ORIGINAL ARTICLE

Chronic Cocaine Exposure During Pregnancy Increases Postpartum Neuroendocrine Stress Responses

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The cycle of chronic cocaine (CC) use and withdrawal results in increased anxiety, depression and disrupted stress-responsiveness. Oxytocin and corticosterone (CORT) interact to mediate hormonal stress responses and can be altered by cocaine use. These neuroendocrine signals play important regulatory roles in a variety of social behaviours, specifically during the postpartum period, and are sensitive to disruption by CC exposure in both clinical settings and preclinical models. To determine whether CC exposure during pregnancy affected behavioural and hormonal stress response in the early postpartum period in a rodent model, Sprague–Dawley rats were administered cocaine daily (30 mg/kg) throughout gestation (days 1–20). Open field test (OFT) and forced swim test (FST) behaviours were measured on postpartum day 5. Plasma CORT concentrations were measured before and after testing throughout the test day, whereas plasma and brain oxytocin concentrations were measured post-testing only. The results obtained indicated increased CORT response after the OFT in CC-treated dams ($P \leq 0.05$). CC-treated dams also exhibited altered FST behaviour ($P \leq 0.05$), suggesting abnormal stress responsiveness. Peripheral, but not central, oxytocin levels were increased by cocaine treatment ($P \leq 0.05$). Peripheral oxytocin and CORT increased after the FST, regardless of treatment condition ($P \leq 0.05$). Changes in stress-responsiveness, both behaviourally and hormonally, may underlie some deficits in maternal behaviour; thus, a clearer understanding of the effect of CC on the stress response system may potentially lead to treatment interventions that could be relevant to clinical populations. Additionally, these results indicate that CC treatment can have long-lasting effects on peripheral oxytocin regulation in rats, similar to changes observed in persistent social behaviour and stress-response deficits in clinical populations.

Key words: cocaine, postpartum, stress, oxytocin, corticosterone.

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Cocaine use, anxiety and dysregulated stress response are highly correlated in clinical populations (1,2), specifically in postpartum women (2,3). Cocaine acutely activates the hypothalamic-pituitary-adrenal (HPA) axis stress response (4), an effect that is intensified by the female sex hormones, which are high during pregnancy. Chronic cocaine treatment can significantly raise the elevated basal corticosterone (CORT) concentrations throughout pregnancy in rats (5). Additionally, HPA reactivity is heightened during acute withdrawal, which can occur within hours of the last exposure to cocaine, and dysregulation persists during protracted abstinence (4,6). Such drug-induced increases in HPA reactivity are partially responsible for increased anxiety and altered behavioural stress-responsiveness in clinical populations and preclinical models (6,7).

Postpartum mood disorders, including increased anxiety and depression, are not well understood and can negatively impact maternal–infant interactions (8–11). Maintaining stress hormones within a strict range is required for optimal maternal behaviour because perturbation in either direction can disrupt these behaviours (12–14). Oxytocin, which is well known for its role in the central nervous system with respect to promoting maternal behaviour, has also been described as an 'anti-stress' signal (15–20). High brain oxytocin concentrations (similar to concentrations observed

during lactation) lower plasma CORT concentrations and mediate some of the HPA hypo-responsiveness observed in the postpartum period (15–20). In addition to its central actions, peripheral oxytocin signalling modulates CORT concentrations; however, these effects are dependent on the length and concentration of circulating oxytocin exposure (21–23), and may be differently regulated during the postpartum period. Recent evidence suggests that, in addition to central actions, peripheral oxytocin signalling is associated with, and may play an important role in response to social interactions in both humans and rodents (24–28), suggesting that peripheral oxytocin affects a variety important behaviours and may serve as a translational biomarker for at-risk women during the postpartum period.

Recent mothers with a history of drug abuse have lower plasma concentrations of oxytocin and cortisol, indicating that neuroendocrine factors in the postpartum period are affected by cocaine use and that the stress response may play an important role in the disrupted maternal behaviour observed in these women (15,29–31). Drug-induced deficits in maternal behaviour may be caused by changes in central or peripheral concentrations of oxytocin or HPA hormones; however, it is difficult to determine whether these changes are caused by cocaine or by other confounding factors, such as low socioeconomic status, social support systems or co-abused drugs.

Preclinical rodent studies that control drug dose and regimen, as well as gestational and postpartum environments, allow for more precise determination of the effects of cocaine on postpartum behaviours. Various cocaine treatment regimens (acute, intermittent or chronic) disrupt mother–infant interaction dynamics at the same time as decreasing oxytocin concentrations in the medial preoptic area, hippocampus and ventral tegmental area in the early postpartum period (32–34). Presently, it is unknown whether plasma concentrations are altered in cocaine-treated rats similarly to that shown in humans (30) or whether cocaine treatment affects central or circulating oxytocin in the postpartum rat at critical points during the stress response. Cocaine-induced disruption of the interactions between oxytocin and CORT in the postpartum period may lead to differential levels of anxiety or depressive-like behaviour, which could partly underlie differences in maternal behaviour.

Although there is an established bidirectional relationship between substance abuse and stress-related symptomatology (4,35,36), little is known about how drug use may alter the behavioural or hormonal stress response during the postpartum period. Because it is known: (i) that cocaine has been shown to disrupt behavioural and HPA activity; (ii) that cocaine can disrupt oxytocin signalling and maternal behaviour in the postpartum; and (iii) given the importance of the interaction between CORT and oxytocin signalling (both central and peripheral) in stress, we proposed several studies aiming to investigate the relationships between these factors in the postpartum period. We designed studies to determine whether CC-treated dams differ from untreated (UN) dams in: (i) measures of anxiety or stress-responsive behaviour in the postpartum period; (ii) HPA axis function; and (iii) plasma and brain oxytocin concentrations after stress. We hypothesised that CC-treated dams would exhibit higher anxiety and greater behavioural and

HPA stress-responsiveness and lower plasma and brain region oxytocin concentrations than UN dams.

Materials and methods

Subjects

All methods used standard procedures advocated by the UNC Division of Laboratory Animal Medicine and approved by UNC-Chapel Hill Institutional Animal Care and Use Committee. Sprague–Dawley nulliparous female rats (approximately 200 g; Charles River, Raleigh, NC, USA) were kept under a 12 : 12 h reversed light/dark cycle (lights off 08.00 h) for at least 1 week and then mated with a single male until conception was noted by the presence of a vaginal plug or sperm in a vaginal smear, and this was defined as gestational day (GD) zero. Seven days after conception (GD7), rats were moved to a colony room and individually housed under a regular 12 : 12 h light/dark cycle (lights on 07.00 h). This procedure typically results in 95% of female rats delivering during the afternoon hours (37). Rats were randomly assigned to either treatment or control groups of nine to 15 each, as they become pregnant. Gestational weight gain was measured daily for all groups. Water and chow were available *ad lib*. Postpartum day (PPD) one was defined as being within 18 h of completed delivery. After parturition, gestational length, litter weight, number of pups per litter and sex ratio were recorded. Dams reared a culled litter of ten of their own biological pups (as close to five males/five females as possible). After testing, dams and their litters were returned to the colony room until the next test session or immediately euthanised.

Chronic cocaine treatment procedure

Dams received 30 mg/kg/day of cocaine HCl (dose calculated as free base, 2 ml total volume; Sigma, St Louis, MO, USA) in a saline solution. Half of the total cocaine dose (15 mg/kg) was injected twice daily, s.c., at approximately 09.00 h and 16.00 h throughout gestation (GD 1–20) and not thereafter. This is the lowest dose for which consistent significant effects on postpartum behaviours have been found (33,38). The absorption rate of subcutaneous injections is relatively analogous to 'snorting' cocaine as commonly reported by humans (39) and this dose (15 mg/kg in rat) is approximately equivalent to 1 g of cocaine in a women weighing 160 pounds (73 kg). The use of a 27-gauge needle and rotating injection sites significantly reduced the number and severity of cutaneous lesions frequently reported with CC injections in rat models. A topical antibacterial ointment (Polymycin–Bacitracin–Neomycin; Glaxo-Wellcome, Raleigh, NC. USA) was applied on any skin lesions as they were discovered. Subcutaneous administration of cocaine should not cause significant behavioural or biochemical stress when carefully monitored (40).

Untreated control procedure

Dams received no drug treatment during gestation or during the postpartum period, although they were weighed daily to control for the effects of handling. Saline-treated dams were not used in these studies because it has been shown previously that their maternal behaviour did not differ from UN dams (41).

Experimental design and methods

Female rats were delivered to the animal colony in cohorts (n = 10–60) and allowed to habituate for 5–10 days. Cohorts included many females that were designated for other studies ongoing in the laboratory, and females from each cohort were randomly assigned to a drug treatment or test type (see below). After 2 days of travel and test room habituation, all members of a cohort

underwent an open field test (OFT) within 2 days of each other. All females were placed in the OFT apparatus 2–5 days before mating to obtain a 'baseline' anxiety measure to account for the natural variation that can occur within large groups of animals (19). On PPDs 1, 3 and 5, dams were brought to a room and pups were removed for weighing and ultrasonic vocalisation (USV) recordings in another room, whereas the dam remained in the home cage for approximately 30 min, after which pups were returned to the home cage. Ultrasonic recording equipment included Med Associates model ANL-932-1 ultrasound detectors (Med Associates, St Albans, VT, USA), sampling at a rate of approximately 30 samples/s, which were connected to transducers, and then to a laptop computer. Med Associates USV software began acquisition of USVs at the session start and terminated 1 min later. Number, duration and mean frequency of USVs were measured.

On the morning of PPD 5, 2 h after pups were returned, CC-treated and UN dams were randomly divided into three test groups or 'types'. Type 1 dams (UN, n = 7; CC, n = 6) were tested for all behaviours and hormonal concentrations (CORT and oxytocin). Tail blood was collected for CORT measurement (10.00 h to 12.00 h) and dams were returned to their home cage in the test room with pups present for 2 h. The dams were then placed in the OFT chamber for 10 min, after which tail blood was again drawn for CORT measurement (12.00 h to 03.00 h), and the dam placed back in her home cage with pups for a second 2-h rest period. Dams were then placed in the forced swim test (FST) tank for a 10-min test after which they were euthanised (14.00 h to 18.00 h).

Type 2 control dams (UN, n = 8; CC, n = 7) were tested for behaviour (OFT and FST) on a similar time schedule, although tail blood was not collected because tail blood collection procedures have been shown to increase CORT concentrations and high CORT concentrations can affect anxiety-like behaviour such as the OFT (42,43).

Type 3 control dams (UN, n = 6; CC, n = 6) were used to measure how plasma CORT concentrations changed throughout the day without having performed the behavioural tasks because CORT exhibits a diurnal rhythm of release that is maintained in lactating rats (44); however, this pattern of release may have been affected by CC treatment (45). All dams were euthanised and trunk blood was collected for CORT and oxytocin measurements. Dam brains were collected and their anterior hypothalamus and amygdala dissected out for oxytocin analysis (see below).

Behavioural testing and analysis

OFT

The OFT chamber (61 × 64 × 38 cm) had dark opaque flooring and walls. Female rats were brought to the testing room and allowed to habituate for at least 30 min before the test, then removed from the home cage and placed in the OFT chamber for 10 min and allowed to explore freely when the experimenters had left the room. After the test, they were returned to the home cage and any urination or defecation was noted. On postpartum testing days, pups remained in the home caged during habituation and testing, and any nursing behaviour was noted before the test. The OFT chamber was cleaned with a nontoxic spray (Greenworks All-purpose Cleaner; Chlorox, Oakland, CA, USA) after each test. A video recorder [either a Panasonic VHS (AG188U; Panasonic, Osaka, Japan) or JVC recorder (JVC, Yokohama, Japan) with low-light sensitivity] placed directly over the test chamber started recording before testing and continued until the session ended for later analysis.

Video analysis for OFT

The OFT chamber was divided into Wall and Centre compartments. The Wall was defined as the area between the outer edges of the chamber up to 10 cm into the chamber. The Centre was defined as the rest of the chamber (54 × 51 cm), comprising approximately 70% of the total space in the chamber. Wall compartment dimensions were chosen based on pilot work determining that this space was typical for female rats to use before changing direction along one side of the chamber. Behavioural coding was performed using ETHOVISION, Version 3.0 software (Noldus Information Technology Inc., Wageningen, the Netherlands). The rat's location was tracked and frequency of entries, duration, total distance travelled and velocity of movement in each chamber (Wall and Centre) was recorded. Ethovision recorded data in 1-min bins that were then summed (duration, frequency and distance) or averaged (velocity) across the 10-min session.

FST

Dams were allowed to rest with pups in the test room before the FST. The FST tank (height 41 cm radius 11 cm) was filled with water (mean temperature 22–25 °C) to a depth so that the rats could not reach the bottom and could not escape the tank. At time of testing, any nursing behaviour was noted and dams were removed from the home cage and placed into the tank for 10 min. The dams were then removed from the tank, towel dried and returned to the home cage.

Video analysis for FST

Two independent scorers who were blind to rat drug treatment or type scored videotapes and their observations were assessed for reliability within 10% for frequency and latency and within 20% for duration of behaviours of interest. Previously described computer software (34,38) was used to record and calculate the frequency, duration, latency and sequence of behaviours displayed by the rat dams as the viewer scored the session. The behaviours of interest were: Dive (dam put entire head under water and swam to the bottom of the tank); Climb (dam pressed rapidly alternating forepaws against the wall above the water line, with a vertical body and ventrum against the wall); Swimming (defined as the number of limbs moving; thus, there were Swim-2 legs, Swim-3 legs and Swim-4 legs designations); and Immobile (animal had no more than one leg moving). Tail movements were consistent across behaviours and thus not considered in the coding. No Swim-3 behaviour was noted in any of the tests.

Endocrine collection and measurement

Decapitation

Rats were euthanised by rapid decapitation. This method was employed so that the neuropeptide levels could be captured with dams experiencing as little stress as possible at the same time as avoiding alteration of neuropeptide levels by anaesthesia. Neuropeptide levels change rapidly in rodents and any behavioural stress can cause a rapid release and thus lead to a false reading of oxytocin levels.

Brain collection

Whole brains were removed from the skull, flash-frozen on dry ice and stored at −80 °C until dissection. Brains were incompletely thawed to allow hand dissection using anatomical landmarks in a standard rat brain atlas (46). The amygdala was collected from −2.12 to −3.14 bregma, ventral to the rhinal fissure, and lateral to the corpus callosum. The anterior hypothalamus was collected from −0.80 to −2.12 bregma, ventral to the anterior commissure and third ventricle, and medial to the lateral ventricles. The amount of time any region was allowed out of −80 °C conditions was < 10 min; therefore, none of the tissue was completely thawed at any point before the time of assay.

Trunk blood collection

Trunk blood was collected into vials containing 500 KIU/ml of aprotonin (Sigma) and 0.0634 M ethylenediaminetetraacetic acid (EDTA; Sigma). Vials were immediately centrifuged at 4 °C at 10 000 *g* for 10–15 min. Plasma was collected, immediately frozen, and stored at −80 °C until further testing. Two UN/Type1 FST blood samples were lost to experimenter error and were not included in the analysis.

Tail blood collection

Rat dam CORT sample collection was accomplished via a tail clip. Briefly, the dam was wrapped in a cloth towel to provide soft restraint. The collection site was swabbed with betadine (povidone iodine) and alcohol, nicked with a surgical blade, and the resulting blood collected into a centrifuge tube containing 0.0634 M EDTA. After collection, direct pressure with sterile gauze and elevation were applied to the site until bleeding stopped. Additionally, Kwik-Stop Styptic (Arc Laboratories, Atlanta GA, USA) powder with benzocaine was applied to the site to hasten clotting. Once bleeding had been controlled, the dam was released from towel restraint and allowed to recover in her home cage. The entire procedure typically lasted < 3 min.

Extraction of oxytocin peptide from plasma

A strata-X 33 μm polymeric reversed phase solid phase extraction sorbent was equilibrated in a 96-well plate containing 60 mg sorbent per well (Phenomenex, Torrance, CA, USA) by adding 1 ml MeOH followed by 1 ml of water. Some 800 μl of plasma was acidified with 0.4 ml of 1.5% trifluoroacetic acid (TFA) and centrifuged at 6000 *g* for 20 min at 4 °C. This supernatant was loaded onto the pre-treated strata-X plate. Wells were washed with 1.5 ml of 0.1% TFA, and then the peptide eluted with 1 ml of 80% acetonitrile. The eluant was collected in a polystyrene tube, evaporated to dryness under a nitrogen stream, and the residue reconstituted in 200 μl of assay buffer. Extraction efficiency was determined by spiking one positive control with a known amount of hormone and extracting with the other samples.

Oxytocin enzymeimmunoassay

Oxytocin concentrations from extracted plasma were measured using an assay kit and protocol from Assay Designs Inc. (Ann Arbor, MI, USA). The endogenous oxytocin hormone competed with oxytocin linked to alkaline phosphatase for the oxytocin antibody binding sites. After overnight incubation at 4 °C, the excess reagents were washed away and the bound oxytocin phosphatase was incubated with substrate and, after 1 h, the enzyme reaction, which generates a yellow colour, was stopped. The optical density (OD) was read on a Sunrise plate reader (Tecan, Research Triangle Park, NC, USA) at 405 nm. The intensity of the colour is inversely proportional to the concentration of oxytocin in the sample. The hormone content (pg/ml) was determined by plotting the OD of each sample against a standard curve. The sensitivity of the assay is 11.6 pg/ml, with a standard range of 15–1000 pg/ml. The intra- and inter- assay variation is 4.8% and 8%, respectively. Assay designs reports cross-reactivity for similar neuropeptides found in mammalian sera at < 0.001%.

CORT radioimmunoassay

Sample CORT measurements were measured using a corticosterone ^{125}I radioimmunoassay kit (MP Biomedicals, Orangburg, NY, USA). Samples were brought to room temperature and steroid diluents, ^{125}I-CORT, and anti-CORT were incubated for 2 h. Precipitant solution was added, vortexed and centrifuged at 1000 *g* for 15 min. The radioactivity in the pellet was measured using a LKB CliniGamma counter (Wizard 1470-005; Perkin Elmer, Boston, MA, USA), which calculates the nanogram content of CORT in each sample from the standard curve. The intra-assay and inter-assay coefficients of variance were 4.4% and 6.5%, respectively.

Oxytocin radioimmunoassay

For brain regions, tissue was processed as described previously (32). Briefly, tissue was homogenised in buffer and centrifuged. Oxytocin immunoreactive content was assayed in the supernatant according to a protocol from Bachem/Peninsula Labs (Belmont, CA, USA). Samples and standards (1.0–128.0 pg) were incubated in duplicate with anti-oxytocin serum. This was followed by incubation with ^{125}I-oxytocin, after which normal rabbit serum and goat anti-rabbit IgG serum were added. The ^{125}I-oxytocin bound to the antibody complex was separated by centrifugation. The radioactivity in the pellet was measured using the LKB CliniGamma counter, which calculates the picogram content of oxytocin in each sample from the standard curve.

Statistical analysis

A one-way ANOVA test was used to evaluate differences amongst cohorts in baseline OFT behaviour. An ANOVA (Treatment × Type) was used to test comparisons of baseline and postpartum OFT tests. Postpartum behaviour was compared with the baseline behaviours for each animal to investigate differences across time, and a two-way ANOVA was used to assess whether CC treatment or Behavioural Testing Type affected this difference. A two-way ANOVA (Type × Treatment) was used to assess differences in FST behaviour and to determine differences between oxytocin plasma or brain concentrations. Given the non-normality of the CORT data, a Kruskal–Wallis test was used. Linear regression analysis was performed to assess relationships between plasma oxytocin–behaviour, plasma oxytocin–plasma CORT, plasma oxytocin–regional brain oxytocin and between region–oxytocin concentrations.

Results

Gestational weight gain and pup weight

All gestational and pup growth data are presented in Table 1. Although gestational length in days was the same across treatment groups, it was observed that CC-treated dams were more likely to start labour earlier (during the dark cycle) compared to UN dams ($\chi^2 = 24.838$, d.f. = 1, P ≤ 0.001). CC-treated dams gained less weight than UN dams during pregnancy (t = −4.146, P ≤ 0.001) and thus weighed significantly less at the end of gestation (t = −3.937, P ≤ 0.05). However, CC-treated dams gained more weight during the first postpartum week compared to UN dams (t = 2.827, P ≤ 0.001). There was a small but significant effect on PND 1, such that CC-exposed pups weighed less than UN pups (t = −3.076, P ≤ 0.001). There were no differences in sex ratio, litter weight, culled litter weight or weight gain during their first neonatal week. Ultrasonic vocalisations (USVs) were recorded from litters of pups on postnatal days 1, 3 and 5. No differences were observed in the number, duration or frequency of litter USVs (data not shown).

Table 1. Gestational Weight Gain and Pup Weight Results.

	Gestational treatment	
	CC	UN
GD 0	239.132 ± 4.041	240.759 ± 4.025
GD 20	367.342 ± 5.142*	387.111 ± 4.201
PPD 1	273.25 ± 3.909*	292.216 ± 3.46
PPD 3	280.139 ± 3.248*	293.566 ± 3.335
PPD 5	286.531 ± 3.55*	301.583 ± 3.241
First week weight gain	21.395 ± 1.795*	26.667 ± 1.09
Second week weight gain	38.605 ± 1.345*	46.093 ± 1.02
Third week weight gain	68.211 ± 2.281*	73.593 ± 1.514
Total pregnancy weight gain	128.211 ± 3.939*	146.352 ± 2.391
Postpartum weight gain	14.969 ± 1.952*	8.082 ± 1.458
Litter weight	86.702 ± 13.998	86.388 ± 20.087
Litter number	14.368 ± 2.421	14.033 ± 1.939
PI culled litter weight	64.8 ± 9.34	67.333 ± 8.098
P 1 pup	6.282 ± 0.466*	6.565 ± 0.425
P 3 litter	75.2 ± 8.591	77.723 ± 9.022
P 3 pup	7.446 ± 0.669	7.74 ± 0.804
P 5 litter	99.231 ± 10.406	99.852 ± 10.065
P 5 pup	9.961 ± 0.918	10.014 ± 0.904
Pup weight gain	4.543 ± 2.339	3.807 ± 2.478

All data presented as the mean ± SEM. Weights were collected every day of pregnancy. *Chronic cocaine (CC)-treated dams differed from untreated (UN) dams ($P < 0.05$). The number of males, females and total number of pups were counted on P1. Regardless of number of pups, litters were culled to ten pups. Pup weight was calculated by averaging litter weight/number of pups. GD, gestational day; PPD, postpartum day; P, postnatal day.

Baseline OFT

Analyses indicated no significant differences between cohorts on a number of behavioural variables including centre duration, centre frequency, centre distance travelled or velocity. Individual cohort comparisons are shown in Table 2. Gestational drug treatment and type were randomly assigned, and a two-way ANOVA revealed there were no significant differences in the baseline centre duration or locomotor activity between treatment or behavioural test types (Fig. 1A,B).

Table 2. Cohort Baseline Open Field Data.

Cohort	Centre duration	Centre locomotor activity
1	70.32 ± 13.34	445.5 ± 34.89
2	55.34 ± 8.13	364.2 ± 49.31
3	57.73 ± 3.31	429.1 ± 26.34
4	40.9 ± 3.33	302.6 ± 24.35
5	49.36 ± 4.8	329.4 ± 32.69

All data presented as the mean ± SEM. Baseline open field test (OFT) behaviour was measured 1 week before pregnancy in the order that animals arrived at the facility. No significant differences were observed between cohorts.

PPD 5 OFT

The blood draw procedure increased centre time in the OFT in Type 1 dams compared to Type 2 dams regardless of CC treatment ($F_{1,24} = 6.021$, $P \leq 0.05$; Fig. 1c). Qualitatively, CC-treated dams were more likely to decrease activity as the session continued compared to UN dams. There were no differences between CC-treated and UN dams on OFT behaviour or urine and feces responses (Fig. 1c,D). However, compared with baseline measures (Fig. 1E,F), ANOVA tests indicated that the CC-treated group (regardless of Type) had a strong trend for shorter centre duration ($F_{1,24} = 3.909$, $P \leq 0.06$). UN dams did not significantly differ from their own baseline measures, however, testing Type qualitatively affected the direction of change.

CORT response to OFT

At baseline (morning), CC-treated dams showed a nonsignificant trend for lower CORT concentrations compared to UN dams ($Z = 1.753$, $P \leq 0.08$). Type 1 UN dams did not exhibit a change in CORT concentrations after the OFT (Fig. 2A); however, CC-treated dams showed a significant increase ($Z = 2.073$, $P \leq 0.05$). Type 3 dams were tested for CORT concentrations at corresponding times of day but without having performed the behavioural tests and there were no differences between treatment groups or within groups between the two time points (Fig. 2B).

FST behaviour

CC-treated dams (Type 1 and Type 2) were immobile for a shorter duration ($F_{1,22} = 5.771$, $P \leq 0.05$; Fig. 3A) compared to UN dams. Experimental procedure type also influenced FST behaviour. Type 1 dams were immobile less often ($F_{1,22} = 9.295$, $P \leq 0.01$) and for a shorter duration ($F_{1,22} = 9.672$, $P \leq 0.01$) compared to Type 2 dams, regardless of treatment.

FST plasma oxytocin and CORT response

All hormonal data are presented in Fig. 3. A two-way ANOVA (Treatment × Type) of plasma oxytocin concentrations revealed FST exposure (Type 1 and Type 2) increased oxytocin concentrations compared to No FST (Type 3) dams ($F_{1,22} = 7.006$, $P \leq 0.01$; Fig. 3B). CC-treated dams had higher oxytocin concentrations than UN dams regardless of Type ($F_{1,22} = 5.145$, $P \leq 0.05$; Fig. 3B). A two-way ANOVA (Treatment × Type) revealed that CORT concentrations were significantly raised in all Type 1 and Type 2 dams by exposure to the FST compared to Type 3/No FST dams ($F_{2,22} = 26.612$, $P \leq 0.001$) with no effect of treatment (Fig. 3c). Correlations between the FST behaviours and plasma concentrations of oxytocin and CORT revealed that a greater amount of total swim time (Swim-2 plus Swim-4) was positively related to plasma oxytocin concentrations within all dams regardless of treatment ($F_{1,12} = 6.047$, $P \leq 0.05$; Fig. 3D). No correlation was observed between plasma CORT and FST behaviours (Fig. 3E). Linear regression analyses indicated that oxytocin and CORT had no direct relationship with each other at this
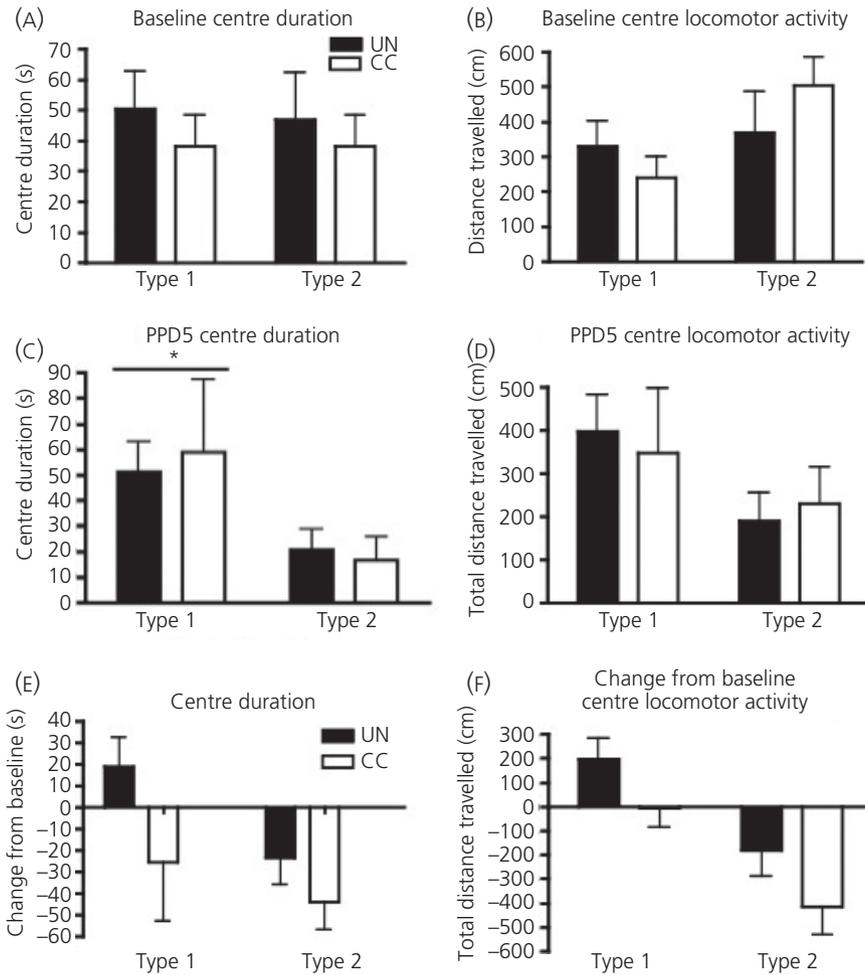
Fig. 1. Open field behaviour. Data are presented as the mean ± SEM. (A, B) Pre-pregnancy (baseline) open field test (OFT) behaviours. Two-way ANOVAs indicate that there were no differences between groups before pregnancy in (A) centre duration or (B) centre locomotor activity. (C) Postpartum day (PPD) 5 OFT behaviour. Centre duration was significantly increased in Type 1 compared to Type 2 dams (*$P \leq 0.005$). (D) PPD 5 OFT Behaviour. No significant difference was observed in centre locomotor activity. (E, F) Change from Baseline. Presented as the group mean of the individual change from baseline (PPD 5 – Baseline). No significant differences were observed. All chronic cocaine (CC) dams showed a negative effect of centre duration in the postpartum (E). Dams did not significantly change the amount of locomotor activity. (F) Untreated (UN)/Type 1, n = 7; UN/Type 2, n = 8; CC/Type 1, n = 6; CC/Type 2, n = 7.
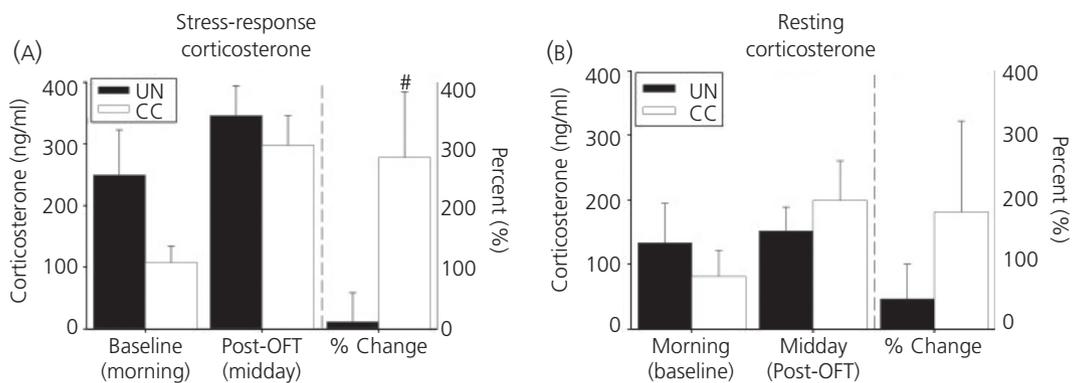

Fig. 2. Corticosterone concentrations at baseline and post-open field test (OFT). Data are presented as the mean ± SEM. Percent (%) change indicates group mean for change between baseline and post-OFT corticosterone (CORT) concentrations. (A) Stress reactivity of Type 1 dams before and after OFT. Chronic cocaine (CC) dams show a significant change CORT concentrations as a group (#$P \leq 0.05$). Untreated (UN), n = 7; CC, n = 6. (B) CORT concentrations of Type 3 dams collected at same time points (morning and midday). UN, n = 6; CC, n = 6.
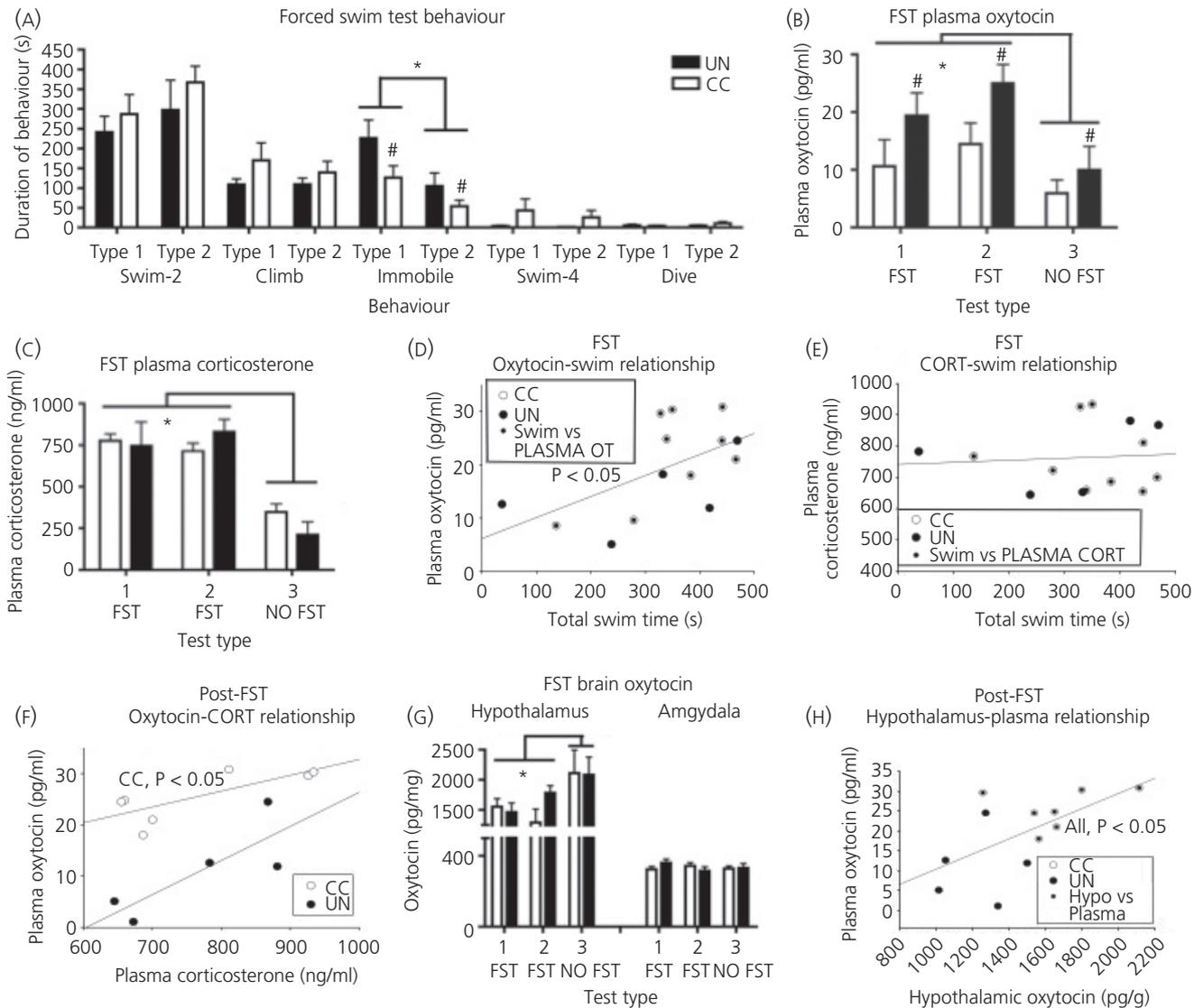
Fig. 3. Forced swim test behaviour and endocrine results. Data presented as the mean ± SEM. (A) Duration of behaviours for Type 1 and Type 2 untreated (UN) and chronic cocaine (CC) dams. CC dams were immobile significantly less than UN dams ([#]$P \leq 0.05$). Type 2 dams were immobile significantly less than Type 1 dams (*$P \leq 0.05$). (B) Plasma oxytocin concentrations. CC-treated dams had higher concentrations compared to UN ([#]$P \leq 0.05$). Forced swim test (FST) exposure (Types 1 and 2) resulted in higher plasma oxytocin concentrations (a and b versus *$P \leq 0.001$) compared to No FST (Type 3). (C) Corticosterone concentrations. FST increased corticosterone (CORT) concentrations (*$P \leq 0.001$). (D) Relationship between plasma oxytocin and swim duration in Type 1 dams. Plasma oxytocin significantly positively correlated with swim duration ($P \leq 0.05$). (E) Relationship between plasma CORT and swim duration in Type 1 dams. No significant effect was observed. (F) Relationship between plasma oxytocin and CORT concentrations in Type 1 dams. CC-treated dams show a significant positive correlation ($P \leq 0.05$) after the FST. (G) Brain oxytocin content. Data presented as the mean ± SEM. FST exposure resulted in lower hypothalamic oxytocin content (Type 1 and 2 versus Type 3; *$P \leq 0.05$). (H) Relationship between plasma oxytocin and hypothalamic concentrations in Type 1 dams. Plasma oxytocin significantly positively correlated with hypothalamic concentrations after FST (Types 1 and 2 presented only) (*$P \leq 0.05$). UN/Type 1, n = 5; UN/Type 2, n = 8; CC/Type 1, n = 6; CC/Type 2, n = 7; UN/Type 3, n = 6; CC/Type 3, n = 6.

time point in UN dams (Fig. 3F). However, CC-treated dams showed a strong positive relationship between the two hormones after the FST (R^2 = 0.76; $F_{1,6}$ = 19.552, $P \leq 0.005$).

Brain oxytocin after FST

There were no differences between Type 1 and Type 2 oxytocin tissue content in dams; thus, their data were pooled for compari-

sons between animals who experienced the FST and those that did not (Type 3) and correlations with behaviours. Type 3 dams showed a trend for higher hypothalamic oxytocin content compared with Type 1 and Type 2 dams regardless of treatment ($F_{2,25}$ = 5.157, $P \leq 0.05$; Fig. 3G). Hypothalamic oxytocin showed a positive relationship with plasma concentrations after the FST (R^2 = 0.318; $F_{1,14}$ = 6.528, $P \leq 0.05$; Fig. 3H). There were no differences in amygdala oxytocin concentrations caused by CC treat-

ment and no significant relationships with behaviour were observed (Fig. 3G).

Discussion

We predicted that CC-treated dams would exhibit behaviours indicating higher levels of anxiety and HPA stress-responsiveness than would UN dams, and also that these differences would correlate with lower plasma and brain oxytocin concentrations. Our hypotheses were partially confirmed because CC treatment resulted in subtle differences in anxiety-like and stress-coping behaviours, as well as important differences in endocrine stress signalling in the early postpartum period.

The peri- and postpartum periods are typically characterised by high basal blood CORT concentrations, and a hypo-responsiveness to stress demonstrated by blunted CORT responses to physiological and psychological stressors (15,18,47–49); however, CC-treated dams showed an increase in CORT in response to the OFT, and this response was absent from UN dams, suggesting that the test was more stressful to CC-treated dams. This change was not the result of a typically occurring diurnal rhythm because CC-treated dams that did not undergo OFT testing did not show a similar increase in CORT. Interestingly, CC-treated CORT concentrations in dams did not change significantly throughout the day, whereas CORT concentrations in UN dams increased by the third blood draw. This rise in CORT concentrations for UN dams may have been in response to the multiple handlings on PPD 5, and a lack of response in the CC-treated dams may suggest that the type of stressors that cause a CORT response differs across drug treatments. Additionally, CC-treated dams show differences in baseline plasma oxytocin (Fig. 3B), which has also been tied to anxiety and CORT levels (50). There was a trend for CC dams to show a relationship between plasma oxytocin and CORT at rest (P < 0.09), suggesting a mechanism for the underlying changes in CORT signalling. However, high levels of peripheral oxytocin can modulate circulating CORT concentrations in the rat dependent on the dose, timing and endocrine state of the animal (21–23), warranting future studies aiming to better understand the interactions between these endocrine systems and their control of anxiety-like and stress-responsive behaviours in the postpartum period.

Unexpectedly, CC dams exhibited significantly less immobility than UN dams. The traditional interpretation of immobility, described as 'despair' that decreases upon antidepressant administration (51), does not appear to be appropriate given the high amount of immobility in PPD 5 dams compared to virgin female rats observed both in the present study as well as in other studies (52). There are a number of physiological reasons why immobility or a 'reactive' coping style would be an advantageous strategy for postpartum females, including changes in fat content, body density, and differences in metabolic and stress mobilisation (53). Interestingly, Type 1 dams were also exhibited more immobility, suggesting that the multiple blood draws may have an important effect on later stress-responsive behaviours. Taking this into consideration, future studies could test depressive-like behaviours in rodents with alternative methods, such as measuring learned helplessness or

sucrose consumption; however, these tests may suffer from similar confounds when measured during lactation. Measuring intracranial electrical self-stimulation behaviour to test anhedonia may also prove useful for understanding depressive-like symptomology in this context. An alternative interpretation of the FST defines greater struggling (i.e. less immobility and greater climbing), as observed in the CC dams, as a 'proactive' coping response to the highly stressful FST (19,51). Proactive coping responses are associated with high levels of aggression and CC dams have consistently shown increased maternal aggression (54–56), suggesting a specific test of coping responses would be highly informative. The neurobiology underlying FST behaviour was altered by CC treatment and, although other cognitive effects of CC treatment may play important roles, neuroendocrine mechanisms are likely major contributors to these behavioural differences.

After the FST, CC-treated dams showed much higher plasma oxytocin concentrations compared to UN dams, perhaps indicating a greater stress response because oxytocin is known to be released into the blood stream in response to stress (57,58). This difference could be a result of altered release concentrations, timing or degradation mechanisms of oxytocin and deserves further study. In the present study, all dams exposed to the FST (Type 1 and 2) exhibited increased plasma oxytocin concentrations compared to control (No FST/Type 3) animals. Although our measurement of plasma oxytocin can only be temporally associated with the end of the FST, previous studies in male rats show that an initial exposure to uncontrollable swimming resulted in a massive release of oxytocin into the plasma, amygdala and supraoptic nucleus (measured by microdialysis) (17,59,60). By contrast, the present study found a lower hypothalamic content after the FST, suggesting that hypothalamic oxytocin was dendritically released and had diffused away from the hypothalamus by the time of collection. These data must be interpreted with caution because they were collected after completion of the test and cannot distinguish between intracellular and extracellular oxytocin concentrations. Supraoptic nucleus oxytocin content during the stressful experience appears to correspond with the degree of uncontrollability of the behavioural context in male rats (17), indicating that future studies using more anatomically and temporally specific measurement techniques, such as microdialysis, may reveal changes in central oxytocin that explain the greater behavioural stress reactivity in CC-treated dams compared to UN dams. Additionally, in FST-exposed animals, a positive correlation was observed between hypothalamic and plasma concentrations (regardless of Type), suggesting a coordination of release during the FST.

In addition to the increased oxytocin after FST, CC-treated dams showed a significant relationship between plasma oxytocin and plasma CORT concentrations that was not observed in UN dams, indicating that, in response to stressful stimuli, the regulation of the hypothalamus, specifically the parvocellular cells of the PVN, may be altered in CC-treated dams; however, a significant relationship may have been found in the UN dams, as well with a larger sample size. Alternatively, these results could represent different set-points for endocrine responsiveness, given that the Type 3 CC-treated dams show differences compared to UN dams in resting

CORT (lower) and oxytocin (higher) concentrations taken at the same time of day. Because CC treatment has a multitude of effects on HPA and PVN signalling during other reproductive stages (4,6,45), future studies may focus more attention on these cells aiming to better understand the changes that may occur in the postpartum period after CC treatment.

An increase in anxiety-like behaviours is observed in postpartum drug-abusing women (30) and, although it is commonly associated with prepartum anxiety, it has been observed that anxiety can spontaneously occur postpartum (61). On PPD 5, CC-treated dams exhibited a strong trend towards an increased anxiety-like profile compared to baseline, with the majority of CC dams showing decreased centre duration. Cocaine withdrawal-induced anxiogenesis typically peaks 3–5 days after the last drug administration in nonlactating rats (62), suggesting that CC-treated dams may have been undergoing withdrawal from cocaine treatments that ended on their GD 20 (4–5 days before testing). One possible mediator of anxiety would be changes in HPA reactivity (43). A previous study indicated that continued cocaine exposure during lactation results in similar deficits in maternal behaviour compared to dams undergoing withdrawal on PPD 1 (63), suggesting that the timing of testing is critical to understanding the underlying mechanisms responsible for the deficits in postpartum behaviours and neuroendocrine signalling. CC dams also exhibited oxytocin concentrations that were two-fold greater than UN dams when taken from rest (Fig. 3B), similar to that observed in depressed women (64), patients with high state anxiety (50) and social anxiety (65).

Significant differences between CC-treated and UN dams were not observed in the direct comparisons of OFT behaviour data, potentially because the variability was very high within the groups. UN dams also showed high variability in the change from baseline, suggesting that there could be normal variation in the progression to the low anxiety typically observed in the second week of lactating rats (15). It was noted in these studies that centre duration was quite low (Fig. 1c), especially in Type 2 dams; however, this could be a habituation effect because pilot work showed that dams without baseline measurements had higher centre duration (S. Williams and J. Johns, unpublished data). Alternatively, Type 2 dams may be reacting to removal from the nest to a greater extent than Type 1 dams. Additionally, the OFT may not be the most sensitive test of anxiety-like behaviour in postpartum rat dams. Future studies using light-dark boxes or elevated plus mazes may reveal any differences. Alternatively, the multiple handling sessions during the first postpartum week may have increased anxiety-like behaviour across all groups, and future studies could determine whether this was the case. Our procedures introduce a light cycle switch on gestation day 7, which may result in subtle long lasting changes in anxiety-like behaviour or hormonal signalling; furthermore, because cocaine has been shown to affect circadian endocrine rhythms (66,67), changes in endocrine signalling during gestation may be perpetuated into the postpartum period, affecting CC-treated dams differently than UN dams.

These data indicate that CC treatment during pregnancy alters peripheral endocrine signalling in a behaviourally context-specific fashion. Such disruptions likely interact and modulate the physio-logical state of dams and thus impact on behaviour. The finding that changes are dependent on the environment suggests a complex tuning of these endocrine systems, and not just a simple knockdown of their function. Stress during pregnancy can reduce maternal behaviour in rodents; however, rats that naturally exhibited low amounts of maternal behaviour are not affected by stress, suggesting that optimal maternal care can be reduced but only in certain populations (68). CC effects on hormonal and behavioural stress response differ from those recently reported after chronic stress during pregnancy (69), suggesting that signalling mechanisms outside of the HPA axis may be involved in mediating the observed effects after CC treatment. Future work directly comparing CC treatment with chronic stress and their combination would be highly informative and perhaps better model the human condition.

Acknowledgements

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