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THE CELLULAR EFFECTS OF ESTROGENS ON NEUROENDOCRINE TISSUES

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Summary—Estrogen action on sensitive neurons in the rat diencephalon has been studied by morphologic techniques; evidence of estrogen action at every level is presented, including tracts, cells, circuitry and subcellular organelles. The demonstration in the arcuate nucleus of estrogen-induced synaptic remodelling, estrogen-induced postsynaptic membrane phenotypes, changes in intracellular membranes and rapid estrogen actions on neuronal endo-exocytosis indicates that cellular estrogen actions may underlie the neuronal control of reproduction.

The development and maintenance of the sexual phenotype of hormone responsive cells is increasingly seen to be dependent upon the type and quantity of sex steroids furnished to sensitive cells. Thus, the actions of these regulatory substances upon neuroendocrine tissues conform with the scheme shown in Fig. 1.

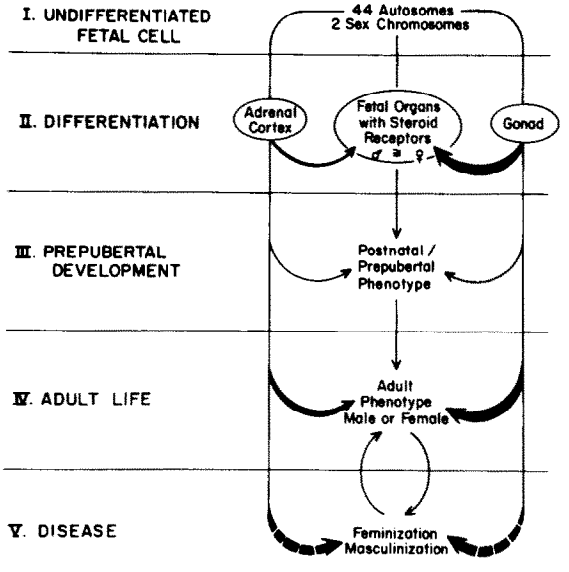

Fig. 1. A general scheme for sexual phenotype expression. Following gonadal differentiation the major controlling factors in development and maintenance of the sexual phenotype are sex steroid actions on responsive cells, rather than direct effects of sex chromosomes.

Estrogen, whether derived from metabolism within the cell or received from outside the cell [1, 2], forms or reforms subcellular components along lines which make up a characteristic phenotype. Recent evidence from our laboratories includes effects of estrogen upon the integrity of hypothalamic neurons and hypothalamic circuitry via actions upon elaboration of neuroregulatory substances, synaptogenesis, denervation, reinnervation and upon glial cells. Examination of the bases of these estrogen-induced neuroendocrine phenotypic effects has focused our attention upon membranes such as the cell nuclear envelope, endoplasmic reticulum, the cell membrane and its specializations such as synapses, intramembranous protein particles (IMP) and endo-exocytotic pits. Evidence will be reviewed which indicates effects of estrogen upon these membranes which may underlie normal neuroendocrine function in developing and adult subjects, and upon processes such as aging and neuroendocrine disease.

HYPOTHALAMIC AND HYPOTHALAMIC-PREOPTIC AREA NEURONS AND THE EFFECTS OF ESTROGEN

This laboratory has been interested for nearly 20 years in the metabolism and actions of gonadal steroids in the brain; in particular, estrogen effects on the mechanisms regulating gonadotrophin release [3]. It is well established that the hypothalamic control of gonadotrophin release is primarily mediated through the secretion of lutenizing hormone releasing hormone (LHRH) into the pituitary–portal circulation, that this activity is sex steroid sensitive and that estrogens are effective steroids in this control [4, 5]. Available evidence suggests that the steroidal feedback control of LHRH secretion is not simply exercised through direct effects of the steroids on LHRH neurons: combined autoradiographic and immunocytochemical studies have failed to demonstrate measurable concentration of [^{3}H]estradiol in LHRH-immunoreactive perikarya in the rat brain, suggesting that these neurons do not themselves contain estrogen receptors [6]. Moreover, a variety of experimental manipulations that give rise to failure of

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the capacity to support cyclic ovarian function do not appreciably affect the ultrastructure or distribution of the LHRH-immunoreactive neurons [7]—see below. These observations are consistent with the hypothesis that the effects of gonadal steroids on LHRH release are primarily mediated through inputs from other steroid-sensitive neuronal systems; however, the nature of the mechanisms responsible for controlling the activity of the LHRH neurons remains poorly understood. For example, a large number of neurotransmitters and neuropeptides including the catecholamines, serotonin, gamma amino butyric acid (GABA) and the endogenous opioid peptides have been implicated in the control of gonadotrophin release [8–13] but the roles played by each of these systems and the manner in which they are integrated remain ill-defined. In addition, the complexity of interneuronal intrahypothalamic connections presents major research problems. Morphologic and electrophysiological studies have clearly demonstrated that there are abundant internal connections between the hypothalamic nuclei [14–22]. Therefore, knowledge of these connections and of the cellular biology of the involved hypothalamic neurons are essential for understanding the hypothalamic control of pituitary gonadotrophins.

During the past decade we have approached this problem by defining and studying the estrogen-sensitive components of the hypothalamus which are concerned with gonadotrophin control. We have focused studies on the AN and the medial preoptic area (MPO), both of which have been shown to concentrate estrogens and to be important in the regulation of reproductive function [3, 23–25], attempting to better define the anatomic interconnections between hypothalamic neuronal populations for which there is evidence of responsiveness to gonadal steroids and to characterize effects of estrogen treatment on AN neurons using biochemical, immunochemical and morphological techniques.

Characterizing estrogen-sensitive neurons

Gonadal steroids affect specific perikaryal constituents in AN neurons. Estrogen treatment increases the observed frequency of neurons containing large, finely granulated cytoplasmic fibrillar bodies termed "nematosomes". In contrast, in either males or females gonadectomy results in the appearance of increased numbers of complex, lamellar cytoplasmic organelles, called "whorl bodies" [26–28]. In addition, these latter structures seem to be induced by a variety of hormonal and pharmacologic manipulations [26, 29–32]. Using markers such as these two estrogen-sensitive organelles we set out to study estrogen effects in the AN using dissections which are precise and independent of the bias which could be imposed by the use of conventional histologic or EM landmarks. To do this, we adapted the microdissection method of Palkovits [33, 34] for use with fixed tissues. Animals were perfused with fixative via the heart and their brains removed. The hypothalamus was blocked by a cut posterior to the mammillary bodies, anterior to the anterior commissure and lateral to each lateral fissure. The pituitary stalk was cut during removal of the brain and a dorsal cut through the thalamus completed the dissection. This block was sectioned into 25-μm sections using the Vibratome, stained with toluidine blue and compared with Palkovits' atlas [33]. Antero-posterior levels of structures during dissection of male and female animals were indistinguishable and met Palkovits' measurements for MPO and AN landmarks. This proof of the method of dissection also validated the comparison of biochemical measurements utilizing

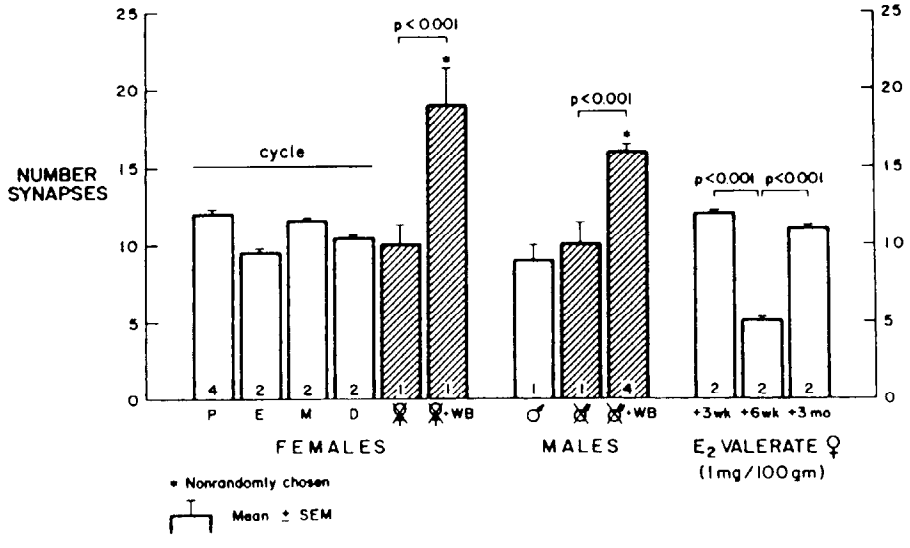

Fig. 2. Adult rat arcuate nucleus—reconstructed random neurons. Number synapses/100 μm neural membrane.

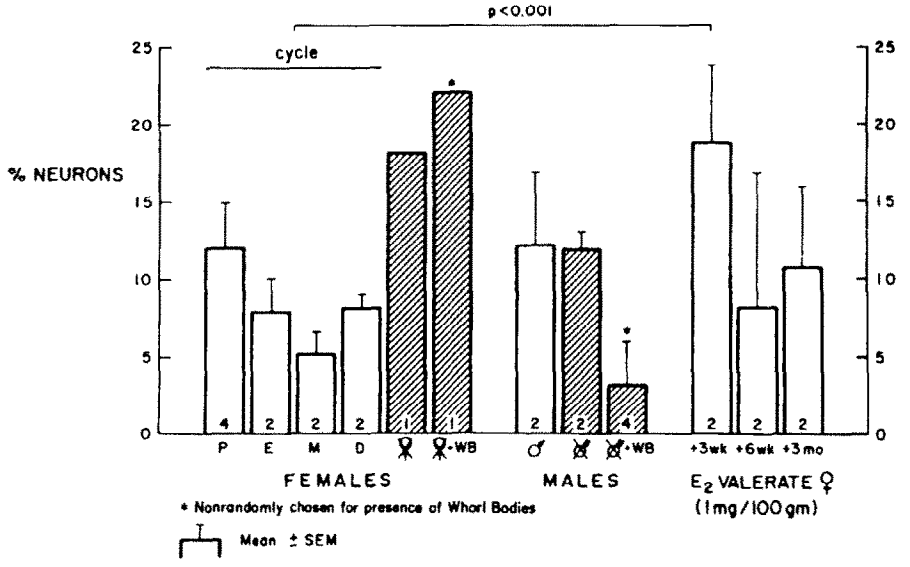

Fig. 3. Adult rat arcuate nucleus. % randomly chosen reconstructed neurons containing nematosomes.

frozen tissues prepared according to Palkovits with our anatomical studies on fixed tissues. Our first set of studies with this methodology demonstrated that AN neurons whose profiles contain characteristic whorled endoplasmic reticulum (whorl bodies) have 1.5–2-fold the number of axosomatic synapses on their perimeter compared to non-whorl body-bearing profiles [34]. Morphometry showed that the difference of synapses on whorl body-bearing cells is an absolute increase since the average perimeter of AN neurons and the average synapse length are the same for whorl body-bearing- and non-whorl body-bearing cells. Thus these estrogen-sensitive cells have more synaptic connections than do neurons which do not demonstrate this characteristic. Figure 2 shows the number of synapses on randomly selected AN neurons (30 neurons per animal studied) to be increased in whorl body-bearing (WB) profiles.

Using the same reconstructed cell profiles (30/animal) we also studied the number of cells containing nematosomes. Figure 3 shows, in addition to confirming the estrogen-induced increase of nematosome-containing cells, that there is a high count of nematosome-containing cells in female castrates with and without whorl bodies. This encouraged further studies into the possible interrelationship of these two estrogen-sensitive organelles. In a follow-up study the sequence of castration followed by estrogen treatment demonstrated the presence of whorl bodies with typical nematosomes in their center, raising the likelihood that nematosomes are associated with whorl bodies, perhaps even being products of estrogen-induced whorl body dissolution [35].

Despite these anatomical studies we have been unsuccessful in determining the functional role of these steroid-sensitive intracellular organelles. However, in collaboration with Dr Nahum Gershon we have made three-dimensional reconstructions of whorl bodies from serial EM sections of neurons and confirmed the confluence of whorl body cisterns with the surrounding rough endoplasmic reticulum, the hollow construction of whorl bodies with cytoplasm in the center and the common association of a Golgi network around the orifice of these spheres of whorled endoplasmic reticulum (unpublished observations).

In attempts to determine the function of whorl bodies and nematosomes we have performed immu-

Table 1. Cytochemical reactions in arcuate nucleus neurons

	Golgi		Whorl body				
	Cis	*Trans*	Outer	Inner	Rough ER	Nucleolus	Nematosome
RNAse*	−	−	−†	(+)†	+	+†	−†
DNAse*	−	−	−	−	−	+	−
OsO_4 impregnation	+	−	+†	−†	+		
Thiamine pyrophphatase	−	+	−	−	−		
Glucose-6-phosphatase	−	−	+	−	+		

*In collaboration with J. Roth, Basel, Switzerland.
†Confirmed by serial sections.

nohistochemical and histochemical studies of nematosome- and of whorl body-containing cells as shown in Table 1.

These results fit with the hypothesis that the whorl bodies are anatomical intermediates between the rough endoplasmic reticulum and the golgi apparatus. As such they could contain enzymes and enzyme products. Thus far, we have used specific antisera to mark the cytoplasm of cells which contain adrenocorticotrophin (ACTH), tyrosine hydroxylase (TH), or glutamic acid decarboxylase (GAD) and looked for immunostained neurons containing whorl bodies or nematosomes. We could find whorl bodies only in GAD positive cells [35]. In those neurons although the antiserum marked the rough endoplasmic reticulum contiguous with the whorl body, neither whorl bodies nor nematosomes were marked by the antiserum. The failure to find neuropeptides or neurotransmitter enzymes in whorl bodies or nematosomes indicates that there are other materials in these organelles. We are presently developing methods for immunostaining steroid hormone metabolizing enzymes in brain cells, which will allow testing for the possibility that whorl bodies are related to changing steroid hormone metabolism in AN neurons.

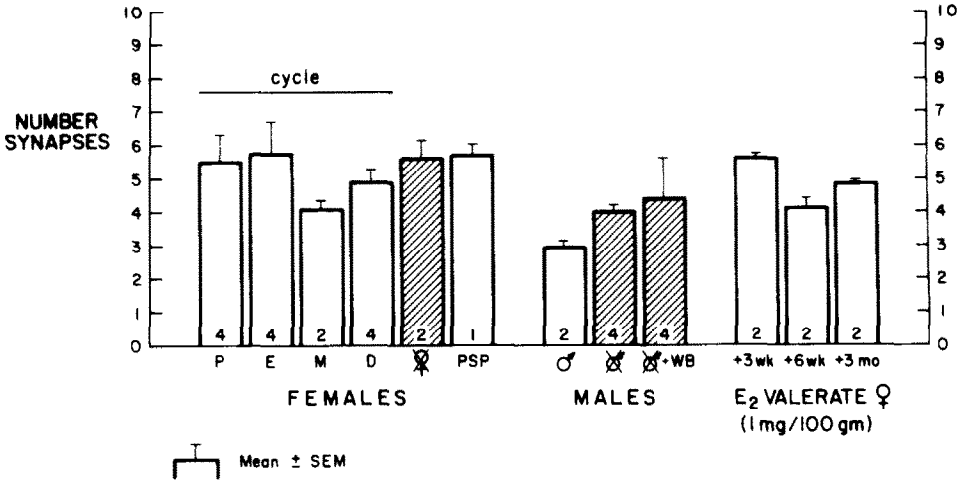

Fig. 4. Adult arcuate nucleus. Number synapses/100 μm^2 of randomly photographed neuropil.

Estrogen-induced AN denervation/reinnervation

In addition to increased numbers of synapses on whorl body-bearing neurons our initial studies indicated possible effects of estrus cycle stage and estrogen treatment on the synaptic complement of arcuate neurons (Fig. 4).

Therefore, female rats were studied 3–32 weeks following an injection of estradiol valerate large enough (1 mg/100 g body wt) to cause AN damage and reproductive failure [36, 37]. Among the animals studied, we found that by 8 weeks there is a significant ($P<0.001$) decrease in axo-somatic synapses and synapses on dendritic shafts, but not on dendritic spines [38]. The time course of synaptic remodelling of AN neurons is illustrated in Fig. 5 by the curve of axosomatic synapses following a single estradiol valerate (1 mg/100 g body wt) injection.

The calculated loss of approx 30% of axo-somatic synapses was matched by an increase of 30% in average synaptic length during this period [38]. In earlier work we demonstrated damage figures and gliosis accompanying estrogen-induced constant estrus [26, 36]. Our newer studies show that the decrease in number of synapses was preceded by the appearance of massive presynaptic fiber degeneration within 3 weeks of the estradiol valerate injection and the appearance of clumped synaptic vesicles in axon terminals which was significantly increased within 3–8 weeks of estrogen injection; both of these mor-

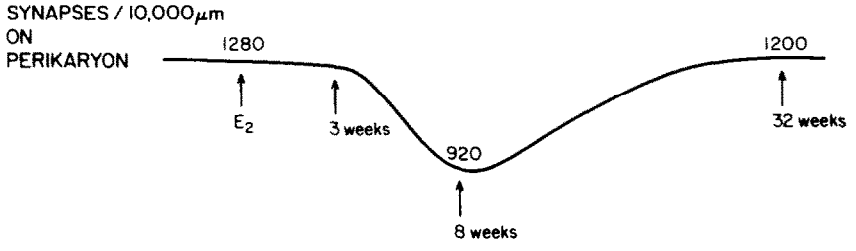

Fig. 5. Chronology of denervation–reinnervation cycle in arcuate nucleus following estradiol valerate administration (E_2, 1 mg/100 g body wt).

phological characteristics had returned to control (proestrus) levels by 16 weeks.

The lack of an effect of estrogen on axon-spine synapses is important because it is an indication that spines may be a non-estrogen-sensitive area of estrogen-sensitive neurons or that neurons which have spines are insensitive to estrogen. Although the question remains unanswered at this time, our freeze-fracture studies of postsynaptic membranes (see below) showed that, in contradistinction to the sexual dimorphism seen in perikaryal and dendrite shaft membranes, spine membranes are not sexually dimorphic in density of intra membrane protein particle (IMP) [39]. In addition, following estrogen treatment spines do not show changes in IMP, while perikaryal membranes and dendritic shafts have sexually dimorphic IMP counts and lower densities of IMP following exposure to estradiol valerate [40].

As can be seen from the representation in Fig. 5, from 8 weeks after estrogen administrations onward the number of synapses gradually increased until the normal density is regained by 16–32 weeks. This picture is consistent with our previous formulation that there are at least two effects of estrogens on AN neurons [26]: under conditions in which synaptogenesis can occur, such as during the perinatal period [41, 42], after surgical disconnection of the AN [43] and following estrogen-induced synaptic loss [38], estrogen causes increased numbers of synapses; however, estrogen and other agents [44] can also cause denervation [38]. Thus, in the perinatal period the predominant effect of estrogen exposure [2] is organization through synaptogenesis [42] without evidence of damage [41], while in adults estrogen causes a full synaptic remodelling cycle which may result in hypothalamus-dependent constant vaginal estrus with failure to respond to an estrogen surge, elevated plasma luteinizing hormone, and active polyfollicular ovaries secreting continuous high levels of estradiol [36, 45]. In the latter case there is residual gliosis and glial thickening around neurons [36–38] while estrogen exposure in the perinatal period is not accompanied by damage figures [41].

In light of the major functional changes following estradiol valerate treatment in adults it is important to realize that one cannot infer that the reinnervation of neurons under the influence of constant estrus has re-established the pretreatment circuitry; in fact, we proposed that, since it no longer supports cyclic gonadotrophin release the post-treatment AN circuitry must differ from that of the normal female. Therefore, in order to characterize the effects of estrogen upon synapses, we have begun to elucidate the synaptic circuitry present in the AN and MPO which is involved in cyclic gonadotrophin release [46].

Lack of an effect of estrogen treatment on LHRH axon density and morphology in the median eminence

Since estrogen treatment causes hypothalmic (AN) neuronal loss of synapses, presynaptic fiber collapse, gliosis and reproductive failure, early on we tested for the possibility of a destructive effect of estrogen upon LHRH axons reaching the median eminence. Our studies in collaboration with Drs J. R. Brawer and K. B. Ruf showed that even during estrogen-induced constant estrus, electrical stimulation of MPO caused LH release, indicating the functional presence of LHRH neurons [47]. We therefore sought morphological estrogen effects on LHRH axons in the area of the pituitary–portal capillaries [44]. Several states known to entail persistently high levels of estrogen and constant vaginal estrus; constant light, neonatal androgenization and EV treatment [48] were compared with normal cycling females. The density of LHRH-positive axons in the median eminence, and the ultrastructure of LHRH immunopositive neurons were examined. Mechanical deafferentation of the MPO from the hypothalamus served to control the techniques employed. In this experiment we studied frontal slices taken through the hypothalamus and median eminence and found that there was no decrease in the density of LHRH axons in any of the induced constant estrus conditions although control bilateral anterior hypothalamic cuts produced an almost complete loss of LHRH fibers. The LHRH-immunopositive fibers in the induced constant estrus groups also showed normal ultrastructural appearances while the mechanically deafferented median eminences had many degenerating LHRH-immunopositive fibers. This study indicated that while estrogen treatment effects LHRH release it does not do so by damage to LHRH neurons sufficient to be detected at the level of the axons in the periportal capillary area. This focused attention upon directly or indirectly estrogen-sensitive AN neurons which control LHRH release. We therefore began a series of studies on estrogen-sensitive hypothalamic connections and the neurons which make them up.

Arcuate-preoptic neurons, synaptic connections and estrogen

Ultrastructural immunocytochemical studies in our laboratory have demonstrated that some of the synaptic contacts on whorl body-containing cells are immunoreactive for the enzyme responsible for GABA synthesis, glutamic acid decarboxylase (GAD) and are therefore presumably GABA-ergic [35]. As mentioned above, after colchicine treatment, we have shown GAD immunoreactivity in the perikarya of whorl body- and of nematosome-containing neurons, but not in these organelles themselves. Total deafferentation of the AN-median eminence, using the Halasz–Pupp knife, leaves the great majority of the GAD immunoreactive axon terminals intact, indicating that this is largely a system of interneurons, as is the case elsewhere in the CNS. Taken together these results suggest that GABA-ergic cells within the AN are morphologically responsive to gonadal steroids and receive synaptic input from intrinsic GABA-ergic

terminals. This conclusion is consistent with previous studies showing that estrogens modulate GAD activity in the AN [49] and with evidence for GABA involvement in neuroendocrine feedback mechanisms, including the control of gonadotrophin and prolactin release [9, 50]. It remains to be established whether the AN GABA-ergic cells are directly responsive to estrogen, or whether they are indirectly affected by gonadal hormones through changes in input from other, estrogen receptor-containing cells. In this regard, light microscopic autoradiographic studies have shown that GAD-immunoreactive neurons in the preoptic area concentrate [^{3}H]estradiol or its metabolites [51]. It is not yet known, however, whether the same is true for the AN and it is possible that the effects of estrogen on AN GABA neurons may be at least partially mediated through interactions with other estrogen target cells.

One of the candidates for such an estrogen-sensitive modulatory input is the hypothalamic dopaminergic system. A subpopulation of hypothalamic dopaminergic neurons (predominantly those in the AN and periventricular nuclei) has been shown to concentrate radioactivity after [^{3}H]estradiol administration [52]. Tyrosine hydroxylase (TH) immunoreactive neurons in the AN are also morphologically and biochemically responsive to estrogen [53]. It therefore seemed possible that the actions of estrogens on the AN GAD-immunoreactive cells might at least in part reflect a change in dopaminergic input. In view of reports indicating effects of GABA on dopamine turnover in the hypothalamus [54], it also seemed possible that such connections, if they existed, might be of a reciprocal nature. To investigate this hypothesis, we attempted to better define the intranuclear connections of the AN dopaminergic and GABA-ergic cells. A study of the distribution and synaptic connections of TH-immunoreactive elements in the AN revealed a number of synaptic connections in which both the pre- and postsynaptic elements were immunopositive for TH, as well as synapses in which only the postsynaptic element was immunopositive [55]. These synapses were essentially unaffected by transection of the afferents from more caudal dopaminergic cell groups. In contrast, when the lateral and medial parts of the AN were separated by a knife cut, degenerating axons terminating on TH-immunoreactive cell bodies in the lateral part of the AN were far more common. In view of the fact that GABA-ergic neurons are predominantly in the medial part of the AN [35, 36], these findings are consistent with the hypothesis that connections exist between arcuate TH- and GAD-immunoreactive cells.

Simultaneous double-labelling technique for two antigens in a single ultra thin section from the hypothalamus

To demonstrate the hypothesized TH–GAD connection, it was necessary to develop a procedure for differential staining of pre- and post-synaptic elements on a single grid for evaluation at the electron microscopic level. This was evident since we had already demonstrated GAD–GAD and TH–TH immunoreactive connections to be present in the AN, indicating that it was not possible to use single label immunochemistry to test for the presence of connections between the GAD and TH systems. Several authors have reported methods for electron microscopic immunocytochemical double labelling, based on labelling the antigens with colloidal gold particles of different sizes [57], or peroxidase combined with gold [58]. The method developed in our laboratory is conceptually similar, but utilizes ferritin and peroxidase as the contrasting electron-dense markers [59]. Using this technique on tissues from the rat AN we have observed GAD-immunoreactive synapses on TH-reactive cells [59], as well as a somewhat more limited number of TH-immunopositive synapses on GAD cells (unpublished observations).

Connections between GAD-, TH- and LHRH-immunoreactive elements in the MPO

The interconnections between the GAD- and TH-immunoreactive elements within the MPO appear to be similar to those in the arcuate nucleus; using electron microscopic double label immunocytochemistry we have identified synapses between GAD-immunoreactive elements, as well as GAD-reactive synapses on TH immunopositive profiles. Both the TH and GAD systems also appear to make direct contact with the LHRH neurons. Using peroxidase labelling for LHRH immunoreactivity and ferritin as a marker for GAD, we have reported the presence of symmetric (Gray II) synapses between GAD-labelled axons and LHRH-reactive dendrites and perikarya in the MPO [60]. More recently, we have obtained similar results after double-label staining for TH and LHRH (unpublished observation).

The origin of the TH-immunoreactive postsynaptic profiles contacting LHRH elements in the preoptic area remains uncertain. They may at least partly represent processes of dopaminergic perikarya in the rostral periventicular area [61]. They could also include the projections of adrenergic, noradrenergic and/or dopaminergic neurons from the brainstem [62]. Further studies utilizing immunostaining for the enzymes dopamine β-hydroxylase and phenyl ethanolamine *N*-methyl transferase responsible for synthesis of adrenaline and noradrenaline, respectively, combined with transection of the ascending catecholaminergic fiber systems will be required to resolve this issue. Likewise, the origins of the GAD-immunoreactive (presumably GABA-ergic) input to the TH-immunoreactive profiles in the preoptic area remains to be defined.

LHRH–LHRH microcircuits within the MPO area

In addition to afferent input to the LHRH-immunoreactive neurons, there is also evidence that LHRH may itself act as a neurotransmitter or neuromodula-

tor within the preoptic area. The existence of intrinsic terminations of LHRH axons in the preoptic area has been demonstrated by a number of authors [63] but the question of whether there are connections within the arborization of individual LHRH cells or between the LHRH cells, perhaps serving to synchronize their activity (micro-feedback), has remained controversial [64, 65]. Our own studies have now confirmed the existence of a direct LHRH to LHRH connection, demonstrating contacts between LHRH-immunopositive presynaptic boutons and LHRH-immunoreactive dendrites and perikarya in the MPO [66]. These contacts are symmetric (Gray II), and appear to differ from LHRH-immunoreactive axons in the median eminence in that they contain fewer dense core vesicles. Thus far, no functional tests have been performed to evaluate this possible microcircuit for LHRH, but they are certainly in order.

A tract composed of projections from proopiomelanocortin-containing neurons in the AN to LHRH neurons in the MPO

The lack of axon boutons synapsing with LHRH axons in the median eminence area [44] indicated that there might be connections from the AN to the LHRH neurons of the MPO. Studies of the connections between hypothalamic nuclei have provided electrophysiological and morphological evidence that a population of AN neurons projects directly to the MPO. Zaborszky and Makara[67] observed degenerated terminals in the MPO following microlesions placed in the medial part of the AN. According to Renaud[21], electrical stimulation in the MPO is associated with orthodromic responses from at least 11.5% of neurons in the AN. Candidates for this projection included axons of proopiomelanocortin (POMC) derived peptide-containing neurons in the AN. In addition to furnishing the first evidence of a direct tract from the AN to LHRH neurons, such a connection would be of considerable interest from a physiological standpoint. This is especially true in view of the evidence supporting a role for POMC-derived peptides in the control of gonadotrophin release [8, 10, 68]. Recently, Mezey *et al.*[69] reported that ACTH-, β-MSH- and β-endorphin-immunoreactive axons originating from the AN form a dense network in the MPO. Our studies sought to address the question of whether—and with what neutrons—the observed POMC-immunoreactive axons do in fact make synaptic contact in the MPO, or if they might simply represent fibers of passage. We first examined the distribution of ACTH-immunoreactive elements in the preoptic area using conventional single-label immunocytochemistry, employing the antibody characterized by Watson and Akil[70]. The majority of the immunolabeled axons were found within the borders of the MPO, at particularly high densities in the medial and the ventrolateral regions of the nucleus [71]. At the electron microscopic level, the labeled axons had characteristic ACTH terminals; containing small, clear spherical vesicles and large (120–130 nm) dense-core vesicles. In sections taken from the medial part of the MPO, ACTH-immunopositive axons were found in synaptic connection with dendritic shafts. In contrast, in the ventrolateral part of the MPO the ACTH terminals synapsed primarily on dendritic spines. The Gray type of the synaptic membrane specializations could not be precisely determined, but appeared to represent a transitional form between symmetric and asymmetric connections.

To determine whether some of these synaptic contacts might represent connections between the ACTH-immunoreactive neurons and LHRH cells in the preoptic area, further studies were performed using double-label immunostaining with peroxidase and avidin–ferritin. Electron microscopic analysis was performed on sections taken from both the medial and ventrolateral parts of the MPO of double-stained Vibratome sections, labeled with peroxidase for LHRH and ferritin for ACTH. Peroxidase-labeled LHRH neurons and axons were examined only in the medial part of the MPO. In this area synaptic connections between ferritin-labeled ACTH axons and peroxidase-labeled LHRH dendrites were frequently observed. Reversing the order in which the primary antibodies were applied to the sections resulted, as expected, in a reversal of the staining pattern; ferritin labelling the LHRH dendrites, with peroxidase labelling the ACTH-immunoreactive synaptic contacts [71].

Finally, using a combination of retrograde horseradish peroxidase (HRP) transport and single label immunocytochemical techniques we examined the origin of ACTH-immunoreactive fibers in the MPO. A modification of the glucose oxidase method was used to visualize the transported HRP [72]. Vibratome sections from both the MPO and the AN were examined. AN sections were taken for further examination only where following of the MPO injection of HRP the extracellular HRP migration from the injection site showed that HRP was essentially limited to the MPO. In the AN sections from these animals, three types of labeled cells could be recognized:

(1) A small number of cells mostly located in the ventromedial portion of the nucleus were found to be double labeled, i.e. they contained very dark, transported HRP granules in ACTH-immunostained cytoplasm. The immunoperoxidase reaction product was easily distinguished from transported HRP granules.

(2) A somewhat larger number of cells homogeneously distributed throughout the AN contained only the transported HRP granules. Synaptic contacts from labelled ACTH axons were frequently observed on these HRP-positive but ACTH-immunonegative neurons.

(3) The majority of labelled cells were found to be immunopositive for ACTH without recognizable

HRP granules. These cells were observed mostly in the ventrolateral parts of the AN, conforming to earlier descriptions [73] of the location of ACTH-immunopositive neurons.

These results establish unequivocally that ACTH-immunoreactive axons make synaptic LHRH contacts in the medial preoptic area. In addition to our findings regarding AN POMC–LHRH axosomatic synapses, ACTH-immunopositive axons synapsing with dendritic shafts were found in the medial part of the MPO. At least some of these contacts appear, from the results of the double label experiments, to be on the dendrites of LHRH-immunoreactive neurons [71]. Since, according to present knowledge, the perikarya of hypothalamic POMC-containing neurons are confined to the AN [62, 74, 75] it seems reasonable to assume that the ACTH-immunoreactive boutons synapsing with LHRH neurons represent direct AN–MPO connections. This hypothesis is consistent with the results of the above HRP experiments and with previous electrophysiological [17, 21] and morphological [67] observations of the existence a direct AN–MPO pathway. In addition, these results suggest that there are two or more different populations of neurons projecting to the MPO, only some of which are ACTH immunopositive. The determination of the neurotransmitter(s) involved will further define this new link in gonadotrophin control which connects the AN with the LHRH system.

The ACTH-immunopositive, HRP-labeled perikarya were found mostly close to the third ventricle. This is in good agreement with the results of the electron microscopic degeneration experiments of Zaborszky and Makara[67], showing that lesions placed in the medial part of the AN cause the largest number of degenerated terminals in the MPO. However, since HRP can be retrogradely transported not only through axons terminating around the injection site but also by fibers of passage, we cannot be sure that all of the HRP-labelled cells in the AN connect to cells in the MPO.

An up-to-date summary of our understanding of AN–MPO circuitry as it pertains to these issues is illustrated in Fig. 6, which is from [71].

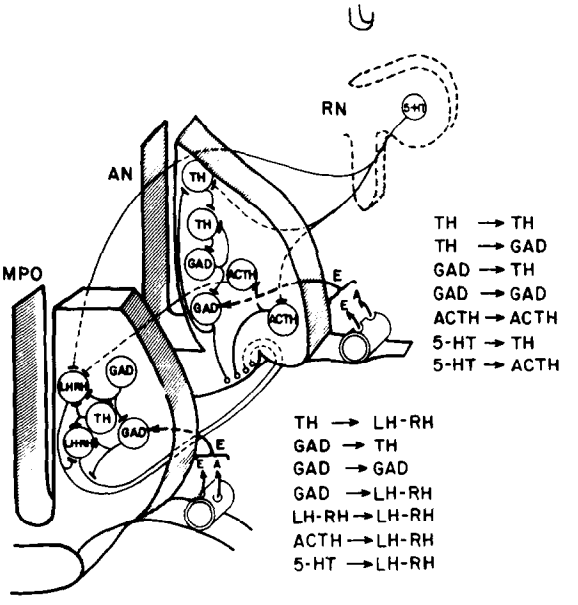

Fig. 6. Pertinent hypothalamic connections. From Ref. [71] by permission.

EFFECTS OF ESTROGENS ON HYPOTHALAMIC CELLULAR STRUCTURE AND FUNCTION

As can be seen from the previous section, estrogen effects upon the developing and adult hypothalamus are well documented and include changes in morphology as well as function [2, 5, 26, 76]. This can be put into the framework of the earlier discussion regarding the organizing and activating effects of sex steroids (Fig. 1). Regardless of the rat's genomic sex, when exposure to estrogen occurs in the perinatal period, the result is a failure of biphasic feedback-dependent gonadotrophin cycling in the adult [77, 78]. In normal adult females there is progression to a similar picture [26]. Ascheim first proposed that ovarian steroids induced reproductive senility in the rat hypothalamus [79]. Finch and colleagues have furnished important supporting evidence for the role of the ovary in aging [45] and Wise and colleagues have correlated these effects with changes in neurotransmitters in the aging rat's brain [80]. We have proposed that the cyclic surges of estrogen from the ovary [46] induce remodelling of the AN which underlies hypothalamic aging and anovulatory hypothalamic failure [26]. There is evidence that protection by androgens [81] and progestins [82] against this estrogen effect could delay the onset of hypothalamic failure in aging female rats. In pursuing the role of estrogen-induced denervation–reinnervation we have studied estrogen-induced AN neuronal changes and found differences in endoplasmic reticulum, in cell membrane protein domains and in endo-exocytotic traffic.

Estrogen effects upon membranes in AN neurotransmitter neurons

As previously indicated, catecholamines and serotonin are believed to be involved in the regulation of gonadotrophin secretion. Dopaminergic cells in the AN are predominantly distributed in the lateral part of the nucleus, in the region where derangement of reproductive function seen after long-term estrogen exposure might be associated with the catecholaminergic or serotonergic systems. We examined changes occurring in the levels of neurotransmitters as well as the activity of the key enzyme involved in catecholamine biosynthesis, tyrosine hydroxylase (TH), during 8 weeks of continuous estrogen exposure (unpublished). As expected [53], dramatic reductions in TH activity and dopamine levels in the mediobasal hypothalamus were seen within 14 days. These changes were sustained throughout the period of estrogen treatment. A progressive fall in serotonin

levels was also seen. No changes were seen in the levels of norepinephrine in the hypothalamus, or of either serotonin, norepinephrine or dopamine in the brainstem. Two weeks after the cessation of estrogen treatment, dopamine concentrations and tyrosine hydroxylase activities in the hypothalamus rebounded to levels above those seen in non-estrogen-treated ovariectomized controls. Hypothalamic serotonin levels, however, remained depressed. In parallel with these biochemical studies we performed ultrastructural immunocytochemical studies which confirmed that estrogen induced large increases in rough endoplasmic reticulum [5], but in these TH-immunopositive cells in the AN there was no substantial overall decrease in TH staining intensity: in fact, before treatment TH-peroxidase reaction product was found evenly distributed through the soma of the immunopositive cells, associated with the rough endoplasmic reticulum, and although characteristic estrogen-induced stacked endoplasmic reticulum was seen in TH-immunopositive cells in the substantia nigra of both ovariectomized and estrogen treated rats, characteristic stacked endoplasmic reticulum in the AN was observed in TH-immunopositive cells only after estrogen treatment. Thus, after 2 weeks of estrogen exposure, patches of minimally labeled, dilated endoplasmic reticulum appeared in the AN TH-immunopositive cells. At this point it is not reasonable to form any conclusions regarding this discrepancy in immunological vs biological potency of TH, but it is of great interest that upon cessation of the estrogen treatment the increased TH-immunonegative stacked rough endoplasmic reticulum began to show TH-immunopositive granules along its cisterns. The apparent dichotomy between structure and function under these circumstances may indicate a cellular basis for estrogen-induced hypothalamic failure and gliosis; however, these studies must be extended to cover longer periods while observing whether the depression of neurotransmitter production becomes permanent with prolonged estrogen exposure, to determine what other morphologic changes accompany the immunohistochemical findings and to identify the content of degenerating fibers.

Freeze fracture characterization of AN neurons: developmental and sex determined differences in membrane components

Sexual dimorphism in synaptic connectivity and protein content have been described in the AN [77], but conventional EM has not shown sex- or developmental differences in AN neuronal membranes [83]. However, we and others have adduced powerful evidence of estrogen effects upon neuronal membranes at all levels of cellular organization. These include effects described above upon synapses, endoplasmic reticulum and other organelles, plus increases in nuclear size [5], and density and number of nuclear pores [84]. As a prelude to studies with sex steroids we performed a quantitative freeze-fracture analysis of AN to assess possible sex differences in neuronal plasma membranes [37]. Male and female rats aged 0 (caesarean-delivered newborns), 10, 20 and 90 days postpartum were studied. Replicated fracture faces of randomly selected dendritic and perikaryal plasma membranes were photographed and the intramembranous protein particles (IMP) in the protoplasmic (P) and external (E) faces of plasma membranes studied [85]. Quantitative evaluation of IMP revealed an increase in the density of P- and E face particles between newborns and adults; results which are similar to what was previously found in cerebellar cortex [86–90]. In addition, at each time point, sex differences in IMP content were found for the P- and E faces of perikarya, and the P faces of dendrites; AN neurons from females contained significantly more IMP than males and added them at a rate which maintained that sex difference throughout development and early adult life [39].

There was little variation in the IMP content within groups of animals. The differences in IMP were due to the presence of greater numbers of small particles (<10 nm) in perikaryal or dendritic membranes from females compared with males ($P<0.001$). In addition, in some cases (perikaryal P face in newborns, dendrite E face in adults), the number of large (≥ 10 nm) particles was increased in males compared with females. Therefore, the progressive rate of protein particle insertion in the developing neuronal plasma membrane differs between males and females. As mentioned and discussed above, the sex difference was present only in perikaryal and dendritic membranes while membranes of dendritic spines had no sex difference [40]. Neuronal membranes from cerebellar Purkinje cells also appeared to be indifferent to sex [40].

The mechanisms underlying sexual dimorphism in the protein particle content of AN post-synaptic plasma membranes are not known; however, since there are estradiol concentrating neurons in AN [91–93] and E administration during the perinatal period changes the number and distribution of AN synapses [42, 43, 77, 94] as well as causing remodeling of the adult AN (see above) we have examined the possibility that estrogen effects on post-synaptic membranes could underlie or at least be associated with changing brain circuitry.

Long-term effects of sex steroids on neuronal plasma membrane organization

In view of the above picture we hypothesized that the sex dimorphism in AN post-synaptic membrane IMP contents was due to estrogen. We therefore performed freeze-fracture analysis to complement our transmission EM studies on the effects of estrogen treatment and to allow interpretation of the finding of sex differences in AN neuronal membrane IMP density and size [40]. Adult (100 days old) cycling Sprague–Dawley female rats were sacrificed at 3, 8, 16, or 32 weeks after estradiol valerate (1 mg/100 g body wt). Freeze-fracture analysis of AN plasma membranes

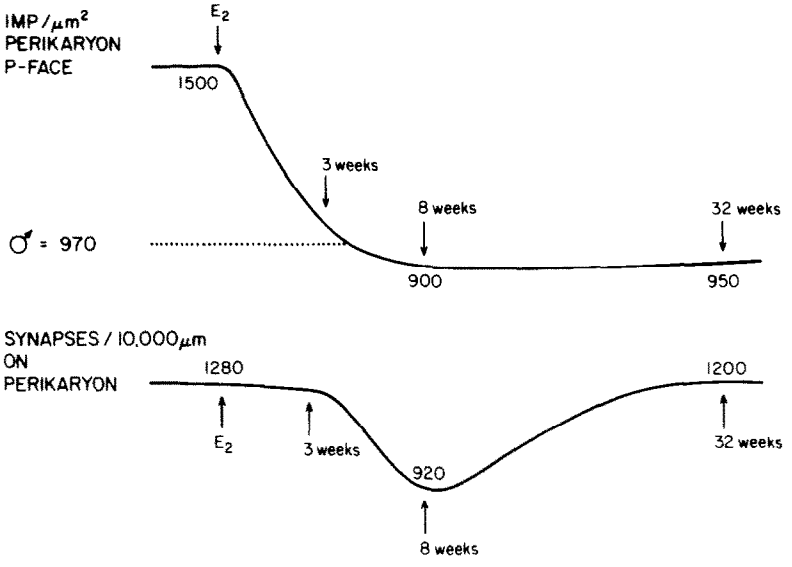

Fig. 7. Chronology of arcuate neuron P face intramembranous protein particle (IMP) changes following estradiol valerate administration (E_2, 1 mg/100 g body wt) in comparison to estrogen-induced axosomatic synapse denervation–reinnervation.

revealed interesting qualitative and quantitative changes compared with untreated controls:

(1) Differences in distribution of IMP were evident by 3 weeks after the injection of EV, with the appearance of aggregates of small IMP (10–20 IMP) on the P face of the AN perikarya.

(2) By 8 weeks large aggregates (150–200 IMP) were frequently observed in the P face of both perikarya and dendrites.

(3) In some cases, gap junction-like structures were observed on dendrites 8 weeks after EV treatment.

(4) There was an apparent increased frequency of endo-exocytotic pits on perikaryal and dendritic faces.

(5) No effect could be found of EV on the IMP of spine E or P faces. During the first weeks after EV treatment the overall density of IMP in the female rat's AN neuronal membranes fell, approaching that seen in males.

In Fig. 7 the chronology of these changes is plotted against the previously described effect of estrogen on AN synapses. Taken together these studies indicate that estrogen-induced membrane changes include insertion and lateral distribution of IMP, actions which have been associated with endo-exocytosis. We therefore studied more directly the estrogen-induced changes in endo-exocytotic pits.

Short-term effects of estrogen on neuronal plasma membranes

We sought to detect the earliest changes in neuronal membranes which could be induced by sex steroids and could contribute to the variation of IMP content [96]. We developed an *in vitro* technique in which brain slices are incubated in artificial CSF (ACSF), with or without added sex steroids, then fixed directly by adding fixative to the ACSF, and processed for freeze-fracture. The ACSF and conditions of incubation are standardized and in keeping with well-known electrophysiological studies, assuring good maintenance of AN tissues during the study period. Studies on AN slices maintained 6 h in the incubation chamber and then rapidly frozen indicate retention of good morphology of the neuronal membranes to be studied in freeze-fracture replicas.

We evaluated the density of endo-exocytotic pits following exposure to estrogens and other compounds. Neuronal perikarya and dendrites from AN slices fixed within 1 min after estradiol (10^{-10}–10^{-6} M) exposure showed a 3–4-fold increase in the number of endo-exocytotic images in their plasma membranes compared with control slices incubated in hormone-free ACSF or ACSF containing 17α-estradiol. Under the conditions studied, the estradiol action was rapid (maximal response by one minute) dose-dependent, blocked by tamoxifen and reversed by estrogen deprivation [96].

In order to detect possible correlations between increased numbers of endo-exocytotic pits and changes in endocytotic activity, we also transferred some mediobasal hypothalamic slices to ACSF containing 1 mg/ml of horseradish peroxidase (HRP) in the presence or absence of estradiol (10^{-10} M). Slices were removed at intervals of 10–160 min, rinsed, homogenized and assayed for HRP as described by Steinman and Cohn[97]. Hypothalamic slices incubated in the presence of estradiol showed an increased uptake of HRP when compared with slices incubated in hormone-free media; this change paralleled the increase in density of endo-exocytotic images. These results show that estradiol induces increased endocytotic activity in hypothalamic cells as soon as 1 min after hormone exposure. They are

consistent with acute membrane-mediated estrogen actions on hypothalamic AN neurons and suggest that estradiol may affect the turnover of some membrane components in sensitive arcuate neurons. The accompanying lateral displacement and aggregation of IMPs in membranes raises the possibility that the clustering of IMPs seen in endocytotic pits (Garcia-Segura and Naftolin, unpublished observations) could, in conjunction with the absence of IMP in the fusion site of exocytotic vesicles [98], result in a net decrease of IMP density in affected membranes.

Since postsynaptic membrane recognition appears to play a critical role in the establishment and maintenance of synaptic contacts, sex differences in synaptic organization and the effects of estrogen on arcuate nucleus synapses could be secondary to an effect of estrogen on postsynaptic membranes [86–90]. We are now assessing AN replicas from cycling female rats in order to determine whether there are cyclic changes in the neuronal membranes which may accompany the variations in circulating estradiol seen during the cycle [46].

In summary, evidence has been presented of cellular estrogen actions upon diencephalic neurons which may underlie the control of reproduction. Although this is a promising beginning, many questions remain unanswered. In any case, it is now clear that morphologic techniques have been successful and should be continued as complements to the other studies in this area of investigation.

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