0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(6):2737–2743

doi: 10.1210/en.2005-1191

The Neuroactive Steroid Pregnenolone Sulfate Stimulates the Release of Gonadotropin-Releasing Hormone from GT1-7 Hypothalamic Neurons, through *N*-Methyl-D-Aspartate Receptors

Martine El-Etr, Yvette Akwa, Etienne-Emile Baulieu, and Michael Schumacher

Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 788, Université Paris 11, 94276, Le Kremlin-Bicêtre, France

Immortalized hypothalamic GT1-7 neurons represent a good model system to investigate the control of GnRH secretion. Using these cells, we observed that the neuroactive steroid, pregnenolone sulfate (PREGS), is able to stimulate the release of GnRH in a dose-dependent manner through *N*-methyl-D-aspartate (NMDA) receptors, because its action is completely blocked by a specific NMDA receptor antagonist and magnesium. GT1-7 neurons express mRNAs for various mouse NMDA receptor subunits (ζ,1, ϵ3, ϵ4, and ϵ2, corresponding to the NR1, NR2C, NR2D, and NR2B rat subunits) and increase their spontaneous release of GnRH when incubated in the presence of exogenous glutamate or NMDA. In addition, we found that these neurons are able to release and synthesize glutamate, as demonstrated by the presence of glutamate accumulated in the defined incubation medium of the neurons, during the experiment and the expression of mRNA coding for vesicular glutamate transporter 2, a specific marker of glutamatergic neurons. The potentiating effect of PREGS on the secretion of GnRH induced by glutamate is consistent with the role of the steroid as a positive allosteric modulator of NMDA receptors. Together these results point to a novel mechanism by which the neuroactive steroid PREGS may potentiate an autocrine excitatory loop in GnRH neurons. (*Endocrinology* 147: 2737–2743, 2006)

HYPOTHALAMIC GnRH NEURONS are central regulators of reproduction; the pulsatile secretion of GnRH is required for fertility and drives the synthesis and release of gonadotropins from the pituitary, which control gametogenesis and steroidogenesis. GnRH neurons, in turn, receive information from several steroid feedback mechanisms and particularly from estrogens, which may act as positive or negative signals, as well as from progesterone and testosterone. Most of the regulations are indirect, through the synaptic release of neurotransmitters and neuropeptides (1, 2) or the astrocytic release of growth factors, such as TGFβ1 (3). However, direct influences of various steroids on GnRH neurons have been reported; this is indeed the case for estrogens, through membrane (4) and genomic mechanisms (5), testosterone and its metabolite 5α-dihydrotestosterone (6), the progesterone metabolite allopregnanolone (7, 8), and dehydroepiandrosterone sulfate (DHEAS), which decreases the electrophysiological responsiveness of GnRH neurons to the activation of γ-aminobutyric acid A ($GABA_A$) receptors (9). Whether the neuroactive steroid pregnenolone sulfate (PREGS) can also modulate the release of GnRH is as yet unknown.

PREGS does not bind to any classical intracellular steroid receptor. Its effects on central nervous system excitability are rapid, mainly through modulation of different channel-gated neurotransmitter receptors; indeed, it negatively interacts with $GABA_A$ receptors (10), a common property of 3β-hydroxysteroid sulfates (11), and counteracts glycine receptor function (12); it also inhibits D,L-α-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) and kainate receptor activities (13), whereas it potentiates most *N*-methyl-D-aspartate (NMDA) receptor-mediated glutamate excitatory responses (14), depending on the subunit composition of the receptors (15). A recent study suggests that PREGS may interact with NMDA receptors at a hydrophobic binding pocket identified on the NR2B subunit (ϵ2 in the mouse) (16).

Among the modulators of hypothalamic GnRH secretion, glutamate plays an important part, because it is involved in the mechanisms of the GnRH pulse generator (17–20), the induction of puberty in males (21) and females (22), including monkeys (23), and the preovulatory GnRH surge (24). Moreover, GnRH responses to glutamate through NMDA receptor stimulation can be modulated according to the age, being significantly decreased in old rats (25, 26). Indeed, compromised reproductive physiology with aging may be related to changes in NMDA receptor subunit composition and stoichiometry in GnRH neurons, with an increase in NR2B subunit affecting functional channel characteristics (27).

Using GT1-7 cells, hypothalamic GnRH neurons of mice, immortalized by genetically targeted tumorigenesis (28), we observed that PREGS, in the absence of exogenous glutamate

First Published Online March 2, 2006

Abbreviations: AMPA, D,L-α-Amino-3-hydroxy-5-methyl-4-isoxazol propionic acid; AP5, D,L-2-amino-5-phosphono-valeric acid; C-DMEM, conditioned-DMEM; CNQX, cyano-3,3-dihydro-7-nitroquinoxaline; DHEAS, dehydroepiandrosterone sulfate (3β-hydroxy-5-androsten-17-one sulfate); GABA, γ-aminobutyric acid; MK-801, dizocilpine maleate; NMDA, *N*-methyl-D-aspartate; GFP, green fluorescent protein; PREGS, pregnenolone sulfate (3β-hydroxy-5-pregnen-20-one sulfate); VGLUT, vesicular glutamate transporter.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

or NMDA, could stimulate the release of GnRH, and this effect involved the activation of NMDA receptors. GT1-7 cells were also found to express vesicular glutamate transporter 2 (VGLUT2) and to release glutamate into the incubation medium. Together, these results strongly suggest that PREGS may potentiate the autocrine stimulatory action of glutamate on GnRH neurons.

Materials and Methods

Cell cultures

GT1-7 cells (provided by R. Weiner and A. Choi, University of California, San Francisco, CA) were cultured in six-well dishes, coated with 3 μg poly-L-ornithine/ml (Sigma-Aldrich Corp., Saint-Quentin-Fallavier, France), in conditioned-DMEM (C-DMEM), which was comprised of a 1:1 mixture of conditioned medium from mouse embryonic astrocytes in primary culture (29) and DMEM with high glucose (Invitrogen Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The use of conditioned medium was essential for the growth of GT1-7 neurons. When cells reached 90% confluence, C-DMEM was replaced by serum-free medium (OptiMEM, Invitrogen Life Technologies, Inc.) for 24 h before the experiment.

GnRH release

On the day of the experiment, confluent GT1-7 cells, seeded on six-well dishes ($4.6 \pm 0.7 \times 10^6$ cells/well) were washed three times for 5 min each time with 2 ml magnesium-free Locke's medium [154 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 6 mM $NaHCO_3$, 10 mM glucose, and 2 mM HEPES (pH 7.4)]. They were then incubated at 37 C for 30 min in 1 ml Locke's medium supplemented with bacitracin (20 μM; Sigma-Aldrich Corp.) and glycine (10 μM; Merck & Co., Rahway, NJ) in the absence or presence of PREGS (5–100 μM), glutamate (5 μM-1 mM), NMDA (5 μM to 1 mM,) and/or the $GABA_A$ receptor channel blocker picrotoxin (100 μM), the NMDA receptor antagonist D,L-2-amino-5-phosphono-valeric acid (AP5; 10 μM), NMDA receptor channel blockers MK 801 (5 μM) and Mg^{2+} (6 mM), or the kainate-AMPA receptor antagonist cyano-3,3-dihydro-7-nitroquinoxaline (CNQX; 10 μM; all from Sigma-Aldrich Corp.). Three wells were used per condition. At the end of the incubation period, media (1 ml) were stored at −20 C until radioimmunoassayed for GnRH.

GnRH RIA

As previously described (7), the concentration of GnRH released into the medium (1 ml/well) was measured by RIA in triplicate by a charcoal precipitation method using [^{125}I]GnRH (2000 Ci/mol; 1 Ci = 37 GBq; Amersham Biosciences, Les Ulis, France), unlabeled GnRH (Fluka, Buchs, Switzerland; Sigma-Aldrich Corp.) as the reference standard, and the rabbit polyclonal antibody R 1245 (30), which is specific for the decapeptide (obtained from T. Nett, Colorado State University, Fort Collins, CO), Briefly, 100 μl antibody (1:76,800 final dilution) was added to each tube already containing 200 μl sample (or unlabeled GnRH for the standard curves) and 200 μl GnRH assay buffer (145 mM NaCl, 25 mM EDTA, 3.3 mM NaH_2PO_4, and 6.7 mM Na_2HPO_4) supplemented with merthiolate (10 mg/liter; Sigma-Aldrich Corp.) and gelatin (1 g/liter; pH 7.4). After 2 h at 4 C, 100 μl [^{125}I]GnRH (12,000 cpm) was added to all tubes, and incubation was continued for an additional 24 h. Seven hundred and fifty microliters of a charcoal/dextran suspension (2.5 and 0.25 g/liter, respectively, in Ca^{2+}- and Mg^{2+}-free PBS) were then added. The tubes were centrifuged at $4000 \times g$ for 10 min, and the supernatants were counted for radioactivity. All samples from an experiment were analyzed in the same assay. The lower limit of detection was 4 pg/ml.

Detection of NMDA receptor subunit mRNAs and VGLUT2 mRNA

The expression of mouse NMDA receptor subunits (ζ1 and ε1–4, corresponding to the rat NR1 and NR2A-D subunits, respectively) was detected by RT-PCR. Total RNA was isolated from GT1-7 cells or from mouse brain regions using TRIzol reagent (Invitrogen Life Technologies, Inc.) and treated with deoxyribonuclease I-ribonuclease-free (Stratagene, La Jolla, CA) to remove potential contaminant DNA. RNAs were controlled on a 1.2% agarose gel stained with ethidium bromide. One microgram of total RNA was incubated for 5 min at 70 C with 100 U of the ribonuclease inhibitor HPRI (Amersham Biosciences) and random hexamer primers (New England Biolabs, Beverley, MA), then reverse transcribed for 90 min at 42 C using 200 U SuperScript II Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Inc.) in the presence of 200 μM of each nucleotide triphosphate (deoxy-NTPs; Invitrogen Life Technologies, Inc.) in a total volume of 26 μl. One height of the RT reaction (3 μl) was used as a template for amplification by PCR using a thermal cycler (Stratagene). The amplification mixture contained cDNA, 25 pmol of each specific forward and reverse primer, 200 μM deoxy-NTPs, 1.5 mM $MgCl_2$, 5 μl buffer, and 1.25 U Hot Star *Taq* DNA polymerase (QIAGEN, Hilden, Germany) in a total volume of 50 μl. Primers were purchased from Sigma-Genosys Ltd. (Pampisford, UK). The nucleotide sequences for sense and antisense primers are shown in Table 1. After an initial denaturation at 94 C for 2 min, each amplification cycle (30 cycles) consisted of denaturation at 94 C for 1 min; annealing at 66 C (ζ1), 48 C (ε1), 50 C (ε2), 68 C (ε3), or 60 C (ε4) for 1 min; and extension at 72 C for 1 min, with a final extension time of 10 min.

The PCR products were analyzed on 1.2% agarose gel and visualized by ethidium bromide staining under UV light. As controls, we performed direct PCR on RNAs treated with deoxyribonuclease I omitting RT to confirm the absence of genomic DNA contamination, RT-PCR using primers for 18S ribosomal RNA (annealing at 58 C) to check the integrity of RNAs, RT-PCR without cDNA template, and RT-PCR of RNAs from adult mouse cortex (ζ,1, ε1, ε2, and ε4) or cerebellum (ε3) as positive controls.

The expression of the glutamate transporter VGLUT2 mRNA was also studied by RT-PCR. Specific nucleotide sequences for sense and antisense primers are shown in Table 1. Five micrograms of total RNA were used for the RT. PCR was performed (40 cycles) in the same way as described above, using a temperature of annealing of 50 C.

Glutamate and glycine release

When cells reached 90% confluence, C-DMEM was replaced by serum-free medium (OptiMEM; Invitrogen Life Technologies, Inc.) for 24 h. On the day of the experiment, GT1-7 cells were carefully washed three times (5 min) with Locke's medium, then incubated in Locke's medium with or without PREGS. This incubation medium was collected at 5 and 30 min and stored at −80 C. Glutamate and glycine concentrations were determined according to a modified version of the method described by Geddes and Wood (31), using a Waters HPLC-fluorometric detection system including a precolumn derivatization with *o*-phtaldialdehyde/mercaptoethanol reagent and a C_{18} (ODS2, 4.6 × 150 mm) Spherisorb column (Waters Corp., Grand Rapids, MI). A nonlinear gradient delivered through a Waters 600 pump was used to separate derivatives [solvent A, 0.1 M potassium acetate and 25% methanol (pH 5.5);

TABLE 1. The nucleotide sequences for sense and antisense primers used in RT-PCR for NMDA receptor subunits, 18S, and VGLUT2 glutamate transporter

Subunit	Sequence (5′–3′)	Fragment size (bp)
ζ1	(843) GGAGCGCGAGATCTCTGGGAAT	576
	(1419) TGTGTCATTAGGCCCCGTAC	
ε1	(597) TATGCAGAACGTGATCACAC	760
	(1357) TCACATTCATCCCTTCGTTG	
ε2	(637) GACTCTAAGATTCAGAATC	514
	(1151) AGGGACTTGTCTTTCCATT	
ε3	(1315) CACACCTTCAGCAGCGGGGAT	665
	(1980) CACAGTGTCGATGTATTGTTCCTGG	
ε4	(849) GGTCCCTGGGGAACCACTTC	375
	(1224) GAGGCGGAGGGTCTGCTGT	
18S	(465) CTACCACATCCAAGGAAGCG	386
	(851) CTCGGGCCTGCTTTGAACAC	
Vglut2	(885) GCGGAGGCAAAGTTATCAA	566
	(1017) CCCAAAGACTCGGTTAGCAG	

solvent B, 0.05 M (13) potassium acetate and 60% methanol (pH 5.5)]. Samples (10 μl) were automatically injected (Waters 717 Plus autosampler) and analyzed using a Waters 474 detector. The limit of detection was 1 pmol/sample. Data were computed with Waters Millenium software (via a Waters bus SAT/IN module) running onto a personal computer system; compound identification and peak quantification were achieved by comparison with standard solutions.

Statistical analysis

Data were expressed as the mean ± SEM of three to five independent experiments, each performed in triplicate. They were analyzed by one-way ANOVA, followed by Fisher's multiple comparison test at the 0.05 level of significance.

Results

PREGS stimulates the release of GnRH from GT1-7 neurons

The spontaneous release of GnRH by $4.2 \pm 0.5 \times 10^6$ GT1-7 cells over 30 min was 33.5 ± 12.6 pg/ml. PREGS (5–100 μM) caused a concentration-dependent increase in GnRH release, up to 4-fold (142.5 ± 22.6 pg/ml) over the basal level after 30 min. The effect was significant between 10 and 100 μM PREGS and reached a maximum value at 50–100 μM (Fig. 1A). PREGS-induced GnRH secretion occurred after a 20-min lag time and reached a maximum after 1 h of incubation (Fig. 1B). After a longer period of 150 min, the basal release of GnRH was increased, but the effect of PREGS remained identical (data not shown).

Studies of steroid specificity showed that the unconjugated steroid pregnenolone was inactive. Among other 3β-sulfated steroids, DHEAS and epiandrosterone sulfate (3β-hydroxy-5α-androstan-17-one sulfate) had no effect, but epiallopregnanolone sulfate (3β-hydroxy-5α-pregnan-20-one sulfate) stimulated GnRH release by 2-fold when used at 10 μM (data not shown). All 3α-sulfated steroids tested, allopregnanolone sulfate (3α-hydroxy-5α-pregnan-20-one sulfate), pregnanolone sulfate (3α-hydroxy-5β-pregnan-20-one sulfate), and androsterone sulfate (3α-hydroxy-5α-androstan-17-one sulfate), were inactive.

PREGS enhancement of GnRH release involves NMDA receptors, but not AMPA/kainate or $GABA_A$ receptors

When GT1-7 cells were incubated with PREGS (10 μM) in the presence of the $GABA_A$ receptor channel blocker picrotoxin (100 μM), GnRH release remained unchanged compared with that induced by PREGS alone (Fig. 2A). Similarly, coincubation with the selective AMPA/kainate receptor antagonist CNQX (10 μM) did not modify the release of GnRH by PREGS (10 μM) (Fig. 2A).

On the contrary, GnRH secretion returned to basal levels when cells were incubated with PREGS in the presence of various inhibitors of NMDA receptor function, namely, AP5 (10 μM) and MK 801 (5 μM), or when the incubation medium contained 6 mM Mg^{2+}, which blocks the NMDA ionic channel (Fig. 2B).

GT1-7 neurons express several NMDA receptor subunit mRNAs

Among the five subunits of the NMDA receptor identified in the mouse, we detected in GT1-7 neurons the expression

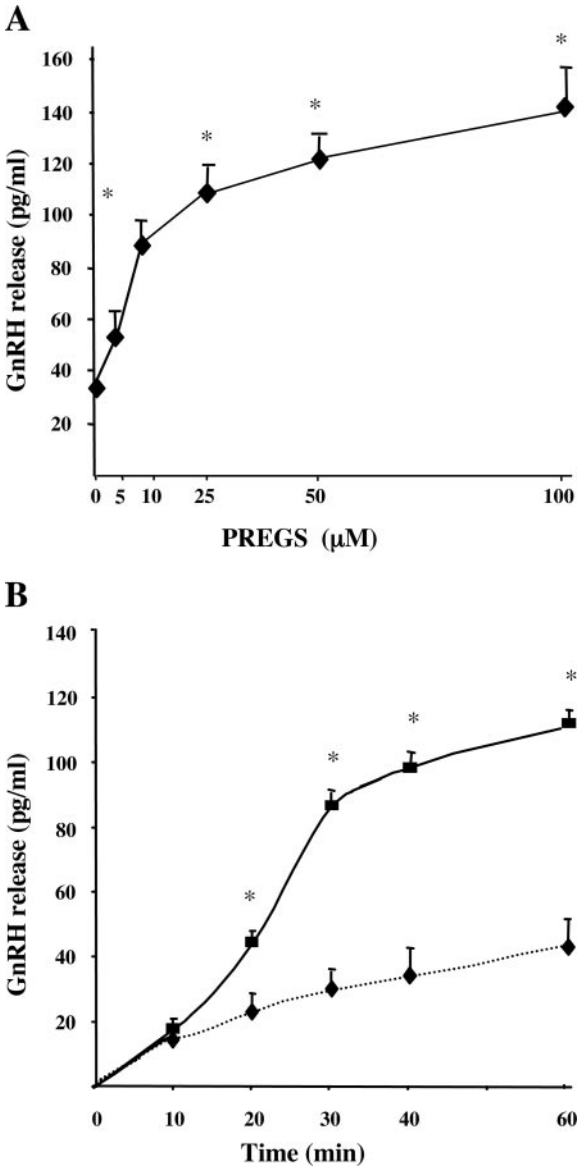

FIG. 1. PREGS stimulates the release of GnRH. A, Dose-response curve. GT1-7 neurons ($4.6 \pm 0.7 \times 10^6$/well) were incubated for 30 min in Locke's medium with 5–100 μM PREGS. Basal GnRH release (33.5 ± 12.6 pg/ml) corresponds to the value on the y-axis (0 μM PREGS). PREGS (100 μM) induced a 4-fold stimulation of GnRH secretion (142.5 ± 22.6 pg/ml). Results correspond to the mean ± SEM of five experiments performed in triplicate. *, $P < 0.05$ compared with the basal release value. B, Time course of PREGS-induced GnRH release. Cells were incubated with 10 μM PREGS for 10, 20, 30, 40, and 60 min. ◆, Basal release; ■, PREGS-induced GnRH release. Results are the mean ± SEM of three experiments performed in triplicate. *, $P < 0.05$ *vs.* corresponding basal release values.

of the obligatory ζ_1 mRNA (NR1 in the rat), but no ϵ_1 mRNA (NR2A). We also found ϵ_3, ϵ_4, and to a lesser extent ϵ_2 subunit mRNAs, which, respectively, correspond to the rat, NR2C, NR2D, and NR2B subunits (Fig. 3).

Glutamate and NMDA stimulate the release of GnRH from GT1-7 neurons

When incubated for 30 min with various concentrations of glutamate or NMDA in the presence of 10 μM glycine in

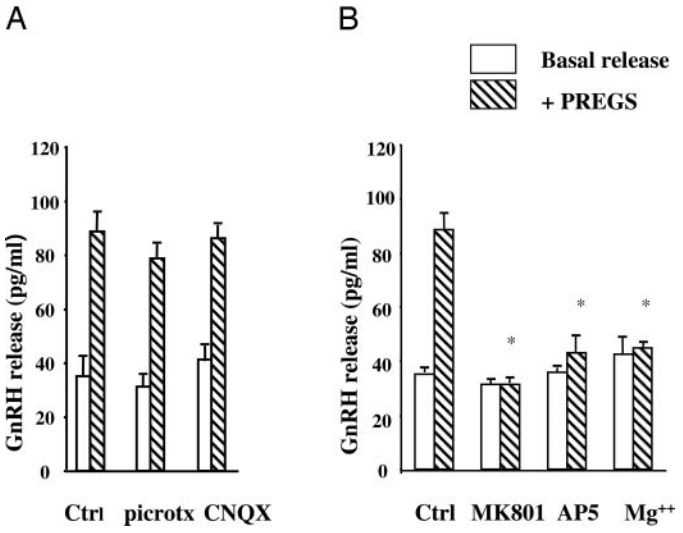

FIG. 2. A, PREGS action does not involve $GABA_A$ receptors or AMPA/kainate glutamate receptors. The respective specific antagonists picrotoxin (100 μM) and CNQX (10 μM) do not modify PREGS (10 μM)-induced GnRH release. Results are the mean ± SEM of two experiments performed in triplicate. B, NMDA receptor antagonists block PREGS-induced GnRH release. GT1-7 cells were incubated with 10 μM PREGS in the presence of the NMDA receptor channel blocker MK 801 (5 μM) or the NMDA competitive antagonist AP5 (10 μM) or when Mg^{2+} (6 mM) was added to the medium. Results correspond to the mean ± SEM of five experiments performed in triplicate. *, $P < 0.05$ *vs.* PREGS alone.

magnesium-free medium, GT1-7 neurons released more GnRH than spontaneously, and NMDA was more effective than glutamate (Fig. 4). The effects of glutamate and NMDA were dose dependent between 10 and 100 μM, with a 3-fold increase in GnRH secretion at 100 μM NMDA. Above this concentration, glutamate and NMDA became toxic and induced cell death, as reflected by decreased GnRH release (data not shown).

GT1-7 neurons release glutamate and glycine and express VGLUT2 glutamate transporter mRNA

The concentrations of glutamate and glycine were measured under basal conditions in the incubation medium of GT1-7 cells at different times of incubation. Although glutamate was absent at the beginning of the experiment, *i.e.* after the 15-min washing with Locke's medium, its concentration increased with time: 2.13 ± 0.07 μM (n = 6) after 5 min of incubation and 3.53 ± 0.16 μM (n = 15) after 30 min of incubation. PREGS did not modify glutamate release. Glycine was also released into the medium: 17.3 ± 2.2 μM (mean ± SEM; n = 6) after 5 min of incubation and 36.7 ± 9.2 μM (mean ± SEM; n = 15) after 30 min of incubation (glycine added to Locke's medium, 10 μM). PREGS did not influence glycine release. The specific marker of glutamatergic neurons, VGLUT2, was expressed at the mRNA level in GT1-7 neurons (Fig. 5).

Discussion

Our data demonstrate that PREGS can stimulate the release of GnRH from immortalized hypothalamic GT1-7 neurons in a dose-dependent manner, with a lag time of 20 min. This action is stereospecific: DHEAS is inactive, and among other 3β-sulfated steroids tested, only epiallopregnanolone stimulates

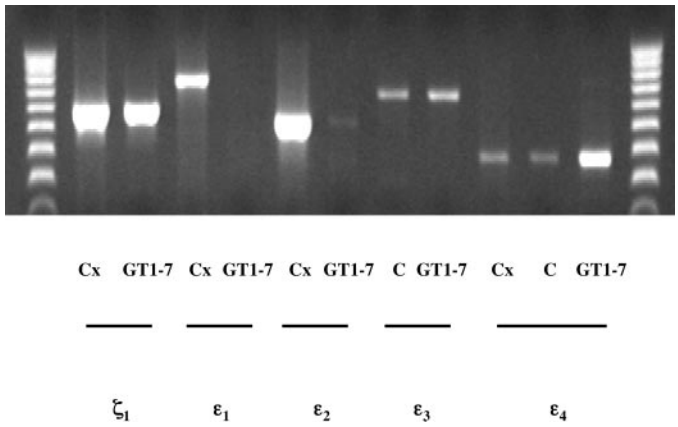

FIG. 3. GT1-7 cells express mRNAs corresponding to different subunits of NMDA receptors. The amplified fragments were specific for a single NMDA subunit (ζ1, ϵ1, ϵ2, ϵ3, and ϵ4). For each subunit, a positive control from mouse adult brain was used, and sequences amplified were as follows: ζ1, 576 bp (cx, cortex); ϵ1, 760 bp (cx), ϵ2, 514 bp (cx); ϵ3, 665 bp (c, cerebellum); and ϵ4, 516 bp (cx). GT1-7 cells express ζ1, ϵ2, ϵ3, and ϵ4, but not ϵ1, NMDA receptor subunit mRNAs.

GnRH secretion. Moreover, PREGS action is prevented by NMDA receptor antagonists (AP5, MK801, and magnesium), suggesting an interaction of PREGS with NMDA receptors.

The previous *in vivo* observations that NMDA elicited pituitary LH release in the prepubertal male monkey (32) and advanced the onset of puberty in the female rat and male monkey (33–35), whereas NMDA receptor antagonists suppressed the pulsatile secretion of LH (36), did not elucidate the precise site of action of NMDA: exclusively in the pituitary gland or also in the hypothalamus. *In vitro* experiments on rat hypothalamic explants demonstrated that glutamate or NMDA perifusion induced GnRH release (37–39), although the cellular target of excitatory amino acids remained unclear. Subsequently, the presence of NMDA receptors at the surface of GnRH neurons was demonstrated (34) and confirmed when transgenic mice expressing green fluorescent protein (GFP) under control of the GnRH promoter (GnRH-GFP mice) were generated and used

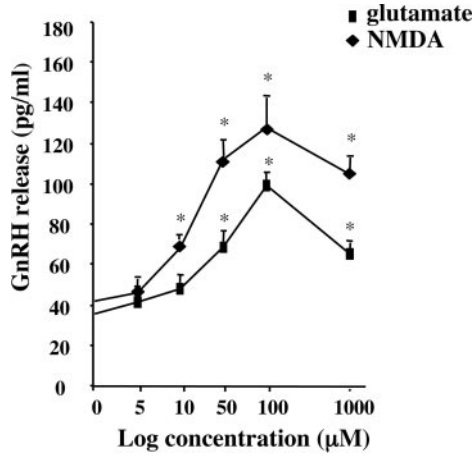

FIG. 4. NMDA and glutamate stimulate the release of GnRH from GT1-7 cells. Cells were incubated for 30 min in Locke's medium without or with increasing concentrations of glutamate or NMDA (5, 10, 50, 100, and 1000 μM). Spontaneous release was 40.25 ± 5.2 pg/ml. Results express the mean ± SEM of three experiments performed in triplicate. *, $P < 0.05$ *vs.* basal release.

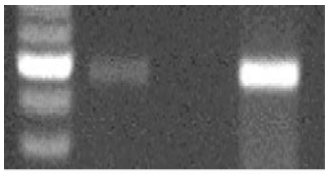

FIG. 5. GT1-7 neurons express mRNAs encoding the glutamate transporter VGUT2 (2). Mouse hippocampal mRNAs (4) and H_2O (3) were used as positive and negative controls, respectively (1). Size marker (100-bp DNA ladder). Sequence amplified, 566 bp.

to determine the electrophysiological properties of identified GnRH neurons in brain slice preparations (40, 41); it was thus clearly observed that functional glutamate receptor channels of the AMPA and NMDA subtypes were present in the membrane of GnRH neurons. Finally, Ottem *et al.* (42) recently demonstrated that the majority of GnRH neurons located in the medial preoptic area of the hypothalamus expressed the NMDA R1 gene.

In immortalized GT1 neurons, conflicting data have been reported. Mahesh *et al.* (43) did not find any electrophysiological or binding characteristics of NMDA receptors in GT1-7 cells despite the expression of NMDAR1 mRNA. In contrast, Mahachoklertwattana (44) reported that NMDA stimulated the release of GnRH from GT1-1 neurons, which expressed NMDAR1 receptor transcripts. Urbanski *et al.* (45) observed the presence of NMDAR1 mRNA in GT1-7 neurons, and Spergel *et al.* (46) showed that glutamate increased intracellular calcium and GnRH release from GT1-7 cells through NMDA receptors. In the present study we report a stimulating effect of glutamate and NMDA on GnRH release and observe that GT1-7 neurons express various NMDA receptor subunit mRNAs. Indeed, among the five subunits identified in the mouse (47), GT1-7 neurons express not only the obligatory ζ_1 mRNA, but also ϵ_3, ϵ_4, and ϵ_2, but not ϵ_1 (NR1, NR2C, NR2D, NR2B, and NR2A, respectively, in the rat). Interestingly, a steroid modulatory domain has recently been identified on the NR2B subunit that is critical for PREGS sensitivity (16).

PREGS has been described as a positive allosteric modulator, not as an agonist of the NMDA receptor. However, in cultures of GT1-7 neurons, the effect of PREGS occurred in the absence of added NMDA or glutamate, suggesting the presence of endogenous excitatory amino acids. When measuring in the incubation medium, the amount of glutamate and glycine, a well-known coagonist of the NMDA receptor (48), their concentrations were found to increase between 5 and 30 min of incubation. The amount of secreted glutamate was too low to induce stimulation of GnRH release and probably did not participate in the basal release of GnRH, because the NMDA receptor antagonists AP5, MK 801, and Mg^{2+} did not lower the spontaneous liberation of GnRH. However, it was probably sufficient to allow positive modulation of the activity of NMDA receptors by PREGS, leading to stimulation of GnRH release.

The question remained whether the release of glutamate occurred after its uptake from OptiMEM medium, in which cells had been cultured 24 h before the experiments or whether GT1-7 cells could synthesize it. For a very long time, no reliable method for detecting glutamatergic neurons existed. This obstacle has recently been overcome with the characterization of VGLUTs, which are specific markers of glutamatergic neurons (49). Indeed, neurons that are able to synthesize glutamate must have the capacity to package and release it via specific VGLUT. Three types of VGLUTs have been described (50): although VGLUT3 is not detected and VGLUT1 is very scarce in the hypothalamus, VGLUT2 is expressed, especially in the arcuate nucleus and median eminence (51). Our data suggest that GT1-7 cells are likely to synthesize glutamate as they express VGLUT2 mRNA and release glutamate into the incubation medium. These data are in accordance with the recent observation by Hrabovsky *et al.* (52) of the expression of VGLUT-2 in GnRH neurons of the adult male rat.

Immortalized GT1–1 and GT1-7 neurons secrete GnRH, but have also been shown to secrete GABA (53), express $GABA_A$ receptors, and release GnRH under muscimol stimulation (Ref. 7 for GT1–1 cells and El-Etr, M., personal unpublished observations for GT1-7 cells), suggesting a possible autocrine influence of GABA on GnRH secretion. The cosecretion of GABA and glutamate by the same neurons has been previously described in the hypothalamus; indeed, some neurons present in the anteroventral periventricular nucleus, a region of the preoptic area involved in the regulation of GnRH neuron function, are able to secrete both amino acids, as demonstrated by their capacity to coexpress VGLUT2, glutamic acid decarboxylase, and vesicular GABA transporter (54). However, to our knowledge, this is the first observation reported of GABA (53) and glutamate (our present data) cosecretion by GnRH neurons.

GnRH neurons represent the final output pathway of a neuronal network that integrates multiple environmental and internal factors, such as steroid hormones, to control fertility in both sexes (55, 56). These regulations by steroids can be indirect, via astrocytes (3, 57) or surrounding interneurons releasing various neurotransmitters and peptides (2), which, in turn, modulate GnRH secretion. However, steroids can directly influence GnRH neurons in a genomic and even nongenomic way; indeed, 17β-estradiol is able to induce a fast nongenomic stimulation of GnRH release through ERβ, which might participate in the positive feedback of estradiol (4, 58) and provoke a rapid inhibition (or stimulation, depending on the dose of estradiol) of GnRH secretion, through G_i-coupled membrane ERα (59).

Besides classical steroid hormones, a few neuroactive steroids have been shown to rapidly modulate GnRH release; the first experiments performed on male rat hemihypothalami had shown an inhibiting effect of allopregnanolone on GnRH release, that involved $GABA_A$ receptors and was counteracted by PREGS, but not by DHEAS. In the same preparations, GABA suppressed GnRH release (60). However, those studies could not ascertain whether these effects occurred at the GnRH neuron itself and/or at the presynaptic cells. Recently, using hypothalamic slices from transgenic GnRH-GFP mice, adult GnRH neurons were shown to maintain high intracellular chloride levels such that the chloride reversal potential was depolarized relative to the threshold for action po-

tential firing, and direct activation of $GABA_A$ receptors on these cells was excitatory regardless of the sex and hormonal state of the animals (61). In agreement with these observations, other studies reported that allopregnanolone enhanced the electrical responses to GABA of juvenile and adult female mouse GnRH neurons from hypothalamic brain slices (8) and increased the electrophysiological responsiveness to GABA of GnRH neurons from adult GnRH-GFP mice, whereas DHEAS reversed this effect (9). In both GT1–1 and GT1-7 cell cultures, allopregnanolone stimulates GnRH release through $GABA_A$ receptors, PREGS counteracts this effect, whereas PREGS alone is able to enhance GnRH release (7; and El-Etr, M., personal unpublished data). Overall, the local concentrations of magnesium and GABA might differentiate between the inhibitory actions of PREGS at the $GABA_A$ receptor and its stimulatory effects through NMDA receptors.

Our present results, which suggest a role of NMDA receptor modulation by PREGS in the control of GnRH release, are likely to be of physiological significance: 1) GnRH neurons indeed express NMDA receptors *in vivo*, and NMDA stimulates GnRH release by acting on the hypothalamus (see above); 2) PREGS is an important positive allosteric modulator of NMDA receptors (15, 16); 3) the positive modulation of NMDA receptors by PREGS has been shown to be involved in the regulation of important brain functions, including memory processes (62, 63). Whether PREGS is an endogenous neurosteroid in the rodent brain has recently been questioned (for a critical evaluation, see Ref. 64). Whatever the outcome of this controversial issue, however, it is well established that PREGS is present in human blood (64) and brain, including the hypothalamus (65).

PREGS stimulated the release of GnRH from GT1-7 neurons at concentrations ranging from 10–100 μM. Whether these concentrations can be locally reached within the nervous system remains a matter of speculation and cannot be solved by currently available assay methods, even by the very sensitive analysis of steroids by mass spectrometry. However, it is known that *in vitro* experimental paradigms sometimes require high concentrations of steroids, and in particular of their sulfated forms. The modulation of NMDA receptors by PREGS using electrophysiological recordings has been extensively studied (15, 66). In their studies the positive allosteric modulation of NMDA receptors by PREGS in various neuronal preparations required very high micromolar concentrations of the steroid (10–100 μM), consistent with our present findings. More recently, the same group identified a PREGS modulatory domain on the NMDA receptor, strongly suggesting that the steroid directly acts on the receptor (16).

In conclusion, in addition to the positive effect of PREGS on GnRH gene expression in rat GnRH neurons (67) the present data show that via NMDA receptors, the neuroactive steroid PREGS can rapidly enhance the release of GnRH by immortalized GT1-7 cells, through the potentiation of the autocrine actions of glutamate, and may thus contribute to a rapid regulation of GnRH release at the level of the GnRH neuron.

Acknowledgments

We are particularly grateful to Jocelyne Cordier for her advice concerning GT1-7 neuronal cultures and for regularly providing us with mouse astrocyte-conditioned medium. We also thank Denise Samuel for HPLC measurements of glutamate and glycine release.

Received September 19, 2005. Accepted February 21, 2006.

Address all correspondence and requests for reprints to: Dr. Martine. El-Etr, Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 788, 80 rue du Général Leclerc, 94276, Le Kremlin-Bicêtre, France. E-mail: eletr@kb.inserm.fr.

M.E.E., Y.A., E.-E.B., and M.S. have nothing to declare.

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