BIOLOGY OF REPRODUCTION **72,** 143–149 (2005)
Published online before print 22 September 2004.
DOI 10.1095/biolreprod.104.033167

Factors Accounting for Perinatal Occurrence of Pulsatile Gonadotropin-Releasing Hormone Secretion In Vitro in Rats[1]

Anne-Simone Parent, Marie-Christine Lebrethon, Arlette Gérard, and Jean-Pierre Bourguignon[2]

Developmental Neuroendocrinology Unit, Research Center of Cellular and Molecular Neurosciences (CNCM), University of Liege, 4000 Liege, Belgium

ABSTRACT

Our aim was to study the inhibitory and facilitatory factors possibly accounting for the undetectable activity of the GnRH pulse generator in late fetal life in vitro and its awakening in early postnatal life. Gamma aminobutyric acid ($GABA_A$) receptor antagonism using SR 95 531 did not cause any secretory pulse in fetal explants, whereas a significant stimulation of GnRH pulse frequency was obtained at 5 and 15 days. GnRH secretory response to repeated N-methyl-D-aspartate (NMDA) stimulation showed progressive disappearance, indicating that the inhibitory autofeedback was operating. GnRH release caused by glutamine was respectively 9% and 20% of that evoked by glutamate in fetal and 5-day-old rats whereas both amino acids were equally active at 15 days. Explants obtained after cesarean section performed at onset of labor did not show any secretory pulse, while pulses could be observed with explants obtained 2 h after vaginal delivery. Incubation of fetal explants with oxytocin (10^{-8} M) or prostaglandin E_2 (PGE_2) (10^{-6} M) resulted in occurrence of GnRH secretory pulses. A facilitatory effect of the oxytocin was shown to persist on Days 1, 5, and 15 and inhibitory effects of an oxytocin receptor antagonist provided some evidence of endogenous oxytocin involvement. We conclude that, in the fetal rat hypothalamus, GnRH inhibitory autofeedback and GABAergic inputs do not account for the absence of pulsatile GnRH secretion in vitro. A low rate of glutamate biosynthesis from glutamine is a possibly limiting factor. Oxytocin and PGE_2 can play a facilitatory role in the postpartal occurrence of pulsatile GnRH secretion.

gonadotropin-releasing hormone, hypothalamus, neuroendocrinology, neurotransmitters, oxytocin

INTRODUCTION

The pituitary gonadotrophin secretion is determined by the pulsatile secretion of GnRH, for which the existence of a hypothalamic pulse generator has been proposed [1]. The observation of episodic gonadotropin-releasing hormone (GnRH) secretion from immortalized murine GnRH neurons [2, 3] and the recent demonstration of pulsatile GnRH release using cultured GnRH neurons from the primate and rat embryonic olfactory placode [4, 5] pointed to the existence of a pulse generator intrinsic to GnRH neurons. In addition, in explants of the retrochiasmatic rat hypothalamus, where no immunocytochemically GnRH cell bodies could be observed, the secretion of GnRH was typically pulsatile in vitro, suggesting the existence of a pulse-generating network extrinsic to the GnRH neurons [6]. Pulsatile GnRH secretion occurred from the first postnatal day and progressively increased in frequency till the age of 25 days, before onset of puberty [7–9]. However, no pulsatile GnRH secretion could be observed with fetal explants obtained on Day 20–21 of gestation [7]. In the present work, we further studied the facilitatory and inhibitory mechanisms possibly accounting for the early postnatal occurrence of pulsatile GnRH secretion.

In previous studies from this laboratory, repeated stimulation of fetal rat hypothalamic explants with N-methyl-D-aspartate (NMDA) was shown to result in intermittent GnRH secretory response [7]. It was therefore concluded that fetal absence of pulsatility did not result from immature synthesis of GnRH or absence of NMDA receptors. An inhibitory autofeedback effect on GnRH secretion at the NMDA receptors is mediated by the $GnRH_{[1–5]}$ subproduct [10]. Such an autofeedback was found to operate already in late fetal life, but prevention of this mechanism with prolyl-endopeptidase antagonists did not cause any pulse to occur at that time [7]. Here, we studied the possible contribution of non-NMDA receptors during perinatal life.

Gamma aminobutyric acid ($GABA_A$) receptors were found to mediate a potent inhibition of the GnRH pulse generator in the juvenile primate in vivo [11, 12] and in the prepubertal rat hypothalamus in vitro [13]. Here, we studied the possible earlier involvement of GABAergic inputs considering both a possible inhibitory role as just mentioned or a possible stimulatory effect of GABA that was reported in the perinatal period and in some in vitro conditions [14, 15]. We showed earlier that increased biosynthesis of glutamate from glutamine through glutaminase could contribute to an increase in frequency of pulsatile GnRH secretion between 15 and 25 days [16]. That mechanism was also studied as a possible factor stimulating pulsatile GnRH secretion earlier in life. Finally, because parturition took place right before pulsatile GnRH secretion became obvious, the possible role of oxytocin and prostaglandin E_2 (PGE_2) was investigated.

MATERIALS AND METHODS

Animals

Male Wistar rats were housed in temperature and light-controlled conditions with water and standard rat pellets ad libitum. The prepubertal pups were with lactating mothers until weaning, which was at 3 wk of age. The protocols were approved by the University Committee on Animal Research. Fetal rats were used irrespective of gender. Postnatally, male rats were used to be consistent with our previous conditions [7]. Fetal age was

[1]Supported by the FRSM (grant 3.4515.01), the Belgian Study Group for Pediatric Endocrinology, and the Faculty of Medicine at the University of Liege. A.S.P. is a research fellow of the Belgian ''Fonds National de la Recherche Scientifique'' (FNRS).
[2]Correspondence: Jean-Pierre Bourguignon, Division of Paediatric and Adolescent Medicine, CHU Sart Tilman, B-4000 Liege, Belgium.
FAX: 00 324 366 7246; e-mail: jpbourguignon@ulg.ac.be

Received: 20 June 2004.
First decision: 8 July 2004.
Accepted: 12 August 2004.

ISSN: 0006-3363. http://www.biolreprod.org

determined based on vaginal plug observed at daily examination after mating. The day of birth was considered as Postnatal Day 1.

Hypothalamic Explant Incubation and GnRH RIA

After decapitation, the retrochiasmatic hypothalamus was rapidly dissected and transferred into a static incubator as described previously [17, 18]. In each experiment, 12–15 explants were studied individually for 4–6 h through collection and renewal of the incubation medium (0.5 ml) every 7.5 min. The incubation medium was MEM (phenol red free minimum essential medium, Gibco, Invitrogen Corporation, Belgium) supplemented with glycine (10 nM), magnesium (1 mM), and glucose (25 mM). The incubation medium was enriched with 20 μM of bacitracin, an inhibitor of GnRH degradation by endopeptidases. We showed previously that pulsatile GnRH secretion was not affected by bacitracin in explants obtained from fetuses and at 5 and 15 days postnatally [7]. No bacitracin was used only when 50 mM of glutamate or NMA were used repeatedly, to not interfere with endopeptidase involvement.

The samples were frozen until the GnRH radioimmunoassay was performed. GnRH was measured in the collected fractions with a highly sensitive RIA [17, 18]. GnRH was measured in duplicate. The intra- and interassay coefficients were 14% and 18%, respectively [17, 18]. The values below the limit of detection (5 pg/7.5 min) were assigned that value. The highly specific GnRH antiserum [19] was generously provided by Dr. Y.F. Chen and V.D. Ramirez (Urbana, IL).

Study Protocols

Glutamate-receptor subtypes and GnRH inhibitory autofeedback. The secretion of GnRH was studied with explants obtained on Fetal Days 20–21 in the absence of any secretagogue (n = 10 explants) or during repeated exposures to 50 mM of NMA (N-methyl-D,L-aspartate; Sigma, Belgium) (n = 5 explants) or glutamate (Sigma) (n = 5 explants). This particular concentration of glutamate and NMDA was used to be comparable with our previous conditions [7–9]. In a second experiment, explants obtained on Fetal Days 20–21 and on Postnatal Days 5 and 15 were used to study the relative contribution of NMDA and non-NMDA receptors at these ages. For this purpose, each explant (n = 5 for each age) was initially exposed to 50 mM of glutamate alone and subsequently to 50 mM of glutamate together with either 1 μM of D-2-amino-5-phosphopentanoic acid (AP-5; Sigma) or 1 μM of 6,7-dinitroquinoxaline-2,3-dione (DNQX; Tocris) to antagonize the NMDA or AMPA/kainate receptors, respectively, or both antagonists together. Glutamate was used for 7.5 min at regular intervals of 37.5 min (five fractions). The inhibitors were used for two fractions of 7.5 min, immediately before and during incubation with glutamate.

Glutamate biosynthesis from glutamine. With explants obtained on Fetal Days 20–21 and on Postnatal Days 5 and 15, the secretion of GnRH was evoked repeatedly every 37.5 min by incubation for 7.5 min with 20 mM of glutamate (n = 5 for each age) or glutamine (n = 5 for each age) (glutamate and glutamine obtained from Sigma). It was been shown earlier that this submaximal concentration allowed demonstration of a developmental difference (15 versus 50 days), whereas such a difference was no longer observed using 50 mM of the two amino acids [16]. At each age studied, the involvement of glutaminase in GnRH secretion evoked by glutamine was studied by coincubation with 10 μM of diazo-oxo-norleucine (DON; Sigma), a glutaminase inhibitor. The inhibitor was used for 2 fractions of 7.5 min, immediately before and during incubation with glutamine (20 mM). To establish that the glutaminase inhibitor was without effect on glutamate stimulated GnRH release, the glutamate-evoked GnRH secretion was studied in presence of 10 μM of DON as well.

GABAergic inputs. With explants obtained on Fetal Days 20–21 and Postnatal Days 5 and 15, pulsatile GnRH secretion was studied without (control) or with 100 μM of SR 95 531, a specific $GABA_A$ receptor antagonist (generously provided by Sanofi-Winthrop, Brussels, Belgium) or with 100 μM of muscimol (Sigma), a specific $GABA_A$ agonist, which were both used throughout the entire experiment. For each age, 5 hypothalamic explants were studied in each condition.

Parturition, oxytocin, and PGE_2. Pulsatile GnRH secretion was studied with hypothalamic explants obtained within 1–2 h after birth by cesarean section performed immediately after the onset of labor (n = 12) or after natural parturition (n = 12).

Hypothalamic explants obtained in fetuses (20–21 days of gestation) were incubated with oxytocin (10 nM, Sigma), PGE_2 (1 μM, Sigma) or both used for the whole experiment. Five hypothalamic explants were studied in each condition. Explants obtained on Postnatal Days 1, 5, and 15 were incubated without or in the presence of oxytocin (10 nM) or an oxytocin antagonist (des-Gly-NH_2d$(CH_2)_2$[D-Tyr^2,Thr^4]OVT, 100 nM), generously provided by Dr. Manning (Toledo, OH). Explants obtained on Days 1 and 15 were incubated without or in the presence of PGE_2 (1 μM), which was used for the whole experiment. For each age, 5 hypothalamic explants were studied in each condition.

Statistical Analysis

The occurrence of significant pulses of GnRH secretion was determined with the Pulsar program, as described previously [20]. The individual interpulse interval and pulse amplitude as well as the mean ± SD were calculated. In some instances, such as with the oxytocin antagonist with explants obtained on Postnatal Day 1, all the interpulse intervals were equal, thus accounting for the SD being equal to zero. With explants obtained in fetuses and neonatal rats on Postnatal Day 1, a GnRH interpulse interval could not be calculated because only one secretory pulse in a period of 4 h could be observed in some experiments. Therefore, pulsatile GnRH secretion was estimated through the number of pulses observed in a period of 4 h. The secretory response of GnRH evoked by the glutamate receptor agonists was calculated as the difference between GnRH secreted during incubation with the agonist and in the fraction collected immediately before. The effect of the different factors on GnRH pulse amplitude and frequency was analyzed by one-way ANOVA followed by Student-Newman-Keuls test. The threshold for significant difference was $P < 0.05$

RESULTS

Glutamate-Receptor Subtypes and Inhibitory Autofeedback

With fetal hypothalamic explants, GnRH secretion was usually found at or close to the detection limit and no pulses were observed (Fig. 1A). In similar conditions, GnRH secretion could be evoked by 50 mM of NMA or glutamate (Fig. 1, B and C). Repeated stimulation with NMA every 37.5 min resulted in a progressive reduction and finally disappearance of the GnRH secretory response (Fig. 1B), indicating that the inhibitory autofeedback mediated through NMDA receptors was already operative. However, with 50 mM of glutamate as secretagogue, the response showed no significant reduction (Fig. 1C), suggesting the capacity of non-NMDA receptors to mediate sustained glutamate-evoked secretion of GnRH at that time.

With hypothalamic explants obtained in fetal and 5- and 15-day-old rats, the GnRH release evoked by 50 mM of glutamate was significantly decreased by coincubation with AP-5 or DNQX used to antagonize the NMDA and AMPA/kainate receptors, respectively (Table 1). While the inhibition obtained with AP-5 was similar at the three ages studied, DNQX accounted for a greater inhibition in the fetal hypothalamus than postnatally, further suggesting the particular contribution of non-NMDA receptors in late fetal life.

Glutamate Biosynthesis and GnRH Secretion

With hypothalamic explants obtained at 20–21 days of gestation as well as 5 and 15 days postnatally, repeated incubation with glutamate every 37.5 min could evoke a GnRH secretory response. This response was maintained with time at the three ages studied (Fig. 2). With explants from fetal rats and 5-day-old rats, glutamine could evoke some GnRH secretory response, which was, however, lower than the response evoked by glutamate and disappeared with repetition of the stimulatory episodes (Fig. 2, A and B). Overall, the glutamine-evoked GnRH release represented 9% and 20% of the glutamate-evoked release in late fetal life and at 5 days, respectively. At 15 days, glutamine was as effective as glutamate in releasing GnRH and the glutamine-evoked GnRH secretion did not show any decrease with time (Fig. 2C).

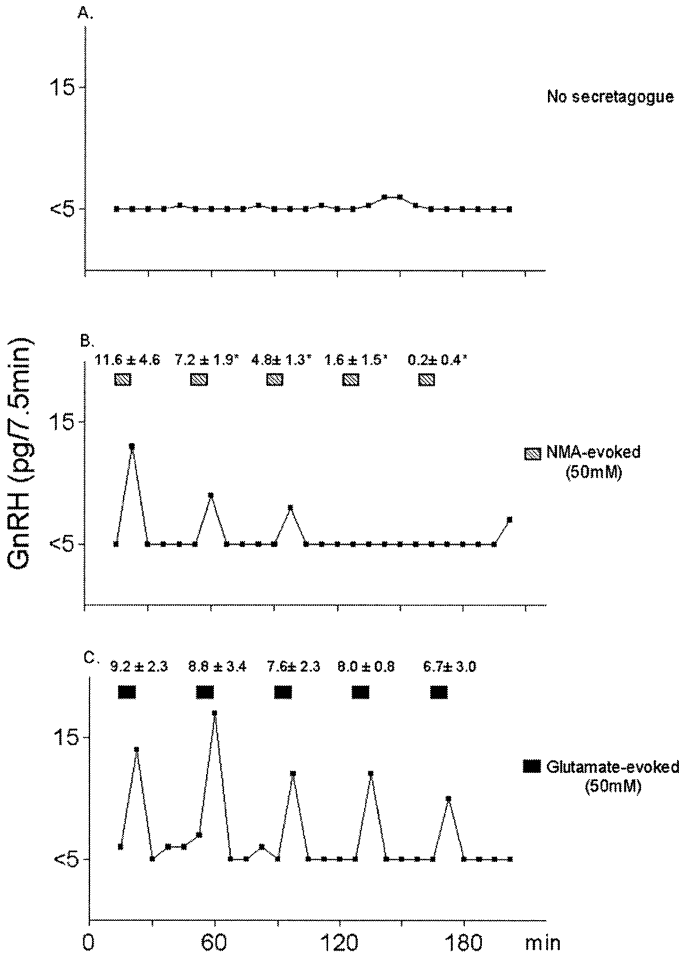

FIG. 1. Representative profiles of GnRH secretion from fetal hypothalamic explants incubated without any secretagogue or with NMA or glutamate used repeatedly in the absence of bacitracin. The mean (±SD) secretory response of GnRH to NMA and glutamate is given for each stimulating episode. The asterisks denote significant differences versus the initial response ($P < 0.05$).

Using 10^{-5} M of DON, a glutaminase inhibitor, the GnRH secretory response evoked by glutamine was nearly abolished at 5 days (0.4 ± 0.9 versus 1.7 ± 1.7 pg/7.5 min) and 15 days (0.8 ± 1.8 versus 9.4 ± 2.4 pg/7.5 min), indicating that glutamine effects were likely to result from transformation into glutamate by glutaminase. DON had no effects on glutamate-evoked secretion at any of the ages studied.

GABAergic Inputs

SR 95 531, a specific $GABA_A$ receptor antagonist, did not induce any pulsatile GnRH secretion when fetal explants were studied (Fig. 3). On Postnatal Days 5 and 15, the GnRH interpulse interval was significantly reduced by the antagonist (74.4 ± 2.1 versus 89.1 ± 2.7, and 48.7 ± 3.9 versus 60.9 ± 2.6 min, respectively, SR 95 531 versus control; Fig. 3), indicating early postnatal GABAergic inhibition of pulsatile GnRH secretion. Incubation of explants with 100 μM of muscimol, a $GABA_A$ receptor agonist, did not account for any pulsatile GnRH secretion in late fetal life nor changes in GnRH pulse frequency at 5 and 15 days (88.1 ± 3.5 versus 89.1 ± 2.7 min, and 59.4 ± 2.2 versus 60.9 ± 2.6 min, respectively, muscimol versus controls; Fig. 3).

TABLE 1. Effect of AP-5 and DNQX on GnRH release induced by glutamate from hypothalamic explants obtained in rats aged 20–21 days of gestation and 5 and 15 days postnatally.[a]

	Secretagogue				
Age	Glu (pg/7.5 min)	Glu (%)	Glu + AP5 (% versus glu)	Glu + DNQX (% versus glu)	Glu + AP5 + DNQX (% versus glu)
Fetal	7.7 ± 2.0	100 ± 26	39 ± 16	23 ± 11	3 ± 6
5 days	7.7 ± 0.8	100 ± 10	49 ± 6	49 ± 11	16 ± 11
15 days	9.8 ± 0.6	100 ± 6	43 ± 9	41 ± 0.0	16 ± 1.1

[a] Data are expresed in percentage of the response evoked by 50 mM of glutamate. Using glutamate (Glu), the data are also provided as pg/7.5 min (mean ± SD).

Effects of Parturition, Oxytocin, and PGE_2

With explants obtained in neonatal rats born by cesarean section performed after the onset of labor, no spontaneous GnRH pulses could be observed (Fig. 4B) such as with explants obtained in fetal rats before labor (Fig. 4A). With explants obtained 2 h after completed parturition and vaginal delivery, spontaneous GnRH secretory pulses were consistently observed (2.1 pulses/4 h) with a mean inter-

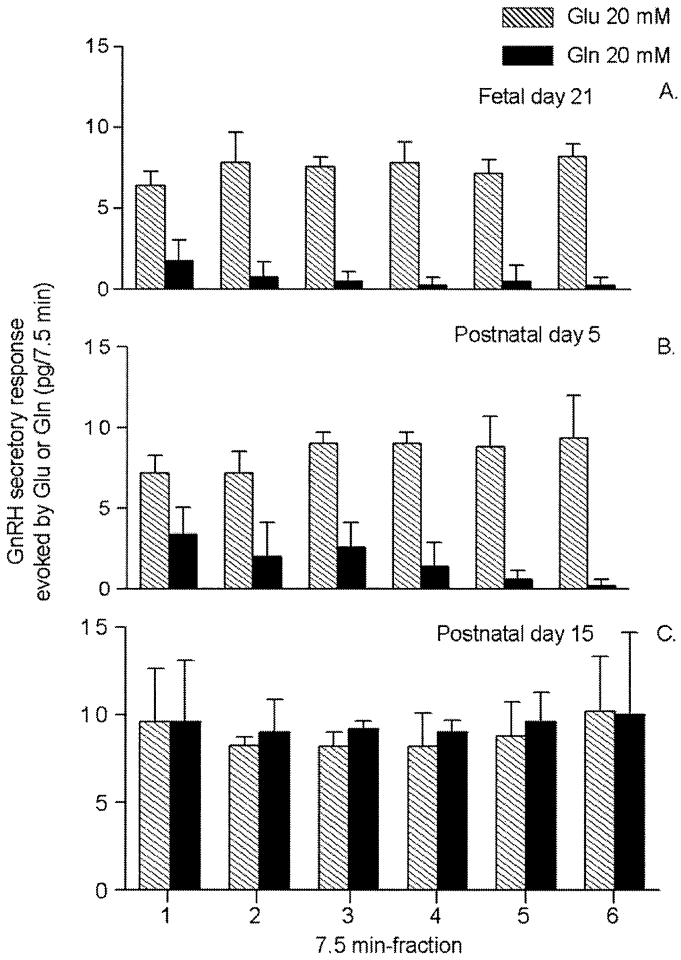

FIG. 2. The GnRH secretory response from hypothalamic explants obtained in late fetal life and at 5 and 15 days postnatally was studied repeatedly (every 37.5 min) using 20 mM of glutamate (Glu) or glutamine (Gln) as secretagogues in the absence of bacitracin. The data are the mean (±SD) secretory response of GnRH to glutamate or glutamine for each stimulating episode.

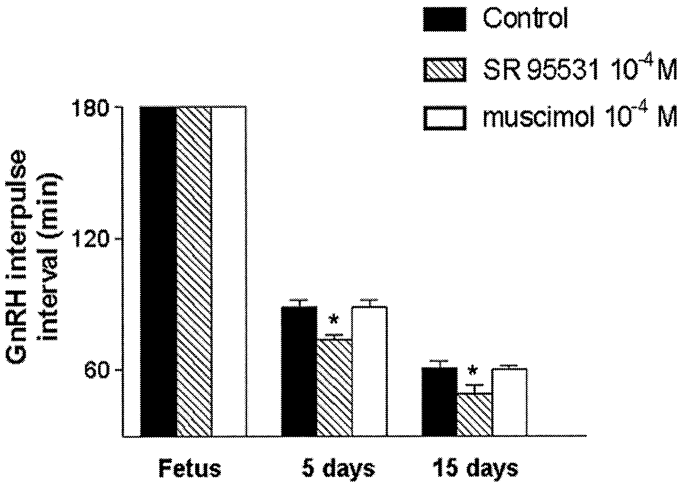

FIG. 3. The mean (±SD) GnRH interpulse interval was studied with hypothalamic explants obtained in late fetal life and at 5 and 15 days postnatally in three conditions: control or incubation with SR 95531 or with muscimol. The data are mean (±SD). The asterisks denote significant differences versus data obtained in control conditions ($P < 0.05$).

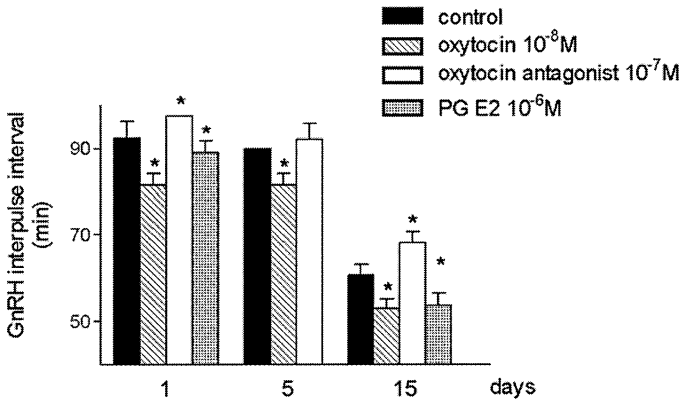

FIG. 5. The mean ± SD GnRH interpulse interval was studied with hypothalamic explants from male rats aged 1, 5, and 15 days in three conditions: control or incubation with oxytocin or with an oxytocin antagonist. Hypothalamic explants from male rats aged 1 and 15 days were also studied in the presence of PGE_2. The data are mean (±SD). The asterisks denote significant differences versus data obtained in control conditions ($P < 0.05$).

pulse interval of 88.0 ± 5.3 min when several pulses were observed using a single explant (Fig. 4C). With explants obtained in late fetal life (20–21 days of gestation), incubation with oxytocin (10 nM) resulted in about one GnRH secretory pulse in a period of 4 h (Fig. 4D). Using 1 μM of PGE_2, a similar effect was observed (Fig. 4E). With fetal explants incubated with oxytocin and PGE_2 both together, two pulses were seen on average in a period of 4 h (Fig. 4F), such as with explants obtained in neonatal rats after natural parturition and incubated in control conditions. Using explants obtained in male rats on Postnatal Day 1 (Fig. 5), the mean GnRH interpulse interval (92.5 ± 3.9 min) was significantly reduced by oxytocin (81.6 ± 2.7 min) and significantly increased by incubation with an oxytocin antagonist (97.5 ± 0.0 min). A facilitatory oxytocin effect on GnRH pulse frequency remained at 5 and 15 days postnatally (81.6 ± 2.7 versus 90.0 ± 0.0 min, and 53.1 ± 2.2 versus 60.8 ± 2.5 min, respectively). The oxytocin antagonist resulted in a significant increase of the GnRH interpulse interval at 15 days (68.3 ± 2.5 min), while this effect was not significant at 5 days (92.1 ± 3.7 min). The antagonist effect suggested a facilitatory effect of endogenous oxytocin on pulsatile GnRH secretion, which was effective perinatally and persisted in postnatal life. As shown in Figure 5, PGE_2 resulted in a significant decrease in GnRH interpulse interval on Day 1 (89.1 ± 2.7 versus 92.5 ± 30.9) and 15 (53.8 ± 2.9 versus 60.0 ± 0.0 min).

DISCUSSION

In this paper, the neonatal occurrence of pulsatile GnRH secretion in vitro has appeared to involve mechanisms different from those accounting for the postnatal increase in frequency of pulsatile GnRH secretion before puberty. In the latter period, reduced inhibitory GABAergic inputs and increased glutamatergic stimulation through NMDA receptors play key roles [13, 16], whereas such mechanisms are not involved neonatally. At this early time, critical factors were found to include glutamate biosynthesis and action through non-NMDA receptors as well as oxytocin and PGE_2 that were studied in relation to parturition.

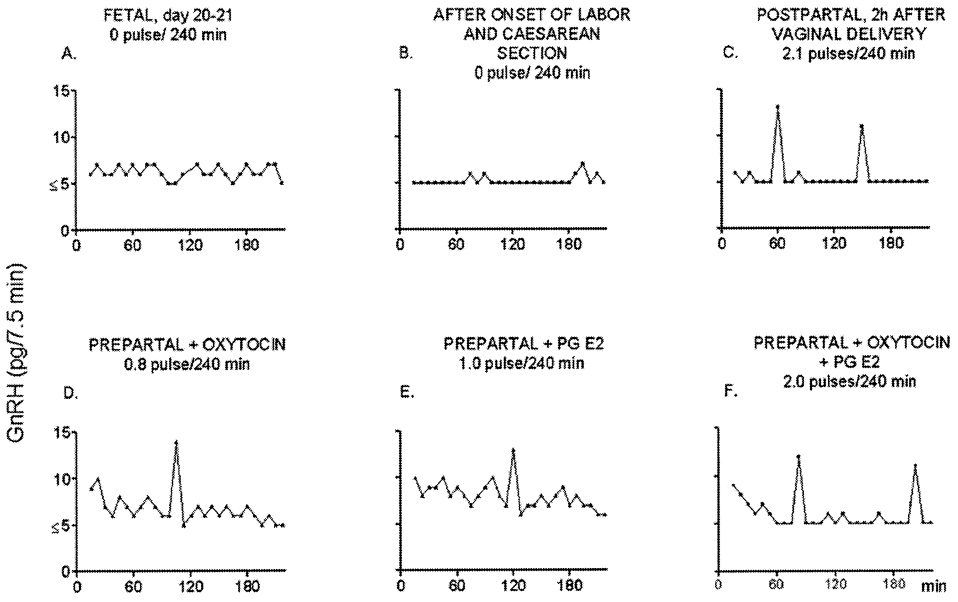

FIG. 4. Representative secretory profiles of GnRH secretion from hypothalamic explants in six different conditions: (**A**) fetal rats, 20–21 days of gestation; (**B**) rats born by cesarean section performed after the onset of labor; (**C**) neonatal rats 2 h after natural parturition; (**d**) explants from fetal rats at 20–21 days of gestation incubated with oxytocin; (**E**) explants from fetal rats at 20–21 days of gestation incubated with PGE_2; (**F**) explants from fetal rats at 20–21 days of gestation incubated with oxytocin and PGE_2.

When fetal rat hypothalamic explants were studied in vitro, the GnRH pulse generator appeared to be silent or below the threshold of detectable activity, whereas it was operating from the day of birth onward. This raised the question as to whether the apparent absence of pulses was linked to true inactivity of the pulse generator or undetectable activity depending on amplitude limitation. It is possible that an ultrasensitive assay could detect some pulses below the threshold of detection in our conditions, supporting an amplitude-modulated process. We did, however, not observe any difference in pulse amplitude, whereas amplitude was found to increase in similar conditions with explants obtained in female rats in the afternoon of proestrus [21]. In addition, obvious changes in pulse amplitude were seen when GnRH secretion was evoked using different secretagogues but not in the absence of such agents.

We have shown that intermittent incubation with glutamate every 37.5 min can cause GnRH release and mimic the spontaneous secretion seen with explants obtained in adult rats. These data indicate that the apparent silence of the GnRH pulse generator in the fetal hypothalamus does not result from immature synthesis of GnRH or absence of glutamate receptors. Indirectly, because the secretory response of GnRH is kept unchanged during 4 h, neuronal damage through NMDA-receptor-mediated excitotoxic effects is unlikely to occur in our conditions. The GnRH inhibitory autofeedback already operates in the fetal hypothalamus and needs to be prevented by the prolyl endopeptidase inhibitor bacitracin to observe intermittent NMDA-evoked GnRH response from fetal hypothalamic explants [7]. This autofeedback can be the reason why repeated NMDA stimulations resulted in progressive disappearance of the GnRH secretory response. Because the glutamate-evoked release of GnRH could be obtained repeatedly and did not decrease in the absence of bacitracin, it could be suggested that non-NMDA receptors, which do not seem to be involved in any inhibitory autofeedback [10], play an important role perinatally. In such conditions, endopeptidase degradation does not appear to be a key factor that restrains the fetal pulse generator because prolyl endopeptidase inhibition does not result in occurrence of pulsatile secretion [7].

Our data indicate that both NMDA and non-NMDA receptors are present and functional in the fetal hypothalamus. Developmental changes in NMDA receptors were observed in several extrahypothalamic regions such as cortex, hippocampus, and cerebellum [22–24]. In the preoptic area, the ratio of NR1 and NR2a and 2b subunits was reported to change as well [25] and could explain variation in sensitivity to NMDA receptor activation. The most dramatic increase in expression of NR1, a subunit that is necessary for a functional NMDA receptor, seemed to take place between Fetal Day 18 and Postnatal Day 10. In the rat gray matter, genes encoding for the kainate receptor subunits were expressed from Fetal Day 14 [26]. Their expression went through a peak in the late fetal/early postnatal period, when the contribution of non-NMDA receptors seems to be predominant in our model. The mRNA editing of different subunits of the non-NMDA receptors, which determines their gating characteristics, is developmentally regulated [27] and may also contribute to the ontogenic changes in glutamate sensitivity.

A major pathway for glutamate biosynthesis is from glutamine under the action of glutaminase [28]. The mechanism of glutamate recycling in the brain involves reuptake by astroglial cells of the glutamate released at the synapse level and transformation into glutamine, which is delivered to glutamatergic neurons where glutaminase causes transformation into glutamate. With DON, a glutaminase inhibitor, we confirmed glutaminase involvement in glutamine effect on GnRH release perinatally and postnatally, in agreement with data reported previously later in life [16]. In such conditions, the glutamine evoked secretion of GnRH compared with that evoked by glutamate provides an indirect assessment of glutamate biosynthesis from glutamine. Our data suggest that a critical increase in glutamate biosynthesis may occur during the early postnatal period. This is in line with our previous observations that inhibition of glutamine-evoked GnRH secretion required higher DON concentrations at 50 days than at 15 days, suggesting an increase in glutaminase activity throughout development [16]. Roth et al. [29], however, did not find any ontogenic change in glutaminase mRNA in the mediobasal hypothalamus and the preoptic area. One limit in the significance of such studies is the regional specificity of changes in the face of the ubiquity of the enzyme. Then, if low glutamate biosynthesis from glutamine partly accounts for absent pulsatile secretion in late fetal life, it is noteworthy that the mechanism could be rather specific of the neuronal-glial apparatus controlling GnRH secretion, as opposed to the involvement of non-NMDA receptors, which appeared to be a general feature of the central nervous system at that particular period.

The involvement of GABA receptors in the control of GnRH secretion is largely supported by in vitro and in vivo studies. In the monkey, tonic inhibition by GABA neurons before onset of puberty was evidenced by push-pull perfusion and infusion in the vicinity of the median eminence of the antagonist bicucullin [11, 30] or an antisense oligodeoxynucleotide for glutamic acid decarboxylase [12, 31]. Using bicucullin or antisense strategies, we observed a GABAergic inhibition of pulsatile GnRH secretion from hypothalamic explants of male rats at 15 days that had disappeared by 25 days [13]. The present data confirm those observations and extend them to the early postnatal period because the $GABA_A$ receptor antagonist SR 95 531 led to an acceleration of GnRH pulse frequency at 5 and 15 days of age. However, incubation with SR 95 531 did not induce any pulsatile GnRH secretion from fetal explants, suggesting that GABAergic inhibition did not account for the prenatal absence of pulsatile GnRH secretion. Such a finding raised the question as to whether the role of GABA could be facilitatory instead of inhibitory at that time. A stimulatory effect of GABA was found in the embryonic hypothalamus [14] as well as in immortalized cell lines retaining the capacity of pulsatile GnRH secretion [15], suggesting that $GABA_A$ receptors could mediate a stimulatory effect on GnRH secretion in the fetal hypothalamus. In the present study, with fetal hypothalamic explants by the end of gestation, the $GABA_A$ receptor agonist muscimol was unable to elicit pulsatile GnRH secretion or to increase the glutamate-evoked GnRH release. From this, however, we cannot conclude that GABA is not involved as a direct stimulator of GnRH neurons because such an effect could coexist with inhibitory effects on an extrinsic GnRH pulse generator and thus not appear in our conditions. The opposing GABA effects during development can involve differences in receptor subtype, changes in the subunit composition of $GABA_A$ receptors, [32], in chloride homeostasis [33], and in the relative importance of glutamate input compared with GABA [34]. The changes in GABA effect may also depend on alterations in the population and sensitivity of interneu-

rons because GABA could act through interneurons [35, 36].

The neonatal occurrence of pulsatile GnRH secretion due to a withdrawal from inhibitory placental factors appeared to be unlikely because occurrence of GnRH pulses should then be expected to occur after both cesarean section and natural parturition, while they were observed in the latter condition only. Thus, we hypothesized a facilitatory role of endocrine events related to parturition being observable only after completion of this process. Oxytocin could be involved because the peptide is synthesized at the uterine as well as hypothalamic level. Oxytocin neurons are located in the paraventricular and supraoptic nuclei, their axons terminating in different areas, including the median eminence and the rostral hypothalamus [37]. In the female, oxytocin is known to play an important role in parturition and lactation [38] and also appears to act centrally to influence maternal and mating behavior [39–41]. Moreover, oxytocin has been shown to stimulate GnRH release from medial basal hypothalamic explants in male rats [42] and to stimulate GnRH secretion on the afternoon of proestrus in the cycling female rat [43]. These effects were observed at low concentrations, consistent with physiologic levels. In the present study, we showed that oxytocin could cause pulsatile GnRH secretion to occur from explants obtained in late fetal life, suggesting a role for this factor in the initiation of pulsatile GnRH secretion neonatally. If oxytocin accounts for such an effect in vivo, the contribution of peptide of maternal versus fetal and peripheral versus central origin remains to be elucidated. Some studies have described an increase in fetal oxytocin during the end of gestation [44], while increased oxytocin of maternal origin during parturition could also be involved in the mechanism. However, some studies provided contradictory data concerning a possible placental transfer of oxytocin [45–47]. With the use of hypothalamic explants of neonatal rats on Day 1, we showed that facilitatory oxytocin effects on GnRH secretion were not limited to late fetal life. Moreover, an oxytocin antagonist was able to decrease pulsatile GnRH secretion by increasing the GnRH interpulse interval, and this effect on GnRH remained in postnatal life at 15 days. These data suggested a possible life-long effect of endogenous oxytocin independent of parturition and thus is likely to originate in the hypothalamus and to involve endogenous peptide because the antagonist is effective in the absence of oxytocin added in the incubation medium. Interestingly, the oxytocin antagonist did not account for any significant effect using explants obtained at 5 days of age. This might suggest that there is an early postnatal maturation process of endogenous oxytocin input to GnRH neurons that is not yet fully completed by the age of 5 days. Indeed, some studies have shown that significant amounts of mature oxytocin are detected in the brain only from the second week of life [48, 49]. Very recently, a reduction of LH secretion was reported following administration of an oxytocin antagonist in adult women, thus indicating that our in vitro data obtained in the rat could be relevant to human physiology or pathophysiology [50]. PGE_2, which is also synthesized at the central as well as the uterine level, has been described as a potent stimulator of GnRH release [51]. Many studies suggest a role for PGE_2 in the median eminence, where it is secreted by astrocytes in response to growth factors such as TGF-alpha [52]. Though we did not exclude that oxytocin concentration or duration of exposure may affect GnRH pulse frequency, the combined effects of PGE_2 and oxytocin suggest that achievement of physiological GnRH pulse frequency requires different neuromodulators. Such a concept is consistent with redundancy of pathways, which characterizes most biological systems involved in homeostasis of important functions in the hypothalamus.

In conclusion, pulsatile GnRH secretion occurs soon after birth in the rat hypothalamus in vitro. There is no evidence of disappearing inhibitors, while our data suggest the involvement of several facilitatory factors, namely oxytocin, PGE_2, and glutamate synthesis from glutamine. A stimulatory oxytocin effect on GnRH secretion persists later in postnatal life and its physiological or pathophysiological significance is still to be elucidated.

ACKNOWLEDGMENT

We are grateful to Dr. V.D. Ramirez for generously providing us with the anti-GnRH antiserum.

REFERENCES

1. Hotchkiss J, Knobil E. The menstrual cycle and its neuroendocrine control. In: Knobil E, Neill JD, (eds.), Physiology of Reproduction. New York: Raven Press; 1994:711–749.
2. Martinez de la Escalera G, Choi AL, Weiner RI. Generation and synchronisation of gonadotropin-releasing hormone (GnRH) pulses: intrinsic properties of the GT-1 GnRH neuronal cell line. Proc Natl Acad Sci U S A 1992; 89:1852–1855.
3. Wetsel WC, Valenca MM, Merchenthaler I, Liposits Z, Lopez FI, Weiner RI, Mellon PL, Negro-Vilar A. Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. Proc Natl Acad Sci U S A 1992; 89:4149–4153.
4. Teresawa E, Keen KL, Mogi K, Claude P. Pulsatile release of luteinizing hormone-releasing hormone (LHRH) in cultured LHRH neurons derived from the embryonic olfactory placode of the rhesus monkey. Endocrinology 1999; 140:1432–1441.
5. Funabashi T, Daikoku S, Shinora K, Kimura F. Pulsatile gonadotropin-releasing hormone (GnRH) secretion is an inherent function of GnRH neurons, as revealed by the culture of medial olfactory placode obtained from embryonic rats. Neuroendocrinology 2000; 71:138–144.
6. Purnelle G, Gérard A, Czajkowski V, Bourguignon JP. Pulsatile secretion of gonadotropin-releasing hormone by rat hypothalamic explants without cell bodies of GnRH neurons. Neuroendocrinology 1998; 66(5):305–312.
7. Yamanaka C, Lebrethon MC, Vandersmissen E, Gerard A, Purnelle G, Lemaitre M, Wilk S, Bourguignon JP. Early prepubertal ontogeny of pulsatile gonadotropin-releasing hormone (GnRH) secretion: I. Inhibitory autofeedback control through prolyl endopeptidase degradation of GnRH. Endocrinology 1999; 140:4609–4615.
8. Bourguignon JP, Gérard A, Mathieu J, Mathieu A, Franchimont P. Maturation of the hypothalamic control of pulsatile gonadotropin-releasing hormone secretion at onset of puberty: I. Increased activation of N-methyl-D-aspartate receptors. Endocrinology 1990; 127:873–881.
9. Bourguignon JP, Gérard A, Alvarez Gonzalez ML, Franchimont P. Neuroendocrine mechanism of onset of puberty. Sequential reduction in activity of inhibitory and facilitatory N-methyl-D-aspartate receptors. J Clin Invest 1992; 90:1736–1744.
10. Bourguignon JP, Alvarez Gonzalez ML, Gérard A, Franchimont P. Gonadotrophin releasing hormone inhibitory autofeedback by subproducts antagonist at N-methyl-D-aspartate receptors: a model of autocrine regulation of peptide secretion. Endocrinology 1994; 134: 1589–1592.
11. Mitsushima D, Hei DL, Teresawa E. Gamma-aminobutyric acid is an inhibitory neurotransmitter restricting the release of luteinizing hormone-releasing hormone before the onset of puberty. Proc Natl Acad Sci U S A 1994; 91:395–399.
12. Terasawa E, Lushansky LL, Kasuya E, Nyberg CL. An increase in glutamate release follows a decrease in gamma aminobutyric acid and the pubertal increase in luteinizing hormone releasing hormone release in female rhesus monkeys. J Neuroendocrinol 1999; 11:275–282.
13. Bourguignon JP, Gérard A, Purnelle G, Czajkowski V, Yamanaka C, Lemaitre M, Rigo JM, Moonen G, Franchimont P. Duality of glutamatergic and GABAergic control of pulsatile GnRH secretion by rat

hypothalamic explants. II. Reduced NR2C- and $GABA_A$ receptor mediated inhibition at initiation of sexual maturation. J Neuroendocrinol 1997; 9:193–199.
14. Kusano K, Fueshko S, Gainer H, Wray S. Electrical and synaptic properties of embryonic luteinizing hormone-releasing hormone neurons in explant cultures. Proc Natl Acad Sci U S A 1995; 92:3918–3922.
15. Hales TG, Sanderson JM, Charles AC. GABA has excitatory actions on GnRH-secreting immortalized hypothalamic (GT1–7) neurons. Neuroendocrinology 1994; 59:297–308.
16. Bourguignon JP, Gerad A, Alvarez Gonzalez ML, Purnelle G, Franchimont P. Endogenous glutamate involvement in pulsatile secretion of gonadotropin-releasing hormone: evidence from glutamine effect and developmental changes. Endocrinology 1995; 136:911–916.
17. Bourguignon JP, Gérard A, Franchimont P. Direct activation of GnRH secretion trough different receptors to neuroexcitatory amino acids. Neuroendocrinology 1989; 49:402–408.
18. Bourguignon JP, Gérard A, Mathieu J, Simons J, Franchimont P. Pulsatile release of gonadotrophin-releasing hormone from hypothalamic explants is restrained by blockade of N-methyl-D,L-aspartate receptors. Endocrinology 1989; 125:1090–1096.
19. Hartter DE, Ramirez VD. Responsiveness of immature versus adult male rat hypothalami to dibutyryl cyclic AMP-and forskolin-induced LHRH release in vitro. Neuroendocrinology 1985; 40:476–482.
20. Bourguignon JP, Gerard A, Debougnoux G, Rose JR, Franchimont P. Pulsatile release of gonadotrophin-releasing hormone (GnRH) from the rat hypothalamus in vitro calcium and glucose dependency and inhibition by superactive GnRH analogs. Endocrinology 1987; 121: 993–999.
21. Parent AS, Lebrethon MC, Gerard A, Vandersmissen E, Bourguignon JP. Leptin effects on pulsatile gonadotropin releasing hormone secretion from the adult rat hypothalamus and interaction with cocaine and amphetamine regulated transcript peptide and neuropeptide Y. Regul Pept 2000; 92:17–24.
22. Franklin SO, Elliott K, Zhu Y-S, Wahlestedt C, Inturrisi CE. Quantitation of NMDA receptor (NMDAR1) mRNA levels in the adult and developing rat CNS. Mol Brain Res 1993; 19:93–100.
23. Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 1994; 368:144–147.
24. Zhong J, Carrozza DP, Williams K, Pritchett DB, Molinoff PB. Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. J Neurochem 1995; 64:531–539.
25. Adams MM, Flagg RA, Gore AC. Perinatal changes in hypothalamic N-methyl-D-aspartate receptors and their relationship to gonadotropin-releasing hormone neurons. Endocrinology 1999; 140:2288–2296.
26. Bahn S, Volk B, Wisden W. Kainate receptor gene expression in the developing rat brain. J Neurosci 1994; 14:5525–5547.
27. Bernard A, Ferhat L, Dessi F, Charton G, Represa A, Ben-Ari Y, Khrestchatiky M. Q/R editing of the rat GluR5 and GluR6 kainate receptors in vivo and in vitro: evidence for independent developmental, pathological and cellular regulation. Eur J Neurosci 1999; 11:604–616.
28. Kvamme E. Glutaminase (PAG). In: Hertz L, Kvamme E, Mc Geer E, Schousboe A (eds.), Glutamine, Glutamate, and GABA in the Central Nervous System. New York: Liss; 1983:51–57.
29. Roth C, Leonhardt S, Theiling K, Lakomek M, Jarry H, Wuttke W. Ontogeny of the GnRH-, glutaminase- and glutamate decarboxylase-gene expression in the hypothalamus of female rats. Brain Res Dev Brain Res 1998; 110(1):105–114.
30. Keen KL, Burich AJ, Mitsuchima D, Kasuya E, Teresawa E. Effects of pulsatile infusion of the $GABA_A$ receptor blocker bicuculline on the onset of puberty in female rhesus monkeys. Endocrinology 1999; 140:5257–5266.
31. Mitsushima D, Marzban F, Luchansky LL, Burich AJ, Keen KL, Durning M, Golos TG, Teresawa E. Role of glutamic acid decarboxylase in the prepubertal of the luteinizing hormone-releasing hormone release in prepubertal female monkeys. J Neurosci 1996; 16:2563–2573.
32. Sim JA, Skynner MJ, Pape JR, Herbison AE. Late postnatal reorganization of $GABA_A$ receptor signaling in native GnRH neurons. Eur J Neurosci 2000; 12:3497–3504.
33. Rivera C, Voipo J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 1999; 397:251–255.
34. Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiars JL. $GABA_A$, NMDA and AMPA receptors: a developmentally regulated ''menage à trois.'' Trends Neurosci 1997; 20:523–529.
35. Feleder C, Jarry H, Leonhardt S, Wuttke W, Moguilevsky J. The GABAergic control of gonadotropin-releasing hormone secretion in male rats during sexual maturation involves effects on hypothalamic excitatory and inhibitory amino acid systems. Neuroendocrinology 1996; 64:305–312.
36. DeFazio RA, Heger S, Ojeda SR, Moenter SM. Activation of A-type γ-aminobutyric acid receptors excites gonadotropin-releasing hormone neurons. Mol Endocrinol 2002; 16:2872–2891.
37. Sawchenko PE, Sawson LW. Relationship of oxytocin pathways to the control of neuroendocrine and autonomic function. In: Aminco JA, Robinson AG (eds.), Oxytocin: Clinical and Laboratory Studies. Amsterdam: Excerpta Medica; 1985:87–103.
38. Crowley WR, Armstrong WE. Neurochemical regulation of oxytocin secretion in lactation. Endocr Rev 1992; 1:13–18.
39. Schulze HG, Gorzalka BB. Oxytocin effects on lordosis frequency and lordosis duration following infusion into the medial preoptic area and ventromedial hypothalamus of female rats. Neuropeptides 1991; 18:99–106.
40. Pedersen CA, Prange JA Jr. Induction of maternal behavior in virgin rats after cerebroventricular administration of oxytocin. Proc Natl Acad Sci U S A 1979; 76:6661–6665.
41. Pedersen CA, Ascher JA, Monroe JA, Prange JA Jr. Oxytocin induces maternal behavior in virgin female rats. Science 1982; 216:648–649.
42. Rettori V, Canteros G, Renoso R, Gimeno M, Mc Cann SM. Oxytocin stimulates the release of luteinizing hormone releasing hormone from medial basal hypothalamic explants by releasing nitric oxide. Proc Natl Acad Sci U S A 1997; 94:2741–2744.
43. Selvage D, Johnston CA. Central stimulatory influence of oxytocin on preovulatory gonadotropin-releasing hormone requires more than the median eminence. Neuroendocrinology 2001; 74:129–134.
44. Higuchi T, Uchide K, Honda K, Negoro H. Functional development of the oxytocin release mechanism and its role in the initiation of parturition in the rat. J Endocr 1985; 106:311–316.
45. Malek A, Blann E, Mattison DR. Human placental transport of oxytocin. J Maternal-Fetal Med 1996; 5(5):245–255.
46. Oosterbaan HP, Swaab DF, Boer GJ. Oxytocin and vasopressin in the rat do not readily pass from the mother to the amniotic fluid in late pregnancy. J Dev Physiol 1985; 7:55–62.
47. Dawood MY, Lauersen NH, Trivedi D, Ylikorkala O, Fuchs F. Studies of oxytocin in the baboon during pregnancy and delivery. Acta Endocrinol 1979; 9:704–718.
48. Almazan G, Lefebvre DL, Zingg HH. Ontogeny of hypothalamic vasopressin, oxytocin and somatostatin gene expression. Dev Brain Res 1989; 45:69–75.
49. Whitnall MH, Key S, Ben-Barak Y, Ozato K, Gainer H. Neurophysin in the hypothalamo-neurohypophysial system. II. Immunocytochemical studies of the ontogeny of oxytocinergic and vasopressinergic neurons. J Neurosci 1985; 5:98–109.
50. Evans J, Reid R, Wakeman S, Croft L, Benny P. Evidence that oxytocin is a physiological component of LH regulation in non-pregnant women. Human Reprod 2003; 18:1428–1431.
51. Sato T, Jyujo T, Kawarai Y, Asai T. Changes in LH-releasing hormone content of the hypothalamus and electron microscopy of the anterior pituitary after prostaglandin E2 injection in rats. Am J Obstet Gynecol 1975; 122:637–641.
52. Ojeda SR, Urbanski HF, Costa ME, Hill DF, Moholt-Siebert M. Involvement of transforming growth factor alpha in the release of luteinizing hormone-releasing hormone from the developing female hypothalamus. Proc Natl Acad Sci U S A 1990; 87:9698–9702.