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Mechanisms of Interaction of Endocrine-Disrupting Chemicals with Glutamate-Evoked Secretion of Gonadotropin-Releasing Hormone

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Abstract

In previous studies, we detected a dichlorodiphenyltrichloroethane (DDT) derivative in the serum of children with sexual precocity after migration from developing countries. Recently, we reported that DDT stimulated pulsatile gonadotropin-releasing hormone (GnRH) secretion and sexual maturation in the female rat. The aim of this study was to delineate the mechanisms of interaction of endocrine-disrupting chemicals including DDT with GnRH secretion evoked by glutamate *in vitro*. Using hypothalamic expiants obtained from 15-day-old female rats, estradiol (E2) and DDT caused a concentration-related increase in glutamate-evoked GnRH release while *p,p′*-dichlorodiphenyldichloroethene and methoxychlor had no effect. The effective DDT concentrations *in vitro* were consistent with the serum concentrations measured *in vivo* 5 days after exposure of immature rats to 10 mg/kg/day of *o,p′*-DDT. Bisphenol A induced some stimulatory effect, whereas no change was observed with 4-nonylphenol. The *o,p′*-DDT effects *in vitro* were prevented partially by a selective antagonist of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtype of glutamate receptors. A complete prevention of *o,p′*-DDT effects was caused by an estrogen receptor (ER) antagonist as well as an aryl hydrocarbon receptor (AHR) antagonist and inhibitors of protein kinases A and C and mitogen-activated kinases. While an intermittent incubation with E2 caused no change in amplification of the glutamate-evoked GnRH release for 4 h, continuous incubation with E2 or *o,p′*-DDT caused an increase of this amplification after 3.5 h of incubation. In summary, DDT amplifies the glutamate-evoked GnRH secretion *in vitro* through rapid and slow effects involving ER, AHR, and AMPA receptor mediation.

Keywords: gonadotropin-releasing hormone ; endocrine-disrupting chemicals ; glutamate receptors ; estrogen receptors ; aryl hydrocarbon receptor.

The persisting secular shift in timing of puberty toward early onset in United States (Herman-Giddens *et al.,* 1997; Lee *et al.,* 2001) raised the issue of the possible role of environmental factors including endocrine-disrupting chemicals (EDCs) (Buck-Louis *et al.,* in press). In other countries such as Belgium and Denmark, a 20-80 times increased risk of sexual precocity in internationally adopted children (Krstevska-Konstantinova *et al.,* 2001; Teilmann *et al.,* 2006) also led to the question of possible EDC involvement (Parent *et al.,* 2003). Based on the measurement of *p,p′*-dichlorodiphenylchloroethene (DDE) levels in the serum of those children, it was hypothesized that migration could account for early puberty after transient exposure to the estrogenic insecticide dichlorodiphenyltrichloroethane (DDT) in the home country (Krstevska-Konstantinova *et al.,* 2001; Parent *et al.,* 2003). That hypothesis was consistent with the early report of precocious puberty in female rats treated neonatally with *o,p′*-DDT by Heinrichs *et al.* (1971) who showed inhibitory effects on pituitary gonadotropin secretion and suggested a facilitatory hypothalamic mechanism (Gellert *et al.,* 1972).

Very recently, we administered *o,p′*-DDT for 5 or 10 days in infantile female rats, and we found pulsatile gonadotropin-releasing hormone (GnRH) secretion *in vitro* to accelerate prematurely and precocious puberty to occur subsequently (Rasier *et al.,* 2007). In that study, the effects on GnRH secretion became significant after several hours, and both the estrogen receptors (ERs) and the orphan dioxin receptor (aryl hydrocarbon receptor [AHR]) appeared to be involved in DDT effects on pulsatile GnRH secretion. In previous studies from our laboratory, estradiol (E2) was shown to cause a rapid (within 7.5-15 min) amplification of the GnRH release evoked by glutamate, a main excitatory neurotransmitter in the brain. This effect involved the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-kainate receptor subtypes, the α-subunit of ERs, and different intracellular kinases (Matagne *et al.,* 2005). Beside those rapid effects, initially inactive concentrations of E2 (Matagne *et al.,* 2005) and DDT (Rasier *et al.,* 2007) were shown to have effects appearing with time, indicating slow and possibly genomic effects. This was the reason why the glutamate-evoked secretion of GnRH was studied over several hours in the present study.

Our aims were as follows: (1) to study whether DDT and derivatives could have rapid effects similar to E2 on the glutamate-evoked release of GnRH *in vitro*; (2) to compare DDT effects with those of other EDCs such as methoxychlor (MXC) and bisphenol A (BPA). MXC is another pesticide that has been developed to replace DDT and to have a similar spectrum of intended effects while being more readily eliminated from the body and less persisting in nature. MXC estrogenic activity is presumably related to the ability of its metabolite hydroxyphenyltrichloroethane to bind to intracellular ERs (Miller *et al.,* 2006). BPA is a common EDC used in the manufacture of a variety of products including reusable milk and food storage containers, baby formula bottles, interior lacquer coating of food cans, or dental sealants. This compound has been found to compete with E2 for binding to ERs though its affinity for those receptors is much less than that of E2 (Kwon *et al.,* 2000). (3) To study whether DDT and derivatives could have effects on the glutamate-evoked release of GnRH over a period of several hours and the receptor mechanisms involved in such effects.

MATERIALS AND METHODS

Animals

Fifteen-day-old female Wistar rats were purchased from the University of Liège. They were housed in standardized conditions with lactating dams (22°C, lights on from 06:30 to 18:30 h, food and water *ad libitum)* till the time of sacrifice. Each litter contained 5-10 pups. The day of birth was considered as postnatal day (PND) 1. All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethical Committee at the University of Liège.

Incubation of Hypothalamic Expiants

After decapitation, the hypothalamus was rapidly dissected. The limits to obtain the retrochiasmatic hypothalamus were the caudal margin of the optic chiasm, the caudal margin of the mammillary bodies, and the lateral hypothalamic sulci. Each expiant was transferred into an individual incubation chamber, as described in detail previously (Bourguignon *et al.,* 1989a; Matagne *et al.,* 2004), which contained 500 µl of phenol red-free minimum essential medium (MEM) supplemented with glucose, magnesium, glycine, and bacitracin to achieve final concentrations of $25 \times 10\text{-}^{3}$, 10^{-3}, 10^{-8}, and 2×10^{-5}M, respectively. The expiants were incubated in an atmosphere of 95% O_2-5% CO_2. The incubation medium was collected and renewed every 7.5 min and kept frozen until assayed. Since some "traumatic" peptide release was observed for the first 7.5-15 min of incubation, the expiants were incubated for 30 min before the first glutamate stimulation.

Assays

The GnRH release in the incubation medium was measured in duplicate using a radioimmunoassay method with intra- and interassay coefficients of variation of 14 and 18%, respectively (Bourguignon *et al.,* 1989a,b). The highly specific CR11-B81 anti-GnRH antiserum (final dilution 1:80,000) was kindly provided by Dr V. D. Ramirez (Urbana, IL) (Dluzen and Ramirez, 1981). The data below the limit of detection (5 pg/7.5-min fraction) were assigned that value.

The identification and quantification of *p,p′*-DDT, *o,p′*-DDT, *p,p′*-DDE, and *o,p′*-DDE in serum were performed using a gas chromatographic analyzer coupled to an ion-trap mass spectrometer (MS) detector (PolarisQ, Thermo Scientific). Sample preparation included a liquid-liquid extraction (petroleum ether:diethylether, 98:2) followed by a solid-phase extraction (Bond Elut Certify, Varian, Harbor City, CA). The eluate was evaporated to dryness, reconstituted with *n*-hexane, and then injected into the gas chromatograph. The column was a HP-5 Trace (30 m × 0.25 mm internal diameter) from Agilent (Waldbronn, Germany). For detection, the MS was operated in the electronic impact mode (70 eV). Multiple reaction monitoring was used for identification and quantification. All solvents were of the highest purity available and pesticide-grade quality (Baker, Phillipsburg, NJ). Reference standards of all pesticides were obtained from Dr Ehrenstorfer (Augsburg, Germany). Aldrine was used as internal standard. The calibration curve was constructed from 0.5 to 20 ppb, and samples dilutions were applied to this concentration range. The limits of quantification were defined as 10 times the SD of the results of a blank sample. These limits were 0.5 ppb for *p,p′*-DDT, *o,p′*-DDT, *p,p′*-DDE, and *o,p′*-DDE, and the coefficients of variation were 7.7, 7.1, 8.2, and 6.1%, respectively. Based on the amount of serum used, the limit of detection was 1 ng/ml serum (Charlier and Plomteux, 2002).

Reagents

The phenol red-free MEM (supplemented with Earle's and L-glutamine) was purchased from Life Technologies Invitrogen Corporation (Merelbeke, Belgium). E2 (17β-estradiol or 3,17β-dihydroxy-l,3,5(10)-estratriene), the two DDT isomers, *o,p′*-DDT (2,4'-DDT) and *p,p′*-DDT (4,4'-DDT), *p,p′*-DDE (4,4'-DDE), MXC (1,1,1-trichloro-2,2-bis-(4-methoxyphenyl)ethane), BPA (2,2-bis-(4-hydroxyphenyl)-propane), 4-nonylphenol (4-NP), bacitracin, the AHR antagonist α-naphthoflavone (7,8-benzoflavone), the protein kinase (PK) A and C inhibitor staurosporine, and the PKC inhibitor chelerythrine chloride (1,2-dimethoxy-N-methyl(1,3)

benzodioxolo(5,6-c)phenanthridinium chloride) were purchased from Sigma-Aldrich (Bornem, Belgium). The AMPA/kainate subtype of glutamate receptor antagonist DNQX (6,7-dinitroquinoxaline-2,3-dione), the AMPA receptor selective antagonist SYM 2206 (4-aminophenyl-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphtalazine) and the ER antagonist ICI 182,780 (7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-1,3,5(10)-estratriene-3,17β-diol) were purchased from Tocris Fisher Bioblock Scientific (Illkirch, France). The mitogen-activated protein kinase (MAPK, extracellular signal-regulated kinase 1/2) inhibitor PD98059 (2'-amino-3'-methoxyflavone) was purchased from Calbiochem (VWR International, Leuven, Belgium). In all experiments, steroid or EDCs were dissolved initially in absolute ethanol (Labonord, Templenars, Belgium) and subsequently in the incubation medium to achieve a final ethanol concentration of 0.01%. All the other drugs were directly diluted in the incubation medium.

Experimental Protocols

In vivo *experiments.* Female rats received a daily sc administration of *o,p′*-DDT (10 mg/kg/day) or vehicle (sesame oil) for 5 days (PND 6-10). The insecticide was first dissolved in absolute ethanol and then diluted in 50 µl of sesame oil for sc injection with 24 rats studied in the treated group in comparison with 24 controls. On PND 15 and 22, on the day of vaginal opening (VO) and on the day of first estrus, six rats from each group were sacrificed to study the serum levels of the DDT isomers. A daily examination for imperforation of the vaginal membrane was achieved to determine age at VO. Thereafter, vaginal smears were taken every day in the afternoon until first estrus. Slides of vaginal smears were colored using the Papanicolaou method to detect the occurrence of estrous cyclicity (Papanicolaou and Traut, 1941). The age at first estrus was considered when vaginal smears contained primary cornified cells after the first proestrous phase which is characterized by both stratified and cornified cells (Ojeda and Urbanski, 1994). The *in vivo* conditions for these experiments were similar to a previous study (Rasier *et al.,* 2007).

In vitro *experiments.* The effects of E2 or EDCs were studied on the glutamate-evoked GnRH release from hypothalamic expiants obtained in 15-day-old female rats. Glutamate was incubated with the expiant for a 7.5-min fraction, at a submaximal concentration (10^{-2}M) which was shown previously to be required to evoke GnRH secretion *in vitro* (Matagne *et al.,* 2005). Such a glutamate challenge was repeated every 37.5 min for a period of 4-5 h. The 10^{-2}M concentration of glutamate was presumably not resulting in any toxic effect since a similar GnRH secretory response could be obtained throughout a 4-h experiment (Bourguignon *et al.,* 1989b; Matagne *et al.,* 2005). Each experiment on the effects of E2 or EDCs started and finished with a control glutamate stimulation in the absence of any other compound. The glutamate-evoked release of GnRH was compared in control conditions and during coincubation with E2 or EDCs added in the incubation medium. These substances were used at increasing concentrations (E2: 10^{-10} to 10^{-6}M; *p,p′*-DDT and *o,p′*-DDT: 10^{-8} to 10^{-4}M; *p,p′*-DDE, MXC, BPA, and 4-NP: 10^{-9} to 10^{-4}M) for two 7.5 -min fractions, before and during incubation with glutamate. In some experiments, the effects of E2 or EDCs were studied in the presence of antagonists or inhibitors that were used for three consecutive 7.5-min fractions so that they were firstly incubated alone, secondly with the steroid or EDCs, and thirdly with the two substances and glutamate. DNQX and SYM 2206 (10^{-6}M) were used to study the involvement of the AMPA/ kainate subtype of glutamate receptors on the amplification of the glutamate-evoked GnRH release caused by E2 (10^{-7}M) or *o,p′*-DDT (10^{-4}M). The implication of ERs was studied using the antagonist ICI 182,780 (10^{-7}M). To investigate the implication of AHR, the antagonist α-naphthoflavone (10^{-7}M) was used. The concentration of these four antagonists was selected based on previous data from this laboratory and another study (Ojeda and Urbanski, 1994; Rasier *et al.,* 2007). To investigate intracellular pathways and kinase involvement in E2 or *o,p′*-DDT effects, the PKA and C inhibitor staurosporine (10^{-7}M), the PKC inhibitor chelerythrine chloride (10^{-5}M), and the MAPK inhibitor PD98059 (5×10^{-5}M) were used.

Statistical Analysis

A minimum of four expiants were used for each condition in an experiment. The secretory response (pg/7.5-min fraction) was calculated as the difference between the levels in the fractions collected immediately prior to and during exposure to the glutamate. Raw data were pooled and analyzed by a one-way analysis of variance test followed by a Newman-Keuls posttest when the threshold for significance of difference ($p < 0.05$) was reached (GraphPad Prism software for PC). In order to compare the results obtained among experiments in which the control data were different due to the use of receptor antagonists in some experiments, the absolute levels (determined by radioimmunoassay as pg/7.5-min fraction) were transformed as percentages of the glutamate-evoked release observed in control conditions and regarded as 100%. The statistical analysis was performed using the absolute as well as transformed data, and identical significance levels were obtained in both conditions. The time-related changes in serum concentrations of *o,p′*-DDT were compared after log transformation of the data because they were lognormally distributed. Geometric means were calculated, and data were represented on a log scale. All other results are expressed as mean ± SD.

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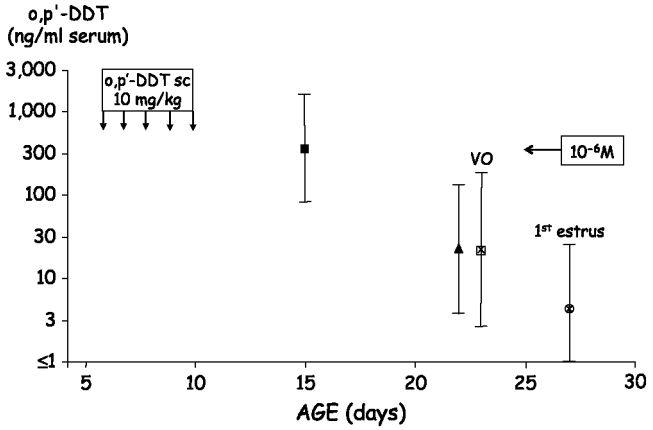
RESULTS

Serum Levels of DDT Isomers and Derivatives After In Vivo *Treatment*

We showed earlier that an early exposure to *o,p′*-DDT between PND 5 and 10 accelerated pulsatile GnRH secretion and induced a precocious puberty in female rats (Rasier *et al.*, 2007). In order to determine if the concentrations of *o,p′*-DDT achieved *in vivo* were consistent with the concentrations used *in vitro* in the present study, we measured DDT isomers and metabolites serum concentrations after *in vivo* administration of *o,p′*-DDT. Five days after a 5-day treatment with *o,p′*-DDT, the mean serum level of this isomer was 361 ng/ml. Seven days later, the level dropped to 23 ng/ml. VO and first estrus occurred early on PND 23 and 27, respectively, confirming our previous findings (Rasier *et al.,* 2007). The mean serum *o,p′*-DDT level was 22 ng/ml at VO and 4 ng/ml at first estrus (Fig. 1). The *p,p′*-DDT isomer and the *p,p′*-DDE and *o,p′*-DDE derivatives were undetectable in the serum of the treated rats at any time of the study, and none of the studied compounds were detectable in the serum of control rats.

FIG. 1. *Geometric mean (± SD) serum levels of o,p′-DDT at four time points after administration of o,p′-DDT for 5 days (PND 6-10): PND 15, PND 22, on the day of VO, and on the day of first estrus. The average serum concentration on PND 15 is equivalent to 10^{-6}M (n = 6).*



Concentration-Response Effects of E2 or EDCs on Glutamate-Evoked GnRH Release

In Figure 2 are illustrated three representative concentration-response profiles of the glutamate-evoked GnRH release from retrochiasmatic hypothalamic expiants obtained at 15 days in female rats. Repeated challenges with glutamate alone for 7.5 min every 37.5 min resulted in a reproducible release of GnRH which was maintained for 4 h (Fig. 2A). Coincubation with increasing concentrations of E2 caused a concentration-related increase in glutamate-evoked GnRH release (Fig. 2B). As shown in Figure 3A, this effect became significant using 10^{-8}M of E2 (128.0 ± 4.9% of control) and further increased to 162.6 ± 15.1% and 195.0 ± 6.4% using 10^{-7}M and 10^{-6}M of E2, respectively. A concentration-response increase in glutamate-evoked GnRH release was also caused by *o,p′*-DDT (Figs. 2C and 3B), 10^{-5} and 10^{-4}M being required for a significant effect (130.8 ± 4.5% and 173.7 ± 7.6%, respectively). After repeated glutamate stimulation in the presence of increasing concentrations of E2 or *o,p′*-DDT, a final stimulatory challenge with glutamate alone could evoke a release similar to that obtained initially (Figs. 2B and C). The *p,p′*-DDT isomer was as effective as *o,p′*-DDT since the 10^{-5} and 10^{-4}M concentrations accounted for a significant increase in GnRH release (135.7 ± 8.8% and 185.3 ± 18.1%, respectively).

In contrast, *p,p′*-DDE, a stable DDT derivative, and MXC, a DDT-related EDC, had no effect (Fig. 3B). BPA was significantly stimulatory when used at 10^{-4}M (201.7 ± 74.2%), whereas the 10^{-5}M concentration did not result in any effect (94.2 ± 22.0%). When used at similar concentrations, 4-NP did not show any effect (Fig. 3C).

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FIG. 2. *Representative profiles of glutamate-evoked GnRH secretion from hypothalamic expiants obtained at 15 days in female rats and incubated in MEM for 4 h. Glutamate was added repeatedly (every 37.5 min) for 7.5 min alone (A) and together with increasing concentrations of E2 (B) or o,p'-DDT (C) which were also incubated for an additional 7.5 min before glutamate stimulation. The limit of detection was 5 pg/7.5 min.*

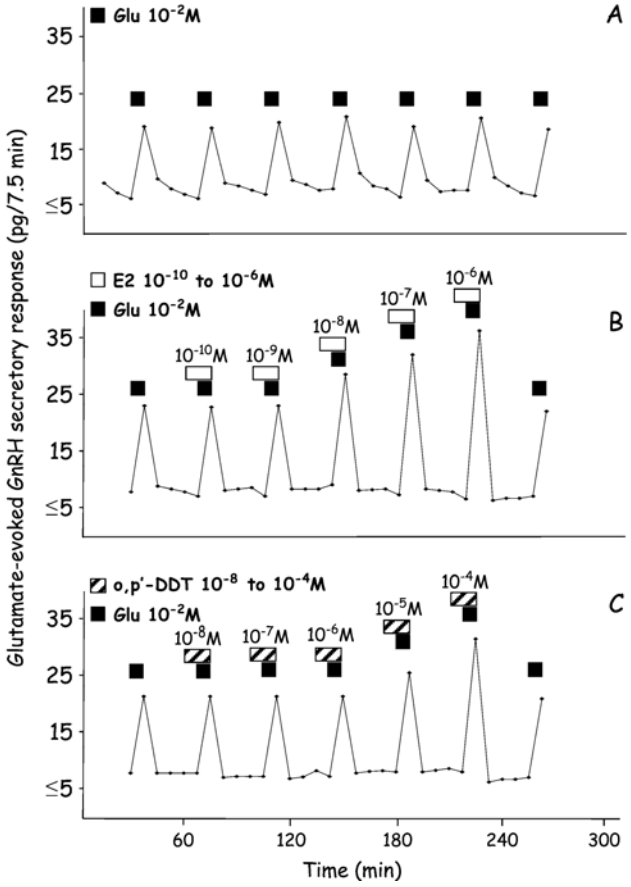
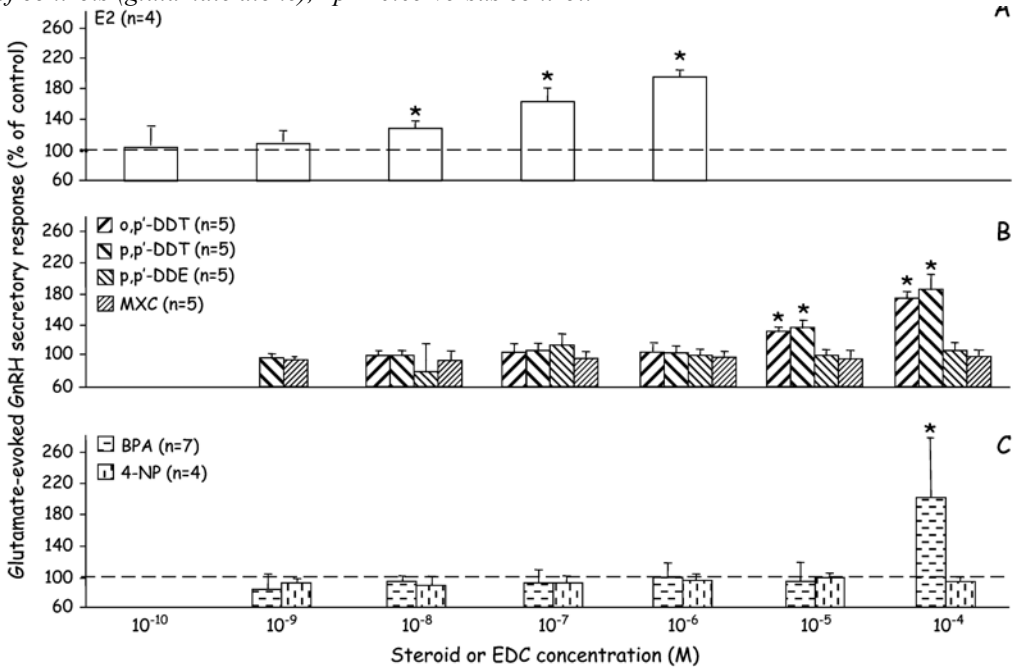


FIG. 3. *Concentration-response effect of E2 (A) and several EDCs (B and C) on glutamate-evoked secretory response of GnRH using hypothalamic expiants from 15-day-old female rats. The GnRH secretory response (mean ± SD) was calculated as the difference between the levels in the fraction collected immediately prior to and during exposure to the glutamate (pg/7.5-min fraction). Those differences were then converted in percentage of controls (glutamate alone), *p < 0.05 versus control.*



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Receptors and Signaling Involved in E2 or EDC Effects on Glutamate-Evoked GnRH Release

When DNQX, an antagonist of the AMPA/kainate subtypes of glutamate receptors, was used, the glutamate-evoked release of GnRH was markedly reduced versus control (5.8 ± 0.4% vs. 11.2 ± 1.3 pg/7.5 min). This was presumably due to suppression of the kainate receptor contribution to the glutamate-evoked release since the selective AMPA antagonist SYM 2206 did not significantly reduce the glutamate-evoked release of GnRH (10.8 ± 0.8 vs. 11.4 ± 1.1 pg/7.5 min). To allow comparison of the amplification by 10^{-7}M of E2 or 10^{-4}M of *o,p'*-DDT of the glutamate-evoked GnRH secretion, the data were calculated as percentages of the secretory response obtained in the presence of the antagonist alone. In the presence of DNQX, both E2 and *o,p'*-DDT could still significantly increase the glutamate-evoked secretion of GnRH though the amplification of the response by E2 was less than that in control conditions. In the presence of the specific AMPA antagonist SYM 2206, both the E2 and *o,p'*-DDT amplification of the glutamate-evoked GnRH release were significant but less marked than in control conditions, indicating a contribution of AMPA receptors (Fig. 4A). When the ER antagonist ICI 182,780 was used (Fig. 4B), the E2 and *o,p'*-DDT amplification of the glutamate-evoked GnRH secretion were completely prevented. When the AHR antagonist α-naphthoflavone was used, only the increase in GnRH secretory response caused by *o,p'*-DDT was prevented, whereas the E2 potentiating effect was unchanged (Fig. 4B). Inhibitors of PKA and C (staurosporine and chelerythrine chloride) as well as MAPK (PD98059) all prevented completely the amplification by E2 and *o,p'*-DDT of the glutamate-evoked GnRH release (Table 1).

When 10^{-8}M of E2 was applied intermittently for 15-min episodes that were repeated every 37.5 min, the GnRH release evoked by glutamate showed a similar increase during a 4-h study period (Fig. 5). After stopping exposure to E2 at the end of the experiment, the expiants recovered a response similar to that obtained initially. When E2 was applied continuously, the glutamate-evoked GnRH release showed a significant increase similar to that observed when E2 was used intermittently. After 3.5 h of incubation, however, a further increase in glutamate-evoked GnRH secretion (148.2 ± 2.1%) was caused by E2 and some significant increase persisted at the end of the experiment (114.9 ± 1.4%), after stopping incubation with E2. A similar time-dependent effect was observed using 10^{-5}M of *o,p'*-DDT, except that the increase in glutamate-evoked secretion was less marked and did not persist after being back to control conditions at the end of the experiment.

FIG. 4. *Effects of antagonists of AMPA/kainate (DNQX) and AMPA (SYM 2206) subtypes of glutamate receptors (A), ERs (ICI 182,780), and AHR (α-naphthoflavone) (B) on the increase in glutamate-evoked GnRH secretion caused by E2 or o,p ' -DDT. The GnRH secretory response (mean ± SD) was calculated as the difference between the levels in the fraction collected immediately prior to and during exposure to the glutamate (pg/7.5-min fraction). Those differences were then converted in percentage of control (glutamate alone or glutamate with antagonist). Hypothalamic expiants from 15-day-old female rats were used, $p < 0.05$ versus glutamate alone (a), versus glutamate + antagonist (b), versus glutamate + E2 (c), versus glutamate + o,p'-DDT (d).*

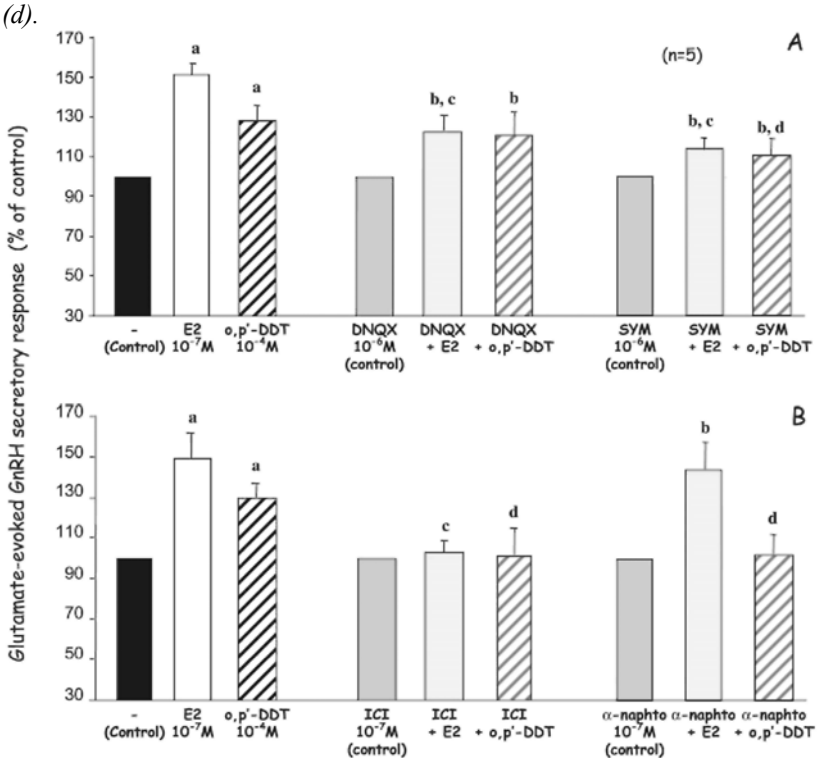

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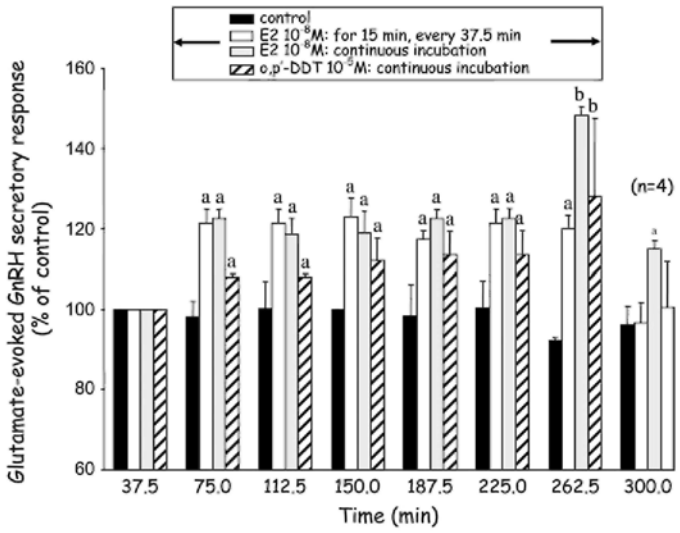
TABLE 1 *Effect of Kinase Inhibitors on the Glutamate-Evoked GnRH Release (% of Controls)*

Inhibitor	Inhibited kinase	Glutamate	+E2 10^{-7}M	+*o,p′*-DDT 10^{-4}M
_	_	100.0	150.4 ± 7.6*	142.0 ± 4.7*
Staurosporine	PKA and PKC	90.6 ± 6.4	95.5 ± 7.6	96.8 ± 4.4
Chelerythrine chloride	PKC	93.8 ± 6.4	90.6 ± 6.4	93.7 ± 6.5
PD98059	MAPKs (ERK1/2)	101.6 ± 7.9	100.0 ± 9.1	96.8 ± 4.4

Note. ERK1/2, extracellular signal-regulated kinase; $n = 5$; *$p < 0.05$ versus glutamate.

FIG. 5. *Secretory response of GnRH to 10^{-2}M of glutamate used for 7.5 min every 37.5 min for 5 h. Starting after 60 min and ending after 262.5 min, the expiants are incubated intermittently (for 15 min, every 37.5 min) or continuously with E2 or o,p′-DDT. a: $p < 0.05$ versus initial glutamate secretory response; b: $p < 0.05$ versus previous E2-potentiated or o,p′-DDT-potentiated glutamate secretory response.*



DISCUSSION

In the present study, we provide evidence that several EDCs can directly stimulate the GnRH secretion evoked by glutamate. Such effects occur rapidly, within 15 min and further increase after several hours of exposure. Emphasis was put on *o,p′*-DDT since we showed recently that early exposure to this chemical resulted in acceleration of pulsatile GnRH secretion and sexual precocity (Rasier *et al.,* 2007). The concentrations used *in vitro* for our study were shown to be consistent with serum levels achieved after *in vivo* administration. We found that the mechanism of *o,p′*-DDT effects involves several receptors (ERs, AHR, and AMPA) and intracellular kinases (A, C, and MAPK).

When compared with other *in vitro* paradigms involving GnRH neurons, the static incubation of hypothalamic expiants maintains GnRH neurons in their original surrounding neurono-glial environment and such expiants retain the capacity of pulsatile secretion of GnRH (Matagne *et al.,* 2004; Rasier *et al.,* 2007). Though these two characteristics may account for some functional relevance of our observations *in vitro*, the required concentrations of some reagents such as glutamate are higher than using cell cultures (Donoso *et al.,* 1990; Kuehl-Kovarik *et al.,* 2002; Matagne *et al.,* 2005; Ojeda and Urbanski, 1994; Rubin *et al.,* 2006). This discrepancy which is still unexplained (reagent diffusion, degradation,...) raises the issue of possible irrelevance of the *in vitro* data as well as concerns regarding possible excitotoxic effects mediated via the N-methyl-D-aspartic acid (NMDA) receptor subtype. However, the capacity of the expiants to respond to repeated glutamate stimulation is sustained for several hours, suggesting the functional integrity of the neurono-glial apparatus involved in the secretory process. The concentrations of E2 and *o,p′*-DDT used *in vitro* for the mechanistic studies are consistent with those used by others (Clark *et al.,* 1998; Diel *et al.,* 2002; Urbanski *et al.,* 1996). They have been selected after a concentration-response study on amplification of the glutamate-evoked secretion of GnRH. For each EDC, the maximal effective concentration has not been determined but a concentration-response effect can be observed. Similar to E2, the two DDT isomers increased rapidly the GnRH secretion evoked by glutamate in a concentration-dependent manner. The E2:EDC ratios of biocapacity observed with DDT isomers and BPA were consistent with other *in vitro* studies (Clark *et al.,* 1998; Desaulniers *et al.,* 2005; Rasier *et al.,* 2006). The other tested EDCs did not show any effect at the studied concentrations. The lack of

effect of DDE can be explained by its prominent anti-androgenic nature. In fact, DDE is considered to be much less estrogenic than DDT. The absence of effect of MXC could be explained by a recent study showing that its estrogenic effects might be mediated by its metabolites, mono-OH MXC and bis-OH MXC (1,1,1 -trichloro-2,2-bis(4-hydroxyphenyl)ethane), after cytochrome P450 metabolization (Miller *et al.,* 2006). It is important that the serum *o,p′*-DDT concentrations measured 5 days after a treatment *in vivo* with the EDC are consistent with the effective concentrations *in vitro*. Using a 10 times higher dose of *p,p′*-DDT *in vivo* given orally for 7 days, others found a plateau plasma concentration of 7.20 µg/l, that is about 20 times higher than the level we have observed 5 days after termination of treatment in our conditions (Mussi *et al.,* 2005). No DDT derivatives, in particular *o,p′*-DDE, could be detected in our conditions after *o,p′*-DDT treatment, suggesting that, in the rat, few transformation of DDT into DDE had occurred. Tomiyama *et al.* (2003), however, found plasma DDE to attain concentrations 10 times lower to those of DDT as soon as 2 days after starting exposure to DDT. Some explanation for this discrepancy could relate to differences in degradation rate depending on the nature of DDT isomer, the treatment dose, and the age at administration since they administered *p,p′*-DDT between PND 36 and 42 while we gave 10 times less of *o,p′*-DDT on PND 6-10.

In previous studies from our laboratory, the effects of E2 on pulsatile GnRH secretion occurring within hours (Matagne *et al.,* 2004) were found to parallel the effects on glutamate- evoked GnRH release occurring within minutes (Matagne *et al.,* 2005). Therefore, after showing that *o,p′*-DDT stimulated frequency of pulsatile GnRH secretion (Rasier *et al.,* 2007), we followed a similar approach toward a mechanistic study based on glutamate-evoked secretion. Along the same line, 15-day-old female rats were used since GnRH secretion *in vitro* was maximally affected by E2 at that age (Matagne *et al.,* 2004, 2005). While BPA was found to be also capable of stimulating the glutamate-evoked GnRH secretion, we put emphasis on DDT because of the possible involvement of the insecticide in sexual precocity of migrating children (Krstevska-Konstantinova *et al.,* 2001; Parent *et al.,* 2003) and the experimental evidence of hypothalamic-pituitary effects in the rat (Gellert *et al.,* 1972; Heinrichs *et al.,* 1971). Overall, the effects of DDT appear to be comparable to those of E2, and ERs play a key role in mediating such effects. The two main mechanisms involved in endocrine disruption are agonistic effects at ERs or estrogenic as shown for DDT, and antagonistic effects at androgen receptors or anti-androgenic as shown for DDE (Clark *et al.,* 1998; Kelce *et al.,* 1995). In our experimental conditions, it was shown previously that both estrogens and androgens were capable of potentiating the GnRH secretion, the effects of androgens being aromatase dependent and ultimately mediated through estrogens (Matagne *et al.,* 2004). Thus, the effects reported here fall within the estrogenic category.

The AMPA receptors are an additional class of receptors involved in DDT effects. The prominent role of NMDA and kainate receptors in glutamate-mediated pulsatile GnRH secretion and the involvement of both NMDA and kainate receptors in the glutamate-evoked release of GnRH have been demonstrated in earlier studies (Bourguignon *et al.,* 1989b; Matagne *et al.,* 2004, 2005; Parent *et al.,* 2005). By contrast, a selective AMPA antagonist does not affect, neither pulsatile GnRH secretion (Rasier *et al.,* 2007) nor the glutamate-evoked release as shown here, indicating no involvement of the AMPA receptor subtype in such conditions. These receptors, however, appear to play some role in the effects of E2 and *o,p′*-DDT on both the pulsatile secretion of GnRH (Rasier *et al.,* 2007) and its release evoked by glutamate. Since those two effects occur, respectively, after 1-2 h and 7.5-15 min, the former were thought to possibly involve genomic mechanisms and the latter nongenomic. In consistency with a time-dependent involvement of two mechanisms, E2 and *o,p′*-DDT cause rapidly an increase in glutamate-evoked GnRH release and a further increase occurring after few hours as a slow effect. Not only time but also continuity of the exposure to the steroid is critical since the slow component of the increased responsiveness to glutamate does not appear when exposure is discontinuous. In contrast, we had no evidence of priming of the response caused by the repeated glutamate stimulation. Herbison (1998), and Herbison and Pape (2001) have reported that E2 exerts complex effects on GnRH neuronal function including long-term or genomic effects through binding to ERα- and/or β-subtypes. Furthermore, *in vitro* rapid nongenomic effects of E2 can occur within seconds to minutes in various conditions to influence cellular events such as kainate-induced currents in hippocampal neurons (Improta-Brears *et al.,* 1999) and second messenger cascades in hippocampal (Gu *et al.,* 1999) or hypothalamic neurons (Abraham *et al.,* 2004; Lagrange *et al.,* 1999). Regarding a possible genomic effect, E2 has been shown to stimulate AMPA glutamate receptor expression in the rat hypothalamus (Diano *et al.,* 1997). This could be one of the mechanisms explaining AMPA involvement in E2 or EDC effects on GnRH secretion. As far as we know, this is the first report showing the involvement of the AMPA subtype of glutamate receptors in rapid EDC effects. A few recent studies showed that AMPA receptors mediate plastic effects induced by E2 in different populations of neurons (Todd *et al.,* 2007; Tsurugizawa *et al.,* 2005). Particularly, AMPA receptors have been shown to be involved in the neuronal morphological changes induced by E2 in the ventromedial nucleus of the hypothalamus in female rats, specifically (Todd *et al.,* 2007). Both E2 and *o,p′*-DDT potentiating effects on the glutamate-evoked GnRH release could be prevented by PKA and C and MAPK inhibitors. These observations confirm our earlier report for E2 and provide further evidence of a rapid intracellular component of EDC effects.

Our data also indicate the possible role of AHR in mediating *o,p′*-DDT effects on GnRH secretion. As reviewed recently, the AHR is an ubiquitous receptor system binding endogenous ligand as well as xenobiotics such as dioxins (Harper *et al.,* 2006). In this study, it appears that *o,p′*-DDT could be a ligand of AHR since its effects on GnRH release are antagonized by α-naphthoflavone. Only the glutamate-evoked GnRH secretion amplified by *o,p′*-DDT is prevented by α-naphthoflavone, suggesting that DDT can stimulate GnRH secretion through a mechanism partially different from that of E2. Beyond the presumably initial step in *o,p′*-DDT effects, the pathway seems to be similar to E2 with involvement of the AMPA receptors in rapid effects and also ERs in rapid and slow effects (Rasier *et al.,* 2007). To integrate these observations, it is conceivable that the *o,p′*-DDT-AHR complex results in a cross talk with the E2 signaling machinery and forms a functional transcriptional complex in the regulatory region of ERα-responsive genes (Ohtake *et al.,* 2003).

Recently, kisspeptin (kiss-1), a tumor suppressing peptide, and its receptor GPR54 have been identified as major actors of the regulation of GnRH secretion (de Roux *et al.,* 2003; Seminara and Kaiser, 2005). Kiss-1 and GPR54 are expressed in the medial basal hypothalamus, and kiss-1 has a direct action on GnRH neurons which express GPR54. Recent studies have shown that kisspeptin neurons in the anteroventral periventricular nucleus express α-subtype of ERs and that E2 might exert a positive feedback on GnRH secretion during LH surge by stimulating kiss-1 expression (Adachi *et al.,* 2007). Knowing that crucial role of kiss-1 in regulation of GnRH secretion and its modulation by E2, we could hypothesize that kiss-1 could be a potential target for estrogenic EDCs.

In summary, this study shows that EDCs, in particular *o,p′*-DDT, can modulate the GnRH secretion *in vitro* in the immature female hypothalamus through both rapid and slow effects with the involvement of estrogen, dioxin, and AMPA receptor pathways.

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