Two Subgroups of Gonadotropin-Releasing Hormone Neurons Control Gonadotropin Secretion in Rats

Fukuko Kimura and Toshiya Funabashi

Two distinct subgroups of gonadotropin-releasing hormone (GnRH) neurons are involved in the control of luteinizing hormone secretion, at least in rats: one subgroup located in the mediobasal hypothalamus constitutes the GnRH pulse generator associated with opioid neurons, and the other located in the preoptic area constitutes the GnRH surge generator associated with γ-aminobutyric acid neurons.

Luteinizing hormone (LH), a gonadotropin of female mammals, is secreted from the anterior pituitary gland via two modes, a tonic and a phasic, i.e., preovulatory, secretion. In rats in which the luteal phase is atypical, the preovulatory period of the estrous cycle is characterized by a growth of ovarian follicles, and tonic secretion stimulates this growth. Serum levels of LH are at their lowest, i.e., basal, from early in the morning on the day of estrus, shortly after ovulation, through the days of diestrus and midday on the day of proestrus. At 1400 in the afternoon of proestrus, showing the involvement of the biological clock, the ovulation-inducing secretion of LH occurs to induce follicular rupture and ovulation. Serum levels of LH begin to increase rapidly and ultimately reach peak levels of ~100 times the level of tonic secretion (thus called the surge of LH secretion) in the late afternoon of proestrus, and thereafter these blood levels begin to decline and reach basal levels by early in the morning of estrus.

Classical model for the neural control mechanism of LH secretion in rats

For the central neuronal mechanism underlying the tonic and phasic (preovulatory) secretion of LH in rats, the classical but fundamental concept is that two neuronal levels control each mode of...
the LH secretion (Fig. 1); the arcuate region of the mediobasal hypothalamus (MBH) regulates the tonic secretion, and the preoptic area (POA) regulates the preovulatory secretion (7). This model for rats was established on the basis of rather classical neuroendocrinological studies utilizing techniques of electrolytic destruction, hormone implantation, electrical stimulation, and hypothalamic deafferentation (for instance, Ref. 8). Later studies clarified that the influence of ovarian hormones on the MBH was inhibitory and responsible for the control of the tonic secretion (negative feedback effect on LH secretion), whereas that of ovarian hormones on the POA was stimulatory and responsible for the control of the preovulatory secretion (positive feedback effect on LH secretion) (see review, Ref. 3). Also, ovarian hormones either lower or increase the responsiveness of the anterior pituitary gland to gonadotropin-releasing hormone (GnRH).

Studies utilizing immunocytochemical procedures had shown, by the early 1980s, that cell bodies of GnRH neurons are distributed rostrocaudally from the diagonal band of Broca to the premammillary region in the hypothalamus (see review, Ref. 3). GnRH axons are observed to terminate at the portal vessels in the median eminence and at the organum vasculosum of the lamina terminalis.

**Discovery of the GnRH pulse generator in rhesus monkeys**

In the course of validating a radioimmunoassay, Knobil and his colleagues (see review, Ref. 9) unexpectedly discovered the pulsatile secretion of LH in ovariectomized rhesus monkeys in 1970. The frequency of LH pulses was approximately one every hour. They next determined that each LH pulse was a response to a bolus of GnRH reaching the anterior pituitary gland from nerve terminals in the median eminence by way of the pituitary portal vessels, and the concept of a GnRH pulse generator responsible for the rhythmic secretion of GnRH from the terminals of GnRH neurons naturally followed. On the basis of the finding that in rhesus monkeys with lesions in the MBH a pulsatile GnRH replacement regimen could sustain ovulatory menstrual cycles, they proposed in 1980 the hypothesis that the menstrual cycle can be normally controlled by GnRH stimulation at a frequency of one pulse per hour in rhesus monkeys (9). Because they had already observed that complete disconnec-
tion of the MBH from the remainder of the central nervous system did not abolish pulsatile and surge secretion of LH in rhesus monkeys, their finding meant that in rhesus monkeys the POA was not involved in the generation of the preovulatory LH surge.

The GnRH pulse generator in rats

In ovariectomized rats, a rhythmic, pulsatile pattern of LH secretion was first described in 1972 (6). This pulsatile secretion was maintained after complete deafferentation of the MBH (2), as in rhesus monkeys. The demonstration of pulsatile secretion in intact cycling rats appeared late (see Ref. 1) and suggested that the tonic LH secretion was actually composed of a pulsatile pattern of LH secretion. The preovulatory LH secretion was, however, reported to be composed of a pulsatile secretion or a single constant, almost unvarying linear pulse of LH secretion (see Ref. 3).

Since then, features of the neuronal mechanism responsible for the pulsatile LH secretion have been intensively studied. The dependence of the pulsatile LH secretion on brain catecholamine was once the subject of much interest. However, the early finding that this mechanism is naloxone sensitive in rats may be more important. Naloxone, an opiate antagonist that binds with a higher affinity to µ-receptors than to other binding sites, was found to increase LH pulse frequency and amplitude in either intact or ovariectomized rats (1, 11, 13). There is now general agreement that endogenous opioids mediate the effect of estrogen, which is inhibitory to pulsatile LH secretion (negative feedback effect of estrogen) (11). Such a function of endogenous opioid peptides is consonant with the fact that amenorrhea and sterility often accompany morphine addiction in human females.

The next object of keen interest was whether the GnRH pulse generator, possibly present in the MBH as mentioned above, could produce the GnRH surge in rats, since it was reported in 1976 that, in rats, the preovulatory LH surge was preceded by a surge of GnRH secretion into the portal vessel, which was not seen at other stages (15). A hypothesis was presented by Kalra and colleagues (11) about a decade ago and since then has dominated investigators in this field: the GnRH pulse generator is stimulated by an abrupt decrease in the inhibitory opioid tone occurring during the afternoon of proestrus. This hypothesis was based on their finding that naloxone infusion in the morning of proestrus prematurely evoked a surgelike LH secretion, which, in terms of amplitude and duration, resembled preovulatory LH surge in the afternoon of proestrus. In addition, when naloxone was implanted directly into the POA or the MBH, LH secretion was rapidly stimulated, but not when implanted into other forebrain regions, in ovariectomized rats experimentally conditioned by estrogen and progesterone to secrete LH in the surge mode (see Ref. 10). It was therefore argued that opioid neurons that reside in the POA are interposed between the biological clock and GnRH neurons and that in the proestrus afternoon the biological clock restrains the inhibitory opioid influence on the GnRH pulse generator.

Electrophysiological approach to the GnRH pulse generator in rats

About 15 years ago, a new technique to record electrical activity of the GnRH pulse generator was developed by the collaboration of the laboratories of Kawakami and Knobil, the latter of which was attempting to record electrical activity in rhesus monkeys (9). Striking increases were found in the multiunit activity (MUA) (called MUA volleys by Knobil’s group) in the MBH of ovariectomized rats and also of ovariectomized rhesus monkeys, which were, at that time, under continuous anesthesia with thiopental (9, 12). Each volley was coincident with the initiation of each LH pulse, suggesting the existence of electrophysiological correlates of the pulsatile pattern of GnRH secretion and thereby of a certain neuronal oscillator. They thought that this neuronal oscillator, indeed, was the GnRH pulse generator, although its neuronal composition and supracellular organization remained in doubt. The technique was later improved and has allowed us to record without anesthesia. We could have monitored the activity of the GnRH pulse generator in real time. It is now assumed that the GnRH pulse generator has an ~20-min periodicity, a considerably shorter period than that under thiopental anesthetics in ovariec-

tomized rats.

By means of this technique, we tested in long-term ovariectomized rats the response of the GnRH pulse generator to naloxone (see Ref. 13). Naloxone clearly increased the frequency of volleys in the MUA, supporting the hypothesis that endogenous opioids inhibit the activity of the GnRH pulse generator tonically (11). However, probably due to the desensitization or downregulation by GnRH released in a large amount, the serum LH level did not increase significantly. This electrophysiological study disclosed the effect of naloxone on the GnRH pulse generator directly but not indirectly through its effect on serum LH (see Ref. 13).
In subsequent experiments, we found that the administration of estrogen to ovariectomized rats decreased the frequency of MUA volleys and that the injection of naloxone into such estrogen-treated ovariectomized rats increased the frequency of volleys (see Ref. 13), together supporting the previous view that endogenous opioids mediate the negative feedback effect of estrogen on LH secretion in rats (see review, Ref. 3).

Evidence that the GnRH pulse generator is not involved in the LH surge in rats

Electrophysiological studies have provided important evidence suggesting that the GnRH pulse generator is not involved in the generation of the LH surge.

First and foremost, it was confirmed that the GnRH pulse generator activity was resistant to barbiturates (4, 13). This feature should have been noticed more than 10 years ago when the MUA volleys as well as LH pulses were first discovered under the continuous infusion of thiopental sodium in experiments to establish the recording technique (12). Very recently, we especially confirmed that MUA volleys and LH pulses were not substantially affected by anesthesia with pentobarbital sodium in ovariectomized rats either with or without estrogen treatment (see Ref. 13).

Second, we observed that the GnRH pulse generator activity was not facilitated but silent around and during the surge of LH secretion experimentally induced by estrogen treatment in ovariectomized rats as mentioned above (13). However, the pulse generator was steadily working even during the LH surge; i.e., MUA volleys became clearer and more frequent when naloxone was infused into proestrous rats in which the preovulatory surge of LH secretion was blocked by pentobarbital sodium (13).

Although not from the electrophysiological study, there is also evidence supporting that the pulse generator is not involved in the LH surge. We found that naloxone infusion in the morning of proestrus, which was reported by Kalra et al. (11) to have induced a premature LH surge, certainly induced an increase in pulsatile LH secretion but did not affect the timing, magnitude, and duration of the preovulatory LH surge that should have occurred in the afternoon (see Ref. 4). This finding suggested that naloxone stimulated the GnRH pulse generator but did not stimulate the neuronal mechanism responsible for the preovulatory LH surge, a GnRH surge generator.

All these features of the GnRH pulse generator are consonant with the idea of the existence of a GnRH surge generator, distinct from the GnRH pulse generator. Among these, however, the feature of barbiturate resistance provided the most important clue supporting the idea that this neuronal mechanism could never be a GnRH surge generator, because, since 1950, it has been known that the preovulatory surge of LH secretion in rats is blocked by a barbiturate, pentobarbital sodium (see Refs. 4, 13).

Search for the GnRH surge generator in rats

Together with the recent pharmacological proof that barbiturates are activators of the γ-aminobutyric acid (GABA), receptor complex (see Ref. 13), the feature of the possible GnRH surge generator, barbiturate sensitivity, suggested to us that this neuronal mechanism involves GABA as an important inhibitory neurotransmitter. The role of GABA was then tested by examining the effect of the GABA receptor antagonist bicuculline administered in the morning, prior to the surge of LH secretion, in proestrous rats (see Ref. 5). The result was clear-cut: no significant changes in LH secretion occurred during the bicuculline infusion, unlike during naloxone infusion, but afterward a rapid and noticeable rise in LH secretion occurred, clearly suggesting the occurrence of a premature LH surge. In addition, this premature LH surge by bicuculline was not due to a phase shift in the biological clock (14). It is possible that the disinhibition of GnRH neurons from GABA results in the LH surge.

In agreement with the hypothesis that inhibitory GABA neurons are involved in the neuronal mechanism of the preovulatory surge of LH secretion, GABA release in the POA decreased with the LH surge, whereas its release in the MBH showed no significant correlations with the LH surge in ovariectomized rats treated with estrogen (10). There are also findings suggesting that the inhibitory action of GABA neurons reaches GnRH neurons directly (see Ref. 10).

We then tried to prove that such a bicuculline-induced LH surge was produced by GnRH neurons that are involved in the spontaneous preovulatory LH surge. Because it is known that GnRH neurons in the POA express Fos protein in association with the LH surge in proestrous or ovariectomized rats treated with estrogen (5), we used dual immunoperoxidase/immunofluorescence staining to test whether bicuculline was effective in inducing Fos protein in the same GnRH neurons that express the protein during the spontaneous preovulatory LH surge (5). We found that the distribution and proportion of GnRH neurons expressing Fos in response to bicuculline were identical to those during the spontaneous LH surge; i.e., ~50% of GnRH neurons in the POA expressed Fos prematurely at 1400 after bicu-
Bicuculline stimulation (Fig. 2B), just as seen at 1700 during the spontaneous LH surge. This confirmed that bicuculline advanced the timing of the activation of GnRH neurons in the POA that were responsible for the preovulatory LH surge.

Two subgroups of GnRH neurons are present in rat forebrains

We found that the Fos response to bicuculline was quite different from that induced by naloxone (4). The proportion of GnRH neurons that expressed Fos during infusion of naloxone in the morning of proestrus was 35–62% in the caudal part of the forebrain including the MBH, and this was larger than that (10%) in the rostral part of the forebrain including the POA (Fig. 2A). Under anesthesia with pentobarbital sodium, naloxone infusion also enhanced Fos expression in those GnRH neurons. Together with the hypothalamic deafferentation experiments (2), it was relevant to consider that the GnRH pulse generator exists in the MBH, which contains GnRH neurons responsive to naloxone.

Here we propose the hypothesis that two distinct subgroups of GnRH neurons are involved in the control of gonadotropin secretion, at least in rats; one subgroup constitutes the GnRH pulse generator associated with opioid neurons, and the other the GnRH surge generator associated...
with GABA neurons (Fig. 3). The GnRH neurons involved in the pulse generator are mainly located in the MBH, whereas those involved in the surge generator are located in the POA. These two groups of GnRH neurons may receive the feedback effects of estrogen mediated by opioid and GABA neurons, respectively. Estrogen exerts an inhibitory action on a group of GnRH neurons via the action on opioid neurons and as a result produces a negative feedback effect on LH secretion throughout the entire estrous cycle. Estrogen also inhibits another group of GnRH neurons via the action on GABA neurons, but this inhibition is disrupted at 1400 on the day of proestrus, probably by the input from the biological clock to cause the surge of LH secretion, and as a result produces what is called a positive feedback effect on LH secretion. For this reason, the classical, rather complicated concept that the estrogen-responsive neurons themselves change phasically (i.e., are at first inhibited and subsequently facilitated) could be improved.

This model looks like a revival of the classical one (7), but it contains much more detail. We

![GnRH Control Mechanism Diagram]

**FIGURE 3.** Schematic illustration showing how a subgroup of gonadotropin-releasing hormone (GnRH) neurons constitutes the GnRH pulse generator with opioid neurons in the mediobasal hypothalamus (MBH) and the other subgroup constitutes the GnRH surge generator with γ-aminobutyric acid (GABA) neurons in the preoptic area (POA). See Figs. 1 and 2 for other abbreviations.

**FIGURE 4.** Schematic illustration showing how 2 distinct neuronal mechanisms are involved in the control of gonadotropin-releasing hormone (GnRH) secretion during the rat estrous cycle.
propose that the GnRH pulse generator works throughout the estrous cycle, producing tonic LH secretion, and that the surge generator is abruptly activated at midday of proestrus for the preovulatory LH secretion (Fig. 4).

References


