α-Difluoromethylornithine Modifies Gonadotropin-Releasing Hormone Release and Follicle-Stimulating Hormone Secretion in the Immature Female Rat (44128)

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Abstract. **Polyamines play an essential role in tissue growth and differentiation, in body weight increment, in brain organization, and in the molecular mechanisms of hormonal action, intracellular signaling, and cell-to-cell communication. In a previous study, inhibition of their synthesis by α-difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ornithine decarboxylase, during development in female rats, was followed by prolonged high follicle-stimulating hormone (FSH) serum level and a delayed puberty onset. Those changes were relatively independent of body mass and did not impair posterior fertility. The present work studies the mechanisms and site of action of polyamine participation in FSH secretion during development. DFMO was injected in female rats between Days 1 and 9 on alternate days. At 10 days of age, hypothalami from control and DFMO rats were perifused *in vitro*, and basal and potassium-induced gonadotropin-releasing hormone (GnRH) release were measured. The response to membrane depolarization was altered in DFMO hypothalami. Increased GnRH release in response to a low K^+ concentration was evidenced. Adenohypophyses of the same treated prepubertal rats were perifused *in vitro* and the response to GnRH pulses was checked. In DFMO-treated rats, higher FSH release was observed, with no changes in LH or PRL secretion. Finally, pituitary GnRH receptor number in adenohypophyseal membranes from treated and control groups was quantified. A significant reduction in specific binding was evident in hypophyses from DFMO-treated rats when compared with binding in the control group. In summary, DFMO treatment in a critical developmental period in the female rat impacts the immature GnRH neuronal network and immature gonadotropes. A delay in maturation is evidenced by a higher sensitivity to secretagogs in both pituitary glands and hypothalamic explants. These events could explain the prolonged high FSH serum levels and delayed puberty onset seen in this experimental model.** [P.S.E.B.M. 1997, Vol 215]

Polyamines (putrescine, spermidine, spermine, and agmatine) are a group of ubiquitous aliphatic amines that play an essential role in tissue growth and differentiation, body weight increment, and brain organization (1–3). In addition, polyamines are also involved in the molecular mechanisms of hormonal action, intracellular signaling and cell-to-cell communication (4, 5). Numerous studies have shown that polyamine levels as well as the activity of ornithine decarboxylase, the limiting enzyme in polyamines biosynthesis, are highest during development and decline after the growth process stops (6, 7). Inhibition of polyamine synthesis during development by α-difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ornithine decarboxylase, impairs brain normal development

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(8, 9), and treatment of newborn rats with polyamines induces precocious somatic and behavioral development (2). Polyamines have also have been shown to influence neuroendocrine interactions during the estrous cycle, and fluctuations in ornithine decarboxylase activity and polyamine concentrations have been reported. Besides, polyamines have been related to gonadotropins release in the adult rat (10–13).

In a previous study, we found that DFMO administered during the first 10 days of life to female rats was followed by prolonged high follicle-stimulating hormone (FSH) serum level and delayed puberty onset. Those changes were relatively independent of body mass, and they did not impair posterior fertility (14). Therefore, it was of interest to determine the mechanisms and site of action involved in polyamine participation in FSH secretion during development.

Materials and Methods

Animals. Sprague-Dawley rats from the Instituto de Biologia y Medicina Experimental were housed in an air conditioned room (22°C) with lights on at 07:00 and off at 19:00 hr. They had free access to laboratory chow and tap water. Mothers with 9–10 pups (approximately 6–7 females and 2–3 males) were each kept in individual cages. Day of birth was considered Day 1.

α-Difluoromethylornithine (Eflornithine HCl, a gift of Marion Merrel Dow Pharmaceuticals, Inc.), a specific and irreversible inhibitor of ornithine decarboxylase, was administered to female rats between Days 1 and 9 on alternate days (Days 1, 3, 5, 7, and 9—five doses) as described previously (14); we choose the present scheme because in this group body weight gain is not altered, vaginal opening is delayed, and serum FSH increased.

DFMO was injected so, between 10:00 and 12:30 hr in a dose of 500 mg/kg body wt. Control rats received an equal volume of saline solution (5 μl/g). Experimental design, doses, and times were chosen according to previous experience (14). Pups were allowed to remain with their mothers until 10 days of age; they were then separated 1 hr before perifusion experiments. To assess the effect of DFMO on body weight and puberty onset, some pups were left undisturbed. Those rats were weighed periodically from the first week of life, and starting on Day 30 they were examined daily for vaginal opening. All DFMO-treated rats showed delayed vaginal opening, and no significant differences in body weight gain were observed, as described earlier (14).

Perifusion Experiments. Rats were decapitated 24 hr after the last injection, at 10 days of life. Trunk blood was collected, and serum was separated, frozen, and kept at −20°C for radioimmunoassay (RIA) determinations. Brains were quickly removed on ice and hypothalami and hypophyses were removed as described below.

Channels that did not exhibit increased hormone release as a result of KCl depolarization were omitted (15, 16).

Hypothalami. Hypothalami, including mediobasal hypothalamus and the suprachiasmatic-preoptic area (MBH), were dissected out as previously described (17), cut in sagittal halves, and transferred into incubation chamber (15, 16). Four MBH were placed into each of the four chambers (two control and two DFMO treated), which were submerged in a temperature-controlled (37°C) waterbath (total number of rats per group:8, belonging to 2–3 mothers). Each experiment was repeated at least three times.

Chambers were perifused with Medium 199 (Sigma-Chemical Co., St. Louis, MO), 25 m*M* HEPES (Sigma), 0.1% bovine seroalbumin (Sigma), pH 7.2, at a rate of 0.05 ml/min by means of a multichannel perifusion Manostat cassette pump. After a 60-min preincubation period, fractions were collected at 10-min intervals for 40 min into tubes containing 10 ml of bacitracin (final concentration in each fraction collected: 200 m*M*). A three-way valve was then switched to infuse a 5-min pulse of 130 m*M* KCl, followed 105 min later by a second pulse of 663 m*M* KCl (which corresponded to a final concentration in the chamber of 11 m*M* and 56 m*M* at the end of each pulse, respectively) to all the chambers, control and DFMO. The variation of drug concentration into the chamber was determined by the formula described in (16). We tested two increasing doses of KCl in order to evaluate different sensitivity in experimental groups.

The response of each channel was normalized by the baseline release of the channel during the first 40 min, in which the release was relatively stable. Normalized response of channels with DFMO-treated hypothalami was compared to the normalized release from control perifusions. Samples were frozen until assayed for gonadotropin-releasing hormone (GnRH).

Hypophysis. Pituitaries from the same treated rats were used. Neurohypophyses were discarded, and four adenohypophyses were placed in each chamber. Four chambers were perifused simultaneously as described for hypothalami. They were perifused with medium at a rate of 0.05 ml/min. Time was considered 0 min at the end of a 210-min stabilization period, and fractions were then collected at 5-min intervals until the end of perifusion. At 20 min, GnRH (Peninsula, Belmont, CA) (4×10^{-8} *M*) was infused to all the chambers for 10 min; concentration rose from zero when the valves were switched on, to 1.2×10^{-8} *M* when valves were switched off. The GnRH pulse was repeated at a higher concentration (4×10^{-7} M) after a lag period of 35 min. Finally, all chambers received a 10-min pulse of 100 m*M* KCl (Ci = 333 m*M*) to test the viability of the tissue. The response of each channel was normalized by the baseline release of the channel during a 20-min control period between 5 and 25 min. Normalized response of channels with DFMO-treated hypophysis was compared with the normalized release from control perifusions. Samples were frozen until assayed for hormone levels.

GnRH Receptor Assay.

Preparation of [^{125}] iodine GnRH analog. [D-Ser (-tBu)^6-des-Gly^{10}]-GnRH-*N*-ethylamide (buserelin) (a gift

from Hoechst, Buenos Aires) was used as tracer and unlabeled hormone in the binding assay (17). Buserelin was iodinated using a chloramine-T method. Briefly, 2 μg of GnRH-a were iodinated in the presence of 2 mCi [^{125}]iodine (New England, MA) and 0.2 μg chloramine-T. Reaction proceeded for 2 min. The procedure was repeated once, and reaction was stopped by transferring to a carboxy-methyl cellulose column and eluting unbound iodine with 0.002 *M* ammonium acetate, and the labeled analog with 0.060 *M* ammonium acetate (18). The iodinated analog was stored at 4°C in this last buffer and used within 3 weeks of preparation. The specific activity of each preparation was assessed by self-displacement in the receptor assay using a crude membrane fraction prepared from pooled pituitaries. Specific activities ranged from 400 to 800 μCi/μg. Maximum binding of the trace, determined by incubation with excess pituitary membranes, was 40%–65%.

Assay of pituitary GnRH receptors. Rats were sacrificed by decapitation, and the pituitaries were quickly removed and stored at −70°C. Pituitaries from 10-day-old DFMO or control rats were thawed and homogenized in Tris-HCL 10 m*M*, pH 7.6, at 4°C in a glass homogenizer (19). Homogenates were prepared immediately before addition to assay tubes. The homogenate from two pituitaries was used to prepare five incubation mixtures (three for total binding and two for nonspecific binding). Each tube had approximately 0.15 mg of tissue in 300 μl of buffer. Homogenates were incubated with 5–8 × 10^4 cpm, ^{125}I buserelin in a total volume of 500 μl assay buffer (Tris-HCL, 10 m*M*, 0.1% bovine seroalbumin (Sigma), and 1 m*M* dithiothreitol (Sigma), pH 7.6. Ligand concentrations were near saturating, representing about 85% receptor occupancy. Nonspecific binding was determined by addition of 10^{-7} *M* of unlabeled buserelin, and represented 5%–8% of total iodinated tracer.

Incubations were performed for 120 min on ice. Free and bound Buserelin were separated by centrifugation at 13,000 rpm in a microfuge for 20 min at 4°C, the supernatant was discarded, and the radioactivity in the pellets was counted in a γ-spectrometer (efficiency, 73%).

RIA Assays. Serum or perifused samples were assayed in duplicate for prolactin (PRL), luteinizing hormone (LH), and FSH concentrations with radioimmunoassay kits supplied by the NIDDK. Results were expressed in terms of PRL RP2, LH RP2, and FSH RP2, respectively.

GnRH concentration was determined in effluent samples of the perifusion experiments, by RIA. Anti-GnRH was provided by Dr. William Ellinwood (Oregon Health Sciences, University Portland, Oregon) and used as reported (20).

Intra- and interassay coefficients of variation were always less than 10% and 13%, respectively, for the four hormones.

Statistical Analysis. In perifusion studies, areas under the curve were analyzed by two-way analysis of variance (ANOVA) for the effects of animal group and drug treatment as described (21), followed by a Scheffé's test for individual comparisons between means when *F* of interaction was significant, or between groups of means if interaction was found not to be significant. For pituitary receptors, Student's *t* test was used. Level of significance was $P < 0.05$.

Results

Serum FSH in Donor Animals. As expected, serum FSH levels in DFMO-pretreated rats were higher than in control animals at 10 days of life (ng/ml: DFMO, 31.72 ± 3.02; controls, 15.97 ± 1.86; $P < 0.001$).

Basal and K^+-induced GnRH Release from Perifused Hypothalami of Control and DFMO-treated Rat. Basal release of GnRH was similar in both groups (pg/ml/hypothalamus:control, 12.83 ± 1.24; DFMO, 11.91 ± 1.10). When the GnRH response was analyzed by two-way analysis of variance, a significant *F* of interaction was detected. When the first pulse of KCl (11 m*M*) was infused, an increased release of GnRH was seen (Fig. 1). The peak was significantly higher in channels with hypothalamus from DFMO-treated rats than in that from control ones ($P < 0.05$). The second pulse of KCl (56 m*M*) produced a second release of GnRH, but in this case the peak of the control channels was significantly higher than in the experimental channels as probable the releasable pool of GnRH had been already secreted with the first low-dose pulse in this group and not in control rats. In the control channels the response was concentration dependent on K^+ ion ($P < 0.05$), but in the experimental channels a significant decrease was seen between the first and second peak.

Basal and GnRH-induced Release of FSH, LH, and Prolactin from Perifused Adenohypophyses of Control and DFMO-treated Rats. Perifusates from hypophysis from both DFMO and control-treated rats showed similar basal release of FSH, LH, and PRL (Table I). Both pulses of GnRH (1.2 × 10^{-8} *M* and 1.2 × 10^{-7} *M*) stimulated FSH and LH, as well as PRL, release (Fig. 2).

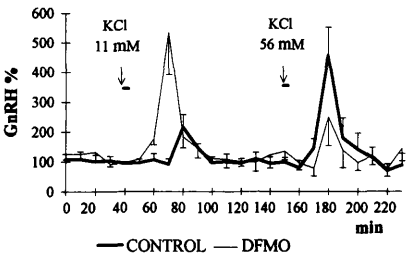

Figure 1. GnRH release from perifused hypothalami of 10-day-old female control or DFMO-treated rats, expressed as percentage of GnRH release, normalized to basal levels. The response to KCl at both doses was significantly different between groups, and the control channels released GnRH in a concentration-dependent manner ($P < 0.05$, two-way ANOVA using the area under the curve for each peak from 50 to 100 min and from 160 to 210 min in three independent experiments, two channels/group). For this and the figures following, the point (or the height of the bar) indicates the mean, and the vertical line 1 SEM. The arrows indicate the beginning of the pulses. The *x* axis corresponds to the time of collection.

Table I. Baseline FSH, LH, and PRL Release from Pituitaries of Control and DFMO-Treated Rats

	Hormone release (ng/ml/hypophysis)		
	FSH	LH	PRL
Control	3.82 ± 0.42	0.86 ± 0.13	1.79 ± 0.14
DFMO	4.13 ± 0.36	0.74 ± 0.09	1.65 ± 0.12

Note. Values are means ± SEM. Baseline release was considered to be from the 20-min control period between 5 and 25 min of perifusion. No significant differences were found.

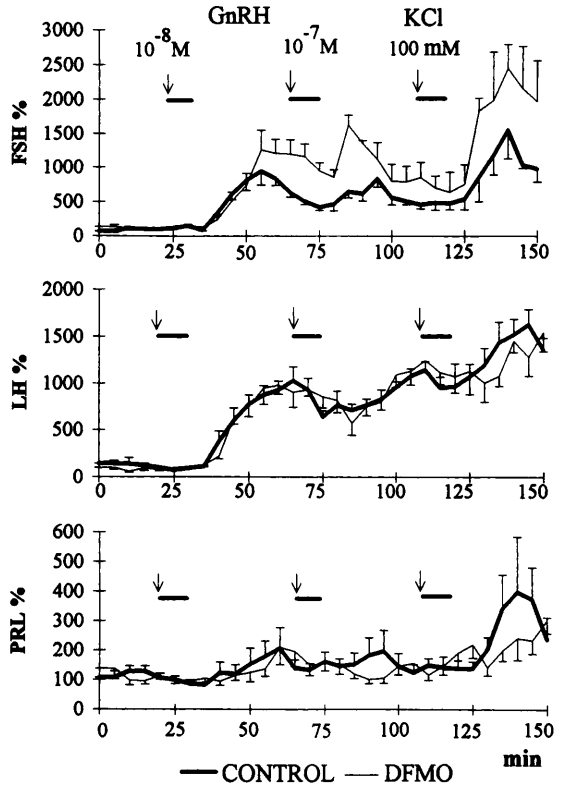

Figure 2. FSH (top), LH (middle), and PRL, (bottom) release in perifused pituitaries of 10-day-old female control or DFMO-treated rats, expressed as a percentage of the release normalized to basal levels. GnRH- and KCl-induced FSH release was significantly higher in DFMO-treated pituitaries ($P < 0.05$, two-way ANOVA using the area under the curve in three independent experiments, two channels/group). No differences were found between groups in either LH or PRL release.

LH and PRL responses to GnRH was similar in both groups, but FSH response (area under the curve from 35 to 100 min) was higher in DFMO hypophyses ($P < 0.05$). When the viability was tested by a depolarizating stimulus of KCl, an important increase in the three hormones was seen, but only a significant difference between groups was found in FSH response from hypophysis from DFMO-treated rats ($P < 0.05$).

GnRH Receptors in Pituitaries of Control and DFMO-treated Rats. Radioiodinated GnRH analog–specific binding to adenohypophyseal membranes from DFMO-treated rats at 10 days of age was significantly lower than binding in control hypophysis (DFMO: 36.30 ± 2.09 fmol/mg tissue; controls: 44.11 ± 1.89 fmol/mg tissue; $P < 0.02$) (Fig. 3).

Discussion

The present study demonstrates that chronic DFMO treatment during the first 10 postnatal days clearly modifies hypothalamic control of FSH secretion in the developing female rat. In a series of *in vitro* experiments, we found that both pituitary glands and hypothalamic explants of animals pretreated with DFMO changed their response to secretagogs. Pituitaries of DFMO-treated rats released more FSH in response to GnRH, and the release of the decapeptide induced by depolarization with K^+ ion was altered in the perifused hypothalami. There was also a marked reduction in ^{125}I-buserelin–specific binding in adenohypophysis from DFMO-treated rats.

The participation of the brain in controlling early events of the development of FSH, LH and PRL secretion, as well as on sexual development in mammals, is well documented (22, 23). FSH secretion is high in immature female rats from Days 10 to 18, and decreases thereafter until adulthood. Those high FSH levels are considered to be important for ovarian maturation and puberty onset (24–26). Temporary elevation of FSH levels in the female is thought to be a relatively steroid-independent event of central origin (27), and several components are involved in such elevation. Furthermore, FSH secretion during development is sexually differentiated, as FSH hormone levels in the male are low in the first postnatal weeks and increase at the time when FSH levels decrease in the female (28, 29). Striking and clear sexual differences have also been described for LH and PRL secretion in the prepubertal rat, and participation of sexual organization of brain structures has been related to the occurrence of such differences (24, 30–33).

The present results indicate that treatment with DFMO during the first 10 days of life impairs GnRH neuron normal development. Although basal release of GnRH was not altered, hypothalami from DFMO-treated rats released more decapeptide than controls after an unspecific depolarization by KCl. It has been described that high FSH levels are due, in part, to sporadic bursts of GnRH associated with immaturity of synaptic connections and that, in the third week of

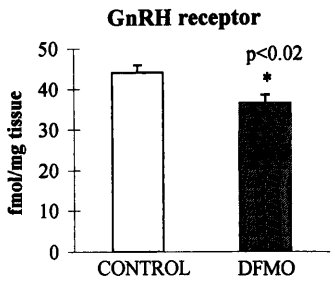

Figure 3. GnRH receptors (fmol/mg tissue) in pituitaries of 10-day-old female control and DFMO-treated rats. (Control: $n = 8$, DFMO: $n = 8$; each sample had two hypophyses of the same group; total number of rats were 16 in each group, belonging to 5–6 different litters).

life, with increasing estradiol titers, those bursts disappear and FSH titers markedly decrease while LH starts to be secreted in a pulsatile and circadian fashion. These results suggest that polyamines play a role in the maturation of the GnRH neuronal network. The high and persistent FSH levels described in the developing female after DFMO treatment could be in part due to the higher releasability of the GnRH neuron associated to the immaturity of the synaptic circuitry which modifies neuronal function. Interestingly enough, quantification of polyamines in hypothalamus and hypophysis by HPLC shows that DFMO treatment significantly decreases putrescine and spermidine (Thyssen *et al.*, unpublished).

Basal release of hormones from intact pituitaries of 10-day-old females was similar in control or DFMO-treated rats, but GnRH-induced FSH release was higher in pituitaries of DFMO-treated animals. High FSH secretory activity in those rats is in good agreement with our previous *in vivo* experiments and may be related to the FSH-releasing activity described for polyamines both *in vivo* and *in vitro* in two independent works (10, 34). Also in agreement with previous work *in vivo,* no differences between groups were observed in LH and PRL release (14). The prolactin-releasing activity of GnRH has been described as related to paracrine interactions in the pituitary and is particularly evident in the immature female rat (21). At this particular age in the female, FSH has a peculiar brain control that seems to be the main target for DFMO action, and FSH seems to be the main hormone involved in DFMO-retarded puberty. In this regard, high infantile levels of FSH have been associated with retarded puberty (35). There was a marked reduction in ^{125}I-buserelin–specific binding in adenohypophysis of DFMO-treated rats. Since secretagogs acting at the hypothalami of those rats release more decapeptide, it could be postulated that the reduction in receptors number at the pituitary is a downregulation effect, which places a limit to the already high FSH release by the gland. On other hand, GnRH receptors increase in females from the fifth day until the end of the third postnatal week (36). Lower values found in DFMO-treated rats could be another consequence of the delay in the maturation process of the hypothalamic hypophyseal unit.

In summary, DFMO treatment in a critical period of the developing female rats impacts the immature GnRH neuronal network and immature gonadotropes. There is a higher sensitivity of the hypothalamus and the pituitary to secretagogs, which can explain the prolonged high FSH serum level and delayed puberty onset found in this experimental model.

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