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$GABA_A$ receptor antagonism in the ventrocaudal periaqueductal gray increases anxiety in the anxiety-resistant postpartum rat

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ABSTRACT

Postpartum mammals show suppressed anxiety, which is necessary for their ability to appropriately care for offspring. It is parsimonious to suggest that the neurobiological basis of this reduced anxiety is similar to that of non-parturient animals, involving $GABA_A$ receptor activity in sites including the midbrain periaqueductal gray (PAG). In Experiment 1, postpartum and diestrous virgin female rats received an intraperitoneal injection of the $GABA_A$ receptor antagonist (+)-bicuculline (0, 2 and 4 mg/kg) and anxiety-related behavior was assessed with an elevated plus maze. The 4 mg/kg dose of (+)-bicuculline significantly increased anxiety-related behavior, particularly in the postpartum females. Experiment 2 revealed that bicuculline's action was within the central nervous system, because anxiety in neither dams nor virgins was significantly affected by intraperitoneal injection of bicuculline methiodide (0, 2 and 6 mg/kg), which does not readily cross the blood–brain-barrier. In Experiment 3, bicuculline methiodide (2.5 ng/side) was directly infused into the ventrocaudal PAG (cPAGv) and significantly increased dams' anxiety compared to saline-infused controls. These studies expand our knowledge of how $GABA_A$ receptor modulators affect anxiety behaviors in postpartum rats to the widely-used elevated plus maze, and indicate that the postpartum suppression of anxiety is in part a consequence of elevated GABAergic neurotransmission in the cPAGv.

Emotional responsiveness in female rodents is tremendously affected by their reproductive state, with lower anxiety often observed during estrus, proestrus, and the early postpartum period compared to other times of the reproductive cycle (Bridges et al., 1972; Fleming and Luebke, 1981; Frye et al., 2000; Hard and Hansen, 1985; Lonstein, 2005, 2007; Marcondes, et al., 2001; Mora et al., 1996; Neumann, 2001; Nomikos and Spyraki, 1988; Zuluaga et al., 2005). In postpartum rats, this change in emotional state is thought to be necessary for some salient behavioral changes occurring during this time, including the display of nurturant responses towards anxiety-generating neonates and heightened aggression towards potentially threatening conspecifics (Fleming and Luebke, 1981; Hard and Hansen, 1985). Because pregnancy, parturition, and lactation involve dramatic fluctuations in ovarian, pituitary, and adrenal hormones it seems reasonable that these hormones would underlie the postpartum suppression of anxiety. However, while hormones may help establish a low-anxiety state in very recently parturient female rats, they have little role thereafter because anxiety remains low in postpartum rats with no ovaries, adrenals, or pituitary glands (Hansen, 1990; Lonstein, 2005). Instead, physical contact with neonates maintains dams' blunted anxiety (Lonstein, 2005) and this is supported by the blunted emotional reactivity found even in nulliparous female rats that are maternally sensitized to care for pups (Agrati et al., 2008; Ferreira et al., 2002; Pereira et al., 2005).

Similar to the neurobiology underlying anxiety in non-lactating rodents (Millan, 2003; Roy-Byrne, 2005), previous research on the postpartum reduction in anxiety implicates the inhibitory neurotransmitter GABA. Interactions with pups increase mothers' cerebrospinal fluid concentration of GABA, whereas separation from pups sharply decreases it (Qureshi et al., 1987). This increase in GABA release is necessary for dams' reduced anxiety, as peripheral administration of drugs that impair GABAergic neurotransmission increase dams' freezing in response to an acoustic stimulus and reduce their punishing drinking (Hansen, 1990; Hansen et al., 1985). Conversely, GABA agonists bring emotional responding in cycling female rats down to a level similar to that found in postpartum females (Ferreira et al., 1989).

Although GABA release during interactions with pups lowers anxiety in mother rats, it is virtually unknown where in the brain GABA produces these effects. It seems reasonable that the neural mechanisms regulating anxiety in postpartum rats would be the same as those regulating anxiety in non-parturient rats, but that these networks are inhibited as a result of females' continual interactions with pups. A large number of neural sites are implicated in anxiety, including the amygdala, bed nucleus of the stria terminalis, cerebral cortex, septum, hippocampus, medial hypothalamus, and periaqueductal gray (PAG) (Millan, 2003). Of these sites, we are particularly interested in how the PAG regulates postpartum anxiety. The PAG is a point of convergence for many forebrain influences on emotionality, and acts as a final common

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pathway for the physiological and behavioral responses to anxiogenic stimuli in animals (Millan, 2003; Vianna and Brandao, 2003). It is extremely rich in GABAergic neurons, terminals, and receptors (Behbehani, 1995; Reichling, 1991). Similar to male rats, the PAG modulates anxiety in postpartum rats, with lesions of the ventrocaudal PAG (cPAGv) even further reducing dams' anxiety-related behavior in an elevated plus maze (Lonstein et al., 1998). The cPAGv of lactating rats is exquisitely sensitive to physical contact with pups (Lonstein and Stern, 1997a,b), and as such may be a neural site where tactile cues from pups increase GABAergic activity to reduce anxiety in mothers. To help evaluate this possibility, we first examined the effects of intraperitoneal administration of the brain-penetrating $GABA_A$ receptor antagonist (+)-bicuculline on anxiety-related behaviors in postpartum and diestrous virgin females rats tested in an elevated plus-maze. Two previous studies have examined the effects of $GABA_A$ receptor inhibition on postpartum anxiety-related behaviors (Hansen, 1990; Hansen et al., 1985), but neither employed the very widely utilized (Canteras and Blanchard, 2008) and pharmacologically reliable (Rodgers et al., 1997) elevated plus maze. To confirm that (+)-bicuculline's effects on anxiety-related behaviors were due to $GABA_A$ receptor blockade within the central nervous system, we also examined elevated plus-maze behavior of postpartum and virgin females given an intraperitoneal injection of bicuculline methiodide, a $GABA_A$ receptor antagonist that does not readily cross the blood–brain barrier (Pong and Graham, 1972; Remler and Marcussen, 1985). Lastly, the possibility that the cPAGv is a locus of $GABA_A$ receptor activity necessary for reduced postpartum anxiety was determined by infusing bicuculline methiodide directly into this site and evaluating dams' elevated plus-maze behavior.

1. Experiment Ia: effects of peripherally administered (+)-bicuculline on anxiety-related behavior in postpartum and diestrous virgin rats

1.1. Subjects

Subjects were 50 female Long–Evans rats, descended from male and female rats purchased from Harlan Laboratories (Indianapolis, IN), born and raised in our colony. After weaning at 21 days of age, subjects were housed in clear polypropylene cages (48×28×16 cm) with wood shavings for bedding, in groups of 2–3 female littermates per cage. Beginning at 75 days old, 24 of the females had their estrous cycles monitored daily with a vaginal impedance meter that measures changes in electrical resistance of the vaginal walls across the estrous cycle (Fine Science Tools, Foster City, CA), and when in proestrus females were placed overnight with a sexually experienced Long–Evans male from our colony. Mated females were removed from males' cages and re-housed with one or two recently inseminated females until 4–5 days prior to the expected day of parturition. Females were then singly housed and remained singly housed with their litters throughout the remainder of the experiment. Litters were culled to contain 4 males and 4 females within 24 h after birth. The day of birth was assigned as day 0 postpartum. The remaining 26 females were not mated, but instead housed alone beginning at approximately 75 days of age, and had their estrous cycles monitored each morning through vaginal swabbing and visualization of vaginal cytology.

Food (Tekland Rodent Diet No. 8640, Harlan) and water were continuously available, lights were on a 12:12 light/dark cycle with onset at 0700 h daily, and the ambient temperature was ~22 °C. To minimize disturbance before testing, entry into the colony rooms housing the subjects was prohibited for anyone other than the experimenters for at least the 4 h before plus-maze testing, and any changing or cleaning of cages occurred no less than ~20 h before testing. All procedures in these experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and the Institutional Animal Care and Use Committee at Michigan State University.

1.2. Drug administration and elevated plus-maze testing

On the afternoon of day 7 or 8 postpartum (1200–1600 h), each dam received an IP injection of vehicle (described below) or 2 or 4 mg/kg of (+)-bicuculline (Sigma, USA) ($n=8$/group). The drug was prepared using methods similar to McDonald et al. (2008): (+)-bicuculline was first dissolved in 45 µl glacial acetic acid, 150 µl propylene glycol, and 200 µl NaOH (50%). That volume was brought up to ~0.8 ml with physiological saline, the solution pHed to 5.0 with sodium hydroxide, and more saline added to bring the final volume to 1 ml. The volume injected was 1 ml/kg of body weight. A group of diestrous virgin females also received an IP injection of vehicle ($n=10$), or 2 or 4 mg/kg of (+)-bicuculline ($n=8$/group) dissolved in vehicle. Because we did not want to handle subjects on the test day, virgins were tested one day after vaginal smears revealed a day of estrus, with the supposition that the next day would be a day of diestrus. This was verified by vaginal smearing immediately after testing and any females not in diestrus were eliminated from the study.

Subjects were returned to their home cages after injection, and 15 min later carried in their home cage to a 10×10 ft room for testing. The plus-maze was constructed of black Plexiglas and fashioned after that of Pellow et al. (1985), as we have described previously (Lonstein, 2005). The testing room was illuminated with a 100-watt light bulb that provided ~28 lx on the center and open arms and ~2 lx at the end of the closed arms. Females were removed from their home cage, placed in the center of the maze facing an open arm, and released for the 10-min test. We used a 10-min test because previous work from our laboratory demonstrated that differences between diestrous virgins and postpartum rats in elevated plus-maze behavior are more salient when a 10-min test, rather than a 5-min test, is used (Lonstein, 2005). Immediately after subjects were released into the maze, the home cage was removed from the testing room. A mirror placed above the plus maze reflected the images into a Panasonic low-light-sensitive video camera interfaced with a Panasonic VCR. Subjects' behavior in the plus-maze was recorded with a custom-made computerized data acquisition system while subjects were being videotaped, or the videotapes were later transcribed. The duration of time spent in the open and closed arms of the maze, and the number of entries made into each, were recorded. A subject was considered to have entered an arm or the central square if at least its head and two forepaws were completely within that space, a criterion previously used by our lab (Figueira et al., 2008; Lonstein, 2005; Smith and Lonstein, 2008) and by others (Beiderbeck et al., 2007; Neumann et al., 2000; Torner et al., 2002; Turgeon et al., 2010; Veenema et al., 2007). The plus maze was cleaned with a 70% ethanol solution and allowed to dry between subjects.

1.3. Data analyses

The number of entries into and duration of time spent in open arms were converted to a percentage of total behavior to account for any differences between groups in time spent in the central square. All data were first analyzed with a 2×2 ANOVA using reproductive state and drug dose as factors, followed by Fisher LSD post-hoc tests. In the case of a significant main effect of drug, separate one-way ANOVAs were then used to conduct *a priori* planned comparisons to compare doses within each reproductive state. Statistical significance was indicated by $p \leq 0.05$.

1.4. Results

Similar to previous experiments in our lab (Lonstein, 2005; Smith and Lonstein, 2008; Figueira et al., 2008), there was a significant main

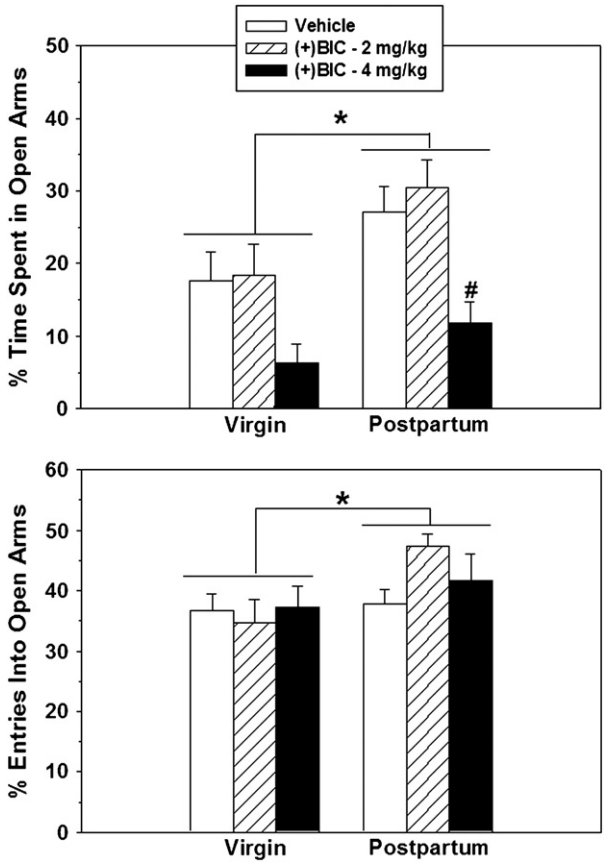

Fig. 1. Percentage of time (Mean ± SEM) spent in the open arms of the elevated plus maze (top) and percentage of open arm entries (bottom) by virgin and postpartum rats after peripheral injection of vehicle or 2 or 4 mg/kg (+)-bicuculline. * = Significant effect of reproductive state, $p<0.05$. # = Significantly different from other two groups within reproductive state, $p<0.05$.

effect of reproductive state on the percentage of time spent in the open arms of the elevated plus-maze, with dams spending a greater percentage of time in the open arms than did virgins ($F(1, 44) = 9.39$, $p<0.004$; Fig. 1). The percentage of arm entries made into the open arms was also higher for the dams ($F(1, 44) = 4.14$, $p<0.05$; Fig. 1). The total number of arms entered (open plus closed) tended to be higher in dams than virgins ($F(1, 44) = 3.99$, $p<0.06$; Table 1), while the number of entries into closed arms did not differ between the groups ($F(1, 44) = 0.74$, $p>0.39$; Table 1). The total duration of time spent in arms of the plus-maze (open duration plus closed duration) did not differ between dams and virgins ($F(1, 44) = 0.01$, $p \geq 0.95$; Table 1).

There were significant effects of (+)-bicuculline on females' elevated plus-maze behavior. Collapsed across reproductive state, the percentage of time spent in the open arms was significantly reduced by the 4 mg/kg dose compared to saline and the 2 mg/kg dose ($F(2, 44) = 10.42$, $p<0.0002$; Fig. 1). One-way ANOVAs within reproductive state revealed that the 4 mg/kg dose of (+)-bicuculline significantly reduced the percentage of time spent in the open arms in dams ($F(2, 21) = 8.74$, $p = 0.002$), but only marginally did so in virgins ($F(2,23) = 3.01$, $p = 0.07$). There was no significant interaction between reproductive state and dose on this variable ($F(2, 44) = 0.44$, $p>0.65$). There was no main effect of (+)-bicuculline on the percentage of entries made into open arms ($F(2, 44) = 0.37$, $p>0.69$), and no interaction between drug and reproductive state on this measure ($F(2, 44) = 2.19$, $p>0.12$; Fig. 1).

The total number of arms entered was reduced by the 4 mg/kg dose of (+)-bicuculline ($F(2, 44) = 7.84$, $p<0.002$; Table 1), as was the number of closed-arm entries ($F(2, 44) = 7.41$, $p<0.002$; Table 1), the latter possibly indicating a reduction in locomotor activity. There were no interactions between drug and reproductive state on either measure ($F(2,44) = 0.29$, $p>0.75$ and $F(2,44) = 0.11$, $p>0.90$, respectively). When these data were analyzed within reproductive state, dams treated with 4 mg/kg (+)-bicuculline showed significantly fewer total arms entered ($F(2, 21) = 5.72$, $p<0.02$) and there was a trend toward this in virgins ($F(2, 23) = 2.86$; $p<0.08$). Similar results were found for the number of entries made into closed arms (dams: $F(2, 21) = 8.08$, $p<0.003$; virgins ($F(2, 23) = 2.31$; $p>0.12$). There was no evidence of general locomotor dysfunction, as indicated by the lack of an effect of (+)-bicuculline on how rapidly subjects entered their first arm after being placed in the elevated plus maze at the beginning of testing ($F(2,44) = 2.48$, $p>0.09$) or their latency to leave the first arm ($F(2,44) = 1.81$, $p>0.17$; Table 1).

2. Experiment Ib: effects of peripherally administered bicuculline methiodide on anxiety-related behavior in postpartum and diestrous virgin rats

2.1. Subjects

Subjects were 74 female Long–Evans rats from our colony, raised and housed as described above. Thirty-four of the females were mated and used in the postpartum groups. The remaining 40 females were not mated, but instead remained as virgins that were housed alone and had their estrous cycles monitored daily.

2.2. Drug administration and plus-maze testing

On the afternoon of day 7 or 8 postpartum, dams received an IP injection of physiological saline ($n = 12$) or 2 mg/kg ($n = 11$) or 6 mg/kg ($n = 11$) of bicuculline methiodide (Sigma, USA) dissolved in physiological saline. The volume injected was 1 ml/kg of body weight. As in Experiment 1a, virgins were tested one day after vaginal smears revealed a day of estrus, and those found not to be in diestrus on the day of testing were removed from the study. Virgins also received an IP injection of saline ($n = 17$), 2 mg/kg bicuculline methiodide ($n = 10$), or 6 mg/kg bicuculline methiodide dissolved in saline ($n = 13$). After

Table 1
Elevated plus-maze behavior of virgin and postpartum female rats after I.P. injection of vehicle or 2 or 4 mg/kg of (+)-bicuculline [(+)-BIC].

	Virgin vehicle	Virgin 2 mg/kg (+)-BIC	Virgin 4 mg/kg (+)-BIC	Postpartum vehicle	Postpartum 2 mg/kg (+)-BIC	Postpartum 4 mg/kg (+)-BIC	Significant effects
# Open arm entries	9 ± 1^a	8 ± 1^a	6 ± 1^b	10 ± 1^a	14 ± 2^a	8 ± 2^b	Reproductive state, Drug
# Closed arm entries	14 ± 2^a	14 ± 2^a	9 ± 1^b	16 ± 1^a	15 ± 1^a	10 ± 1^b	Drug
Total arm entries	23 ± 3^a	23 ± 3^a	15 ± 1^b	26 ± 2^a	29 ± 3^a	18 ± 3^b	Drug
Time in open arms (s)	88 ± 20^a	90 ± 21^a	32 ± 12^b	136 ± 17^a	149 ± 16^a	60 ± 14^b	Reproductive state, Drug
Time in closed arms (s)	426 ± 27^a	405 ± 27^a	495 ± 21^b	367 ± 20^a	351 ± 30^a	476 ± 29^b	Reproductive state, Drug
Time in all arms (s)	514 ± 11	495 ± 13	527 ± 12	503 ± 10	500 ± 21	536 ± 17	–
Latency enter 1st arm (s)	0.15 ± 0.01	0.25 ± 0.8	0.18 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.21 ± 0.01	–
Latency exit 1st arm (s)	8 ± 2	19 ± 10	10 ± 4	7 ± 2	6 ± 1	28 ± 12	–

Significant main effects of drug indicated by different superscript letters, collapsed across reproductive state, $p \leq 0.05$.

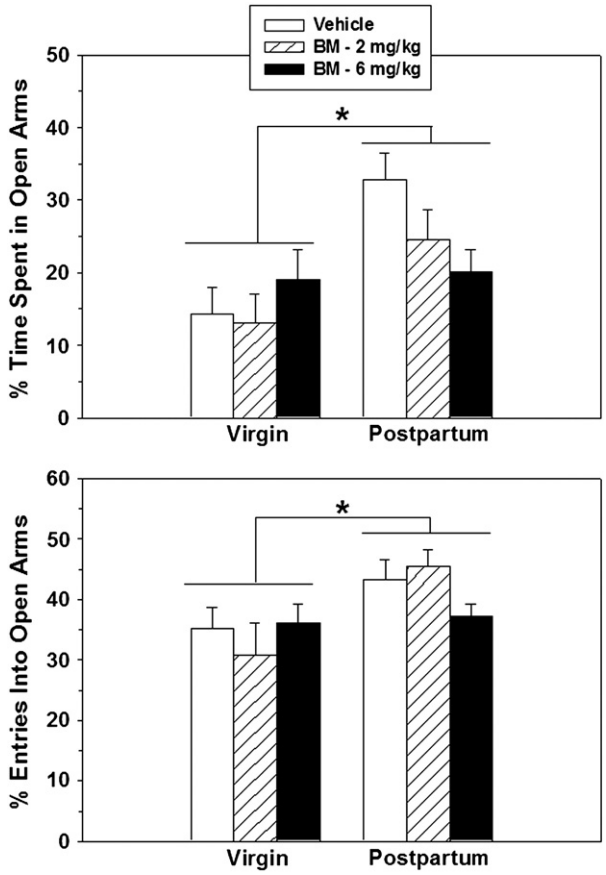

Fig. 2. Percentage of time (Mean ± SEM) spent in the open arms of the elevated plus maze (top) and percentage of open arm entries (bottom) by virgin and postpartum rats after peripheral injection of saline or 2 or 6 mg/kg of bicuculline methiodide (BM).

injection, subjects were returned to their home cages, and 15 min later tested in an elevated plus-maze as described in Experiment 1a.

2.3. Data analyses

Statistical analyses were conducted as described in Experiment 1a. There were no significant main effects of drug on any variable, so planned comparisons to compare the three doses within each reproductive state were unnecessary. Statistical significance was indicated by $p \leq 0.05$.

2.4. Results

Similar to Experiment 1a, there was a significant main effect of reproductive state on the percentage of time spent in the open arms, with dams spending a greater percentage of time there than did virgins ($F(1, 68) = 10.50$, $p < 0.002$; Fig. 2). The percentage of arm entries that were made into open arms was also higher in dams than virgins ($F(1, 68) = 7.90$, $p < 0.007$; Fig. 2). Dams entered all arms (open plus closed) ($F(1, 68) = 12.57$, $p \leq 0.0007$) and closed arms ($F(1, 68) = 8.83$, $p \leq 0.005$; Table 2) more often than virgins. The total duration of time spent in arms of the plus-maze (open duration plus closed duration) did not significantly differ between the reproductive states ($F(1, 68) = 0.00$, $p \geq 0.99$; Table 2).

Unlike (+)-bicuculline in Experiment 1a, there was no main effect of bicuculline methiodide on the percentage of time spent in open arms ($F(2, 68) = 0.89$, $p > 0.41$), but there was a trend toward an interaction between bicuculline methiodide and reproductive state on this measure ($F(2, 68) = 2.66$, $p > 0.07$), with dams showing a somewhat reduced percentage of time in open arms with increasing dose of bicuculline methiodide (Fig. 2). There was no significant main effect or interaction found for the percentage of entries made into open arms ($F(2, 68) = 0.32$, $p > 0.72$; interaction: $F(2, 68) = 1.80$, $p > 0.17$; Fig. 2), and the same was true for most of the other variables. An exception was the total number of arms entered ($F(2, 68) = 3.73$, $p < 0.03$), with dams showing fewer entries with increasing dose of bicuculline methiodide, whereas virgins showed somewhat more entries with increasing dose (Table 2).

3. Experiment II: effects of cPAGv infusion of bicuculline methiodide on anxiety-related behavior in postpartum rats

3.1. Subjects

Subjects were 36 postpartum female rats from our colony, mated and housed as described in Experiment Ia.

3.2. Intracranial cannulation

Between days 16 and 19 of pregnancy, rats were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg; Butler Co., Dublin OH) followed by an intramuscular injection of xylazine (4 mg/kg; Butler Co, Columbus, OH) and placed in a Kopf stereotaxic instrument with the tooth bar set at zero. The scalp was shaved, cleaned with ethanol and Nolvasan©, and anesthetized with a SC injection of 3% lidocaine (Sigma, USA). After a 1.5 cm incision was made in the scalp, two holes were drilled in the skull above each side of the cPAGv (A/P −7.6, M/L ±0.75 mm from bregma). A 5-mm long, 22-gauge bilateral guide cannula (Plastics One, Roanoake, VA) was slowly lowered until the cannula base was flush with the skull. Cannulae were permanently secured with jeweler's screws implanted into the skull and dental cement surrounding them and the cannula pedestal. The guide cannulae were kept patent with the insertion of dummy cannulae extending 2 mm beyond the guide cannulae, and the entire apparatus was covered with a dust cap. The scalp was closed around the protruding portion of the cannulae with surgical staples.

3.3. Drug administration and elevated plus-maze testing

On day 6 postpartum, subjects were habituated to the handling and infusion procedures by being transported in a clean carrying cage to a nearby room where they were gently handled by an experimenter for approximately 5 min, during which their dust cap and dummy

Table 2
Elevated plus-maze behavior of virgin and postpartum female rats after I.P. injection of saline or 2 or 6 mg/kg of bicuculline methiodide (BM).

	Virgin saline	Virgin 2 mg/kg BM	Virgin 6 mg/kg BM	Postpartum saline	Postpartum 2 mg/kg BM	Postpartum 6 mg/kg BM	Significant effects
# Open arm entries	7 ± 1	7 ± 2	8 ± 1	14 ± 2	13 ± 2	8 ± 1	Reproductive state, Reproductive state × Drug
# Closed arm entries	11 ± 1	12 ± 2	13 ± 1	16 ± 1	15 ± 1	13 ± 1	Reproductive state
Total arm entries	18 ± 2	19 ± 3	22 ± 2	30 ± 2	28 ± 3	21 ± 2	Reproductive state, Reproductive state × Drug
Time in open arms (s)	67 ± 14	65 ± 20	93 ± 19	169 ± 20	127 ± 22	97 ± 14	Reproductive state, Reproductive state × Drug
Time in closed arms (s)	442 ± 25	444 ± 27	403 ± 24	343 ± 17	390 ± 23	389 ± 17	Reproductive state
Time in all arms (s)	509 ± 13	509 ± 12	496 ± 10	512 ± 6	516 ± 9	487 ± 7	–

cannula were removed. A 28-gauge injection cannula extending into the cPAGv (~14 mm in length) was inserted into the guide and left for 4–5 min; nothing was infused. The injection cannula was then slowly removed, and the dummy cannula and dust cap replaced. Dams were then returned to their home cage in the colony room. The next day, dams were again brought in a clean carrying cage to the nearby habituation/infusion room and received a cPAGv infusion of 250 nl/side of physiological saline ($n=14$) or 2.5 ng bicuculline methiodide dissolved in 250 nl of saline/side (total infusion of 5 ng; $n=22$). Dams were returned to their home cage in the colony room, and 10 min later transported in their home cage to the nearby testing room and tested in the elevated plus-maze for 10 min.

3.4. Verification of infusion sites

Within a week after testing, subjects were overdosed with sodium pentobarbital and perfused through the heart with 200 ml isotonic saline. Brains were removed and postfixed overnight in 10% formalin, followed by submersion in 30% sucrose in sodium phosphate buffer (pH = 7.6). Brains were sectioned at 40 µm on a freezing microtome, stained with Neutral Red, dehydrated, and coverslipped. Infusion sites were verified microscopically at 100× magnification. Some subjects were infused bilaterally with 250 nl methyl green immediately before perfusion, and subjects with correctly placed cannulae never had methyl green in their ventricular system, similar to what we previously found (Figueira et al., 2008).

3.5. Data analyses

Of the 14 dams with saline infused into the cPAGv, 4 had asymmetrical infusions with one prong in the cerebral aqueduct and one prong at the lateral border of the cPAGv; these subjects were removed from the study. Of the 22 dams with infusions of bicuculline methiodide directed at the cPAGv, 12 were asymmetrical; because of the large number of these subjects, they were not removed from the study but instead placed into the separate "Missed" comparison group. One bicuculline-infused dam rapidly circled and jumped out of the holding cage during infusion, so was removed from the study.

3.6. Results

Infusions of bicuculline methiodide in the bilateral cPAGv group were within the ventrolateral and lateral columns of the intercollicular PAG (Fig. 3). These dams showed a significantly lower percentage of time spent in the open arms of the plus-maze compared to dams infused with saline into the cPAGv or the "Missed" dams with asymmetrical bicuculline methiodide infusions near the cPAGv ($F(2, 28)=4.50$, $p<0.03$; Fig. 4). The percentage of arm entries made into open arms also significantly differed among groups in the same pattern ($F(2, 28)=4.61$, $p<0.02$; Fig. 4). The total number of arms entered (open plus closed) was significantly lower ($F(2, 28=6.18$, $p<0.007$), and the number of closed-arm entries tended to be lower ($F(2, 28)=3.33$, $p<0.06$), in dams with bilateral cPAGv bicuculline methiodide infusions (Table 3). Similar to Experiment 1a, there was no evidence of general locomotor dysfunction because the latencies from the beginning of testing to enter ($F(2, 28)=1.75$, $p>0.19$) and exit ($F(2, 28)=0.11$, $p>0.89$; Table 3) the first arm were not affected by bicuculline methiodide. Groups did not significantly differ in the duration of time spent in all arms of the elevated plus maze (open plus closed) ($F(2, 28)=0.80$, $p>0.46$; Table 3).

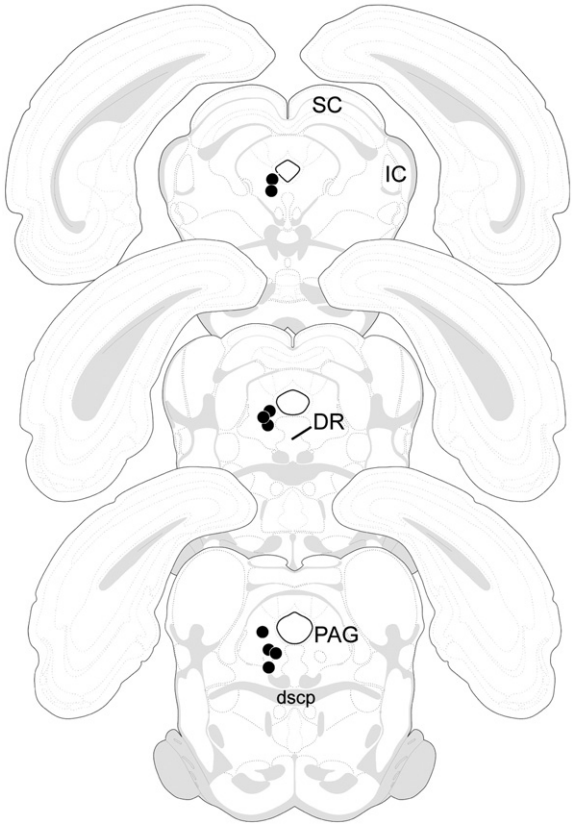

Fig. 3. Schematic representation of infusion sites for dams receiving bicuculline methiodide into the cPAGv (black circles). The bilateral infusion sites are depicted unilaterally. Modified from Swanson (1998). SC — superior colliculus, IC — inferior colliculus, DR — dorsal raphe, PAG — periaqueductal gray, dscp — decussation of the superior cerebellar peduncle.

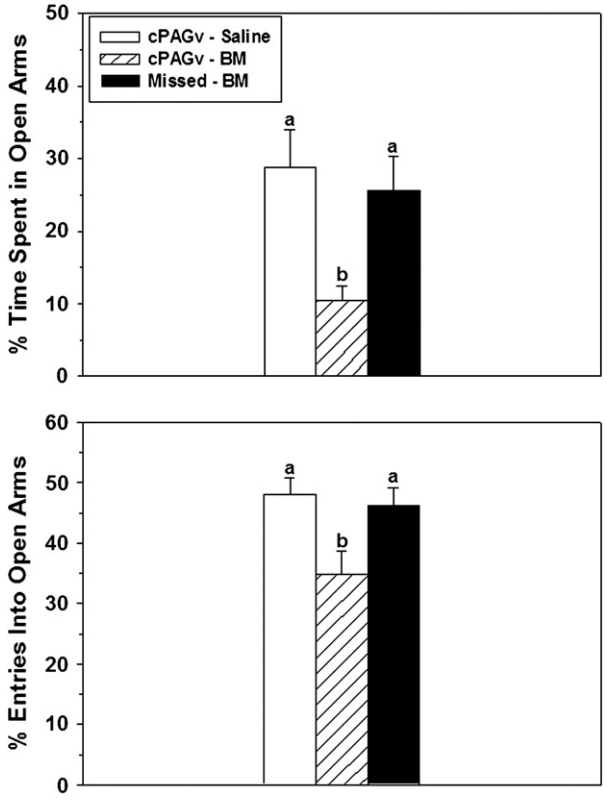

Fig. 4. Percentage of time (Mean ± SEM) spent in the open arms of the elevated plus maze (top) and percentage of open arm entries (bottom) after dam received bilateral infusion of saline or bicuculline methiodide (BM) into the cPAGv, or "missed" infusions of BM. Significant differences among groups indicated by different letters above bars, $p \leq 0.05$.

Table 3
Elevated plus-maze behavior of postpartum females after infusion of saline or 2.5 ng bicuculline methiodide (BM) into each hemisphere of the cPAGv, or bicuculline methiodide infused asymmetrically in the midbrain (Missed).

	cPAGv Saline	cPAGv BM	Missed BM	$F(2,28)$
# Open arm entries	15 ± 2^a	6 ± 1^b	12 ± 2^a	6.79
# Closed arm entries	15 ± 1	11 ± 1	13 ± 1	3.33
Total arm entries	29 ± 3^a	17 ± 1^b	25 ± 3^a	6.18
Time in open arms (s)	134 ± 24^a	50 ± 10^b	122 ± 22^a	4.64
Time in closed arms (s)	335 ± 26	443 ± 27	375 ± 32	3.11
Time in all arms (s)	470 ± 9	493 ± 23	497 ± 15	0.80
Latency enter 1st arm (s)	1.5 ± 0.5	0.5 ± 0.1	0.8 ± 0.4	1.75
Latency exit 1st arm (s)	12 ± 3	13 ± 3	11 ± 4	0.11

Groups with different superscript letters significantly differ from each other, post-hoc $p \leq 0.05$.

4. Discussion

Anxiety-related behaviors in non-parturient animals are regulated by a tremendous number of neurochemicals, with the GABAergic system being particularly important. Blockade of $GABA_A$ receptors typically increases anxiety, whereas $GABA_A$ agonists invariably reduce anxiety (Millan, 2003; Roy-Byrne, 2005). $GABA_A$ receptor activity is also essential for the suppressed anxiety-related behavior of mother rats. Qureshi et al. (1987) demonstrated that physical contact with pups maintains high cerebrospinal fluid concentrations of GABA in dams, and suggested that this increase was responsible for the mothers' low anxiety. Dams' anxiety-related behaviors do increase within hours after the litter is removed (Figueira et al., 2008; Lonstein, 2005; Smith and Lonstein, 2008), which may reflect the decrease in central GABA levels. Furthermore, peripheral administration of negative $GABA_A$ receptor modulators, such as FG-7142 or pentylenetetrazol, increases dams' freezing in response to a sudden acoustic stimulus and reduces their punished drinking (Hansen, 1990; Hansen et al., 1985). The present experiments are the first to examine how $GABA_A$ receptor modulators affect postpartum anxiety in the very widely-used and pharmacologically reliable elevated plus-maze, and the results are consistent with previous findings. We found that peripheral administration of (+)-bicuculline significantly increased dams' anxiety-related behavior to levels observed in control diestrous virgins. A significant effect was not found in even larger groups of rats peripherally injected with a relatively high dose of bicuculline methiodide, which does not readily cross the blood–brain barrier (Pong and Graham, 1972; Remler and Marcussen, 1985), suggesting that the $GABA_A$ receptor activity modulating postpartum anxiety lies primarily within the central nervous system. Even so, there was a trend toward a reduced percentage of time spent in the open arms for dams (but not virgins) treated with bicuculline methiodide. This could be due to a small effect of reproductive state on feedback from peripheral organs that express $GABA_A$ receptors (Ong and Kerr, 1990; Watanabe et al., 2002) to the areas of the central nervous system regulating anxiety (Damasio, 1998; Katkin et al., 2001; Pollatos et al., 2007). It might also reflect a small reproductive state difference in blood–brain-barrier permeability for some, but certainly not most, molecules (Abbud and Smith, 1993; Aschner and Clarkson, 1987; Nyberg et al., 1989; Oztaş et al., 1993; Rubin and Bridges, 1989).

We also found that reproductive state seemed to influence females' locomotor activity, as indicated a greater number of closed arm entries in dams compared to virgins (Pellow et al., 1985). This is apparently consistent with one other report comparing the behavior of postpartum and virgin females in an elevated plus maze (Vinogradova, 1999), but there are other reports of lower activity in dams compared to virgins when assessed in other paradigms (Barnett and McEwan, 1973; Silva et al., 1997) or no effects of reproductive state on activity (Stern et al., 1973; Zuluaga et al., 2005). The effects of reproductive state on locomotor activity even within an elevated plus maze could benefit from further examination, as experiments from within our own laboratory do not consistently find differences between postpartum and virgin rats in the number of closed arm entries (Figueira et al., 2008; Lonstein, 2005).

Dams' number of closed arm entries was significantly reduced by (+)-bicuculline in Experiment 1a, and tended to be reduced in those receiving cPAGv infusion of bicuculline methiodide in Experiment 2. These effects were not due to gross motor impairments, because the latencies to enter and exit the first arm were not altered in either experiment. The PAG is part of the "mesencephalic locomotor region", a collection of brain sites from which stepping and other limb movements can be elicited via activation of reticulospinal pathways (Jordan, 1998; Parker and Sinnamon, 1983), making the PAG a prime target for the locomotor-altering effects of pharmacological agents. The apparent locomotion-reducing effects of bicuculline might be surprising, though, because bicuculline has been seen to either have no effect on locomotion or cause bursts of activity when given peripherally, into the ventricular system, or directly into the PAG of male rats (Agmo and Giordano, 1985; Bandler et al., 1985; Carrive et al., 1986; Marazioti et al., 2009; Morgan and Clayton, 2005; Sainati and Lorens, 1983; Zarrindast et al., 2001). Disinhibiting or stimulating the cPAGv produces a wide range of responses, though, including immobility and behavioral inhibition (Depaulis and Vergnes, 1986; Depaulis et al., 1994; Zhang et al., 1990). The PAG is also a locus of control for behavioral switching in rats (Sukikara et al., 2006, 2010), and its role in balancing conflicting motivational states probably extends to whether animals freely explore a novel environment (such as an elevated plus maze) or inhibit movement in response to potential threats. Because brain sites such as the PAG modulate both exploration and anxiety, and exploration is suppressed by anxiety-elicited defensive responses, it has been questioned whether the number of closed-arm entries in an elevated plus maze could possibly be an unconfounded measure of locomotor activity (for review see Wall and Messier, 2001; Weiss et al., 1998).

Only the relatively high dose of (+)-bicuculline (4 mg/kg) was found to increase dams' anxiety-related behavior, whereas others have shown that lower doses alter plus-maze behavior in male rats (Cruz et al., 1994; Cole et al., 1995; Pellow and File, 1986; Zarrindast et al., 2001). There are sex differences in responsiveness to bicuculline and other $GABA_A$ receptor drugs, with females requiring higher doses than males to induce physiological and behavioral effects (Manev et al., 1987; Meng and Drugan, 1993; Pericic et al., 1999; Wilson, 1992; although see Devaud et al., 1998). (+)-Bicuculline did not notably increase the already high anxiety behavior of diestrous virgins, probably because antagonizing the receptor when endogenous ligand levels are relatively low would have little effect. It may also reflect a floor effect, such that the low duration of time diestrous virgins spent in the open arms could not readily be further decreased. Perhaps it would be easier to increase virgins' anxiety-related behaviors with peripheral (+)-bicuculline during proestrus or estrus, when GABA tone in brain areas influencing emotional responsiveness is high (*e.g.*, Felton and Auerbach, 2004; Lovick, 2008; Smith et al., 2007) and anxiety is low (Frye et al., 2000; Marcondes, et al., 2001; Mora et al., 1996; Nomikos and Spyraki, 1988; Zuluaga et al., 2005). It should be mentioned that the dose of (+)-bicuculline we found effective in altering anxiety-related behavior (4 mg/kg) did not cause any adverse effects, but was similar or higher than the doses another study reported to produce convulsions and even death in cycling female rats after intraperitoneal administration (Pericic and Bujas, 1997). This difference may be related to the strain of rat used (Long–Evans vs. Wistar; *e.g.*, Shephard et al., 1982), housing conditions (individually housed vs. group housed; *e.g.*, Rilke et al., 1995), or an interaction between bicuculline and the very large volume of solution Pericic and Bujas reported they injected into the peritoneal cavity of their rats (1 ml solution per 100 g of body weight).

Our data reveal that the cPAGv is one of surely many sites in the traditional anxiety network (Millan, 2003) where $GABA_A$ receptor

activity acts to suppress anxiety-related behaviors in mother rats. $GABA_A$ receptor activity in the cPAGv is also required for dams' postpartum estrus copulatory behavior and nursing of pups, but not their maternal aggression (Salzberg et al., 2002), indicating widespread function of $GABA_A$ receptors in the cPAGv for postpartum behavior. With its dense connections to forebrain sites involved in emotional responding, the PAG is thought to be a final common pathway for the behavioral and physiological responses to an aversive stimulus (Bandler and Shipley, 1994; Brandão et al., 2008). Even so, bicuculline infused into two forebrain sites densely connected with the cPAGv (central/basolateral amygdala and ventromedial hypothalamus) was previously found to not affect dams' suppressed freezing in response to a sudden acoustic stimulus (Hansen and Ferreira, 1986). Thus, other forebrain sites projecting densely to the cPAGv may be more critical for the postpartum suppression of emotional behaviors, and we have suggested that the ventral bed nucleus of the stria terminalis (BSTv) is one of them (Lonstein, 2007; Lonstein and Miller, 2008; Smith and Lonstein, 2008). The BSTv is strongly implicated in anxiety-related behaviors (Davis et al., 2010), is responsive to pup-related sensory cues (Numan and Numan, 1994, 1995), and in postpartum rats shows an immediate-early gene response to anxiogenic stimuli that is uniquely affected by recent contact with pups (Smith and Lonstein, 2008). Integrating anxiety- and offspring-related cues within the BSTv, and relaying that information to the cPAGv, may be essential for the appropriate behavioral and physiological responses in postpartum animals. This pathway may also involve input to the BSTv from regions of cerebral cortex (Balfour et al., 2006; Spencer et al., 2005; White et al., 1991) that show increased GABAergic tone when dams interact with pups (Abbud et al., 1993; Kornblatt and Grattan, 2001).

The PAG can be divided into subregions dorsoventrally and rostrocaudally based on its cytoarchitecture, anatomical connections, neurochemistry, and function (Depaulis and Bandler, 1991). In male rats, the dorsal PAG at relatively rostral levels (rPAGd) is almost exclusively studied with regards to anxiety. This may be due to historical precedent, rather than a greater importance of this PAG subregion over others. Early studies using electrical stimulation to discover areas of the brain from which aversive responses could be elicited (a model of anxiety or fear) identified the rPAGd as one such site (Olds and Olds, 1963; Stein, 1965; Valenstein 1965). These early studies did not examine the cPAGv in detail, or at all, but a later and more thorough study did find that the effects of cPAGv stimulation were similar to rPAGd stimulation (Schmitt et al., 1977). In one of the few anxiety studies directly comparing the dorsal and ventral PAG, unilateral NMDA receptor antagonism in either subregion increased open arm time in an elevated plus maze and punished licking in male rats (Molchanov and Guimaraes, 2002). Even so, a more recent study (De Luca-Vinhos et al., 2006) reported that midazolam infused unilaterally into the cPAGv did not affect elevated plus-maze behavior in male rats, as is found after unilateral midazolam infusion into the rPAGd (Motta and Brandao, 1993). Our current and previous work (Lonstein et al., 1998; Figueira et al., 2008) does not address if the rPAGd is involved in suppressing postpartum anxiety, but does demonstrate that the cPAGv is absolutely essential. Furthermore, some preliminary work in our lab suggests that bicuculline methiodide infused into the dorsal cPAG or deep layers of the superior colliculus has no significant effects on dams' plus-maze behavior (Peabody and Lonstein, preliminary data). It is intriguing that the PAG is sexually dimorphic in its neurochemistry, neuroanatomy, and function (Krzanowska and Bodnar, 2000; Loyd and Murphy, 2009; Normandin and Murphy, 2008; Tershner et al., 2000; Tsukahara and Yamanouchi, 2002). A sex difference in how the numerous PAG subregions mediate behavioral responses to aversive stimuli may be associated with sex differences in anxiety behaviors in rodents, the higher prevalence of anxiety disorders in women compared to men (Palanza, 2001), and the etiology of anxiety disorders in susceptible peripartum women (Levine et al., 2003).

In conclusion, we have provided additional direct evidence that increased $GABA_A$ receptor signaling is necessary for the reduced anxiety-related behavior of postpartum rats. We believe that these data support the idea that postpartum and male rat brains employ similar neural systems to modulate anxiety, but that this network is inhibited by $GABA_A$ receptor stimulation as a result of mothers' continual interactions with pups. Dams require recent physical contact with pups, but not suckling, for their reduced anxiety (Lonstein, 2005, 2007). This may reflect the ability of tactile cues from offspring to stimulate GABA release in the postpartum rat brain, including in the cPAGv. Changes in $GABA_A$ receptor subunit expression in the maternal brain is also likely to contribute to their suppressed anxiety (Byrnes et al., 2007; Fénelon and Herbison, 1996; Follesa et al., 1998). Of course, GABA is not the only neurochemical affected by infant contact to suppress emotional reactivity during the postpartum period. For example, oxytocin and prolactin signaling are high in postpartum rats and contribute to their reduced anxiety (Figueira et al., 2008; Neumann et al., 2000; Torner et al., 2002). Similar to GABA, the anxiolytic properties of these peptides is not unique to females (*c.f.*, Blume et al., 2008; Torner et al., 2001). It is possible that oxytocin and prolactin are anxiolytic, at least in part, by increasing central GABA release or potentiating the effects of $GABA_A$ receptor ligands (see Breton et al., 2008; Brussaard, 1995; Duvilanski et al, 1987a,b; Israel et al., 2008; Locatelli et al., 1985; Nicoletti et al., 1983; Viviani et al., 2010). Indeed, we recently reported that a highly selective oxytocin receptor antagonist infused into the cPAGv produces the same anxiogenic effects in dams as those produced by (+)-bicuculline (Figueira et al., 2008), indicating possible synergy or redundancy between anxiety-modulating neurochemicals in the postpartum brain.

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