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Time course of estrogen and progesterone induced lordosis in mice

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Introduction

The neuronal circuitry within the hypothalamus and limbic system that regulates sexual and reproductive behavior in mice relies on hormonal interactions. Specifically, the actions of estrogen and progesterone in the medial preoptic nucleus (MPN) plays an important role in facilitating lordosis, a reflex indicating sexual receptivity, wherein the female arches her back, slightly lifts her head, and moves her tail to the side. Estrogen acts through estrogen receptor-**α** (ER-**α**) which signals through metabotropic glutamate receptor in the arcuate nucleus to release endogenous opioids that bind to m-opioid receptors (MOR) in the MPN (Eckersell et. al., 1998; Long et. al., 2014; Sinchak and Micevych, 2001). Binding of these ligands induces internalization of MOR which can be modelled as activation of MOR in the MPN (Eckersell et. al., 1998; Sinchak and Micevych, 2001). Studies have demonstrated the translocation of MOR from the plasma membrane to the interior of the cell following estrogen treatment and the accentuation in MOR fiber density, suggesting that estrogen stimulates the release of endogenous peptides that further causes MOR internalization. (Eckersell et. al., 1998). Subsequently, activation of the MPN inhibits lordosis (Sinchak and Micevych, 2001).

The transient inhibition of lordosis is relieved by progesterone which reverses the activation of MOR (Sinchak and Micevych, 2001). Interestingly, estrogen increases the expression of progesterone receptors (PR) and neuroprogesterone (neuroP) through binding with ER-**α**, dimerization of the receptor, and inducing gene transcription after binding DNA (Fitzpatrick et. al., 1999; Micevych and Sinchak, 2008a; Micevych et. al., 2008b). Estrogen induction of PR mRNA expression has been demonstrated abundantly in the anteroventral periventricular nucleus of the hypothalamus (AVPV), a region that has also been shown to be important in regulating gonadotropin-releasing hormone and luteinizing hormone (LH) (Simerly et. al., 1996). This mechanism not only increases expression of progesterone to facilitate lordosis, but also correlates with estrogen positive feedback in the estrous cycle. Prior to the LH surge, rising estrogen levels in proestrus allow for the increasing levels of progesterone in the hypothalamus, leading into estrogen positive feedback and the LH surge that induces ovulation (Micevych et. al., 2008). Without this increase in hypothalamic progesterone, the LH surge fails to occur, suggesting that neuroP is necessary for estrogen positive feedback which in turn stimulates the LH surge (Micervych et. al., 2008).

Similarly, increased progesterone expression in the MPN facilitates sexual receptivity in the form of lordosis in mice. Treatment with progesterone significantly decreases the estrogen-induced MOR fiber density, signifying deactivation of MOR in the MPN, and allowing for lordosis behavior (Sinchak and Micevych, 2001). In parallel, comparison between male and female mice exhibit higher levels of progesterone in the MPN of males than females (Quadros and Wagner, 2008). This finding is due to the highly estrogen-dependent progesterone expression in the MPN and the corresponding differential estrogen exposure in females (Quadros and

Wagner, 2008). Essentially, the temporary inhibition of sexually receptive behaviors through estrogen and the following relief by progesterone allows for the expression of lordosis.

While the circuitry between estrogen and progesterone in the MPN has been well-studied in rats, the specific time course of these actions in mice has not yet been elucidated. This study aims to understand the duration of estrogen's effects in the MPN, as well as the length of time required before progesterone can reverse the inhibition on lordosis. As follows, we performed lordosis testing in ovariectomized female mice with hormone treatment at various time points and observed PR and MOR expression in the AVPV and MPN regions.

Methods

Adult C57BL/6J male and female mice (Postnatal day 60 (P60), Jackson Laboratories #000664) were used. Female mice were ovariectomized and allowed two weeks to recover before beginning hormone treatment. All mice were housed in a climate-controlled room, two per cage, on a 12:12 light/dark cycle, and provided ad libitum food and water. All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles.

Female mice were randomly assigned to one of three groups (Table 1): 1) 24 hours between estrogen and progesterone administration, 2) 16 hours between estrogen and progesterone administration, or 3) 48 hours between estrogen and progesterone administration. All female mice were subcutaneously (sc) injected with 20ug estradiol dissolved in safflower oil 24 hours apart on days 1 and 2, and 500ug progesterone dissolved in safflower oil either 24 hours, 16 hours, or 48 hours later, depending on group condition (Table 2). Five hours after progesterone administration, females began behavior testing. Adult male mice were placed in a chamber and allowed ten minutes to adapt to the new environment. Subsequently, the female mouse was placed with the male for a duration of fifteen minutes, or until the male mounted the female ten times. The lordosis quotient (LQ) (the number of times the female displayed lordosis out of 10 mounts, multiplied by 100) was calculated. Each set of the four-day hormone treatment with behavioral testing was executed three times for each female and the results averaged.
One hour after the third behavioral test, females were heavily anesthetized and perfused transcardially with saline followed by 4% paraformaldehyde in Sorenson's buffer (pH 7.4), and their brains harvested. The brains were post-fixed in paraformaldehyde for 24 hours at 4C before being switched into a solution of 30% (w/v) sucrose in a phosphate buffer for 48 hours at 4C, and then flash frozen in hexanes cooled on dry ice before being sectioned with a cryostat (Leica Biosystems, CM1950). Sections were cut coronally at 25um and stored in a cryoprotectant solution at -20C until use.

Table 1 Experimental Group

Group	Hormone Treatment	Interval between day 2 EB and P4	*n*
Group 1	EB+P4	24 hours	4
Group 2	EB+P4	16 hours	4
Group 3	EB+P4	48 hours	4

Table 2 Hormone Treatment

Group 1	Day 1 20 ug EB → 24 hours→ 20 ug EB → 24 hours→ 500 ug P4
Group 2	Day 1 20 ug EB → 24 hours→ 20 ug EB → 16 hours→ 500 ug P4
Group 3	Day 1 20 ug EB → 24 hours→ 20 ug EB → 48 hours→ 500 ug P4

Sections identified with the medial preoptic nucleus and anteroventral periventricular nucleus intact were processed for MOR or PR immunohistochemistry (IHC) (See Table 3). Sections were washed three times for 10 minutes each in Tris-buffered saline (TBS, pH 7.4) on a rotating table at room temperature (RT), then placed in a blocking solution of 2% normal donkey serum (NDS, Equitech-Bio #SG30-0500) and 0.03% Triton-X 100 (Sigma-Aldrich, #X100-100ML) for one hour at RT. Sections were placed in a solution containing either rabbit anti-MOR or rabbit anti-PR and allowed to incubate on a rotating table for 72 hours at 4C. Sections were washed as before and then placed in a corresponding fluorescent secondary antibody solution (See Table 4) on a rotating table for 24 hours at 4C. Finally, sections were again washed in TBS then mounted onto SuperFrost slides (Fisher Scientific, #12-550-15). Once dry, slides were applied with a mounting medium containing DAPI (DAPI Fluoromount-G, Southern Biotech #0100-20) before being coverslipped, sealed with nail polish, and stored in the dark at -20C.

Sections were analyzed using a Leica Aperio VERSA Slide Scanner equipped with LAS X Life Science software suite (Leica Biosystems). Sections were imaged with a 20x objective (HC PL APO 20x/0.80), resulting in a final optical magnification of 200x.

Table 3 Primary Antibody

Section	Primary	Dilution
MPN	Rabbit anti-MOR	1:4000
AVPV	Rabbit anti-PR	1:8000

Table 4 Secondary Antibody

Antibody	Dilution
Alexa Fluor 488 Goat anti rabbit IgG	1:2000

Results

Lordosis Behavior

A one-way ANOVA was performed to compare the LQ results of the 24-,16-, and 48- hour time groups. There was a statistically significant difference in LQ scores between mice that received progesterone treatment 16 hours after estrogen displaying a reduction in the LQ (7 ± 8.16) and mice that received progesterone treatment 24 hours after estrogen (71 ± 8.29; $p<0.005$; Fig. 1), but no statistically significant difference in LQ scores between the 16 and 48 hour (25 ± 24.09) time groups ($p=0.46$; Fig. 1). Despite a lack of significance, the 48 hour time group LQ scores did seem to trend higher than the 16 hour time group (Fig. 1). Between the 24 and 48 hour time groups, there was a statistically significant difference ($p=0.0095$; Fig. 1), with the 48 hour group displaying reduced LQ scores.

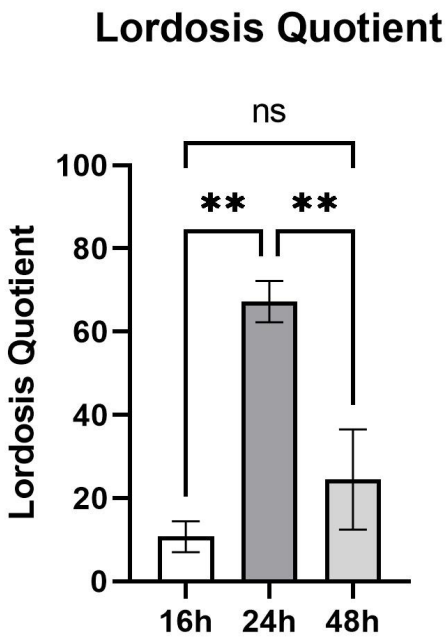

Figure 1 Lordosis quotient for 16-, 24-, and 48-hour time group. Significantly increased lordosis score for 24-hour time group in comparison to 16- and 48- time group; no significant difference between 16- and 48- hour time group

PR Cell Count

Cells were counted within the boundaries drawn for the AVPV region in each image. No significant differences in the areas of the AVPV regions between the 16-, 24-, and 48-hour time group were detected using a one-way ANOVA.

A one-way ANOVA was performed to compare the cell counts of the AVPV sections stained with PR antibody of the 24-, 16-, and 48- hour time groups. No statistically significant difference was found between either of the groups (16 vs 24 hour, $p=0.14$; 16 vs 48 hour, $p=0.15$; 24 vs 48 hour, $p=0.10$; Fig. 2). However, the PR cell count of the 16 hour group (138 ± 45.96) trended upwards in comparison to both the 24 (65 ± 24.82) and 48 (67 ± 53.63) hour time groups.

MOR Cell Count

Cells were counted within the boundaries drawn for the MPN region in each image. No significant differences in the areas of the AVPV regions between the 16-, 24-, and 48-hour time groups were observed using a one-way ANOVA.

A one-way ANOVA was performed to compare the cell counts of the MPN sections stained with MOR antibody of the 24-, 16-, and 48- hour time groups. No statistically significant difference was found between either of the groups (16 vs 24 hour, $p=0.77$; 16 vs 48 hour, $p=0.97$; 24 vs 48 hour, $p=0.86$; Fig. 3).

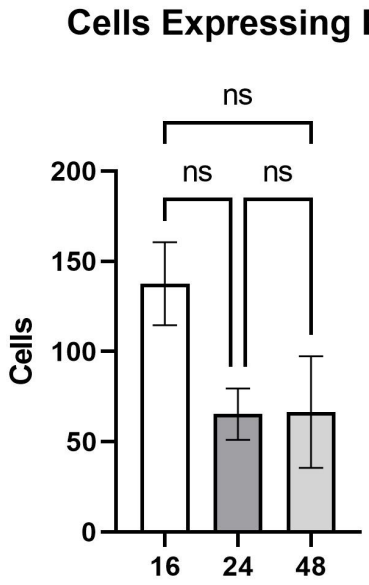

Figure 2 PR cell counts for 16-, 24-, and 48-hour time group in AVPV region. No significant difference between 16- , 24-, and 48-hour time group, yet apparent upward trend in 16-hour time group

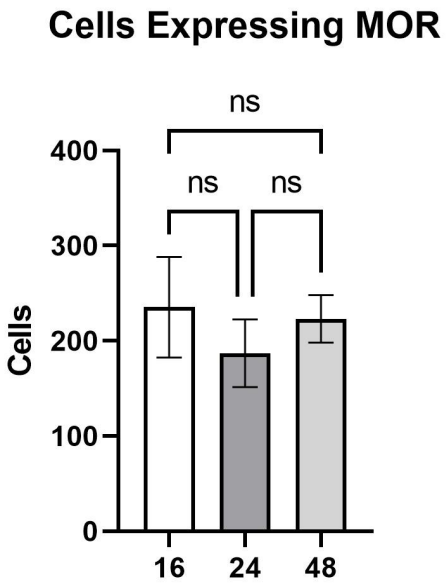

Figure 3 MOR cell count for 16-, 24-, and 48-hour time group in MPN region. No significant difference between 16-, 24-, and 48-hour time group

Discussion

From previous studies, it is evident that estrogen induces both the activation of MOR—which inhibits lordosis behavior—as well as the production of PR to which progesterone binds in order to alleviate the inhibition of sexual receptivity (Eckersell et. al., 1998; Sinchak and Micevych, 2001). Estrogen-induced PRs are found only in neurons containing the estrogen receptor, while mutations in estrogen responsive elements of the PR promoter region results in the attenuation of PR production, further supporting estrogen's role in the formation of PR (Shughrue et. al., 1997).

Studies done in rats through nuclear run-on assays exhibit PR gene transcription following estrogen injection beginning to increase at 1 hour with a value 3.5 times larger at 24 hours, and PR mRNA levels appearing only after 6 hours post-estrogen treatment and continuing to rise until 18 hours (Lee and Gorski, 1996). This 6 hour period agrees with the time point required for anisomycin—an antibiotic that inhibits the synthesis of proteins in the brain—injected into the preoptic area and medial basal hypothalamus of rats to reduce lordosis activity (Rainbow et. al., 1980). Implicated in this study is the transcriptional and translational processes caused by estrogen for the production of PR. As lordosis behavior is seen given an 18 to 24 hour time period between estrogen and progesterone treatment, the time gap between the appearance of PR mRNA and the behavioral response can be explained by post-translational processing or intracellular movement of proteins (Rainbow et. al., 1980).

Results of this study demonstrate that lordosis is significantly reduced when progesterone is administered 16 hours after estrogen treatment in female mice in comparison to the 24 hour group, suggesting that this refractory period is due to the time required for the estrogen-induced

production of PR (Figure 1). However, the corresponding upward trend in PR cell count as compared to the 24 hour group does not agree with the absence of lordosis behavior (Figure 2). These results also contrast previous studies done in guinea pigs that demonstrate an increase in PR concentration appearing 24 hours after estrogen, roughly the same time as the onset of sexual receptivity (Blaustein and Feder, 1979).

This study also demonstrates a significantly reduced lordosis score for the 48 hour time group in comparison to the 24 hour time group, with no significant difference to the 16 hour time group (Figure 1). Similar to the 16 hour time group, the reduction in sexual receptivity may be due to the inability of progesterone to reverse the estrogen-induced inhibition of lordosis mediated by the internalization of MOR. In contrast, the 48 hour time group did demonstrate a slightly elevated lordosis score in comparison to the 16 hour time group, although not significant, perhaps explaining the PR cell count of the 48 hour time group being comparable to that of the 24 hour time group. In the rat ventromedial hypothalamus and arcuate nucleus, PR mRNA values and grains per cell—estimated as the number of messages per cell—begin to decline at 48 hours ensuing estrogen treatment, also in accordance with the decrease in sexual receptivity for rats (Romano et. al., 1989). The decrease in PR mRNA values in rats suggests a reduced production in PR to respond to progesterone in order to facilitate lordosis, with the 48 hour time point agreeing with the current data. As 48 hours is the first time point at which Romano et. al. (1989) noted a decrease in PR mRNA, the lack of difference between the 24 and 48 hour PR cell counts may be explained by the delay from translation, from mRNA to the protein product. In a similar manner, PR concentration in guinea pigs reached maximal values at 64 hours, with a significant reduction by 88 hours, guiding the peak of lordosis behavior at 40 hours and a significant decline measured at 91 hours (Blaustein and Feder, 1979). While these maximal times points of sexual receptivity do not agree with our results, this variability could be due to species differences.

The increased PR expression in the 16 hour time group may be due to the preparation for the luteinizing hormone (LH) surge rather than a marker for lordosis. Negative feedback control on gonadotropin releasing hormone (GnRH) secretion is maintained with low levels of estrogen (Stephens et. al., 2015). During proestrus, estrogen levels begin to rise, leading into estrogen positive feedback and the induction of the preovulatory LH surge (Stephens et. al., 2015). This point also coincides with the highest PR-B expression and elevated Kiss1-expressing cells--neurons that encode kisspeptin--in the afternoon of proestrus in comparison to the morning of proestrus or estrus (Guerra-Araiza et. al., 2000; Smith et. al., 2006). *Kiss1* neuron density is modulated by estrogen, as *Kiss1* neurons express ERα, such that estrogen increases Kiss1 levels in the AVPV (Stephens et. al., 2015). In turn, *Kiss1* neurons in the AVPV regulate the LH surge, as *Kiss1* knockout female mice abolishes the LH surge, though projections to GnRH neurons in the preoptic area (Smith et. al., 2006; Stephens et. al., 2015;). During proestrus in the AVPV of female rats, 80% of the total neurons were *Kiss1* neurons that expressed Fos activation and 60% of the total neurons in the AVPV were *Kiss1* with Fos activation during the LH surge (Smith et. al., 2006). From this data, it is clear that *Kiss1* neurons modulate the stimulation of the LH surge. In addition, *Kiss1* neurons of the AVPV largely coexpress PR with the co-localization of PR in

Kiss1 neurons within the rostral periventricular continuum of the third ventricle (RP3V) only apparent during proestrus of mice and absent during diestrus (Stephens et. al., 2015; Mittelman-Smith et. al., 2017). Knockout of PR specific to kisspeptin neurons in female mice resulted in the reduction of *Kiss1* neuronal activation during the expected period of the LH surge, which lead to fewer corpora lutea, suggesting decreased ovulation, and the absence of LH surges at 12 weeks and 6 months of age (Stephens et. al., 2015).

Further, female rats infused with PR antisense oligonucleotides following estrogen treatment failed to elicit an LH surge with reduced PR-immunoreactive cells in the periventricular regions (Chappell and Levine, 2000). These findings indicate the requirement of progesterone and PR to mediate estrogen positive feedback and the following LH surge for reproductive and sexual behavior. As follows, neuroP, rather than ovarian progesterone, is necessary for the LH surge, as a decrease in ovarian progesterone in ovx and intact female rats occurs preceding the LH surge (Chappell and Levine, 2000). Further, the LH surge is blocked by progesterone synthesis inhibitors, suggesting that hypothalamic neuroP is the source of LH surge induction (Micevych and Sinchak, 2008b). This data highlights the idea that estrogen induces the production of neuroP and PR which allows for the LH surge necessary for ovulation and sexual receptivity. The absence of lordosis in the 16 hour time group suggests that the corresponding PR increase is due to the estrogen-induced elevation of *Kiss1* neurons expressing PR just preceding the LH surge, rather than PR receptors that are responding to exogenous progesterone to allow for lordosis behavior.

No significant differences were found in the MOR cell counts between the 16-, 24-, and 48-hour time groups (Figure 3). The lack of significant differences could be due to the short period of hormonal treatment. While estrogen stimulates MOR mRNA expression 48 hours following treatment, estrogen does not modulate the density of MOR in the short term (Eckersell et. al., 1998). Further, the combination of estrogen and progesterone treatment in ovx female rats does not upregulate MOR labeling in the medial preoptic area until 27 hours after progesterone treatment, and remains lower three hours after progesterone (Hammer et. al., 1994). In this study, female mice were tested behaviorally 5 hours after progesterone treatments, and sacrificed in another 1 hour for the last test. As follows, these 6 hours do not meet the 27 hour delay seen before estrogen and progesterone alter MOR concentrations. Given this information, we are going to next analyze these sections for MOR internalization, with the expectation of increased MOR internalization in the 16- and 48- hour time groups in comparison to the 24-hour group to parallel the behavior results.

Together, this study establishes a general time course for sexual behavior in female mice treated with estrogen and progesterone, in conjunction with PR and MOR cell alterations. A significantly reduced lordosis score was found for the 16 and 48 hour time points as compared to the 24-hour time point, with no significant differences in MOR expression, and an upward trend for PR expression in the 16-hour group. Future studies that implement vaginal smears to solidify the estrous cycle stages during this time course would be beneficial in understanding whether the time differences between estrogen and progesterone treatment elongate particular estrous stages.

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