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GABA enhancement of maternal defense in mice: Possible neural correlates

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Abstract

Previous studies have shown that low doses of $GABA_A$ receptor agonists facilitate maternal defense of offspring (maternal aggression), without significantly affecting other maternal behaviors. In addition, it has been demonstrated that endogenous changes in GABAergic neurotransmission occur in association with lactation. This study investigated the effects of $GABA_A$ receptor agonist, chlordiazepoxide (CDP), a benzodiazepine (BDZ), on maternal behaviors including aggression, and identified brain regions with altered activity in association with treatment. Another aim of the study was to determine whether CDP injections could prevent decreases in maternal aggression that occur with pup separation. Intraperitoneal injections of 1 mg/kg of CDP significantly increased maternal defense without affecting other maternal behaviors, although a trend towards elevated nursing was noted. CDP significantly reduced c-Fos in lateral septum (LS) and caudal periaqueductal gray (cPAG) in behaviorally-experienced mice relative to vehicle-injected controls. In behaviorally-naïve subjects, CDP also decreased c-Fos in LS, but in cPAG this decrease was just above significance ($p=0.051$). CDP was not sufficient to "rescue" maternal aggression when pup stimulus was removed. Overall, these studies provide further insights into the role for GABA in maternal behaviors, including aggression, and how and where BDZs may act to modulate behavior.

Keywords: Maternal aggression; Nursing; Benzodiazepine; Chlordiazepoxide; GABA; Lateral septum; Caudal periaqueductal gray

1. Introduction

Lactation is associated with a suite of behavioral and physiological alterations in female mammals. For instance, in addition to producing milk and nursing, a lactating female displays a range of maternal behaviors, including defense of offspring, found in low or no levels in non-lactating females. Changes in gene expression and neurotransmission within the CNS play an important role in supporting the physiology and behavior of lactation (Numan and Insel, 2003).

Previous studies have identified altered neurotransmission of gamma-aminobutyric acid (GABA), the principle inhibitory neurotransmitter in the central nervous system (CNS), as supporting maternal physiology and behavior. For example, in the supraoptic nucleus (SON), there is increased clustering of $GABA_A$ receptors (Koksma et al., 2005), increased GABAergic synapses (El Majdoubi et al., 1997; Gies and Theodosis, 1994; Kornblatt and Grattan, 2001; Theodosis et al., 1995; Theodosis and Poulain, 2001) and increased GABA release onto oxytocin neurons (de Kock et al., 2003) in lactating versus non-lactating females. Additional brain regions, including the medial preoptic area (MPA) (Kendrick et al., 1992), the bed nucleus of the accessory olfactory tract (BAOT) (Rodriguez et al., 2004), the cingulated cortex (Cg) (Kornblatt and Grattan, 2001), and other hypothalamic areas (Kornblatt and Grattan, 2001), also exhibit altered GABAergic transmission with lactation. In addition, the BAOT has increased levels of glutamate decarboxylase (GAD), an enzyme that synthesizes GABA, in postpartum rats (Rodriguez et al., 2004). Thus, natural changes of GABAergic transmission in particular neural regions are strongly correlated with a female converting from a non-lactating to lactating state.

In addition to observations of endogenous changes of GABA, altering GABAergic signaling using GABA agonists, including benzodiazepines (BDZs), modulates maternal

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behaviors and maternal aggression (defense of offspring). Previous studies showed that BDZ agonists (which increase the binding affinity of GABA onto $GABA_A$ receptors) alter maternal aggression in rats (Ferreira et al., 2000; Mos and Olivier, 1989; Olivier et al., 1985) and mice (Palanza et al., 1996; Yoshimura and Ogawa, 1989, 1991) with low doses increasing and higher doses inhibiting aggression. Where and how BDZs act centrally to modify maternal defense, though, is not known.

In some studies, systemic injections of BDZ agonists have been shown to impair pup retrieval and nest building, but insignificantly alter other maternal traits in rats (D'Amato et al., 1997; Ferreira et al., 2000). Furthermore, site-directed injections into the CNS of muscimol (a $GABA_A$ receptor agonist) reduced active maternal traits like pup retrieval (Arrati et al., 2006), nest building (Arrati et al., 2006; Ferreira et al., 2000; Numan et al., 2005), and high arched-back nursing (Arrati et al., 2006; Numan et al., 2005; Salzberg et al., 2002), as well as passive maternal behaviors, including low arched-back nursing, and licking and grooming (Arrati et al., 2006). Conversely, postpartum ewes injected with a BDZ agonist were more willing to accept alien offspring and allowed suckling compared to those injected with saline (Ferreira et al., 1992). Thus, GABA can impair or enhance aspects of maternal care, but its actions are likely complex and site-specific.

Although there is a rich history of research about neural alterations of the GABAergic system associated with lactation, to date no study has addressed where in the CNS specific BDZ treatments are acting to alter maternal aggression and behavior. One aim of this study was to examine the GABAergic modulation of maternal aggression and other maternal behaviors using the BDZ agonist, chlordiazepoxide (CDP), and then to link changes in brain activity (using c-Fos as an indirect marker) with behavioral alterations to gain insights into how CDP regulates maternal behaviors. We hypothesized that intraperitoneal (i.p.) injections of CDP will augment maternal aggression at a low dose and that CDP-induced changes in brain activity would identify critical nodes of the maternal aggression circuit on which CDP acts to modify behavior. We also hypothesized that CDP treatment would modestly impair other maternal behaviors. CDP was chosen because it has previously been shown to enhance maternal aggression in mice and rats. The effect of CDP on other maternal behaviors, except latency to retrieve pups in association with a predatory odor (Mandillo and D'Amato, 1999), had previously not been examined.

Another aim of these studies was to examine whether CDP could prevent decreases in maternal aggression that occur with separation of pups (Gandelman, 1972; Gandelman and Simon, 1980; Stern and Kolunie, 1993; Svare et al., 1981). Because increases in GABAergic neurotransmission is associated with sensory input from offspring (Qureshi et al., 1987), we hypothesized that CDP treatment would prevent decreases in maternal aggression due to separation from pups. To date, no study has examined whether BDZs can prevent decreases in maternal aggression due to pup separation. Together, these experiments provide further insights into the role of the GABAergic system in modulating maternal aggression and other maternal behaviors, and provide information on how and where BDZs may act to modulate behavior.

2. Materials and methods

2.1. Mice

Outbred hsd:ICR (Harlan, Madison, WI), sexually naïve mice were used. Females, 7 weeks old, were housed singly in polypropene cages with access to tap water and breeder chow ad libitum. Intruder males were grouped housed. Intruder males (~ 2 months old) were given ad lib access to regular chow. Intruder males were never used more than once per day and used for ~ 3 tests each. Cages were changed weekly prior to parturition and after which they were unchanged for the remainder of the experiments. All animals were housed on a 14:10 light/dark cycle with lights on at 06:00 CST.

A female was paired with a breeder male and after impregnation (~ 14 days), the male was removed and precut nesting material was added into the female's cage. The day after birth (postpartum day 1), litters were culled to ten pups. 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. Pharmacological treatment and injection

Females were divided into two treatment groups ($n=9$ per group): one receiving vehicle (0.9% saline) and the other receiving chlordiazepoxide hydrochloride (CDP) (Sigma Chemical, St. Louis, MO) dissolved in saline (1 mg/kg). Dose was based on previous literature (D'Amato et al., 1997; Mos and Olivier, 1989; Olivier et al., 1985; Palanza et al., 1996; Yoshimura and Ogawa, 1989). All injections were interperitoneal (i.p.). Before each injection (sham and treatment), each mouse was lightly anesthetized under isoflurane to minimize the stress effects of injection. Previous work had found that acute i.p. injections can impair maternal aggression in mice (S.C. Gammie, unpublished observations). However, light isoflurane in association with injection (either i.c.v. or i.p.) 30 min prior to testing does not impair production of maternal aggression or other maternal behaviors (D'Anna et al., 2005; D'Anna and Gammie, 2006; Gammie et al., 2004). Immediately after injection, each mouse was placed back in the homeroom for 30 min prior to behavioral testing. To establish baseline levels of behavior, mice were anesthetized lightly with isoflurane and given a sham injection (inserting a needle attached to an empty syringe into the dam's body) on postpartum day (PPD) 4 and then tested. Treatments for the two groups were conducted on PPDs 5 and 7.

2.3. Maternal behavior testing

Behavioral testing occurred between 0800 and 1700 h on PPD 4, 5, and 7. 30 min following injection, females were moved into the testing room, pups were separated from the dam,

and a male intruder was placed into the female's cage for 5 min. CDP and vehicle mice were always alternately tested on the same day such that the intruder mice from the same cage were used equally to test both groups. Thus, any previous fighting experience of intruders that may have affected outcome was evenly divided among the two groups. Removal of pups from the home cage of a dam before an aggression test does not diminish the expression of maternal aggression in mice (Svare et al., 1981). After the intruder male was removed, the pups were scattered evenly away from the nest allowing the female to retrieve pups and perform maternal behaviors for 55 min. Total behavioral testing lasted 1 h. Each test session was recorded on videotape and subsequently analyzed off-line to quantify maternal behaviors by individuals blind to testing conditions. For quantification of maternal aggression the following features were measured: latency to first attack, number of attacks, and total duration of attacks (Gammie and Nelson, 1999; Gammie et al., 2000). Pup retrieval was quantified by measuring the time elapsed to retrieval of the first and fourth pup. Other maternal behaviors were surveyed every 30 s and quantified, which included nursing (including all forms, such as high and low arched-back nursing); licking and grooming of pups by the female; nest building activity; and time on and off nest.

2.4. Immunohistochemistry for c-Fos in behaviorally-experienced mice

Mice examined above were injected on PPD 9 with either vehicle or CDP according to their assigned treatment, reunited immediately after injection with their pups, and returned to the homeroom. 120 min later (±5 min), mice were anesthetized, decapitated, and their brains removed. Brains were postfixed overnight in 6% acrolein in phosphate buffered saline (PBS) and cryoprotected in 30% sucrose in PBS for 2 days. Brains were frozen on a platform and cut into 40 μm thick sections using a sliding microtome (Leica, Microsystems, Heidelberg, Germany) and stored in a cryoprotectant solution at −20 °C until processing for immunohistochemistry. Sections were washed in PBS in the presence of 0.2% Triton-X-100 (PBS-X), blocked in 5% normal goat serum for 1 h, and incubated for 2 days at 4 °C with rabbit anti-c-Fos antibodies (1:15,000; Oncogene Research Products, San Diego, CA). After washes in PBS-X, the sections were incubated for 90 min at room temperature in biotinylated goat anti-rabbit secondary antibodies (1:500, Vector Laboratories, Burglingame, CA), washed in PBS-X, exposed to an avidin–biotin complex (Vector) for 1 h, washed again in PBS-X, and stained using diaminobenzidine (Sigma) as a chromagen, enhanced with 0.008% nickel chloride. The sections were then mounted, dehydrated in a series of ethyl alcohols and xylenes, and cover slipped.

2.5. Immunohistochemistry for c-Fos in behaviorally-naive mice

Because mice examined above for c-Fos had previously been exposed to testing and injection, it was possible that previous experience altered how CDP affected CNS activity. To gain additional insights into the effects of CDP on c-Fos activity, we examined the effects of CDP injection on c-Fos in behaviorally-naïve lactating mice. All mice were bred and housed as described above and were caring for their first litter. Brains were collected on postpartum Day 5 because this was the day on which CDP significantly alters aggression (see below). For this study, 3 groups were used: one received no handling or injection ($n=9$); one received i.p. saline under light isoflurane anesthesia 2 h before brain collection ($n=9$); and one received i.p. CDP (1 mg/kg) ($n=9$) under light isoflurane anesthesia 2 h before brain collection.

2.6. Analysis of c-Fos immunoreactivity

Bright field microscopy was used for counting c-Fos-positive cells. The images of brain sections were projected from an Axioskop Zeiss light microscope (Zeiss, Gottingen, Germany) through an Axiocam Zeiss high-resolution digital camera attached to the microscope and interfaced with a computer. Counting was based on a previously used paradigm (Gammie and Nelson, 2001; Gammie et al., 2004). Using boxes, cells in a specific region on a specific section were automatically counted. For details on approximate sizes and locations of boxes used in this study, see (D'Anna et al., 2005; Hasen and Gammie, 2005). One section per brain region was used to quantify c-Fos-IR in each animal to maximize consistency for a given region. Averaging of counts from multiple sections could have been used, but was avoided because alternate sections were used (each section was 40 μm thick) and in mice some brain regions cannot be sampled multiple times with these limits. To ensure c-Fos-IR was measured consistently between samples; 1) all sections were exposed to diaminobenzidine for 10 min, 2) the backgrounds were normalized by adjusting light levels, 3) a threshold of staining levels was used to automatically distinguish between c-Fos-positive cells, 4) all slides were coded and the counting for each specific brain region was performed by one individual, blind to the experimental conditions, 5) only c-Fos-positive nuclei within a specified size range were counted, and 6) all sections for a given study were run in one batch.

2.7. Statistical analysis

To incorporate individual variability into the statistical model, effect of treatment on behaviors was analyzed by first establishing differences or proportion change from baseline for each mouse with each treatment. A one-way analysis of variance (ANOVA) on difference scores was then performed between the vehicle and CDP groups. This approach is modeled after standard statistical method used in other behavioral paradigms (Toufexis et al., 2004; Walker et al., 2003). In cases were data were not normally distributed, a non-parametric Kruskal–Wallis One-Way ANOVA on Ranks was used. Results presented are based on difference change analysis. In all cases, the statistical results using proportion change mirrored those found with difference score. Due to poor taping quality, the results from a small number of mice could not be evaluated. The

final numbers for behavioral analysis were: PPD 4: vehicle, $n=8$; CDP, $n=9$; PPD 5: vehicle, $n=8$; CDP, $n=8$; PPD 7: vehicle, $n=9$; CDP, $n=9$; PPD 7: vehicle, $n=9$; CDP, $n=9$. Additionally, to examine a possible interaction of drug by time, a two-way ANOVA and repeated measures ANOVA was run on data from PPDs 5 and 7 using treatment (e.g. CDP or vehicle) and time (either PPD 5 or 7) as the two variables.

Brain regions associated with BDZ action were examined in this study. Prior to decoding three brains were eliminated from the study in behaviorally-experienced mice due to poor histological quality. The final numbers used for behaviorally-experienced immunohistochemical analysis per group were: vehicle, $n=7$; CDP, $n=8$. The final numbers used for behaviorally-naïve immunohistochemical analysis per group were: unhandled, $n=9$; vehicle, $n=9$; CDP, $n=9$. c-Fos was analyzed using a one-way ANOVA to identify differences in c-Fos activity separately for behaviorally-naïve and behaviorally-experienced groups. If data were not normally distributed, then non-parametric Kruskal–Wallis One-Way ANOVA on Ranks was used.

2.8. Effects of CDP on maternal aggression in dams without pup stimulation

To determine whether CDP could elevate or prevent the normal drop in maternal aggression that occurs with pup removal for 24 h, two groups were used: one receiving vehicle and the other receiving CDP as above.

2.8.1. Mice

Females with one previous maternal experience were used. Dams with second litters show aggression that is either consistent or elevated from the levels during the first litter (Svare and Gandelman, 1976).

2.8.2. Treatment injection and maternal aggression test

Behavioral tests were performed between 0800 and 1000 on PPDs 4, 5, and 7. On PPD 4, mice received a sham injection and were given a 3 min aggression test followed by a 2 min pup retrieval test. Once testing was completed, pups were immediately removed from the dam and cross-fostered with surrogate (non-test) dams for 24 h. Pups need to be separated from the mother for at least 5 h in order to reduce maternal aggression, but aggression can be restored within minutes of reunion (Svare and Gandelman, 1973). On PPD5 (1 day after pup separation), each mouse was given a 3 min aggression test (without pup retrieval), following sham injection, to establish baseline aggression in the absence of pups. Immediately after the testing, each dam was injected with either vehicle or CDP, replaced in the homeroom (still without pups), and then tested for aggression after 30 min. Immediately following this test, 6 pups were returned to each dam and they were allowed to interact for 30 min prior to a final 3 min aggression test (followed by retrieval testing). One hour before pups were returned to focal dams they were removed from the surrogate dams and placed underneath a heating lamp to ensure suckling by the pups when they were returned to the focal dam.

2.8.3. Statistical analysis

Statistics were performed as described above, where difference scores from baseline were first established and then one-way ANOVAs between the two groups were run.

3. Results

3.1. Effects of CDP on dam and pup weight

Neither dam weight [H (1,16)=0.216, $p=0.642$] (ANOVA on Ranks) nor pup weight [F (1,16)=2.537, $p=0.131$] (one-

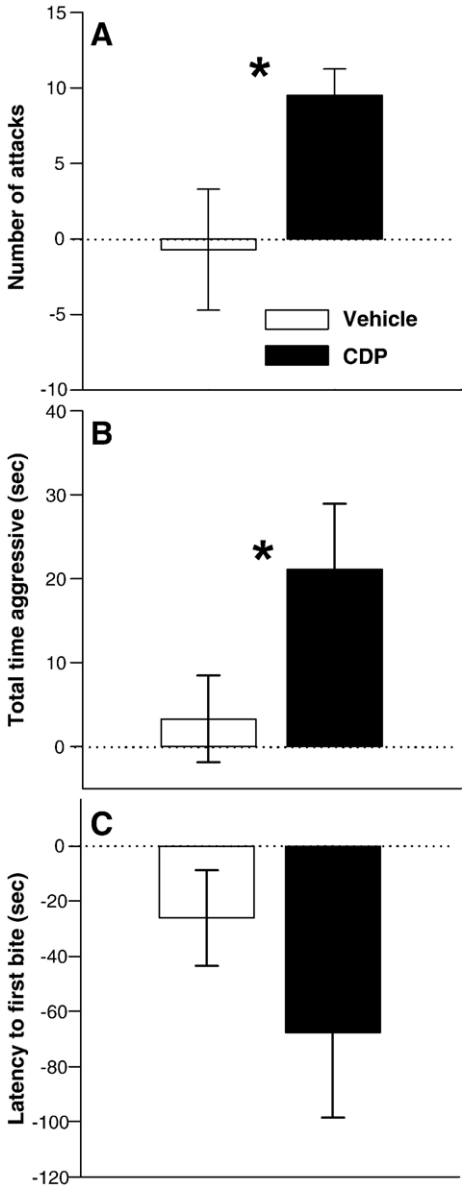

Fig. 1. Effects of CDP on maternal aggression. Bars represent mean differences ± SEM between treatment (PPD 5) and baseline (PPD 4). A: There was a significant increase of the number of attacks in CDP-treated dams. B: Total time being aggressive significantly increased in CDP-treated dams relative to vehicle-treated dams. C: No significant difference occurred between vehicle and CDP in terms of latency to first bite. Asterisks indicate significant difference between groups ($p<0.05$).

Table 1
Effects of CDP (1 mg/kg) on maternal aggression

	Vehicle			CDP		
	Baseline	PPD 5	PPD 7	Baseline	PPD 5	PPD 7
FB latency	53.88±35.5	27.88±21.8	37.33±32.9	77.22±33.6	14.63±9.8	12.78±3.6
ATT	12.25±3.8	13.00±2.2	11.78±2.6	9.89±2.5	**18.36±3.3**	14.11±2.6
AGG	15.63±4.4	20.50±4.3	18.44±5.4	12.11±3.3	**32.38±9.6**	24.22±5.8

Mean (±SEM) of raw data.
Bold indicate $p<0.05$ relative to baseline.
FB = first bite, ATT = number of attacks, AGG = total time aggression.

way ANOVA) showed a response to treatment when examined on PPD 7.

3.2. Effects of CDP on maternal aggression

When aggression was examined during the baseline period (PPD 4), no differences in latency to first bite [$H=0.671$, $p=0.413$], number of attacks [F (1,15)=0.282, $p=0.603$] and total time aggression [F (1,15)=0.423, $p=0.525$] were found between vehicle and CDP mice. As expected, CDP (1 mg/kg) augmented maternal aggression when compared to vehicle. CDP triggered significant increases in both number of attacks [F (1,13)=5.989, $p=0.029$] (Fig. 1A) and total time aggressive [$H=5.102$, $p=0.021$] (Fig. 1B) when examined on PPD 5, but not on PPD 7. There was a trend toward decreased latency to first attack, but these differences did not reach significance on PPD 5 ($H=1.211$, $p=0.281$) (Fig. 1C). The mean levels of aggression for baseline (PPD 4), PPD 5, and PPD 7 are shown in Table 1.

Two-way ANOVA revealed no significant interaction between day (PPD 5 and PPD 7) and treatment (CDP and vehicle) in terms of latency to first bite [F (1,30)=0.0734, $p=0.788$], number of attacks [F (1,30=0.316), $p=0.578$], or total time aggressive [F (1,30)=0.219, $p=0.643$].

3.3. Effects of CDP on maternal behaviors

No effect of treatment was found for any of the maternal behaviors observed on PPD 5 or 7 (Table 2). However, when proportion change (rather than difference scores) was used, there was a trend towards increased nursing in CDP relative to vehicle-injected dams on PPD 5 [F (1,13)=3.338, $p=0.091$]. Moreover, 88% of CDP-injected dams displayed an increase in nursing, whereas 43% of the saline dams exhibited an increase in nursing on PPD 5.

Two-way ANOVA analysis revealed number of bouts on the nest significantly decreased from PPD 5 to PPD 7 [F (1,30), $p=0.011$] and latency to nest building significantly increased from PPD 5 to PPD 7 [F (1,30), $p=0.045$]. No significant interactions between day and treatment were found. However, a trend towards an interaction was found for pup retrieval whereby CDP-treated females tended to retrieve the fourth pup faster and vehicle-treated females retrieved pups more slowly on PPD 7 relative to PPD 5 [F (1,30)=3.016, $p=0.093$].

Repeated measure ANOVA analysis revealed similar results as two-way ANOVA. For example, number of bouts on the nest significantly decreased from PPD 5 to PPD 7 [F (1,14), $p=0.010$], latency to nest building significantly increased from PPD 5 to PPD 7 [F (1,14), $p=0.012$], and a trend towards an interaction between day and treatment was found for pup retrieval whereby CDP-treated females tended to retrieve the fourth pup faster and vehicle-treated females retrieved pups more slowly on PPD 7 relative to PPD 5 [F (1,14)=3.442, $p=0.085$]. In addition, latency of retrieving the first pup significantly increased from PPD 5 to PPD 7 [F (1,140), $p=0.022$], self-grooming significantly decreased from PPD 5 to PPD 7 [F (1,14), $p=0.034$], and number of bouts of nest

Table 2
Effects of CDP (1 mg/kg) on maternal behaviors

	Vehicle			CDP		
	Baseline	PPD 5	PPD 7	Baseline	PPD 5	PPD 7
PR1 latency	80.5±19.2	57.1±18.0	69.9±19.8	53.9±17.3	41.9±17.2	84.7±17.7
PR4 latency	99.1±13.7	90.3±12.6	75.9±17.5	107.9±7.2	74.5±13.7	107.7±9.2
NUR latency	1106±190.5	1260±364.9	833±313.4	1466±405.7	795±136.7	858±321.6
Total NUR	59.6±5.3	59.6±11.7	69.9±9.8	43.3±9.6	69.3±4.4	70.8±9.4
LG latency	1083±307.1	1009±280.2	2173±410.7	1920±362.3	1680±464.2	2005±418.7
Total LG	8.0±2.1	7.1±1.5	3.7±1.8	3.6±1.8	3.3±1.3	2.8±1.1
NB latency	2213±516.7	1806±512.2	2483±440.8	2573±400.7	2168±536.1	3247±14.8
Total NB	1.3±1.8	2.1±1.2	0.9±0.5	0.6±0.3	0.9±0.3	0.0±0.0
On latency	428±102.0	420±174.8	600±343.6	487±206.9	158±50.6	287±135.7
Total on	24.3±4.2	17.3±5.4	5.3±1.4	18.1±2.9	10.7±1.4	7.4±1.4
Total off	11.1±2.2	20.4±8.9	8.0±4.8	27.7±9.0	19.7±4.8	16.9±9.6
Total SG	17.4±2.7	10.1±2.0	4.8±2.0	12.9±2.21	9.6±1.4	7.2±2.3

Mean (±SEM) of raw data.
PR1 = 1st pup retrieval, PR4 = 4th pup retrieval, NUR = nursing, LG = licking and grooming, NB = nest building, ON = on nest, OFF = off nest, SG = self grooming.

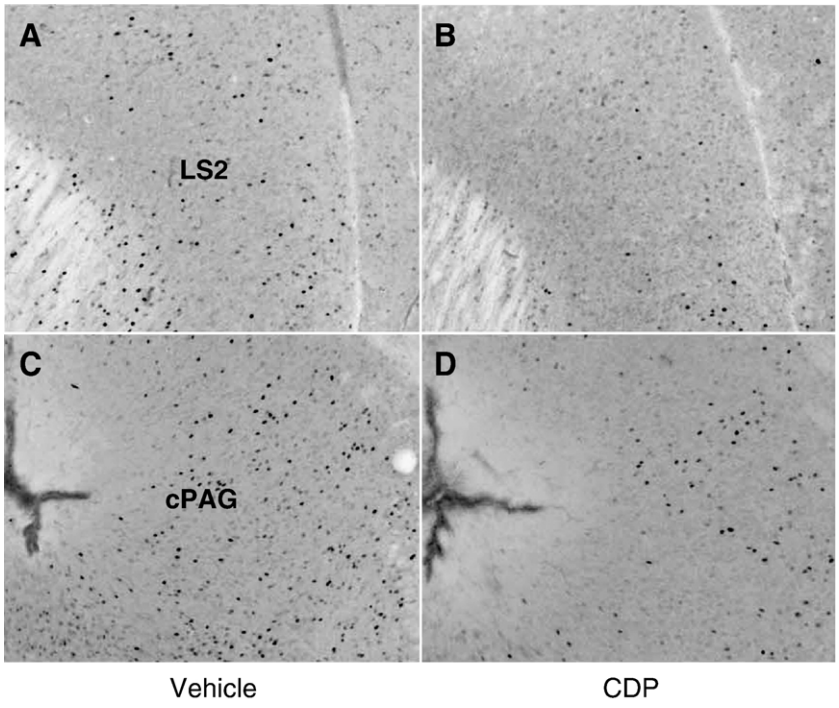

Fig. 2. Examples of c-Fos immunoreactivity in behaviorally-experienced mice. LS2 and cPAG 2 h after i.p. injection of vehicle (A, C) and CDP (B, D).

building significantly decreased from PPD 5 to PPD 7 [F (1,14), $p=0.021$].

3.4. Effects of CDP on c-Fos-IR in behaviorally-experienced mice

Injections of CDP (1 mg/kg) (in the absence of aggression testing on PPD 9) resulted in significant decreases in c-Fos counts (relative to injection of vehicle) in three brain regions, including lateral septum 2 (LS2) ($H=5.959$, $p=0.015$) (Fig. 2A, B), dentate gyrus (Dg) [F (1,13)=5.519, $p=0.035$], and periaqueductal grey, caudal (cPAG) [F (1,13)=6.267, $p=0.026$] (Fig. 2C, D) (Table 3). An additional portion of LS approached significance with treatment. c-Fos counts for additional regions examined (some of which approach significance) are shown in Table 3.

3.5. Effects of CDP on c-Fos-IR in behaviorally-naïve mice

Injections of CDP (1 mg/kg) on PPD 5 in behavioral-naïve mice resulted in significant decreases in c-Fos counts (relative to injection of vehicle) in four brain regions: medial preoptic nucleus (MPOM) [F (1,16)=6.483, $p=0.022$], lateral septum, ventral (LSv) [F (1,14)=9.512, $p=0.008$] (Fig. 3), anteroventral thalamic nucleus (AVA) [F (1,16)=4.886, $p=0.042$], and lateral hypothalamus (LH) [F (1, 16)=8.582, $p=0.010$] (Table 4). In addition, cPAG was close to significant decreases in c-Fos counts relative to vehicle [F (1,16)=4.465, $p=0.051$] (Fig. 3) (Table 4). Other brain regions that approached significance with CDP were LS2, lateral septum 3 (LS3), ventrolateral hypothalamic nucleus (VMH), and ventral tegmental area (VTA) (Table 4). In many cases, vehicle or CDP injection triggered significant increases in c-Fos relative to unhandled controls as shown in Table 4.

3.6. Maternal aggression with injection but without pup stimulation

As expected, 24 h without pup stimulation decreased maternal aggression from baseline equally for mice in both groups (with sham injections) (data not shown). 30 min following treatment, aggression remained low for both groups and no differences between groups were found in terms of either latency to first bite ($H=1.535$, $p=0.215$), number of attacks ($H=1.164$, $p=0.281$), or total time aggression ($H=1.054$, $p=0.305$) (Fig. 4). When pups were reunited with dams, aggression increased for both groups (Fig. 4) and again no differences between groups were found in terms of either latency to first bite [F (1, 15)=1.040, $p=0.324$], number of attacks [F (1,15)=1.490, $p=0.241$], or total time aggressive [F (1, 15)=1.195, $p=0.291$] (Fig. 4).

4. Discussion

In this study, we examined maternal behavior and brain responses to treatment with CDP in mice. We also tested whether CDP could elevate decreases in maternal aggression produced by the absence of pups. As expected, CDP significantly increased maternal defense. Identified changes in brain activity with CDP treatment (in behaviorally-experienced and -naïve subjects) in LS suggest this may be a critical site for CDP modulation of aggression. cPAG showed a significant response in behaviorally-experienced mice and this effect was just above significance in behaviorally-naïve mice ($p=0.051$), so it is also

Table 3
Effects of CDP (1 mg/kg) on c-Fos cell counts in behaviorally-experienced mice

Brain region	Vehicle	CDP	*p*-value
Cg1	34.9±6.7	34.5±11.2	0.979
NaCc	14.7±3.4	21.3±6.6	0.417
NaCs	12.4±2.8	18.5±5.8	0.387
LS	60.9±16.8	43.0±7.3	0.946
MS/VDB	7.6±2.5	8.1±3.4	0.955
Pir	60.7±16.2	62.1±12.9	0.946
M2	9.7±1.5	16.6±4.2	0.169
LS2	**10.9±3.3**	**3.4±1.3**	**0.014**
MPOM	57.1±13.9	45.4±8.6	0.471
MPA	36.9±7.8	32.6±5.3	0.653
LPO	13.0±3.2	12.3±2.9	0.864
BNSTd	20.7±5.6	16.9±6.9	0.189
BNSTv	8.9±2.0	8.1±3.0	0.397
LS3	24.4±4.3	15.3±5.4	0.072
LSv	61.1±13.5	35.4±9.0	0.127
Cg 1 & 2	50.4±11.0	53.8±25.1	0.463
PVA	279.4±46.3	241.0±31.1	0.493
PVN	40.5±10.9	69.6±22.2	0.282
AHA	15.0±3.7	10.6±1.8	0.284
AAV	14.7±6.5	11.8±2.4	0.660
SON	10.0±3.5	26.9±11.9	0.233
MeA	54.7±16.2	37.9±9.7	0.536
CeA	16.9±1.4	11.8±3.7	0.242
BLA	8.3±3.0	4.0±1.5	0.232
AHP	75.1±12.5	62.9±11.3	0.478
LH	45.3±10.1	35.1±9.6	0.481
VMH	15.1±4.8	9.1±2.4	0.265
Dg	**20.0±5.8**	**6.9±1.4**	**0.035**
Ca1	3.0±1.6	0.8±0.8	0.613
Ca2	6.0±0.6	6.4±1.9	0.862
Ca3	13.0±2.5	10.4±3.8	0.586
VTA	58.6±10.9	61.4±10.3	0.855
DRD	61.4±6.7	60.3±13.5	0.942
cPAG	**134.6±14.5**	**87.6±12.1**	**0.026**
LDTg	17.6±3.8	38.0±25.4	0.613
LC	68.7±8.6	65.9±23.5	0.916
PB	49.4±9.2	46.6±20.7	0.232

Mean(±SEM) of raw data.
Bold indicates $p<0.05$ between vehicle and CDP.
Cg1 = Cingulated cortex; NaCc = Nucleus accumbens, core; NaCs = Nucleus accumbens, shell; LS = Lateral septum; MS/VDB = Medial septal nucleus/ nucleus of vertical limb of diagonal band; Pir = Piriform cortex;
M2 = Motor cortex 2; MPOM = Medial preoptic nucleus, medial part; MPA = Medial preoptic area; LPO = Lateral preoptic area; BNSTd = Bed nucleus of stria terminalis, dorsal; BNSTv = Bed nucleus of stria terminalis, ventral; LS3 = Lateral septum 3; LSv = Lateral septum, ventral; Cg 1 & 2 = Cingulate cortex 1 and 2; PVA = Paraventricular thalamic nucleus, anterior; PVN = Paraventricular thalamic nucleus; AHA = Anterior hypothalamic area, anterior; AVA = Anteroventral thalamic nucleus; SON = Supraoptic nucleus; MeA = Medial amygdaloid nucleus, anterior part; CeA = Central amygdaloid nucleus; BLA = Basolateral amygdaloid nucleus, anterior part; AHP = Anterior hypothalamic area, posterior part; LH = Lateral hypothalamus; VMH = Ventrolateral hypothalamic nucleus; Dg = Dentate gyrus; Ca1 = Field CA1 of hippocampus; Ca2 = Field CA2 of hippocampus; Ca3 = Field CA3 of hippocampus; VTA = Ventral tegmental area; DRD = Dorsal raphe nucleus; cPAG = Periaqueductal grey, caudal; LDTg = Lateral dorsal tegemental nucleus; LC = Locus coeruleus; PB = Parabrachial nucleus.

possible this region contributes to CDP actions. The finding that CDP could not elevate maternal aggression in the absence of pups suggests that altered BDZ activity alone is not sufficient for production of maternal aggression.

The increase of maternal defense by CDP supports the involvement of the GABAergic system in maternal aggression. One striking detail was that for each dam injected with CDP (8/8), aggression was increased relative to baseline. Our findings are consistent with previous studies showing that lower doses of CDP, as well with other BDZs, such as diazepam and oxazepam, facilitate maternal aggression (Ferreira et al., 2000; Grimes et al., 2006; Mos and Olivier, 1989; Yoshimura and Ogawa, 1991). Although CDP could be elevating normal levels of maternal aggression, an alternative hypothesis is that experimental treatment (e.g., isoflurane, handling, injection) induce some impairment of maternal aggression and that CDP is mitigating that effect. CDP is an anti-anxiety drug, and could possibly reverse the stressor effects on maternal aggression. We previously have found that injections in the absence of light isoflurane impairs maternal aggression (S.C. Gammie, unpublished observations), but injections with light isoflurane could also have a minor negative impact. Previous studies have demonstrated that slight stimuli like injections (Asanuma et al., 1992; Asanuma and Ogawa, 1994; Morgan et al., 1987; Nakajima et al., 1989; Ryabinin et al., 1999; Sharp et al., 1991), anesthesia (Bullitt, 1990; Dragunow et al., 1990; Morgan et al., 1987), and handling (Asanuma et al., 1992; Asanuma and Ogawa, 1994; Ryabinin et al., 1999) can induce c-Fos mRNA or protein in the male rodent brain. The c-Fos-IR in behaviorally-naïve mice supports the idea that injection with isoflurane itself is a stressor. For 22 brain regions a significant increase in c-Fos was found in vehicle-injected versus unhandled controls (Table 4). Some of these regions showing heightened activity with injection, such as

Table 4
Effects of CDP (1 mg/kg) on c-Fos cell counts in behaviorally-naïve mice

Brain region	Unhandled	Vehicle	CDP
NaCc	11.63±2.90	35.56±7.81*	31.00±6.33*
NaCs	11.88±3.39	32.33±8.23*	34.00±10.50
LS	50.25±6.65	89.89±11.88*	79.44±9.55*
MS/VDB	6.63±1.22	34.22±7.14*	20.44±6.87
Pir	39.13±9.76	117.67±18.16*	108.44±17.15*
MPOM	47.56±6.16	**90.11±12.43***	**52.56±7.93**
BNSTd	17.44±5.10	32.11±7.09	45.00±6.95*
BNSTv	6.00±1.491	14.44±2.44*	15.33±2.20*
LS3	14.63±3.50	32.75±6.61*	17.25±4.28
PVA	159.13±25.48	311.67±18.32*	289.00±33.58*
PVN	21.33±2.96	121.78±23.28*	132.78±19.67*
AHA	21.25±5.32	**21.33±3.19**	**12.89±2.11**
SON	10.29±1.60	35.22±4.95*	53.11±10.16*
MeA	52.56±9.05	93.33±12.56*	74.33±10.29
CeA	24.13±3.87	47.11±6.68*	42.00±6.63*
BLA	8.63±1.98	24.44±2.63*	17.44±3.489
AHP	73.50±16.80	116.00±15.17*	95.11±10.16
LH	49.50±4.76	**66.11±7.65**	**40.33±4.35**
VMH	10.63±2.46	29.22±6.57*	19.33±5.37
VTA	23.86±4.24	53.33±11.51*	28.00±4.48
DRD	59.78±6.91	97.89±13.34*	88.78±14.21
LDTg	12.25±2.41	27.67±6.86*	17.44±3.46
LC	20.50±4.66	51.00±5.15*	46.56±11.09
PB	8.63±2.58	30.22±7.14*	35.00±7.51*

Mean(±SEM) of raw data.
Bold indicates $p<0.05$ between vehicle and CDP.
* indicates $p<0.05$ relative to unhandled.

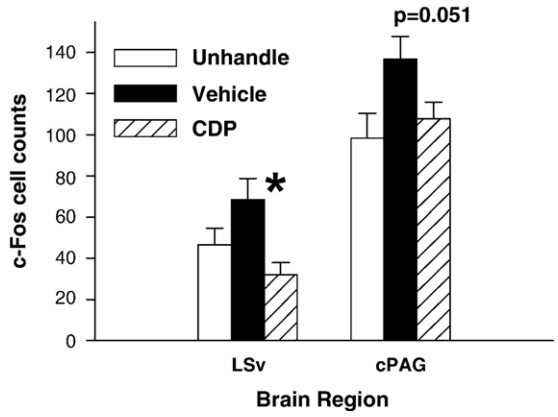

Fig. 3. Effects of CDP on c-Fos cell counts in behaviorally-naïve subjects. Bars represent mean ± SEM. LSv = lateral septum, ventral has significant decreases of c-Fos counts in the CDP-treated dams when compared to vehicle-treated. cPAG = periaqueductal grey, caudal approached significance with treatment. Asterisk indicates significant differences between groups ($p<0.05$).

PVN and LC, suggest injection is eliciting a stress response. CDP significantly decreases c-Fos relative to vehicle injection and it is possible that it is mitigating the effect of injection. Behaviorally, then, CDP could elevate aggression by mitigating the possible inhibitory effects of injection. If CDP does elevate aggression by minimizing stress-induced impairment of aggression, then this suggest the interesting possibility that maternal aggression can be adjusted by altering central reactivity to stressors. Also, if CDP is mitigating c-Fos increases due to injection paradigm, then the insights into where CDP is altering neuronal activity under these circumstances is useful.

A related issue is possible interactions of CDP and isoflurane. Isoflurane can activate corticosterone increases (Altholtz et al., 2006), alter acetylcholine release (Dong et al., 2006; Jansson et al., 2004), and also exert benzodiazepine-like actions on the GABA system (Antkowiak, 1999; Flaishon et al., 2003; Gyulai et al., 2001; Harris et al., 1993; Harrison et al., 1993; Moody et al., 1993). Hence, isoflurane and CDP could be acting in a complementary manner. Indeed, recent work found that both isoflurane and midazolam (a BDZ) increased the frequency of spontaneous inhibitory postsynaptic potentials (Verbny et al., 2005). Previous studies have found that isoflurane can depress c-Fos in the spinal cord (Jinks et al., 2002), but elevate it in the hypothalamus in aged rats (Jansson et al., 2004), so it is not clear how isoflurane may have acted in this study, if at all on c-Fos expression. As indicated above, the use of isoflurane in association with injection here was aimed to reduce stress effects of injection and its previous use in association with injection has not been found not to overtly impair aggression or other maternal behaviors (D'Anna et al., 2005; D'Anna and Gammie, 2006; Gammie et al., 2004). Exactly whether or how CDP and isoflurane may interact at the doses used in this study would need to be examined in subsequent experiments.

To gain insights into where BDZ may positively regulate aggression, we examined brains following treatment in behaviorally-experienced and -naïve lactating mice. In both cases, CDP significantly decreased c-Fos activity in the LS relative to vehicle-injected controls, although slightly different portions of LS were affected (LS2 in behaviorally-experienced and LSv in behavioral-naïve mice). CDP significantly decrease cPAG activity in behaviorally-experienced mice and this difference was just above significance in behaviorally-naïve mice ($p=0.051$). Thus, either or both of these sites could be critical for CDP action. Over 30 additional regions examined did not show altered c-Fos with treatment (relative to vehicle) in either behaviorally-experienced subjects (Table 3) or behaviorally-naïve subjects (Table 4), suggesting the effects of CDP were not ubiquitous. Given that GABA treatment has previously been found to diminish c-Fos labeling (van Luijtelaar et al., 2001) and we and others see decreases in c-Fos with CDP (Hitzemann and Hitzemann, 1999; Imaki et al., 1995), it is possible that the effects seen here are due to direct actions of CDP on neurons containing $GABA_A$ receptors. However, in the absence of double-labeling for this GABA receptor and c-Fos or of site-directed injections, it is not possible to know whether the effects were direct or indirect.

LS is a prime candidate for modulation of maternal defense by BDZs because it is associated with fear and anxiety (Numan and Insel, 2003; Pesold and Treit, 1996; Singewald et al., 2003) and previously has been hypothesized to be a critical site of negative regulation for maternal aggression (D'Anna et al., 2005; Gammie et al., 2004). For example, for three peptides that inhibit maternal aggression, CRF (Gammie et al., 2004), urocotin (Ucn) 1, and Ucn 3 (D'Anna et al., 2005), c-Fos activity in LS was elevated with treatment at the dose that impaired aggression. Also, in association with lactation, LS shows a blunted reactivity to stressors and centrally administered CRF (da Costa et al., 1996, 1997), suggesting altered LS activity supports maternal behaviors, including defense. An intriguing possibility is that GABA signaling in LS gates reactivity to the intruder and alters behavioral output in the dams.

cPAG is another interesting candidate for the critical site of CDP action. Previous work indicates cPAG lesions in rats enhances maternal defense (Lonstein and Stern, 1997a, 1998). pCREB activity in the cPAG is increased in aggressive relative

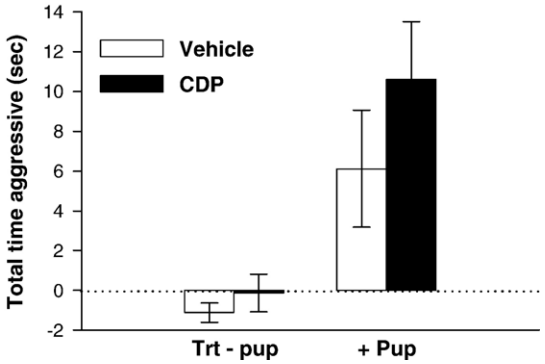

Fig. 4. Effects of CDP-treatment without (−) or with (+) pup stimulation on maternal aggression. Neither CDP nor vehicle was able to elevate maternal aggression in pup-less dams (−) relative to baseline. See Materials and methods for additional details. However, maternal aggression increased in both groups following 30 min with pups.

to non-aggressive lactating females (Gammie and Nelson, 2001), suggesting a role for this region in maternal defense. Our c-Fos results here suggest the interesting possibility that site-direct injections of CDP (or another $GABA_A$ agonist) into the LS or cPAG could enhance maternal defense, but this would need to be tested.

Other brain regions identified as having altered activity with CDP in naïve or experienced mice could also be important sites for how CDP alters aggression. Given that behavioral testing and repeated exposure to injections can affect c-Fos, the finding from the behaviorally-naïve test group may have a higher level of validity for examining CDP actions. An important point is that just because c-Fos changes are found in a region does not necessarily mean that region is involved in the behavior of interest. Specific testing is required. Conversely, just because a region does not show alterations in c-Fos activity with CDP does not necessarily mean that these regions are not involved in CDP's modulation of behavior. This point is relevant because c-Fos as an indirect marker of neuronal activity has limits in that not all neurons express the protein and it is does not indicate whether a neuron exhibited increased or decreased excitability. Here we view c-Fos as a tool, but not an endpoint in understanding possible sites of action of drug. Use of additional indirect markers of neuronal activity (e.g., Egr-1) and site-directed injections of drug will be useful research to develop an understanding of mechanism of action.

CDP enhanced maternal aggression when dams were in recent contact with pups, but it was unable to elevate the decreases in aggression that occurred with 24 h of separation of pups. This result suggests that without pup stimulation, CDP is not sufficient to elevate maternal aggression. One explanation is that although CDP alters GABAergic neurotransmission by acting on the BDZ binding site, a lack of GABA signaling induced by pups removes the signal on which CDP can act. Thus, CDP action is ineffective because the necessary GABA signal is gone. Consistent with this idea that GABA is actively released by the presence of pups, a previous study indicated that concentrations in the cerebrospinal fluid of GABA in lactating rats significantly jumped when pups were reunited with female (Qureshi et al., 1987).

For all other maternal behaviors examined, no effect of CDP was found. However, a trend towards an increase in nursing was noted. The trend towards enhancing nursing with CDP is consistent with some earlier work showing a general enhancement of maternal behaviors with BDZs. For example, early postnatal injections of diazepam enable virgin rats to display higher levels of maternal behaviors relative to vehicle-injected controls (Del Cero et al., 1995). In postpartum ewes diazepam-injected females were more willing to accept alien offspring and allowed suckling compared to those injected with saline (Ferreira et al., 1992). However, evidence of BDZs impairing maternal care also exists. For example, diazepam impairs some maternal behaviors in lactating rats, such as retrieval and nest building (Ferreira et al., 2002).

Although speculative, the altered c-Fos activity in cPAG with CDP could also help explain possible trends towards elevated nursing. For example, there have been numerous studies that associate cPAG to be the sensorimotor integration site for nursing. c-Fos studies indicate that there is an increase of activity in the cPAG when lactating rats are nursing (Lonstein and Stern, 1997b) and lesions of the cPAG severely reduced kyphosis (for review see, (Stern and Lonstein, 2001). Although increased activity of GABA in the cPAG severely reduces kyphosis, this could represent a dose response profile for GABA (see below).

Recent work indicates that direct injections of muscimol (a GABA agonist) into MPA in rats impair maternal care and aggression (Arrati et al., 2006). The simplest interpretation of our increases in aggression with CDP is that in this study we are triggering a moderate enhancement of ongoing GABAergic neurotransmission that specifically affects maternal defense. Impairment of maternal behaviors at higher GABA signaling levels would be consistent with GABA having an inverted U-shaped effect on some maternal behaviors, with intermediate levels enhancing and higher and lower levels impairing the behaviors. Indeed, high levels of BDZs are associated with sedative side effects. In pilot studies we found a higher dose of CDP (15 mg/kg) to impair both maternal aggression and other maternal behaviors and this may have been a result of non-specific sedation of the mice. Furthermore, postpartum mice injected with the high dose of CDP were observed to be more sedentary in the homecage. The enhancement of aggressive behaviors with CDP here suggests a lack of sedative effect. At the very least, the lack of overt change in a number of maternal behaviors examined suggests CDP did not overtly affect motor behaviors. In addition, past literature support that low doses of CDP (0.5–5 mg/kg) have no effect on locomotion (Kennett et al., 1997; Leveleki et al., 2006; Nicholls et al., 1992) or increased mobility (Choleris et al., 2001; Czech and Quock, 1993; Varty et al., 2003) in rodents. Although rodents in the above studies were not in the postpartum period, the results do support the idea that 1 mg/kg of CDP was not producing sedative side effects in this study. Residual accumulation of drug in the female's body most likely did not affect behaviors because the half-life of CDP is 30 h (Rall, 1990) and injections were given every other day. However, the decreased effectiveness of injection on behavior with the second injection could reflect a change in responsiveness of the dams due to prior treatment.

CDP is an anxiolytic drug (Rall, 1990). The finding of elevated aggression with CDP is consistent with the model that maternal aggression is inversely related to levels of fear and anxiety. For example, relative to virgin females lactating rodents show decreased anxiety in a number of tests, such as the elevated plus maze (Ferreira et al., 2002; Kellogg and Barrett, 1999), freezing behavior (Ferreira et al., 2002), light–dark choice test (Lonstein and Gammie, 2002), acoustic startle stimulus (Hard and Hansen, 1985; Toufexis et al., 1999), and open field test (Fleming and Luebke, 1981). It has been suggested that a decrease in fear/anxiety may facilitate aggression such that a dam is now more likely to attack a normally fear-evoking intruder male (Lonstein and Gammie, 2002). However, a number of discrepancies among a variety of studies, such as fear/anxiety changes not always being associated with heightened maternal aggression, suggests a

clear link between fear/anxiety and maternal aggression may not exist (Lonstein, 2005). From the perspective of GABA, though, decreasing anxiety is associated with elevated aggression. Further, for three peptides that are considered to be anxiogenic, CRF, Ucn 1, and Ucn 3, each impairs aggression when injected centrally (D'Anna et al., 2005; Gammie et al., 2004). In addition, oxytocin has been found to both elevate maternal aggression (Bosch et al., 2005) and to decrease anxiety (Ring et al., 2006). Thus, for five modulators known to alter anxiety, in each case maternal aggression is inversely regulated relative to fear/anxiety. Another approach to address this issue is to induce stress and examine the effect on aggression. Recent work indicates that with acute restraint, maternal aggression is dramatically reduced (Gammie and Stevenson, 2006). Hence, examining whether CDP could rescue the inhibitory effects of anxiety on aggression could help an understanding of the relationship between fear/anxiety and aggression. Certainly understanding the underlying basis of maternal aggression in greater detail will be the critical step in understanding whether or how maternal aggression and anxiety are linked.

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