Effects of Polyamines on the Release of Gonadotropin-Releasing Hormone and Gonadotropins in Developing Female Rats

SANDRA M. THYSSEN,* PABLO F. HOCKL,* ASTRID CHAMSON,*† VICTORIA A.R. LUX-LANTOS,* AND CARLOS LIBERTUN*†,[1]

*Laboratorio de Neuroendocrinología, Instituto de Biología y Medicina Experimental-Consejo Nacional de Investigaciones Científicas y Técnicas, V. Obligado 2490, (1428) Buenos Aires, Argentina; and †Departmento Physiology, University of Buenos Aires Medical School, Argentina

Polyamines, putrescine (PUT), spermidine (SPD), spermine (SPM), and agmatine (AGM), are polycationic amines related to multiple cell functions found in high concentrations during the development of hypothalamus and pituitary. In previous works, we demonstrated that α-difluoromethylornithine (DFMO), an inhibitor of polyamines biosynthesis, induced a delay in puberty of female rats, accompanied by high, sustained follicle-stimulating hormone (FSH) levels during the infantile period. Also, DFMO treatment induced changes in polyamine concentration both in hypothalamus and pituitary of rats, mainly a decrease of PUT and SPD, an increase in SPM, and no change in AGM. In the present work, we investigated the direct effects of polyamines on the secretion of hypothalamic GnRH and pituitary gonadotropins in 6- and 15-day-old female rats. In 6-day-old animals, *in vitro* incubations with PUT, SPD, and AGM of hypothalami or anterior pituitaries were able to inhibit GnRH, FSH, and leutinizing hormone (LH) secretion, respectively. SPM showed a nonspecific transient inhibitory effect on FSH. When challenged with either high K^+ (hypothami) or GnRH (pituitaries), the tissues incubated in the presence of polyamines showed no differences when compared with their controls. No effects of polyamines in 15-day-old rats in either tissue were observed. Pituitary cell cultures of 6-day-old animals incubated with DFMO for 4 days showed a significant increase in FSH, but not in LH. We conclude that high PUT, SPD, and AGM levels during the first 10 days of life are important for the development of the hypothalamic-hypophyseal unit, probably related to an inhibitory effect on GnRH and gonadotropins. Therefore, polyamine participation, especially PUT and SPD, is of importance in the regulation of GnRH and gonadotropin secretion in the neonatal and infantile periods, critical stages in the establishment of sexual differentiation.

[Exp Biol Med Vol. 227(4):276–281, 2002]

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT; no. BID 802 OC AR PICT 0043), Universidad de Buenos Aires, and Ministerio de Salud de la Nacion, Argentina.

[1] To whom requests for reprints should be addressed at Laboratorio de Neuroendocrinología, Instituto de Biología y Medicina Experimental, V. Obligado 2490. (1428) Buenos Aires, Argentina. E-mail: libertun@dna.uba.ar

Received July 16, 2001.
Accepted December 3, 2001.

1535-3702/02/2274-0276$15.00

Key words: hypothalamus; adenohypophysis; polyamines; GnRH; gonadotropins

Polyamines are a group of aliphatic amines with multiple physiological roles in tissue growth and differentiation, body weight increment, brain organization, molecular mechanisms of hormonal action, intracellular signaling, and paracrine communication (1–3). The most classical components of this group are putrescine (PUT), spermidine (SPD), and spermine (SPM); recently, agmatine (AGM) was also described in brains of mammals (4, 5).

In the rat, several studies have shown that polyamine levels, as well as the activity of ornithine decarboxylase, the rate limiting enzyme for polyamine biosynthesis, were highest during development and declined after the growth process stopped; furthermore, inhibition of their synthesis during development by α-difluoromethylornithine (DFMO) impaired normal brain development (6, 7). In contrast, treatment of newborn rats with polyamines induced precocious somatic and behavioral development (8). In adults, polyamines have also been shown to influence neuroendocrine interactions during the estrous cycle, and fluctuations in ornithine decarboxylase activity and polyamine concentrations have been reported (9). In addition, in a pioneer work, White *et al.* (10) showed that polyamines were related to gonadotropin release and this was later confirmed by other authors (11, 12). Polyamines, specifically putrescine, were also related to γ-aminobutyric acid (GABA) synthesis, a neurotransmitter critically involved in the regulation of gonadotropin secretion (13). In previous studies, we described that DFMO administered during the first 10 days of life in female rats, but not at later ages, was followed by prolonged high follicle-stimulating hormone (FSH) serum levels and a delayed puberty onset (14). Those changes were relatively independent of body mass and they did not impair posterior fertility. DFMO treatment in a critical developmental period in the female rat impacted on the immature

GnRH neuronal network and immature gonadotropes. A delay in maturation was evidenced by a higher sensitivity to secretagoges in both pituitary glands and hypothalamic explants (14, 15). More recently, we identified and measured the four polyamines mentioned above in relevant neuroendocrine areas, i.e., hypothalamus and pituitary, of the developing rat, comparatively in males and females (Thyssen SM, Libertun C, unpublished data). Briefly, each polyamine showed its own pattern during development in the rat. Some sex differences were observed where males always exhibited higher values; PUT, SPD, and SPM titers were higher in the pituitary, Whereas AGM was higher in the hypothalamus. In addition, in the above-mentioned work, we demonstrated that *in vivo* treatment with DFMO effectively diminishes PUT and SPD production in both hypothalamic and pituitary tissue.

In the present work, we determined the direct *in vitro* effects of each polyamine, PUT, SPD, SPM, or AGM, on hypothalamic GnRH secretion and pituitary gonadotropin secretion in developing female rats. Also, the *in vitro* effect of DFMO on cell cultures of female pituitaries of 6- and 15-day-old animals was investigated.

Materials and Methods

Animals. Pregnant Sprague Dawley rats were housed in an air-conditioned room with lights on at 0700 hr and off at 1900 hr. They were given free access to laboratory chow and tap water. Mothers with eight pups each were kept in individual cages. Day of birth was considered as Day 1. Female pups of 6 and 15 days of age were separated from their mothers; they were quickly decapitated between 0900 and 1000 hr to avoid stress and to prevent variations due to the circadian pattern, and the brains were quickly removed. Hypothalami, including mediobasal hypothalamus and the suprachiasmatic-preoptic area, were dissected as described (16); pars distalis of the adenohypophyses (neurohypophyses were discarded under dissection microscopy) from the same rats were used.

Incubations. Tissues were immediately transferred to vials containing 2 ml of Medium 199 (Sigma, St. Louis, MO) and 0.1% bovine serum albumin (BSA; Sigma), pH 7.2, and were incubated in a Dubnoff metabolic shaker at 37°C, 60 cycles/min, under 95% O_2, 5% CO_2 atmosphere. Hypothalami or adenohypophyses were divided longitudinally into halves and were randomized among vials. Two hemi hypothalami were used in 6-day-old rats and one hemi hypothalamus was used in 15-day-old animals to attain similar tissue weight. Four hemi pituitaries were used in 6-day-old rats and two hemi pituitaries were used in 15-day-old animals.

After a 1-hr preincubation, the medium was discarded and the tissue was washed twice with the same medium. Finally, 2 ml of fresh medium (Control) or medium containing 1.10^{-5} *M* of either PUT, SPD, SPM, or AGM was added to each vial followed by a 150-min incubation. Samples were taken at 30 and 120 min, and the same volume of medium was replaced after each sampling; after the second sample, the medium was supplemented with GnRH (1.10^{-8} *M* final concentration) for pituitaries, or KCl (11 m*M* final concentration) for the hypothalami. The GnRH concentration used was selected from previous works from our laboratory (17) and those of others (18), where it was demonstrated that lower concentrations do not induce significant effects on gonadotropin release in this experimental model.

Thirty minutes later, i.e., after the 150-min incubation, a third sample was taken. All samples were stored at –20°C for radioimmunoassay (RIA) determinations. Experiments were repeated four times for pituitaries and five times for hypothalami.

Pituitary cell dispersion and culture were done as previously described (19). Briefly, pituitary fragments of 6- or 15-day-old female rats were digested with trypsine and cells were resuspended in a medium that consisted of Dulbecco's modified Eagle's medium supplemented with horse and fetal calf sera. Cells were plated in tissue culture plates, 50,000 cell/well, and were incubated at 37°C with 95% air and 5% CO_2 . Cells were incubated for 4 days in the presence or absence (controls) of 5 m*M* of DFMO. Thereafter, cells were washed and incubated in serum-free medium M199 alone, or with GnRH (1.10^{-11}, 1.10^{-9}, or 1.10^{-7} *M*). After 1 hr, medium was taken and frozen at –20°C for hormone determinations. Experiments were repeated three to four times, and each group consisted of four to eight wells.

DFMO studies were conducted in dispersed pituitary cells because preincubations lasted for 4 days before stimuli were added, and pituitary halves cannot be kept *in vitro* for so long. The other experiments were done in pituitary halves to maintain the gland architecture, keeping cell connections in place.

RIA. GnRH concentration in media was determined in duplicate using the Anti-GnRH antiserum (EL 14, final dilution 1:500,000) kindly provided by Dr. William Ellinwood (Oregon Health Sciences, Portland, OR). Intra- and interassay coefficients of variation were 9.3% and 14.3%, respectively. Two hundred-microliter samples were tested, and the detectability limit was 8 pg.

Samples (100 μl of culture medium) were measured in duplicate for leutinizing hormone (LH) and FSH with kits kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases. Results were expressed in terms of LH-RP2 and FSH-RP2 standards. Intra- and interassay coefficients of variation were 7.2% and 11.4% and 8.0% and 13.2% for LH and FSH, respectively. The detectability limit was 0.15 ng for LH and 1.2 ng for FSH.

Statistical Analysis. Results were expressed as mean percentage of variation ± SE from three to five experiments taking control values as a 100%. The mean GnRH

values of 6-day-old control hypothalami were 622 ± 118 pg/mg DNA after 30-min incubations, and 927 ± 226 pg/mg DNA after 120-min incubations. The mean GnRH values of 15-day-old control hypothalami were 310 ± 46 pg/mg DNA after 30-min incubations, and 747 ± 219 pg/mg DNA after 120-min incubations. The mean FSH values of 6-day-old pituitaries were 50 ± 8 ng/mg DNA after 30-min incubations, and 79 ± 10 ng/mg DNA after 120-min incubations. The mean FSH values of 15-day-old pituitaries were 24 ± 13 ng/mg DNA after 30-min incubations, and 85 ± 26 ng/mg DNA after 120-min incubations. The mean LH values of 6-day-old pituitaries were 8 ± 4 ng/mg DNA after 30-min incubations, and 66 ± 25 ng/mg DNA after 120-min incubations. The mean LH values of 15-day-old pituitaries were 4 ± 2 ng/mg DNA after 30-min incubations, and 27 ± 14 ng/mg DNA after 120-min incubations.

To compare hormone effects for each polyamine against its control, one-way analysis of variance (ANOVA) was performed on transformed data (x′ = arcsin of the square root(x)/100), as data are expressed in percentages (20). The analysis was followed by Dunnet test. $P < 0.05$ was considered significant.

Results

***In Vitro* Effect of Polyamines on Basal and GnRH-Stimulated FSH Secretion.** PUT, SPD, SPM, and AGM decreased pituitary FSH secretion in 6-day-old rats after a 30-min incubation (Fig. 1). After 120 min, the inhibitory effect was still present, although it did not reach statistical significance for SPM. In contrast, no effect was observed in 15-day-old animals.

When pituitaries were stimulated with GnRH (1.10^{-8} *M*) in the presence of polyamines, no differences were detected at either age with regard to controls (data not shown).

***In Vitro* Effect of Polyamines on Basal and GnRH-Stimulated LH Secretion.** PUT, SPD, and AGM, but not SPM, decreased pituitary LH secretion in 6-day-old rats, after 30- and 120-min incubations (Fig. 2). No effects were observed in 15-day-old rats. LH response to GnRH was not altered in polyamine-treated pituitaries (data not shown).

***In Vitro* Effect of Polyamines on Basal and KCl-Stimulated GnRH Release.** PUT, SPD, and AGM, but not SPM, decreased hypothalamic GnRH release in 6-day-old rats after 30 min (Fig. 3). Under unspecific stimulation (11 m*M* KCl), no differences were observed with regard to controls (data not shown).

Effect of DFMO on Basal and GnRH-Stimulated FSH and LH Release in Pituitary Cell Cultures. In basal conditions, cells incubated for 4 days with 5 m*M* DFMO released more FSH, but not LH, than controls (Fig. 4). This effect was only observed in 6-day-old animals. Under specific GnRH stimulation, the response was similar in DFMO- and medium-treated pituitary cells. In all cases, a good correlation between GnRH concentration-hormone release was observed; interestingly enough, in the DFMO group, the decapeptide at 1.10^{-11} *M* did not further increase the already high basal FSH levels.

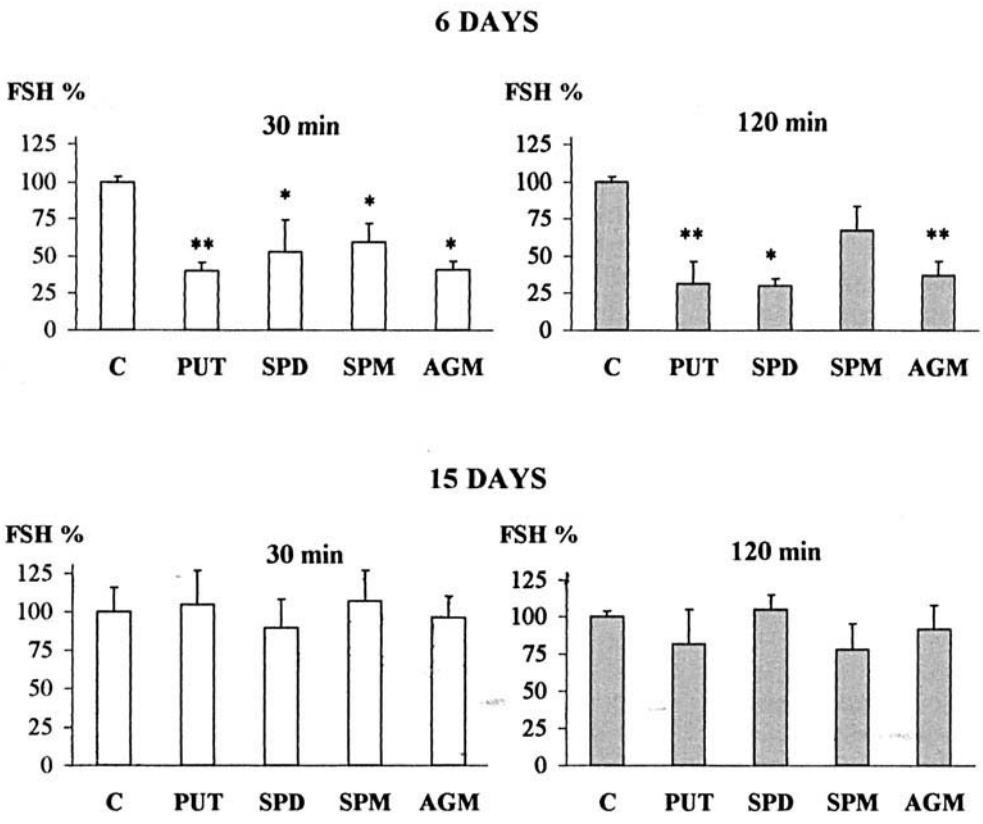

Figure 1. *In vitro* effect of polyamines (1.10^{-5} M) on basal FSH secretion in 6- and 15-day-old female rat anterior pituitaries after 30 and 120 min of incubation. For this and the following figures: PUT, putescine; SPD, spermidine; SPM, spermine; AGN, agmatine. Control values were taken as 100%. $^{*}P < 0.05$, $^{**}P < 0.01$.

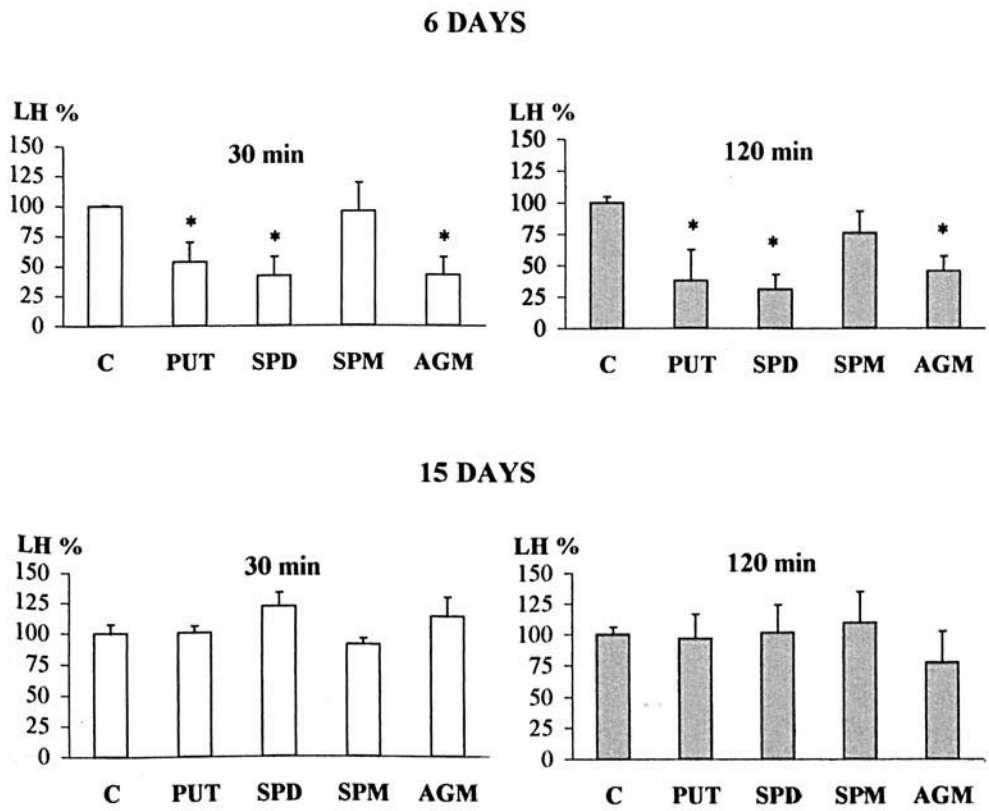

Figure 2. *In vitro* effect of polyamines on basal LH secretion in 6- and 15 day-old female rat anterior pituitaries after 30 and 120 min of incubation.

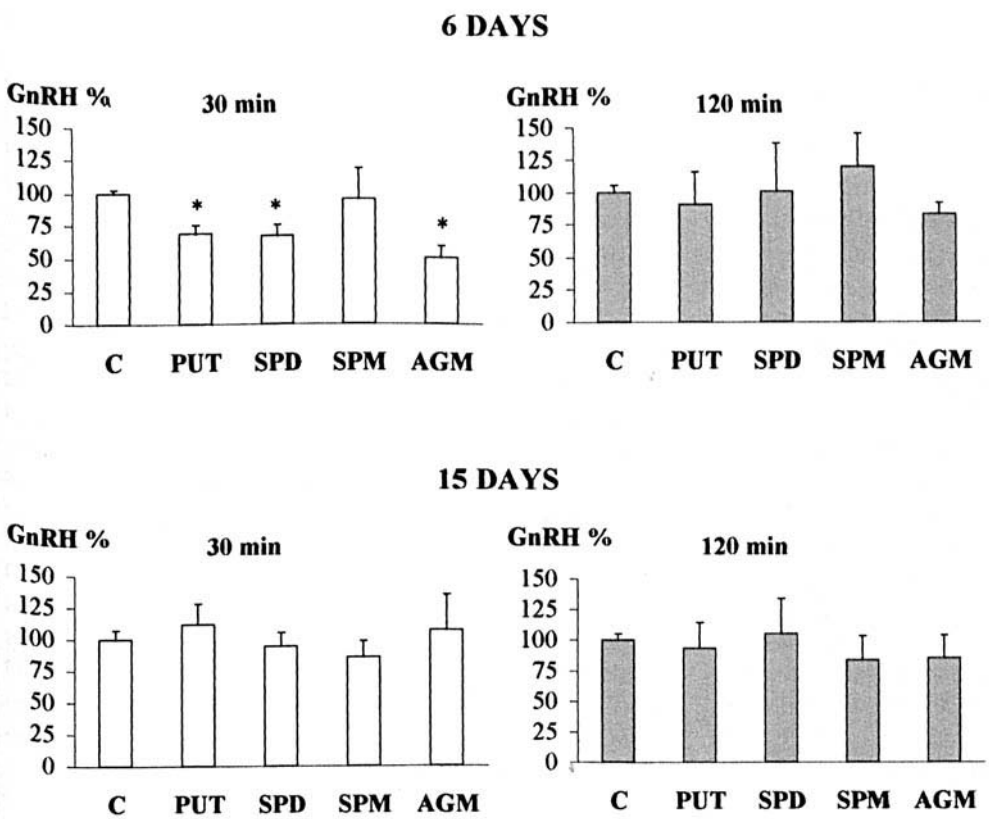

Figure 3. *In vitro* effect of polyamines on basal GnRH release in 6- and 15 day-old female rat hypothalami after 30 and 120 min incubation.

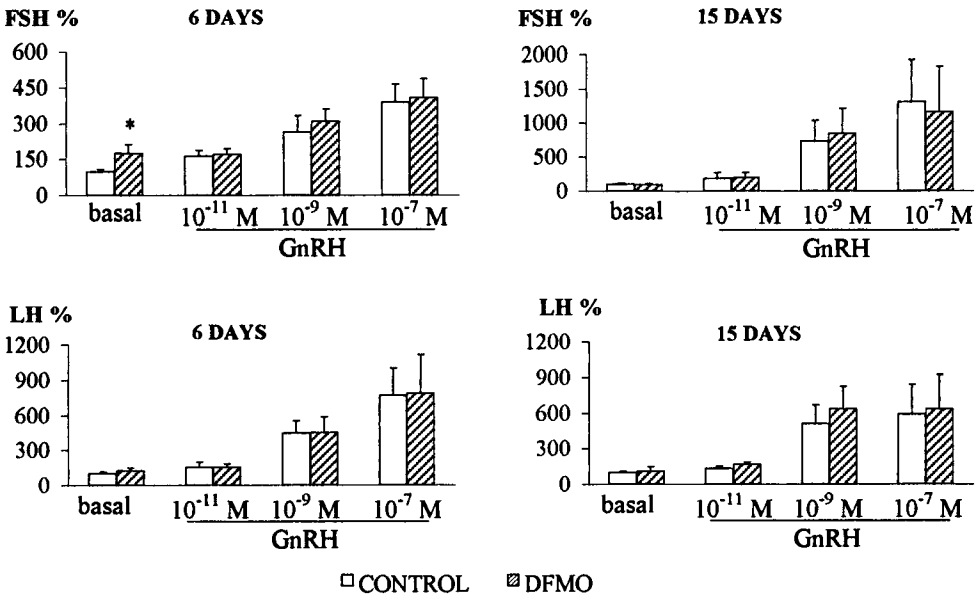

Figure 4. *In vitro* effect of 5 m*M* DFMO on basal and GnRH stimulated FSH and LH secretion in anterior pituitary cell cultures from 6- and 15-day-old female rats.

Discussion

These results show that some polyamines such as PUT, SPD, and AGM suppressed hypothalamic GnRH release and hypophyseal FSH and LH secretion by acting directly on the respective tissues in 6-day-old female rats, but not in 15-day-old animals. The only effect found for SPM, the other polyamine tested, was that it inhibited FSH secretion at 30 min of incubation in the younger group, and this effect was transient, as it was no longer observed after 120 min, even though SPM degradation is slower than that of the other polyamines (21). The lack of specificity of the different polyamines could be due to the high degree of interconversion that occurs among them (2). In addition, DFMO acting directly on pituitary cells in culture increased FSH selectively only in 6-day-old animals.

Taking into consideration the present and previous results in female developing rats, there is a critical period during the first 10 postnatal days in which polyamines play an important role in GnRH and gonadotropin release. The present results show a direct effect of some polyamines on hypothalamic and adenohypophyseal tissues. They are in agreement with previous results using DFMO *in vivo*: the lower concentration of PUT and SPD achieved, under DFMO treatment (Thyssen SM, Libertun C, unpublished data), was followed by an increase of FSH secretion and a delayed puberty onset (14, 15). Therefore, from our *in vitro* and *in vivo* results, a relationship between polyamine levels and the output of the GnRH-gonadotropin system is demonstrated in early stages of development.

These are the first evidences that some polyamines act tonically, suppressing the basal release of the hypothalamic decapeptide and FSH and LH from the pituitary. No effects were observed under KCl or GnRH-stimulated release.

The inhibition on LH release induced by PUT, SPD, and AGM *in vitro* was in good correlation with the *in vivo* treatment with DFMO injected on alternate days between Days 1 and 9, which resulted in high LH titers at 20 days. Interestingly enough, SPM was the only polyamine that evoked just a rapid and transient inhibition of FSH release and had no effect on LH or GnRH. In addition, this was the only amine that was not inhibited by DFMO, and it was the only one that remained constant in both hypothalamus and anterior pituitary during postnatal development, as determined in a parallel work. These results indicate that SPM was not responsible for the endocrine changes previously determined with the *in vivo* treatment with DFMO, where the female rats presented high FSH release and an associated puberty delay (14, 15).

AGM partially suppressed FSH, LH, and GnRH in *in vitro* release in newborn rats, but *in vivo* treatment with DFMO did not modify its concentration. *In vivo*, in adult ovariectomized estrogen-progesterone-treated rats, AGM released GnRH and LH, suggesting that this polyamine may have different endocrine effects in mammal brains according to the experimental model utilized (22).

It is important to note that no changes were observed when the same treatment was applied in tissues from 15-day-old female rats showing a regulatory role of polyamines only in early stages of development. This indicates that during the neonatal and the beginning of the infantile periods, the polyamines PUT and SPD are essential for the normal maturation of the GnRH-gonadotropin system. They would participate by inhibiting GnRH and FSH release. In agreement, when both polyamines are decreased by DFMO treatment *in vivo*, an increase in GnRH and FSH release is observed when the tissues are stimulated specifically by GnRH or unspecifically by KCl (14). Furthermore, DFMO acting directly on pituitary cells of 6-day-old females increased FSH release, precisely when polyamine concentrations are higher.

With regard to a possible mechanism by which polyamines could be altering GnRH and gonadotropin secretion, it has been demonstrated that polyamine levels, especially PUT, are critical for GABA synthesis in early postnatal life, at least in certain tissues, before the expression of GAD becomes evident (23). In addition, depletion of polyamines prevents the neurotrophic activity of GABA-A agonists in cultured rat cerebellar granule cells (24). On the other hand, GABA has been shown to modulate hypothalamic GnRH and pituitary gonadotropin secretion (13, 19, 25, 26). Therefore, this neurotransmitter is a candidate for mediating the present polyamine actions, because an increase in GABA production in 6-day-old tissues, induced by polyamine addition, could be responsible for the decrease in GnRH and gonadotropins observed in the present experiments. In addition, GABA has been demonstrated to be involved in the process of sexual differentiation induced by estradiol (27) and, therefore, a decrease in GABA levels, induced by DFMO treatment, which inhibits polyamine synthesis, could be the cause of the delay in puberty onset observed in *in vivo* neonatally DFMO-treated female rats in our previous experiments (14). In addition, taking into account the metabolic pathways for GABA synthesis from polyamines (2, 3), PUT and its direct precursors, SPD and AGM, would clearly be expected to be the most active polyamines followed by SPM, which is the furthest away in the pathway, as in effect occurs in the experiments here described. The lack of response in 15-day-old animals to polyamine or DFMO treatment may be due to the fact that at this stage of development, polyamines no longer participate in GABA biosynthesis. The participation of GABA in the effects of polyamine on GnRH and gonadotropin release in infantile female rats will be matter of future studies.

In summary, a relationship between polyamine levels and the output of the GnRH-gonadotropin system is demonstrated in the female rat during early postnatal life, a critical period of neuroendocrine organization.

We wish to thank the National Institute of Diabetes and Digestive and Kidney Diseases' National Hormone and Pituitary Program and Dr. A.F. Parlow for FSH and LH RIA kits.

1. Morgan DML. Polyamine Protocols. Totowa, NJ: Humana Press, pp1–186, 1998.
2. Scalabrino G, Lorenzini EC, Ferioli ME. Polyamines and mammalian hormones: Part I: Biosynthesis, interconversion and hormone effects. Mol Cell Endocrinol **77:**1–35, 1991.
3. Scalabrino G, Lorenzini EC. Polyamines and mammalian hormones: Part II: Paracrine signals and intracellular regulators. Mol Cell Endocrinol **77:**37–56, 1991.
4. Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ. Agmatine: an endogenous clonidine-displacing substance in the brain. Science **263:**966–969, 1994.
5. Raasch W, Regunathan S, Li G, Reis DJ. Agmatine, the bacterial amine, is widely distributed in mammalian tissues. Life Sci **56:**2319–2330, 1995.
6. Sltokin TA, Bartolome J. Role of ornithine decarboxylase and the polyamines in nervous system development: a review. Brain Res Bull **17:**307–320, 1986.
7. Najm I, Vanderklish P, Lynch G, Baudry M. Effect of treatment with α-difluoromethylornithine on polyamine and spectrin breakdown levels in neonatal rat brain. Dev Brain Res **63:**287–289, 1991.
8. Gilad GM, Dornay M, Gilad VH. Polyamines induce precocious development in rats: possible interaction with growth factors. Int J Devl Neurosci **7:**641–653, 1989.
9. Persson L, Nilsson M, Rosengren E. Ornithine decarboxylase activity and polyamines in the anterior pituitary gland during the rat oestrous cycle. J Endocrinol **107:**83–87, 1985.
10. White WF, Cohen AI, Rippel RH, Story JC, Schally AV. Some hypothalamic polyamines that deplete pituitary follicle stimulating hormone. Endocrinology **82:**742–752, 1968.
11. Aslam M, Nicholson S, Gillham B, Jones M. Permissive role for ornithine decarboxylase and putrescine in the luteinizing hormone surge. Neuroendocrinology **45:**473–478, 1987.
12. Nicholson SA, Wynne-Jones GA. Differential effect of α-difluoromethylornithine on the increases in plasma concentrations of reproductive hormones on the afternoon of pro-oestrus in the rat. J Endocrinol **121:**495–499, 1989.
13. Kordon C, Drouva SV, Martinez de la Escalera G, Weiner RI. Role of classic and peptide neuromediators in the neuroendocrine regulation of luteinizing hormone and prolactin. In: Knobil E, Neill JD, Eds. The Physiology of Reproduction. New York: Raven Press, pp1621–1681, 1994.
14. Thyssen SM, Becu-Villalobos D, Lacau-Mengido IM, Libertun C. α-Difuoromethylornithine modifies gonadotropin-releasing hormone release and follicle-stimulating hormone secretion in the immature female rat. Proc Soc Exp Biol Med **215:**192–197, 1997.
15. Thyssen SM, Libertun C. α-Difluoromethylornithine modifies FSH secretion and puberty onset in the female rat. Proc Soc Exp Biol Med **211:**76–80, 1996.
16. Becu-Villalobos D, Libertun C. Ontogenesis of 3(H) serotonin binding sites in the hypothalamus of the female rat: relation to serotonin induced LH release in moxestrol pretreated rats. Dev Brain Res **25:**111–116, 1986.
17. Lux-Lantos V, Becu-Villalobos D, Bianchi M, Rey-Roldán EB, Chamson-Reig A, Pignataro O, Libertun C. GABAB receptors in anterior pituitary cells. Neuroendocrinology **73:**334–343, 2001.
18. Yu WH, Karanth S, Walczewska A, Sower SA, McCann SM. A hypothalamic follicle-stimulating hormone-releasing decapeptide in the rat. Proc Natl Acad Sci U S A **94:**9499–9503, 2000.
19. Lux-Lantos VAR, Rey EB, Libertun C. Activation of GABA B receptors in the anterior pituitary inhibits prolactin and luteinizing hormone secretion. Neuroendocrinology **56:**687–693, 1992.
20. Sokal RR, Rohlf FJ. Biometry. New York: W.H. Freeman and Co., 1995.21. Pegg AE, McCann PP. Polyamine metabolism and function. Am J Physiol **243:**C212–C221, 1982.
22. Kalra SP, Pearson E, Sahu A, Kalra PS. Agmatine, a novel hypothalamic amine, stimulates pituitary luteinizing hormone release in vivo and hypothalamic luteinizing hormone-releasing hormone release in vitro. Neurosci Lett **194:**165–168, 1995.
23. Yamasaki EN, Barbosa VD, De Mello FG, Hokoc JN. GABAergic system in the developing mammalian retina: dual sources of GABA at early stages of postnatal development. Int J Dev Neurosci **17:**201–213, 1999.
24. Abraham JH, Hansen GH, Seiler N, Schoubsoe A. Depletion of polyamines prevents the neurotrophic activity of the GABA-agonist THIP in cultured rat cerebellar granule cells. Neurochem Res **18:**153–158, 2001.
25. Bilger M, Heger S, Brann DW, Paredes A, Ojeda SR. A conditional tetracycline-regulated increase in γ-amino butyric acid production near luteinizing hormone-releasing hormone nerve terminals disrupts estrous cyclicity in the rat. Endocrinology **142:**2102–2104, 2001.
26. Leonhardt S, Böning B, Luft H, Wuttke W, Jarry H. Activation of gene expression of the γ-aminobutyric acid rather than the glutamatergic system in the preoptic area during the preovulatory gonadotropin surge of the rat. Neuroendocrinology **71:**8–15, 2000.
27. Perrot-Sinal TS, Davis AM, Gregerson K, Kao J, McCarthy MM. Estradiol enhances excitatory gammabutyric acid mediated calcium signalling in neonatal hypothalamic neurons. Endocrinology **142:**2238–2243, 2001.