Elevated Corticosterone Levels in Stomach Milk, Serum, and Brain of Male and Female Offspring After Maternal Corticosterone Treatment in the Rat

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ABSTRACT: **Early influences such as maternal stress affect the developmental outcome of the offspring. We created an animal model of postpartum depression/stress based on giving high levels of corticosterone (CORT) to the rat dam, which resulted in behavioral and neural changes in the offspring. This study investigated whether highly elevated levels of maternal CORT during pregnancy or the postpartum result in higher levels of CORT in the stomach milk, serum, and brain of offspring. Dams received daily injections of CORT (40 mg/kg) or oil (control) either during pregnancy (gestational days 10–20) or the postpartum (Days 2–21). Pups that were exposed to high gestational maternal CORT had higher CORT levels in serum, but not in stomach milk or brain, on postnatal day (PND) 1. However, on PND7, pups that were exposed to high postpartum maternal CORT had higher CORT levels in stomach milk and brain, but not in serum. Conversely on PND18, pups that were exposed to high postpartum maternal CORT had higher CORT levels in serum, but not in brain (prefrontal cortex, hypothalamus, or hippocampus). Moreover, 24 h after weaning, there were no significant differences in serum CORT levels between the groups. Thus, CORT given to the dam during pregnancy or the postpartum results in elevated levels of CORT in the offspring, but in an age- and tissue-dependent manner. Developmental exposure to high CORT could reprogram the HPA axis and contribute to the behavioral and neural changes seen in adult offspring.** © 2010 Wiley Periodicals, Inc. Develop Neurobiol 70: 714–725, 2010

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INTRODUCTION

Chronic stress can have a profound impact on well-being and health via changes in hypothalamic-pituitary-adrenal (HPA) axis function. Stress or exposure to elevated glucocorticoids during early development can reprogram the HPA axis and permanently change the behavioral, cognitive, and neuroendocrine outcome of the individual. In humans, high levels of cortisol during gestation are associated with higher infant death rates and maternal and neonatal complications (Bevan et al., 1987; Pricolo et al., 1990). However, glucocorticoids are also indispensable for early maturation processes (Henning, 1981) such as lung maturation (Morishige and

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Joun, 1982) and are vital for both maternal memory and maternal behavior (Rees et al., 2004; Graham et al., 2006). Indeed, antenatal or postnatal glucocorticoid treatment of children is a common clinical practice to promote lung development, although repeated doses of glucocorticoids may have serious side effects on somatic and brain growth (Klinger and Koren, 2000; Park-Wyllie et al., 2000; Bolt et al., 2001; Koenen et al., 2007). In fact, multiple course antenatal glucocorticoids for women with risk of preterm birth did not improve preterm-birth outcomes, but were associated with decreased body weight, length, and head circumference of the children at birth (Murphy et al., 2008). In rats, neonatal administration of corticosterone (CORT) can cause permanent changes in the HPA axis, behavior, and cognition (Nyakas and Endroczi, 1972; Leshner and Schwartz, 1977; McCormick et al., 2001).

Maternal and fetal levels of glucocorticoids can influence each other. Cohen et al. (1990) showed that adrenalectomy during the second or third week of a rat's pregnancy did not eliminate maternal serum CORT, suggesting that CORT produced by the fetus influences maternal concentrations of glucocorticoids. The fetus is partially protected from elevated maternal glucocorticoid levels by the placental enzyme 11βHSD-2, which converts active CORT and cortisol to inert 11 keto-products (11-dehydrocorticosterone and cortisone; Seckl, 1997), but nonetheless maternal glucocorticoids can influence offspring outcome. Manipulations of maternal glucocorticoids during pregnancy by prenatal stress (e.g., Darnaudery and Maccari, 2008), injections of CORT (e.g., Wilcoxon and Redei, 2007), or synthetic glucocorticoids such as dexamethasone (e.g., O'Regan et al., 2004) can influence the offspring in a dose-, time-, and sex-specific way. In particular, offspring subjected to prenatal stress have impaired HPA axis function, which is prevented by maternal adrenalectomy (Weinstock, 2005). Thus, the maternal hormonal state during pregnancy can crucially influence offspring phenotype, despite the downregulation of maternal HPA axis during pregnancy (Johnstone et al., 2000) and the presence of placental 11βHSD-2 (Seckl, 1997).

The level of maternal glucocorticoids postpartum can also profoundly affect the offspring. For instance, a low dose of CORT administered to the dam through the drinking water reduces anxiety, improves learning and stress coping, and protects the adult offspring against ischemia (Catalani et al., 1993, 2000; Casolini et al., 2007). In contrast, high levels of CORT administered to the dam via s.c. injection result in reduced postweaning hippocampal cell proliferation and anxiety-like behavior in adulthood (Brummelte et al., 2006). During the postpartum period, maternal hormones can be transferred via the mother's milk (Zarrow et al., 1970; Angelucci et al., 1983; Yorty et al., 2004) and can have a direct impact on the offspring's glucocorticoid levels. Fetal reprograming could occur via transfer (direct or indirect) of maternal glucocorticoids to the offspring or via glucocorticoid-induced changes in maternal behavior (for review see: Fleming et al., 1999).

We developed an animal model of postpartum depression or stress based on high levels of CORT given to dams during pregnancy or the postpartum period (Brummelte et al., 2006; Brummelte and Galea, 2010). The administration of CORT (40 mg/kg) from Days 10 to 20 of pregnancy or from Days 2 to 24 postpartum led to reduced maternal care, increased depressive-like behavior in the dam (only postpartum group), and several behavioral and neuroendocrine changes in the adult offspring (Brummelte et al., 2006; Brummelte and Galea, submitted). The current studies examined whether elevated maternal CORT levels during gestation (Experiment 1) or the postpartum (Experiment 2) result in elevated CORT concentrations in the offspring. We investigated CORT concentrations in the stomach milk, serum, and brain of offspring at different ages (from postnatal day 1 (PND1) to weaning). CORT concentrations in the whole brain (PND7) or in the prefrontal cortex (PFC), hypothalamus, and hippocampus (PND18) were measured using solid-phase extraction (SPE) coupled with radioimmunoassay. This extraction method successfully extracts steroids from the lipid-rich brain tissue (Newman et al., 2008a; Schmidt and Soma, 2008; Newman and Soma, 2009). We hypothesized that high maternal CORT treatment during gestation would increase serum and brain levels of CORT in the offspring on the first-day postpartum and that high CORT treatment to dams during the postpartum would increase CORT levels in the stomach milk, serum, and brain of male and female offspring.

METHODS

All protocols were in accordance with ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. For breeding, two females were paired with one sexually experienced male overnight. Vaginal lavage was conducted every morning, and the presence of sperm on the slides was considered proof of impregna-

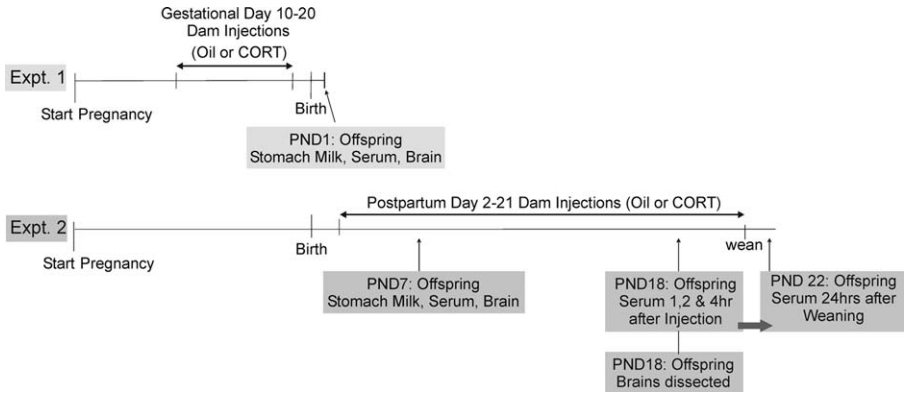

Figure 1 Timeline of the two experiments. Dams in Experiment 1 (Exp. 1) received daily corticosterone (CORT) or vehicle (oil) injections from Days 10 to 20 of gestation, whereas dams in Experiment 2 (Exp. 2) received CORT or oil injections from Days 2 to 21 postpartum. Offspring were sacrificed on postnatal day 1 (PND1) for Experiment 1. For Experiment 2, stomach milk, brain, and serum were collected on PND7. Brain samples were collected on PND18. Serum samples were collected from the remaining offspring on PND18 and PND22 (The bolded gray arrow indicates that the same animals were used for both collection time points).

tion, and thereafter the females were housed singly following common procedures (Rees et al., 2004; Brummelte et al., 2006; Bagot et al., 2009; Pawluski et al., 2009). Rats were handled daily for 4 days before the start of injections.

Experiment 1: Prenatal Administration of High Exogenous CORT to the Mother

A total of 16 dams ($n = 8$/group) and 30 pups were used in this experiment. Approximately 3-month-old female Sprague Dawley rats were received from Charles River (Saint-Constant, QC, Canada) and initially housed in pairs in opaque polyurethane bins (48 cm $\times$ 27 cm $\times$ 20 cm) with absorbent bedding for 1 week. They were given Purina rat chow and tap water *ad libitum* and maintained in a 12:12 h light/dark cycle (lights on at 7:30 a.m.).

Rats were handled daily for 4 days before the start of injections. From Days 10 to 20 of gestation, dams received a daily s.c. injection of either CORT ($n = 8$; 40 mg/kg; 1 mL/kg injection volume) or vehicle ($n = 8$; 0.2 mL sesame oil). We discontinued CORT treatment to the dam before term to avoid inducing a preterm birth. All injections were performed between 10 and 11 a.m. The day of parturition was considered PND0, and time of parturition was noted as either in the morning, afternoon, or night. To ensure basal CORT levels, pups were marked with a pen in the morning of PND1 and then returned to the nest for 4 h before they were quickly removed and sacrificed by rapid decapitation within 3 min of touching the cage. Blood, stomach content, and brains were collected for each pup. To avoid litter effects, no more than one male and female pup per litter were included in this study, resulting in the following numbers per group: Oil: females $n = 7$, males $n = 8$; CORT: females $n = 7$, males $n = 8$. For an overview of the timelines of Experiments 1 and 2, see Figure 1.

Experiment 2: Postnatal Administration of High Exogenous CORT to the Mother

Twenty-six female Sprague Dawley rats, $\sim$3 months of age, were used in this study. Rats were received from the UBC Animal Care Facility (Vancouver, BC) and housed and mated in the same way as in Experiment 1. Rats were handled from Days 17 to 20 of pregnancy, and litters were culled to five male and five female pups on PND1. Dams were randomly assigned to receive a daily s.c. injection of either CORT ($n = 14$; 40 mg/kg) or sesame oil ($n = 12$) from PND2 to 21. To avoid litter effects, no more than two male and female pups per litter were included for each time point. The dose and injection schedule was based on previous studies (Kalynchuk et al., 2004; Brummelte et al., 2006).

On PND7, $\sim$6 h after the dam had received her injection, pups were quickly removed from the nest and sacrificed via rapid decapitation within 3 min, and blood, stomach contents, and brains were collected (oil: females = 6, males = 6; CORT: females = 8, males = 8). PND7 was chosen because it is during the stress hyporesponsive period (Sapolsky and Meaney, 1986).

On PND18, blood samples from pups were taken at three different time points after the maternal injection (1, 2, and 4 h, different pup each time). For this, one male and one female pup from each litter were quickly and gently removed from the cage at 1 h after the dam's injection; blood was collected via tail nick within 3 min, the tail was marked, and then they were returned to their nest. At 2 and 4 h after the dam's injection, a different (unmarked) male and female were used for blood collection and also returned to their home cage afterward (oil: females = 3, males = 3; CORT: females = 4, males = 4; for each time point). Further, 16 male and 16 female pups were briefly anesthetized with CO_2 for 1 min and then sacrificed via rapid decapitation at 4 h after the dam's injection. Time from touching the cage until decapitation was no more than 3 min. Brains

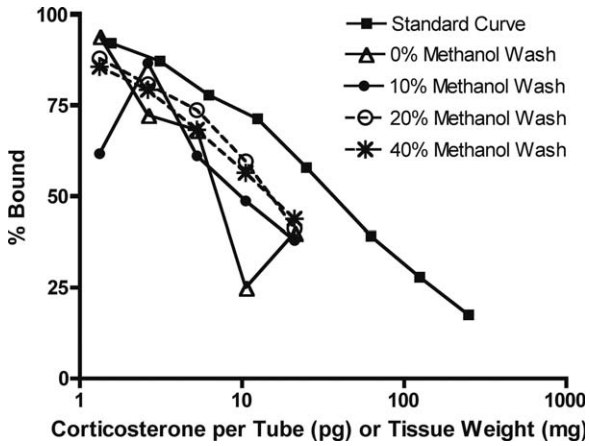

Figure 2 Serially diluted samples of rat brain tissue extracted with solid-phase extraction (SPE). After loading samples onto primed and equilibrated columns, columns were washed with varying concentrations of HPLC-grade methanol. Serially diluted samples washed with 20 or 40% methanol were parallel to the standard curve, whereas serially diluted samples washed with 0 or 10% methanol were not. Thus, 40% methanol wash was used to remove interfering substances from experimental samples.

were rapidly removed and processed as described below (oil: females = 8, males = 7; CORT: females = 8, males = 8). PND18 was chosen because it is the latest time point during lactation when pups are still suckling but also eat normal rat chow (Doerflinger and Swithers, 2004). Thus, the stomach content was not collected on PND18 as pups eat rat chow as well as drink milk at this age.

The remaining pups were weaned on PND21 with their same-sex siblings. Weaning took place in the afternoon, ~4 h after the last injection to the dam. On PND22, 24 h after weaning and thus ~28 h after the last injection to the dam, blood was collected from the pups via tail nick, within 3 min of touching the home cage (oil: females = 6, males = 6; CORT: females = 8, males = 8; Fig. 1).

Corticosterone Preparation

CORT (Sigma-Aldrich, St. Louis, MO) was mixed every few days at a concentration of 40 mg/mL with 10% ethanol in sesame oil and injected s.c. to achieve a dose of 40 mg/kg. The dose was chosen on the basis of our previous work (Brummelte et al., 2006) and the fact that this dose increases depressive-like behavior in the forced swim test in both male and female rats (Kalynchuk et al., 2004; Brummelte et al., 2006). Previous data from our laboratory indicate that this protocol increases serum CORT levels in the dam to ~400 ng/mL at 24 h after administration (unpublished data).

Serum Collection

Trunk and tail nick blood was collected in EDTA-free tubes and immediately stored on ice and then overnight at 4°C. The next morning, samples were centrifuged at 10,000*g* for 10 min, and serum was removed and stored at −20°C until further processing.

Stomach Milk Collection

After decapitation of the neonatal pups (PND1 and PND7), the stomach milk was removed from the stomach and collected into 1.5-mL centrifuge tubes. Milk samples were diluted 1:1 with unsupplemented Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA; Yorty et al., 2004). The diluted milk was centrifuged for 10 min at 10,000*g*. The supernatant was collected from below the lipid layer and stored at −20°C until further processing.

Brain Collection and Dissection

Brains were quickly extracted from the skull, briefly rinsed with distilled water to remove surface blood, and then either immediately frozen on dry ice (PND1 and PND7) or quickly dissected into PFC, hippocampus, and hypothalamus (PND 18) before freezing on dry ice. Brains were then stored at −80°C.

Steroid Extraction Using Solid-Phase Extraction

We conducted a series of validations of the SPE procedure. This was done by comparing serial dilutions of brain tissue to the standard curve of the radioimmunoassay (Newman et al., 2008a). Displacement curves that are parallel to the standard curve indicate that the extraction procedure effectively removes interfering substances. After the samples are loaded onto the C18 SPE columns, removal of interfering substances can be performed by washing columns with deionized water (dH_2O; Newman et al., 2008a,b) or methanol in dH_2O (Belanger et al., 1990; Vallee et al., 2000). After loading serial dilutions of brain tissue onto primed and equilibrated columns, columns were washed with either 0, 10, 20, or 40% HPLC-grade methanol in dH_2O (Fig. 2). Washing columns with either 20 or 40% methanol improved parallelism to the standard curve (Fig. 2), and thus for the extraction procedure, we chose to wash columns with 40% methanol after samples were loaded onto columns.

Steroids were extracted from brain tissue using SPE with C18 columns (nonendcapped, UCTCUC18156, United Chemical Technologies; Newman et al., 2008a). For PND1, the whole brain was used; for PND7, only the right hemisphere was used to avoid an excess of tissue; and for PND 18, three different dissected brain areas (PFC, hippocampus, and hypothalamus) were used. Brain tissue was homogenized in ice-cold dH_2O (3× weight of tissue). Then, HPLC-grade methanol was immediately added (4× volume of homogenate), and samples were incubated overnight at 4°C. The following day, C18 columns were primed with 3 mL HPLC-grade ethanol and equilibrated with 10 mL dH_2O. Samples were centrifuged at 3000*g* for 10 min at

Table 1 Body Weights of Dams During Gestation and the Postpartum

	Group	GD10	GD20	PPD1	PPD12	PPD20
Treatment during gestation (Exp. 1)	Oil ($n = 8$)	316 ± 4	413 ± 6	320 ± 2	–	–
	CORT ($n = 8$)	314 ± 7	377 ± 12*	285 ± 10*	–	–
Treatment during postpartum (Exp. 2)	Oil ($n = 12$)	–	–	344 ± 7	374 ± 7	373 ± 9
	CORT ($n = 14$)	–	–	355 ± 5*	335 ± 6*	311 ± 6*

Maternal treatment with CORT (40 mg/kg) during pregnancy resulted in significantly lower body weights in dams on gestational day (GD) 20 and postpartum day (PPD) 1 compared with oil-treated dams. Maternal treatment with CORT during the postpartum resulted in significantly lower body weights on PPD12 and PPD20 compared with oil-treated dams. We also found that the CORT dams weighed more than oil dams before treatment despite random assignment.

* $p < 0.05$.

2°C. Supernatant was decanted, and 250 μL was transferred to a 16 mm × 100 mm glass tube and brought up to 10 mL with dH_2O. Samples were then loaded onto primed and equilibrated columns. Columns were washed with 40% HPLC-grade methanol (2 × 5 mL), and steroids were eluted with 5 mL 90% HPLC-grade methanol. Eluates were dried under a steady stream of medical-grade nitrogen at 37°C. Dried extracts were resuspended in 125 μL of assay buffer provided with the CORT radioimmunoassay kit. Absolute ethanol (5% of resuspension volume) was used to aid in resuspension of CORT (Newman et al., 2008a). Recovery of CORT in brain tissue was determined by spiking brain tissue pools with 50 pg of CORT and comparing spiked samples to unspiked samples ($n = 3$ spiked and 3 unspiked). Recovery of CORT was ~111% from brain samples.

Radioimmunoassay for CORT

CORT for all samples (serum, milk, and brain) was assayed using a commercial radioimmunoassay kit (MP Biomedicals, Orangeburg, NY) with inter- and intra-assay coefficients of variation less than 6.3%. All kit reagents and standards were halved, and samples were run in duplicate.

Data Analyses

Litter characteristics, serum, stomach milk, and brain levels of CORT, and brain weights were analyzed separately for each experiment using a factorial ANOVA with sex (male, female) and treatment (CORT, control) as between-subject factors. Dams' body weights were analyzed with a repeated measures ANOVA with days (Exp. 1: gestational day (GD) 10, GD20 and postpartum day (PPD) 1; Exp. 2: PPD1, PPD12, and PPD20) as a within-subject factor. Pups' serum CORT levels on postnatal (PND)18 were analyzed using an ANOVA with time (1, 2, or 4 h) as a between-subject factor, and brain CORT levels on PND18 were analyzed with areas (PFC, hippocampus, and hypothalamus) as a within-subject factor. If appropriate, a Fisher LSD *post hoc* test was performed. Significance level was set at $p \leq 0.05$.

RESULTS

Experiment 1: Prenatal Administration of High Exogenous CORT to the Mother

There were no significant differences in litter size ($p = 0.67$) or body weight of pups ($p = 0.17$) on PND1 between treatment groups. We noted a significant effect of CORT treatment on the length of pregnancy, with CORT dams giving birth earlier than controls (CORT: 21.80 ± 0.09 days; controls: 22.13 ± 0.08 days; $F(1, 14) = 9.00$; $p = 0.01$). Maternal body weight changed significantly during pregnancy and after parturition ($F(2, 28) = 423.14$, $p < 0.0001$), but there was also a significant effect of treatment during pregnancy ($F(1, 14) = 5.93$, $p = 0.029$) and an interaction effect of days and treatment during pregnancy ($F(2, 28) = 16.67$, $p < 0.0001$). *Post hoc* tests revealed that dams did not differ on the first day of injection (GD10: $p = 0.80$), but CORT dams weighed less on GD20 and on PPD1 ($p < 0.0001$; Table 1). Administration of 40 mg/kg of CORT to dams from Days 10 to 20 of pregnancy significantly increased serum levels of CORT in pups on PND1 compared with pups from control dams (main effect of treatment: $F(1, 24) = 13.0$, $p = 0.001$; Fig. 3). However, there was no main effect of sex ($p = 0.73$) and no significant interaction effect between sex and treatment ($p = 0.17$). Because of the effect of CORT on pregnancy length, we also performed analyses of covariance for the pups' serum CORT concentrations with the time between the last maternal injection and culling as a covariate, but results showed no significant effect of the covariate ($p = 0.27$). Levels of CORT in stomach milk and brain were both low and did not significantly differ between treatment groups at this time (treatment: $p = 0.68$; $p = 0.39$, respectively; sex: $p = 0.43$; $p = 0.91$, respectively; interaction: $p = 0.52$; $p = 0.39$, respectively; Fig. 3). There was also no significant difference in brain weight between the groups ($p = 0.12$) or sexes ($p = 0.60$; data not shown).

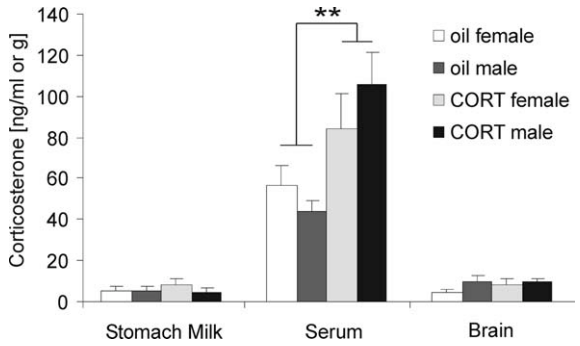

Figure 3 Mean concentration (±SEM) of corticosterone (CORT) in stomach milk, serum, and brain (ng/g) of pups ($n = 5$–8 per group and sex) on postnatal day 1 (PND1) after dams were exposed to daily CORT injections or vehicle (oil) during pregnancy (Exp. 1). There was no significant difference in CORT levels in stomach milk or brain between the groups or sexes, but pups from CORT-treated dams during pregnancy had significantly higher serum levels compared with controls. $^{**}p = 0.01$.

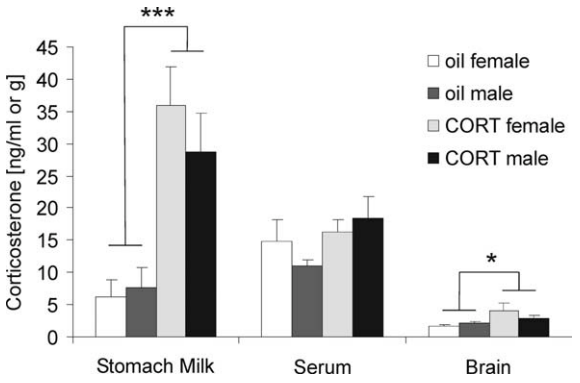

Figure 4 Mean concentration (±SEM) of corticosterone (CORT) in stomach milk, serum, and brain (ng/g) of pups ($n = 5$–8 per group and sex) on postnatal day 7 (PND7) after dams were exposed to daily CORT injections or vehicle (oil) during the postpartum period (Exp. 2). There was no significant difference in serum CORT levels ($p = 0.11$) between the groups, but CORT-exposed pups had significantly higher CORT levels in stomach milk and brain compared with controls. $^{*}p = 0.05$, $^{***}p = 0.001$.

Experiment 2: Postnatal Administration of High Exogenous CORT to the Mother

Maternal body weight changed significantly during the postpartum ($F(2, 48) = 6.99$, $p = 0.002$), but there was also a significant effect of treatment ($F(1, 24) = 11.79$, $p = 0.002$) and an interaction effect of days and treatment during the postpartum ($F(2, 48) = 64.74$, $p < 0.0001$). *Post hoc* tests revealed that CORT dams weighed significantly less on PPD12 and on PPD20 ($p < 0.0001$), and despite random assignment CORT dams weighed more on PPD1 than oil dams (before treatment; $p = 0.019$; Table 1).

Postnatal Day 7

Postpartum maternal treatment with CORT did not significantly affect body or brain weight of pups on PND7 (body weight: $p = 0.73$; brain weight: $p = 0.15$). Figure 4 shows the CORT levels on PND7 in stomach milk, serum, and brain across treatment groups. There was a weak trend for higher serum levels of CORT in pups from CORT-treated dams compared with pups from control dams (main effect of treatment: $F(1, 24) = 2.81$, $p = 0.11$), but no main effect of sex ($p = 0.75$) or interaction effect ($p = 0.27$). For CORT levels in stomach milk, there was a significant main effect of treatment, with pups from CORT-treated dams showing higher CORT values than pups from control dams ($F(1, 21) = 20.10$, $p = 0.0002$). Similarly, brain concentrations of CORT were significantly higher in pups from CORT-treated dams compared with controls (main effect of treatment: $F(1, 24) = 4.16$, $p = 0.05$; no other significant main or interaction effects, $0.27 < p < 0.75$).

Postnatal Day 18

On PND18, blood samples were taken from pups 1, 2, or 4 h after the dam's injection. An ANOVA revealed a main effect of treatment ($F(1,30) = 7.35$; $p = 0.01$; Fig. 5), indicating that offspring from CORT-treated dams had higher levels of CORT in their serum compared with offspring from vehicle-treated dams. There were no other significant main or interaction effects (sex: $p = 0.59$; time since injection: $p = 0.55$; interaction of sex × treatment: $p = 0.18$; interaction of treatment × time since injection: $p = 0.0\times7$; sex × time since injection: $p = 0.56$; and treatment × sex × time: $p = 0.46$).

Further, on PND18, brains were dissected into PFC, hippocampus, and hypothalamus. An ANOVA revealed no main effect of treatment ($p = 0.65$), sex ($p = 0.94$), or interaction ($p = 0.29$). There was a significant effect of area ($F(2,60) = 18.59$, $p < 0.0001$). *Post hoc* tests showed that CORT levels in the hypothalamus were significantly lower than CORT levels in the PFC or hippocampus ($p < 0.0001$), but there was no significant difference between CORT levels in the PFC and hippocampus ($p = 0.46$). There were no other significant interaction effects (all ps > 0.05). Data are presented in Figure 6.

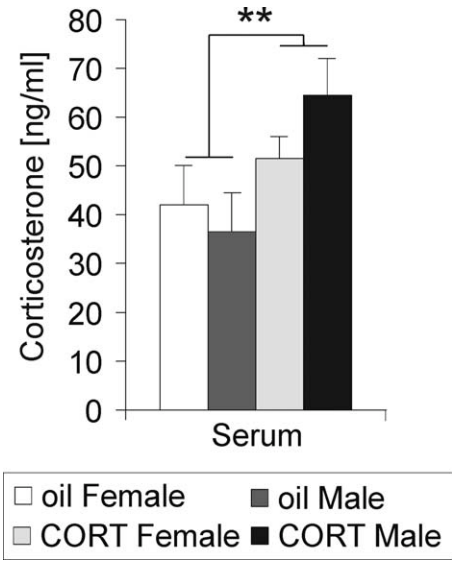

Figure 5 Serum concentrations of corticosterone (CORT) on postnatal day 18 (PND18) were significantly higher in pups from CORT-treated dams (during the postpartum period; $n = 24$) compared with controls ($n = 18$), mean ± SEM, $^{**}p = 0.01$.

Postnatal Day 22

Twenty-four hours after weaning and ~28 h after the last maternal injection, there was a weak trend for a main effect of treatment ($F(1, 23) = 2.36$, $p = 0.13$), with the serum CORT levels in the offspring from CORT-treated dams slightly higher than offspring from vehicle-treated dams (Table 2). There were no significant sex ($p = 0.52$) or interaction effects ($p = 0.50$), but serum CORT levels of control females were twice as high as in control males, and the difference in serum levels between controls and CORT-treated pups was more obvious in males than females (though not significantly different).

DISCUSSION

The results from our experiments demonstrate for the first time that increased maternal CORT levels during pregnancy are associated with elevated serum CORT levels 1 day after birth in male and female pups, but not with elevated CORT levels in stomach milk or brain. Furthermore, we provide evidence that postpartum administration of very high levels of CORT to the dam (1) significantly increased levels of CORT in stomach milk and brain, but not in serum, in PND7 pups; (2) significantly increased levels of CORT in serum, but not in the PFC, hypothalamus, or hippocampus of PND18 pups; and (3) did not affect serum

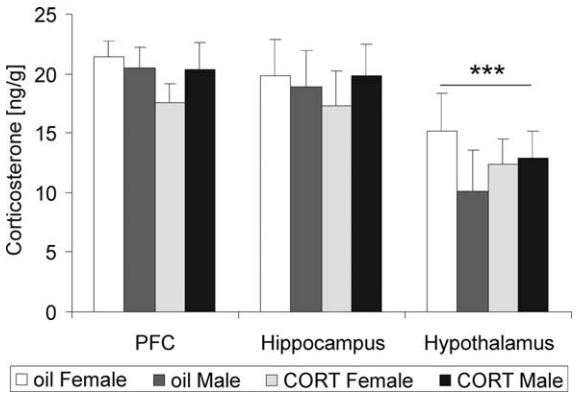

Figure 6 Mean CORT levels (ng/g) ± SEM for the prefrontal cortex, hippocampus, and hypothalamus from offspring on PND18. All pups were sacrificed 4 h after the daily maternal administration of 40 mg/kg of CORT (oil: females = 8, males = 7; CORT: females = 8, males = 8). There was no significant difference between the groups, but CORT levels in the hypothalamus were significantly lower than levels in the prefrontal cortex and hippocampus ($p < 0.001$).

CORT levels in pups after weaning. Furthermore, we found that CORT levels vary across different areas within the brain on PND18. These results demonstrate that CORT levels in the offspring vary with maternal treatment in a tissue- and age-dependent manner.

Prenatal Administration of High CORT to the Mother Results in Elevated Levels of CORT in the Serum of PND1 Offspring

CORT administration to the dam during pregnancy increased serum CORT levels in offspring but did not change brain or stomach milk CORT levels on PND1, suggesting that the higher serum CORT levels were not the result of elevated CORT in maternal milk. Basal and stress levels of CORT are altered in adult rats that were exposed to prenatal stress (for review see: Maccari and Morley-Fletcher, 2007), and stress during pregnancy also influences the HPA axis

Table 2 Serum Concentrations (Mean ± SEM) of Corticosterone in Pups on Postnatal Day 22 (~28 h After the Last Maternal Injection)

	Maternal Treatment	
Offspring	Oil	Corticosterone
Females ($n = 14$)	80.32 ± 27 ng/mL	101.06 ± 16 ng/mL
Males ($n = 13$)	48.35 ± 14 ng/mL	101.96 ± 32 ng/mL

Corticosterone levels in offspring from corticosterone-treated dams were not significantly higher ($p = 0.13$).

in human infants (Matthews et al., 2004; Rieger et al., 2004; Lazinski et al., 2008; O'Donnell et al., 2009). Further, exogenous CORT administration to adrenalectomized dams can alter the offsprings' HPA axis function (Wilcoxon and Redei, 2007) and intrauterine exposure to synthetic glucocorticoids reduces offspring HPA activity under unstimulated conditions in humans (Tegethoff et al., 2009). Together these results suggest that it is likely that exposure to high maternal CORT during pregnancy caused a reprograming of the offspring HPA axis.

We did not find increased levels of CORT in the brain or stomach milk of PND1 offspring from dams given gestational CORT on Days 10–20 of gestation. In all likelihood, the elevated levels of maternal CORT from the gestational injections would have been cleared from the system at this time point, and elevated serum levels in the PND1 offspring might reflect reprograming of the HPA axis. Thus, the lack of an increase in CORT stomach milk levels on PND1 is not very surprising, and brain levels of CORT may be independently regulated from serum levels at this age unlike what is seen in adulthood (Droste et al., 2008, 2009). It is also possible that elevated CORT levels would have been detected in the pup brains earlier for example on Day 20 or 21 of gestation. However, fetuses are protected from high maternal glucocorticoids by placental 11βHSD2 (Seckl, 1997), and so it is also possible that elevated CORT levels would not have been detected during late gestation. In addition, we examined levels of CORT in the whole brain, and thus region-specific changes in CORT might have been obscured in this data.

The fact that CORT administration significantly reduced the length of pregnancy could also play a role in the effects on serum CORT levels of PND1 offspring. Shorter gestational times would result in less time between the last maternal injection and PND1. However, the gestational length was only reduced by half a day, and an analysis of covariance did not reveal a significant effect of time between the last maternal injection and culling on pups' serum CORT levels, but caution must be used because our measure of gestational length was not exact.

Postnatal Administration of High CORT to the Mother Results in Elevated Levels of CORT in the Stomach Milk and Brain of PND7 Offspring

CORT concentrations are significantly increased in the stomach milk of both male and female PND7 pups from CORT-treated dams. Previous studies show a transfer of corticosteroids from the mother to the pups through the milk (Angelucci et al., 1983, 1985), but it is not clear at what age stomach milk was analyzed (Angelucci et al., 1983) or if there were differences between males and females (Angelucci et al., 1983; Yorty et al., 2004). Furthermore, in the previous studies, CORT was administered via the drinking water, which resulted in relatively normal levels of CORT in the dam (Angelucci et al., 1983), and blood and milk samples were collected after anesthesia (Yorty et al., 2004), which may have affected CORT levels (Vahl et al., 2005).

In this study, brain CORT levels on PND7 were significantly higher in offspring from CORT-treated dams. To our knowledge, only one other study has examined CORT levels in the brain of pups after maternal glucocorticoid manipulation (Angelucci et al., 1983). Unfortunately, there are not enough details in that article to compare with our results. For instance, no information is given about how CORT was measured in the brain, the sensitivity of their method, the age, sex, or number of pups that were used, nor is it clear whether control groups were used. Here, we show an increase in CORT levels in the brains of both male and female PND7 pups that were exposed to high maternal glucocorticoid levels relative to vehicle-injected controls.

Postnatal Administration of High CORT to the Mother Does Not Affect Levels of CORT in the Serum of PND7 Offspring During the Stress Hyporesponsive Period

Although increased levels of CORT were seen in stomach milk and brains from PND7 offspring of CORT-treated dams, serum CORT levels were not significantly higher on PND7. This may be due to the fact that PND7 is during the stress hyporesponsive period. During this period, serum CORT levels are extremely low at baseline and show little increase after stress or ACTH challenge (Diez et al., 1976; Sapolsky and Meaney, 1986; Levine, 2002; Yoshimura et al., 2003). In this study, serum CORT levels on PND7 may have been too low to detect a significant difference between groups, perhaps as a result of high clearance of CORT in the bloodstream and/or altered negative feedback during the stress hyporesponsive period (Sapolsky and Meaney, 1986). We examined serum 6 h after the maternal treatment, and thus, an earlier transient increase in serum CORT levels might have been missed in this group because of

the long interval of time between the CORT administration to the dam and the time of the sampling. In contrast, Yorty et al. (2004) reported higher serum CORT levels on PND6 after dams received 200 μg/mL CORT in drinking water (a much lower dose than in this study) in mice. However, they used isoflurane anesthesia for sacrificing the pups, which likely caused an increase in circulating CORT levels (Vahl et al., 2005), whereas our values reflect basal levels of CORT. Further, CORT in the drinking water of the dam can produce discrepant results as slightly higher basal and stress-induced serum CORT levels were observed in PND11 male rat pups (Casolini et al., 1997), but not in PND10 (Angelucci et al., 1983) or PND16 pups (Casolini et al., 1997), of dams receiving CORT in their drinking water. The administration method, dose, and age of the pups likely each play a role in mediating the amount of glucocorticoids observed in the serum of the offspring.

Possible Mechanisms of Altered CORT Levels in the Offspring Across the Postpartum

Although serum CORT levels were not significantly increased on PND7, we found that maternal CORT treatment postpartum caused an elevation of serum CORT in the offspring on PND18. Interestingly, there was no significant difference in serum CORT levels 1 day after weaning on PND22, suggesting that the increase in serum CORT on PND18 might, at least partially, be due to a direct transfer of maternal CORT. This is in line with a study by McCormick et al. (2001) that showed that offspring of dams receiving CORT in their drinking water for 4 days had higher serum levels of CORT at the end of maternal treatment but not at weaning. However, to truly assess whether maternal CORT was transferred to the offspring, we would have to use radioactively labeled CORT. Thus, in this study, we cannot exclude other possibilities besides a direct transfer from the mother to the pup through the maternal milk.

Changes in absorption of CORT, corticosteroid-binding globulin levels, pup adrenal adaptation, or local production of CORT could explain why we see increased levels of CORT in the stomach milk and brain but not in the serum on PND7, and increased levels of serum CORT but not brain CORT on PND18. For example, increased CORT in the stomach milk on PND7 might have suppressed adrenal function of the offspring or increased corticosteroid-binding globulin in the neonatal offspring (Zhao et al., 1997), potentially explaining the lack of an effect on serum CORT levels on PND7. As mentioned above, PND 7 is during the stress hyporesponsive period, which might also explain the low and unaltered CORT levels in the offspring at this time point. Further, the absorption of CORT from the intestine differs with age. In the third week of life, the intestinal tract changes to prepare for the switch from milk to adult food, and glucocorticoid sensitivity of the jejunum ceases abruptly at PND 17-18 (Henning and Sims, 1979). Exogenous CORT might affect the maturation of the intestine and thus the absorption of CORT. Further, a change in prolactin transport from the gastric tract to plasma is seen during development (Whitworth and Grosvenor, 1978), and the prolactin content of the milk influences the behavioral outcome of the offspring (Melo et al., 2009). Taken together, these data suggest that the absorption of CORT from the intestine might change with age and increased CORT exposure.

Glucocorticoid binding to receptors in the brain increases throughout the first 4 weeks of life in the rat (Olpe and McEwen, 1976; Meaney et al., 1985). Similarly, corticosteroid-binding globulin levels rise dramatically from PND12 onward in rats (Henning, 1978), which might limit the access of CORT to the brain during later development. Note that we saw higher levels of CORT in the PFC, hippocampus, and hypothalamus on PND18 compared with the levels for the one hemisphere on PND7. These differences in CORT may be due to either the different regions of the brain used or a natural rise in cerebral CORT levels during development. The observed differences between the brain regions on PND18 (i.e., lower levels of CORT in the hypothalamus compared with hippocampus and PFC) could be due to the lower retention of this hormone in the hypothalamus (McEwen et al., 1968). Further steroids such as dehydroepiandrosterone can be produced locally in the brain (neurosteroids; Baulieu, 1997; Soma et al., 2004; Newman et al., 2008b). Thus, it is possible that our observed increase in brain CORT levels on PND7 or the lack of an increase on PND18 might result from altered endogenous local production of neurosteroids in the brain. Taken together, it is clear that there are developmental changes in the transfer, availability, function, and regulation of CORT, which could explain the differences between PND7 and PND18 and apparent discrepancy between serum and brain levels of CORT.

Developmental Consequences of Elevated Maternal CORT Levels

Elevated maternal glucocorticoids can have long-lasting effects on the offspring. For instance, elevated

maternal glucocorticoids during the postpartum period suppress hippocampal cell proliferation in male offspring at PND30 and change locomotor activity in the open field test in adult males and females (Brummelte et al., 2006). In addition, CORT in the dam's drinking water decreases 5HT1A receptors in the hippocampus of adult offspring (Meerlo et al., 2001) and alters performance in hippocampus-dependent learning and memory tasks (Casolini et al., 1997; Catalani et al., 2002). Therefore, early exposure to CORT can have enduring effects on the hippocampus, in addition to reprograming of the HPA axis.

Maternal care is also affected by glucocorticoids (Rees et al., 2004; Brummelte et al., 2006; Casolini et al., 2007), and changes in maternal care can in turn affect the development and function of the HPA axis in the progeny (Meaney, 2001). Treatment with 40 mg/kg of CORT during the postpartum period reduces maternal care, with dams spending more time off the nest (Brummelte et al., 2006). Therefore, it is conceivable that changes in offspring CORT levels might be mediated, in part, through maternal care.

Measurement of CORT Levels in the Brain

SPE is an alternative to traditional organic solvent extraction methods, as it removes interfering substances such as lipids more efficiently and exhibits better recovery rates (for details see: Newman et al., 2008a). Our high recovery rate and the parallelism between a serial dilution of brain tissue and the assay standard curve confirm the efficacy of our methodology. Washing the samples with 40% methanol increased the removal of interfering substances. To our knowledge, this is the first study to use SPE to measure CORT in the brain of neonatal and postnatal male and female rats.

CONCLUSIONS

Taken together, our data show that elevated maternal CORT levels result in higher levels of CORT in the offspring in an age-, tissue-, and possibly sex-dependent manner. It is plausible that increased serum and brain concentrations of CORT during early development contribute to a reprograming of the HPA axis and result in life-long effects on behavioral and cognitive systems.

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