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Estradiol Stimulation of Pulsatile Gonadotropin-Releasing Hormone Secretion *in Vitro*: Correlation with Perinatal Exposure to Sex Steroids and Induction of Sexual Precocity *in Vivo*

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Our aim was to study the effect of estradiol (E2) on pulsatile GnRH secretion *in vitro* in relation to sex and development. When hypothalamic explants obtained from 5- and 15-d-old female rats were exposed to E2 (10^{-7} M), a reduction of GnRH interpulse interval (IPI) occurred but not at 25 and 50 d of age. This effect was prevented by the estrogen receptor antagonist ICI 182.780 and the AMPA/kainate receptor antagonist DNQX but not by the AMPA and *N*-methyl-D-aspartate receptor antagonists SYM 2206 and MK-801. E2 did not affect GnRH IPI in hypothalamic explants obtained from male rats. Therefore, the possible relation between the female-specific effects of E2 *in vitro* and perinatal sexual differentiation was investigated. When using explants obtained from female rats masculinized through testosterone injection on postnatal d 1, E2 was no longer effective *in vitro* at 5 and 15 d. In addition, with explants obtained from male rats demasculinized through perinatal aromatase inhibitor treatment, E2 became capable of decreasing GnRH IPI *in vitro* at 15 d. To study the possible pathophysiological significance of early hypothalamic E2 effects, female rats received a single E2 injection on postnatal d 10. This resulted in reduced GnRH IPI *in vitro* on d 15 as well as advancement in age at vaginal opening and first estrus. In conclusion, E2 decreases the GnRH IPI in the immature female hypothalamus *in vitro* through a mechanism that depends on perinatal brain sexual differentiation and that could be involved in some forms of female precocious puberty. (*Endocrinology* 145: 2775–2783, 2004)

IN CONDITIONS SUCH AS congenital adrenal hyperplasia (1, 2), sex-steroid-secreting tumors (3), or gonadotropin-independent sexual precocity (4, 5), the secondary occurrence of central precocious puberty has led to the hypothesis that sex steroids could promote hypothalamic maturation. In addition, the different forms of central precocious puberty are remarkably more frequent in the female than in the male (6), suggesting that the female hypothalamus could be more sensitive to triggering signals such as sex steroids. Hypothalamic mechanisms, however, are difficult to study in humans because they can only be assessed indirectly through pituitary gonadotropin secretions that are directly inhibited by a potent negative feedback, particularly in the immature individual (7). In the rat, evidence has been provided that sexual maturation could be advanced after estrogen administration before puberty (8). Since we have set up a rat hypothalamic explant paradigm in which developmental changes in frequency of pulsatile GnRH secretion can be observed (9), we first aimed at studying the developmental and sex-related effects of sex steroids in the hypothalamus *in vitro*.

In previous studies, we found that the developmental changes in frequency of pulsatile GnRH secretion *in vitro* were associated with an increase in stimulatory glutamatergic inputs (10–12), an increase in activity of glutaminase (an enzyme involved in glutamate biosynthesis) (13), and a reduction in prolyl endopeptidase activity that resulted in a decreased inhibitory GnRH autofeedback mediated by the $GnRH_{1-5}$ degradation product through interaction at the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors (12, 14). Moreover, *in vivo* studies in rats and monkeys have shown the involvement of glutamate receptors in the experimental induction of precocious puberty using NMDA receptor agonists and delayed puberty using NMDA receptor antagonists (15–18). Our second aim was to study the possible involvement of glutamate receptors in estradiol (E2) effects on GnRH secretion *in vitro*.

The impact of E2 on adult reproductive function depends on brain sexual differentiation during the perinatal period (19, 20). In females, neonatal injection of testosterone (T) disrupts estrous cyclicity in the adult and induces male sexual behavior (19). In males orchidectomized at birth, an LH surge (20) can be induced by E2 treatment in adulthood, and the male brain can also be demasculinized using an aromatase inhibitor within the first hours after birth (21, 22). Our third aim was to determine whether perinatal sexual differentiation could influence the effects of E2 on pulsatile GnRH secretion *in vitro*. In addition, we aimed to study whether the effects of E2 on GnRH pulse frequency *in vitro* could be observed after E2 treatment of immature female rats *in vivo*

Abbreviations: AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; ATD, 1,4,6-androstatrien-3,17-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; E2, estradiol; ER, estrogen receptor; GABA, γ-aminobutyric acid; IPI, interpulse interval; NMDA, *N*-methyl-D-aspartate; T, testosterone; V.O., vaginal opening.

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and whether this effect could possibly be associated with subsequent precocious puberty.

Materials and Methods

Animals and reagents

Pregnant rats and 5-, 15-, 25-, and 50-d-old male and female Wistar rats were purchased from the University of Liège and housed under standardized conditions (22 C, lights on from 0630 to 1830 h, food and water *ad libitum*). Each litter contained nine pups, and weaning occurred at the age of 3 wk. The day of birth was considered as postnatal d 1. All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethical Committee at the University of Liege.

The incubation medium MEM (phenol red-free MEM) was purchased from Life Technologies, Inc. (Invitrogen Corp., Merelbeke, Belgium). Sesame oil was purchased from VWR international (Leuven, Belgium). The aromatase inhibitor ATD (1,4,6-androstatrien-3,17-dione) was purchased from Steraloids Inc. (Newport, RI). The NMDA receptor antagonist MK-801 was obtained from Merck Sharp & Dohme research laboratory (Rahway, NJ). The AMPA/kainate receptor antagonist DNQX (6,7-Dinitroquinoxaline-2,3-dione), the AMPA receptor antagonist SYM 2206 (4-aminophenyl-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphtalazine) and the estrogen receptor (ER) antagonist ICI 182.780 were purchased from Tocris (Fisher Bioblock Scientific, Illkirch, France). 17β-E2 and T were purchased from Sigma-Aldrich (Bornem, Belgium); and R76713, racemic Vorozole, an aromatase inhibitor, was obtained from Janssen Pharmaceutica (Beerse, Belgium). All steroids were dissolved initially in absolute ethanol and subsequently in incubation medium to achieve a final ethanol concentration of 0.01%. R76713 was initially diluted in 20% polyethyleneglycol (3.35 mg/ml) and subsequently in the incubation medium to achieve a 10^{-5} M solution containing 0.02% polyethyleneglycol. All the other drugs were diluted directly in the incubation medium.

Hypothalamic explant incubation and hormone assays

The animals were rapidly decapitated between 1000 and 1100 h, and trunk blood was collected. After decapitation, the retrochiasmatic hypothalamus was rapidly dissected and transferred into a static incubator as described in detail previously (23, 24). The medium consisted of phenol red free MEM supplemented with glucose, magnesium, glycine, and bacitracin (25 mM, 1 mM, 10 nM, 20 μM, respectively). The incubation medium was collected and renewed every 7.5 min and kept frozen until assayed. Each experiment included 12–16 hypothalamic explants, which were incubated individually, each in a separate chamber. In each experiment, at least two explants were incubated in MEM alone and used as controls. GnRH was measured in duplicate using a RIA method with intra- and interassay coefficients of 14 and 18%, respectively (24, 25). The CR11-B81 anti-GnRH antiserum (final dilution, 1:80,000) was kindly provided by Dr. V. D. Ramirez (Urbana, IL) (26). The data below the limit of detection (5 pg/7.5 min) were assigned that value. After 4 h clotting at room temperature, trunk blood was centrifuged (10 min at 1500 $\times$ *g*). Serum was collected and stored at −20 C until assayed. The LH assay material was kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases program. The samples were measured in duplicate.

Study protocols

In vitro experiments. The effect of E2 on pulsatile GnRH secretion was studied using explants obtained from female and male rats at 5, 15, 25, and 50 d of age. The steroid was used for the whole 4-h experimental period. The explants were incubated with 10^{-9}–10^{-7} M E2 in initial experiments. Subsequently, explants obtained from 15-d-old male and female rats were used and incubated using 10^{-7} M E2. To study the implication of ionotropic glutamate receptor subtypes, the antagonists MK-801 (3.10^{-6} M), DNQX (10^{-6} M), or SYM2206 (10^{-6} M) were used together with E2 or alone as control. The dosage of the antagonists was selected based on previous experiments (23, 24). The effect of T was studied by incubation with T (10^{-7} M). The aromatase involvement in the T effect was studied through coincubation of T with the aromatase inhibitor R76713 (10^{-5} M), which was also used alone as control. The involvement of ER was studied using the ER antagonist ICI 182.780 (10^{-7} M) in the incubation medium together with E2 or alone as control.

In vivo experiments. In Fig. 1, a schematic summary of the *in vivo* experiments is shown on an age scale.

Protocol A. Masculinization of female rats. On postnatal d 1, female pups received either a single sc injection of 1.25 mg T in 100 μl sesame oil (27) or vehicle only, for control purposes. The animals were killed either on postnatal d 5 or on d 15, and pulsatile GnRH secretion was studied *in vitro* in the presence of 10^{-7} M E2 or in MEM alone.

Protocols B and C. Demasculinization of male rats. In protocol B, pregnant rats were injected daily sc with the aromatase inhibitor ATD (5 mg in

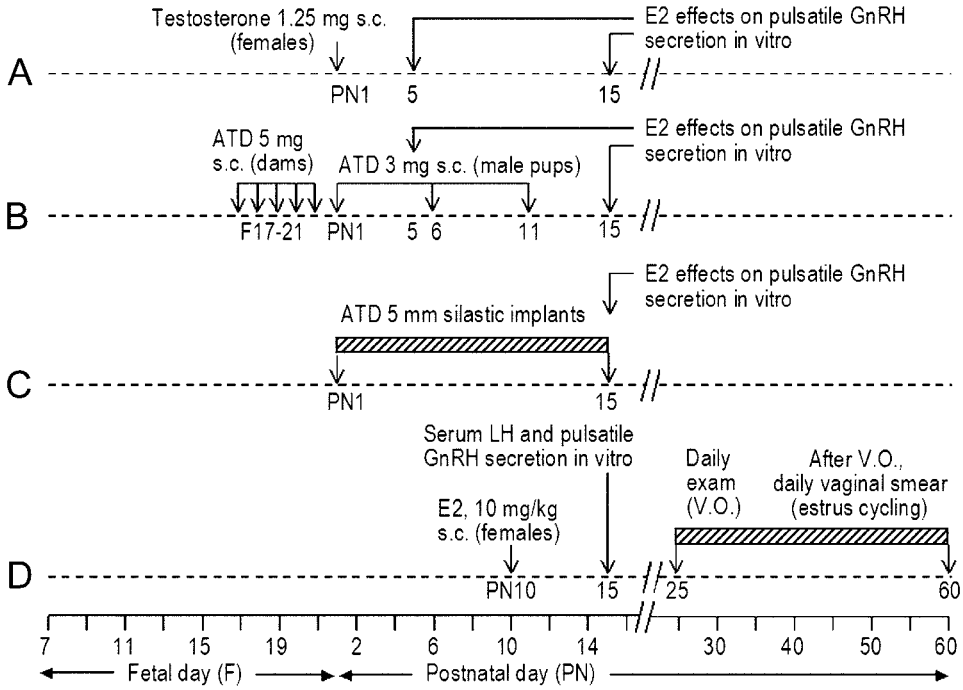

FIG. 1. Schematic illustration of the *in vivo* experiments, with treatment and investigations represented on an age scale.

100 μl sesame oil) from d 17–21 of gestation. In the control group, pregnant rats were injected with vehicle. In the offspring, the male pups were injected sc with 3 mg ATD in 50 μl sesame oil on postnatal d 1, 6, and 11 (28). The control rats were injected with the vehicle only. The animals were killed either on postnatal d 5 or 15, and pulsatile GnRH secretion was studied *in vitro* with or without the addition of 10^{-7} M E2. In protocol C, ATD was administered as silicone implants (length, 5 mm; inner diameter, 1.6 mm; outer diameter, 2.4 mm; Degania Silicone Silclear tubing, Degania Bet, Israel). Within 5 h after birth, the pups were anesthetized through ice-cooling and surgically implanted in the neck (29). The animals were killed on d 15 to obtain the hypothalamic explants and to study pulsatile GnRH secretion with or without 10^{-7} M E2.

Protocol D. Single E2 administration in immature female rats. Ten-day-old female rats received a single sc injection of E2 (10 mg/kg given in 100 μl sesame oil) (30, 31), which is comparable to the dose used by others (32). This particular age was selected to be outside of the critical period for sexual differentiation of the brain (31), and a single injection was given to avoid the persisting negative feedback effect that could result from repeated or prolonged administration. To increase the likelihood of an effect after a single administration, we used a massive dose that was greater than in the previous experiments showing induction of precocious puberty (8). There were three groups of 14 rats injected either with E2 or with sesame oil or saline. On d 15, five rats from each group were killed to study pulsatile GnRH secretion *in vitro* in the absence of any steroid in the incubation medium. Serum LH was also measured using trunk blood. In the remaining animals, sexual maturation was subsequently evaluated by daily examination for imperforation of the vaginal membrane (vaginal opening, V.O.). Thereafter, vaginal smears were taken every day in the afternoon until postnatal d 60. Slides of vaginal smears were colored following the papanicolaou method and examined to detect the occurrence of estrus cycle. The age at the first estrus was considered when vaginal smears contained primary leukocytes after the first proestrus phase, which was characterized by cornified cells (33).

Statistical analysis

The mean LH levels (ng/ml) were calculated after log transform and expressed as geometric mean.

GnRH secretory pulses were detected using the PULSAR program for PC (basic version by S. Rosberg) (34). The cut-off criteria for peak detection were determined empirically and were G1 = 2.5 and G2 = 2 (35). Peak splitting parameter was set at 2.7, and the intraassay coefficient was used as B coefficient (35). Data concerning the interpulse interval (IPI) from different experiments were pooled and expressed as mean ± SD. The mean (±SD) amplitude of GnRH secretory pulses was calculated (pg/7.5 min fraction), and the data from different experiments were analyzed separately by *t* test to avoid biases due to interassay variations between experiments. In several instances, all the explants in a group showed a similar GnRH IPI. Then, SD was zero and could not be represented in the figures. Similarly, when V.O. occurred at the same age in all the rats in a control group, the SD was zero and could not be represented. Significant effect of treatment was determined by *t* test (control *vs.* E2 incubation). The effect of antagonist on control and treated groups was determined by ANOVA test (one-way ANOVA) followed by Newman-Keuls post test when the threshold for significance of difference ($P < 0.05$) was reached.

Results

As shown in Fig. 2, incubation of individual hypothalamic explants obtained in 15-d-old female rats with different E2 concentrations (10^{-9}–10^{-7} M) resulted in a significant dose-related decrease of GnRH IPI. E2 effectiveness occurred in the nanomolar range as observed in other studies (36, 37). In subsequent experiments, we used the highest concentration because it was the most efficient in our paradigm. In explants from 15-d-old male rats, no significant decrease of GnRH IPI was caused by 10^{-7} M of E2 (Fig. 2). With explants from 5-d-old female rats, E2 (10^{-7} M) caused a significant decrease in GnRH IPI, whereas no effect of E2 on pulsatile GnRH secretion could be observed at 25 or 50 d in the female or at any age studied in the male (Table 1). GnRH pulse amplitude (mean ± SD, pg/7.5 min) was not affected by E2 at 5 d or 25 d in both sexes. In 15-d-old female rats, discrepant data were obtained because GnRH pulse amplitude was slightly, but significantly, decreased in one experiment (mean amplitude ± SD; controls, 9.9 ± 0.2 pg/7.5 min; E2, 7.4 ± 0.4 pg/7.5 min; t = 5.657; *df* = 16), whereas a significant increase in amplitude was seen in a second experiment (controls, 10.3 ± 0.4 pg/7.5 min; E2, 15.6 ± 2 pg/7.5 min; t = 7.7; *df* = 19). In a third experiment, amplitude was found to be higher and more variable with no significant changes.

As previously reported (23, 35), the NMDA receptor antagonist MK-801 resulted in a significant increase of GnRH IPI (59 ± 3 *vs.* 68 ± 4 min, control *vs.* MK-801, Fig. 3) when hypothalamic explants of 15-d-old rats were used. In contrast, the AMPA/kainate receptor antagonist (DNQX) or the specific AMPA receptor antagonist (SYM 2206) did not affect the GnRH IPI in similar conditions (control, 59 ± 3 min;

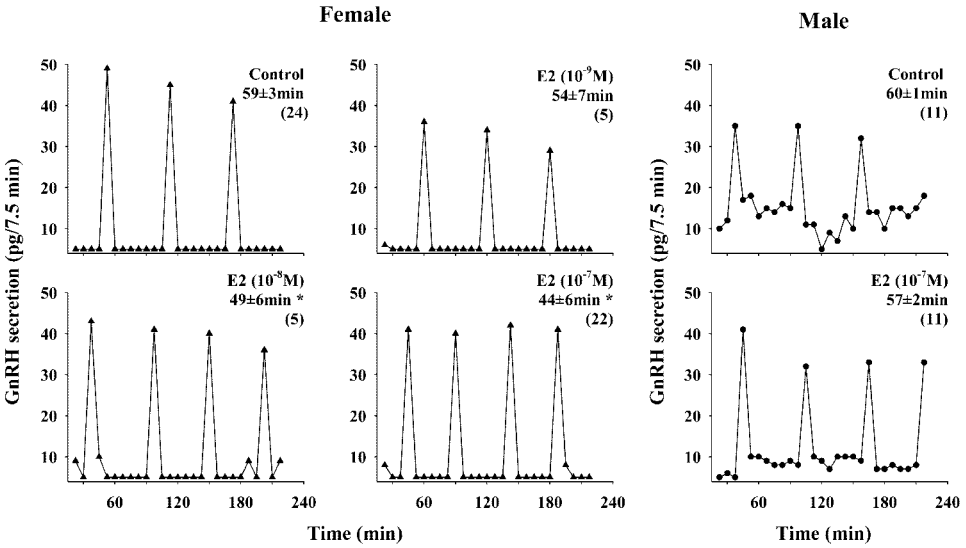

FIG. 2. Representative profiles of pulsatile GnRH secretion from six individual hypothalamic explants obtained at 15 d in female (*left and middle panels*) and male (*right panels*) rats and incubated in control conditions or together with different concentrations of E2 (mean ± SD). GnRH IPI is given together with the number of explants studied in each condition (n). *, $P < 0.05$ (controls *vs.* E2 10^{-8} M, t = 6.127, *df* = 27; controls *vs.* E2 10^{-7} M, t = 12.4, *df* = 44). The limit of detection was 5 pg/7.5 min.

DNQX, 60 ± 4 min; SYM2206, 62 ± 1 min). When similar experiments were performed in the presence of 10^{-7} M of E2 (Fig. 3), the reduction of GnRH IPI caused by E2 was not observed anymore in the presence of DNQX (59 ± 2 min). Using SYM 2206, the E2 effect was still observed (62 ± 1 min *vs.* 48 ± 2 min, SYM 2206 *vs.* E2/SYM 2206). Likewise, the E2 effect was still observed in the presence of MK-801 (68 ± 4 *vs.* 58 ± 1 min, MK-801 *vs.* E2/MK-801). With hypothalamic explants obtained from female rats and incubated in the presence of the ER antagonist ICI 182.780, the reduction of the GnRH IPI caused by E2 was absent (Table 2). T was as effective as E2 in decreasing GnRH IPI in female explants, and this effect was significantly prevented by an aromatase inhibitor (R76713, 10^{-5} M) that had no effect when used alone. When explants obtained from male rats were incubated either with E2 or with T, no change in GnRH IPI was observed (Table 2).

Because E2 could affect the GnRH IPI in the female only, we studied the involvement of steroid imprinting in the perinatal period. As shown in Fig. 4, after female rats were androgenized through a sc injection of T on postnatal d 1, the hypothalamic explants studied on d 5 and 15 did not show any significant change in GnRH IPI when incubated with E2 (84 ± 2 *vs.* 90 ± 0 min at 5 d, 58 ± 1 *vs.* 61 ± 2 min at 15 d, E2 *vs.* control). With control explants from female rats injected neonatally with the vehicle, E2 could significantly decrease the GnRH IPI *in vitro* at both ages (75 ± 0 *vs.* 86 ± 10 min at 5 d, 45 ± 6 *vs.* 56 ± 7 min at 15 d, E2 *vs.* control). When male rats were demasculinized through perinatal administration of the aromatase inhibitor ATD, E2 caused a significant decrease in GnRH IPI at 15 d (48 ± 4 min *vs.* 60 ± 0 min, E2 *vs.* control). A nonsignificant effect was observed at 5 d (83 ± 5 *vs.* 86 ± 6 min, E2 *vs.* control). Using explants from male rats treated perinatally with vehicle, E2 had no effect on the GnRH IPI. To decrease the stress due to manipulation, we implanted male pups with a capsule containing ATD within 5 h after birth. In this case, the results obtained were similar to those obtained in ATD-injected pups (Table 3), which indicates that neonatal inhibition of aromatase activity is sufficient for E2 effect to occur.

After a single massive injection of E2 on d 10 *in vivo*, the GnRH IPI studied *in vitro* on d 15 was found to be significantly reduced (Fig. 5). On the same day, serum LH was decreased in the E2-treated animals. In the E2-injected female rats, the age at V.O. (27 ± 1 d) and at first estrus (33 ± 1 d) was significantly advanced in comparison with controls injected with vehicle (V.O., 31 ± 1 d; first estrus, 37 ± 2 d) or saline (V.O., 33 ± 0 d; first estrus, 38 ± 1 d). Age at V.O. was also advanced after vehicle injection when compared with saline, but age at first estrus was not significantly different between these two groups.

TABLE 1. Effect of E2 (10^{-7} M) on the IPI of pulsatile GnRH secretion *in vitro* using male and female rat (n) hypothalamic explants

Age (d)	Females		Males	
	Control	Estradiol	Control	Estradiol
5	85 ± 15 (15)	75 ± 7[a] (14)	83 ± 15 (11)	87 ± 2 (11)
15	59 ± 3 (24)	44 ± 6[a] (22)	60 ± 1 (11)	56 ± 4 (11)
25	35 ± 3 (6)	32 ± 2 (7)	41 ± 2 (6)	45 ± 10 (8)
50	34 ± 0 (3)	34 ± 3 (3)	34 ± 3 (3)	35 ± 2 (3)

Data are mean ± SD (min, [a] $P < 0.05$, E2 *vs.* control, 5-d-old female rats: t = 2.16, df = 27; 15-d-old female rats: t = 12.4, df = 44).

Discussion

The developmental increase in GnRH pulse frequency is an important event for the pubertal changes in gonadotropin secretion and has been observed *in vivo* in the female (38) and the male rat (39) as well as in the female monkey (40) and *in vitro*, using our rat explant paradigm (9). The critical age period for such changes, however, was earlier *in vitro* (9) than

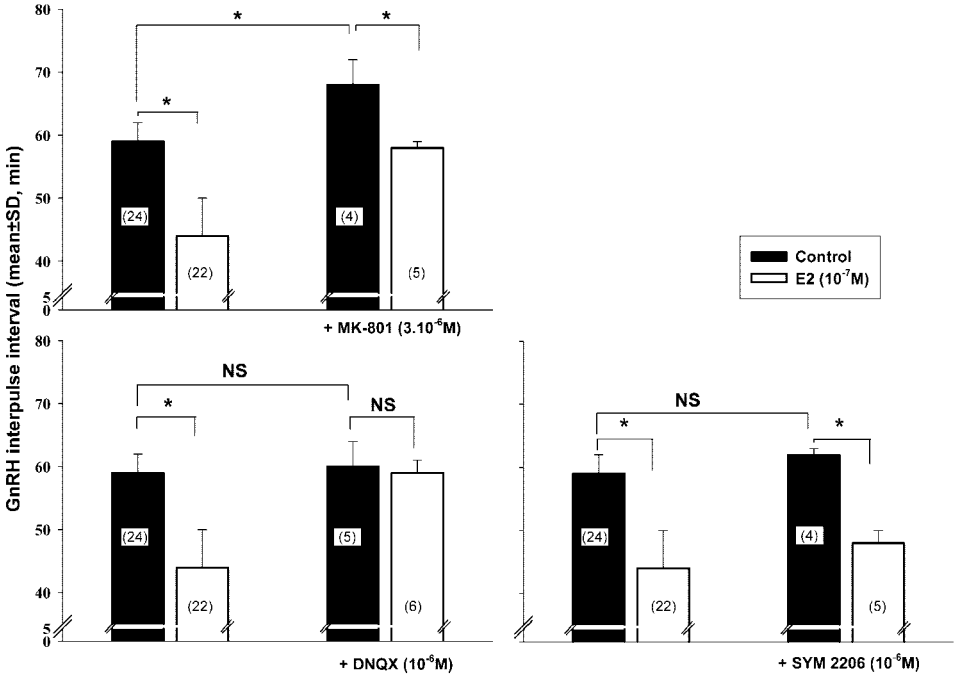

FIG. 3. Effect of antagonists of NMDA (MK-801), AMPA/kainate (DNQX), and AMPA receptors (SYM 2206) on GnRH IPI (mean ± SD, min) using (n) hypothalamic explants obtained from 15-d-old female rats and incubated in control conditions or with E2. NS, Nonsignificant; *, $P < 0.05$ (MK-801, F = 79.27, *df* = 3; DNQX, F = 68.23, *df* = 3; SYM2206, F = 67.69, *df* = 3.

in vivo (38, 39). Isolation of the hypothalamus could suppress extrahypothalamic inhibiting inputs and account for earlier manifestation of the accelerated GnRH pulse generator. In this study, the developmental acceleration of pulsatile GnRH secretion *in vitro* was found to be stimulated by E2 specifically in the immature female hypothalamus and through a mechanism dependent on perinatal brain sexual differentiation. In addition, E2 administration to immature female rats *in vivo* resulted in both stimulation of pulsatile GnRH secretion in the hypothalamus and subsequent precocious puberty. The physiological significance of our data in the normal immature rat was uncertain due to normally limited or absent exposure to sex steroids in such young animals. However, ultrasensitive E2 assay showed much higher plasma levels in prepubertal girls than in boys, which was proposed to be responsible for the faster maturation in girls than in boys (41). Considering the abnormal early exposure to sex steroids, our observations could provide a pathophysiological basis for central precocious puberty occurring as a complication of peripheral sexual precocity.

Using the rat model, we explored the possible pathophysiological significance of accelerated hypothalamic maturation induced by abnormal early exposure to sex steroids, a putative mechanism of central precocious puberty occurring

TABLE 2. Effect of E2 and testosterone and ER antagonist (ICI 182.781) and aromatase inhibitor (R76713) on the IPI of pulsatile GnRH secretion *in vitro* using (n) 15-d-old rat hypothalamic explants

Steroid antagonist	Sex	Control	E2 (10^{-7} M)	T (10^{-7} M)
None	F	59 ± 3 (24)	44 ± 6[a] (22)	46 ± 4[a] (6)
ICI 182.780 (10^{-7} M)	F	61 ± 1 (4)	57 ± 1 (5)	
R76713 (10^{-5} M)	F	59 ± 6 (4)		56 ± 6 (4)
None	M	60 ± 1 (11)	57 ± 2 (11)	51 ± 4 (4)

Data are mean ± SD (min, [a] $P < 0.05$, control *vs.* E2: t = 12.4, df = 44; control *vs.* T: t = 11.36, df = 28)

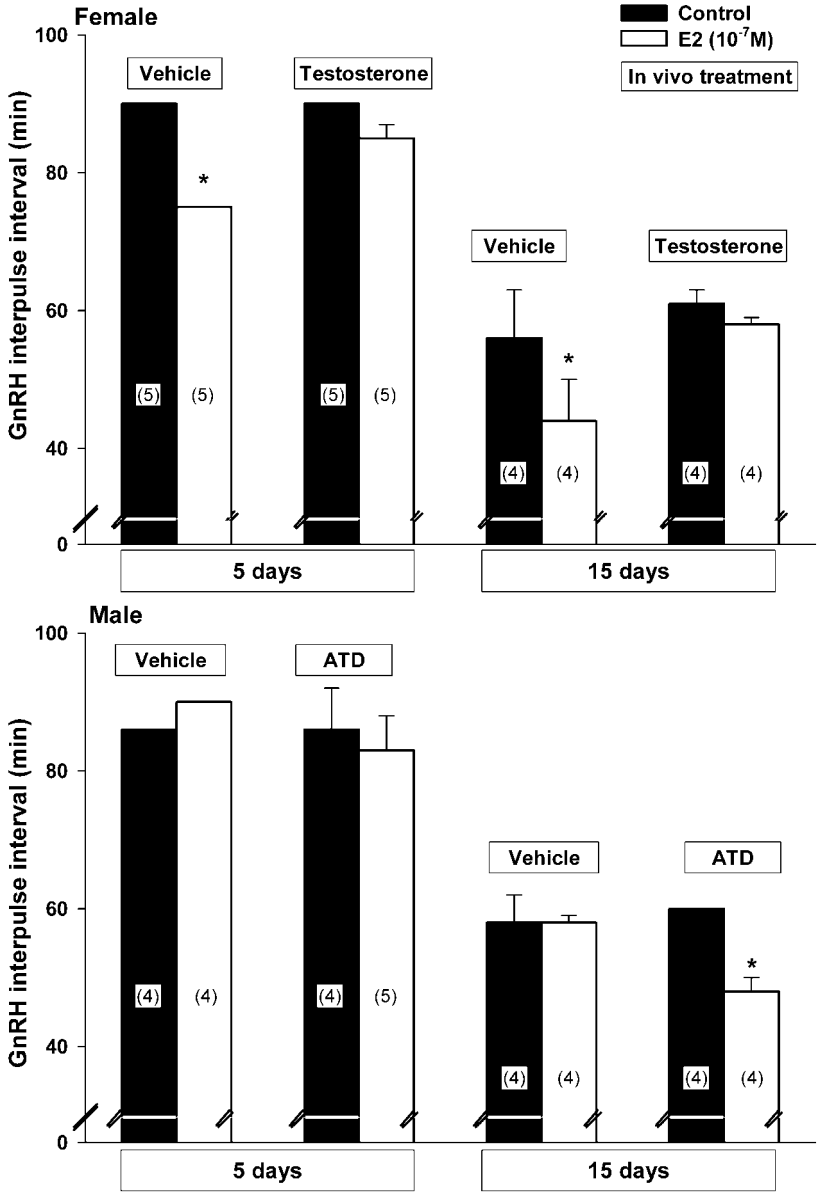

FIG. 4. E2 effect *in vitro* on the GnRH IPI (mean ± SD) obtained using (n) hypothalamic explants from female rats androgenized neonatally through sc injection of 1.25 mg T on postnatal d 1 (*upper panel*) and from male rats treated perinatally using 5 mg of the inhibitor of aromatase ATD injected daily sc to the dams on d 17–21 of gestation and 3 mg to the pups on postnatal d 1, 6, and 11 (*lower panels*). *, $P < 0.05$, control *vs.* E2 (5-d-old female rats, vehicle, t = 2.379, *df* = 8; 15-d-old female rats, vehicle, t = 2.484, *df* = 6; 15-d-old male rats, ATD, t = 11;54, *df* = 6).

secondarily to peripheral precocity (1–4). In the female rat, chronic treatment with low doses of E2 between postnatal d 5 and 30 resulted in early peripheral signs of puberty as shown by early V.O. (42). Because administration of high doses of E2 before d 10 caused brain masculinization with loss of estrus cyclicity (19, 31), we elected to use a single E2 administration on d 10 to avoid interfering with this sexually differentiated process, so that the timing of first estrus cyclicity, a presumably centrally driven pubertal event, could be studied. The E2 administration *in vivo* resulted in a reduction of serum LH 5 d after E2 injection, which is in agreement with pituitary negative feedback effects that characterize immature female rats (42, 43). A decrease in the age at V.O., as well as at first estrus, was also observed. These results are in agreement with other findings (32) and point out that the hypothalamic-pituitary-gonadal axis is sensitive to E2 maturational effect *in vivo* both peripherally (V.O.) and centrally (first estrus). In addition, the study of pulsatile GnRH secretion *in vitro* provided evidence of concomitant advance in the developmental acceleration of GnRH pulse frequency that could account for subsequent occurrence of central precocious puberty. This pattern was similar to several clinical conditions such as congenital adrenal hyperplasia (1, 2), sex steroid secreting tumors (3), and gonadotropin-independent sexual precocity (4, 5), where central precocious puberty occurred after treatment suppressing the peripheral excess of sex steroids. So, these conditions account for withdrawal of sex steroids after a period of exposure (6) such as obtained here after an acute massive E2 administration.

E2 effects on GnRH neurons or GnRH secretion in the hypothalamus were shown in several conditions using different paradigms. Using hypothalamic explants from adult female monkeys, an increase in GnRH release was observed *in vitro* 12 and 48 h after E2 treatment *in vivo*, whereas LH secretion was reduced after 12 h and increased after 48 h (44). Such a discrepancy between E2 effects on GnRH secretion *in vitro* and gonadotropin secretion *in vivo* were consistent with our *in vivo* data showing reduced serum LH levels and increased frequency of pulsatile GnRH secretion 5 d after a single E2 administration. This suggested a possible coexistence of negative feedback effects at the pituitary level and stimulating effects in the hypothalamus. Because E2 commonly resulted in inhibition of pulsatile LH secretion, particularly in immature rodents (45, 46), a concomitant stimulating hypothalamic effect could be difficult to demonstrate through the study of LH secretion. Noteworthy were some possible inhibitory effects of E2 on the GnRH pulse generator that could also occur as shown through electrophysiological recording in the hypothalamus (47). A stimulating effect of E2 on GnRH output *in vivo* was described in female rats (48) and ewes (49). In these experiments, the mass of GnRH release increased in possible relation to the preovulatory GnRH and/or LH surge. However, these results were obtained in adult ovariectomized animals (44, 48, 49), a condition different from the present study in intact immature rats. It is possible that stimulating effects of E2 observed *in vitro* persist in the adult female rat but could not be observed in the conditions of the present study. We showed earlier that the amplitude of GnRH secretion by hypothalamic explants obtained around 1600 h in cycling female rats was increased in the afternoon of proestrus, presumably in relation with the sex steroid induced preovulatory surge (50). For consistency and practical reasons, all the experiments reported here were carried out starting at 1000 h and in the absence of the preoptic area. Further experiments that start at 1600 h with explants obtained from adult female rats and including the preoptic area are warranted to study the possible effects of E2 on either GnRH pulse frequency and/or amplitude *in vitro*. In this study, E2 increased GnRH pulse frequency within minutes after starting incubation *in vitro*. Such a rapid effect suggests a mechanism different from the preovulatory surge and also deserves further studies. A rapid effect of E2 on LH secretion was reported in the ovariectomized female guinea pig that was attributed to direct E2 effects on the pituitary responsiveness rather than on the GnRH output (51). Our data raises the question as to whether E2 acts directly on GnRH neurons or on other target structures in the hypothalamus to increase GnRH pulse frequency. Recently, GnRH neurons were shown to express ERα protein (52) and ERβ protein and mRNA (53). Using hypothalamic slices from GnRH-GFP transgenic mice, E2 was able to decrease the

TABLE 3. Effect of perinatal inhibition of aromatase activity in 15-d-old male rats; ATD was delivered through silastic implants with or without (control) aromatase inhibitor

Implants	Control	E2 (10^{-7} M)
Empty	59 ± 1 (3)	58 ± 1 (3)
ATD	60 ± 0 (5)	48.9 ± 2[a] (6)

Data are mean ± SD (min, [a] $P < 0.05$, E2 *vs.* control, ATD implants: t = 10.73, df = 3). N (n) explants were used in each condition.

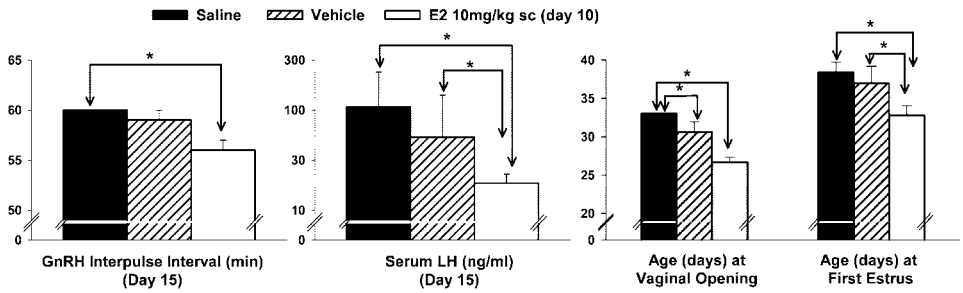

FIG. 5. Effect of a single sc injection of E2 (10 mg/kg, postnatal d 10) in female rats on GnRH IPI *in vitro* (n = 4 in each group, F = 21.50, *df* = 2). Measurement of serum LH (n = 5 in each group, F = 7.094, *df* = 2) as well as age at V.O. (n = 10 in each group, F = 133.2, *df* = 2) and age at first estrus (n = 10 in each group, F = 31.85, *df* = 2). The control groups were injected sc either physiological saline (saline) or vehicle (sesame oil). Data are mean ± SD. *, $P < 0.05$.

GnRH neuron firing rate (54) and increase the number of quiescent GnRH neurons (55), whereas membrane excitability was increased as well (56). Although these observations support a direct involvement of GnRH neurons in hypothalamic effects of E2, we used retrochiasmatic explants where GnRH cell bodies were virtually absent (57). Therefore, an indirect E2 effect on GnRH secretion through interneurons or glial cells could occur. Such cells have been shown to play an important role in the control of GnRH secretion throughout development (58). In addition, an E2 effect could occur in the terminal part of GnRH neurons because, using median eminence explants, an increased output of basal GnRH release was observed within 5–10 min of incubation with E2 (36).

Since the onset of puberty involves an activation of hypothalamic glutamatergic inputs to GnRH neurons (59), we evaluated glutamate receptor involvement in the E2-induced decrease in GnRH IPI. As expected from the earlier demonstration implicating NMDA receptor activation in pulsatile GnRH/LH secretion (16, 23), the NMDA receptor antagonist MK-801 resulted in increased GnRH IPI *in vitro*. However, MK-801 did not affect E2 stimulation of an increased GnRH pulse frequency, suggesting that NMDA receptors were not primarily involved in the mechanism of E2 effects. Previous studies showed that non-NMDA receptors are involved in adult female reproductive life, because nearly half of activated GnRH neurons expressed kainate receptor subunits in the afternoon of proestrus (60). In addition, the AMPA/kainate receptor antagonist DNQX can block the E2-induced LH surge in OVX rats (61). In the present study, E2 stimulation of GnRH pulse frequency was shown to involve specifically the kainate receptor subtype. As reported previously (24), DNQX alone had no effect on pulsatile GnRH secretion *in vitro*. Based on the evidence of an E2-kainate receptor interaction involved in the control of GnRH secretion, the absence of kainate receptor antagonist effect on the timing of puberty could be explained by the silent role of such receptors in the physiological absence of sex steroids in the immature animal. The implication of kainate receptor in E2 effect was further supported by hypothalamic colocalization of kainate receptor with ER (62). The preferential involvement of kainate receptors in E2 effect on GnRH secretion *in vitro* was consistent with the potentiating effect of E2 on kainate-evoked currents in hippocampal neurons (63) or in oxytocinergic neurons (37). The developmental disappearance of E2 effect in the female rat hypothalamus could also be linked to the developmental increase in activity of glutamatergic inputs and/or decrease of GABAergic inputs shown in the primate (64) and the rodent (10, 11, 35, 65). E2 was reported either to increase γ-aminobutyric acid (GABA) turnover in relation with negative feedback effects (66) or to decrease GABA levels just before the E2-induced LH surge (67). In addition, kainate down-regulates GABA release in mediobasal hypothalamic explants from ovariectomized female rats treated with E2 but not in untreated animals (68). It could be hypothesized that E2 effects were involving the GABAergic pathway because they were seen only in a period of high activity of that inhibitory amino acid. Such indications of involvement of the GABAergic pathway in E2 effects with developmental changes point to the need of further studies using our *in vitro* paradigm.

An important finding was that the E2 stimulatory effects on GnRH pulse frequency were only seen using hypothalamic explants obtained from female rats but not using explants obtained from male rats. Such a sex-related effect was not simply dependent on E2 because T was as effective as E2 in the female, provided that T could be aromatized into E2. Further confirmation of the key role of E2 came from experiments showing the inhibitory effect of ICI 182.780 on E2 stimulation of GnRH pulse frequency. Such an involvement of ER was not inconsistent with the rapid, possibly nongenomic effect of E2, because ICI 182.780 was shown to prevent some rapid E2 effects such as those involving NO (36). The sexual dimorphism of E2 effects could be related with the sexually divergent effects of kainate on LH secretion that was found to be inhibited in female rats and stimulated in males (69). In addition, the occurrence of E2 effects in the immature female rat could be related to the sexually dimorphic expression of ERβ that was reported in the rat hypothalamus before 21 d of age (70). These developmental variations in ERβ expression are consistent with our observation that this age period is characterized by a particular sensitivity to E2 for increase in GnRH pulse frequency.

The dimorphic effects of E2 could depend also on sexual brain differentiation. If so, we postulated that the E2 effect should occur in hypothalamic explants from demasculinized male rats, whereas this effect should be absent using hypothalamic explants obtained from masculinized females. It was well established that neonatal exposure to T or E2 induced masculinization of female rats, resulting in disappearance of female sexual behavior, absence of the E2-induced LH surge (30), and the occurrence of a delayed anovulatory syndrome in adult life (19, 31). In the male, blockade of aromatase activity can induce appearance of female sexual behavior (21, 28). Therefore, we used these two strategies to investigate the possible early programming of the E2 effect on GnRH pulse frequency that we had confirmed was occurring. Such findings raised the issue of pre- or perinatal programming of sexually dimorphic events that can be different among species. For instance, in the ovine species, the timing of puberty is sexually dimorphic and is clearly affected by fetal exposure to T (71).

In summary, evidence is provided in this paper that E2 can rapidly stimulate an increase in GnRH pulse frequency in the immature female hypothalamus. It is suggested that such a mechanism can contribute to some form of sexual precocity, a hypothesis which deserves further studies.

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