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RU486 BLOCKS EFFECTS OF ALLOPREGNANOLONE ON THE RESPONSE TO RESTRAINT STRESS

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Abstract

These experiments were designed to provide information about the potential involvement of progesterone receptors in the ability of allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one) to reduce the lordosis-inhibiting effects of restraint stress. Ovariectomized Fischer rats were hormonally primed with 10 μ g estradiol benzoate and 4 mg/kg allopregnanolone or vehicle. One hr before allopregnanolone, rats were injected with the progesterone receptor antagonist, RU486 (11 β -(4-dimethylamino)phenyl-17 β -hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), or vehicle. Four hr after allopregnanolone or vehicle, sexual behavior was examined before and after a 5-min restraint stress. Lordosis behavior of rats primed only with estradiol benzoate declined after the 5 min of restraint while allopregnanolone prevented this decline. RU486 attenuated the ability of allopregnanolone to prevent the restraint-induced decline in lordosis behavior. These findings are consistent with earlier suggestions that progesterone receptors are involved in allopregnanolone's ability to reduce the effects of restraint stress.

Keywords

lordosis behavior; proceptivity; progesterone metabolites; progesterone receptors; female rats

1.0 Introduction

Female rat sexual behavior is temporally coordinated with ovulation to maximize reproductive fitness (Blaustein, 2008; Sodersten, 1981). Both estrogens and progestins influence this reproductive synchrony (Auger, 2004; Mani et al., 1997; Pfaff, 1970) so that reproductive behavior abruptly ceases following ovariectomy. However, sexual behavior can be restored in ovariectomized rats by exogenous treatment with gonadal hormones (Auger, 2004; Pfaff, 2005). The full repertoire of female sexual behavior includes appetitive, precopulatory and copulatory activities and gonadal hormones appear to differentially influence these various events (Blaustein, 2008; Erskine, 1989; Frye, 2007). The lordosis reflex (required for copulation) is thought to depend exclusively on estradiol (Auger, 2004; Blaustein, 2008). Progesterone is not required, but the probability and frequency of lordosis

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behavior can be increased by progesterone; and progesterone may be required for the occurrence of precopulatory activity such as hopping and darting behavior (Erskine, 1989; Frye and Vongher, 1999; Sodersten, 1981). Progesterone also reduces measures of anxiety (Auger and Forbes-Lorman, 2008; Gomez et al., 2002) and we have previously suggested that progesterone's anxiolytic action contributes to its facilitation of female rat sexual behavior (Truitt et al., 2003; Uphouse et al., 2008; White and Uphouse, 2004).

When ovariectomized Fischer inbred rats are hormonally primed with 10 μ g estradiol benzoate, lordosis behavior is comparable to that obtained following hormonal priming with estradiol benzoate and progesterone. However, when subjected to a mild 5-min restraint stress, rats primed only with estradiol benzoate show disruption of lordosis behavior (Hassell et al., 2011; White and Uphouse, 2004). The lordosis disruption is transient, lasting 5–10 min after the restraint experience (Uphouse et al., 1993; White and Uphouse, 2004). The restraint experience amplifies the negative effect of the serotonin 1A (5-HT_{1A}) receptor agonist, 8-OH-DPAT [(+/-)-8-hydroxy-2-(di-n-propylamino)tetralin] on lordosis behavior (Uphouse et al., 2007) but is attenuated by the 5-HT₂ receptor agonist DOI [(+/-)-2,5-dimethoxy-4-iodophenyl-2-aminopropane HCl]. We have suggested that this dual regulation by 5-HT is important to the female's continuity of mating in the presence of a mild stressor. Increased 5-HT release, precipitated by stress (Chaouloff, 2000), can activate all 5-HT receptors. Activation of the higher affinity 5-HT_{1A} receptors produces a rapid decline in lordosis behavior; activation of slower acting, lordosis facilitating 5-HT₂ receptors attenuates the decline in behavior and allows mating to continue (Uphouse, 1997; Wolf et al., 1999; Wolf et al., 1998). Progesterone attenuates this response to stress by reducing the lordosis-inhibitory effects of 5-HT_{1A} receptor activation, in part by reducing extracellular 5-HT (Farmer et al., 1996; Maswood et al., 1995; Maswood et al., 1999; Truitt et al., 2003; White and Uphouse, 2004). Therefore, one of the effects of progesterone in the modulation of female rat sexual behavior includes its attenuation of the negative effects of mild stress on lordosis behavior. However, the relative roles of the parent molecule and its metabolites in the mechanisms leading to this attenuation are unknown.

Progesterone modulates reproductive and nonreproductive behaviors through classical progesterone-receptor-mediated mechanisms that can be initiated via both ligand-dependent and ligand-independent pathways (Conneely et al., 2003; Dressing et al., 2011; Mani et al., 1997; Pluchino et al., 2009). Progesterone can also be metabolized by 5 α -reductase into 5 α -dihydroprogesterone and then by 3 α -hydroxysteroid dehydrogenase (3 α -HSD) into allopregnanolone (Rupprecht, 2003; Schule et al., 2011). Progesterone metabolites, such as allopregnanolone, contribute to the regulation of female rat sexual behavior (Blaustein, 2008; Frye et al., 1998; Mani and Portillo, 2010) and are especially important mediators of progesterone's anxiolytic effects (Dubrovsky, 2006; Eser et al., 2008). However, these progesterone metabolites do not appear to be required for progesterone's attenuation of the negative effects of 5 min restraint on lordosis behavior (Hassell et al., 2011; Miryala et al., 2011).

In previous studies, we questioned if progesterone's reduction of the lordosis-inhibiting effect of restraint stress resulted from the parent molecule, progesterone, or if progesterone metabolites were responsible (Hassell et al., 2011; Miryala et al., 2011). Progesterone's attenuation of the response to restraint stress (a) was mimicked by the nonmetabolizable progestin, medroxyprogesterone; (b) was not attenuated by the 5 α -reductase inhibitor, finasteride, or the 3 α -HSD inhibitor, indomethacin; but (c) was attenuated by the progesterone /glucocorticoid receptor antagonist, RU486, and by the more selective progesterone receptor antagonist, CDB4124, that is devoid of glucocorticoid receptor action (Hassell et al., 2011; Miryala et al., 2011; Uphouse and Hiegel, 2012). These data, therefore, were consistent with a requirement for progesterone-receptor mediated events in

progesterone's attenuation of the lordosis-inhibiting effect of restraint. However, the progesterone metabolite, allopregnanolone, was also effective in reducing the effects of restraint (Miryala et al., 2011). Since inhibition of progesterone metabolism with finasteride or indomethacin did not eliminate progesterone's ability to attenuate the negative response to restraint, allopregnanolone's ability to reduce the response to restraint could mean that redundant mechanisms (e.g. progesterone-receptor mediated or progesterone-metabolite initiated events) were capable of reducing the lordosis-inhibiting effect of restraint. However, this seems unlikely since blocking progesterone receptors blocked progesterone's attenuation of restraint while blocking progesterone metabolism did not (Hassell et al., 2011; Miryala et al., 2011). An alternative explanation is that allopregnanolone is also able to lead to activation of progesterone receptors.

Allopregnanolone does not bind directly to intracellular progesterone receptors (Raynaud et al., 1974; Smith et al., 1974) but may indirectly activate progesterone receptors through a variety of intracellular signaling pathways (Etgen et al., 2006; Frye and Walf, 2008; Gonzalez-Flores et al., 2010; Mani et al., 2000). PKC, MAPK, and Src kinase-dependent signaling are modulated by allopregnanolone (Etgen and Acosta-Martinez, 2003; Frye and Walf, 2008; Gonzalez-Flores et al., 2006; Mani et al., 2000) and each of these mechanisms can contribute to ligand-independent activation of progesterone receptors (Boonyaratanakornkit et al., 2007; Gonzalez-Flores et al., 2006; Li and Shang, 2007; Mani and Portillo, 2010; Tetel, 2009). Such an indirect activation of progesterone receptors by allopregnanolone has been suggested previously (Auger and Forbes-Lorman, 2008; Beyer et al., 1995; Gonzalez-Flores et al., 2010; Gonzalez-Flores et al., 2006; Miryala et al., 2011). Moreover, at least 1 hr of priming by allopregnanolone was required for the progesterone metabolite to reduce the lordosis-inhibiting effect of restraint (Miryala et al., 2011). Therefore, the collective data are consistent with the hypothesis that progesterone receptors contribute to allopregnanolone's attenuation of the lordosis-inhibiting effect of restraint. If so, RU486 should be able to block the effect of allopregnanolone. The following experiment was designed to test this hypothesis.

2.0 Methods

2.1 Materials

Estradiol benzoate, allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one), RU486 (11 β -(4-dimethylamino)phenyl-17 β -hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), dimethyl sulfoxide (DMSO) and sesame seed oil were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Propylene glycol was obtained from Eastman Kodak Company (Rochester, NY). Isoflurane (AErrane[®]) was purchased from Butler Schein Animal Health (Dublin, OH). Decapicone[®] restrainers were from Braintree Scientific, Inc. (Braintree, MA). Other supplies came from Fisher Scientific (Houston, TX).

2.2 Animals, housing and surgical procedures

Adult Fischer (F-344) female rats were purchased from Charles River Laboratories (Wilmington, MA) and were housed in polycarbonate shoebox cages in a colony room with lights on from 12 midnight to 12 noon. Food and water were available *ad lib*. After a two week acclimation to the animal facility, when 60 to 90 days of age, females were anesthetized with AErrane[®] and ovariectomized as previously described (White and Uphouse, 2004). Two weeks after ovariectomy, rats were injected subcutaneously (sc) with 10 μ g estradiol benzoate. Two days later, rats were injected sc with vehicle (13.5% DMSO + propylene glycol) or the progesterone receptor antagonist, RU486 (5 mg/kg). One hr later, rats were injected sc with vehicle (sesame seed oil) or 4 mg/kg allopregnanolone. This led to 4 treatment conditions: estradiol benzoate, vehicle, vehicle (EB/VEH rats); estradiol

benzoate, RU486, vehicle (EB/RU486 rats); estradiol benzoate, vehicle, allopregnanolone (EB/ALLO rats); and estradiol benzoate, RU486, allopregnanolone (EB/RU486/ALLO rats). Testing occurred 4 hr after allopregnanolone or vehicle. All procedures were approved by the TWU IACUC committee in accordance with the PHS Guide.

2.3 Testing for sexual behavior

Four hr after sesame seed oil or allopregnanolone treatment, females were pretested for sexual behavior. Pretesting took place in the home cage of a sexually active male and behavior was monitored until the male had achieved 10 mounts or for a maximum of 30 min. Sexual receptivity (L/M ratio; number of lordosis responses divided by number of male mounts) and lordosis quality (measured by the magnitude of the lordosis response) were scored as previously described (White and Uphouse, 2004). Proceptivity (defined as the presence of hopping and darting) and resistance (defined as fighting, boxing, rolling over, trying to escape the cage) were measured as present or absent. When females showed at least one hop/dart sequence or one fighting/boxing sequence, that behavior was categorized as present.

2.4 Restraint procedures

Immediately after completion of the pretest, females were restrained for 5 min as previously described (White and Uphouse, 2004). The female was placed head first into a Decapicone® so that her nose was flush with the small opening at the tip of the cone. The base of the cone was gathered around the female's tail and secured tightly with tape. Immediately after the restraint experience, females were placed back into a male's cage for 15 consecutive min of behavioral testing, as described in 2.3.

2.5 Statistical procedures

Data for L/M ratios and lordosis quality were grouped into 5 min intervals and were evaluated by two-way repeated measures ANOVA with time relative to restraint as the repeated factor and with RU486 and allopregnanolone as independent factors. Post-hoc comparisons were made with Tukey's test. Proceptivity and resistance were compared by Chi-Square and Fisher's Exact Test procedures. Data were analyzed with SPSS and the statistical reference was Zar (1999).

3.0 Results

3.1 Behavior before restraint

With the exception of a single rat that had been treated only with estradiol benzoate and vehicles, every rat showed L/M ratios greater than 0.7 during the pretest. This single rat was omitted from data analyses. L/M ratios from the remaining rats are shown in Table 1. There were significant effects of both allopregnanolone ($F_{1,55} = 12.86$, $p = 0.001$) and RU486 ($F_{1,55} = 9.12$, $p = 0.004$) and their interaction ($F_{1,55} = 8.80$, $p = 0.004$) on L/M ratios during the pretest before restraint. These differences were due to lower L/M ratios of the vehicle-treated rats that were significantly different from all other treatments (Tukey's, all $q_{55,1} = 5.95$, $p = 0.05$). For lordosis quality, there was a significant main effect for allopregnanolone ($F_{1,55} = 4.91$, $p = 0.031$) resulting in significant post-hoc comparisons between rats treated with allopregnanolone or vehicle (Tukey's $q_{55,1} = 4.09$, $p = 0.05$).

Only rats treated with allopregnanolone showed evidence of proceptivity leading to significant treatment effects (Chi Square = 11.54, $df = 3$, $p = 0.009$) due to an increase in proceptivity in all allopregnanolone-treated groups (Chi Square = 10.07, $df = 1$, $p = 0.002$). Rats treated with RU486 and allopregnanolone (EB/RU486/ALLO rats) did not differ from rats treated with vehicle and allopregnanolone (EB/ALLO rats) ($p > 0.05$).

3.2 Behavior after restraint

Thirteen rats would not allow the male to mount during the 10 min after restraint so that effects on lordosis behavior could not be examined for these rats. Eight of these 13 rats were from the EB/RU486/ALLO condition leading to a significant difference between EB/RU486/ALLO rats and all other groups (Chi Square = 5.56, $df = 1$, $p = 0.018$).

Both RU486 and allopregnanolone influenced the response to restraint. L/M ratios for rats that allowed mounting after restraint are shown in Figure 1. In agreement with the report by Miryala et al. (2011), allopregnanolone significantly attenuated the restraint-induced decline in the L/M ratio (significant main effect of allopregnanolone; $F_{1,43} = 4.42$, $p = 0.041$). There was not a significant main effect of RU486 ($p > 0.05$), but there was a significant interaction between RU486 and allopregnanolone ($F_{1,43} = 9.15$, $p = 0.004$). Consistent with prior studies (Hassell et al., 2011; White et al., 2007), group differences were most evident in the first 5 min after restraint leading to both a significant effect of time ($F_{1,129} = 22.32$, $p = 0.001$) and a significant three-way interaction between time, RU486, and allopregnanolone ($F_{3,129} = 3.36$, $p = 0.021$). The restraint-induced decline in L/M ratios of rats treated with estradiol benzoate and vehicles (EB/VEH rats) seen after restraint was attenuated in rats treated with allopregnanolone (Tukey's $q_{120,4} = 7.92$, $p = 0.05$) but not in rats treated with both RU486 and allopregnanolone (Tukey's, $p > 0.05$). Rats given RU486 plus allopregnanolone (EB/RU486/ALLO rats) had significantly lower L/M ratios 5 min after restraint than did rats treated with allopregnanolone (EB/ALLO rats) ($q_{120,4} = 4.40$, $p = 0.05$). Surprisingly, rats treated with estradiol benzoate and RU486 (EB/RU486 rats) had significantly higher L/M ratios than the EB/VEH group 5 min after restraint (Tukey's, EB/VEH vs EB/RU486 rats, $q_{120,4} = 5.03$, $p = 0.05$).

There were only minor effects of restraint on lordosis quality and group differences reflected primarily the comparatively lower lordosis quality of the vehicle-treated rats. Lordosis quality scores after restraint for EB/RU486/ALLO, EB/RU486, EB/ALLO, and EB/VEH groups, respectively, were: 2.61 ± 0.08 , 2.73 ± 0.09 , 2.81 ± 0.96 , and 2.41 ± 0.11 . There was a significant main effect of allopregnanolone ($F_{1,33} = 4.35$, $p = 0.045$) and a significant two-way interaction between allopregnanolone and RU486 ($F_{1,33} = 12.131$, $p = 0.001$). No other effects were significant.

There was almost no proceptivity after restraint and no group differences were present, indicating a lack of attenuation by allopregnanolone of the restraint-induced decline in proceptivity.

4.0 Discussion

These experiments were designed to provide information relevant to the hypothesis that allopregnanolone's reduction of the lordosis-inhibiting effect of restraint could include an indirect activation of the progesterone receptor. If this hypothesis were correct, then the progesterone receptor antagonist, RU486, should reduce allopregnanolone's effect. The results of this study are consistent with this hypothesis.

Allopregnanolone's neural effects have often been attributed to positive effects at GABA_A receptors (Auger and Forbes-Lorman, 2008; Barbaccia, 2004; Eser et al., 2008; Frye et al., 2008; Picazo and Fernandez-Guasti, 1995; Reddy et al., 2005; Sullivan and Moenter, 2003; Walf et al., 2006). However, RU486's ability to block allopregnanolone's reduction of the effects of restraint implicates the progesterone receptor. Even though allopregnanolone does not bind to the progesterone receptor (Raynaud et al., 1974; Smith et al., 1974), it is possible that allopregnanolone was metabolized via 3 α -hydroxysteroid dehydrogenase into dihydroprogesterone, which can bind to the progesterone receptor (Rupprecht, 2003;

Rupprecht et al., 1993). This is an unlikely explanation for the current findings since, in a prior study, indomethacin (which blocks 3 α -HSD) did not alter the effect of allopregnanolone on the response to restraint (Miryala et al., 2011).

A variety of intracellular signaling pathways (e.g. PKC, PKA, MAPK, Src kinase) are altered by allopregnanolone (Blackmore, 2008; Etgen and Acosta-Martinez, 2003; Frye and Walf, 2008; Gonzalez-Flores et al., 2006; Mani et al., 2000; Pluchino et al., 2006) and we have suggested that allopregnanolone could lead to progesterone receptor activation by one or more of these mechanisms (Miryala et al., 2011). These same signaling pathways contribute to ligand-independent activation of progesterone receptors (Boonyaratanakornkit et al., 2007; Boonyaratanakornkit et al., 2001; Mani, 2006) and may account for the apparent role of the progesterone receptor in the beneficial effects of allopregnanolone against restraint stress.

Although allopregnanolone has been reported to increase estrogen-facilitated lordosis behavior (Beyer et al., 1995; Frye et al., 1998; Mani et al., 1997), neither allopregnanolone nor RU486 significantly altered the female's lordosis behavior prior to the restraint experience. This is consistent with the findings by Miryala et al. (2011) and reflects the relatively high lordosis responding of ovariectomized Fischer rats after priming with 10 μ g estradiol benzoate. Allopregnanolone did, though, significantly increase the proportion of females that showed proceptivity before restraint. In contrast to allopregnanolone's attenuation of the lordosis-inhibiting effects of restraint, allopregnanolone did not prevent the decline in proceptivity that occurred following restraint. As evident from the current study, treatment with estradiol benzoate is sufficient to induce lordosis behavior but is not sufficient for induction of high levels of proceptive behavior. Therefore, the differential effect of allopregnanolone on L/M ratios versus proceptivity after restraint could underscore the differential dependencies of these behaviors on exogenous administration of estradiol and progesterone (Blaustein, 2008; Sodersten, 1981). However, it is important to note that Fischer females do not exhibit high levels of proceptivity in the nonpaced mating paradigm used in the current studies, and we have found that the percentage of females that do show proceptivity (before or after allopregnanolone) can be variable across experiments (Hassell et al., 2011; Miryala et al., 2011).

RU486 is thought to antagonize the effects of progesterone by inhibiting the interaction between transcriptional factors and the progesterone receptor (Leonhardt et al., 2003) and has previously been reported to attenuate allopregnanolone's facilitation of lordosis behavior (Beyer et al., 1995). In the current experiment, RU486's attenuation of allopregnanolone's ability to reduce the negative effects of restraint on lordosis behavior is consistent with these earlier findings. Interestingly, compared to rats treated with estradiol benzoate and vehicles, estradiol benzoate plus RU486 offered partial protection from the effects of restraint. This could reflect the partial agonist action of RU486 (Leonhardt et al., 2003; Meyer et al., 1990; Taylor et al., 1998) and is consistent with an earlier report that RU486 could facilitate lordosis behavior in ovariectomized rats hormonally primed with a low dose of estradiol benzoate (Pleim et al., 1990). However, it is also important to note that RU486 has antiglucocorticoid, as well as antiprogesterone, activity (Lee et al., 2009; Zhang et al., 2007) so we cannot rule out the possibility that RU486 acted through such a mechanism or that allopregnanolone, by facilitating GABA_A receptors, reduced the response to stress (Barbaccia, 2004; Compagnone and Mellon, 2000). However, allopregnanolone attenuates glucocorticoid receptor-mediated events (Basta-Kaim et al., 2007) so it would be puzzling if the additional antiglucocorticoid activity of RU486 reduced the effect of allopregnanolone. Nevertheless, further studies with a progesterone receptor antagonist that does not have antiglucocorticoid activity will be needed to definitively conclude that RU486 reduces the

effect of allopregnanolone through an antiprogesterone, rather than an antiglucocorticoid, action.

5.0 Conclusions

Consistent with prior research, 5 min restraint reduced lordosis behavior of ovariectomized rats hormonally primed with 10 μ g estradiol benzoate. Addition of allopregnanolone priming attenuated the response to restraint and RU486 reduced the effect of allopregnanolone. Estradiol benzoate priming, alone, was sufficient to induce lordosis behavior but did not produce proceptive behavior. Neither allopregnanolone nor RU486 altered estradiol's induction of lordosis behavior before restraint but allopregnanolone increased the proportion of females showing proceptivity. Neither allopregnanolone nor RU486 altered the effect of restraint on proceptivity but RU486 blocked allopregnanolone's ability to reduce the effects of restraint on L/M ratios. These findings are consistent with prior suggestions that progesterone receptors are involved in allopregnanolone's attenuation of the lordosis-inhibiting effect of restraint.

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Highlights

1. Five min of restraint stress reduced lordosis behavior of Fischer female rats hormonally primed with 10 μ g estradiol benzoate.
2. Allopregnanolone reduced the inhibitory effects of restraint on lordosis behavior.
3. The progesterone receptor antagonist, RU486, attenuated allopregnanolone's protection against restraint stress.

Figure 1. Effects of restraint on L/M ratios

Ovariectomized rats were primed with 10 μ g estradiol benzoate. Two days later they were injected sc with either vehicle (13.5% DMSO, propylene glycol) or 5 mg/kg of the progesterone receptor antagonist, RU486. One hr later, rats were injected with either sesame seed oil or 4 mg/kg allopregnanolone. This led to 4 different treatments after estradiol benzoate: vehicle/vehicle (EB/VEH), RU486/vehicle (EB/RU486), vehicle/allopregnanolone (EB/ALLO) and RU486/allopregnanolone (EB/RU486/ALLO). Four hr later, rats were pretested for sexual behavior and immediately restrained for 5 min. They were then returned to the male's cage for 15 consecutive min of sexual behavioral testing. Data are the mean $\pm$ S.E. lordosis to mount ratios before and for 3 consecutive 5-min intervals after restraint. Ns for each group were EB/RU486/ALLO = 14, EB/RU486 = 10, EB/ALLO = 15, and EB/VEH = 8. * indicates a significant difference from the pretest, within treatment condition. ** indicates a significant difference from EB/VEH within time. \$ indicates a significant difference from EB/ALLO within time interval.

Table 1

Characteristics of animals before and after restraint

Group	N	L/M ratio (mean $\pm$ S.E.) Before Restraint	Quality (mean $\pm$ S.E.) Before Restraint	% Proceptive Before Restraint	% Proceptive After Restraint
EB/RU486/ALLO	22	0.97 $\pm$ 0.01	2.86 $\pm$ 0.04	8/22 (36.4%) ^c	3/22 (13.6%)
EB/RU486	11	0.96 $\pm$ 0.02	2.84 $\pm$ 0.05	1/11 (9.1%)	1/11 (9.1%)
EB/ALLO	15	0.97 $\pm$ 0.01	2.89 $\pm$ 0.04 ^b	8/15 (53.3%) ^c	1/15 (6.6%)
EB/VEHICLE	11	0.86 $\pm$ 0.03 ^a	2.67 $\pm$ 0.10 ^b	0/11 (0%)	0/11 (0%)

^a significant difference from all other groups^b significant difference between ALLO and VEHICLE^c significant effect of allopregnanolone