Anxiogenic Effects of Neurosteroid Exposure – Sex Differences and Altered GABA$_A$-Receptor Pharmacology in Adult Rats

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List of abbreviations

3α,5α-THP     allopregnanolone or 3α-OH-5α-pregn-20-one
ASR         acoustic startle response
PWD         progesterone withdrawal
Abstract

Acute exposure to progesterone (P) or its neurosteroid derivative allopregnanolone (3α,5α-THP), is anxiolytic, consistent with the GABA-modulatory effects of 3α,5α-THP at the GABA$_A$-receptor. However, continuous exposure to progesterone increases anxiety in association with increased expression of the benzodiazepine-insensitive GABA$_A$-receptor α4 subunit. Furthermore, negative mood symptoms and altered GABA$_A$-receptor pharmacology in patients with premenstrual dysphoric disorder occur in the early luteal phase in association with peak circulating levels of progesterone and 3α,5α-THP. Because sex differences have been reported in steroid-regulated anxiety responses, the present study investigated the role of sex and development in the regulation of anxiety following short-term exposure to 3α,5α-THP. To this end, we compared the effects of hormone administration in adult male, adult female and juvenile female rats. Increased anxiety in the elevated plus maze was evident in all groups following 48 hr exposure to either 3α,5α-THP or progesterone. At this time point, alterations in the anxiolytic profile of benzodiazepine agonists and antagonists were also observed in both adult males and females in the elevated plus maze. However, sex differences in the acoustic startle response (ASR) were observed after short-term hormone treatment such that only female rats displayed the increased ASR indicative of higher anxiety levels. These results suggest that although neurosteroid exposure may influence both the pharmacological properties of the GABA$_A$-receptor and the manifestation of anxiety in both sexes, the effects of neurosteroids may be modulated in a sex and task-specific manner.

Keywords: allopregnanolone, neurosteroid, progesterone, GABA$_A$ receptor, anxiety, benzodiazepine, flumazenil elevated, plus maze, premenstrual dysphoric disorder, acoustic startle response
The regulation of anxiety is integrally associated with the function of several neurotransmitter systems, including the GABA\textsubscript{A} receptor system (Sanders and Shekhar, 1995; Sundström et al., 1997; Crestani et al., 1999; Low et al., 2000; Serra et al., 2000). There is, however, a marked difference between the acute effects of agents acting via the GABA\textsubscript{A}-receptor and the effects of longer-term treatment on the GABA\textsubscript{A}-receptor system and on relevant behavioral outcomes. Acute treatments with several classes of drugs that positively modulate the GABA\textsubscript{A}-receptor are anxiolytic (Sanders and Shekhar, 1995; Brot et al., 1997), however chronic treatment with and/or withdrawal from these substances can have the opposite effect, leading to increased anxiety levels in association with altered expression and function of GABA\textsubscript{A}-receptor (File et al., 1987; Saunders et al., 1990; Rassnick et al., 1992; Gallo and Smith, 1993; Holt et al., 1996; Moy et al., 1997; Follesa et al., 2001; Follesa et al., 2002). Endogenous modulators of the GABA\textsubscript{A}-receptor, such as the neuroactive metabolites of steroid hormones, are also known to affect GABA\textsubscript{A}-receptor function and expression. Therefore, the regulation of GABA\textsubscript{A}-receptor gene expression and function by neurosteroids may be essential for understanding the etiology and treatment of anxiety.

The GABA\textsubscript{A}-receptor is a ligand-gated chloride channel, the functional properties of which depends on its subunit composition (Wafford et al., 1996). Potentiation of the GABA\textsubscript{A}-receptor by several of its modulators, including benzodiazepines and the benzodiazepine antagonist, flumazenil (FLU) is dependent on the isoform of the receptor. Benzodiazepines, such as lorazepam, for example, are generally positive modulators of GABA-gated current when the GABA\textsubscript{A}-receptor contains a $\gamma$ subunit in combination with $\alpha$1-3 or 5 (Wafford et al., 1996). However, GABA\textsubscript{A}-receptors containing $\alpha$4 subunits are insensitive to lorazepam and are instead positively modulated by flumazenil (FLU, a.k.a. RO 15-1788) (Wafford et al., 1996).

The neurosteroid, allopregnanolone ($3\alpha,5\alpha$-THP) is also potent positive modulator of GABA-gated
current and, in common with several other classes of drugs which act via the GABA$_A$-receptor, is anxiolytic when acutely applied (Bitran et al., 1993; Akwa et al., 1999). In contrast, prolonged exposure to this neurosteroid produces time-dependent anxiogenic effects. Continuous exposure to progesterone or 3$\alpha$-5$\alpha$-THP for 48-72 increases anxiety in association with insensitivity to benzodiazepine agonists and increased expression of the $\alpha$4 GABA$_A$-receptor subunit (Gulinello et al., 2001). However, by 5 days of prolonged steroid exposure, anxiety responses, $\alpha$4 levels and benzodiazepine pharmacology have returned to control values and remain unaltered during continuous steroid exposure until steroid “withdrawal” (Gulinello et al., 2001) at which time these parameters are again altered 8-48 hrs. after cessation of steroid administration. Benzodiazepine insensitivity associated with chronic, short-term 3$\alpha$,5$\alpha$-THP treatment has also been replicated in cell culture (Friedman et al., 1993; Yu et al., 1996). In addition, chronic treatment with other GABA-modulatory agents also increases expression of the $\alpha$4 subunit (Holt et al., 1996; Ramsey-Williams and Carter, 1996; Devaud et al., 1998), although the time course of these effects are not identical between different classes of drugs and other groups have reported divergent findings which may be a function of brain region, dosing paradigm, gender and cell line in addition to other factors (Devaud et al., 1998; Grobin et al., 2000; Arnot et al., 2001; Follesa et al., 2001; Follesa et al., 2002).

A change in anxiety state in association with neurosteroid exposure may be pertinent to patients with premenstrual mood disorders, who also demonstrate increased anxiety and insensitivity to benzodiazepines in association with peak levels of progesterone in the luteal phase (Wang et al., 1996; Sundström et al., 1997). Furthermore, several studies demonstrate that 3$\alpha$,5$\alpha$-THP may be a relevant modulator of both GABA$_A$-receptor subunit expression and behavior in males as well as females (Corpechot et al., 1993; Steimer et al., 1997; Concas et al., 1998; Gomez et al., 1998; Concas et al., 1999; Serra et al., 2000; Gulinello et al., 2002). Levels of 3$\alpha$,5$\alpha$-THP are also correlated with symptoms of mood disorders in males (Uzunova et al., 1998; Strohle et al., 1999). These data are corroborated by animal models of mood disorders that also demonstrate altered levels of 3$\alpha$,5$\alpha$-THP in conjunction with changes in GABA$_A$-receptor subunit expression and altered...
GABA<sub>A</sub>-receptor pharmacology in males (Drugan et al., 1989; Park et al., 1993; Steimer et al., 1997; Serra et al., 2000).

Therefore, although there is evidence to suggest that 3α-5α-THP levels may be involved in the regulation of GABA<sub>A</sub>-receptor subunit expression and relevant behavioral outcomes, there are however some issues which remain to be clarified. It is a matter of some controversy whether increases or decreases in the levels of 3α-5α-THP are associated with mood disorders in humans. Symptoms of depression and anxiety and are associated with decreased levels of 3α-5α-THP in major unipolar depression and in rodent models of depression and anxiety (Uzunova et al., 1998; Guidotti et al., 2001 Romeo, 1998 #142) while in contrast increased levels of 3α-5α-THP are associated with anxiety disorders and negative mood symptoms in premenstrual dysphoric disorder (Wang et al., 1996; Spivak et al., 2000). Some of these differences may reflect the differing modes of action of acute increases in neurosteroids, which are generally anxiolytic (Bitran et al., 1993; Brot et al., 1997; Frye and Walf, 2002) and have a negative feedback effect on the stress responses (Drugan et al., 1994; Patchev et al., 1994; Guo et al., 1995), and longer-term exposures, which can regulate atypical GABA<sub>A</sub>-receptor subunit levels, thus resulting in altered GABA<sub>A</sub>-receptor kinetics and GABAergic transmission (Smith et al., 1998a; Smith et al., 1998b; Gulinello et al., 2002) It has also been suggested that the effects of neurosteroid exposure may be sex-dependent (Wilson and Biscardi, 1997; Fernández-Guasti and Picazo, 1999; Zimmerberg et al., 1999), which may further complicate attempts to elucidate the role of neurosteroids in affective syndromes.

We therefore investigated the effects of short-term neurosteroid exposure on anxiety and the predicted changes in the anxiolytic profile of GABA<sub>A</sub>-receptor modulators in the elevated plus maze. In order to determine if the effects of 3α-5α-THP and its parent compound, progesterone, are dependent on ovarian status or sex, we employed adult male rats, adult female rats and juvenile female rats. Since the effects of several anxiolytic and anxiogenic agents can be task dependent (Johnston and File, 1991) we also assessed anxiety levels in the acoustic startle response (ASR).
The ASR is a whole body response to acoustic stimuli which has a similar circuitry and pharmacology in humans (Koch, 1999). Altered ASR has been demonstrated in anxiety and depressive disorders in humans (Allen et al., 1999) and in animal models of these disorders (Schwegler et al., 1997).
Methods

Animals

Adult male, adult female and juvenile female Long-Evans rats (Charles River, NC) were housed in single sex pairs in the same room under a 14-hour light and 10 hour dark cycle with food and water ad libitum. Adult rats (≈2 months old) were 200±25 g, and juvenile female rats were 23-25 days old (60-75 g, after weaning but before puberty) at the start of each experiment. All animals were tested during the light portion of the circadian cycle in between 9:00 AM and 2:00 PM. In adult female rats, estrous cycle stage was determined by microscopic examination of the vaginal lavage and by measures of vaginal impedance as described previously (Gulinello et al., 2001). Male rats and juvenile female rats were handled for the same amount of time. Animals were randomly assigned to hormone and treatment groups, and animals not in diestrus were excluded from the experiments, which eventually resulted in unequal numbers of subjects per group in some experiments. All animal care was conducted in accordance with guidelines provided by the Institutional Animal Care and Use Committee.

Drugs and Hormone Administration

Animals were injected (intraperitoneally) with either progesterone (P, 5 mg/kg in sesame oil), allopregnanolone (3α,5α-THP, or 3α-OH-5α-pregnan-20-one 10 mg/kg in sesame oil) once each morning (between 9:00 and 10:00 AM) over a 48 hour period for a total of 3 injections during this period. Injection volumes were 250 ml per adult animal and 60-75 ml per juvenile animal (depending on body weight). These doses of progesterone and 3α,5α-THP result in hippocampal levels of the neurosteroid which are physiological (6-7 ng/gm) (Moran and Smith, 1998; Frye and Bayon, 1999). Control animals were given the same number of injections of vehicle (sesame oil).

Animals were tested 3-4 hours after the final hormone injection. On the day of testing, animals
were injected intraperitoneally with flumazenil (10 mg/kg) or lorazepam (0.75 mg/kg) 12-15 min before testing in the case of FLU or 45 min before testing in the case of lorazepam. Control animals were injected similarly with vehicle (VEH, 1.8% polyethylene glycol 400 in propylene glycol with 4 drops of TWEEN 80). Due to developmental differences in GABA<sub>A</sub>-receptor pharmacology and subunit expression between pre-pubertal and adult animals, only adult animals were utilized to test the pharmacological profile of lorazepam and flumazenil (Barr and Lithgow, 1983; Araki et al., 1996). Chemicals were obtained from Sigma, Inc., unless otherwise indicated. Lorazepam (injectable) was obtained from Wyeth Laboratories and flumazenil from Tocris/Cookson.

**Behavioral Testing**

**Elevated Plus Maze**

Rats were tested on the plus maze, elevated 50 cm above the floor, in a room with low, indirect lighting and low noise levels. The plus maze consists of 2 enclosed arms (50 x 10 x 40 cm) and 2 open arms (50 x 10 cm). The apparatus was thoroughly cleaned with 70% ETOH after each trial. The open arms had a small rail outside the first half of the open arm as previously described (Gulinello et al., 2001). The floor of all four arms was marked with grid lines every 25 cm. On the day of testing each rat was transferred to the testing room and acclimatized for 1 hour prior to testing, then placed in a start box in the center of the plus maze and tested for 10 minutes after exiting the start box into the plus maze. To be considered as an entry into any arm, the rat must pass the line of the open platform with all four paws. The duration (in seconds) of time spent in the open arm was recorded from the time of entry into the open arm. Decreased time spent in the open arm generally indicates higher levels of anxiety since rodents have an intrinsic preference for closed rather than open elevated spaces (Handley and Mithani, 1984; Pellow and File, 1986; Cruz et al., 1994). In order to measure general locomotor activity, the number of total grid crosses was counted. The percentage of open arm entries compared to total entries is a further measure of anxiety-like behavior indicated in the relevant results sections as is % open arm entries (Pellow et
al., 1985). Data from adult males and females in the elevated plus maze were first analyzed in a 2-way ANOVA (hormone condition x sex) followed by a post hoc Fishers PLSD t-test. Data from juvenile females were analyzed separately in a one way ANOVA (hormone condition) followed by a post hoc Fisher's PLSD t-test. Data from each graph represents a separate group of animals tested in either the elevated plus maze or the acoustic startle test since multiple trials in the elevated plus maze do not reliably result in the same pattern of responses (File et al., 1993; Bertoglio and Carobrez, 2002) and prior exposure to stressful experiences can also influence performance on subsequent tests of emotional behavior (DaCunha et al., 1992; Andrews and File, 1993; Bertoglio and Carobrez, 2002). .

**Acoustic Startle**

Because of the gross differences in body weights and in the development of the ASR between adult and juvenile animals, juvenile animals were not tested in the startle paradigm (Gallager et al., 1983). Acoustic startle magnitude (Fleshler, 1965; Szabo, 1967) was assessed using an S-R Lab (San Diego Instruments, San Diego, CA). Rats were placed in a 20 cm x 32 cm plexiglass cylinder attached to a piezoelectric transducer platform to detect the motion of the rat. Movement of the platform results in a voltage change in the transducer which was digitized and analyzed by the S-R Lab program on an attached computer. After a 5 minute period of acclimatization to 65 dB background noise, rats were presented with 10 consecutive trials of 120 dB sound pulses of 40 ms duration in a habituation trial. Immediately thereafter startle magnitude and threshold were assessed by presentation of broadband noise of varying intensities (0, 90, 110 or 120 dB) a total of 5 times in random order with random time intervals separating each trial. Startle magnitude was defined as an average of responses to each stimulus intensity and is illustrated in the results section as the maximum startle response. Habituation trials were performed for several reasons. This protocol results in reliable responses to the stimuli, whereas the responses to the first several presentations are more variable (Hoffman and Stitt, 1969; Schwarzkopf et al., 1993). Some groups
have indicated that habituation and acclimatization periods are important factors in sex comparisons (Schwarzkopf et al., 1993; Lehmann et al., 1999; Faraday and Grunberg, 2000).
Results

48 hr Hormone Treatment Increases Anxiety in the Elevated Plus Maze in Male, Female and Juvenile Female Rats

Administration of either progesterone or 3α,5α-THP for 48 hr significantly increased anxiety in adult rats of both sexes and in juvenile female rats. Both 48 hr progesterone (P) and 48 hr neurosteroid treatment (3α,5α-THP) significantly decreased the time spent in the open arm by roughly 2-fold in comparison to vehicle injected controls (fig 1, p< 0.01 for male, female and juvenile females).

Exposure to 3α,5α-THP or progesterone also decreased the absolute number of open arm entries (fig 1), and the percentage of open arm entries (fig 1) by roughly 50 %. There were no significant effects of sex across treatment conditions in any plus maze measures. There were no significant differences in locomotor activity across treatment conditions as measured by total number of grid crosses (fig 1), number of closed arm entries and total number of entries. Treatment with 3α,5α-THP was not significantly different from progesterone in any plus maze parameter.

Data from the elevated plus maze were first analyzed in a 2 way ANOVA (hormone condition x sex, df condition = 2, df sex = 2, df condition x sex = 2, df residual = 54 ) for adult animals (males and females).

There were significant effects of hormone condition on time spent in the open arm(F= 52.847, p < 0.001), however, there were no significant effects of sex (p< 0.2436) and no interaction of hormone condition and sex (p< 0.8081) on time spent in the open arm. A post hoc t-test was then performed.

Adult control animals of both sexes spent significantly more time in the open arm than adult animals injected with either progesterone (female and male, Fisher’s PLSD p< 0.001) or 3α,5α-THP (Fisher’s PLSD female and male, p< 0.001). Juvenile female control animals (F= 19.540, df condition = 2, df residual = 24, p< 0.001) similarly spent more time in the open arm than either progesterone (Fisher’s PLSD, p< 0.001) or 3α,5α-THP (Fisher’s PLSD, p< 0.001) injected juvenile females. The number of open arm entries (F= 30.519, p< 0.001) and the percent open arm entries (F= 19.540, p< 0.001) were similarly affected by 48 hr steroid exposure. Adult animals of either sex injected with
vehicle exhibited a significantly higher number and percentage of open arm entries than animals injected with either progesterone or 3α-5α-THP (p< 0.002 for all conditions in both males and females). In contrast, neither grid crossings (F= 2.755, p< 0.7) or closed arm entries (F= 1.724, p< 0.19) were significantly altered by hormone treatments.

48 hr Progesterone Treatment Alters the Anxiolytic Effects of Flumazenil and Lorazepam in the Elevated Plus Maze

Administration of progesterone significantly altered the anxiolytic effects of lorazepam and flumazenil (ANOVA hormone condition x sex x drug condition; df hormone condition = 1, df drug condition = 2, df sex = 2, df residual = 86; F hormone condition = 35.412, p< 0.001; F drug condition = 18.717, p< 0.001 ). There were no significant sex differences across treatment groups as indicated by a lack of significant interactions of sex with hormone condition (F= 0.35, p< 0.85) or drug condition (F= 1.853, p< 0.16). In contrast, there were significant interactions between progesterone treatment and drug condition (F= 59.934, p< 0.001). Injections of lorazepam (LZM) following 48 hr administration of progesterone (P / LZM) were significantly less anxiolytic than lorazepam injections in control rats (fig 2). Injections of lorazepam following progesterone exposure decreased the time spent in the open arm, the number of open arm entries and the % open arm by approximately 50% relative to lorazepam injections in control rats of both sexes (LZM vs. P / LZM, p< 0.01, fig 2).

In contrast, FLU was without effect in control animals but it became significantly anxiolytic in progesterone injected animals of both sexes (FLU vs. P / FLU p< 0.01, fig 2). FLU injections following progesterone exposure increased the time spent in the open arm, the number of open arm entries and the % of open arm entries by 50-60%. This alteration of the anxiolytic effect of flumazenil and lorazepam is consistent with up-regulation of the α4 subunit of the GABA_A receptor, since α4-containing GABA_A receptor isoforms are insensitive to modulation by lorazepam and are instead positively modulated by flumazenil.
48 hr Hormone Treatment Increases the Acoustic Startle Response in Female Rats

Exposure to elevated neurosteroid levels following 48 hr administration of P, and following 48 hr administration of 3α,5α-THP significantly increased the peak acoustic startle response (fig 3 ANOVA F=6.921 p< 0.002 df condition = 2, df residual = 102). Exposure to either progesterone (p< 0.001) or 3α-5α-THP (p< 0.007) in female rats increased the peak ASR 2-3 fold over vehicle injected controls. There was no significant difference in peak ASR between 3α,5α-THP or progesterone treated groups. In contrast to the increased ASR demonstrated by female rats after hormone exposure, male rats exposed to progesterone did not demonstrate an altered ASR relative to vehicle injected controls (fig 3)
Discussion

These data demonstrate that short-term exposure to the neurosteroid, 3α,5α-THP, increases anxiety and alters the anxiolytic potential of the benzodiazepine ligands, lorazepam and flumazenil. This pharmacological profile is highly indicative of increased expression of the GABA\(_{\alpha}\)-receptor \(\alpha4\) subunit. Elevated anxiety levels following 48 hr. administration of progesterone or 3α,5α-THP were evident in both sexes when anxiety was assessed in elevated plus maze and in the acoustic startle paradigm in female rats.

It is likely that the role of progesterone in the regulation of anxiety is mediated via 3α,5α-THP. These data demonstrate that anxiogenic effects of progesterone exposure are replicated by direct exposure to 3α,5α-THP in female rats in 2 separate anxiety measures – the elevated plus maze and the acoustic startle paradigm. Furthermore, previous studies indicate that inhibition of neurosteroid synthesis during progesterone exposure prevents the up-regulation of the GABA\(_{\alpha}\)-receptor \(\alpha4\) subunit and changes in anxiety levels (Bitran et al., 1993; Smith et al., 1998a; Follesa et al., 2000; Frye et al., 2000). However, we cannot rule out the possibility that prolonged exposure to progesterone also alters GABAergic transmission and/or anxiety-like behavior via its classic al actions on gene transcription.

Although acute alterations in brain 3α,5α-THP concentrations can alter anxiety (Bitran et al., 1993; Frye et al., 2000); it is unlikely that the alterations in anxiety levels evident here are a function of the 3α,5α-THP concentration at the time of testing. Firstly, animals were tested several hours after the final treatment with 3α,5α-THP or progesterone. Secondly, even if total 3α,5α-THP levels were not substantially decreased at the time of testing, high levels of 3α,5α-THP are generally anxiolytic (Bitran et al., 1993; Akwa et al., 1999; Frye et al., 2000). Therefore if endogenous levels of 3α,5α-THP at the time of testing were the major factor regulating anxiety at the time of these tests, one would expect progesterone and 3α,5α-THP injected rats to be significantly less anxious than
controls, which was not the case. In contrast, we suggest that changes in physiology, such as altered GABA_α-receptor expression and function due to hormone exposure, may underlie the increased anxiety evident following 48 hr exposure to elevated 3α,5α-THP levels.

We have previously demonstrated that treatment with either progesterone or 3α,5α-THP for 48 hours increases hippocampal expression of the α4 subunit of the GABA_α-receptor (Smith et al., 1998a; Gulinello et al., 2001). α4-containing GABA_α-receptor have a distinctive pharmacology such that they are insensitive to the modulatory effects of benzodiazepine agonists such as lorazepam, but are instead positively modulated by the benzodiazepine antagonist, flumazenil. The increase in functional α4-containing GABA_α-receptor was confirmed here at a behavioral level by a comparative insensitivity to anxiolytic effects of the benzodiazepine agonist lorazepam and agonist-like properties of the benzodiazepine antagonist, flumazenil, following 48 hr treatment with progesterone in both male and female rats in the elevated plus maze.

We have previously published similar results obtained following administration of progesterone via a subcutaneously implanted progesterone capsule (Gulinello et al., 2001). These data would argue against the possibility that the injection protocol utilized in the present study results in a short withdrawal paradigm. In the case of progesterone implants, steroid levels remain at high, steady-state concentrations during the anxiety testing procedure at which time we have previously demonstrated similarly altered anxiety levels and benzodiazepine responses as are reported in the present study. Our earlier studies (Smith et al., 1998a; Smith et al., 1998b) also demonstrate that the effects of steroid withdrawal do not occur until 8 hrs. after termination of progesterone treatment, and animals in the present study were tested 3-4 hrs. after the last steroid injection. Therefore, it is unlikely that the altered anxiety like behavior that we have demonstrated here is solely the result of cessation of hormone treatment, but rather due to continuous exposure to neurosteroids. Furthermore, similar pharmacological changes occur after 48 hr neurosteroid exposure in cell cultures (Friedman et al., 1993).
Several other studies have also demonstrated that changes in GABA<sub>α</sub>-receptor function and expression following exposure to 3α,5α-THP (Bitran et al., 1991; Finn and Gee, 1993; Friedman et al., 1993; Yu et al., 1996; Belmar et al., 1998). Furthermore, altered anxiolytic effects of GABA<sub>α</sub>-receptor modulators have also been demonstrated following these relatively short-term exposures to neurosteroids (Bitran et al., 1991; Fernandez-Guasti and Picazo, 1997). In fact, GABA<sub>α</sub>-receptor subunit switching may also occur very rapidly during hormone exposure (Brussaard et al., 1997) or after exposure to stressors which substantially increase brain 3α,5α-THP concentrations (Orchinik et al., 1995; Barbaccia et al., 1996). Taken together, these data suggest that the manifestation of anxiety-like behavior and the altered modulatory effects of GABA<sub>α</sub>-receptor ligands may be regulated by the common mechanism of subunit selective expression.

There is ample evidence that acute secretion of progesterone and neurosteroids are protective against the damaging effects of stressors (Drugan et al., 1994; Guo et al., 1995; Patchev and Almeida, 1996); and this may be one mechanisms by which altered neurosteroid levels could occur in males (Barbaccia et al., 1996; Serra et al., 2000). However prolonged exposure to 3α,5α-THP may dysregulate these responses and render the GABA<sub>α</sub>-receptor insensitive to neurosteroids, and may thus predispose subjects to negative effects of stressors (Drugan et al., 1994; Serra et al., 2000).

In fact, several studies have linked negative mood symptoms in both sexes to alterations in neurosteroid levels in association with altered GABA<sub>α</sub>-receptor function (Sundström et al., 1997; Uzunova et al., 1998; Serra et al., 2000). These data suggest that manifestation of negative mood symptoms may be correlated with alterations of GABA<sub>α</sub>-receptor subunit expression during exposure to neurosteroids.

There are, however, indications that females may be more susceptible to modulation of anxiety-like behavior by neurosteroids, or that these effects may be more widespread in females. These data
are important in light of the fact that there are notable sex differences in the prevalence of mood disorders in humans (Kessler et al., 1994; Pigott, 1999). Although there were no sex differences in anxiety-like behavior, or in the pharmacological effects of GABA<sub>α</sub>-receptor modulators in the elevated plus maze following hormone treatments, there were sex differences in the ASR following hormone exposure. Females startle significantly more after 48 hour exposure to either progesterone or 3α,5α-THP, while, in contrast, males do not.

It has elsewhere been demonstrated that GABA<sub>α</sub>-receptor expression is regulated by exposure to GABA<sub>α</sub>-receptor modulators in a sex-specific manner in specific brain regions important in the regulation of the ASR, such as the amygdala (Papadeas et al., 2001). There are also notable sex differences in the hormonal regulation of several other major neurotransmitter systems which also regulate the ASR, including the glutamatergic system (Cyr et al., 2000) and the serotonergic system (Maswood et al., 1999; Zhang et al., 1999). Recent evidence also suggests that continuous neurosteroid exposure in females also regulates neuropeptide expression in the amygdala (Ferrara et al., 2001). Therefore, several other factors may also contribute to the sex differences observed here and in sexually dimorphic responses to stressors in general (Akinci and Johnston, 1993; Figueiredo et al., 2002).

These factors may include other steroid hormones in addition to progesterone. In contrast to progesterone exposure, chronic exposure to testosterone decreases anxiety and increases GABA-stimulated chloride flux (Bitran et al., 1996). Furthermore, the higher estrogen levels in females may also be important in the manifestation of anxiety following progesterone exposure in females (Shors et al., 1999); since estrogen can interact with progesterone and 3α-5α-THP to regulate neuronal excitability and anxiety (Cyr et al., 2000; Laconi et al., 2001) and may also have independent effects on synaptic transmission (Woolley and McEwen, 1994; Cyr et al., 2000). These data suggest that both the acute and organizational effects of hormones may be involved in the divergent responses of males and females to neurosteroid exposure in specific tasks, via
differential actions in different brain regions and/or neurotransmitter systems.

The results from the present study demonstrate a change in behavior in two widely used animal models of anxiety, the elevated plus maze and the acoustic startle response. Increases in “anxiety-like” behavior have been demonstrated in both tasks following withdrawal from GABA-modulatory drugs (Ryan and Boisse, 1983; File et al., 1987; Rassnick et al., 1992; Moy et al., 1997). In fact, the ASR may be increased in subjects suffering from posttraumatic stress disorders (Morgan et al., 1996; Shalev et al., 2000) and during anticipatory anxiety (Grillon et al., 1991) and in several classes of anxiety disorders (Jetty et al., 2001; Kumari et al., 2001). Use of both tests, as in the present study, provides a more complete analysis of the behavioral state to both proximal and distal threats produced by 48 hr. steroid exposure (Rodgers, 1997).

In summary, these data indicate that relatively short exposures to elevated neurosteroid concentrations can result in increased anxiety. Furthermore, neurosteroids can alter the anxiolytic effects of several GABA<sub>A</sub>-receptor modulators, which strongly indicates altered GABA<sub>A</sub>-receptor subunit expression. These phenomena occur in both sexes, suggesting that neurosteroid regulation of GABA<sub>A</sub>-receptor expression may be relevant not only to premenstrual mood symptoms, but also to affective disorders in males. The anxiogenic effects of short-term neurosteroid exposure also appear to be more widespread in females, which may have implications for the observed sex differences in the prevalence of human mood disorders.
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Figure Legends

Figure 1: 48 Hour Exposure to Progesterone or 3α,5α-THP Increases Anxiety in the Elevated Plus Maze

A-C  Anxiety Measures. 48 hr exposure to progesterone (P, 5 mg/kg) or 3α,5α-THP (10 mg/kg) significantly increased anxiety in female (open bars), male (dark bars) and juvenile female (gray bars) rats. For this and the following graphs, sample size is indicated at the base of the bars in panel D and significant effects relative to controls at p< 0.01 is indicated by *. Panel A illustrates anxiety-like behavior assessed as time spent in the open arm (sec). Panel B shows the absolute number of open arm entries. Panel C shows the number of open arm entries (OA Entries) corrected for locomotor activity by presenting these data as a percentage of total arm entries (% OA Entries).

D - E  Locomotor activity. General locomotor activity is illustrated as total number of grid crosses in a 10 min test period in Panel D. There were no significant effects of locomotor activity across treatment conditions in adult male or female rats. 3α,5α-THP significantly decreased locomotor activity in juvenile female rats. Steroid administration did not alter the number of closed arm entries (CA Entries, Panel E).
Figure 2: 48 Hour Exposure to Progesterone Alters the Anxiolytic Profile of Lorazepam and Flumazenil in the Elevated Plus Maze

A  Following 48 hr exposure to progesterone (P), the anxiolytic effects lorazepam (LZM) were significantly decreased as indicated by decreased time (in seconds) spent in the open arm in female (open bars) and male (dark bars) rats. In contrast to the lack of anxiolytic efficacy of LZM following P exposure, flumazenil (FLU) was significantly anxiolytic only following P administration. There were no significant sex differences in any parameter assessed in the plus maze in this and the following graphs. Sample size is indicated at the base of the bars in panel D and significance effects relative to controls injected with the same drug at p< 0.005 is indicated by (*).

B & C  When anxiety was assessed as open arm entries (OA Entries, panel B) or as % open arm entries (% OA Entries, panel C), significantly altered pharmacology was evident following P exposure. LZM injections following P exposure resulted in significantly fewer entries and a lower % OA Entries relative to LZM injections in controls. In contrast, FLU injections following P administration significantly increased open arm entries and % OA Entries relative to controls.

D & E  General locomotor activity is illustrated as total number of grid crosses in Panel D, as number of closed arm entries (CA Entries) in Panel E. There were no significant effects of locomotor activity across treatment conditions in male or female rats.
Figure 3: Administration of Progesterone or 3α,5α-THP Increases the Acoustic Startle Response in Female Rats

A Higher levels of anxiety and arousal were indicated by an elevated peak startle response (ASR – peak, y-axis) to varying intensities of acoustic stimuli (x-axis) in female rats following administration of progesterone (P-Fem, n = 9 closed circles) or 3α,5α-THP (THP Fem, n = 7, shaded circles) relative to vehicle injected controls (n =11, open circles). Significant differences between progesterone (P)-treated and control groups are indicated by (*) and between 3α,5α-THP and controls indicated by (+) at p < .02.

B In contrast to the increased ASR demonstrated in female rats following hormone treatments, the peak ASR was not significantly different in male hormone treated rats (P Male, dark triangles, n = 6) relative to vehicle injected controls (control Male, open triangles, n = 6).