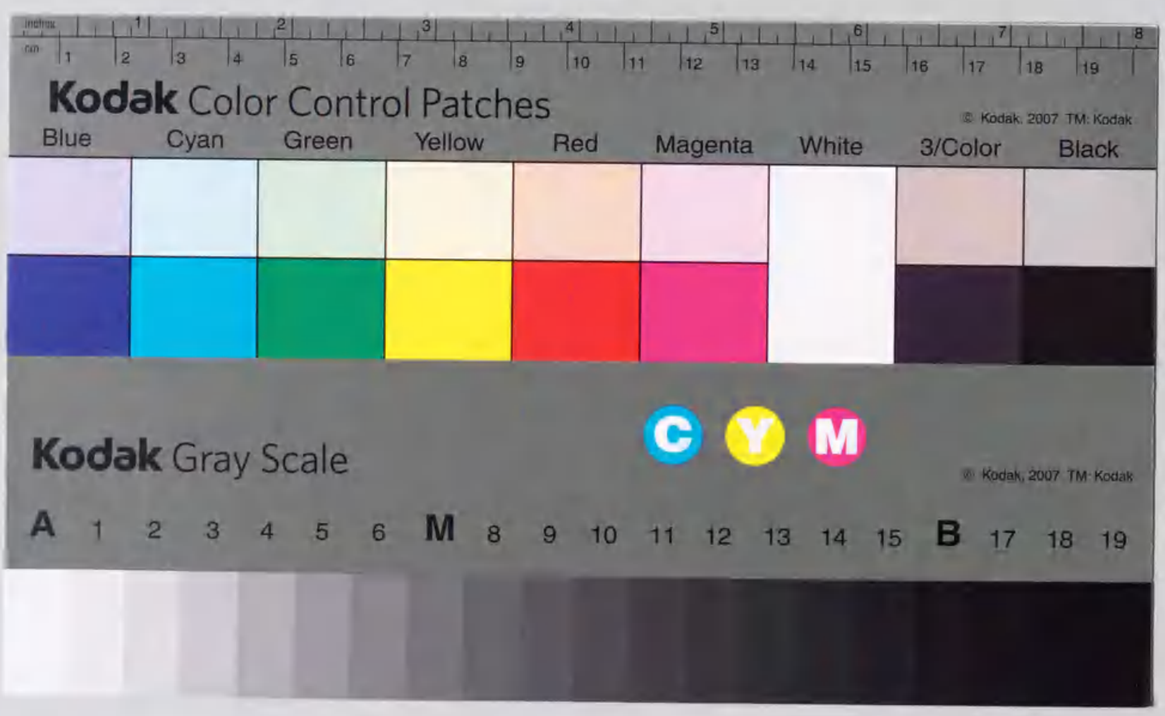
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The Hypothalamic Mechanisms Controlling Gonadotropin-Releasing Hormone Release in Rats: Pulse-Generating and Estrogen Feedback Mechanisms

by

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ABSTRACT

The present dissertation demonstrated the hypothalamic mechanisms controlling gonadotropin-releasing hormone (GnRH) secretion in rats. Two principal mechanisms for regulation of GnRH release are inherited in the hypothalamus. In Chapters 3 and 4, GnRH pulse-generating system was investigated to determine how pulsatile GnRH release is generated. The other principal mechanism is the feedback mechanisms of estrogen to modulate GnRH release. Chapters 5 and 6 was designed to determine the feedback mechanisms in the hypothalamus.

It has been considered that two functional components for GnRH pulse, synchronization of GnRH release and generation of an episodic pattern of GnRH release, are important to release the peptide in a pulsatile manner. It is reasonable that GnRH secretion is synchronized at the nerve terminal level in a non-synaptic fashion, since GnRH neuronal cell bodies are scattered over the hypothalamus and the nerve terminals are concentrated in the median eminence (ME), where few synaptic contacts with GnRH nerve terminals are found. Chapter 3 first describes to reveal morphological relationships between gap junctions and GnRH nerve terminals in castrated male rats to explore the possibility that an intercellular signal transmission through gap junction plays a role in the synchronization of GnRH pulse at the terminal level. In the ME, gap junctions composed of connexin 43 protein were found in most astrocytes, and some of which in GnRH nerve terminals. In the second experiment of Chapter 3, local administration of gap junction blocker into the ME slowed down pulsatile luteinizing hormone (LH) secretion, but did not disrupt LH pulses in castrated male rats. These results suggests that gap junctional communication at the GnRH nerve terminals is involved in the synchronization of GnRH release but not in the pulse generation.

Chapter 4 deals with another important issue: How are GnRH pulses generated? It is still unknown whether the mechanism generating GnRH pulse is in GnRH neurons themselves or is composed of non-GnRH neurons in the hypothalamus. In the chapter, the primary culture containing fetal GnRH neurons was established as an *in vitro* model for the GnRH pulse-generating system and GnRH secretory pattern was monitored. GnRH secretory pattern showed an episodic pattern in the primary culture originating from either the rat fetal mediobasal hypothalamus, whole hypothalamus, medial preoptic area, or cerebral cortical regions. This finding suggests that GnRH neurons themselves have an intrinsic activity to release GnRH in a pulsatile manner regardless of the difference in the origin of GnRH neurons. The activity of GnRH neurons themselves could be important for GnRH pulse generation.

Taken all findings in Chapters 3 and 4 together, the GnRH pulse-generating system in the hypothalamus could be composed of the GnRH neurons having an intrinsic activity for episodic release of GnRH and the mechanism synchronizing the activities in each GnRH neuron at the nerve terminal level.

Beside of the GnRH pulse-generating system, positive and negative feedback mechanisms of estrogen are important for stimulation and inhibition of GnRH release, respectively. Chapter 5 was designed to reveal the estrogen feedback mechanisms in the arcuate nucleus (ARC), one of the estrogen feedback sites to modulate GnRH release at the nerve terminal level. Specific aims in this chapter are to determine whether nitric oxide (NO), a messenger molecule mediating glutamate actions, mediates the stimulatory effect of glutamate on *in vitro* GnRH release from the ARC-ME tissue fragment, and whether estrogen modulates the NO action. Glutamate induced GnRH release from the tissue fragments taken from estradiol (E_2)-primed ovariectomized

(OVX) rats. The glutamate-induced GnRH release was reduced by blocking NO production or actions. To the contrary, in the OVX group, glutamate did not increase GnRH release, but the release was stimulated by blocking NO production or actions. These results demonstrated that NO has both stimulatory and inhibitory effects on glutamate-induced GnRH release, and that estrogen alters the NO actions. These results suggest that the NO actions involved in the dual feedback effects of estrogen in the ARC.

In addition to the dual estrogen feedback effects to monitor reproductive cycles, estrogen has other functions involving the increased response of hypothalamic mechanisms suppressing GnRH release to stressful factors. The functions of estrogen are called novel feedback effects of estrogen, which is important for adaptation of reproductive functions to stressful conditions. Chapter 6 is aimed to determine the novel feedback actions in the paraventricular nucleus (PVN), an action site of estrogen. The PVN contains many corticotropin-releasing hormone (CRH) neurons mediating suppressive effects of stressful factors on pulsatile GnRH/LH secretion. In the chapter, effects of intracerebroventricular injection of CRH antagonist on the glucoprivic suppression of pulsatile LH secretion are investigated in both OVX rats and E_2-treated OVX rats. Pulsatile LH secretion was suppressed by glucoprivation in control animals bearing E_2 implants. The CRH antagonist blocked the suppressive effect of glucoprivation on pulsatile LH secretion in the E_2-treated OVX animals. On the other hand, in the absence of estrogen, the effect of glucoprivation was not blocked by the CRH antagonist. These results suggest that the mechanisms mediating glucoprivic suppression of LH secretion involve two components: One is estrogen-dependent and the other estrogen-independent. Estrogen could activate the estrogen-dependent component, in which CRH plays a role in mediating glucoprivic suppression of GnRH/LH secretion.

Chapters 5 and 6 suggest that estrogen widely affects the hypothalamic mechanisms to modulate GnRH release, and estrogen alters and/or activates the functions of the already-inherited hypothalamic mechanisms. Accordingly, the role of the estrogen feedback actions is to give additional functions to them. GnRH secretion is, thus, modulated by estrogen feedback actions.

Estrogen feedback mechanisms have cooperative functions with the GnRH pulse-generating system to control neuroendocrine functions for reproductive events in the hypothalamus. Pulsatile GnRH secretion is regulated by the GnRH pulse-generating system. Although the system fulfills its function even without influence of gonadal steroids, GnRH/LH release is modulated by the estrogen feedback mechanisms. Both GnRH pulse-generating system and estrogen feedback mechanisms contribute to homeostasis of the reproductive system and flexibility of neuroendocrine control of reproduction in the brain.

CONTENTS

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LIST OF ABBREVIATIONS

ARC	arcuate nucleus
cGMP	cyclic guanosine 3', 5'-monophosphate
CRH	corticotropin-releasing hormone
2DG	2-deoxyglucose
E_2	estradiol-17β
EAA	excitatory amino acid
ER	estrogen receptor
GFAP	glial fibrillary acidic protein
GnRH	gonadotropin-releasing hormone
Hb	hemoglobin
icv	intracerebroventricular
LH	luteinizing hormone
MBH	mediobasal hypothalamus
ME	median eminence
mPOA	medial preoptic area
NDS	normal donkey serum
NGS	normal goat serum
NMDA	*N*-methyl-D-aspartate
NMMA	N^G-monomethyl-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide-Y
OVX	ovariectomized
PB	phosphate buffer

PBS	phosphate buffered saline
PVN	paraventricular nucleus
RIA	radioimmunoassay
SD	standard deviation
SNP	sodium nitroprusside

CHAPTER 1

General introduction

Neuroendocrine control of reproduction

It has been well established that the brain controls endocrine glands such as the gonads and thyroid and adrenal glands and their regulation is achieved through the pituitary hormones. During 1930s, endocrine control of the endocrine glands is still unknown. The first scientist who predicted that the brain controls the endocrine glands is G. W. Harris. He described a concept that the neurohumoral factors in the brain regulate the pituitary gland functions and in turn control the endocrine glands in his book "Neural Control of the Pituitary Gland" in 1955 (Harris, 1955). It is not too much to say that the field of the neuroendocrinology has started with his works.

Harris's work is followed by the discovery of some neuropeptide regulating the pituitary hormone secretion. Of these hypothalamic peptides, gonadotropin-releasing hormone (GnRH) was simultaneously discovered as the neuropeptide to stimulate gonadotropin release from the anterior pituitary by both Guillemin's and Schally's laboratories in 1971. They isolated and purified the neuropeptide from the porcine (Matsuo *et al.*, 1971; Schally *et al.*, 1971) and ovine (Amoss *et al.*, 1971) hypothalamus. GnRH-producing neurons have some distinguished characteristics: GnRH neurons migrate from the olfactory placode to the forebrain at the late phase of embryogenesis (Schwanzel-Fukuda & Pfaff, 1989); and they are distributed in the septo-preoptic region or hypothalamus without forming an apparent nucleus (Kawano & Daikoku, 1981; Witkin *et al.*, 1982). However, our knowledge are not enough for understanding the neuroendocrine control of reproduction in the central nervous system.

Our understanding of the neuroendocrine control of reproduction obtained from basic research is not only important to expose the mystery of the life but also useful for establishing therapy for reproductive disorders, which are caused

by failure of the functions of the reproductive center in the brain, in domestic animals and human.

Estrogen, a sex steroid hormone modulating neuroendocrine control of reproduction in the brain

A. A. Berthold had first succeeded experiments in reproductive neuroendocrinology in 1849 (Berthold, 1849). He found that male chickens developed as capons do not show several characteristic sexual behavior of roosters and the sexual behavior disrupted is recovered by transplantation of testis to castrated cockerels. Berthold's experiment suggests that testis produces a humoral substance involving male sexual behavior. The substance is one of sex steroid hormones produced by gonads. It has been well demonstrated that sex steroid hormones synthesized by gonads have functions to modulate the neuroendocrine mechanism for reproduction. The steroids are also important hormone for reproductive functions as well as GnRH and gonadotropin, but the brain mechanisms modulated by sex steroids are still unclear.

Estrogen is one of the sex steroid hormones synthesized in the ovary. Estrogen is involved not only in the neuroendocrine control of reproduction, but also a variety of brain functions regulating growth, differentiation, and memory processes (Kawata, 1995). Estrone is the first purified estrogen in 1929 (Doisy *et al.*, 1929), and the finding was followed by the identification of estriol (Marrian, 1930a; Marrian, 1930b) and estradiol (Schwenk & Hildebrandt, 1933) .

In addition to the discovery of these bio-active estrogen from organisms, some of numerous chemical products artificially synthesized are known to mimic estrogen-like actions in the body and be endangering continuation of species. Recently, our anxiety about the disturbance of endocrine regulation for

reproduction with these chemicals called "endocrine disrupter" has been growing, since T. Colborn *et al.* has published a book, "Our Stolen Future", in 1996 (Colborn *et al.*, 1996). We must, therefore, think back on our life style and reveal mechanism of estrogenic actions to devise a countermeasure against these substances.

The hypothalamus is the center of the hypothalamo-pituitary-gonadal axis

Reproductive events in mammals are regulated by a neuroendocrine system called the hypothalamo-pituitary-gonadal axis. The activity of the axis is further regulated by the higher brain regions. The hypothalamus produces and releases GnRH, a key peptide to maintain gonadal activity by regulating pituitary functions. Most of the GnRH neuronal cell bodies are located in the medial preoptic area (mPOA), diagonal band of Broca and medial septum in the rat brain and were scattered over these regions without forming an apparent nucleus (Kawano & Daikoku, 1981; Witkin *et al.*, 1982). Most of the GnRH neuronal terminals are projecting to the median eminence (ME) and organum vasculosum of the lamina terminalis. GnRH is secreted from the nerve terminals in the ME and transported to the anterior pituitary through the hypophysial portal system to facilitate gonadotropin secretion from gonadotrophs in the anterior pituitary.

Gonadotropin is involved in various gonadal activities, such as follicular development, ovulation and luteinization in ovary, spermatogenesis in testis and steroidogenesis in both gonads. The sex steroids are released to the general circulation to act on the hypothalamus and pituitary to modulate GnRH/gonadotropin secretion. Gonadotropin also modulate GnRH secretion from the hypothalamus. The actions of the sex steroids and gonadotropin are called "feedback action". The regulatory functions of the hypothalamus in the

hypothalamo-pituitary-gonadal axis is, thus, modulated by hormones released from both the pituitary and gonads.

The functions of the hypothalamus are also modulated by external and internal environmental factors such as pheromone (Mori & Takeuchi, 1998), photoperiod, nutrition and suckling stimulus (Maeda, 1998). A variety of information emanating from the environmental factors is finally conveyed to the hypothalamus. The hypothalamus integrates the information and transduces them to the secretory pattern of GnRH. Consequently, reproductive functions would be adapted to a new environment. For example, malnutrition is known to suppress reproductive functions: Luteinizing hormone (LH), a gonadotropin, secretion from the pituitary is suppressed by fasting in humans (Cameron *et al.*, 1991), rhesus monkeys (Campbell *et al.*, 1977), and rats (Maeda & Tsukamura, 1996) followed by a reduction of mating and pregnancy rates (McClure & Saunders, 1985). One of the pathways conveying fasting signals emanating from the upper digestive tract to the hypothalamus involves the noradrenergic neuron originating in the medulla oblongata and projecting to the paraventricular nucleus (PVN) to stimulate corticotropin-releasing hormone (CRH) release and suppress LH secretion (Maeda *et al.*, 1994).

Pulsatile GnRH release is essential for the regulation of reproductive functions

It has been well demonstrated that tonic or basal secretion of GnRH shows a pulsatile pattern in many mammalian species including rats (Levine & Ramirez, 1980) and sheep (Clarke & Cummins, 1982; Moenter *et al.*, 1992). It has been also reported that LH is secreted from the pituitary in a pulsatile fashion in rats (Gay & Sheth, 1972), monkey (Dierschke *et al.*, 1970) and sheep (Clarke & Cummins, 1982; Moenter *et al.*, 1992). The pulsatile release of GnRH and

LH is well correlated with each other (Clarke & Cummins, 1982; Moenter *et al.*, 1992). The pulsatile pattern of GnRH secretion has been considered to be indispensable for the maintenance of the pulsatile LH secretion and consequent gonadal activities, such as follicular growth, spermatogenesis and steroidogenesis, since pulsatile LH secretion impaired by a hypothalamic lesion was restored by an intermittent systemic administration of an exogenous GnRH, but not by a constant infusion in rhesus monkeys (Belchetz *et al.*, 1978). This result clearly indicates that GnRH pulse is important for controlling reproductive functions rather than the level of GnRH released. It has been postulated that pulsatile GnRH secretion is generated by a hypothalamic mechanism called "GnRH pulse generator" (Dyer & Robinson, 1989; Knobil, 1992; Maeda, 1990), the details of which are largely unknown.

GnRH pulse-generating mechanism in the hypothalamus

The GnRH pulse generator is considered to be located in the mediobasal hypothalamus (MBH), since pulsatile LH secretion is not impaired by the isolation of MBH from the rest of the brain by means of deafferentation in ovariectomized (OVX) rats (Blake & Sawyer, 1974; Ohkura *et al.*, 1991; Soper & Weick, 1980). *In vitro* GnRH release from the MBH tissue fragment shows pulsatile pattern in human (Rasmussen *et al.*, 1989) and guinea pig (Giri & Kaufman, 1994). It was further reported that GnRH is released from the rat MBH tissue fragment in a pulsatile manner, but not from the rat mPOA tissue fragment (Purnelle *et al.*, 1997). Pulsatile LH secretion abolished by the posterior-anterior hypothalamic deafferentation, which cuts off the anterior part of the arcuate nucleus (ARC) from the MBH, was restored by transplanting the fetal MBH tissue into the third cerebral ventricle (Ohkura *et al.*, 1992). These

reports strongly support the idea that GnRH pulse-generating mechanism exists in the MBH.

It is likely that GnRH pulse is synchronized at the nerve terminal level rather than the neuronal cell body level. Most of the GnRH nerve terminals are concentrated in the lateral portion of the external layer of the ME and GnRH neuronal cell bodies are scattered over the mPOA (Kawano & Daikoku, 1981; Witkin *et al.*, 1982). LH pulses are abolished by either bilateral electrolytic lesions of the anterior part of the ARC combined with anterior hypothalamic deafferentation (Soper & Weick, 1980) or the posterior-anterior hypothalamic deafferentation (Ohkura *et al.*, 1991). It has been further reported that *in vitro* GnRH release from the rat ME fragment still shows an episodic pattern (Rasmussen, 1993). All of these reports suggest that synchronization of GnRH pulse is regulated at the GnRH nerve terminals in the ME by a mechanism in the MBH. Since GnRH-immunopositive nerve terminals, or GnRH-immunopositive nerve terminal and other nerve terminal has been observed to make few synaptic contacts in the rat ME (Kawakami *et al.*, 1998), synchronization of GnRH pulses may be regulated at the nerve terminal level in a non-synaptic fashion.

Two different possibilities on the mechanism generating GnRH pulses have been proposed: (1) The GnRH neuron itself has an intrinsic rhythm to generate GnRH pulses; (2) Non-GnRH neurons periodically stimulate GnRH neurons for the pulsatile GnRH release. The first possibility is supported by the following evidence. *In vitro* GnRH release from the GT1-7 cells, an immortalized GnRH neuronal cell line, (Krsmanovic *et al.*, 1992; Wetsel *et al.*, 1992) or primary culture of GnRH neurons purified from the rat hypothalamus (Melrose *et al.*, 1987) shows a pulsatility. On the other hand, several lines of evidence indicate the second possibility. Several neurotransmitters were reported to play an important role in regulation of GnRH pulse. Pulsatile LH secretion is

suppressed by an intracerebroventricular (icv) injection of selective excitatory amino acids (EAAs) receptor antagonist (Ping *et al.*, 1995) or nicotinic acetylcholine receptor antagonist (Kalash *et al.*, 1989) in gonadectomized rats. Transplantation of the fetal MBH, which dose not contain GnRH neurons, restored LH pulse in OVX rat bearing the posterior-anterior hypothalamic deafferentation (Ohkura *et al.*, 1992). These reports suggest the possibility that GnRH pulse is regulated by non-GnRH neurons.

In addition to two possible mechanisms generating GnRH pulses, it is also possible that an ultra short feedback action of GnRH neurons to themselves is involved in GnRH pulse generation, since the multiple unit volleys in the ME, an indicator of the activity of pulsatile GnRH release, are evoked by microinjection of GnRH peptide into the ME but not into the mPOA (Hiruma & Kimura, 1995). This result suggests that pulsatile GnRH release is modulated by an ultra short feedback action of the peptide at the nerve terminals.

Feedback effects of estrogen on the modulation of GnRH/LH secretion

The secretion of GnRH/LH is controlled by the feedback actions of sex steroid hormones. In addition to basal and tonic GnRH/LH secretion showing a pulsatile pattern, another mode of GnRH/LH secretion, the surge mode, is important for the regulation of reproduction in female mammals. In females, estrogen synthesis in granulosa cells increases as the follicles growing during the estrous cycle. The high level of estrogen produced by the mature follicles plays a critical role in inducing a large amount of GnRH/LH release known as "GnRH/LH surge" (Fox & Smith, 1985; Hauger *et al.*, 1977; Moenter *et al.*, 1992; Norman *et al.*, 1984; Rahe *et al.*, 1980; Sarkar *et al.*, 1976), which is followed by ovulation. The GnRH/LH surge is known to also be induced by the

exogenous estrogen treatment in monkeys (Pau *et al.*, 1988; Xia *et al.*, 1992), sheep (Clarke, 1993; Moenter *et al.*, 1992) and rats (Sarkar *et al.*, 1976). The stimulatory effect of this high level of estrogen on GnRH/LH release has been called the "positive feedback action".

On the other hand, the low level of estrogen synthesized by developing follicles tonically inhibits the pulsatile GnRH/LH secretion during the luteal or diestrous phase of the reproductive cycles. The pulsatile GnRH/LH release is increased by ovariectomy, and estrogen replacement reverses the effect of ovariectomy by decreasing GnRH/LH secretion (Chongthammakun *et al.*, 1993; Freeman, 1994; Goodman, 1994; Goodman & Knobil, 1981). This inhibitory action of estrogen tonically suppressing GnRH/LH secretion is called "negative feedback action". The hypothalamus thus employs the positive and negative feedback actions of estrogen to monitor ovarian status which shows drastic changes throughout the reproductive cycles.

Estrogen is considered to have another type of feedback actions to modulate the activity of the reproductive axis in addition to the above-mentioned dual feedback actions of the steroid. The feedback actions would be involved in the modulation of GnRH/LH secretory responses to environmental factors. The existence of this kind of feedback action is based on the following evidence: The suppressive effect of fasting stress on LH secretion is estrogen-dependent (Cagampang *et al.*, 1991; Cagampang *et al.*, 1990), and pharmacological reduction of glucose availability suppresses LH secretion in the presence or absence of sex steroids in both genders with steroid-treated animals being more sensitive to the glucoprivation (Nagatani *et al.*, 1996). The action of estrogen in these cases is defined as "novel feedback action of estrogen" (Maeda *et al.*, 1996). In the light of the evidence, the novel feedback action of estrogen in female mammals would contribute to obtain a strategy for reproduction. In other

words, reproductive functions are ceased in order to take precedence of their survival when individuals encounter stressful conditions such as malnutrition.

Estrogen feedback sites and its actions in the hypothalamus and anterior pituitary

It is generally considered that feedback actions of estrogen are exerted through the binding of the steroid to the estrogen receptor (ER). Two isoforms of ER identified in the brain and anterior pituitary belong to the nuclear receptor superfamily. In this regard, genomic actions of estrogen, thereby, act as the feedback actions of the steroid. Recently, an isoform has been cloned from the cDNA library of rat prostate (Kuiper *et al.*, 1996), mouse ovary (Tremblay *et al.*, 1997), and human testis (Moore *et al.*, 1998). The novel receptor is termed as ERβ to distinguish it from the classical ER, ERα, previously cloned from rat uterus (Koike *et al.*, 1987). Amino acid sequences of both ERs show a high homology (Kuiper *et al.*, 1996), but the distributions of both receptors show different pattern. A large number of ERβ-immunoreactivities is distributed in the hypothalamic regions such as the PVN and supraoptic nucleus (Li *et al.*, 1997; Österlund *et al.*, 1998; Shughrue *et al.*, 1997). On the other hand, hypothalamic ERα-immunoreactivities are mainly located in the ventromedial hypothalamic nucleus and periventricular nucleus (Österlund *et al.*, 1998; Shughrue *et al.*, 1997). The other hypothalamic regions such as the mPOA and ARC have both types of ER with the number of ERβ-immunoreactive cells being less than that of ERα-immunoreactive ones. Region-specific expression of ERα and/or ERβ suggests that estrogen acts in a variety of the hypothalamic region through both receptors to modulate GnRH secretion. In the anterior pituitary, gonadotrophs

have ERα and/or ERβ with ERβ expression being slightly less than ERα expression (Mitchner *et al.*, 1998).

The anterior pituitary is one of the most important target sites of the estrogen feedback actions, where it has both positive and negative feedback actions. Indeed, estrogen is known to have stimulatory and inhibitory effects on LHβ subunit gene transcription (Abbot *et al.*, 1983; Shupnik *et al.*, 1989), indicating that genomic actions of estrogen are involved in dual feedback mechanisms at the anterior pituitary level. It is also possible that responsiveness of the anterior pituitary to GnRH changed by estrogen are related with the feedback mechanisms, since it has been demonstrated that the responsiveness to GnRH are related to the stage of the estrous cycle with maximal pituitary responsiveness occurring on proestrus in rats (Cooper *et al.*, 1975).

In the hypothalamus, it has been generally accepted that the primary site for the positive feedback action of estrogen is the mPOA, since local estrogen implant into the mPOA induces LH surge in OVX rats (Goodman, 1978). The negative feedback site is thought to be located in the MBH in rats and monkeys (Goodman & Knobil, 1981), since estrogen administration into this area has been reported to decrease LH secretion in OVX rhesus monkeys (Ferin *et al.*, 1974). Recently, Caraty *et al.* has reported that a local implantation of estrogen into the ventromedial hypothalamic nucleus induces GnRH surge as well as LH surge, but the implantation into the mPOA dose not induce these surges in the ewe (Caraty *et al.*, 1998). In this context, the positive or negative feedback sites of estrogen in the hypothalamus are still not clearly determined yet. In addition, the brain regions, where ERβ-immunoreactivities were recently found, could also be the sites of estrogen feedback action.

While estrogen activates some gene transcriptions followed by increases in protein synthesis, several researchers have proposed that estrogen has not only genomic actions but also non-genomic actions. Non-genomic actions of estrogen could be characterized as a more rapid process without gene transcriptions (McEwen, 1991; Schumacher, 1990). Estrogen directly changes the excitability of rat medial amygdala neurons within minutes without protein synthesis (Nabekura *et al.*, 1986). Furthermore, it has been reported that estrogen directly inhibits electrical activity of GnRH neuron by opening a potassium channel in guinea pig hypothalamic slice culture (Lagrange *et al.*, 1995). Thus, both genomic and non-genomic actions of estrogen could mediate its positive and negative feedback effects to control GnRH/LH secretion at the level of the hypothalamus and anterior pituitary.

The ARC is one of the estrogen feedback sites to modulate GnRH release at the nerve terminal level

The ARC is well known as one of the estrogen feedback sites, because both ERα and ERβ are located in the nucleus (Österlund *et al.*, 1998; Shughrue *et al.*, 1997). Estrogen seems to modulate the neuronal outputs from the ARC to regulate GnRH secretion at the nerve terminal level, because most of the ER-immunoreactive neurons projecting to the ME are located in the nucleus in the ewe (Jansen *et al.*, 1996). Feedback actions of estrogen in the ARC could be brought about by non-GnRH neurons expressing ERs, since few GnRH neurons locate in the rat ARC (Kawano & Daikoku, 1981; Witkin *et al.*, 1982). Indeed, few GnRH neurons express ERs (Shivers *et al.*, 1983; Watson *et al.*, 1992), suggesting that modulation of GnRH release with estrogen is carried out by indirectly actions of estrogen.

Several neurotransmitters has been considered to be involved in mediating the feedback action of estrogen in the ARC. Opioid peptides (Jirikowski *et al.*, 1986; Morrell *et al.*, 1985) and tyrosine hydroxylase (Sar, 1984), a rate-limiting enzyme for the catecholamines synthesis, are localized in ER-containing ARC neurons in rats. It was reported that dopamine turnover rate in the ARC is elevated by estrogen compared with OVX rats (Wise *et al.*, 1981), suggesting that activities of dopaminergic neurons in the ARC are modulated by estrogen. It is likely that estrogen affects opioidergic neurons through ER in the ARC to induce LH surge, since opioid peptides have an inhibitory effect on LH surge induced by estrogen (Gabriel *et al.*, 1986). Neuropeptide-Y (NPY) neurons in the ARC could also be involved in inducing preovulatory LH surge, since NPY-immunoreactivities are located in the ARC neurons having ERs in rats (Sar *et al.*, 1990); NPY mRNA level in the ARC increases during proestrus in female rats (Bauer-Dantoin *et al.*, 1992); and *in vitro* GnRH release from the rat ME is induced by NPY in an estrogen dose-related manner (Sabatino *et al.*, 1989). Furthermore, galanin neurons in the ARC is considered to play an important role in the effects of NPY on preovulatory LH surge in rats. Galanin itself was reported to be involved in preovulatory LH surge, because icv injection of galantide, a specific galanin receptor antagonist, blocks LH surge during proestrus in female rats (Sahu *et al.*, 1994), and galanin neurons also express ERs in the rat ARC (Horvath *et al.*, 1995). It has been revealed that most of the NPY neuronal terminals establish synaptic connections with galanin neurons in the rat ARC, and LH secretion increased by icv injection of NPY is attenuated by galantide in estrogen-primed OVX rats (Horvath *et al.*, 1996). This report indicates that effects of NPY on LH surge are mediated by galanin neurons in the ARC. Taken together, these reports support that a part of the estrogen feedback

actions is to modulate these neurotransmitter releases from the ARC in order to modulate GnRH release at the GnRH nerve terminal in the ME.

EAA is considered as one of the most important neurotransmitters involved in the estrogen feedback actions in the hypothalamus. It has been reported that subcutaneous administration of MK801, a specific antagonists for *N*-methyl-D-aspartate (NMDA) receptor, led to a attenuation of both preovulatory and gonadal steroid-induced LH surge in female rats (Brann & Mahesh, 1991). EAA is also involved in pulsatile LH secretion, since icv infection of a specific NMDA receptor antagonist or non-NMDA receptor antagonist suppresses pulsatile LH secretion in both genders (Ping *et al.*, 1994; Ping *et al.*, 1995). These actions of EAA would be exhibited by controlling GnRH secretion at the hypothalamic level but not by directly controlling LH secretion in the anterior pituitary, since EAA agonists do not stimulate *in vitro* LH release from the rat pituitary (Schainker & Cicero, 1980), and pretreatment of GnRH antagonist invalidates the stimulatory effect of EAA on LH secretion in rhesus monkeys (Gay & Plant, 1987).

It is most likely that the ARC is an action site of EAA involving feedback actions of estrogen to modulate GnRH release at the nerve terminal level. It was revealed that kainate receptors (Diano *et al.*, 1998) and *dl*-α-amino-3-hydroxy-5-methyl-4-isoxazone-propionate receptors (Diano *et al.*, 1997), subtypes of EAA receptor, are colocalized with ER in the rat ARC neurons. In addition, estrogen has a stimulatory effect on the expression of EAA receptors in the hypothalamus (Diano *et al.*, 1997). These facts might support the idea that estrogen alters EAAergic neuronal activity in the ARC to modulate GnRH release from the nerve terminals. It was well demonstrated that EAAs themselves have potent actions to facilitate GnRH release, since glutamate, an endogenous EAA, and its

agonists induce *in vitro* GnRH release from ARC-ME fragments taken from intact male rats and the glutamate-induced GnRH release is blocked by its antagonists *in vitro* (Donoso *et al.*, 1990; López *et al.*, 1992). Of the stimulatory effects of EAA on GnRH release, it is included that EAAs directly act on the GnRH nerve terminals to release the peptide, since NMDA receptor- and kainate receptor-immunoreactivities are colocalized with GnRH-immunoreactivity in nerve terminals of the ME (Kawakami *et al.*, 1998), and glutamate and its agonists stimulate GnRH release from the rat ME fragments (Maeda *et al.*, 1995).

In the light of all findings, EAAergic neurotransmission could play an important role in estrogen feedback actions to modulate GnRH release at the nerve terminal level. It is possible that EAAs activate stimulatory and/or inhibitory neuronal activities to release neurotransmitters including the above-mentioned neurotransmitters in the ARC for estrogen to modulate GnRH release at the nerve terminals. However, signal transduction mechanisms of EAAergic neuronal activities in the region and an involvement of estrogen on the mechanism are remained to be revealed.

The novel feedback effects of estrogen on the modulation of GnRH/LH secretion with stressful factors

It is well considered that reproductive functions of mammalian species are strongly depressed when animals are exposed to a variety of stressful factors. It has been demonstrated that starvation, a kind of the stress for animals, apparently decrease mating, pregnancy, and fertility rates in female rats (McClure & Saunders, 1985) and shows loss of body weight and organ weights of the anterior pituitary and accessory reproductive organs in male rats (Campbell *et al.*, 1977). These effects of stressful factor on the reproductive axis

are due to the suppression of GnRH/gonadotropin secretion, since it has been reported that pulsatile LH secretion is inhibited by 48-h fasting stress in monkey (Cameron *et al.*, 1991) and by electric foot shock stress in rats (Rivier *et al.*, 1986). Furthermore, it is well accepted that the suppression of GnRH/LH secretion is due to the CRH release activated by stressful factors, since electric foot shock stress-induced inhibition of pulsatile LH secretion is blocked by icv injection of α-helical CRF, a CRH antagonist (Rivier *et al.*, 1986), and the antagonist also reinstates suppressed pulsatile LH release in 48-h fasted rats (Maeda *et al.*, 1994). CRH mRNA in the PVN also increased in response to hypertonic saline injection, restraint and swim stress (Harbuz & Lightman, 1989). In this context, CRH is one of the primary physiological mediators of stress responses. It was further revealed that CRH actually inhibits GnRH/LH release (Gambacciani *et al.*, 1986; Nikolarakis *et al.*, 1986; Petraglia *et al.*, 1987; Rivier & Vale, 1984), followed by inhibition of ovulation and pregnancy (Rivier & Vale, 1984).

Several lines of evidence suggest that the CRH neuronal activities are modulated by gonadal steroids. It was reported that the primary transcript for CRH is increased by endotoxin lipopolysaccharide and *c-fos* mRNA is increased in CRH-immunoreactive cells on the morning of proestrus in female rats (Nappi *et al.*, 1997). Furthermore, both corticotropin release and corticosterone release in response to restraint stress (Viau & Meaney, 1991) and electric foot shock stress (Burgess & Handa, 1992) were greater in estrogen-primed OVX rats rather than OVX rats. These results indicate that estrogen is involved in activating CRH production and/or release in the PVN.

In the PVN, mRNA and protein for the ERβ, a new isoform of ER, are strongly expressed (Li *et al.*, 1997; Österlund *et al.*, 1998; Shughrue *et al.*,

1997), suggesting that the PVN is considered to be one of the feedback action sites of estrogen. In addition, ERα was reported to be involved in the feedback action of estrogen in the PVN, since fasting and immobilization stress increases ERα expression in the rat PVN and A2 region of the brain stem (Estacio *et al.*, 1996). Accordingly, a feedback action of estrogen in the PVN or A2 region could play an important role in the response of LH-releasing system to stressful conditions such as fasting and immobilization stress. The action of estrogen in these brain regions, therefore, could be different from the classical one monitoring the ovarian conditions.

The presence of estrogen could make the brain more sensitive to stresses. Indeed, 48-h fasting stress is known to inhibit pulsatile LH secretion in estrogen-treated OVX rats and intact female rats but not in OVX rats (Cagampang *et al.*, 1991; Cagampang *et al.*, 1990) and local treatment of estrogen into the PVN or A2 region suppresses LH secretion in OVX rats when the animal was fasted for 48 h (Nagatani *et al.*, 1994; Nagatani *et al.*, 1996). In addition, it was demonstrated that pharmacological reduction of glucose availability with 2-deoxyglucose (2DG) interrupts estrous cyclicity in Syrian hamsters (Schneider *et al.*, 1993), and suppresses pulsatile LH secretion in lambs (Bucholtz *et al.* 1996) and rats (Murahashi *et al.*, 1996; Nagatani *et al.*, 1996; Nagatani *et al.*, 1996). 2DG suppresses LH secretion in the presence or absence of sex steroids in both genders, with steroid-treated animals being more sensitive to the glucoprivation (Nagatani *et al.*, 1996). These reports suggest that the response of the reproductive axis to glucoprivation is also modulated by estrogen as well as fasting stress.

The information of reduced glucose availability may be finally sent to the PVN via the noradrenergic pathway, since it was reported that glucoprivation

stimulates noradrenaline release in the PVN and the blockade of catecholamine synthesis in the PVN nullifies the glucoprivic suppression of LH secretion (Nagatani *et al.*, 1996). In this context, it is likely that the increases in the sensitivity to glucoprivation in the presence of estrogen is, at least in part, due to the feedback effect of estrogen in the PVN. Taken these together, it is possible that CRH would be involved in the increases in the sensitivity to glucoprivation with the novel feedback effect of estrogen in the PVN, although it is still unclear how estrogen increases the sensitivity to the effect of 2DG in the PVN.

Objectives

The present dissertation focuses on the hypothalamic mechanisms controlling GnRH secretion in rats, which are used as an experimental model for mammalian species. In Chapters 3 and 6 in the dissertation, LH secretion from the anterior pituitary is monitored as an *in vivo* indicator of GnRH secretion from the hypothalamus.

The first specific aim is to reveal the GnRH pulse-generating system existing in the brain. Based upon a large number of reports concerning GnRH pulse-generating system, two functional components for GnRH pulse, synchronization of GnRH release and generation of an episodic pattern of GnRH release, could be important to release the peptide in a pulsatile manner. However, the mechanisms of the GnRH pulse-generating system are largely unknown. Since GnRH neurons do not make apparent nucleus and the axonal terminals are concentrated to the ME without synaptic connections, synchronization of GnRH release would be carried out at the nerve terminal level in a non-synaptic fashion. In Chapter 3, an involvement of gap junction at the ME on GnRH pulse-generating mechanism is, thereby, investigated to test the hypothesis that an intercellular signal transmission through gap junctions plays

an important role in synchronization of GnRH release at the nerve terminal. It is still controversial about two contradict possibilities about the generation of GnRH pulses: One is that an intrinsic property for episodic-releasing pattern is inherent in GnRH neurons themselves and the other is that non-GnRH neuronal components stimulate intermittent GnRH release. In Chapter 4, GnRH secretory pattern in primary cultured cells originating from the rat fetal MBH are compared with that originating from either the fetal rat whole hypothalamus, mPOA or cerebral cortical regions, all of which are known to contain GnRH neurons, to determine whether GnRH neuron has an intrinsic activity to generate pulsatile GnRH release.

Chapters 5 and 6 focus on mechanisms modulating GnRH secretion. These chapters aim to reveal the feedback mechanisms of estrogen on GnRH secretion in the hypothalamus. OVX female rats and estrogen-treated OVX rats are used for experiments in both chapters in order to clarify estrogen actions. Chapter 5 investigates effects of estrogen on glutamate-induced *in vitro* GnRH release from the ARC-ME fragments to reveal the estrogen feedback mechanism modulating GnRH release at the nerve terminal level. In Chapter 6, effects of CRH antagonist on the glucoprivic suppression of pulsatile LH secretion are investigated in both OVX rats and estrogen-treated OVX rats to determine the novel feedback mechanism of estrogen in the PVN.

CHAPTER 2

General procedures

Animals

Adult male and female Wistar-Imamichi strain rats, weighing 270-350 g and 195-275 g respectively, were used. Animals were kept in a light (14 h light: 10 h dark, lights on at 0500 h)- and temperature (22±2 ˚C)- controlled room with free access to water and food (Labo-MR-stock; Nihon Nosan Kogyo Co., Yokohama, Japan). In female rats, the daily pattern of vaginal smear was observed in each animal to confirm estrous cycles. Animals showing at least two consecutive estrous cycles were used in experiments.

Surgeries

Animals were anesthetized with ether and then subjected to the following surgeries under an aseptic condition. After surgeries, animals were injected with antibiotics (Mycillin Sol Meiji; Meiji Seika Kaisha, Ltd., Tokyo, Japan).

Gonadectomy

Male rats used in the experiments described in the Chapter 3 were castrated. In experiments of the Chapters 5 and 6, female rats were bilaterally OVX. Some of the OVX animals were implanted with Silastic tubing (inner diameter, 1.5 mm; outer diameter, 3.0 mm; length, 25 mm; Dow Corning, Midland, MI, USA) containing estradiol-17β (E_2; Sigma Chemical Co., St. Louis, MO, USA) dissolved in peanut oil at 20 μg/ml immediately after ovariectomy to serve as the OVX+E_2 group. This E_2 treatment has been previously reported to produce physiological plasma E_2 levels corresponding to

those found on diestrous day 2 in cycling rats (35.8±1.20 pg/ml) (Cagampang *et al.*, 1991).

Implantation of a guide cannula for push-pull perfusion

Animals were placed in a stereotaxic instrument (SP-6; Narishige Scientific Instrument Laboratory, Tokyo, Japan) with the bregma and lambda at the same dorsoventral level. The skull was exposed and then an unilateral stainless-steel outer guide cannula for push-pull perfusion (21 gauge; Plastic Products Co., Roanoke, VA, USA) was implanted with its tip in the ME. Stereotaxical coordinates for the tip of the push-pull guide cannula were 3.1 mm caudal, 10.4 mm ventral and 0.5 mm lateral to the bregma according to a rat brain atlas (Paxinos & Watson, 1986). The implanted guide cannula was fixed by dental acrylic resin (Quick Resin; Shofu Inc., Kyoto, Japan) with screws anchored to the skull. A dummy cannula (28 gauge; Plastic Products Co.) was then inserted into the guide cannula until push-pull perfusion.

Push-pull perfusion was carried out by an apparatus shown in Fig. 2-1. An inner cannula (28 gauge; Plastic Products Co.) was inserted into the guide cannula, and then the ME was perfused using two peristaltic pumps (Minipuls 3 Peristaltic Pump; Gilson Medical Electronics, Villiers le Bel, France) at a flow rate of 15 μl/min.

Implantation of a guide cannula for icv injection

A stainless-steel guide cannula for icv injection (22 gauge; Plastic Products Co.) was stereotaxically implanted into the third cerebral ventricle by using the stereotaxic instrument. Stereotaxic coordinates for the tip of icv cannula were 0.8 mm caudal and 7.0 mm ventral to the bregma at the midline according to a rat brain atlas (Paxinos & Watson, 1986). After fixing the guide

cannula to the skull, a dummy cannula (28 gauge; Plastic Products Co.) was inserted into the guide cannula to prevent a block of tubing until icv injection.

Atrial cannulation for the blood sampling

One day before blood sampling, a Silastic catheter (silicone tubing; inner diameter, 0.5 mm; outer diameter, 1 mm; Shin-Etsu Polymer Co., Tokyo, Japan) was inserted into the right atrium through the right jugular vein according to the method established by K.-I. Maeda and H. Tsukamura (Maeda & Tsukamura, 1989). The catheter was filled with the solution of 40% polyvinilpyrrolidone (PVP-10; Mol. Wt. 10,000; Sigma Chemical Co.) dissolved in saline which contains heparin sodium (200 units/ml; Shimizu Pharmaceutical Co., Ltd., Shimizu, Japan), cephalosporin (2 mg/ml; Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) and streptomycin sulfate (2 mg/ml; Meiji Sekia Kaisha, Ltd.) to prevent blood coagulation and bacterial infection.

Animals were allowed to freely move in each cage and bled (100 μl) through the Silastic catheter. Frequent blood sampling was started from 13:00 at 6-min interval for 3-4 h. An equivalent volume of rat red blood cells taken from donor animals was suspended in saline and replaced through the Silastic catheter after each blood sampling. Blood samples were centrifuged at 12,000 rpm for 10 min, and 50 μl plasma was taken and kept at -30 ˚C until assayed for plasma LH concentrations.

Primary cell culture

All glassware used in primary cell cultures were autoclaved and solutions were filtrated with a cellulose membrane filter (pore size: 0.20-0.22 μm) for

sterilization before use. Other instruments were cleaned with 70% ethanol and sterilized under ultraviolet rays at least overnight.

Embryonic 17-day fetuses were obtained from pregnant female rats. Pregnant female rats were deeply anesthetized with ether and then the abdominal region was incised to take the pregnant uterus out. The fetuses were taken from the uterus and placed in a glass dish filled with a phosphate buffered saline (in mM: NaCl, 136.9; KCl, 2.7; $Na_2HPO_4 \cdot 12H_2O$, 8.1; KH_2PO_4, 1.5, pH 7.4). The fetus brains were removed with microscissors and forceps.

The fetal hypothalamic regions were dissected out according to a rat fetal brain atlas (Paxinos *et al.*, 1994) as follows (Fig. 2-2): (1) The borders of the dissected tissue were delineated by the anterior margin of the olfactory tubercle, the posterior margin of the mammillary bodies, the lateral margin of the hypothalamic sulci, and the dorsal margin of the anterior commissure to get the region including the whole hypothalamus (AH+PH); (2) the AH+PH tissue fragments were separated by the midline of the optic chiasma to get the anterior part of the hypothalamic region including the mPOA (AH) and the posterior part of the hypothalamic region including the MBH (PH). The cerebral cortical region (CX) described in Fig. 2-2 was also excised.

These fetal brain tissue fragments were put in an ice-cold serum-free culture medium, Dulbecco's Modified Eagle Medium (high glucose with L-glutamine without sodium pyruvate and sodium bicarbonate; GIBCO BRL, Life Technologies, Inc., Rockville, MD, USA) containing 44 mM $NaHCO_3$, 50 U/ml liquid mixture of penicillin-streptomycin (GIBCO BRL), and 1 mM sodium pyruvate. The brain tissue fragments were transferred to and treated with 2 ml of a papain solution, which is phosphate buffered saline containing 0.15 units/ml papain (Worthington Biochemical Co., NJ, USA), 0.02% bovine serum albumin, 5 mg/ml D-glucose, and 0.2 mg/ml L-cysteine·HCl·H_2O, at 37

°C for 15 min at 2 times in order to dissociate tissues into single cells. The papain-treated tissues were suspended in the culture medium containing 5% newborn calf serum (Cat. No. 16010-159; Lot No. 37k7149; GIBCO BRL) and 5% horse serum (Cat. No. 14-403E; Lot No. 5M0477; BioWhittaker, MD, USA). To remove the enzymatic activity of papain completely, the cell suspension was centrifuged at 1,000 rpm for 5 min, and then the resulting pellets of dissociated cells was washed with the serum-containing culture medium at 2 times. The concentration of the cell suspension was finally adjusted to 2×10^6-4×10^6 cells/ml. Two hundred μl of the suspension was placed on a glass-bottom culture dish (Matsunami Glass Ind., Ltd., Osaka, Japan) coated by polyethylenimine (Sigma Chemical Co.). Culture dishes were placed for 10 min at room temperature to allow the cells attached to the glass bottom of the culture dishes. The attached cells were cultured with 2 ml of the serum-containing culture medium in an incubator under 5% CO_2-95% air at 37 °C.

Half of the culture medium in the cultures dishes was replaced with the same volume of fresh medium every 3 or 4 days as long as the culture lasts. The culture medium removed from the dishes was treated with 50 μl 2 N HCl for 1-2 h at 4 °C to inactivate enzymes degrading GnRH peptide. Reaction mixture was centrifuged at 12,000 rpm for 10 min, and supernatant was kept at -30 °C. The supernatant were neutralized with 50 μl of 2 N NaOH when assayed for GnRH.

In vitro superfusion of primary cultured cells

Cultured cells were superfused with a serum-free culture medium containing 0.1% bovine serum albumin, 0.142 mg/ml bacitracin, and 5 μg/ml insulin, gassed with 95% O_2-5% CO_2 at 37 °C by using a superfusion system (Tokai Hit Co., Shizuoka, Japan) described in Fig. 2-3. Cultured cells were first

superfused with the medium at a flow rate of 30 µl/min for 1 h to establish a stable condition of primary cultured cells in the superfusion system. After presuperfusion period for 1 h, samples were collected every 10 min for 3 h. Samples were treated with 15 µl of 2 N HCl for 1-2 h at 4 °C to inactivate enzymes. Reaction mixtures were centrifuged at 12,000 rpm for 10 min, and supernatant was taken and kept at -30 °C. Samples were neutralized with 25 µl of 1.2 N NaOH before assayed for GnRH.

In vitro static incubation of ARC-ME tissue fragments

Animals were decapitated and the brains were immediately removed from the skull. The ARC-ME region were dissected out of the brain. The tissue fragments were placed in a clean plastic chambers filled with 500 µl of an incubation medium, Yamamoto's solution (in mM: NaCl, 145; KCl, 5; $MgCl_2$, 2; $NaHCO_3$, 5; $CaCl_2$, 2, pH 7.3-7.4) containing 10 mM HEPES, 1 g/l D-glucose, 0.05% bovine serum albumin, and 142 mg/l bacitracin, for 30 min. The incubation chambers were kept in a water bath at 37 °C with a constant shaking in an atmosphere of 95% O_2-5% CO_2. Schematic drawing of the static incubation system was shown in Fig. 2-4.

The incubation medium collected after each incubation was centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatant was stored at -30 °C until assayed for GnRH.

Assays

GnRH

GnRH were measured by a double-antibody radioimmunoassay (RIA) using a rabbit anti-GnRH serum (RIX-419) provided by Dr. Hasegawa of Gunma University and [^{125}I]GnRH purchased from Amersham (Tokyo, Japan). Authentic GnRH obtained from Sigma Chemical Co. was used as a standard. The least detectable level was 0.39 pg/tube. The intra- and interassay coefficients of variation were 9.97% at level of 11.82 pg/tube and 14.0% at level of 13.04 pg/tube, respectively.

LH

Plasma LH concentrations were determined by a double antibody RIA with a rat LH RIA kit provided by the National Hormone and Pituitary Program (Baltimore, MD, USA). The LH concentrations were expressed in terms of the NIDDK rat LH RP-3. The least detectable level of LH was 0.156 ng/ml for 50 µl of plasma. The intra- and interassay coefficients of variation were 6.71% at level of 0.76 ng/ml and 11.2% at level of 1.26 ng/ml, respectively.

Cyclic guanosine 3', 5'-monophosphate (cGMP)

ARC-ME tissue fragments were homogenized with 600 µl of ice-cold 6% trichloroacetic acid, and then the homogenate was centrifuged at 5,300 rpm for 15 min at 4 °C. Five hundred µl of the supernatant was washed four times with 2.5 ml of water-saturated diethyl ether and then upper ether layer was discarded at each time. The aqueous extracts were dried up and were stored at -30 °C until assayed for cGMP.

The dried extracts were dissolved with 250 µl of 0.05 M acetate buffer (pH 5.8). cGMP contents were determined by a cGMP RIA kit (Amersham) with acetylation standard. The least detectable level was 2.5 fmol/ml for 100 µl of the extract solution.

Nitric oxide synthase (NOS) enzymatic activity

The enzymatic activity of NOS was determined according to the method described by Kadowaki *et al.* (Kadowaki *et al.*, 1994) with a slight modification. NOS activity was determined by measuring [^{3}H]citrulline formation from [^{3}H]arginine (Amersham).

ARC-ME fragments dissected out of the whole brain were immediately frozen by dry ice to prevent the degradation of the enzyme activity. The frozen tissue fragments were homogenized in 80 µl ice-cold 50 mM HEPES buffer (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged at 10, 000 rpm for 1 min at 4 °C, and 50 µl of supernatant was incubated for 20 min at 37 °C in 50 mM HEPES buffer containing 1 mM βNADPH (Sigma Chemical Co.), 1 mM EDTA, 1.25 mM $CaCl_2$, 1 mM dithiothreitol (Sigma Chemical Co.), 10 µM tetrahydrobiopterin (Research Biochemical International, Natick, MA, USA), 10 µg/ml calmodulin (Sigma Chemical Co.), 3 µM arginine, and 1 µCi [^{3}H]arginine. Reaction was stopped by adding 900 µl of 100 mM HEPES solution (pH 5.5) containing 10 mM EDTA. To separate [^{3}H]citrulline, the reaction mixture was loaded onto a resin (Dowex-50W; Sigma Chemical Co.) column. [^{3}H]Citrulline was eluted with distilled water and its radioactivity was counted. The protein concentrations of homogenates were measured by protein-dye binding assay according to the method described by Bradford (Bradford, 1976).

Histological techniques

Immunohistochemistry for confocal laser scanning microscopy

Animals were deeply anesthetized by pentobarbital sodium and perfused intracardially with 0.05 M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde-0.05 M phosphate buffer (PB). Brains were postfixed with the same fixative for 2 h and immersed in 30% sucrose-PB for 3 days at 4 ˚C. Coronal hypothalamic sections (50 μm) were made with a cryostat.

Dual immunostaining with GnRH and connexins

Incubations were performed at room temperature unless otherwise stated. Free-floating sections were sequentially incubated with (1) 5% normal donkey serum (NDS; Sigma Chemical Co.)-0.05 M PBS for 1 h, (2) a mixture of rabbit anti-GnRH (1:5000; LR1 A-LHRH), kindly provided by Dr. Robert Benoit (Montreal General Hospital Research Institute, Montreal, Canada) and either mouse anti-connexin 26 (1:500; Zymed Laboratories, Inc., CA, USA), mouse anti-connexin 32 (1:500; Zymed Laboratories, Inc.) or mouse anti-connexin 43 (1:200; Zymed Laboratories, Inc.) in 5% NDS-0.1% Triton X-100-0.05 M PBS at 4 ˚C for 96 h, and (3) a mixture of donkey anti-mouse IgG labeled with FITC (1:200; Jackson ImmunoResearch Laboratories, Inc., PA, USA) and donkey anti-rabbit IgG labeled with Cy3 (1:400; Jackson ImmunoResearch Laboratories, Inc.) in 5% NDS-0.05 M PBS for 2 h. Sections were rinsed in 0.05 M PBS at 3-4 times after each step except between steps 1 and 2. Some sections were dual immunostained with connexin 43 antibody (1:200; Zymed Laboratories, Inc.) and rabbit anti-glial fibrillary acidic protein (GFAP; 1:5; Dako, Denmark) with the same protocol as mentioned above. Sections were

mounted on slide grasses and coverslipped with FluoroGuard (Bio-Rad Laboratory, CA, USA).

Immunostaining with brain NOS or macrophage NOS

Brain NOS or macrophage NOS was immunostained according to the above-mentioned procedure for dual immunostaining with GnRH and connexins except steps 2 and 3.

Free-floating sections were incubated with rabbit anti-brain NOS (1:25; Transduction Laboratories, Lexington, KY, USA) or mouse anti-macrophage NOS (1:1000; Transduction Laboratories) in 5% NDS-0.1% Triton X-100-0.05 M PBS at 4 °C for 96 h at step 2, and then with donkey anti-mouse IgG conjugated with FITC (1:200; Jackson ImmunoResearch Laboratories, Inc.) or donkey anti-rabbit IgG conjugated with Cy3 (1:400; Jackson ImmunoResearch Laboratories, Inc.) in 5% NDS-0.05 M PBS for 2 h at step 3, respectively.

Immunocytochemistry of connexin 43 for electron microscopy

Animals were perfused with 0.1 M PBS (pH 7.4), and then with 4% paraformaldehyde (TAAB Laboratories Equipment Ltd., Berks, UK) containing 0.2% glutaraldehyde (TAAB Laboratories Equipment Ltd.) in 0.1 M PB. Tissues were postfixed with 4% paraformaldehyde-0.1 M PB overnight at 4 °C. Coronal hypothalamic sections (50 μm) were cut on a microslicer.

Incubations were performed at room temperature unless otherwise stated. Free-floating sections were incubated with (1) 5% NDS-0.1 M PBS for 1 h, (2) mouse anti-connexin 43 (1:20; Zymed Laboratories, Inc.) in 5% NDS-0.1 M PBS at 4 °C for 96 h, (3) rabbit anti-mouse immunoglobulins labeled with biotin (1:50; Dako) in 0.1 M PBS for 90 min, and (4) streptavidin labeled by horse radish peroxidase (1:80; Dako) in 0.1 M PBS for 90 min. Sections were rinsed

in 0.1 M PBS at 3-4 times after each step except between steps 1 and 2. After step 4 followed by rinsing in Tris-HCl buffer (pH 7.6), sections were reacted with 0.05% 3, 3'-diaminobenzidine (Sigma Chemical Co.)-0.01% H_2O_2 solution for visualization of immunoreactivities. Immunostained-sections were then exposed to 1% OsO_4 in 0.1 M PBS for 90 min at 4 °C and dehydrated through a graded series of ethanols followed by immersion in propylene oxide (TAAB Laboratories Equipment Ltd.). They were then flat-embedded in Araldite (Nisshin EM Co., Ltd., Tokyo, Japan). After polymerization, ultrathin sections were cut on an ultramicrotome and mounted on 250-mesh copper grids followed by staining with uranyl acetate and lead citrate.

Immunohistochemistry of GnRH in fetal brain sections

The fetal brain tissues were fixed with 4% paraformaldehyde in 0.05 M PBS (pH 7.4) for 2 h at 4 °C, and then immersed in 30% sucrose-PB for 3 day at 4 °C. Tissues were cut with a cryostat at 50 μm, and then mounted on a slide grass coated gelatin.

Incubations were performed at room temperature unless otherwise stated. Sections were incubated with (1) 0.3% H_2O_2-0.05 M PBS for 30 min to remove endogenous peroxidase activities, (2) 5% normal goat serum (NGS)- 0.05 M PBS for 1 h, and (3) rabbit anti-GnRH (1:5000, LR1 A-LHRH) containing 5% NGS-0.05 M PBS for 96 hr at 4 °C. Sections were rinsed in 0.05 M PBS at 3-4 times at 4 °C after each step except between steps 2 and 3.

GnRH-immunoreactivities were visualized by the ABC method using Vectastain Elite Kit (Vector Laboratories, Inc., Burlingame, CA, USA). Briefly, the sections were incubated with (1) 0.2% biotinylated goat anti-rabbit IgG in 0.05 M PBS containing 0.6% NGS for 1 h, (2) avidin-biotinylated horseradish peroxidase complex in 0.05 M PBS for 1 h, and (3) 0.1 M Tris-HCl buffer (pH

7.2) containing 0.05% 3, 3'-diaminobenzidine (Sigma Chemical Co.) and 0.01% H_2O_2 was applied for a few minutes. The sections were washed with 0.05 M PBS between steps 1 and 2 and with 0.1 M Tris-HCl buffer for 1h at 4 °C between steps 2 and 3.

Immunohistochemistry of GnRH in primary cultures

Primary cultured cells were washed with 0.05 M PBS (pH 7.4) and fixed with 4% paraformaldehyde-0.05 M PB for 2 h at 4 °C. After washing with 0.05 M PBS, fixed cells were dried up and kept at room temperature until GnRH immunostaining.

Incubations were performed at room temperature unless otherwise stated. Cultured cells were incubated with (1) 1% Triton X-100-0.05 M PBS for 30 min, (2) 5% NDS-0.05 M PBS for 1 h, (3) rabbit anti-GnRH (1:5000, LR1 A-LHRH) in 5% NDS-0.05 M PBS at 4 °C for 96 h, (4) 0.2% biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc.) in 0.05 M PBS containing 0.6% NGS for 1 h, and (5) 20 μg/ml of fluorescein streptavidin (Vector Laboratories, Inc.) in 10 mM HEPES buffered saline (pH 8.2) for 1 h. Sections were rinsed with 0.05 M PBS after each step except between steps 2 and 3. After step 5, cultured cells were washed with 10 mM HEPES buffered saline and coverslipped with a water-soluble embedding.

Data analysis

LH pulse

LH pulses were identified by the PULSAR computer program provided by Drs. G. R. Merriam and K. W. Wachter (Merriam & Wachter, 1982). The criteria for LH pulse detection were as follows. If the difference between a single

LH concentration and the baseline concentration was 3 times greater than the standard deviation (SD) at the level of the LH concentration, it was considered to be a part of a LH pulse. If the differences between 2, 3 or 4 consecutive LH concentrations and the corresponding baseline values were 2.4, 1.85 or 1.52 times greater than the SD at these LH concentrations, they were also considered to be parts of a LH pulse. The SD at each hormone concentration was calculated from the equation, y=(6.9819x+2.8748)/100, where x was the plasma LH concentration and y was the SD for each plasma LH concentration determined by assaying five series of control plasma in ten replicates.

GnRH pulse

GnRH pulses were also identified by the PULSAR computer program. The criteria for GnRH pulse detection were as follows. If the difference between a single point and the corresponding baseline value was 1.5 times greater than the SD at the level of the GnRH release, it was considered to be a part of a GnRH pulse. If the differences between 2 or 3 consecutive points and the corresponding baseline values were 1.25 or 1.15 times greater than the SD at these GnRH release, they were also considered to be parts of a GnRH pulse. The SD at each hormone release was calculated from the equation, y=(7.321x+14.22)/100, where x was the GnRH release and y was the SD for each GnRH release determined by assaying three series of control sample in ten replicates.

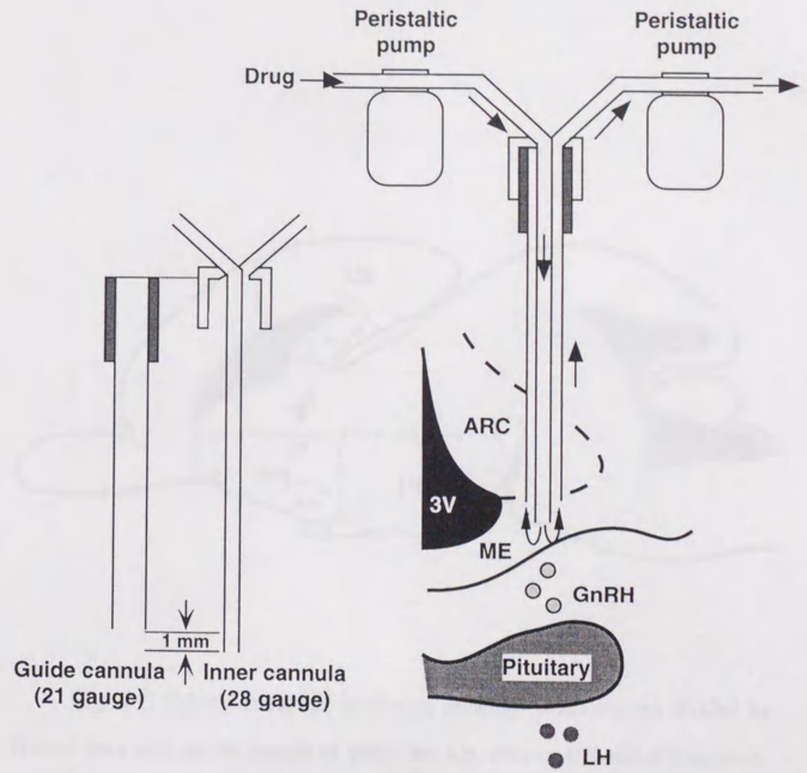


Fig. 2-1. Schematic diagram of push-pull perfusion system. Animal was inserted an inner cannula (28 gauge) into a guide cannula (21 gauge) implanted with its tip in the ME. Perfusate containing drug was perfused through both inner and guide cannula by two peristaltic pumps. Arrows indicate the flow of perfusate. ME, median eminence; ARC, arcuate nucleus; 3V, third cerebral ventricle.

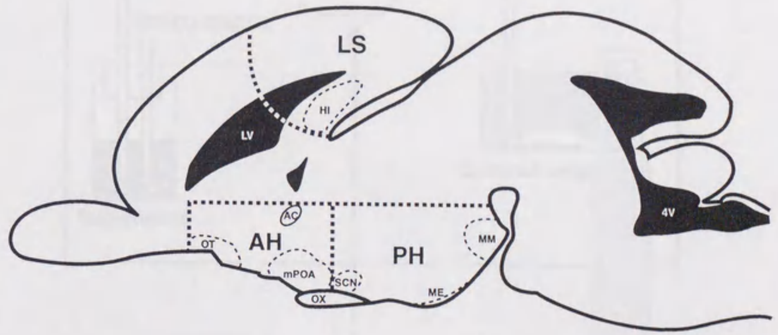


Fig. 2-2. Schematic sagittal fetal brain drawing. Brain regions divided by broken lines indicate the margin of either the AH, PH, or CX tissue fragments. The margin of the AH+PH tissue fragment is indicated by broken line surrounding both AH and PH regions. AC, anterior commissure; HI, hippocampus; LV, lateral cerebral ventricle; 4V, fourth cerebral ventricle, ME, median eminence; MM, mammillary bodies; OT, olfactory tubercle; OX, optic chiasma; mPOA, medial preoptic area; SCN, supraoptic nucleus.

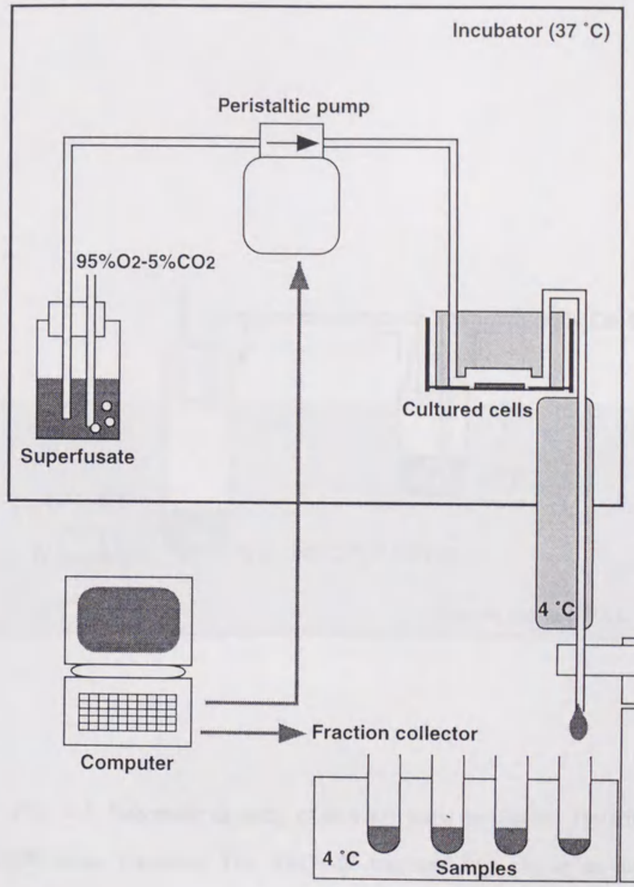


Fig. 2-3. Schematic diagram of *in vitro* superfusion system for primary cultured cells. Cultured cells in culture dishes were put in an incubator at 37 ˚C, and superfused with superfusate gassed with 95% O_2-5% CO_2. Movements of peristaltic pump and fraction collector are controlled by a computer.

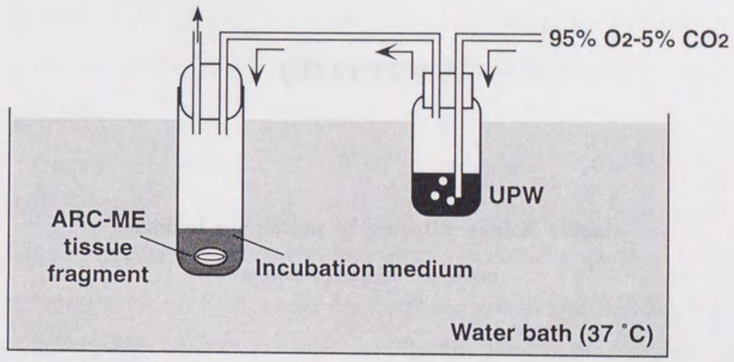


Fig. 2-4. Schematic drawing of *in vitro* static incubation system for the ARC-ME tissue fragment. The ARC-ME fragment was put in an incubation chamber filled with incubation medium. They were kept in a water bath at 37 ˚C with a constant shaking in an atmosphere of 95% O_2-5% CO_2.

CHAPTER 3

Terminal regulation of pulsatile GnRH release in non-synaptic fashion

-Involvement of gap junction in the ME on the synchronization of GnRH pulse-

Introduction

GnRH pulses have been suggested to be synchronized at the nerve terminals by a mechanism located in the MBH (Blake & Sawyer, 1974; Ohkura *et al.*, 1991; Soper & Weick, 1980). The terminal regulation of pulsatile GnRH release may, at least in part, be due to a non-synaptic fashion, since it was reported that few synaptic contacts are found on GnRH nerve terminals in the rat ME (Kawakami *et al.*, 1998). It, therefore, is possible that gap junction is one of the candidates involving the synchronization of GnRH pulse at the nerve terminal level.

Gap junction is composed of connexin, a transmembrane protein forming hexamer called as connexon, and two connexons located on the plasma membrane in neighboring cells are coupled with each other to form a channel. In the central nervous system, connexin 43 mRNA and protein have been reported to be expressed in astrocytes, connexin 32 mRNA and protein are located in oligodendrocytes and neurons (Dermietzel *et al.*, 1989; Micevych & Abelson, 1991; Micevych *et al.*, 1996) and connexin 26 protein is localized in leptomeningeal cells, ependymal cells and pineal gland (Dermietzel *et al.*, 1989). It has been reported that astrocytic-neuronal or -astrocytic excitatory signal transmission is mediated by Ca^{2+} mobilized through gap junctions in primary culture of rat forebrain cells (Nedergaard, 1994). These reports suggest that gap junctions play an important role in cell-to-cell communication in the brain.

To explore the possibility that an intercellular signal transmission through gap junction plays a role in the synchronization of GnRH pulse at the terminal level, this chapter first describes to reveal morphological distributions of connexin 26, 32, and 43 and relationship to GnRH nerve terminals in the ME. Effects of local administration of gap junction blocker into the ME on pulsatile

LH secretion is also tested to determine a role of gap junctional communications in the ME on GnRH pulse generation.

Materials and Methods

Exp. 1

Adult male Wistar-Imamichi strain rats were castrated for two weeks. Coronal hypothalamic sections (50 μm) of four castrated animals were dual immunostained with GnRH and either connexin 26, connexin 32, or connexin 43. Some sections were dual immunostained with GFAP and connexin 43. The secretions were observed under a confocal laser scanning microscope (MRC 1024; Bio-Rad Laboratory).

Three castrated animals were used for immunoelectron microscopy of connexin 43. The ultrathin sections, which were made of coronal hypothalamic sections immunostained with connexin 43, were examined with an electron microscope (JEM-1210; Jeol Ltd., Tokyo, Japan).

Exp. 2

Adult male Wistar-Imamichi strain rats were castrated for two weeks. One week after castration, brain surgery was carried out to implant the guide cannula for push-pull perfusion into the ME. The animals were allowed to recover from brain surgery for one week before blood sampling.

The push-pull perfusion was simultaneously carried out during 4-h blood sampling. The ME was perfused with an artificial cerebrospinal fluid (in mM: NaCl, 145; KCl, 3.5; $MgCl_2$, 1; $CaCl_2$, 0.3), which was filtrated and degassed to avoid a block of tubing, for at least 1 h before push-pull perfusion with drugs. In the first 2 h of the sampling period, the ME was perfused with vehicle, 14% 2-hydroxypropyl-β-cyclodextrin (Research Biochemical International) in the

artificial cerebrospinal fluid, and then with 50 mM octanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution for the last 2 h of the sampling period.

Histology

After blood sampling, animals were perfused intracardially with saline followed by 10 % formalin to confirm whether tip of the push-pull cannula was located on the ME. Brains were postfixed with the same fixative overnight and immersed in 30 % sucrose-PB for 3 days. Coronal hypothalamic sections (50 μm) were made with a cryostat, stained with buffered thionin, and were observed under a microscope. Only the results obtained from animals implanted with the cannula into the ME were used.

Statistical analysis

LH pulses were identified by the PULSAR computer program (Merriam & Wachter, 1982). Mean LH concentration, inter-peak interval, and pulse amplitude for the first 2 h of blood sampling and that for the next 2 h were calculated for each experimental animal and then for each group. Statistical differences in the three parameters were analyzed by Mann-Whitney U-test.

Results

Exp. 1

Morphological relationships between GnRH nerve terminals and connexins in the ME

Confocal laser scanning microscopy showed that most GnRH-immunoreactive fibers were located in the external zone of the lateral portion of the ME (Fig. 3-1a, b, c, d). Connexin 43-immunoreactive puncta were distributed in the ME and ARC (Fig. 3-1a, d, e). They were distributed between GnRH nerve terminals and some of the connexin 43-immunoreactive puncta were colocalized with GnRH-immunopositive fibers (Fig. 3-1a, d arrows). To the contrary, only a few connexin 32-immunoreactivities were found in the ME (arrowheads) and were not associated with GnRH-immunoreactivities (Fig. 3-1b). Connexin 26-immunoreactivities were observed only in the ependymal cell layer of the third cerebral ventricle (arrowhead) without colocalization with GnRH-immunoreactive fibers (Fig. 3-1c).

Location of connexin 43-immunoreactivities in the ME

In the results of dual immunostaining with GFAP and connexin 43, most of the connexin 43-immunoreactivities were found in GFAP-immunoreactive cells in the ME (Fig. 3-2 a, b) and ARC (Fig. 3-2 c). Some of the colocalization of connexin 43- and GFAP-immunoreactivities were indicated by arrows.

The electron microscopy showed that connexin 43-immunoreactivities (arrowheads) were located on the neighboring plasma membranes of the glial-like processes (arrows), one of which contains a bundle of filaments, in the ME (Fig. 3-3 a). Some of the immunoreactivities were found on the lamella

structures of the glial-like processes surrounding an axonal terminal (asterisk) (Fig. 3-3 b).

Exp. 2

Implantation site of push-pull perfusion cannula

A photomicrograph shows that an implantation site of a push-pull cannula (four arrowheads) locates in the lateral portion of the ME in a representative animal used (Fig. 3-4).

Effect of octanol on pulsatile LH secretion in castrated male rats

Fig. 3-5 shows representative profiles of plasma LH concentrations in the control and octanol (50 mM)-treated groups. In the control group, both representative animals showed regular LH pulses during 4-h sampling period. The regular LH pulse was also found during the first 2 h of blood sampling in animals before octanol (50 mM) treatment. However, the pulsatile LH secretion was slowed down by octanol treatment for the last 2 h of sampling period compared with that for the first 2 h of sampling period.

In the octanol-treated group, the mean inter-peak interval of LH pulses during the last 2 h of the sampling period was significantly ($p<0.05$, Mann-Whitney U test) increased by the local administration of octanol to the ME compared with that during the first 2 h of the sampling period perfused with vehicle (Fig. 3-6). On the other hand, there was no significant difference in the inter-peak interval of LH pulses between the first and second 2-h periods in the control group. Mean plasma LH concentrations and LH pulse amplitude were not significantly altered by octanol. LH pulse amplitude significantly ($p<0.05$, Mann-Whitney U test) decreased during the last 2 h vehicle perfusion in the control group.

Discussion

The present result showed that the local administration of a gap junction blocker into the ME increased inter-peak intervals of LH pulses in castrated male rats. This suggests that gap junctions in the ME are involved in the regulation of the frequency of GnRH pulses at the nerve terminal level. Octanol, which was used in the present experiment, is a gap junction blocker and has been known to close gap junction channels followed by blocking intercellular signal transmissions through gap junctions (Pappas *et al.*, 1996; Rose & Ransom, 1997; Saez *et al.*, 1989). It has been reported that octanol decreases gap junctional electrical conductance in canine heart muscle (Joyner & Overholt, 1985) and rat myometrium (Blennerhassett & Garfield, 1991). The Ca^{2+} mobilization through gap junctions is inhibited by the gap junction blocker in rat forebrain primary culture (Nedergaard, 1994). Effects of octanol on pulsatile LH secretion could, therefore, be due to a blockade of an intercellular signal transmission through gap junctions in the ME. The intercellular signal transmission seems to be involved in the synchronization of GnRH release at the GnRH nerve terminals but not in the generation of GnRH pulses, since LH pulse frequency was decreased by octanol but the pulsatility of LH secretion was not disrupted by the administration of octanol. It has been reported that an intracellular Ca^{2+} propagate through gap junctions to neighboring cells is followed by an induction of Ca^{2+} wave in connexin 43-transfected cells (Charles *et al.*, 1992; Toyofuku *et al.*, 1998), suggesting that gap junctional communication plays a role in synchronizing activities of the cells.

Immunohistochemical studies demonstrated that most gap junctions in the ME were composed of connexin 43 protein, some of which were expressed in GnRH nerve terminals. On the other hand, neither connexin 26 nor connexin 32

protein was found on GnRH nerve terminals. These immunohistochemical results indicate that octanol treatment into the ME interrupts an intercellular signal transmission through gap junctions composed of connexin 43 in the ME followed by a reduction of GnRH pulse frequency.

In addition to connexin 43 in some GnRH nerve terminals, a large number of the gap junctions located on the plasma membrane of astrocytes. It has been well demonstrated that astrocytes express connexin 43 (Dermietzel *et al.*, 1989; Micevych & Abelson, 1991; Micevych *et al.*, 1996) in the brain. Astrocytes are considered to communicate with each other through gap junctions, since Ca^{2+} transportation from an astrocyte to another was blocked by octanol in rat forebrain primary culture (Nedergaard, 1994). Since some connexin 43 were expressed in GnRH nerve terminals, it is possible that astrocytes communicate not only with each other but also with GnRH nerve terminals through gap junctions. Astrocytes are considered to be involved in neuroendocrine functions, because astrocytes produce some neurotransmitters such as somatostatin and opioid peptides (Shinoda *et al.*, 1989) and express glutamate receptors (Steinhäuser & Gallo, 1996). It was also suggested that glial glutamate transporters take up extracellular glutamate released from presynaptic neurons followed by the termination of excitatory synaptic neurotransmission of glutamate in cultured rat hippocampal cells (Mennerick & Zorumski, 1994). In addition, astrocyte networks through gap junctions are considered to play an important role in neurotransmission (Jensen & Chiu, 1993). Taken these results together with the present results, an interaction between GnRH nerve terminals and astrocytes through gap junctions composed of connexin 43 may be involved in the synchronization of GnRH release at the GnRH nerve terminal level.

Neither connexin 32- nor connexin 26-immunoreactivity was found in the GnRH nerve terminals. It has previously been reported that GnRH neurons

express connexin 32 mRNA, and most of the connexin 32 protein are found on the cell bodies, although a few connexin 32 protein are found in the nerve terminals (Hosny & Jennes, 1998). Connexin 32 may be involved in regulating GnRH-neuronal activity at the cell body level rather than the terminal level. On the other hand, connexin 43, which was found in the close proximity with the GnRH immunoreactive terminals in the ME in this study, seems to be involved in regulating GnRH release at the terminal level. Connexin 26 in the ME were expressed in the ependymal cells in the present study. This result is consistent with previous reports that most connexin 26 in the brain are distributed in the ependyma (Dermietzel *et al.*, 1989). It is, therefore, unlikely that connexin 26 is involved in the regulation of GnRH release at the ME, since no connexin 26-immunoreactivity was found in any of the GnRH-immunoreactive fibers examined in the present study. Conversely, it was reported that only the connexin 26 protein is found in the GT1-7 cells, an immortalized GnRH neuronal cell line, but neither connexin 32 nor connexin 43 is found in the cell line (Matesic *et al.*, 1993). This contradiction in the expression of connexins could be due to the differences in properties between hypothalamic GnRH neurons and immortalized GnRH neurons.

In conclusion of this chapter, an intercellular signal transmission through gap junctions between GnRH nerve terminals and astrocytes might be involved in the synchronization of GnRH release at the nerve terminal level.

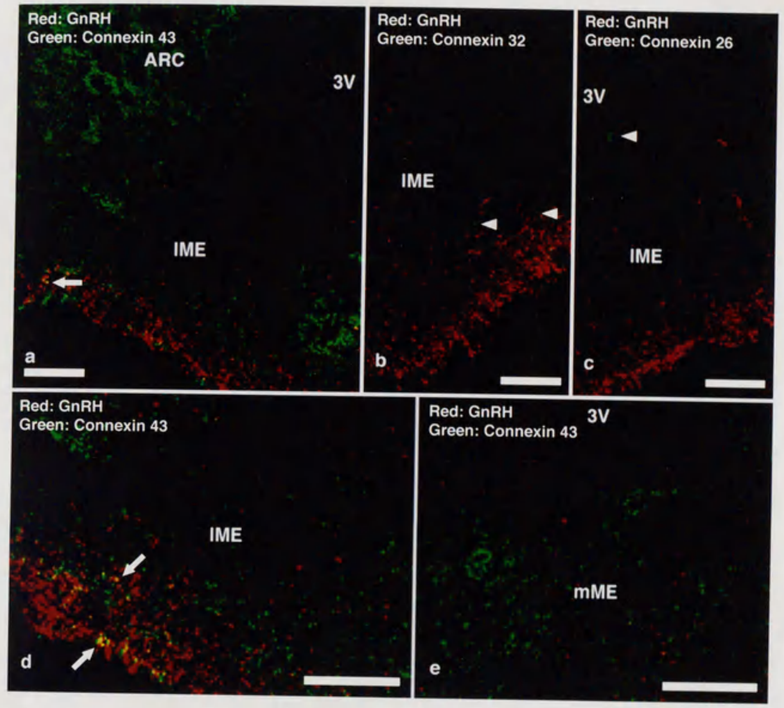


Fig. 3-1. Digital images of confocal laser scanning microscopy showing GnRH (red)- and connexin 43 (green)-immunoreactivities (a, d, e), GnRH (red)- and connexin 32 (green)-immunoreactivities (b), and GnRH (red)- and connexin 26 (green)-immunoreactivities (c). Yellow field shows colocalization of GnRH and connexin 43 (a, d) and arrows indicate some of the colocalization. Arrowheads indicate connexin 32- (b) or connexin 26-immunoreactivities (c). Scale bar, 40 μm; 3V, third cerebral ventricle; mME, medial portion of the median eminence; lME, lateral portion of the median eminence; ARC, arcuate nucleus.

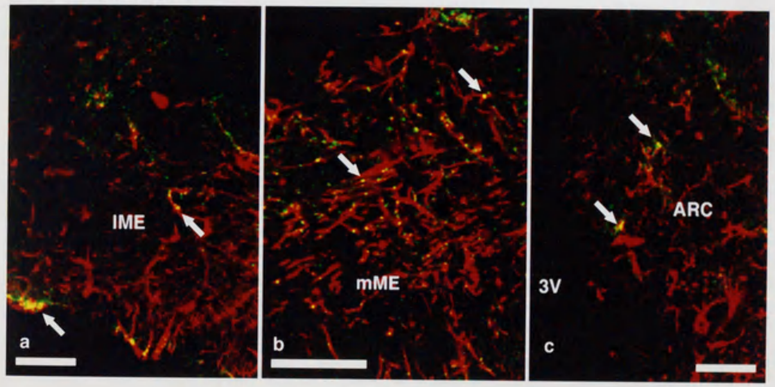


Fig. 3-2. Digital images of confocal laser scanning microscopy showing GFAP (red)- and connexin 43 (green)-immunoreactivities in the ME (a, b) and ARC (c). Yellow field shows colocalization of GFAP and connexin 43, some of which are indicated by arrows. Scale bar, 40 μm. See Fig. 3-1 for details.

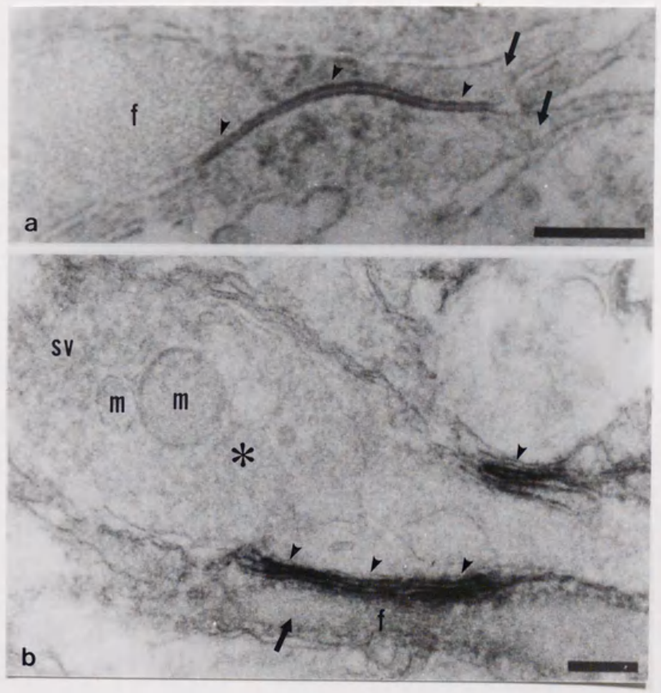


Fig. 3-3. Electron micrograph of connexin 43-immunoreactivities (arrowheads) on the plasma membranes of the glial-like processes in the ME (a). Some connexin 43-immunoreactivities are on the lamella structures of the glial-like processes surrounding an axonal terminal (asterisk) (b). Scale bar, 200 nm; f, bundle of filaments; arrows, glial-like processes; sv, synaptic vesicles; m, mitochondrion.

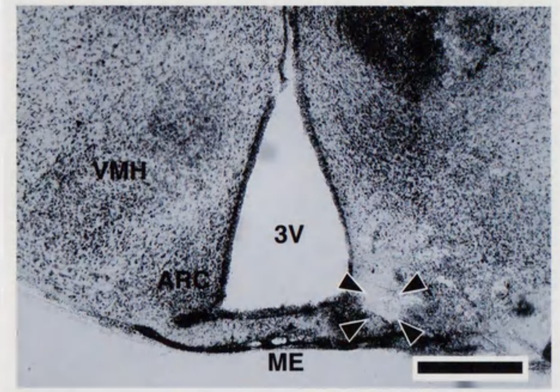


Fig. 3-4. Photomicrograph of thionin-stained frontal section of the brain taken from a castrated male rat implanted with a push-pull cannula into the ME. Four arrowheads indicate the site of the cannula tip. Scale bar, 500 μm, ME, median eminence; 3V, third cerebral ventricle; ARC, arcuate nucleus; VMH, ventromedial hypothalamic nucleus.

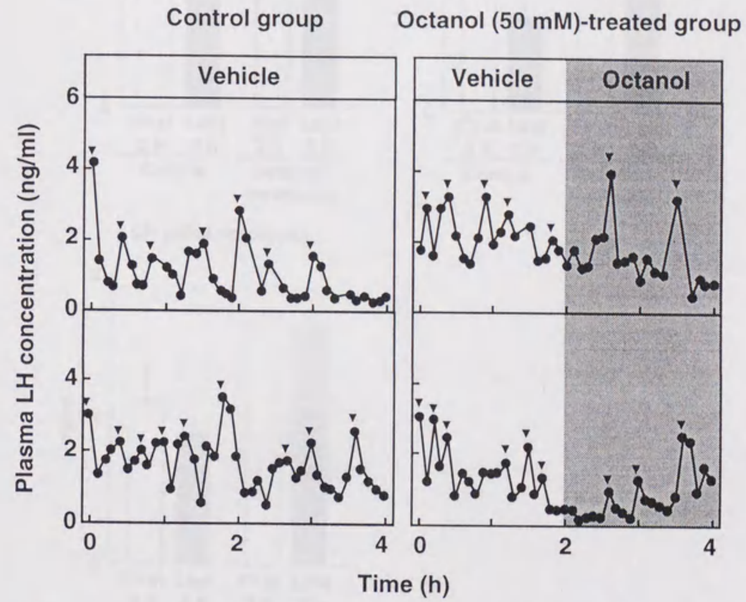


Fig. 3-5. Representative profiles of plasma LH concentrations in control and octanol (50 mM)-treated groups. Male rats have been castrated for two weeks. The ME was perfused with vehicle for the first 2 h and with octanol for the last 2 h in the octanol-treated group, or with vehicle for 4 h in the control group. Blood samples were collected every 6 min for 4 h. Arrowheads indicate LH pulses identified with PULSAR computer program.

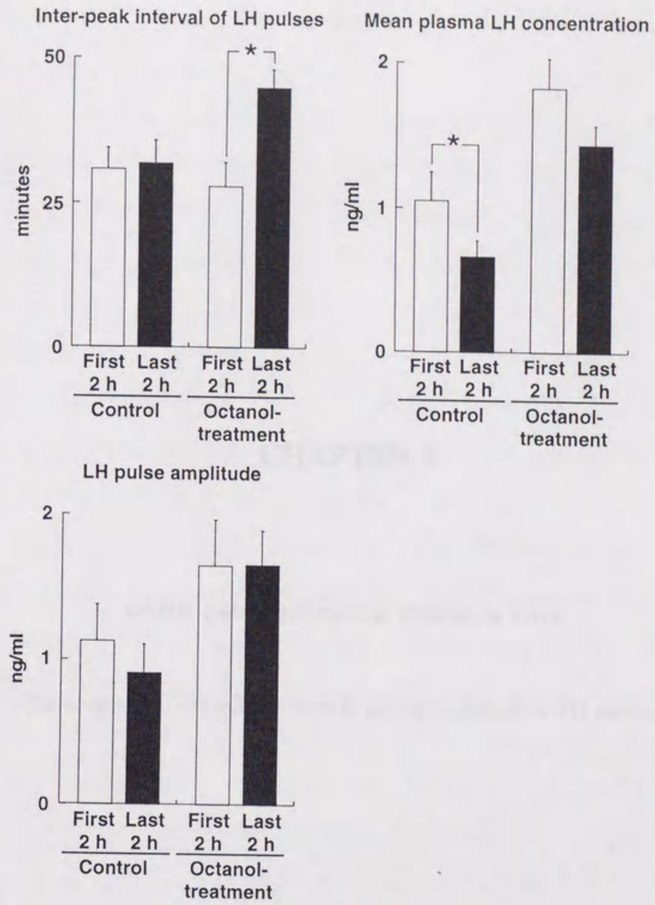


Fig. 3-6. Inter-peak intervals and amplitudes of LH pulses and the mean plasma LH concentrations in castrated male rats. The ME was perfused with vehicle (control group) or octanol (octanol-treated group) during the last 2 h of the sampling period. Values are the mean±SEM (n=7, control group; n=5, octanol-treated group). Open and closed columns indicate the mean values for the first and last 2 h of sampling periods, respectively. *, $p<0.05$ vs the first 2 h of sampling period.

CHAPTER 4

GnRH pulse-generating system *in vitro*

-Characterization of GnRH release in primary cultured GnRH neurons-

Introduction

An intercellular signal transmission through gap junctions in the ME is suggested to be involved in the synchronization of GnRH release from the nerve terminals in Chapter 3. The Chapter 4 deals with another important issue: How are GnRH pulses generated? It is still unknown whether the mechanism generating GnRH pulse is in GnRH neurons themselves or the mechanism is composed of non-GnRH neurons.

The fetal hypothalamus could already have functional components for the GnRH pulse-generating system, since it has been reported that transplantation of the fetal MBH graft containing few GnRH neurons into the third cerebral ventricle maintains pulsatile LH secretion in the OVX female rat bearing the posterior-anterior hypothalamic deafferentation, which abolishes LH pulses (Ohkura *et al.*, 1992). In the mutant hypogonadal mice, which is characterized by a deficiency of GnRH neurons (Cattanach *et al.*, 1977), transplantation of the normal fetal mPOA tissue can restore the reproductive functions such as mating, pregnancy, and delivery of healthy litters in adult females as well as spermatogenesis and testicular development in adult males (Gibson *et al.*, 1984; Krieger *et al.*, 1982). These recovery of reproductive functions has been suggested to be due to an increase in GnRH secretion from GnRH neurons originating from fetal mPOA, which indicates that fetal GnRH neurons have the same functional properties as adult animals to release GnRH. Furthermore, it has been reported that pulsatile GnRH secretion in an adult animal could be mimicked by the primary culture of rat fetal hypothalamic cells (Krsmanovic *et al.*, 1992), indicating that the primary culture originating from the fetal hypothalamic region is considered as one of the *in vitro* models for the GnRH pulse generator.

Taken the above-mentioned evidence together, the fetal hypothalamic region including GnRH neurons has the same GnRH-releasing activity as the adult has, and the primary culture originating from the fetal hypothalamus seems to have an ability to construct GnRH pulse-generating system *in vitro*. However, a numerous number of attempts have failed to construct a successful *in vitro* GnRH pulse-generating system. In this chapter, the secretory pattern of GnRH release from primary cultured cells, that originate from the fetal brain region including either the MBH, mPOA, both MBH and mPOA, or cerebral cortex, are characterized. If GnRH neurons themselves have an intrinsic activity for an episodic GnRH release, the release would be detected in any preparation. To the contrary, if the hypothalamic regions, especially the MBH, have non-GnRH neuronal components for generating pulsatile GnRH release, only the primary culture originating from the brain region containing the MBH would be able to release GnRH peptide in a pulsatile manner. Thus, the present chapter aims to test the above-mentioned hypothesis using an *in vitro* system.

Materials and Methods

Exp. 1

The fetal brain tissue fragments containing the whole hypothalamus (AH+PH), the anterior part of the hypothalamic region including the mPOA (AH), the posterior part of the hypothalamic region including the MBH (PH), or the cerebral cortical region (CX) were dissected out of the fetal brain at gestational day 17 for the primary cell cultures. Some of them were immunostained with GnRH.

The primary cell cultures originating from either the AH+PH, AH, PH, or CX were maintained for 2 weeks by replacing the half volume of the culture medium in the dish with same volume of the flesh one every 3-4 days. The medium taken were assayed for GnRH.

Exp. 2

At day 3-4 of the culture (day 0 is designated the day of the onset of the culture), some of the dishes were used for *in vitro* superfusion to monitor a secretory pattern of GnRH or immunostained with GnRH to count the number of GnRH-immunopositive cells in them.

Statistical analysis

GnRH pulses were identified by the PULSAR computer program (Merriam & Wachter, 1982). Mean and baseline level of GnRH release, and the frequency and amplitude of GnRH pulses were calculated for each experimental animal and then averaged for each group. The number of GnRH-immunoreactive cells were counted for each dish and averaged for each group. Statistical

differences in all parameters were analyzed by one-way factorial ANOVA. Fisher's least significant difference test was used as a post-hoc test.

Results

Exp. 1

GnRH-immunoreactive neurons in the fetal AH, PH, and CX tissue sections

GnRH-immunopositive neuronal cell bodies were found in the fetal AH, PH, and CX tissue fragments (Fig. 4-1). A large number of the cell bodies were observed in the AH tissue sections with scattering over the tissue. In the PH tissue sections, GnRH-immunopositive fibers (arrows) were also found. A small number of GnRH-immunoreactive neurons in the CX tissue sections located in the dentate gyrus of the hippocampus.

Changes in GnRH content in culture medium during 2-weeks culturing

GnRH content in culture medium is the highest level at day 3-4 of the culture in all groups (Fig. 4-2). GnRH contents remarkably decreased from day 6-7 of the culture in all groups but the AH group in which the content was still kept at a high level.

Exp. 2

Number of GnRH-immunoreactive cells in primary cultures

Fig. 4-3 A shows GnRH-immunoreactive cells in the AH+PH, AH, PH, and CX groups. The numbers of the cells in the AH group were significantly ($p<0.05$, ANOVA followed by Fisher's least significant difference test) higher than that in the CX group (Fig. 4-3 B). In the AH+PH and PH groups, numbers of GnRH-immunoreactive cells stayed in an intermediate level and were not significantly different from that in the AH or CX group.

Secretory pattern of GnRH release from primary cultures

Fig. 4-4 shows profiles of GnRH release in the AH+PH, AH, PH, and CX groups. GnRH were released in an episodic manner in all groups. The parameters for GnRH pulses, such as the mean GnRH level and the frequency and amplitude of GnRH pulses, were not significantly different between groups, although GnRH pulse amplitude in the CX group had a relatively low level compared with the other groups (Fig. 4-5). The baseline level of GnRH release is significantly ($p<0.05$, ANOVA followed by Fisher's least significant difference test) higher in the CX group than in any of the other groups.

Discussion

In the present experiment, the GnRH contents showed the highest values at day 3-4 of the culture in all groups, suggesting that the primary culture established in this study has the highest capacity to produce GnRH during this period. In the AH group, GnRH contents were still kept at a high level until 6-7 days after the onset of the culture. The high level of GnRH release could be due to a large number of GnRH neurons in the AH group compared with those in the other groups. The primary cultures originating from the fetal AH tissue fragment have a potential to produce a large amount of GnRH peptide.

Since the primary culture has the highest capacity to produce GnRH peptide day 3-4 of the culture, these primary cultured cells were used for *in vitro* superfusion to monitor secretory pattern of GnRH release. The release showed an episodic pattern in all groups. This result indicates that GnRH neurons themselves have an intrinsic activity to release the peptide in an episodic manner regardless of the origin of GnRH neurons in the fetal brain. While most GnRH neurons were found in the fetal hypothalamic regions and in the primary culture originating from the fetal hypothalamus, several GnRH neurons were distributed in the fetal extrahypothalamic region such as the dentate gyrus of the hippocampus and in the primary culture originating from the cerebral cortex. In this context, GnRH neurons originating in any regions of the brain could have a potential to release GnRH in a pulsatile manner at least during prenatal phase. Although GnRH neurons themselves are essential for release the peptide in an episodic manner, the number of GnRH neurons does not seem to be essential for pulsatile release. In the CX group, the number of GnRH-immunopositive cells is smaller than the others, but GnRH was intermittently released with significantly higher mean basal GnRH level than the other groups. To the contrary, any

parameters for the pulsatile GnRH release in the AH group, which contains the most GnRH-immunopositive neurons in culture dishes, were not significantly different from that in the AH+PH and PH groups.

The present experiment demonstrated that GnRH neurons have an intrinsic activity to release GnRH peptide intermittently, but the activity does not seem to be enough for generating pulsatility of GnRH release. Episodic secretory pattern of GnRH release from the primary cultured cells originating from the CX tissues could be different from that originating from the hypothalamic regions, because mean baseline value of GnRH pulses in the CX group was significantly higher than that in other three groups originating from the fetal hypothalamic regions. In the CX group, some GnRH neurons might release the peptide with the individual releasing activity. The mean baseline of GnRH level was, therefore, increased compared with the other groups. It is likely that non-GnRH neuronal components in the hypothalamus are involved in the synchronization of GnRH pulse, because Chapter 3 demonstrated that intercellular communications between each astrocyte and/or between astrocyte and GnRH nerve terminal through gap junctions play a role in the synchronization of GnRH pulse. Furthermore, it has been reported that transplantation of the fetal MBH graft, which dose not contain GnRH neurons, recovers pulsatile LH secretion in OVX rats, but not in the animals with transplantation of the fetal cerebral cortical graft (Ohkura *et al.*, 1992). In addition, GnRH release from the GT1-7 cells was also reported to be increased by co-culturing with type 1 rat astrocytes (Melcangi *et al.*, 1995).

In conclusion of this chapter, GnRH neurons themselves have intrinsic proprieties for an intermittent GnRH release. The intrinsic properties are essential for generating GnRH pulses, but the number of GnRH neurons is not important for GnRH pulse generation.

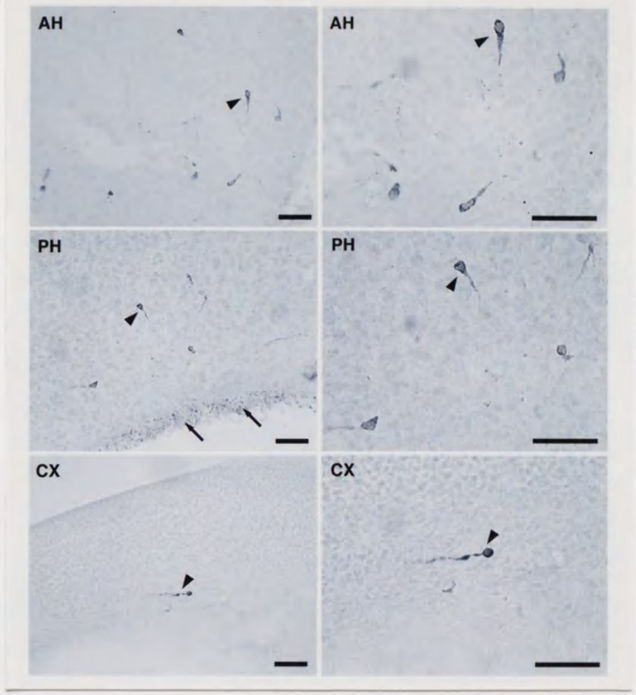


Fig. 4-1. Photomicrographs of GnRH-immunostained section of the fetal AH, PH, and CX tissue. Photomicrographs in the right hand side show higher magnification of the left hand side photomicrographs. Arrowheads indicate same GnRH-immunopositive neurons showing at each side. Scale bar, 50 μm; arrows, GnRH-immunoreactive fibers.

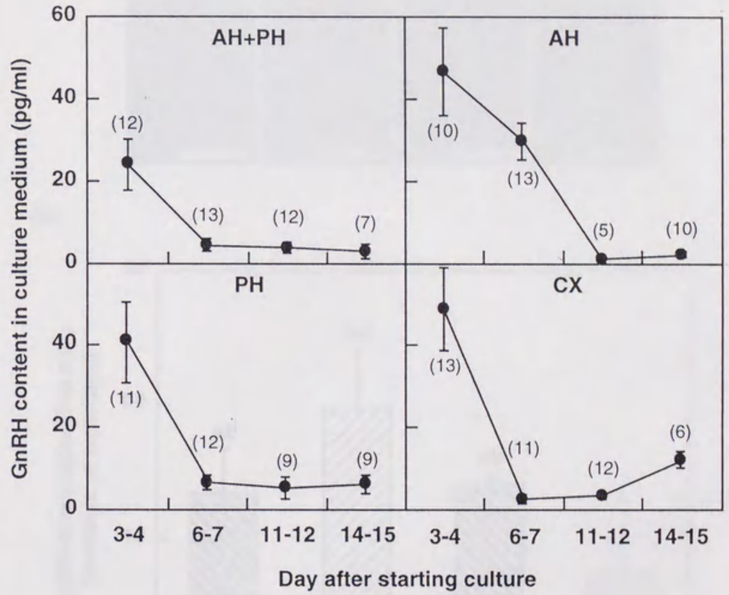
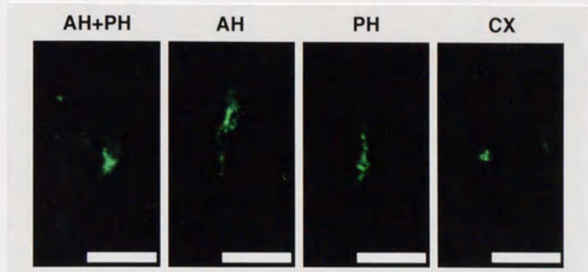


Fig. 4-2. Changes in the GnRH content in the culture medium in the AH+PH, AH, PH, and CX groups. Values are the mean±SEM at each stage and group. Numbers in each parentheses indicate the numbers of samples used for calculating each value.

(A)



(B)

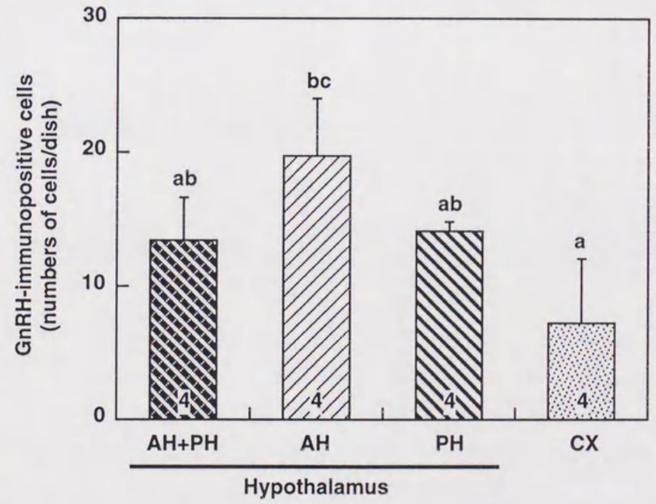


Fig. 4-3. GnRH-immunopositive cells in culture dishes of the AH+PH, AH, PH, and CX groups (A). Scale bar, 20 μm. Numbers of GnRH-immunopositive cells in culture dishes of each group (B). Values are the mean±SEM. Numbers in columns indicate the number of culture dishes used in each group. Values with different letters are significantly ($p<0.05$) different from each other.

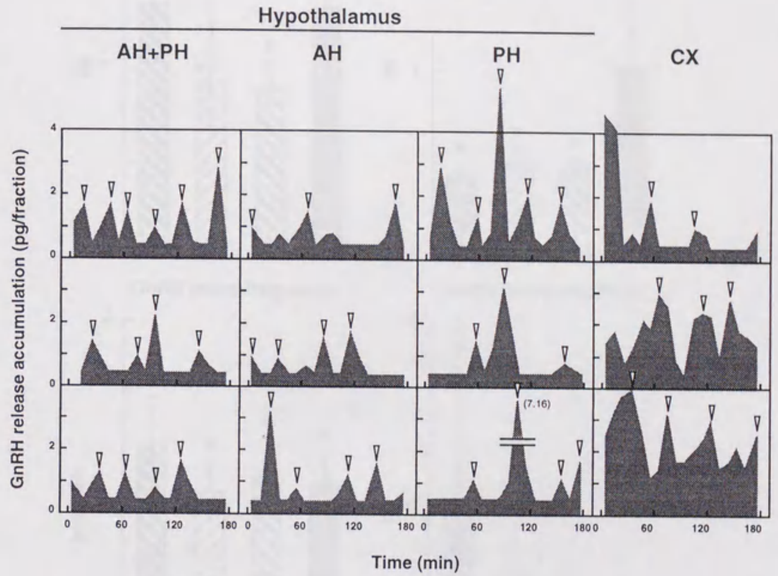


Fig. 4-4. Three representative profiles of GnRH release in primary cultured cells of the AH+PH, AH, PH, and CX groups. Superfusate was collected every 10 min for 3 h. Arrowheads indicate GnRH pulses identified with PULSAR computer program.

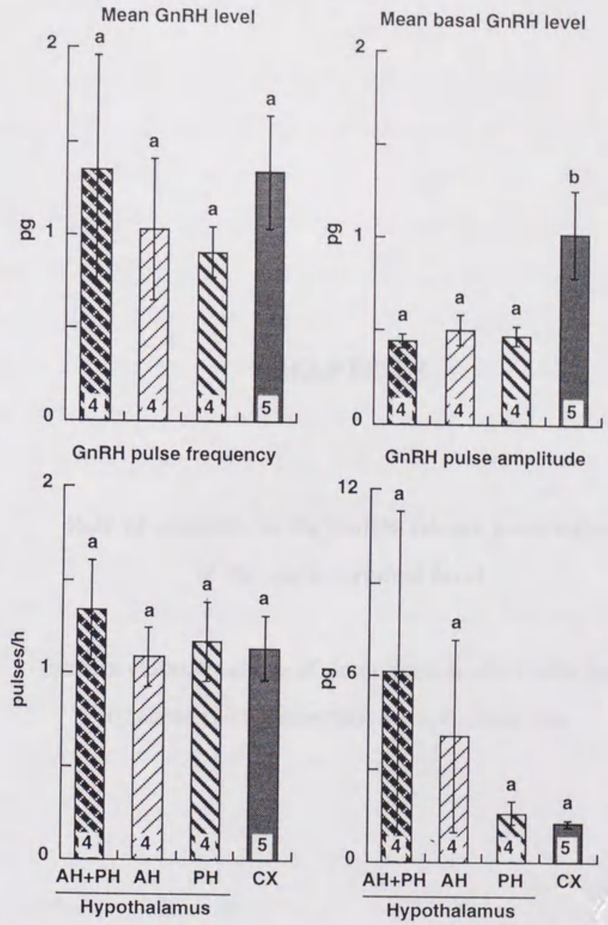


Fig. 4-5. Mean GnRH level, mean basal GnRH level, GnRH pulse frequency, and GnRH pulse amplitude in the primary cultured cells originating from the rat fetal AH+PH, AH, PH, and CX tissue fragments. Values are the mean±SEM. Numbers in columns indicate the number of culture dishes used in each group. Values with different letters are significantly ($p<0.05$) different from each other.