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Post-coital Ultrastructural Changes In Neurons Of Three Nuclei And The Median-eminence Of The Rabbit Hypothalamus

Robert Earle Clattenburg

POST-COITAL ULTRASTRUCTURAL CHANGES IN NEURONS OF THREE NUCLEI AND THE MEDIAN EMINENCE OF THE RABBIT HYPOTHALAMUS

by

Robert Earle Clattenburg, B.Sc., M.Sc.

Department of Anatomy

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ABSTRACT

The effects of coitus on the structure of three hypothalamic nuclei and the median eminence of the rabbit brain were studied with the electron microscope.

Discrete post-coital ultrastructural changes were observed within 19.2% and 31.5% of the neurons located near capillaries of the preoptic and suprachiasmatic nuclei respectively. These changes were characterized by an increase in neuronal and nuclear diameters, a remarkable development of the rough endoplasmic reticulum and the Golgi systems, an increase in the number of ribosomes, mitochondria and lysosome-like bodies, and the appearance of numerous dense-core vesicles within the soma of these neurons.

These intraneuronal changes first became apparent at one-half hour and one hour after coitus within suprachiasmatic and preoptic nuclei respectively; they became even more pronounced at one and two hours post-coitus, then continued without significant change for 10 hours. Neurons with similar morphological features were never observed within the suprachiasmatic and preoptic nuclei of the controls. Clearly defined changes within the neurons of the arcuate nuclei of the rabbit following coitus were, likewise, not observed.

The post-coital ultrastructural changes observed within the neurons of the preoptic and suprachiasmatic nuclei reflect high synthetic activity; the products of this synthesis being packaged by the Golgi system into the large dense-core vesicles which began to

appear within the soma of these neurons at one-two hours post-coitus.

Four populations of 'cored' vesicles, two with cores of a light, granular material, and two with typical electron-dense cores, were observed. The large, light granular vesicles with a mean diameter of approximately 2000 Å and which were observed within the internal layer of the ME of both the control and experimental animals probably contain the neurohypophyseal hormones. A similar type of light cored vesicle with a mean diameter of 1900 Å was only observed within the soma of the suprachiasmatic neurons of the experimental animals. The nature of these vesicles is unknown. That this type of vesicle represents the immature form of the large dense-core vesicles observed within these neuron soma is suggested; however, morphological evidence supporting this hypothesis is inconclusive.

The two populations of dense-core vesicles had mean diameters of approximately 1000Å and 1400 Å respectively. The smaller (1000 Å) dense-core vesicles, which are probably aminergic in nature, were a common finding within the axon profiles and their terminals of all three hypothalamic nuclei and the median eminence. The occasional small dense-core vesicles with identical morphological features also occurred within the soma of preoptic and suprachiasmatic neurons of the control animals, as well as within the perikarya of the 'dark' neurons observed within the arcuate nuclei of both the control and experimental groups. Evidence that these small vesicles within the preoptic, suprachiasmatic and arcuate-median eminence may contain the biogenic amines noradrenalin, 5-Hydroxytryptamine and dopamine

respectively, is discussed.

The large dense-core vesicles with a mean diameter of 1400 Å were only observed within the neuropil and neuron soma of the preoptic and suprachiasmatic nuclei of the experimental animals, and within a number of axon profiles and their terminals of the median eminence. This vesicle type was not observed within the neuron soma of the control animals. Their appearance in significant numbers within the soma of many neurons located near capillaries of the preoptic and suprachiasmatic nuclei first became evident at one-two hours post-coitus. Evidence for their origin from the Golgi system is presented.

The small and the large dense-core vesicles were also observed within separate terminals in contact with the perivascular spaces of the external layer of the median eminence. The loss of the electron-dense material from only the larger population of these dense-core vesicles following coitus suggests a depletion of the vesicle content. The presence of 'ghosts' of uniform size and with a mean diameter of 1368 Å within these terminals of animals killed at 10 minutes post-coitus, suggests that this depletion is induced by coitus.

Since the large (1400 Å) dense-core vesicles were only observed within the soma of certain neurons of the preoptic and suprachiasmatic nuclei of the mated experimental animals and within a number of the terminals of the median eminence of the controls, we believe that they represent the luteinizing hormone-releasing factor (LH-RF), or this neuro-hormone in combination with a carrier protein

or peptide.

This work provides the most convincing morphological evidence to date that the site of synthesis of LH-RF in the rabbit is within the preoptic and suprachiasmatic hypothalamic nuclei.

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to

MOTHER

and

Dawn, Roy, Rob, Jay and Jeff

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure

Page

Figure Page

LIST OF TABLES

LIST OF APPENDICES

LIST OF FIGURE ABBREVIATIONS

A	Axon
AC	Anterior commissure
Arc.	Arcuate nucleus
Ast	Astrocyte
Ast-f	Astrocytic end-feet
Ast-p	Astrocytic process
At	Axon terminal
bl_1	endothelial basal lamina
bl_2	perivascular basal lamina
BS	Brain stem
bt	bouton termineau
C	Capillary
Ce	Cerebellum
CH	Cerebral hemisphere
Col	Collagen
cv	coated vesicle
Cw	Capillary wall
Cy	Cytoplasm
D	Dendrite
DCV	Dense-core vesicle
DCV_1	Small dense-core vesicles
DCV_2	Large dense-core vesicles
De	Desmosome

DN	Dark neuron
Ds	Dendritic spine
E	Endothelial cell
Ed	Ependyma
Ep	Ependymal process
ER	Endoplasmic reticulum
f	fiber
Fb	Fibroblast
G	Golgi system
gh	'ghosts'
Gr	Granule
Hb	Herring body
ir	intranuclear rodlet
IS	Infundibular stalk
jc	junctional complex
L	Lysosome-like body
LCV	Light-core vesicle
LPO	Lateral preoptic nucleus
M	Mitochondrion
mA	myelinated axon
MB	Mammillary body
ME	Median eminence
MI	Massa intermedia
MPO	Medial preoptic nucleus
MVB	Multivesicular body

N	Neuron
Nc	Nucleolus
NE	Nuclear envelope
Npl	Neuropil
NS	Neuron soma
Nt	Neurotubule
Nu	Nucleus
O	Oligodendrocyte
OCh	Optic chiasma
ON	Optic nerve
OT	Optic tract
P	Pericyte
PC	Posterior commissure
pf	pars fibrosa
pg	pars granulosa
PPA	preoptic periventricular area
PN	Pale neuron
PO	Preoptic nucleus
psm_1	presynaptic membrane
psm_2	postsynaptic membrane
pvs	perivascular space
r	ribosome
SCH	Suprachiasmatic nucleus
SO	Supraoptic nucleus
sv	synaptic vesicle
Tj	Tight junction

V_{III}	Third ventricle
VMN	Ventromedian nucleus

I INTRODUCTION

A) The Concept of Neurosecretion

Neurons with the characteristics of gland-like cells were first observed in the caudal part of the spinal cord of sharks and rays by Dahlgren (1914). Many of the early studies, which confirmed the presence of similar cells within the hypothalamus of vertebrates and the central nervous system of invertebrates, are sited in the review by Hofer (1968).

Bargmann and Scharrer's (1951) concept of neurosecretion is presently widely accepted. According to this concept, the site of origin of the neurosecretory material (NSM) is the perikaryon, from where it is transported through the processes of the neurosecretory nerve cells to their terminals, where it may either be delivered directly into a body fluid, or be stored. When compared with other endocrine organs, the uniqueness of the metazoan neurosecretory system is proven by two facts: the NSM is, in general, not released directly from the cell body in which it is produced, but is delivered to, and stored within, an organ adapted for this purpose. This organ, termed a 'neurohemal organ' by Knowles and Carlisle (1956), is also situated at some distance from the origin of the NSM.

The neurosecretory cell has been described as a neuron having all the characteristics of both nerve and endocrine cells (Bern, 1963).

Because of their cellular metabolism during the production of the NSM, neurosecretory neurons have a rich blood supply. That this relationship may serve as a pathway for the hormonal feed-back from target organs to the neurosecretory cell is also generally accepted. Until a few years ago it was also assumed that the processes of neurosecretory cells do not synapse with other neurons or effector organs. Whether or not the recently described synapses within neurohemal organs were those of true neurosecretory neurons, is currently a matter for speculation. Further, conventional neurons influence other neurons or effector organs by the release of transmitter substances (neurohumors) at specific synaptic junctions; the time involved for the onset of a response following excitation is of the order of milliseconds. By contrast, the onset of response following excitation of neurosecretory cells can usually be measured on a time scale ranging from minutes to hours, or even days. In addition, neurohormones contained within the cytoplasm of secretory neurons can be demonstrated at the light microscopic level.

Since the introduction of the electron microscope to the study of neurosecretory systems, the cytological criteria for the identification of a neurosecretory cell have been further refined to include those neurons that contain electron-dense secretory granules. It is generally assumed that those granules having a diameter of 1000 Å to 3000 Å contain the neurohormones (see reviews of Sachs, 1969; Ishii, Iwata and Kobayashi, 1969; Kobayashi, Matsui and Ishii, 1970), while the morphologically similar granules of smaller diameter (less than 1000 Å) contain the biogenic amines (Matsui, 1967; Rinne, 1966;

Knowles, 1967; Zambrano and De Robertis, 1967, 1968). However, the Scharrers (1963) have cautioned on the labelling of neurons as 'neurosecretory' simply on the basis of their content of electron-dense granules. They argue for example, that certain neurons of the arcuate or infundibular nucleus display the cytological characteristics of neurosecretory cells, but that they elaborate the neurotransmitter dopamine (DA). Since it is now well established that DA does not directly influence the synthesis and/or release of the adenohypophyseal hormones (Porter and Mical, 1969; Kamberi, Mical and Porter, 1969, 1971a; Porter, Kamberi, Goldman, Mical and Grazia, 1970), these neurons should not be considered neurosecretory.

In this thesis, the term 'neurosecretory neuron' is used with reference to a neuron which is engaged directly with an endocrine function. The use of the term 'neurohormone' refers to the secretory products of these cells and conforms to the conventional definition of a hormone - a unique chemical mediator produced by a specific group of cells and carried via the blood stream to target organs. The major portion of the central nervous system is excluded by definition therefore on the grounds that these neurons produce conventional transmitter substances (neurohumors) as opposed to neurohormones. The hypothalamus is one exception.

B) Neurosecretory Cells in the Mammalian Hypothalamus

It has become widely known that many of the neurons within the various nuclei of the hypothalamus are neurosecretory in nature. Of the neurons designated as neurosecretory, those of the

hypothalamo-neurohypophyseal complex have been studied most extensively. That the neurons of this system lie within the supraoptic and paraventricular nuclei of the anterior hypothalamus, and that their axons project caudally into the neurohypophysis, has been repeatedly demonstrated. Because the structure and functions of this system are now well accepted, and because it is not particularly germane to the text of this thesis, no further comment need be made on the neurohypophyseal system.

During the past two decades an increasing number of investigators have turned their attention to the hypothalamo-adenohypophyseal axis. With the development of the concept of hypothalamic releasing and inhibiting factors and their control over the release of pituitary trophic hormones, many investigators have attempted to identify the hypothalamic centers in which these factors are synthesized. The functional importance of the hypothalamus as a controlling center for ovulation has been recognized primarily as a result of studies in which carefully placed hypothalamic lesions, hypothalamic deafferentation, or electrical and chemical stimulation of precise hypothalamic areas have altered the ovulation patterns in a number of laboratory animals. Three hypothalamic areas have been implicated — the preoptic-anterior hypothalamic area (PO-AHA), the suprachiasmatic region, and the arcuate nucleus-median eminence (Arc-ME) complex.

It is presently widely accepted that the release of ovulatory surges of luteinizing hormone (LH) from the adenohypophysis is controlled by the neurohormone luteinizing hormone-releasing factor (LH-RF). In the rabbit, the release of LH-RF from its storage site and

consequent release of pituitary LH is induced by the physiological stimulus of coitus. Hilliard, Hayward and Sawyer (1964) have shown that measurable increases in the plasma levels of LH occur as early as 10 to 15 minutes following coitus in this animal. Assuming that this release of LH is mediated by LH-RF, depletion of the median eminence stores of this neurohormone must occur immediately after the stimulus of coitus. Throughout this investigation it was also assumed that the depletion of median eminence (ME) stores of LH-RF would be followed by the synthesis of this neurohormone in an attempt to replenish the lost stores.

If this synthesis does occur within the perikarya of neurons located some distance from the 'storage sites', as suggested by Halász (1969), Crighton, Schneider and McCann (1970), and Tima (1971), intra-neuronal changes reflecting enhanced synthetic activity within these cells should be apparent at the ultrastructural level. Palay and Palade (1955) have reported that the ultrastructural changes observed within other types of secretory cells and which have been interpreted to represent elevated synthesis, apply equally well to the neuron. Furthermore, that secretory cycles do, in fact, occur within cells of the central nervous system has been repeatedly observed both at the light (Scharrer and Scharrer, 1940; Bargmann, 1949) and electron microscopic levels (Sloper and Bateson, 1965; Zambrano and De Robertis, 1966; Flament-Durand, 1971).

The purpose of this investigation therefore was to study the ultrastructure of three nuclei of the rabbit hypothalamus; two of these, the preoptic (PO) and suprachiasmatic (SCH) nuclei being located

within the anterior hypothalamus and, the third, the arcuate nucleus located more caudally within the infundibular region of the hypothalamus. If the neurons of these hypothalamic nuclei are associated with the replacement of lost LH-RF stores from the ME following coitus in the rabbit, ultrastructural changes suggesting enhanced secretory activity might be observed with the electron microscope. Also, if the neurohormone LH-RF is stored within the dense-core (DC) vesicles of axons terminating in the ME, ultrastructural evidence suggesting the loss of the electron-dense material from these vesicles may represent its depletion. These findings would provide rather convincing morphological evidence for the present tenet regarding the functional roles played by hypothalamic neurons and the median eminence in the synthesis and storage of the neurohormone LH-RF.

II LITERATURE REVIEW

A) Brain-Pituitary Gland Relationships

A review of the literature for possible brain-pituitary relationships brings to our attention two widely separated periods in history - the second century A.D. and the past 40 years of the present century.

The first account of possible functional integrity between the brain and the pituitary gland is found in the Galenic doctrine (Galen ca 130-210 A.D.). The Greeks believed that vital spirits in the blood were transformed into animal spirits in the brain, and that the waste products from this reaction then passed to the pituitary gland through the funnel-shaped infundibular stalk and thence by 'ducts' to the nasal cavity as the 'pituita' or nasal mucus.

The modern views of brain-pituitary relationships, in particular, the role played by the central nervous system (CNS) in regulating gonadotrophin secretion, developed largely from the work of F.H.A. Marshall (1936, 1942). Although a number of earlier investigators were aware of the reflex nature of ovulation in certain mammalian species (Heape, 1905), Fee and Parkes (1929) were the first to mention the possible role of the pituitary gland in this endocrine function. They concluded that the immediate effects of coitus (in the rabbit)

were nervous in nature and that the mechanisms resulting in ovulation acted directly upon the anterior pituitary gland and not upon the CNS. The reviews of Marshall (1936, 1942) provided the first convincing evidence for internal endocrine sexual rhythms in mammals, and that the factors controlling these rhythms act through the intermediation of the CNS and the anterior pituitary gland. Thus, the pituitary gland was no longer looked upon as the 'conductor of the endocrine orchestra', rather, it was viewed as part of an integrated functional unit which also included the CNS.

Because of the close spatial relationship between the anterior pituitary and the hypothalamus, it is not surprising therefore that experiments were specifically designed to provide evidence for the possible functional relationships between these two endocrine centers. The most common neurophysiological techniques used were electrical stimulation and ablation of the hypothalamus, stalk sectioning and pituitary gland transplantation. The possible hypothalamic control of the anterior pituitary was largely determined by observing target organ responses to an increase or a decrease in the circulating levels of the various hypophyseal hormones.

Electrical stimulation of the hypothalamus was reported to stimulate the release of several adenohypophyseal hormones (luteinizing hormone - Harris, 1936, 1937, 1948a; Marshall and Verney, 1936; Haterius and Derbyshire, 1937, corticotrophin - de Groot and Harris, 1950; Hume and Wittenstein, 1950, and the thyrotrophic hormone - Harris and Woods, 1958, D'Angelo and Snyder, 1963).

The loss of gonadal function with subsequent atrophy following hypothalamic lesions has been confirmed repeatedly in a variety of laboratory animals including the dog (Camus and Roussy, 1920; Ganong, Frederickson and Hume, 1954), the rat (Heatherington and Ranson, 1940; Bogdanove, Spirtos and Hamli, 1955), the guinea-pig (Dey, 1941, 1943) and the cat (Robinson and Sawyer, 1957). Although the results of these lesions were often inconsistent, they did provide early support for possible hypothalamic influence over the activity of the anterior pituitary gland. The nature of this controlling influence was, however, unknown.

The effects of interrupting possible hypothalamic influence on pituitary function by cutting the pituitary stalk or by transplanting the hypophysis to a region remote from the hypothalamus have been investigated. Gonadal atrophy following stalk section has been reported in the rabbit (Harris, 1937; Westman and Jacobsohn, 1937) and in the rat (Westman and Jacobsohn, 1940). Conflicting reports of the recurrence of estrus in rabbits (Brooks, 1938) and of the resumption of estrous cycles in the rat following stalk section (Demsey and Uotila, 1940) probably resulted, at least in part, from the regeneration of the portal veins (Harris, 1949; Jacobsohn, 1954). More recently, excellent reviews of these early experiments on the effects of hypothalamic-hypophyseal separation on gonadotrophic functions have been compiled by Harris (1955a), Benoit and Assenmacher (1955), Nikitovitch-Winer and Everett (1958) and Greep (1961).

The transplantation of the hypophysis to a site distant from the base of the hypothalamus results in practically complete loss of

gonadotrophic function in both the male and female rat (Harris, 1955b; Everett, 1961a). Even hypophyseal transplants to such well nourished areas as the subarachnoid space or beneath the renal capsule showed no evidence of gonadotrophic secretion. If, however, the anterior pituitary is transplanted in close proximity to the hypothalamus, restoration of gonadotrophic function is restored in both sexes (Harris and Jacobsohn, 1952; Nikitovitch-Winer, 1960; Knigge, 1962; Halász, Pupp and Uhlarik, 1962).

In an attempt to elucidate the significance of secreto-motor fibers passing to the anterior pituitary gland from the area of the hypothalamus, a number of investigators (Brooks, 1937; Westman and Jacobsohn, 1937; Brooks, Beadenkopt and Bojar, 1940) independently succeeded in sectioning the infundibular stalk of the rabbit. Since post-coital ovulation was never observed in these animals, it was assumed that the loss of this endocrine-controlled function was the result of interrupting the secreto-motor innervation to the anterior lobe. It was later demonstrated, however, that the failure of these rabbits to ovulate was the result of interruption of the portal veins in the infundibular stalk, and not from the loss of secreto-motor fibers (Dey, Fisher and Ranson, 1941; Dey, 1943; Markee, Sawyer and Hollinshead, 1946). The absence of a secreto-motor control of hypophyseal activity has also since been supported by Markee, Sawyer and Hollinshead (1948), and Donovan and Harris (1956). For a summary of the early evidence for possible neural control of adenohypophyseal activity, the reader is referred to the review by G. W. Harris (1948b).

The notion of a 'humoral' control of the anterior pituitary gland emerged from the laboratories of Hinsey and Markee (1933), Harris (1937) and Brooks *et al.* (1940). The discovery of a system of portal veins (Popa and Fielding, 1930, 1933) provided the obvious route by which mediators of hypothalamic origin might reach the anterior lobe of the pituitary. That the direction of blood flow within the portal veins is in fact from the hypothalamus toward the pituitary gland, and not in the reverse direction as previously suggested by Popa and Fielding (1930, 1933), was first demonstrated in the Rhesus monkey by Wislocki and King (1936) and Wislocki (1937, 1938). However, it was not until 1947 that Green and Harris fully recognized the significance of this vascular link and proposed the 'neurohumoral hypothesis' for the control of anterior pituitary gland secretion. Their view held that "..... substances formed in or near the median eminence, due to the effect of environmental influences, pass along the portal vessels and so exert a dominating effect upon the secretory activity of the 'leader of the endocrine orchestra' - they conduct, if you will, the glandular symphony composed of the environment". The physiological evidence for this hypothesis was provided by the numerous experiments involving hypothalamic lesions, the electrical stimulation of the hypothalamus, interrupting of the infundibular stalk and pituitary transplants just described.

Thus, by the 1950's, many investigators had already recognized the hypothalamic-pituitary axis, and the vascular linkage afforded by the hypophyseal vessels, as a significant functional unit which integrates, at least in part, the activities of the endocrine system.

It seems logical therefore that attention now be directed to obtaining evidence suggesting the nature of the proposed hypothalamic neurohumors, their mechanisms of action, as well as to the areas of the hypothalamus associated with their synthesis.

B) Hypothalamic Releasing Factors

1. Terminology

The hypothalamic principles (neurohormones) proposed by Green and Harris (1947) were termed 'releasing factors' (RF's) by Saffran and Schally (1955) and Saffran, Schally and Benfey (1955). This terminology was suggested following the demonstration that rat stalk-median eminence (SME) possessed the property of releasing corticotrophin from the anterior pituitary gland *in vitro*. Since it was not known then (and the evidence is still incomplete today) whether these hypothalamic agents might also affect the 'synthesis' of the anterior pituitary hormones, the terms 'hypothalamic hypophysiotrophic principles' have also been used (Guillemin, 1971). Although inconvenient when applied to certain of the hypophyseal hormones (e.g., gonadotrophic-hypothalamic hypophysiotrophic hormone), the term 'hypophysiotrophic' alone is commonly used to describe the effects of a substance of hypothalamic origin which influences the activity of the anterior lobe. Also, when making general reference to the hypothalamic principles which are transferred to the adenohypophysis via the portal vessels, such terms as 'neurohormone', 'regulating substances', 'hypophysiotrophins', or 'neurochemical mediators' may be conveniently substituted for the term 'releasing factors' (B. Scharrer, personal communication).

More recently, Schally, Arimura, Bowers, Kastin, Sawano and

Redding (1968b) proposed that the hypothalamic releasing factors be designated as 'hormones' on the basis that the releasing factors, like other hormones, have the property of acting in minute amounts to regulate the release, and in some cases, the 'synthesis' of the trophic hormones. This new nomenclature would therefore have as its basis the abbreviation of the respective adenohypophyseal principle, followed by the terms 'releasing' or 'inhibiting', and then the word 'hormone'. For example, the originally suggested terminology 'corticotrophic-releasing factor' and abbreviation CRF (Saffran and Schally, 1955) would be subsequently replaced by CRH (corticotrophic-releasing hormone).

Although both systems of nomenclature appear in the literature, the terminology proposed by Saffran and Schally (1955) is convenient and is the one most frequently used. This terminology has also been adopted for the present study.

2. The first hypothalamic releasing factor.

The first direct evidence supporting the neurohumoral theory for the control of the anterior pituitary gland (Green and Harris, 1947) came from the work of Saffran and Schally (Saffran and Schally, 1955; Saffran *et al.*, 1955; Schally, Saffran and Zimmerman, 1958). These investigators used rat anterior pituitary tissue incubated *in vitro* as a test system to detect substances affecting the release of the corticotrophic hormone (ACTH). In this method (later modified and adapted for the detection of other hypothalamic hormones), a rat's anterior pituitary gland was halved and each half incubated separately in Krebs-Ringer bicarbonate medium. The material being assayed was added to one half before incubation, and to the other half after incubation. The

activity of the CRF in their crude hypothalamic extracts was then determined by the difference in corticotrophic activity in the media bathing the two pituitary halves.

Since the work of Saffran and his colleagues (1955-1958), at least ten other factors of hypothalamic origin and which affect the activity of the anterior pituitary gland have been reported. It should be appreciated that convincing evidence for the existence of each of these hypothalamic mediators was slow and unusually difficult to obtain. First, bioassays had to be developed with the specificity and sensitivity to measure the slightest changes in the circulating levels of each of the anterior pituitary hormones and, secondly, purification of the hypothalamic extracts was limited by the extremely low yields from available brain material. Furthermore, the results obtained by many of the earlier investigators in an attempt to elucidate the specific effects of 'releasing factors' on anterior pituitary gland activity were inconsistent due to the crude nature and variability of the hypothalamic extracts being used. However, with the recent development of highly sensitive assays for many of the hypophyseal hormones (Parlow, Monroe and Midgley, 1967; Niswender, Midgley, Monroe and Reichert, 1968) and the availability of certain of the releasing factors in highly purified form (Schally, Bowers, White and Cohen, 1967a; Vasova and Ginodman, 1968), it is presently accepted that for each of the hormones synthesized within the anterior pituitary gland, there is a corresponding hypothalamic 'releasing' and/or 'inhibiting' factor.

It is not the intention of the author to review the voluminous literature dealing with each of the known releasing factors. Since the present investigation is intended, at least in part, to provide ultrastructural evidence suggesting the intraneuronal synthesis of LH-RF within the rabbit hypothalamus, only the literature dealing with this hypothalamic principle will be reviewed.

C) Evidence for the Existence of Luteinizing Hormone-Releasing Factor (LH-RF)

The concept of CNS control over the regulation of LH secretion was based upon extensive physiological studies summarized in various books and reviews (Harris, 1955a, 1961; Greep, 1961; Flérko, 1962, 1963; Bogdanove, 1963; Schally *et al.*, 1968b; Everett, 1969; McCann and Porter, 1969; Campbell, 1970; Burgus and Guillemin, 1970; Everett, 1972). It is now well established that the mechanism of hypothalamic control of LH-release is neurohormonal (Guillemin, 1964; Campbell, Feuer and Harris, 1964; McCann and Ramirez, 1964; Harris, Reed and Fawcett, 1966; McCann, Dhariwal and Porter, 1968; Kamberi *et al.*, 1971a). The substance of hypothalamic origin and which has been repeatedly shown to stimulate the release of the trophic hormone LH, was designated luteinizing hormone-releasing factor (LH-RF) by McCann, Taleisnik and Friedman (1960).

1. Evidence for LH-RF using crude acid extracts of the stalk median eminence (SME)

The first direct evidence for the existence of a hypothalamic neurohormone which regulates the release of LH from the anterior pituitary gland was provided independently by McCann *et al.* (1960),

and the group led by the late Professor G. W. Harris (Harris, 1961; Campbell, Feuer, Garcia and Harris, 1961). It was noted that when crude acid extracts of rat SME tissues were tested *in vivo*, plasma LH levels were elevated (hence its release from the anterior pituitary) as determined by the depletion of ascorbic acid from the ovary. This technique, the ovarian ascorbic acid depletion (OAAD) test for the release of endogenous LH, was first described by Parlow (1958), and later confirmed for specificity and sensitivity by Parlow (1961), Guillemin, Jutisz and Sakiz (1963), Persky and Norton (1964), and many others.

Elevated plasma LH levels were also reported by Harris (1961) and Campbell *et al.* (1961) following direct infusions of acetic and HCl extracts of rabbit or beef SME into the anterior pituitary gland of the rabbit. The SME-induced release of LH was confirmed by the fact that ovulation was evoked in a high percentage of these animals. This was, incidentally, the first demonstration that separate hypothalamic releasing factors probably existed (see Campbell, 1970). It also provided early evidence for the absence of species specificity among the releasing factors. That hypothalamic extracts of at least seven animal species are capable of stimulating the release of LH has since been clearly demonstrated by several other groups of investigators (Courrier, Guillemin, Jutisz, Sakiz and Aschheim, 1961; Nikitovitch-Winer, 1962; McCann, 1962).

The trans-hypophyseal activity of acid extracts of hypothalamic origin was demonstrated by McCann *et al.* (1960) and McCann (1962). Intravenous injections of SME extracts were not active in hypophysectomized rats, even though the sensitivity of these animals to LH

release was similar to that of control animals. In normal rats, on the other hand, such injections were followed within 10 minutes by the appearance in the plasma of the test animals of significantly large amounts of LH. Further evidence that the hypothalamic agent acted directly on the pars distalis was obtained from rats with ME lesions and by injecting SME extracts into rats in which LH levels were depressed by estrogen or combinations of estrogen and progesterone. McCann and his colleagues also reported that the SME extracts yielded a linear response (stimulation of the secretion of LH) as a function of the log of the dose injected.

The effect of hypothalamic extracts on rat anterior pituitary tissue or single cell cultures incubated *in vitro* has been investigated by Kobayashi and his collaborators (Kobayashi, Kobayashi, Kigawa, Mizuno and Amenomori, 1963b). They found that crude extracts added to the culture medium, after the pituitary cells had lost their gonadotrophic activity, caused the reappearance of gonadotrophins in both the culture medium and the cells, as assayed in the uterine weight test in the immature mouse. The hypothalamic extracts themselves were inactive in this test. The *in vitro* effects of acid extracts of hypothalamic tissue were also studied by Schally and Bowers, 1964.

Thus, the LH-hypophysiotrophic activity of the acid extracts of SME origin, first described by McCann and his associates (McCann *et al.*, 1960), met most of the important criteria outlined by Guillemin (1964) as prerequisites for the characterization of a substance as a hypothalamic mediator of a pituitary function. Since these initial reports, the detection of LH-releasing activity of crude

hypothalamic extracts from a variety of mammalian species including the rat, mouse, guinea-pig, rabbit, dog, sheep, cattle, pig, monkey and man has been claimed by workers in many laboratories (Guillemin *et al.*, 1963; Endröczi and Hilliard, 1965; Dhariwal, Naller, Batt and McCann, 1965; Jutisz, Berault, Novelle and Ribot, 1967; Schally, Arimura, Müller, Saito and Bowers, 1968a).

2. Evidence for LH-releasing activity of partial and highly purified LH-RF

In view of the rather crude nature of the hypothalamic extracts used, the validity of many of the reports cited in section C-1 (above) was very much in doubt. In fact, the very existence of an LH-releasing factor of hypothalamic origin was, at least temporarily questioned. The then most widely used method of determining LH-releasing activity of a test substance was to measure the depletion of ovarian ascorbic acid. However, it has since been independently shown that ovarian ascorbic acid is also reduced by such contaminants of crude hypothalamic extracts as vasopressin (Courrier *et al.*, 1961; Parlow, 1961; McCann and Taleisnik, 1961). More recently, it has been demonstrated that vasopressin can be removed from these extracts by treating them with reducing agents such as thioglycollate (Ramirez, Naller and McCann, 1964a) or by further purification using chromatographic techniques (Guillemin *et al.*, 1963; Ramirez *et al.*, 1964a; Schally *et al.*, 1967a). The failure of vasopressin to stimulate the release of LH from the anterior pituitary has also been demonstrated by Fink, Naller and Worthington, 1967. Two other contaminants; namely,

LH and copper salts, which also have an effect on ovarian activity (Guillemin *et al.*, 1963; Courrier, Jutisz and Cologne, 1963), are likewise removed by further purification with chromatography (Vasova and Ginodman, 1968).

The first partial purification of LH-RF from acid extracts of bovine (beef) and porcine (pig) hypothalamic tissue was obtained by gel filtration with Sephadex G-25 followed by ion-exchange chromatography (Schally and Bowers, 1964; Fawcett, Harris and Reed, 1965; Guillemin *et al.*, 1963). Biological activity was determined by the OAAD test (Parlow, 1961), or by the property of the partially purified factor to induce ovulation in the rabbit or rat following intrapituitary infusion. According to the OAAD assay, the LH-RF obtained by these purification methods, was active at 3.0 ug/dose (Schally and Bowers, 1964); it also stimulated ovulation in animals in which the spontaneous cyclic release of LH had previously been inhibited by hypothalamic lesions (Schiavi, Jutisz, Sakiz and Guillemin, 1963), as well as elevating plasma LH levels in ovariectomized rats pretreated with estrogen and progesterone (Ramirez and McCann, 1963). Furthermore, the samples of LH-RF obtained by these methods were also shown to release LH *in vitro* (Schally, Bowers and Loche, 1964)

Since 1967, at least several groups of investigators have independently isolated highly purified LH-RF from tissue fragments of pig, sheep, cattle and human hypothalami (Schally, Carter, Arimura, and Bowers, 1967b; Schally, Arimura, Bowers, Sawano, Kastin, Redding, Saito, White and Cohen, 1968c; Schally, Arimura, Kastin, Reeves, Bowers, Baba and White, 1970a; Schally, Arimura, Bowers, Kastin,

Wakabayashi, Mittler, Redding and Pizzolato, 1970b; Fawcett, Reed, Charlton and Harris, 1968; Vasova and Ginodman, 1968; Reeves, Arimura and Schally, 1970a; Amoss, Burgus, Blackwell, Vale, Fellows and Guillemin, 1971). The LH-releasing activity of purified LH-RF, both *in vivo* and *in vitro*, has largely been determined by using ultrasensitive radioimmunoassay techniques developed recently by Niswender *et al.*, 1968. Elevated plasma LH following injections of purified LH-RF (ovine, bovine and porcine) were observed in pseudopregnant rats pretreated with estrogen and progesterone, and in ovariectomized female and castrated male rats pretreated with testosterone (Schally *et al.*, 1967b). More recently, Hilliard, Schally and Sawyer (1971) induced ovulation in the estrous rabbit by infusing a highly purified preparation of porcine LH-RF directly into the adenohypophysis through stereotaxically implanted cannulae.

Significant increases in serum LH in response to purified LH-RF of ovine or porcine origin were also reported in the sheep (Amoss and Guillemin, 1969; Reeves *et al.*, 1970a). Also, in studies designed to test pituitary responsiveness to purified LH-RF during various stages of the estrous cycle in the sheep, Reeves, Arimura and Schally (1970b) observed that maximum increases in LH after LH-RF administration were far below preovulatory LH levels previously reported (Goding, Catt, Brown, Kaltenbach, Cumming and Mole, 1969). Moreover, the responses to LH-RF in terms of increase in plasma LH in the castrated ram were much higher than in intact ewes. These findings suggest that pituitary responsiveness to LH-RF differs under various physiological conditions and thus could be important in the

surge of LH release prior to ovulation (Reeves *et al.*, 1970b; Reeves, Arimura and Schally, 1971).

The effects of purified LH-RF on the release of LH *in vitro* has also been widely reported (Schally *et al.*, 1967a; Jutisz, 1967; Jutisz *et al.*, 1967; Crighton, Watanabe, Dhariwal and McCann, 1968; Jutisz, Kerdelhué and Bérault, 1970a; Jutisz, de la Llosa, Bérault and Kerdelhue, 1970b). Vasova and Ginodman (1968) also observed an increased release of LH from rat anterior pituitary *in vitro* using purified human LH-RF. For a review of the bioassays of the hypophysiotrophic hormones in both *in vitro* and *in vivo* systems, the reader is referred to the reviews by Guillemin and Vale (1970), and McCann (1970a).

D) The Biogenic Amines and the Release of LH

A number of investigators have independently suggested that aminergic mechanisms might be involved in the regulation of gonadotrophin release from the anterior pituitary (Sawyer, Markee and Townsend, 1949; Sawyer, 1959; Everett, 1964; Coppola, 1969, Ahrén, Fuxe, Hamberger and Hökfelt, 1971). In 1952, Markee, Everett and Sawyer, and more recently Sawyer (1963) observed that amines, when injected into the third ventricle, induced ovulation in the rabbit. This concept has since been supported by the fact that the monoamine content of the hypothalamus varies during the estrous cycle, during pregnancy, and following castration in the rat (Kobayashi, Kobayashi, Kato and Minaguchi, 1964; Zolovich, Pearse, Boehlke and Eleftheriou, 1966; Fuxe, Hökfelt and Nilsson, 1967, 1969a,b; Stefano and Donoso, 1967; Ahrén *et al.*, 1971). Using fluorescent techniques, Falck (1962),

Fuxe (1964), Fuxe and Hökfelt (1967) and Hökfelt (1967) have shown that monoamine concentrations are very high in certain regions of the hypothalamus, particularly the ME — the proposed hypothalamic center for the synthesis and/or storage of the releasing factors. For a review of the fine structural location of the biogenic monoamines in nervous tissue, the reader is referred to the work of Bloom (1970).

Further support for the possible amine control of LH release from the anterior pituitary came from the work of Schneider and McCann (1969a). It was noted that when DA was injected into the third ventricle of the rat, the plasma levels of LH rose significantly as determined by the radioimmunoassay techniques of Niswender *et al.*, 1968. This seemed to suggest that the factor controlling LH release from the pituitary might be one of the hypothalamic amines.

However, it has recently been shown that if DA, epinephrine (E), norepinephrine (NE), serotonin (5-HT) or melatonin are injected directly into the portal veins, or into the anterior pituitary, the release of LH is not affected (Kamberi *et al.*, 1969, 1971a; Kamberi, Mical and Porter, 1970a,b; Porter *et al.*, 1970). Furthermore, it has been observed that if DA is injected directly into the third ventricle, the concentration of LH-RF within samples of the hypophyseal portal blood (as determined by its property to release LH both *in vivo* and *in vitro*) is greatly increased (Kamberi *et al.*, 1969, 1970a; Schneider and McCann, 1970a,b,c; Porter, Mical, Ondo and Kamberi, 1972). Kamberi and workers noted that pituitaries incubated in stalk plasma from donor rats that had received an intraventricular injection of DA

released 450% more LH than did pituitaries incubated in femoral artery plasma. On the other hand, pituitaries incubated in stalk plasma from donor rats that were not injected with DA released only 70% more LH than did pituitaries incubated in peripheral blood plasma. These studies provide the basis for the present widely accepted view that the role of DA in the CNS is to regulate the release of LH-RF from storage sites in the ME. The LH-RF in turn activates the release of LH from the anterior pituitary (Schneider and McCann, 1970c; Rubenstein and Sawyer, 1970; Porter *et al.*, 1970, 1972; Kamberi *et al.*, 1971a; Kamberi, Mical and Porter, 1971b).

E) LH-RF in the Hypophyseal Portal Blood

The presence of LH-RF in the blood of the hypophyseal portal system was first demonstrated by Fink (1967a,b) and Fink *et al.* (1967). Samples of hypophyseal-portal plasma from both intact and hypophysectomized rats were highly active in releasing LH from most animals as determined by the OAAD test (Parlow, 1961). These findings have recently been confirmed by Fink and Harris (1970), Porter *et al.* (1970, 1972) and Harris and Ruf (1970). However, it was the elegant work of Porter and Mical (1969) and Kamberi *et al.* (1969, 1971a) which convincingly demonstrated the role of the hypothalamic-hypophyseal portal veins as a transport system for the hypothalamic hormones. These investigators independently described a technique for microcannulation of the stalk-portal veins and thereby making it possible to infuse (downstream) test substances into the anterior pituitary gland *in vivo*. This technique made it possible for investigators to calculate precisely the rate at which a test substance

is delivered into the anterior pituitary; also whether or not test substances act directly on the pituitary gland or indirectly through the hypothalamus. Samples of LH-RF infused into the stalk vessels of adult rats resulted in a significant increase in plasma LH over a 20 minute period. Other substances (5-HT, E, NE, melatonin and DA), injected at the rate of 2 µl/min, had no effect on the release of LH (Porter and Mical, 1969; Porter *et al.*, 1970; Kamberi *et al.*, 1969, 1971a).

Further evidence for the hypophyseal-portal vein concept of hypothalamic hormone 'transport' has been provided by recent studies in which the LH-RF content of the portal blood varied with physiological conditions. Kamberi *et al.* (1969) demonstrated that pituitary halves incubated with stalk plasma of untreated animals released 1.8 times more LH than those incubated in peripheral plasma. Similarly, pituitary halves incubated with portal plasma following DA injection into the third ventricle released 4.3 times as much LH as did pituitary halves incubated in untreated stalk plasma. LH levels in the incubation media were determined by the radioimmunoassay techniques of Niswender *et al.* (1968). More recently, Kamberi *et al.* (1971b) have observed that LH-RF, as well as follicle stimulating hormone-releasing factor (FSH-RF) and prolactin-inhibiting factor (PIF) activity in stalk blood are altered following the injection of DA into the third ventricle of the rat brain. Fluctuating levels of LH-RF in the rat hypothalamus have also been reported during various phases of the estrous cycle (lowest during late proestrous and early estrus) by Schally *et al.* (1968c). Furthermore, the LH-RF activity

reported by McCann and Porter (1969) in rats following hypophysectomy, disappeared following lesions in the ME (Schneider and McCann, 1970b).

F) LH-RF - Chemical Nature and its Effects on the Release of LH and FSH

The chemical nature and effects of LH-RF on the possible release of FSH, as well as LH, are poorly defined (Fawcett *et al.*, 1968; Schally *et al.*, 1968a,c, 1970a; Kastin, Schally, Gual, Midgley, Miller and Cabeza, 1971a). Since the early reports in 1955 which brought about good evidence for the releasing factors, it was proposed that these mediators would prove to be polypeptides of low molecular weight. This proposal was primarily based on the fact that it was already known that other areas of the hypothalamus secrete vasopressin and oxytoccin, both of which are octapeptides. Indeed, as already reviewed, the attempts to purify the hypothalamic factors were conducted by methods classically used for the purification of polypeptides.

The polypeptide nature of LH-RF received early support from the work of Schally and Bowers (1964) on the basis of the high amino acid content of their crude hypothalamic extracts. Similar findings were independently reported by Guillemin (1964, 1967a) and Fawcett *et al.* (1965). More recently, however, the polypeptide nature of LH-RF has been questioned, particularly on the basis of the fact that inactivation with various proteolytic enzymes (trypsin, pepsin and ninhydrin) often resulted in the loss of LH-releasing activity of this hypothalamic mediator (Fawcett *et al.*, 1968; Gregory, Walpole, Charlton and Harris, 1968; Burgus and Guillemin, 1970; Currie,

Sievertson, Bogentoft, Chang, Folkers, Bowers and Doolittle, 1971). However, the results of such studies have not been consistent.

It is now generally accepted, at least by those investigating the chemistry of LH-RF, that it is a decapeptide. For a recent review of the techniques used to determine the amino acid content and sequence of the LH-RF molecule, the reader is referred to the work of Amoss *et al.* (1971). Concurrently, Matsuo, Baba, Nair, Arimura and Schally (1971) succeeded in isolating a decapeptide from porcine hypothalamic extracts with the following amino acid sequence: (pyro) Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. This decapeptide has also been synthesized (Sievertson, Chang, Bogentoft, Currie, Folkers and Bowers, 1971) and assayed for the *in vivo* release of LH in rats using the methods of Ramirez and McCann (1963). Test samples of this decapeptide also significantly increased the levels of plasma LH as determined by radioimmunoassay methods.

The molecular weight of this decapeptide is not definitely known. Earlier Schally and Bowers (1964) proposed a molecular weight of 1200-2000. More recently, however, most of the molecular weights reported for LH-RF are below 600 (Schally *et al.*, 1968a; White, 1970). The most recent accounts dealing with the chemistry of LH-RF are those of Schally *et al.* (1970a); Schally, Baba, Arimura, Redding and White (1971a); Amoss *et al.* (1971); Currie *er al.* (1971); Chang, Sievertson, Bogentoft, Currie, Folkers and Bowers (1971) and Guillemin (1972).

Recently, White (1970), proposed a hypothalamic 'monotrophic'

control for the hypophyseal hormones LH and FSH on the basis that LH-RF activated the release of both LH and FSH from the anterior pituitary. Similar findings were concurrently reported by Schally *et al.* (1970a) and Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk and White (1971b) while studying the effects of purified LH-RF on the *in vitro* release of LH in the rat. It was also noted that inactivation of the test samples of LH-RF resulted in both a loss of LH and FSH from their incubation media (hence a loss of LH and FSH from the adenohypophysis) as determined by radioimmunoassay methods. Similarly, Kastin, Zarate, Midgley, Canales and Schally (1971b) observed an increase in the circulating levels of both LH and FSH in the human following administration of purified LH-RF. The concomitant release of LH and FSH has also been reported by Kamberi, Mical and Porter (1971a). However, the release of LH induced by LH-RF *in vitro* was considerably higher than that of FSH (Amoss *et al.*, 1971).

Thus, whether or not there is a common hypothalamic mediator for the release of both LH and FSH, or whether two separate factors for LH and FSH actually exist but share the property of releasing both hypophyseal hormones, are current matters of speculation and obviously must await further study.

G) The Mammalian Hypothalamus

The hypothalamus is the most ventral of the four regions of the mammalian diencephalon. Its rather diffuse mass is divided into similar right and left halves by the vertically oriented third ventricle (V_{III}).

The boundaries of the hypothalamus are not clearly defined. In higher vertebrates, and particularly in mammals, the hypothalamus extends dorsally at the ventricular surface to the dorsal thalamus, from which it is separated by the hypothalamic sulcus. Ventrally, the hypothalamus forms the only free surface of the diencephalon and extends from behind the optic chiasma caudalward to include the mammillary bodies. These bodies, along with the tuber cinereum, a small ME with attached infundibular stalk, form the only certain landmarks visible from the ventral surface. Laterally the hypothalamus is bounder by the various nuclear groups of the subthalamus and by the optic tract. Caudally, the hypothalamus continues backward as the ventral part of the diencephalon and merges (without demarcation) with the rostral limits of the midbrain tegmentum. The caudal boundary of the hypothalamus is, however, usually established, in those forms having mammillary bodies, by an imaginary, slightly concave, transverse plane which touches upon the caudal end of the mammillary bodies and continuing dorsalward to the region of the posterior commissure. Rostrally, the hypothalamus ends beneath the caudal extension of the preoptic area. Any demarcation or separation of these two areas of the mammalian brain is, again, purely arbitrary. Whether or not one regards the preoptic area as a part of the diencephalon, as Kuhlenbeck (1954) has suggested, or as part of the telencephalon (Le Gros Clark, 1936), these areas and the hypothalamus are intimately interrelated functionally and must be considered together (Crosby and Showers, 1969).

In most vertebrates, the preoptic area lies at levels immediately rostral to and over-lapping the rostral end of the optic

chiasma. Dorsally it reaches to the anterior commissure, with the diagonal band of Broca separating the preoptic area from the tuberculum olfactorium ventrally. Caudally, the preoptic area passes into the hypothalamus at levels dorsal to the optic chiasma. In the medial region of the preoptic area, this separation is often along an oblique plane, such that the caudal end of the dorsal part of the preoptic area overlaps the more ventrally situated rostral pole of the hypothalamus. The lateral preoptic area merges with the lateral hypothalamic area (Crosby and Showers, 1969).

Any attempt to divide the mammalian hypothalamus into dorsal and ventral regions would be forced and would provide only a convenient approach for the description of the hypothalamus. However, both the preoptic and hypothalamic areas have been subdivided in various other ways. Longitudinally, two zones - a medial and a lateral - have been described by Crosby and Woodburne (1940). More recently, three such longitudinal zones are generally recognized; namely, a periventricular zone (largely fibrous and lying nearest the third ventricle), a middle or medial zone (containing many of the hypothalamic and preoptic nuclei) and a lateral zone (largely fibrous with only a few clearly identifiable nuclear groups). The preopticohypothalamic portion of the diencephalon can also be subdivided arbitrarily into several major coronal regions (e.g., the <u>posterior hypothalamus</u> which includes the mammillary and tuberal regions, the <u>infundibular level</u> in the region of the infundibular stalk, and the <u>anterior hypothalamus</u> which extends forward from the

caudal border of the optic chiasma to include the chiasmatic and preoptic areas). It should be mentioned that these are vague descriptive terms only, and should not be used with reference to the functional significance of any part of the hypothalamus.

A large number of studies using cytoarchitectonic and/or topographical methods have clearly demonstrated the presence of many distinct neuron groupings or nuclei within the mammalian hypothalamus (Ingram, Hannett and Ranson, 1932; Le Gros Clark, 1936; Bodian, 1939; Christ, 1969). The location of these nuclei within the hypothalamic and preoptic areas have been described (Crosby, Humphrey and Lauer, 1962; Barr, 1969, 1972; Bossy, 1970). Perhaps one of the most recent and complete reviews of the hypothalamic nuclei is that of Christ (1969). This review not only describes the cytoarchitecture and extent of each of the known nuclear groups, but also includes a useful atlas of the structural organization of the hypothalamus of the rat, rabbit, cat, dog and monkey brain. Crosby and Showers (1969) have also surveyed the general trends in the structural development of the preoptic and hypothalamic areas of representative submammalian and mammalian forms, including man. Reviews dealing specifically with the rabbit brain include those of Rioch, Wislocki and O'Leary (1940) and Rose (1942), and more recently that of Sawyer, Everett and Green (1954).

Of the hypothalamic nuclei described in the works of Sawyer *et al.* (1954), Christ (1969) and Nauta and Haymaker (1969), only three are of interest in the present study. Two of these, the PO and the

SCH nuclei lie within the anterior hypothalamus, and the third, the arcuate nucleus, is located within the infundibular region.

H) Areas of the Mammalian Hypothalamus Associated with the Synthesis of LH-RF

The existence of a hypothalamic mediator which acts specifically on the anterior pituitary to stimulate the release of LH appears to be established beyond reasonable doubt (McCann and Porter, 1969). It has also been suggested that the hypophysiotrophic principles have a discrete localization within the hypothalamus (McCann *et al.*, 1968). However, as reported recently by Guillemin (1971), there is no convincing evidence that the hypophysiotrophic principles are synthesized within neuron soma of the known hypothalamic nuclei.

1. Median Eminence - a possible synthesis site of LH-RF

It is well documented that a large number of the early investigations done in an attempt to provide evidence for the very existence of LH-RF, were studies using extracts of ME tissue, or the placement of lesions within, and/or electrically stimulating, this area of the hypothalamus. Since many of these studies significantly altered the release of LH both *in vitro* and *in vivo*, it was generally concluded that the ME may be the site of synthesis of the hypothalamic mediator LH-RF (McCann *et al.*, 1960; Campbell *et al.*, 1961; McCann, 1962; Flérko, 1962; D'Angelo, 1963).

Early evidence for the presence of LH-RF within the ME region of the hypothalamus was provided by Halász *et al.* (1962). It was observed that anterior pituitary implants, when placed into a

relatively wide area of the medial basal hypothalamus (MBH), retained their normal histology despite lack of contact with the ME capillary loop system. Previously, Nikitovitch-Winer and Everett (1958, 1959) had emphasized the essential role of the hypophyseal vessels in maintaining their anterior pituitary implants. The hypothalamic area which supported the functional integrity of the anterior lobe was termed the 'Hypophysiotrophic Area' (HTA) by Halász *et al.* (1962) and defined as the horseshoe-shaped region of tissue at the base of the hypothalamus and which included the whole of the arcuate nuclei and extending forward to the posterior border of the optic chiasma or, more commonly, the retrochiasmatic area.

To further elucidate the hypophysiotrophic capacity of the HTA and to provide possible evidence for the synthesis of LH-RF within this region, Halász and Pupp (1965) and Halász and Gorski (1967) developed a technique for interrupting all the nervous afferents to the HTA in such a way that the 'hypothalamic islands' thus formed remained connected to the pituitary gland through the infundibular stalk. The capacity of the HTA to maintain normal secretory activity of the anterior lobe was then assessed by observing the changes which occurred in the target organs three to five weeks following deafferentation. It was concluded that the isolated HTA was capable of maintaining fairly normal structure and function of the thyroid, adrenal and testis, but not that of the ovary. Female rats with neurally isolated HTA's developed polyfollicular ovaries and persistent vaginal cornification. Ovulation failure, however, was only observed in those animals in which the afferents from the anterior regions of

the hypothalamus were destroyed; all afferents into the HTA from the lateral and posterior areas of the hypothalamus were left intact. Also, these preparations did not interfere with the maintenance of the pituitary gland, nor result in gonadal atrophy (Halász and Gorski, 1967).

These findings strongly suggested that the neurally isolated HTA could not release LH-RF in amounts necessary to induce ovulation, and were in agreement with the earlier hypothesis of a dual hypothalamic mechanism for the control of gonadotrophin secretion, proposed independently by Barraclough and Gorski (1961) and Flérko (1962, 1963). According to this hypothesis, one hypothalamic mechanism controls the basal or 'tonic' release of gonadotrophins from the anterior pituitary, while the other, or higher level of control, is responsible for the 'cyclic' release of gonadotrophins (LH) necessary for ovulation to occur. It was further suggested (Barraclough and Gorski, 1961) that the center controlling the 'tonic' levels of gonadotrophins is located within the arcuate-ventromedian nuclear region of the hypothalamus, while the neuronal center controlling the release of ovulatory surges of LH lies within the more rostral areas of the hypothalamus. That the anterior hypothalamus is in some way associated with the cyclic release of LH has received support from studies in which electrical stimulation (Critchlow, 1958; Everett, 1964; Hagino, 1969) or electrolytic lesions (Barraclough, 1963) of the PO-AHA has induced and abolished ovulation respectively.

Rather convincing evidence that the HTA is in fact associated with the synthesis of at least low levels of LH-RF has emerged from the

recent deafferentation studies of Tima (1971). Using the Halász technique, neurally isolated hypothalamic islands were prepared and the ME assayed eight to 15 days later for hypophysiotrophic activity. It was assumed that if the anterior hypothalamus was totally responsible for the synthesis of this neurohormone, hypophysiotrophic activity should disappear completely from the HTA. Since this did not occur, as determined by the OAAD test (Parlow, 1961), it was concluded that measurable levels of LH-RF within the ME were being maintained by neurons located within this region. These findings were in agreement with the earlier studies of Ramirez, Abrams and McCann (1964b) in which lesions placed into the ME of the rat prevented the rise of plasma LH following ovariectomy.

For a review of the feed-back effects of ovarian steroids on the cells of the HTA, the reader is referred to the work of Flérko (1967), Gorski (1968) and Stumpf (1971).

2) The anterior hypothalamus and the synthesis of LH-RF

Since the dual mechanism of hypothalamic control of LH release was first proposed, it has been demonstrated beyond reasonable doubt that the more anterior regions of the hypothalamus, directly or indirectly, control the release of ovulatory surges of LH from the anterior pituitary. Many investigators, however, have failed to clearly define the precise area, or areas, of the hypothalamus incorporated into their studies, therefore the precise roles played by the PO and SCH nuclei in the synthesis of LH-RF are difficult to assess. For example, Everett (1965) on the basis of the results obtained from a series of electrochemical studies, concluded that the neural substrate

for the ovulatory release of LH occupies a funnel-shaped region of tissue which includes the septal-PO area rostrally, and continues caudally through the anterior hypothalamus to terminate in the MBH. Others make reference to the PO-AHA without clearly indicating whether only the PO nuclei, or both the PO and SCH nuclei were involved in their studies. Occasionally the SCH nucleus, one of the anterior hypothalamic nuclei, has also been included with the HTA of the hypothalamus. Aware of the rather inconsistent way in which these nuclei have been treated, the author will now attempt to briefly summarize the evidence that both the PO and SCH nuclei of the anterior hypothalamus are associated with the synthesis of the LH-RF which controls the release of ovulatory surges of LH from the adenohypophysis.

a) The preoptic (PO) nucleus and the synthesis of LH-RF

That the PO area of the anterior hypothalamus is associated with the release of ovulatory surges of LH from the anterior pituitary has been suggested since the 1930's. Marshall and Verney (1936), Harris (1937) and Haterius and Derbyshire (1937) independently reported ovulation in the rabbit following electrical stimulation of the anterior hypothalamus. The region most sensitive to electrical stimulation was described by Haterius and Derbyshire (1937) as the circumscribed area located directly above and anterior to the optic chiasma. Furthermore, these workers also reported that electrolytic lesions placed a short distance from this area had no effect on ovulation, suggesting that the release of ovulatory surges of LH from

the anterior lobe of the pituitary is controlled by a localized area of the hypothalamus.

Since these early studies, the onset of ovulation in response to electrical stimulation of the PO area of the anterior hypothalamus has been demonstrated in a variety of laboratory animals (Markee *et al.*, 1948; Christian and Markee, 1958; Everett, 1961b,1964; Quinn, 1969; Clemens, Sharr, Kleber and Tandy, 1971a,b; Breed and Charlton, 1971; Kalra and McCann, 1971; Kalra, Ajika, Krulich, Fawcett, Quijada and McCann, 1971). Ovulation following electrochemical stimulation of this area has also been reported (Everett and Radford, 1961; Everett, 1964; Terasawa and Sawyer, 1969). That the PO-AHA of the hypothalamus is associated with the release of ovulatory surges of LH is further supported by studies in which electrochemical stimulation of this area induced ovulation in rats pretreated with such blocking agents as sodium pentobarbital (Quinn, 1966; Hagino, 1969) and nembutal (Kalra *et al.*, 1971). On the basis of these findings, it was suggested that the role of the PO area in regulating ovulation is that of 'amplifying' the afferent impulses to the HTA. These impulses were thought to then activate the release of LH in amounts necessary to induce ovulation. Although no mention was made of the actual synthesis sites of LH-RF, such a scheme strongly suggests that the arcuate-ME area of the MBH is most intimately associated with this function.

The effects of electrolytic lesions placed within the PO-AHA have been studied in a number of laboratory animals (D'Angelo and Kravatz, 1960; Barraclough and Gorski, 1961; Taleisnik and McCann, 1961; Tejasen and Everett, 1967; Kalra and Sawyer, 1970; Prezekop

and Domanski, 1970). The results of these investigations were, in general, consistent in that the electrolytic destruction of this area usually resulted in anovulatory, persistent estrus. These findings, however, were not consistent with those of Prezekop and Domanski (1970). Using the sheep as their experimental animal, these workers reported that animals with similar lesions showed regular estrous cycles and ovulations. On the basis of these observations, Prezekop and Domanski (1970) suggested that either their lesions did not destroy specific hypothalamic nuclei, or that the fibers which control the release of LH from the anterior pituitary, merely pass through the anterior hypothalamic area (AHA) and are not fibers of the neurons located within this area. That such a pathway might exist for the regulation of cyclic LH-release had, in fact, already been suggested by Everett (1965). Based on a series of studies in which electrochemical stimulation was applied to the rat hypothalamus, it was proposed that the mechanism controlling the 'cyclic' release of LH may be located within, or merely pass through the PO area. Upon reaching the HTA, these fibers stimulate the neurons of this area which, in turn, send their fibers to the ME where they terminate on the capillary system of the hypophyseal portal vessels. Although this concept has received recent support (Mess, Fraschini, Motta and Martini, 1967; Crighton *et al.*, 1970; Kalra *et al.*, 1971; Clemens *et al.*, 1971b) the precise location of the neurons which synthesize LH-RF (if in fact they do) has never been described. In general, these investigators agree that the LH releasing factor is localized to a long medial basal zone of the hypothalamus extending from the optic chiasma caudally to the pituitary stalk, and not within the more

anterior areas 'sensitive' to LH release. Clemens *et al.* (1971a), on the other hand, report that nearly any site along an area of the brain extending from the arcuate nucleus rostrally through the AHA and PO areas and ending in the septal area, when stimulated electrochemically, will increase the plasma levels of LH.

b) The suprachiasmatic nucleus (SCH) and the synthesis of LH-RF

The notion that at least part of the synthesis of LH-RF which controls the 'cyclic' release of LH from the anterior lobe lies within the SCH area has received support from studies in which electrolytic lesions have been placed within this area of the hypothalamus (D'Angelo and Kravatz, 1960; Taleisnik and McCann, 1961; Barraclough, Yrarrazaval and Halton, 1964). More recently, Antunez-Rodriques and McCann (1967) observed that similar lesions, when placed within the SCH area of the rat, blocked ovulation, but failed to alter the basic circulating levels and pituitary content of LH as compared with non-lesioned, diestrous rats. Furthermore, these lesions did not prevent the increase of plasma LH levels which normally follow ovariectomy, but were successful in blocking progesterone-induced ovulation in the rat as reported earlier by Barraclough *et al.* (1964).

In an attempt to further investigate the SCH area as a possible site for LH-RF synthesis, Schneider, Crighton and McCann (1969) placed electrolytic lesions in the SCH area of both intact and ovariectomized rats and studied the changes in LH-RF stores at the level of the ME. This was done by preparing ME extracts of both lesioned and non-lesioned animals and comparing their LH releasing

activity *in vitro*. Since the ME extracts of lesioned animals failed to activate the release of LH in amounts equal to those prepared from non-lesioned animals, it was concluded that some of the LH-RF secreting neurons were destroyed by the lesions. Concurrently, Crighton and Schneider (1969) and Crighton *et al.* (1970), demonstrated that extracts prepared from blocks of tissue which contained the SCH area, significantly increased the *in vitro* release of LH.

Similarly, changes marked by a decreased concentration of LH-RF in the ME area following neural isolation of the HTA, have been reported by Tima (1971) and Butler and Donovan (1971). It was observed that, when the SCH area was excluded from their 'hypothalamic islands', a significant reduction occurred in the ME stores of LH-RF (Tima, 1971). On the other hand, Butler and Donovan (1971), reported that animals with 'hypothalamic islands' which included the SCH area, or in which the anterior connections to the hypothalamus were severed by a cut placed just anterior to the SCH nucleus, developed persistent vaginal estrous. Interrupting of the posterior connections to the hypothalamus did not interfere with the estrous cycles in these animals. These facts, according to Crighton *et al.* (1970), suggested that the more rostral regions of the hypothalamus make an appreciable contribution to the level of LH-RF in the SME area, although the ME and associated nuclei may themselves constitute a site for the synthesis of LH-RF.

That the SCH and PO areas of the hypothalamus should perhaps be considered together when discussing the neuronal mechanisms controlling ovulation has been suggested (Crighton *et al.*, 1970). This

concept was based on the results of previous studies in which electrical stimulation of the suprachiasmatic-preoptic area (SCH-POA) resulted in ovulation in the intact, pentobarbital-blocked female rat (Everett, 1964). The implantation of estrogen in this area of the intact, immature female rat causes ovulation, whereas lesions in the SCH-POA block the response (Döcke and Dörner, 1965; Smith and Davidson, 1968). Also, labelled estradiol is taken up preferentially in this area (Kato and Villee, 1967). These findings all support the concept of a dual hypothalamic mechanism for the control of LH from the anterior pituitary (Barraclough and Gorski, 1961; Flerko, 1962, 1963). Based on the LH-releasing activity of their crude extracts prepared from SCH cubes and the changes observed in the ME stores of LH-RF following the placement of lesions into the SCH area, Schneider *et al.* (1969) concluded that the LH-RF secreting neurons may be located in the SCH area and be responsible for the discharge of ovulatory amounts of LH. Similarly, based on the LH-releasing activity of male rat hypothalamic extracts, Crighton *et al.* (1970) summarized their findings as follows: '.....from our work on the localization of LH-RF as far rostrally as the suprachiasmatic region, it appears likely that the ovulatory surge of LH is brought about by the rostral LH-RF neurons whose axons presumably project to the median eminence, there to discharge the releasing factor which triggers the ovulatory discharge of LH from the adenohypophysis....'.

III MATERIALS AND METHODS

A) The Experimental Animal

The rabbit is perhaps the best known of the species which ordinarily requires coital stimulus to induce ovulation. It is also commonly known that the sexual excitement associated with mating - even in the absence of vaginal penetration, or on being mounted by another female, will induce ovulation and pseudopregnancy in the estrous female. Although the neural afferents responsible for the release of ovulatory surges of LH from the adenohypophysis are not clearly understood, it is generally accepted that the sensory or psychic stimulation accompanying copulation in the rabbit acts through the hypothalamus (Sawyer and Markee, 1959).

The rabbit was chosen as the experimental animal for the present study because of its reflex nature of ovulation (Heape, 1905; Donovan and Harris, 1965) which normally occurs nine to 11 hours post-coitus (Sawyer and Markee, 1959; Everett, 1961a). The ME and hypothalamic nuclei could, therefore, be removed at specific times after coitus and processed for study with the electron microscope. Furthermore, the effects of coitus on the release of LH from the anterior pituitary gland could easily be determined by checking the ovaries for rupture sites. However, since many of the rabbits used during

this investigation were sacrificed before ovulation could occur, vaginal stimulation could only be confirmed by examining vaginal smears for the presence of sperm.

The brains of 85 adult female New Zealand rabbits of the species *Oryctolagus cuniculus L.* were studied. Of these, nine were processed for light microscopy; the remaining 76 brains were prepared and subsequently studied with the electron microscope. Of the 76 animals investigated ultrastructurally, 53 were mated and sacrificed at the following post-coital intervals: 10 minutes, 1/2 hour, one hour, two hours, four hours, six hours, eight hours and 10 hours. A minimum of four rabbits were included in each of these post-coital groups. Twenty-one non-mated animals served as the controls.

All of the rabbits used in this investigation were at least six months of age, with a body weight of 3.5 kg to 4.3 kg. These animals were purchased from Riemens Fur Ranches Ltd., R. R. # 1 St. Agatha, Ontario, and housed individually for a minimum period of 25 days within 25" x 12" x 17" wire cages*. Food (Rabbit Pellets, Master Feeds) and water were provided *ad libitum*. The room temperature was maintained at 72 ± 2°F, with a 12-hour cycle of light and darkness.

One buck of the same species was also housed under similar environmental conditions. This animal was used for mating purposes only.

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* Camm Research, Wayne, New Jersey, U.S.A.

B) The Perfusion Apparatus

The perfusion apparatus designed and used throughout this study consisted simply of two 2-liter pyrex aspirator bottles placed on a shelf located approximately 160 centimeters above a stainless steel operating table. Each bottle was fitted with a length of ½ inch-bore polyethylene tubing which served to carry the perfusate distally to a glass 'Y' joint. From here, a third length of the same tubing, further conducted the perfusate distally to a steel cannula prepared from a #13 hypodermic needle.

This apparatus provided an open, gravity-fed system with a pressure equivalent to approximately 100-110 mm Hg. The rate of flow of the perfusate (35-40 ml/min) was controlled by two clamps placed distally around each length of polyethylene tubing attached to the perfusion bottles.

A nearby oxygen tank served as the source of oxygen with which the perfusate was saturated.

C) Surgical Procedure for Perfusion Fixation

The animals sacrificed at 10 minutes post-coitus were prepared for perfusion by cervical dislocation. All other animals used in this study were anesthetized for perfusion fixation with an aqueous solution of 25% Urethane (1,200 mg/kg body weight) introduced into one of the marginal ear veins.

The fur was removed from the thoracic and abdominal midline areas and the abdominal cavity opened by making a longitudinal incision through the skin and linea alba. A hemostat was clamped over the

descending aorta and inferior vena cava at the approximate level of the renal arteries, thus limiting the flow of blood and perfusate only to the head, cervical and thoracic regions, the forelimbs and the abdominal viscera. This procedure reduced the volume of fixative required for satisfactory fixation of the brain.

The thoracic cavity was opened by cutting the left costal cartilages and intercostal tissues with a pair of blunt scissors. The pericardium was opened carefully to expose the heart. A small incision into the apex of the left ventricle served as an opening through which the steel cannula of the perfusion apparatus was inserted and carefully pushed forward through the aortic valve and up into the ascending aorta. A similar incision into the wall of the right atrium provided an 'outlet' for the blood and perfusate. Air was prevented from entering the blood vascular system by maintaining a flow of perfusate while the steel cannula was being inserted into the ascending aorta. Once in position, the cannula was secured with a hemostat placed around the myocardium near the point at which it was inserted through the wall of the heart. Approximately 20 seconds from the time the thoracic cavity was opened, the perfusate was flowing to the brain.

D) Preparation of Tissues for Light Microscopy

At the outset of this investigation, the brains of nine rabbits were fixed by vascular perfusion of 1,250 ml of a 10% solution of buffered neutral formalin. Whole brains, or their hypothalamic blocks, were removed and fixation continued for 48 hours with a volume of the same fixative maintained at room temperature. Subsequent dehydration,

clearing and embedding were carried out in tetrahydrofuran (THF) - paraffin according to the techniques of Haust, 1959 (Appendix I). Thick (12 μ) sections were stained with thionin (Appendix I) and studied carefully with the light microscope to ensure accurate identification and removal of the ME and hypothalamic nuclei for the definitive electron microscopic study to follow.

The ovaries of a one centimeter-long section of each uterine horn from these animals, as well as from the 74 animals used for the electron microscopic part of this study, were also removed and fixed in 10% buffered neutral formalin for 48 hours. Dehydration, clearing and embedding were carried out in a slightly modified version of Haust's THF-paraffin technique for processing brain tissue (Appendix II), or in dioxane and paraffin as described in the Manual of Histologic and Special Staining Technics (Appendix II). Thick (12 μ) sections were stained with hematoxylin, phloxine and saffron (Appendix II). Prepared sections of the ovaries from animals sacrificed at 10 hours post-coitus were examined carefully for rupture sites - an indication that ovulation had occurred.

All conventional light micrographs were taken with a Zeiss photomicroscope and recorded on Kodak Panatomic-X film using a green filter. The film was developed for nine minutes in Kodak Microdol-X at 20°C.

E) Preparation of Tissues for Electron Microscopy

1) Fixation

The 76 rabbit brains prepared for study with the electron

microscope were fixed by vascular perfusion of 1,500 ml of a modified Karnovsky's paraformaldehyde-glutaraldehyde fixative (Karnovsky, 1965) or an equal volume of Reese and Brightmann's dual paraformaldehyde-glutaraldehyde fixative as described by Alan Peters, 1970 (see Appendix III, sections A and B, respectively). The Karnovsky method provided satisfactory fixation of the hypothalamic nuclei, but not of the ME. For this part of the present study, the dual fixatives of Reese and Brightmann were used. These fixatives, because of their relatively low osmolarity (i.e., between 500 and 600 mOsM/kg), eliminated much of the shrinkage previously observed within the ME when using the Karnovsky fixative. The pH of both fixatives was 7.2-7.4.

All fixatives were prepared on the day of the perfusion as outlined in Appendix III, and heated to 41°C and saturated with oxygen before being introduced into the blood vascular system. Exsanguination with phosphate buffer alone (Karlsson and Schultz, 1965) was only carried out when using the Karnovsky fixative.

2) Excision of the hypothalamic nuclei and median eminence

Immediately after each perfusion, the rabbit was decapitated and the skin removed from the head. The brain was exposed by carefully chipping away the surrounding bone with rongeurs. The cerebral hemispheres were transected coronally at the level of the orbits and the optic nerves sectioned near their exit through the optic foramina. The more rostral part of the brain was then carefully lifted from its bony floor, and the infundibular stalk transected by placing a # 11 surgical blade horizontally into the sphenoid bone. Once the optic chiasma and the ventral surface of the hypothalamus were free, the remaining cranial

nerves were cut and the brain lifted from the remains of its bony floor. The brain was then placed in a volume of the appropriate fixative, and fixation continued overnight at 4°C.

The following morning, a block of tissue approximately 1 cm^3 consisting of the hypothalamus, ME and attached infundibular stalk, was removed from each brain. Photographs of the ventral surface of the rabbit brain and the hypothalamic block dissected from the same brain are shown in Figures 1 and 2, respectively. Each hypothalamic block was limited caudally by the posterior border of the mammillary bodies, and rostrally by a coronal section made about 1/8-inch in front of the rostral border of the optic chiasma. Laterally and superiorly, each block was limited by sections through the lateral hypothalamic area and the thalami, respectively. A single-edged razor blade was used for this procedure.

The small pieces of tissue containing the hypothalamic nuclei, as well as the ME, were excised from the hypothalamic blocks using one centimeter-long knives cut from a thin, double-edged razor blade and placed into a steel blade holder*. All tissues were carefully removed with the aid of a Zeiss dissecting microscope. A line diagram of a hypothalamic block in sagittal section is shown in Figure 3. The location of the tissues for electron microscopic study, as well as the coronal planes used to locate each of the hypothalamic nuclei, are also illustrated.

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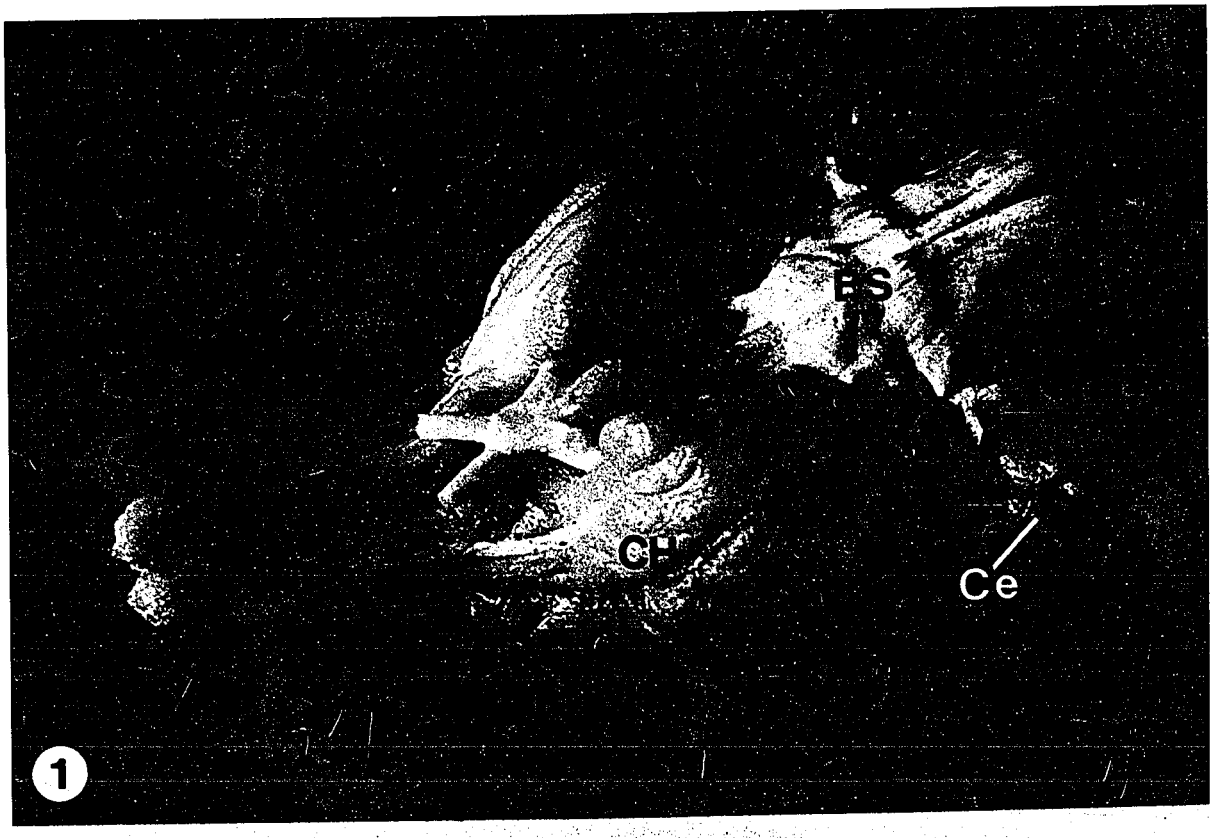
* Irex German Surgicals, Toronto, Ontario.

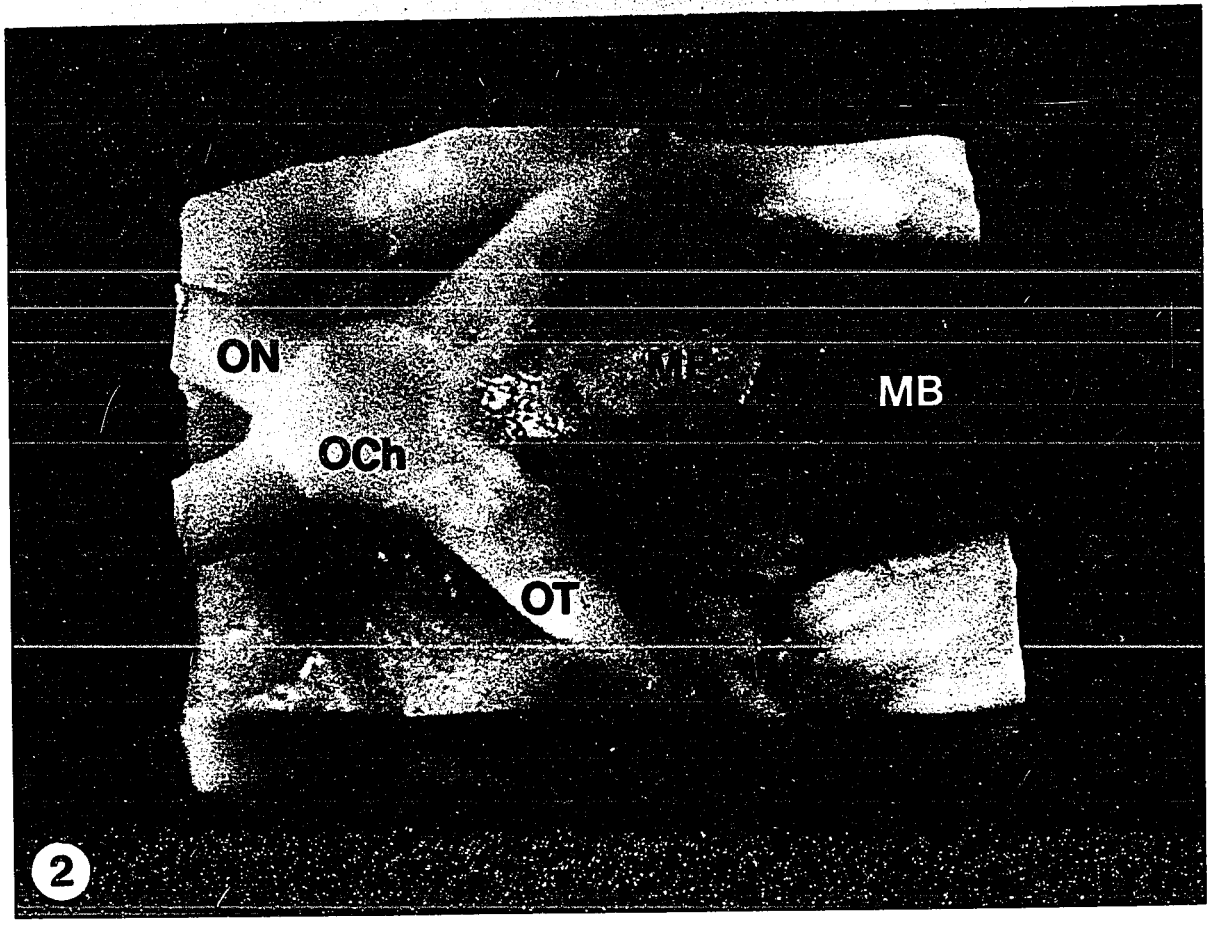
Figure 1. Ventral view of the rabbit brain.
BS, Brain stem; Ce, Cerebellum; CH, Cerebral hemisphere.

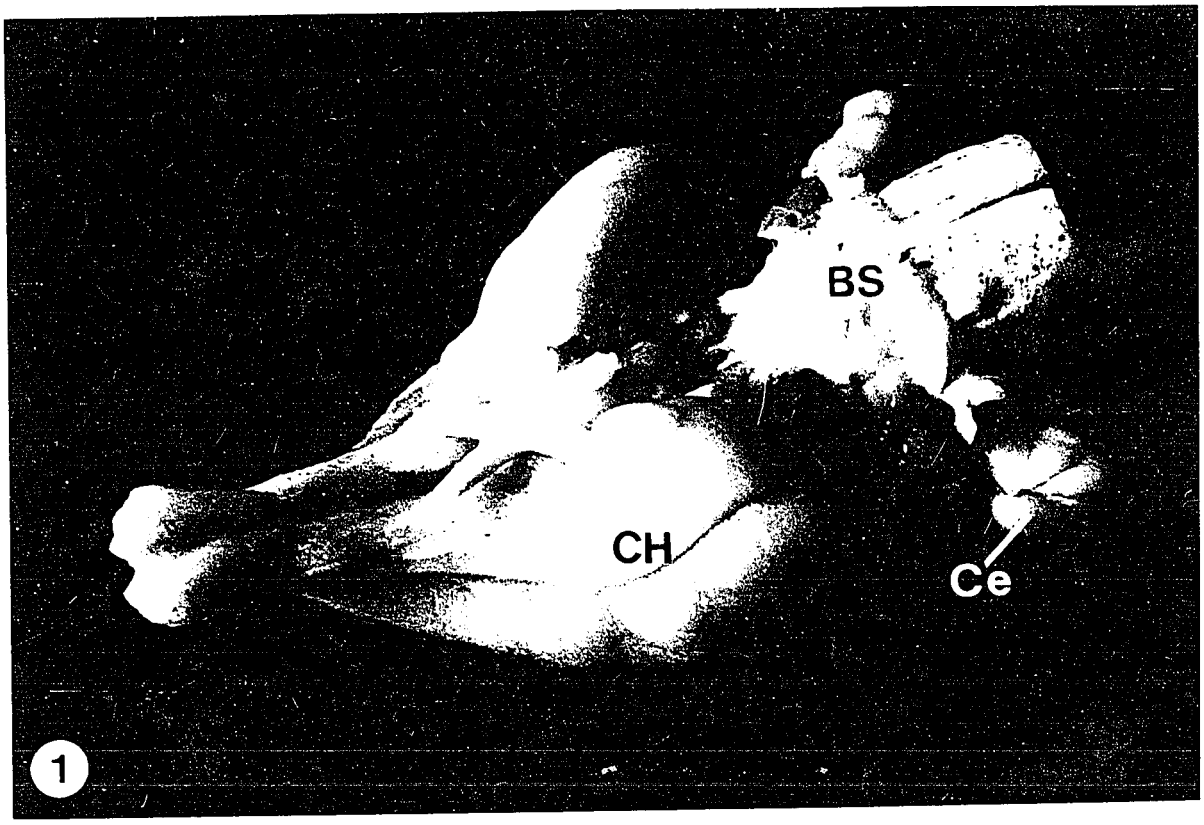
X 2.7

Figure 2. Ventral view of the hypothalamic block removed from the rabbit brain shown in Figure 1.
MB, Mammillary body; ME, Median eminence; OCh, Optic chiasma; ON, Optic nerve; OT, Optic tract.

X 9.2


BS
Ce
1


ON
MB
OCh
OT
2


BS
CH
Ce
1

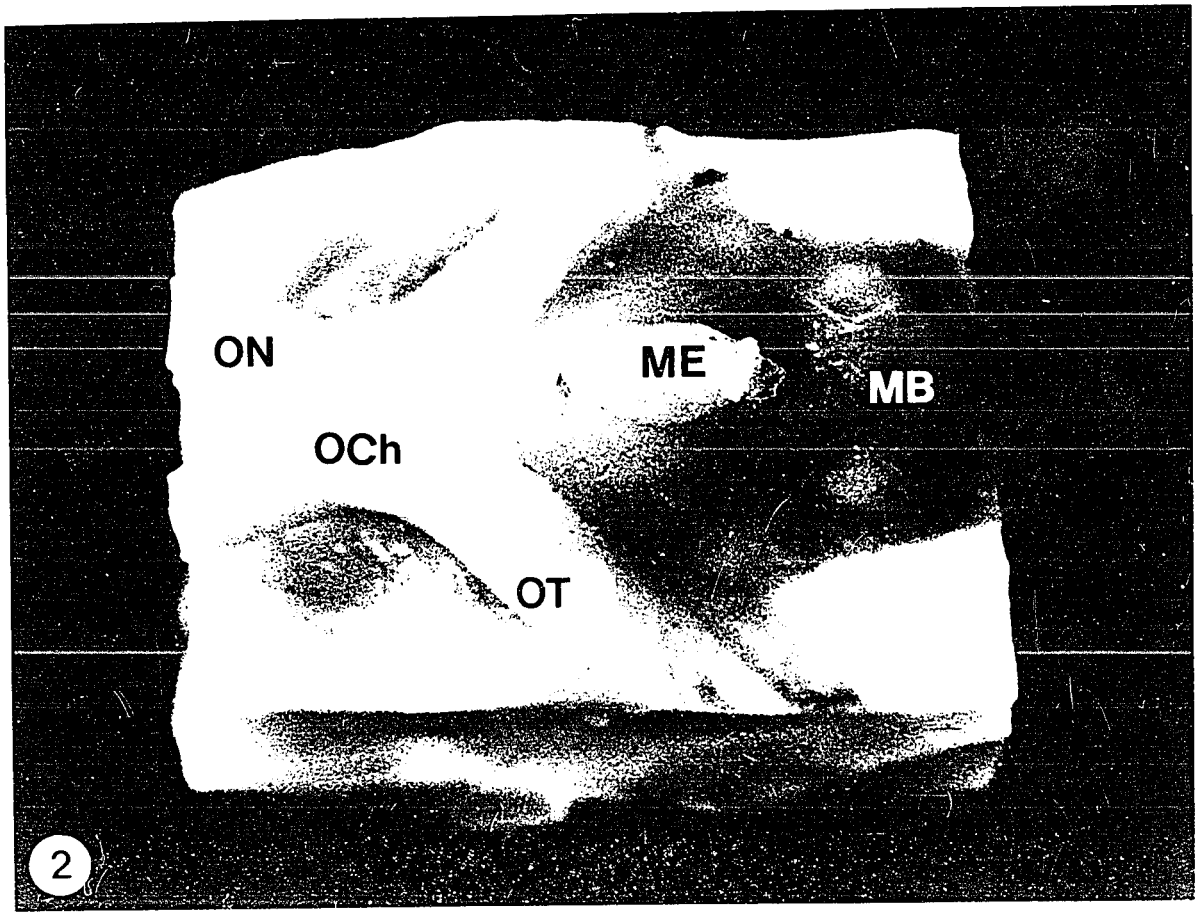

ON
ME
MB
OCh
OT
2

Figure 3. A line diagram of a sagittal section through the rabbit hypothalamus showing the location of the preoptic (PO), suprachiasmatic (SCH), and arcuate (Arc.) nuclei. The three coronal places indicated by the arrows and labelled 1, 2, and 3, represent the sections made through the hypothalamus for removing the PO, SCH and Arc nuclei, respectively. AC, Anterior commissure; IS, Infundibular stalk; MB, Mammillary body; ME, Median eminence; MI, Massa intermedia; OCh, Optic chiasma; PC, Posterior commissure.

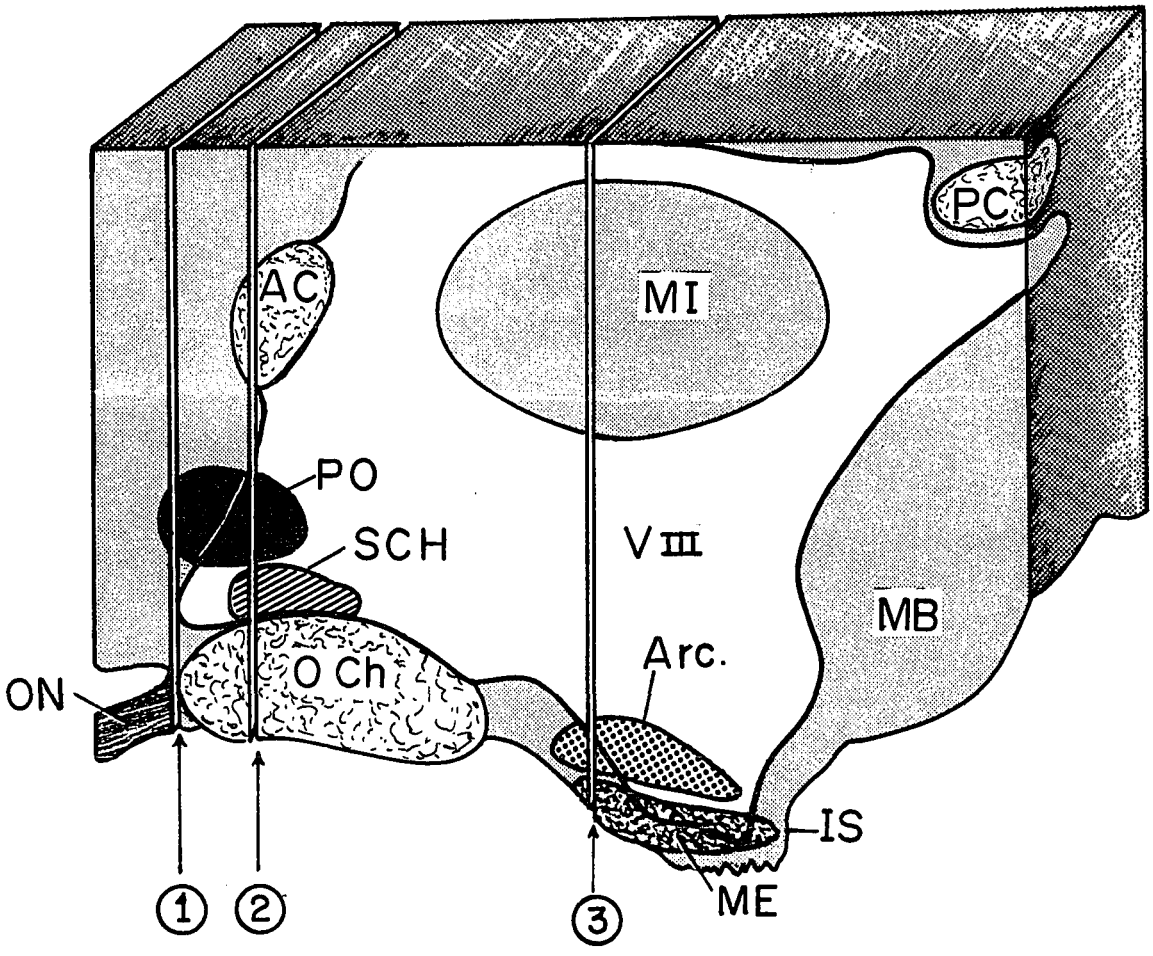


Figure 3

3) Subsequent processing of the median eminence and hypothalamic nuclei for electron microscopy

Pieces of tissue containing the ME and hypothalamic nuclei were washed in 5.4% sucrose prepared in phosphate buffer and post-fixed in an aqueous solution of 1% osmium tetroxide in the same buffer (Appendix IV). The tissues were then rinsed in distilled water, stained 'en block' with a saturated solution of uranyl acetate, and subsequently dehydrated and cleared in alcohols and propylene oxide, or in alcohols only, depending on the embedding medium used.

The most extensively used embedding medium was Epon 812. Because of the superior infiltration and cutting qualities of Spurr, this embedding medium was chosen for the median eminence. However, this procedure was soon discontinued since considerable difficulty was experienced in staining Spurr embedded tissues. The embedding procedure for both Epon 812 and Spurr are outlined in Appendix IV, sections D and E, respectively.

For orientation purposes, particularly when studying the median eminence, the Epon-embedded material was sectioned at 0.5 μ on the Reichert RU2 automatic microtome. Sections were mounted on glass slides and routinely stained with a 1% solution of Mallory's Azur II - methylene blue stain (Richardson, Jarett and Finke, 1960) for one minute at 60-70°C.

Ultrathin sections for transmission electron microscopy were also cut on a Reichert RU2 automatic microtome. These were placed on 75 x 75 or 100 x 100 mesh copper grids coated with formvar and

stained with Reynolds lead citrate (Reynolds, 1963). All sections were examined with an AEI, EM6B or EM801 electron microscope* operated at 60 KV. All electron micrographs taken on these instruments were recorded on Ilford Type E N4E50 70 mm roll film or Electron Microscope Type EM-5 8.2 x 8.2 cm glass plates and developed in Kodak D-19 for six minutes at 20°C.

F) Measurements and Computer Analysis

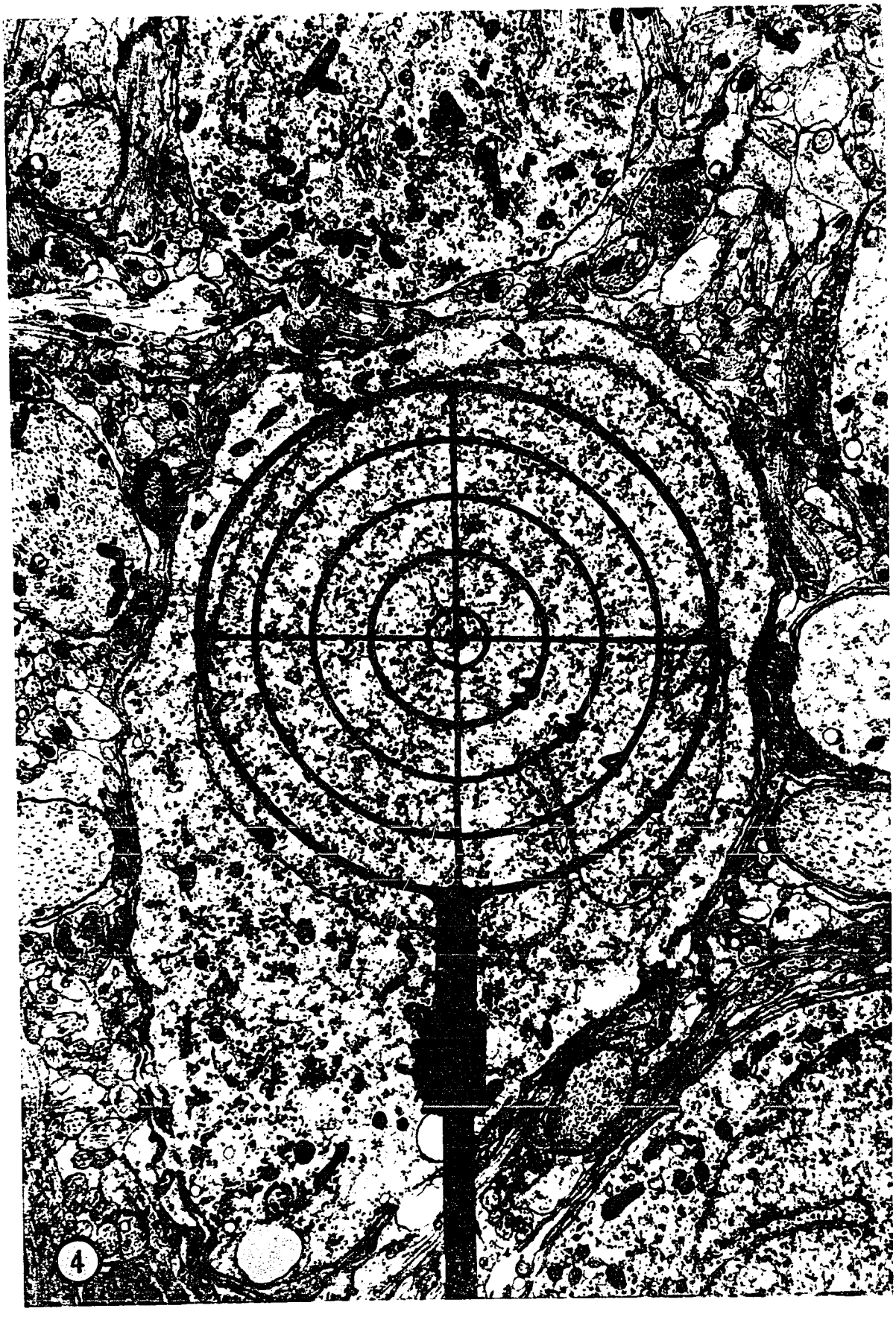
In an attempt to provide statistical evidence for the ultrastructural changes observed during this investigation, measurements of neuron diameters, their nuclear profiles and nucleoli, as well as diameters of the vesicle types observed within the neuron soma, axons and axon terminals, were recorded and processed for computer analysis. All measurements were obtained by using a 'gunsight' mounted into the viewing chambers of the AEI, EM6B or EM801, or directly from micrographs.

The 'gunsights' were designed and etched from a thin plate of copper by Dr. D. G. Montemurro. These were mounted in the viewing chamber of each microscope in a manner such that they could be readily moved into position over the viewing screen for recording measurements, or away from the electron beam when recording on plates or film. An electron micrograph of the shadow cast by the 'gunsight' mounted on the AEI, EM801 is shown in Figure 4. The diameter of an object, as represented by the distance across each of the five rings of the 'gunsight', or by the space between the rings in two of the four

* AEI (Associated Electrical Industries Ltd.) Scientific Apparatus Division, P.O. Box 1, Harlow, Essex, England.

Figure 4. Electron micrograph showing the shadow cast by the 'gunsight' mounted into the viewing chamber of the AEI, EM801. This 'gunsight' was used to measure the diameters of neurons, as well as the diameters of their nuclear profiles. The large neuron shown on the micrograph has a diameter of 8.9μ.

X 5,800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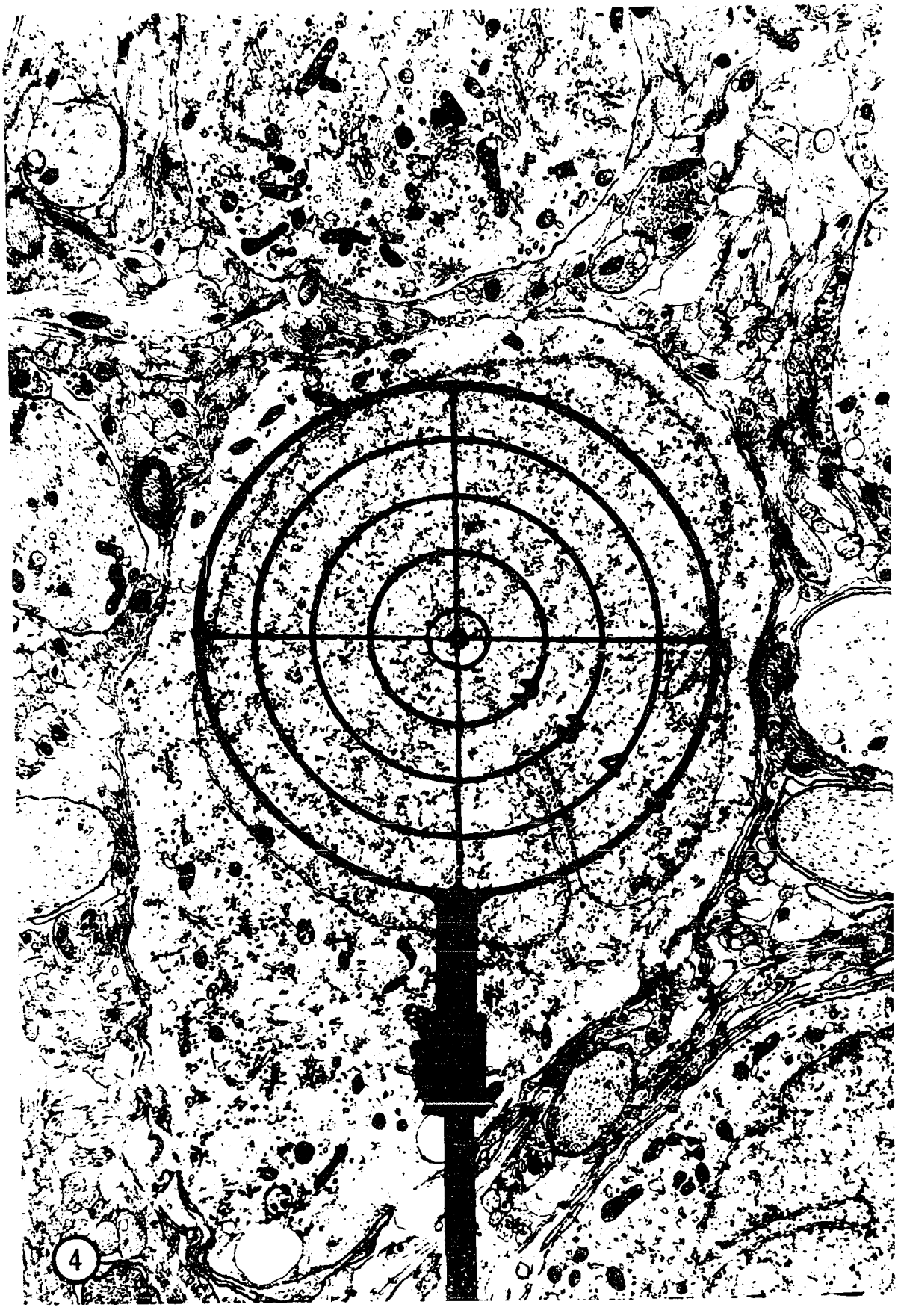

4

quadrants at any given magnification, was determined by using a 2,160 lines/mm diffraction grating. The tables prepared and used while measuring objects directly from the viewing screen of the EM6B and EM801 are shown in Appendix V. These 'gunsights' provided an accurate technique for recording measurements of neurons, as well as their nuclear and nucleolar profiles. This method was not used to obtain diameters of smaller structures such as vesicles, mitochondria and lysosome-like bodies. Measurements of these and other cytoplasmic organelles were recorded directly from the electron micrographs. To this end, two tables were prepared and used to convert measurements recorded in millimeters or centimeters, to micron (μ) or angstrom (Å) units. These 'conversion tables', for both the EM6B and EM801 microscopes, are included in Appendix VI. Each negative used in this investigation was enlarged exactly 3.5 times. Knowing the negative magnification, it was then possible (with the aid of the 'conversion tables') to quickly convert diameters recorded in centimeters or millimeters from the micrographs, to micron and/or angstrom units. All measurements from micrographs were made with a hand lens calibrated to 100 lines/cm. In all cases, whether the 'gunsight' or hand lens-micrograph technique was used, only the shortest diameters were recorded.

The diameters of representative samples of the neurons and their nuclei, as well as the DC vesicles were recorded for each of the hypothalamic nuclei investigated in both the control and experimental groups. Measurements were also recorded for the light-core (LC) vesicles and the nucleoli of the SCH neurones. These data were

subsequently processed for computer (PDP-10) analysis. All parametric data were compared using Fisher's 'Student t Test' (with Bessels corrections) and 'P' values determined.

IV RESULTS

PREOPTIC NUCLEUS

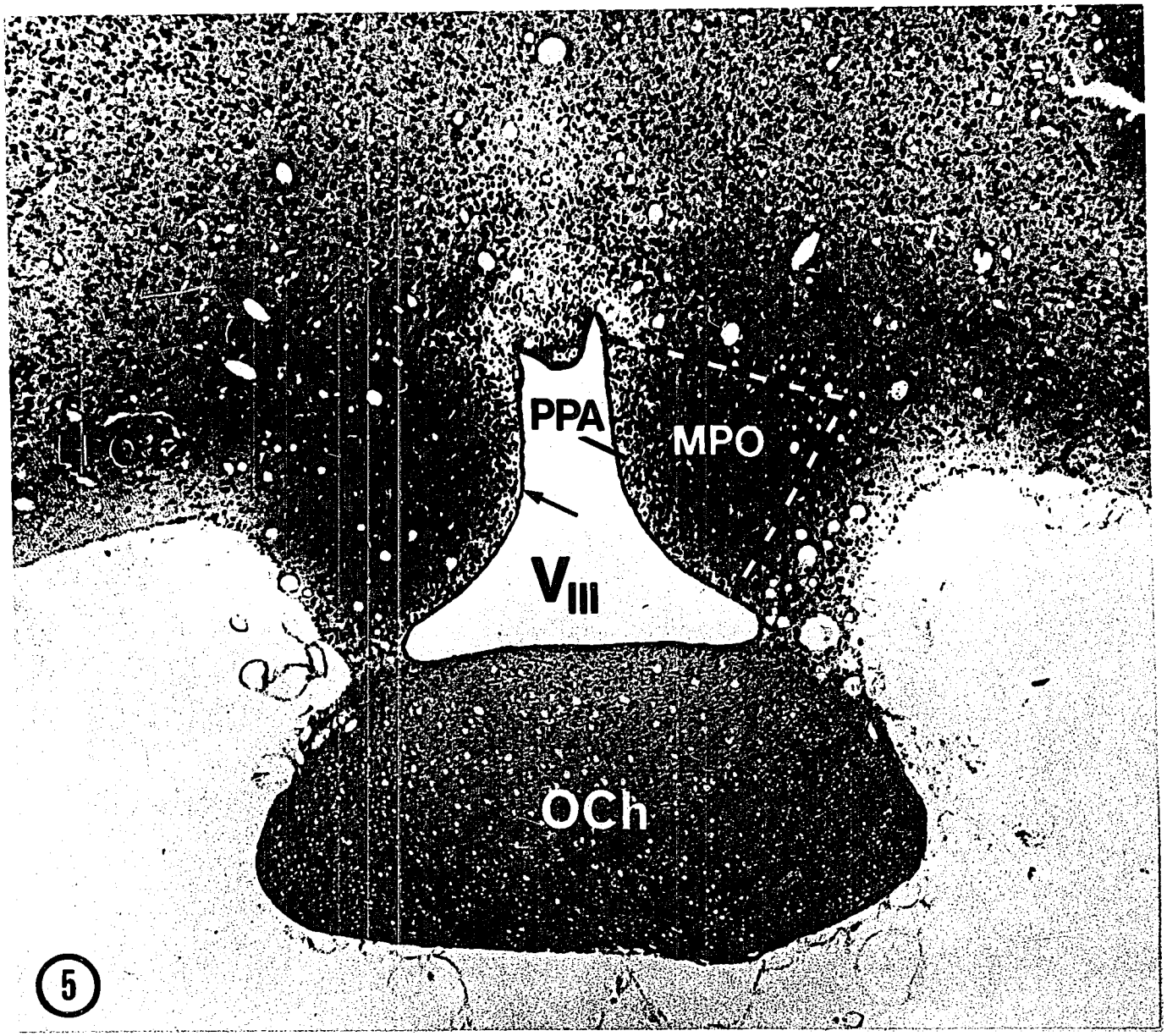
A) Light Microscopy

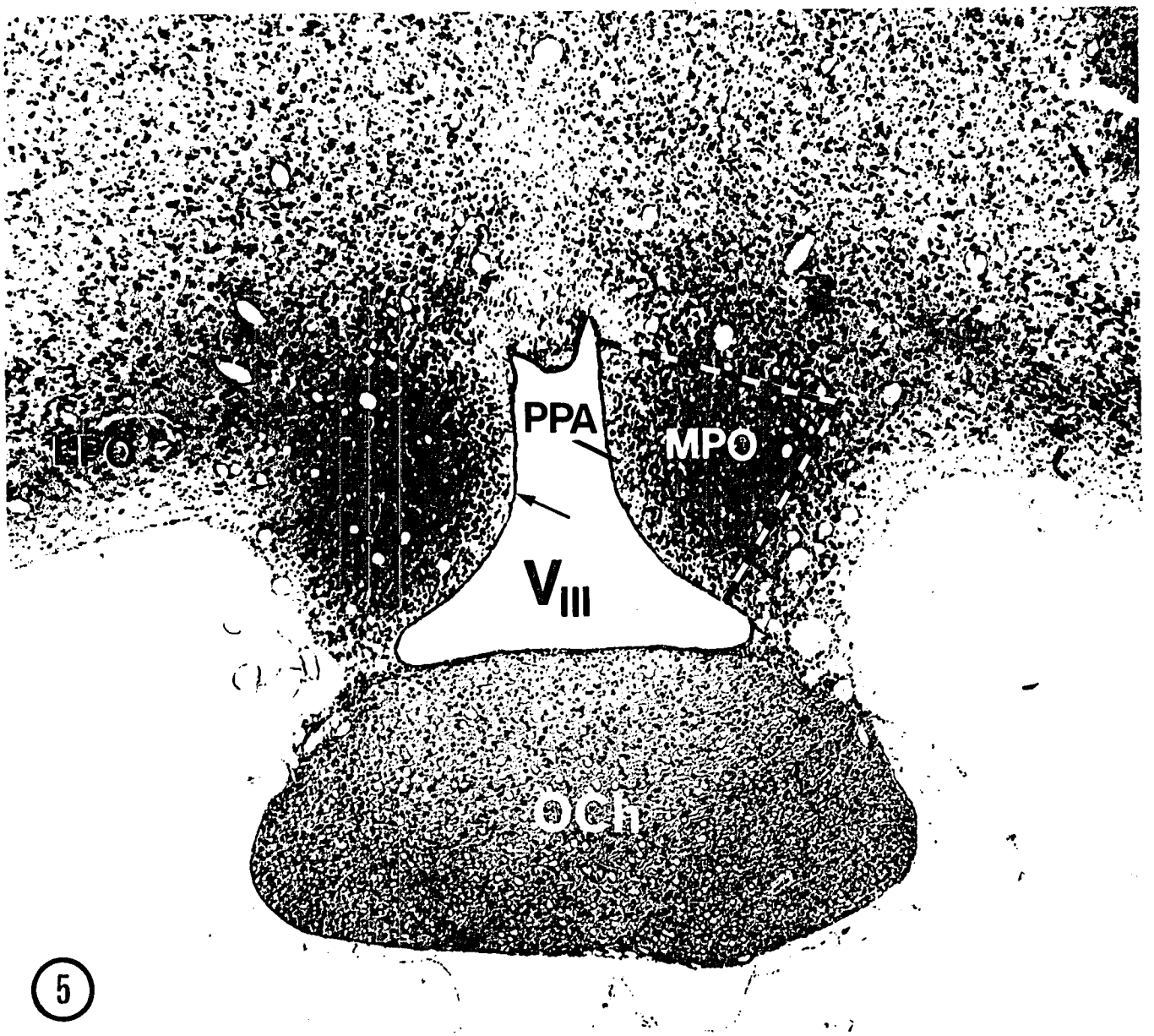
The preoptic area of the rabbit brain is clearly visible in frontal sections made at the rostral tip of the recessus preopticus. The tissue excised from each side of the third ventricle (V_{III}) at this level, and subsequently processed for electron microscopic study is represented by the broken line in Figure 5. Ultrathin sections were cut from the large medial preoptic nucleus which, in the rabbit brain, consists of the spherical to oval group of small cells lying immediately lateral to the dorsal portion of the preoptic periventricular area. In Figure 5, this area is represented by the narrow pale region occupying the wall of the V_{III} just lateral and superior to the ependymal lining of the recessus preopticus. Laterally, the medial preoptic nucleus is not clearly demarcated from the neurons of the lateral preoptic nucleus.

Rupture sites were observed in seven of the 12 ovaries removed from animals sacrificed at 10 hours post-coitus, with hemorrhagic and/or mature follicles observed in each of the remaining five.

Figure 5. Frontal section of the rabbit brain made at the level of the rostral tip of the recessus preopticus. The block of tissue removed from each side of the third ventricle (V_{III}) and processed for electron microscopy is represented by the broken line. It includes a portion of the ependyma (arrow), the underlying preoptic periventricular area (PPA), and most of the large medial preoptic nucleus (MPO). LPO, Lateral preoptic nucleus; OCh, Optic chiasma. Thionin.

X 370


PPA
MPO
V III
OCh
5



5

B) Electron Microscopy

1) Ultrastructure of the preoptic nucleus

The preoptic nucleus of the rabbit brain is composed of neurons and neuroglial elements, a dense network of interwoven cytoplasmic processes of these cell types (i.e., the neuropil), and numerous capillaries. In general, the neurons of the PO nucleus were distributed uniformly throughout the sections studied (Figure 6). Their close spatial relationship with capillaries was also a common finding (Figure 7). The neurons were ovoid to spherical in shape, and were characterized by their large eccentric, typically vesicular nuclei (Figures 6 and 7). A more detailed description of the ultrastructure of the neurons of the PO nucleus is included in the following section of the results. Based on carefully obtained measurements of 120 neurons located near capillaries of the controls, these neurons had a mean diameter of 12.0 ± 0.2 μ (Figure 8); the mean diameter of their nuclear profiles was 9.1 ± 0.1 μ (Figure 9). The minimum and maximum diameters for each of these parameters are shown in Tables 1 and 2, respectively. The distribution curves for these measurements are included in Figure 10.

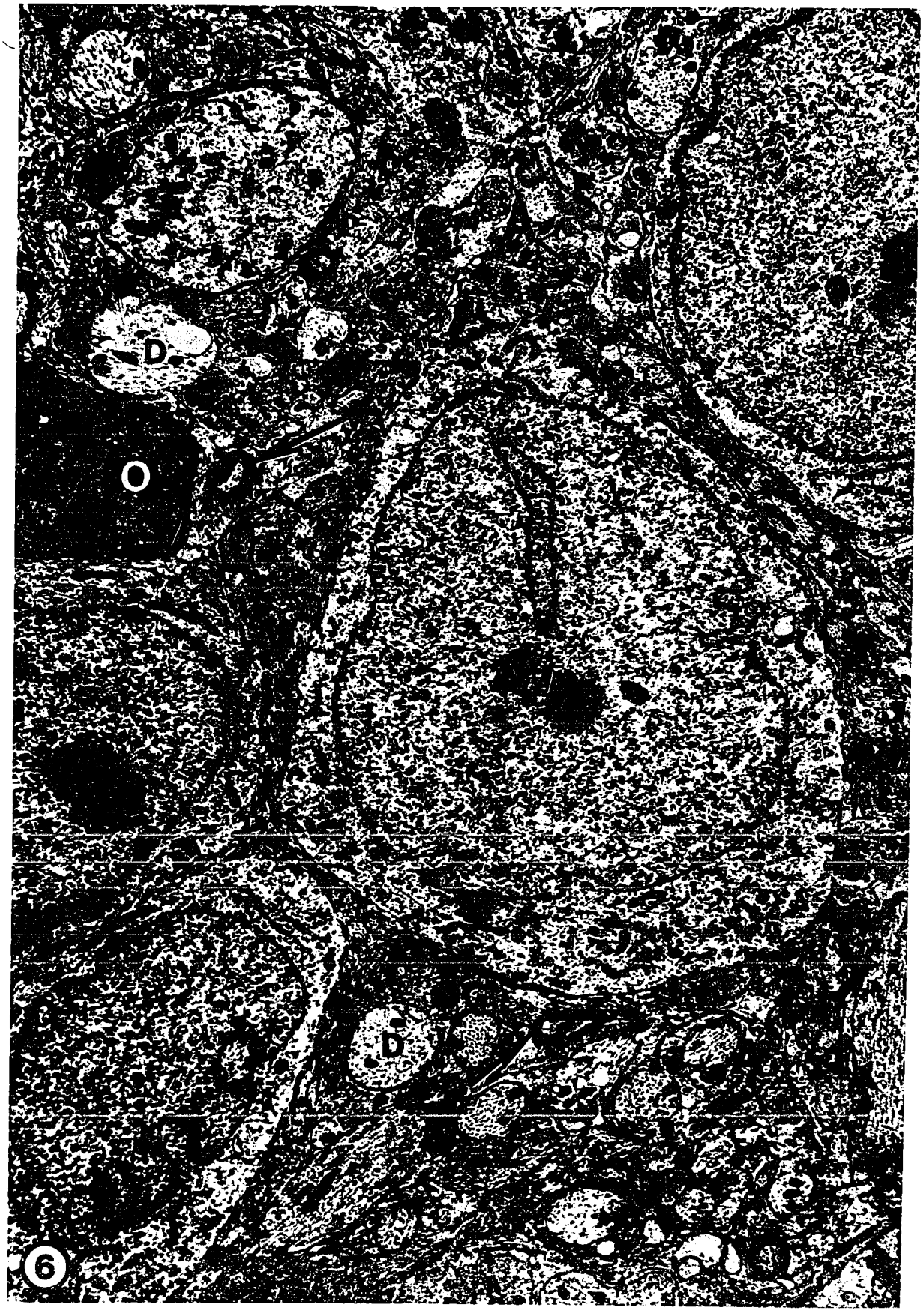
Neuroglial elements were largely represented by oligodendrocytes and astrocytes. The oligodendrocytes were either completely surrounded by the neuropil (interfascicular), or occupied a 'satellite' position to the larger neurons. The astrocytes were frequently in close spatial relationship with the capillaries. The ultrastructural features of one 'satellite' oligodendrocyte are shown in Figure 11. The small size, angular shape and generally dark appearance of these

cells were the basic features affording their identification. The nucleus was typically eccentric, and characterized by the clumping of chromatin into a narrow zone adjacent to the nuclear envelope. Nucleoli were seldom observed within these cells. The rough ER was relatively well developed. Dense populations of free ribosomes contributed largely to the dense, granular appearance of the cytoplasm. Mitochondria, a small Golgi system, vesicular structures and filamentous profiles were identified, but showed no unusual features. The astrocytes were identified on the basis of the relatively homogeneous nature of the karyoplasm, the presence of fiber bundles within the cytoplasm and the irregular nature of the cell soma (Figure 12). The usual cytoplasmic organelles such as rough ER, ribosomes, Golgi system, mitochondria and vesicles, were sparsely represented. For the most part, these organelles were interspersed among the bundles of fibers within the perikarya, with the exception of the mitochondria and vesicles which frequently followed the fibers out into the neuropil. Junctional complexes identified as tight junctions (Figure 12) and desmosome-like structures or puncta adherens (Figure 12, inset), were also observed between the membranes of these cells.

The compact neuropil was composed of axons, axon terminals, dendrites and processes of glial cells (Figures 6, 7 and 11). In general, the axons were small with smooth, spherical profiles, and were unmyelinated. A few scattered axons of larger diameters displayed a thin myelin sheath (Figure 6). Dendrites, on the other hand, were usually larger than the axons, had irregular profiles, and when viewed in transverse section, showed many uniformly arranged neurotubules.

Figure 6. Low magnification electron micrograph of a portion of the preoptic nucleus of a control animal showing the general distribution and structure of neurons and nature of the neuropil. Only a thin ring of cytoplasm surrounds the conspicuous neuronal nuclei. The neuropil is composed largely of small unmyelinated axons (A) and large dendrites (D). Myelinated axons (arrows) are sparsely represented. A portion of a small 'satellite' oligodendrocyte (O) is also shown.

X 6,400


D
O
D
6

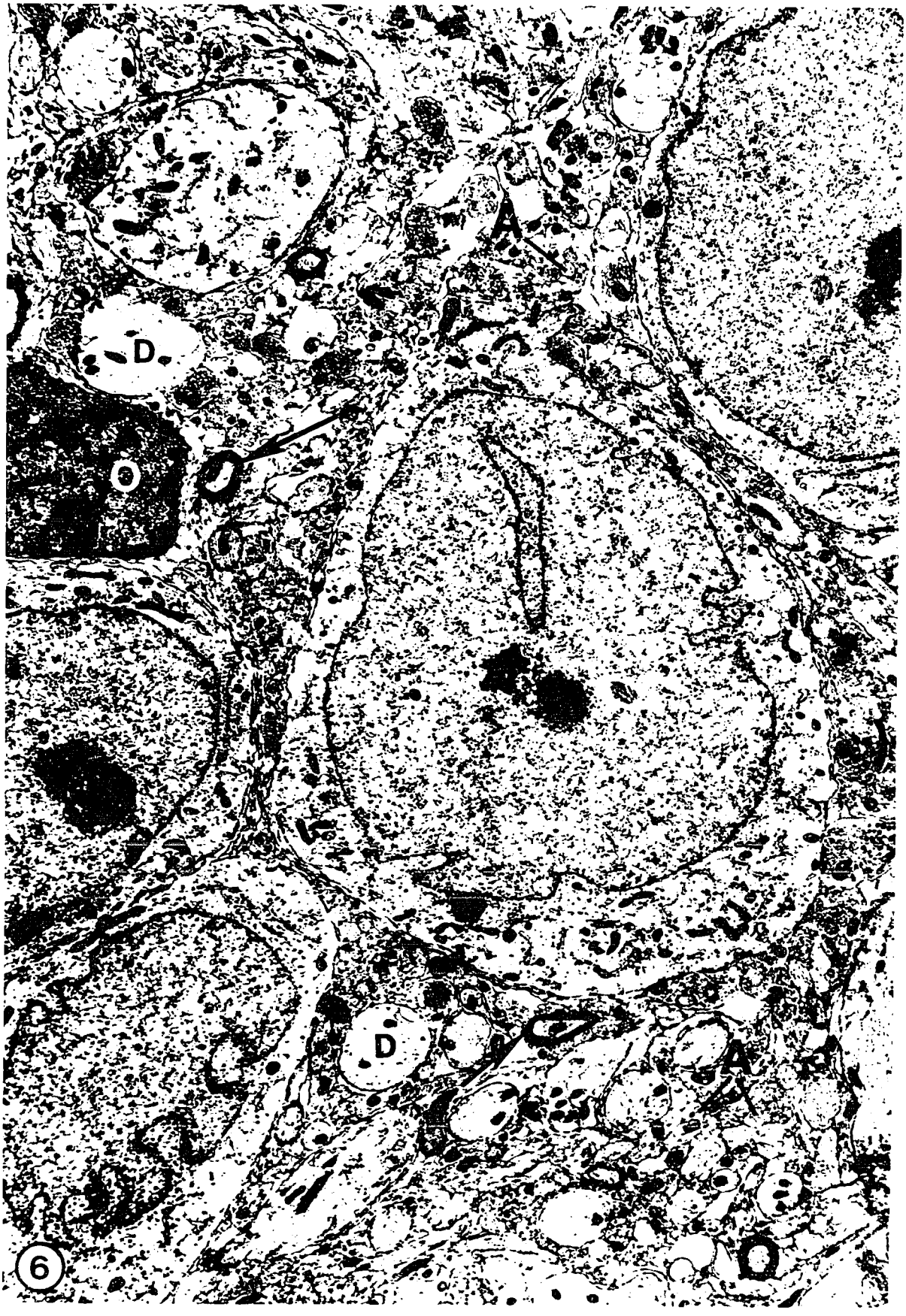
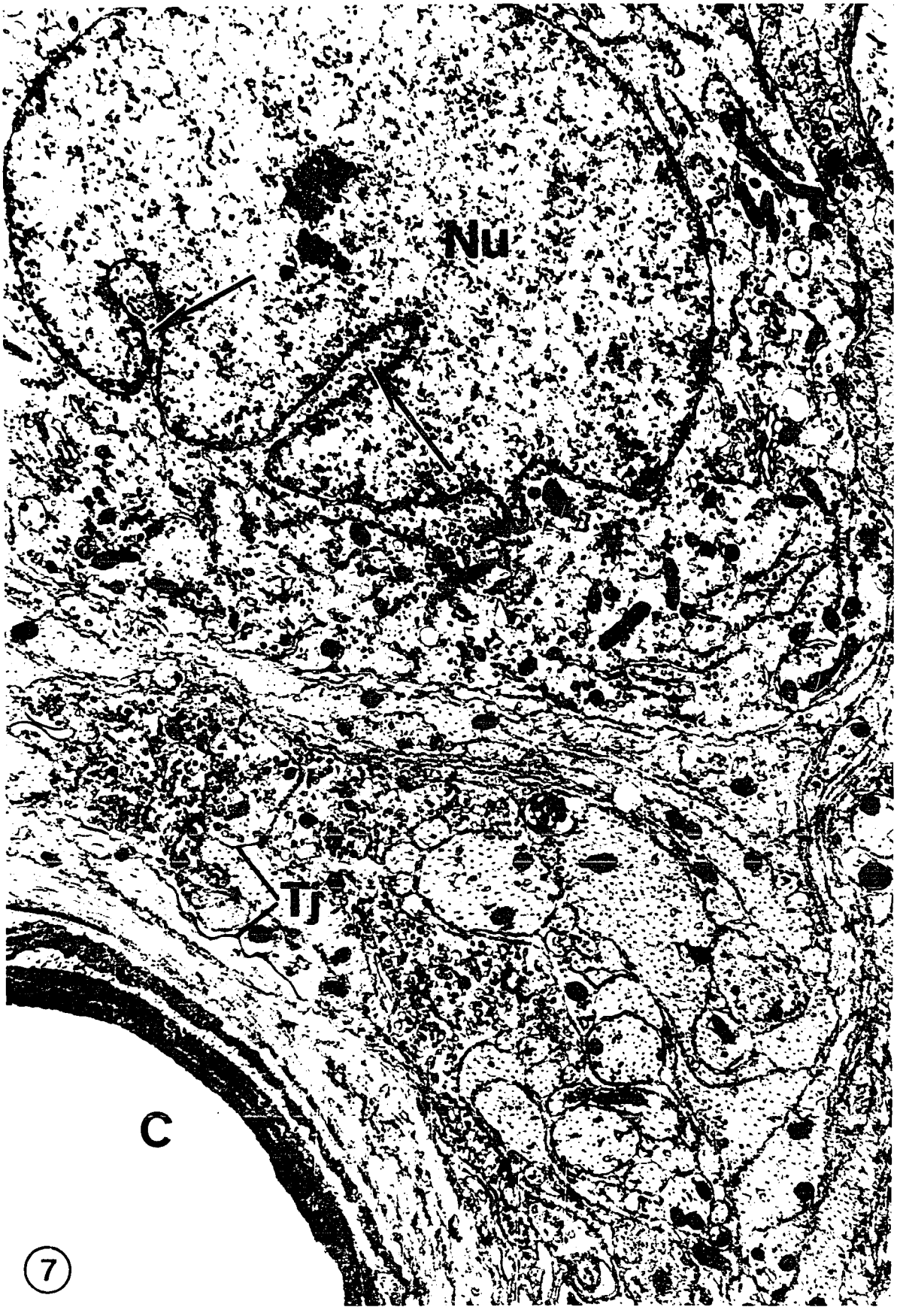

A
D
D
D
A
D
6

Figure 7. Electron micrograph showing the morphological features of a neuron of the control group and its close spatial relationship with a capillary (C). Note the large typically vesicular nucleus (Nu) with several deep indentations or clefts (arrows). The cell organelles are uniformly distributed throughout the cytoplasm but, in general, are sparsely represented. Tj, Tight junction. Preoptic nucleus.

X 20,500


C
7



(7)

Figure 8. Histograms showing the mean diameters of neurons of the rabbit hypothalamus: preoptic nucleus (), suprachiasmatic nucleus (), and arcuate nucleus ().

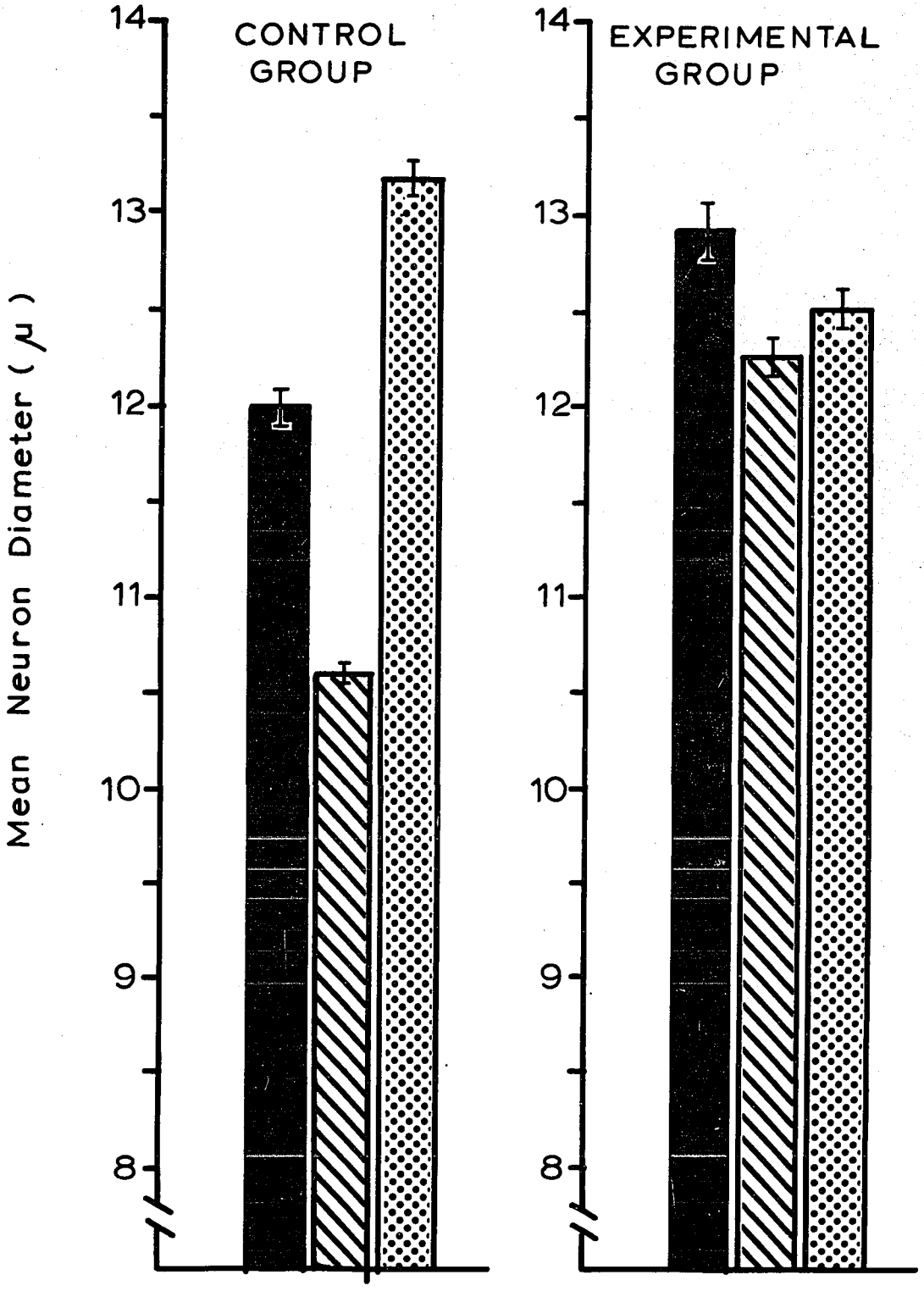

CONTROL GROUP
EXPERIMENTAL GROUP
Mean Neuron Diameter (μ)
14
13
12
11
10
9
8

Figure 8

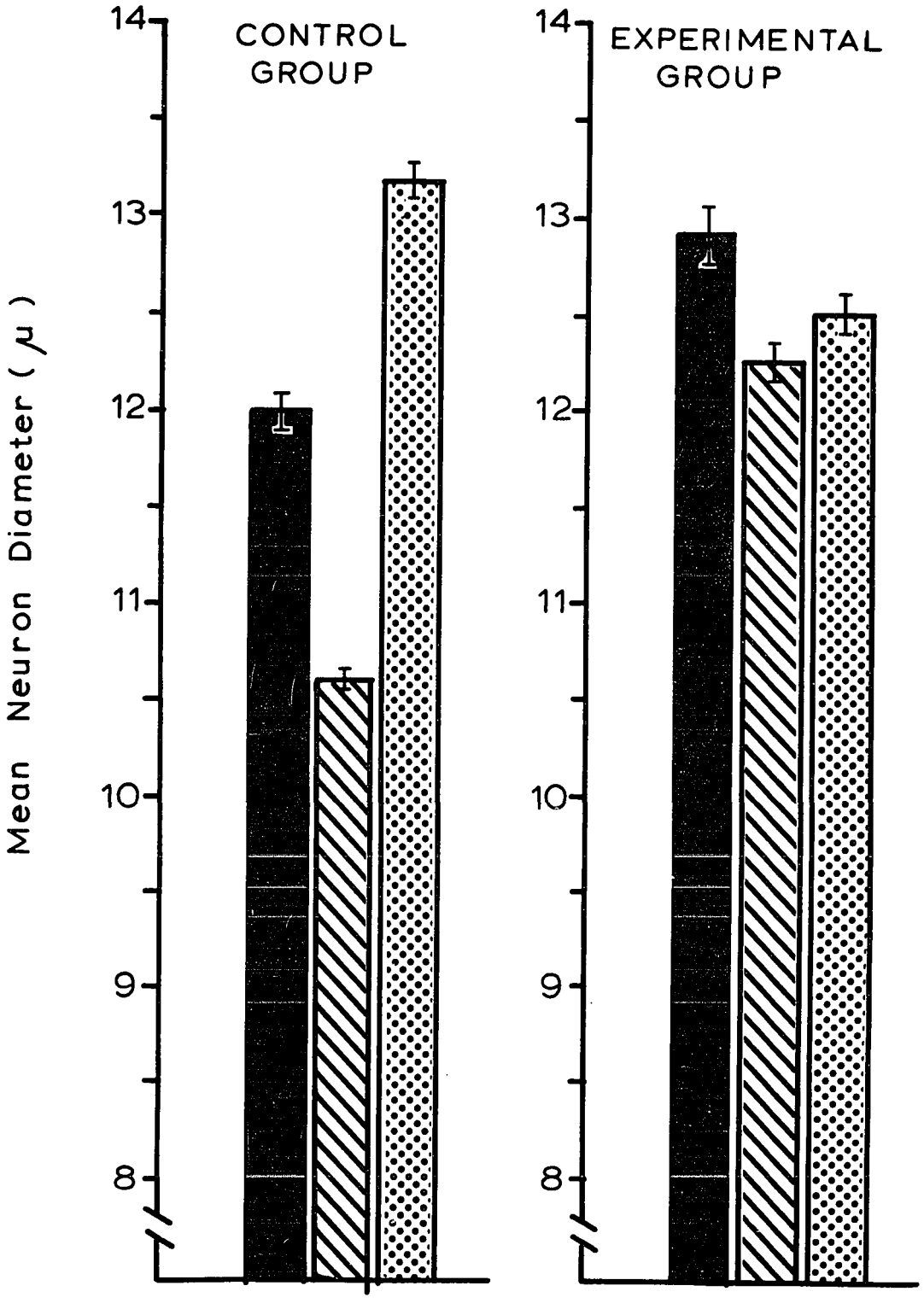

CONTROL GROUP
EXPERIMENTAL GROUP
Mean Neuron Diameter (μ)
14
13
12
11
10
9
8

Figure 8

Figure 9. Histograms of the mean diameters of the neuronal nuclei of both the control and experimental animals: preoptic nucleus (), suprachiasmatic nucleus (), and arcuate nucleus ().

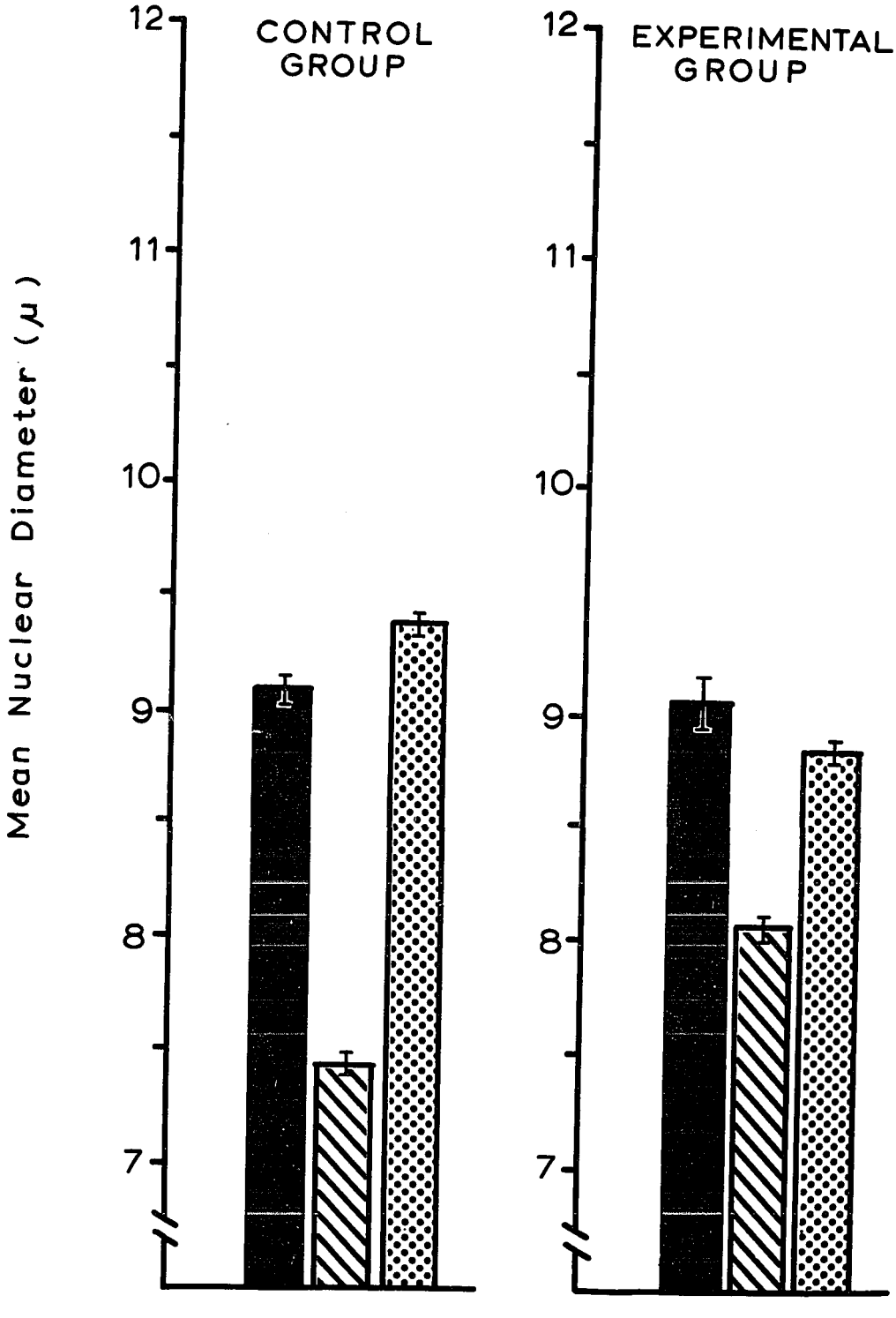

CONTROL GROUP
EXPERIMENTAL GROUP
Mean Nuclear Diameter (μ)
12
11
10
9
8
7

Figure 9

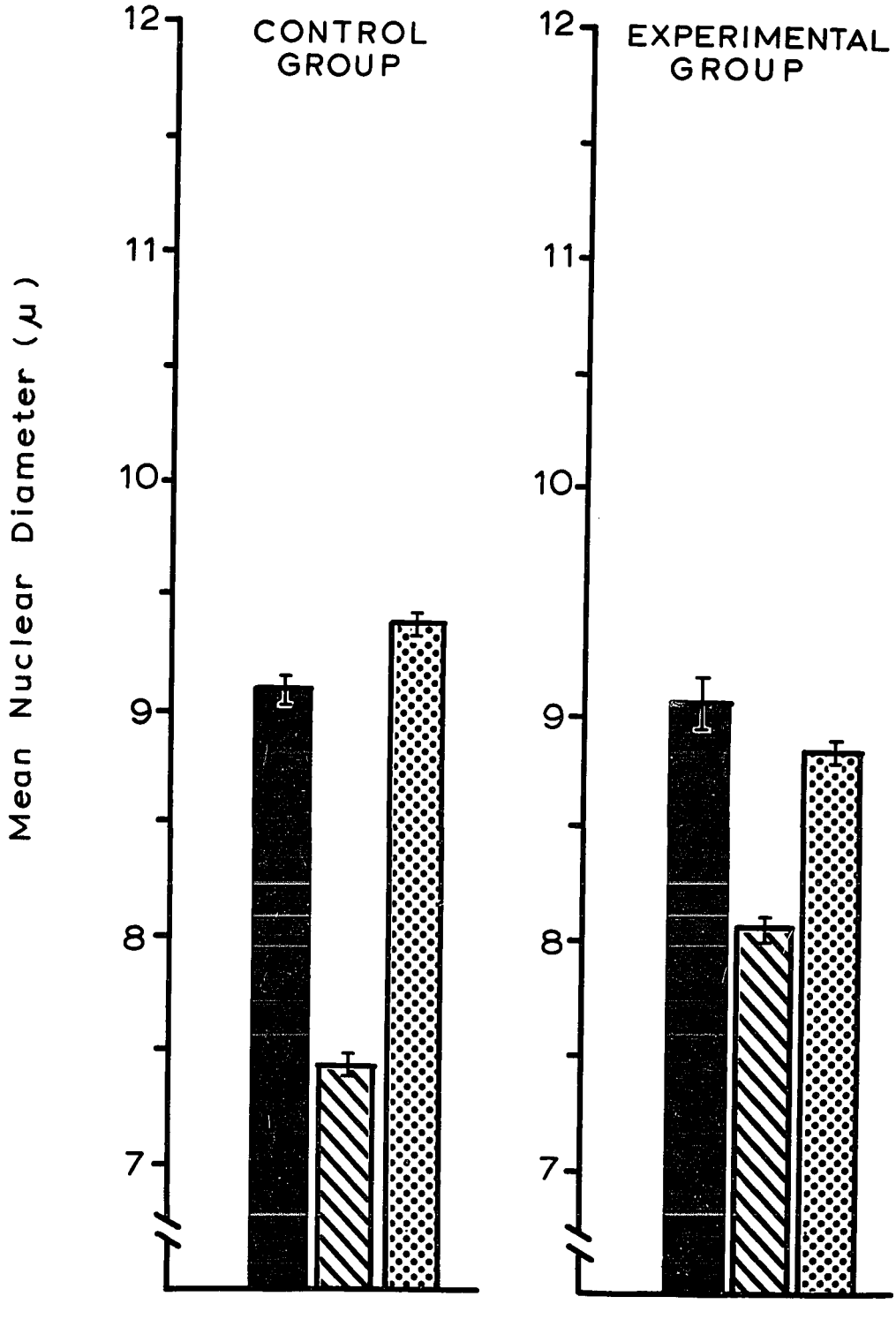

CONTROL GROUP
EXPERIMENTAL GROUP
Mean Nuclear Diameter (μ)
12
11
10
9
8
7

Figure 9

TABLE 1

SUMMARY OF COMPUTER ANALYSIS OF THE DIAMETERS OF NEURONS WITHIN THE PREOPTIC, SUPRACHIASMATIC AND ARCUATE NUCLEI

Hypothalamic Nucleus	Group	N	Minimum Diameter (μ)	Maximum Diameter (μ)	$\bar{x}$ ± S.E.M. (μ)	t	P value
Preoptic	Controls	120	8.4	16.0	12.0 ± 0.2	2.62	< .01
	Experimental	120	8.0	24.0	12.9 ± 0.3		
Suprachiasmatic	Controls	200	6.7	14.6	10.6 ± 0.1	7.00	< .001
	Experimental	200	6.8	22.0	12.3 ± 0.2		
Arcuate	Controls	100	9.4	17.0	13.2 ± 0.2	2.50	< .02
	Experimental	100	8.9	16.1	12.5 ± 0.2		

TABLE 2

SUMMARY OF COMPUTER ANALYSIS OF THE NUCLEAR DIAMETERS OF THE NEURONS OBSERVED WITHIN THE PREOPTIC, SUPRACHIASMATIC AND ARCUATE NUCLEI

Hypothalamic Nucleus	Group	N	Minimum Diameter (μ)	Maximum Diameter (μ)	$\bar{x}$ ± S.E.M.(μ)	t	P value
Preoptic	Control	120	6.4	12.0	9.1 ± 0.1	0.00	not significant
	Experimental	120	5.4	14.6	9.1 ± 0.2		
Suprachiasmatic	Control	200	3.4	12.0	7.4 ± 0.1	4.62	< .001
	Experimental	200	3.6	11.0	8.1 ± 0.1		
Arcuate	Control	100	7.0	12.0	9.4 ± 0.1	3.23	< .001
	Experimental	100	6.0	12.2	8.9 ± 0.1		

Figure 10. Distribution curves of the measurements obtained for the preoptic neurons and their nuclear profiles of both the control and experimental animals: controls - neurons (—x—), nuclei (——o——); experimental animals - neurons (—●—), nuclei (——△——). The stippled area represents the 23 (19.2%) neurons in which discrete ultrastructural changes were observed.

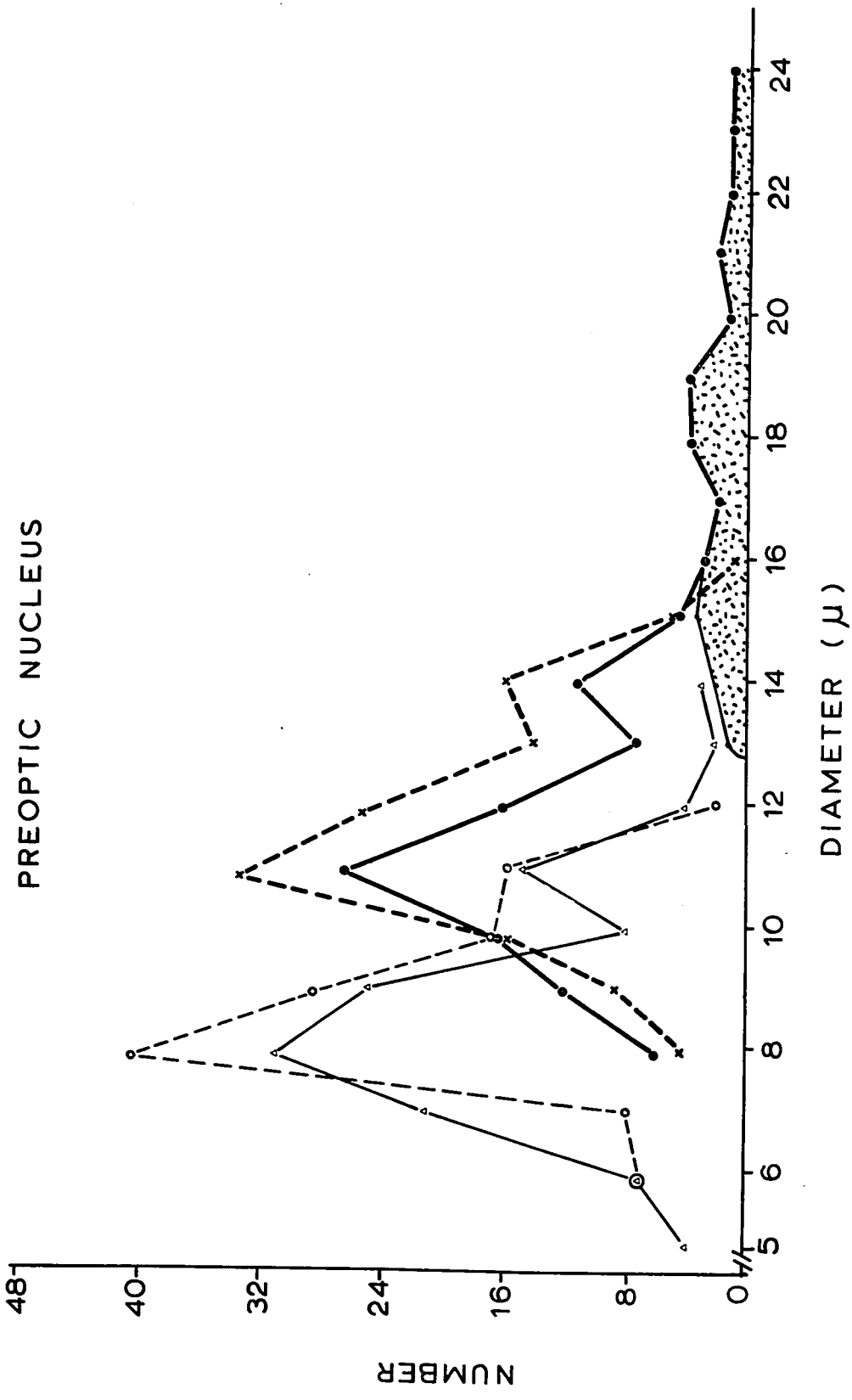

PREOPTIC NUCLEUS
NUMBER
DIAMETER (μ)
0
8
16
24
32
40
48
5
6
8
10
12
14
16
18
20
22
24

Figure 10

Figure 11. A 'satellite' oligodendrocyte observed within the preoptic nucleus of a control animal. Their relationship with the larger neuron soma (NS), their angular sharp, eccentric nuclei with clumped chromatin (arrow), and dark, granular cytoplasm are the characteristic features of these cells. The small axons (A) in the field are arranged in bundles between the larger dendrites (D). A number of the axons contained dense-core vesicles (DCV) as shown in the inset.

X 21,500
inset X 37,500

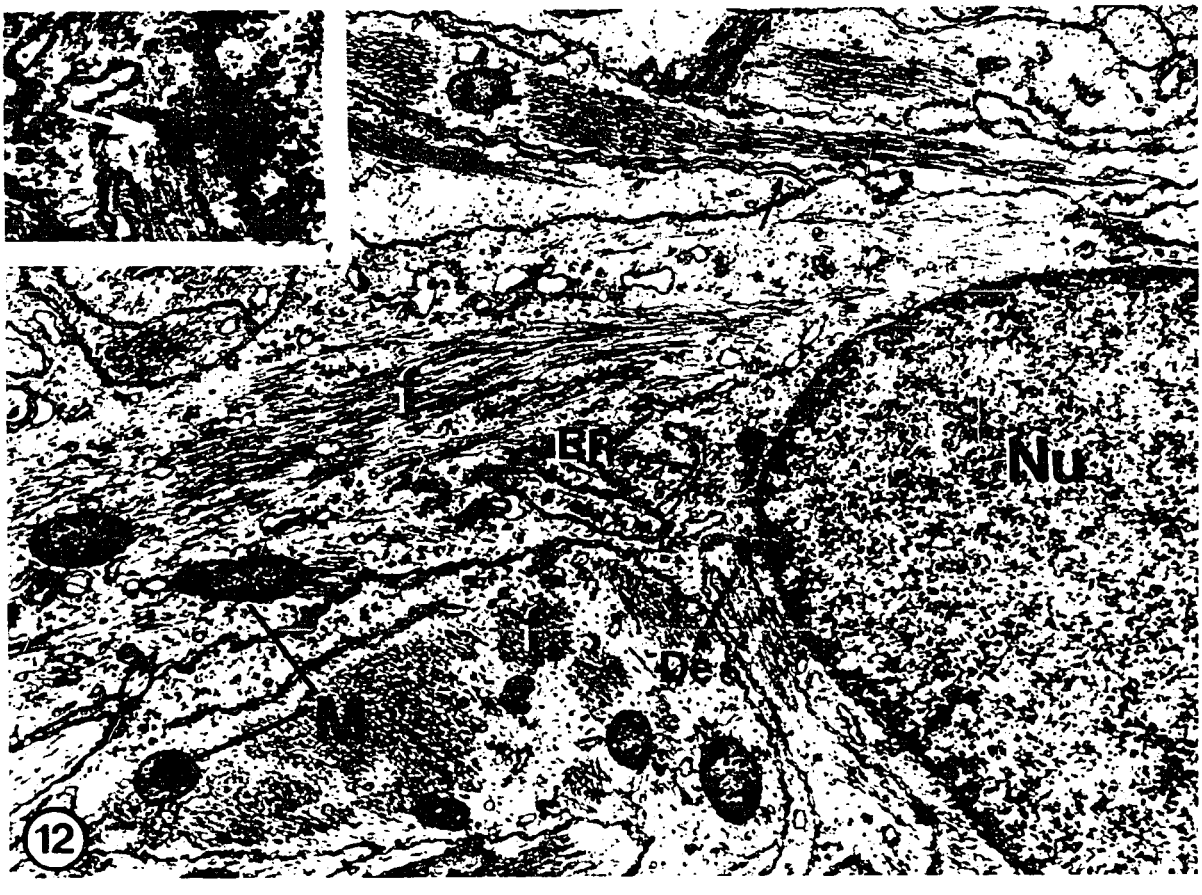
Figure 12. Portion of the nucleus (Nu) and perinuclear zone of an astrocyte. The chromatin is evenly distributed; bundles of fibers (f) and mitochondria (M) occur within the cytoplasm and processes. The cisternae of the rough endoplasmic reticulum (ER) are short and irregular. Junctions between the membranes of the astrocytic profiles resemble zonula occludens (black arrow) and desmosomes (De). A desmosome-like structure (white arrow) is featured in the inset. Preoptic nucleus, control animal.

X 15,000
inset X 27,500

D
NS
DCV
11

I
EL
N
12


D
NS
Dcv
11


ER
Nu
12

The regular arrangement of neurotubules was, however, frequently interrupted by the presence of scattered cisternae of the rough ER, mitochondria, saccules of the Golgi system and a variety of vesicular structures. These organelles, with the exception of mitochondria and vesicles, were not observed within the axon profiles. Also, the axons tended to course through the neuropil as small bundles of profiles in which the closely apposed units were arranged parallel to one another (Figure 11). In contrast, the dendrites usually occurred individually.

A sample of 200 dense-core (DC) vesicles observed within the axons of the control group had a mean diameter of 895 ± 10 Å (Figure 13), with minimum and maximum diameters of 570 Å and 1372 Å, respectively (Table 3). A histogram showing the frequency distribution of these measurements is included in Figure 14. These vesicles were characterized by their core of electron-dense material that was typically separated from a limiting membrane by a distinct clear area or 'halo' (Figure 11, inset).

The processes of glial cells, in particular those of the astrocytes, were commonly observed near, or terminating as end-feet and forming the perivascular glia limitans around the perivascular spaces of a number of the capillaries. The presence of large compact bundles of fibers, similar to those of the astrocytes (Figure 12) assisted in the identification of these processes (Figure 15). Tight junctions between the glial cell processes were common (Figure 15). Localized cytoplasmic densities resembling hemidesmosomes characterized the free surface of these end-feet which, in turn, were separated from

Figure 13. Histograms of the mean diameters of the dense-core vesicles observed within the axon profiles of the control and experimental animals: preoptic nucleus (), suprachiasmatic nucleus (), and arcuate nucleus ().

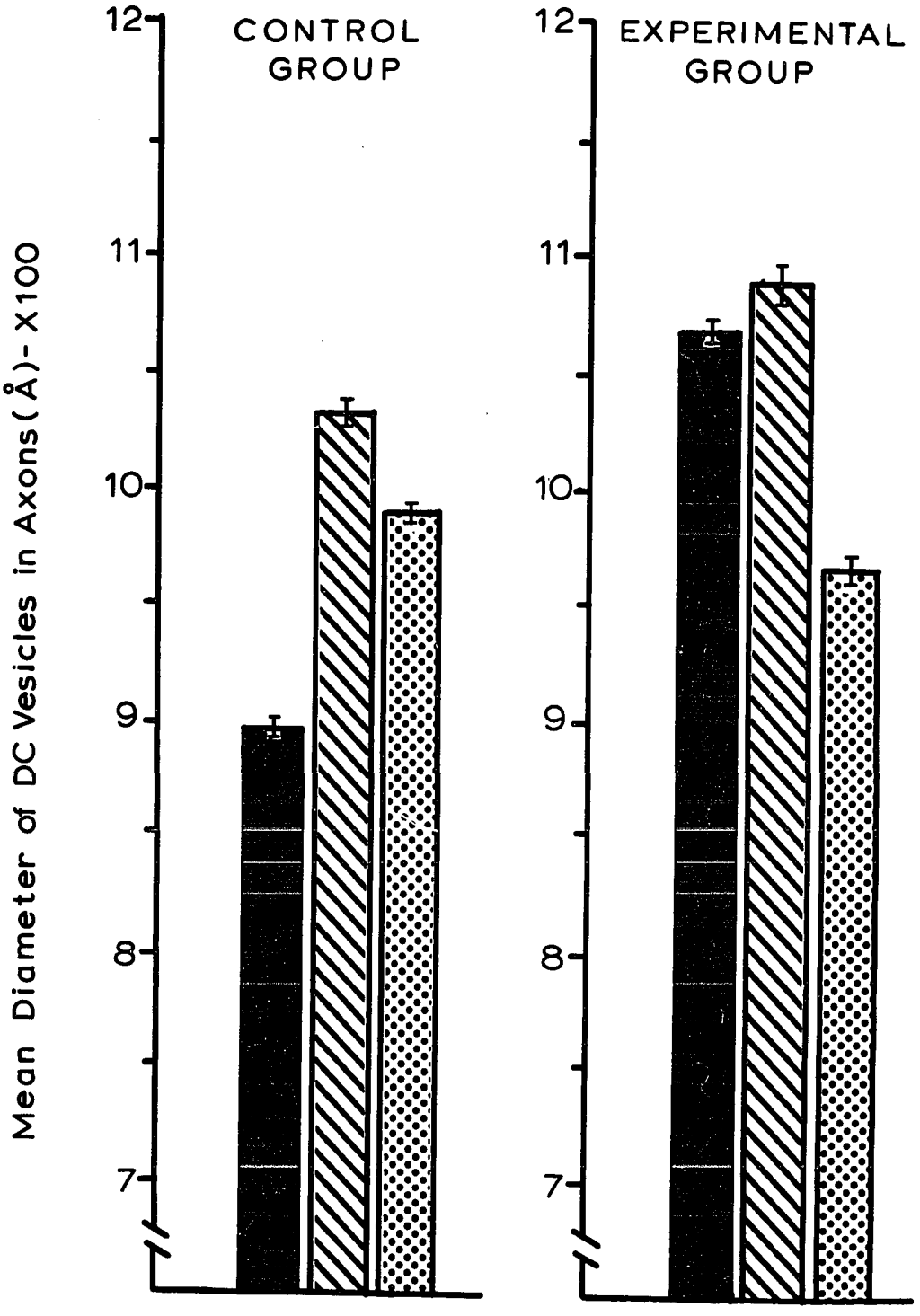

CONTROL GROUP
EXPERIMENTAL GROUP
Mean Diameter of DC Vesicles in Axons (Å)- X100
12
11
10
9
8
7

Figure 13

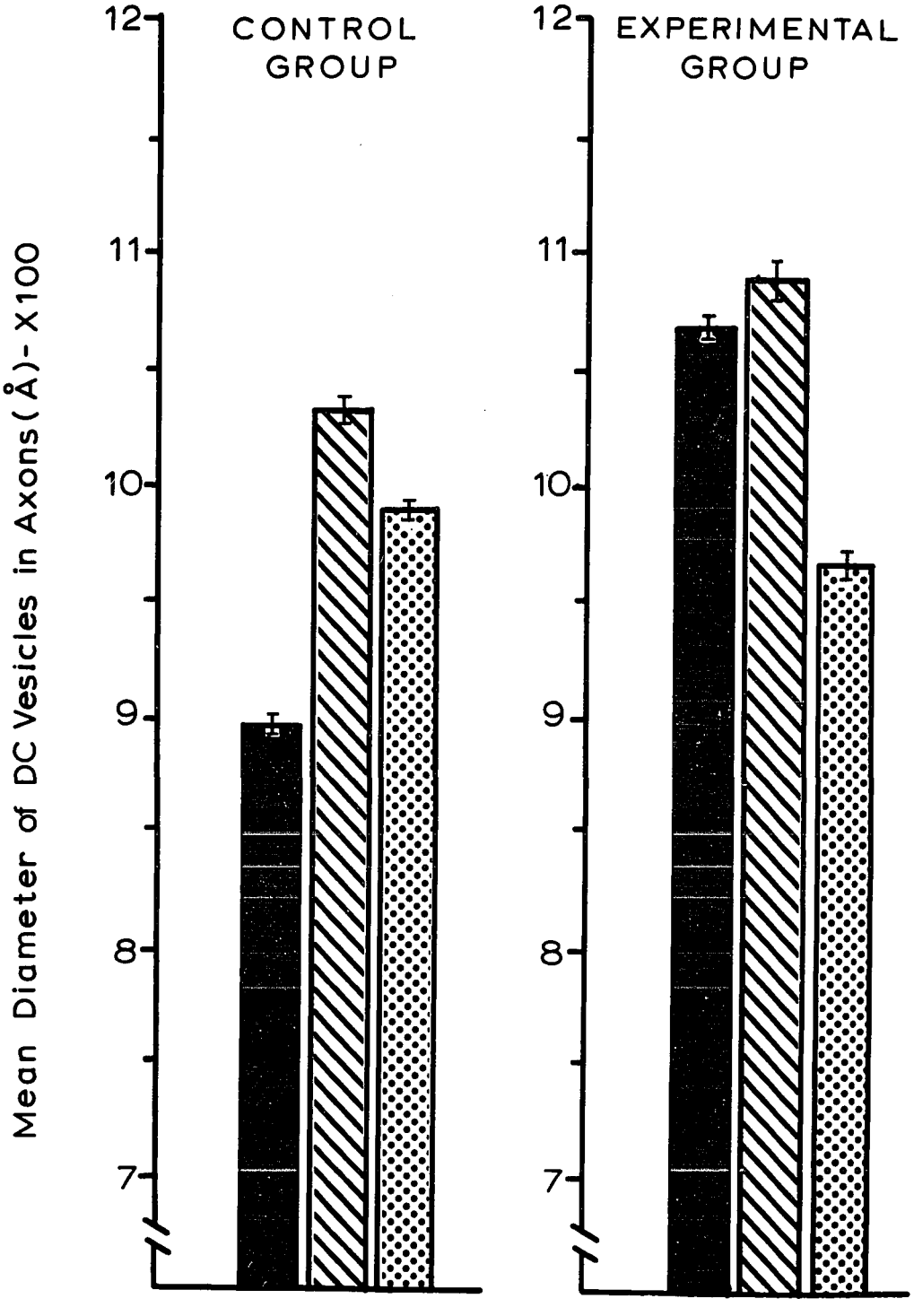

CONTROL GROUP
EXPERIMENTAL GROUP
Mean Diameter of DC Vesicles in Axons (Å) - X100
12
11
10
9
8
7

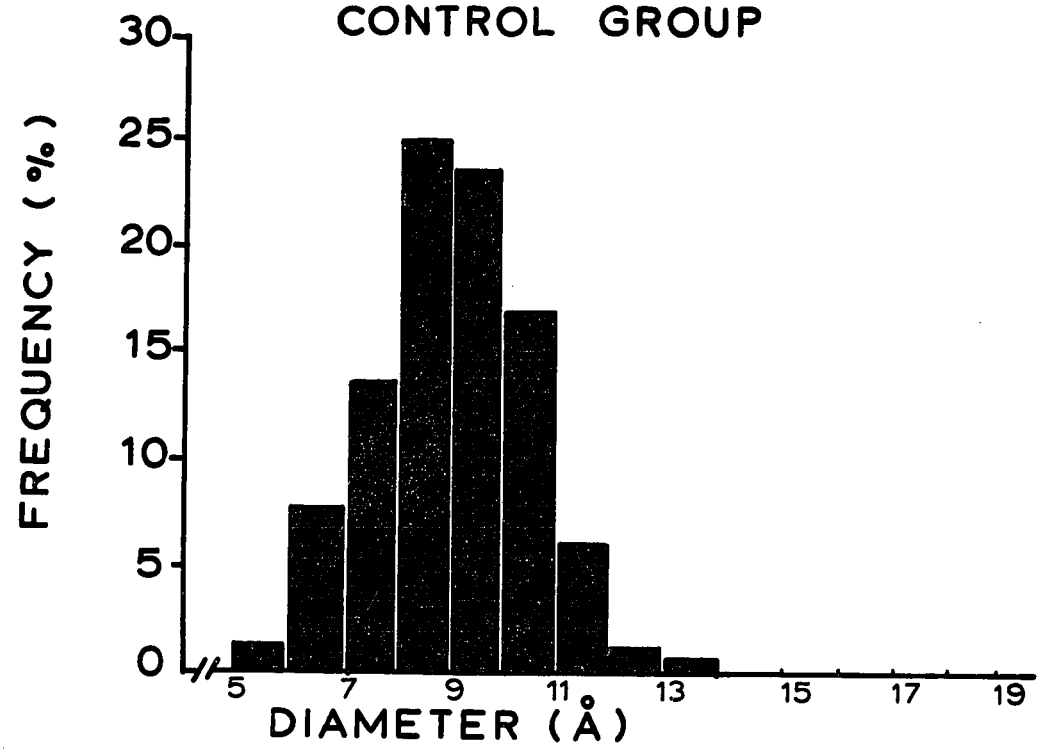
Figure 13

TABLE 3

SUMMARY OF COMPUTER ANALYSIS OF THE DIAMETERS OF DC VESICLES WITHIN AXONS OF THE PREOPTIC, SUPRACHIASMATIC AND ARCUATE NUCLEI

Hypothalamic Nucleus	Group	N	Minimum Diameter (Å)	Maximum Diameter (Å)	$\bar{x}$ ± S.E.M. (Å)	t	P value
Preoptic	Controls	200	570	1372	895 ± 10	10.0	< .001
	Experimental	445	600	1710	1072 ± 11		
Suprachiasmatic	Controls	200	600	1550	1032 ± 13	2.58	< .01
	Experimental	293	570	1900	1089 ± 16		
Arcuate	Controls	315	628	1800	990 ± 9	1.28	> .10
	Experimental	312	500	1600	972 ± 11		

Figure 14. Histograms showing the frequency distribution of the dense-core vesicles contained within the axon profiles of the preoptic nuclei.


CONTROL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
DIAMETER (Å)

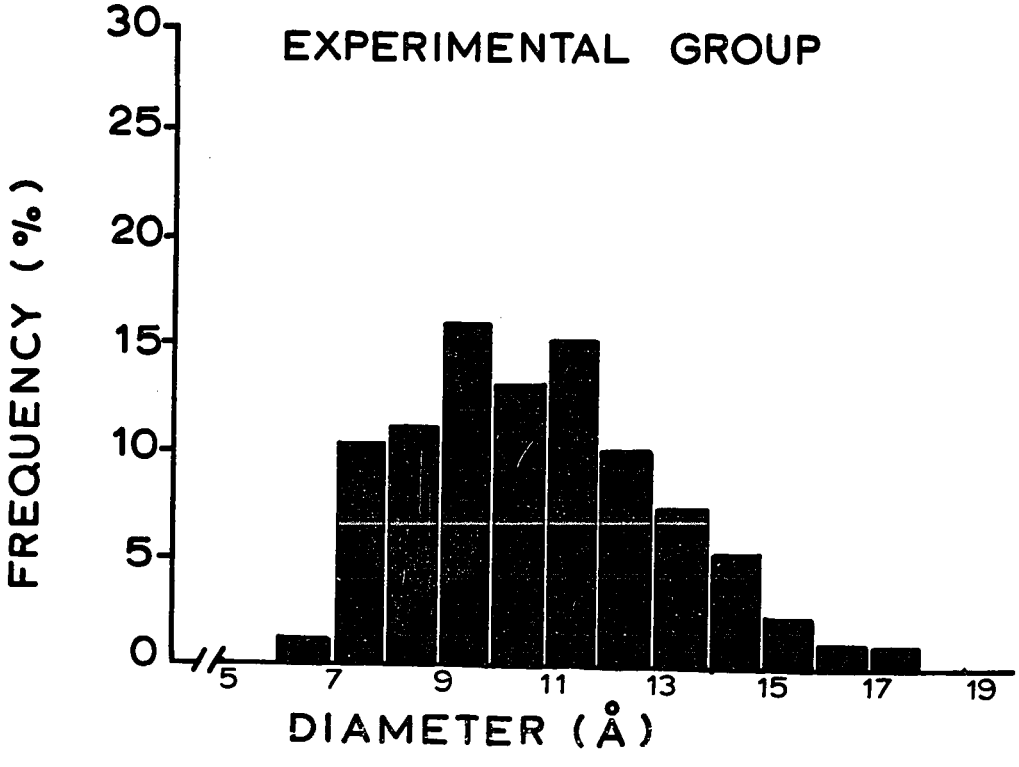

EXPERIMENTAL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
DIAMETER (Å)

Figure 14

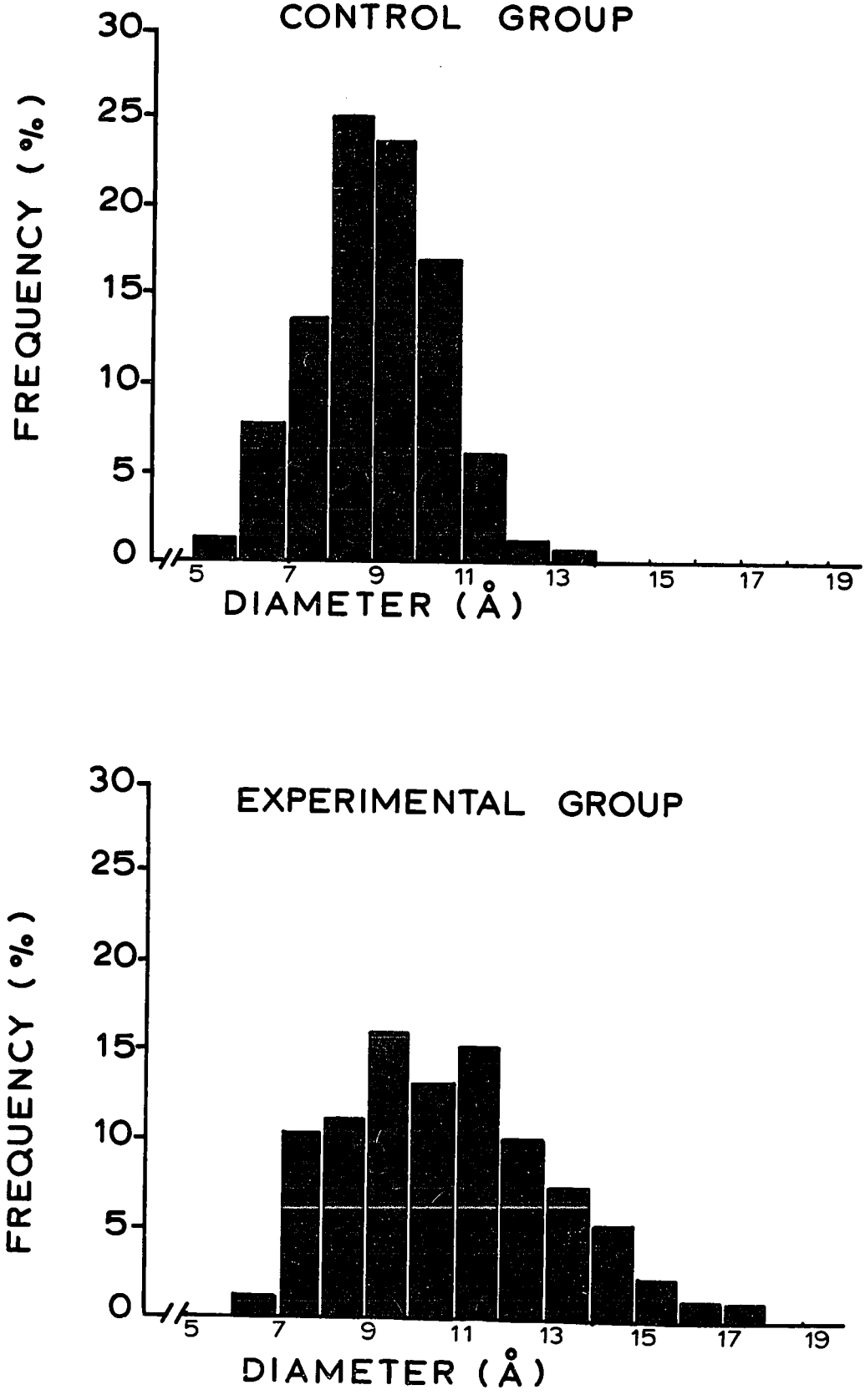
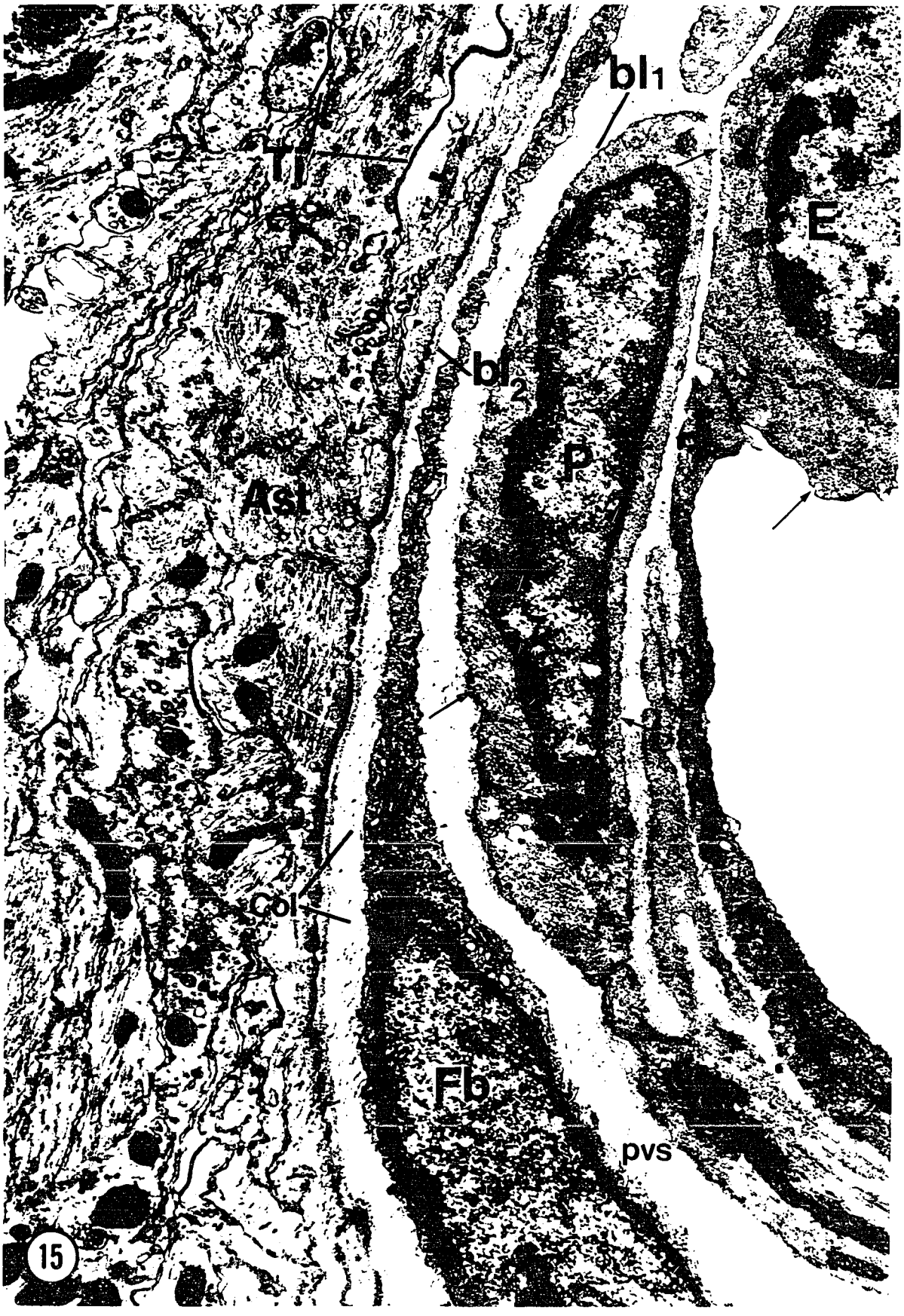

CONTROL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
DIAMETER (Å)
EXPERIMENTAL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
DIAMETER (Å)

Figure 14

Figure 15. High magnification electron micrograph showing portion of a capillary wall, perivascular space, and the surrounding neuropil. Endothelial cells (E) and pericytes (P) form the capillary wall proper. Note the numerous pinocytotic vesicles (black arrows) in these cells. The pericytes appear to be embedded within the basal lamina (bl_1) surrounding the capillary wall. Fibroblasts (Fb) and collagen (Col) fibers are shown within the perivascular space (pvs). Foot processes of astrocytes (Ast) terminate on, but are separated from the perivascular space proper by, a thick basal lamina (bl_2). Protoplasmic condensations (white arrows) occur under the free surface of these processes. Tight junctions (Tj) are seen between the membranes of the astrocytic processes.
Preoptic nucleus - control animal.

X 20,200

blh
pvs
15


bl1
Tf
E
bl2
P
Ast
Col
Fb
pvs
15

the perivascular spaces by a wide (400 Å to 700 Å) basal lamina (Figure 15). Tight junctions between opposing membranes of the end-feet were also observed.

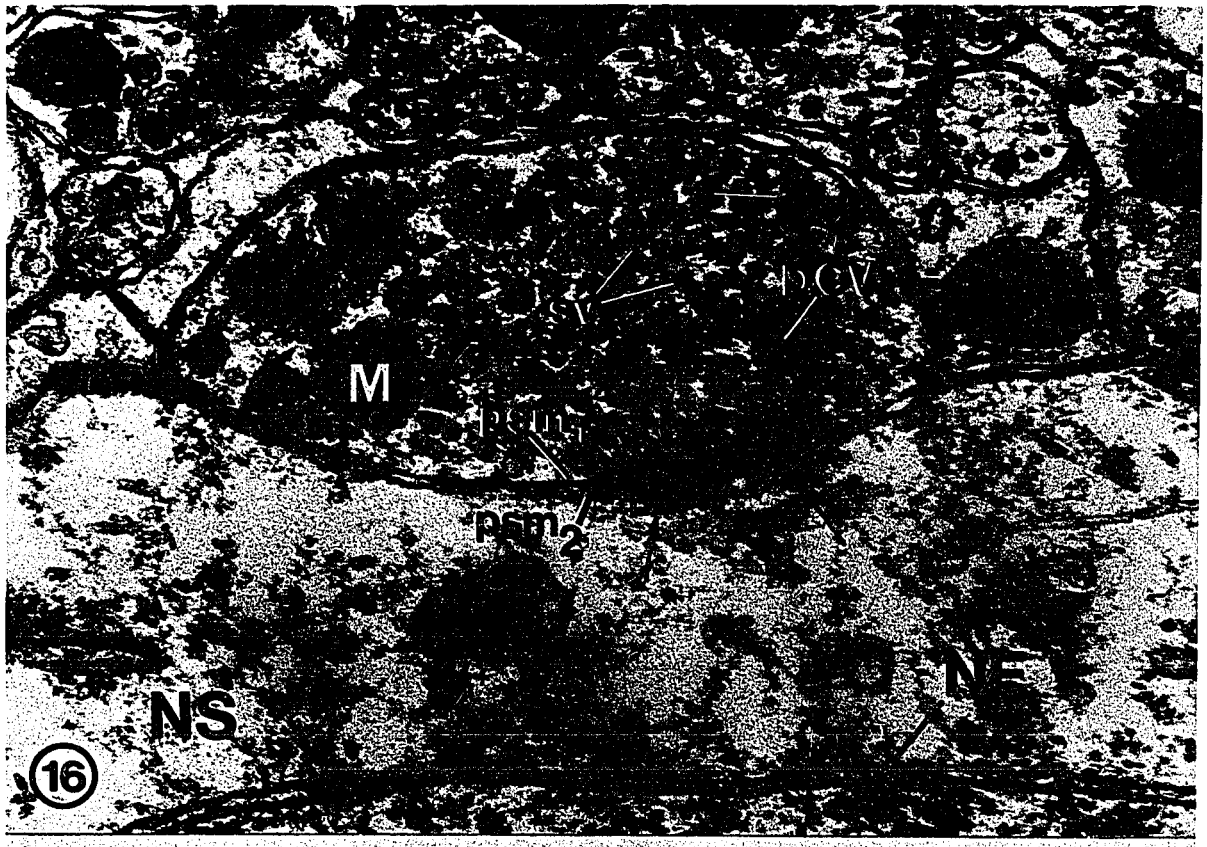
Axon terminals and the synaptic junctions between them and other structures of the PO nucleus were observed. Axo-somatic and axo-dendritic junctions were the most prominent. The morphological features of these synapses are shown in Figures 16 and 17 respectively. In each type, the axon terminal was readily identified by the presence of numerous membrane-bound vesicles ranging in diameter from 350 Å to 600 Å, mitochondria and a number of larger DC vesicles similar to those observed within the axon profiles. The mean diameter of these DC vesicles, based on 200 measurements, was 923 ± 10 Å (Figure 18), with minimum and maximum diameters of 620 Å and 1360 Å respectively (Table 4). The synaptic complexes associated with these axon terminals consisted of closely apposed presynaptic and postsynaptic membranes separated one from the other by a narrow (150 Å to 200 Å) space (Figures 16 and 17). The cytoplasm adjacent to each membrane showed an electron-dense, granular or filamentous material which continued for some distance along the length of the junction. The close association of the smaller, clear, membrane-bound vesicles with the electron-dense material within the axon terminal was a common finding. A variety of structures, including mitochondria, elongated vesicular structures, cisternae of the rough ER, free ribosomes, neurotubules and occasionally several regularly spaced 'button-like' condensations of electron-dense material, were observed in close spatial relationship with the junctional zone of the postsynaptic membrane. The 'button-like'

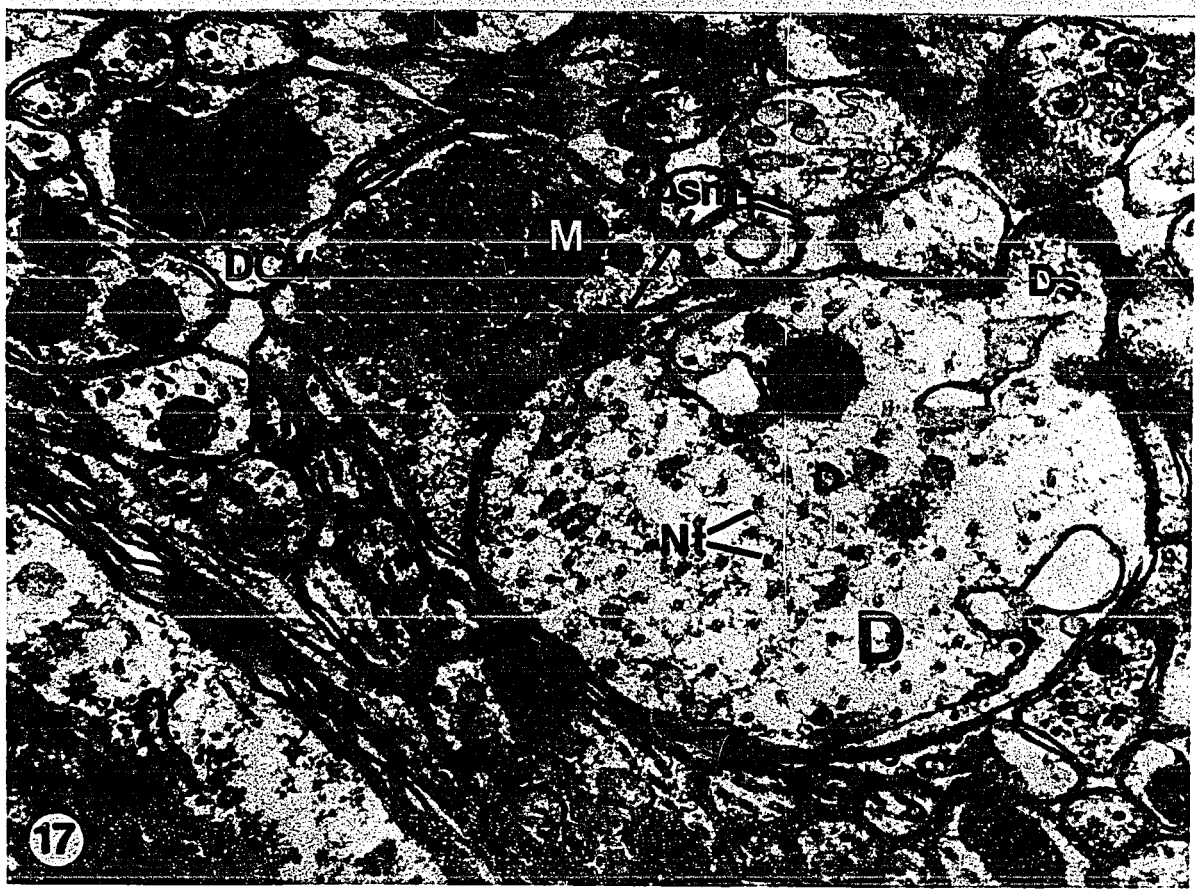
Figure 16. Electron micrograph of an axo-somatic junction. Note the dense population of synaptic vesicles (sv), and scattered dense-core (DCV) and mitochondria (M). 'Button-like' condensations under the postsynaptic membrane (psm_2) are clearly shown (arrows). A number of the synaptic vesicles are in close spatial relationship with the presynaptic membrane (psm_1) only in the region of the synaptic zone. Preoptic nucleus, control animal. NS, Neuron soma; NE, Nuclear envelope.

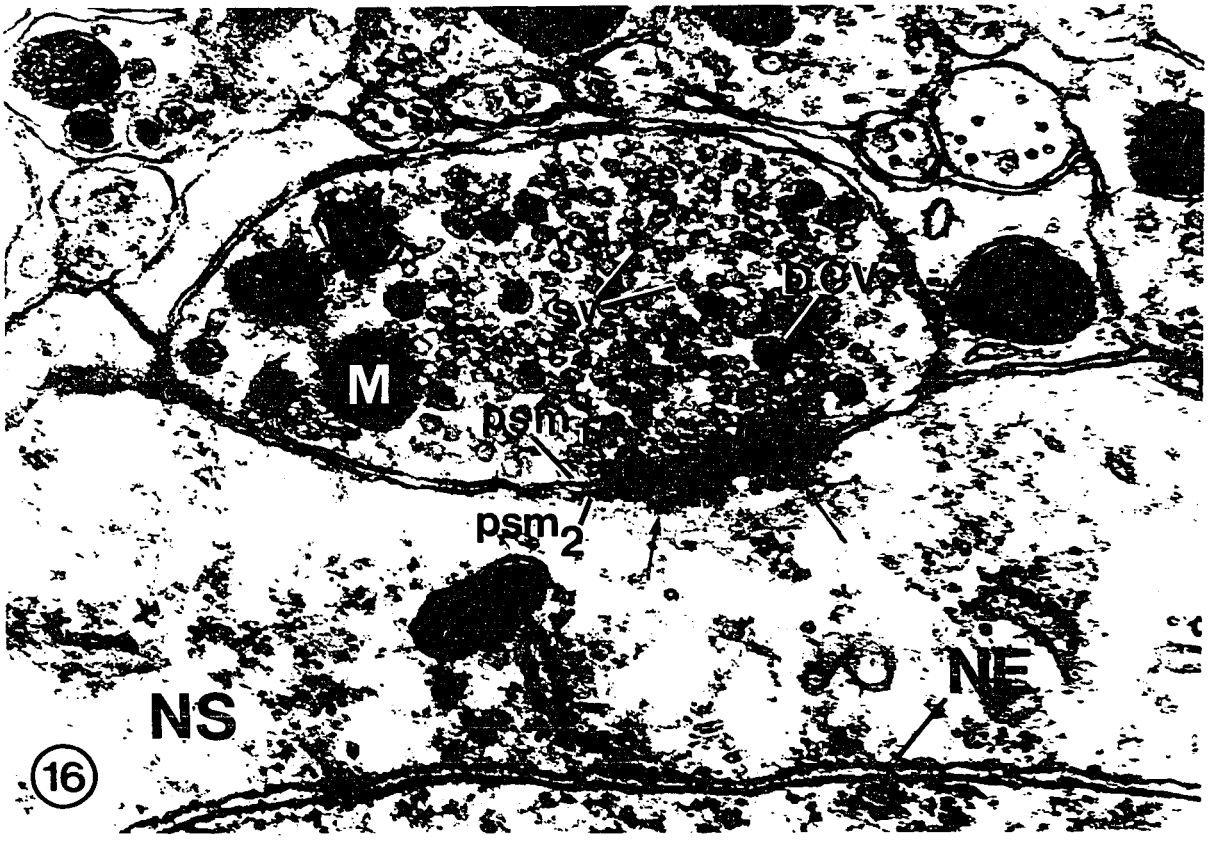
X 60,300

Figure 17. High magnification electron micrograph of an axo-dendritic junction observed within the preoptic nucleus of a rabbit sacrificed at 10 hours post-coitus. Again note the dense population of synaptic vesicles (sv), a number of which appear to be in contact with the presynaptic membrane (psm_1). Mitochondria (M) and dense-core vesicles (DV) were also common within the axon terminals forming these junctions. D, Dendrite; Ds, Dendritic spine; Nt, Neurotubule.

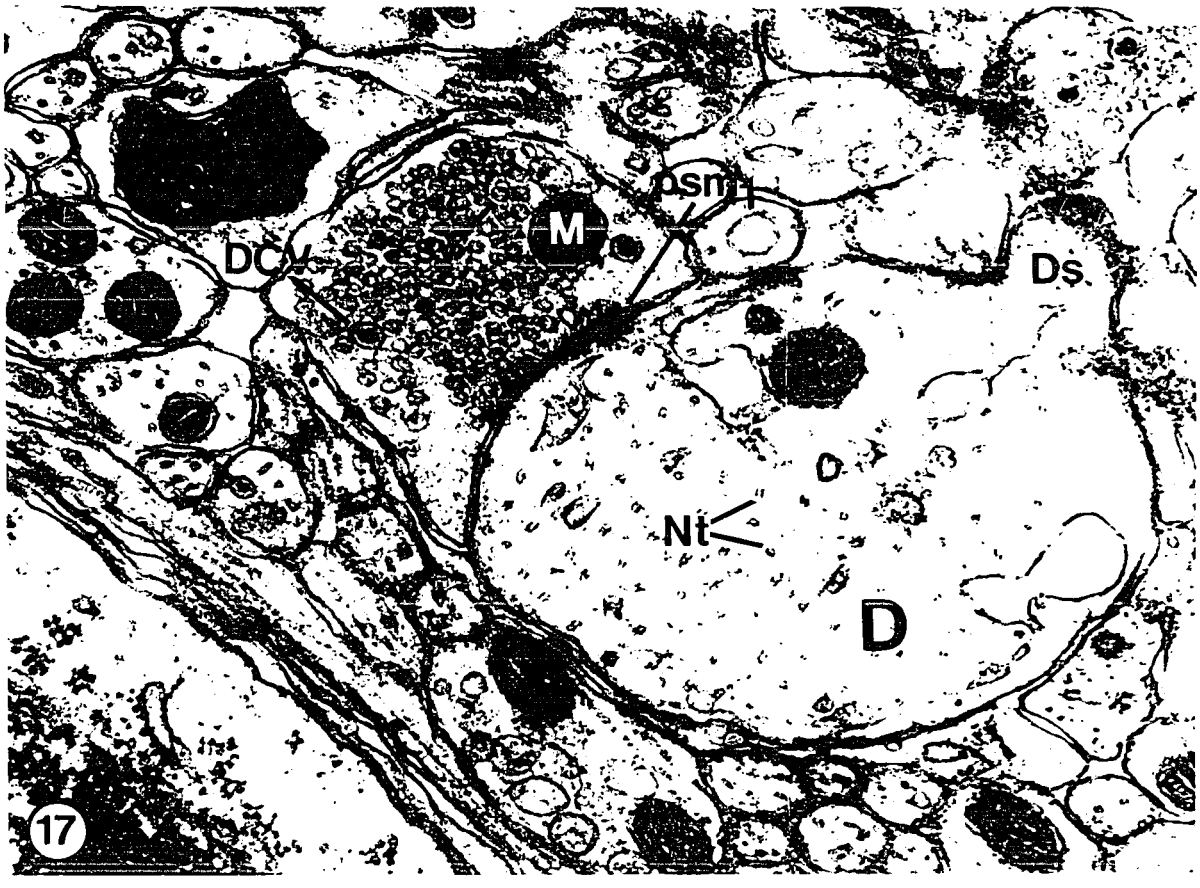
X 45,000


M
PSm1
PSm2
NS
N
16


M
Dc
Ds
Nf
D
17



16



17

Figure 18. Histograms of the mean diameters recorded for the dense-core vesicles located within the axon terminals of the control and experimental groups: preoptic nucleus (), suprachiasmatic nucleus (), and arcuate nucleus ().

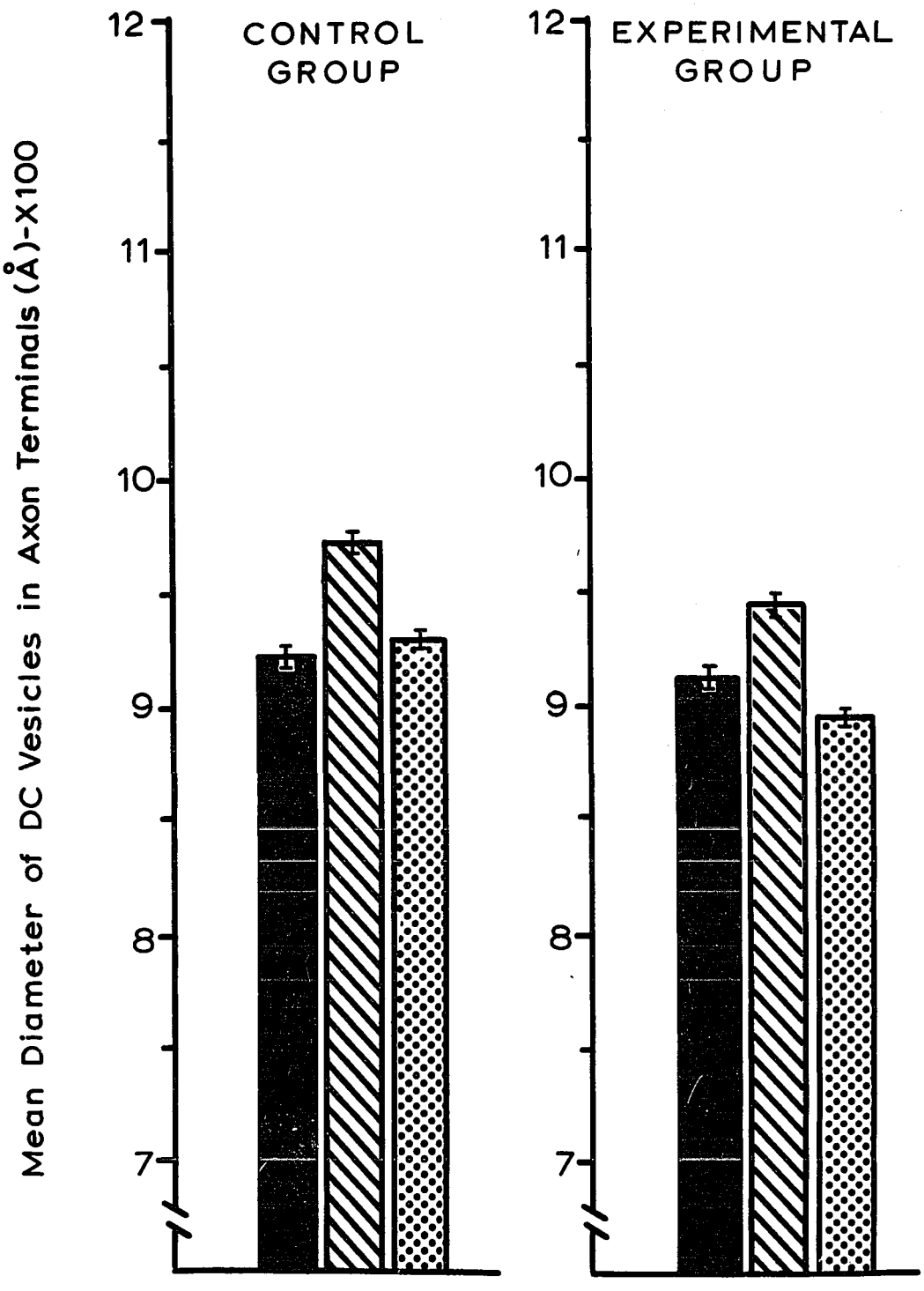

CONTROL GROUP
EXPERIMENTAL GROUP
Mean Diameter of DC Vesicles in Axon Terminals (Å)-X100
12
11
10
9
8
7

Figure 18

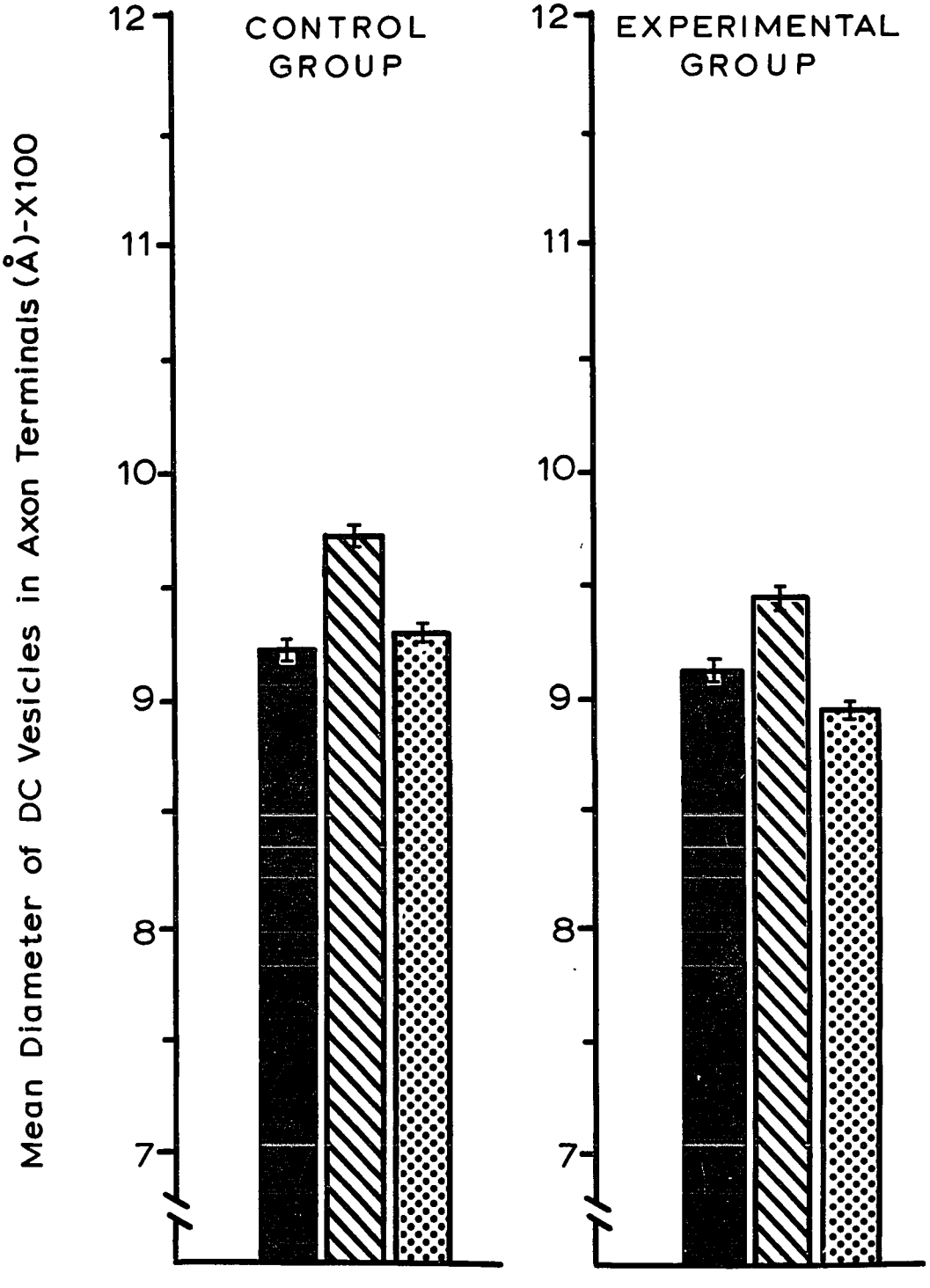

Mean Diameter of DC Vesicles in Axon Terminals (Å)-X100
12
11
10
9
8
7
CONTROL GROUP
EXPERIMENTAL GROUP

Figure 18

TABLE 4

SUMMARY OF COMPUTER ANALYSIS OF THE DIAMETERS OF DC VESICLES WITHIN AXON TERMINALS OF THE PREOPTIC, SUPRACHIASMATIC AND ARCUATE NUCLEI

Hypothalamic Nucleus	Group	N	Minimum Diameter (Å)	Maximum Diameter (Å)	$\bar{x}$ ± S.E.M. (Å)	t	P value
Preoptic	Controls	200	620	1360	923 ± 10	0.47	not significant
	Experimental	220	450	1370	917 ± 10		
Suprachiasmatic	Controls	200	620	1700	972 ± 12	0.67	not significant
	Experimental	228	600	1700	946 ± 11		
Arcuate	Controls	315	570	1428	933 ± 8	0.36	> .10
	Experimental	318	500	1450	891 ± 8		

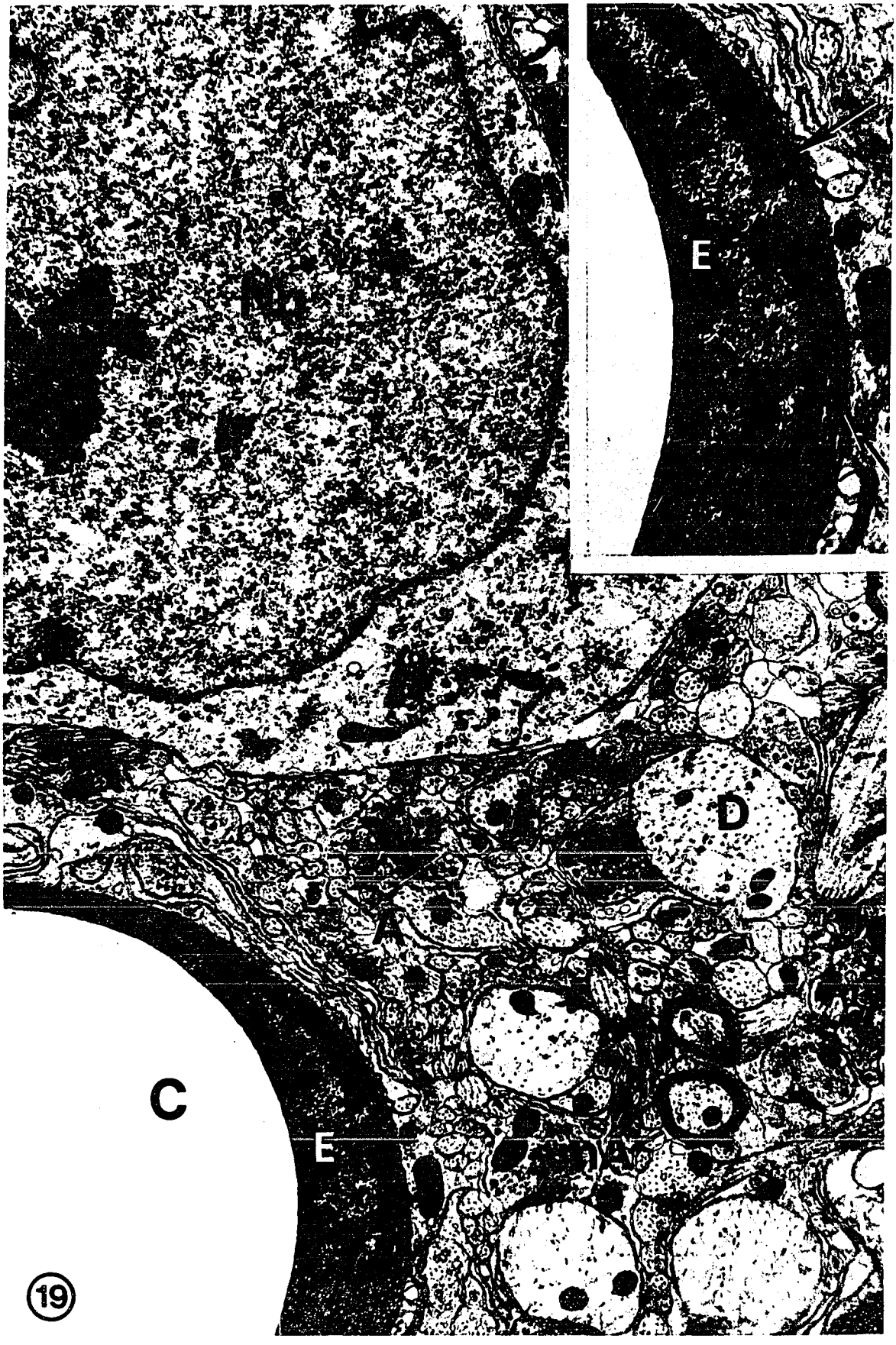
condensations of electron-dense material shown in Figure 16 were also observed occasionally below the postsynaptic membranes of the axodendritic junctions (Figure 17).

Capillaries were common throughout the PO nucleus. The ultrastructural features of portions of the wall of two capillaries, as well as their relationship with the surrounding neuropil, are shown (Figures 7 and 15). Most commonly, the lumen of these capillaries were separated from the brain parenchyma only by one or more endothelial cells and a thick (400 Å to 700 Å) basal lamina. Several dark cells identified as pericytes were usually embedded within the basal lamina adjacent to the endothelial cells (Figure 15). However, pericytes were occasionally absent (Figure 19). The capillary lumen was typically lined by one or more endothelial cells characterized by an elongated nucleus and dark, granular cytoplasm. The pericytes embedded within the surrounding basal lamina had a similar dark appearance. Numerous pinocytotic vesicles were observed within both the inner and outer surfaces of the endothelial cells (Figure 15). Pinocytotic vesicles were also observed in the surrounding pericytes, but were less numerous. Large numbers of pinocytotic vesicles were not a common finding within the endothelial cells forming the walls of the smaller capillaries (Figure 19, inset). Junctional zones between adjacent endothelial cells were also observed (Figure 15). Occasionally, the capillary wall was separated from the neuropil by a wide perivascular space that invariably contained fibroblasts and collagen fibers (Figure 15). It was of interest to note that in many instances where the neurons were observed in close spatial relationship

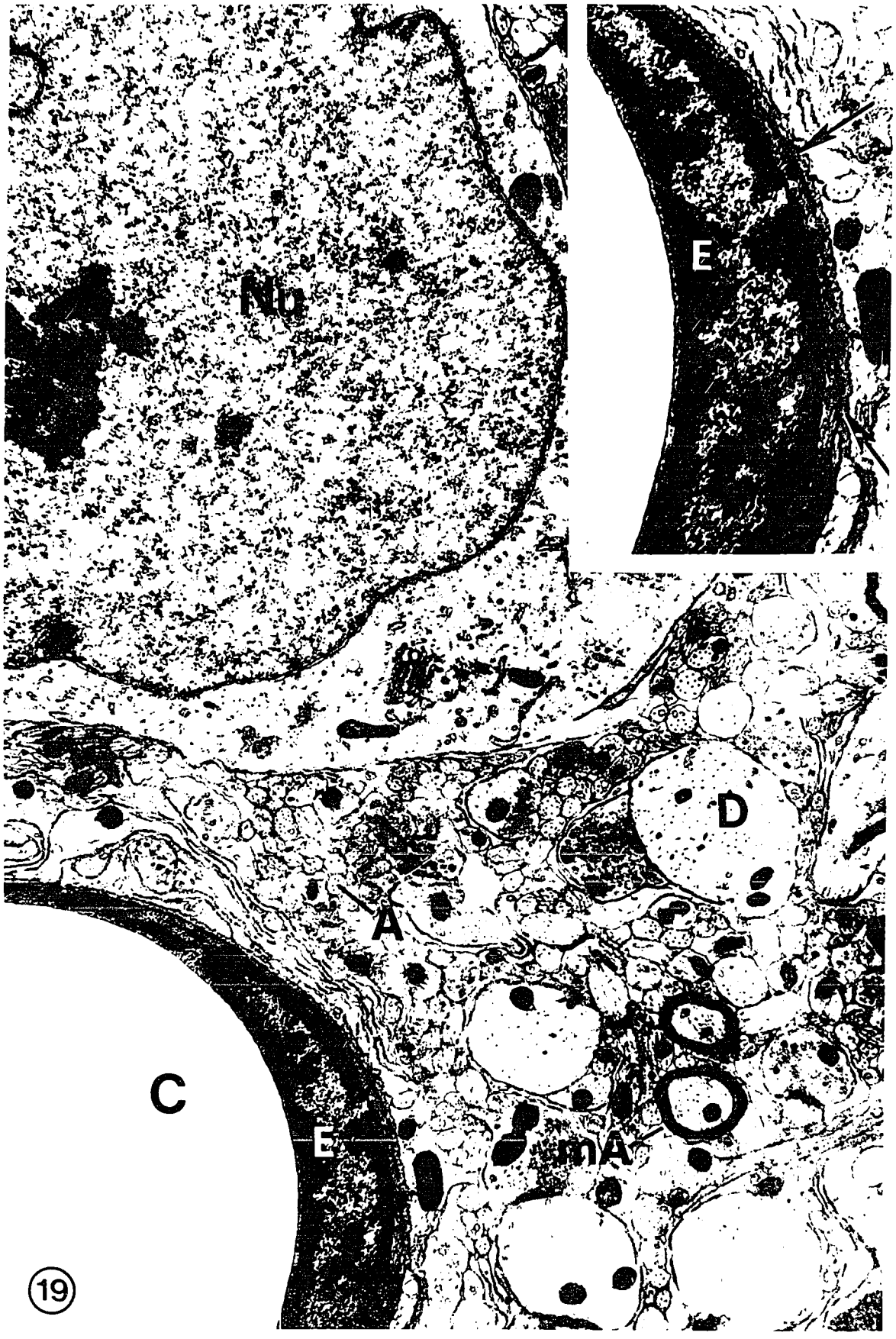
Figure 19. Electron micrograph of portion of a large neuron within the preoptic nucleus of the control group. The structural details of the nucleus (Nu), and characteristic sparsity of cell organelles within the cytoplasm are clearly illustrated. Note the close spatial relationship between the neuron and the capillary (C). The capillary wall consists of a single, large endothelial cell (E), which is separated from the neuropil by a thick basal lamina (see inset - arrows). The components of the neuropil: - axons (A), dendrites (D) and myelinated axons (mA) are also clearly shown.

X 13,500

inset X 21,600



19



19

with capillaries, perivascular spaces of the type shown in Figure 15 were usually absent; only a narrow zone of the neuropil consisting primarily of processes identified as axon and dendritic profiles separated the neuron soma from the capillary wall (Figure 19). On the other hand, the majority of profiles intimately associated with the perivascular spaces, were those of the astrocytes.

2) Ultrastructure of PO neurons of the control animals

Many of the ultrastructural features that characterized the neurons of the controls, as well as their close spatial relationship with the capillaries, are shown in Figures 20 and 21. Much of the central area of these neurons was occupied by large, eccentric, vesicular nuclei with several deep indentations or clefts that frequently faced the more central zone of cytoplasm. The single nucleolus, readily identified by its large size and electron-density, was usually free within the karyoplasm, although contacts between this organelle and the inner member of the nuclear envelope were observed. The nucleolar components; namely, the pars fibrosa and pars granulosa, are also shown in Figures 20 and 21. Intranuclear rod-like structures were also a common finding within the neurons of the control group. The ultrastructure of these inclusions is described in a separate section of the results.

The nucleus was surrounded by a thin shell of cytoplasm which, on the basis of the distribution and sparsity of the cytoplasmic organelles, could not be divided arbitrarily into 'marginal' and 'central' zones. The Nissl substance was not well represented,

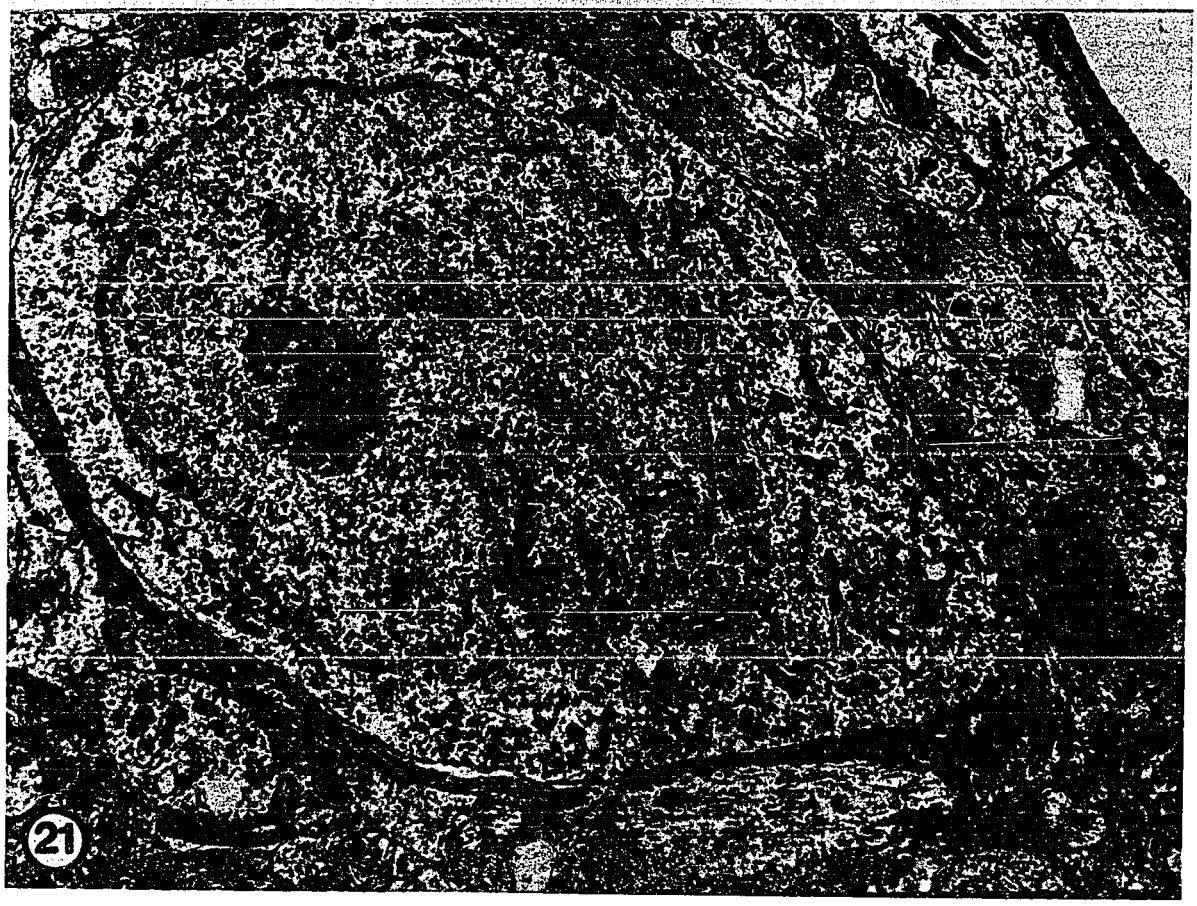
Figure 20. Electron micrograph showing the morphological features of a neuron removed from the preoptic nucleus of a control animal. The large nucleus (Nu) is typically vesicular and eccentric, and contains a single, large nucleolus (Nc). Note that the deep nuclear clefts (arrow) are facing the more central zone of the cell. Membrane profiles of the rough endoplasmic reticulum (ER) and Golgi system (G) are illustrated; these organelles are not well developed.

X 10,000

Figure 21. Field showing a neuron of the preoptic nucleus and the close spatial relationship between the soma of these cells and the capillary wall (arrow). Ultrastructurally, this neuron is similar to the one shown in Figure 20. The pars fibrosa (pf) and pars granulosa (pg) of the nucleolus are shown. Note the absence of a wide perivascular space between the capillary wall proper and the surrounding brain parenchyma. Control animal

X 7,500


Nc
Mu
ER
20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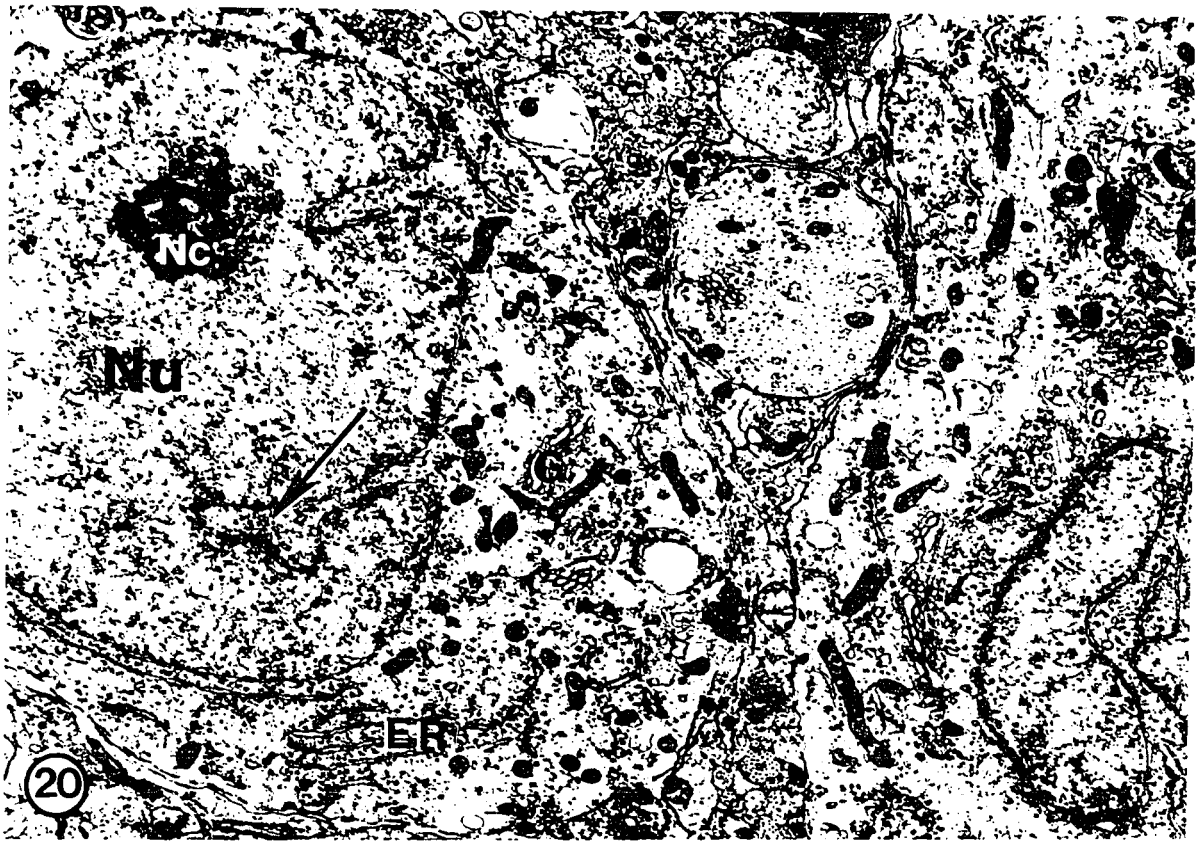
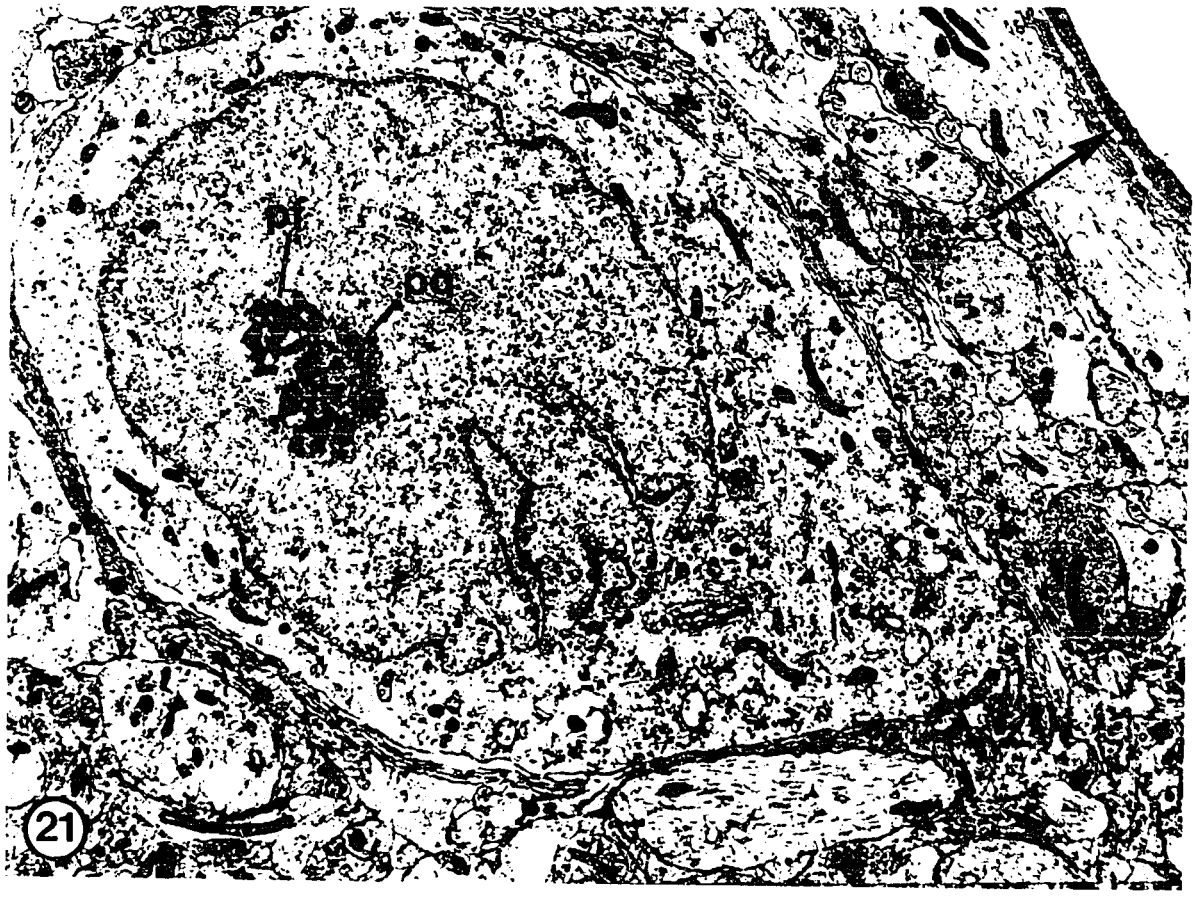
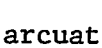
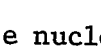

Nc
Nu
ER
20


pl
pg
21

consisting of only a few scattered, ribosome-studded cisternae of rough ER and isolated patches of free ribosomes. The free ribosomes were usually arranged in small clusters or rosettes which occupied much of the space between the cisternae of the rough ER (Figure 20). In these neurons, the Golgi system was likewise poorly developed. Both the saccular and vesicular components of this organelle appeared empty; accumulations of electron-dense material within the Golgi were never observed. The other cytoplasmic organelles such as mitochondria, lysosome-like structures, multivesicular bodies and neurotubules, were sparsely represented. Similarly, the DC vesicles were only present in very low numbers within the soma of these neurons. A total of 77 clearly identified DC vesicles occurred within the soma of some 200 neurons located near capillaries. These vesicles had a mean diameter of 1037 ± 18 Å (Figure 22), with a diameter range of 650 Å to 1450 Å (Table 5). The frequency distribution of these measurements is shown in Figure 23.

Throughout this study, the DC vesicles were distinguished from the lysosome-like bodies on the basis of their spherical shape and central core of electron-dense material that was typically separated from a limiting membrane by a clear area of 'halo' (Figure 24, inset B). The lysosome-like bodies were also much larger than the DC vesicles observed (Figure 24).

The multivesicular bodies were spherical in shape and bounded by a smooth membrane. No spatial relationship between this organelle and the Golgi was observed. Contained within the parent membrane were

Figure 22. Histograms of the mean diameters of the dense-core vesicles located within the neuron soma of the control and experimental groups: preoptic nucleus (), suprachiasmatic nucleus (), and arcuate nucleus ().

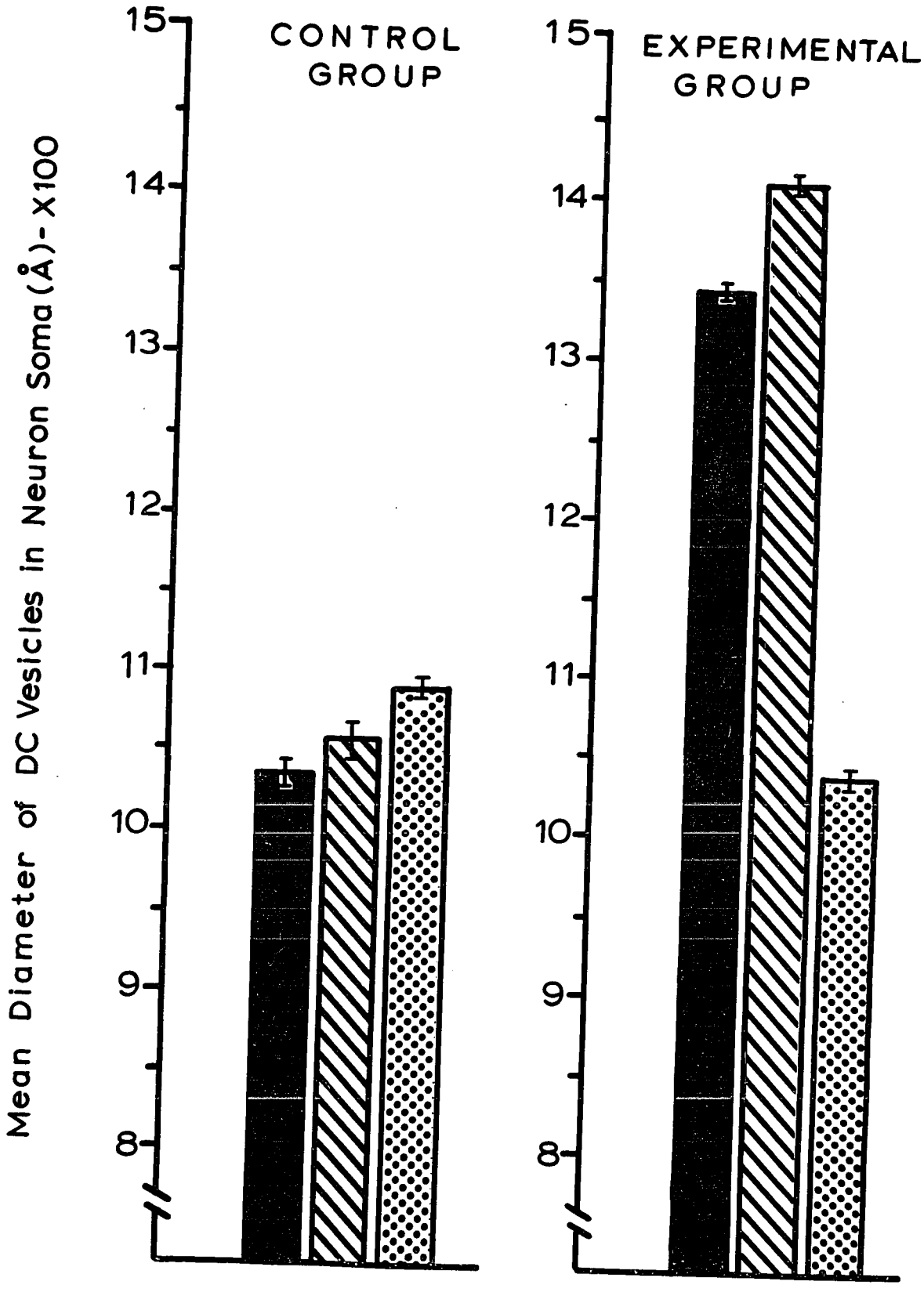

Mean Diameter of DC Vesicles in Neuron Soma (Å)- X100
15
14
13
12
11
10
9
8
CONTROL GROUP
EXPERIMENTAL GROUP

Figure 22

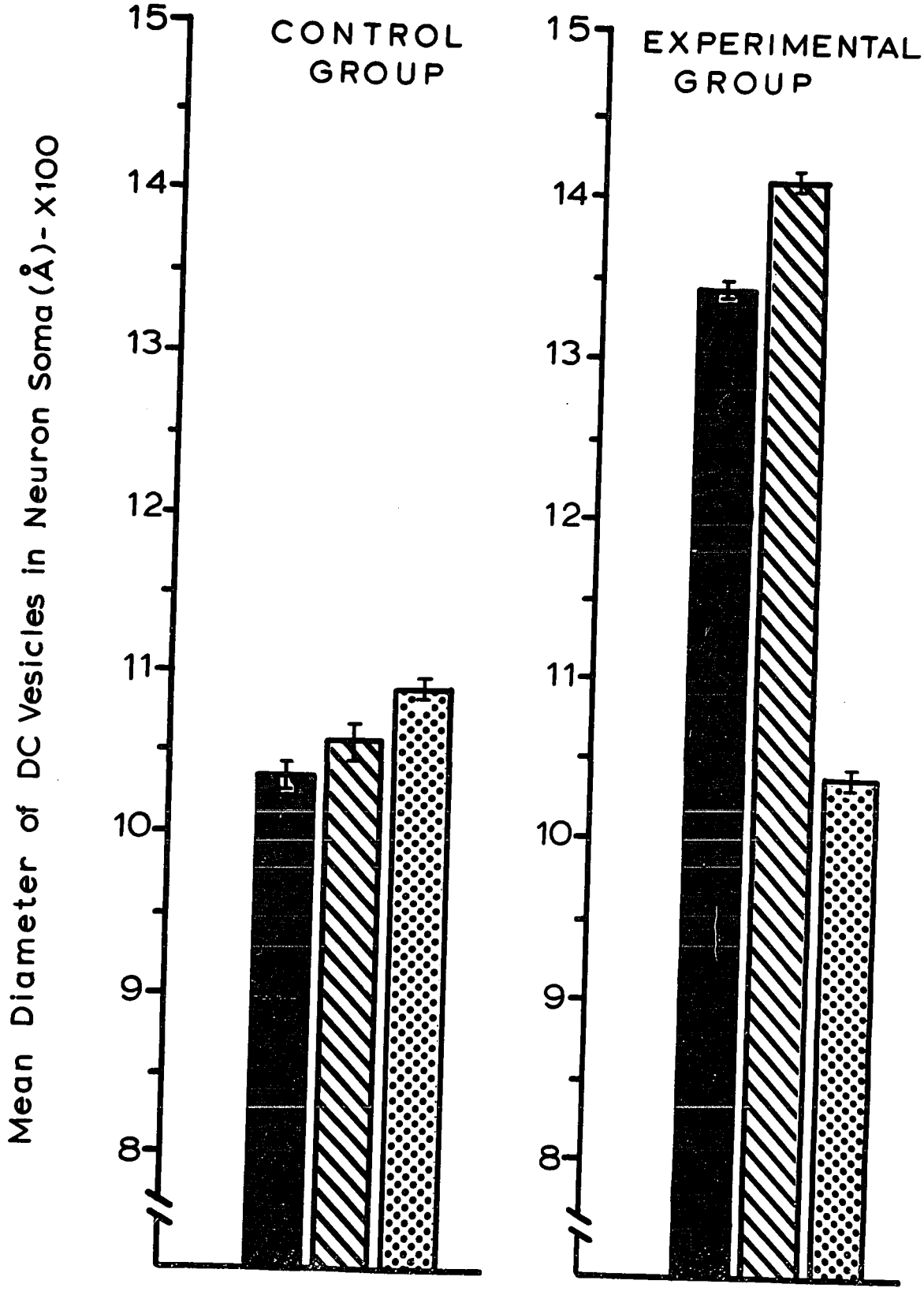

Mean Diameter of DC Vesicles in Neuron Soma (Å)- X100
15
14
13
12
11
10
9
8
CONTROL GROUP
EXPERIMENTAL GROUP

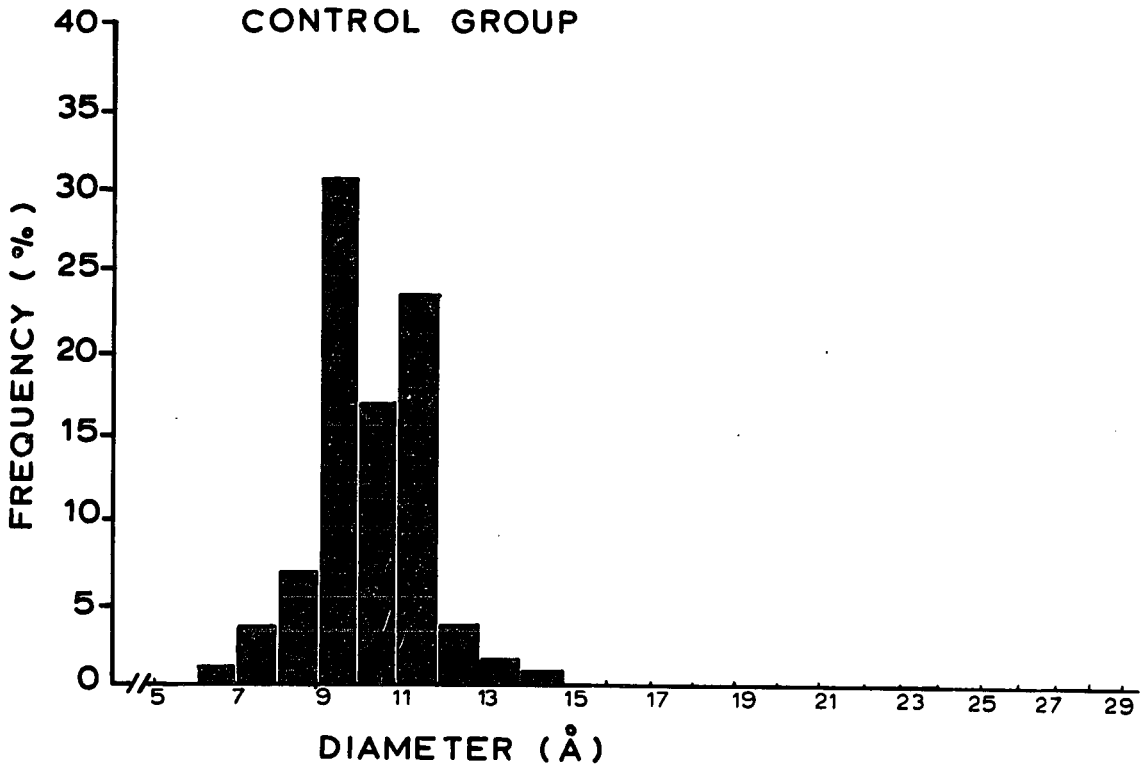
Figure 22

TABLE 5

SUMMARY OF COMPUTER ANALYSIS OF THE DIAMETERS OF DC VESICLES WITHIN THE SOMA OF NEURONS OF THE PREOPTIC, SUPRACHIASMATIC AND ARCUATE NUCLEI

Hypothalamic Nuclei	Group	N	Minimum Diameter (Å)	Maximum Diameter (Å)	$\bar{x}$ ± S.E.M. (Å)	t	P value
Preoptic	Controls	77	650	1450	1037 ± 18	7.81	< .001
	Experimental	696	500	2850	1343 ± 13		
Suprachiasmatic	Controls	73	750	1700	1059 ± 22	8.63	< .001
	Experimental	744	629	2470	1416 ± 13		
Arcuate	Controls	324	628	2050	1089 ± 13	2.71	< .01
	Experimental	336	500	2000	1039 ± 14		

Figure 23. Bar-diagrams showing the frequency distributions of the dense-core vesicles measured within the soma of neurons of the preoptic nucleus. The presence of large vesicles with diameters exceeding 1400 Å in the soma of the preoptic neurons following coitus is clearly evident.


CONTROL GROUP
FREQUENCY (%)
40
35
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
21
23
25
27
29
DIAMETER (Å)

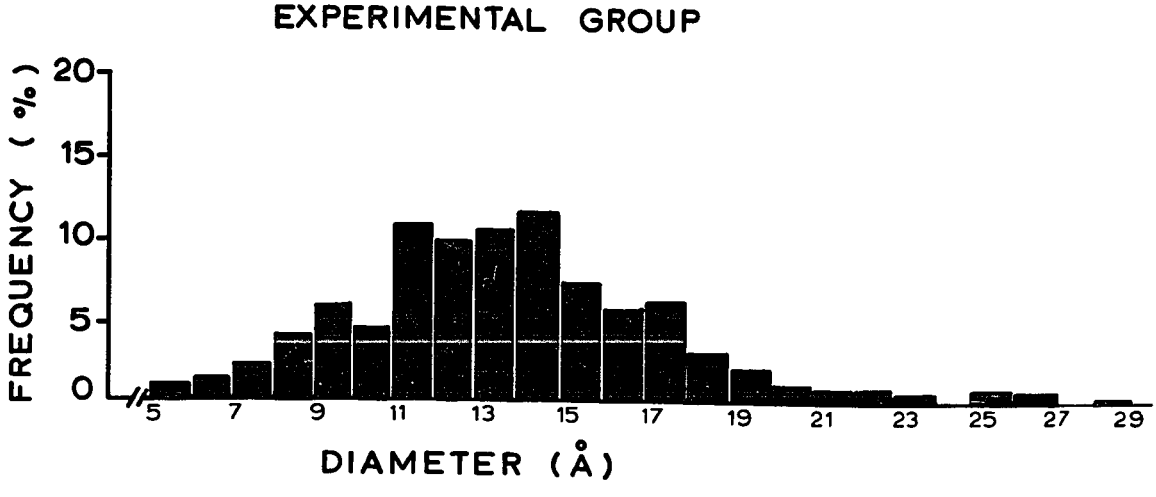
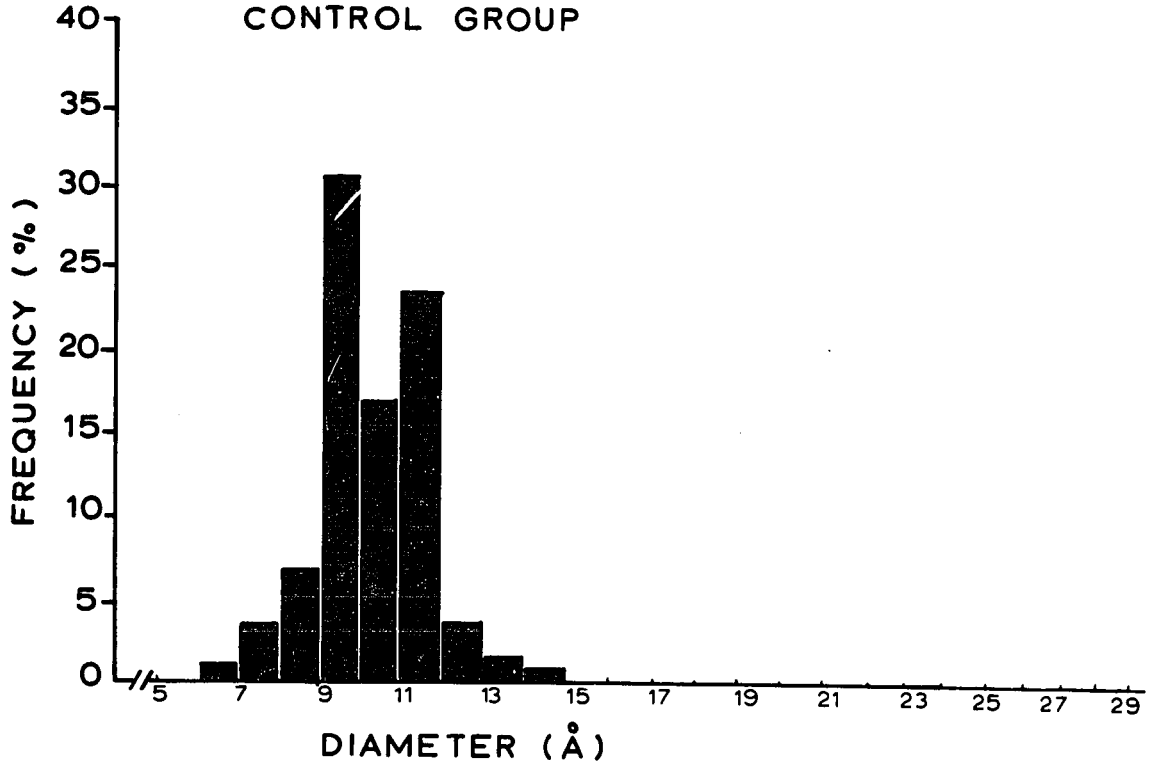

EXPERIMENTAL GROUP
FREQUENCY (%)
20
15
10
5
0
5
7
9
11
13
15
17
19
21
23
25
27
29
DIAMETER (Å)

Figure 23


CONTROL GROUP
FREQUENCY (%)
40
35
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
21
23
25
27
29
DIAMETER (Å)

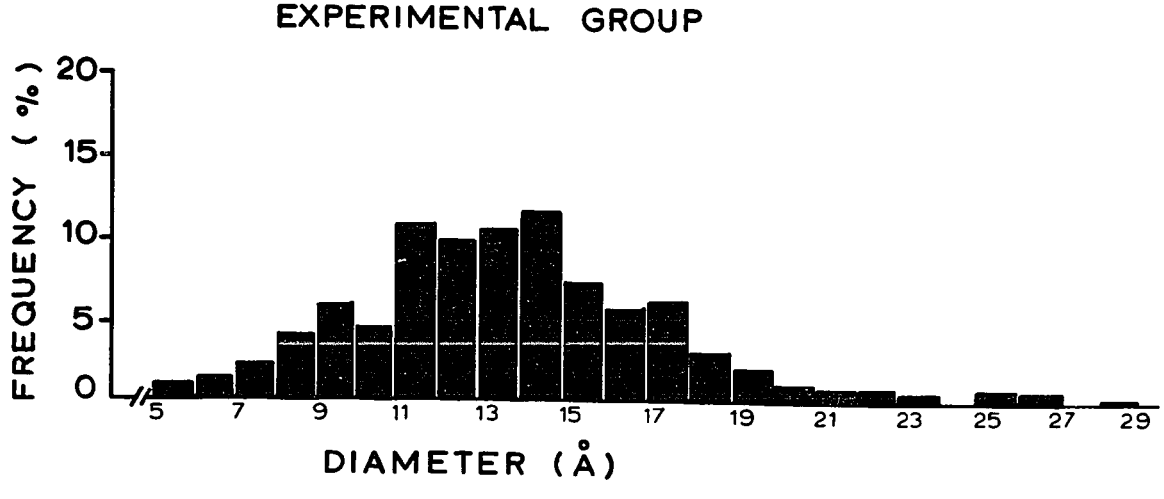

EXPERIMENTAL GROUP
FREQUENCY (%)
20
15
10
5
0
5
7
9
11
13
15
17
19
21
23
25
27
29
DIAMETER (Å)

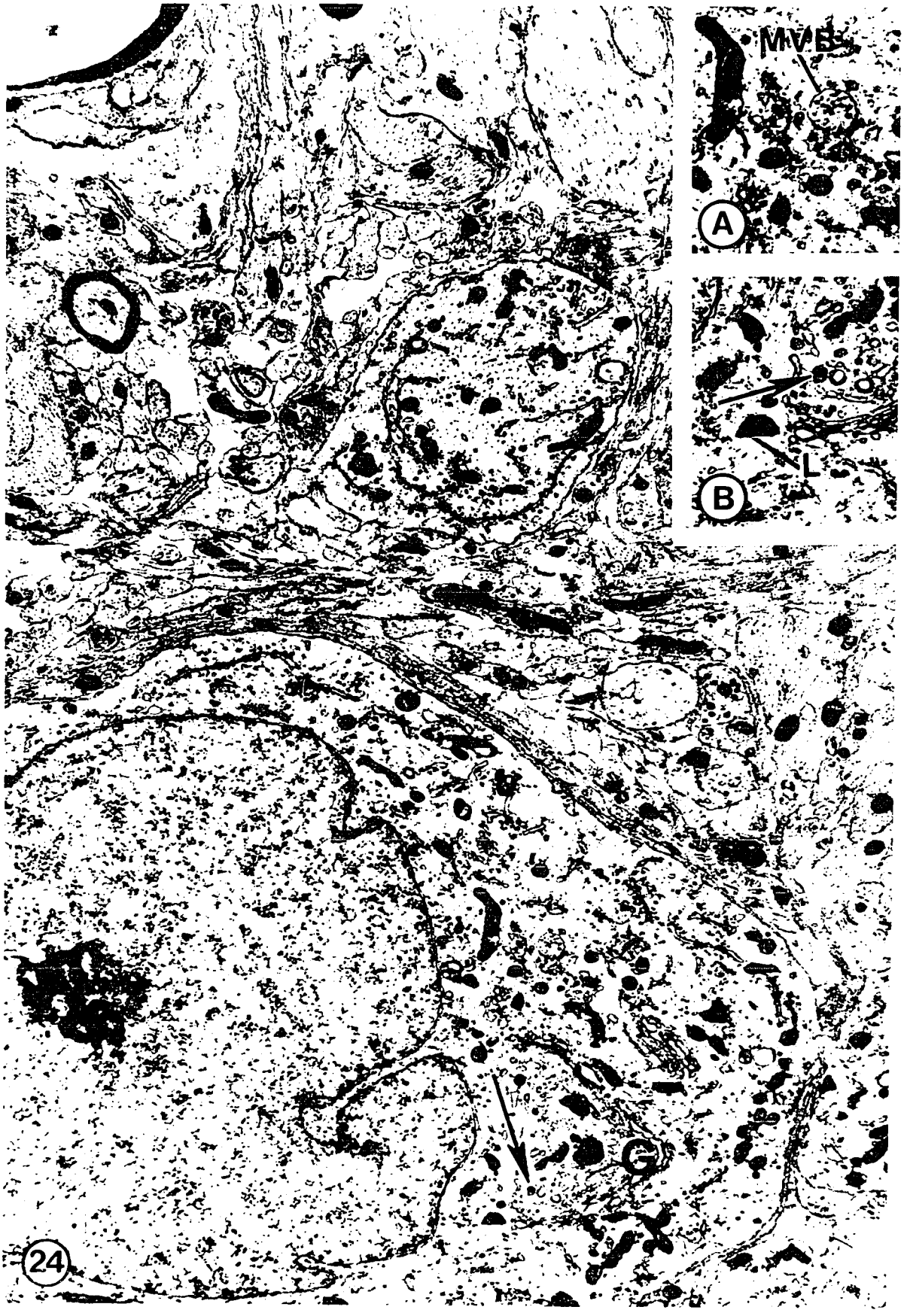
Figure 23

Figure 24. Electron micrograph of a neuron removed from the preoptic nucleus of a control animal. Only the occasional dense-core vesicle (arrow) was observed within the soma of the control neurons (inset B). The larger, electron-dense, irregular shaped bodies in inset B are lysosomes (L) or lysosome-like structures. A multivesicular body (MVB) is featured in inset A. G, Golgi system.

X 10,700

insets X 18,700

MVB
A
B
24


MVB
A
B
L
G
24

three to 20 smaller, clear vesicles with diameters ranging between 450 Å and 700 Å (Figure 24, inset A). Accumulations of electron-dense material within the smaller vesicles was not a common finding. The space between the smaller vesicles was occupied by a granular material not unlike that of the surrounding cytoplasm. Occasionally, membrane continuations with the parent membrane of the multivesicular bodies were also observed.

3) Ultrastructural changes in PO neurons of the experimental groups

Many of the neurons observed within the PO nucleus of the experimental animals were morphologically similar to those of the controls. However, discrete and obvious changes in ultrastructure were observed in a number of neurons located near the capillaries of the PO nucleus. These changes occurred in a total of 23 (19.2%) of 120 neurons, and were characterized by an increase in neuron diameter, the presence of elaborate rough ER which occupied much of the 'marginal' cytoplasm, several large crescent-shaped Gogli systems, as well as the presence of numerous large DC vesicles.

The mean diameter of the 120 neurons (including the 23 in which the discrete ultrastructural changes were observed) measured near capillaries of the experimental groups, was 12.9 ± 0.3 μ (Figure 8); the mean of their nuclei was 9.1 ± 0.2 μ (Figure 9). The ranges of these measurements as obtained from computer analysis are shown in Tables 1 and 2. When compared with the same parameters for the PO control animals, the mean diameter of the experimental neurons was significantly larger ($P < .01$); there was no significant difference

between the means recorded for their nuclear profiles (Table 2). However, when the 23 neurons in which discrete ultrastructural changes occurred were compared with those of the controls, both the neurons and their nuclei showed a significant ($P < .001$) increase in diameter.

The neuropil of the experimental groups was also studied carefully for the presence of DC vesicles. The mean diameter of 445 DC vesicles observed within the axon profiles in the PO nuclei of all the animals sacrificed from one hour through to 10 hours post-coitus was 1072 ± 11 Å (Figure 13). This measurement is significantly greater ($P < .001$) than that recorded for the same parameter within the control animals (Table 3). The ultrastructure of several large DC vesicles observed within an axon of the experimental group is shown in Figure 25.

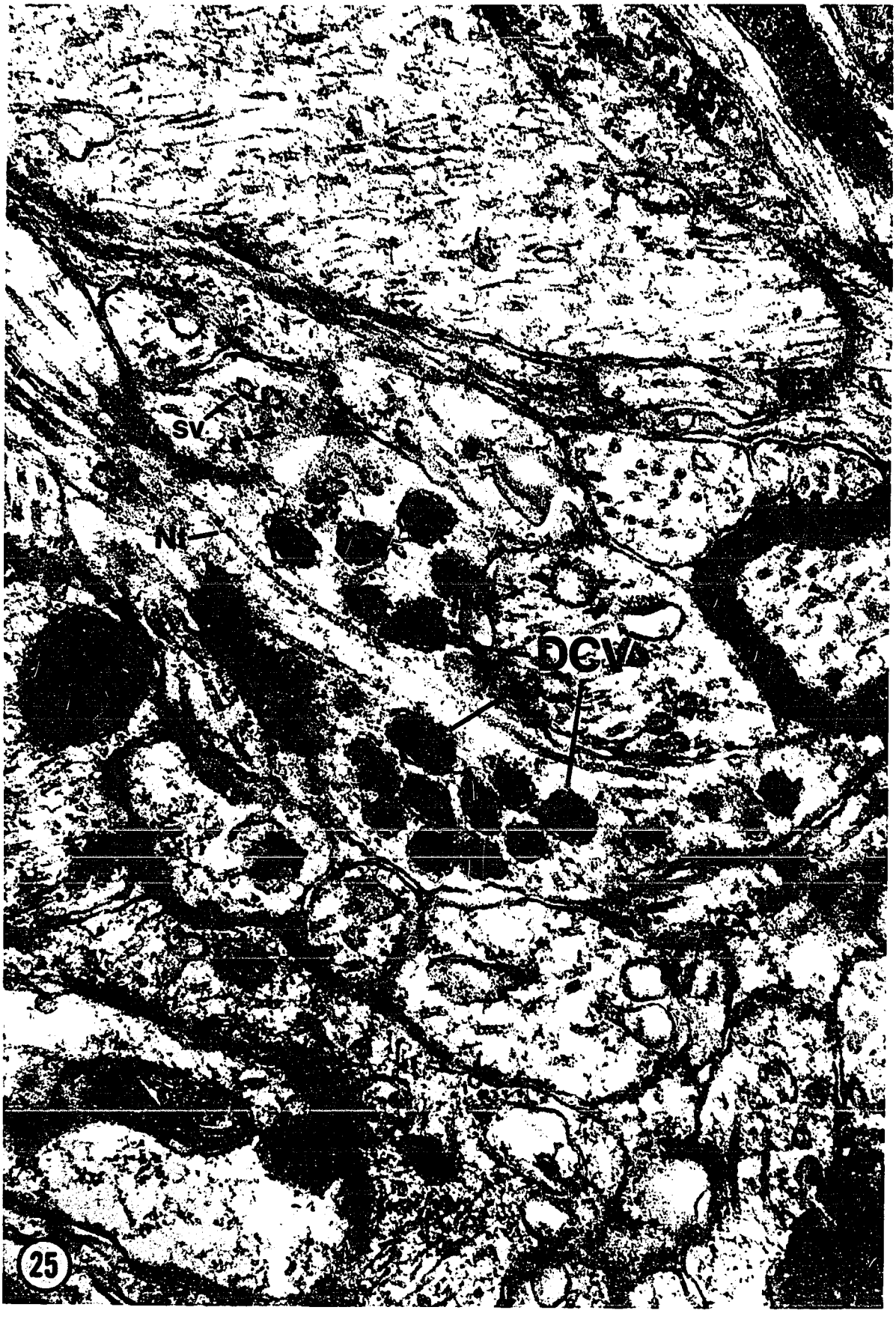
Similarly, the mean diameter of 200 DC vesicles recorded from the axon terminals, was 917 ± 10 Å (Figure 18). This mean was not significantly larger than that obtained for a similar sample of DC vesicles within the axon terminals of the controls (Table 4). The diameter ranges of these vesicles are also given in Table 4.

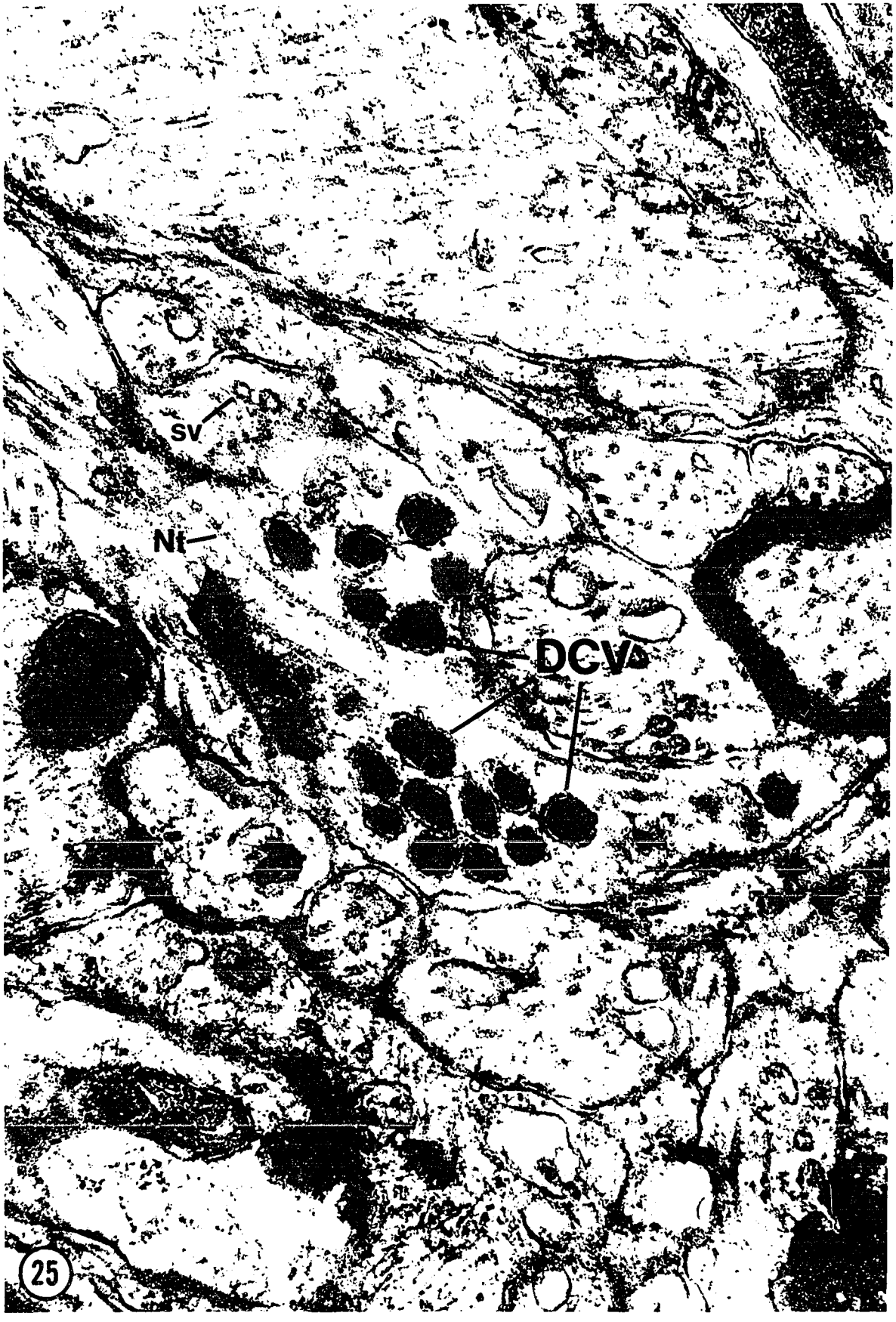
On the basis of the ultrastructural changes observed within the neurons of the experimental groups, it is convenient to present our observations under the following headings: (a) One Hour Post-Coitus, and (b) Two Hours to 10 Hours Post-Coitus.

a) <u>One Hour Post-Coitus</u>

Electron micrographs of the PO nuclei of animals sacrificed as early as one hour after coitus provided the first evidence of

Figure 25. High magnification electron micrograph showing portion of the neuropil of an animal sacrificed at 10 hours post-coitus. The ultrastructure of several large dense-core vesicles (DCV) within an axon profile is shown. The 'halos' separating the electron-dense cores of these vesicles from their limiting membranes are clearly visible. Neurotubules (Nt) and membrane profiles resembling synaptic vesicles (sv) are also shown.
Preoptic nucleus. X 66,700


SV
NI
DCV
25

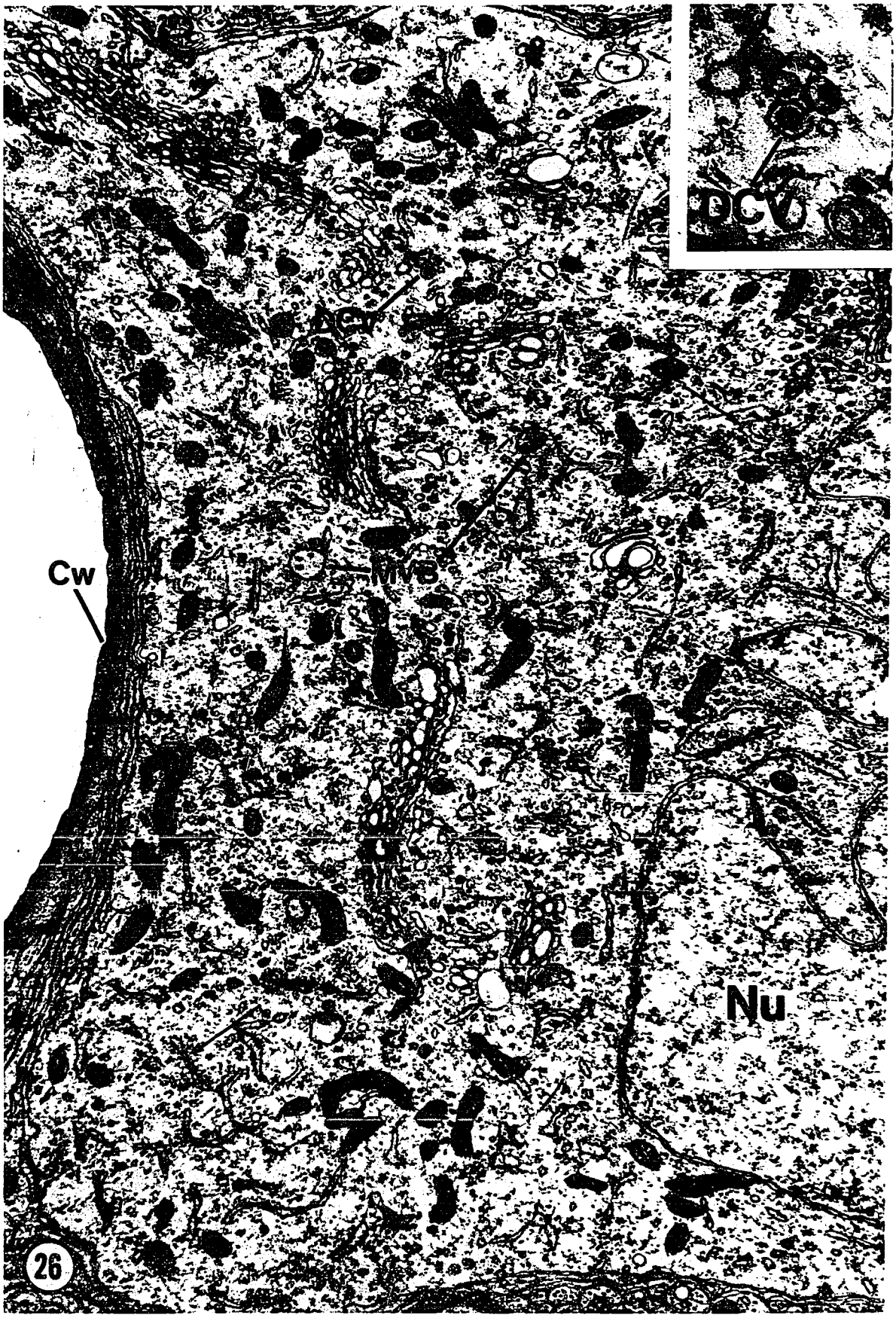

SV
Nt
DCV
25

intracellular reorganization. These changes were most obvious in neurons located in close spatial relationship with capillaries (Figures 26 and 27). In general, the ultrastructural changes observed were characterized by an increase in the neuron diameter and a proliferation of several of the cytoplasmic organelles. No changes were observed within the nuclei of these cells, except possibly for an increase in the degree of folding of the nuclear envelope (Figures 26 and 27). The rough ER of many of these neurons was more prominant that that of the control neurons. The irregular cisternae of this organelle were either scattered throughout the cytoplasm (Figure 26) or occurred in rather orderly arrays of parallel, broad profiles stacked one upon the other. Occasionally, a fine granular material was resolved within the cisternae of this organelle (Figure 27). The external surface of the membranes limiting the ER cisternae were now more richly studded with ribosomes; there was also an apparent increase in the number of free ribosomes (Figure 27). The Golgi profiles were also much larger and more numerous than those previously observed within the neuron soma of the control animals (compare Figures 26 and 20). Many of the neurons removed at one hour following coitus also showed an increase in the number of mitochondria. This organelle was diffusely distributed throughout the cytoplasm; no apparent changes were observed in its ultrastructure. Other cytoplasmic organelles, including the multivesicular bodies, neurotubules and the larger lysosome-like bodies, were only sparsely represented. Several small DC vesicles were also observed (Figure 26, inset).

Figure 26. Portion of the perinuclear zone of a neuron removed from the preoptic nucleus of an animal sacrificed at one hour post-coitus. Note the close spatial relationship between the neuron soma and the capillary wall (Cw). There is an obvious change in the cytoplasm-nuclear volumes. Several relatively well developed Golgi profiles (G), and many scattered, irregular cisternae of the rough endoplasmic reticulum (arrows) now appear in the cytoplasm. However, only a few dense-core vesicles (DCV) were observed within the soma of the preoptic neurons removed at this time post-coitus. One of these vesicles is shown in the inset. MVB, Multivesicular body, Nu, Nucleus.

X 2,200

inset X 6,700


DCV
Cw
MVB
Nu
26

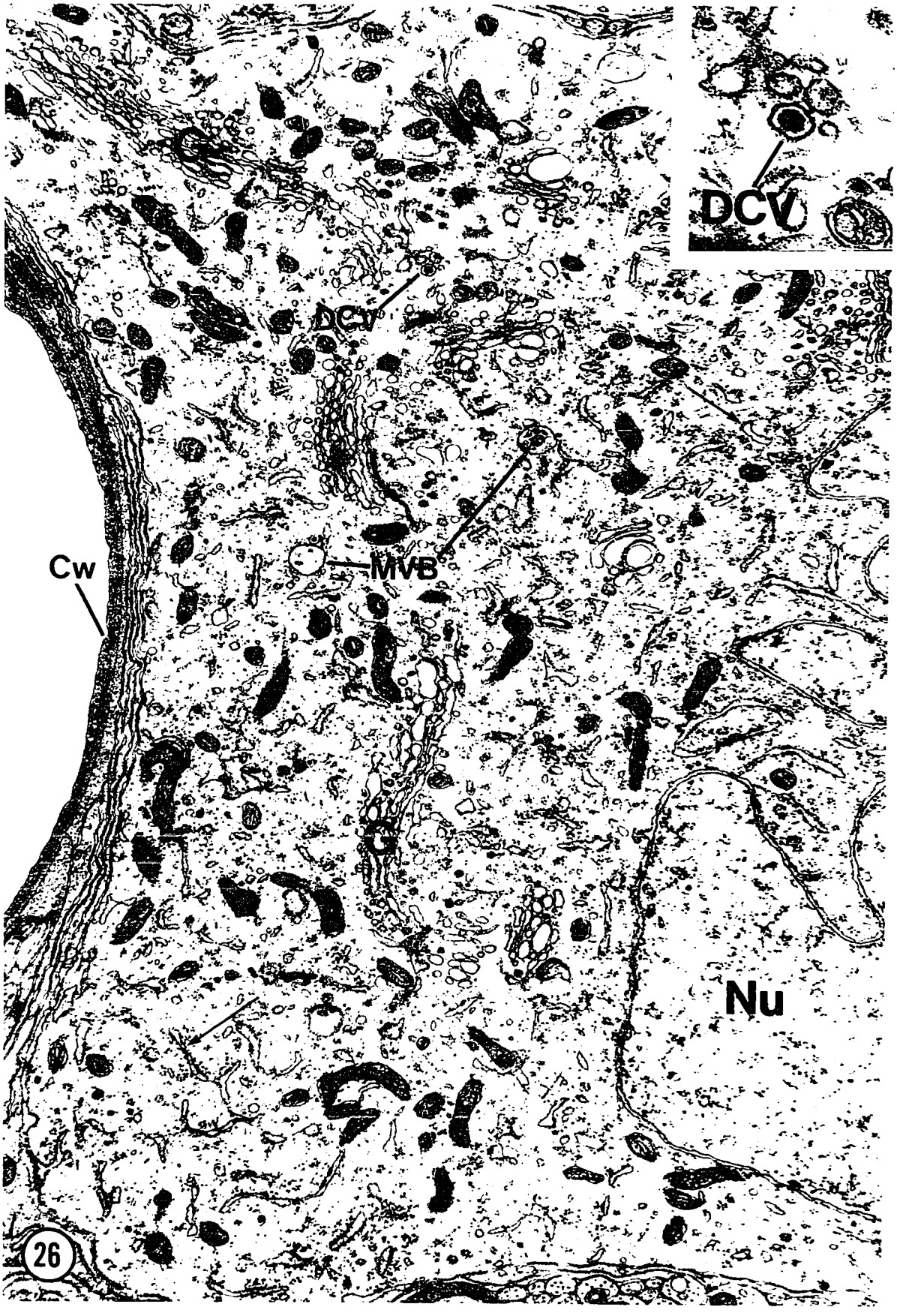
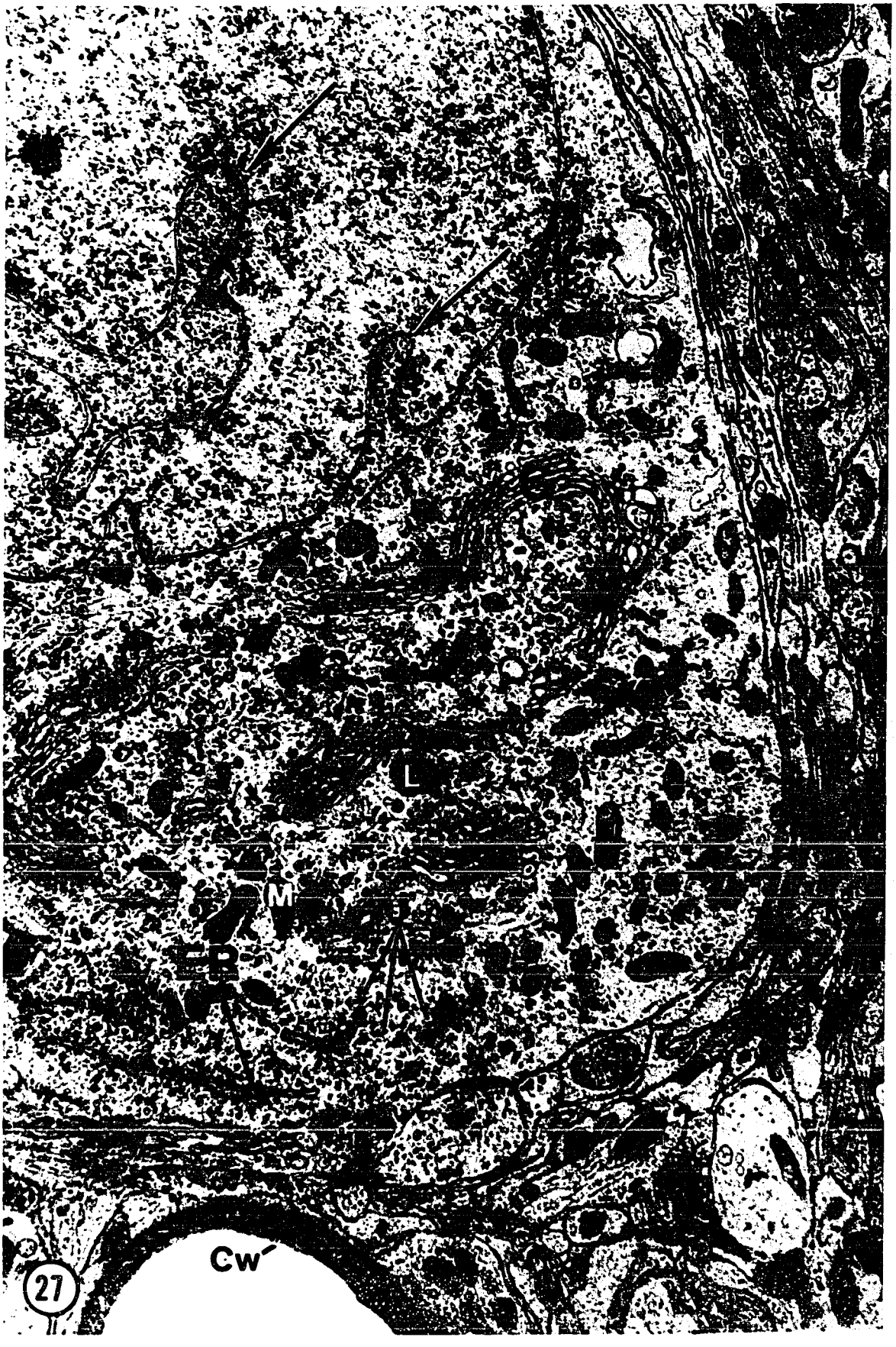
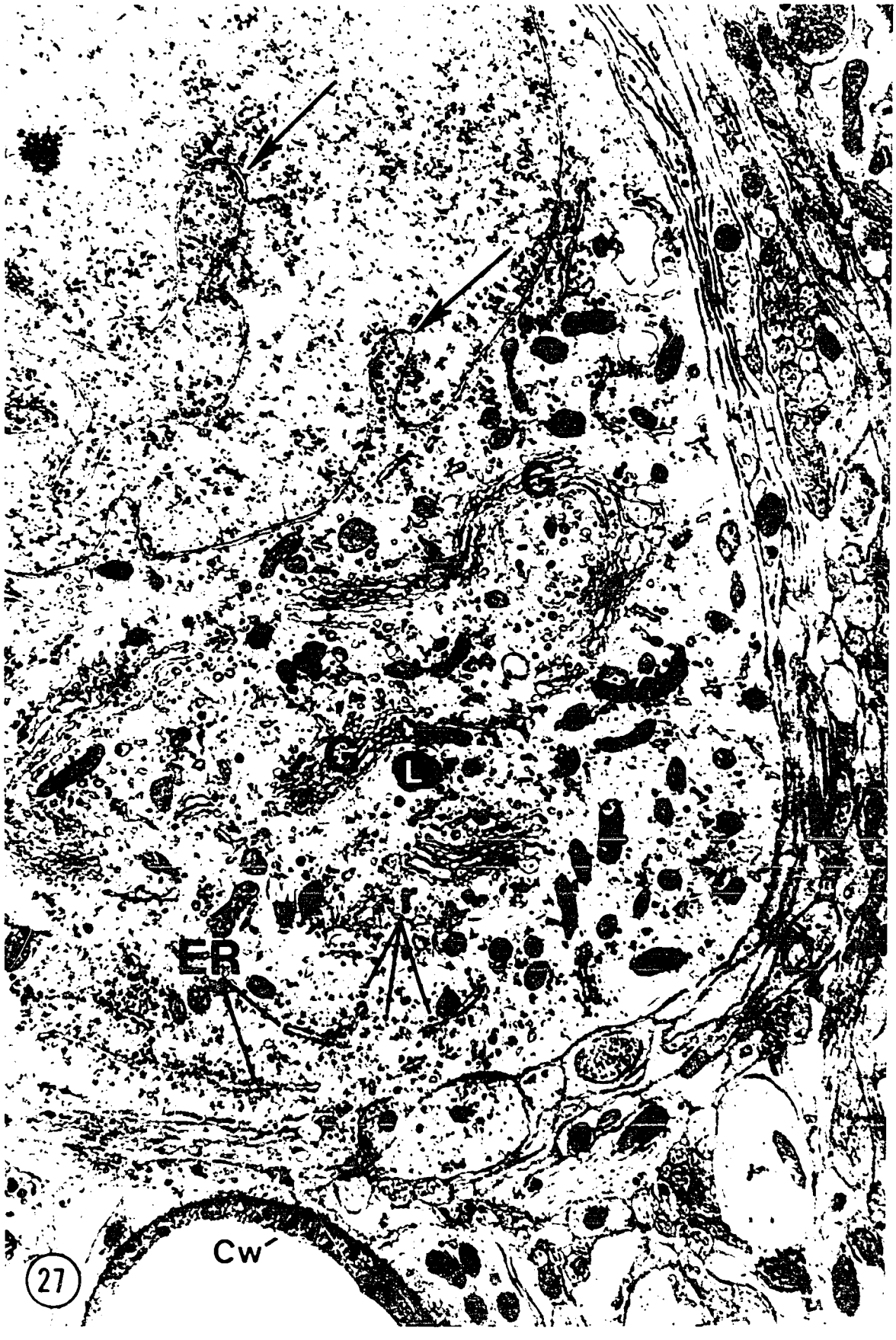

DCV
DCV
Cw
MVB
Nu
26

Figure 27. Electron micrograph of a large neuron removed from the preoptic nucleus at one hour post-coitus. Note the extensive infoldings (arrows) of the nuclear envelope. The multiple, large Golgi profiles (G) are located within the more central region of the cell; several long cisternae of the rough endoplasmic reticulum (ER) and numerous ribosomes (r) are more peripherally located. Again note the close spatial relationship between the neuron soma and the capillary wall (Cw). L, Lysosome-like body; M, Mitochondrion.

X 16,700


L
M
B
Cw'
27


L
ER
Cw´
27

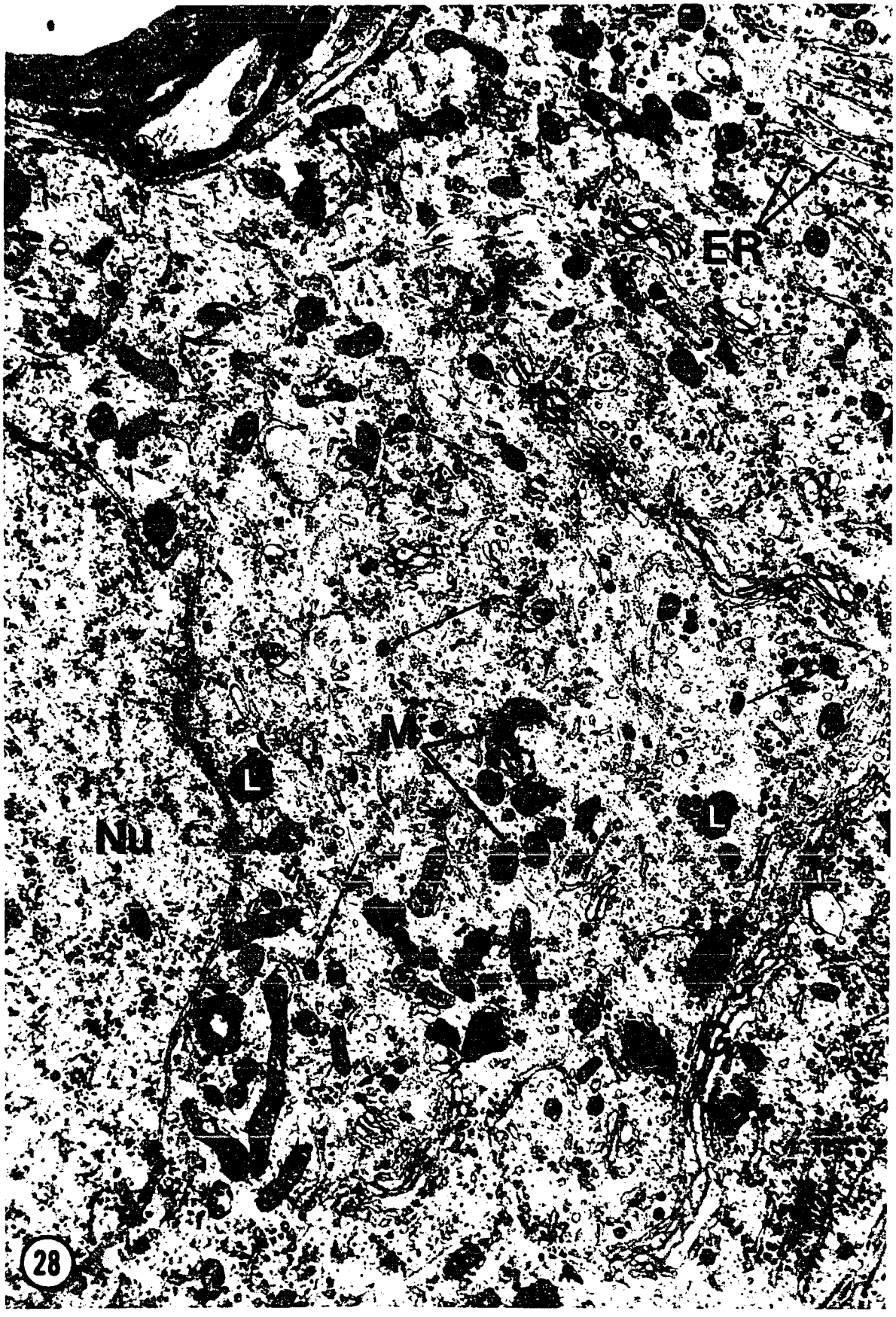
b) Two Hours to 10 Hours Post-Coitus

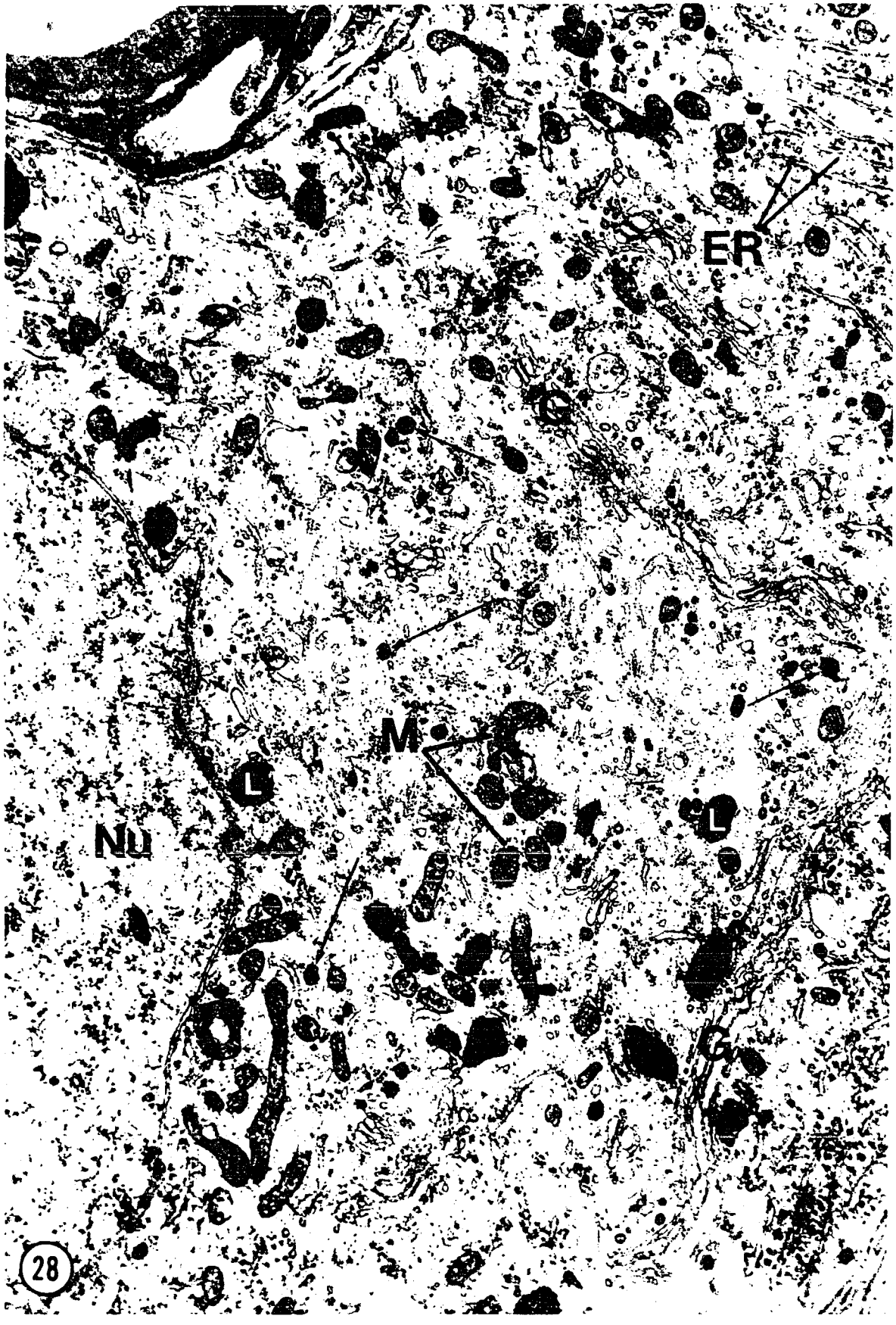
Portions of the perinuclear zones of three large PO neurons removed from the rabbit brain at two hours, four hours and six hours following coitus are shown (Figures 28, 29 and 30). The large neuron shown in Figure 31 is representative of the 10 hours post-coitus group. These large neurons were readily identified under the electron microscope by their darker appearance, due largely to the extensive accumulation of organelles within their perikarya. The Nissl substance of these cells now occupied much of the marginal cytoplasm, and thus provided the morphological basis for arbitrarily dividing the perikarya of these neurons into 'marginal' and 'central' zones. Such a division served merely as a convenient basis for describing the distribution of the other organelles.

The rough ER and Golgi systems of these neurons were extensive (Figures 28, 29, 30 and 31). The cisternae of the rough ER were arranged in parallel one upon the other and frequently followed the contours of the cell membrane. Shorter, more irregular cisternae also occurred within the 'central' zone of cytoplasm. The commonly observed fine granular material within the cisternae of the rough ER is shown in Figures 29 and 30. This macromolecular substance also appears more abundant here than was previously observed within neurons of the one hour post-coitus animals (compare Figures 27 and 29). A further increase in the number of free ribosomes and/or their rosettes was also apparent. These attained their greatest density within the 'marginal' cytoplasm where they occupied much of the space between the cisternae of the rough ER (Figures 30 and 31). Several Golgi profiles were

Figure 28. Field showing portion of the perinuclear zone of a large neuron of the preoptic nucleus removed at two hours post-coitus. The cytoplasm of these cells can now be clearly divided into 'marginal' and 'central' zones on the basis of the distribution of the cell organelles. Both the rough endoplasmic reticulum (ER) and Golgi system (G) are well developed. Mitochondria (M) are abundant. Large, irregular lysosome-like bodies (L) are also common. Note the increased number of dense-core vesicles (arrows). Nu, Nucleus.

X 22,300

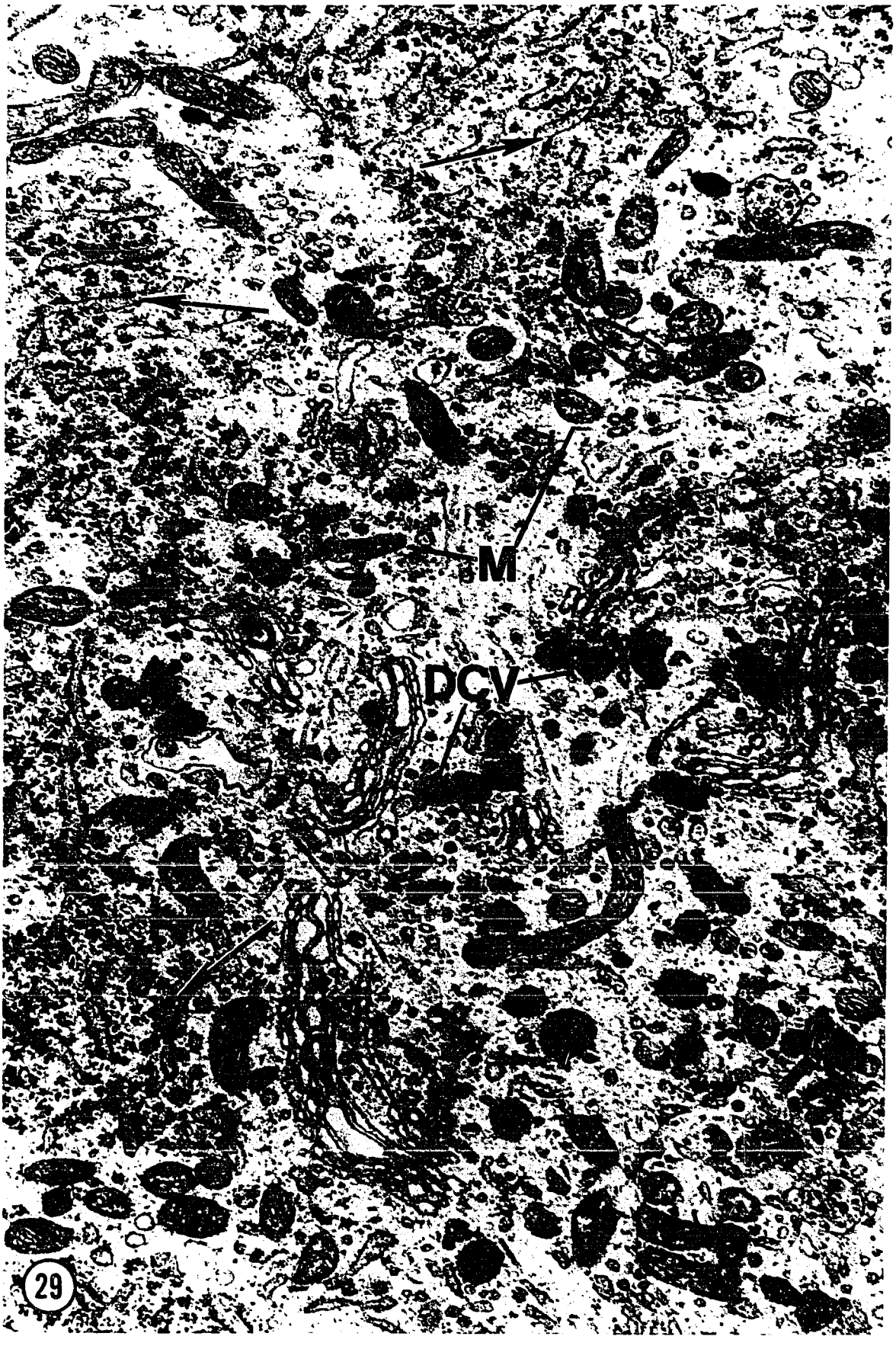

ER
M
L
NU
L
28

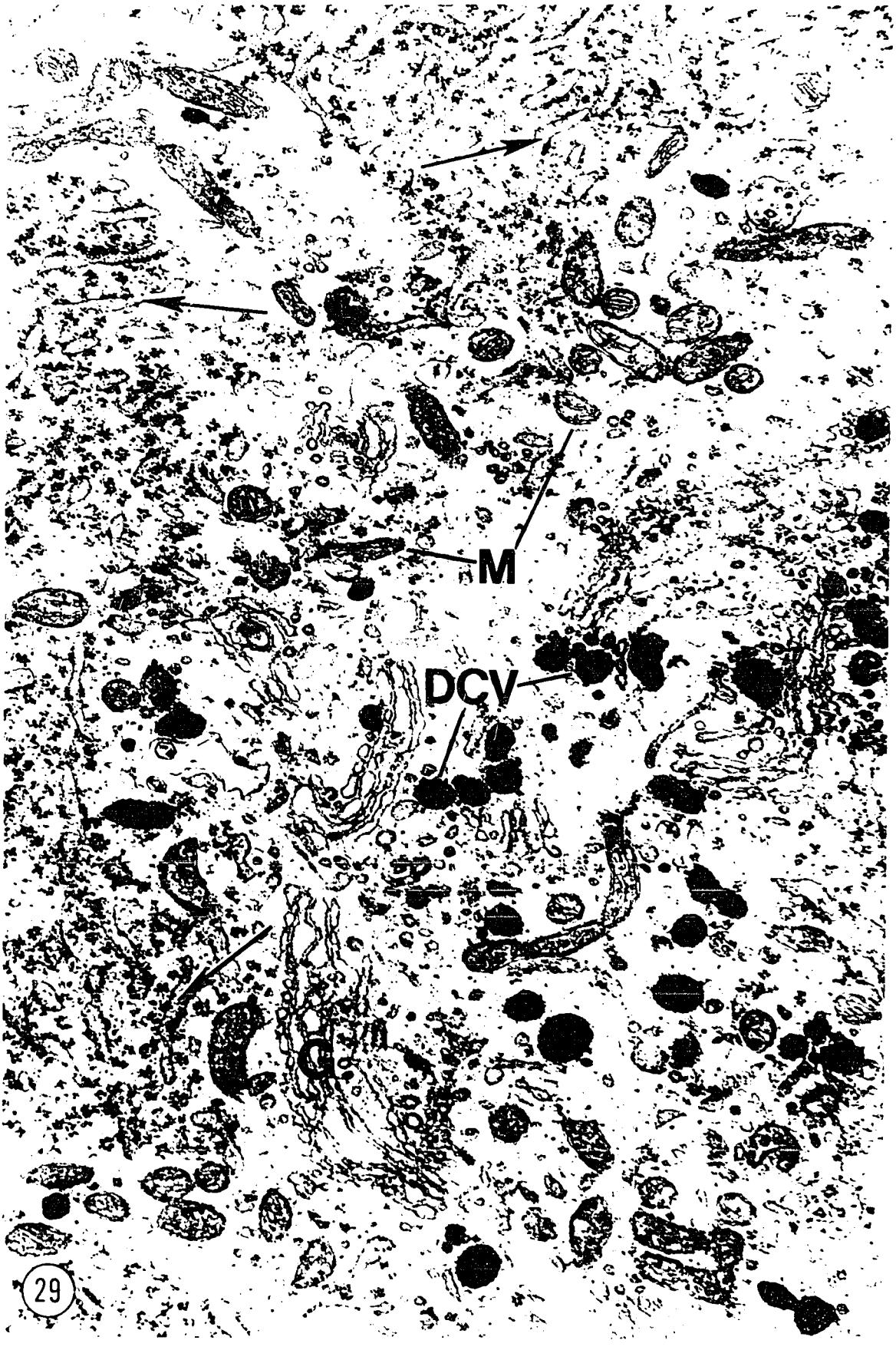


28

Figure 29. High magnification electron micrograph of the perinuclear zone of a preoptic neuron removed from an animal sacrificed at four hours post-coitus. Note the elaborate rough endoplasmic reticulum occupying much of the 'marginal' cytoplasm, and the macromolecular material (arrows) contained within the cisternae of this organelle. Small polysomes are abundant. Several well developed Golgi systems (G) with closely related electron-dense vesicles (DCV) are shown. The mitochondria (M) are also numerous and uniformly distributed throughout the field.

X 28,000

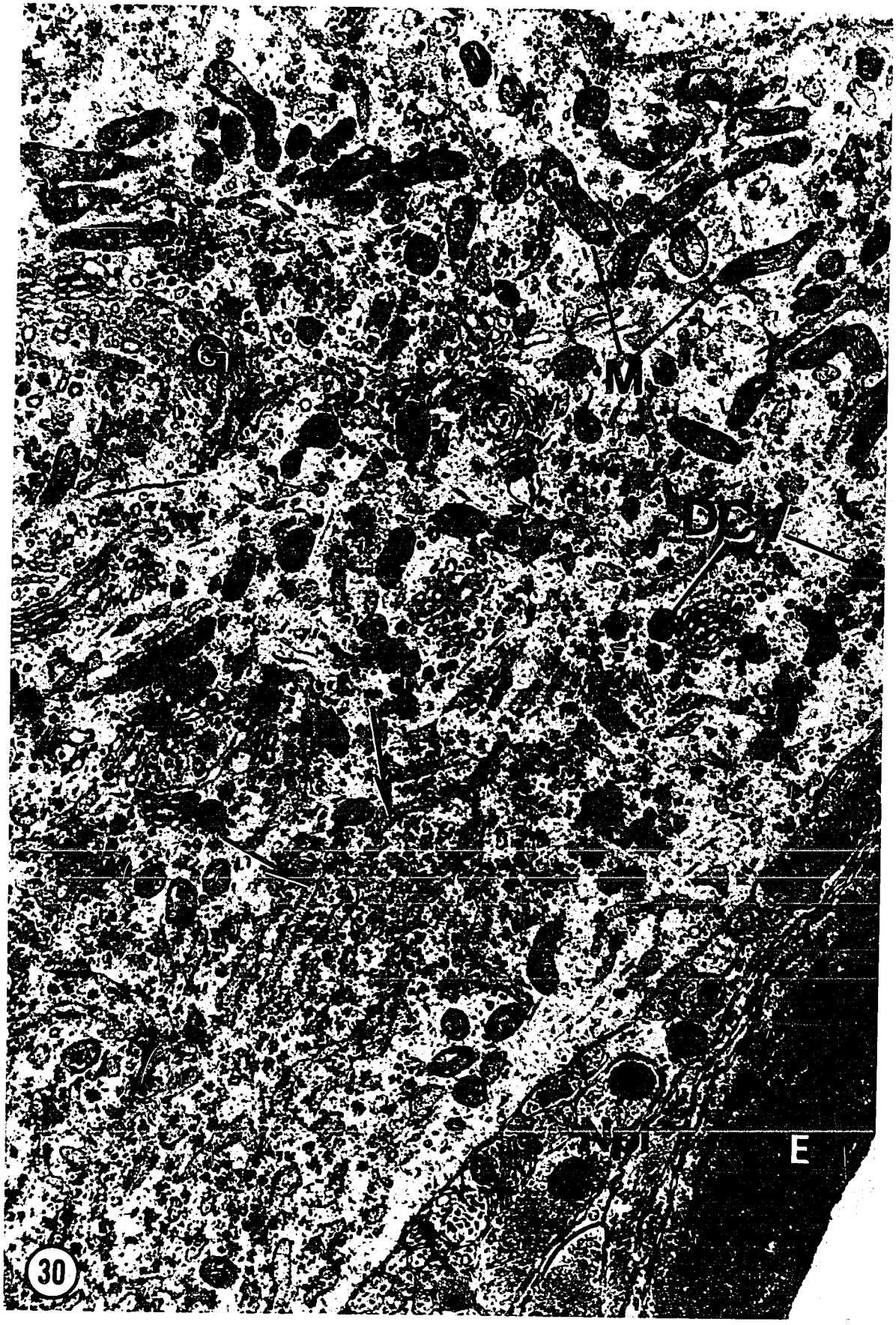

M
DCV
29



29

Figure 30. Electron micrograph showing a portion of the perinuclear zone of a large preoptic neuron removed at six hours post-coitus. Again note the presence of elaborate rough endoplasmic reticulum and the dense population of polysomes. The macromolecular material (arrows) contained within the cisternae of this organelle is clearly illustrated. The neurons showing these morphological features were usually in close spatial relationship with an endothelial cell (E) forming the capillary wall; only a narrow layer of the neuropil (Npl) intervened. Again note the absence of a perivascular space. DCV, Dense-core vesicle; G. Golgi system; M, Mitochondrion.

X 26,700

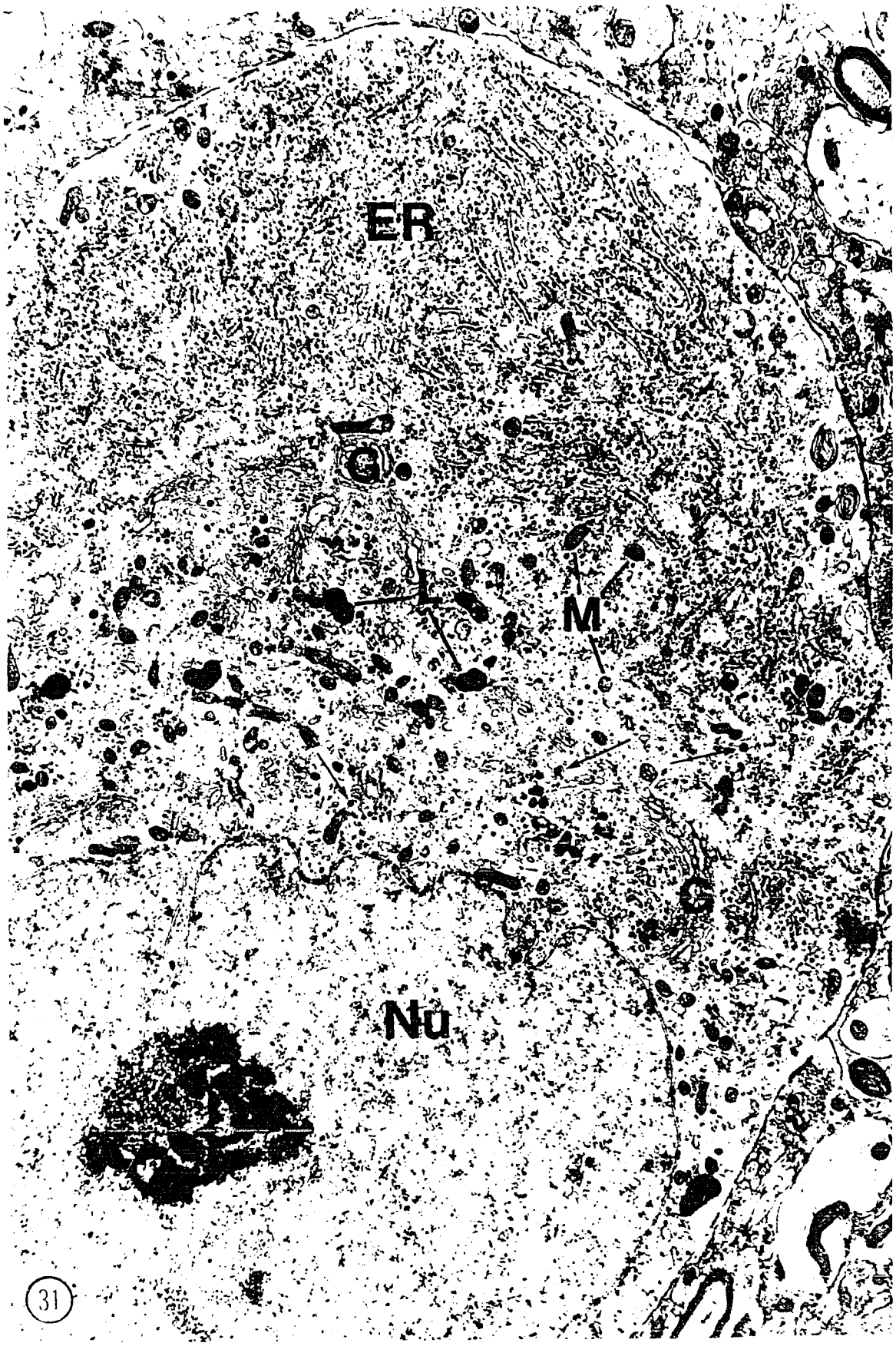

M
DCT
E
30

30

Figure 31. Electron micrograph of a large neuron of the preoptic nucleus excised at 10 hours post-coitus. Note that the rough endoplasmic reticulum (ER) occupies nearly the entire 'marginal' cytoplasm of the cell. Several large Golgi systems (G), lysosome-like bodies (L), mitochondria (M), and dense-core vesicles (arrows) occur in the 'central' cytoplasm. The morphological features of the typically large vesicular and eccentric nuclei (Nu) of the neurons showing ultrastructural changes following coitus are also clearly illustrated.

X 12,000

31



31

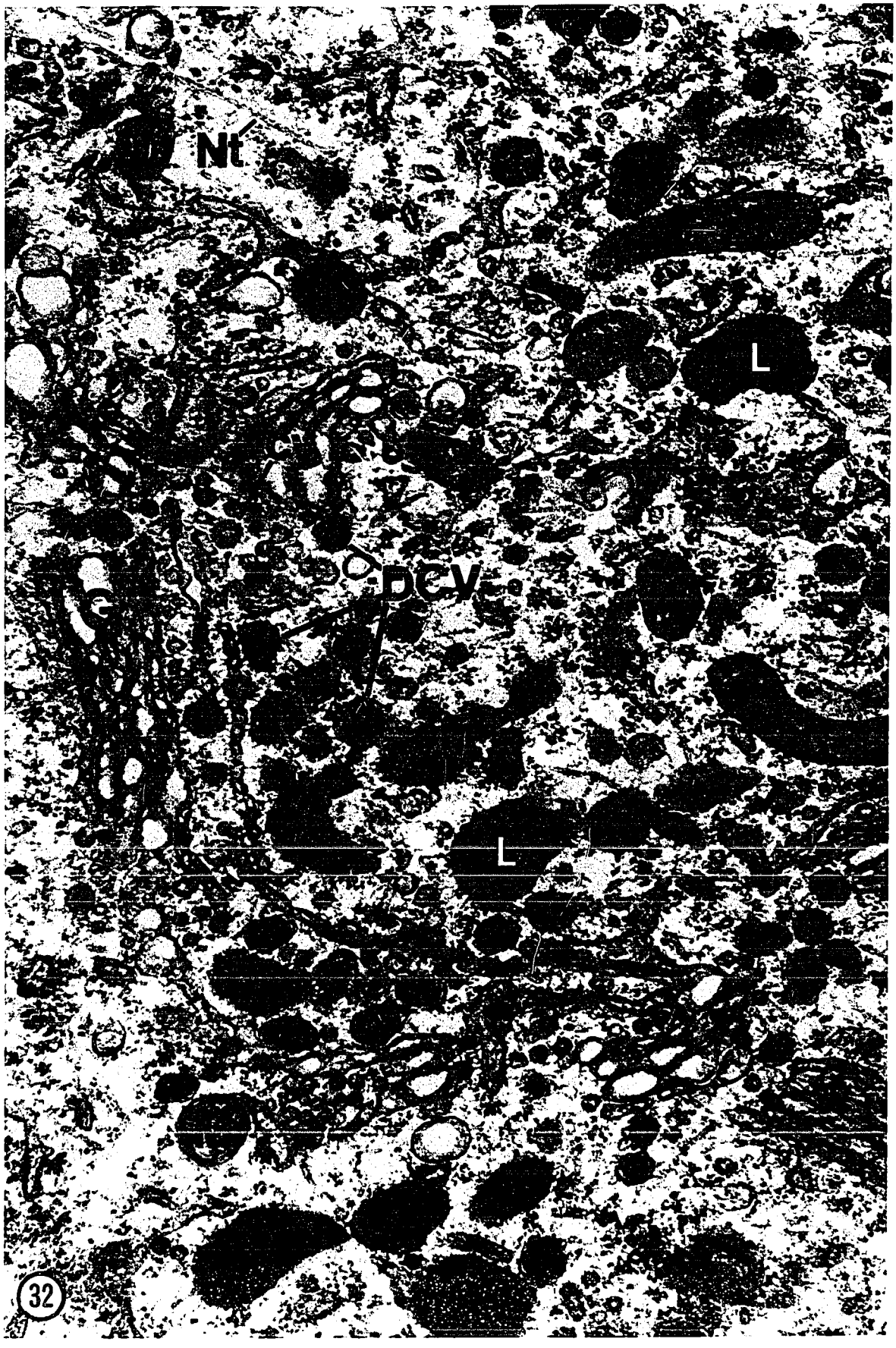
usually observed within the 'central' zone of cytoplasm of each neuron (Figures 28, 29 and 31). The saccules and vesicular components of this organelle appeared as crescent-shaped profiles, frequently orientated such that their outer convex or immature face was directed toward the peripherally located Nissl substance, while their concave or maturing face was directed toward the more central area of the cell (Figure 32). The presence of electron-dense material within saccules of the Golgi system was a common finding (Figures 33 and 34).

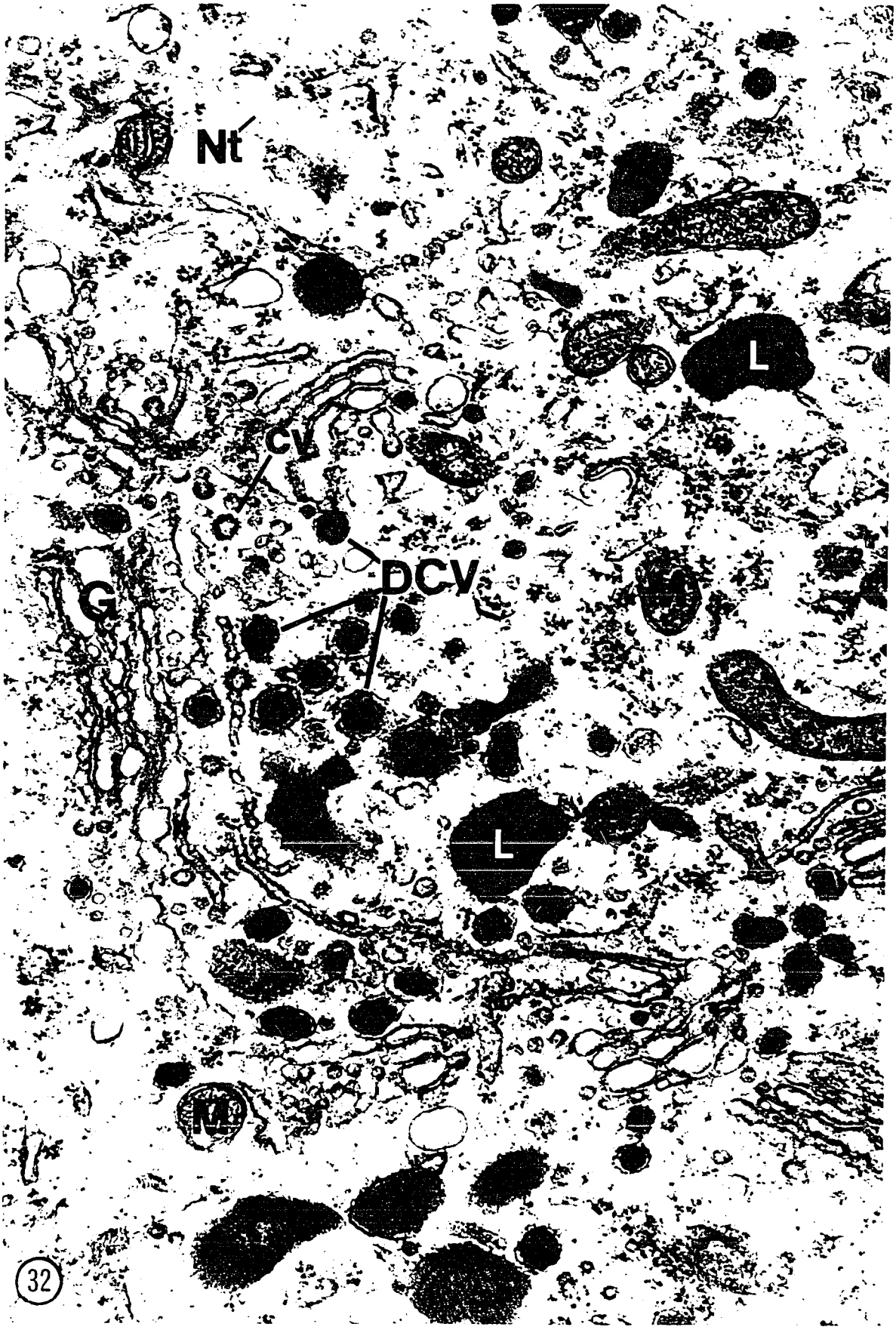
Large neurons of the experimental groups were also characterized by the presence of numerous DC vesicles. These large vesicles were first observed within the soma of neurons removed at two hours post-coitus (Figure 28). Their presence in greater numbers was a consistent finding in the neurons removed from animals sacrificed at four hours through to 10 hours following coitus (Figures 29, 30 and 31). In general, the large DC vesicles were located within the 'central' zone of cytoplasm, and frequently in close spatial relationship with the saccules of the Golgi system (Figures 31 and 32). Morphological evidence supporting their possible origin from the Golgi saccules is shown in Figure 34.

The DC vesicles found within the neuron soma of the experimental groups had a mean diameter of 1343 ± 13 Å (Figure 22). The diameter range of the 696 DC vesicles measured is included in Table 5. These vesicles were significantly larger ($P < .001$) than the morphologically identical DC vesicles observed within the neuron soma of the control neurons (Table 5). The frequency distribution of these measurements is presented graphically in Figure 23.

The cytoplasm of these same neurons was also characterized by dense populations of mitochondria and, to a lesser extent, an increase in the number of lysosome-like bodies. These findings were even more pronounced within the neurons observed at four hours through to 10 hours following coitus (Figures 29, 30 and 31). The morphological features of these organelles are shown in Figure 35. The other cytoplasmic organelles such as neurotubules and a variety of vesicular structures are also shown, but present no identifiable changes in their morphology, distribution or numbers.

Figure 32. High magnification electron micrograph showing a large Golgi profile (G) with a number of large dense-core vesicles (DCV) closely associated with the concave or maturing face of this organelle. Several 'coated' vesicles (cv), mitochondria (M), lysosome-like bodies (L) and neurotubules (Nt) are also visible. Preoptic nucleus. Experimental animal. X 42,700


Nt
L
pcv
L
32



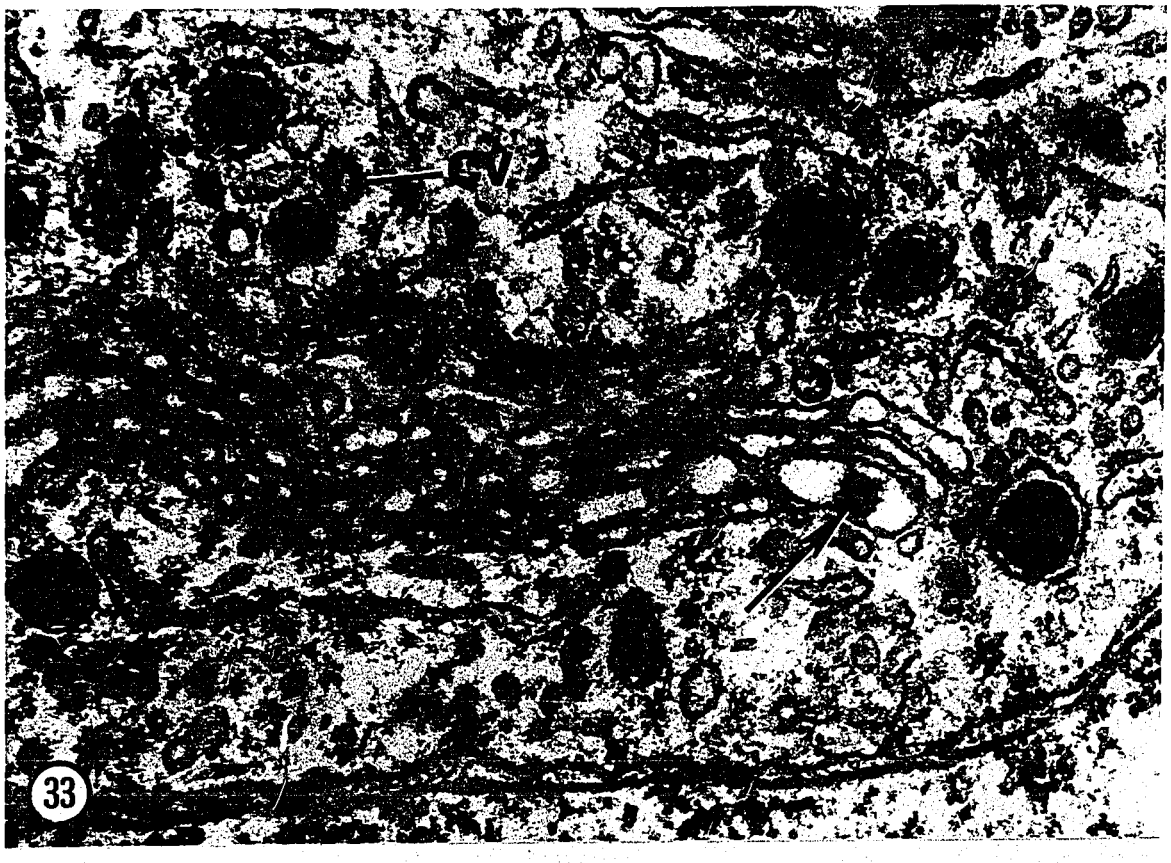
32

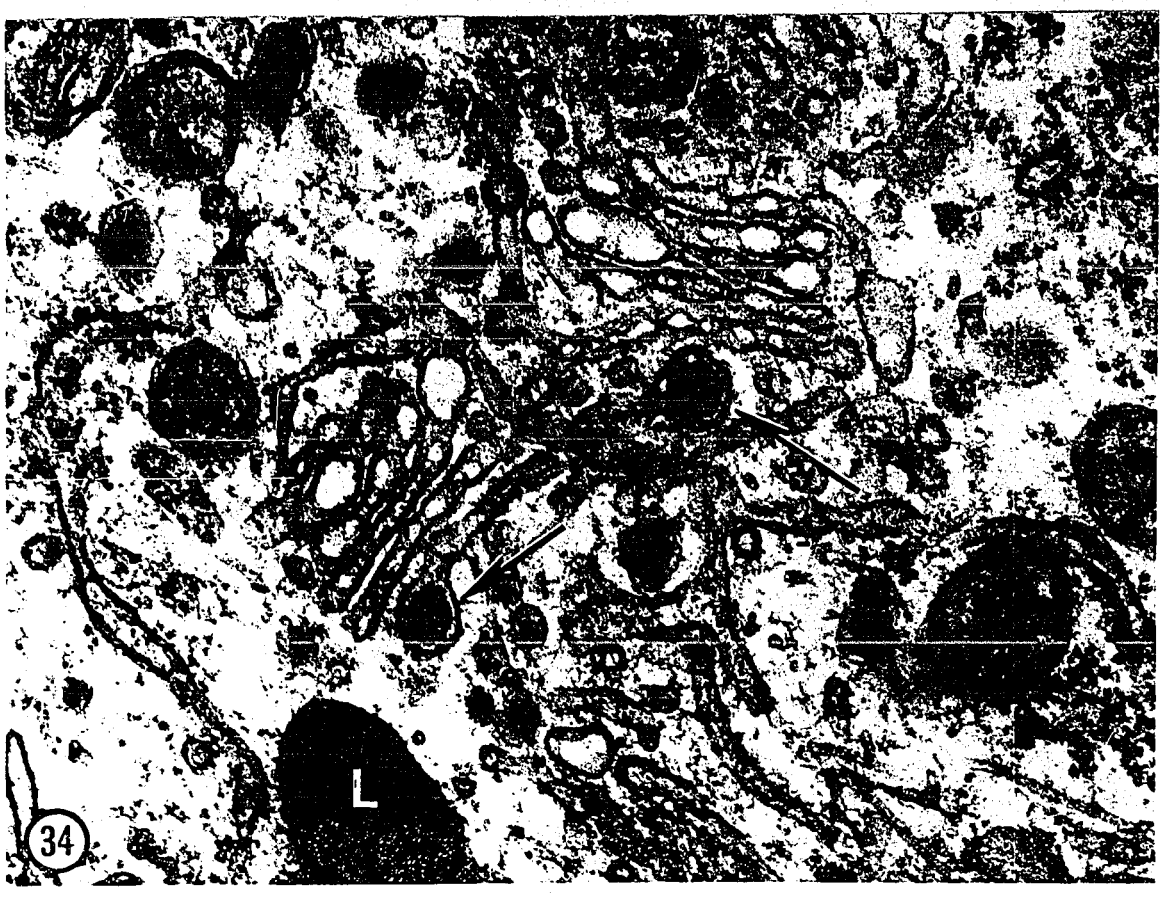
Figure 33. High magnification electron micrograph of a Golgi profile showing electron-dense material (arrow) within a saccule terminal. The two large dense-core vesicles seen at the right of the field are approximately 2000 Å in diameter. Preoptic nucleus, eight hours post-coitus. cv, coated vesicle.

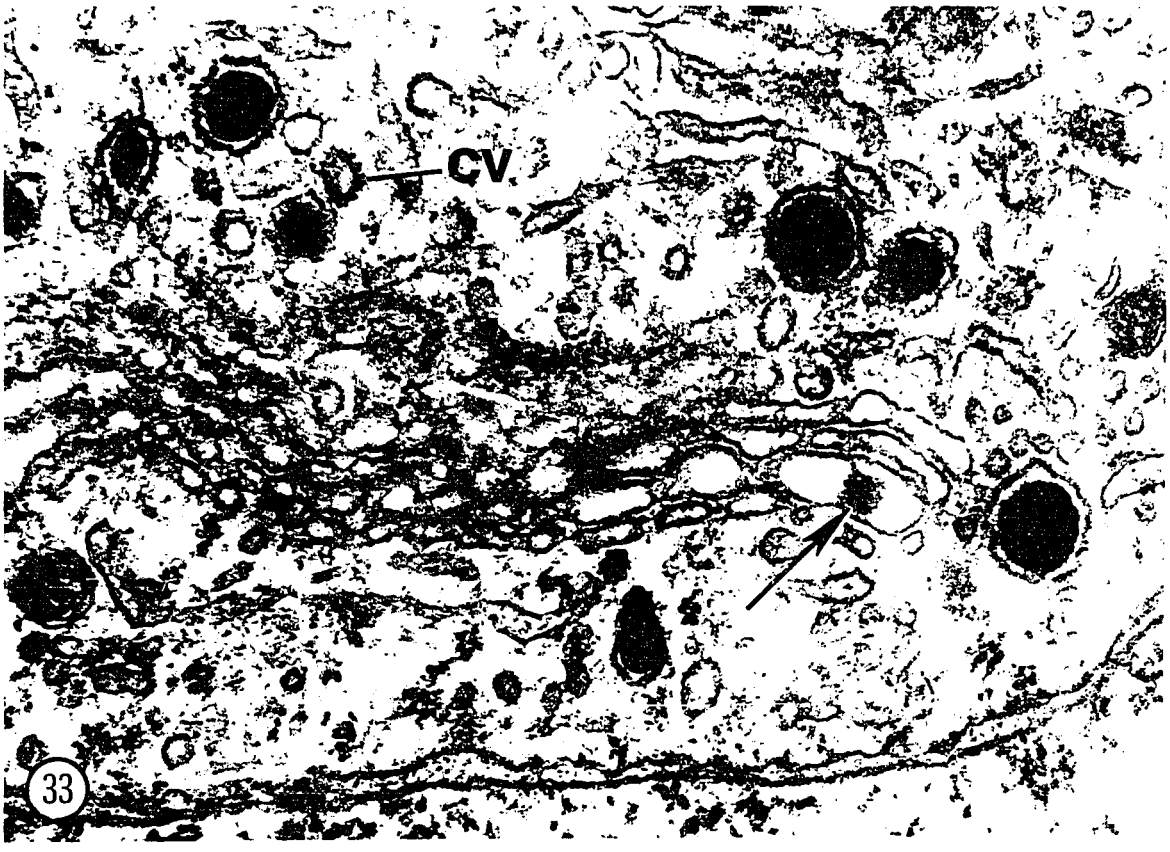
X 60,100

Figure 34. Electron micrograph showing two large dense-core vesicles (arrows) in the process of pinching off from the Golgi system. L, Lysosome-like body; M, Mitochondrion. Preoptic nucleus, four hours post-coitus.

X 50,200


GV
33


L
34


CV
33

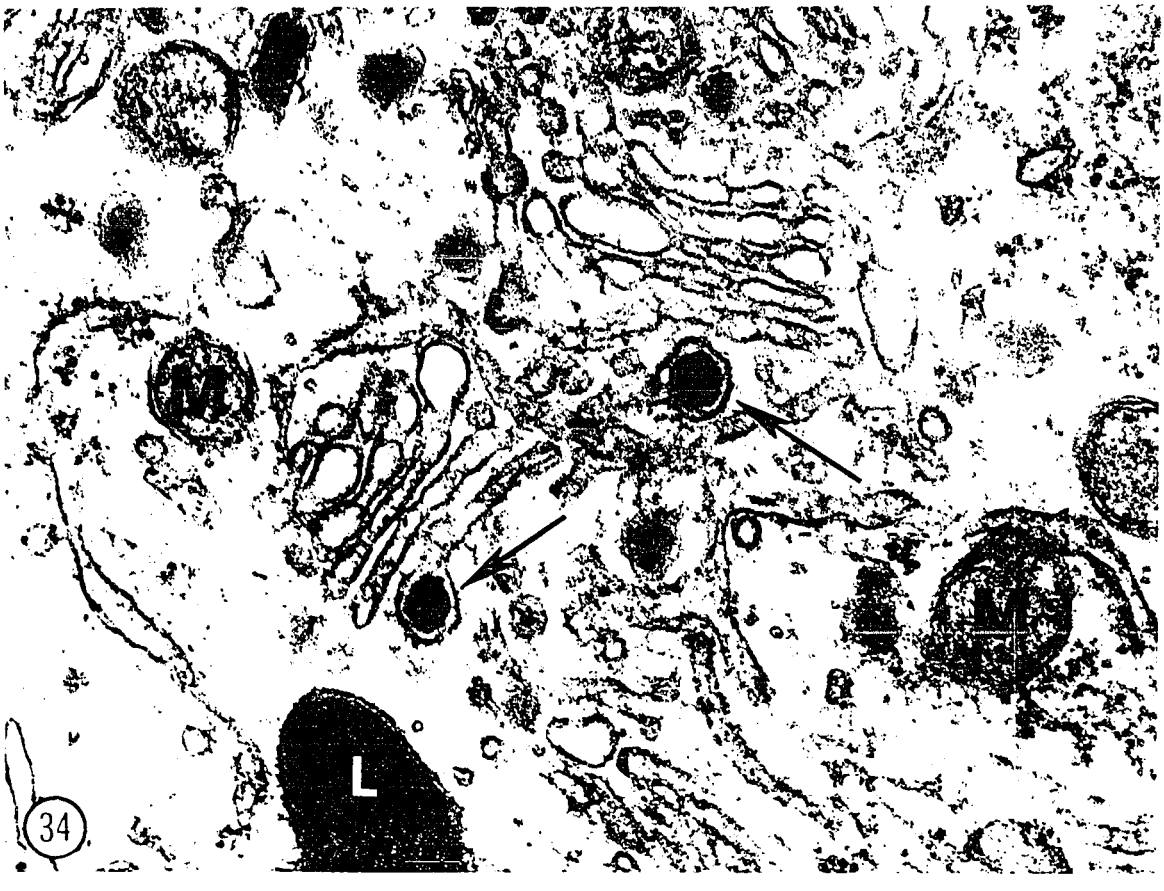
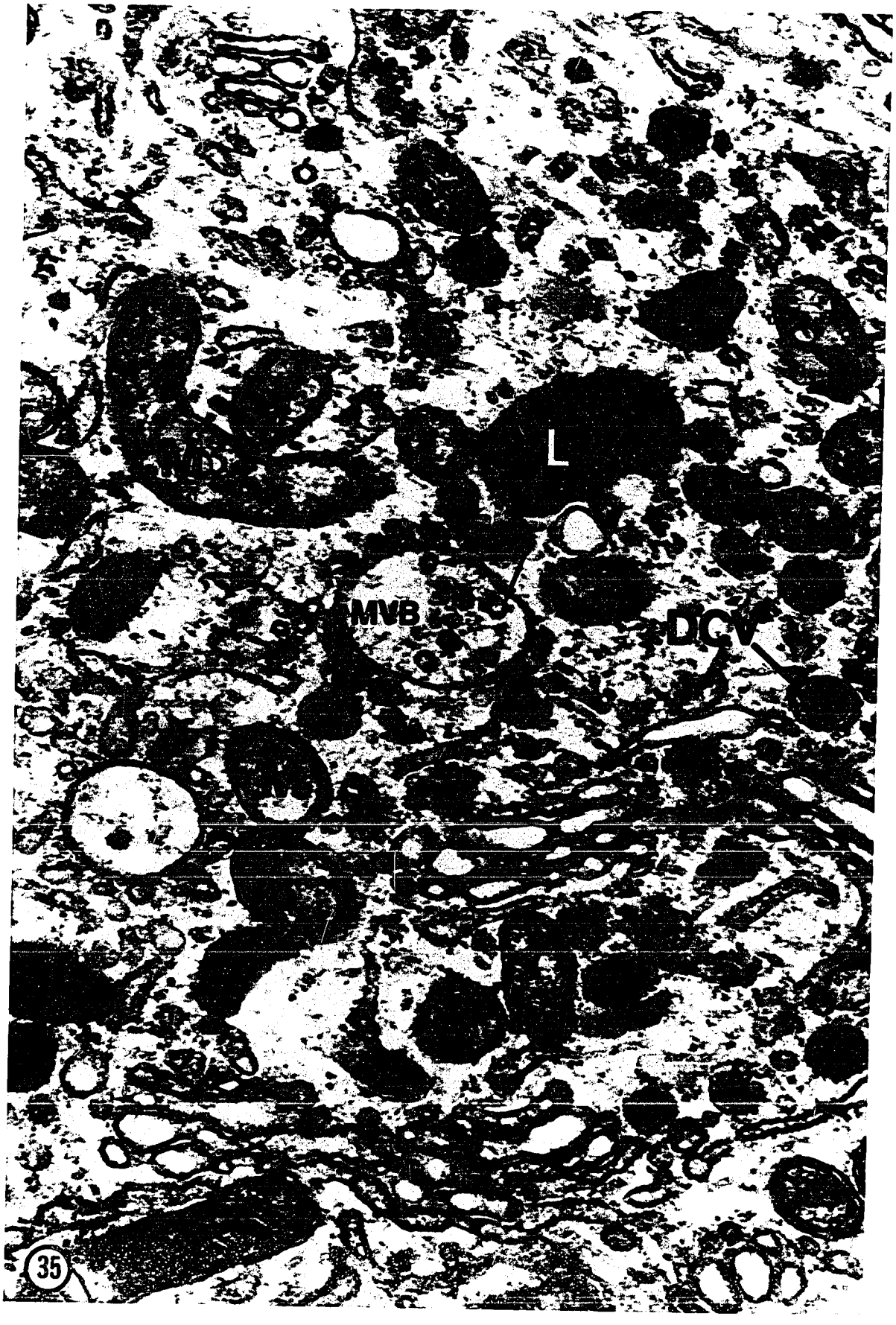
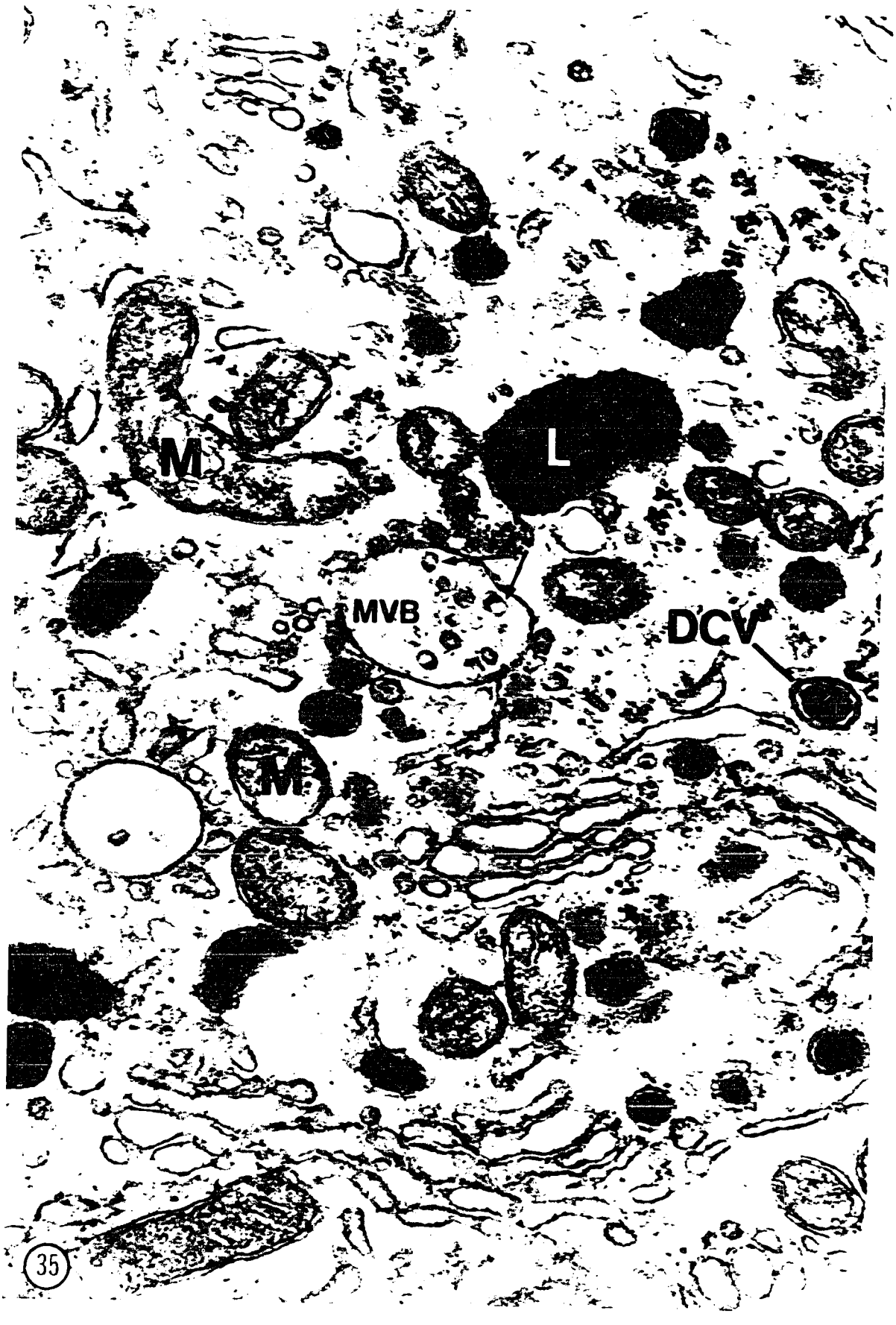

M
M
L
34

Figure 35. High magnification electron micrograph showing a portion of the perinuclear zone of a large neuron removed from the brain of a rabbit sacrificed at 10 hours post-coitus. The large multivesicular body (MVB) near the center of the field with eight to ten small vesicles (arrows) contained with a parent membrane is typical of those observed throughout this study. Several large dense-core vesicles (DCV) are shown. Compare the ultrastructure of the large dense-core vesicle located at the right of the figure, with the nearby lysosome-like body (L). M, Mitochondrion.

X 52,500


L
MVB
DCV
35



35

SUPRACHIASMATIC NUCLEUS

A) Light Microscopy

The SCH nucleus of the rabbit brain consists of a densely packed group of small, oval or fusiform cells lying immediately dorsal to the optic chiasma and close to the ventral aspect of the V_{III} (Figure 36). The area outlined by the broken line in Figure 37 is representative of the tissue removed from each side of the V_{III} and processed for electron microscopic study.

B) Electron Microscopy

1) Ultrastructure of the suprachiasmatic nucleus

Ultrathin sections of the SCH nucleus of the rabbit brain contained essentially the same structures as those of the PO nucleus. The neurons and neuroglial elements, as well as the general nature of the neuropil, are shown in Figure 38. The compact arrangement of the neurons, their ovoid to fusiform shape and intermediate size are illustrated in Figure 39. On the basis of the measurements obtained from 200 neurons located near capillaries of the control group, the SCH neurons had a mean diameter of 10.6 ± 0.1 μ (Figure 8). The mean diameter of the nuclear profiles of these cells was 7.4 ± 0.1 μ (Figure 9). The distribution of both the neuronal and nuclear measurements are plotted in Figure 40.

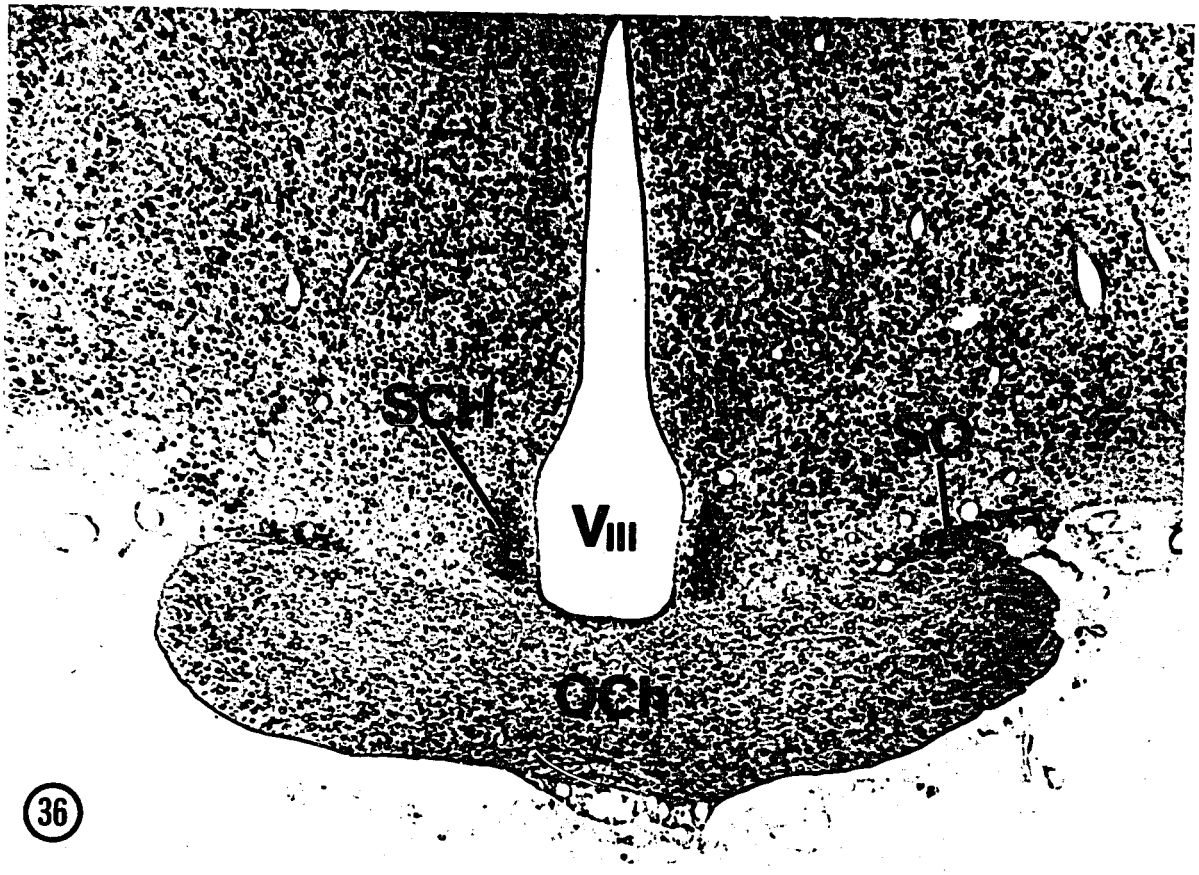
The neuroglial elements were represented by a very diffuse population of small cells identified as oligodendrocytes and astrocytes (Figure 38). Ultrastructurally, these cells were identical to

Figure 36. Photograph of a frontal section of the rabbit brain made at the level of the optic chiasma (OCh) and showing the location of the suprachiasmatic nucleus (SCH). SO, Supraoptic nucleus; V_{III}, Third ventricle. Thionin

X 290

Figure 37. Photograph of the suprachiasmatic area of the rabbit brain. The area outlined by the broken line is representative of the tissue removed from each side of the third ventricle (V_{III}) and processed for electron microscopy. Ed, Ependyma; OCh, Optic chiasma; SO, Supraoptic nucleus. Thionin

X 1290



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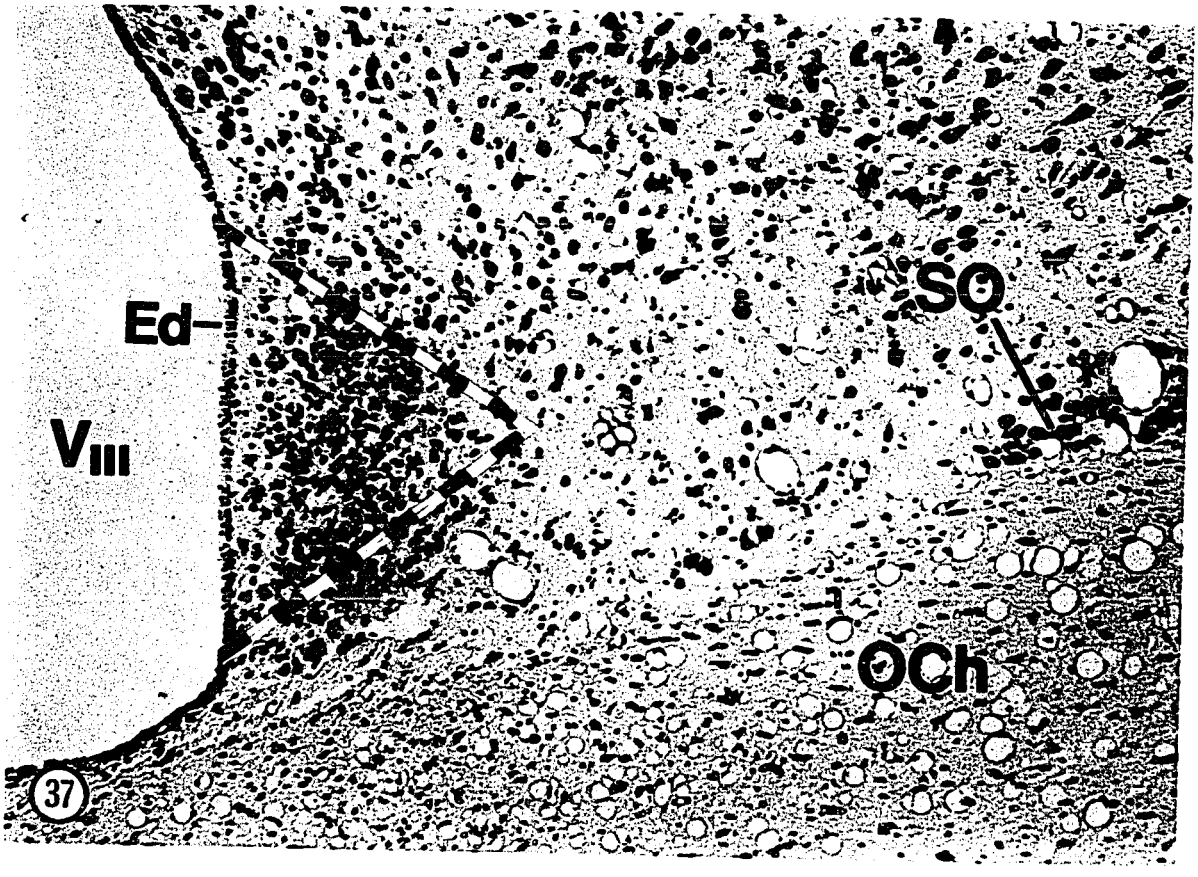

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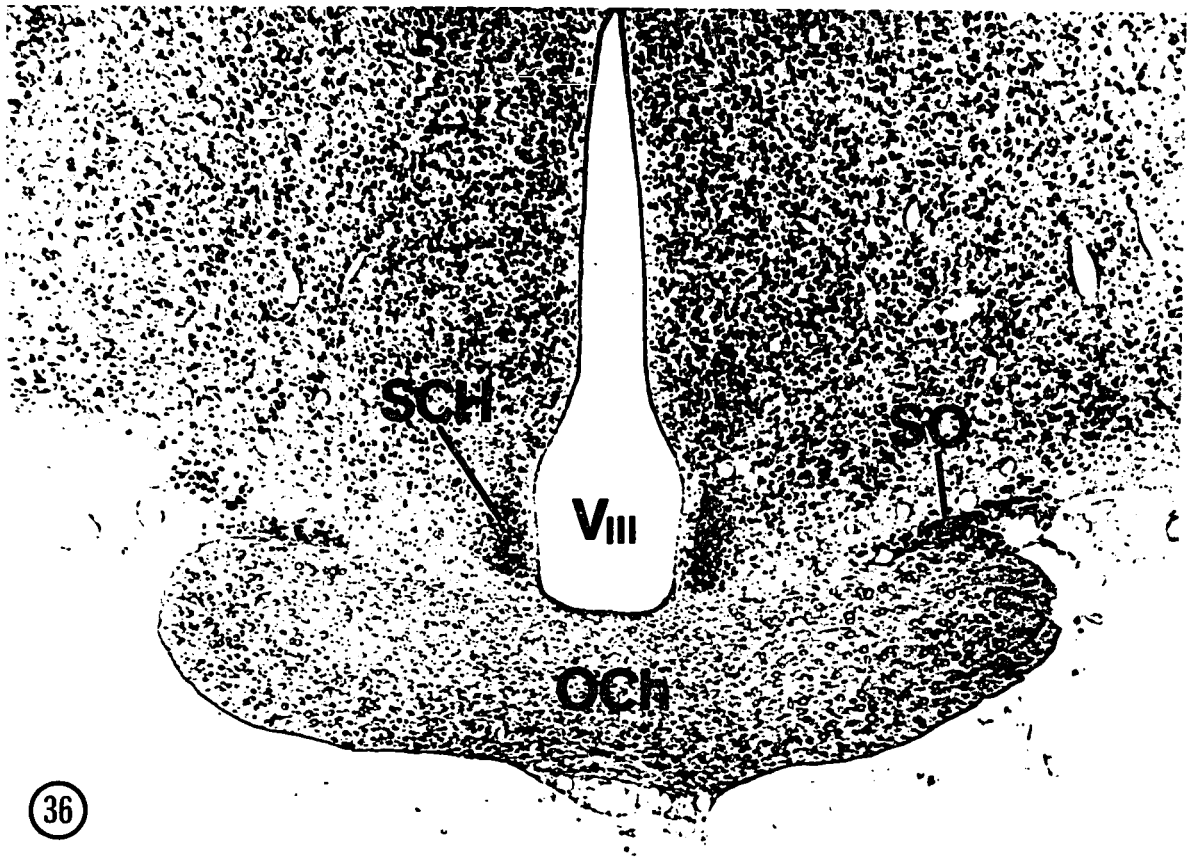

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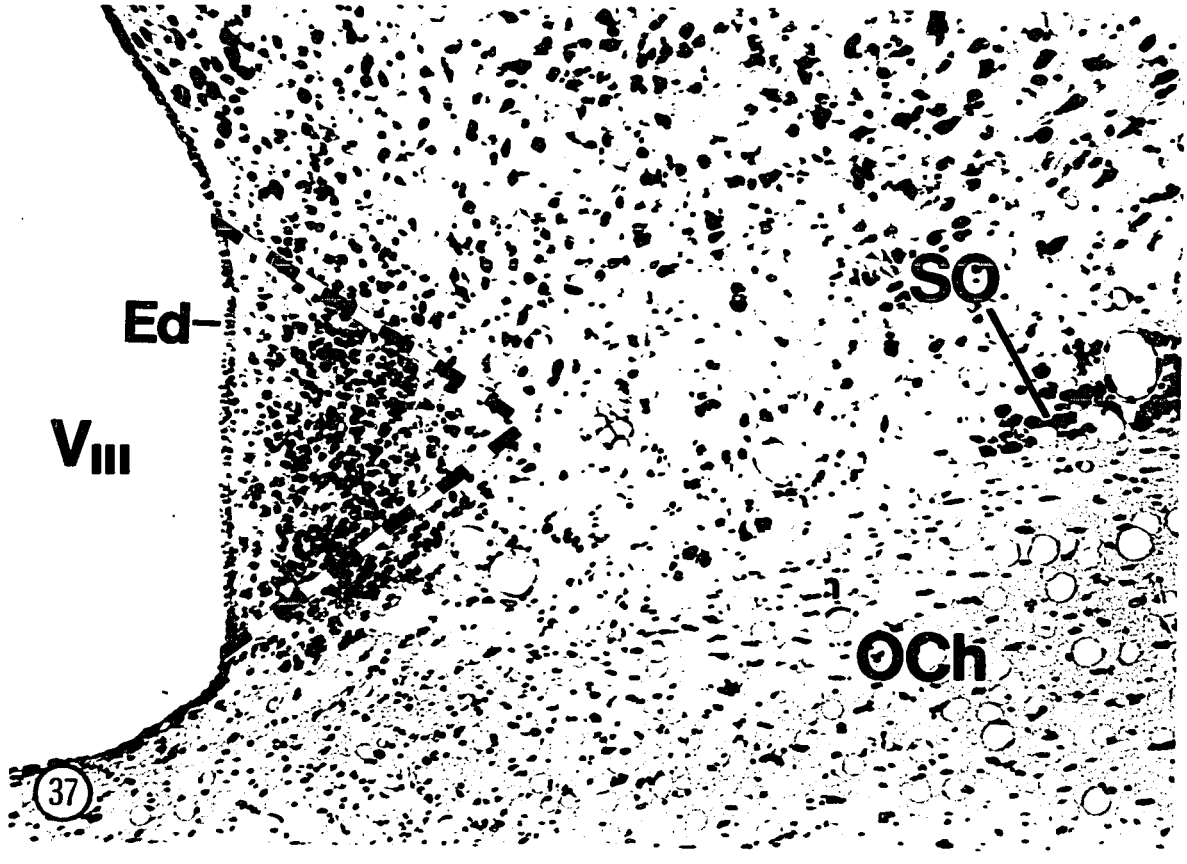
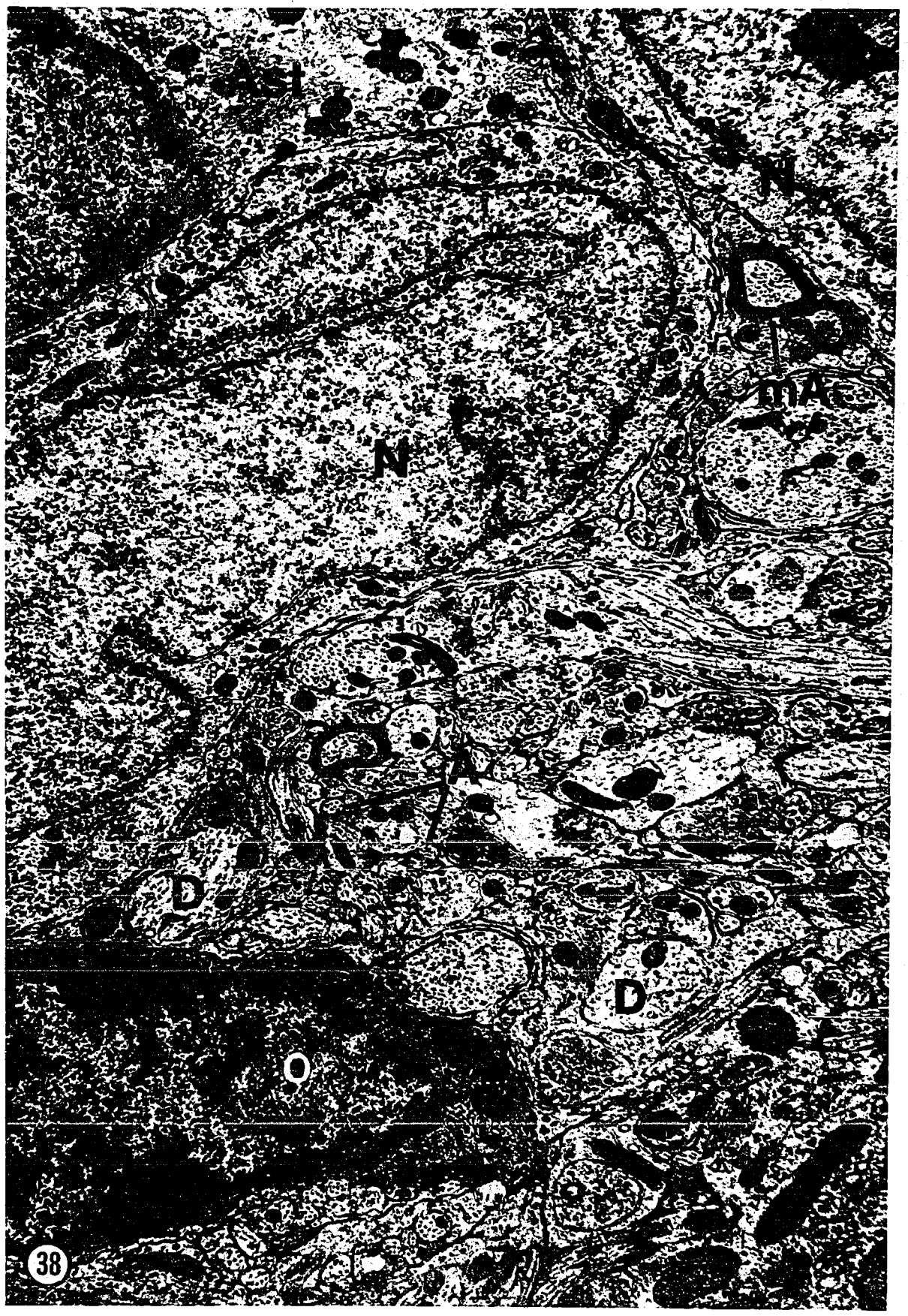


37

Figure 38. Electron micrograph showing the ultrastructure of several cellular components and the general nature of the neuropil of the suprachiasmatic nucleus. Portions of the nuclei and perinuclear zones of two neurons (N), an astrocyte (Ast) and one oligodendrocyte (O) are shown. Small unmyelinated axons (A), several larger axons with thin sheets of myelin (mA) and dendrites (D) compose the neuropil. Control animal.

X 12,700


D
MA
N
D
D
D
38

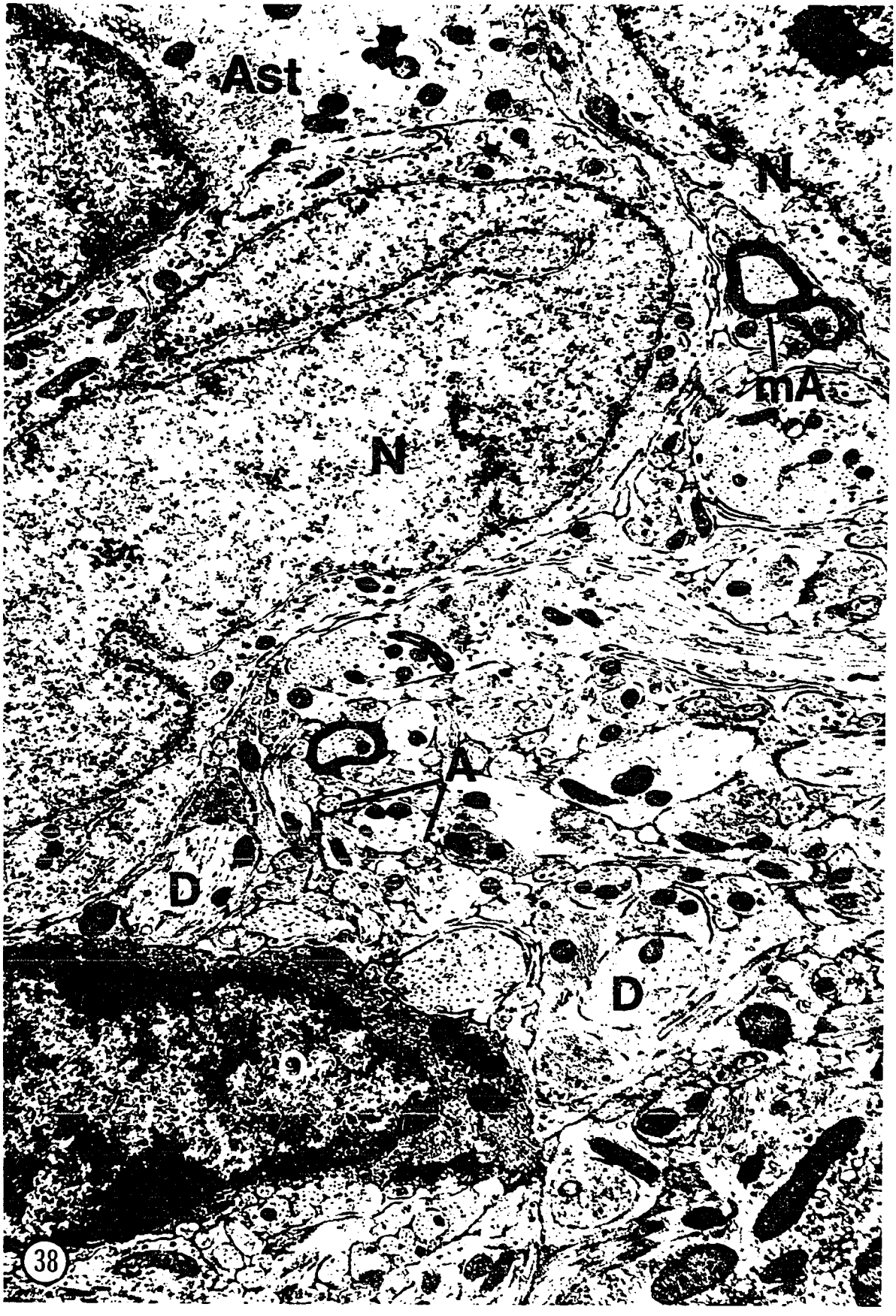

Ast
N
mA
N
A
D
D
9
9
38

Figure 39. Electron micrograph showing the fusiform shape and compact distribution of the suprachiasmatic neurons. The frequently observed close spatial relationship between many of the neurons and the capillaries of this nucleus is illustrated. Again, the compact nature of the neuropil is evident. Control animal.

X 6,300

39

39

Figure 40. Distribution curves of the measurements made of the neurons and their nuclear profiles within the suprachiasmatic nucleus of the rabbit hypothalamus. Legend: controls - neurons (—×—), nuclei (—△—); experimental animals - neurons (—o—), nuclei (—o—). The stippled area represents the 63 (31.5%) neurons of the suprachiasmatic nucleus in which discrete ultrastructural changes were observed following coitus.

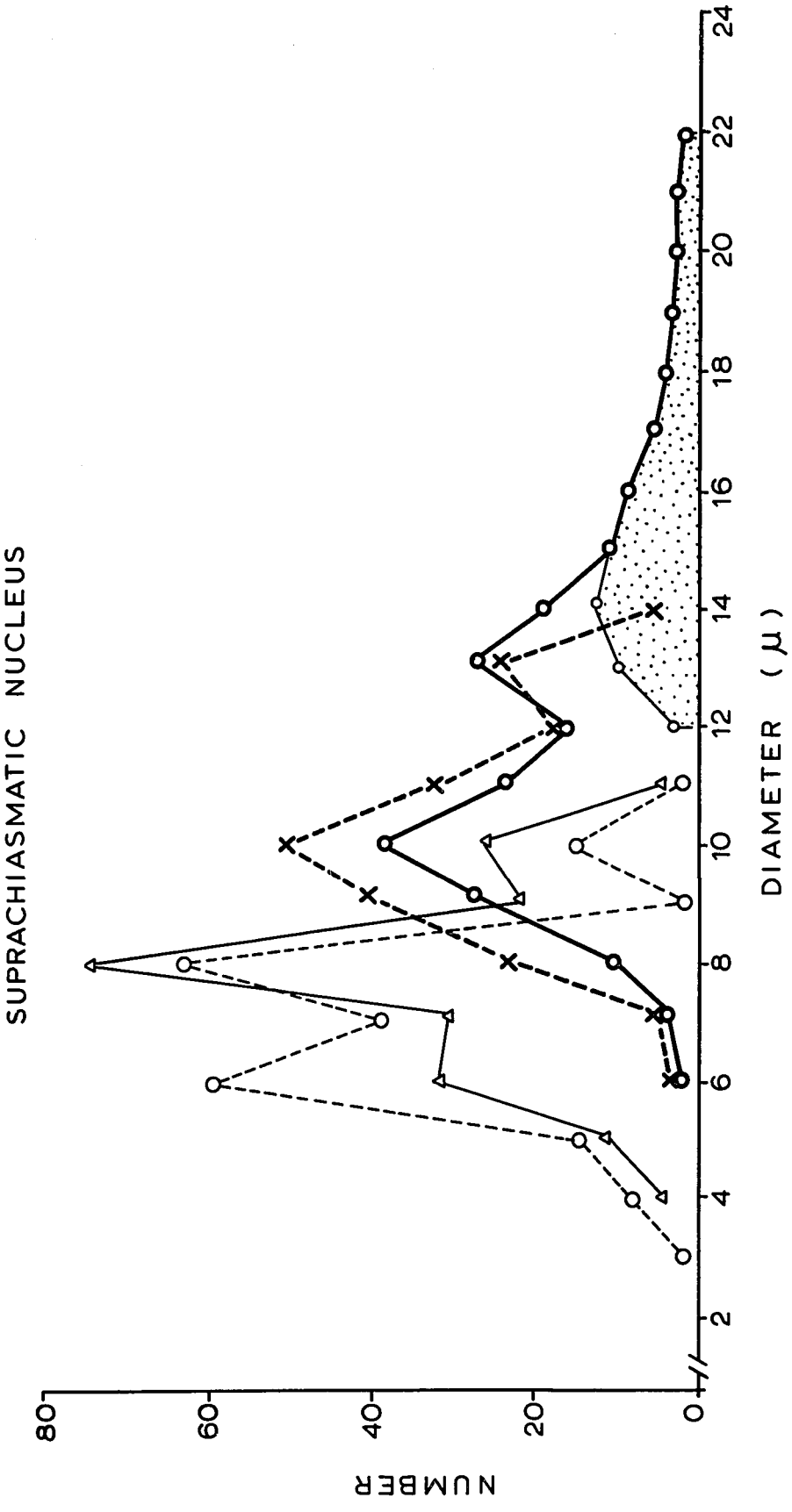

SUPRACHIASMATIC NUCLEUS
NUMBER
DIAMETER (μ)
0
20
40
60
80
2
4
6
8
10
12
14
16
18
20
22
24

Figure 40

the oligodendrocytes and astrocytes observed within thin sections of the PO nucleus.

Scattered, large dendritic profiles, bundles of small unmyelinated axons, the occasional myelinated axon, and fiber-containing profiles of astrocytes composed the neuropil (Figures 38 and 29). The DC vesicles observed within the axon profiles of the control animals had a mean diameter of 1032 ± 13 Å (Figure 13). The mean diameter obtained for a similar sample of DC vesicles located within the axons of the experimental groups was 1089 ± 16 Å (Figure 13) and was significantly larger ($P < 0.1$) than that of the controls (Table 3). The number of DC vesicles included in each sample, as well as their diameter ranges are also recorded in Table 3. Frequency distributions of these measurements are presented graphically in Figure 41. The ultrastructure of several large (1300 Å - 1500 Å) DC vesicles located within the axons of the experimental groups is shown in Figure 42.

The DC vesicles contained within the axon terminals of the control animals had a mean diameter of 972 ± 12 Å while those identified within the axon terminals of the experimental groups had a mean of 946 ± 11 Å (Figure 18). The diameter ranges of these vesicles, for both the control and experimental groups, was 600 Å to 1700 Å (Table 4). However, only the occasional DC vesicle had a diameter exceeding 1400 Å. The difference between these two samples of DC vesicles was not significant (Table 4).

Capillaries were common and uniformly distributed throughout most of the sections studied, suggesting a rich vascular supply to

Figure 41. Histograms showing the frequency distributions of the dense-core vesicles measured within the axons of the suprachiasmatic nucleus - control and experimental groups. Note that in the mated experimental groups, there is an increase in the number of dense-core vesicles with diameters ranging between 1300 Å and 1900 Å.

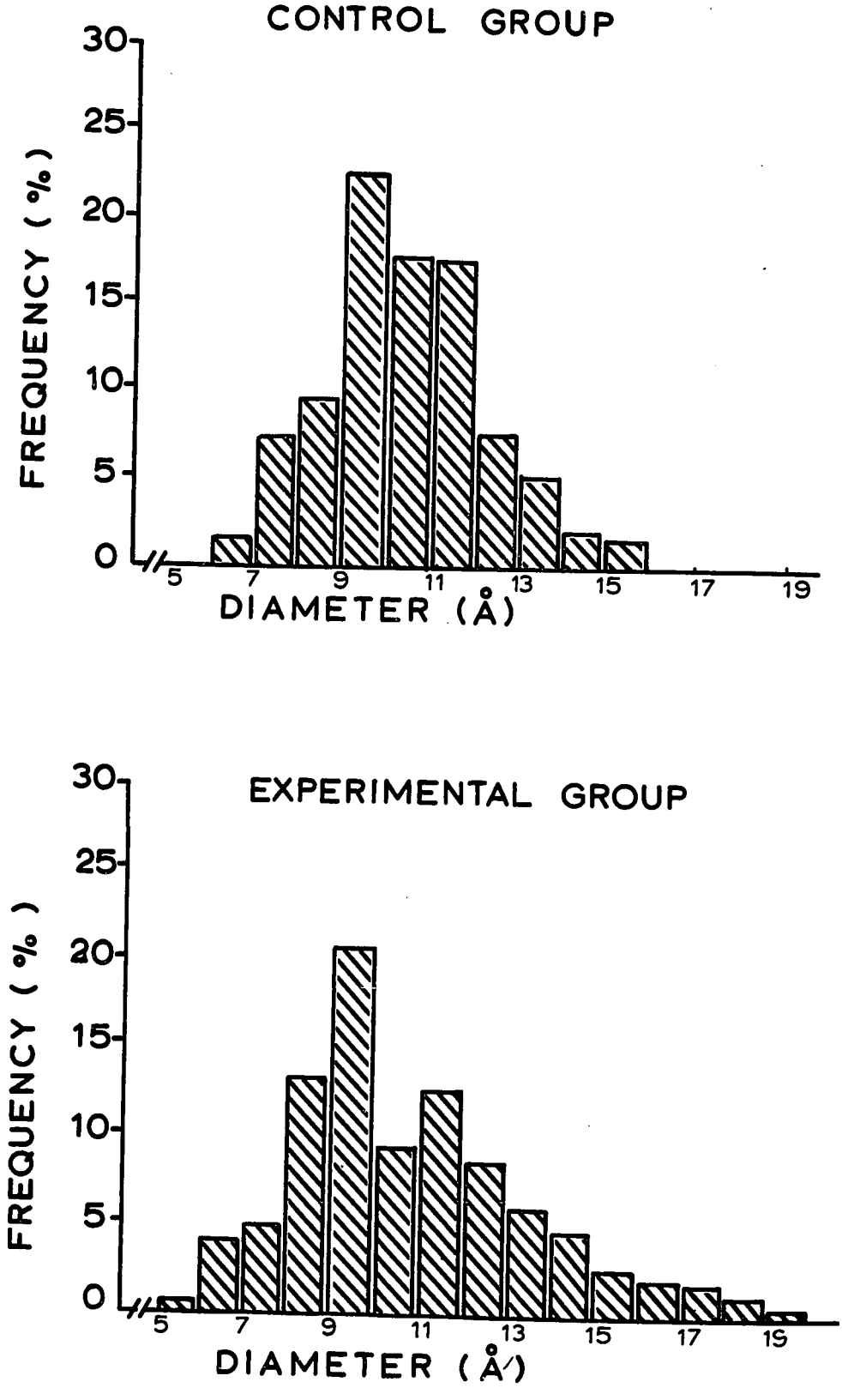

CONTROL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
DIAMETER (Å)
EXPERIMENTAL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
DIAMETER (Å)

Figure 41

Figure 42. High magnification electron micrograph of a small area of the neuropil of a rabbit sacrificed at 10 hours post-coitus. The large dense-core vesicles (DCV) within the axon profiles just below the middle of the field have a maximum diameter of 1500 Å. Compare these vesicles with the smaller ones (arrows) contained within the other axons shown. Suprachiasmatic nucleus. M, Mitochondrion; Nt, Neurotubule.

X 53,000


Nt
M
42

42

this area. Ultrastructurally, the capillaries observed within the SCH nucleus were identical to those of the PO nucleus; their morphological features, therefore, need not be described again.

2) Ultrastructure of SCH neurons of the control animals

The ultrastructural features of one SCH neuron and a portion of the perinuclear zone of another are shown in Figure 43. The close spatial relationship between many of the SCH neurons and elements of the blood vascular system was also evident (Figures 39 and 44). Generally, except for the usual fusiform shape (Figures 39 and 43), the neurons of the SCH nucleus were ultrastructurally similar to those of the PO nucleus. No discrete ultrastructural differences were noted in their nuclei. One of the commonly observed contacts between the nucleolus and the nuclear envelope is illustrated in Figure 44. Note that these contacts most intimately involve the granular component of the nucleolus.

Similarly, as observed in neurons of the PO nucleus, those of the SCH nuclei of the control animals were characterized by a thin ring of cytoplasm containing very sparse populations of the cell organelles. The rough ER and Golgi systems were both poorly developed (Figures 43 and 44). Dense-core vesicles were not common; only 73 occurred within the soma of approximately 240 neurons studied. The mean diameter of these vesicles was 1059 ± 22 Å (Figure 22), with a diameter range of 750 Å to 1700 Å (Table 5). The frequency distribution of these measurements is shown in Figure 45.

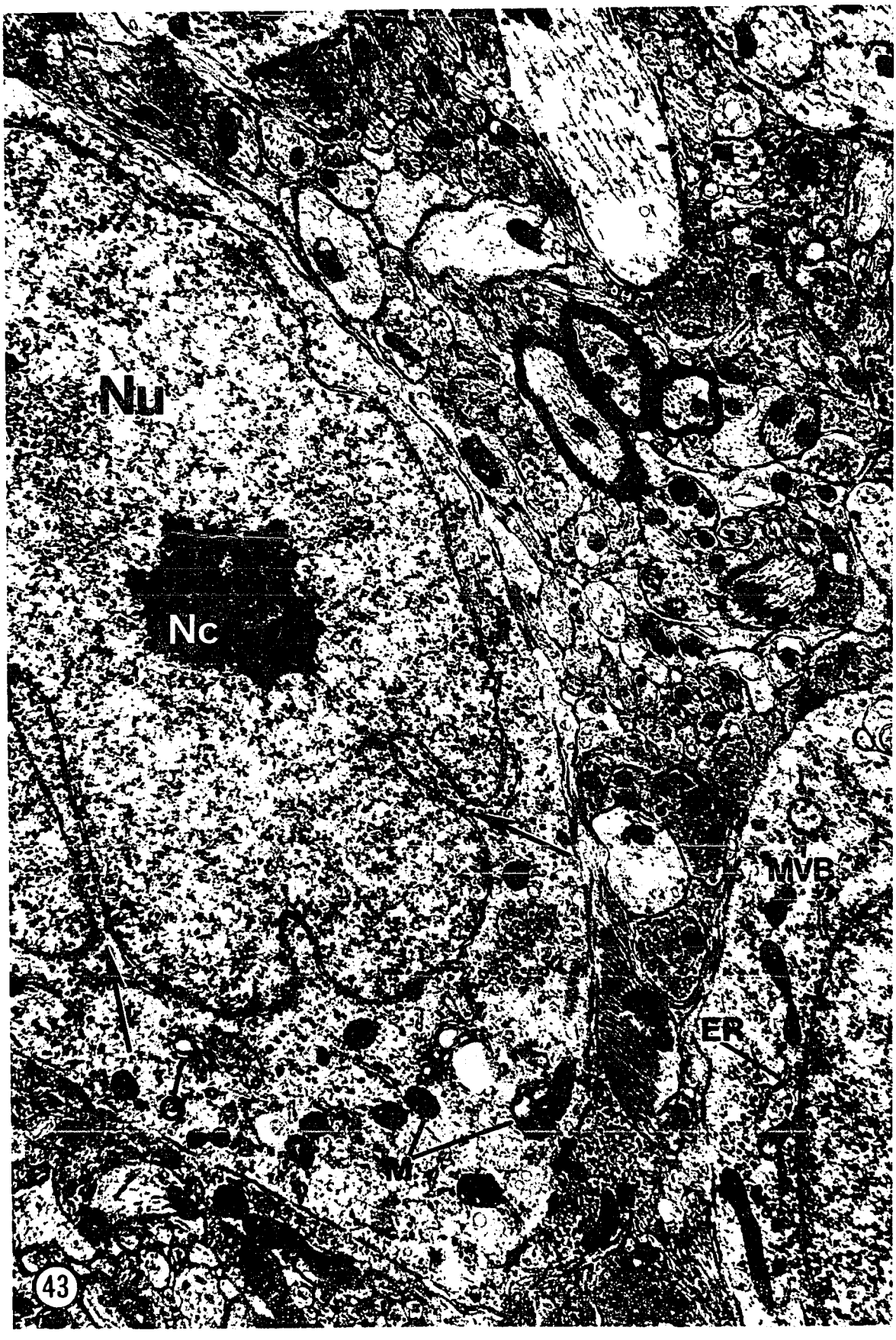
3) Ultrastructural changes in SCH neurons of the experimental groups

As observed in the PO nucleus, discrete and readily identifiable changes also occurred in certain neurons of the SCH nucleus following coitus. The ultrastructural changes, similar to the post-coital changes observed within the PO nucleus, were characterized by an increase in both neuronal and nuclear diameters, by the presence of large, very elaborate Golgi systems, by the tremendous increase in the amount of rough ER, and by the occurrence of relatively dense populations of mitochondria, lysosome-like bodies, and numerous large DC vesicles. These changes occurred within a total of 63 (31.5%) of 200 neurons measured near capillaries. The other neurons observed within the SCH nuclei following coitus were morphologically identical to those of the control animals.

The 200 neurons of the experimental groups, including those in which discrete ultrastructural changes were observed, had a mean diameter of 12.3 ± 0.2 μ (Figure 8). The mean diameter of their nuclear profiles was 8.1 ± 0.1 μ (Figure 9). The minimum and maximum measurements for each of these parameters are shown in Tables 1 and 2, respectively. Also, as shown in these Tables, both the means obtained for the neuronal and nuclear diameters of the experimental groups were significantly larger ($P < .001$) than the same parameters of the controls. The distribution curves for the neuronal and nuclear measurements for both the control and experimental animals are included in Figure 40.

Figure 43. Electron micrograph of portions of the perinuclear zones of two neurons of the non-mated control animals. Note the characteristically large vesicular nucleus (Nu) with its conspicuous nucleolus (Nc) and several deep clefts (arrows). Only a few mitochondria (M), multivesicular bodies (MVB), scattered, short cisternae of the rough endoplasmic reticulum (ER) and several small Golgi profiles (G) can be identified within the cytoplasm. The ultrastructure of the various components of the neuropil is also clearly shown. Suprachiasmatic nucleus.

X 13,100


Nu
Nc
MVB
ER
G
M
43

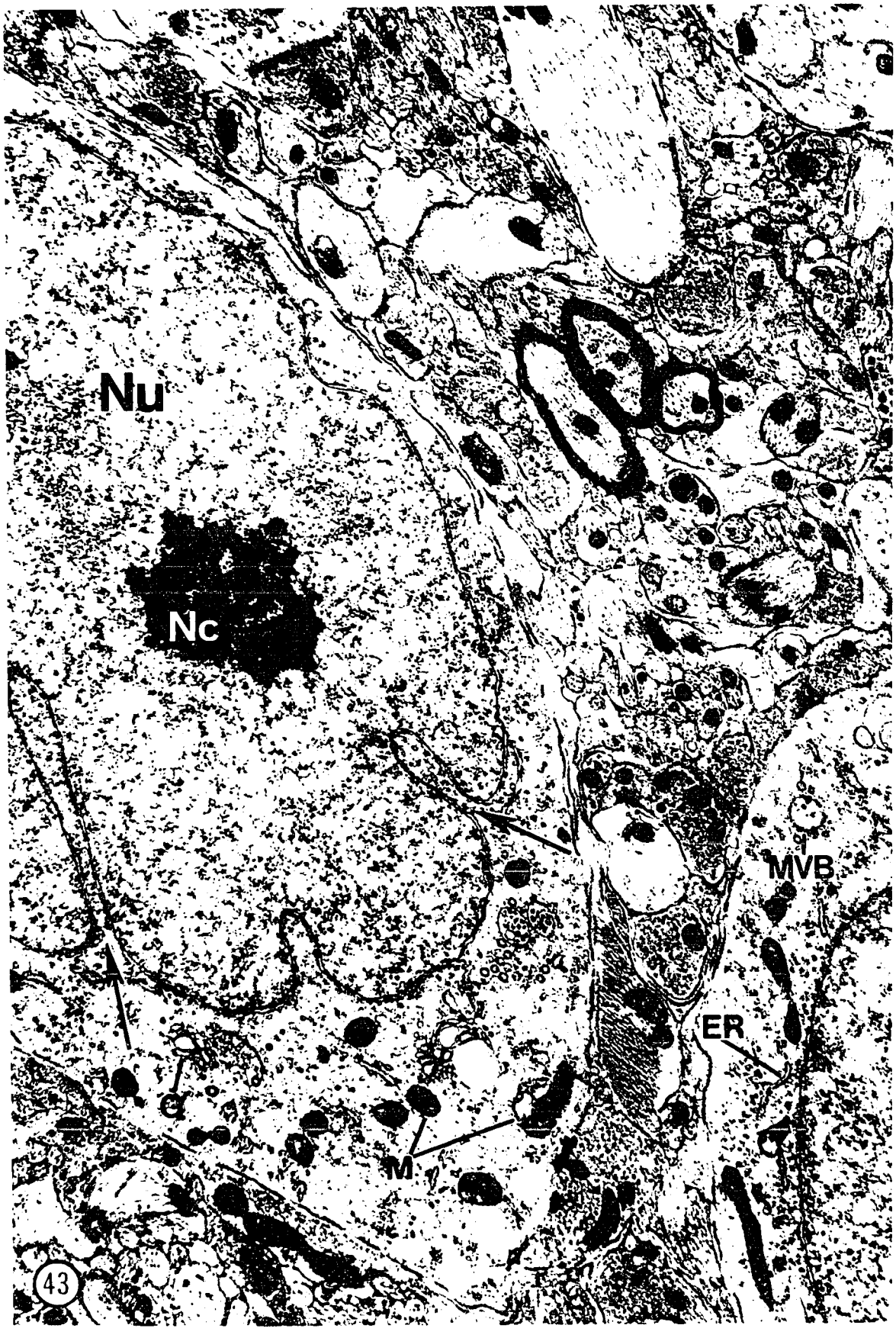

Nu
Nc
MVB
ER
M
43

Figure 44. Electron micrograph of portions of three supra-chiasmatic neurons of the non-mated control animals. The large nucleolus (Nc) is in contact with the nuclear envelope (arrows). Again note the generally sparse populations of the cytoplasmic organelles. Only a thin layer of the neuropil (Npl) separates the soma of the large neuron in the field from the capillary wall. E, Endothelial cell; ir, intranuclear rodlet; P, Pericyte.

X 11,800

Figure 44. Electron micrograph of portions of three supra-chiasmatic neurons of the non-mated control animals. The large nucleolus (Nc) is in contact with the nuclear envelope (arrows). Again note the generally sparse populations of the cytoplasmic organelles. Only a thin layer of the neuropil (Npl) separates the soma of the large neuron in the field from the capillary wall. E, Endothelial cell; ir, intranuclear rodlet; P, Pericyte.

X 11,800

44

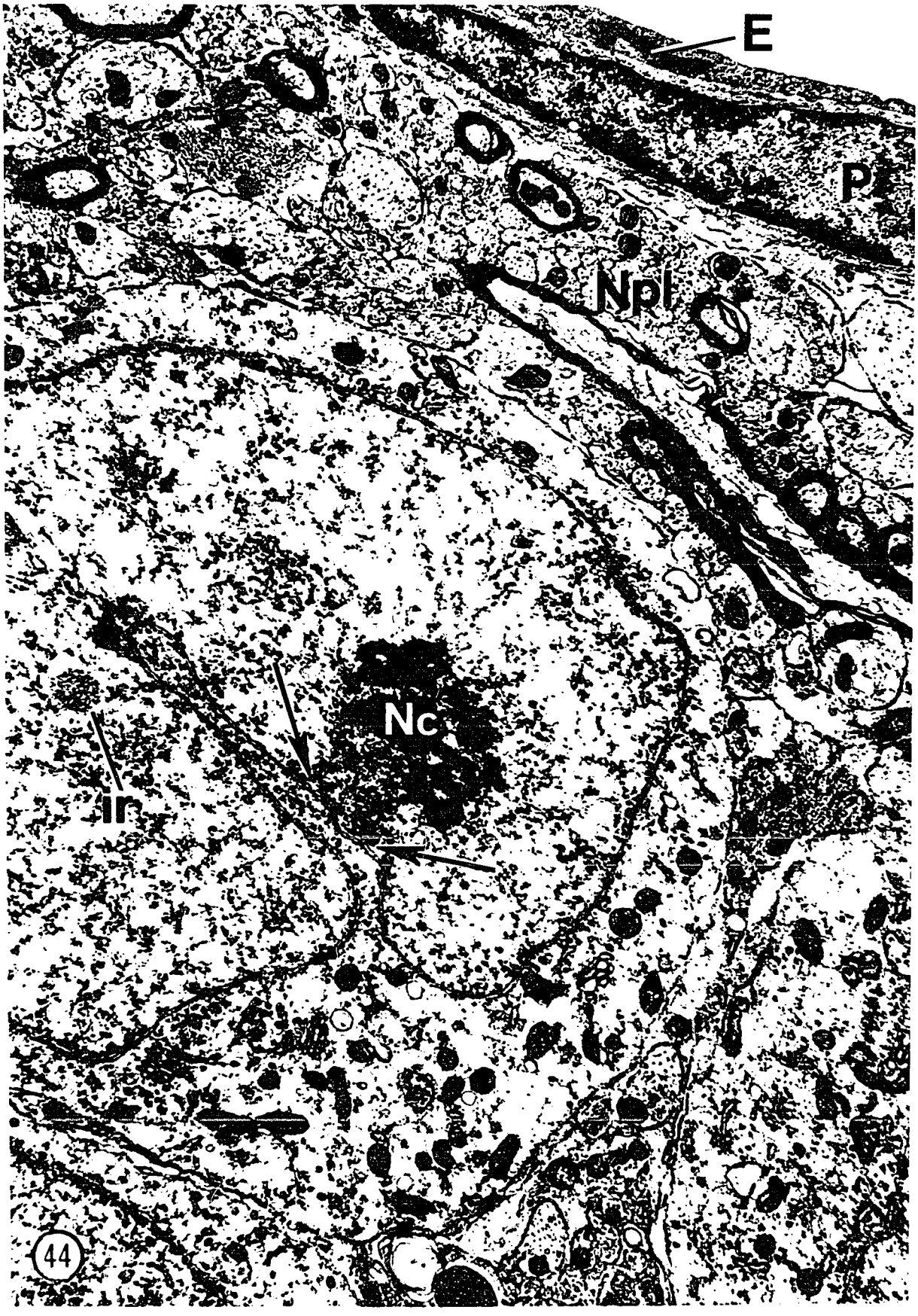
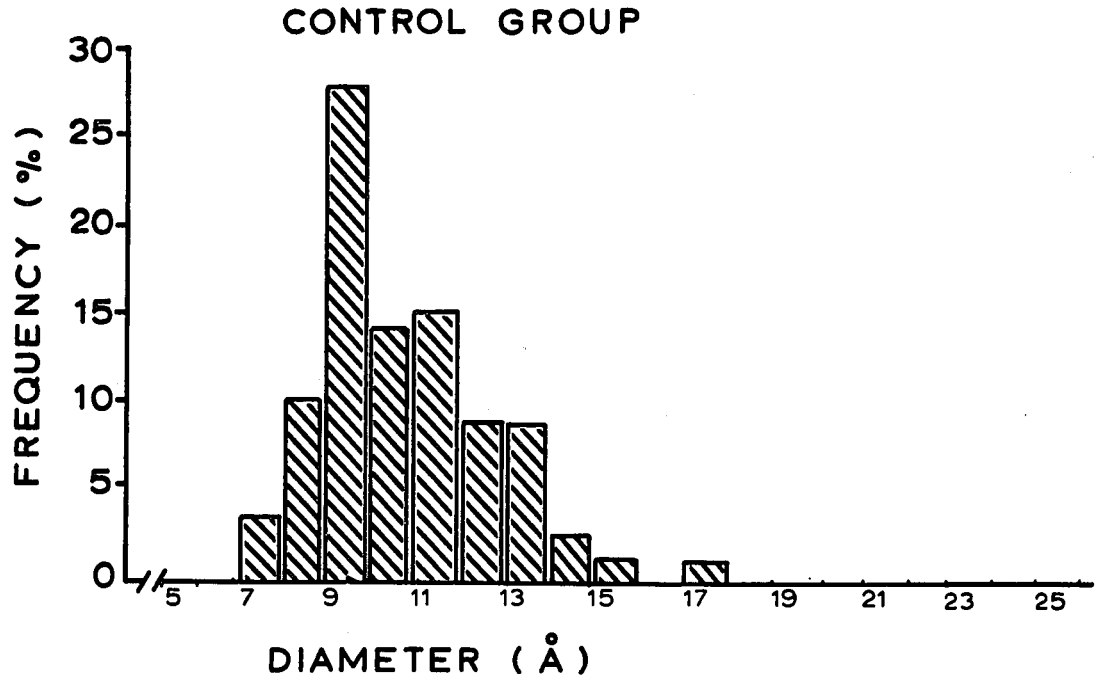

E
P
Npl
Nc
ir
44

Figure 45. Histograms showing the frequency distribution of the measurements obtained for the dense-core vesicles observed within the soma of suprachiasmatic neurons of both the non-mated controls and mated experimental groups. Within the soma of suprachiasmatic neurons of the mated experimental animals there was a significant increase in the numbers of dense-core vesicles with diameters exceeding 1300 Å.


CONTROL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
21
23
25
DIAMETER (Å)

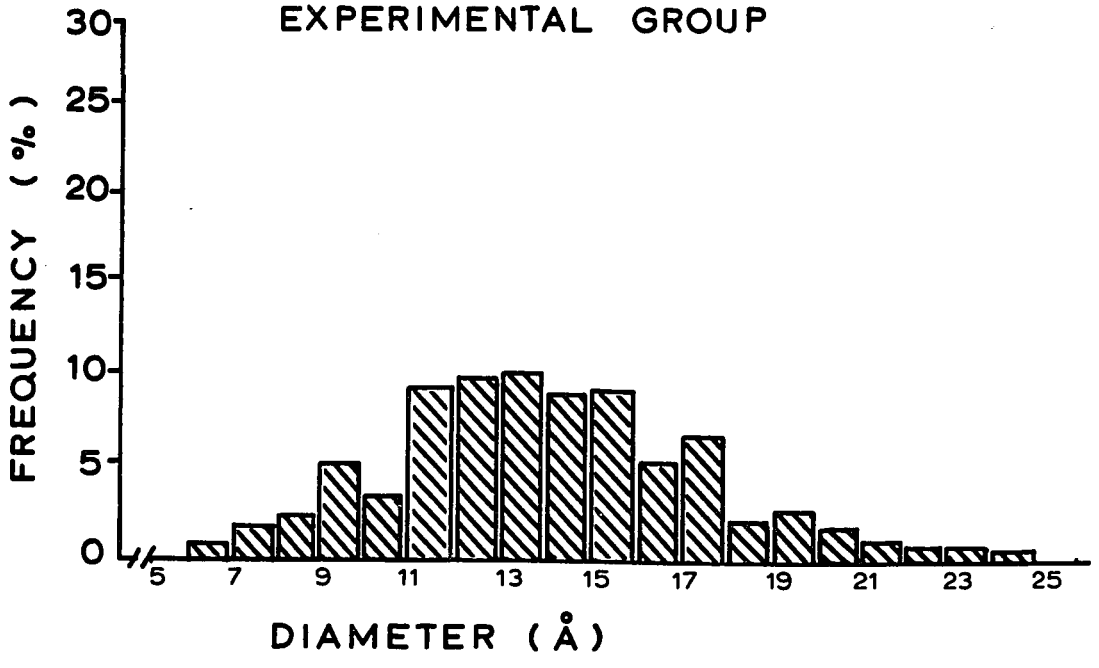

EXPERIMENTAL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
21
23
25
DIAMETER (Å)

Figure 45

a) Ultrastructure of SCH neurons of animals sacrificed at 1/2 hour post-coitus

Evidence of intracellular reorganization within the neurons of the SCH nucleus became apparent as early as 1/2 hour post-coitus. Changes in ultrastructure were first observed in large neurons located near capillaries as shown in Figure 46. These changes were expressed primarily by an increase in neuron diameter, and by the degree of development of certain of the cell organelles. Neurons with relatively well developed rough ER, consisting of long, regular cisternae stacked one upon the other and lying in parallel with the contours of the cell membrane were frequently observed (Figure 46). Dense populations of free ribosomes and their rosettes were common. The rather ill-defined central zone of cytoplasm contained several medium-sized Golgi systems, mitochondria, a variety of vesicles, lysosome-like bodies and a few scattered neurotubules. DC vesicles of the type already described, and with diameters up to 1700 Å, were also observed. This, however, was not a common finding within these neurons, and when present, the DC vesicles were very infrequent.

b) Ultrastructure of SCH neurons of animals sacrificed at one hour to 10 hours post-coitus

More pronounced ultrastructural changes were observed in neurons of the SCH nucleus removed and examined at one hour, two hours, four hours, six hours, eight hours and 10 hours post-coitus (Figures 47, 48, 49 and 50). These changes were again common only within the large neurons located near capillaries. The close spatial relationship between these neurons and the capillary wall is shown in

Figures 47, 48 and 49. Much of the 'marginal' cytoplasm is now occupied by the well developed rough ER and dense populations of small polysomes. The presence of a fine macromolecular material of low electron density within the rough ER cisternae was also a common finding. As observed in similar neurons of the PO nucleus, this material appeared to be most abundant in the neurons removed at two hours through to 10 hours post-coitus. Multiple, well developed Golgi systems, numerous mitochondria, lysosome-like bodies, a variety of vesicles, scattered neurotubules and many large DC vesicles characterized the 'central' zone of cytoplasm (Figures 37, 48, 49 and 50). Scattered short, irregularly arranged cisternae of the rough ER were also observed in this region of the cytoplasm; however, membrane continuations between this organelle and the Golgi profiles were never found. The structure and orientation of the Golgi systems within these neurons were identical to those described for similar neurons of the PO nucleus (compare Figures 32 and 51). The presence of electron-dense material within saccules of the maturing face of this organelle was observed (Figures 47 and 51). The close spatial relationship between the large DC vesicles and the Golgi membranes, as well as evidence for their origin from the saccule tips were frequently observed (Figure 52). The appearance of DC vesicles in relatively large numbers first became evident in neurons removed at one hour post-coitus (Figure 47). Their occurrence in greater numbers was a consistent finding in the large neurons of the two hours through to 10 hours post-coitus groups (Figures 47, 49, 50 and 51). An increase in the number of lysosome-like bodies was also apparent within

Figure 46. Electron micrograph of a suprachiasmatic neuron removed one-half hour following coitus. There is an increase in the cytoplasmic-nuclear ratios. The rough endoplasmic reticulum (ER) is relatively well developed and stacked in parallel with the cell membrane. Ribosomes are abundant. Several 'islands' of Golgi membrane (G) are seen. The neuron soma is closely related with the capillary wall (arrow).

X 11,000


ER
46

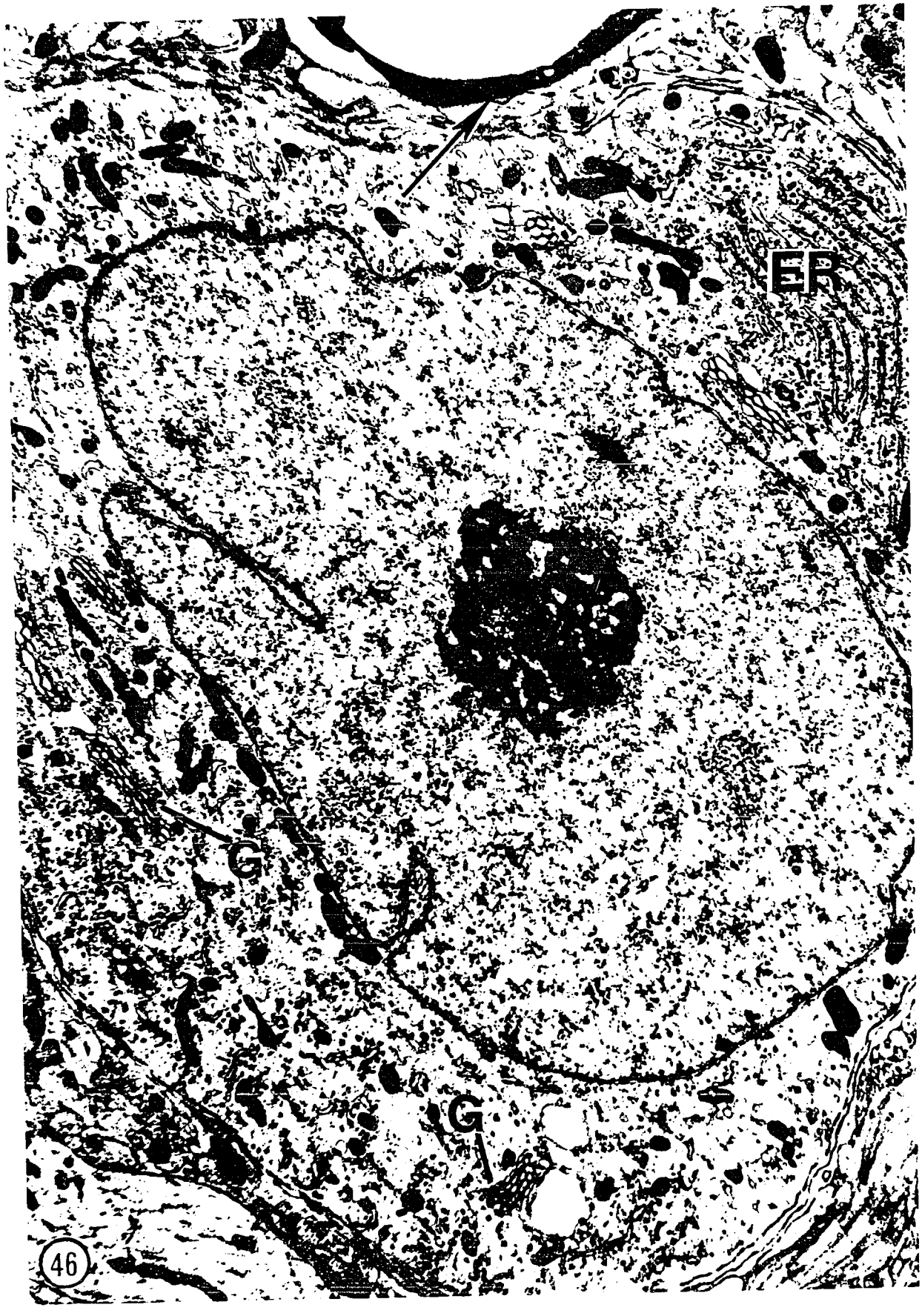
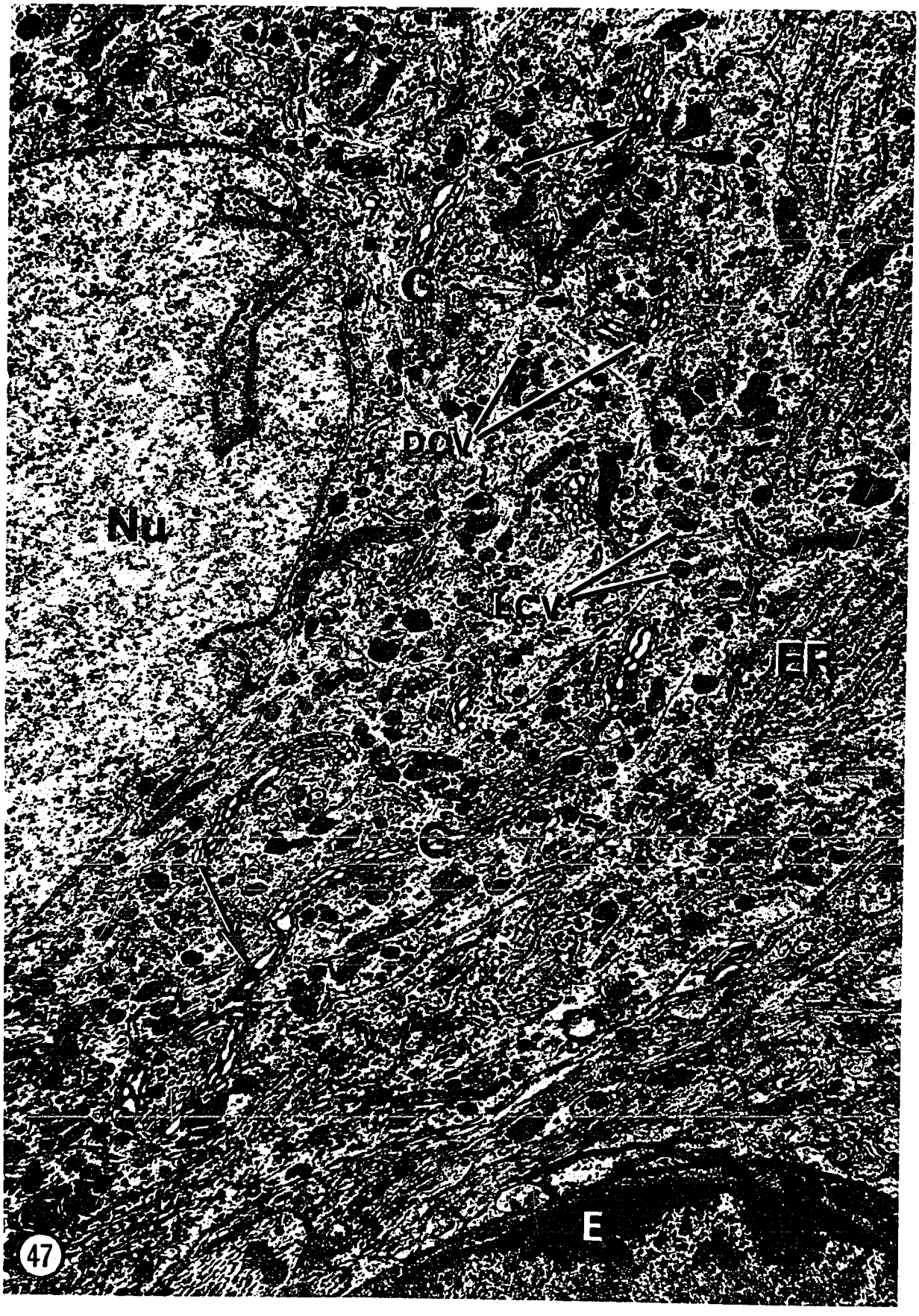

ER
G
G
46

Figure 47. Electron micrograph showing portion of the perinuclear region of a large suprachiasmatic neuron removed at one hour post-coitus. Much of the 'marginal' cytoplasm is occupied by the well developed rough endoplasmic reticulum (ER) and dense populations of small polysomes. Several large Golgi profiles (G) contain electron-dense material (arrows). Large dense-core vesicles (DCV) are in close relationship with the Golgi. Mitochondria are common. Note the dense population of vesicles with light cores (LCV). This is somewhat atypical of most large neurons observed within the suprachiasmatic nucleus. E, Endothelial cell; Nu, Nucleus.

X 12,000


G
DCV
Nu
LCV
ER
G
E
47

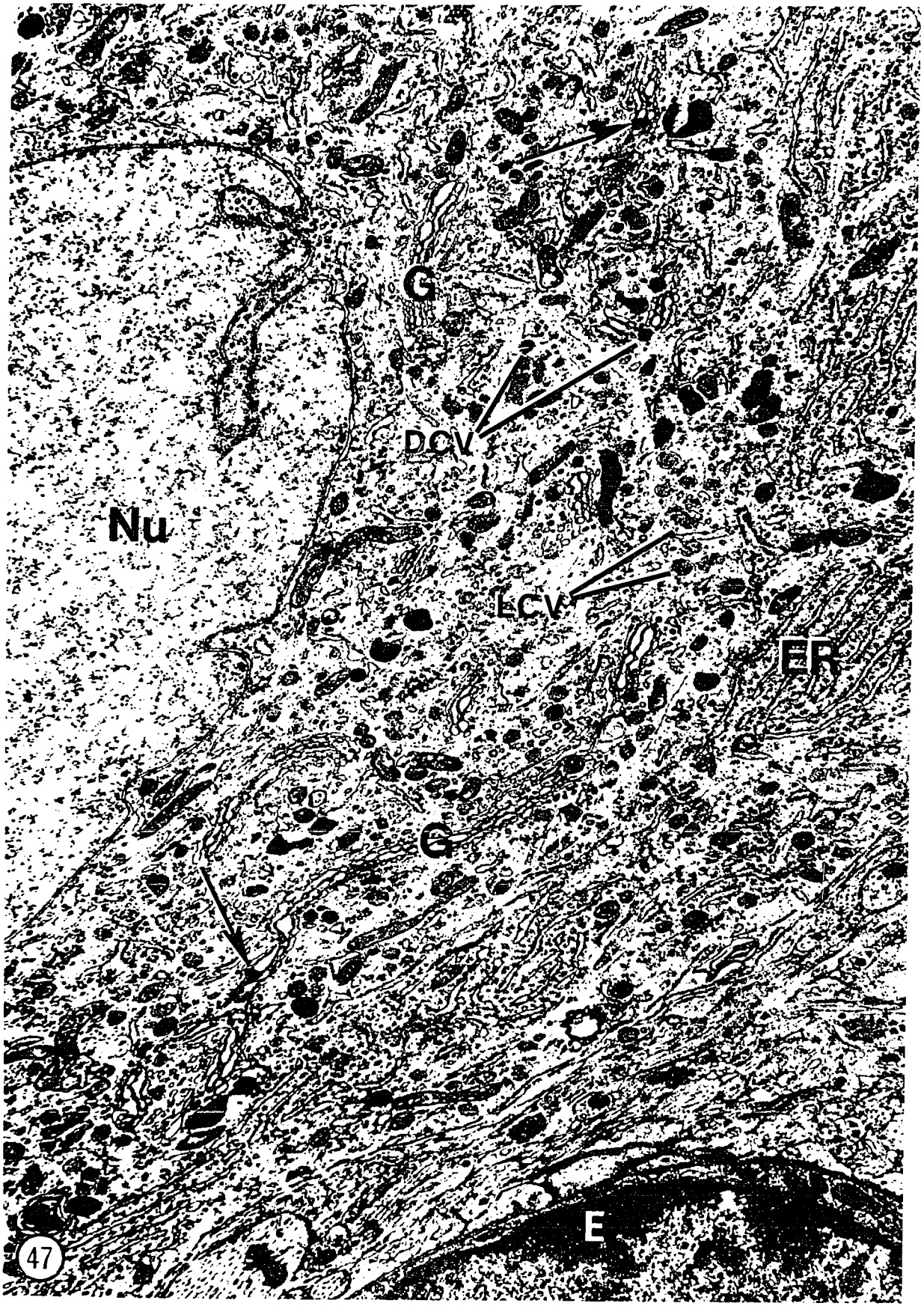
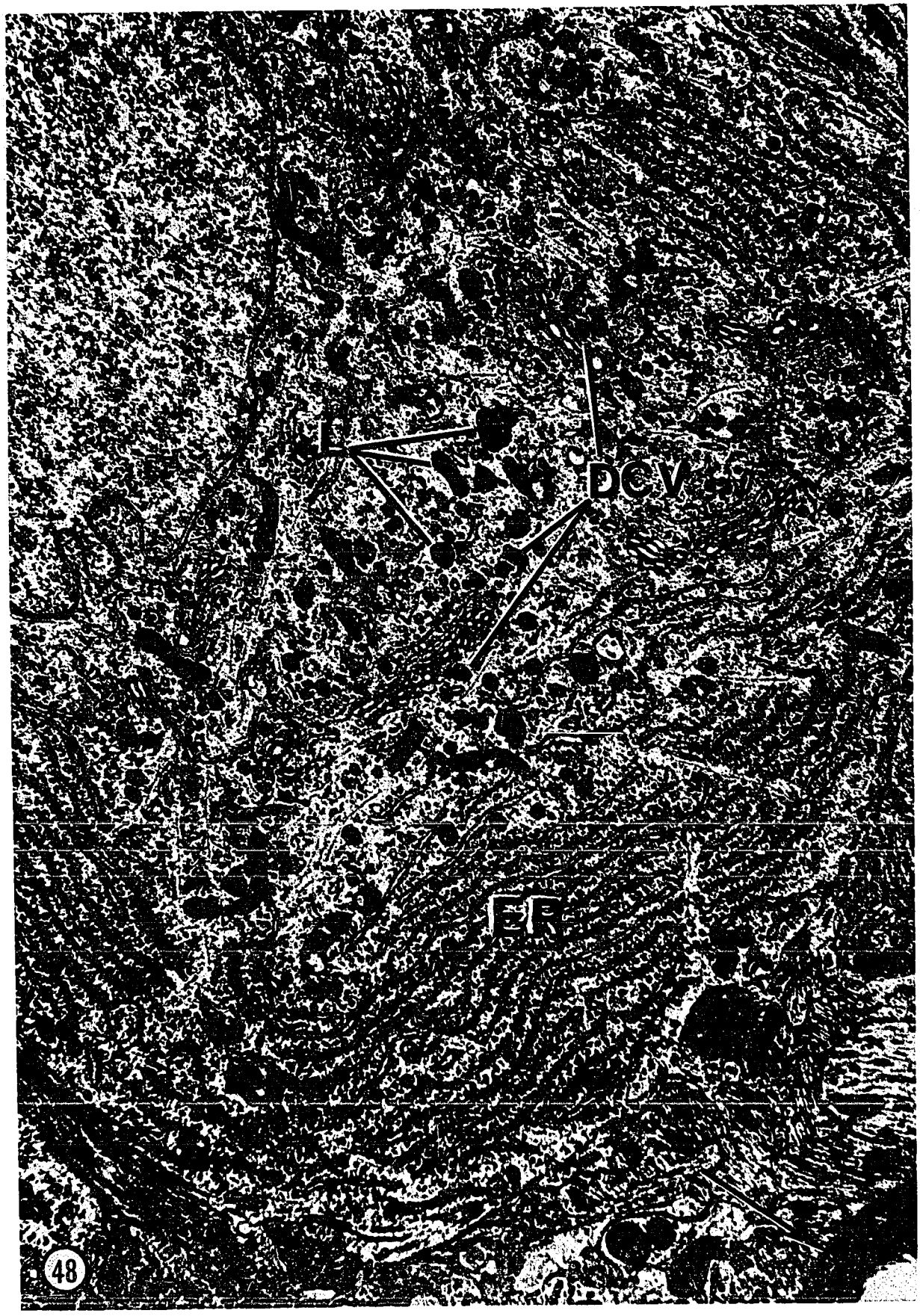
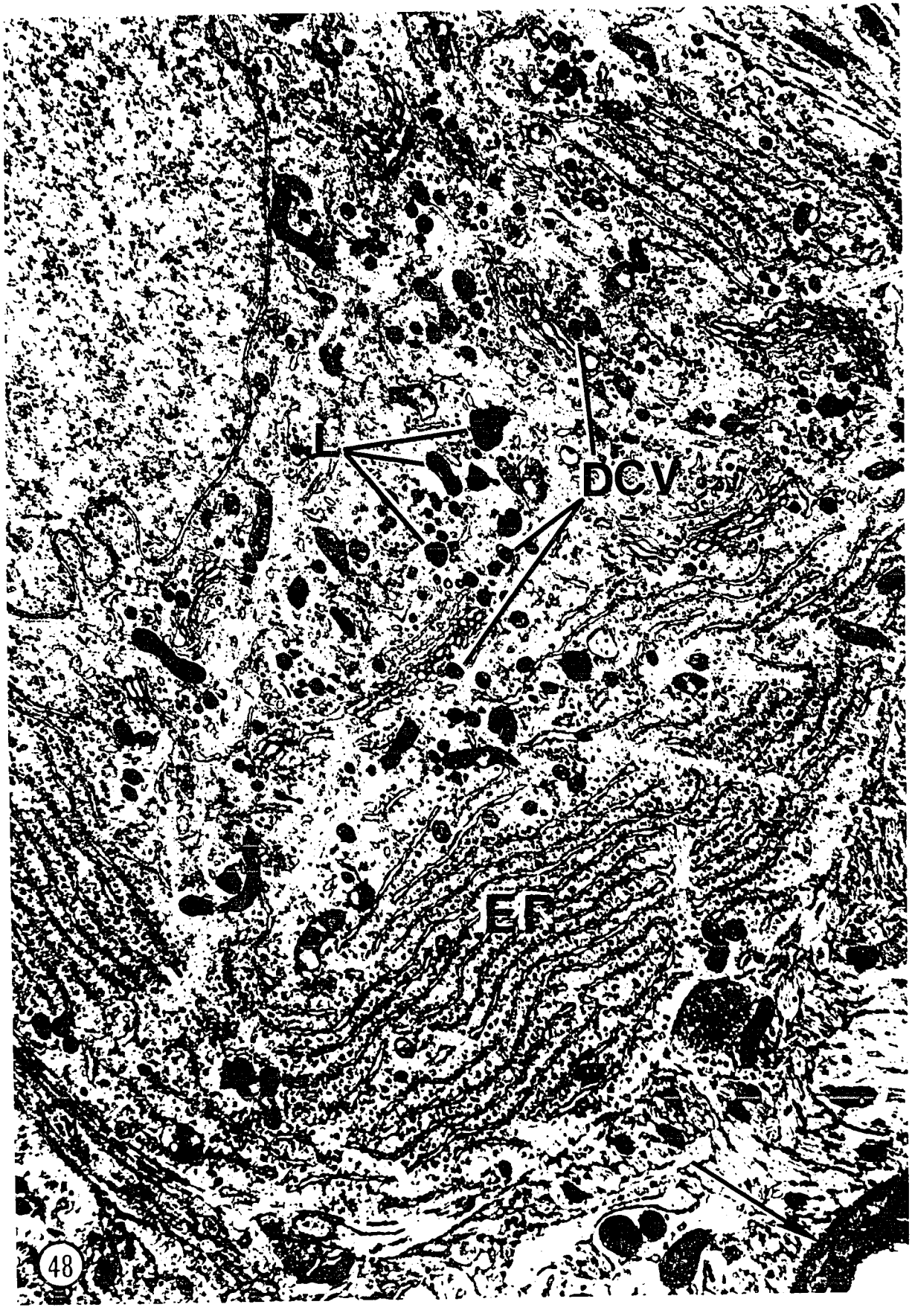

G
DCV
Nu
LCV
ER
G
E
47

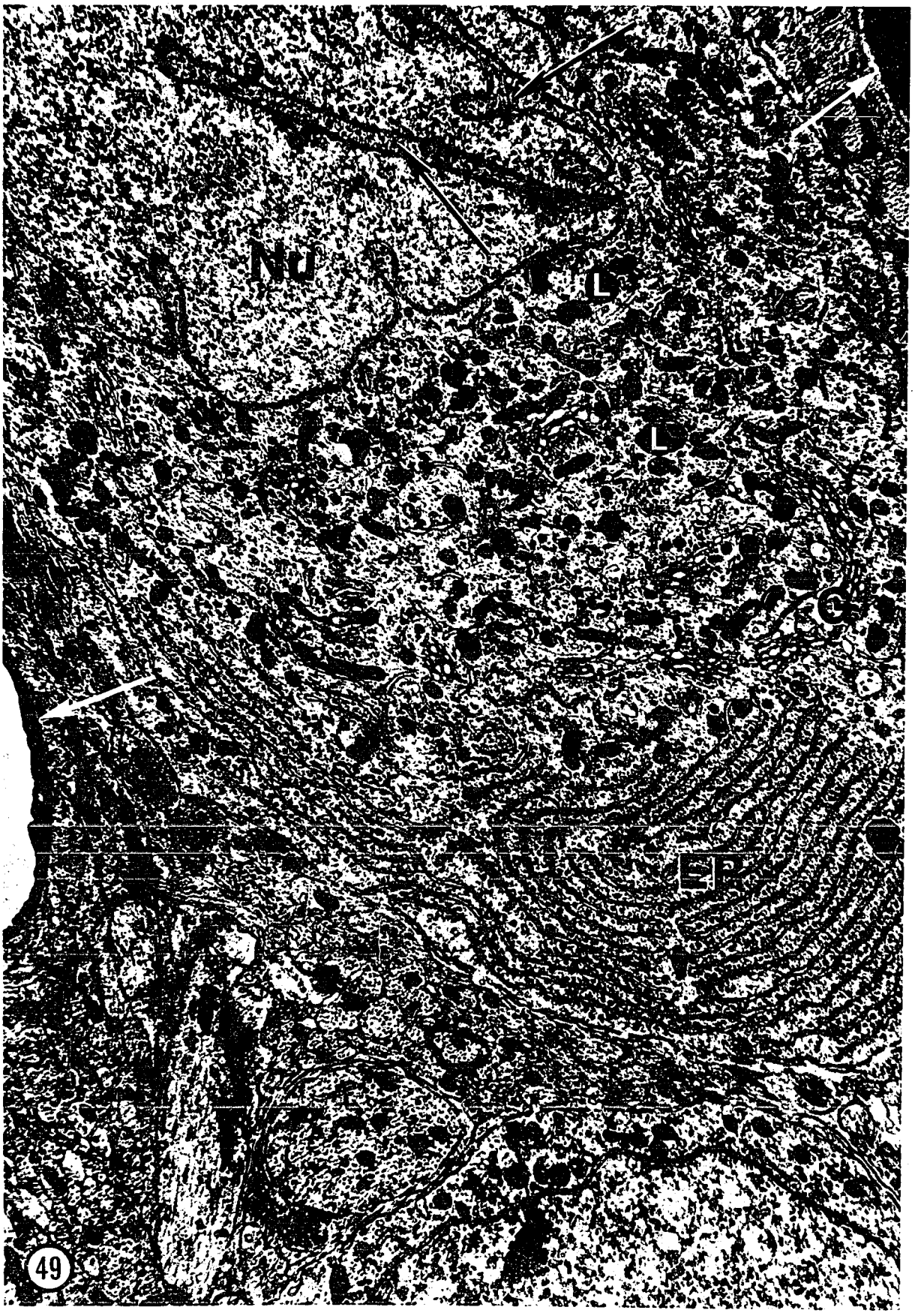
Figure 48. Electron micrograph of part of the perinuclear zone of a large neuron removed from the rabbit hypothalamus at two hours post-coitus. Mary large dense-core (DCV) vesicles are present in the 'central' cytoplasm. The rough endoplasmic reticulum (ER) is very well developed. Numerous free polysomes occur between the cisternae of this organelle. The neuron soma is closely related to a capillary wall (arrow).
L, Lysosome-like body.

X 14,000



48


DCV
EF


Nu
L
L
G
EP
49

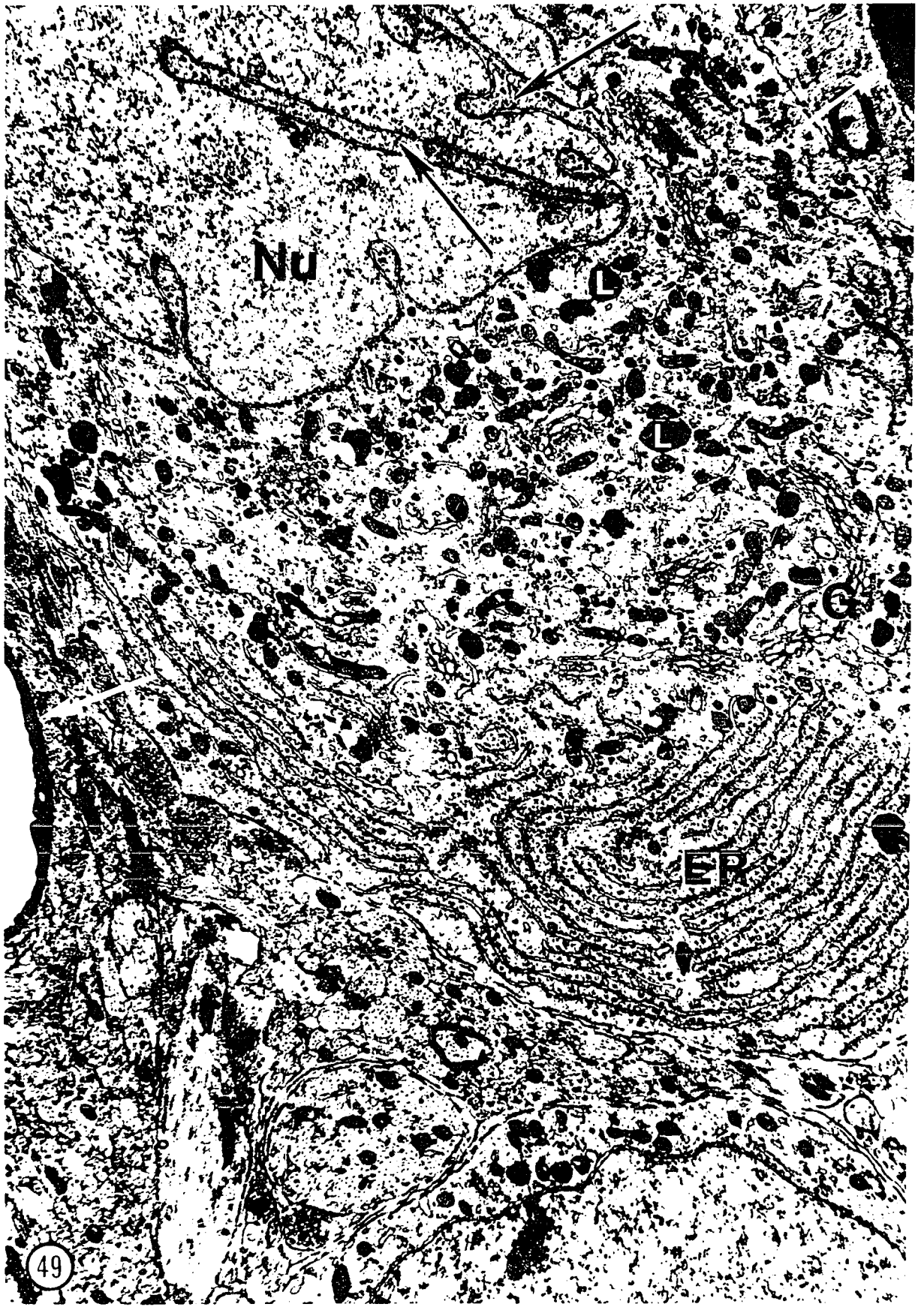
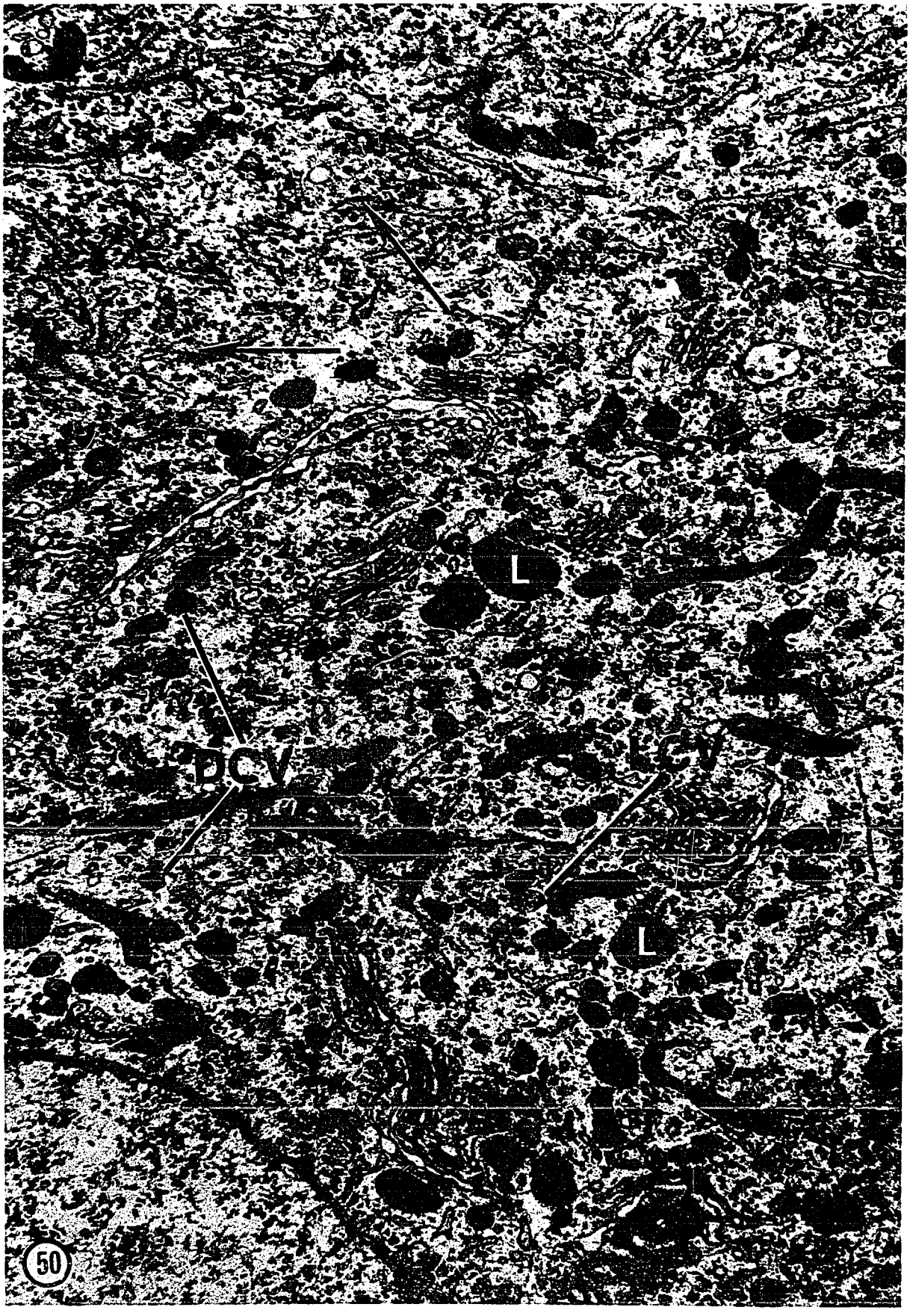

Nu
L
L
G
ER
49

Figure 50. High magnification electron micrograph of the perinuclear zone of a suprachiasmatic neuron removed at 10 hours post-coitus. Macromolecular material (arrows) is present within the rough endoplasmic reticulum cisternae. Large lysosome-like bodies (L) are numerous. Only a few dense-core vesicles (DCV) and those with lighter cores (LCV) are seen in the cytoplasm.

X 21,000


L
PCV
PCV
L
50

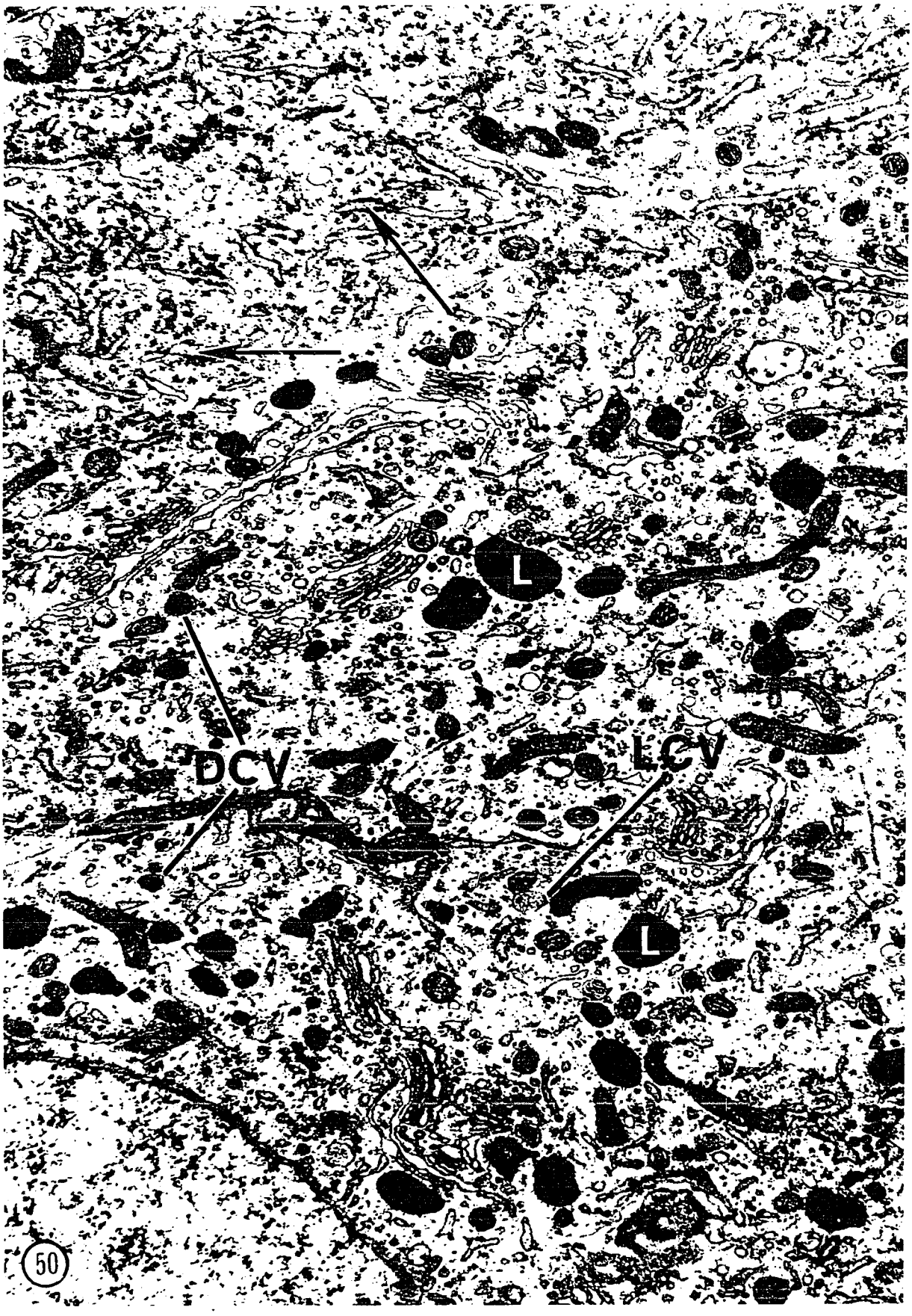
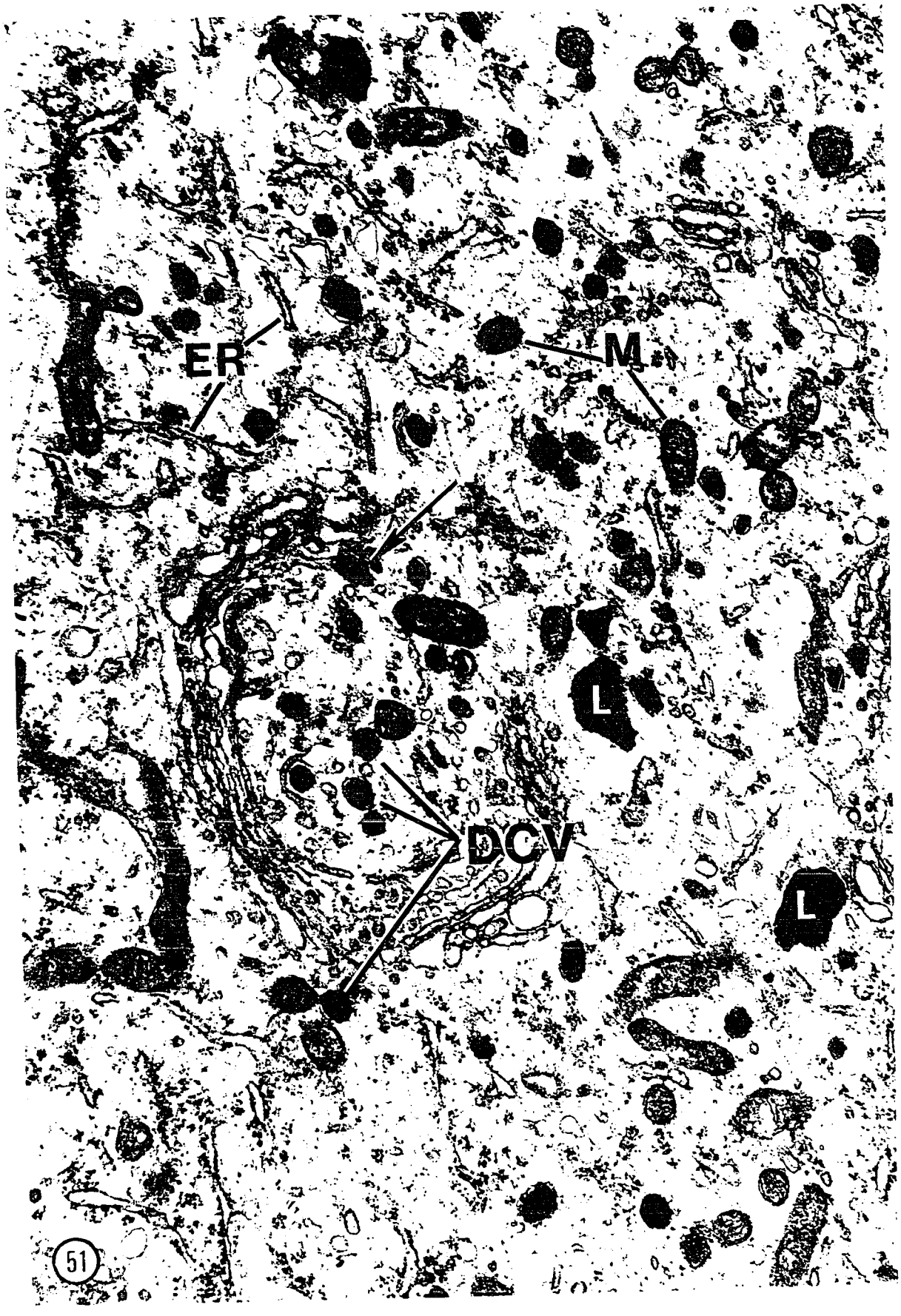

L
DCV
LCV
L
50

Figure 51. High magnification electron micrograph of a Golgi system. Electron-dense material is seen (arrow) within a saccule terminal at the concave or forming face of this organelle. Several large dense-core vesicles (DCV) are closely associated with the Golgi system. Scattered cisternae of the rough endoplasmic reticulum (ER), mitochondria (M) and several large lysosome-like bodies (L) are shown. Suprachiasmatic nucleus, six hours post-coitus.

X 25,200

ER
M
L
PCV
L
51


ER
M
L
DCV
L
51

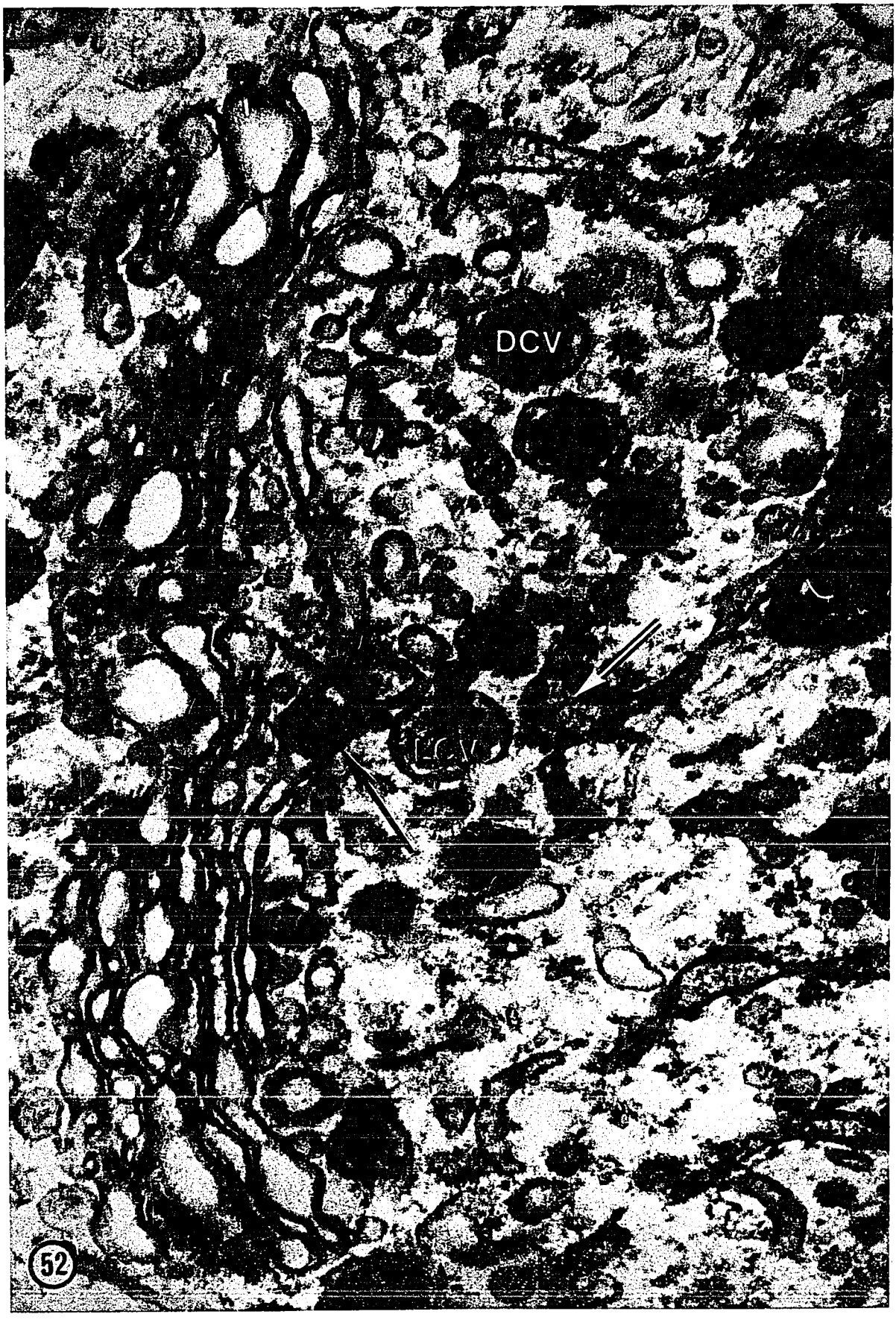
the soma of neurons removed at these times post-coitus.

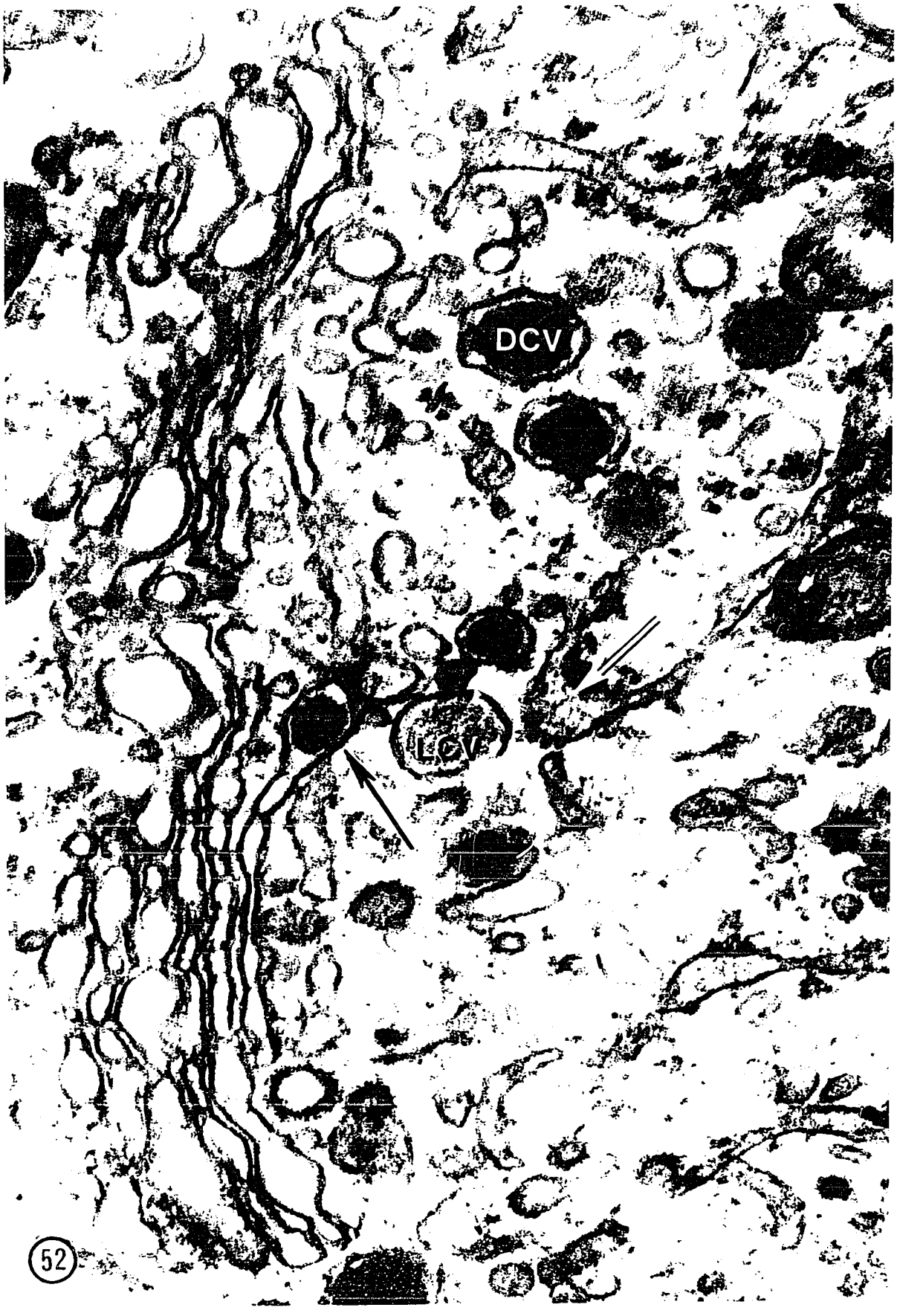
A sample of 744 DC vesicles located within the neuron soma of the experimental groups had a mean diameter of 1416 ± 13 Å (Figure 22). This was significantly larger ($P < .001$) than the mean obtained for the DC vesicles observed within the neuron soma of the control animals (Table 5). The minimum and maximum measurements recorded for these vesicles is also included in Table 5. The increase in the frequency of the large DC vesicles within the neuron soma of the experimental animals is clearly shown graphically in Figure 45.

Another type of vesicle, having a granular content of low electron-density that typically was not separated from the surrounding membrane by a 'halo' was also observed (Figures 52 and 53). Possible intermediate forms of this vesicle type are also shown (Figure 53). These light-cored (LC) vesicles were distributed widely throughout the cytoplasm, but were only occasionally observed in close association with the Golgi system (Figure 52). The mean diameter of the LC vesicles, based on 210 measurements, was 1937 ± 27 Å with a diameter range of 1140 Å to 2960 Å.

Figure 52. High magnification electron micrograph showing a dense-core vesicle (black arrow) pinching off from the Golgi system (G). Note the large light-core vesicle (LCV) near the middle of the field. The granular material it contains is similar to that within the nearby 'swollen' cisternae (white arrow), and may support the origin of the LCV from this organelle. The large dense-core vesicle (DCV) near the top of the field is 2000 Å in diameter. Suprachiasmatic nucleus, 10 hours post-coitus.

X 73,500

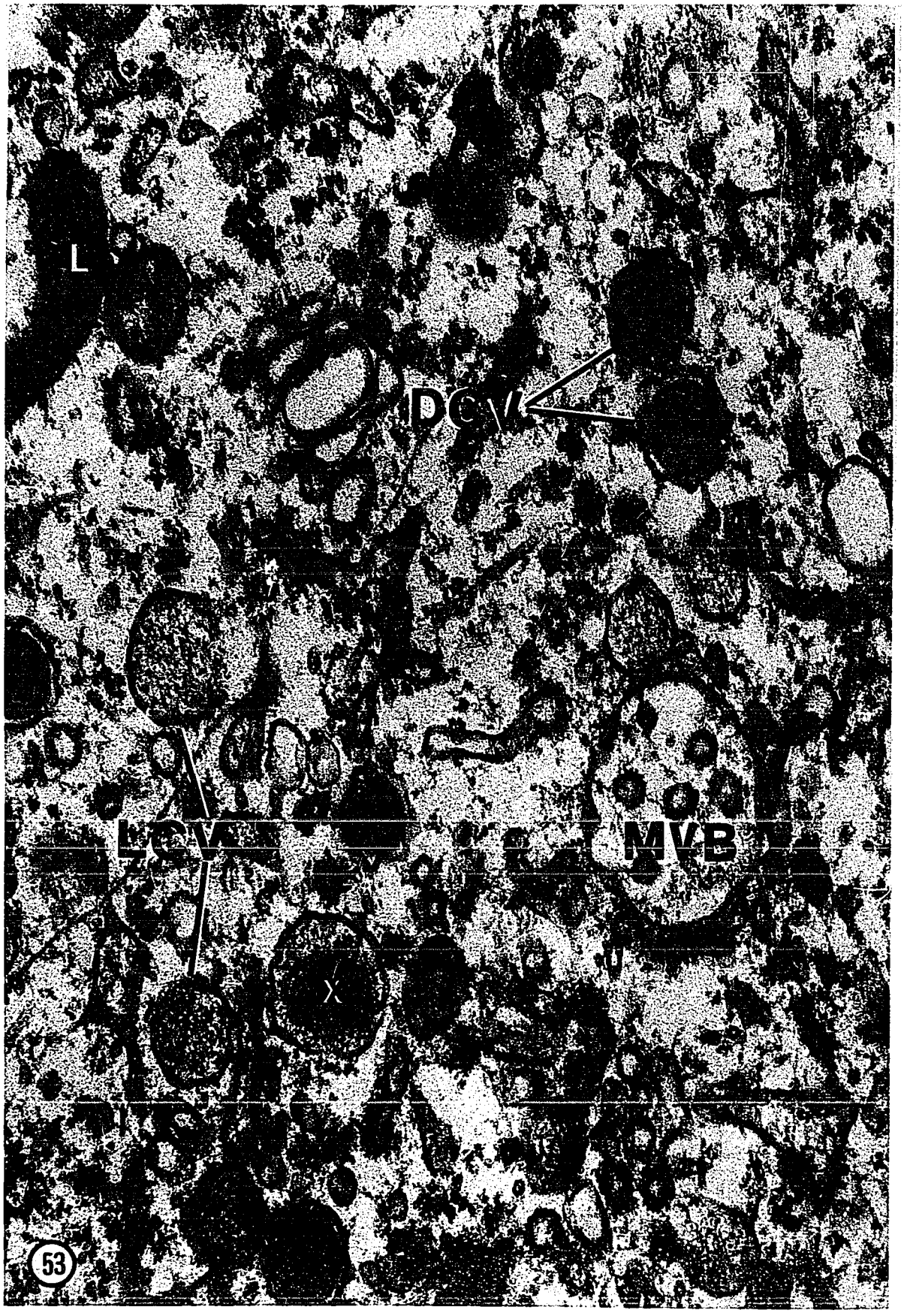

DCV
LCV
52



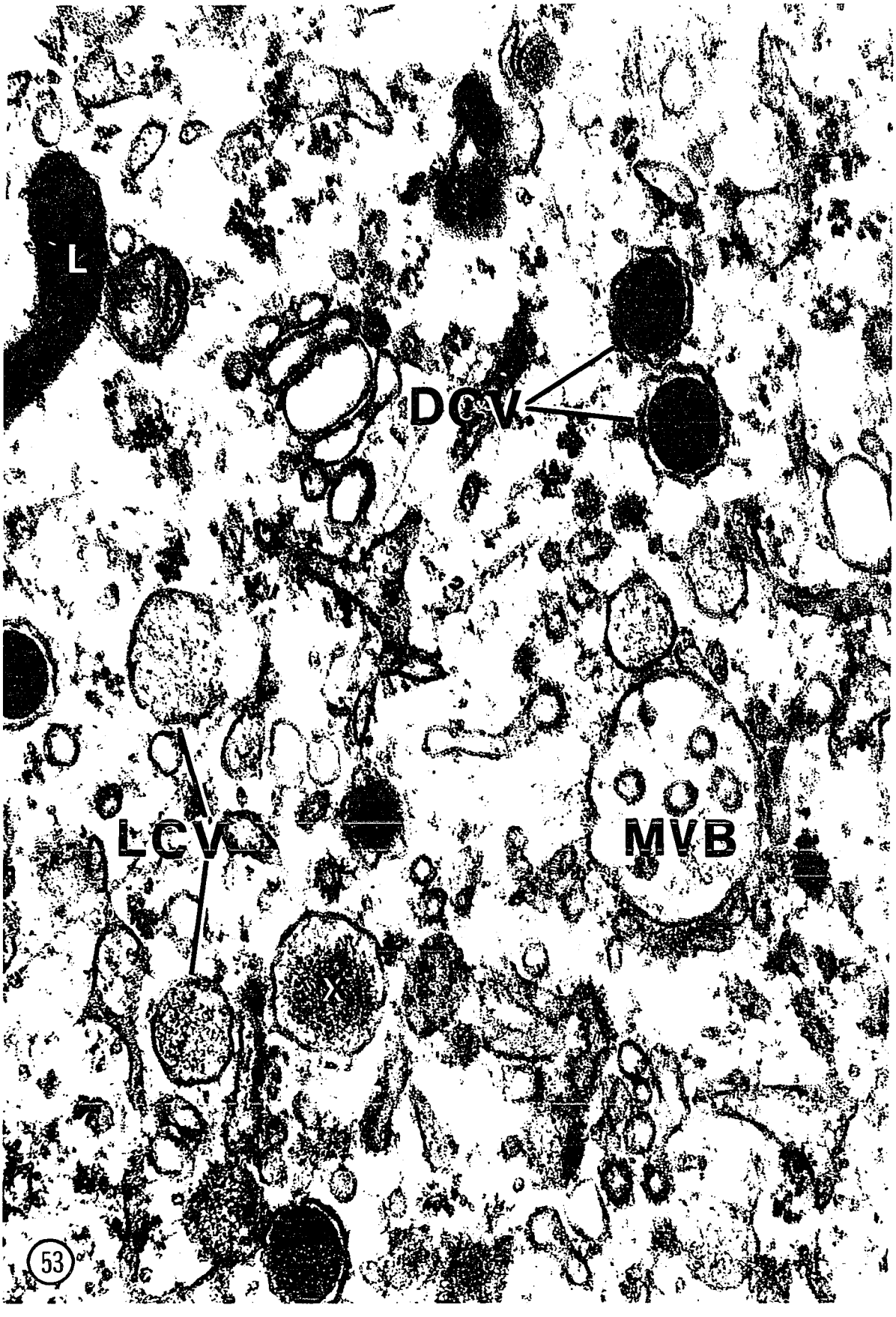
52

Figure 53. High magnification electron micrograph of the various vesicle types encountered within the soma of suprachiasmatic neurons. The large vesicle (x) may represent an intermediate form of the large dense-core vesicles (DCV). These or similar light-core vesicles were never observed pinching off from the Golgi system. LCV, Light-core vesicles; MVB, Multivesicular body; L, Lysosome. Ten hours post-coitus.

X 80,000



53


L
DCV
LCV
MVB
X
53

ARCUATE NUCLEUS

A) Light Microscopy

The arcuate nucleus of the rabbit brain is located in the mediobasal region of the tuber cinereum, surrounding the most ventral portion of the V_{III} and the entrance to the infundibular recess (Figure 54). The cells of this nucleus are in close contact with the ependymal lining medially, and extend ventrally into the median eminence. Dorsolaterally, a cell-poor zone separates the concave border of the arcuate nucleus from the ventromedial nucleus. That part of the rabbit hypothalamus representing the arcuate nucleus and which was removed and processed for electron microscopy is represented by the broken line in Figure 54.

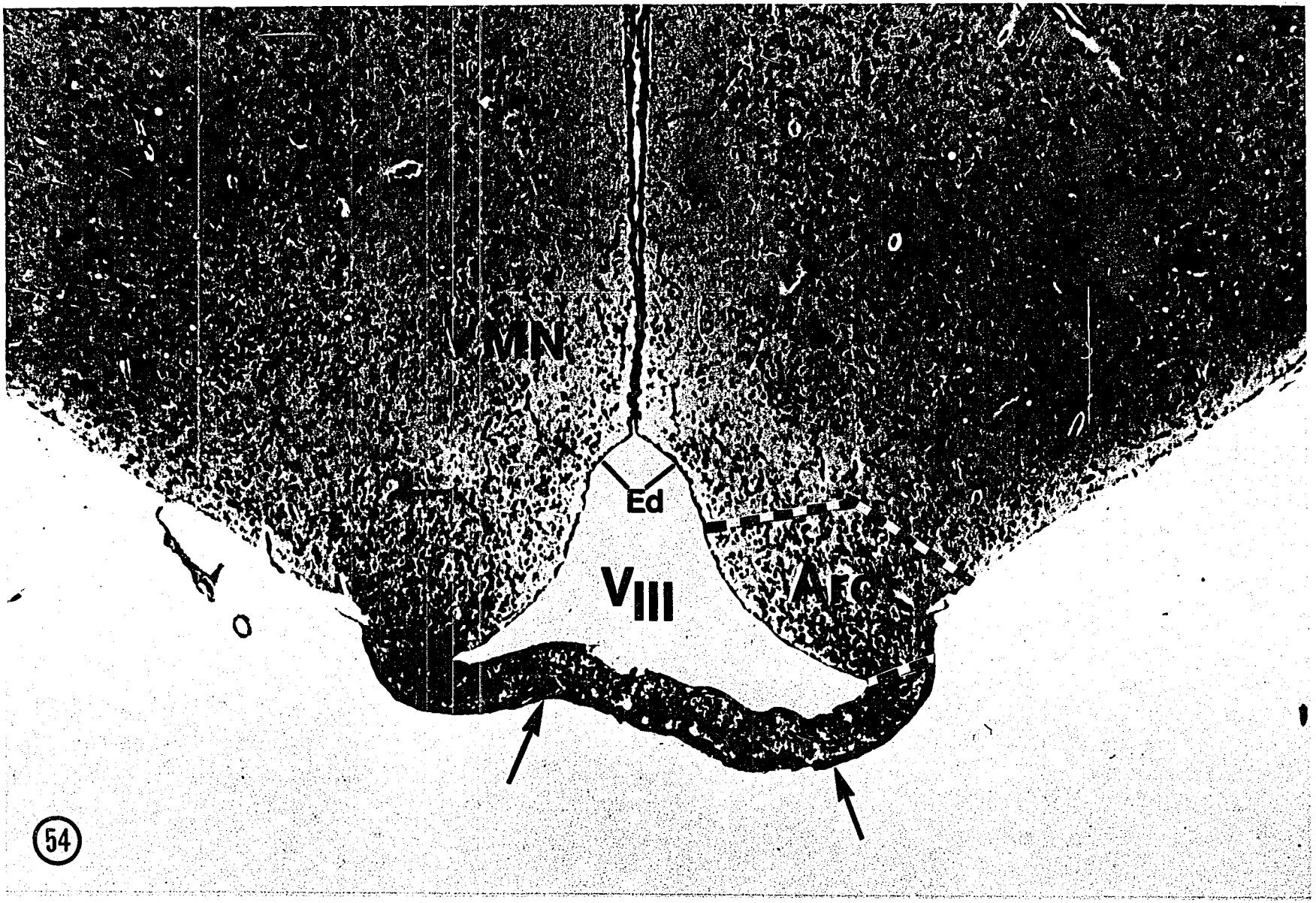
B) Electron Microscopy

1) Ultrastructure of the arcuate nucleus

Neurons, scattered glial cells, capillaries and the densely interwoven neuropil characterized the thin sections cut from the arcuate nucleus (Figure 55). The glial cells featured in Figures 56 and 57 are representative of those frequently observed within the arcuate nuclei of both the control and experimental groups. Ultrastructurally, these cells were identical to the glial elements observed within the PO and SCH nuclei (compare Figures 11, 12 and 38 with Figures 56 and 57). The neuropil was characterized by the presence of axon profiles which contained relatively large numbers of DC vesicles (Figure 58), the occasional myelinated axon (Figure 55) and

Figure 54. Photo micrograph of a frontal section of the rabbit brain showing the location of the arcuate nucleus (Arc). The tissue surrounded by the broken line is representative of that removed from each side of the third ventricle (V_{III}) and processed for electron microscopic study. Ed, Ependyma; VMN, Ventromedian nucleus.
Arrows, Median eminence.

X 200


VMN
Ed
VIII
ARC
54

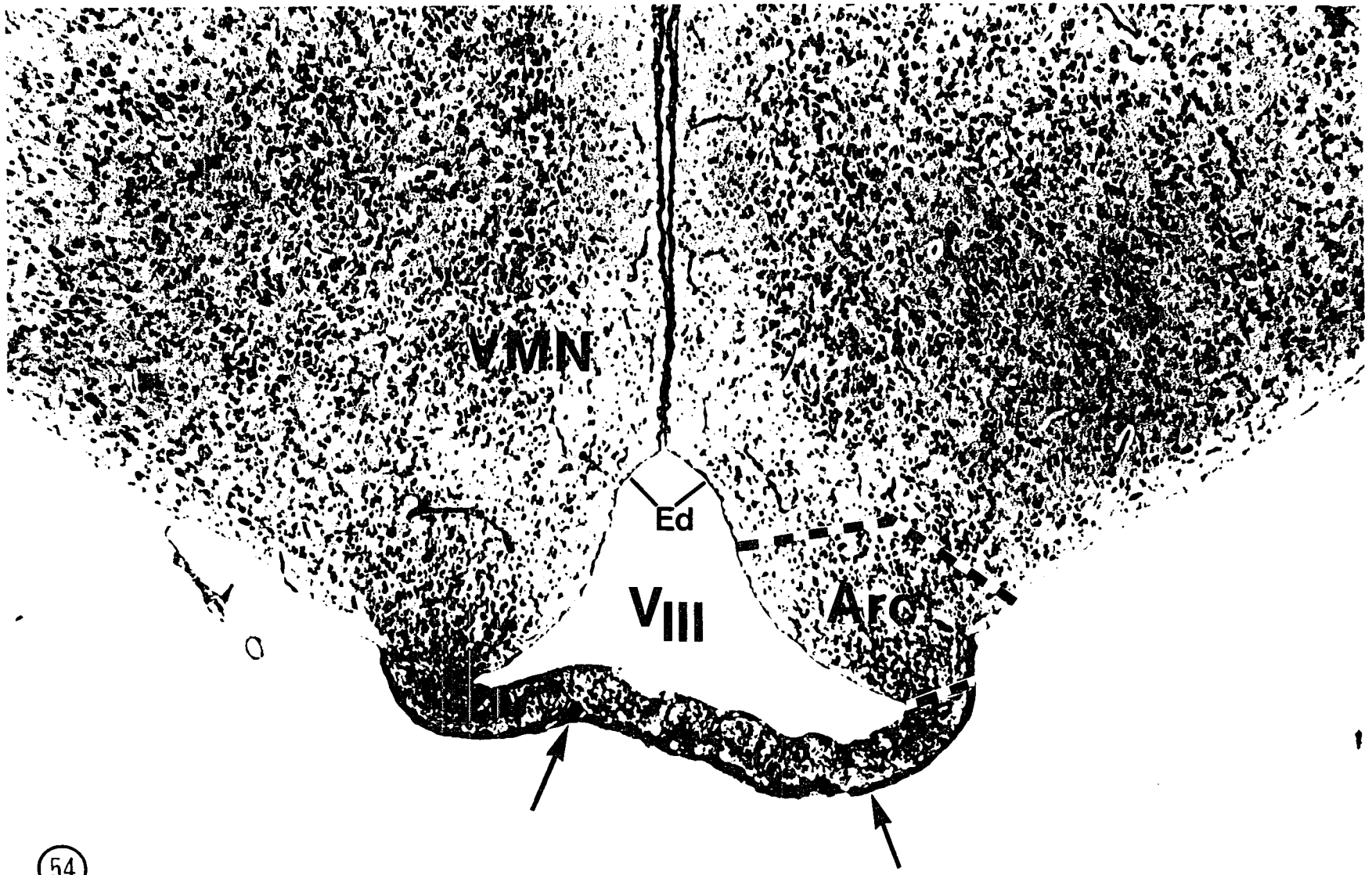
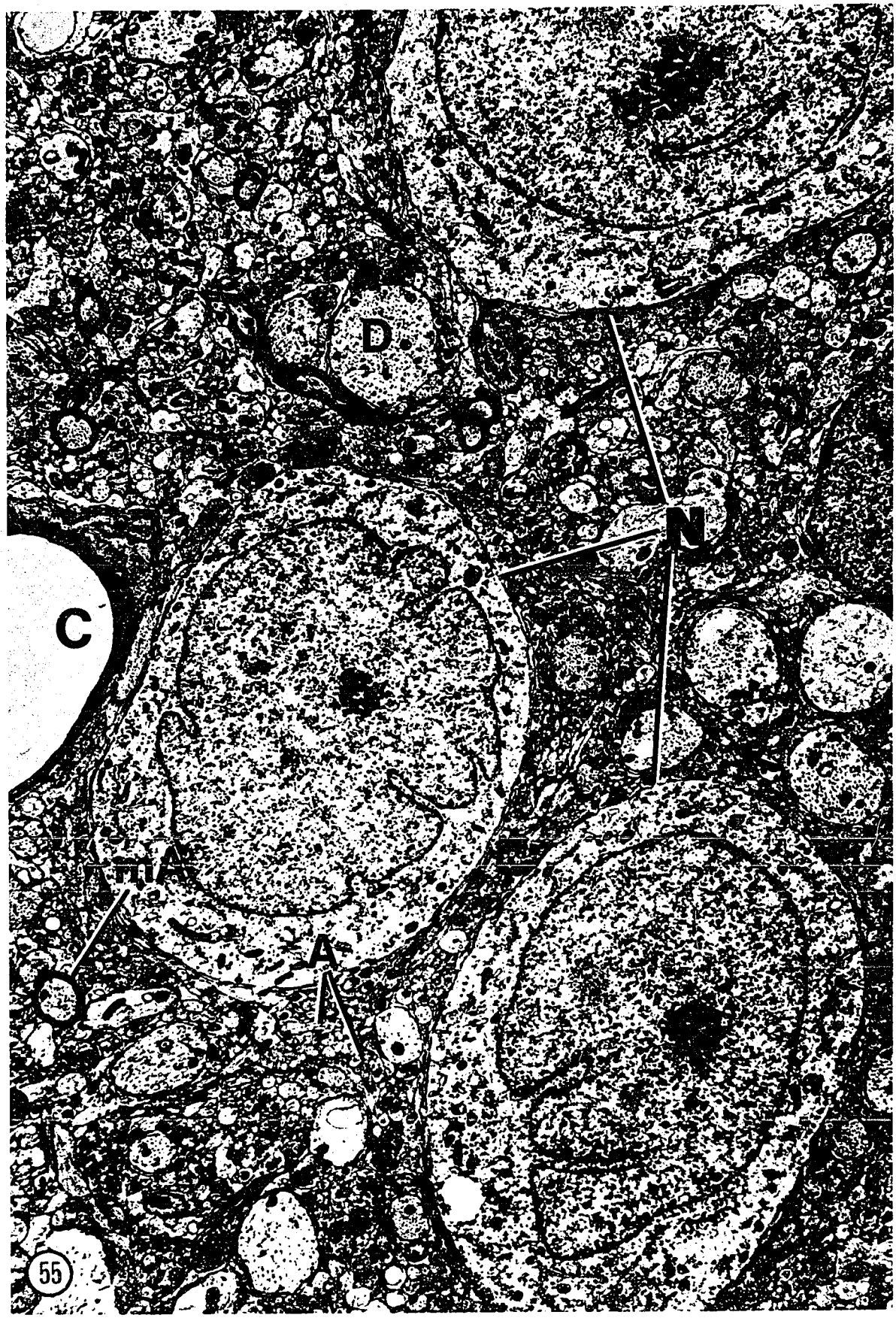

VMN
Ed
VIII
ARC

Figure 55. Electron micrograph showing the general nature and distribution of the cellular components and fibrous processes of the arcuate nucleus - control animal. A number of the neurons are in close spatial relationship with the capillary (C). D, Dendrite; mA, myelinated axon; N, Neuron.

X 7,250


D
N
C
mA
A
55

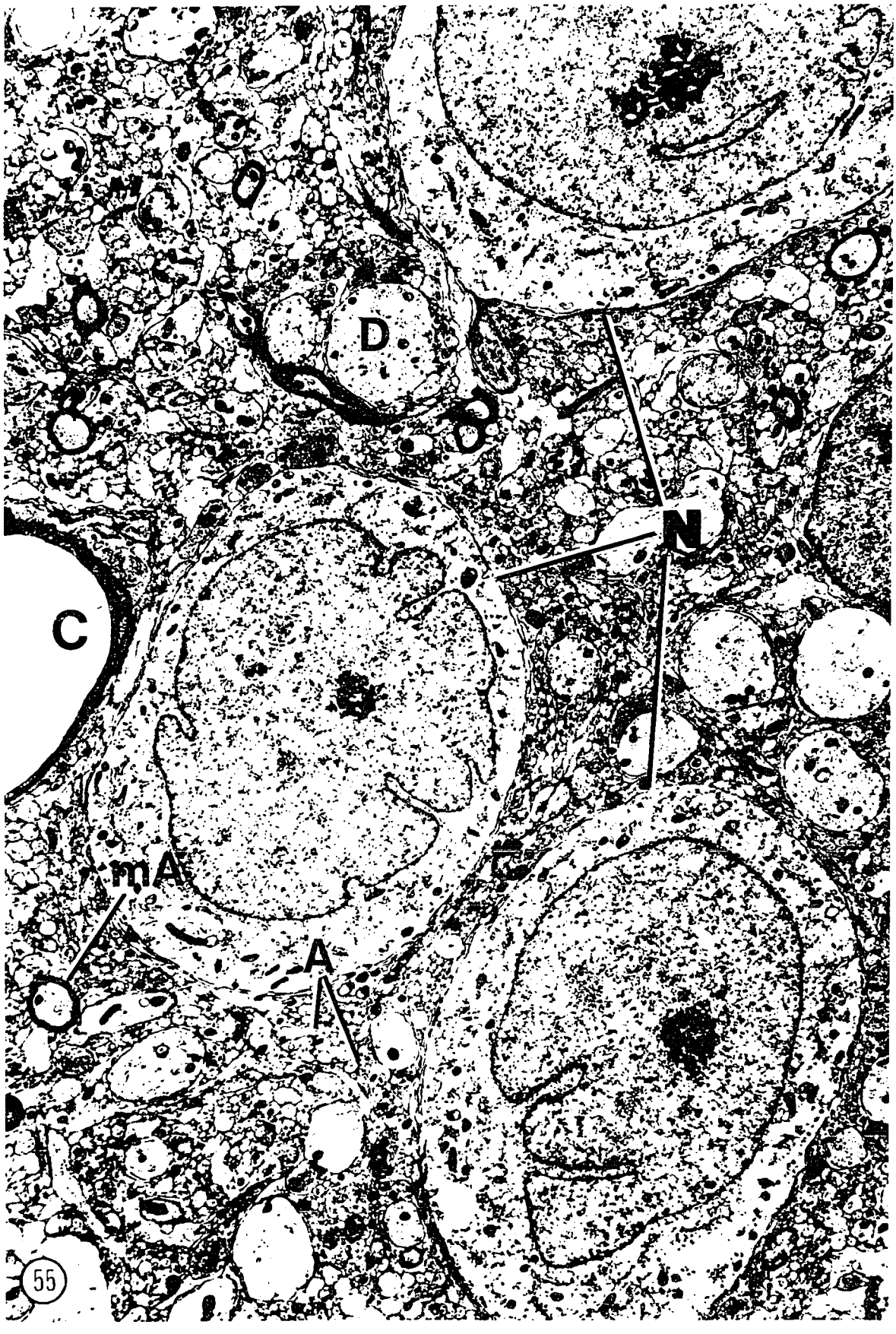
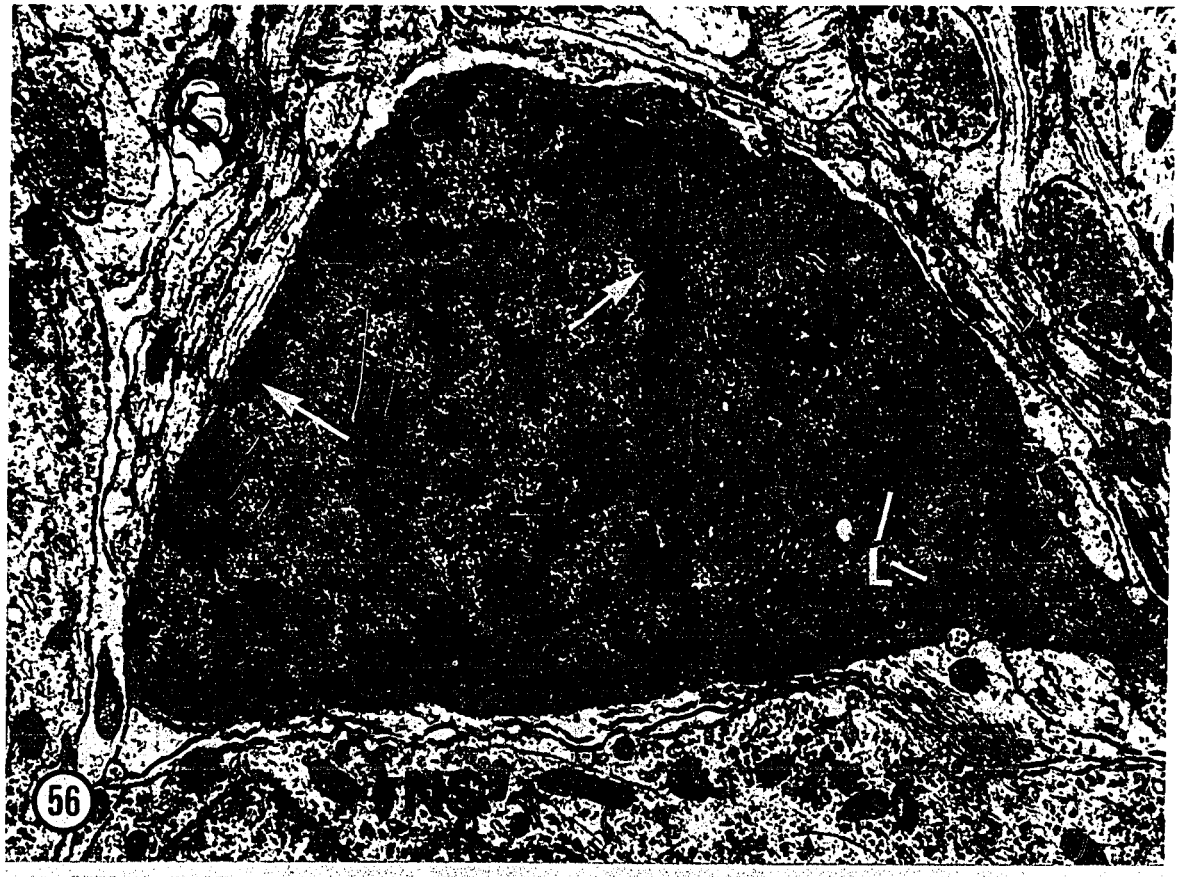

D
N
C
mA
A
55

Figure 56. Field showing a 'satellite' oligodendrocyte in the arcuate nucleus of a control animal. The eccentric nucleus is pyknotic with clumped chromatin (arrows); the cytoplasm is typically dark and granular. NS, Neuron soma; L, Lysosome-like body.

X 17,250

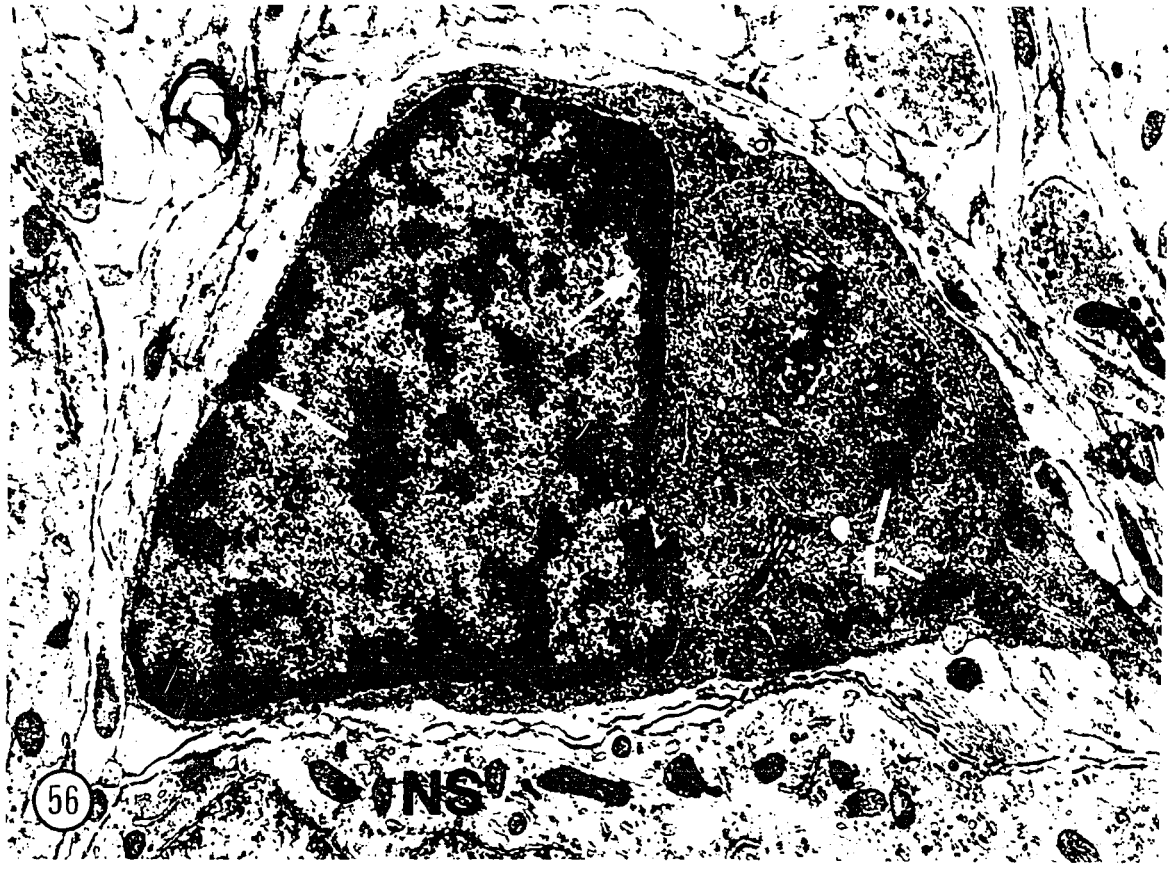
Figure 57. Electron micrograph of a portion of the nucleus (Nu) and perinuclear region of an astrocyte. The cytoplasmic organelles are sparsely represented. Broad bands of fibers (f) are common within the perikarya and processes of these cells. The nucleus is typically vesicular and oval in shape. Arcuate nucleus, experimental group. M, Mitochondria.

X 14,250



56

57


NS
56

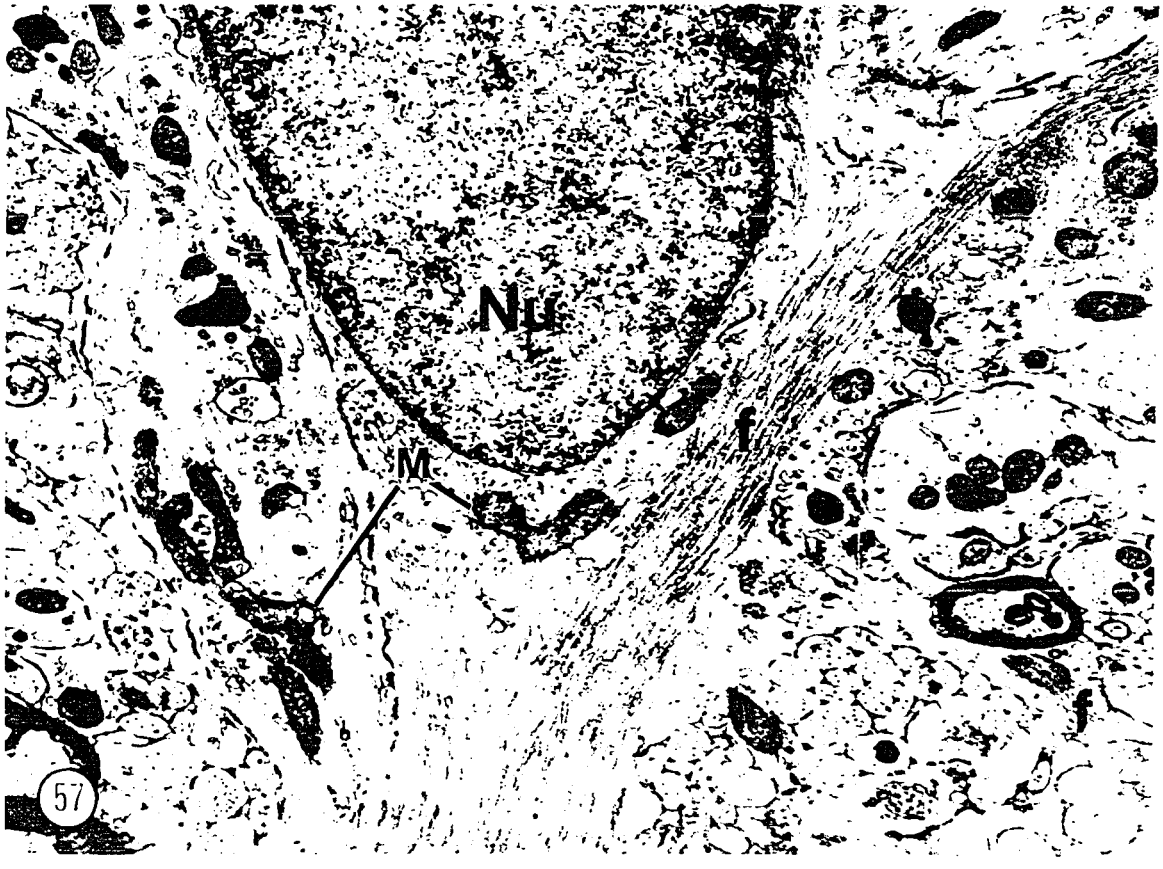
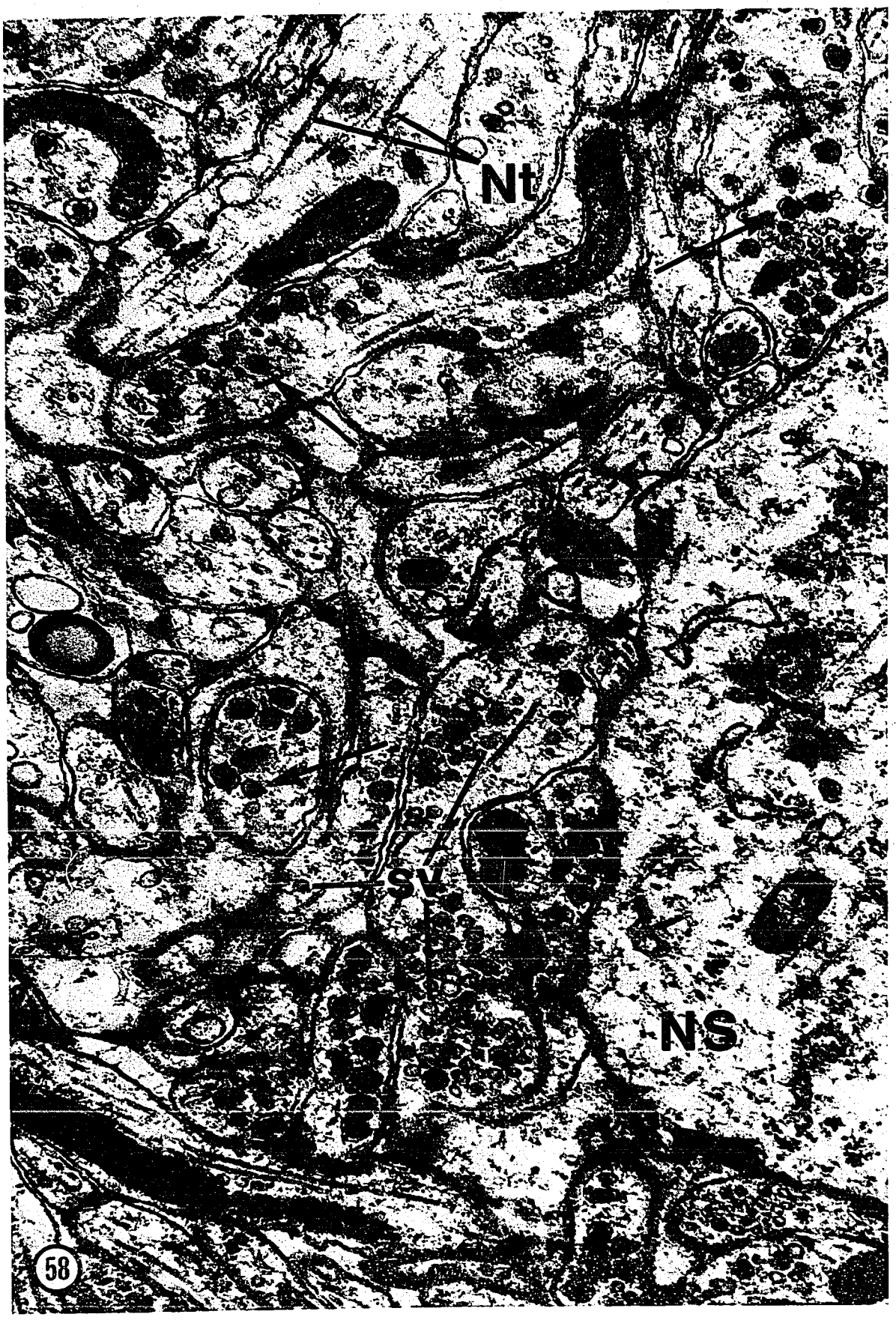
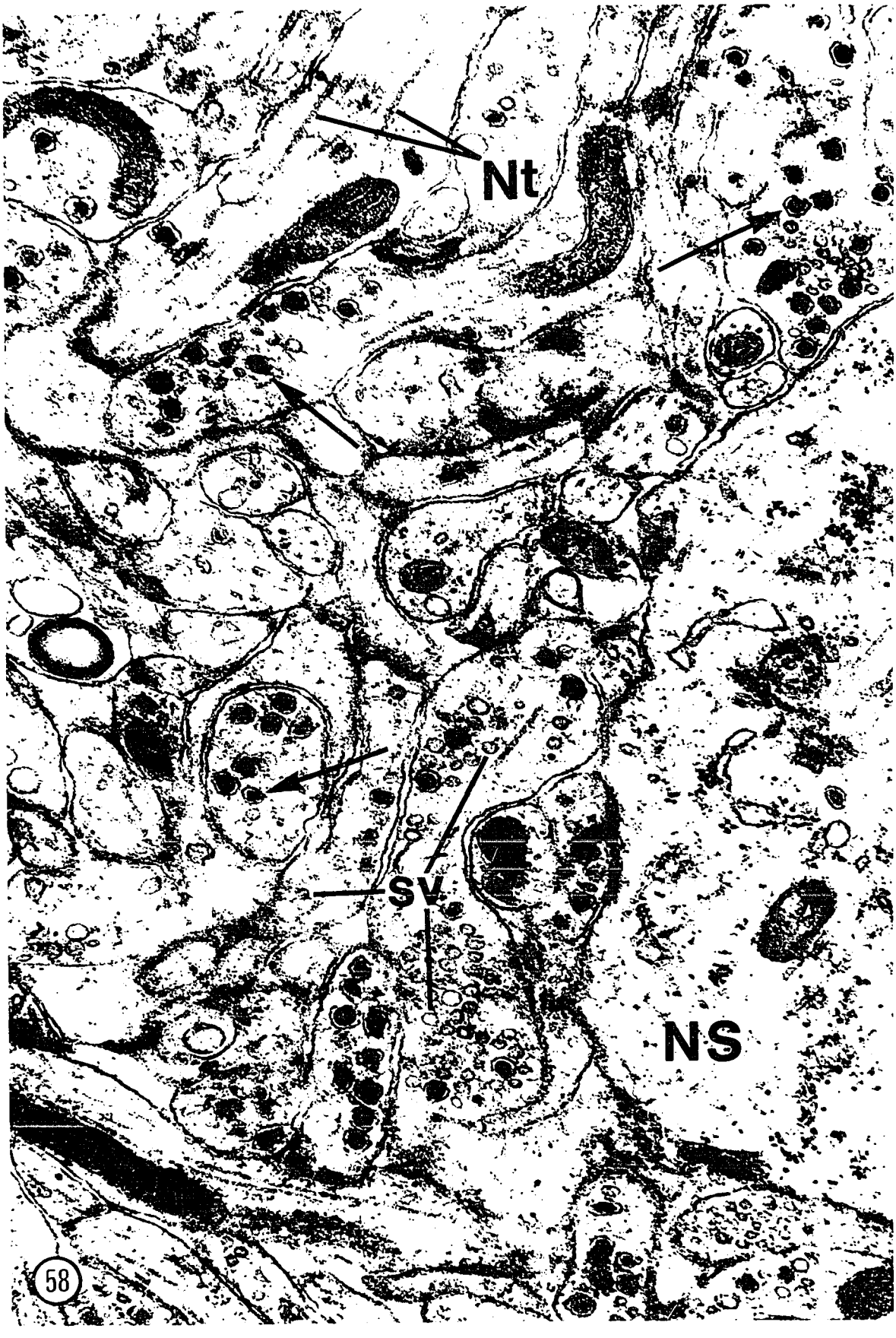

Nu
M
57

Figure 58. Electron micrograph showing an area of the neuropil of the arcuate nucleus - control group. Axon profiles with relatively large numbers of smaller (700 Å - 1150 Å) dense-core vesicles (arrows) were abundant. Neurotubules (Nt) and synaptic-type vesicles (sv) were also common. NS, Neuron soma.


Nt
SV
NS
58

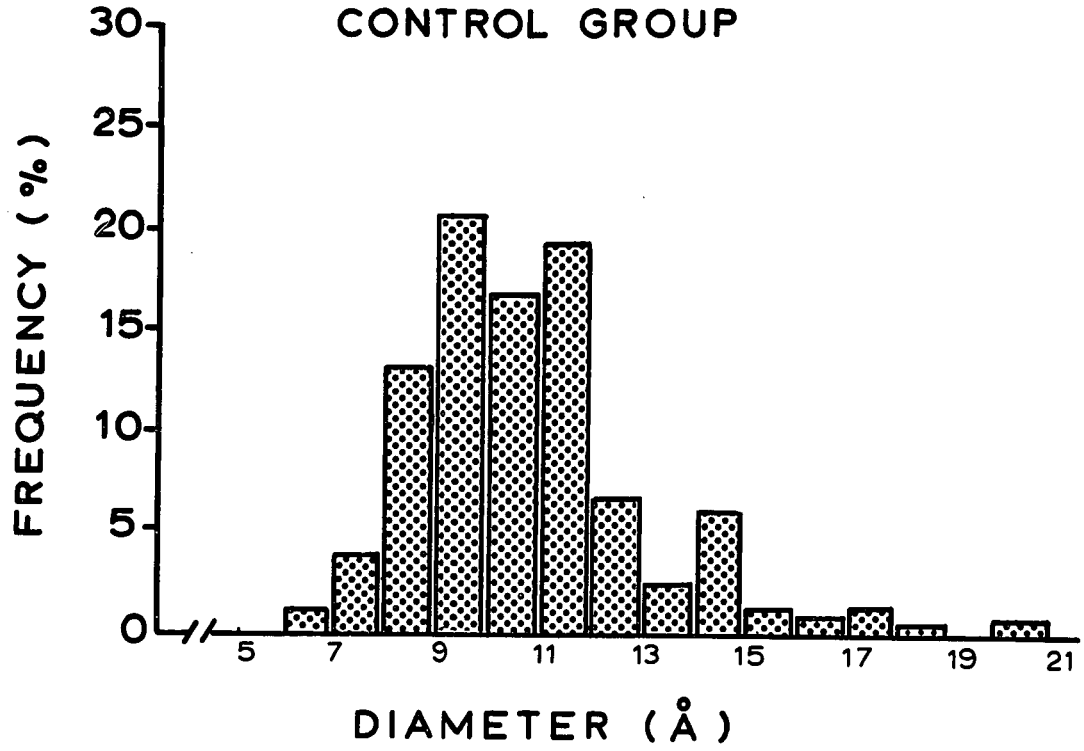

Nt
SV
NS
58

scattered large dendrites. The mean diameter of the DC vesicles observed within the axons of the controls, based on 315 measurements, was 990 ± 9 Å (Figure 13), while that for a similar sample of DC vesicles observed within the axon profiles of the experimental animals was 972 ± 11 Å (Figure 13). The diameter ranges and P values for these measurements are shown in Table 3. The frequency distributions of these diameters are presented graphically in Figure 59. The difference between these means was not significant (Table 3).

Axon terminals forming junctional complexes with the neuron soma (Figure 60) and with the dendritic profiles (Figure 61) were common. Generally, these terminals were characterized by their large numbers of both the small synaptic vesicles with a diameter range of 350 Å to 750 Å, and the larger vesicles with typical electron-dense cores. Terminals containing only the smaller, clear synaptic vesicles were also observed. The DC vesicles within the axon terminals of the controls, based on 315 measurements, had a mean diameter of 933 ± 8 Å (Figure 18). The mean diameter of a similar sample of 318 DC vesicles observed within the axon terminals of the experimental groups was 891 ± 8 Å (Figure 18). A similar diameter range of 500 Å to 1450 Å was recorded for both of these samples (Table 4). Also, as shown in Table 4, there is no significant statistical difference between the means for these two samples of DC vesicles.

The ultrastructural features of capillaries and their relationship with the surrounding neuropil are illustrated in Figures 55 and 62. What appears to be a contact zone between the membranes of

Figure 59. Histograms showing the frequency distribution of the dense-core vesicles measured within the axons of the arcuate nuclei of both the control and experimental animals. The two histograms are almost identical.


CONTROL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
21
DIAMETER (Å)

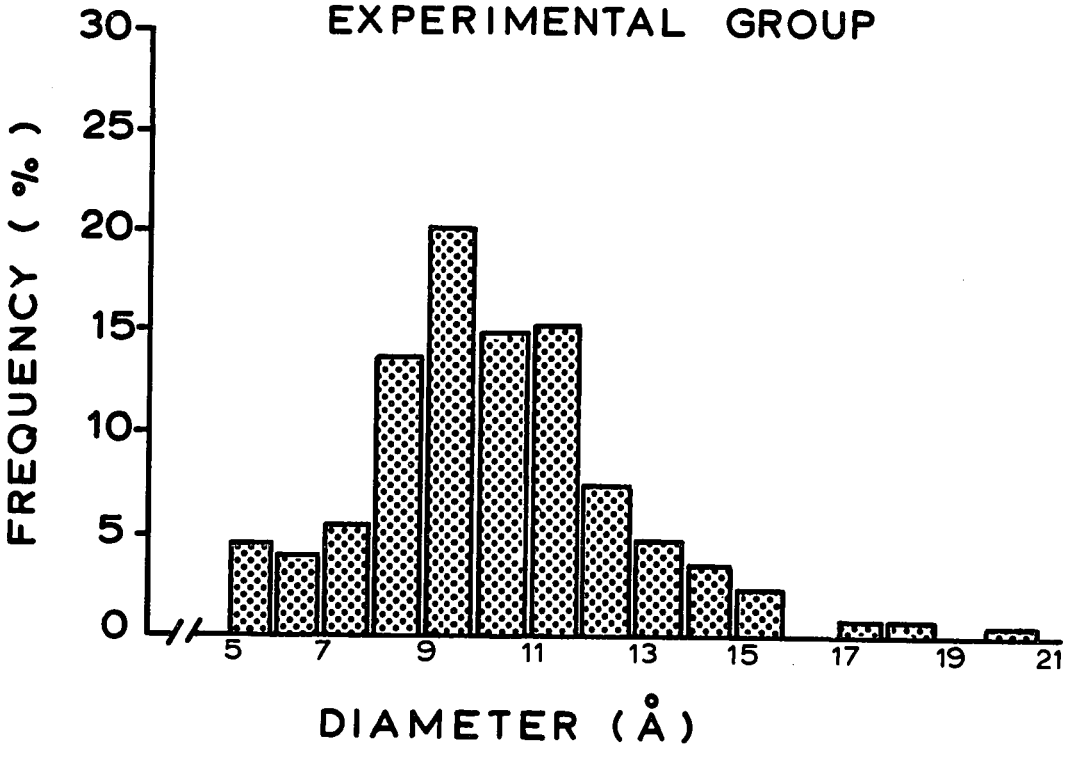

EXPERIMENTAL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
21
DIAMETER (Å)

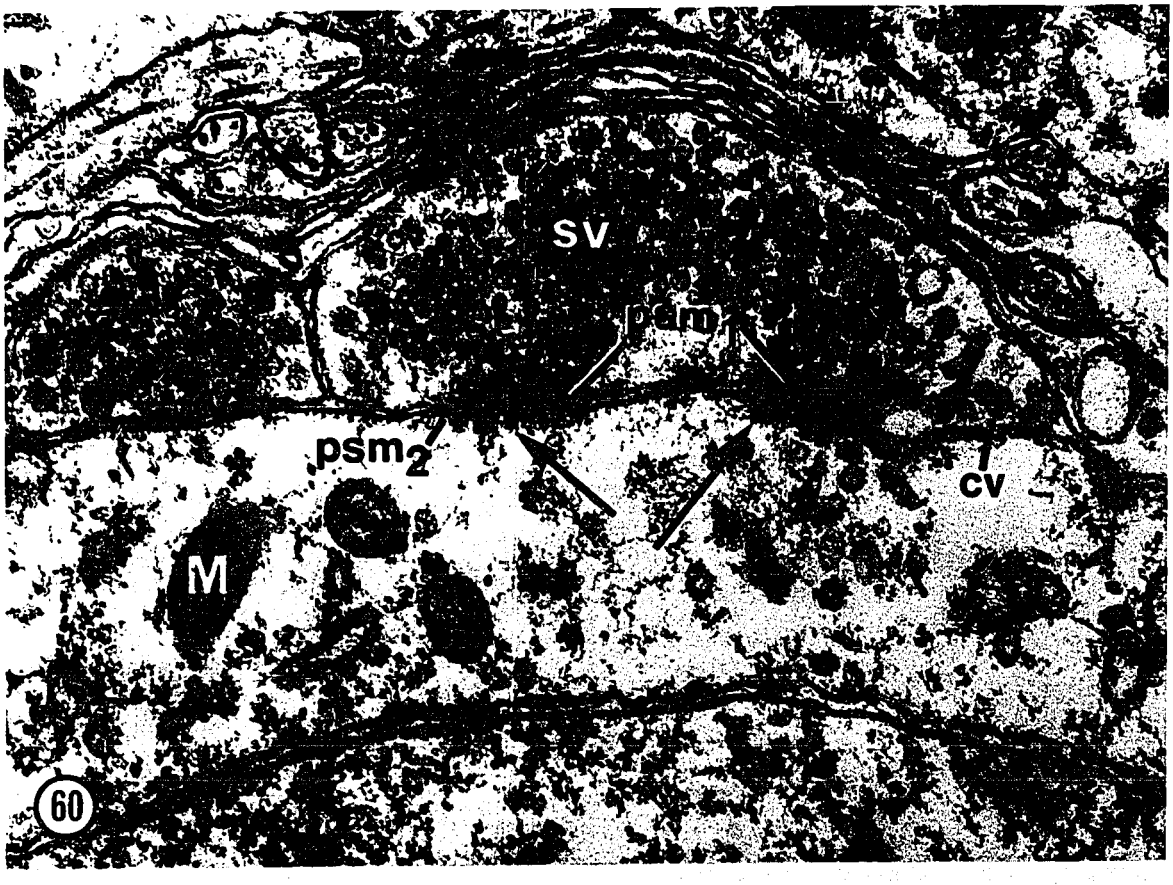
Figure 59

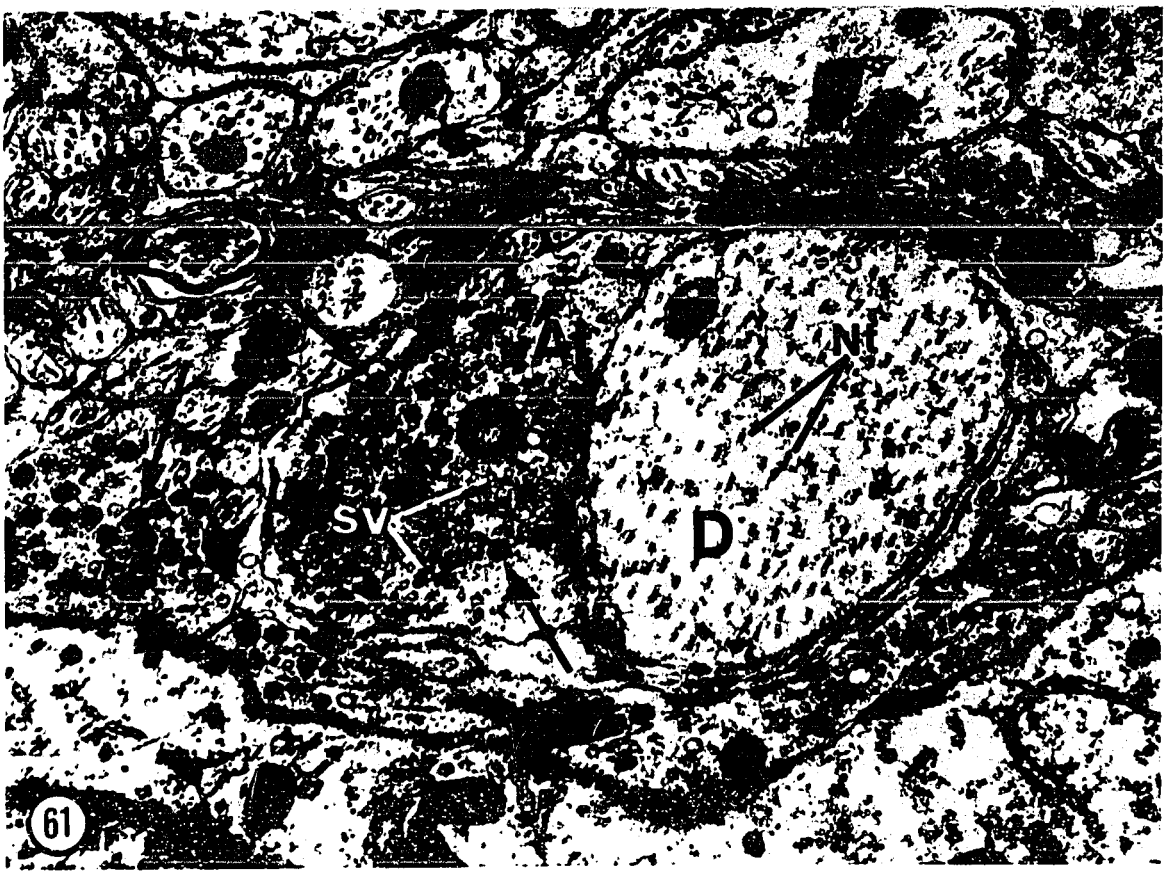
Figure 60. Electron micrograph of an axo-somatic junction within the arcuate nucleus of a control animal. Synaptic vesicles (sv) were abundant and approach the presynaptic membrane (psm_1) at the junctional zones. Coated vesicles (cv) and larger dense-core vesicles were less numerous. A thick electron-dense "plaque" occurs under the postsynaptic membrane (psm_2). The button-like condensations (arrows) were a common finding. M, Mitochondria.

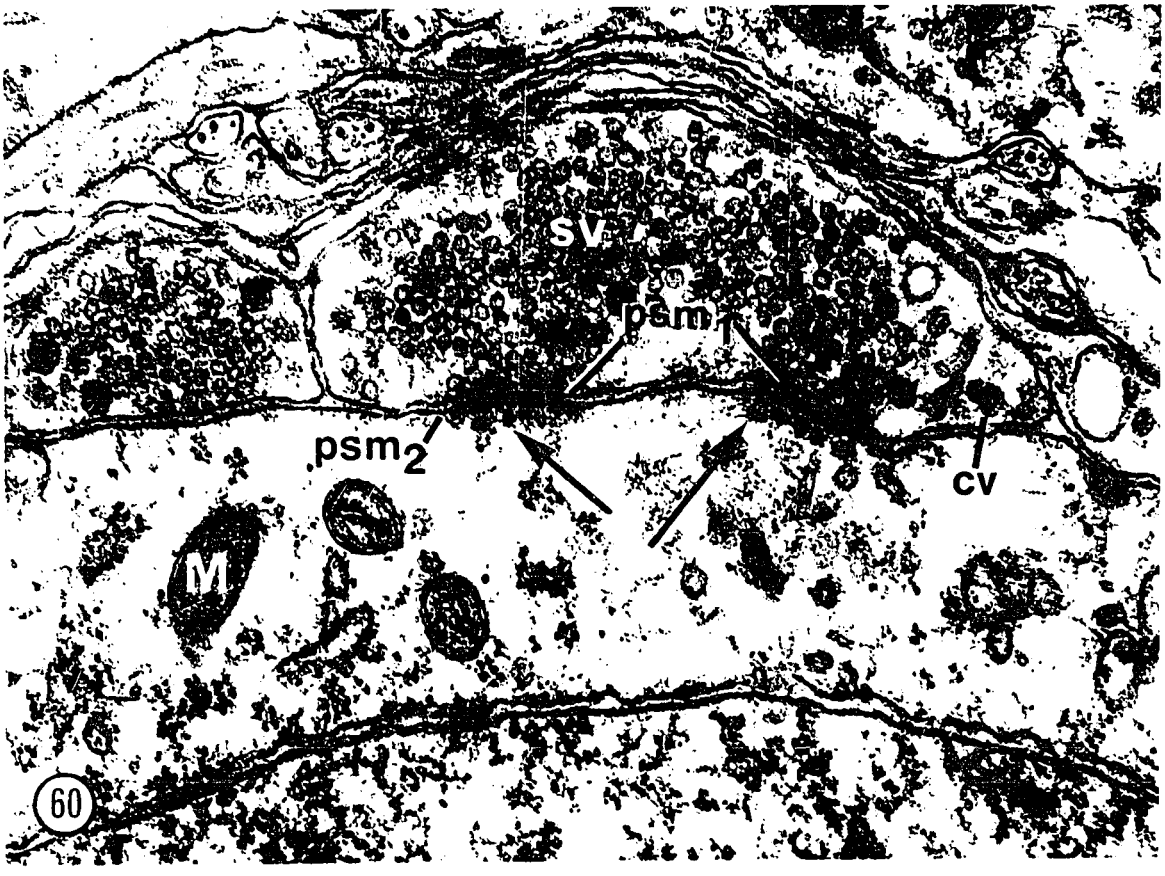
X 44,200

Figure 61. Electron micrograph showing an axo-dendritic junction within the arcuate nucleus of an experimental animal. Synaptic vesicles (sv) and small dense-core vesicles (arrows) are shown in the axon terminal (At). Neurotubules (Nt) were uniformly spaced within the dendrite (D).

X 28,200


SV
psm
psm2
cv
M
60


A
N
SV
P
61


SV
psm1
psm2
cv
M
60

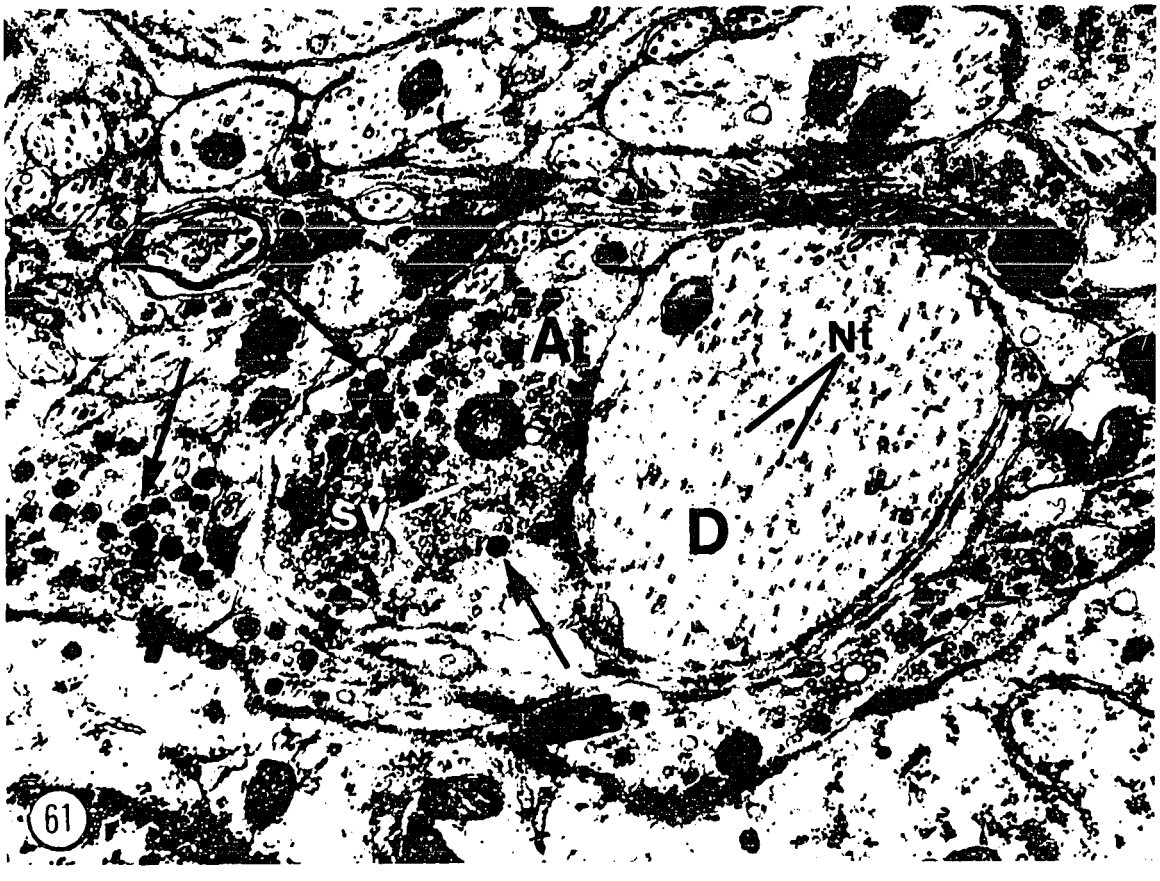
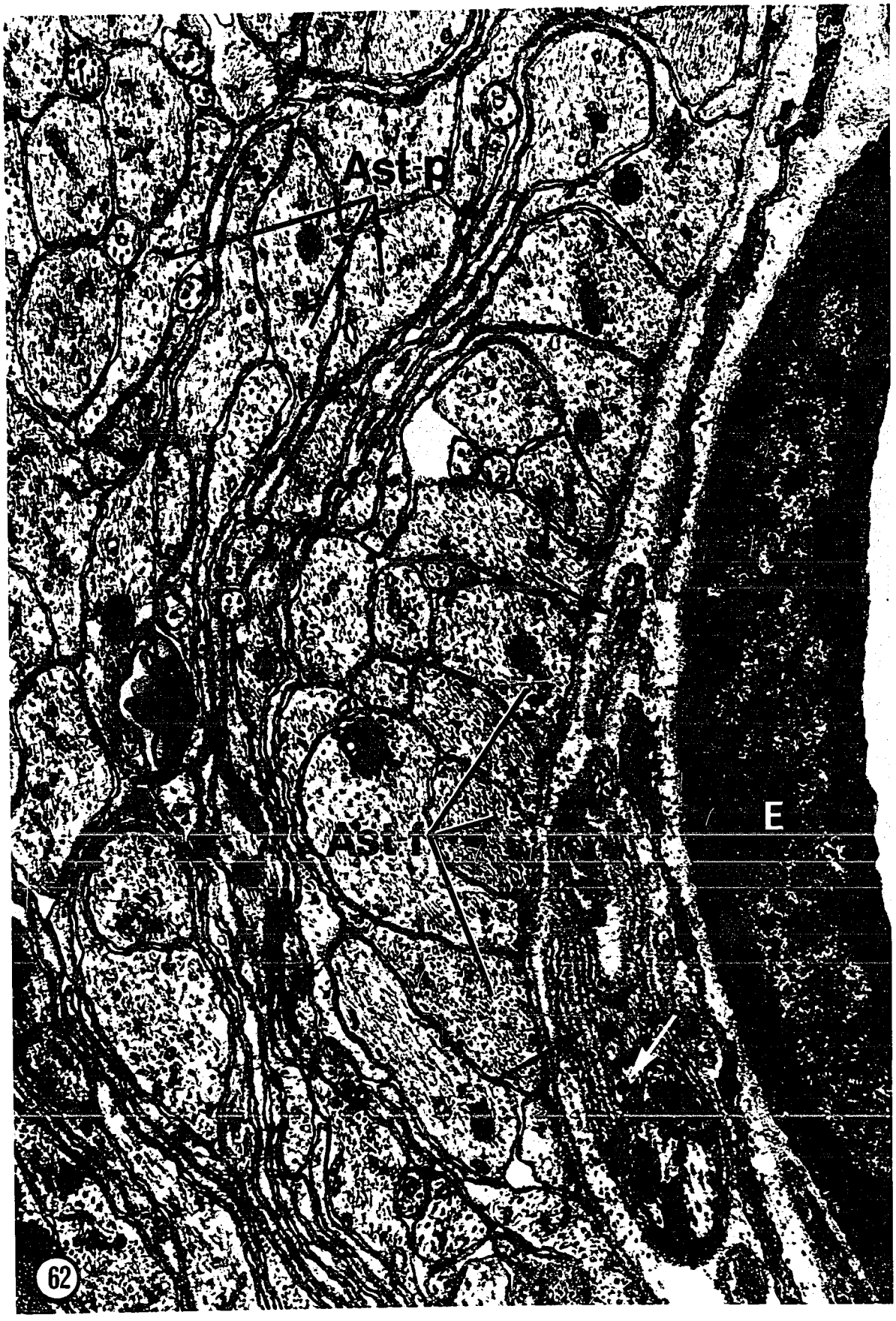
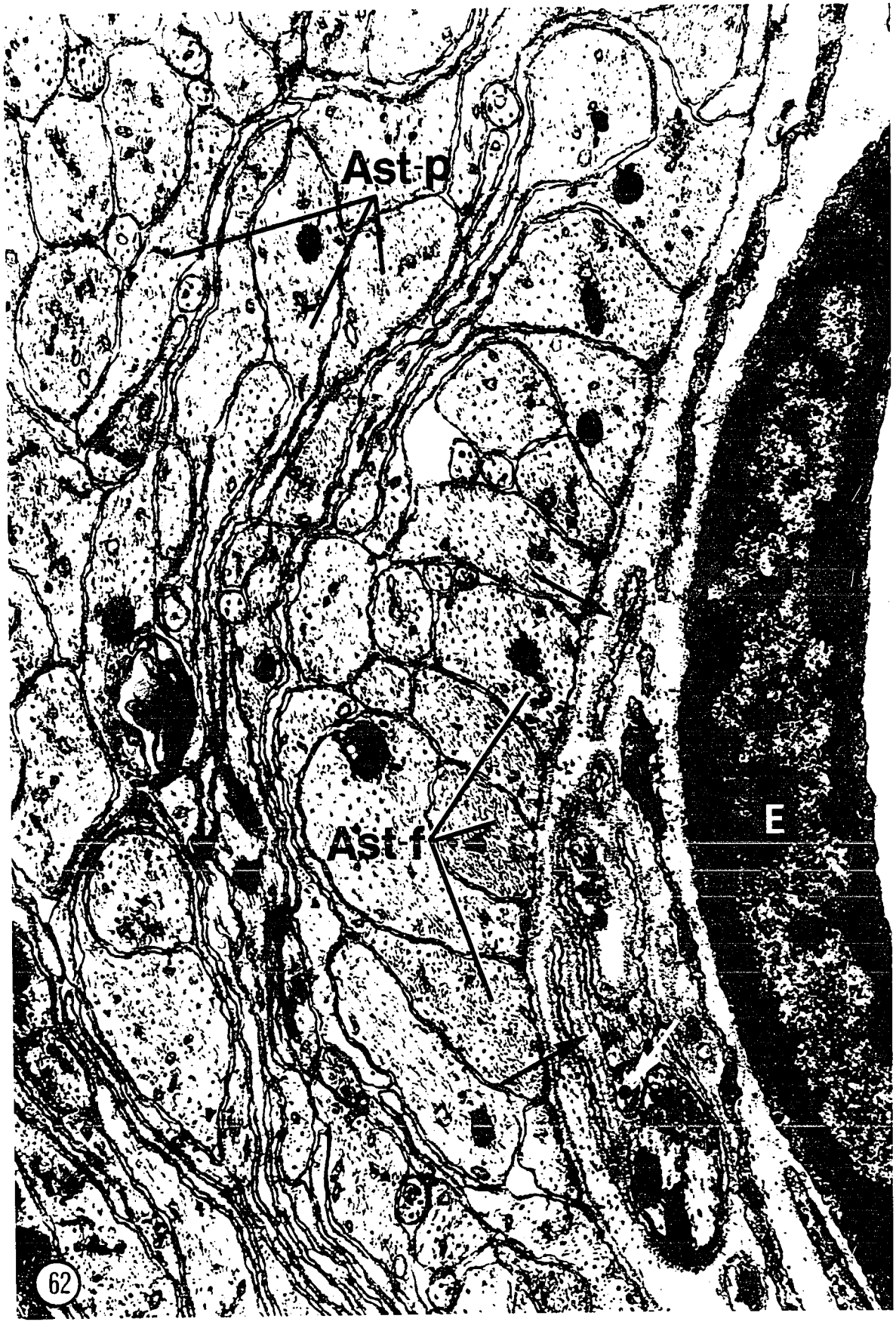

At
Nt
SV
D
61

Figure 62. Electron micrograph showing portion of a capillary wall, perivascular space and nature of the surrounding neuropil. Axon profiles containing the occasional small dense-core vesicles (white arrow) were observed within the perivascular space. These processes are surrounded by the basal lamine (black arrow). The surrounding brain parenchyma or neuropil is composed primarily of astrocytic processes (Ast-p). Arcuate nucleus, control animal. E, Endothelial cell; Ast-f, Astrocytic end-feet.

X 27,500


Ast-B
E
Ast-I
62


Ast-p
Ast-f
E
62

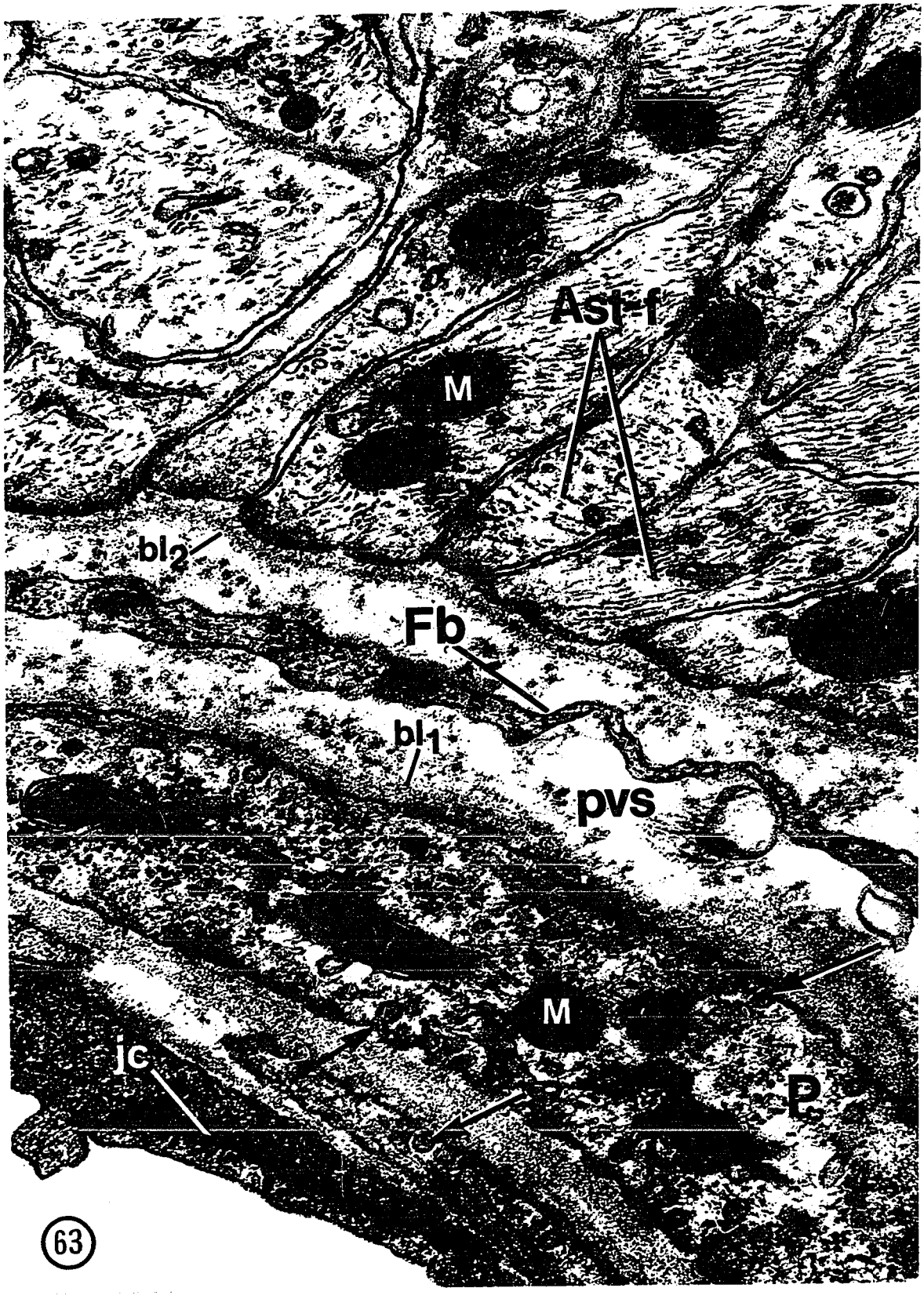
the endothelial cells of a capillary, a portion of a pericyte showing many pinocytotic vesicles, as well as the nature and arrangement of the astrocytic end-feet around the perivascular space, are featured in Figure 63. Both the inner and outer surfaces of the pericytes were typically coated by the basal lamina. A similar coating of fine granular material also occurred in the perivascular space adjacent to the astrocytic end-feet. Occasionally, axon profiles were observed within the perivascular spaces (Figure 62). These profiles, without exception, were also surrounded by the basal lamina. The brain parenchyma around the perivascular spaces was composed chiefly of astrocytic processes (Figures 62 and 63). However, tight junctions were generally less common here than between similar processes which surrounded the capillaries of the PO nucleus (compare Figures 15 and 62).

2) Ultrastructure of arcuate neurons of the control animals

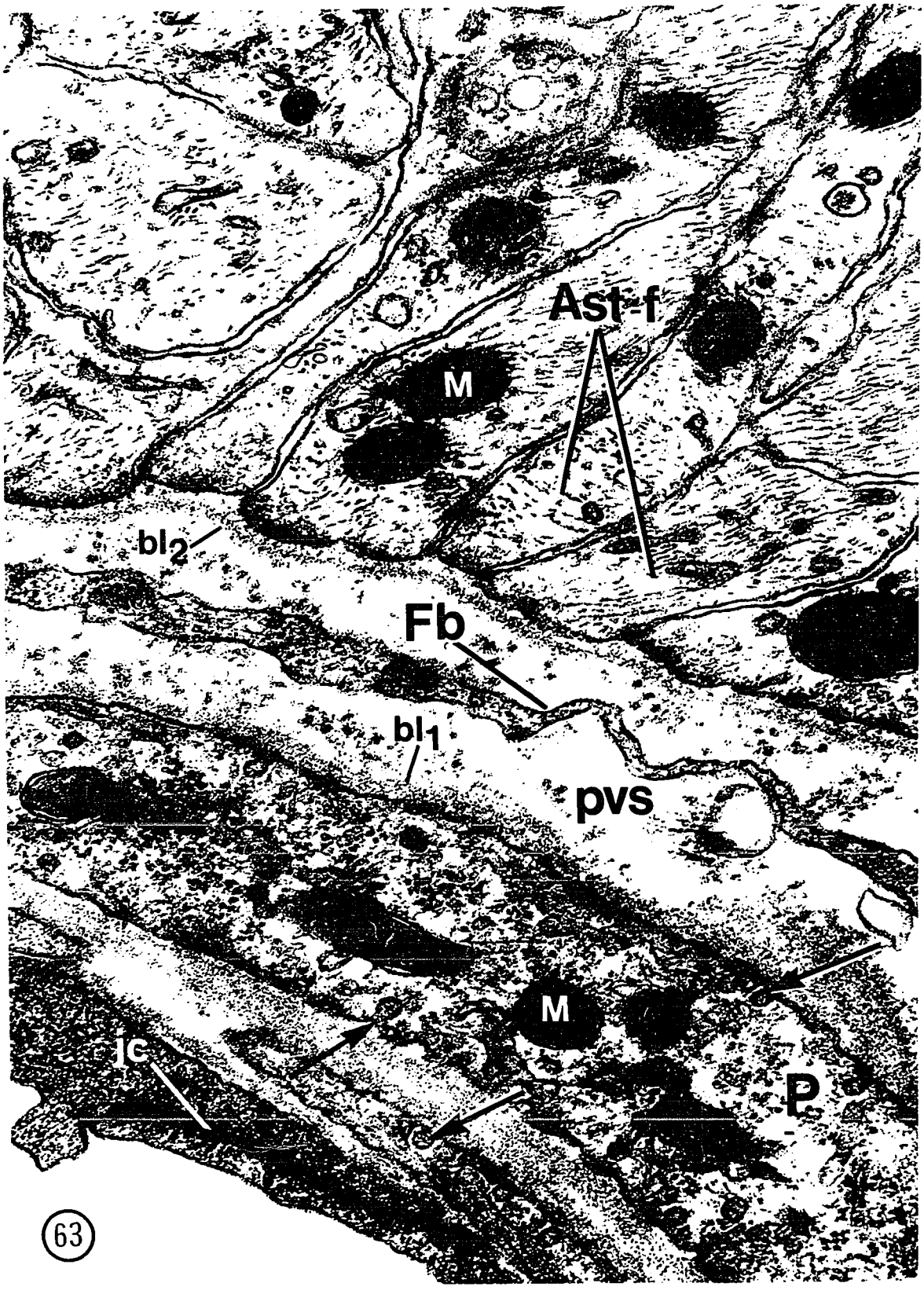
The compact distribution of the neurons, their relatively large size and spherical shape are shown in Figures 55 and 64. What appears to be two types of neurons on the basis of certain morphological parameters, have been identified within the arcuate nuclei of both the control and experimental groups (Figures 64 and 65). The morphological features of a neuron of the non-mated control animals, and the frequently observed close spatial relationship between the neurons and the capillaries of the arcuate nucleus are shown in Figure 66. These neurons had a mean diameter of 13.2 ± 0.2 μ (Figure 8). The distribution of the diameters recorded for these neurons is shown in Figure 67.

Figure 63. High magnification electron micrograph showing a small area of the capillary wall and surrounding brain tissue. A junctional complex (jc) is shown between the processes of two endothelial cells. The cytoplasm of the pericyte (P) is generally paler than that of the ependymal cells. Note the numerous pinocytotic vesicles (arrows) in these cells. Fibroblasts (Fb) occur within the perivascular space (pvs). A thick basal lamina (bl_2) separated the perivascular space from the astrocytic end-feet.(Ast-f). The protoplasm under the free surface of these processes appears condensed. Arcuate nucleus, experimental group. bl_1, perivascular basal lamina; M, Mitochondrion.

X 58,200



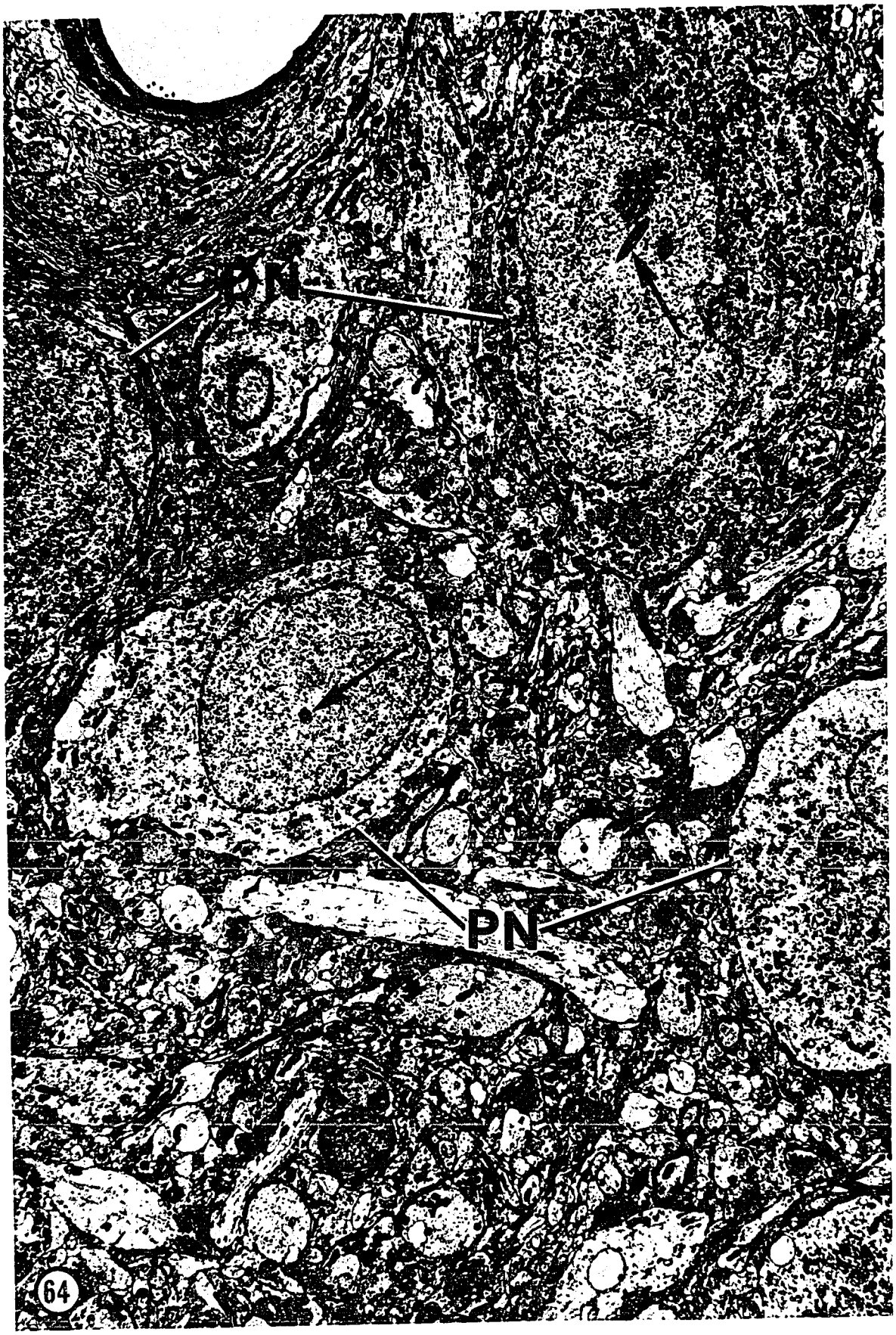
63

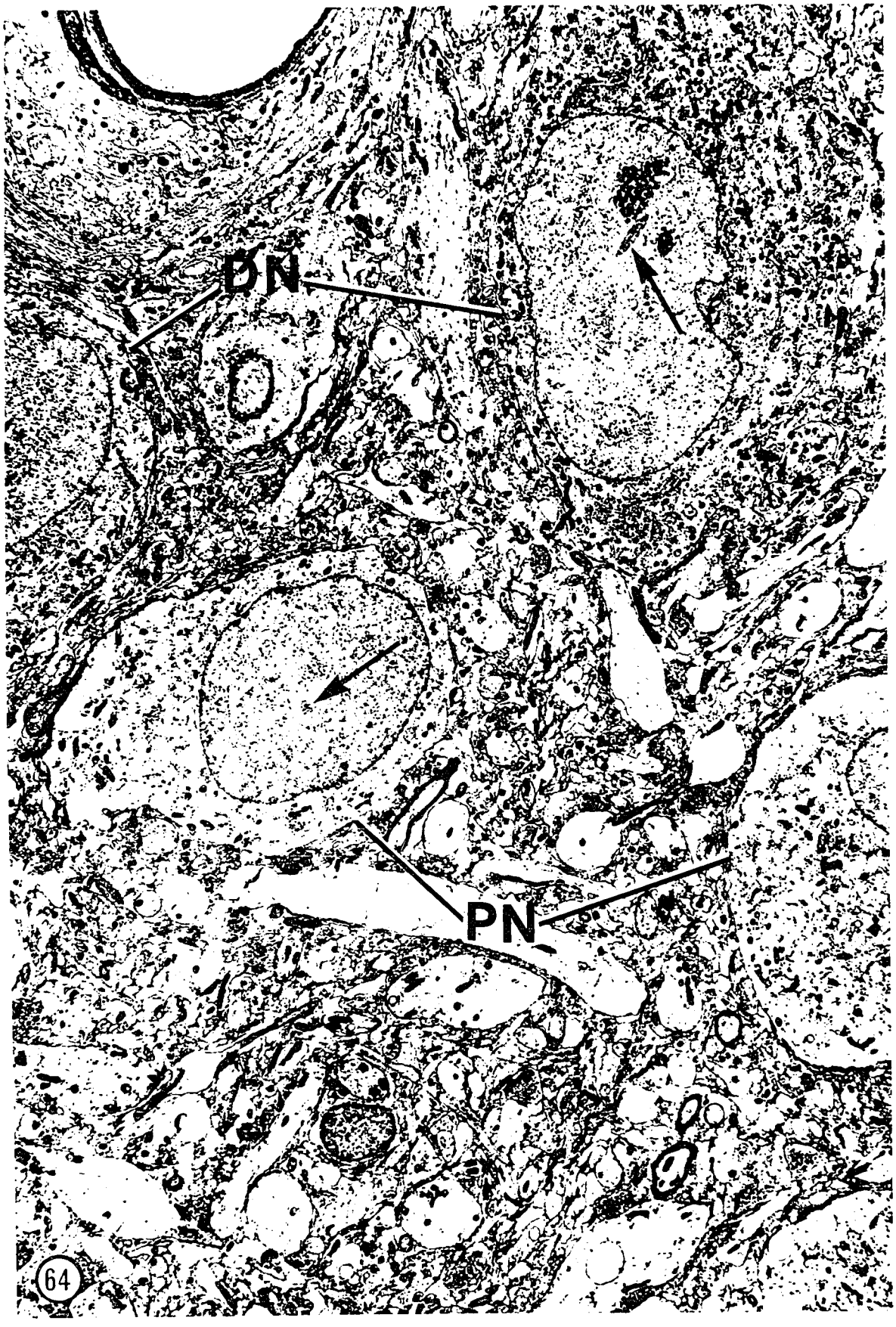


63

Figure 64. Electron micrograph showing the general nature and distribution of the neurons of the arcuate nucleus, and their frequently observed close spatial relationship with a capillary. Two types of neurons, 'dark' (DN) and 'pale' (PN) were identified on the basis of certain morphological criteria. Control animal.

X 3,600

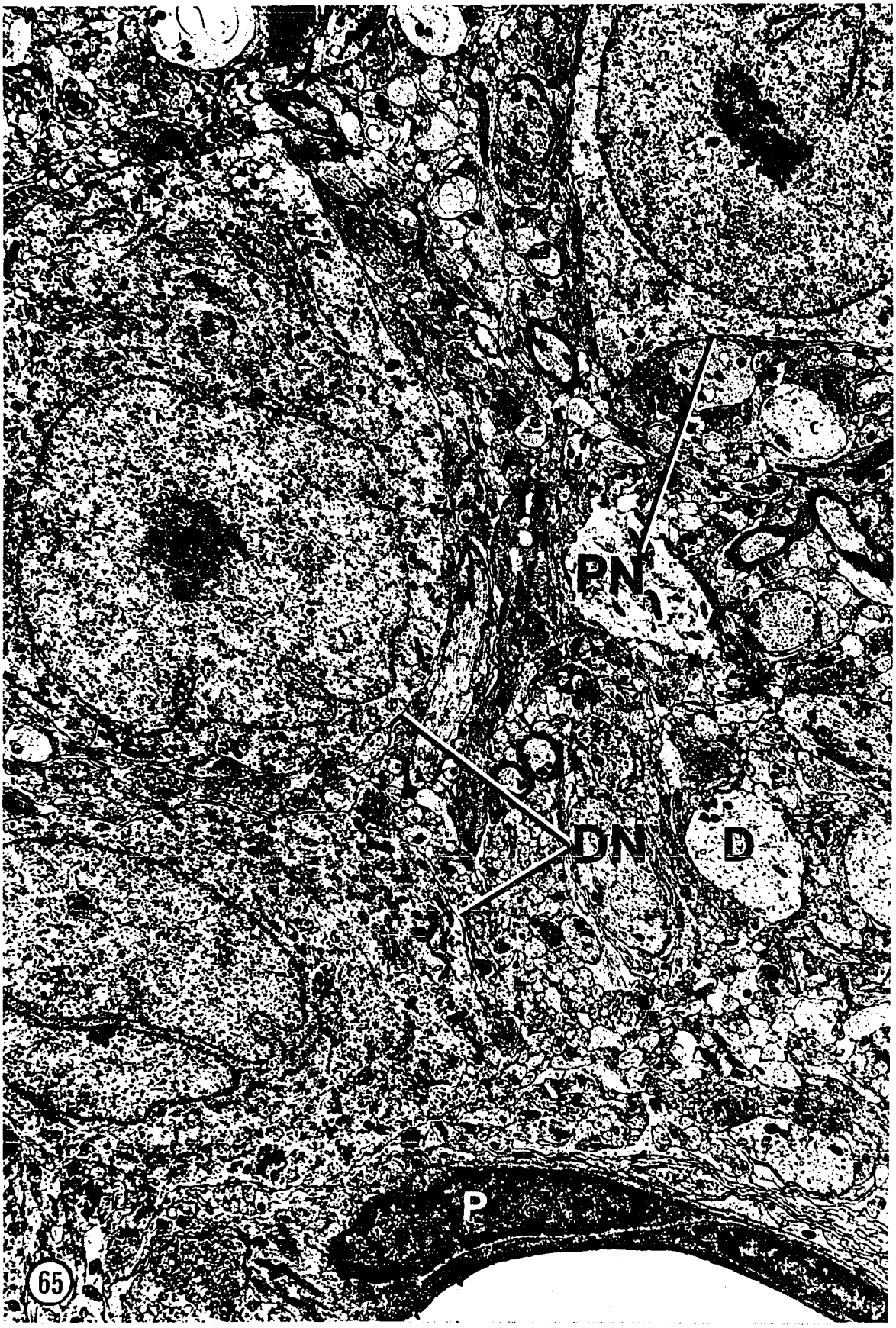

PN
PN
64

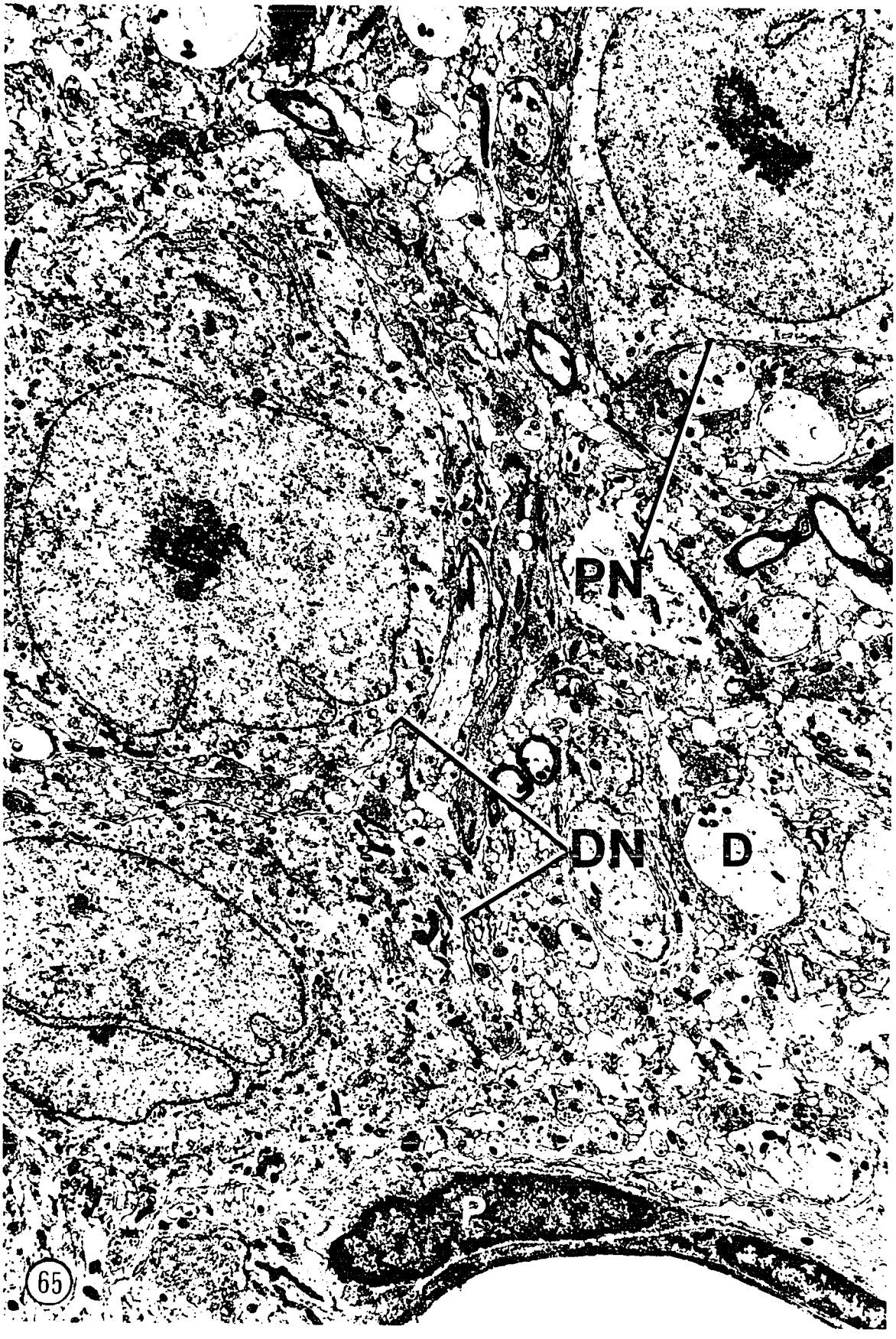


64

Figure 65. Electron micrograph showing portions of the perinuclear zones of two 'dark' neurons (DN) and one 'pale' neuron (PN) of the arcuate nucleus removed from an experimental animal. D, Dendrite; P, Pericyte.

X 6,400

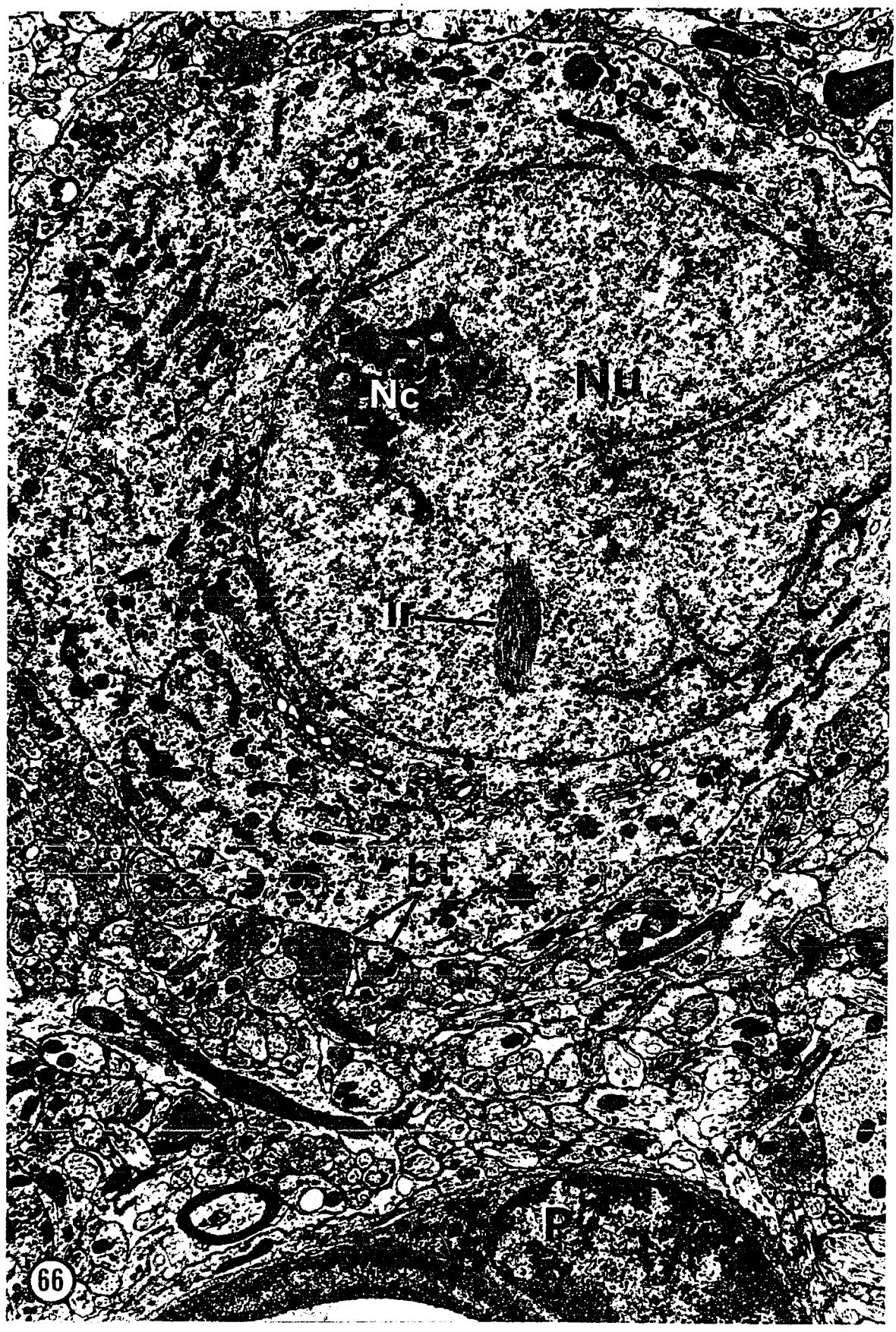

PN
DN
D
P
65



65

Figure 66. Electron micrograph showing the ultrastructural features of a large arcuate neuron removed from a non-mated control animal. The neuron is typically large, oval to spheroid in shape, and with a large eccentric, vesicular nucleus (Nu). The nucleolus (Nc) is in contact with the nuclear envelope (arrow). Dense populations of cytoplasmic organelles characterize the perinuclear region. Bouton termineau (bt) or axo-somatic junctions are numerous. P, Pericyte; ir, intranuclear rodlet.

X 9,700



66

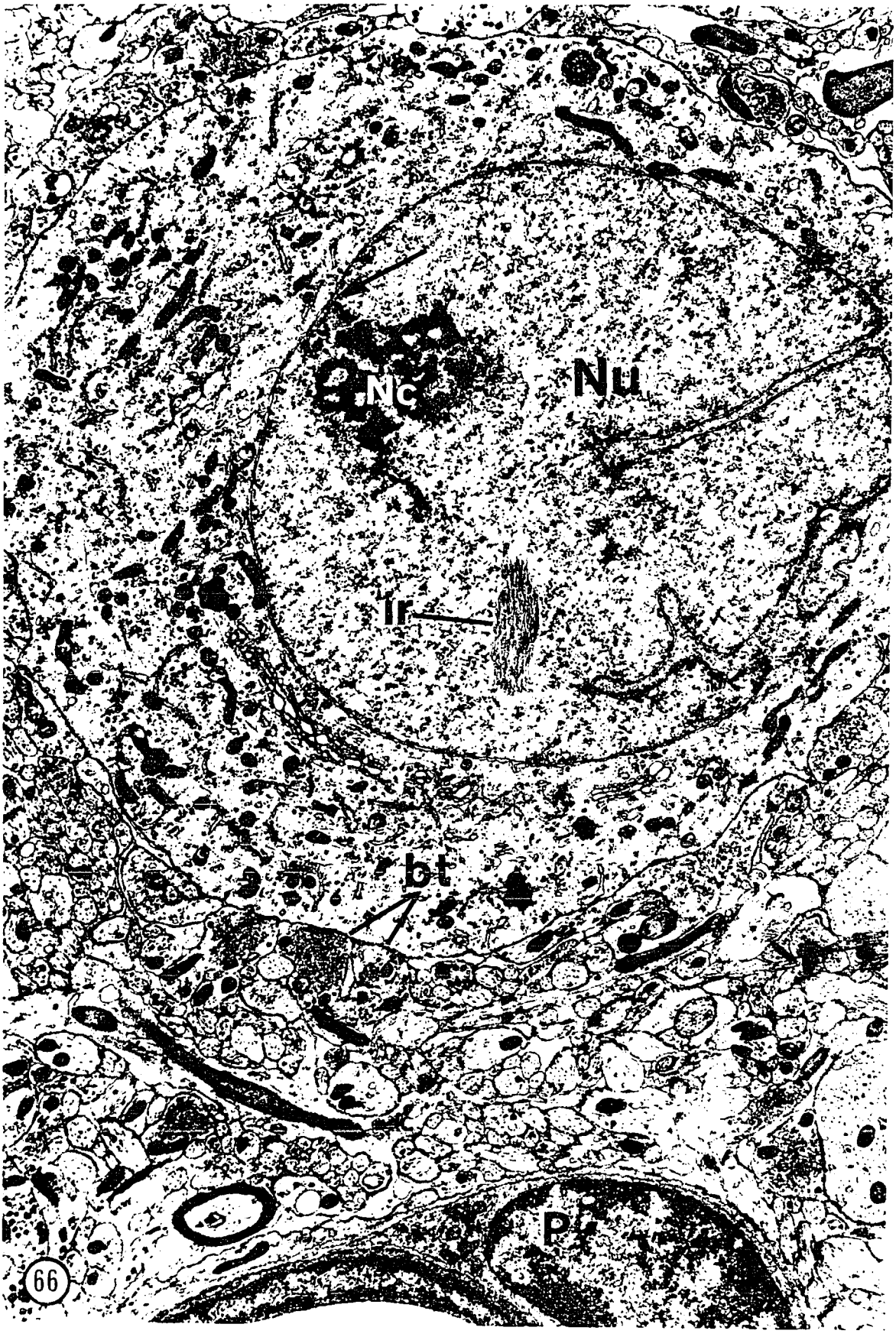

NC
Nu
Ir
bt
P
66

Figure 67. Distribution curves of the measurements made of the arcuate neurons and their nuclear profiles within the control and experimental groups: controls - neurons (—·—x—·—), nuclei (— — o — —); experimental animals - neurons (——●——), nuclei (——▲——). Note that there is no difference in the distribution of these measurements for the non-mated controls and the mated experimental animals.

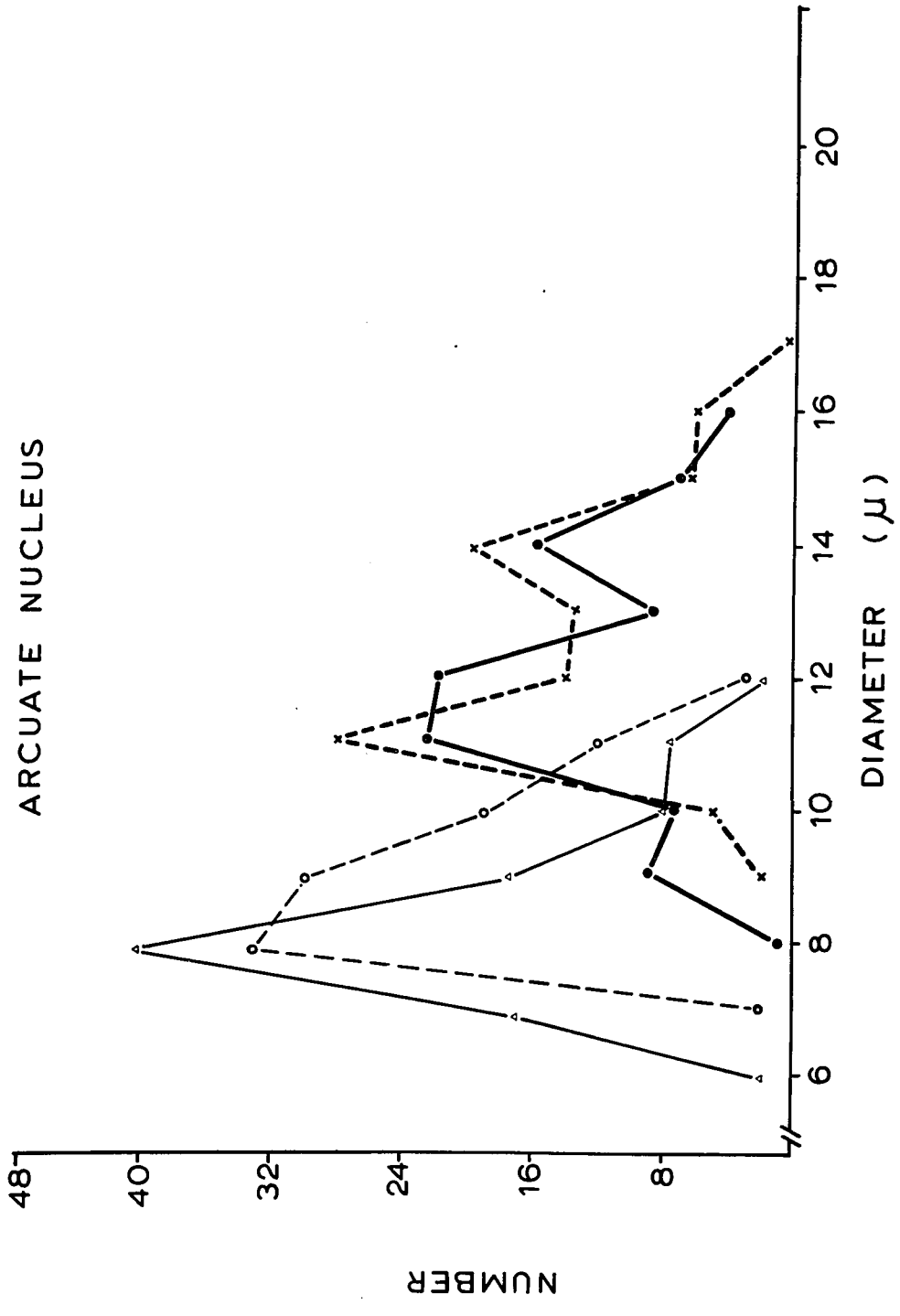

ARCUATE NUCLEUS
NUMBER
48
40
32
24
16
8
6
8
10
12
14
16
18
20
DIAMETER (μ)

Figure 67

The large spherical to ovoid nuclei were typically eccentric, with one or more deep nuclear clefts (Figure 66). The vesicular karyoplasm frequently contained a single nucleolus which was free or in contact with the nuclear envelope. Intranuclear rod-like structures of the type shown in Figure 66 were common components of these neurons. The nuclear profiles of the 100 neurons measured near capillaries had a mean diameter of 9.4 ± 0.1 μ (Figure 9) and a range of 7.0 μ to 12.0 μ (Table 2). A distribution curve of these measurements is also included in Figure 67.

The cytoplasm of many neurons of the control animals was characterized by the presence of rather dense populations of mitochondria and lysosome-like bodies, as well as the presence of relatively well developed rough ER and Golgi systems (Figures 68 and 69). Numerous free ribosomes distributed throughout the cytoplasm also gave these neurons a characteristic granular appearance. In other neurons of the arcuate nucleus, these organelles were very sparsely represented, leaving the cell with a comparatively light, agranular appearance (Figure 70). These morphological differences provided the ultrastructural basis for the identification of the two neuron types; namely, 'dark' and 'pale', within this hypothalamic nucleus.

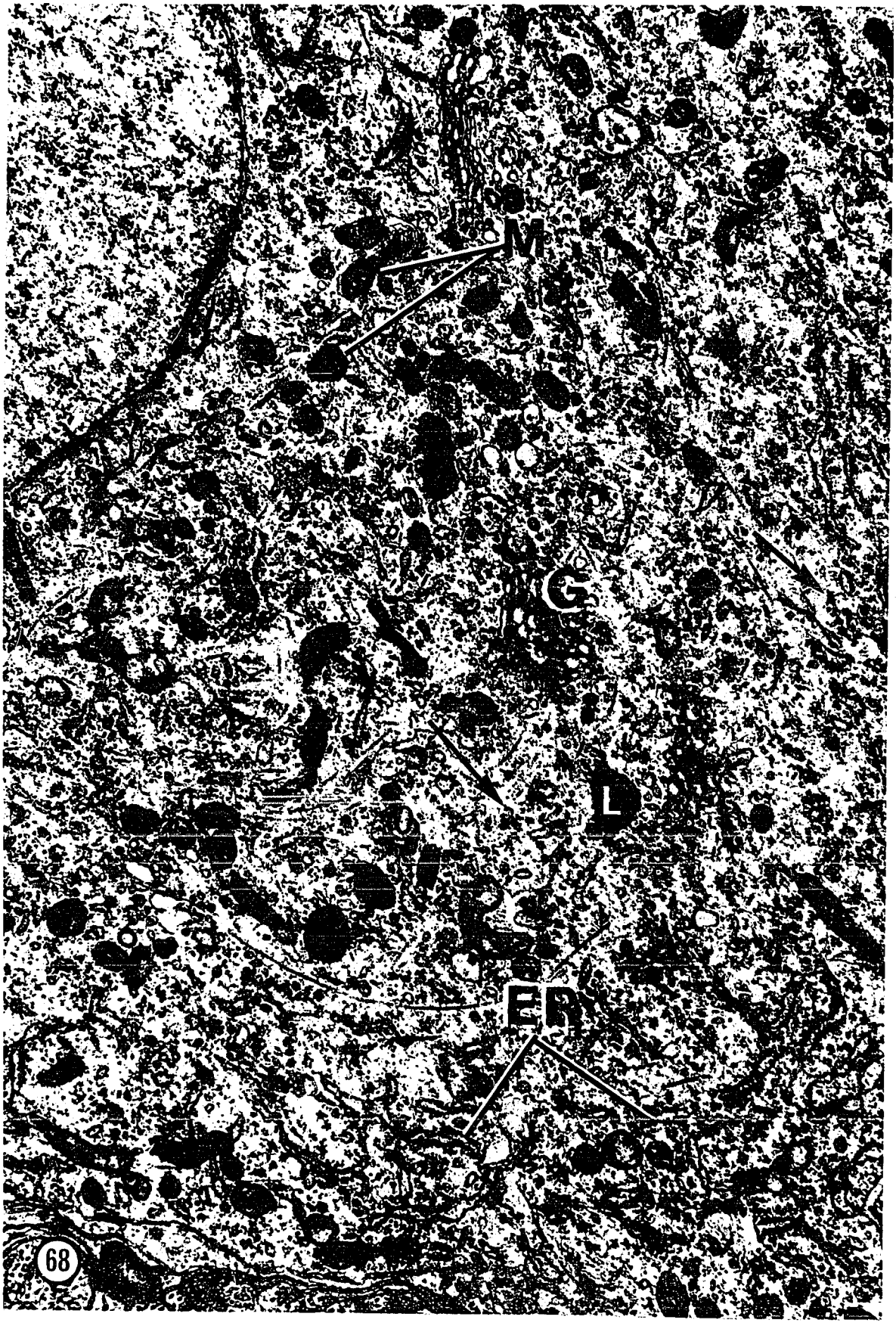
The rough ER of the 'dark' neurons was usually diffuse (Figure 68); well developed rough ER with cisternae confined to the 'marginal' cytoplasm was not a common finding. The frequently observed fine granular material within the cisternae of this organelle is also shown in Figure 68. Several well developed profiles of the Golgi system

were identified within most of these neurons (Figure 69), but presented no unusual morphological features. The mitochondria, lysosome-like bodies, neurotubules and vesicular structures were well represented and uniformly distributed throughout the cytoplasm. Free ribosomes and their rosettes were particularly abundant, affording the granular nature of the perikarya of these neurons (Figures 68 and 69). The cytoplasm of the 'dark' neurons also invariably contained a number of DC vesicles (Figures 68 and 69). The mean diameter of these vesicles, based on 324 measurements, was 1089 ± 13 Å (Figure 22), with a diameter range of 628 Å to 2050 Å (Table 5). The frequency distribution of these measurements is shown in Figure 71.

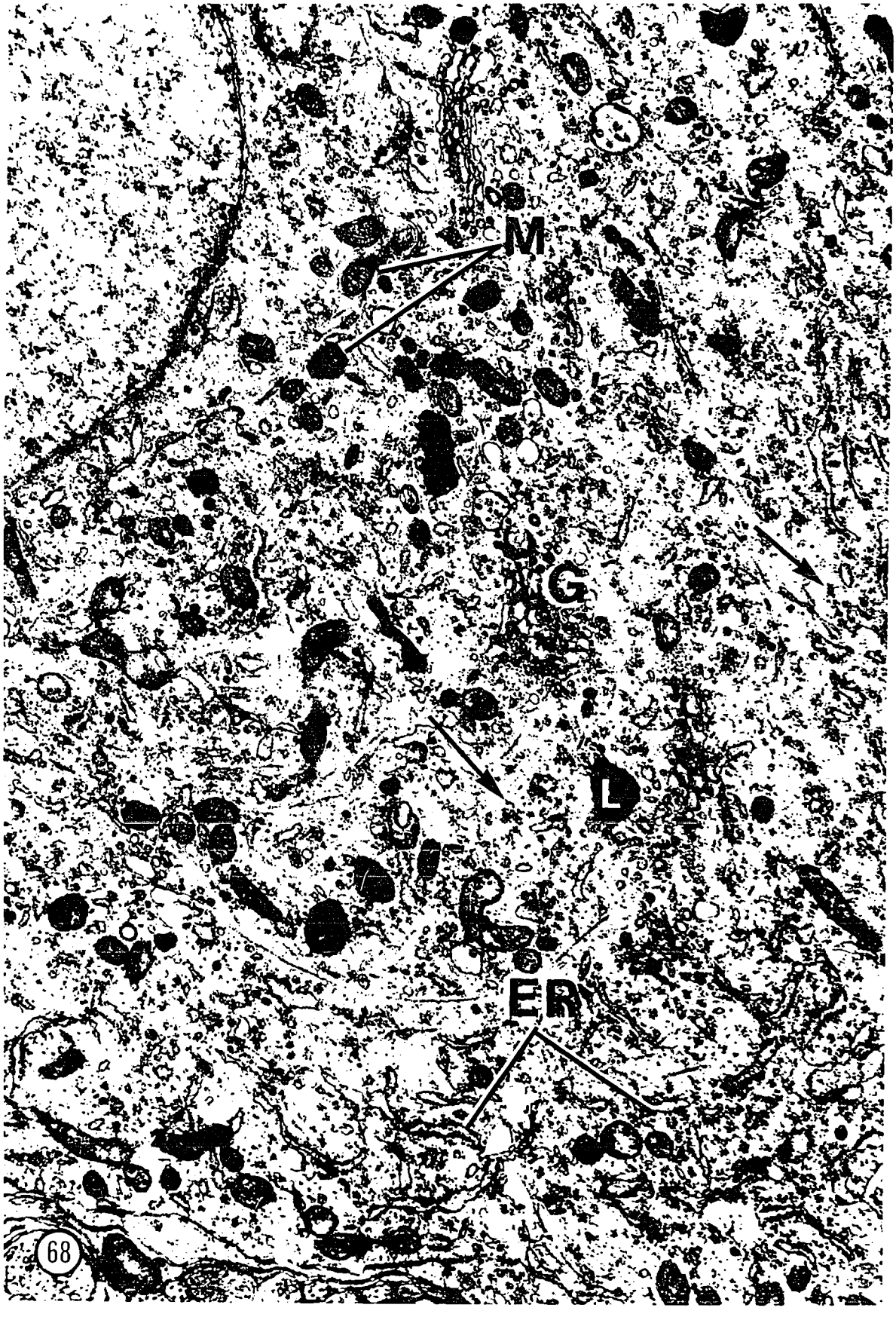
The large neuron shown in Figure 70 is representative of the 'light' neurons of the arcuate nucleus. Only a few scattered cisternae of the rough ER can be found in these cells. The free ribosomes were likewise sparsely represented. Short saccules and the associated vesicles of the Golgi system were resolved near the nuclei, but were, without exception, much smaller than the Golgi profiles of the 'dark' neurons (compare Figures 69 and 70). The consistently few mitochondria, lysosome-like bodies and tubular structures also contributed to the light appearance of these neurons. DC vesicles identical to those of the 'dark' neurons were also few in number. These however, when clearly identified, were included within the sample of 324 DC vesicles measured from the neuron soma of the control animals.

Figure 68. Electron micrograph showing portion of the perinuclear zone of a 'dark' neuron observed in the arcuate nucleus of a control animal. The rough endoplasmic reticulum (ER) is relatively well developed. Numerous small polysomes (arrows) give the cytoplasm a granular appearance. G, Golgi system; L, Lysosome-like body; M, Mitochondrion.

X 27,000



68



68

Figure 69. Electron micrograph showing portion of a 'dark' neuron; arcuate nucleus, control animal. Dense populations of cytoplasmic organelles give the cytoplasm a dark appearance. Several large Golgi systems (G) are seen in many of these cells. The numerous small polysomes give the cytoplasm a granular appearance. DCV, Dense-core vesicles; L, Lysosome-like bodies; M, Mitochondria; Nu, Nucleus.

X 20,800

PEV
G
M
N
69

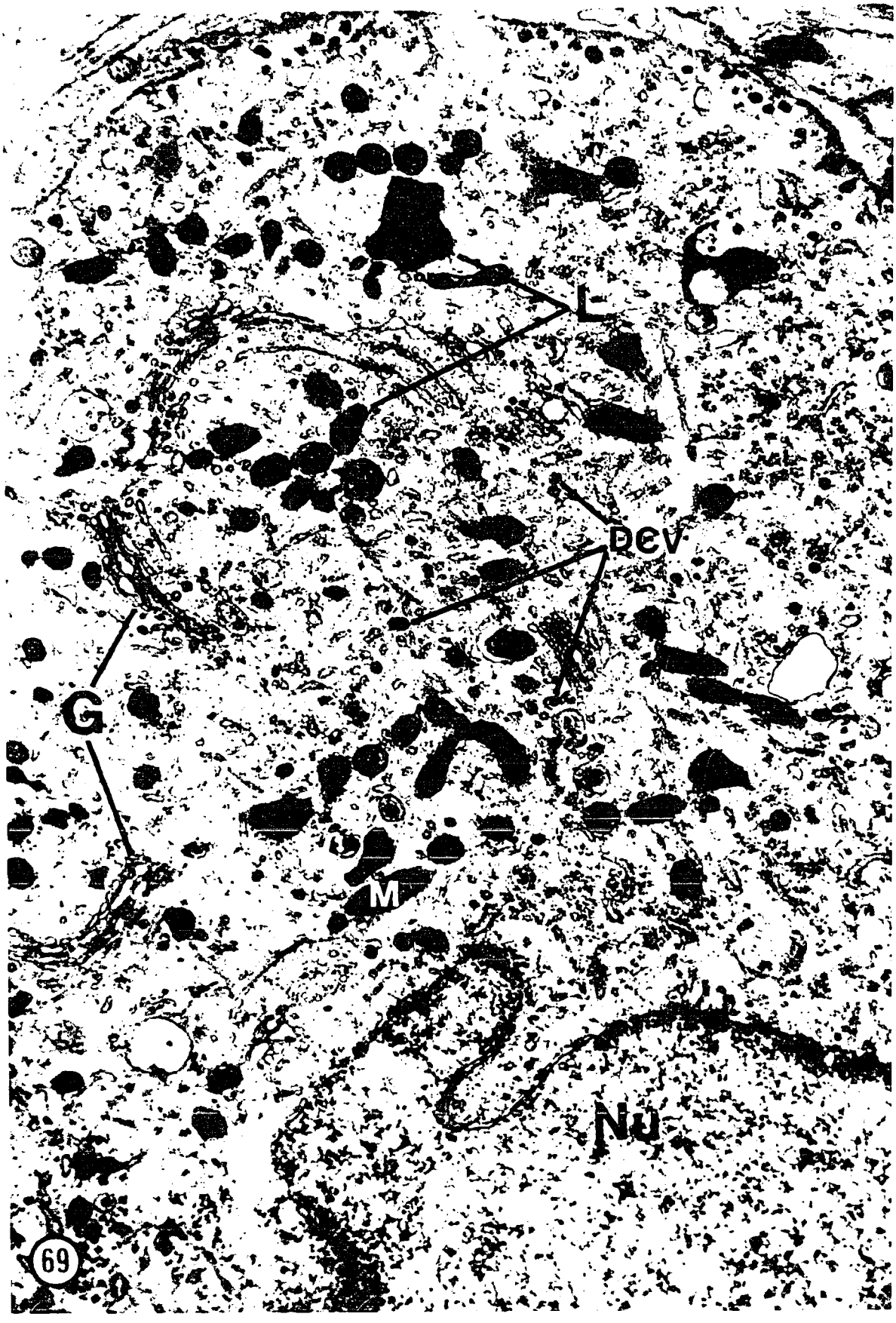

L
Rev
G
M
Nu
69

Figure 70. Electron micrograph of a 'pale' neuron of the arcuate nucleus. Note the conspicuous absence of dense populations of cell organelles. Only the occasional cisterna of the rough endoplasmic reticulum (ER) and mitochondria (M) can be identified. The Golgi system (G) is not well developed. Control animal. D, Dendrite; Nc, Nucleolus; Nu, Nucleus.

X 10,500


Dg
Nu
Nc
ER
M
G
D
70

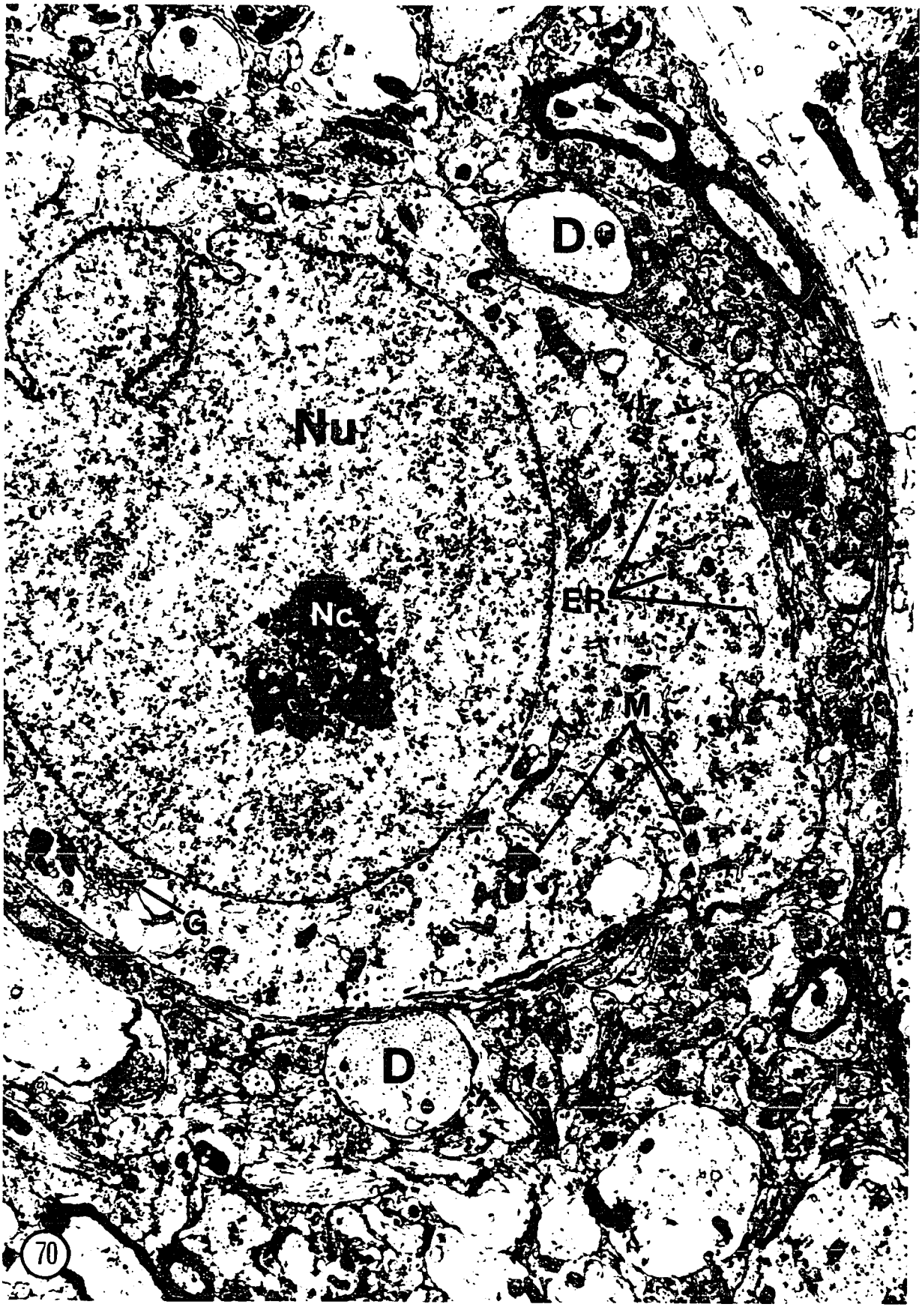
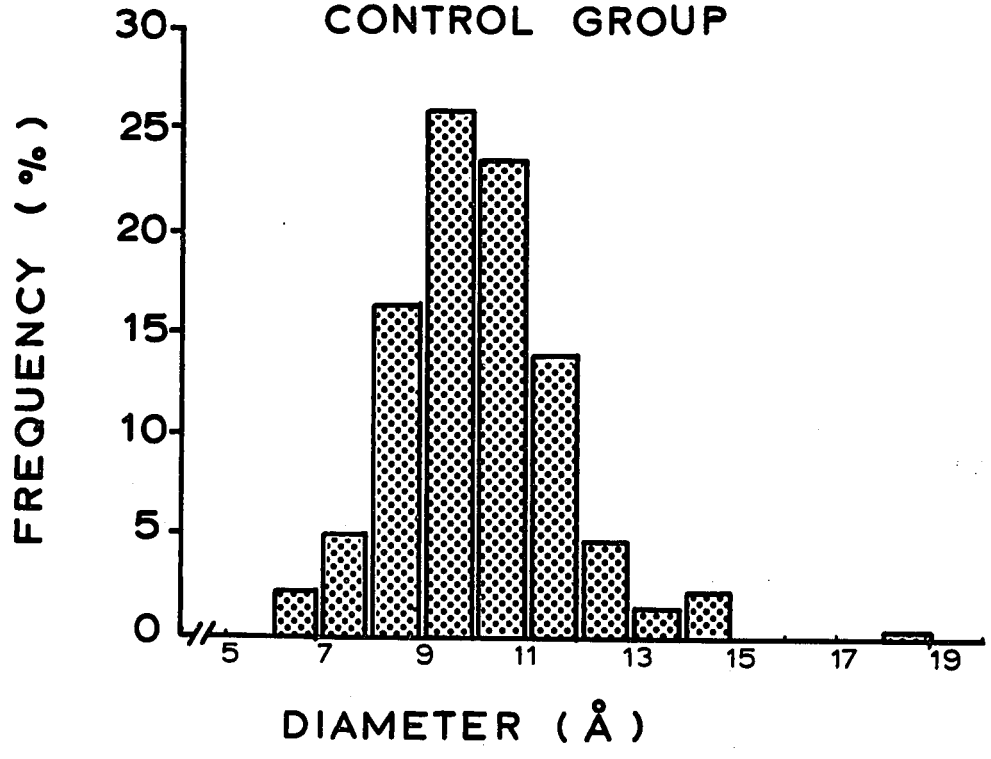

D
Nu
Nc
ER
M
G
D
70

Figure 71. Histograms showing the frequency distributions of the measurements made of the dense-core vesicles within the soma of arcuate neurons of both the non-mated controls and mated experimental animals. Note that dense-core vesicles within the neuron soma of the experimental group had a size distribution similar to those of the controls; the two histograms are similar.


CONTROL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
DIAMETER (Å)

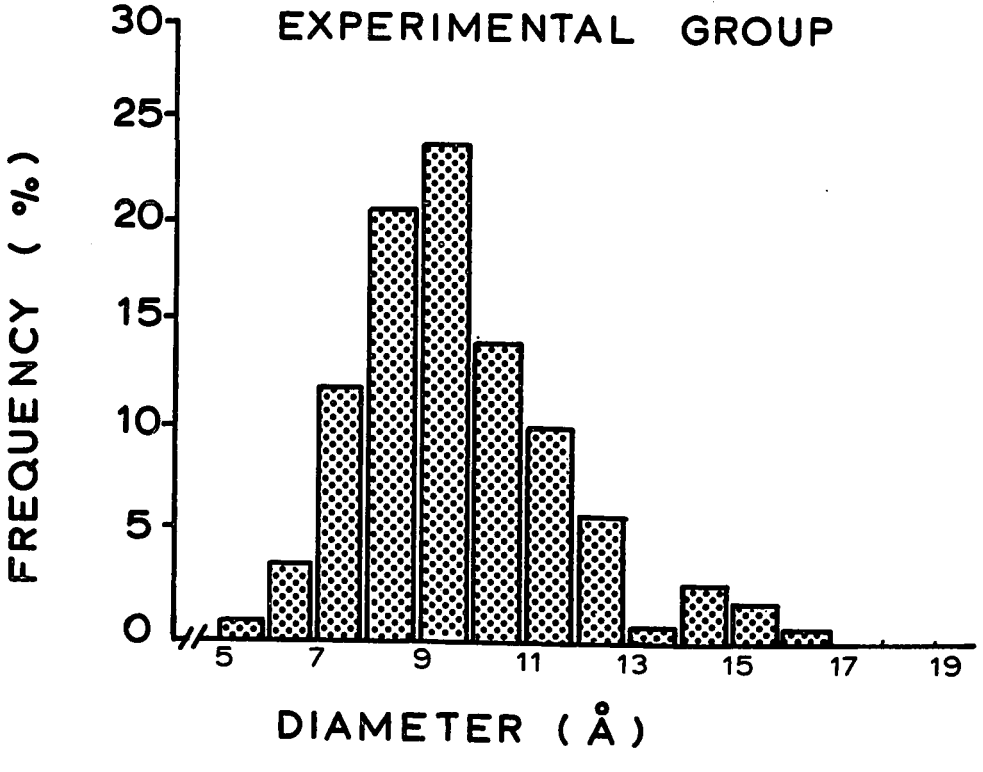

EXPERIMENTAL GROUP
FREQUENCY (%)
30
25
20
15
10
5
0
5
7
9
11
13
15
17
19
DIAMETER (Å)

Figure 71

3) Ultrastructural changes in arcuate neurons of the experimental groups

The two neuron types, as described for the control animals, were also observed within the ultrathin sections of each of the experimental groups. Portions of the perinuclear zones of two 'light' neurons and part of the perinuclear zone of one 'dark' neuron removed from the rabbit hypothalamus at eight hours post-coitus are presented in Figure 72.

The perinuclear areas of three 'dark' neurons excised from animals sacrificed at one hour, six hours and 10 hours following coitus are shown in Figures 73, 74 and 75. It is clearly evident that the ultrastructural changes that characterized the neurons of the PO and SCH nuclei following coitus have not occurred. Generally, the ultrastructural features of these neurons were identical to the 'dark' neurons observed throughout the control animals (compare Figures 68 and 69, and Figures 73, 74 and 75). Only the occasional neuron showed rough ER with long cisternae stacked one upon the other, and oriented in parallel with the cell membrane and thus occupying much of the 'marginal' cytoplasm (Figure 75). No apparent changes were observed in the numbers and distribution of the Golgi profiles, mitochondria, lysosome-like bodies or free ribosomes. A number of small DC vesicles, morphologically similar to those of the 'dark' neurons of the controls, were also a consistent component of the perikarya of the 'dark' neurons of the experimental animals (Figures 73, 74 and 75). Evidence for the origin of these DC vesicles from the saccules of the Golgi systems is shown in Figure 76 (inset).

The 'pale' neurons identified within each of the post-coital groups were also ultrastructurally similar to those of the non-mated conrols (compare Figures 70 and 72).

The mean diameter of the neurons of the experimental groups, based on measurements of 100 'dark' and 'pale' neurons located near capillaries, was 12.5 ± 0.2 μ (Figure 8) with a diameter range of 8.9 μ to 16.1 μ (Table 1). The nuclear profiles of these neurons had a mean diameter of 8.9 ± 0.1 μ (Figure 9). As shown in Tables 1 and 2, both the neuronal and nuclear diameters of the experimental groups were smaller ($P < .02$ and $P < .001$) than those of the control animals. The distribution curves for both the neurons and their nuclei within the experimental groups are also included in Figure 67.

The DC vesicles within the soma of both neuron types of the experimental animals had a mean diameter of 1039 ± 14 Å (Figure 22), with a range of 500 Å to 2000 Å (Table 5). The frequency distribution of these measurements is presented graphically in Figure 71. The mean diameter of the DC vesicles measured within the soma of the experimental groups was slightly, but significantly smaller ($P < .01$) than those of the control animals (Table 5).

Figure 72. Electron micrograph showing portions of the perinuclear zones of two 'pale' (PN) and one 'dark' neuron (DN) of the arcuate nucleus. Arcuate nucleus, eight hours post-coitus. D. Dendrite; O, Oligodendrocyte.

X 10,600


PN
DN
O
D
72

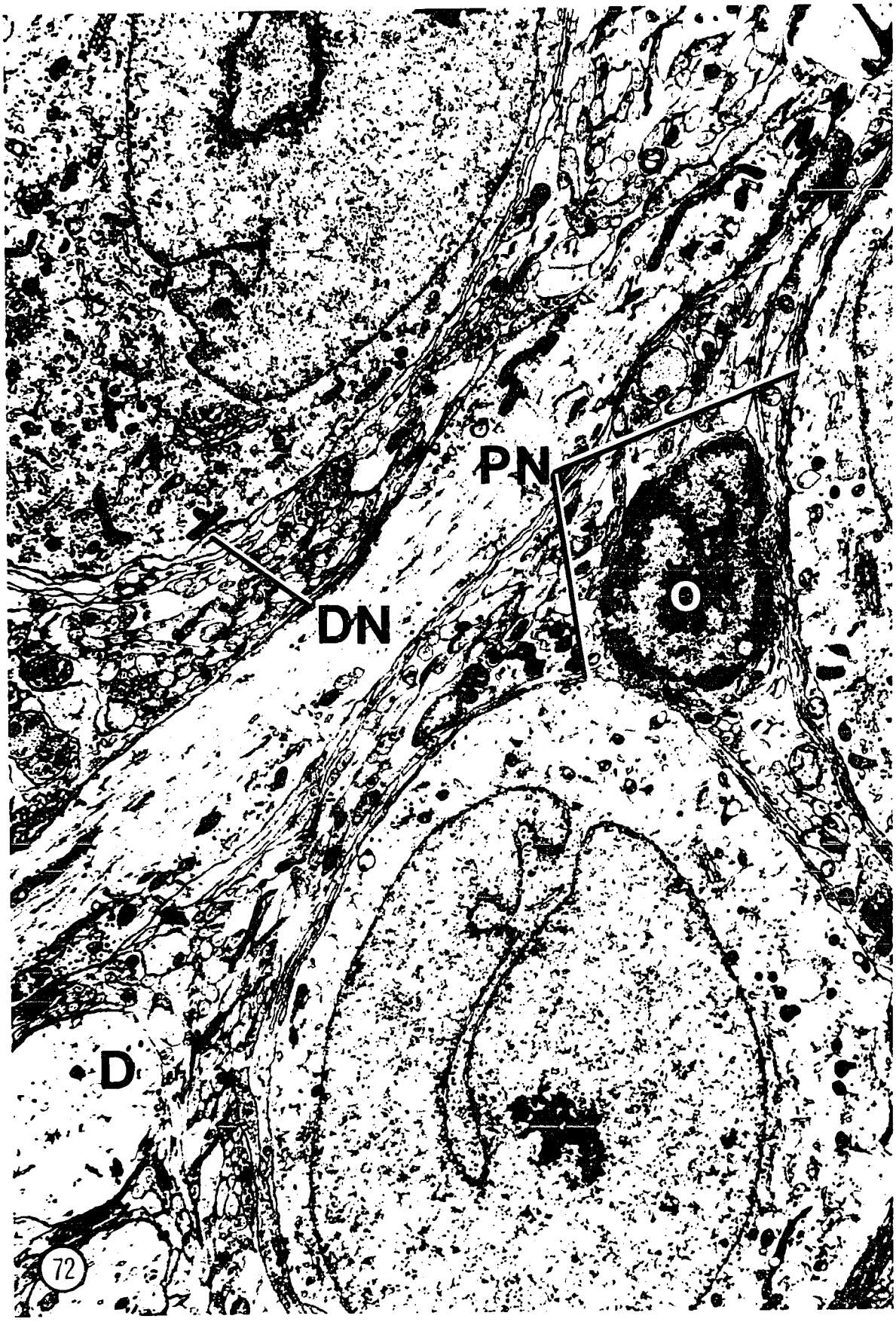
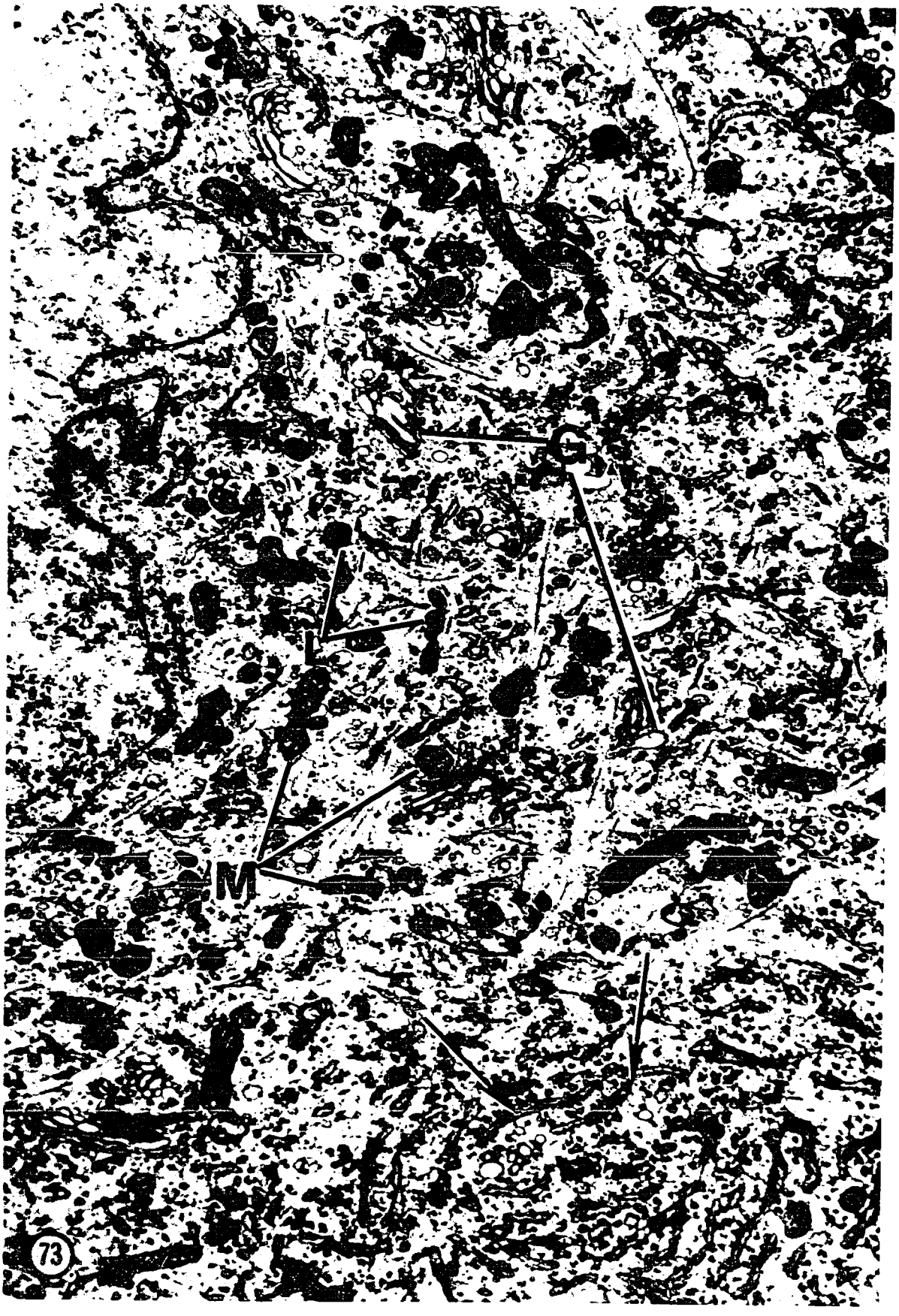

PN
DN
O
D
72

Figure 73. Electron micrograph of a portion of the perinuclear zone of a 'dark' neuron observed within the arcuate nucleus of a rabbit sacrificed at one hour post-coitus. The rough endoplasmic reticulum is well developed and contains a granular material (arrows). The cytoplasm near the nucleus contains several small Golgi profiles (G), numerous mitochondria (M) and scattered lysosome-like bodies (L).

X 19,500

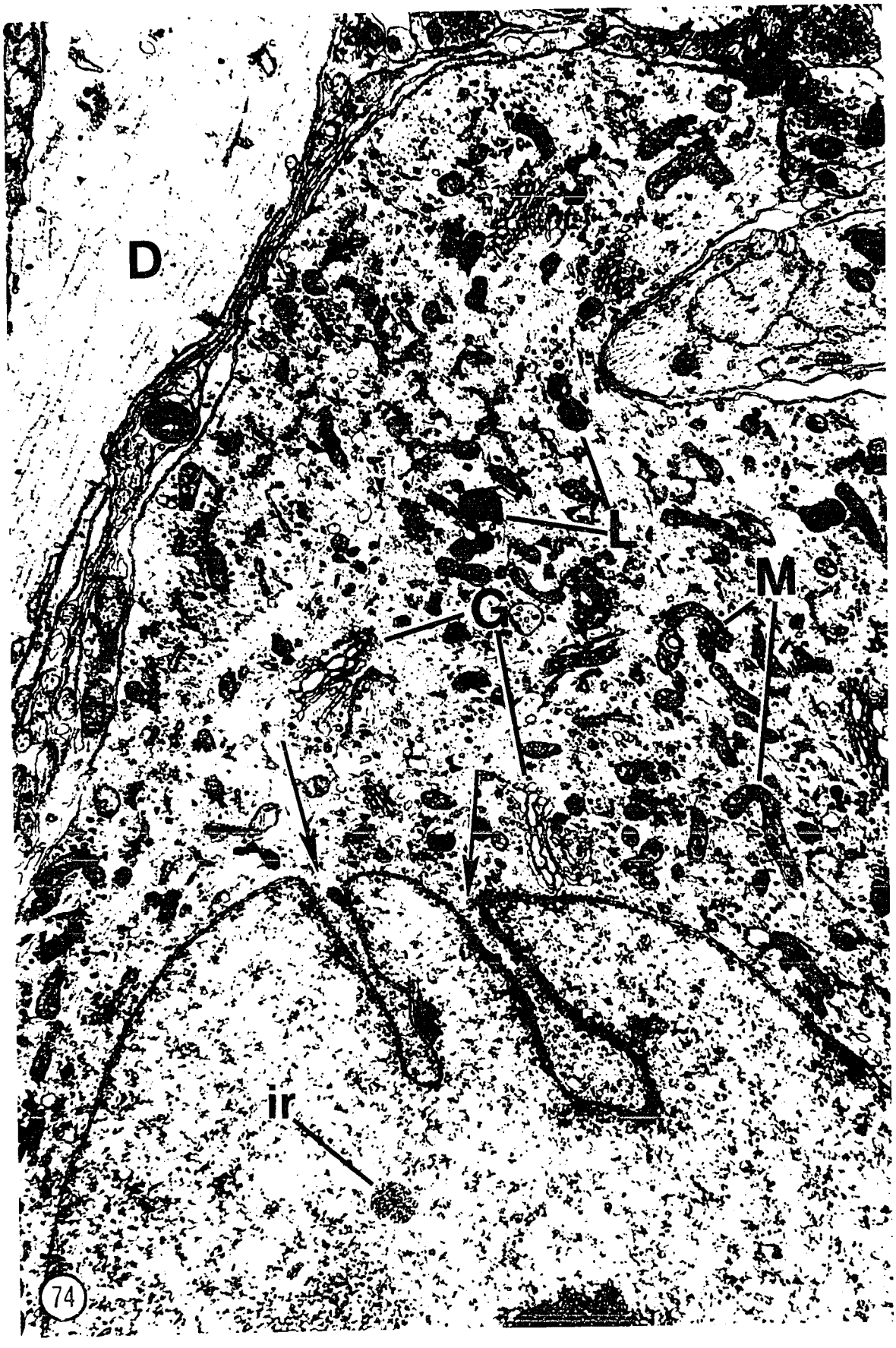

M
73


G
M
73

Figure 74. Electron micrograph of a 'dark' arcuate neuron observed at six hours post-coitus. The cytoplasmic organelles are well represented. The intranuclear rodlet (ir) is cut in cross section. Several deep nuclear clefts (arrows) face the central region of the cell. D, Dendrite; G, Golgi system; L, Lysosome-like body; M, Mitochondrion.

X 14,400


D
C
M
Ir
74



74

Figure 75. Electron micrograph showing a portion of the nucleus and perinuclear region of a 'dark' neuron of the arcuate nucleus observed at 10 hours post-coitus. The cisternae of the rough endoplasmic reticulum (ER) are arranged one upon the other and oriented in parallel with the cell membrane. The Golgi system (G) is well developed. Mitochondria (M) are numerous. Nu, Nucleus.

X 19,400


G
75

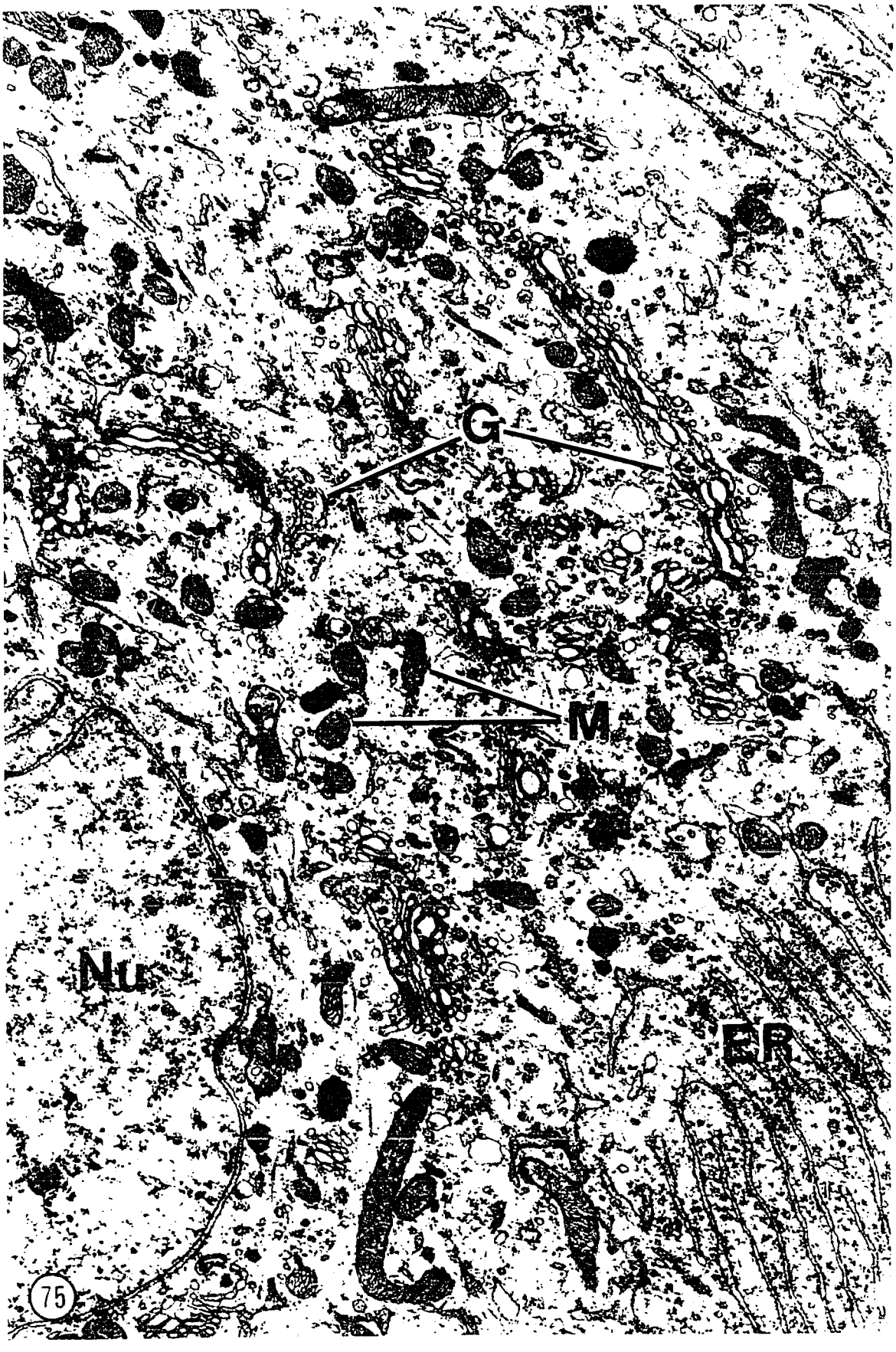
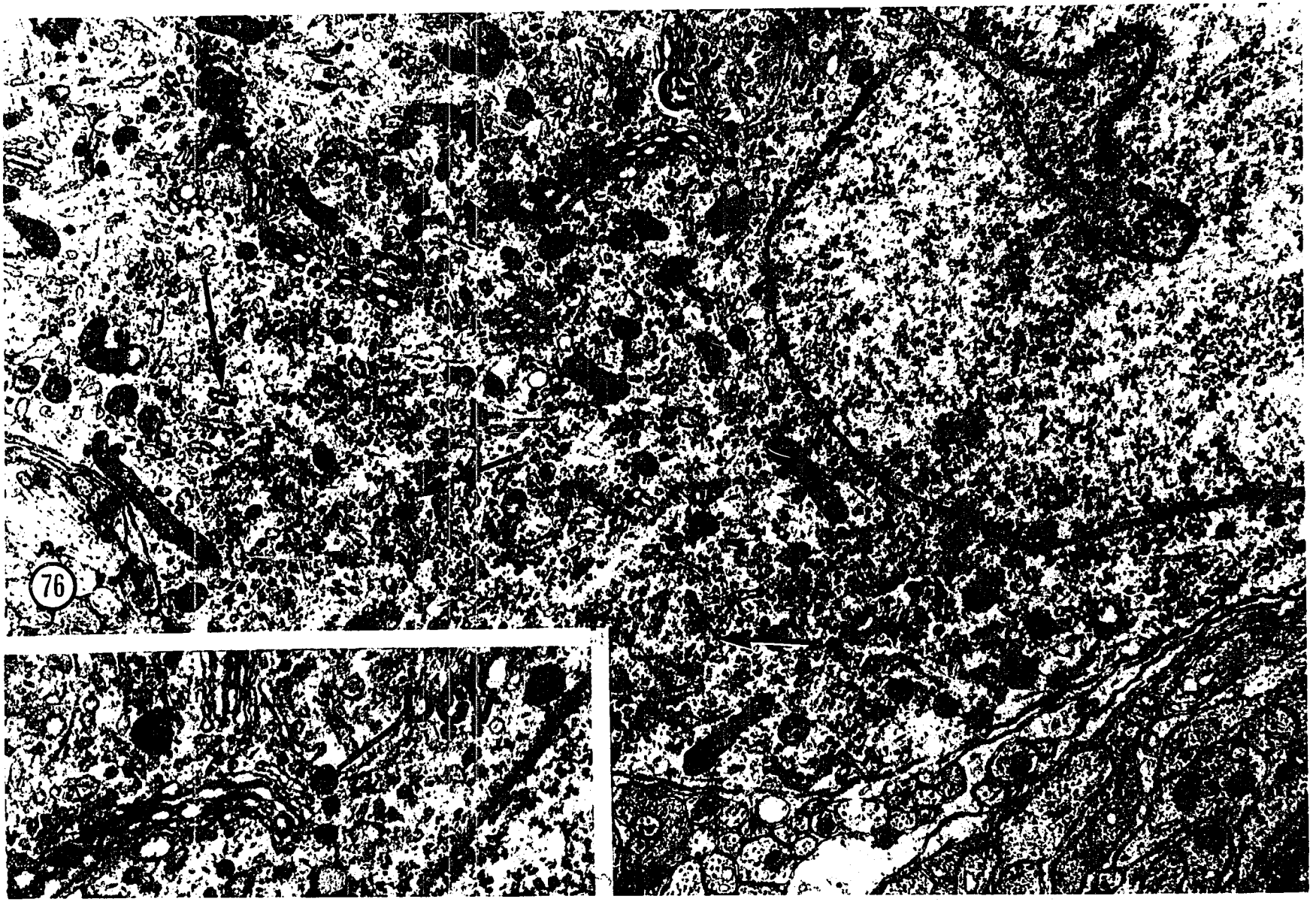
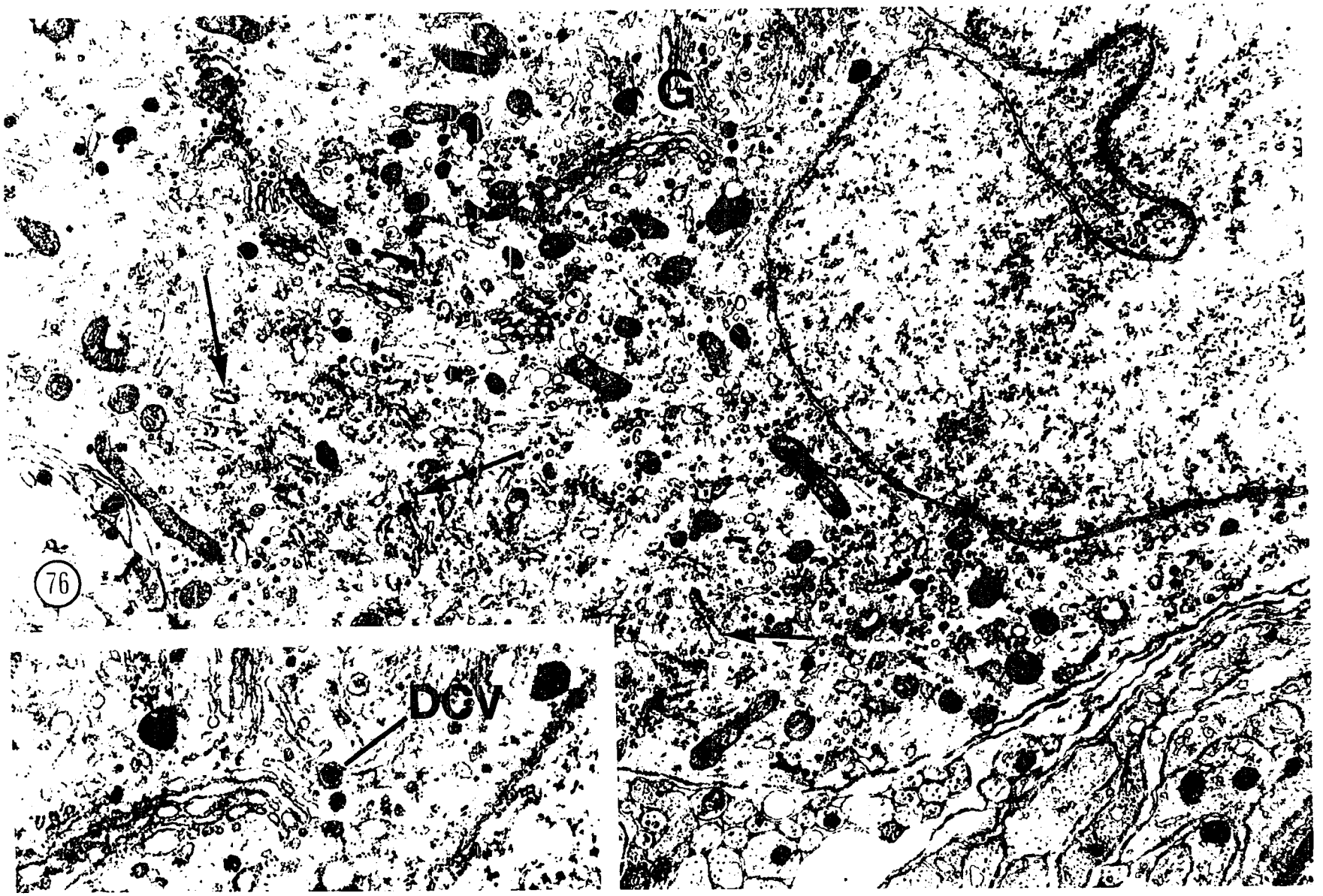

G
M
Nu
ER
75

Figure 76. Field showing a large 'dark' arcuate neuron observed at two hours post-coitus. The rough endoplasmic reticulum is well developed and contains a macromolecular material (arrows). Several large Golgi systems (G) are in the central cytoplasm. Small dense-core vesicles (DCV) occur in the neuron soma; their origin from the Golgi system is shown in the inset.

X 16,900

inset X 29,300


76


G
76
DCV

INTRANUCLEAR RODLETS

A) Electron Microscopy

Intranuclear rodlets have been widely observed within neurons of the PO, SCH and arcuate nuclei of the rabbit brain (Table 6). This nuclear inclusion appeared as single, rod-shaped filamentous bands up to 9.6 μ in length and from 0.1 μ to 0.45 μ in width (Figure 77), or as oblong to spherical filamentous bundles according to the plane of sectioning (Figures 78 and 79). Doublets or branching forms of these rodlets were never observed. As shown in the inset in Figure 77, the rodlet was composed of closely packed filaments approximately 60 Å to 70 Å in diameter, and was of indeterminate length. In longitudinal section, the rodlet had a relatively uniform diameter as it extended from one pole of the nucleus to the other as a straight filamentous band (Figure 77). Slightly curved forms were also observed; these, however, did not lie close to, or follow, the contours of the nuclear envelope. The tips of these rodlets were usually tapered, with the component filaments becoming less compact in their arrangement. Actual contacts between the rodlet tips and the nuclear envelope, or extensions of this inclusion out into the cytoplasm were not observed. A clear area or 'halo' appeared to separate many of the rodlets from the surrounding granular karyoplasm (Figures 77 and 78). Direct contacts with the other components of the nucleus were not a common finding; however, what appears to be close spatial relationships between the rodlet and the nucleolus were occasionally observed (Figures 78 and 79).

Also, as clearly shown (Table 6), these filamentous rodlets not only occurred within neuronal nuclei of the non-mated control animals, but were also common within morphologically similar neurons of the mated experimental groups. These, or similar inclusions, were never observed within the nuclei of the large PO and SCH neurons which displayed discrete ultrastructural changes following coitus (Figures 31, 46 and 49). The presence of this rodlet within the nuclei of both the 'dark' and 'pale' neurons of the arcuate nucleus is shown in Figure 57. This nuclear inclusion was not observed within any of the glial or ependymal cells associated with these hypothalamic nuclei.

TABLE 6

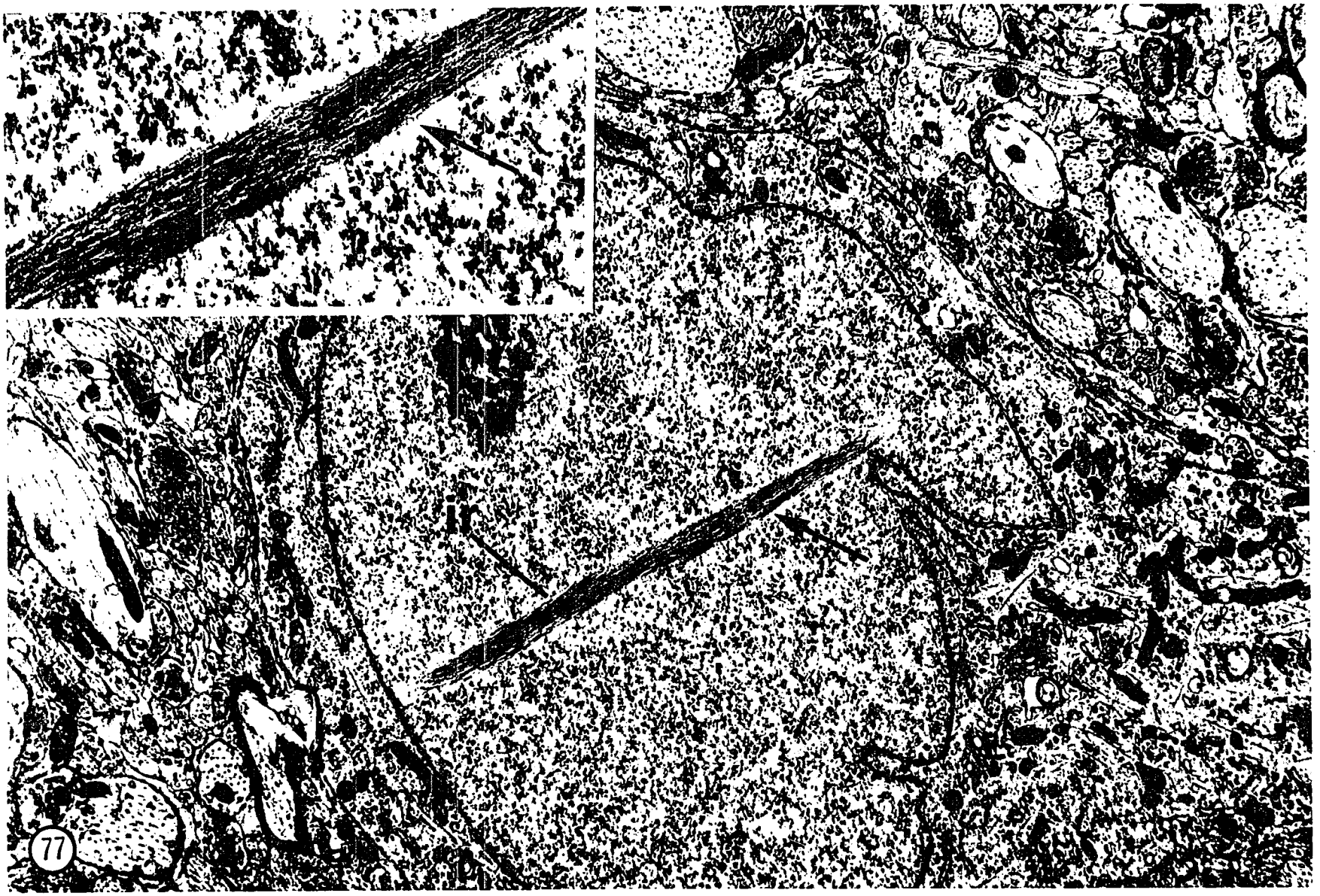
INCIDENCE OF INTRANUCLEAR RODLETS IN HYPOTHALAMIC NUCLEI OF NORMAL AND MATED RABBITS

Hypothalamic Nucleus	Controls (non-mated rabbits)			Experimental Animals (0.5 hours to 10 hours post-coitus)					
				I. Neuron population not showing ultrastructural changes following coitus			II. Neuron population showing ultrastructural changes following coitus		
	Number of neuronal nuclei	Number of rodlets	Frequency (%)	Nuclei	Rodlets	Frequency (%)	Nuclei	Rodlets	Frequency (%)
Suprachiasmatic	100	25	25.0	68	20	29.4	86	0	0
Preoptic	83	31	37.3	84	19	22.6	62	0	0
Arcuate	64	20	31.3	82	21	25.6	0	0	0

Figure 77. Electron micrograph showing a large neuron removed from the suprachiasmatic nucleus at eight hours post-coitus. The large intranuclear rodlet (ir) extends from one pole of the nucleus to the other. The fibrous nature of the rodlet is shown in the inset. A clear area (arrows) tends to separate the rodlet from the surrounding karyoplasm.

X 12,000

inset X 30,000


77

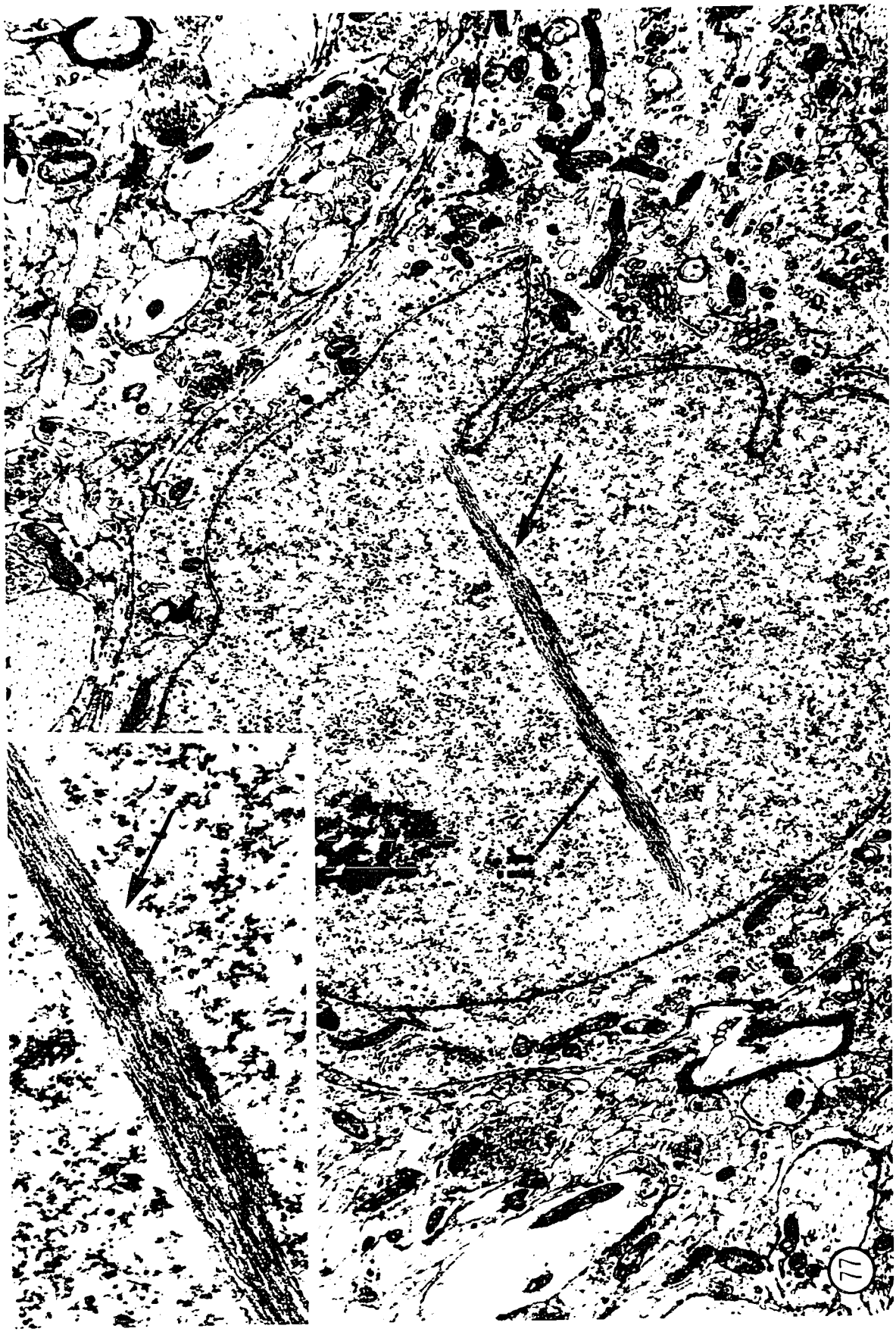
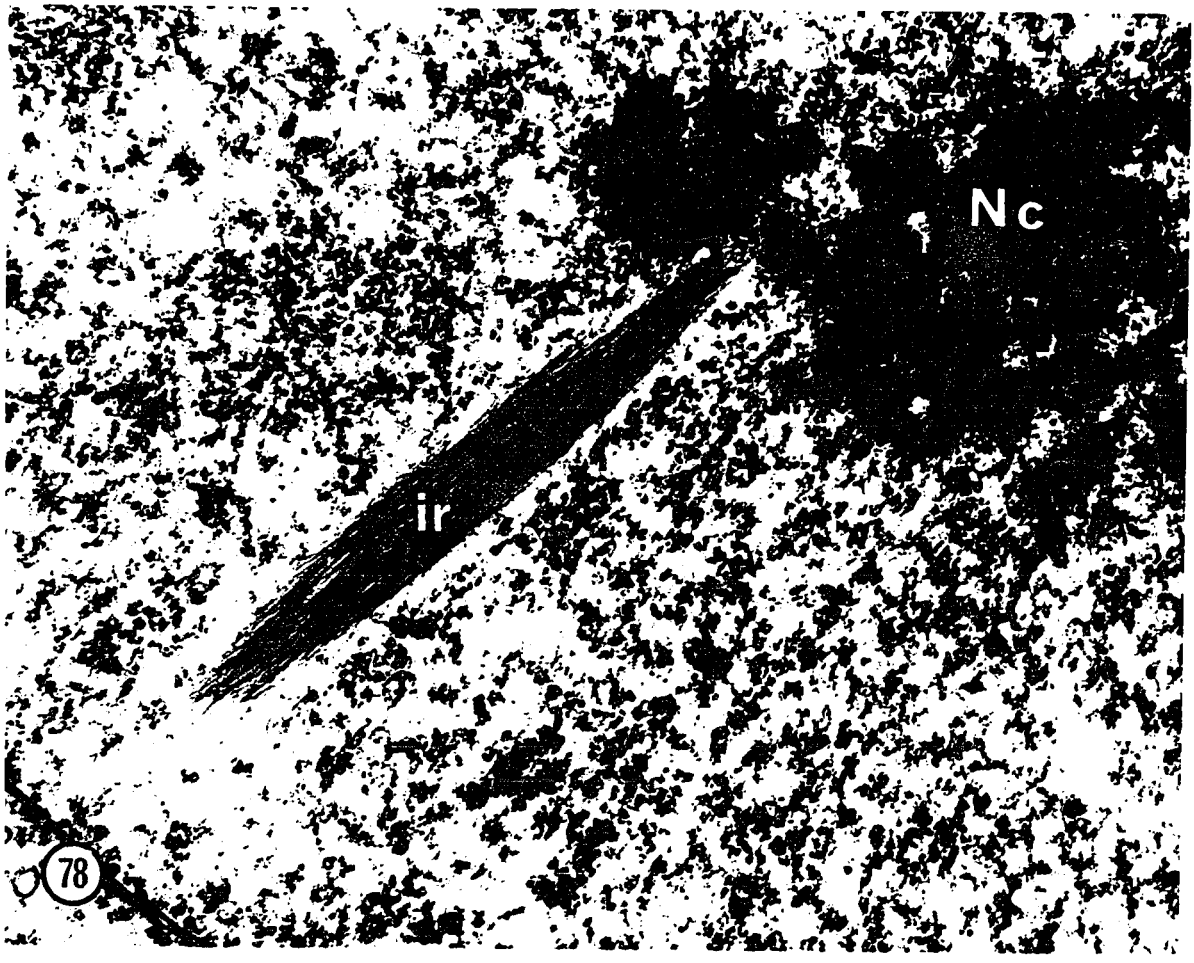


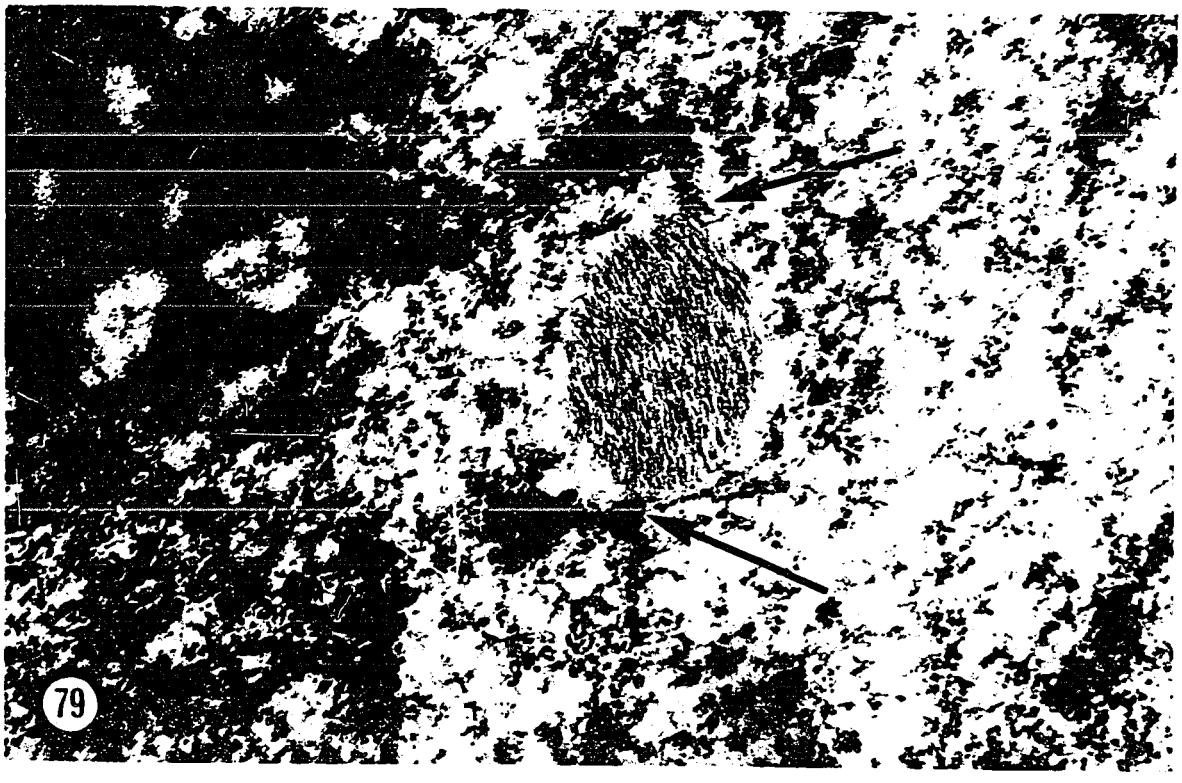
Figure 78. Electron micrograph showing the fibrous nature of an intranuclear rodlet (ir) cut in tangential section. The rodlet is in close spatial relationship with the nucleolus (Nc). Arcuate nucleus, experimental animal.

X 32,200

Figure 79. High magnification electron micrograph of an intranuclear rodlet within a neuron of the preoptic nucleus excised at four hours post-coitus. The rodlet is cut in cross-section and appears to be suspended (arrows) by projections of the nucleolar material.

X 49,100


Nc
ir
78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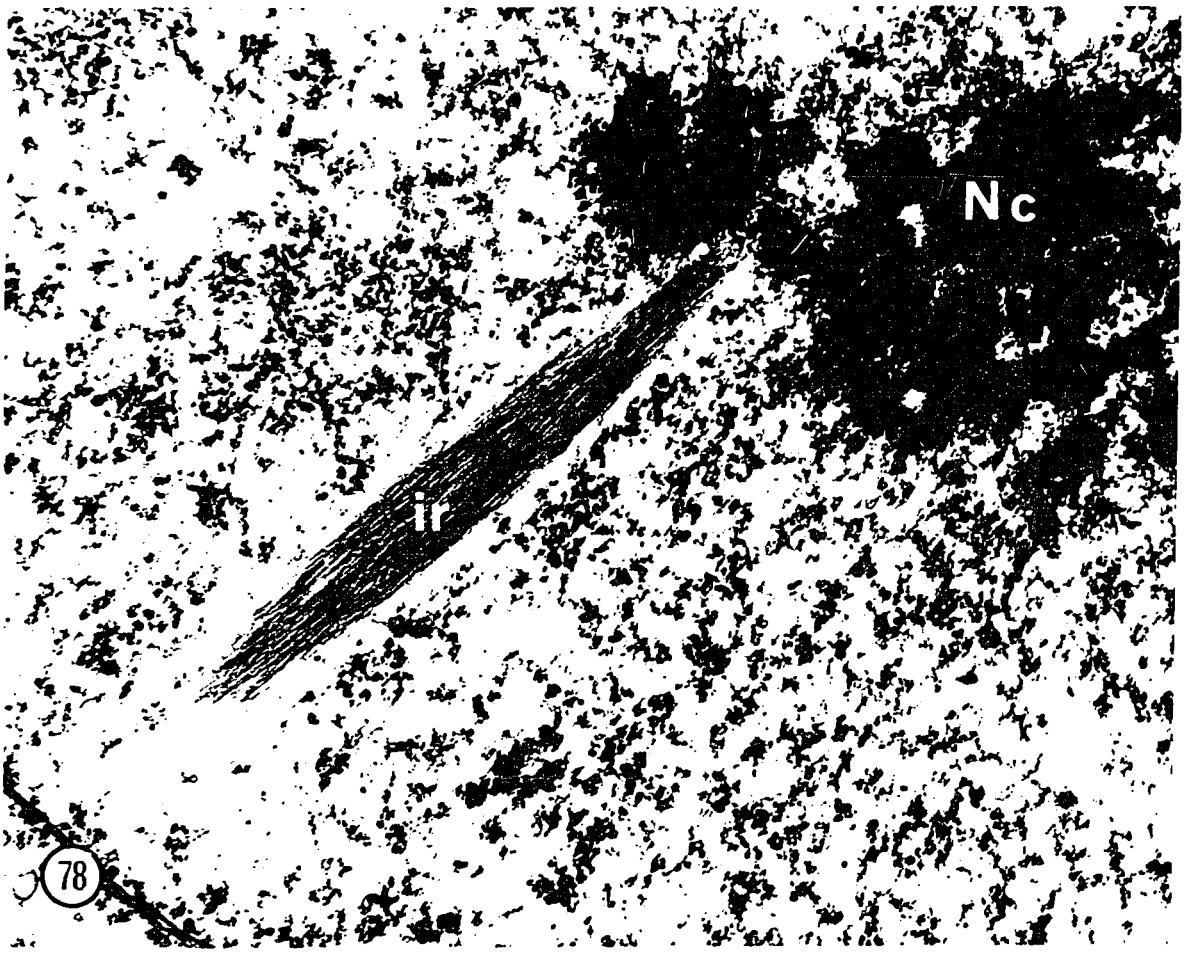
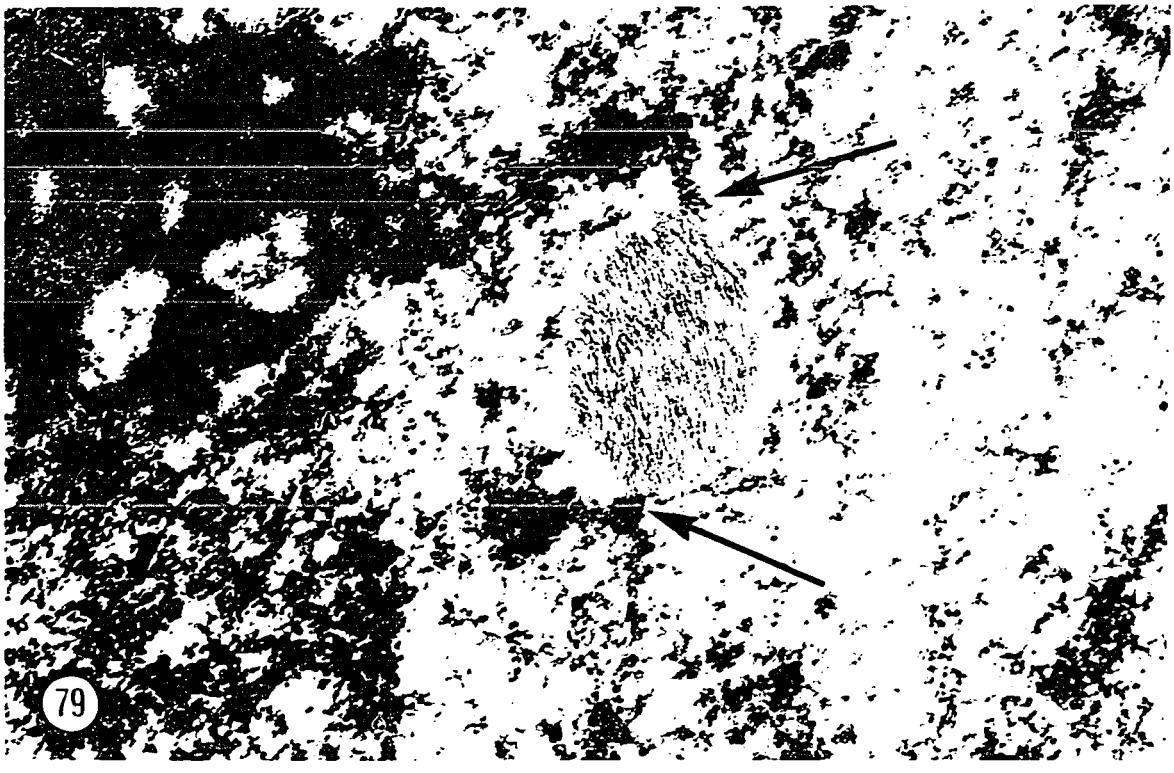

Nc
ir
78


79

MEDIAN EMINENCE

A) Light Microscopy

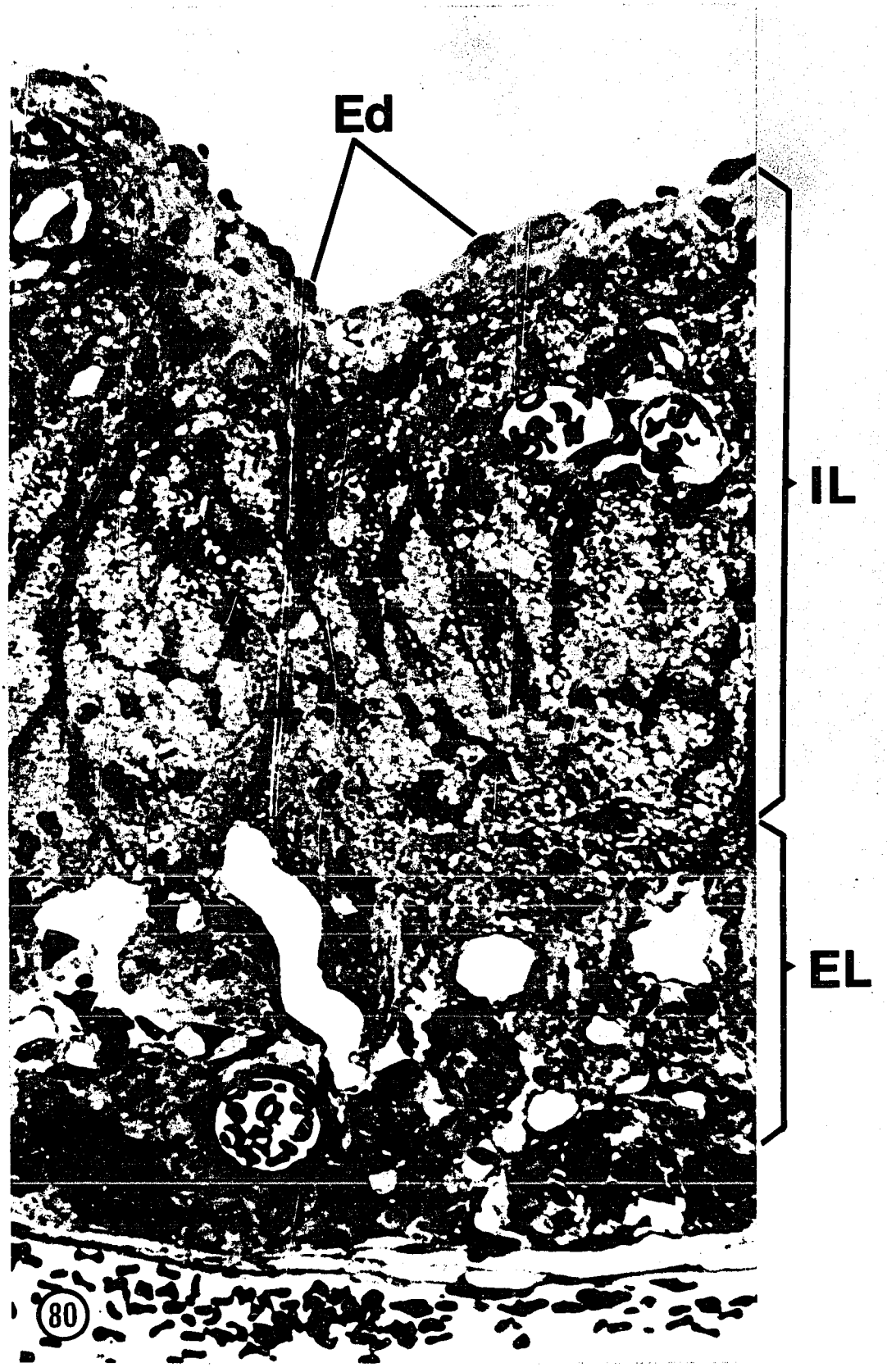
A frontal section of the anterior median eminence of the rabbit tuber cinereum is shown in Figure 80. The approximate extent of the internal and external layers is clearly illustrated. A single ependymal cell layer covers the ventricular surface, and a 'cuff' or small, darkly stained cells of the pars tuberalis surrounds the outer surface of the external layer. At the light microscopic level, the internal layer appears to be composed largely of bundles of fibers in close apposition and oriented perpendicular to the surface. These fibers, probably those of the neurohypophyseal tract, were usually interrupted by nuclear profiles and narrow bands of fibers which appeared to course downward toward the external layer of the ME. By contrast, the external layer contained a very sparse population of nuclei, the fiber profiles were irregular in their orientation, and were typically interrupted by capillaries of the portal circulation.

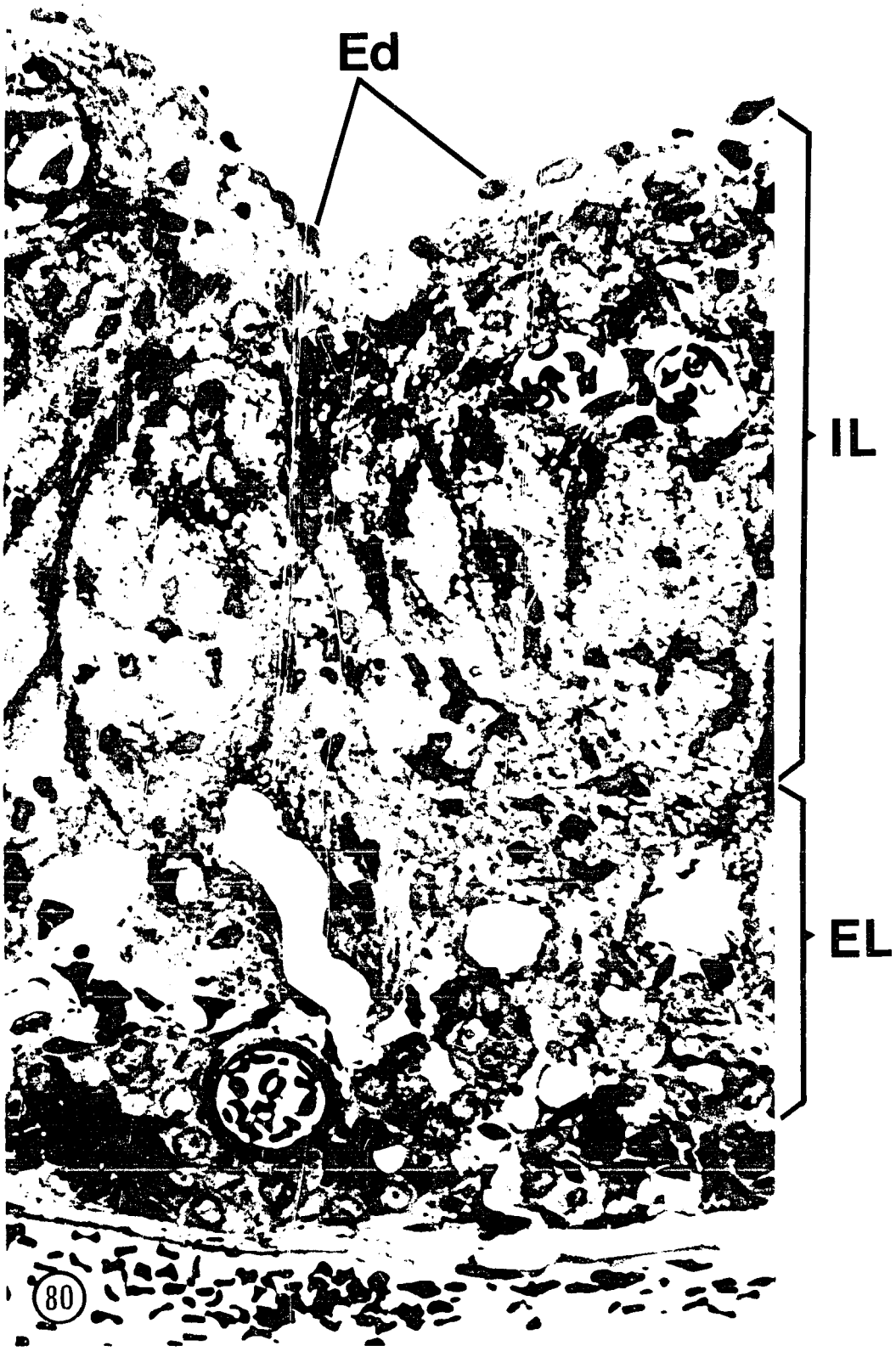
B) Electron Microscopy

Many of the axon profiles of the internal layer contained a number of large vesicles that were characterized by a core of light, granular material typically separated from a limiting membrane by a relatively wide clear area or 'halo' (Figure 81, inset). Similar vesicles were also observed within large (3.0 μ to 8.5 μ wide) Herring bodies. These vesicles were not consistently uniform in size or in their electron density, although extremes of electron lucency or electron density were not observed. Based on 218 measurements, the

Figure 80 Light photograph of a coronal section cut from the anterior median eminence of the rabbit brain. The approximate extents of the internal layer (IL) and the external layer (EL) are shown. The ventricular surface of the median eminence is formed by the monolayered ependyma. The dark cells near the bottom of the field represent the pars tuberalis. Epon section, Mallory's Azur II - Methylene Blue stain.

X 1,850


Ed
IL
EL
80


Ed
IL
EL
80

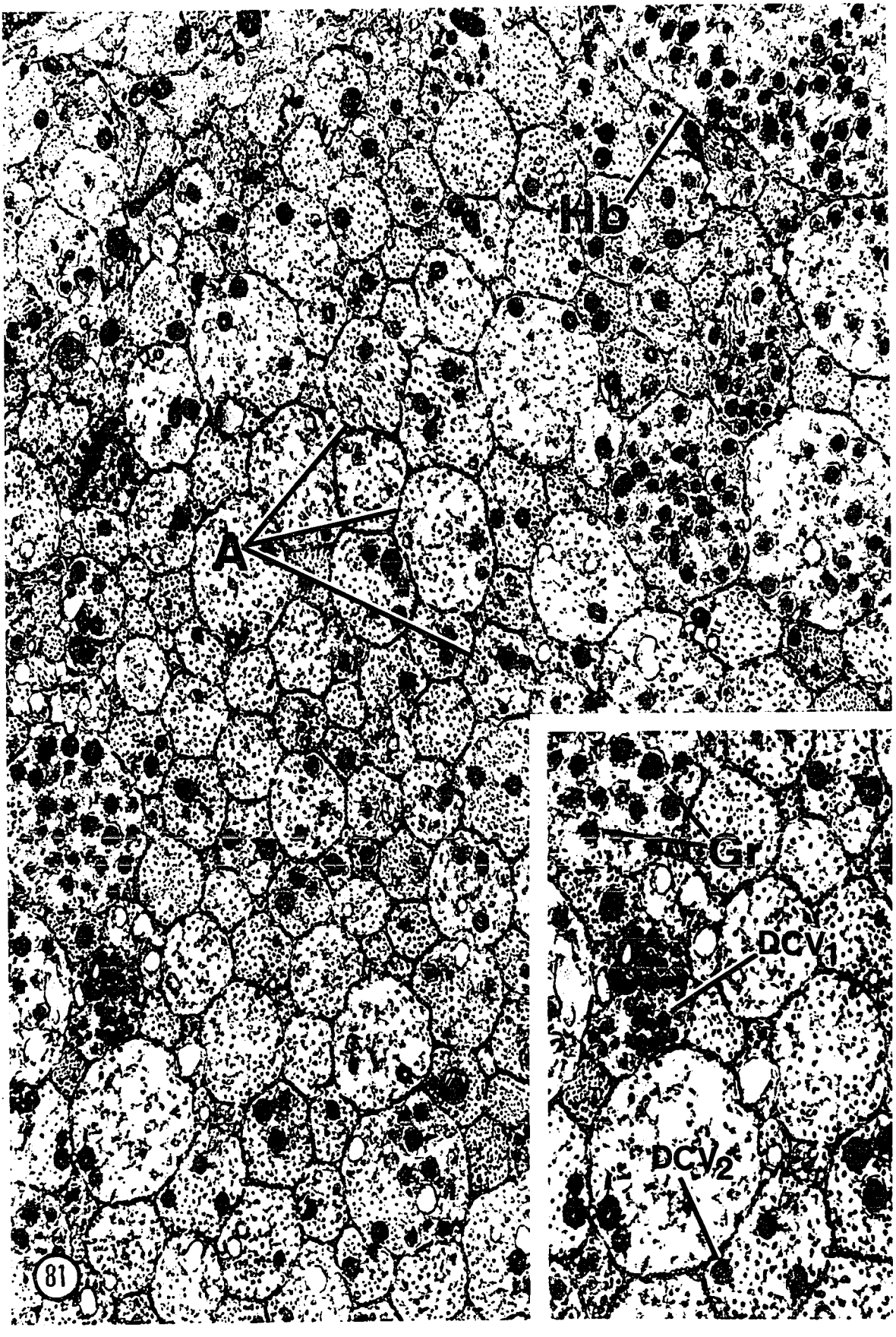
large, lighter-cored vesicles observed within the internal layer of the ME of the non-mated control animals, had a mean diameter of 2017 Å and a diameter range of 1265 Å to 2850 Å. A sample of 200 of these vesicles located within the Herring bodies and axon profiles of the internal layer of the experimental animals had a mean of 2030 Å with a diameter range of 1260 Å to 2990 Å. The distribution curves of these measurements for both the control and experimental groups are included in Figures 82 and 83, respectively. Smaller vesicles were also a common finding within the axons of the internal layer of both the non-mated controls and the mated experimental animals (Figure 81, inset). These vesicles could readily be distinguished from the lighter-cored vesicles on the basis of their size, homogeneous core of electron-dense material and typically narrow 'halo'. Also, the smaller DC vesicles, and those of intermediate size, occurred in separate profiles.

A portion of the external layer is shown in Figure 84. This region of the ME was composed primarily of neuronal processes, many of which are shown terminating on the perivascular spaces of the capillaries. Axon profiles containing DC vesicles were common, only the occasional axon carried vesicles identical to the large, light granular vesicles commonly observed within the axons of the internal layer. Other processes, identified as those of ependymal cells on the basis of their dark, fibrous content, were common and frequently terminated as end-feet on the perivascular spaces. What appears to be condensations of the cytoplasm under the membrane of the ependymal end-feet in contact with the perivascular spaces were common (Figure 84).

Figure 81. Electron micrograph showing a portion of the internal layer of the median eminence. Axon profiles (A) and a segment of a large Herring body (Hb) fill the field. Representatives of the vesicle types observed within these axons are shown in the inset. The large granules (Gr) with cores of varying densities and separated from an irregular limiting membrane by wide 'halos' are distinguished from the smaller vesicles with consistent electron-dense cores (DCV). The DCV's are represented by two size populations - DCV_1 and DCV_2.

X 18,800

inset X 27,300


Hb
A
Gr
DCV1
DCV2
81

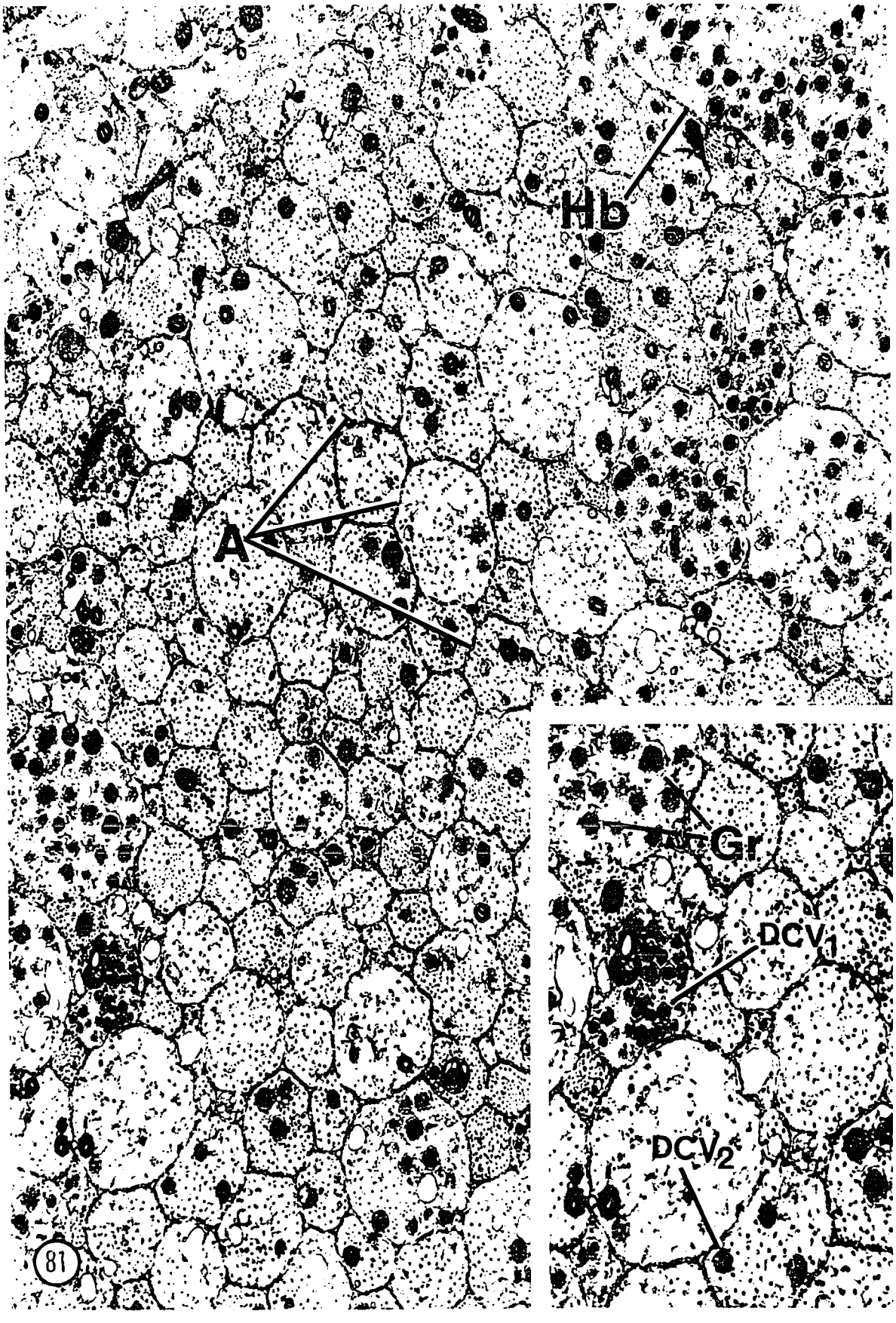

Hb
A
Gr
DCV1
DCV2
81

Figure 82. Distribution curves of the measurements made of the vesicle types located within the median eminence of the control animals. Legend: (---o---) - light-cored vesicles observed within axon profiles and Herring bodies of the internal layer; (—▼—) - dense-core vesicles observed within axons near, and axon terminals in contact with, the perivascular spaces of the external layer; (—o—) - 'ghosts' observed within axon profiles near, and within axon terminals in contact with the perivascular spaces of the external layer. Number of measurements: light-cored vesicles = 218; dense-cored vesicles = 642; 'ghosts' = 60.

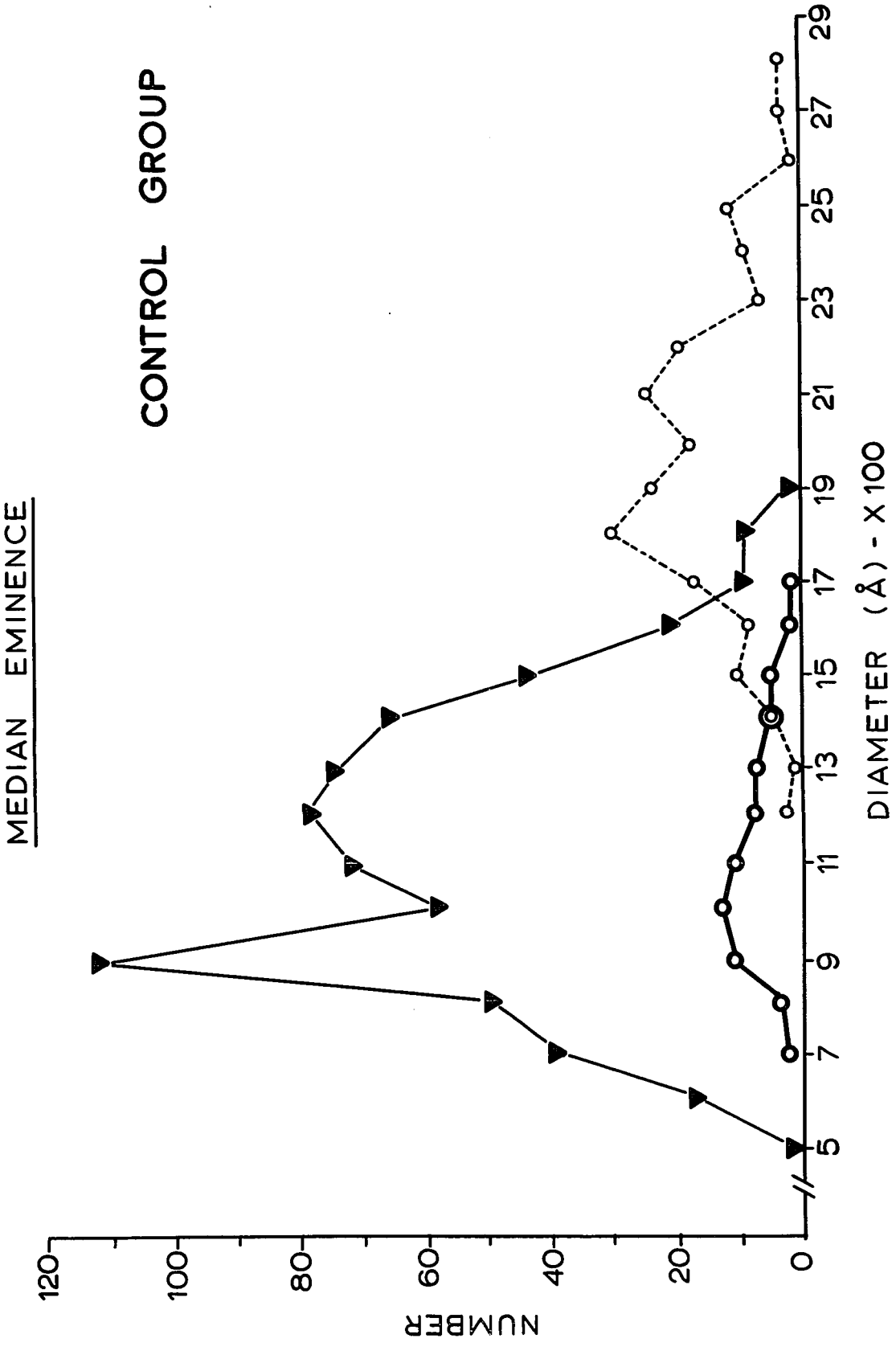

MEDIAN EMINENCE
CONTROL GROUP
NUMBER
0
20
40
60
80
100
120
5
7
9
11
13
15
17
19
21
23
25
27
29
DIAMETER (Å) - X 100

Figure 82

Figure 83. Distribution curves of the measurements obtained for the vesicle types observed within the median eminence of the experimental animals.

Legend: (---O---) - light-cored vesicles observed within axon profiles and Herring bodies of the internal layer; (—▼—) - dense-core vesicles observed within the axons near, and the axon terminals in contact with, the perivascular spaces of the external layer; (—O—) - 'ghosts' observed within axon profiles near, and terminating in contact with, the perivascular spaces of the external layer of the median eminence. Number of measurements: light-cored vesicles = 200; dense-cored vesicles = 205; 'ghosts' = 205.

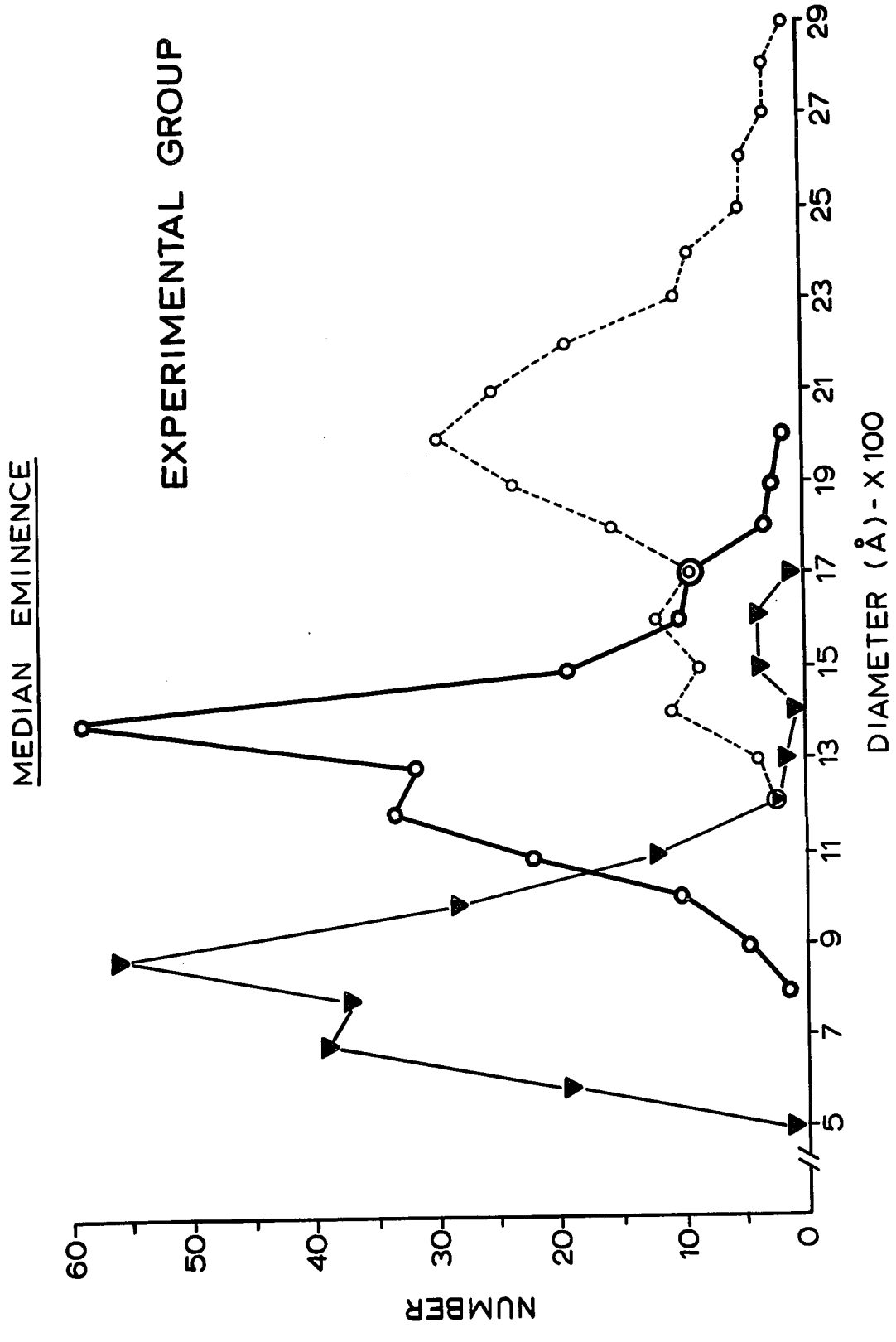
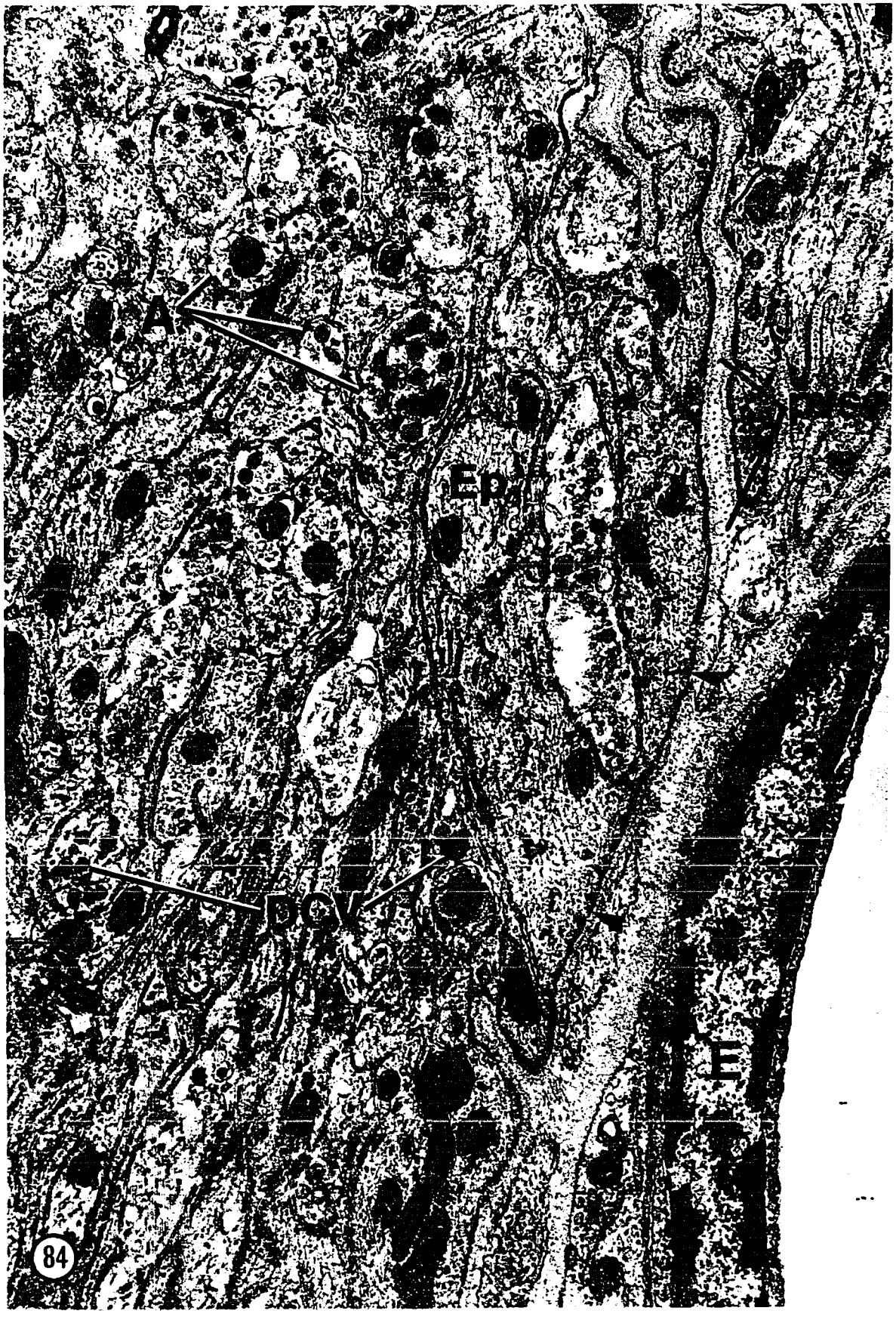
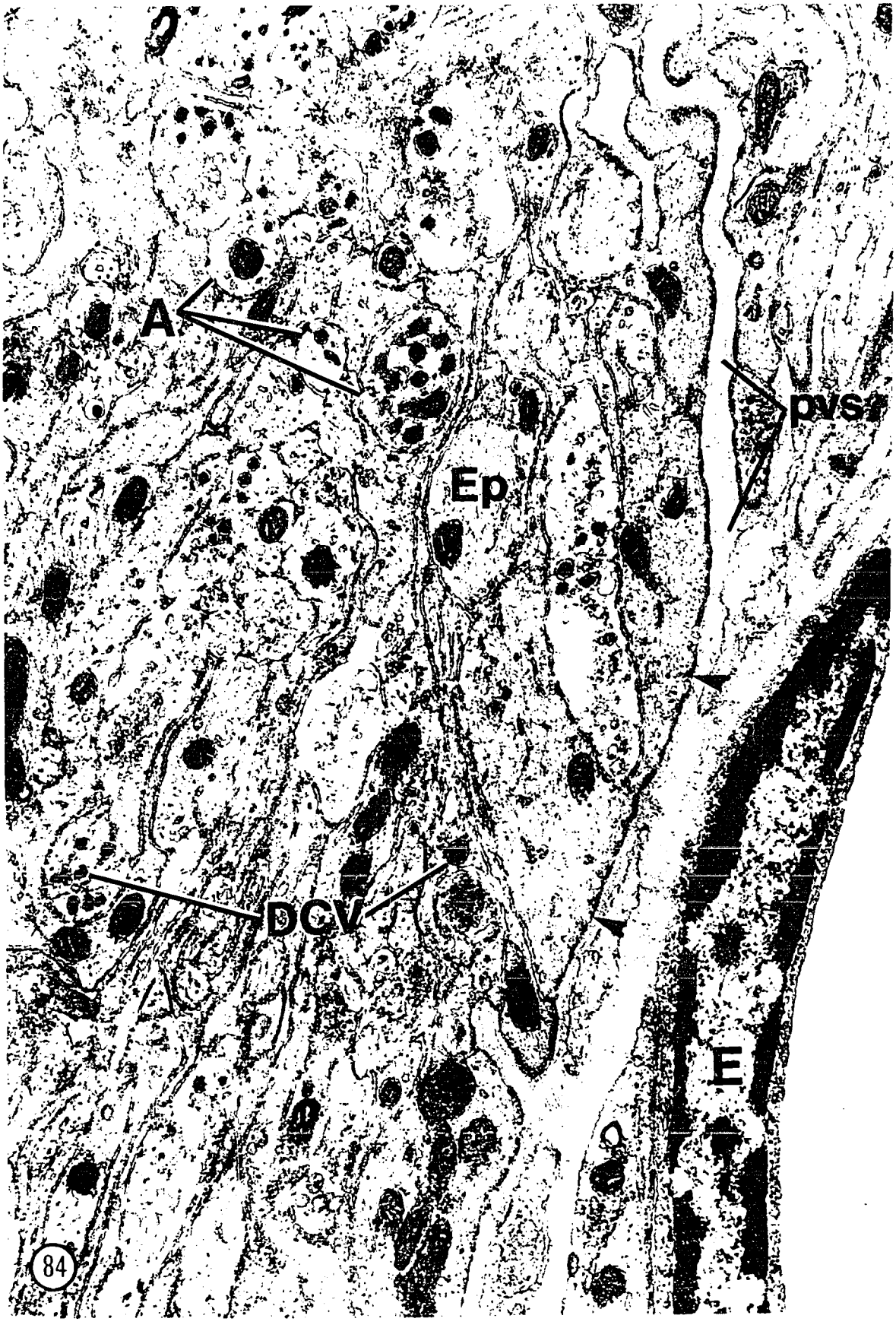

MEDIAN EMINENCE
EXPERIMENTAL GROUP
NUMBER
0
10
20
30
40
50
60
5
7
9
11
13
15
17
19
21
23
25
27
29
DIAMETER (Å) - X 100

Figure 83

Figure 84. Electron micrographs showing a portion of the external layer of the median eminence. The perivascular space (pvs) projects out into the surrounding tissue. Several dark ependymal processes (Ep) terminate on the pvs. A number of axons (A) contain dense-core vesicles (DCV). The protoplasm under the free surface of the ependymal end-feet appears to be condensed (arrows). Ten minutes post-coitus.
E, Endothelial cell.

X 20,100


A
EP
PC
E
84


A
Ep
PVS
DCV
E
84

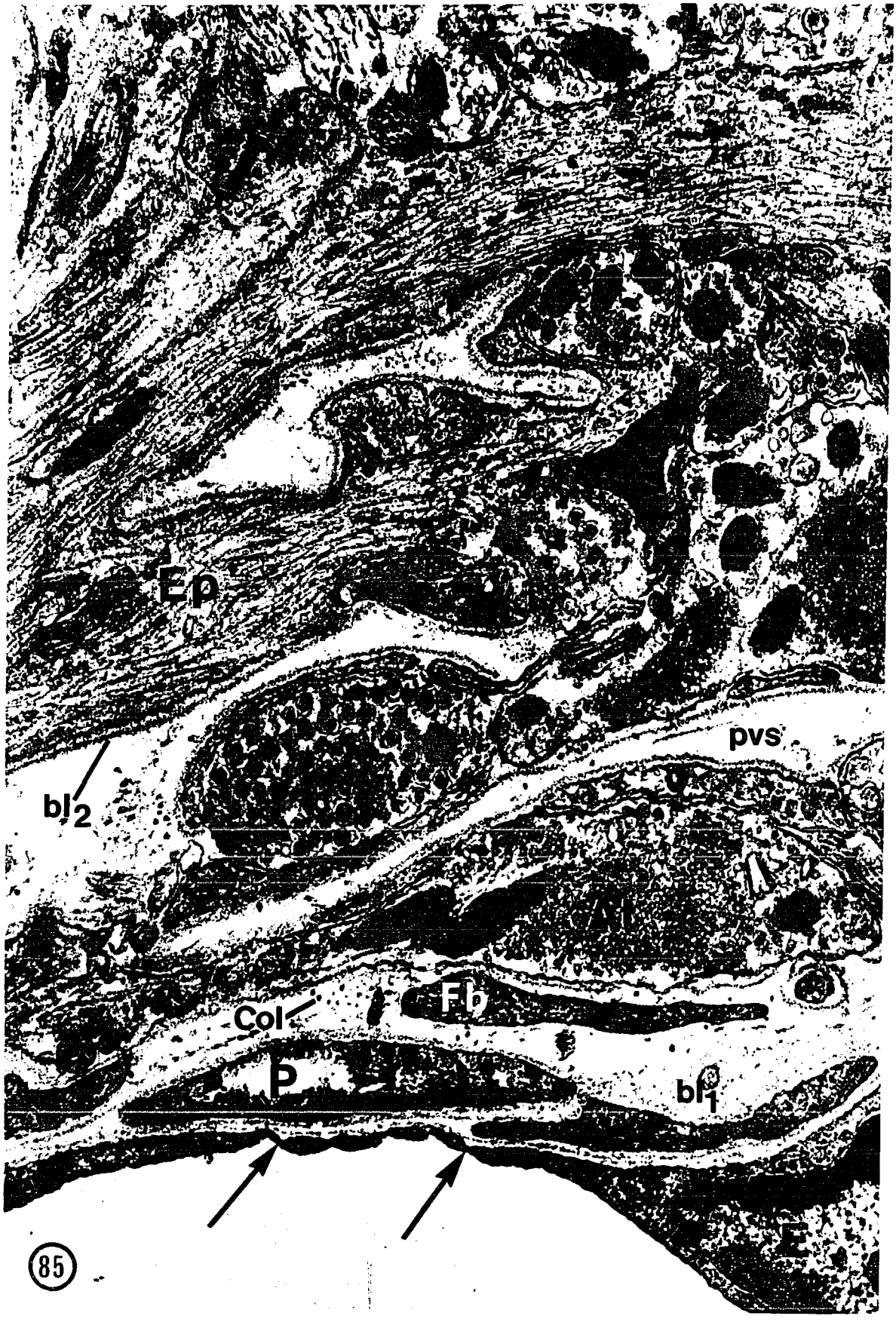
The morphological features of a portion of the wall of a large capillary is shown (Figure 85). The endothelial cells were characterized by numerous pinocytotic vesicles and fenestrations. Endoplasmic invaginations into the lumen of some vessels in the form of villi were not uncommon. The wide, somewhat irregular, perivascular spaces invariably contained cellular profiles identified as those of fibroblasts, as well as scattered clusters of collagen fibers. Axons containing DC vesicles within the perivascular spaces or within the lumen of the capillaries, were not observed. The axon profiles, unlike those of the fibroblasts, were usually surrounded by the 300 Å to 650 Å basal lamina. Elongated cells identified as pericytes are also shown (Figure 85). These elements were typically surrounded by, or contained within the thick basal lamina which coated the perivascular surface of the endothelial cells. Like the latter, the pericytes showed numerous pinocytotic vesicles in both their luminal and perivascular surfaces. The often loosely formed wall of tissue around the perivascular spaces was composed largely of axon terminals that contained dense populations of DC vesicles. Extensions of the basal lamina outward between the processes surrounding the perivascular spaces were common (Figures 84 and 85).

1) Axon terminals of the control animals

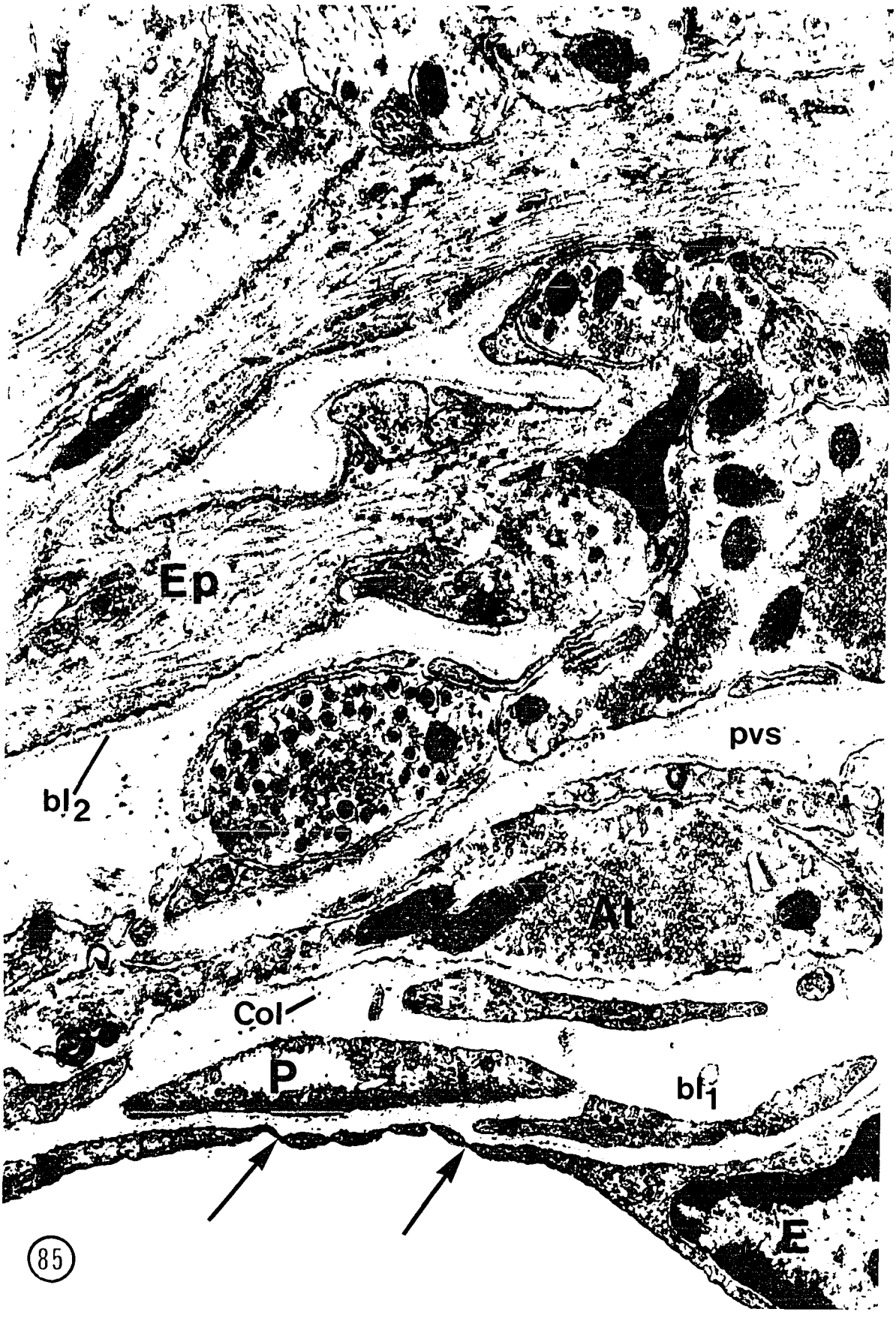
The axon terminals shown in Figures 86 and 87 are representative of those observed lying close to, or in contact with, the perivascular spaces of the non-mated control animals. These terminals were characterized by the presence of relatively homogeneous populations

Figure 85. Electron micrograph of a portion of the wall of a large capillary. Fenestrations (arrows) in the endothelial cells (E) were common. Pericytes (P) are embedded within the endothelial basal lamina (bl_1). A thick perivascular basal lamina (bl_2) separates the perivascular space (pvs) proper from the surrounding neuropil. Fibroblasts (Fb) and collagen (Col) occupy the pvs. Axon terminals (At) and Ependymal processes (Ep) terminate on the pvs. Median eminence, 10 minutes post-coitus.

X 29,500



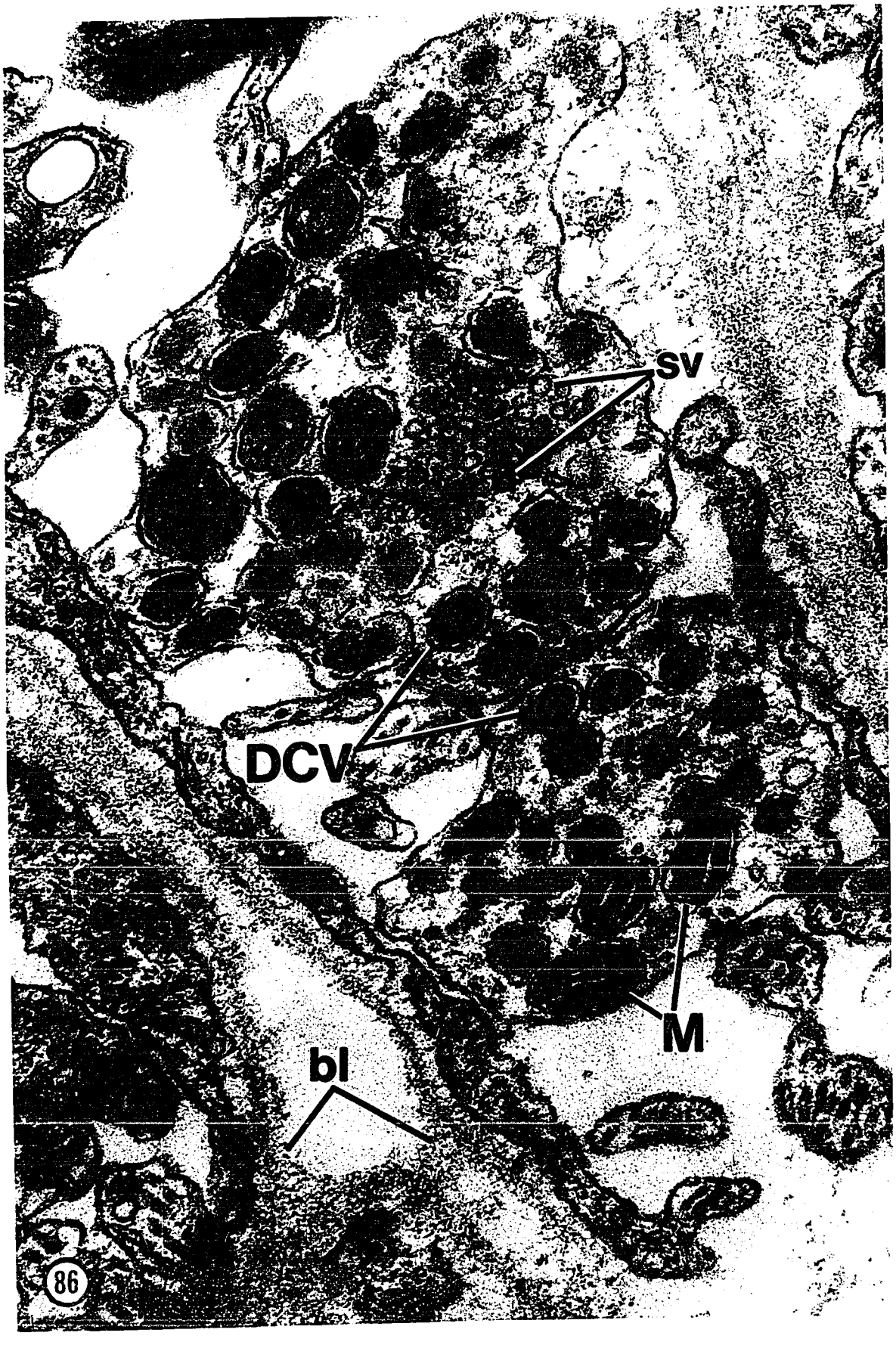
85



85

Figure 86. High magnification electron micrograph of the median eminence external layer showing two axon terminals with relatively homogeneous populations of large dense-core vesicles (DCV). Synaptic vesicles (sv) and mitochondria (M) are common in these terminals. Few synaptic vesicles are in contact with the terminal membrane. Control animal. bl, basal lamina.

X 75,000


SV
DCV
M
bl
86

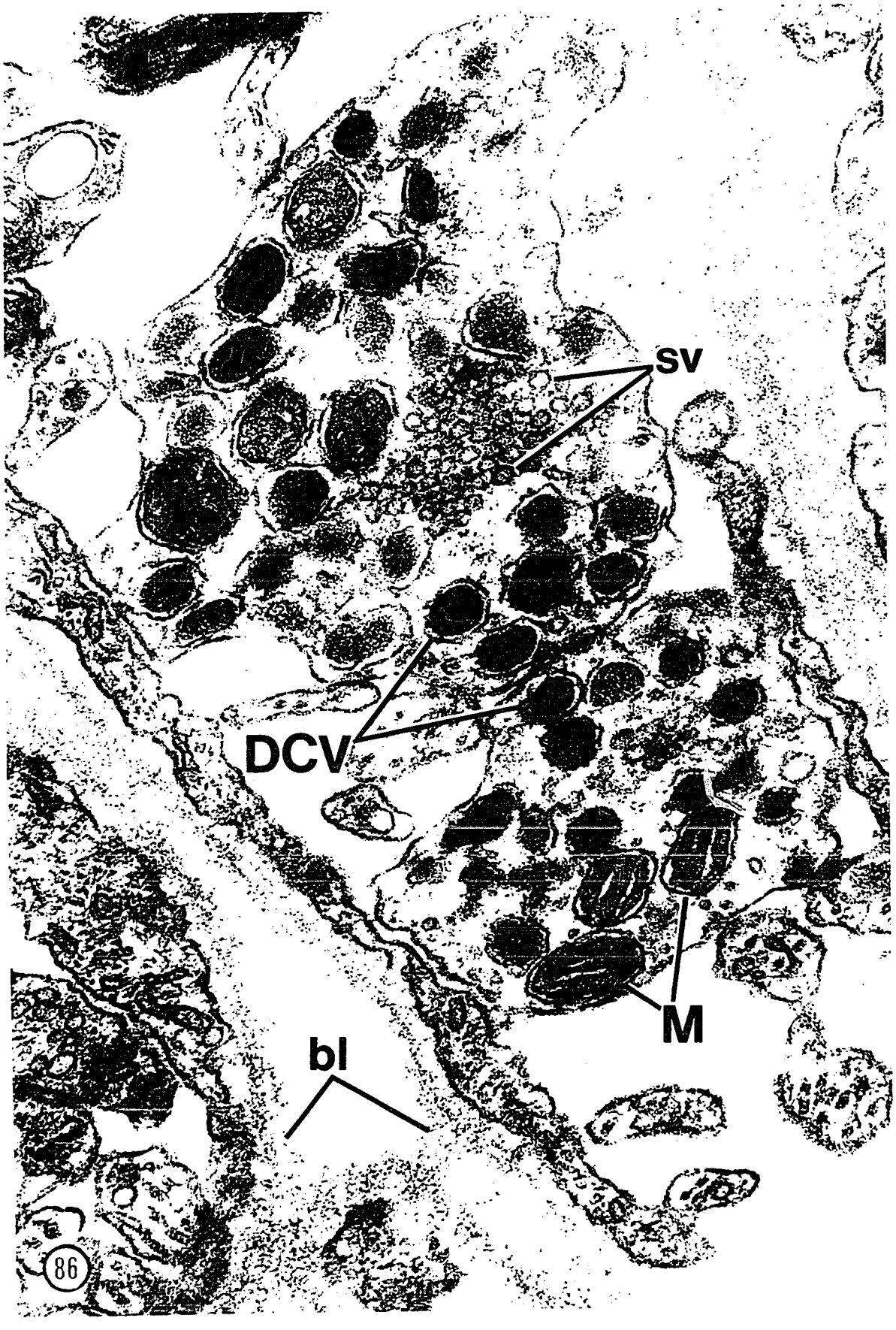
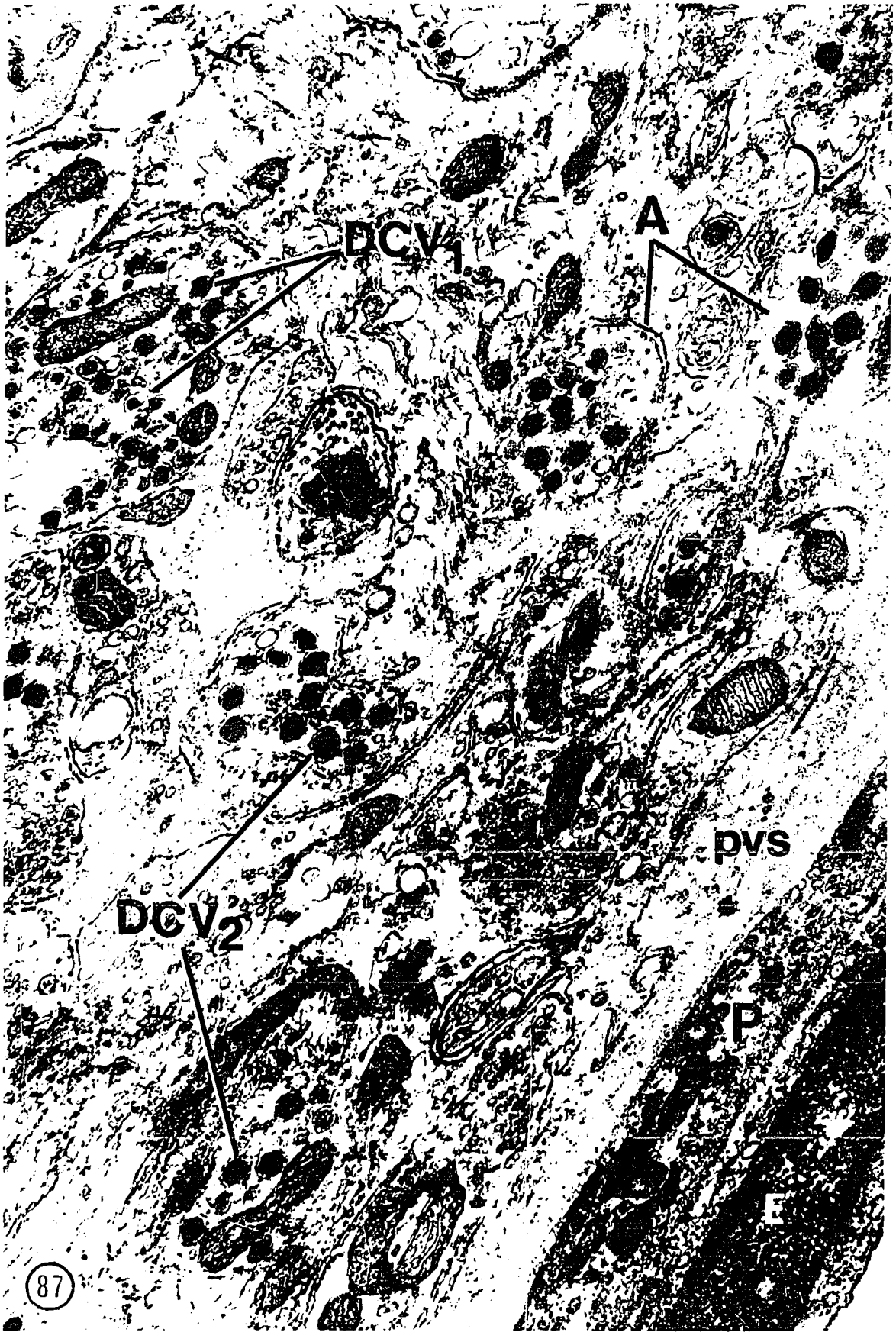

SV
DCV
M
bl

Figure 87. Electron micrograph showing a portion of the median eminence of a control animal. Several large axons (A) located near-, and several terminals in contact with -, the perivascular space (pvs) are shown. Two populations of dense-core vesicles are apparent (compare DCV_1 and DCV_2) and occur within separate axons or their terminals. E, Ependymal cell; P, Pericyte.

X 42,000


PCV
A
PVS
PCV2
E
87


DCV1
A
pvs
DCV2
P
E
87

of either small or large DC vesicles, synaptic vesicles with a diameter range of 350 Å to 700 Å, and mitochondria. Accumulations of electron-dense material under the portion of the terminal membrane exposed to the perivascular spaces were not apparent. The close association between the small synaptic vesicles and this portion of the terminal membrane was also an infrequent finding (Figure 86). Occasionally, membrane profiles, referred to here as vesicle 'ghosts', were observed within the axon terminals lying close to, or in contact with, the perivascular spaces. The DC vesicles within these profiles had a mean diameter of 1205 Å with a range of 540 Å to 1910 Å. A sample of 60 'ghosts' which also occurred within these axons had a mean of 1102 Å, with minimum and maximum diameters of 790 Å and 1710 Å respectively. The distribution curves for the diameters of both the DC vesicles and the 'ghosts' are included in Figure 82.

2) Axon terminals of the experimental animals

The ultrastructural features of the axons and axon terminals shown in Figure 88 are representative of those observed in close spatial relationship to, or in actual contact with, the perivascular spaces of animals sacrificed at 10 minutes post-coitus. As is clearly illustrated, these profiles are now characterized by the presence of large numbers of 'ghosts' which appeared empty or, more typically, contained varying amounts of a fine granular or electron-dense material (Figure 88, inset). These 'ghosts' were, in general, uniformly distributed throughout the axon profiles; accumulations of these structures near the segment of the axon terminal in contact with

the perivascular spaces were not observed. Also, there was no morphological evidence suggesting the coalescence of the 'ghosts' with the terminal membranes.

The smaller vesicles were likewise diffusely distributed throughout these axons and axon terminals. Accumulations of these vesicles under the terminal membrane in contact with the perivascular spaces was only an occasional finding. A number of the axon profiles lying near and, less frequently, in actual contact with the perivascular spaces, contained either relatively homogenous populations of the small or the larger DC vesicles (Figure 89). In these axons, membrane profiles resembling the 'ghosts' commonly observed within the axon terminals in contact with the perivascular spaces, were sparsely represented.

The sample of 205 DC vesicles, primarily those within axon terminals in contact with the perivascular spaces, were measured and recorded. The distribution curve for these diameters is included in Figure 83. Based on this sample, the DC vesicles of the experimental animals had a mean diameter of 906 Å. The minimum and maximum diameters of these vesicles were 570 Å and 1910 Å, respectively. The 'ghosts', based on 205 measurements, had a mean diameter of 1368 Å and a range of 860 Å to 2000 Å.

No changes were apparent in the numbers and/or distributions of the mitochondria and synaptic vesicles within the axons or axon terminals of the experimental groups.

Figure 88. High magnification electron micrograph of several axon terminals in contact with the perivascular spaces (pvs) of the external layer; median eminence, experimental group. Numerous 'ghosts' (gh) representing the limiting membranes of vesicles which have apparently lost their cores of electron-dense material characterize these terminals. Dense-core vesicles (DCV) at various phases of 'emptying' are featured in the inset. The 'ghosts' are uniformly distributed; there is no evidence for their fusion with the terminal membrane. The axon terminal (At) containing the smaller or DCV_1-type dense-core vesicle does not contain 'ghosts'. A desmosome-like structure (arrow) is also shown. These were not common.

X 31,000

DCV
DCV
pvs

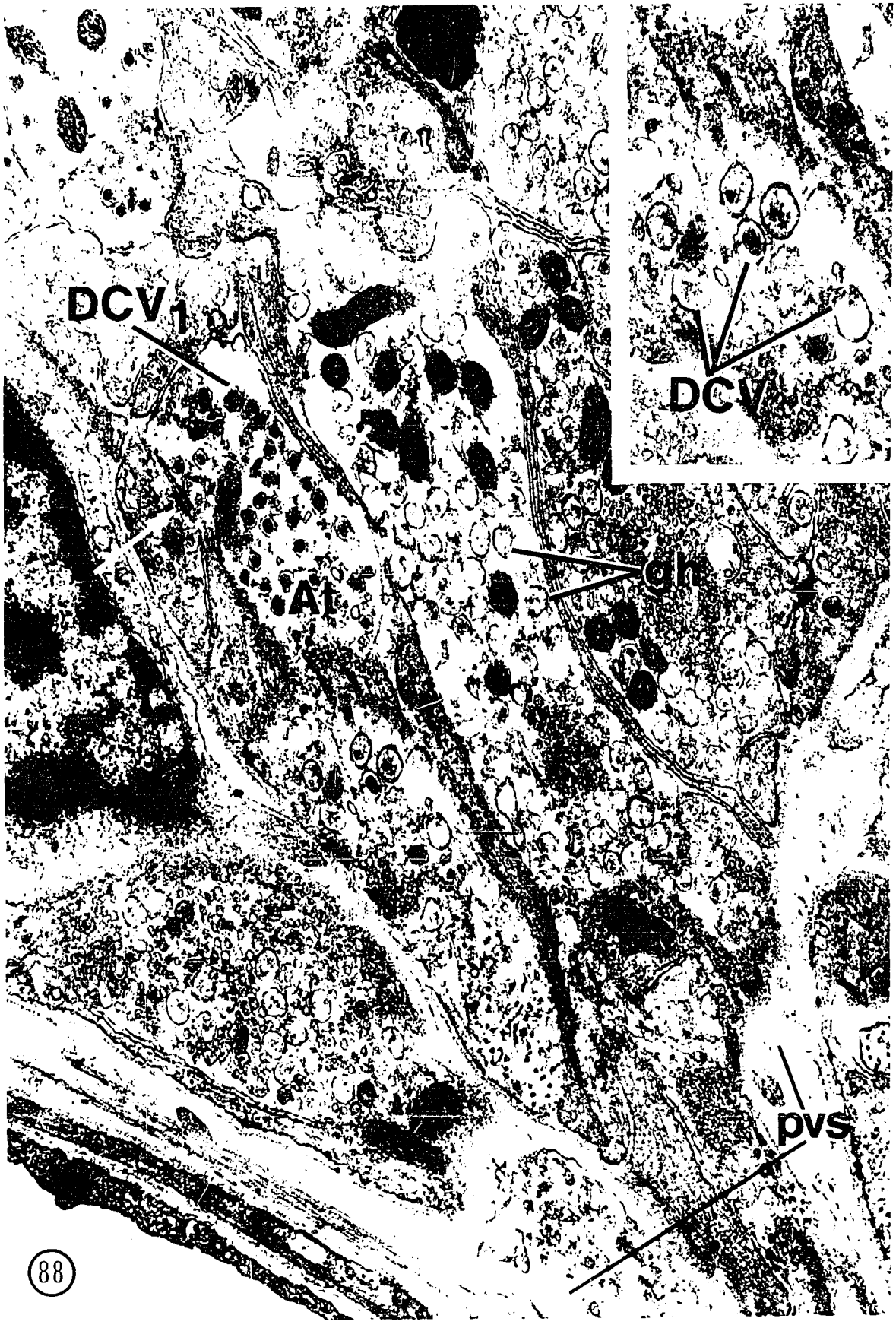
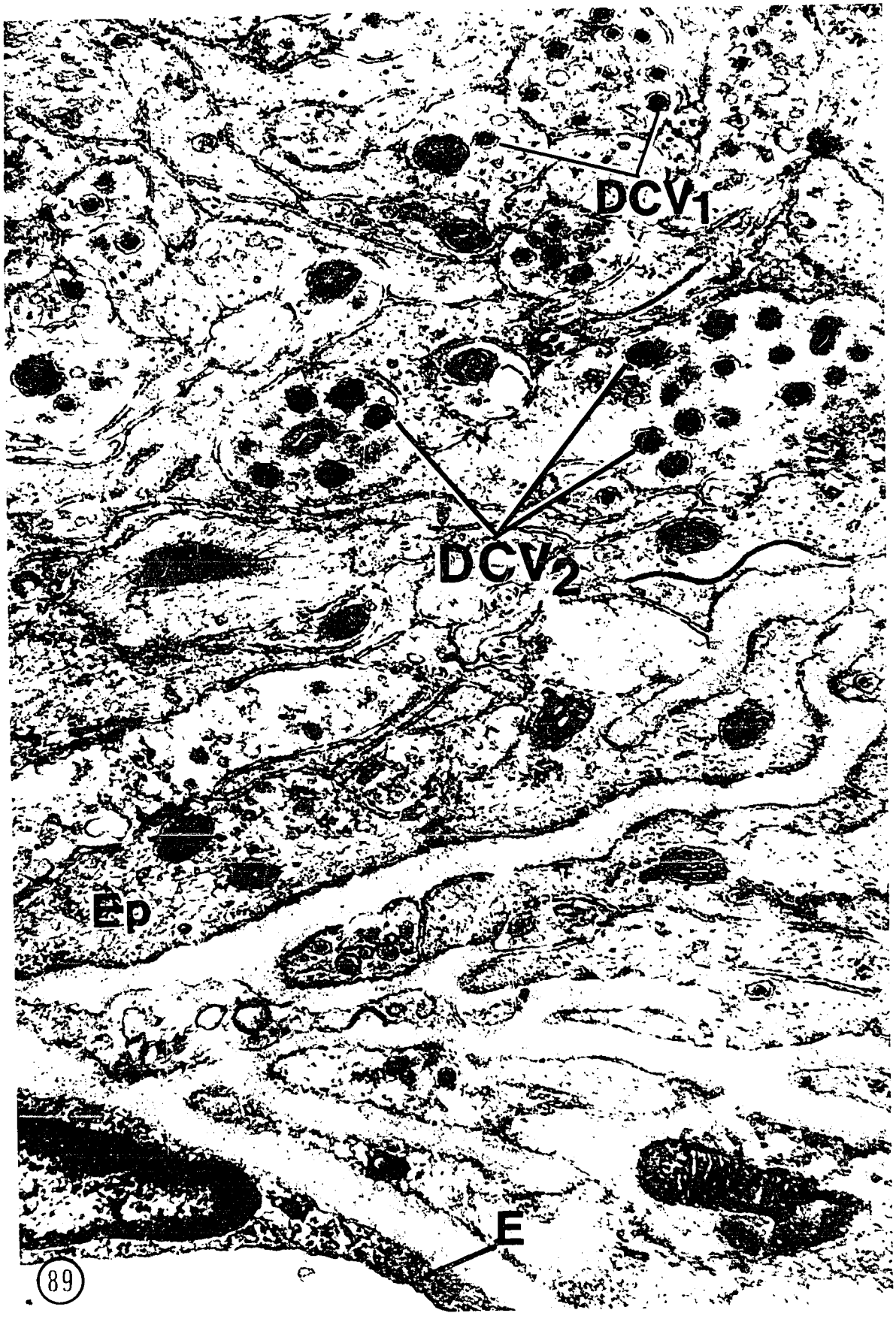

DCV
DCV
gh
At
pvs

Figure 89. Electron micrograph showing a small area of the median eminence external layer of an animal sacrificed 10 minutes following coitus. The perivascular space (arrows) is irregular. A number of large axons not in contact with the perivascular spaces contain a homogeneous population of the DCV_2-type dense-core vesicles. Other axon profiles contain the smaller dense-core vesicles (DCV_1). Ep, Ependymal cell process; E, Endothelial cell.

X 29,900

89



89

V DISCUSSION

The results of this study have clearly demonstrated that ultrastructural changes do occur in the mammalian central nervous system following the physiological stimulus of coitus. These changes, as observed within neurons of the rabbit hypothalamus, were: an increase in neuronal and nuclear diameters, remarkable increases in the amount of rough ER and Golgi membrane, and the occurrence of numerous large DC vesicles within the neuron soma. Increases in the numbers of mitochondria and lysosome-like bodies were also apparent. These changes were observed within large neurons located in close spatial relationship with the capillaries of the PO and SCH nuclei of the experimental animals. Morphologically similar neurons were never observed within these same nuclei removed from the non-mated controls. Similarly, the discrete post-coital ultrastructural changes observed within the PO and SCH nuclei were never observed within the neurons of the arcuate nucleus. We are interpreting the changes observed throughout the present investigation to reflect enhanced neurosecretory activity and that the products of this synthesis are contained within the large DC vesicles.

Changes in the ultrastructure of the SCH and PO neurons first became apparent at one-half hour (Figure 46) and one hour (Figures 26 and 27) respectively, following coitus and became more pronounced at one hour (Figure 47) and two hours (Figure 28)

post-coitus. Except for the apparent increase in the number of lysosome-like structures within neurons of the SCH and PO nuclei studied at four through 10 hours post-coitus, other ultrastructural changes (i.e., the amount of rough ER and Golgi membrane, and the number of large DC vesicles) remained relatively constant. Collectively, the early ultrastructural changes are interpreted as representing a 'preparation' stage for the more 'active' period of synthesis which followed within these neurons. According to our observations on the SCH nucleus, the elaboration of secretory products in the form of DC vesicles, requires a period of approximately one hour. Maximum synthesis occurred between two and six hours post-coitus then continued with possible decline, through to 10 hours. Since we did not study animals that were mated and sacrificed at later intervals (i.e., 15 hours, 20 hours and 30 hours) post-coitus, we cannot provide evidence for the duration of this synthetic activity.

The close association between neurons and the capillaries of the central nervous system has been reported by Scharrer and Scharrer (1940) and interpreted as a manifestation of the synthetic activity in neurosecretory neurons. Zambrano and De Robertis (1967) also observed that many of the 'active' cells within the SO nucleus of the dog were in close spatial relationship with capillaries. Similar findings have likewise been clearly demonstrated in the SO and PV nuclei of the opossum (Brooks, Koizumi and Zeballos, 1966) and in the PV nucleus of the rat (Morris, 1971). The precise functional

significance of this relationship is difficult to determine because of the static nature of electron microscopic studies. We can only speculate that such a relationship might facilitate the exchange of materials or 'information' between neurons and the blood, as suggested earlier by the Scharrers (1940). To date, ultrastructural studies which have dealt specifically with neurosecretory systems of the mammal (Zambrano and Mordoh, 1966; Zambrano and De Robertis, 1966, 1968; Zambrano, 1969; Suburo and Pellegrino de Iraldi, 1969; Morris, 1971; Brawer, 1971; Ratner and Adamo, 1971) seem to provide little evidence that such exchanges do, in fact, occur. Indeed, that this relationship between neurons and capillaries provides a pathway for the release of material contained within DC vesicles of neurosecretory cells, has never been demonstrated. In the present study, large DC vesicles have occasionally been observed near the limiting membrane of neurons. However, pits or other irregularities of the plasma membrane suggesting an exocytotic phenomenon, were never observed.

In the present study, discrete ultrastructural changes were observed within a total of 63 (31.5%) of the 200 neurons measured near capillaries of the SCH nucleus (Figure 40, stippled area), and in 23 (19.2%) of 120 neurons located near capillaries of the PO nucleus (Figure 10, stippled area). Are we in fact observing two different populations of neurons, or different functional states of the same kind of cell? (Zambrano and De Robertis, 1968). If we consider the possibility of observing two functionally different populations of cells, the first type of cell would be represented by those neurons that did not show changes following coitus, while the

second type would include all those neurons in which post-coital changes were observed.

Two distinct neuron types, commonly referred to as 'pale' and 'dark' on the basis of certain morphological criteria, have been observed in control neurons of the rat arcuate nucleus (Jaim Etcheverry and Pellegrino de Iraldi, 1968; Zambrano and De Robertis, 1968; Ratner and Adamo, 1971). 'Pale' and 'dark' neurons have also been observed in the rat SCH nucleus (Suburo and Pellegrino de Iraldi, 1969), SO nucleus (Zambrano and De Robertis, 1966; Zambrano and Mordoh, 1966; Rechardt, 1969), and PV nucleus (Morris, 1971), the SO nucleus of the dog (Zambrano and De Robertis, 1966), and in the ganglion cell layer of the retina (Pellegrino de Iraldi and Jaim Etcheverry, 1967). To our knowledge, no one has previously described the ultrastructure of the mammalian PO nucleus. According to Jaim Etcheverry and Pellegrino de Iraldi (1968), the functional significance of these neuron types is obscure. Zambrano and De Robertis (1968), on the other hand, have provided substantial evidence that the morphological differences observed between these cells do not represent two distinct neuron types, but are functional states of the same kind of cell. This concept was based on the fact that following castration in the rat, the perikarya of the arcuate neurons hypertrophied and showed a marked increase in the amount of rough ER and Golgi membrane, as well as an increase in the number of granulated vesicles. Similar changes within arcuate neurons of the rat following castration have also been reported by Brawer (1971). That these changes are in fact associated with gonadotrophic activity is strongly

supported by the work of Zambrano (1969) on the cycling female rat. Similarly, other investigators (Zambrano and De Robertis, 1966; Zambrano and Mordoh, 1966; Suburo and Pellegrino de Iraldi, 1969; Morris, 1971; Brawer, 1971), who have observed morphologically similar neurons within other hypothalamic nuclei, have correlated their findings with neurosecretory phenomenon.

Throughout the present study, neurons with distinct morphological differences were observed within the arcuate nuclei of both the non-mated control and mated experimental groups, and within certain neurons of the PO and SCH nuclei of the experimental animals. Since two distinct types of neurons were never observed within the PO and SCH nuclei of the non-mated control animals, it is likely that the ultrastructural changes observed within these nuclei following coitus represent functional states of the same kind of cell and not two functionally different cell populations.

If the post-coital ultrastructural changes observed within neurons of the PO and SCH nuclei do represent functional states of the same kind of cell, why were they evident within only 19.2% and 31.5% of the neurons located near capillaries of the PO and SCH nuclei respectively? Supposing that the synthetic activity within these neurons is controlled by hormonal feed-backs through the systemic circulation, one would expect to find a greater number of these neurons showing evidence of enhanced synthesis. This, however, is purely speculation and may be explained by the fact that the neurons controlling the hypothalamo-adenohypophyseal axis exhibit some degree

of functional asynchrony, with only a portion of the neuron population responding at any one time to a given stimulus. This, of course, cannot be concluded with the data obtained from the present study, but is supported by the observations of others. Zambrano and De Robertis (1966) noted that within the SO nucleus of the rat, extreme types of neurons (those with well developed rough ER and Golgi systems, etc., and those without) were present within both their control and experimental animals. These findings were interpreted to represent a steady state of activity among the SO neurons, with the level of secretory activity being conditioned to the neurohormonal needs of the organism. From our findings within the PO and SCH nuclei of the rabbit, a similar 'steady state of activity' obviously does not occur at all times among the neurons of the PO and SCH nuclei since discrete ultrastructural changes suggesting synthetic activity were never observed within our control animals. This concept, however, might be applied to the neurons of the rabbit arcuate nucleus since both neuron types (pale and dark) were observed in approximately equal numbers within both the control and experimental animals.

There is no doubt in our minds that the neuronal changes we describe do represent enhanced synthetic activity. Many studies, both at the light and the electron microscopic levels, have related increases in neuronal, nuclear and nucleolar sizes, as well as increases in the amounts of Nissl material and granules with enhanced neurosecretory activity (Ortmann, 1951; Kovács, Backrach, Jacobits, Haváth and Korápssy, 1954; Gabe and Arvy, 1960; Rensing, 1964; Bloch,

Thomsen and Thomsen, 1966; Zambrano and Re Robertis, 1966, 1968; Zambrano and Mordoh, 1966; Suburo and Pellegrino de Iraldi, 1969; Morris, 1971). Conversely, decreases in both neuronal and nuclear sizes have been related to a reduction in synthetic activity within neurons (Leveque, 1953; Wolter, 1956).

We could find no evidence of enhanced synthetic activity within the neurons of the arcuate nucleus following coitus. If anything, both the neurons and their nuclear profiles here were smaller than those of the controls (Tables 1 and 2). These findings are not in agreement with those of Zambrano and De Robertis (1968) and Brawer (1971) who observed that following castration in the rat, the neurons of the arcuate nucleus and their nuclei undergo considerable hypertrophy. The significance of these observations on the arcuate nucleus will be referred to again later in this discussion.

Morphologically, the neuronal nuclei observed throughout this study conform to the conventional descriptions of nerve cell nuclei as described by Fawcett (1967) and Peters, Palay and Webster (1970). Several findings of interest within the nuclei of these cells were the frequently observed contacts between the nucleolus and the inner membrane of the nuclear envelope, the incidence of the intranuclear rodlet and the deep nuclear indentations of clefts.

Contacts between the nucleolus and the nuclear envelope have been previously reported both at the light (Scharrer and Scharrer, 1940) and electron microscopic levels (Zambrano and De Robertis, 1968). These investigators have interpreted this

relationship to reflect elevated synthesis within neurons. Within the present study, such contacts (Figures 44 and 66) were commonly observed within the neuronal nuclei of both the control and experimental animals. Such nucleolar contacts were in fact more common among the neurons of the experimental animals not showing changes following coitus, than in those neurons that did. We find it difficult, therefore, to accept this morphological observation as evidence of enhanced synthetic activity within neurosecretory neurons.

Intranuclear rodlets of the type shown in Figures 44, 64, 66, 77, 78 and 79 have been previously described in a variety of cells under both normal and pathological conditions (Chandler and Willis, 1966; Karlsson, 1966; Magalhaes, 1968; Boquist, 1969; Lane, 1969; Masurovsky, Benitez, Kim and Murray, 1970; Kim, Masurovsky, Benitez and Murray, 1970; Dahl, 1970). In the present study, this intranuclear inclusion occurred within neurons of all of the hypothalamic nuclei we have investigated with the electron microscope. As this was an incidental observation made while studying the effects of coitus on the ultrastructure of the rabbit hypothalamus, we can offer no conclusive evidence for its origin or possible functional significance. That the intranuclear rodlet is formed by the nucleolus has been suggested (Boquist, 1969; Masurovsky *et al.*, 1970; Kim *et al.*, 1970). Although close spatial relationships between these rodlets and the nucleolus were occasionally observed (Figures 78 and 79), we saw no convincing ultrastructural evidence suggesting the origin of the rodlet from this organelle.

The functional significance of these intranuclear rodlets is likewise unknown. That they may represent 'viral footprints' has been suggested (Bernhard and Granboulan, 1963; Patrizi and Middelkamp, 1969; Stefani and Tonak, 1970; Buck, 1971). Others (Sotelo and Palay, 1968; Popoff and Stewart, 1968; Boquist, 1969; Dahl, 1970; Seite, 1970), have related their presence to the metabolic activity of the cell. Throughout the present study, the neurons we were studying were, to the best of our knowledge, normal, healthy cells. We have no reason therefore to suggest, or even consider, that the rodlets observed represented present or previous viral infections. On the contrary, the frequency with which these rodlets were observed within the neurons of the arcuate, PO and SCH neurons of the rabbit (Table 6) strongly suggests that they are in fact normal components of these cells. Furthermore, our observations show quite clearly that there is an inverse relationship between the synthetic activity of these neurons and the occurrence of the intranuclear rodlet. Note (Table 6) that the intranuclear rodlet was never observed within the nucleus of a neuron displaying the ultrastructural changes normally observed following coitus. Although our findings are negative in nature, they do strongly suggest that the incidence of the intranuclear rodlet in neurons is related to functional activity (Clattenburg, Singh and Montemurro, 1972).

Nuclear clefts or deep invaginations of the nuclear envelope of neurons has also been associated with enhanced synthetic activity (Scharrer and Scharrer, 1940; Zambrano and De Robertis, 1968). Clefting of the nuclear envelope has been a common finding throughout

the present study within the neurons of both the non-mated and mated animals (Figures 7 and 26). The fact that these clefts were generally more elaborate within many neurons of the experimental groups displaying other morphological features of enhanced neurosecretory activity (compare Figures 24 and 26), tends to support the view that they may be related also to enhanced synthetic activity. According to the Scharrers (1940) "....it is understandable that nerve cells with an additional secretory activity may have nuclei, the surface of which is enlarged by lobation or subdivision, since it is commonly assumed that the vitally important interactions between nucleus and cytoplasm increase with an increased rate of metabolism. The observation of lobulated or muptiple nuclei is in itself no proof of a secretory activity of the cells, but it is in favour of an interpretation such as has been suggested....".

Throughout this investigation, one of the most consistent findings within the perikarya of the large neurons located near capillaries of the PO and SCH nuclei of the experimental animals, was the presence of very elaborate rough ER and Golgi systems (Figures 28, 29, 47, 48 and 49). These observations are presently interpreted as morphological evidence for the elevated synthetic activity within these neurons and are in agreement with the findings of many previous investigators (Palay, 1960; Scharrer and Brown, 1961; Caro and Palade, 1964; Zambrano and De Robertis, 1966, 1968; Zambrano and Mordoh, 1966; Jamieson and Palade, 1967; Suburo and Pellegrino de Iraldi, 1969; Iijima, 1970; Morris, 1971) who have also studied neurosecretory neurons and/or a variety of other protein secreting cells. Furthermore,

that rough ER and Golgi systems undergo an apparent period of development before the secretory products actually appear within the soma of these neurosecretory cells is evident from our findings within SCH and PO neurons removed at one-half hour (Figure 46) and one hour (Figures 26 and 27) post-coitus, respectively.

Similar ultrastructural changes within the rough ER and Golgi systems following coitus were never observed within neurons of the arcuate nucleus. Although well developed rough ER and Golgi profiles did characterize many of the 'dark' neurons observed within this hypothalamic nucleus (Figures 68, 69 and 75), the fact that these organelles were more highly developed within the 'dark' neurons of the experimental animals than within those of the controls, was not apparent. Furthermore, we have no evidence for a possible increase in the number of 'dark' neurons within the arcuate nuclei of the mated groups. We can only conclude that the neurons of this hypothalamic nucleus do not undergo ultrastructural changes within the first 10 hours following the stimulus of coitus. Certainly our findings within the arcuate nucleus of the rabbit are not in agreement with the elaborate development of rough ER and Golgi systems within arcuate neurons as reported independently by Zambrano and De Robertis (1968) and Brawer (1971) following castration in the rat.

How do the times at which intracellular changes were observed within the SCH and PO neurons of the rabbit compare with those reported within secretory cells by other investigators? Zambrano and De Robertis (1966), while studying the secretory cycle

of the rat SO nucleus following dehydration, observed that changes in the rough ER, the number of ribosomes and the Golgi profiles began to appear at 48 hours. Also, at 48 hours, dense accumulations of granular material became evident within the ER cisternae. Neurosecretory granules, however, did not reappear until approximately 60 hours after the rats were placed on dry food diets. Although the events leading to the formation of membrane-bound vesicles are similar within the two systems, we probably should not compare the time differences for these changes on the basis of the nature of the stimuli controlling the onset of secretion within these systems. Similarly, elaboration of the rough ER and Golgi systems, and a marked increase in the number of ribosomes were observed within rat arcuate neurons at one month following castration (Zambrano and De Robertis, 1968). By six months after castration, all of the neurons within the arcuate nucleus had greatly increased in diameter, the ER was even more highly developed and contained an abundance of granular material, and numerous DC vesicles (800 Å - 1300 Å in diameter) were present within the cytoplasm. That ultrastructural changes within these neurons do undergo relatively early changes following castration has since been confirmed by Brawer (1971).

Perhaps the most accurate accounts of the events which occur during the secretory process, have resulted from studies using labelled amino acids. Kent and Williams (1971), using EM autoradiographic techniques, observed that ^{3}H-cysteine labelling within secretory neurons of the rat PV and SO nuclei was maximum at one to two hours, and fell to a minimum at 17 to 20 hours. The fact that

synthesis continued for at least 10 hours from the point of labelling agrees with our findings within the neurons of the PO and SCH nuclei of the rabbit. Certainly one of the most recent studies describing the uptake of labelled leucine (^{3}H-leucine), its movement from the points of uptake through the cisternae of the rough ER and saccules of the Golgi system, and subsequent incorporation into secretory granules, is that of Jamieson and Palade (1967). After seven minutes labelling appeared over the periphery of the Golgi, and at 37 minutes it began to appear over the condensing saccules of this organelle. At minute 57, many labels occurred over the membrane-bound granules, especially those in close spatial relationship with the Golgi profiles. According to Palay and Palade (1955), the sequence of events which occur in other protein secreting cells can also be applied to the neuron. Assuming this to be so, our findings in the present study with regard to the sequence of events leading to the presence of the large DC vesicles, correspond very closely with times reported by Jamieson and Palade (1967).

Although the nature of our study does not provide morphological evidence for the functional role of the ER membranes and attached ribosomes, it is generally accepted that the raw materials ultimately packaged within membrane-bound vesicles of secretory cells are manufactured within these organelles (Siekevitz and Palade, 1958, 1960; Jamieson and Palade, 1967). The macromolecular material commonly observed within the ER cisternae throughout this study is interpreted to represent newly synthesized materials for packaging by the Golgi system and is in agreement with the interpretations of

others regarding the nature of this material (Palay, 1960; Scharrer and Brown, 1961; Dalton, 1961; Jamieson and Palade, 1966; Zambrano and De Robertis, 1966, 1968; Beams and Kessel, 1968; Holtzman and Dominitz, 1968). Furthermore, Zambrano and De Robertis (1966) observed that puromycin inhibited the formation of the granular material normally seen within the rough ER cisternae of neurons within their dehydrated rats.

It seems that our observations on the rough ER and Golgi system within the present investigation are in agreement with the current widely held view that the raw materials for export are synthesized by the membranes and associated ribosomes of the rough ER, pass through the cisternae of this organelle for transfer to the saccules of the Golgi system where it is condensed and packaged into the secretory granule. We conclude therefore that the DC vesicles having a mean diameter of 1343 Å and 1416 Å, respectively, and which were first observed within the large neurons of the SCH and PO nuclei at one and two hours post-coitus, do represent the Golgi condensations of the macromolecular material synthesized by these cells.

The mechanism by which the granular content of the rough ER cisternae reaches the Golgi saccules is uncertain. Direct membrane continuations between these organelles within neurosecretory cells have, to date, not been clearly demonstrated. It would seem therefore that the transfer of the newly synthesized materials from the rough ER to the Golgi system is a physiological one, and that the vehicle for this transport is the agranular vesicle of the type shown in Figures 32, 51 and 52. These structures, because of their proposed transport

function, have been termed 'transition elements' (Ziegal and Dalton, 1962) or 'shuttle carriers' (Caro and Palade, 1964; Jamieson and Palade, 1967). Whether they originate from the rough ER and move to the forming face of the Golgi where they coalesce to form the saccules of this organelle (Ziegal and Dalton, 1962; Caro and Palade, 1964; Redman, Siekevitz and Palade, 1966; Jamieson and Palade, 1967), or whether they originate from the Golgi system and serve as the transport vehicles (Novikoff, Essner and Quintana, 1964; Hirsch and Cohn, 1964; Friend and Farquhar, 1967; Peters *et al.*, 1970) is currently a controversial issue.

Another type of small vesicle, commonly referred to as 'alveolate' or 'coated vesicles' and distinguished from the smooth vesicles just described by the presence of a granular material attached to their limiting membrane, have also been observed near the Golgi profiles of many neurons (Figure 32). This vesicle type, however, was only observed in very low numbers. Whether or not they represent an immature form of the smooth vesicles observed within the same area is only speculation. The function of the 'coated vesicles' is likewise uncertain. According to the reports of other investigators (Novikoff, 1967; Holtzman, Novikoff and Villaverde, 1967; Holtzman, 1969; Peters *et al.*, 1970), this vesicle type originates from the Golgi system. Their function in the transport of hydrolytic enzymes from this organelle to primary lysosomal structures such as the multivesicular body has been suggested (Friend and Farquhar, 1967). The presence of reaction product within the 'coated vesicle' of neurons suggesting acid phosphatase activity (Holtzman *et al.*, 1967)

tends to support this view.

During the present investigation, the lysosome-like body was a common finding within the neuron soma of all hypothalamic nuclei studied from both the non-mated control and mated experimental animals (Figures 24 and 35, respectively). There was, however, an apparent increase in the numbers and size of these electron-dense bodies, particularly within the large synthetically active neurons of the PO and SCH nuclei removed at six hours through to 10 hours post-coitus (compare Figures 46 and 50). The lysosome-like body was also more abundant within the soma of the 'dark' neurons of the arcuate nuclei of both the control and experimental animals (Figures 68, 69 and 74). According to Holtzman (1969), the specific function of the neuronal lysosome is largely unknown. That they provide the enzyme systems for the 'clean-up' of excess membrane, secreted materials, etc., following periods of enhanced activity within the neuron has been suggested (Zambrano and De Robertis, 1968). That the lysosome is associated with similar activities within all neurons has also been reported (Holtzman, 1969; Koenig, 1969). Our observations on the occurrence of lysosome-like bodies within the neurons of the rabbit hypothalamus tend to support the views of these investigators.

The large MVB shown in Figures 35 and 53 are morphologically identical to those previously observed in other neurons (Palay and Palade, 1955; Palay, 1960, 1963; Holtzman *et al.*, 1967; Koenig, 1969; Holtzman, 1969). According to Holtzman (1969), the origin of the MVB is problematic. There is presently little morphological evidence supporting their origin from the Golgi system or from the cisternae

of the rough ER. Several investigators (Palay, 1963; Rosenbluth and Wissig, 1964; Holtzman *et al.*, 1967) have suggested their origin from the cell membrane. As far as we can determine, our observations do not support or contradict either of these views. Obviously, the origin of this organelle requires further study.

The function of the MVB is also unknown. Sotelo and Porter (1959) have suggested that the small, agranular vesicles commonly observed near the Golgi system of many cell types, originate from this organelle. Our findings do not support this concept and are in agreement with the observations of Palay (1960) on the PO nucleus of the goldfish. It seems that the current and most widely held view regarding the function of the MVB is that they represent lysosomes and therefore participate in the enzymatic digestion of endogenous and/or exogenous materials within the cell (Novikoff, 1967; Holtzman *et al.*, 1967).

Increases in the number of mitochondria first became evident in SCH and PO neurons removed at one-half hour (Figure 46) and one hour (Figures 26 and 27), respectively. Dense populations of this organelle also characterized the large neurons located near capillaries of the SCH and PO neurons removed at all subsequent times post-coitus, as well as within the 'dark' neurons of the arcuate nuclei of both the control and experimental animals. These findings are also interpreted to reflect the enhanced synthetic activity within these neurons - the energy for this enhanced synthetic activity supplied by the oxidative reactions which occur within this organelle.

During the present investigation, four distinct populations of 'cored' vesicles were observed. Of these, two populations clearly showed cores of a light, granular material, while the other two vesicle populations were characterized by their cores of electron-dense material. Furthermore, the vesicles with cores of light, granular material were generally larger (i.e., with mean diameters exceeding 1900 Å) than those with well defined cores of electron-dense material. The mean diameters of these vesicles were less than 1500 Å.

The large, light core (LC) vesicles identical to those shown in Figure 52 were only observed within the large neurons of the SCH nucleus which clearly demonstrated other ultrastructural changes following coitus. The chemical nature of these vesicles is currently unknown. We have considered the possibility that these LC vesicles may represent the immature form of the large (1343 Å) DC vesicles observed within the SCH neurons after coitus. However, the fact that the LC vesicles were never observed in the process of 'pinching-off' from the Golgi systems of these neurons, tends to oppose this view. Furthermore, the material we observed within the saccules of the Golgi systems was always more electron-dense than the material forming the cores of the light vesicles. The fact that the content of the LC vesicles was not unlike the material commonly observed within the cisternae of the rough ER may suggest their origin from this organelle (Figure 52). The presence of intermediate forms (Figure 53) may also suggest that the large DC vesicles attain their mature morphological features by a 'condensation' process not unlike that reported to occur within the Golgi system.

The second population of vesicles characterized by their core of a light, granular material was observed within the axon profiles and Herring bodies of the internal layer of the ME (Figure 81). Although similar in size, these vesicles differed from those observed within the SCH neurons of the experimental animals in that their cores of light, granular material were typically separated from a limiting membrane by a wide 'halo'. Whether or not these vesicles are formed within the soma of SCH neurons and develop their 'halo' as they migrate from the SCH area toward the ME cannot be concluded from our observations. The fact that LC vesicles with distinct 'halos' were not observed within axon profiles of the SCH nucleus seems to suggest that this is not so. Furthermore, there is presently unequivocal evidence that the large vesicles within the internal layer of the mammalian ME carry the neurohypophyseal hormones, and that these vesicles have their origin within the neuron soma of the PV and SO nuclei (Rodriques, 1972). This concept is further supported by electron microscopic studies in which LC vesicles (commonly called 'elementary secretory granules' for this system) identical to those we have observed within the internal layer of the rabbit ME, have also been described within the soma of the PV and SO nuclei (Zambrano and De Robertis, 1966; Recherdt, 1969; Morris, 1971).

The two populations of DC vesicles had mean diameters of approximately 1000 Å and 1400 Å, respectively. The smaller DC vesicles were observed within the neuron soma, axon profiles and axon terminals of all three hypothalamic nuclei we have studied with the

electron microscope. These vesicles were also observed within the axons of both the internal and external layers of the ME (Figures 81 and 85). The large DC vesicles only occurred within the soma of the PO and SCH neurons which clearly displayed other ultrastructural changes following coitus, and within a relatively small number of the axon profiles of these nuclei removed at four hours through to 10 hours post-coitus (Figures 25 and 42). Among the control animals, similar DC vesicles were only observed within the axon terminals of the external layer of the ME (Figures 86 and 87).

The small (1000 Å) DC vesicles likely contain the biogenic amines. The presence of NA and 5-HT terminals within the anterior regions of the mammalian hypothalamus and PO area seems to be well established (Pellegrino de Iraldi, Duggan and De Robertis, 1963; Fuxe, 1965; Hökfelt, 1967, 1970; Fuxe and Hökfelt, 1968, 1969, 1970; Zambrano, 1970). Whether or not the 77 small DC vesicles we have observed within the soma of PO neurons of the control animals (Table 5) also contain one or both of these amines, cannot be concluded on the basis of our findings. According to others (Andén, Dahlström, Fuxe and Larsson, 1965; Andén, Dahlström, Fuxe, Olson and Ungerstedt, 1966a; Andén, Dahlström, Fuxe, Larsson, Olson and Ungerstedt, 1966b) the NA fibers and terminals within the hypothalamus and PO area, probably originate from NA nerve cell bodies located within the pons and medullary regions of the brain stem.

The small DC vesicles we observed within the neuropil and terminals of the SCH nucleus may carry the indolamine 5-HT. Using histochemical techniques, Fuxe and Hökfelt (1970) and others (Fuxe,

1965; Aghajanian, Bloom and Heard, 1969) have demonstrated high concentrations of this amine within axon terminals of the rat SCH nucleus. Again, whether or not the 73 DC vesicles with a mean diameter of 1059 Å (Table 5) and located within the soma of the SCH neurons, also contain or carry this amine, is only speculation. Several investigators have independently reported that the 5-HT terminals within the hypothalamus, as well as within the anterior region of the ME, arise mainly from 5-HT cell bodies located within the mesencephalon (Dahlström and Fuxe, 1964; Fuxe, 1965). Furthermore, according to Dahlström and Fuxe (1964), there are no monoaminergic neurons within the SCH nuclei of the rat. Whether this applies to the SCH nuclei of all mammals is not known.

Certainly, to date, the hypothalamic nucleus studied most extensively, both histochemically and morphologically, has been the arcuate. Probably the close spatial relationship between this hypothalamic nucleus and the ME, and the possible role it may play in the control of gonadotrophic function stimulated many of these studies. It has been demonstrated beyond reasonable doubt that the axons of arcuate neurons end in the outer layer of the ME (Fuxe and Hökfelt, 1967; Szentagothai, Flérko, Mess and Halász, 1968) and in contact with the vessels of the portal system (Zambrano and De Robertis, 1968). Although the DC vesicles observed within the soma of arcuate neurons throughout the present study had a greater diameter range (500 Å - 2000 Å, see Table 5), the majority of these vesicles fell within the 800 Å - 1300 Å (Figure 71) described by Jaim Etcheverry and Pellegrino de Iraldi (1968) and Zambrano and De Robertis (1968).

Whether or not the axons of the arcuate neurons we have observed project ventrally into the external layer of the ME is difficult to conclude. However, the presence of axon terminals within this layer of the ME with homogeneous populations of similar DC vesicles (Figures 85 and 88) tends to support this view.

In the present investigation, axons with large populations of similar DC vesicles (Figure 58) were also observed within the neuropil of the arcuate nuclei of both control and experimental animals. Whether these DC vesicles eminate from the neurons of the arcuate nucleus or from neurons located elsewhere in the CNS cannot be concluded from our findings. However, Fuxe (1964), using fluorescence techniques for catecholamines, showed that the neurons of this nucleus have catecholamines located in their perikarya and nearby axons. Since the early work of Fuxe (1964), many studies have demonstrated that the arcuate neurons and axons, as well as the outer zone of the ME, are rich with the catecholamine DA (Aghajanian and Bloom, 1966; Fuxe and Hökfelt, 1967; Matsui, 1967; Sano, Odake and Takemoto, 1967; Fuxe and Hökfelt, 1969, 1970). That the arcuate nucleus and the DA-containing vesicles within the external layer of the ME are associated with gonadotrophic activity has been demonstrated by Zambrano and De Robertis (1968). It was observed that following castration in the rat, the number of small DC vesicles within the soma of the arcuate neurons and within the terminals located near blood vessels of the external layer of the ME greatly increase in number. Similar changes were not observed in these areas following coitus in the rabbit.

The large DC vesicles with a mean diameter of approximately 1400 Å were only observed within the soma of PO and SCH neurons of the experimental animals (Figures 35 and 52, respectively), and within a number of axon profiles and their terminals located within the ME of both the control (Figure 86) and experimental groups (Figure 89). Although the occasional large DC vesicle was also observed within the soma of arcuate neurons (Table 5 and Figure 71), we have little evidence that their numbers increased following coitus (Figure 71). That the large DC vesicles were not observed within the soma of the PO and SCH neurons of the control animals strongly suggests that their synthesis is related to the physiological stimulus of coitus in the rabbit, and that they may contain the neurohormone LH-RF and probably also its carrier peptide.

It is well known that measurable increases in plasma LH levels occur within 10-15 minutes following coitus in the rabbit (Hilliard *et al.*, 1964). The rapid nature of this response implies that a direct neuronal stimulus is responsible for the depletion of LH-RF stores from the ME, which subsequently induces the release of ovulatory surges of LH from the adenohypophysis.

The LH-releasing activity of both natural and synthetic LH-RF seems to be established beyond reasonable doubt (Fawcett *et al.*, 1968; Amoss and Guillemin, 1969; McCann, 1970b; Schally *et al.*, 1970a; Harris and Naftolin, 1970; Chang *et al.*, 1971; Sievertsson *et al.*, 1971; Arimura, Matsuo, Baba and Schally, 1971). There is also unequivocal evidence that this neurohormone is transported to the adenohypophysis by way of the hypophyseal portal circulation

(Kamberi *et al.*, 1969, 1970a, 1971a; Porter *et al.*, 1970; Harris and Ruf, 1970). That the ME functions primarily as a neurohemal organ for the adenohypophyseal factors also seems well established following countless studies which have employed a variety of neurological techniques. Although not supported by unquestionable morphological evidence, the most current and widely held view is that the specific storage site of the releasing factors is the axon terminals located within the external layer of the ME (see reviews by Kobayashi, Matsui and Ishii, 1970; Knigge and Scott, 1970) and that the soma of the neurons that secrete these factors are located at distances quite removed from this portion of the tuber cinereum (Mess, 1968; Crighton and Schneider, 1969; Schneider *et al.*, 1969; Mess, Zanisi and Tima, 1970; Gorski, 1970; Tima, 1971; Motta, Piva, Tima, Zanisi and Martini, 1971). The relationship between the hypothalamic releasing factors and the various vesicle types commonly observed within the external layer of the ME is uncertain. However, according to Kobayashi *et al.* (1970), since the neurohypophyseal hormones and other biologically active substances such as acetylcholine (ACh) and the monoamines are carried by membrane-bound granules, it is also assumed that the releasing factors are likewise associated with granules that move toward the ME through axons and are stored therein. That the large DC vesicles within the vertebrate ME with a mean diameter of the order of 1350 Å may, in fact, contain the releasing factors, has recently received considerable support (Ishii *et al.*, 1969; Kobayashi and Matsui, 1969; Ishii, 1970; Peczely and Calas, 1970; Ishii, 1972). With this information at hand, therefore,

to what extent can our findings within the soma of PO and SCH neurons and those made at the level of the ME following coitus be related to the synthesis and the depletion of the neurohormone LH-RF?

If we assume that the electron-dense material forming the cores of the large vesicles observed within the external layer of the ME (Figures 86 and 87) contain the neurohormone LH-RF, the loss of the electron-dense cores would suggest that depletion of the vesicle content has occurred. Assuming also that LH-RF is solely responsible for the release of LH from the anterior lobe, and that plasma LH levels do occur as early as 10-15 minutes post-coitus (Hilliard *et al.*, 1964), the depletion of LH-RF must occur within minutes following coitus. As shown in Figure 82, two populations of DC vesicles with distribution peaks at approximately 900 Å and 1200 Å are apparent. The minimum and maximum diameters recorded for these vesicles was 540 Å and 1910 Å, respectively. Duffy and Menefee (1965) have reported only one type of DC vesicle within the ME of the rabbit, suggesting that they contain monoamines and possibly also the releasing factors. The diameter range of the DC vesicles observed by these investigators was 700 Å to 1200 Å. We also agree that the DC vesicles of this diameter range probably contain the monoamines. However, in the present investigation the small and large DC vesicles were observed in separate axon terminals (Figures 87 and 89). This observation supports our interpretation of two populations of DC vesicles within the ME of the rabbit, and that the larger population of DC vesicles contains the releasing factors.

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In the present study, no attempt was made to separate the two populations of DC vesicles while recording their diameters. As indicated in the results, the mean diameter of all the DC vesicles included within the sample studied from the external layer of the ME was 1205 Å. This mean diameter is intermediate to that (i.e., 1000 Å) generally accepted as representing the biogenic amines, and the mean diameter of 1350 Å proposed recently by Ishii *et al.* (1969) for the releasing factors. It was also felt that if only one of these populations of DC vesicles is associated with LH-RF, morphological evidence suggesting depletion phenomenon would provide further knowledge regarding the nature of the different sized DC vesicles contained within the axon terminals of the mammalian ME.

There was a significant increase in the number of 'ghosts' within the axons and their terminals observed within the external layer of the ME of the mated experimental animals (compare Figures 82 and 83). Also, as clearly demonstrated in Figure 88, the 'ghosts' occurred within terminals located in contact with the perivascular spaces. These findings are interpreted to reflect depletion phenomenon and that this depletion has occurred from the larger of the two populations of DC vesicles represented in the distribution curves shown in Figure 82. The presence of membrane profiles referred to in the present investigation as 'ghosts', have also been observed by others (Palay, 1957; Monroe and Scott, 1966; Scott, 1968) following specific stimuli and have been independently associated with the loss of materials from membrane-bound vesicles. That these observations represent the loss of the electron-dense cores from the

large DC vesicles (Scharrer, 1969) is further supported by the fact that membrane profiles of equal size were also observed to contain varying amounts of this material (Figure 88, inset).

Does the apparent loss of the electron-dense cores from these vesicles reflect the depletion of the neurohormone LH-RF? If the depletion of stored LH-RF is the event directly responsible for the rapid surges of plasma LH levels which, as previously mentioned, occur at 10-15 minutes post-coitus (Hilliard *et al.*, 1964), then our observations may indeed represent the depletion of this factor in response to the stimulus of coitus. Furthermore, since most of the 'ghosts' observed contained little resolvable material or appeared empty, the actual mechanism of depletion must be a rapid one and is, for the most part, completed prior to the time at which significant increases of LH can be measured within the circulating blood.

If these findings within the ME do represent the depletion of stored materials from the large DC vesicles, what is the nature of the mechanism(s) responsible for this release to occur? Since our observations reflect a very rapid phenomenon, the mechanism is probably neuronal in nature. Whether the depletion observed was controlled by aminergic mechanisms is uncertain. That the brain amines play some role in the control of gonadotrophin release has been previously suggested (Coppola, Leonardi and Lippmann, 1966; Labhsetwar, 1967; Coppola, 1968, 1969; Schneider and McCann, 1969b; Rubenstein and Sawyer, 1970). Furthermore, that this control occurs at the level of the ME, thus effecting the release of the neurohormones, has also been reported (Kamberi *et al.*, 1970). Although terminals

containing homogeneous populations of the small DC vesicles were observed in contact with the larger terminals containing 'ghosts' of the larger DC vesicles (Figure 88), changes among these terminals suggesting the loss of the smaller (1000 Å) DC vesicle content were not apparent. Whether or not the 300 Å - 700 Å clear synaptic vesicles commonly observed within all terminals of the ME and which probably contain acetylcholine (Wurtman, 1970), were associated with this depletion is also a possibility. Attempts to explain precisely how the content of the DC vesicles reaches the perivascular spaces of the portal capillaries is hampered again by the lack of morphological evidence. Our findings do not support exocytotic phenomenon or the movement of intact DC vesicles from the axon terminals into the perivascular spaces. Intact, free DC vesicles were likewise never observed within the perivascular spaces of the portal capillaries. Our findings suggest that the material contained within the DC vesicles either is changed chemically, or perhaps separated from its carrier, thus permitting it to move freely across membrane barriers, or that changes occur in the permeability of the membrane which permit unopposed movement of the electron-dense material out into the perivascular spaces (Scharrer, 1969).

How do the observations made at the level of the ME correspond with the post-coital changes described within the large neurons of the PO and SCH nuclei? Although the present study provides no information regarding the nature of the feed-back mechanisms which regulate the synthesis of LH-RF, our findings indirectly indicate that such mechanisms do exist. As mentioned previously,

the occurrence of the large DC vesicles within the soma of PO and SCH neurons did not become apparent until one hour and two hours, respectively, post-coitus. Whether or not the feed-back responsible for this elevated synthesis is hormonal in nature (possibly a positive feed-back of LH on these neurons) is only speculation. However, the fact that synthesis was usually observed in neurons located in close spatial relationship with the capillaries of the PO and SCH nuclei, seems to support this view. The stimulus for the observed enhanced synthesis may also be intraneuronal in nature. If so, the ultrastructural changes observed would represent a direct attempt by the PO and SCH neurons to replenish the lost stores of secreted materials from their axon terminals located within the ME. The other alternative is that the elevated synthesis we have observed reflects a direct or indirect neuronal feed-back onto the soma of these neurons. From our evidence, it is impossible to determine which one, or if all three of these possible mechanisms are operative.

That axons of neurons which secrete LH-RF reach the external layer of the ME had been suggested (Tejasen and Everett, 1967; Schneider *et al.*, 1969). That at least some of the LH-RF secreting neurons lie within the more anterior areas of the hypothalamus has also been supported by deafferentation studies following which the concentration of the LH-RF stores within the ME was greatly reduced (Mess, 1968; Tima, 1971). In addition, Tima (1971) observed that extracts prepared from small pieces of tissue removed from the SCH area of the rat brain maintained a relatively high level of LH-releasing activity. In the present study, the absence of evidence

suggesting the large DC vesicles produced within the synthetically active neurons of the PO and SCH nuclei, are being stored within the soma of these neurons (Figures 31 and 50), suggests their immediate transport to storage sites within the ME. It would be interesting to observe whether or not the large DC vesicles observed within the external layer of the ME reappear with time following coitus. If so, this would provide further evidence for the presently held view that the axons of these neurons do terminate within the ME. This view is currently supported by the occurrence of large DC vesicles within the axon profiles of both the PO and SCH nuclei of animals sacrificed at four hours through to 10 hours post-coitus. Also, the earlier suggestion that the membrane-bound vesicles of neurosecretory cells undergo maturation changes which include and increase in their diameter as they migrate from source to storage sites (Gerschenfeld, Tramezzani and De Robertis, 1960; Monroe, 1965; Sachs, Portanova, Haller and Share, 1967) is not supported by the observations made during the present investigation.

VI SUMMARY AND CONCLUSIONS

The effects of coitus on the ultrastructure of neurons within three nuclei and the median eminence of the female rabbit hypothalamus were investigated. The post-coital ultrastructural changes observed within certain neurons of the preoptic and suprachiasmatic nuclei are interpreted to reflect elevated synthetic activity; the products of this synthesis being contained within large dense-core vesicles with a mean diameter of approximately 1400 Å. The changes observed at the level of the median eminence suggest the depletion of the electron-dense material from the large (approximately 1400 Å) membrane-bound vesicles located within the axon terminals in contact with the perivascular spaces of the median eminence external layer.

1. Fifty-three mature, adult female rabbits (*Oryctolagus cuniculus L.*) were mated and sacrificed at 10 minutes, one-half hour, one hour, two hours, four hours, six hours, eight hours and 10 hours post-coitus. Twenty-three non-mated rabbits of the same species served as controls.
2. The rabbits were killed by viviperfusion through the left ventricle and ascending aorta of either a modification of Karnovsky's (1965) glutaraldehyde-paraformaldehyde fixative, or the dual glutaraldehyde-paraformaldehyde perfusates of Reese and Brightman as described by Peters (1970).
3. The brains were removed from the cranium and the following

hypothalamic nuclei excised: the arcuate, preoptic, suprachiasmatic, paraventricular, supraoptic, ventromedian and dorsomedian. The anterior portion of the median eminence was also removed from each animal.

4. Only our observations on the preoptic, suprachiasmatic and arcuate nuclei and the median eminence are included within this thesis.

5. The this sections obtained from each of these hypothalamic tissues were routinely stained with uranyl acetate and lead citrate, and examined with the electron microscope.

6. The quantitative data were obtained directly from the viewing screen of the electron microscope using a calibrated 'gunsight' mounted into the viewing chamber of this instrument, or directly from electron micrographs using a calibrated hand-lens. These data were subjected to computer analysis.

7. Post-coital ultrastructural changes occurred within 19.2% and 31.5% of the neurons located near the capillaries of the preoptic and suprachiasmatic nuclei respectively. Similar morphological changes were not observed within the preoptic and suprachiasmatic neurons of the control animals, nor within the neurons of the arcuate nucleus. The changes observed within the suprachiasmatic and preoptic neurons first became apparent at one-half hour and one hour post-coitus respectively; became even more pronounced at one hour and two hours following coitus, and continued without significant change for 10 hours.

8. The post-coital ultrastructural changes observed within the preoptic and suprachiasmatic nuclei and reported herein are as follows: a) - a significant increase in neuronal diameter as well as that of their nuclear profiles, b) - a remarkable increase in the development of certain cell organelles, particularly the rough endoplasmic reticulum and the Golgi system, c) - an increase in the numbers of ribosomes, mitochondria and lysosome-like bodies, d) - the occurrence of numerous large (1400 Å) dense-core vesicles within the soma of neurons located in close spatial relationship with the capillaries of these hypothalamic nuclei.

9. Since these changes were never observed among the neurons of these nuclei within the control animals, they suggest elevated synthetic activity in response to coitus. Similar discrete morphological features within neurons of the arcuate nuclei following coitus in the rabbit were not observed.

10. Several populations of membrane-bound vesicles on the basis of their size and nature of the material they contained, were observed within the neuropil of the hypothalamic nuclei and within the axons and their terminals located within the median eminence. Evidence supporting the biogenic nature of the smaller (1000 Å) dense-core vesicles is presented. The nature of the large light-cored vesicles with mean diameters of approximately 2000 Å is uncertain; however, those contained within the axon profiles of the median eminence internal layer probably carry the neurohypophyseal hormones.

11. Evidence is presented supporting the presence of two distinct populations of the dense-cored vesicles within axon terminals of the

median eminence external layer. Duffy and Menefee (1965) have described only one population of this vesicle type within the external layer of the rabbit median eminence.

12. Ultrastructural evidence supporting the depletion of only one population of dense-core vesicles from the axon terminals of the median eminence external layer following coitus is also presented.

13. We believe that the large (1400 Å) dense-core vesicles observed within the soma of the preoptic and suprachiasmatic neurons and within certain terminals of the external layer of the rabbit median eminence do represent the luteinizing hormone-releasing factor (LH-RF), or this neurohormone in combination with a carrier protein or peptide.

14. These observations provide the most convincing data yet available that LH-RF (at least in the rabbit) is synthesized within the neurons of the preoptic and suprachiasmatic nuclei, and that the depletion of this hypothalamic factor from axon terminals in contact with the capillary spaces of the external layer of the median eminence occurs within minutes following the stimulus of coitus in this animal.

VII POSSIBLE AVENUES OF FUTURE STUDY

1. Examine the remaining hypothalamic nuclei (supraoptic, paraventricular, ventromedial and dorsomedian) already excised from these animals.
2. Continue our observations on the PO and SCH nuclei at post-coital intervals exceeding 10 hours to determine when the neurons of these nuclei return to their normal state.
3. Do hypothalamic changes occur within these hypothalamic nuclei following the induction of ovulation with HCG?
4. Can one inhibit the neuronal response to ovulation in the rabbit by intraventricular or systemic administration of ovulation inhibitors?
5. What is the function of the ventricular system in this phenomenon?
6. Continue our observations on the ultrastructure of the rabbit median eminence following intravenous and/or intraventricular injection of dopamine.
7. What are the effects of castration in the rabbit on the ultrastructure of the arcuate neurons?
8. To study depletion phenomenon within the axon terminals of the rabbit median eminence external layer following reserpine treatment.
9. To examine the ultrastructural changes which might occur within anterior hypothalamic neurons of a spontaneous ovulator such as the rat.

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Addendum

Manual of Histologic and Special Staining Technics (1960), 2nd Ed. McGraw-Hill Book Co., New York, 1960. Page 10.

APPENDIX I

Histological Procedure for Brains

(Light Microscopy)

A) Fixation:

10% buffered neutral formalin

- vascular perfusion of 1,200 ml
- continue fixation by immersion for 48 hours after the brain is removed from the skull

transfer directly to 70% ETOH

wash overnight in running water

B) Dehydration, Clearing and Infiltration (after Haust, 1959):

1.	Tetrahydrofuran (THF)/water (1:1)	two hours
2.	THF	two hours
3.	THF	two hours
4.	THF	two hours
5.	THF/paraffin (1:1)	60°C for two hours
6.	Paraffin	60°C for two hours
7.	Paraffin (in vacuum)	60°C for two hours

C) Embedding and Cutting:

Embed tissues in paraffin using disposable embedding moulds (Peel-A-Way Scientific, W. Glenn Wunderly Co., Calif.)

Cut paraffin sections at 12 microns

D) Staining Procedure (Thionin):

1.	Xylol (two changes)	each five min
2.	Absolute ETOH/xylol (1:1)	five min
3.	Absolute ETOH (two changes)	each five min
4.	Parlodion (1.0%) in 50:50 ETOH and diethyl ether	dip
5.	70% ETOH	five min
6.	Distilled water	five min
7.	Thionin (0.1%) in oven at 56°C	20-25 min
8.	Wash thoroughly in tap water	five min
9.	Differentiate in 90% ETOH (check for color change with microscope)	10-30 sec
10.	Rinse in Absolute ETOH	1- 2 min
11.	Clear in xylol	
12.	Mount in Depex (George T. Gurr Ltd., London, England)	
13.	Coverslip	

Results

Nissl substance. . . . . .deep blue to mauve

Nucleoli and chromatin. . . . . blue

Other tissue elements. . . . . colorless

Solutions

1. Parlodion (1.0%)

Parlodion	1 gm
Absolute ETOH	50 ml
Diethyl ether	50 ml

2. Thionin (0.1%)

Thionin	.1 gm
Distilled water	to 100 ml

APPENDIX II

Histological Procedure for Body Tissues
(Light Microscopy)

A) Fixation:

10% buffered neutral formalin	48 hours
store in 70% ETOH	

B) Dehydration, Clearing and Infiltration:

- using tetrahydrofuran (THF) - paraffin (after Haust, 1959)

1. 70% ETOH/THF (1:1)	two hours
2. THF	four hours
3. THF	four hours
4. THF	four hours
5. THF/paraffin (1:1) two changes	each two hours
6. Paraffin	four hours
7. Paraffin (in vacuum)	three-four hours

- using dioxane - paraffin (Manual of Histologic and Special Staining Technics, 1960)

1. Dioxane/water (1:1)	overnight
2. 100% dioxane (three changes)	each three-four hours
3. Paraffin (three changes)	each one hour
4. Paraffin (in vacuum)	two hours

C) Embedding and Cutting:

Embed tissues in paraffin using disposable embedding moulds (Peel-A-Way Scientific, W. Glenn Wunderly Co., Calif.)

Cut paraffin sections at 12 microns

D) Staining Procedure (Hematoxylin-Phloxine-Saffron):

1.	Toluol	two-five min
2.	100% ETOH (three changes)	each two-five min
3.	50% ETOH	five min
4.	Wash in tap water	five min
5.	Stain with hematoxylin	five min
6.	Wash in tap water	five min
7.	Differentiate in 2% acid alcohol	one dip
8.	Wash in tap water	five min
9.	Blue in 2% ammonia water	one dip
10.	Wash in tap water	five min
11.	Stain in phloxine	five min
12.	Wash in tap water	five min
13.	Differentiate in 75% ETOH	10-20 sec
14.	Differentiate in 85% ETOH	10-20 sec
15.	Differentiate in 95% ETOH	10-20 sec
16.	Stop differentiation in several changes of absolute ETOH	
17.	Stain in saffron	five-10 min
18.	Differentiate in Absolute ETOH (three-four changes)	
19.	Toluol (four changes)	three-four min

Results

Nuclei. . . . . blue

Connective tissue. . . . . .yellow

Red blood cells. . . . . .bright red

Muscle fibers, cell content. . . . . .pink

Background. . . . . .colorless

Solutions

1. Hematoxylin

Distilled water	500 ml
Potassium aluminum sulphate	60 gm
Hematein	1.25 gm
Glacial acetic acid	5 ml

Make up potassium alum by adding 60 gm of potassium aluminum sulphate to 500 ml of distilled water. Let it come to a boil and add 1.25 gm of hematein (remove from flame when adding hematein). Boil very gently for 10 min, then cool and shake gently. Add 5 ml of glacial acetic acid. When cool, add a crystal of thymol.

2. Ploxine

Distilled water	500 ml
Phloxine B (2% solution)	10 gm
Thymol	1 crystal

3. Saffron

Absolute ETOH	1000 ml
Saffron	30 gm

Add 500 ml of absolute ETOH to 30 gm of saffron and let it ripen in an oven at 60°C for 48 hours. Pour off the ETOH and add 500 ml of unused absolute ETOH. Let ripen in an oven at 60°C for 48 hours. Both amounts of ETOH are used.

4. Acid alcohol

95% ETOH	100 ml
Concentrated HCl	1 ml

5. Ammonia water

Distilled water	200 ml
Ammonium hydroxide	4 ml

APPENDIX III

Fixatives Used for Electron Microscopy

A) Formaldehyde-Glutaraldehyde Fixative of Karnovsky (1965) - modified

Distilled water	1000 ml
Paraformaldehyde	80 gm

Heat the distilled water to 60°C, add the paraformaldehyde and stir. Add 25-30 drops of 1N NaOH (dropwise) until solution clears. Cool to room temperature and add an equal volume of phosphate buffer (Karlsson and Schultz, 1965)*.

Procedure for preparing the 1500 ml of fixative perfused through each rabbit:

1. Karnovsky's formaldehyde-glutaraldehyde fixative	503 ml
2. Phosphate buffer	907 ml
3. 50% glutaraldehyde (Biological Grade, Fisher Scientific Company)	75 ml
4. Filter	
5. Heat to 41°C	
6. Saturate with oxygen	

The final fixative -

Concentration of glutaraldehyde	2.5%
Concentration of paraformaldehyde	4.0%
pH	7.2 - 7.4
Osmolarity (modified)	approx. 1,200 mOsM/kg

Solutions

*1. Phosphate buffer (Karlsson and Schultz, 1965)

Distilled water	2780 ml
Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$)	5.40 gm
Sodium phosphate dibasic (anhydrous) (Na_2HPO_4)	36.93 gm
Sodium chloride (NaCl)	18.00 gm

May be stored for several months in a refrigerator at 4°C

B) The Two-Stage Formaldehyde-Glutaraldehyde Fixatives of Reese and Brightmann as described by Alan Peters (1970)

To prepare 1,500 ml of fixative for each perfusion -

1. Dilute solution (1% paraformaldehyde - 1.25% glutaraldehyde)

Distilled water	500 ml
Add 1N NaOH	1.9 ml
Bring to boil	
Remove from heat and add paraformaldehyde	10 gm
Add 50% glutaraldehyde (Biological Grade, Fisher Scientific Company)	25 ml
Add 0.2M Na Cacodylate	400 ml
Add 5% $CaCl_2$	1.9 ml

Adjust pH to 7.2 - 7.4 with 0.1N HCl

Add distilled water to bring final volume to 1000 ml

Filter through #44 filter paper

Heat to 41°C and saturate with oxygen before using

2. Concentrated Solution (4% paraformaldehyde - 5% glutaraldehyde)

Distilled water	285 ml
Add 1N NaOH	4.5 ml
Bring to boil	
Remove from heat and add paraformaldehyde	24 gm
Add 50% glutaraldehyde (Biological Grade, Fisher Scientific Company)	60 ml
Add 0.2M Na Cacodylate	240 ml
Add 5% $CaCl_2$	2.25 ml
Adjust pH to 7.2 - 7.4 with 0.1N HCl	
Add distilled water to bring the final volume to 600 ml	
Filter through #44 filter paper	
Heat to 41°C and saturate with oxygen before using	

The final fixative -

Osmolarity:

Dilute solution	520 mOsM/kg
Saturated solution	580 mOsM/kg

Solutions

1. Cacodylic Acid (0.2M)

Distilled water	200 ml
Cacodylic acid	5.52 gm

2. Calcium Chloride (5%)

Distilled water	96 ml
Calcium chloride	5.0 gm

Add distilled water to bring final volume to 100 ml

3. Sodium Cacodylate (0.2M)

Distilled water	1000 ml
Sodium cacodylate	42.8 gm

4. Hydrochloric acid (0.1N)

Distilled water	100 ml
Hydrochloric acid (concentrated)	10 ml

APPENDIX IV

Routine Procedure for Processing Brain Tissue

(Electron Microscopy)

A) Fixation:

- using Karnovsky's formaldehyde-glutaraldehyde fixative

1. Vascular perfusion of 1,500 ml
2. Remove hypothalamic block and continue fixation overnight in a volume of the same fixative
3. Remove hypothalamic nuclei and median eminence
4. Wash in 5.4% sucrose prepared in phosphate buffer (three changes) each four hours (or overnight)
5. Post-fix in 1% osmium tetroxide (prepared in the same buffer) four hours
6. Wash in distilled water (three changes) each 10 min

- using Reese and Brightmann's two-stage formaldehyde-glutaraldehyde fixatives

1. Vascular perfusion of 1,500 ml
2. Remove hypothalamic block and continue fixation overnight in a volume of the concentrated fixative
3. Remove the hypothalamic nuclei and median eminence
4. Wash in 5.4% sucrose prepared in cacodylate buffer (three changes) each four hours (or overnight)
5. Post-fix in 1% osmium tetroxide prepared in cacodylate buffer four hours

6. Wash in distilled water (three changes) — each 10 min

B) Staining - 'en block'

Stain 'en block' with an aqueous solution of saturated uranyl acetate — three- four hours

C) Dehydration and Clearing (All Tissues)

1. 30% ETOH (one or two changes, or until no color change occurs) — each five - 10 min
2. 50% ETOH — 10 - 15 min
3. 70% ETOH — 10 - 15 min
4. 90% ETOH — 10 - 15 min
5. Absolute ETOH stored over copper sulphate (two changes) — each 10 - 15 min

D) Subsequent procedure for embedding in Epon 812

- continuing from Step 5 - Dehydration and Clearing

6. Propylene oxide (two changes) — each 10 - 15 min
7. Propylene oxide/Epon 812 (1:1) — on rotator - overnight
8. Pure Epon 812 — on rotator - 24 hours
9. Pure Epon 812 (in capsules)
10. Polymerize for 24 hours at 37°C
11. Polymerize for 72 hours (three days) at 60°C
12. Dissolve gelatin capsules in warm water

E) Subsequent procedure for embedding in Spurr

- continuing from Step 5 - Dehydration and Clearing

6. Spurr/Absolute ETOH (1:1) — on rotator - 3 hours
7. Pure Spurr mixture — on rotator - overnight

8.	Change Spurr mixture	six hours
9.	Spurr (in capsules)	
10.	Polymerize at 60°C	12 - 14 hours
11.	Dissolve capsules in warm water	

F) Staining of ultrathin sections

- using Epon 812 as the embedding medium

1.	Reynolds lead citrate	five min

- using Spurr as the embedding medium

1.	Uranyl acetate	15 - 20 min
2.	Reynolds lead citrate	15 min

Solutions

1. Sucrose (5.4%)

Sucrose	5.4 gm
Buffer (0.2 sodium cacodylate or phosphate)	to 100 ml

2. Osmium tetroxide (2% stock solution)

Distilled water	50 ml
Osmium tetroxide (Scientific and Industrial Chemicals Corp., New York)	1 gm

Store stock solution at 4.0 C in refrigerator

Allow 48 hours for osmium crystals to dissolve

3. Osmium tetroxide (1% solution)

Buffer (0.2M sodium cacodylate or phosphate)

Add an equal volume of 2% stock solution of osmium tetroxide

4. Sodium cacodylate buffer (0.2M) - for 1000 ml

0.2M sodium cacodylate [$(CH_3)_2AsO_2Na \cdot 3H_2O$]	240 ml
0.2M cacodylic acid [$(CH_3)_2AsO \cdot OH$]	100 ml
0.2M calcium acetate [$Ca(CH_3COO)_2 \cdot H_2O$]	10 ml

Make up to 1000 ml with isotonic sodium sulphate (Na_2SO_4 - anhydrous)

Stock solutions for 0.2M sodium cacodylate buffer

1. Cacodylic acid (0.2M)

Distilled water	200 ml
Cacodylic acid	5.52 gm

2. Calcium acetate (0.2M)

Distilled water	100 ml
Calcium acetate (dried)	3.16 gm

3. Calcium chloride

Distilled water	100 ml
Calcium chloride	5.0 gm

4. Sodium cacodylate (0.2M)

Distilled water	1000 ml
Sodium cacodylate	42.8 gm

5. Sodium sulphate - isotonic

Distilled water	1000 ml
Sodium sulphate (anhydrous)	16.76 gm

G) Procedure for preparing stains:

1. Uranyl acetate (saturated)

Boiled water	100 ml
Uranyl acetate [$UO_2(CH_3\ COO)_2 \cdot 2H_2O$]	
(B.D.H. Laboratory Chemicals Division, England)	5 gm

2. Reynolds lead citrate

Distilled water	30 ml
Lead citrate [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$] (Fisher	
Scientific Company, New Jersey, U.S.A.)	1.33 gm
Sodium citrate [$Na_3C_6H_5O_7 \cdot 2H_2O$] (The McArthur	
Chemical Co. Ltd., Montreal)	1.76 gm
Shake well	
Let stand for 30 minutes	
Add 1N NaOH (carbonate free)	8 ml
Dilute with distilled water	50 ml

Keep container (a narrow neck bottle with ground glass top) tightly stoppered. Do not disturb container before use.

H) Procedure for preparing embedding media:

1. Epon 812 (for hard blocks)

Mixture A:

Epon 812 resin (Fisher Scientific Co.)	37 ml
D.D.S.A. (Dodecenyl-succinic anhydride)	
[$C_{12}H_{23}CHCH_2COOCO$]	60 ml

Mixture B:

Epon 812 resin	95 ml
N.M.A. (Nadic methyl anhydride) [$C_{10}H_{10}O_3$]	85 ml

Thoroughly mix 90 ml of Mixture A with 180 ml of Mixture B (1:2 ratio)

Add DMP-30 (2,4,6, -Tri(dimethylaminomethyl) phenol) [$C_{15}H_{27}N_3O$] 0.17 ml/10 ml

For final volume of 270 ml (Mixtures A and B), add 4.6 ml of DMP-30 (activator).

Stir thoroughly (15 - 20 min) with a glass stirring rod.

Store in 10 ml polyethylene disposable syringes in freezer.

Bring to room temperature before using.

2. Spurr (the B or hard mixture as suggested by Polysciences, Inc., Warrington, Pa.)

VCD (Vinylcyclohexene dioxide)	10 gm
D.E.R. 736 (Diglycidyl ether of polypropylene glycol)	4 gm
NSA (Nonenyl-succinic anhydride)	26 gm
Dimethyllaminoethanol (activator)	.4 gm

Mix thoroughly

Place in 50 ml containers and store in freezer

Bring to room temperature before using

APPENDIX V

AEI, EM6B 'GUNSIGHT' CALIBRATION (2,160 lines/mm. grating)

MAG. (X1000)		1.5	3	5	7.5	10	15	20	30	30'	40	60	80	100	150
RING NUMBER	1	2.8μ	1.5μ	926mμ	671mμ	500mμ	310mμ	245mμ	253mμ	162mμ	120mμ	790Å	650Å	510Å	370Å
	2	8.9μ	4.6μ	2.9μ	1.9μ	1.4μ	972mμ	704mμ	463mμ	509mμ	324mμ	232mμ	194mμ	153mμ	111mμ
	3	14.8μ	7.6μ	4.7μ	3.2μ	2.4μ	1.6μ	1.2μ	787mμ	880mμ	588mμ	384mμ	324mμ	255mμ	185mμ
	4	20.4μ	10.6μ	6.6μ	4.4μ	3.5μ	2.3μ	1.7μ	1.1μ	1.2μ	847mμ	542mμ	463mμ	361mμ	255mμ
	5	25.5μ	13.4μ	8.3μ	5.0μ	4.4μ	2.9μ	2.2μ	1.4μ	1.5μ	1.0μ	764mμ	532mμ	463mμ	333mμ
BETWEEN RINGS	1 & 2	2.8μ	1.5μ	926mμ	670mμ	500mμ	310mμ	240mμ	153mμ	162mμ	116mμ	790Å	650Å	510Å	370Å
	2 & 3	2.8μ	1.5μ	926mμ	670mμ	500mμ	310mμ	240mμ	153mμ	162mμ	116mμ	790Å	650Å	510Å	370Å
	3 & 4	2.8μ	1.5μ	916mμ	650mμ	470mμ	300mμ	232mμ	153mμ	162mμ	116mμ	790Å	650Å	510Å	370Å
	4 & 5	2.8μ	1.5μ	910mμ	630mμ	460mμ	300mμ	225mμ	150mμ	160mμ	116mμ	790Å	650Å	510Å	370Å

Calibrations recorded from the 'upper left' and 'lower right' quadrants of the gunsight

AEI, EM801 'GUNSIGHT' CALIBRATION (2,160 lines/mm. grating)

MAG. (X1000)		1	1.6	2.5	4	6.3	10	16	25	40	63	100	160
RING NUMBER	0	1.4μ	787mμ	463mμ	312mμ	280mμ	185mμ	926Å	570Å	460Å	--	--	--
	1	4.6μ	2.8μ	1.7μ	1.0μ	602mμ	463mμ	324mμ	185mμ	926Å	463Å	--	--
	2	13.4μ	8.1μ	5.1μ	3.0μ	2.2μ	1.4μ	880mμ	509mμ	324mμ	185mμ	926Å	--
	3	22.0μ	13.2μ	8.3μ	5.0μ	3.0μ	2.2μ	1.4μ	880mμ	509mμ	278mμ	139mμ	--
	4	30.6μ	18.3μ	11.6μ	7.0μ	4.8μ	3.1μ	2.0μ	1.2μ	787mμ	417mμ	232mμ	185mμ
	5	38.0μ	23.2μ	14.7μ	8.9μ	5.3μ	3.9μ	2.3μ	1.6μ	926mμ	556mμ	324mμ	231mμ

APPENDIX VI

TABLE FOR CONVERTING MEASUREMENTS FROM MICROGRAPHS TAKEN WITH THE AEI,EM6B TO ANGSTROMS (Å) AND MICRONS (μ)

MICROGRAPH MAGNIFICATION

Millimeter Units:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
5,250X -	1905Å	3810Å	5715Å	7620Å	9525Å	1.14μ	1.33μ	1.54μ	1.71μ	1.90μ	2.10μ	2.28μ	2.47μ	2.66μ	2.99μ	3.08μ	3.25μ	3.42μ	3.61μ	3.80μ	----	----	----	----	----	----	----	----	----	----
10,500X -	952Å	1904Å	2860Å	3808Å	4764Å	5720Å	6668Å	7716Å	8572Å	9620Å	1.05μ	1.15μ	1.24μ	1.34μ	1.44μ	1.54μ	1.63μ	1.72μ	1.82μ	1.91μ	2.00μ	2.10μ	2.20μ	2.30μ	2.40μ	2.49μ	2.58μ	2.68μ	2.78μ	2.88μ
17,500X -	571Å	1143Å	1714Å	2286Å	2857Å	3428Å	4004Å	4572Å	5127Å	5714Å	6285Å	6856Å	7432Å	8008Å	8576Å	9144Å	9700Å	1.03μ	1.08μ	1.14μ	1.20μ	1.26μ	1.31μ	1.37μ	1.43μ	1.49μ	1.54μ	1.60μ	1.66μ	1.72μ
26,250X -	381Å	762Å	1143Å	1524Å	1905Å	2286Å	2667Å	3048Å	3429Å	3810Å	4191Å	4572Å	4953Å	5334Å	5715Å	6096Å	6477Å	6858Å	7239Å	7620Å	8001Å	8382Å	8763Å	9144Å	9525Å	9906Å	1.03μ	1.07μ	1.10μ	1.14μ
35,000X -	286Å	571Å	857Å	1142Å	1428Å	1714Å	2000Å	2284Å	2570Å	2856Å	3142Å	3428Å	3714Å	4000Å	4284Å	4568Å	4854Å	5140Å	5426Å	5712Å	5998Å	6284Å	6570Å	6856Å	7142Å	7428Å	7714Å	8000Å	8284Å	8568Å
52,500X -	190Å	380Å	570Å	760Å	950Å	1140Å	1330Å	1520Å	1710Å	1900Å	2090Å	2280Å	2470Å	2660Å	2850Å	3040Å	3230Å	3420Å	3610Å	3800Å	3990Å	4180Å	4370Å	4560Å	4750Å	4940Å	5130Å	5320Å	5510Å	5700Å
70,000X -	143Å	286Å	428Å	571Å	714Å	857Å	1000Å	1143Å	1286Å	1428Å	1571Å	1714Å	1857Å	2000Å	2143Å	2286Å	2429Å	2572Å	2714Å	2856Å	3000Å	3142Å	3285Å	3428Å	3571Å	3714Å	3857Å	4000Å	4143Å	4286Å
105,000X -	95Å	190Å	285Å	380Å	475Å	570Å	665Å	760Å	855Å	950Å	1045Å	1140Å	1235Å	1330Å	1425Å	1520Å	1615Å	1710Å	1805Å	1900Å	1995Å	2095Å	2185Å	2280Å	2375Å	2470Å	2565Å	2660Å	2755Å	2850Å
140,000X -	---	143Å	214Å	286Å	357Å	428Å	500Å	572Å	643Å	714Å	785Å	856Å	928Å	1000Å	1072Å	1144Å	1215Å	1286Å	1357Å	1428Å	1499Å	1570Å	1641Å	1712Å	1784Å	1856Å	1928Å	2000Å	2072Å	2144Å
210,000X -	---	---	143Å	190Å	239Å	286Å	333Å	380Å	429Å	478Å	525Å	572Å	619Å	666Å	713Å	760Å	809Å	858Å	907Å	956Å	1003Å	1056Å	1097Å	1144Å	1191Å	1238Å	1285Å	1332Å	1349Å	1426Å
280,000X -	---	---	---	143Å	179Å	214Å	250Å	286Å	322Å	358Å	393Å	428Å	464Å	500Å	536Å	572Å	608Å	644Å	680Å	716Å	751Å	786Å	821Å	856Å	892Å	928Å	964Å	1000Å	1036Å	1072Å

0.2 mm Units:	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.0	5.2	5.4	5.6	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8
17,500X -	1142Å	1256Å	1370Å	1485Å	1599Å	1713Å	1827Å	1941Å	2056Å	2170Å	2284Å	2398Å	2512Å	2627Å	2741Å	2855Å	2969Å	3083Å	3198Å	3312Å	3426Å	3540Å	3654Å	3769Å	3883Å	3994Å	---	---	---	---
26,250X -	762Å	839Å	914Å	991Å	1067Å	1143Å	1219Å	1296Å	1372Å	1448Å	1524Å	1600Å	1676Å	1753Å	1829Å	1905Å	1981Å	2057Å	2143Å	2210Å	2286Å	2362Å	2438Å	2515Å	2591Å	2667Å	2743Å	2819Å	2896Å	---
35,000X -	571Å	629Å	686Å	744Å	801Å	858Å	915Å	972Å	1030Å	1087Å	1144Å	1201Å	1258Å	1316Å	1373Å	1430Å	1487Å	1544Å	1602Å	1659Å	1716Å	1773Å	1830Å	1888Å	1945Å	2002Å	2059Å	2116Å	2173Å	2231Å
52,500X -	---	---	---	---	532Å	570Å	608Å	646Å	684Å	722Å	760Å	798Å	836Å	874Å	912Å	950Å	991Å	1026Å	1067Å	1102Å	1143Å	1178Å	1219Å	1254Å	1296Å	1330Å	1372Å	1406Å	1448Å	1482Å

0.5 mm Units:	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	11.5	12.0	12.5	13.0	13.5	14.0	14.5	15.0	15.5	16.0	16.5
70,000X -	286Å	358Å	429Å	501Å	572Å	644Å	715Å	787Å	850Å	930Å	1001Å	1073Å	1144Å	1216Å	1289Å	1359Å	1430Å	1502Å	1573Å	1645Å	1716Å	1788Å	1859Å	1931Å	2002Å	2074Å	2145Å	2217Å	2288Å	2360Å
105,000X -	---	---	---	---	380Å	428Å	475Å	523Å	570Å	618Å	665Å	713Å	760Å	808Å	855Å	903Å	950Å	998Å	1045Å	1093Å	1140Å	1188Å	1235Å	1283Å	1330Å	1378Å	1425Å	1473Å	1520Å	1568Å

TABLE FOR CONVERTING MEASUREMENTS FROM MICROGRAPHS TAKEN WITH THE AEI,EM801 TO ANGSTROMS (Å) AND MICRONS (μ)

MICROGRAPH MAGNIFICATION	Millimeter Units:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
3,500X	-	2858Å	5718Å	8573Å	1.14μ	1.43μ	1.71μ	2.00μ	2.31μ	2.57μ	2.85μ	3.15μ	3.42μ	3.71μ	4.00μ	4.49μ	4.62μ	4.88μ	5.13μ	5.42μ	5.70μ	--	--	--	--	--	--	--	--	--	--
5,600X	-	1785Å	3570Å	5363Å	7140Å	8932Å	1.07μ	1.25μ	1.45μ	1.61μ	1.80μ	1.97μ	2.16μ	2.33μ	2.51μ	2.70μ	2.89μ	3.06μ	3.23μ	3.41μ	3.58μ	3.75μ	3.94μ	4.13μ	4.31μ	4.48μ	4.65μ	4.84μ	5.03μ	5.21μ	5.40μ
8,750X	-	1142Å	2286Å	3428Å	4572Å	5714Å	6856Å	8008Å	9144Å	1.03μ	1.14μ	1.26μ	1.37μ	1.49μ	1.60μ	1.72μ	1.83μ	1.94μ	2.06μ	2.16μ	2.28μ	2.40μ	2.52μ	2.65μ	2.74μ	2.86μ	2.98μ	3.08μ	3.20μ	3.32μ	3.44μ
14,000X	-	714Å	1428Å	2143Å	2858Å	3571Å	4285Å	5005Å	5715Å	6409Å	7143Å	7856Å	8571Å	9290Å	1.00μ	1.07μ	1.14μ	1.21μ	1.29μ	1.35μ	1.35μ	1.50μ	1.58μ	1.64μ	1.71μ	1.79μ	1.86μ	1.93μ	2.00μ	2.08μ	2.15μ
22,050X	-	453Å	907Å	1360Å	1814Å	2267Å	2720Å	3174Å	3627Å	4081Å	4534Å	4987Å	5441Å	5894Å	6347Å	6801Å	7254Å	7708Å	8161Å	8614Å	9068Å	9521Å	9975Å	1.04μ	1.09μ	1.13μ	1.18μ	1.23μ	1.27μ	1.31μ	1.36μ
35,000X	-	286Å	571Å	857Å	1142Å	1428Å	1714Å	2000Å	2284Å	2570Å	2856Å	3142Å	3428Å	3714Å	4000Å	4284Å	4568Å	4854Å	5140Å	5426Å	5712Å	5998Å	6284Å	6570Å	6856Å	7142Å	7428Å	7714Å	8000Å	8284Å	8568Å
56,000X	-	178Å	357Å	535Å	714Å	893Å	1071Å	1250Å	1429Å	1607Å	1785Å	1964Å	2143Å	2321Å	2500Å	2679Å	2858Å	3036Å	3215Å	3392Å	3570Å	3750Å	3927Å	4106Å	4285Å	4464Å	4642Å	4821Å	5000Å	5179Å	5358Å
86,500X	-	114Å	229Å	342Å	457Å	571Å	686Å	800Å	914Å	1029Å	1142Å	1257Å	1371Å	1486Å	1600Å	1714Å	1829Å	1943Å	2058Å	2171Å	2285Å	2400Å	2514Å	2628Å	2742Å	2937Å	2971Å	3086Å	3200Å	3429Å	3559Å
140,000X	-	67Å	143Å	214Å	286Å	357Å	428Å	500Å	572Å	643Å	714Å	785Å	856Å	928Å	1000Å	1072Å	1144Å	1215Å	1286Å	1357Å	1428Å	1499Å	1570Å	1641Å	1712Å	1784Å	1856Å	1928Å	2000Å	2072Å	2144Å
220,000X	-	45Å	90Å	136Å	181Å	226Å	272Å	317Å	362Å	408Å	453Å	498Å	544Å	589Å	634Å	680Å	725Å	770Å	816Å	861Å	906Å	952Å	997Å	1043Å	1088Å	1133Å	1179Å	1226Å	1273Å	1309Å	1357Å

0.2 mm Units:		1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.0	5.2	5.4	5.6	5.8	6.0	6.2	6.4	6.6	6.8
14,000X	-	714Å	857Å	999Å	1142Å	1284Å	1428Å	1569Å	1712Å	1854Å	1999Å	2143Å	2286Å	2429Å	2571Å	2713Å	2858Å	2998Å	3140Å	3283Å	3425Å	3571Å	---	---	---	---	---	---	---	---	---
22,050X	-	--	--	--	727Å	817Å	907Å	997Å	1088Å	1177Å	1264Å	1360Å	1450Å	1542Å	1631Å	1720Å	1814Å	1912Å	2000Å	2089Å	2178Å	2267Å	2359Å	2447Å	2538Å	2629Å	2720Å	2811Å	2900Å	2991Å	3080Å
35,000X	-	--	--	--	--	--	571Å	628Å	685Å	742Å	799Å	857Å	910Å	965Å	1020Å	1057Å	1142Å	1199Å	1256Å	1313Å	1370Å	1428Å	1485Å	1542Å	1599Å	1656Å	1714Å	1771Å	1828Å	1885Å	1942Å