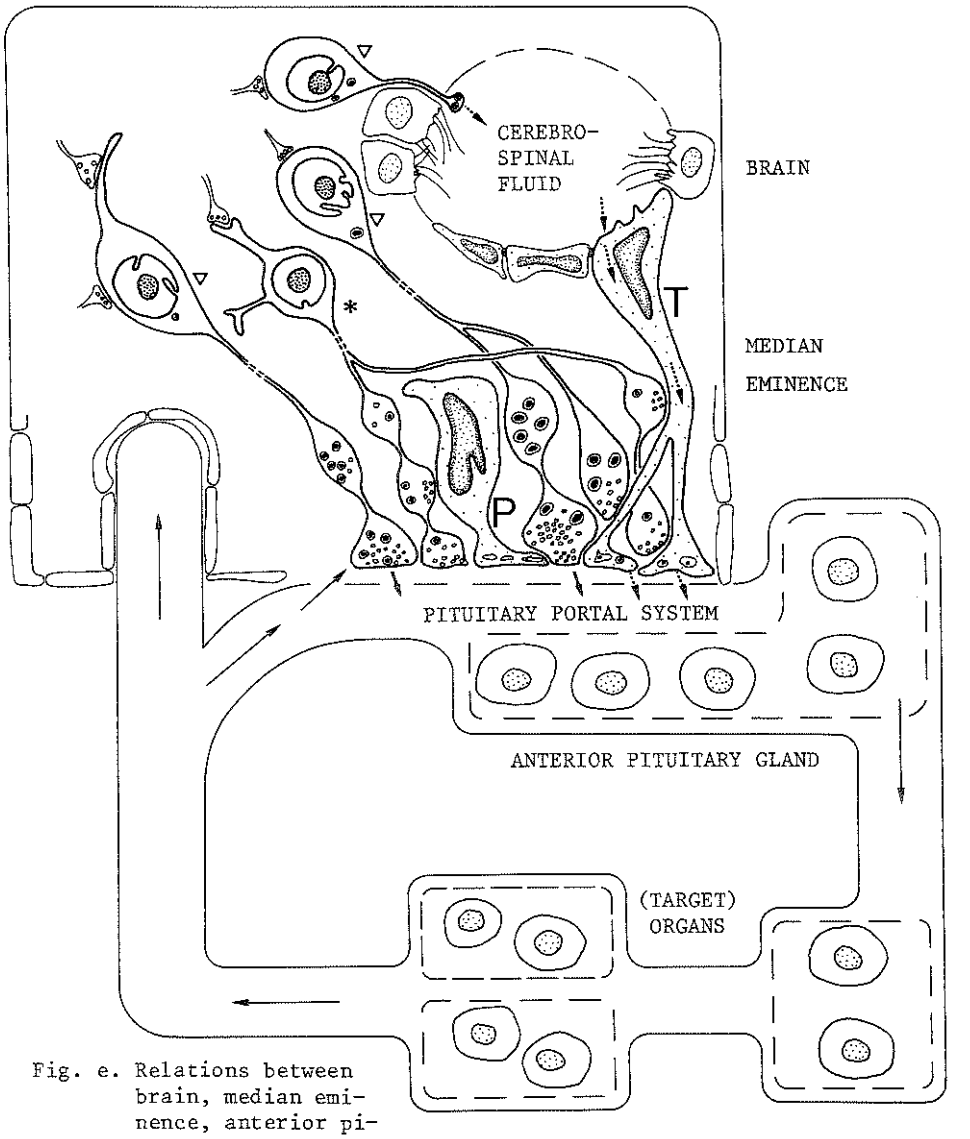


Fig. e. Relations between brain, median eminence, anterior pituitary gland, and (target) organs, with emphasis on two presumed functions of the m.e.: 1. release into the primary plexus of the pituitary portal system of hypophysiotropic substances from the neuronal endfeet of (peptidergic) hypophysiotropic neurons (thick arrows), 2. uptake of hypophysiotropic substances by tanycytes (T) after their release into the cerebrospinal fluid, and subsequent transport to the primary plexus (interrupted arrows). In addition to the (peptidergic) hypophysiotropic neurons (▽), a monoaminergic neuron (*) is shown with several axonal swellings in the m.e. The size of the axonal swellings present in the m.e. is exaggerated.
◉◉ granular vesicles; ◦◦ microvesicles; P pituicyte-like cell

ependymal cells transport hypophysiotropic substances from infundibular recess to portal blood. Thus, the m.e. serves as a transporting system (I 3.3.).
Both views may be correct and have in common that the main task of the m.e. is release of biologically active substances into the primary plexus.

3. *Factors involved in release of hypophysiotropic substances*
A multitude of factors is involved in the ultimate release of a hypophysiotropic substance like Gn-RH into portal blood. Factors thought to be of major importance are listed below.

3.1. neuronal inputs
If we accept that hypophysiotropic cells are neurons, a multitude of neurons probably makes synaptic contacts with these cells. These neurons participate in neuronal circuits by which e.g. visual, olfactory, tactile and hormonal stimuli, as well as built-in behavioural patterns and biological rhythms affect endocrine function and behaviour. In addition, a direct neural pathway between gonads and hypothalamus has been suggested by Gerendai and Halász (1976).
Effects on gonadotropin release of (putative) neurotransmitters like acetylcholine, noradrenaline, dopamine and serotonin have amply been demonstrated (I 4.2. - 4.4.). In view of their presence in m.e. and/or hypothalamus, several other putative neurotransmitters and modulators may be involved (Table III), although the functions of these substances are, as yet, far from clear. Angiotensin II, enkephalins and histamine are of special interest, because of their high concentration near the primary plexus. The high concentration of histamine, however, can in part be explained by the presence of mast cells.

3.2. hormonal inputs
The area where hypophysiotropic cells are thought to be present (I 4.1.1.) is an important target of hormones secreted by peripheral glands like the gonads (Table IV). Although the hormonal effects may be exerted directly at hypophysiotropic cells (Stumpf and Sar, 1977; Warembourg, 1977), part of the effects are exerted at interneurons, like serotoninergic neurons (Kordon and Glowinski, 1972; Fuxe et al,

Table III Putative neurotransmitters[1] and modulators possibly involved in control of hypophysiotropic neurons

acetylcholine, noradrenaline, dopamine, adrenaline, serotonin	for lit. , see I 4.2.-4.4.
γ-aminobutyric acid	Yagi and Sawaki, 1975; Fuxe et al, 1976; Tappaz and Brownstein, 1977
histamine	Brownstein, 1977; Rivier and Vale, 1977
substance P	Nilsson et al, 1974; Brownstein et al, 1976b; Fuxe et al, 1976; Davies and Dray, 1977
angiotensin II	Fuxe et al, 1977a
enkephalins and endorphins	Bowers et al, 1977; Bruni et al, 1977b; Cocchi et al, 1977; Guillemin, 1977b
α-melanocyte-stimulating hormone	Pelletier and Dube, 1977
vasoactive intestinal polypeptide	Fuxe et al, 1977b

[1] For information on neuronal localization, see also Otsuka et al, 1975; Garbarg et al, 1976; Simantov et al, 1976; Chan-Palay and Palay, 1977; Frederickson, 1977; Fuxe et al, 1977a; Giachetti et al, 1977; Lane et al, 1977

1974c; Grant and Stumpf, 1975) and especially catecholaminergic neurons (Fuxe et al, 1972, 1974b, 1977c; Hökfelt and Fuxe, 1972b; Zolovick and Labhsetwar, 1973; Endersby and Wilson, 1974; Gunaga et al, 1974; Kizer et al, 1974; Grant and Stumpf, 1975; Luine et al, 1975; Chiocchio et al, 1976; Drouva and Gallo, 1976; Gudelsky et al, 1977).

For the regulation of gonadotropin release three feedback loops have been proposed, long, short and ultra-short (fig. f), reflecting actions at the level of the brain by gonadal hormones, gonadotropins and Gn-RH, respectively (Table V). Gonadal substances show, in addition, feedback effects at the level of the anterior pituitary gland (Table V).

Table IV Medial basal hypothalamus and preoptic area as sites of hormonal action

1. effects of implantation of hormones on gonadotropin release	Davidson and Sawyer, 1961; Lisk, 1962; Bloch and Davidson, 1967; Chowers and McCann, 1967; Davidson, 1969; Schuiling et al, 1974; Smith and Davidson, 1974; Cheung and Davidson, 1977
2. effects of, mainly intraventricularly, injected hormones	Knigge et al, 1973; Orías et al, 1974; Chiappa et al, 1975; McEwen et al, 1975
3. presence of enzymes which aromatize or reduce androgens	Rommerts and van der Molen, 1971; Naftolin et al, 1972; Selmanoff et al, 1977
4. presence of hormone receptors	Jouan et al, 1971; Kato and Onouchi, 1973, 1977; Kato et al, 1974, 1975; McEwen et al, 1975; Naftolin and Ryan, 1975; Stumpf, 1975; Vreeburg et al, 1975; Seiki et al, 1977; Stumpf and Sar, 1977
5. hormonal effects on neuronal electrical activity	Yagi and Sawaki, 1973; Dufy et al, 1976; Moss et al, 1976; Kelly et al, 1977a,b

Table V Different types of feedback in control of gonadotropin secretion

1. long feedback loop (at the brain)	Davidson, 1969; Bishop et al, 1972; Schwartz and McCormack, 1972; Yagi and Sawaki, 1973; see further Table IV
(at the pituitary)	Moore and Price, 1932; Debeljuk et al, 1972; Mittler, 1972; Kingsley and Bogdanove, 1973; Negro-Vilar et al, 1973; Libertun et al, 1974; Van Dieten et al, 1974; Vreeburg et al, 1975; Aiyer et al, 1976; Drouin and Labrie, 1976; Ferland et al, 1976; Gnodde, 1976; Porter et al, 1976, Labrie et al, 1977; De Koning, 1978
2. short feedback loop[1)]	David et al, 1966; Davies et al, 1975; McEwen et al, 1975; Petrusz, 1975; Molitch et al, 1976; but see Gay, 1974
3. ultrashort feedback loop[2)]	Hyyppa et al, 1971; Krieg et al, 1976; Moss et al, 1976

[1)] pituitary hormones other than gonadotropins, e.g. prolactin, may also influence release of Gn-RH (Fuxe et al, 1969, 1972; Leonardelli, 1977; Wuttke and Beck, 1977)

[2)] whether a direct action of Gn-RH at the brain reflects a hormonal action can be questioned (I 4.1.1.)

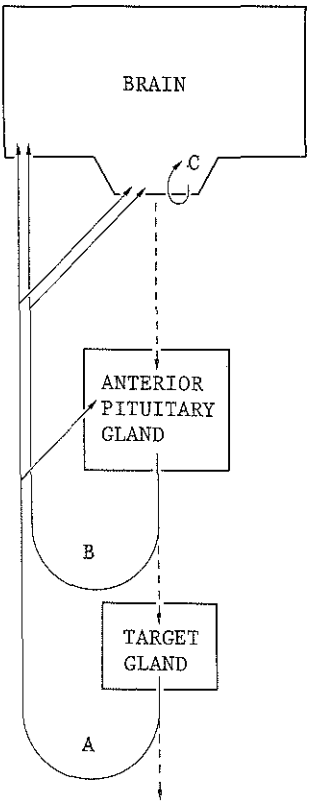


Fig. f. Diagram of long (A), short (B) and ultrashort (C) feedback loops.

In addition to the hormones of the hypothalamo-hypophyseal-gonadal system, other hormones or hormone-like substances may be involved in the control of gonadotropin release. To these belong e.g. the polypeptide arginine vasotocin and the indole melatonin, both present in the pineal gland (Moguilevsky et al, 1976; Reiter et al, 1976; Reiter and Vaughn, 1977). Furthermore, the prostaglandins, "until recently, ... a group of hormonally-active substances in search of a function" (Hedge, 1977), are involved in stimulating gonadotropin release. Apparently, one site of action of prostaglandins, in rat especially prostaglandin E_2, is the brain (Chobsieng et al, 1975; Eskay et al, 1975; Ojeda et al, 1975a, b; Harms et al, 1976; Porter et al, 1976; Carlson et al, 1977; Gallardo and Ramirez, 1977).

Enzymes which degrade hypophysiotropic substances (Reichlin et al, 1976) may be involved in several of the actions mentioned above. These enzymes are especially present in the hypothalamus and their activity can be influenced by LH (Kuhl and Taubert, 1975), Gn-RH (McKelvy and Grimm-Jorgensen, 1977), prostaglandin E_2 (Kuhl et al, 1976) and gonadal steroids (Hooper, 1968; Griffiths and Hooper, 1973, 1974; Kuhl and Taubert, 1975; Griffiths, 1976; McKelvy and Grimm-Jorgensen, 1977). The cellular localization of these enzymes has not been established.

3.3. ependymal cells

The infundibular recess, is lined by atypical ependymal cells (Kobayashi et al, 1970; Raisman, 1971; Millhouse, 1971, 1972; Scott et al, 1972), as far as typical ependymal cells exist (Mitro, 1976). The

atypical cells lack e.g. the normal abundance of cilia, but may show bleb-like formations protruding into the recess. The ependymal cells of the m.e. show junctional complexes including tight junctions (zonulae occludentes), which seem to preclude intercellular transport between cerebrospinal fluid and interstitial fluid of the m.e. (Brightman et al, 1975). Certain ependymal cells, the tanycytes (Horstmann, 1954), are characterized by a long basal process ending with several endfeet near the primary plexus at the base of the brain (Kobayashi et al, 1970; McArthur, 1970; Raisman, 1971; Bara and Böti, 1974). Tanycytes lining the (lateral) wall of the third ventricle frequently end at capillaries in the periventricular region of the medial basal hypothalamus (Brawer, 1972) and at the base of the brain lateral to the m.e. (Millhouse, 1972).
The ependymal cells of the m.e. show several features which possibly are related to a rôle in neuroendocrine control, especially in transport (see Table VI, in which also literature is mentioned on hormones in the cerebrospinal fluid).
On the basis of the observations referred to in Table VI, it has been hypothetized that hormones released into the cerebrospinal fluid are taken up by the tanycytes of the m.e. and are transported to the primary plexus (Löfgren, 1959; Knowles and Anand Kumar, 1969; Porter et al, 1970; Kobayashi et al, 1970; Knigge and Silverman, 1972; Ondo et al, 1973b; Joseph et al, 1975; Zimmerman et al, 1975; Knigge et al, 1976, 1977; Naik, 1976).

4. *Neuronal systems distributing to the median eminence*

In this paragraph, a survey is given of the main neuronal systems distributing to the m.e. and thought to be involved in control of the anterior pituitary gland.

4.1. peptidergic systems [1)]

4.1.1. the hypophysiotropic system

The peptidergic hypophysiotropic system comprises cells which influence the function of the anterior pituitary gland by releasing peptides into

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1) 'The age of the hypothalamic peptide has arrived' (Kastin et al, 1976)

Table VI Observations possibly related to a (transport) role of certain ependymal cells of the m.e. in neuroendocrine control

1. unicellular link between cerebrospinal fluid in third ventricle and that around primary plexus	Kobayashi et al, 1970
2. ultrastructural changes after intraventricular injection of catecholamines (a), Gn-RH (b), after castration (c) during oestrous cycle (d)	a. Schechter and Weiner, 1972; Hökfelt, 1973; Scott et al, 1974 b. Bruni et al, 1977a c. Kobayashi and Matsui, 1969; Knowles and Anand Kumar, 1969; McArthur, 1970; Oksche et al, 1972; d. Bruni, 1974
3. presence of synaptoid contacts at ependymal cells	Matsui, 1966; Wittkowski, 1967, 1968; Kobayashi et al, 1970; Güldner and Wolff, 1973; Nozaki, 1975
4. presence of hormones like Gn-RH and TRH in cerebrospinal fluid (a), modification by castration (b), during oestrous cycle (c)	a. Cramer and Barraclough, 1974; Knigge and Joseph, 1974; Morris, 1975 b. Joseph et al, 1975 c. Knigge et al, 1977
5. uptake of intraventricularly injected substances (a) and modification by steroids (b); uptake in vitro (c)	a. Kobayashi et al, 1972b; Silverman et al, 1973b; Ben-Jonathan et al, 1974; Léranth and Schiebler, 1974; Scott et al, 1974; Joseph et al, 1975; Uemura et al, 1975 b. Recabarren and Wheaton, 1977 c. Knigge and Silverman, 1972; Silverman et al, 1973a; Joseph et al, 1975
6. passage of Gn-RH and TRH from ventricle to pituitary portal blood, leading to increased release of pituitary hormones	Gordon et al, 1972; Weiner et al, 1972b; Knigge et al, 1973; Ondo et al, 1973b; Ben-Jonathan et al, 1974; Uemura et al, 1975
7. possible presence of Gn-RH in tanycytes [1]	Zimmerman et al, 1974, 1975; Naik, 1976; Silverman, 1976a

[1] for an opposite view, see Baker et al, 1974; Kordon et al, 1974; Pelletier et al, 1974a; Goldsmith and Ganong, 1975; Barry, 1976; Sétaló et al, 1976a

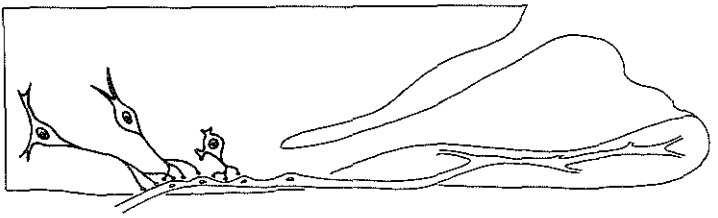

Fig. g. Drawing of hypophysiotropic cells with several endfeet at the primary plexus of the pituitary portal system (parasagittal section). The size of the cells is exaggerated.

the primary plexus of the pituitary portal system (fig. g). Since 'classical' hypophysiotropic substances like Gn-RH, TRH and somatostatin appeared to be small peptides, the peptidergic hypophysiotropic system is generally viewed upon as *the* hypophysiotropic system. It is, however, emphasized that probably also cells exist which function as hypophysiotropic cells but which release another substance, e.g. dopamine, into the primary plexus (I 4.2.). Furthermore, it can not be excluded that certain neurons which produce the peptides oxytocin and vasopressin and which are classified as hypothalamo-neurohypophyseal system (I 4.1.2.) also function as hypophysiotropic cells. For the sake of simplicity, the term hypophysiotropic system will be used in its classical sense, excluding cells producing oxytocin, vasopressin and non-peptides. Non-neuronal cells are also excluded.
Histologically, the hypophysiotropic system is 'anything but well defined' (Dellmann, 1973) . The majority of the endings of the hypophysiotropic system are located near the primary plexus, where concentrations of hypophysiotropic substances are maximal (Brownstein et al, 1976a), but there is much confusion about the location of the cell bodies. According to Halász et al (1962) and Szentágothai (1964), the cell bodies of this system are located in an area called hypophysiotropic, an area capable of maintaining a rather normal structure and function of pituitary tissue transplanted into it. This hypophysiotropic area (fig. h) is a region at the base of the hypothalamus, located at

both sides of the third ventricle. The region was said to include the whole arcuate nucleus, the ventral part of the periventricular nucleus and the parvicellular region of the retrochiasmatic area (cf. Réthelyi and Halász, 1970).

From cell bodies located in the hypophysiotropic area, especially in the arcuate nucleus, unmyelinated axons could be traced to the surface of m.e. and infundibular stem (Spatz, 1951; Nowakowski, 1951; Metuzals, 1959; Martinez, 1960; Szentágothai et al, 1968). There, grape-like terminals were described, located close to the primary plexus (Szentagothai et al, 1968). The axons form the so-called tubero-infundibular tract (Szentágothai, 1964). It is emphasized that certain axons of the tubero-infundibular tract belong to dopaminergic neurons (I 4.2.).

Electrophysiologically, the presence in the medial basal hypothalamus of neuronal cell bodies projecting to the m.e. has been confirmed (Harris et al, 1971; Makara et al, 1972; Sawaki and Yagi, 1973; Yagi and Sawaki, 1973; Renaud, 1976). They are mainly located in the arcuate and ventromedial nuclei. In addition, neurons located outside the medial basal hypothalamus appeared to project to the arcuate/m.e. region. The cell bodies were predominantly located in suprachiasmatic, preoptic and

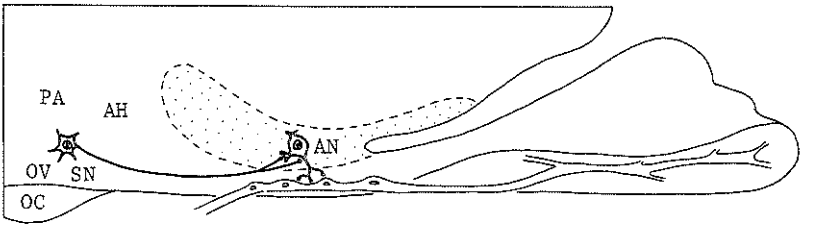

Fig. h. Diagram of approximate location of the optic chiasm (OC), organum vasculosum laminae terminalis (OV), preoptic area (PA), suprachiasmatic nucleus (SN), anterior hypothalamic area (AH), arcuate nucleus (AN) and of the hypophysiotropic area (stippled) as defined by Halász et al (1962). The rostral neuron located in the 'cyclic' centre shows synaptic contacts with a neuron in the 'tonic' centre, which neuron projects to the primary plexus.

septal areas, including the organum vasculosum laminae terminalis (fig. h; Dyer, 1973; Kawakami et al, 1975; Kelly et al, 1977a,b).

Especially neurons located in the ventral portion of the anterior hypothalamus showed an increase in firing rate during the afternoon of prooestrus (Dyer et al, 1972; Cross and Dyer, 1972). Furthermore, numerous neurons in the above mentioned areas appeared to respond to gonadal steroids, LH and FSH (Moss et al, 1975, 1976; Dyer, 1977; Hayward, 1977). These effects might be interpreted as feedback effects (fig. f) on neurons producing Gn-RH (see p.25), but these electrophysiologically identified neurons are not necessarily hypophysiotropic. Kelly et al (1977b) showed, for instance, that in the medial preoptic and septal areas especially those neurons were responsive to oestrogen which did not project to the m.e. (cf. Dyer, 1973).

Functions

As described in I 1.1. hypophysiotropic cells are considered as 'the final common pathway for the neuroendocrine control of pituitary function' (Nauta, 1963), or shorter 'the final common neuroendocrine pathway' (Scharrer, 1965). However, hypophysiotropic substances and immunoreactive varicosities have not only been detected in the hypothalamus and the m.e. but also in regions of the central nervous system remote from the hypothalamus (Barry et al, 1973; Barry, 1976; Fuxe et al, 1976; Brownstein, 1977; Hökfelt et al, 1977b; Jackson, 1977). These substances may be present either in collaterals of hypophysiotropic cells (Yagi and Sawaki, 1975; Wilber et al, 1976; Renaud, 1976; Barry, 1976) or in cells which are not (primarily) hypophysiotropic (cf. Parsons et al, 1976; Jackson and Reichlin, 1977; Goldschmidt, 1977). In view of the responsiveness of hypothalamic neurons to application of Gn-RH, TRH and somatostatin, hypophysiotropic substances may act here as neurotransmitter, whereas also extrahypothalamic effects have been described (Renaud and Martin, 1975; Sawyer, 1975; Wilber et al, 1976; Moss, 1977). In addition, several behavioural effects of hypophysiotropic substances have been described (Pfaff, 1973; McCann and Moss, 1975; Renaud and Martin, 1975; Wilber et al, 1976; Moss, 1977). In this respect it is noteworthy that Guillemin (1977a) showed a table with 17 'CNS mediated actions of TRH'; this table was dated november 1976.

Location of neurons synthetizing Gn-RH

Neurons involved in control of gonadotropin secretion are not confined to the hypophysiotropic area as defined by Halász et al (1962). There is evidence from stimulation, deafferentiation, lesion and implantation studies that, in the female rat, neurons located more rostrally in preoptic area, anterior hypothalamic area and especially suprachiasmatic nuclei are concerned with the cyclic release of LH necessary for ovulation (Everett, 1964; Halász and Pupp, 1965; Halász and Gorski, 1967; Davidson, 1969; Kaasjager et al, 1971; Bishop et al, 1972; Brown-Grant and Raisman, 1977; Raisman and Brown-Grant, 1977). Therefore, a dual hypothalamic control of gonadotropins was proposed (Barraclough, 1966; Taleisnik et al, 1970; Flerkó, 1972), implying that neurons located in a rostral 'cyclic' centre project to Gn-RH producing neurons in the 'tonic' centre of the hypophysiotropic area (fig. h). In addition, modulating effects on hypothalamic neurons and gonadotropin release are exerted by neurons located in amygdala and hippocampus (Lawton and Sawyer, 1970; Raisman and Field, 1971; Brown Grant and Raisman, 1972; Schuiling and Van Rees, 1974; Renaud, 1976; Carillo et al, 1977).

Data on the content of Gn-RH in the brain showed that, in addition to its preferential localization near the primary plexus in the m.e., Gn-RH also occurred in the suprachiasmatic and preoptic areas, especially in the organum vasculosum laminae terminalis (Kawakami et al, 1975; Weiner et al, 1975; Neill et al, 1977; Okon and Koch, 1977; for other literature Table I, point 2). Presence of Gn-RH in cell bodies located outside the hypophysiotropic area was for several species shown by immunohistochemical studies, notwithstanding some doubts with respect to specificity (Barry et al, 1973; Leonardelli et al, 1974; Pelletier et al, 1974a; Barry and Dubois, 1974, 1975; Barry and Carette, 1975; Naik, 1975a; 1976; Barry, 1976, 1977; Sétaló et al, 1976a; Silverman, 1976a; Bugnon et al, 1977a; Mazzuca, 1977; Paulin et al, 1977; Silverman et al, 1977). In the rat, several groups failed to demonstrate such immunoreactive cell bodies (see Barry, 1976), but according to Fuxe et al (1976) some are present in the most rostral part of the preoptic area and Sétaló et al (1976a) described such cells in the suprachiasmatic area and near the organum vasculosum. Such a rostral location would be in line with the dramatic reduction in Gn-RH content of the medial basal hypothalamus after deafferentiation (Weiner et al, 1975; Brownstein et al, 1976c; Palkovits et al, 1976; Sétaló et al, 1976b). Nevertheless, these observations do not exclude that certain Gn-RH producing cells in the rat are located in or near the arcuate nucleus, as claimed by Naik (1975b, 1976; cf. Brawer and Van Houten, 1976). Obviously, the area containing cell bodies which synthetize Gn-RH is much larger than the hypophysiotropic area as defined by Halász et al (1962). An explanation for this discrepancy is offered by Sétaló et al (1976b) who assign the special capacities of the hypophysiotropic area to hypophysiotropic substances present in capillaries draining the m.e.

Furthermore, it can not be excluded that e.g. Gn-RH has peripheral effects (Bernardo et al, 1977; Corbin et al, 1977).

Consequently, the cells of the anterior pituitary gland are not the only targets of hypophysiotropic substances and thus, the hypophysiotropic cells may fulfill tasks other than implied by their name (cf. Gorski, 1977)

4.1.2. the hypothalamo-neurohypophyseal system[1)]

By using the chrome alum haematoxyline method (Gomori, 1941) which stains the disulphide-rich neurosecretory material, Bargmann (1949) visualized neurosecretory cells, with cell bodies located in the supraoptic and paraventricular nuclei (or preoptic nucleus in anamniotes), axons passing through the m.e. and terminals located in the infundibular process (fig. i) Synthesis of oxytocin, vasopressin (antidiuretic hormone) and neurophysins (cystine-rich proteins that specifically bind oxytocin and vasopressin) appeared to occur mainly in the cell bodies, storage and release in the terminals (Sachs and Takabatake, 1964;

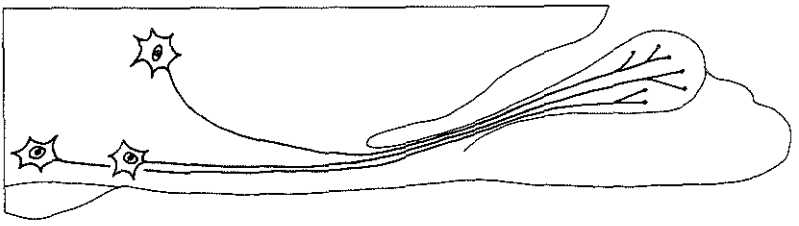

Fig. i. The hypothalamo-neurohypophyseal system with cell bodies in the (basal) supraoptic nucleus and (dorsal) paraventricular nucleus and main termination in the infundibular process (parasagittal section).

[1)] Since the neurons of this system project preferentially to a *part* of the neurohypophysis, this term is somewhat misleading.

Bargmann, 1966; Ginsburg and Ireland, 1966; Sachs et al, 1969; Norström, 1975; Vandesande and Dierickx, 1975; Brownstein and Gainer, 1977). It was furthermore demonstrated that oxytocin and vasopressin are present in separate neurons (Swaab et al, 1975; Vandesande and Dierickx, 1975; Vandesande et al, 1975) and that in the cell bodies oxytocin, vasopressin and their respective neurophysins are packed in 'elementary (neurosecretory) granules', or more accurately neurosecretory vesicles (Palay, 1957; Bargmann and Knoop, 1957; Heller and Lederis, 1961; LaBella et al, 1962; Barer et al, 1963; Dean et al, 1968; Bridges and Lederis, 1973; Cannata and Morris, 1973). An extravesicular pool of neurohypophyseal hormones and neurophysins can, however, not be excluded (Norström, 1973, 1975; Grainger and Sloper, 1974; Krisch, 1974, 1977; Silverman and Zimmerman, 1975; Leclerc and Pelletier, 1976).

Although the infundibular process represents the major site of termination of the hypothalamo-neurohypophyseal system, some collaterals proceed to the primary plexus in the m.e., as suggested by the observations of Arko and Kivalo (1958) and Rinne (1960) and affirmed by immunohistochemical studies (Leclerc and Pelletier, 1974; Vandesande et al, 1974, 1977; Silverman and Zimmerman, 1975). Moreover, certain cells of this system project to the choroid plexus (Kozlowski et al, 1976b) and into the ventricular system (Zimmerman et al, 1975).

Functions

Apart from the well known effects of oxytocin on the smooth muscles of uterus and mammary glands, and of vasopressin on reabsorption in the kidney, especially vasopressin may have modulating effects on a variety of other structures, such as vascular smooth muscles and epididymis and, in addition, on ACTH, catecholamines, cerebrospinal fluid and memory processes (Van Wimersma Greidanus et al, 1975; Altura and Altura, 1977; Hib, 1977; Saffran, 1977; Schwabedal et al, 1977; Tanaka et al, 1977).

4.2. catecholaminergic systems

The m.e. has an extremely rich catecholaminergic innervation, as is apparent from

1. chemical estimates (Clementi et al, 1971),

2. Falck-Hillarp fluorescence technique (Fuxe and Hökfelt, 1969; Ungerstedt, 1971; Björklund et al, 1973; Fuxe et al, 1976; Lofström et al, 1976a), 3. immunocytochemistry (Fuxe et al, 1974a, 1976; Swanson and Hartman, 1975) and 4. measurement of enzyme activities (Palkovits et al, 1974b; Brownstein et al, 1976a, Saito et al, 1977).
Although in mammals several catecholaminergic systems may be involved (Björklund et al, 1970, 1973; Löfström et al, 1976a; Kizer et al, 1976c), two main subsystems have been proposed, the tuberoinfundibular dopaminergic system and the ascending noradrenergic system.

4.2.1. the tuberoinfundibular dopaminergic system
This system seems confined to the medial basal hypothalamus (Weiner et al, 1972a; Cuello et al, 1973), with the cell bodies in arcuate nucleus and anterior periventricular nucleus and a projection preferentially to the primary plexus at the lateral side of the m.e. (Fuxe and Hökfelt, 1969; Jonsson et al, 1972; Ajika and Hökfelt, 1973, 1975 ; Björklund et al, 1973; Lofström et al, 1976a; Scott et al, 1976).
Dopaminergic neurons project, in addition, through the infundibular stem to the pars intermedia and the infundibular process (Björklund et al, 1973; Ben-Jonathan et al, 1977).

According to recent data a second dopaminergic system projecting to the m.e. arises in the mesencephalon (Kizer et al, 1976c; cf. Fuxe et al, 1977c; Nemeroff et al, 1977).

4.2.2. the ascending noradrenergic system
Practically all cell bodies which supply noradrenergic varicosities and terminals to the m.e. are located outside the hypothalamus (Jonsson et al, 1972; Weiner et al, 1972a; Cuello et al, 1973; Swanson and Hartman, 1975; Brownstein et al, 1976a), probably in medulla and pons (Hökfelt and Fuxe, 1972b; Björklund et al, 1973; Broadwell and Brightman, 1976). The neurons of this system project to several hypothalamic nuclei and to the internal zone of the m.e., especially subependymally. In addition, some noradrenergic varicosities and terminals are present in the external zone, especially in the medial portion (Löfström et al, 1976a). Furthermore, near the pial membranes noradrenergic nerves and varico-

sities are present, which are assumed to be exclusively involved in vascular innervation. Their cell bodies are probably located in the superior cervical ganglia.

In view of the presence of the enzyme which converts noradrenaline to adrenaline (Brownstein et al, 1976a), it may be expected that also adrenergic neurons project to the m.e. The concentration of adrenaline in the m.e. is, however, quite low (Brownstein, 1977) and adrenaline seems mainly to be present in the internal zone (Fuxe et al, 1976). Most adrenergic cell bodies seem to be located outside the hypothalamus (Brownstein, 1977).

Functions

Catecholamines, especially noradrenaline and dopamine, have important rôles in the control of gonadotropin secretion (Fuxe and Hökfelt, 1969; Porter et al, 1972; Kizer et al, 1974; McCann and Moss, 1975; Sawyer, 1975; Kalra, 1977b; Smythe, 1977; Yen, 1977). Their effects are in part exerted within the m.e. (Kizer et al, 1974, 1975; Löfström et al, 1976a,b). With respect to the rôles of dopamine and noradrenaline, there is a continuing controversy (see Table VII). Evidence for a stimulating effect of noradrenaline and an inhibiting one of dopamine is, at least as far as LH is concerned, growing. Data on effects of adrenaline are scanty (Table VII).

The possibility that catecholamines act as hypophysiotropic substances for LH and FSH is not likely (Schneider and McCann, 1969; Kamberi and McCann, 1969; Kamberi et al, 1970a,b; Porter et al, 1970, 1972; Hökfelt and Fuxe, 1972a,b; McCann et al, 1972). However, dopamine may well be the prolactin-inhibiting factor. Dopamine does occur in portal blood (Ben-Jonathan et al, 1977), inhibits prolactin release at the pituitary level (Donoso et al, 1973; Blake, 1976) and has been implied in the inhibitory short feedback action of prolactin (Hökfelt and Fuxe, 1972a; Advis et al, 1977; Fuxe et al, 1977c; Gudelsky et al, 1977).

Dopaminergic neurons projecting to the pars intermedia may be responsible for the action ascribed to melanocyte-stimulating hormone-release-inhibiting factor (Tilders et al, 1975; Tilders and Smelik, 1977).

Table VII Rôles ascribed to different catecholamines in control of gonadotropin secretion

dopamine	
- inhibitory	Fuxe and Hökfelt, 1969; Uemura and Kobayashi, 1971; Hökfelt and Fuxe, 1972b; Craven and McDonald, 1973; Miyachi et al, 1973; Sawyer et al, 1974; Fuxe et al, 1976, 1977; Gnodde and Schuiling, 1976; Löfström et al, 1976b
- stimulatory	Kordon and Glowinski, 1969, 1972; Schneider and McCann, 1969, 1970a,b; Kamberi et al, 1969, 1970a,b; McCann et al, 1972; Clemens et al, 1976; Kordon et al, 1976; Rotsztejn et al, 1976; Vijayan and McCann, 1977
noradrenaline	
- inhibitory	Uemura and Kobayashi, 1974
- stimulatory	Cramer and Porter, 1973; Kalra and McCann, 1973 Ojeda and McCann, 1973; Cocchi et al, 1974; Sawyer et al, 1974; Tima and Flerkó, 1974; Terasawa et al, 1975; Drouva and Gallo, 1976, 1977; Gnodde and Schuiling, 1976; Gudelsky et al, 1976; Leblanc et al, 1976; Fuxe et al, 1977c; Hall et al, 1977; Kalra 1977a,b
adrenaline	
- inhibitory	Blake, 1976
- stimulatory	Rubinstein and Sawyer, 1970; Sawyer et al, 1974

4.3. indole-aminergic system(s)

Serotonin (5-hydroxytryptamine), the principle neuronal indole-amine, is present in moderate concentrations in m.e. and infundibular stem (Saavedra et al, 1974), as is tryptophan hydroxylase (Brownstein et al, 1976a), the serotonin forming enzyme. Serotonin is not necessarily present in neuronal elements (Knigge et al, 1975) but according to Baumgarten and Lachenmayer (1974) indole-aminergic nerve fibres do occur in the external zone of the m.e. These fibres seem mainly to be derived from extrahypothalamic areas (Brownstein et al, 1976a), especially the nucleus raphe dorsalis (Palkovits et al, 1977a).

Functions

The effect of serotonin on gonadotropin release has been described mostly as inhibitory (Kordon et al, 1968; Kamberi et al, 1970a; Schneider and McCann, 1970a; Kordon and Glowinski, 1972; Labhsetwar,

1972; Zolovick and Labhsetwar, 1973; Fuxe et al, 1974c; Ladosky and Wandscheer, 1975; Gallo, 1976). According to recent data on the oestrous cycle serotonin has, in addition, a (long term) stimulating effect (Kordon et al, 1976; Wuttke et al, 1977). The site of action of the stimulating effect may be the suprachiasmatic and/or preoptic area, whereas the more acute inhibiting effect may be exerted at the level of the arcuate/m.e. region (Kordon et al, 1976). Serotonin is also thought to be stimulatory in prolactin release (Meites, 1977).

4.4. cholinergic system(s)

Presence of acetylcholinesterase (Kobayashi et al, 1970) and, more importantly, a high concentration of choline acetyltransferase (Brownstein et al, 1975), the enzyme responsible for synthesis of acetylcholine, are indicative of cholinergic neurons projecting to the m.e. The cell bodies of these neurons are probably located in the medial hypothalamus (Brownstein et al, 1976a), especially in the arcuate nucleus (Carson et al, 1977).

Functions

Several experiments suggest that acetylcholine is involved in control of gonadotropin release (Luine et al, 1975; McEwen et al, 1975). It may in fact facilitate release of Gn-RH (Everett, 1964; Libertun and McCann, 1973, 1976; Fiorindo and Martini, 1975; Gnodde and Schuiling, 1976).

5. *Relevance and objectives*

> 'I am optimistic about the eventual practical value of almost any finding dealing with mammalian reproduction. Nevertheless, practical value should not be the sole reason or even a very important criterion in judging the value of an investigation, since the esthetic value of the discovery of the unknown is at least as important'
>
> (quotation from anonymous referee, commenting on a grant application)

The motive to study a part of the brain which represents on weight base only 0.02% of the rat brain resides in the extraordinary rôle of the m.e. in neuroendocrine regulation. As expressed by Harris and Campbell (1966): 'The median eminence is the key link in neuroendocrine relationships'. Indeed, changes in the internal milieu and in neural signals derived from higher integrative centres converge upon the m.e.

where the multitude of signals is converted into release of quota of hypophysiotropic substances influencing the function of the anterior pituitary gland.

The importance for life of a proper functioning of the anterior pituitary gland in controling the major endocrine glands and of the brain in controling the anterior pituitary gland, is too well known from effects of brain or pituitary defects and of emotional, environmental and seasonal factors, to eleborate upon here. The importance of the hypophysiotropic substances themselves is indicated by the profound effects of immunization against Gn-RH (Table I) and is suggested by their extensive clinical use, both as diagnostic and as therapeutic tools. Perspectives concern, in the case of Gn-RH or its analogues, both birth prevention and promotion of pregnancy (Schally et al, 1973a, 1976b; De la Cruz et al, 1976; Kastin et al, 1976; Nillius, 1976; Schally and Coy, 1977; Vale et al, 1977). Notwithstanding optimistic expectations, therapeutic use of Gn-RH in females appears to be faced by severe problems (Corbin et al, 1977; Vale et al, 1977; Zárate et al, 1977); effects in males are more promising (Mortimer and Besser, 1977). Diagnostic value of Gn-RH is limited up to now, although it is of value in assessing the functional state of the hypothalamo-hypophyseal system (Mortimer and Besser, 1977; Schneider et al, 1977).

Inherent to its presumed key position, the m.e. represents the last opportunity for the central nervous system to influence directly and specifically the function of the anterior pituitary gland. In that respect, it would be surprising if the m.e. would be just a pool of nerve endings 'passively waiting' for a signal, delivered far away to dendrites or cell body, to release stored material into the extracellular space. Obviously (I 3.), numerous complex mechanisms are operating in the m.e. which together are responsible for the ultimate release of the hypophysiotropic substances. These mechanisms are at best vaguely understood and a correlation of structure and function is far from established.

This study is an attempt to correlate structure and function in the m.e. and is focussed on the following subjects, which are related to the involvement of neuronal and non-neuronal elements of the m.e. in control of anterior pituitary function, especially with respect to gonadotropin release:

1. quantitative data on neurons projecting to the primary plexus, with special reference to release of gonadotropin releasing hormone,
2. granular vesicles as parameter to distinguish different types of nerve endings,
3. mechanisms of release and the fate of remnants of the release

process in relation to the origin and function of microvesicles,
4. rôles of the typical non-neuronal cells,
5. plasticity near the primary plexus.

It seemed necessary to obtain at first detailed information on certain features of the ultrastructural organization of the m.e. They concern qualitative and quantitative aspects, mainly of the area near the primary plexus (III 1.; see also App. 2 and 3).

A second part of the results (III 2.; see also App. 1 and 4) deals with the mechanism(s) by which biologically active substances stored in the m.e. are released. Since release will inevitably lead to formation of (membranous) by products, which somehow have to be removed, attention is also given to events subsequent to release. For this purpose an extracellular tracer has been used since one mechanism of release (exocytosis) will necessitate recapture of membranous material from the plasma membrane. It may be expected that during this process extracellular material will be incorporated into the nerve ending.

In the third part (III 3.1.; see also App. 5 and 6), effects will be described of disturbing the hormonal equilibrium by castration, which leads to a marked increased release of gonadotropins, and by subsequent steroid treatment which, in part, counteracts the effects of castration. The chance of documenting (increased) release of Gn-RH at the ultrastructural level will be considered.

Castration is generally assumed to lead to increased release of Gn-RH, although not all attempts to establish such a relationship are convincing. Data of Shin et al (1974a) on the concentration of Gn-RH in peripheral blood were even indicative of a decreased release. However, their data have to be doubted seriously since concentrations found were much higher than those found by others in pituitary portal blood. In the latter, increased concentrations of Gn-RH were found after castration (Ben-Jonathan et al, 1973; Eskay et al, 1974; Porter et al, 1975, 1976, 1977; Ching, 1976), as was also reported for peripheral blood by Saito et al (1976).
Moreover, treatment of castrated rats with anti-serum to Gn-RH resulted in a reduction of serum LH and FSH level (Arimura, 1976). After castration, there was, furthermore, a pronounced decrease in content of Gn-RH in m.e. (Joseph et al, 1975) and (medial basal) hypothalamus (Moguilevsky et al, 1975; Root et al, 1975; Asai and Wakabayashi, 1976; Baram and Koch, 1977). There is also evidence that castration stimulates synthesis of hypothalamic Gn-RH (Moguilevsky et al, 1974, 1975). Castration leads in the m.e. also to increased protein synthesis (Ter Haar and McKinnon, 1972).

In certain experiments a division will be made between a lateral and medial part of the m.e., in view of the preferential localization of

Gn-RH (as well as dopamine) at the lateral side of the m.e. (Barry et al, 1973; King et al, 1974; Kizer et al, 1976a; Pelletier et al, 1976 ; Kozlowski et al, 1976a; Löfström et al, 1976a). For other, basically qualitative studies on the effect of castration on the ultrastructure of the m.e., see T. Kobayashi et al (1969), H. Kobayashi and Matsui (1969), H. Kobayashi et al (1970), McArthur (1970), as well as a study of Zambrano and De Robertis (1968).

In the final part of the results (III 3.2.), effects will be described of electrochemical stimulation of the medial preoptic area, which rapidly results in a raised serum LH level.

> This increased release of LH is at least in part mediated by an increased release of Gn-RH into portal blood (Eskay et al, 1974; 1977; Fink and Jamieson, 1974, 1976; Porter et al, 1977). Although the mechanisms involved are not well understood (Dyer and Burnet , 1976; Dyer, 1977), release of Gn-RH may be subsequent to an increase in synthesis (Kalra et al, 1973).

I am well aware that this study is confined to limited aspects of neuroendocrinology. To make things worse, the study is dealing exclusively with the rat. However, this species has been used more than any other species in neuroendocrine studies.[1)]

It may be added that the opportunity to obtain properly fixed normal human material of the m.e. is virtually absent, as is the possibility of experimenting with such material.

In view of the abundance of submicroscopical vesicles as one of the most conspicious organelles in the structural elements composing the m.e., electron microscopy was chosen as method of approach, notwithstanding certain disadvantages of this choice.

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1) 'I feel that our clinical colleagues have a tremendous tendency to pooh-pooh the rat data as being irrelevant and immaterial to what is going on in the human and yet the more we see of the data obtained from experiments with humans, the more obvious it becomes that rat data are giving similar, if not identical results' (Barraclough, 1973).

II. MATERIALS AND METHODS

1. animals, anaesthesia

Over 1000 adult male rats (R-Amsterdam, a highly inbred Wistar substrain) were used. They were kept under controlled conditions (see App.1). The animal room was lighted from 5.00 to 19.00 hours. Data on female rats refer to those with a regular 5-day cycle for at least two cycles, as judged by daily vaginal smearing.
In the standard procedure, rats were removed from the animal room to the laboratory. There they remained 1-1½ hours before they received an intraperitoneal injection of Avertin (Winthrop; 0.18 gr/kg body weight). In case of brain stimulation or intraventricular perfusion, they were instead anaesthetized with an intraperitoneal injection of urethane (2 gr/kg body weight).

2. fixation, dehydration and embedding procedures

For general references, see Hopwood (1972), Pfenninger (1973) and Glauert (1974).

2.1. fixation

Primary fixation occurred in the standard procedure by vascular perfusion (see App.5).
In some experiments, rats were decapitated by a guillotine, without prior anaesthesia. Fixation occurred in situ with cooled fixative.
To obtain direct fixation of the ventricular lining, fixative solution was infused into the third ventricle. The outflow was from the lateral ventricle. A flow rate of 37.5 µl/min was obtained by using a Braun-Melsungen pump (Type 871020).
As standard fixative a mixture was used of 1% formaldehyde, prepared from paraformaldehyde, 2% glutaraldehyde (TAAB), 0.02% $CaCl_2$ and 2% polyvinyl pyrrolidone in 0.1M sodium cacodylate buffer, pH 7.4.
In case of in situ and immersion fixation, polyvinyl pyrrolidone was omitted and in certain experiments 2,4,6, trinitrophenol (see App.1) was added. After (pre) fixation, the brain was removed from the skull. The hypothalamus, including m.e. and infundibular stem, was excised and immersed into the same fixative solution.
Temperature: 4°C. Total fixation time: in case of perfusion, 2-7 hours, in case of immersion, 5-18 hours.
In certain experiments, tissue was fixed by immersion in potassium permanganate in Locke's solution according to Douglas and Poisner (1964), pH 7.0. Temperature: 0°C. Duration: 45 min.

2.2. wash

Aldehydes were removed by rinsing in the same buffer, to which 0.02% $CaCl_2$ and in addition sucrose was added to the same osmolality as that of the fixative solution. Temperature: 4°C. Duration: at least 18 hours.
In case of fixation with potassium permanganate, the tissue was rinsed in Locke's solution. Temperature: 0°C. Duration: 10 min. During the rinse period, the hypothalamus was sliced into coronal sections in most cases, using a tissue chopper or occasionally a vibratome. Selection of the slices occurred on the basis of the shape of third ventricle, m.e. and infundibular stem.

2.3. postfixation

In the standard procedure, aldehyde fixed tissue was postfixed in cacodylate buffered 2% osmium tetroxide, to which 0.02% Ca_2Cl_2 and 1% sucrose

was added, pH 7.4. Temperature: 4°C. Duration: 2 hours.

2.4. en bloc treatment with uranyl acetate
Part of the material fixed by aldehyde-osmium tetroxide and all material fixed by potassium permanganate was, after a brief rinse in distilled water, treated with half-saturated uranyl acetate in distilled water (pH 4.1). Temperature: 4°C. Duration: 18 hours.

2.5. dehydration
After a brief rinse in distilled water, the tissue was treated with 70% acetone. Temperature: 4°C. Duration: 30 min. The tissue was subsequently dehydrated in 90% acetone (room temperature, duration: 30 min) and 100% acetone (room temperature, duration: 2 x 60 min).

2.6. embedding
After an impregnation period in a medium containing a 1:1 mixture of 100% acetone and Epon 812 (room temperature, duration: 2 hours), the tissue was transferred to pure embedding medium (temperature 37°C, duration: 2 hours). After embedding in gelatine capsules, polymerization occurred at 60°C (duration 48 hours).

3. sectioning and 'staining'
Coronal and sagittal sections of the m.e. and infundibular stem were made using a Reichert Om U2 or a LKB ultratome III. For orientation, 1 µm sections were stained with alkaline methylene blue. For electron microscopy, ultrathin sections of silver interference colour (about 80 nm in thickness) were collected on carbon coated formvar grids. Coating occurred with an Edwards Speedivac Coating Unit. Ultrathin sections were contrasted with uranyl acetate and/or lead citrate. Uncontrasted sections were also used; they were collected on bare grids.

4. electron microscopy
For (transmission) electron microscopy, a Philips EM 300 was used, operating at 40 or 60 KV and with objective apertures of 10 or 20 µm. Enlargement was checked with a carbon grating replica (Fullam, type 1002x, 2160 lines/mm).

5. electrochemical stimulation
Four min after injection of urethane, the rats were placed into a stereotaxic apparatus. Two electrodes were oriented bilaterally in the medial preoptic area, 1.0 mm from the midsagittal plane. The atlas of De Groot (1959) was used as guide for placement of the electrodes. Bipolar concentric stainless steel electrodes were used (Rhodes medical instruments, model SNE-100, outer diameter 0.25 mm, anodal central lead). Beginning nine min after injection of urethane, the medial preoptic area was stimulated by passing a continuous direct current of 30 µA for 60 sec through each electrode. A Grass stimulator (S-8) and constant current unit was used. For the sham-stimulated rats, the procedure was similar, but no current was passed.

6. treatment with horseradish peroxidase (HRP)
For studying micro-pinocytotic activity, HRP was injected intravenously (see App.4-6). HRP (Sigma, type 2, or in special cases Boehringer, grade I) was dissolved in Locke's solution. In controls, solvent only was injected.

Peroxidase activity was demonstrated according to Graham and Karnovsky (1966). Tissue chop sections were used instead of frozen sections. It appeared that the sections were thicker than the dial of the tissue chopper indicated (50 um). In view of the limited depth of penetration of the incubation medium, care was taken to use exclusively superficial ultrathin sections. Incubation occurred at room temperature, during 40 min. After 20 min the medium was replenished.

7. identification of monoaminergic elements
To visualize neuronal elements which are able to accumulate exogenous monoamines, the m.e. was removed from decapitated rats and incubated in 2 ml culture medium Difco 199 at 37°C in a Dubnoff metabolic shaker under 95% oxygen and 5% carbon dioxyde. Six min after decapitation, the medium was replaced for medium with or without 5-hydroxydopamine (5-OHDA; 100 µg/ml). This 'false' neurotransmitter, or its metabolites, can be accumulated by noradrenergic, dopaminergic and, at the concentration used, serotoninergic varicosities, due to the active (re)uptake mechanism of these neurons (see Tranzer and Thoenen, 1967, 1968; Hökfelt, 1968, 1970; Ehinger et al, 1970; Richards and Tranzer, 1970; Ajika and Hökfelt, 1973).
Fixation followed after 20 min, with either potassium permanganate or aldehyde-osmium tetroxide.

8. in vitro treatment with steroids
Male rats, castrated 2 or 4 weeks earlier, were treated as in 7., but after incubation with 5-OHDA, the medium was replaced with medium 199 with or without testosterone (17β-hydroxy-4-androstene-3-one). Therefore, 0.70 mg testosterone was dissolved in 7.0 ml absolute ethanol. To 1 ml of this solution 99 ml of Locke's solution was added. Of this solution 0.1 ml was added to 1.9 ml of medium 199 to yield a final concentration of 50 ng testosterone/ml. Apart from the addition of testosterone, the same procedure was followed for control medium.
Fixation with potassium permanganate followed after an incubation period of 60 min.

9. treatment with ethanolic phosphotungstic acid
To impregnate synaptic junctions and cores of granular vesicles (App.3), part of the aldehyde fixed material was treated with ethanolic phosphotungstic acid according to Bloom and Aghajanian (1968a).

10. sizes of granular vesicles
Sizes of granular vesicles were measured with a Zeiss TGZ 3 (App.3).

11. X/Y plots
To registrate the distribution of neuronal and non-neuronal endfeet directly in the electron microscope, a X/Y recorder was connected to the mechanical stage of the electron microscope through two linear potentiometers (design, Dr. J. Dekker, Department of Anatomy).

12. radioimmunoassay

Serum levels of LH and FSH were measured according to Welschen et al (1975). In the standard procedure, blood was collected from the right atrium at the start of the perfusion procedure.
For convenience, data on LH and FSH, obtained in experiments described in III 3.1., are collectively reported here. Serum levels of LH and FSH were significantly raised in castrated rats, if compared with sham-operated controls (6 adult males and 5 adult females per group). Time indicates the period between operation and sacrifice. Data (± SEM) are expressed on the basis of reference preparation NIAMD rat-LH RP-1 and NIAMD rat-FSH RP-1.

	ng LH/ml serum		ng FSH/ml serum	
	castrates	controls	castrates	controls
males, 30 days[1)]	194.5±14.68	15.7±0.67	292.0±21.47	157.3± 8.76
males, 30 days[1,2)]	192.0±22.62	18.8±4.87	322.3±21.85	132.2±14.35
females, 18 days[1,2)]	171.6±17.51	12.6±2.27	335.4±66.48	44.8±22.56
males, 24 hours	61.7± 6.48	10.7±2.11	214.0±14.22	146.7± 6.37

[1)] withdrawal of blood, 20 min after intravenous injection of HRP under ether anaesthesia

[2)] controls refer to females sacrificed at di-oestrous I

In male rats, castrated 30 days earlier and treated during 3 days with oestradiol benzoate in arachid oil or with oil only (see III 3.1.), serum LH levels (± SEM) were resp. 138.2 ± 12.38 and 210.6 ± 30.69 ng/ml and serum FSH levels 364.5 ± 17.28 and 359.6 ± 7.05 ng/ml (for both groups, n=5).

13. statistics

The Wilcoxon two-sample test was used for statistical analysis.
The means were considered significantly different if the double tail probability was smaller than 0.05.

III. RESULTS

Unless otherwise stated, the following applies.
Data refer to coronal sections of the midcentral region (see fig. 7) of the m.e. of avertin anaesthetized, adult male rats of varying age, which had been perfused between 11.00 and 16.00 hours with a mixture of formaldehyde and glutaraldehyde. The m.e. was postfixed with osmium tetroxide. When rats were treated with horseradish peroxidase (HRP), they received an intravenous injection of 25 mg HRP/100 gr body weight.
The term varicosity indicates an axonal swelling, the term neuronal endfoot, a varicosity in contact with the outer basement membrane. The term neuronal profile indicates a cross-section of an axon, of a varicosity or, exceptionally, of a dendrite. A non-neuronal profile indicates a cross-section of a non-neuronal cell body, process or endfoot.
Quantitative data on neuronal and non-neuronal endfeet include all endfeet at the outer part of the palisade zone (sub-zone A) which endfeet are present at the base of the infundibular recess in an ultrathin section of silver interference colour. Data refer to one half of the m.e.
Means are given with the standard error of the mean.

For convenience of the reader, the rather detailed data of each paragraph are preceeded by a summary.

1. General qualitative and quantitative data on the ultrastructural organization of the median eminence

1.1. differences between neuronal and non-neuronal profiles

SUMMARY Axonal swellings in the external zone of the m.e. are characterized by synaps-like microvesicles and, frequently, numerous granular vesicles; lipid droplets, ribosome-like particles and granular endoplasmic reticulum are absent. These characteristics are in contrast to those of non-neuronal profiles (see Table I).

Fig. 1. Neuronal and non-neuronal profiles of the m.e., located near the outer basement membrane of the primary plexus (see Table 1 for explanation of numbers and letters). Material was fixed in aldehyde-osmium tetroxide, as in the other electron micrographs (fig. 5,6 excepted). With the exception of material treated for demonstration of peroxidase activity (fig. 20-24), these electron micrographs were derived from tissue treated en bloc with uranyl acetate.
If not specified, electron micrographs concern areas close to the primary plexus as frequently shown by the presence of the outer basement membrane (arrowhead).
F processes of fibroblasts x 30.000

Table 1 Characteristics of neuronal and non-neuronal profile

neuronal

1. close to the basement membrane[1)]

1. either a varicosity measuring up to 5 µm in width or an axon*
2. cytoplasm relatively electron-lucent
3. electron-lucent microvesicles* about 50 nm in diameter
4. granular vesicles about 60-160 nm in diameter
5. mitochondria oval or rounded, mostly larger than 0.2 µm in width
6. invaginations of the plasma membrane about 50 nm in diameter
7. microtubules
8. small cisterns of agranular reticulum
9. synaptoid contacts* with non-neuronal profiles
10. dense, frequently myelinated bodies; autophagic vacuoles

2. away from the basement membrane

1. either an axon* or a varicosity measuring up to 30 µm in width
2. microtubules, visible especially in axons
3. granular vesicles
4. microvesicles*
5. agranular reticulum
6. synaptoid contacts*

non-neuronal

1. close to the basement membrane[1)]

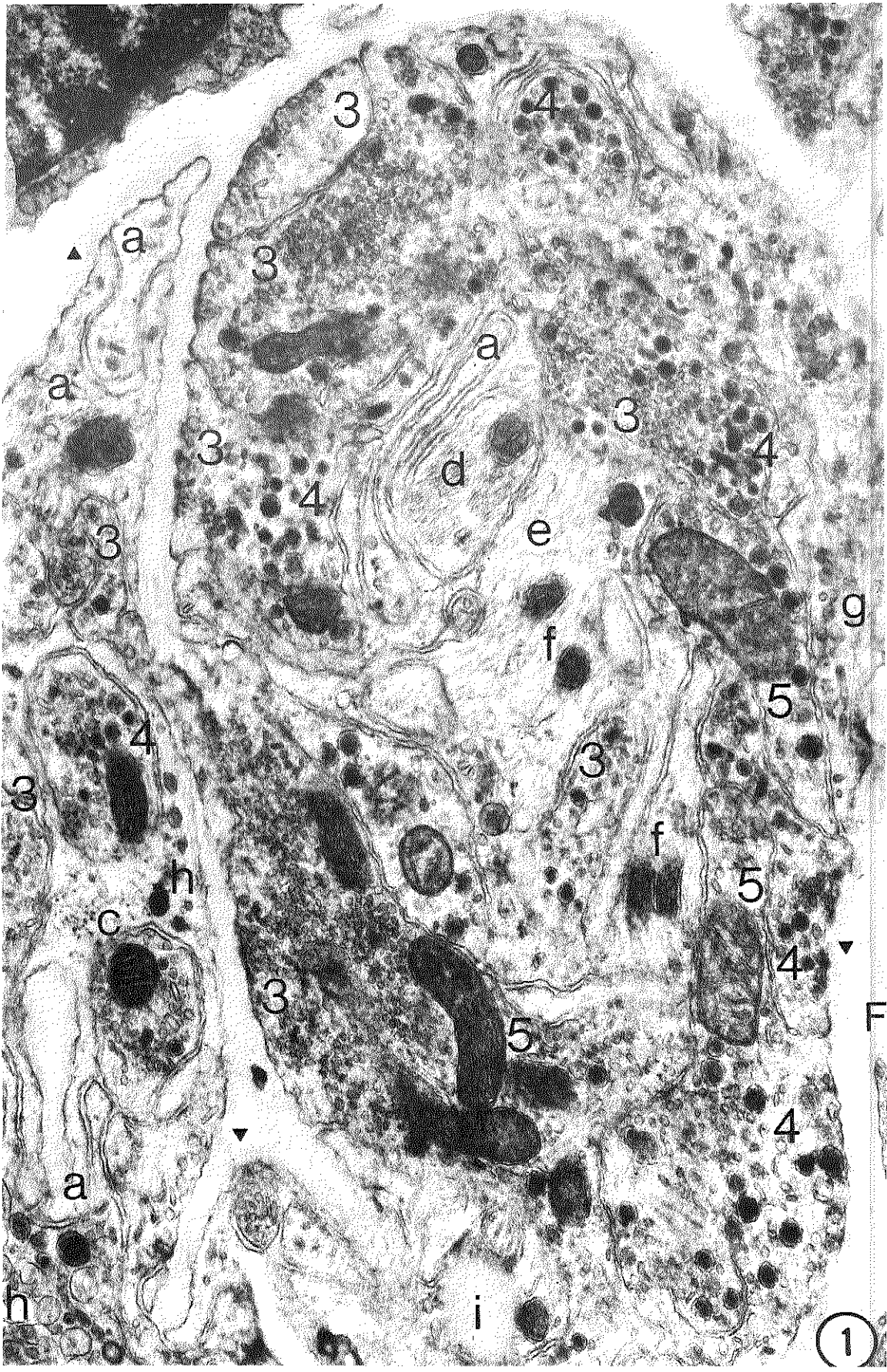
a. profiles highly variable in size and shape
b. cytoplasm variable in electron-density
c. ribosome-like particles*
d. microfilaments
e. microtubules
f. mitochondria elongated, mostly smaller than 0.2 µm in width
g. tubular or rounded agranular endoplasmic reticulum, frequently with material of high electron-density and highly variable in size
h. vesicular material of variable size and of irregular shape, with and without electron-dense material
i. lipid droplets*
j. granular endoplasmic reticulum*
k. invaginations of the plasma membrane larger than 60 nm in diameter
l. tight junctions with other non-neuronal profiles

2. away from the basement membrane

a. profiles mostly fibre-like, of considerable width, and oriented from the infundibular recess towards the primary plexus
b. ribosome-like particles*
c. abundant microtubules
d. microfilaments
e. abundant agranular endoplasmic reticulum
f. lipid droplets*
g. granular endoplasmic reticulum*

1) see fig. 1

* of determinative value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Two types of ectodermal profiles are present in the m.e.: neuronal and non-neuronal. Their characteristics are summarized in Table 1 (see also fig. 1). A division is made between profiles located close to the outer basement membrane of the primary plexus (say, within 5 µm) and those away from the basement membrane. The characteristics are arranged roughly in decreasing order of frequency. They refer to material fixed in aldehyde-osmium tetroxide.

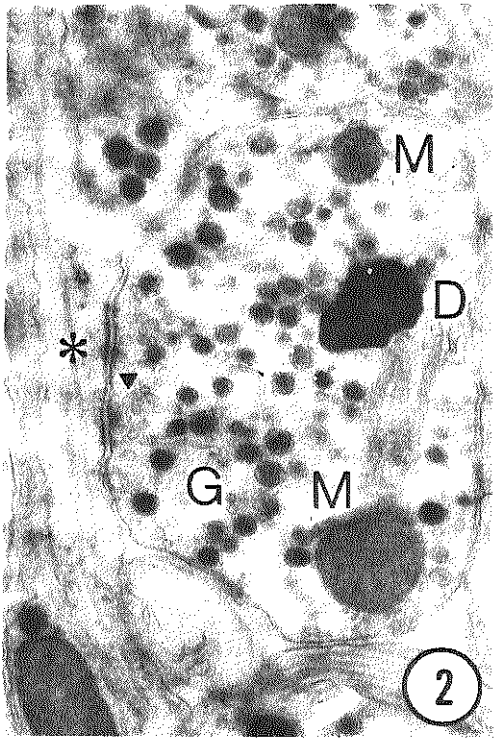
1.2. special features of the neuronal elements

SUMMARY Throughout the m.e., synaptoid contacts with non-neuronal elements were present. Certain varicosities showing such contacts were thought to belong to peptidergic neurons. Synaptic specializations present between axons, were practically absent but close contacts were numerous.
Microvesicles constituted a heterogeneous group of organelles as shown by pretreatment with the 'false' transmitter 5-OHDA, since only microvesicles of certain varicosities acquired electron-dense material. This was most pronounced if fixation occurred with potassium permanganate. These varicosities were assumed to belong to monoaminergic neurons. The monoaminergic varicosities were present throughout the m.e. but were preferentially located at the lateral side of the m.e. near the primary plexus.
Certain non-monoaminergic profiles were, after fixation with potassium permanganate, characterized by numerous electron-lucent vesicles of the (variable) sizes of the granular vesicles (GV) seen after aldehyde-osmium tetroxide fixation. These profiles, probably, belong to peptidergic neurons. In comparing preterminal and terminal parts of their axons, there was no evidence for a difference in size of GV. GV and microvesicles were preferentially located near the primary plexus.

Fig. 2. Neuronal profile containing a dense body (D), mitochondria (M), numerous GV (G), and microvesicles (▼) accumulated near a synaptic contact. In all probability, the postsynaptic structure (✱) is an axon. x 32.000

Fig. 3. Broad tanycyte-like process, with lipid droplet (L) and elongated mitochondria (M), and adjoining varicose axons, all oriented from infundibular recess towards primary plexus (palisade zone). Note two synaptoid contacts (✱) with accumulations of microvesicles in varicosities containing, in addition, GV about 100-130 nm in diameter. x 25.000

Fig. 4. Monoaminergic and non-aminergic profiles located near the basement membrane (▲) after incubation with 5-OHDA and fixation with aldehyde-osmium tetroxide. Note the presence of small electron-dense organelles in several mono-aminergic (✱) profiles. Two nerve profiles containing several GV are without such small electron-dense organelles. These non-aminergic, probably peptidergic profiles contain large numbers of electron-lucent microvesicles. Lateral part of sub-zone A; four weeks post-castration. x 30.000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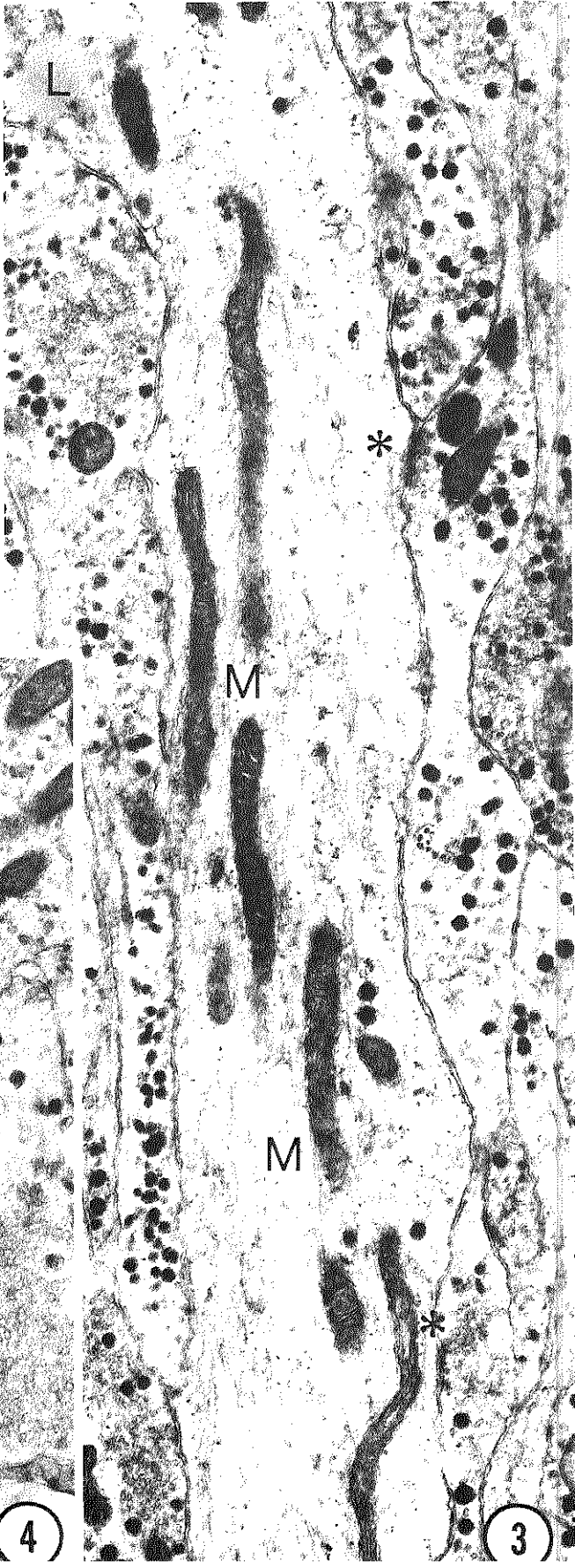
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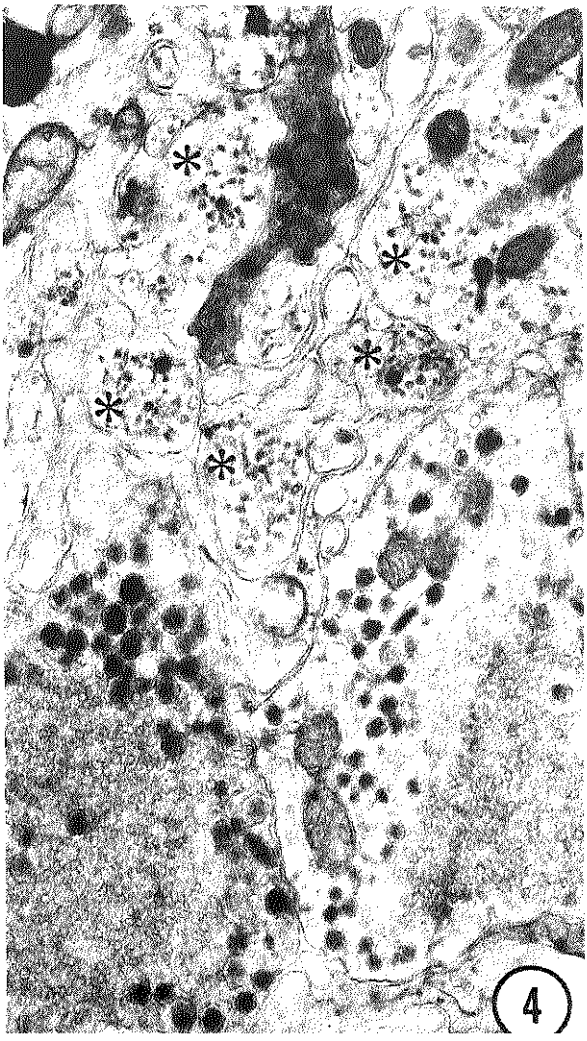
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1.2.1. synapses and synaptoid contacts

Axosomatic and axodendritic synapses were small in number and restricted to the internal zone. In the external zone, synapses were, with one possible exception (fig. 2), not observed. Absence was supported by negative results after aldehyde-ethanolic phosphotungstic acid, a procedure used to reveal synaptic junctions. It has, however, to be mentioned that adjacent varicosities without interposed non-neuronal processes are a common feature of the external zone.

Synaptoid contacts with non-neuronal elements (fig. 3) were present throughout the m.e. Postsynaptic specializations were generally completel absent and the contacts were not well revealed by aldehyde-ethanolic phosphotungstic acid. The vesicular content of varicosities showing synap toid contacts varied. Occasionally, large numbers of GV were present, mostly 80-110 nm in diameter, and sometimes larger in sizes (fig. 3).

Since GV present in monoaminergic profiles of the external zone appeare to be relative small in size and number, whereas GV in peptidergic profiles can be large and numerous (III 1.2.4.), part of the above mentioned varicosities showing synaptoid contacts may belong to peptidergic neurons.

1.2.2. granular vesicles (GV)

Distribution - Passing through the palisade zone in the direction of the primary plexus, thus from sub-zone D to A (fig. d), the relative numb of nerve profiles with GV increased (20.8, 29.3, 41.1 and 48.7% respectively), as calculated from data mentioned in App. 3, Table 2. This phenomenon is further supported by data mentioned in III 1.4. (Table 2), where GV were present in 74.8% of the nerve profiles in contact with the outer basement membrane of the primary plexus. Furthermore, certain varicositie located near the primary plexus appeared to contain over 2000 GV.

Apparently, GV are in the external zone preferentially located near the primary plexus.

Sizes - In App. 3, it was mentioned that, in the palisade zone, GV were relatively small near the basement membrane. One possible cause was a diminution in size of GV in the terminal part of the axons. Therefore, sizes of GV were measured at different distances from the basement membra in serial sections of axonal profiles with numerous (in one case 297) GV. Lengths were mostly confined to 10 µm. No consistent differences in mean sizes of GV were found between preterminal and terminal parts of the axon studied.

This indicates that at least in the last 10 µm of an axon abrupt change in sizes of GV are uncommon.

1.2.3. microvesicles

Distribution - Microvesicles were present in all varicosities showi synaptic or synaptoid contacts and were especially numerous near the primary plexus. Deep in the palisade zone (sub-zone D), less than 25% of the nerve profiles contained microvesicles (App. 2, Table 1), against 65% of the nerve profiles localized within 10 µm from the outer basement membrane (App. 5, Table 1), and against 99.5% of the nerve profiles contactin the outer basement membrane (App. 6).

Thus, microvesicles are a prominent feature of the varicosities contacting the outer basement membrane of the primary plexus.

Heterogeneity - The frequency distribution of the microvesicles showed a sharp peak at 45-50 nm but they ranged roughly between 30 and 60 nm. Between different varicosities differences in size, shape and distribution of the microvesicles was occasionally apparent. Microvesicles of only certain varicosities acquired electron-dense material after incubation with 5-hydroxydopamine (III 1.2.4.)

These observations indicate that microvesicles represent a heterogeneous group of organelles.

1.2.4. monoaminergic and non-aminergic varicosities

Certain nerve profiles located near the primary plexus were characterized by small electron-dense organelles if fixation with aldehyde-osmium tetroxide was preceded by incubation with 5-OHDA (II 7.). These organelles resembled small and flat microvesicles as well as tubular membrane-bound reticulum (fig. 4). Such organelles were absent after incubation without 5-OHDA. Characteristically, nerve profiles containing numerous GV were devoid of such small electron-dense organelles (fig. 4).

Nerve profiles which, after incubation with 5-OHDA, become characterized by such small electron-dense organelles probably belong to monoaminergic neurons, in view of the documented accumulation of 5-OHDA in this type of neuron (II 7.). Nerve profiles which are, after such an incubation, characterized by the presence of numerous GV and absence of small electron-dense organelles represent probably varicosities of non-(mono) aminergic, presumably peptidergic neurons (see below).

Differentiation between presumed monoaminergic and non-aminergic varicosities appeared to be easier if incubation with 5-OHDA was followed by fixation with potassium permanganate. Then, the following was observed in the external zone.

1.2.4.1. differences between monoaminergic and non-aminergic varicosities

Due to accumulation of 5-OHDA, there were two different types of varicosities, as based on presence or absence of distinct electron-dense material in microvesicles (fig. 5-6). The respective profiles are called monoaminergic and non-aminergic (for references: II 7.). In thus defined monoaminergic profiles, practically all microvesicles contained electron-dense material; such material was in addition present in GV of about 80-100 nm and in membrane-bound organelles resembling smooth endoplasmic reticulum. Such electron-dense material was completely absent from the other, viz. non-aminergic profiles.
The monoaminergic profiles differed in more respects from the non-aminergic ones:
a. the microvesicles with electron-dense material, the so-called small granular vesicles[1]), were distributed rather homogeneously in their varicosities, whereas the electron-lucent microvesicles of the non-aminergic profiles were occasionally present in central clusters of over 100 micro-

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[1]) Note that in aldehyde-osmium tetroxide fixed material, another distinction between small and large granular vesicles was made (App. 2,3)

vesicles, especially after castration (compare fig. 4),
b. in contrast to monoaminergic profiles in which GV were scanty or absent, non-aminergic profiles frequently contained numerous vesicles of the size of GV (fig. 5). These vesicles will be denoted as 'GV' because of their low electron-density. The characteristic vesicles of 150-220 nm, localized in the axons of the hypothalamo-neurohypophyseal system, were also of this low electron-density after permanganate fixation.
c. monoaminergic profiles seemed relatively small. Their mean cross-sectional area was laterally in sub-zone A 0.46 ± 0.07 and medially 0.39 ± 0.03 μm^2, versus 0.60 ± 0.03 and 0.67 ± 0.12 μm^2 for non-aminergic profiles (means derived from 5 rats; number of profiles involved: 483).

1.2.4.2. distribution of monoaminergic profiles
a. The following example gives an impression of the multitude of monoaminergic profiles present in different layers of the m.e. Profiles located laterally to the floor of the infundibular recess were excluded. The data show, form dorsal to basal, the relative numbers of varicosities belonging to monoaminergic neurons:

internal zone	20% (n=164)
dorsal palisade zone (sub-zone D and C)	13% (n=375)
basal palisade zone (sub-zone B and A)	25% (n=550)
zona granulosa	22% (n=237)

Nerve profiles of sub-zone A located at the outer basement membrane were for 33% (n=311) monoaminergic in this preparation, whereas in two other preparations figures of 18% (n=521) and 21% (n=470) were found. This indicates that roughly 25% of the neuronal endfeet at the outer basement membrane were monoaminergic.
b. In the lateral part of sub-zone A, the percentage of monoaminergic profiles tended to be larger than medially (41 ± 7.5 and 24.9 ± 4.5, respectively means derived from 5 rats).
c. Most monoaminergic profiles in the internal zone differed from those in the external zone by the presence of several large GV and limited numbers of small granular vesicles.
d. In direct vicinity of the pial vessels, small clusters of monoaminergic and non-aminergic varicosities were present. The small granular vesicles in the monoaminergic varicosities were characterized by electron-dense cores, considerably larger than those in the external zone.

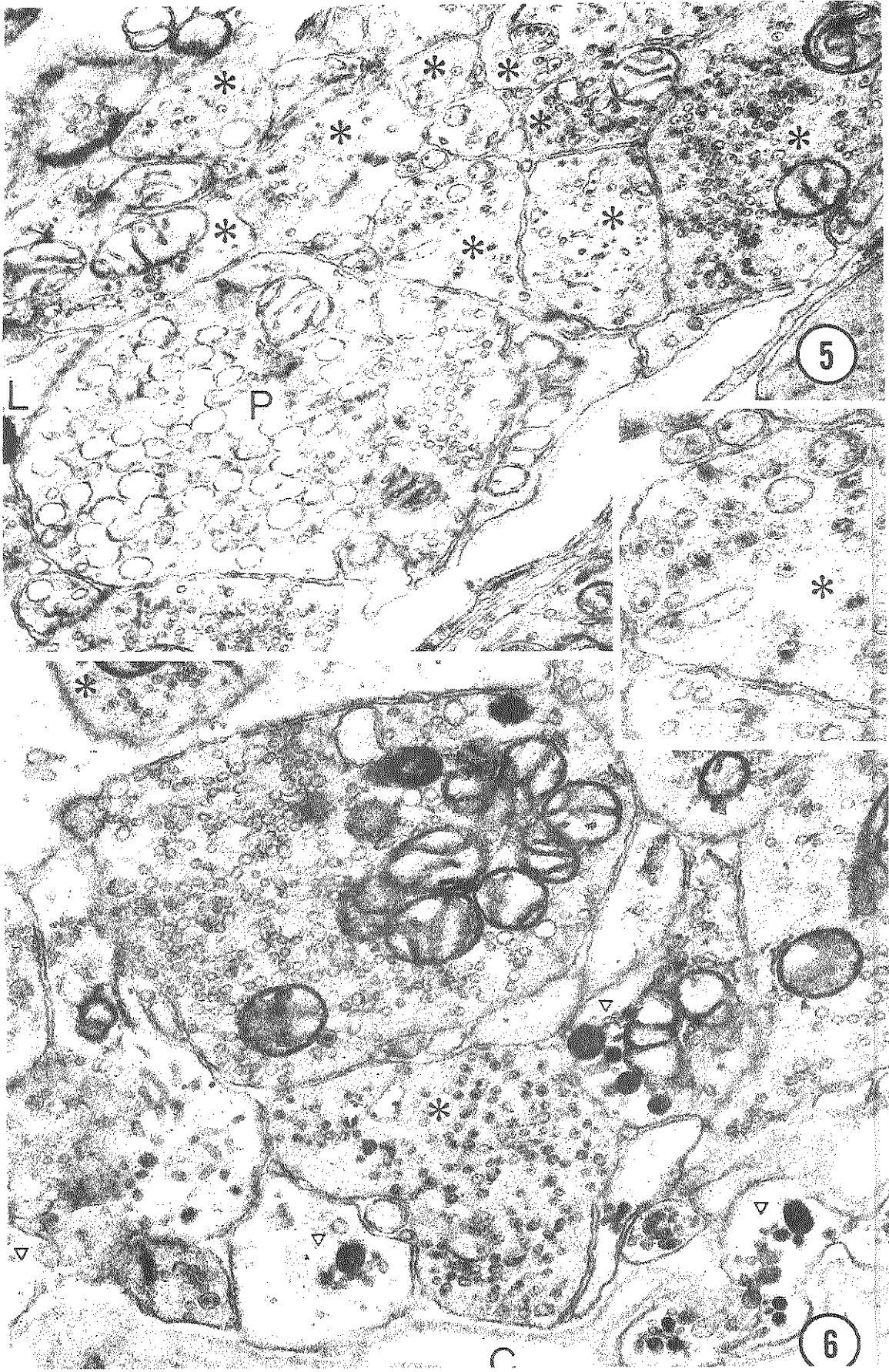
Obviously, these monoaminergic and non-aminergic varicosities reflect respectively, the noradrenergic and cholinergic innervation of pial vessels (Edvinsson et al, 1972; Hartman, 1973).

e. Frequently, monoaminergic and non-aminergic profiles were located in proximity, without interposing non-neuronal processes (fig. 4-6).

Fig. 5-6. Incubation with 5-OHDA and fixation with potassium permanganate (compare with fig. 4). Monoaminergic profiles (*) show electron-dense material especially in microvesicles (small granular vesicles), while microvesicles in non-aminergic profiles are electron-lucent.

Fig. 5. Note the large electron-lucent vesicles ('GV') in a typical peptidergic profile (P), without small granular vesicles.
L distorted lipid droplet in non-neuronal profile.
x 40.000; inset x 80.000

Fig. 6. Note electron-dense material in non-neuronal profiles (▽).
C collagen fibril in perivascular space x 35.000


L
P
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6

Probably, the monoaminergic profiles seen near the primary plexus (especially those laterally) mainly belong to dopaminergic neurons, since

1. there is a massive projection of dopaminergic neurons, and not, as far as known yet, from other catecholaminergic neurons, to the external zo of the m.e. (I 4.2.); there monoaminergic profiles were numerous (1.2.4.2.a),
2. dopaminergic neurons project preferentially to the lateral side of the external zone (I 4.2.1.); there the percentage of monoaminergic profiles was particularly high (1.2.4.2.b),
3. the monoaminergic varicosities present in the external zone differed in ultrastructure from the presumed noradrenergic ones located near the pial vessels and in the internal zone (1.2.4.2. c,d).

It is added that also serotoninergic neurons may accumulate 5-OHDA (II 7.).

Probably, the numerous nerve profiles in the external zone which, after 5-OHDA and potassium permanganate fixation contain neither small granular vesicles nor large GV, but instead numerous electron-lucent vesicles of the sizes of GV, belong to peptidergic neurons, since

1. there is no evidence for accumulation of 5-OHDA by peptidergic varicosities (Ajika and Hökfelt, 1973; II 7.),
2. peptidergic varicosities are numerous in the external zone (I 4.1.1., IV 1.1.),
3. it is not to be expected that small peptides like the known hypophysiotropic substances nor carrier proteins (if present) are retained by permanganate fixation; this would explain the electron-lucent appearance of the 'GV' in the non-aminergic profiles,
4. on the basis of immuno-electromicroscopical studies (IV 2.1.), numerous electron-lucent 'GV' are to be expected in varicosities of (peptidergic) hypophysiotropic neurons,
5. also 'GV' larger than 110 nm are expected to be present in such varicosities, whereas GV of such size were practically absent in monoaminergic profiles,
6. the large vesicles localized in the axons of the hypothalamo-neurohypophyseal system, a peptidergic system (I 4.1.2.), had the same electron-lucent appearance as the 'GV' present in the presumed peptidergic profiles of the external zone.

Non-aminergic profiles without 'GV', or with very limited numbers of 'GV', may represent varicosities of peptidergic neurons in a special functional state (App. 6), but may as well represent other (non-aminergic) neurons.

For material fixed by aldehyde-osmium tetroxide, the observations imply that profiles with many GV, especially in case of GV larger than 110 nm, belong to peptidergic neurons. Those containing many microvesicles and small numbers of GV may represent monoaminergic neurons but peptidergic ones as well. The nature of profiles with (almost) exclusively microvesicles will be diverse, including terminals of cholinergic neurons (see e.g. Lentz and Chester, 1977).

1.3. distribution of neuronal and non-neuronal endfeet

SUMMARY Fig. 8-13 give a visual impression of the distribution of neuronal and non-neuronal endfeet at the primary plexus. At the boundaries of the m.e. (pars oralis) as well as in the m.e. pars caudalis neuronal endfeet were scarce. In these areas non-neuronal endfeet were relatively large. Most non-neuronal endfeet were characterized by the presence of electron-dense organelles, which were practically absent in the zona granulosa and were not seen outside the m.e. Non-neuronal endfeet were relatively numerous at sites where the pars infundibularis was absent.

The m.e. is a far from homogeneous structure. In addition to distinct horizontal layers (I 2.3.; App. 2,3), there are differences between medial and lateral areas (III 1.2.3.). Therefore, the distribution of neuronal and non-neuronal endfeet along the outer basement membrane of m.e. and infundibular stem was analyzed using a X/Y device (II 11.). The observations concern 3490 profiles (fig. 7-13), all derived from one male rat.

1.3.1. neuronal endfeet

Of the profiles analyzed, 43% was neuronal in nature. Neuronal endfeet (circles) were concentrated in the central part of the m.e., rostral to the infundibular stem (fig. 8,11). Here, the bulk of the zona granulosa was located (open symbols). At the lateral and rostral side of the m.e. neuronal endfeet gradually diminished in number and in the m.e. pars caudalis neuronal endfeet only occurred close to the rostral infundibular stem. At the dorsal side of the infundibular stem, neuronal endfeet were relatively scarce.

1.3.2. non-neuronal endfeet

There were, between medial and lateral areas, differences in presence, size and shape of the non-neuronal profiles. In the lateral part, non-neuronal endfeet were relatively numerous and large (triangles in fig. 11, at right).

This suggests that the function of the non-neuronal endfeet laterally and medially in the m.e. is not identical. As a consequence, data on non-neuronal endfeet have to be specified for location.

It is emphasized that the appearance of the non-neuronal endfeet at the lateral side of the m.e. is quite different from the non-neuronal profiles lateral to the sulcus infundibularis, i.e. outside the m.e. There, numerous thin non-neuronal profiles are present, arranged in parallel to the outer aspect of the brain.

A preponderance of non-neuronal endfeet was not only apparent at the lateral side of the m.e. but also at the rostral side, in the m.e. pars caudalis and, less outspoken, in the infundibular stem. It is noteworthy that, in general, non-neuronal endfeet were relatively numerous at sites where the pars infundibularis was absent.
Non-neuronal endfeet with electron-dense organelles (▲) were present throughout the m.e. (and not outside of it). Such endfeet were numerous at the lateral side of fig. 11e. They were scarce in the infundibular stem, except rostrally, and practically absent in the zona granulosa.

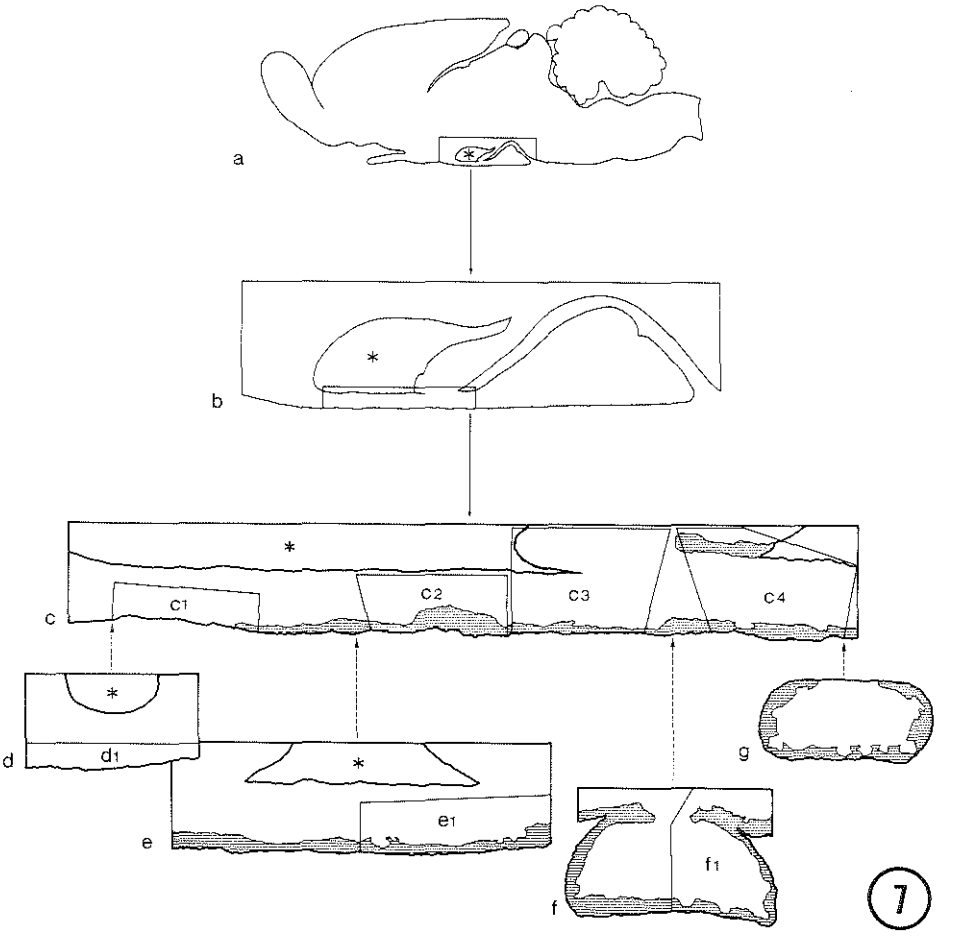


Fig. 7. Schematic drawing of a rat brain to illustrate the locations of the sections used for fig. 8-13. Fig. a,b and c represent sagittal sections and d,e,f and g coronal sections, from rostral to caudal. The infundibular stem is at right in fig. c. In fig. c-g, the pars infundibularis is hatched. * (infundibular recess) of third ventricle The area corresponding to c2 and e is referred to as the midcentral region of the m.e.

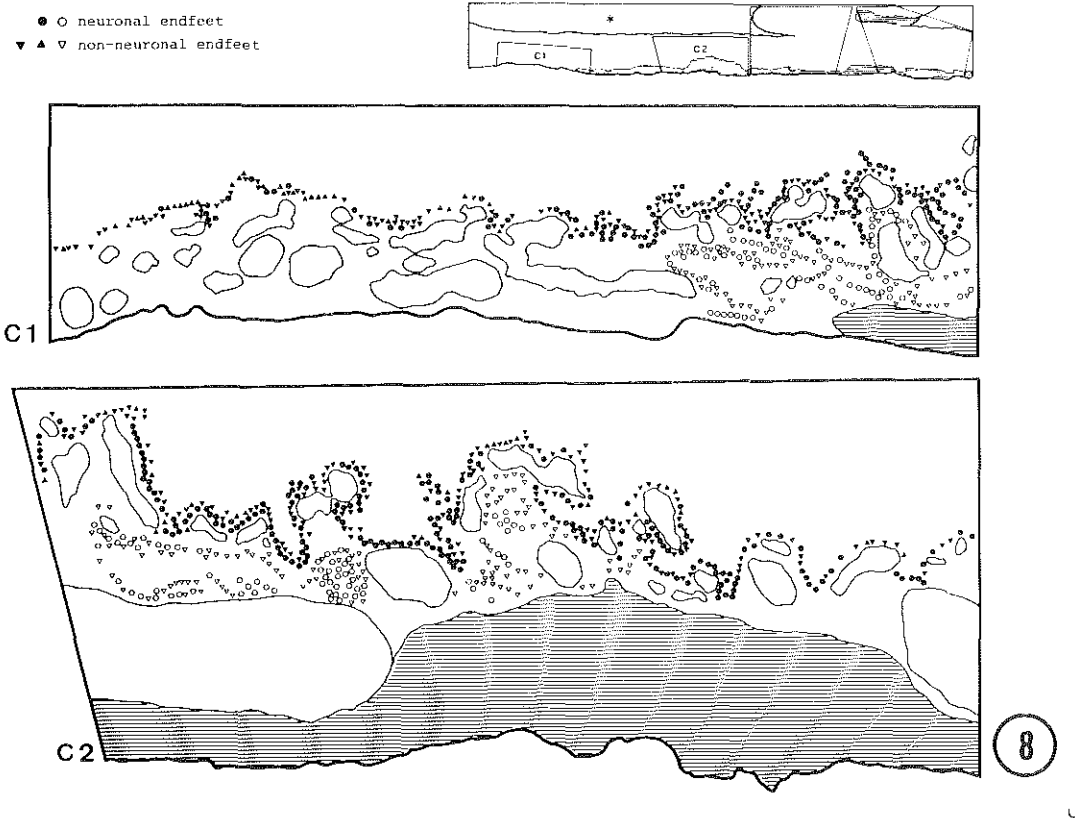
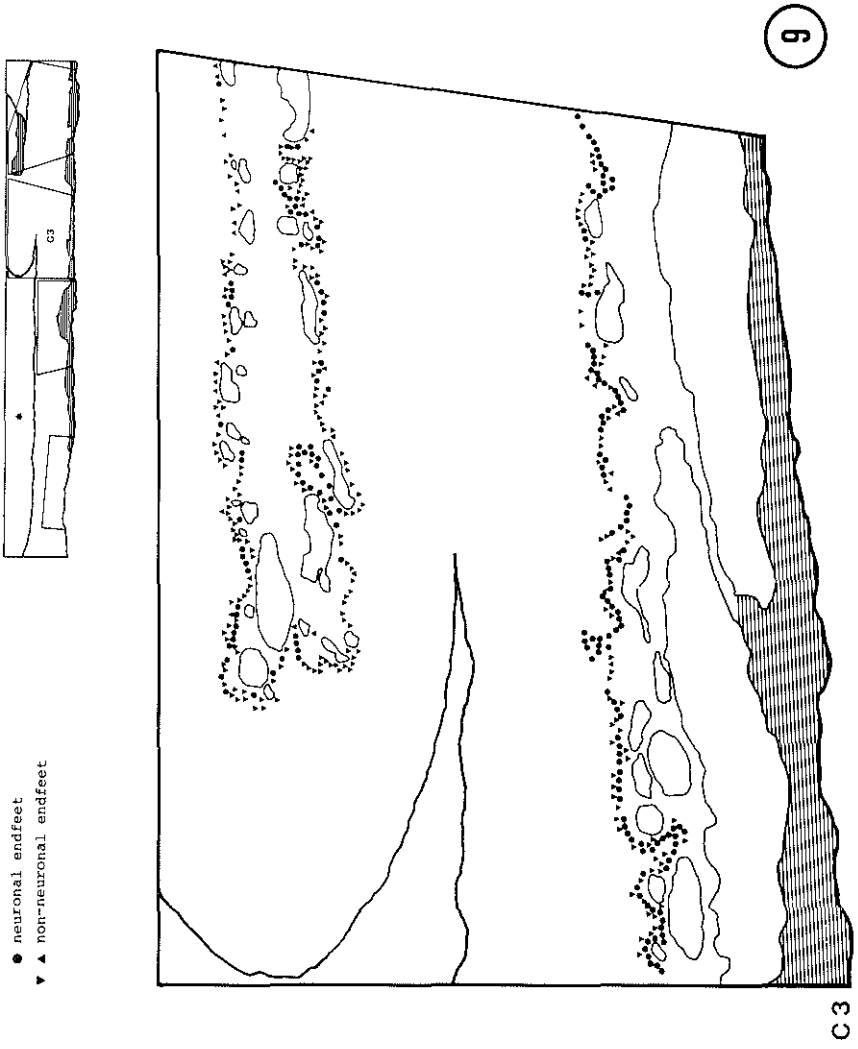


Fig. 8-13. Distribution of neuronal and non-neuronal endfeet along the outer basement membrane of the primary plexus.
Figures are arranged from rostral to caudal (see fig.7).
Neuronal profiles are indicated with circles, non-neuronal ones with triangles.
Symbols in sub-zone A are filled, those in zona granulosa open. Non-neuronal profiles without electron-dense material are indicated with ▼ or ▽, those containing electron-dense material with ▲ (symbols with Δ are absent since such profiles were not observed in the zona granulosa).
Empty structures of variable size and shape represent capillaries.
The pars infundibularis is hatched.
Magnification approximately x 500


C3
● neuronal endfeet
▼▲ non-neuronal endfeet
C3
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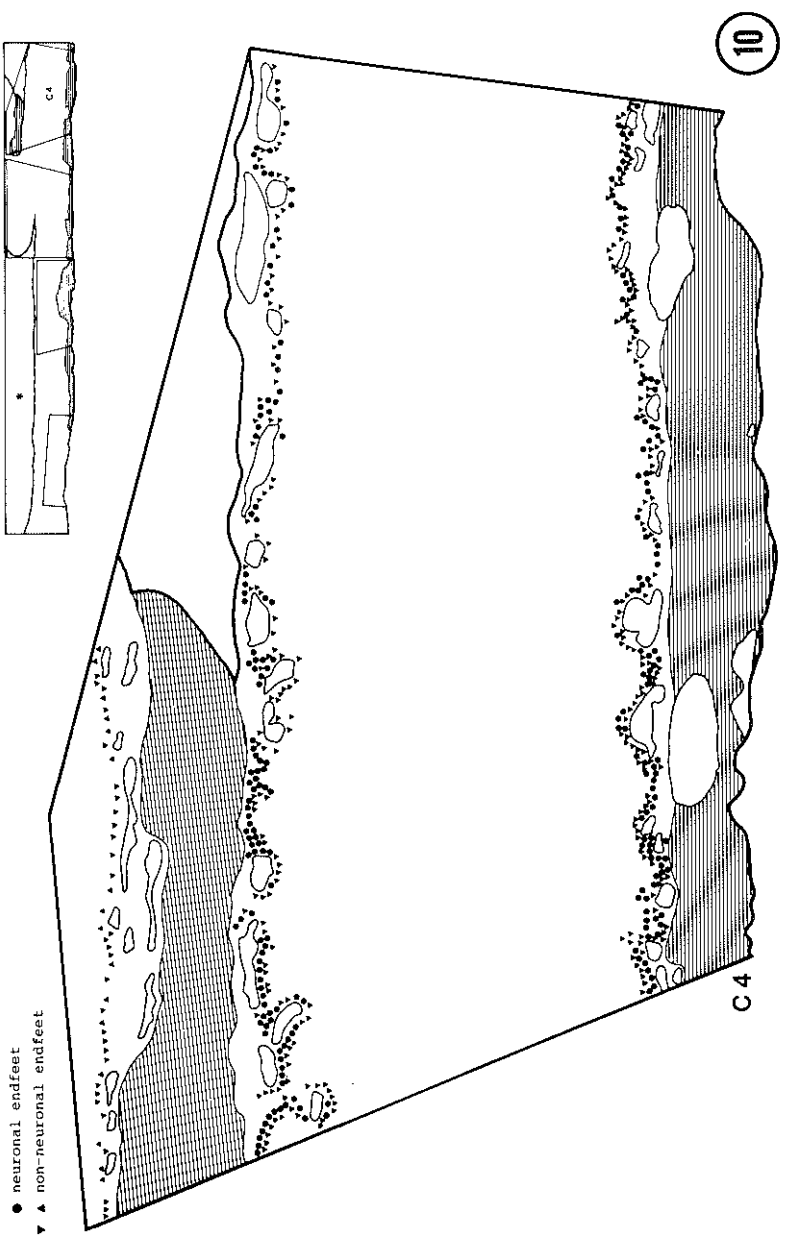
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▼ ▲ non-neuronal endfeet
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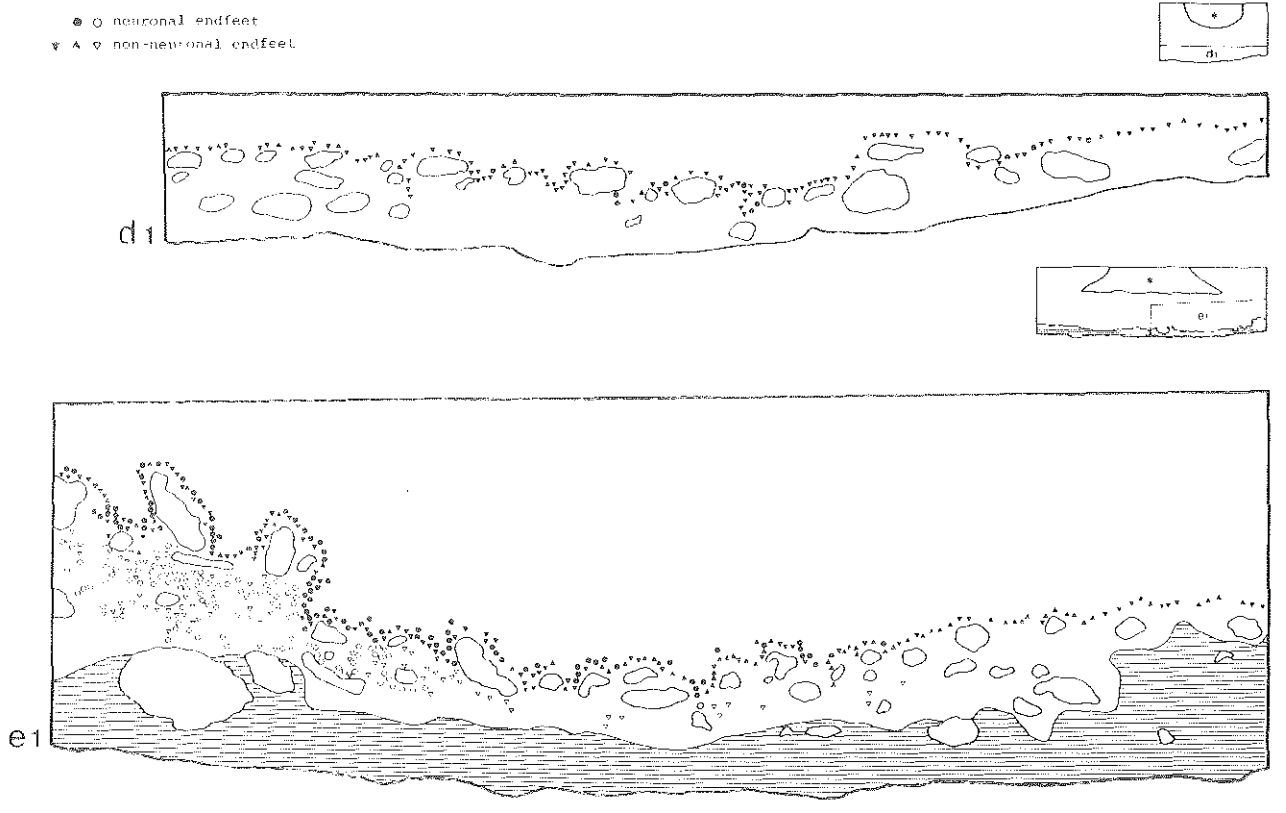
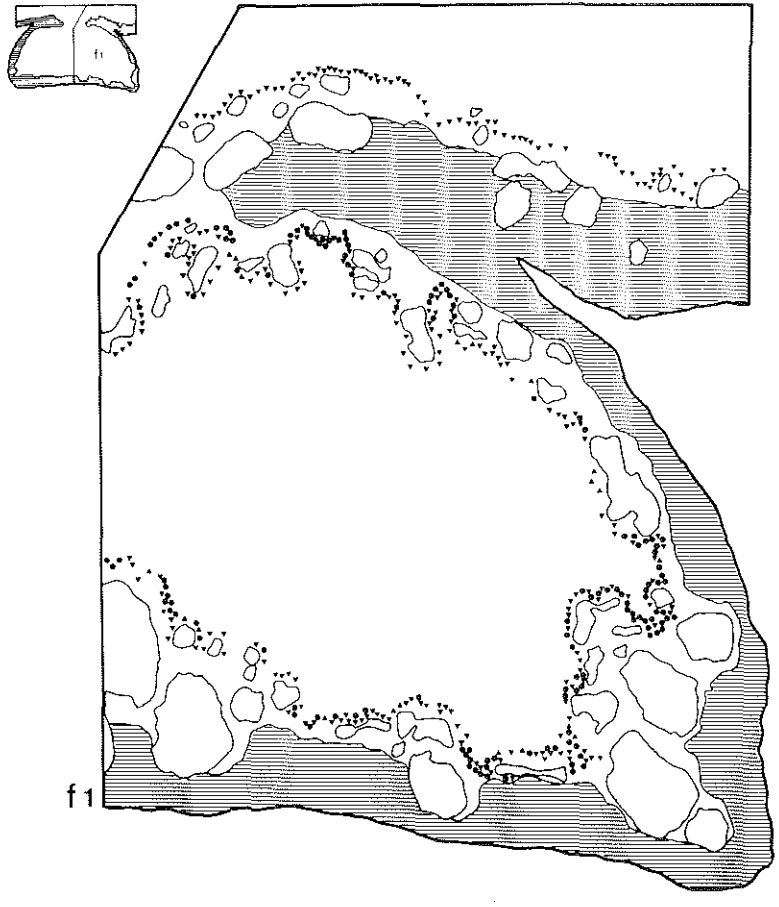


11

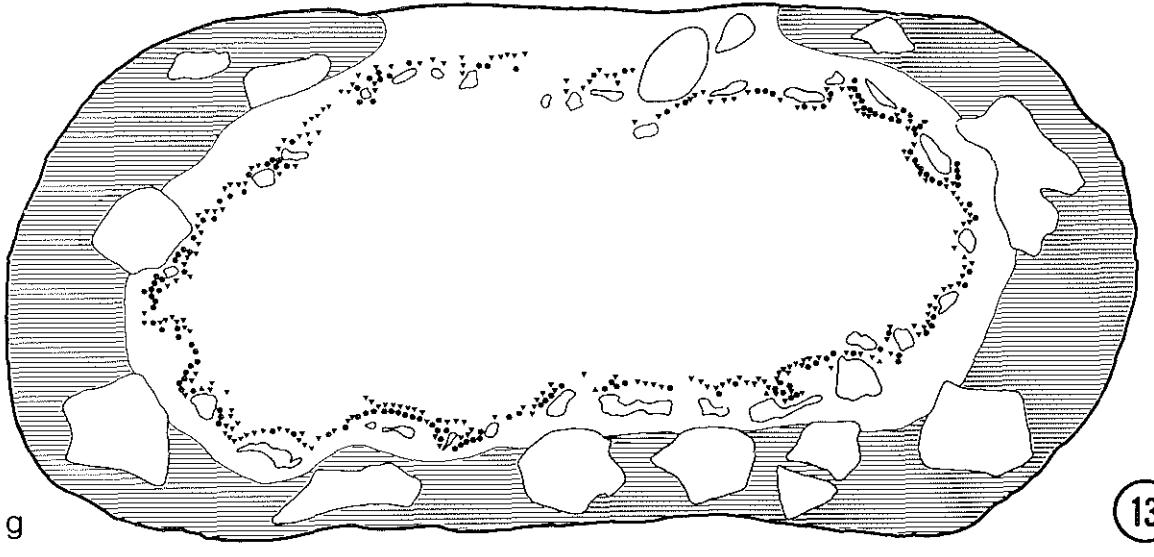
● neuronal endfeet

▼ ▲ non-neuronal endfeet



12

● neuronal endfeet
▼ ▲ non-neuronal endfeet



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1.4. some quantitative data on vesicular content of neuronal endfeet

SUMMARY Especially nerve profiles which contained 31-40 microvesicles and relatively small numbers of GV were numerous at the outer basement membrane of the primary plexus. There, nerve profiles without microvesicles were absent, in contrast to profiles without GV. There was no inverse numerical relationship between microvesicles and GV.

One of the most striking differences between most varicosities present in the m.e. and those characteristic for nervous tissue outside the neurohypophysis is the abundance of large GV in the m.e. Microvesicles are present in varicosities in and outside the m.e. but whether their functions are comparable is not known. Therefore, the presence of both microvesicles and GV was analyzed in nerve profiles contacting the outer basement membrane. Data from one rat are reported. They concern 1510 profiles containing roughly 120.000 vesicles.

Calculated over all neuronal profiles, about 75 microvesicles and 5.6 GV were present per profile. In profiles containing exclusively microvesicles, per profile about 64 microvesicles were present, versus about 79 microvesicles and 7.5 GV in the remaining profiles.

This indicates that, at the outer basement membrane, the number of microvesicles is relatively high in profiles containing GV. This was a general tendency in our material of both males and females, and possibly is related to release by exocytosis.

Profiles with 31-40 microvesicles were numerous at the basement membrane, practically independent of the number of GV (Table 2). There was no inverse numerical relationship between microvesicles and GV. Nerve profiles without microvesicles were not identified at the basement membrane, in contrast to profiles without GV (25.2%)

Assuming that in the m.e. biologically active substances are stored in GV, then microvesicles seem somehow indispensable for release, since profiles with GV but without microvesicles were practically absent at the basement membrane (see IV 3.).
The tremendous variation in ratios of microvesicles and GV does not support the inverse numerical relationship mentioned by several authors (Daniel and Lederis, 1966); Monroe and Scott, 1966; Bergland and Torack, 1969. Absence of such a relation is at least in part due to different types of endfeet (III 1.2.3.).

1.5. estimate of the numbers of neuronal and non-neuronal endfeet and of the number of granular vesicles

SUMMARY According to a rough estimate, 1.4 x 10^6 neuronal and non-neuronal endfeet were present at the outer basement membrane of the m.e. About 45% was neuronal in nature. In the whole m.e., roughly 1.6 x 10^9 GV were estimated to be present.

Table 2 Relative numbers of individual nerve profiles as characterized by their respective numbers of microvesicles and granular vesicles

numbers of gran.ves. / microves.	0	1	2	3	4	5	6-10	11-20	21-30	31-40	>40	
1- 10	2.3	2.1	1.7	0.5	0.7	0.3	1.1	0.1	0.1	-	-	*8.9*
11- 20	3.5	1.8	1.7	0.4	0.7	0.3	1.1	0.5	0.1	-	0.1	*10.2*
21- 30	2.7	1.1	1.0	0.7	0.7	0.3	1.7	0.8	0.1	0.1	-	*9.2*
31- 40	5.5	1.8	2.7	1.5	1.5	0.7	2.8	1.1	0.3	0.1	-	*18.0*
41- 50	1.9	1.3	0.5	0.3	0.4	0.5	1.5	0.9	0.3	-	-	*7.6*
51- 60	2.0	1.2	1.0	0.4	0.5	0.3	1.6	0.9	0.1	-	-	*8.0*
61-100	4.4	2.5	2.9	1.4	2.0	1.0	4.7	4.5	1.0	0.3	-	*24.7*
101-200	2.1	0.5	0.5	0.4	0.8	0.7	2.1	1.7	0.8	0.1	0.2	*9.9*
201-300	0.7	0.1	0.2	0.2	0.2	0.1	0.5	0.5	0.3	-	0.3	*3.1*
301-400	0.1	0.1	-	-	0.1	-	-	-	0.1	0.2	-	*0.6*
>400	-	0.1	-	-	-	-	0.1	-	0.1	0.1	0.1	*0.5*
	25.2	*12.6*	*12.2*	*5.8*	*7.6*	*4.2*	*17.2*	*11.0*	*3.3*	*0.9*	*0.7*	

All profiles (n=1510; 100%) were located at the outer basement membrane of the primary plexus.

In italics, total percentages per group.

number of endfeet

There are no data on the number of neuronal and non-neuronal endfeet located at the basement membrane of the primary plexus of any species. Here follows, for the rat, an admittedly rough estimate, which is based on the following assumptions derived from light microscopical and electron microscopical estimates:
1. a total contact area along the basement membrane of the primary plexus of 2×10^6 μm^2, 2. a contact area occupied for 35% by neuronal and for 65% by non-neuronal tissue, 3. a mean contact area for neuronal endfeet of 1 μm^2 and for non-neuronal endfeet of 2 μm^2. Then, 0.70×10^6 neuronal endfeet and 0.65×10^6 non-neuronal endfeet are present at the basement membrane.

numbers of granular vesicles

In material treated with ethanolic phosphotungstic acid, cores of GV were

markedly electron-dense (App.3, fig. 3), which enables visualization at low magnification. By counting, at a magnification of 10.000 x, the cores present in a coronal section, about 80 nm in thickness, taken from level e (fig. 7) of the m.e., the total number of GV present appeared to be about 10^5. A figure in the same order of magnitude was obtained if the mean numbers of GV/μm^2 (App.2), were used for calculation. If it is assumed that 1. the estimated number of GV is more or less representative for the m.e., 2. the volume of such a section is 0.0057% of the volume of the m.e. and 3. for 10% of the GV counted the centre was located in an adjacent section, then 1.6 x 10^9 GV were present in the m.e. Notwithstanding the enormous numbers of GV present in certain varicosities of the internal zone, most GV were present in the external zone.

2. *Mechanisms of release and possible events subsequent to release*

2.1. mechanisms of release

SUMMARY Fusions of GV with the plasma membrane and omega-shaped configurations of the plasma membrane, occasionally enclosing core material located in the extracellular space, were indicative of release by exocytosis. Such configurations were exclusively present near the primary plexus, but sites of release were not restricted to that part of the endfoot in contact with the outer basement membrane of the primary plexus. In certain endfeet, such membrane configurations were numerous.

In addition to the observations reported in App. 1, the following is mentioned.
Configurations suggestive of release by exocytosis (fig. 14-19) were especially found in nerve profiles contacting the basement membrane of the primary plexus but the configurations were not preferentially located *at* the outer basement membrane. In a collection of 159 of such configurations, which included fusions of GV with the plasma membrane and similar sized omega-shaped configurations of the plasma membrane with and without extracellular core material, only 15% was located at the basement membrane.

Some other features were the following:
1. in case GV were located near the plasma membrane, delicate filaments were frequently present between the respective membranes,
2. a common feature of the omega-shaped membrane configurations was the presence of a bristle coat at the cytoplasmic side (fig. 15,16,18; App. 1 fig. 1b),
3. occasionally, features compatible with exocytotic release were associated with profound invaginations of the plasma membrane (fig. 14,15;App. 5, fig. 3),
4. extracellular core material was localized between plasma membrane and basement membrane and was never seen at the vascular side of the outer basement membrane,
5. in some cases, subunits could be discerned in extruded material (fig. 17,18,19; App. 1, fig. 5),
6. certain neuronal endfeet were characterized by several omega-shaped configurations of the plasma membrane and/or by several extruded cores (fig. 14; App. 5, fig. 3), although the total number of such phenomena seen was small ,
7. membrane phenomena suggestive of release by exocytosis were observed in nerve profiles containing different mean sizes of GV,
8. exceptionally a microvesicle seemed to arise directly from an omega-shaped configuration of the plasma membrane (App. 1, fig. 6).

The observations indicate that at least certain neuronal endfeet can release the contents of the GV by exocytosis. By such a mechanism massive release of hormones seems possible.

2.2. 'uptake' of an extracellular tracer (horseradish peroxidase, HRP)

SUMMARY Already one minute after intravenous injection of HRP, peroxidase activity could be demonstrated in microvesicles present in neuronal endfeet at the primary plexus. Subsequently, HRP became located in larger organelles. Certain varicosities showed accumulations of such large HRP-loaded organelles indicating pronounced uptake of HRP. Part of the incorporated HRP appeared to be transported in tubular membrane-bound organelles to perikarya located in the arcuate nucleus and the internal zone of the m.e.

Uptake and accumulation of HRP was in non-neuronal endfeet slower and less pronounced than in neuronal endfeet. There was evidence for accumulation of HRP in the apical part of the ependymal cells, thus near the cerebrospinal fluid.

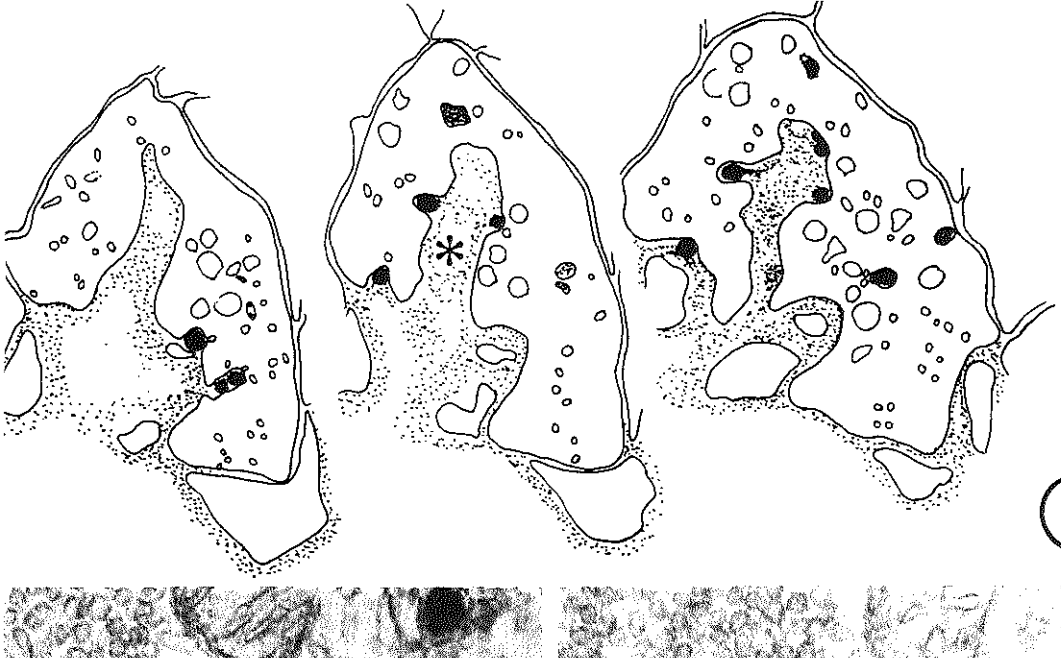
Fig. 14. Drawing of three consecutive sections of a neuronal endfoot. Note profound invagination of the plasma membrane and several granules in an extension of the perivascular space (✻). The granules are assumed to be cores of GV after release by exocytosis (see further, legenda to fig. 3, App. 5). GV have been drawn without core.
Fixation 30 min after sham-stimulation of the medial preoptic area and 20 min after intravenous injection of HRP (III 2.2.).
x 25.000

Fig. 15a,b. Consecutive sections of a neuronal endfoot in the zona granulosa with a coated (arrow) invagination of the plasma membrane Possibly, one GV (✻), is in the process of exocytosis.
Female rat; oestrus; nembutal anaesthesia. Fixation 24 hours after electrochemical stimulation of the medial preoptic area. x 75.000

Fig. 16-19. Examples of neuronal profiles with omega-shaped configuration of the plasma membrane, occasionally enclosing extracellular material. This material is assumed to represent the extruded content of GV, in which subunits can be discerned (✻). Certain omega-shaped configurations show a coat (arrow) at the cytoplasmic side of the plasma membrane.

Fig. 16. Note also GV (Ⓞ) possibly fused with the plasma membrane of a neuronal profile characterized by the presence of dense bodies and relatively flat microvesicles. x 55.000

Fig. 17. Note also small omega-shaped configurations of the plasma membrane (▽), suggestive of micropinocytotic activity.
E extension of the perivascular space, lined by the outer basement membrane (▼) See further p.64. x 35.000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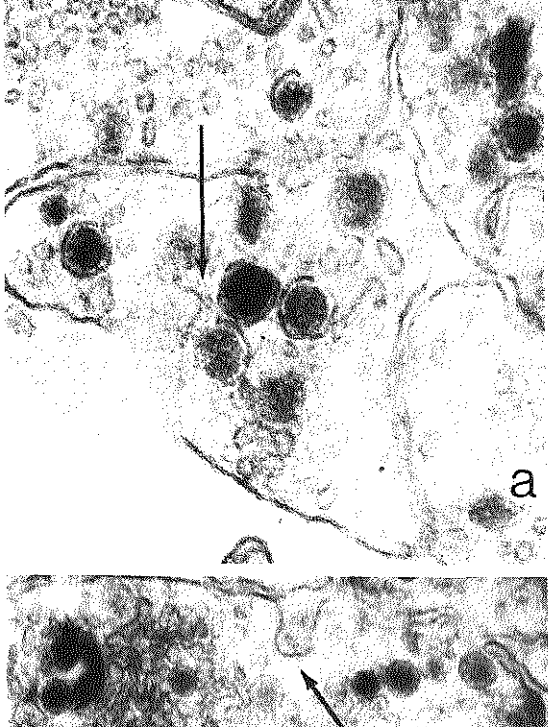
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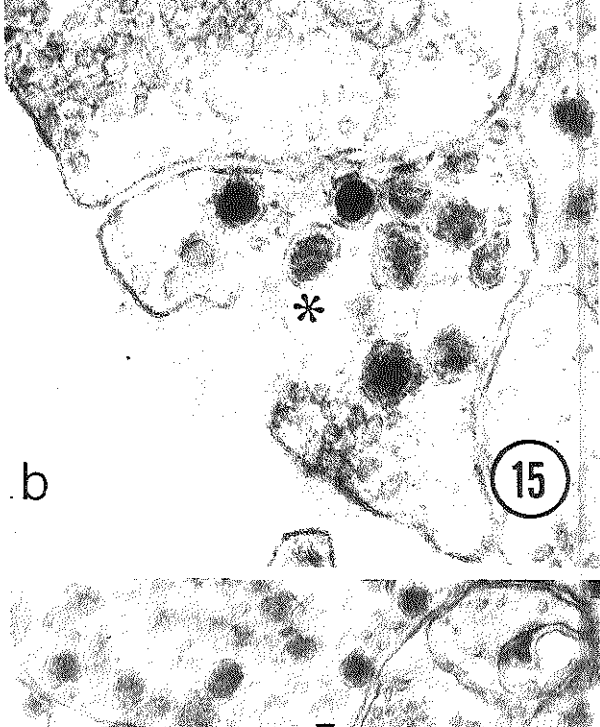
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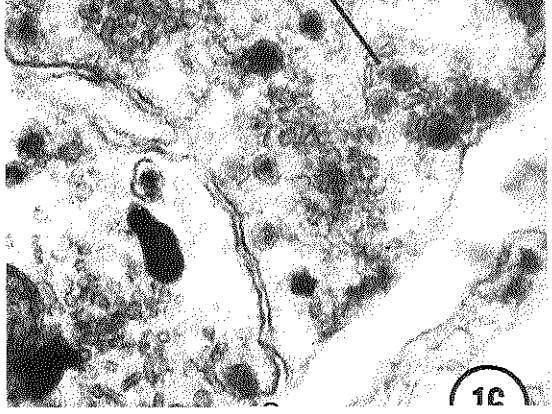
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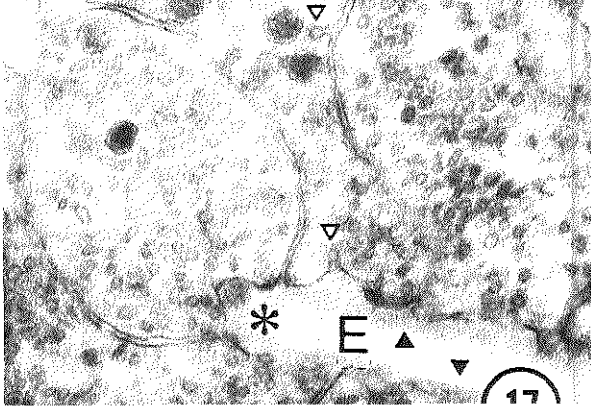
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Since part of the synaps-like microvesicles were thought to arise by micropinocytosis to compensate for the effect of release by exocytosis (App. 1), it was attempted to study micropinocytotic activity by uptake of HRP (for a preliminary note, see App. 4). HRP is an enzyme, which is not able to pass intact membranes and which is used as extracellular tracer.
In addition, attention was given to the penetration of HRP into the m.e. and to a possible role of the non-neuronal cells of the m.e. in transporting foreign material.

2.2.1. endogenous peroxidase activity
In tissue chop sections treated for demonstration of peroxidase activity (II 6.), accumulations of reaction product were observed in glial cells located in a restricted area lateral and dorsal to the m.e. This area mainly comprises the arcuate nucleus. At the ultrastructural level, pronounced endogenous peroxidase activity was only observed in these cells, which on the basis of abundance of microfilaments probably are a specific type of astroglial cells (cf. Sherlock et al, 1975).

2.2.2. localization of (exogenous) HRP

2.2.2.1. extracellular space
Male rats were fixed by perfusion at different intervals after injection of HRP into a tail vein (from one min. to 24 hours). Tissue chop sections derived from rats fixed one min. after injection showed, after incubation, a diffuse brown reaction product in the basal part of the m.e. With longer periods between injection and fixation the brown area became progressively larger, but was no longer visible 7 hours after injection. Fixation 60 min after injection resulted in a brown area which probably included the whole arcuate nucleus as well as parts of the ventromedial nucleus.

The observations fit in with the concept that the m.e. is located outside the blood-brain barrier. As a consequence, HRP can penetrate the extracellular space of the m.e. and subsequently reaches even areas outside the m.e, thus inside the blood-brain barrier.

At the ultrastructural level, traces of reaction product, mostly adhering to the plasma membrane, were in the extracellular space already present one minute after injection. They were seen in the external zone, in the internal zone and in nearby parts of the arcuate nucleus up to 135 min after injection, but no longer after 7 hours. Occasionally, it was evident that HRP could not pass the tight junctions which seal the apical parts of the ependymal cells of the m.e.

Thus, there is an extremely rapid transport of HRP from the capillaries through the m.e. Passage from the extracellular space of the m.e. into the infundibular recess seems restricted by tight junctions. Within 7 hours HRP is removed again from the extracellular space.

The extracellular space showed after decapitation and immersion fixation much more reaction product than after perfusion fixation. Obviously, part of the HRP is removed from the extracellular space during the perfusion.

2.2.2.2. neuronal profiles
After intravenous injection of HRP, certain microvesicles became loaded with reaction product (App. 4, fig. 1,2; App. 5, fig. 2). These vesicles,

about 50 nm in diameter, will be called HRP-microvesicles (for examples, see also fig. 20).
Small omega-shaped configurations of the plasma membrane frequently showed reaction product at the side of the extracellular space (see fig. 21 for an exceptional case).
Peroxidase containing organelles larger than HRP-microvesicles were absent from rats fixed one minute after fixation, but were regularly observed at longer time intervals (fig. 20, 24). These organelles were generally rounded, up to 350 nm in diameter. Mostly, reaction product was located in small clumps of high electron-density against the inner side of the limiting membrane. The ultrastructure of these organelles was frequently badly preserved. Also tubular and cup-shaped organelles with reaction product were seen.
In rare cases, peroxidase positive material resembling a core of a GV was found in the extracellular space. Generally, such core-like material, which lacked a limiting membrane, was located near membrane configurations suggestive of release by exocytosis (fig. 14; App. 5, fig. 3).
In (unmyelinated) axons, occasionally membrane-bound peroxidase positive material was observed. It was generally contained in tubular organelles and was especially seen if fixation occurred at least 20 min after injection of HRP.

time course of 'uptake' of HRP
To get an impression of the time course of HRP-'uptake', in fact HRP-accumulation, two series of rats were perfused 1, 5, 20 and 60 min after injection of HRP. The percentage of nerve profiles with at least one HRP-microvesicle was calculated as well as the percentage of profiles containing other peroxidase positive organelles and no HRP-microvesicles. The analysis concerned nerve profiles of sub-zone A which contacted the basement membrane of the primary plexus (first 'layer') and the adjacent deeper nerve profiles (second 'layer'; see App. 6, fig. 1). Notwithstanding the small number of rats involved, the following tendencies seem apparent (fig. 23):
Already one min after injection of HRP, many profiles in contact with the basement membrane contained HRP-microvesicles, whereas profiles containing exclusively other HRP positive organelles did not yet occur. Up to about 20 min, there was for the first 'layer' an increase in percentage of nerve profiles with HRP-microvesicles. For the second 'layer', the percentage of these profiles lagged behind. There was no apparent difference between rats anaesthetized with Avertin and urethane in accumulation of peroxidase containing organelles.
Six urethane anaesthetized rats were fixed at longer time intervals after injection of HRP. In one rat fixed 105 min after injection, the percentages of profiles with HRP positive organelles was somewhat higher than after 60 min. Certain nerve profiles showed large accumulations of such organelles, which were relatively large. In one rat perfused 135 min after injection, the percentage of nerve profiles with HRP positive organelles seemed to have declined and 7 and 10 hours after injection peroxidase positive organelles were only rarely observed. In two rats perfused 24 hours after injection HRP-microvesicles were absent but small numbers of other peroxidase positive organelles were occasionally observed.

In rats sacrificed 7 and 10 hours after injection of HRP certain neuronal perikarya in the arcuate nucleus and the internal zone showed particularly

electron-dense lysosome-like organelles, suggesting retrograde transport of HRP.

frequency of HRP containing profiles and organelles
In rats perfused 20 min after injection of HRP, 30-35% of the nerve profiles at the basement membrane contained one or more peroxidase positive organelles (fig. 23). Of course, these percentages underestimate the percentage of varicosities with such organelles.
The mean number of HRP-microvesicles per nerve profile was, independent of the interval between injection of HRP and fixation, small (App. 5, Table 4). Certain neuronal varicosities, however, were characterized by an accumulation of HRP containing organelles, as indicated by the presence of nerve profiles with over 30 and even over 50 HRP positive organelles (fig. 24). Such profiles were observed in three males (one 30 days post-castration, one stimulated in the medial preoptic area 30 before fixation, one fixed shortly after ether anaesthesia) and two females (decapitated at pro-oestrus between 15.00 and 17.00 hours). The number of HRP microvesicles in these profiles was, generally, small.

Fig. 18-19. See legend on page 60 .

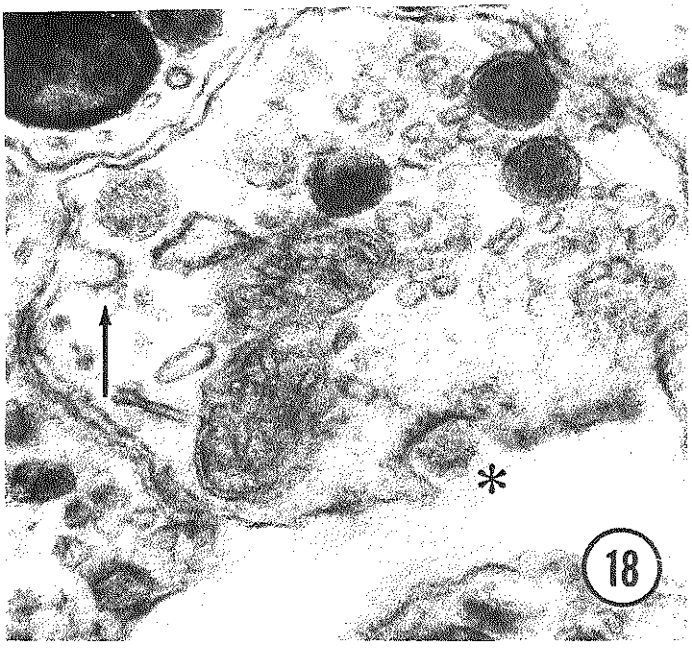
Fig. 18. Note two omega-shaped configurations of the plasma membrane, one of which (*) encloses subunits located in the extracellular space. Note also a myelinated body in the upper left corner which contains GV and which probably represents an autophagic body.
x 85.000

Fig. 19. Note omega-shaped configuration of the plasma membrane of a varicosity rich in GV. In the extracellual space, subunits can be discerned (*). x 80.000

Fig. 20. Neuronal profiles separated from the outer basement membrane (▲) by non-neuronal profiles. In one neuronal profile (*), several HRP containing microvesicles are present. In two other neuronal profiles (Δ), large organelles are present with reaction product mainly localized at the periphery. Some of these organelles are disrupted. Fixation 24 hours after castration and 20 min after injection of HRP. x 60.000

Fig. 21. Neuronal profile (*) located against the outer basement membrane with several HRP containing omega-shaped configurations of the plasma membrane. Fixation 20 min after injection of HRP and 15 min after electrochemical stimulation of the medial preoptic area. x 35.000

Fig. 22. Part of a neuronal endfeet which contains numerous microvesicles only some of which contain reaction product (*). Two larger HRP containing organelles can also be observed. Note cristal-like accumulation of microvesicles (▽), in another neuronal profile at left. Fixation 20 min after injection of HRP, urethane anaesthesia.
x 30.000



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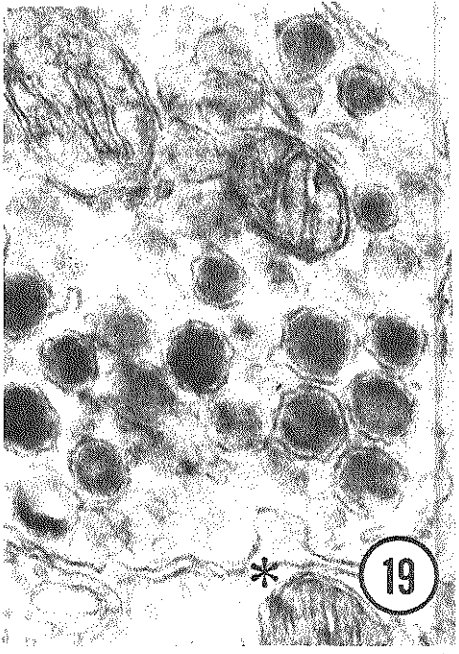

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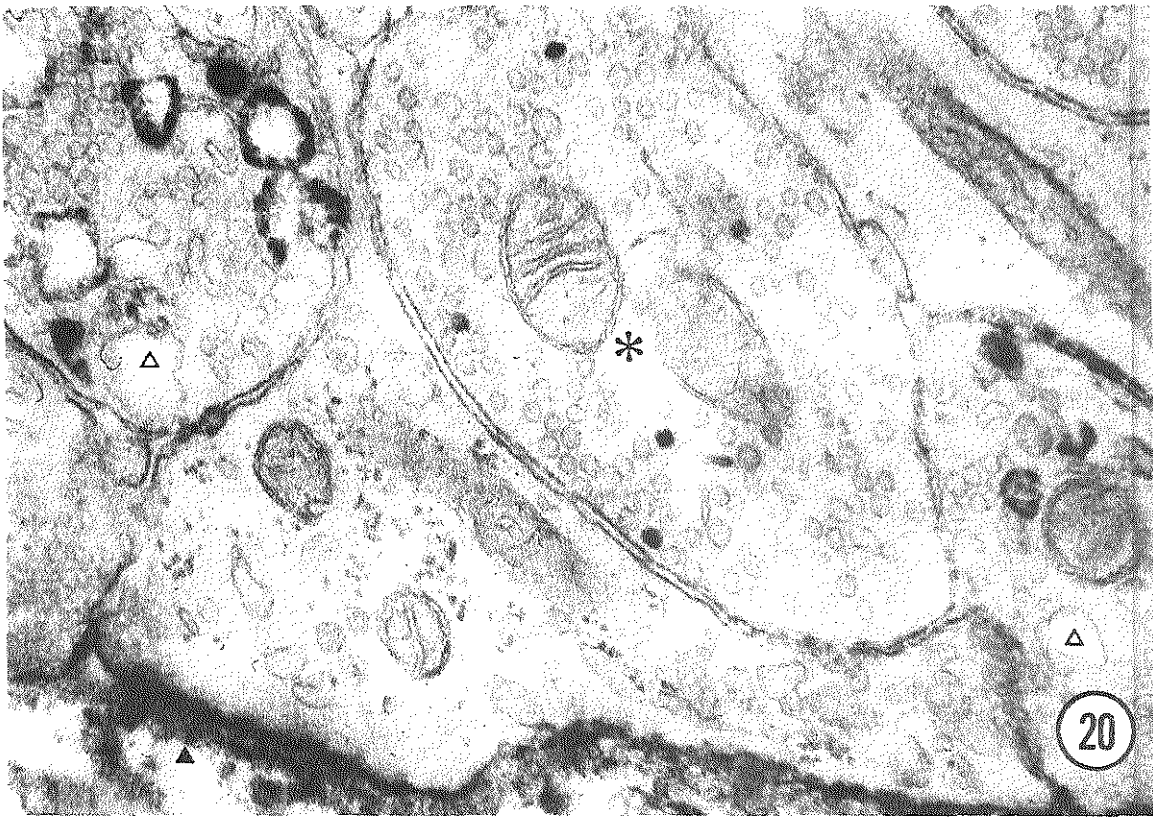

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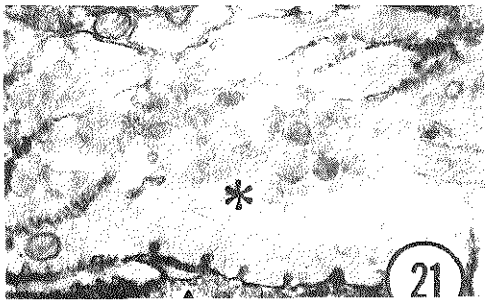

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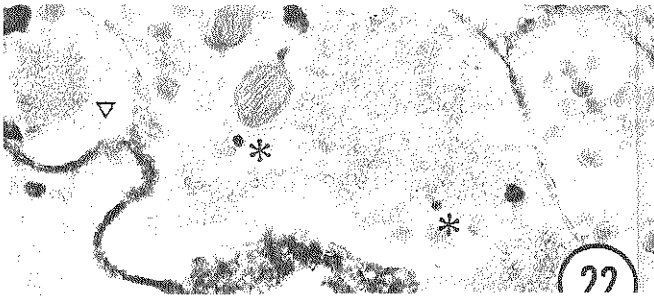

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The examples show that, may be especially under stimulated conditions, certain varicosities contain several hundreds of peroxidase positive organelles. These neurons must have a very active uptake mechanism for exogenous materials like HRP.

2.2.2.3. non-neuronal profiles

'Uptake' of HRP by non-neuronal endfeet was less evident than by neuronal endfeet. HRP containing organelles resembled the large ones of neuronal endfeet.
In rats perfused one min after injection of HRP, no HRP positive organelle were seen in the non-neuronal elements of the m.e. Five min after injectio such organelles were occasionally present in endfeet. In ependymal and hyp endymal cell bodies, HRP positive organelles were numerous 60, 105 and 135 min after injection. After the two latter intervals, reaction product was seen, rarely, in blebs protruding into the cerebrospinal fluid; signs of actual release were not observed.

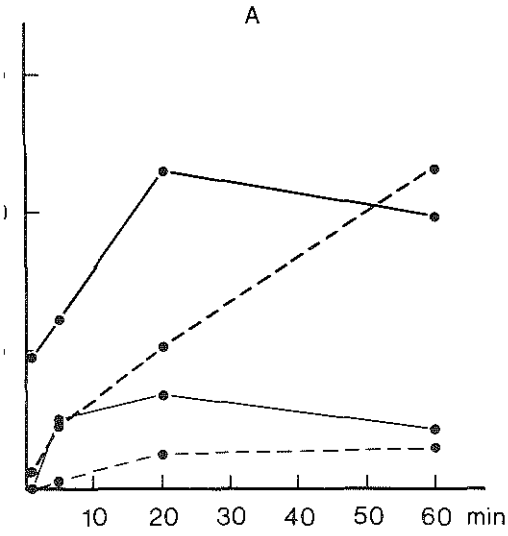
3. *Effects of disturbing the hormonal equilibrium*

3.1. effects of castration and steroids

SUMMARY Changes in the ultrastructure of nerve profiles located near the primary plexus were most striking, 24 hours and 30 days after castration, if the observations were restricted to neuronal endfeet contacting the outer basement membrane of the primary plexus. The observations were suggestive of depletion of GV, which after long term castration seemed to occur preferentially at the lateral side of the m.e.
There was no evidence for changes in size of GV, 24 and 48 hours after castration.
The outer basement membrane of the primary plexus, became, after castratio preferentially contacted by neuronal endfeet. Thirty days after castration the changes were especially prominent at the lateral side of the m.e.
After treatment of castrated rats with oestradiol benzoate, there was in

Fig. 23. Presence of HRP containing organelles at different intervals (1, 5, 20 and 60 min) after injection of HRP.
Uninterrupted lines indicate the percentages of nerve profiles with HRP containing microvesicles, interrupted lines that of nerve profiles with exclusively HRP containing organelles other then HRP-microvesicles.
Thick lines refer to profiles in contact with the other basement membrane thin lines to adjacent deeper profiles. Figure A refers to four rats anaesthetized with Avertin, figure B to four urethane anaesthetized rats.
Number of profiles involved: 2379.

Fig. 24. Large neuronal and slender non-neuronal profiles located near a capillary (△) with an electron-dense inner and outer basement membrane (▼). Two neuronal profiles (✻) are crowded with microvesicles and GV. HRP containing organelles are numerous, but among them microvesicles are scarce.
Fixation 30 days after castration and 20 min after injection of HRP.
x 30.000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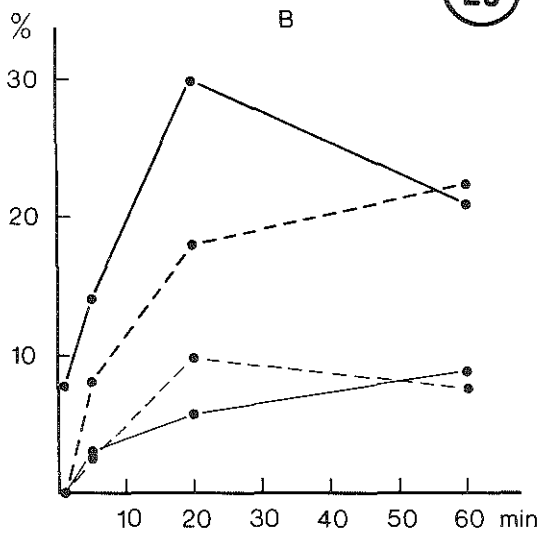
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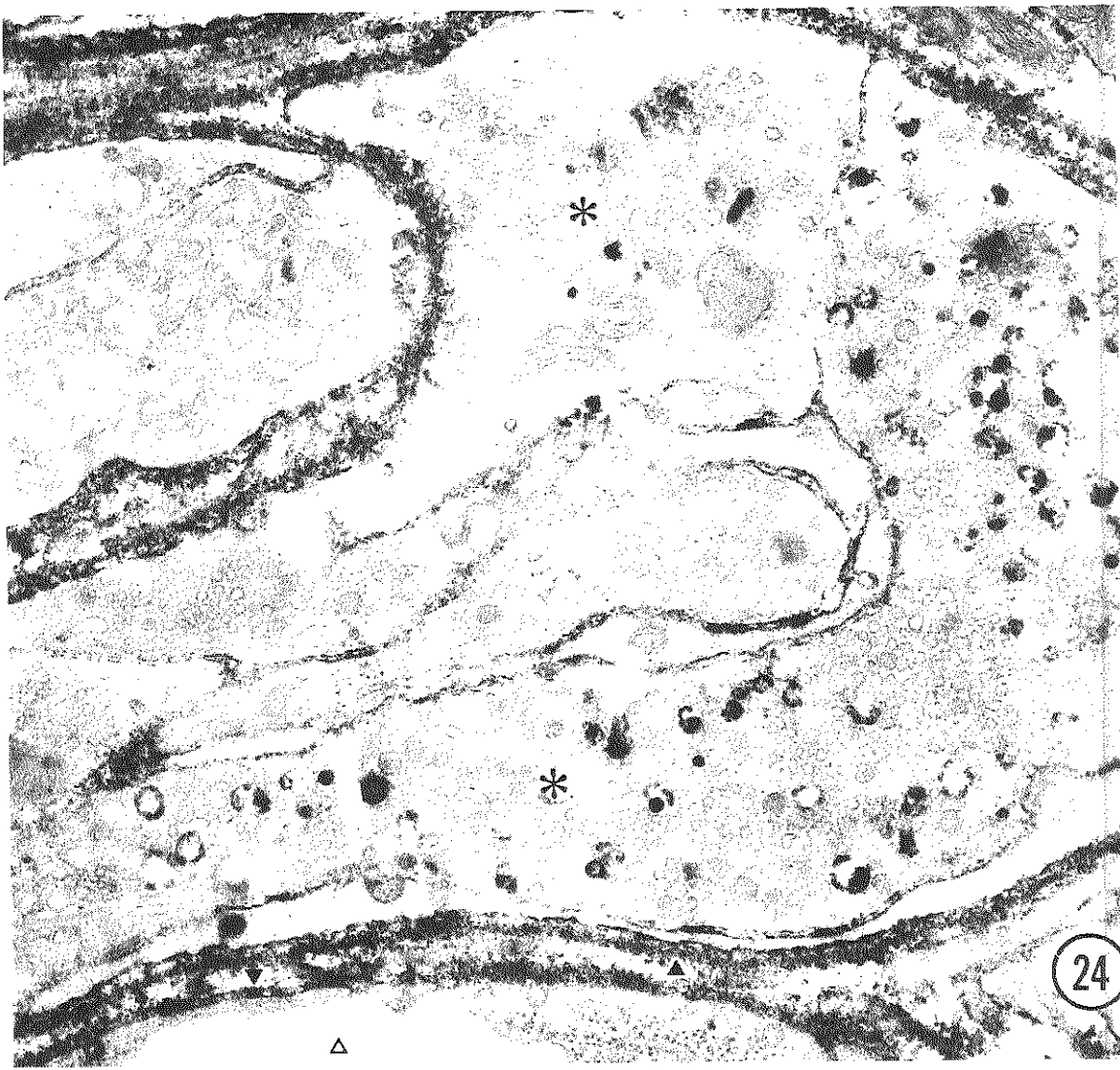

the (sub)ependymal layer, the palisade zone and the zona granulosa a significant increase in the number of non-neuronal profiles with lipid droplets.
Data obtained in in vitro studies suggest the following: a. a differential effect of castration on monoaminergic and non-monoaminergic varicosities near the primary plexus, b. a direct, acute effect of testosterone at the level of the m.e. and c. a more pronounced effect of castration and of treatment with testosterone at the lateral part of the m.e. than at the medial part.
Twenty four hours after castration (and 20 min after intravenous injection of HRP), the percentage of nerve profiles with HRP-loaded microvesicles but without GV was significantly increased. This was not the case after long term castration of males or females.

3.1.1. effects of castration (see App. 5 and 6)

general aspects

In contrast to the data mentioned in App. 5, which were not exclusively dealing with nerve profiles in direct contact with the outer basement membrane, there was, 24 hours after castration, at the basement membrane a significant shift from profiles containing microvesicles and GV to profiles containing exclusively microvesicles (App. 6). A comparable change at the basement membrane was found 30 days after castration, although exclusively at the lateral side of the m.e. There, the percentage of profiles with exclusively microvesicles increased significantly from 33.8 $\pm$ 4.17 to 51.6 $\pm$ 1.93 and that of profiles with microvesicles and GV decreased from 66.2 $\pm$ 4.17 to 44.5 $\pm$ 2.54 (for both groups, n=6; serum LH and FSH levels were significantly raised after castration, II 12.).

Such changes suggest that in the direct vicinity of the primary plexus endfeet with microvesicles and GV transform after castration into endfeet containing exclusively microvesicles. This may occur especially at the lateral side of the m.e. Varicosities away from the basement membrane are apparently less affected.

The experiments in App. 5 and 6, dealing with incorporation of HRP and showing after short term castration an increase in percentage of nerve profiles containing HRP-microvesicles and no GV, were repeated for male rats castrated or sham-operated 30 days before perfusion (both groups, n=6). In addition, female rats ovariectomized 18 days before perfusion were compared with 5-day cycling rats perfused between 12.00 and 14.00 hours at di-oestrous I (both groups, n= 5). Only those nerve profiles were analyzed, which were in direct contact with the basement membrane. Serum LH and FSH levels were in the castrated rats significantly raised (II 12.).
In contrast to the short term castrated male rats, there was no significant difference between castrated rats and controls in the presence of HRP-microvesicles.

This suggests that differences exist between short term and long term castrated rats in accumulation of HRP by certain nerve endings at the outer basement membrane of the primary plexus.

plasticity at the outer basement membrane
Rats castrated 48 hours before fixation showed, in comparison with sham-operated rats, a significant decrease in mean contact length of the non-neuronal endfeet with the outer basement membrane (from 1.4 ± 0.08 to 1.1 ± 0.04 µm; n=4). Neuronal endfeet did not show a significant change (0.8 ± 0.06 and 0.9 ± 0.08 µm)
Moreover, the relative extent of the basement membrane occupied by non-neuronal tissue was, 30 days after castration, decreased if compared with that of sham-operated rats (from 55.3 ± 3.42 to 41.4 ± 2.06 %; n=6; $P < 0.01$). This change was especially manifest at the lateral side of the m.e. (from 66.5 ± 1.69 to 50.8 ± 3.67 %; $P < 0.01$; for a significant increase after castration in serum LH and FSH levels, see II 12.).

The observations indicate a marked plasticity near the primary plexus: as a consequence of castration, the primary plexus becomes preferentially surrounded by neuronal endfeet.

sizes of GV
In order to see whether castration caused changes in the sizes of the GV near the primary plexus, sizes were compared 24 and 48 hours after castration or sham-operation (four rats per group; total number of neuronal profiles involved: 4143)
There was after castration no significant change in mean sizes of GV.

3.1.2. effect of steroid treatment on lipid droplets in non-neuronal elements

If the non-neuronal elements of the m.e. are involved in control of gonadotropin release, it may be expected that a disturbance in the hormonal equilibrium will influence their ultrastructure. Therefore, the effect was studied of treating castrated rats with oestradiol benzoate. As parameter, the presence of lipid droplets was chosen since they are a characteristic feature of the typical non-neuronal cells of the neurohypophysis and are, at least in the infundibular process thought to be involved in the release process (for literature, see IV 4.1.).

Rats, three months of age, castrated four weeks earlier, received at 11.00 hours, at three successive days, an intramuscular injection of 0.5 ug oestradiol benzoate (Dimenformon, Organon) in 50 µl arachid oil. Controls received solvent only (both groups, n= 5; mean weight, resp. 228 ± 6.1 and 245 ± 11.1 gr). The rats were fixed 24 hours after the last injection. In ultrathin sections of silver interference colour, the number of non-neuronal profiles containing one or more lipid droplets was assessed. The region studied was restricted to the floor of the infundibular recess and was divided into four areas (see Table 3).
The relative sizes of these areas differ markedly.
There was, after steroid treatment, for the whole area a significant increase in the mean number of profiles containing lipid droplets (from 185 ± 21.8 to 396 ± 17.8; $P \leq 0.01$). This increase was evident in the ependymal and subependymal layer, in the palisade zone and in the zona granulosa (Table 3). The total number of lipid droplets was, after steroid treatment, also significantly increased in each of these areas.

Table 3 Effect of oestradiol benzoate on the mean number of non-neuronal profiles containing lipid droplets

	oil			steroid		
(sub) ependymal	77	±	13.2[1)]	166	±	3.9[2)]
fibre layer	5	±	1.0	7	±	1.3
palisade zone	94	±	9.4	187	±	16.1[2)]
zona granulosa	10	±	2.1	37	±	2.4[2)]

1) S.E.M.; n=5

2) $P \leq 0.01$

The data show that at least part of the non-neuronal cells of the m.e., which include pituicyte-like cells, ependymal cells and subependymal cells, are markedly influenced by the steroid treatment.

3.1.3. effects of steroids, in vitro

To obtain information on a possible direct effect of steroids on the m.e., the m.e. of rats castrated 2-4 weeks earlier, were incubated with or without testosterone (4-6 rats per group; see II 8.). To differentiate between monoaminergic and non-aminergic varicosities, tissue of castrated rats, as well as that of controls, was preincubated with 5-OHDA (II 7.; III 1.2.4.). Since from such a preparation arcuate nucleus and other nuclei were excluded, most axons and varicosities were severed from their cell bodies. The data refer to the outer 3 µm of the lateral part of the sub-zone A and are obtained from montages of electron micrographs (x 35.000), each covering about 100 μm^2.

For non-aminergic profiles the following was observed:
1. the mean area of the profiles was after castration significantly increased (from 0.6 ± 0.03 μm^2 in the controls to 0.9 ± 0.09 μm^2 in the castrated rats).
2. the mean number of microvesicles per profile was after castration significantly increased (from 25.4 ± 1.9 to 39.6 ± 4.2).
This increase occurred in profiles with both microvesicles and 'GV' since the number of microvesicles in non-aminergic profiles with exclusively microvesicles remained constant. Treatment with testosterone (20 ng/ml) reversed the effect of castration completely (23.7 ± 4.5).
3. the mean number of 'GV' per um^2 of nerve profile, which was not changed by castration (7.4 ± 0.9 and 7.5 ± 0.5), was significantly decreased after incubation with 20 ng or 50 ng testosterone /ml (5.5 ± 0.6 and 5.9 ± 0.4, resp.).
For monoaminergic profiles such changes were not found. The only significant effects were, after castration, a decrease in number of monoaminergic profiles/100 μm^2 (from 21.3 ± 2.0 to 5.9 ± 0.5), whereas after incubation with 20 ng testosterone/ml this number was significantly increased (to 12.4 ± 1.8), if compared with material of castrated rats incubated without testosterone.

In the medial part the same tendencies were observed as in the lateral part but differences were not significant.

The observations suggest the following:
1. a differential effect of castration upon non-aminergic and monoaminergic varicosities in the external zone of the m.e.,
2. a direct acute effect of testosterone at the level of the m.e., involving both monoaminergic and non-aminergic elements,
3. a more pronounced effect of castration and treatment with testosterone at the lateral part of the external zone than at the medial part.

3.2. effects of electrochemical stimulation of the medial preoptic area

SUMMARY *There was, 30 and 60 min after electrochemical stimulation of the medial preoptic area at the primary plexus an increase in the relative extent of the basement membrane contacted by non-neuronal tissue. Changes in the ultrastructure of neuronal varicosities seemed to be restricted to limited numbers.*

After electrochemical stimulation of the medial preoptic area, serum levels of LH increased rapidly (Table 4). Since it might be expected that the increase was caused by an increased release of Gn-RH (I 5.), the

Table 4 Serum LH levels (ng NIAMD-rat-LH RP-1/ml serum) at different times after electrochemical stimulation of the medial preoptic area

	sham stimulation	stimulation
15 min	21 ± 1.4[1)]	71 ± 8.5[2)]
30 min	12 ± 4.1	149 ± 15.0[2)]
60 min	12 ± 5.1	220 ± 56.0[2)]

1) S.E.M.; for all groups, n=6
2) $P \leq 0.02$

Table 5 Percentage of the outer basement membrane at the primary plexus occupied by non-neuronal tissue, at different times after electrochemical stimulation of the medial preoptic area

	sham stimulation	stimulation
15 min	43 ± 1.9[1)]	48 ± 2.9
30 min	45 ± 0.5	64 ± 1.1[2)]
60 min	43 ± 2.4	52 ± 0.4[2)]

1) S.E.M.; for all groups. n=6
2) $P \leq 0.02$

ultrastructure near the primary plexus was analyzed at different times after stimulation. Remarkably, there was at the primary plexus a significan increase, 30 and 60 min after stimulation, in the relative extent of the outer basement membrane occupied by non-neuronal tissue (Table 5), which comprises endfeet of pituicyte-like cells, ependymal cells and sub-ependymal cells.

The observations suggest a marked plasticity at the primary plexus of the neuronal and non-neuronal endfeet. It is noteworthy that the relative extent of the non-neuronal endfeet was increased.

It was also studied whether stimulation affected, at the basement membrane, the percentages of different categories of nerve profiles (see App. 5), the 'uptake' of intravenously injected HRP and the relative number of neuronal and non-neuronal endfeet. However, rats perfused 30 min after stimulation and 20 min after injection of HRP did not differ from similarly treated sham-stimulated rats (both groups, n= 6; total number of profiles involved: 7250). Furthermore, an analysis of 900 profiles containing over 20.000 microvesicles and GV, did not yield evidence for a change in number of microvesicles or GV per profile, neither for nerve profiles in contact with the outer basement membrane nor for the adjacent profiles.

A limited number of neuronal profiles showed after stimulation a multitude of membrane configurations and/or peroxidase positive organelles suggestive of high exocytotic and micro-pinocytotic activity.

The limited effect may indicate that relatively few varicosities present in the m.e. are affected by preoptic stimulation, which may be related to release of Gn-RH from the organum vasculosum laminae terminalis (I 4.1.1.), a circumventricular organ close to the site of stimulation (cf. Phelps et al, 1976).

IV. DISCUSSION

1. *Quantitative data on neurons projecting to the median eminence, with special reference to release of gonadotropin releasing hormone*

1.1. number of perivascular endfeet, hypophysiotropic neurons and monoaminergic neurons

The number of neuronal endfeet present at the outer basement membrane of the primary plexus was estimated to be 7 x 10^5 (III 1.5.). These endfeet belonged for about 25% to monoaminergic neurons (III 1.2.4.). This percentage agrees well with that found for the (whole) zona granulosa (III 1.2.4.) and also agrees reasonably with the data on the perivascular area mentioned by Ajika and Hökfelt (1973) and Richards and Tranzer (1974). Thus, it can be estimated that roughly 2 x 10^5 perivascular endfeet belong to monoaminergic neurons and 5 x 10^5 to non-monoaminergic, probably mainly peptidergic neurons.

Data on the relative numbers of different types of neuronal endfeet present at the primary plexus are absent but for the varicosities present in the external zone, the following picture arises; data shown represent the percentages of the neuronal varicosities said to contain the substance(s) mentioned.

Gn-RH	10-20%	Pelletier et al, 1974a; Naik, 1976
somatostatin	30 %	Pelletier et al, 1974b
serotonin	14 %	Ajika and Okinaga, 1976
monoamines (serotonin presumably included)	25-30%	Ajika and Hökfelt, 1973; Richards and Tranzer, 1974; III 1.2.4.
other substances	6-35%	

It can be questioned whether these data are correct, since they are based on small and mostly not rigidly specified parts of the external zone and regional differences are in the m.e. clearly present (III 1.2.4.; III 1.3.; Kizer et al, 1976a; Löfström et al, 1976a; Silverman and Desnoyers, 1976). Yet, the data suggest that Gn-RH and somatostatin producing neurons contribute almost 50% of the varicosities present in the external zone. In view of the additional presence of TRH -with an amount much larger than that of Gn-RH (Brownstein, 1977) - and the possible presence of varicosities belonging to still other peptidergic hypophysiotropic neurons, it may be expected that by far the majority of the non-aminergic varicosities belong to peptidergic hypophysiotropic neurons.
In the foregoing, it was taken for granted that different neurohormones and neurotransmitters are present in different neurons. This, however, is disputed. Co-existence in the same neuron of Gn-RH and monoamines, especially dopamine, has been suggested by several authors (Ishii, 1970b; Clementi et al, 1970, 1971; Rodríguez, 1972), but according to Kizer et al (1975) long lasting destruction of catecholaminergic neurons did not result in decreased amounts of Gn-RH. Therefore, an interaction of (separate) dopaminergic and peptidergic neurons seems more likely (I 3.1.). In this respect, Ochs (1977) boldly stated that 'the tuberoinfundibular dopaminergic neurons terminate in the external zone of the m.e. by

axo-axonic endings on peptidergic neurons', but axo-axonic contacts have not been demonstrated convincingly (Ajika and Hökfelt, 1973; see III 1.2.1., for one possible exception). It can, however, not be excluded that the close anatomical relations between monoaminergic and 'peptidergic' varicosities, seen in the external zone (III 1.2.4.), reflect 'synapses' which are morphologically not specialized.

Palkovits (1977) estimated that in the rat medial basal hypothalamus 2-3 x 10^5 neurons are present. Only a small percentage of these neurons belongs to hypophysiotropic neurons, since

1. retrograde labelling of neuronal cell bodies projecting to the m.e. (and to the other areas located outside the blood-brain barrier) showed that such neurons were present in very restricted areas of the hypothalamus (Broadwell and Brightman, 1976), and

2. by immunohistochemical studies on hypophysiotropic substances only few cell bodies could be demonstrated (Barry and Dubois, 1975; Barry and Carette, 1975; Alpert et al, 1976; Barry, 1976, 1977; Sétáló et al, 1976a; Silverman, 1976a; Bugnon et al, 1977a,b). Their total number may be in the order of 4 x 10^3, which admittedly is a rough estimate.

Data obtained by electrophysiological studies, using antidromic stimulatic yielded for neurons projecting to the m.e. a higher percentage, especially when recording from the arcuate nucleus (Moss et al, 1976). Dufy et al (1976) reported for the rabbit that 3% of the hypothalamic neurons studied responded to stimulation of the m.e. By stimulating the rat m.e.-pituitary stalk junction, Renaud (1976) obtained evidence of antidromic stimulation for at least 15% of the neurons studied in the middle and anterior hypothalamus. This percentage, however, overestimates the percentage of (peptidergic) hypophysiotropic neurons considerably, since

a. the identified neurons will include other types of neurons, e.g. dopaminergic ones,

b. recording probably occurred preferentially from areas rich in neurons projecting to the m.e. and

c. possibly areas outside the m.e. were affected by current spread.

If the estimates of the numbers of hypophysiotropic neurons and perivascular endfeet are more or less correct, then an 'average' hypophysiotropic neuron terminates with at least 125 endfeet at the outer basement membrane of the primary plexus. Branching of hypophysiotropic neurons is also suggested by other morphological observations (Szentágothai et al, 1968; Barry, 1972, 1976).

In view of the enormous number of varicosities calculated to be present per dopaminergic neuron of the nigro-striatal system, viz. 10^5-10^6 (Andén et al 1966), it would not be surprising if the number of monoaminergic, probably mainly dopaminergic endfeet estimated to be present near the primary plexus, 2 x 10^5, belongs to only a small number of monoaminergic neurons.

1.2. tentative calculation on release of Gn-RH

Data on a relation between the amounts of hypophysiotropic substances stored in the m.e. and the amounts released are not available. On the basis of the following calculation, the amount of Gn-RH daily released seems to be much smaller than the amount present, a phenomenon also reported for the neurohypophyseal hormones of the infundibular process (Jones and Pickering, 1972)

In 1 ml portal plasma of anaesthetized male rats, 30 pg radioimmunoassayable Gn-RH is present according to data of Eskay et al (1974). Given a flow rate of 7 µl/min. and assuming portal blood to consist for 60% of plasma (Porter et al, 1976), 0.126 pg Gn-RH is released per min., which means 181 pg per day. However, calculated on the basis of data presented by Fink and Jamieson (1974), viz. 18.2 pg Gn-RH released per 30 min, in urethane anaesthetized male rats, 874 pg would be released per day.
Data on the total amount of radioimmunoassayable Gn-RH present in the m.e. vary also markedly. According to Knigge et al (1977), 8200 pg is present per m.e., whereas Swann and Pickering (1976) found 7000 pg, Morris et al (1975) 2600-4300 pg and Kawakami et al (1975) 30.000 pg for a block containing the m.e. and the arcuate nucleus as well. According to Palkovits (1975) only 521 pg is present in the m.e. but his illustrations suggest that the lateral part of the m.e., where most Gn-RH is present, was excluded from the analysis.

If Gn-RH is stored in GV (IV 2.1.1.), then the following calculation may throw some light on a quantitative aspect of release of Gn-RH, although it is emphasized that the data should only be considered as an order of magnitude. Taking into consideration ,
a. the preponderance of GV in the external zone (III 1.5.),
b. the presence of Gn-RH positive granules in 10-20% of the nerve profiles of the external zone (IV 1.1.; IV 2.1.1.) and
c. the relatively small number of GV in monoaminergic profiles (III 1.2.4.; Cuello and Iversen, 1973), then roughly 15% of the estimated number of 1.6×10^9 GV (III 1.5.) may contain Gn-RH. In that case, 3.42×10^{-5} pg Gn-RH would be present per GV in varicosities belonging to Gn-RH producing neurons, taking as a basis the data reported by Knigge et al (1977), viz. 8200 pg Gn-RH per m.e.

From 3.42×10^{-5} pg Gn-RH per GV it follows, for a molecular weight of 1182, that 2.9×10^{-20} moles and 1.7×10^4 molecules of Gn-RH would be present in one GV. Other speculations on the amount of Gn-RH or other hypophysiotropic substances present in GV are not available but the number of molecules of oxytocin and vasopressin in GV of the infundibular process are of the same order of magnitude (Morris, 1976), viz. 8.4×10^4 molecules of hormone .

A release rate of 0.126 pg Gn-RH per min. (Eskay et al, 1974), i.e. 2.1×10^{-3} pg per second, and presence of 3.42×10^{-5} pg Gn-RH per GV would necessitate release of about 60 GV per second, or, taking the data of Fink and Jamieson (1974) as basis, 300 GV per second, assuming that all Gn-RH released would reach the portal blood. For the whole m.e., it would imply that during one second 60-300 Gn-RH containing GV would be released in a total number of 1.6×10^9 G V , thus roughly $1 : 10^7$.
The possibility of observing such an event in case of release by exocytosis (IV 3.) , which probably is of very short duration (Normann, 1970, 1976; Heuser et al. 1976), seems extremely small, even if it is assumed that, say 0.1% or 1.0% of the total number of GV is in a 'releasable position'.
Prospects to increase the chance of observing such phenomena by increasing release of Gn-RH are rather sad, since, for instance, castration hardly causes a two-fold increase in Gn-RH release (Eskay et al, 1974; Ching,

1976; Porter et al, 1976). As a consequence, the hope expressed in App. I of identifying fibres with different biologically active substances by way of stimulation-induced exocytosis, was rather optimistic, as also indicated by data of Morris (1976) on the infundibular process. He calculated that during maximal release of oxytocin at maximum two exocytotic images could be expected in 10^6 'granules' studied.

2. *Granular vesicles as parameter to distinguish different types of nerve endings*

Differentiation between nerve profiles in the mammalian m.e. seems more difficult than in non-mammalian species where marked ultrastructural differences have been described in vesicular content of different profiles (Peczely and Calas, 1970; Rodríguez, 1972; Dierickx et al, 1973). In a study on the subcellular structure of the rat m.e., Clementi et al (1971) even state 'from a morphological point of view, the median eminence nerve endings are similar to each other...'. This, however, is not the case (III 1.2.4.).

2.1. contents of granular vesicles

GV are the most conspicuous organelles, after fixation by aldehyde-osmium tetroxide, of most varicosities present in the m.e. With respect to their contents, there are numerous candidates, viz. a large variety of neurohormones and (putative) neurotransmitters (Brownstein, 1977; Palkovits, 1977). Hypophysiotropic substances and monoamines are the main candidates for the contents of GV located in the external zone of the m.e.

2.1.1. hypophysiotropic substances

For the following reasons, it is concluded that hypophysiotropic substances are stored in GV, localized in (neuronal) varicosities:

1. the preponderance of GV near the primary plexus, where also the bulk of the hypophysiotropic substances is present (I 1.1.; I 4.1.1.),
2. the presence near the primary plexus of fusions of GV with the plasma membrane, suggesting release into portal blood (App. 1; III 2.1.; IV 3.),
3. the presence in neuronal profiles of granules, resembling cores of GV and immunoreactive to anti-Gn-RH and anti-somatostatin (Pelletier et al, 1974b; Goldsmith and Ganong, 1975; Naik, 1975b; Kozlowski et al, 1976a; Pelletier, 1976; Silverman and Desnoyers, 1976; Goldsmith, 1977),
4. the presence of axonal swellings and/or granular vesicles in subcellular fractions with high concentrations of hypophysiotropic substances (Shin et al, 1974b; Taber and Karavolas, 1975; Barnea et al, 1977; Gautron et al, 1977; Parker et al, 1977; Warberg et al, 1977),
5. the comparable ultrastructural features of the infundibular process where oxytocin and vasopressin are, mainly or exclusively, stored in (large) GV (I 4.1.2.).

It is not known whether the electron-dense core of the GV, seen in 'peptidergic' profiles after aldehyde-osmium tetroxide fixation or after aldehyde fixation followed by ethanolic phosphotungstic acid (App. 3), reflects the presence of peptide hormones and/or of associated substances like e.g. carrier proteins.

2.1.2. monoamines

Both in the peripheral and the central nervous system, microvesicles seem to be the major storage site for monoamines (Hökfelt, 1968, 1970; Bloom, 1970, 1972; Ajika and Okinaga, 1976). Yet, presence of a limited number of (large) granular vesicles, generally said to be 80-100 nm in diameter, is a common feature of varicosities and endings presumed to contain monoamines, as shown by

1. autoradiography (Aghajanian and Bloom, 1967a,b; Bloom and Aghajanian, 1968b; Doerr-Schott and Follenius, 1970; Calas, 1973; Cuello and Iversen, 1973; Palay and Chan-Palay, 1976),
2. loading with ('false') monoamine transmitters or precursors (Hökfelt, 1967, 1968; Pellegrino de Iraldi and Jaim Etcheverry, 1967; Tranzer and Thoenen, 1967, 1968; Richards and Tranzer, 1970; Ajika and Hökfelt, 1973; Mazzuca and Poulain, 1974; Smith and Helme, 1974) and
3. cytochemical and pharmacological techniques (Wood, 1966, 1977; Tranzer and Thoenen, 1968; Lorez and Richards, 1973; Richards and Tranzer, 1974).

The electron-dense core seen after aldehyde-osmium tetroxide fixation reflects mainly the presence of proteins (Bloom and Aghajanian, 1968b; Bloom, 1970; Rinne, 1970; Hökfelt, 1970; Klein and Thureson-Klein, 1974; Till and Banks, 1976; De Potter and Chubb, 1977). However, monoamines are present as well (Hökfelt, 1968, 1970; Bisby and Fillentz, 1970; Richards and Tranzer, 1970; De Potter and Chubb, 1977; cf. III 1.2.4.). In view of the above mentioned data and of the abundance of monoaminergic varicosities in the external zone (I 4.2.; III 1.2.4.), it may be expected that in this area numerous GV contain monoamines.

2.1.3. neurohypophyseal hormones

Since certain collaterals of hypothalamo-neurohypophyseal neurons seem to terminate near the primary plexus (I 4.1.2.), some GV may contain oxytocin, vasopressin and neurophysins.

2.1.4. acetylcholine and other (putative) transmitters

Small numbers of GV, roughly 100 nm in diameter, are rather universally present in nerve endings, e.g. in those of cholinergic neurons (see e.g. Ehinger et al, 1970). Thus, it can be expected that also in the m.e. some GV are present in neurons other than mentioned above. The significance of these GV has not been established.

2.1.5. acid hydrolases

Some lysosomes in the infundibular process had, after dehydration, the appearance of GV (Whitaker and LaBella, 1972). This implies that also in the m.e. a limited number of GV(-like organelles) contain acid hydrolases.

Notwithstanding this large variety of candidates, the majority of the GV present in the external zone of the m.e. is located in varicosities of peptidergic hypophysiotropic neurons since

1. most varicosities are non-aminergic (III 1.2.4., Ajika and Hökfelt, 1973; Cuello and Iversen, 1973; Mazzuca and Poulain, 1974),
2. the number of GV in non-aminergic varicosities is much higher than in monoaminergic ones (III 1.2.4.; Cuello and Iversen, 1973) and
3. semi-quantitative data based on immuno-electron microscopy suggest a high percentage of Gn-RH and somatostatin containing varicosities, with numerous CV (Goldsmith, 1977).

2.2. sizes of GV

In view of the large size of the vasopressin and oxytocin containing GV in the internal zone and in the infundibular process (Leclerc and Pelletier, 1974) if compared for example with monoaminergic GV (Bloom, 1972), a correlation may be expected between size of GV and content (App. 3; Wittkowski, 1973). The following observations indicate, however, that sizes of GV present in the m.e. are of limited value as indicator of their content (cf. Winkler, 1977, for the adrenal medulla).

1. Data based on subcellular fractionation of the m.e. were not conclusive in correlating sizes of GV and hormonal activity (Andreoli et al, 1970; Ishii, 1970a; Kobayashi et al, 1970; Mulder et al, 1970; Fink et al, 1972).

2. Data obtained by immuno-electron microscopy were not very informative. According to Pelletier et al (1976) somatostatin containing granules are in the rat slightly larger than those containing Gn-RH (90-110 nm and 75-95 nm, resp.), whereas according to Naik (1976) Gn-RH was stored in granules of 90-130 nm. Besides, Silverman and Desnoyers (1976) stated that in the guinea pig Gn-RH was present in axons of the palisade zone in granules of 90-120 nm and in nerve endings abutting the portal plexus in granules of 40-70 nm. This observation seems in harmony with the relatively small size of the GV in the vicinity of the primary plexus (App. 2 and 3; Monroe et al, 1972) and seems to support a reduction in size of GV during transit to the terminal, proposed by Monroe (1967). However, evidence for a diminution in size of GV in individual endings could not be obtained in the present study (III 1.2.1.; cf. Kazawa, 1976).

3. Reports on castration-induced changes in sizes of GV (Kobayashi et al, 1967, 1969; Kobayashi and Matsui, 1969) could not be confirmed in the present study (III 3.1.1.); it may be added that the conclusions in the first two reports were based on one ovariectomized rat and one control, whereas the other report was based on qualitative observations. Also during the female cycle no change in sizes of GV was found (Kazawa, 1976). In contrast, bilateral adrenalectomy caused in the external zone an increased number of relatively large GV (Rinne, 1970; Bock et al, 1976), which were considered organelles storing CRF and "CRF-associated neurophysin". In view of the associated increase in content of neurohypophyseal hormones (Pelletier et al, 1976; Vandesande et al, 1977), this remains to be proven.

3. *Mechanisms of release and fate of remnants of the release process in relation to the origin and function of microvesicles*

3.1. mechanisms of release

Events like dehydration and haemorrhage, known to induce release of vasopressin from the infundibular process, caused loss of electron-dense material from GV present in the peptidergic endings, as shown after fixation by osmium tetroxide (Palay, 1957). It appeared, however, that the dehydration-induced loss of electron-dense material could not be related to

the amount of hormones released (Daniel and Lederis, 1966). Besides, this loss could be largely prevented, provided aldehydes were used as primary fixative (Reinhardt et al, 1969; Boudier, 1974), especially if the aldehydes were used at low pH (Morris and Cannata, 1973). The latter authors showed that release of vasopressin was parallelled by a significant reduction in *number* of GV, an observation supported by data of Reinhardt et al (1969), Boudier et al (1970), Whitaker and LaBella (1972), Dempsey et al (1973) and Nordmann and Morris (1976). Most authors failed to detect ultrastructural features compatible with release by 'reverse micro-pinocytosis' or exocytosis (Kurosumi et al, 1961; Röhlich et al, 1965; Monroe and Scott, 1966; Rinne, 1966; Monroe, 1967; Picard, 1969; Rodríguez and La Pointe, 1969; Kurosumi, 1971; Nakai, 1971; Clattenburg, 1974). Therefore, it was proposed that hormone release from the infundibular process and the m.e. occurred by 'molecular dispersion', i.e. extrusion through morphologically intact membranes (Kurosumi et al, 1961) or by fragmentation of GV (Table VIII).

Table VIII Proposed mechanisms for release of hormones from granular vesicles present in the neurohypophysis.

Mechanism	infundibular process	median eminence
'molecular dispersion' or diffusion	Hartmann, 1958; Kurosumi et al, 1961; Kurosumi, 1971; Krisch et al, 1972	Bern et al, 1966; Rinne, 1966; Rodríguez, 1969; Nakai, 1971; Monroe et al, 1972; Clattenburg, 1974
fragmentation	Holmes and Knowles, 1960; Bern, 1963; Daniel and Lederis, 1966; Streefkerk, 1967	Rodríguez, 1969; Nakai, 1971; Kobayashi et al, 1972a; Dierickx et al, 1973
exocytosis		
-on the basis of thin-sectioned material	Nagasawa et al, 1970; Douglas et al, 1971; Santolaya et al, 1972; Boudier, 1974	App. 1; III 2.1.
-on the basis of freeze-fracture studies	Santolaya et al, 1972; Dempsey et al, 1973; Dreifuss et al, 1973; Dreifuss, 1975; Sandri et al, 1977	
-on the basis of biochemical data	Fawcett et al, 1968; Uttenthal et al, 1971; Edwards et al, 1973; Matthews et al, 1973	

However, more recent ultrastructural observations on the infundibular process have shown that release can occur by exocytosis, as was also demonstrated for the m.e. in the present study (Table VIII). In both areas of the neurohypophysis, the number of exocytotic images was very small (see Orci, 1974; Winkler, 1977, for comparable findings in the endocrine pancreas and the adrenal medulla). This may at least in part be due to the chemical fixatives used, since membrane configurations interpreted as sites of release by exocytosis, or otherwise as sites of exocytosis-endocytosis, were more readily observed in freeze-fracture studies (Table VIII; for another cause, see IV 1.2.)
Release from the infundibular process by exocytosis is supported by biochemical data, since neurohypophyseal hormones and intravesicular proteins appear to be released simultaneously, whereas cytoplasmic enzymes are retained (Table VIII). For the m.e. such data are not available (protein carriers or other intravesicular substances are not known) but for intravesicular proteins present in other types of cells, e.g. the noradrenergic neurons and the chromaffin cells of the adrenal medulla, the same has been reported (Smith et al, 1970; Weinshilsboum et al, 1971; De Potter and Chubb, 1977; Winkler, 1977). Such observations fit in with the concept that exocytosis is a rather general mechanism for discharge of macromolecular secretory products (Dreifuss, 1975; Palade, 1975; Normann, 1976; Tixier-Vidal et al, 1976; Holtzman, 1977; Winkler, 1977).
Thus, it is assumed that release of the content of the GV present in the neurohypophysis occurs by exocytosis, although a spectrum of release mechanisms (Thorn, 1970), dependent upon intensity and duration of the stimulus, can not be excluded.

3.2. Events subsequent to release

In case of release by exocytosis, the vesicular membrane of the GV is incorporated into the plasma membrane and retrieval of membrane material seems necessary. Indeed, retrieval of material from the plasma membrane has been described for a large variety of secretory cell types, nerve endings included (Diner, 1967; Holtzman et al, 1971; Ceccarelli et al, 1972; Nagasawa and Douglas, 1972; Abrahams and Holtzman, 1973; Heuser and Reese, 1973; Orci et al, 1973; Jorgensen and Mellerup, 1974; Pelletier and Puviani, 1974; Geuze and Kramer, 1974; Nordmann and Dreifuss, 1974; Whittaker, 1974; Nordmann and Morris, 1976; Tixier-Vidal et al, 1976; Zimmermann and Denston, 1977a,b).
For the infundibular process, Douglas and co-workers (Nagasawa et al, 1970, 1971; Douglas et al, 1971) proposed that release by exocytosis is followed by micro-pinocytosis ('vesiculation') leading to formation of microvesicles. Such a simple sequence of events would explain the frequently reported, but rarely quantified increase in number of microvesicles after stimulation of vasopressin or oxytocin release (Palay, 1957; Gerschenfeld et al, 1960; Bern et al, 1966; Monroe and Scott, 1966; Dempsey et al, 1973; Boudier, 1974; Kodama and Fujita, 1975; Livingston , 1975).

In the present study changes in numbers of microvesicles were shown in the external zone of the m.e. in an in vitro experiment (III 3.1.3.). At the lateral side, non-aminergic, probably peptidergic profiles present in tissue derived from castrated rats contained after incubation significantly more microvesicles than profiles present in identically treated tissue derived from control rats. Tissue derived from castrated rats and

incubated in the presence of testosterone did not show an increase in number of microvesicles. In view of
a. the preferential localization of Gn-RH at the lateral side of the external zone (I 5.),
b. the increase in release of Gn-RH after castration (I 1.1.) and
c. the suppression of Gn-RH release by testosterone (Cheng, 1976),
it seems likely that these observations are related to respectively raised and depressed release of Gn-RH.

The hypothesis of a coupling of exocytosis and micro-pinocytosis was supported by the uptake of a macromolecular extracellular tracer like HRP into microvesicles of the infundibular process (Nagasawa et al, 1971). Such an uptake of HRP was in the present study demonstrated for neuronal endfeet present in the m.e. (III 2.2.2.2.; App. 4).
Of course, formation of HRP-loaded vesicles is not necessarily related to release, since micro-pinocytosis is a very general phenomenon. The following observations are, however, suggestive of a relation between micro-pinocytosis and release by exocytosis.

1. Several omega-shaped configurations of the plasma membrane, with and without electron-dense material resembling the core of GV, were characterized by a distinct coat at the cytoplasmic side of the membrane (III 2.1.; Douglas et al, 1971; Boudier, 1974). Such a coat is frequently seen around caveolae or vesicles incorporating foreign, especially proteinaceous, material from the extracellular space; it is thought to play a rôle in the mechanism of infolding and fission of the membrane (Roth and Porter, 1964; Daems et al, 1969; Kanaseki and Kadota, 1969; Kristensson et al, 1974).
In the adrenal medulla of the guinea pig (Diner, 1967; Grynszpan-Winograd, 1971; Benedeczky and Smith, 1972; Nagasawa and Douglas, 1972), small coated vesicles were seen 'budding off' from exocytotic images, a phenomenon only seen exceptionally in our material.
Both phenomena may indicate that release by exocytosis is followed very rapidly by membrane retrieval. This can be expected since in various cell types the composition of the vesicular membrane and that of the plasma membrane seems different (Smith, 1971; Winkler, 1977).

2. Uptake of extracellular tracers by non-stimulated nerve endings is, generally, negligible, whereas electrical stimulation or stimulation by a depolarizing concentration of potassium ions leads to pronounced accumulation of tracer inside organelles (peripheral nervous system: Holtzman et al, 1971; Ceccarelli et al, 1972; Heuser and Reese, 1973; Basbaum and Heuser, 1976; Zimmermann and Denston, 1977a,b; Zimmermann and Whittaker, 1977; infundibular process: Nordmann and Dreifuss, 1974; Theodosis et al, 1976, 1977). Also in (other) secretory cell types, stimulation of release can lead to enhanced endocytosis of extracellular material, e.g. in the adrenal medulla (Abrahams and Holtzman, 1973), the endocrine pancreas (Orci et al, 1973) and in certain cell types of the anterior pituitary gland (Pelletier and Puviani, 1974; Tixier-Vidal et al, 1976).
For the external zone of the m.e. it was shown (App. 5,6) that castration caused an increase in the percentage of nerve profiles characterized by presence of HRP-loaded microvesicles and absence of GV. The mean number of HRP-loaded microvesicles remained small. After electrochemical stimulation of the medial preoptic area, there was also no clear increase in number of HRP-loaded microvesicles. However, the number of nerve profiles characterized by a large accumulation of relatively large HRP-containing organelles tended to be increased under conditions of stimulated release(III2.2.2.2.).

Such relatively large HRP-containing organelles were, in contrast to HRP-loaded microvesicles, absent one minute after injection of HRP (III2.2.2.2.). Thus, the HRP-loaded microvesicles may represent short-lived intermediates in the sequence of incorporation and subsequent removal of extracellular material, possibly related to removal (or reuse) of retrieved membrane material. Such a rôle as intermediate would explain that the number of HRP-loaded microvesicles remained small, independent of the interval between injection of HRP and sacrifice, whereas, apparently, the number of relatively large HRP-containing organelles increased with time (III2.2.2.2.). For the infundibular process, Theodosis et al (1977) did show an increase in number of HRP-loaded microvesicles after stimulation, but exclusively shortly after electrical stimulation of the pituitary stalk, a very intense stimulus, and not after haemorrhage.

3.3. Fate of endocytosed material and associated membranes

For a large variety of cells, it has been demonstrated that the limiting membranes of endocytotic vesicles fuse with membranes of lysosomes, after which the endocytosed material can be digested (Daems et al, 1969; Nagasawa and Douglas, 1972; Abrahams and Holtzman, 1973; Geuze and Kramer, 1974; Pelletier and Puviani, 1974; Tixier-Vidal et al, 1976; Winkler, 1977). In neurons, digestion seems to occur mainly in the perikaryon (Holtzman, 1977), which necessitates, for material endocytosed by terminals, axonal retrograde transport. For an extracellular tracer like HRP, there is ample evidence for such a transport (Kristensson et al, 1971; La Vail and La Vail, 1975; Kievit and Kuypers, 1975); it has also been demonstrated for neurons of the hypothalamo-neurohypophyseal and the tuberoinfundibular system (Sherlock et al, 1975; Broadwell and Brightman, 1976; cf. III 2.2.2.2.)

The fate of the limiting membrane of the endocytotic vesicles is less clear. It may for instance be digested but, in certain cells, it may, after transport to the Golgi area, participate in the packaging of secretory products (Pelletier, 1973; Orci et al, 1973; Meldolesi, 1974; Gagnon et al, 1976; Winkler, 1977). For endocytotic microvesicles which arise in nerve terminals, there is presumably the additional possibility of local reloading with neurotransmitter and subsequent release by exocytosis, a sequence possibly repeated in several cycles (Heuser and Reese, 1973; Bennett et al, 1976; De Potter and Chubb, 1977; Holtzman, 1977; Zimmermann and Whittaker, 1977). For endfeet of peptidergic neurons, such a sequence is not likely, since there is no evidence for the presence of a substantial amount of neurohormone in microvesicles (Swann and Pickering, 1976; Goldsmith, 1977). More likely seems a rôle in rapid retrieval of material from the plasma membrane, after release of GV by exocytosis. In view of the high surface/volume ratio of the microvesicles, these organelles are particularly suited for such a process (see further IV 3.2.).

It is not known whether the membranes of the microvesicles present in peptidergic neurons are, like endocytosed HRP, transported to the perikaryon. Possibly, digestion occurs at the level of the neurohypophysis, since increased release from the infundibular process is associated with an increased activity of lysosomal enzymes (Hiroshige et al, 1966; Whitaker and LaBella, 1972; Boer and Jongkind, 1974; Boer, 1976). See further, IV 4.

4. *Rôles of the typical non-neuronal cells of the neurohypophysis*

Pituicytes and pituicyte-like cells are generally viewed upon as modified (astro) glial cells, thus they may share certain functions. This does not throw much light on their function since Somjen (1975) stated 'More than hundred years of research brought us to this point where each proposition suggesting one or another function for neuroglia can be countered by a valid point of criticism or doubt'. Nevertheless, it can not be excluded that the pituicytes and the pituicyte-like cells are involved in the trophic and supportive functions, ascribed to astroglial cells especially by regulating the ionic composition of the extracellular fluid. In addition, the typical non-neuronal cells of the neurohypophysis may have specific functions in digestion (4.1.), phagocytosis (Dellmann, 1973) and secretion (4.3.). Possibly, the modified ependymal cells of the m.e. share certain of these functions but in addition they may especially be involved in transport between cerebrospinal fluid and portal vessels (4.2.).

4.1. lipid droplets and digestion

The pituicytes, pituicyte-like cells, ependymal cells and subependymal cells of the neurohypophysis share the presence of the so-called lipid droplets or lipoprotein granules, which according to Kurosumi et al (1964) mainly consist of phospholipids (cf. Giesing, 1971), especially lecithin, and according to Raviola and Raviola (1963) of unsaturated neutral fat, although presence of lecithin could not be excluded.
Involvement of these non-neuronal cells of the neurohypophysis in hormone release, for example in case of dehydration, is suggested by hypertrophy of pituicytes, increase in mitotic activity, increase in RNA and protein synthesis and accumulation of lipid droplets (Kurosumi et al, 1964; Streefkerk, 1967; Krsulovic and Brückner, 1969; Sachs et al. 1971; Sunde et al, 1972a,b; Léranth and Schiebler, 1975; Livingston, 1975). Therefore, it was hypothetized (Kurosumi et al, 1964; see also Boer and Van Rheenen-Verberg, 1976; Boer, 1976) that the membranous remnants of the release process passed, after partial degradation in the nerve endings, into the non-neuronal cells for final digestion. The lipid moiety of the remnants was thought to be retained as lipid droplets prior to final break-down, or might represent the residues of lysosomal activity (Reinhardt et al, 1969; Pilgrim et al, 1974; Wagner and Pilgrim, 1974). In addition, the neurohormones and soluble lipoproteins present in the GV (Mylroie and Koenig, 1971; Meyer-Grass and Pliška, 1974) may be additional sources for the lipid droplets (see Giesing, 1971). After release by exocytosis, these substances directly reach the nearby non-neuronal cells. Uptake may occur by micro-pinocytosis, in view of the uptake of HRP (III 2.2.2.3.; Pilgrim et al, 1974; Wagner and Pilgrim, 1974). For the m.e. also, a relation may exist between hormone release and presence of lipid droplets as suggested by their increased numbers after steroid treatment of castrated rats (III 3.1.2.).

4.2. transport

The ependymal cells of the m.e. are by most authors classified as tanycytes (see e.g. Brawer et al, 1974; Bruni, 1974; Akmayev and Popov, 1977), which for mammals is not correct since only certain ependymal cells of the m.e. show the long basal process characteristic of tanycytes (I 3.3.; McArthur, 1970; Raisman and Field, 1971; Bara and Böti, 1974). The tanycytes of the m.e. are frequently assumed to transport biologically active substances from cerebrospinal fluid to portal blood (I 3.3.) but the functional significance of such a transport is debated (Gordon et al, 1972;

Weiner et al, 1972b; Dierickx et al, 1973; Dierickx, 1974; Uemura and Kobayashi, 1977). Doubts can e.g. be raised by the observation that most tanycytes are not located at the floor of the infundibular recess but at the (lateral) wall and that their basal processes, in contrast to the general opinion (Kobayashi et al, 1970), mainly end at the base of the brain lateral to the primary plexus or at capillaries outside the m.e. (Millhouse, 1971; Raisman and Field, 1971; Bleier, 1972; Bara and Böti, 1974). However, certain tanycytes of the m.e., mainly present at the lateral side, do have contacts with the primary plexus.
It is added that transport ascribed to tanycytes is not necessarily part of an endocrine pathway. The absence of a blood-brain barrier necessitates some manner of removal of blood-borne substances (Wagner and Pilgrim, 1974), as well as of waste products from the cerebrospinal fluid.
Although the non-tanycyte ependymal cells of the m.e. do not contact the primary plexus at the base of the palisade zone, they also may have a transport function. Substances released from their basal parts, would rapidly reach the portal blood, in view of the absence of tight junctions in the external zone and the rapid extracellular transport of HRP (III 2.2.2.1.).

In this respect it is surprizing that some authors emphasize the presence of a 'cuff' of non-neuronal endfeet separating the neuronal endfeet from the outer basement membrane and imply that neurosecretory products have to pass through the non-neuronal endfeet to reach the primary plexus (see e.g. Rodríguez and LaPointe, 1969; Oota et al, 1974).

For the non-tanycyte ependymal cells of the m.e., another transport function seems possible. Most subependymal capillaries are contacted by non-neuronal tissue, which largely consists of ependymal and subependymal cells (I 2.3.). These capillaries mainly belong to the subependymal tangentional plexus by which blood can pass from the neurohypophysis to certain hypothalamic areas (Török, 1964; Szentágothai et al, 1968; Duvernoy, 1972; Oliver et al, 1977; Porter et al, 1977). Thus, it seems possible that the (these) ependymal cells form a link between the cerebrospinal fluid in the infundibular recess and the hypophysiotropic area (cf. Sétálő et al, 1976b).

Three points, however, have to be raised. 1. Microvilli and bleb-like protrusions were found to be relatively scarce at the floor of the infundibular recess (with the exception of lateral and rostral areas), if compared with those on ependymal cells lining the (lateral) wall of the third ventricle (cf. Millhouse, 1972). Since the presence of microvilli generally is related to absorption, most ependymal cells of the floor do not seem to be very active in this respect. Moreover, signs of micro-pinocytosis are not conspicious. 2. Because of the absence of tight junctions between ependymal cells located outside m.e. and other circumcentricular organs (Weindl and Joynt, 1972), substances present in the cerebrospinal fluid have a relatively free access to the extracellular space surrounding the periventricular cells (Rapoport, 1976). This seems to make a route, as described, rather superfluous, unless the composition of the cerebrospinal fluid in the infundibular recess is quite distinct from that elsewhere. 3. It may be questioned whether substances present in these ependymal cells can reach the lumen of the subependymal capillaries which only exceptionally show fenestrations.

In addition to 'orthograde' transport in tanycytes of the m.e., retrograde transport (i.e. transport from endfeet to cerebrospinal fluid) was proposed by Wittkowski (1967). After intravenous injection, HRP was indeed incorporated by non-neuronal endfeet lining the primary plexus (III 2.2.2.3.). Uptake was rapid if compared with that described by Wagner and Pilgrim (1974) after intraventricular injection of HRP. The subsequent accumulation of HRP-loaded organelles in the ependymal cell bodies near the cerebrospinal fluid may be indicative of transport to the infundibular recess. This might e.g. be a route for transport of pituitary hormones, implicated in short feedback actions (I 3.2.). Evidence that HRP was released into the infundibular recess, as reported for the frog (Nakai and Naito, 1974, 1975), was not obtained in the present study.

4.3. secretion

The typical non-neuronal cells of the m.e. have also been claimed to be involved in secretory processes (Lévêque et al, 1965; Kobayashi et al, 1970; McArthur, 1970; Porter et al, 1973; Akmayev and Popov, 1977). In view of the presence of synaptoid contacts (Wittkowski, 1968; Kobayashi et al, 1970; Güldner and Wolff, 1973), this might well be an additional possibility. The varicosities showing synaptoid contacts are generally thought to belong to monoaminergic neurons (Kobayashi and Matsui, 1969; Kobayashi et al, 1970; Baumgarten and Lachenmayer, 1974) but an additional peptidergic source seems possible (III 1.2.1.).

Of course, synaptoid contacts may be involved in phenomena other than stimulation of secretion, for instance in transfer of materials from peptidergic varicosities to non-neuronal cells (IV 4.1.).

Secretion may be directed to the infundibular recess and/or to the portal blood, but ultrastructural evidence for release is poor. Discharge into the infundibular recess of cytoplasm (or even whole nuclei) by pinching off bulbous protrusions, as reported by Kobayashi et al (1970), seems very unlikely.

In view of the many doubts, it is obviously too early for a coherent picture of the typical non-neuronal cells of the m.e.

5. *Plasticity near the primary plexus*

If it would be possible to view in the living animal the external zone of the m.e. by electron microscopy, there might well be an astonishing wriggling, protruding and withdrawal of neuronal and non-neuronal processes, supplemented with expulsion of material from vesicles fused with the plasma membrane and inward bubbling of microvesicles - phenomena admittedly somewhat difficult to envisage for an electron microscopist. Such a plasticity at the primary plexus is e.g. suggested by the significant increase in the relative extent of the outer basement membrane occupied by non-neuronal tissue 30 and 60 min after electrochemical stimulation of the medial preoptic area (III 3.2.), a procedure generally leading to increased release of Gn-RH (IV 1.).
Such a relative increase in non-neuronal tissue along the outer basement membrane has been described also by Hökfelt (1973) shortly after intraventricular injection of dopamine. During long term castration, the opposite appeared to occur, viz. a relative increase in neuronal tissue along the outer basement membrane (III 3.1.1.), a phenomenon also described one month after adrenalectomy (Wittkowski, 1973; Wittkowski and Scheuer,

1974) and, for the infundibular process, three days after dehydration (Wittkowski and Brinkmann, 1974).
The increase in relative extent of the basement membrane occupied by neuronal tissue was interpreted, by Wittkowski and co-workers, as increased release from nerve endings, which seems logical. However, the relative decrease in neuronal tissue found in the short term experiments creates a problem, in view of the rapid increase in serum LH level after preoptic stimulation (III 3.2.) and, possibly, after intraventricular injection of dopamine (Kamberi et al, 1970a; Schneider and McCann, 1970a; Kamberi, 1973), although Cramer and Porter (1973) could not confirm such an increase. Release of Gn-RH seemed to be increased after both procedures (IV 1.; Kamberi et al, 1969; Schneider and McCann, 1970b; Porter et al, 1972). Thus, the question arises whether the increase in relative extent of non-neuronal tissue along the basement membrane of the primary plexus, in analogy with the interpretation after long term stimulation, reflects increased release from non-neuronal endfeet.
Regarding the seemingly comparable effects of intraventricular injection of dopamine and preoptic stimulation on the relative distribution of neuronal and non-neuronal tissue along the basement membrane, it would be interesting to know whether preoptic stimulation leads to an increased level of dopamine (or of other catecholamines) in the cerebrospinal fluid. The concentration of Gn-RH in cerebrospinal fluid does not seem to be increased after preoptic stimulation (Cramer and Barraclough, 1975).

V. SUMMARY AND CONCLUSIONS

Several aspects of the ultrastructural organization of the rat median eminence were analyzed in an attempt to correlate its ultrastructure and function, especially with respect to release of gonadotropin-releasing hormone (Gn-RH).

1. A first aspect concerns estimates of the numbers of various morphological elements, more specifically of the numbers of

a. peptidergic hypophysiotropic neurons projecting to the primary plexus,
b. different types of neuronal (and non-neuronal) endfeet at the primary plexus,
c. neuronal granular vesicles present in the median eminence,
d. Gn-RH containing vesicles, with their amount of Gn-RH present, and
e. Gn-RH containing vesicles released per unit of time.

It is estimated that at the outer basement membrane of the primary plexus up to 5×10^5 endfeet belong to peptidergic hypophysiotropic neurons and that at least 100 endfeet are present per 'average' hypophysiotropic neuron. In the whole median eminence, almost 2×10^9 granular vesicles were estimated to be present, mainly in varicosities of peptidergic neurons. The amount of Gn-RH present in granular vesicles of Gn-RH producing neurons is estimated to be about 10^{-5} picogram. A consideration of the possibility of documenting release of Gn-RH containing vesicles, and increased release after castration, offered a gloomy prospect.
For the 2×10^5 monoaminergic endfeet estimated to be present at the outer basement membrane, the possibility is raised that they belong to only a limited number of neurons.

2. The distribution of neuronal and non-neuronal tissue lining the outer basement membrane of the primary plexus could be altered. Long term castration was accompanied by a relative increase in neuronal tissue lining the outer basement membrane, a shift compatible with increased release from neuronal endfeet into portal blood. Acute electrochemical stimulation of the medial preoptic area showed, however, an opposite effect. The possibility of a marked plasticity at the outer basement membrane of the neuronal and non-neuronal endfeet is emphasized.

3. Another effect of castration on the median eminence is an alteration in vesicular content of the nerve profiles. The relative number of profiles

with granular vesicles and microvesicles decreased, whereas that of profiles with exclusively microvesicles increased. Such a shift, caused by withdrawal of gonadal substances, could only be demonstrated in the direct vicinity of the outer basement membrane.

4. Non-monoaminergic, presumably mainly peptidergic varicosities located near the primary plexus showed changes in their ultrastructure, if the median eminence derived from castrated rats was incubated with testosterone. This indicates a rapid action of testosterone at the level of the median eminence. Ultrastructural changes were most pronounced at the lateral side of the median eminence. This is in line with the presence of Gn-RH preferentially at the lateral side of the m.e. and supports the view that in the median eminence functionally different areas are present.

5. A fifth aspect concerns sizes of granular vesicles present near the primary plexus in neuronal varicosities. No evidence has been obtained for a change in size after castration, nor during transport to the terminal part of the axon.

6. A major aspect concerns the mechanism by which biologically active substances are released from their storage vesicles in the m.e. The ultrastructural data indicate that release by exocytosis, during which the vesicular membrane fuses with the plasma membrane, can be an efficient mechanism in the median eminence for simultaneous release of large quantities of stored products. In this respect it is noteworthy that release is not restricted to that part of the neuronal plasma membrane in contact with the basement membrane; release seems possible along the whole surface of the endfeet. The plasma membranes of certain neuronal profiles present near the primary plexus showed profound invaginations, which were thought to be due to massive release of granular vesicles by exocytosis. In view of the exceptional presence of such deep invaginations, it is assumed that they are rapidly eliminated by membrane retrieval.

7. Microvesicles, which are a characteristic feature of the neuronal endfeet contacting the outer basement membrane of the primary plexus, were thought to reflect, in part, retrieved membrane material. Loading with an extracellular tracer (horseradish peroxidase), showing their origin from the plasma membrane, supports this possibility.

However, the heterogeneity of the microvesicles is emphasized. One aspect of their heterogeneity was revealed by incubation of the median eminence with the 'false' neurotransmitter 5-hydroxydopamine; then, only microvesicles of certain varicosities acquired an electron-dense content.

Certain varicosities showed a profound accumulation of organelles containing horseradish peroxidase, especially under conditions of increased release. This indicates that such varicosities can incorporate large amounts of extracellular material, which may well be related to massive release by exocytosis.

8. At least part of the endocytosed horseradish peroxidase is transported retrogradely to perikaryon. In addition, digestion of endocytosed material and/or of remnants of the release process may occur at the level of the median eminence. It may be assumed that the peculiar non-neuronal cells of the median eminence, viz. modified ependymal cells, subependymal cells and pituicyte-like cells, are involved in such a process. These cells show, after steroid treatment of castrated rats, a dramatic increase in number of lipid droplets. Possibly, these organelles represent remnants of intraneuronal material.

VI. SAMENVATTING EN CONCLUSIES (met toelichting)

De eminentia mediana is een gebied aan de basis van de hersenen waar hormonen afgegeven worden aan een speciaal bloedvatsysteem, een portaalsysteem, dat uitmondt in de voorkwab van de hypophyse (het hersenaanhangsel). Deze (hypophysiotrope) hormonen worden geproduceerd door speciale cellen gelegen in en nabij de hypothalamus, een basaal gelegen deel van de hersenen.
In het algemeen wordt aangenomen dat de cellen die deze hypophysiotrope hormonen produceren gespecialiseerde neuronen, zenuwcellen, zijn waarvan de uitlopers eindigen bij het portaal systeem. De (meeste) hypophysiotrope hormonen behoren chemisch tot de peptiden. De betreffende cellen worden dan ook aangeduid als peptiderge hypophysiotrope neuronen. De uitlopers van deze neuronen zijn gekenmerkt door grote aantallen zogenaamde granulaire blaasjes, blaasjes ongeveer 10^{-4} mm in doorsnede, die in de electronen microscoop een electronendichte inhoud vertonen - na bepaalde voorbehandeling van het weefsel.
Verder komen in de eminentia mediana ook talloze uitlopers voor van andere typen neuronen, met name van monoaminerge neuronen, zo genoemd omdat zij als werkzame stoffen monoaminen, zoals dopamine en noradrenaline, produceren.
De hypophysiotrope hormonen kunnen, na transport door het portaal systeem, bepaalde cellen in de hypophyse aanzetten tot verhoogde (of verlaagde) activiteit, meestal resulterend in verhoogde (of verlaagde) afgifte van hypophysaire hormonen. De hypophysaire hormonen op hun beurt kunnen, na afgifte aan het bloed, weer andere organen beinvloeden. Zo hebben bijvoorbeeld de hypophysaire gonadotrope hormonen een stimulerend effect op de testis, de mannelijke geslachtsklier. Ook deze klier produceert weer hormonen - met name testosteron - die, na afgifte aan het bloed, via 'terugkoppeling' een remmende invloed kunnen uitoefenen op de afgifte van gonadotrope hormonen uit de hypophyse. Deze terugkoppeling komt tot stand, deels door een direct effect op de hypophyse, deels door een effect op de hersenen, via een verminderde afgifte van het gonadotropine-afgevend hormoon, het hypophysiotrope hormoon dat stimulerend werkt op de afgifte van de gonadotrope hormonen.

Om inzicht te kunnen krijgen in de ultrastructuur van de bouwelementen van de eminentia mediana van de rat, die betrokken zijn bij de afgifte van het gonadotropine-afgevend hormoon, zijn verschillende aspecten geanalyseerd.

1. Een aspect van de analyse betreft een kwantitatieve benadering van de bouwelementen van de eminentia mediana. Een schatting is gemaakt van

a. het aantal peptiderge hypophysiotrope neuronen,
b. het aantal verschillende neuronale (en niet-neuronale) uitlopers naar het portaalsysteem,
c. het aantal granulaire blaasjes,

d. het aantal granulaire blaasjes dat gonadotropine-afgevend hormoon bevat, met de hoeveelheid van deze stof in elk blaasje en

e. het aantal van dergelijke blaasjes afgegeven per minuut.

Hierbij blijkt ondermeer dat een peptiderg hypophysiotroop neuron met meer dan honderd uitlopers contact maakt met de basaal membraan die de haarvaten van het portaalsysteem omgeeft, dat bijna twee miljard granulaire blaasjes aanwezig zijn in de eminentia mediana en dat deze blaasjes voornamelijk voorkomen in uitlopers van peptiderge hypophysiotrope neuronen. De hoeveelheid gonadotropine-afgevend hormoon per blaasje is geschat op ongeveer 10^{-5} picogram (1 picogram = 10^{-12} gram). Het blijkt dat het relatieve aantal van dergelijke blaasjes, afgegeven per minuut, uiterst gering is, ook na castratie. Dit maakt de aantoonbaarheid van (verhoogde) afgifte op ultrastructureel nivo uiterst moeilijk.
Ten aanzien van het grote aantal monoaminerge uitlopers bij het portaalsysteem, geschat op 2×10^5, wordt de mogelijkheid geopperd dat zij behoren tot slechts een gering aantal neuronen.

2. Na ingrepen in het hormonale regelsysteem, zoals castratie of electrochemische stimulatie van het preoptisch gebied, een basaal gelegen deel van de hersenen, worden in de directe nabijheid van het portaalsysteem veranderingen waargenomen in de verdeling neuronaal/niet-neuronaal weefsel. Beide ingrepen resulteren in verhoogde afgifte van gonadotrope hormonen. Benadrukt wordt dat de 'bekleding' van het portaalsysteem niet statisch is maar afhankelijk van de fysiologische toestand. Zo veroorzaakt castratie langs de basaal membraan van het portaalsysteem een toename in neuronaal oppervlak, een verandering die hormoon-transport van neuronale uitlopers naar portaalbloed kan vergemakkelijken.

3. Een ander gevolg van castratie, zowel na een dag als na dertig dagen, is een verandering in de blaasjes-samenstelling van neuronale uitlopers: uitlopers met granulaire blaasjes en microblaasjes (blaasjes ongeveer 5×10^{-5} mm in doorsnede) nemen in percentage af, uitlopers met uitsluitend microblaasjes nemen toe. Een dergelijke verschuiving, gevolg van het verdwijnen van door de testis geproduceerde stoffen, was alleen aantoonbaar in de directe nabijheid van het portaalsysteem en wordt geinterpreteerd als een verhoogde afgifte van granulaire blaasjes uit bepaalde neuronale uitlopers.

4. Bij in vitro experimenten werd de eminentia mediana ge(pre)incubeerd met 5-hydroxydopamine, een 'pseudo'-neurotransmitter die opgenomen wordt door uitlopers van monoaminerge neuronen en gebruikt wordt ter onderscheiding van monoaminerge en niet-monoaminerge uitlopers. Bij verge-

lijking van weefsel afkomstig van gecastreerde en controle dieren blijken verschillen aantoonbaar in de ultrastructuur van uitlopers behorende tot niet-monoaminerge, waarschijnlijk voornamelijk peptiderge neuronen. Zo is na castratie ondermeer de diameter van deze uitlopers toegenomen, evenals het aantal microblaasjes. Incubatie met testosteron doet dit laatste effect teniet, hetgeen suggereert dat testosteron een terugkoppel-effect heeft op de eminentia mediana. Opvallend is dat veranderingen in ultrastructuur na castratie en na incubatie met testosteron lateraal in de eminentia mediana veel duidelijker zijn dan mediaal. Dit verschil in effect is in overeenstemming met de relatief hoge concentratie Gn-RH aan de laterale kant van de eminentia mediana en steunt de opvatting dat binnen de eminentia mediana functioneel verschillende gebieden voorkomen.

5. Ingegaan wordt op de diameters van de granulaire blaasjes aanwezig in neuronale uitlopers van de eminentia mediana. Aanwijzing voor een verandering in diameter na castratie of tijdens transport naar het uiteinde is niet gevonden.

6. Een ander aspect betreft de wijze waarop hypophysiotrope hormonen vrij komen. Hierbij werd er van uitgegaan dat deze hormonen opgeslagen liggen in granulaire blaasjes, waarvoor goede aanwijzingen bestaan. Afgifte door middel van exocytose, een proces waarbij de membraan van het granulaire blaasje versmelt met die van de neuronale uitloper, wordt op grond van ultrastructurele waarnemingen aannemelijk geacht. Aangezien bij het portaalsysteem afgifte door middel van exocytose plaats lijkt te vinden over het gehele oppervlak van de uitloper kan een dergelijk mechanisme leiden tot snelle afgifte van grote hoeveelheden opgeslagen hormoon.
Bij het portaalsysteem vertoont de membraan van bepaalde neuronale uitlopers diepe instulpingen. Zij worden geinterpreteerd als het gevolg van intense hormoon-afgifte door exocytose, waarbij immers de membraan van het granulaire blaasje wordt opgenomen in de membraan van de uitloper. Gezien het beperkte aantal van dergelijke instulpingen is het aannemelijk - er van uitgaande dat afgifte inderdaad door exocytose plaats vindt - dat een dergelijke toename in membraanoppervlak snel gecompenseerd wordt door afsnoering van het teveel aan membraanoppervlak.

7. Verondersteld wordt dat de microblaasjes, die in enorme aantallen voorkomen in de neuronale uitlopers bij het portaalsysteem, voor een deel ontstaan door zo'n afsnoering. Na intraveneuse toediening van mierikswortel peroxidase (horseradish peroxidase) - een eiwit te groot om intacte membranen te passeren en gebruikt ter 'markering' van de extracellulaire vloeistof - kan deze stof inderdaad aangetoond worden in bepaalde microblaasjes. Dit geeft aan dat deze microblaasjes, direct of indirect, ontstaan uit de membraan van de uitloper.

Benadrukt wordt dat microblaasjes een heterogene verzameling organellen vormen. Dit blijkt ondermeer na toediening van 5-hydroxydopamine, dat alleen opgenomen blijkt te worden in microblaasjes van bepaalde neuronale uitlopers.

8. Omtrent het lot van opgenomen mierikswortel peroxidase is waargenomen dat althans een deel naar het cellichaam wordt getransporteerd. Daarnaast kunnen de niet-neuronale cellen van de eminentia mediana, die opvallen door de aanwezigheid van karakteristieke 'vet druppeltjes' , een rol spelen bij de afbraak van opgenomen stoffen en van stoffen die als afvalproducten van het secretieproces ontstaan. Zo is na steroidbehandeling van gecastreerde dieren het aantal van deze 'vet druppeltjes' toegenomen.

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Renée, Harry, Arie - wat moet ik, als voorbeelden (!), memoreren? De 41.394 ultradunne coupes die Renée uit het water viste, de duizend en een karweien en karweitjes waar Harry zijn hand niet voor om leek te draaien, de 100.000 eindigingen en 1.000.000 blaasjes die Arie verwerkte? Het zal duidelijk zijn, de meeste dank gaat uit naar jullie.

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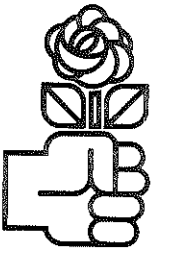

Curriculum vitae

When I was young, I used to howl at
the moon every night ..
I was wild and ignorant those days ..
I had a lot of fun, though.
Now, I don't have any fun, and I'm
still ignorant!

(Snoopy)

In de Watergraafsmeer (Amsterdam oost) genoot ik een rimpelloze jeugd. Na de kleuterschool ging ik direct naar de tweede klas van de lagere school - dat krijg je in een gezin van louter onderwijzenden. Het gewonnen jaar verspeelde ik moeiteloos op het Barlaeus gymnasium waar mijn voorkeur vooral naar gymnastiek uit ging. Ook volgens mijn moeder hield ik mij in die jaren voornamelijk met (korf)ballen bezig.
Gehecht aan Amsterdam als ik was, ging ik daar, aan de GU, biologie studeren. Bij het Zoölogisch Lab. werd ik student-, later kandidaatsassistent. Ik vergaderde veel. Dat bleek voor de toen geldende normen geen beletsel om bij het doctoraal een cum te krijgen. Morfologische dierkunde was mijn hoofdvak, biochemie en dierfysiologie waren bijvakken.
De geringe motivatie om de dienstplicht te vervullen, enerzijds, en de verwachting dat de jonge, toen nog dynamische medische faculteit te Rotterdam goed zou zijn voor een onmisbaarheidsverklaring, anderzijds, waren aanleiding om daar de dag na het doctoraalexamen, tegen het al te forse salaris van een wetenschappelijk medewerker, te beginnen. Daar ik in het Dijkzigt Ziekenhuis te gast was bij de electronenmicroscopiegroep van de Pathologie, zag ik de medewerkers van mijn eigen afdeling, de Anatomie, weinig, in die pre-WUB en pre-hoogbouw periode. Pas later leerde ik deze vakgroep met zijn vele bijzondere trekjes beter kennen. Nu maak ik deel uit van Anatomie I, waarvan J. Moll beheerder is.
Een zekere voorliefde voor Mirthe, Joeke, onderwijs en 'randverschijnselen' als studium generale, welzijnscommissie en faculteitsraad zijn er mede oorzaak van dat dit stukje eerst nu is geschreven.

When God created man
She was only joking

VIII. APPENDIX PAPERS

Z. Zellforsch. 131, 99—107 (1972)

Ultrastructural Evidence for Exocytosis in the Median Eminence of the Rat

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Summary. Exocytosis has been demonstrated by electron microscopy in the external zone of the median eminence of the rat. Exocytotic profiles have been observed in nerve fibres characterized by the presence of granular vesicles with median diameters of 90–130 nm and agranular vesicles of about 50 nm. In addition to the small agranular vesicles, coated vesicles of the same size have been found in many nerve fibres, suggesting that at least part of the agranular vesicles in the median eminence originate by micro-pinocytosis. The nature of the fibres showing exocytosis is discussed. Attention is drawn to the possibility of identifying types of fibres in the median eminence by the occurrence of exocytosis.

Key words: Median eminence — Rat — Exocytosis — Micro-pinocytosis — Releasing factors.

A large part of the hormones secreted by endocrine cells is stored in vesicles and may be liberated by exocytosis. In this process the membrane of the vesicle fuses with the plasma membrane of the cell, the fused membranes open and the contents of the vesicle are extruded into the extracellular space. Examples of this release mechanism have been described, e.g., in the rat anterior pituitary gland (Farquhar, 1961; Pelletier *et al.*, 1971) and the adrenal medulla (Diner, 1967).

A comparable release of the membrane-limited elementary (neurosecretory) granules in the posterior pituitary gland is suggested by the finding of Fawcett *et al.* (1968) that vasopressin and its carrier protein are released simultaneously. Recently, ultrastructural evidence has also been brought forward for the occurrence of exocytosis in the neurosecretory terminals in the posterior pituitary gland (Nagasawa *et al.*, 1970, 1971; Douglas *et al.*, 1971; Krisch *et al.*, 1972; Santolaya *et al.*, 1972).

The ultrastructural features of the external zone of the median eminence are in many respects comparable to those of the posterior pituitary gland. In this zone many nerve fibres abut on the basement membranes of the hypothalamo-hypophysial portal capillaries. These tubero-infundibular fibres are thought to contain biogenic amines and releasing- and inhibiting factors (RF and IF), stored mainly in granular (GV) and agranular (AV) vesicles. Hitherto, evidence for the release of material from these GV by way of exocytosis has not been presented (Röhlich *et al.*, 1965; Bern *et al.*, 1966; Rinne, 1966; Monroe, 1967; Rodríguez, 1969; Nakai, 1971). We are not aware of data on the existence of carrier proteins for RF and IF, but a proteinaceous component of the matrix of GV is surmised

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(Scott *et al.*, 1972). It seems possible that the release of RF and IF can also occur by exocytosis. Since evidence of exocytosis might be used to identify active nerve terminals, the median eminence was examined for signs of such a mechanism.

After release by exocytosis plasma membrane has to be recaptured to prevent the terminals from swelling. Nagasawa *et al.* (1970) hypothesize that the small AV, which occur in the posterior pituitary gland in addition to the neurosecretory granules, are the products of this membrane recapture. Therefore, exocytosis might explain the origin and the significance of at least part of the millions of small AV in the median eminence.

Materials and Methods

Brains of adult male rats (R-Amsterdam strain) were fixed either by perfusion through the heart under ether anaesthesia or by immersion fixation after rapid decapitation. A few animals of the latter category were subjected to acute cold of about 5° C. For the first procedure we used as primary fixative a solution of 0.5% paraformaldehyde and 2% glutaraldehyde (TAAB laboratories) in 0.1 M cacodylate buffer, pH 7.4, to which 2% polyvinyl pyrrolidon was added (Kalimo, 1971). At room temperature 400–500 ml fixative was infused over 15–20 min. The hypothalamus was dissected out immediately after the perfusion. Slices of 1 mm were cut and immersed in fresh fixative at 4° C up to a total fixation time of 2 hr.

For the material fixed by immersion we used 1.5% paraformaldehyde, 2% glutaraldehyde and 0.02% 2, 4, 6, trinitrophenol (picric acid) in cacodylate buffer (Ito and Karnovsky, 1968), to which 0.05% $CaCl_2$ was added. In addition some brains were fixed in buffered 3% glutaraldehyde. Within 30 sec after decapitation the chilled solution was injected into the cranial cavity through the perimedullary space, after which it took 5 min for the immersion of the hypothalamus into fresh fixative at 4° C. The sliced material was kept in this solution for at least 5 hr.

The perfusion and immersion fixed material was rinsed in buffer to which sucrose was added to obtain the same osmolality as the primary fixative. The material was post-fixed for 2 hr in 2% osmium tetroxide in sucrose-cacodylate buffer. The perfusion fixed material was treated with uranyl acetate and phosphotungstic acid in acetone (Wohlfarth-Bottermann, 1957), or with aqueous uranyl acetate. The immersion fixed material was treated with aqueous uranyl acetate. The tissue was dehydrated in graded acetone, embedded in Epon 812 and sectioned on a Reichert Om U2 ultramicrotome. For light microscopy coronal and sagittal sections (1–2 μm) were stained with methylene blue and used for selection of the mesa (Galey, 1964). Ultrathin sections, collected on bare grids or on carbon coated formvar films, were occasionally contrasted with a lead citrate solution (Venable and Coggeshall, 1965). The sections were examined with a Philips EM 300 electron microscope operating at 40 KV.

Observations

In 12 rats connections have been observed between the membranes of GV and the neuronal plasma membranes (Figs. 1 d, 2, 3, 4), connections named exocytotic fusions in the terminology of Santolaya *et al.* (1972). They occur in nerve fibres in the external zone of the median eminence and of the rostral infundibular stem. A limited number of vesicles shows an open connection with the extracellular space and still contains an electron dense content (exocytotic vesicles, Figs. 1 b, 5, 6). Both signs of exocytosis, which will be referred to as exocytotic profiles, are found in brains fixed by perfusion as well as in brains fixed by immersion. They occur predominantly in membranes close to, but rarely in contact with, the perivascular basement membrane.

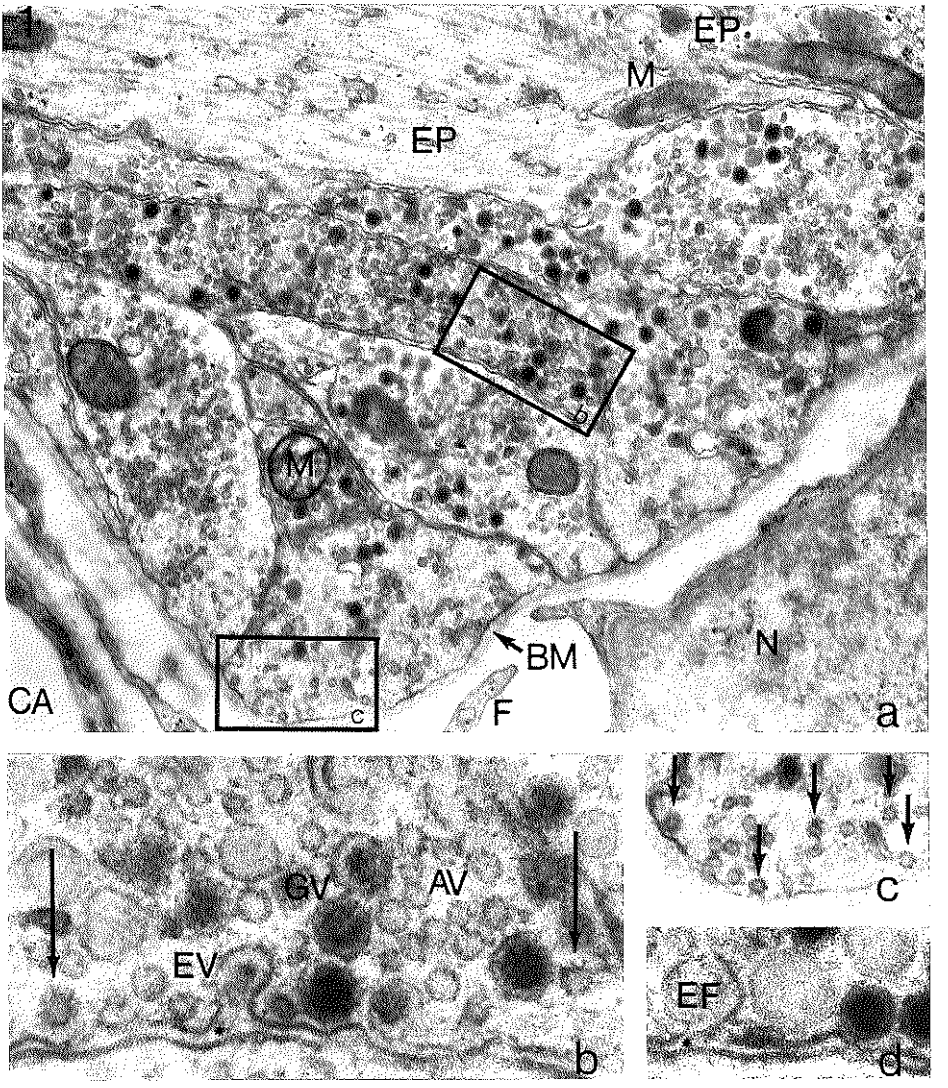

Fig. 1. a Perivascular layer of the external zone of the median eminence, showing nerve fibres and terminals near the perivascular basement membrane (*BM*). Perfusion fixation, uranyl acetate and phosphotungstic acid. × 22000. b Enlargement of the area enclosed in rectangle b of Fig. 1a. Exocytotic vesicle (*EV*) and coated vesicles (arrows). × 80000. c Higher magnification of rectangle c in Fig. 1a, showing coated vesicles (arrows). × 39000. d Serial section of another part of the nerve fiber shown in Fig. 1b. Exocytotic fusion (*EF*). × 80000. *AV* small agranular vesicles; *BM* basement membrane; *CA* lumen of capillary; *EP* ependymal, hypendymal or glial element; *F* fibrocyte; *GV* granular vesicles; *M* mitochondria; *N* nucleus of fibrocyte; * intercellular space

The median diameters of the GV in individual fibres with distinct exocytotic profiles differ considerably. They range from 90 to 130 nm. These GV are much smaller than the neurosecretory granules in the hypothalamo-hypophysial tract.

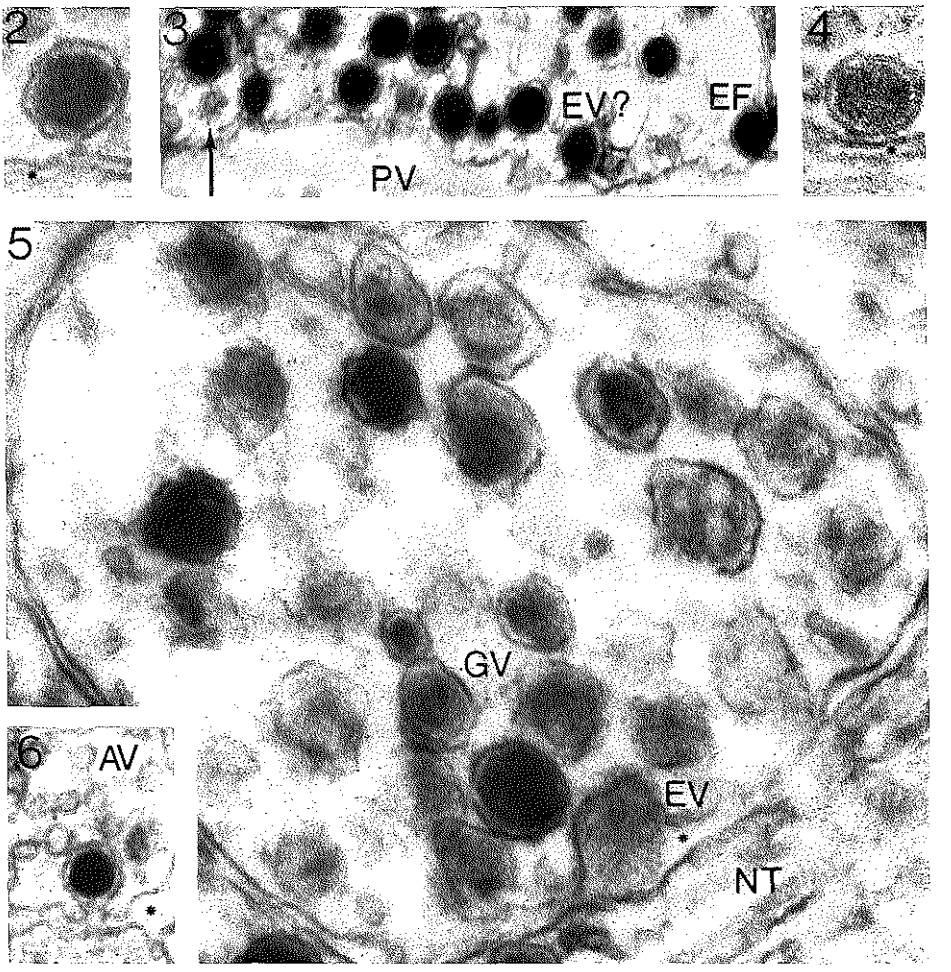

Fig. 2. Exocytotic fusion. Perfusion fixation, uranyl acetate. × 100000

Fig. 3. Part of a nerve terminal in contact with the perivascular space (*PV*), showing an exocytotic fusion (*EF*), an indication of an exocytotic vesicle (*EV* ?) and a coated vesicle (arrow). Immersion fixation: glutaraldehyde. Experimental animal (acute cold). × 70000

Fig. 4. Exocytotic fusion. Immersion fixation: formaldehyde, glutaraldehyde, picric acid. × 95000

Fig. 5. Ventral layer of the external zone of the median eminence. Cross-section of a nerve fibre with predominantly large *GV*, one of which shows exocytosis (exocytotic vesicle, *EV*). Immersion fixation as Fig. 4. × 100000

Fig. 6. Exocytotic vesicle. Immersion fixation as Fig. 4. Experimental animal (acute cold). × 70000

AV small agranular vesicles; *EV* exocytotic vesicle; *EF* exocytotic fusion; *GV* granular vesicles; *NT* neurotubuli; *PV* perivascular space; * intercellular space

The median diameters of the neurosecretory granules in the majority of these fibres are 165–180 nm.

In the fibres showing exocytosis the usual AV of about 50 nm occur among GV, but generally in small numbers. Coated vesicles (Figs. 1b, 1c, 3) of a size comparable to the small AV have also been found in these fibres, and in other nerve fibres as well. Coated vesicles of a larger size have been observed both in nervous and in ependymal, hypendymal or glial elements. In the latter elements neither exocytotic profiles nor small coated vesicles have been observed.

Discussion

According to the present findings, exocytosis occurs in the external zone of the median eminence. Until now the occurrence of exocytosis has not been reported in the median eminence (Röhlich *et al.*, 1965; Bern *et al.*, 1966; Rinne, 1966; Monroe, 1967; Rodríguez, 1969; Nakai, 1971) and exocytosis has not been proposed as a release mechanism in this area (Rodríguez, 1969; Nakai, 1971). The infrequent occurrence of distinct exocytotic vesicles and their preferential localization in membranes not in contact with basement membrane material might explain why they have been overlooked. Because stimuli, inducing rapid release of hormones, are able to increase the frequency of exocytosis in different endocrine tissues (Normann, 1969; Couch *et al.*, 1969; Nagasawa *et al.*, 1971; Santolaya *et al.*, 1972), it is possible that increased release of certain RF, IF and/or biogenic amines causes in the external zone of the median eminence an increase in the numbers of exocytotic vesicles.

A remarkable feature of the exocytotic profiles observed in the median eminence is their frequent localization in the non-terminal zones of the nerve fibres. As a consequence their content is released into the intercellular space between neuronal and ependymal, hypendymal or glial processes. This does not exclude that material released by exocytosis reaches the nearby portal vessels via the intercellular space, considering 1. RF are small polypeptides (Folkers *et al.*, 1969; Schreiber, 1969), 2. the relatively large intercellular space in the median eminence and 3. the complete lack of membrane specializations, resembling tight junctions, in the external zone of the median eminence. However, it is also possible that the material acts directly on the neighbouring processes or is ingested into the non-nervous fibres by micro-pinocytosis (Rodríguez and La Pointe, 1969; Kobayashi and Ishii, 1970) and is stored in tubular and vesicular organelles.

Our observations, on the presence of coated vesicles of a size comparable to the small AV in fibres with exocytosis as well as in other nerve fibres, support the hypothesis of release by exocytosis followed by membrane recapture, as postulated by Douglas *et al.* (1971). According to these authors, coated caveolae, believed to indicate micro-pinocytosis (Roth and Porter, 1964; Kanaseki and Kadota, 1969), are formed as a consequence of exocytosis. The pinched off coated vesicles are assumed to transform into the smooth small AV by shedding their coat. The membranes of 5–6 vesicles of 50 nm diameter are theoretically required to compensate for the membrane of one GV of, say, 120 nm, which has been incorporated into the plasma membrane. It can therefore be expected that coated

vesicles are more readily found than exocytotic vesicles, assuming that the life-span of an exocytotic vesicle does not markedly exceed that of a coated vesicle. Coated vesicles thus offer in principle an even better criterion for identification of nerve fibres, actively involved in hormone release, than exocytotic profiles.

It is not necessary to assume that all small AV of the external zone of the median eminence originate from membrane recapture. Several authors (Kobayashi *et al.*, 1966; Hökfelt, 1967; Kobayashi and Matsui, 1969; Clementi *et al.*, 1971; Rodríguez, 1972) believe that the small AV contain transmitter substances, as also indicated by the frequent use of the term "synaptic" vesicles. Evidence presented by Hökfelt (1967, 1968) and Rodríguez (1972) suggests that small AV in many nerve terminals in the external zone of the median eminence store monoamines. Therefore, our observations strengthen the idea that different types of small AV exist in the median eminence (Kobayashi *et al.*, 1968), one of which is due to exocytosis.

Which materials are contained in the exocytotic vesicles in the median eminence is a question of considerable interest. By analogy with the adrenal medulla and the posterior pituitary gland a protein component seems likely. As active principles several RF, IF, neurohypophysial hormones, dopamine, noradrenaline, serotonin and small amounts of acetylcholine have been demonstrated in the median eminence (Carlsson *et al.*, 1962; Fuxe, 1964; Guillemin *et al.*, 1965; McCann and Dhariwal, 1966; Szentágothai *et al.*, 1968; Iwata and Ishii, 1969; Clementi *et al.*, 1970, 1971). Data on the correlation of these substances with special vesicles are far from conclusive. There is some evidence (Pellegrino de Iraldi and Etcheverry, 1967; Matsui, 1967; Kobayashi *et al.*, 1968; Hökfelt, 1968; Oehmke *et al.*, 1969; Doerr-Schott and Follenius, 1970; Rinne, 1970; Ishii, 1970; Grofova and Rinvik, 1971) that after conventional fixation procedures fibres containing catecholamines are characterized by small AV of about 50 nm and GV of about 100 nm or smaller. Serotonin is, at least in the frog median eminence, predominantly localized in fibres with GV of 60–100 nm (Nakai, 1971). Studies with density gradient centrifugation suggest that RF are contained in GV (Kobayashi and Ishii, 1969; Mulder *et al.*, 1970; Clementi *et al.*, 1971). According to Ishii (1972) gonadotrophin releasing factors are contained in GV with modal diameters of 110 and 120 nm.

Considering these data and the size range of the median diameters of the GV in the median eminence fibres showing exocytotic profiles, we hypothesize that both biogenic amines and RF can be released by exocytosis. The much larger size of the neurosecretory granules and their nearly complete limitation to the internal zone of the median eminence and to the posterior pituitary gland makes oxytocin and vasopressin less likely as the content of the exocytotic vesicles. Acetylcholine is also ruled out since it is localized in terminals with almost exclusively small AV (see Livingston, 1971).

If it could be demonstrated that different stimuli, each releasing a specific releasing factor of neurohumor, induce increased numbers of exocytotic profiles and coated vesicles in fibres with different ultrastructural contents, this would provide a method for identifying fibres containing different RF, IF and/or biogenic amines by way of exocytosis. Experiments to test this are in progress.

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Z. Zellforsch. 136, 111—120 (1973)

Quantitative Data on the Fine Structural Organization of the Palisade Zone of the Median Eminence

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Summary. In order to characterize different sub-zones in the palisade zone of the rat median eminence, quantitative ultrastructural parameters were applied to brains fixed in aldehyde-osmium tetroxide. The palisade zone has been subdivided in 4 successive sub-zones. Increasing numbers of granular vesicles (GV), especially those smaller than 110 nm, are observed from dorsal to ventral sub-zones. There also are more GV per unit area of nervous tissue in the perivascular than in the more dorsal sub-zones. The individual nerve profiles exhibit a larger size in the perivascular layer than in the more dorsal areas, whereas the number and size of nerve profiles devoid of vesicles diminish from dorsal to ventral. As a consequence more GV occur in the perivascular nerve profiles. In the GV containing nerve profiles the concentration of GV is, however, constant in the different sub-zones. A fluctuating size of the preterminal and terminal parts of the nerve fibres is suggested.

Key words: Median eminence — Rat — Granular vesicles — Ultrastructure — Quantitative evaluation.

Most studies on the vesicular inclusions in the nerve fibres of the external zone of the rat median eminence are confined to the area bordering immediately upon the portal capillaries (Akmayev *et al.*, 1967; Pellegrino de Iraldi and Etcheverry, 1967; Hökfelt, 1967; Zambrano and de Robertis, 1968; Zambrano, 1969; Rinne, 1970, 1972; Daikoku *et al.*, 1971). This area is characterized by the occurrence of nerve terminals and non-nervous processes abutting on the basement membranes of the hypothalamo-hypophysial portal capillaries.

In some studies the vesicular inclusions in the perivascular layer have been compared with those in a more dorsal area in the external zone of the median eminence (Rinne, 1966; Matsui, 1966; Kobayashi *et al.*, 1967, 1968; Monroe *et al.*, 1972). Most of the authors have noted a difference in size of the granular vesicles (GV) between these two areas. The implication of this difference, however, is not clear.

Because of the possible physiological significance of GV in the storage of releasing factors, inhibiting factors and biogenic amines, a systematic study has been made of the distribution of GV in successive sub-zones of the external zone of the median eminence.

Materials and Methods

After rapid decapitation the brains of 3 male adult rats (R-Amsterdam strain) were fixed by immersion. Two additional brains were fixed by aldehyde perfusion (see Discussion).

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As primary fixative we used a solution of paraformaldehyde, glutaraldehyde and 2, 4, 6, trinitrophenol, with an osmolality of 880 mOsM. The material was kept in this solution for 4 hr. The material was post-fixed in osmium tetroxide and treated with uranyl acetate. For details, see Stoeckart *et al.* (1972).

Sections of 1–2 μm were stained with methylene blue for selection of the ultrathin sections. To determine the rostro-caudal level of the sections the decrease in thickness of the median eminence rostro-caudally was used. The part of the median eminence showing a third ventricle-portal capillary distance of 140–160 μm was selected. Here, the distance between third ventricle and pial membrane is 190–200 μm. Ultrathin sagittal sections of silver interference colour were made of the median eminence of rats 1 and 2. Coronal sections were made in rat 3. They were collected on bare grids.

In the external zone of the median eminence, areas with portal capillaries exclusively at the ventral side were selected. On the dorsal side of these capillaries a series of electron micrographs was taken over a distance of at least 42 μm. The areas studied had to be free of glial cell bodies and capillary loops (Fig. 1). This area of the external zone of the median eminence is referred to as palisade zone and thus excludes the part of the external zone situated between, and ventral to, the portal capillaries (zone granulosa in the terminology of Martinez, 1960). No special attention was paid to the distance of the areas studied to the mid-sagittal plane.

In the montages of the electron micrographs (magnification 11500×) GV were counted in areas of 508 μm². Such areas were sub-divided in 4 sub-zones, from dorsal to ventral D, C, B and A, respectively (Fig. 1). Each sub-zone had a dorsal-ventral width of 10.4 μm. The area in these sub-zones is occupied by: 1. nerve profiles with neither GV nor agranular vesicles (AV); these profiles are predominantly axons, 2. nerve profiles with one or more GV (with and without AV), 3. nerve profiles with exclusively AV, 4. ependymal, hypendymal and glial processes. The area occupied by these 4 elements, present in the same areas in which GV were counted, was estimated by a method of differential point counting (Weibel, 1969). As the mean extracellular space amounted to less than 4% of the total area, this constituent has been neglected.

Observations

In the following description the terms increase and decrease refer to changes which occur in observed values going from the dorsal sub-zone D in the direction of the perivascular sub-zone A (Fig. 1).

The Nervous Elements of the Palisade Zone (Table 1)

1. Nerve Profiles. The relative area occupied by nervous tissue in the various sub-zones does not show a definite trend. However, the size (*i.e.* the mean area occupied by the individual profiles) of the nerve profiles increases. As might be expected, this increase is paralleled by a decrease in the number of nerve profiles.

Further quantitative changes become apparent when the vesicular content of the nerve profiles is considered:

2a) Nerve Profiles without GV and AV. The relative area occupied by nerve profiles devoid of GV as well as AV shows a striking decrease, due to both the smaller size and the smaller number of these profiles in the more perivascular layers. Between individual rats the absolute number of these profiles—the majority of which could be classified as axons—varies, however, markedly.

2b) Nerve Profiles with GV (irrespective of the occurrence of AV). The size of the nerve profiles characterized by the presence of GV is evidently larger in the perivascular sub-zone than in the more dorsal sub-zones. The area occupied by these profiles shows a tendency to increase.

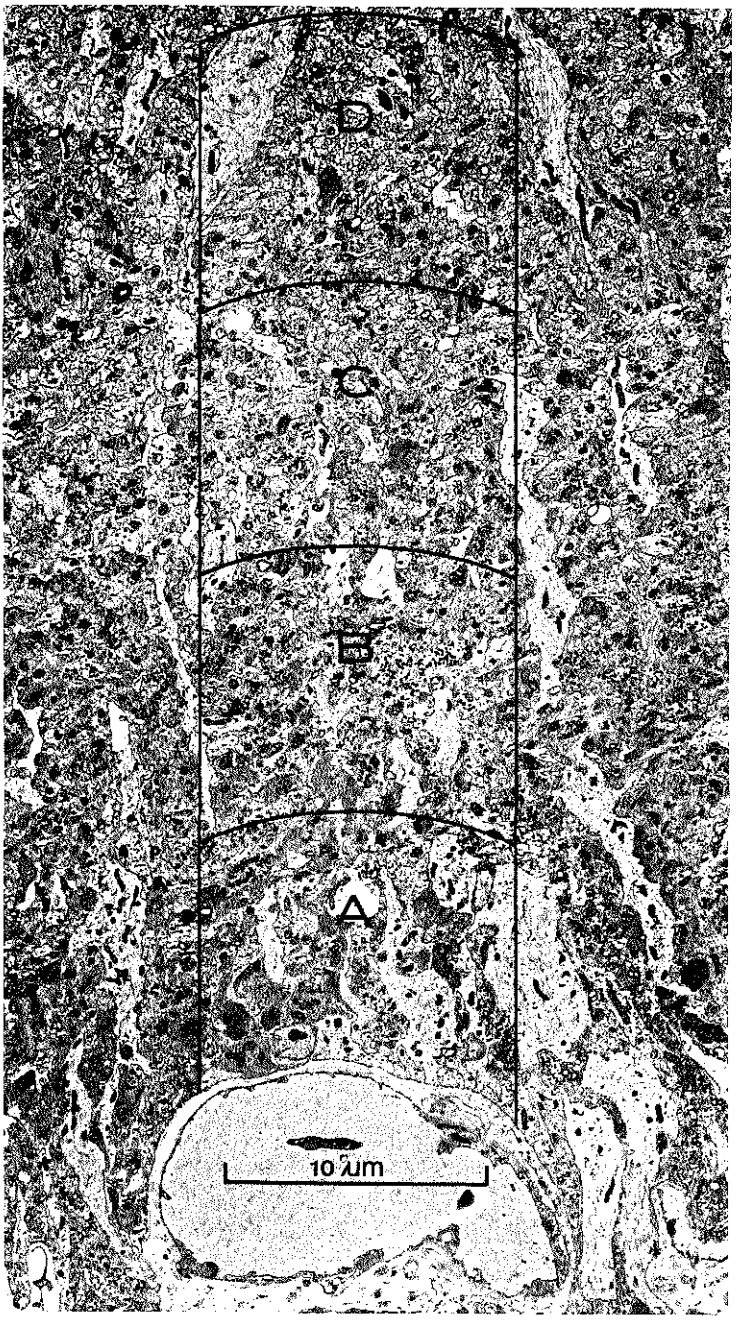

Fig. 1. Montage of low power electron micrographs of the palisade zone of the median eminence to demonstrate the localization of the sub-zones *D*, *C*, *B* and *A*. The most ventral part of sub-zone A is bordering the outer basement membrane of a portal capillary, in which a platelet is noted. Coronal section, Rat 3. ×3500

Table 1. The nervous elements in successive sub-zones of the palisade zone of the median eminence

Sub-zone	Nerve profiles			Nerve profiles without GV and AV		
	Relative area [a]	Mean area[b]	Mean number[c]	Relative area	Mean area	Mean number
D	65.7[aa]±0.8[bb]	0.56±0.04	135.8±15.9	34.7± 7.6	0.43±0.01	101.3±23.3
C	68.8 ±4.8	0.68±0.12	138.7±24.4	24.3±10.5	0.31±0.04	92.3±30.6
B	64.5 ±4.7	0.84±0.13	105.9±21.7	13.0± 6.8	0.28±0.04	56.1±25.6
A	60.0 ±4.3	1.35±0.24	63.1±13.1	3.5± 2.3	0.17±0.02	24.1±13.0
	Total number counted: 3991			Total number counted: 2464		

Sub-zone	Nerve profiles with GV			Nerve profiles with only AV		
	Relative area	Mean area	Mean number	Relative area	Mean area	Mean number
D	24.8± 7.1	1.13±0.13	28.2±6.6	5.8±1.6	1.21±0.22	6.3±1.4
C	41.1±12.5	1.23±0.15	40.7±6.7	5.6±1.2	1.37±0.27	5.7±2.2
B	45.1± 6.1	1.40±0.08	43.5±4.1	6.3±1.9	1.41±0.54	6.3±1.4
A	47.2± 6.2	2.02±0.09	30.7±3.8	9.3±2.2	1.37±0.12	8.3±1.0
	Total number counted: 1287			Total number counted: 240		

[a] % of the total surface area of a sub-zone occupied by nerve profiles.
[b] mean area occupied by individual nerve profiles in μm^2.
[c] mean number of nerve profiles per 127 μm^2.
[aa] mean for 3 rats; the values of the individual rats are the means of 3 different areas of 127 μm^2 each.
[bb] SEM.

2c) Nerve Profiles with AV only. Nerve profiles with exclusively AV occur throughout the palisade zone, but occupy even in sub-zone A only a minor proportion of the total area. In sub-zone A they constitute 10–15% of the total number of nerve profiles, in the dorsal sub-zones less than 5%.

The Granular Vesicles of the Palisade Zone (Table 2)

The tendency towards an increase of the area occupied by nerve profiles which contain GV (Table 1) is reflected by an increase in number of GV per surface area (Table 2). Since the relative area of nervous tissue is constant, as already mentioned, the number of GV per area nervous tissue increases too. As the size of the nerve profiles increases, a marked increase in number of GV per nerve profile can be expected in the perivascular sub-zone. Indeed, a fivefold increase is noted (Table 2). In the GV containing profiles, especially from sub-zone B to A, an increase occurs in number of GV (Table 2). This can only be due to the increase in size of the GV containing profiles (Table 1), since the number of GV per unit area GV-profile remains unchanged (Table 2).

Table 2. Numbers of GV in successive sub-zones of the palisade zone of the median eminence

Sub-zone	Rat	Mean number of GV per 1 μm^2 of			Mean number of GV per	
		Total surface area	Nervous tissue	GV containing tissue	Nerve profile	GV containing profile
D	1	1.8[a]±0.4[b]	4.5±1.7	5.2±1.0	2.1±0.4	6.9±1.8
	2	1.9 ±0.2	2.9±0.2	6.8±0.4	1.9±0.3	7.0±0.3
	3	1.3 ±0.2	1.8±0.2	10.5±0.7	0.9±0.1	10.1±1.6
		1.6 ±0.3	3.0±0.8	7.5±1.6	1.6±0.4	8.0±1.1
C	1	3.3 ±0.4	4.7±0.7	5.7±0.8	4.4±1.0	8.8±2.3
	2	2.8 ±0.3	4.7±0.4	9.0±0.6	2.9±0.1	10.6±1.5
	3	2.3 ±0.9	3.1±0.2	9.4±1.7	1.5±0.1	8.8±0.5
		2.8 ±0.3	4.2±0.5	8.0±1.2	2.9±0.8	9.4±0.6
B	1	3.8 ±0.1	5.9±0.6	7.0±0.8	6.3±0.5	9.9±1.2
	2	3.4 ±0.8	6.0±1.0	7.4±0.6	4.8±1.0	9.6±1.4
	3	3.6 ±0.4	5.0±0.5	10.3±1.4	3.2±0.6	13.3±2.2
		3.6 ±0.1	5.6±0.3	8.3±1.0	4.8±0.9	10.9±1.2
A	1	3.6 ±0.2	5.3±0.2	6.1±0.2	9.1±1.7	13.0±1.9
	2	3.5 ±0.7	6.8±1.1	9.7±0.7	9.8±1.8	20.3±4.9
	3	4.1 ±0.4	7.0±0.6	9.2±0.9	6.4±1.2	16.9±1.9
		3.7 ±0.2	6.4±0.5	8.3±1.1	8.4±1.1	16.7±2.1

[a] mean for 3 different areas of 127 μm^2 each
[b] SEM

Total number of GV counted in rat 1, 2 and 3: 4778, 4377 and 4226, respectively.

The Size of the Granular Vesicles (Fig. 2)

If 110 nm is taken as boundary between small and large GV, the increase in number of GV per surface area (Table 2) holds especially for the small GV. The ratio small GV/large GV (data ± SEM from 3 rats) thus increases from sub-zone D to A: 1.2±0.1, 1.7±0.3, 2.1±0.4, 5.9±0.5, respectively. When individual GV containing profiles are roughly divided into small-GV-profiles and large-GV-profiles, on the basis of the mean size of their GV, the ratio profiles with small GV/profiles with large GV also shows an increase: 1.8±0.4, 1.7±0.4, 2.6±0.6, 3.6±0.9, respectively.

Discussion

The following aspects of our observations will be discussed:

I. The number of GV in different sub-zones in comparison to other investigations.

II. The increased number of small GV in the perivascular sub-zones.

III. The possibility of a variation in the size of the nerve terminals.

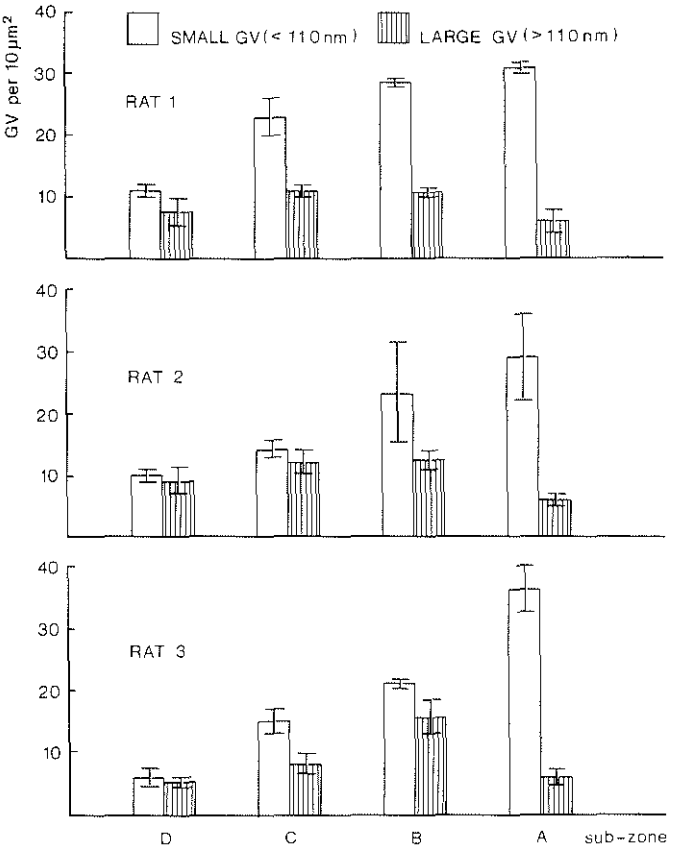

Fig. 2. Mean number of small (< 110 nm) and large (> 110 nm) GV in successive sub-zones of the palisade zone of the median eminence. Values represent mean numbers (± SEM) for 3 areas of 127 μm^2 each. Total number of GV counted: 13381

I. Absolute numbers of GV are not meaningful, because they depend on factors such as fixation, section thickness, localization of the areas studied and criteria according to which para-median sections of GV are included in the sample. Thus, the number of GV in the rat perivascular layer, reported by different authors (Pellegrino de Iraldi and Etcheverry, 1967; Kobayashi *et al.*, 1967; Zambrano and de Robertis, 1968; Rinne, 1970) varies between 18 and 80 GV/10 μm^2. When, however, our data on GV in different sub-zones are compared with the data of Monroe *et al.* (1972) a remarkable difference is noted. They found 54 GV/10 μm^2 in the direct perivascular zone, but 122 GV at a distance of approximately 10–20 μm from the portal capillaries. In roughly comparable sub-zones in our material (A and B) 37 and 36 GV/10 μm^2, respectively, are found (Table 2). To exclude the possibility of important differences in number of GV between dorsal and ventral parts of our sub-zone A we further sub-divided A

in a dorsal and ventral zone. This yielded, however, about equal numbers of GV. Thus, in contrast to our data, Monroe *et al.* (1972) found a relatively small number of GV in the direct perivascular layer of the adult female rat.

The data of Monroe *et al.* (1972) on anaesthetic, fixation and localization of the areas are not completely clear, but our additional data on perfusion fixed, anaesthetized rats do not show more GV in the dorsal sub-zones: Rat 4, adult female, diestrous, Avertin anaesthesia i.p., mean number (± SEM) of 4 montages from sub-zone D to A resp. 14.9 ± 1.9, 18.3 ± 2.1, 29.8 ± 7.0, 43.0 ± 4.9; Rat 5, adult male, ether anaesthesia, mean number of 3 montages resp. 16.5 ± 2.6, 22.6 ± 8.7, 21.2 ± 3.4, 18.1 ± 1.1. The areas used were of the same size as mentioned in Materials and Methods, but the localization of these areas extended over a rostro-caudal distance of about 1000 μm.

Our data are more in agreement with those of Kobayashi *et al.* (1967) and Matsui (1966). The former authors found 18.6 GV/10 μm^2 in the palisade zone and 18.1 in the perivascular zone. The distance between their zones is, however, not mentioned and the total area involved is small. The same holds for the data of Matsui. He found about 15% more GV in the perivascular layer than in the palisade zone.

II. Obviously, nerve fibres and terminals in the perivascular 10 μm wide sub-zone at the dorsal side of the portal capillaries contain relatively small[1] GV (Fig. 2). This suggests that either GV diminish in size in the distal part of the fibre or that a special type of nerve fibre, characterized by relatively small GV, is preferentially localized in this area (or both). The first possibility is suggested by Rinne (1966) and Monroe *et al.* (1972), the second by Kobayashi *et al.* (1968). The second view, that of a preponderance of special fibres with small GV occurs in the perivascular layer, seems, at least partly, correct on the basis of the following: 1. A considerable part of the terminals in the perivascular zone of the median eminence seems to originate from the arcuate nucleus (Lichtensteiger and Langemann, 1966; Fuxe and Hökfelt, 1966; Sano *et al.*, 1967; Szentágothai *et al.*, 1972; Björklund *et al.*, 1970; Réthelyi and Halász, 1970; Raisman, 1972), 2. In our material the GV in the neuronal perikarya of the arcuate nucleus are of a small diameter with a mean size of about 90 nm (Klootwijk, unpublished). If one accepts these findings, the opposite view, a diminution of size of the GV in the distal part of the fibre, does not seem necessary. Moreover, if exocytosis, demonstrated in the external zone of the median eminence (Stoeckart *et al.*, 1972), is the sole release mechanism in this area, there is no compelling reason for a decrease in the size of the GV in the terminal part of the nerve fibre.

According to our observations, the majority of the axons in the palisade zone do not proceed from dorsal to ventral areas in a coronal plane, but more in a rostro-caudal direction. In this respect, the name palisade zone is a misleading one, because this palisade appearance is caused by non-nervous processes. Only a limited number of terminals, therefore, belongs to axons and preterminal parts observed immediately dorsally. Thus, observations on the distribution of GV refer to axons and terminals in general and not to axons with their own terminals. Serial sections will be necessary to establish if changes in the size of the GV occur along the axon.

1 In different reports the terms small and large GV designate entirely different categories. The term small GV, as used in this article, refers to GV of 60–110 nm.

No direct evidence on the content of the GV is provided by our observations. However, micrographs published in several articles (Sano *et al.*, 1967; Smith and Simpson, 1970; Hökfelt and Fuxe, 1972) demonstrate an increase in fluorescent material from dorsal to ventral in the palisade zone. This increase is in agreement with the increase in numbers of small GV and the relative increase in numbers of profiles with small GV, noted here. Therefore, fibres characterized by small GV are likely to contain catecholamines (Kobayashi *et al.*, 1968; Hökfelt 1968; Fuxe and Hökfelt, 1969; Barry, 1970; Doerr-Schott and Follenius, 1970).

The diameters of the GV in the different sub-zones of the external zone of the median eminence and the localization of the terminals with relatively large GV are now being studied.

III. Evidently, the nerve fibres enlarge into preterminal and terminal swellings especially in the sub-zone closest to the portal capillaries (Table 1). Since profiles devoid of GV decrease in size from the dorsal to the more perivascular subzones and the size of the exclusively AV containing profiles remains unchanged, the swellings are characterized by the presence of GV. In these GV containing profiles increased numbers of GV are found in the more perivascular sub-zones (Table 2). However, the concentration of the GV in these profiles (*i.e.* the number of GV per unit area of GV containing tissue) remains unchanged. It seems, therefore, that an increased supply of GV does not result in a larger concentration of GV, but in a larger terminal. This suggests that the size of the preterminal and terminal parts of the nerve fibres may fluctuate under normal physiological circumstances. In that case, the size will depend on the synthesis of GV in the perikaryon, the transport velocity in the axon and, possibly, on local synthesis or refilling of vesicles in the terminal and also on release and/or degradation of the GV. It cannot be excluded that exocytosis and micro-pinocytosis (Douglas *et al.*, 1971; Stoeckart *et al.*, 1972) play a role in this process.

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Sizes of Granular Vesicles in the Rat Median Eminence, with Special Reference to the Zona Granulosa

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Summary. A quantitative evaluation has been made of the sizes of neuronal granular vesicles (GV) in different (sub-)zones of the rat median eminence. On the basis of the frequency distribution of the mean sizes of the GV in the individual nerve profiles, at least 4 tentative categories of nerve profiles may be distinguished. However, available data do not permit characterization of nerve fibre categories in terms of neurohormone and/or neurotransmitter storage.

From dorsal to ventral the palisade zone shows a decrease in mean size of GV; furthermore, the relative number of nerve profiles characterized by small GV (mean size smaller than 112 nm) increases. In contrast, the GV in the zona granulosa, that part of the external zone of the median eminence ventral to the palisade zone, are of relatively large size.

Qualitative observations indicate that the zona granulosa may be regarded as an area for both storage and release of biologically active substances.

Key words: Median eminence — (Rat) — Granular vesicles — Ultrastructure — Quantitative evaluation.

Granular vesicles (GV) are thought to contain specific biologically active substances. Releasing factors, inhibiting factors and biogenic amines have been proposed as active substances in the GV of the non-myelinated nerve profiles of the tuberoinfundibular system (review by Kobayashi *et al.*, 1970). In addition, a correlation between size and content of GV has been suggested (Ishii, 1970; Kobayashi *et al.*, 1970; Rinne, 1970; Rodríguez, 1971; Ishii, 1972).

In a previous article (Stoeckart *et al.*, 1973) attention was drawn to differences in the fine structural organization of successive sub-zones of the palisade zone of the median eminence where a progressive decrease in size of GV from dorsal to ventral was noted. As the data were obtained from observations at a magnification too low to measure precisely the sizes of the GV, measurements were made at a higher magnification. In addition to the palisade zone, we studied areas at the dorsal and ventral side of this zone: the zona interna and the zona granulosa. The zona interna is traversed by the neurosecretory axons which arise from the cell bodies of the supraoptic and paraventricular nuclei and terminate in the posterior pituitary gland, while the nature of the zona granulosa is unclear.

Materials and Methods

For general observations brains of adult male and female rats (R-Amsterdam strain) were fixed by intravascular perfusion (Figs. 1, 2, 3). Animals were perfused under Avertin

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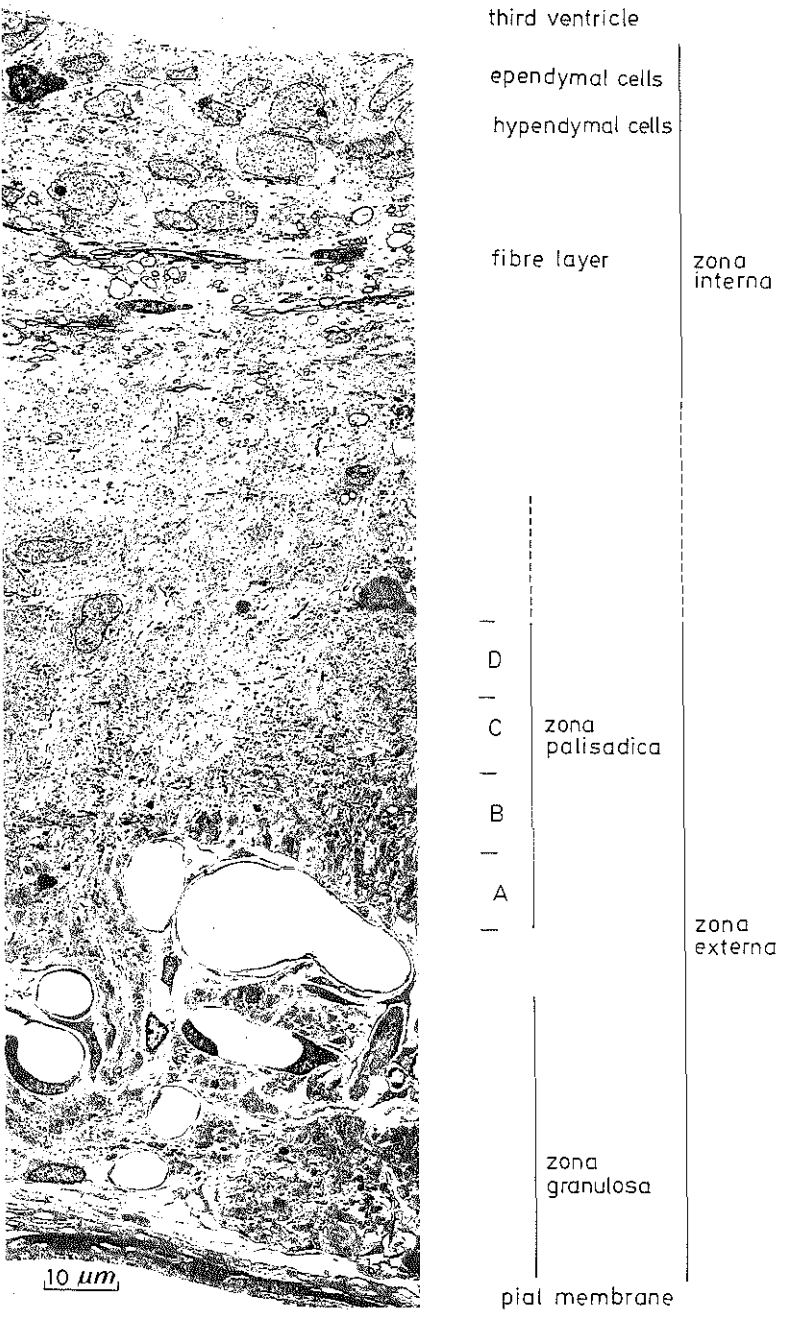

Fig. 1. Montage of low power electron micrographs derived from the rostral part of the median eminence, to demonstrate the different (sub-)zones. Coronal section. Perfusion fixation; osmium tetroxide; uranyl acetate block staining. In the terminology of Kobayashi *et al.* (1970) a zona reticularis is located between the fibre layer and the palisade zone. ×1100

anaesthesia with a mixture of 1% formaldehyde and 2% glutaraldehyde in cacodylate buffer, to which polyvinyl pyrrolidon was added. For further details, see Stoeckart *et al.* (1972). In cases where post-fixation with osmium tetroxide was omitted (Fig. 3), the material was treated with ethanolic phosphotungstic acid (Bloom and Aghajanian, 1968). For the quantitative study, three immersion fixed brains, used in previous work (Stoeckart *et al.*, 1973), were investigated in greater detail. The area of the median eminence studied was situated 0.35–0.85 mm rostral to the infundibular stem and as close to the midline as possible. In sagittal sections of silver interference colour we compared the zone interna, the palisade zone and the zona granulosa. The palisade zone was arbitrarily sub-divided into 4 sub-zones (from ventral to dorsal A, B, C and D) with a dorso-ventral width of 10.4 μm each (Fig. 1). The zona granulosa (Hagen, 1955; Martinez, 1960) is part of the external zone of the median eminence and will be referred to as zona gr. In each rat 7 electron micrographs of 31 μm^2 (magnification 35000×) were used, taken from the 6 mentioned (sub-)zones. Areas characterized by large non-nervous processes were not used. With the aid of a particle size analyzer TGZ 3 (Zeiss) the sizes of GV were measured (interval scale 5.25 nm). Only GV characterized by clearcut membranes were included in the sample. As a consequence, about 40% of the, mainly tangentially sectioned, GV were excluded.

In an attempt to characterize nerve profiles by the size of their GV, we determined the mean size of the GV in the individual nerve profiles. Because of the size variation of the GV within one profile, we excluded nerve profiles containing less than 5 GV.

For significance of difference between the means of two size distributions Student's t-test was used.

Observations

Sizes of GV in the Median Eminence

The following lists the determinations carried out and their rationale:

I. the mean sizes of GV in different (sub-)zones, in order to determine which overall changes in size of GV occur from dorsal to ventral in the median eminence.

II. the frequency distribution of sizes of GV in different (sub-)zones, in order to determine if GV of a special size occur preferentially in special (sub-)zones.

III. the frequency distribution of sizes of all GV measured, in order to determine if one or more peak values occur.

IV. the frequency distribution of mean sizes of GV in individual nerve profiles (containing 5 GV at least), in order to establish whether categories of nerve profiles exist, as far as mean size of GV is concerned.

V. the relative numbers of nerve profiles, grouped into categories on the basis of IV., in the different (sub-)zones, in order to compare the (sub-)zones on the basis of the mean sizes of the GV in the individual nerve profiles.

VI. the sizes of the GV occurring in nerve profiles with at least 5 GV, compared to those in profiles with less GV, in order to establish if the GV of the nerve profiles selected for IV. and V. are representative for all GV.

I. Mean Sizes of GV in Different (Sub-)Zones (Table 1)

In the external zone, a decrease in the mean sizes of the GV was observed from the dorsal sub-zone D to the perivascular sub-zone A. However, GV in the most ventral area of the external zone, the zona gr., did not follow this trend. On the contrary, in rat 1 and 2 the mean sizes of the GV in the zona gr. appeared to be significantly larger ($P<0.001$) than those in sub-zone A. The mean sizes of the GV in the internal zone were, however, much larger than those in the zona gr.

II. Frequency Distribution of Sizes of GV in Different (Sub-)Zones (Fig. 4)

For Fig. 4 the data of the individual rats were combined for each of the (sub-) zones. Marked peak values within a (sub-)zone were lacking. The mean size of the

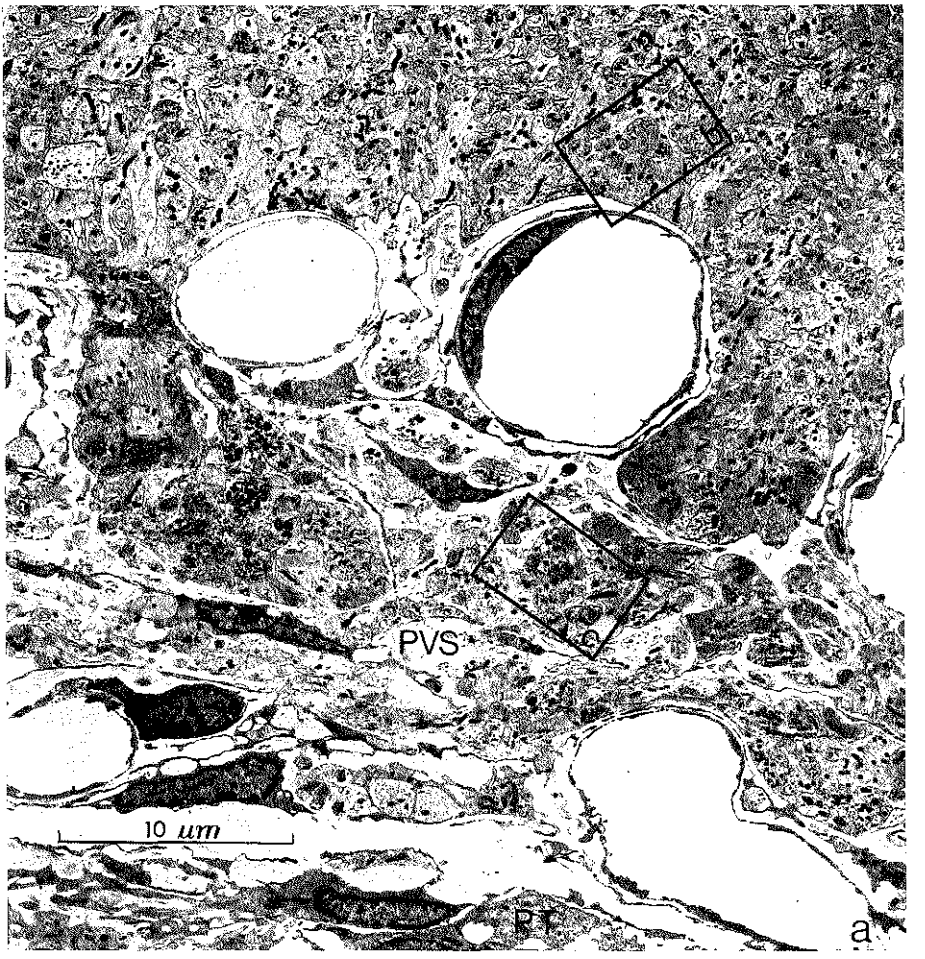

Fig. 2a

Fig. 2a. Ventral part of the external zone of the median eminence. Coronal section. Capillaries are empty as a consequence of a perfusion fixation; osmium tetroxide; uranyl acetate block staining. ×3300. b detail of sub-zone A ×20000. c detail of zona granulosa ×20000. *AV* agranular vesicles; *GV* granular vesicles; *L* lipid droplet in non-nervous process near to synaptoid contact; *P* non-nervous process; *PT* dorsal part of pars tuberalis; *PVS* (extension of) perivascular space; arrows outer basement membrane; *omega-shaped profile suggestive of exocytosis

GV decreased from 117.6 nm in sub-zone D to 98.4 nm in sub-zone A, while the mean size of the GV in the zona gr. appeared again to be relatively large (110.3 nm).

The large size range of the GV in the internal zone will be caused by the occurrence of markedly different types of GV, the largest of which (the "classical" neurosecretory granules) apparently range from about 140 nm to 260 nm.

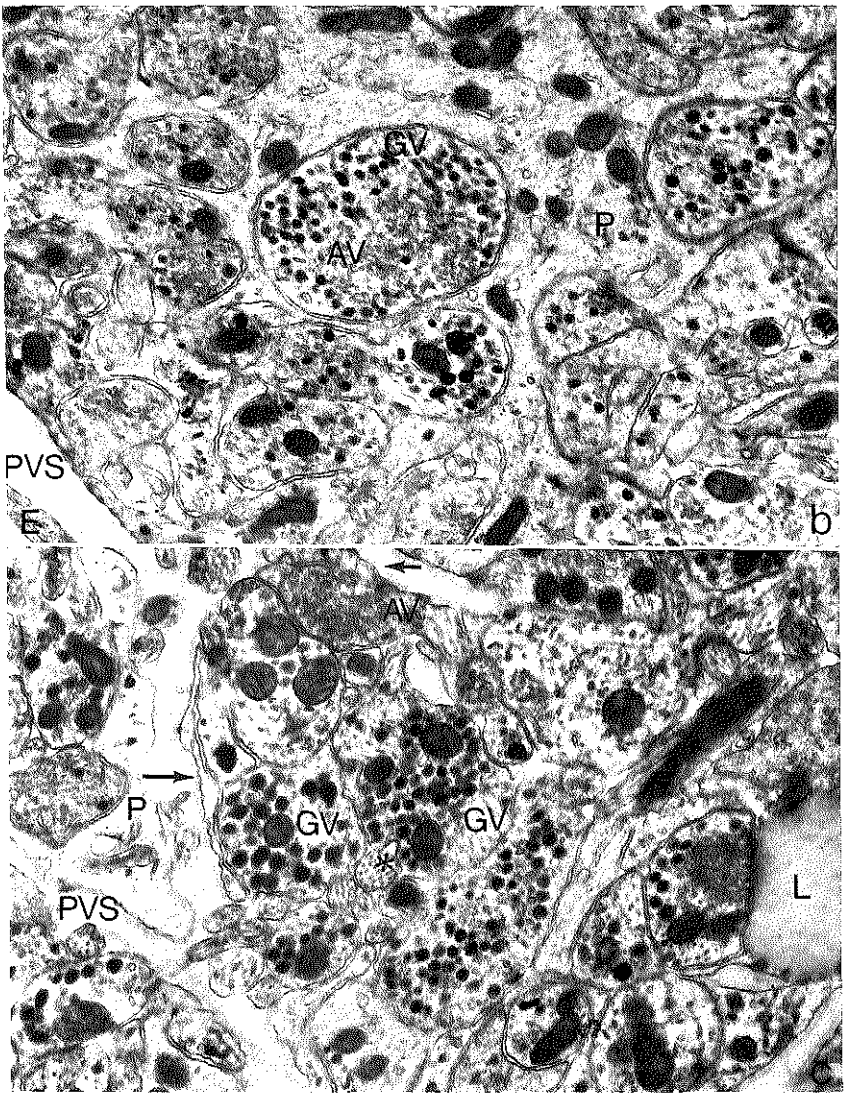

Fig. 2b and c

III. Frequency Distribution of Sizes of all GV Measured (Fig. 5, open Area)

The main peak in frequency of GV occurred at 90–116 nm, with indications of minor ones at 59–64 and 122–127 nm. It should be noted that the neurosecretory granules which appeared to occur almost exclusively in the internal zone (Fig. 4) do not reach a peak frequency. This is due to a predominance of electron micrographs derived from the external zone. In the series of electron micrographs of the external zone the various sub-zones are equally represented.

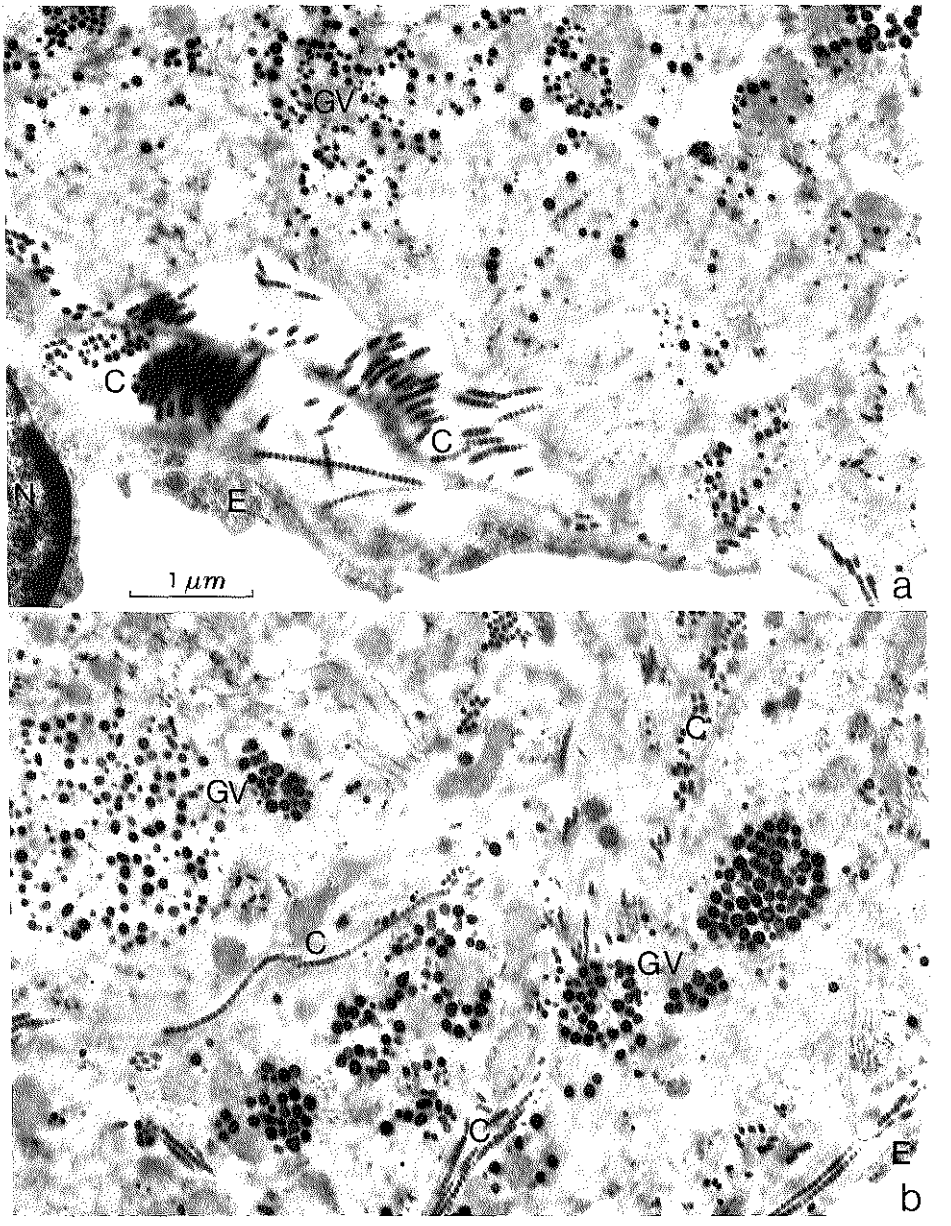

Fig. 3a and b. Ventral part of the external zone of the median eminence. Coronal section. As a consequence of the procedure (ethanolic phosphotungstic acid after perfusion fixation) the cores of the GV are electron dense. Protein containing components like collagen fibrils are also markedly electron dense. a detail of sub-zone A ×18000. b detail of zona granulosa ×18000. *E* endothelium; *C* collagen fibrils in perivascular space; *GV* cores of granular vesicles; *N* endothelial nucleus

Table 1. Mean sizes of GV (nm) in different (sub-)zones of the median eminence

	Rat 1			Rat 2			Rat 3		
(sub-)zone	size[1]	SEM[2]	n[3]	size	SEM	n	size	SEM	n
interna	168.7	1.9	424	161.4	2.0	195	169.8	3.1	120
D	105.9	0.8	477	124.7	1.2	371	125.0	1.4	348
C	106.6	1.0	395	121.4	1.2	473	110.3	1.0	374
B	101.7	0.8	570	109.9	1.3	356	108.9	0.9	431
A	91.0	0.6	563	102.3	0.9	559	102.2	0.9	498
granulosa	111.7	0.7	741	116.4	1.1	372	103.5	0.9	486

[1] mean for GV in 7 areas of 31 μm^2 each
[2] standard error of mean (SEM)
[3] number of GV

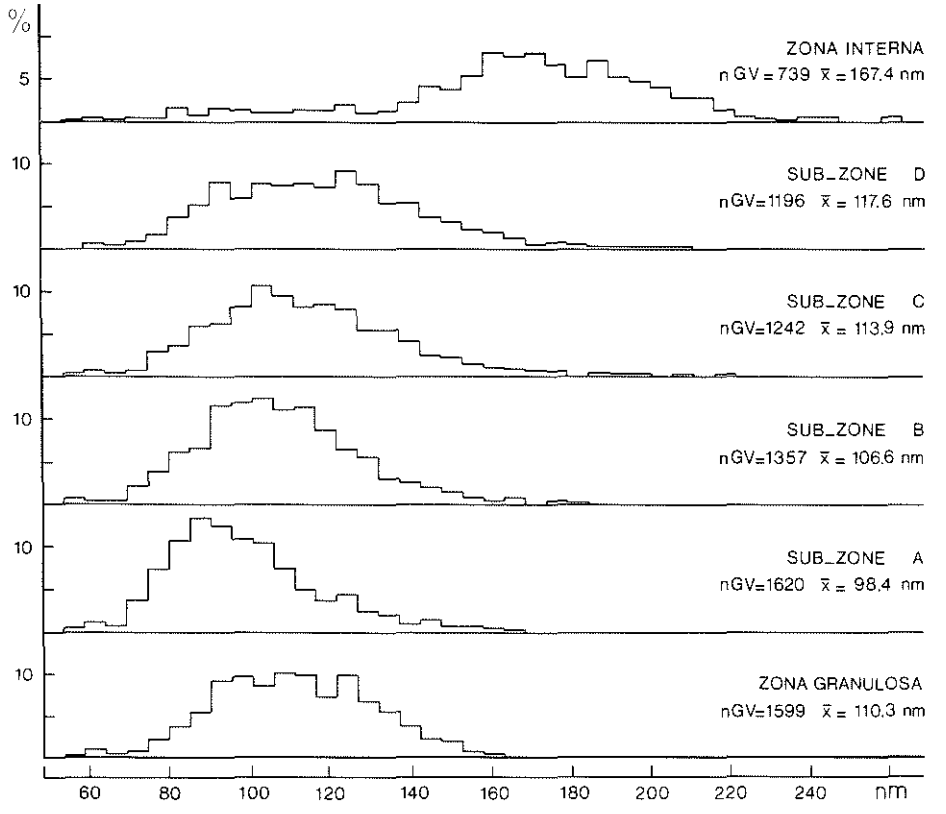

Fig. 4. Frequency distribution of sizes of GV in different (sub-)zones of the median eminence. Data are derived from 3 rats; 7 areas of 31 μm^2/(sub-)zone/rat

Fig. 5. Frequency distribution of sizes of GV (open area) in all nerve profiles and of means of sizes of GV (hatched area) in nerve profiles with 5 GV or more. Data for GV are derived from 7753 GV, occurring in 1827 GV containing nerve profiles, located in the same 126 areas as used for fig. 4. Of these nerve profiles 480 contained 5 GV or more. Vertical interrupted lines indicate boundaries of tentative categories

IV. Frequency Distribution of Mean Sizes of GV in Individual Nerve Profiles (Fig. 5, Hatched Area)

Mean sizes of GV in individual nerve profiles containing at least 5 GV peaked at 90–95, 101–106, 117–127 and 169–174 nm. Taking into consideration data of others, also indicating that different categories of nerve terminals exist as far as size of GV is concerned (Rodríguez, 1971; Ishii, 1972), this suggests that at least 4 categories of nerve profiles may be distinguished. The mean sizes of the GV in these *tentative* categories of nerve profiles are: smaller than 96 nm, 96–111 nm, 112–148 nm and larger than 148 nm. Nerve profiles with GV of about 60–70 nm may represent an additional category. Partly because of the relatively small number of small GV/nerve profile (see VI.), they hardly show up in the hatched frequency distribution of Fig. 5.

V. Relative Numbers of Nerve Profiles, Grouped into Categories on the Basis of IV., in the Different (Sub-)Zones (Table 2)

The proportion of nerve profiles with a mean size smaller than 96 nm increased from 6.1% in the zona interna to 50.9% in sub-zone A. More than 80% of the nerve profiles in sub-zone A were characterized by a mean size smaller than 112 nm. The predominance of profiles with, thus defined, small GV is also found in sub-zone B but not in the other (sub-)zones. Nerve profiles containing GV of a mean size larger than 148 nm hardly occurred in the ventral (sub-)zones of the external zone of the median eminence.

Table 2. Relative number[1] of nerve profiles[2], grouped into categories on the basis of the mean size of GV, in different (sub-)zones of the median eminence (total number of nerve profiles[2]/(sub-)zone = 100%)

(sub-)zone	categories <96[3]	96–111	112–148	>148	number of profiles
interna	6.1	3.0	6.1	84.8	33
D	18.0	16.7	58.3	7.0	73
C	13.9	26.6	54.5	5.0	79
B	25.3	39.8	33.7	1.2	83
A	50.9	33.0	16.1	—	106
granulosa	19.8	28.3	50.9	—	106

[1] calculated over total number of nerve profiles/(sub-)zone in 3 rats; the values of the individual rats are derived from 7 areas of 31 μm^2/(sub-)zone
[2] only nerve profiles with $\geq$ 5 GV
[3] nm

VI. Comparison between Profiles Containing 5 GV at Least and those Containing Less GV

It can be calculated that, by selection of nerve profiles with 5 GV or more, 74% of the nerve profiles were disregarded; these disregarded profiles contained 36% of the GV. However, if the data of Fig. 4 were used to group the GV in the separate (sub-)zones on the basis of the categories of IV., these relative numbers in the different (sub-)zones, obtained for all GV, appeared to agree remarkably well with the data of Table 2, which represent the selected GV only. Nevertheless, the possibility remained that the GV in the selected nerve profiles were not representative for all GV. Therefore, we compared the sizes of GV in profiles with 1–4 GV (group a) and those in profiles with more GV (group b). It turned out that GV of group a (2854 GV) were significantly smaller ($P < 0.001$) than those of group b (4899 GV): mean $\pm$ SEM, 110.3 $\pm$ 0.5 and 117.2 $\pm$ 0.4 nm, respectively.

As in all separate (sub-)zones GV in nerve profiles with 5 GV or more were relatively large, a correlation between the number of GV/profile and the size of GV holds probably for several parts of the nerve fibre (axon, varicosity and/or terminal).

As the number of GV/nerve profile is caused by two factors, the size of the nerve profile and the concentration of the GV (number of GV/unit area of GV containing axoplasm), we roughly estimated for rat 1 these two factors for the profiles of group a and b. In the profiles with 5 GV or more, the mean size of the nerve profiles was 2.6$\times$ larger than that of the nerve profiles containing less GV, while the concentration was 1.8$\times$ larger. These observations suggest that nerve profiles with 5 GV or more, 1. are relatively large, 2. contain a relatively large concentration of GV and 3. contain relatively large GV.

The Zona granulosa

Part of the nervous and non-nervous processes in the palisade zone are observed to pass by the portal capillaries which border the palisade zone and enter the zona gr. (Figs. 1, 2a). The non-nervous processes in the zona gr. are not oriented as in the palisade zone. The processes in the zona gr. are located in an extensive collagen containing perivascular space (Figs. 2a, 2c, 3b). At many places, cells of the pars tuberalis (pars infundibularis) of the anterior pituitary gland border ventrally the zona gr. The thickness of the zona gr. is maximal in the mid-central region of the median eminence and varies greatly rostro-caudally. The dorso-ventral width may amount to 40 μm but at the most rostral part of the median eminence and at the level of the hypophysial stem this zone is almost non-existent.

The nerve profiles of the zona gr. impress as relatively large and containing large numbers of GV. The GV seem, generally, to be larger than those in sub-zone A (Fig. 2; compare Fig. 4). This observed difference also holds for the core of the GV, which can be seen well after treatment with ethanolic phosphotungstic acid, by which especially protein containing components like collagen become electron dense (Fig. 3). In a small proportion of the nerve profiles images have been observed compatible with exocytotic release of the core of the GV (Fig. 2c). Agranular vesicles occur especially in nerve profiles observed to be in contact with the outer basement membrane of the perivascular space.

The quantitative data on the median eminence obtained at low magnification (Stoeckart *et al.*, 1973) were extended to the zona gr. by determining the ratio small GV/large GV (borderline at about 110 nm). This ratio was for the zona gr. of rat 1, 2 and 3: 0.7 ± 0.1, 1.4 ± 0.3 and 1.3 ± 0.3, respectively (mean $\pm$SEM; the values are the means of those obtained in 3 different areas of 127 μm^2 each; total number of GV included: 3449). These ratios are comparable to those for the dorsal sub-zones of the palisade zone, while values fo sub-zone A are much higher (Stoeckart *et al.*, 1973). Consequently, GV in the zona gr. are, generally, larger than those of the nearby sub-zone A, as shown also in Fig. 4.

Discussion

Categories of Nerve Fibres

Data on "types" of nerve fibres in specific (sub-)zones of the external zone of the median eminence, are almost completely lacking. Quantitative data are available only on the perivascular layer of the rat (Rinne, 1966) and the frog (Nakai, 1971) and on the rostral palisade zone of the pigeon (Peczely and Calas, 1970). Their data on the perivascular layer are comparable to our data for sub-zone A, with regard to the large proportion of fibres containing GV smaller than 112 nm. Their data on the palisade zone show a high proportion of fibres with GV of 120–140 nm, comparable to that of nerve profiles with GV of 112–148 nm in our sub-zone C and D (Table 2).

The data suggest that the median eminence of different vertebrate classes has more features in common than the general organization only.

For the rat median eminence, most authors recognized two "types" of nerve fibres, on the basis of size of GV (Röhlich *et al.*, 1965; Kawashima *et al.*, 1966; Kobayashi, 1966; Oota, 1966; Matsui, 1966; Monroe, 1967; Scott and Knigge, 1970; Fink and Smith, 1971; Raisman, 1973). The predominant sizes of the GV in these two "types" varied, however, markedly in the different studies: 77–120 nm, and 110–200 nm or larger, respectively. GV of the first "type" were thought to contain monoamines and, possibly, releasing factors; the second "type" was assumed to contain neurosecretory material.

In contrast, Rinne (1966), Kobayashi *et al.* (1968, 1969) and Kobayashi *et al.* (1970) concluded to 3 categories, with main values at about 80–100, 110–133 and 150–200 nm. According to them the first 2 categories may contain monoamines, releasing factors and, possibly, neurohypophysial hormones; the last category may contain neurohypophysial hormones, but other active substances could not be excluded.

On the basis of our findings on the frequency distribution of the mean sizes of GV in individual nerve profiles one could assign at least 4 tentative categories

of nerve profiles: mean size smaller than 96 nm, 96–111 nm, 112–148 nm and larger than 148 nm. The first category may represent two sub-categories, one of which contains GV of about 60–70 nm.

It should be mentioned that a reliable categorization of nerve profiles is neither possible on the basis of the limited material of this study nor on the basis of the material used in other studies on the rat median eminence. Mode values as proposed by Rodríguez (1971) can not be obtained, since the number of GV per cross-sectional nerve fibre is small (in our material 4.2 in 1800 nerve profiles containing GV). Median values, or mean values as used by us, characterize the nerve profiles only roughly and underestimate the real size of the GV, since in many cases the center of the GV will be located outside the ultrathin section.

Besides, the validity of the values obtained for mean (or median) sizes of GV in individual nerve profiles is low if all GV containing nerve profiles are used, because of the size variation within one profile and the large proportion of GV containing profiles with a small number of GV (in our material 74% of these profiles contain 1–4 GV). Therefore, we excluded nerve profiles with 4 GV or less, while Ishii (1972) selected profiles with 6 GV or more. A drawback of this kind of selection is the difference in size of GV, noted by us, between nerve profiles containing 1–4 GV and those containing more GV. Serial sections will be necessary to solve these problems.

On the basis of an extensive quantitative study on the stalk-median eminence of two horses, Ishii (1972) concluded to five groups of GV containing axons. Four of these groups are, roughly, comparable to our categories. A fifth category at about 145 nm is not recognized in our material. However, in our material GV of this size are mainly found in the internal zone of the median eminence from which area, in comparison to the external zone, only a limited number of electron micrographs has been derived. According to another quantitative study (Rodríguez, 1971), 4 types of GV containing nerve fibres occur in the median eminence of the toad.

It is of little use to speculate on the possible biological significance of the tentative categories, since our material is limited and since our categories are based on relatively small peaks in the frequency distribution of the mean sizes of the GV in individual profiles. Besides, the available data do not permit to characterize the categories in terms of neurohormone and/or neurotransmitter storage. The reasons for this are the following: 1. the sizes of the GV may change along the preterminal and terminal parts of the nerve fibre (see below), 2. according to several studies varicosities and terminals of aminergic neurones are, after aldehyde-osmium tetroxide fixation, characterized by agranular vesicles of about 50 nm and GV of 80–120 nm (Hökfelt, 1967, 1968; Rinne *et al.*, 1967; Pellegrino de Iraldi and Jaim-Etcheverry, 1967; Aghajanian and Bloom, 1967; Doerr-Schott and Follenius, 1970; Hökfelt and Fuxe, 1972) or smaller (Rinne, 1970; Nakai and Shinkawa, 1971). This size range includes the sizes of the GV in three of our tentative categories, 3. differential and gradient centrifugation of stalk-median eminence tissue indicates that releasing factors and biogenic amines are contained in vesicular material, but a correlation between size and content of vesicles remains uncertain (Kobayashi and Ishii, 1968; Kobayashi *et al.*, 1970; Mulder *et al.*, 1970; Clementi *et al.*, 1971), 4. the effects of ovariectomy (Kobayashi *et al.*, 1967, 1968; Kobayashi and Matsui, 1969) as well as of adrenalectomy (Akmayef *et al.*, 1967; Rinne, 1970, 1972; Wittkowski and Bock, 1972) on the frequency distribution of sizes of GV in the median eminence are not conclusive for a correlation of size and content of GV.

It has to be stated that also for the median eminence of the rat the functional significance of types of nerve fibres is still open to discussion. In this context one should bear in mind that if all neurohormones and neurotransmitters, hypothesized to be present in the median eminence, are synthesized by different types of neurones (which is by no means established—Clementi *et al.*, 1971; Rodríguez, 1972), it would be necessary to differentiate among as many as 16 types of fibres and their terminals. One or more of them may exclusively contain agranular vesicles, while nerve profiles containing GV of comparable sizes do not, necessarily, share other ultrastructural characteristics. Besides, neurones with the same ultrastructural characteristics do not necessarily synthesize the same biologically active substance(s).

The Zona granulosa

The external zone of the median eminence comprises, in addition to the palisade zone, the zona granulosa. Some authors, however, do not mention this zone (see Kobayashi *et al.*, 1970). Both in the rat (this article) and in several other mammalian species (Hanström, 1952; Hagen, 1955; Martinez, 1960; Wittkowski, 1967) the zona gr. is, in contrast to the palisade zone, not characterized by dorso-ventrally oriented processes.

Functional Significance. Practically all nerve profiles observed in the zona gr. will belong to nerve fibres which either deliver neurohormones to the fenestrated portal capillaries or release neurotransmitters to act on the surrounding elements. The following observations are in favour of this view:

1. the large nerve swellings, which contain large numbers of GV and/or agranular vesicles. The agranular vesicles are generally viewed upon as indicative of release phenomena (De Robertis, 1964; Streefkerk, 1967; Wendelaar Bonga, 1970; Douglas *et al.*, 1971; Stoeckart *et al.*, 1972),

2. the occurrence of exocytotic profiles,

3. the peculiar arrangement of an extensive perivascular space. The arrangement is comparable to that in other endocrine organs and is supposed to facilitate release (Palay, 1957; Barer and Lederis, 1966; Wittkowski, 1967; Rodríguez, 1969) In the palisade zone of the rat, only the areas immediately adjacent to the portal capillaries show a comparable arrangement.

In view of the close anatomical relationship between the zona gr. and the pars tuberalis and in view of the presence of terminals on pars tuberalis cells (Unsicker, 1971; Cameron and Foster, 1972), it can not be ruled out that some nerve profiles in the zona gr. belong to fibres proceeding to the pars tuberalis (Metuzal, 1959; contrast Szentágothai *et al.*, 1972). As the number of axons (defined as nerve profiles without agranular vesicles and/or GV) in the zona gr. is very small, at most a very small number of nerve fibres will proceed from the zona gr. to the pars tuberalis.

In conclusion, assuming that releasing factors, inhibiting factors and/or biogenic amines are at least partly stored in GV, the zona gr. is in all probability an area for both storage and release of biologically active substances (compare Hagen, 1955–p. 28).

Sizes of GV. The reported diminution in size of GV from dorsal to ventral in the palisade zone (Stoeckart *et al.*, 1973) does not hold for the zona gr., situated

most ventrally. This observation might be explained by the occurrence of specific fibres characterized by relatively small GV and projecting mainly to the capillaries bordering upon the palisade zone (Stoeckart *et al.*, 1973). The zona gr., then, fulfills the requirements for a termination of fibres with relatively large GV. However, preliminary quantitative data indicate that GV of different sizes are not homogeneously distributed in the zona gr.: the sizes of the GV in nerve profiles, observed to be in contact with the outer basement membrane, seem generally to be smaller than those in other nerve profiles. As a consequence, a diminution in size of GV in those parts of the nerve fibre, which are in contact with the basement membrane might be a second explanation for the decrease in size of GV from dorsal to ventral in the palisade zone. It can, however, not be excluded that specific fibres, characterized by relatively small GV, contact preferentially the outer basement membrane. In that case, they may represent neurohormone containing fibres, whereas fibres containing larger GV (may be containing neurotransmitters) do not preferentially contact the basement membrane. On the basis of data obtained in the palisade zone Monroe *et al.* (1972) assumed a diminution in size of GV to occur in the distal part of the axon. Rodríguez (1969), in contrast, reported on an increase in size of GV in terminals of the toad median eminence. For full clarification of this matter serial sections will be necessary.

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Micro-Pinocytosis and Exocytosis in Nerve Terminals in the Median Eminence of the Rat

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INTRODUCTION

The concept, put forward by Douglas and co-workers (1971), that posterior pituitary hormones and their carrier-proteins are released by exocytosis, is gaining support (Dreifuss *et al.*, 1972; Santolaya *et al.*, 1972; Matthews *et al.*, 1973). Ultrastructural evidence has been presented that the content of the granular vesicles in nerve terminals of the median eminence can also be released by exocytosis (Stoeckart *et al.*, 1972). It has been hypothesized that these granular vesicles contain releasing factors, inhibiting factors and/or neurotransmitters (Kobayashi *et al.*, 1970). As a consequence of release by exocytosis, during which the membranes of the granular vesicles fuse with the nerve membranes, membrane material has to be disposed of. According to Nagasawa *et al.* (1971), recapture of excess membrane occurs by micro-pinocytosis in the posterior pituitary. This phenomenon may result in coated vesicles which are supposed to transform into agranular "synaptic" vesicles (Nagasawa *et al.*, 1971). In this study, horseradish peroxidase (HRP), a marker substance capable of demonstrating micro-pinocytosis in a variety of cells, has been used to establish whether the agranular vesicles in the nerve terminals of the median eminence may arise by micro-pinocytosis. As the median eminence is located outside the blood–brain-barrier, HRP has been injected intravenously.

METHODS

Ten adult male rats (R-Amsterdam strain), anesthetized with Avertin, were given injections of either 50 mg HRP (Calbiochem B grade) in 0.5 ml Locke or 100 mg HRP in 1.0 ml Locke by way of the tail vein. In addition, 5 male rats castrated about 20 h before were used. In control experiments HRP was omitted. Intravascular perfusion fixation was performed 5–60 min after the administration of HRP or of the solvent, using a mixture of 1% formaldehyde and 2% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4), to which 2% polyvinylpyrrolidone was added. The tissue of the median eminence was processed for demonstration of HRP (Graham and Karnovsky, 1966) before post-fixation with osmium tetroxide. Part of the material was impregnated

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with uranyl acetate (examples in Fig. 2). The ultra-thin sections were stained with lead citrate.

OBSERVATIONS

Within 5 min after the intravenous administration of HRP, electron-dense reaction product indicative of HRP could be observed in the perivascular space (Fig. 1, at the right). Small amounts of the reaction product occurred in the intercellular spaces around the plasma membranes. A small part of the neuronal vesicles measuring 30–65 nm in diameter also appeared to be filled with electron-dense reaction product. The proportion of small vesicles characterized by the reaction product was not markedly increased if fixation occurred either 10, 20 or 60 min after administration of HRP. In the control experiments none of the small vesicles contained reaction product. In many of the nerve profiles, characterized by the small "labeled" vesicles, granular vesicles of different diameters (70–130 nm) also occurred, but these granular vesicles rarely contained reaction product. Preliminary results suggest that in the castrated rats the incorporation of HRP into small vesicles of some nerve terminals is increased (Fig. 2).

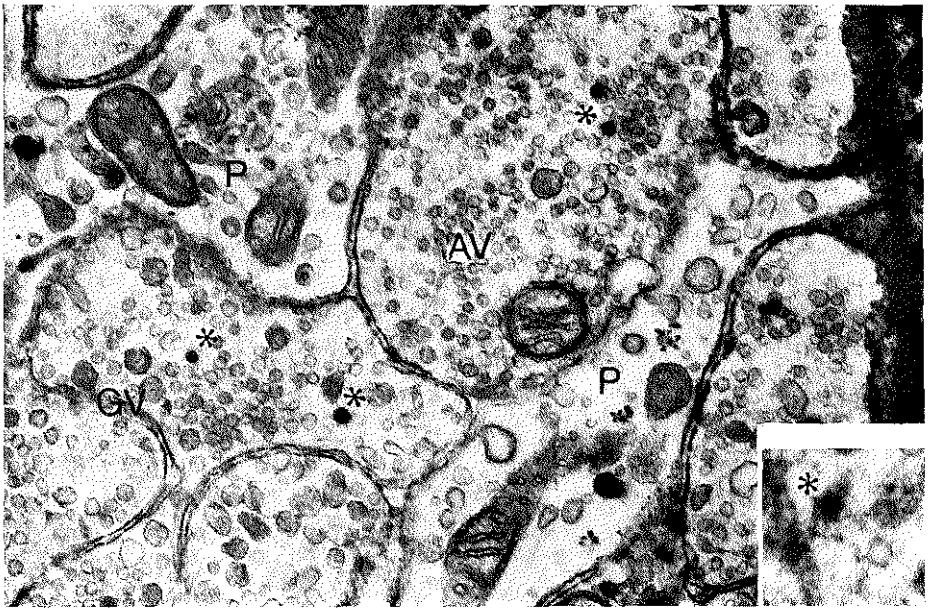

Fig. 1. Perfusion fixation 5 min after intravenous injection of 50 mg horseradish peroxidase (HRP). Nerve profiles in the external zone of the median eminence are characterized by granular vesicles (GV) of low electron-density and small agranular vesicles (AV). Some small vesicles (*) contain an electron-dense reaction product indicative of HRP. Whether the electron-dense material in the cisternae of the endoplasmic reticulum of non-nervous processes (P) represents HRP is uncertain. × 40,000. Inset: immersion fixation; coated vesicle (*) filled by HRP, close to the nerve membrane. × 70,000.

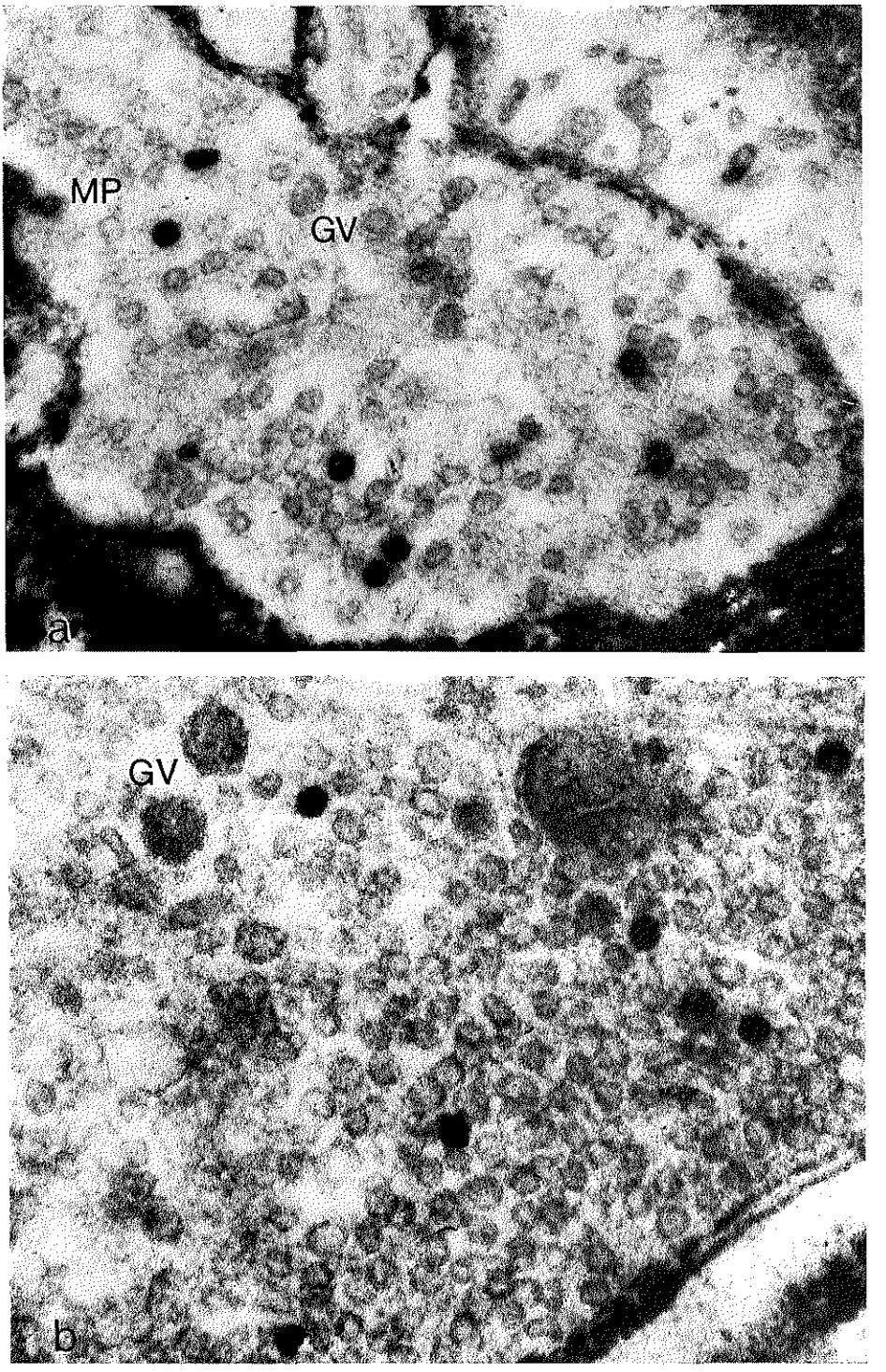

Fig. 2. Perfusion fixation 10 min after intravenous injection of 100 mg HRP; male rat, 1 day post castration. a: some vesicles of 45–55 nm contain a reaction product indicative of HRP; granular vesicles (GV) are of very low electron-density. Note micro-pinocytosis-like image (MP), containing HRP. × 75,000. b: small vesicles marked by HRP, in addition to similar but unmarked small agranular vesicles and large granular vesicles (GV). × 95,000.

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DISCUSSION

The results of the present study demonstrate that at least part of the agranular "synaptic" vesicles of the median eminence arises by micro-pinocytosis. The preliminary results on incorporation of HRP in castrated rats, *i.e.* under conditions of increased release of releasing factors, suggest that a marker substance like HRP may be used to identify functional types of nerve terminals.

Although these observations do not rule out other release mechanisms, they are compatible with the exocytotic release mechanism proposed by Douglas and coworkers (1971). Moreover, evidence has been presented that the content of neuronal granular vesicles of the median eminence can be released by exocytosis (Stoeckart *et al.*, 1972). The proportion of granular vesicles observed in the process of exocytosis is small (less than 1 %). However, we calculated that at least a million granular vesicles are involved in the process of exocytosis at the moment of fixation. These granular vesicles represent the most conspicuous organelles of the preterminal and terminal nerve profiles of the median eminence which is generally accepted to be the final common pathway of the hypothalamic component of the hypothalamo–hypophyseal system. Direct evidence for the nature of the materials stored in the granular vesicles is lacking. However, studies on differential and gradient centrifugation suggest that median eminence fractions with high activity of releasing factors and biogenic amines are electron microscopically characterized by small agranular and larger granular vesicles, sometimes still contained in synaptosomes (Clementi *et al.*, 1970; Ishii, 1970; Kobayashi *et al.*, 1970; Mulder *et al.*, 1970).

SUMMARY

Incorporation of intravenously administered horseradish peroxidase into small neuronal vesicles of the rat median eminence demonstrates that at least part of these small "synaptic" vesicles of the median eminence arises by micro-pinocytosis. Preliminary studies under conditions of increased release of releasing factors suggest that horseradish peroxidase may be used to identify functionally different types of nerve terminals. The observations are compatible with an exocytotic release mechanism for neuronal granular vesicles.

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DISCUSSION

MONROE: First of all, I would like to say that your pictures on peroxidase uptake are very convincing. However, there have been so many people lately showing that a variety of cells will take up peroxidase, that I wonder what your reasons are for feeling that this is quite specific for exocytosis instead of a rather general tendency of cells to take up peroxidase, just because it is there.

STOECKART: What I meant to say is that *if* exocytosis occurs, uptake of peroxidase has to occur. I certainly did not want to say that the uptake of peroxidase is proof of exocytosis. In fact, other release mechanisms cannot be ruled out as yet. However, even if micro-pinocytosis is not the consequence of release by exocytosis, it nevertheless reflects the stage of activity of the terminal.

GUILLEMIN: I personally know of no evidence whatsoever to prove that the releasing factors are really produced in neurons and not *e.g.* in tanycytes. I would like to know if the speaker or anybody in this audience is able to give me such evidence!

STOECKART: Although I am not aware of any final evidence, indirect evidence is derived from density gradient centrifugation experiments.

PILGRIM: There is some evidence in analogy to the classical hypothalamo–neurohypophyseal system. There is very good evidence for this system that the neurophysins and the posterior lobe hormones are localized in these granules. So I think it is fair that we assume in the first place that other peptides like releasing factors are synthesized in nerve cells and are present in such granules.

GUILLEMIN: I would like to say in public a few things, particularly for the young people. Because they are the ones who are once going to give us the answer to these important questions. These density gradient experiments are fine as far as the density gradients are concerned. However, I want to say that no chemistry, no simple technique of centrifugation, of separation of subcellular organs, or centrifugation of fractions of the extract of a tissue, are any better than the bio-assay that you finally use to prove your biological activity. Therefore, if the assay that is used is questionable, the conclusion as to the biochemical substrate is just as questionable.

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Influence of Castration on Incorporation of Exogenous Peroxidase into "Synaptic" Vesicles of the Median Eminence

I. The Basal Part of the Palisade Zone in Male Rats

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Summary. Nerve profiles in the basal part of the palisade zone of rat median eminence have been divided into 4 categories on the basis of their vesicular content: profiles *a* with agranular vesicles of about 50 nm, *b* with similar agranular vesicles and also with granular vesicles of mainly 70–140 nm, *c* with granular vesicles alone and *d* without vesicles. After intravenous injection of exogenous peroxidase, castrated male rats showed, in comparison to sham-operated controls, a 3–5 fold increase in the percentage of nerve profiles of category *a* which contained peroxidase loaded vesicles of about 50 nm. In contrast, no significant change was observed for nerve profiles of other categories. It is concluded that exogenous peroxidase may be used to demonstrate changes in the activity of tuberoinfundibular nerve terminals at the ultrastructural level.

Key words: Median eminence — Agranular and granular vesicles — Horseradish peroxidase — Micro-pinocytosis — Ultrastructure.

Involvement of the medial basal hypothalamus in the regulation of gonadotropin release is well established (Harris, 1955; Halász and Gorski, 1967; McCann and Porter, 1969; Szentágothai *et al.*, 1972; Barry *et al.*, 1973). This involvement requires that changes in secretion of gonadotropin releasing hormone (LRF) are accompanied by changes in activity of certain neuronal and/or non-neuronal cells in this area, as has indeed been demonstrated at the light microscopical level for dopaminergic neurones (Hökfelt and Fuxe, 1972).

At the ultrastructural level it has been demonstrated that, as a consequence of micro-pinocytosis, "synaptic" vesicles in the tuberoinfundibular nerve terminals of the median eminence can be loaded by exogenous peroxidase (Stoeckart *et al.*, 1974). On the basis of the marked uptake of peroxidase by stimulated nerve terminals of the peripheral nervous system (Holtzmann *et al.*, 1971; Ceccarelli *et al.*, 1972) and of the posterior pituitary gland (Nagasawa *et al.*, 1971), it might be expected that an increase in activity of tuberoinfundibular nerve terminals is reflected in an increased uptake of peroxidase. As data on ultrastructure and histophysiology

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of the *hypothetical* LRF containing tuberoinfundibular nerve terminals are lacking, it seemed of interest to study the incorporation of exogenous peroxidase by nerve terminals of the median eminence under conditions of expected increase in release of LRF. Since the concentration of LRF in the hypothalamo-hypophysial portal vessels is increased following withdrawal of gonadal steroids (Ben-Jonathan *et al.*, 1973), castrated and sham-castrated rats were compared.

Methods

Adult male rats (R-Amsterdam, a Wistar substrain) were used. The animals were kept in a controlled temperature (22–25°C) and under controlled light conditions (14 hr light, 10 hr darkness). They had free access to food and water. Two parallel experiments were carried out. In each experiment, 5 rats were castrated and 5 were sham castrated under ether anaesthesia between 9 a.m. and 1.30 p.m. In Experiment 1, the mean weight (±SEM) of the castrated rats was 305.8±7.2 g and that of the sham-castrated rats 312.8±6.9 g. In Experiment 2, the mean weights were 280.0±14.5 and 270.8±9.7 g, respectively.

Twenty-four hr after (sham)castration the rats were anaesthetized with Avertin (tribromoethanol plus amylene hydrate); 4 min later 100 mg horseradish peroxidase (HRP, Sigma II; molecular weight ca. 40000) in 1 ml Locke solution was injected into a tail vein. The animals were killed by perfusion with fixative 20 min after administration of HRP. This time period was chosen, since according to a pilot study of nerve profiles in direct contact with the outer basement membrane, the proportion of nerve profiles with HRP loaded vesicles was higher after this period than after 1, 5 or 60 min. The perfusion procedure was performed as follows. After opening the thoracic cavity a cannula was pushed via the left ventricle into the ascending aorta where the cannula was secured with a hemostat. An incision in the wall of the right atrium provided the outlet for blood and fixative (1% paraformaldehyde, 2% glutaraldehyde and 2% polyvinyl pyrrolidon in 0.1 M cacodylate buffer, pH 7.4). The perfusion lasted for 15 min during which about 120 ml of fixative was used.

In Experiment 2, 1 ml of blood was collected immediately after opening the right atrium. Serum LH was estimated by double antibody radioimmunoassay (Welschen *et al.*, 1974). For the sham-operated rats the mean value (±SEM) was 20±3.5 ng LH/ml ($n=5$). For the castrated rats, from which only 3 samples were available, the mean value was 56±9.1 ng LH/ml.

After perfusion, hypothalamus and median eminence were removed from the cranial cavity and dropped into the fixative, in which they remained for 3–7 hr. Sections of about 50 μm, prepared by a tissue chopper, were incubated for 2×20 min in Tris-maleate buffer containing 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Graham and Karnovsky, 1966). The sections were post-fixed in buffered 2% osmium tetroxide, dehydrated in aceton and embedded in Epon. Coronal sections (see Fig. 1a) were made 0.7–1.0 mm rostral to the infundibular stem. Ultrathin sections of silver interference colour were contrasted with lead citrate (Reynolds 1963). Unaware of the experimental group of rats from which the sections were derived, the ventral 8.6 μm of sub-zone A (see Fig. 1a, hatched area) was analyzed. This sub-zone represents the basal part of the palisade zone (Stoeckart *et al.*, 1973).

For the analysis a Philips EM 300 was used at a magnification of 15000×. The primary image was viewed with a binocular microscope (10×). An imaginary dorso-ventral line was drawn at the junction of the lateral and ventral wall of the third ventricle (Fig. 1a). Between this line and the mid-sagittal plane parallel lines were drawn at distances of 5.6 μm (Fig. 1a and b). Going from dorsal to ventral along these lines, nerve profiles (*i.e.* cross-sections of axons, varicosities or terminals) were selected according to a principle depicted in Fig. 1c. By this principle, 3 profiles along each line were selected from each of the subsequent "layers" of profiles. By this procedure about 500 nerve profiles were included in the sample of each rat.

Irrespective of the occurrence of HRP loaded vesicles, the nerve profiles were divided into 4 categories on the basis of their vesicular content in the ultrathin sections: *a.* only agranular vesicles (AV) of about 50 nm present, *b.* both AV and granular vesicles (GV) of mainly 70–

140 nm present, *c*. only GV present, *d*. no vesicles present. For the different categories, the percentage of nerve profiles characterized by the occurrence of small HRP loaded vesicles was determined, as well as the number of HRP loaded vesicles per profile. No distinction has been made between coated vesicles with HRP and "smooth" vesicles with HRP.

The Wilcoxon two-sample test was used for statistical analysis.

Results

Categories of Nerve Profiles Based on Their Vesicular Content

About 50% of the nerve profiles were characterized by the occurrence of both AV and GV (Table 1). Nerve profiles containing either AV or GV amounted each to about 10%, while 25% did not contain vesicles. After a 24 hr period of castration no significant change occurred for any category, neither in Experiment 1 nor in Experiment 2 (Table 1). Also between Experiment 1 and 2 there were no significant differences.

Incorporation of Horseradish Peroxidase

I. General Observations. Twenty min after injection of HRP, peroxidase activity was found in the extracellular space of the median eminence up to the third ventricle. Part of the "synaptic" vesicles in certain tuberoinfundibular nerve profiles were characterized by a markedly electron dense reaction product (Fig. 2). For 50 of these HRP loaded vesicles, measured on 15 electron micrographs and derived from 8 rats, the size range was 30–65 nm and the mean size 49 nm $\pm$ 8.3 (SEM). The reaction product generally filled completely the area within the vesicular membrane (Figs. 2, 3).

Nerve profiles with HRP loaded vesicles were most numerous in the direct vicinity of the outer basement membrane. There were no apparent differences in the distribution of these nerve profiles between lateral and medial parts of subzone A.

Part of the nerve profiles of category *a* and *b* contained membrane-bound structures of various size and shape with a generally peripheral deposit of reaction product (see Ceccarelli *et al.*, 1972; Heuser and Reese, 1973). In addition, some nerve profiles (about 0.1%) occurred in which the cores of the GV were markedly electron dense.

A small number of images consistent with exocytosis as a release mechanism was observed (Fig. 3). In these cases, electron dense core material of former GV was located in the extracellular space and a vesicular membrane was lacking.

II. Quantitative Observations. In sham-operated rats, only 0.4–0.5% of the nerve profiles contained both HRP loaded vesicles and AV without GV (Table 2). In both experiments castration did induce a significant (3–5 fold) increase in this percentage. No significant change was observed for the percentage of nerve profiles with both HRP loaded vesicles and AV+GV (Table 2). By definition, nerve profiles of category *c* and *d* did not contain HRP loaded vesicles.

For sham-operated rats, the percentage of the nerve profiles of category *a* with HRP loaded vesicles (Table 3) was about the same as that for the nerve profiles of category *b* with HRP loaded vesicles. Exclusively the percentage of the nerve profiles of category *a* with HRP loaded vesicles was increased by castration.

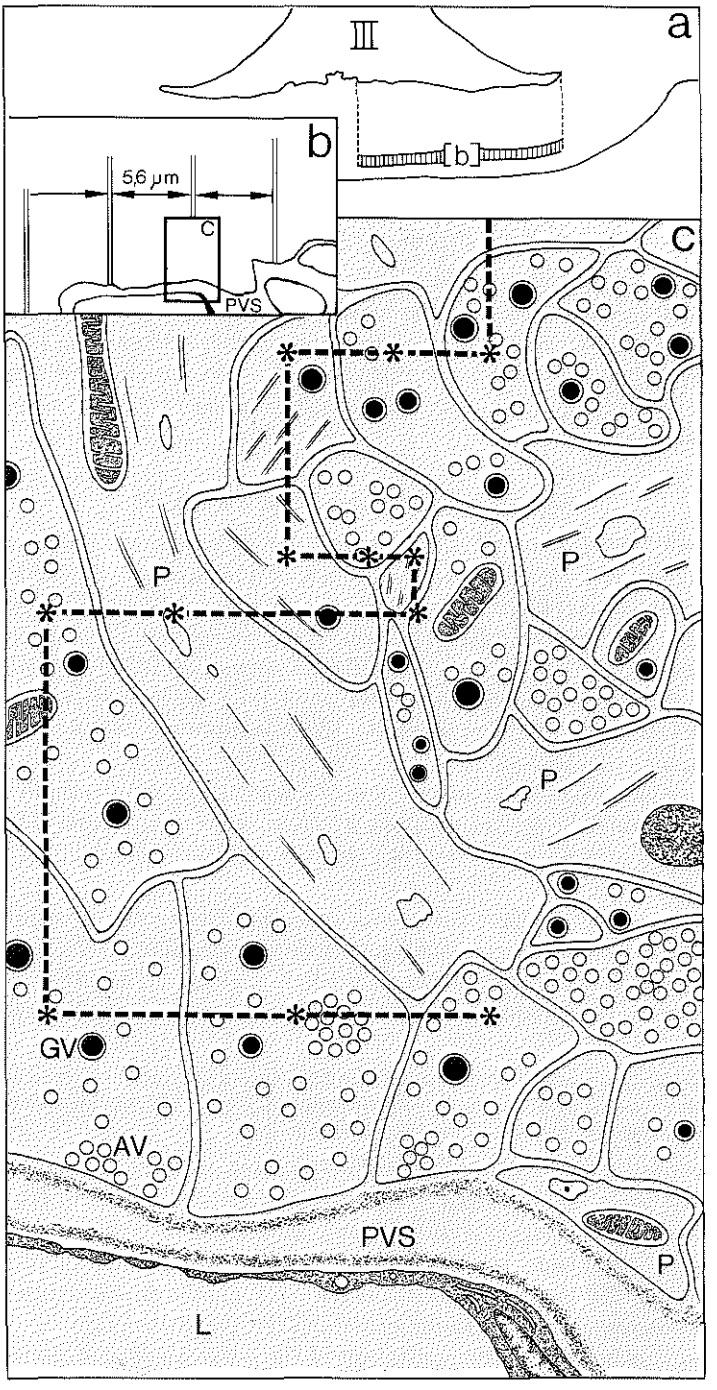

Fig. 1a —c. Diagram of the procedure according to which nerve profiles were selected. (a) The area of the median eminence studied; the hatched area represents sub-zone A, coronal section.

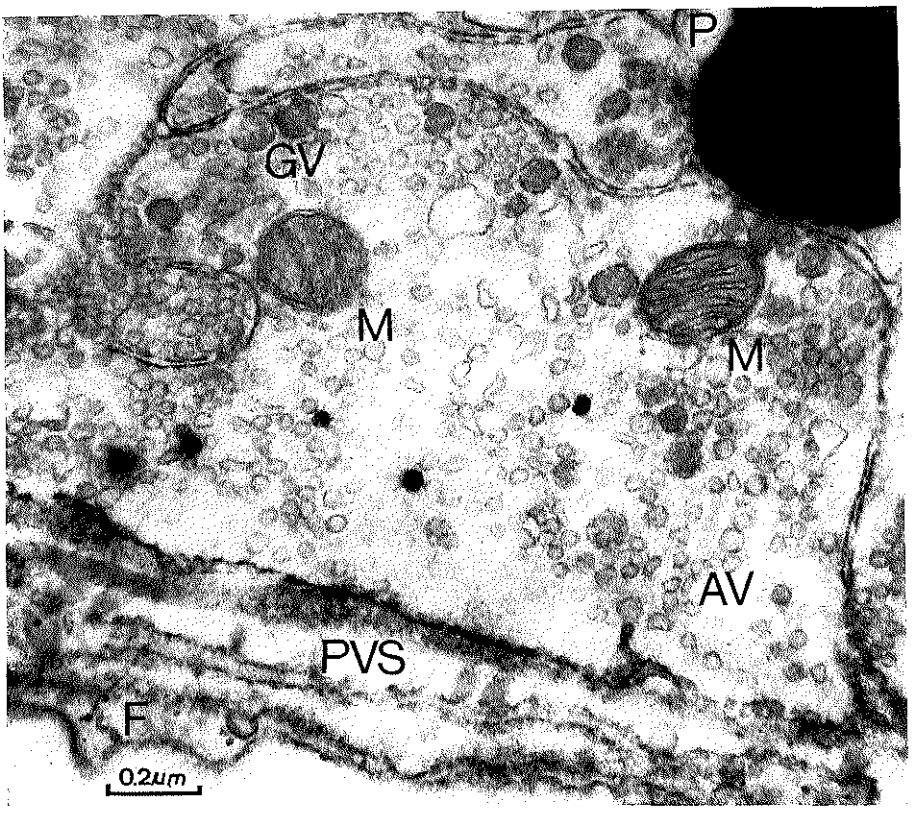

Fig. 2. Nerve profile in direct contact with the outer basement membrane. Male rat, 24 hr post-castration, administration of HRP 20 min before fixation. Reaction product can be observed in 5 small vesicles. The other small AV and the larger GV do not contain reaction product. Possibly reaction product occurs also in the large lipid-like inclusion in the non-nervous process P. ×60000. *AV* agranular vesicles; *GV* granular vesicles; *F* fibroblast; *M* mitochondria; *P* non-nervous process; *PVS* perivascular space

Even for nerve profiles with HRP loaded vesicles, the mean number of HRP loaded vesicles per profile was small: 1.6 ± 0.07 and 1.6 ± 0.20 in the sham operated animals of Experiment 1 and 2. No change was observed after castration (1.7 ± 0.19 in Experiment 1 and 1.4 ± 0.11 in Experiment 2). A change was also not observed if profiles of category *a* and *b* were considered separately (Table 4).

×160. (b) Detail of (a), representing the dorso-ventral lines in sub-zone A, along which nerve profiles were selected. ×2000. (c) Detail of (b). Nerve profiles were selected according to a "hit-system", an example of which is shown by the interrupted line. The procedure was followed irrespective of the kind of profile hit (nervous or non-nervous). Non-nervous processes were, however, not subject of this study. ×35000. III third ventricle; *AV* agranular vesicles; *GV* granular vesicles; *L* lumen of portal capillary; *P* non-nervous process; *PVS* perivascular space

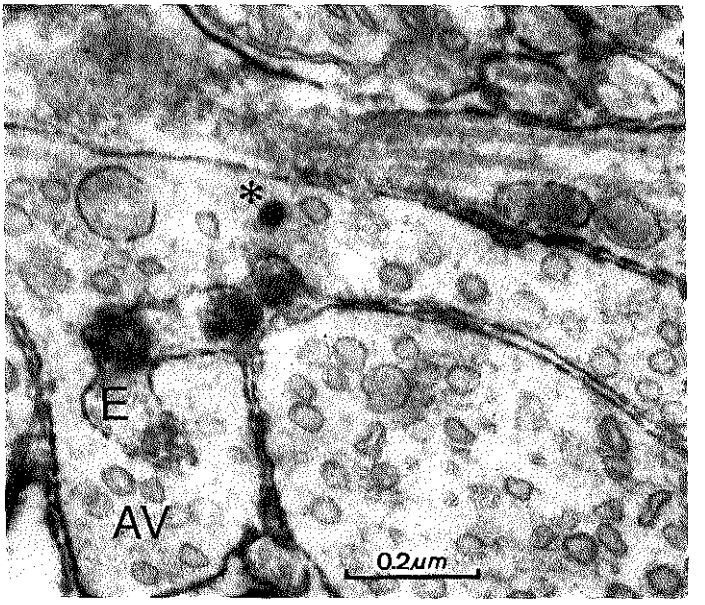

Fig. 3. Nerve profiles in sub-zone A, not in direct contact with the outer basement membrane. Male rat, 24 hr post-castration, 20 min HRP. AV are, with one exception (*), not characterized by reaction product. Three GV seem to be in the final process of exocytosis: the cores of the former GV are devoid of their vesicular membrane and are located in an extension of the extracellular space (*E*). The sizes of the cores are about 100 nm, which indicates that the former GV were about 110–130 nm. Probably, the electron density of the cores represents peroxidase activity, acquired after expulsion into the HRP containing extracellular space. Traces of peroxidase activity also adhere locally to plasma membranes. ×90000. *AV* agranular vesicles; *E* extension of extracellular space; * peroxidase loaded vesicle

Table 1. Percentages of different categories of nerve profiles

	Experiment 1		Experiment 2	
Category	sham operated	castrated	sham operated	castrated
a (AV)	10.0[a] ±1.03[b]	10.6±1.11	11.8±1.00	11.5±0.99
b (AV+GV)	54.6 ±1.68	50.8±1.41	54.1±1.50	56.9±0.91
c (GV)	11.3 ±1.79	11.5±2.27	9.2±0.72	8.9±1.13
d (--)	24.1 ±2.44	27.1±2.17	24.9±1.85	22.7±0.86
Number of profiles	2131	2101	2788	2891

[a] Mean of 5 rats.
[b] SEM; Differences not significant.

Discussion

The present study demonstrates that, by use of exogenous peroxidase, changes can be detected in certain tuberoinfundibular nerve terminals and varicosities

Table 2. Percentages of nerve profiles with both HRP loaded vesicles and either AV or AV + GV

Category	Experiment 1		Experiment 2	
	sham operated	castrated	sham operated	castrated
a (AV)	0.4[a] ±0.14[b]	2.1[c] ±0.25	0.5 ±0.18	1.5[c] ±0.18
b (AV + GV)	2.5 ±0.21	3.7 ±0.52	3.3 ±0.37	3.4 ±0.50

[a] Mean of 5 rats.
[b] SEM
[c] $p \leq 0.02$; Other differences not significant.

Table 3. Percentages of the nerve profiles of category *a* and category *b* with HRP loaded vesicles

Category	Experiment 1		Experiment 2	
	sham operated	castrated	sham operated	castrated
a (AV)	3.7[a] ±1.09[b]	19.2[c] ±0.78	4.1 ±1.84	12.9[c] ±1.00
b (AV + GV)	4.6 ±0.38	7.3 ±0.99	6.0 ±0.58	6.1 ±0.85

[a] Mean of 5 rats.
[b] SEM
[c] $p \leq 0.02$; Other differences not significant.

Table 4. Mean number of HRP loaded vesicles in the nerve profiles of category *a* and *b* with HRP loaded vesicles

Category	Experiment 1		Experiment 2	
	sham operated	castrated	sham operated	castrated
a (AV)	1.1[a] ±0.13[b]	1.4 ±0.16	1.3 ±0.14	1.3 ±0.08
b (AV + GV)	1.7 ±0.09	1.7 ±0.20	1.6 ±0.20	1.5 ±0.14

[a] Mean of 5 rats.
[b] SEM; Differences not significant.

following castration. Previously described changes in the ultrastructure of these terminals (Kobayashi and Ishii, 1969; Kobayashi and Matsui, 1969; Kobayashi *et al.*, 1967, 1969, 1970) lacked a quantitative base, with the exception of a study by Zambrano and De Robertis (1968). For perivascular nerve profiles, they found an overall increase in the number of neuronal GV, 1 month after castration.

The precise location of the cell bodies from which the described nerve terminals derive is unknown, but following castration changes have been described in the ultrastructure of cell bodies in the arcuate nucleus (Zambrano and De Robertis, 1968; Brawer, 1971).

Interpretation. Results of combined electrophysiological and morphological studies (Holtzmann *et al.*, 1971; Ceccarelli *et al.*, 1972, 1973; Heuser and Reese, 1973) suggest that an increase in the activity of nerve terminals will be reflected in increased uptake of HRP which may result in increased numbers of HRP loaded vesicles. It has to be stated, however, that under the conditions of the present experiment, fate nor lifetime of the HRP loaded vesicles is known. Notwithstanding the unknown lifetime, we are tempted to interpret the increase in the percentage of nerve profiles with HRP loaded vesicles after castration as a result of increased uptake of HRP by those terminals which became stimulated due to castration. The question, then, remains, which physiological type(s) of neurones is (are) involved.

Two main possibilities will be discussed: A. An increase in activity of LRF containing nerve terminals may certainly be expected: 1. according to Davidson (1969) an inhibitory feedback of testosteron is exerted directly on the hypothalamus, 2. according to Barry *et al.* (1973) the synthesis of LRF is, at least in guinea pig, increased following castration, 3. for female rats the LRF concentration in hypophysial portal blood has been demonstrated to increase after (longterm) ovariectomy (Ben-Jonathan *et al.*, 1973) and 4. in our experiment serum LH was increased after castration, while in another experiment, where HRP was omitted, both LH and FSH levels were significantly elevated 24 hr after castration (see also Gay and Midgley, 1969; Swerdloff *et al.*, 1970; Yamamoto *et al.*, 1970; Tapper *et al.*, 1972). Therefore, since increase in incorporation of HRP was restricted to profiles with AV, it might be supposed that LRF is stored in terminals (and varicosities) which contain especially AV. However, if one assumes that LRF is at least partly stored in GV (Kobayashi and Ishii, 1969; Clattenburg *et al.*, 1973) and that its release may occur by means of exocytosis (Stoeckart *et al.*, 1972; contrast Clattenburg *et al.*, 1973), followed by micro-pinocytosis (Stoeckart *et al.*, 1974), the increased proportion of AV-profiles with HRP loaded vesicles may, more probably, be caused by depletion of GV which contained LRF on one hand and by increased micro-pinocytosis on the other. This would result in nerve profiles depleted of GV and recognized as AV-profiles with HRP loaded vesicles. As following castration the increase in percentage of AV-profiles with HRP loaded vesicles is restricted to less than 2% of the total number of profiles studied (Table 2), such a change cannot be expected to be detected in the percentages of nerve profiles of different categories (Table 1).

In this view, the increased proportion of HRP "labeled" AV-profiles is at least partly caused by a causally related occurrence of exocytosis and micro-pinocytosis (see Douglas, 1973).

B. Apart from an expected influence of castration on the activity of the hypothetical LRF containing terminals, the effects of castration may be more widespread (Moguilevsky, 1971; Ter Haar and McKinnon, 1972). They may involve, in addition to non-neuronal elements (Kobayashi and Matsui, 1969; Oksche *et al.*, 1972), neuronal systems different from the hypothetized LRF containing system—assuming that different biologically active substances are synthetized by and stored in separate neurones. The increase in incorporation of HRP may *e.g.* concern stimulated dopaminergic terminals (Schneider and McCann, 1970; Kordon and Glowinski, 1972; Kamberi, 1973; contrast Fuxe *et al.*, 1969; Fuxe and Hök-

felt, 1969; Kobayashi and Matsui, 1969; Cramer and Porter, 1973; Miyachi *et al.*, 1973; Quijada *et al.*, 1973/74) or noradrenergic terminals (Donoso *et al.*, 1969; Rubinstein and Sawyer, 1970; Anton-Tay and Wurtmann, 1971; Coppola, 1971; Ojeda and McCann, 1973; contrast Kobayashi and Matsui, 1969; Cramer and Porter, 1973; Quijada *et al.*, 1973/74). Dopaminergic terminals in particular are numerous in the perivascular part of the median eminence (Hökfelt, 1967; Ajika and Hökfelt, 1973) and AV of about 50 nm are the main organelles after aldehyde/osmium tetroxide fixation, generally in addition to a limited number of larger GV.

In this view, micro-pinocytosis is regarded as a general phenomenon of nerve terminals (see Ceccarelli *et al.*, 1972; Heuser and Reese, 1973; Turner and Harris, 1973), although it may also be caused by an exocytotic release mechanism.

The following general conclusions have been drawn.

1. Exogenous peroxidase may be used to demonstrate at the ultrastructural level changes in the activity of tuberoinfundibular nerve terminals.

2. The castration induced increase in the percentage of stimulated tuberoinfundibular nerve terminals (and varicosities) may concern LRF containing neurones; other neurones, *e.g.* catecholaminergic ones, cannot, however, be excluded.

3. The observations support the hypothesis of a functional relationship between the activity of certain tuberoinfundibular nerve terminals and the level of gonadotropin release.

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Influence of Castration on Incorporation of Exogenous Peroxidase into "Synaptic" Vesicles of the Median Eminence

II. The Perivascular Part of the Palisade Zone in Male Rats

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Summary. In the median eminence of male rats, nerve profiles in the immediate vicinity of portal capillaries have been divided into 4 categories on the basis of their vesicular content: profiles *a* with agranular "synaptic" vesicles of about 50 nm, *b* with similar agranular vesicles and also with granular vesicles of mainly 60–140 nm, *c* with granular vesicles alone and *d* without vesicles.

Twenty-four hours after castration, the percentage of profiles of category *a* was significantly increased when compared with sham-operated animals, whereas the percentage of profiles of category *b* was significantly decreased. After intravenous injection of exogenous peroxidase, especially the nerve profiles located in direct contact with the outer basement membrane of the portal capillaries contained peroxidase positive "synaptic" vesicles. Injection of peroxidase after castration resulted in a significant increase in the percentage of nerve profiles containing both peroxidase positive and peroxidase negative "synaptic" vesicles (category *a*). It is suggested that, in certain nerve terminals, castration may lead to release of the content of granular vesicles, which may contain gonadotropin releasing factor. This release then may cause that nerve terminals with both agranular vesicles and granular vesicles (category *b*) change in their vesicular content and are therefore classified as terminals with only agranular vesicles (category *a*). An increased turnover rate in the ultrastructurally affected terminals may be reflected in increased uptake of exogenous peroxidase. The observations imply that certain neurones projecting to portal capillaries in the median eminence are, directly or indirectly, sensitive to changes in the level of gonadal steroids.

Key words: Median eminence — Agranular and granular vesicles — Horseradish peroxidase — Castration — Ultrastructure.

Releasing factors are generally assumed to be stored in granular vesicles (GV) located in preterminal and terminal parts of certain tuberoinfundibular neurones (McCann and Porter, 1969; Kobayashi *et al.*, 1970, 1972; Scott *et al.*, 1972; Clattenburg, 1974). For luteinizing hormone-releasing hormone this assumption has been substantiated by Pelletier *et al.* (1974) who observed, using an immunohistochemical technique at the ultrastructural level, that this hormone is contained in secretory granules of some nerve endings in the palisade zone of the median eminence.

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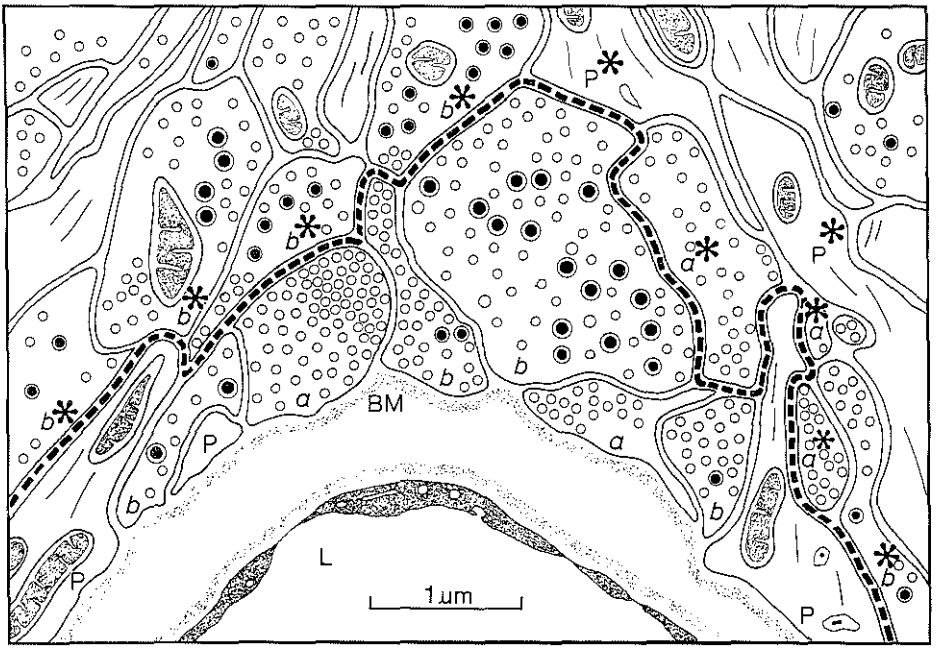

Fig. 1. Diagram of the perivascular part of the palisade zone of the median eminence, showing profiles in direct contact with the outer basement membrane (first "layer") and profiles in the adjacent (second) "layer". The borderline between first and second "layer" is indicated by heavy interrupted line. Profiles in the second "layer" are characterized by an *. Vesicles containing horseradish peroxidase are not shown. *a* nerve profile with AV only; *b* nerve profile with AV + GV; *BM* outer basement membrane; *L* lumen of portal capillary; *P* non-neuronal process

Following castration and injection of exogenous peroxidase (Stoeckart *et al.*, 1974b), the basal part of the median eminence showed, in comparison with sham-operated rats, an increase in the percentage of nerve profiles containing both peroxidase positive "synaptic" vesicles and agranular vesicles (AV), without GV. The increase was, however, very small and was not accompanied by a decrease in percentage of profiles containing GV. Therefore, it could not be decided whether this increase in percentage of peroxidase "labeled" AV-profiles was due to a castration induced depletion of GV from nerve terminals which contained either AV and GV or GV alone under non-stimulated conditions. However, changes induced by castration in the ultrastructure of the terminal system of the tuberoinfundibular neurones might be most pronounced in the direct vicinity of portal capillaries. Therefore, the *perivascular* nerve terminals have been analyzed in the present study.

Methods

The ultrathin sections of the rat brains which have been studied previously (Stoeckart *et al.*, 1974b; Experiment 2) were reexamined in greater detail. These brains were derived from 10 male rats which had been sham operated or castrated 24 hours before fixation. The

Table 1. Percentages of different categories of nerve profiles

Category	First "Layer"		Second "Layer"	
	sham operated	castrated	sham operated	castrated
a (AV)	28.2[a] ± 2.95[b]	51.1[c] ± 1.99	17.6 ± 2.74	37.7[c] ± 1.37
b (AV + GV)	71.3 ± 3.02	48.0[c] ± 1.56	75.6 ± 1.43	53.4[c] ± 4.17
c (GV)	0.52 ± 0.33	0.66 ± 0.66	5.2 ± 2.80	5.9 ± 2.40
d (—)		0.26 ± 0.26	1.5 ± 1.11	3.0 ± 2.98
Number of profiles	640	700	700	750

[a] Mean of 5 rats.
[b] SEM.
[c] $P < 0.02$; Other differences between sham-operated rats and castrated rats are not significant.

rats were killed by perfusion 20 min after intravenous injection of horseradish peroxidase (HRP). This enzyme can be incorporated by micro-pinocytosis into tuberoinfundibular nerve terminals, which results in nerve profiles containing peroxidase positive "synaptic" vesicles (Stoeckart *et al.*, 1974a).

In the same area which was studied previously, *i.e.* the basal part of the palisade zone of the median eminence, a further analysis was carried out on each nerve profile which was in direct contact with the outer basement membrane of portal capillaries (Fig. 1, first "layer"). In addition, each nerve profile was analyzed which was directly bordering the profiles of the first "layer", with the exception of those profiles located further than 2 μm from the outer basement membrane. The "layer" of profiles adjacent to the first "layer" will be designated as second "layer" (Fig. 1). With this method about 140 nerve profiles per "layer" were analyzed in each rat.

Results

I. Categories of Nerve Profiles Based on Their Vesicular Content (Table 1)

1. In the *first* "layer" of the sham-operated rats, 28.2% of the nerve profiles contained AV only (category *a*) and 71.3% contained both AV and GV (category *b*). Thus, 99.5% of the profiles contained AV.

Following castration, the percentage of all nerve profiles containing AV remained the same (99.1%), but the percentage of profiles of category *a* significantly increased (from 28.2 to 51.1%) while at the same time that of nerve profiles of category *b* significantly decreased (from 71.3 to 48.0%). There was no difference in the percentages of profiles of category *c* (GV only) and *d* (no vesicles), which categories constituted less than 1% of the profiles in the first "layer".

2. In the *second* "layer" of the sham-operated animals, 17.6% of the profiles contained AV and 75.6% contained both AV and GV. Thus, 93.2% contained AV.

Following castration, the percentage of profiles containing AV remained the same (91.1%). Castration induced, however, also in this "layer" a significant increase in percentage of nerve profiles of category *a* (from 17.6 to 37.7%) and a decrease in that of profiles of category *b* (from 75.6 to 53.4%). No significant

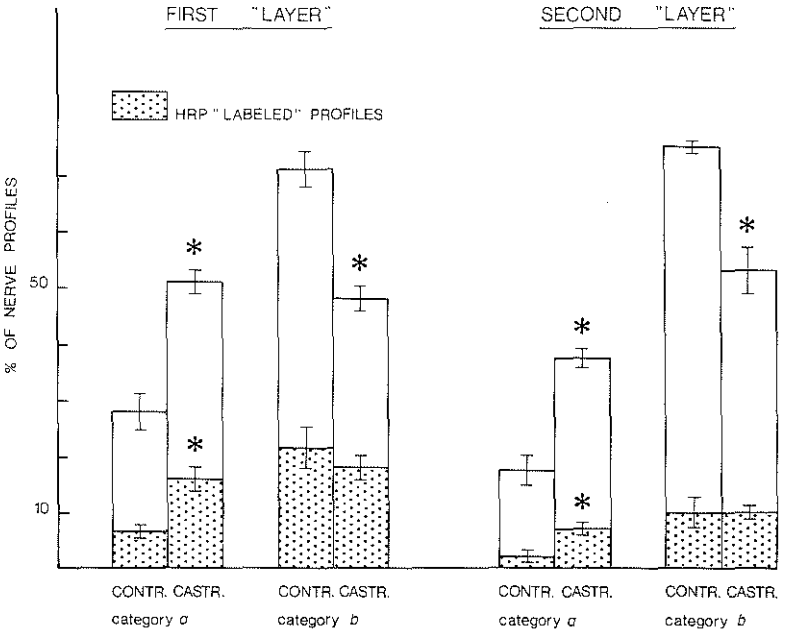

Fig. 2. Percentages of the nerve profiles of category *a* and category *b* in the first and second "layer", 24 hours after castration or sham operation, and the percentages of the nerve profiles ▨ containing both HRP positive vesicles and either AV (category *a*) or AV + GV (category *b*). Significant differences between sham-operated and castrated rats are marked by an *. For details, see Table 1 and 2

change was observed for category *c* and *d*, which categories constituted together about 8% of the nerve profiles in the second "layer".

II. Incorporation of Horseradish Peroxidase

A (Table 2, Fig. 2). 1. In the *first* "layer" of the sham-operated rats, 6.4% of the total number of analyzed nerve profiles contained both peroxidase positive "synaptic" vesicles and AV (category *a*). Castration significantly increased this percentage (to 16.1%).

In the sham-operated rats 21.3% of the profiles contained both peroxidase positive vesicles and AV + GV (category *b*). No change was observed after castration.

2. In the *second* "layer", 1.9% of the nerve profiles in the controls contained both peroxidase positive vesicles and AV (category *a*). Castration significantly increased also this percentage (to 6.8%).

In the sham-operated rats, 10.1% of the profiles contained both peroxidase positive vesicles and AV + GV (category *b*). After castration this percentage remained unchanged.

3. In the sham-operated as well as in the castrated rats, the percentages of the nerve profiles which contained both peroxidase positive vesicles and either

Table 2. Percentages of nerve profiles with both HRP positive vesicles and either AV or AV + GV

$$\left(\frac{\text{n of profiles with HRP positive vesicles and AV, or AV + GV}}{\text{total n of profiles}} \times 100\right)$$

Category	First "Layer"		Second "Layer"	
	sham operated	castrated	sham operated	castrated
a (AV)	6.4[a] ± 0.78[b]	16.1[c] ± 2.05	1.9 ± 0.66	6.8[c] ± 0.83
b (AV + GV)	21.3 ± 2.96	18.5 ± 2.08	10.1 ± 2.40	10.1 ± 0.84

[a] Mean of 5 rats.
[b] SEM.
[c] $P < 0.02$; Other differences between sham-operated rats and castrated rats are not significant.

Table 3. Percentages of the nerve profiles with AV that contain HRP positive vesicles and of the profiles with AV + GV that contain HRP positive vesicles

$$\left(\frac{\text{n of profiles with HRP positive vesicles and AV}}{\text{n of profiles with AV}} \times 100\right)$$

and

$$\left(\frac{\text{n of profiles with HRP positive vesicles and AV + GV}}{\text{n of profiles with AV + GV}} \times 100\right)$$

Category	First "Layer"		Second "Layer"	
	sham operated	castrated	sham operated	castrated
a (AV)	24.0[a] ± 3.76[b]	31.7 ± 3.87	12.0 ± 4.29	18.3 ± 2.68
b (AV + GV)	30.5 ± 4.88	38.8 ± 4.81	13.5 ± 3.42	19.2 ± 1.86

[a] Mean of 5 rats.
[b] SEM.
Differences between sham-operated rats and castrated rats are not significant.

AV or AV + GV were in the first "layer" significantly greater ($P \leq 0.02$) than the corresponding percentages in the second "layer".

B (Table 3). The percentages of the profiles of category *a* and *b* which contained peroxidase positive vesicles were not significantly different from each other, neither in the first "layer" (24.0% of *a* versus 30.5% of *b*) nor in the second (12.0% of *a* versus 13.5% of *b*). There was no significant difference in the percentages after castration.

Discussion

The present study demonstrates that, 24 hours after castration (and 20 min after injection of HRP), the percentage of nerve profiles containing both AV and GV is decreased in direct vicinity (mostly within 2 μm) of the outer basement

membrane of the portal capillaries. In contrast, the percentage of nerve profiles with AV alone is increased (Table 1). In a study in which injection of HRP was omitted comparable changes were observed in the perivascular nerve profiles (unpublished results). The described changes could not be detected in a previous study (Stoeckart *et al.*, 1974b) dealing with a larger area of the palisade zone (the basal 8.6 μm). This discrepancy indicates that changes in the vesicular content of tuberoinfundibular varicosities and terminals are, 24 hours after castration, most prominent in the direct vicinity of portal capillaries. So, apparently, the preterminal parts of tuberoinfundibular neurones represent ultrastructurally a relatively static component as far as the occurrence of AV and GV is concerned.

The above mentioned castration induced increase in percentage of profiles with AV alone and the corresponding decrease in that of nerve profiles with AV + GV may be a result of retraction of terminals with AV + GV, away from the portal capillaries, or/and extension of terminals with AV alone in the direction of the capillaries. However, it seems more likely that, due to castration, GV disappear from a huge number of terminals, which disappearance may be detected only if formation of GV (or their transport to the terminal) stays, at least temporarily, behind their release (or degradation). The disappearance of GV may occur by exocytosis followed by micro-pinocytosis (Stoeckart *et al.*, 1972, 1974a; Douglas, 1974) and/or by fragmentation (see *e.g.* Streefkerk, 1967; Dierickx *et al.*, 1973). Both mechanisms, probably, result in the formation of AV.

This explanation would imply that, depending on the functional state of the terminal, a nerve profile may show up either as a profile with AV and GV or as a profile with AV alone. Therefore, we do not favour the view (Kobayashi and Matsui, 1969; Rodríguez, 1969; Nakai, 1971) that nerve profiles of the median eminence which contain exclusively AV belong to one functional category (*i.e.* cholinergic). AV as well as GV represent in all probability functionally heterogenous groups of vesicles. In this respect, it is necessary to emphasize that the categorization used in the present study is a crude one, since it is exclusively based on the presence or absence of AV and GV (for a further categorization of nerve profiles in the rat, see Rinne, 1966; Stoeckart *et al.*, 1973; Wittkowski, 1973, and in amphibians Rodríguez, 1969; Belenky *et al.*, 1973 and Dierickx, 1973).

If we assume that the shift from terminals of category *b* to those of category *a* results from release of GV and is accompanied or followed by increased micropinocytotic activity, then consequently the increase in percentage of peroxidase "labeled" AV-profiles (Table 2) may concern terminals which contained AV and GV under non-stimulated conditions.

The castration induced shift in percentage of nerve profiles of category *a* and *b* amounted to about 23% (Table 1), whereas the increase in percentage of profiles which contained both peroxidase positive vesicles and AV (category *a*) was, even in the first "layer", only 9.7% (Table 2, Fig. 2). If, for the sake of simplicity, we speculate that castration did not influence the dynamics of the original population of terminals from which the profiles of category *a* are derived, then the 9.7% increase represents exclusively part of the terminals thought to be depleted of GV. In that case, the 9.7% increase represents $9.7/23 \times 100 = 42\%$ of the population of terminals depleted of GV.

This reasoning suggests that by injecting HRP 20 min before fixation only part of the population of terminals which changed ultrastructurally during the 24 hour period of castration is "labeled" by HRP. This may well be the consequence of the parameter used (the presence of one or more peroxidase positive vesicles per profile), as the number of peroxidase positive vesicles *per profile* is very small (Stoeckart *et al.*, 1974b). Therefore, it may be assumed that both in castrated rats and in controls only a certain percentage of the active terminals is characterized by the presence of one or more peroxidase positive vesicles per profile. In that case, an increase in percentage of stimulated terminals is only partly reflected in an increase in percentage of HRP "labeled" profiles. Hence, serial sections would probably give a more complete answer.

Another cause for the observed difference may be the different time periods upon which the two parameters are based (the percentages of the categories are based upon a 24 hour period and the percentages of HRP "labeled" profiles upon the last 20 min of this period), especially if there would be a phasic secretion of gonadotropin releasing factor (see Gay and Sheth, 1972; Blake and Sawyer, 1974) under the conditions of the present experiment.

Thus, the 9.7% increase in HRP "labeled" AV-profiles probably represents an underestimation of the percentage of nerve terminals stimulated by castration. In this respect it is worth noting that according to Pelletier *et al.* (1974) luteinizing hormone-releasing hormone is present in about 10 to 20% of nerve endings in the median eminence.

That increased release of hormones may be associated with an increase in numbers of AV has been frequently reported, especially for the posterior pituitary gland (see *e.g.* Lederis, 1974) but also for the median eminence (Harris and Campbell, 1966; Kobayashi and Matsui, 1969; Péczely and Calas, 1970). These reports concern, however, qualitative observations, with the exception of a report by Santolaya *et al.* (1972). They showed a significant increase in the ratio of AV: elementary granules in nerve endings of the posterior pituitary gland from rats subjected to severe acute haemorrhage. That increased release of neurotransmitters may be associated with increased incorporation of exogenous peroxidase has been shown by the studies of Ceccarelli *et al.* (1972) and Heuser and Reese (1973). As far as incorporation of HRP by nerve terminals secreting neurohormones is concerned, Castel (1974) observed in a qualitative study a change in the rate of uptake in the posterior pituitary gland of dehydrated mice.

Of the different physiological types of neurones to which the terminals, ultrastructurally affected by castration, may belong, five will be mentioned:

A. It may be expected that at least part of the terminals belong to neurones which synthetize and secrete releasing (and inhibiting) factors. The observation that the shift from nerve profiles of category *b* to those of category *a* occurs preferentially in direct vicinity of portal capillaries is in harmony with this expectation. After all, the most strategic place for terminals secreting releasing factors will be close to the portal system draining towards the anterior pituitary gland.

The possibility that these neurones synthetize and secrete gonadotropin releasing factor is an obvious one:

1. following castration both synthesis (Moguilevsky *et al.*, 1974) and release (Eskay *et al.*, 1974) of gonadotropin releasing factor seems to be increased in male rats,

2. gonadotropin releasing factor may be stored, at least under conditions of non-stimulation, in GV of certain nerve terminals near the portal capillaries (Clattenburg, 1974; Pelletier *et al.*, 1974); this does not exclude the possibility that under conditions of stimulation gonadotropin releasing factor is transported

and released in an extravesicular form; this neither excludes the possibility of storage in non-neuronal elements of the median eminence (Zimmerman *et al.*, 1974),

3. according to the preliminary data of Pelletier *et al.* (1974), the percentage of luteinizing hormone-releasing hormone containing nerve profiles is in the same range as the percentage of the profiles which are, according to our study, affected by castration. For further discussion, see Stoeckart *et al.* (1974b).

We are not aware of data which indicate that castration causes changes in the secretion of other releasing and inhibiting factors, but this certainly can not be excluded.

B. The possibility that the affected nerve terminals are dopaminergic terminals certainly cannot be excluded (Kizer *et al.*, 1974; for further references, see Stoeckart *et al.*, 1974b). However, the decrease of about 23% in nerve profiles with AV and GV and the comparable increase in profiles with AV suggest that large numbers of terminals, affected by castration, store their biologically active substance in GV. Dopamine, in contrast, is by several authors (Hökfelt, 1967; Rinne, 1970; Ajika and Hökfelt, 1973; Cuello and Iversen, 1973) assumed to be stored mainly in small, after our procedure agranular, vesicles (AV), although GV certainly may occur in catecholaminergic varicosities and terminals (Pellegrino de Iraldi and Etcheverry, 1967; Ajika and Hökfelt, 1973). Moreover, Ajika and Hökfelt (1973) reported that the percentage of catecholaminergic, probably dopaminergic, terminals at the lateral side of the median eminence is 2–3 times higher than the percentage at the medial side. The changes, observed in our study, were not specifically associated with the lateral side (unpublished results). So, the possibility that the described changes concern especially dopaminergic terminals is not likely.

C. The possibility that the terminals concern mainly noradrenergic terminals (for references, see Stoeckart *et al.*, 1974b) is not likely as, according to Ajika and Hökfelt (1973), only few noradrenergic terminals occur in the area studied.

D. Serotoninergic terminals are not a likely candidate, if the assumption is correct that the observed changes reflect increased activity of the terminal. According to many studies (Kamberi *et al.*, 1970, 1971; Schneider and McCann, 1970; Kordon and Glowinski, 1972; Labhsetwar, 1972; Zolovick, 1973; Fuxe *et al.*, 1974; contrast Cramer and Porter, 1973), increased release of LH and FSH is accompanied by a decreased activity of central serotoninergic neurones.

E. The possibility that the terminals concern cholinergic terminals cannot be excluded since they probably are also involved in the control of gonadotropin release (Libertun and McCann, 1973). Whether they occur in the area studied has, however, not been established.

Conclusions

1. Ultrastructural studies on the vesicular content of nerve profiles in the basal part of the median eminence may be focussed on the area in direct vicinity of portal capillaries, since that area shows, at least 24 hours after castration, a marked change.

2. Twenty-four hours after castration, about 23% of the nerve profiles in direct vicinity of portal capillaries may be transformed from profiles with AV

and GV to profiles with AV alone. This could occur if synthesis (or supply) of GV stays behind release (or degradation) and suggests that nerve profiles which contain exclusively AV do not constitute one functional category.

3. Especially the nerve profiles in direct contact with the outer basement membrane of the portal capillaries are characterized by peroxidase positive "synaptic" vesicles, 20 min after intravenous injection of HRP.

4. Horseradish peroxidase injected 20 min before fixation seems to label only part of the population of tuberoinfundibular nerve terminals altered by castration.

5. The castration induced increase in percentage of nerve profiles containing both peroxidase positive and peroxidase negative "synaptic" vesicles (and not GV) may be due to loss of GV, which may store gonadotropin releasing factor, from nerve profiles containing both AV and GV under conditions of non-stimulation.

6. The castration induced changes in the ultrastructure of tuberoinfundibular nerve terminals imply that certain neurones projecting to portal capillaries in the median eminence are, directly or indirectly, sensitive to changes in the level of gonadal steroids.

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