Published in final edited form as:

Pharmacol Biochem Behav. 2015 October ; 137: 1–6. doi:10.1016/j.pbb.2015.07.009.

REPEATED ESTRADIOL BENZOATE TREATMENT PROTECTS AGAINST THE LORDOSIS-INHIBITORY EFFECTS OF RESTRAINT AND PREVENTS EFFECTS OF THE ANTIPROGESTIN, RU486

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Abstract

The following experiment was designed to test two specific questions: (1) Does the antiprogesterin, RU486, reduce emergence of lordosis behavior and/or proceptivity in rats given repeated treatment with 10 µg estradiol benzoate (EB) and/or a single high dose (40 µg) of EB? (2) Does RU486 accentuate the effects of a 5 min restraint experience on sexual behaviors in rats given repeated treatment with estradiol benzoate (EB) and/or a high dose of EB? RU486 was used to determine if a high dose and/or repeated treatment with EB enhanced proceptivity and reduced the response to mild stress through an intracellular progesterone receptor-mediated process.

Ovariectomized Fischer rats were injected with a single dose of 10 or 40 µg estradiol benzoate (EB) or received 4 consecutive weeks of treatment with 10 µg EB. Forty-eight hours after the last treatment with EB, rats were injected with 5 mg/kg of the antiprogesterin, RU486, or the RU486 vehicle. That afternoon, rats were monitored for sexual behaviors. Sexually-receptive rats were then restrained for 5 min and again tested for sexual behaviors. A separate set of rats received 4 consecutive weeks of 10 µg EB treatment before treatment with a higher (5 mg/rat) dose of RU486. Lordosis to mount ratios, lordosis quality, proceptivity, and resistance were monitored. RU486 had no effect on the emergence of sexual behaviors but did accentuate the lordosis-inhibitory effect of restraint in rats given a single treatment with EB. Rats treated for 4 consecutive weeks with EB showed no effect of restraint and were unaffected by RU486. These findings lead to the suggestion that repeated EB initiates select behavioral effects that are not mimicked by acute EB treatment and that the intracellular progesterone receptor may not be involved.

Keywords

lordosis behavior; proceptivity; stress; female rats; progesterone receptor; Fischer rats

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1. Introduction

The following experiment was designed to test two separate, but related, hypotheses: (1) Does RU486 reduce emergence of lordosis behavior and/or proceptivity in rats given repeated treatment with estradiol benzoate (EB) and/or a high dose of EB? (2) Does RU486 accentuate the effects of a 5 min restraint experience on lordosis behavior and/or proceptivity in rats given repeated treatment with estradiol benzoate (EB) and/or a high dose of EB? RU486 was used to determine if EB enhanced proceptivity and reduced the response to mild stress through an intracellular progesterone receptor-mediated process.

Female rat sexual behavior includes approach/incentive behaviors, proceptivity (behaviors that draw the male's attention to the female), and the consummatory response (dorsoflexion of the back; lordosis) that is essential for successful impregnation (Blaustein, 2008; Pfau et al., 1999). The expression of the entire set of these behaviors is thought to require the sequential effects of estradiol and progesterone (Blaustein, 2008; Pfaff et al., 2002; Sakuma, 2008). Only estradiol is required for the lordosis response (Blaustein, 2008; Frye et al., 1998; Sodersten, 1981), but proceptivity, often viewed as evidence of the female's motivation to mate, usually requires the addition of progesterone (Erskine, 1989; Mani et al., 1994b; Ogawa et al., 1994). Given the general belief that emergence of proceptivity in ovariectomized female rats requires sequential priming with estrogen and progesterone (Blaustein, 2008; Sodersten, 1981), it is surprising that repeated EB and/or a high dose of EB can elicit proceptivity even without progesterone (Micevych and Dewing, 2011; Uphouse et al., 2014). How such priming only with estrogen is able to mimic the effects of estrogen and progesterone treatment is currently unclear.

In ovariectomized rats, when progesterone is given after EB priming, a lower dose of EB is required to facilitate sexual behaviors than when EB, alone, is given (Blaustein, 2008; Sodersten, 1981). Progesterone enhances sexual behavior through binding to intracellular progesterone receptors and membrane progesterone receptors as well as through progesterone metabolites (Blaustein, 2008; Conneely et al., 2003; Frye et al., 1998; Frye et al., 2013; Mani et al., 1997; Pluchino et al., 2009). For lordosis behavior, involvement of intracellular progesterone receptors has been inferred from the lordosis-inhibitory effects of the antiprogesterin, RU486 (11 β -(4-dimethylamino)phenyl-17 β -hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), (Beyer et al., 1995; Blaustein et al., 1987; Brown et al., 1987), from the failure of progesterone to enhance lordosis behavior in progesterone receptor knock-out mice (Lydon et al., 1995; Mani et al., 1997; Mani et al., 2006), and from the inhibitory effect of antisense oligonucleotides to the intracellular progesterone receptor (Mani et al., 1994a; Mani et al., 1994b). Whether such intracellular progesterone receptors are responsible for high dose/repeated EB effects on the emergence of lordosis behavior and proceptivity remain unclear. The current experiments were designed to determine (a) if the antiprogesterin, RU486, can block these effects (especially proceptivity) of EB and (b) if repeated EB treatment and a high dose of EB have equivalent effects on sexual behaviors.

A second objective of the experiment was to test the hypothesis that repeated EB treatment reduces the effects of a mild restraint experience in a progesterone receptor-dependent manner. When ovariectomized Fischer rats are injected with 10 μ g EB, the emergence of

lordosis behavior (but not proceptivity) can be as high as observed in rats given EB and 500 µg progesterone, but when these 10 µg EB treated rats are given a brief 5 min restraint experience, lordosis behavior is rapidly reduced. The addition of progesterone completely prevents this restraint-induced decline (Hassell et al., 2011; Miryala et al., 2011; White and Uphouse, 2004) and the antiprogestins, RU486 or CDB4124, reduce progesterone's ability to attenuate the effect of restraint (Hassell et al., 2011; Uphouse and Hiegel, 2013). However, when rats were given 4 consecutive weeks of treatment with 10 µg EB, the repeated treatment with estradiol benzoate was as effective as the addition of progesterone in reducing the lordosis-inhibiting effect of a 5 min restraint stress (Uphouse et al., 2014). Moreover, rats given such repeated treatment differed from rats given a single EB treatment by the occurrence of proceptivity in addition to lordosis behavior. In a prior report, (Micevych and Dewing, 2011), proceptivity induced by a high dose of EB was reduced by RU486. In addition, multiple 50 µg EB treatments increased the amount and activity of the enzyme, 3-β-hydroxysteroid dehydrogenase (which converts pregnenolone to progesterone), and also increased progesterone receptors in the hypothalamus (Soma et al., 2005). The occurrence of proceptivity in the absence of exogenous progesterone treatment was hypothesized by the authors to result from an estrogen-induced amplification of neuroprogesterone. If the effects of repeated, lower dose EB also result from such amplification of neuroprogesterone and consequent activation of intracellular progesterone receptors, then proceptivity as well as the response to the 5 min restraint should be reduced by the antiprogestin, RU486.

In the following experiment, ovariectomized Fischer females received a single treatment with either 10 µg or 40 µg EB or received four consecutive weeks of treatment with 10 µg EB. On the day of testing, rats were injected with RU486 or vehicle and the emergence of lordosis behavior and proceptivity and the response to restraint was examined. It was hypothesized that RU486 would prevent emergence of proceptivity (but not lordosis behavior) and would enhance effects of a mild, 5 min restraint, on both lordosis behavior and proceptivity. Of special interest was a comparison between rats given a single 40 µg dose of EB and those given 4 consecutive weeks of 10 µg EB treatment.

2. Materials and methods

2.1 Materials

Estradiol benzoate, RU486 (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Propylene glycol was purchased from Eastman Kodak Company (Rochester, NY). Isoflurane (AErrane®) came from Butler Schein Animal Health (Dublin, OH). Decapicone® restrainers were from Braintree Scientific, Inc. (Braintree, MA). All other supplies came from Fisher Scientific (Houston, TX).

2.2 Methods

Ovariectomized Fischer rats were injected for 4 consecutive weeks with 10 µg EB. Additional rats received a single treatment with either 10 µg or 40 µg EB. Forty-eight hours after the last EB treatment, rats were injected with RU486 or vehicle. Sexual behavior

testing after RU486 or vehicle was used to assess the effects of treatments on the emergence of lordosis behavior and proceptivity. If rats were sexually receptive (e.g. L/M ratio = 0.7), they were then restrained for 5 min and immediately retested for sexual behaviors. A diagrammatic representation of the procedures is shown in Table 1.

2.2.1 Animals—Eighty-one, adult Fischer (F-344) female rats were purchased from Charles River Laboratories (Wilmington, MA) and were housed 2 or 3 per cage in polycarbonate shoebox cages (45.72 × 24.13 × 2.59 cm) in a colony room maintained at 25 °C and 55% humidity, with lights on from 12 midnight to 12 noon. Food and water were available *ad lib*. All procedures were conducted according to PHS policy and were approved by the IACUC at Texas Woman's University.

2.2.2 Surgical procedures and hormone treatments—Females were anesthetized with AErrane® and ovariectomized as previously described (White and Uphouse, 2004). For the repeated EB treatment, four-week condition, beginning two weeks after ovariectomy, rats received 3 weekly subcutaneous (s.c.) injections with 10 µg EB (in sesame seed oil). On the 4th week, rats were injected again with 10 µg EB. For the single hormonal priming condition, either 10 µg or 40 µg EB was injected two weeks after ovariectomy. Forty-eight hours after the final EB treatment, rats were injected s.c. with 5 mg/kg RU486 or the RU486 vehicle (15% DMSO-propylene glycol). Four to six hours later, sexual behavior was tested in the home cage of a sexually-experienced male. In one additional group, rats given the 4 weeks of EB were injected with vehicle or a higher dose (5 mg/RAT, approximately 30 mg/kg) of RU486 before testing.

For hormonal priming, estradiol benzoate (EB) was dissolved in sesame seed oil and injected s.c. in a volume of 0.1 ml/rat. 5 mg/kg RU486 (dissolved in 15% DMSO and propylene glycol) was injected s.c. in a volume of 0.1 ml/100 gram body weight. The higher dose of RU486 was administered s.c. in a volume of 0.3 ml/100 gram body weight. Females were approximately 139 days of age at the time of testing.

2.2.3 Restraint procedures—Restraint procedures were as previously described (Uphouse et al., 2007). The female was restrained in a Decapicone® with the female's nose flush against a small opening on the cone to enable the female to breathe. The base of the cone was secured tightly with tape. The female was then set aside for 5 min of restraint.

2.2.4 Testing for sexual receptivity—Females were tested during the dark portion of the light/dark cycle. Red lighting was used to allow visibility by the experimenter. The emergence of sexual behavior, as previously described (Hassell et al., 2011; White and Uphouse, 2004), was initiated within 1 to 3 hours after colony lights off. L/M ratios (the number of lordosis responses divided by the number of mounts), lordosis quality scores (the sum of lordosis quality scores divided by the number of lordosis responses), proceptivity (hopping and darting), and resistance (rolling over, fighting, escape) were scored. The lordosis quality scale was modified from the scale described by Hardy and DeBold (Hardy and DeBold, 1972). The absence of a lordosis response was given a score of 0.0; minimal arching of the back was given a score of 1.0. A normal reflex was scored as 3.0 and an intermediate response was given a score of 2.0. An exaggerated reflex, with front feet off the

floor, was scored as 4.0. We summed the quality scores and divided by the number of lordosis responses. Since in our testing paradigm with Fischer female rats, the total number of hops/darts and resistive behaviors varies as a function of the male's attention, proceptivity and resistance were measured as present or absent and the percentage of females showing the behaviors was computed for each individual treatment condition.

In the test for emergence of sexual behavior, females were placed into the home cages of sexually experienced Sprague-Dawley male rats for 10 min or until the male accomplished 10 mounts. If females showed L/M ratios ≥ 0.7 , they were immediately restrained for 5 min. After restraint, the female was returned to the male's cage for an additional 10 min.

2.2.5 Statistical procedures—Data were evaluated in two separate ways. First, for emergence of L/M ratios, lordosis quality and number of mounts, data for all animals during the initial test were evaluated with univariate ANOVA with prior hormonal treatment and RU486 treatment as independent factors. These procedures were performed to determine if type of hormonal treatment and/or RU486 had influenced emergence of sexual behavior.

After this test for emergence of the behaviors, those rats with L/M ratios ≥ 0.7 were used to assess the effects of restraint (See Table 1). For evaluation of effects of restraint, L/M ratios, lordosis quality scores, and number of mounts by the male were compared for the same animals before and after restraint. Data were examined before and after restraint by repeated measures ANOVA with prior treatment and RU486 treatment as main factors and time relative to restraint (e.g. before or after restraint) as the repeated factor. Post-hoc comparisons were made with Tukey's test.

Proceptivity and resistance were compared with Chi-Square procedures. Statistical analyses were conducted with SPSS 17 for Macintosh or SPSS 15 for PC. Independent pair-wise comparisons were performed manually (Zar, 2010). An alpha level of at least 0.05 was required for rejection of the null hypothesis.

3.0 Results

3.1 Effects of differential hormonal priming on the emergence of sexual behaviors after 5 mg/kg RU486

During the initial test for emergence of sexual behaviors, most rats showed relatively high levels of lordosis responding, but only rats given 4 consecutive weeks of EB treatment showed high proceptivity and low resistance (Table 2). The lowest L/M ratios were in rats given a single priming with 10 μ g EB, but even in this group, 10/12 rats showed L/M ratios of at least 0.7. Nevertheless, there was a significant effect of the hormonal treatment on L/M ratios ($F_{2,68} = 5.61$, $p = 0.006$) due to the comparatively lower L/M ratios of the 10 μ g EB group. Similarly, a slightly lower lordosis quality of rats given a single treatment with EB led to an overall significant effect of type of hormonal treatment on lordosis quality ($F_{2,62} = 4.43$, $p = 0.001$). There was no difference in the number of mounts received by the females ($p > 0.05$).

Overall, type of hormonal treatment significantly affected proceptivity and resistance due to higher proceptivity and lower resistance in rats given 4 consecutive weeks of EB treatment (all Chi-Square, $p \leq 0.05$). However, when vehicle and RU486-treated rats were examined within hormonal treatment conditions, there were no significant differences between vehicle and RU486-treated rats on either proceptivity or resistance (all Chi-Square, $p > 0.05$).

3.2 Effects of differential hormonal priming on the restraint-induced change in sexual behaviors after 5 mg/kg RU486

Only rats showing an L/M ratio of at least 0.7 during the initial emergence test were restrained. Of rats given a single 10 μ g EB treatment, 2 vehicle and 3 RU486-treated rats failed to meet this criterion. Of rats given a single 40 μ g EB, one rat from each of the treatment conditions failed the 0.7 L/M ratio criterion. All rats in the 4-week EB condition continued into the restraint part of the study.

As would be expected from prior studies (Hassell et al., 2011; Miryala et al., 2011), there was an overall effect of restraint on L/M ratios ($F_{1,59} = 82.56$, $p \leq 0.01$) (Figure 1). Restraint led to a significant decline in L/M ratios in rats given a single EB injection (either 10 μ g or 40 μ g) but not in rats given 4 consecutive weeks of 10 μ g EB. There was also a significant interaction between restraint and RU486 treatment ($F_{1,59} = 10.76$, $p \leq 0.002$) and a significant type of hormonal treatment by restraint interaction ($F_{2,59} = 16.27$, $p \leq 0.001$). RU486 amplified the effect of restraint in rats given a single treatment with 10 μ g EB (Tukey's $q_{59,6} = 5.80$, $p \leq 0.05$) and in rats given a single 40 μ g EB treatment (Tukey's $q_{59,6} = 4.45$, $p \leq 0.05$), but not in rats given the multiple EB injections. As a consequence, the three-way interaction was also significant ($F_{2,59} = 4.74$, $p \leq 0.012$).

Relative to the effects of restraint on L/M ratios, there were smaller effects on lordosis quality (Table 3). Nevertheless, there was a significant effect of restraint ($F_{2,57} = 30.59$, $p \leq 0.001$) and a significant restraint by type of hormonal treatment interaction ($F_{2,57} = 5.63$, $p \leq 0.006$). Relative to their initial test, the effect of restraint on lordosis quality was significant only for rats given a single EB treatment of 10 μ g or 40 μ g (respectively, Tukey's $q_{57,6} = 5.97$ and 6.08 , $p \leq 0.05$). These were also the only rats where RU486-treated rats differed significantly from their vehicle control ($q_{57,6} = 4.32$, $p \leq 0.05$). There were no significant effects on number of mounts received by the female ($p > 0.05$). The percentage of rats given 10 μ g or 40 μ g EB that showed proceptivity was reduced by restraint, but the low percentage of females showing proceptivity in these groups before restraint precluded a significant effect (all Chi-Square, $p > 0.05$). Similarly, resistance in these two groups was high before restraint and was not significantly reduced by the experience (all Chi-Square, $p > 0.05$) (See Table 3).

3.3 Effects of 4 weeks of EB priming on the emergence of sexual behaviors and response to restraint after a higher dose of RU486 (5 mg/RAT)

To determine if the absence of effects of RU486 treatment on lordosis behavior of rats treated for 4 consecutive weeks with 10 μ g EB was the result of the relatively low dose of RU486, an additional set of rats was tested with vehicle or a higher dose of RU486 (5 mg/RAT). As was true for the lower dose, there was no effect of RU486 (all $p > 0.05$) (Table 4).

4.0 Discussion

The current studies were designed to determine (1) if RU486 would reduce the emergence of proceptivity that is initiated by multiple treatments with EB and (2) if rats given multiple treatments with EB and then treated with RU486 would exhibit vulnerability to the effects of a 5 min restraint experience. It was of additional interest to determine if the effects of 4 weeks of EB treatment resulted from an accumulation of EB after the repeated treatment or if a time-dependent exposure to EB was required.

4.1 Emergence of behaviors

Although there is a generally accepted belief that estrogen, alone, can elicit lordosis behavior in ovariectomized rats, proceptivity is thought to require progesterone (Blaustein, 2008). However, a higher dose of estradiol benzoate and/or repeated treatments with estradiol benzoate can elicit proceptivity, but the mechanisms responsible for this induction of proceptivity have not been clarified. Because a high dose of estrogen was reported to increase synthesis of progesterone (Soma et al., 2005) and elicit proceptivity that was attenuated by the progesterone receptor antagonist, RU486 (Micevych and Dewing, 2011), we hypothesized that treatment with RU486 would prevent the emergence of proceptivity occurring in rats given repeated EB and/or a high dose of EB. However, so few rats given acute 10 µg or 40 µg EB showed proceptivity that no effects of RU486 were evident for emergence of proceptivity. Rats treated for 4 consecutive weeks of EB did, though, show high proceptivity and this also was not reduced by RU486. There also was no effect of RU486 on the emergence of lordosis behavior.

4.2 Response to restraint

Lordosis behavior was reduced after the 5 min restraint experience in rats given either a single 10 µg or 40 µg EB. In contrast, none of the behaviors were altered either by restraint or by RU486 in rats given 4 consecutive weeks of EB. These findings appear to indicate clear differences between rats given an acute treatment with EB and those given protracted EB treatment. The number of such EB treatments required for this resistance to RU486 and/or restraint was not investigated in the current study, but we previously reported that rats given a single 10 µg EB exposure one week before a second 10 µg EB treatment testing was sufficient to reduce the response to restraint (Uphouse et al., 2014).

The absence of an effect of RU486 on the emergence of proceptivity is in contrast to the findings by Micevych and Dewing (Micevych and Dewing, 2011) where 4 consecutive daily injections (over a 4–5 day period) with 10 µg EB in Long-Evans rats induced both lordosis behavior and proceptivity and where proceptivity (but not lordosis behavior) was reduced by RU486 treatment. These investigators argued that the EB treatment had initiated synthesis of neuroprogesterone that was then available for binding to intracellular progesterone receptors that could, therefore, be blocked by the RU486 treatment. Since RU486 was not able to block proceptivity of 4-week EB treatment, it is possible that proceptivity of these animals was not dependent on intracellular progesterone receptors. However, this does not rule out an action of progesterone at such intracellular receptors prior to the day of testing.

In addition to its action at intracellular progesterone receptors, progesterone also influences sexual behavior by binding to membrane progesterone receptors (Frye et al., 2013) and by metabolism to compounds such as allopregnanolone (Frye and Vongher, 1999). Either or both of these mechanisms could have contributed to the current findings. Alternatively, even in the absence of progesterone, female rat sexual behavior can be increased in estrogen-primed rats by neurotransmitters that increase cAMP (Mani, 2006).

In prior experiments, we were able to suggest that progesterone metabolites were not essential for the exogenous progesterone-mediated reduction of the response to restraint (Hassell et al., 2011; Miryala et al., 2011) in rats with an acute 10 µg EB treatment. In these experiments, finasteride (which blocks 5-alpha reductase) and hence derivation of allopregnanolone from the exogenous progesterone, did not reduce the effect of progesterone (Miryala et al., 2011). Medroxyprogesterone (which is not readily metabolized to progesterone metabolites but does bind to the intracellular progesterone receptor) did mimic effects of progesterone (Hassell et al., 2011). Both RU486 (Hassell et al., 2011) and CDB4124 (Uphouse et al., 2013) did reduce the effect of the exogenous progesterone. However, allopregnanolone was as effective as progesterone but not on a short time scale (e.g. allopregnanolone required at least 30 or more minutes to attenuate the response to restraint) and the effect of allopregnanone was reduced by RU486 or CDB4124 (Miryala et al., 2011; Uphouse et al., 2013; Uphouse and Hiegel, 2014) suggesting an involvement of the intracellular progesterone receptor. Similar studies have not been performed after the protracted EB treatment. Consequently, it is possible that EB-induced neuroprogesterin had already been synthesized and interacted with the intracellular progesterone receptor prior to the injection with RU486. If intracellular progesterone receptor-mediated events had already been initiated, an acute RU486 treatment would not be expected to have interfered with sexual behavior. Future studies will be needed to assess this possibility.

It will be interesting to determine if the effects of protracted EB treatment on female rat sexual behaviors are truly independent of the intracellular progesterone receptor. Ligand-independent, neurotransmitter mediated, activation of progesterone-receptor effects is well recognized (Mani et al., 1994a; Mani et al., 1996). A common mediator of this ligand-independent action appears to be an increase in cAMP and/or MAPK and phosphorylation either of the intracellular progesterone receptor or nuclear steroid coactivators such as SRC-1 (Dewing et al., 2007; Mani, 2006; Micevych and Mermelstein, 2008; Rowan et al., 2000). Interestingly, of the multiple membrane progesterone receptors that have been identified, several reduce cAMP but the membrane progesterone receptor delta (which has a high presence in neural tissue) increases cAMP (Pang et al., 2013). Therefore, there are multiple mechanisms whereby repeated EB could be affecting sexual behavior either via a ligand-dependent (via neuroprogesterone synthesis) or ligand-independent manner.

However, there is an additional factor to consider regarding RU486's lack of effect in the rats given 4 consecutive weeks of EB. While RU486 effectively blocks progesterone-interaction with its intracellular receptor, RU486 also has partial agonist action at the intracellular PR (Leonhardt et al., 2003; Meyer et al., 1990; Taylor et al., 1998); and such agonist action is amplified in the presence of cAMP (Rowan et al., 2000). It is, therefore, possible that prior EB treatment could amplify these agonist effects of RU486 and thereby

fail to demonstrate any effects of RU486 on the emergence of lordosis behavior on proceptivity or on the response to restraint.

This latter point is especially interesting since RU486 did reduce proceptivity in naturally cycling proestrous rats (Uphouse, 2015). Although proestrous rats have a history of protracted exposure to endogenous estradiol (albeit at a lower dose than the 10 µg of the current study), these rats also have exposure to both estradiol and progesterone. The presence of this endogenous progesterone may have been sufficient to dampen any agonist action of RU486.

In summary:

1. A high dose of estradiol benzoate and/or repeated treatments with estradiol benzoate not only elicits lordosis behavior but also proceptivity. Neither proceptivity nor lordosis behavior were sensitive to inhibition by the progesterone receptor antagonist, RU486.
2. RU486 amplified the lordosis-inhibiting effects of restraint in all groups except those given 4 consecutive weeks of EB. Coupled with our earlier studies (Hassell et al., 2011; Miryala et al., 2011), the current findings further accentuate a potential role of the intracellular progesterone receptor in the antistress effects of the hormone.
3. Finally, the differential responsivity of rats given acute versus repeated EB treatment indicates that repeated EB initiates select behaviors that are not mimicked by acute EB treatment.

Acknowledgments

Appreciation is expressed to Ms. Karolina Blaha-Black for animal care and to members of the Uphouse lab for reading prior versions of the manuscript. Research supported by NIH HD28419, by NIH R25GM058397, and by the TWU Microgrant Program for undergraduate research.

References

- Beyer C, Gonzalez-Flores O, Gonzalez-Mariscal G. Ring A reduced progestins potently stimulate estrous behavior in rats: paradoxical effect through the progesterone receptor. *Physiol Behav.* 1995; 58:985–993. [PubMed: 8577898]
- Blaustein JD. Neuroendocrine regulation of feminine sexual behavior: lessons from rodent models and thoughts about humans. *Annu Rev Psychol.* 2008; 59:93–118. [PubMed: 17678443]
- Blaustein JD, Finkbohner R, Delville Y. Estrogen-induced and estrogen-facilitated female rat sexual behavior is not mediated by progestin receptors. *Neuroendocrinology.* 1987; 45:152–159. [PubMed: 3574601]
- Brown TJ, Moore MJ, Blaustein JD. Maintenance of progesterone-facilitated sexual behavior in female rats requires continued hypothalamic protein synthesis and nuclear progestin receptor occupation. *Endocrinology.* 1987; 121:298–304. [PubMed: 3595521]
- Conneely OM, Mulac-Jericevic B, Lydon JP. Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms. *Steroids.* 2003; 68:771–778. [PubMed: 14667967]
- Dewing P, Boulware MI, Sinchak K, Christensen A, Mermelstein PG, Micevych P. Membrane estrogen receptor- α interactions with metabotropic glutamate receptor 1 α modulate female sexual receptivity in rats. *J Neurosci.* 2007; 27:9294–9300. [PubMed: 17728443]

- Erskine MS. Solicitation behavior in the estrous female rat: a review. *Horm Behav.* 1989; 23:473–502. [PubMed: 2691387]
- Frye CA, Bayon LE, Pursnani NK, Purdy RH. The neurosteroids, progesterone and 3alpha,5alpha-THP, enhance sexual motivation, receptivity, and proceptivity in female rats. *Brain Res.* 1998; 808:72–83. [PubMed: 9795145]
- Frye CA, Vongher JM. Progesterone has rapid and membrane effects in the facilitation of female mouse sexual behavior. *Brain Res.* 1999; 815:259–269. [PubMed: 9878774]
- Frye CA, Walf AA, Kohtz AS, Zhu Y. Progesterone-facilitated lordosis of estradiol-primed mice is attenuated by knocking down expression of membrane progesterin receptors in the midbrain. *Steroids.* 2013; 81:17–25. [PubMed: 24269738]
- Hardy DF, DeBold JF. Effects of coital stimulation upon behavior of the female rat. *J Comp Physiol Psychol.* 1972; 78:400–408. [PubMed: 5067071]
- Hassell J, Miryala CS, Hiegel C, Uphouse L. Mechanisms responsible for progesterone's protection against lordosis-inhibiting effects of restraint I. Role of progesterone receptors. *Horm Behav.* 2011; 60:219–225. [PubMed: 21635894]
- Leonhardt SA, Boonyaratanakornkit V, Edwards DP. Progesterone receptor transcription and non-transcription signaling mechanisms. *Steroids.* 2003; 68:761–770. [PubMed: 14667966]
- Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM, O'Malley BW. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev.* 1995; 9:2266–2278. [PubMed: 7557380]
- Mani SK. Signaling mechanisms in progesterone-neurotransmitter interactions. *Neuroscience.* 2006; 138:773–781. [PubMed: 16310962]
- Mani SK, Allen JM, Clark JH, Blaustein JD, O'Malley BW. Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. *Science.* 1994a; 265:1246–1249. [PubMed: 7915049]
- Mani SK, Allen JM, Lydon JP, Mulac-Jericevic B, Blaustein JD, DeMayo FJ, Conneely O, O'Malley BW. Dopamine requires the unoccupied progesterone receptor to induce sexual behavior in mice. *Mol Endocrinol.* 1996; 10:1728–1737. [PubMed: 8961281]
- Mani SK, Blaustein JD, Allen JM, Law SW, O'Malley BW, Clark JH. Inhibition of rat sexual behavior by antisense oligonucleotides to the progesterone receptor. *Endocrinology.* 1994b; 135:1409–1414. [PubMed: 7925102]
- Mani SK, Blaustein JD, O'Malley BW. Progesterone receptor function from a behavioral perspective. *Horm Behav.* 1997; 31:244–255. [PubMed: 9213138]
- Mani SK, Reyna AM, Chen JZ, Mulac-Jericevic B, Conneely OM. Differential response of progesterone receptor isoforms in hormone-dependent and -independent facilitation of female sexual receptivity. *Mol Endocrinol.* 2006; 20:1322–1332. [PubMed: 16484336]
- Meyer ME, Pornon A, Ji JW, Bocquel MT, Chambon P, Gronemeyer H. Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor. *EMBO J.* 1990; 9:3923–3932. [PubMed: 2249658]
- Micevych PE, Dewing P. Membrane-initiated estradiol signaling regulating sexual receptivity. *Front Endocrinol (Lausanne).* 2011; 2:26. [PubMed: 22649369]
- Micevych PE, Mermelstein PG. Membrane estrogen receptors acting through metabotropic glutamate receptors: an emerging mechanism of estrogen action in brain. *Mol Neurobiol.* 2008; 38:66–77. [PubMed: 18670908]
- Miryala CS, Hassell J, Adams S, Hiegel C, Uzor N, Uphouse L. Mechanisms responsible for progesterone's protection against lordosis-inhibiting effects of restraint II. Role of progesterone metabolites. *Horm Behav.* 2011; 60:226–232. [PubMed: 21621542]
- Ogawa S, Olazabal UE, Parhar IS, Pfaff DW. Effects of intrahypothalamic administration of antisense DNA for progesterone receptor mRNA on reproductive behavior and progesterone receptor immunoreactivity in female rat. *J Neurosci.* 1994; 14:1766–1774. [PubMed: 8126569]
- Pang Y, Dong J, Thomas P. Characterization, neurosteroid binding and brain distribution of human membrane progesterone receptors delta and {epsilon} (mPRdelta and mPR{epsilon}) and mPRdelta involvement in neurosteroid inhibition of apoptosis. *Endocrinology.* 2013; 154:283–295. [PubMed: 23161870]

- Pfaff D, Frohlich J, Morgan M. Hormonal and genetic influences on arousal--sexual and otherwise. *Trends Neurosci.* 2002; 25:45–50. [PubMed: 11801338]
- Pfaus JG, Smith WJ, Coopersmith CB. Appetitive and consummatory sexual behaviors of female rats in bilevel chambers. I. A correlational and factor analysis and the effects of ovarian hormones. *Horm Behav.* 1999; 35:224–240. [PubMed: 10373335]
- Pluchino N, Cubeddu A, Giannini A, Merlini S, Cela V, Angioni S, Genazzani AR. Progestogens and brain: An update. *Maturitas.* 2009; 62:349–355. [PubMed: 19167174]
- Rowan BG, Garrison N, Weigel NL, O'Malley BW. 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. *Mol Cell Biol.* 2000; 20:8720–8730. [PubMed: 11073973]
- Sakuma Y. Neural substrates for sexual preference and motivation in the female and male rat. *Ann N Y Acad Sci.* 2008; 1129:55–60. [PubMed: 18591469]
- Sodersten, P. Estradiol-progesterone interactions in the reproductive behavior of female rats. In: Ganten, D.; Pfaff, D., editors. *Current Topics in Neuroendocrinology: Actions of Progesterone on the Brain.* New York: Springer-Verlag; 1981. p. 141-174.
- Soma KK, Sinchak K, Lakhter A, Schlinger BA, Micevych PE. Neurosteroids and female reproduction: estrogen increases 3beta-HSD mRNA and activity in rat hypothalamus. *Endocrinology.* 2005; 146:4386–4390. [PubMed: 16020475]
- Taylor RN, Savouret JF, Vaisse C, Vigne JL, Ryan I, Hornung D, Seppala M, Milgrom E. Promegestone (R5020) and mifepristone (RU486) both function as progestational agonists of human glycodeclin gene expression in isolated human epithelial cells. *J Clin Endocrinol Metab.* 1998; 83:4006–4012. [PubMed: 9814484]
- Uphouse L. Dose-dependent effects of the antiprogestin, RU486, on sexual behavior of naturally cycling Fischer rats. *Behav Brain Res.* 2015; 282:95–102. [PubMed: 25591479]
- Uphouse L, Adams S, Miryala CS, Hassell J, Hiegel C. RU486 blocks effects of allopregnanolone on the response to restraint stress. *Pharmacol Biochem Behav.* 2013; 103:568–572. [PubMed: 23046854]
- Uphouse L, Hiegel C. Allopregnanolone's protection against lordosis-inhibiting effects of restraint is blocked by the antiprogestin, CDB4124. *Pharmacol Biochem Behav.* 2014; 122:16–19. [PubMed: 24650591]
- Uphouse L, Hiegel C. An antiprogestin, CDB4124, blocks progesterone's attenuation of the negative effects of a mild stress on sexual behavior. *Behav Brain Res.* 2013; 240:21–25. [PubMed: 23153933]
- Uphouse L, Hiegel C, Adams S, Murillo V, Martinez M. Prior hormonal treatment, but not sexual experience, reduces the negative effects of restraint on female sexual behavior. *Behav Brain Res.* 2014; 259:35–40. [PubMed: 24172220]
- Uphouse L, Hiegel C, Perez E, Guptarak J. Serotonin receptor involvement in effects of restraint on female rat lordosis behavior. *Pharmacol Biochem Behav.* 2007; 86:631–636. [PubMed: 17368527]
- White S, Uphouse L. Estrogen and progesterone dose-dependently reduce disruptive effects of restraint on lordosis behavior. *Horm Behav.* 2004; 45:201–208. [PubMed: 15047015]
- Zar, J. *Biostatistical Analysis.* Upper Saddle River, New Jersey: Pearson Prentice Hall; 2010.

Highlights

1. 5 mg/kg RU486 had no effect on lordosis of ovariectomized rats primed with estradiol benzoate.
2. 5 mg/kg RU486 plus 5 min restraint reduced lordosis behavior in rats with a single hormone injection.
3. Neither 5 mg/kg nor 5 mg/RAT RU486 affected sexual behavior in rats given 4 weeks of hormone treatment.
4. Restraint had no effect in rats given 4 weeks of hormone treatment.

Figure 1.

Restraint and RU486 effects on lordosis behavior

Data are the mean $\pm$ lordosis to mount (L/M) ratios before and after a 5 min restraint experience in rats treated with a single (10 or 40 µg) or repeated estradiol benzoate (EB) (4 weeks of 10 µg; 4 EB) and then injected with 5 mg/kg RU486 or vehicle. Data are for (a) 10 and 8 rats given 10 µg EB followed by vehicle or RU486, respectively; 10 rats for vehicle and RU486 given 40 µg EB; and 14 and 10 rats given 4 weeks hormonal treatment followed by vehicle or RU486, respectively. * indicates a significant effect relative to the same hormonal treatment. ** indicates a significant effect relative to the vehicle within the same hormonal treatment.

TABLE 1

Diagrammatic representation of experimental procedures

Day 1	Day 7	Day 14	Day 21	Day 23 10:00 am	Day 23 2:00 pm initial test ¹	Day 23 restraint ²	Day 23 test ³
HORMONAL TREATMENT				5 mg/kg RU486			
10 µg EB group							
NO EB (n = 12)	NO EB	NO EB	10 µg EB	vehicle	TEST	RESTRAIN (n = 10)	TEST
NO EB (n = 11)	NO EB	NO EB	10 µg EB	5 mg/kg RU486	TEST	RESTRAIN (n = 8)	TEST
40 µg EB group							
NO EB (n = 11)	NO EB	NO EB	40 µg EB	vehicle	TEST	RESTRAIN (n = 10)	TEST
NO EB (n = 11)	NO EB	NO EB	40 µg EB	5 mg/kg RU486	TEST	RESTRAIN (n = 10)	TEST
4-week EB ⁴ group							
10 µg EB (n = 14)	10 µg EB	10 µg EB	10 µg EB	vehicle	TEST	RESTRAIN (n = 14)	TEST
10 µg EB (n = 10)	10 µg EB	10 µg EB	10 µg EB	5 mg/kg RU486	TEST	RESTRAIN (n = 10)	TEST
TREATMENT				5 mg/RAT RU486			
4 week EB group							
10 µg EB (n = 5)	10 µg EB	10 µg EB	10 µg EB	vehicle	TEST	RESTRAIN (n = 5)	TEST
10 µg EB (n = 7)	10 µg EB	10 µg EB	10 µg EB	5 mg/RAT RU486	TEST	RESTRAIN (n = 7)	TEST

¹ behavioral testing for the initial (emergence) test of sexual behaviors

² only rats with L/M 0.7 in emergence test were restrained

³ behavioral testing for effects of restraint on sexual behaviors

⁴ EB = estradiol benzoate

TABLE 2

Effects of 5 mg/kg RU486 on the emergence of sexual behavior

HORMONAL TREATMENT	N	L/M ¹ ratio ² ± S.E.	Lordos is quality ² ± S.E.	Percentage receptivity	Percentage ² showing proceptivity	Percentage ² showing resistance
Single 10 µg EB³						
Vehicle	12	0.83 ± 0.05	2.7 ± 0.11	83.3	25.3	91.6
RU486 (5 mg/kg)	11	0.84 ± 0.09	2.7 ± 0.08	81.8	9.1	63.6
Single 40 µg EB						
Vehicle	11	0.88 ± 0.06	2.9 ± 0.05	90.9	54.5	72.7
RU486 (5 mg/kg)	11	0.94 ± 0.02	2.8 ± 0.08	100	36.3	63.6
4 weeks 10 µg EB						
Vehicle	14	0.99 ± 0.01	3.01 ± 0.03	100	85.7	28.5
RU486 (5 mg/kg)	10	1.00 ± 0.0	3.02 ± 0.01	100	100	0

¹ L/M = lordosis to mount² significant ANOVA for type of hormonal treatment³ EB = estradiol benzoate

Table 3

Effects of restraint on lordosis behavior

HORMONAL TREATMENT	N	Lordosis Quality ¹ ± S.E.		Percentage ² Proceptive		Percentage ² Resistive	
		Before Restraint	After Restraint	Before Restraint	After Restraint	Before Restraint	After Restraint
Single 10 µg EB³							
Vehicle	11	2.8 ± 0.07	2.6 ± 0.10	18.2	18.2	90.1	90.9
RU486 (5 mg/kg)	8	2.7 ± 0.06	2.1 ± 0.23	11.1	0	100	100
Single 40 µg EB							
Vehicle	10	2.9 ± 0.06	2.6 ± 0.26	60.0	20.0	70.0	100
RU486 (5 mg/kg)	10	2.8 ± 0.09	2.2 ± 0.13	36.4	18.1	63.6	100
4 weeks 10 µg EB							
Vehicle	14	3.0 ± 0.02	2.9 ± 0.02	85.7	71.4	28.5	57.1
RU486 (5 mg/kg)	10	3.0 ± 0.01	2.9 ± 0.05	100	90.0	0	20.0

¹ = significant effect of restraint

² percentage refers to the percentage of females within the same treatment condition

³ EB = estradiol benzoate

TABLE 4

Effects of 5 mg/RAT RU486

HORMONAL TREATMENT	N	L/M ¹ ratio ± S.E.		Lordosis Quality ± S.E.		Percentage Proceptive		Percentage Resistance	
		Before Restraint	After Restraint	Before Restraint	After Restraint	Before Restraint	After Restraint	Before Restraint	After Restraint
4 weeks 10 µg EB ²									
Vehicle	5	1.0 ± 0	0.97 ± 0.02	2.9 ± 0.2	2.9 ± 0.25	100	100	0	20.0
RU486 (5 mg/rat)	7	1.0 ± 0	0.99 ± 0.01	3.0 ± 0.14	2.8 ± 0.06	85.7	71.4	28.6	42.9

¹L/M = lordosis to mount²EB = estradiol benzoate