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Journal of Pharmacology & Clinical Toxicology

Research Article

Intracerebral Infusion of Levonorgestrel, but no other Synthetic Progestins, Induces Estrous Behavior Entirely through Progesterone Receptor

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Submitted: 23 October 2018

Accepted: 30 October 2018

Published: 31 October 2018

ISSN: 2333-7079

Keywords

- Levonorgestrel
- Medroxyprogesterone acetate
- Megestrol acetate
- Progesterone receptor
- Lordosis
- Proceptivity

Abstract

Synthetic progestins (SPs) Levonorgestrel (LNG), Medroxyprogesterone Acetate (MPA) and Megestrol Acetate (MGA) are more potent than progesterone to induce estrous behavior (lordosis and proceptive behaviors) in estrogen-primed rats. To test the role of progesterone receptor (PR) and estrogen receptor (ER) in the sexual response in rodents induced by SPs, three experiments were designed. In experiment 1, four dose levels (range 1.3-1300 ng) of the three SPs were infused into the right lateral ventricle (icv) to estrogen primed rats. All SPs induced significantly sexual behavior, and calculated ED50s showed that MPA was the most effective progestin to elicit the estrous behavior, followed by LNG and MGA. In experiment 2, the estrous behavior stimulating effects of all three SPs (at a dose of 1300 ng) were significantly reduced by the antiprogestin RU486, indicating that binding to the PR is required for these effects. Interestingly, tamoxifen (TMX) blocked the sexual behavior induced by MPA and MGA, but failed to block significantly the sexual behavior response induced by LNG. In experiment 3, was assessed the role of the Src/MAPK system in the facilitation of estrous behavior induced by the three SPs. Icv infusion of either PP2 (Src inhibitor) or PD98059 (MAPK inhibitor) prior of 1300 ng of SPs infusion fail to inhibit both lordosis and proceptive behaviors induced by the three SPs. The results point clearly toward an active role of the PR in the sexual behavior display elicited by the three SPs. Besides, the ER seems to be required only for the progesterone derivatives MGA and MPA and unnecessary for the androgen derivative LNG which is readily reduced at C5. The active system Src/MAPK related in some extent with the PR in neoplasic cells is not linked with the present results in the central nervous system.

INTRODUCTION

The participation of estrogen and progesterone (P) receptors are essential for the induction of female estrous behavior elicited in rodents by several agents with varied chemical structures, among them: steroids such as estradiol (E_{2}), progestins, and corticoids [1-3], peptides and proteins [4-8], biogenic amines [9,10], acetylcholine [11-13], cyclic nucleotides [14-19] and prostaglandins [7,20-23]. Sensory stimulation is also included, as occurs with the vaginocervical stimulation [24]. In addition, synthetic progestins (SPs) such as R5020, levonorgestrel (LNG), medroxyprogesterone acetate (MPA) or megestrol acetate (MGA), which are compounds that show high affinity for P receptors [25,26], and more agonistic effect than P, as LNG [27], and MGA [28], for inducing estrous behaviors (lordosis and proceptivity) in estrogen-primed rats.

SPs are substances structurally related to natural androgens and progestins and are functionally similar, but exhibit longer biological half-live than P. Chemically, like P, the three SPs studied bears a C3-carbonyl group at the A-ring. Interestingly, MPA and MGA are derivatives of 17α-hydroxyprogesterone, while LNG is a 19-nortestosterone derivative. Some of these progestins are metabolized to yield active compounds. Both MPA and LNG can be metabolized at the A-ring [29,30]. However, MGA is not biochemically reduced at C5 due to a double bond at C6 [31]. LNG yields readily to A-ring reduced compounds when incubated with rat pituitary and hypothalamus [29]. However, we have demonstrated that neither the C5 nor the C3-carbonyl group reductions are needed for the induction of estrous behavior by SPs [28].

The effects exerted by SPs on different aspects of reproduction have been widely studied, especially those actions related to the

development and growth of the uterus, i.e., progestogenic activity [32], and as contraceptives by inhibiting ovulation [26,33,34]. Biological effects at the cellular level are principally mediated by activation of the intracellular steroid receptors. However, the capacity of each SP to bind the P receptor (PR) varies; for example, LNG shows the greatest affinity for the PR, followed by MPA and then MGA [25,26,31]. Additionally, LNG has been reported to have no binding or transcriptional activity via the estrogen receptor (ER) [26], while MPA binds to the ER in the rat uterus and *in vitro* [35]. On contrary, other studies, have demonstrated that this progestin lacks of binding to the ER and therefore, devoid of estrogenic activity [36, 37].

At the present, it is unknown whether SPs exert membrane effects capable of activating second messenger signaling systems such as the Src/MAPK pathway or other kinases within brain regions involved in the induction of estrous behavior in rodents. It is known however, that MPA and LNG can modulate second messenger pathways in association with some physiological process [38,39]. For example, MPA increases activation of ERK while prevents its translocation to the cell nucleus, the latter is a necessary process by which estrogens and P induce neuroprotection [39]. In addition, it has been described that LNG stimulates cyclic AMP induction in veins, a process involved in relaxation in several models of constricted veins [40].

Interestingly, both PR and ER elicit membranal responses, independent of transcriptional mechanisms [41-43], by activating the Src/MAPK pathway. The interaction of the PR and ER with c-Src tyrosine kinase produces the stimulation of the Src/ER/PR/MAPK pathway in different intracellular systems [44-49]. We recently showed, that P and some of its A-ring reduced P metabolites induce lordosis behavior through the activation of Src kinase [50].

SPs have been used as an important tool to explore probable cellular mechanisms exerted by steroid hormones; thus, to assess the importance of reduction at C5 for stimulation of estrous behavior in the rat, three experiments were designed. Briefly: in experiment 1, was tested the capacity of various ICV dosages of MPA and LNG which eventually, may be biochemically reduced at C5. Similarly, MGA, which lacks of C5-reduction due to an additional double bond at C6 [31], was also evaluated for comparison to stimulate estrous behavior in ovariectomized (OVX) estrogen-primed rats. In experiment 2, were assessed the participation of both ER and PR in the estrous behavior induced by the ICV administration of these SPs. To accomplish this, their respective antagonists, tamoxifen (TMX) or RU486 were also administered. In experiment 3, was explored the possible role of the Src tyrosine kinase pathway in the estrus-facilitating actions of MPA, LNG, and MGA by concurrently administering PP2 and PD98059, which are inhibitors of Src kinase and MAPK respectively [51-53].

MATERIAL AND METHODS

Two hundred forty-two sexually inexperienced Sprague Dawley female rats (230-270 g body weight), bred in our colony in Tlaxcala City, were used. Females were maintained under controlled temperature (23 + 2°C) and light conditions (14h light: 10h dark; lights off at 1000 h). They were fed with Purina rat chow and water *ad libitum*.

Surgical procedure

Female rats were bilaterally ovariectomized (OVX) under anesthesia with xylazine (4mg/kg) and ketamine (80mg/kg) and group housed (4/cage). One week later, the females were anesthetized with xylazine (4mg/kg) and ketamine (80mg/kg) and placed in a Kopf stereotaxic instrument (Tujunga, CA) for implantation into the right lateral ventricle of a stainless-steel cannula (22 gauge, 17-mm length) following the Paxinos and Watson [54], atlas coordinates: anteroposterior +0.80 mm, mediolateral−1.5 mm, dorsoventral −3.5 mm with respect to bregma. A stainless-steel screw was fixed to the skull, and both the screw and cannula were attached to the bone with dental cement. A dummy cannula (30 gauge) provided with a cap was introduced into the guide cannula to prevent clogging and contamination. Immediately after each surgical procedure, the rats were injected with penicillin (165,000 IU/kg of procaine benzyl penicillin and 55,000 IU/kg of crystalline benzyl penicillin), and this continued for 3 days after surgery. After surgery, rats were housed individually in plastic cages with food and water available *ad libitum* for recovery until the test day. All of the experiments were performed under the guidelines of the Mexican Law of Animal Protection (NOM-062-ZOO-1999) under the approval and supervision of the Institutional Committee for the use and care of laboratory animals of Centro de Investigación y de EstudiosAvanzados.

Drugs

Estradiol benzoate (EB), and three SPs: medroxyprogesterone acetate (6α-methyl-3,20-di-oxopregn-4-en-17-yl acetate); levonorgestrel (13-β-ethyl-17α-ethynyl-17β-hydroxygen-4-en-3-one) and megestrol acetate (17α -acetyloxy-6-methyl-pregnan-4.6-diene-3,2O-dione) were used in this experiment. Steroids were dissolved in carthamus oil and EB was injected subcutaneously (sc, 0.1ml), while the SPs were injected ICV. All steroids were purchased from Sigma (St. Louis, MO). The ER antagonist TMX was dissolved in sesame oil vehicle and injected ICV , while the PR antagonist RU486 was dissolved in sesame oil: benzyl benzoate: benzoic alcohol (80:15:5) and injected sc. Both TMX and RU486 were purchased from Sigma Chemicals (St. Louis, MO). The specific inhibitor of the Src kinase family 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP2; [51,52]) and the MAPK inhibitor, PD98059 (2′-amino-3′-methoxyflavone; [53]) were prepared in 10% dimethylsulfoxide (DMSO) and purchased from Calbiochem (San Diego, CA). All protein kinase inhibitors were administered ICV.

Experiment 1. Establishment of dose-response curves and effective dose 50 (ED50) for MGA, LNG, and MPA administered ICV on estrous behavior of OVX EB-primed rats

The objective of this experiment was to test the effect of SPs on estrous behavior and to establish a dose response relationship. One week after intracerebral cannula implant, the females were primed with 5μg EB (sc, in 0.1ml), 40 h before SPs or vehicle (oil; n=9) injections. Dosages used for MPA were; 1.3ng (n= 11), 13ng (n= 9), 130ng (n= 10) and 1300ng (n= 11); for LNG; 1.3ng (n= 11), 13ng (n= 9), 130ng (n= 9) and 1300ng (n= 9); and MGA; 1.3ng (n= 9), 13ng (n= 8), 130ng (n= 10) and 1300ng (n=

9). These dosages were selected from previous studies published by us [27].

Experiment 2. Effect of TMX and RU486 on the induction of lordosis behavior by SPs

In this experiment, we tested the idea that lordosis induced by SPs are mediated by ER and PR. One week after cannula implantation rats were primed with 5µg/100µl of EB, administered sc. At 39.5 hr after EB priming, we administered one of the following treatments, in order to respectively test the effects of TMX or RU486 on SPs-induced lordosis: (1) 5µg TMX combined with 1300ng of MPA (n= 10), LNG (n= 10), or MGA (n= 10). (2) 5mg RU486 combined with 1300ng of MPA (n= 12), LNG (n= 10) and MGA (n= 8). Dose, schedule and via of injection of TMX and RU486 were selected from previous results showing the optimal inhibitory effect of these compounds [55,56].

Experiment 3. Effect of PP2 and PD98059 on the induction of lordosis by SPs

As in the previous experiments, OVX animals were primed 5µg of EB. At 39.5hr later, the following treatments were initiated: (1) 30µg of PP2 was infused ICV, followed 15 min later by 1300ng of MPA (n= 10), LNG (n= 9) or MGA (n= 8). (2) 3.3µg of PD98059 was infused ICV, followed 15 later by 1300ng of MPA (n= 11), LNG (n= 10), or MGA (n= 10). Doses of the inhibitors and the schedule of injection were established based on previous results that showed the optimal inhibitory effect of these treatments to counteract the estrous behavior induced by several agents [50].

Testing procedures

The tests for estrous behavior (receptivity and proceptivity) were conducted 60, 120, and 240 min after infusion of SPs, by an experimenter blind to treatment groups. Thus, the female rat was placed in a circular Plexiglas arena (53 cm diameter) with a sexually active male rat. The lordosis quotient [LQ = (number of lordosis / 10 mounts) × 100] and lordosis score (LS) were used to assess receptive behavior in response to the first 10 mounts. LS refers to the intensity of lordosis, which is quantified according to Hardy and DeBold [57]. This scale ranges from 0 to 3 for each individual response and consequently, from 0 to 30 for each female that received 10 mounts. Proceptivity behavior was studied by determining the incidence of hopping, darting, and ear-wiggling across the whole receptivity test. The proportion of females displaying at least two of these behavioral patterns was analyzed.

Statistical analysis

Regression lines for the dose-response curves of the three SPs explored in this study and ED50s were calculated according to Tallarida and Murray [58].

The effects of the TMX, RU486, PP2, and PD98059 on the induction of estrous behavior by MPA, LNG, and MGA (experiments 2 and 3) were assessed by comparing the LQs obtained with these agents alone versus those obtained when inhibitor agents were co-administered. Since the distribution of LQ values in same groups was not normal, the Wilcoxon–Mann–Whitney test was used to compare two independent groups [59, 60]. This test is an excellent alternative to the t-test with a power efficiency of 95.5% of the parametric test [59,60]. Fischer's exact probability test was used to compare the proportions of proceptive females between experimental groups [59,60].

RESULTS

The three SPs showed similar effect inducing sexual behavior display in both lordosis and proceptivity but at different extent. In addition the antagonists TMX and RU486 which binds the ER and PR respectively, gave important data on the action mechanism of the SPs.

Experiment 1: Determination of dose-response curves and effective dose 50 (ED50) for MGA, LNG, and MPA.

Figure 1 shows dose-response relationships for lordosis and proceptive behaviors induced by each of the SPs, tested at 60, 120, and 180 min after the ICV SP administration. The control (oil, black bar) group showed very low levels of lordosis and did not display proceptivity. A linear relationship was observed for MGA and LNG at each of the three times tested, excepting MPA. A regression analysis for each of the three compounds at 240 min, which was the time at which the best response was obtained, showed that MPA was the most potent of the SPs with respect to lordosis induction. The ED50 values for lordosis behavior were as follows: MGA= 31.5ng, LNG= 6.4ng and MPA= 1.5ng while the proceptivity ED50s were: MGA= 89ng, LNG= 66ng and MPA= 2.5ng.

Experiment 2. Effect of the antagonists TMX and RU 486 on the effectiveness of SPs for eliciting lordosis Behavior

Figure 2 shows the effects of TMX and RU486 on lordosis and proceptive behaviors induced by MPA, LNG, and MGA. The lordosis response induced by MPA was significantly reduced by both TMX and RU486, at 60 ($p< 0.05$), 120 ($p< 0.01$) and 240 min ($p< 0.001$). Proceptive behavior induced by MPA was also inhibited at 120 ($p< 0.05$) and 240 min ($p< 0.01$). Likewise, MGA lordosis and proceptivity were inhibited by both TMX and RU486 at 120 and 240 min. Interestingly, lordosis induced by LNG was inhibited by RU486 at 120 ($p< 0.5$) and 240 ($p< 0.01$) min, but not by TMX which was clearly ineffective. However, the proceptive behavior induced by LNG was significantly inhibited by both RU486 and TMX at 120 and 240 min ($p<0.01$, $p< 0.05$ respectively). (See Figure 2 for the values of significance).

Experiment 3. Effect of PP2 and PD98059 on estrous behavior induced by SPs in EB-primed rats

Table 1 shows the effect of PP2 and PD98059 on lordosis and proceptive behaviors induced by MPA, LNG, and MGA. Neither PP2 nor PD98059 inhibited SPs-induced lordosis or proceptive behaviors to the different times tested.

DISCUSSION

The present data show that the ICV administration of all SPs to EB primed rats, induced estrous behavior, with different extent. MPA for instance, was the most effective to induce estrous behavior, followed by LNG. Interestingly, when these compounds are administered subcutaneously the opposite occurs, being LNG which showed the most agonist effect to induce these

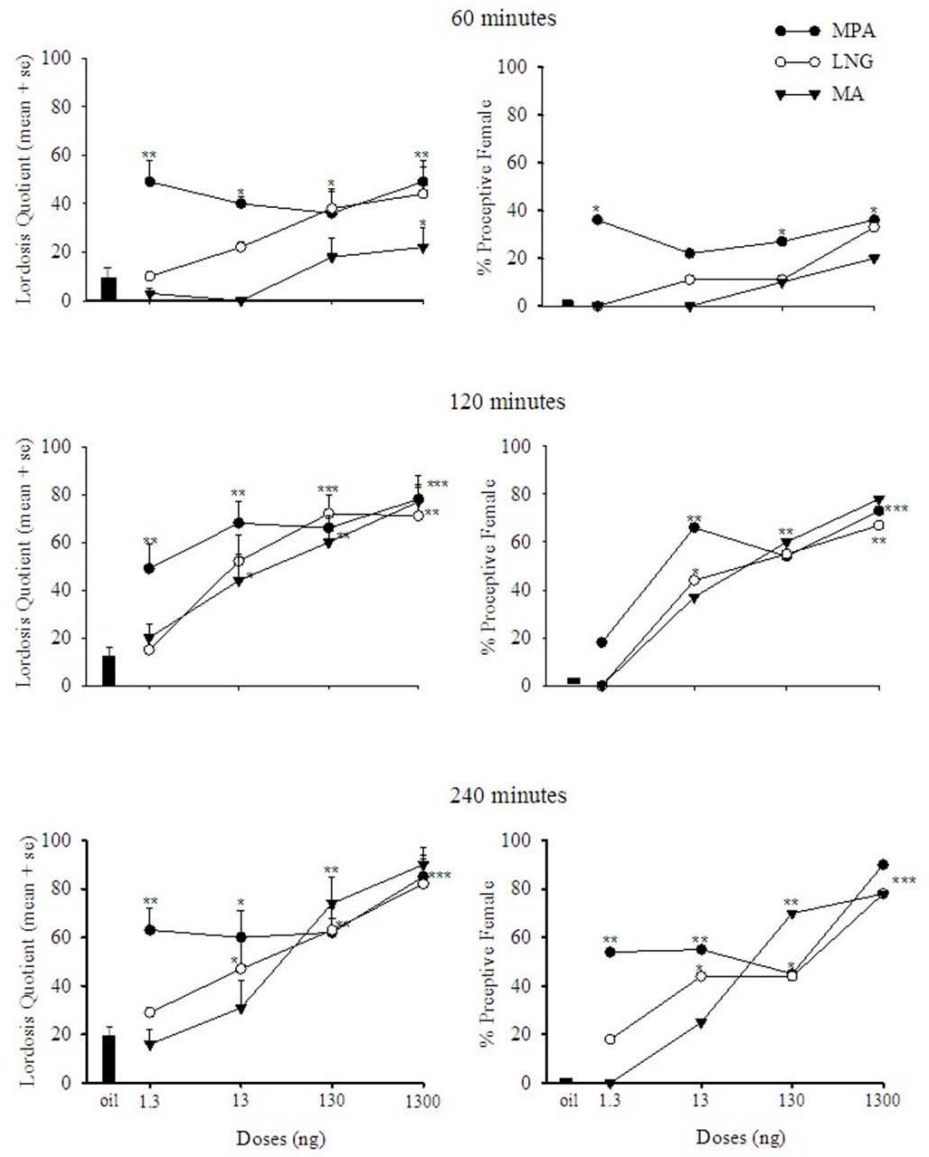

Figure 1 Effect of icv injection of Medroxyprogesterone Acetate (MPA), Levonorgestrel (LNG) and Megestrol Acetate (MGA); 1.3, 13, 130 and 1300 ng, or oil, inovx, EB-primed rats on: lordosis quotient (LQ; panel A) and % Proceptive behavior (LS; panel B). Females were tested at 60, 120, and 240 min after injection of progestins or oil. ***p< 0.001, **p< 0.01, *p< 0.05 vs oil.

behaviors in estrogen-primed rats [27]. In addition, we also find that full lordosis and proceptive behaviors can be displayed by those SPs that can be reduced at C5, that is: LNG and MPA. In fact, the A-ring reduction favors the expression of female estrous behavior, as showed with the intravenous injection of C5-reduced P metabolites such as: dihydroprogesterone and allopregnanolone, which induce intense estrous behavior in estrogen-primed rats [55]. Notably, MGA was the progestin with the lowest behavioral effect, likely because the C5 reduction is unfavorable sterically because its double bond at C6. According to Kincl [61], chlormadinone with similar C6=C7 double bond as MGA, possessed less than 25% of the potency of P to induce estrous behavior in estrogen-primed rats.

All SPs have bind human, rabbit and rat PR with higher relative binding affinity than progesterone [25,62, 63]. Regarding this work, the following binding affinities order can be considered LNG> MPA> MGA [64, 65]. This is important since the present results support the participation of PR in the induction of estrous behavior by SPs, because of the antiprogestin RU486 reduced estrous behavior induced by the three SPs. Moreover, lordosis induced by natural progestins is significantly inhibited by intracerebral or systemic injection of RU486 [55,66] and the combination of RU486 with some SPs in reproductive organs such as: hyperplasia of mammary glands induced by MPA, or inhibition in the prolactin secretion induced by LNG both are reduced markedly by RU486 [67,68]. Regarding the ER, it has been reported that MPA, LNG, and MGA do not bind to this receptor, therefore, transcriptional effects mediated by this receptor are improbable [36,37]. Surprisingly, in the present study, estrous behavior induced by MPA and MGA was blocked by TMX (selective modulator of ER), while lordosis induced by

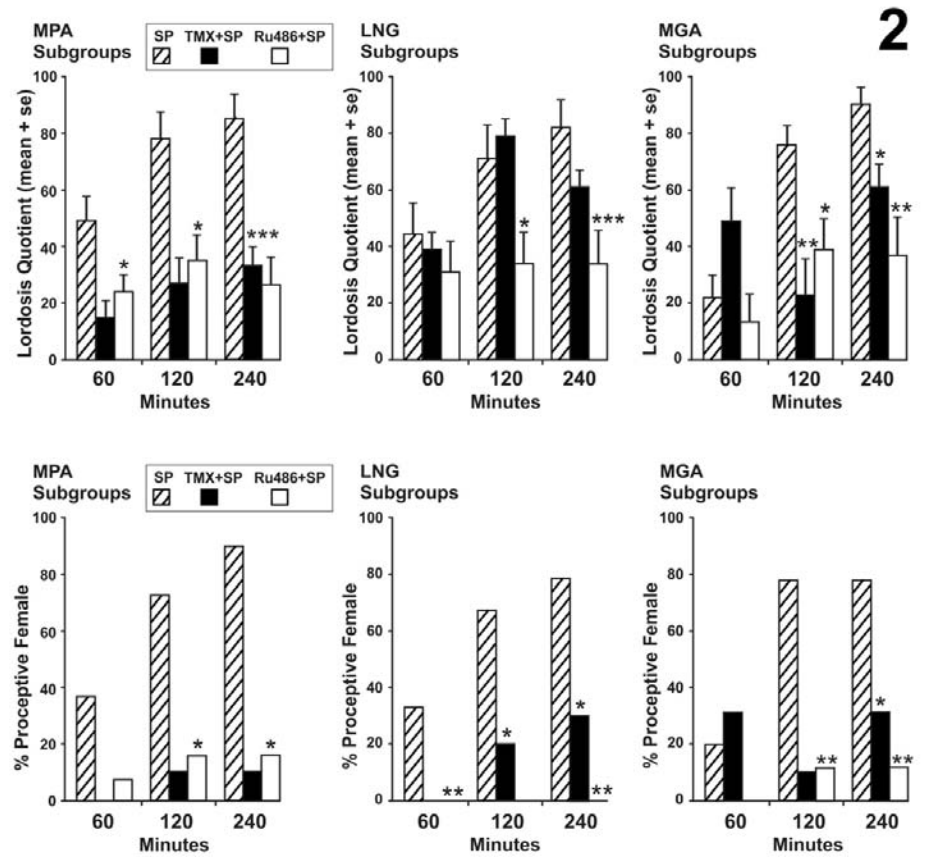

Figure 2 Icv effect of tamoxifen (TMX) and RU486 on lordosis and proceptivity behaviors induced by icv infusion of 1300 ng of MPA, LNG and MGA, in ovx, EB primed rats at 60, 120 and 240 min after SP administration. RU486 significantly blocked estrous behavior induced by the three progestins, while TMX did not block lordosis or proceptivity induced by LNG. ***p< 0.001, **p< 0.01, *p< 0.05 vs progestin synthetic (PS) + antagonist.

Table 1: Effect of PP2 (Src tyrosine kinase inhibitor) and PD98059 (MAPK inhibitor) on the facilitation of estrous behavior by Medroxyprogesterone Acetate (MPA), Levonorgestrel (LNG) and Megestrol Acetate (MGA) in ovariectomized estrogen primed (5 mg EB) rats.

Progestina	n	1h		2h		4h	
		LQ	% Proceptivity	LQ	% Proceptivity	LQ	% Proceptivity
MPA	11	49 ± 9	36	78 ± 10	72	85 ± 9	91
MPA+PP2	10	26 ± 6	0	54 ± 11	40	42 ± 13	40
MPA+PD	11	28 ± 11	36	76 ± 12	72	63 ± 14	63
LNG	9	38 ± 8	11	72 ± 8	55	63 ± 5	44
LNG+PP2	9	68 ± 9	55	76 ± 6	78	91 ± 5	89
LNG+PD	10	19 ± 9	20	67 ±12	20	68 ± 10	0
MA	9	22 ± 8	22	76 ± 8	78	90 ± 7	78
MA+PP2	8	34 ± 7	0	59 ± 9	25	69 ± 6	37
MA+PD	10	44 ± 10	30	78 ± 10	60	73 ± 12	70

LQ: lordosis quotient; MPA: medroxyprogesteroneacetate; LNG; levonorgestrel; MA: megestrolacetate; PP2; Srckinaseinhibitor; PD: PD98059, MAPK inhibitor.

LNG was not inhibited by TMX. Some, *in vitro* studies however, have showed that TMX inhibited MPA-induced cell proliferation of breast cancer cells [69], indicating that, at least *in vitro*, TMX can inhibit MPA-mediated processes. Additional experiments on this issue are needed to address this doubtful finding.

Regarding the proceptive behavior, which appears immediately after the lordosis response, it is known that is largely dependent upon the action of P [70-73], involving the binding of the hormone with its receptor. The present results agree with this possibility, since RU486 lowered statistically the proceptivity induced by the SPs treatment. In addition, the proceptive behavior induced by several P metabolites is reduced by intracerebral and intravenously administration of RU486 [55,66].

Unexpectedly, proceptive behavior induced by the three SPs was significantly reduced by TMX administration, pointing toward an interaction of TMX with the PR. Since PR and ER belong to the same receptor family in our experimental condition an interaction steroid receptors-TMX interaction may occur, however experimental work in this regard is needed.

On other side, the fact that several steroids can exert effects on membrane receptors generates an additional level of complexity in the mechanism of action of steroid hormones. Cross-talk between a membranal (non-genomic) and genomic signaling via steroid receptors is suggested to play a role in the induction of female estrous behavior. For example, progestins have been shown to activate various pathways such as the Src and MAPK signaling cascades. This non-genomic signaling by progestins is primarily thought to be mediated by the classic PR and ER [44-49]. The Src system is an important integrator of steroid receptor signaling because it has an SH2 domain that binds directly to ERα as well as an SH3 domain which interacts with the PR. This interaction, in turn, activates MAPK, for example, in breast cancer cells. Migliaccio et al. [49], found that E2 activated both Src and MAPK, and that Src interacts with both ER and PR. Based in such data, it seems appealing to explore the SPs, which show good affinity for the PR, might exert some physiological effects by modulating the Src/ER/PR'MAPK intracellular mechanism in the central nervous system. Oppositely, the estrous behavior induced by SPs was not blocked by either PP2 or PD98059 (Src and MAPK inhibitors respectively), showing that the Src signaling pathway is not involved for the expression of proceptive and lordosis behaviors. However in oncology this signaling appears to be important since in an experimental model of hormonal carcinogenesis, MPA treatment induced rapid Src tyrosine phosphorylation [71], while immunoblotting and kinase assay in MCF-7 cells, MPA induced a late decrease in MAPK activities [74]. Regardless of the previous data, we recently showed that several agents that do not bind the PR, like ring A reduced progestins, GnRH and prostaglandin E2, are capable to induce lordosis behavior by activating the Srcsystem, since both inhibitors PP2 and PD98059 reduced the facilitating effects of these compounds [50, 70].

In conclusion, the present results support the fact that strong binding to the PR and A-ring reduction, two important bio-pharmacological properties of MPA and LNG, are adequate to facilitate normal estrous behavior in estrogen-primed rats. MGA with the lowest potency to induce the sexual response is as well, resistant to the enzymatic A-ring reductions. An important difference observed that MPA and MGA both progesterone derivatives were ER dependent since the TMX administration blocked the sexual behavior display induced by both progestins. On contrary, the sexual induction produced by LNG the androgen derivative was not hampered by TMX resulting efficacious to elicit the behavioral response. Finally, the Src/MAPK pathway functioning along with the PR in neoplastic cells, is not active in the present behavior model, dependent of the central nervous system.

ACKNOWLEDGEMENTS

Proyecto apoyado por el fondo sectorial de investigación para la educación. Proyecto 255936 CONACYT CB-2015-01.

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Cite this article

González-Flores O, Gómora-Arrati P, García-Juárez M, Encarnación-Sánchez JL, Lima-Hernández FJ, et al. (2018) Intracerebral Infusion of Levonorgestrel, but no other Synthetic Progestins, Induces Estrous Behavior Entirely through Progesterone Receptor. J Pharmacol Clin Toxicol 6(6):1124.