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GABA_A receptor signaling in caudal periaqueductal gray regulates maternal aggression and maternal care in mice

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Abstract

Maternal aggression (maternal defense) is exhibited by lactating females towards intruders and contributes to the protection of offspring. Enhancement of GABA_A receptor signaling by benzodiazepines elevates maternal aggression, and we previously found indirect evidence (via c-Fos immunohistochemistry) that caudal periaqueductal gray (cPAG) and lateral septum (LS) could be sites where benzodiazepines increase aggression. We recently found that GABA_A receptor signaling in LS modulates maternal aggression, and in this study, we tested the hypothesis that GABA_A receptor signaling in cPAG also regulates this behavior. Site-directed injections to cPAG were made in lactating mice using the GABA_A receptor antagonist, bicuculline (3–9 ng) or the GABA_A receptor positive modulator, chlordiazepoxide (CDP), a benzodiazepine (2.5–20 µg). Maternal aggression, other maternal behaviors, and anxiety-like measures (using the light-dark box) were then examined. GABA_A receptor positive modulator did not increase aggression, which could have resulted from a ceiling effect. However, 8 ng and 9 ng of bicuculline in cPAG significantly decreased maternal aggression without altering other maternal behaviors or light-dark box performance, suggesting some GABA_A receptor signaling in cPAG is required for full maternal aggression expression. Additionally, 7 ng of bicuculline significantly increased licking/grooming of pups, and decreased the number of transitions between the light and dark compartments of the light-dark box without affecting aggression. Given these results indicating that antagonizing GABA_A-receptor in cPAG dose-dependently promotes offspring grooming behavior while impairing aggression, it is possible that the cPAG represents a key site for decision making (aggression versus other behaviors) in the lactating female.

Keywords

Maternal aggression; Caudal periaqueductal gray (cPAG); GABA; Maternal behaviors; Bicuculline; Chlordiazepoxide

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1. Introduction

The neural region, periaqueductal gray (PAG), is a key regulator of motor output for a number of forms of aggressive behaviors, such as male aggression and defensive behavior (for review see, [76] [9;30;33;77]). Maternal aggression (maternal defense), a component of maternal behavior, is a fierce protective behavior whereby lactating females attack an intruder to protect offspring, and may also be regulated by PAG. Lesions in the caudal (c)PAG increase maternal aggression in rats [51;52] and injections of serotonin (5-HT) 1 receptor antagonists and 5-HT (2a/2c) receptor agonists into cPAG decrease maternal aggression [14;15]. Brain activity studies using immediate early genes also indicate an association of cPAG with maternal aggression regulation [25;26;28;34]. Collectively, this evidence suggests that cPAG may play a critical role in the regulation of maternal aggression.

Gamma-Aminobutyric acid (GABA)_A receptors are expressed at high levels in cPAG [6;55;78]. Additionally, pup stimulation increases immunoreactivity of glutamate decarboxylase (GAD), an enzyme that produces GABA, by 61% in the PAG of lactating females [48]. These findings suggest a role for GABA_A receptor signaling in cPAG in behaviors expressed during the postpartum period. GABA has been previously linked to maternal aggression as benzodiazepine (BDZ) agonists (which increase the binding affinity of GABA onto GABA_A receptors) alter maternal aggression in rats [19;57;58] and mice [66;84;85]. For example, chlordiazepoxide (CDP), a BDZ, triggers lactating females to be significantly more aggressive towards heavier male intruders [59]. We previously replicated earlier studies, and found CDP increases maternal aggression in mice [39]. As part of that study, we found that injections of CDP in the absence of behavioral testing decreased Fos activity in cPAG and lateral septum (LS). This result suggested that either LS, cPAG, or both regions acting together could be critical for how GABA modulates maternal aggression. We recently found that antagonizing GABA_A receptor in LS decreases maternal aggression without affecting other components of maternal behaviors [40], but a role for the GABA_A receptor in cPAG regulating maternal defense remains unclear.

An earlier study in rats examined the role of GABA_A signaling in cPAG on reproductive posture behaviors (lordosis and kyphosis), and also monitored maternal aggression, but no effects of agonist or antagonist were found [72]. However, baseline levels of maternal aggression were suppressed as pups were removed for three hours prior to testing, so a lack of effect could have resulted from low levels of the dependent measure. To date, a study of cPAG-GABA_A receptor activity regulation of maternal aggression in animals showing full maternal aggression has not been performed.

The aim of this study was to investigate how GABA_A modulation in the cPAG alters maternal aggression in mice. We hypothesized that GABA_A positive modulator, CDP, injected into cPAG would increase maternal aggression, whereas the GABA_A antagonist, bicuculline, would decrease maternal aggression. Because cPAG [29;38;79] and GABA_A receptor signaling [5;16;20;31;46;67] have been linked to anxiety control, in this study we also wanted to evaluate whether treatments affected anxiety-like behavior using the light-dark box. Also, because previous studies have linked cPAG to nursing behavior, and studies suggest offspring protection and other maternal behaviors could include some shared overlapping neural circuitry components [21], we observed other maternal behaviors here to evaluate the specificity of our treatment on maternal aggression. This study was designed to provide insights into whether cPAG is a critical neural region for GABA_A receptor regulation of offspring protection in lactating mice.

2. Materials and methods

2.1. Animals

Female mice selected for high maternal aggression [23] and male outbred hsd:ICR (Harlan, Madison, WI) mice were used in this study. Mice were housed in polypropylene cages with access to tap water and breeder chow (for females) or regular chow (for males) ad libitum. Females were paired with a breeder male and after 10 days, the male was removed from the cage. The day after birth [postpartum day (PPD) 1], litters were culled to eleven pups. Outbred, sexually-naïve male mice were grouped housed and were used as intruders. Intruder males were never used more than once per day and were used for ~ 3 tests each. Cages were changed weekly prior to parturition after which they were unchanged for the remainder of the experiment. All animals were housed on a 14:10 light/dark cycle with lights on at 06:00 CST. All procedures followed the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of the University of Wisconsin.

2.2. Cannulae surgeries

On PPD 3, under isoflurane anesthesia and using a stereotaxic apparatus (David Kopf Instruments, Tunjunga, CA), a midline incision across the top of the skull was made. Prior to the cut, the hair above the skull was shaved and the skin cleaned and treated with alcohol and Betadine (Purdue Frederick, Stamford, CT). We targeted a subregion of cPAG based on our previous work with CDP [39]. For cPAG unilateral injections, a small hole was drilled +5.0 mm posterior and ± 0.15 mm lateral to Bregma. In the hole, a 26 gauge stainless-steel indwelling cannula (Plastics One, Roanoke, VA) was implanted to -2.1 mm below the skull surface. In some cases, the cannula hit the aqueduct (Aq), and these results were analyzed separately. Two small screws were drilled into the skull lateral and anterior to the guide cannula, and each cannula was secured to the skull using dental cement (Plastics One). A dummy cannula was inserted to maintain patency until injections were made. Injections were made using a 33 gauge stainless-steel cannula attached to PE-50 tubing (Becton Dickinson, Sparks, MD) fitted to a 10 μ L Hamilton syringe (Hamilton, Reno, NV). On PPD 3 and 4, a cannula was briefly put into the guide to prepare the tissue for later injections on test days. This approach was used to ensure more consistent conditions of tissue and subsequent injections across test days. Before and after surgery, all animals received s.c. injections of ketoprofen (1 mg/kg) to minimize any discomfort.

2.3. Pharmacological treatment and site-directed injections

Separate groups of mice were used for evaluating bicuculline methiodide (Sigma Chemical, St. Louis, MO), and CDP hydrochloride (Sigma). For any given group, vehicle and two doses of a drug were used. The following groups were used: vehicle, 3 ng and 7 ng bicuculline; vehicle, 8 ng and 9 ng bicuculline; vehicle, 2.5 μ g and 5 μ g CDP; and vehicle, 10 μ g and 20 μ g CDP. Within each group, a within-subjects repeated measures design was used whereby each animal received vehicle and the two levels of a given drug treatment. Order of injections was always counterbalanced. This approach was used because if there were any effect of multiple injections or multiple behavioral testing, then those effects would be equally distributed across treatments. Final N's for the separate groups and intra-cPAG injections are provided below. The bicuculline and CDP doses were chosen based on our previously studies [39;40] and pilot observations. Beginning on PPD 6, single injections were delivered each day for up to 3 consecutive days to animals under light isoflurane anesthesia. All unilateral injections were made using 0.25 μ L volume. Based on previous studies and our histological analysis following injection of Chicago sky blue dye (see below), it was expected that with each site-specific injection, that the fluid would have diffused ~ 200 μ m from the injector tip at the time of behavioral testing. Although most

injections were clearly within cPAG, we cannot exclude the possibility that diffusion from some injections to adjacent regions had an impact on behavior. Additionally, there would be a trend for some fluid to follow up along the cannula [36;47;60]. Given diffusion of the small volume of drug (0.25 μ l) at body temperature and previous studies examining effects of site-specific injections of muscimol (which has a high binding affinity) [61], it was expected that the effect of any given treatment lasted no longer than 6 hours. Infusions were verified by following movement of an air bubble in the tubing and the cannula remained in place for 60 seconds following each injection. 20 minutes after injection, a female was exposed to a 5 minute aggression test, a 5 minute light-dark box test, and then observed for maternal behaviors for 30 minutes. No mice displayed stereotypic behavior following the injections.

2.4. Behavioral testing

All behavioral testing occurred between 0800 and 1200 hours on PPD 6, 7, and 8. In previous studies, we have found that maternal aggression and other maternal behaviors are expressed consistently across this time period [11;12;13]. Light phase measures were taken because maternal aggression levels do not change during the active or inactive phase of the daily photo cycle (N.S. Hasen, S.C. Gammie, in preparation) and mice show high levels of active maternal behaviors in normal light phase [32;42;43;80]. Each test session was recorded on videotape and subsequently analyzed off-line to quantify maternal behaviors and anxiety-like behaviors by individuals blind to testing conditions. Total behavioral testing lasted for 40 min. To maintain consistency among scorers, all scorers had to pass a calibrator aggression test just prior to scoring. Further, each mouse was always evaluated by the same scorer to ensure that any lingering rater bias was equally spread across each treatment. Each test day, the same order of behavioral testing was used and because the order of injections was counterbalanced, any carry-over effect would have been equally distributed among groups. It was not expected that performance of the LD test just prior to maternal behaviors would have affected the latter because in previous work we found that application of a strong stressor (restraint) did not affect maternal behaviors [28].

2.4.1. Maternal aggression testing—Twenty minutes following injection, females were moved into the testing room, and the pups were separated from the dam. A male intruder was placed into the female's cage for 5 minutes. Intruder mice from the same cage were used equally to test different treatments. Removal of pups from the home cage of a dam before an aggression test does not diminish the expression of maternal aggression in mice [81]. For quantification of maternal aggression, the following features were measured: latency to first attack, number of attacks, and total duration of attacks [24;27].

2.4.2. Light-dark box testing: After the intruder male was removed, the female was exposed to a light-dark box test for 5 minutes. The apparatus consisted of a large box (30 $\times$ 24.5 $\times$ 25.5 cm) with a black plastic box in one half with an opening to connect the dark compartment (~9.3 lux) to the light compartment (~250 lux). At the beginning of the test, the female was placed directly in front of the opening to the dark box. For quantification of light-dark box test, the following features were measured: latency to enter the dark compartment, number of transitions between light and dark compartments, and total duration spent in the light and dark compartments [5].

2.4.3. Maternal behavior observations—Immediately after the light-dark box test, the female was placed back into her home cage, pups were scattered evenly away from the nest allowing the female to retrieve pups, and the female was allowed to perform maternal behaviors for 30 minutes. Pup retrieval was quantified by measuring the time elapsed to retrieve the first and fourth pup. Other maternal behaviors were surveyed every 30 seconds

and quantified. Maternal behaviors include nursing (all forms, such as high and low arched-back nursing), licking/grooming of pups by the female, nest building activity, self-grooming, and time on and off nest. Latency to nursing, nest building, and grooming of pups, and rates of each maternal behavior were quantified in each observation.

2.5. Nissl staining for cannulae placement

On PPD 9 and prior to brain collection, a 0.25 μ L volume of 0.01% Chicago sky blue in saline was injected into the brain to verify cannula placement. Mice were anesthetized, decapitated and their brains were removed from the skull. Brains were fixed overnight in 6% acrolein in phosphate buffered saline (PBS) and stored in 30% sucrose in PBS for 2 days. Brains were frozen on a platform and cut into 40 μ m thick section using a sliding microtome (Leica, Microsystems, Heidelberg, Germany) and stored in cryoprotectant solution at -20 degrees C until processing. Brain sections were mounted, stained with Thionin, cover slipped, and images of the section were projected from an Axioskop Zeiss light microscope through an AxioCam Zeiss high-resolution digital camera attach to the microscope and interfaced with a computer.

2.8. Analysis of maternal and anxiety-like behaviors

All behavioral testing variables were analyzed using a one-way repeated measures (RM) analysis of variance (ANOVA). In the cases where data were not normally distributed, data were transformed to achieve normality (using a 0.3 power transform, 0.7 power transform, or square root transform) and one-way RM ANOVA was performed. If normality was not found after transformations, then a non-parametric Friedman RM ANOVA on Ranks test was performed. For maternal behavior proportions, if data were not normally distributed, data were transformed to $2\arcsin\sqrt{x}$ [41], and then a one-way RM ANOVA was performed. If an overall significant effect of treatment was found, the subsequent Holm-Sidak (parametric) or Dunn's method (nonparametric) post hoc tests were performed. In the case of latency to first attack, if an animal was not aggressive, a time of 300 seconds (the maximum time of the test) was assigned. The following indicate sites of cannula placement and sample size for behavioral analysis (Fig. 1): low dose of bicuculline (3 ng and 7 ng) relative to vehicle: cPAG, $n = 8$; high dose of bicuculline (8 ng and 9 ng) relative to vehicle: cPAG, $n = 10$; low dose of CDP (2.5 μ g and 5 μ g) relative to vehicle: cPAG, $n = 9$; high dose of CDP (10 μ g and 20 μ g) relative to vehicle: cPAG, $n = 4$; low dose of bicuculline relative to vehicle: Aq, $n = 9$; high doses of bicuculline relative to vehicle: Aq, $n = 10$; high dose of CDP relative to vehicle: Aq, $n = 10$; low dose of CDP relative to vehicle: Aq, $n = 3$. In a few cases, the hits to cPAG were near to Aq and we cannot exclude the possibility of diffusion to Aq.

3. Results

3.1. Effects of bicuculline injections on maternal aggression

The high doses of bicuculline (8 ng and 9 ng) infused into cPAG had an overall significant effect on total duration of attacks ($F(2,15) = 5.601$, $p = 0.015$) (Fig. 2A). Holm-Sidak post hoc tests revealed that 8 ng ($t(9) = 2.928$, $p = 0.010$) and 9 ng ($t(5) = 2.733$, $p = 0.015$) of bicuculline significantly decreased the total time spent being aggressive towards an intruder relative to vehicle. However, low doses of bicuculline (3 ng and 7 ng) did not alter total time of aggression ($F(2, 13) = 1.259$, $p = 0.316$) (Fig. 2A). In terms of number of attacks, high dose group of bicuculline had an overall significant effect ($F(2, 14) = 4.876$, $p = 0.025$) (Fig. 2B). Holm-Sidak post hoc tests revealed that 8 ng of bicuculline significantly decreased number of attacks relative to vehicle ($t(9) = 3.093$, $p = 0.008$). No difference was found in number of attacks between low dose group of bicuculline ($F(2, 13) = 0.216$, $p = 0.808$), and

vehicle (Fig. 2B). Both high doses ($F(2, 14) = 2.103, p = 0.159$) and low doses ($\chi^2(2, 8) = 1.000, p = 0.620$) of bicuculline did not significantly alter latency to first attack (Fig. 2C).

With Aq injections, the high dose group did not affect total duration of attacks ($\chi^2(2, 10) = 0.667, p = 0.954$), number of attacks ($\chi^2(2, 10) = 2.667, p = 0.522$), or latency to first attack ($\chi^2(2, 10) = 0.500, p = 0.9054$) (Not shown). Furthermore, the low dose group did not affect total duration of attacks ($F(2, 14) = 0.0631, p = 0.939$), number of attacks ($F(2, 14) = 0.0222, p = 0.978$), or latency to first attack ($\chi^2(2, 9) = 0.519, p = 0.768$) (Not shown).

3.2. Effects of CDP injections on maternal aggression

Intra-cPAG injections of CDP had no effect on total duration of aggression, number of attacks or the latency to first attack. The mean levels of aggression for treatment groups are shown in Table 1. The p -values for low doses are as followed: total time duration of aggression ($p = 0.394$); number of attacks ($p = 0.328$); latency to first attack ($p = 0.377$). The p -values for high doses are as followed: total time duration of aggression ($p = 0.262$); number of attacks ($p = 0.574$); latency to first attack, ($p = 0.800$).

In addition, Aq injections of high doses of CDP (10 μg and 20 μg) did not alter total time of aggression ($F(2, 5) = 0.415, p = 0.681$), number of attacks ($F(2, 5) = 1.422, p = 0.324$), or latency to first attack ($F(2, 5) = 1.015, p = 0.427$) (Not shown). In terms of the low dose group (2.5 μg and 5 μg), Aq injections did not alter total time aggression ($F(2, 3) = 1.772, p = 0.310$), number of attacks ($F(2, 3) = 1.621, p = 0.333$), or latency to first attack ($F(2, 3) = 1.791, p = 0.308$). The sample size of the intra-Aq CDP dose groups are too low to be interpreted with confidence. However, injections of these doses into the lateral ventricles did not have an effect in a previous study [40].

3.3. Effects of bicuculline and CDP injections on light-dark box test

For intra-cPAG injections of bicuculline and CDP, no effect on light-dark box performance was found (See Table 2), except for low doses of bicuculline. There was an overall significant difference on the number of transitions from light to dark compartments ($F(2, 13) = 4.426, p = 0.034$) (Fig. 3A). Holm-Sidak post hoc tests revealed that 3 ng ($t(7) = 2.329, p = 0.37$) and 7 ng ($t(8) = 2.736, p = 0.017$) of bicuculline significantly decreased the number of transitions relative to vehicle.

For Aq injections, the only overall significant effect was for high dose bicuculline treatment on number of transitions ($F(2, 12) = 9.994, p = 0.003$) (Not shown). Holm-Sidak post hoc tests showed that subjects injected with 9 ng crossed less between compartments relative to vehicle ($t(9) = 3.398, p = 0.025$), and 8 ng of bicuculline ($t(9) = 4.108, p = 0.001$). For all other Aq injections, high doses of bicuculline did not affect total time in light ($p = 0.284$) or latency to enter the dark ($p = 0.225$). Low doses of bicuculline did not affect either time in the light ($p = 0.378$), number of transitions ($p = 0.306$), or latency to enter the dark ($p = 0.768$). High doses of CDP did not affect total time in the light ($p = 0.834$), number of transitions ($p = 0.474$), or latency to enter the dark ($p = 0.657$). Low doses of CDP did not affect total time spent in the light ($p = 0.650$), number of transitions ($p = 0.469$), or latency to enter the dark ($p = 0.208$).

3.4. Effects of bicuculline and CDP injections on maternal behaviors

For intra-cPAG injections, neither bicuculline nor CDP had any effect on any of the maternal behaviors monitored (See Table 3), except for low doses of bicuculline. Low doses of bicuculline had an overall significant effect on the frequency of licking/grooming of pups behavior ($F(2, 13) = 8.692, p = 0.004$) (Fig. 3B). Holm-Sidak post hoc tests revealed that 7 ng of bicuculline significantly increased the rate of licking/grooming pups relative to vehicle

($t(7) = 3.315$, $p = 0.006$) and 3 ng ($t(7) = 3.801$, $p = 0.002$). In addition, there was a non-significant trend for high doses of CDP to increase nursing behavior frequency ($F(2,6) = 4.610$, $p = 0.061$).

Aq injections of bicuculline and CDP did not alter maternal behaviors (Not shown).

4. Discussion

We previously found that CDP injected systemically via i.p. injections elevates maternal aggression and decreases Fos counts in LS and cPAG, suggesting these brain regions may be critical neural sites for GABA_A regulation of maternal defense in mice [39]. Other studies have suggested that cPAG is associated with maternal defense [51;52] and the purpose of this study was to investigate whether the cPAG is a crucial brain region for GABA_A modulation of maternal aggression in mice. We found that 8 ng and 9 ng of bicuculline, the GABA_A receptor antagonist, significantly lowered the total time spent being aggressive towards an intruder while similar injections into the Aq had no effect on aggression. Because the doses of bicuculline that impaired aggression had no effect on active motor behaviors (line crossing in light/dark box, pup retrieval, and pup grooming), it is suggested that these doses did not have any non-specific motor effects. These findings highlight a role for GABA_A receptor neurotransmission in cPAG in the regulation of maternal aggression. Altogether, our results suggest that some level of GABA_A receptor activity in cPAG is required for optimal expression of offspring protection.

One previous study found that bicuculline injections in cPAG did not alter maternal defense [72], but as noted above, the focus of that study was lordosis and kyphosis in rats and baseline maternal aggression levels were suppressed due to pup removal manipulations. Thus, significant effects could have been missed due to a floor effect. The present study was the first to date to examine the effects of GABA_A receptor manipulations in cPAG on maternal aggression in animals showing full levels of the behavior.

Although a wide range of CDP doses were applied (2.5–20 µg), CDP did not affect any measures of defense. One possible explanation for a lack of BDZ action is a ceiling effect, whereby a baseline of GABA_A receptor signaling exists in cPAG that promotes aggression and further increases in signaling do not alter the behavior. We used unilateral injections in this study to minimize tissue damage in cPAG and because in previous work we were able to modulate maternal aggression with unilateral injections [11;40]. However, we cannot exclude the possibility that if we had used bilateral injections, then an effect would have been found.

Past literature found that CDP has pro-aggressive effects when there are low baseline levels of maternal aggression [59;63] and if we had lowered our baseline aggression by using a different opponent or testing situation, then it is possible that we might have seen an effect with our doses of CDP. Although we cannot rule out that using this particular strain of mice limited the effects of CDP, in two recent studies we found treatments that enhance aggression in this line [22] (M.A. Scotti and S.C. Gammie, unpublished observations), suggesting this line is appropriate for testing modulators that may increase or decrease aggression. Our rationale for using this line of mice is that they provide a consistent baseline of maternal aggression, unlike the outbred mice. Further, we feel that results from the selected mice can be generalized to other non-selected females because they were derived from outbred mice and our selection approach minimizes inbreeding, so there should be high genetic similarity to outbred hsd:ICR mice. However, as for any work in a given mouse strain, it is useful to be cautious in assuming our results will necessarily generalize to all other strains.

Another explanation for a lack of CDP actions is that a different portion of PAG is involved in promoting maternal aggression. In males, defense behavior is suggested to be associated with dorsal PAG [9;74] and the GABA_A receptor may be involved in regulating defensive behavior in this region [74]. In the present study, all intra-cPAG CDP injections were infused in the lateral region, so it is possible that injections into other cPAG region would have amplified maternal aggression. Finally, it is possible that for benzodiazepines to elevate aggression that multiple regions must be affected at the same time. In our previous study, we found that bicuculline impaired aggression when injected into LS while CDP was without effect [40]; findings similar to those found in this study. We observed that intracerebroventricular (ICV) (lateral ventricle) injections of CDP (5 µg/1µl) had a trend towards enhancing maternal aggression ($p=0.054$) (Unpublished observations). Here, Aq-CDP injections did not promote maternal aggression and possible explanations are that levels of CDP in the Aq were too low to affect aggression and that flow of CSF from anterior to posterior regions would limit the action and movement of CDP rostrally. Thus, it is possible that for CDP to promote aggression that it would need to act in LS, cPAG, or possibly other regions at the same time to elicit an effect. This possibility has yet to be tested.

cPAG is tonically inhibited by GABA neurotransmission [3;4]. For example, in vitro and in vivo experiments have demonstrated that baseline firing of neurons in cPAG increased when bicuculline was administered into the cPAG with the medial and medioventral part of the cPAG showing the strongest response to GABA manipulations [4]. One possible mechanism of bicuculline decreasing maternal defense is that inhibition of the GABA_A receptor is altering the excitability of neurons in cPAG that project elsewhere. The cPAG has several different projection targets, including cortical regions [1;10;17;37], nucleus accumbens [18;44;45], ventral tegmental area [7], substantia nigra [7], locus coeruleus [8], medulla [35;54;56;62;69], pons [8;54;62], and various regions in the spinal cord (see review, [82]). Some of the brain regions receiving input from cPAG, including the hypothalamus [7;18;53], amygdala [18;44;64;70], medial preoptic area [7;18;71], bed nucleus of the stria terminalis [7;18], and LS [18;75], have previously been linked to maternal aggression [11;13;25;40;49].

The identity of the neurons in cPAG responding to our GABA_A receptor treatment is not known. The cPAG contains GABAergic neurons [2;4;65;68;73;83], and it has been suggested that GABA-positive interneurons within PAG themselves contain GABA_A receptors [4]. Some GABA-positive neurons within cPAG project to rostral ventromedial medulla [56] and medullary nucleus raphe magnus [69], but it is unknown whether these regions could play a role in regulating maternal aggression.

In terms of light-dark box, intra-PAG injections of the low dose bicuculline (3 ng and 7 ng) significantly decreased the number of transitions between the light and dark compartments. This result suggests a change in exploratory behavior, but not necessarily in anxiety-like behavior [5]. One study discovered that prepartum lesions in lateral and ventrolateral cPAG decreased anxiety-like behaviors when a postpartum female is exposed to elevated plus maze [50], suggesting that cPAG plays a role in anxiety-like behavior regulation in lactating females. However, our results may indicate that GABA_A modulation in the cPAG of postpartum mice may not play as great of a role in anxiety, at least as evaluated by this one measure.

The cPAG has been linked to regulating maternal behaviors, and we wanted to evaluate whether any of our treatments could alter behaviors other than aggression. 7 ng of bicuculline infused in cPAG increased the frequency of licking/grooming of the pups, and this study is the first study to show that intra-cPAG bicuculline increased this measure.

Other studies that have manipulated the cPAG regions have shown no changes in licking/grooming behavior of postpartum females [50;51;72]. In a recent study, we found that ICV injections of bicuculline (7 ng) increased the frequency of licking/grooming of pups [40]8. Based on the present results, it is possible that ICV bicuculline elevated maternal licking of pups due to action within cPAG.

The cPAG may regulate the final motor output in the maternal aggression neural pathway [21]. In the present study, we demonstrated that bicuculline infused into the cPAG dose-dependently decreased offspring protection (8 and 9 ng of bicuculline), increased the frequency of maternal licking (7 ng of bicuculline), and reduced exploratory behavior in the light-dark box paradigm (3 and 7 ng of bicuculline). These results suggest our narrow dose range is capturing a dose-response curve. Given the previous work and this study showing that removing GABA_A - receptor inhibition in cPAG (via bicuculline) promotes some maternal behaviors while dose-dependently impairing maternal aggression, it is possible that the cPAG represents a key site for decision making (aggression versus other behaviors) in the postpartum female.

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Fig. 1. Cannulae placement for all treatment groups. Grey dots represent the location of the tips of cannula injections in cPAG. See Methods section for information on likely area of diffusion for each injection. White dot represents Aq injections. (A) Cannula placement for bicuculline treatments. Aq injections = 19. (B) Cannula placement for CDP treatments. Aq injections = 7. The two coronal brain sections represent Bregma -4.60 mm and -4.72 mm. Each injection was within 0.12 mm of the shown section. (C) Representative photomicrograph of cPAG tissue following cannula implantation and injections. Arrows surrounding darker staining in cPAG indicates Chicago sky blue dye and asterisk indicates approximate location of the tip of the cannula. Diagrams are adapted from *The Mouse Brain in Stereotaxic Coordinates* (2nd. Ed), G. Paxinos and K.B.J. Franklin, 2001. Aq = aqueduct, DMPAG = dorsomedial periaqueductal gray, DLPAG = dorsolateral periaqueductal gray,

LPAG = lateral periaqueductal gray, VLPAG = ventrolateral periaqueductal gray, DRD = dorsal raphe nucleus, dorsal part, RC = raphe cap.

Fig. 2. Effects of cPAG-bicuculline injection on maternal aggression. (A) 8 ng and 9 ng of bicuculline significantly lowered the mean total time spent being aggressive towards an intruder relative to vehicle. (B) Only 8 ng of bicuculline significantly decreased the mean of number attacks relative to vehicle. (C) No treatments of bicuculline affected the mean latency to first bite. Bars represent means + SEM between treatments. * = $p \leq 0.05$; ** = $p \leq 0.01$.

Fig. 3. Effects of cPAG- bicuculline injection on light-dark box and maternal behaviors. (A) 3 ng and 7 ng of bicuculline significantly decreased the number of transitions between the light and dark compartments. (B) 7 ng of bicuculline significantly increased the frequency the female licked/groomed her pups relative to vehicle and 3 ng of bicuculline. Bars represent means + SEM between treatments. * = $p \leq 0.05$; ** = $p \leq 0.01$.

Effects of cPAG-CDP injection on maternal defense. No significant effects were found for any treatments on any measures.

Table 1

	vehicle	2.5 µg	5 µg	vehicle	10 µg	20 µg
AGG	35.0±4.7	42.5±6.1	39.2±5.6	44.8±14.7	28.0±3.5	24.3±7.7
ATT	21.2±2.0	23.4±2.5	23.6±3.0	22.3±7.3	16.8±2.1	18.0±4.9
FA Latency	5.0±1.4	7.9±2.6	4.3±0.7	5.0±1.1	7.5±3.9	7.5±2.1

Mean (±SEM) of raw data in seconds.

AGG = total time of aggression, ATT = number of attacks, FA latency = latency to first attack.

Table 2

Effects of treatment (cPAG injection) on light-dark box test. No significant effects were found in any treatments on any measure except for number of transitions.

Bicuculline		3 ng	7 ng	8 ng	9 ng
	vehicle		vehicle		
D Latency	0.1±0.1	0.0±0.0	6.1±6.1	0.4±0.4	50.8±49.8
Light	108.5±31.4	83.9±33.0	68.9±26.6	116.4±29.4	81.7±46.6
Dark	180.5±29.6	165.7±40.0	210.8±34.1	164.6±25.9	197.0±43.4
Transition	5.6±1.4	3.1±0.8	2.4±0.8	6.6±1.7	3.5±1.2
CDP		2.5 µg	5 µg	10 µg	20 µg
	vehicle		vehicle		
D Latency	12.3±8.3	23.6±21.2	10.5±9.5	0.0±0.0	75.0±75.0
Light	95.3±22.0	84.2±19.2	73.0±12.8	19.5±8.2	107.0±67.1
Dark	191.0±21.6	204.1±18.2	215.0±11.5	272.8±5.5	185.8±67.9
Transitio	7.2±1.4	7.3±0.9	7.3±0.8	2.0±0.8	4.0±1.1

Mean (± SEM) of raw data in seconds.

D latency = latency to enter dark compartment, Light = total time spent in light compartment, Dark = total time spent in dark compartment, Transition = number of transitions between compartments. Bold = p < 0.05 relative to vehicle.

Table 3

Effects of treatment (cPAG injection) on maternal behavior. No significant effects were found in any treatment on any measure except for licking/grooming of pups.

Bicuculline		vehicle	3 ng	7 ng	vehicle	8 ng	9 ng
PRI latency		53±20	38±15	45±18	69±18	61±13	74±19
PR4 latency		90±15	89±16	95±14	97±12	100±12	107±11
NUR latency		296±81	540±229	274±118	426±124	468±129	416±206
LG latency		1046±265	1191±242	724±228	1800±0	1641±152	1800±0
NB latency		1800±0	1800±0	1680±120	1623±177	1800±0	1800±0
NUR		0.55±0.09	0.63±0.12	0.74±0.06	0.68±0.07	0.67±0.07	0.45±0.12
LG		0.02±0.01	0.02±0.01	0.05±0.01*	0.00±0.00	0.00±0.00	0.00±0.00
NB		0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
SG		0.10±0.04	0.14±0.04	0.10±0.03	0.06±0.02	0.04±0.01	0.05±0.01
OFF		0.26±0.05	0.21±0.09	0.16±0.04	0.27±0.06	0.28±0.06	0.50±0.12
ON		0.10±0.04	0.04±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.02±0.01
CDP		vehicle	2.5 µg	5 µg	vehicle	10 µg	20 µg
PRI latency		61±18	39±18	41±18	37±28	74±27	41±28
PR4 latency		84±15	73±14	69±16	72±28	86±20	74±27
NUR latency		754±164	758±219	613±139	570±175	563±259	375±166
LG latency		1620±136	1395±265	1256±250	893±351	1283±390	803±336
NB latency		1433±241	1744±56	1590±182	1800±0	1800±0	1800±0
NUR		0.49±0.09	0.37±0.09	0.43±0.07	0.43±0.03	0.34±0.07	0.64±0.09
LG		0.01±0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.05±0.04	0.02±0.01
NB		0.01±0.01	0.01±0.01	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00
SG		0.10±0.04	0.13±0.03	0.13±0.02	0.12±0.05	0.16±0.05	0.11±0.06
OFF		0.32±0.08	0.45±0.08	0.40±0.06	0.29±0.05	0.40±0.10	0.22±0.05
ON		0.10±0.03	0.06±0.02	0.06±0.03	0.16±0.06	0.10±0.01	0.04±0.01

Latency of maternal behavior is expressed by the mean (±SEM) of raw data in seconds.

Maternal behaviors are expressed by the mean ($\pm$ SEM) of raw data in rates. PR1 = retrieval of first pup, PR4 = retrieval of fourth pup, NUR = nursing, LG = licking/grooming of pups, NB = nest building, SG = self grooming, OFF = off nest, ON = on nest.

* $p < 0.01$ relative to 3ng and vehicle.