Distribution of Brain Cells with Estrogen Receptor and Their Response to Estrogen

—A Possible Role of Estrogen Receptor in Coordination of Reproductive and Ingestive Behaviors—

エストロゲン受容体を持つ脳細胞の分布と それらの細胞のエストロゲンに対する反応に関する研究

—繁殖行動と摂食行動の協調に果たす エストロゲン受容体の役割について—

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エストロゲン受容体を持つ脳細胞の分布と

Distribution of Brain Cells with Estrogen Receptor and Their Response to Estrogen

— A Possible Role of Estrogen Receptor in Coordination of Reproductive and Ingestive Behaviors —

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Abstract

In order to localize estrogen receptor in the rat brain, a polyclonal antibody was produced against proteins expressed in *Eschelichia Coli* cells bearing a complementary DNA for the rat estrogen receptor. The polyclonal antibody specifically recognized the estrogen receptor in rat tissues, and was found to be a powerful tool to apply on immunohistochemistry. Using this antibody, it was demonstrated that estrogen receptor immunoreactivity distributes in selected nuclei in the brain and its expression is under control of estrogen. Double-labeling immunohistochemistry for estrogen receptor and substance P revealed that subsets of neurons in the medial preoptic area and ventromedial nucleus of the hypothalamus contain both materials in the same cell body, and that activity of substance P in these two nuclei was largely dependent on the presence of estrogen in circulation. Co-localization of other substances with estrogen receptor was also demonstrated not only in the brain but also in the pituitary. When estrogen receptor immunohistochemistry was combined with NADPH diaphorase histochemistry, a striking co-localization of estrogen receptor and NADPH diaphorase, which is nitric oxide synthase in the brain, was observed in the medial preoptic area and the ventromedial nucleus of the hypothalamus. Activity of nitric oxide synthase in the receptor containing neurons remarkably increased by administration of estrogen. Since both nuclei are thought as essential centers for regulation of reproductive and ingestive behaviors, a possible involvement of substance P and nitric oxide in estrogen-mediated those functions was suggested.

Preface

Each chapter of this thesis bears its own Summary, Introduction, Materials and Methods, Results, and Discussion sections. The references, however, have been listed together at the end of the thesis. A common Abstract, Introduction and General Discussion of the Results have been included.

A part of each Chapters 3 and 4, and a main body of Chapter 5 have been taken and modified from original papers already published in learned journals. In addition, some unpublished results are included in all three Chapters in order to complete a story of the thesis. The published manuscripts are as follows:

Chapter 3 Okamura H, Yamamoto K, Hayashi S, Kuroiwa A and Muramatsu M. (1992) A polyclonal antibody to the rat oestrogen receptor expressed in *Escherichia coli*: characterization and application to immunohistochemistry. Journal of Endocrinology, 135: 333-341.

Chapter 4 Okamura H, Yokosuka M and Hayashi S. (1994) Induction of substance P-immunoreactivity by estrogen in neurons containing estrogen receptors in the anteroventral periventricular nucleus of the female but not male rats. Journal of Neuroendocrinology, *in press*.

Chapter 5 Okamura H, Yokosuka M, McEwen BS and Hayashi S. (1994) Colocalization of NADPH-diaphorase and estrogen receptor immunoreactivity in the rat ventromedial hypothalamic nucleus: stimulatory effect of estrogen on NADPH-diaphorase activity. Endocrinology, 135: 1705-1708.

Okamura H, Yokosuka M and Hayashi S. (1994) Estrogenic induction of NADPH-diaphorase activity in the preoptic neurons containing estrogen receptor immunoreactivity in the female rat. Journal of Neuroendocrinology, *in press*.

In Chapter 3, although the author was responsible for the design of experiments and the preparation of manuscript, some portions of experiments were carried out by people other than the author. A rat estrogen receptor cDNA was a gift by Dr. Muramatsu. The ligation of the cDNA into the plasmid and the construction of the cDNA into the expression vector were performed by Dr. Kuroiwa. Preparation of cell lysates and the experiments of Figs. 3-2, -3 and -5 were carried out by Dr. Yamamoto. Immunization of antigens into rabbits and following blood sampling and screening were done by Dr. Hayashi with the help of Ms. Ueda. Fig. 3-8A was prepared by Mr. Yokosuka, a graduate student in Tokyo University of Agriculture and Technology.

All experiments in Chapters 4 and 5 were carried out by the author. Mr. Yokosuka helped the author for preparing animals and steroid injections. Colchicine injections into animals were performed by Dr. Hayashi. Dr. McEwen, who spent for one month in this laboratory, participated in the discussion of Chapter 5 and gave critical comments for the manuscripts.

All experiments were carried out in this laboratory, Anatomy and Embryology in Tokyo Metropolitan Institute for Neuroscience, except those done by Dr. Yamamoto in Waseda University. Upon the preparation of all published manuscripts, Dr. Hayashi carefully checked author's drafts and gave a large amount of critical comments.

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Hormones, Neurotransmitters and Receptors

ANP : atrial natriuretic peptide
CCK : cholecystkinin
CGRP : calcitonin gene related peptide
DYN : dynorphin
EB : estradiol benzoate
END : β-endorphin
ENK : methionine-enkephalin
ER : estrogen receptor
FSH : follicle stimulating hormone
GAL : galanin
GABA : gamma aminobutylic acid
GH : growth hormone
GRF : growth hormone-releasing factor
LH : luteinizing hormone
LHRH : luteininzing hormone-releasing hormone
NPY : neuropeptide Y
NT : neurotensin
OT : oxytocin
P : progesterone
PR : progesterone receptor
PRL : prolactin
SOM : somatostatin
SP : substance P
TH : tyrosine hydroxylase
TSH : thyroid stimulating hormone
VIP : vasoactive intestinal polypeptide

Chemicals, Buffers and Others

BSA : bovine serum albumine
cDNA : complementary DNA
DAB : 3,3'-diaminobenzidine
DTT : dithiothreitol
EDTA : ethylenediaminetetraacetic acid
Mr : relative molecular weight
NADPH : nicotinamide adenine dinucleotide phosphate
NBT : nitro blue tetrazolium
PAGE : polyacrylamide gel electrophoresis
PB : phosphate buffer
PBS : phosphate buffered saline
SDS : sodium dodecyl sulfate
-ir : -immunoreactivity or -immunoreactive

Brain structures

ADP : anterodorsal preoptic nucleus
AH : anterior hypothalamus
ARC : arcuate nucleus
AVP : anteroventral preoptic nucleus
AVPv : anteroventral periventricular nucleus
BST : bed nucleus of the stria terminalis
COA : cortical nucleus amygdala
DM : dorsomedial nucleus of the hypothalamus
LHA : lateral hypothalamic area
LPO : lateral preoptic area
LSi : lateral septal nucleus, intermediate part
ME : median eminence
MEA : medial nucleus amygdala
MePO : median preoptic nucleus
MPOA : medial preoptic area
MPN : medial preoptic nucleus

MPNc : central part of the MPN

MPO : medial preoptic area

MS : medial septal nucleus

PH : posterior hypothalamic nucleus

PSCh : suprachiasmatic preoptic nucleus

PVi : intermediate periventricular nucleus of the hypothalamus

PvPO : periventricular preoptic nucleus

RE : nucleus runiens

SCH : suprachiasmatic nucleus

SO : supraoptic nucleus

VMN : ventromedial nucleus of the hypothalamus

VMNc : central part of the VMN

ZI : zona incerta

ac : anterior commissure

cpd : cerebral peduncle

fx : columns of the fornix

mtt : mammillothalamic tract

och : optic chiasma

V3 : third ventricle.

VL : lateral ventricle

Chapter 1. Review of Literature and General Introduction

1. Introductive Part

An individual has to rely on and adapt to continuously changing environment such as food availability, weather, the presence of mates, and body stores of food, water and other nutrients. Brain is an organ that controls the individual to adapt such numerous surrounding conditions. External and internal cues are conveyed neuronally or hormonally to the brain. They are integrated in the neural circuit to predict what lies ahead and to prepare the body for it. Subsequent outcome from the brain is an induction of behaviors that involve the elaboration of complex sequences of movements and postures that require a great deal of somatomotor integration for the expression of the appropriate adaptive response. In some case, different stimuli each trigger a single behavior at the same time. Alternatively, a single stimulus often evokes complex behaviors. In either case, the display of behavioral responses must be arranged in a stereotyped temporal order to ensure the proper adaptation.

Among numerous adaptive responses, reproductive and ingestive behaviors cloud be the best described ones by a variety of people such as physiologists, endocrinologists, anatomists, zoologists, human and animal clinicians, and animal-breeders. Each of these behaviors may have its own regulatory machinery in the brain. It has been known that fluctuating levels of gonadal steroids hormone are able to alter both reproductive (Pfaff, 1980, Short, 1984) and ingestive (Tarttelin & Gorski, 1973, Wade, 1975) behaviors in a variety of mammalian females. Thus, it may be possible that neural machineries that govern respective behaviors cross-talk each other through estrogen-mediated actions to operate in a coordinated manner in the female. An evolutionary scenario proposed by O'Malley (1989) may give some implication for such a concordant interaction to occur; " It is likely that evolution of the steroid receptor superfamily of gene regulators began a thousand million years ago in very primitive organisms. -------- Their needs were simple: 1) to survive in the environment and 2) to replicate. ------ Primitive members of the steroid receptor superfamily were intracellular receptors which bound environmental nutritional agents and/or metabolic substrates (e.g. amino acids or sugars) and by-products as ligands. ------ The ligands were likely to be absorbed from the environment or produced within the same cells which contained these receptors, thereby providing apparent constitutive activity."

It may not be curious, therefore, to imagine that two essential responses, ingestive behavior "to survive" and reproductive behavior "to replicate" are simultaneously regulated at

least in part by one of members of steroid receptor superfamily in the present animals. Likely candidate is a receptor for estrogen. The estrogen receptor is a gene regulatory protein (O'Malley & Means, 1974, Yamamoto, 1985), whose actions are exerted following the binding of estrogen. Since levels of circulating estrogen precisely reflect reproductive stages of the female, the estrogen receptor can presumably activate the brain machineries so that the female adapts her own reproductive status. In the brain, the estrogen receptor localize in specific nuclei (Pfaff & Keiner, 1973), and some of them are thought to be centers for reproductive (Pfaff, 1980) and ingestive (Brobeck, 1941, Heatherington & Ranson, 1942, Anand & Brobeck, 1951) behaviors in the brain.

2. Reproductive behavior

In many species females display ovulatory or estrous cycles (Short, 1984), and estrous behavior is an integral part of this cycle. The female exhibits well-circumscribed periods of heat when she is receptive to the male, while at all other times she is not receptive. Its expression is strictly restricted in a particular time point during estrous cycle that favors fertilization and gestation (Carter,1992). It is evident, therefore, that the behavioral changes that prepare the female for mating must coincide with the physiologic changes that prepare her for starting a successful pregnancy.

During estrus, a cow becomes to sensitive and walk around with a dreadful look, and she often stands still and allow herself to be ridden by other cows; a mare will raise her tail, urinate, and wink - a rhythmic contraction of the vulva and eversion of the clitoris, when a teaser stallion is introduced to her; a sow behaves to be nervous, sniffs, often mounts on another sow and becomes immobile to allow boars to mount her when she is pushed her back strongly by hand; a bitch becomes disobedient to her owner when she is in estrous, and highly attractive to all the male in the neighborhood.

Feminine sexual behavior can be divided into two components, courtship and copulatory behaviors (Carter, 1992), and both behaviors are under control of gonadal hormones (Dewsbury, 1979). Sexual receptivity in rodents has been operationally defined by the occurrence of lordosis. Lordosis is usually characterized by immobility and an arching of the back, which in conjunction with hindleg extension elevates the rump and head. Since the earlier

scientists (Beach, 1942, Young *et.al.*, 1961) found that lordosis behavior is strongly controlled by gonadal steroid hormones, a number of works have established the effect of the steroid on this behavior. The amount and intensity of lordosis behavior in ovariectomized rats increase with higher estrogen doses (Know & Pfaff, 1975). They show lordosis response after estrogen treatment alone, but usually show better responses with estrogen treatment followed by progesterone (Pfaff,1970a). The actions of these sex hormones on lordosis behavior are not necessarily routed the pituitary gland, because normal behavioral effects of estrogen and progesterone can be obtained in hypophysectomized animals (Pfaff, 1970b).

While sexual behavior in the female is clearly controlled by the ovarian steroid hormones, it also can be regulated by environmental stimuli such as photoperiod, weather, food and water. In addition, substances that are released prior to ovulation, including monoamines, LHRH, oxytocin, prolactin and endogenous opiates (for a review, Pfaff & Schwartz-Giblin, 1988), are all capable of inducing or facilitating female sexual behavior.

Accumulated evidence indicates that an important anatomical site of estrogenic regulation of female reproductive behavior resides within the VMN. Local implants of estrogen into the VMN facilitates lordosis, whereas estrogen implants elsewhere have little effect (Davis *et. al.*, 1979). Lesion of the VMN disrupts female sexual behavior (Law & Meagher, 1958). Electrical stimulation of the VMN facilitates the lordosis reflex of ovariectomized, estrogen-primed female rats (Pfaff & Sakuma, 1979). Moreover, ultrastructural studies suggest a profound effect of estrogen on the protein synthetic apparatus in neurons in the VMN (Jones *et. al.*, 1985).

3. Ingestive behavior

Although there had been early clinical observations that the hypothalamus had a role in eating, a concept that this region is responsible for the ingestive center had not been established until the findings in the early 1940s that lesions in the VMN produced overeating and obesity (Brobeck, 1941), while those in the LHA resulted in aphagia and adipsia (Heatherington & Ranson, 1942, Anand & Brobeck, 1951). Other types of experiments, such as electrical stimulation (Heatherington & Ranson, 1942, Bray, 1974), systemic injections of gold thioglucose (Mayer & Marshall, 1956), and elctrophysiological measurement of firing rate in

the hypothalamic single cells (Oomura *et. al.*, 1964), confirmed the earlier suggestions that the VMN is responsible for the inhibition of ongoing feeding while the LHA is involved in the activation of food seeking behavior. Consequently, the VMN have been designated as the satiety center while the LHA as the feeding center.

A variety of hormones and nutrients have been implicated in the regulation of ingestive behavior through direct or indirect actions on these ingestive centers in the brain. Those substances include bombesin (Gibbs *et. al.*, 1979), gastrin related peptide (Stein & Woods, 1982), somatostatin (Lotter *et. al.*, 1981), cholecystokinin (Morley, 1982), calcitonin (Freed *et. al.*, 1979), insulin (Bray, 1974), glucose (Russek, 1970), and lipid (Kennedy, 1972) as well as gonadal steroids (Brobeck *et. al.*, 1947, Tarttelin & Gorski, 1973, Wade, 1975). We empirically know phenomena that gonadal steroids influence food uptake in the female. For example, domestic animals such as the cow and swine become hypophagia in concordance with the initiation of estrous behavior following the proestrous peak of plasma estrogen, and the ovariectomy of dogs and cats renders them hyperphagia and body-weight gain. These events have been more experimentally examined in laboratory animals. Female rats show a decreased food intake and body weight (Wang, 1924, Brobeck *et. al.*, 1947, terHaar, 1972) at estrus when estrogen levels are high (Butcher *et. al.*, 1974). At diestrus, when estrogen levels are low, food intake and weight gain elevate (Brobeck *et. al.*, 1947). Withdrawal of ovarian hormones by ovariectomy induces a hyperphagia, rapid weight gain (Tarttelin & Gorski, 1973) and suppressed running-wheel activity (Wang, 1923). The effect of ovariectomy on eating (Tarttelin & Gorski, 1973) and running-wheel activity (Mook *et. al.*, 1972) can be reversed by treatment with estrogen alone. Progesterone has no effect on food intake, voluntary exercise or body weight (Hervey & Hervey, 1966, Rodier, 1971, Tarttelin & Gorski, 1973), but in the presence of estrogen, treatment of high dose of progesterone increases food intake and body weight (Wade, 1975).

4. Estrogen receptor

Hormone is a molecule which carries a given type of information. The information is received by a cellular counterpart structure, receptor, which mediates it into the cell body. Thus

the information embodies when the hormone binds to a given receptor, and then the signal molecule acquires a "sense" which is expressed as cellular responses. A central dogma has been developed to describe the general mechanism of action of steroid hormones. First, systemic steroid penetrates most tissues cells (Miligrom *et. al.*, 1970, Williams & Gorski, 1974). Studies have shown that retention of steroid appears only in certain tissues referred to as target tissues for the particular steroid (Stumpf & Sar, 1976). In the cells of these tissues, specific steroid binding proteins (receptors) have been found for the respective steroid hormones (Toft & Gorski, 1966, Miligrom *et. al.*, 1970). History of ER initiated by the discovery of specifically retained tritiated estradiol in target tissues such as rat uterus (Jensen & Jacobson, 1962). Careful studies revealed the presence of ERs in discrete areas of the brain (Pfaff, 1968, Stumpf & Sar, 1976, Goy & McEwen, 1980). Characteristics of estrogen binding and biochemical nature of estrogen receptor have been reviewed in detail (Gorski, 1987, Gorski *et. al.*, 1986, Jensen, 1987, MacLusky, 1987).

4-1. Structure of the estrogen receptor

The ER belongs to a large group of gene regulatory proteins so called as the steroid and thyroid hormone super family, which includes receptors for glucocorticoid, mineralocorticoid, progesterone, vitamin D and retinoic acid, and thyroid hormone as well as viral oncogene *erb*A (Evans, 1988). All receptors analyzed thus far are structured in a similar way: they exhibit a variable N-terminal region, a short and well-conserved cysteine-rich central domain, which is responsible for the DNA binding activity of the receptors, and a relatively well-conserved C-terminal half (Beato, 1989). The cDNA for ER has been cloned in various animal species such as human (Walter, 1985, Green *et. al.*, 1986, Green *et. al.*, 1986), chicken (Krust *et. al.*, 1986), mouse (White *et. al.*, 1987), rat (Koike *et. al.*, 1987), *Xenopus* (Weiler *et. al.*, 1987) and rainbow trout (Pakdel *et. al.*, 1989). The comparison of the deduced amino acid sequences of ERs revealed that they can be divided into 6 regions having high degree of homology across species in the DNA binding domain and steroid binding domain (Koike *et. al.*, 1987, Kurmar *et. al.*, 1987). These two domains have been found to be essential for the receptor to activate efficiently the transcription of estrogen-responsible genes (Kurmar *et. al.*, 1987).

4-2. Mechanism of action of estrogen receptor

Classically, estrogenic action was explained by the long-held "two-step" hypothesis: The ER was assumed to be cytoplasmic protein, which, when complexed with estrogen, translocated to the nucleus where it modified nuclear function (Jensen *et. al.,* 1968, Gorski *et. al.,* 1968). In 1984, a new model of the estrogen receptor was reported by two groups. King and Green (1984) demonstrated that a monoclonal antibody (H222) against the ER in human breast cancer exclusively interacts with nuclei of target cells by immunohistochemical method. Welshons *et. al.* (1984) have also shown by biochemical enucleation assay that the unoccupied estrogen receptor is not present in the cytosol. Although at present there is still some controversy to subcellular distribution of the estrogen receptor (Ramm *et. al.,* 1988, Blaustein & Turcotte, 1989, Blaustein 1992 &1993, Lehman *et. al.,* 1993), it is widely accepted that the estrogen receptor is predominantly located in the nucleus of target cells regardless of hormonal milieu (for reviews, Jensen, 1987, MacLusky, 1987).

A schematic illustration of estrogen action on gene expression is shown in Fig. 1-1 (modified from Rories & Spelsberg, 1989). Estrogen molecules diffuse in and out of all cells but are retained only target cells with the receptor. Once the steroid bound, the receptor molecule undergoes a conformational change that allows the hormone-receptor complex to bind to a specific DNA sequence termed estrogen responsive element (Seiler-Tuyns *et. al.,* 1986). These events occur as fast as 5 min after the steroid injection into an animal (Rories & Spelsberg, 1989). The binding of the hormone receptor complex to DNA in turn triggers the transcription of specific genes (1-4 hrs) depending on the presence of additional transcription factors (Nelson *et. al.,* 1988), leading to the specific protein products (6-24 hrs) (O'Malley & Means, 1974, O'Malley, 1984). The subsequent physiological effects of the steroid in cells can be observed 12 to 24 hours after steroid treatment (Rories & Spelsberg, 1989).

Along with this general mechanism of estrogen action, a number of estrogen-iduced changes in the hypothalamus have been reported at each step of the cascade. Administration of estrogen to ovariectomized rats has been shown to enhance the synthesis of tRNA or mRNA with peak values between 6 and 24 hours after treatment (Jones *et. al.,* 1986, Yahr & Ulibarri, 1986) as well as to induce specific proteins (Scouten *et. al.,* 1985, Mobbs *et. al.,* 1990) in the preoptic area and hypothalamus. These changes in cellular functions in response to estrogen are reflected in ultrastructural changes of neurons such as nuclear and somal hypertrophy (Jones

et. al., 1985, Leranth *et. al.*, 1991), and synaptic organization (Carrer & Aoki, 1982, Garcia-Segura *et. al.*, 1987, Frankfurt *et. al.*, 1990) as well as changes in neurochemical characteristics as described later.

In contrast to the delayed genomic actions of estrogen, rapid and specific effects of estrogen on hypothalamic neurons also have been demonstrated. For example, estrogen rapidly modulates the excitability of neurons (Nabekura *et. al.*, 1986, Nishihara & Kimura, 1989), changes neuronal membrane ultrastructure (Garcia-Segura *et. al.*, 1987), and modulates activity of neurotransmitter receptors (Lévesque & Di Paolo, 1988, Kelly *et. al.*, 1992). Moreover, specific binding sites for estrogen on synaptic plasma membrane have been shown (Towel & Sze, 1983). These observations clearly indicate that estrogen acts through non-genomic mechanisms by binding to the neuronal membrane (for reviews, Duval *et. al.*, 1983, Haukkamaa, 1987). However, unlike the nuclear receptor, the membrane receptor for any steroid hormones has yet been cloned so far and its physiological function is far from clear.

4-3. Distribution of estrogen receptor

The earlier evidence for the existence of ER in the brain relied on biochemical assays of hormone binding in extracts from relatively large, dissected samples (for a review, McEwen , 1976.) Improvement in microdissection techniques enhanced the anatomical resolution of this method (Rainbow *et. al.*, 1982). After Michael (1965) established an autoradiographic technique, which relies on the capacity of steroid hormone receptor to bind radiolabeled ligand, the estrogen-sensitive neurons have been localized in a variety of species, such as mammals (Pfaff & Keiner, 1973, Sheridan *et. al.*, 1974, Stumpf & Sar, 1976), bird (Arnold *et. al.*, 1976), amphibian (Morrell *et. al.*, 1975), fish (Davis *et. al.*, 1977), and reptile (Halpern *et. al.*, 1982). Estrogen-concentrating cells are found in the medial preoptic area, medial hypothalamus, and limbic forebrain areas in the vertebrate brain. In general, these neurons are found in brain regions that participate in behavioral and pituitary reproductive functions (McEwen *et. al.*, 1979).

5.Anatomical substrates involved in reproductive and ingestive behaviors

5-1. Medial preoptic area

The MPOA forms the walls of the supraoptic recess of the third ventricle. The border of the MPOA and AH is ill defined, and is often referred to as a part of the hypothalamus. The rostal boundary does not occupy a neat ventricle plane. Several rostal telencephalic structures, including the nucleus of the diagonal band, olfactory tubercle, and ventral striatum, are found in the most rostal coronal plane of the MPOA. The MPOA contains two representative nuclei, rostally the AVPv and caudally the MPN, in its ventral aspect.

In the rat, increasing concentrations of circulating estrogen are required for the occurrence of the LH surge (Ferin *et. al.*, 1969, Caligaris *et. al.*, 1971, Brown-Grant, 1974, Freeman *et. al.*, 1976, Blake, 1977, Kalra, 1993). Several reports have suggested that at least in part of stimulatory actions of estrogen on the LH release are exerted through the AVPv and adjacent MPN (Goodman, 1978, Terasawa *et. al.*, 1980, Wiegand *et. al.*, 1980, Peterson & Barraclough, 1989). Estrogen implanted into the MPN elicits the LH surge (Goodman, 1978), whereas it is suppressed by microimplants of anti-estrogens (Peterson & Barraclough, 1989). It has also been reported that electrolytic lesion of the AVPv/MPN blocks the occurrence of the LH surge (Wiegand *et. al.*, 1980).

The MPOA as a whole is thought to play an important role in various components of reproductive behaviors and the associated neuroendocrine and autonomic responses, including copulatory behavior (Clemens & Gladue, 1979, Larson, 1979), maternal behavior (Numan, 1983), and the control of prolactin release (Neill, 1980, Kalra & Kalra, 1983). In addition, the MPOA is implicated in mechanisms underlying thermoregulation (Boulant, 1981) and hypovolemic thirst (Swanson & Mogenson, 1981) as well as ingestive behavior (Simerly & Swanson, 1988).

The MPOA contains a high percentage of estrogen binding neurons throughout vertebrate phylogeny. In the rat (Pfaff & Keiner, 1973), the highest percentage of estrogen concentrating neurons are found near the midline, below the anterior commisure. The laterai POA contains scattered labeled cells, including some cells in the medial forebrain bundle.

5-2. Ventromedial hypothalamic nucleus

The largest nucleus in the tuber cinereum of the hypothalamus with ovoid-shape is the

VMN, which lies between the caudal edge of the optic chiasma and the mammillary body. The nucleus contains cells with small to medium size, and separated from the adjacent structures by the fiber-rich narrow capsule. The VMN consists of three subdivisions, dorsomedial, central and ventrolateral portions, and they differ each other in neurochemical specificity (Romano *et. al.*, 1988) and neural projections (Dornan *et. al.*, 1990).

The VMN has been shown to be an essential integrative neural substrate for a variety of functions. Among them, sexual and ingestive behaviors have been well-documented. Both functions are sensitive to gonadal steroids (Pfaff, 1980, Brobeck *et. al.*, 1947, Tarttelin & Gorski, 1973). Lesion of the VMN of female rats causes rats to become hyperphagic (Anand & Brobeck, 1951, Albert *et. al.*, 1971) and disrupts sexual behavior (Law & Meagher, 1958, Kennedy & Mitra, 1963). Implants of estradiol to the VMN blocks hyperphagia induced by ovariectomy (Wade, 1975), and facilitates lordosis behavior (Davis & Barfield, 1979).

The ERs are found throughout the VMN, and are densest in the ventrolateral, posterior part. Positive neurons are also observed outside the VMN, scattering toward the ARC, DMN and fornix, and the LHA. In the LHA, labeled cells are mingled with the medial forebrain bundle (Pfaff & Keiner, 1973).

5-3. Neural connection between the MPOA and VMN

Studies of the retrograde tracers demonstrated that injections of the tracers into the region of the MPOA resulted in dense clusters of labeled cells in the VMN (Saper *et. al.*, 1976, Kreiger *et. al.*, 1979, Simerly & Swanson, 1988). Most of the projections from the VMN to the MPOA arise from its ventrolateral part (Saper *et. al.*, 1976, Simerly & Swanson, 1988) where the ER exists in abundant (Pfaff & Keiner, 1973). The majority of those fibers course rostally through ventral and lateral regions of the anterior hypothalamic area, just medial to the definitive medial forebrain bundle (Simerly & Swanson, 1986). On the other hand, studies of injections of anterograde tracer into the MPOA found that fibers from the MPOA that descend in the medial forebrain bundle give rise to the terminal fields in the VMN and LHA (Conrad & Pfaff, 1976, Simerly & Swanson, 1988). The MPOA (Simerly *et. al.*, 1986) and VMN (Yamano *et. al.*, 1986, Micevych *et. al.*, 1988, Dornan *et. al.*, 1990) contain a variety of molecules, which are thought to be participate in signal transductions in these nuclei. By combining SP immunohistochemistry and retrograde tracer labeling, Yamano *et. al.*, (1986) have identified the existence of a prominent SP containing pathway from the VMN and LHA to the

MPOA. Estrogen concentrating neurons that project to the MPN are found to be numerous in the hypothalamic cells including the VMN (Akesson *et. al.*, 1988).

6. Neurochemical Identity of Neurons Containing Estrogen Receptor

One essential step in determining how estrogen influences neuronal functioning is to identify neurochemicals synthesized in neurons sensitive to the steroid. In this regard, a number of substances have been examined whether they co-localize with the ER in the same cells. They are summarized in Table 1-1.

In addition, estrogen has been shown to modulate activities of a number of neurotransmitters in the brain (for a review, Harlan, 1988). For example, protein concentrations of SP and CCK (Parnet *et. al.*, 1990, Micevych *et. al.*, 1988, Oro *et. al.*, 1988) as well as levels of mRNAs for OT (Miller *et. al.*, 1986), NT (Alexander *et. al.*, 1989), proenkephalin (Romano *et. al.*, 1988), proopiomelanocortin (Treiser & Wardlaw, 1992) and GAP 43 (Shughrue & Dorsa, 1993) in the preoptic area and hypothalamus change during the estrous cycle or by experimental estrogen treatment. Enzymes responsible for neurotransmitter degradation such as monoamine oxidase and acetylcholine esterase are under control of estrogen (Luine & McEwen, 1983). Estrogen is also known to influence the expression of a nuclear transcription factor, c-*fos*, gene in the central nervous system (Cattaneo & Maggi, 1990). Further, it has been demonstrated that the numbers and binding characteristics of some receptors are modulated by estrogen. Oxytocin (Coirini *et. al.*, 1989) and CCK (Akesson *et. al.*, 1987) bindings in the VMN vary depending on the stage during estrous cycle. Dopaminergic (Roy *et. al.*, 1990, Chiodo & Caggiula, 1983), β-adrenergic (Weiland & Wise, 1989) and muscarinic receptors (Lauber & Whalen, 1988) are regulated at least in part by estrogen. In specific regions of the brain, regulations of μ opioid (Martini *et. al.*, 1989) and high-affinity $GABA_A$ receptors (Schumacher *et. al.*, 1989) by estrogen also have been shown.

These numerous actions of estrogen on hormones, neurotransmitters and their receptors could be reflecting a wide variety of functions of this steroid in the brain. Although a number of substances have been shown either to co-localize with ER as listed in Table 1-1 or to be affected by estrogen as mentioned above, they have never examined under various hormonal conditions except for PR (Blaustein & Turcotte, 1989, Warembourg *et. al.*, 1989). Therefore, in

order to understand more in detail the mechanism of action of estrogen, it is necessary to identify neurochemicals that are modulated by estrogen within neurons containing ER.

7. Introduction to Experimental Section

As discussed above, it is very likely that reproductive and ingestive behaviors are, in part, regulated in a coordinated manner through actions of estrogen. Although these two behaviors have been independently well-studied, few reports are available studying a neural mechanism underlying such cross-talk between two systems. This kind of information may help to better understanding of not only individual behaviors but also integrated neural circuitry.

The aim of the thesis, therefore, is to find and elucidate a mechanism involved in the estrogen-dependent coordination of reproductive behavior with ingestive one mainly by morphological methods. In the MPOA and VMN, information of the circulating levels of estrogen could be translated as activities of neurotransmitters/neuromodulators in neurons containing the ER, which may, in turn, act as messengers to evoke physiological responses and to integrate neural circuitry. In this context, it will be informative to identify the substances whose production is directly regulated by estrogen.

At first, it is necessary to obtain a tool to localize the ER in the brain at a single cell level. In Chapter 3, development of either *in situ* hybridization histochemistry to localize mRNA for ER or ER immunohistochemistry by producing an antibody against the rat ER is described. Among these two techniques, the latter one was found to be satisfactory for purposes of the subsequent studies; Using this antibody, a role of down-regulation of ER by estrogen, and co-localization of several substances in ER containing cells are presented in Chapter 4-1. In Chapter 4-2, an effect of estrogen on SP activity in the ER containing neurons is examined and a possible involvement of this peptide in reproductive and ingestive functions is described. In Chapter 5, co-localization of nitric oxide synthase and ER in the MPOA and VMN is demonstrated, and it is suggested that nitric oxide may act as a novel second messenger which accounts for a wide variety of phenotypes induced by estrogen actions on these two nuclei.

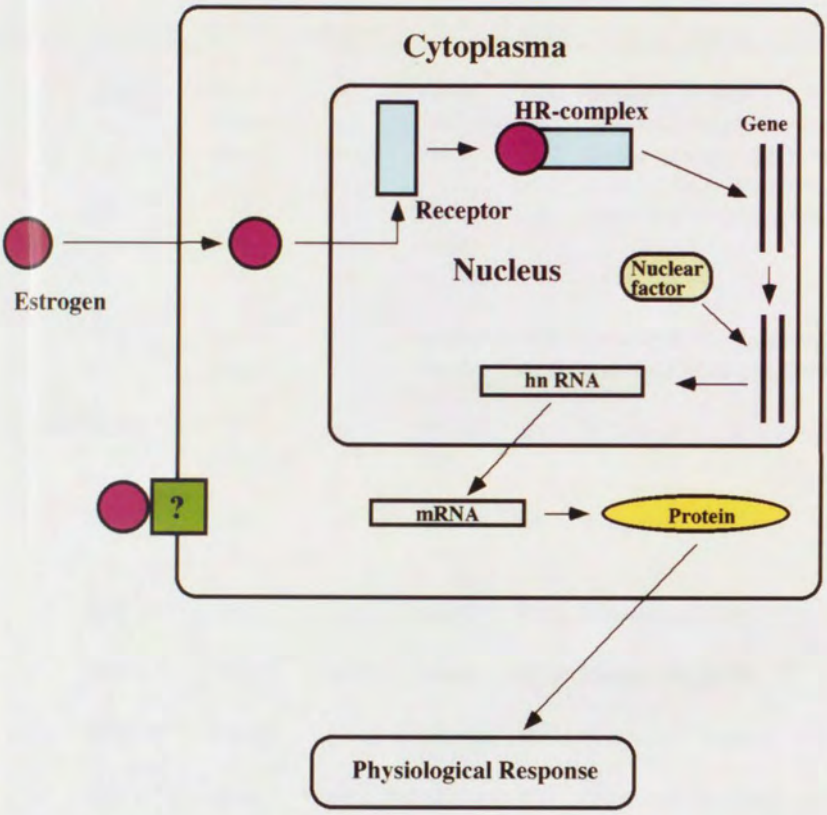


Figure 1-1. A model of the basic mechanism of estrogen action

modified from Rories & Spelsberg (1989)

Table 1-1. Neurochemical identity in neurons containing estrogen receptor

Region	Substance	% of* colocalization with ER	Animal	Sex	detection** of ER	Author
MPOA	LHRH	absent	rat	female	ARG	Shivers et.al., 1983
		absent	rat	female/male	IHC	Herbison & Theodosis, 1992
		absent	sheep	female	IHC	Lehman & Karsch,1993
	NT	50	rat	female	IHC	Herbison & Theodosis, 1992
		25		male		
		25	rat	female	IHC	Axelson et. al., 1992
	CCK	absent	rat	female/male	IHC	Herbison & Theodosis, 1992
		absent	rat	female	ARG	Akesson & Micevych,1988b
	CGRP	80	rat	female	IHC	Herbison & Theodosis, 1992
		30		male		
	GAL	20	rat	female	ARG	Bolch et.al., 1992
		8		male		
	ANP	15	rat	female	IHC	Watson et.al., 1994
	GABA	35	rat	female	ARG	Flügge et. al., 1986
	TH	absent	rat	female/male	IHC	Herbison & Theodosis, 1992
	SOM	absent	rat	female/male	IHC	Herbison & Theodosis, 1993
	PR	almost all	guinea pig	female	IHC	Blaustein & Turcotte,1989 Warembourg et.al.,1989

Region	Substance	% of colocalization with ER	Animal	Sex	detection of ER	Author
BST	CCK	absent	rat	female	ARG	Akesson & Micevych,1988b
	VIP	almost all	rat	male	IHC	Axelson & Leeuwen,1990
PVN	OT	almost all	guinea pig	female	IHC	Warembourg & Poulain,1991
	SP	8 to 26	rat	female	ARG	Akesson & Micevych,1988a
ARC	NPY	10 to 20	rat	female	ARG	Sar et.al.,1990
	GRF	20 to 30	rat	female	ARG	Shirasu et.al.,1990
	END	4	rat	female	ARG	Morrell et.al.,1985
		25	rat	female	ARG	Jirikowski et.al.,1986
		25	mouse	female	ARG	Jirikowski et.al.,1986
		15 to 20	sheep	female	IHC	Lehman & Karsch,1993
	DYN	10	rat	female	ARG	Morrell et.al.,1985
	TH	30	rat	female	ARG	Sar,1984
		3 to 5	sheep	female	IHC	Lehman & Karsch,1993
	PR	alomost all	guinea pig	female	IHC	Blaustein & Turcotte,1989 Warembourg et.al.,1989
	SP	42	rat	female	ARG	Akesson & Micevych,1988a

Region	Substance	% of colocalization with ER	Animal	Sex	detection of ER	Author
VMN	CCK	absent	rat	female	ARG	Akesson & Micevych,1988b
	GAL	absent	rat	female/male	ARG	Bloch et.al.,1992
	PR	almost all	guinea pig	female	IHC	Blaustein & Turcotte,1989 Warembourg et.al.,1989
	VIP	almost all	rat	male	IHC	Axelson & van Leeuwen,1990
	SOM	56 52	rat	female male	IHC	Herbison,1994
Amygdala	PR	absent	guinea pig	female	IHC	Warembourg et.al.,1989

*: Percentage of cells containing both ER and neurochemical to the total cells containing each neurochemical.

**: ARG represents autoradiography using tritium-labeled estradiol, while IHC represents immunohistochemistry

Chapter 2. General Materials and Methods

1. Animals

Sprague-Dawley rats maintained and bred in this laboratory were used. They were housed in a light-controlled room (lights on 07:00-19:00 h) and received food and tap water *ad libitum*. Newborn pups within 24 hrs after birth were designated as PD1. The neonates were weaned at PD21, and thereafter females and males were separately maintained. When necessary, vaginal smears were daily examined in mature female rats (after ~PD45) to determine the stage of the estrous cycle.

2. Steroids Treatment

Some male and female Sprague-Dawley rats (weight 230-320 g, 60-80 days) were gonadectomized under light ether anesthesia. After recovery, EB (Sigma, St.Louis, MO, USA; 10 μg/0.1 ml sesame oil/100 g body weight) or progesterone (Sigma, 2 mg/ 0.1 ml sesame oil/animal) were subcutaneously injected to the animals according to the experimental schedule. Corresponding amounts of sesame oil were given into control animals.

3. Protein Concentration

Unless otherwise stated, protein concentrations in the sample were determined by the method of Lowry *et. al.* (1951) using BSA as standard.

4. Tissue Preparation

Adult animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (40-50 mg/kg body weight), while the neonates were anesthetized on ice. They were perfused intracardially with PBS followed by a fixative in 0.1 M PB, pH 7.4. The brain section was dissected out, and post fixed for 2 h at 4 °C, and then it was immersed in 20

% sucrose in 0.1 M PB, pH 7.4, overnight at 4 °C. Unless otherwise noted frontal sections were cut at 30 µm thickness on a freezing microtome and free floating sections were kept in 0.1 M PB, pH 7.4, containing 30% sucrose, 1% polyvinyl pyrrolidone and 30% ethylene glycol (Watson *et. al.*, 1986) at -20 °C until used. The fixative and post fixative were changed depending on experiments.

Paraffin-embedded sections were prepared as follows. After anesthetized, animals were perfused with PBS followed by 0.4% paraformaldehyde in 0.1 M PB, pH 7.4. The brain was removed and post fixed in the same fixative at 4 °C overnight. They were dehydrated through increasing concentrations of ethanol, 50% toluene in ethanol, and 100% toluene, and then paraffin embedded. Sections with 5 µm thickness were cut and mounted BSA-coated slide glasses.

5. Immunohistochemistry

Free Floating Section

Immunohistochemistry for ER was performed according to the method described by Hoffman *et al.* (1990) with minor modifications. In brief, sections were rinsed with PBS containing 0.5% Triton X-100 (PBST), and then treated with 3% hydrogen peroxide in methanol for 15 min. They were rinsed extensively with PBST, and incubated with 8 % normal goat serum in PBST containing 1% BSA and 0.02% sodium azide (BSA-PBST) for 2 hrs at room temperature. Then sections were subsequently incubated with the anti-rat ER serum (AS-408 (Chapter 3), 1:30,000 in BSA-PBST) for 72 hrs at 4 °C, biotinylated goat anti-rabbit IgG (1.8 µl/ml of PBST containing 1 % normal goat serum) for 1 hr at room temperature, avidin-biotin complex solution (4.5 µl each/ml PBST) for 1 hr at room temperature. Each step was followed by three 15 min washes with PBST. After the last wash, sections were immersed in 0.175 M sodium acetate, pH 5.6, followed by reaction with the chromogen solution consisted of 25 mg/ml nickel-sulfate, 0.2 mg/ml DAB and 0.0025 % hydrogen peroxide in 0.175 M sodium acetate for 8 min. The reaction was stopped by immersing sections in the sodium acetate solution. After intensive washing with PBST, sections were mounted on the coated slide glass, air dried, dehydrated through graded ethanol solutions and xylene, and then cover slipped.

Immunohistochemistry for PR was done by a slightly different procedure from that for

ER. Free floating sections were rinsed with 50 mM PBS for 10 times, and then incubated an anti-PR monoclonal antibody (1:500 in 50 mM PBS containing 0.4% Triton X-100) for 72 hrs at 4 °C. After 10 times rinse with 50 mM PBS, sections were incubated with biotinylated horse anti-mouse IgG (1.8 µl/ml of 50 mM PBS containing 0.4% Triton X-100) for 1 hr at room temperature, and then avidin-biotin complex solution (4.5 µl each/ml 50 mM containing 0.4% Triton X-100) for 1 hr at room temperature. Each step was followed by three 15 min washes with 50 mM PBS. After this step, PR-ir was visualized with the same procedure as that for ER. In a preliminary experiment, it was found that the treatment of sections with hydrogen peroxide or normal horse serum prior to the incubation with the anti-PR antibody, or the use of the Triton X-100 containing buffer in the washing procedure, drastically reduced the intensity of positive staining, although all of these procedures were successfully utilized to reduce the background staining without affecting staining for ER.

When double-labeling immunohistochemistry was performed, sections were extensively washed with PBST following to the DAB reaction for ER or PR. Then sections were incubated with one of the second antisera being appropriately diluted with BSA-PBST for 72 hrs at 4 °C. The staining procedure for the second antiserum was similar to that for the ER except that the sodium acetate solution was substituted with 50 mM Tris-HCl, pH 7.4, and nickel-sulfate was omitted from the chromogen solution. Positive reaction products for the second antiserum were observed as brown materials in contrast to blue-black products for ER or PR.

Paraffin Embedded Section

Paraffin sections were deparaffinized through xylene and graded alcohols. After this step, sections were processed with an essentially similar method for free floating sections except followings. The titer of AS-408 was increased to 1:10,000. Triton X-100 was omitted from all solutions used. The concentration of hydrogen peroxide was reduced from 3 to 0.5%, and it was diluted with PBS instead of methanol.

6. Statistics

Cells which demonstrated histochemically stained materials varied in their strength among cells. In the evaluation of the numbers of those cells, however, no distinction between

weak and strong staining was made. Statistical comparisons between each group were done using the Mann-Whitney U test. P values less than 0.05 were considered as significant. (*: $p<0.05$, **: $p<0.01$).

7. List of Antibodies Used in the Present Study

Anti-ER polyclonal antibody:	Generated in the experiment of Chapter 3.
Anti-PR monoclonal antibody:	Affinity Bio Reagents, NJ, USA.
Anti-LHRH polyclonal antibody:	UCB-Bioproducts, SA, Brussels, Belgium.
Anti-TH polyclonal antibody:	Eugene Tech International, Inc., NJ, USA.
Anti-END polyclonal antibody:	UCB-Bioproducts, SA, Brussels, Belgium.
Anti-ENK polyclonal antibody:	UCB-Bioproducts, SA, Brussels, Belgium.
Anti-SP polyclonal antibody:	UCB-Bioproducts, SA, Brussels, Belgium.
Anti-FSH polyclonal antibody:	National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK), USA
Anti-LH polyclonal antibody:	National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK), USA
Anti-PRL polyclonal antibody:	National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK), USA
Anti-TRH polyclonal antibody:	National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK), USA
Anti-GH polyclonal antibody:	Gunma University, Institute of Endocrinology.

Chapter 3. Localization of Estrogen Receptor by *In Situ* Hybridization and Immunohistochemistry

Summary

At first, *in situ* hybridization technique was developed to detect ER mRNA in the rat tissues. A cDNA clone coding the rat ER was inserted into the plasmid Bleuscript. This construct was transcribed by RNA polymerases using [^{35}S]UTP, and resulting cRNA probes were utilized to localize ER mRNA in paraffin-embedded sections from female rats. Selected subpopulations of cells in the brain, pituitary gland, and uterus, all of them are known to be estrogen sensitive, were found to contain ER mRNAs. Labeling was specific, since no positive signals were observed when sense probe was hybridized. Although *in situ* hybridization histochemistry allowed to localize ER mRNA in several cells, this technique was not sufficient to further characterize ER neurons with a combination of another histochemistry. Therefore, next attempt was focused on production of an antibody to the rat estrogen receptor.

A rat ER-β-galactosidase fusion protein was expressed in *Escherichia Coli* using a pEX-2/rat ER cDNA construct. Scatchard analysis of [^{3}H]estradiol binding to the cell lysate revealed that the fusion protein had functional binding sites specific for estradiol with a dissociation constant of 1.49 nM. A molecular weight of the fusion protein was determined as 180 kilodalton by immunoblot analysis. The protein was isolated by means of SDS-PAGE and subsequent electroblotting. By immunizing the purified materials on nitrocellulose membranes, a polyclonal antibody to the rat ER, designated as AS-408, was raised in a rabbit. Binding of [^{3}H]estradiol to the estrogen receptor in the rat uterus was inhibited by AS-408 in a dose response manner. The antibody was also able to recognize the [^{3}H]estradiol-ER complexes. Thus, the antibody AS-408 could react with both forms of the solubilized receptor being occupied and unoccupied with the hormone. In immunoblot analysis of the cytosol fraction of the rat uterus, a single band of 67 kilodalton, the size of the estrogen receptor, was detected by AS-408. Moreover, when the antibody was applied to immunohistochemical examination of rat brain sections, positive staining was observed in neurons in specific regions of the brain known to contain ER.

These results demonstrate that the polyclonal antibody obtained in the present study directed specifically to the rat ER, and it would be a tool to detect and analyze the receptors in a various target tissues for estrogen.

Introduction

Distribution of ERs in the estrogen target tissues has been examined by biochemical assays of hormone binding (see McEwen, 1976), and autoradiographic observations of tritium-labeled estradiol uptake (Pfaff & Keiner, 1973, Sheridan *et. al.*, 1974). The later technique has been widely used to localize the estrogen sensitive cells in a variety of species (Chapter 1, 4-3). Although it satisfies all of the criteria of receptor binding (Stumpf *et. al.*, 1980), the technique includes several disadvantages. For example, the method requires the very long processing time (some hundreds days!). Although recent development of [^{125}I] estrogen autoradiography (Stumpf *et. al.*, 1987, Toran-Allerand *et. al.*, 1992) has partially resolved this problem, there still remains considerable methodological disadvantages that animals have to be gonadectomized in order to reduce competitive binding of endogenous estrogen and that only ligand-bound receptors can be demonstrated by autoradiography.

Over the past two decades, the knowledge of molecular biology has expanded dramatically with the development of recombinant DNA techniques and sensitive methods for detection of specific DNA or RNA sequences by molecular hybridization. *In situ* hybridization was first introduced in 1969 (Boungiorno-Nardeli & Amaldi, 1969, Gall & Pardue, 1969, John *et. al.*, 1969). Since then, *in situ* hybridization histochemistry has become a powerful tool to study the localization and regulation of selected DNA or mRNA species in single neurons. A recent success of molecular cloning of cDNA for the rat ER (Koike *et. al.*, 1987) enabled to prepare a molecular probe to detect ER. Because this technique does not rely on the hormonal status in given animals, an attempt was made to establish a method to localize ER mRNA by *in situ* hybridization in this chapter.

Another attempt was focused on production of antibody to the rat ER, since antibodies to the receptor have been shown to offer direct and accurate localization of the receptors by an immunohistochemical method. Several groups have produced antibodies directed to the receptor protein from human mammary tumors (Green *et. al.*, 1984) or from calf uterus (Moncharmont *et. al.*, 1982), or directed to synthesized peptides which correspond to preselected regions in the receptor sequence (Traish *et. al.*, 1989, Furlow *et. al.*, 1990). Of those antibodies, the monoclonal antibody to the human ER, H222, which is commercially available, has widely been used to detect the ERs in a variety of species including human (King & Green, 1984, Walker *et. al.*, 1988), mouse (Korach *et. al.*, 1988, Koch, 1990),

guinea pig (Blaustein & Turcotte, 1989, Blaustein *et. al.*, 1992, DonCarlos *et. al.*, 1991), rabbit (Zaino *et. al.*, 1989), sheep (Lehman *et. al.*, 1993, Lehman & Karsh, 1993) and bird (Balthazart *et. al.*, 1989, Gahr, 1990). However, although this rat monoclonal antibody has been used to examine the rat ER in several laboratories (Blaustein, 1992, Herbison & Theodosis, 1992a,b, Yuri & Kawata, 1992), it may not be suitable to localize the ER in rat tissues, since the monoclonal antibody was raised in the rat and thus during the immunohistochemical procedure the secondary antibody (anti-rat IgG) binds throughout the tissue to endogenous immunoglobulins and produces widespread background staining (Gee *et. al.*, 1990, Blaustein, 1993). Thus, to study the precise localization of the ER in the rat, antibodies to the ER raised in any species other than the rat have been desired.

In order to produce an antibody, sufficient amount of receptor preparation is necessary. In general, the steroid receptors constitute less than 0.01% of the total mass of protein in the cell, and it is thought that they are extremely difficult to purify (Alberts *et. al.*, 1989). The ER proteins account for approximately 0.01% of the total cellular protein in the chick oviduct (Maxwell *et. al.*, 1987). The hypothalamus contains approximately 100-fold less ER than the uterus (Peck & Kelner, 1983). Therefore, in the present study, the ER protein was obtained in a large amount by expressing the ER in the *Escherichia Coli* (*E. Coli*) cells to which the cDNA clone of the rat ER (Koike *et. al.*, 1987) was introduced.

Materials and Methods

1. *In situ* hybridization

The paraffin-embedded sections were deparaffinized in xylene, hydrated through graded alcohols, rinsed with 0.1 M Tris-HCl, pH 7.5, and then air dried. Radio-labeled probe was prepared as follows. The cDNA insert encoding rat ER (Koike *et. al.*, 1987) was ligated into the *Eco*RI site of plasmid Bleuscript. The orientation was confirmed by restriction mapping. After linearization with *Bam*HI, the plasmid was transcribed by T7 RNA polymerase using [α-^{35}S]UTP to produce an antisense probe. A sense probe was transcribed using T3 polymerase after linearization with *Hind*III. Both probes were truncated to approximately 75 nucleotides by alkaline treatment. Hybridization was carried out as described (Ishii *et. al.*, 1990) with 2 x 10^7 cpm/ml of the ^{35}S-labeled probes at 50 °C overnight in the solution containing

50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 10 mM DTT, 0.5 mg/ml tRNA, 0.5 mg/ml sonicated salmon sperm DNA, 1 x Denhardt's solution, and 10% dextran sulfate. The sections were washed in 2 x SSC for one hr at 50 °C and then treated with cooked RNase A (20 μg/ml) in 0.5 M NaCl, 10 mM Tris-HCl, pH, 8.0, for 30 min at 37 °C. They were subsequently washed with 2 x SSC for 1.5 hrs at 50 °C, 1 x SSC for 1.5 hrs at 50 °C, and then 1 x SSC for 30 min at room temperature. All washing solutions contained 50% formamide. After dehydration, the slides were dipped in emulsion, air dried in the dark, and then exposed for 10 days at 4 °C. They were developed in Konicadol X, fixed, and then counter stained with hematoxylin.

2. Production of antibody to the rat estrogen receptor

Expression of the rat ER in E.Coli : The cDNA for the rat ER (Koike *et al.*, 1987) was modified to introduce an *Eco*RI site at the 5' terminus of the sequence for the construction to produce the β-galactosidase-rat ER fusion protein. The fragment encompassing from codon 68, through translational termination codon to some additional 3' non-coding sequence was excised by *Sac*II and *Eco*RI double digestion. The *Eco*RI site at the 3' end of the fragment was artificially introduced during cDNA cloning (Koike *et. al.*, 1987). To introduce another *Eco*RI site at the 5' end of the fragment, oligonucleotides corresponding to a region of 391 to 416 in the cDNA (5'-AATTCAACGCCGCCGCCGCCGCCGCG and 5'-GCGGCGGCGGCG-GCGTTG) were synthesized with a single base substitution G at position of 393 of the cDNA sequence to A. This substitution creates the *Eco*RI sites at 5' end of the oligonucleotide without changing the amino acid sequence of the rat ER portion. The oligonucleotides were annealed to form a double strand, ligated with the *Sac*II-*Eco*RI fragment and the resulting chimeric fragment was cloned into the *Eco*RI site of pUC18. The sequence of the exchanged region was then determined. The *Eco*RI fragment was then excised, transferred to the *Eco*RI site of pEX-2 vector and a clone containing the cDNA with the sense orientation for β-galactosidase was selected. The final construct was introduced into N4830-1 host cells for protein production. The rat ER portion in the fusion protein corresponds to amino acid 61 to carboxyl terminus of native rat ER and contains DNA binding and ligand binding domains. The protein was expressed in the host cells according to the method described by Maniatis *et. al.* (1989). Briefly, the cells were incubated in two x YT broth (bacto-trypton, 16 mg/ml; bacto-yeast extract, 10 mg/ml; NaCl, 5 mg/ml; pH 7.0) containing 50 μg/ml ampicillin at 30 °C with

constant agitation. Absorbance at 650 nm was monitored. When it reached 0.8, the culture was transferred to 42 °C and further incubated for 2 hrs with constant agitation. The cell pellet was stored at -80 °C until further processing.

Biochemical characterization of the fusion protein : The *E.Coli* cells were sonicated and centrifuged at 10000 x g for 15 min. The pellet was washed three times with 10 mM Tris.HCl, pH 7.4, containing 1 mM EDTA, 1 mM DTT and 10 mM sodium molybdate (TEDM buffer) and resuspended in the buffer. The cell lysate (400 µg protein) was incubated with increasing concentrations of [^{3}H]estradiol in a total volume of 0.25 ml with or without a 100-fold excess of unlabeled estradiol for 20 min at 37 °C. The mixture was then cooled on ice and centrifuged at 10000 x g for 30 min. After washing the pellets three times with 3 ml of TEDM buffer, radiolabeled materials were extracted with 1 ml of absolute ethanol. Three ml of scintillation fluid was added to each extract and the radioactivity was measured. Specific binding was calculated as the difference between the radioactivity bound in the absence and that bound in the presence of unlabeled ligand. Binding data were calculated by the method of Scatchard (Scatchard, 1949) and analyzed using a suitable computer program.

In order to examine the cross reactivity of the expressed ER to other steroid hormones, the cell lysate (400 µg protein) was incubated with 10 nM [^{3}H]estradiol in the presence of various amount of unlabeled estrogen, progesterone, corticosterone and testosterone. Binding of [^{3}H]estradiol to the ER in each incubation was measured as described above.

The molecular weight (Mr) and the quantity of the fusion protein in the cell lysate were determined by means of the immunoblot analysis. The *E.Coli* cells were sonicated, dialyzed , and then lyophylized. Samples were resuspended and boiled in the SDS-PAGE sample buffer under reducing conditions. Proteins were separated on discontinuous 7.5% SDS-PAGE (Laemmli, 1970), followed by electroblotting onto nitrocellulose membranes (Towbin *et. al.*, 1979). The blot was immunostained employing the anti-human ER monoclonal antibody and reagents kit according to the manufacture's instruction. A band specific for the ER was visualized by incubating the membranes for one min with DAB solution. Alternatively, the membrane was stained with Amido Black and staining density of the band corresponding to the fusion protein was compared to that of a standard protein in order to estimate the amount of the fusion protein in the cell lysate.

Immunization : The lyophylized *E.Coli* cell lysate was subjected to SDS-PAGE and electroblotting as described above. The blots were briefly stained with Amido Black, destained with water, and a band corresponding to the Mr of the fusion protein, 180K, was cut. The strips were pooled, and kept at 4 °C until used for first immunization. Total amount of proteins on the strips were roughly estimated at 180 μg by silver staining of the minigel (Phast system).

For the booster, solubilized antigen was prepared. Proteins in the cell lysate were separated on SDS-PAGE, and the gel was stained with 4 M sodium acetate. A band around 180 K was cut and pooled. The fusion protein was eluted by immersing the gel slices in 10 mM Tris·HCl, pH 7.4, including 0.1 % SDS at 4 °C for 48 h with constant rotation. The eluate was concentrated on a Centricon-30 filter and dialyzed against 10 mM $NaHCO_3$ overnight at room temperature. Protein concentration in the solution was estimated at 250 μg/ml by silver staining of the mini gel. The solubilized antigen was kept frozen until used for booster injections.

The membrane strips containing the fusion protein were inserted subcutaneously at the back of a male New Zealand White rabbit. At the same time, the Ribi adjuvant system (RAS, R-700, Ribi Immuno Chem Res. Inc.,Hamilton, Montana) emulsified with physiological saline only was given to the cut skin. Four weeks after the initial immunization, the mixture of the solubilized protein (25 μg) and the R-700 (1 ml) was injected to the animal according to the manufacture's protocol. The booster shots were repeated every second week then on.

Blood sampling : Blood samples were collected prior to the immunization and once a week after the first booster injection. Production of antibodies was monitored by immunohistochemical examination of brain sections from adult female rat.

Biochemical characterization of the antiserum : The uterus cytosol (400 μg protein), prepared from immature Sprague-Dawley rats as described previously (Aihara *et. al.*, 1988), was incubated with various amounts of IgGs obtained from either antiserum or control serum in the presence of10 nM [^{3}H]estradiol at 4 °C overnight, and specific binding of [^{3}H]estradiol to the ER was determined as described before (Watson & Clark, 1980).

To examine whether the antiserum reacts with the ER occupied with estrogen, the uterus

cytosol (400 µg protein) was incubated with 10 nM [^{3}H]estradiol in the presence or absence of a 100-fold molar excess of unlabeled estradiol at 4 °C for 3 hours. IgG fraction (800 µg protein) was added and further incubated at 4 °C overnight. At the end of the incubation, 0.25 ml of TN buffer (10 mM Tris-HCl, pH 7.4, containing 0.45 M NaCl) was added and the sample was applied to Protein A-Sepharose CL-4B in a column (0.5 ml bed volume). After washing with 20 ml of TN buffer, the hormone-receptor-antibody complex was eluted with 5 ml of 0.1 M citric acid, pH 3.0. The radioactivity in an aliquot (2 ml) of the eluate was measured.

The rat uterus cytosol was run on SDS-PAGE followed by electroblotting as described above. After quenching nonspecific binding sites, the blot was subsequently incubated with either the antiserum or the control serum in a final dilution of 1:5000 and ^{125}I-labeled F(ab')$_2$ (2 µCi/ml) according to the method of Maniatis *et. al.* (1989). The membrane was subjected to autoradiography using Kodak X-OMAT film with an intensifying screen for 18 hours at -80 °C.

Immunohistochemistry : Animals were perfused with 4% paraformaldehyde in 0.1 M PB, pH 7.4. Sections were processed according to the method in Chapter 2.

Immunohistochemical controls : The specificity was established by incubating the sections with the antiserum preabsorbed with lysates from either the control cells or the cells which contain the fusion protein. Additional controls were the incubation with the preimmune serum or the omission of primary or the secondary antibody in the staining procedure.

Results

1. *In situ* hybridization

In situ hybridization histochemistry revealed that ER mRNA containing neurons localize in selected nuclei of the brain including the ARC (Fig. 3-1A), VMN (Fig. 3-1A), AVPv (Fig. 3-1C), MPN, and the amygdala of proestrous females. The labeling appeared to be specific because within the equivalent regions few silver grains were observed over cells when sections were hybridized with a sense-strand probe (Fig. 3-1B). However, distribution of ER mRNA

containing neurons was obscure in other regions such as the habenula nucleus and BST where estrogen binding sites have been reported (Pfaff & Keiner, 1973). It can be seen in Fig. 3-1D that the ependymal cells and glia-like cells (small arrows) were not labeled and that some neurons containing abundant ER mRNA (large arrows) could be distinguished from those containing less ER mRNA by the number of silver grains around the cell nucleus.

Outside the central nervous system, the pituitary gland and uterus were also found to contain ER mRNA. The specific labeling in the pituitary occurred exclusively in the anterior lobe, while the intermediate or posterior lobes showed few signals (Fig. 3-1E). In the uterus from 5-days old female rat, positive signals were observed throughout the stroma but not in the luminal epithelial cells (Fig. 3-1F). In the adult uterus, the distribution of ER mRNA containing cells was reversed to that in the neonate, *i.e.* they were localized in the glandular and luminal epithelial cells, while the stroma cells avoided positive reaction (Fig. 3-1G).

2. Production of antibody to the rat estrogen receptor

Expression of the functional ER in the transfected cells was confirmed by specific binding of [^{3}H]estradiol. Scatchard analysis of the binding data revealed that a single class of high affinity binding sites with a dissociation constant (Kd) and a receptor density of 1.49 nM and 118 fmol/mg protein, respectively, for the crude fraction prepared from cells containing the ER cDNA with sense orientation (Fig. 3-2). In contrast, that from cells inserted the control plasmid showed no specific binding. The Kd value was slightly higher than that of the ER of uterine origin (Kd: 0.80 nM, a value obtained in a parallel experiment employing the nucleus fraction of the rat uterus), which is probably due to conformational change or a steric hindrance of the estrogen binding site by the fused β-galactosidase in the expressed ER. The binding was specific for estrogen. The binding of radiolabeled estradiol to the fusion protein was inhibited by increasing concentrations of unlabeled estradiol, while progesterone, corticosterone and testosterone, failed to displace bound [^{3}H]estradiol (Fig. 3-3). Thus, the competition studies confirmed that the fusion protein containing the functional ER was expressed in the *E.Coli* cells.

The fusion protein in the lysates was electrophoretically separated and blotted on the nitrocellulose membrane. Lane 1 in Fig. 3-4 shows the membrane which was stained with Amido Black. By immunoblot analysis employing the anti-human ER antibody, two bands were specifically detected (Fig. 3-4, lane 2). The Mr of the major band was around

180K which agrees with the estimated Mr of the fusion protein, *i.e.* 65K for the ER and 116K for β-galactosidase. The minor species may represent its truncated form. From these observations, it was considered that the highest band around Mr of 180 K in lane 1 is representing the fusion protein. The band on the membrane was cut, pooled and used for immunization.

Three months after the start of immunization, the blood sample showed a positive reaction in the screening. The serum was designated as AS-408. The specificity of the serum to the ER was confirmed by three methods, *i.e.*, binding assay, immunoblot analysis and histochemical examination.

The effect of the antiserum on [^{3}H]estradiol binding to the ER in the rat uterus is shown in Fig. 3-5a. The IgG fraction obtained from the antiserum inhibited estrogen binding to the receptor in a dose response manner, while that of the control serum had no effect. The antiserum was also able to recognize the receptor preoccupied with estrogen. The antiserum AS-408 was also able to recognize the [^{3}H]estradiol-ER complex. When the labeled hormone receptor complexes were reacted with the IgG fraction from AS-408 and then passed through the Protein A-Sepharose CL-4B column, 170.7 out of 206.2 fmol ER/mg protein applied was recovered in the eluate (recovery 82.8%), while only 1.5 fmol ER/mg protein were eluted (recovery 0.7%) when the complexes were reacted with the IgG fraction of the preimmune serum (Fig. 3-5b).

The uterus cytosol was subjected to immunoblot analysis using the antiserum and ^{125}I-labeled anti-rabbit F(ab')$_2$ fraction. The autoradiogram shows that only the antiserum detected a single band, Mr 67K (Fig. 3-6, lane 2), which is comparable to that of the native ER of the rat (Lubahn *et. al.*, 1985, Koike *et al.*, 1987).

Histochemical examination was performed on free floating coronal sections from the rat brain. Representative sections containing the AVPv, MPN and the medial basal hypothalamus are shown in Fig. 3-7. The densest staining was observed in the middle portion of the AVPv with a cluster of positive neurons around the third ventricle (Fig. 3-7A). Some immunoreactive materials were scattered in the MPO and area extending from the BST to LSi. At caudal portion of the MPOA (Fig. 3-7B), the PvPO and MPN contained a large population of ER positive neurons. They were extended dorsolaterally to the MPO and further to the BST. The SCH and SO avoided ER staining. In the medial basal hypothalamus (Fig. 3-7C), dense immunoreactivity existed in the ARC and VMN. Within the VMN, positive neurons were

exclusively localized in the ventrolateral portion of the nucleus. The LHA contained a relatively large number of positive neurons in its ventrolateral aspect. A few signals were able to be seen in the PVi and DMN. In the amygdala, the medial nucleus showed a heavy staining. Moderately stained neurons were also present in other part of the amygdala.

In adsorption test, preincubation of the antiserum with the lysate of the *E.Coli* cells expressing the β-galactosidase-ER fusion protein failed to show positive labeling in immunohistochemistry, while preincubation with that of the control cells had little effect on the staining.

Discussion

1. *In situ* hybridization

In situ hybridization histochemistry has widely been used to detect and localize mRNAs in interest in tissue sections. A detailed distribution of ER mRNA in the rat has been made by Simerly *et. al.* (1990), and thereafter several groups have reported changes in levels of ER mRNA utilizing *in situ* hybridization (Lauber *et. al.*, 1990, 1991, Shughrue *et. al.*, 1992). In the present study, it was also found that ER mRNA containing cells can be identified by this technique. In the brain, positive neurons were observed in the selected nuclei, the MPOA, VMN and ARC, which are involved in reproductive and/or ingestive functions (Pfaff, 1980, Brobeck, 1947). However, they were not apparent in other regions such as the LHA and the hippocampus, which are also shown to contain ER mRNA (Simerly, *et. al.*, 1990). In the other estrogen-sensitive tissues, such as the pituitary gland and the uterus, unique distributions of ER mRNA were shown, which are comparable to the previous findings by labeled estradiol uptake (Stumpf, 1968) or immunohistochemistry (Sar & Parikh, 1986, Yamashita *et. al.*, 1989). However, although *in situ* hybridization is advantageous when a quantitative analysis of ER mRNA is required (Sato *et. al.*, 1994) or when a confirmation of the expression of ER mRNA is necessary (Orikasa *et. al.*, 1994), this technique does not seem suitable to examine the materials that are directly regulated by estrogen within estrogen sensitive cells due to its tedious procedure and relatively low resolution of ER positive neurons at a single cell level (see Fig. 3-1D). Therefore, subsequent attempt was made to produce an anti-ER antibody that can be applied in immunohistochemistry.

2. Production of antibody to the rat estrogen receptor

A polyclonal antibody to the rat ER was produced utilizing an antigen produced by a molecular biological technique. Since the cDNA employed here encodes a full length of the rat ER protein, it could be possible that the polyclonal antibody generally cross-reacts with other members of the steroid-thyroid hormone receptor superfamily that share the common amino acid sequence with the ER (Evans, 1988). However, several lines of evidence suggest that it can be the antibody that directs exclusively to the ER. First, the binding of estrogen to the receptor in the uterus was inhibited in a dose response manner by the antibody, and the estradiol-ER complex was recognized by the antibody. Secondly, in immunoblot analysis of the uterus cytosol, the antibody detected a single band of Mr 67K which is in good agreement with the Mr of the rat ER (Lubahn *et al.*, 1985, Koike *et al.*, 1987). Finally, the distribution of the immunoreactivity in the rat tissue (Fig. 3-7) detected by the antibody was comparable to those demonstrated by autoradiography (Pfaff & Keiner, 1973, Sheridan *et al.*, 1974), by *in situ* hybridization (Simerly *et. al.*, 1990, Fig. 3-1) and by immunohistochemistry (Sar & Parikh, 1986).

Precise regions to which the polyclonal antibody direct are unclear. Recently, Furlow *et al.* (1990) produced antisera to a synthetic peptide which corresponds to an amino acid sequence found in the hinge region of the rat ER. Those antisera also bind to both denatured and native receptors, and interact with unoccupied ER as well as those bound estrogen. Therefore, it is possible that the antibody AS-408 recognizes the same sequence as above. Further, the antibody may direct to the N-terminal part in the receptor sequence, because this region is hypervariable in size and amino acid composition among the steroid and thyroid receptor superfamily (Evans, 1988) and is known to be highly antigenic (Wrang & Gustafsson, 1978).

When applied to immunohistochemistry, it was found that AS-408 is able to recognize ER molecule in tissue sections with very low background staining. The distribution of ER demonstrated in the present study is in good agreement with that shown by autoradiography (Pfaff & Keiner, 1973, Sheridan *et. al.*, 1974), *in situ* hybridization (Simerly *et. al.*, 1990) and immunohistochemistry using H222 (Axelson *et. al.*, 1992, Blaustein, 1992, 1993, Herbison & Theodosis, 1992a, b, Yuri & Kawata, 1992) or using a monoclonal antibody directed to the calf uterine cytosolic ER (Sar & Parikh, 1986). The titer of AS-408 is sufficient to detect small

amounts of the ER, which allowed to identify the ER in tissues such as the facial nucleus (Yokosuka & Hayashi, 1992) and cerebral cortex (Fig. 3-8A) (Yokosuka *et.al., in press*) in the neonates, and the hippocampus (Fig. 3-8B), in which the presence of ER immunoreactivity had not been shown previously. Although physiological functions of ER in such nuclei are unclear, the widespread distribution of ER imply a variety of actions of estrogen in the brain.

In most experiments performed here, the specific staining was exclusively confined to the nuclei of estrogen target cells. This result supports previous findings that the receptor protein resides in the nuclear compartment regardless of the hormonal environment (King & Green, 1984, Welshons *et. al.*, 1984). The nuclear localization of ER immunoreactivity has also been confirmed immunohistochemically by several groups (Axelson *et. al.*, 1992, Herbison & Theodosis, 1992a, b, Yuri & Kawata, 1992). However, Ramm *et al.* (1988) have suggested that the preferential localization of ERs in the nuclei obtained with immunohistochemical techniques is due to a loss of loosely bound receptor in the cytosol during fixation step. Recently, the presence of ER immunoreactivity in cytoplasm and neurites has been reported in the rat (Blaustein, 1992, 1993), guinea pig (Blaustein *et. al.*, 1992) and sheep (Lehman *et. al.*, 1993). Using the antibody AS-408, the existence of ER immunoreactivity outside of the cell nucleus was also observed in a few cases (Fig. 3-9). However, at the present it is not convincing whether this staining is reflecting the presence of true ER protein or it is false positive due to some experimental procedures, because the cytoplasmic staining was observed only in a small number of sections and it has never occurred in paraffin sections.

In summary, *in situ* hybridization technique using the cRNA probe biochemically transcribed from the rat cDNA was established, which allows to detect ER mRNA in the specific cells in the rat. Employing the same cDNA, the polyclonal antibody to the rat ER was produced. Several lines of evidence demonstrate that this antibody directs exclusively to the ER. By applying the antibody to immunohistochemistry, it was found that areas known to be involved in some aspect of reproductive and ingestive functions, such as the MPOA, VMN and LHA, contain relatively large populations of ER immunoreactive cells. Thus, these ERs may participate in the control of expression and coordination of reproductive and ingestive behaviors.

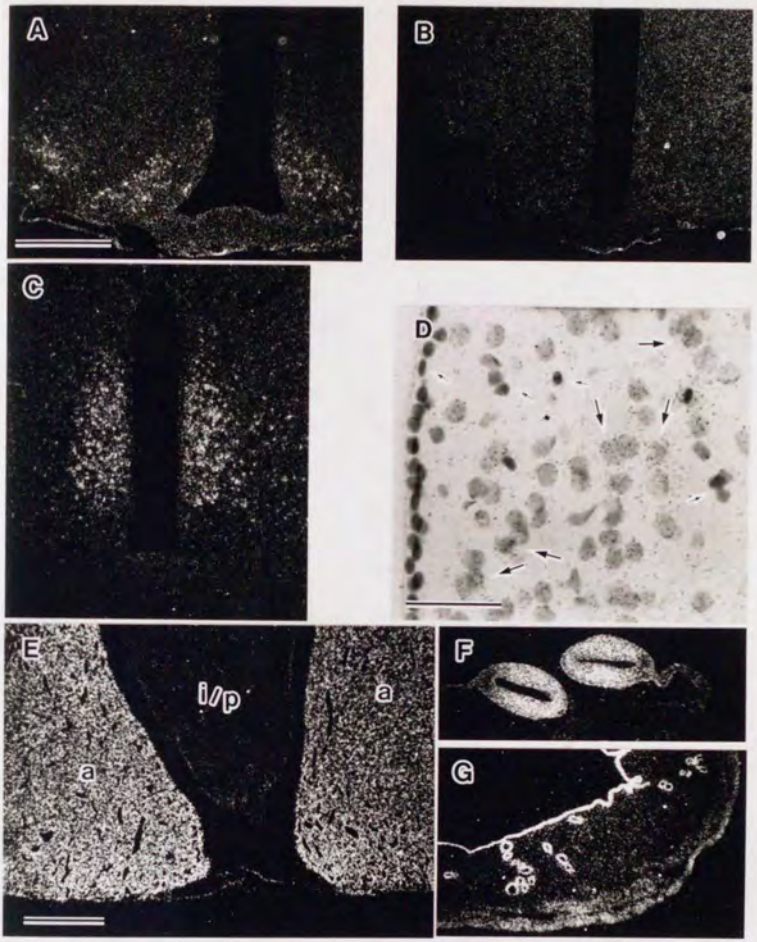


Figure 3-1. ***In situ*** **hybridization histochemistry in the brain, pituitary and uterus.** **A, B**: dark-field photomicrograms of sections in the medial basal hypothalamus hybridized with the antisense (**A**) or sense (**B**) probes. **C**: section containing the AVPv. **D**: bright-field photomicrogram of **C**. **E**: the pituitary section. a= anterior lobe, i/p= intermediate and posterior lobes. **F, G**: sections from 5-days old (**F**) and adult (**G**) rats uterus. It can be seen in **D** that some neurons (large arrows) are identifiable as ER neurons by dense silver grains, while the ependymal and glia-like cells (small arrows) are not labeled. Scale bars = 300 μm (**A, B, C, F, G**), 100 μm (**E**), 30 μm (**D**).

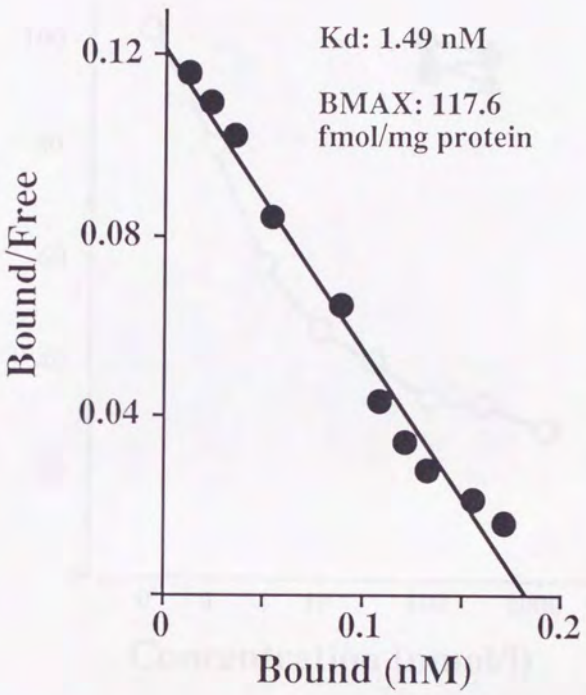


Figure 3-2. Scatchard analysis of [^{3}H]estradiol binding to the fusion protein. Lysate (400 μg protein) of cells containing the β-galactosidase-ER fusion protein was incubated with increasing concentrations of [^{3}H]estradiol in the absence (total binding) or in the presence (nonspecific binding) of a 100-fold excess of unlabeled ligand for 20 min at 37 °C. Nonspecific binding was 12.4-26.1% of the total radioactivity added. The binding data were analyzed according to the method by Scatchard (Scatchard, 1949).

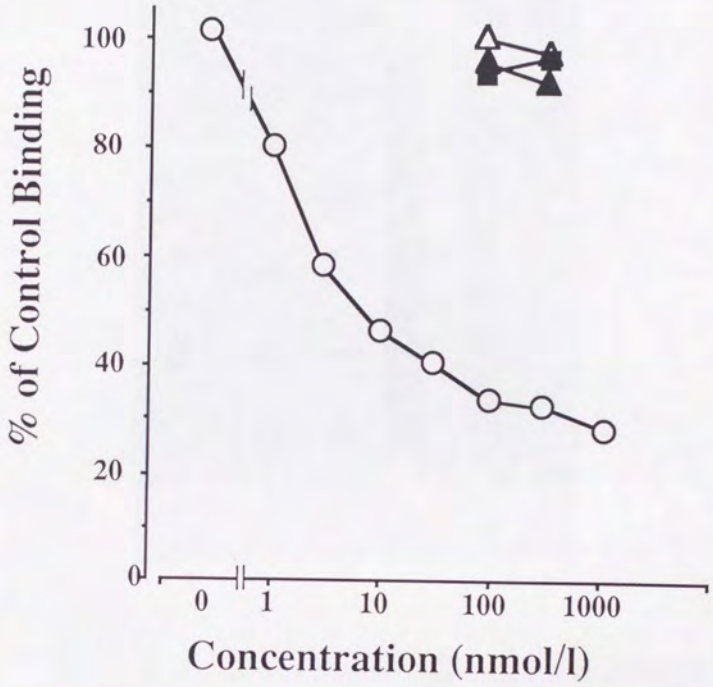


Figure 3-3. Effects of steroid hormones on [^{3}H]estradiol binding to the fusion protein. Lysate (400 μg protein) of cells containing the fusion protein was incubated with 10 nM of [^{3}H]estradiol in the presence of indicated amounts of unlabeled steroid hormones (open circles; estradiol, open triangles; progesterone, closed squares; corticosterone, closed triangles; testosterone). Specific binding of [^{3}H]estradiol, represented as a percentage of control binding (without steroid hormone), is shown.

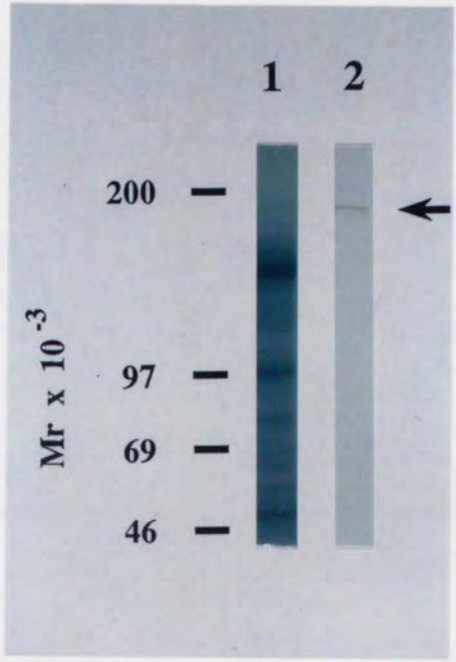


Figure 3-4. Immunoblot analysis of the fusion protein. The cell lysate (80 µg/lane was run on 7.5% SDS-PAGE under the reducing conditions, and electroblotted on a nitrocellulose membrane. The blot was stained with Amido Black (lane 1) or immunostained employing H222 monoclonal antibody (lane 2). Two bands were detected in lane 2, a major band at around 180K (arrow) and a minor one just below it. Relative molecular weights (Mr) of the standard proteins are shown on the left.

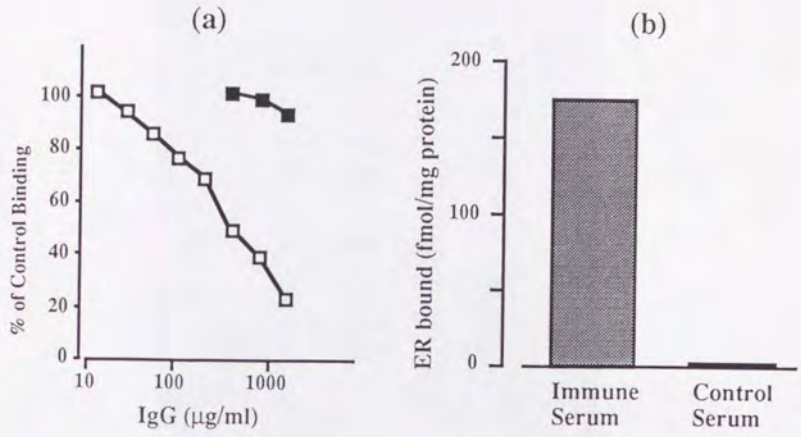


Figure 3-5. (a) **Effects of the antiserum on [^{3}H]estradiol binding to the ER in the rat uterus.** Cytosol fraction (400 μg protein) of the rat uterus was incubated with 10 nM of [^{3}H]estradiol in the presence of various concentrations of IgG purified from either the immune serum (open square) or the preimmune serum (closed square), and specific binding was determined as described under Materials and Methods. Specific binding, represented as a percentage of control binding (without IgG), is shown. Control specific binding and nonspecific binding were 3.9 % and 1.0 %, respectively.

(b) Binding of the antiserum to estradiol-ER complexes.

Cytosol fraction (400 μg protein) of the rat uterus was incubated with 10 nM of [^{3}H]estradiol for 3 hrs at 4 °C. The mixture was incubated with IgG (800 μg) obtained from the antiserum or the preimmune serum and further incubated at 4 °C overnight. The hormone-receptor-antibody complexes were isolated using a Protein A-Sepharose CL-4B column, and radioactivity was measured. Each values represents the mean of duplicate determinations.

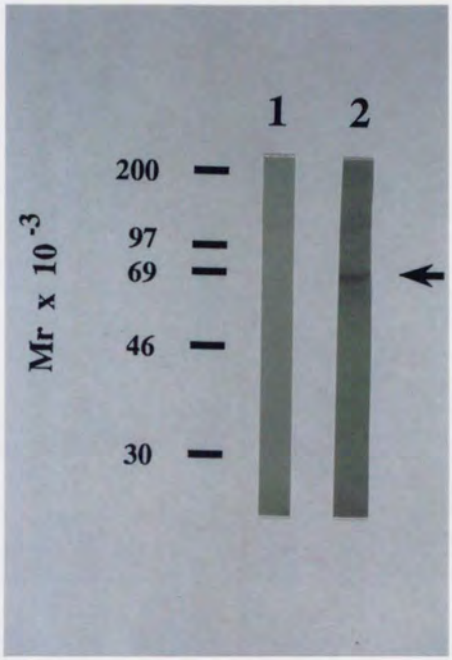


Figure 3-6. Immunoblot analysis of the ER in the rat uterus using AS-408.

Cytosol fractions (16 μg/lane) were subjected 10% SDS-PAGE under the reducing conditions, and blotted onto the membrane. The blot was incubated with the preimmune serum (lane 1) or AS-408 (lane 2) at a dilution of 1: 5000 for 2 hrs at 4 °C, and was further incubated with ^{125}I-labeled F(ab')$_2$ fragment of anti-rabbit IgG (2 μCi/ml) for 2 hrs at room temperature. After extensive washing, the membrane was dried and autoradiographed at -80 °C for 18 hrs. A single band at 67K was detected in lane 2 as indicated by an arrow.

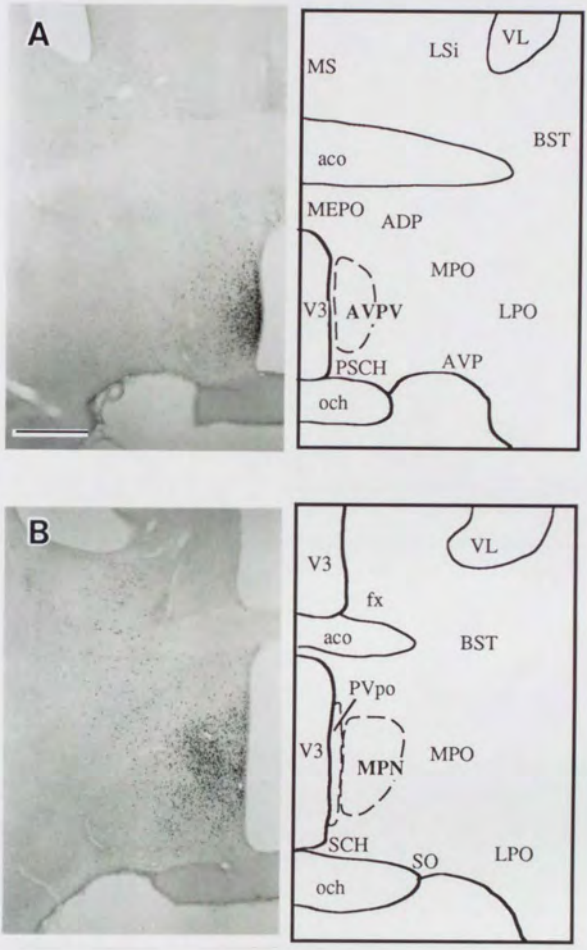
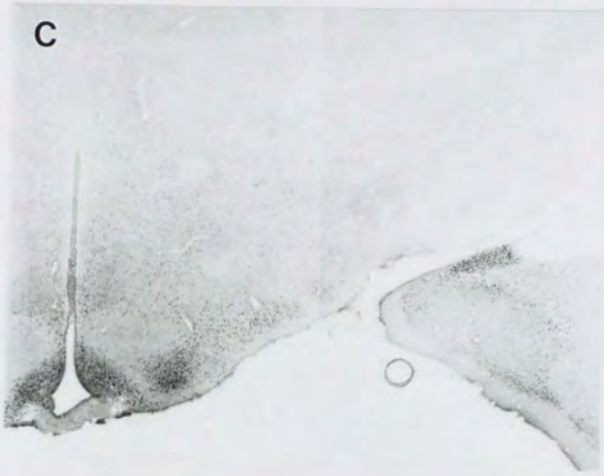


Figure 3-7. Photomicrograms of sections processed for ER immunohistochemistry and schematic illustrations. Sections containing the AVPv (**A**), MPN (**B**) and VMN (C) with 30 μm thickness were processed for immunohistochemistry using AS-408. Positive reaction products for ER are seen as black dots.

Scale bar = 300 μm. For abbreviations see page xii.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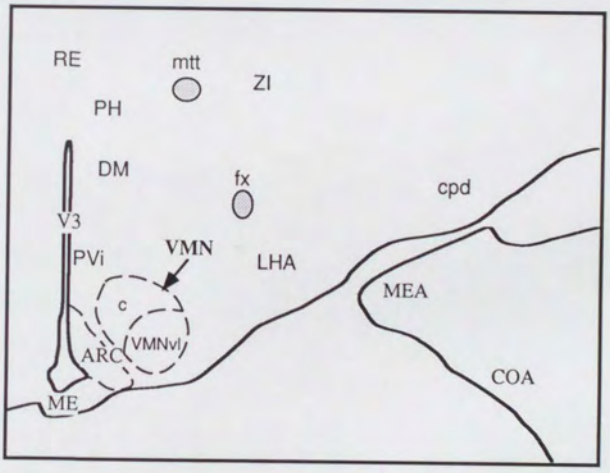
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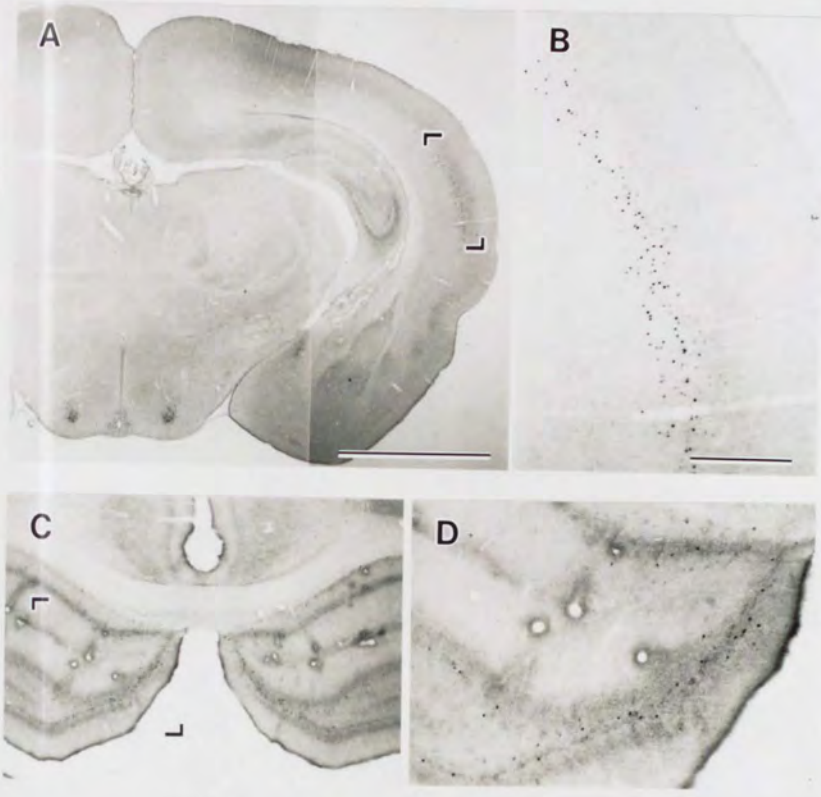

Figure 3-8. ER-ir neurons outside the hypothalamus. **A**: transiently expressed ER-ir in the layer V of the auditory cortex of 10-days old rat. **B**: a large magnification of **A**. **C**: ER-ir in the hippocampus of adult rat. **D**: a large magnification of **C**. Free floating sections with 30 μm thickness (**A, B**) or vibratome sections with 50 μm (**C, D**) were processed for ER immunohistochemistry using AS-408. Scale bars = 1000 μm (**A, C**), 300 μm (**B, D**).

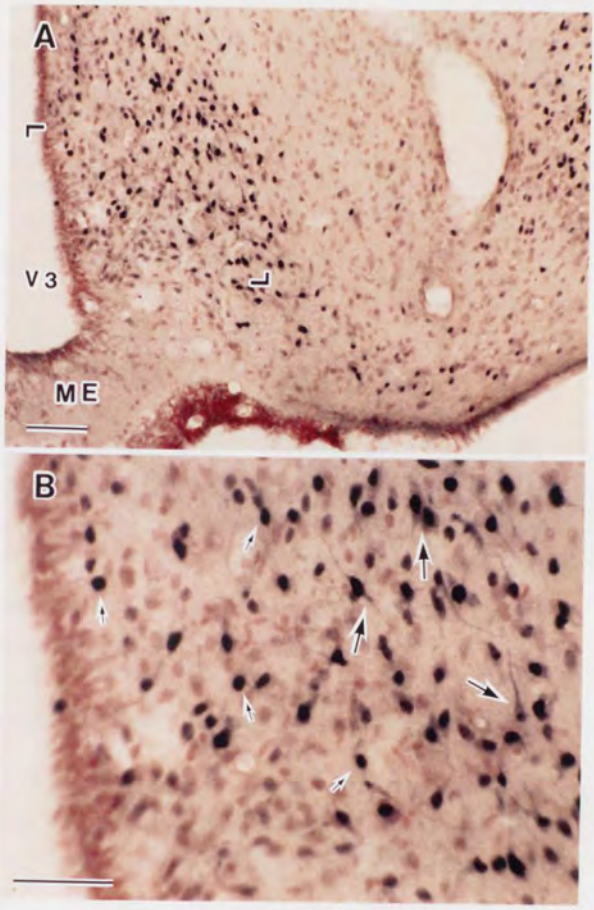

Figure 3-9. ER-ir in the cytosol and neurites of cells in the ARC.

Free floating sections with 30 μm was processed for ER immunohistochemistry. The section was counter stained with Neutral Red (**A**). In a large magnification (**B**), while most ER-ir localized in cell nuclei (small arrows), some cytoplasmic staining (large arrows) were also observed. However, they occurred only in a few cases. Scale bars = 100 μm (**A**), 30 μm (**B**).

Chapter 4. Applications of Polyclonal Antibody AS-408 to Immunohistochemistry

4-1. Regulation of Estrogen Receptor by Estrogen and Identification of Chemicals in Estrogen Receptor Containing Cells

4-2. Induction of Substance P-Immunoreactivity by Estrogen in Female Rats

4-1. Regulation of Estrogen Receptor by Estrogen and Identification of Chemicals in Estrogen Receptor Containing Cells

Summary

Effects of estrogen on numbers of neurons containing ER in the AVPv and VMN, and chemical specificity of cells with ER in the brain and the anterior pituitary gland were examined by immunohistochemistry employing the antibody specific for ER.

In female ovx rats (female control group), a large population of ER-ir cells were found clustered throughout the AVPv. They were counted more than 2,000 in total of four sections in this nucleus. Administration of EB to ovx female animals decreased the total number of ER-ir cells to 67% of the control group. In females received subsequent injections of EB and progesterone, the number of ER-ir neurons also decreased to 79% of the control group. Male gonadectomized group contained a smaller population of ER-ir cells relative to the female ovx group. Administration of EB to the gonadectomized male also decreased the number of ER-ir cells in a manner similar to that in the female. In the VMN, while ER-ir cells were observed throughout the rostro-caudal extent of this nucleus, sections from the caudal portion had the largest number of positive neurons. When compared at this level of the VMN, it was found that the number of ER-ir neurons varies depending on the circulating levels of estrogen. In ovary intact animals, the highest value was observed during proestrous and the lowest one during estrous. The treatment of ovx animals with EB decreased the population of ER-ir neurons by approximately 66% of the control group as in the AVPv.

By double-labeling immunohistochemistry it was revealed that relatively large populations of neurons with TH-ir in the ARC and ENK-ir in the VMN contain ER-ir in their nuclei, while very few END-ir co-exist with ER-ir in the ARC. In the septo-preoptic area, ER-ir was not observed within LHRH-ir cell bodies under any hormonal conditions in both sexes. In the anterior pituitary gland of ovx rats, ER-ir existed in the most of gonadotropes and lactotropes, in some somatotropes, and in a few thyrotropes.

The results demonstrate that there exists a sexual dimorphism in the number of ER-ir neurons in the AVPv and that estradiol reduces the number of ER-ir neurons in the AVPv and VMN in a similar extent. Since contents of ER in given tissues could influence sensitivity of those tissues to estrogen, the sexual dimorphism in the number of ER and estrogenic regulation of ER may contribute to activity of estrogen sensitive neural circuitry. In addition, the data of double-labeling immunohistochemistry suggest that pituitary hormones and some neurotransmitters but not LHRH in the brain are directly regulated by estrogen in ER containing cells.

Introduction

In the previous chapter, it was found that large numbers of ER containing cells concentrate in specific nuclei, such as the MPOA and VMN by *in situ* hybridization and immunohistochemistry. Because these nuclei are known to be essential anatomical substrates for controlling gonadotropin secretion (Kalra, 1993), and sexual (Pfaff, 1980) and ingestive (Brobeck, 1941) behaviors, and because they are modulated by estrogen (Kalra, 1993, Pfaff, 1980, Tarttelin & Gorski, 1973), it is possible that the ERs in the MPOA and VMN contribute to changes in these functions. Therefore, a mode of regulation of ER and a chemical specificity of ER containing cells were investigated by immunohistochemistry.

Because the physiological response to estrogen requires that the ER be present, the concentration of ER protein is supposed to be a major determinant of the sensitivity of neurons. Several neurotransmitters and neuroactive drugs have shown to modulate estrogen action by regulating synthesis and/or degradation of the receptor (for a review, Nock & Feder, 1981). For example, the muscarinic cholinergic agonist, bethabechol, increases the number of hypothalamic estradiol binding sites in ovariectomized rats (Lauber & Whalen, 1988), and agents which interact with dopaminergic (Thompson *et. al.*, 1983) and noradrenergic (Blaustein, 1987, Tetel & Blaustein, 1991) systems alter the concentrations of ER in the hypothalamus.

Among numerous mechanisms concerning to the regulation of ER, action of

homologous ligand, estrogen, on the ER levels is thought to be of primary importance. Treatment of female rats with estradiol increases ligand binding in the brain tissue, as determined by nuclear exchange assay (Brown *et. al.*, 1988, Lustig *et. al.*, 1989). On the other hand, a recent study by *in situ* hybridization has reported that ovariectomy increases ER mRNA expression in the hypothalamus compared with that in intact animals (Lauber *et. al.*, 1990). When ovx animals were treated with estrogen, a time-dependent reduction of ER mRNA expression was observed (Lauber *et. al.*, 1991, Simerly & Young, 1991). Moreover, levels of ER mRNA vary during the estrous cycle and the magnitude of change observed during the estrous cycle are region specific (Shughrue *et. al.*, 1992). By immunohistochemistry, changes in ER-ir in response to estrogen treatment have been shown using H222 monoclonal antibody (Koch, 1990, Blaustein, 1993) or other antibodies (Blaustein, 1993). Because polyclonal antibody AS-408 recognizes both occupied and unoccupied forms of ER as described in Chapter 3, it might be useful to identify changes in ER-ir under a variety of hormonal conditions.

On the other hand, one essential step in determining how estrogen influences cellular functioning is to elucidate chemicals synthesized in estrogen target cells. In this regard, a number of substances have been examined as summarized in Table 1-1. Here, LHRH, TH, an enzyme responsible for dopamine synthesis, and opiates were examined whether they co-localize with ER in neurons of the MPOA, ARC or VMN by double-labeling immunohistochemistry instead of the previously used autoradiographic technique. All of these substances have been implicated in estrogen-regulated neuroendocrine functions (Kalra, 1993, McCann & Krulich, 1981, Sirinathsinghji, 1983, Pfaus & Gorzalka, 1988).

Outside of the central nervous system, one of major target organs for estrogen is the anterior pituitary gland. Estrogen is thought to regulate pituitary hormone secretion through its hypothalamic actions as well as by acting directly at the level of the pituitary (Karsch, 1984). The anterior pituitary gland consists of many different cell types classified on the basis of their size, shape, and histological staining characteristics. In order to identify the cell types upon which estrogen directly acts, double-labeling immunohistochemistry was performed on paraffin sections from the ovx female rat using antibodies to ER, and FSH, LH , PRL, GH and TSH.

Materials and Methods

Animals

The number of ER-ir neurons in the MPOA and VMN were evaluated in different groups, respectively. For the evaluation of the MPOA, sections from animals used for double-labeling for ER and SP in the later section (Chapter 4-3) were utilized. They were comprised of female control (n=4), female EB (n=6), female EB+P (n=5), male control (n=3), and male EB (n=5) groups, and all animals received a colchicine injection (Chapter 4-3). Anatomical levels of the AVPv in which the numbers were counted are shown in Fig. 4-2.3. On the other hand, for the evaluation of ER-ir population in the VMN, ovary intact and ovx rats were used. In the cycling rat, animals (two rats in each stage) were perfused on the afternoon of proestrus, estrus and diestrus. The ovx animals were injected either EB (n=4) or sesame oil (n=4) in the morning of day 1 and day 2, and sacrificed on the afternoon of day 4. All animals were perfused with 4% paraformaldehyde in 0.1 M PB, pH 7.4, immersed in the same fixative. Thereafter, sections from the cycling rats were processed for ER immunohistochemistry by the ER staining method as described in Chapter 2, while those from the ovx animals were processed by the method for PR staining using AS-408. Thus, the numbers of ER-ir cells between two groups can not be accurately compared each other.

Double-labeling immunohistochemistry for LHRH and ER was performed in free floating sections from the cycling rat as well as gonadectomized and gonadectomized EB-treated male and female rats. Co-localization of TH, END or ENK with ER was examined in free floating sections from ovx rats treated with colchicine (Sigma, 50 μg/ 5 μl saline) 24 hrs prior to perfusion, while that of pituitary hormones (FSH, LH, PRL, GH, TSH) with ER was investigated in paraffin sections from ovx rats received no colchicine.

Results

Regulation of ER-ir by estrogen

The AVPv: In the MPOA, effects of estradiol on the number of ER-ir neurons were examined in gonadectomized females and male rats. Although a large population of ER-ir neurons were observed throughout the AVPv and adjacent MPN as shown in Fig. 3-7, the number of ER-ir was counted only in the AVPv. Labeling was consistently restricted to the cell nucleus. A few of ER-ir cells were also found scattered in the lateral and dorsal areas adjacent to the AVPv, but they were clearly distinguishable from the clusters of positive cells in the nucleus. While there was no distinct difference in their distribution between sexes, the male control group showed a significantly smaller population of ER positive cells than the female control group (1497±166 *vs.* 2143±260 in total, $p<0.05$). The difference was predominant at the caudal half of the nucleus (437±28 *vs.* 713±85, $p<0.05$ at section 3 and 605±37 *vs.* 890±111, $p<0.05$ at section 4 in Fig. 4-2.3) (Fig. 4-1.1).

Administration of EB to the female rat resulted in a decrease in recognizable ER-ir cells (Fig. 4-1.1). The reduction was observed in all four sections being predominant at the caudal half of the AVPv. When compared with the corresponding values in the control group, the numbers of ER neurons in the EB-treated group decreased to 67% (477±115, $p<0.05$) at the level of section 3, 59% (526±122, $p<0.05$) at section 4, and 67% (1435±308, $p<0.05$) in total. The down-regulation by estrogen also occurred in the male in a manner similar to that in the female. The EB-treated male rats contained 41% and 31% fewer ER-ir cells than the male control group at the levels of section 3 and 4, respectively (258±73 *vs.* 437±28 and 416±83 *vs.* 605±37, respectively, $p<0.05$).

Whereas the numbers of ER-ir cells in the EB+P group tended to exceed those in the EB+Oil group (Fig. 4-1.1), there was no statistically significant difference between them. When compared to the control rats, the EB+P group showed a slightly smaller population of ER-ir cells (79% of the control in total), and a significant difference was observed only at the level of section 3 (529±75 *vs.* 713±85, $p<0.05$). In the present study no distinction between weak and strong staining was made when the positive

neurons were counted, but in general, the intensity of labeling was the highest in the control and the lowest in the EB+Oil groups.

The VMN: Sections from the proestrous rats were firstly examined in order to determine the distribution of ER-ir in the VMN. As shown in Fig. 4-1.2, while ER-ir was observed throughout the rostro-caudal extent of the VMN, its number differed among each level with two peak values at rostral and caudal portions. Because the caudal peak was higher and sharper than the rostral one, and can be easily identified by its appearance (see Fig. 4-1.4A), following data evaluations in the VMN was performed at this level (section No. 10 in Fig. 4-1.2). Within the VMN, while few ER-ir cells were found in the dorsal and central portions, positive cells were exclusively localized in the ventrolateral portion of the nucleus at any level.

A variation in the population of ER-ir cells was found during the estrous cycle (Fig. 4-1.3). Although statistical comparison could not be made due to a small animal number (n=2 in each stage), the number of ER-ir cells in the proestrous rats (402.6: average of two animals) appeared to be higher than that in the estrous animals (323.6), and that in the diestrous rats showed an intermediate value (370.6).

The EB treatment of ovx animals reduced the number of ER-ir neurons in the caudal portion of the VMN (Fig. 4-1.3) as in the AVPv (Fig. 4-1.1). The difference in the number of ER-ir between OVX and OVX+EB groups was statistically significant (276 ± 79 *vs.* 181 ± 67, $p<0.05$). The reduction was observed throughout the rostro-caudal extent of the VMN. Although EB reduced ER-ir in the ARC and LHA too, the suppressive effect of estradiol appeared to be greater in the VMN than other two nuclei (Fig. 4-1.4), suggesting a regional difference in the responsiveness to estrogen.

Co-localization of chemicals in ER containing cells

As expected or disappointedly, AS-408 failed to show any evidence for co-localization of ER within LHRH neurons under any hormonal conditions in both sexes. Representative sections from the EB-treated female rat are shown in Fig. 4-1.5. It can be seen that distributions of ER (arrow heads) and LHRH (large arrows) neurons in the anterior preoptic area are close but apparently separated each other (Fig. 4-1.5A,B). In

contrast, some LHRH fibers in this area coursed in direct proximity to ER cells (Fig. 4-1.5C, D, small arrows) in the AVPv.

Representative sections double-stained for ER and TH, ENK or End are shown in Fig. 4-1.6. A number of TH positive cells were distributed in the ARC but not in the VMN in the ovx rat (Fig. 4-1.6A). In a higher magnification, it can be seen that a substantial population of those neurons contain ER-ir in their cell nuclei (Fig. 4-1.6B, arrows). Although distribution of END-ir overlapped to that of ER-ir, occurrence of co-existence of both materials (arrows) was very low (Fig. 4-1.6C). When counted on several sections, it was roughly estimated to less than 3%. In the EB-treated female, dense fibers with ENK-ir throughout the VMN and ENK positive cell bodies in the ventrolateral portion of the nucleus were observed (Fig. 4-1.6D), and the majority of these neurons also contained ER-ir (arrows).

In the pituitary, ER-ir localized only in the anterior lobe but not in the intermediate or posterior lobes (Fig. 4-1.7A). It can be seen from Fig. 4-2.3 that ER exists in all cell types examined, while the relative degree of co-localization is distinct for each cell type (Figs. 4-1.7B-F).

Discussion

The number of ER-ir neurons exhibited a sexual dimorphism in the AVPv. Castrated male rats contained fewer labeled neurons than ovx females (see Table 4-2.1). The sex difference in labeled estrogen binding sites (Rainbow *et. al.*, 1982, Brown *et. al.*, 1988, Bloch *et. al.*, 1992) as well as mRNA for ER (Lauber *et. al.*, 1991, Simerly & Young, 1991, Shughrue *et. al.*, 1992) have been demonstrated in the rat. While in the present study it was evaluated that in the control group the population of ER-ir cells was 1.4-fold larger in females relative to that in males, Bloch *et al.* (1992) have reported a more predominant sex difference in the number of estrogen concentrating cells in the AVPv (3.3-fold greater in the female than the male). The difference between these two experiments might be due to difference in the method used (autoradiography *vs.* immunohistochemistry) or due to different areas selected for data evaluation. By immunohistochemistry employing the monoclonal antibody H222, Herbison and

Theodosis (1992a) have failed to show a significant sex difference in the number of labeled cells in the AVPv/MPN but they also have observed slightly larger population of ER-ir neurons in the female (see results in Herbison & Theodosis, 1992a). In the present study, all animals received colchicine in order to enhance immunoreactivity for SP in cell bodies as presented in Chapter 4-2. Since it has been reported colchicine suppresses ER-ir (Herbison & Theodosis, 1992a,b, Yuri & Kawata, 1992), a possibility cannot be denied, although unlikely, that colchicine influenced differently between sexes on the number of ER-ir cells. The abundance of ER-ir neurons in the female may account for a sex difference in the responsiveness of neurons to hormonal activation in the MPOA (Luine & McEwen, 1983, Brown *et. al.*, 1990), and thus contribute to sexually different functions including feeding, maternal, and copulatory behaviors (Wade, 1976, Clemens & Glaude, 1979, Larson, 1979, Numan, 1985).

Injection of estradiol to the castrated animals resulted in not only lowered signal intensity of labeled cells but also more than 35% decrease in the number of ER-ir neurons in the AVPv both in the female and male rats (see, Table 4-2.1) and in the VMN of the female (Fig. 4-1.3,4). This is consistent with the previous findings (Koch, 1990, Herbison & Theodosis, 1992a, Blaustein *et. al.*, 1992, Blaustein, 1993) showing the reduction of ER-ir after the estrogen treatment. It also has been demonstrated that estrogen down-regulates not only mRNA for ER (Lauber *et. al.*, 1990, Lauber *et. al.*, 1991, Simerly & Young, 1991) but also the receptor protein content (McGinnis *et. al.*, 1981, Brown *et. al.*, 1988, Lustig *et. al.*, 1989) in the rat brain. Blaustein (1993) has reported for other antibodies a reduced immunoreactivity of the ER occupied with estrogen, and has suggested that the decrease in cell nuclear ER-ir is due at least in part to a conformational change in the receptor. Although the antibody used in the present study recognizes both occupied and unoccupied forms of the solubilized ER (Chapter 3), it is possible that in tissue sections from perfused animal it failed to detect the occupied ER. Thus, observed decrease in the number of ER-ir cells could be reflecting following two effects of estrogen treatment on the ER: (i) a conformational change in the receptor molecule or steric hindrance evoked by the steroid occupation; (ii) suppression of synthesis of ER molecules and/or enhancement of their degradation. The result that the reduction in the number of ER-ir neurons occurred in a similar manner in both female and male suggests a common mechanism of ER regulation in the AVPv. Lauber *et al.*

(1991) have reported, however, that estrogen does not down-regulate ER message levels in the male hypothalamus. This discrepancy may be due to a regional sex difference in the regulation of ER. Regionally specific effects of estrogen on ER mRNA (Lauber *et. al.*, 1990, Shughrue *et. al.*, 1992) as well as regional sex differences in estrogen binding capacity (Brown *et. al.*, 1988) have been reported.

Down-regulation of steroid receptors by homologous ligand has been well documented for progesterone (Read *et. al.*, 1988) and thyroid hormone (Lazar & Chin, 1988), along with tissue-specific down-regulation of glucocorticoid receptor (Kalinyak *et. al.*, 1987). Positive regulation has been observed for vitamin D receptors, which are increased after vitamin D treatment of chickens (McDonnell *et. al.*, 1987)

In the ovary intact female, a phase-dependent change in the number of ER-ir neurons in the VMN were observed with the highest value during proestrus, which is consistent with the previous report showing increased levels of nuclear binding of [^{3}H] during proestrus in cycling animals (McGinnis *et. al.*, 1981). Shughrue *et. al.* (1992) also have demonstrated a change in ER mRNA expression during the estrous cycle, and that the expression of mRNA is the highest during proestrous in the VMN. All of these data indicate enhanced ER levels during proestrous when circulating estrogen levels increase (Butcher *et. al.*, 1974). This regulation pattern of ER by estrogen is in contrast to that in the ovx rat, *i.e.* when the ovx rat treated with estrogen, both mRNA (Shughrue *et.al.* 1992) and protein for ER (Fig. 4-1.3) decreased. The discrepancy in the action of estrogen between two groups implies that a more complex mechanism of action may be present in the ovary intact animal.

Because the staining intensity of a labeled cell by immunohistochemistry can be correlated its content of ER protein, observed fluctuation in the staining and the number of ER-ir in response to circulating estradiol levels might be responsible for the change in ER protein concentrations. The levels of ER in given tissues could influence sensitivity of those tissues to estrogen. Thus, the regulation of ER and the sexual dimorphism in the number of ER may contribute to actions of estrogen on neuroendocrine functions. The MPOA and VMN have been implicated in the hormonal regulation of gonadotropin secretion and reproductive behavior (Kalra, 1993, Pfaff, 1980). Although in the female rat, administration of estrogen is capable of inducing the preovulatory LH surge, estrogen is unable to elicit a similar effect in the male (Brown-Grant, 1974). Similarly, activation

of the female pattern of reproductive behavior in the male requires a much higher dose of estrogen than is required in the female (Goy & McEwen, 1980, Pfaff, 1980). Other estrogen-regulated sexually differentiated behaviors including feeding (Wade, 1976), maternal (Numan *et. al.*, 1983), and male copulatory (Davis & Barfield, 1979) behaviors, may be controlled by these brain regions.

As described in Chapter 1, estrogen plays a critical role in the occurrence of the LH surge in the afternoon of proestrus. Because the release of LH from the pituitary is strictly regulated by LHRH (Sarkar *et. al.*, 1976, Levine & Ramirez, 1982), the primary action of estrogen might be on the synthesis and/or release of LHRH. However, it has been shown that LHRH neurons contain neither estrogen binding sites (Shivers *et. al.*, 1983) nor ER-ir (Herbison & Theodosis, 1992, Lehman & Karsch, 1993). Nevertheless, a few questions in terms of this important issue had remained to be answered. Since autoradiography requires to use only gonadectomized animals, it was possible that ER may appear in LHRH neurons depending on the circulating estrogen levels. Since polyclonal antibody AS-408, seems to be more powerful than the previously used monoclonal antibody, H222, (Herbison & Theodosis, 1992, Lehman & Karsch, 1993) in detection of ER in fixed tissues as discussed in Chapter 3, this antibody was expected to detect a scarce amount of ER within LHRH neurons. Using cycling female rats and gonadectomized EB-treated animals, these possibilities were re-examined. However, no evidence that suggests a direct interaction of estrogen on LHRH neurons was obtained under any hormonal conditions in both sexes. The results support the previous hypothesis that certain populations of neurons in the MPOA must monitor ovarian steroid levels and relay this information on the LHRH neurons (Herbison & Theodosis, 1992). In contrast, some LHRH-ir fibers coursed in direct proximity to ER cells, this is comparable to the result by Langub *et. al.* (1991). They have reported abundant appositions of LHRH-ir varicosities on ER-ir neurons, and suggested that LHRH synaptic input to ER neurons may be a means by which feedback information can be conveyed between the two systems (Langub *et. al.*, 1991).

One essential step in determining estrogenic actions in the brain is to identify neurochemicals used by neurons with ER. In this regard, three substances were examined whether they exist in ER neurons. A relatively large population of TH positive cells were found to contain ER. Although the number of double-labeled neurons was not

counted in this study, the extent of co-localization seems to be similar to the reported value (30%) using tritium-labled estradiol binding in the ovx rat (Sar, 1984) but different from the value in the ewe (3-5%) (Lehman & Karsch). Since dopamine β-hydroxylase does not exist in the ARC (Sar, 1984), the observed co-existence of TH- and ER-irs in the same neurons indicates that estrogen directly affects activity of the dopamine system. Dopamine is most commonly characterized as a neurotransmitter that inhibits LH and PRL, and female sexual behavior in rodents (for reviews, Crowley & Zemlan, 1981, McCann & Krulich, 1981), and thus actions of estrogen on those physiological responses may be, in part, mediated by dopamine.

Previously, Morrell *et. al.* (1985) have reported that 4% of the END-ir neurons in the basal hypothalamus of ovx rats concentrate estradiol in their nuclei, while Jirikowski *et. al.* (1986) have shown to be 25% using the same technique. The present result support the former report. In spite of the fact that END is a potent mediator of estrogen actions on the gonadotropin secretion (Schulz *et. al.*, 1981) and lordosis behavior (Sirinathsinghji, 1983), the ratio of co-localization of END and ER was very small. This implies that a major part of action of estrogen on this opiate could be mediated through other ER containing cells in some regions.

Although ER has not yet been directly proved to localize in ENK neurons in the VMN, anatomical evidence to support this is available. At least 40% of neurons in the ventrolateral portion of the VMN contain ER (Morrell *et. al.*, 1986), and approximately 80-90% of neurons in this area contain proenkephalin mRNA (Romano *et. al.*, 1988). Therefore, some of ENK neurons must contain ER. This was directly demonstrated by double-staining in the present study. In the ventrolateral portion of the VMN, a number of ENK positive neurons were identified to contain ER in the EB-treated females but not in the ovx control animal, which received no estrogen. These results suggest that the synthesis of ENK is directly enhanced by estrogen in the VMN. It has been shown that the levels of proenkephalin mRNA in this nucleus increase 3.1 fold after 2 weeks of estrogen treatment (Romano *et. al.*, 1988). Since opioid δ-receptor-specific agonists facilitate lordosis behavior in the rat (Pfaus & Gorzalka, 1988) and ENK selectively binds to δ-receptors, estrogen-induced ENK could act to facilitate lordosis by binding to this receptor subtype.

ER-ir was also existed in various cells of the anterior pituitary gland. This result

is comparable with the previous study reporting that ^{3}H-estradiol binds to these four cell types with varying extent of labeling intensity (Keefer *et. al.*, 1976). The order of degree of co-localization was: gonadotropes ≥ lactotropes > somatotropes > thyrotropes. Under the present experimental condition, the ER was observed in almost all gonadotropes and lactotropes, suggesting a strong association of estrogen actions with synthesis and/or secretion of those hormone.

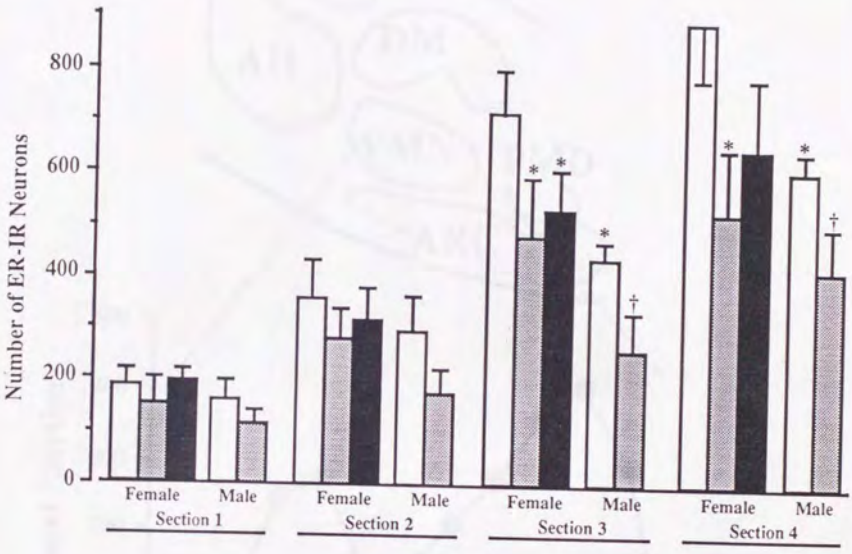

Figure 4-1.1. Effect of estrogen on numbers of ER-ir neurons in the AVPv of the female and male rats The numbers of ER-ir neurons were counted in four sections (sections 1-4) in each animal and represented as average values (± s.e.) as described in Materials and Methods. Open bars: Control group (female, n=4; male, n=3). Shaded bars: EB+Oil group (female, n=6; male, n=5). Closed bars: EB+P group (n=5). *, †: significant difference ($p<0.05$) compared with the female control group (*) or the male control group (†). Sections 1-4 are corresponding to those indicated in Fig. 4-2.3.

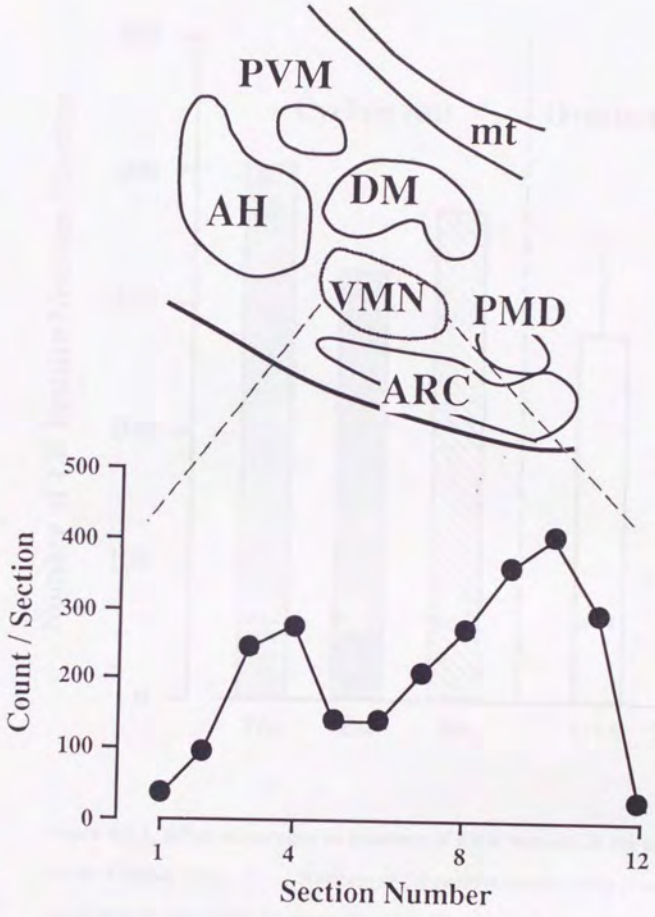

Figure 4-1.2. Numbers of ER-ir neurons in the VMN of proestrous rat.

Numbers of ER positive neurons were counted in two rats bilaterally. Twelve sections, separated each other by 90 μm, in the rostro-caudal extent of the VMN were analyzed, and data are represented as mean values. Schematic drawing of a sagital section of the hypothalamus including the VMN is shown in the upper position of Figure. See page xii for abbreviations.

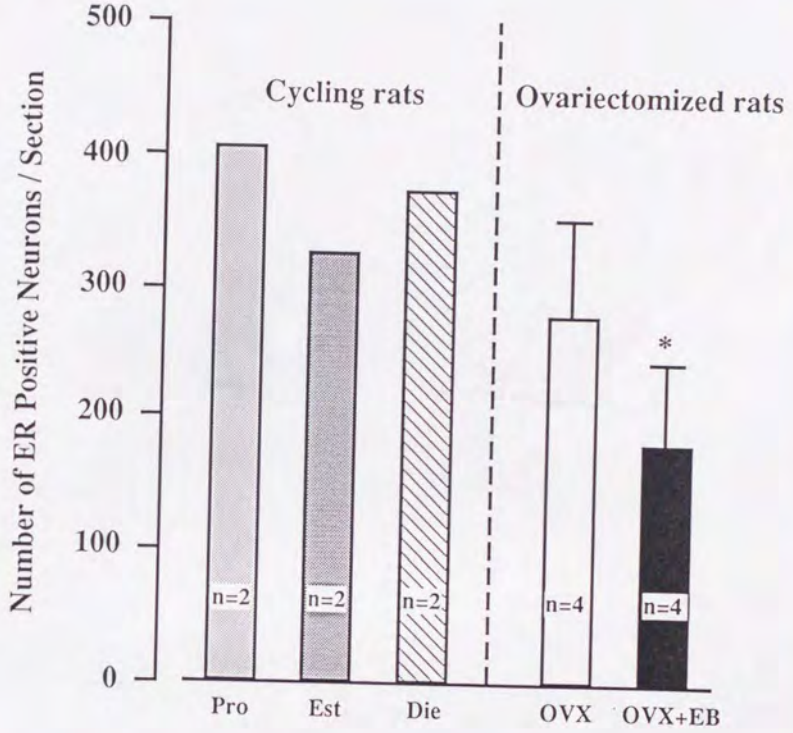

Figure 4-1.3. Effect of estrogen on numbers of ER-ir neurons in the caudal portion of the VMN of female rats. Numbers of ER positive neurons were counted bilaterally in the caudal portion (equivalent to section No. 10 in Fig. 4-1.2) of the VMN of cyclic (left side) and ovariectomized (right side) rats. Although all sections from the cyclic rats were processed simultaneously, those from ovariectomized rats were stained in separate run. Numbers in the bars represent number of animals in each group. Data are shown as mean (cycling rats) or mean ± s.e. (ovx rats). Pro = Proestrus, Est = Estrus, Die = Diestrus. The ovx rats were treated either oil or EB as described Materials and Methods. *: Stastically different ($p<0.05$) from OVX group.

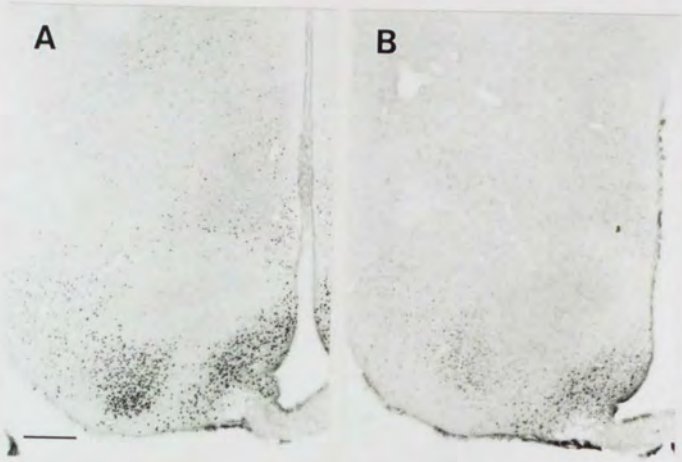

Figure 4-1.4. Effect of estrogen on ER-ir in the medial basal hypothalamus.
Free floating sections from female control (**A**) or EB-treated (**B**) rats were processed for ER immunohistochemistry. While estrogen reduces ER-ir in all areas in the medial basal hypothalamus, the reduction is most prominent in the ventrolateral portion of the VMN. Scale bar = 150 μm.

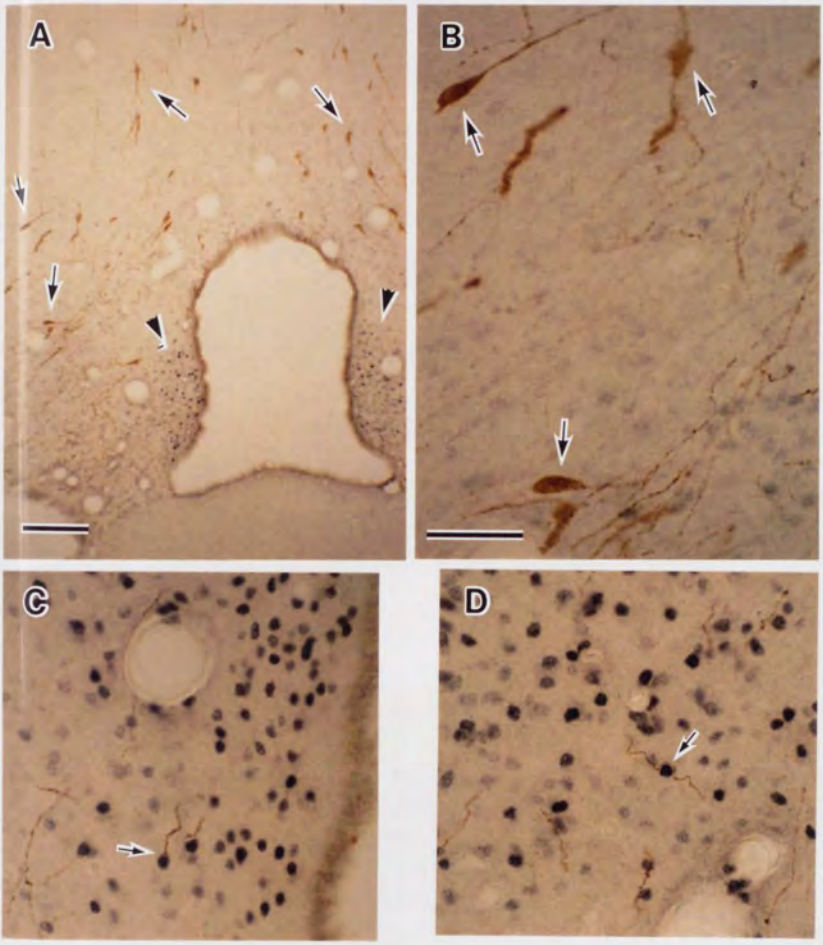

Figure 4-1.5. Double-labeling of ER- and LHRH-irs in the MPOA. Free floating sections of female rat treated with EB for 2 days were processed for double-labeling using AS-408 and anti-LHRH antibody (1:50,000) according to the method in Chapter 2. In the anterior preoptic area, distribution of LHRH neurons (large arrows) is close to but apparently different from that of ER neurons (blue black products, arrow heads)(**A**). In a higher magnification of **A**, it can be seen that ER-ir is not contained in cell bodies of any LHRH neurons (**B**). On the other hand, some LHRH fibers seem to appose on ER cells (small arrows)(**C, D**).
Scale bars = 200 μm (**A**), 30 μm (**B - D**).

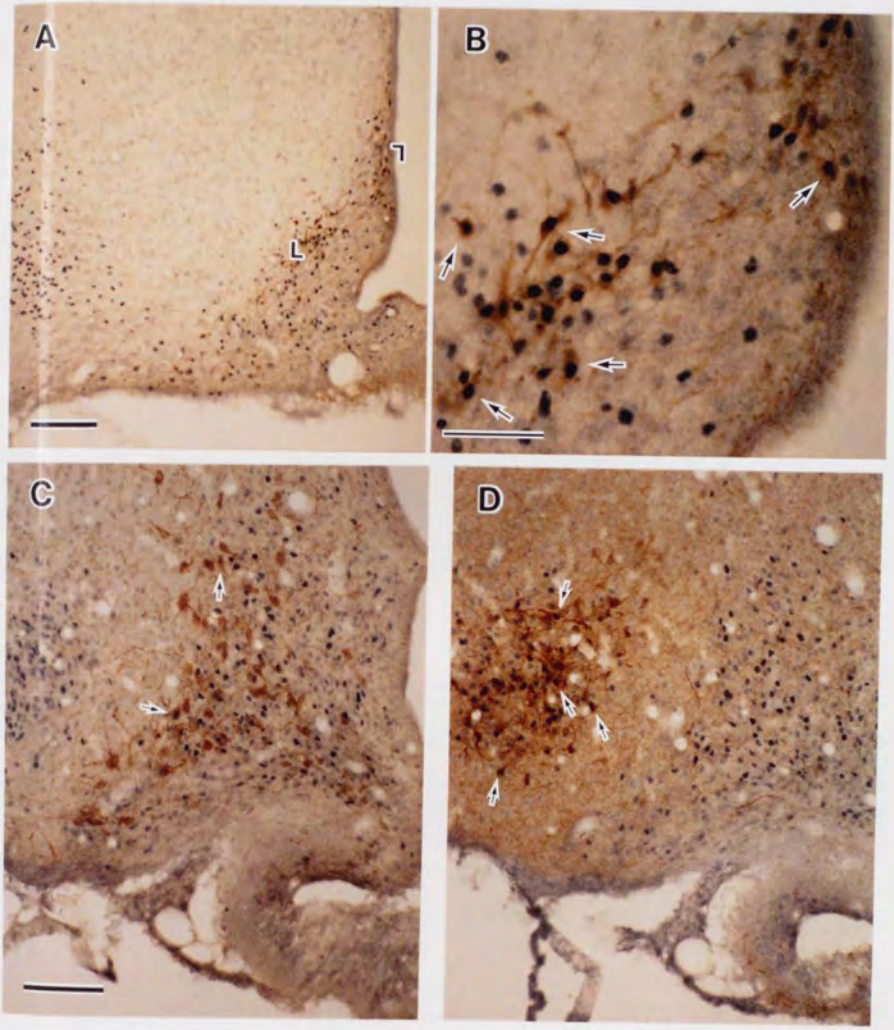

Figure 4-1.6. Double-labeling of ER- and TH-, END- or ENK-irs in the ARC and VMN. Free floating sections of female rats were processed for double-labeling immunohistochemistry using AS-408 and anti-TH (ovx rat, 1:60,000, **A**, **B**), anti-END (EB-treated rat, 1:30,000, **C**) or anti-ENK (EB-treated rat, 1:40,000, **D**). Arrows indicate double-labeled neurons. Scale bars = 200 μm (**A**), 30 μm (**B**), 100 μm (**C**, **D**).

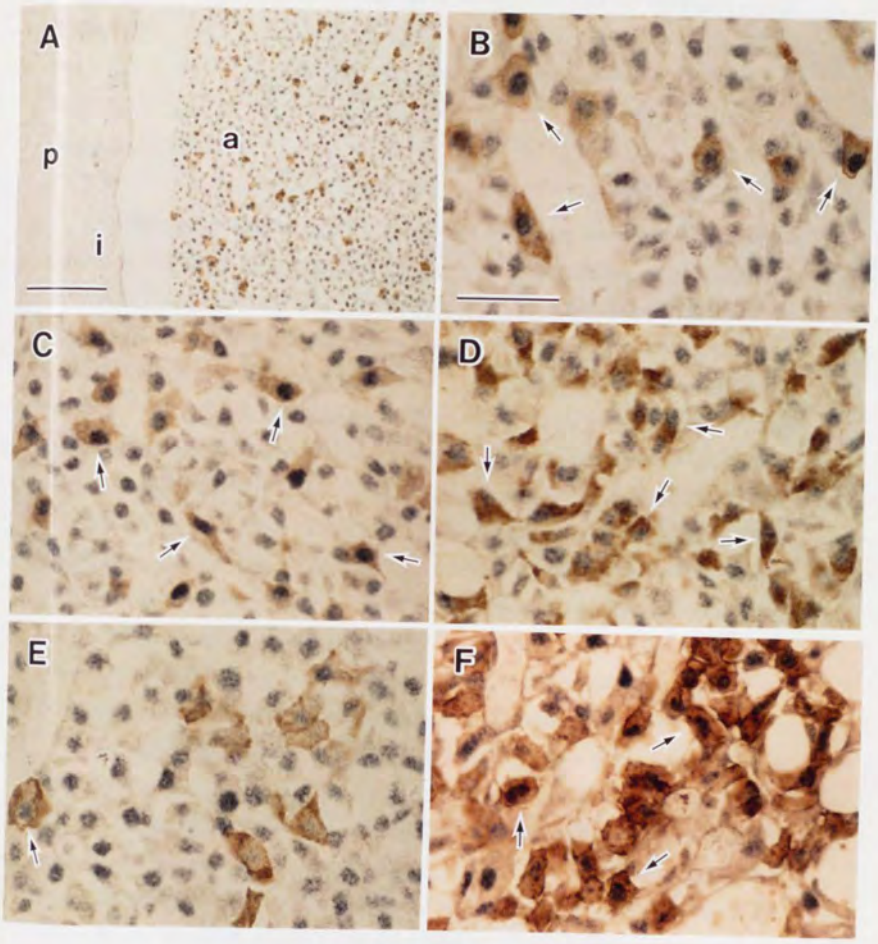

Figure 4-1.7. Double-labeling of ER- and FSH-, LH-, PRL-, TSH- or GH-irs in the female pituitary. Paraffin sections with 5 μm thickness from ovx rats were processed for double-labeling immunohistochemistry using AS-408 and anti-FSH (1:10,000, **A**, **B**), anti-LH (1:12,000, **C**), anti-PRL (1:12,000, **D**), anti-TSH (1:8,000, **E**) or anti-GH (1:6,000, **F**) as described in Chapter 2. While a majority of cells in the anterior lobe (a) contain ER-ir, cells in the intermediate (i) and posterior (p) lobes do not (**A**). Arrows indicate double-labeled cells. Scale bars = 100 μm (**A**), 30 μm (**B** - **F**).

Chapter 4-2. Induction of Substance P Immunoreactivity by Estrogen in Female rats

Summary

Effects of gonadal steroids on substance P (SP) were examined in the MPOA and VMN of adult rats by double-labeling immunohistochemistry employing antibodies specific for ER and SP. Animals were gonadectomized and received subcutaneously either oil alone (control group), sequential injections of estradiol benzoate and oil (EB+Oil group), or those of EB and progesterone (EB+P group).

In the AVPv, SP-ir neurons were scarcely observed in the control group of both sexes. Administration of estrogen induced SP-ir neurons in the AVPv of the female. Approximately 50-80 SP-ir neurons were counted in four sections within the nucleus, and 59% of these neurons expressed ER-ir material in their nuclei. In the female EB+P group, although the number of SP-ir neurons decreased to 32% of that in the EB+Oil group, a ratio of co-existence of ER-ir material in these neurons increased to 75%. Unlike the female, estrogen failed to induce SP-ir neurons in the AVPv of male rats. The treatment of the female with EB also induced SP-ir neurons in the dorsal aspect of the MPN, but the number was relatively low compared to that in the AVPv.

In areas adjacent to the VMN including the LHA, estrogen remarkably enhanced SP-ir fibers in the female. SP-ir was also induced by estrogen in cell bodies of the ventrolateral portion of the VMN. Double-labeling immunohistochemistry revealed that a majority of those estrogen-induced SP-ir neurons contain ER-ir. However, only a few fibers with SP-ir were observed in the dorsomedial and central portions of the VMN. The control animals showed few SP-ir in the medial basal hypothalamus.

These results demonstrate sexual dimorphism in the AVPv in the responsiveness of SP neurons to estrogen. They further provide anatomical evidence that activity of SP in the MPOA and VMN are regulated by estradiol in estrogen sensitive neurons in the female rat. The data also suggest that this peptide is involved in mechanisms which underlie actions of estrogen on gonadotropin secretion as well as estrogen-dependent behaviors.

Introduction

Substances that mediate estrogenic actions on reproductive and ingestive behaviors might exist within neurons containing ER in the MPOA and VMN, and their synthesis and/or release could be controlled by estrogen. Among a variety of neurochemicals, a brain-gut peptide, substance P, was choosed as one of such candidates and examined in the present study. SP was initially isolated from the equine intestine and brain as a factor with smooth muscle stimulating and hypotensive effects (VonEuler & Gaddum, 1931). Leeman and her coworkers succeeded in a complete purification of SP from bovine hypothalamic extracts and determined its amino acid sequence (Chang *et.al.*, 1971), which made it possible to develop a sensitive radioimmunoassay for detection of SP (Powell *et.al.*, 1973). Subsequent studies have revealed a marked regional distribution of SP in the brain and spinal cord, and have found a diverse actions of this gut peptide.

SP-ir cell bodies and fibers can be found in most areas in the central nervous system including the MPOA and VMN (Ljungdahl *et. al.*, 1978). It has been demonstrated that SP neurons in the rat hypothalamus change in number and ultrastructure depending on the estrous cycle and sex (Tsuruo *et. al.*, 1984), and the concentration of SP in the MPOA varies according to the estrous cycle (Parnet *et. al.*, 1990). An involvement of SP in the control of neuroendocrine function has been well-documented (Aronin *et. al.*, 1986). Jarry *et. al.* (1988) have reported that the release of SP from the preoptic area increases significantly prior to the LH surge in the rat treated with estrogen. Stimulatory effects of this peptide on gonadotropin release have been demonstrated *in vivo* (Vijayan & McCann, 1979, Arisawa *et. al.*, 1990) or *in vitro* (Vijayan & McCann, 1979, Ohtsuka *et. al.*, 1987). Since the MPOA is believed to be the major anatomical substrate in which estrogen exerts its effect on the LH surge (Kalra, 1993, Chapter 1), SP is likely to be involved in mechanisms of the LH surge.

SP also has been implicated in the modulation of lordosis (Dornan *et. al.*, 1987) and male copulatory (Dornan & Malsbury, 1989) behaviors. A pathway from the ventrolateral portion of the VMN to the dorsal midbrain central gray, which is known to be involved in the regulation of lordosis behavior (Pfaff, 1980), includes fibers with SP (Dornan *et. al.*, 1990). Since 43% of SP neurons in this portion concentrate estrogen (Akesson & Micevych, 1988a), SP might participate in the mechanism of estrogenic

regulation of sexual behavior.

On the other hand, several reports suggest that SP may be involved in some aspect of ingestive behavior. For example, SP was originally purified as a sialogen (Chang *et.al.*, 1971), which stimulates salivary secretion in a dose response manner when injected into anesthetized rats (Leeman & Hammerschlag, 1967, Leeman & Mroz, 1974). Administration of SP inhibits vaguely induced gastric acid secretion in the rat (Okuma & Osumi, 1991). Boyer *et.al.*, (1994) have reported that bilateral intranigral injections of colchicine in the rat results in not only a marked enhancement of SP-ir in the MPOA and VMN but also drastic disturbances in motor and ingestive behaviors. Further, infusion of SP into the ventral tegmental area is demonstrated to increase latency to eat in rats (Cador *et. al.*, 1986), while an intraperitoneal injection of SP has been found to reduce food intake probably by modulating mesolimbic dopamine activity (Hasenöhrl, et.al., 1994). In turkeys, a possible involvement of SP receptors in food intake has been suggested (Denbow, 1989). Moreover, effects of SP on water intake also reported. For example, SP injected into the male MPOA induces a significant decrease in urinary sodium, potassium and water excertion (Picanco & Antunes, 1989), and synthetic SP has been shown to induce drinking behavior in the pigeon (Everred *et. al.*, 1977).

Taken together, all of these observations indicate that SP is a likely candidate which mediates estrogenic actions on reproductive and ingestive behaviors. The present study was designed, therefore, to address an anatomical association between neurons containing SP and ER, and to examine effect of estrogen on SP activity in the MPOA and VMN by employing double-labeling immunohistochemistry with antibodies specific for ER and SP.

Materials and Methods

Animals

The female rats were divided into three groups and steroids or vehicle were subcutaneously injected according to the following schedule. A EB+Oil group (n=6) received estradiol benzoate (EB) at 0 h (at 09:00 h of the colony time) and 0.1 ml sesame oil at 72h. In a EB+P group (n=5), the EB treatment at 0 h was followed by an injection

of progesterone at 72 h . Corresponding amounts of sesame oil were given at 0 and 72 h into a control group (n=4). Male rats were assigned to two groups consisted of the control (n=3) and EB+Oil groups (n=5). The progesterone treatment was not performed in the male. All animals received a colchicine (Sigma, 50 μg/5 μl saline) injection into the third ventricle at 80 h under sodium pentobarbital. Amounts of steroids and timing of their injections were chosen so that the EB+P treatment induces the LH surge in the female (Brown-Grant, 1974, Wiegand *et. al.*, 1980).

Immunohistochemistry

Twenty four hours after the colchicine treatment, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital and perfused intracardiaclly with PBS followed by a fixative consisted of 4% paraformaldehyde, 0.1% gultaraldehyde and 0.2% picric acid in 0.1 M PB, pH 7.4.

Every fourth section was selected for double-labeling immunohistochemistry of ER and SP, while the remaining ones were processed for control staining as described below or staining with cresyl violet for determination of areas to be measured.

Immunohistochemical controls included substitution of the first antibody with non-immune rabbit serum or omission of either the first or second antibody, which resulted in single staining with respective color and manner, *i.e.* blue-black products were always observed in cell nuclei while brown products in cell somata and fibers. No staining was observed in the case when both antibodies were omitted at the same time. The specificity of anti-SP serum was checked by pre-absorbing the diluted serum with a 25 μM of SP peptide (Sigma).

Data evaluation

Numbers of SP-ir neurons were counted in the AVPv of both sexes. Sections were carefully matched across animals under a microscope according to the appearance of ER-ir materials as well as the morphological structures in sections stained with cresyl violet. Among double-stained sections, four successive ones, separated each other by 90 μm, were selected so that the most rostal section contains the caudal structure of the vascular organ of the laminae terminalis (which is at the level of Plate 17 of the Swanson brain maps (Swanson, 1992)), and they were designated as sections 1-4 from the rostal

to caudal direction (Fig. 4-2.2). The numbers of neurons containing SP alone or both SP and ER (SP/ER) were directly counted on the sections under the microscope. SP-ir in the VMN was examined only in the control and EB+Oil female groups.

While it has been described that a 48-72 hrs colchicine exposure is necessary to substantially enhance immunoreactivity for SP (Simerly *et.al.*, 1986), the prolonged colchicine treatment is known to decrease immunoreactivity for ER (Herbison & Theodosis, 1992a,b, Yuri & Kawata, 1992). In the present study, however, animals were perfused 24 hrs after the colchicine injection, since this protocol had been found to be optimal for the double-staining in the preliminary experiment in Chapter 4-1. Thus, the numbers of SP- and SP/ER-ir cells might be underestimated in this study.

Results

Effects of steroids on the number of SP- and SP/ER-ir neurons in the AVPv

In the control female rat, while some fibers that contain moderate amount of SP immunoreactivities were observed in the AVPv, SP-ir cell bodies were scarcely detected at any level of the nucleus (Fig. 4-2.1, Table 4-2.1). On the other hand, the treatment of female rats with estrogen resulted in not only dense labeling of SP-ir fibers throughout the nucleus but also remarkable induction of SP-ir neurons in the central and lateral aspects of the AVPv (Figs. 4-2.1B, 4-2.3). As can be seen in Figures 4-2.2 and 4-2.3, while the induction occurred at any level, it was more predominant in the caudal half of the nucleus. Approximately 50-80 SP-ir neurons were observed in total of the four sections (Table 4-2.1). In Fig. 4-2.1F, neurons that contain both SP- and ER-irs are apparent. They comprised 59% of the SP neurons and 3% of the total ER neurons in total (Fig. 4-2.2, Table 4-2.1).

Administration of progesterone to the estrogen treated rat resulted in a reduction of the number of SP-ir neurons (Fig. 4-2.1C). It was found in all sections that the population of SP-ir neurons was remarkably smaller relative to that in the EB+Oil group ($p<0.05$) (Fig. 4-2.2, Table 4-2.1). However, the ratio of co-existence of ER- and SP-ir materials increased to 75% in the EB+P group, which was significantly higher than that in the EB+Oil group ($p<0.05$) (Table 4-2.1). In the MPN, estrogen also induced SP-ir in

some neurons located in the dorsal portion of this nucleus of the female (Fig. 4-2.4). Although the number of those neurons was relatively smaller than that in the AVPv, it was observed that ER exist in most of the SP neurons (Fig. 4-2.4).

The AVPv of the control male rat contained denser SP-ir fibers compared with that of the female, and which made a distinct boundary against the surrounding structures (Fig. 4-2.1D). While neurons containing faint SP immunoreactivity were found occasionally (0-2 per section) in this group, their number was remarkably low as was in the female control group (Table 4-2.1). However, a marked difference existed between sexes in terms of the responsiveness of SP neurons to estrogen. Unlike the female, the steroid failed to induce SP-ir neurons in the castrated male rat (Fig. 4-2.1E, Table 4-2.1). The population of SP-ir neurons in the EB treated male group was comparable to that in the control.

Effect of estrogen on SP-ir in the medial basal hypothalamus

In the medial basal hypothalamus, the effect of estrogen on SP-ir was examined in the female control and EB+Oil group at the level of No. 10 in Fig. 4-1.2 The control animals showed few SP-ir in the medial basal hypothalamus (Fig. 4-2.5A). In contrast, the EB treatment remarkably enhanced SP-ir. It can be seen from Fig. 4-2.5B that a plexus of fibers containing SP-ir spread throughout areas adjacent to the VMN including the LHA and DM. Within the VMN, although only a few SP-ir fibers were observed in the dorsomedial and central portions where few ER exists, estrogen drastically induced SP-ir neurons in the ventrolateral portion of the VMN (Fig. 4-2.5B). It appears that a majority of those estrogen-induced SP-ir neurons in the female VMN contain ER-ir. However, due to the appearance of weakened ER-ir by estrogen and the presence of dense SP-ir fibers which overlaid those ER-ir and SP-ir cell bodies, it was unable to determine the number of SP- and SP/ER-ir neurons in this area.

Discussion

The AVPv/MPN, which is containing a large population of estrogen concentrating cells (Fig. 2-7A,B), is known to be profoundly involved in the stimulatory

action of estrogen on the LH secretion as introduced in Chapter 1. Administration of estrogen markedly induced SP neurons in the female AVPv. Because 59% of those neurons contained ER, SP activity could be regulated by direct genomic action of estrogen. Absence of ER in the rest of SP neurons might be due to the reduction in ER immunoreactivity in those cells by the estrogen treatment which also caused a 32% decrease in the number of ER-ir cells (Chapter 4-1). A variety of neurotransmitters have been identified in subpopulations of preoptic neurons with ERs as shown in Table 1-1, which include CGRP, galanin, GABA and NT (see references therein). Although the ratio of SP/ER-ir neurons to the total ER-ir cells was quite smaller than the values reported for other neurotransmitters, SP may co-localize in the preoptic neurons with those neurotransmitters and estrogen may regulate them in a common mechanism. Indeed, NT (Shimada *et. al.*, 1988) and prodynorphin (Neal *et. al.*, 1989) have been reported to co-exist in SP neurons in the MPOA. Moreover, as is observed in the SP neurons in this study, it has been demonstrated that NT, CGRP and galanin neurons are distributed in a sexually dimorphic manner (Herbison & Theodosis, 1992a,b, Bloch *et. al.*, 1992) and that cells expressing mRNA for NT are markedly induced by estrogen in the MPOA of female rats (Alexander *et. al.*, 1989).

Administration of progesterone in estrogen-primed females, the treatment well-known to induce the LH surge in ovariectomized rats (Brown-Grant, 1974, Wiegand *et. al.*, 1980), resulted in a considerable decrease in SP-ir neurons compared with the treatment of EB plus oil (Table 4-2.1). The result suggests that progesterone enhances the release and/or inhibits the synthesis of SP molecules in the AVPv of female rats. Since estrogen has been shown to be able to induce PRs in subpopulations of hypothalamic and preoptic neurons with ERs (Warembourg *et. al.*, 1989, Blaustein & Turcotte, 1989), one can imagine that progesterone acts through those PRs to facilitate the release of estrogen-induced SP in the AVPv. This possibility was partially proved by double-labeling of SP and PR (Fig. 4-2.6). Although immunohistochemistry for PR brought about a high background staining (Chapter 2), it is apparent that in the EB+Oil group a majority of estrogen-induced SP neurons contained PR in their nuclei (Fig. 4-2.6). The released SP by progesterone may in turn directly stimulate the production and/or secretion of LHRH. This speculation is supported by the previous findings that the release of SP from the preoptic area increases by estrogen (Jarry *et. al.*, 1988), this

peptide stimulates gonadotropin secretion (Vijayan & McCann, 1979, Ohtsuka *et. al.*, 1987), and further SP-ir fibers directly appose to LHRH neurons (Hoffman, 1985).

Another outstanding feature of the action of estrogen was a distinct sexual dimorphism in terms of induction of SP neurons in the AVPv. While the number of SP neurons markedly increased by estrogen in the female, the steroid failed to induce SP cells in the male (Table 4-2.1). The fact that intact or gonadectomized male rats are unable to exhibit the LH surge in response to exogenously injected steroid hormones (Brown-Grant, 1974, Feder, 1981) may be in part due to the lack of this estrogenic action on SP in the AVPv/MPN. Since the establishment of the positive feedback mechanism in the rat does not depend on the genetic sex but on the existence of gonadal steroids at the neonatal period (Feder, 1981, Goy & McEwen, 1980), it is of interest to examine whether orchidectomy of newborn rats is able to restore the action of estrogen in the adult.

In the medial basal hypothalamus, estrogen also induced a marked increase of SP in the female. This result is consistent with the finding by Tsuruo *et. al.* (1984) demonstrating that the number of SP-ir cells in the ARC increases during proestrus and estrus when plasma estrogen levels are high. In the medial basal hypothalamus of ovariectomized rats, a substantial proportion (26.1-42.9%) of SP cells have been identified to concentrate radio-labeled estrogen (Akesson & Micevych, 1988a). Together with this observation, present results provide anatomical evidence that a subset of SP neurons in the rat brain are regulated by estrogen in neurons containing ER. Since estrogen has been demonstrated to enhance not only SP-ir proteins (Tsuruo *et. al.*, 1984) but also mRNA for SP in the hypothalamus (Brown *et. al.*, 1990a, Rance & Young III, 1991), regulation of this peptide could occur at the transcriptional level. The existence of a putative estrogen-responsive element up-stream from the SP promoter has been suggested (see discussion in ref. Brown *et. al.*, 1990a).

Hypothalamic regulation of reproductive behavior is thought to be, in part, dependent on estrogen modulation of gene products, which may eventually serve as neurotransmitters or their regulatory enzymes. Ultrastructural studies of the effects of estrogen demonstrated that this steroid induces the elaboration of a neurosecretory apparatus in neurons of the VMN (Meisel & Pfaff, 1985a) and, indeed, the lordosis reflex has been shown to be dependent on estrogen induction of protein synthesis

(Meisel & Pfaff, 1985b). Thus, observed induction of SP in the VMN by estrogen strongly suggests that SP participates in the mechanism of estrogen-dependent lordosis. It has been demonstrated that SP fibers project from the VMN to the midbrain of central gray (Dornan *et. al.*, 1990), and that injections of SP in the periaqueductal central gray produces a rapid, dose-related facilitation of lordosis behavior (Dornan *et. al.*, 1987).

Unlike other gut peptides, such as CCK (Morley *et.al.*, 1985) and bombesin (Denbow, 1989), there is no information which indicates a direct interaction of SP with the ingestive centers. However, several lines of evidence suggest that SP might involve in the neural mechanism which control ingestive behavior.

It has been reported that intranigral injections of colchicine result in not only enhancement of SP-ir in the MPOA and VMN but also drastic disturbance in ingestive behavior (Boyer *et.al.*, 1994). Because activities of dopamine neurons in the substantia nigra are influenced by estrogen (Chido & Caggiula, 1983) in spite of the fact that these neurons are devoid of ER (Heritage *et.al.*, 1977), the estrogenic action on dopamine neurons may be mediated by SP in the estrogen-sensitive neurons in the MPOA and/or VMN. Thus, it can be speculated that SP induced by estrogen in the MPOA and VMN as shown in the present study is conveyed to the substantia nigra to modulate the dopaminergic system, and then dopamine acts to reduce food intake. The facts that dopamine terminals and receptors are rich in the LHA (Leibowitz & Brown, 1980) and the dopaminergic system acts to suppress feeding at the level of the LHA (Leibowitz, 1986) support this hypothesis. Moreover, alterations of ingestive behavior by administration of SP intraperitoneally (Hasenöhrl, *et.al.*, 1994) or into ventral tegmental area (Cador *et.al.*, 1986) are thought to occur through its interaction with the dopaminergic system.

Another possible neural substrate involved in the regulation of feeding behavior by SP may reside in the nucleus of the solitary tract. It has been demonstrated that SP fibers terminate, although their origins are unknown, on neurons in the nucleus of the solitary tract (Ljungdahl *et.al.*, 1978), in which glucose-sensitive neurons functionally linked with hepatoportal afferents (Adachi *et.al.*, 1984) and CCK cell bodies (Takagi *et.al.*, 1984) are present. Because both glucose (Oomura *et.al.*, 1974) and CCK (Morley *et.al.*, 1985) are thought to be major determinants of ingestive behavior, SP may change ingestive responses by modulating activities of those cells.

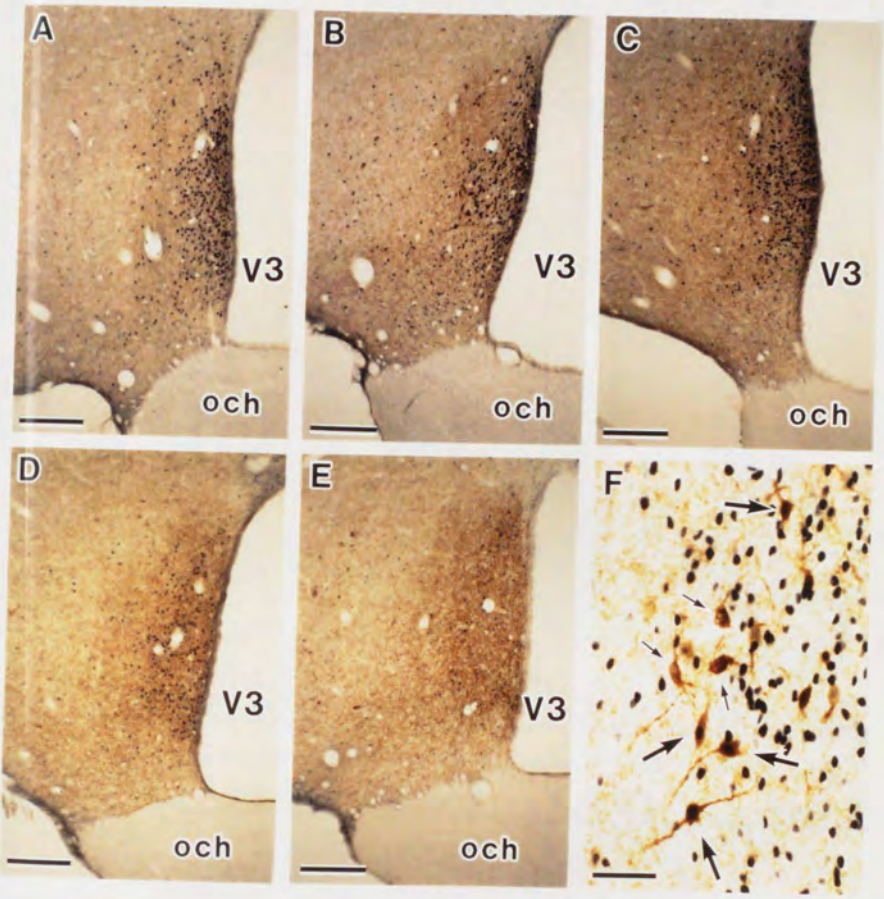

Figure 4-2.1. Photomicrographs of double immunohistochemistry for ER and SP in the female and male AVPv. Sections of the female (**A**, **B**, **C**, **F**) and male (**D**, **E**) rats, corresponding to the level at section 3 in Fig, 4-2.3, are shown. **F**: An enlargement of **B**. Four double labeled neurons (large arrows) and three SP-ir neurons without ER-ir material (small arrows) are clearly distinguishable. The presence or absence of ER-ir is not obvious in the rest of three SP-ir neurons. Bars=200 μm (**A** - **E**), 50 μm (**F**). For abbreviations, see page xii.

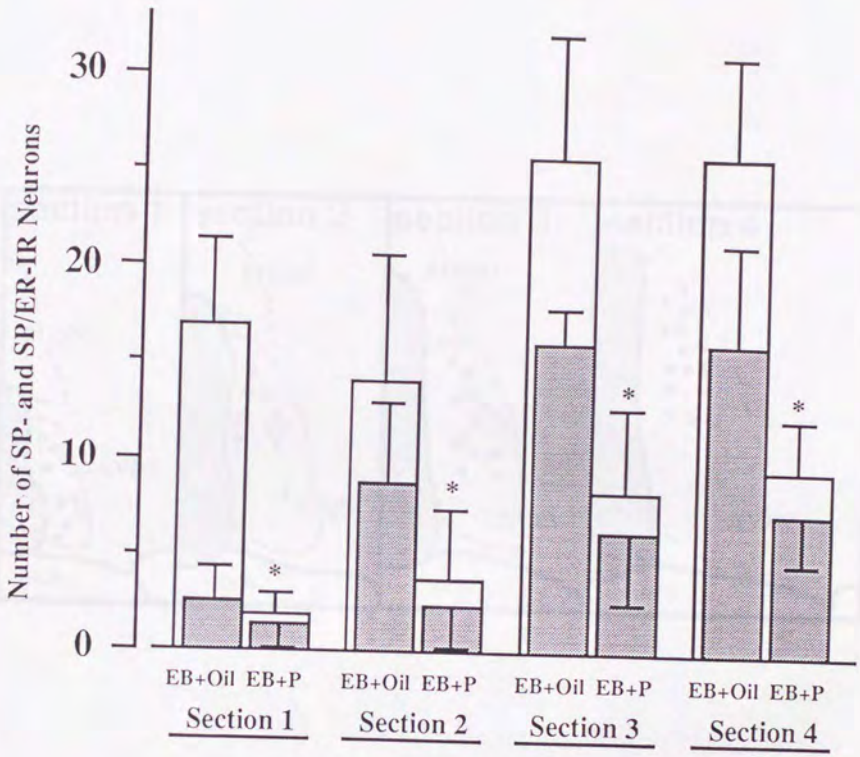

Figure 4-2.2. Effect of estrogen on number of SP- and SP/ER-ir neurons in the female AVPv. The numbers of SP-ir neurons and those containing both SP and ER (SP/ER)-ir in the AVPV of the estrogen-treated female groups were counted in four sections in each animal and represented as average values (±s.e.). Open bars: SP-ir neurons. Shaded bars: SP/ER-ir neurons. *: significant difference ($p<0.05$) compared with the EB+Oil group.

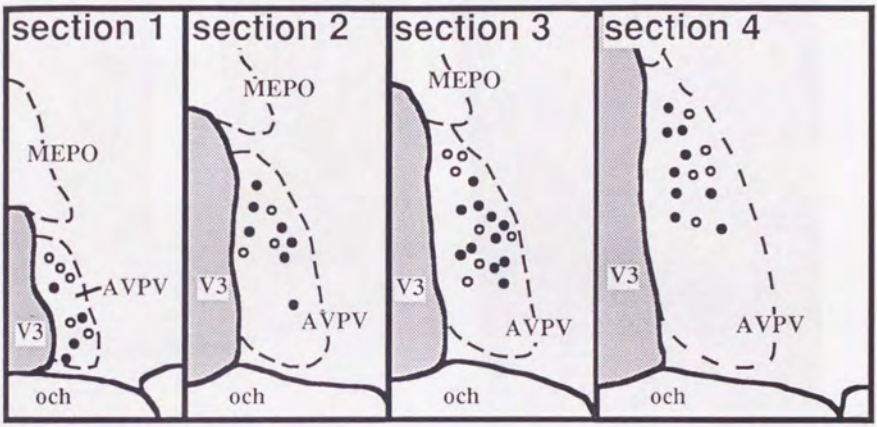

Figure 4-2.3. Schematic drawings illustrating the distribution of SP-ir neurons in the female AVPv. The distribution of SP-ir (open circles) and SP/ER-ir (closed circles) neurons at sections 1-4 (see Materials and Methods for details) of the AVPv from a representative female EB+Oil animal are illustrated schematically. The boundaries of the AVPV and MEPO were defined from cresyl violet and ER-IR staining.

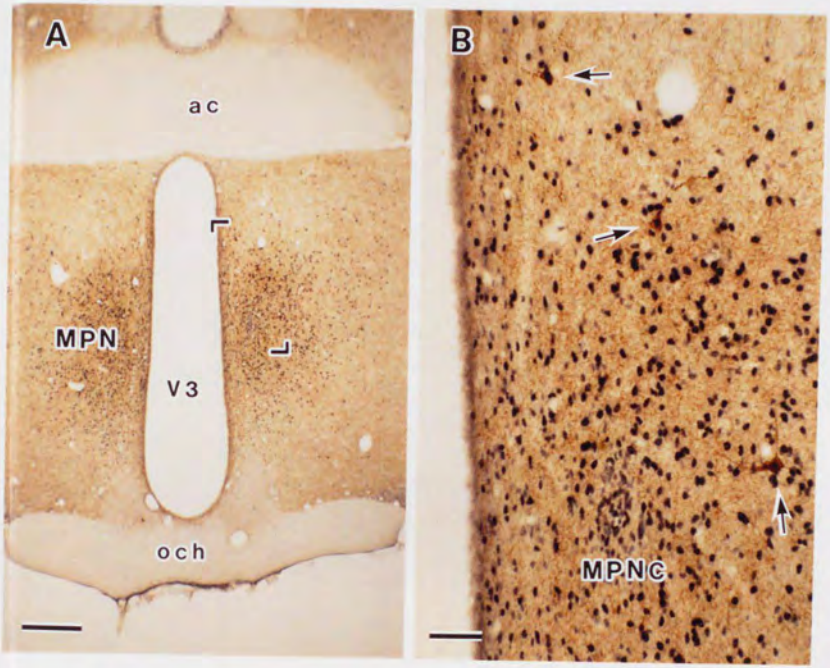

Figure 4-2.4. Photomicrographs of double-labeling of ER- and SP-irs in the EB-treated female MPN. In the middle portion of the MPN, a large population of ER-ir can be seen (**A**). In a higher magnification (**B**), it is observed that several estrogen-induced SP-ir neurons contain ER-ir in their nuclei (arrows). However, the number of SP-ir neurons in the MPN was much smaller than that in the AVPv (Fig. 4-2.1).
Scale bars = 300 μm (**A**), 50 μm (**B**)

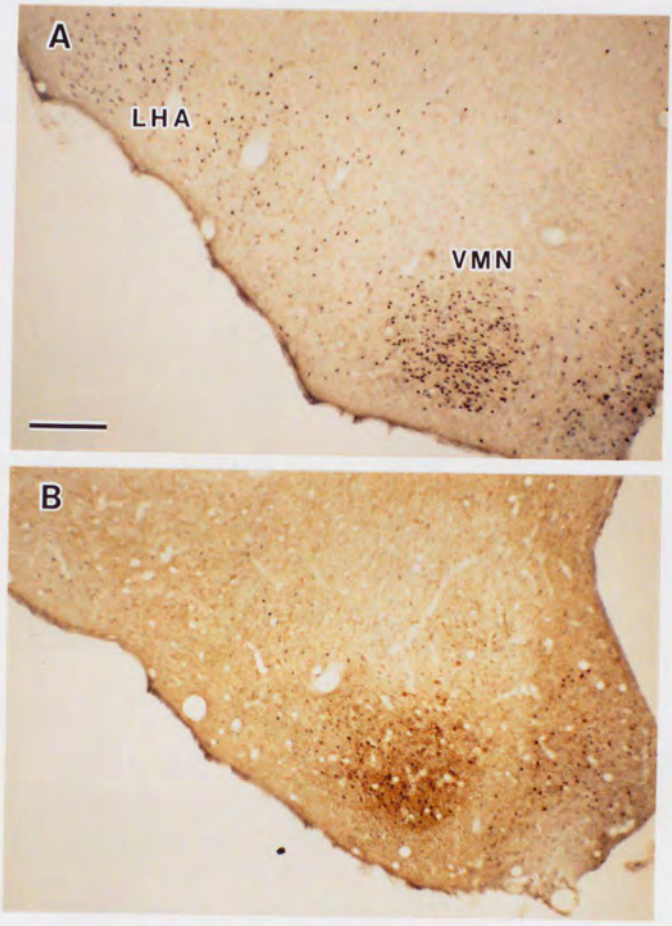

Figure 4-2.5. Photomicrographs of double-labeling of ER- and SP-irs in the female VMN. Free floating sections from control (A) and EB-treated (B) female rats processed for double-labeling immunohistochemistry are shown. In the control rat, the medial basal hypothalamus show little SP-ir (A). On the other hand, the EB treatment remarkably induced SP-ir in this area except in the dorsal part of the VMN(B). Scale bar = 150 μm.

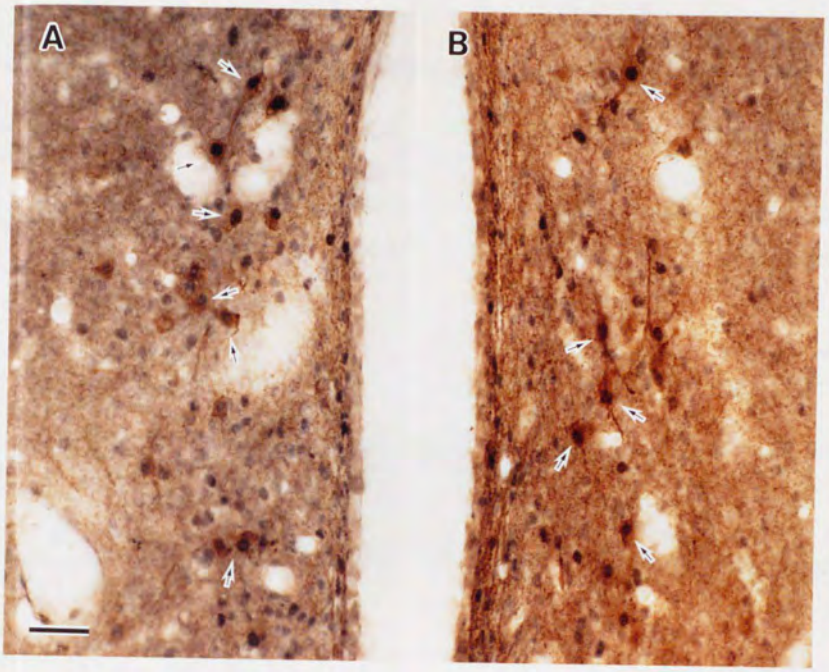

Figure 4-2.6. Photomicrographs of double-labeling of PR- and SP-irs in the EB-treated female AVPv. Sections adjacent to those shown in Fig. 4-2.1B were processed for PR and SP double-labeling immunohistochemistry according to the method in Chapter 2. Estrogen induced not only SP-ir as presented in Fig. 4-2.1 but also a large population of PR-ir in the female AVPv. Although PR immunohistochemistry resulted in a high background staining, it still can be seen that most of SP-ir neurons contain PR-ir (arrows) in sections from two different animals (**A**, **B**). Scale bar = 30 μm.

Table 4-2.1. Numbers of ER- , SP- and SP/ER*-ir neurons in the AVPV

Group		Section				Total
		1	2	3	4	
Female						
Control (n=4)	ER	184.5 ± 32.0	355.5 ± 73.8	712.5 ± 84.6	890.0 ±111.3	2142.5 ± 259.9
	SP	0.3 ± 0.4	0.5 ± 0.9	0.5 ± 0.9	0	1.3 ± 1.3
	SP/ER	0	0	0	0	0
EB+Oil (n=6)	ER	152.3 ± 47.3	280.3 ± 57.3	477.3 ± 115.3[a]	525.7 ±121.8[a]	1435.3 ± 307.9[a]
	SP	17.0 ± 4.3	14.0 ± 6.7	25.7 ± 6.4	25.8 ± 5.3	73.3 ±13.2
	SP/ER	2.5 ± 1.7	8.8 ± 4.1	16.0 ± 1.8	16.0 ± 5.4	43.0 ± 6.2
EB+P (n=5)	ER	195.0 ± 22.8	316.0 ± 64.8	529.2 ± 75.4[a]	649.2 ±132.5	1694.4 ± 259.5
	SP	1.8 ±1.2[c]	3.8 ± 3.8[c]	8.4 ± 4.4[c]	9.6 ± 2.7[c]	23.6 ±10.3[c]
	SP/ER	1.4 ±1.4	2.4 ± 2.4	6.4 ± 3.6	7.4 ± 2.4	17.6 ± 6.9
Male						
Control (n=3)	ER	161.3 ± 36.1	293.3 ± 70.1	436.7 ± 27.9[a]	605.3 ± 36.5[a]	1496.7 ±166.0[a]
	SP	1.3 ± 0.5	0.7 ± 0.9	0.7 ± 0.9	0.7 ± 0.5	3.3 ± 2.1
	SP/ER	0	0.3 ± 0.5	0	0	0.3 ± 0.5
EB+Oil (n=5)	ER	114.3 ± 24.3	174.8 ± 46.1	257.6 ± 73.1[b]	416.4 ± 83.0[b]	963.2 ± 189.0[b]
	SP	1.0 ± 0.6	0.6 ± 0.8	1.0 ± 0.6	0.4 ± 0.5	3.0 ±1.8
	SP/ER	0.2 ± 0.4	0	0.2 ± 0.4	0	0.4 ± 0.5

*: Number of neurons which contain both ER and SP immunoreactivities in the same cell bodies.
Numbers of animals in each group are shown in parentheses, and values are represented as mean ± s.e..

Significantly different ($p<0.05$) from
the corresponding female control value : a.
the corresponding male control value : b.
the corresponding value in the female EB+Oil group : c.

Chapter 5. Nitric Oxide as a Novel Messenger that May Mediate Actions of Estrogen in the MPOA and VMN: An Anatomical Evidence for a Direct Interaction of Nitric Oxide and Estrogen Receptor

Summary

NADPH diaphorase histochemistry was combined with ER immunohistochemistry in order to study effect of estrogen on NADPH diaphorase activity and establish an anatomical relationship between NADPH diaphorase and ER-ir containing neurons in the MPOA and VMN. After gonadectomy, rats received either oil (control group) or 10 μg of EB injections for two successive days and were sacrificed on day 4.

NADPH diaphorase histochemistry stained specific subpopulations of neurons in both MPOA and VMN. In the MPOA of female rats, while only a few neurons stained very weakly for NADPH diaphorase were found in control rats, a drastic increase in NADPH diaphorase activity was observed in the MPN of EB-treated animals. The total number of NADPH diaphorase neurons in the MPN of female EB group enhanced 3-fold relative to the control, and more than 80% of those neurons contained ER-ir in their nuclei.

In the VMN, neurons in the ventrolateral portion of all groups were stained for NADPH diaphorase. However, there was a marked sex difference in the effect of EB treatment. EB elevated the number of NADPH diaphorase positive neurons in females but had no significant effect in males. Double-labeling histochemistry revealed that more than 70% of the NADPH diaphorase positive neurons contained ER-ir in the VMN of all groups, with EB-treated females having a significantly higher frequency than all other groups.

Since neuronal NADPH diaphorase is nitric oxide synthase (NOS), these results provide anatomical evidence for an association of estrogen with NOS in selected nuclei in the rat and suggest that NOS activity can be modulated by estradiol in estrogen sensitive neurons. Because the induction of NADPH diaphorase activity by estradiol was observed in the nuclei essential for neuroendocrine events in the female, the results strongly suggest that nitric oxide acts as a second messenger in estrogen-dependent functions.

Introduction

Nitric Oxide (NO) is a highly reactive and unstable free radical gas. The first evidence that NO may participate in some neural functions arose from the observation by Furchgot & Zawadski (1980), in which they have demonstrated that the acetylcholine-induced relaxation of rabbit aorta requires the presence of a diffusible factor produced in endothelial cells. Further studies have shown that the smooth muscle relaxation by nitroglycerine and other nitrate vasoladiators such as sodium nitroprusside involves as active metabolite, NO, whose properties are very much like those endothelium-derived factor (Murad *et. al.*, 1978, Feelisch & Noack, 1987). Eventually, it has been identified that chemically detectable NO released from endothelial cells account for all endothelium-derived relaxing factor activity (Palmer *et. al.*, 1987, 1988). Since then, NO has been implicated in several other systems (for review, Bredt & Synder, 1991, Schuman & Madison, 1994) including macrophage cytotoxity (Marletta, 1989), nonadrenergic noncholinergic intestinal relaxation (Desai *et. al.*, 1991), penile erection (Rajfer *et. al.*, 1992), neurotoxity (Dawson *et. al.*, 1991), and plasticity in the hippocampus (O'Dell *et. al.*, 1991) and cerebellum (Shibuki & Okada, 1991). In addition, it has become apparent that NO is also involved in regulation of a variety of neuroendocrine functions such as the hypothalamo-pituitary adrenal axis (Yasin *et. al.*, 1993, Costa *et. al.*, 1993), hypothalamo-hypophysial system (Kadowaki *et. al.*, 1994) and hypothalamo-pituitary-gonadal axis (Moretto *et. al.*, 1993, Bonavera *et. al.*, 1993, Sortino *et. al.*, 1994).

On the other hand, since Scherer-Singler *et. al.* (1983) have established that neurons containing an highly active endogenous enzyme, NADPH diaphorase, can reduce the dye nitro blue tetrazolium to a bright blue reaction product, histochemistry for this enzyme has been used to stain a few well-defined cell groups in the brain (Vincent, 1986, Sagar & Ferriero, 1987, Scott *et. al.*, 1987, Croul-Ottman & Brunjes, 1988, Hope & Vincent, 1989, Vincent & Kimura, 1992), although function of NADPH diaphorase had been unknown. Recently, it has been identified that neuronal NADPH diaphorase is NO synthase (NOS) (Hope *et. al.*, 1991, Bredt *et. al.*, 1991, Dawson *et. al.*, 1991a), an enzyme responsible for the generation of NO. Thus NADPH diaphorase histochemistry provides a useful method to localize NOS neurons in the brain (Vincent & Kimura, 1992, Pow, 1992, Kadowaki *et. al.*, 1994). Fig. 5-1 shows a biochemical pathway of the NO generation as well as the NADPH dependent reduction of tetrazolium salt by "NOS" or "NADPH diaphorase".

Since NO is shown to be involved in mechanisms which underlie neuroendocrine events in reproduction (Moretto *et. al.*, 1993, Sortino *et. al.*, 1994) and may mediate the action of gonadal steroids (Bonavera *et. al.*, 1993), it is of interest to examine whether estrogen modulates NOS activity in specific nuclei responsible for estrogen sensitive behaviors. In the present experiment, therefore, the effect of estrogen on NOS activity and an anatomical relationship between NOS and ER containing neurons were investigated by combining NADPH diaphorase histochemistry and ER immunohistochemistry in the rat MPOA and VMN. As presented in Chapter 3 (Fig. 3-7), these two nuclei contain a large population of ER and are thought to play critical roles in reproduction such as LHRH release (Kalra, 1993), and lordosis (Pfaff, 1980) and ingestive (Brobeck, 1941) behaviors.

Materials and Methods

Animals: Adult female and male rats were bilaterally gonadectomized and 7 to 10 days later were injected either 10 μg estradiol benzoate (EB) in 0.1 ml sesame oil (EB group) or 0.1 ml sesame oil (control group) on day 1 and day 2 at 09:00. On the afternoon of day 4, the rats were perfused with 4% paraformaldehyde in 0.1 M PB, pH 7.4. Frontal sections were cut at 30 μm on a freezing microtome.

Histochemistry: The free-floating sections were rinsed in 0.1 M PB and incubated in 0.1 M PB, containing 0.3% Triton-X 100, 0.1 mg/ml NBT and 1.0 mg/ml β-NADPH at 37°C (Vincent & Kimura, 1992). The sections from each group were always reacted in parallel under identical conditions for the same time (30 min). Following the reaction, the sections were rinsed in 0.1 M PB and some of them were mounted on the coated slides and cover-slipped as previously described (Vincent & Kimura, 1992), while remaining sections were further processed for ER immunohistochemistry as described in Chapter 2.

Data evaluation: Numbers of neurons containing NADPH diaphorase alone or both NADPH diaphorase and ER-ir were counted in hemisections of the MPOA and the caudal region of the VMN under the microscope.

Results

1. MPOA

In the MPOA, only female rats were examined. NADPH diaphorase histochemistry stained a specific subpopulation of neurons in the MPOA of female rats. In the ovx animals that received no estradiol benzoate (EB), a large number of ER immunoreactive neurons were found in the MPN (Fig. 5-2A). Only a few neurons which showed weak staining for NADPH diaphorase were found scattered in the MPN and its surrounding area (Fig. 5-2A,C) of this group. In contrast, while the intensity of ER-ir decreased (Chapter 4-1), the treatment of ovx rats with EB resulted in a drastic increase in the number of NADPH diaphorase neurons in the MPN (Fig. 5-2B). The staining intensity of those estradiol-induced NADPH diaphorase neurons was moderate and lighter compared to that of intensely stained neurons in other brain regions such as the supraoptic nucleus, striatum and paraventricular nucleus, but appeared higher than that of NADPH diaphorase neurons in the MPN of the control ovx rats (Fig. 5-2,C *vs.* D). It can be seen in Fig. 5-2D that the majority of estradiol-induced NADPH diaphorase neurons contain ER-ir in their nuclei. The number and activity of NADPH diaphorase neurons in the supraoptic nucleus, which lacks ER-ir, did not change in response to the EB treatment (at the levels corresponding to Fig. 5-3B or E, the number of NADPH diaphorase neurons were 18.6±2.1 and 18.4±2.3, respectively in the control and EB group, 5 rats for each group).

Distribution of neurons containing NADPH diaphorase alone or both NADPH diaphorase and ER-ir in the MPOA was depicted from one representative animal in each group and illustrated in Fig. 5-3. Numbers of those neurons were counted in 5 animals in each group at the corresponding levels of sections and summarized in Table 5-1. In the AVPv, a few weakly stained NADPH diaphorase neurons with and without ER-ir were observed in the control (Fig. 5-3A). The EB treatment did not change the number and stainability of these cells in this nucleus (Fig. 5-3D). On the other hand, NADPH diaphorase activity was markedly enhanced by EB at the two levels of the MPN (Fig. 5-3E and F, for rostal and middle portions, respectively). In these areas, although the numbers of neurons containing NADPH diaphorase alone did not differ from those in the corresponding areas of the control, the total numbers of NADPH diaphorase positive neurons increased more than 3-fold in response to the EB treatment (Table 5-1). At both levels (Fig. 5-3E,F) more than 80 % of those neurons contained ER-ir (Table 5-1). While the population of NADPH diaphorase neurons in the EB-treated

animal did not significantly differ between these two levels of the MPN (Table 5-1), there was a dissimilarity in the distribution of double-labeled cells (Fig. 5-3,E *vs.* F). Many of NADPH diaphorase neurons were clustered in the ventromedial aspect of the nucleus at the rostal level of the MPN (Fig. 5-3E). At the middle level of the MPN, on the other hand, they were evenly distributed within the nucleus (Fig. 5-3F) except the central part of the MPN (MPNc) and some of them extended in the area dorsolateral to the MPN (Fig. 5-3F). In the MPNc appearance of NADPH diaphorase neurons was very rare in both groups.

2.VMN

A subpopulation of neurons throughout the rostro-caudal extent of the VMN of both female and male was stained for NADPH diaphorase histochemistry. Within the VMN, while a few weakly stained neurons were found in the dorsal and central portions, most positive cells were localized and clustered in the ventrolateral portion of the VMN (Fig. 5-4). NADPH diaphorase activity in the female markedly changed in response to the EB treatment. While ER-ir decreased as in the MPOA, the number of NADPH diaphorase stained neurons and their staining intensity were significantly higher in the EB treated female rat (Fig. 5-4B,D) compared to those in the control animal (Fig. 5-4A,C). The stimulatory effect was observed throughout the rostro-caudal extent of the VMN, being most prominent in a caudal region (Fig. 5-4D) where many ER exist. The number of NADPH diaphorase neurons was counted in 8 females and 4 males in each group. In this region, the female EB group contained a 1.8-fold larger population of NADPH diaphorase neurons relative to the control group (Table 5-2). In the male, the apparent increase in the number of NADPH diaphorase neurons (Fig. 5-5) was not statistically significant (Table 5-2). Double-labeling of NADPH diaphorase and ER-ir revealed that more than 70% of NADPH diaphorase stained neurons contained ER-ir in their nuclei in all groups (Table 5-2). It also can be found from Table 5-2 that the increase in the total number of NADPH diaphorase cells in females after estrogen treatment was due to the increase in double-labeled cells while no significant difference in the populations of neurons containing only NADPH diaphorase were found among groups. In addition to the MPN and VMN, NADPH diaphorase co-localizaed with ER in a subpopulation of neurons in the LHA (Fig. 5-6), and their activities were also enhanced by the EB-treatment (Fig. 5-6, A *vs* B). In all groups, estrogen had no effect on NADPH diaphorase activity in the amygdala (Fig. 5-6), although a number of neurons containing ER exist in this area (Fig. 3-7).

Discussion

In the present study, the effect of estrogen on NADPH diaphorase activity in the rat MPOA and VMN was examined by double-labeling histochemistry for NADPH diaphorase and ER-ir. In the female, the total number of NADPH diaphorase neurons in the MPN increased 3-fold in the EB-treated animals relative to the control. This increase was due to the increase in double-labeled cells that accounted for more than 80% of the total NADPH diaphorase neurons (Table 5-1). The numbers of NADPH diaphorase neurons that did not contain ER-ir were not different between two groups throughout the three levels of the MPOA examined. Therefore, there may be two different populations of NADPH diaphorase neurons in the MPN, one is sensitive to estrogen and the other not. The observation that the staining intensity for NADPH diaphorase in the double-labeled neurons was generally higher than that in the cells without ER-ir support this possibility. The NADPH diaphorase neurons containing ER-ir in the AVPv seems to belong to a different subpopulation from that in the MPN. Different functions of estrogen between these two nuclei have been reported (Kalra, 1993). A variety of neurotransmitters, such as NT (Herbison & Theodosis, 1992a), galanin (Bloch *et al.*, 1992), natriuretic peptide (Watson *et al.*, 1994) and GABA (Flügge *et. al.*,1986), and CGRP (Herbison & Theodosis, 1992b), have been shown to co-localize with ER in the MPN to the extent of 3 to 35%; the present finding of up to 80% co-localization of NADPH diaphorase in ER-ir neurons of EB-treated rats implies that NADPH diaphorase also co-localizes with al least some of these neurotransmitters.

The MPOA is a sexually dimorphic nucleus in the cytoarchitecture (Simerly, 1984) and neurochemical identity (Herbison & Theodosis, 1992a,b, Bloch *et. al.*, 1992, Simerly *et. al.*, 1984). This may account for the fact that neither NADPH diaphorase neurons (Bredt *et. al.*, 1991, Vincent & Kimura, 1992) nor neurons containing NOS-ir or mRNA (Bredt *et. al.*, 1990, Bredt *et. al.*, 1991) has been shown in the MPOA of male rats. In this study, the MPN of EB-treated male was not examined. However, since estrogen enhanced the NADPH diaphorase activity in a sexually dimorphic manner in the VMN (Table 5-2), it is possible that the estrogen sensitive NADPH diaphorase neurons in the MPN distribute differently between sexes and that they may contribute to a sex difference in the mechanism of action of estrogen.

In the VMN, estrogen also stimulated NADPH diaphorase activity throughout the rostro-caudal extent of this nucleus. In the caudal region of VMN where ER exists in

abundance (Fig. 4-2), while the female EB group contained a 1.8-fold larger population of NADPH diaphorase neurons relative to the female control (Table 5-2), the apparent increase in the number of those neurons in the male EB group (Fig. 5-5) was not statistically significant (Table 5-2). This result is qualitatively similar to the reported sex difference in progestin receptor induction by estradiol (Rainbow *et. al.*, 1982) and is different from the very similar estrogen-induction of oxytocin receptors in male and female VMN (Coirini *et. al.*, 1989). The sex differences in NADPH diaphorase induction may be a reflection of reported differences in estrogen receptor levels in the VMN (Brown *et. al.*, 1992).

Double-labeling of NADPH diaphorase and ER-ir revealed that more than 80% and 88% of the NADPH diaphorase neurons, respectively, in the MPN and VMN of the female EB-treated rat contained ER in their cell nuclei. In a preliminary experiment, it was found that the effect on NADPH diaphorase activity can be identified at 24 hrs but not 8 hrs after the estrogen treatment. Together with that result, the striking co-localization indicates that estrogen acts to regulate NADPH diaphorase via a delayed action that may reflect an effect on gene expression (Fig. 1-1). Several neurotransmitters have been shown to co-exist in NADPH diaphorase containing neurons, such as neuropeptide Y and somatostatin in the striatum (Kowall *et. al.*, 1987) and galanin in the basal forebrain (Pasqualotto *et. al.*, 1991). The present study has shown for the first time that NADPH diaphorase co-localize with ER in a specific subpopulation of neurons in the rat MPN and VMN, and its activity can be modulated by estradiol in neurons sensitive to estrogen. Changes in NADPH diaphorase activity in response to experimental manipulation, such as axotomy (Gonzalez *et. al.*, 1987) and salt loading (Kadowaki *et. al.*, 1994), have been reported previously.

It has been demonstrated that neuronal NADPH diaphorase and NOS are identical (Hope *et. al.*, 1991, Bredt *et. al.*, 1991) and NADPH diaphorase staining reflects NOS activity (Pow, 1992, Kadowaki *et. al.*, 1994). Therefore, the increase in NADPH diaphorase activity observed here indicates that estrogen enhances the NOS synthesis and thus may enhance the generation of NO in the selected neurons sensitive to estrogen in the rat. Moreover, since the majority of NADPH diaphorase neurons contained ER-ir, the data provide anatomical evidence for a direct association of the gonadal steroid with NOS activity in the MPOA and VMN. The effects of NO are known to be mediated via activation of soluble guanylate cyclase and subsequent formation of cGMP in the cerebellum (Bredt & Snyder, 1989), some neural cells (Moretto *et. al.*, 1993, Sortino *et. al.*, 1994) and peripheral tissue (Yallampalli *et al.*, 1994).

Yallampalli *et al.* (1994) have demonstrated that a rise in circulating estradiol inhibits the NO-cGMP system in the uterus, although in the present study it was observed that estrogen acts stimulatory on NOS activity. This reciprocal effect of the steroid may indicate that the action of NO is coupled with other second molecules than cGMP in the MPN and VMN as has been suggested (Bredt *et. al.*, 1991, Bredt & Snyder, 1992). Indeed, very little guanylate cyclase has been identified in the hypothalamus (Matsuoka *et. al.*, 1992).

Roles of NO in the MPN and VMN are far from clear so far. As mentioned, the MPN contains a number of neurotransmitters including NT (Herbison & Theodosis, 1992a), galanin (Bloch *et. al.*, 1992), natriuretic peptide (Watson *et. al.*, 1994), CGRP (Herbison & Theodosis, 1992b), GABA (Flügge *et. al.*, 1986) and other molecules (Simerly *et. al.*, 1986). NO may mediate the actions of estrogen on the release and/or synthesis of those substances. NOS does not necessarily co-localize in the same cells with them, since NO can diffuse through the membrane without specific receptors (Goretski & Hollocher, 1988) and may, therefore, act in neighboring cells as well as in the cells in which it is formed (Hope *et. al.*, 1991). In the VMN, a number of estrogenic actions also have been reported, *e.g.* synthesis, release, turnover, metabolism and availability of receptors for a variety of neurotransmitters (Dohanich *et. al.*, 1985) and alteration of synaptic organization (Frankfurt *et. al.*, 1990). Although highly speculative, NO in the MPN and VMN may participate in such diverse actions acting as a second messenger to modulate cellular functions (Bredt & Snyder, 1992). NO also has been known to be involved in synaptic plasticity in the hippocampus (O'Dell *et. al.*, 1991).

Morley & Flood (1991) have reported that an injection of NO synthesis inhibitor inhibits food intake in mice, which suggests that a decrease in the amount of NO is required for the suppression of food intake. This result is opposite to the present observation that NOS activities in the MPOA and VMN were enhanced by high levels of circulating estrogen, which might induce hypophagia (Tarttelin & Gorski, 1973) in the same animal. The discrepancy between two experiments may be explained by different functions of NO between the central nervous system and peripheral tissues. Because they injected the NO synthesis inhibitor subcutaneously, its sites of action probably reside in peripheral tissues such as intestine. Appetite regulation is a complex process involving a variety of neurotransmitters and hormones (Morley, 1980). In the brain, it is possible that NO participates in the control of their activities through which ingestive responses are changed.

The dose and mode of EB administration employed in this study have been shown,

when the treatment was followed by progesterone injection, to facilitate lordosis behavior in the ovx rat (Pfaff, 1980), and the treatment of estrogen is known to suppress food intake in the ovx rat (Tarttelin & Gorski, 1973). Since the induction of NOS activity by estradiol was observed in three nuclei essential for neuroendocrine events, the present results suggest that NO plays a critical role in mechanism through which actions of estrogen on reproductive and ingestive functions are exerted. Future studies of influence of NOS inhibitors on estrogen-dependent activities would provide clues for the physiological functions of NO in these nuclei.

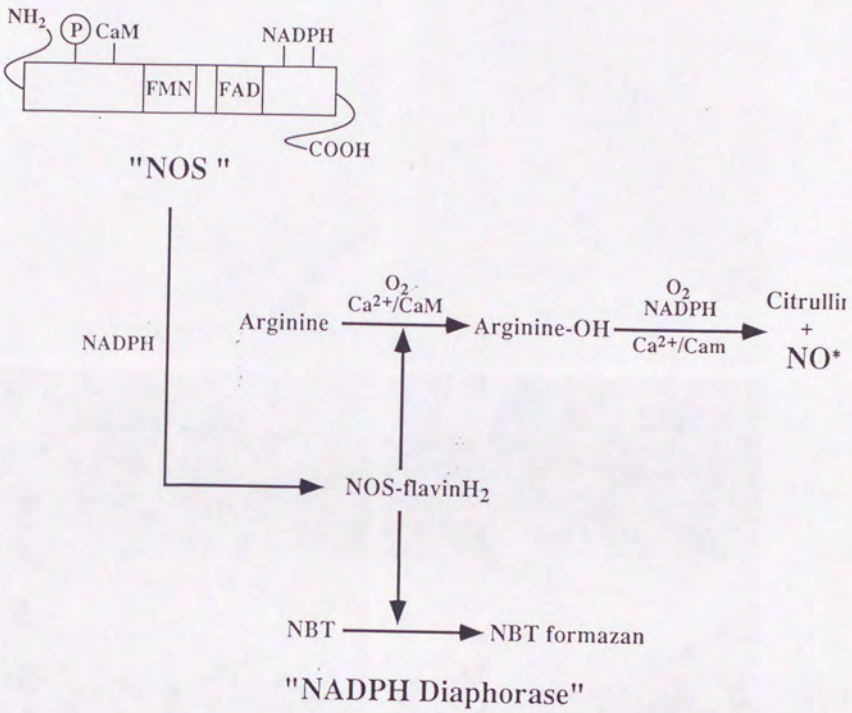

Figure 5-1. Biochemical pathway of the NO generation and the NADPH dependent reduction of tetrazolium salt.

Modified from Bredt & Synder, 1992

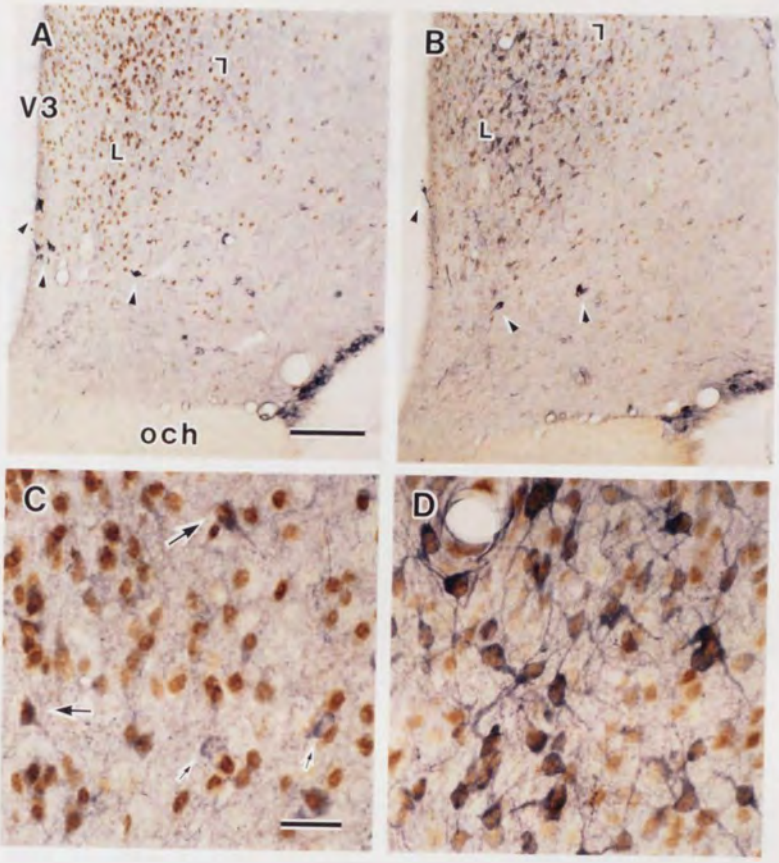

Figure 5-2. Photomicrographs of representative sections of the female MPN double-stained for NADPH diaphorase and ER-ir. Sections including the MPN of control (**A**) and EB-treated (**B**) animals are shown. **C** and **D** are large magnifications of the areas indicated by two markers in **A** and **B**, respectively. The sections were double-labeled for NADPH diaphorase (blue products) and ER-ir (brown dots). Arrows in **C** indicate NADPH diaphorase neurons with (large arrows) or without (small arrows) ER-ir. Although several neurons intensely stained for NADPH diaphorase (arrow heads) were observed within the MPOA, especially beneath the ependymal layer, they were omitted from the following data evaluation, because they were obviously different from other NADPH diaphorase neurons in shape and staining. Scale bars =150 μm in **A**, **B** and 30 μm in **C**, **D**.

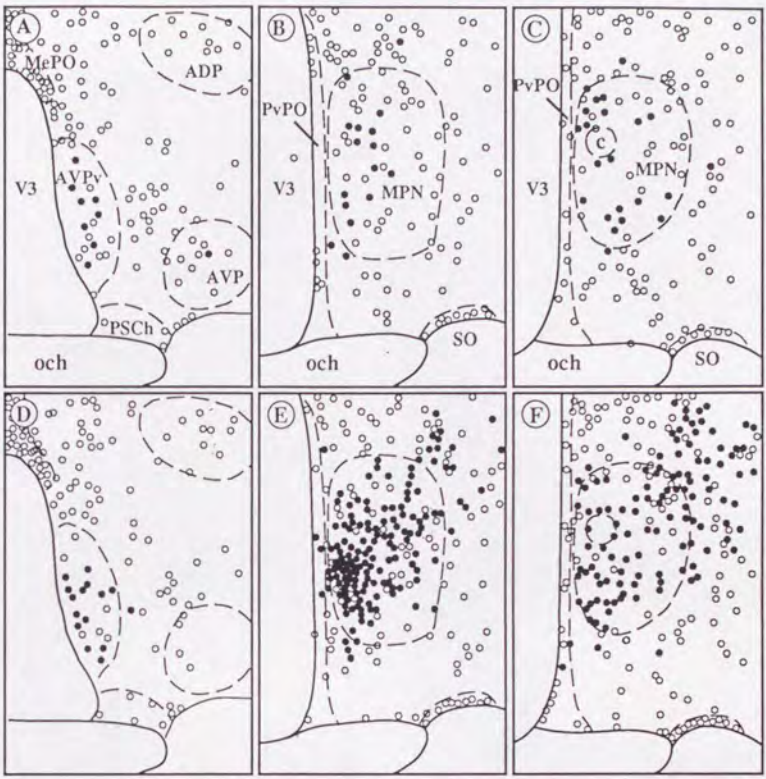

Figure 5-3. Mapping of NADPH diaphorase neurons in the female MPOA

NADPH diaphorase stained neurons with (filled circles) and without (open circles) ER-ir in the MPOA of control (A - C) and EB-treated (E - F) female rats. Levels corresponding to the AVPv (A, D) and at the rostal (B, E) and middle (C, F) portions of the MPN depicted from one representative animal are shown. The boundaries of the AVPv, MPN and central part of MPN were delineated by ER-ir staining. The numbers of NADPH-d neurons with and without ER-ir did not differ in the AVPv of both groups (A, D). In contrast, the numbers of NADPH-d neurons containing ER-ir remarkably increased in the MPN of EB rat (E, F) compared to the control (B, C). The double-labeled neurons were found clustered at the ventromedial portion of the rostal aspect of the MPN (E), while they were scattered throughout the nucleus and extended to the dorsolateral area to the MPN at the middle level of the MPN (F). See page xii, for abbreviations.

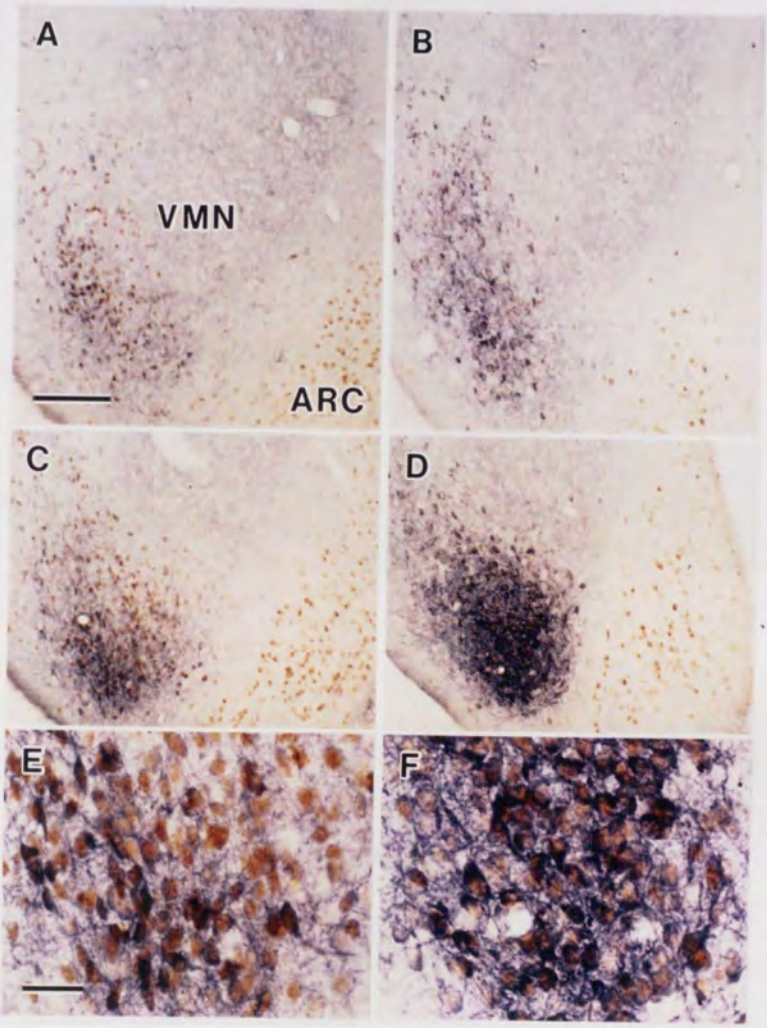

Figure 5-4. Photomicrographs showing representative sections double-stained for NADPH diaphorase and ER-ir in the female VMN. The middle (**A**, **B**) and caudal (**C**, **D**) regions of the VMN are shown. **A**, **C**: control group. **B**, **D**: EB group. **E** and **F** are large magnifications of **C** and **D**, respectively. NADPH diaphorase activity is markedly higher in the EB group (**B**, **D**) than the control animal (**A**, **C**) in both regions. The ARC is unstained for NADPH diaphorase histochemistry. Scale bars = 150 μm (**A** - **D**), 30 μm (**E**, **F**).

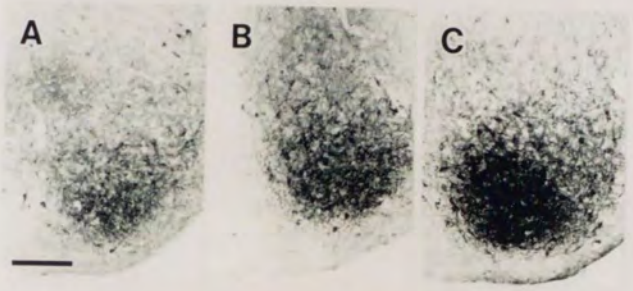

Figure 5-5. Photomicrographs of the caudal region of the VMN stained with NADPH diaphorase histochemistry. **A**: male control group. **B**: male EB group. **C**: female EB group. NADPH diaphorase activity in the male is higher in the EB group (**B**) than the control animal (**A**). However, the stimulatory effect of estrogen on NADPH diaphorase activity is more predominant in the female (**C**) than in the male (**B**). The third ventricle is left side of the photograph. Scale bar = 150 μm.

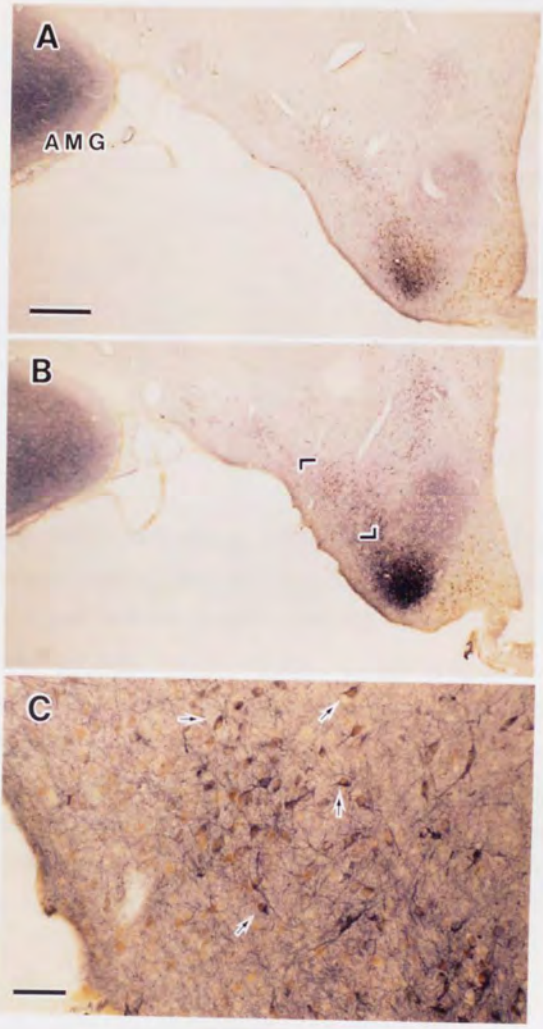

Figure 5-6. Photomicrographs of double-labeled sections for NADPH diaphorase and ER in the VMN and LHA of the female rat. Neurons containing NADPH diaphorase extend from the ventrolateral portion of the VMN to the LHA in the control rat (**A**). However, their activities are relatively weaker than those in the EB-treated rat (**B**). In a larger magnification of **B**, it can be seen a majority of those neurons contain ER (arrows) in the LHA (**C**) as in the VMN. Note that NADPH diaphorase activities in the amygdala (AMG) are not different between two groups (**A** *vs.* **B**). Scale bars = 300 μm (**A, B**), 50 μm (**C**).

Table 5-1. Numbers of NADPH diaphorase neurons in the MPOA

		Neurons with NADPH-d† alone	Neurons with NADPH-d and ER-ir	Total number of NADPH-d neurons	% of NADPH-d neurons with ER-ir
Cont	AVPv (A)	12.4±1.5	12.0±1.9	24.4±2.1	49.1±5.2
	MPN-1(B)	19.0±3.5	15.0±2.5	34.0±4.7	43.8±11.7
	MPN-2(C)	18.0±2.3	14.4±4.1	32.4±5.6	42.7±6.8
EB	AVPv (D)	10.0±1.7	12.2±1.6	22.2±2.5	43.7±3.9
	MPN-1(E)	18.2±3.0	94.2±7.6**	112.4±9.3**	84.0±2.2**
	MPN-2(F)	17.4±3.5	76.0±3.6**	93.4±6.3**	81.8±3.2**

Sections were carefully matched across animals under a microscope according to the morphological structures. Numbers of neurons containing NADPH-d alone or both NADPH-d and ER-ir were counted under the microscope in hemisections of the AVPv and MPN at the levels shown in Fig. 5-3. Alphabets in parentheses are corresponding to those in Fig. 5-3. Boundaries of areas to be measured were determined on the basis of ER-ir staining. Although the NADPH-d staining intensity varied among cells, no distinction between weak and strong staining was made in this evaluation. Values obtained from 5 animals in each group are represented as mean ± s.e. †: NADPH diaphorase.

**: significantly different from corresponding control values. $p<0.01$

Table 5-2. Numbers of NADPH diaphorase neurons in the female and male VMN

		Neurons with NADPH-d† alone	Neurons with NADPH-d and ER-ir	Total number of NADPH-d Neurons	% of NADPH-d Neurons with ER-ir
Female	Control	23.1 ± 6.5	67.9 ± 9.6	90.8 ± 15.3	77.4 ± 3.4
	EB	21.5 ± 3.8	143.5 ± 7.2**	165.1 ± 9.4*	87.5 ± 1.8*
Male	Control	24.3 ± 4.0	61.5 ± 11.1	85.8 ± 15.1	71.5 ± 1.0
	EB	33.0 ± 8.1	79.5 ± 5.8	112.5 ± 5.4	71.0 ± 6.2

Numbers of neurons containing NADPH diaphorase alone or both NADPH diaphorase and ER-ir were counted at the caudal region of the VMN under the microscope by three observers who knew neither sex nor treatment, and three counts were averaged. Values taken from 8 animals in each female group and 4 animals in each male group are represented as mean ± s.e.

†: NADPH diaphorase.

*: Significantly different from others. $p<0.05$. **: $p<0.01$

Chapter 6. General Discussion

1. Distribution and Regulation of Estrogen Receptor in the Rat Brain

Neurons that contain the ER can be found in a variety of brain regions that participate in behavioral and pituitary reproductive functions (McEwen *et. al.*, 1979). At present, four methods are available to localize the ER in discrete regions; i) biochemical assays of hormone binding (McEwen, 1976, Rainbow *et. al.*, 1982), ii)autoradiographic observations of radio-labeled estradiol uptake (Pfaff & Keiner, 1973, Stumpf *et. al.*, 1987), iii) detection of mRNA by *in situ* hybridization (Simerly *et. al.*, 1990, Lauber *et. al.*, 1990), and iv) detection of ER-ir using antibody to the ER (Blaustein, 1992, Herbison & Theodosis, 1992a, Okamura *et. al.*, 1992). Each method contains advantages and disadvantages in its own methodological process. For example, although autoradiography satisfies all of the criteria of receptor binding (Stumpf *et. al.*, 1980), and thus the presence of functional ER can be identified by this method, it includes some disadvantages as described in Chapter 3. On the other hand, while immunohistochemistry has been shown to offer direct and accurate localization of the receptor, it always holds a suspicion whether antibodies specifically recognize the ER or whether they do not recognize other molecules which have a similar sequence or structure to the ER. Therefore, caution should be exercised when distribution of ER is discussed.

A polyclonal anti-rat ER antibody, AS-408, was generated in the experiments of Chapter 3. Due to its polyclonal nature, it cannot be ruled out a possibility that materials other than the ER are recognized by AS-408. However, this is very unlikely. The antibody could recognize solely the ER, because the distribution of ER demonstrated using AS-408 (Figs. 3-7) is comparable with those shown by biochemical assays (Rainbow *et. al.*, 1982), autoradiography (Pfaff & Keiner, 1973) and *in situ* hybridization (Simerly *et. al.*, 1990). Further, immunoreactivity of the ER was down-regulated by estrogen in all areas examined here.

Dense concentrations of the ER were found in the MPOA and VMN, and a relatively large population of the ER in the LHA (Fig. 3-7), areas known to be involved in some aspect of reproductive and ingestive behaviors. This suggests that influences of estrogen on such functions may be exerted, in part, at these neural substrates. Because there are massive reciprocal connections between the MPOA and the VMN and LHA (Chapter 1, 5-3), it appears that these ER containing areas form a neural network to integrate the influences of circulating estrogen on a number of physiological and behavioral responses. Connections between regions

that contain estrogen sensitive neurons have been reviewed (Pfaff, 1980, Cottingham & Pfaff, 1986). Such network system may allow amplification of signal, permit greater stability and impose a selective filtering upon inputs, channeling some to the hypothalamus or to an output line from the network. A schematic model of the neural circuit between three estrogen-sensitive nuclei is shown in Fig. 6-1.

Because the physiological response to estrogen requires ER, the concentration of ER protein is supposed to be a major determinant of the sensitivity of neurons. Among a number of mechanisms for the ER regulation, action of estrogen is thought to be primary importance. The characteristic of AS-408 that it recognizes both occupied and unoccupied ERs enabled to examine levels of the ER under a variety of hormonal conditions.

Estrogen down-regulates its own receptor levels with a similar extent in the AVPv and VMN (Chapter 4-1), which is in good agreement with the previous demonstrations in the rat brain by immunohistochemistry (Blaustein, 1993), receptor assay (McGinnis, *et. al.*, 1981) and *in situ* hybridization (Simerly & Young, 1991). In addition, estrogen has been shown to decrease levels of both ER mRNA and protein in MCF-7 cancer cells (Read *et. al.*, 1989) as well as in the rat uterus (Shupnik *et. al.*, 1989). However, this steroid also has been shown to significantly induce ER mRNA in T47-D cells, similar to that found in the rat liver and pituitary (Read *et. al.*, 1989, Shupnik, 1989). Moreover, regionally specific effects of estrogen on ER mRNA has been reported in the rat brain (Lauber *et. al.*, 1990, Shughrue, *et. al.*, 1992). Taken together with the fact that an estrogen responsible element in the 5' flanking region of the ER gene has yet to be identified, the cell and tissue specific regulation of the ER implies a possibility that a primary action following to the estrogen binding is to express regulatory factors which suppress ER gene expression. In a putative model by Rories & Spelsberg (1989), the actions of steroid have been explained by a regulatory gene as an early gene and a structural gene as a late gene; the binding of steroid-receptor complex to the nuclear acceptor site regulates the transcription of the regulatory gene within minutes after the steroid enters the target cell, which in turn could regulate chromatin structure or structural gene transcription as transcription factor occurring one to four hours after the initiation of the cascade.

In the ovary intact rats, the highest expression of ER-ir in the VMN was observed during proestrus (Chapter 4-1) when circulating estrogen levels are high, even though estrogen down-regulates ER protein and mRNA in the ovx rat. It may be possible that some factors secreted by the ovary influence the activity of such regulatory gene as mentioned above to

maintain ER levels in the VMN. The highest expression of ER may contribute to the maximum action of estrogen in the VMN during proestrus, a period which requires complex responses to the steroid such as lordosis and ingestive behaviors.

2. Role of Estrogen Induced Substance P in the MPOA and VMN

In the experiments of Chapter 4-2, it was observed that estrogen induces SP in ER containing neurons of the MPOA (Fig. 4-2.1) and VMN (Fig. 4-2.5). The enhanced SP activity also found in the LHA (Fig. 4-2.5). It has been reported that fibers from the MPOA give rise to the terminal fields in the VMN and LHA (Conrad & Pfaff, 1976, Simerly & Swanson, 1988). On the other hand, a number of ER containing cells in the VMN have been shown to project to the MPN (Akesson *et. al.*, 1988). Further, the existence of a prominent SP containing pathway from the VMN and LHA to the MPOA has been demonstrated (Yamano *et. al.*, 1986). Therefore, SP could be conveyed between estrogen sensitive nuclei in the neural circuit. Functional significance of the existence of SP in the reciprocal connections is not clear. However, if both directions are excitatory, the possibility of positive feedback mechanism arises, producing an extremely sharp rise in system-output; rapid changes in LHRH secretion and behavioral responses. This system should be equipped with ending machineries, otherwise it works to be catastrophic. In this case, they could be ovulation and satiety, respectively in reproductive and ingestive responses. Alternatively, SP may play a modulatory role in the neural circuit. Barchas *et. al.* (1978) have proposed that SP, endorphin and some other neuropeptides act as neuromodulators, which affect transmitter synthesis, release, uptake, receptor interaction, and metabolism. Some results by Magnusson *et. al.* (1976) and Starr *et. al.* (1978) have shown that such postulated interaction could really exist between SP and the monoaminergic neurons. Since SP neurons contain NT (Shimada *et. al.*, 1988), prodynorphin (Neal *et. al.*, 1989) and possibly some other neurotransmitter including CGRP and galanin as suggested in Chapter 4-2, it is probable that SP modulates synthesis and/or release of these substances whereby a fine turning of the neural circuit is made.

Since the estrogenic induction of SP in the AVPv was observed only in female but not male rats, physiological significance of SP in this circuit seems to correlate to sexually different

functions. Most of reproductive responses have been known to be sexually dimorphic (Goy & McEwen, 1980). While intact rats show the LH surge on the afternoon of proestrus, or the surge can be induced by programmed steroids treatment in ovx rats, intact or gonadectomized rats are unable to exhibit the LH surge in response to exogenously injected steroid hormones (Brown-Grant, 1974, Feder, 1981). The present results strongly suggest that SP is involved in the mechanism of the LH surge probably acting on the LHRH neurons. Another representative sexually dimorphic response is lordosis behavior (Pfaff, 1980). Accumulated evidence indicates that the VMN is an important anatomical site of estrogenic regulation of lordosis behavior (Davis *et. al.*, 1976, Pfaff & Sakuma, 1979, Pfaff, 1980). Because fibers containing SP project from the VMN to the center of lordosis, the midbrain central gray (Dornan *et. al.*, 1990), and a direct injection of SP induces a rapid facilitation of lordosis behavior (Dornan *et. al.*, 1987), the estrogenic modulation of SP activity shown in this study and by others (Tsuruo *et. al.*, 1984, Parnet *et. al.*, 1990) probably play a key role in the establishment of this behavior.

In contrast to reproductive behavior, a sexual dimorphism of action of estrogen on feeding behavior has not been well-documented. However, withdrawal of androgens by orchidectomy has been shown to decrease weight gain and food intake in male rats (Leshner & Collier, 1973, Gentry & Wade, 1976), and these effects of orchidectomy can be reversed by treatment with testosterone (Gentry & Wade, 1976). These responses of male ingestive behavior to androgens is opposite to that of female rats, *i.e.* ovariectomy induces food intake and body weight gain (Tarttelin & Gorski, 1973) and the effects of ovariectomy are blocked by the estrogen replacement (Tarttelin & Gorski, 1973). Therefore, while effects of estrogen on male ingestive behavior are unclear, it is possible that it also shows a sexual dimorphism in terms of action of estrogen.

Although evidence for a direct action of SP on ingestive behavior within the VMN or LHA is not available, several reports have shown that this behavior is influenced by SP (Boyer *et.al.*, 1994, Cador *et.al.*, 1986, Hasenöhrl, *et.al.*, 1994). The effects of SP seems to be mediated through other neural structures than the VMN or LHA, which are implicated in feeding behavior such as the substantia nigra and the nucleus of the solitary tract as discussed in Chapter 4-2. Overall putative actions of SP on reproductive and ingestive functions in response to estrogen are summarized in Fig. 6-2. By acting as a neurotransmitter and/or neuromodulator, SP may relay information of estrogen to a variety of neural substrates that are involved in the expression and coordination of estrogen-induced responses.

CCK, another representative brain-gut peptide, has been known to be involved in the regulation of food intake (Della-Ferra & Baile, 1979, Morley *et. al.*, 1985) and reproductive function (Mendelson & Gorzarka, 1984, Vijyan *et. al.*, 1979). Injections of CCK into sheep cerebral ventricle suppress food intake (Della-Ferra & Baile, 1979), and hypothalamic CCK produces inhibition of lordosis behavior in female rats (Mendelson & Gorzarka, 1984). In the VMN, high levels of CCK have been observed (Beinfeld & Palkovits, 1981). The CCK content of the VMN varies between female and male rats and fluctuates during the estrous cycle (Frankfurt *et. al.*, 1985), suggesting that levels of CCK in the VMN may be affected by fluctuating levels of gonadal steroids. Thus, it is possible that CCK participates in the neural circuit between the estrogen sensitive nuclei shown for SP in Fig. 6-2. This is likely the case, since cell bodies containing CCK are present in the PVpo and central part of MPN (Simerly *et. al.*, 1986) and high levels of CCK were found in the VMN (Beinfeld & Palkovits, 1981). However, neither the MPOA nor the VMN contain neurons with both ER and CCK as presented in Table 1-1. Therefore, CCK cannot be a messenger which is directly regulated by estrogen in the neural circuit. The mechanism by which estrogen affect on activity of this peptide is not clear regardless of its estrogen-dependent effects on reproductive and ingestive behaviors. However, Akesson *et. al.* (1987) have been demonstrated that CCK binding in the VMN changes during estrous cycle, and suggested that ER neurons may also have receptors for CCK and estrogen directly modulates activity of these receptors in the VMN. They also have suggested that ER neurons may release a modulating factor which influences the binding of CCK in another population, since decreased binding of CCK was found throughout the VMN (Akesson *et. al.*, 1987) while ER neurons concentrated exclusively in the ventrolateral portion within the nucleus (Fig. 3-7B, Chapter 4-1). In this thesis, next interest was focused on NO as one of such modulating factors in the neural circuit.

3. Nitric Oxide as a Possible Modulator that Organizes Effects of Estrogen on Reproductive and Ingestive Behaviors

Nitric oxide is a peculiar substance, which has been recently identified as a novel messenger in the brain (for reviews, Bredt & Snyder, 1992, Schuman & Madison, 1994). It is

an unconventional transmitter which is not packed in vesicles but rather diffuses from its site of production in the absence of any specialized release machinery. As a gas lacking electrical charge and extremely membrane permeant (Goretski & Hollocher, 1988), NO can bypass normal signal transduction routes involving interaction with synaptic membrane receptors. Thus, effects of NO does not necessarily remain within the cell in which it is formed (Hope *et. al.*, 1991). It also can affect neighboring cells by the type of action known to be "volume transmission". Based on this, it should be clarified how the NO system exerts specificity of action in its regulatory tasks.

One of the critical components for the system to achieve such tasks might be the selective presence of the intracellular effector system as well as of the NOS. In Chapter 5, it was demonstrated that NOS is selectively localized in ER neurons in the MPOA, VMN and LHA. This selective localization of NOS and estrogen-dependent regulation of its activity within the hypothetical neural circuit strongly suggest that NO plays some essential roles by which information of circulating estrogen levels is translated into physiological responses (Fig. 6-3).

It has been shown that NO is involved in excitatory amino acid-induced cGMP production not only in the cerebellum (Bredt & Snyder, 1989) but also in neural cell line that represents a pure population of LHRH-secreting neurons (Moretto *et. al.*, 1993, Sortino *et. al.*, 1994). These observations indicate a link between excitatory amino acids and NO in regulating cGMP levels and suggest that NO is involved in the regulation of LHRH secretion by mediating the actions of excitatory amino acids (Moretto *et. al.*, 1993, Sortino *et. al.*, 1994). Bonavera *et. al.* (1993) also have demonstrated evidence *in vivo* that NO mediates the ovarian steroid-induced LH surge through excitatory amino acids actions, and in which they have proposed a following scheme; ovarian steroids facilitate a neural trigger for LHRH surge which, in turn, activates excitatory signals including excitatory amino acid release, and then the amino acid activates *N*-methyl-D-asparate (NMDA) receptors to produce an increase in NO efflux (Bonavera *et. al.*, 1993). This model seems to offer a functional significance to the association of ER with the NO system presented in this thesis. However, observed induction of NOS by estrogen in the MPN, VMN and LHA requires neither the mediation of "neural trigger" nor NMDA receptors but rather activity of NOS is induced by direct action of estrogen within ER containing neurons. Moreover, because very little guanylate cyclase is present in the hypothalamus (Matsuoka *et. al.*, 1992), the estrogen-dependent NO system in the neural circuit shown in Fig. 6-3 could be different from that participating in the control of LHRH release. In

this regard, it is noteworthy that progesterone but not estradiol enhances the vetridine-induced release of glutamate from synaptosomes in the MPOA (Flischmann *et. al.*, 1990), suggesting that progesterone can regulate the release of excitatory amino acid from this neuroendocrine tissue. Further, because function of SP has been shown to be linked with the NO-cGMP system in vascular endothelial cells (Chinkers & Garbers, 1991), the effect of estrogen on LHRH release may be mediated by this peptide in some regions of the brain.

As discussed above, the action of NO seems to be coupled with other second molecules than cGMP in the neural circuit proposed here. Those include kinases, enzymes, receptors for neurotransmitters and regulatory factors of gene translation (Fig. 6-3). For example, estradiol has been shown to elevate the level of cAMP concentration in the hypothalamus (Gunaga *et.al.*, 1974), and administration of cAMP elicits lordosis in ovx and estrogen-primed rats (Beyer & Gonzalez-Mariscal, 1986). Thus, one of target molecules for estrogen-induced NO in the hypothalamus may be adenylate cyclase. Moreover, because of its highly reactive properties, NO could interact with many chemical groups as well as with iron in molecules (Bredt & Snyder, 1992). This may account for a variety of actions of estrogen in the regulation of reproductive and ingestive behaviors, *e.g.* modulation of a number of neurotransmitters (see, Chapter 1-6), synthesis, release, turnover, metabolism and availability of receptors for those neurotransmitters (Dohanich *et. al.*, 1985). Although a putative estrogen responsible element up-stream the SP promoter has been suggested to exist (see discussion in ref. Brown *et. al.*, 1990a), at present any conclusive evidence for the presence of such estrogen responsible element in the 5' flanking region of genes for the neurotransmitters or receptors has yet to be identified. This further support the present hypothesis that actions of estrogen are mediated by molecules having a basic, multi-functional property, such as NO.

The multi-functionality must be necessary for the coordination of behaviors, which requires the elaboration of complex sequences of movements and postures, and a great deal of somatomotor integration. Because of its highly active nature, it may be possible that NO can modulate neural activities in the circuit by affecting on the membrane components such as hormone and metabolite receptors and ion channels by one hand, and on the synthesis/release of neurotransmitters such as SP and ENK by the other. The action of NO does not necessarily remain within the cell in which it is formed. Nitric oxide can affect neighboring cells by so-called volume transmission. This may be the case in the estrogenic modulation of CCK binding sites throughout the VMN as discussed above. Since estrogen is able to induce NO in the

MPOA, VMN and LHA, the components of the circuit and its surrounding structures can be simultaneously influenced by NO during estrus. Thereby the coordination of two behaviors may be achieved.

In summary, in order to explain the role of estrogen in the coordination of reproductive and ingestive responses, the structures in the brain cannot be divided into a mosaic center for respective behavior but rather the presence of the neural circuit which monitors and integrates estrogenic information should be necessary. As such a model, the neural connections between nuclei containing ER neurons was proposed (Fig. 6-1), in which the information may be amplified, stabilized, filtrated, and channeled. Although SP seemed to be one of potent candidates that constitute of the neural circuit (Fig. 6-2), the coordination of behaviors that requires the complex sequences of movements and neuroendocrine events cannot be explained solely by the action of SP. Molecule with more basic and multi-functional properties must participate in the circuit. With this concept, the involvement of NO in the hypothetical neural circuit was demonstrated (Fig. 6-3). By employing NO as a second messenger in the neural circuit, estrogen could simultaneously coordinate reproductive and ingestive behaviors.

In adult females, estrogen is periodically secreted from ovarian follicles in response to a series of changes such as growth of the follicles and ovulation in the ovary. Estrogen released in the circulation not only acts on peripheral tissues to induce their morphological and functional changes but also acts on the central nervous system to control the synthesis and/or release of hormones and neurotransmitters, and the expression of a variety of behaviors. These include reproductive behavior to preserve species and ingestive one to maintain individual. During estrus, when the estrogen levels are high, both behaviors are simultaneously regulated in a coordinated manner by estrogen. The coordination of two behaviors might be a reproductive strategy in the female to concentrate all elaborations on reproduction to successfully achieve the mating and thus the preservation of species. The present thesis provided morphological basis of neural mechanism underlying such coordination by estrogen, and suggests that the ERs in the neural circuit among the MPOA, VMN and LHA play essential roles in the coordination of two behaviors by means of inducing neurotransmitters and second messengers, such as SP and NO, in response to the circulating estrogen levels.

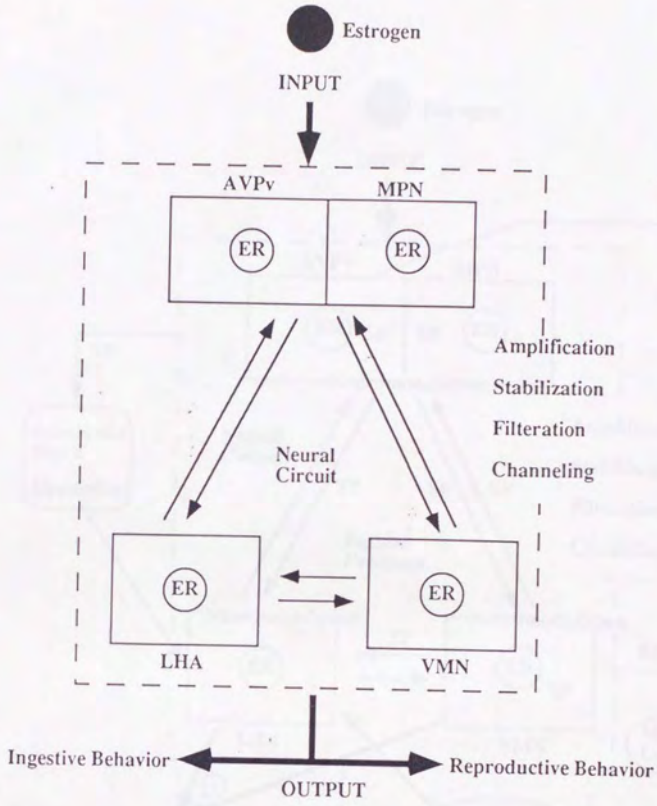

Figure 6-1. A model of neural circuit between ER containing nuclei

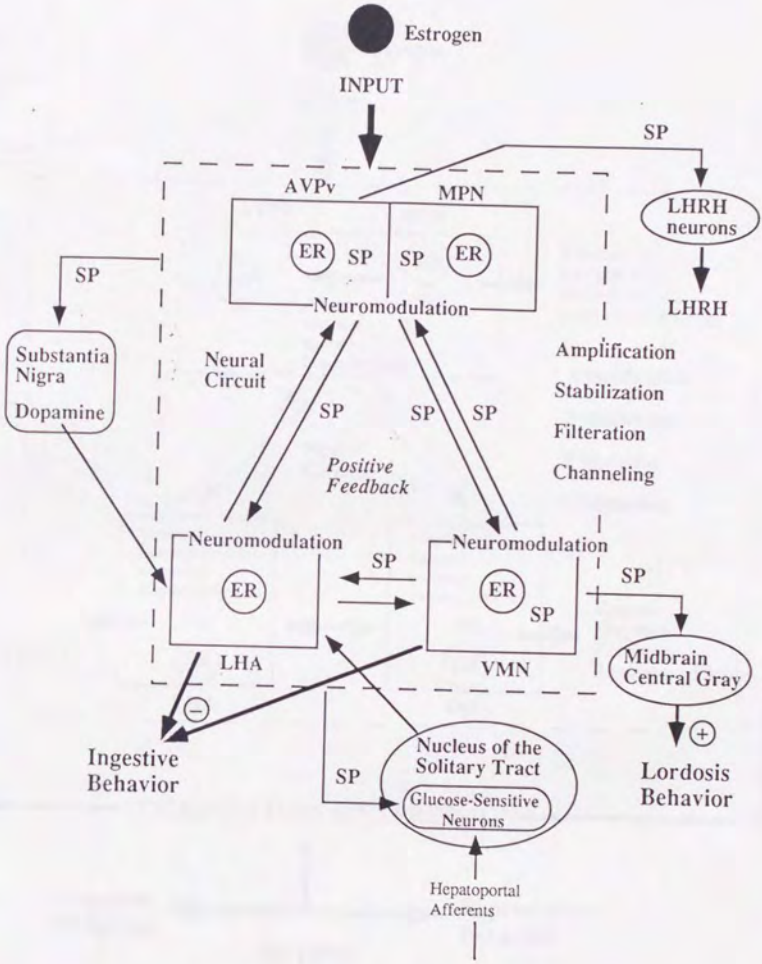

Figure 6-2. A model of estrogen mediated actions of SP in the neural circuit

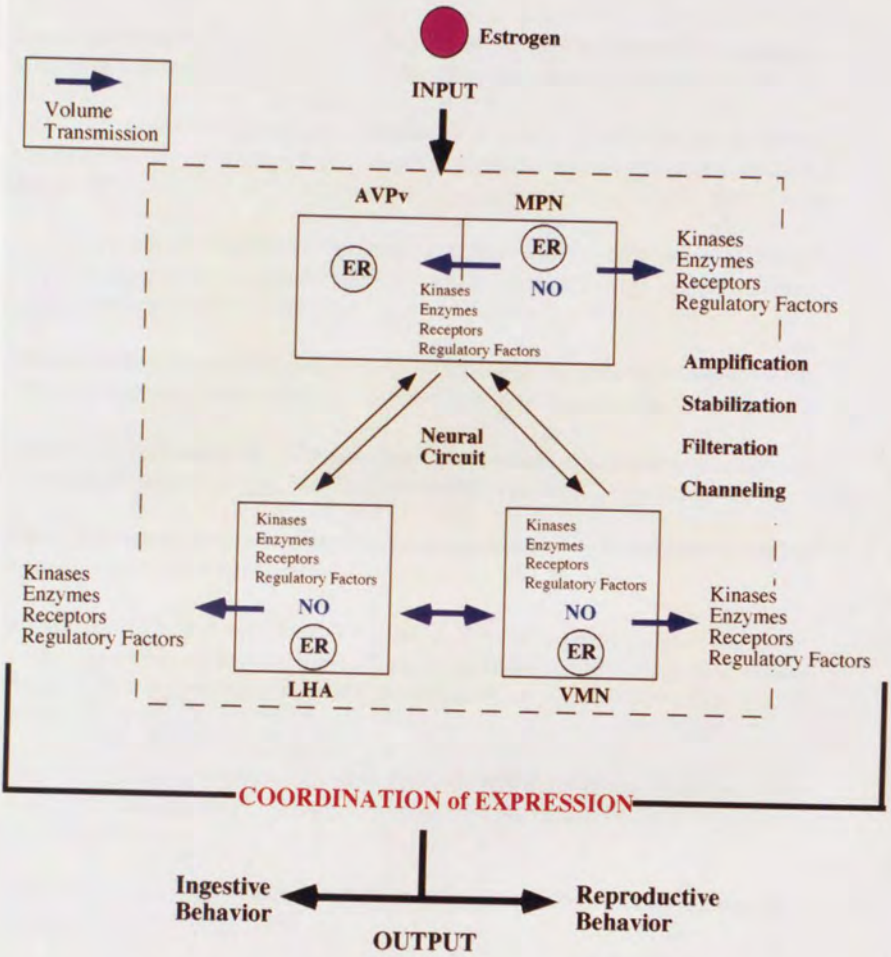

Figure 6-3. A model of estrogen mediated actions of NO in the neural circuit

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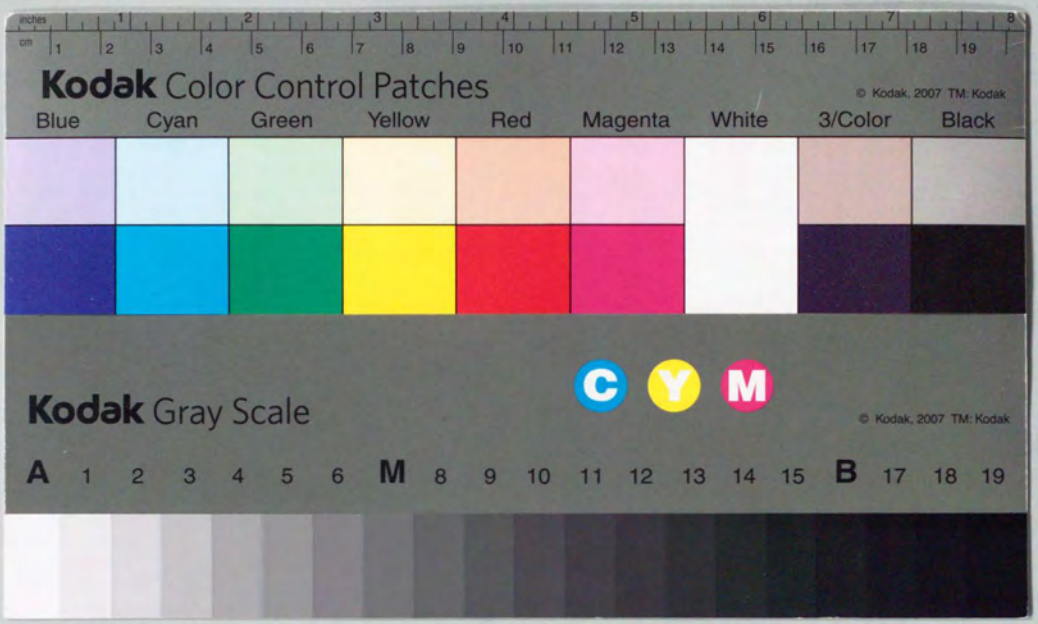
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