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Oxytocin Facilitates Female Sexual Maturation through a Glia-to-Neuron Signaling Pathway

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It has been earlier proposed that oxytocin could play a facilitatory role in the preovulatory LH surge in both rats and humans. We here provide evidence that oxytocin also facilitates sexual maturation in female rats. The administration of an oxytocin antagonist for 6 d to immature female rats decreased GnRH pulse frequency *ex vivo* and delayed the age at vaginal opening and first estrus. The *in vitro* reduction in GnRH pulse frequency required chronic blockade of oxytocin receptors, because it was not acutely observed after a single injection of the antagonist. Hypothalamic explants exposed to the antagonist *in vitro* showed a reduced GnRH pulse frequency and failed to respond to oxytocin with GnRH release. Prostaglandin E_2 (PGE_2) mimicked the stimulatory effect of oxytocin on GnRH pulse frequency, and inhibition of PG synthesis blocked the effect of oxytocin, suggesting that oxytocin accelerates pulsatile GnRH release via PGE_2. The source of PGE_2 appears to be astrocytes, because oxytocin stimulates PGE_2 release from cultured hypothalamic astrocytes. Moreover, astrocytes express oxytocin receptors, whereas GnRH neurons do not. These results suggest that oxytocin facilitates female sexual development and that this effect is mediated by a mechanism involving glial production of PGE_2. (*Endocrinology* 149: 1358–1365, 2008)

OXYTOCIN PLAYS a crucial role in reproduction. The peptide plays a pivotal role in parturition and lactation in many species (1) and acts centrally to influence maternal and mating behavior in rodents (2–4). In addition to this involvement in reproductive behavior, oxytocin has been shown to stimulate GnRH secretion from medial basal hypothalamic explants of adult male rats (5) and of cycling female rats on the afternoon of proestrus (6). Using hypothalamic explants from male rats, one of our laboratories recently showed that neonatal pulsatile GnRH secretion *in vitro* is facilitated by oxytocin and that this stimulatory effect is mimicked by prostaglandin E_2 (PGE_2) (7).

Sexual maturation involves an acceleration of pulsatile GnRH secretion (8–10). This activation is elicited by neuronal as well as astroglial factors produced by cells functionally connected to GnRH neurons (11). The neuronal networks involved in the transsynaptic regulation of GnRH secretion mainly comprise neurons that use excitatory and inhibitory amino acids for neurotransmission in addition to the newly discovered kisspeptin-GPR54 signaling system (12, 13). However, additional neuronal systems that either stimulate or inhibit GnRH secretion have been described, including noradrenergic, dopaminergic, and opiatergic neurons (14). More recently, oxytocin neurons have been involved in the facilitatory control of GnRH secretion (5–7). The recent findings that oxytocin stimulates GnRH secretion in prepubertal male rats (7) and that administration of an oxytocin antagonist blunted the preovulatory LH peak in women (15) prompted us to study the role of oxytocin in female puberty. Thus, we aimed at studying *in vivo* the possible delaying effects of an oxytocin antagonist on female sexual maturation and used an explant paradigm to define *in vitro* the mechanism underlying this effect. In particular, we aimed at determining whether PGE_2 mediates the facilitatory effect of oxytocin on pulsatile GnRH secretion, a pathway suggested by the ability of oxytocin to stimulate PGE_2 release from the rat hypothalamus (5), and the effectiveness of PGE_2 to stimulate GnRH release (16) via PGE_2 receptors expressed in GnRH neurons (17).

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Abbreviations: $AACOCF_3$, Arachidonylfluoromethyl ketone; OTR, oxytocin receptor; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2.

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Materials and Methods

Animals

Female Wistar rats used for *in vivo* studies and *in vitro* experiments to measure pulsatile GnRH release were housed in temperature- and light-controlled conditions and were given *ad libitum* access to water and standard rat pellets. The prepubertal animals were housed with their mothers until weaning at 3 wk of age. Except on d 1 when rats were used irrespective of gender, only female rats were used. The day of birth was considered as postnatal d 1. Two-day-old female rats of the Sprague Dawley strain purchased from Charles River Laboratories (Wilmington, MA) were used for RNA extraction and preparation of astrocyte cultures. For comparative purposes, RNA was also extracted from the hypothalamus of 2-d-old female mice (FVB/NTAC strain; Taconic, Hudson, NY). The use of rats and mice was approved by the University of Liege and the Oregon National Primate Research Center Animal Care and Use Committees in accordance with the National Institutes of Health guidelines for the use of animals in research.

Incubation of hypothalamic explants and GnRH RIA

The animals were decapitated between 1000 and 1100 h, and tissue fragments that included the preoptic region and the medial basal hypothalamus were rapidly dissected and transferred into a static incubator. 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. This procedure has been described in detail in previous publications (7, 10, 18). The incubation medium was phenol red-free MEM (Life Technologies, Inc., Invitrogen Corp., Merelbeke, Belgium) supplemented with glycine (10 nM), magnesium (1 mM), and glucose (25 mM). The incubation medium was supplemented with 20 μM bacitracin, an inhibitor of GnRH degradation by endopeptidases. The medium samples were frozen until the GnRH RIA was performed. GnRH was measured in duplicate samples using a highly sensitive RIA (18, 19) and an antiserum (20) generously provided by Dr. Y. F. Chen and V. D. Ramirez (Urbana, IL). The intra- and interassay coefficients of variation were 14 and 18%, respectively (18, 19). Values below the limit of detection of the assay (5 pg/7.5 min) were assigned that value.

Cell culture

Astrocytes were isolated from the hypothalamus of 1- to 2-d-old rats and cultured as described previously (21, 22). After a growth period of 8–10 d in 75-cm^3 culture flasks containing DMEM-F12 medium supplemented with 10% calf serum, the astrocytes were isolated from contaminant cells by overnight shaking at 250 rpm and were replated on 10-cm^3 dishes for RT-PCR or 12-well plates for PGE_2 release experiments. After reaching 90% confluence, the medium was replaced with a serum-free, astrocyte-defined medium consisting of DMEM devoid of phenol red, supplemented with 2 mM L-glutamine, 15 mM HEPES, 5 μg/ml insulin, and 100 μM putrescine. The cells were used 2 d later for RT-PCR or PGE_2 release experiments. For RT-PCR, the cells were snap-frozen on dry ice before extraction of total RNA. To assess the effect of oxytocin on PGE_2 release, the cells were incubated for 16 h with oxytocin acetate (10^{-8} M; Sigma Chemical Co., St. Louis, MO). TGFα (100 ng/ml; PreproTech Inc., Rocky Hill, NJ) was used as a positive control. After stimulation, the medium was collected and stored at −85 C before PGE_2 assay.

The immortalized GnRH-producing cells GT1-7 (kindly provided by Dr. R. Weiner, University of California, San Francisco, CA) were cultured in 10-cm dishes in DMEM containing 10% fetal calf serum. After reaching 70–80% confluence, the cells were washed with PBS and frozen on dry ice before RNA extraction.

Measurement of PGE_2 release

PGE_2 released from astrocytes in response to TGFα or oxytocin was measured by RIA as described previously (23).

RNA extraction and RT-PCR

Total RNA from rat and mouse hypothalami was prepared by the acid phenol-extraction method. Tissues were homogenized (100 mg/ml) in Tri Reagent (Molecular Research Center, Cincinnati, OH), and the aqueous and organic phases were separated by the addition of 0.1 vol bromochloropropane (Sigma) followed by centrifugation at 4 C. One volume of isopropanol was added to the aqueous phase, and RNA was precipitated by overnight incubation at −20 C. Samples were centrifugated at 14,500 × *g* for 15 min at 4 C. The pellets were washed in 70% ethanol and then air dried for 5 min. The RNA pellets were then resuspended in diethylpyrocarbonate-treated H_2O, and the suspension was incubated with DNA-free DNase I (two units per reaction) from Ambion (Austin, TX) for 30 min at 37 C. RNA concentrations were determined spectrophotometrically, and RNA integrity was verified on denaturing agarose gels.

RT2-PCR was used to detect oxytocin receptor (OTR) mRNA in cultured hypothalamic astrocytes, GT1-7 cells, and hypothalami obtained from 2-d-old female rats and mice. Five hundred nanograms of total RNA were reverse transcribed using Omniscript RT Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. PCR was performed in a volume of 25 μl containing 1 μl RT product, 2.5 μl 10× buffer (HotStar *Taq* Polymerase Kit; QIAGEN), 1 μl 10 mM dNTPs (HotStar *Taq* Polymerase Kit; QIAGEN), 0.15 μl HotSar *Taq* Polymerase (QIAGEN), and 0.5 μl OTR primers (50 μM) or 0.5 μl of a set of primers (50 μM) that amplify cyclophilin mRNA, a constitutively expressed mRNA. After an initial incubation at 95 C for 15 min, the samples were amplified for 35 cycles consisting of 30 sec at 94 C (denaturing), 30 sec at 64 C (annealing), and 1 min at 72 C (extension) and then incubated 10 min at 72 C (final extension). The primers were designed using the Primer Select software (DNASTAR Inc., Madison, WI) and were as follows: mouse OTR (XM_144956.6) sense 5′-TTCTACGGGC-CCGACCTGCTGTGT-3′ and antisense 5′-CTGTGCGGATTTTGGCCTT-GGAGA-3′, rat OTR (NM_012871.2) sense 5′-TTCTATGGGCCCGACCT-GCTGTGT-3′ and antisense 5′-CCGTGCGGATTTTGGCCTTGGAGA-3′, mouse cyclophilin (NM_008907) sense 5′-GGCAAATGCTGGACCAAA-CACAA-3′ and antisense 5′-GGTAAAATGCCCGCAAGTCAAAAG-3′, and rat cyclophilin (M19533) sense 5′-CTTTGCAGACGCCGCTGTCT-CTTTTCGCCG-3′ and antisense 5′-GCATTTGCCATGGACAAGATGC-CAGGA-3′. PCR products were resolved on a 2% agarose gels and visualized by ethidium bromide staining. Both rat and mouse OTR primers amplify a 505-bp PCR product.

Combined immunohistochemistry-in situ hybridization

To determine whether OTR mRNA is expressed in GnRH neurons of the rat hypothalamus, we used a combined immunohistochemistry-*in situ* hybridization procedure described earlier in detail (17, 24). GnRH neurons were stained with a monoclonal antibody to GnRH (25) diluted 1:3000, and the reaction was developed to a brown color with 3,3′-diaminobenzidine. After completing the GnRH immunohistochemical procedure, the sections were mounted on glass slides and dried overnight under vacuum before hybridization with a [^{35}S]UTP-labeled rat (r)OTR cRNA probe described below. After an overnight hybridization at 55 C, the slides were washed and processed for cRNA detection. After a final dehydration step in graded alcohols, the slides were dipped in NTB-2 emulsion and were exposed to the emulsion for 3 wk at 4 C. At this time, the slides were developed, counterstained with 0.1% methyl green, quickly dehydrated, dried, and coverslipped for microscopic examination. All reagents used for the immunohistochemical procedure were prepared in diethylpyrocarbonate-treated water.

The OTR cRNA probe used was prepared by *in vitro* transcription of a cDNA template cloned into the pGEM-T vector (Promega, Madison, WI) and that derived from the 505-bp PCR product described above.

Study protocols

Effect of an oxytocin antagonist on sexual maturation. From d 15–20 of age, 16 immature female rats received a daily ip injection of 200 μg/kg of an oxytocin antagonist, des-Gly-NH_2d$(CH_2)_2$[D-Tyr^2, Thr^4]vasotocin (10^{-7} M), generously provided by Dr. Maurice Manning, Medical University of Ohio, Toledo, OH. This antagonist is selective for the OTR with respect to vasopressin receptors, in particular the closely related vasopressin receptor V1a (26). Such selectivity allowed us to consider the effect of the antagonist as specifically due to functional disruption of the intended target.

The antagonist was diluted in saline. The control group consisted of 16 immature female rats receiving an ip injection of the saline vehicle from d 15–20 at 0800 h. On d 20, 2 h after the last injection, eight of the oxytocin antagonist-treated rats and eight of the control rats were killed, and the hypothalamus was dissected and incubated for 4 h either in regular medium or in the presence of oxytocin (10^{-8} M). The other half of the control and oxytocin antagonist-treated groups were inspected daily from d 20 on for vaginal opening. Subsequently, vaginal lavages were obtained daily for 6 wk to determine the age at first estrus (27), and the cells were visualized after staining using the Papanicolaou method.

The second experiment followed the same protocol, except that the rats were injected daily between d 10 and 20 of age to determine whether an earlier neutralization of oxytocin actions was more effective to delay sexual maturation.

To determine whether the effect of the oxytocin antagonist administration *in vivo* on GnRH pulse frequency *in vitro* resulted from an acute effect of the last dose injected or a chronic effect of the 6-d treatment, a group of 16 20-d-old female rats received only one injection of oxytocin antagonist or saline at 0800 h and were killed 2 h later. The hypothalami were dissected and incubated as above.

Dose dependency of oxytocin stimulatory effect on pulsatile GnRH secretion in vitro. Because the above *in vitro* paradigm allowed us earlier to detect an increase in frequency of pulsatile GnRH secretion between 10 and 25 d of age in male rats (8), the present studies were performed using explants of an intermediate age (15 d). Pulsatile GnRH secretion was evaluated after exposing the explants to 10^{-10}, 10^{-9}, 10^{-8}, or 10^{-7} M oxytocin for

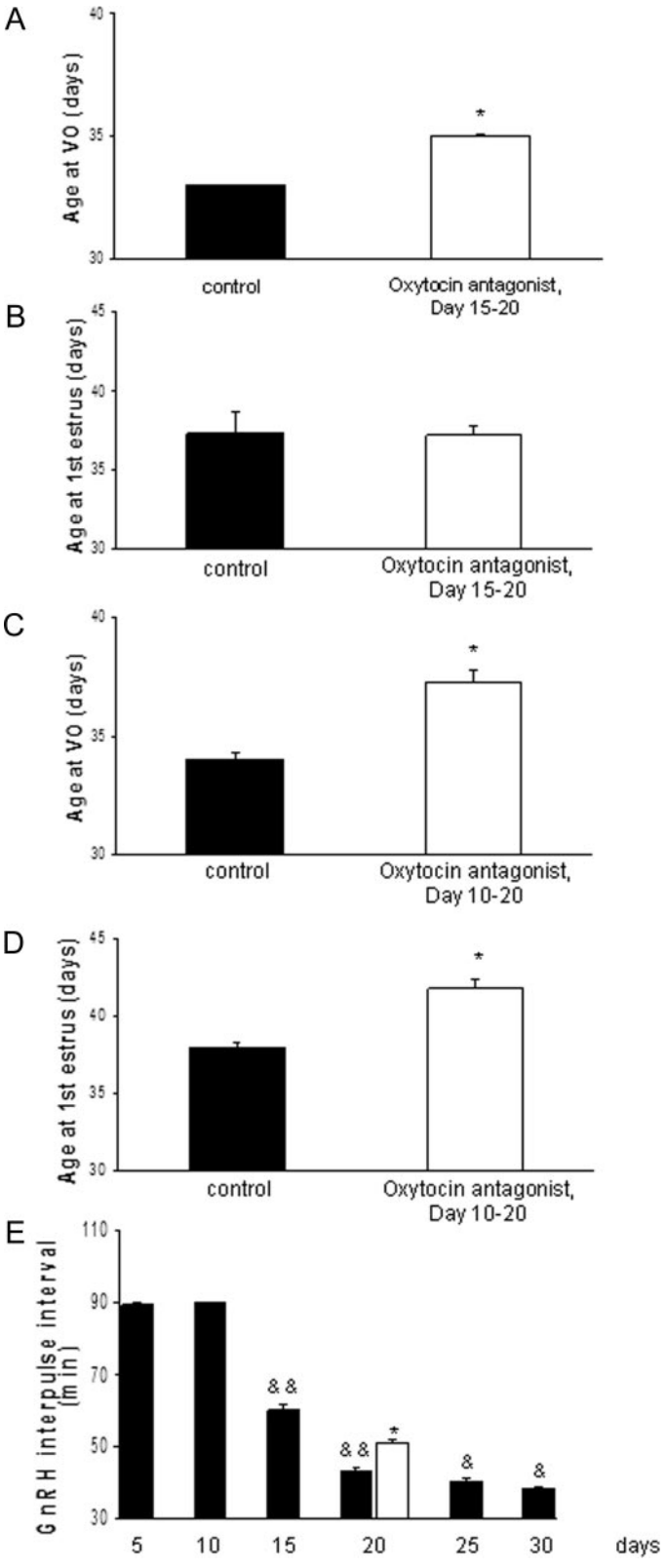

FIG. 1. Effect of *in vivo* administration of an oxytocin antagonist on the onset of puberty in female rats and on pulsatile hypothalamic GnRH release. A, Age at vaginal opening in vehicle-treated rats and rats treated with an oxytocin antagonist from postnatal d 15–20. B, Age at first estrus of the same animals. *Bars* are means, and *vertical lines* are SEM. Each group represents the mean of seven to 10 animals. C, Age at vaginal opening in female rats treated with the oxytocin antagonist or vehicle from postnatal d 10–20. D, Age at first estrus of the same animals. *Bars* are means, and *vertical lines* are SEM. Each group represents the mean of seven to 10 animals. E, GnRH interpulse interval during female rat development using hypothalamic explants from 5-, 10-, 15-, 20-, 25-, and 30-d-old rats as well as hypothalamic explants of 20-d-old rats incubated *in vitro* after a 6-d treatment (d 15–20) with an oxytocin antagonist. *Bars* represent the mean ± SEM of four to five explants for each age. *, $P < 0.001$ *vs.* vehicle-treated group; &&, $P < 0.001$ *vs.* preceding age; &, $P < 0.05$ *vs.* preceding age.

4 h (four explants per concentration) in comparison with eight explants incubated under control conditions.

Effect of oxytocin on GnRH pulse frequency in vitro. Pulsatile GnRH secretion was studied for 4 h, starting at 1000 h, using explants obtained from 1-, 5-, 15-, and 50-d-old female rats. The 50-d-old rats were used irrespective of the phase of the estrous cycle. In a previous study, we showed that changes in pulsatile GnRH secretion related to the phase of the estrous cycle were detected only when the experiments were started around 1600 h (28). The explants were incubated with saline vehicle, oxytocin alone, or a combination of oxytocin and the oxytocin antagonist (n = 5 for each condition), as outlined above.

Oxytocin-PGE_2 interactions in vitro. Pulsatile GnRH secretion was studied for 4 h using explants obtained from 1- and 15-d-old female rats. Pulsatile GnRH secretion was studied under control conditions, in the presence of oxytocin (10^{-8} M) or PGE_2 (10^{-6} M) or in the presence of arachidonylfluoromethyl ketone ($AACOCF_3$, 25 μM; Biomol Research Laboratories, Plymouth Meeting, PA), a blocker of phospholipase A_2 (PLA_2) that results in inhibition of PGE2 synthesis. This inhibitor was used alone or together with oxytocin (10^{-8} M).

Statistical analysis

Pulses of GnRH secretion were identified using the Pulsar program, as described previously (29). The individual interpulse interval as well as the mean ± SEM interpulse interval was calculated. In several instances, all the interpulse intervals were equal, thus accounting for an SEM equal to zero.

The effect of the different agents on GnRH pulse amplitude and frequency was analyzed by one-way ANOVA followed by the Student-Newman-Keuls test. The effect of the oxytocin antagonist on the age at vaginal opening and first estrus was analyzed by unpaired *t* test. The threshold for significant difference was $P < 0.05$.

Results

Effect of an oxytocin antagonist on female sexual maturation

In rats treated with an oxytocin antagonist for 6 d (d 15–20), vaginal opening was delayed compared with rats injected with the vehicle (35 ± 0 *vs.* 33 ± 0 d, $P < 0.0001$; Fig. 1A). The age at first estrus, which defines the age of first ovulation, was not affected (Fig. 1B). When treatment with the oxytocin antagonist started earlier (d 10 instead of 15), both vaginal opening and the age at first estrus were significantly delayed ($P < 0.0001$; Fig. 1, C and D).

In vitro study of GnRH pulse frequency under control conditions using hypothalamic explants from female rats at 5, 10, 15, 20, 25, and 30 d of age showed a decrease of GnRH interpulse interval between d 10 and 20 (Fig. 1E). When the hypothalamic explants of the rats treated *in vivo* with the oxytocin antagonist were studied *in vitro* on d 20, the GnRH interpulse interval was significantly increased with respect to explants obtained from vehicle-treated animals (51 ± 3 *vs.* 44 ± 3 min, respectively, $P < 0.001$; Figs. 1E and 2, A and B). This increase resulted from the chronic administration of the

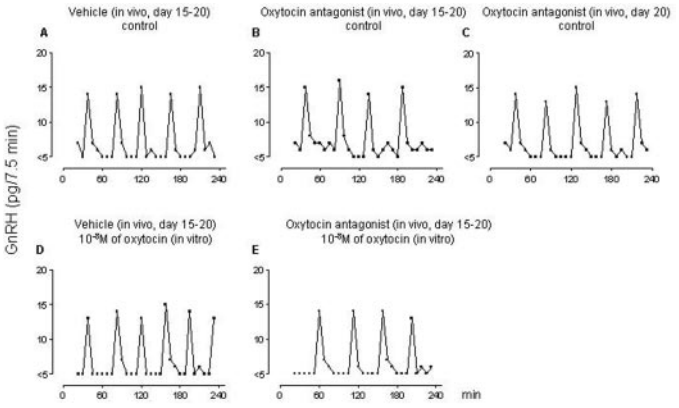

FIG. 2. Representative profiles of GnRH secretion from hypothalamic explants of 20-d-old female rats. The rats received a daily injection of vehicle (A and D) or 200 μg/kg of an oxytocin antagonist (B and E) from d 15–20 or only one injection of oxytocin antagonist on d 20 (C). The explants were incubated with 10^{-8} M oxytocin (D and E) or without the hormone (A–C). Each group consists of five explants.

antagonist because the GnRH interpulse interval was not affected by a single *in vivo* administration of the antagonist on d 20 (45 ± 0 min; Fig. 2C). Exposure of the explants from rats injected with the vehicle to oxytocin *in vitro* resulted in a significant reduction in GnRH interpulse interval (40 ± 4 *vs.* 44 ± 3 min, $P < 0.01$; Fig. 2, A and D). This was consistent with our previous data showing a facilitatory effect of oxytocin on pulsatile GnRH secretion from male hypothalami (7). Administration of the oxytocin antagonist for 5 d *in vivo* did not affect this *in vitro* effect of oxytocin (48 ± 4 *vs.* 51 ± 3 min, $P < 0.05$; Fig. 2, B and E), likely due to displacement of decreasing antagonist concentrations by an excess of oxytocin from common binding sites.

Dose dependency and ontogeny of oxytocin and oxytocin antagonist effect on pulsatile GnRH secretion in vitro

Incubation of hypothalamic explants from 15-d-old female rats with oxytocin resulted in a dose-dependent reduction in GnRH interpulse interval, which was significant at concentrations of 10^{-9} to 10^{-7} M (Fig. 3). When explants from 1-, 5-, and 15-d-old rats were examined, the mean GnRH interpulse interval was significantly ($P < 0.001$) reduced by incubation with 10^{-8} M oxytocin (Fig. 4). Conversely, with the exception of d 5, GnRH pulse frequency was significantly decreased by addition of the oxytocin antagonist to the incubation medium, in the absence of exogenous oxytocin. This effect is consistent with previous results obtained using explants from male rats (7). Control hypothalami showed a GnRH interpulse interval that decreased gradually throughout sexual maturation, reaching minimal values at 50 d of age (Fig. 4). At this time, neither oxytocin nor the antagonist was any longer effective in altering pulsatile GnRH release. When explants from 5-d-old rats were incubated simultaneously with oxytocin and its antagonist, the effect of oxytocin on GnRH interpulse interval was totally inhibited (Fig. 4).

Oxytocin-PGE_2 interactions in vitro

Because oxytocin has been shown to induce PGE_2 release from medial basal hypothalamic explants (5), we hypothesized that PGE_2 might mediate the stimulatory effect of oxytocin on pulsatile GnRH secretion. As shown in Fig. 5, both oxytocin and PGE_2 were able to significantly decrease GnRH interpulse interval at the two ages studied (1 and 15 d). $AACOCF_3$, an inhibitor of PLA_2, was more effective in blocking the effect of oxytocin on d 1 than on d 15. When used alone $AACOCF_3$ had no effect.

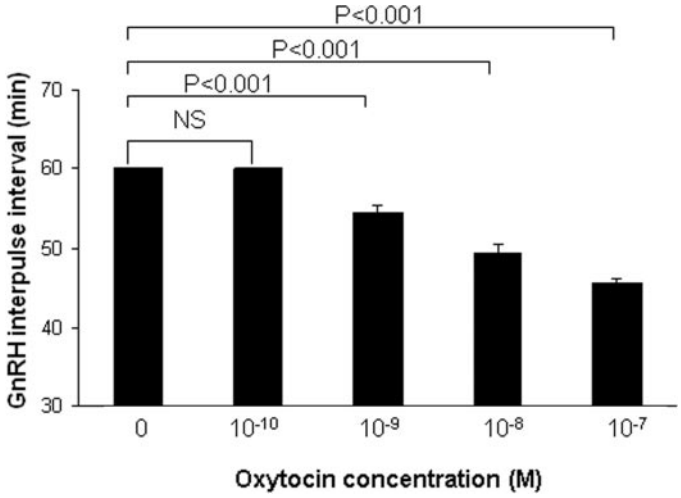

FIG. 3. Effects of increasing oxytocin concentrations on the GnRH interpulse interval (mean ± SEM) of hypothalamic explants from 15-d-old female rats (n = 4 explants per group). NS, Not significantly different from vehicle-treated group.

Study of OTR expression in GnRH neurons

The actions of oxytocin are mediated by activation of G protein-coupled receptors (30). To determine whether GnRH neurons express the OTR gene, we examined the preoptic region of two immature 28-d-old female rats. GnRH neurons were identified by immunohistochemistry and OTR mRNA by *in situ* hybridization. In agreement with earlier findings (31), OTR transcripts were expressed at low abundance in cells scattered throughout the suprachiasmatic region. However, they were not detected in GnRH neurons. Instead,

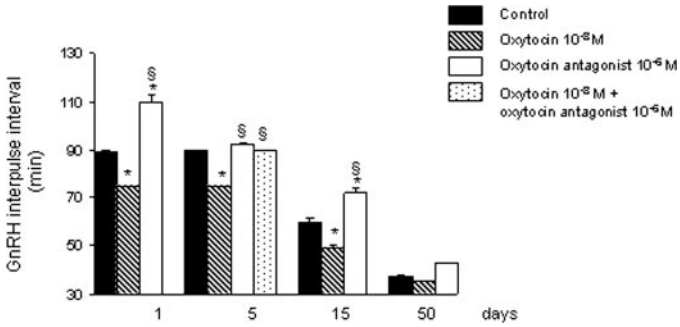

FIG. 4. Effect of an oxytocin antagonist on GnRH interpulse interval and on the increase in GnRH pulse frequency elicited by oxytocin during postnatal development of the female rat hypothalamus. Hypothalamic explants from 1-, 5-, 15-, and 50-d-old rats were used. *Bars* represent means (n = 4), and *vertical lines* are SEM. A group of hypothalamic explants from 5-d-old rats was incubated in presence of both oxytocin and oxytocin antagonist. *, $P < 0.001$ *vs.* group incubated with vehicle alone; §, $P < 0.001$ *vs.* group treated with oxytocin alone.

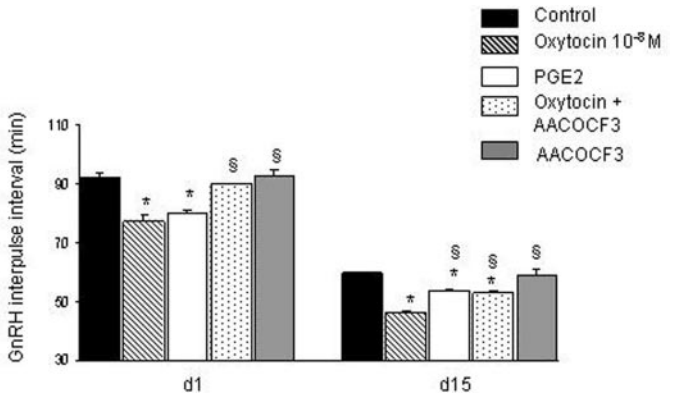

FIG. 5. Effects of PGE_2 on GnRH pulse frequency and of an inhibitor of PLA_2 synthesis ($AACOCF_3$) on the increase in GnRH pulse frequency elicited by oxytocin on hypothalamic explants from 1- and 15-d-old female rats (n = 4 per group). *Bars* are means, and *vertical lines* represent SEM. *, $P < 0.001$ *vs.* control group incubated with vehicle; §, $P < 0.001$ *vs.* group treated with oxytocin.

hybridization signals were seen in cells adjacent (Fig. 6, A and B) or near (Fig. 6C) these neurons.

Study of OTR mRNA in hypothalamic astrocytes and GT1-7 cells

Detection of OTR mRNA by RT-PCR showed that the transcripts are abundant in cultured rat hypothalamic astrocytes and in the rat medial basal hypothalamus (Fig. 7A). In contrast, they were absent in immortalized mouse GnRH neurons but clearly evident in cultured mouse hypothalamic astrocytes and mouse medial basal hypothalamus (Fig. 7B), suggesting that oxytocin might act on astrocytes to stimulate GnRH release indirectly, instead of acting on GnRH neurons.

PGE2 release by oxytocin from hypothalamic astrocytes

TGFα stimulates GnRH release via a glial intermediacy that involves activation of astrocytic erbB1 receptors and the

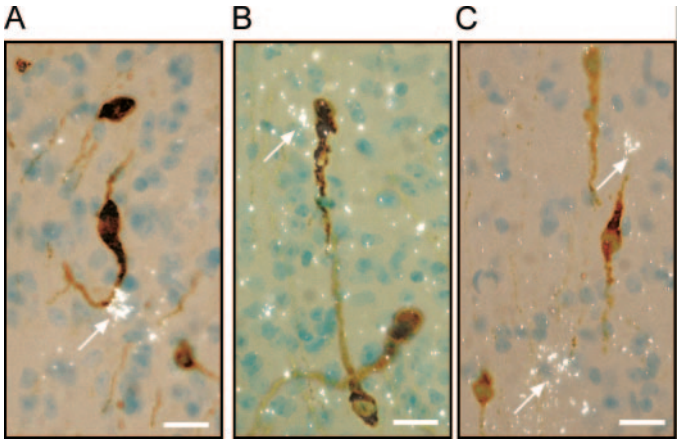

FIG. 6. Absence of OTR mRNA in GnRH neurons of the immature (28-d-old) female rat preoptic region, as assessed by combined immunohistochemistry (GnRH)/*in situ* hybridization (OTR) using a [^{35}S]UTP-labeled OTR cRNA. A and B, Examples of OTR mRNA-positive cells (*arrows*) adjacent to GnRH neurons (*brown* staining) lacking detectable OTR mRNA transcripts. C, OTR mRNA-positive cells (*arrows*) in the vicinity of GnRH neurons lacking OTR mRNA. *Bars*, 20 μm.

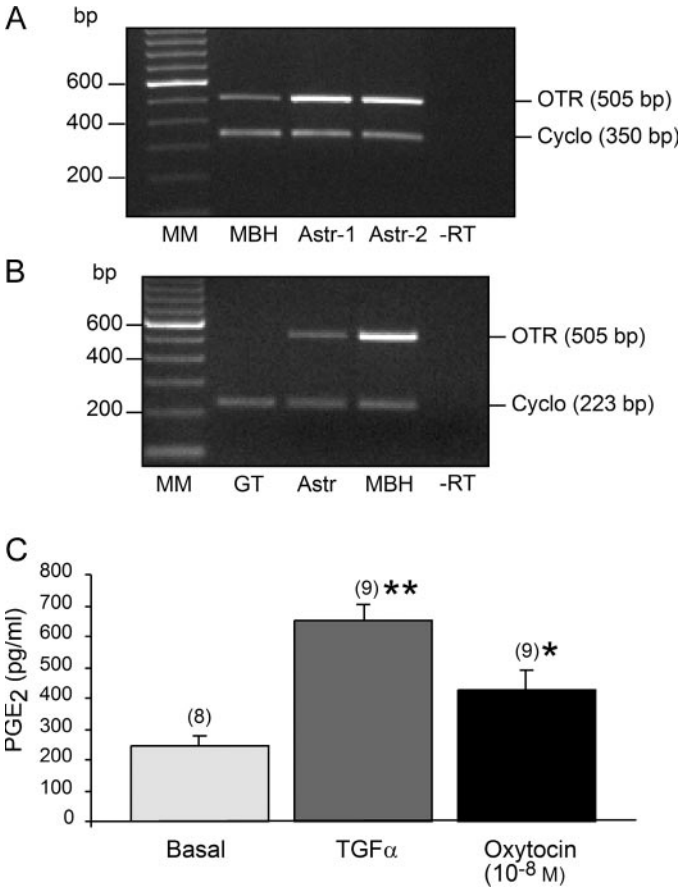

FIG. 7. OTR mRNA can be detected by RT-PCR in hypothalamic astrocytes but not in immortalized GnRH-producing cells. A, OTR mRNA is present in cultured rat hypothalamic astrocytes (Astr-1 and Astr-2) and rat medial basal hypothalamus (MBH). B, Absence of OTR mRNA in GT1-7 cells (GT), in contrast with its presence in both cultured mouse hypothalamic astrocytes (Astr) and mouse MBH. C, Stimulation of PGE_2 release from rat hypothalamic astrocyte cultures by oxytocin (10^{-8} M) or TGFα (100 ng/ml). *Bars* are means, and *vertical lines* are SEM. *Numbers in parentheses* are number of wells per group. **, $P < 0.01$; *, $P < 0.05$ *vs.* basal release. Cyclo, Cyclophilin mRNA; MM, molecular markers; −RT, no RT reaction.

subsequent release of PGE_2 (32). We hypothesized that oxytocin action on GnRH neurons could be similarly mediated by astrocytic PGE_2. We measured PGE_2 release from rat hypothalamic astrocytes in primary culture after 16 h of exposure to oxytocin (10^{-8} M) or TGFα (100 ng/ml). As shown in Fig. 7C, oxytocin induced a significant ($P < 0.01$) increase in PGE_2 release, which was, however, lower than that elicited by TGFα.

Discussion

In this paper, we provide evidence that oxytocin facilitates sexual maturation in the female rat. We also show that oxytocin accelerates GnRH pulse frequency *in vitro* from hypothalamic explants obtained from female rats of different prepubertal ages and that this effect is mostly lost in adulthood. Using medial basal hypothalamic explants obtained from adult male rats, acceleration of GnRH pulsatile release by oxytocin concentrations as low as 10^{-10} M was previously

reported (5). The present study, using hypothalamic explants from female rats, is in agreement with those earlier findings.

That endogenous oxytocin is physiologically involved in the control of female puberty is suggested by the delayed sexual maturation resulting from blocking oxytocin actions during the infantile period of postnatal development. Of note, the oxytocin antagonist did not affect pulsatile GnRH secretion from 5-d-old explants, in contrast to the inhibitory effects observed earlier on d 1 and later on d 15. This ineffectiveness at 5 d may be related to a low availability of endogenous oxytocin at this age, instead of a reduced OTR response, because oxytocin was as effective on d 5 as on d 1 or 15 to increase GnRH pulse frequency. It is possible that after the effect of oxytocin of maternal origin dissipates shortly after birth, a maturational process is required for endogenous oxytocin to be released at physiologically relevant amounts in the offspring. Earlier studies have shown that significant amounts of mature oxytocin are detected in the hypothalamus only after the second week of postnatal life (33, 34). In agreement with this hypothesis, a study reported the absence of effect of the oxytocin antagonist on age at vaginal opening and first estrus when administered between 1 and 7 d of age. In contrast, they observed a delayed vaginal opening and first estrus after exposure to oxytocin during the same period (35). Our *in vitro* data suggest that the stimulatory effect of oxytocin on GnRH secretion decreases with age. This could be due to an age-related increase in oxytocin clearance or a reduced activity of its receptor. No study has specifically reported the ontogeny of oxytocin expression in the rat hypothalamus during pubertal maturation. However, Chibbar *et al.* (36) have shown that oxytocin mRNA levels increase in rats after puberty, after sex steroids stimulation. The regulation of the OTR appears to be complex as well. Although the receptor is expressed prenatally in the rat brain (37), the same study described a progressive decrease in receptor expression in certain regions, such as the hippocampus and the parietal cortex during postnatal development (37). Autoradiography experiments have shown the appearance of OTRs in the rat ventromedial hypothalamus at the time of puberty, probably under the stimulation of estrogens (38). To our knowledge, expression of the OTR in the hypothalamus during pubertal development has never been studied. The age-related decrease in oxytocin effectiveness in our model might be related to a decrease in expression of its receptor, as suggested by the aforementioned studies. Alternatively, a change in receptor affinity for oxytocin may also occur as the animal matures. However, only a limited subset of oxytocin neurons project to targets located within the hypothalamus (39). Thus, changes of oxytocin expression affecting the overall population of oxytocin neurons might make it difficult to identify changes occurring only in those subsets of oxytocin neurons innervating the hypothalamus.

Beside its role in puberty, oxytocin has been suggested to play a role in the preovulatory LH surge. Using explants obtained at different phases of the estrous cycle, it has been shown that oxytocin can stimulate GnRH release in the afternoon of proestrus only (6). In our study, neither oxytocin nor the antagonist was any longer effective in significantly altering the frequency of pulsatile GnRH secretion in adult female rats. However, these data were obtained using explants studied in the morning, whereas we have shown earlier that the amplitude of GnRH secretion was increased in the afternoon of proestrus (28). Data concerning oxytocin effects on the human menstrual cycle are controversial. In contrast to the data showing an effect of oxytocin and an oxytocin antagonist on the endogenous LH surge (15, 40), a recent study showed that neither oxytocin nor its antagonist had any effect on basal and GnRH-induced gonadotropin secretion in the late follicular phase of the normal menstrual cycle (41).

A major finding of the present study is that blockade of endogenous oxytocin actions by *in vivo* administration of a specific antagonist delays the initiation of female puberty. The onset of puberty is characterized by an increase in GnRH pulse generator activity (7). The main hypothalamic neurotransmitters/neuromodulators involved in this activation have been extensively characterized (14). They include excitatory and inhibitory amino acids such as γ-aminobutyric acid and glutamate (11) and the newly described neuropeptide kisspeptin (12, 13). Our results suggest the existence of an independent oxytocin-mediated pathway contributing to the central regulation of the pubertal process. When endogenous oxytocin actions are blocked transiently (single injection of the antagonist), the timing of puberty is not affected. A delay is observed only when the antagonist is given for 6 d at the end of the infantile period, and becomes more evident when the treatment is initiated at an even earlier age. The age at which the antagonist is effective and the need for a sustained blockade for the antagonist to act effectively suggests that oxytocin regulates the pubertal process by modifying hardwiring events that take place during infantile development. The nature of these events has not been elucidated, but it may be related to the ability of oxytocin to cause morphological changes in glial cells and neurons (42). Other studies have shown that both neuronal remodeling induced by oxytocin (43) and permanent sex-related changes in glial morphology that occur during early postnatal development are mediated by PGE_2 (44). Thus, our *in vivo* and *in vitro* data suggest the convergence of different regulatory mechanisms. The stimulatory effect of oxytocin on PGE_2 release from astrocytes in primary culture as well as the stimulatory effect of oxytocin on GnRH secretion from hypothalamic explants *in vitro* suggest a rapid stimulatory effect of oxytocin. Oxytocin stimulation leads to a release of PGE_2 that is able to directly stimulate GnRH release (17). The necessity of repeated *in vivo* injections suggests a long-term effect potentially involving morphological changes, as discussed above.

The oxytocin antagonist was administered by ip injection, leading to the question of its transfer across the blood-brain barrier. The common understanding is that oxytocin antagonists cross the blood-brain barrier in small amounts (45–47). However, oxytocin antagonists have been shown to induce behavioral changes when administered peripherally, implying a rate of brain transfer sufficient to induce central effects (48–50). Because these behavioral effects were observed shortly after a single peripheral injection of the oxytocin antagonist (50), it would appear unlikely that the antagonist failed to cross the blood-brain barrier in our experiments. In the present study, the concordant reduction in GnRH pulse frequency observed both *ex vivo* after systemic administra-

tion of the antagonist and *in vitro* demonstrates that the antagonist is acting centrally after peripheral administration. However, a peripheral effect of the injected oxytocin antagonist cannot be excluded. Oxytocin and its receptor are both expressed in rodent, human. and nonhuman primate ovaries (51–53), and the local production of oxytocin seems to play a role in the regulation of ovarian function during the estrous cycle (reviewed in Ref. 54). To our knowledge, no effect of oxytocin on ovarian maturation has been yet shown.

Oxytocin neurons are located in the supraoptic and the paraventricular nuclei, and their axons terminate in several areas including the median eminence and the rostral hypothalamus (39). Our results suggest that oxytocin does not stimulate pulsatile GnRH release by acting directly on GnRH neurons. Instead, the stimulatory effect of oxytocin on GnRH secretion appears to be mediated by PGE_2 released from astrocytes. These findings are in keeping with earlier reports showing the involvement of PGE_2 in mediating oxytocin actions in other cellular systems (55). PGE_2 is a potent GnRH secretagogue (16), which upon release from glial cells, binds to specific receptors located on GnRH neurons to elicit GnRH release (17) (reviewed in Ref. 56) However, a very recent study using double-label immunofluorescence reported the expression of OTR in 10% of the GnRH neurons in female rat hypothalamus (57), suggesting a possible direct action of oxytocin on GnRH neurons. Our results did not reveal the presence of OTR mRNA in GnRH neurons. Our combined immunohistochemistry/*in situ* hybridization procedure might not be sensitive enough to detect low levels of transcripts in such a small fraction of neurons.

Cytosolic PLA_2 is a major enzyme involved in prostaglandin production by generating arachidonic acid, the precursor of the prostaglandins, from membrane glycerophospholipids. Blocking PLA_2 with $AACOCF_3$ prevented the effect of oxytocin on GnRH pulse frequency, implicating prostaglandins in this process. That the prostaglandin involved is PGE_2 of glial origin was evidenced by the presence of OTRs in astrocytes but not GnRH neurons and the ability of hypothalamic astrocytes to release PGE_2 in response to oxytocin. Because the effect of oxytocin was more evident after 16 h, it is possible that the effect of oxytocin on PGE_2 release may involve an increase of cyclooxygenase 2 expression, because it is observed in supraoptic neurons and astrocytes (43). Although the stimulatory effect of PGE_2 on GnRH release appears to be more prominent in the median eminence (16), the main terminal field for GnRH axons, PGE_2 is also able to stimulate GnRH neurons when directly applied to the preoptic region (58). That this is an important site of action in mediating oxytocin-induced GnRH release is suggested by the previous observation that during the preovulatory GnRH release, a stimulatory effect of oxytocin requires the presence of the preoptic area in addition to the median eminence (6).

Spontaneous mutations in the oxytocin gene have not been reported in either rodents or humans. With the exception of an inability to eject milk, no reproductive defects have been reported to occur in oxytocin knockout mice (59). Because it frequently occurs when describing knockout animals, a detailed analysis of potential defects in pubertal maturation resulting from oxytocin deficiency has not been undertaken, but is necessary. Given the complexity of the neuroendocrine mechanisms involved in controlling puberty, the failure of unconditional gene targeting to verify findings made via other means can be best explained as due to early compensatory mechanisms set in motion in response to the gene deletion.

In summary, our results show that oxytocin can act on the immature female hypothalamus to accelerate pulsatile GnRH release and to advance the onset of female puberty. Our results also show that this effect requires the intermediacy of OTR-containing astroglial cells that respond to oxytocin with PGE_2 release.

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