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Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3α,5α-THP

Cheryl A. Frye*, Sandra M. Petralia, Madeline E. Rhodes

Department of Psychology, SUNY-Albany, 1400 Washington Avenue, Albany, NY 12222, USA

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Abstract

Sex differences and estrous cycle variations in anxiolytic-like behaviors and progestin concentrations were examined. Proestrous ($n=22$), estrous ($n=19$), diestrous ($n=20$), and male ($n=18$) Long–Evans rats were tested in horizontal crossing, open field, elevated plus-maze, emergence, holeboard, social interaction, tailflick, pawlick, and defensive burying tasks. Concentrations of plasma and hippocampal progesterone and 5α-pregnan-3α-ol-20-one (3α,5α-THP) were measured by radioimmunoassay in behaviorally tested (proestrus $n=11$, estrus $n=8$, diestrus $n=9$, male $n=7$) and yoked non-tested rats (proestrus $n=11$, estrus $n=8$, diestrus $n=10$, male $n=8$). Proestrous females exhibited more anxiolytic-like behavior than all other groups on the elevated plus-maze, social interaction, and defensive burying tasks. Proestrous females had significantly shorter latencies to emerge from a cylinder than did estrous and diestrous females, but not males. Proestrous and estrous females entered significantly more peripheral and total squares in a brightly-lit open field than did males. While proestrous females had a tendency to make more beam breaks than did males in the horizontal crossing task, there were no differences between groups on the holeboard task. There was a tendency for proestrous females to have longer tailflick latencies than diestrous and male rats; however, on the pawlick task there were no differences among the groups. Plasma and central progesterone and 3α,5α-THP of tested and non-tested rats were not different. Proestrous females had significantly higher plasma and hippocampal progesterone and 3α,5α-THP levels than all other groups. These data demonstrate that proestrous increases in anxiolytic-like behavior coincide with elevated circulating and hippocampal progestin concentrations.

Keywords: Progestin; 5α-Pregnan-3α-ol-20-one (3α,5α-THP); Hippocampus; Non-genomic; Neurosteroid; Allopregnanolone

During the proestrous phase of the estrous cycle, when estradiol and progesterone peak, there are many physiological changes. For example, on proestrus there are increases in physiological activity [41], plasticity [54], long-term potentiation [43,58], and synaptic density [33,60] in the hippocampus, which are not seen during the estrous phase of the cycle (characterized by a decrease in estradiol and progesterone relative to proestrus). The physiological changes that are most recognized over the estrous cycle are fluctuations in the steroid hormones, estradiol, and progesterone; however, estrous changes in other endocrine factors, such as progesterone metabolites, may influence performance on anxiety tasks.

Fluctuations in progesterone metabolites may mitigate performance on anxiety tasks. Progesterone metabolites are increased peripherally and centrally on proestrous compared to other phases of the estrous cycle. Administration of progesterone and its metabolites increases anxiolytic behaviors [7,8,48,59]. The similar effects of progesterone and its metabolites on anxiety-like behavior suggest a common action. The discrepant affinities of progesterone and its metabolites for intracellular progestin receptors (PRs) and γ-aminobutyric acid $(GABA)_A$/benzodiazepine receptor complexes (GBRs) indicate their common behavioral effects may require metabolism. Peripherally and centrally, progesterone and its 5α-reduced metabolite dihydroprogesterone (DHP) are converted readily to 5α-pregnan-3α-ol-20-one (3α,5α-THP). 3α,5α-THP is the most effective endogenous compound at enhancing GBR function [35,45], but is devoid of affinity for PRs. Progesterone and DHP have high affinities for PRs [38],

* Corresponding author. Tel.: +1-518-442-4836; fax: +1-518-442-4867.

E-mail address: cafrye@cnsunix.albany.edu (C.A. Frye).

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but are only weakly active at GBRs [45]. Progestins can have benzodiazepine-like effects [45,46,61], produce analgesia [26,27], anxiolysis [7,8], and generalize to pentobarbital in drug discrimination tasks [36]. These similar effects of progestins, in conjunction with their discrepant affinities for PR and GBRs, suggest progestins' ability to be metabolized to 3α,5α-THP and have actions at GBRs may underlie progestin-induced variations in anxiety behaviors.

Progestin actions in the hippocampal complex may be integral in mediating performance on anxiety tasks. Evidence in support of this includes the following. First, progestin increases in the hippocampal complex, an area of the brain that is particularly important for anxiety-like behaviors, are evident on proestrus [23]. Second, infusions of pregnanolone, a precursor of progesterone, and 3α,5α-THP, into the hippocampus of rats increases open arm time on the elevated plus-maze and decreases defensive burying in the shock-probe test [5]. Third, infusions of 3α,5α-THP into the central nucleus of the amygdala of rats increases punished responding in the Geller–Seifter conflict task and increases open arm time in the elevated plus-maze [1].

Taken together, these data strongly suggest that increases in progesterone and 3α,5α-THP in the hippocampal complex may modulate changes in performance on anxiety tasks in rodents. The purpose of this experiment is to test the hypothesis that estrous cycle and sex differences in performance on anxiety tasks occur concomitant with endogenous changes in peripheral and hippocampal progesterone and 3α,5α-THP.

1. Method

These methods were pre-approved by the Institutional Animal Care and Use Committee.

1.1. Animals and housing

Long–Evans rats ($N = 116$; 90 females and 26 males), approximately 55 days of age, were obtained from Taconic Farms (Germantown, NY) and were group housed (four per cage) in polycarbonate cages ($45 \times 24 \times 21$ cm) in a temperature-controlled room (21 ± 1°C) in the laboratory animal care facility. The rats were maintained on a 12/12-h reversed light cycle (lights off 8:00 am) with access to Purina Rat Chow and tap water in their home cages.

Vaginal epithelium was obtained at 8:00 am via lavage and examined daily from female rats ($n = 90$) to determine the day of the estrous cycle. Estrus was determined by the characteristic cornification of the vaginal epithelium at this stage [28,44]. Rats were cycled through two normal estrous cycles (4–5-day cycle) and then randomly assigned to be tested during the proestrous, estrous, or diestrous phases of the estrous cycle ($n = 61$) based on vaginal cytology. Male rats were handled daily to account for the effects of cycling procedures in females.

1.2. Behavioral testing

After cycling/handling, the vaginal cytology was examined and estrous cycle phase was determined. Rats in proestrus ($n = 22$), estrus ($n = 19$), diestrus ($n = 20$), and males ($n = 18$) were randomly assigned to be tested once, with counterbalancing across groups on and between days, to prevent any possible order effects. Three to five rats were tested daily, each for 90 min, in a single, brightly lit, room adjacent to the animal housing facility, between 1000 and 1700 h, in the behavioral tasks described below. All of the rats were exposed to all of the tasks consecutively and without any rest periods in the order indicated.

1.2.1. Horizontal crossings

Rats were placed in a brightly lit $39 \times 39 \times 30$-cm Digiscan Optical Animal Activity Monitor (Accuscan Instruments, Columbus, OH). The number of beam breaks that occurred in a 5-min test was mechanically recorded.

1.2.2. Open field

The open field task [10] was used in accordance to the methods described by McCarthy et al. [47]. The open field ($76 \times 57 \times 35$ cm), is a box with a 48-square grid floor (6×8 squares, 9.5 cm/side), and an overhead light illuminating the central squares (all but the 24 perimeter squares were considered central). Rats were placed in the box and observed for 5 min while the number of central and peripheral squares (summed for total) entered was recorded during the test period by an observer.

1.2.3. Elevated plus-maze

The elevated plus-maze paradigm described by Pellow and File [50] and as per Dunn et al. [17] was employed. The elevated plus-maze consisted of four arms, 49 cm long and 10 cm wide, elevated 50 cm off the ground. Two arms were enclosed by walls 30 cm high and the other two arms were exposed. Rats were placed at the junction of the open and closed arms of the maze and were observed for 5 min. The number of entries and the amount of time spent on the open and closed arms were recorded during the test by an observer. Rats were considered to be either in the closed or open arms of the maze and open arm entry and time were recorded only when the rat had all four paws on the open arm of the elevated plus-maze.

1.2.4. Emergence test

Rats were placed in a closed opaque cylinder ($21 \times 7 \times 7$ cm), that was set in an open field and secured to prevent rolling. The lid of the cylinder was removed and the latency for the rat to emerge completely from the cylinder was recorded by an observer (maximum latency: 15 min).

1.2.5. Holeboard

The holeboard procedure utilized was according to the methods of Zangrossi and File [62]. The holeboard was a wooden box ($60 \times 60 \times 35$ cm) with four holes (3.8 cm diameter) equally spaced on the floor. The incidence of head dips and rearings were recorded at the time of the test by an observer for a period of 5 min.

1.2.6. Social interaction

The social interaction test previously described by Ge et al. [32] was conducted in a wooden box ($60 \times 60 \times 35$ cm) similar to that used for the holeboard test. Testing involved placing each member of a pair of rats in opposite corners of the box and then leaving them undisturbed for 10 min while an experimenter recorded the total duration of time (seconds) that the test rat engaged the stimulus animal in crawling over and under partner, sniffing of partner, following with contact, genital investigation of partner, tumbling, boxing, and grooming. Male rats were tested with intact male conspecifics, female rats were tested with diestrous female conspecifics, and all conspecifics were randomly selected from a pool of stimulus rats.

1.2.7. Tailflick

The tailflick paradigm used was described by D'Amour and Smith [16] and followed the methods of Frye and Duncan [26,27]. Rats were handled, covered with a towel, placed on the platform of the tailflick apparatus (San Diego Instruments, San Diego, CA) and held in place while their tails were smoothed above the radiant heat source. The mean latency of three tailflick trials was used as an index of nociception (maximum latency: 10 s).

1.2.8. Pawlick

The pawlick procedures employed by Smythe et al. [53] and Bardo and Valone [4] were utilized. The apparatus consisted of a slide warming tray (model #77; Fisher Scientific, Swanee, GA) upon which a clear, floorless, plastic chamber ($28.5 \times 17.5 \times 12.5$ cm) was placed. The plastic chamber was used to confine the animal to the hotplate surface that was heated to 53°C. Rats were placed in the chamber on the hotplate and the latency to lick the hindpaw was recorded (maximum latency: 180 s).

1.2.9. Defensive burying

The test chamber ($26.0 \times 21.2 \times 24.7$ cm) utilized for the defensive burying procedure was constructed of clear Plexiglas. A pedestal (2.5 cm in diameter and 10.0 cm in height) was placed in the right rear corner of the chamber, 3.0 cm from the back wall and 2.5 cm from the right wall. Wood chip bedding was placed 5.0 cm deep from the bottom of the chamber, such that the pedestal extended 4.5 cm above the material. The pedestal was wrapped by two wires that were connected to a shock source (Lafayette Model A615B, Lafayette, IN) set to deliver 6.66 mA of unscrambled shock, initiated by the experimenter and terminated by the animals withdrawal of its paws from the pedestal.

The defensive burying procedure utilized was according to the modified methods of Gallo and Smith [30]. Briefly, rats were placed in the chamber and an observer recorded the duration of freezing and burying activity for 15 min after the rat had touched the electrified prod.

1.3. Tissue collection

After 11 rats per group had been tested, the remaining 72 animals, 35 that underwent behavioral testing (proestrus $n = 11$, estrus $n = 8$, diestrus $n = 9$, male $n = 7$) and 37 that did not (proestrus $n = 11$, estrus $n = 8$, diestrus $n = 10$, male $n = 8$) but were in the same hormonal condition (proestrus, estrus, diestrus, or male), were killed by rapid decapitation. Tested and non-tested subjects were yoked. The animal that was behaviorally tested was terminated immediately after testing in the necropsy facility that is adjacent to the behavioral testing laboratory. The non-tested animals remained in their home cages and were brought to the necropsy room and decapitated (after their yoked partner). Trunk blood was collected and remained on ice until refrigerated centrifugation (4°C at $3000 \times g$ for 8 min). Serum was aliquoted and stored at $-70°$ C until radioimmunoassay for progesterone and 3α,5α-THP. Brains were rapidly removed, the hippocampus was dissected bilaterally, placed in dry ice, and stored at -70°C until radioimmunoassay for progesterone and 3α,5α-THP.

1.4. Radioimmunoassay of plasma and brain progesterone

Concentrations of progesterone were determined by radioimmunoassay according to previously published methods [22,29]. Briefly, progesterone was extracted from plasma samples by diethyl ether; the solvent was removed using a speed drier, and samples were resuspended in assay buffer (pH = 7.4). For brains, hippocampal tissue was homogenized with a glass/Teflon homogenizer in distilled water. Steroids were extracted from the homogenate with 50% MeOH, 1% acetic acid, dried down in an evaporator drier, and the pellet was reconstituted in trimethyl pentane (TMP) to half the homogenate volume. Progesterone was extracted from the reconstituted plasma and brain extracts using Celite column chromatography. The progestin fraction was collected using a 100% TMP wash. Fractions were dried using a speed drier and then reconstituted in phosphate assay buffer.

Radioimmunoassay was performed using [^{3}H] P (NET-208, specific activity 48.4 Ci/mmol, New England Nuclear (NEN), Boston, MA) and antisera (P#337 from Dr. G.D. Niswender, Colorado State University). The progesterone antibody was used in a 1:30,000 dilution and bound between 30% and 50% of [^{3}H] P.

The standard curve was prepared in duplicate to give a range of nine concentrations from 50 to 8000 pg/ml; total

Table 1
Estrous cycle and sex differences in total number of beam breaks in the horizontal crossing and mean tailflick latencies

	Condition			
	Proestrus ($n=22$)	Estrus ($n=19$)	Diestrus ($n=20$)	Male ($n=18$)
Total number of beam breaks	975 ± 59	937 ± 50	807 ± 77	771 ± 74
Mean tailflick latency (s)	4.5 ± 0.4	3.5 ± 0.4	3.1 ± 0.3	4.1 ± 0.4

volume 800 μl. Incubation (4°C for 24 h) was terminated by the addition of charcoal. Following a 15-min incubation on ice, samples were centrifuged at $1200 \times g$ for 10 min. Sample concentrations were calculated using the logit–log method [52], interpolation of the standards, and correction for recovery. The minimum detectable limit of the assay was 50 pg, and the intra-assay and inter-assay coefficients of variance were 10.7% and 9.2%, respectively.

1.5. Radioimmunoassay of plasma and brain 3α,5α-THP

3α,5α-THP was measured according to previously established methods [20,23,24,51]. Briefly, steroids were extracted from plasma samples using diethyl ether. Steroids were extracted from homogenized brain samples in 50% MeOH, 1% acetic acid through a series of centrifugation and filtrations. Three hundred microliters of 0.1 M phosphate assay buffer (pH = 7.4) was added to test tubes containing steroid extracts and equilibrated.

The antibody, purchased from Dr. Robert Purdy (Veterans Medical Affairs, La Jolla, CA), is very specific to 3α,5α-THP [20]. The 1:5000 dilution of this antibody bound between 40–60% of [^{3}H] 3α,5α-THP (NET-1047, 51.3 Ci/mmol; NEN).

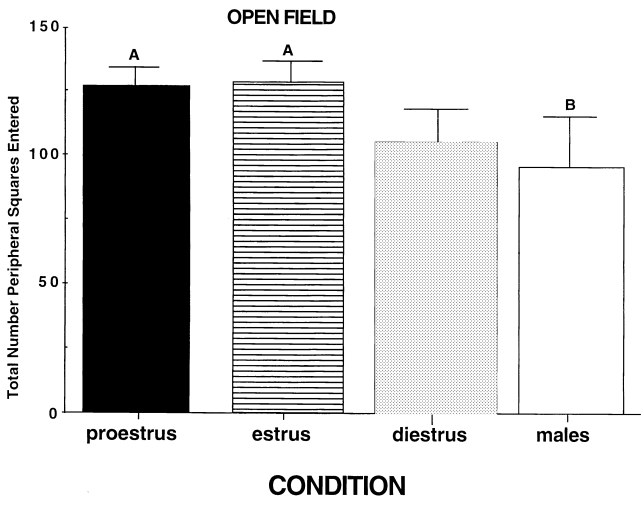

Fig. 1. Represents the total number of peripheral squares entered (± S.E.M.) of proestrous (black bars), estrous (striped bars), diestrous (gray bars), and intact male rats (open bars). Bars with different letters are significantly different from each other ($P<.05$).

The standard curve was prepared in duplicate with a range of nine concentrations from 50 to 8000 pg/ml; total volume 950 μl. Incubation at 4°C for 24 h was terminated by charcoal separation of bound and free. Sample tube concentrations were calculated using the logit–log method of Rodbard and Hutt [52], interpolation of the standards, and correction for recovery. The minimum detectable limit of the assay was 100 pg. The intra-assay and inter-assay coefficients of variance were 12.1% and 15.6%.

1.6. Statistical analyses

Multiple one-way analyses of variances (ANOVAs) were used to examine effects of hormone condition on behavior. Two-way ANOVAs were used to examine how

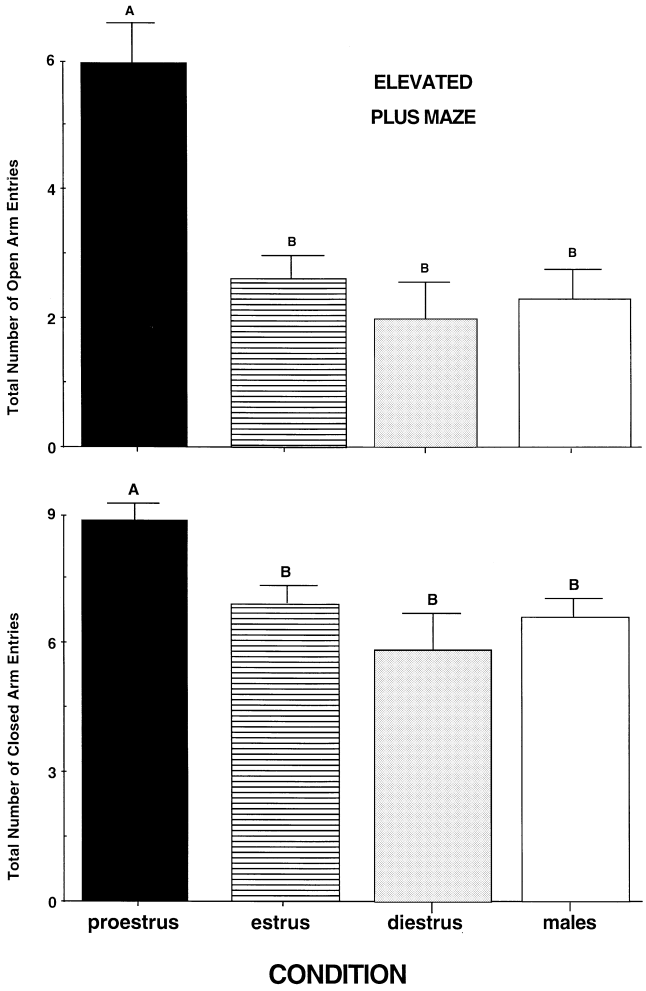

Fig. 2. The top panel represents the total number of open arm entries (± S.E.M.) on the elevated plus-maze of proestrous (black bars), estrous (striped bars), diestrous (gray bars), and intact male rats (open bars). Bars with different letters are significantly different from each other ($P<.05$). Bottom panel represents the total number of closed arm entries (± S.E.M.) on the elevated plus-maze of proestrous (black bars), estrous (striped bars), diestrous (gray bars), and intact male rats (open bars). Bars with different letters are significantly different from each other ($P<.05$).

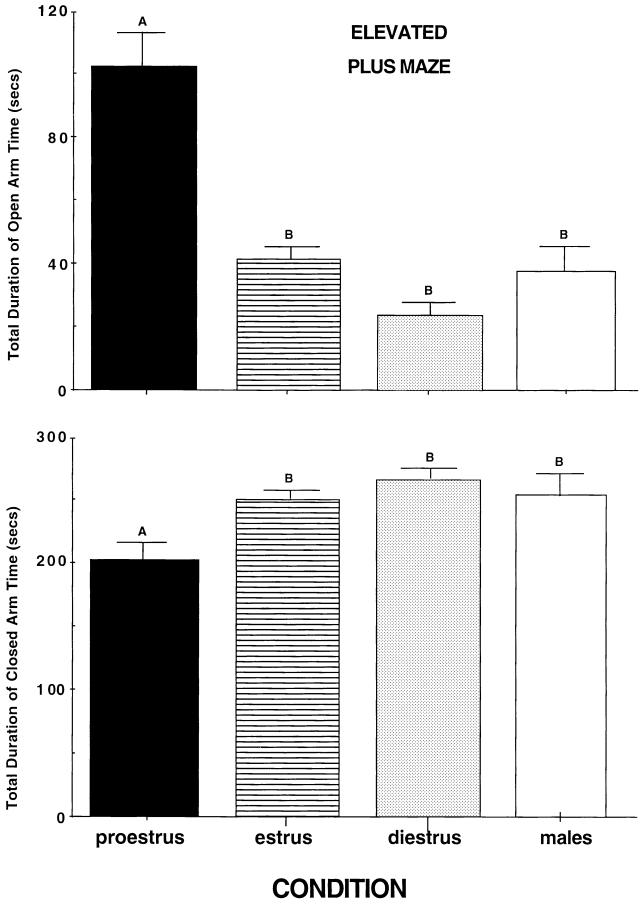

Fig. 3. The top panel represents the total duration of open arm time (±S.E.M.) on the elevated plus-maze of proestrous (black bars), estrous (striped bars), diestrous (gray bars), and intact male rats (open bars). Bars with different letters are significantly different from each other ($P<.05$). Bottom panel represents the total duration of closed arm time (±S.E.M.) on the elevated plus-maze of proestrous (black bars), estrous (striped bars), diestrous (gray bars), and intact male rats (open bars). Bars with different letters are significantly different from each other ($P<.05$).

hormone conditions and testing altered steroid concentrations. Where appropriate, ANOVAs were followed by Fisher's post-hoc tests and least squares mean comparisons between groups.

2. Results

2.1. Horizontal crossings

There was a tendency for proestrous females to make more beam breaks in the horizontal crossing task than males [$F(3,75)=2.271$, $P<.087$; see Table 1].

2.2. Open field

The proestrous and estrous females entered significantly more peripheral [$F(3,75)=3.082$, $P<.03$; see Fig. 1] and total squares [$F(3,75)=3.456$, $P<.03$] than did males.

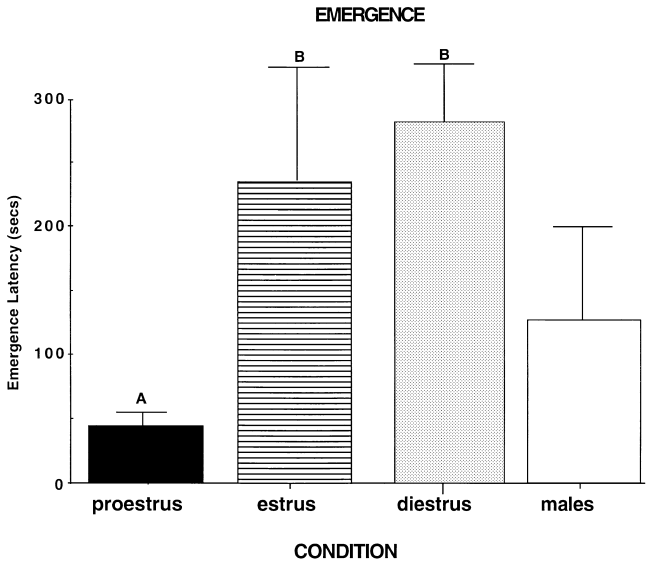

Fig. 4. Represents the latency to emerge from a tube in seconds (±S.E.M.) of proestrous (black bars), estrous (striped bars), diestrous (gray bars), and intact male rats (open bars). Bars with different letters are significantly different from each other ($P<.05$).

There was no significant difference between groups in the total number of central squares entered. The average number of central squares entered by each group was proestrus 33 (±4), estrus 37 (±6), diestrus 27 (±6), male 20 (±4).

2.3. Elevated plus-maze

The number of entries to the open arms [$F(3,75)=17.554$, $P\leq.0001$], the number of entries to the closed arms [$F(3,75)=3.556$, $P\leq.01$], the amount of time spent on the open arms [$F(3,75)=19.926$, $P\leq.0001$], and the amount of time spent on the closed arms [$F(3,75)=16.571$, $P\leq.0001$] of the elevated plus-maze varied between the groups.

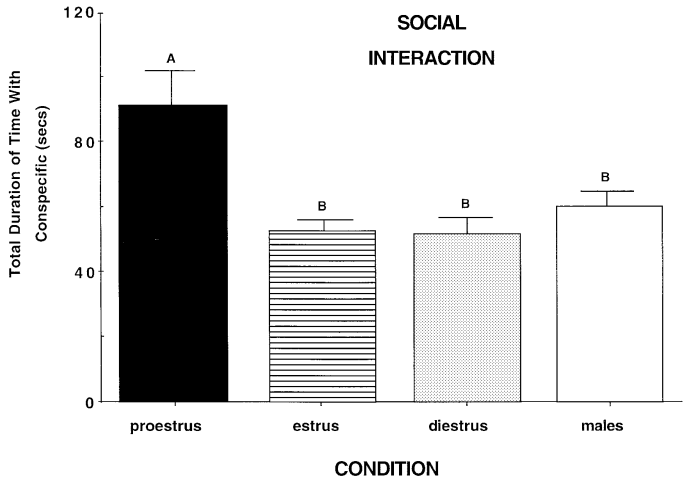

Fig. 5. Figure represents total duration of time spent interacting with conspecific in seconds (±S.E.M.) of proestrous (black bars), estrous (striped bars), diestrous (gray bars), and intact male rats (open bars). Bars with different letters are significantly different from each other ($P<.05$).

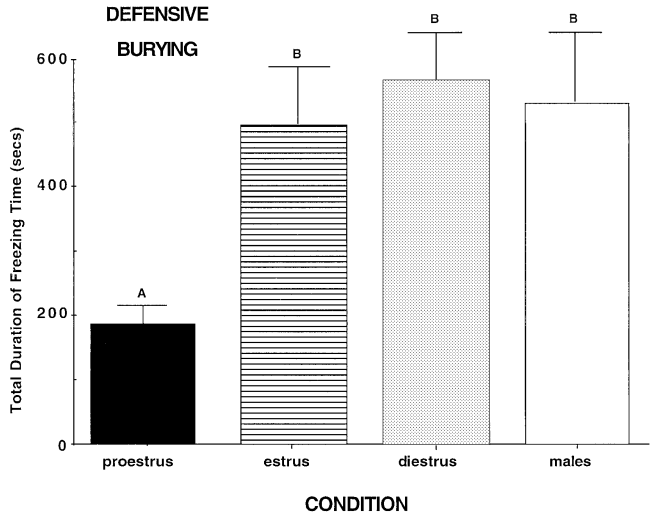

Fig. 6. Represents total duration of freezing in seconds (±S.E.M.) of proestrous (black bars), estrous (striped bars), diestrous (gray bars), and intact male rats (open bars). Bars with different letters are significantly different from each other ($P<.05$).

Proestrous rats had significantly more entries to the open arms and closed arms (and likewise the total number of arm entries, $F(3,75)=11.033$, $P\leq.0001$) than did estrous, diestrous, or male rats (see Fig. 2). The duration of time spent on the open arms was significantly greater in the proestrous rats compared to all other groups (see Fig. 3, top). The duration of time spent on the closed arms was significantly less in the proestrous rats compared to estrous, diestrous, and male rats (see Fig. 3, bottom).

2.4. Emergence test

The latency for the rats to emerge completely from the cylinder was significantly shorter for proestrous than for estrous and diestrous but not male rats [$F(3,75)=2.810$, $P\leq.04$; see Fig. 4].

2.5. Holeboard

The incidence of head dips did not vary among the groups. The average number of head dips for each group was proestrus (3.2±0.6), estrus (2.6±0.6), diestrus (2.7±0.7), and male (1.2±0.4). The total number of rearings was not different across conditions and the total rearings for each group were proestrus (14.3±1.8), estrus (13.5±1.7), diestrus (14.8±2.5), and male (9.5±1.8).

2.6. Social interaction

The total duration of time spent in contact with a conspecific in genital investigation, sniffing, crawling over and under, tumbling, boxing, and grooming was significantly higher [$F(3,75)=11.285$, $P\leq.0001$] in proestrous rats compared to all other groups (see Fig. 5).

2.7. Tailflick

There was a tendency for proestrous females to have longer tailflick latencies than diestrous and male, but not estrous, rats (see Table 1).

2.8. Pawlick

The latency to lick the hindpaw did not vary as a function of cycle phase or sex. The pawlick latencies for each group were proestrus (117.8±11.9), estrus (102.0±12.3), diestrus (107.8±11.0), male (140.8±12.3).

2.9. Defensive burying

The duration of freezing following shock was significantly less in proestrous compared to estrous, diestrous, and male rats [$F(3,75)=11.426$, $P\leq.0001$; see Fig. 6]. There

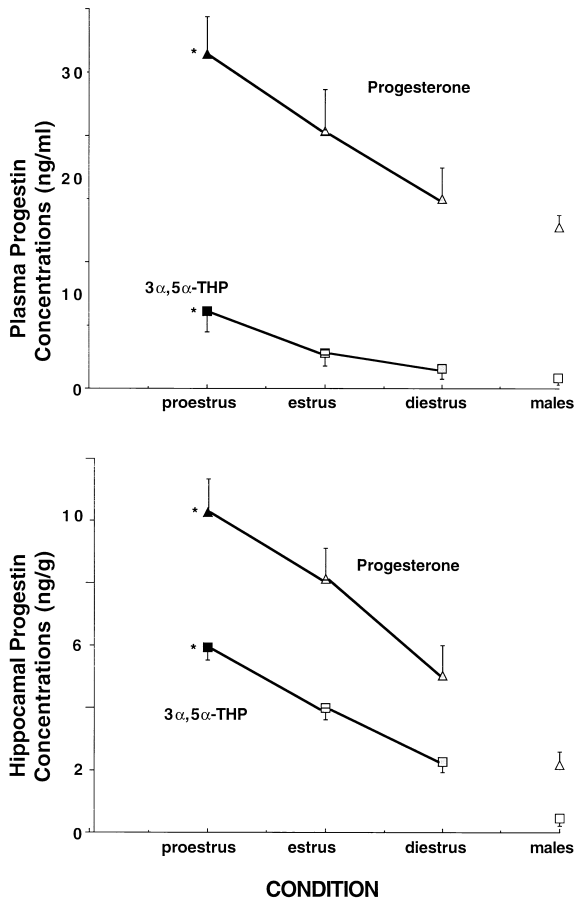

Fig. 7. The top panel depicts plasma progestin concentrations (mean ng/ml±S.E.M.) for proestrous (black symbols), estrous (striped symbols), diestrous (gray symbols), and male rats (open symbols). Asterisk indicates a significant difference ($P<.05$). Bottom panel depicts progestin concentrations in hippocampal tissues (mean ng/g±S.E.M.) for proestrous (black symbols), estrous (striped symbols), diestrous (gray symbols), and male rats (open symbols). Progesterone concentrations are denoted by triangles and 3α,5α-THP is indicated by squares. Asterisk indicates a significant difference ($P<.05$).

was no significant difference in burying time for proestrus (228.0 ± 30.4), estrus (147.9 ± 37.6), diestrus (161.0 ± 40.6), or males (161.4 ± 40.4). The height of sawdust did not vary among the groups and the sawdust height for each group was proestrus (4.8 ± 0.2), estrus (4.1 ± 0.3), diestrus (3.9 ± 0.3), and males (4.2 ± 0.3).

2.10. Radioimmunoassay of plasma and brain progesterone and 3α,5α-THP

Proestrous females had significantly higher plasma progesterone [$F(3,64) = 14.463$, $P \leq .0001$] and 3α,5α-THP [$F(3,64) = 5.071$, $P \leq .003$] than all other groups (see Fig. 7, top). Hippocampal progesterone [$F(3,64) = 11.046$, $P \leq .0001$] and 3α,5α-THP [$F(3,64) = 12.098$, $P \leq .0001$] were significantly higher in proestrous females compared to all other groups (see Fig. 7, bottom). There were no differences between behaviorally tested and non-tested rats for plasma or hippocampal progesterone or 3α,5α-THP. Hormone condition and testing did not interact to influence plasma or hippocampal progestin concentrations.

3. Discussion

Proestrous females exhibited more anxiolytic-like behavior than other groups on some, but not all, of the tasks and had significantly higher hippocampal and plasma progesterone and 3α,5α-THP levels than did all other females and males. The increased anxiolytic-like behavior of the proestrous rats, compared to all other groups, was demonstrated by significantly more open arm entries and time in the elevated plus-maze, longer social interactions with conspecifics, and less freezing time in response to shock. The number of closed arm entries and total amount of closed arm time also varied across groups with proestrous females having significantly more closed arm entries and less closed arm time than all other groups. In addition to this, proestrous females had significantly shorter emergence latencies than did estrous and diestrous, but not male, rats. Proestrous and estrous females exhibited significantly more peripheral square entries in the open field than did males. There was a trend for the proestrous females to make more beam breaks than the males in the horizontal crossing; however, there were no significant differences among the groups in the holeboard task. Although, there were no differences among the groups in the pawlick task, proestrous females tended to have longer tailflick latencies than diestrous or male, but not estrous, rats. Tested and non-tested animals were not different in concentrations of plasma or hippocampal progesterone or 3α,5α-THP. Progesterone and 3α,5α-THP levels were significantly increased in proestrous females compared to all other groups. Together, these data indicate that proestrous increases in some anxiolytic-like behaviors coincide with elevated levels of endogenous circulating and hippocampal progestin concentrations.

The present findings are consistent with the literature that suggests progesterone metabolism to 3α,5α-THP may mediate changes in behavior on tasks of anxiety. For example, administration of progesterone or 3α,5α-THP induces analgesia [25–27] and anxiolytic-like behavior in male and female rodents [8,11,14,48]. Progesterone's anxiolytic effect is attenuated by co-administration of a 5α-reductase inhibitor, which blocks progesterone's conversion to 3α,5α-THP [9]. In addition to this, there are estrous cycle variations in females' responses to aversive stimuli; proestrous females engage in less defensive burying compared to rats in other phases of the estrous cycle [19]. Further, precipitating withdrawal from progesterone by administering the metabolism inhibitor, indomethacin, increases defensive burying compared to vehicle-administered rats [30].

It is likely that peripheral progesterone and 3α,5α-THP mediate changes in anxiolytic-like behaviors through central actions of progestins at GBRs. 3α,5α-THP release elicited in the hippocampus by activation of peripheral benzodiazepine receptors produces anxiolytic-like effects in rats [6]. As well, RU38486, the PR blocker, which would be expected to attenuate progesterone but not 3α,5α-THP's action, does not prevent the anxiolytic action of progesterone [9]. Further, GBR antagonists prevent progestin-induced anxiolysis. For example, co-administration of the GBR blocker, picrotoxin, attenuates pregnanolone-induced increases in open arm time in the plus-maze [7]. Progestins' ability to enhance GBR function correlates well with their anti-nociceptive effects [26,27]. Taken together, previous findings and the present data suggest that the actions of 3α,5α-THP at GBRs may account for the anxiolytic-like behavioral changes seen in proestrous rats.

This study is among the first to show increases in endogenous 3α,5α-THP in the hippocampal complex coincident with changes in anxiolytic-like behaviors. The increased levels of hippocampal and plasma progesterone and 3α,5α-THP observed in the proestrous (behavioral estrus) females of this study are consistent with findings previously reported by this and other labs [12,21–23,37,51]. Cyclic changes in progestins were observed both dependent and independent of behavioral testing, consequently, it is unlikely that an anxiolytic state caused the observed increases in central and circulating steroid levels. Also, it is improbable that a third variable caused changes in both behavior and steroid levels because tested and non-tested animals had similar progestin levels.

The concurrent proestrous increases in anxiolytic-like behavior and progestin concentrations presently demonstrated should be interpreted with caution for several reasons. First, this study does not demonstrate a causal relationship that alterations in peripheral or hippocampal progestin concentrations induce the behavioral variations observed over the estrous cycle and between the sexes.

Indeed, there are estrous variations in a number of behaviors that progestin fluctuations may influence directly or indirectly to produce some of the changes seen here. In particular, phase of the estrous cycle influences activity [3,13], appetite [31,40,56,57], sensitivity to environmental stimuli [25], stress responsiveness [15,55], and anxiety [2,3,8,34,39]. Indeed, in the present study differences in activity and pain sensitivity may have influenced performance. For example, proestrous rats demonstrated an increase in total entries in the plus-maze and open field, as well as beam breaks. Also, proestrous rats had longer tailflick latencies and decreased freezing following shock. Because activity and pain sensitivity varied, it is possible that performance on these "anxiety" tasks may be due to changes in anxiety, as well as other contributing factors. Second, there also may be other environmental factors that mediate the dramatic changes in anxiety-like behavior seen across hormonal and reproductive states. For example, one environmental factor that may have influenced our results is that the order in which these tests were administered was not validated, e.g., the effects of one or more of these tasks may have confounded animals' behavior on subsequent tasks. Other researchers have used a series of behavioral tasks to assess more than one model of animal anxiety in a single investigation [39,49] as there is not one single index of anxiety-like behavior in animals. NB: All of the animals were tested in all of the tasks and in the same order, consequently any possible order effects were uniformly distributed across all subjects and all conditions. Third, there also may be other endogenous physiological factors that mediate anxiety-like behaviors. A recent study indicates 3α,5α-THP concentrations are not the *sine qua non* for anxiolytic-like behavioral changes. Lactating rats with low progestin levels show increased open arm time in the plus-maze; administration of a 5α-reductase inhibitor, to limit even further the already low 3α,5α-THP concentrations, fails to reduce open arm time [42]. One interpretation of these findings is that dramatic increases in 3α,5α-THP during pregnancy change the sensitivity of GBRs for 3α,5α-THP, such that GBR sensitivity may be a more important mediator of anxiety in lactating rats than are 3α,5α-THP concentrations. However, it is important to consider that changes in other physiological parameters may also mediate such effects. Fourth, it has not been clearly established that traditional tests of anxiety measure the same variables in both sexes [39]. A recent factor analysis of male and female behavior in anxiety tasks suggests that male rats are motivated by sex and anxiety, whereas female rats are driven by activity [18]. In addition to this, males and females or females in different phases of the estrous cycle may vary on changes in other behaviors/states, responsiveness to other environmental factors, and/or other physiological parameters.

In summary, these data demonstrate that increases in some anxiolytic behavior of proestrous rats occur coincident with elevations in circulating and central progesterone and 3α,5α-THP in the hippocampus. These findings suggest that these increases in endogenous progestins may modulate the observed behavior changes. Endogenous changes in progesterone and 3α,5α-THP and their subsequent actions at GBRs in the hippocampus may mitigate endogenous variations in anxiolytic-like behavior.

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