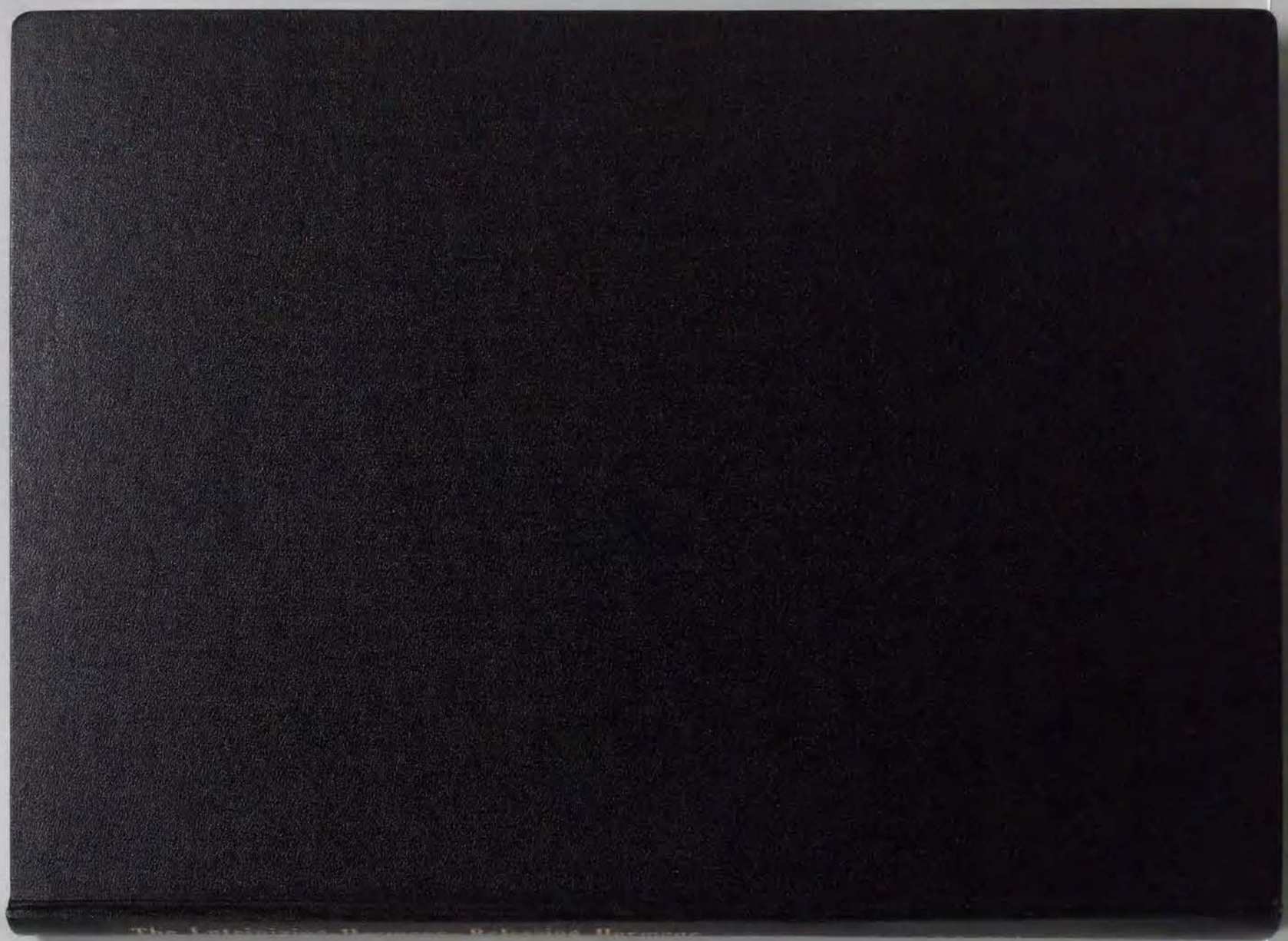

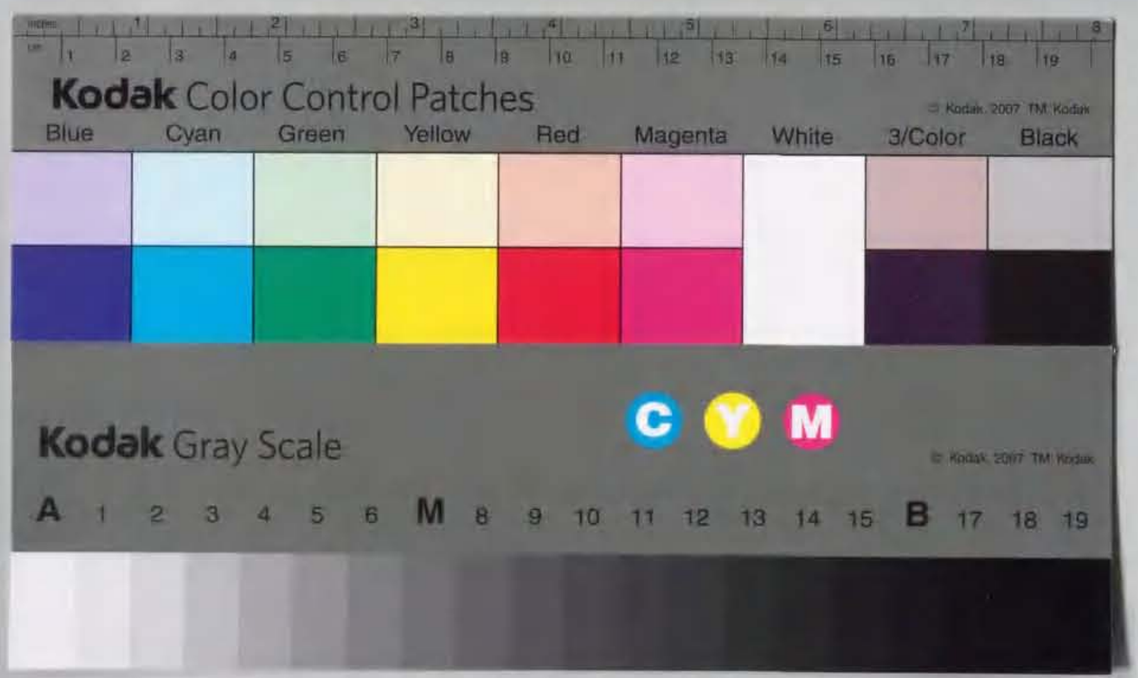
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The Luteinizing Hormone-Releasing Hormone Pulse Generator in Female Rats

by

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ABSTRACT

The present dissertation describes the neuroendocrine mechanism generating pulsatile luteinizing hormone-releasing hormone (LHRH) release in female rats.

In Chapter 3, the pulsatile luteinizing hormone (LH) secretion in chronically ovariectomized rats bearing various types of hypothalamic deafferentation was examined to determine whether the LHRH pulse generator is located in the mediobasal hypothalamus (MBH). The pulsatile LH secretion was observed in rats bearing complete (CD), anterolateral or anterior hypothalamic deafferentation and these types of deafferentation did not affect the frequency of LH pulses. Since the frequency is a parameter of LH pulses which directly reflects the activity of the LHRH pulse generator, all these deafferentations do not seem to affect its activity. These results suggest that the LHRH pulse generator resides within the hypothalamic island made by CD.

In the second experiment of Chapter 3, animals were subjected to posterior anterior-hypothalamic deafferentation (PAD), which separated the anterior part of the arcuate nucleus (ARC) from the MBH. Animals showed an irregular fluctuating pattern in plasma LH concentration 1 week after bearing PAD. LHRH-immunopositive neuronal fibers were still found in the external layer of the median eminence caudal to the incision of PAD, implying that perturbation of LH secretion is not ascribed to the damage to the LHRH neuronal fibers. These results suggest that the LHRH pulse generator consists of neurons that are different from LHRH-producing neurons.

In Chapter 4, to further confirm the existence of the LHRH pulse generator in the MBH, animals that had been transplanted with fetal brain tissue containing the MBH into the third ventricle were subjected to PAD. Rats bearing PAD without transplantation showed irregular pulsatile fluctuation of plasma LH, whereas LH pulses were maintained in rats bearing transplantation of the fetal MBH tissue. In rats which

had been transplanted with the cerebral cortex, LH pulses were less apparent after PAD than in the MBH-transplanted or sham-deafferentated animals. Parameters of pulsatile LH secretion in the MBH-transplanted rats were comparable to that in sham-deafferentated animals. These findings indicate that the graft containing the fetal MBH tissue prevented the effects of PAD and played a role in generating regular LH pulses after PAD. Moreover, no cell bodies of LHRH neurons were found immunohistochemically in the MBH grafts. These results, therefore, give further evidence that the LHRH pulse generator consists, at least in part, of a group of non-LHRH neurons in the MBH.

The pulsatile LH secretion after bilateral radiofrequency lesions of the ARC was examined in Chapter 5 to clarify the localization of the LHRH pulse generator within the MBH. The pulsatile LH secretion was observed in rats bearing bilateral lesions restricted to the rostral part of the ARC without the damage to the median eminence. This result suggests that the rostral part of the ARC is not directly involved in generating LH pulses. It is likely that the LHRH pulse generator is situated in the hypothalamic island made by CD, probably in the area between the rostral edges of the incisions of CD and the PAD.

The other experiment of Chapter 5 was designed to evaluate the involvement in generating LHRH pulses of the catecholaminergic neuronal system projecting to or in the arcuate nucleus-median eminence (ARC-ME) region. Animals suffered a neurotoxic depression of the catecholaminergic neuronal activity in the ARC-ME region from the intracranial microinjection with catecholaminergic neurotoxin, 6-hydroxydopamine (6-OHDA) and tyrosine hydroxylase inhibitor, α-methyl-p-tyrosine (α-MPT). Although the mean contents of norepinephrine and dopamine in the ARC-ME region in the 6-OHDA/α-MPT-treated rats were significantly reduced by 75.5% and 51.8%,

respectively, compared to those in the controls injected with vehicles, regular LH pulses were still apparent. These results suggest that the catecholaminergic neuronal system projecting to or in the ARC-ME region is not involved in the LHRH pulse generating mechanism.

In Chapter 6, the role of LHRH-producing neurons in the preoptic area (POA) in generating LHRH pulses was evaluated in rats by placing the electrolytic lesion in the POA. A regular pattern of pulsatile LH secretion was observed in rats with bilateral lesions of the POA. The frequency and amplitude of LH pulses were not affected by the bilateral damage restricted to the POA. These results suggest that the POA is not crucially involved in generating LH pulses and that the LHRH-producing neurons *per se* are not equipped with the mechanism generating LHRH pulses.

In conclusion, the findings in the present thesis demonstrate the neuroendocrine mechanism by which the pulsatile nature of LHRH release is generated in ovariectomized rats. The results in the present study suggest that (1) the LHRH pulse generator is located in the MBH, especially in the adjacent area of the anterior part of the ARC, but not within the ARC (2) the LHRH pulse generator consists of a group of neurons distinct from the LHRH-producing neurons, and (3) the catecholaminergic system projecting to or in the ARC-ME region is not involved in generating LHRH pulses.

It has been revealed anatomically that most neuronal cell bodies of LHRH neurons are widely distributed over the area anterior to the hypothalamus and project their axons to the median eminence and that there are few synaptic contacts between neuronal fibers in the median eminence. I, therefore, propose the following mechanism: The LHRH pulse-generating neurons located in the MBH transmit a signal to the neuronal terminals of LHRH neurons in the median eminence by hypothetical non-

synaptic "volume transmission" mechanism and generate pulsatile LHRH release in female rats.

CONTENTS

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The Author

LIST OF ABBREVIATIONS

AD	anterior hypothalamic deafferentation
ALD	anterolateral hypothalamic deafferentation
ARC	arcuate nucleus
ARC-ME	arcuate nucleus-median eminence
α-MPT	α-methyl-p-tyrosine
CD	complete hypothalamic deafferentation
DA	dopamine
HPLC-ECD	high performance liquid chromatography-electrochemical detection
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
MBH	mediobasal hypothalamus
MUA	multiple unit activity
NDS	normal donkey serum
NE	norepinephrine
NMDA	*N*-methyl-D,L-aspartate
6-OHDA	6-hydroxydopamine
PAD	posterior-anterior hypothalamic deafferentation
PAP	peroxidase-antiperoxidase
PBS	phosphate-buffered saline
POA	preoptic area
POA/AHA	preoptic/anterior hypothalamic area
RIA	radioimmunoassay
SCN	suprachiasmatic nucleus
SD	standard deviation

CHAPTER 1

General introduction

Reproductive functions are regulated by the brain

In mammals, the reproductive processes, such as sexual differentiation, reproductive cycles and sexual behavior, are remarkably complex but are efficiently organized. For instance, the timing of the occurrence of the mating behavior well coincides with that of ovulation to allow animals to be pregnant. During lactation, ovulation is blocked as long as mothers nurse their babies, so that mothers are allowed to spend all of their energy to yield milk but not to maintain the pregnancy. One should note that those reproductive processes, like all other physiological processes, are under the brain control.

The information derived from various external or internal environmental factors alters the reproductive function through the brain mechanisms (Gilmore & Cook, 1981). An annual change in daylength, or the photoperiod, is the predominant external cue for establishing annual reproductive cyclicity in both sexes of most mammals (Karsch, Bittman, Foster *et al.*, 1984; Lincoln & Short, 1980; Turek & Campbell, 1979). The long-day condition reduces the gonadal activity in short-day breeders, such as sheep, goats, deer, mink and some species of primates, whereas it increases the activity in long-day breeders, such as hamsters, ferrets, horses and most temperate-zone birds. Exposure to stress is known to induce the disruption of reproductive activities in many mammalian species (Briski & Sylvester, 1988; Rivier, Rivier & Vale, 1986; Rose, Gordon & Bernstein, 1972). Gonadal activity is also depressed under the condition of malnutrition (Cagampang, Maeda, Yokoyama & Ôta, 1990; Campbell, Kurcz, Marshall & Meites, 1977; McClure & Saunders, 1985; Pirke & Spyra, 1981). In addition, several kinds of odors deriving from animal's body serve as chemosignals, *i.e.* pheromones, between individuals to time the reproductive events: Some of these odors alter the activity of physiological mechanisms controlling reproductive events

such as sexual behavior, ovarian cycle or puberty through the olfactory system (Vandenbergh, 1988). The gonadal function is also affected by the physiological events in animals: Lactation is one of the events suppressing the reproductive activity (Howie & McNeilly, 1982; Maeda, Tsukamura & Yokoyama, 1987; McNeilly, 1988; Smith & Neill, 1977).

The information derived from these environmental factors is accepted by the various sensory systems and conveyed to the brain by neural pathway. The brain then integrates these sensory inputs and converts them into humoral signals, such as hypothalamic hormones, which in turn regulate the gonadal function by facilitating or suppressing the secretion of gonadotropins or other hormones from the anterior pituitary gland.

Thus, the brain should be put in the higher place in the complexity of the network managing the reproductive system in higher vertebrates. The brain mechanism regulating gonadotropin secretion has, therefore, attracted much our attention to the network of the reproductive system in mammals.

The dawn of the neuroendocrinology

The term "neuroendocrinology" is based on the concept that the neural and endocrine systems are closely related to and influenced by each other. It is well accepted that neuroendocrine mechanisms, involving the interaction of neural and endocrine systems, regulate a variety of body functions. This interaction is reciprocal: The neural system regulates, directly or indirectly, the function of the endocrine glands, such as the anterior pituitary, posterior pituitary, ovary, testis, thyroid gland and adrenal gland. Hormones derived from these glands feed back to the central nervous system and control the activity of neurons (McEwen, Biegon, Davis *et al.*, 1982).

Neuroendocrinology, therefore, may be defined as a field that deals with mechanisms converting the neural information into the humoral one, and vice versa.

Geoffrey W. Harris first described the concept of neurohumoral control of the pituitary gland based on a series of his excellent and skillful experiments (Harris, 1955). He found that disconnecting the pituitary stalk results in an atrophy in the gonad and the thyroid and adrenal glands, the functions of which are now known to be regulated by pituitary hormones. He also revealed that the recovery of function of these glands was directly proportional to the extent of regeneration of hypophysial portal vessels in stalk-sectioned animals. Eventually, the electrical stimulation of the hypothalamus was found to evoke gonadotropic or adrenocorticotropic secretion from the anterior pituitary, whereas direct stimulation of the gland itself was ineffective. Thus it became evident that the hypothalamus regulates the secretion of the anterior pituitary gland. Harris assumed from his findings that nerve fibers from the hypothalamus must liberate some humoral substance into the capillaries of the primary plexus in the median eminence and that this substance must be carried by the hypophysial portal vessels to excite the function of the anterior pituitary. In other words, he concluded that the portal vessel system represents a final common pathway from the brain to the anterior pituitary.

Luteinizing hormone is secreted from the pituitary in an episodic manner

Of the gonadotropins, luteinizing hormone (LH) has a critical role in the regulation of reproductive function in both sexes. In the female, it has been established that there are two modes of LH secretion from the anterior pituitary gland: One is the tonic mode, which is responsible for follicular development and ovarian hormone

synthesis or its secretion; the other is the cyclic mode, which induces ovulation followed by luteinization. It has been considered that these two modes of LH secretion are regulated at two specific sites of the brain, which are called the tonic center located in the mediobasal hypothalamus (MBH) and the cyclic center located in the preoptic area (POA) (Gorski, 1968; Halász & Gorski, 1967; Halász & Pupp, 1965).

Both modes of LH secretion are characterized by an episodic fluctuation. The pulsatile LH secretion was first demonstrated in the ovariectomized monkeys (Dierschke, Bhattacharya, Atkinson & Knobil, 1970) and then found in rats (Gay & Sheth, 1972), ewes (Butler, Malven, Willett & Bolt, 1972), cows (Forrest, Fleeger, Long *et al.*, 1980) and cyclic women (Midgley & Jaffe, 1971). The striking feature of LH secretion is observed in any phase of estrous cycle (Fox & Smith, 1985; Gallo, 1981b; Hauger, Karsch & Foster, 1977; Higuchi & Kawakami, 1982; Norman, Lindstrom, Bangsberg *et al.*, 1984; Rahe, Owens, Fleeger *et al.*, 1980) and even in the preovulatory (Gallo, 1981a; Rahe *et al.*, 1980) or gonadal steroids-induced (Terasawa, Krook, Eman *et al.*, 1987) LH surge. In females, the frequency and amplitude of pulsatile LH secretion dramatically change from phase to phase, indicating that the ovarian activity during the estrous cycle is controlled by the pattern of pulsatile LH secretion.

Neuroendocrine regulation of pulsatile LH secretion

The LH secretion from the anterior pituitary is controlled by the neurohumoral mechanism in the hypothalamus via hypophysial portal vessels. Harris's prediction (Harris, 1955), that the hypothalamus yields and liberates some humoral substances to regulate the function of gonadotrophs, was proved in the early 70's by Schally's and Guillemin's groups; they isolated a decapeptide, luteinizing hormone-releasing

hormone (LHRH) from porcine (Matsuo, Baba, Nair *et al.*, 1971; Schally, Arimura, Baba *et al.*, 1971) and ovine (Amoss, Burgus, Blackwell *et al.*, 1971; Burgus, Butcher, Amoss *et al.*, 1972) hypothalamus and determined its amino acid sequence.

It has been reported that blockade of the LHRH actions by immunoneutralizing the endogenous decapeptide (Ellis, Desjardins & Fraser, 1983; Fraser, Jeffcoate, Gunn & Holland, 1975) or antagonizing its receptor (Ellis *et al.*, 1983; Grady, Shin, Charlesworth *et al.*, 1985) reduces LH secretion and results in disruption of gonadal function leading to infertility. Moreover, mutant mice that are deficient in LHRH-producing ability shows hypogonadism (Cattanach, Iddon, Charlton *et al.*, 1977). These results indicate the importance of LHRH in regulation of LH secretion and then, of gonadal function.

It is well documented that LHRH is produced in the hypothalamus and released into the hypophysial portal vessels in a pulsatile manner (Carmel, Araki & Ferin, 1976; Levine & Ramirez, 1980), and that its rhythmic release leads to the pulsatile secretion of LH from the anterior pituitary into the peripheral circulation (Clarke & Cummins, 1982; Levine, Pau, Ramirez & Jackson, 1982; Moenter, Brand, Midgley & Karsch, 1992). Temporal relationships between LHRH and LH pulses have been well documented in monkeys (Levine, Norman, Gliessman *et al.*, 1985; Pau, Hess, Kaynard *et al.*, 1989; Van Vugt, Diefenbach, Alston & Ferin, 1985; Xia, Van Vugt, Alston *et al.*, 1992), sheep (Caraty & Locatelli, 1988; Clarke & Cummins, 1982; Karsch, Cummins, Thomas & Clarke, 1987; Levine *et al.*, 1982), rabbits (Pau, Orstead, Hess & Spies, 1986), and rats (Levine & Duffy, 1988; Urbanski, Pickle & Ramirez, 1988): In all these works, each LH pulse corresponds to a LHRH pulse.

The physiological importance of pulsatile LHRH release in maintaining the function of the hypothalamo-pituitary-gonadal axis has also been demonstrated. In

monkeys with hypothalamic lesions that deplete the endogenous LHRH release, continuous infusion of the exogenous LHRH fails to restore the gonadotropin secretion, whereas an intermittent administration with a frequency as is seen in intact monkeys reestablishes pituitary gonadotropin secretion (Belchetz, Plant, Nakai *et al.*, 1978). A poor responsiveness of pituitary LH secretion to LHRH stimulus is also observed after continuous infusion of LHRH in rat anterior pituitary cells cultured *in vitro* (Badger, Loughlin & Naddaff, 1983). These results suggest that constant exposure to LHRH leads to desensitization of the anterior pituitary. Moreover, the ovulatory menstrual cycles are maintained by mimicking the pulsatile LHRH release in the rhesus monkey bearing a hypothalamic lesion (Knobil, 1980). In the hypogonadal mouse, the pulsatile administration of LHRH is much more effective than a single large bolus injection of LHRH in stimulating the synthesis and release of gonadotropin and thereby establishing the normal function of the gonad (Charlton, Halpin, Iddon *et al.*, 1983).

External environmental factors as described above are known to induce the change in the pattern of the pulsatile LH secretion, and subsequently the change in the gonadal activity (Karsch *et al.*, 1984; Lincoln & Short, 1980; McNeilly, 1988). On the other hand, internal environmental factor, such as hormonal milieu, also has influence on the pulsatile LH secretion. For instance, ovarian steroids secreted from ovaries affect the pulsatile secretion of LH by the negative or positive feedback mechanism during the estrous cycle (Freeman, 1988; Weick, 1981). In ovariectomized animals, treatment of exogenous ovarian steroids, such as estrogen and progesterone, inhibit LH pulses in monkeys (Yamaji, Dierschke, Bhattacharya & Knobil, 1972), rabbits (YoungLai, 1978) and rats (Blake, Norman & Sawyer, 1974; Leipheimer, Bona-Gallo & Gallo, 1984). Goodman and Karsch (1980) have provided the evidence that

progesterone treatment lowers the frequency of LH pulses in the ovariectomized ewe without changing the amplitude, whereas estrogen treatment decreases the amplitude of LH pulses without affecting the frequency. On the contrary, estradiol can enhance the frequency of LH pulses in ewes during the follicular phase (Karsch, Foster, Bittman & Goodman, 1983) and in long-term ovariectomized ewes (Kaynard, Follett & Karsch, 1988). Thus, the steroid-hormonal milieu plays a role in determining the frequency and amplitude of LH pulses as one of the internal environmental factors.

The pattern of the pulsatile LH secretion is, therefore, a key determinant regulating the gonadal activity in mammals. Signals originating in external or internal environmental factors and affecting the reproductive system would be finally fed to a putative mechanism governing a pulsatile discharge of LHRH, the LHRH pulse generator. Although the existence of the LHRH pulse generator has been postulated in the brain, the location and the components of the LHRH pulse generator is still under debate (Dyer & Robinson, 1989; Levine, Bauer-Dantoin, Besecke *et al.*, 1991; Lincoln, Fraser, Lincoln *et al.*, 1985; Ramirez & Dluzen, 1987).

LHRH pulse generator

Halász and Pupp (1965) first reported that female rats bearing "complete hypothalamic deafferentation" showed either persistent estrus or persistent diestrus, and suggested that the center for the tonic LH secretion is located inside the hypothalamic island, namely the MBH, which is isolated by the complete hypothalamic deafferentation from the rest of the brain. The MBH includes the arcuate nucleus (ARC), periventricular nucleus and the medial part of the retrochiasmatic area. Subsequently, Blake and Sawyer (1974) reported that the pulsatile LH secretion is still apparent in ovariectomized rats after bearing complete hypothalamic deafferentation. These are the first reports suggesting that the putative LHRH pulse generator, the

mechanism generating the pulsatile LH secretion, is located in the MBH.

Several lines of evidence support the idea mentioned above that the LHRH pulse generator exists in the MBH. Multiple unit activity (MUA) recording technique revealed that MUA volleys in the arcuate nucleus-median eminence (ARC-ME) region correspond with peaks of LH pulses in anesthetized or conscious monkeys (Knobil, 1981; Wilson, Kesner, Kaufman *et al.*, 1984), rats (Kawakami, Uemura & Hayashi, 1982; Kimura, Nishihara, Hiruma & Funabashi, 1991; Nishihara, Hiruma & Kimura, 1991) and goats (Mori, Nishihara, Tanaka *et al.*, 1991). Blake and Sawyer (1974) have reported that post-castration rise in plasma LH levels and the pulsatile LH secretion were blocked in rats bearing anterior hypothalamic deafferentation with the lesion of the anterior part of the ARC. Furthermore, similar results were obtained, in which anterior hypothalamic deafferentation combined with the electrolytic lesion of the anterior part of the ARC abolished the pulsatile LH secretion (Soper & Weick, 1980). All these observations suggest that certain neural circuits involved in generating LHRH pulses are located within the MBH.

However, the advancement of immunocytochemical technique brought about a discovery of unexpected distribution of the LHRH-producing neurons in the brain. In the rat, most cell bodies of LHRH neurons are distributed extensively in the region anterior to the hypothalamus, *i.e.* the medial septal area, diagonal band of Broca and medial POA, with most of their axons projecting to the external layer of the median eminence (Kawano & Daikoku, 1981; Sétáló, Vigh, Schally *et al.*, 1976; Witkin, Paden & Silverman, 1982). Some cell bodies of LHRH neurons exist in the hypothalamic island made by complete hypothalamic deafferentation, but have never been found in the ARC. Therefore, several workers, who consider the LHRH release to be regulated at the level of the LHRH neuronal cell bodies or dendrites, assume that

the LHRH pulse generator is located in the preoptic/anterior hypothalamic area (POA/AHA) or in the extrahypothalamic structure. They argue that the maintenance of LH pulses after complete hypothalamic deafferentation could be ascribed to the subchiasmatic LHRH projection to the median eminence, which left intact by the hypothalamic deafferentation (Coen, 1987; Hoffman & Gibbs, 1982). In addition, local application of a specific α_1-receptor antagonist into the POA, but not into the MBH, caused a reduction of LH pulsatility (Jarry, Leonhardt & Wuttke, 1990). The inverse pattern of plasma LH concentrations and γ-aminobutyric acid release from the POA/AHA was reported in rats using the push-pull perfusion technique (Jarry, Perschl & Wuttke, 1988; Wuttke, Jarry, Demling *et al.*, 1987). Furthermore, Meyer (1987) reported that the superfused MBH fragments showed less apparent periodicity of LHRH release than the MBH fragments with preoptic-suprachiasmatic area *in vitro*. All these data support the idea that the LHRH pulse generator is located outside the MBH.

Other researchers think that the LHRH neurons themselves are capable of generating pulsatile LHRH release. Isolated LHRH neurons from the POA exhibited spontaneous LHRH release which continued in a regular repetitive manner *in vitro* (Melrose, Gross, Cruse & Rush, 1987). Clonal LHRH-producing neuronal cell lines, which were immortalized by genetically directed tumorigenesis in transgenic mice (Mellon, Windle, Goldsmith *et al.*, 1990; Weiner, Wetsel, Goldsmith *et al.*, 1992), show a pulse-like pattern of LHRH release *in vitro* (Martínez de la Escalera, Choi & Weiner, 1992). In addition, perifused primary cultures of fetal rat hypothalamic neurons, as well as the LHRH neuronal cell line, spontaneously exhibited episodic LHRH release; the frequency of which was comparable to that observed in perifused adult or fetal hypothalamic tissues (Krsmanovic, Stojilkovic, Merelli *et al.*, 1992).

Thus, there has still been a controversy over the location and nature of the brain mechanism generating LHRH pulses. Although the concept of the LHRH pulse generator has been widely accepted, it has been in the "black-box" state in the reproductive physiology. The physiological importance of the pulsatile LHRH release in mammalian reproduction motivates us to put a definition into what is still an amorphous nature.

Objectives

The aim of this dissertation is to elucidate the neuroendocrine mechanism which generates LHRH pulses in mammals: Particularly, the location and components of the putative LHRH pulse generator are discussed. Ovariectomized rats are used as a model for the domestic animals and human through a series of experiments. The pulsatile LH secretion from the anterior pituitary gland is monitored as an *in vivo* indicator of the activity of the LHRH pulse generator in the brain.

In the first approach in Chapter 3, effects of the hypothalamic deafferentation on the pulsatile secretion of LH are examined to determine whether the LHRH pulse generator is located in the MBH. In the second place, in Chapter 4, existence of the LHRH pulse generator in the MBH are confirmed by fetal brain transplantation technique. Thirdly, in Chapter 5, the participation of catecholaminergic system within the ARC-ME region in generating LHRH pulses is estimated: The system was damaged by radiofrequent or chemical lesion technique. Finally, in Chapter 6, the role of LHRH-producing neurons existing in the POA in generating LHRH pulses is evaluated in rats by placing the electrolytic lesion in the POA.

CHAPTER 2

General procedures

Animals

Female Wistar-Imamichi strain rats weighing 250 to 300 g were used. They were housed under conditions of 14 h light/10 h dark (lights on at 0500 h) and 22±2 °C with free access to food (Labo-MR-Stock; Nihon Nohsan Kogyo Co., Yokohama, Japan) and water.

The daily pattern of vaginal smear was observed in each animal to determine the estrous cycle before receiving various experimental treatments. Animals showing two or more consecutive regular 4-day estrous cycles were bilaterally ovariectomized.

Surgery

All surgical procedures were performed under ether anesthesia and aseptic conditions. Animals were injected with antibiotics (Mycillin Sol Meiji; Meiji Seika Kaisha, Ltd., Tokyo, Japan and Gentacin; Schering-Plough Co., Osaka, Japan) after receiving any surgical operations.

Hypothalamic deafferentation

Four types of hypothalamic deafferentation, *i.e.* complete (CD), anterolateral (ALD), anterior (AD) and posterior-anterior (PAD) hypothalamic deafferentation were employed by the procedure previously described (Blake & Sawyer, 1974; Halász & Pupp, 1965; Tsukamura, Maeda, Ohkura & Yokoyama, 1990). The bayonet-shaped knives used had the following dimensions: knife A: vertical, 1.8 mm; radius, 1.0 mm; knife B: vertical, 1.8 mm; radius, 1.8 mm (Figure 2.1). Animals were placed in a stereotaxic instrument (SR-6; Narishige Scientific Instrument Laboratory, Tokyo,

Japan) with the dorsoventral level of bregma 1.6 mm below lambda to make the base of the brain horizontal.

CD was made by lowering knife A into the brain to the base of the skull with the tip end at 1.5 mm posterior to bregma. Then the knife was rotated 90° to the left and moved 2.5 mm posteriorly. After the knife was raised 0.5 mm, it was moved 1.5 mm further posteriorly and rotated 180° to make a posterior half circle. The knife was then moved 4.0 mm anteriorly in two steps (the knife was lowered 0.5 mm after moving 1.5 mm), rotated 90° to the left, and was removed from the same site at which it had entered the brain.

Knife B was used for ALD, AD and PAD. The operating procedure for ALD and AD was identical to that of CD except that posterior and latero-posterior portions were not cut in ALD and AD, respectively. Briefly, in ALD, the knife was rotated 90° to the left after lowering to the base of the skull and moved 4.0 mm posteriorly in two steps (the knife was raised 0.5 mm after moving 2.5 mm) and then anteriorly. The knife was rotated 180° to the right and moved 4.0 mm posteriorly and then anteriorly in the same manner as in the opposite side. Finally, the knife was rotated 90° to the left and removed. AD was made by rotating the knife 90° to both the right and left. PAD was performed in a similar manner to AD except that the knife was lowered into the brain 3.0 mm posterior to bregma.

In the sham-deafferentated animals, only the outer cannula of the knife (outer diameter 0.7 mm) was inserted into the brain at 2.5 mm and 4.3 mm posterior to bregma for CD and PAD, respectively. The tip end of the outer cannula was positioned at 8.0 mm below bregma in each case. The outer cannula was either moved 4.0 mm posteriorly and then 4.0 mm anteriorly as in CD or not moved as in PAD.

Fetal brain transplantation

The brain tissue was grafted by the procedure described previously (Matsumoto, Kobayashi, Murakami & Arai, 1984) with a slight modification. A grafting block of the brain tissue including either the MBH or the cerebral cortex was dissected with microscissors from the forebrain of a fetus on day 17 of gestation (day 0 = the day when a vaginal plug was found). The graft was immediately loaded into a stainless-steel cannula (internal diameter 0.7 mm; outer diameter 1.2 mm) under an aseptic condition. In order to position the graft within the bottom of the third ventricle of the recipient, the cannula was stereotaxically (Paxinos & Watson, 1986) lowered to 8.8 mm ventral to and 2.8 mm posterior to bregma in the midline. The stylet (outer diameter 0.7 mm) was then depressed to push out the graft into the third ventricle.

Electrolytic lesion

Bilateral lesions of the POA were produced electrolytically by passing a cathodal direct current of 1 mA for 30 seconds through a monopolar stainless-steel electrode (outer diameter 0.25 mm). The electrode was insulated by epoxide except for its tip end. The current was generated with an electric stimulater (DPS-151; Dia Medical System Co., Tokyo, Japan) which was connected to an isolation unit (DPS-122; Dia Medical System Co.). The stereotaxic coordinates for the POA lesion were as follows: 0.6 mm posterior to and 8.8 mm below bregma; ±0.4 mm from the sagittal sinus (Paxinos & Watson, 1986). In the sham-lesioned animals, the electrode was lowered into the POA without passing a current.

Radiofrequency lesion

Bilateral thermocoagulative lesions of the ARC were achieved by passing a

radiofrequency current (4 to 5 mA, 500 kHz), which was generated with a lesion generator system (Model RFG-4A; Radionics, Inc., Burlington, MA, USA), through an electrode (Type TCZ, tip diameter 0.25 mm, tip exposure 0.25 mm; Radionics, Inc.). The current controlled to keep the temperature at the tip end of the electrode at 60 °C was passed for approximately 60 sec to make the size of lesion constant. Two lesions were made on each side of the brain for each animal to ensure to destroy the rostral half of the nucleus. Stereotaxic coordinates were as follows: 2.4 and 3.2 mm posterior to and 10.0 and 10.2 mm below bregma, respectively; ±0.3 mm lateral to the midsagittal sinus (Paxinos & Watson, 1986). Sham lesions were made by lowering the electrode without passing the current.

Hypothalamic microinjection cannula implantation

Bilateral intrahypothalamic microinjections of the drug solution were performed through a guide cannula that had been chronically implanted in the ARC. Animals were placed in a stereotaxic apparatus with the levels of the bregma and lambda equal. A bilateral guide cannula, which consisted of two 26-gauge needles with a distance of 1.0 mm between the needles, was implanted into the brain with the coordinate at 2.4 mm posterior to bregma and ±0.5 mm lateral to the midline according to the brain atlas of Paxinos and Watson (1986). The guide cannula was fixed to the skull with dental acrylic resin (Quick Resin; Shofu Inc., Kyoto, Japan). The tip end of the 33-gauge internal cannula (C315I; Plastic Products Co., Roanoke, VA, USA) for microinjection protruded into the ARC-ME region at the depth of 10.0 mm from the surface of the skull. Dummy cannulae (31 gauge) were then inserted into the guide cannula to prevent a block of tubing.

Blood sampling

An indwelling Silastic cannula (No. 00; inner diameter 0.5 mm, outer diameter 1.0 mm; Shinetsu Polymer Co., Tokyo, Japan) for collecting blood samples was inserted into the right atrium through the right jugular vein (Maeda & Tsukamura, 1989) 1 to 5 days before blood sampling under ether anesthesia. The cannula was filled with the solution of 40% (W/V) polyvinylpyrrolidone (PVP-10; Mol. Wt. 10,000, Sigma Chemical Co., St. Louis, MO, USA) dissolved in physiological 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 Seika Kaisha, Ltd.) to prevent blood coagulation and bacterial infection.

Blood samples (80 μl or 100 μl) were collected every 6 min for 3 h from 1300 h. Each sample was replaced with an equal volume of rat red blood cells that had been collected from other rats and suspended in saline. Plasma was separated by immediate centrifugation at 4 °C for 20 min and stored at -20 °C until assayed for LH.

Brain histology

After blood sampling in each experiment, animals were anesthetized with pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL, USA) and perfused with physiological saline followed by 0.05 M phosphate-buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde and 0.5% glutaraldehyde. Brains were removed from the skull and stored in 0.05 M PBS containing 4% paraformaldehyde. Frozen coronal sections (30 or 50 μm) were obtained from each brain with a cryostat

(Type MTE; SLEE Technik GmbH, Mainz, Germany).

Some of the sections were stained with cresyl violet (Chroma Gesellschaft Schmid & Co., Stuttgart, Germany) or thionin (E. Merck AG, Darmstadt, Germany) to determine the position of deafferentation, the location and extent of lesions, the site of microinjection and the location of the graft according to the brain atlas of Paxinos and Watson (1986).

Some sections mounted on gelatin-coated glass slides were immunostained for LHRH according to the peroxidase-antiperoxidase (PAP) method. The rabbit anti-LHRH serum, LR1 A-LHRH, was kindly provided by Dr. R. Benoit (Montreal General Hospital Research Institute, Montreal, Canada) and its characteristics have previously been described (Schwanzel-Fukuda & Pfaff, 1989). The sections were rinsed overnight in 0.1 M PBS, pH 7.4, and were then prepared as follows: 1) PBS containing 2% lysine monohydrochloride (Sigma Chemical Co.) and 0.1% NaN_3 was applied for 72 h; 2) rabbit anti-LHRH serum at 1:5000 dilution in PBS containing 5% normal donkey serum (NDS; Chemicon International Inc., El Segundo, LA, USA) and 0.1% NaN_3 was applied for 96 h; 3) goat anti-rabbit γ-globulin serum (Nihon Shiba-yagi Center, Gunma, Japan) at 1:50 dilution in PBS containing 5% NDS and 0.1% NaN_3 was applied for 2 h; 4) PAP complex (Dakopatts, Glostrup, Denmark) at 1:200 dilution in PBS containing 1% NDS was applied for 2 h; 5) for visualization, 0.1 M Tris buffer, pH 7.6, containing 0.0167% 3,3'-diaminobenzidine (Sigma Chemical Co.) and 0.0045% hydrogen peroxide was applied for 10 to 20 min. Steps 1 to 3 were followed by rinsing the sections in PBS for 1 h, while steps 4 and 5 followed by rinsing in 0.1 M Tris buffer for 45 min. The first two steps were performed at 4 °C and the rest at room temperature, in a moist chamber. The sections were then dehydrated and mounted with Canada balsam.

Assays

Luteinizing hormone (LH)

In experiment 1 of Chapter 3, LH concentrations in single aliquots of 25 µl of plasma samples were determined by a heterologous radioimmunoassay (RIA) as previously described (Maeda, Tsukamura, Uchida *et al.*, 1989). The antiserum to ovine LH, GDN #15, was generously provided by Dr. G.D. Niswender of Colorado State University, Fort Collins, CO, USA (Niswender, Midgley, Monroe & Reichert, 1968). The rat LH for reference and iodination was supplied by the National Hormone and Pituitary Program (Baltimore, MD, USA). The least detectable level of LH was 1.9 pg/tube. The intra- and interassay coefficients of variation were 14.4% at the level of 30.9 pg/tube and 9.2% at the level of 29.0 pg/tube, respectively.

In the rest of the experiments, LH concentrations in single aliquots of 50 µl of plasma samples were measured by a homologous RIA with a rat LH kit supplied by the National Hormone and Pituitary Program. The least detectable level of LH was 7.8 pg/tube. The intra- and interassay coefficients of variation were 6.19% at the level of 121.4 pg/tube and 13.6% at the level of 212.3 pg/tube, respectively.

The values were expressed in terms of the NIDDK-rLH-RP-2 reference preparation in all assays. All plasma samples were assayed as a single determination.

Catecholamines

After blood sampling in experiment 2 of Chapter 5, contents of norepinephrine (NE) and dopamine (DA) in the ARC-ME region of the hypothalamus were measured to validate the effects of catecholaminergic depressant. Animals were killed by decapitation and the brain was quickly removed from the skull. A coronal slice of the

brain, which is about 2.5 mm thick from the rostral edge of the median eminence, was made with a razor blade and a brain mold kept on an ice bath. The ARC-ME region was then dissected out from the slice on an ice-cold dish under a microscope: The cuts were made approximately 1 mm lateral to the midline and 1 mm from the ventral surface of the brain. The brain tissue was homogenized with a teflon/glass homogenizer in 1 ml of ice-cold 0.1 N HCl and then kept on an ice for 30 min. After centrifugation at 20,000×*g* for 20 min at 4 °C, supernatant was taken and filtered with 0.20-μm cellulose membrane filter (Minisart RC 4; Sartorius AG, Göttingen, Germany).

Each sample obtained from the ARC-ME tissue was applied to a reverse-phase high performance liquid chromatography-electrochemical detection (HPLC-ECD) system (Eicom Co., Kyoto, Japan) to measure contents of NE and DA. Ten microliters of each extracted sample were injected on a column (Eicompack MA-5ODS, 4.6 mm × 150 mm; Eicom Co.) with a mobile phase consisting of 0.1 M citric acid-sodium acetate buffer, pH 3.5, containing 8% methanol, 230 mg/liter sodium 1-octanesulfonate (Nacalai Tesque Inc., Kyoto, Japan), and 5 mg/liter disodium ethylenediaminetetraacetate (EDTA). The mobile phase was filtered with 0.2-μm cellulose nitrate filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) prior to use and pumped at a rate of 1 ml/min with a HPLC pump (LKB2150; LKB-Produkter AB, Bromma, Sweden). The applied voltage in an electrochemical detector (ECD-100; Eicom Co.) was maintained at 0.65 V with respect to a Ag/AgCl reference electrode. A series of standard samples consisted of a mixture of NE and DA, which were prepared from (-)arterenol (Sigma Chemical Co.) and 3-hydroxytyramine (Sigma Chemical Co.) dissolved in 0.1 N HCl, respectively. The minimum detectable level of catecholamines was 25 pg/10 μl. The values were expressed in ng of catecholamine per ARC-ME region.

LH pulse analysis

The frequency and amplitude of LH pulses were calculated with the PULSAR computer program provided by Drs. G.R. Merriam and K.W. Wachter (Merriam & Wachter, 1982). The following criteria were chosen in the identification of LH pulses. If the difference between a single LH concentration and the base line concentration was 3.0 times greater than the standard deviation (SD) at the level of the LH concentration, it was considered to be a part of an LH pulse. If the differences between 2 or 3 consecutive hormone concentrations and the base line were 1.0 or 0.4 times greater than the SD at the hormone concentrations, they were also considered to be parts of a pulse. The SD for each hormone concentration was calculated from the linear regression line. The equations, $y = (13.04x + 1.41)/100$ or $y = (4.47x + 4.25)/100$, $y = (4.47x + 4.25)/100$ or $y = (5.43x + 8.13)/100$, and $y = (6.34x + 5.08)/100$ were used in Chapters 3, 4, and 5 & 6, respectively; where x was the LH level and y was the SD for each hormone level determined by assaying four or five series of control sample in ten replicates.

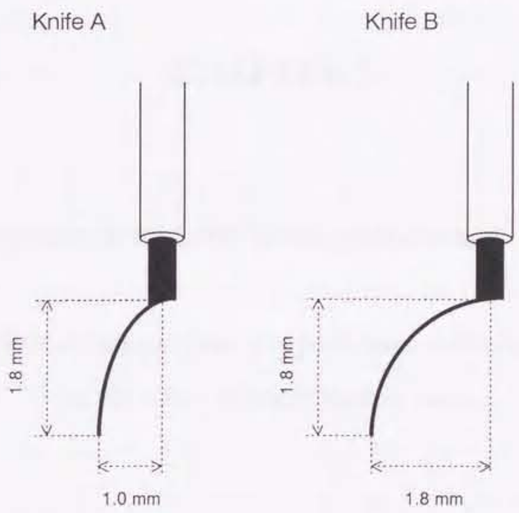


Figure 2.1. Schematic illustrations of the knife A (left) and knife B (right) used in the present experiment.

CHAPTER 3

Topography of the LHRH pulse generator in the brain

—Effects of various types of hypothalamic deafferentation
on LH pulses in ovariectomized rats—

Introduction

Halász and Pupp (1965) first reported that female rats bearing CD showed either persistent estrus or persistent diestrus, and suggested that the center for the tonic LH secretion was located inside the hypothalamic island, namely the MBH. It has also been reported that the pulsatile LH secretion is still apparent in ovariectomized rats bearing CD (Blake & Sawyer, 1974; Tsukamura *et al.*, 1990). Soper and Weick (1980) have reported that AD combined with the electrolytic lesion of the anterior part of the ARC abolished the pulsatile LH secretion. All these reports suggest that the putative LHRH pulse generator, the mechanism regulating the pulsatile LH secretion, is located in the MBH.

In the rat, most cell bodies of LHRH neurons are located extensively in the region anterior to the hypothalamus, and some cell bodies of LHRH neurons exist in the hypothalamic island made by CD (Kawano & Daikoku, 1981; Sétáló *et al.*, 1976; Witkin *et al.*, 1982). Therefore, several workers assume that the LHRH pulse generator is located in the preoptic/anterior hypothalamic area (POA/AHA): The local application of a specific α_1-receptor antagonist into the POA/AHA, but not into the MBH, caused a reduction of LH pulsatility (Jarry *et al.*, 1990); the inverse pattern of LH and γ-aminobutyric acid release from the POA/AHA was found with push-pull perfusion technique (Jarry *et al.*, 1988; Wuttke *et al.*, 1987). Furthermore, subchiasmatic LHRH neuronal fibers (Coen, 1987; Hoffman & Gibbs, 1982), which may be left intact after any type of hypothalamic deafferentation employed to date, could maintain the LH pulses after CD. Thus, there has been a controversy over the location of the LHRH pulse generator.

In the present chapter, the effect of various types of hypothalamic deafferentation

on the pulsatile LH secretion was elucidated to determine whether the LHRH pulse generator is located in the MBH in ovariectomized rats.

Materials and Methods

Experiment 1

Animals were subjected to CD, ALD, AD or sham-deafferentation 1 week after ovariectomy. Blood samples were collected every 6 min for 3 h 5 days after deafferentation.

Experiment 2

Animals subjected to PAD 2 weeks after ovariectomy were divided into three groups and blood samples were collected every 6 min for 3 h 1, 3 and 5 weeks after the deafferentation, respectively. Sham-deafferentated rats were bled 1 week after surgery.

Brain histology

After blood sampling was finished, frozen coronal sections (30 μm) were obtained from each brain. Some of the sections were stained with cresyl violet to determine the position of deafferentation and some sections were immunostained for LHRH according to the PAP method.

Data analysis

The mean and standard deviation (SD) for a series of LH concentrations for 3-h sampling period were calculated in each animal as indicators for the pulsatility of LH secretion. The frequency and amplitude of LH pulses were identified by the PULSAR computer program (Merriam & Wachter, 1982).

Statistical differences in each parameter for the pulsatile LH secretion between groups were determined by Duncan's multiple range test.

Results

Experiment 1

No necrosis was found in the deafferentated hypothalamus in any animals used (Figure 3.1A). In animals with CD, the ARC and the medial part of the ventromedial hypothalamic nuclei, the ventral part of the dorsomedial hypothalamic nuclei and the medio-rostral part of the mammillary body were included in the hypothalamic island. The knife cuts of AD and ALD were similar to that of CD except for the latero-posterior and the posterior cut, respectively. The anterior end of the incision was positioned at or just posterior to the suprachiasmatic nuclei (SCN) in all groups. LHRH-immunopositive neuronal fibers were found in the external layer of the median eminence in rats bearing AD, ALD or CD at a density similar to that seen in sham-deafferentated rats (Figure 3.1B).

Pulsatile LH secretion was observed in all rats bearing AD, ALD or CD (Figure 3.2). The frequency of LH pulses was not significantly different between any groups (Figure 3.3). The mean LH levels during the 3-h sampling period in rats with AD, ALD and CD were significantly ($P<0.05$) lower than that in sham-operated rats (Figure 3.3, Duncan's multiple range test). The level in rats with CD was the lowest and that in rats with ALD was between AD and CD. The mean amplitude of LH pulses was significantly ($P<0.05$) lower in rats with ALD and CD than in rats with sham-deafferentation (Figure 3.3, Duncan's multiple range test). The amplitude decreased as the incision extended postero-laterally.

Experiment 2

In animals bearing PAD, the anterior part of the ARC was severed by the knife

(Figure 3.4A). LHRH-positive neuronal fibers were identified immunohistochemically in the external layer of the median eminence (Figure 3.4B). No difference in LHRH immunoreactivity was seen between rats that had been deafferentated for 1, 3 and 5 weeks.

Regular LH pulses were less apparent in most deafferentated rats 1 week after PAD than sham-deafferentated controls (Figure 3.5). The baseline levels, however, were not completely depressed in rats 1 week after PAD. LH pulses were reinstated in some animals 3 weeks after surgery, and pulsatile LH secretion with high frequency and amplitude was observed in seven out of eight rats 5 weeks after PAD (Figure 3.5).

The levels of the mean and SD of LH concentrations over the 3-h sampling period and the frequency and amplitude of LH pulses were significantly ($P<0.05$) reduced 1 week after PAD, compared with those in sham-deafferentated rats (Figure 3.6, Duncan's multiple range test). The mean SD for the 3-h sampling period, indicating the pulsatility of LH secretion, was markedly reduced 1 week after PAD. The level of these four parameters gradually increased as the time progressed after PAD and reached sham levels 5 weeks after surgery. The mean LH level in rats 3 and 5 weeks after PAD was significantly ($P<0.05$) higher than that in rats 1 week after PAD. The highest value of the mean SD, which was comparable to the level found in the sham-deafferentated animals, was obtained 5 weeks after PAD. The frequency and amplitude of LH pulses 5 weeks after PAD were significantly ($P<0.05$) higher than those 1 week after PAD, and the amplitude of LH pulses in rats 5 weeks after PAD was comparable to the level found in the sham-deafferentated animals.

Discussion

The results in the present chapter demonstrated that the pulsatile LH secretion persisted in all rats bearing AD, ALD or CD and that these types of deafferentation did not affect the frequency of LH pulses, which reflects directly the activity of the LHRH pulse generator (Clarke & Cummins, 1982). This suggests that the LHRH pulse generator is located within the hypothalamic island made by CD as has been previously suggested (Blake & Sawyer, 1974; Soper & Weick, 1980; Tsukamura *et al.*, 1990). Kawano and Daikoku (1981) have reported that most cell bodies of the LHRH neurons are located extensively in the region anterior to the hypothalamus in the rat and their fibers project to the external layer of the median eminence, and that some cell bodies of LHRH neurons exist in the MBH. In the present chapter, LHRH-positive neuronal fibers were identified immunohistochemically in the median eminence after CD. LHRH might be supplied by the LHRH neurons located in the base of the tuberal hypothalamic area and spared by CD (Kawano & Daikoku, 1981). Another possibility that should be considered is that the subchiasmatic LHRH fibers that were spared by CD (Hoffman & Gibbs, 1982) supply LHRH.

It should be noted that the amplitude of LH pulses decreased as the deafferentation extended postero-laterally in the present experiment. It has been reported that the amplitude of LH pulses is closely correlated with that of LHRH pulses (Levine *et al.*, 1982). Since the laterobasal LHRH fiber tract enters the MBH laterally and extremely posteriorly (Kawano & Daikoku, 1981), more fibers of LHRH neurons could be spared after AD and more LHRH could be released at the same time from nerve terminals than after ALD.

There is a possibility that LHRH neurons in the tuberal hypothalamic area or the

subchiasmatic LHRH neuronal fibers, which would presumably be spared by CD, have an intrinsic mechanism producing pulsatile LHRH release. From the findings in the present chapter, however, the LHRH pulse generator could be assumed to consist of neurons that are independent of LHRH-producing neurons. Pulsatile release of LHRH was perturbed by PAD, possibly impairing the mechanism for synchronizing the pulsatile release of LHRH but not the LHRH-producing neuron. The results in the present chapter support this notion; pulsatility of LH release in rats 1 week after PAD was less apparent although plasma levels of LH were not completely depressed compared to sham-operated controls and immunoreactive LHRH neuronal fibers were still found in the median eminence. It has been shown that no LH pulses are observed in rats bearing CD together with a lesion of anterior part of the ARC (Soper & Weick, 1980) and an increase in the MUA in the ARC is noted at the peak of LH pulses in ovariectomized rats (Kawakami *et al.*, 1982; Nishihara *et al.*, 1991). These data suggest that the LHRH pulse generator is located in the anterior part of the ARC within the MBH. In the present chapter, PAD would disconnect the neural pathway between the LHRH pulse generator and LHRH neuronal terminals in the median eminence.

All parameters of the pulsatile LH secretion gradually recovered after PAD. In particular, the mean SD in rats 5 weeks after PAD was the highest and comparable to the level found in the sham-deafferentated animals. This restoration of LH pulses in the present experiment might be ascribed to a functional reorganization of the neurons controlling pulsatile LHRH release in the hypothalamus. Merchenthaler, Sétáló, Horváth & Flerkó (1980) have demonstrated that transected LHRH-containing fibers in the basal hypothalamus grew along the line of incision more than 6 weeks after deafferentation, demonstrating the ability of LHRH axons to regenerate. In addition, Rose and Weick (1987) reported that rats bearing AD and electrolytic lesions of the

anterior part of the ARC, which had been treated with monosodium-L-glutamate during the neonatal period, continued to show pulsatile LH secretion. They suggested that some other generating system could be at work in the rat in which the intrinsic LHRH pulse generator within the ARC had been destroyed.

In summary, I conclude that at least one pulse generator, which consists of a group of non-LHRH neurons, might be located within the MBH in the rat.

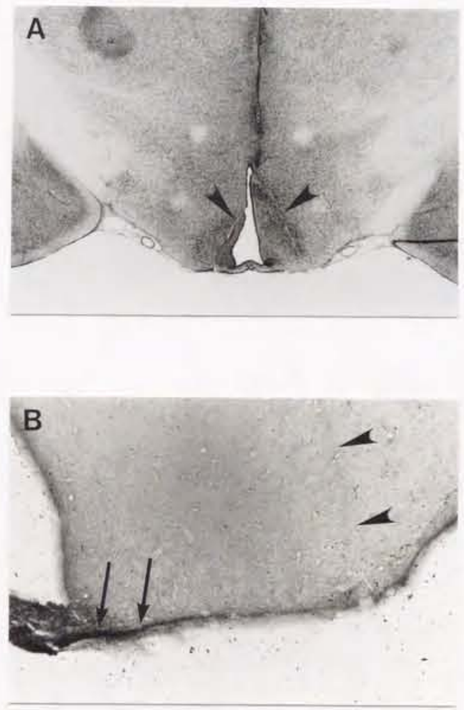


Figure 3.1. Photomicrographs of the coronal section of an ovariectomized rat brain with complete hypothalamic deafferentation. Sections were stained with cresyl violet (A) or were immunostained with anti-LHRH serum (B). Arrowheads and arrows indicate the location of the incision and LHRH-positive neuronal fibers in the median eminence, respectively. (A) × 9; (B) × 75.

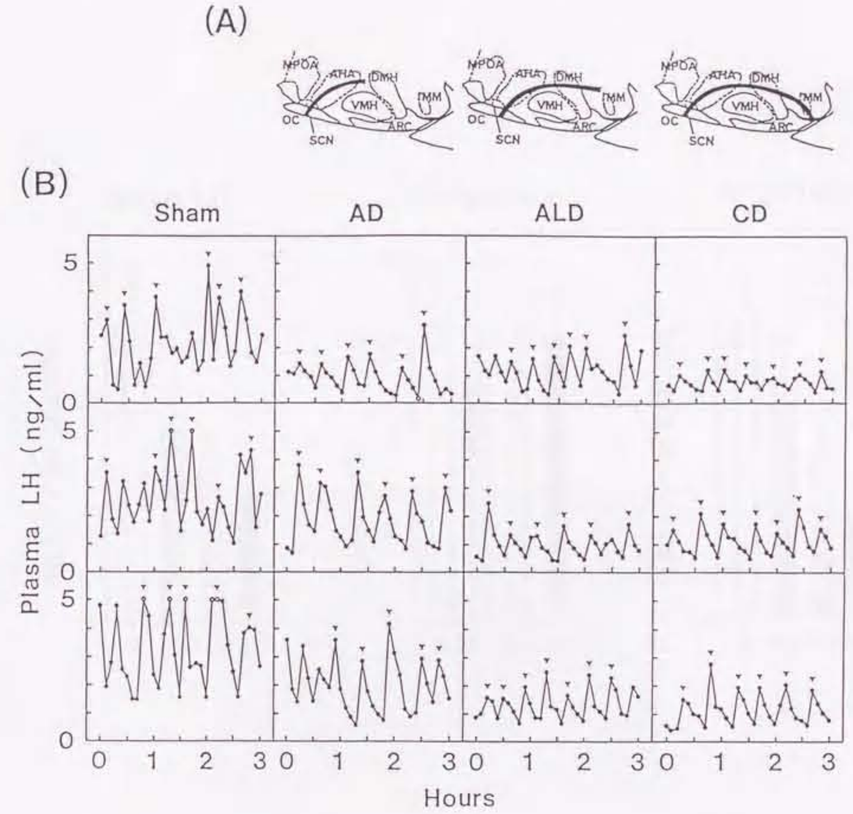


Figure 3.2. (A) Schematic illustrations of a sagittal section of the hypothalamus showing the position of anterior (AD), anterolateral (ALD) and complete (CD) hypothalamic deafferentation. AHA—anterior hypothalamic area; ARC—arcuate nucleus; DMH—dorsomedial hypothalamic nucleus; MM—medial mammillary nucleus; MPOA—medial preoptic area; OC—optic chiasm; SCN—suprachiasmatic nucleus; VMH—ventromedial hypothalamic nucleus. (B) Pulsatile LH secretion in three representative ovariectomized rats of each group bearing sham-deafferentation (Sham), AD, ALD and CD. The values were expressed in terms of the NIDDK-rLH-RP-2. Open circles indicate LH values which were out of the range of the assay: they were taken for the assay limit (5 ng/ml and 0.039 ng/ml). Arrowheads represent the LH pulse identified by the PULSAR computer program.

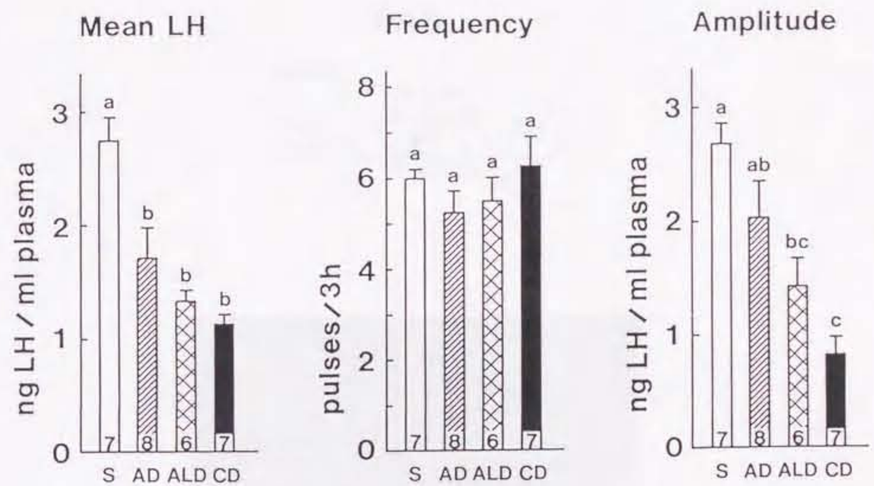
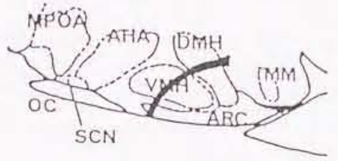


Figure 3.3. Parameters of the pulsatile LH secretion in rats bearing sham (S), anterior (AD), anterolateral (ALD) and complete (CD) hypothalamic deafferentation. Mean LH levels were calculated for a series of plasma LH concentrations during the 3-h sampling period. The frequency and amplitude of LH pulses were identified by the PULSAR computer program. Values are means ± SEM. Numbers in each column indicate the number of animals used. Values with different letters are significantly different from each other ($P<0.05$, Duncan's multiple range test).

A



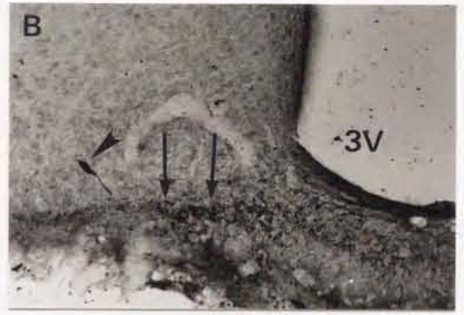


Figure 3.4. (A) Schematic illustration of a sagittal section of the hypothalamus showing the position of posterior-anterior hypothalamic deafferentation (PAD). See Figure 3.2 for other details. (B) A photomicrograph showing LHRH-positive neuronal fibers (arrows) in the median eminence of an ovariectomized rat with PAD. An arrowhead indicates the LHRH-positive neuronal cell body adjacent to the lateral border of the median eminence. 3V—third ventricle. (× 112.5).

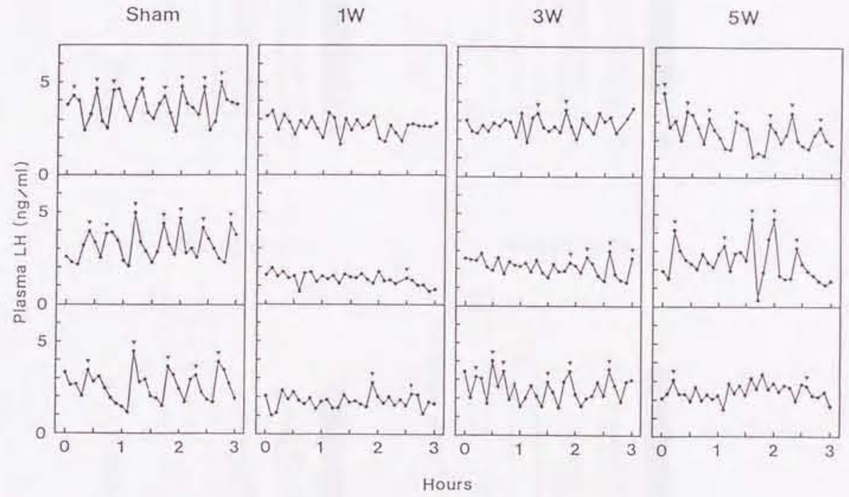


Figure 3.5. Profiles of plasma LH concentrations in three representative ovariectomized rats bearing sham-deafferentation (Sham) and in rats 1 (1W), 3 (3W) or 5 (5W) weeks after PAD. Arrowheads represent the LH pulse identified by the PULSAR computer program.

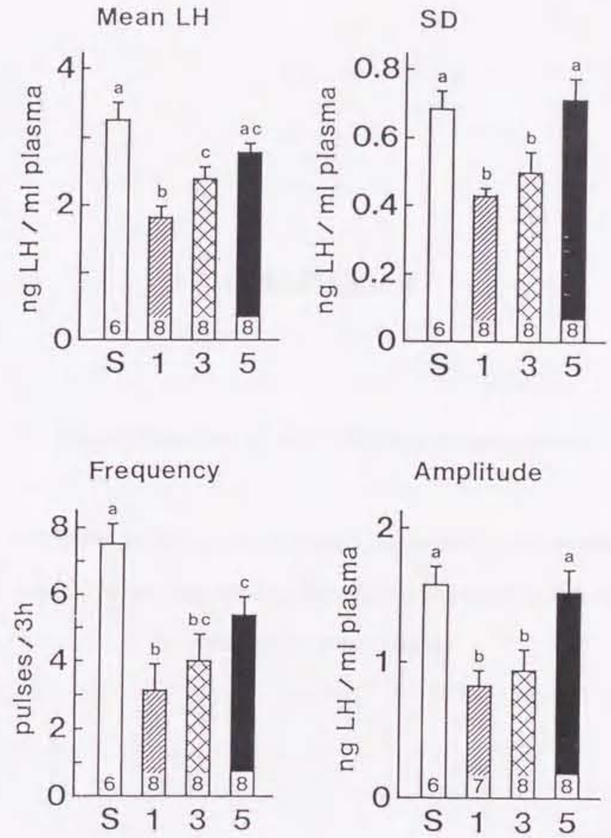


Figure 3.6. Parameters of the pulsatile LH secretion in rats bearing sham-deafferentation (S) and in rats 1, 3 or 5 weeks after PAD. Mean LH levels and SD were calculated for a series of plasma LH concentrations during the 3-h sampling period in each rat. See Figure 3.3 for other details.

CHAPTER 4

Transplantation of the LHRH pulse generator

—Effects of transplants of fetal mediobasal hypothalamus
on LH pulses impaired by hypothalamic deafferentation
in adult ovariectomized rats—

Introduction

According to the findings in Chapter 3, the hypothesis that the putative LHRH pulse generator is located inside the MBH was raised. The aim of the experiment in the present chapter is to verify the above hypothesis.

Transplantation of fetal or neonatal brain tissue into the brain has been employed to restore the functions which have been impaired by lesions in the brain or to compensate for genetic deficiencies in synthesizing neuropeptides. For example, circadian rhythms in locomotor activity can be restored by the transplantation of the neonatal and fetal SCN in rats (Sawaki, Nihonmatsu & Kawamura, 1984) and in hamsters (Ralph, Foster, Davis & Menaker, 1990), respectively, with SCN lesions. Moreover, reproductive functions recovered in congenitally hypogonadal mice (Gibson, Kokoris & Silverman, 1988; Kokoris, Lam, Ferin *et al.*, 1988) and in aged rats (Matsumoto *et al.*, 1984) after the transplantation of the fetal POA and the neonatal MBH, respectively. Thus, transplantation of brain tissue is a useful tool to determine the function of a discrete area of the brain.

In the present chapter, the effect of the transplantation of fetal brain tissue into the third ventricle of adult ovariectomized rats on pulsatile LH release which had been impaired by PAD was examined. Because it may take more than 4 weeks for the transplanted brain to function in the host brain, and it is shown in Chapter 3 that LH pulses are not apparent 1 week after PAD and gradually restored thereafter, pulsatile LH secretion 1 week after PAD in ovariectomized rats that had been transplanted with brain tissues 4 weeks before PAD was examined.

Materials and Methods

Treatments

Animals were transplanted 2 weeks after ovariectomy with a piece of the brain tissue including either the MBH or the cerebral cortex taken from the 17-days-old fetal brain. Rats with or without the brain transplantation were subjected to PAD or sham PAD 4 weeks after the brain transplantation. One week after the deafferentation, blood samples (100 μl) were collected every 6 min for 3 h. Other groups of rats transplanted with the fetal brain tissue containing the MBH (n=5) or the cerebral cortex (n=6) were bled 5 weeks after the transplantation without deafferentation.

Brain histology

After blood sampling was finished, rats were injected with colchicine (100 μg/30 μl saline) into the lateral ventricle by stereotaxic surgery. Twenty-four hours later, animals were anesthetized with pentobarbital and perfused. Frozen coronal sections (30 μm) were obtained and histological examination was carried out.

Data analysis

Statistical differences in each parameter for the pulsatile LH secretion between groups were determined by Duncan's multiple range test.

Results

Histological examination of each brain revealed that the grafted tissue containing the fetal MBH or the cerebral cortex survived and attached to the third ventricular wall in all recipients (Figure 4.1A). No LHRH-positive neurons were detected immunohistochemically either in the MBH or cerebral-cortex grafts (Figure 4.1B), but many LHRH-positive fibers were found in the median eminence in all animals.

LH pulses were generally frequent and occurred with a regular rhythm 1 week after PAD in MBH-transplanted rats. In rats which had received no transplants or transplants of cerebral cortex, LH pulses were infrequent and occurred at irregular intervals 1 week after PAD (Figure 4.2).

LH pulse characteristics (Figure 4.3) show that PAD significantly ($P<0.05$, Duncan's multiple range test) reduced the mean LH level and LH pulse frequency, and partially diminished LH pulse amplitude in nontransplanted rats. Prior transplantation of fetal MBH prevented the effect of PAD on LH pulse amplitude and mean LH and partially, but not significantly, reversed the effect on pulse frequency. The mean LH level in cerebral cortex-transplanted rats was significantly ($P<0.05$) lower than that in MBH-transplanted rats and in sham-deafferentated rats (Duncan's multiple range test). Levels of the other parameters in cortex-transplanted animals were lower than those in MBH-transplanted rats, but differences were not significant. Transplantation of the MBH or the cerebral cortex tissues alone did not affect the pulsatile LH secretion (Figures 4.4 and 4.5).

Discussion

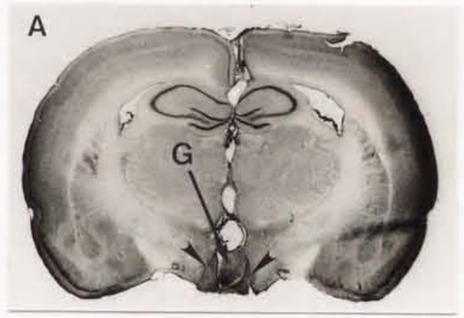
The results in the present chapter clearly demonstrated that transplantation of the fetal brain tissue containing the MBH is most effective in maintaining regular pulsatile LH secretion after PAD. The mean LH level in MBH-transplanted rats was significantly higher than that in cerebral cortex-transplanted rats and nontransplanted rats, and was comparable to that in sham-deafferentated animals. The frequency and amplitude of pulsatile LH secretion in MBH-transplanted rats showed the highest, but non-significant, value compared to that in other animals bearing PAD except for the difference in the amplitude between MBH- and nontransplanted rats. These results indicate that the graft containing the fetal MBH affects LH pulses and might play a role in generating them, supporting the view of Blake and Sawyer (1974) that the putative LHRH pulse generator is located within the MBH.

The results in Chapter 3 demonstrated that LH pulses disappeared for 1 week after PAD, but then returned gradually after PAD. This restoration of pulsatile LH secretion could be ascribed to either the regeneration of nerve fibers or the functional reorganization of the mechanism generating LH pulses. In the present chapter, PAD was performed in rats that had been transplanted with the fetal brain tissue. Since blood samples were collected 1 week after PAD, the pulsatile LH secretion after PAD in MBH-transplanted rats was most likely due to the transplantation of MBH and not to the spontaneous restoration of LH pulses. In contrast, LH pulsatility in rats with the graft of the fetal cerebral cortex was not comparable to that in MBH-transplanted rats. The graft containing the fetal cerebral cortex could have some effects in maintaining regular LHRH pulses, because typical LH pulses were observed in some of these animals.

No LHRH-immunoreactive neuronal cell bodies were detected in the MBH grafts despite the fact that these grafts were able to maintain regular LH pulses after PAD. On the other hand, LH pulses were impaired by PAD in nontransplanted rats despite the presence of LHRH immunoreactivity in the median eminence posterior to the incision of PAD. Therefore, it can be assumed that the LHRH pulse generator consists of a group of neurons other than the LHRH neurons. An electrophysiological study in ovariectomized rats has reported that peaks of LH pulses correspond with volleys of MUA in the anterior portion of the ARC (Kawakami *et al.*, 1982; Nishihara *et al.*, 1991) where few LHRH cell bodies are found (Kawano & Daikoku, 1981; Sétáló *et al.*, 1976; Witkin *et al.*, 1982). LH pulses disappeared in rats in which the anterior portion was severed from the rest of the ARC and the median eminence (Blake & Sawyer, 1974; Soper & Weick, 1980; see Chapter 3). In addition, the recovery of ovarian function in aged female rats has also been observed by the transplantation of the neonatal MBH tissue containing the ARC (Matsumoto *et al.*, 1984). All these findings support the hypothesis that non-LHRH-containing neurons in the anterior part of the ARC could have a neural connection with LHRH axons at the median eminence and could play a role in regulating pulsatile LHRH release.

On the contrary, it is also possible that the pulse generator exists in the host brain and that the graft affects it as a modulator after impairing regular LH pulses by PAD. Although this interpretation is not excluded in the present experiment, it is most likely that the MBH has a mechanism which is involved in generating LHRH pulses in rats.

In conclusion, the results in the present chapter suggest that the LHRH pulse generator may consist, at least in part, of a group of neurons in the MBH other than LHRH-producing neurons.



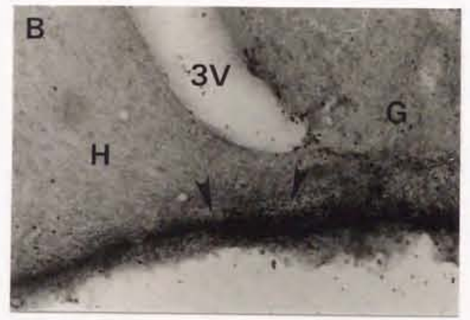


Figure 4.1. Photomicrographs of coronal sections from the brain of an ovariectomized rat which had received a transplant of fetal MBH tissue and PAD. (A) MBH graft in the third ventricle. The arrowheads indicate the location of the knife cuts. × 4.5. (B) LHRH-positive fibers (arrowheads) in the median eminence. No LHRH-positive neuronal cell body was found in the MBH graft. × 125. G—graft; H—host; 3V—third ventricle.

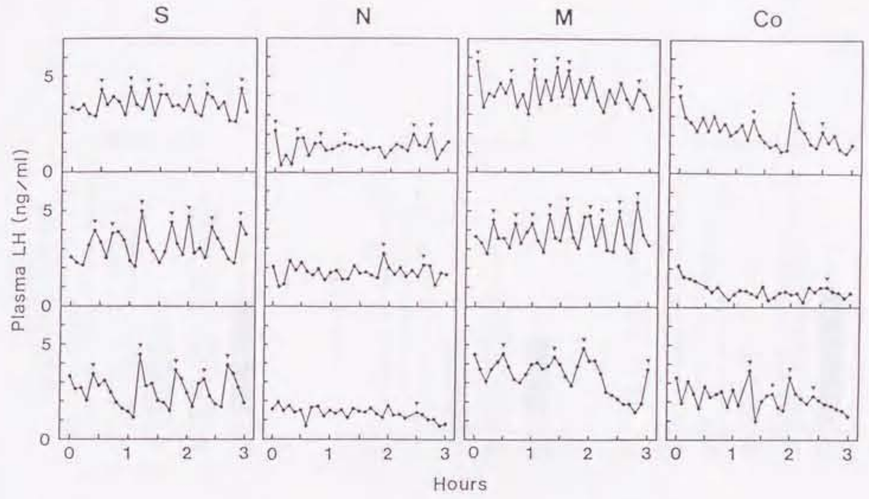


Figure 4.2. Profiles of plasma LH concentrations in 3 representative ovariectomized rats following sham deafferentation (S) or PAD (N, M and Co). Prior to PAD, rats received either no transplant (N), fetal MBH (M) or fetal cerebral cortex (Co) transplants. The values were expressed in terms of the NIDDK-rLH-RP-2. Arrowheads represent the LH pulse identified by the PULSAR computer program.

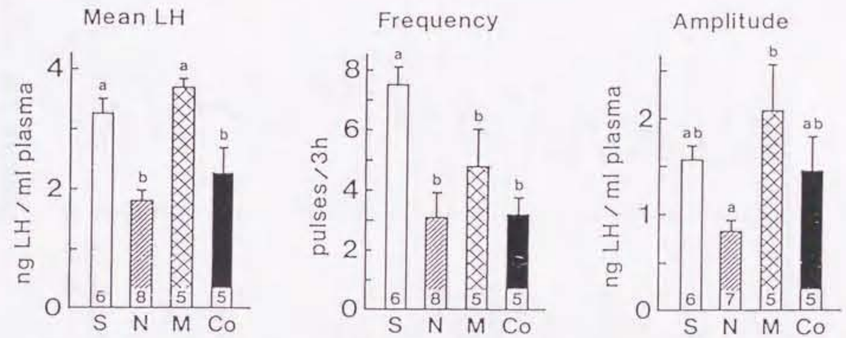


Figure 4.3. LH pulse characteristics in ovariectomized rats following sham deafferentation (S) or PAD (N, M and Co). Prior to PAD, rats received either no transplant (N), fetal MBH (M) or fetal cerebral cortex (Co) transplants. Mean LH levels were calculated for a series of plasma LH concentrations during the 3-h sampling period. The frequency and amplitude of LH pulses were identified by the PULSAR computer program. Values are means ± SEM. Numbers in each column indicate the number of animals used. The amplitude of LH pulses was calculated only in rats in which LH pulses were identified by the PULSAR computer program. Values with different letters are significantly different from each other ($P<0.05$, Duncan's multiple range test).

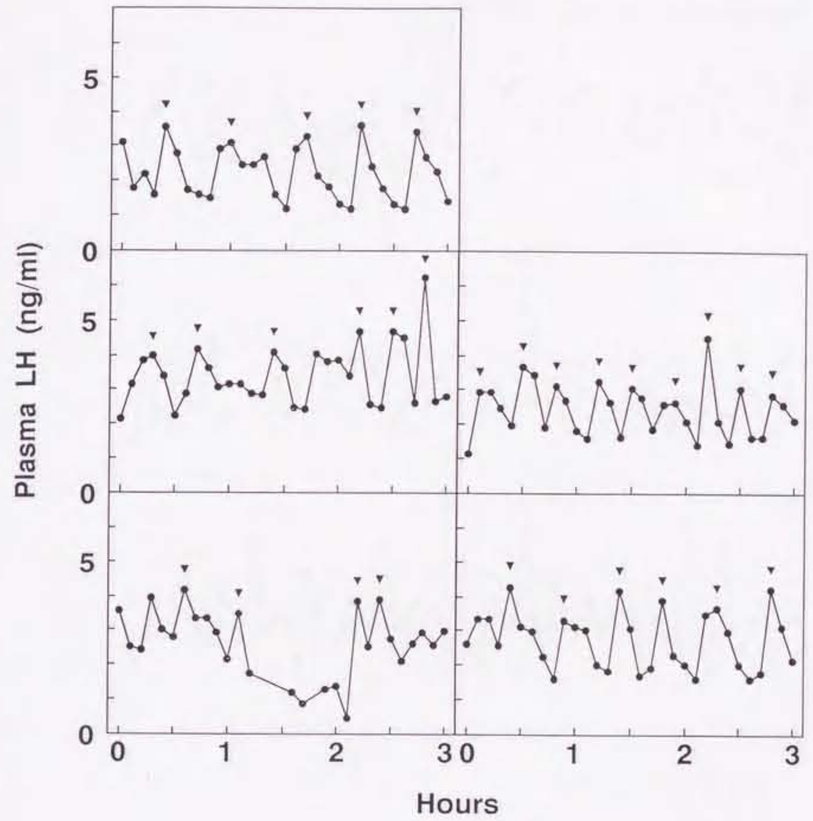


Figure 4.4. Profiles of plasma LH concentrations in ovariectomized rats with fetal MBH transplants. Animals did not receive PAD. Arrowheads represent the LH pulse identified by the PULSAR computer program.

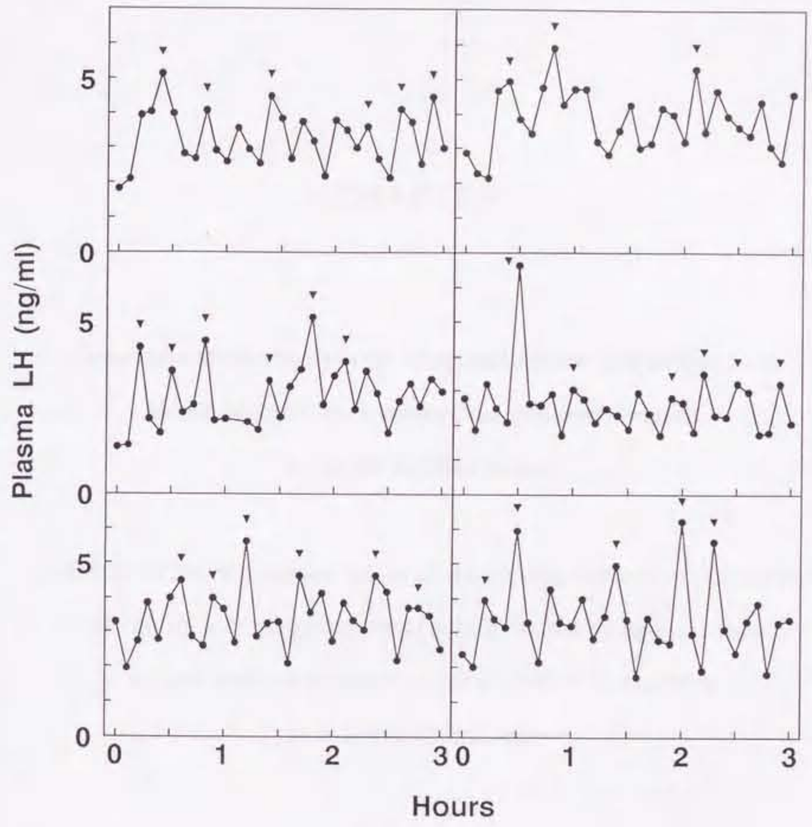


Figure 4.5. Profiles of plasma LH concentrations in ovariectomized rats with fetal cerebral cortex transplants. Animals did not receive PAD. Arrowheads represent the LH pulse identified by the PULSAR computer program.

CHAPTER 5

Does the catecholaminergic neuronal system projecting to or in the arcuate nucleus-median eminence region generate LHRH pulses?

—Effect of radiofrequency lesion of the arcuate nucleus or depression of catecholaminergic neuronal activity in the arcuate nucleus-median eminence region on the pulsatile LH secretion in ovariectomized rats—

Introduction

The results in the previous chapters demonstrate that the LHRH pulse generator is composed of a group of neurons other than LHRH-producing neurons and located inside the MBH, especially in the anterior part of the ARC.

The hypothalamus are abundantly innervated by catecholaminergic neurons (Fuxe & Hökfelt, 1969; Jonsson, Fuxe & Hökfelt, 1972; Palkovits & Jacobowitz, 1974). In particular, there is one of the largest groups of dopaminergic neurons in the ARC, namely A_{12} or tuberoinfundibular dopaminergic neurons (Fuxe & Hökfelt, 1966; Palkovits, 1981). Peripheral administration of dopamine (DA) agonist (Drouva & Gallo, 1976; Gnodde & Schuiling, 1976) or intraventricular infusion of DA (Gallo & Drouva, 1979) has been reported to suppress the pulsatile LH secretion in ovariectomized rats, suggesting an inhibitory role of DA in the pulsatile LH secretion. However, it does not appear that dopaminergic neurons are crucially involved in generating LH pulses in ovariectomized rats, since blockade of DA receptors with specific antagonists has no effect on pulsatile LH secretion (Drouva & Gallo, 1976; Drouva & Gallo, 1977; Weick, 1978). In ovariectomized monkeys, moreover, peripheral injection of DA receptor antagonists does not reduce the pulsatile release of LHRH from the stalk-median eminence (Gearing & Terasawa, 1991).

Several lines of evidence suggest that noradrenergic neurons have both stimulatory and inhibitory roles in the maintenance of pulsatile LH secretion. Inhibition of norepinephrine (NE) synthesis (Drouva & Gallo, 1976; Gnodde & Schuiling, 1976; Negro-Vilar, Advis, Ojeda & McCann, 1982), blockade of α-adrenergic receptors (Weick, 1978), or destruction of the ventral noradrenergic bundle with 6-hydroxydopamine (6-OHDA) (Hancke, Beck, Baumgarten *et al.*, 1977) suppresses

LH pulses in ovariectomized rats. On the other hand, stimulating the adrenergic receptors by infusing NE (Bergen & Leung, 1986; Gallo, 1984; Gallo & Drouva, 1979; Leung, Arendash, Whitmoyer *et al.*, 1982) or α- or β-adrenergic agonists (Leung *et al.*, 1982) into the third ventricle also results in the suppression of pulsatile LH secretion, indicating the inhibitory role of NE. Thus, the noradrenergic system may be involved in controlling LH pulses.

In these pharmacological studies, the site of action of drugs has not been taken into much account. The experiments in the present chapter were, therefore, designed to determine whether catecholaminergic systems innervated to the arcuate nucleus-median eminence (ARC-ME) region are involved in generating LH pulses in chronically ovariectomized rats. First, the effect of radiofrequency lesion of the ARC on pulsatile LH secretion was examined to evaluate the involvement of the nucleus in generating LH pulses. Second, the effect of the depression of the catecholamine contents by a combination of local injection with neurotoxin and catecholamine-synthesizing enzyme inhibitor into the ARC-ME region on LH pulses was investigated to evaluate the involvement of catecholaminergic neurons projecting to or in the ARC-ME region in generating LH pulses. Since the receptors may become supersensitive and the tyrosine hydroxylase, the rate limiting enzyme for catecholamine synthesis, may become more active after denervation (Bacha & Donoso, 1974; Kostrzewa, 1989; Ramirez, Feder & Sawyer, 1984; Scapagnini, Annunziato, Clementi *et al.*, 1977), the tyrosine hydroxylase inhibitor, α-methyl-p-tyrosine (α-MPT), was administered after neurotoxic lesion of catecholaminergic neurons with 6-OHDA to ensure the reduction of catecholaminergic activity.

Materials and Methods

Experiment 1

Effect of lesioning the ARC on pulsatile LH secretion was examined. Ovariectomized rats received bilateral radiofrequency lesions of the ARC or sham operation on the day before blood sampling. Blood samples were collected every 6 min for 3 h.

Experiment 2

Effect of bilateral intrahypothalamic microinjections of both 6-OHDA hydrobromide (Research Biochemicals Inc., Natick, MA, USA) and α-MPT methyl ester hydrochloride (Sigma Chemical Co.) on the pulsatile LH secretion were examined. Animals were injected with 10 μg of 6-OHDA dissolved in 0.5 μl of saline containing 1 mg/ml ascorbic acid at each side immediately after implanting chronic guide cannula. One week after the injection of 6-OHDA, conscious animals were administered through the implanted cannula with 50 μg of α-MPT dissolved in 0.2 μl of saline in each side 2 h before the initiation of blood sampling. Drug solutions were freshly prepared at each time of injection. Control animals received equal volumes of vehicles 1 week and 2 h before bleeding, respectively. Injections were performed with 25-μl microsyringe (Hamilton Co., Reno, NV, USA) attached to a micro-syringe pump (EP-60; Eicom Co.) at a flow rate of 0.1 μl/min and internal cannula was left in place 2 min after infusion to minimize withdrawal of the drug solution.

On the next day of blood sampling, animals were injected again with the same amount of α-MPT or saline and decapitated 2 h later to determine the contents of NE and DA in the ARC-ME region.

Brain histology

After blood sampling in Experiment 1, frozen coronal sections (50 μm) were obtained from each brain and were stained with thionin to determine the location and extent of the ARC lesions.

Data analysis

Statistical differences between ARC- and sham-lesioned groups, or 6-OHDA/α-MPT injected and vehicle/saline injected groups were determined by Student's *t*-test or Cochran-Cox test.

Results

Experiment 1

Histological examination of each brain revealed that rostral half of the ARC was damaged bilaterally in animals bearing radiofrequency lesions (Figure 5.1). The extent of lesions was restricted to the ARC and the median eminence was spared by the lesion (Figure 5.2).

A regular pattern of pulsatile LH secretion was observed in sham-lesioned animals (Figure 5.3). LH pulses also occurred regularly in rats after bilateral lesions of the ARC (Figure 5.4). Table 5.1 shows mean plasma LH level and the frequency and amplitude of LH pulses in rats with or without bilateral damage to the ARC. The levels of these parameters of pulsatile LH secretion were not significantly ($P>0.05$, Student's *t*-test) different between two groups.

Experiment 2

Eleven rats were bilaterally injected with both 6-OHDA and α-MPT into the ARC-ME region, and 8 animals with both vehicle and saline. The combination of 6-OHDA and α-MPT injection resulted in a depression of both NE and DA contents in the ARC-ME region (Figure 5.5); the mean NE and DA contents in the 6-OHDA/α-MPT-treated group were significantly ($P<0.001$) depressed by 75.5% and 51.8%, respectively, compared to those in the control group (Student's *t*-test or Cochran-Cox test).

LH pulses were apparent in all animals with the 6-OHDA/α-MPT (Figure 5.6) injection as were seen in control animals (Figure 5.7). Some animals in the 6-OHDA/α-MPT-injected group showed irregular pulsatile fluctuations of plasma LH

levels (Figure 5.6).

Table 5.2 gives parameters of pulsatile LH secretion in animals bearing the vehicle/saline or 6-OHDA/α-MPT injection into the ARC-ME region. All these parameters were not significantly (P>0.05, Student's *t*-test or Cochran-Cox test) affected by depressing the contents of catecholamines in the ARC-ME region.

Discussion

The results of the present chapter demonstrated that bilateral radiofrequency lesion restricted to the rostral part of the ARC did not affect the pulsatile LH secretion in ovariectomized rats. In these animals, the median eminence was revealed to be spared the damage and to remain intact by the histological examination. These results suggest that the rostral part of the ARC is not directly involved in generating LH pulses. In the experiment of Chapter 3, CD did not affect the frequency of LH pulses and PAD, which separated the rostral part of the ARC from the more caudal part of the MBH, abolished LH pulses. Moreover, transplantation of the fetal MBH tissue prior to the PAD overcame such perturbation of LH pulses as was caused by PAD (Chapter 4). It was, therefore, assumed that the LHRH pulse generator is located within the MBH, particularly in the rostral part of the ARC. However, the results in the present chapter are not consistent with this notion: The ARC would not be crucially involved in generating LH pulses. Together with these results, it is likely that the LHRH pulse generator is situated in the hypothalamic island made by CD, probably in the area between the rostral edges of the incisions of CD and the PAD.

In the present experiment, depletion of the contents of not only DA but also NE in the ARC-ME region caused by intrahypothalamic injection of 6-OHDA and α-MPT had no effect on the pulsatility of LH secretion: The respective levels of NE and DA were depressed by a quarter and a half of control levels. These results suggest that the catecholaminergic system projecting to or in the ARC-ME region is not involved in the LHRH pulse generator in ovariectomized rats. The depletion of catecholamine contents would be restricted to neurons projecting to the ARC-ME region, because the drugs were locally injected into this region and are known to be taken up preferentially by the

neuronal terminals to perikarya (Kostrzewa, 1989). Moreover, animals were locally administered into the region with α-MPT after the neurotoxic lesion with 6-OHDA to overcome the supersensitization of catecholaminergic receptors after denervation (Bacha & Donoso, 1974; Kostrzewa, 1989; Ramirez *et al.*, 1984; Scapagnini *et al.*, 1977). Therefore, it could be concluded that the catecholaminergic system projecting to or in the ARC-ME region is not necessarily involved in generating LH pulses.

In primates, pulsatile release of LHRH and NE estimated *in vivo* with push-pull perfusion technique in the stalk-median eminence was reported to be synchronized (Terasawa, Krook, Hei *et al.*, 1988). Anatomical studies have, however, revealed that the cell bodies of NE-containing neurons innervating the hypothalamus are situated in the extrahypothalamic region, the lower brain stem (Palkovits, Záborszky, Feminger *et al.*, 1980; Swanson & Hartman, 1975) but not in the MBH where the LHRH pulse generator would be located. It has also been shown that deafferentation of the MBH resulted in the reduction of NE levels by approximately 70% in the median eminence and the ARC (Palkovits, 1981) and this type of deafferentation did not obstruct the pulsatile LH secretion in rats (Blake & Sawyer, 1974; Soper & Weick, 1980; Tsukamura *et al.*, 1990). These results also support the idea that the NE-containing neurons are not necessary to maintain LH pulses. It is also likely that dopaminergic neurons in the ARC are not crucially involved in generating LH pulses in ovariectomized rats, since blockade of DA receptors has no effect on pulsatile LH secretion (Drouva & Gallo, 1976; Drouva & Gallo, 1977; Weick, 1978). Therefore, NE and DA play only modulatory roles in controlling the LHRH pulse generator activity. An alternative but less likely interpretation of the results in the present chapter is that the remaining levels of these catecholamines are still sufficient to generate LH pulses.

In the present study, regular LH pulses were observed after lesioning the rostral part of the ARC without damaging the median eminence. The radiofrequency lesions employed in the present study produced a fairly restricted damage in the ARC. It has been reported that pulsatile LH secretion was disrupted after electrolytic lesion of the ARC accompanied by a damage to the median eminence, whereas LH pulses did not altered by the axon-sparing chemical lesions of the ARC with *N*-methyl-D,L-aspartate (NMDA) (Sisk, Nunez & Thebert, 1988). Soper and Weick (1980) reported that the "electrolytic" lesion of the anterior part of the ARC combined with anterior hypothalamic deafferentation perturbed the pulsatile LH secretion, but the lesioned area was not clearly shown in their paper and it may be difficult to make a restricted lesions to the ARC by a direct current. Since the pulsatile LH secretion is preserved after CD (Blake & Sawyer, 1974; Soper & Weick, 1980; Tsukamura *et al.*, 1990), it can be assumed that some other structure in the MBH than the ARC would be responsible for maintaining LH pulses after lesions restricted to the ARC.

In summary, the result in the present chapter suggests that the rostral part of the ARC does not participate in generating LH pulses. It is proposed that the LHRH pulse generator is located in the structure near to, but other than the anterior part of the ARC and projects to the median eminence in order to regulate LHRH pulses in rats. In addition, the catecholaminergic neuronal system projecting to or in the ARC-ME region would not be involved in the LHRH pulse generating mechanism.

Table 5.1. Mean plasma LH level and the frequency and amplitude of LH pulses in ovariectomized rats with radiofrequency lesions of the ARC.

	n	Mean LH level (ng/ml)	Pulse frequency (pulses/3 h)	Pulse amplitude (ng/ml)
Sham lesion	6	1.34 ± 0.13	5.17 ± 0.54	1.08 ± 0.11
ARC lesion	6	1.29 ± 0.16	6.33 ± 0.80	0.99 ± 0.14

Values are means ± SEM; n, Number of animals used. Mean LH levels were calculated for a series of plasma LH concentrations during the 3-h sampling period. The frequency and amplitude of LH pulses were identified by the PULSAR computer program. No significant difference in each parameter was determined between two groups ($P>0.05$, Student's *t*-test).

Table 5.2. Mean plasma LH level and the frequency and amplitude of LH pulses in ovariectomized rats injected with 6-hydroxydopamine/α-methyl-p-tyrosine (6-OHDA/α-MPT) or vehicle/saline (Veh/Sal) into the ARC.

	n	Mean LH level (ng/ml)	Pulse frequency (pulses/3 h)	Pulse amplitude (ng/ml)
Veh/Sal	8	2.61 ± 0.09	4.75 ± 0.88	1.17 ± 0.05
6-OHDA/α-MPT	11	2.58 ± 0.14	4.55 ± 0.51	1.35 ± 0.10

Values are means ± SEM; n, Number of animals used. Mean LH levels were calculated for a series of plasma LH concentrations during the 3-h sampling period. The frequency and amplitude of LH pulses were identified by the PULSAR computer program. No significant difference in each parameter was determined between two groups ($P>0.05$, Student's *t*-test or Cochran-Cox test).

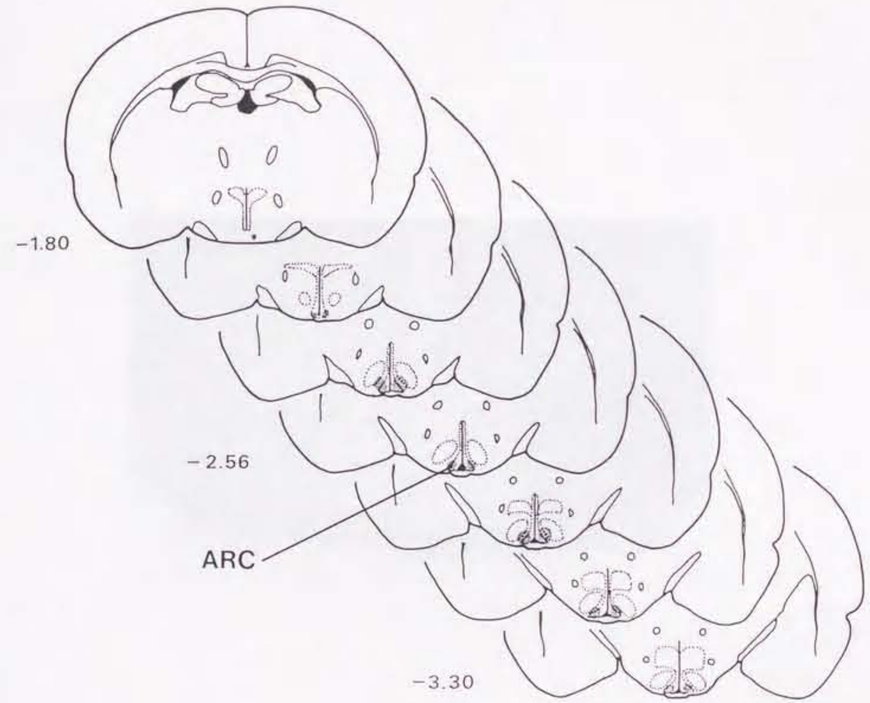


Figure 5.1. Schematic illustrations of coronal sections of the brain showing the extent of the radiofrequency lesion of the ARC. Line drawings are modified from the atlas of Paxinos and Watson (1986). Shaded areas designate the areas damaged by lesions.

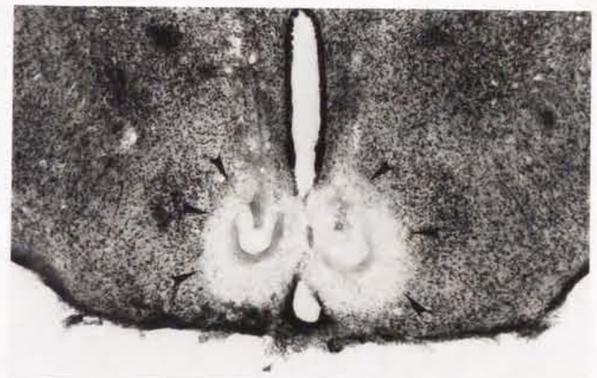


Figure 5.2. A photomicrograph of a coronal section of an ovariectomized rat brain with bilateral radiofrequency lesions of the ARC. The section was stained with thionin. Arrowheads indicate the area damaged by lesion. × 30.

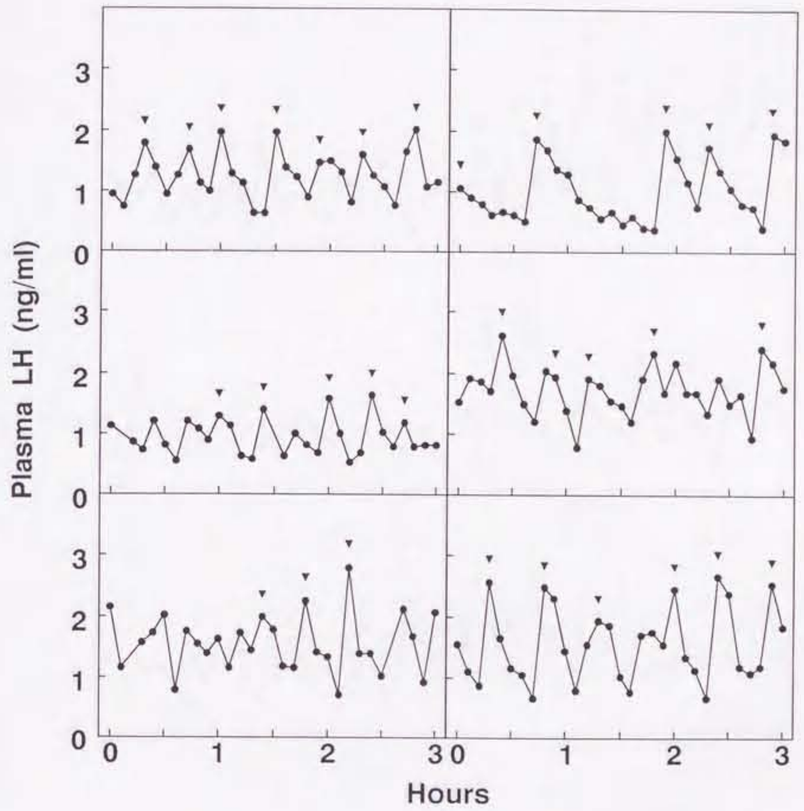


Figure 5.3. Profiles of plasma LH concentrations in ovariectomized rats bearing sham-lesion of the ARC. The values are expressed in terms of the NIDDK-rLH-RP-2. Arrowheads represent the LH pulse identified by the PULSAR computer program.

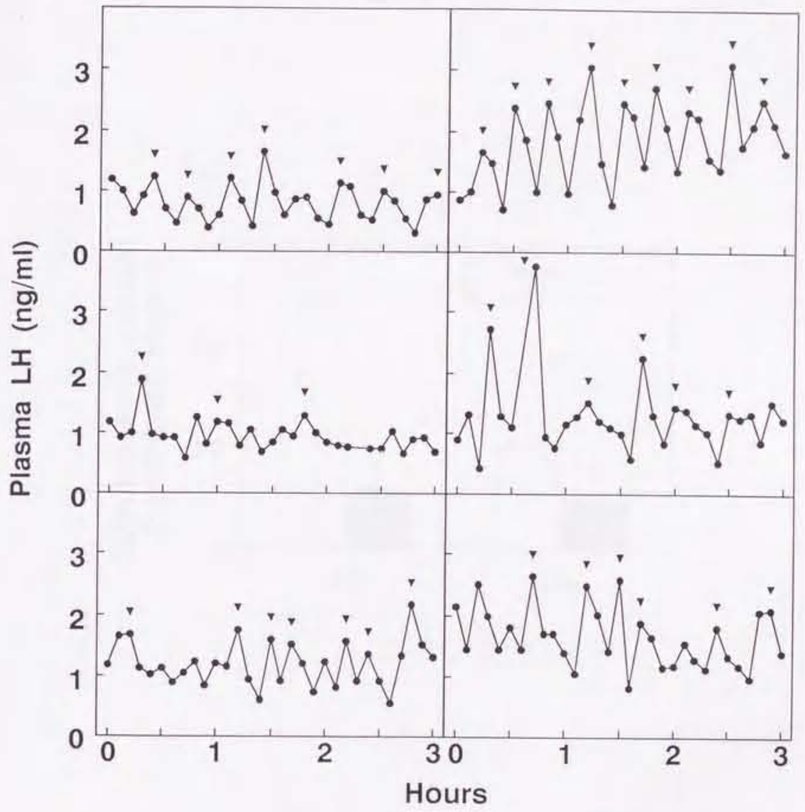


Figure 5.4. Profiles of plasma LH concentrations in ovariectomized rats bearing radiofrequency lesion of the ARC. Arrowheads represent the LH pulse identified by the PULSAR computer program.

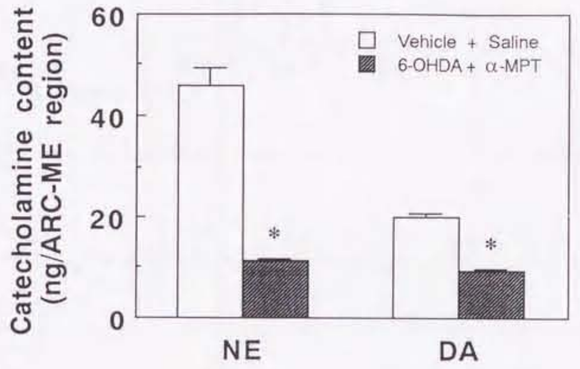


Figure 5.5. Contents of norepinephrine (NE) and dopamine (DA) in the arcuate nucleus-median eminence (ARC-ME) region of ovariectomized, 6-hydroxydopamine (6-OHDA)- and α-methyl-p-tyrosine (α-MPT)-treated rats. Open and hatched columns represent the control (n=8) and 6-OHDA/α-MPT-injected (n=11) groups, respectively. Values are means ± SEM. *P<0.001, compared with the control group (Student's *t*-test or Cochran-Cox test).

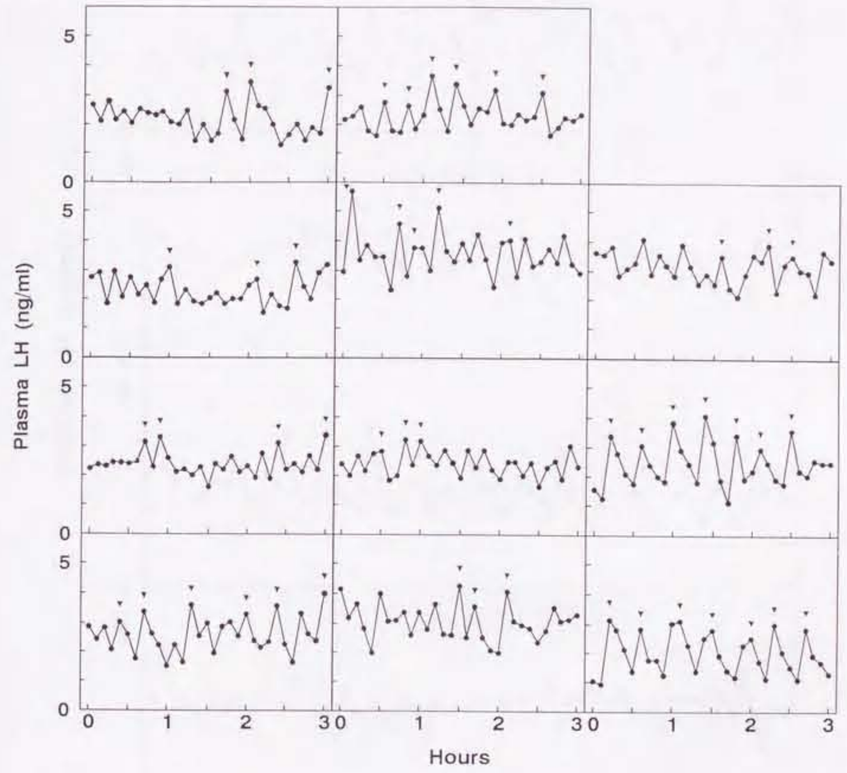


Figure 5.6. Profiles of plasma LH concentrations in ovariectomized rats with microinjections of 6-OHDA and α-MPT into the ARC-ME region. Animals were injected with 10 μg of 6-OHDA into each side 1 week before the day of bleeding. Two hours before the start of blood sampling, animals were injected with 50 μg of α-MPT into each side. Arrowheads represent the LH pulse identified by the PULSAR computer program.

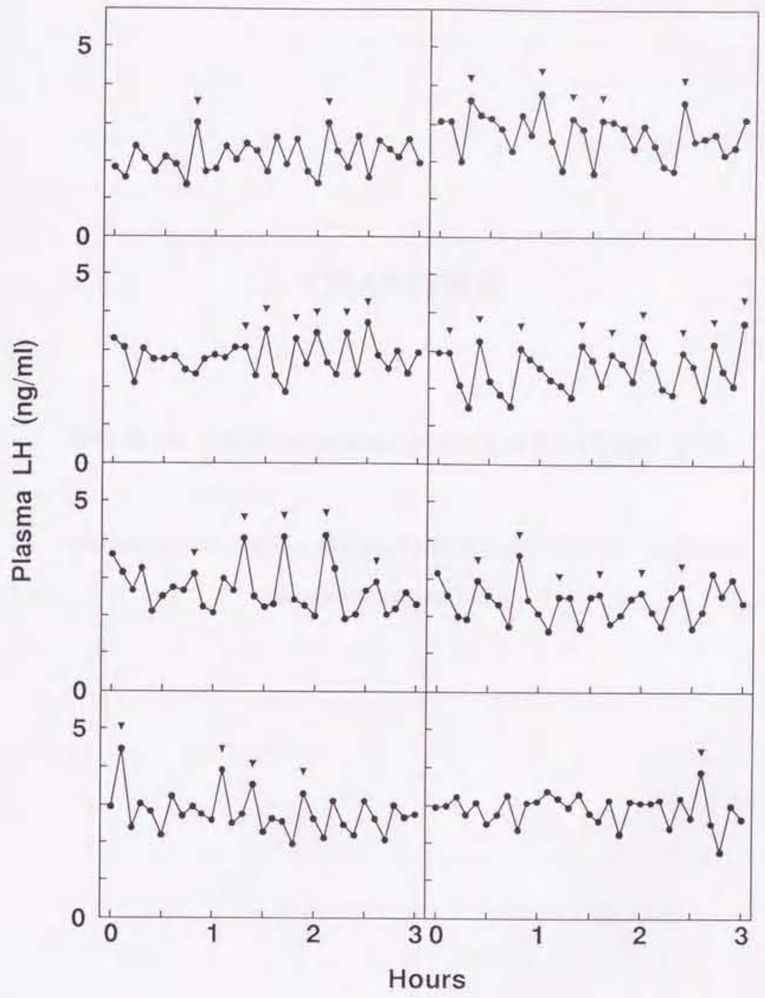


Figure 5.7. Profiles of plasma LH concentrations in ovariectomized rats injected with vehicles into the ARC-ME region. Animals were injected with 0.5 μl of saline containing 1 mg/ml ascorbic acid in each side 1 week before the day of bleeding. Two hours before the start of blood sampling, animals were injected with 0.2 μl of saline in each side. Arrowheads represent the LH pulse identified by the PULSAR computer program.

CHAPTER 6

Role of the LHRH-producing neurons in the preoptic area

—Effects of electrolytic lesion of the preoptic area on LH pulses in ovariectomized rats—

Introduction

The POA in the brain has been proposed as a center for regulating the cyclic mode of LH secretion which induces ovulation (Gorski, 1968; Halász & Gorski, 1967; Halász & Pupp, 1965). Indeed, previous reports revealed that the lesions restricted to the medial POA resulted in disappearance of LH surge and a subsequent anovulatory persistent estrus in female rats (Kawakami, Yoshioka, Konda *et al.*, 1978; Popolow, King & Gerall, 1981; Wiegand, Terasawa & Bridson, 1978; Wiegand, Terasawa, Bridson & Goy, 1980). However, the role of the POA on another mode of LH secretion, the tonic mode, has not been fully described.

Most of the cell bodies of LHRH-producing neurons are located in a region anterior to the hypothalamus, the medial POA, the diagonal band of Broca, and the septum in rats (Kawano & Daikoku, 1981; Sétáló *et al.*, 1976; Witkin *et al.*, 1982). Most of axons of these neurons terminate in the median eminence or the organum vasculosum of the lamina terminalis. Recently, clonal LHRH-producing neuronal cell lines, which were derived from the POA of transgenic mice, are reported to exhibit a pulse-like pattern of LHRH release into the medium *in vitro* (Krsmanovic *et al.*, 1992; Martínez de la Escalera *et al.*, 1992), suggesting that the LHRH-producing neurons in the POA are equipped with a mechanism synchronizing the LHRH release from the nerve terminals.

On the other hand, results of experiments in Chapters 3 and 4 suggest that the LHRH-producing neurons are not involved in the pulse-generating mechanism, since PAD perturbed the pulsatile LH secretion without depression of the LHRH immunoreactivity in the median eminence, and transplanted fetal MBH tissue, which contained no immunoreactive LHRH neurons, maintained LH pulses after PAD.

According to the hypothesis which has been proposed in previous chapters, it is postulated that the pulsatility of LH secretion is not affected by destroying LHRH neuronal cell bodies in the POA. In the experiment of the present chapter, bilateral electrolytic lesions were placed in the POA to evaluate the role of LHRH-producing neurons in this area on LHRH-pulse generation in ovariectomized rats.

Materials and Methods

Treatments

Ovariectomized animals were received bilateral lesion of the POA (n=6) or sham operation (n=6) on the day before blood sampling. Blood samples were collected every 6 min for 3 h.

Brain histology

After blood sampling, frozen coronal sections (50 μm) were obtained from each brain and were stained with cresyl violet to determine the location and extent of the POA lesions.

Data analysis

Statistical differences in each parameter of the pulsatile LH secretion between POA-lesioned and sham-operated groups were determined by Student's *t*-test or Cochran-Cox test.

Results

Histological examination of each brain revealed that the medial POA was almost completely destroyed in animals bearing electrolytic lesions (Figure 6.1). The boundaries of the lesions were generally as follows: In the rostral-caudal plane from the emergence of the optic chiasm to about 1.0 mm caudal, in the medial-lateral plane from the midline to about 1.0 mm lateral, and in the dorsal-ventral plane from the anterior commissure to the optic chiasm (Figure 6.1A). The SCN was spared in POA-lesioned rats except for one animal (#11), in which the lesion extended to the rostral part of the SCN (Figure 6.1B). Figure 6.2 shows a photomicrograph of typical lesion of the POA.

A regular pattern of pulsatile LH secretion was observed in sham-lesioned animals (Figure 6.3). LH pulses with regular intervals also occurred in 5 out of 6 rats with bilateral lesions of the POA, but was repressed completely in one animal (#11) with the POA lesion extended to the SCN (Figure 6.4).

Table 6.1 shows mean plasma LH level and the frequency and amplitude of LH pulses for rats with or without bilateral damage restricted to the POA. The levels of these parameters of pulsatile LH secretion were not significantly ($P>0.05$, Student's *t*-test or Cochran-Cox test) different between two groups.

Discussion

The results in the present chapter demonstrated that bilateral electrolytic lesions restricted to the POA, where the most cell bodies of LHRH-producing neurons are located, did not affect the pulsatile LH secretion in ovariectomized rats. The levels of parameters of pulsatile LH secretion in rats with bilateral lesions of the POA were comparable to those in sham-lesioned animals. These results suggest that the POA does not crucially participate in generating LH pulses and that the LHRH-producing neurons in the POA *per se* are not involved in the mechanism generating LHRH pulses.

In the present experiment, LH pulses in rats with lesion of the POA would be maintained by the pulsatile LHRH release from neuronal terminals in the median eminence, suggesting that a sufficient amount of LHRH to release was still stored at least one day after the lesion. It has been reported that rats with bilateral lesions of the POA/AHA had substantially reduced mean LH levels and made LH pulses undetectable 7 days after the lesion (Jarry *et al.*, 1990). The depression of LH pulses in their results might be ascribed partly to the exhaustion of LHRH which had stored in the neuronal terminals in the median eminence, because blood samples were collected 7 days after the POA/AHA lesion and the lesion would completely abolish the supply of LHRH into the median eminence.

Pulsatile LH secretion was reduced completely in one animal (#11), in which the lesion extended to the rostral part of the SCN (Figure 6.4). This result suggests that the SCN may be involved in the mechanism generating LHRH pulses. Previous studies showed, however, that bilateral lesion restricted to this nucleus did not change the pulsatility of LH secretion (Arendash & Gallo, 1979; Soper & Weick, 1980). This discrepancy between the present experiment and previous studies might be attributed to

the extent of the damage: Combined SCN lesion with the POA lesion may result in the LH depression. Further studies are required to clarify this point.

In summary, the results found in the present chapter showed that the LHRH-producing neurons in the POA are not involved in the mechanism generating LHRH pulses. The cell bodies of LHRH neurons located in the POA would only play a role in supplying LHRH to the neuronal terminals in the median eminence where the LHRH pulse generator may regulate its release.

Table 6.1. Mean plasma LH level and the frequency and amplitude of LH pulses in ovariectomized rats with electrolytic lesions of the POA.

	n	Mean LH level (ng/ml)	Pulse frequency (pulses/3 h)	Pulse amplitude[a] (ng/ml)
Sham lesion	6	1.61 ± 0.06	6.50 ± 0.43	1.04 ± 0.09
POA lesion	6	1.46 ± 0.28	5.00 ± 1.03	1.24 ± 0.13

Values are means ± SEM; n, Number of animals used. Mean LH levels were calculated for a series of plasma LH concentrations during the 3-h sampling period. The frequency and amplitude of LH pulses were identified by the PULSAR computer program. No significant difference in each parameter was determined between two groups ($P>0.05$, Student's *t*-test or Cochran-Cox test). [a]The amplitude of LH pulses was calculated only in rats in which LH pulses were identified by the PULSAR computer program.

A

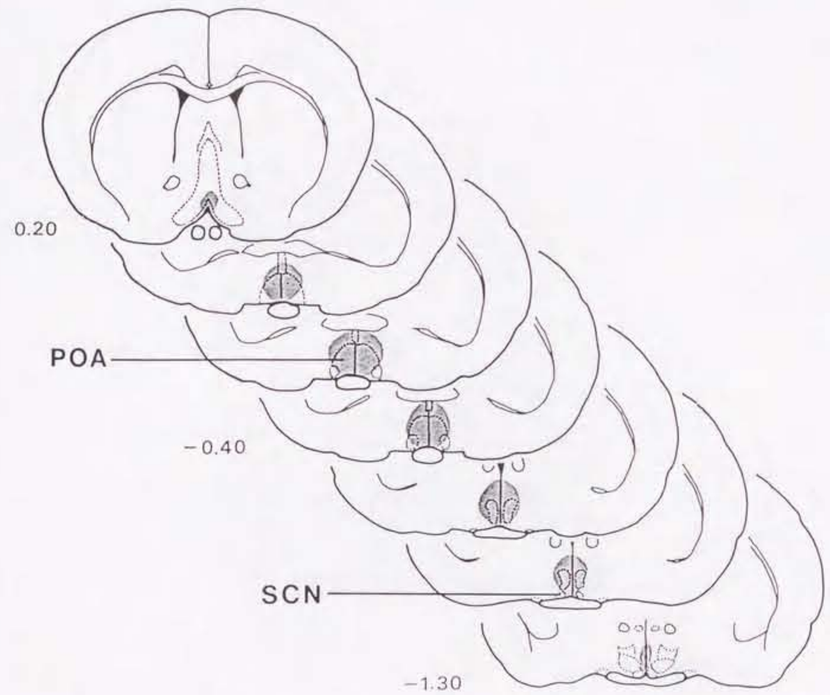


Figure 6.1A. Schematic illustrations of coronal sections of the brain showing the electrolytic lesion restricted to the preoptic area (POA). Line drawings are modified from the atlas of Paxinos and Watson (1986). Shaded areas designate the areas damaged by lesions. SCN—suprachiasmatic nucleus.

B

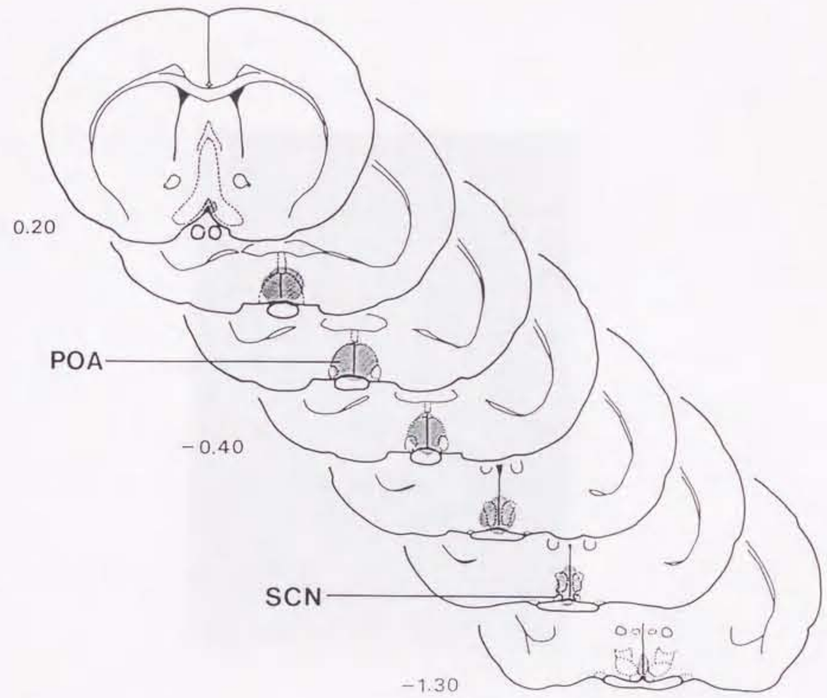


Figure 6.1B. Schematic illustrations of coronal sections of the brain showing the electrolytic lesion of the POA. Lesion was extended to the rostral part of the SCN. Line drawings are modified from the atlas of Paxinos and Watson (1986). Shaded areas designate the areas damaged by lesions.

Figure 6.2. A photomicrograph of a coronal section of an ovariectomized rat brain with bilateral electrolytic lesions of the POA. The section was stained with cresyl violet. Arrowheads indicate the area damaged by lesion. × 30.

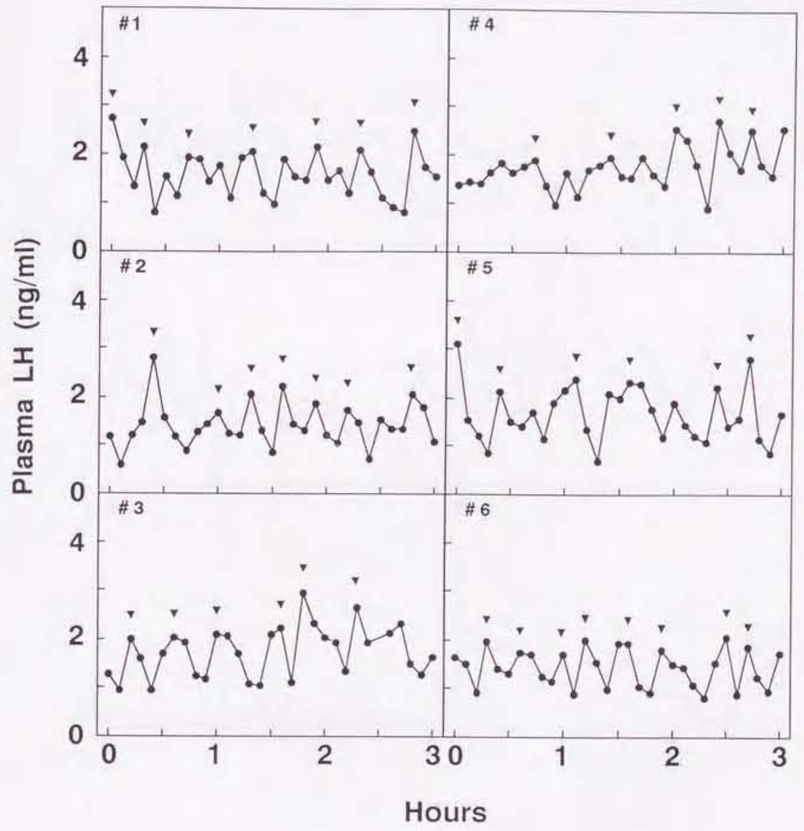


Figure 6.3. Profiles of plasma LH concentrations in ovariectomized rats bearing sham lesion of the POA. The values were expressed in terms of the NIDDK-rLH-RP-2. Arrowheads represent LH pulses identified by the PULSAR computer program.

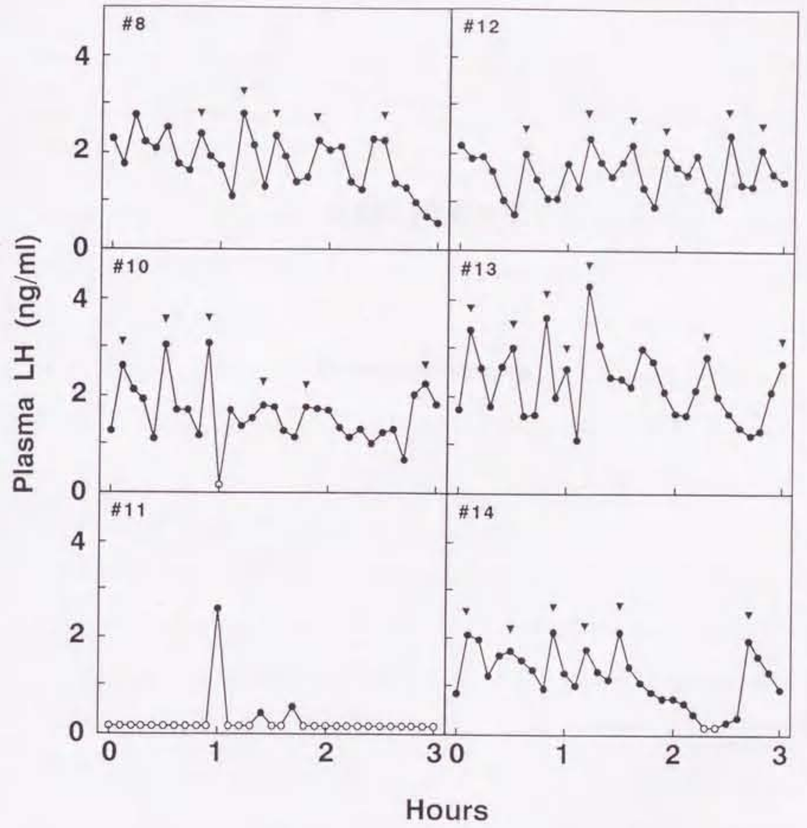


Figure 6.4. Profiles of plasma LH concentrations in ovariectomized rats bearing electrolytic lesion of the POA. The values were expressed in terms of the NIDDK-rLH-RP-2. Open circles indicate LH values which were lower than the limit of assay (0.156 ng/ml plasma). Arrowheads represent LH pulses identified by the PULSAR computer program.

CHAPTER 7

General discussion

The location and components of the hypothalamic LHRH pulse generator in rats

The MBH has been considered to be a center governing the tonic release of LH in rats (Gorski, 1968). Following complete deafferentation of the MBH in the adult female rat, animals showed constant vaginal estrus with polyfollicular ovaries or constant vaginal diestrus with persistent corpora lutea without showing ovarian atrophy (Halász & Gorski, 1967; Halász & Pupp, 1965). When the prepubertal female rat was subjected to CD, anovulatory persistent vaginal estrus ensued and, additionally, the animal displayed precocious ovarian maturation (Ramaley & Gorski, 1967). These earlier studies indicate that the MBH separated from the other part of the brain is still capable of stimulating the anterior pituitary to maintain the ovarian activity. Subsequently, Blake and Sawyer (1974) reported that rats bearing complete deafferentation of the MBH showed pulsatile fluctuations of plasma LH levels after the ovariectomy. The results obtained in Chapter 3 also clearly demonstrate that the LHRH pulse generator exists within the MBH, because the frequency of LH pulses was not affected by CD. The frequency of LH pulses directly reflects the bursting activity of neurons constituting the LHRH pulse generator, irrespective of the responsiveness of the pituitary gland to LHRH. In fact, it is revealed that each LH pulse corresponds to a LHRH pulse (Clarke & Cummins, 1982; Levine & Duffy, 1988; Levine *et al.*, 1982; Urbanski *et al.*, 1988). Therefore, unchanged level of the frequency of LH pulses suggests that the mechanism generating LHRH pulses is preserved after complete isolation of the MBH. Taking these and the previous studies into consideration, the MBH is a unit to maintain the pulsatile release of LH without any afferent neural inputs from the other structures of the brain.

Anatomical evidence in rats has raised a controversy over the location and

components of the mechanism generating LHRH pulses. Immunohistochemical studies revealed that most neuronal cell bodies of LHRH neurons are scattered over the region anterior to the hypothalamus, *i.e.* the medial septal area, diagonal band of Broca and medial POA, with most of their axons projecting to the median eminence (Kawano & Daikoku, 1981; Sétáló *et al.*, 1976; Witkin *et al.*, 1982). In addition, there are few cell bodies of LHRH neurons in the MBH. Therefore, the first question to be answered is whether the LHRH-producing neurons themselves are capable of generating pulsatile LHRH release. The results in Chapters 3 and 4 revealed that the LHRH pulse generator consists of a group of neurons other than LHRH-producing neurons: Pulsatility of LH secretion in rats 1 week after PAD was less apparent although immunoreactive LHRH neuronal fibers were still found in the external layer of the median eminence (Chapter 3); in addition, transplantation of fetal MBH tissue, which has no cell bodies of LHRH neurons, into the third ventricle maintained regular pulsatile LH secretion after PAD (Chapter 4). These results suggest that the fetal MBH tissue contains a mechanism driving the periodic release of LHRH from the median eminence and that the mechanism is independent of the LHRH-producing system. Matsumoto *et al.* (1984) reported that the transplantation of neonatal brain tissue containing the MBH which does not have cell bodies of LHRH neurons restored the ovarian function in aged female rats, supporting the hypothesis that non-LHRH neurons in the MBH play a role in regulating pulsatile LHRH release in rats. The results in Chapter 6 also suggest that the LHRH neurons *per se* are not included in the mechanism generating LHRH pulses because the electrolytic lesions of the POA, where the most cell bodies of LHRH-producing neurons are located, do not affect the pulsatile LH secretion. Moreover, the pulsatile nature of LHRH release from the retrochiasmatic hypothalamic fragment in rats, which contains no cell bodies of LHRH neurons, has been demonstrated by the

static incubation *in vitro* (Bourguignon & Franchimont, 1984; Bourguignon, Gerard, Debougnoux *et al.*, 1987). All these studies indicate that the LHRH pulse generator would be distinct from the LHRH neurons themselves.

On the contrary, several researchers assert their view that the pulsatile LHRH release is generated by the intrinsic mechanism of the LHRH neurons. In particular, their assertion is based on the fact that superfused LHRH-producing cell line exhibits the pulsatile LHRH release spontaneously *in vitro* (Krsmanovic *et al.*, 1992; Martínez de la Escalera *et al.*, 1992). However, it is not likely that the results obtained from this cell line reflect the physiological events *in vivo* concerning the LHRH-releasing mechanism, because the cell line was established from artificially-tumored LHRH neurons. Moreover, the density of these tumored cells employed in *in vitro* superfusion study is larger than *in vivo*, because cultured LHRH cells were grown to confluency. However, LHRH-producing neurons *in vivo* are found to be scattered over the region anterior to the hypothalamus and close contacts between LHRH neurons are not often found in the brain (Pelletier, 1987; Silverman, 1988).

In Chapter 6, the pulsatile LH secretion was not affected by the electrolytic lesions of the POA where it has been considered that most of LHRH neurons and the center for cyclic secretion of LH are located (Gorski, 1968). This result suggests that the POA is not involved in generating LHRH pulses. It has been previously reported that the lesions restricted to the medial POA induced an anovulatory persistent estrus in female rats (Kawakami *et al.*, 1978; Popolow *et al.*, 1981; Wiegand *et al.*, 1978; Wiegand *et al.*, 1980). This anovulatory condition was characterized by persistent vaginal cornification and polyfollicular ovaries, which was obviously caused by the abolishment of LH surges (Wiegand *et al.*, 1980) but not the tonic LH secretion or LH pulses. In sheep, the placement of the hypothalamic deafferentation between the ARC

and the SCN did not change the pulsatile LH secretion but blocked the estradiol-induced LH surge (Jackson, Kuehl, McDowell & Zaleski, 1978). Two independent mechanisms for regulating tonic and cyclic modes of LH secretion were also suggested by the electrophysiological studies in monkeys (Kesner, Wilson, Kaufman *et al.*, 1987; O'Byrne, Thalabard, Grosser *et al.*, 1991) and goats (Tanaka, Mori & Hoshino, 1992): The increment in the MUA, *i.e.* MUA volleys, in the MBH continued with decreased frequency during the spontaneous or estradiol-induced LH surge, indicating that preovulatory LH surges could not be caused by an increased frequency of LHRH pulses. Suckling stimulus suppresses the pulsatile LH secretion (Maeda *et al.*, 1989; Tsukamura *et al.*, 1990) but not the estradiol-induced LH surge in lactating rats (Tsukamura, Maeda & Yokoyama, 1988), suggesting that the mechanism regulating pulsatile LH secretion is independent of LH surge-generating mechanism. It is most likely, therefore, that the neuronal system governing pulsatile LH secretion is different from that governing the LH surge.

The findings in the present dissertation and the previous morphological evidence implies that the LHRH neurons would play only a role in supplying the decapeptide and that its release from nerve terminals of the LHRH neurons would be regulated by other mechanism. The mechanism would reside within the MBH and send signals to the LHRH neurons to cause LHRH pulses: In other words, the LHRH pulse generator is independent of the LHRH-producing system and synchronizes the firing of the LHRH neurons and the subsequent the release of LHRH from the neuronal terminals. The LHRH contents in the ARC-ME region were significantly reduced in cycling rats 2 weeks after bearing AD of the MBH using a knife with a large radius (2.0 mm), and CD caused a more marked decrease in its contents than AD (Taketani, Nozaki, Taga *et al.*, 1980), suggesting that the LHRH detected in the external layer of the median

eminence is mainly supplied from the LHRH-synthesizing neurons located outside the MBH. These results are in agreement with the results in Chapter 3 showed that the amplitude of LH pulses decreased as the incision of the hypothalamic deafferentation extended. It has been reported that the amplitude of LH pulses is closely correlated with that of LHRH pulses (Levine *et al.*, 1982). Although a few cell bodies of LHRH neurons exist in the MBH, they would be insufficient to maintain pulsatile release of LHRH with normal amplitude.

Which part of the MBH is indispensable for the generation of LHRH pulses? Previous works with the electrophysiological technique revealed that the MUA volleys in the ARC-ME region invariably associated with the peaks of LH pulses in rats (Kawakami *et al.*, 1982; Kimura *et al.*, 1991; Nishihara *et al.*, 1991). Since this temporal correlation between the MUA volleys and LH pulses was observed only in the animals with the recording electrode placed in the anterior part of the ARC-ME region (Kawakami *et al.*, 1982), it was assumed that the LHRH pulse generator is located in the anterior part of the ARC within the MBH. In addition, decreased frequency of the MUA volleys during the LH surge indicates that the MUA volley represents the excitation of neurons responsible for generating LHRH pulses but not that of LHRH fibers. Soper and Weick (1980) reported that electrolytic lesions of the anterior part of the ARC combined with the anterior deafferentation of the MBH blocked LH pulses in ovariectomized rats. Moreover, the results in Chapter 3 as well as in previous reports in rats (Blake & Sawyer, 1974) and sheep (Jackson *et al.*, 1978) demonstrated that the PAD, which cut off the anterior part of the ARC, perturbed the pulsatile LH secretion, supporting the view that the LHRH pulse generator exists in this nucleus, especially in the region cut off from the posterior part.

On the other hand, the findings in Chapter 5 were not in agreement with this

notion, because the restricted damage to the anterior part of the ARC did not affect the regular LH pulses. Soper and Weick (1980) also reported that the electrolytic lesions of the anterior part of the ARC alone did not affect the pulsatile LH secretion. In addition, rats with neonatal administration of monosodium-L-glutamate, in which up to 90% of the neuronal cell bodies in the ARC were destroyed (Nemeroff, Konkol, Bissette *et al.*, 1977), showed pulsatile pattern of LH secretion (Rose & Weick, 1986). It has been demonstrated that pulsatile LH secretion was disrupted after electrolytic lesions of the ARC with the damage to the median eminence, whereas LH pulses did not altered by the axon-sparing chemical lesions of the ARC (Sisk *et al.*, 1988). These results imply that the ARC is not involved in generating normal pulsatile secretion of LH in rats.

The location of the LHRH pulse generator is deduced by the results of deafferentation study of the present dissertation (Figure 7.1). Since the pulsatile LH secretion was maintained after CD but not after PAD, it is most likely that the LHRH pulse generator is located in the hypothalamic island made by CD, especially in the adjacent area of the anterior part of the ARC that is separated from the caudal part of the MBH by PAD. It is also assumed from these results that the putative LHRH pulse-generating neurons project to the terminals of LHRH neurons in the median eminence, but not to cell bodies of LHRH neurons, to generate LHRH pulses regularly.

Another explanation is derived from the work of Soper and Weick (1980). They reported that the electrolytic lesion of the ARC combined with AD perturbed the pulsatile LH secretion, whereas any of those treatments alone does not affect LH pulses. They hypothesized that two pathways can stimulate the pulsatile LH secretion independently of each other: One of these pathways involves the ARC or adjacent structures, while the other includes extrahypothalamic structures cut off by AD. Rose

and Weick (1987) demonstrated that the electrolytic lesions of the area postero-dorsal to the anterior ARC, but not the anterior part of the ARC, resulted in blockade of LH pulses in rats with neonatal monosodium-L-glutamate treatment, suggesting that the other generating system could work in the rat in which intrinsic LHRH pulse generator had been destroyed by the neurotoxin. These reports imply that several mechanisms generating LHRH pulses might exist in the brain. It is well known in the heart that the autonomous activity of sino-atrial node in the cardiac conduction system causes heartbeats as a cardiac pacemaker. However, the other pacemaker, atrioventricular node, also has an autonomous activity intrinsically with its own periodicity (Ganong, 1989). Moreover, a set of oscillators have been proposed to be arranged in a hierarchical manner in the mechanism regulating the mammalian circadian rhythms. In this model, the "driving oscillator" will entrain secondary and tertiary oscillators (Moore, 1978). It is also possible, therefore, that there are several LHRH pulse generators in the brain and that one of them plays a role of a driving oscillator in generating LHRH pulses.

At all events, the results in the present dissertation clearly demonstrate that at least one pulse generator, which consists of a group of non-LHRH neurons, is located within the MBH in the rat.

Possible mechanisms generating pulsatile LHRH release

How does the LHRH pulse generator regulate the periodic activation of the LHRH-producing neurons? In order to make up a clear pulse, a large amount of LHRH should be released simultaneously from the neuronal terminals of LHRH-producing neurons into the pituitary portal vessels. Therefore, the LHRH pulse generator should excite a lot of LHRH neurons at the same time, resulting in synchronized LHRH

release from most of LHRH neurons. The following question should be answered: Where does the LHRH pulse generator act on the LHRH neurons? For regulating pulsatile LHRH release, two sites of action of the LHRH pulse generator is considered: One is at the level of LHRH cell bodies or dendrites in the POA and the other is that of neuronal terminals in the median eminence.

In the lactating animals, oxytocin neurons evolve a synchronized electrical activation which permits pulsatile oxytocin release and then the milk ejection (Higuchi, Honda, Fukuoka *et al.*, 1983; Higuchi, Honda, Fukuoka *et al.*, 1985; Wakerley, Clarke & Summerlee, 1988). During lactation, in both the supraoptic and paraventricular nucleus, glial processes between oxytocinergic neurons diminish, so that large numbers of oxytocinergic neurons become directly in contact with adjacent oxytocinergic neurons, and the number of double synapses, which make synaptic contacts with each of two adjacent neurons, shows a great increase (Theodosis, Chapman, Montagnese *et al.*, 1986; Theodosis & Poulain, 1984). Likewise, close contacts between LHRH neurons or many inputs from other neurons should be found at LHRH perikarya, if pulsatile release of LHRH is regulated at the level of cell bodies or dendrites in the POA. Although synaptic contacts between LHRH neurons and some other neuronal processes have been demonstrated in the POA (Leranth, MacLusky, Sakamoto *et al.*, 1985; Leranth, MacLusky, Shanabrough & Naftolin, 1988; MacLusky, Naftolin & Leranth, 1988; Pelletier, 1987), the density of these synapses on the LHRH neurons has been found to be fairly low (Silverman, 1988). In the POA, moreover, neuronal cell bodies of LHRH-producing neurons are distributed diffusely (Kawano & Daikoku, 1981; Sétáló *et al.*, 1976; Witkin *et al.*, 1982). Therefore, a dense network between LHRH neurons or LHRH neurons and other neurons could not be easily constructed in the POA and hence the synchronized release of LHRH not be

ensured.

On the other hand, neuronal terminals of LHRH neurons are concentrated at the median eminence. The convergence of LHRH neuronal terminals could enable the LHRH pulse generator to easily contact most of LHRH terminals and to synchronize LHRH release in the median eminence. Therefore, it seems most likely that LHRH release is regulated at the level of the neuronal terminals in the median eminence rather than at the level of cell bodies or dendrites in the POA. The stimulatory action of both NE and DA (Negro-Vilar, Ojeda & McCann, 1979) or of excitatory amino acid mediated by non-NMDA type receptors (Donoso, López & Negro-Vilar, 1990; López, Donoso & Negro-Vilar, 1992) on the release of LHRH from the median eminence fragments incubated *in vitro* has been reported. These results suggest that neuronal terminals of LHRH neurons in the median eminence are capable of being excited by the stimulatory input from other neurons and strongly support the above notion that pulsatile LHRH release is regulated at the level of neuronal terminals. However, morphological studies of the median eminence of the rat demonstrate that few synaptic contacts between neuronal fibers are observed in this region (Ajika, 1980; Kobayashi & Matsui, 1969), though synaptic contacts between tyrosine hydroxylase- and LHRH-containing axons were found in the median eminence only in the ewe (Kuljis & Advis, 1989). On the basis of these anatomical evidence, the LHRH pulse generator is postulated to regulate LHRH pulses in a non-synaptical manner in the median eminence.

Fuxe and Agnati (1991) recently proposed a novel hypothesis on the communication between neurons, called the "volume transmission". Volume transmission refers to the variety of means by which the extracellular space of the nervous system may serve as a conduit for informational substances, as distinct from

classical synaptic communication or "wiring" transmission. The leakage of the neurotransmitter often occurs at the non-synaptic site and the substance diffuses the distance and reaches the receptors on the neighboring neurons at a high concentration enough to stimulate receptors. For instance, norepinephrine and epinephrine could inhibit the release of acetylcholine from the electrically stimulated guinea pig ileum, despite the fact that there is no synaptic contact between noradrenergic and cholinergic neurons in the gut (Vizi, 1991). Mismatches between the distributions of various neurotransmitters and their receptors visualized by immunohistochemistry and receptor autoradiography were observed in the brain, suggesting that sites of release of neurotransmitters and its receptors have discrete and heterogeneous distributions in the nervous system (Herkenham, 1987). In the external layer of the rat median eminence, exocytotic profiles have been observed in the "non-terminal" zones of the nerve fibers (Stoeckart, Jansen & Kreike, 1972), implying that the neurotransmitters are released into the intercellular space and acts directly on the neighboring processes. At present, volume transmission seems to be the sole mechanism to explain how the LHRH pulse generator regulates pulsatile LHRH release in the median eminence non-synaptically.

Figure 7.2 gives a summary of the possible mechanism generating pulsatile LHRH release in rats. The LHRH pulse generator resides within the MBH and consists of a group of neurons other than the LHRH-producing neurons. Periodic activity of the LHRH pulse generator is transmitted to the LHRH neuronal terminals in the median eminence via axo-axonal volume transmission mechanism and causes pulsatile release of LHRH, and then pulsatile LH secretion.

Possible neurotransmitter driving the LHRH pulse generator

The results found in Chapter 5 failed to demonstrate the participation of the catecholaminergic systems in the ARC-ME region in generating LHRH pulses, because the depression of the catecholaminergic activity in that region did not affect the pulsatile LH secretion. In primates, *in vivo* release of LHRH and NE estimated with push-pull perfusion technique in the stalk-median eminence was reported to be pulsatile and synchronous with each other (Terasawa *et al.*, 1988). However, since the cell bodies of NE-containing neurons are situated in the lower brain stem (Palkovits *et al.*, 1980; Swanson & Hartman, 1975), but not in the MBH in which the LHRH pulse generator would be located, it is less likely that the NE-containing neurons play a crucial role in generating LHRH pulses. Jarry *et al.* (1990) demonstrated in rats that local administration of a α_1-receptor antagonist into the POA/AHA,but not into the MBH, by push-pull perfusion technique resulted in reduction of LH pulses. Since the action site of the LHRH pulse generator on LHRH neurons is postulated to be at the level of the neuronal terminals in the median eminence (Figure 7.2), the catecholaminergic system is not crucially involved in generating LH pulses, but only plays a permissive role in regulating LH pulses.

Which neuronal system drives the pulsatile signal for LHRH release? There are abundant literature concerning the action of various peptidergic and aminergic neuromediators on hypothalamic regulation of LH secretion (Kalra & Kalra, 1983; Weiner, Findell & Kordon, 1988). Since the LHRH pulses with regular intervals are observed even in castrated animals, the LHRH pulse generator should be composed of a group of neurons that are able to stimulate LHRH release even under the steroid-free condition. In this notion, several transmitters or neuropeptides that affect the LHRH release in a steroid-dependent manner or consistently inhibit the release of LHRH

would be ruled out to play a crucial role in generating LHRH pulses. For instance, noradrenergic neurons have both stimulatory and inhibitory roles in the maintenance of pulsatile LH secretion. Blockade of NE synthesis (Drouva & Gallo, 1976; Gnodde & Schuiling, 1976; Negro-Vilar *et al.*, 1982) or α-adrenergic receptors (Weick, 1978) suppresses LH pulses in ovariectomized rats, but central infusion of NE (Bergen & Leung, 1986; Gallo, 1984; Gallo & Drouva, 1979; Leung *et al.*, 1982) or α- or β-adrenergic agonists (Leung *et al.*, 1982) also results in the suppression of pulsatile LH secretion. Injection of neuropeptide Y into the third cerebral ventricle increases plasma levels of LH in ovariectomized rats treated with estrogen and progesterone (Kalra & Crowley, 1984) but decreases LH levels in steroid-untreated ovariectomized rats (Kalra & Crowley, 1984; McDonald, Lumpkin & DePaolo, 1989; McDonald, Lumpkin, Samson & McCann, 1985). The third ventricular injection of both α-bungarotoxin, an antagonist of the nicotinic cholinergic receptors, and nicotinic receptor agonists decreases the frequency of LH pulses in ovariectomized rats (Kalash, Romita & Billiar, 1989), suggesting the biphasic actions of acetylcholine. Moreover, central administration of γ-aminobutyric acid (Nishihara & Kimura, 1987), corticotropin-releasing factor (Rivier & Vale, 1984) or β-endorphin (Kinoshita, Nakai, Katakami *et al.*, 1980) causes inhibition of the LH secretion in castrated rats. All these neuromediators may not be involved in the LHRH pulse generator, but in modulating its activity.

It has been reported that systemic administration of the agonists of neuroexcitatory amino acids, such as NMDA and kainate, activate hypothalamic receptors and stimulate LH secretion independently of gonadal steroids in rats (Price, Olney & Cicero, 1978; Schainker & Cicero, 1980) and primates (Gay & Plant, 1987) at subtoxic doses. The *in vitro* release of LHRH from the hypothalamic explants was

also stimulated by both NMDA and kainate (Bourguignon, Gérard & Franchimont, 1989a). Blockade of NMDA type receptors by intravenous injections of a specific antagonist results in suppression of pulsatility of LH secretion *in vivo* (Arslan, Pohl & Plant, 1988). The pulsatile LHRH release from the hypothalamus incubated statically *in vitro* was abolished by antagonism of NMDA receptors (Bourguignon, Gérard, Mathieu *et al.*, 1989b). All these results suggest that neuroexcitatory amino acids receptor system might be one of the candidates involving in the mechanism generating LHRH pulses. However, one can not conclude that the LHRH pulse generator is composed of excitatory amino acids system at present. Since the LHRH pulse generator is postulated to regulate the LHRH pulses at the median eminence (Figure 7.2), the existence of NMDA or non-NMDA type receptors on the LHRH neuronal terminals in the median eminence should be demonstrated: It has never yet achieved. It has only been demonstrated that non-NMDA receptor sites would be preferential to stimulate LHRH release from the ARC-ME fragments *in vitro* (Donoso *et al.*, 1990; López *et al.*, 1992). In addition, recently, intracerebroventricular administration of a specific NMDA receptor antagonist has been reported to have no effects on LH secretion in ovariectomized lambs (Hileman, Schillo & Estienne, 1992). Therefore, further studies are required to identify the components of the mechanism generating LHRH pulses.

Conclusion

The present dissertation demonstrate the neuroendocrine mechanism by which the pulsatile LHRH release is generated in ovariectomized rats. The results in the present study suggest that (1) the LHRH pulse generator is located in the MBH, especially in the adjacent area of the anterior part of the ARC, but not within the ARC (2) the LHRH

pulse generator consists of a group of neurons distinct from the LHRH-producing neurons, and (3) the catecholaminergic system in the ARC-ME region is not involved in generating LHRH pulses. The LHRH pulse generator sends the pulsatile signal to the neuronal terminals of LHRH neurons in the median eminence by hypothetical non-synaptic "volume transmission" mechanism.

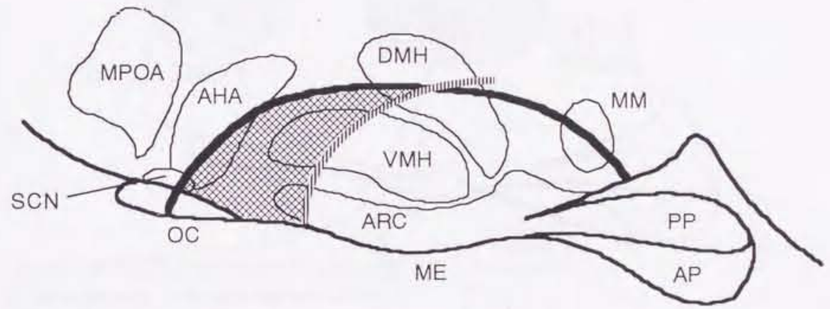


Figure 7.1. Schematic illustration of the possible location of the LHRH pulse generator in rats (hatched area). Solid and broken lines indicate incisions of complete and posterior-anterior hypothalamic deafferentation, respectively. AHA—anterior hypothalamic area; AP—anterior pituitary; ARC—arcuate nucleus; DMH—dorsomedial hypothalamic nucleus; ME—median eminence; MM—medial mammillary nucleus; MPOA—medial preoptic area; OC—optic chiasm; PP—posterior pituitary; SCN—suprachiasmatic nucleus; VMH—ventromedial hypothalamic nucleus.

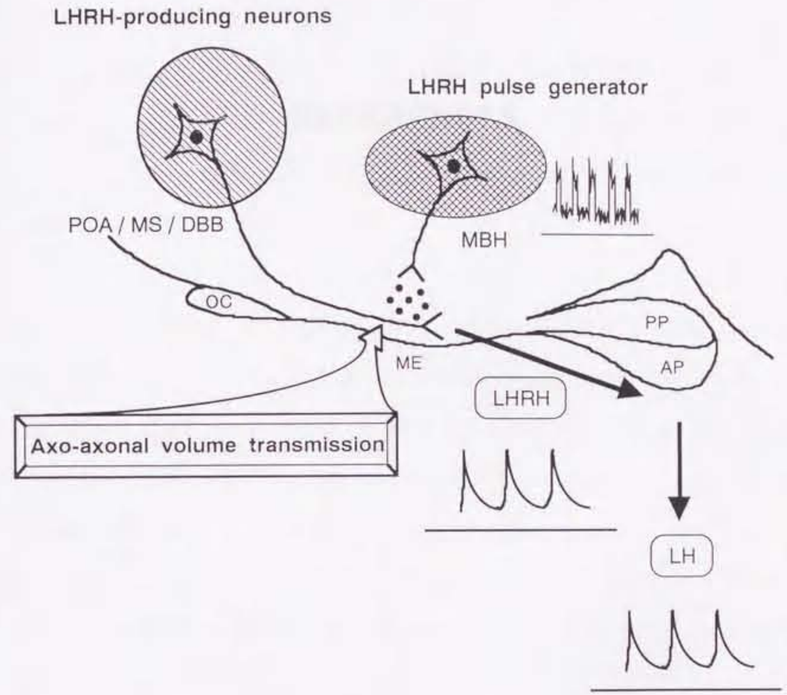


Figure 7.2. Schematic illustration of the possible neuroendocrine mechanism generating LHRH pulses in rats. AP—anterior pituitary; DBB—diagonal band of Broca; MBH—mediobasal hypothalamus; ME—median eminence; MS—medial septal area; OC—optic chiasm; POA—preoptic area; PP—posterior pituitary.

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LIST OF PUBLICATIONS CONCERNING THIS DISSERTATION

1. Ohkura, S., Tsukamura, H. and Maeda, K.-I.

   Effects of various types of hypothalamic deafferentation on luteinizing hormone pulses in ovariectomized rats.

   Journal of Neuroendocrinology **3**, 503-508 (1991).

2. Ohkura, S., Tsukamura, H. and Maeda, K.-I.

   Effects of transplants of fetal mediobasal hypothalamus on luteinizing hormone pulses impaired by hypothalamic deafferentation in adult ovariectomized rats.

   Neuroendocrinology **55**, 422-426 (1992).

3. Ohkura, S., Kawakami, S.-I., Nagabukuro, H., Tsukamura, H. and Maeda, K.-I.

   Depression of catecholaminergic neuronal activity in the arcuate nucleus-median eminence region or lesion of the arcuate nucleus do not affect the LHRH pulse generator activity in ovariectomized rats.

   Neuroendocrinology, submitted.

Pulse generator in female rats