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CHAPTER 40

Puberty in the Rat

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HISTORICAL ASPECTS

The first recorded use of the rat for experimental purposes was published in 1856 (1). Since its domestication in the early part of this century, the rat has probably contributed more substantially to the advancement of the biological sciences than any other laboratory species. Clearly, the story of the rat, as portrayed by Lindsey (2), is one of "ascendancy from the gutter to a place of nobility."

The use of the rat for the study of sexual development and puberty can be traced to the beginning of the 20th century, when investigators initiated studies to test the hypothesis that the ovaries contain a substance(s) responsible for the advent of sexual maturation. Essential to these undertakings was the pioneering work of Long and Evans, who in 1922 published a classic monograph (3) describing in detail the physiology of the rat estrous cycle and the initiation of female reproductive capacity. Long and Evans (3) demonstrated, for the first time, that the ovaries of immature rats develop rapidly when transplanted into adult rats. It was, however, the work of Allen and Doisy (4), published in 1924, that showed conclusively that extracts of follicular fluid injected into immature rats were capable of advancing vaginal opening. A year later Frank et al. (5) presented evidence that a "lipoid extract" of placental tissue was not only able to induce precocious vaginal opening but, more importantly, to also advance the first ovulation. These authors concluded that "puberty results from the elaboration, in sufficient amount, of the female hormone, and that the advent of puberty is not due to the removal of an inhibitory influence. . . ."

That immature ovaries can modify the secretion of gonadotropin hormones from the anterior pituitary and that, in turn, these hormones can hasten the initiation of puberty by accelerating ovarian maturation were first demonstrated by Kallas in 1929 (6). This author found that when two immature female rats were joined parabiotically and one of them was castrated, the other one underwent precocious puberty. These experiments indicated that a dynamic relationship between the anterior pituitary and the ovaries is operative well before puberty; it was not until 3 years later, however, that the participation of the brain in this relationship was postulated by Hohlweg and Junkmann (7). Surprisingly, this concept found little immediate support. This was perhaps because of the widespread acceptance of a hypothesis formulated by Moore and Price (8), also in 1932, that stated that a pituitary–gonadal interrelationship was sufficient

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to explain the maturation and initiation of reproductive capacity.

It was not until 20 years later that the work of Harris and Jacobson (9) provided the basis for our current understanding of the pivotal role played by the central nervous system (CNS) in the control of reproductive maturation. These authors showed that pituitaries from immature rats transplanted into adult animals were able to sustain estrous cyclicity. Subsequent experiments performed by Donovan and van der Werff ten Bosch (10) in the mid-1950s led to the concept that there are certain hypothalamic areas that exert inhibitory influences on gonadotropin secretion and that removal of these influences results in the initiation of puberty. A few years later, Elwers and Critchlow (11) found that lesions of the amygdala resulted in precocious ovarian activation, thereby providing evidence that extrahypothalamic structures are also involved in modulating gonadotropin secretion in developing rats. Although these early research efforts provided much insight into the neuroendocrinology of puberty, further significant progress was hampered by the lack of sensitive and accurate methods to measure changes in serum hormone levels. The 1970s witnessed an explosion of reports that, utilizing the novel technique of radioimmunoassay (RIA), probed the neuroendocrine system from different angles in search for more definitive answers. In recent years, the development of recombinant DNA technology has provided a new set of powerful tools with which to explore the mechanisms underlying the initiation of puberty (12,13).

Beginning with the comprehensive review of Donovan and van der Werff ten Bosch (14), published in 1965, the physiology of rat puberty has been systematically reviewed over the years (15–20). It is the purpose of this chapter to discuss the pubertal process in the rat and provide the reader with an update of developments in this field. Similarities to and differences from sexual maturation in other species will become apparent when this chapter is considered in conjunction with the chapters by Foster and Plant which deal with puberty in the sheep and monkey, respectively.

THE RAT AS A MODEL

The rat is a convenient animal to use for the study of sexual development. It grows rapidly, reproduces frequently, is relatively inexpensive, and is easy to handle, and external signs of sexual maturity, though scanty, are readily detectable. In working with this animal, one may also assume that most of the basic mechanisms underlying the process of sexual maturation are conserved across species. If such an assumption is correct, then many of the results obtained in the rat may be extrapolated to other species, including the human. Among such mechanisms, the control of luteinizing hormone-releasing hormone (LHRH) by neurotransmitters, the initiation of gonadotropin secretion, the cellular components of steroid positive and negative feedback, and the control of ovarian follicular development represent only a few examples. Perhaps the most striking difference between the rat and the primate is the relative absence in the former of the juvenile hiatus of gonadotropin secretion that is characteristic of the latter (21). This difference severely handicaps the rat as an animal model in which to investigate gonadal-independent, CNS-originated events that might be responsible for the decline in hypothalamic activity during the human juvenile period. Species such as the rhesus monkey appear to be the logical choice to examine this outstanding issue. However, the demonstration that the rat, like the primate, exhibits a diurnal change in the mode of luteinizing hormone (LH) secretion at the end of juvenile development (22) reiterates the possibility of using the rat as a model for the analysis of at least some aspects of this early event, which appears to be the primary hormonal manifestation of puberty onset.

From a developmental point of view the rat is born at the stage comparable to 150 days of human gestational life (23). The gestational period of the rat lasts for 22 to 23 days. The first ovulation in most laboratory stocks occurs 35 to 45 days after birth. In males the first spermatozoa are seen in the lumen of seminiferous tubules by 45 days of age (24), and they reach the vas deferens 13 to 14 days later (25). Testicular descent occurs after day 15. Externally, the progression of puberty in the male rat can be followed by the growth of the testes and more precisely defined by the separation of the foreskin of the penis from the glans, known as balanopreputial separation (26). In females the only signal that puberty has occurred is canalization of the vagina, which normally is imperforate before puberty and later becomes patent as a consequence of estrogenic stimulation. Vaginal opening usually occurs on the day after the first preovulatory surge of gonadotropins has taken place (15,16,27,28). In most cases, vaginal lavages at opening show a majority of cornified cells (estrus), a condition that is followed within 1 to 2 days by the appearance of leukocytes, which soon become the predominant cell type (first diestrous phase of puberty).

Male rodents, in general, do not display a postnatal period of testicular quiescence analogous to that observed during the human juvenile period but, rather, show initiation and progression of testicular development at a very early age. Thus, terms such as "infantile" and "juvenile," when applied to male rats, should be treated with caution, as they are not directly analogous to these phases in humans. Whereas developmental phases in the female rat have usually been defined in relation to the maturational stages of the ovary (15,29–31), sexual development of male rats has generally not been divided into specific maturational stages for the

purpose of its study. Ramirez (16) suggested a classification of the phases of rat puberty based on physiological parameters such as the changes in circulating gonadotropin levels and the alterations in steroid feedback mechanisms occurring at different postnatal ages. This classification was subsequently modified to include morphological and physiological parameters for both males and females (18). Accordingly, in this classification, sexual development in the male rat can be divided into four phases: a neonatal period that comprises the first week after birth, an infantile period that extends from days 8 to 21, a juvenile period that ends around day 35, and finally the peripubertal period that ends at about 55 to 60 days of age, i.e., with the appearance of mature spermatozoa in the vas deferens (25). Details of the functional and morphological parameters utilized to define these developmental periods are provided in ref. 18.

Postnatal development of the female rat can also be divided into four phases, which have been described in detail earlier (18). They are a neonatal period that is initiated at birth and ends on postnatal day 7, an infantile period that extends from days 8 to 21, a juvenile period that ends around days 30 to 32, and a peripubertal period that has a variable duration but that culminates with the occurrence of first ovulation (around day 38 for most laboratory stocks). Defining the end of juvenile development has been difficult; morphologically, the appearance of uterine fluid signals the beginning of the peripubertal period (18) but tells us little about the hormonal events responsible for the change. It now appears that the end of the juvenile period can be more precisely defined as the time when morning–afternoon differences in pulsatile LH release become established (22).

An additional developmental phase that needs to be included (32) is a fetal period, which for mechanistic purposes can be considered to be initiated at gestational day 12. It is around this time that LHRH can first be detected in the fetal brain (33).

THE FEMALE

Fetal Development

Initiation of LHRH and Gonadotropin Secretion

Although LHRH neurons of adult rats are located primarily in the septal medial preoptic and hypothalamic areas of the brain (34,35), they are embryonically derived from cells in the olfactory placode (36–38). Most of these subsequently migrate caudally during fetal development and assume a position in the forebrain. It is now clear that the appearance of LHRH in the hypothalamus precedes that of gonadotropins in the anterior pituitary by several days. The study of Aubert et al. (33) showed that immunoreactive LHRH can be detected in the brain of rat fetuses as early as day 12, at which time LHRH-binding sites can also be found in the primordial anterior pituitary. If anterior pituitary anlagen are collected after day 13 and cultured *in vitro*, gonadotropes will differentiate spontaneously (39,40). However, if the glands are removed at an earlier age, the gonadotropes will fail to differentiate unless they are exposed to exogenous LHRH (40,41). Although the pituitary gland may not yet be vascularly connected to the brain at fetal days 12 to 14 (42,43), the above findings suggest that hypothalamic LHRH plays a trophic function essential for the differentiation of pituitary gonadotropes.

How the differentiation of LHRH neurons themselves is controlled remains unknown, but it would not be unreasonable to assume that the process is controlled by genes encoding tissue-specific classes of transcription factors that, on coordinated activation, determine the LHRH neuronal phenotype. Levels of hypothalamic LHRH remain low until around fetal days 17 to 18, at which time they begin to increase, showing a substantial elevation by the day of birth (33,39,44). Pituitary LH can be detected around fetal day 17 (33,39,45), and a response to LHRH can be observed by days 17 to 18 (45). Pituitary follicle-stimulating hormone (FSH), on the other hand, becomes detectable much later, by days 19 to 21 (33,39,46). An earlier discrepant report failed to detect either gonadotropin until the day of birth (47). In any event, circulating gonadotropin levels remain at low values until the day of birth (33,39,44,45,47–49).

Initiation of Ovarian Function: Pituitary and Extrapituitary Regulation

Substantial evidence exists that the fetal testis is responsive to gonadotropins (*vide infra,* subsection entitled "Initiation of Testicular Function"). On the other hand, it does not appear that plasma gonadotropins are involved in the development of the fetal ovary. Several reports have failed to detect LH or FSH receptors in ovaries of rats younger than 4 to 5 days of age (50–54). That initiation of follicular growth is gonadotropin independent is suggested by the findings that neither administration of PMSG or FSH (55,56) nor immunoneutralization of endogenous gonadotropins (57) is able to alter the number of follicles that begin to grow during the first few days of postnatal life (58,59).

Recent studies have demonstrated that initiation of follicular formation in the rat is a dramatic event that takes place shortly after birth (60,61). Although very few, if any, primordial follicles are seen within the first 24 hours of birth, a marked increase occurs within the next 24 hours (Fig. 1). Before development of the first primordial follicles takes place, the ovary consists of three main components: germ cells, mesenchymal cells, and epithelial cells (62). As the germ cells migrate into the primitive

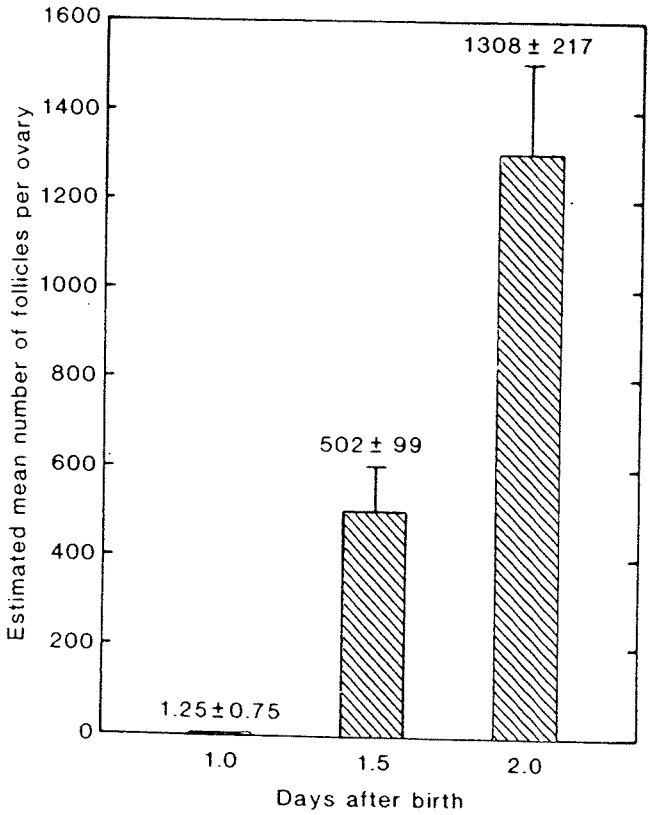

FIG. 1. Development of primordial follicles in the neonatal rat ovary. *Columns* represent mean values. *Vertical lines* indicate standard errors of the means (SEM). $N = 4$ for each column. (From ref. 61, with permission.)

gonad, they are surrounded by epithelial cells of the ovarian rete, which is derived from the mesonephric tubules (see the chapter by Byskov and Høyer). It is now believed that these cells, and not those derived from the coelomic epithelium, are destined to form the granulosa cells. Formation of primordial follicles is preceded by migration of mesenchymal cells that form stromal "pockets" containing groups of presumptive granulosa cells and clusters of oocytes. Subsequently, the mesenchymal cells encircle single oocytes surrounded by a single layer of pregranulosa cells to form primordial follicles. Recent evidence suggests that this differentiation may be influenced by neurotropins, a family of target-derived growth factors that include nerve growth factor (NGF) and three other members (63). Mesenchymal cells have been found to contain a class of neurotropin receptors known as p75, or low-affinity NGF receptors, long before the formation of the first primordial follicles; p75 NGF receptors are recognized by all members of the NGF family. A selective increase in the gene expression of neurotropin 4, the most recently identified neurotropin, occurs in oocytes shortly after birth, during the hours preceding the formation of the first primordial follicles, suggesting a causal relationship between the two events. *In vitro* experiments utilizing neonatal ovaries in organ culture have shown that antibodies to NGF (which presumably also antagonize the biological activity of the other neurotropins) caused widespread mesenchymal death and disrupted follicular formation (64).

Although these observations suggest that neurotropins —and not pituitary gonadotropins—may be able to affect the initiation of folliculogenesis, there is also evidence that the early steroidogenic activity of the ovary is gonadotropin independent. Fetal ovaries cultured *in vitro* fail to respond to either FSH or LH with increases in aromatase activity (65). Nevertheless, exposure to exogenous cAMP or activation of their adenylate cyclase system with forskolin results in increased aromatase activity, suggesting that a first messenger other than FSH or LH may operate within the ovary before follicular growth is initiated. The identity of such a messenger(s) is not clear at present, but it is noteworthy that ovarian nerves contain vasoactive intestinal polypeptide (VIP) (66), which is present in the rat ovary before the appearance of primary follicles (67). Moreover, VIP can stimulate cAMP production and induce aromatase activity in fetal ovaries (65) well before acquisition of responsiveness to FSH. These observations raise the possibility that initiation of follicular growth in the rat may be influenced by neurotransmitters reaching the ovary via the extrinsic ovarian innervation. Such a possibility is supported by the finding that the sympathetic innervation of the ovary develops before the formation of primordial follicles (61).

Postnatal Development

The Prepubertal Period

This section first discusses the maturational events that occur at the hypothalamic–pituitary level and within the ovary during the neonatal, infantile, and juvenile periods. Second, consideration is given to the changing interrelationships among these three basic components of the neuroendocrine reproductive axis.

The Hypothalamic–Pituitary Unit: Changes in Gonadotropin Secretion and Their Relationship to LHRH Release

Beginning shortly after birth, plasma FSH levels start to increase, reaching maximum values by day 12 (68,69). Thereafter, levels decline gradually, so that by the end of the juvenile period they are about one-fifth the values present at day 12 (68,70). Plasma LH is also more elevated in neonatal–infantile rats than in juvenile animals (68,70,71), but the elevation is less evident than that of FSH. Release of LH in infantile rats appears to

)nform to a pattern of moderately elevated levels of the)rmone interrupted by sporadic surges of release 0,72). Such bursts appear to become less evident as the iimal grows and disappear completely with the advent juvenile development (73).

How do these patterns of gonadotropin release relate) developmental changes in LHRH secretion? Little is nown regarding the functional development of the HRH-releasing system. The hypothalamic content of HRH increases markedly between the day of birth and ie end of juvenile development (33,39,74). Although HRH content is substantially lower during the eonatal–infantile period than during juvenile develop- ient, *in vitro* experiments have shown that the LHRH- leasing system is more responsive to a depolarizing imulus around the second week of postnatal life than uring the juvenile period (75). On the other hand, *in itro* experiments using hypothalamic explants have hown that LHRH is secreted in a pulsatile fashion iroughout prepubertal development (76,77). This sug- ests that the infantile LHRH neuronal system already as the capacity to generate LHRH secretory episodes in he absence of extrahypothalamic inputs. That pulsatil- ty may be an intrinsic property of LHRH neurons is uggested by the finding that isolated LHRH neurons in ulture release LHRH in a pulsatile fashion in the ab- ence of any neural connections (78).

Although no published data exist regarding the devel-)pmental changes in LHRH release from the hypothala- mus of female rats, the study of Bourguignon and Franchimont (76) in developing male rats indicates that the frequency of LHRH pulses increases during prepubertal maturation. Demonstration of a similar increase in LHRH pulse frequency in females could help to explain why the secretion of FSH during the infantile phase of development differs so markedly from that of LH. It is conceivable that as the reproductive hypothalamus matures during the first two weeks of postnatal life, LHRH is released as infrequent discharges that are sufficient to sustain a high level of FSH secretion but only generate transient bursts of LH release. Such a differential pattern of gonadotropin release has actually been observed in ovariectomized rhesus monkeys bearing hypothalamic lesions that were given low-frequency pulses of exogenous LHRH (79).

By the same reasoning, the hypothesis also implies that the decrease in FSH levels that follows the peak of secretion on day 12 results from an increase in LHRH discharges. Support for this concept is provided by (a) our unpublished observation that subcutaneous infusion of LHRH as 1-hour pulses every other hour to infantile, ovariectomized rats via osmotic minipumps decreases plasma levels of FSH but not LH (Fig. 2). Moreover, plasma FSH in untreated rats ovariectomized on postnatal day 6 becomes greatly elevated on day 16 and then decreases to an intermediate level. The decrease is observed at approximately the age at which serum FSH levels normally begin to decline in intact rats. (b) Studies

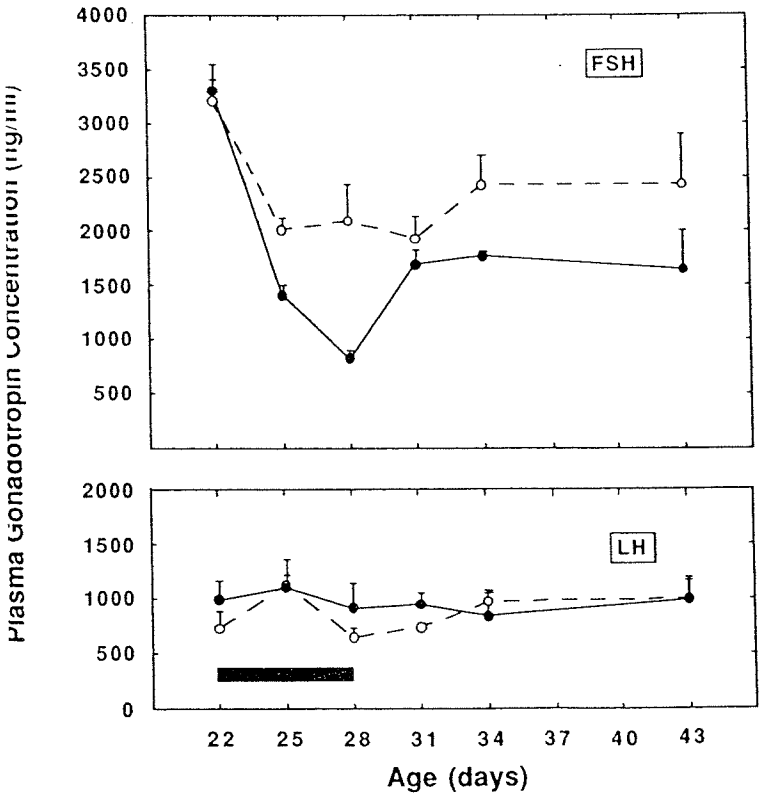

FIG. 2. Effect of exogenous LHRH on plasma FSH (**upper panel**) and LH (**lower panel**) levels in female rats. All of the animals were ovariectomized on day 10. Between days 22 and 28 (*horizontal bar*) they received either a 1-hour s.c. infusion of LHRH (1 μg) every other hour (*solid circles*) or served as untreated controls (*open circles*). The procedure for intermittent infusion of LHRH via osmotic minipump was based on that described by Lynch et al. (547). Each point represents the mean of five to seven animals, and the SEM are depicted as *vertical lines*. $^{*}p < 0.05$; $^{**}p < 0.01$. (H. F. Urbanski and S. R. Ojeda, *unpublished data*.)

showing that pulses of LHRH administered every 120 to 480 minutes increase the mRNA levels of FSHβ but not LHβ; on the other hand, LHRH pulses administered at a high frequency (e.g., every 8 minutes) produce an increase in the mRNA levels of LHβ but not FSHβ (80). Taken together, these results suggest that the frequency of LHRH discharge may play an important role in determining the pattern of FSH release during infantile days. They also provide evidence that such changes may be, at least in part, the consequence of steroid-independent events of central origin.

Another factor that may contribute to the pattern of gonadotropin release observed in infantile female rats as compared with juvenile animals is a different pituitary responsiveness to LHRH. It is now well established that pituitary responsiveness to LHRH is much greater in infantile rats (81–83) than in juvenile animals. This increased responsiveness may be related to the observation that the pituitaries of the younger rats contain a greater percentage of gonadotropes (84), and it may also be related to a direct facilitatory effect exerted by nonaromatizable androgens and progesterone (P) on the pituitary gland. Ovariectomy has been found to reduce the FSH response to LHRH, whereas 5d-dihydrotestosterone (DHT) or P treatment restores it (83). A stimulatory effect of DHT on FSH secretion has also been observed within 24 hours of its administration to immature female rats (85). A physiological role for DHT is further indicated by the fact that, although its plasma levels are low in female rats, 5α-reductase activity is elevated in the anterior pituitary of infantile rats (86). Moreover, the developmental pattern of pituitary 5α-reductase activity closely follows that of serum FSH (86).

Still another factor that contributes to the elevated gonadotropin levels in infantile rats is the relative ineffectiveness of estradiol (E_2) negative feedback. Administration of E_2 has been repeatedly shown to be less effective in depressing gonadotropin levels in ovariectomized infantile rats than in juvenile animals (87–89). Of particular interest in this regard is the finding (90) that immunoneutralization of serum E_2 in infantile rats fails to induce a rise in circulating gonadotropin levels, which would be expected if E_2 negative feedback is operative at this time. The demonstration that RU2858, a synthetic estrogen that does not bind to α-fetoprotein (AFP, vide infra), strikingly inhibits the postovariectomy rise of serum gonadotropins in infantile rats (91) firmly established the concept that E_2 negative feedback fails to operate in infantile rats because of the presence of AFP and not because of a lack of specific E_2 receptors. By day 16, AFP levels have decreased sufficiently, allowing free E_2 levels to be detected for the first time (92–94). Coinciding with this shift, the high serum FSH levels begin to decrease. Thus, it appears that the infantile increase in serum FSH levels may depend on at least three factors: (a) a slow frequency of LHRH release, (b) a facilitatory effect of 5α-reduced androgens produced in the anterior pituitary on LHRH-induced gonadotropin secretion, and (c) the failure of E_2 negative feedback to operate at full capacity.

Additional developmental changes include a shift in the molecular forms of pituitary FSH from forms with low biological activity to those with high ones (95). However, both pituitary and serum FSH from infantile rats are biologically active, as they can induce ovarian growth and follicular development (96). As with FSH, there is some evidence to suggest that LH also changes in nature during puberty (97). With regard to LH secretion, infantile rats show sporadic bursts of LH secretion, which appear to result from activation of noradrenergic neurons, since the norepinephrine (NE) turnover in the POA has been found to increase at the time of the LH secretory discharges (98). On the other hand, the concept that these bursts of LH release are caused by an early expression of E_2 positive feedback (99) has not been supported by the observations that (a) E_2 is unable to induce an LH surge before postnatal day 15 (94,100), (b) passive immunization against E_2 fails to inhibit the LH surges (90), and (c) environmental disturbance inhibits their occurrence (101).

By the end of the infantile period, plasma FSH levels have decreased substantially, and the bursts of LH secretion have begun to disappear. Throughout juvenile development, plasma FSH continues to decrease, though much less noticeably, and plasma LH levels remain at low values (68–73). The mode of LH release, however, is clearly pulsatile (22,102,103), with an interpulse interval of about 30 minutes (22).

The low circulating gonadotropin levels seen in juvenile rats may not faithfully reflect the changes in hypothalamic activity that appear to occur during this period of development. This may be, at least in part, because of a low pituitary responsiveness to LHRH (81,83,104), which does not increase until the day of the first proestrus (105,106) (vide infra). This is in contrast with the marked activation of LHRH gene expression observed between the infantile and peripubertal periods, a time when proLHRH mRNA levels increase almost threefold (107). Perhaps one of the most striking events that takes place within the POA–MBH region at this time is the morphological maturation of LHRH neurons (108). During neonatal–infantile days the soma of the majority of LHRH neurons has a smooth surface. In contrast, LHRH cells with an irregular surface ("spiny cells") become predominant between weeks 4 and 5 of postnatal life. Remarkably, it appears that this transformation occurs independently of the ovaries (109). However, E_2 may also play a role, because in the sheep E_2 treatment has been found to increase the number of neuronal processes from LHRH neurons (110). Since in the prepubertal rat the total number of LHRH cells does not

change despite an increase in the number of spiny cells, it is evident that smooth LHRH cells have developed into spiny ones. The physiological mechanisms underlying these morphological changes are not understood, but they may involve an increase in the number of synaptic connections (111,112) and/or reflect an increase in cellular activity (113). This interpretation is indirectly supported by the finding that in the arcuate nucleus of the hypothalamus (114), as well as in other brain areas of the developing rat (115), the greatest increase in synaptic formation occurs before the fifth week of postnatal life. The relevance that these morphological changes may have for the initiation of puberty is unclear. Neither gonadectomy (109) nor food restriction (116) alters the formation of spiny neurons in spite of affecting in opposite directions the activity of the LHRH-LH secreting system. Nevertheless, spiny neurons have been shown to be more metabolically active than smooth neurons (113), a feature that correlates well with the increase in LHRH neuronal responsiveness that appear to occur during juvenile development, i.e., at the time when formation of spiny neurons is reaching completion. At this time, the hypothalamic–pituitary unit becomes fully responsive to the positive feedback effect of estradiol (94; vide infra). Also, the capacity of LHRH neurons to release LHRH, estimated by the *in vitro* challenge of median eminence nerve terminals with prostaglandin E_2 (PGE_2), increases gradually between postnatal days 22 and 34 (104). The LHRH response to PGE_2 may be considered a reliable index of LHRH neuron function, since a sizable body of evidence indicates that PGE_2 is a physiological component in the process of NE-induced LHRH release (for a review see ref. 117).

Interestingly, the age-related increase in the capacity of LHRH neurons to release LHRH in response to PGE_2 appears to be, at least partially, maintained by circulating E_2 levels. When juvenile rats were ovariectomized on day 22, the subsequent LHRH response to PGE_2 was found to be blunted on day 34. On the other hand, restoration of juvenile E_2 levels via E_2-containing Silastic capsules significantly reversed the effect of ovariectomy (118).

The activity of neurotransmitter systems involved in the control of LHRH secretion also changes during juvenile development. Thus, the turnover rates of both dopamine (DA) and NE have been shown to increase at this time (119,120). Interestingly, the ability of DA to stimulate adenylate cyclase activity decreases, suggesting a loss in receptor sensitivity to the catecholamine (121). Although this change may be functionally related to the increasing prolactin (Prl) levels observed during development (70,73,122), it may also reflect a loss of an inhibitory DA tone on LHRH release (121). Pertinent to this issue is the observation of a change in the influence that monoamines exert on gonadotropin secretion during infantile–juvenile development (123,124). Blockade of catecholamine synthesis in 16-day-old rats resulted in elevation in plasma LH levels, but the same treatment reduced LH levels in late juvenile 30-day-old rats, suggesting that whereas catecholamines inhibit LHRH secretion in young animals, they have a stimulatory effect as the animal approaches the initiation of puberty.

Maturation of the Hypothalamic–Pituitary–Ovarian Interrelationship: Modulatory Role of Ovarian Steroids

A predominant feature in the maturation of steroid feedback mechanisms in the female rat is the relative inability of E_2 negative feedback to operate during neonatal–infantile development (87–91). That a feedback relationship is not operative at all during the first few postnatal days is suggested by the finding that ovariectomy of neonatal rats fails to activate gonadotropin release (125). When ovariectomy is performed during the infantile period, serum gonadotropin levels increase, but, as already mentioned, the capacity of E_2 to suppress this elevation is much reduced (87–91). It is now well established (89–91) that the relative inability of E_2 negative feedback to operate is not related to the lack of specific hypothalamic–pituitary estrogen receptors but rather to the presence, in serum and tissues, of extremely high levels of AFP, which binds estrogen avidly (126,127).

It seems that during the infantile period of female development, aromatizable androgens play a leading role in the steroid negative feedback control of gonadotropin secretion. Both testosterone (T) and androstenedione can be detected in the blood of infantile rats (73,128,129). When T was administered via Silastic capsules to ovariectomized rats to mimic precastration levels of the androgen, it was found that physiological levels of T were effective in preventing the postcastration rise in serum gonadotropins (128). Whether the effect of T is related to its androgenic capacity per se or to prior local aromatization to estrogens is unknown. Estradiol, if given at sufficiently high doses that presumably overcome binding to AFP, can effectively inhibit gonadotropin release in infantile rats (88,89,91,94,130).

As levels of *AFP* decline, the capacity of E_2 to suppress gonadotropin release increases (87–89), so that it becomes maximally effective during the juvenile period (87–89,128). This enhanced effectiveness is maintained throughout juvenile development (128,130). In contrast to these changes in E_2 negative feedback, the capacity of aromatizable androgens to inhibit gonadotropin release remains relatively constant throughout infantile and juvenile development (128). It can, therefore, be concluded that the steroid negative feedback regulation of gonadotropin release changes from a predominantly

androgenic control during infantile days to a dual estrogenic–androgenic control during the juvenile period.

Development of Estradiol Positive Feedback

Although administration of sufficiently high doses of E_2 to infantile rats suppresses circulating gonadotropin levels, a stimulatory effect of E_2 on LH release cannot be demonstrated before the third week of postnatal life (94,131). Early experiments found that injections of E_2 could not evoke a surge of LH in rats younger than 21 to 24 days of age (131,132). A more detailed study, in which E_2 was administered via Silastic capsules and the resulting serum E_2 levels were measured by RIA, demonstrated that before day 15, E_2 levels as high as 400 pg/ml are unable to stimulate LH release (94). Between days 16 and 20, E_2 was found to be effective in inducing an LH surge, but the serum levels needed to be twice as high as those observed on proestrus. After day 20, however, proestrus E_2 levels were sufficient. At all of these ages the LH surge occurred 54 hours after implantation of the E_2 capsules, but when E_2 was administered to animals older than 28 days of age an LH surge occurred within 30 hours. It is therefore evident that as the animal matures, the hypothalamic–pituitary unit becomes more sensitive to the stimulatory effect of E_2. This increased sensitivity may be, at least in part, the result of exposure to E_2, since pretreatment of juvenile rats with E_2 prior to an E_2 challenge advances the age at which LH responds to the steroid with a surge of secretion (132). In addition to these temporal changes, the magnitude of the LH response to E_2 also increases during the juvenile period (94,133), so that by days 30 to 32 the surge of LH induced by E_2 is indistinguishable from that normally seen at first proestrus. As discussed later in this review, by the end of the juvenile period the LH-releasing system is so sensitive to E_2 that serum levels of the steroid even less than 50% of those seen at proestrus suffice to elicit large, preovulatory-like LH discharges.

There is no doubt that, in the rat, the stimulatory effect of E_2 on LH release involves the activation of LHRH secretion from the hypothalamus (106,134). Although more information is now known about the mechanism(s) underlying E_2-induced LHRH release (vide infra), much remains to be learned regarding the inability of E_2 to induce LH release in infantile rats. It seems clear, however, that even though the capacity of E_2 to stimulate LH release becomes established quite early, a precocious preovulatory surge of gonadotropins fails to occur because the ovary is not yet capable of producing E_2 in sufficient amounts, and for a sufficient period of time, to stimulate LH release.

The Ovary: Hormonal Control

Evaluation of parameters such as E_2 production (135), cAMP formation (136), and the number of gonadotropin receptors (50–54) have revealed that the ovary is relatively insensitive to gonadotropin stimulation during the first week of postnatal life. Nevertheless, primary follicles can already be observed by day 4 (58,59), and, although initiation of follicular growth is gonadotropin independent (55–59), there is ample evidence that maintenance of follicular development depends on the continuous presence of gonadotropins (55–59,137,138).

Interestingly, neonatal (1-day-old) ovaries cultured *in vitro* develop responsiveness to LH in the absence of any hormones (137). This may indicate that responsiveness to gonadotropins is acquired after the formation of primordial follicles, which—as indicated earlier in this review—appears to be a gonadotropin-independent, neurotropin-mediated process (for a review see ref. 63). The mechanisms underlying the acquisition of responsiveness to gonadotropins by the newly formed follicles are not known. Since cAMP is able to induce granulosa cell differentiation (139), the possibility needs to be considered that the acquisition of responsiveness to gonadotropins by the neonatal ovary is, at least in part, a cAMP-dependent phenomenon. As such, it may involve neurotransmitter molecules such as VIP and/or catecholamines, which are known to stimulate cAMP formation. Both VIP and norepinephrine are present in the feto–neonatal ovary (67,140), and VIP has been shown to stimulate cAMP formation and induce aromatase activity in fetal ovaries (65). In early experiments, gonadotropins failed to stimulate ovarian steroidogenesis or cAMP formation, even at as late a time as postnatal day 10 (135,136). Because FSH receptors may be present by day 4 (52,53), it would appear that uncoupling of newly formed receptors from adenylate cyclase (141) may occur during neonatal ovarian development. The control of ovarian follicular development by FSH may, indeed, be initiated during the first 5 days of life, as suggested by the findings that by day 4, FSH can stimulate the conversion of T to E_2 (64,137). Moreover, suppression of gonadotropin release by daily subcutaneous injections of DHT propionate (DHTP) during postnatal days 1 to 5, but not between days 5 and 11, markedly decreases ovarian FSH receptor content on day 12 (142). This effect of DHTP can be reversed by administration of FSH from days 1 through 5, suggesting that once follicular growth is initiated, the presence of FSH is important for the subsequent acquisition of FSH receptors by the developing follicles.

Substantial evidence now exists that ovarian follicles become subjected to strong gonadotropin control during the second week of postnatal life (57–59). Almost twice

as many small follicles begin to move into a more advanced developmental stage during the second postnatal week than at later ages (143). Suppression of serum gonadotropin levels by either DHTP treatment or immunoneutralization disrupts follicular and interstitial cell development at this time (57,144–146). The level of plasma FSH necessary for maintenance and/or formation of FSH receptors during the infantile period is no greater than that observed during the late juvenile period (~200 ng rat LH-RP-1/ml); suppression of serum FSH from the high infantile levels to the low juvenile values, using DHTP, failed to affect ovarian FSH receptor content even when serum LH was reduced to undetectable levels (142). During the infantile period, ovarian production of estrogen from exogenous precursors increases markedly (147,148), and FSH becomes able to induce aromatase activity (65). In addition, the ovary unequivocally demonstrates the capacity to respond to endogenous increases in serum gonadotropins with steroid release (149).

It has been difficult to determine the actual impact that the high infantile serum FSH levels have on subsequent sexual maturation (144,145). Nevertheless, early follicular growth is enhanced at this time, and completion of follicular growth takes 15 to 19 days (143), which suggests that many of the follicles that begin to grow during this period may be destined to reach a preovulatory, or even an ovulatory, condition at puberty.

The development of the neonatal–infantile ovary appears to be regulated by an additional modulatory mechanism of maternal origin that becomes established shortly after birth. Rat milk, like that of several other species, contains an LHRH-like substance that, as judged from its chromatographic behavior in Sephadex G-25 and high-pressure liquid chromatography (HPLC), is indistinguishable from hypothalamic LHRH (150,151). After suckling, LHRH-like immunoreactivity can be detected in the stomach content of the pups, and an increase in LHRH levels can be observed in plasma. Moreover, available LHRH receptors in the pup's ovaries decrease, an effect that can be prevented by prior intravenous administration of an antiserum to LHRH (151). The decrease in available receptors is not a consequence of suckling per se or of stomach distention because intragastric administration of milk, but not saline, reproduces the decrease in ovarian LHRH receptors associated with suckling. These observations have led to the conclusion that LHRH of maternal origin is transferred to the pup via the milk; it crosses the gastrointestinal epithelium and reaches the ovary via the bloodstream, where it binds to specific receptors (151).

Milk LHRH behaves like hypothalamic LHRH in that it is able both to stimulate gonadotropin release from the anterior pituitary *in vitro* (151,152) and to inhibit gonadotropin-induced E_2 and P release from granulosa cells in culture (151). Since the rat pup suckles frequently throughout the entire day, it would be expected that milk LHRH is almost continuously available for binding to the infant ovary. Chronic exposure to continuous levels of LHRH is known to depress ovarian function (for a review see ref. 153), and thus the suggestion may be made that milk LHRH plays a physiological role in restraining neonatal–infantile development of the pup ovary. This phenomenon may represent an evolutionary mechanism by which the mother rat regulates gonadal development of its offspring beyond intrauterine life. Indeed, available ovarian LHRH receptor content increases after postnatal day 15 (154,155), i.e., at the time when pups begin to eat pelleted food and when nursing episodes become less frequent or cease altogether.

During juvenile development the ovary grows under the influence of low serum levels of LH and FSH. Several excellent articles have reviewed the morphology (59,143,145,156) and the hormonal control (157) of the immature ovary. The interested reader is therefore referred to these articles for more detailed information. It is important to emphasize that throughout the juvenile period the ovary undergoes waves of follicular development and atresia (143,145,158), though in no instance does a crop of follicles reach the ovulatory stage. This is probably because of the relative lack of stimulatory inputs because the juvenile ovary can ovulate if challenged with sufficiently large amounts of exogenous gonadotropins (159,160).

Under normal conditions there appear to be a multiplicity of factors in addition to gonadotropins that contribute to regulating the gradual, orderly maturation of the ovary. The responsiveness of the ovary to gonadotropins is negligible during the early neonatal period, rises during infantile days, and becomes most prominent during the juvenile period (161,162). This change in responsiveness appears to be related, at least in part, to an increase in the number of gonadotropin receptors (53,54). Although the most pronounced change in FSH receptors appears to occur between postnatal days 4 and 16, hCG (LH) receptors increase more rapidly during juvenile days (53).

Whether the hypothetical inhibitory control exerted by milk LHRH remains operative throughout infantile development remains to be determined. Whether an LHRH-like peptide, produced locally or transported to the ovary via the nerves, contributes to the regulation of juvenile ovarian function is not clear at present. However, its presence is suggested by the changes in LHRH receptors observed at this time. The highest content of LHRH receptors occurs around day 25 (151,154,155). Thereafter, the content declines gradually toward the

first proestrus, with the sharpest decrease being observed during early proestrus 1 (163) [i.e., the phase of puberty when uterine fluid becomes apparent for the first time (164)]. This decline in receptor content represents a true loss of receptors and not a reduced availability for binding, because dissociation (by $MgCl_2$) of endogenous ligand(s) from ovarian membranes of late juvenile rats fails to uncover additional binding sites (163). Although the prepubertal decrease in receptor content has been interpreted as being indicative of a declining LHRH inhibitory influence on the ovary, the validity of this hypothesis remains to be established.

In addition to gonadotropins, two anterior pituitary hormones appear to be involved in the regulation of prepubertal ovarian function. Both Prl and growth hormone (GH) have been shown to support ovarian maturation by facilitating the effects of gonadotropins. The secretion of both somatomamotropins is low at the beginning of the juvenile period and increases gradually thereafter (73,122,165,166). An adult-like pattern of GH release becomes established around the time of puberty (166), whereas a quotidian pattern of Prl release, characterized by Prl discharges occurring approximately every 3 hours, has been reported to occur by the beginning of the juvenile period (167). The most prominent Prl secretory episodes occur at midafternoon and during the early morning hours (168). As the animal approaches the end of the juvenile period, the nocturnal increases in Prl levels disappear, but the afternoon surge becomes even more pronounced (168). The afternoon increase in Prl levels can already be detected by the third week of postnatal life, i.e., during the infantile period (169).

It has been known for several years that Prl accelerates the onset of puberty in females (170). This effect is observed following the systemic (99,170) or intrahypothalamic (171,172) administration of Prl. It appears that one of the mechanisms by which Prl exerts this effect is by enhancing ovarian responsiveness to gonadotropins (161). Prolactin may also act directly to enhance aromatase P-450 gene expression, as shown in ovaries from pregnant rats (173). Chronic stimulation of endogenous Prl release by blockade of dopaminergic receptors advances the onset of puberty and increases the P and E_2 response of the ovary to hCG and FSH (161,174). The ovaries from hyperprolactinemic rats exhibit an increased number of LH receptors (174), suggesting that a significant part of the stimulatory effect of Prl on the immature ovary is the facilitation of LH actions. In contrast to these results, some authors have failed to find an advancing effect of Prl on the timing of puberty in female rats (175).

Other studies have shown that pharmacological inhibition of Prl secretion by administration of bromoergocriptine (CB-154), an ergot alkaloid that activates DA receptors, delays the onset of puberty, and reduces the steroidal response to the ovary to gonadotropins (176). Hypoprolactinemic rats also show a reduced number of ovarian hCG (LH) receptors. Although concomitant administration of Prl can reestablish the inhibitory effect of CB-154, the possibility that subtle changes in LH release may play a role in these effects cannot be completely ruled out. Such changes in plasma LH may have not been detected by the heterologous LH assay employed in these studies.

Experiments involving removal of the adrenal gland have revealed that Prl requires the presence of this gland in order to fully facilitate the ovarian steroidogenic response to gonadotropins (174,177). Adrenalectomy in hyperprolactinemic rats blunts the enhanced P and E_2 response of the ovaries to hCG, whereas corticosterone restores the P, but not the E_2, response (174,177). Since hyperprolactinemic–ovariectomized rats do not show increased serum levels of adrenal P or aromatizable androgens, it does not appear that the adrenal gland mediates the facilitatory effects of Prl on the ovary. Rather, Prl and adrenal products may act at different steps in the sequence of events leading to formation of preovulatory follicles (177).

Like Prl, GH has been shown to exert a facilitatory influence on sexual maturation of the female rat. Suppression of GH release by implantation of GH into the medial basal hypothalamus (178) or by inoculation with the somatomedin-producing worm *Spirometra mansonoides* (179) can delay the onset of puberty, as determined by the age at vaginal opening and at first ovulation. Part of the facilitatory effect of GH on sexual maturation is likely to be exerted at the level of the ovary because GH treatment *in vivo* can increase the ovarian P response to gonadotropins *in vitro* (178). Jia et al. (180) showed that GH facilitates the capacity of FSH to induce LH receptors and to stimulate P secretion from cultured granulosa cells of immature hypophysectomized rats. Moreover, GH also facilitates the stimulatory effect of cAMP and forskolin on P secretion, indicating that the hormone acts at more than one biochemical step to positively regulate granulosa cell function (180). Although some of the actions of GH in the ovary may be exerted directly, there is now substantial evidence indicating that most of them are mediated by insulin-like growth factor I (IGF-I). The IGF-I is produced by granulosa cells and facilitates a number of FSH-dependent effects such as induction of aromatase activity, progesterone secretion, and formation of LH receptors (for a review see ref. 181).

Neural Control

It is now clear that ovarian function is regulated not only by hormones but also by direct neural influences (for reviews see refs. 182–185) that might provide fine,

minute-to-minute control. These neural inputs may also participate in the initiation of ovarian function (see section entitled "Initiation of Ovarian Function: Pituitary and Extrapituitary Regulation"). The immature rat ovary is innervated not only by adrenergic but also by peptidergic nerves. With regard to the adrenergic control, the developing ovary exhibits both readily measurable amounts of NE (140,186) and a well-defined population of adrenergic receptors of the β_2 subtype, the content of which varies in relation to the phases of puberty (187). The β_2-adrenergic receptors are coupled to P and androgen release, an effect that can be demonstrated both by short-term incubation of whole ovaries and by culture of ovarian cells (187–190).

The source of adrenergic input to the ovary appears to be twofold: the adrenergic nerves and circulating epinephrine (EPI) of adrenal medullary origin. Adrenal medullectomy of juvenile rats depresses plasma EPI levels and delays the onset of puberty without altering serum corticosterone levels (191). On the other hand, EPI at nanomolar concentrations was found to stimulate P secretion and to amplify the stimulatory effect of hCG and FSH on P secretion from granulosa cells in culture (187,191). These findings have led to the suggestion that, under physiological conditions, circulating EPI may facilitate the effect of gonadotropins on P secretion and stimulate P secretion on its own (191).

The ovary is innervated by two main adrenergic nerves: the superior ovarian nerve (SON), which carries most of the noradrenergic fibers to the steroidogenic tissue of the gland, and the plexus nerve, which primarily innervates the ovarian vasculature (192). Electrical stimulation of the SON increases P concentration in the ovary of diestrus rats (193). Conversely, sectioning of the SON, when performed in proestrus animals, results in an acute drop in P and E_2 levels in the ovarian vein effluent (194). This suggests that activation of neural inputs reaching the ovary via the SON contributes to maintaining and/or enhancing the increased steroid secretion that occurs on the day of proestrus. In a longer time frame, SON secretion results in a compensatory increase in β-adrenergic receptors (195). This increase is likely to be accompanied by hypersensitivity of steroid secretion in response to β_2-adrenergic stimulation. *In vitro* experiments designed to test this notion have shown that granulosa cells from juvenile ovaries, primed with FSH and then incubated in the absence of NE, release P when exposed to zinterol, a β_2-adrenergic agonist (195). If the cells are preincubated with a dose of NE that is high enough to down-regulate the receptors, zinterol is no longer effective in stimulating P secretion.

The noradrenergic control of ovarian function is initiated early in life, as suggested by the presence of tyrosine-hydroxylase-containing nerves in fetoneonatal ovaries (61). Norepinephrine itself is detected in the ovaries of newborn rats (140,186). The catecholamine content decreases at the time when serum FSH is elevated (second to third week of life) but increases again during juvenile development (140,186). That these fluctuations may be related to changes in circulating FSH levels is suggested by the finding that PMSG injection elicits a decrease in NE content within a few hours of its administration (196). This latter report, however, has not been verified by other authors, who found that NE content in the ovary increased rather than decreased after PMSG injection (186).

The ovarian content of β_2-adrenergic receptors decreases abruptly in the afternoon of the first proestrus and remains at low levels during estrus (187), but neither LH nor FSH can evoke a similar decrease *in vitro* (197). This indicates that catecholamines, rather than gonadotropins, are responsible for the decrease in receptor content seen in the afternoon of first proestrus. Interestingly, corticosterone at physiological levels can depress the β-adrenergic receptor content of granulosa cells *in vitro* by 60% (197), suggesting that ovarian β-adrenoreceptors may be tonically inhibited by corticosterone during prepubertal development.

Although the bulk of available evidence suggests that NE plays a facilitatory role in ovarian steroidogenesis, the turnover rate of ovarian NE has been found to decrease 48 hours after PMSG administration (186), prior to the preovulatory surge of gonadotropins. This implies that the activity of the ovarian NE system declines before the gonadotropin surge. However, measurement of follicular NE during the normal proestrus showed that NE content decreases only after the preovulatory surge of gonadotropins (198). Perhaps the discrepancy results from to the inability of PMSG treatment to faithfully reproduce the changes in ovarian function at puberty (199).

That an activation of noradrenergic neurons projecting to the ovary indeed occurs on the day of proestrus is indicated by the finding of a rise in ovarian NE release at the time of the preovulatory surge of gonadotropins detected in push–pull perifusates of the ovary of freely moving animals (200). Furthermore, other experiments demonstrated that electrical stimulation of ovaries *in vitro* causes a greater increase in [^{3}H]NE release from the ovary of animals in proestrus and estrus than at other phases of the estrous cycle (201), indicating that the activity of ovarian sympathetic nerves is enhanced during the hours encompassing ovulation.

In addition to these observations, evidence exists that the ovarian sympathetic innervation exerts a facilitatory influence on follicular growth (for a review see ref. 183). Support for this concept has been provided by experiments in which development of the ovarian sympathetic innervation was prevented by immunosympathectomy (202,203). Active immunosympathectomy was achieved

by the administration of the adrenergic blocking agent guanethidine (203), which, on chronic administration, initiates an autoimmune response that selectively destroys adrenergic nerves. Passive immunosympathectomy was achieved by treating newborn rats with antibodies to nerve growth factor (NGF) (202). Since the ovary, as a target organ for sympathetic neurons, produces NGF (204), it was expected that blockade of NGF actions would prevent development of the ovarian innervation. This was indeed the case, as the treatment almost completely eliminated the sympathetic nerves of the ovary and reduced the sensory innervation of the gland (202). Importantly, follicular development was significantly delayed (Fig. 3), estradiol release in response to gonadotropins was reduced, puberty was delayed, and the animals exhibited marked irregularities of the estrous cycle. Since these abnormalities were observed long after administration of the antibodies, the inevitable conclusion is that loss of the innervation abolished the sympathetic input to developing follicles and, therefore, deprived them of a facilitatory influence. Without negating the validity of this conclusion, recent experiments suggesting a participation of members of the NGF family in the initiation of folliculogenesis (63) have indicated that destruction of the ovarian sympathetic nerves by immunosympathectomy may not be the only explanation for the alteration in follicular development observed. The interested reader is referred to pertinent reviews for a more detailed discussion of the matter (12,63,205).

The neural inputs arriving at the ovary are not limited to noradrenergic fibers. Examination of ovarian sections using immunohistofluorescence demonstrated the presence of delicate nerve fibers containing either substance P (SP), VIP, or neuropeptide Y (NPY). These fibers innervate the ovarian vasculature and interstitial tissue and are associated with the thecal layers of developing follicles (66,206,207). All three ovarian peptides are immunologically and chromatographically indistinguishable from the authentic peptides, as revealed by their cross-reactivity in the respective RIAs and their behavior in Sephadex G-25 or HPLC. Interestingly, the peptidergic innervation of the ovary reaches the gland via different routes: nerve fibers containing SP and NPY are carried by the plexus nerve (207,208), and VIPergic nerves travel within the superior ovarian nerve (208). In vitro experiments have shown that neither SP nor NPY affects ovarian steroidogenesis in the rat (209,210), although they may do so in porcine ovaries (211). Neuropeptide Y, on the other hand, has been shown to reduce the release of NE from ovarian nerves via activation of prejunctional autoreceptors of the Y2 subtype (210), suggesting that one of the functions of NPY in the ovary

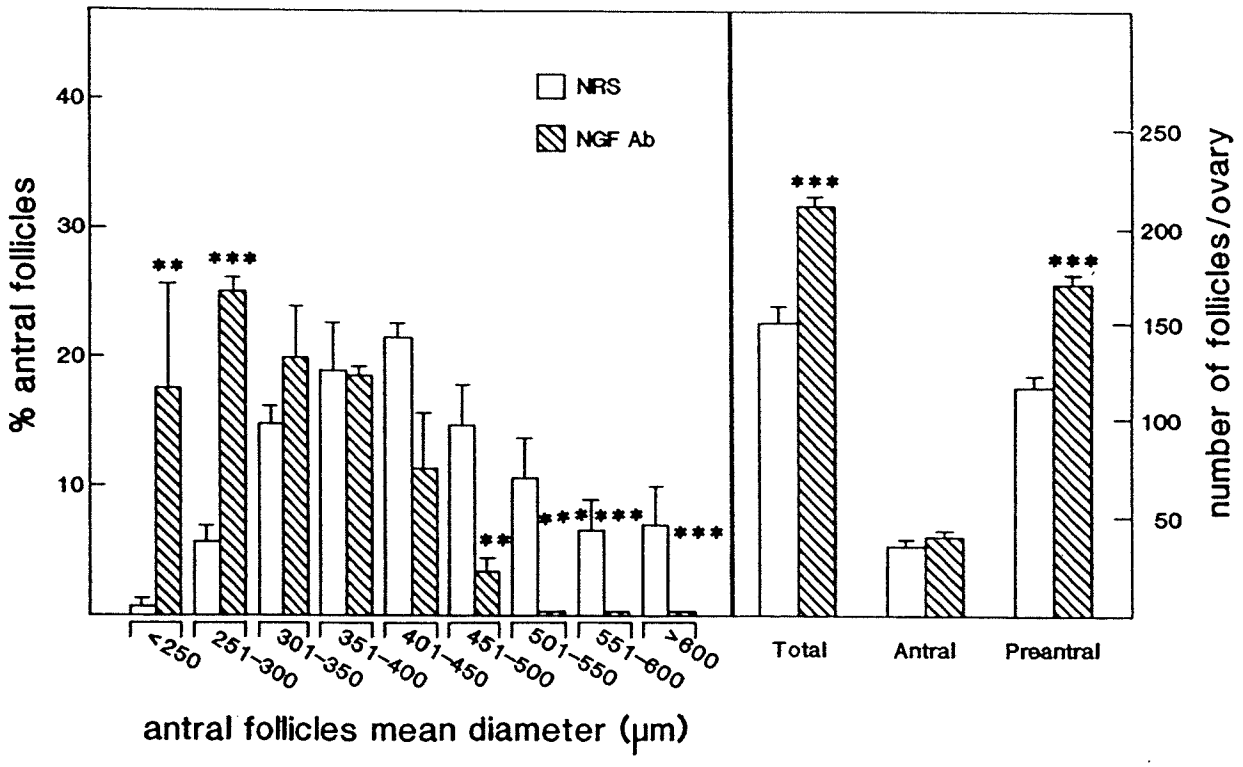

FIG. 3. Effect of neonatal administration of antibodies to NGF (NGF Ab) on follicular development of juvenile 29- to 30-day-old rats. Morphometric analysis of follicular size was performed as described in ref. 202. $^{**}p < 0.02$; $^{***}p < 0.001$ (versus normal rabbit serum, NRS-treated controls). (From ref. 202, with permission.)

is to regulate the availability of NE to its ovarian receptors.

In contrast to SP and NPY, VIP is a potent stimulator of ovarian steroidogenesis, as it elicits progesterone, estradiol, and androgen release from either whole ovaries or ovarian cells in culture (66,212). The molecular mechanisms underlying these effects involve an enhanced synthesis of all three components of the cholesterol side-chain cleavage (SCC) enzyme complex (213), the rate-limiting enzyme in steroid biosynthesis, as well as stimulation of aromatase enzyme activity (65,212). VIP appears to affect SCC synthesis by up-regulating SCC cytochrome P-450 mRNA levels (214). That the peptide may play a role in early granulosa cell function is suggested by the finding that VIP targets a subpopulation of granulosa cells that is unresponsive to FSH (215). Moreover, VIP is an effective inducer of aromatase activity in feto-neonatal prefollicular ovaries (65), which are unresponsive to gonadotropins. Conceivably then, VIP may contribute to the process of granulosa cell differentiation, a possibility inferentially supported by the facts that the actions of VIP are exerted via activation of cAMP formation, and that cAMP itself induces granulosa cell differentiation (216).

In addition to this role, VIPergic nerves may contribute to facilitating the stimulatory effect of gonadotropins on ovarian steroid secretion at proestrus. This is suggested by the rapid drop in estradiol (E_2) and progesterone (P) secretion that results from transection of the SON in the afternoon of proestrus (194), at the time of elevated plasma gonadotropin levels, and in the absence of measurable changes in blood flow. Although part of this effect may be caused by the loss of NE inputs, the drop in estradiol secretion may be best attributed to the transection of VIPergic fibers because VIP, but not catecholamines, stimulates estradiol secretion (66,187,188, 195,212).

An additional piece of evidence supporting an involvement of ovarian nerves in the developmental regulation of ovarian function was provided by the demonstration that transplanted ovaries fully recover their ability to keep gonadotropin secretion in check around the time when their reinnervation is completed (217). These experiments also showed that if the reinnervation is prevented by neonatal immunosympathectomy, the negative feedback loop controlling gonadotropin secretion is disrupted because of the inability of the transplanted ovary to maintain FSH secretion at basal levels.

In summary, the foregoing observations permit the conclusion that the nervous system directs the maturation of the ovary via two main routes, a hormonal and a neural one (Fig. 4). Whereas the former involves the secretion of hypothalamic factors that control the secretion of LH, FSH, Prl, and GH from the adenohypophysis, the latter directly links the CNS to the ovary via peptidergic and adrenergic nerves. Moreover, EPI of adrenal medullary origin may facilitate ovarian function after reaching the gland via the bloodstream. It appears that most of these regulatory mechanisms become firmly established during the juvenile period.

The Onset of Puberty

The Initial, Gonadal-Independent Activation of Pituitary Gonadotropin Secretion

It is now clear that the onset of puberty is determined by a multiplicity of interrelated events, some of which originate during the infantile period. Nevertheless, the first unambiguous hormonal manifestation that puberty is under way occurs only after the fourth postnatal week of development and is expressed as a diurnal change in the mode of LH release. The experiments of Meijs-Roelofs et al. (218) were the first to demonstrate an unequivocal prepubertal increase in mean LH levels, which became apparent 8 to 9 days before the expected day of first proestrus and appeared to be greater in the afternoons than in the mornings. An earlier characterization of the mode of LH release in peripubertal rats (102) showed that, starting around day 30 (vaginal opening occurs around day 38), a diurnal pattern of release developed in the female rat. This pattern was characterized by an afternoon increase in LH pulse amplitude. In subsequent experiments the mode of LH release was more precisely characterized in blood samples obtained every 5 min (22) by means of an automated blood-sampling technique (219). The results clearly indicated that, during the fifth postnatal week of life, both basal LH levels and LH pulse amplitude become greater in the afternoon than in the morning (Fig. 5). That this diurnal change in the mode of LH release is physiologically relevant to the functional development of the ovary is evidenced by experiments in which peripubertal ovaries were perifused with LH regimens designed to mimic either morning or afternoon pulses of LH secretion (220). The results demonstrated that LH pulses of an amplitude similar to that seen in the afternoon of the peripubertal period elicited significantly more E_2 and P release than did morning-type LH pulses. In addition to showing an afternoon change in LH pulse amplitude, some peripubertal animals also exhibited a more sustained midafternoon episode of LH secretion, which has been termed a "minisurge" of LH (22). Mimicking such a secretory episode *in vitro* also led to enhanced E_2 and P secretion from the ovary (220), thus suggesting that LH minisurges may also be important for the peripubertal activation of ovarian function.

With regard to the mechanisms underlying the two types of change in the mode of LH release, evidence ex-

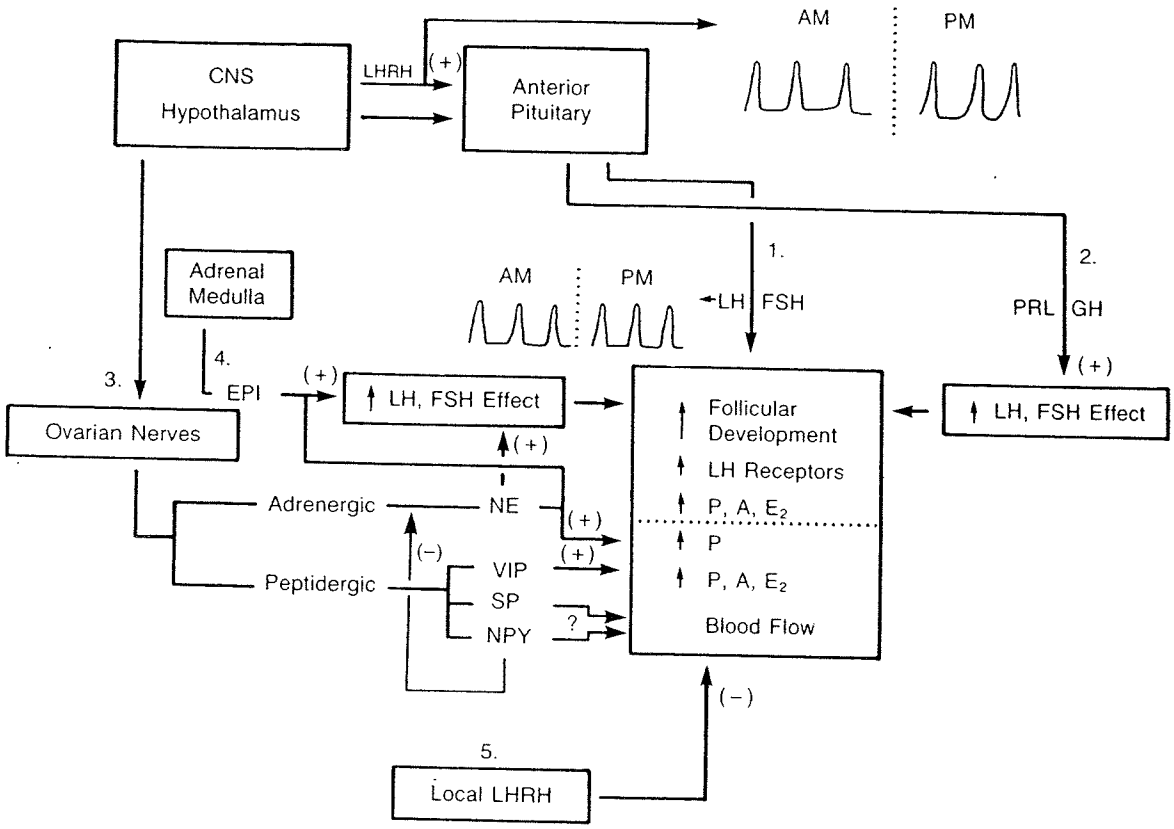

FIG. 4. Hormonal and neurogenic factors controlling ovarian development during the juvenile period of the female rat. A similar pattern of LH release in the mornings and afternoons is assumed to reflect similar patterns of LHRH release. Numbers indicate the different control mechanisms involved in regulating ovarian function; +, facilitatory; −, inhibitory; ?, effect not known; AM, morning; PM, afternoon; CNS, central nervous system; LHRH, luteinizing hormone-releasing hormone; EPI, epinephrine; NE, norepinephrine; VIP, vasoactive intestinal polypeptide; SP, substance P; NPY, neuropeptide Y; P, progesterone; E_2, estradiol; A, androgens; PRL, prolactin; GH, growth hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone. (Modified from ref. 32.)

ists that the afternoon increase in LH pulse amplitude is not ovarian dependent (221) and, more specifically, is not E_2 induced (222). On the other hand, the minisurges of LH secretion appear to be caused by subtle increases in serum E_2 levels (222). These conclusions are based on experiments in which short-term (48-hour) ovariectomy of prepubertal rats was found to result in greater plasma LH levels in the afternoon than in the morning (221). Detailed examination of the LH release pattern at this time, using an automated 5-minute bleeding paradigm (219), revealed that these high LH values were not caused by a sustained surge of LH release but were, instead, the consequence of the increased amplitude of the LH pulses. When mean precastration serum E_2 levels were produced in short-term ovariectomized juvenile rats via subcutaneous Silastic capsules, pulsatile LH release was inhibited rather than enhanced. The use of larger doses of exogenous E_2 consistently failed to induce an increase in LH pulse amplitude but instead resulted in the appearance of minisurges and proper surges of LH secretion (222).

Additional support for the concept that the diurnal changes in LH secretion observed at the end of the juvenile period are centrally driven comes from the results of experiments examining Prl release. It is known that Prl secretion occurs episodically (167) and that during development of the female rat Prl levels become more elevated in the afternoons than in the mornings (27,168,169). Kimura and Kawakami (223) first observed that ovariectomy of early juvenile rats did not abolish the afternoon surges of Prl, indicating that these secretory episodes could occur in the absence of the ovaries. In other studies it was found that when neonatal female rats were ovariectomized and their plasma patterns of Prl subsequently examined at different ages, at least 50% of the animals exhibited a midafternoon secretory episode of Prl release, even as late as 40 days after ovariectomy (224). Restoration of juvenile serum E_2 levels via subcutaneous E_2-containing Silastic capsules resulted in amplification of the surge, which still occurred at the same time of the day. Thus, these results (223,224) demonstrate that the neural mechanism responsible for

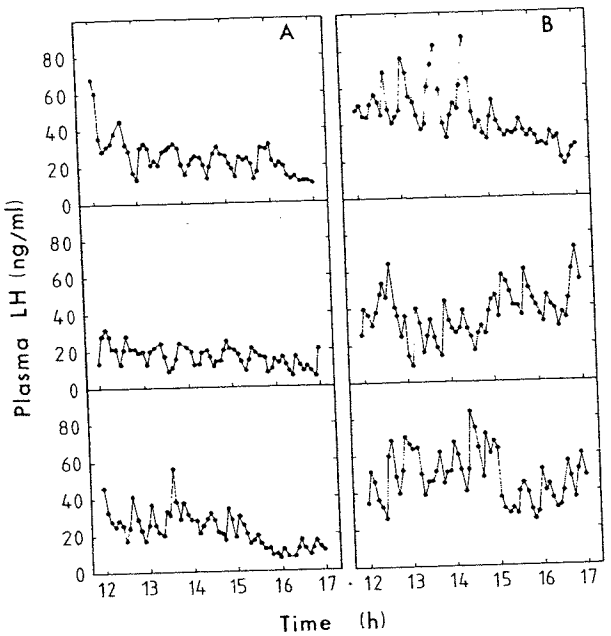

FIG. 5. Representative afternoon plasma LH profiles from juvenile (**A**: 27- to 29-day-old) and peripubertal (**B**: 30- to 38-day-old) female rats bled continuously for 5 hours. Six individual profiles from a total of 16 are depicted. Pulses of LH secretion are indicated by *arrows*. (From ref. 22, with permission.)

the afternoon appearance of a Prl surge can develop in the absence of ovarian influences and that ovarian E_2 amplifies the magnitude of the Prl surge.

A quite different conclusion has been drawn from studies performed to elucidate the mechanisms determining the peripubertal "minisurges" of LH secretion (222). Such sustained episodes of release could not be detected in either short- or long-term ovariectomized rats. Only when circulating E_2 levels were slightly increased over juvenile values (via subcutaneous E_2-containing Silastic capsules) did a minisurge of LH secretion occur, indicating that they are E_2 dependent.

Another factor that may play a role in accelerating ovarian maturation at the end of juvenile development is a change in the biological activity of circulating gonadotropins. As already mentioned, evidence exists that as the female rat matures, the biological activity of pituitary FSH increases (95). With respect to LH it appears that the hormone exists as different molecular forms in the bloodstream of prepubertal rats; when PMSG was administered to 27-day-old females, two different patterns of serum LH emerged, depending on which of two antisera was used in the RIA (225). Interestingly, this divergence was observed more clearly in the lighter animals (<60 g body weight), which also failed to ovulate. The same laboratory reported that LH released in PMSG-treated rats lighter than 60 g was measurable by RIA but was inactive in a cytochemical bioassay (97). In contrast, LH released in PMSG-treated rats heavier than 60 g was active in both the RIA and the cytochemical bioassay employed. Surprisingly, however, both types of LH were equally effective in stimulating T secretion from testicular interstitial cells.

There is little doubt that the diurnal changes in pulsatile LH release detected at the initiation of puberty result from the activation of LHRH secretion. The potential mechanisms underlying this activation are discussed later in this review. It appears clear, however, that the resulting changes in LH secretion are important for the continuation of puberty because, under the influence of these LH secretory episodes, the ovary is stimulated to produce more E_2 (220). In turn, subtle increases in E_2 levels appear to be able to evoke minisurges of LH secretion (222), which can induce further ovarian activation (Fig. 6).

The Precipitation of Events: Activation of the Ovary

Once the diurnal pattern of LH release becomes established, a new cascade of events develops that culminates with the first preovulatory surge of gonadotropin and the first ovulation. The necessity of examining the many components of this cascade has made it important to divide the process of puberty into different phases. According to this classification, which is mainly based on morphological criteria (27,164), puberty in the female rat can be divided into the following phases:

1. *Anestrus (A).* Originally this phase was meant to correspond to the late juvenile phase, but it now appears to correspond to the phase during which the changes in the mode of LH release begin to occur (22). Animals in this phase are about 30 days of age, their uteri are small (wet weight less than 100 mg), and, importantly, no intrauterine fluid can be detected. The vagina is always closed.
2. *Early proestrus (EP).* Animals in this phase have larger uteri with intraluminal fluid; their vagina is closed. A further division into EP-1 and EP-2 subphases has been proposed, based on the amount of uterine fluid and the uterine weight observed (164).
3. *Late proestrus (LP).* This phase corresponds to the day of first proestrus. Animals have large "ballooned" uteri full of fluid, with a wet weight greater than 200 mg. Their ovaries have large follicles. Most animals in this phase show closed vaginae.
4. *Estrus (E).* This is the day of first ovulation, when uterine fluid is no longer present, fresh corpora lutea can be readily discerned, the vagina is open, and vaginal cytology shows a predominance of cornified cells.
5. *First diestrus (D).* This phase of puberty is characterized by a vaginal cytology showing a predominance of leukocytes as well as by the presence of mature corpora lutea within the ovaries.

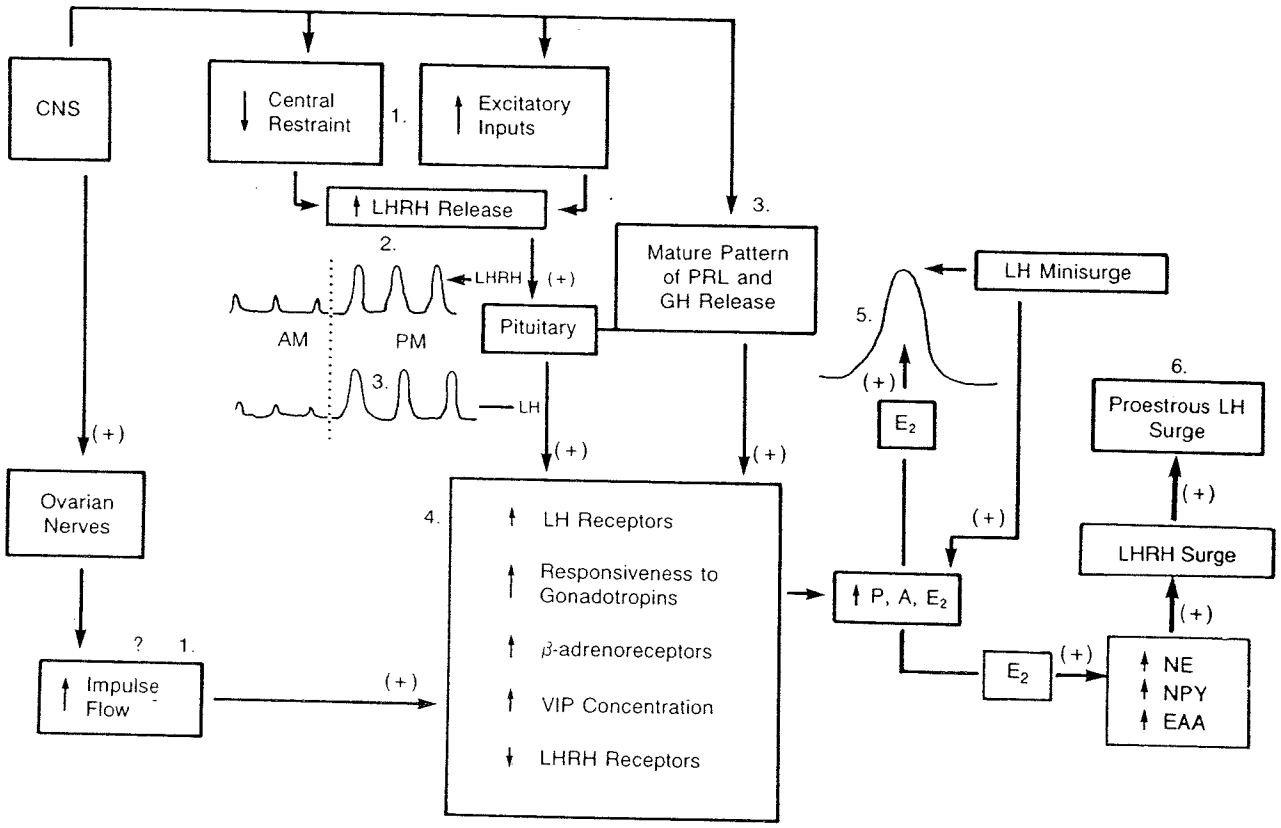

FIG. 6. Postulated cascade of initial events during the onset of puberty in the female rat. Activation of LHRH release in the afternoon is proposed to be determined by an increase in excitatory inputs to LHRH neurons coupled to a reduction in transsynaptic inhibitory influences. Whether or not the activity of ovarian nerves also increases at this time is not known, but it is suggested by the changes in ovarian β-adrenergic receptors and ovarian VIP concentration. The numbers indicate the sequence in which these events may occur. NPY, neuropeptide Y; NE, norepinephrine; EAA, excitatory amino acids. For other abbreviations see Fig. 4. (Modified from ref. 32.)

With the onset of puberty already determined by the activation of pulsatile LH release, the single most important event that remains to be defined is the timing of the first preovulatory surge of gonadotropins, which, in itself, represents the climax of female neuroendocrine reproductive maturation. There is little doubt, in our view, that both the occurrence and the timing of this final event depend on the completion of ovarian maturation. Only when the ovary becomes capable of producing E_2 levels of sufficient magnitude, and for a sufficiently long period of time, will the preovulatory LH surge occur.

A multitude of maturational changes appears to be involved in hastening the acquisition of preovulatory competence by the ovary (Fig. 6). Whereas FSH receptor content is already maximal by the end of juvenile development, the number of LH receptors in granulosa cells increases dramatically between the A and LP phases of puberty (53,54). Concomitant with this increase, a decline in LHRH receptor content occurs, and the magnitude of the decrease is more pronounced between A and EP than at later times (163). The implications that these two divergent changes in hormone receptor may have for ovarian function have already been discussed. It is noteworthy that during the days preceding the preovulatory gonadotropin surge, the steroidal responsiveness of the ovary to gonadotropins increases dramatically (162,164), most likely reflecting the progressive development of the follicles destined to ovulate at the first estrus.

The neurogenic component of the ovary also undergoes noticeable changes. The content of β-adrenergic receptors increases between A and LP and then declines abruptly at the time of the proestrus surge (187). Paralleling the increase in receptor content, the release of P in response to β-adrenergic stimulation becomes more prominent at LP. However, the greatest increase in response occurs after ovulation, more specifically during the first E. Surprisingly, at this time the receptor content is low.

The concentration of VIP in the ovary, which remains almost unchanged between the second postnatal day and the end of juvenile development, increases significantly during the early part of the peripubertal period (days 30 to 35) (67). Moreover, the steroidogenic response to VIP undergoes profound changes at the time of puberty (66).

The E_2 response to VIP, already distinct in juvenile rats, increases noticeably during the EP and LP phases of puberty. The P response to the peptide increases only moderately at this time, then strikingly after ovulation. Radioimmunoassayable SP content in the ovary also increases between A and LP (209). Although the function of these changes in SP content is unknown, it would not be unreasonable to suspect that they may be implicated in the edematization of the ovary that occurs at puberty (226) and/or in the changes in blood flow that occur during the estrous cycle (227,228).

Presumably as a consequence of this marked enhancement in facilitatory inputs to the ovary, the pattern of steroid production changes dramatically. Serum E_2 levels increase markedly between A and LP (28,164,229, 230), reaching about 80 pg/ml during the morning of LP (230). Serum P increases moderately before the LH surge, but serum T levels do so more prominently (229, 230). This increase in T (or, in more general terms, aromatizable androgens) appears to be relevant to the mechanism of vaginal opening. It has been shown that the production of early proestrus plasma levels of T, via T-containing Silastic capsules, in late juvenile rats results in precocious vaginal opening but does not advance the first ovulation (129). Examination of serum E_2 levels in animals treated with T shows that E_2 is not increased by the exposure to elevated T levels. Nevertheless, the vaginal epithelium is able to metabolize androgens via an aromatase-like reaction (231), suggesting that the hastening effect of physiological levels of T on vaginal opening may result, at least in part, from local estrogen production by aromatization.

While the secretion of E_2, P, and T increases, the secretion of 3α-androstanediol diminishes (232,233), a change that becomes much more pronounced during the hours encompassing the first preovulatory LH surge (234,235) and that seems to be elicited, at least in part, by the rising Prl levels (234). Based on the findings that ovarian 5α-reductase activity decreases markedly at puberty (236) and that administration of 3α-diol delays the timing of first ovulation (237), Eckstein and colleagues (236,237) have proposed that 3α-diol is involved in restraining the onset of puberty. A decrease in serum 3α-diol, however, does not appear to have major consequences on gonadotropin release, since neither blockade of 5α-reductase activity nor administration of physiological levels of 3α-diol was found to affect the time of puberty in female rats (235). Moreover, the prepubertal decrease in ovarian production of 3α-diol is not maintained because levels of the steroid increase again on the second proestrus (233).

The First Preovulatory Surge of Gonadotropins

The acquisition by the ovary of the capacity to secrete sufficient E_2 for an adequate period of time represents the key event that determines the timing of puberty in the female rat. Passive immunoneutralization of circulating E_2 levels prevents the LH discharge and ovulation (238), underscoring the importance of the steroid for the occurrence of the first gonadotropin surge. Estrogen acts on both the anterior pituitary and the hypothalamus to bring about the proestrus surge. In the hypothalamus, it evokes a discharge of LHRH release (106); in the pituitary, it sensitizes the gonadotropes to the stimulatory effect of LHRH (239). A direct stimulatory effect of P on LHRH release in immature rats has also been demonstrated (240). This finding suggests that the two- to three-fold increase in serum P observed before proestrus (28,164,229,230) may have a role in facilitating the stimulatory effect of E_2 on LHRH release.

The stimulatory effect of P on LHRH release may be mediated, at least in part, by an increase in LHRH gene expression, since P markedly increases steady-state LHRH mRNA levels within 4 to 6 hours of its administration to immature rats (241,242). In the female rat, an increase in pituitary responsiveness to LHRH is only observed on the day of proestrus (105,106), indicating that some elevation in basal LHRH output may be necessary for the responsiveness of the pituitary to increase in the presence of elevated E_2 levels. As a consequence of the release of LHRH and/or LHRH-related peptides (243), available pituitary LHRH receptors decline in the afternoon of proestrus (244,245). This phenomenon can be reversed by preventing the expression of neural events leading to LHRH release (244,245). The decrease in LHRH receptors appears to reflect ligand-induced unavailability of receptor for binding, rather than representing a true loss of receptors (245).

Little is known regarding the hypothalamic mechanism by which E_2 activates an LHRH surge at puberty. Although no E_2 receptors have been detected in LHRH neurons (246), molecular characterization of the 5′ flanking region of the human LHRH gene revealed the presence of an estrogen response element approximately 500 base pairs upstream from the transcription initiation site (247). Transient transfection experiments utilizing a human choriocarcinoma cell line as the host and either the luciferase or chloramphenicol acetyltransferase gene as reporter genes demonstrated the ability of E_2 to stimulate the transcriptional activity of the LHRH gene (247), suggesting that, at least in humans, LHRH gene expression may be directly regulated by E_2. In contrast to this, no estrogen response elements were detected in the 5′ regulatory region of the rat gene (248). These observations suggest that a significant part of the stimulatory action of E_2 on LHRH gene expression (249) and secretion is exerted via a neurotransmitter system functionally and anatomically coupled to LHRH neurons. Early reports implicated catecholaminergic and serotoninergic pathways in the process. More recently, evidence has accumulated suggesting an involvement of two additional

neuronal systems: one that uses NPY as a transmitter and another that employs excitatory amino acids (see below).

In regard to serotoninergic neurons, it has been shown that blockade of serotonin synthesis depresses FSH secretion (250), delays vaginal opening (251), and inhibits the gonadotropin surge induced by PMSG in immature rats (252). Conversely, pharmacological activation of serotoninergic transmission facilitated E_2-induced LH surges (253,254) and restored the surge response of LH to E_2 in ovariectomized rats that lost the response after chronic E_2 treatment (255). Estradiol also increases serotoninergic receptors in brain (256). Although these observations suggest a facilitatory role of serotonin on gonadotropin secretion, other results indicate that the serotoninergic system may both facilitate and inhibit steroid-induced gonadotropin release in immature rats. Thus, blockade of serotonin synthesis advances the onset of E_2/P positive feedback, whereas administration of the serotonin precursor 5-hydroxytryptophan to rats younger than 26 days of age stimulates LH release (123). Clearly, more work is needed to define the role of serotonin in the initiation of puberty.

A stimulatory effect of catecholamines on LHRH release at puberty is consistently supported by the available evidence. For instance, inhibition of catecholamine biosynthesis prevents ovarian compensatory hypertrophy in prepubertal rats (257), and microinjection of α-methyldopa, which results in the formation of "false" catecholamines, blocks PMSG or hCG-induced LH release when injected during the critical period of proestrus (258). These authors have concluded that DA is the catecholamine involved in facilitating the proestrus LHRH discharge, but others have contended that DA plays an inhibitory role instead (121,259).

An involvement of NE in stimulating LHRH release at puberty has been shown directly by the finding that selective destruction of NE terminals by intraventricular injection of 6-hydroxydopamine blunts the LHRH surge induced by PMSG in immature rats (260). Noradrenergic and serotoninergic turnover increases before the proestrus surge of gonadotropins occurs, with the former increasing more noticeably during early proestrus and the latter increasing on the day of first proestrus (261). The peripubertal enhancement in NE activity may, at least partially, result from an E_2 action, because E_2 has been shown to increase NE turnover in the hypothalamus (for reviews see refs. 262 and 263) and to promote NE release from hypothalamic slices *in vitro* (264). Furthermore, noradrenergic neurons have been found to contain E_2 receptors (265), and NE-containing neurons in the area of the nucleus tractus solitarius (A2 cells), which project to the median eminence and rostral hypothalamus, have been shown to respond to E_2 with genomic activation as shown by an increase in *c-fos* expression (266).

A sizable body of evidence has accumulated in recent years implicating NPY in the control of gonadotropin secretion (for reviews see refs. 267–269). Its participation in the maturational process that leads to the initiation of puberty is suggested by the marked increase in hypothalamic NPY content that occurs during the infantile–juvenile phases of development (270). That NPY is also involved in the genesis of the first preovulatory surge of gonadotropins was demonstrated by the findings that the secretion of NPY into the portal blood increases in the afternoon of the first proestrus (270) and that immunoneutralization of NPY on the day of first proestrus inhibited the LHRH surge and attenuated the preovulatory increase in plasma LH (271). The presence of E_2 receptors in NPY neurons of the arcuate nucleus (272) suggests that E_2 acts directly on NPY neurons to affect their secretory activity (273). The NPYergic neurons involved in stimulating LHRH secretion may be those located in the hypothalamus rather than catecholaminergic neurons of the brainstem that coexpress the peptide with NE (268,269,274). Of potential relevance for the understanding of the mechanisms underlying the diurnal change in pulsatile LH release that characterizes the initiation of puberty is the observation that NPY levels in the suprachiasmatic and arcuate nucleus—but not in other hypothalamic nuclei—show a diurnal rhythm, with peak levels occurring at the end of the light phase of the photoperiod (275).

Several other reports have provided rather compelling evidence for the participation of still another neurotransmitter system in the mechanism by which estradiol elicits the first preovulatory surge of gonadotropins. This stimulatory system uses excitatory amino acids (EAA) as neurotransmitters. Experiments conducted independently in rhesus monkeys and rats first demonstrated that pulsatile administration of *N*-methyl-D-aspartic acid (NMDA) was able to elicit LH release and advance the onset of puberty (276,277). That endogenous EAA acting via activation of NMDA receptors are physiologically involved in the genesis of the first preovulatory surge of gonadotropins was demonstrated by the finding that blockade of NMDA receptors with either competitive or noncompetitive receptor antagonists delayed the initiation of puberty and inhibited the LH surge induced by estradiol (278). A similar conclusion was reached by other authors in subsequent reports on the matter (279–285). Furthermore, treatment with E_2 and P, which by itself increases plasma gonadotropin levels, has been shown to potentiate the stimulatory effect of NMDA on LH and FSH release (286). It thus appears that E_2 activates more than one neurotransmitter system functionally coupled to LHRH neurons to elicit the first preovulatory surge of gonadotropins. Whether activation of these systems is a synchronized, interdependent phenomenon or simply represents a high degree of redundancy aimed at ensuring the occurrence of the surge is not known.

With regard to the intracellular mechanisms underly-

ing the first preovulatory discharge of LHRH, evidence exists that E_2 may alter different steps in the sequence of events leading to LHRH release. The capacity of the hypothalamus to synthesize PGE_2 from arachidonic acid increases during the days preceding the preovulatory LH surge (287). This increase can be mimicked by the administration of E_2 to juvenile rats at a dose that evokes a premature surge of LH.

Incubation of median eminences from animals exposed *in vivo* to proestrus levels of E_2 with various concentrations of NE or PGE_2 indicated that E_2 facilitates LHRH release by acting at two different biochemical steps (118). On the one hand, it permits PGE_2 formation to be stimulated by lower doses of NE. On the other hand, it increases both the sensitivity and the responsiveness of the LHRH terminals to PGE_2.

These results suggest that E_2 exerts part of its stimulatory action on LHRH release by activating a NE and PGE_2-dependent pathway. On the other hand, evidence exists that cAMP is involved in the process of LHRH secretion and that formation of cAMP occurs at a step subsequent to NE-induced synthesis of PGE_2 (288,289). Thus, PGE_2 increases cAMP production in hypothalamic fragments *in vitro*, and methoxamine, an α_1-adrenergic agonist, enhances both cAMP formation and LHRH release, an effect that is blocked by indomethacin, an inhibitor of prostaglandin synthesis (288). Indomethacin also blocks NE-induced LHRH release without altering the stimulatory effect of PGE_2 on LHRH output (290). When adenylate cyclase activity is stimulated by forskolin, cholera toxin, or pertussis toxin, LHRH release is enhanced without an increase in PGE_2 formation (289). Since simultaneous exposure of median eminence nerve terminals to PGE_2 and forskolin does not result in a greater LHRH response than that elicited by either agent individually, PGE_2 and cAMP might act along a common pathway (288).

Because E_2 can also increase cAMP formation in the hypothalamus (291), it can be suggested that the preovulatory surge of LHRH at puberty involves the activation of a PGE_2–cAMP pathway. This mechanism, however, may not be the only one that operates at the first proestrus, as suggested by the finding that activation of protein kinase C, a Ca^{2+}-activated phospholipid-dependent kinase (for a review see ref. 292), by either a synthetic diacylglycerol or a phorbol ester results in LHRH release (293). Moreover, phospholipase C, which in intact cells catalyzes the hydrolysis of membrane polyphosphoinositides to generate diacylglycerol, also induces LHRH release. Activation of this protein-kinase-C-dependent pathway was found to induce release of LHRH independently of the PGE_2–cAMP pathway. Thus, blockade of PG synthesis fails to suppress diacylglycerol-induced LHRH release or a phorbol-ester-induced LHRH release without affecting PGE_2 formation. Coexistence of both pathways is indicated by the finding that exposure of median eminences to maximally effective doses of diacylglycerol or phorbol ester together with NE, PGE_2, or

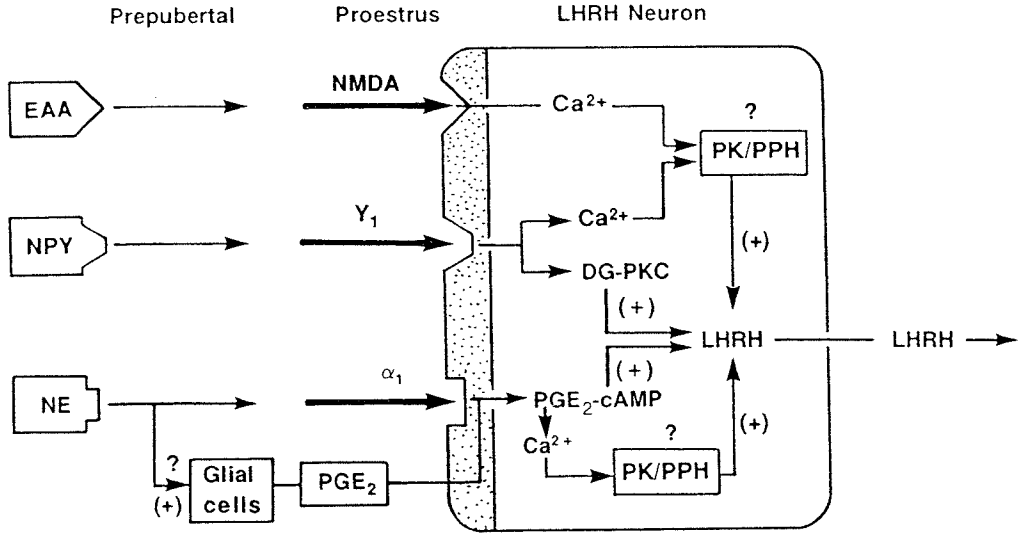

FIG. 7. Postulated intracellular pathways involved in the transduction of neurotransmitter signals leading to LHRH release during puberty in the female rat. During prepubertal days all pathways may be operative, but the intensity of the extracellular inputs activating them is low (*thin arrows*). In the afternoon of proestrus, an increased activity (*thick arrows*) of neurons that use NE or EAA as neurotransmitters, and of another neurotransmitter system (NPY?) that presumably activates the PKC pathway, induces the preovulatory discharge of LHRH. The effect of NE may be exerted via activation of glial PGE_2 release. NE, norepinephrine; PKC, protein kinase C; PGE_2, prostaglandin E_2; Ca^{2+}, calcium; ?, unknown. (Modified from ref. 20.) For simplicity, the cellular events leading to intracellular Ca^{2+} mobilization (e.g., phospholipase C activation, phosphatidylinositol hydrolysis) are not represented. The assumption is also made that the stimulatory effect of Ca^{2+} on LHRH release involves activation of protein kinases (PK) and protein phosphorylation (PPH).

forskolin results in an additive effect on LHRH release (293). It appears that activation of both cAMP and protein-kinase-C-dependent intracellular pathways result in up-regulation of LHRH gene expression (294) in addition to the changes in LHRH output.

The aforementioned observations suggest that simultaneous activation of both pathways may be required for the proestrus surge of LHRH to occur (Fig. 7). Such a mechanism implies that NE, by itself, acts on two subtypes of adrenergic receptors or that NE and another neurotransmitter (NPY?) act on different receptors to provide the extracellular dual signal that increases LHRH release. That NPY may be a neurotransmitter involved in this process is suggested by the ability of NPY to stimulate LHRH release in an intracellular Ca^{2+}-dependent, PG-independent manner via activation of Y-1 receptors (295,296). In addition, a third pathway that requires extracellular Ca^{2+} and is activated by the interaction of EAA with NMDA receptors is postulated to contribute to the proestrous LHRH surge. The effect of NPY and NE may be exerted directly on LHRH neurons (297,298), but it is unclear whether EAAs act directly or via NE neurons (299) to facilitate LHRH release. An additional intracellular event triggered by preovulatory levels of E_2 is an increased expression of the early immediate genes *c-fos* and *c-jun* in LHRH neurons (300 and references therein). Although well documented, it is unclear whether such a change is required for the LHRH surge to occur or is a consequence of the preovulatory LHRH discharge.

The model depicted in Fig. 7 assumes that both PGE_2 and cAMP operate within the LHRH neuron itself, but this may not be the case. Recent evidence obtained while studying the ability of growth factors to affect LHRH release suggests an alternative explanation, namely, that PGE_2 is produced by glial cells morphologically associated with LHRH neurons and that on release it acts on the neurons to activate the intracellular steps that lead to LHRH release (12) (Fig. 7). Though not yet firmly documented, this interpretation is supported by the finding that the neuronal LHRH-producing cell line GT1-7 responds to PGE_2 with LHRH release but is unable to produce the PG in response to several secretagogues (301).

THE MALE

Fetal Development

Initiation of LHRH and Gonadotropin Secretion

As mentioned earlier, Aubert et al. (33) detected traces of LHRH in whole-brain extracts as early as gestational day 12. However, in this study, male and female tissue was pooled, and so it is unknown whether sex differences exist during this early period of development. Likewise, even though it is clear that the hypothalamus contains radioimmunoassayable LHRH by gestational day 15 (44), it is unknown whether any sex difference exists at this time. Significantly, LHRH receptors are already detectable in the anterior pituitary of male rats at gestational day 16 (33), suggesting an active involvement of LHRH in the control of fetal pituitary function. The number of receptors then increases in parallel with hypothalamic LHRH content, but with a phase delay of a few days. Studies using pooled pituitary homogenates from males and females have found radioimmunoassayable LH and FSH as early as gestational days 15 and 19, respectively (39). However, studies in which the sexes were segregated before use have yielded conflicting results with regard to sex differences. In one report, pituitary LH could be detected in male rats from gestational day 17 onward, but not in females (47). The bulk of the evidence, however, indicates that pituitary LH can be detected in both sexes at this age and that pituitary LH content tends to be greater in the female (33,39,45).

Initiation of Testicular Function

Sexual differentiation of the male gonad begins very early in life, with the seminiferous cords being formed at gestational day 13 (for a review see ref. 302). Testicular LH receptors have been detected as early as gestational day 15.5 (303), and it is noteworthy that by this time LH stimulation causes an increase in cAMP and testosterone (T) production (304–306). The LH receptor content increases further at gestational day 18.5, and maximum levels are attained around the time of birth. Coincident with this rise, the number of interstitial cells increases (307), as does testicular T content (303,308). [Note: The adult population of interstitial cells appears to be functionally different from the population found in the fetal testis (307,309).] This fetal T is thought to play an important role in male sexual differentiation (310); its concentration declines soon after birth, most likely because of alterations within the steroidogenic pathway. Sertoli cells are present within the seminiferous tubules before birth and undergo rapid division toward the end of gestation; within the first few weeks of postnatal life, however, they cease to divide (311–314). The Sertoli cells play a key role in the initiation of spermatogenesis, and they have been shown to possess FSH receptors from as early as gestational day 17.5 onward, rising markedly just before birth (303,315,316). These findings suggest that FSH and LH are functional in controlling testicular development even before birth.

Postnatal Development

This section discusses postnatal development of the hypothalamic–pituitary–testicular axis. Initially, a sepa-

rate description is given of the changes that occur in each of the system's components. An attempt is then made to show how the basic components are integrated to produce a functional neuroendocrine unit as well as how this unit operates during the onset of puberty.

The Hypothalamic–Pituitary Unit

In the male rat, hypothalamic LHRH levels continue to increase throughout postnatal development (310, 317–319). However, unlike the situation in the female, where levels reach a maximum just before proestrus, in the male they increase even during adulthood. This ultimately results in a significant sex difference between the adult LHRH levels (44,74,320).

Similarly, the pituitary content of LH and FSH increases gradually with age, as does the responsiveness of the gland to LHRH stimulation (44,319,321–323). In the male, the maximum FSH response occurs between 25 and 35 days of age, whereas the maximum LH response occurs between 35 and 45 days of age (82,324). This is several days later than the peak gonadotropin response observed in females (81,83; also see subsection entitled "The Hypothalamic–Pituitary Unit: Changes in Gonadotropin Secretion and Their Relationship to LHRH Release"). Examination of the ontogeny of pituitary LHRH receptors in the male rat has revealed a close correlation between the number of LHRH receptors and the pituitary content of LH. Both appear to stabilize when the animals enter the peripubertal phase of development (~30 days) (325,326). When expressed as a concentration rather than by content, pituitary LHRH receptor levels show an increase during the first 4 weeks of life, reaching a peak at around 30 days, and then decline to the lower adult levels seen between 60 and 80 days of age (154,325). This decline during the latter part of sexual development is inversely correlated with rising serum T levels and, therefore, suggests an increased negative-feedback action of testicular steroids on hypothalamic–pituitary function (vide infra).

During the neonatal period, serum gonadotropin levels are high, though significantly lower than in females. Within a few days, however, the levels fall drastically (70,319). Numerous studies have attempted to characterize the subsequent developmental changes in gonadotropin secretion in the male rat, but a consistent pattern has not been forthcoming, especially for LH. Some reports suggest that serum LH concentrations increase as puberty approaches (70,319,327,328), whereas at the other extreme it has been suggested that they actually decrease (325,329). In the majority of cases, however, no significantly consistent alterations have been observed in mean serum LH levels (44,68,73,154,317,320). Such inconsistency most probably arises because of the pulsatile manner in which LH is released from the pituitary gland (330–332), even before puberty (103). In the female, this problem of fluctuating serum LH levels has been overcome either by using a very large number of animals in the study (218) or by examining an individual's pulsatile LH release pattern in detail (22,102). Whether or not definite puberty-related changes in the pulsatile LH release pattern occur in the male has yet to be convincingly established (see ref. 262 for a comprehensive review of factors that regulate LH secretion in the rat).

In contrast to the discrepant reports on LH secretion, there is a general consensus that sexual maturation in the male rat is associated with an increase in FSH secretion. Serum FSH levels rise during postnatal life and reach a maximum usually between 30 and 40 days of age. They then fall gradually as serum testosterone concentrations increase and attain relatively low adult levels (44,68,70, 73,317,319,325,327,328,333). Whether or not a peak of FSH secretion also occurs around day 12, as is the case for females, is uncertain, but this has been observed by some investigators (70,154,320,334).

In addition to changes in the amount of gonadotropin released, or even possible changes in pulsatile pattern, the chemical nature of the hormones may also change during sexual development. This latter possibility is supported by a finding that the isoelectric focusing pattern of FSH changed in male rats undergoing sexual maturation. Unfortunately, it was not demonstrated whether or not such chemical changes resulted in a form of the hormone with a greater biological activity (319). In this context it is relevant to reiterate that pleomorphism of pituitary FSH during prepubertal maturation in the female rat has been shown to be associated with the appearance of molecular forms of FSH that have greater biological activity (95).

The Testis

Morphometric techniques have been used to quantify developmental changes in the number of Sertoli cells and germ cells within the rat testis (312,335). Details of such changes as well as a review of physiological aspects of testicular development are presented in the chapter by de Kretser and Kerr. In the present section, discussion is focused on the two major routes by which testicular spermatogenesis and steroidogenesis in the rat are controlled by the hypothalamic–pituitary unit.

Hormonal Control

The most important androgen produced by the male gonad is T. It plays a pivotal role in several aspects of sexual maturation, including behavior, spermatogenesis, and differentiation and maintenance of accessory sex organs. Testosterone also exerts a tight control over gonadotropin secretion by a negative feedback loop.

During the infantile–juvenile period, however, T is not the primary androgen produced by the rat testis. Marked prepubertal changes in testicular androgen production arise because of differences in the development of various enzymes (317,336–339). Most significantly, the activity of 5α-reductase develops during the infantile–juvenile period (336–339). Consequently, the primary androgens produced by the immature rat testes are androstenedione, 5α-androstanediol, and 5α-reduced steroids such as DHT. From around day 25 onward, the activity of other enzymes also becomes apparent, most notably 17α-hydroxylase, C_{17-20} lyase, and 17β-hydroxysteroid dehydrogenase. Therefore, as the testes develop, more of the C_{21} precursors pass along the T-synthesizing pathway. The activity of 5α-reductase declines after about 40 days of age, leaving T as the major testicular androgen in the adult (337,340–342). Aromatase activity in the Sertoli cells declines during maturation but increases in the interstitial cells (343). Nevertheless, testicular production of estrogen is considerably lower than that of the androgens.

During early postnatal development, serum T levels are low. Although the levels begin to increase around the first week of life, the most pronounced rate of increase occurs much later, between 50 and 60 days of age (73,328,342,344–346). Furthermore, it has been shown that the actual pattern of T secretion is highly correlated, but phase-delayed, with pulses of LH secretion (330). With regard to puberty, it is clear that changes in the secretion of the pituitary hormones precede the maturation of the testes. It is well established that FSH binds within the seminiferous tubules to facilitate spermatogenesis, whereas LH stimulates T secretion by a direct action on the interstitial cells. It is also clear that prepubertal increases in serum FSH levels promote the formation of gonadotropin receptors within the testes (17,309,328, 347). Additionally, FSH has been shown to enhance the production of steroid biosynthetic enzymes (348). Overall, testicular growth proceeds in parallel with the FSH increase (68). The effect of LH on the release of steroids from immature testes can be blocked by hypophysectomy, but treatment of the hypophysectomized animals with FSH restores the effectiveness of LH (347). This is probably because of the capacity of FSH to increase testicular LH receptors (347). In view of these findings it appears that a puberty-related increase in the production of T might theoretically occur irrespective of whether or not the tonic levels of serum LH actually change (assuming that the prepubertal increase in serum FSH levels has primed the gonad to the actions of LH). It remains to be established whether subtle changes in the pulsatile pattern of LH secretion play a role in modulating prepubertal steroidogenesis in the male as they do in the female (22,222).

Follicle-stimulating hormone is not the only hormone that induces testicular responsiveness to LH. This ability appears to be shared, to some extent, with GH and Prl (17,349,350), both of which show a progressive rise in their serum levels during sexual development (70,73, 320,327). On the other hand, some reports have shown that the seminiferous tubules of GH-deficient rats have a qualitatively normal morphology during pubertal development; testicular endocrine function is also normal in these animals despite the small size of their testes (351). The involvement of thyroid hormones in testicular development is also unclear. In support of its active role is the observation that thyroidectomy of immature rats severely inhibits gametogenesis and interstitial cell development (352,353). Interestingly, however, the induction of transient hypothyroidism during neonatal life, using a reversible goitrogen, ultimately results in a lasting enlargement of the testes and other reproductive organs when the animals become adults (354). LHRH has also been shown to exert some control over prepubertal steroidogenesis. Receptors to LHRH have been demonstrated in testicular interstitial tissue, and a finding suggests that their binding capacity increases between 30 and 40 days of age, subsequently falling to a stable level by day 60 (154,355). Moreover, it has been shown that LHRH can directly inhibit steroidogenesis in the rat testis (153,356), although the precise chemical identity of gonadal LHRH remains to be elucidated (for reviews see refs. 153,357,358).

Testicular steroidogenesis is, therefore, influenced by at least two important factors: the secretory pattern of hypothalamo–hypophyseal hormones and the responsiveness of the testes to these hormones. An analogous situation exists for the physiological actions of T during puberty, at which time these actions are exerted only on specific target tissues. Since steroid hormones circulate in the plasma predominantly in a protein-bound form, it is important to establish whether or not steroid–protein binding changes during prepubertal development. Apparently it does not (359,360), but the metabolic clearance rate of T might. For instance, when Smith et al. (361) gave male rats subcutaneous Silastic capsules containing T, they found a progressive, age-related decrease in the clearance rate of T from the circulation. On the other hand, in an earlier study by Ulrich and Kent (362), the half-life for the disappearance of radiolabeled T was found to be similar in immature and adult animals. Besides changes in the steroid production, the developing testes also show an alteration in the capacity to secrete certain proteins (363). One of the most important is androgen-binding protein (ABP), which specifically binds testosterone and 5-DHT and is thought to be under the direct control of FSH. Serum levels of ABP rise sharply after birth, reaching a maximum at around 3 weeks of age. Its decline in the circulation seems to be related to the formation of the blood–testis barrier, after which ABP is released primarily into the epididymis (302).

Neural Control

There is some evidence to suggest that testicular function in the rat is regulated not only by hormonal factors but also directly by autonomic innervation (364,365). Testicular denervation in the immature rat has been shown to cause a significant reduction in testicular weight, especially if performed during the infantile period, when gonadotropic stimulation is still relatively low (366). Severe disruption of the seminiferous tubules, but not the interstitial tissue, was also found. Furthermore, essentially similar results were obtained when testicular sympathetic nerve terminals were destroyed with 6-hydroxydopamine (366). It remains to be established whether such neural regulation of testicular development simply reflects modulation of testicular blood flow or, indeed, represents a direct neuroendocrine action.

The Hypothalamic–Pituitary–Testicular Interrelationship

One of the classic examples of a negative feedback loop in endocrine systems is that of T and the hypothalamic–pituitary unit. The existence of such a loop in the rat has been demonstrated by surgical intervention. It is well known, for example, that orchidectomy leads to an immediate increase in the level of gonadotropin secretion, which can readily be suppressed by the administration of exogenous T. Interestingly, such a response is observed early in life, even during the neonatal period (125,367–371). The increase in gonadotropin secretion immediately after castration appears to be greatest in sexually mature animals (371), although a week later serum gonadotropin levels are the same irrespective of age (369). It should perhaps be emphasized that this lack of marked age-related response to castration contrasts notably with the observations made in agonadal humans (see the chapter by Plant).

The notion that the hypothalamic–pituitary unit becomes progressively less sensitive to testicular negative feedback has been the basis for a popular hypothesis that tries to explain the initiation of sexual maturation in the male rat. The reasoning behind this hypothesis is that, as feedback sensitivity decreases, the hypothalamic–pituitary unit will become more effective at stimulating testicular development. Also, serum T levels will be able to increase without completely suppressing the secretion of the gonadotropins. Initial support for this hypothesis was provided by Ramirez and McCann (367), who used the ovarian-ascorbic-acid-depletion bioassay to demonstrate an age-related decrease in T feedback sensitivity in orchidectomized rats. Numerous other studies, using RIAs, have now either confirmed or extended these findings (361,371–374). Interestingly, when the steroid treatment was delayed for 5 days following orchidectomy, an age-related alteration in feedback sensitivity was not found (17). It has subsequently been suggested that removal of gonadal feedback might elevate the sensitivity threshold of the hypothalamic–pituitary axis (371,375). To date, however, actual changes in sensitivity (e.g., at the receptor level) have not been demonstrated, nor is it clear whether such changes are relevant to the processes involved in the initiation of sexual development. Indeed, Nazian and Piacsek (376,377) have shown that the exposure of male rats to low ambient temperatures delays the onset of puberty, even though a corresponding change in negative feedback sensitivity was not detected (see subsection entitled "The Gonadostat Hypothesis" for further discussion of this subject).

A second negative feedback loop has been hypothesized for numerous years to account for the testicular control of FSH secretion. The need for such a hypothesis is quite evident when one considers the findings that, as testicular development progresses, serum FSH levels fall, even though mean LH levels increase little or remain relatively unchanged. Evidence exists supporting the involvement of inhibin in the feedback regulation of FSH secretion in maturing rats (378,379). Interestingly, the distribution of inhibin appears to change from a predominantly extratubular to an intratubular pattern as the testes mature (379). Circulating inhibin levels are especially high during the neonatal period, most likely because the blood–testis barrier is not yet fully complete (380,381) and therefore allows passage of the hormone out of the seminiferous tubules. Since serum inhibin levels decrease between 15 and 25 days of age (378), it is possible that this decline is involved in determining the increase in serum FSH levels that occur after day 25. Another possibility is that pituitary binding sites for inhibin decrease with age (378). Neither case, however, explains the subsequent fall in serum FSH that occurs after day 40.

The Pubertal Activation of the Hypothalamic–Pituitary–Testicular Axis

The current consensus is that the number of pituitary LHRH receptors reflects, at least on a short-term basis, hypothalamic secretion of LHRH (357,358). Since both the hypothalamic content of LHRH and the pituitary content of LHRH receptors begin to increase early in life, it is very probable that developmental changes within the LHRH-releasing centers provide one of the earliest stimuli for initiating sexual maturation. Several lines of evidence obtained from the male rat support this view. Similarly to those in females, LHRH neurons in males undergo morphological changes as puberty approaches (108,109). As already discussed, these changes consist of a significant increase in the proportion of cells with spiny-like processes as opposed to cells with a

smooth surface. Also, as in the case of the female, such morphological changes in the LHRH neurons might reflect an increase in puberty-related synaptic inputs to the cells and be associated with an increase in central, gonadal-independent drive. Likewise, this increase in central drive may be the primary factor responsible for the initiation of male puberty.

Another approach used to investigate the role of LHRH in the initiation of puberty has been to block the action of LHRH by passive or active immunization. If this blockade is initiated during the infantile period (before postnatal day 15), then testicular function is permanently impaired (382–384). Still another approach is that employed by Bourguinon and Franchimont (77,385), who examined the pulsatile release of LHRH from the medial basal hypothalami of male rats *in vitro*. A puberty-related increase in pulse frequency was detected, although a concomitant increase in pulse amplitude cannot be completely ruled out.

The possibility that the responsiveness of the pituitary gland to LHRH stimulation changes during maturation has already been mentioned, and it is particularly interesting that the peak gonadotropin responses occur during the peripubertal period of development (82,324). Furthermore, androgens have been shown to have direct effects on the pituitary gland; the studies of Nazian and Mahesh (386,387) imply that T can potentiate the pituitary response to LHRH in immature but not adult animals.

Another important developmental step in the pubertal activation of the hypothalamic–pituitary–testicular axis is the change in responsiveness of the testes to hypothalamic–pituitary stimulation (vide supra). The role of such changes in the initiation of sexual development is, however, more likely to be secondary to neuroendocrine changes first occurring at the central level. The same is probably also true for hypothetical alterations in the hypothalamic–pituitary sensitivity to testicular negative feedback (discussed later).

MODULATORY INFLUENCES REGULATING THE TIMING OF PUBERTY

The Adrenal Gland

A variety of experiments reported over the years have demonstrated that removal of the adrenal gland delays the age at which both vaginal opening and first ovulation occur (for reviews see refs. 388,389). Most, if not all, of the studies on the involvement of the adrenal gland in the onset of puberty have been performed using female rats, perhaps because of the inherent difficulties encountered in trying to alter the timing of puberty in the male rat (15,16).

Adrenalectomy may delay puberty by at least four different, but not necessarily exclusive, mechanisms. They are (a) retardation of bodily growth, (b) loss of circulating corticosterone levels, (c) elimination of circulating EPI caused by the removal of the adrenal medulla, and (d) increased secretion of ACTH resulting from the loss of corticosteroid negative feedback.

Adrenalectomized rats do not grow as much as intact controls (389), probably because of the loss of corticosteroid support to intermediate metabolism. Although a low rate of bodily growth may by itself contribute to the delay in puberty (133,390), corticosterone replacement alone has been shown to normalize the time of vaginal opening and first ovulation (388). This effect of corticosterone appears to be exerted, at least in part, at the level of the ovary. Corticosterone facilitates the stimulatory effect of FSH on ovarian steroidogenesis (391) and supports the amplifying effect of Prl on the response of the ovary to gonadotropins (174,177).

Further evidence that the adrenal gland contributes to the regulation of ovarian function is provided by the findings that adrenalectomy decreases the ovarian response to PMSG (392), reduces the number of medium to large-size follicles (393), and inhibits ovarian compensatory hypertrophy (394). Earlier experiments in which adrenalectomy was followed by reimplantation of the adrenal glands indicated that the medulla did not have any influence on the timing of puberty (395). However, evidence has been presented that selective removal of the adrenal medulla, leaving the cortex intact, delays the age of vaginal opening and first ovulation without altering plasma corticosterone levels or body weight (191). Because plasma EPI levels were found to be depressed, and, at nanomolar concentrations, EPI stimulated granulosa cells to produce P, the conclusion was drawn that a loss of medullary products, presumably EPI, is responsible for the delay in puberty (191).

Reports suggesting that ACTH may exert inhibitory effects on the reproductive system have been published (396,397). Nevertheless, no agreement has been reached as to whether this effect of ACTH is direct or is mediated by the adrenal gland.

Somatic Growth

The influence of metabolic cues on the timing of puberty has been suspected for many years. The early work of Kennedy (398) indicated that vaginal opening was more related to body weight than to chronological age. Subsequently, Kennedy and Mitra (390) postulated that the state of somatic growth is reported to the hypothalamus by some metabolic cue that acts as a signal to initiate puberty. Years later, Frisch and Revelle (399) made a proposal, based on epidemiological data collected on human females, that the attainment of a critical body weight is essential for puberty to occur. This hypothesis was subsequently modified to state that a particular ratio of lean to fat tissue and a minimum percentage of body

fat are necessary for the occurrence of puberty (400). Experiments performed in the rat by Frisch and colleagues (401) showed that females fed a high-fat diet had their first estrus earlier than rats fed a low-fat diet, but that the caloric intake at vaginal opening or first estrus was similar in both cases. These authors postulated that the constant caloric intake per unit of body weight at estrus may indicate that puberty occurs at a particular composition of fat/lean mass or fat/body weight. Further support for this hypothesis was provided by a subsequent report from the same laboratory (402) demonstrating that the relative percentages of body water, protein, and fat do not vary at the first estrus in female rats fed either a high-fat or a low-fat diet.

The hypothesis, however, has been disputed by other investigators. Some have concluded that, for the timing of puberty, growth rate is more important than attainment of a particular body weight (403). These authors have also presented evidence showing that the attainment of a certain percentage of body fat does not represent a signal for puberty onset and have contended that sexual maturation and increasing body fat are parallel, rather than causally related, phenomena (403). Others have further examined this issue and concluded that attainment of a specific percentage of body protein is more likely to be the metabolic signal for puberty (404).

In spite of these divergent conclusions it is clear that metabolic disturbances associated with weight loss or decreased rate of body growth are inhibitory to the reproductive system (for a review see ref. 405). This is in keeping with the hypothesis of Kennedy and Mitra (390) that chemical signals derived from the body's metabolic activity influence the reproductive hypothalamus and contribute to its activation at puberty. The nature of these chemical signals is unknown, but experiments conducted by different investigators indicate that there may, in fact, exist an array of such substances able to alter the release of LHRH via direct or indirect means. Insulin, amino acids, and essential fatty acids have been implicated in this role. Insulin has been shown to increase estrogen binding to certain areas of the brain (406) and to facilitate LHRH-induced gonadotropin release (407); availability of amino acids that are precursors for neurotransmitter synthesis has been shown to affect the formation of brain serotonin, acetylcholine, and catecholamines (408). Also of interest in this context is the observation that fasting reduces the turnover of DA and NE in the hypothalamus (409).

That essential fatty acids may also play a role is indicated by the observation that when they are made deficient during fetal life, the age at vaginal opening and first ovulation is delayed (410). Formation of PGE_2 in response to appropriate stimuli is decreased in both hypothalamic and ovarian tissue. Moreover, the LH response to E_2 is delayed, and the E_2 response of the ovary to hCG is diminished.

These observations suggest that both dietary amino acids essential for synthesis of brain neurotransmitters and essential fatty acids necessary for formation of arachidonate metabolites may be metabolic factors that contribute to regulate the timing of puberty. The relative importance that these factors may have in influencing the timing of puberty is, however, open to question, because neither amino acids nor essential fatty acids have been shown to change appreciably during the days antedating the initiation of puberty. In contrast, plasma levels of insulin-like growth factor I (IGF-I), a trophic factor that mediates the biological effects of growth hormone, increase strikingly during the onset of puberty in both rodents and primates (411–413). At least in rats, this increase occurs independently of the gonads (411). *In vitro* exposure of median eminences from juvenile rats to IGF-I resulted in a dose-related increase in LHRH release (414), suggesting that IGF-I may be physiologically involved in facilitating the changes in LHRH secretion that occur at puberty. Since synthesis of IGF-I in the postnatal hypothalamus is limited (415), the low levels of IGF-I mRNA detected in the medial basal hypothalamus do not appear to vary during puberty (W. L. Dees and S. R. Ojeda, unpublished data), and IGF-I receptors are highly concentrated in the median eminence (416–418), the view has been advanced that IGF-I of peripheral origin constitutes the predominant source of IGF-I available to the median eminence during peripubertal sexual development (414). This hypothesis also states that the elevation in plasma IGF-I levels that accompanies the pubertal process may contribute to enhancing LHRH release, and thus implicates IGF-I as one of the elusive metabolic "signals" involved in regulating the timing of mammalian puberty.

The Pineal Gland

At the present time there is little doubt that the pineal gland of mammals functions as a true endocrine organ that transduces neuronal information about day length into endocrine secretions (for a review see refs. 419–425). Melatonin is an indoleamine produced and released by the pineal gland (426). Alterations in the secretory pattern of this hormone mediate many, if not all, of the important effects of the pineal. Perhaps one of the most impressive demonstrations of the pineal's function, pertaining to the reproductive system, is found in seasonally breeding rodents such as hamsters. Normally, when sexually mature male Syrian hamsters (*Mesocricetus auratus*) are transferred from long to short photoperiodic conditions (i.e., <12.5 hours of light per day), their testes regress within 8 to 10 weeks, and the animals become sexually quiescent (427–430). Surgical removal of the pineal gland, however, or its sympathetic denervation completely blocks these photoperiod-induced

changes (431). On the other hand, when exogenous melatonin is administered to such animals in an appropriate mode, pinealectomy can be functionally reversed (432).

In contrast, the influence of the pineal gland on the reproductive system of the laboratory rat appears to be much less dramatic. This is not unexpected, because the rat does not have a well-defined breeding season, even in the feral condition. Under typical laboratory conditions of ad libitum feeding, constant ambient temperature, and constant long photoperiods, the pineal gland is essentially impotent from a functional point of view (420), and its contribution to the control of sexual maturation is greatly diminished. Nevertheless, there are certain experimental procedures that can render the reproductive system more sensitive to the pineal gland. For example, the coupling of potentiating factors such as anosmia, neonatal steroid treatment, or underfeeding with a reduced lighting schedule significantly delays the onset of puberty in the rat; the same effect occurs in rats that have been blinded (433,434). Furthermore, pinealectomy can, on the whole, remove most of the inhibition. Some effects of the pineal gland and melatonin can, however, be detected even under typical laboratory environmental conditions. Unfortunately, many of the earliest studies of this phenomenon produced conflicting results, and since details of important environmental factors, such as the photoperiod, are frequently not reported, evaluation of these data from a contemporary standpoint is often difficult.

Overall, there is a general consensus that pinealectomy advances vaginal opening and can cause earlier ovulation after PMS treatment. Although some researchers have consistently been unable to show an effect of melatonin on sexual maturation in the rat (435), others have clearly shown that daily melatonin injections given at an appropriate time of day can suppress the growth and functional activity of the ovary, delay vaginal opening, and inhibit PMS-induced ovulation in immature rats (436–446). In the male rat, pinealectomy advances sexual maturation, whereas daily afternoon injections of melatonin cause a reduction in the weight of the testes and seminal vesicles, decrease plasma levels of testosterone, LH and FSH, and decrease the number of pituitary LHRH receptors. Interestingly, these inhibitory effects are observed only when melatonin is administered to animals during the juvenile–peripubertal period and not earlier or later (447–450).

At this point in time, it is unclear exactly where the action of melatonin is exerted. A growing body of evidence indicates that the neuroendocrine centers of the brain are the most likely primary target and that these develop before birth (451–455). Additionally, a few *in vitro* studies have suggested that melatonin may also affect the pituitary gland (456,457) and even the gonads directly (458,459).

Pheromones

Pheromones are chemicals released from the body specifically to serve as a means of communication between members of the same species (see the chapter by Vandenbergh). Since the rat does not possess specialized external sweat glands, the most likely sources of its pheromones are the urine, the feces, and the skin that covers the mammary glands (460–462). A classic example of how pheromones can affect the reproductive system of rodents is the so-called "Whitten effect" (463). Exposure of female mice to male mice or to their excreta induces estrus in approximately half of the females three nights later. In the context of puberty, it is well established that sexual maturation in the female mouse can be accelerated by the presence of an adult male or even by introducing the female to a cage previously occupied by the male. On the other hand, if female mice are reared in groups, the onset of puberty is delayed compared to that of singly caged animals (for reviews see refs. 464–466). Similar studies in the rat are less well documented but suggest that advancement or retardation of sexual maturation by male or female pheromones, respectively, is nowhere near as impressive as in the mouse and is, at best, only marginally significant (467,468).

THE MECHANISM OF THE ONSET OF PUBERTY

The "Gonadostat" Hypothesis

The popular "gonadostat-resetting" hypothesis, formulated to explain the onset of puberty, is based on experiments originally performed in the rat. This hypothesis (367,469) proposes that, as the animal matures, the sensitivity of the hypothalamic–pituitary unit to steroid negative feedback decreases. Thus, a gradual increase in gonadotropin levels results and, in turn, stimulates further release of gonadal steroids.

The hypothesis of the gonadostat resetting originated in the early 1930s when Hohlweg and Dohrn (470) reported that formation of castration cells in the pituitary gland could be prevented in immature rats by a dose of E_2 that is approximately 1% of that required in the adult. Ramirez and McCann coined the phrase "resetting of the gonadostat" and, based on their results using castrated male rats and T replacement therapy, developed the hypothesis as it is known today (367,469).

Once RIA technology became available and permitted the measurement of circulating gonadotropin levels, these early findings were confirmed in both females (471,472) and males (361,371,377,378). Steele and Weisz (471) demonstrated that when prepubertal ovariectomized rats were infused with E_2 for several days,

plasma LH levels were suppressed initially and "escaped" from E_2 inhibitory control at the time of vaginal opening. Further support for the gonadostat hypothesis was provided by the observation that E_2 implanted into the hypothalamus was more effective in suppressing the postovariectomy rise of gonadotropins in immature, as opposed to adult, rats (473).

It is evident, therefore, that immature rats are more sensitive than postpubertal animals to the inhibitory effect of gonadal steroids on gonadotropin release. However, the concept that this change in sensitivity is responsible for the onset of puberty has been questioned on the basis of experiments in which the inhibitory effect of E_2 was examined at several intervals before and after the first ovulation (474). In these experiments the animals were ovariectomized at different ages or physiological phases, and E_2 was provided immediately after ovariectomy via subcutaneous Silastic capsules. A very low concentration of E_2 was equally effective in suppressing serum gonadotropin levels in juvenile and peripubertal rats, even when ovariectomy and E_2 therapy were instituted as late as the morning of the first proestrus. Once ovulation had taken place, the effectiveness of E_2 was lost, so that the same dose of the steroid became unable to suppress gonadotropin levels unless the higher P levels observed in postpubertal rats were concomitantly replaced (474). These results led to the conclusion that the resetting of the gonadostat occurs after the first ovulation, and thus it cannot be implicated as the cause of puberty. It appears that resetting is an event associated with the first ovulation; in fact, resetting may be a consequence of the initiation of reproductive cyclicity. The possibility still remains, however, that lower doses of E_2 may have uncovered a gradual loss in sensitivity to the steroid before first ovulation. It may also be contended that E_2 replacement in rats ovariectomized at proestrus cannot provide an accurate estimation of the relative sensitivity to E_2 inhibitory control because of the elevated endogenous E_2 levels present at this time. Although one would expect the contribution of the high proestrous levels of E_2 to be minimal because these levels decline very rapidly after ovariectomy, a possible residual effect cannot be ruled out.

A further indication that resetting of the gonadostat occurs mostly after the first LH surge was provided by Döcke et al. (473), who found that intrahypothalamic implants of E_2 became ineffective in suppressing postovariectomy LH levels when the implants were placed on the day of the first estrus. In a subsequent study from the same laboratory, it was concluded that the decrease in estrogen negative feedback effectiveness does occur prior to the first preovulatory surge of gonadotropins (475). However, in these experiments no significant differences were found between the groups treated with estradiol, but instead there was a marked difference in gonadotropin response to ovariectomy, which was two- to threefold greater in juvenile animals than in rats ovariectomized on the day of first proestrus or first estrus. Other investigators have reported that the effectiveness of E_2 in suppressing gonadotropin release changes little before puberty (476).

The concept that "resetting of the gonadostat" is responsible for the onset of puberty cannot, therefore, any longer be considered a tenable hypothesis. Of special relevance in this regard are the results, obtained in humans, demonstrating that, in both males and females, the gonadotropin-secreting system is remarkably sensitive to the inhibitory effect of gonadal steroids during both early and midpuberty, i.e., when gonadotropin secretion has already begun to increase (477,478). In searching for alternative explanations for the initiation of puberty, one must consider the most obvious possibility, i.e., that the onset of puberty depends on a gonadal-independent, centrally originating mechanism(s) that activates LHRH release.

The Loss of a Central Restraint

The concept that certain areas of the brain exert a tonic inhibitory control of sexual development originated from the early work of Donovan and van der Werff ten Bosch (10). These authors demonstrated that electrolytic lesions of the anterior hypothalamic area resulted in precocious puberty in female rats and postulated that the lesions initiated puberty because they eliminated an important area for steroid inhibitory control. These initial findings have been amply confirmed by other authors (479–482); moreover, evidence has been provided that rats with lesion-induced precocious puberty are able to mate and rear litters (482,483), indicating that the intrahypothalamic lesion induces a true precocious puberty.

A puzzling finding is that localization of the lesion in a precise site, or the age at which it is made, has little influence on the ability of the lesion to advance puberty. Similar results were obtained when anterior hypothalamic lesions were placed at 3 to 4, 14 to 15, or 23 days of age (14,482,484). In some cases, lesions in the posterior hypothalamus were also effective in advancing the time of puberty (481).

Inhibitory influences originating within the limbic system and in the cerebral cortex have also been evoked as playing a role in restraining the initiation of puberty. Elwers and Critchlow (11) first demonstrated that bilateral electrolytic lesions in the medial portion of the amygdaloid complex induce precocious ovarian activation. They postulated that this portion of the amygdala exerts an inhibitory influence on gonadotropin secretion. Other authors have argued, however, that the re-

sults were caused by an irritative effect of the electrolytic lesion, because electrochemical stimulation of the amygdala induces, rather than suppresses, gonadotropin release (485). In contradiction with this view, chronic bilateral electrochemical stimulation of the corticomedial portion of the amygdala was found to delay, rather than advance, puberty (486). Part of the confusion may derive from the site of the lesion/stimulation within the amygdaloid complex. This is suggested by the finding that lesions made with platinum electrodes induce precocious puberty only if they are placed in the anterior part of the medial amygdaloid complex (487). In spite of these divergent findings, there is general agreement that amygdaloid influences reach the hypothalamus via the stria terminalis, since both amygdaloid-induced inhibition and stimulation of gonadotropin release are prevented by transection of the stria terminalis (485,488).

Additional extrahypothalamic structures considered to exert a restraining effect on puberty are the hippocampus (489–491) and the cerebral cortex (492). Although stimulation of the hippocampus inhibits gonadotropin release (493,494), a specific inhibitory role of the hippocampus in the onset of puberty is debatable because hippocampal lesions actually delay puberty (489–491). This delay, however, may be more related to a reduction in body growth than to a specific effect on gonadotropin secretion (490). Furthermore, electrochemical stimulation of the hippocampus has been found to advance, rather than delay, the onset of puberty (495). Hemidecortication, on the other hand, though effective in inducing precocious puberty, does not permit identification of the specific area(s) involved (492).

The advancement of puberty brought about by hypothalamic lesions has been attributed for many years to the destruction of a center inhibitory to gonadotropin secretion (10,496). However, the validity of this concept has been challenged by the findings that the lesions do not eliminate the ability of ovarian steroids to suppress gonadotropin release (497) and do not result in elevated basal levels of plasma gonadotropins (482). It thus appears that the mechanisms underlying the advancing effect of hypothalamic lesions on puberty must involve mechanisms different from those originally postulated. Although an "irritative" mechanism has been invoked to explain the effect of lesions on other hypothalamic functions (498), a more accurate interpretation is obviously required. Since brain injury is followed by accumulation of mitogenic/neurotropic activities near the site of injury (499), a recent study considered the possibility that one of these activities is involved in the process by which lesions advance the initiation of female puberty (500). Attention was focused on transforming growth factor α (TGFα), a mitogenic polypeptide with neurotropic activity that is produced in the hypothalamus and is able to stimulate LHRH release from median eminence nerve terminals *in vitro* (501). The results demonstrated that lesions of the anterior hypothalamic area that advanced puberty also resulted in activation of TGFα gene expression in reactive astrocytes surrounding the lesion site (500).

That an increased production of TGFα contributes to the acceleration of puberty induced by the lesion was suggested by the ability of an inhibitor of epidermal growth factor (EGF) receptors infused into the lesion site to prevent the advancing effect of the lesion on puberty. An inhibitor of EGF receptors was used because most of the biological effects of TGFα are thought to be exerted via activation of EGF receptors (502). Further characterization of the hypothalamic response to injury demonstrated that reactive astrocytes also displayed an increase in EGF receptor gene expression (503), a change accompanied by an increase in biologically active EGF receptor protein, as determined by the ability of the receptor to autophosphorylate on dimerization. Experiments using double immunohistochemistry demonstrated that in intact animals EGF receptor immunoreactivity is located in glial cells of the hypothalamus and tanycytes of the third ventricle but not in LHRH neurons (504). Puberty-advancing lesions, which markedly increased EGF receptors in reactive astrocytes, did not result in the appearance of EGF receptors in LHRH neurons (503), suggesting that the stimulatory effect of TGFα on LHRH neurons is exerted indirectly via the intermediacy of glial cells. Since TGFα has been shown to stimulate LHRH release through activation of EGF receptors and requires the intermediacy of PGE_2 (501), the hypothesis has been proposed that TGFα produced in astrocytes acts in a paracrine/autocrine fashion to stimulate the glial release of prostaglandins, which, in turn, act on the LHRH nerve terminals to stimulate release of the neuropeptide (for a review see ref. 12).

Other experiments provided additional insights into the possible sequence of events by which hypothalamic lesions enhance the secretory activity of LHRH neurons (505). The information thus far obtained in this and other recent studies may be summarized as follows: Within 20 hours after injury there is a marked genomic activation of cells adjacent to the lesion site, including astrocytes, as revealed by an increase in *c-fos* expression (505). This activation does not occur in LHRH neurons, since they show no increase in *c-fos* levels at this time. Nevertheless, LHRH neurons do respond to the injury with a loss in spinyness. This phenotypic change probably represents reversal to a more immature morphological type, since spiny LHRH neurons have been shown to be more densely innervated (111) and to be metabolically more active than smooth neurons (113). These initial changes are followed by an intermediate phase (24 hours to 4 days) during which the astrocytes near the lesion site become reactive and exhibit an increased expression of the genes encoding TGFα and its receptor (500,503), the morphology of LHRH neurons returns to

normal (505), and TGFα may begin to stimulate glial production of PGE_2, which would then act on LHRH neurons to stimulate LHRH release (501). It appears that this effect requires neither activation of LHRH gene expression nor enhanced processing of the LHRH precursor peptide (505). The increase in LHRH output that presumably would result from PGE_2 action would then stimulate LH release, which would begin to stimulate the ovary to produce estradiol. Finally, a resolution phase ensues when estradiol levels become sufficiently elevated to acutely activate the secretory activity of LHRH neurons via their associated neuronal–glial circuitry. The resulting precocious preovulatory increase in LHRH output, presumably induced by estradiol, is accompanied by cell-specific genomic activation as determined by the selective appearance of *c-fos* protein in LHRH neurons on the day of the precocious first proestrus (505). This sequence of postulated events is depicted in Fig. 8.

These findings make it clear that the advancing effect of hypothalamic lesions on puberty is not caused by removal of an inhibitory tone on gonadotropin release, as postulated in earlier reports (10,496,506). They also cast doubts on the hypothesis that loss of a central restraint is responsible for the initiation of puberty. There is, however, evidence for the existence of an opioid inhibitory mechanism operating during prepubertal days. Opiatergic neurons may directly influence LHRH neuronal activity because they synapse with LHRH neurons (507). In females, at least, opiates appear to play an active role in inhibiting gonadotropin release well before puberty (508). Such an ability has been found to decrease during the days preceding the first ovulation, although it should be emphasized that in males it increases (509) during sexual development (510). The decreased effectiveness in opioid tone seen in peripubertal female rats has been interpreted as being indicative of an involvement of the opiate system in mediating the pubertal "resetting of the gonadostat" (510), a conclusion based on the earlier suggestions that opiates mediate the negative feedback effect of gonadal steroids on LH release (511,512). The same authors have reported that the capacity of the opiate system to suppress gonadotropin release depends on the presence of gonadal steroids (513) and have suggested that the coupling of hypothalamic opiate receptors to the LH regulatory mechanisms is dependent on gonadal steroids. A similar conclusion has been reached by other investigators (514). The interaction between the opiate system and gonadal steroids appears to be more complex because, in intact prepubertal rats, E_2 has been found to inhibit the capacity of naloxone to elicit LH release (508).

Although the relative preponderance of opiate-mediated mechanisms in the initiation of puberty has not been elucidated, it appears that the opiate system does, indeed, affect the pace of sexual maturation. This has been shown by the finding that naloxone administered to female rats during the first 10 postnatal days results in precocious puberty (515). A sex difference exists in that naloxone is very effective in inducing LH release in infantile females but not in males. Conversely, as the animals mature, naloxone becomes less effective in the female but more effective in the male (508,509, 516–518). A transient diurnal loss of opiate inhibitory tone may also contribute to the initiation of the peripubertal afternoon increase of LH release in females. This is suggested by the finding that the effectiveness of naloxo-

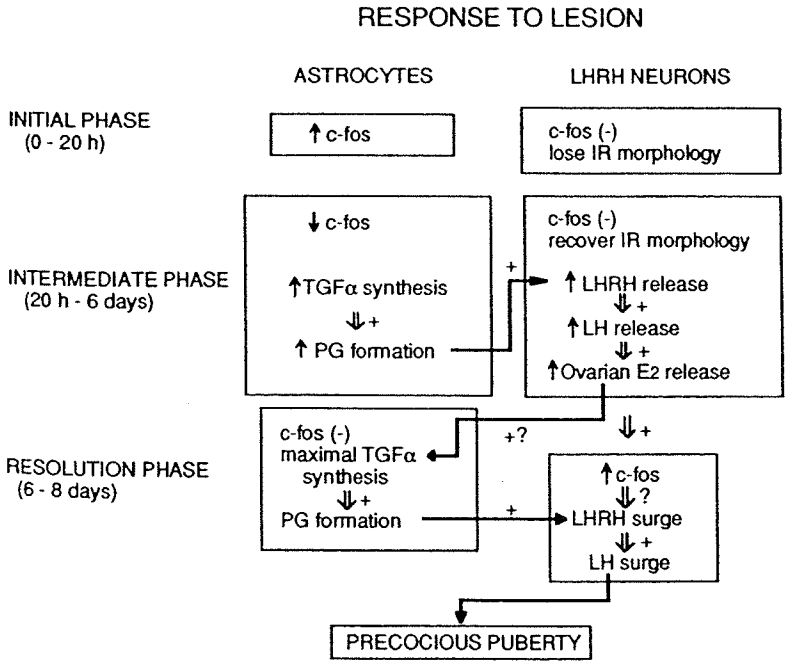

FIG. 8. Hypothetical sequence of events involved in the advancement of puberty induced by hypothalamic lesions. The duration of each response phase is measured in hours or days after injury. ↑, increase; ↓, decrease; —, no change; +, stimulation; ?, insufficient information; IR, irregular. For details see text. (From ref. 505, with permission.)

one in enhancing LH release decreases in the afternoon of the peripubertal period (519).

Gamma-aminobutyric acid (GABA)ergic neurons constitute another major inhibitory neurotransmitter system that may contribute to restraining the prepubertal secretory activity of LHRH neurons. GABAergic neurons synapse onto LHRH neurons (520) and appear to inhibit LHRH secretion via GABA receptors of the B subtype (521). In peripubertal female rats, however, activation of $GABA_A$ receptors has been shown to suppress gonadotropin secretion (522). In spite of the effectiveness of these inhibitory systems, it is unlikely that the actual initiation of puberty is determined only by the removal of opiate or GABAergic restraining influences. A more plausible view is that the initiation of puberty is facilitated by a loss of such restraining influences, but that it is primarily dependent on the activation of excitatory inputs (13). The concept of an increasing prevalence of excitatory inputs overriding a declining inhibitory opiate tone has been discussed by other authors (515,523).

The Activation of Excitatory Inputs

Although a decreased sensitivity to steroid negative feedback and a reduction in inhibitory neurotransmitter inputs to LHRH neurons may be modulatory components of the pubertal process, the initiation of puberty appears to depend on the activation of a still poorly understood series of events collectively known as the "central drive." According to this concept, prepubertal LHRH neurons have a low level of activity that is enhanced at the end of the juvenile period by a change in stimulatory inputs, resulting in the activation of the pituitary–gonadal axis. Although LHRH neurons undergo a series of maturational changes during juvenile development (see the section on "Postnatal Development"), it does not appear that the neurons themselves constitute a limiting factor for puberty to occur, because their secretory activity can be prematurely enhanced by experimental manipulations. Examples of such manipulations are the electrical stimulation of the hypothalamus (524–526) and the administration of NMDA (276,280), both of which have been shown to be effective in accelerating the onset of puberty. It would then appear that puberty is initiated by the activation of stimulatory neural pathways functionally and anatomically connected to LHRH neurons. The increases in LHRH mRNA level (107) and neuropeptide content (74) that occur during the juvenile-peripubertal transition period, the greater LHRH secretory response to secretagogues observed in late juvenile animals as compared with early juveniles (118), and the increased number of LHRH neurons with a more mature, "spiny," morphological appearance detected in juvenile–peripubertal animals as compared with younger rats (108) may be maturatio changes that facilitate the secretory response of LHF neurons to the pubertal increase in central drive.

As already discussed in the section on "The Onset Puberty," there are at least three neuronal systems t may be implicated in the pubertal activation of LHF neurons. They are those that use NE, NPY, and EAAs neurotransmitters. At least two of these neuronal s tems (NE, NPY) have been shown to be associated w LHRH via direct synaptic contacts (297,298). In ad tion to this transsynaptically mediated control syste there is now evidence (500,501,503,505) that LHR neuronal function may be regulated by trophic mo cules, such as TGFα, that do not act as neurotransm ters, but rather mediate cell-to-cell interactions betwe neurons and glia, the two major cell types in the nervo system (vide infra).

Although rather compelling evidence exists suppo ing a facilitatory involvement of NE, NPY, and EA that operate via NMDA receptors in the initiation a progression of puberty (261,270,271,276,278–284,52 nothing is known about the molecular mechanisms th set the process in motion, i.e., that enhance the transsy aptic activity of these stimulatory neuronal system Equally unresolved is the question of whether the thr systems function independently or are related to ea other by some form of hierarchy. The demonstratic that the activity of NMDA receptors increases before t onset of puberty in a gonadal-independent mann (527,528) does not resolve this issue but further suppor the view that the pubertal increase in central drive is, to significant extent, a gonadal-independent phenomeno Furthermore, although NMDA receptors appear to pla a physiological role in mediating the effects of endog nous EAAs in the pubertal process (278–282), it does n appear that other EAA receptor subtypes such as kainat or AMPA, shown to release LHRH in adult ra (529,530), are also involved in the pubertal pr cess (531).

The postnatal changes in LHRH mRNA levels (10 and the striking ability of E_2 and P to increase LHR gene expression within a few hours of their administra tion (241,242) raise the question of whether the neur transmitter systems stimulatory to LHRH secretion a able to affect LHRH gene expression, or, more directl stated, whether or not a change in LHRH gene expres sion is required for them to enhance LHRH secretor output. Evidence exists that activation of NMDA recep tors results in a rapid increase in LHRH mRNA level that parallels that in plasma LH (532). It is unclear, how ever, if the increase in LHRH mRNA is a direct result o NMDA receptor activation or represents a homeostati adjustment triggered by the activation of peptide release Other experiments have shown that blockade of norad renergic α_1 receptors in ovariectomized rats decrease LHRH mRNA levels (533), thus suggesting that a norad

energic tone operating through α_1 adrenoreceptors contributes to maintaining LHRH gene expression. Again, t is not known whether such an effect on LHRH gene xpression is required for NE to stimulate LHRH ecretion.

As indicated above, evidence now exists that trophic factors may also contribute to the peripubertal activation of LHRH neurons. The involvement of one such polypeptide factor, TGFα, in the mechanism by which hypothalamic lesions induce sexual precocity has been proposed (vide supra), and recent studies have provided evidence that TGFα may also play a role in the initiation of normal female puberty (242). In this study, TGFα mRNA and protein were detected in several hypothalamic nuclei concerned with LHRH release, including the suprachiasmatic, arcuate, and ventromedial nuclei, and in the median eminence and tanycytes of the third ventricle. Although some neurons were immunoreactive, most of the TGFα immunoreactivity was localized to astroglial cells. Intriguingly, the highest TGFα levels were seen in astrocytes present in the abovementioned hypothalamic nuclei, indicating that glial expression of the TGFα gene in the hypothalamus is predominant in subpopulations of glial cells associated with specialized neuronal subsets. TGFα mRNA levels, quantitated with a sensitive RNase protection assay, increased significantly during the second week of postnatal development, declined during the late infantile–juvenile phases of development, and increased again on the day of the first preovulatory surge of gonadotropins in both the preoptic region and median eminence–arcuate nucleus area. Treatment of ovariectomized late juvenile rats with estradiol and progesterone at doses that increased LHRH mRNA levels was also effective in elevating TGFα mRNA levels in both the preoptic region and the median eminence–arcuate nucleus region (242). Pharmacological blockade of EGF-like receptors targeted to the median eminence delayed the onset of puberty, suggesting that a site-specific activation of EGF receptors is an essential component of the neuroendocrine process that leads to reproductive capacity.

Based on these considerations, it may be suggested that the LHRH neuronal network develops under the influence of both excitatory and inhibitory inputs (Fig. 9). The excitatory inputs appear to be provided via both transsynaptic stimulation and glial–neuronal interactions. Thus far, only inhibitory inputs of neuronal origin have been described. With regard to the neuronal systems that may contribute to the initiation of puberty via transsynaptic stimulation of LHRH neurons, it appears that the neurons that utilize EAAs, NPY, and NE are the most promising candidates to fulfill this role. The glial influence on LHRH neurons, on the other hand, is mediated by at least one polypeptide trophic factor, TGFα, which appears to affect mainly the LHRH release mechanism and utilizes PGE_2 as an intermediate. The morphological basis for this interaction is provided by the tight relationship that exists between LHRH nerve terminals and glial cells—tanycytes of the median eminence (534).

The main inhibitory neuronal systems that may participate in the control of puberty are the opiatergic and GABAergic systems. In female rats, opiate tone appears to be most intense during infantile days (508) but continues to operate throughout postnatal development (508,517,518). A decrease in its inhibitory effectiveness per se may not result in the pubertal activation of LHRH release unless there is a concomitant increase in excitatory inputs to the LHRH neuronal network (Fig. 9). Furthermore, the relevance of a decreasing opiate tone to the onset of puberty is equivocal, because in male rats the sexual development is associated with an increase, rather than a decrease, in opiate tone (509). The predominance of these excitatory inputs at the end of juvenile development and its coupling to the ongoing rhythmic activity of a hypothalamic circadian oscillator (535) may result in the diurnal synchronization of LHRH release. Indeed, the activity of the NE (536) and NPY (269) systems has been shown to be higher in the afternoon than in the morning, as opposed to the activity of the opiate and GABAergic system, which is lower in the afternoon than in the morning (519,537). In addition to the establishment of a functionally appropriate integration between excitatory neurotransmitter systems and LHRH neurons, the synaptic interconnectivity of LHRH neu-

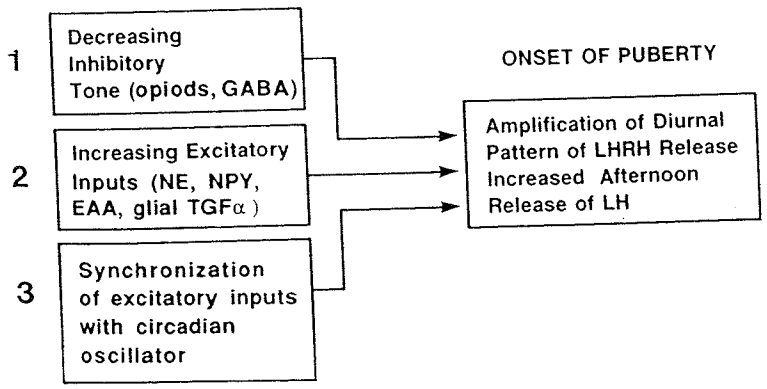

FIG. 9. Possible mechanisms underlying the onset of puberty in the female rat. Available evidence suggests that the simultaneous occurrence of events 1 and 2 and their synchronization with the hypothalamic circadian oscillator (suprachiasmatic nucleus?) may be the underlying mechanisms responsible for the development of the afternoon increase in LH release that signals the initiation of puberty. (Modified from ref. 20.)

rons (538,539) may be a critical factor that ensures the synchronized activation of the LHRH neuronal network to secrete physiologically effective pulses of LHRH secretion and then a preovulatory discharge of the neuropeptide.

Estradiol may facilitate maturation of the overall process through its neurotropic effects. Estradiol enhances neurite growth (540) and has been shown to induce synaptogenesis in the hypothalamus of juvenile rats at doses that elicit a preovulatory surge of gonadotropins (541). The neurotropic effects of E_2 appear to involve an increase in gene expression of the cytoskeletal proteins GAP-43 (542), class II-β-tubulin (543), and Tau (544). GAP-43 is a phosphoprotein predominantly found in developing axons, Class II-β-tubulin isotype is a microtubule protein predominantly expressed in developin and injured neurons, and Tau is also a microtubule associated protein found in elongating axons. In addi tion to these neurotropic effects, E_2 has recently bee found to act on hypothalamic astrocytes to increas TGFα gene expression (545).

CONCLUSIONS

The Female

The developmental process that leads to puberty i the female rat is based on an extraordinarily comple series of interrelated events (Fig. 10). The CNS plays

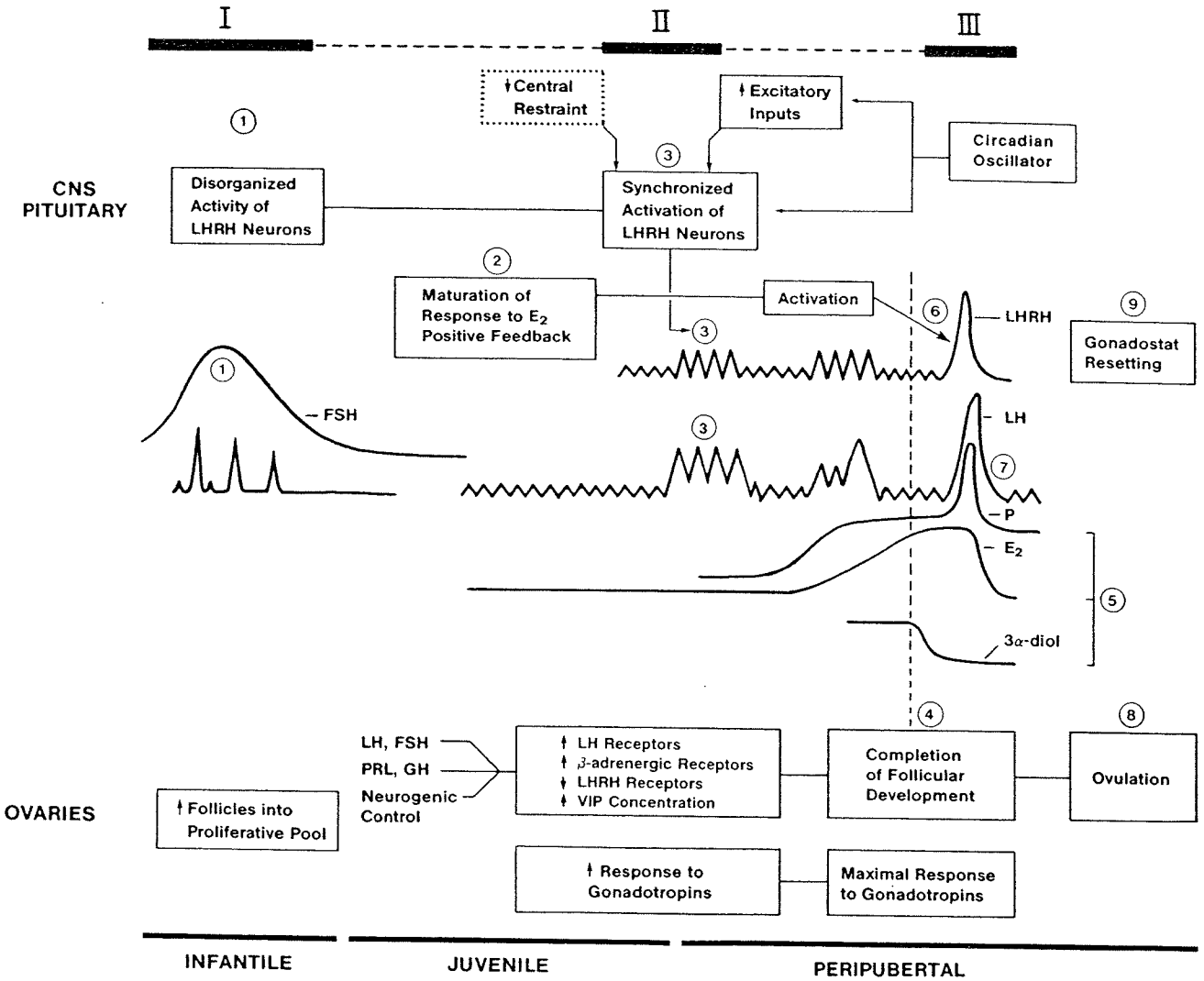

FIG. 10. Proposed sequence of developmental events leading to the first preovulatory LH surge in the female rat. The Roman numerals represent the various activational periods of the hypothalamic–pituitary unit identified during prepubertal postnatal development. The numbers indicate the sequence in which the events may occur. The *vertical dashed line* represents 12:00 p.m. on the day of the first proestrus. The *box* outlined by *dotted lines* indicates that a loss in central restraint may contribute but may not be the predominant factor for the synchronized activation of LHRH-secreting neurons. (Modified from ref. 19.)

critical role by controlling both anterior pituitary function, through the secretion of hypothalamic factors, and the ovary, via pituitary hormones and direct neural inputs. Gonadotropins play a decisive role at all stages of sexual development with the exception of the fetal period, during which ovarian development is gonadotropin independent. Three major periods of activation of gonadotropin secretion can be identified during postnatal development.

The first activational period occurs during infantile development and is manifested as an enhancement in FSH secretion with sporadic elevation in LH levels. Although this strikingly different pattern of gonadotropin release may result, to a significant extent, from the interplay of steroidal influences, its primary determinant appears to have a central origin. It may, in fact, reflect a "disorganized" activity of LHRH neurons, which, under the influence of nascent excitatory inputs, fail to discharge synchronously. Infrequent, randomly occurring synchronization may result in low-frequency LHRH discharges, which, in turn, would maintain high plasma FSH levels. A strong inhibitory (opiate) influence capable of slowing down the frequency of LHRH discharges may also contribute to the infantile pattern of gonadotropin release. During the second week of postnatal life, ovarian development comes under firm gonadotropin control. Driven by the increased FSH levels, a large crop of primordial follicles, some of which are destined to ovulate at puberty, is incorporated into a proliferative pool and begins to grow.

The second activational period denotes the end of juvenile development and results in the first overt hormonal manifestation of the onset of puberty. It is initiated by the ovarian-independent activation of transsynaptic inputs (NE, NPY, EAA) to, and perhaps glial interactions (TGFα?) with, LHRH neurons and is manifested as a diurnal change in basal LH levels and pulsatile LH release. A decrease in neuronal inhibitory influences (opioids, GABA) on LHRH secretion may also contribute to the process. Both basal release of LH and the magnitude of LH secretory episodes increase in the afternoon. These changes stimulate the ovary to produce more E_2, which then evokes minisurges of LH secretion. In turn, the surge further stimulates ovarian development and steroidogenesis. Establishment of this diurnal rhythm in LH release may reflect coordination of the hypothalamic circadian oscillator that resides within the suprachiasmatic nucleus with excitatory neuronal systems stimulatory to LHRH release and the synchronization of LHRH discharges. This perhaps results from completion of the synaptic circuitry impinging on LHRH neurons, the interconnections between these neurons, and their morphological and functional maturation. The overall process may be facilitated by the neurotropic effects of E_2 but is not determined by a decrease in hypothalamic sensitivity to negative feedback.

The third and final activational period occurs more abruptly and is predominantly determined by an increased output of ovarian steroids, especially E_2. This period corresponds to the preovulatory discharge of LHRH, which evokes the first surge of gonadotropins. The sequence of changes in LH release postulated to occur at puberty is shown in Fig. 11, which depicts individual profiles of LH release observed in peripubertal rats.

It is clear that this last activational phase is a result of the expression of E_2 positive feedback. Estradiol positive feedback develops by the end of the infantile period. At this time the ovary is unable to secrete sufficient levels of E_2 to produce a surge of LH. Completion of ovarian maturation is, therefore, essential for the preovulatory gonadotropin discharge to occur. The ovaries grow under the influence of pituitary gonadotropins. During neonatal–infantile development, this influence may be modulated by milk LHRH; afterwards it is facilitated by GH and Prl. An additional facilitatory control mechanism appears to be provided by adrenergic and peptidergic nerves, neurotropic factors produced within the ovary, and also EPI of adrenal medullary origin. The adrenergic inputs to the ovary facilitate P secretion either directly or by amplifying the effect of gonadotropins. VIPergic nerves appear to be involved in stimulating the secretion of all the main steroids (E_2, P, and aromatizable androgens). NPYergic nerves appear to modulate the availability of NE to its receptors and, in conjunction with SP, may also be involved in regulation of blood flow.

Under the influence of all these factors, the steroid output of the ovary increases more rapidly as proestrus approaches. The pace of follicular development is accelerated by an increase in LH receptors and possibly by a decrease in local LHRH inhibitory influences. The increased serum E_2 levels then set in motion the central component of E_2 positive feedback, possibly by enhancing the transsynaptic activity of stimulatory neuronal systems associated with the LHRH neuronal network and the secretory activity of associated glia. The intracellular mechanisms underlying the resulting surge of LHRH release may involve activation of a PGE_2 and cAMP-dependent pathway that appears to mediate the stimulatory effect of NE on LHRH release. The PGE_2 component of this pathway may not be located within LHRH neurons but rather on adjacent (glial?) cells. The function of an independent diacylglycerol and protein kinase C-mediated mechanism set in motion by neurotransmitters other than NE (DA, EAA?) may also increase at this time. Simultaneous stimulation of both pathways may then trigger the proestrus surge of LHRH, which, in turn, by acting on a pituitary sensitized by E_2, elicits the first preovulatory surge of gonadotropins. The gonadotropin discharge induces the first ovulation, which occurs on the early morning of estrus. On this day, estrogenic stimulation of the vaginal epithelium results in canalization of the vagina, an event that provides the

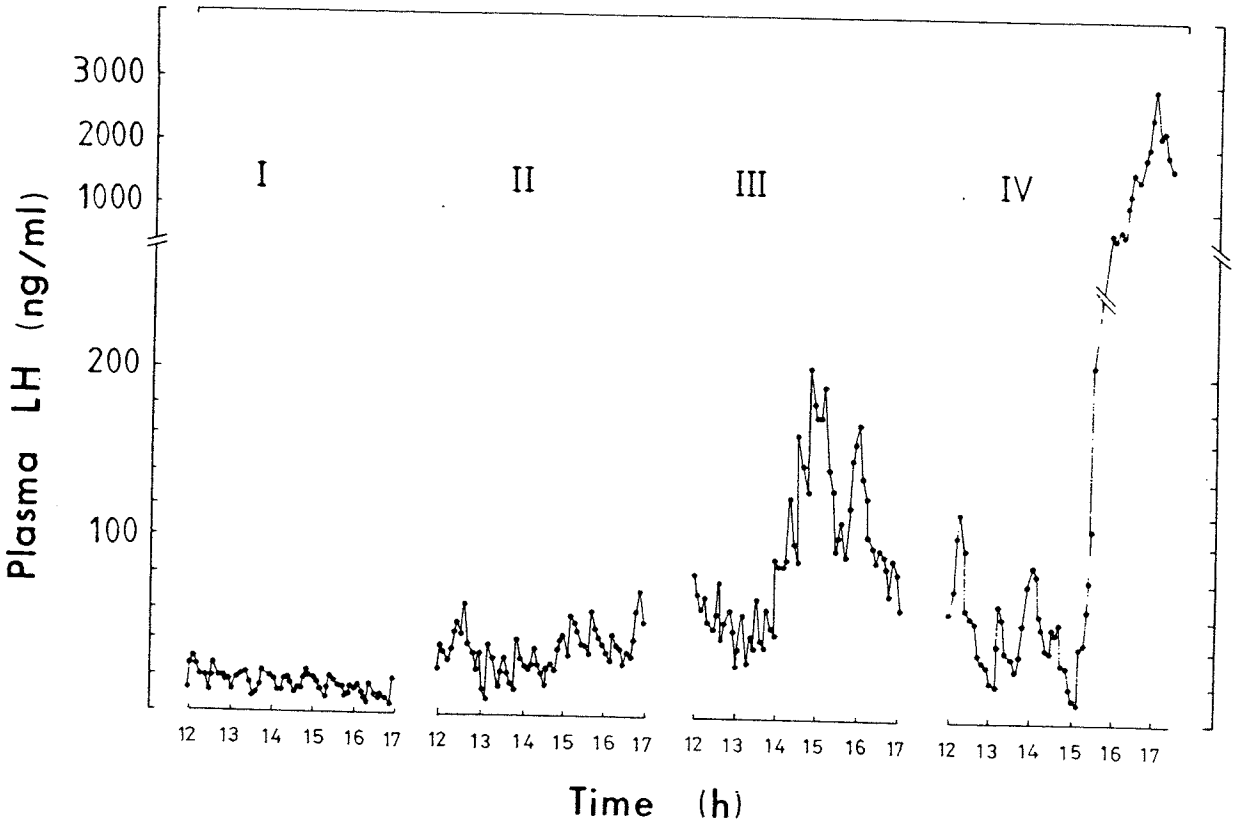

FIG. 11. Postulated sequence of changes in the mode of LH release during the onset of puberty in the female rat. Roman numerals indicate the phases in which different afternoon patterns of LH release were observed in conscious, free-moving peripubertal animals bled every 5-minutes using an automated bleeding technique. Each profile is derived from a different animal. (I) Low-amplitude pulses similar to those seen in the morning; (II) increased basal LH release and LH pulse amplitude; (III) minisurge of LH secretion; (IV) proper proestrus surge of LH. (From ref. 20, with permission.)

first somatic manifestation that reproductive competence has been attained.

The Male

Much less is known regarding the mechanisms that underlie the onset of puberty in male rats than in female rats. It is clear, however, that males attain sexual maturity in a much less climactic manner than females. Though well defined, the developmental changes observed at each of the three levels of the hypothalamic–pituitary–testicular axis are, relatively speaking, very gradual. Contrary to the female, the interrelations between the hypothalamic–pituitary unit and the gonads of the male are already functional before birth. Thus, the first few weeks of postnatal life may be envisaged as a period of synchronization or fine-tuning of the various interrelated processes rather than as a period during which these interrelationships become established. Available evidence permits the suggestion that, as in the female, the primary events that set into motion the onset of male puberty originate within the central nervous system. Studies using male hamsters have shown that the major endocrine events associated with puberty are preceded by an increase in the size of LHRH neuronal perikarya (546). These changes may reflect changes in the synthesis and/or alteration in the secretory pattern of LHRH, leading to the initiation or enhancement of the pubertal rise of gonadotropin secretion. Such endocrine developments, and possibly also direct neural factors, in turn influence the growth and maturation of the testes.

One of the most significant testicular developments occurring at this time involves changes in the steroidogenic pathways so that T becomes the predominant testicular androgen. Furthermore, as the animal matures the testes become more sensitive to the stimulatory actions of the gonadotropins, primarily because of elevated FSH secretion, and so the level of T production becomes enhanced. Meanwhile, both T and the gonadotropins provide the basic stimulus for initiating spermatogenesis. Each wave of sperm production may actually take several weeks to be completed, and it is therefore not surprising that attempts to advance the onset of puberty experimentally in the male rat have been largely unsuccessful. The increased output of T also produces the physical alterations that are typically asso-

ciated with puberty, namely, the development and maintenance of the accessory sex organs. In addition, T and inhibin act as negative feedback agents to attenuate gonadotropin secretion. It has previously been hypothesized that the onset of male puberty results from a decrease in sensitivity of the hypothalamic–pituitary unit to negative feedback. This change in sensitivity would permit gonadotropin secretion to increase in spite of an enhanced production of androgens by the developing testes. The hypothesis, however, does not explain the divergent patterns of FSH and LH secretion observed in developing males. Several observations, including the finding that the pattern of LHRH release (assessed *in vitro*) changes as the animal matures, suggest that an activation of LHRH release, independent of steroid negative-feedback control, may play the primary, if not the most decisive, role in the initiation of puberty in the male rat.

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