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Hormone-like behavioral effects of levonorgestrel and its metabolites in the male rat

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Abstract

Levonorgestrel (LNG), a contraceptive progestin, exhibits, besides its progestational activity, other hormone-like effects at the peripheral level. To assess whether LNG and its metabolites exert androgenic and/or estrogenic actions at the central nervous system (CNS), their effects on male sexual behavior in castrated rats were examined. LNG, 5α-dihydro LNG (5αLNG), and the 3α,5α- and 3β,5α-tetrahydro derivatives of LNG (3αLNG and 3βLNG, respectively) were administered for 3 weeks either alone (1000 μg/day) or in combination (300 μg/day) with 5α-dihydrotestosterone (DHT, 300 μg/day) or with estradiol-17β (E_2, 5 μg/day). Copulatory behavior was assessed twice per week and sex accessory organs weights recorded at the end of treatments. LNG restored full copulatory behavior comparable to that of testosterone treated animals, although with a slight delay, whereas 5αLNG induced male sexual behavior in a significantly lower number of subjects. 3βLNG and 3αLNG induced mounting but failed to restore intromission and ejaculation. Combined LNG + E_2 treatment fully activated mounting and intromission, but ejaculation was only partially restored. Combined 5αLNG + E_2 treatment and the combinations of 3αLNG or 3βLNG with E_2 were significantly less effective, activating fewer intromissions and ejaculations. 3αLNG and 5αLNG, in combination with DHT, restored male sexual behavior. LNG, but not its metabolites, induced a significant increase on the weight of sex accessory organs. The overall results demonstrated that high doses of LNG induce a potent androgen agonistic behavioral effect and that its A-ring reduction diminishes this potency and enables a shift towards a weak estrogen-like effect.

Keywords: Male sexual behavior; Levonorgestrel; Contraceptive progestins; Levonorgestrel metabolites; Androgenic effects of levonorgestrel

1. Introduction

Levonorgestrel (D-(l-norgestrel), LNG) is a totally synthetic 13β-ethyl-substituted 19-nor progestin (Smith et al., 1963) widely used either alone or combined with ethinylestradiol in a variety of contraceptive formulations including pills, injectables, medicated intrauterine devices, subdermal implants, vaginal rings, and more recently, emergency contraceptive pills (Ball et al., 1991; Cravioto et al., 1997; Garza-Flores et al., 1991; Piaggio et al., 1999; Sivin and Stern, 1994; Thau and Jackanicz, 1994; WHO, 1998). LNG induces hormone agonistic effects other than that of its progestational activity. Indeed, LNG specifically binds with high affinity to the mineralocorticoid (Kuhl, 1996; Rebar and Zeserson, 1991) and androgen intracellular receptors (Cabeza et al., 1995; Lemus et al., 1992; Phillips et al., 1990) and exerts the corresponding effects with a relatively high potency. On the contrary, the estrogen-like actions of LNG have remained as a controversial issue. It has been well documented that LNG, at high concentrations, induces cell growth and proliferation of breast cancer cells (Catherino et al., 1993; Schoonen et al., 1995a,b; Van der Burg, 1991; Van der Burg et al., 1992)—an effect that is precluded by steroidal anti-estrogens but not by anti-progestins (Catherino et al., 1993)—suggesting that the estrogen-like effects of LNG are mediated by the estrogen receptor (ER).

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However, a number of experimental evidences have demonstrated that the LNG molecule does not interact at all with ERs (Kuhl, 1996; Rebar and Zeserson, 1991) indicating that this synthetic progestin does not induce estrogen agonistic effects.

A number of studies have shown that LNG undergoes extensive peripheral metabolism (Stanczyk and Roy, 1990) and biotransformation to A-ring-reduced derivatives at hormone-sensitive organs (Cabeza et al., 1995; Lemus et al., 1992), in a similar manner to that reported for naturally occurring androgens (Martini, 1982; Pérez et al., 1975; Pérez-Palacios et al., 1970) and for other synthetic 19-nor progestins (Larrea et al., 1987; Lemus et al., 2001). The observation that A-ring reduction of progestins of the 19-nor series may modulate the expression of their hormone-like effects (Lemus et al., 2000; Pérez-Palacios et al., 1993) at

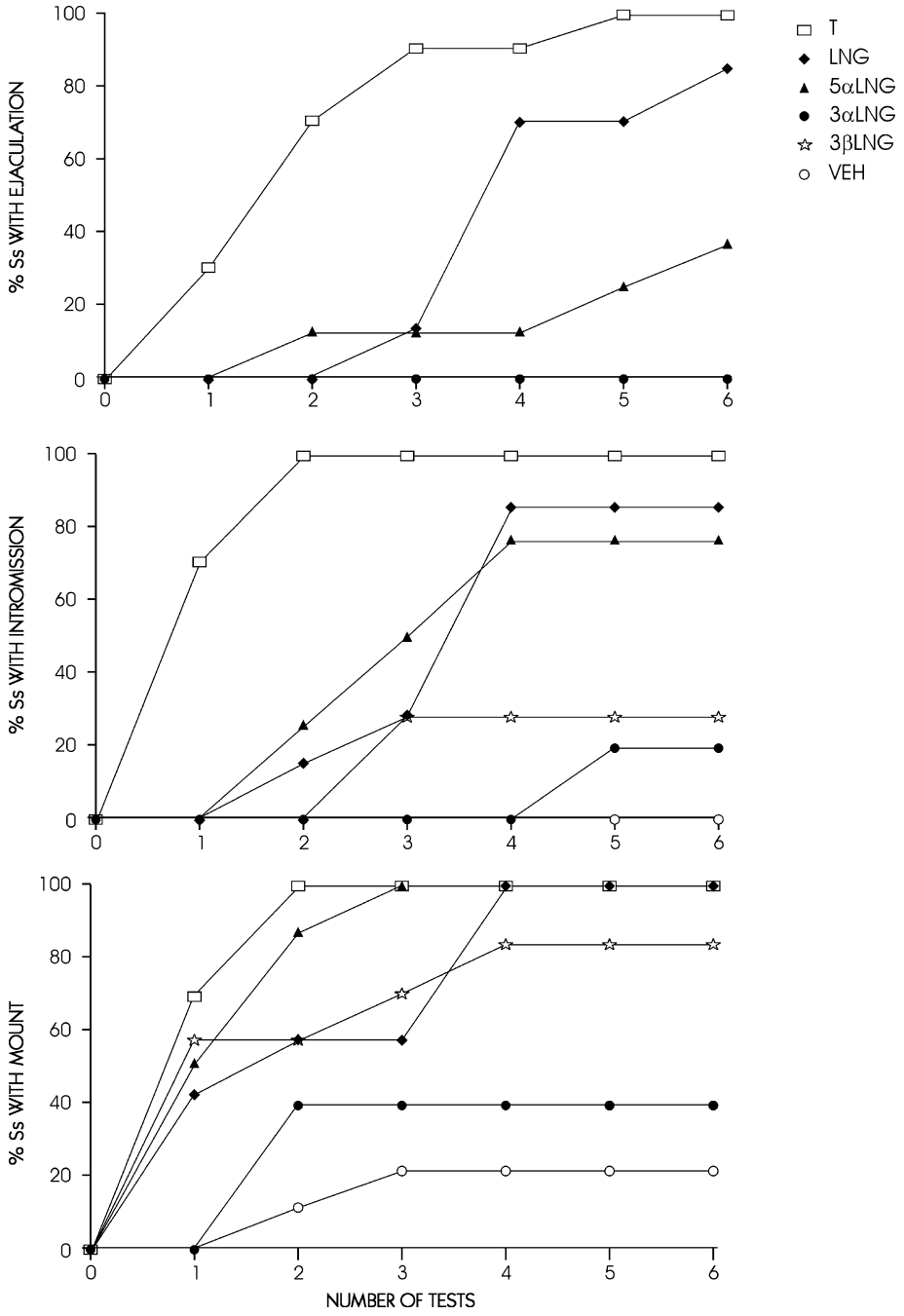

Fig. 1. Intrinsic behavioral effects of LNG and its A-ring-reduced metabolites. Results are presented as cumulative percentages of castrated male rats showing mount, intromission, and ejaculation during 21 days of treatment (1000 μg/day) with LNG, 5αLNG, 3αLNG, or 3βLNG. Castrated rats treated with T (1000 μg/day) or vehicle (VEH) served as controls. LNG restored full copulatory behavior in the treated Ss ($P < .001$, as compared to VEH), while 5α-reduction of LNG resulted in a decrease of its behavioral effect (LNG metabolites, percentage of Ss with ejaculation: $P < .01$, as compared to LNG or T, Fisher Exact Probability Test).

both peripheral and central nervous system (CNS) levels prompted us to investigate the androgen and/or estrogen agonistic behavioral effects and potency of LNG and three of its A-ring-reduced metabolic conversion products in the long-term castrated male rat. Activation of masculine sexual behavior was used in this study as a screening experimental model, taking advantage of its well-documented hormone-regulated expression in rodents. The behavioral effects of LNG and its metabolites were assessed by giving them alone, as well as by their capability to synergize with either estradiol-17β (E_2) or 5α-dihydrotestosterone (DHT). The peripheral hormone-like activity of the synthetic steroids was assessed by their effects on the ventral prostate and seminal vesicles of the castrated animals.

Further interest in the conduction of this study stemmed from the observation that enzyme-mediated 5α-reduction of the molecule of norethisterone (NET), another 19-nor contraceptive progestin, enhances its binding affinity for the androgen receptor (AR) (Chávez et al., 1985), but unexpectedly diminishes and almost abolishes its androgen agonistic effects (Lemus et al., 1997).

2. Materials and methods

2.1. Steroids

Testosterone (T), DHT, and E_2 were supplied by Sigma (St. Louis, MO). LNG was kindly provided by Schering Mexicana, S.A. de C.V. (Mexico City). The 5α-reduced derivative of LNG, 5α-dihydro LNG (5αLNG), was synthesized by lithium-ammonia reduction of LNG, crystallized from ethyl acetate-hexane, and purified by flash chromatography (Still et al., 1978). The 3α,5α-tetrahydro derivative of LNG (3αLNG) was prepared from reduction of 5αLNG with L-selectride under anhydrous conditions (Brown and Krifhnamurthy, 1972). A mixture of 3αLNG (98%) and 3β,5α-tetrahydro LNG (3βLNG) (2%) reduced derivatives was obtained. Synthesis of 3βLNG was carried out by sodium borohydride reduction of 5αLNG (Bowers et al., 1958). The resulting epimeric alcohols were separated by flash chromatography using the system ethyl acetate–hexane (3:7). The chemical purity of LNG and its derivatives was assessed by their melting points, HPLC behavior, infrared absorption, and H-nuclear magnetic resonance. The physical and spectroscopic constants of the A-ring-reduced LNG derivatives have been previously described (Lemus et al., 1992).

2.2. Animals

Subjects (Ss) were 80- to 90-day-old (260–290 g body weight) male Wistar rats bred in our laboratory and selected on the basis of their display of the whole pattern of sexual behavior including ejaculation, in at least three screening tests. Animals were housed in individual cages with food and water available ad libitum, and were maintained under a reversed light–dark cycle (0830 h off, 1830 h on). All rats

Table 1
Parameters of sexual activity displayed by castrated male rats under the various steroid daily treatments for 21 days

	T, 1000 μg ($n=10$)	LNG, 1000 μg ($n=7$)	5αLNG, 1000 μg ($n=8$)	3αLNG, 1000 μg ($n=10$)	3βLNG, 1000 μg ($n=7$)	Vehicle ($n=10$)
% Ss with mounting, intromission, and ejaculation	100***, 100***, 100***	100***, 86***, 86***	100***, 75***, 38**,††	40††, 20†††, 0†††	85**, 28††, 0†††	20†††, 0†††, 0†††
% Tests with mounting, intromission, and ejaculation	83***, 83***, 75***	62***,†, 29***,†††, 24***,†††	69***, 33***,†††, 13*,†††	27†††, 3†††, 0†††	48*,†††, 17*,†††, 0†††	10†††, 0†††, 0†††
Mount latency (min)[a]	1.7 ± 1.1**	2.6 ± 0.7**,†	5.0 ± 1.6*,†	10.8 ± 0.7†††	3.6 ± 0.8***	13.4 ± 0.5††
Intromission latency (min)[a]	2.0 ± 1.1	2.8 ± 0.8	3.7 ± 1.0	11.9 ± 0.6††	1.2 ± 0.1	–
Ejaculation latency (min)[a]	9.3 ± 1.4	11.3 ± 3.0	10.7 ± 3.5	–	–	–
Postejaculatory interval (min)[a]	7.4 ± 0.8	16.7 ± 2.6†††	12.0 ± 1.8†	–	–	–
Interintromission interval (min)[a]	0.9 ± 0.1	1.8 ± 0.4†	2.4 ± 0.4††	2.7 ± 0.5†††	4.1 ± 1.0†††	–
Hit rate[a]	0.68 ± 0.04	0.53 ± 0.07	0.39 ± 0.07††	0.32 ± 0.04††	0.24 ± 0.01†††	–

[a] Mean ± S.E.M. of the last two behavioral tests.
* $P<.05$, as compared to the vehicle-treated group.
** $P<.01$, as compared to the vehicle-treated group.
*** $P<.001$, as compared to the vehicle-treated group.
† $P<.05$, as compared to the T-treated group.
†† $P<.01$, as compared to the T-treated group.
††† $P<.001$, as compared to the T-treated group.

were castrated under ether anesthesia at least 90 days before experiments. Evidence that no intromission and ejaculation were retained before the onset of steroid treatments was obtained in at least two behavioral tests. All experiments were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Research Ethics Committee of the Instituto Mexicano del Seguro Social.

2.3. Experimental design

All steroids were subcutaneously administered dissolved in 10% ethanol–corn oil. Injection volumes were 0.1–0.2 ml. Groups of rats were subjected to one of the following treatments: Group 1: One of the following steroids alone (1000 μg/day): T ($n=10$), LNG ($n=7$), 5αLNG ($n=8$), 3αLNG ($n=10$), and 3βLNG ($n=7$); Group 2: E_2 (5 μg/

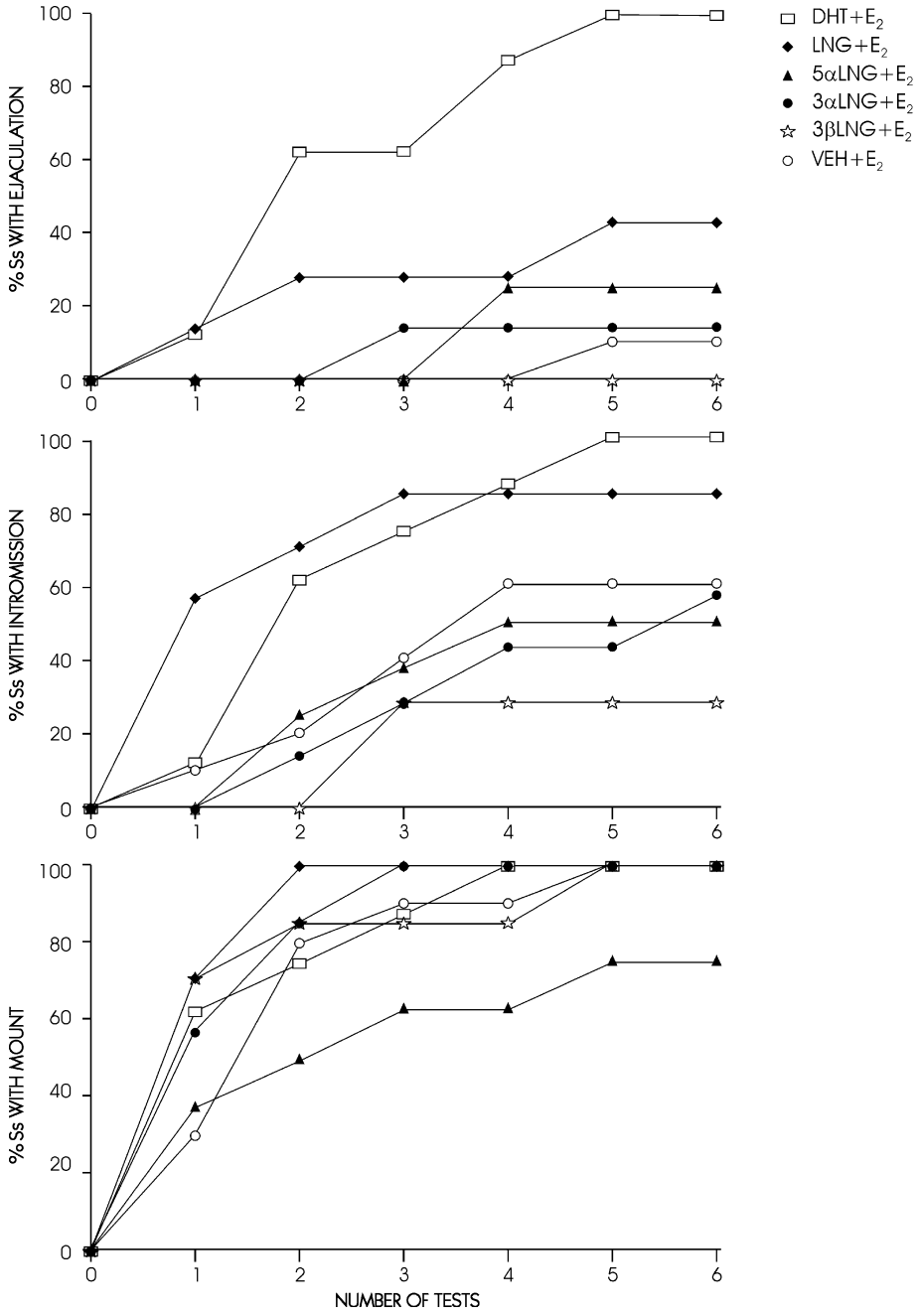

Fig. 2. Androgen-like behavioral effects of LNG and its A-ring-reduced metabolites, as assessed by their ability to synergize with E_2. Results are presented as cumulative percentages of castrated male rats showing mount, intromission, and ejaculation during 21 days of treatment with LNG, 5αLNG, 3αLNG, or 3βLNG (300 μg/day) in combination with E_2 (5 μg/day). Castrated rats treated with either DHT (300 μg/day) or vehicle, in combination with E_2 (5 μg/day) served as controls. LNG restored intromission behavior in most Ss (86%) and ejaculation in 43% ($P<.05$, as compared to VEH + E_2, Fisher Exact Probability Test), while its 5α-reduced metabolites were less effective ($P<.01$, as compared to DHT + E_2, Fisher Exact Probability Test).

day) plus one of the following steroids (300 μg/day): LNG ($n=7$), 5αLNG ($n=8$), 3αLNG ($n=7$), 3βLNG ($n=7$); Group 3: combination of DHT (300 μg/day) with one of the following steroids (300 μg/day): LNG ($n=8$), 5αLNG ($n=8$), 3αLNG ($n=8$), or 3βLNG ($n=8$). Additional groups of animals that received E_2 (5 μg/day) ($n=10$), DHT (300 μg/day) ($n=12$), or vehicle alone ($n=10$) for 21 consecutive days, were used as negative controls; animals ($n=8$) receiving DHT (300 μg/day) plus E_2 (5 μg/day) served as positive controls. The doses of steroids used in this study were chosen on the basis of previous studies using naturally occurring androgens (Moralí et al., 1994) or synthetic progestins (Moralí et al., 1990), which exhibited androgenic and/or estrogenic behavioral effects when administered in similar dose schemes.

2.4. Behavioral assessment

Male sexual behavior was evaluated by standard techniques (Beyer et al., 1973; Larsson, 1979; Moralí et al., 1990, 1993, 1994; Meisel and Sachs, 1994). Tests began on the day of the onset of treatment (Day 0) and continued thereafter twice a week until Day 21. Tests were done during the dark phase of the cycle under dim red light. Rats were placed in Plexiglas observation cages (60 × 60 × 42 cm), and after a 5-min adaptation period, each subject was presented with a receptive female. Stimulus females received 5 μg E_2 benzoate three times per week and 0.5 mg progesterone, 4 h before testing. The results are presented as cumulative percentage of responsive animals to facilitate comparison with previous studies in which the androgenic and/or estrogenic behavioral activities of naturally occurring androgens (Beyer et al., 1974; Moralí et al., 1993) and synthetic progestins (Moralí et al., 1990) were assessed in castrated male rats.

The number of mounts and intromissions as well as the mount, intromission, and ejaculation latencies, and the postejaculatory interval, were recorded and measured. The test was ended in one of the following circumstances: (1) 15 min after the presentation of the female to the male if no intromission occurred, (2) 30 min after the first intromission if no ejaculation had occurred, or (3) after the first intromission following ejaculation. The rates of copulation and its efficiency were evaluated as the interintromission intervals and the hit rates, respectively. The interintromission interval results from dividing the ejaculation latency by the number of intromissions, or by dividing 30 min by the number of intromissions when no ejaculation occurs; the hit rate results from dividing the number of intromissions by the total number of mounts plus intromissions displayed by the subject in each test; it renders an estimation of the efficiency of the consummatory mechanism (Larsson, 1979; Sachs and Barfield, 1972). The results of the behavioral parameters analyzed were expressed as the average values obtained in the last two tests (Days 18 and 21 of the experiments) to avoid changes frequently observed in the course of steroid treatments. After completion of the last

Table 2
Parameters of sexual activity displayed by castrated male rats under the various steroid daily treatments for 21 days

	DHT + E_2, 300 and 5 μg ($n=8$)	LNG + E_2, 300 and 5 μg ($n=7$)	5αLNG + E_2, 300 and 5 μg ($n=8$)	3αLNG + E_2, 300 and 5 μg ($n=7$)	3βLNG + E_2, 300 and 5 μg ($n=7$)	Vehicle + E_2, 0.2 ml and 5 μg ($n=10$)
% Ss with mounting, intromission, and ejaculation	100, 100, 100 *	100, 86, 43†	75, 50†, 25††	100, 57, 14†††	100, 28††, 0†††	100, 60, 10†††
% Tests with mounting, intromission, and ejaculation	96 *, 77***, 63***	90, 67***, 24***,†††	54*,†††, 29†††, 8†††	74††, 21†††, 5†††	86, 10†††, 0†††	77†, 23†††, 1†††
Mount latency (min)[a]	0.6 ± 0.3 *	2.2 ± 0.9†	4.2 ± 2.0†	2.4 ± 0.7†	3.1 ± 0.8†	3.9 ± 1.0†
Intromission latency (min)[a]	2.0 ± 1.1	2.7 ± 0.4	3.0 ± 1.3	7.6 ± 2.2†	8.4 ± 6.2†	3.0 ± 1.1
Ejaculation latency (min)[a]	11.3 ± 2.2	19.2 ± 2.4†	19.1 ± 3.8	16.2	–	29.0
Postejaculatory interval (min)[a]	9.5 ± 1.2	12.2 ± 1.3 *,†	8.6 ± 0.7 *	8.8 *	–	8.7
Interintromission interval (min)[a]	1.0 ± 0.1 *	2.5 ± 0.4††	5.4 ± 2.1††	7.2 ± 0.8†††	8.0 ± 2.0†††	5.1 ± 2.6†
Hit rate[a]	0.68 ± 0.03***	0.38 ± 0.04†††	0.24 ± 0.06†††	0.23 ± 0.04†††	0.15 ± 0.01†††	0.32 ± 0.05†††

[a] Mean ± S.E.M. of the last two behavioral tests.
* $P<.05$, as compared to the vehicle + E_2-treated group.
*** $P<.001$, as compared to the vehicle + E_2-treated group.
† $P<.05$, as compared to the DHT + E_2-treated group.
†† $P<.01$, as compared to the DHT + E_2-treated group.
††† $P<.001$, as compared to the DHT + E_2-treated group.

behavioral test, Ss were killed by overexposure to ether, and ventral prostate and seminal vesicles were removed and weighed to the nearest 0.1 mg.

2.5. Statistics

The proportions of sexually active animals were analyzed by the Fisher's Exact Probability Test, while analysis of the proportions of tests in which Ss were active was done by χ^2 tests. Numbers and latencies of behavioral responses were compared between groups by repeated measures analysis of variance followed by Tukey tests or Mann–Whitney U tests when appropriate. Organ weights were analyzed by the Student's t test. Group differences were considered significant when $P < .05$ was reached (two-tailed test) (Montgomery, 1991).

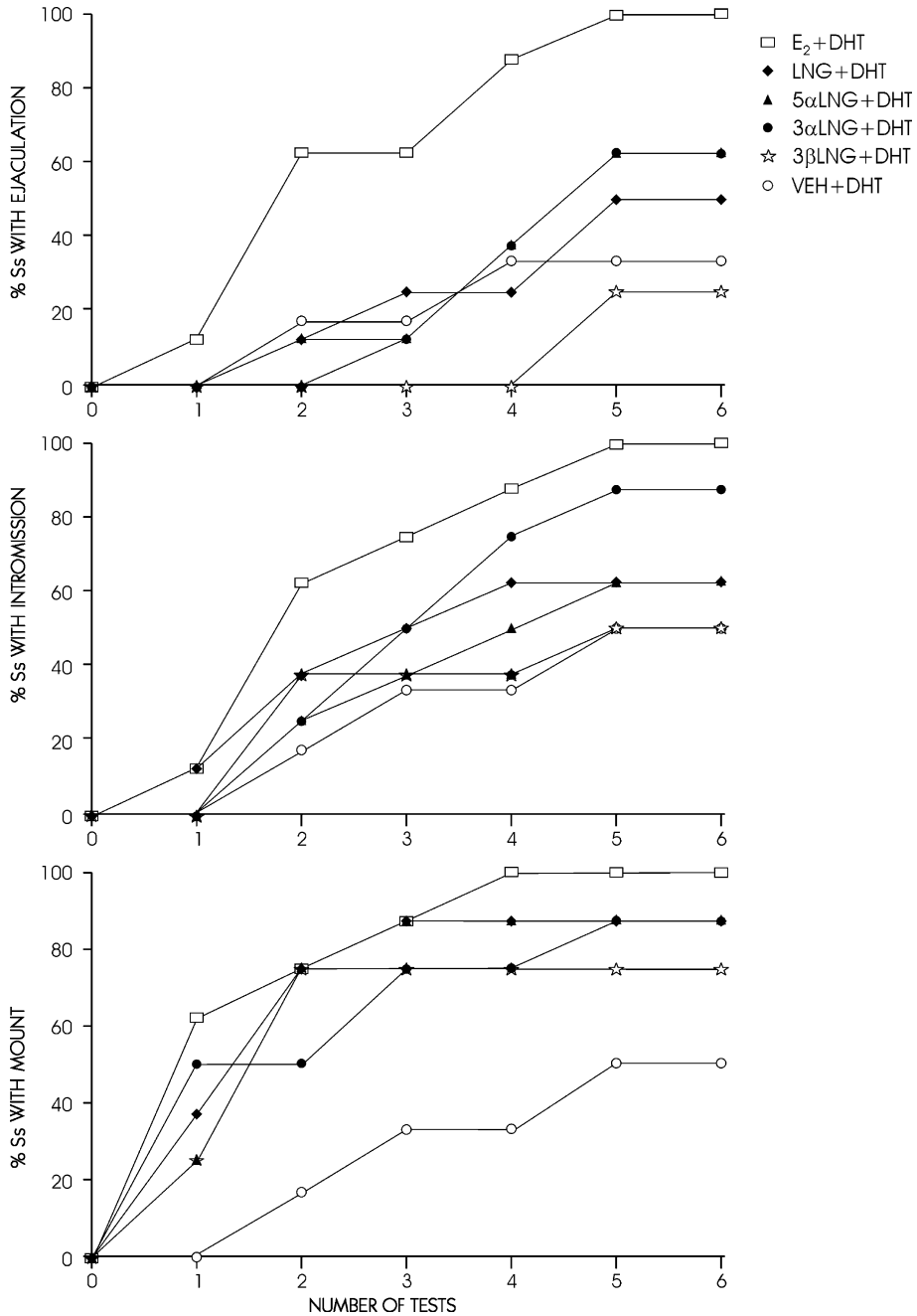

Fig. 3. Estrogen-like behavioral effects of LNG and its A-ring-reduced metabolites, as assessed by their ability to synergize with DHT. Results are presented as cumulative percentages of castrated male rats showing mount, intromission, and ejaculation during 21 days of treatment with LNG, 5αLNG, 3αLNG, or 3βLNG (300 μg/day) in combination with DHT (300 μg/day). Castrated rats treated with either E_2 (5 μg/day) or vehicle, in combination with DHT (300 μg/day) served as controls. Slight estrogenic effects were exerted by 5α-reduced metabolites of LNG (5α- and 3αLNG), since non-significant differences were found in the percentages of Ss with ejaculation as compared to E_2 + DHT (Fisher Exact Probability Test).

3. Results

3.1. Effects of LNG and its derivatives given alone

Highly significant differences ($P<.01$) between groups were noticed in terms of the incidence, the number, and temporal characteristics of behavioral responses (Fig. 1; Table 1).

LNG treatment fully restored copulatory behavior in long-term castrated rats. All Ss treated with LNG presented mounting behavior and most of them (86%) displayed intromission and ejaculatory behavior, although with a slight delay over the course of treatment (Fig. 1). The delayed onset of intromission and ejaculatory behaviors conditioned a low percentage of tests with intromission (29%) and with ejaculation (24%) in this group (Table 1); however, once the Ss started to ejaculate, the incidence of ejaculatory responses increased significantly (45%), as its was recorded in the last three tests. LNG-treated animals presented hit rates and intromission and ejaculation latencies similar to those of control animals treated with T (Table 1).

5αLNG partially restored the copulatory behavior of castrated animals (Fig. 1), but only a proportion of them (38%) displayed ejaculatory responses in a lower percent of tests than LNG or T-treated rats (Table 1). Animals treated with 5αLNG exhibited significantly longer interintromission intervals ($P<.01$) and lower hit rate values ($P<.01$) but similar response latencies than those of animals receiving LNG or T (Table 1).

Treatment with the 3αLNG or 3βLNG derivatives stimulated mounting and intromission responses in a low proportion of tests (27% and 3%; 48% and 17%) and it was totally ineffective to restore ejaculatory behavior in castrated rats (Fig. 1, Table 1). Mounting latencies of animals treated with 3βLNG were similar to those of LNG- or T-treated rats (Table 1).

3.2. Effects of LNG and its derivatives given in combination with E_2

LNG in combination with E_2 restored mounting and intromission behaviors in most Ss (100% and 86%, respectively) with a similar timing over the course of treatment and in a similar percent of tests as those of the DHT + E_2 control group (Fig. 2). However, ejaculation behavior in LNG + E_2-treated animals was activated in only 43% of Ss in 23% of tests, values significantly lower ($P<.05$; $P<.001$) than those of the DHT + E_2 control group (Fig. 2; Table 2). Nevertheless, the incidence of ejaculatory responses exhibited by LNG + E_2-treated rats was significantly higher ($P<.001$) than that observed in control animals receiving vehicle + E_2 (Table 2).

The combination 5αLNG + E_2 was less effective to restore copulatory behavior in castrated rats, as compared

Table 3
Parameters of sexual activity displayed by castrated male rats under the various steroid daily treatments for 21 days

	E_2 + DHT, 5 and 300 μg ($n=8$)	LNG + DHT, 300 and 300 μg ($n=8$)	5αLNG + DHT, 300 and 300 μg ($n=8$)	3αLNG + DHT, 300 and 300 μg ($n=8$)	3βLNG + DHT, 300 and 300 μg ($n=8$)	Vehicle + DHT, 0.1 ml and 300 μg ($n=12$)
% Ss with mounting, intromission, and ejaculation	100*, 100*, 100**	88, 63, 50†	88, 63, 63	88, 88, 63	75, 50†, 25††	50†, 50†, 33††
% Tests with mounting, intromission, and ejaculation	96***, 77***, 63***	50*,†††, 35*,†††, 17†††	58*,†††, 31*,†††, 23*,†††	58*,†††, 42**,††, 23*,†††	46*,†††, 21†††, 6†††	19†††, 19†††, 14†††
Mount latency (min)[a]	0.6 ± 0.3 *	4.5 ± 1.2†	6.6 ± 1.2††	4.5 ± 0.9†	8.0 ± 1.0†††	3.5 ± 1.5†
Intromission latency (min)[a]	2.0 ± 1.1 *	4.6 ± 0.5†	7.0 ± 2.0†	5.3 ± 1.1†	6.8 ± 0.5†	6.7 ± 1.6†
Ejaculation latency (min)[a]	11.3 ± 2.2	23.1 ± 3.3†	21.7 ± 2.5†	12.9 ± 1.4	19.2 ± 4.3†	14.5 ± 1.3
Postejaculatory interval (min)[a]	9.5 ± 1.2 *	13.2 ± 2.5†	13.7 ± 1.5†	14.4 ± 1.1†	23.9 ± 9.7††	14.6 ± 1.6†
Interintromission interval (min)[a]	1.0 ± 0.1 *	3.6 ± 1.4†	3.1 ± 0.6††	2.6 ± 0.7†	3.0 ± 0.7††	1.8 ± 0.4†
Hit rate[a]	0.68 ± 0.03 *	0.41 ± 0.04††	0.52 ± 0.06†	0.66 ± 0.05	0.40 ± 0.10††	0.58 ± 0.09†

[a] Mean ± S.E.M. of the last two behavioral tests.
* $P<.05$, as compared to the vehicle + DHT-treated group.
** $P<.01$, as compared to the vehicle + DHT-treated group.
*** $P<.001$, as compared to the vehicle + DHT-treated group.
† $P<.05$, as compared to the E_2 + DHT-treated group.
†† $P<.01$, as compared to the E_2 + DHT-treated group.
††† $P<.001$, as compared to the E_2 + DHT-treated group.

with the combined treatment of LNG + E_2 (Fig. 2; Table 2). Thus, the number of 5αLNG + E_2-treated Ss achieving intromission and ejaculation (50% and 25%, respectively) and the number of successful tests (29% and 8%, respectively) were significantly lower ($P < .05$) than those of animals treated with LNG + E_2, as well as of the control group receiving DHT + E_2 ($P < .001$). The incidence and number of behavioral responses shown by animals treated with 5αLNG + E_2 were not significantly different to those of the control group receiving vehicle + E_2 (Fig. 2; Table 2).

Administration of 3αLNG + E_2 or 3βLNG + E_2 activated mounting behavior in all Ss, but failed to restore ejaculatory activity. Indeed, the number of Ss in these groups displaying intromission and ejaculation and the number of successful

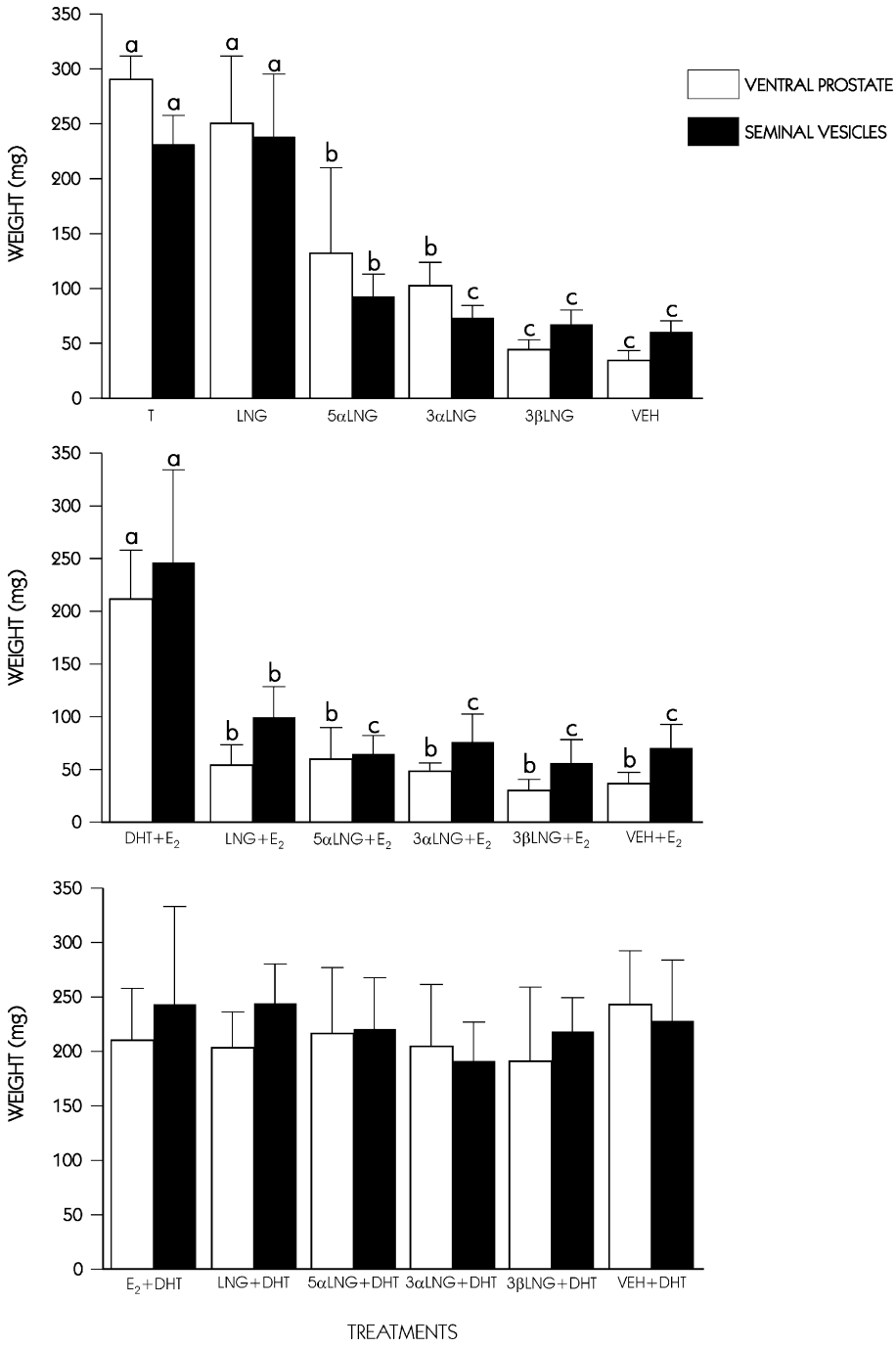

Fig. 4. Peripheral androgenic effects of LNG and its A-ring-reduced metabolites. Effects of 21 days of daily treatment with LNG, 5αLNG, 3αLNG, 3βLNG (1000 μg), or with their combination (300 μg) with either E_2 (5 μg) or DHT (300 μg), on accessory sex organ weight. Castrated rats treated with T (1000 μg), DHT (300 μg) + E_2 (5 μg), E_2 (5 μg) + vehicle (VEH), DHT (300 μg) + VEH, or VEH alone, served as controls. Data are presented as means ± S.D. Bars with different letters are significantly different from one another ($P < .05$, Student's t test) when comparing either ventral prostate or seminal vesicles weights.

tests were significantly lower than those of animals treated with LNG + E_2 ($P < .05$) or DHT + E_2 ($P < .001$) and similar to those of the vehicle + E_2 control group (Fig. 2; Table 2).

3.3. Effects of LNG and its derivatives given in combination with DHT

LNG + DHT treatment only partially restored copulatory behavior in castrated rats, in a similar manner to that observed in control animals receiving vehicle + DHT (Fig. 3; Table 3). The incidence of mounting, intromission, and ejaculatory responses was significantly lower ($P < .001$) than that of control, E_2 + DHT-treated animals.

On the contrary, treatments with 5αLNG + DHT or 3αLNG + DHT activated a higher level of copulatory behavior than that exhibited by control animals treated with vehicle + DHT (Fig. 3; Table 3). Indeed, 63% of Ss treated with these combinations displayed ejaculatory behavior, although in a lower proportion of tests (23%) than that observed in control E_2 + DHT-treated rats.

The 3βLNG + DHT combined treatment was able to activate mounting behavior in 75% of Ss, but it had very limited effects on intromission and ejaculatory responses, which were not significantly different to those observed in control animals receiving vehicle + DHT (Fig. 3; Table 3).

3.4. Peripheral effects of LNG and its derivatives

LNG treatment significantly increased ($P < .001$) the ventral prostate and seminal vesicles weights in castrated rats, as compared with those of control Ss receiving vehicle alone. The effects of LNG on rat sex accessories were similar to those observed in T-treated animals (Fig. 4). Treatment with 5αLNG induced a significantly lower effect in ventral prostate and seminal vesicles weights, as compared with LNG or T treatments. 3αLNG or 3βLNG induced very little, if any, effect upon the weights of ventral prostate and seminal vesicles of castrated rats (Fig. 4).

Combined administration of LNG or its A-ring-reduced derivatives with E_2 did not induce a significant modification on sex accessory organs weights, as compared with control Ss treated with vehicle + E_2 (Fig. 4). When LNG and its A-ring-reduced metabolites were simultaneously given with DHT to castrated animals, the ventral prostate and seminal vesicles weights were similar to those found in Ss treated with vehicle + DHT.

4. Discussion

The results presented herein indicate that the integrated analysis of male copulatory behavior in rodents is a suitable screening model to assess the hormone-like effects and potency of synthetic contraceptive progestins and their metabolic conversion products. The results also provide information on the mode of behavioral action of LNG and its metabolites at the CNS.

The restoration of sexual behavior in castrated male rats observed after the administration of large doses of LNG (1000 μg/day) was interpreted as demonstrating an androgen-like agonistic effect of this synthetic progestin (Fig. 1), particularly since the values of androgen-regulated behavioral parameters as the hit rate and the intromission and ejaculation latencies in LNG-treated animals were identical to those of T-treated animals used as a control group (Table 1). Lower doses of LNG (300 and 500 μg/day), although still effective, were less potent to activate copulatory behavior (data not shown). The androgenic effect of LNG at the CNS correlated well with its capability to restore the weight of the ventral prostate and seminal vesicles as noticed in the castrated LNG-treated rats (Fig. 4). These observations are in line with the relatively high binding affinity of LNG to the AR, at both central and peripheral hormone-sensitive tissues (Lemus et al., 1992).

The finding that 5αLNG was significantly less effective than LNG to restore copulatory behavior in castrated rats indicates that enzyme-mediated A-ring reduction of LNG diminishes its androgenic activity at the CNS in a similar manner to that observed following 5α-reduction of naturally occurring androgens (Meisel and Sachs, 1994; Morali et al., 1994; Whalen et al., 1985). Even more, 5αLNG was also significantly less potent than LNG to increase the weights of sex accessory organs in the castrated rats. The fact that 5α-reduction of LNG diminished its androgen-like potency at peripheral organs was unexpected, since 5αLNG binds with a relative higher affinity to the AR than LNG, its parent compound (Lemus et al., 1992). This striking finding is in a sharp contrast with the classic concept that 5α-reduction of naturally occurring androgens not only enhances their binding affinity to the AR (Liao et al., 1973; Martini, 1982) but also amplifies their androgenic effects at peripheral androgen-dependent organs (Dorfman and Shipley, 1956; Liao et al., 1973). The paradoxical effect of 5α-reduction upon the LNG molecule is rather similar to that reported for the NET molecule (Lemus et al., 1997; Morali et al., 1990). Since the nature of this unexpected phenomenon has been attributed, in the case of NET, to the presence of the ethynyl moiety at C-17 (Lemus et al., 1997) it seems plausible to extrapolate this explanation to LNG and other 19-nor progestin molecules bearing a 17α-ethynyl group.

Further 3α or 3β reduction of the 5αLNG molecule resulted in a complete abolishment of its androgen like effects at both central and peripheral levels. Indeed, 3αLNG or 3βLNG induced neither activation of copulatory behavior nor increase of sex accessory organs weights in the castrated rats, an observation that correlates with the lack of interaction of both LNG tetrahydro reduced derivatives with the AR (Lemus et al., 1992).

Treatments of LNG and its derivatives at a lower dose (300 μg/day) combined with E_2 were used to assess their capability to synergize as androgens with the behavioral

effects of E_2. The finding that only the LNG + E_2 combination was able to fully activate the copulatory behavior pattern in castrated animals, although with lower effectiveness than the control DHT + E_2 combination, demonstrated that the unchanged LNG molecule possesses intrinsic androgen-like activity. The observations that 5αLNG + E_2 failed to restore full copulatory behavior and that 3αLNG or 3βLNG + E_2 were completely ineffective suggest that A-ring reduction of the LNG molecule results in a diminution of its intrinsic androgenic potency, confirming the results obtained following the administration of LNG and its metabolites alone.

To assess whether LNG and its metabolites may induce estrogen-like behavioral effects, they were administered in combination with DHT to castrated rats. 5αLNG + DHT and 3αLNG + DHT were the only effective combinations capable to activate sexual behavior at a level higher than that of control animals receiving vehicle + DHT. These results suggest that A-ring-reduced LNG metabolites exert estrogen-like behavioral effects with a significantly lower potency than E_2, a finding that is in line with a recent report (Santillán et al., 2001) demonstrating that tetrahydro reduced LNG metabolites interact with the ER with a significantly low relative binding affinity and are able, at very high doses, to transactivate an estrogen-dependent yeast system co-transfected with the human ER gene and estrogen-responsive elements fused to a β-galactosidase reporter vector.

The potency of estrogen-like behavioral effects exerted by high doses of A-ring-reduced LNG derivatives was significantly lower than those previously reported for the corresponding NET metabolites (Moralí et al., 1990), an observation that correlates with their different binding affinities to the ER (Chávez et al., 1985; Santillán et al., 2001) and also with the well-known estrogenic effects observed after NET administration (Etchegoyen et al., 1983; Larrea et al., 1984; Pérez-Palacios et al., 1981; Vilchis et al., 1986).

Interestingly, the observation that 33% of castrated animals treated with vehicle + DHT used as a control group, exhibited full copulatory behavior, confirms previous reports indicating that DHT alone may induce sexual behavior in sexually experienced, castrated individuals of several mammalian species (Meisel and Sachs, 1994; Whalen et al., 1985). We have suggested that the behavioral effects of DHT are exerted by its own androgenic potency and the estrogen-like effects of its nonphenolic metabolites, the 3β,5α- and 3α,5α-androstanediols (Moralí et al., 1994).

The overall results demonstrate that LNG at high doses induces androgenic effects at both central and peripheral levels and a weak estrogenic activity mediated by its metabolic conversion products. All in all, the data underline the role of metabolism of LNG to explain some effects induced by nonpharmacological doses of this synthetic progestin.

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References

Ball MJ, Ashwell E, Gillmer MDG. Progestagen-only oral contraceptives: comparison of the metabolic effects of levonorgestrel and norethisterone. Contraception 1991;44:223–33.

Beyer C, Larsson K, Pérez-Palacios G, Moralí G. Androgen structure and male sexual behavior in the castrated rat. Horm Behav 1973;4:99–108.

Bowers A, Ringold HJ, Derot E. Steroids CL. 19-nordihydrotestosterone derivatives. J Am Chem Soc 1958;80:6115–21.

Brown HC, Krifhnamurthy S. Lithium-tri-*sec*-butyl borohydride. A new reagent for the reduction of cyclic and bicyclic ketones with superstereo selectivity. J Am Chem Soc 1972;94:7159–61.

Cabeza M, Vilchis F, Lemus AE, Díaz de León L, Pérez-Palacios G. Molecular interactions of levonorgestrel and its 5α-reduced derivative with androgen receptors in hamster flanking organs. Steroids 1995;60:630–5.

Catherino WH, Jeng MH, Jordan VC. Norgestrel and gestodene stimulate breast cancer cell growth through and oestrogen receptor mediated mechanism. Br J Cancer 1993;67:945–52.

Chávez BA, Vilchis F, Pérez AE, García GA, Grillasca I, Pérez-Palacios G. Stereospecificity of the intracellular binding of norethisterone and its A-ring reduced metabolites. J. Steroid Biochem. 1985;22:121–6.

Cravioto MC, Alvarado G, Canto-de-Cetina T, Bassol S, Oropeza G, Santos-Yung R, Valencia J, Palma Y, Fuziwara JL, Navarrete T, Garza-Flores J, Pérez-Palacios G. A multicenter comparative study on the efficacy, safety, and acceptability of the contraceptive subdermal implants Norplant® and Norplant®-II. Contraception 1997;55:359–67.

Dorfman RI, Shipley RA, editors. Androgens, biochemistry, physiology, and clinical significance. New York: Wiley, 1956 (590 pp.).

Etchegoyen G, Wolpert E, Galvan E, Landeros J, Pérez-Palacios G. Effects of synthetic steroid contraceptives on biliary lipid composition of normal Mexican women. Contraception 1983;27:591–603.

Garza-Flores J, Hall PE, Pérez-Palacios G. Long-acting hormonal contraceptives for women. J Steroid Biochem Mol Biol 1991;40:697–704.

Kuhl H. Comparative pharmacology of newer progestogens. Drugs 1996; 51:188–215.

Larrea F, Moctezuma O, Pérez-Palacios G. Estrogen-like effects of norethisterone on the hypothalamic pituitary unit of ovariectomized rats. J Steroid Biochem 1984;20:841–7.

Larrea F, Vilchis F, Chávez B, Pérez AE, Garza-Flores J, Pérez-Palacios G. The metabolism of 19-nor contraceptive progestins modulates their biological activity at the neuroendocrine level. J Steroid Biochem 1987; 27:657–63.

Larsson K. Features of the neuroendocrine regulation of masculine sexual behavior. In: Beyer C, editor. Endocrine control of sexual behavior. New York: Raven Press, 1979. p. 77–163.

Lemus AE, Vilchis F, Damsky R, Chávez BA, García GA, Grillasca I, Pérez-Palacios G. Mechanism of action of levonorgestrel: in vitro metabolism and specific interactions with steroid receptors in target organs. J Steroid Biochem Mol Biol 1992;41:881–90.

Lemus AE, Enríquez J, García GA, Grillasca I, Pérez-Palacios G. 5α-reduction of norethisterone enhances its binding affinity for androgen receptors but diminishes its androgenic potency. J Steroid Biochem Mol Biol 1997;60:121–9.

Lemus AE, Zaga V, Santillán R, García GA, Grillasca I, Damián-Matsumura P, Jackson KJ, Cooney AJ, Larrea F, Pérez-Palacios G. The oestrogenic

effects of gestodene, a potent contraceptive progestin, are mediated by its A-ring reduced metabolites. J Endocrinol 2000;165:693–702.

Lemus AE, Santillán R, Damián-Matsumura P, García GA, Grillasca I, Pérez-Palacios G. In vitro metabolism of gestodene in target organs: formation of A-ring reduced derivatives with oestrogenic activity. Eur J Pharmacol 2001;417:249–56.

Liao S, Liang T, Fang S, Castañeda E, Shao T.-C. Steroid structure and androgenic activity. J Biol Chem 1973;248:6154–62.

Martini L. The 5α-reduction of testosterone in the neuroendocrine structures. Biochemical and physiological implications. Endocr Rev 1982; 3:1–25.

Meisel RL, Sachs BD. The physiology of male sexual behavior. In: Knobil E, Neill JD, editors. The physiology of reproduction, vol. 2. New York: Raven Press, 1994. p. 3–106.

Montgomery DC. Design and analysis of experiments. Belmont: Wiley, 1991 (537 pp.).

Moralí G, Lemus AE, Oropeza MV, García GA, Pérez-Palacios G. Induction of male sexual behavior by norethisterone: role of its A-ring reduced metabolites. Pharmacol Biochem Behav 1990;37:477–84.

Moralí G, Lemus AE, Munguía R, Arteaga M, Pérez-Palacios G, Sundaram K, Kumar N, Bardin CW. Induction of male sexual behavior in the rat by 7α-methyl-19-nortestosterone, an androgen that does not undergo 5α-reduction. Biol Reprod 1993;49:577–81.

Moralí G, Oropeza MV, Lemus AE, Pérez-Palacios G. Mechanisms regulating male sexual behavior in the rat: role of 3α- and 3β-androstanediols. Biol Reprod 1994;51:562–71.

Pérez AE, Ortiz A, Cabeza M, Beyer C, Pérez-Palacios G. In vitro metabolism of ^{3}H-androstenedione by the male rat pituitary, hypothalamus, and hippocampus. Steroids 1975;25:53–62.

Pérez-Palacios G, Castañeda E, Gómez-Pérez F, Pérez AE, Gual C. In vitro metabolism of androgens in dog hypothalamus, pituitary, and limbic system. Biol Reprod 1970;3:205–13.

Pérez-Palacios G, Fernández-Aparicio MA, Medina M, Zacarías-Villareal J, Ulloa-Aguirre A. On the mechanism of action of progestins. Acta Endocrinol (Copenhagen) 1981;97:320–8.

Pérez-Palacios G, Moralí G, García GA, Cruz ML, Lemus AE. Behavioral effects of synthetic progestins. In: Mornex R, Jaffiol C, Leclère J, editors. Progress in endocrinology. Lancs: Parthenon, 1993. p. 155–9.

Phillips A, Demarest K, Hahn DW, Wong F, McGuire JL. Progestational and androgenic receptor binding affinities and in vivo activities of norgestimate and other progestins. Contraception 1990;41:399–410.

Piaggio G, von Hertzen H, Grimes DA, Van Look PFA. Timing of emergency contraception with levonorgestrel or the Yuzpe regimen. Lancet 1999;353:729.

Rebar RW, Zeserson K. Characteristics of the new progestogens in combination oral contraceptives. Contraception 1991;44:1–10.

Sachs BD, Barfield RJ. Functional analysis of masculine copulatory behavior in the rat. In: Rosenblatt JS, Hinde RA, Shaw E, Beer C, editors. Advances in the study of behavior, vol. 7. New York: Academic Press, 1972. p. 91–154.

Santillán R, Pérez-Palacios G, Reyes M, Damián-Matsumura P, García GA, Grillasca I, Lemus AE. Assessment of the oestrogenic activity of the contraceptive progestin levonorgestrel and its non-phenolic metabolites. Eur J Pharmacol 2001;427:167–74.

Schoonen WGEJ, Joosten JWH, Kloosterboer HJ. Effects of two classes of progestagens, pregnane and 19-nortestosterone derivatives, on cell growth of human breast tumor cells: I. MCF-7 cell lines. J Steroid Biochem Mol Biol 1995a;55:423–37.

Schoonen WGEJ, Joosten JWH, Kloosterboer HJ. Effects of two classes of progestagens, pregnane and 19-nortestosterone derivatives, on cell growth of human breast cells: II. T47D cell lines. J Steroid Biochem Mol Biol 1995b;55:439–44.

Sivin I, Stern J. Health during prolonged use of levonorgestrel 20 μg/d and the copper TCu 380Ag intrauterine contraceptive devices: a multicenter study. Fertil Steril 1994;61:70–7.

Smith H, Hughes GA, Douglas GH, Hartley D, McLoughlin BJ, Siddall JB, Wendt GR, Buzby GC, Herbst DR, Ledig KW, McMenamin JR, Pattison TW, Suida J, Tokolics J, Edgren RA, Jansen ABA, Gadsby B, Watson DHR, Phillips PC. Totally synthetic (±)-13-alkyl-3-hydroxy and methoxy-gona-1,3,5(10)-trien-17-ones and related compounds. Experientia 1963;19:394–6.

Stanczyk FZ, Roy S. Metabolism of levonorgestrel, norethindrone, and structurally related contraceptive steroids. Contraception 1990;42:67–95.

Still WC, Kahan M, Mitra A. Rapid chromatographic technique for preparative separation with moderate resolution. J Org Chem 1978;43: 2923–5.

Thau R, Jackanicz T. Contraceptive rings—a user-controlled long-acting method for family planning. In: Van Look PFA, Pérez-Palacios G, editors. Contraceptive research and development 1984 to 1994. The road from Mexico City to Cairo and beyond. Oxford: Oxford Univ. Press, 1994. p. 107–20.

Van der Burg B. Sex steroids and growth factors in mammary cancer. Acta Endocrinol (Copenhagen) 1991;125:38–41.

Van der Burg B, Kalkhoven E, Isbrücker L, de Laat SW. Effects of progestins on the proliferation of estrogen-dependent human breast cancer cells under growth factor-defined conditions. J Steroid Biochem Mol Biol 1992;42:457–65.

Vilchis F, Chávez B, Pérez AE, García GA, Angeles A, Pérez-Palacios G. Evidence that a non-aromatizable metabolite of norethisterone induces estrogen-dependent pituitary progestin receptors. J Steroid Biochem 1986;24:525–31.

Whalen RE, Yahr P, Luttge WG. The role of metabolism in hormonal control of sexual behavior. In: Adler N, Pfaff D, Goy RW, editors. Handbook of behavioral neurobiology. New York: Plenum, 1985. p. 609–63.

World Health Organization. Task force on post-ovulatory methods of fertility regulation. Randomized controlled trial of levonorgestrel versus the Yuzpe regimen of combined oral contraceptives for emergency contraception. Lancet 1998;352:428–33.