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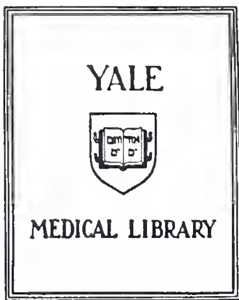
THE EFFECTS OF ESTROGEN ON GLUTAMIC ACID DECARBOXYLASE (GAD)-CONTAINING NEURONS IN THE ARCUATE NUCLEUS AND MEDIAL PREOPTIC AREA OF THE RAT: AN ELECTRON MICROSCOPIC IMMUNOCYTOCHEMICAL STUDY

CAROL F. FARVER

1985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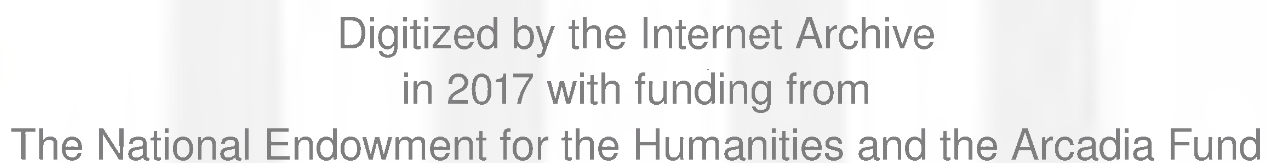

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THE EFFECTS OF ESTROGEN ON GLUTAMIC ACID DECARBOXYLASE (GAD)-CONTAINING NEURONS IN THE ARCUATE NUCLEUS AND MEDIAL PREOPTIC AREA OF THE RAT: AN ELECTRON MICROSCOPIC IMMUNOCYTOCHEMICAL STUDY

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Carol F. Farver

1985

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ABSTRACT

The arcuate nucleus and MPOA (medial preoptic area) of the rat brain, implicated as sites of estrogen's action in controlling gonadotropin release, are known to contain significant amounts of GABA (gamma-aminobutyric acid). Pharmacologic, electrophysiologic and anatomic studies show GABA is involved in the control of LH release and recent evidence in both the MPOA and the arcuate nucleus suggests these GABA neurons may be estrogen-sensitive targets in a neuroendocrine loop controlling ovulation. In an immunocytochemical electron microscopic study, we examined the effects of ovariectomy and estrogen treatment on the ultrastructure of these GABA neurons. We found no significant difference in the cellular morphology of the GABA neurons from either region under the two experimental conditions, but in the MPOA under both conditions, previously unreported glutamic acid decarboxylase(GAD)-GAD axosomatic synapses and whorl body formation were observed. These results are discussed within the context of the technical difficulties imposed by the labeling methodology used in this study and speculations on the functional role of the GAD-GAD synapses in the MPOA are offered.

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INTRODUCTION

It has been well-documented that estrogen binds to and affects the brain both structurally and functionally in physiologic and pathologic ways. The keen interest with which these effects have been studied is, for the most part, a result of the important role they are known to play in all stages of development from sexual differentiation of the brain through initiation of puberty to the control of sexual function in the mature organism. Perhaps the most studied estrogen-CNS interactions have been those involved in the control of gonadotropin release. Here, specific areas of the brain involved in the positive and negative feedback of estrogen have been localized anatomically and the neurotransmitters involved are gradually being defined. One substance strongly implicated in the neuronal circuitry controlling GnRH release is GABA (gamma-aminobutyric acid). From this laboratory, evidence based on light and electron microscopic studies suggests that GAD-immunoreactive perikarya in two areas of the brain well-known to be involved in the control of gonadotropin release--the arcuate nucleus and the medial preoptic area (MPOA)--are estrogen-responsive. The purpose of this study is to describe in the rat brain, the morphologic effects ovariectomy and high dose estrogen treatment have on GABA neurons in these two estrogen-sensitive areas. It is our hope that this information together with that collected from electrophysiologic and biochemical studies under similar conditions, may help to formulate for a given neuron population specific structural and functional correlates of estrogen's effect on neural tissue, and perhaps help in deciphering GABA's role in both the positive and negative feedback estrogen exerts on gonadotropin release.

ESTROGEN'S EFFECT ON THE BRAIN--Studies of Neuroendocrine Function

There is ample evidence that estrogen can both stimulate (Everett'48;Shirley et.al.'68;Ferin et.al.'69;Labsetwar'70;and Naftolin'72) and suppress (Ramirez&McCann'63;Brown-Grant'71;Gay&Midgley'69) gonadotropin release from the pituitary. Evidence for the localization of this effect was first suggested by Hohlweg and Junkmann ('32) when they postulated the existence of a 'sex center' in the hypothalamus after noticing that castration cells did not appear in transplanted pituitaries after ovariectomy. Other investigators had supported the concept of a neural control over other anterior pituitary hormones (Selye'34;F.H.A. Marshall'42) and the anatomical basis for this functional connection had been established earlier by Popa and Fielding ('30;'33),who first described the anatomical detail of the hypophyseal portal blood supply, but because they assumed a blood flow from hypohysis to hypothalamus were unaware of its functional significance. Not until Green and Harris ('49) provided a direct visualization of the portal vessels in a living rat, was the blood flow shown to go from hypothalamus to hypophysis, supporting Harris's concept of a neurovascular control of the pars distalis and the necessity of portal blood flow to pituitary function (Harris'47). In subsequent studies Harris demonstrated the existence of this neural control by inducing ovulation with direct brain stimulation (for review see Harris'55) and the humoral nature by showing that animals made acyclic by removal of the pituitary gland could recover ovarian function if the pituitary was transplanted back into the sella turcica and revascularized (Harris&Jacobsen'52).

Localization of this control to the hypothalamus and suggestions of this area's role as the 'final common pathway' from the CNS to the pituitary was

demonstrated by implantation, lesion and electrical stimulation studies in its various areas. The estrogen sensitivity of the MBH (medial basal hypothalamus) was demonstrated first by crude implantation experiments showing that estrous cycles returned when median eminence extracts where infused into pituitaries transplanted to various areas in the animal's body (Nikitovitch-Winer'62;Campbell'64). Halasz et.al. ('65) transplanted pituitary fragments into this area and found that within a certain region, these fragments retained their normal cytological characteristics and ovarian cycles were maintained. This crescent-shaped hypothalamic region that could maintain the pituitary cells was named the 'hypophysiotrophic area' (HTA). Subsequent work using estrogen implants in this area and monitoring either ovarian atrophy (Flerko&Szentagothai'57) or pituitary function (Lisk'60,'63;Davidson&Sawyer'61;Kanematra&Sawyer'64;Ramirez et.al.'64;Chowers &McCann'67;and Martini et.al.'68) demonstrated that increased levels of estrogen produced a decrease in gonadotropin secretion and lesioning this area (Bogdanov'57;Flerko&Bardos'61;Bishop'71k'72a,72b;Everett&Tyer'77) or local electrical stimulation to it (Markee'52;Gallo'76)inhibited LH release and produced ovarian atrophy.

Confounding results were reported by some investigators who found increases in LH with estrogen implants into the MBH (Palka'66;Weick&Davidson'70;Billard&McDonald'73) but these data, in general, were attributed to the implanted steroid being carried by the hypophyseal portal system to the pituitary from the MBH and exerting a direct effect on the pituitary (Bogdanov'63). However, some discrepancies were not so easily explained and it soon became apparent that another area within the hypothalamus may be involved in gonadotropin secretion. Halasz pursued this

notion and, using a hypothalamic deafferentation technique, isolated the MBH from the rest of the brain by a knife cut that left in place only its connection to the pituitary. He found that though the ovaries were maintained in a nonatrophic state, ovulation did not occur (Halasz&Pupp'65). Anterior cuts placed just caudal to the optic chiasm also induced persistant estrus, but when cuts were made rostral to the preoptic area, cyclic ovulation could be maintained (Halsaz&Gorski'67;Koves&Halasz'70). As expected, lesions in this rostral hypothalamic-preoptic area blocked ovulation, but did not produce ovarian atrophy (Hillarp'49;Flerko&Bardos'60)and electrical stimulation of this area was able to induce ovulation in previously acyclic rats (Everett'61;Terasawa&Sawyer'69;Kawakami&Terasawa'70;Cramer&Barraclough'71;Kalra et.al.'71;Dyer'73;Turgeon&Barraclough'73;Everett'76;Clemens'76). A number of studies showing that high circulating levels of estrogen produced an enhanced responsiveness or firing rate of the neurons in the MPOA (Bueno&Pfaff'76;Cross&Dyer'72;Yagi'73;Dufy'76)supported the concept of an activation or positive feedback by ovarian steroids to this area.

Thus, the inhibitory effects estrogen has on the arcuate nucleus in regulating tonic release of gonadotropin secretion and its stimulatory effect on the MPOA in controlling the phasic release have been well-established in the rat. (This is probably not true in the primate as evidence in rhesus monkeys currently suggests that the MBH may work autonomously in controlling ovulation --for review see Knobil'78). This gross anatomical localization of areas involved in gonadotropin secretion in the rat brain has helped to define the arcuate and MPOA as functionally sensitive to estrogen and has led the way for

research into the neurotransmitters and neuropeptides which may be involved in estrogen's feedback control.

ESTROGEN'S EFFECT ON THE BRAIN--The Role of GABA in the Control of Gonadotropin Release

The gradual identification of various neuroregulatory messengers involved in neuroendocrine functions has been underway ever since the existence of neural control of the anterior pituitary was first postulated. Identifying the substances that influence endocrine function has been done mainly by pharmacologic manipulation of known CNS neurotransmitters and by mapping their distribution in areas of the brain, in particular the hypothalamus, that are known to affect pituitary responses. This has been done extensively for the biogenic amines and has led to the suggestion of dopamine as a prolactin (releasing) inhibitory factor (for review see Leong'83) and norepinephrine as a stimulator of LHRH release (for review see Meites&Sonntag'81). More recently, a possible involvement of GABA, a recognized inhibitory neurotransmitter in the CNS (Baxter&Roberts'60;Schmidt'71;Storm-Mathisen'74) has emerged and anatomical as well as physiologic investigations are underway to decipher its actual role in controlling gonadotopin release.

The anatomical evidence that provides the basis for the GABAergic control of anterior pituitary function in the hypothalamus is much more scarce than for either the monoaminergic or dopaminergic systems. GABA and its biosynthetic enzyme, GAD (glutamic acid decarboxylase) are reported to be concentrated in the hypothalamus (Fahn'76); hypothalamic slices preferentially take up GABA (Hokfelt'70); hypothalamic synaptosomes release it after electrical stimulation (Edwardson'72). Within the median eminence, 3H-GABA is taken up

by both neuronal cell bodies and their processes (Makara'75) and concentrated in the external layer of the median eminence (Tappaz'80). A GAD-immunoreactive density is prominent over both the medial and lateral part of the median eminence external layer (Tappaz'83) and according to studies combining deafferentiation with biochemical mapping, the source of this GABA is probably from neurons within the arcuate nucleus or the VMN (Tappaz'77;Wallas&Funnum'78). The possibility that GABA is secreted into the hypophyseal portal system and directly affects the anterior pituitary is supported by studies that show GABA terminals on the pericapillary space of these vessels (Tappaz'83) and the presence of GABA without GAD activity in the anterior pituitary (Oertal'82;Vincent'82) suggesting it is of hypothalamic origin (Racagni'79). Schally suggests GABA as having significant prolactin (releasing) inhibitory activity both in vivo and in vitro (Schally'77) and, in general, GABA's direct effect on the anterior pituitary has been most strongly implicated in the control of prolactin release (Casanueva'81).

Outside of the median eminence, GAD activity has been shown in highest concentrations in the lateral hypothalamus (especially those areas traversed by the rostral median forebrain bundle), the VMN and MPOA (Fonnum'77), with somewhat less activity in the arcuate (Tappaz et.al.'76;Tappaz'77;Perez et.al.'81;Mansky'82;Kimura'75). The source of this GAD activity, at least in the MBH is thought to be intrinsic though some input to the arcuate and VMN from the lateral and posterior hypothalamus is possible (Brownstein et.al.'76;Tappaz&Brownstein'77). The source of the GAD activity in the MPOA has not been studied but possibilities include: mesencephalic reticular formation input from the mamillary peduncle via the MFB; hippocampal input via

the pre-commissural fornix; and perhaps, amygdaloid input to the lateral preoptic area from the stria terminalis (Brodal'81).

To correctly interpret these studies, one must be aware that problems remain in assuming that levels of GABA or GAD activity has functional significance. For example, Tappaz states that in measuring 3H-GABA in neural tissues, the artifact incurred by the ability of the glial elements to readily take up and sequestor GABA must be eliminated (Tappaz et.al.'80). He does this by measuring only initial uptake as the glial mechanisms are much slower and take significantly longer to accumulate the labeled GABA. In addition, in earlier studies, he questioned GABA levels as any indicator of its concentration in various hypothalamic nuclei since within minutes after a rat is sacrificed, GABA accumulates very rapidly due to its metabolizing enzymes (GABA-transaminase and succinic-semialdehyde dehydrogenase) denaturing much more quickly than GAD, its synthetic enzyme (Tappaz et.al.'77). This has led to erroneous reporting of GABA levels throughout the brain, but has also been used by some (Mansky'82) as a way of measuring GABA turnover and correlating this with LH levels in the MPOA. It is also important to recall that in cerebral glucose oxidative metabolism, GABA (via the GABA shunt) plays a critical role in providing a defnse mechanism against hypoxic conditions (Wood'67) and may, in fact, vary in amount depending on the physiologic state of the organism. Therefore as Brownstein notes, in evaluating this literature it is not possible to determine to what extent GABA exists in the tissue as a neurotransmitter or as a result of intermediary metabolism (Brownstein'77) and conclusions drawn from it should be done so cautiously.

Measuring GAD activity has similar drawbacks and artifactual problems should be considered. A significant problem until recently was mapping GABAergic

fibers since a specific antibody to GAD was not available, making it difficult to preferentially label only GABA terminals. Oertel et.al. (81) have helped to eliminate this problem by developing a very specific anti-serum to rat brain GAD using anion exchange chromatography and a polyvalent anti-serum, raised from partially purified GAD, to 'trap' GAD. With this GAD antibody, a more accurate picture of GAD activity in discrete areas of the hypothalamushas emerged. But quantifying GAD activity and correlating it to functional activity is still questioned by some since enzyme activity levels (TH and DBH) have been shown to be poor indicators of dopamine levels and could, likewise, be poor indicators for GABA levels (Kreiger&Wuttke'80). For example, GAD activity could represent neuronal or glial synthesis since both are known to occur in some parts of the CNS (Csillik'70), or, even if localized only to the neurons,be a function of neuronal synthetic rates and not neuronal numbers. Kravitz's classic studies in the crustacean nervous system have showm that inhibitory fibers are capable of producing GABA at about eleven times the rate of excitatory fibers (Kravitz et.al.'68), and indeed, they may contain over one hundred times as much GAD as excitatory fibers. What does high GAD activity in the MPOA then mean--a few inhibitory fibers or many excitatory ones? Clearly, the meaning of GAD activity in the CNS remains to be defined.

The physiologic relevance of GAD activity within the hypothalamus and the role it plays in controlling LH release also remain quite vague. The plethora of GABAergic endings surrounding unlabeled neurons, especially in the MPOA, suggests that a great many neurons, including the LHRH neurons, may be under GABAergic control but the evidence as to what this control may be is sketchy. GABA in other brain areas has a predominently inhibitory effect (Schmidt'71) and in the hypothalamus, iontophoretic application of GABA inhibits firing of

cells (Curtis&Johnston'74). In addition, the demonstration of a recurrent picrotoxin-sensitive inhibitory loop which controls the firing of tubero-infundibular neurons has prompted the speculation that neurosecretory cells, such as the LHRH neurons, may have a Renshaw-cell-type GABAergic inhibitory neuron (Renaud'76;Yagi&Sawaki'75). The existence of local GABAergic cells in the hypothalamus as shown by deafferentiation studies (Tappaz&Brownstein'77) argues for this possibility. Unfortunately, the results of studies on the effects that GABAergic drugs have on LH release have been equivocal. A number of studies, for the most part from the same laboratory, report the ability of intraventricularly injected GABA to stimulate LH release (Vijayan&McCann'78a;'78b;McCann et.al.'81;McCann et.al.'84;Ondo'74) an action blocked by bicuculline, a GABA blocker, but unaffected by dopamine blockers (Vijayan&McCann'79). In addition, they report an elevation of LHRH in the suprachiasmatic-preoptic region after intraventricular GABA injection accompanied by an elevation in LH, and suggest the action of GABA may be via a direct efffect on rostral LHRH neurons (McCann et.al.'82). Conversely, other investigators attribute this stimulatory effect to a permissive effect of pentobarbital on the GABA-induced secretion of LH in anesthetized rats and maintain that in free-moving rats, GABA, in fact, has no direct effect on LH release (Pass&Ondo'77). However, studies show that when LH levels are high under conditions of low-circulating estrogen, GAD activity in the hypothalamus is reduced (Lamberts et.al.'83;McGinnis'80;Gordon'77; Wallis&Luttge'80; Early&Leonard'78), supporting an inhibitory GABA effect.

Present speculation as to how GABA exerts this purported inhibitory effect centers around an indirect effect on the LHRH neurons in the MPOA via

pre-synaptic inhibition of the norepinephrine input to these cells (Lamberts et.al.'83) which is known to stimulate LHRH release (Vijayan&McCann'78a). Anatomical evidence supports this schema--LHRH and NE terminals do synapse in this area (Jennes'82)--and pharmacologic evidence shows that GABA produces a decrease in both LH and norepinephrine levels in the MPOA (Wuttke et.al.'81). But, as Tappaz notes, if GABAergic cells within the hypothalamus directly or indirectly participate in the physiological regulation of pituitary function, they must be expected to be targets of circulating estrogen (Tappaz '84). Preliminary evidence in this regard shows that in the MPOA, GABA neurons do concentrate 3H-estradiol (Sar et.al.'83) and GABA neurons in the arcuate are estrogen-responsive (Leranth et.al.'85). What remains is to more precisely define the relationship of GABAeric synapses to the monoaminergic axons in the MPOA as well as the relationship of the monaminergic input to the LHRH neurons.

ESTROGEN'S EFFECT ON THE BRAIN--3H-Estradiol Binding Studies

If, in fact, GABA neurons are key estrogen-responsive neurons in both the arcuate and the MPOA, by what cellular mechanism does estrogen affect these neurons? Is it a 'typical' steroid and act at the gene level or does it work differently in an excitable cell and exert its effect at the membrane level inducing electrophysiologic instead of biochemical changes? To define estrogen's effect on neurons, early researchers first sought to map, within the brain the neurons that actually bind estrogen; to correlate this anatomical distribution with functionally-responsive areas; and, to localize within these areas, those neurons in which subsequent cellular effects then might be studied.

Initial studies of the distribution of estrogen in the brain used cell fractionation techniques to measure labeled estrogen in carefully dissected areas of the brain. Anterior pituitary tissue had very high levels of radioactivity, comparable to those found in the uterus and vagina and the anterior and mediobasal hypothalamus showed significantly higher uptake than either cerebral or cerebellar tissue (Eisenfeld&Axelrod'65,'66;Kato&Villee'67a,'67b;McGuire&Lisk'68;Luttge&Whalen'72). Subsequent studies using finer dissections and more extensive brain sampling revealed estrogen binding in the MPOA and limbic system in addition to the anterior pituitary and hypothalamus (McEwen&Pfaff'70;McEwen et.al.'75) and significant binding in the septum and midbrain but no apprecialbe estrogen in the cerebral cortex. McEwen showed preinjection of nonradioactive estrogen inhibited uptake in the areas of highest estrogen (anterior pituitary, MBH, MPOA, and limbic system) and described these structures showing both high levels of estrogen and good binding site competition effects as 'a limbic-preoptic-hypothalamic group of structures'(McEwen&Pfaff'70).

Cell fractionation studies provided estimates of the regional distribution of estrogen, but more anatomical detail could be gained by using an approach which could show within each brain region, the specific neurons to which estrogen was binding. Autoradiographic localization of systemically injected tritiated estrogen concentrated in specific brain areas provided this anatomical detail. Using this technique, Pfaff localized estrogen-binding to neurons within the MBH, MPOA, amygdala, hippocampus, ventral medial nucleus of the hypothalamus, pre-piriform cortex and septum (Pfaff'68a,'68b). But controversy arose over this methodology with some investigators claiming it introduced significant diffusion artifacts and declaring his results erroneous

(Stumpf&Sar'70). Modifying this technique, Pfaff repeated his early work but used unembedded, unfixed frozen sections and produced similar results (Pfaff'73). Stumpf, using a technique based on dry-mounting of freeze-dried sections designed to eliminate diffusion artifacts, produced an estrogen-binding map very similar to Pfaff and, noting that the distribution of these neurons followed known terminations of the stria terminalis, offered this as support for the concept of an endocrine-amygdaloid-hypothalamic-hypophyseal axis (Stumpf'68).

In general, there is good agreement among most autoradiographic studies showing estrogen in a limbic-hypothalamic distribution which correlates well with the regional distribution of estrogen concentration shown by scintillation counting of dissected brain regions and functional localization of neuroendocrine function in the brain. This apparent congruity of hormone localization and known neuroendocrine function suggests autoradiographic studies are relevant to defining the 'sex center' of the brain and have continued to be a useful experimental tool. Current work using autoradiography in combination with other labeling techniques has further characterized the distribution of these estrophilic neurons within each of the above anatomical loci and their relationship to neurotransmitters(Sar&Stumpf'81;Sar'84'Grant et.al.'77) and to releasing factors (Shivers'83). This has been especially useful in suggesting possible functional interactions steroids and neurotransmitters may have in controlling reproductive function.

ESTROGEN'S EFFECT ON THE BRAIN--Receptor Studies

Estrogen-sensitive cells in the brain have been localized with respect to the putative receptor sites as discussed above, making it possible to study in the particular cell groups involved in neuroendocrine responses, the specific cellular changes that occur under estrogen's influence and to define those necessary for estrogen to affect a neural response. This study of the cellular and subcellular mechanism of estrogen in the brain is founded in the work that had been done in the uterus where it is known that estrogen binds to a receptor that is recovered in the soluble fraction of the cytoplasm following tissue homogenization and is subsequently bound in the nucleus, where it alters the pattern of gene expression (Welshons,et.al.'84;King,et.al.'84). This estrogen receptor may also be found in the plasma membrane fraction as has been recently suggested but its functional significance is still questionable. The existence in neural tissue of a similar highly specific, limited capacity binding system for estrogen was first demonstrated by a number of workers using ovariectomized and estrogen-treated rats (Eisenfeld&Axelrod'65,'66;Kato&Ville'67;Luttge'72). Preferential uptake by the anterior pituitary, hypothalamus, preoptic area and limbic system was a constant feature in all of these studies but investigators questioned whether these receptors represented a physiological phenomenon or an artifact of treatment. McGuire et.al. ('69) were the first to demonstrate this preferential accumulation of labeled estrogen in the intact rat and also suggested that this binding changed with varying titers of estrogen. The question of changing hypothalamic receptor binding during different phases of the estrus cycle was addressed by a number of studies some suggesting there was no change and such data were a result of methodological error,

(Whalen'69), but most concluding that estrogen binding by the hypothalamus does vary with the circulating estrogen titers, increasing during estrus with low circulating steroid levels and decreasing during proestrus when estrogen levels are at their highest (Kato'70;Luttge'72;Ginsburg'75;Vertes et.al.'77). This response, the opposite of that seen in the anterior pituitary and uterus, could be explained by either increased occupation of receptors in the hypothalamus by the endogenous hormone or a decrease in synthesis of receptors by the hypothalamus in response to increasing estrogen levels (Vertes'77) and, as Eisenfeld speculates, may control the level of a neuron's response to estrogen during the estrous cycle (Eisenfeld'69).

Subsequent receptor studies using more refined isolation techniques further characterized the biochemical properties of the receptor protein (Kahwango'69; Verte'73) including its binding characteristics (Davies'75;McEwen'79) such that, to date, brain estrogen receptors are seen as 'highly stereospecfic, with very little cross-talking occurring and resembling very closely those found in other target tissues of the body' (McEwen'79).

Controversies, however, still remain concerning the specifics of this receptor system. It is well-known that effects the androgens have on the CNS, at least in the developing rodent, depend on their prior aromatization to estrogen (McEwen'74). These aromatizing enzymes are found throughout the brain regions that have estrogen receptor sites, including the hypothalamus and MPOA , but according to Krey et.al. only one-half of the neural estrogen receptor sites bind this estrogen, suggesting the existence of subsets of estrogen receptor systems in estrogen-sensitive neurons of the brain (Krey et.al.'80). In addition, the involvement of another steroid transformation, the formation of catecholestrogen (2- or 4-hydroxylation of estradiol) may be

involved in brain-estrogen interactions. Cuatrecasas suggests they may act at the membrane of the neuron by actually fitting into the catecholamine receptor and acting either as a catecholamine or blocking the action of the catecholamines (Cuatrecasas '81). Others have speculated an effect on the intracellular receptor, but both in vivo (Jellinick'81) and in vitro(Merriam'80) studies have shown that the degree of binding of these metabolites to intracellular estrogen receptors is significantly less than estradiol, though in situ formation of these compounds makes it possible that their local concentrations may be high enough to cause an effect (Fishman'82,'75). Of note when considering the extent to which estrogen metabolites are involved in the cellular action of estrogen, are the intial receptor studies wherein most investigators reported that a majority of the labeled hormone was recovered bound to the receptor in an unmetabolized form (Kato&Ville'67a;Michales'65,McEwen'79;Zigmond'70)arguing against a prior metabolism of estrogen in neural target tissue. Finally, the existence of progestin receptors in some estrogen-sensitive areas including the arcuate and MPOA (Sar'73), has led to the speculation that perhaps estrogen-induced progestin receptors are responsible for some of estrogen's effects, e.g. induction of sexual receptivity (Davies'79). However,because not all estrogen-sensitive neurons display this progestin receptor induction, including those of the amygdala (MacLusky'80), it is difficult to implicate this in the general schema of estrogen's cellular mechanism of action.

ESTROGEN'S EFFECT ON THE BRAIN--Cellular Mechanism of Action

Events in the neuron after estrogen binds to its receptor, either at the cytoplasmic or plasma membrane level, are obscure, but speculation centers around two mechanims: 1)direct action on the membrane, or 2)indirect action at the gene level as has been well-documented in the uterus (Katzenellenbogen&Gorski'75). Direct effects of estrogen on neural tissue have not been extensively examined, but studies of other steroids have shown them capable of neuromodulation by affecting ATPase activity and, thereby, modifying membrane potentials in neuroendocrine cells (Schade'71). The studies previously cited involving the changing electrophysiologic activity of MPOA neurons under varying estrogen levels suggest a direct effect of estrogen on neuronal activity though in situ studies such as this do not allow one to differentiate if the effect is related to the direct actions of estrogen at the monitoring site or to its influence on other brain areas that then affect this site. As McEwen discusses, in evaluating indirect versus direct effects of a hormone, one must consider latency and duration e.g. direct actions usually have short latency and brief duration and indirect just the opposite (McEwen'81) and protocols using electrophysiologic recordings during estrus cycles or estrogen treatment make it difficult to evaluate this temporal function. Kelly et.al. circumvented this problem by applying estrogen iontophoretically while monitoring cell firing in the MPOA and found an immediate decrease in neuronal firing, suggesting a direct, membrane action of estrogen (Kelly et.al.'78), by mechanisms currently unknown.

A much larger body of evidence has been gathered for the indirect mechanism of estrogen involving the genome. Early investigations by Lisk ('63) and Ifft ('64) showed that nucleolar size in neurons in the rat hypothalamus, including

the arcuate, is responsive to the amount of estrogen circulating in the system and though their data imply opposite effects on the hypothalamus, they both conclude that such structural changes may suggest that estrogen is affecting the nuclear constituents of neural tissue in some way. Subsequent receptor studies showing the labeled hormone-receptor complex translocating to the nucleus (Peck'79) and cell fractionation (McEwen&Pfaff'70;Zigmond&McEwen'70;McEwen'75) and autoradiographic studies (Stumpf'68;'70) demonstrating the labeled hormone bound by cell nuclei in the hypothalamus and the preoptic area led to the idea the estrogen works by activation (though transient) of an RNA polymerase (Peck'79). In this way it induces transcription of new RNA (Belajev et.al.'67;Foreman'77) and translation of new proteins (Litteria'73). Most investigators agree with this schema but conclusions as to the functional role of the newly synthesized proteins in either neuroendocrine function (Schally'69) or sexual behavior (Hugh'74) remain quite speculative. It is known only that oxidative activity (Moguilevsky'65) and some enzyme activities (Zolvik'66;Luine'74;Luine et.al.'75) vary with changing circulating estrogen levels, but specific enzymes are not known though obvious candidates include those involved in synthesis and degradation of neurotransmitters (Luine'75;Anton-Tay et.al.'68;Stefano et.al.'67;Lichtensteiger'69;Gudelsk'77;Donoso'67;Fuxe et.al.'67;Fuxe et.al.'72) and releasing factors, especially LHRH (Palkovits'74). Obviously, a number of biochemical effects of estrogen on neurons have been described, but definitive conclusions concerning cause-and-effect relationships between specific biochemical changes and estrogen-induced alterations in neural function in the CNS currently do not exist.

ESTROGEN'S EFFECT ON THE BRAIN--Morphologic Studies

Assigning biochemical causes to the morphologic effects estrogen has on neurons is a difficult task at best, but studying the morphologic changes estrogen can induce in neurons, in both physiologic and pathologic states may help to localize where within the cellular machinery estrogen is having its effect and perhaps offer insight into the long-held speculations concerning the plasticity of the adult brain.

Initial studies that implicated estrogen in affecting CNS tissue ultrastructure were done in developing rat brains in an effort to show the factors that ultimately determine the sexual maturation of the rat brain (more specifically, the hypothalamus). Dorner and Staudt ('68;'69) found effects of neonatal castration or neonatal androgenization on nuclear sizes in hypothalamic preoptic anterior areas and the ventromedial nucleus of the rat. More recently, Toran-Allerand ('76;'80) has shown that estradiol can enhance the outgrowth of neurites from newborn mouse hypothalamic and preoptic area explants *in vivo* which is consistent with older reports that estrogen administration to young rats *in vivo* markedly stimulates myelinization and functional maturation of the brain (Heim&Timiras'63;Curry&Hein'66). But perhaps the most striking evidence for steroid-induced structural changes in the developing CNS comes from the work of Raisman and Field ('71;'73) who demonstrated that the distribution of synapses in the preoptic area is sexually dimorphic in rats (males having a higher proportion of synapses on dendritic shafts than on dendritic spines) and that neonatal castration of males results in a female-type pattern of distribution in this area. An effect of sex steroids on synaptogenesis was also suggested by Matsumoto and Arai ('76A;'76b) who found that treatment of female rats with estradiol during

the first thirty days of life resulted in twice the number of axodendritic synapses in the arcuate nucleus compared to oil-treated control though the steroid treatment did not seem to alter the rate of formation of axosomatic synapses in the arcuate suggesting that only particular axon systems are susceptible to the hormonal effect. MacLusky et. al. ('81) speculating as to the role synaptogenesis plays in development state "Several lines of evidence suggest that the survival of neurons may to some extent be dependent on the formation of synaptic contacts, cells that form from only a limited number of synaptic connections being preferentially eliminated during CNS maturation...Gonadal steroids could alter this process by selectively stabilizing some connections or enhancing the role of degradation of others (or both)."

In addition to the strong evidence for morphologic effects of gonadal steroid treatment on the developing CNS, there is a growing body of evidence that suggests this effect may not be limited to early development. Arai et.al. report that if the MBH is deafferentiated in adulthood resulting in degeneration of some presynaptic elements, administration of estrogen will stimulate the formation of axodendritic synapses, an effect not seen in the normal adult (Arai et.al.'78). Naftolin supports the idea of synaptogenesis in the hypothalamus and raises the possibility that this steroid effect may be focused on growth cones in the hypothalamus, such as those found in the PVM that are cytologically identical to those commonly seen in developing prenatal or neonatal nervous tissue (Naftolin&Brawer'77). Pathologic effects reported on synapses of adult neurons, when estrogen is introduced in pharmacologic amounts suggest that it can also behave as a neurotoxic pathogen. In a series of studies using large doses of administered estrogen, Brawer describes the development of acyclicity and demonstrable (by light and electron miscroscopy)

lesions and gliosis in the arcuate nucleus of the adult male and female rat (Brawer&Sonnenschein'75;Brawer et.al.'78;Brawer et.al.'80). This effect is predominently glial with an increased number of microglia and astrocytes with inclusions while the neurons demonstrated no real changes from controls and only a mild axonal degeneration of the neuropil. Brawer postulates this response may represent a functional chemical deafferentiation of the MPOA from the MBH producing acyclicity and multicystic ovaries and suggests it as the basis for age-related hypothalamic failure (Brawer et.al.'80).

Though some believe the influence of estrogen on both the mature brain and the developing brain is predominantly at the synaptic level (Naftolin&Brawer'76), as these studies seem to indicate, most believe that plasticity of the adult brain remains a questionable issue. Studies have shown it possible to promote a functional plasticity in a damaged nervous system through a variety of electrical, biochemical and surgical techniques (Freed et.al.'85) while others have declared that, at least in primates, there is no real evidence for post-developmental neurogenesis (Rakic'85), though this does not rule-out synaptogenesis. It is probably best to conclude that in the adult, the evidence to date suggests that steroid hormones exert an 'activational' and not an 'organizational' effect; they exert profound but transitory effects and activate or inhibit the function of existing neural circuits without making fundamental changes in the neuronal circuitry (Gorski'79).

To establish this activational effect of estrogen on neurons, investigators have looked for ultrastructural evidence but instead have discovered cellular changes whose functional significance remains quite speculative. An early study by Lisk and Newton ('63) shows that estrogen implanted in the arcuate nucleus of female adult rats results in a decrease in the size of the nucleoli

of the neurons in this nucleus and produces a dramatic ovarian atrophy which they speculate is a result of decreased synthetic activity by the arcuate in response to the increased estrogen. Conversely, Ifft ('64) shows a decrease in nucleoli size in neurons of the arcuate in castrated animals and , more recently, Zimmerman ('82) shows an increase in the nuclear area of these neurons in the mouse describing the effect as a 'functional nuclear edema', a result of increased cell permeability which occurs with the activation of the nuclear metabolism by sex steroids (McEwen et.al.'78). Studies of the neuronal cytoplasmic ultrastructure have yielded similarly conflicting results. Zambrano and DeRobertis, looking at the effects of castration, found the arcuate nucleus as well as the supraoptic and paraventricular ('68b) to show signs they ascribed to hyperactivity: increased numbers of ribosomes organized into polysomes; dilated cisternae of endoplasmic reticulum; a well-developed nucleolus; and, increased numbers of granulated vesicles in the surrounding neuropil. This correlates well with a later study, where granulated vesicle content in arcuate neuron terminal endings was measured at different stages of the estrus cycle demonstrating that at the end of proestrus and especially during estrus the system has the lowest content of granulated vesicles, with a considerable increase in the number beginning at proestrus (Zambrano'69). Citing previous studies which correlate granulated vesicles and catecholamine content in the arcuate (Jaim-Etcheverry'68), Zambrano suggests that this cyclic change in the number of granulated vesicles supports the notion that catecholamines may play a role in the control of gonadotropin secretion and estrogen may be affecting their synthesis. Looking at his data, one can question the significance of the difference in vesicle content among the four time periods and the statistical analysis he uses to determine this, as well as his assumption that these types of vesicles contain

primarily catecholamines. Moreover, he ignores the possibility that they could contain other substances such as releasing factors or both releasing factors and neurotransmitter. Such work is illustrative of the inherent difficulty one has in drawing conclusions about cellular function from ultrastructural changes in cellular constituents whose function has not been fully characterized. This is even more clearly illustrated in the following studies.

Knowing that the neurons of the arcuate nucleus projected to the pericapillary space and may function as neurosecretory cells similar to the supraoptic or paraventricular nuclei, Brawer et.al. sought to characterize more fully the ultrastructure of these neurons. Using both normal and castrated male rats, they discovered an increased occurrence of whorl bodies, previously described endoplasmic reticulum formations (Palay and Palade'55) in castrated animals (Brawer'71,'72). King and Williams thought these formations to be dependent on circulating steroid levels and studied their rate of occurrence at various stages of the estrous cycle and in ovariectomized animals (King et.al.'74). They, too, concluded that low plasma steroid levels induced the formation of these whorls and speculated that perhaps they represent a hypersynthetic activity involved in LHRH production in these neurons. In fact, Naik, in an immunocytochemical electron microscopic study of the arcuate, found whorl bodies in labeled LHRH neurons (Naik'75) though the presence of LHRH neurons in the arcuate has been questioned by many investigators as has the ability of these neurons to bind estradiol (Shivers et.al.'83). Many other investigators have described the increased incidence of these formations in castrated animals, male (Price et.al.'76;Ford et.al.'73;Ford et.al.'74) and female (Cohen&Pfaff'81) and in a multiplicity of other organs in both pathologic and physiologic states (for review see King et.al.'74). But their functional

significance still remains obscure. Possibilities range from a response to toxic insult (for review see Steiner et.al.'63) to a center of hypersynthetic activity (King et.al.'74) with a very recent study postulating their occurrence as a response to decreased cytoplamic concentrations of calcium (Kallenbach'84). Until the functional nature of these organelles is more clearly established, their presence or absence in varying hormonal states provides little help in elucidating the role of various hypothalamic neurons in neuroendocrine function.

The presence of other cytoplasmic organelles has been described to be hormonally-sensitive also, the most notable being the nematosomes (Anzil et.al.'73;Hindelang-Gerner et.al.'74). Like the whorl bodies, these are not found exclusively in neurons, but unlike them, castration has no effect on their incidence (Santolaya'73) and instead, recent reports suggest an increase in their number correlates with increased steroid levels (King et.al.'74;Brawer'71; Leranth et.al.'85). Do they represent a storage or transit form of readily available material piled up in perikaryon or moving along processes prior to being used (Anzil'73) or is their increased presence in perikaryon a result of colchicine-treatment which blocks their normal migration into axons where they transport ribosomal material to be utilized at synaptic terminals(Hindelang-Gertner'74)? As with the whorl bodies, the precise role, if any, nematosomes play in the activational effects of estrogen on neural tissue must await further characterization of their biochemical functions within the cell.

SUMMARY

There is a large body of evidence for induction of both functional and structural changes by estrogen in the adult rat brain but the relationship

between the structural and biochemical effects of estrogen on neural tissue and the eventual neuroendocrine effects are still not completely defined. Is estrogen acting by changing thresholds for neuroendocrine responses or by grossly changing the course of neural pathways? The idea that steroids change the essential structure of the nervous system is questionable and it is probably more correct to assume, as was proposed even by the earliest investigators, that the effects of steroids would be subtle rather than gross (Phoenix'59). In this respect, narrowing studies of estrogen's effects to a specific neuronal population such as the GABA neurons, seems an appropriate approach in attempting to find the functional and structural correlates of its action in the CNS.

METHODS AND MATERIALS

Six female Sprague-Dawley rats (200-220 g. Charles River, Wilmington, MA; CD (SD) BR strain) were used in these experiments. The animals were kept under standard laboratory conditions: 24 C, 12 h light-dark period, with food and water ad libitum. Rats were divided into two treatment groups of three animals each: animals in Group I were bilaterally ovariectomized and immediately received a single dose of estradiol valerate (EV; 2 mg. in 200 ul of sesame oil, s.c.), 21 days before sacrifice; Group II rats were ovariectomized 21 days before sacrifice and received no estrogen. Both Groups I and II received 80 ug of colchicine (dissolved in 20 ul saline) into the lateral ventricle 24h prior to sacrifice.

Animals were perfused under ether anesthesia with 50 ml of saline, followed by 200 ml of fixative containing 4% paraformaldehyde, 0.08% glutaraldehyde and 150 ml. saturated picric acid in 1000 ml. 0.1 M phosphate buffer, pH 7.35 (PB) (Somogyi&Takagi'82). After rapid removal of the brains, the MBH and the anterior hypothalamus, including the MPOA, were dissected out and postfixed for 24h at 4 C in glutaraldehyde-free fixative. Coronal sections (40um) were cut on a Vibratome (Lancer, St.Louis,MO) and washed for 24h at 4 C in several changes of PB. The sections were transferred to a vial containing 0.5 ml PB with 10% normal rabbit serum to saturate non-specific protein binding sites.

The anti-GAD antibody used was raised in sheep against GAD purified from rat brain symaptosomes, and has been previously characterized (Oertel et.al.'81). For immunostaining, Sternberger's peroxidase-anti-peroxidase (PAP) technique (Sternberger et.al.'70) was used according to a previously described protocol

(Leranth&Feher'83). Incubation times and dilutions were as follows: anti-GAD 1:1500, 24h at 4 C; anti-sheep immuno-gamma-globulin (Cappel Laboratories, Cochranville, PA) 1:40 dilution, 1.5 h at 20 C; sheep PAP (Cappel Laboratories) 1:1000 dilition, 1.5 h at 20 C. All dilutions were made in PB containing 1% normal rabbit serum and 0.1% NaN3. The tissue-bound peroxidase was visualized by using 0.17% 3,3' diaminobenzidine with 0.002% H2O2 in Tris-HCl buffer (0.05 M, pH 7.6 for 7 min. at 20 C). The sections were then post-osmicated, dehydrated and embedded in EM-BED-812 between a glass cover slip and aluminum foil to keep them flat. After 24 h polymerization, the aluminum foil was removed from the 'sandwich' and the sections were fixed on an araldite block. Ultrathin sections were cut from the outer surface (2-3um) of the 40um Vibrotome sections and were counter-stained for 30 sec. with lead citrate prior to the electron microscopic analysis of the stained profiles.

RESULTS

ARCUATE NUCLEUS

Fine Structure

Tissue preservation using intracardiac perfusion appeared very good throughout the hypothalamic region with easy cytological differentiation of neuronal and glial elements. All sections were taken from an area of the arcuate nucleus adjacent to the ependymal layer near the median eminence (Fig.1). The same ultrastructural features cited by other investigators (Brawer'71;Santoloya'73) are observed in this study with no differences noted between experimental groups. The nuclei of the labeled and unlabeled arcuate neurons are ellipsoidal or round and usually display either shallow or very deep indentations. Usually each neuron contains one prominent nucleolus, the cytology of which is exactly as described for neuron nuclei in general (Peters et.al.'71). The cytoplasm of the unlabeled neurons show no remakarble features with Golgi bodies (1-2) scattered in the cytoplasm close to the nucleus and granular endoplasmic reticulum, diffuse and loosely arranged throughout the periphery of the cell. Other structures in these neurons are identical to those described in standard texts, e.g. mitochondria, lysosomes, multivesicular bodies and neurotubules. Glial elements are unremarkable, showing no signs of gliosis. The nucleus of the oligodendrocyte is oval, contains more chromatin than a neuronal nucleus and has a characteristic clumping of the chromatin around the nuclear envelope with nuclear pores between the clumps. Astrocytes, seen less frequently, have cytoplasm which is electron-lucent and relatively free of organelles. The neuropil is characteristic for the sporadic appearance of myelin figures (Fig.2), a term

applied to dense osmiophilic pools of phospholipid which typically appear in glutaraldehyde-fixed specimens as dark lamellae in compact spirals (Fawcett&Susumo'58) and probably represent artifacts of fixation. In addition, an occasional tanycyte process is seen running through the field (Fig.2) from the adjacent ependymal layer.

GAD-Postive Neuronal Perikaryon

The poor penetration of immunoreagents allowed a reliable evaluation of GAD-positive profiles only in the most superficial few micrometers of the Vibratome sections. This necessitated observation of numerous ultrathin sections in order to study a reasonable number of labeled neurons. Only 3-4 labeled neurons were visible in a 50um x 50um section. GAD-positive neurons in both experimental groups appeared essentially similar, except for a slightly increased synthetic activity in neurons from the castrated group as seen by occasional areas of extensive RER stacking (Fig.3A,B). GAD-positive neurons in both groups had a charaacteristic 'clover-shaped' nucleus (Fig.4). Axo-somatic synapses with GAD-positive terminals (Fig.5) and terminals containing large, clear vesicles (Fig.6) are seen in both groups. Nematosomes are quite prevalent in neurons from both groups, but in the estrogen-treated group more than one nematosome (either cytoplasmic or nuclear) per neuron was more likely to occur.

GAD-Positive Axons Terminals

The numerous immunoreactive dots visible at the light-microscopic level (Fig.1) can be identified, in the ultrastructural analysis, as labeled axon terminals. Labeled axons are much more numerous than labeled soma, usually comprising about 25% of the total axonal population of a section. Typical

synapses are found between GAD-positive endings and unlabeled perikaryon or dendrites and labeled neurons and dendrites. No synapses are seen between labeled axons.

Whorl Bodies

Whorl bodies as shown in Fig.7 are found only in estrogen-treated rats, contrary to previous accounts (Brawer'71) and only in unlabeled neurons, though the neurons in which these formations are found may be beyond the reach of the immunoreagent and, therefore, may, in fact, be GABA neurons. A total of three whorl bodies are found in approximately 150 (50um x 50um) sections viewed, and all consist of smooth endoplasmic reticulum and contain no vesicles or mitochondria.

MPOA

Fine Structure

As in the arcuate nucleus, tissue preservation was very good throughout the MPOA. Sections were taken from an area of the MPOA as indicated in Fig.8(A,B) where most GAD activity appeared by light microscopy and where the LHRH neurons are known to be located. There is no difference in the cytology of this area between the two experimental groups and both show characteristics similar to that previously described in detail (Prince&Jones-Witters'74). The nuclei of the unlabeled neurons have a variety of shapes, but are, for the most part, oval or elliptical with some indentations. In one or two sections, rare fusiform neurons are seen, probably representing the LHRH neurons. The unlabeled neurons contain the usual cytoplasmic organelles in normal amounts including a granular endoplasmic reticulum, more frequently perinuclear, and a singular Golgi apparatus. Glial cells are as described in the arcuate nucleus

with characteristic clumping of the chromatin around the nuclear envelope. As in the arcuate, the neuropil is remarkable only for occasional extracellular myelin figures.

GAD-Positive Neuronal Perikaryon

Again, poor penetration of the immunoreagent limited evaluation of the GAD-positive neurons in the MPOA. Labeled neurons are very scarce with an average of 0-1 per (50um x 50um) section viewed and appearing the same in both experimental groups, showing no differences in degree of neuronal synthetic activity and having numerous axo-somatic synapses with both GAD-positive terminals and terminals containing dense-core and large, clear vesicles (Fig.9). GAD-positive neurons in the MPOA in both experimental groups show many more axo-somatic synapses (3-5/GAD-positive profile) than those of the arcuate nucleus. Nematosomes are seen in labeled and unlabeled (Fig.10) neurons in equal proportions, with more than one per neuron seen very rarely. There is no difference in the number of nematosomes per section viewed between experimental groups. The nuclei of the labeled neurons show the characteristic indentations as seen in the arcuate (Fig.4).

GAD-Positive Axon Terminals

A very high density of immunoreactive dots is present at the light microscopic level (Fig.8A,B) and is identified as axon terminals at the ultrastructural level. Labeled axon terminals are more numerous than in the arcuate nucleus, comprising approximately 30-35% of the total axonal population and synapse predominantly on GAD-positive and unlabeled somas. No GAD-GAD synapses between axons are seen.

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Whorl Bodies

One whorl body as shown in Fig.11 is present, found after viewing approximately 200 (50um x 50um) sections. This is present in the estrogen-treated group in an unlabeled neuron, but as in the arcuate, at a level where the GAD label is lost. The whorl body consists of smooth endoplasmic reticulum and numerous vesicles of electron-dense material at its center. No mitochondria are seen within the whorl body.

RESULTS--Addendum

AMYGADALA

GAD-Positive Neuronal Perikaryon and Axon Terminals

Ultrathin sections from the corticomedial nucleus of the amygadala show numerous GAD-positive neuronal profiles with numerous GAD-positive axosomatic synapses (Fig.12). Myelin figures are presently in large numbers throughout the neuropil; no whorl bodies or nematosomes are seen in 75 (50um x 50um) sections viewed.

FIGURES

Fig.1: Arcuate nucleus; estrogen-treated rat: 40um section stained for GAD; A: Demonstrating area of GAD-positive estrogen-sensitive neurons (arrow). (Magnification x10). B: Demonstrating GAD-positive neuronal profile (arrow). (Magnification x40).

Fig.2: Arcuate nucleus; estrogen-treated rat: Neuropil with tanycyte process (T) running through field; (m)myelin figures. (Magnification x12,000).

Fig.3: Arcuate nucleus; ovariectomized rat: A: Unlabeled neuron with extensive rough endoplasmic reticulum (arrow). (Magnification x3,000). B: Extensive stacking of rough endoplasmic reticulum. (Magnification x15,000).

Fig.4: Arcuate nucleus; ovariectomized rat: GAD-positive neuron with characterteristic 'clover-shaped' nucleus. (Magnification x9,000).

Fig.5: Arcuate nucleus; ovariectomized rat: GAD-GAD axo-somatic synapse (arrows); (a)axon; (s)soma; (Magnification x34,000).

Fig.6: Arcuate nucleus; ovariectomized rat: Clear vesicle axon terminal (a) making synapse (arrow) with GAD-positive soma (s). (Magnification x42,000).

Fig.7: Arcuate nucleus; estrogen-treated rat: Whorl body in cytoplasm of unlabeled neuron. (Magnification x12,000).

Fig.8: MPOA; estrogen-treated rat: 40um section stained for GAD; A: Area from which ultrathin sections were taken (arrow). (Magnification x10). B: Dark dots representing GAD-positive axon terminals (arrow). (Magnification x40).

Fig.9: MPOA; estrogen-treated rat: GAD-GAD axo-somatic synapse (arrow); clear vesicle (c), dense-core vesicle (d), and, both clear and dense-core vesicle (b) axon terminals making synapses with labeled soma (s). (Magnification x 9,000).

Fig.10): MPOA; estrogen-treated rat: Unlabeled neuron with nematosome (n). (Magnification x18,000).

Fig.11: MPOA; estrogen-treated rat: Whorl body in unlabeled meuron with electron-dense material in center. (Magnification x7,000).

Fig.12: Amygdala; estrogen-treated rat: A: GAD-GAD axo-somatic synapse (arrow). (Magnification x12,000). B: GAD-GAD axo-somatic synapse (arrow). (Magnification x36,000).

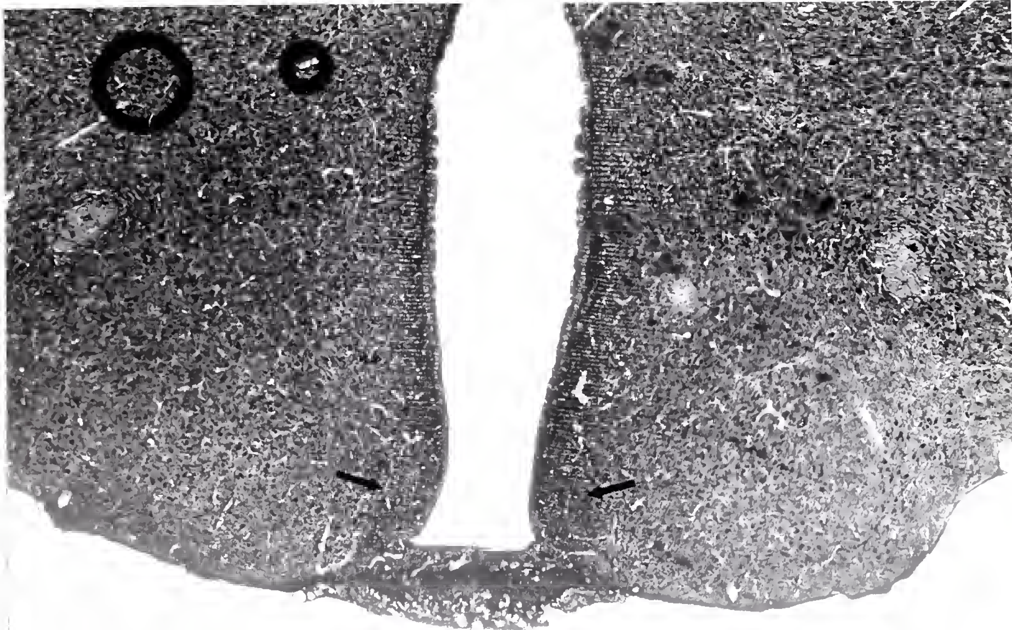


Figure 1-A.

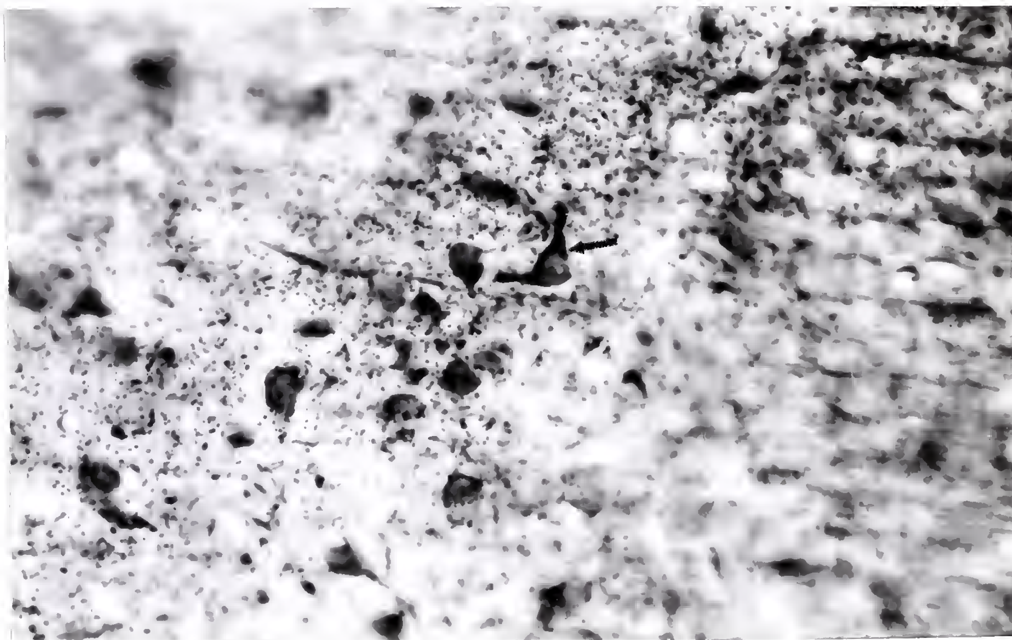


Figure 1-B.

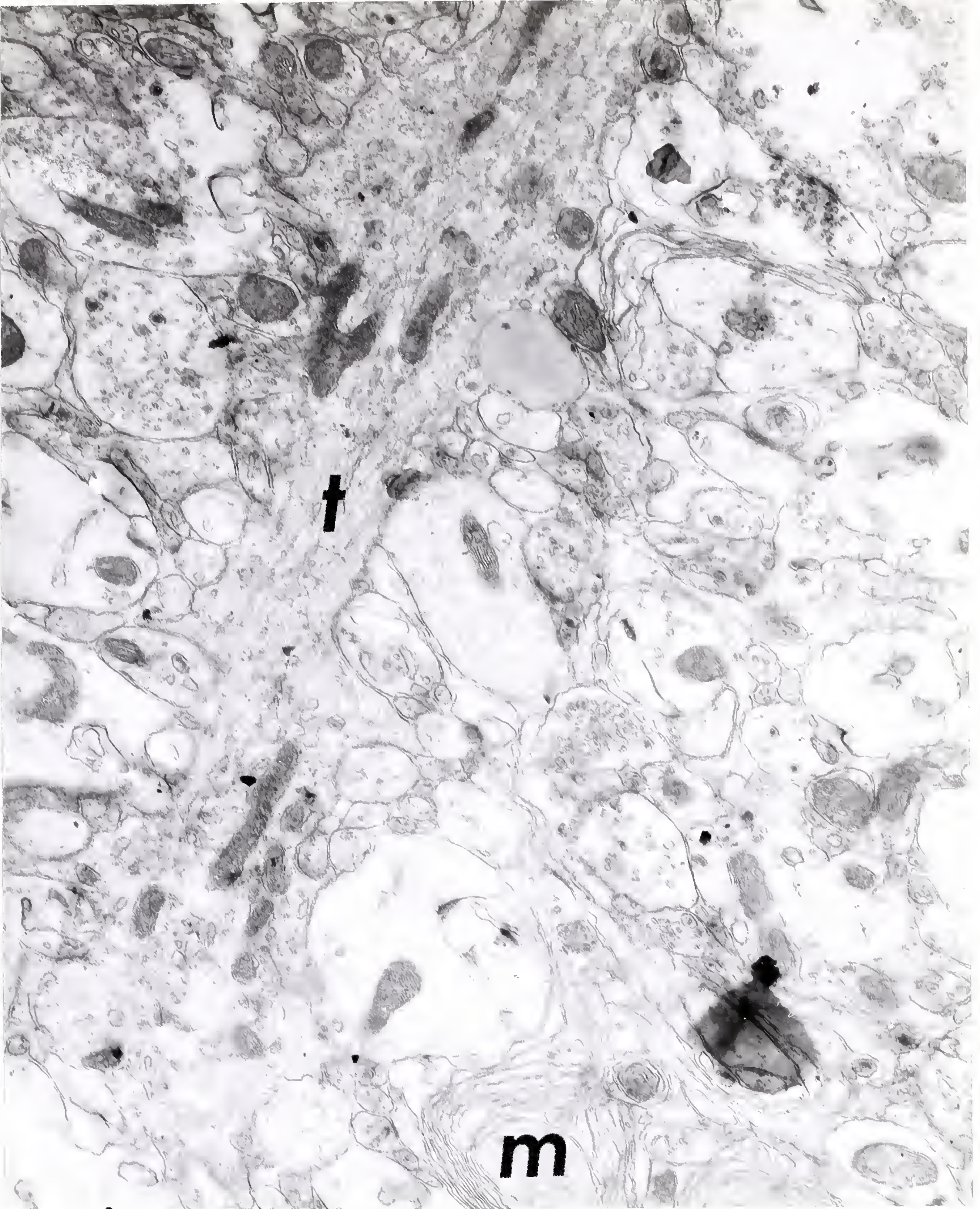


Figure 2.

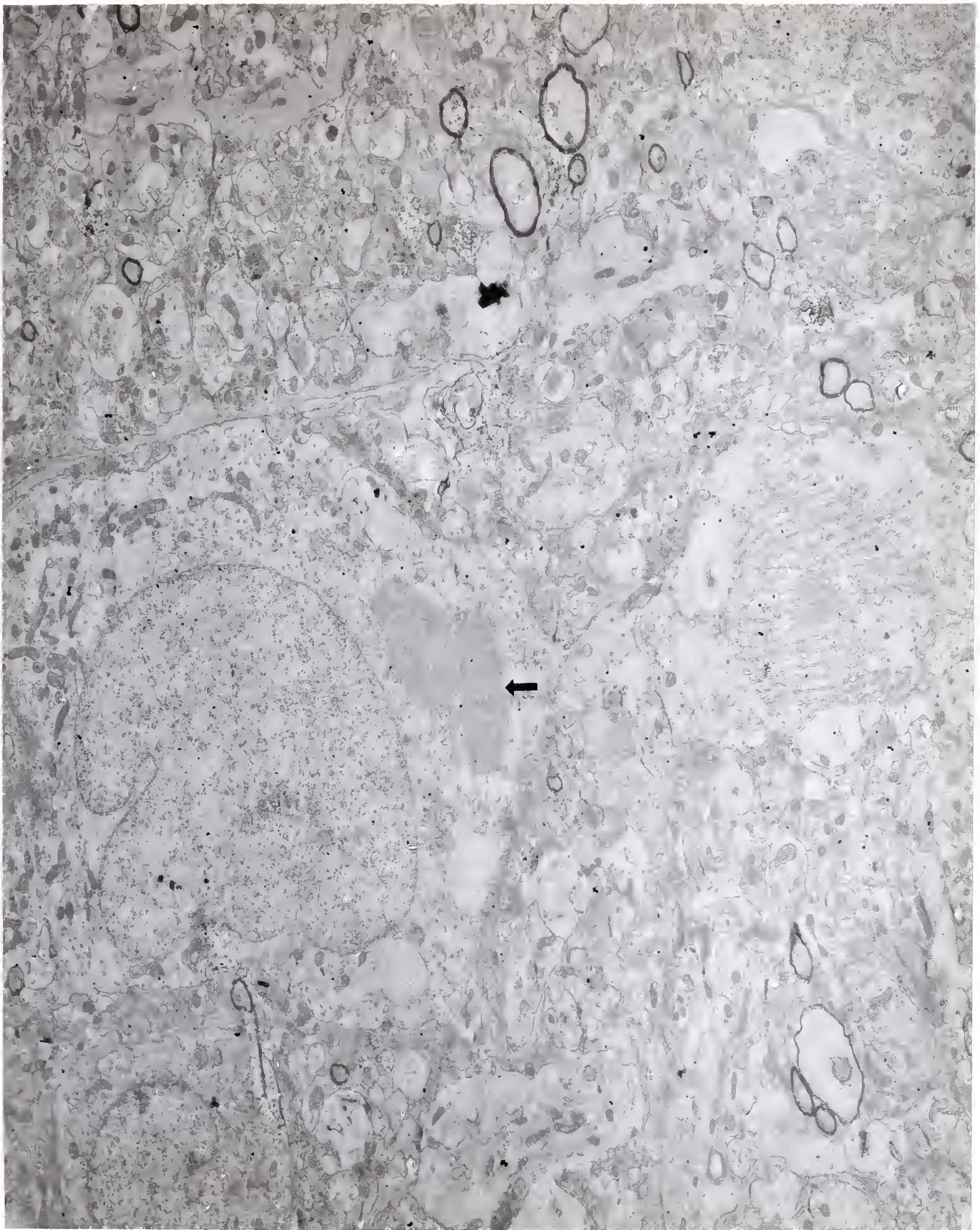


Figure 3-A.

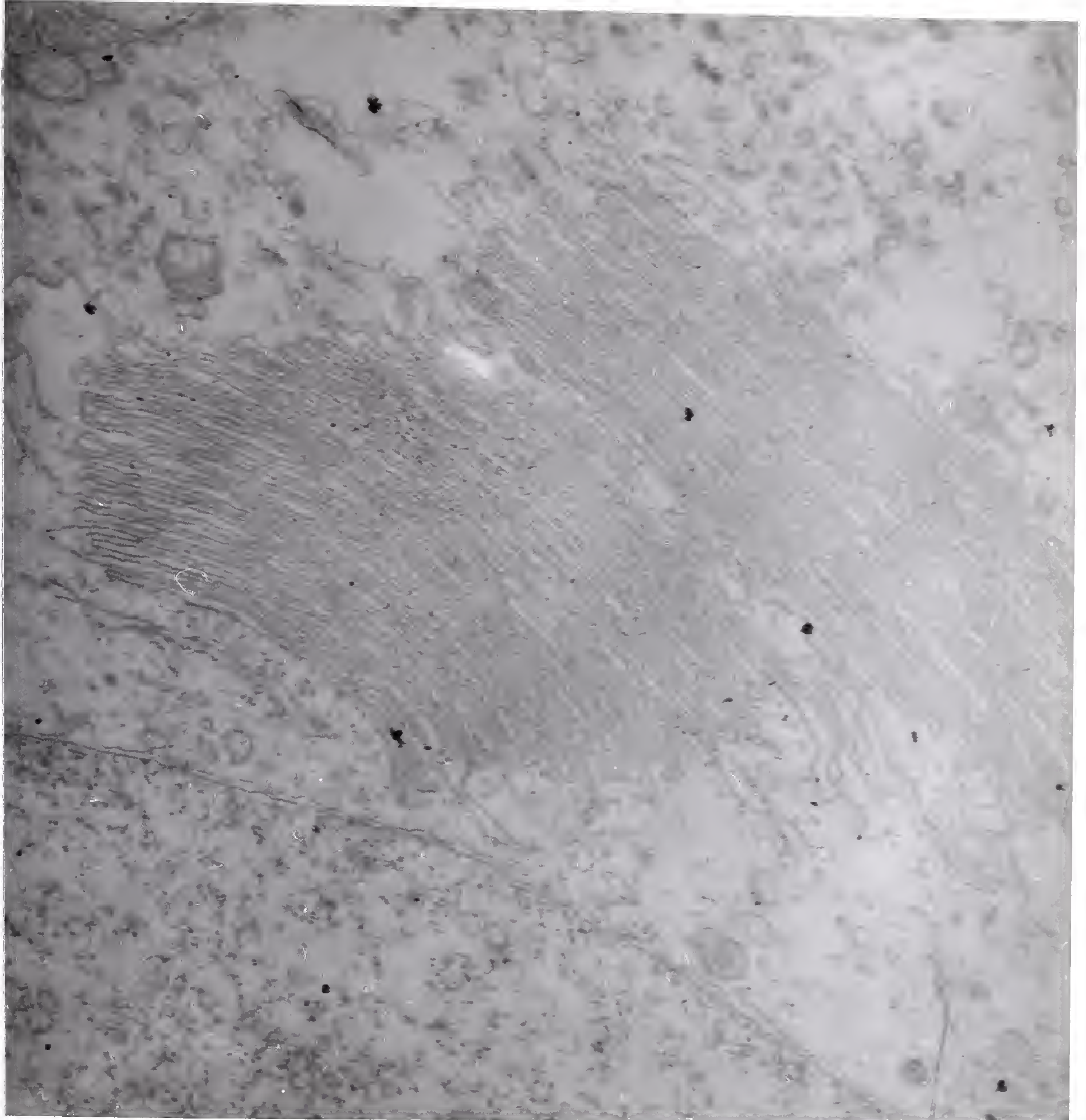


Figure 3-B.

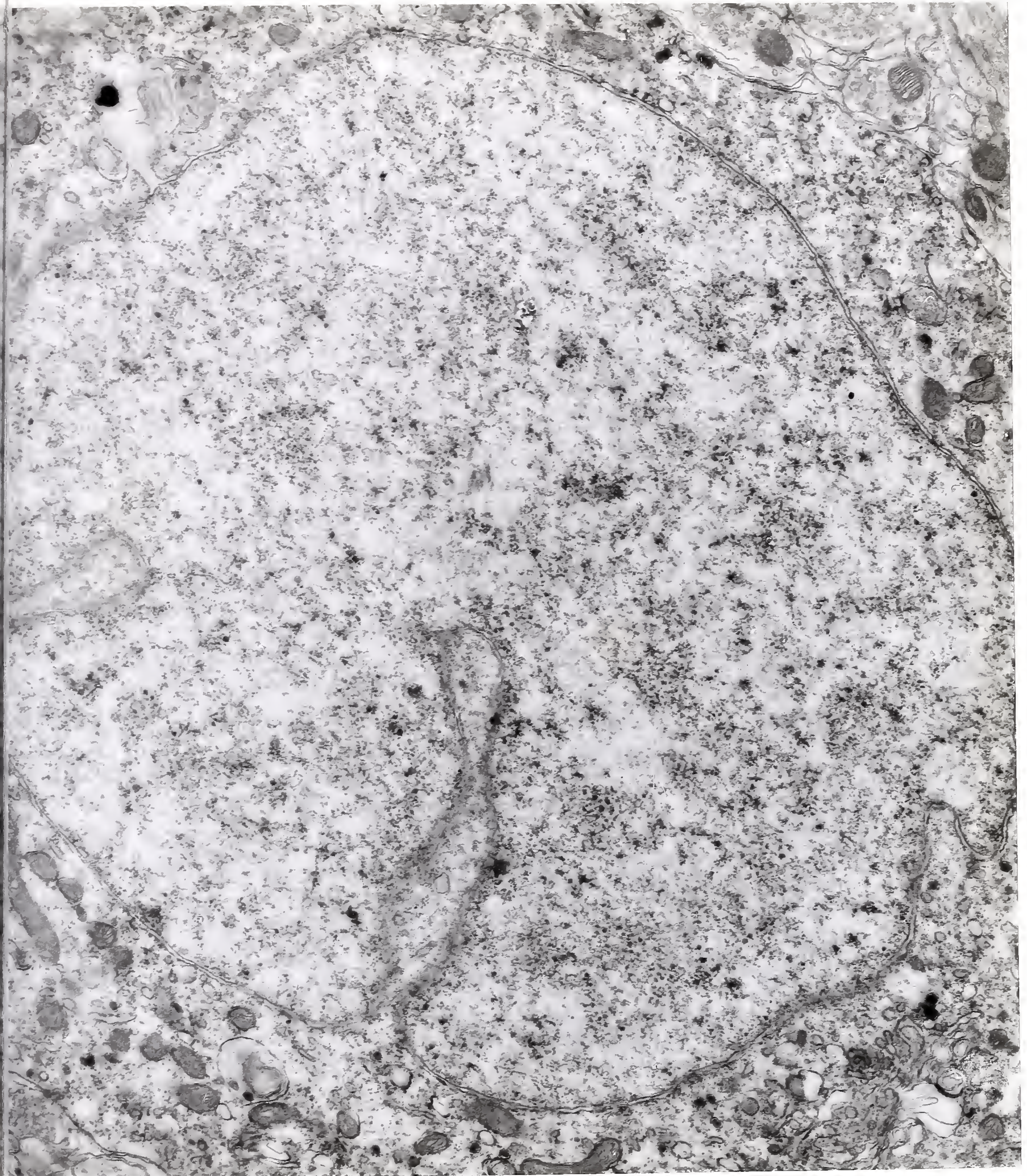
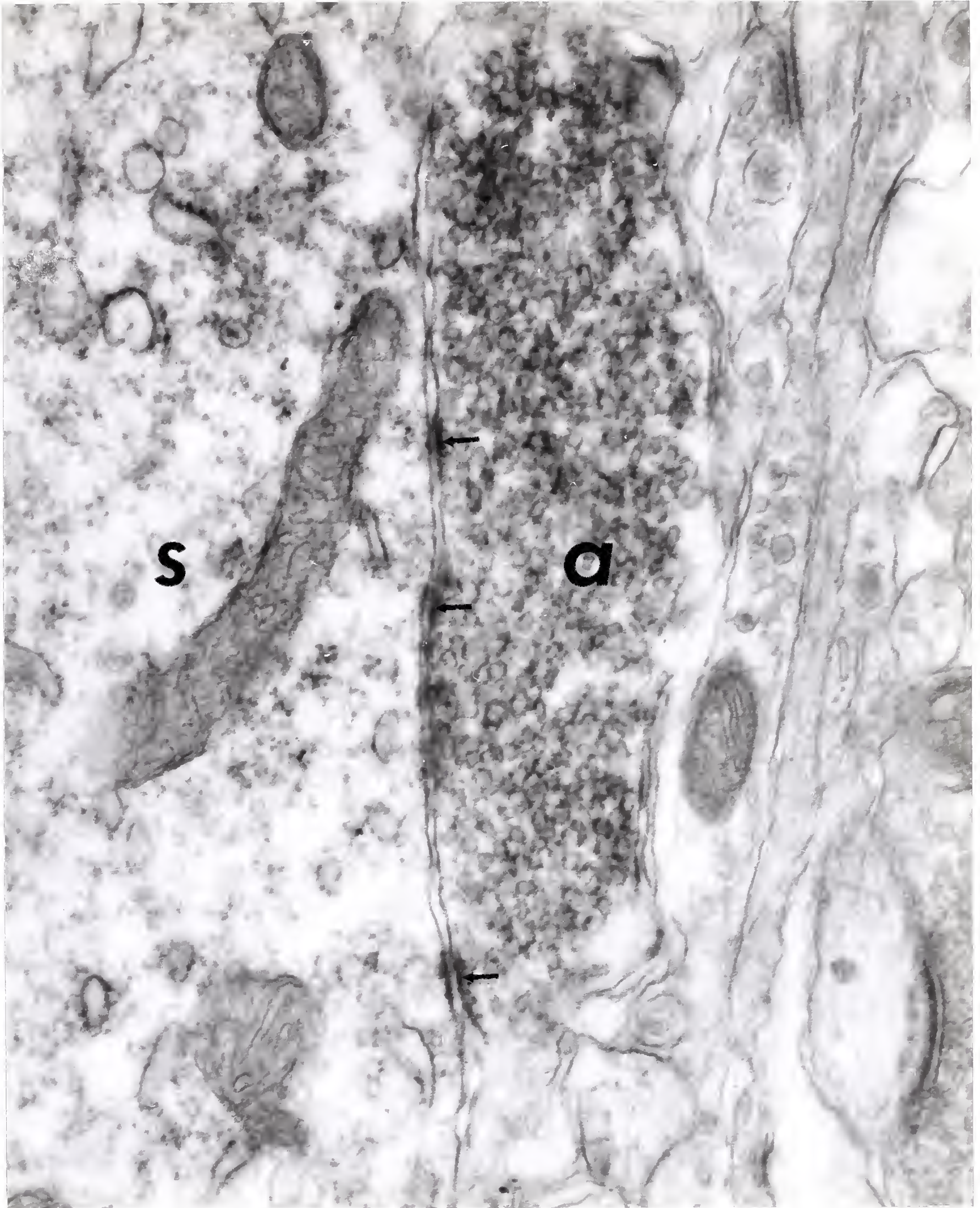


Figure 4.



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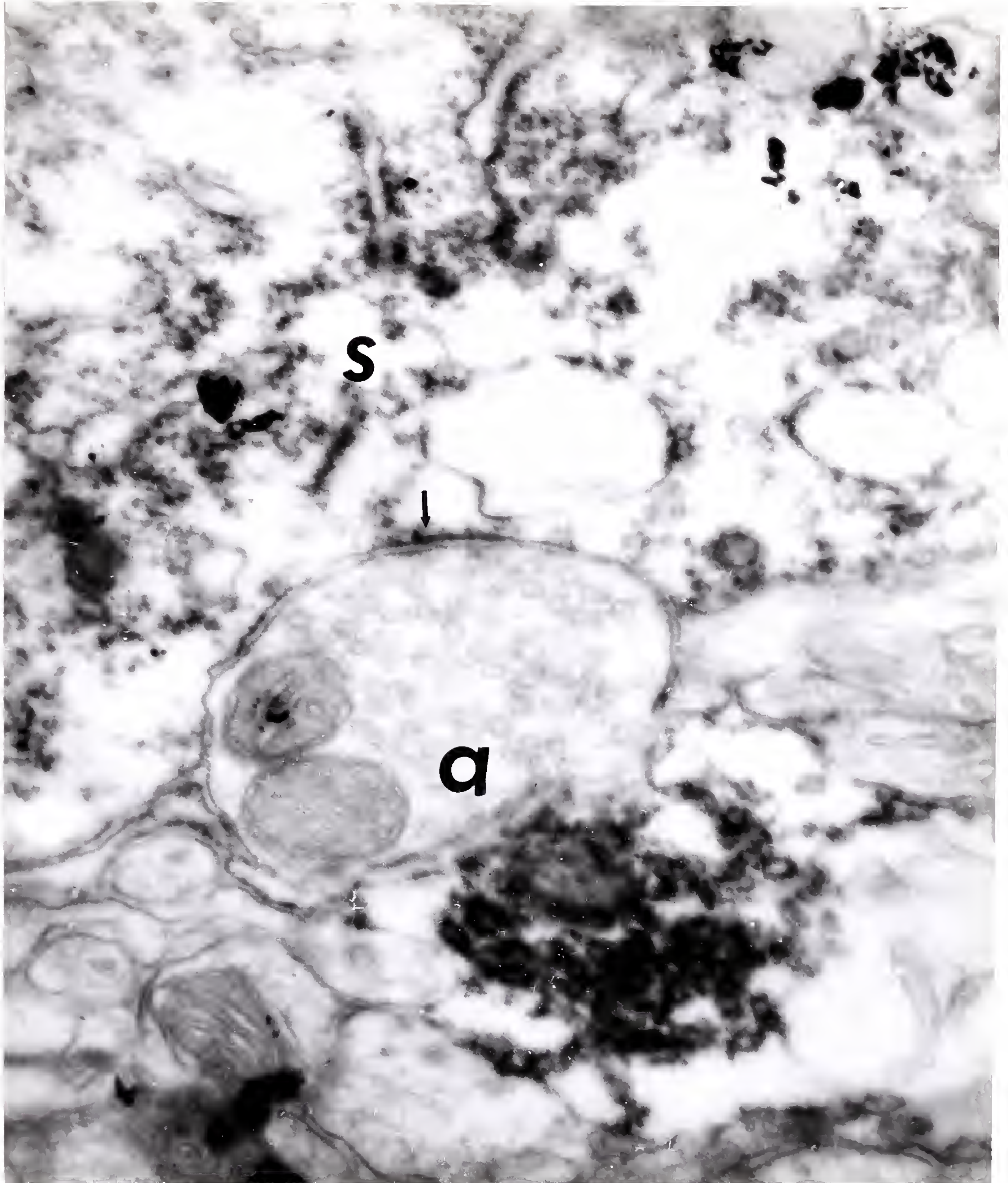

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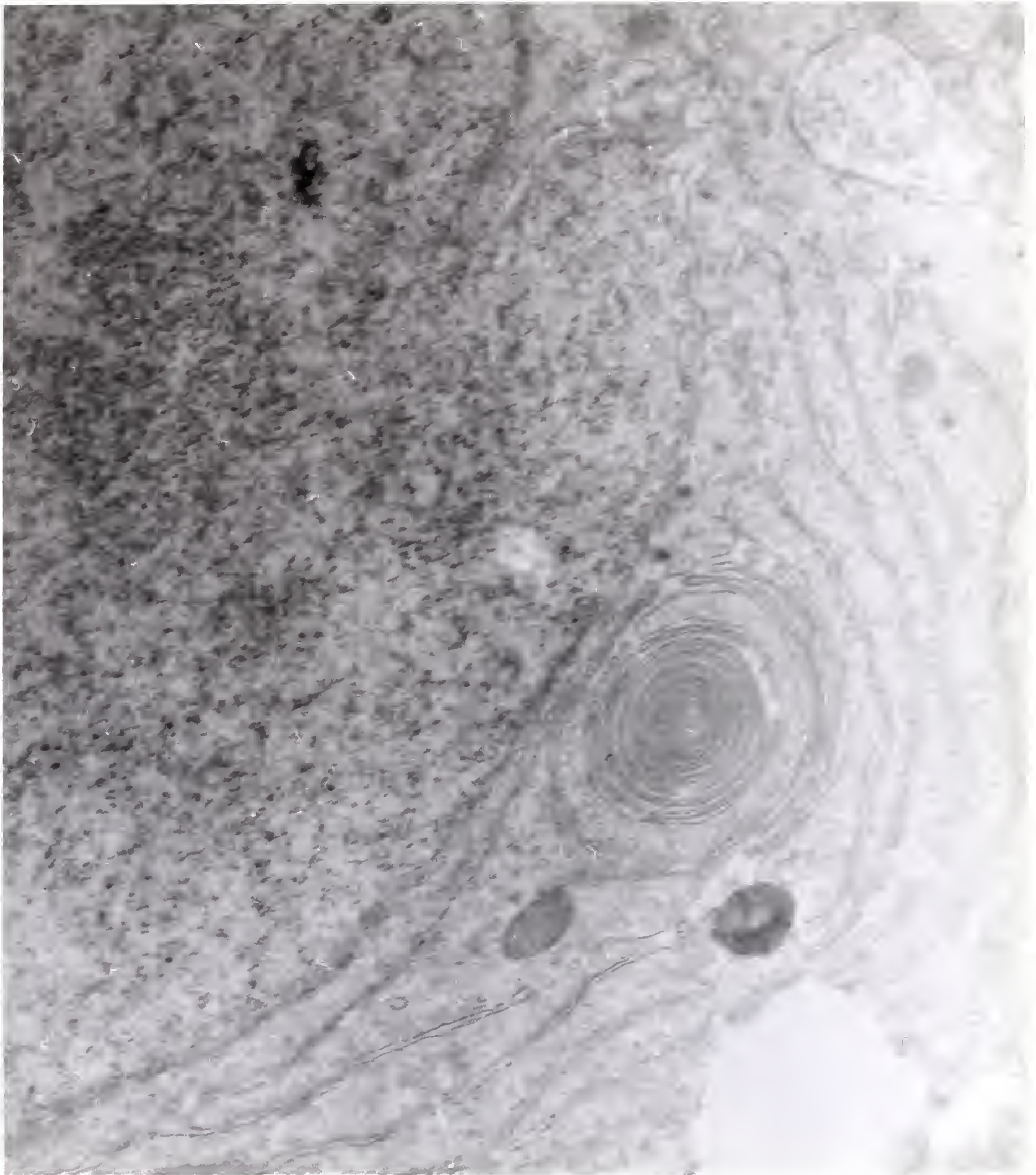

Figure 7.

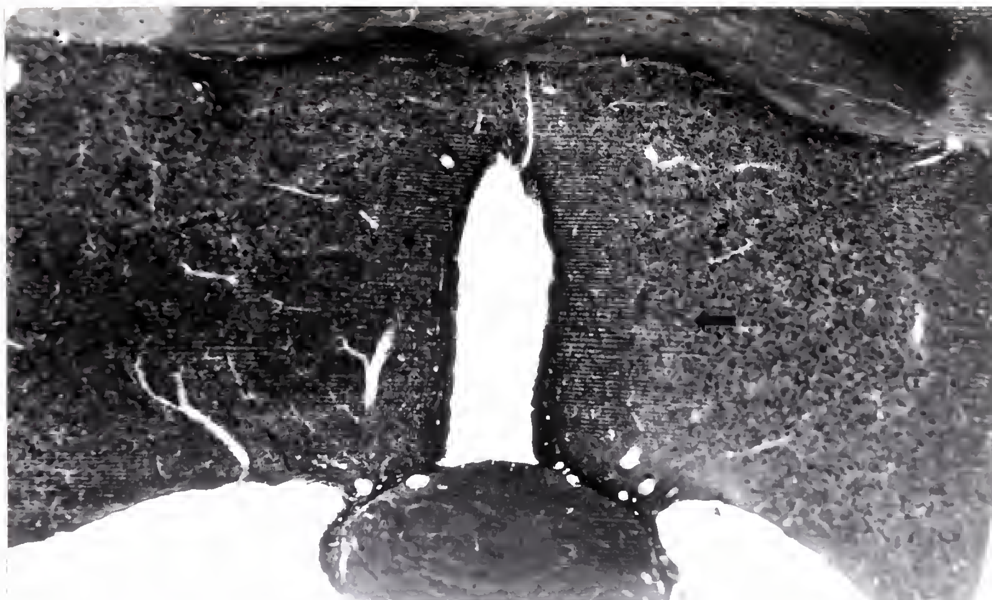

Figure 8-A.

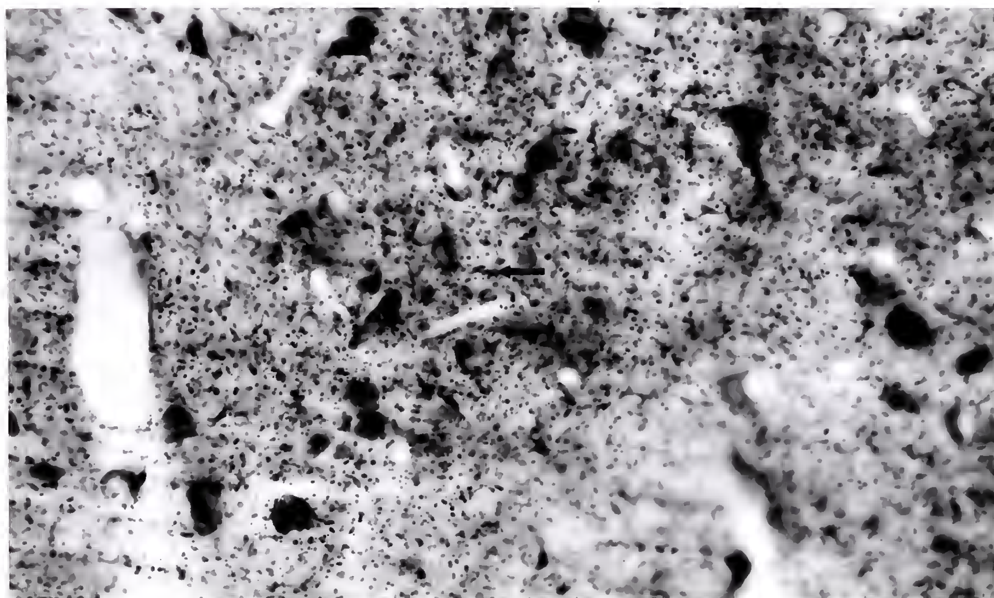

Figure 8-B.

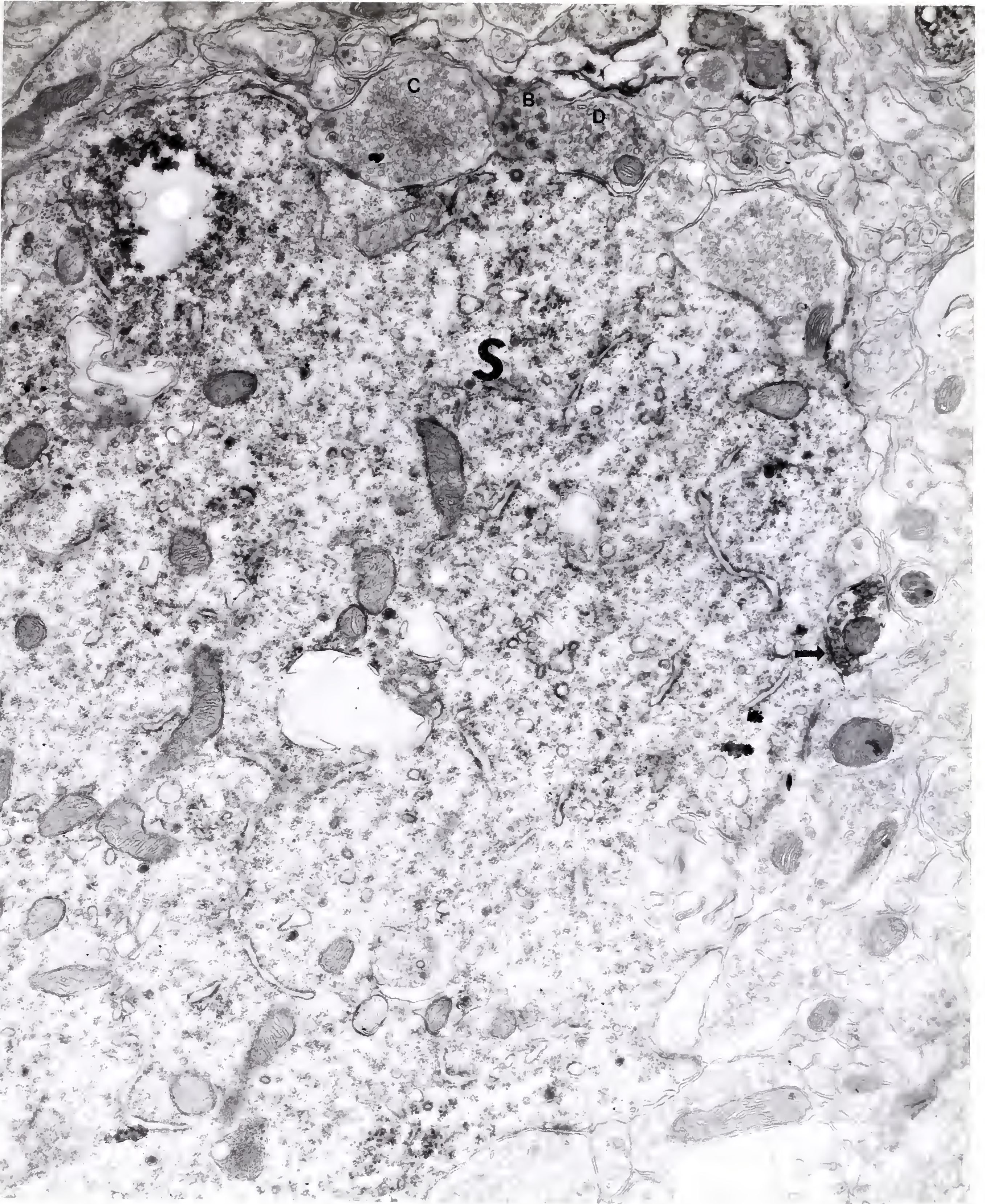

Figure 9.

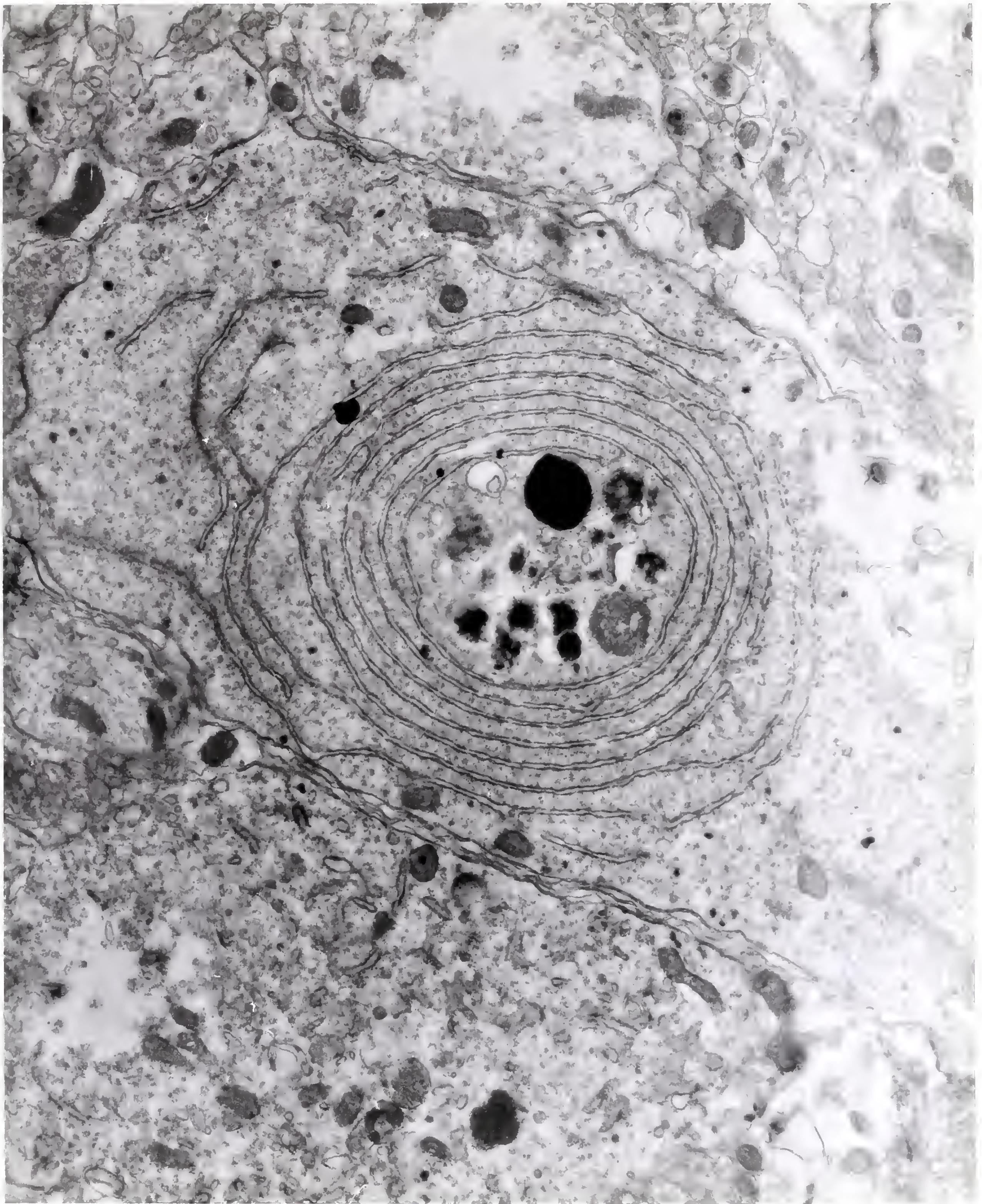

Figure 11.

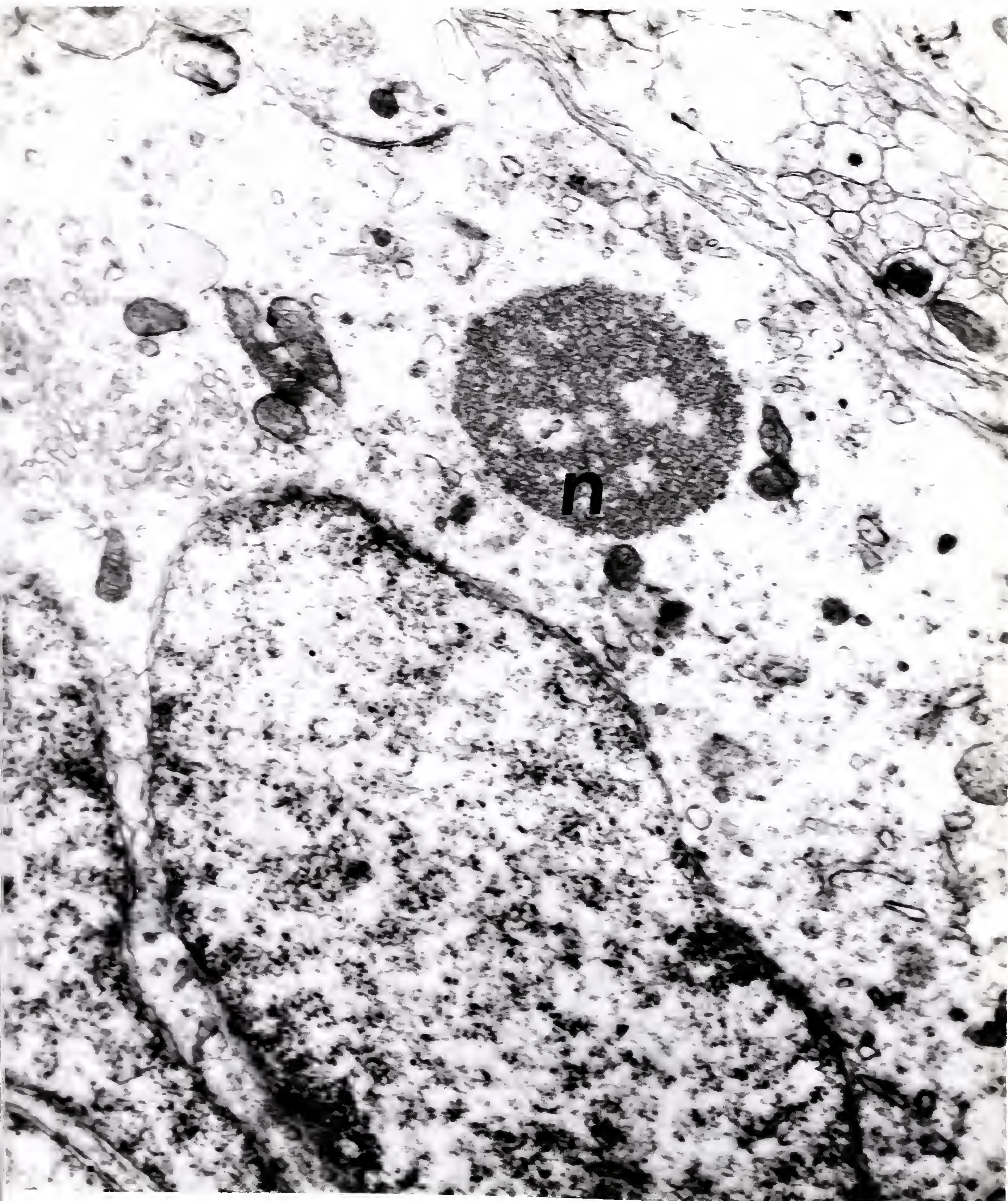

Figure 10.

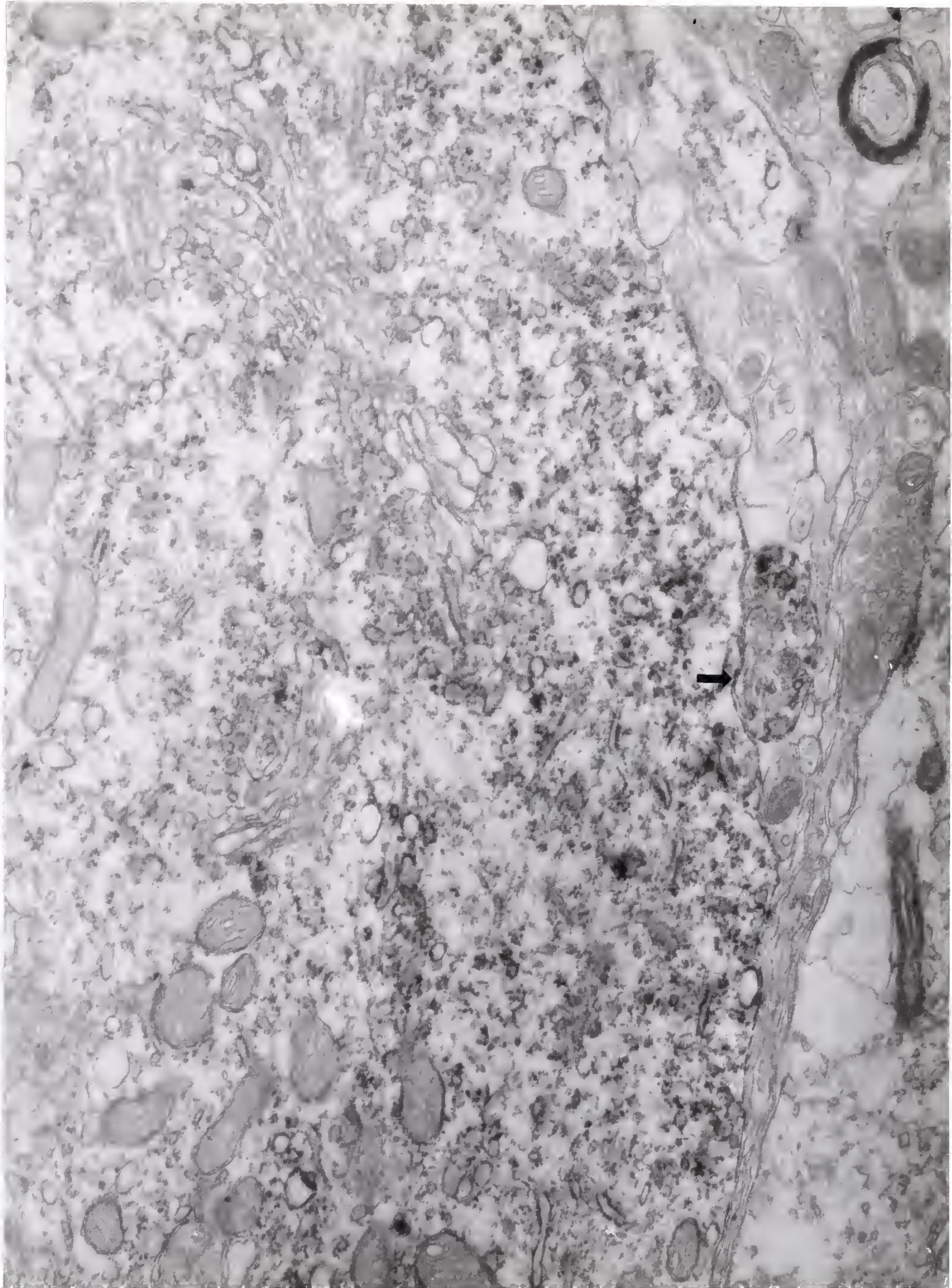

Figure 12-A.

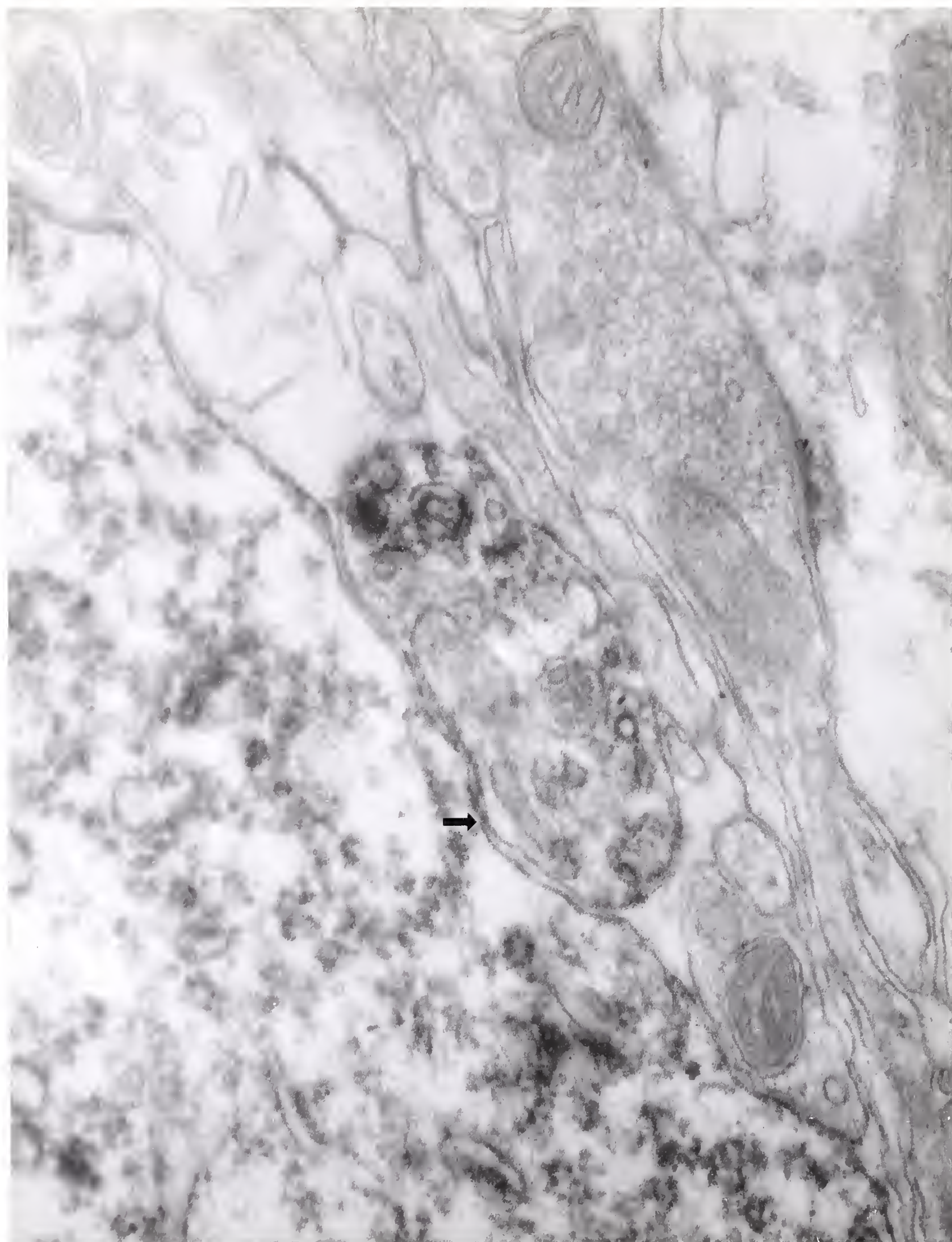

Figure 12-B.

DISCUSSION

Anatomical, biochemical and electrophysiologic studies reviewed previously argue overwhelmingly for an affect by estrogen on gonadotropin release in two regions of the brain known to control it--the arcuate nucleus and the MPOA. Further, the evidence for GABA as a neurotransmitter affecting this release and GABA neurons in either or both of these areas as targets of estrogen in this feedback control is gradually growing. This study sought to assess the morphologic effects of estrogen on these neurons with the hope this might provide clues to the role GABA plays in the release of LH. Clearly the data from this study reveal no such effects. The overall fine structure of the arcuate and the MPOA did not differ significantly between the two experimental groups and cellular morphology of the GABA neurons showed no change under varying circulating estrogen levels. Further, neither whorl bodies nor nematosomes showed significant changes in numbers under the two conditions, contrary to what is known from the literature (Brawer et.al.'71;Leranth et.al.'85a; King et.al.'74). Technical difficulties rather than physiologic phenomena are probably responsible for this data and will be considered briefly before proceeding from what was not found to the significance of what was found in this investigation.

Three major problems in this study made the collection of reliable data very difficult. First, using immunocytochemical methods with electron microscopy in investigations of cellular ultrastructure presents the investigator with a difficult compromise: tissue preservation vs. antibody penetration. The nature of this study made it critical to discern even the most subtle ultrastructural changes and necessitated weighing the decision heavily in favor of preservation over penetration. Unfortunately, this limited our

investigation to the top 2-3um of a Vibratome section and greatly reduced the number of actual labeled parikarya that were studied. This resulted in numbers of whorl bodies and nematosomes far to small from which to derive any accurate, statistically significant conclusions. Thus, comparing numbers of these organelles in experimental groups (ovariectomy vs. estrogen treatment) from one region or in one experimental group (e.g.ovariectomy) from both regions is meaningless, i.e. finding whorl bodies in only the estrogen-treated animals is may well be a sampling error. Some investigators have circumvented this problem by matching labeled neuronal profiles from the superficial layers of the section with the unlabeled profile in deeper cuts (Leranth et.al.'85a). This allows a number of planes of section to be examined for each labeled neuron, increasing the probability of finding whorl bodies, nematosomes or other ultrastructural changes of significance. Alternatively, if readily recognizable cellular features such as whorl bodies or nematosomes are used as markers of a neuron's response to estrogen, one may be able to decrease the quality of the tissue fixation as these organelles can be easily seen even in less well-preserved sections, and allow increased penetration of the immunoreagent used to label the GABA neurons. Under these conditions, one may be able to derive larger numbers of whorl bodies and to make possible studies from which more accurate conclusions can be drawn. Of interest in this regard would be a comparison of numbers of whorl bodies in the arcuate vs. the MPOA in ovariectomized animals since these two regions are known to have opposite effects on LH release and, therefore, may show opposite changes in numbers of whorl bodies. Such studies may help in determining if whorl bodies do, indeed, positively label the neurons in which they are found as gonadotropin-controlling cells.

The second problem involved actually localizing the GABA neurons within the estrogen-sensitive regions. In the arcuate nucleus, this is not difficult as previous studies from this laboratory (Leranth et.al.'85a) found estrogen-responsive GABA neurons in the area adjacent to the ependymal cells as noted in Fig.1(arrow). In the MPOA, much less information is available. Previous studies by Sar et.al. ('83) have localized GABA neurons that concentrated tritiated-estradiol to the MPOA, but their exact location within this region has not been documented. In effect, this necessitated random sampling from various parts of this region in hopes of finding evidence of ultrastructural effects (e.g.whorl bodies) and in actuality, it may have resulted in missing the estrogen-sensitive area completely. The presence of one whorl body in the area of the MPOA indicated in Fig.2(arrow) suggests this may be the most worthwhile area for future studies to investigate. Also, LHRH neurons can be found here (Jennes et.al.'83) and, if as suggested, the GABA neurons are involved in controlling norepinephrine activity to them, a juxtaposition of these two neuronal populations would seem appropriate.

Finally, the paucity of GABA neurons in the MPOA presented a problem both unexpected and insoluble. Investigators have suggested that though GAD-activity is extremely high in the MPOA, this is probably the result of GABA fibers from neurons in more rostral suprachiasmatic or septal nuclei traversing or impinging on this area (Leranth, personal communication). If this is true, one's sampling size is naturally restricted, underscoring the importance of future studies to maximize the labeling and ultrastructural analysis of those GABA neurons that are present. Even under these conditions, one may still be restricted to descriptive rather than quantitative studies.

Despite these problems, significant morphologic findings were gleaned from this study, of which the GAD-GAD axosomative synapses in the MPOA are clearly the most interesting. In the arcuate, GAD-GAD axosomatic synapses had been previously reported (Leranth et.al.'85) with the likely source of the GABA terminals coming from within the MBH (Tappaz&Brownstein'77). It is known that the MPOA has a very high concentration of GAD-activity (Fonnum'77) but its source has not been determined. Are these GABA terminals part of a population of GABA interneurons intrinsic to this area or are they from outside sources in the midbrain, hippocampus or more rostral areas including the suprachiasmatic or septal nuclei? Or, more importantly, do they represent input from the GABAergic system in the arcuate and affect the tonic control of LH release? Hypothalamic deafferentiation studies including total MBH cuts, as well as selective anterior (both rostral and caudal to the MPOA), posterior and lateral cuts would be especially helpful in defining the source of the GABAergic input to the GABA neurons of the MPOA.

Defining the source of the GABAergic input to the GABA neurons in the MPOA is an important step in constructing a workable schema as to GABA's role in the control of LH release. A local source from within the MPOA may suggest inhibitory interneurons involved in a neuronal loop which integrates different input from various origins and transmits the resulting signal as an inhibitory input to the neurosecretory cells (Tappaz et.al.'83). The concept of the MPOA GABA neurons as key integrators is supported by the unusually large number of synapses found on their soma (see Fig.9). However, if the source is from the MBH, speculations may center around an interaction between the GABAergic system of the arcuate and MPOA. A short, local recurrent picrotoxin-sensitive inhibitory loop thought to control the firing of the tubero-infundibular

neurons has been reported (Renaud'76). Perhaps collaterals from these neurons invade the MPOA
to coordinate the tonic release of the LHRH neurons found there? Finally, the GAD-GAD axosomatic synapses may represent GABAergic elements from a remote location involved in neuroendocrine feedback loops impinging on key GABA integrator neurons and affecting LHRH release in this way. This would explain the numerous effects on various endocrine parameters seen with central pharmacological manipulation of GABAergic transmission.

Having deciphered the input to and the output from these GABA neurons one can then more clearly examine how they may fit into the neuronal circuitry that controls LH release. Do they act, as many have speculated (Mansky et.al.'82;Lamberts et.al.'83), as pre-synaptic inhibitors to norepinephrine? Morphologic evidence from this study (Fig 9) and others (Mansky et.al.'82;Vijayan&McCann'79) showing GABA terminals juxtaposed with terminals of clear or dense-core vesicles would argue for GABA as a local neuromodulator. Or do they act directly on the neurosecretory neurons in the MPOA as McCann et.al. have proposed (McCann et.al.'82). Leranth et.al. ('85b) have shown recently through double-labeling techniques that the GAD-positive terminals in the MPOA do, in fact, synapse on the LHRH neurons there. These double-labeling techniques are making possible studies examining the inter-relationship of the GABA, dopamine, norepinephrine, and LHRH neurons in the control of LH release and will undoubtedly play a vital part in unraveling the increasingly more intriguing mystery of how the brain controls ovulation.

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