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# Infusions of bicuculline to the ventral tegmental area attenuates sexual, exploratory, and anti-anxiety behavior of proestrous rats

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ABSTRACT

Actions of 5α-pregnan-3α-ol-20-one (3α,5α-THP), in the midbrain ventral tegmental area (VTA) modulate sexual receptivity of female rats. Actions of 3α,5α-THP at GABAergic substrates in the VTA are known to modulate consummatory aspects of sexual behavior among rodents, such as lordosis. However, the extent to which $GABA_A$ receptors in the VTA are important for appetitive (exploratory, anti-anxiety, social) aspects of sexual receptivity is not well-understood. Proestrous rats were bilaterally-infused with saline or bicuculline (100 ng), a $GABA_A$ receptor antagonist, to the VTA or missed control sites. Rats were assessed for exploratory/anti-anxiety (open field/elevated plus maze), social (social interaction), and sexual (paced-mating) behavior. Compared to saline or missed site controls, intra-VTA bicuculline significantly reduced the number of central entries in an open field, time spent on the open arms of an elevated plus maze, frequency and intensity of lordosis, anti-aggression towards a male, pacing of sexual contacts, and 3α,5α-THP concentrations in midbrain and hippocampus. Bicuculline-infused rats also displayed less affiliation with a novel conspecific, fewer sexual solicitations, and had lower 3α,5α-THP concentrations in diencephalon and cortex, albeit these were not significant differences. Thus, actions at $GABA_A$ receptors in the midbrain VTA are essential for appetitive and consummatory aspects of sexual receptivity among rats.



## 1. Introduction

Progesterone ($P_4$) plays a critical role in mediating sexual receptivity of female rodents (Powers, 1972) largely through actions in the ventromedial hypothalamus (VMH) and the ventral tegmental area (VTA) of the midbrain (DeBold and Malsbury, 1989; Frye et al., 1992; Malsbury et al., 1977; Pleim et al., 1990; Takahashi and Lisk, 1985). However, within these regions, $P_4$ acts via different mechanisms. There are many intracellular progestin receptors in the VMH at which $P_4$ can alter gene transcription through classical genomic action (Blaustein et al., 1994). Notably, in this region, rodent sexual behavior is also dependent on actions of estradiol ($E_2$; Meisel et al., 1987; Takahashi and Lisk, 1988). In the VTA, very few progestin receptors exist (Warembourg, 1978) and $P_4$ acts through its metabolism to the neurosteroid 5α-pregnan-3α-ol-20-one (3α,5α-THP). Unlike $P_4$, 3α,5α-THP acts at cell membranes of neurotransmitter targets and is dependent on their downstream signal transduction processes to modulate lordosis (the stereotypical female posture in response to male mounting; Frye et al., 2006a). Actions of 3α,5α-THP in the VTA are essential for $P_4$'s lordosis-enhancing effects (Frye et al., 2006a).

One mechanism underlying 3α,5α-THP's effects in the VTA involves actions at $GABA_A$ receptors. GABAergic neurons have been identified using *in vivo* extracellular and intracellular recordings in rat VTA (Steffensen et al., 1998). GABAergic neurons play a primary role in the local inhibition of mesocorticolimbic dopamine neurons which are plentiful in this region and can modulate GABA release (Kalivas and Duffy, 1995). 3α,5α-THP is the most potent known endogenous modulator of $GABA_A$ receptors (Majewska et al., 1986) and can act at these substrates to facilitate consummatory sexual behavior (lordosis; Frye, 2001; McCarthy et al., 1995) by enhancing GABAergic function (Twyman and Macdonald, 1992). Further, inhibiting glutamic acid decarboxylase, the enzyme that catalyzes GABA formation from glutamate, in this region reduces lordosis among rats and hamsters (Frye and Vongher, 1999; McCarthy et al., 1994). Thus, actions at $GABA_A$ receptors in the VTA are important for consummatory aspects of mating in the female rodent.

In addition to using lordosis as a bioassay, or index of the capacity for steroids to mediate consummatory aspects of sexual behavior, we are also interested in how progestogens mediate appetitive aspects of mating. When in the sexually-receptive phase of the estrous cycle (proestrous), female rats demonstrate less species-typical anxiety-like behavior and conspecific-avoidance, and spend more time in affiliation (Frye and

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Rhodes, 2008; Mora et al., 1996; Reddy and Kulkarni, 1999). Actions of progestogens at GABAergic substrates in the hippocampus have been implicated in such appetitive behaviors. For instance, soporific and anxiolytic effects associated with benzodiazepines (which target $GABA_A$ receptors) have long been demonstrated to act in hippocampus (Haefely, 1979; Jahnsen and Laursen, 1981). As well, 3α,5α-THP administration to hippocampus is anxiolytic (Bitran et al., 1991, 2000). While, $GABA_A$ receptor activation may underlie affective effects in hippocampus, the role of allosteric action in midbrain for anti-anxiety and associated appetitive sexual behavior is not well-understood. As such, we aimed to assess the necessity of $GABA_A$ receptors in the midbrain VTA for appetitive (exploratory, anti-anxiety, social, sexual approach and solicitation) and consummatory (lordosis) aspects of mating behavior. We anticipated that infusion of the $GABA_A$ receptor antagonist, bicuculline, to the VTA would attenuate proestrous-typical behavior among female rats.

## 2. Materials and methods

These methods were pre-approved by the Institutional Care and Use Committee at The University at Albany-SUNY.

### 2.1. Animals and housing

Adult, intact, female Long–Evans rats ($N=35$) were obtained from the breeding colony of the Life Sciences Research Laboratory Animal Care Facility at The University at Albany-SUNY (original stock Charles River, Raleigh, NC). Rats were group-housed in a temperature- and humidity-controlled room on a 12/12 h reverse light cycle (lights off at 0800 h) with *ad libitum* access to water and rat chow in their cages.

### 2.2. Surgery

Rats were stereotaxically implanted with bilateral guide cannulae aimed at the medial aspect of the VTA (from bregma: AP = −5.3, ML = ±0.4, DV = −7.0; Paxinos and Watson, 1986) under xylazine (12 mg/kg) and ketamine (60 mg/kg) anesthesia. Guide cannulae consisted of modified 23-gauge thin-wall stainless steel needles. Following surgery, rats were monitored for loss of weight, righting response, flank stimulation response, and/or muscle tone (Marshall and Teitelbaum, 1974).

### 2.3. Evaluation of sexual receptivity

Estrous cycle phase was determined by daily examination of vaginal epithelium (between 0700–0800 h), per previous methods (Frye et al., 2000; Long and Evans, 1922). Rats with cytology characterized by the presence of many nucleated epithelial cells were considered to be in the proestrous phase of their cycle. During this phase $E_2$ levels are declining, but progestogen levels are high (Feder, 1984; Frye and Bayon, 1999). Rats with proestrous vaginal smears were vaginally masked to prevent alterations in neuroendocrine status that can arise from vaginocervical stimulation (Frye and Bayon, 1999; Meerts and Clark, 2007, 2009; Pfaus et al., 1994) and briefly paired with a sexually-vigorous male. Sexual receptivity was determined by the response of experimental females to stimulus male investigation. Rats that demonstrated receptive (lordosis) and proceptive behaviors (hopping, darting, and ear wiggling) were considered to be in behavioral estrus, while those that exhibited aggressive behaviors (vocalizing, defensive posturing, boxing, and avoidance) were considered to not be in behavioral estrus. Only rats that demonstrated both proestrous cytology and sexually-receptive behavior were tested.

### 2.4. Behavioral testing

Rats in behavioral estrus were tested in the battery of tasks described below. All testing apparatus were brightly lit from above. All behavioral data were collected with the ANY-Maze data collection program (Stoelting Co., Wheat Dale, IL) and were hand-collected by one of two observers. There was a 97% concordance rate between data that was collected by ANY-Maze and that collected by observers.

#### 2.4.1. Open field

Behavior in the open field has been shown to be an index of exploration, anxiety, and motor behavior (Blizard et al., 1975; Frye et al., 2000). The open field (76×57×35 cm) had a 48-square grid floor (6×8 squares, 9.5 cm/side): there was an overhead light illuminating the central squares (all but the 24 perimeter squares were considered central). Per previous methods, rats were placed in the open field and the path of their exploration was recorded for 5 -min. The number of central, peripheral, and total entries was then calculated from these data as indices of anxiolysis and motor behavior, respectively.

#### 2.4.2. Elevated plus maze

Behavior in the elevated plus maze has been utilized to assess exploration and anxiety in the past (File, 1993; Frye et al., 2000). The elevated plus maze was elevated 50 cm off the ground and consisted of four arms (49 cm long and 10 cm wide). Two arms were enclosed by walls 30 cm high and the other two arms were exposed. As per previous methods, rats were placed at the juncture of the open and closed arms and the number of entries into, and the amount of time spent on, the open and closed arms were recorded during a five-minute test. Time spent on the open arms was used as an index of anxiety and the total number of arm entries was a measure of motor activity.

#### 2.4.3. Social interaction

The social interaction task assessed exploratory and anxiety behavior associated with interacting with a novel conspecific (File and Seth, 2003; Frye et al., 2000). Each member of a pair of rats (1 experimental, 1 stimulus) was placed in opposite corners of an open field (76×57×35 cm). The total duration of time that experimental rats engaged an ovariectomized stimulus rat in crawling over and under, sniffing, following with contact, genital investigation, tumbling, boxing, and grooming was recorded during a five-minute test (Frye et al., 2000). An ovariectomized rat was utilized as the stimulus animal in order to avoid the possibility of vaginocervical stimulation of experimental rats, which might occur if a male had been used as the stimulus animal. Duration of time spent interacting with a conspecific is an index of anxiety behavior.

#### 2.4.4. Paced mating

Paced mating was utilized over standard mating because of its greater ethological relevance and procedures were carried out as previously reported (Erskine, 1985; Frye and Erskine, 1990; Gans and Erskine, 2003; McClintock and Adler, 1978). Paced mating tests were conducted in a chamber (37.5×75×30 cm), which was equally divided by a partition that had a small (5 cm in diameter) hole in the bottom center, to allow a female free access to both sides of the chamber, but which prevented the larger stimulus male from moving between sides. Females were placed in the side of the chamber opposite the stimulus male. Rats were behaviorally tested for an entire ejaculatory series. Behaviors recorded were the frequency of mounts and intromissions that preceded an ejaculation. As well, the frequency (lordosis quotient = incidence of lordosis/number of mounts) and intensity (lordosis rating) of lordosis, quantified by rating of dorsiflexion on a scale of 0–3 (Hardy and DeBold, 1972), was recorded. The percentage of proceptive (i.e. hopping, darting, ear wiggling; proceptivity quotient) and aggressive (i.e. vocalizations, defensive postures; aggression quotient) behaviors prior to contacts was also recorded. Pacing measures included the percentage of times the female left the compartment containing the male after receiving a particular copulatory stimuli (% exits after mounts,

intromissions, and ejaculations) and latencies in seconds to return to the male compartment after these stimuli. The normal pattern of pacing behaviors for percent exits and return latencies to be longer after more intensive stimulation (ejaculations>intromissions>mounts) was observed in the present study.

### 2.5. Intracranial drug administration

Proestrous rats were infused (1 μl, bilaterally) with vehicle (saline; $n=12$) or the $GABA_A$ receptor antagonist, bicuculline (100 ng; $n=23$). Bicuculline acts by non-competitively, inhibiting ion channel opening of $GABA_A$ receptors that are targets for neuroactive steroids (Ueno et al., 1997). Intra-VTA administration of this dose of bicuculline has previously been demonstrated to attenuate lordosis among intact, proestrous or OVX, $E_2$- and $P_4$-primed rats and hamsters (Frye et al., 2006a; Frye and Vongher, 1999).

### 2.6. Tissue collection

Immediately following testing, trunk blood and whole brains were collected and stored at −80° for later measurement of corticosterone, $E_2$, $P_4$, dihydroprogesterone (DHP), and 3α,5α-THP. Trunk blood was centrifuged at 3000×*g* for 10 min and serum was stored at −80 °C. Brains were rapidly frozen on dry ice and stored at −80 °C for approximately three months prior to radioimmunoassay.

### 2.7. Tissue preparation

Serum was thawed on ice and steroids were extracted as described below. Assessing brains for endocrine analyses precluded histological analyses. As such, brains were coronally sectioned at the cannulation site so that they could be visually-inspected for placement in the intended region. Eight out of 23 brains in the bicuculline condition presented with cannula placement that was discrepant with a bilateral hit to the VTA as previously reported (Frye and Petralia, 2003; Frye and Seliga, 2003a,b). Brains were then thawed on ice and midbrain, hippocampus, hypothalamus, cortex, were grossly dissected as previously described and the remaining tissue without cerebellum was used as a control ("inter-brain") (Frye and Rhodes, 2006). Following dissection, steroids were extracted from brain tissue as described below.

### 2.8. Radioimmunoassay for steroid hormones

Corticosterone, $E_2$, $P_4$, DHP, and 3α,5α-THP concentrations were measured as described below, using previously reported methods (Choi and Dallman, 1999; Frye and Bayon, 1999; Frye et al., 1996).

#### 2.8.1. Radioactive Probes

[$^3$H]corticosterone (NET 182: specific activity = 48.2 ci/mmol), [$^3$H]$E_2$ (NET-317, 51.3 Ci/mmol), [$^3$H]$P_4$ (NET-208: specific activity = 47.5 Ci/mmol), and [$^3$H]3α,5α-THP (used for DHP and 3α,5α-THP, NET-1047: specific activity = 65.0 Ci/mmol), were purchased from Perkin Elmer (Boston, MA).

#### 2.8.2. Extraction of steroids from serum

Corticosterone was extracted from serum by heating at 60 °C for 30 min (Choi and Dallman, 1999). $E_2$, $P_4$, DHP, and 3α,5α-THP were extracted from serum with ether following incubation with water and 800 cpms of $^3$H steroid (Frye and Bayon, 1999). After snap-freezing twice, test tubes containing steroid and ether were evaporated to dryness in a speed drier. Dried down tubes were reconstituted with phosphate assay buffer to the original serum volume.

#### 2.8.3. Extraction of steroids from brain tissues

$E_2$, $P_4$, DHP, and 3α,5α-THP were extracted from brain tissues following homogenization with a glass/glass homogenizer in 50% MeOH, 1% acetic acid. Tissues were centrifuged at 3000×*g* and the supernatant was chromatographed on Sepak-cartridges. Steroids were eluted with increasing concentrations of MeOH (50% MeOH followed by 100% MeOH). Solvents were removed using a speed drier. Samples were reconstituted in 500 μl assay buffer.

#### 2.8.4. Antibodies

The corticosterone antibody (#B3-163, Endocrine Sciences), which typically binds 40–60% of [$^3$H]corticosterone was used in a 1:20,000 dilution. The $E_2$ antibody (E#244, Dr. G.D. Niswender, Colorado State University, Fort Collins, CO), which generally binds between 40% and 60% of [$^3$H]$E_2$, was used in a 1:40,000 dilution. The $P_4$ antibody (P#337 from Dr. G.D. Niswender, Colorado State University) used in a 1:30,000 dilution typically binds between 30% and 50% of [$^3$H]$P_4$. The DHP (X-947) and 3α,5α-THP antibodies (#921412-5, purchased from Dr. Robert Purdy, Veterans Medical Affairs, La Jolla, CA) used in a 1:5000 dilution binds between 40 and 60% of [$^3$H]3α,5α-THP.

#### 2.8.5. Set-up and incubation of radioimmunoassays

The range of the standard curves was 0–4 ng for corticosterone, 0–1000 pg for $E_2$, and 0–8000 pg for $P_4$, DHP, and 3α,5α-THP. Standards were added to assay buffer followed by addition of the appropriate antibody (described above) and [$^3$H] steroid. Total assay volumes were 900 μl for corticosterone, 800 μl for $E_2$ and $P_4$, 950 μl for DHP, and 1250 μl for 3α,5α-THP. All assays were incubated overnight at 4 °C, except for corticosterone which incubated at room temperature for 60 min.

#### 2.8.6. Termination of binding

Separation of bound and free steroid was accomplished by the rapid addition of dextran-coated charcoal. Following incubation with charcoal, samples were centrifuged at 3000×*g* and the supernatant was pipetted into a glass scintillation vial with 5 ml scintillation cocktail. Sample tube concentrations were calculated using the logit–log method of Rodbard and Hutt (1974), interpolation of the standards, and correction for recovery with Assay Zap. The inter- and intra-assay reliability co-efficients were: corticosterone 0.05 and 0.11, $E_2$ 0.07 and 0.05, $P_4$ 0.11 and 0.01, DHP 0.11 and 0.04, and 3α,5α-THP 0.09 and 0.09.

### 2.9. Analyses

Separate one-way analyses of variance (ANOVAs) were conducted to determine effects of inhibitor infusion (intra-VTA vehicle, intra-VTA bicuculline, or missed site bicuculline) on behavioral and neuroendocrine endpoints. All pairwise contrasts via Fisher's Protected Least Significant Difference *post-hoc* tests were conducted to assess group differences. Alpha level for statistical significance was $p<0.05$. Trends towards significance were noted in text when $p<0.10$.

## 3. Results

### 3.1. Behavioral endpoints

#### 3.1.1. Open field

Infusions of bicuculline to VTA, but not missed sites, significantly reduced the number of entries rats made into the center of the open field compared to infusions of vehicle [$F(2,32)=5.64$, $p<0.05$] (Fig. 1, top). Total entries made in the open field did not significantly differ between intra-VTA bicuculline- (272 ± 23) or vehicle-infused rats (315 ± 31) or missed site bicuculline-infused rats (358 ± 25).

#### 3.1.2. Elevated plus maze

Compared to intra-VTA infusions of vehicle or missed site infusions of bicuculline, intra-VTA infusions of bicuculline significantly reduced the amount of time rats spent on the open arms of the elevated plus

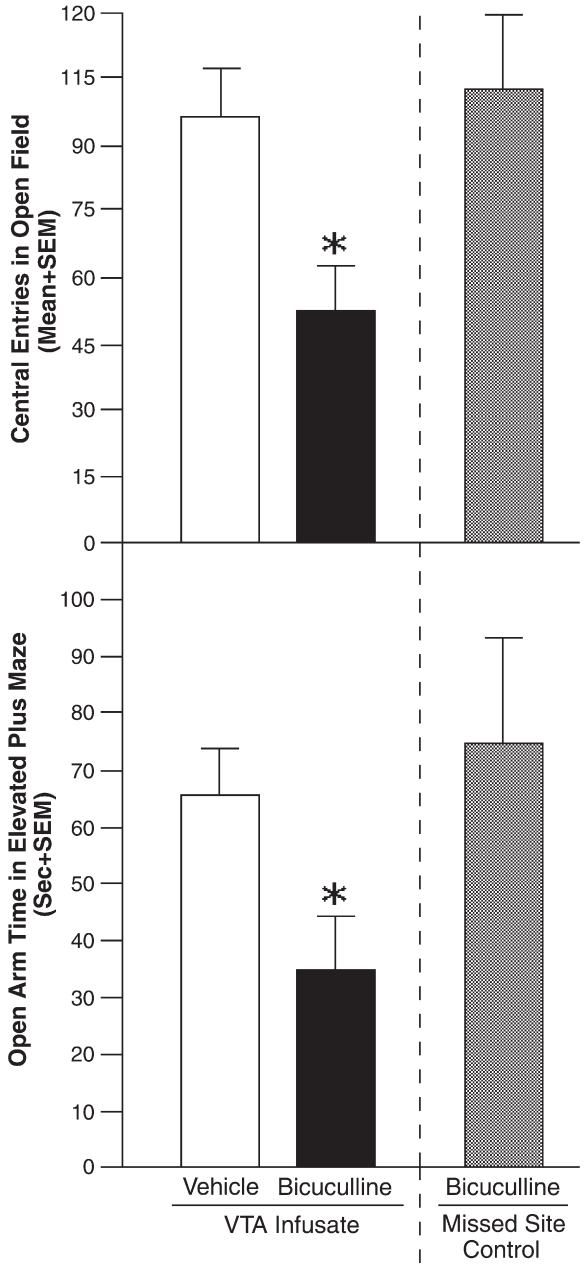


**Fig. 1.** Proestrous rats infused with bicuculline (100 ng; $n=15$) to the ventral tegmental area (VTA) of the midbrain made fewer entries into the center of a brightly lit open field (top) and spent less time on the open arms of an elevated plus maze (bottom) than did proestrous rats infused with vehicle ($n=12$) to VTA or bicuculline to missed sites (100 ng; $n=8$). * indicates significant difference between rats infused with bicuculline to VTA compared to those infused with either vehicle to VTA or bicuculline to missed sites, $p<0.05$.

maze [$F(2,32)=3.63$, $p<0.05$] (Fig. 1, bottom). As well, bicuculline-infused rats made fewer arm entries ($9\pm1$) than did vehicle ($15\pm1$) or missed site bicuculline-infused control rats ($14\pm2$) [$F(2,32)=4.80$, $p<0.05$].

### 3.1.3. Social interaction

Rats infused with bicuculline to the VTA tended to spend less time ($76\pm8$ s) interacting with a novel conspecific compared to those infused with vehicle to the VTA ($97\pm9$ s) and spent significantly less time interacting compared to those infused with bicuculline to missed sites ($128\pm22$ s) [$F(2,32)=4.31$, $p<0.05$].

### 3.1.4. Paced mating

Infusions of bicuculline to the VTA significantly reduced the frequency [$F(2,32)=4.75$, $p<0.05$] and intensity [$F(2,32)=9.05$, $p<0.05$] of lordosis among rats compared to infusions of vehicle to the VTA or bicuculline to missed sites (Fig. 2). As well, compared to either vehicle-infused or missed site controls, rats infused with bicuculline to

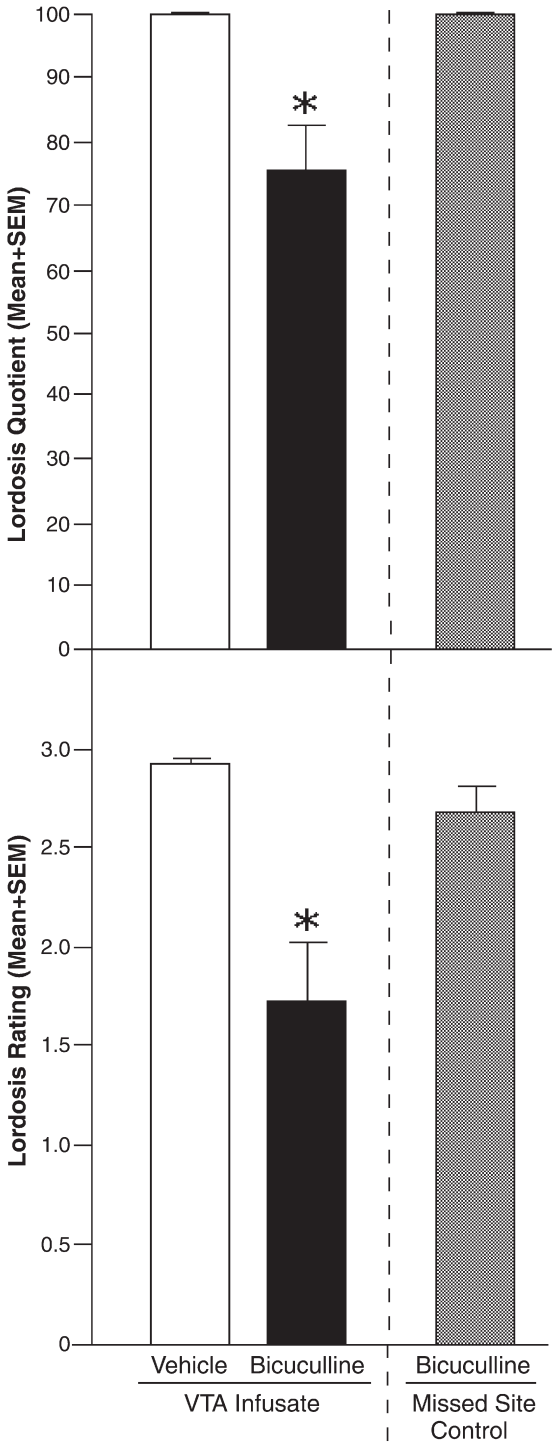


**Fig. 2.** Frequency (top) and intensity (bottom) of lordosis was reduced among proestrous rats receiving intra-ventral tegmental area (VTA) infusions of bicuculline (100 ng; $n=15$) compared to those infused with vehicle to VTA ($n=12$) or bicuculline to missed sites (100 ng; $n=8$). * indicates significant difference between rats infused with bicuculline to VTA compared to those infused with either vehicle to VTA or bicuculline to missed sites, $p<0.05$.

the VTA demonstrated less proceptive behavior (Fig. 3, top), significantly more aggression towards stimulus males [$F(2,32)=3.73$, $p<0.05$] (Fig. 3, middle), and significantly less pacing of their mating contacts [$F(2,32)=3.42$, $p<0.05$] (Fig. 3, bottom).

### 3.2. Neuroendocrine endpoints

There were no differences between groups in concentrations of $E_2$, $P_4$, or DHP in any tissue examined, nor did plasma corticosterone levels significantly differ (Table 1). Likewise, 3α,5α-THP levels did not significantly differ in plasma, diencephalon, or interbrain. However, there was a significant reduction of 3α,5α-THP among rats infused with bicuculline to the VTA in midbrain [$F(2,32)=3.70$, $p<0.05$] and hippocampus [$F(2,32)=4.62$, $p<0.05$] compared to those infused with vehicle to the VTA or bicuculline to missed sites (Table 1). Rats

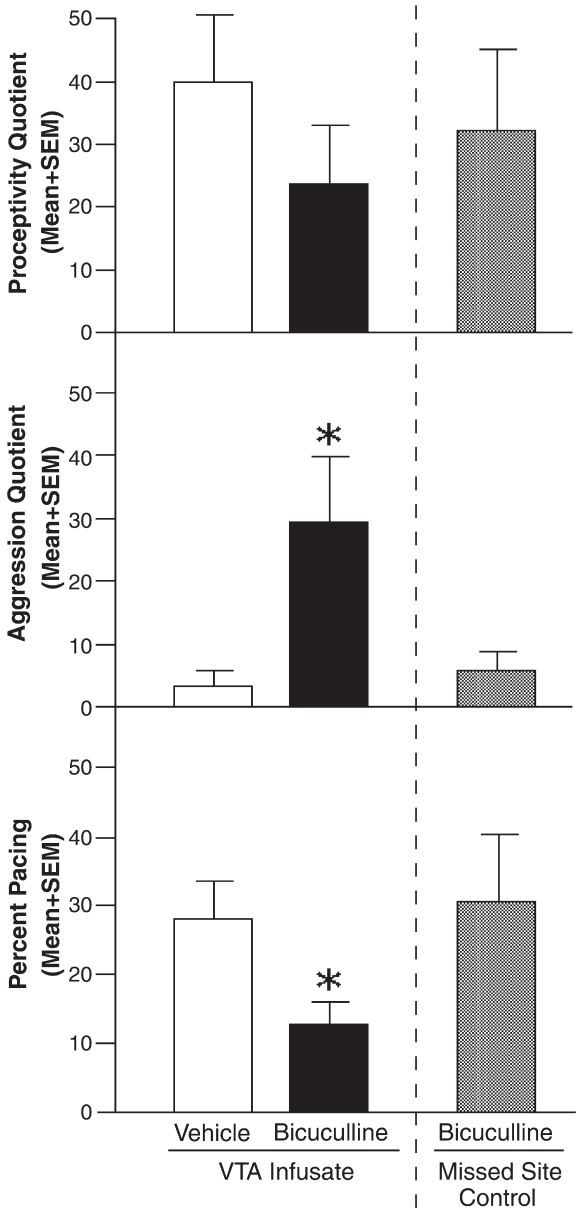


**Fig. 3.** Compared to proestrous rats infused with vehicle to the ventral tegmental area (VTA) of the midbrain ($n=12$) or bicuculline to missed sites (100 ng; $n=8$), rats infused with bicuculline to VTA (100 ng; $n=15$) demonstrated fewer proceptive behaviors (top), significantly more aggressive behaviors in response to male mounting (middle), and significantly less pacing of their mating contacts (bottom). * indicates significant difference between rats infused with bicuculline to VTA compared to those infused with either vehicle to VTA or bicuculline to missed sites, $p<0.05$.

**Table 1**
Depicts concentrations (mean ± SEM) of estrogen ($E_2$), progesterone ($P_4$), dihydroprogesterone (DHP), and 5α-pregnan-3α-ol-20-one (3α,5α-THP) in serum, midbrain, hippocampus, diencephalon, cortex, and interbrain, as well as serum corticosterone, among proestrous rats infused with saline vehicle or bicuculline (100 ng) to the ventral tegmental area (VTA) of the midbrain or bicuculline (100 ng) to missed sites.

| | Intra-VTA infusate | | Missed site controls |
|---|---|---|---|
| | Vehicle ($n=12$) | Bicuculline ($n=15$) | Bicuculline ($n=8$) |
| *Corticosterone* | | | |
| Serum (μg/dl) | 2.4 ± 0.7 | 3.4 ± 0.7 | 3.8 ± 1.2 |
| *$E_2$* | | | |
| Serum (pg/ml) | 12.5 ± 1.6 | 16.4 ± 2.9 | 13.9 ± 1.8 |
| Midbrain (pg/g) | 1.7 ± 0.1 | 1.7 ± 0.2 | 1.7 ± 0.1 |
| Hippocampus (pg/g) | 3.3 ± 0.2 | 3.4 ± 0.3 | 3.7 ± 0.5 |
| Diencephalon (pg/g) | 1.3 ± 0.1 | 1.3 ± 0.1 | 1.5 ± 0.2 |
| Cortex (pg/g) | 1.6 ± 0.1 | 2.0 ± 0.3 | 1.8 ± 0.1 |
| Interbrain (pg/g) | 1.1 ± 0.1 | 1.1 ± 0.1 | 1.2 ± 0.1 |
| *$P_4$* | | | |
| Serum (ng/ml) | 12.8 ± 2.7 | 15.7 ± 2.1 | 10.0 ± 2.6 |
| Midbrain (ng/g) | 0.9 ± 0.1 | 1.0 ± 0.1 | 0.7 ± 0.2 |
| Hippocampus (ng/g) | 2.3 ± 0.1 | 2.4 ± 0.2 | 2.1 ± 0.1 |
| Diencephalon (ng/g) | 0.7 ± 0.1 | 0.8 ± 0.1 | 0.6 ± 0.2 |
| Cortex (ng/g) | 1.1 ± 0.1 | 1.2 ± 0.1 | 0.9 ± 0.2 |
| Interbrain (ng/g) | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.1 |
| *DHP* | | | |
| Serum (ng/ml) | 23.5 ± 9.7 | 24.2 ± 10.2 | 14.7 ± 3.8 |
| Midbrain (ng/g) | 3.4 ± 1.1 | 2.4 ± 0.4 | 4.3 ± 2.0 |
| Hippocampus (ng/g) | 8.5 ± 2.4 | 7.7 ± 1.9 | 8.9 ± 4.1 |
| Diencephalon (ng/g) | 1.5 ± 0.4 | 1.3 ± 0.2 | 2.1 ± 1.1 |
| Cortex (ng/g) | 3.1 ± 0.7 | 2.2 ± 0.4 | 3.1 ± 1.2 |
| Interbrain (ng/g) | 1.3 ± 0.4 | 1.4 ± 0.3 | 2.0 ± 0.5 |
| *3α,5α-THP* | | | |
| Serum (ng/ml) | 19.8 ± 4.6 | 16.5 ± 4.7 | 8.7 ± 3.3 |
| Midbrain (ng/g) | 11.9 ± 2.7 | 4.2 ± 2.3* | 13.8 ± 3.4 |
| Hippocampus (ng/g) | 20.2 ± 4.7 | 5.9 ± 2.4* | 17.5 ± 4.5 |
| Diencephalon (ng/g) | 7.9 ± 1.8 | 3.3 ± 1.2 | 7.1 ± 3.3 |
| Cortex (ng/g) | 12.6 ± 3.7 | 4.8 ± 1.9 | 11.5 ± 2.6 |
| Interbrain (ng/g) | 1.5 ± 0.3 | 1.2 ± 0.3 | 1.3 ± 0.4 |

* indicates significant difference between rats infused with bicuculline to VTA compared to those infused with either vehicle to VTA or bicuculline to missed sites, $p<0.05$.

receiving intra-VTA infusions of bicuculline also tended to have lower 3α,5α-THP levels in cortex [$F(2,32)=2.56$, $p<0.10$], albeit this was not significant (Table 1).

## 4. Discussion

The present findings supported the hypothesis that infusion of the $GABA_A$ antagonist, bicuculline, to the midbrain VTA would attenuate exploratory, anti-anxiety, and consummatory/appetitive aspects of mating behavior that are typical of proestrous rats. Intra-VTA infusion of bicuculline significantly attenuated anti-anxiety behavior in an open field and an elevated plus maze and reduced duration of interaction time with a novel conspecific compared to control infusions. Bicuculline significantly attenuated consummatory aspects of sexual behavior characterized by lordosis, as well as appetitive sexual behavior processes, such as sexual solicitation, reduction of aggression, and approach–avoidance behavior in the paced-mating paradigm. Notably, these attenuations in behavior never exceeded ~50% reduction indicating the importance of alternate mechanisms in these processes. Thus, $GABA_A$ receptors in the VTA appear to play an important role in mediation of appetitive and consummatory aspects of mating among rats, albeit, other mechanisms must also be critical for these behaviors.

The present investigation supports and extends prior findings. We have previously observed that inhibition of $GABA_A$ receptors in VTA via bicuculline can attenuate enhancement of consummatory aspects of mating, such as lordosis frequency and intensity, among sexually-

receptive rodents (Frye and Vongher, 1999; Frye et al., 1993, 2006b). The current investigation confirms these findings and extends them to appetitive measures associated with natural rodent receptivity, including exploratory/anti-anxiety behavior and pacing of sexual contacts. These processes may be involved in many aspects of engagement in motivated behavior given that anxiety must be reduced and social approach must be enhanced in order to locate and engage in copulation with a mate. Others have found that intra-VTA blockade of $GABA_A$ receptors via bicuculline can attenuate engagement in some motivated behaviors including opioid-induced feeding (Echo et al., 2002) and alcohol consumption among alcohol preferring rats (Nowak et al., 1998). However, other reports suggest that bicuculline administration to VTA (particularly the anterior region) can be reinforcing (Ikemoto et al., 1997a,b; Xi and Stein, 2000). Together, these data support findings that resolve $GABA_A$ receptors to mediate bidirectional reward signals in VTA dependent on endogenous reward state (Laviolette and van der Kooy, 2001, 2004). Thus, actions at $GABA_A$ receptors in VTA may contribute to many aspects of natural reward and these processes are likely dependent on interactions between endogenous reward systems (such as opioid-dependent processes) and hormonal state.

In the present study, infusions of bicuculline were also associated with some alteration of pregnane neurosteroid formation. In the past, we have found that mating-enhanced 3α,5α-THP formation in brain is not dependent on peripheral steroid production and can be blocked or enhanced by central administration of neurosteroidogenesis inhibitors or enhancers, respectively (Frye et al., 2008, 2009). As such, engaging in paced mating may trigger *de novo* synthesis of pregnane neurosteroids in brain. In the current study, the observed reduction of 3α,5α-THP in midbrain and hippocampus among bicuculline-infused rats is intriguing and has several implications. First, it does not seem plausible that central 3α,5α-THP attenuation can be attributable to direct effects of the infusate, bicuculline, particularly given that 3α,5α-THP was not reduced in any tissue among missed site bicuculline-infused controls. Second, reductions in 3α,5α-THP may be indirectly due to actions of bicuculline. We have demonstrated that engaging in paced mating can readily enhance 3α,5α-THP in midbrain and hippocampus (and to a lesser extent in diencephalon and cortex; Frye and Rhodes, 2006; Frye et al., 2007) and bicuculline reduces engagement in mating. As such, reductions in 3α,5α-THP in these regions may be the result of reduced engagement in paced mating compared to proestrous controls. Third, bicuculline may also have effects on 3α,5α-THP synthesis via actions at novel sites that underlie neurosteroid formation. For instance, pregnane xenobiotic receptor (PXR) is a promiscuous nuclear transcription factor that is both a target of 3α,5α-THP (Kliewer et al., 2002; Langmade et al., 2006) and is associated with upregulation of CYP3A enzymes which is a rate-limiting step in 3α,5α-THP biosynthesis (Masuyama et al., 2005). Actions of 3α,5α-THP may enhance PXR expression in rodents (Mellon et al., 2008) and this may further enhance neurosteroid biosynthesis. Attenuating 3α,5α-THP actions at $GABA_A$ receptors may have effects to dampen PXR enhancement. As well, it could be that bicuculline is a modulator of PXR, given that PXR is a promiscuous xenobiotic receptor. These and other questions regarding this receptor need to be explored. However, bicuculline may also affect neurosteroidogenesis by virtue of being a pharmacological stressor. The midbrain VTA and hippocampus are important regions in the modulation of stress (Herman et al., 2003) and acute stressors can enhance neurosteroid-promoting factors, such as PXR (Narang et al., 2008). Thus stress factors that may underlie bicuculline's effects must be considered.

An important factor associated with neurosteroid production and pharmacological manipulations of steroid targets is the parasympathetic tone of the organism. Acute stress readily activates the hypothalamic–pituitary–adrenal (HPA) axis and promotes rapid pregnane neurosteroid formation (Drugan et al., 1995; Erskine and Kornberg 1992; Purdy et al., 1991). When elevated 3α,5α-THP can act as a homeostatic modulator in hypothalamus to attenuate corticotrophin releasing hormone formation, thus, dampening HPA arousal (Patchev et al., 1994). Notably, the hippocampus is an important target of stress effects and stress-related disorders are associated with GABAergic dysregulation in this region (Linthorst and Reul, 2008). While, the results of the current investigation do not reveal the mechanism by which intra-VTA actions of bicuculline may alter stress effects mediated by the hippocampus, they do add to what is known regarding the interplay between these structures. Dopaminergic neurons from the VTA project to the hippocampus (Gasbarri et al., 1997; Swanson, 1982) and inhibition of $GABA_A$ receptors in the VTA can enhance DA release from projecting neurons (Ikemoto et al., 1997a,b). As well, we and others have found that exposure to lordosis-relevant stimuli is associated with enhancement of dopamine in rodent midbrain, striatum, and/or cortex (Frye, 2001; Meisel et al., 1993). Apart from a direct connection from VTA to hippocampus, a multi-synapse pathway has been proposed wherein GABAergic innervations from the ventral pallidum to the VTA can be activated by the subiculum to create a feedback loop between the hippocampus and the VTA (Lisman and Grace, 2005). We have found that inhibition of 3α,5α-THP in VTA can increase HPA response to stressors associated with task performance in the behavioral battery described (Frye et al., 2008) and others have demonstrated that lesions to the hippocampus can enhance the gluccocorticoid response to open field exposure (Herman et al., 1998; Nyakas et al., 1983). Whether, these differences are due, in part, to GABAergic actions in midbrain and/or hippocampus is an intriguing question. Indeed, electrophysiological and dopaminergic responses to novelty in the hippocampus and midbrain VTA occur concomitantly, indicating that they comprise an important circuit underlying these effects (Lisman and Grace, 2005). Microdialysis studies have also demonstrated that acute stress in rats is associated with increases in extracellular GABA in hippocampus (De Groote and Linthorst, 2007; Bianchi et al., 2003). These data support the notion that GABAergic activity in the VTA may alter stress responses that are mediated by hippocampus.

Notably, there are sex differences in both HPA responding (Luine, 2002) and exploratory behavior of rats (Luine, 2002; Sutcliffe et al., 2007) that are hippocampally-mediated. While, the current investigation found only a modest increase in corticosterone among bicuculline-infused females compared to control females, it is an intriguing question as to whether sex differences would be observed in males. Others have found that bicuculline administration to the VTA can enhance the dopamine-stress response of male rats to restraint (Doherty and Gratton, 2007). As well, stress associated with inter-male aggression results in an enhancement of dopamine production in nucleus accumbens and cortex of threatened rats (Miczek et al., 2008). It is also possible that engaging in the present behavioral paradigm may not have been a salient enough stressor to dissociate between bicuculline- and vehicle-infused females. A recent investigation demonstrates that mild stress associated with exercise in an acute paradigm is not associated with changes in basal corticosterone levels (Droste et al., in press) and, in our study, the corticosterone levels obtained with bicuculline infusion were scarcely beyond what is typical. Future investigations may aim to utilize males and females with exposure to more salient behavioral stressors.

This investigation demonstrates that GABAergic signaling in the midbrain VTA is important for not only consummatory aspects of rodent mating behavior, but also appetitive aspects of mating that include affective and motivational processes. Given that intra-VTA blockade of $GABA_A$ receptors did not abolish any behavior observed, future investigations will aim to assess other factors that may regulate neurosteroid production as well as affective, social, and sexual behaviors.

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