Antireproductive Effects of a Potent Gonadotropin-Releasing Hormone Antagonist in the Male Rat

Abstract. *Administration of a potent antagonist of gondadotropin-releasing hormone (GnRH) antagonist [Ac-dehydro-Pro[1], pCl-D-Phe[2],D-Trp[3,6]]-N[α]-MeLeu[7]-GnRH to adult male rats for 2 weeks resulted in decreased testosterone production and sexual organ weights and in disrupted spermatogenesis. The results demonstrate the essential role of gonadotropin-releasing hormone in the maintenance of reproductive functions and have implications for the regulation of male fertility.*

The key role played by gonadotropin-releasing hormone (GnRH) in controlling reproductive functions has been demonstrated by studies showing that active or passive immunization to GnRH leads to infertility and gonadal atrophy in both male and female animals (*1*). An alternative approach to studying the consequences of immunoneutralizing endogenous GnRH involves the administration of GnRH antagonists under various circumstances. Until recently, however, such studies have been hindered by the low potency and short biological duration of action of these peptides and have mostly been restricted to the ability of GnRH antagonists to block ovulation or modify hormone levels for short periods (*2*). The GnRH antagonist [D-pGlu[1],D-Phe[2],D-Trp[3,6]]GnRH (antagonist 1) (*3*), which reduces the GnRH-induced secretion of luteinizing hormone (LH) at an inhibitory concentration ratio (ICR_{50}) (*4*) of 3:1 (*5*), interfered with pregnancy when administered to female rats for the first 7 days after mating (*6*), but the effective doses required were large and led to dermatological lesions at the injection sites.

Recently, much more potent GnRH antagonists have been developed (*7, 8*). We found that [Ac-dehydro-Pro[1],pCl-D-Phe[2],D-Trp[3,6]]-N^{α}-MeLeu[7]-GnRH (antagonist 2) has an ICR_{50} of 0.043:1 and completely blocks ovulation when administered at a dose of 20 μg at noon on the day of proestrus. Furthermore, this antagonist has a 25 times higher affinity for GnRH hypophyseal binding sites than GnRH has (*7*). We therefore investigated the effect of long-term administration of this peptide on spermatogenesis, sexual organ weights, and testosterone production in adult male rats.

Antagonist 2, synthesized as described in (*5, 9*), was dissolved in corn oil and administered subcutaneously in a volume of 0.2 ml daily for 14 days to mature male rats. Control animals received the vehicle only. When used, human chorionic gonadotropin (Calbiochem) was dissolved in saline and injected subcutaneously in a volume of 0.2 ml. Blood samples were obtained periodically from the jugular vein with the animals under light ether anesthesia. At the end of the experiment, the appropriate organs were removed and processed.

All animals remained healthy throughout the experiment and no macroscopic lesions, dermatological or otherwise, were observed. There were significant decreases in plasma concentrations of testosterone and follicle-stimulating hormone (FSH) in all rats treated with antagonist 2 (testosterone, 22 percent of control values on days 4 and 14; FSH, 48 percent on day 4 and 25 percent on day 14). Changes in plasma LH concentration could not be documented because values were at the limit of sensitivity of the assay. The amounts of LH and FSH in the pituitary were significantly lower in antagonist 2-treated animals (LH, 60 percent and FSH, 27 percent of control values), suggesting that LH as well as FSH production was decreased. These data indicate that potent GnRH antagonists inhibit the synthesis as well as the secretion of gonadotropins. Consistent with the dramatic decrease in circulating testosterone, the weights of testes and androgen-dependent organs were also reduced (testes, 40 percent; prostate, 9 percent; and seminal vesicles, 12 percent of control values) (Fig. 1), and histological examination of the testes showed atrophic Leydig cells (Fig. 2). Tubular diameters in the testes of rats treated with antagonist 2 were greatly reduced [control, 0.270 ± 0.031 (standard error) mm; treated, 0.143 ± 0.009 mm)]. The germinal epithelium of the treated rats was depleted, with no signs of mitosis. There were no germinal cells that had developed beyond spermatogonia and a few spermatocytes. These data emphasize that the effects of antagonist 2 on spermatogenesis are of rapid onset, since they occur within one series of division of the stem cells (*10*).

It has been reported that GnRH and its agonists act directly on the testis to inhibit steroidogenesis (*11*) and to bind specifically and with high affinity to testicular membranes (*12*). Since GnRH antagonists reverse the inhibitory effects of GnRH on the ovary (*13*), it is conceivable that antagonist 2 could have stimulatory effects on the testis if that organ were exposed to LH-releasing factor from any endogenous source. We have observed no effects of antagonist 2 on sex organ weights in hypophysectomized male rats, with or without gonadotropin replacement therapy (Fig. 3). Furthermore, the observation that antagonist 2 profoundly inhibits gonadal function in the intact rat suggests either that the effects of GnRH antagonists on the pituitary supersede any putative positive go-

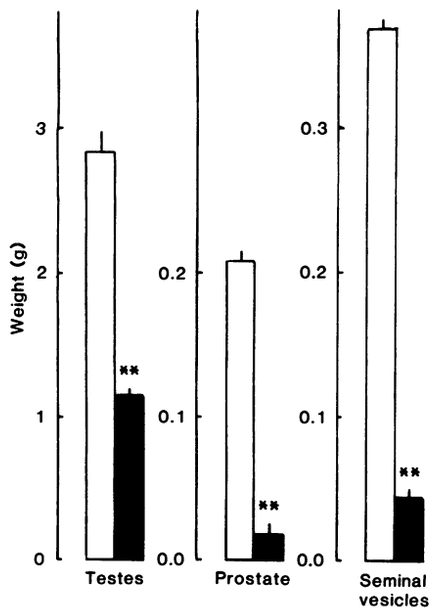

Fig. 1. Effect of a 14-day treatment with 1 mg daily of antagonist 2 on sexual organ weights. Mature (70 to 72 days old) Sprague-Dawley rats were used. At the end of the experiment, the animals were decapitated, and their sexual organs were dissected free of fat and weighed to the nearest 0.1 mg. Open bars, control; solid bars, antagonist 2-treated. Each bar represents the means ± standard error for five rats. Data were evaluated by analysis of variance. (**) Significantly different from control values at $P < .01$.

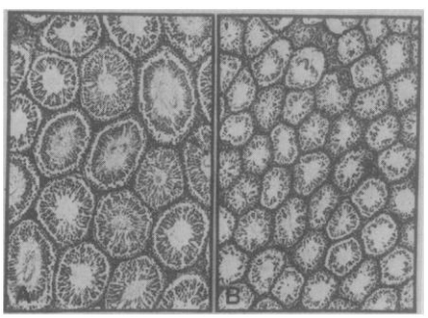

Fig. 2. Testicular morphology of intact adult male rats treated with (A) corn oil and (B) 1 mg of antagonist 2 daily for 14 days. The testes were embedded in paraffin. Sections (7 μm thick) were stained with hematoxylin-eosin. Three slides per testis were examined for the presence of ongoing spermatogenesis, and five tubules were measured in each testis.

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nadal effects or that, under the circumstances of our studies, the testis is not under the direct inhibitory influence of endogenous GnRH. However, antagonist 2 has six times lower affinity for gonadal than for pituitary binding sites and hence has greater organ dissociation than any other peptide we tested (*7, 12*). An antagonist of higher testicular affinity would be more appropriate for the evaluation of direct gonadal effects of GnRH antagonists.

These data provide evidence from long-term experiments that the action of endogenous GnRH in the male can be successfully inhibited by the administration of one of its antagonists, and emphasize the importance of endogenous GnRH in maintaining the integrity of male reproductive functions.

Our results have implications for the regulation of male fertility. Methods for dissociating the effects of antagonist 2 on spermatogenesis from those on androgen-dependent secondary sex characteristics need to be developed. In short-term experiments we have shown that testosterone enanthate can maintain ventral prostate weight without reversing the antagonist 2-mediated decreases in spermatogenesis and in the weight of the testes. Alternatively, advantage might be taken of probable differential rates in recovery of the functions of Leydig cells and seminiferous tubules after the administration of antagonist 2 is terminated; we have shown that testosterone concentrations returned to control values within 1 week after cessation of treatment with GnRH agonists, whereas spermatogenesis and the weight of the tests were suppressed for more than 1 month (*14*).

Long-term treatment with potent GnRH agonists is also associated with reduced androgen production and disrupted spermatogenesis (*14, 15*) and has therefore been considered as a possible means of regulating fertility. However, the mechanisms through which GnRH agonists interfere with fertility appear to be complex, with numerous possible sites of action (*16*), whereas those governing the effects of GnRH antagonists seem to be more direct. Furthermore, morphological examination of the testes of agonist-treated rats show disruption of the tubular membranes and the presence of polynucleated cells, qualitative changes that are not seen in the testes of antagonist 2-treated animals and that may be of concern if GnRH agonists are considered as potential contraceptives in the human male. The tubules of rats treated with antagonist 2 show consistently greater quantitative and more uniform changes in tubular morphology than those of GnRH agonist-treated animals.

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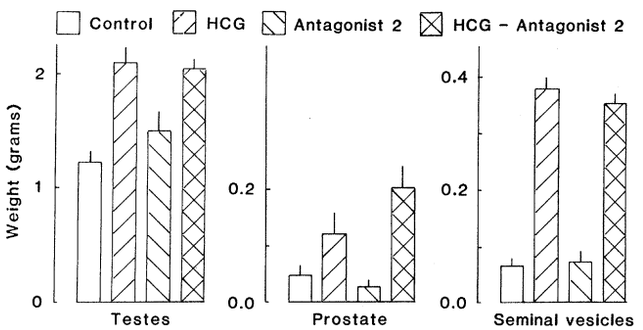

Fig. 3. Interaction of antagonist 2 (2 × 250 μg) and human chorionic gonadotropin (*HCG*) (30 I.U.) on sexual organ weights in hypophysectomized male rats with or without gonadotropin replacement therapy. Treatments were initiated within 5 days of surgery and administered for 14 days.

Table 1. Adult male rats were given daily injections of 1 mg of antagonist 2 for 14 days. Blood samples were obtained periodically just before an injection, with animals under light ether anesthesia. At the end of the experiment, the pituitaries were removed and homogenized in 0.1*M* Na_2CO_3; appropriate dilutions were made in 0.1*M* phosphate buffer (*p*H 7.3) containing 0.5 percent bovine serum albumin. Concentrations of LH and FSH in both plasma and pituitary homogenates were obtained with radioimmunoassay kits from the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD), and results are expressed in terms of the NIAMDD RP-1 rat LH and FSH standards. Testosterone was assayed as described in (*9*). Blood samples were obtained immediately before each injection. All values are the means ± standard error for eight rats.

Day of treatment	Concentration in plasma (ng/ml)		Concentration in anterior pituitary (μg per gland)	
	Testosterone	FSH	LH	FSH
		Control		
1	2.72 ± 0.51	221.3 ± 13.9		
4	2.95 ± 0.46	226.2 ± 15.1		
9	3.01 ± 0.39	218.7 ± 16.8		
14	2.89 ± 0.27	215.6 ± 17.6	51.4 ± 6.7	72.6 ± 2.6
		Antagonist 2		
1	3.41 ± 0.62	225.4 ± 14.3		
4	0.75 ± 0.04*	107.3 ± 11.2*		
9	0.84 ± 0.05*	61.2 ± 8.9*		
14	0.72 ± 0.04*	57.7 ± 11.6*	30.9 ± 4.1	20.1 ± 2.2*

*Significantly different from control values at $P < .01$.

References and Notes

1. A. Arimura, H. Sato, T. Kumasaka, R. B. Worobec, L. Debeljuk, J. Dunn, A. V. Schally, *Endocrinology* **93**, 1092 (1973); A. Arimura, L. Debeljuk, A. V. Schally, *ibid.* **95**, 323 (1974); H. M. Fraser, A. Gunn, S. L. Jeffcoate, D. T. Holland, *J. Endocrinol.* **63**, 399 (1974); B. Kerdelhue, S. Catin, C. Kordon, M. Jutisz, *Endocrinology* **98**, 1539 (1976); A. de la Cruz, A. Arimura, K. G. de la Cruz, A. V. Schally, *ibid.*, p. 490; N. Nichi, A. Arimura, K. G. de la Cruz, A. V. Schally, *ibid.*, p. 1024; J. T. McCormack, T. M. Plant, D. L. Hess, E. Knobil, *ibid.* **100**, 663 (1977).
2. A. de la Cruz, D. H. Coy, J. A. Vilchez-Martinez, A. Arimura, A. V. Schally, *Science* **191**, 195 (1976); C. W. Beattie, A. Corbin, T. J. Foell, V. Garsky, W. A. McKinley, R. W. A. Rees, D. Sarantakis, J. P. Yardley, *J. Med. Chem.* **18**, 1247 (1975).
3. The abbreviations for the amino acid substituents are: D-pGlu, D-pyroglutamic acid; D-Phe, D-phenylalanine; D-Trp, D-tryptophan; Ac-dehydro-Pro, acetyldehydroproline; *p*Cl-D-Phe, *p*-chloro-D-phenylalanine; and N^{α}-MeLeu, N^{α}-methylleucine.
4. ICR_{50} is the molar ratio of antagonist to GnRH that inhibits the GnRH-induced LH release by 50 percent in vitro.
5. J. E. Rivier and W. W. Vale, *Life Sci.* **23**, 869 (1978); the primary structure of GnRH is pGlu-His-Tyr-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (His, histidine; Tyr, tyrosine; Ser, serine; Gly, glycine; and Arg, arginine).
6. C. Rivier, J. Rivier, W. Vale, *Contraception* **19**, 185 (1979).
7. M. Perrin, J. Rivier, W. Vale, in preparation.
8. K. Channabasavaiah and J. M. Stewart, *Biochem. Biophys. Res. Commun.* **86**, 1266 (1979); D. H. Coy, I. Mezo, E. Pedroza, M. V. Nekola, J. Vilchez, P. Piyachaturawat, A. V. Schally, J. Seprodi, I. Teplan, *Peptides Structure and Biological Function*, E. Gross and J. Meinhofer, Eds. (Pierce Chemical Co., Rockford, Ill., 1979), p. 775.
9. J. Rivier, W. Vale, R. Burgus, N. Ling, M. Amoss, R. Blackwell, R. Guillemin, *J. Med. Chem.* **16**, 545 (1973).
10. Y. Clermont and S. C. Harvey, *Ciba Found. Colloq. Endocrinol. Proc.* **16**, 172 (1967).
11. A. J. W. Hsueh and G. F. Erickson, *Nature (London)* **281**, 66 (1979).
12. M. Perrin, J. Vaughan, J. Rivier, W. Vale, *Life Sci.* **26**, 2251 (1980).
13. A. J. W. Hsueh and N. C. Ling, *ibid.* **25**, 1223 (1979).
14. C. Rivier, J. Rivier, W. Vale, *Endocrinology* **105**, 1191 (1979).
15. G. Pelletier, L. Cusan, C. Auclair, P. A. Kelly, L. Desy, F. Labrie, *ibid.* **103**, 641 (1978); C. P. Auclair, A. Kelly, F. Labrie, D. H. Coy, A. V. Schally, *Biochem. Biophys. Res. Commun.* **76**, 855 (1977); C. P. Auclair, A. Kelly, D. H. Coy, A. V. Schally, F. Labrie, *Endocrinology* **101**, 1890 (1977).
16. A. J. W. Hsueh, M. L. Dufau, K. J. Catt, *Biochem. Biophys. Res. Commun.* **72**, 1145 (1976); M. Conti, J. P. Harwood, A. J. W. Hsueh, M. L. Dufau, K. J. Catt, *J. Biol. Chem.* **251**, 7729 (1976); K. J. Catt, A. J. Baukal, T. F. Davies, M. L. Dufau, *Endocrinology* **104**, 17 (1979); F. Haour and J. M. Saez, in *Structure*

and Function of the Gonadotropins, K. W. McKerns, Ed. (Plenum, New York, 1978), p. 497; F. Chasalow, H. Marr, H. Haour, J. M. Saez, *J. Biol. Chem.* **254**, 5613 (1979).
17. We are indebted to the NIAMDD rat pituitary hormone distribution program for supplying the radioimmunoassay for rat LH and FSH. The excellent technical assistance of H. Laurent, N. Keating, J. Boone, K. von Dessonneck, J. Porter, and R. Kaiser is gratefully acknowledged. Supported by NIH grants HD09690, RFP CD-78-5, the Rockefeller Foundation, the Clayton Foundation for Research, California Division, and the Texas Salk Institute Foundation.

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Excitatory and Inhibitory Effects of Opiates in the Rat Vas Deferens: A Dual Mechanism of Opiate Action

Abstract. *Both natural (−)-morphine and its unnatural enantiomer (+)-morphine exert an excitatory action on electrically stimulated contractions of rat vas deferens. Preexposure to (−)-morphine results in cross-tolerance to the inhibitory action of β-endorphin. (−)-Naloxone and its stereoisomer (+)-naloxone also exert an excitatory action, but only (−)-naloxone blocks the inhibitory action of β-endorphin. Thus morphine exerts a dual action on a peripheral organ: one an inhibitory action mediated by the stereospecific endorphin receptor that is blocked stereospecifically by naloxone, the other an excitatory action mediated by a nonstereospecific receptor that is not blocked by naloxone. The opiate abstinence syndrome is seen as due to the unmasking of the excitatory action of opiates when its concomitant inhibitory influence is removed by selective blockade by naloxone or weakened by selective tolerance. The view that the rat vas deferens is devoid of morphine receptors is now seen as arising from a reverse example of morphine's dual action: the masking of the inhibitory action of morphine by its concomitant and more potent excitatory action.*

We previously proposed a dual mechanism model of morphine action in the central nervous system (CNS) and presented evidence that morphine actions in the CNS are mediated by two classes of receptors (*1*). One, stereospecific for opiates and antagonized by naloxone, mediates the inhibitory actions of opiates (analgesia, sedation, and immobility); the other, not stereospecific for opiates and not antagonized by naloxone, mediates the excitatory actions of opiates (such as hyperreactivity) (*2*). This dual action mechanism implies that the opiate abstinence syndrome is due to the unmasking of the excitatory action of the opiate when its simultaneous inhibitory influence is removed by selective blockade by naloxone or weakened by the development of selective tolerance. We now present evidence for a dual mechanism of opiate action in a peripheral organ, the rat vas deferens.

The rat vas deferens has been reported to be devoid of morphine receptors (*3*), yet β-endorphin, the opiate peptide that is the putative endogenous ligand for the classical morphine receptor, was reported to exert a potent inhibitory action on this tissue (*4*). Morphine was found to exert a stimulatory action (*5*), whereas the potent opiate etorphine was found to exert an inhibitory action (*6*). This seeming contradiction led us to examine the effects of morphine on this tissue. We now report that morphine exerts a dual action—both excitatory and inhibitory—on this preparation, with the excitatory effect masking the inhibitory one.

To demonstrate the inhibitory action of morphine (which is masked by its concomitant and more potent excitatory action), we investigated cross-tolerance to the inhibitory action of β-endorphin after exposure to morphine. This effect was observed to be stereospecific, occurring only after exposure to (−)-morphine, not its unnatural enantiomer (+)-morphine (*7*). The excitatory action was nonstereospecific, occurring after both (−)- and (+)-morphine, and was not blocked by the opiate antagonist (−)-naloxone. While the excitatory action also occurred after exposure to (−)-naloxone or its stereoisomer (+)-naloxone (*7*), only (−)-naloxone blocked the inhibitory action of β-endorphin (Fig. 1) (*8*). These results extend to a peripheral organ our previous findings for the CNS that morphine exerts dual actions that are mediated by stereospecific inhibitory receptors and nonstereospecific excitatory receptors, with the former being blocked stereospecifically by (−)-naloxone.

Vasa deferentia were dissected from freshly killed Sprague-Dawley rats (250 to 300 g), carefully stripped of surrounding tissue, and pressed to remove their seminal contents. Each tissue sample was suspended in a 5-ml bath containing Krebs-Ringer solution (*9*) maintained at 37°C and *p*H 7.4 and gassed with a mixture of 95 percent O_2 and 5 percent CO_2. The resting tension was approximately 1 g; the contractions induced by electrical stimulation (30 V, 0.1 Hz, 0.5 msec) (*6*) were measured isometrically. All strips were equilibrated for 1 hour, with washes every 15 minutes. This was followed by a pretreatment period of 30 minutes, during which the tissue was continuously exposed to one of four drugs or no drug while the electrical stimulation continued. Immediately afterward, the strips

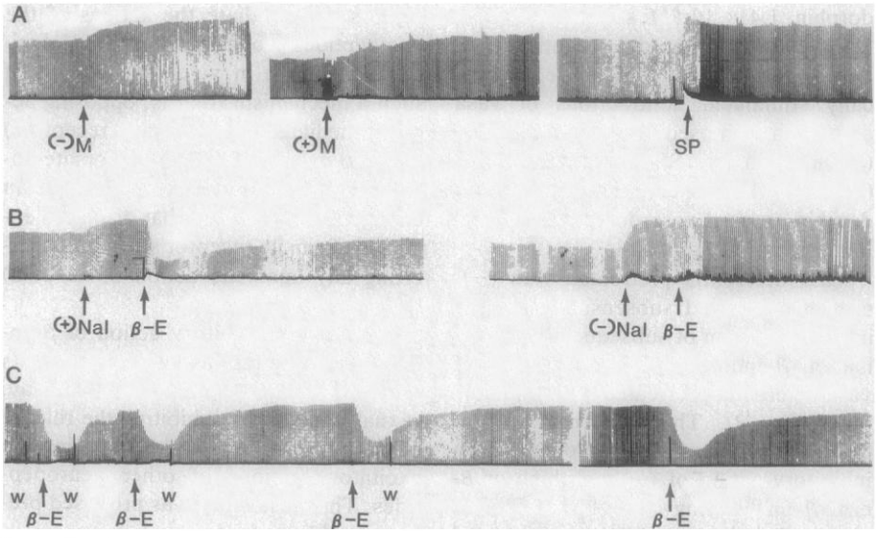

Fig. 1. Tracings showing excitatory and inhibitory actions of opiates and peptides on the electrically stimulated contractions of rat vas deferens. (A) Left, excitation after addition of (−)-morphine (*M*) (final dilution, $1 \times 10^{-4}M$); center, after (+)-morphine ($1 \times 10^{-4}M$); right, after substance P (*SP*) ($2.5 \times 10^{-6}M$). (B) Left, excitation after addition of (+)-naloxone (*Nal*) ($1 \times 10^{-4}M$) followed by inhibition (no blockade) when β-endorphin (*β-E*) ($1.4 \times 10^{-6}M$) was added in its presence; right, excitation after addition of (−)-naloxone ($1 \times 10^{-4}M$); no inhibition (blockade) followed when β-endorphin ($1.4 \times 10^{-6}M$) was added in its presence. (C) Inhibition in a vas deferens preexposed to (+)-morphine ($1 \times 10^{-4}M$); β-endorphin ($1.4 \times 10^{-7}M$) was added 1, 10, 30, and 60 minutes later. Three minutes after the first three additions, the strips were washed (*w*).

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