BIOLOGY OF REPRODUCTION 36, 59–76 (1987)

Release of Luteinizing Hormone-Releasing Hormone (LHRH) and Neuroactive Substances in Unanesthetized Animals as Estimated with Push-Pull Cannulae (PPC)[1]

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ABSTRACT

In this report, we have reviewed recent information gathered by probing with a push-pull cannula (PPC) the in vivo activity of the suprachiasmatic nucleus (SCN), hypothalamus, and anterior pituitary gland of freely moving animals. In male and female rats, probing of the SCN with the PPC revealed distinct oscillatory patterns of 5-hydroxy indolacetic acid (5-HIAA) output very much dependent on the position of the cannula. In males, it was also possible to demonstrate, for the first time, in vivo output of immunoreactive vasopressin (VP) most likely from the SCN. Interestingly, the output of VP was stimulated by local activation of probable 5-hydroxytryptamine (5-HT) terminals with 5-hydroxytryptophan (5-HTP), a precursor of 5-HT synthesis. Probing the hypothalamus of rats and rabbits revealed that the in vivo release of luteinizing hormone-releasing hormone (LHRH) (frequency and amplitude of the LHRH signal) can be altered by administration of estrogen to ovariectomized rats; in both species, progesterone stimulated the amplitude of the LHRH signal, but only when this steroid was infused in pulses—the physiological mode of circulating progesterone in the rat. Further, in male rabbits, pulses of progesterone did not stimulate LHRH release. Last, probing the anterior pituitary with the PPC revealed that a series of push-pull perfusions could be performed in the same animal under different experimental conditions for nearly 60 days of experimentation. It also resolved the apparent paradox that after castration, decreased instead of increased activity of the neural LHRH apparatus was noticed when the PPC was positioned in the hypothalamus. Moving the PPC to the anterior pituitary revealed that castration was accompanied by an increase in the amplitude and frequency of the LHRH signals arriving in the anterior pituitary of castrated male rats. This mode of operation of the LHRH pulse generator is clearly compatible with the mode of luteinizing hormone (LH) release in gonadectomized animals. Finally, based on these results, a hypothetical model of the operation of the LHRH pulse generator has been proposed.

INTRODUCTION

To realize the goal of understanding the functioning of the central nervous system (CNS) in awake, unrestrained animals requires noninvasive techniques capable of (1) discriminating between several neuromessengers such as neuropeptides, neurotransmitters, and other neuroactive substances that are produced in vivo by different neuronal systems; and (2) measurement of the neurosecretory release process of these substances that proceeds along milliseconds, minutes, hours, days, or months. Such a goal is far from our present technical capabilities, although some efforts in that direction currently are being vigorously pursued. In particular, in positron emission tomography (Phelps et al., 1986), we have a technique capable of identifying the site of actions of hormones and drugs in the brain of conscious humans and animals, and even of characterizing the binding properties of receptor molecules (Farde et al., 1986). However, even this technique is far from being able to measure the release process of endogenous neuroactive substances, information basic to understanding the communication capacities of neurons.

An alternative to this dilemma is the current use of invasive techniques that can accomplish some of the measurements mentioned above. The two tools currently available to neuroscientists to probe the brain of freely behaving animals are in vivo voltammetry (Adams, 1976) and in vivo push-pull perfusion (PPP) (Ramirez, 1985). In effect, both techniques should be viewed as complementary to each other in

[1] This work has been made possible in part by NSF Grant 85-09064 to Victor Ramirez.

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the pursuit of learning how a specific neuronal system functions in vivo under approximate physiological conditions. The interested reader can seek detailed information on this subject from three recently published monographs (Marsden, 1984; Knott and Myers, 1987; Levine and Ramirez, 1986).

The push-pull cannula (PPC) was introduced in 1980 by Levine and Ramirez (1980) to estimate the in vivo functions of the neural luteinizing hormone-releasing hormone (LHRH) apparatus in the rat. Since then, rapid progress has been made in this area. This technique has been extended to probe other neuronal systems (Chen et al., 1984, 1985) and the original method has been modified and improved (Dluzen and Ramirez, 1986a). The major technical improvements have been (1) a reduction in the size of the cannulae (from an outer cannula of #22 gauge to #24 gauge using a push cannula of #33 gauge dimensions), (2) a reduction in flow rate (from 20 μl/min to 7–10 μl/min), and (3) a coating of the outer cannula and stylette to insulate the chronically implanted device from the CNS extracellular fluid. The sum of all these changes has contributed to a marked improvement in the overall performance and success of the push-pull perfusion, and has reduced the technical pitfalls of this method considerably.

The intent of the present article is to reiterate the potential applications and usefulness of the PPC as a reliable CNS probe to estimate the output of neuroactive substances in freely moving animals. To this end, data gathered from two different neuronal systems, as probed from several distinct hypothalamic sites and from PPC placed into the pituitary, will be discussed as bona fide examples of the applications of the PPC in neuroendocrinology. We will begin our report with recent studies on the in vivo activity of the serotonergic system as probed at the level of the suprachiasmatic nucleus-preoptic area (SCN-POA) region of the brain from unrestrained, unanesthetized rats; continue with a detailed description of the neural LHRH apparatus as probed in the hypothalamus of rats and rabbits, as well as in the pituitary of male rats; and conclude with a summary of our current view of the in vivo functions of the neural LHRH apparatus.

THE PPP OF THE SCN

The first neuronal system that we examined with the PPC in freely behaving rats comprised the SCN, a fundamental biological neural structure involved in photoperiod time measurement (Pittendrigh, 1972). This work has been carried out in my laboratory by A. Ramirez, who has been capable of placing the tip of the PPC in, between, or near the two small bilateral oval masses of neurons lying dorsal to the optic chiasm and lateral to the third ventricle. As a background to our results, it seems appropriate to refer briefly to the organization of the SCN in rodents. According to Moore (1983), at least three components can be considered: a small rostral portion and two larger caudal areas—the ventrolateral and dorsomedial divisions, respectively. The rostral portion of the nucleus lacks retinohypothalamic projections, which are confined almost exclusively to the ventrolateral region. Of the many neurotransmitters and peptides localized immunocytochemically in the rat SCN (Moore, 1983), our studies are confined to a peptide, vasopressin, and a neurotransmitter, serotonin (5-HT). Vasopressin-containing neurons are located exclusively in the rostral and dorsomedial subdivisions, and they form extensive axonal plexuses in this area that are also interconnected with the ventrolateral subdivision of the nucleus. Two recent abstracts (Earnest and Sladek, 1985; Reppert and Gillette, 1985) indicate that these vasopressin-containing neurons can release this peptide in vitro. More importantly, they undergo robust changes in the amount of vasopressin messenger RNA in vivo, with high levels during light at 1000 h and low levels during dark at 2200 h, as visualized and quantified by in situ hybridization techniques. Interestingly, these photoperiodic changes in hybridizable vasopressin messenger RNA were detected in the SCN but not in the paraventricular or supraoptic nuclei (Uhl and Reppert, 1986). These selective changes in the SCN suggest that this structure is responsible for the circadian fluctuations in vasopressin concentrations in the cerebrospinal fluid (CSF) previously reported, and that are known to be independent of osmotic regulation of plasma vasopressin (Reppert et al., 1986, personal communication). However, the precise role of vasopressin-containing neurons in the SCN is completely unknown.

There is general concensus that the SCN has high levels of 5-HT that are contained in nerve terminals projecting from cell bodies located in the midbrain raphe complex (Azmitia and Segal, 1978). Recently, by using differential pulse voltammetry, it was shown that these terminals undergo circadian variation during the rest-activity cycle of freely behaving rats (Faradji et al., 1983). According to Groos et al. (1983), microphoretically applied 5-HT inhibits the

firing in 71% of cells of the SCN. Although there is good evidence that this transmitter may be related to the control of sleep-wake cycles (Puizillout et al., 1979; Cespuglio et al., 1981) and reproductive functions (Turek and Campbell, 1979; Kordon and Ramirez, 1975; Gallo, 1980; Turek et al., 1984), its precise function(s) in controlling biological rhythm is far from clear.

Our present results, in cycling female rats (Ramirez and Meyer, 1986), indicate clear disruption of estrous cyclicity but not major SCN lesions after PPC implantation in this area, since all animals continually show diestral but not estral vaginal smears (Brown-Grant and Raisman, 1977). In these animals, two major patterns of 5-hydroxy indolacetic acid (5-HIAA) output, herein considered an index of increase in serotonergic activity, can be discriminated as a function of the position of the PPC. The first is a stationary but oscillatory pattern of 5-HIAA release characterized by a rather constant and relatively low release rate of 5-HIAA (5—20 pg/min). The second pattern is an ascending rise characterized by a 2- to 3-fold increase in the output of 5-HIAA during the afternoon of the photoperiod, and by apparent maximal levels reached around 1900 h, the onset of the subjective night for these animals. In animals exhibiting the first pattern of output, the PPC perfused mainly the retrochiasmatic portion of the SCN, whereas in animals exhibiting the second pattern, the rostral-preoptic portion of the nucleus underwent perfusion. Figure 1 (upper inset) shows, schematically, the main position of the cannulae as determined histologically in the SCN in these two examples (lower inset), and the clear differences in the serotonergic activity as measured from these two sites by high-performance liquid chromatography-electrochemical detector (HPLC-ECD) coupled to the PPP procedure as previously described (Chen et al., 1984; 1985).

To circumvent, in females, the apparently unavoidable effect of estrous-cycle disruption after lesion of this area (Wiegand et al., 1978), Ramirez recently determined the activity of 5-HT raphe neurons projecting to the SCN of freely behaving male rats by positioning the tip of the PPC in, between, or close to the SCN. In addition, immunoreactive arginine-vasopressin (A-VP) was measured in the remainder of the perfusate samples by a specific radioimmunoassay using an antiserum, generously supplied by Dr. P. M. Conn, with a sensitivity of 10 pg per tube at 90% binding. From the data available (Ramirez and Ramirez, 1986), it seems that PPCs placed near the rostral portion of the SCN detect the ascending oscillatory pattern of 5-HIAA seen in females, since the output of 5-HIAA clearly increases during the afternoon of the photoperiod and reaches maximal levels close to 1800 h. However, in this case, contrary to what was observed in females, the 5-HIAA output declined and reached basal levels thereafter as shown for rat #27 in Figure 2. In this same animal, detectable levels of immunoreactive-like vasopressin molecules were measured in 9 out of 29 samples. This demonstrates for the first time the endogenous release of this neuropeptide most likely from the SCN of freely behaving male rats. A second perfusion in this same animal revealed levels of 5-HIAA (between 20 to 50 pg/min) and vasopressin (about 2 pg/min) similar to those observed two days earlier (Fig. 3). In this rat, infusion of 10^{-4} M 5-hydroxytryptophan (5-HTP), a precursor of 5-HT, through the push side of the PPC evoked a robust in vivo biochemical response in 5-HIAA and A-VP outputs, the former preceeding the latter by 20 min. Interestingly, 10^{-4} M iproniazid, an inhibitor of monoamine oxidase activity, markedly reduced the output of 5-HIAA. Unfortunately, the levels of A-VP were already undetectable.

In another animal (rat #28, Fig. 4) with detectable levels of A-VP and 5-HIAA, but with the tip of the PPC in a more rostral location with respect to the anterior limit of the SCN than in rat #27, infusion of 10^{-4} M 5-HTP also elicited a clear increase in the 5-HIAA output and a 4-fold change in A-VP release; however, the peak response of A-VP occurred 60 min after the 5-HIAA peak in this case. These preliminary but robust responses suggest an interesting in vivo relationship between these neuronal systems. Unmistakably, the position of the PPC plays an important role in the outcome of these descriptive results, since placing the PPC several hundred μm off the target site—in this case, the SCN—can result in a different pattern of release, as mentioned above, or absence of detectable levels of 5-HIAA, as shown in Figure 5, rat #29. In rat #29, the PPC crossed the base of the brain to lie in the optic chiasm at the level of the SCN, as shown in the inset. Interestingly, in this case, detectable levels of vasopressin were also recorded, which suggests a tracking down of this neuropeptide along the cannula from the SCN into the area of perfusion.

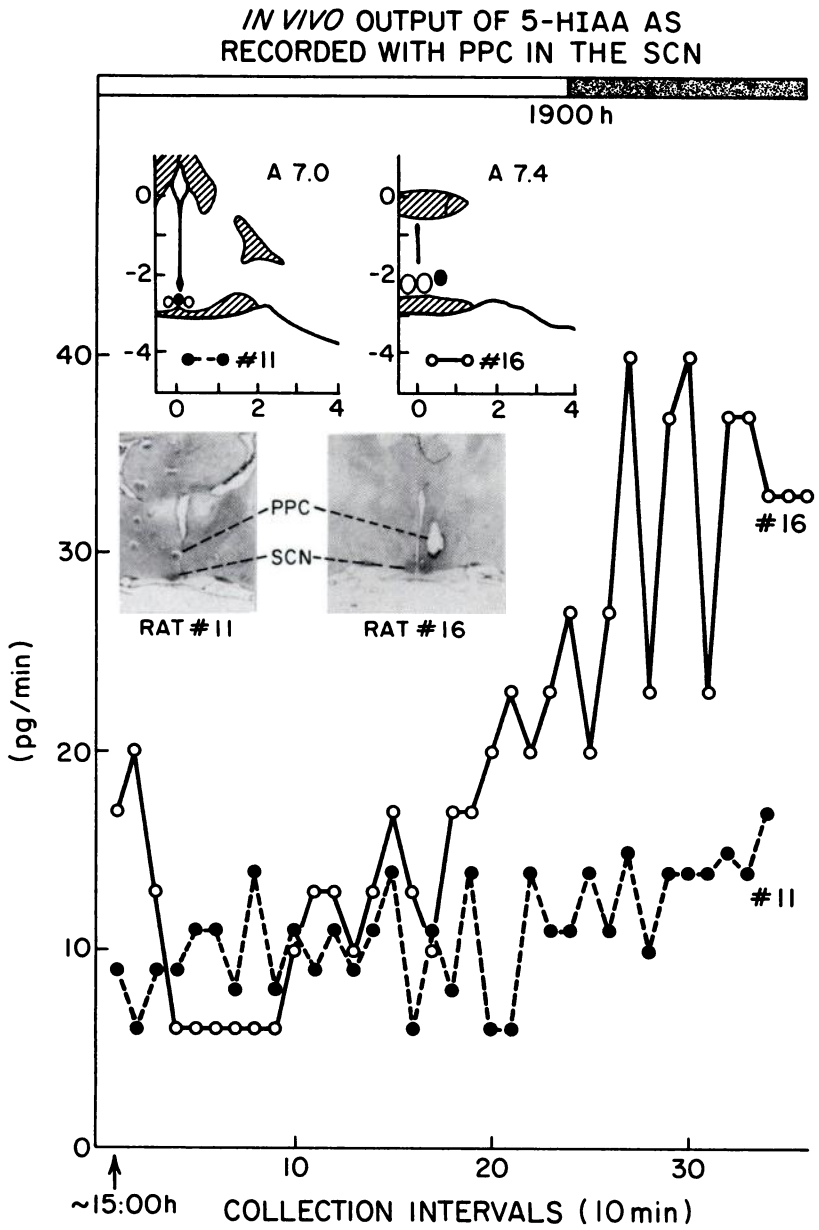

FIG. 1. First description of differential in vivo changes in 5-HIAA levels in female rats as probed with a push-pull cannula (*PPC*) placed close to or between the suprachiasmatic nuclei (*SCN*) at A 7.4 and 7.0, respectively (DeGroot's Rat Atlas). When the cannulae tips were positioned close to the rostral portion of the left SCN of a female rat, a clear ascending oscillatory pattern in 5-HIAA output was revealed during the push-pull perfusion (PPP) as measured in 20-μl samples of artificial medium perfusing that area at 7 μl/min (Rat #16). In contrast, Rat #11, bearing a PPC that perfused both SCN, but in a more caudal or retrochiasmatic plane, no changes in the mean release rate of 5-HIAA were noticed, although the release was clearly oscillatory. Histological section of the hypothalamus of Rats #16 and #11 at the level of the preoptic area (*POA*) depicting the position of the PPC with respect to the SCN is shown in the *lower inset* (X10). Fifty-μm frozen sections were stained with toluidine blue. Notice the normal preservation of the SCN and the close position of the cannulae laterodorsal and in between the SCN in Rats #16 and #11, respectively.

Collectively, these in vivo data reinforce the presumption of functional selectivity in the innervation of the SCN by serotonergic afferent pathways from the midbrain raphe neurons and illustrate some biochemical functions of these serotonergic terminals. The initial critical demonstration that the PPC technique, as worked out by our group, can be applied successfully to examine in vivo the biological functions of the SCN—a crucial component of the circadian system in mammals—has been achieved. Future work will be required to examine specific questions with broader implications.

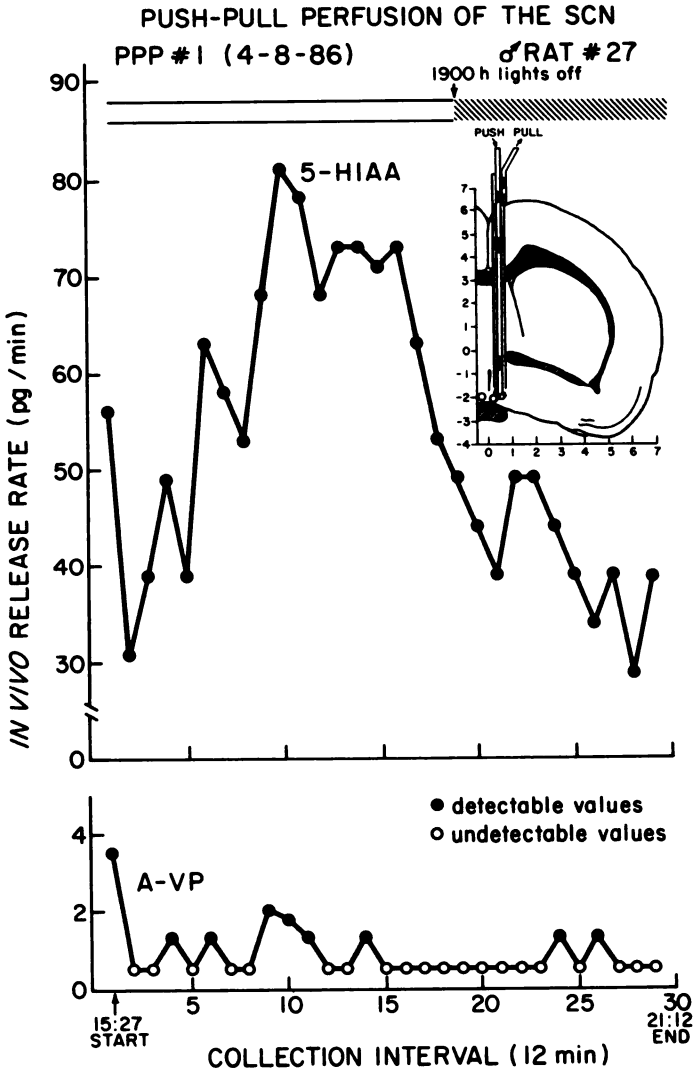

FIG. 2. In vivo profile of *5-HIAA* output as estimated with a *PPC* perfusing the rostral portion of SCN in a male rat. In contrast to female Rat #16, the mean release rate of 5-HIAA rose to the highest level before the subjective night for this animal, and it declined thereafter to levels similar to those found at the beginning of the *PPP*. In this rat, detectable levels of immunoreactive arginine-vasopressin molecules (*A-VP*) were also measured in 9 out of 29 samples with evidence of episodic discharges. A-VP was measured by RIA in approximately 50 μl of perfusates that were immediately acidified with HCl to 0.1 N final concentration. (Abbreviations defined in *legend to Fig. 1.*)

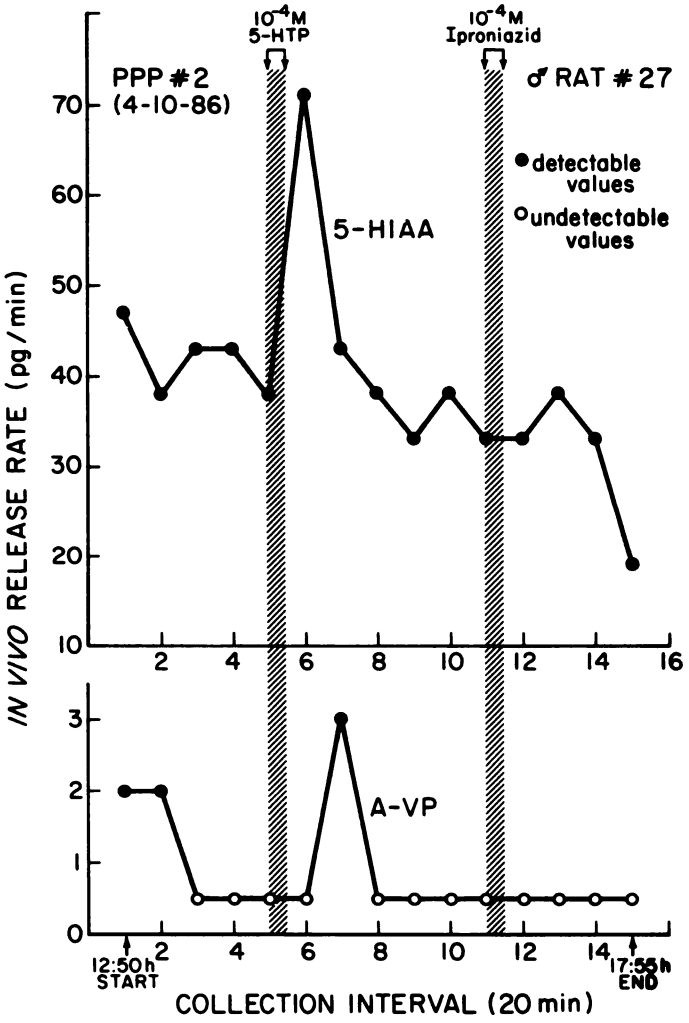

FIG. 3. Changes in *5-HIAA* and arginine-vasopressin molecules (*A-VP*) outputs in Rat #27 after local pharmacological manipulation of *5-HT* metabolism at the perfusion site shown in the *inset* of *Figure 2*. In this rat, two days after determining the spontaneous 5-HIAA and A-VP outputs, 5-HTP (10^{-4} M) and iproniazid (10^{-4} M), a precursor of 5-HT synthesis and an inhibitor of monoamine oxidase (MAO), respectively, were infused through the push side of the PPC for 10 min, as indicated in the figure. 5-HTP elicited a rather rapid response, presumably of 5-HT terminals since a peak in 5-HIAA output was observed within the first 20 min of collection. Interestingly, A-VP from undetectable levels rose to 3 pg/min about 40 min after the infusion of 5-HTP to become undetectable again, thereafter. Iproniazid clearly decreased the output of 5-HIAA, and apparently did not increase A-VP levels.

The Luteinizing Hormone-Releasing Hormone (LHRH) Pulse Generator of the Rat: Role of Estrogen and Progesterone

To continue with our objectives, we will now examine some recent new data obtained with PPC placed into the hypothalamus of rabbits and rats. Inasmuch as we have described in several recent reviews (Ramirez, 1985; Ramirez et al., 1987) and articles (Dluzen and Ramirez, 1985, 1986b; Ramirez et al., 1986) the in vivo function of the neural LHRH apparatus in the rat and the rabbit—a spontaneous and a reflex ovulator, respectively—herein we will consider only two main issues: first, the role of progesterone and estrogen in the control of in vivo LHRH release; and second, our recent experimental attempts to resolve the paradox that after castration,

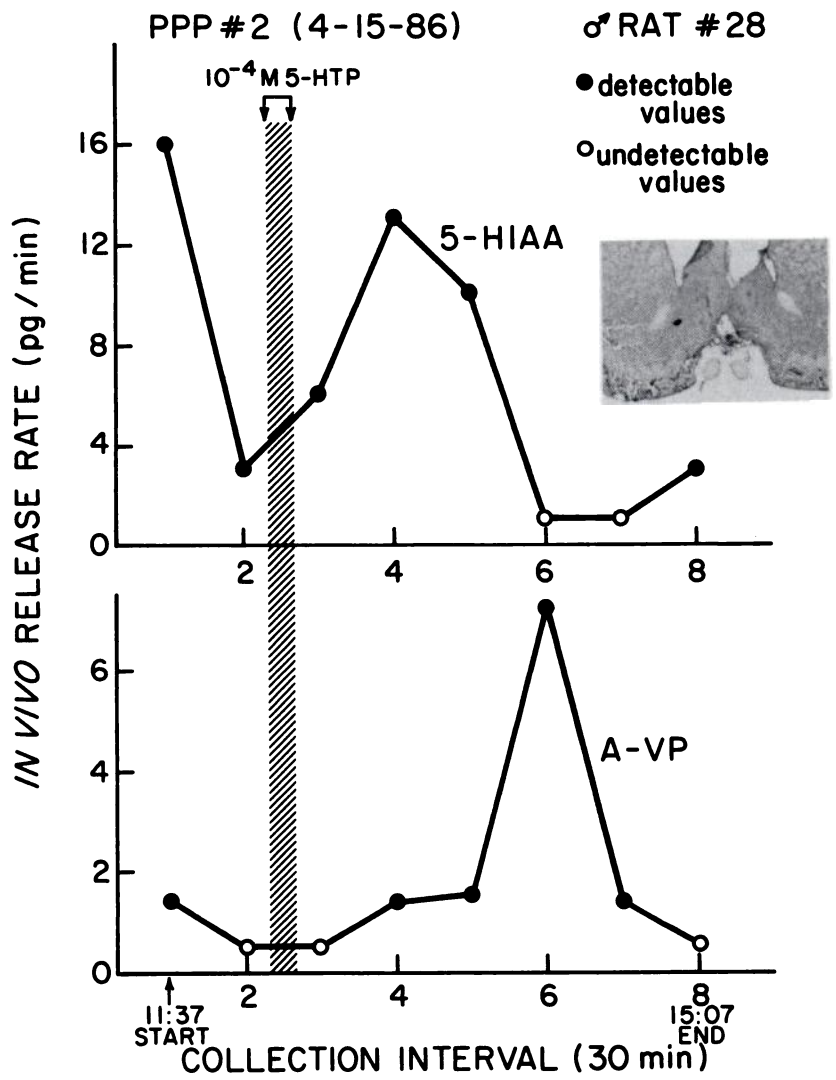

FIG. 4. Another example of the effect of local perfusion of 10^{-4} M 5-HTP on the in vivo activity of 5-HT and vasopressin terminals. In this case, the PPC lies in a more rostral position relative to that in Rat #27, as shown in the inset (X10), and the A-VP peak occurred 60 min after the 5-HIAA peak.

the activity of the LHRH pulse generator as probed in the hypothalamus apparently decreases instead of increases. This occurred in spite of the high levels of plasma luteinizing hormone (LH) released at a rapid rate from the pituitary of castrated animals, a well-documented finding (Ellis and Desjardins, 1984a,b; Kalra, 1986).

To begin, perhaps it will be useful to view the LHRH pulse generator as part of a neural LHRH apparatus that also comprises an LHRH surge generator. Although pulse and surge generators may reside in the same pool or field of LHRH neurons, there is evidence, particularly in rodents (Barraclough and Gorski, 1961; Gorski and Barraclough, 1962), that the LHRH surge generator ("phasic center") may reside within the POA-SCN structure of the rostral part of the hypothalamus, whereas the LHRH pulse generator as such may be anatomically restricted to the medial basal hypothalamus (MBH; "tonic center"), mainly the ventromedial hypothalamic (VMH)-arcuate region (for review, see Barraclough, 1973). Morphological and functional circumstantial evidence tends to support this concept, although a convincing demonstration has not yet been provided. For instance, LHRH cell bodies are abundant in the rostral hypothalamus, particularly the POA, and organum vasculosum of lamina terminalis (OVLT) (Clarke and Joseph, 1982; Witkin et al., 1982; Wray and Hoffman,

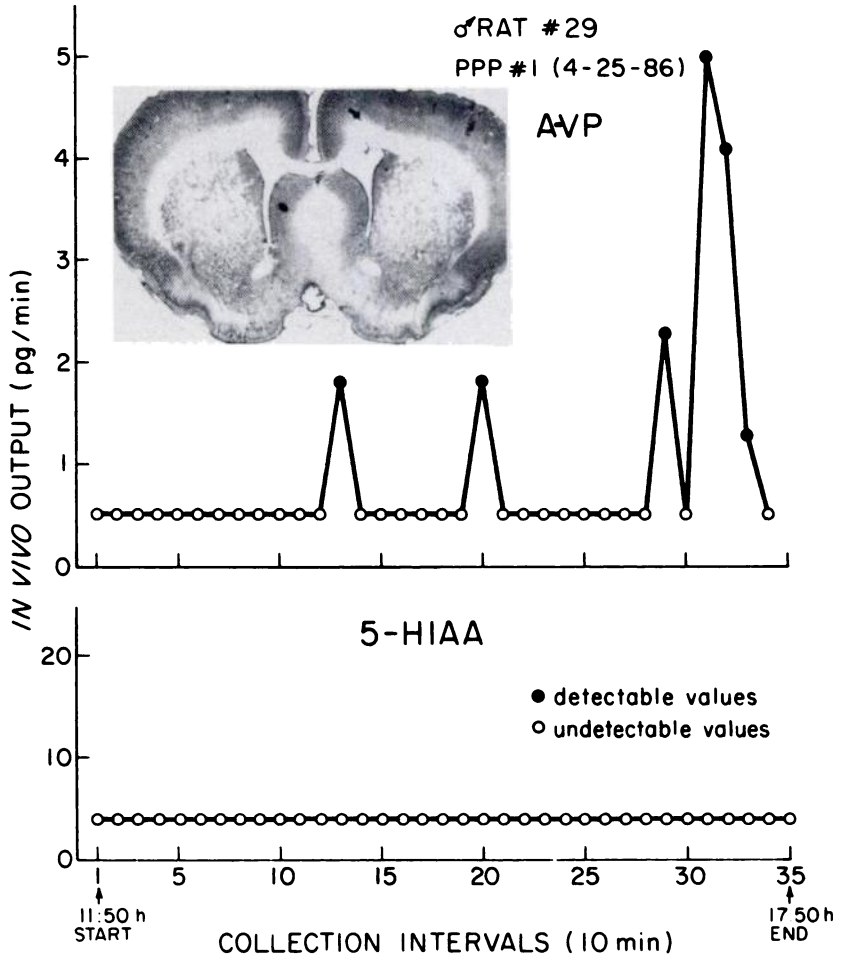

FIG. 5. This rat illustrates an example of a PPC placement that crosses at the plane of the SCN but lies in the optic chiasm (inset X 10). Notice the absence of detectable *5-HIAA* levels in the perfusates, although *A-VP* levels were measured in several samples. (Abbreviations defined in *legend to Figure 1.*)

1986), with projections to the MBH and SCN as well as to other hypothalamic structures. On the other hand, a scarce number of neurons are visualized in the MBH, although some authors question their existence (King and Gerall, 1976; Terasawa and Gary, 1983). An example of an LHRH neuronal field close to the OVLT in the rat is illustrated in Figure 6, as shown by J. Gitzen in my laboratory. From very early experiments, it was clear that complete surgical MBH islands in the rat (Halász and Pupp, 1965; Blake et al., 1973) were capable of supporting the so-called normal tonic levels of gonadotropins as well as the pulsatile release of LH in gonadectomized animals (Krey et al., 1975; Blake and Sawyer, 1974). In contrast, in intact rats bearing such islands or anterior but not posterior MBH deafferentations, complete cessation of the ovulatory surge of LH occurs (Halász and Gorski, 1967; Köves and Halász, 1970).

The in vitro activity of the neural LHRH apparatus from superfused hypothalamic units (HU) comprising the POA-AHA-SCN-MBH regions and derived from cycling female rats behaved markedly differently, as a function of the estrous cycle, from hypothalamic fragments containing only the MBH, as clearly shown by D. Meyer in this meeting (Meyer, 1986). Moreover, in vitro pulsatile LHRH release from HU is very much dependent on the time interval after ovariectomy: initially there is a drop, as compared to diestrus controls, and an increase thereafter (Dluzen and Ramirez, 1986c). Previously, we published that the entire hypothalamic unit expresses changes in LHRH release as a function of the estrous cycle (Kim and Ramirez, 1986). Overall, these data indicate that the pulse generator resides within the HU and that it can be markedly altered by the gonadal milieu of the animal.

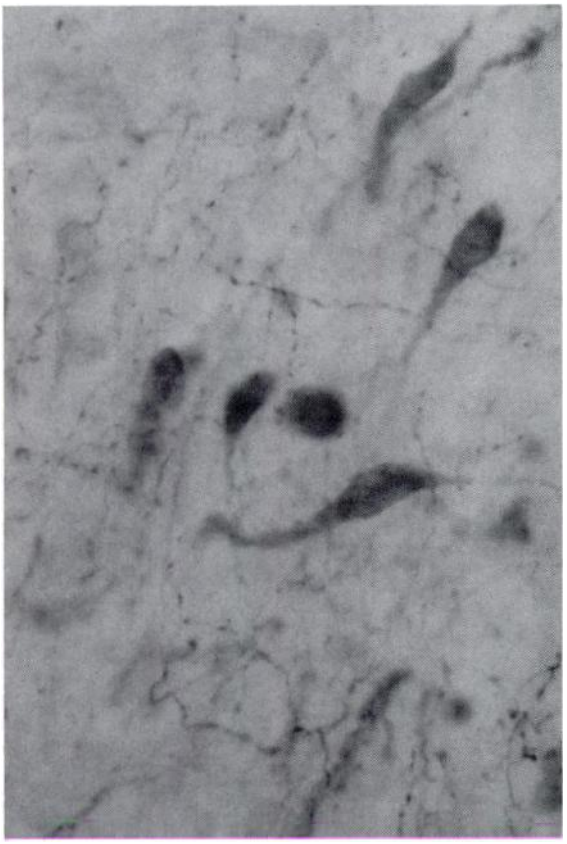

FIG. 6. Examples of a network of LHRH neurons at the level of the organum vasculosum of lamina terminalis (OVLT) in the female rat as recognized by an anti-LHRH antiserum (Chen-Ramirez Antibody) at 8000:1 dilution. ×65.

In our first description of the in vivo functional changes in the activity of the neural LHRH apparatus during the rat estrous cycle (Levine and Ramirez, 1982), we noticed the appearance, in some animals, of two types of LHRH output: (1) a pulse-dominated type consisting of a prominent baseline of pulses whose amplitude changed only moderately during periods of LHRH output, and (2) a peak-dominated mode characterized by abrupt, multiple increases in amplitude over nondetectable or low amplitude basal pulses. Admittedly highly speculative, these two modes of operation of the neural LHRH apparatus may reflect the activity of the LHRH pulse generator and the LHRH surge generator, respectively. The LHRH apparatus may be controlled by different hormonal mechanisms and even perhaps reside in different pools of LHRH neurons as seems to be the case in the rat hypothalamus.

Recent work from Dr. Knobil's laboratory (Wilson et al., 1984; Kesner et al., 1986) demonstrated remarkable electrical correlates between multiunit activity (MUA) recorded from the arcuate-ventromedial portion of the hypothalamus of ovariectomized (OVX) rhesus monkeys and spontaneous rises in blood LH levels. This tight correlation suggests that in ovariectomized animals, the now classical rhythmic pulsatile release of LH functions in tandem with electrical volleys corresponding to the LHRH pulse generator. Similar work in the rat (Kawakami et al., 1982) and sheep (Thiéry and Pelletier, 1981) has shown close correlates between hypothalamic electrical volleys and LH pulses in castrated animals. Previous work from our group at Urbana (Levine et al., 1982) and that of Drs. Clarke and Cummins' group in Australia (Clarke and Cummins, 1982), using simultaneous measurements of LH and LHRH in unanesthetized, OVX sheep, led to similar conclusions, although there were cases of ineffective LHRH pulses, as there were of electrical volleys. Overall, these findings indicate that the LHRH pulse generator is the main structure responsible for controlling LH pulses in ovariectomized animals. We shall see later that after gonadectomy, the LHRH pulse generator of the male rat appears to function at its maximal at intervals of about 40 min.

As mentioned above and as previously reported, gonadectomy markedly reduces the activity of the neural LHRH apparatus as determined by PPC probing the hypothalamus of male (Dluzen and Ramirez, 1985) and female rats (Levine and Ramirez, 1982). An example of an LHRH release-rate profile of an OVX rat is shown in Figure 7, rat #13. Under the artificial condition of ovariectomy, it is possible to examine the role of estrogen and estrogen plus progesterone on the activity of the neural LHRH apparatus. It should be borne in mind that none of these steroid-replacement therapies used so far reproduces the normal secretory events of these steroids during the rat estrous cycle. An example of one such artificially treated rat is illustrated in Figure 7, rat #5. In this case, an OVX animal received two subcutaneous injections of estradiol benzoate (EB, 25 μg/rat) at 48 and 24 h prior to PPP. The animal was lightly anesthetized with ether, and a blood sample was obtained from the jugular vein just before (1000 h) and at the completion (1800 h) of the PPP. The figure illustrates the low levels of serum LH-RP2 in the morning relative to the high levels in the evening, a known effect of this type of estrogen treatment (Ramirez and Sawyer, 1974). With regard to our LHRH determinations, there were two salient changes that occurred in this OVX + E-treated rat. First, the

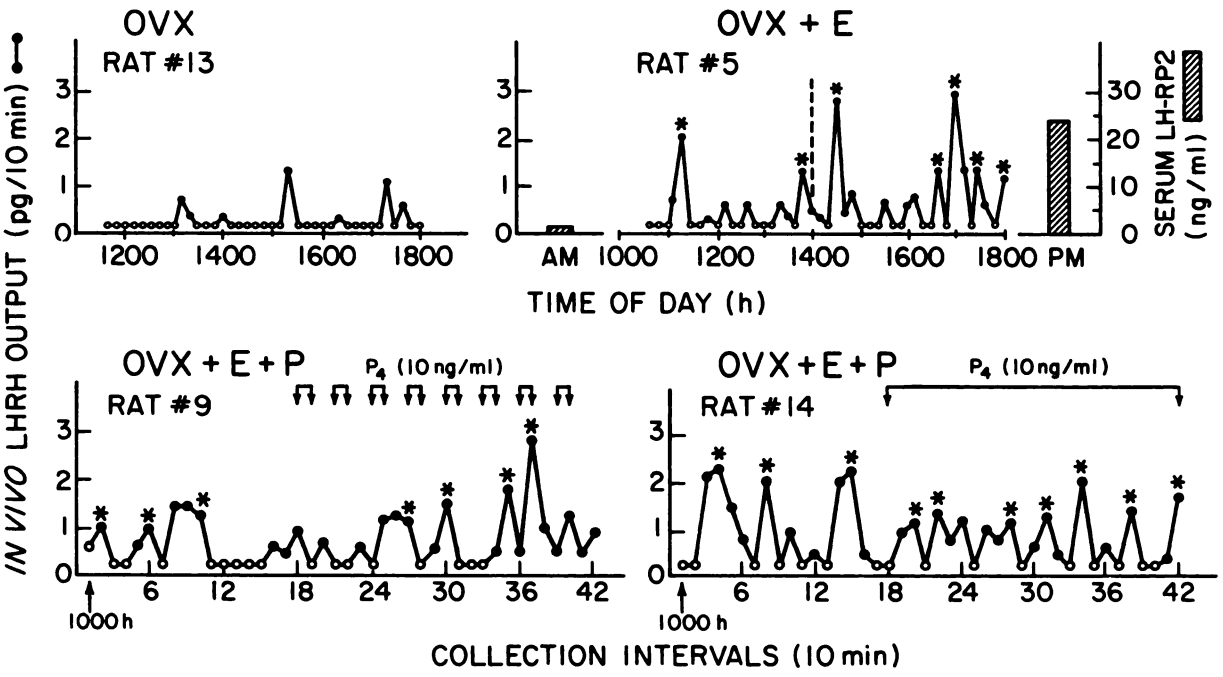

FIG. 7. Composite diagram depicting in vivo characteristics of the LHRH pulse generator as probed by PPC placed within the ventromedial-arcuate region of the hypothalamus of an ovariectomized (*OVX*) rat (*#13*), an OVX rat primed with estrogen (*#5*), an OVX rat primed with estrogen (*E*) and infused locally with pulses of progesterone (P_4)(*#9*) or an OVX rat primed with estrogen and infused continuously with an identical concentration of the steroid (*#14*). Notice the low activity of the LHRH pulse generator in the OVX rat (about 7 days), which is, however, clearly stimulated after 2 days of estrogen priming. In this condition, the frequency of the LHRH signal was low before the critical period (1000–1400 h), but it increased at least 2-fold during the critical period (1410–1800 h). In OVX + E rats, progesterone infusion in an intermittent mode increased only the amplitude of the LHRH signal and not its frequency. In contrast, progesterone continuously infused did not change the spontaneous activity of the LHRH pulse generator. For complete details see *text* and *Table 1*.

overall activity of the neural LHRH apparatus was markedly increased, as determined by LHRH measurements, in the perfusates from PPC perfusing the arcuate-ventromedial region as compared to those in oil-treated, OVX animals with PPC located similarly (Fig. 7, rat #13). In these OVX animals, in spite of these apparently low LHRH levels, high levels of plasma LH were detected (Levine and Ramirez, 1982). In addition to this increase in amplitude of the LHRH signal in OVX + E rats, the second salient change was a clear shift in the frequency of the signal between the pre-critical hours (1000–1400 h) and the post-critical hours (1410–1800 h), as identified by the pulsar program of Merriam and Wacther (1982) and adopted to an IBM-PC by J. Gitzen in our laboratory (Gitzen and Ramirez, 1986). While only two pulses were identified during the first four hours of PPP, five pulses were obtained during the succeeding four hours of push-pull perfusion. Lastly, when progesterone was administered in pulses (Fig. 7, rat #9)—but not when infused continuously (Fig. 7, rat #14)—through the push side of the PPC into the arcuate-ventromedial area of the hypothalamus of estrogen-primed OVX rats, clear changes were observed in the amplitude of the LHRH signal, without changes in frequency. Table 1 summarizes these data as taken from three published articles (Levine and Ramirez, 1982; Ramirez et al., 1985; Dluzen and Ramirez, 1986b). The data clearly demonstrate an active LHRH pulse generator in OVX rats treated with estrogen, and that under this condition of estrogen-priming, the rate at which the LHRH pulse generator functions between the pre- vs. post-critical period is remarkably different. This suggests that perhaps the photoperiod most likely working through the SCN underlies the rate function of this oscillator. In addition, progesterone appears capable only of increasing the amplitude of the LHRH signal, which fits well with previously reported data that indicated an increase in MBH-LHRH content within 2 h after progesterone treatment (Simpkins et al., 1980), and in vitro LHRH release rates of progesterone-treated OVX + E-treated immature rats (Ramirez et al., 1980).

TABLE 1. Changes in frequency or amplitude of the LHRH signal in ovariectomized (OVX) rats primed with estrogen (E) and in OVX-E rats treated with progesterone (P)

Condition	n	Activity of the LHRH pulse generator		
		Mean Release (pg/10 min)	Period (min)	Amplitude (pg)
A. OVX	7	<0.5	ND	ND
B. OVX + E	5			
1. Pre-critical period (1000–1400 h)		0.5 ± .05	79 ± 19 ↓ ↓	0.95 ± .08
2. Post-critical period (1400–1800 h)		0.63 ± .08	46 ± 5	0.90 ± .07
C. OV + E + P	5			
1. Control period		0.72 ± .08	48 ± 6	1.14 ± .18 ↓ ↓
2. P-treatment period		1.12 ± .23	42 ± 5	1.99 ± .53

From our in vivo and in vitro work, it was evident that progesterone was effective in stimulating the release of LHRH only when infused in an intermittent mode, since continuous infusion of the steroid at similar concentrations was either ineffective or had a tendency to inhibit the spontaneous release of this neuropeptide (for review, see Ramirez et al., 1985). This very robust difference between the two modes of progesterone administration was unexpected and was received with a certain degree of skepticism by some of our colleagues who referee our papers on the basis of the physiological significance of our results. Therefore, we decided to test the hypothesis of whether progesterone fluctuates in an episodic manner during the estrous cycle in the blood of unanesthetized rats. Figure 8 shows blood progesterone levels in three individual rats during three different phases of the estrous cycle (D-1, E and early proestrous). These rats received jugular cannulae at least 3–4 h before bleeding, and samples were collected at 10-min intervals for a 6-h period. Our results indicate that plasma progesterone does indeed fluctuate in an episodic manner throughout the rat estrous cycle, with a mean frequency of approximately 1 pulse/50-60 min. Another interesting fact from this work was that maximal levels of progesterone were obtained in the blood of D-1 animals (30 ng/ml) and minimal levels in early proestrous (approximately 5 ng/ml). That previous investigators (Kalra and Kalra, 1977; Barraclough, 1973; Kaneko et al., 1986) have failed to demonstrate such episodic changes in plasma progesterone can be explained by the fact that less frequent bleeding intervals have been used in the rat, or that animals have been killed by decapitation. Indeed, this is true, because if the same data that reveal episodic fluctuations in plasma progesterone when collected at 10-min intervals are plotted at one-h intervals, no such phenomenon is detected (Fig. 9). On the contrary, a rather linear, plasma progesterone concentration is observed. The completed study of episodic fluctuation of progesterone during the rat estrous cycle has been presented to this meeting by Park and Ramirez (1986); this study confirms in rats what was previously demonstrated in monkeys (Healy et al., 1984), cattle (Schallenberger et al., 1985; Hixon et al., 1983), hamsters (Ridley and Greenwald, 1975), and humans (Crowley et al., 1985).

THE LHRH PULSE GENERATOR IN THE RABBIT: ROLE OF PROGESTERONE

These findings in the rat, a spontaneous ovulator, led us to investigate the role of progesterone in the activity of the neural LHRH apparatus of a reflex ovulator, the New Zealand White rabbit. Young, virgin, intact, female rabbits and young, mature males were isolated at least 1–2 wk in single cages in our animal quarters. These animals were implanted with PPC aiming to the tuberal portion of the hypothalamus. After at least one wk post-surgery, the rabbits were perfused for several hours in a cage that allowed the animals to move freely, eat, drink, and rest quietly on the ventral side over the floor of

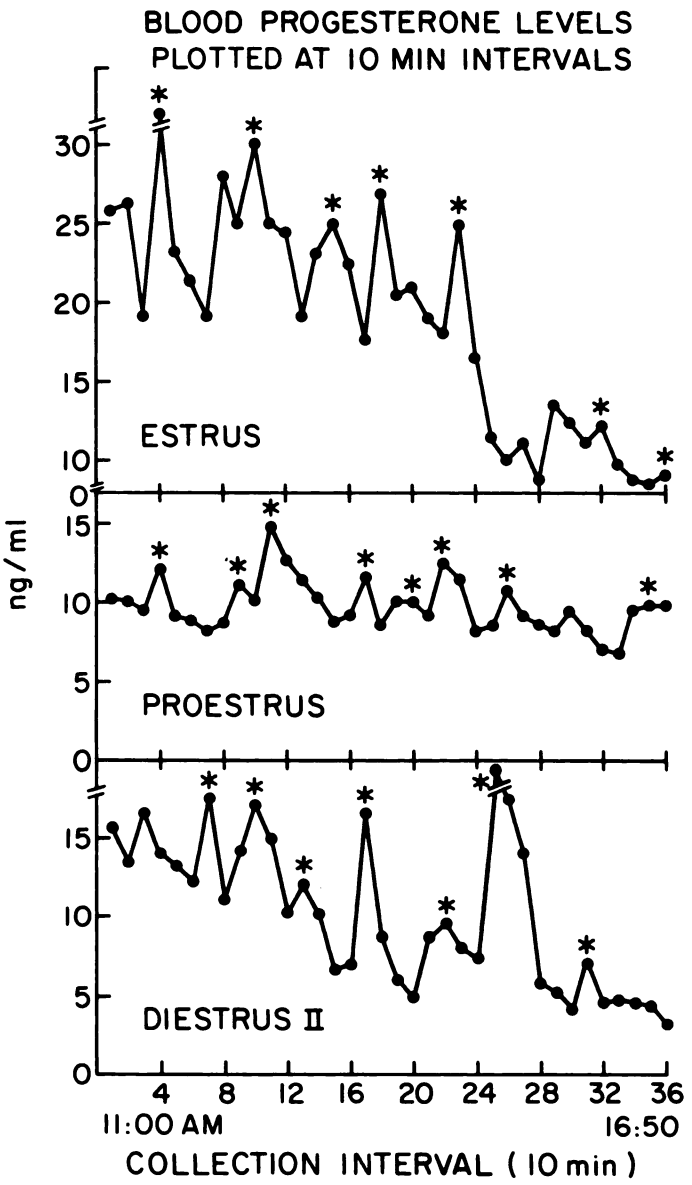

FIG. 8. Blood progesterone levels measured by RIA in samples withdrawn at 10-min intervals for 6 h in three rats in three different phases of the estrous cycle. Distinct pulses of progesterone were identified by the IBM-PC pulsar program (Gitzen and Ramirez, 1986) in the three animals with the lowest levels of progesterone measured during early proestrus.

the cage. For details of this procedure, see our recent paper describing the spontaneous changes of the LHRH pulse generator in intact female rabbits (Ramirez et al., 1986). Under these conditions, we examined whether progesterone administered in pulses would be as effective in stimulating the neural LHRH apparatus as in the female rat. Moreover, to test for stimulus-parameter specificity and sex specificity, progesterone was infused in an intermittent or a continuous mode in females or in pulses in male rabbits. As shown in the examples of Figure 10, progesterone administered in pulses (10 ng/ml, 10 min on-30 min off) into the hypothalamus of female rabbits was extremely powerful in stimulating the release of LHRH (Female rabbit #1). The continuous mode of progesterone infusion (Female rabbit #24) lacked the capacity to stimulate significantly the neural LHRH apparatus, thereby demonstrating results similar to those obtained in the rat. Moreover, this phenomenon appeared to be sex-specific, since pulsatile progesterone administration to the male rabbit (Male rabbit #27) was clearly ineffective in altering LHRH release. A complete, detailed study was presented to this meeting by W. Lin and Ramirez (1986), and a full paper describing these results is in preparation.

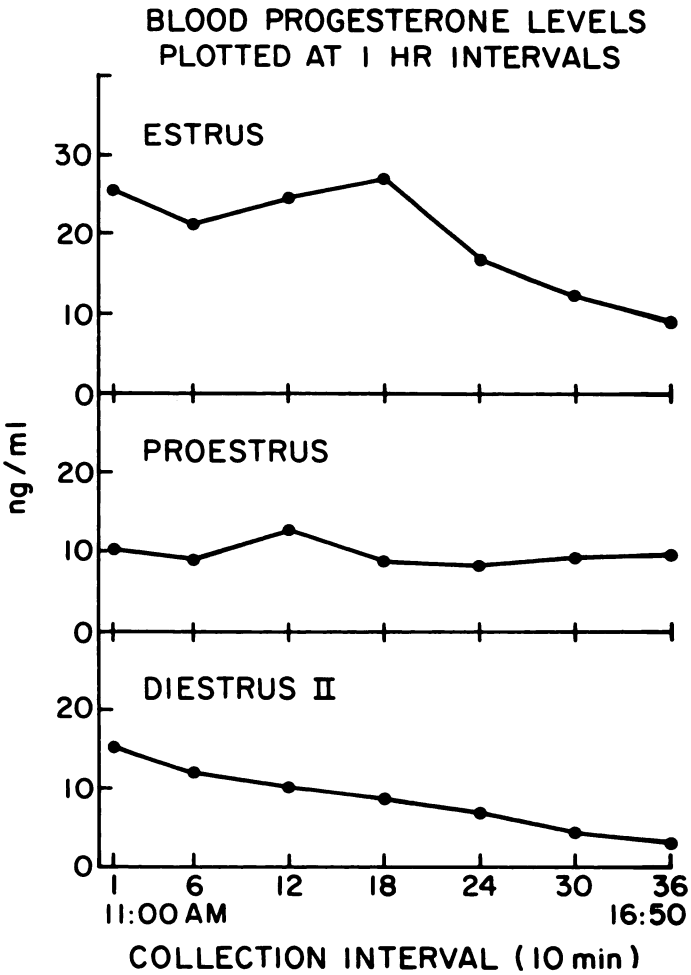

FIG. 9. Identical data to those of *Figure 8*, but plotted at 1-h intervals, reveal the importance of frequent sampling to demonstrate pulsatile changes of progesterone, since use of this time computation reveals clearly the linear concentration levels of progesterone.

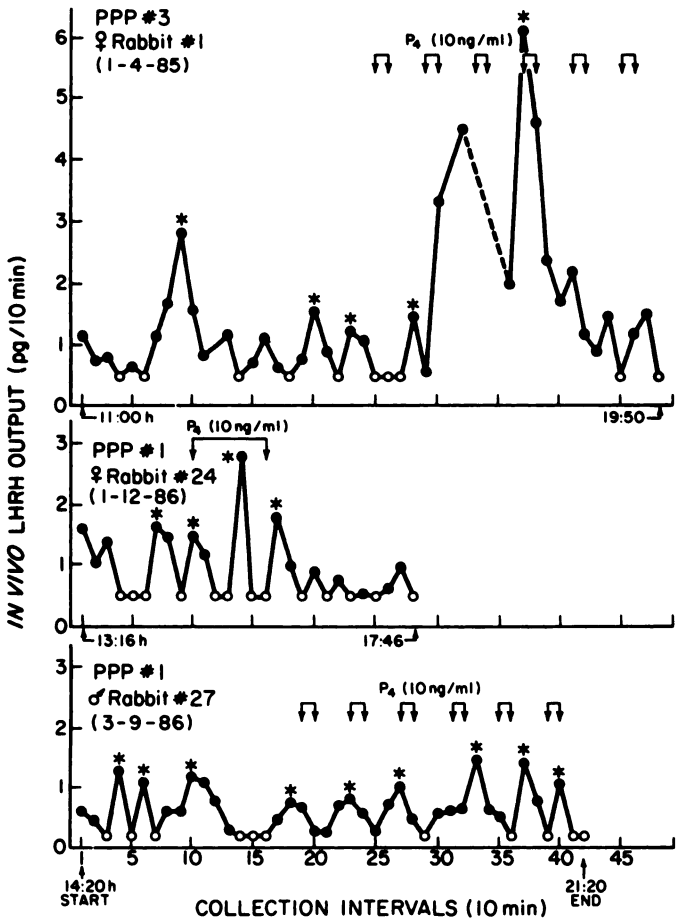

FIG. 10. Effect of pulsatile infusion of progesterone (P_4) on the activity of the *LHRH* pulse generator of a young virgin intact female rabbit as compared to a young adult male rabbit. A clear sex difference was revealed in the response to pulses of P_4 infused directly into the hypothalamus. In the female, a marked increase in the amplitude of the LHRH signal occurred, whereas in the male no changes were detected. Besides this sex-specificity for progesterone action there was also a stimulus-parameter specificity in the response of the LHRH pulse generator in the female, since continuous infusion of the steroid at the same concentration and total amount of that used in the upper figure was ineffective in Rabbit #24.

In summary, in the female rabbit, a reflex ovulator, and in the female rat, a spontaneous ovulator, pulses of progesterone are highly effective in eliciting increases in the amplitude of the LHRH signal, but apparently are not effective in increasing the frequency of the LHRH discharges. In contrast, at least in the rat, estrogen also appeared to control somehow the rate (frequency) at which the LHRH pulse generator operates (Dluzen and Ramirez, 1986b). The fact that male rabbits do not respond to pulses of progesterone is an argument in favor of hypothalamic sex differentiation of the mechanism that governs the neural LHRH apparatus in this species. Further studies will be required to clarify these initial findings.

THE PPP OF THE ANTERIOR PITUITARY

Lastly, we will discuss our recent data on push-pull perfusion of the anterior pituitary as a means for resolving the apparent paradox that in castrated animals, low levels of LHRH were measured with PPC positioned in the hypothalamus. In these PPP studies of the anterior pituitary, we simultaneously measured the output of neurotransmitters with the aid of an HPLC-EC detector by taking 20 μl of perfusate for the HPLC-EC and using the remainder, (approximately 180 μl) to determine LHRH output at 10-min intervals. For details, see legends to Figures 11 and 12. Initially, we compared the activity of the neural

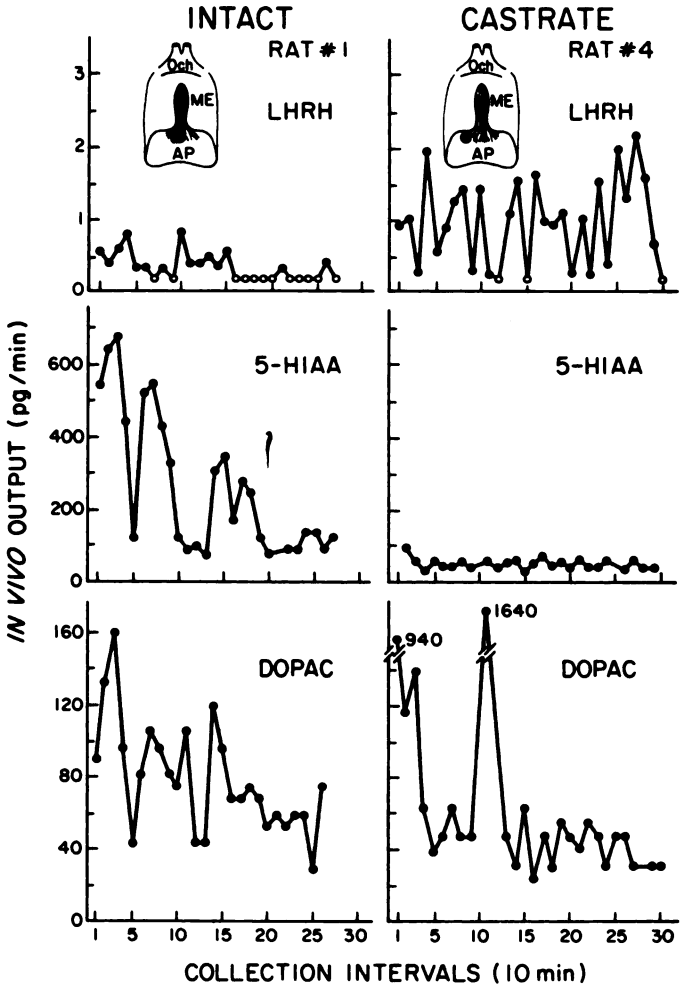

FIG. 11. Two examples demonstrating, in an *intact male rat* (*#1*) and a *long-term castrated rat* (*#4*, over 12-mo-old), dramatic differences in the in vivo input of LHRH signals reaching the anterior pituitary, as estimated with push-pull perfusion (*PPP*) of this gland at a site indicated in the *inset*. (In this case, the perfusion medium contained bacitracin.) Twenty μl of perfusates were used to measure levels of 5-HIAA and DOPAC, the two principal metabolites of 5-HT and dopamine (DA) neurons, respectively. Notice that these metabolites reached the anterior pituitary gland and that the levels of 5-HIAA but not of DOPAC dropped to very low values in the castrated rat.

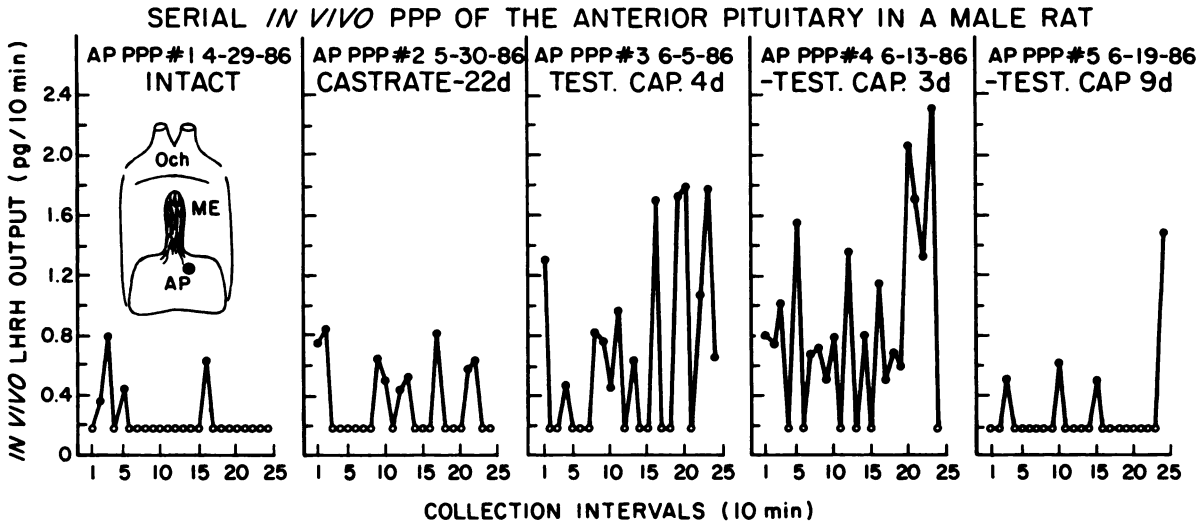

FIG. 12. Serial push-pull perfusions (PPPs) of the anterior pituitary in a male rat performed during an interval of approximately 60 days. In this case, this animal underwent 5 separate PPPs under different experimental conditions: INTACT, 22 days after castration (CASTRATE-22d), during testosterone replacement therapy with s.c. testosterone inplants for 4 days (TEST.CAP.4 d), and after 3 and 9 days of testosterone implant removal (–TEST.CAP.3d and –TEST.CAP.9d), respectively. Notice the remarkable effect of testosterone on the amplitude and frequency of the LHRH pulses, which persisted for 3 days after testosterone removal but were not evident after 9 days. For details, see *text*.

LHRH apparatus in intact rats vs. long-term castrated animals (greater than 4 wk; 12 mo in the example depicted in Fig. 11). Under these conditions, we were able to measure LHRH consistently, as well as 5-HIAA and dihydroxyphenylacetic acid (DOPAC), these last two substances comprising the main metabolites of serotonergic and dopaminergic neurons, respectively. These results represent the first time, under in vivo conditions, that levels of 5-HIAA and DOPAC reaching the anterior pituitary gland of freely moving rats have been measured. Interestingly, the levels of 5-HIAA dropped very low in the long-term castrated rat, though DOPAC levels remained within the range of previously measured values. Independently, Craig et al. (1983) detected 5-HIAA in the pituitary portal blood of anesthetized rats, which indicates that this metabolite of serotonin reaches the anterior pituitary gland from hypothalamic sources. These intriguing data require confirmation, but once more indicate the potential use of this technique to examine biological events in vivo in unrestrained, unanesthetized animals.

To our surprise, intact animals bearing a PPC in the anterior pituitary gland had a pattern of LHRH output rather similar to that obtained in rats bearing PPC in the hypothalamus, as estimated by mean level of LHRH, and frequency and amplitude of the LHRH signal as shown in the example of Figure 11. However, and quite interestingly, in long-term castrated rats (greater than 4 wk), the output of LHRH was of high frequency, as expected, but of unexpectedly large amplitude. These data partially support our previously published thesis (Dluzen and Ramirez, 1985) in which we postulated an LHRH signal of high frequency but low amplitude in castrated animals. Our prediction, therefore, was only partially ratified in this experiment, since the amplitude of the LHRH signal as recorded from PPC perfusing the anterior pituitary gland was of high instead of low amplitude, as is clearly demonstrated in Figure 11. A complete report on these experiments was presented to the first International Congress in Neuroendocrinology (Dluzen and Ramirez, 1986d), in San Fransisco this year.

Since two different animals were used in these studies with PPCs (although similarly positioned in the anterior pituitary as illustrated in the inset of the Figure 11), the differences in LHRH profiles might still have been caused, among several possibilities, by differences in the magnitude of the lesion in the pituitaries of these two different animals, thereby generating artificial changes in LHRH levels. Hence, to partially override such a criticism, we decided to use the same animal for a series of experimental manipulations. The current data on these exciting ongoing experiments are illustrated in Figure 12. In this example, a male rat was implanted with a PPC aimed to the anterior pituitary, and the LHRH output was recorded under five different conditions

during a two-mo interval. For details of the experimental paradigm, see the legend of Figure 12. Several important features are evident from this preliminary experiment that so far have been confirmed in a second animal. First, and the foremost finding from a technical point of view, is the clear demonstration that in vivo PPP can be carried out in the same animal for an extended period of time, which makes this technique an attractive and powerful experimental approach. The second important finding is that castration for a short-term period—in this case, for 22 days—is accompanied by a clear increase in the rate at which a bolus of LHRH reaches the pituitary gland. Curiously, the amplitude of the LHRH signal did not appear to be modified in this case, but an increase in the frequency was apparent, which is in marked contrast to the increased amplitude and frequency that we observed in animals castrated over 27 days, as indicated above (see Figs. 11 and 12 for comparison, and our paper, Dluzen and Ramirez, 1986d). Such remarkable differences between these two conditions, short- versus long-term castrated animals, needs, however, to rule out possible differences in PPC placement within the anterior pituitary gland. This is an issue that can be resolved by the current approach of using the same animal to study the effect of different treatments. Third, testosterone replacement therapy for four days with implants containing the steroid in concentration and conditions aimed to generate mean constant blood levels similar to those found in intact males as shown by Steiner et al. (1982) markedly increased the amplitude and the frequency of the LHRH signal, with mean secretory values above those detected under intact conditions. Interestingly, an acute increase in plasma testosterone concentration at low doses does not reduce pulsatile LH secretion, (Clifton and Steiner, 1986). Fourth, and quite intriguingly, removal of the testosterone implant for three days was not sufficient to decrease the large amplitude or the frequency of the LHRH signals arriving in the pituitary. Last, but not least, when the absence of exogenous testosterone was extended for another six days, i.e. a total of nine days, the frequency and magnitude of the LHRH signals reaching the anterior pituitary gland dramatically dropped to pretreatment levels, which clearly indicates that testosterone treatment can markedly and for a prolonged time alter the activity of the neural LHRH apparatus.

HYPOTHETICAL MODEL OF THE OPERATION OF THE LHRH PULSE GENERATOR IN THE MALE RAT

These preliminary but robust data, along with our previous findings in which we used the PPC to probe in vivo the hypothalamus of intact and short-term castrated rats (Dluzen and Ramirez, 1985), stimulated us to construct a hypothetical model of the operation of the LHRH pulse generator in the intact male rat and in the short- (less than 25 days) and long-term (greater than 25 days) castration-condition, as schematically presented in the last Figure 13.

First, we assume that the total amount of LHRH released per pulse results from the interaction of three probable factors: 1) The quantum of LHRH available for release per neuron. This is a function of the size of the readily releasable LHRH pool present in that neuron at a particular instant of time. 2) The frequency of firing (spike/s) of that particular neuron, which governs the release process. Here we imply that every action potential is accompanied by a quantum of LHRH release that is, however, very much dependent on a certain critical frequency of discharge, as shown by Dyer et al. (1980). 3) The number of neurons that are discharging in tandem at that particular instant.

Therefore, the amplitude of an LHRH signal (pulse) within the hypothalamus is very much dependent on the firing rate of the neurons involved in such an event and on the size of the readily releasable LHRH pool. A pulse of LHRH in the rat represents a very minute amount (0.5—20 pg) of the total LHRH measured in the hypothalamus of an intact male rat (2—4 ng/MBH). The frequency at which these pulsatile LHRH signals are repeated (number of LHRH pulses/h, for instance) is a function of the interval at which these neural secretory volleys are repeated. Several factors can alter these intervals (increasing or decreasing it), such as hormones, light, inhibitory interneurons, etc. Therefore, the total LHRH output/unit of time represents the interaction among these diverse neural events.

Our next premise assumes that the LHRH firing type (i.e., activation of several neuronal pools of functional LHRH neurons) can fluctuate between two extremes: in the intact male rat, these neuronal pools are desynchronized—i.e. discharging at different times—whereas in long-term castrated rats, the LHRH pulse generator is synchronized with short-term

castrated animals showing an intermediate mode, that is, a partial asynchronous LHRH firing type. From the data gathered from Figures 11 and 12, the input pattern of LHRH reaching the pituitary is depicted for each of these three conditions in Figure 13. In the intact physiological condition, the activity of the LHRH pulse generator is characterized by bursts of activity with silent intervals, as shown in the diagram. Its activity is similar when probed with PPC in the hypothalamus, as previously reported (Dluzen and Ramirez, 1985), or in the pituitary, as shown here. In the intact condition, we assume that only one field of LHRH terminals is active. A fair approximation of the LHRH release during the 4 h of the experiment can be estimated by the LHRH frequency (3 pulses/4 h in the example) times the average amplitude of the pulse (0.75 pg/pulse in the example) times the one field of LHRH terminals active during this period of sampling. It should be perfectly clear that these calculations are relative and have value only if the relationships between different conditions are compared, particularly if they are obtained from the same animal. After short-term castration, three main changes affect the LHRH pulse generator. The amplitude of the LHRH pulses markedly decreases (from 0.75 to 0.3 pg in the example), the pulse

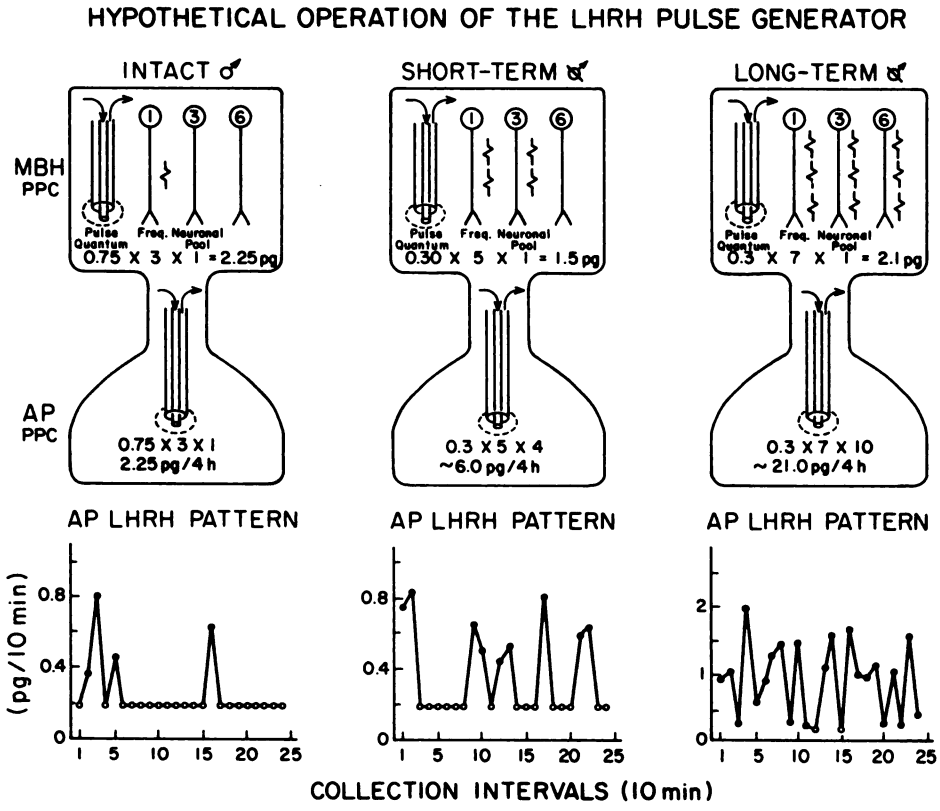

FIG. 13. Hypothetical operation of the LHRH pulse generator under three conditions: *INTACT*, *SHORT-TERM* (22 days after castration), or *LONG-TERM* (over 12 mo after castration). The *upper figures* depict the medial basal hypothalamus (MBH) connected to the anterior pituitary with a push-pull cannula (PPC) placed in either of these two structures. The *bottom figure* depicts the actual LHRH profile as recorded from the anterior pituitary. The total output of LHRH during the 4-h period is a function of three factors: LHRH pulse quantum X frequency X number of active LHRH terminals. For details, see *text*.

Since in the intact condition, similar profiles were observed with PPC placed in the MBH or in the anterior pituitary, we have assumed an asynchronous discharge of LHRH involving only a unit LHRH neuronal pool.

In short-term castrated rats (less than 22–25 days), three changes in the operation of the LHRH pulse generator are assumed to occur: (1) increase in frequency as shown from the LHRH profile derived from PPP of the pituitary; (2) decrease in amplitude of the LHRH signal, as reported for the LHRH profile derived from PPP of the MBH (not shown, but see references: Dluzen and Ramirez, 1985; Ramirez, 1985); and (3) an assumed increase in the number of active neuronal LHRH pools (from 1 to 4) to explain the greater total output of LHRH detected in the pituitary of a rat in this condition as compared to that of an intact animal (~6.0 vs 2.25 pg LHRH /4 h).

Last, in the long-term castrates (> than 27 days; paper in press, Dluzen and Ramirez, 1987) high amplitude and frequency of the LHRH pulses reaching the pituitary are evident (note change in scale). These results are clearly compatible with those reported for serum LH, i.e. an increase in both the frequency and amplitude of LH release (Ellis and Desjardins, 1984a,b). Therefore, those changes are indicative of further increases in frequency of discharges and, more importantly, further recruiting of LHRH terminals that discharge in a synchronized manner.

frequency increases (from 3 to 5), and a partial synchronous discharge of several LHRH terminals occurs (from 1 to 4). The resulting summated effect of these changes are two: since the PPC in the hypothalamus can only perfuse a restricted area, the LHRH output is barely detectable as previously shown to be the case (Ramirez, 1985). However, since the anterior pituitary gland collects all the LHRH molecules discharged into the portal vessels, the perfusion of the anterior lobe successively collects enough LHRH molecules to be detected in the radioimmunoassay (RIA) in spite of a marked decrease of at least 50% in the content of LHRH after castration (Badger et al., 1978; Gross, 1980; Kalra, 1986). This permits experimental measurement of the type of changes observed after short-term castration, that is, an increase in frequency, and since the total output of LHRH is greater, we have to conclude, on the reasonable assumption of a decrease in the amplitude of the LHRH pulse, that new fields of LHRH terminals are, therefore, active under this particular condition.

In a long-term castrated rat, the LHRH profile, as detected in the anterior pituitary, is depicted in the bottom right of our diagram. Our predictions are that under these conditions, the LHRH pulse generator is operating close to maximum with almost all of the LHRH terminals fields active (synchronized) and the intervals between pulses reduced to a minimum, i.e., LHRH pulses are being discharged at high frequency. These two predictions are supported from the data obtained while perfusing the anterior pituitary, since the frequency rose to 7 pulses/4 h, and the magnitude of the pulses increased considerably in spite of low LHRH content in the MBH, as discussed before. This leads us to propose that new fields of LHRH neurons are discharging but in a synchronized manner. These results are clearly consistent with those reported for serum LH in the castrated male rat, i.e. an increase in both the frequency and the amplitude of LH release (Ellis and Desjardins, 1984a,b) and they resolve the apparent paradox resulting from probing only the MBH with the PPC. The pattern of LHRH output, as probed with the PPC in the MBH of long-term castrated animals, remains to be demonstrated. We predict that in this case we will be able to detect a higher mean release rate of LHRH than in short-term castrated animals, although the amplitude of LHRH pulses still will be approximately the same. In support of this prediction is our finding that in short-term OVX rats in vitro, pulsatile LHRH release from the HU were reduced, while in long-term OVX animals, the in vitro LHRH release was significantly higher, and large pulses were observed during the beginning of the superfusion period, as pointed out before (Dluzen and Ramirez, 1986c).

Admittedly, these concepts are highly speculative, but they reflect the kind of information and analysis that can be constructed by using PPP. Most importantly, these concepts produced hypotheses that can be tested experimentally. In summary, we have presented experimental evidence demonstrating the usefulness of the PPC in gaining further insights on the "know-how" of the functions of different neuronal brain systems in awake animals in which their natural physiological conditions are almost retained.

ACKNOWLEDGMENTS

We wish to thank Kathy Roberts for her skillful help in preparing this manuscript. Also, we want to express our sincere appreciation to our current students who have contributed so much to make this present work a reality: W. W. Lin, O.-K. Park, J. Gitzen, and A. D. Ramirez. Also, our appreciation to Norma Ramirez for her contribution as a reliable and dedicated technician.

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