HHS Public Access

Author manuscript

Psychoneuroendocrinology. Author manuscript; available in PMC 2019 February 26.

Published in final edited form as:

Psychoneuroendocrinology. 2017 August ; 82: 98–106. doi:10.1016/j.psyneuen.2017.05.011.

Intergenerational accumulation of impairments in maternal behavior following postnatal social stress

B.C. Nephew[a], **L.M. Carini**[a], **S. Sallah**[b], **C. Cotino**[b], **R.A.S. Alyamani**[b], **F. Pittet**[a], **S. Bradburn**[b], **C. Murgatroyd**[b,*]

[a]Tufts University Cummings School of Veterinary Medicine, North Grafton, MA, USA

[b]Manchester Metropolitan University, School of Healthcare Science, Manchester, UK

Abstract

Early adversity such as depressed maternal care can have long-term physiological and behavioral effects on offspring and future generations. Exposure to chronic social stress (CSS), an ethologically model of postpartum depression and anxiety, during lactation impairs maternal care and exerts similar effects on the F1 dam offspring of the stressed F0 dams. These changes associate with increased corticosterone and neuroendocrine alterations. CSS F2 offspring further display decreased social behavior as juveniles and adults and decreased basal levels of corticosterone. This current study investigates the intergenerational inheritance of alterations in maternal behavior in F2 CSS dams together with neuroendocrine and immune markers to explore whether aspects of maternal behavior are intergenerationally inherited through immune and neuroendocrine mechanisms. We find that defects in maternal care behavior persist into the F2 generation with F2 dams exhibiting a pervasively depressed maternal care and increased restlessness throughout lactation. This occurs together with reduced basal cortisol (in contrast to an increase in F1 dams), a lack of changes in neuroendocrine gene expression, and reduced serum ICAM-1 (intercellular adhesion molecule-1) levels - a marker for inflammation and blood–brain barrier integrity. The data support the hypothesis that the effects of chronic social stress can accumulate across multiple generations to depress maternal care, increase restlessness and alter basal functioning of the immune system and hypothalamic pituitary adrenal axis.

Keywords

Postnatal depression; Maternal care; Corticosterone; Intergenerational inheritance; ICAM-1; Social behavior

1. Introduction

Social stressors, such as impaired maternal care and maternal depression are associated with adverse behavioral (Lee and Gotlib, 1991) and emotional outcomes (*for review* Beardslee et al. 2011). Children of depressed parents are at risk for developing depressive disorders

*Corresponding author at: School of Healthcare Science, Manchester Metropolitan University, Chester Street, Manchester M1 5GD, UK., c.murgatroyd@mmu.ac.uk (C. Murgatroyd).

themselves and other internalizing and externalizing disorders (Lyons-Ruth et al., 1997; National Research Council (US) and Institute of Medicine (US) Committee on Depression et al., 2009). Whether these effects are transmitted beyond 2 generations is not established with few 3-generation studies of major depression (Olino et al., 2008; Pettit et al., 2008). One study of 800 depressed and never-depressed women together with information from their children (at 15 yrs) and grandmothers detected an intergenerational transmission of depression, whereby the grandmother's depression affected the mother's depression and her own stressful life context, and maternal and grandmother depression affected youth depression. This was mediated by interpersonal stress processes, which in turn affected parenting and children's social functioning (Hammen et al., 2004). A further recent study on 251 sets of grandchildren, parents and grand-parents found those with 2 previous generations affected with major depression were at highest risk for major depression; though the study was too small to test for sex effects (Weissman et al., 2016).

Intergenerational and transgenerational transmission – defined either by the presence or absence of a direct exposure to the stressor of the parental (F0) and subsequent generations – of parental depression involves environmental risk factors and heritable components of depression, and most likely the interplay of these elements. A challenge in clinical studies is proving that parental depression has true environmental risk effects on offspring outcomes, due to the fact that genetic transmission of depression may confound the environmental effects of parental depression. Mendelian randomization and twin studies are advancing our understanding of genetic and environmental contributions toward depression. However, these techniques are yet to be integrated into a transgenerational study design (Natsuaki et al., 2014). As such, most studies based on clinical samples either represent unusually adverse contexts that are difficult to generalize and, for the most part, have not been large enough and/or included multiple collected environmental and biological variables. Animal models allow to control for genetics and the environment. One prenatal stress study in rats, measuring tail chasing behavior as an indicator of postnatal maternal care found a reduction in 3 generations, reflective of inter-generational programming (Ward et al., 2013). Tail chasing is suggested to be indicative of preparations for pup retrieval, where the pregnant female is attempting to retrieve her tail to the nest area. To our knowledge, we are the first to investigate intergenerational inheritance of the effects of postnatal stress on maternal behavior across 3 generations, in clinical or animal studies.

The chronic social stress (CSS) rat model, which has a robust impact on maternal behavior, allows for the intergenerational testing of the effects of maternal social stress across three generations, F0–F2. Exposure of F0 rat dams to chronic stress during lactation impacts maternal behavior including reductions in pup grooming and nursing and increased maternal aggression (Murgatroyd et al., 2015a; Nephew and Bridges, 2011). The female F1 generation of the CSS F0 dams also show decreases in maternal care accompanied by impaired lactation, decreased saccharine intake (a measure of abstract anhedonia), decreased maternal aggression and increased restlessness/anxiety-related behaviors (Carini and Nephew, 2013; Murgatroyd and Nephew, 2013; Murgatroyd et al., 2015a,b). These behavioral effects are associated with decreases in hypothalamic Oxytocin (*Oxt*), Vasopressin (*Avp*), and Prolactin (*Prl*) gene expression (Murgatroyd and

Nephew, 2013), as well as decreases in basal plasma concentrations of estradiol and PRL and increased corticosterone during lactation (Carini and Nephew, 2013).

Analysis of the F2 females as juveniles and as adults also reveal altered social behavior, specifically diminished allogrooming during adult social interactions. Allogrooming is a common form of direct mammalian social contact that is necessary for establishing mammalian social bonds at multiple life history stages (Insel, 1997). This was accompanied by decreases in basal corticosterone, elevated juvenile Oxt and decreased adult Prl (Babb et al., 2014) and changes in several immune markers, particularly a significant reduction in inter-cellular adhesion molecule-1 (ICAM-1), a cell-adhesion molecule expressed on macrophages, lymphocytes and endothelial cells which plays an important role in immune-mediated cell–cell adhesive interactions and blood–brain-barrier (BBB) permeability (Murgatroyd et al., 2016). This suggests that the CSS has long-term generational effects on the immune system together with the changes in behavior and the hypothalamic–pituitary–adrenal (HPA) axis.

The behavioral changes in F2 juveniles and adults indicate that F0 CSS effects multiple generations. The developmental elements of the CSS model include the potential effects of behavioral and hormonal or immune changes in the F0 and F1 dams on F2 dams; either through pup-dam interactions, milk constituents, or germline exposure of the F2 generation during early-life stress exposure in the F1 animals. The current study investigated the generational inheritance of alterations in maternal behaviors in F2 CSS dams together with endocrine, immune, gene regulation and epigenetic markers to investigate whether aspects of maternal care are intergenerationally inherited through potential immune and neuroendocrine mechanisms.

2. Methods and materials

2.1. Animals and CSS model

Sprague-Dawley rats (Charles River Inc., Kingston, NY) in this study were maintained in accordance with the guidelines of the Committee of the Care and Use of Laboratory Animals Resources, National Research Council, and the research protocol was approved by the Tufts Institutional Animal Care and Use Committee.

The F0 CSS dams were subjected to a CSS protocol from days 2 to 16 of lactation involving exposure to a novel male intruder for one hour each day (Carini et al., 2013; Nephew and Bridges, 2011). The control and CSS F1 dams were the offspring of the F0 control and CSS dams; the differences between the treatments of the control and early-life CSS F1 females were limited to the exposure of the early-life CSS F1 females to attenuated maternal care and conflict between their F0 mothers and the male intruders during age 2–16 days. The F1 control and early-life CSS animals were treated identically after the age of 16 days. After weaning all F1 pups on day 23, the female offspring from the twelve control and twelve CSS dams were housed in groups of four until 70 days old when two from each litter were mated with 6 proven breeder males. Total F2 pup number and litter weights were recorded on the day of parturition, and litters were then culled to four females and four males. The F2 control and CSS animals were treated identically throughout the study; the only difference

between the two groups was the attenuated maternal care and increased restlessness and anxiety-related behavior expressed by their respective CSS and control F1 dams (Carini and Nephew, 2013). Importantly, there were no treatment effects on litter size or bodyweights of the F2 pups (day 2 of lactation: F2 litter size, control 15.6 ± 0.5, CSS 15.8 ± 10.4, $p = 0.7$; F2 mean pup body-weight (g), control 6.8 ± 0.2, CSS 6.9 ± 0.2, $p = 0.7$; F1 dam body-weight (g), control 340.9 ± 10.4, CSS 345.3 ± 8.0, $p = 0.7$) juvenile or adults (all p's > 0.2) (Carini and Nephew, 2013).

All F2 dams were euthanized on day 23 of lactation; brains were extracted and stored at −80 °C until micropunched, relative to bregma, for PVN (bilateral Ø0.5 mm, −2.0 to −1.5, 0.5 mm lateral to midline, SON (bilateral Ø0.5 mm, −1.5 to −1.0, 1.8 mm lateral to midline) and hippocampus (bilateral Ø1.0 mm, −1.8 to −1.3, 2.5 mm lateral to midline) and trunk blood was collected for serum. The same F2 animals were used for all brain, cytokine and immune assays. Finally, the design of this model ensures that the F1 and F2 generations derive from 10 separate dams each for CSS and control at each generation with only 1–2 pups taken from each litter to avoid litter effects. See Fig. 1 for an overview of the intergenerational model.

2.2. Maternal care and aggression testing

Maternal care and maternal aggression were assessed in all F2 dams between 0800 and 1000 h on days 2 (early), 9 (mid), and 16 (late) of lactation, as previously described for F1 dams (Carini et al., 2013; Murgatroyd and Nephew, 2013). After a 1-h pup removal, maternal care testing was performed, which included reintroduction of all pups into the home cage and video recording of the dam for 30 min. Frequencies and durations of pup retrieval, pup grooming, nursing, nesting, self-grooming, and general locomotor activity were scored by an observer who was blind to the treatment, using ODLog behavioral analysis software (Macropod Inc.). Scoring of nursing behavior was started when the dam had been motionless over the litter for longer than 10 s and stopped whenever she moved off the pups. Nursing duration is the cumulative nursing behavior during the 30-min maternal care observation. Total maternal care included the combined durations of pup grooming and nursing. Nesting was defined as manipulation of the nesting material with the mouth or paws. The expression of elevated levels of self grooming and locomotor activity (which have been associated with chronic social stress induced anxiety in other models (Denmark et al., 2010; Venzala et al., 2012)) during maternal care testing is referred to as maternal restlessness. Following the 30-min maternal care video recording, the pups were left undisturbed for another 90 min (without video recording) to provide an undisturbed 2-h nursing period to assess milk intake by weighing the litter.

Following the maternal care testing and nursing period, the pups were weighed, returned to the cage, and a similarly sized (225–300 g) novel virgin male Sprague Dawley intruder was introduced for a 30-min maternal aggression test. Dams were never exposed to the same male twice. The latency to initiate aggression and the frequency and durations of attacking (boxing or tackling), biting, kicking, pinning to the bottom of the cage, self-grooming, and locomotor activity were scored. Total aggression consisted of the combined durations of attacks, bites, kicks, and pins. Importantly, both controls and CSS were tested for maternal

aggression on days 2, 9, and 16 of lactation while only CSS F0 dams were chronically exposed to male intruders each day from days 2 to 16.

2.3. Saccharin intake

At 1600, the day before maternal behavioral testing, 0.02% saccharin bottles were added to the cages (alternating between right and left side of the cage with each animal) and percentage intake of saccharin versus water in a 16-h period was measured by weighing the bottles at 0800 on testing days (lactation days 2, 9, 16) and dividing the weight of saccharin intake by total fluid intake.

2.4. Milk

Milk intake was calculated by subtracting the litter weight at the start of maternal care observation from the litter weight 2 h later. Milk corticosterone levels were measured, using a commercial RIA kit (Siemens), from pups (one per litter, per day, using the same litters as for the behavior) killed by decapitation at the end of the nursing period on days 2, 9, and 16, and stomach contents collected through an incision at the anterior end of the stomach.

2.5. RNA expression analyses

Total RNA was extracted from paraventricular nucleus (PVN) and supraoptic nucleus (SON) (Bettscheider et al., 2011) and reverse transcription reactions (Bioline) were performed on 200 ng RNA using random primers. Quantitative PCR (qPCR) was performed on a StepOne Plus (Applied Biosystems) using SensiFast SYBR Green (Bioline). Primer sequences and conditions for qPCR reactions are as previously described (Murgatroyd et al., 2015a). Expression levels for *Oxt*, *Oxt receptor* (*Oxtr*), *Avp*, *Avp V1a receptor*, the long form of the *Prl receptor*, GR (*Nr3c1*) and MR (*Nr3c2*) were normalized against three combined housekeeping genes, *β-actin, hypoxanthine phosphoribosyltransferase (Hprt) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh)*.

2.6. DNA methylation analysis

Genomic DNA was extracted from hippocampi, as previously described (Bettscheider et al., 2011) from hippocampus and 200 μg DNA was bisulfite-modified using the EpiTect Bisulfite Kit (Qiagen). Amplification of a 137-bp region within the rat Nr3c1 promoter was performed with specific primers (F-biotin TTGGTTTGGGAGGGAAAT; R-AACTATCCCCTCCAAAACTCTAACTAC), using the PyroMark PCR Kit (without Q solution); the conditions were: 40 cycles of 94° C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. Single- stranded biotinylated product was purified by mixing 10 μl of the amplification mixture, 2 μl of streptavidin sepharose HP (Amersham Biosciences), and 40 μl of binding buffer. The sepharose beads containing the immobilized biotinylated product were purified, washed, and denatured in 0.2 mol/l NaOH and washed again using the Pyrosequencing Vacuum Prep Tool (Qiagen). The biotinylated DNA was resuspended in 12 μl of annealing buffer containing 0.3 μmol/l pyrosequencing primer (CCCT-CCAAAACTCTAACTACC). DNA methylation of seven CpG residues within the Nr3c1 gene promoter (RGSC 5.0/rn5; chr18: 32351349–32351579) were quantified by pyrosequencing using the PSQ 24MA

system with the PyroGold SQA reagent kit (Qiagen). The percentage methylation for each of the CpG sites was calculated using Pyro Q-CpG software (Qiagen).

2.7. Immune and Endocrine Assays

Assay panels of selected (following results in F2 adults (Murgatroyd et al., 2016)) pro- and anti-inflammatory cytokines (GM-CSF, ICAM-1, IFN-gamma, IL-10, IL-6, TNF-alpha, VEGF) (R & D Systems) and a Milliplex MAP Rat/Mouse Neuropeptide Mag Bead Panel (Acth, Bdnf, TSH and prolactin) (Millipore Ltd) were analyzed on a Luminex 200 Bio-Plex Platform. Samples were thawed directly on the day of analysis. Working wash solutions and protein standards were prepared within 1 hr of beginning the assay. A magnetic plate washer was utilized during washing stages. Following processing, protein concentrations were calculated and analyzed with the xPONENT software (Luminex, v.3.1.871). The majority of the samples were run in duplicate in an individual assay to eliminate interassay variation. Oxt was measured by commercial ELISA (Enzo Life Sciences).

2.8. Statistics

Behaviors and hormone levels in control vs. CSS F2 dams were compared with 1- or 2-tail *t*-tests, with individual lactation days being assessed independently as in previous studies due to differences in the expression, relevance, and interpretation of maternal behaviors across days (e.g., pup retrieval on day 2 vs. day 16). A priori decreases in maternal care and serum markers in previous studies of the CSS F0 and F1 dams and F2 adults females (Carini and Nephew, 2013; Nephew and Bridges, 2011; Murgatroyd et al., 2016) were used to justify the use of one-tailed *t*-tests for behaviors and immune markers. All assumptions of normality and equality of variance between groups were met. All graphical results are presented as mean + SEM, and the level of statistical significance was $p \leq 0.05$.

3. Results

3.1. Maternal behavior testing and milk intake

There were significant differences in total maternal care on all three days of lactation with F2 CSS dams (i.e. the 3rd generation of CSS-exposed dams) showing reduced levels (1-tail t-test, d2, $P = 0.0185$; d9, $P = 0.004$; d16, $P = 0.0034$) (Fig. 2A). This was reflected in F2 CSS dams spending significantly less time grooming their pups on days 2, 9 and 16 (1-tail *t*-test, d2, $P = 0.045$; d9 $P = 0.004$; d16 $P = 0.009$) (Fig. 2B) and a reduction in F2 CSS dam nursing on days 9 and 16 (1-tail *t*-test, d2, $P = 0.13$; d9, $P = 0.044$, d16, $P = 0.033$) (Fig. 2C). There was significantly reduced duration of nesting in the F2 CSS on day 9 (1-tail *t*-test, d2, $P = 0.225$; d9, $P = 0.023$; d16, $P = 0.075$) (Fig. 2D). There were no significant differences in time spent retrieving pups back to the nest (1-tail *t*-test, d2, $P = 0.41$, d9, $P = 0.14$; d16, $P = 0.44$). Regarding maternal restlessness behaviors, there was a significant elevation in self-grooming on day 16 (1-tail *t*-test, d2, $P = 0.17$; d9, $P = 0.45$; d16, $P = 0.037$) (Fig. 2E) and increased locomotor activity on days 2 and 16 (1-tail *t*-test, d2, $P = 0.027$; d9, $P = 0.3$; d16, $P = 0.028$) (Fig. 2F). Total maternal aggression did not differ between CSS and control dams (1-tail *t*-test, d2, $F1,29 = 0$, $P = 1.0$; d9 $F1,30 = 1.6$, $P = 0.17$; d16; $F = 0.01$, $P = 0.92$).

Milk intake by the F3 pups of the F2 control and F2 CSS dams did not significantly differ on days 2, 9 or 16 (2-tail *t*-test, d2, $F1,31 = 1.13$, $P = 0.34$; d9, $F1,31 = 0.06$, $P = 0.1$; d16, $F1,31 = 0.34$, $P = 0.15$). There were also no significant differences in saccharin preference between F2 CSS and Control Dams during lactation (2-tail *t*-test, d2, $F1,31 = 0.95$, $P = 0.3$; d9, $F1,31 = 0.10$, $P = 0.8$; d16, $F1,31 = 0.15$, $P = 0.56$).

3.2. Endocrine measures

Levels of milk corticosterone, taken at day 16 from F2 pups were reduced in the CSS group ($F1,22 = 7.6$, 2-tail *t*-test, $P = 0.01$) (Fig. 3A). Serum levels of corticosterone were also significantly reduced in the CSS F2 dams compared to the controls at day 23 of lactation (2-tail *t*-test, $P = 0.03$) (Fig. 3B). There were no significant correlations between corticosterone in the milk from F1 Dams and in the serum of F2 Dams ($P = 0.965$, Pearsons = −0.11) or between serum corticosterone levels in F1 (from Carini and Nephew, 2013) and F2 dams ($P = 0.199$, Pearsons = 0.241). There were no significant differences in serum levels of ACTH, BDNF, Prolactin or TSH between F2 CSS and control Dams (Fig. 3C). Oxt levels also did not differ between the F2 CSS and controls ($P = 0.37$) (Fig. 3D).

3.3. Neuroendocrine gene expression

There were no significant differences in the expression of Oxt, Oxtr, Avp, MR or GR in either the PVN or SON between F2 CSS and control Dams (Fig. 3E and F).

3.4. Epigenetic regulation of Nr3c1

Methylation was investigated in the hippocampus. The seven CpGs studied cover Exon 1.7 promoter of the Nr3c1 gene (CG(1)ACCCACG(2)GGGCG(3)GGCTCCCG(4)AGCG(5)GTTCCAAGCCT-CG(6)GAGCTGGGCG(7)GGG) with CpG2 and CpG3 spanning a previously described NGF1-A binding site with CpG2 being specifically regulated in a rat model of maternal care (Weaver et al., 2004). There were significant reductions in DNA methylation at multiple CpG sites across the Nr3c1 promoter 1.7 in hippocampi of the F1 CSS dams compared to F1 control. Average methylation across the region was also significantly reduced. There were no significant differences in hippo-campus CpG methylation between the F2 control and CSS dams. DNA methylation also did not significantly differ between the two generations of controls (Fig. 4A and B) and there were no associations between average DNA methylation and cortisol for either group in either generation (F1 Con $r = -.308$, $p = .331$; F1 CSS $r = -.042$, $p = .887$; F2 Con $r = -.154$, $p = .633$; F2 CSS $r = -.327$, $p = .185$).

3.5. Immune markers

Serum levels of ICAM-1 were significantly lower in F2 CSS Dams compared to controls (1-tail *t*-test, $P = 0.049$) (Fig. 5D). However, levels of ICAM-1 in the F1 Dams were not significantly lower (1-tail *t*-test, $P = 0.22$) (Fig. 5A). Levels of GM-CSF, IL-6, IL-10 and VEGF did not significantly differ between CSS and controls in either the F1 dams or the F2 dams (Fig. 5). In light of the significant reductions of ICAM-1 in the F2 adults and F2 dams, the F0 Dams were tested. This revealed no significant difference between CSS and control ($P = 0.9$) (data not shown).

4. Discussion

Our results demonstrate that exposure of F0 dams to CSS induces significant deficits in maternal care that persist into the F2 generation. This provides support for the intergenerational transmission of mater-nal care behavior (Curley et al., 2012). When comparing these results with our previous studies from F0 and F1 dams we find that the maternal care in the F2 dams is depressed during all stages of lactation, i.e. early, mid and late (days 2, 9 and 16) in F2, compared to day 9 and day 2 only in the F0 and F1 dams respectively (Table 1). This suggests that the duration of depressed maternal care increases with generations. Where both the F0 and F1 dams returned to expressing typical control levels of maternal care by mid or late lactation, F2 dam maternal care was depressed throughout lactation. We see a similar pattern in the increased maternal restlessness with more extensive effects in the F2, where increased non-maternal behavior expression during maternal care testing is confined to mid and early lactation days in the F0 and F1 dams, respectively. While it is possible that the depressed maternal care and increased restlessness are related, the expression of these behaviors during the 30 min maternal care observations was not temporally limited and an increase in maternal care does not require an increase in restlessness. Since these F2 offspring were never directly exposed to the CSS paradigm, we postulate that it is the reduced levels of maternal care of the F1 dams, particularly during early lactation, (Carini and Nephew, 2013) that mediate the intergenerational effects of CSS on the F2 maternal care behavior. Importantly, while there are numerous factors that may introduce variation in studies across several years, and this are always a concern. Therefore, many factors have been carefully considered in the CSS studies, including using the same rodent supplier, training and controlling behavioral observation scorers and consistency in handling by experimental and animal husbandry staff. Further, the behavioral testing paradigms focus on robust, easily identifiable duration measures, such as pup grooming and nursing, over a suffcient observation period. Excessive behavioral testing, as well as blood sampling, is avoided to limit the introduction of unwanted confounds from the procedures. Maternal behavior testing is conducted in the same room where the animals are housed, and these are used solely for the CSS study. Behavioral observations are always done in home cages, eliminating the potential confounds associated with a novel environment. Perhaps most importantly, the novel male intruder stress is ethologically relevant and has substantial, reliable, and repeatable effects on the behavior of dams and future generations.

Current clinical research indicates that chronic maternal depressive states tend to associate with hypocortisolemia (e.g. Groer and Morgan, 2007), while hypercortisolemia is found more with immediate post-partum mood states (e.g. Ehlert et al., 1990) (for review Seth et al., 2015). Within this study, the reduced basal corticosterone in the F2 dams, together with depressed maternal care and increased restlessness persisting across the entire span of lactation support the above hypothesis in regards to chronic maternal depressive states. This is in contrast to the increased corticosterone and more attenuated duration of behavioral changes we previously reported in the F1 and F0 dams (Murgatroyd and Nephew, 2013). Hypocortisolinemia has also been previously observed after gestational social stress in dams and their female offspring, in a paradigm that likewise leads to depressive-like behavior (Cordero et al., 2012). In a clinical study with strong parallels to the current

project, the early-life stress of postnatal depression exposure combined with family conflict (similar to the F1) induced elevated basal cortisol levels in children, where exposure to the early-life stress of postnatal depression alone results in decreased basal cortisol (Essex et al., 2011). In regards to mechanisms, it has been postulated that an initial stress-induced hypercortisolemia may trans- form over time during chronic stress into hypocortisolemia, to protect the brain and metabolic processes from prolonged exposure to excess cortisol levels (Fries et al., 2005; Miller et al., 2007). This highlights that HPA axis function is dependent on length of exposure to stress and nature of the stressor, and in the case of the CSS generations, that HPA axis function may depend on duration of exposure to depressed maternal care and restlessness.

Mechanisms underlying the intergenerational impact of CSS on F2 dam hypocorticosolemia may relate to the reduced maternal care that the F2 dams received as pups. It is possible that the exposure of the control animals to maternal aggression made it more difficult to detect effects of CSS on some variables due to ceiling effects, but we have not observed this in previous studies of the CSS model using the same protocol. Cross-fostering experiments would be needed to dissect postnatal maternal care from prenatal endocrine and lactrocrine mechanisms. Nursery-reared Rhesus monkeys exposed to reduced parental care have lower basal cortisol levels compared to mother raised controls (Capitanio et al., 2005), while human offspring exposed to the early-life stress of maternal depression have lower basal cortisol concentrations compared to control populations exposed to moderate levels of early-life stress (Essex et al., 2011; Morrison et al., 2016). This is further supported by rodent studies demonstrating attenuated corticosterone levels in response to early-life exposure to maternal separation (e.g. Faure et al., 2006; Morrison et al., 2016).

A potentially related finding is the lower milk corticosterone levels of F1 CSS dam mothers, i.e. F2 dams received lower levels as pups. Interestingly, juvenile offspring of dams given corticosterone in the drinking water also display decreased basal corticosterone concentrations (McCormick et al., 2001), similar to the effect observed in the F2 offspring in the present study, where their F1 mothers exhibited elevated levels. Several other studies in rodents have shown that ingestion of glucocorticoids via mother's milk has beneficial programming effects in offspring that persist into adulthood, including better spatial memory (e.g. Casolini et al., 1997) and reduced anxiety and altered HPA axis response to stress (Catalani et al., 2000). This suggests that the reduced corticosteroid in the F1 CSS milk could mediate the depressed maternal care, increased restlessness and hypocorticosolemia of the F2 dams.

The relative lack of neuroendocrine changes in F2 dams also contrasts with results from previous studies of F0 and F1 dams. F0 dams had reductions in amygdala Oxt (Murgatroyd et al., 2015b), while the CSS F1 dams show reduced levels of Prolactin together with reduced activities of genes coding for PRLR, Oxt, Oxtr and Avp in the PVN, SON and amygdala (Murgatroyd and Nephew, 2013) (Table 1). This may relate to the lack of changes in maternal aggression in the F2 dams, compared to F0 and F1 dams, with respect to the role of OXT and AVP in this behavior (Bosch, 2013), as these changes may be more directly involved with changes in maternal aggression. Furthermore, the suppressed milk secretion (as measured by pup intake) within the F0 and F1 dams is no longer evident in

the F2 mothers and may be mediated by the lack of differences in PRL levels. However, the normalization of these endocrine factors do not explain the F2 dam's increased levels of depressed maternal care and restlessness; behaviors that are known to be associated with these neuropeptides (Bosch, 2013) as well as with changes in F2 juveniles and adults (Carini and Nephew, 2013). It is possible that changes in receptor levels or other neuroendocrine systems play more of a role in the F2 dam behavior distinct to the F0 and F1, and/or that changes in corticosterone, OXT, AVP, and/or PRL at earlier life history stages program behavior changes that are not dependent on differential plasma levels or these particular genes at the maternal stage (Bales et al., 2007).

We next tested the hypothesis that DNA methylation in the 1.7 promoter region of Nr3c1 might be altered by alterations in maternal care. Previous studies have demonstrated epigenetic mechanisms underlying transmission of early-life stress across generations of male rats (Franklin et al., 2010) and studies have shown CpG sites in male rat offspring exposed to high licking and grooming are hypomethylated when compared with those reared with low licking and grooming (Weaver et al., 2004). Interestingly, we only find significant differences in methylation in between in CSS and control in the F1 animals and not in the F2. Furthermore the lower levels of Nr3c1 exon1.7 DNA methylation in the F1 CSS dams appear contrary to other previous studies (e.g. Weaver et al., 2004). However, most other studies have focused on male rat offspring; we focused on female offspring and particularly dams. One other study on female rats exposed to high licking and grooming also found hypermethylation of particular CpG sites in the highest licked offspring of high licking dams relative to the lowest licked offspring among low licking dams (Pan et al., n.d.). This may relate to the higher baseline corticosterone levels that females have been shown to have (Beiko et al., 2004). One human study has reported a prenatal stress and gender interaction, whereby exposed males had heightened NR3C1 methylation and females had hypomethylation (although this did not reach significance in females) (Braithwaite et al., 2015) in line with the emerging idea that there may be gender-specific mechanisms that mediate effects of early life stress (Glover Vivian, 2012). The current variation in DNA methylation at Nr3c1 exon1.7 between the maternal environments of the F1 and F2 is hypothesized to mediate the differences in basal corticosterone and pattern of depressed maternal care between the two generations of dams.

The social signal transduction theory of depression proposes that exposure to social adversity, especially during early life, alters immune responses, and these changes mediate depression symptoms (Slavich and Irwin, 2014). Stressors during the perinatal period can induce persistent changes in the immune system and offspring behavior (Bilbo and Schwarz, 2012). For example, deficient maternal care alters immune associated gene expression (Cole et al., 2012). A further rodent study has shown that chronic perinatal stress can alter BBB function (Gómez-González and Escobar, 2009). It has therefore been postulated that inflammation may mediate the adverse effects of early-life stress on mental health (Carpenter et al., 2010; Danese et al., 2007). Analysis of immune markers in F2 adults revealed reduced serum levels of ICAM-1 in CSS animals, though this did not differ in F1 or F0 dams. ICAM-1 levels have been positively associated with stressed-induced cortisol levels (Heinz et al., 2003) so the reduction seen in the F2 animals may relate to the hypocorticosolemia. A marker of inflammation that is involved in regulating BBB function

and structure, decreased ICAM-1 serum levels have been found in schizophrenia (Schwarz et al., 2000) and there is decreased ICAM-1 gene regulation in choroid plexus in depression and suicide cases compared to controls. The current alteration in ICAM-1, specific only to the F2 generation dams and adults would again support the accumulation of stress theory through the F0 to F2 dams.

The current data present several avenues to explore in future studies. Three major factors involved in the extensive depression of F2 maternal care are direct transmission of maternal care, the related effects of CSS on endocrine pathways (including understudied lacto-trine programming), and the differential exposures to intruder and deficient maternal care stressors at specific developmental stages (germline, gestational, early life, juvenile, adult). The presence of depressed maternal care throughout F2 lactation may be due to F2 exposure to all three of these factors at some stage. Implications for future generations, such as F3, could include either a similar pattern of maternal care in the F3's due to programming by F2 maternal care, or a relative increase in maternal care. The lack of changes in several F2 dam endocrine factors and maternal aggression, which were altered in earlier generations, may indicate that F3 and future generations will display increased maternal care. Multigenerational models such as the CSS paradigm also represent an ideal framework to study multi-hit models (Feigenson et al., 2014) and the match/mismatch hypothesis (Santarelli et al., 2014), which states that early life experiences prepare individuals for similar environments at later stages, due to the natural developmental variation in stress exposure and opportunities for a variety of stress combinations. Exploring these avenues may elucidate transgenerational mechanisms underlying changes in overall prevalence rates of stress-associated disorders. Finally, it should be considered that, due to the nature of this study, the behavioral data for the F0–F2 studies were performed at different times. Though this was all performed in the same animal house we cannot …

This study reports the pervasive depression of maternal care and increased restlessness in the F2 offspring of dams exposed to chronic social stress, supporting the accumulation of intergenerationally-inherited stress. The presence of hypocorticosolemia and increased levels of a marker of inflammation in the F2 dams suggest environmentally sensitive mechanisms functioning to regulate maternal behavior and responses to varying levels of chronic and acute stress. Evidence of the pervasive effects of stress across generations in the etiology of stress-associated disorders has significant implications for future research on the prevention and treatment for these maladies. A better under-standing of intergenerational etiology will improve efforts at prevention as well as allow for the improvements in biological marker and patient history informed efforts at enhanced personalization of treatments.

Acknowledgments

The authors would like to thank the Tufts University Cummings School of Veterinary Medicine Laboratory Animal Medicine Services for providing exceptional care for the study animals and cooperative logistical support for the experiments. The work in this study was supported by NIH/NICHD Award R00 HD056643, a Tufts CTSI Award UL1 RR025752 and a Tufts University Collaborates Award to BN and a Manchester Metropolitan University accelerator grant to CM.

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Fig. 1.

Schematic of the 3-generational chronic social stress model for maternal behavior. F0 Dams are exposed to stress during lactation (red box). Her offspring (F1) are then allowed to grow and their offspring (F2) are tested for maternal behavior (MB) and endocrine and immune measures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

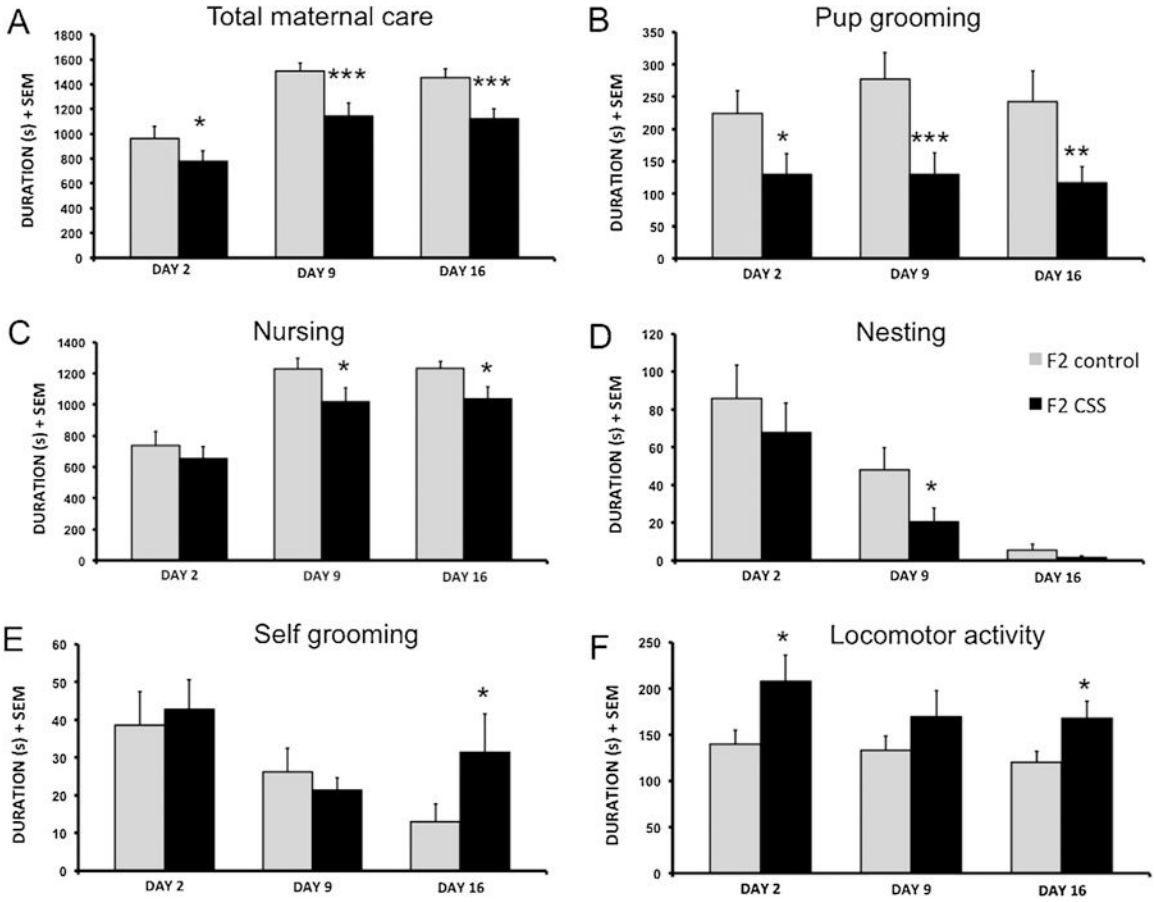

Fig. 2.

Behavior and maternal care. Mean + SEM of duration of total maternal care (pup grooming, nursing) (A), pup grooming (B), nursing (C), nesting (D), self-grooming (E) and locomotor activity (F) on days 2, 9, and 16 of lactation during a 30 min maternal care observation in control and early life CSS (stress) dams. Significant effects of treatment using a 1-tailed *t*-test are indicated $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

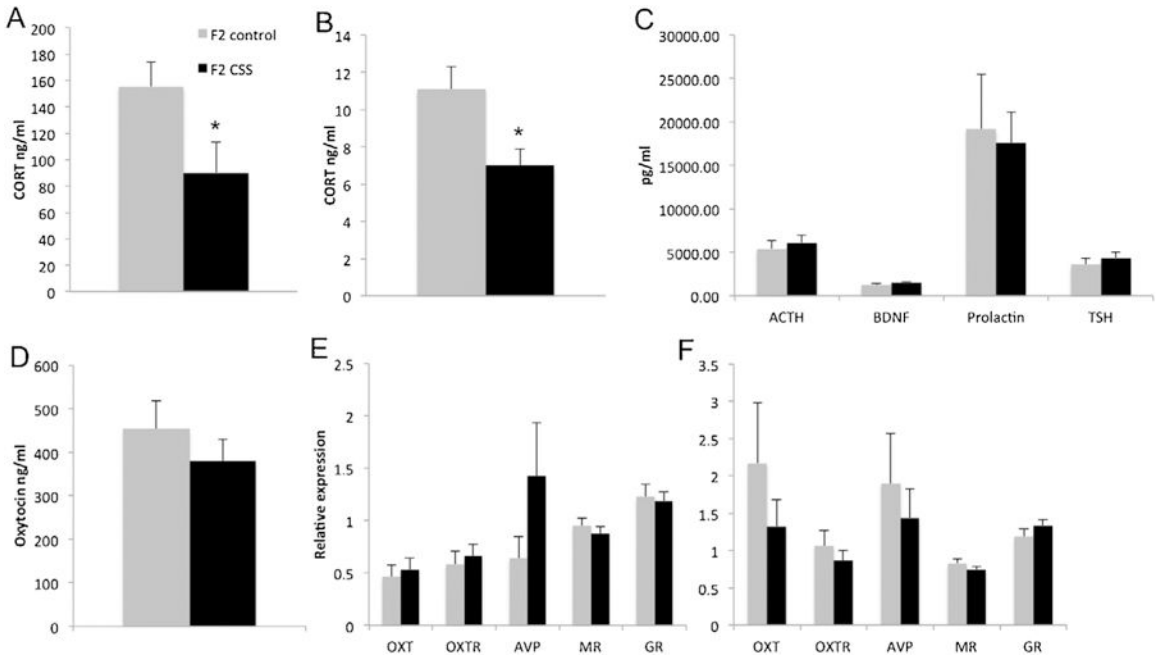

Fig. 3.

Neuroendocrine analysis. Mean + SEM of corticosterone levels in milk from F2 offspring at day 16 (A), corticosterone levels in serum from F2 dams at day 23 of lactation (B), serum levels of ACTH, BDNF, prolactin TSH (C) and OXT (D), and relative mRNA expression levels of *Oxt*, *Oxtr*, *Avp*, *MR* and *GR* in the PVN (E) and SON (F) of F2 CSS and control dams. Significant effects of treatment using a 2-tailed *t*-test are indicated $*P < 0.05$. $n = 13$ per group.

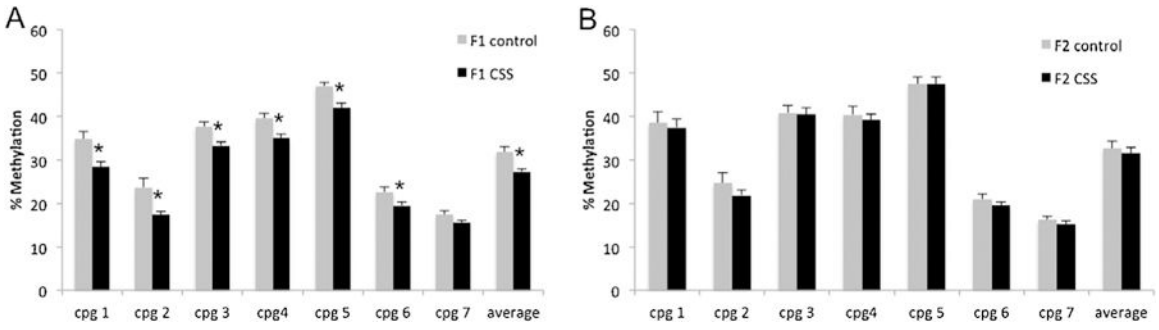

Fig. 4.

Glucocorticoid receptor methylation. Mean + SEM of DNA methylation and at Nr3c1 promoter 1.7 in F1 (A) and F2 (B) control and CSS dams. Significant effects of treatment using a 2-tailed *t*-test are indicated $*P < 0.05$. $n = 14$ control, 14 CSS (F1 group) and 13 control, 13 CSS (F2 Group).

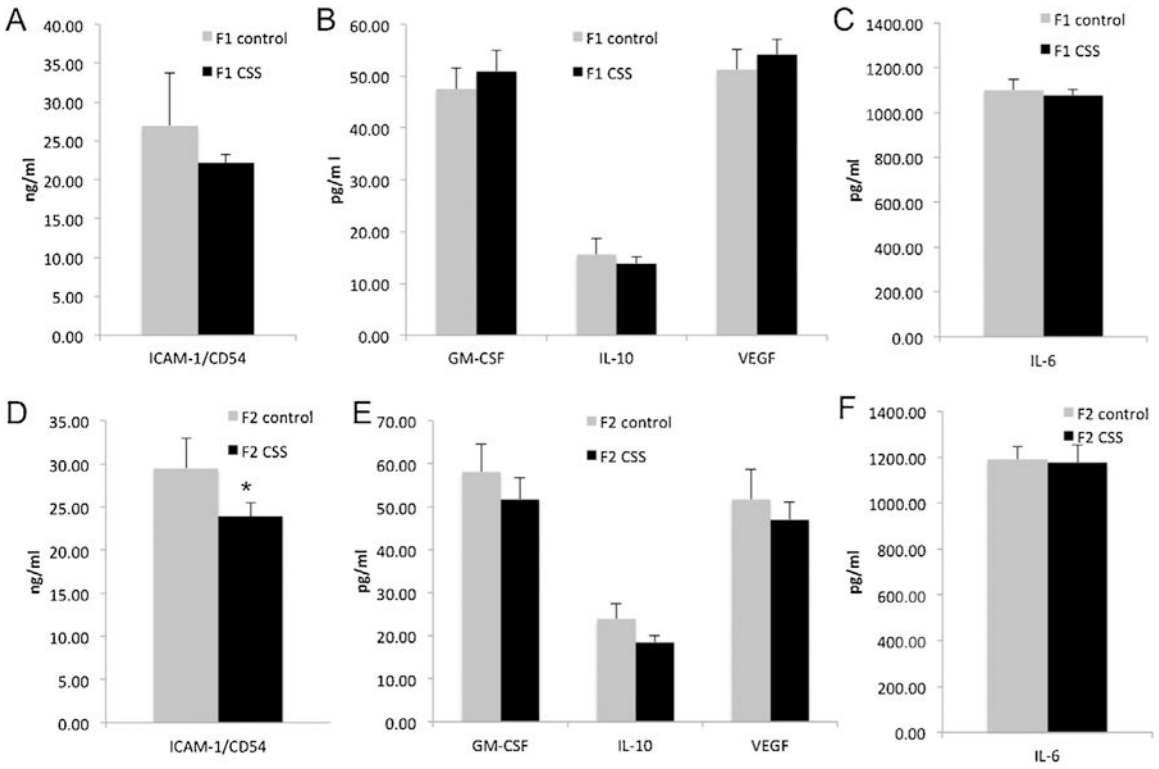

Fig. 5.

Immune markers. Mean + SEM of serum levels in F1 CSS and control dams of ICAM-1 (A), GM-CSF, IL10, VEGF (B) and IL6 (C). Serum levels in F2 CSS and control dams of ICAM-1 (D), GM-CSF, IL-10, VEGF (E) and IL-6 (F). Significant effects of treatment using a 1-tailed *t*-test are indicated $*P < 0.05$. $n = 6$ Control, 10 CSS (F1 group) and 10 Control, 13 CSS (F2 group).

Table 1

Transgenerational transmission of Maternal measures through F0 to F2 dams.

Maternal factors	F0 (§Nephew and Bridges, 2011; §§Murgatroyd et al.,2015a,b)			F1 (§Carini and Nephew, 2013; §§Murgatroyd and Nephew,2013)			F2		
	Day 2	Day 9	Day 16	Day 2	Day 9	Day 16	Day 2	Day 9	Day 16
Maternal care		§		§					
	–	↓	–	↓	–	–	↓	↓	↓
Maternal restlessness		§		§					
	–	↑	–	↑	–	–	↑	–	↑
Maternal aggression		§		§					
	–	↑	↑	↑	–	–	–	–	–
Milk intake		§		§					
	–	↓	↓	↓	–	–	–	–	–
Corticosterone (Day 23)	§§			§§					
	–			↑			↓		
Prolactin (Day 23)	§§								
	–			↓			–		
ICAM-1 (Day 23)	–			–			↓		
Gene regulation	§§			§§					
	PVN: ↑GR			PVN, SON, CeA, MeA: ↓OXT PVN: ↓OXTR, PRLR, AVP			–		
	MeA: ↓OXT								
Nr3c1 exon 1.7 methylation	§§			§§					
	PVN: ↑CpG2			PVN: ↑CpG3					
				Hipp: ↓ CpG1–6			–		