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Progesterone withdrawal increases the $\alpha 4$ subunit of the GABA_A receptor in male rats in association with anxiety and altered pharmacology — a comparison with female rats

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Abstract

Withdrawal from the neurosteroid 3 α ,5 α -allopregnanolone after chronic administration of progesterone increases anxiety in female rats and up-regulates the $\alpha 4$ subunit of the GABA_A receptor (GABA_A-R) in the hippocampus. We investigated if these phenomena would also occur in male rats. Progesterone withdrawal (PWD) induced higher $\alpha 4$ subunit expression in the hippocampus of both male and female rats, in association with increased anxiety (assessed in the elevated plus maze) comparable to effects previously reported. Because $\alpha 4$ -containing GABA_A-R are insensitive to the benzodiazepine (BDZ) lorazepam (LZM), and are positively modulated by flumazenil (FLU, a BDZ antagonist), we therefore tested the effects of these compounds following PWD. Using whole-cell patch clamp techniques, LZM-potentiation of GABA_(EC20)-gated current was markedly reduced in CA1 pyramidal cells of male rats undergoing PWD compared to controls, whereas FLU had no effect on GABA-gated current in control animals but increased it in PWD animals. Behaviorally, both male and female rats were significantly less sensitive to the anxiolytic effects of LZM. In contrast, FLU demonstrated significant anxiolytic effects following PWD. These data suggest that neurosteroid regulation of the $\alpha 4$ GABA_A-R subunit may be a relevant mechanism underlying anxiety disorders, and that this phenomenon is not sex-specific.

Keywords

Neurosteroid; GABAA receptor alpha-4 subunit; Benzodiazepine; Gender; Flumazenil

1. Introduction

The regulation of anxiety is integrally associated with function of the GABA_A receptor (GABA_A-R) system (Bremner et al., 2000; Crestani et al., 1999; Serra et al., 2000; Sundström et al., 1998). Furthermore, modulation of the GABA_A-R system is the primary mechanism of many anxiolytics and anti-panic drugs (for review see (Mehta and Ticku, 1999). Therefore, the regulation of GABA_A-R gene expression and function by endogenous modulators may be essential for understanding the etiology and treatment of anxiety in both males and females (Crestani et al., 1999; Gulinello et al., 2001; Mehta and Ticku, 1999; Serra et al., 2000; Smith et al., 1998a).

The GABA_A-R system is actually a homologous family of ligand-gated chloride channel receptor isoforms. The functional properties of each GABA_A-R isoform depend on its subunit

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composition (Benke et al., 1997; Wafford et al., 1996; Wisden et al., 1991). Accordingly, the binding and efficacy of different classes of ligands vary according to the isoform of the receptor (Benke et al., 1997; Mehta and Ticku, 1999; Wafford et al., 1996; Wisden et al., 1991). Benzodiazepines (BDZ), such as lorazepam (LZM), for example, are generally positive modulators of GABA-gated current when the GABA_A-R contains a γ subunit in combination with $\alpha 1$ –3 or 5 (Benke et al., 1997; Mehta and Ticku, 1999; Wafford et al., 1996). However, GABA_A-R containing $\alpha 4$ subunits are insensitive to LZM and are instead positively modulated by flumazenil (FLU, a.k.a. RO 15-1788), which is otherwise a BDZ antagonist (Benke et al., 1997; Wafford et al., 1996; Wisden et al., 1991).

The neurosteroid, 3α - 5α -THP, (allopregnanolone) is a potent positive modulator of GABA-gated current (Majewska et al., 1986) and is thus anxiolytic when acutely applied (Bitran et al., 1999). However, chronic exposure to and withdrawal from neurosteroids can regulate specific GABA_A-R subunit expression (Follesa et al., 2001; Smith et al., 1998a; Smith et al., 1998b) similarly to chronic exposure to and withdrawal from other GABA_A-R modulators (Devaud et al., 1997; Follesa et al., 2001; Holt et al., 1996; Mahmoudi et al., 1997). Withdrawal from 3α - 5α -THP after chronic exposure to its precursor, progesterone (P), increases the $\alpha 4$ subunit of the GABA_A-R in the hippocampus and in cell culture models (Follesa et al., 2001; Smith et al., 1998a; Smith et al., 1998b). Neurosteroid withdrawal results in a syndrome typified by increased susceptibility to seizures (Frye and Bayon, 1997; Reilly et al., 2000; Smith et al., 1998a), increased anxiety (Gallo and Smith, 1993; Smith et al., 1998a) and a distinctive pharmacological profile that includes decreased sensitivity to BDZs (Moran et al., 1998; Smith et al., 1998a; Smith et al., 1998b) and agonist-like effects of inverse agonists and antagonists (FLU) (Smith et al., 1998a). Similar pharmacological changes are observed after chronic exposure to and withdrawal from other GABA-modulatory agents (Buck and Harris, 1990; Follesa et al., 2002). A change in anxiety state in association with hormone fluctuations may be pertinent not only to premenstrual syndrome (PMS) but also to mood disorders resulting from chronic stress, suggesting that regulation of GABA_A-R subunit expression may be relevant to anxiety disorders in both sexes.

There are several lines of evidence which suggest that 3α - 5α -THP may be a relevant modulator of both GABA_A-R subunit expression and behavior in males as well as females (Barbaccia et al., 1996; Ladurelle et al., 2000; Steimer et al., 1997; Strohle et al., 1999). 3α - 5α -THP has similar potency as a positive modulator of GABA_A-R current in both sexes (Kellogg and Frye, 1999; Wilson and Biscardi, 1997). Furthermore, both males and females express in the adrenal gland and brain the enzymes necessary for de novo synthesis of 3α - 5α -THP (Poletti et al., 1997). The production of 3α - 5α -THP has been documented endogenously and after exogenously administered, physiological doses of progesterone in both sexes (Corpechot et al., 1993; Echaute et al., 1999), where the characteristic behavioral effects occur rapidly (Bitran et al., 1999; Brot et al., 1997). Finally, levels of both P and 3α - 5α -THP increase dramatically in both sexes after physiologically relevant stimuli, such as stress (Barbaccia et al., 1996; Purdy et al., 1991; Steimer et al., 1997; Vallee et al., 2000). Brain levels of 3α - 5α -THP in males rise from pre-stress levels of approximately 2–4 ng/g (similar to females in diestrus) (Kellogg and Frye, 1999; Purdy et al., 1991) to 7–12 ng/g following a stressful stimulus (Barbaccia et al., 1996; Purdy et al., 1991; Vallee et al., 2000), which is similar to proestrous values (Kellogg and Frye, 1999). Stressors can induce brain levels of 3α - 5α -THP as high as 20–30 ng/g depending on the brain region, the type of stressor and time after stress (Barbaccia et al., 1996; Vallee et al., 2000).

Therefore, the progesterone withdrawal (PWD) paradigm may provide a useful model in order to investigate the effects of neurosteroids on behavior in males as well as females. There are relevant circumstances in which elevated neurosteroid levels subsequently decline in the male in association with increased anxiety. Social stress, for example, results in decreased response

to GABA-modulatory drugs, cognitive dysfunction and anxiety that persists after cessation of the stressor in correlation with the decline of elevated neurosteroid levels (Dong et al., 2001; Frisone et al., 2002; Guidotti et al., 2001; Kehoe et al., 2000; Serra et al., 2000). Therefore, this syndrome may be a type of endogenous neurosteroid withdrawal in the male. Furthermore, several commonly used drugs, such as alcohol, initially raise neurosteroid levels (Morrow et al., 2001) and after chronic use and withdrawal, significantly decrease neurosteroid levels and result in a similar GABA_A-R pharmacology as we have demonstrated here (Buck et al., 1991; Moy et al., 1997; Romeo et al., 1996). Taken together, this body of evidence suggests that the PWD model may be relevant to the elucidation of neurosteroid influences on behavior in males in addition to females.

We therefore compared the effects of PWD in male and female rats. To this end, we quantified the levels of the $\alpha 4$ subunit in the hippocampus (by Western blots) in male and female rats following PWD. In addition, we used patch clamp techniques in isolated hippocampal neurons to identify the potential alterations in the GABA-modulatory effects of LZM and FLU that characteristically result from changes in GABA_A-R subunit composition. Finally, we investigated the anxiety profiles (in the elevated plus maze) of male and female rats undergoing PWD, and in response to LZM and FLU.

2. Methods

2.1. Animals

Male and female Long-Evans rats (Charles River) were housed in single-sex pairs under a 14 hour light and 10 hour dark cycle with food and water ad libitum. In female rats, estrous cycle stage was determined by microscopic examination of the vaginal lavage, as described previously (Montes and Luque, 1988) and by measures of vaginal impedance (Taradach, 1982) throughout one entire cycle prior to testing. Male rats were handled for the same amount of time. All animal care was conducted in accordance with guidelines provided by the Institutional Animal Care and Use Committee.

2.2. Drugs and hormone administration

Progesterone was administered rather than 3α - 5α -THP because it is known that elevated circulating levels of P, such as found during the estrous (or menstrual) cycle or after stress, (Barbaccia et al., 1996; Kellogg and Frye, 1999; Purdy et al., 1991; Steimer et al., 1997; Vallee et al., 2000; Wilson and Biscardi, 1997) are readily converted to 3α - 5α -THP in the brain and result in 3α - 5α -THP levels sufficient to potentiate GABAergic inhibition (Smith, 1994) and modulate GABA_A-R subunit expression (Smith et al., 1998a; Smith et al., 1998b).

P implants were made from silicone tubing as previously described and implanted s.c. under anesthesia in the abdominal area of the rat for 21 days (Moran et al., 1998; Smith et al., 1998b). This method has been shown to result in CNS levels of 3α - 5α -THP in the high physiological range (7–12 ng/gm hippocampal tissue) in association with increased circulating levels of P (40–50 ng/ml plasma, approximately 130–160 nM) (Moran et al., 1998; Smith et al., 1998b). These levels are roughly equivalent to proestrous levels of 46 ng/ml of plasma progesterone and 7.75 ng/g of 3α - 5α -THP in brain tissue (Kellogg and Frye, 1999). Control animals were implanted in an identical manner with empty (sham) silicone capsules. 24 hrs after removal of the implant (P withdrawal), animals were either tested or sacrificed, the hippocampi removed and frozen on dry ice for isolation of plasma membrane fractions and subsequent Western Blot analysis. Female rats weighed 200 ± 20 g ($\approx$ 60–70 days old) and male rats weighed 250 ± 20 g ($\approx$ 60–70 days old) at the time of testing.

On the day of testing, animals were injected with either LZM (0.75 mg/kg), FLU (20 mg/kg) or vehicle (1.8% polyethylene glycol 400 in propylene glycol with 4 drops of TWEEN 80). This resulted in 6 groups, with both sham-implanted and PWD animals receiving one of each of the 3 drug treatments. Animals were tested either 10–15 min after injection in the case of FLU or 50–60 min after testing in the case of LZM. These times and doses were chosen on the basis of experiments that established the effective behavioral time window and dose of both drugs (Baldwin and File, 1988; DaCunha et al., 1992b; Lapin, 1995; Lee and Rodgers, 1991; Saldivar-Gonzalez et al., 2000).

2.3. Western blots

$\alpha 4$ levels were measured in hippocampal plasma membranes using Western Blot procedures explained in detail elsewhere (Smith et al., 1998b). Immunoreactivity of the $\alpha 4$ band (67 kDa) was probed with an antibody developed against a peptide sequence of the rat $\alpha 4$ subunit (amino acids 517–523) (Kern and Sieghart, 1994) using ECL (enhanced chemiluminescence) detection and quantified using One-Dscan software (Smith et al., 1998a). The results were standardized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 36 kDa) control protein and were then expressed as a ratio of the average optical density of control values (Gulinello et al., 2001).

2.4. Electrophysiology

Pyramidal neurons were acutely isolated from CA1 hippocampus following PWD, with stages standardized at proestrus, using a procedure described previously with trypsin digestion at 32 °C (Smith et al., 1998a). GABA-activated current was recorded at room temperature (20–25 °C) in a 120 mM NaCl buffer and a pipette solution containing 120 mM N-methyl-D-glucamine. The ATP regeneration system *Tris* phosphocreatinine (20 mM) and creatine kinase were added as previously described (Smith et al., 1998a). GABA-gated current (10 μ M GABA, EC₂₀) was recorded with whole-cell patch clamp techniques at a holding potential of –50 mV using an Axopatch-1D amplifier. Current was filtered at 1–2 kHz (–3dB, eight-pole low-pass Bessel filter) and digitally sampled at a 500 Hz sampling frequency using pClamp 5.51. Drug delivery was accomplished via a solenoid-activated gravity-feed superfusion system positioned within 50 μ m of the cell and triggered by the pClamp program. This system releases drugs for 20 msec at 1–3 min intervals to result in exposure times in the 40–100 msec range and has been described in detail elsewhere (Smith et al., 1998b). A background perfusion system (4 ml/min) provides a washout flow in the opposite direction. The percent potentiation of GABA-gated current was calculated for all drug concentrations using peak GABA-gated current according to the following formula $(\text{GABA}_{\text{drug}} - \text{GABA}_{\text{control}}) / (\text{GABA}_{\text{control}})$. LZM and FLU were applied across a range of concentrations between 0.01 and 100 μ M.

2.5. Behavioral testing

Animals were randomly assigned to hormone and treatment groups. Of the 130 implanted animals, 11 lost their implants during the three-week exposure period and were therefore not included in the behavioral tests. Animals not in diestrus were also excluded from the experiment before testing, which eventually resulted in unequal numbers of animals in each treatment group. All animals were tested during the light portion of the circadian cycle between 9:00 am and 2:00 pm. Rats were tested on the plus maze, elevated 50 cm above the floor, in a room with low, indirect incandescent lighting and low noise levels. The plus maze consists of two enclosed arms (50 $\times$ 10 $\times$ 40 cm) and two open arms (50 $\times$ 10 cm) and is validated in detail elsewhere (Pellow et al., 1985). The open arms had a small rail outside the first half of the open arm as described in Fernandes and File (1996)). The floor of all four arms was marked with grid lines every 25 cm. Each rat was placed in the testing room for 30–40 minutes prior to testing in order to acclimatize the animal. At the time of testing, each animal was evaluated

for 10 minutes after exiting a start box in the center platform of 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 (Pellow et al., 1985). Other behavioral measures recorded included the duration of time spent (in seconds) beyond the rail. The amount of time that subjects spend in the open portion of the plus maze in the absence of rails is considered to be more sensitive to anxiolytic agents (i.e., agents that would increase the amount of time spent in the open arm) than the amount of time spent in the open arms with rails (Fernandes and File, 1996). The number of total grid crosses and total arm entries was counted as a measure of locomotor activity. Percent time spent in the open arm is indicated in the relevant figures and is calculated as a percent of the time spent in the open arm (in seconds) divided by the amount of time spent in the closed arm and in the center.

2.6. Statistical analysis

Differences between groups in Western Blots were assessed using an unpaired Student's *t* test (two-tailed). Data from the plus maze were analyzed in a MANOVA (condition $\times$ sex, significance level $p < 0.01$) followed by a one-way ANOVA (condition for each sex individually) and post hoc *t*-tests (Fishers PLSD) (enumerated in Table 1). Statistical significance for each analysis is indicated in the relevant results section. Electrophysiological data were analyzed using a one-way ANOVA followed by a Tukey test for unequal sample size.

2.7. Source of materials

Except where indicated, most chemicals were obtained from Sigma, Inc. The $\alpha 4$ antibody was produced by Genosys Inc., and the GAPDH antibody by Chemicon. Pierce Chemical Co. provided ECL supplies. Silicone tubing and adhesive were obtained from Nalgene Co. and Dow Corning, respectively. LZM was obtained from Wyeth Laboratories (injectable, used in plus maze) or RBI/Sigma (powder, used in patch clamp). FLU was obtained from Tocris/Cookson.

3. Results

3.1. PWD increases the $\alpha 4$ subunit of the GABA_A-R in male and female rat hippocampus

The levels of GABA_A-R $\alpha 4$ subunit in the hippocampus increased by approximately 50% after PWD in female and male rats (Fig. 1; male control vs. male PWD, *df* 28; $t = -3.628$, $p < 0.01$; female control vs. female PWD, *df* 12; $t = -4.14$, $p < 0.01$). In contrast, there was no change in GAPDH levels in any treatment group. These results in both males and females in diestrus are similar to data we have previously reported in females after PWD (Smith et al., 1998a).

3.2. PWD in male and female rats alters the effects of LZM and FLU on GABA-gated current in hippocampal neurons

The changes in pharmacology that have been previously reported in female rats following PWD (Smith et al., 1998a) were closely paralleled by a similar pharmacological profile in male rats during PWD assessed using whole-cell voltage clamp techniques. In acutely isolated hippocampal CA1 pyramidal neurons from control males, LZM (0.01–100 μ M) significantly potentiated GABA(*EC*₂₀)-gated current (10 μ M, Fig. 2) as a function of concentration to a maximum of 40% at 10 μ M LZM. However, in neurons isolated from PWD rats, LZM did not significantly alter GABA-gated current at any concentration tested (Fig. 2). In contrast, the BDZ antagonist, FLU, was ineffective as a modulator of GABA-gated current under control conditions, but resulted in robust potentiation of GABA-gated current following PWD, where

FLU potentiated GABA-gated current by a maximum of 50% in a dose-dependent manner (Fig. 2). These pharmacological effects are consistent with increased $\alpha 4\beta\gamma 2$ expression (Benke et al., 1997; Wafford et al., 1996) and are similar to the results previously reported in females following PWD (Smith et al., 1998a)

3.3. PWD Increases anxiety in male and female rats

We compared several groups of implant and injection conditions to determine the anxiety levels and the anxiolytic profiles of LZM and FLU after PWD in the elevated plus maze. To this end, PWD and sham-implanted animals received one of each of the three possible drugs (vehicle 250 μ l; LZM 0.75 mg/kg, i.p.; or FLU 20 mg/kg, i.p.). Twenty-four hours after removal of the P implant (PWD) both male and female rats were significantly more anxious (decreased time spent in the open arm) than animals receiving only sham implants (Fig. 3A, Table 1; MANOVA Condition, $df_{(condition)} 5$; $F_{(condition)}=40.513$, $p<0.0001$). Male rats did not significantly differ from female rats in time spent in the open arm (Fig. 3A, Table 1; MANOVA Sex, $df_{(sex)} 1$, $F=1.142$, $p<0.2876$; Condition $\times$ Sex, $df_{(condition)} 5$; $df_{(sex)} 1$ $df_{(condition \times sex)} 5$; $F_{(condition \times sex)}=0.316$, $p<0.9026$). Relevant ANOVA and post-hoc t-test values are indicated in Fig. 3 and Table 1 for all plus maze data. PWD decreased the percent open arm entries in both sexes (Fig. 3C, Table 1) which is an additional assessment of anxiety levels (MANOVA Condition $\times$ Sex, $df_{(condition)} 5$; $df_{(sex)} 1$ $df_{(condition \times sex)} 5$; Condition, $F=12.039$, $p<0.0001$; Sex, $F=1.408$, $p<0.2381$; Condition $\times$ Sex, $F=1.719$, $p<0.1363$). There were no differences in baseline anxiety levels (absolute time in seconds spent in the open arm or percent time spent in the open arm) between sham-implanted males and females (tested in diestrus), nor were there any significant differences between sexes in anxiety levels (time open arm) after PWD.

3.4. PWD does not alter locomotor activity in male or female rats

MANOVA analysis revealed a general effect of locomotor activity between the sexes (Fig. 4 and Table 1), such that females overall have higher numbers of grid crosses (Fig. 4A). There were no other significant effects of locomotor activity across drug or implant conditions. (MANOVA Condition $\times$ Sex, $df_{(condition)} 5$; $df_{(sex)} 1$ $df_{(condition \times sex)} 5$; Condition, $F=2.130$, $p<0.06$; Sex, $F=6.949$, $p<0.01$; Condition $\times$ Sex, $F=1.0$, $p<0.4214$). Higher activity levels in females have also been reported by other groups (Meng and Drugan, 1993; Nasello et al., 1998). There was no significant effect of total arm entries in males in any condition (Fig. 4B). Females undergoing PWD and injected with FLU had a higher number of total arm entries compared to FLU injected rats or to PWD rats injected with vehicle (Fig. 4B). (MANOVA Condition $\times$ Sex, $df_{(condition)} 5$; $df_{(sex)} 1$ $df_{(condition \times sex)} 5$; Condition, $F=4.630$, $p<0.0007$; Sex, $F=2.94$, $p<0.09$; Condition $\times$ Sex, $F=0.73$, $p<0.6028$).

3.5. PWD changes the anxiolytic profile of benzodiazepines and flumazenil in both sexes

As has been well documented, LZM was highly anxiolytic when injected into sham-implanted animals of either sex (see Fig. 3). Injections of LZM relative to vehicle-injected rats (sham implants) significantly increased the time spent in the open arm and the percent time spent in the open arm in both sexes (Fig. 3A 3C and Table 1). Female rats injected with LZM also spent significantly more time beyond the rail of the open arm and had a significantly higher percentage of open arm entries (Fig. 3B and D, and Table 1) than vehicle-injected controls (MANOVA Condition $\times$ Sex, $df_{(condition)} 5$; $df_{(sex)} 1$ $df_{(condition \times sex)} 5$; Condition, $F=24.128$, $p<0.0001$; Sex, $F=3.323$, $p<0.0711$; Condition $\times$ Sex, $F=2.517$, $p<0.04$, Fig. 3, Table 1). In contrast, LZM was no longer anxiolytic following PWD in either sex (Fig. 3, Table 1). In both sexes, LZM treatment of PWD animals resulted in significantly less time spent in the open arm, a lower percentage of time spent in the open arm and a lower percentage of open arm entries than LZM treatment in sham implanted rats (Fig. 3, Table 1). Therefore, rats of both

sexes undergoing PWD exhibited insensitivity to the anxiolytic effects of LZM in association with up-regulation of the BDZ-insensitive $\alpha 4$ subunit.

FLU injections were not significantly different than vehicle injections in sham implanted rats (Fig. 3 and Table 1) with regard to any behavioral measures in the plus maze, consistent with its mechanism as a BDZ antagonist. However, after PWD, FLU was highly anxiolytic (Fig. 3, Table 1). FLU injections in PWD animals of both sexes markedly increased time spent in the open arm relative to sham implanted, FLU injected rats and in comparison to PWD rats injected with vehicle (Fig. 3A, Table 1). FLU injections following PWD also increased the percentage of open arm entries (Fig. 3C, Table 1) and time spent beyond the rail of the open arm (Fig. 3B, Table 1) in both sexes. FLU injections were also significantly more anxiolytic compared to LZM following PWD, in that PWD animals of both sexes injected with FLU spent significantly more time in the open arm and beyond the rail of the open arm than PWD animals injected with LZM (Fig. 3).

4. Discussion

The results from this study clearly demonstrate that the PWD syndrome, typical of withdrawal from GABA modulators, occurs in male rats and is similar to what has been previously reported in females (Smith et al., 1998a). Withdrawal from the GABA-modulatory neurosteroid, 3α - 5α -THP, after 21 days exposure to its precursor, progesterone, increased anxiety in male rats in conjunction with up-regulation of the $\alpha 4$ subunit of the GABA_A-R in the hippocampus. The increase in functional $\alpha 4$ -containing GABA_A-R was confirmed at a behavioral and a neuronal level by a comparative insensitivity to the benzodiazepine, LZM, and agonist-like properties of the BDZ antagonist, FLU. The use of exogenous hormone administration produced levels of 3α - 5α -THP in the high physiological range in both sexes, thus facilitating a comparison of the withdrawal syndrome between sexes.

Although other limbic regions, notably the amygdala (Akwa et al., 1999), have been demonstrated to play a role in anxiety, several lines of evidence also point to the hippocampus as both a target and a modulator of physiological events associated with withdrawal from GABA_A-R modulators and anxiety, which is consistent with its role as a major integrator of limbic circuitry (Andrews et al., 1997; Bitran et al., 1999; Harro et al., 1990; Mahmoudi et al., 1997; Nazar et al., 1999). First, acute, direct hippocampal infusions of either BDZ or 3α - 5α -THP decrease anxiety (Bitran et al., 1999; Nazar et al., 1999). Endogenous levels of neurosteroids and neuropeptides in the hippocampus are also correlated with anxiety (Frye et al., 2000; Thorsell et al., 2000). In addition, the percentage of time spent in the open arms of the elevated plus maze is correlated with altered GABA_A-R levels and function in the hippocampus (DaCunha et al., 1992a). Last, human patients with anxiety and/or panic disorders have decreased GABA_A-R levels and/or function in the hippocampus (Bremner et al., 2000; Malizia et al., 1998). While these data indicate that hippocampal GABAergic tone may play a role in the regulation of anxiety, the contribution of other brain regions and other major neurotransmitter and neuropeptide systems in the regulation of anxiety is also worthy of note. In fact, several of these systems, including the serotonergic system, the neuropeptide Y (NPY) system and the major stress neuropeptides, such as ACTH and CRF, engage in a substantial amount of cross-talk with the GABA_A-R and neurosteroid systems (Ferrara et al., 2001; Keim and Shekhar, 1996; Matsubara et al., 2000; Nazar et al., 1999; Oberto et al., 2000; Sibille et al., 2000; Torres et al., 2001; Zhang and Jackson, 1994).

Several lines of evidence suggest that the increases in $\alpha 4$ -containing GABA_A-R following PWD are correlated with the specific phenomena typical of PWD. We have previously demonstrated that the time course of the rise and fall of $\alpha 4$ subunit expression in the hippocampus closely parallels the rise and fall of anxiety levels in rats after both short-term

progesterone treatment and following PWD (Gulinello et al., 2001; Smith et al., 1998b). PWD clearly results in faster decay times and decreases the total GABA-gated current in isolated hippocampal CA1 neurons (Smith et al., 1998b), an effect which is prevented by the administration of anti-sense oligonucleotides that prevent $\alpha 4$ subunit up-regulation. Therefore, increased expression of $\alpha 4$ subunit and shortened-duration GABA_A-R-mediated synaptic potentials could lead to hyperexcitability and to the relevant behavioral outcomes of hippocampal hyperexcitability (Kapur, 2000; Mangan and Bertram, 1997; Smith et al., 1998b). We have also previously shown that the pharmacological and behavioral changes that are characteristic of increased expression of the $\alpha 4\beta\gamma 2$ GABA_A-R in the hippocampus following PWD in female rats are also prevented by suppression of $\alpha 4$ expression with anti-sense oligonucleotides, suggesting that the $\alpha 4\beta\gamma 2$ GABA_A-R may be a predominant isoform during neurosteroid withdrawal (Benke et al., 1997; Smith et al., 1998a; Wafford et al., 1996).

The characteristic LZM insensitivity following PWD is consistent with increased expression of the $\alpha 4\beta\gamma 2$ isoform (Benke et al., 1997; Wafford et al., 1996) and is also typical of withdrawal from other GABA modulators, including alcohol, and BDZ (Buck and Harris, 1990; Follesa et al., 2001; Toki et al., 1996). In fact, although we have already established that female rats undergoing PWD are insensitive to the sedative and anti-seizure effects of BDZ (Moran et al., 1998), this is the first report of insensitivity to the anxiolytic effects of BDZ in rats during PWD. These data may be important in light of recent evidence demonstrating that the anxiolytic, sedative and anti-seizure effects of GABA_A-R modulators are mediated by different GABA_A-R isoforms and different genes (Lilly and Tietz, 2000; Low et al., 2000; Mathis et al., 1995; McKernan et al., 2000).

The anxiolytic effect of FLU following progesterone withdrawal is consistent with reports that this BDZ antagonist behaves as a BDZ agonist at $\alpha 4\beta\gamma 2$ receptors, which are increased following PWD (Benke et al., 1997; Smith et al., 1998a; Wafford et al., 1996). In addition, other withdrawal models result in anxiety which is insensitive to BDZ and sensitive to the potentiating and/or anxiolytic effects of FLU in male rats (Baldwin and File, 1988; Buck and Harris, 1990; File and Baldwin, 1987; File et al., 1989; Moy et al., 1997; Toki et al., 1996). This is, however, the first report of the anxiolytic actions of FLU following P administration in either sex, which may have implications for the management of BDZ-resistant forms of anxiety (File and Baldwin, 1987; Saxon et al., 1997). The fact that positive modulation of the GABA_A-R by FLU occurs in isolated pyramidal cell argues against this outcome being mediated via the effects of an endogenous benzodiazepine site ligand (Baldwin and File, 1988; Moy et al., 1997).

This is also the first report of P treatments resulting in anxiety in males, although similar protocols of P administration and withdrawal affect cognitive function and seizure susceptibility in male rodents (Johansson et al., 2002; Ladurelle et al., 2000; Reilly et al., 2000). The question remains whether or not neurosteroid modulation of GABA_A-R expression is relevant to anxiety in males as well as females. In females, it has been well documented that depression, anxiety and altered GABA_A-R pharmacology and function are related to endogenous fluctuations in neurosteroid levels (Jenkins et al., 2000; Sundström et al., 1998; Wang et al., 1996; Bitran et al., 1999). Rodent and human models (using male subjects) of withdrawal, stress, anxiety and depression also typically demonstrate altered GABA_A-R expression, function and a pharmacology consistent with altered $\alpha 4$ subunit expression (Drugan et al., 1989; Kram et al., 2000; Moy et al., 1997; Orchinik et al., 2001; Serra et al., 2000; Sibille et al., 2000). This includes changes in sensitivity to GABA_A-R ligands, such as BDZ and FLU (Baldwin and File, 1988; Cowley et al., 1993; File et al., 1989; Moy et al., 1997; Roy-Byrne et al., 1996; Serra et al., 2000). These data corroborate human clinical data which suggest that alterations in the GABA_A-R system by neurosteroids may play a role in the BDZ insensitivity

and dysregulation of mood and cognitive function in male patients. Neurosteroid levels and GABA_A-R function are correlated with the severity of negative symptoms in both male and female patients with a variety of psychiatric and affective disorders and with levels of anxiety and depression in male rodents (Bremner et al., 2000; Dong et al., 2001; Serra et al., 2000; Steimer et al., 1997; Strohle et al., 1999; Uzunova et al., 1998). In addition, antidepressants that are effective in reducing the symptoms of anxiety and depression may also directly affect the enzymes that synthesize neurosteroids (Dong et al., 2001; Romeo et al., 1998; Strohle et al., 2002; Uzunova et al., 1998). These data suggest that a rodent model of neurosteroid fluctuations would indeed be relevant in males. Furthermore, while male animals do not exhibit the cyclic variation in neurosteroid levels typical of the estrous or menstrual cycle, the levels of 3 α -5 α -THP increase profoundly after relevant environmental stimuli, such as stress, and are differentially increased in high- and low-anxiety subjects (Barbaccia et al., 1996; Purdy et al., 1991; Steimer et al., 1997; Vallee et al., 2000).

In fact, the effects of stressors on neurosteroid levels and GABA_A-R function and expression can persist for long periods of time after cessation of the stressor (Dong et al., 2001; Guidotti et al., 2001; Serra et al., 2000). Stress hormone and peptide administration likewise directly raise levels of 3 α -5 α -THP (Torres et al., 2001), whereas repeated exposure to stressors subsequently decrease the initially elevated neurosteroid levels and dysregulate the neurosteroid response to stress (Dong et al., 2001; Frisone et al., 2002; Girdler et al., 2001; Guidotti et al., 2001; Kehoe et al., 2000; Serra et al., 2000). It is plausible that chronically fluctuating or elevated neurosteroid levels in males may also play a role in regulating GABA_A-R subunit expression and function (Miller et al., 1987; Orchinik et al., 2001), and may result in tolerance to neurosteroids and/or uncoupling of sensitivity of the GABA_A-R to its modulators (Follesa et al., 2000; Kellogg et al., 1993; Yu and Ticku, 1995).

Therefore, clarifying the gender differences and similarities in the behavioral and molecular responses to neurosteroids may help to elucidate the etiology of mood disorders. In addition, the use of males provides a control for the potential confounding effects of other steroid hormones and their derivatives that profoundly fluctuate in cycling females. In fact, we did not find any significant sex differences in behavior or pharmacology in either control rats or following PWD. This suggests that changing levels of estrogens or androgens (and other gonadal or pituitary hormones) are not substantially confounding factors with regard to anxiety levels in the PWD syndrome. However, several groups have reported sex differences in anxiety and GABA_A-R function (Frye et al., 2000; Imhof et al., 1993; Johnston and File, 1991; Nasello et al., 1998; Rodriguez-Sierra et al., 1986; Wilson, 1992; Wilson and Biscardi, 1997) while others have reported a lack of sex differences (Stock et al., 2000). These discrepancies may be due to the fact that some groups use ovariectomized and castrated rather than intact animals as we did in this case (Wilson, 1992). Furthermore, observed sex differences in anxiety are dependent on the type of test (Johnston and File, 1991) and the stage of estrous cycle during which females are tested (Frye et al., 2000) as well as the age of the animals (Imhof et al., 1993) and environmental variables immediately preceding the test (Nasello et al., 1998).

Finally, it is worth noting that neurosteroid regulation of GABA_A-R subunit levels also occurs in vitro (Follesa et al., 2000; Friedman et al., 1993; Grobin and Morrow, 2000; Yu and Ticku, 1995). Although the results of these studies have not always been consistent (Follesa et al., 2000; Friedman et al., 1993; Grobin and Morrow, 2000; Yu and Ticku, 1995), the majority of these data are consistent with the results presented here. Primary cultures of brain neurons exposed chronically to neurosteroids exhibit similarly altered pharmacology as we have demonstrated, including insensitivity to BDZ and an alteration in the response to inverse agonists and antagonists of the GABA_A-R (Follesa et al., 2001; Friedman et al., 1993; Yu and Ticku, 1995). Exposing adult rat cerebellar granule cells to PWD results in increased expression of α 4 subunit mRNA in conjunction with a positive receptor response to FLU, and reduced

responsiveness to BDZs (Follesa et al., 2001). However, when embryonic teratocarcinoma cells (P19) are exposed to 3α - 5α -THP for 4 days, a decrease in $\alpha 4$ subunit mRNA expression is observed, which is reversed upon withdrawal from the steroid (Grobin and Morrow, 2000). There are several methodological variable and issues that may account for these differences. Regulation of $\alpha 4$ subunit expression is highly brain-region-specific, dependent on the developmental stage and the time course of the treatment (Buck and Harris, 1990; Devaud et al., 1997; Follesa et al., 2001; Holt et al., 1996; Ma and Barker, 1998; Mahmoudi et al., 1997; Tietz et al., 1999). Therefore, it is difficult to compare results from different types of cells derived from different tissues at different developmental stages.

In summary, withdrawal from neurosteroids produces effects in male rats similar to those reported in females. Anxiety levels and the pharmacological profile of GABA-modulatory agents are consistent with the up-regulation of the GABA_A-R $\alpha 4$ subunit demonstrated in both sexes. These data suggest that the increase in the $\alpha 4$ GABA_A-R subunit after PWD may be a relevant mechanism underlying mood disorders associated with changes in levels of neurosteroids, and that this phenomenon is not sex-specific. The clarification of patterns of specific GABA_A-R subunit expression in anxiety has implications not only for the etiology of anxiety disorders, but for drug treatments as well.

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Nomenclature

3α - 5α -THP	allopregnanolone or 3α -OH- 5α -pregnan-20-one
ACTH	adrenocorticotropin
BDZ	benzodiazepine
CNS	central nervous system
CRF	corticotropin releasing factor
ECL	enhanced chemiluminescence
FLU	flumazenil, RO 15-1788
GABA _A -R	GABA _A receptor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
LZM	lorazepam
NPY	neuropeptide Y
P	progesterone
PMDD	Premenstrual Dysphoric Disorder
PMS	Premenstrual Syndrome
PWD	progesterone withdrawal
s.c.	subcutaneous
SSRI	specific serotonin re-uptake inhibitor

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Fig. 1. PWD increases GABA_A-R α4 subunit levels in male and female rat hippocampus. **A** Representative Western Blot. This figure illustrates α4 subunit immunoreactivity (left panel, Lanes 1 and 2 —PWD implanted (female or male), lane 3 and 4 — sham implanted (female or male). GAPDH control immunoreactivity protein (right panel) is indicated by arrows and does not change in any condition. **B** 24 hours after removal of a chronic P implant both male (indicated by +) and female (indicated by *) rats have significantly ($p < 0.001$) higher levels of the α4 subunit in isolated hippocampal membranes than sham-implanted rats. Data are represented as integrated optical densities relative to control. The numbers inside the bars are the sample size for each condition.

Fig. 2.

Pharmacological changes after PWD. The response to GABA (10 μ M, EC₂₀) in CA1 pyramidal neurons when applied in combination with LZM or FLU and analyzed using whole-cell patch clamp techniques. Results are expressed as the percent increase in peak GABA-gated current. **A** Reduction of LZM potentiation after PWD is not sex specific. LZM (100 μ M) potentiates GABA-gated current in CA1 neurons isolated from sham-implanted animals, but there was almost total insensitivity to LZM potentiation in neurons isolated from male PWD rats, similar to previously reported data in female rats. For both panels A and B, female control — open bars, female PWD — light closed bars, male control — hatched bars, male PWD — closed bars. Statistical significance in this and the following graphs is indicated by (*) at $p < 0.01$ when comparing PWD to sham-implanted controls. Sample size is indicated by numbers at the bottom of the bars in figure A and B. **B** Potentiation of GABA-gated current by FLU following PWD is not sex dependent. FLU (10 μ M) is without effect on GABA-gated current in control rats, but significantly increases peak current following PWD in male rats as has been demonstrated in female rats. **C** Concentration curve of LZM in male control and male PWD rats. GABA-gated current is increased in male control rats (open circles) across a range of concentrations of LZM (0.1–100 μ M) while the same concentrations do not effectively

potentiate GABA-gated current after PWD (closed circles). For Figure C and D, sample size is indicated by the numbers beside the circles. **D** Concentration curve of FLU in male control and male PWD rats. GABA-gated current is unaffected in male control rats (open circles) across a range of concentrations of FLU (0.1–100 μ M) while the same concentrations effectively potentiate GABA-gated current after PWD (closed circles).

Fig. 3.

PWD Increases anxiety that is insensitive to LZM but is positively modulated by FLU in both sexes. **A** Anxiety levels — time open arm. Bars indicate the mean time spent in the open arm (sec) of the elevated plus maze for either male (closed bars) or female (shaded bars) animals. Sham-implanted rats (in this figure and Fig. 4) are denoted by the type of injection they received (i.e., vehicle, LZM or FLU). Sample sizes are indicated in Fig. 4. PWD significantly decreased time spent in the open arm in both sexes. Some subjects received injections of LZM (0.75 mg/kg), which is significantly anxiolytic (increases time spent in the open arm) in sham-implanted rats of both sexes. In contrast, LZM is not anxiolytic in animals undergoing PWD. FLU (20 mg/kg) significantly increased time in the open arm only in PWD animals. FLU-injected

animals with sham implants were not significantly different than vehicle-injected animals. There were no significant effects of sex and no interaction of sex and either drug or implant condition. Significant effects ($p < 0.009$) are indicated by (*), in comparison to sham implanted animals, by (+) in comparison to PWD animals, by (#) in comparison to LZM and by (–) in comparison to FLU. Full details, additional statistical comparisons and the relevant p values are enumerated in table 1. **B** Time outside the rail. LZM significantly increased the percent time beyond the rail of the open arm in females relative to vehicle-injected rats. LZM injections following PWD are significantly less anxiolytic than LZM injections alone for females with regard to time spent beyond the rail. Although FLU injections have no significant effect in sham-implanted rats, they significantly increase the time spent beyond the rail in both sexes following PWD, relative to vehicle-injected rats and relative to LZM injections during PWD. **C** Percent time open arm. PWD significantly decreased the percent time spent in the open arm (relative to closed arm) in both sexes. LZM significantly increased the percent time spent in the open in comparison to vehicle-treated, sham-implanted rats (vehicle) of both sexes. However, LZM is not significantly anxiolytic following PWD in either sex. FLU injections had no significant effects on the percent time spent in the open arm in sham-implanted rats. However, after PWD, FLU injections are anxiolytic relative to sham-implanted subjects of both sexes injected with either vehicle or FLU. **D** Open arm entries/total arm entries. PWD significantly decreased the percent open arm entries relative to total entries in both sexes. LZM significantly increased the percent open arm entries in comparison to vehicle-treated, sham-implanted rats (vehicle). In females, however LZM is significantly less anxiolytic following PWD in both sexes. FLU injections had no significant effects on the percent open arm entries in sham-implanted rats. However, after PWD, FLU injections were anxiolytic relative to sham-implanted subjects of both sexes injected with either vehicle or FLU.

Fig. 4.

PWD does not alter locomotor activity. **A** Grid crosses. Indicates the total number of grid crosses in a ten-minute test session in the elevated plus maze in either male (closed bars) or female (shaded bars) rats. There were no significant effects of implant condition or drug treatment on locomotor activity. Females had a general higher activity level than males, which did not interact with either drug or implant condition. Sample size for this figure and for Fig. 3 is indicated by the numbers at the base of the bars. **B** Total entries. Although ANOVA tests indicate a significant effect of total arm entries in females, post hoc comparisons (enumerated in Table 1) reveal that the only significant effects were an increase of total entries in female PWD rats receiving FLU injections compared to PWD rats and to FLU-injected sham-implanted controls (vehicle).

Table 1

Statistical tables of significance. This table refers to Figs. 3 and 4, and indicates ANOVA values for each sex individually by condition and post-hoc comparisons as assessed with a Fishers PLSD post hoc t-test between treatment conditions in the elevated plus maze. Significant results are indicated between the treatment groups in boldface and by the letter "S" in the significance column. The main treatment effects are abbreviated as follows: sham-implanted rats are identified by the type of injection only (i.e. vehicle, LZM or FLU) whereas animals undergoing PWD are so indicated in combination with the injection type (i.e., PWD/LZM and PWD/FLU). The group indicated by PWD was also injected with vehicle

Female		Male	
Time Open Arm	P-Value	Time Open Arm	P-Value
Anova (df 5,61) F=27.545	0.001	S Anova (df 5,46) F=16.350	0.001 S
vehicle vs. LZM	0.001	S vehicle vs. LZM	0.001 S
vehicle vs. PWD	0.004	S vehicle vs. PWD	0.009 S
vehicle vs. PWD/FLU	0.001	S vehicle vs. PWD/FLU	0.001 S
vehicle vs. PWD/LZM	0.740	vehicle vs. PWD/LZM	0.204
FLU vs. LZM	0.001	S FLU vs. LZM	0.001 S
FLU vs. PWD/FLU	0.001	S FLU vs. PWD/FLU	0.001 S
LZM vs. PWD/LZM	0.001	S LZM vs. PWD/LZM	0.001 S
PWD vs. PWD/FLU	0.001	S PWD vs. PWD/FLU	0.001 S
PWD vs. PWD/LZM	0.005	S PWD vs. PWD/LZM	0.124
PWD/FLU vs. PWD/LZM	0.001	S PWD/FLU vs. PWD/LZM	0.001 S
% Time Open Arm	P-Value	% Time Open Arm	P-Value
Anova (df 5,61) F=27.55	0.001	S Anova (df 5,46) F=16.35	0.001 S
vehicle vs. LZM	0.001	S vehicle vs. LZM	0.001 S
vehicle vs. PWD	0.005	S vehicle vs. PWD	0.009 S
vehicle vs. PWD/FLU	0.001	S vehicle vs. PWD/FLU	0.001 S
vehicle vs. PWD/LZM	0.740	vehicle vs. PWD/LZM	0.204
FLU vs. LZM	0.001	S FLU vs. LZM	0.001 S
FLU vs. PWD/FLU	0.001	S FLU vs. PWD/FLU	0.001 S
LZM vs. PWD/LZM	0.001	S LZM vs. PWD/LZM	0.001 S
PWD vs. PWD/FLU	0.001	S PWD vs. PWD/FLU	0.001 S
PWD vs. PWD/LZM	0.005	S PWD vs. PWD/LZM	0.124
PWD/FLU vs. PWD/LZM	0.001	S PWD/FLU vs. PWD/LZM	0.001 S

Time Outside Rail	P-Value	Time Outside Rail	P-Value
Anova (df 5,61) F=22.384	0.001	S Anova (df 5,46) F=7.587	0.001
vehicle vs. LZM	0.001	S vehicle vs. LZM	0.034
vehicle vs. PWD	0.559	S vehicle vs. PWD	0.451
vehicle vs. PWD/FLU	0.001	S vehicle vs. PWD/FLU	0.001
vehicle vs. PWD/LZM	0.241	S vehicle vs. PWD/LZM	0.670
FLU vs. LZM	0.001	S FLU vs. LZM	0.017
FLU vs. PWD/FLU	0.001	S FLU vs. PWD/FLU	0.001
LZM vs. PWD/LZM	0.001	S LZM vs. PWD/LZM	0.078
PWD vs. PWD/FLU	0.001	S PWD vs. PWD/FLU	0.001
PWD vs. PWD/LZM	0.055	S PWD vs. PWD/LZM	0.258
PWD/FLU vs. PWD/LZM	0.001	S PWD/FLU vs. PWD/LZM	0.001
Open Arm Entries/Total Entries	P-Value	Open Arm Entries/Total Entries	P-Value
Anova (df 5,61) F=14.024	0.001	S Anova (df 5,46) F=9.967	0.001
vehicle vs. LZM	0.001	S vehicle vs. LZM	0.533
vehicle vs. PWD	0.004	S vehicle vs. PWD	0.001
vehicle vs. PWD/FLU	0.003	S vehicle vs. PWD/FLU	0.994
vehicle vs. PWD/LZM	0.356	S vehicle vs. PWD/LZM	0.020
FLU vs. LZM	0.001	S FLU vs. LZM	0.001
FLU vs. PWD/FLU	0.001	S FLU vs. PWD/FLU	0.002
LZM vs. PWD/LZM	0.004	S LZM vs. PWD/LZM	0.008
PWD vs. PWD/FLU	0.001	S PWD vs. PWD/FLU	0.001
PWD vs. PWD/LZM	0.001	S PWD vs. PWD/LZM	0.003
PWD/FLU vs. PWD/LZM	0.017	S PWD/FLU vs. PWD/LZM	0.043
Total Entries	P-Value	Total Entries	P-Value
Anova (df 5,61) F=5.276	0.001	S Anova (df 5,46) F=1.074	0.387
vehicle vs. LZM	0.137	S vehicle vs. LZM	0.735
vehicle vs. PWD	0.075	S vehicle vs. PWD	0.101
vehicle vs. PWD/FLU	0.112	S vehicle vs. PWD/FLU	0.632
vehicle vs. PWD/LZM	0.218	S vehicle vs. PWD/LZM	0.296

FLU vs.PWD/FLU	0.003	S	FLU vs.PWD/FLU	0.459
FLU vs. PWD/LZM	0.329		FLU vs. PWD/LZM	0.661
LZM vs. PWD/LZM	0.003	S	LZM vs. PWD/LZM	0.204
PWD vs. PWD/FLU	0.001	S	PWD vs. PWD/FLU	0.308
PWD vs. PWD/LZM	0.516		PWD vs. PWD/LZM	0.471
PWD/FLU vs. PWD/LZM	0.005	S	PWD/FLU vs. PWD/LZM	0.673