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Within- and Between-Litter Maternal Care Alter Behavior and Gene Regulation in Female Offspring

Pauline Pan, Alison S. Fleming, Daeria Lawson, Jennifer M. Jenkins, and Patrick O. McGowan
University of Toronto

Rat dams show natural variations in maternal care, licking and grooming (LG), that are associated with distinct behavioral and neural phenotypes in offspring. However, there has been limited research on the effects of differences in LG received by female pups and of variations in maternal care within the litter. Here, we investigated LG received by measuring active maternal care after pup retrieval of female offspring. We then examined locomotor activity, open field exploration, and restraint stress reactivity in adult female offspring. We also investigated the expression of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) and DNA methylation of the GR1₇ promoter in the hippocampus. High compared with low LG siblings and female offspring from high compared with low LG dams showed increased locomotor activity. High compared with low LG siblings also showed reduced anxiety behavior regardless of the overall level of LG received in the litter. Unexpectedly, both the lowest licked offspring from low LG litters and the highest licked offspring from high LG litters showed suppressed corticosterone (CORT) responses to stress. However, high LG offspring within litters also showed increased expression of the GR gene, which was negatively correlated with the CORT response to restraint. DNA methylation at 2 CpG sites within GR1₇ promoter was significantly higher in high LG offspring. These differences in the response to maternal care both within- and between-litters were distinct in part from previous reports of between litter effects, potentially a result of the sex studied or the methods used to observe maternal care.

Keywords: epigenetics, gene expression, glucocorticoid receptor, hippocampus, maternal care

Parental care has a major impact on a child's behavioral and brain development (Belsky & de Haan, 2011; Tolan, Dodge, & Rutter, 2013). This is true not only in the context of pathology, as in instances of severe abuse, neglect, or family conflict (Andersen et al., 2008; McGowan et al., 2009), but also in the context of variations of parenting style within the normal range (Barrett & Fleming, 2011; Ellis & Boyce, 2011; Maccoby, 2000; Whittle et al., 2009). Research in this area indicates that children receive

different amounts and kinds of parental attention and, as a result, show differences in the risk for behavioral problems (Feinberg, Neiderhiser, Simmens, Reiss, & Hetherington, 2000; Meunier, Boyle, O'Connor, & Jenkins, 2013). This inherent 'plasticity' in the offspring's response to parental care in early life is conserved across a wide phylogenetic range. Rodent models have offered important insights into biological mechanisms by which natural variations in maternal behavior can lead to the nongenomic transmission of traits from parent to offspring (F. A. Champagne, Francis, Mar, & Meaney, 2003; Meaney, 2001).

In rodents, an important component of maternal care is the licking and grooming (LG) of pups, and there is natural variation in the amount of LG that dams provide to their offspring between litters (F. A. Champagne, Francis, et al., 2003). Within a given population, dams that provide levels of LG one standard deviation above and below the population mean frequency of LG may be characterized as high and low LG dams, respectively. Males and females reared in high and low LG litters show long-term changes in adult behavior, gene expression, and epigenetic signatures (N. M. Cameron et al., 2005; F. A. Champagne et al., 2006; F. A. Champagne, Weaver, Diorio, Sharma, & Meaney, 2003; McGowan et al., 2011; Weaver et al., 2004).

Variations in maternal care are associated with a number of specific behavioral outcomes. For example, male and female adult offspring reared in low LG litters spend less time in the center of an open field (Caldji et al., 1998; F. A. Champagne & Meaney, 2007; Weaver, Meaney, & Szyf, 2006) and male adult offspring show greater immobility in a forced swim test compared with offspring reared in high LG litters (Weaver et al., 2005). These

Pauline Pan, Department of Cell and Systems Biology, University of Toronto, Toronto; Department of Psychology, University of Toronto, Mississauga; Department of Biological Sciences, University of Toronto, Scarborough; and Centre for Environmental Epigenetics and Development (CEED), University of Toronto, Toronto; Alison S. Fleming and Daeria Lawson, Department of Psychology, University of Toronto, Mississauga; Jennifer M. Jenkins, Department of Applied Psychology and Human Development, University of Toronto, Toronto; Patrick O. McGowan, Department of Cell and Systems Biology, University of Toronto, Toronto; Department of Biological Sciences, University of Toronto, Scarborough; and Centre for Environmental Epigenetics and Development (CEED), University of Toronto, Toronto.

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Correspondence concerning this article should be addressed to Pauline Pan, Department of Cell & Systems Biology, University of Toronto, 1265 Military Trail, Science Wing, Room SW328A, Toronto, Ontario, Canada M1C1A4. E-mail: pauline.pan@mail.utoronto.ca

results suggest that offspring from low LG litters generally display increased anxiety- and depressive-like behaviors relative to offspring from high LG litters. In the context of an artificial rearing paradigm where nutrition and tactile stimulation are provided by an experimenter, rats deprived of maternal care show hyperactive locomotor activity in adulthood (Belay et al., 2011; Gonzalez & Fleming, 2002; Gonzalez, Lovic, Ward, Wainwright, & Fleming, 2001; Lovic, Gonzalez, & Fleming, 2001). Likewise, the adult male and female offspring of 'high responder' rats, which show increased novelty-induced exploration and lower levels of maternal care, show higher levels of spontaneous locomotor activity (Clinton et al., 2007).

Together with alterations in stress-related behaviors, male adult offspring from high LG litters exhibit a reduced hypothalamic-pituitary-adrenal (HPA) stress response, showing lower levels of plasma corticosterone in response to a restraint stress (Weaver et al., 2005). These effects of maternal LG on the HPA response in offspring are thought to be mediated in part by changes in the expression of the Glucocorticoid Receptor (GR) gene, as high LG-reared rat and mice offspring show higher mRNA expression of the GR gene in the hippocampus than do low LG reared offspring (Belay et al., 2011; Chourbaji et al., 2011; Francis, Diorio, Liu, & Meaney, 1999; Franklin et al., 2010; McGowan et al., 2011; Meaney, 2001; Weaver et al., 2004). Furthermore, the effects of elevated LG on behavior, the stress response, and gene expression are associated with reduced DNA methylation of the promoter region of the GR1₇ untranslated splice variant in rats and mice (Liberman, Mashoodh, Thompson, Dolinoy, & Champagne, 2012; Weaver et al., 2004) as well as additional transcriptional and epigenetic alterations throughout the genomic locus containing GR (McGowan et al., 2011).

Although much of the existing literature has focused on inter-litter differences in maternal care, recent findings show that there is significant variation in the level of maternal care received by individual rats within the same litter. Studies of within-litter effects have found that higher LG siblings showed increase expression of the GR gene in the hippocampus and increased dentate synaptic plasticity, including increased potentiation in response to CORT in hippocampal slices from male offspring (van Hasselt, Boudewijns, van der Knaap, & Joels, 2012; van Hasselt, Cornelisse, et al., 2012). These data support previous studies of between-litter effects (Liu et al., 1997; Meaney, 2001). In addition, there is evidence from behavioral studies that male offspring that receive higher LG than their siblings are less active in adolescence than their low licked siblings (Cavigelli, Ragan, Barrett, & Michael, 2010) and that higher licked female offspring are slower to approach novel objects in adulthood (Ragan, Loken, Stifter, & Cavigelli, 2012). Notably, the effects of maternal LG on offspring behavior reported in these studies were the opposite of those observed between litters, where the male offspring in high LG litters showed *increased* locomotor activity (Cavigelli et al., 2010), and *increased* exploratory behavior (Caldji et al., 1998). These data indicate that within-litter effects associated with maternal LG may be partially distinct from between-litter effects, and may be to some extent sex specific.

The impact of variations in maternal care on stress and stress-related behaviors has been extensively studied in males, however several recent lines of evidence point to sex-specific effects on behavioral outcomes and hippocampal plasticity (Kundakovic,

Lim, Gudsnuik, & Champagne, 2013; Peña, Neugut, & Champagne, 2013; van Hasselt, Cornelisse, et al., 2012). In addition, females can transmit behavioral phenotypes, including maternal behavior, intergenerationally (D. L. Champagne et al., 2008; Francis, Diorio, et al., 1999). These observations underline the necessity for a more complete understanding of the effects of maternal care on stress-related behavior and neurophysiology in females.

The present study had several aims. First, we investigated the effects of differential maternal care received by individual offspring within litters on their activity, anxiety behaviors, HPA reactivity, gene expression, and DNA methylation. We hypothesized that female offspring that receive higher LG than their siblings would show reduced anxiety-like behaviors, a more attenuated rise in CORT in response to a stressor, higher GR gene expression, and reduced DNA methylation levels in the GR1₇ splice variant in adulthood. Based on the extant literature, we predicted increased activity in the offspring of high- compared with low-licking dams (i.e., the between-litter comparison) and lower activity in offspring that were more frequently licked than their siblings (i.e., the within-litter comparison). We also predicted that higher levels of GR gene expression would be associated with reduced anxiety-like behaviors and a less reactive stress response. Second, we investigated additive and interactive effects of intralitter and interlitter variations in maternal care. We predicted that the high versus low LG effects would be greatest among the offspring that receive the highest licking across litters (highest licking received within litter and highest LG dam) in comparison with those that receive the lowest (lowest-licked offspring in lowest LG litter). Burt, McGue, Iacono, & Krueger, 2006; Meunier, Bisceglia, & Jenkins, 2012.

Method

Subjects, Breeding Procedures, and Female Offspring

Forty virgin female and 20 virgin male Long-Evans (LE) rats were obtained from Charles River Farms (St. Constant, Quebec) at 55–65 days of age. Animals were allowed to acclimatize in the vivarium at the University of Toronto at Mississauga for seven days before experimental conditions were applied. Males were singly housed after acclimatization and allowed to gain sexual experience from existing breeding stock females for five days. Experimental females were kept pair-housed in standard sized cages until mating. Animals were housed in standard Plexiglas cages (20 cm × 43 cm × 22 cm) under 12:12 h light: dark cycle with lights on at 0800 h. Temperature and humidity were kept constant at 22°C and 60%, respectively. Food and water were available ad libitum. All procedures were approved by the University of Toronto's Animal Care Committees at UTM and UTSC.

At mating, each male was paired with one virgin LE female for 7 days. Females were single-housed after mating and for the duration of gestation. Females were monitored for parturition beginning on gestation Day 18. For each litter, if pups were delivered before 1500 h, that day was designated as postnatal day (PND) 0.

At PND 1, each mixed sex litter was culled to six female pups. Litters were included in the study if they contained at least 4 female offspring. Litters were excluded from the study because of cannibalism ($n = 4$), the presence of male pups that were mistaken

for female pups ($n = 2$), or unsuccessful impregnation ($n = 1$). In total, 189 pups in 33 litters were assessed individually for maternal LG received.

Maternal Care Observations of Female Offspring

Maternal care was examined using standardized methods that have been employed in many other studies to examine active maternal behavior (e.g., Melo et al., 2006; Palombo, Nowoslawski, & Fleming, 2010). Specifically, maternal care was observed for 30 min from PND 1 to PND 10 between 0900 hr and 1300 hr. On each observation day, pups were removed from the nest for 10 min and colored. Each pup was marked using odorless and taste-free food coloring (Club House, London, Ontario, Canada) daily to differentiate them during observations. The pups were then returned to the corner diagonally opposite to the nest and maternal care was observed for 30 min. Two experimenters with high interrater reliability (>0.9) coded the dams' behaviors live using Behavioral Evaluation Strategy and Taxonomy (BEST) software (Educational Consulting Inc., Florida). The duration of time the dam spent engaged in the following behaviors were coded for each pup: pup retrieval, pup mouthing, anogenital licking, and body licking. Hovering and crouching over the pups were coded for each litter as a whole, as dams primarily hover and crouch over entire litters and not individual pups. Anogenital and body licking for each of the six pups were recorded separately, that is, separate sets of keys were designated to each pup. Cages were not changed during the 10-day observation period.

The duration of combined anogenital licking, body licking, and grooming was used as an index of maternal care (F. A. Champagne, Francis, et al., 2003). Grouping based on standard deviation from the group mean, the method used by Champagne et al. (2003), left only four litters in the low licking group because of a smaller subject size in our study. Therefore, we used quartile grouping to designate equal numbers of high and low litters. Eight litters in the top 25% of the entire cohort's total duration of LG across 10 observation days were characterized as high LG litters, and eight litters that displayed overall LG levels in the bottom 25% were designated as the low LG litters. The remaining 17 litters in the middle 50% of the cohort's LG were designated as mid LG litters.

Behavior and Stress Reactivity in Adult Female Offspring

The order of testing was as follows: locomotor activity, open field, and stress reactivity. For the locomotor activity test, the two highest and two lowest LG female offspring from each litter type ($n = 4 \times 8$ high LG litters = 32, $n = 4 \times 17$ middle LG litters = 68, and $n = 4 \times 8$ low LG litters = 32) were tested in adulthood (PND75-PND105). Next, for the open field test, the highest LG offspring and the lowest LG female offspring from each litter type, previously examined for locomotor activity, were tested ($n = 2 \times 8$ high LG litters = 16, $n = 2 \times 17$ middle LG litters = 34, and $n = 2 \times 8$ low LG litters = 16). To measure the CORT response to restraint stress, the same subjects from high LG and low LG litters tested in the open field were examined ($n = 2 \times 8$ high LG litters = 16, $n = 2 \times 8$ low LG litter = 16). Animals were given at least one day's rest between different behavioral tasks.

Locomotor activity. Adult female offspring were tested over a 30-min session in a locomotor activity box (47 cm \times 26 cm \times 20 cm). Activity levels were measured by an automated monitoring system that consisted of 16 parallel test boxes with infrared photocells mounted on a metal assembly into which a standard cage without bedding was placed. Activity levels were quantified as the number of total photocell interruptions (i.e., 'beam breaks') over the test session (Lynch, Castagne, Moser, & Mittelstadt, 2011). The test boxes were cleaned with 70% ethanol and dried before each trial and the number of boli produced by each animal was recorded for each test session. All female offspring were tested between 11 a.m. and 3 p.m. in a pseudorandomized order by experimenters blinded to litter and offspring high/low status.

Open field task. We used the open field test as a validated measure of anxiety-related behavior (Belzung & Griebel, 2001) that has been used in many previous studies of the effects of interlitter differences in maternal care (e.g., Caldji et al., 1998; F. A. Champagne & Meaney, 2007; Weaver et al., 2006). Behaviors in the open field were examined in a 5-min session in a 100 cm \times 100 cm \times 35 cm arena. The arena was divided into 49 equal grid squares. The nine central squares were designated as the 'center,' four squares situated in the corners of the box were designated as 'corners,' and six squares between two adjacent corners were designated as 'sides.' Adult female offspring were placed in the center facing the experimenter and allowed to explore the apparatus. The experimenter recorded the time spent in the center, corners, and sides using BEST software. The arena was cleaned with 70% ethanol and dried before each trial and the number of boli produced by each adult female offspring was recorded for each test session. Anxiety levels were quantified as the proportion of total time animals spent in the center of the apparatus (Hall & Ballachey, 1932). All adult female offspring were tested between 11 a.m. and 3 p.m. in a pseudorandomized order by experimenters blinded to litter and offspring high/low status.

Stress response test. We next measured CORT levels, an important component of the response to stress shown to vary in offspring as a function of differences in maternal care (Francis, Champagne, Liu, & Meaney, 1999). Adult female offspring were first hand-restrained under a cotton towel and blood was collected from a nick in the tail as previously described (Belay et al., 2011; Flutterm, Dalm, & Oitzl, 2000) into a nonheparinized 0.6-ml centrifuge tube (baseline, approximately 100 μ l per rat) and immediately placed on ice. Female offspring were then placed into Plexiglas restrainers (8 cm diameter \times 20 cm length) and, 20 minutes later, a second sample of blood (peak, approximately 100 μ l per rat) was collected as described above immediately before female offspring were released from the restraint. Female offspring were then returned to their home cages without their cage mates and left undisturbed for 70 minutes in the same room where the stress and blood collection occurred. Ninety minutes after the first blood collection, a third sample of blood was collected (return to baseline, approximately 100 μ l per rat). Blood was left on ice for at least 30 min before the samples were centrifuged at 4°C and 4000 rpm for 25 min. Blood serum was extracted and stored at -80 °C. Serum levels of CORT for each female offspring at each of the three time-points were measured using a commercial rat/mouse corticosterone ELISA kit (ALPCO Diagnostics, Cat # 55-CORMS-E01, Salem, NH). Given that our goal was to

examine both the sensitivity of the CORT response to restraint stress as well as the intensity of the response, we calculated the area under the curve with respect to the ground (AUC_G) as a measure of adrenal cortical activity according to the following formula $AUC_G = \sum (m_{(i+1)} + m_i)/2$, with m_i denoting the individual measurement (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003).

Hippocampal Gene Expression and DNA Methylation Analysis in Adult Female Offspring

One of the highest LG and one of the lowest LG offspring that were tested for locomotor activity but not for open field and stress reactivity tests from 6 high and 6 low LG litters ($n = 2 \times 6$ high LG litters = 12 and $n = 2 \times 6$ low LG litters = 12) were selected for gene expression and DNA methylation analyses. Female offspring were euthanized one week after the conclusion of behavioral testing, and glucocorticoid receptor (GR) gene expression, mineralocorticoid receptor (MR) gene expression, and $GR1_7$ promoter DNA methylation levels were determined in the hippocampus of each female offspring (see below).

Brain extractions and tissue punches. Adult female offspring were euthanized via CO_2 inhalation and their brains were rapidly extracted within approximately 4 min, snap frozen in isopentane on dry ice, and stored at $-80^\circ C$. Brains were cryosectioned into 300- μm slices using a Leica CM3050S cryostat and the whole hippocampus (HPC) was punched out and collected bilaterally for each subject using stereotaxic coordinates targeting -1.80 to -5.20 bregma (Paxinos & Watson, 1997).

Gene expression. Total RNA was extracted along with genomic DNA using an DNA/RNA/Protein Mini kit (Qiagen, Toronto, Ontario, Canada). Total RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Burlington, ON, Canada), and gene-specific real-time quantitative PCR (RT-qPCR) was performed using a StepOne Plus (Life Technologies, Grand Island, NY) thermocycler. A standard curve was generated from 11 serial dilutions of a mixture of cDNA from all animals using the Sybr-Green method (Fast SYBR Green Master Mix, Life Technologies, Burlington, Ontario, Canada; see Sasaki, de Vega, St-Cyr, Pan, & McGowan, 2013). Expression levels of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) genes were quantified relative to the ribosomal RNA (18S) housekeeping gene. Samples were plated in triplicate and average gene expression levels were

calculated for each subject. Product specificity was verified by the presence of a single peak. Table 1 shows the primer sets designed to interrogate the MR and GR genes, and GR exon-1 splice variants previously shown to be expressed in the rat hippocampus (McCormick et al., 2000; McGowan et al., 2011; Sasaki et al., 2013).

DNA methylation. DNA samples extracted from the same brain tissues analyzed for gene expression were examined for DNA methylation using sodium bisulfite mapping (Clark, Harrison, Paul, & Frommer, 1994). Briefly, genomic DNA was bisulfite converted (EpiTect Bisulfite Kit, Qiagen, Toronto, Ontario, Canada) and amplified by PCR using primers targeting 17 CpGs within the $GR1_7$ promoter region (Weaver et al., 2004). Bisulfite-treated DNA was subjected to two cycles of PCR using the primers listed in Table 1. The nested PCR product was subcloned, transformed, and at least 10 different clones per subject were miniprep (Original TA cloning kit, Invitrogen). The ligated DNA fragments were then sequenced using standard Sanger sequencing by synthesis (Eurofins Operon MWG, Huntsville, AL).

Statistical Analysis

Statistical analyses were conducted using SPSS v.21 and v.22 for Mac. Measures of maternal LG were analyzed by within \times between Analysis of Variance (ANOVA), with female offspring (pup1–6) or time (PND1–10) as the repeated measures and litter type (low 25%, mid 50%, and high 25%) as the between groups measure. Specific comparisons within and between litters were examined using Bonferroni-controlled post hoc testing. Measures from the locomotor activity box, open field task, GR and MR gene expression, GR exon 1 splice variants expression, and $GR1_7$ DNA methylation were analyzed by repeated measure 2×2 or 2×3 (offspring: low LG, high LG; litter: low 25%, high 25% or low 25%, mid 50%, high 25%) within \times between ANOVA, where appropriate. DNA methylation levels were assessed for each of the 17 CpG sites contained within $GR1_7$ promoter as percent methylation, as well as for the promoter region as a whole as percent of sequenced clones containing at least one methylated CpG site (McGowan et al., 2009). Results are presented as means and SEMs.

Correlations between offspring's LG-specific deviation from litter's LG mean (calculated using the formula: offspring LG received $-$ litter mean LG) behavioral measures, stress reactivity (CORT levels at all time points and percent change across restraint

Table 1
Primer Sequences Designed to Interrogate Levels of Gene Expression of MR and GR, and DNA Methylation (DName) of the $GR1_7$ Splice Variant

Gene name	Forward primer	Reverse primer
MR	ggcagctgcaaagtcttct	gacagttcttccgccgaatc
GR(total)	ctcgaaggctccacaagcaatgt	gcaatgcttctccagaagccga
$GR1_5$	ctgagagggtttgcatcgc	tcctcccctcaggcttttat
$GR1_6$	cgggctcacattaatattgccaatggac	ggcgaagatgcagaaccttgact
$GR1_7$	ggagcctgggagaagagaaa	ctaccagggggagactaagga
$GR1_{10}$	acgccgactgtttatctgg	tcctcccctcaggcttttat
$GR1_{11}$	gggctgttaggggtgattg	taccagggggagactaagga
$GR1_7$ DName (outside)	tttttaggttttttagaggc	atttcttaattctctctcc
$GR1_7$ DName (nested)	tttttttaggtgtgatattt	ttctccaaactcctcc

stressor), and gene expression were calculated using two-tailed bivariate Pearson correlations. Correlations between DNA methylation levels of each of the 17 GR1₇ CpG sites, total GR1₇ DNA methylation, GR1₇ expression, total GR gene expression, CORT levels, as well as LG received by offspring were also analyzed in this manner. As GR expression levels and CORT levels were collected in different offspring within litters, GR expression and CORT correlations were conducted between siblings matched for within litter LG ranking.

Results

Maternal Care

There was variation in the amount of maternal care (duration of anogenital and body LG; F. A. Champagne, Francis, et al., 2003) provided by dams between high and low LG litters, $F_{(1, 23)} = 931.481$, $p < .001$, as well as provided to individual female offspring within litters, $F_{(5, 115)} = 70.969$, $p < .001$ (Figure 1a) across the 10-day observation period. To investigate differences in

the relative amount of LG received, the amount of LG received by each pup was calculated as a percentage of their litters' mean LG levels. Dams licked and groomed their pups differentially, as the relative percentage of LG received was significantly different within litters, $F_{(5, 189)} = 104.562$, $p < .001$ (Figure 1b). To assess consistency in maternal levels of LG across days, LG was analyzed separately for each day during the postnatal observation period (PND 1–10). This analysis revealed that low, mid, and high LG dams licked significantly differently from one another on each of PNDs 2, 3, 4, and 5 ($ps < 0.05$), high dams licked significantly more than mid and low dams on each of PNDs 6, 7, and 8 ($ps < 0.05$), and there were no differences between litter types on PNDs 1, 9 and 10 ($ps > 0.05$; Figure 1c). Pups' level of LG received across the 10 observation days was generally consistent, as pups' LG ranking did not change significantly across days (Figure 1d). Using both parametric and nonparametric tests, we found that between-litter effects were comparable between analyses using either standard deviation characterized or quartile characterized LG statuses (data not shown). Importantly, the specific color used to identify pups within the same litters (i.e., coloring) did not alter

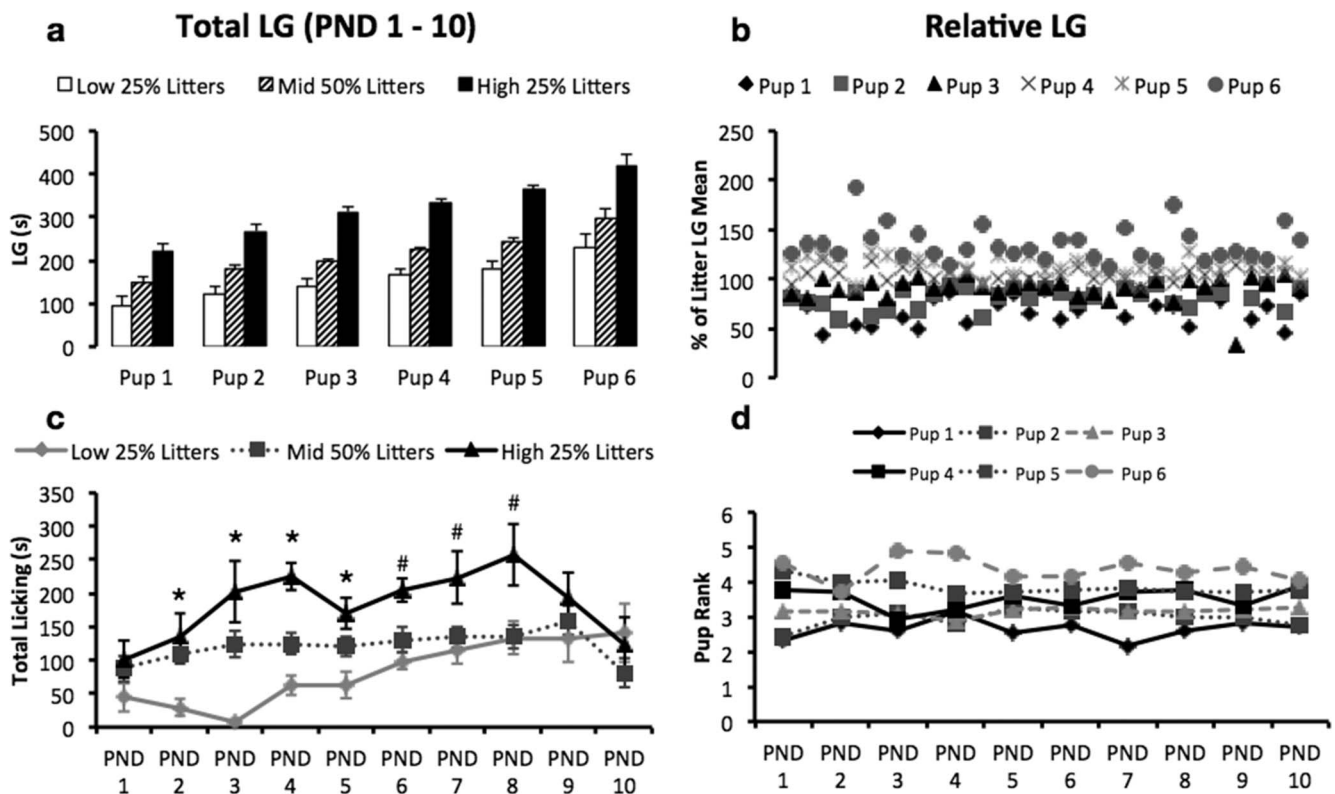


Figure 1. Duration of licking and grooming (LG) received by female offspring during the first 10 postnatal days. (a) Dams differed in the amount of LG they provided to their female offspring overall. Dams also treated female offspring differently within litters, as siblings received significantly different levels of LG. (b) LG received by female offspring within each litter is shown (symbols; $n = 189$ offspring in 33 litters), expressed as a percentage of litter LG mean (horizontal line). Female offspring differed in the amount of LG received within the litter. (c) The amount of LG differed between all 3 litter types on PNDs 2, 3, 4, and 5 (* $ps < 0.05$); on PND 6, 7, and 8, high LG litters were significantly different from mid and low LG litters (# $ps < 0.05$), but mid and low LG litters were not different. There were no differences between litter types on PNDs 1, 9, and 10. (d) The level of LG received by each female offspring was generally consistent, as offspring's LG ranking did not change across days.

the level of licking received by individual pups ($p = .148$). Similar methods of coloring pups have been used in previous studies (Cavigelli et al., 2010; van Hasselt, Cornelisse, et al., 2012) and our methods were chosen to ensure accuracy when identifying pups in the nest, especially if dams were crouching over them.

Behavior and Stress Reactivity in Adult Female Offspring

Locomotor activity task. We found main effects both within and between litters on locomotor activity. High LG adult female offspring were significantly more active than low LG offspring from the same litters, $F_{(1, 26)} = 4.368, p = .047$ (Figure 2a). Activity levels also differed between litters, $F_{(2, 26)} = 5.49, p = .01$, and post hoc analyses showed that offspring from the low 25% litters and the mid 50% litters were significantly less active than offspring from the high 25% litters ($ps < 0.05$).

Open field task. We predicted that female offspring that receive higher levels of LG would be less anxious in adulthood, and that this effect would occur within and between litters. Results from this task showed a significant within litter effect, but no between litter effects. High LG offspring spent a significantly higher proportion of the total time in the center of the arena than their low LG siblings, $F_{(1, 30)} = 5.415, p = .027$ (Figure 2b). This effect persisted after covarying locomotor activity, $F_{(1, 57)} = 4.249, p = .044$, suggesting that the observed differences in open field task behavior were not a result of differences in general activity levels.

Stress response test. We predicted that female offspring receiving higher levels of LG would show attenuated CORT in adulthood in response to a physical restraint stressor compared to female offspring receiving lower levels of LG. There were no differences in basal CORT levels within or between litters (high compared with low offspring, $p = .552$; high compared with low litters, $p = .802$). We also found no significant effect of differential maternal care within or between litters on overall CORT levels ($p = .844$ and $p = .890$, respectively). However, there was a significant main effect of time, $F_{(2, 48)} = 32.696, p < .01$, indicating an increase in CORT as a function of restraint in all groups. When CORT levels at 20 and 90 minutes were calculated as a percentage of baseline levels (0 minutes), there was a significant three-way interaction between time, litter type, and offspring type, $F_{(2, 50)} = 3.195, p = .049$ (Figure 2c). High LG offspring from the high LG litters and low LG offspring from the low LG litters had a significantly inhibited rise in CORT levels in response to restraint stress.

Gene Expression and DNA Methylation in Adult Female Offspring

Gene expression of MR, GR, and GR exon 1 splice variants. We hypothesized that female offspring that receive higher levels of LG would display higher levels of GR expression in adulthood. Analysis of GR expression in the hippocampus showed that offspring that received high LG compared with their low LG siblings had higher levels of GR, $F_{(1, 20)} = 4.862, p = .039$ (Figure 3a); but there was no main effect of being reared in a high compared with low LG litter. In support of our predictions, there was a significant negative correlation between total GR expression and the CORT

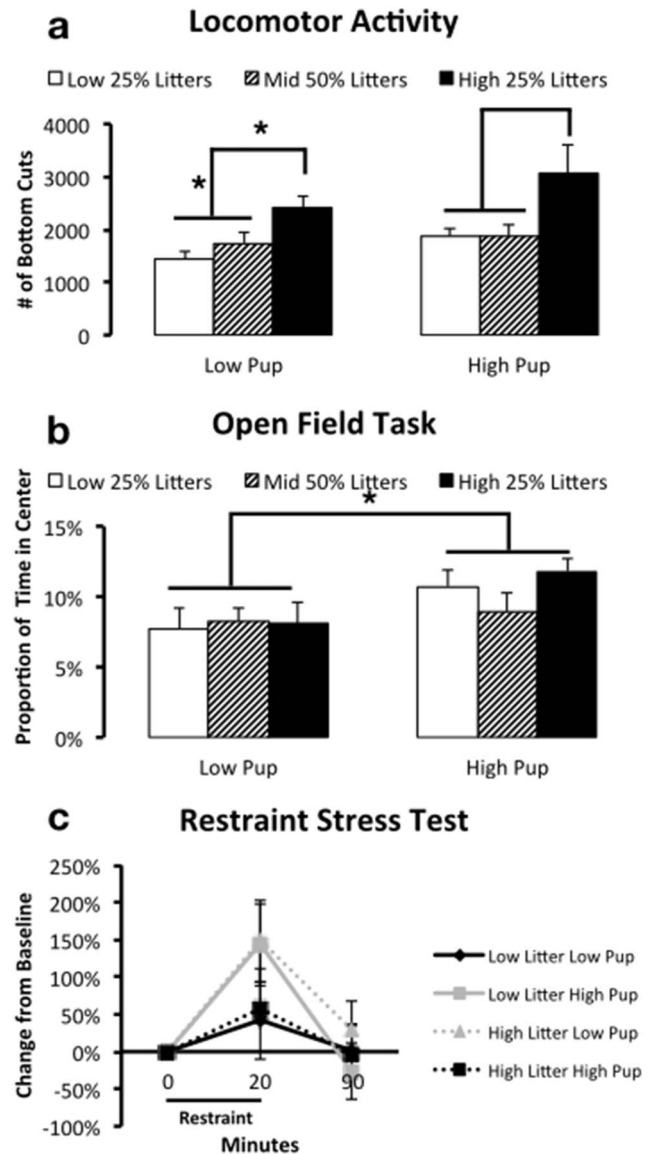


Figure 2. Behaviors and the corticosterone (CORT) response to stress in adult female offspring as a function of LG received. (a) Female offspring that received the highest LG compared with their siblings that received the lowest LG within the litter were more active in a locomotor activity test ($* p < .05$). In addition, there was a main effect of litter type, such that offspring born to high LG mothers were more active than offspring born to mid or low LG mothers ($** ps < 0.05$). (b) Female offspring that received the highest LG compared with their siblings that received the lowest LG within the litter spent more time in the center of the open field ($* p < .05$). There were no differences between offspring reared in high versus low LG litters. (c) There was a significant main effect of time on CORT levels of female offspring exposed to a physical restraint stressor and a significant three-way interaction between time, litter type, and pup type. High licked offspring from the high LG litters and low licked offspring from the low LG litters had an inhibited rise in CORT levels in response to restraint stress. There were no overall differences between offspring receiving different levels of LG within litters or offspring born to high and low LG dams.

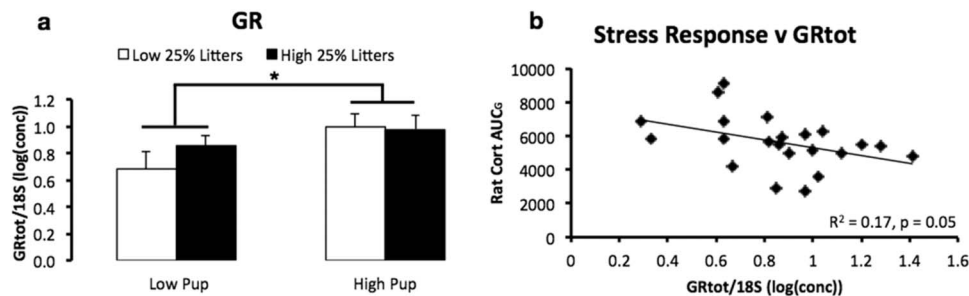


Figure 3. Expression of the glucocorticoid receptor in the hippocampus of adult female offspring. (a) Within litters, female offspring that received high LG compared with their low LG siblings had higher levels of total GR expression (GRtot; * $p < .05$). There were no differences in total GR expression between offspring reared by high or low-licking dams. (b) CORT response to restraint stress measured by area under the curve was negatively and significantly correlated with total GR expression.

response to stress as determined by area under the curve analysis (AUC_G; $R = -0.414$, $p = .028$; Figure 3b).

We next examined gene expression of GR1 splice variants shown to be expressed in the hippocampus (McCormick et al., 2000; McGowan et al., 2011; Sasaki et al., 2013; Figure 4a). GR1₆, GR1₁₀, and GR1₁₁ expression levels were significantly and positively correlated with total GR expression levels (GR1₆: $R = 0.394$, $p = .028$, Figure 4b; GR1₁₀: $R = 0.355$, $p = .044$, Figure 4d; GR1₁₁: $R = 0.403$, $p = .026$, Figure 4e). GR1₅ (data not shown) and GR1₇ (Figure 4c) expression levels did not show a significant correlation with GR total expression levels. MR expression did not differ significantly between female offspring that received different levels of LG within or between litters (data not shown).

DNA methylation of GR1₇. We hypothesized that female offspring that received higher levels of LG would display lower levels of DNA methylation of the GR1₇ splice variant in adulthood. Contrary to our predictions, there were no differences within, $F_{(1, 20)} = 0.021$, $p = .888$, or between litters, $F_{(1, 20)} = 0.151$, $p = .703$, in the overall percentage of methylated clones (Figure 5a). However, there was a significant positive correlation between the total amount of LG female offspring received and their GR1₇ DNA methylation levels ($R = 0.345$, $p = .049$). Detailed analysis of percent DNA methylation levels at each of the 17 GR1₇ CpG sites examined revealed differences in DNA methylation as an effect of LG at sites 7 and 17 (Figure 5b–5d). Within litters, DNA methylation levels at CpG site 7 were significantly different between high LG and low LG siblings, $F_{(1, 20)} = 5.603$, $p = .039$ (Figure 5b). Post hoc analysis showed that low LG offspring from low LG litters had significantly lower DNA methylation levels than high LG offspring in low LG litters and high LG offspring from high LG litters ($p = .021$ and 0.039 , respectively). DNA methylation levels at CpG site 17 were also significantly different between offspring reared by low and high LG dams, $F_{(1, 20)} = 7.481$, $p = .021$ (Figure 5c). Post hoc analysis revealed that low LG offspring from low LG litters had significantly lower levels of DNA methylation than high LG offspring from high LG litters ($p = .05$). CpG site 5 showed a trend toward lower DNA methylation between offspring reared in high LG litters compared to offspring reared in low LG litters, $F_{(1, 20)} = 3.02$, $p = .098$ (Figure 5d).

Discussion

In this study, we investigated between and within litter differences in maternal care and their effects on adult female offspring stress and anxiety phenotypes, as well as potential mechanisms underlying these effects. Our results in female offspring support previous observations, predominantly in male offspring, that there is substantial naturally occurring variation in the level of LG both between and within litters (Cavigelli et al., 2010; F. A. Champagne, Francis, et al., 2003; Ragan et al., 2012; van Hasselt, Boudewijns, et al., 2012; van Hasselt, Cornelisse, et al., 2012; van Hasselt, Tieskens, et al., 2012). Female pups that received relatively more LG than their siblings during early development were more active and less anxious in adulthood. As adults, higher licked offspring had increased expression of the GR gene, a lower CORT response to stress, and higher levels of DNA methylation at CpG sites 7 and 17 of the GR1₇ promoter.

Our analysis focused on variations in active maternal care as a result of pup retrieval both between and within the lowest 25%, mid 50%, and highest 25% litters on each of the 10 PNDs. Dams showed significantly different pup-directed behavior between PNDs 3 and 8, with the high 25% dams licking significantly more than the low and mid dams. In addition, individual pups generally maintained their relative ranking of high versus low in terms of the licking that they received across the 10-day period. Thus, dams showed stable preferences toward individual female offspring, likely driven by characteristics of the individual pups. Maternal female rats are known to show a particular responsiveness to pup vocalizations that prompts them to actively orient toward a vocalizing pup when it is in close proximity (Farrell & Alberts, 2002a, 2002b). It is possible that different pups emit distinct levels of ultrasounds.

To monitor both between- and within-litter effects of maternal care, we studied a relatively large cohort (33 litters with a total of 189 pups) in which observations of the active maternal response to female offspring occurred after handling and over one 30-minute observation session per litter per day during the light cycle. It is possible that the method we used to monitor the maternal response resulted in the characterization of high and low LG litters in a manner distinct from previous studies of undisturbed litters monitored across the light–dark cycle. A recent study using undis-

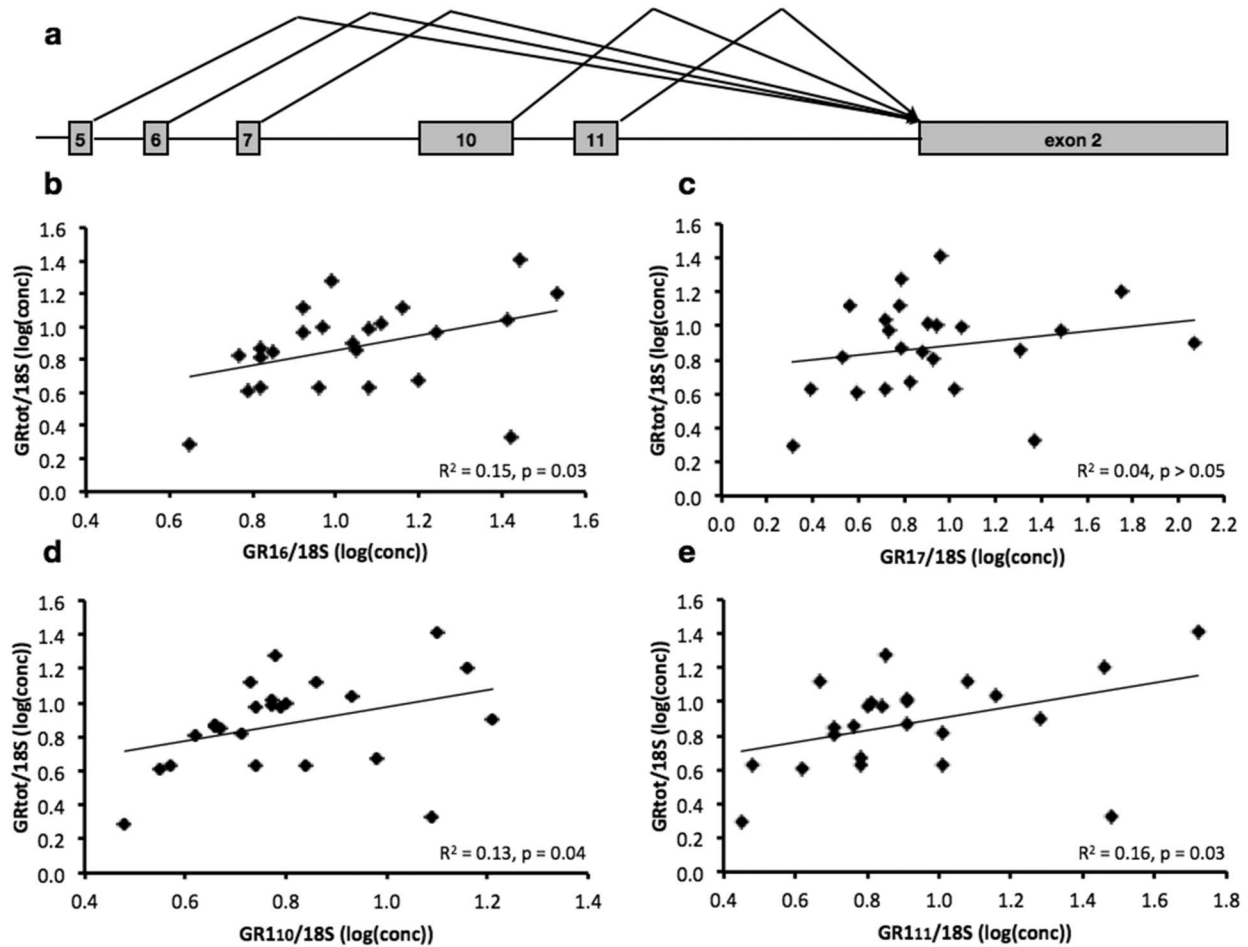


Figure 4. Relationship between total GR expression and GR exon 1 splice variant gene expression in the hippocampus of adult female offspring. (a) Relative genomic positions of GR exon 1 splice variants previously shown to be expressed in the rat hippocampus (GR₁₅, GR₁₆, GR₁₇, GR₁₀, and GR₁₁) and GR exon 2. Sizes and distances between exons are not to scale. (b) GR₁₆ expression was positively and significantly correlated with total GR expression (GR_{tot}). (c) GR₁₇ expression was not significantly correlated with total GR gene expression. (d) GR₁₀ expression was significantly and positively correlated with total GR expression. (e) GR₁₁ expression was positively and significantly correlated with total GR expression.

turbed monitoring (Peña et al., 2013) indicated that relatively small deviations in maternal behavior procedures, such as monitoring only in the light phase of the circadian cycle, can influence the determination of relative LG levels between litters. Future studies involving repeated monitoring, including in undisturbed conditions, will enable a comparison between the effects detected in this study and other studies using undisturbed monitoring. In addition, in future studies, it would be important to understand the relationship between the estrous cycle and levels of maternal care received on adult phenotype, as differences in the timing of puberty onset were reported in association with between litter differences in LG (Cameron et al., 2008).

This study was designed to characterize at an individual subject level the effects of pup-directed maternal care in female offspring. In considering the potential impact of the removal of males from the litter, it should be noted that a seminal study in this area found no sex differences in the effects of naturally occurring differences

in maternal care on gene expression and HPA development (F. A. Champagne, Francis, et al., 2003). In addition, male/female composition and litter size did not influence frequency of LG, although males received slightly more LG than females (F. A. Champagne, Francis, et al., 2003). A recent study also indicated that levels DNA methylation in the GR₁₇ promoter methylation do not differ significantly between mixed-sex litters and female-only litters, suggesting that our epigenetic data may be informative for studies of mix-sex litters (Kosten, Huang, & Nielsen, 2014). Some previous studies, however, have reported that mothers preferentially lick male pups (Moore & Morelli, 1979), potentially as a result of a shorter latency in male offspring to extend their legs and thereby facilitate licking (Moore, 2004). It is thus possible that removal of the male offspring in this study affected the dynamics of interactions between dams and offspring.

We hypothesized that rats that receive higher LG than their siblings would show reduced anxiety-like behaviors in adulthood,

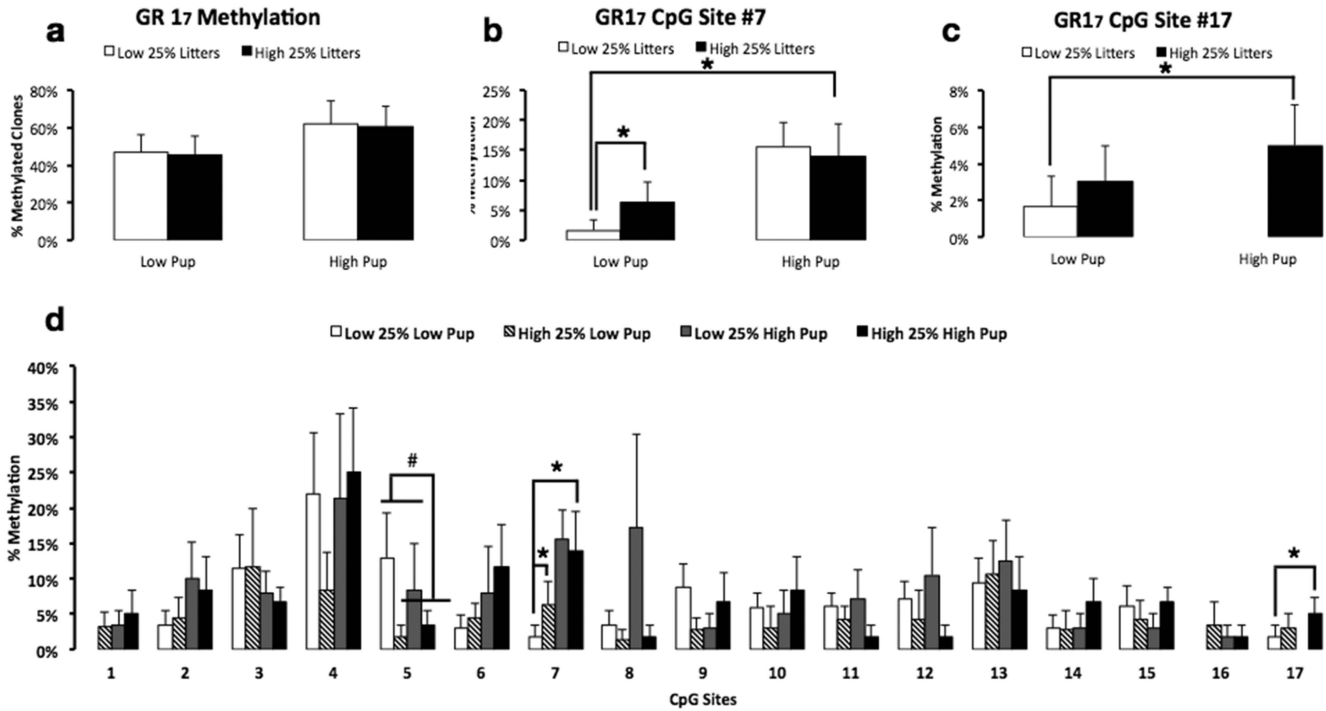


Figure 5. DNA methylation of GR exon 17 promoter in the hippocampus of adult female offspring. (a) Overall, DNA methylation percentage across the entire GR17 promoter was not significantly different within or between litters. (b) DNA methylation percentage at GR17 CpG site 7 was significantly higher within litters in high LG compared with low LG female offspring (bar * $p < .05$). Post hoc testing revealed that low LG offspring from low LG litters had significantly lower levels of methylation when compared with high LG offspring from low LG litters and high LG offspring from high LG litters (* $ps < 0.05$). (c) DNA methylation percentage at GR17 CpG site 17 was significantly higher between high LG litters compared with low LG litters (** $p < .05$), but there was no within-litter effect. Post hoc testing revealed that the lowest LG offspring from low LG litters had significantly less methylation than the highest LG offspring from high LG litters (* $p < .05$). (d) DNA methylation percentage at each of the 17 CpG sites in the GR17 promoter. In addition to differences found in CpG sites 7 and 17, CpG site 5 showed a trend toward lower DNA methylation between offspring reared in high LG litters compared with offspring reared in low LG litters (# $p = .098$). Missing bars indicate 0% DNA methylation.

and that rats receiving the highest absolute levels of LG (i.e., high LG offspring reared in high LG litters) would be the least anxious. Consistent with this prediction, we found that higher LG offspring were less anxious than their lower LG siblings within litters in adulthood, as demonstrated by a significantly longer duration of time spent in the center of the open field arena as a proportion of the total time spent in the arena. Importantly, this effect was not a result of differences in overall activity levels. However, we found no differences between rats reared in high and low LG litters, in contrast to what has been reported previously (Meaney, 2001). This within-litter, but not between-litter, effect of LG on anxiety behaviors has been reported before (Kosten et al., 2014; Kurata, Morinobu, Fuchikami, Yamamoto, & Yamawaki, 2009). These results may indicate that receiving more licking than a sibling reduces offspring anxiety or that certain pups elicit less licking as a function of their anxiety behaviors. Total LG received was not significantly correlated with time spent in the center of the open field, suggesting that the absolute level of LG received is not a strong determinant of anxiety behavior in the open field task, at least in our cohort of female offspring.

As adults, female offspring showed differences in activity levels as a function of maternal care received. Within litters, female offspring that received high LG were more active in adulthood than their low LG siblings. Between litters, offspring reared in high LG litters were significantly more active in adulthood than offspring reared in low and mid LG litters, though offspring reared in low and mid LG litters did not differ significantly. Generally, deficits in maternal care have been linked with hyperactivity, as seen in rats reared artificially without their mothers (Belay et al., 2011; C. Burton, Lovic, & Fleming, 2006; Weaver et al., 2004). A recent study found no differences in activity among female offspring that received more LG (Cavigelli et al., 2010). However, our findings suggest that within the normal range of variations in maternal behavior, those that receive more LG are more active—perhaps a result of less anxiousness, as demonstrated from the results of the open field task.

We also predicted that high LG female offspring would show reduced levels of CORT in response to physical restraint stress compared with their low LG siblings within litters, and that offspring receiving the highest absolute levels of LG would show the most

attenuated CORT response to restraint in adulthood. Our results indicated an unexpected relationship between the amount of LG received and offspring stress reactivity. We found an interaction between litter LG status and within-litter offspring LG rank. As adults, low licked offspring from low LG litters and high licked offspring from high LG litters showed a reduced stress response in comparison to high licked offspring from low LG litters and low licked offspring from high LG litters. Total LG received was not significantly correlated with percent change in CORT attributable to the stressor, suggesting that absolute levels of licking are not driving CORT reactivity in this cohort of female offspring. This finding may indicate that simply receiving differential LG than one's siblings can significantly influence the female offspring's stress reactivity, regardless of whether an individual offspring actually receives more or less care. Interestingly, in an analogous situation in humans, it has been shown that siblings receiving significantly differential care report lower self-worth and higher emotionality, regardless of whether they were receiving more or less care than their siblings (Burt, McGue, Iacono, & Krueger, 2006; Feinberg et al., 2000; Meunier, Biscaglia, & Jenkins, 2012; Meunier et al., 2013). Although some animal studies have shown an association between maternal care and stress reactivity for male offspring (Weaver et al., 2004; Weaver et al., 2007), other studies in female offspring have not shown this effect (C. L. Burton et al., 2007).

We hypothesized that offspring receiving higher LG would show higher GR expression in adulthood than their lower LG siblings and that offspring receiving the highest levels of absolute LG would show higher GR expression compared with offspring receiving lower absolute LG levels, as levels of GR in hippocampus are known to be important for the negative feedback effects of CORT after a stress response (Sapolsky, Krey, & McEwen, 1984). We found that, within litters, high LG offspring had significantly higher levels of GR gene expression than their low LG siblings. Total LG received did not correlate significantly with GR expression or MR expression, suggesting that absolute levels of LG were not a major driver of GR and MR gene expression in our cohort of female offspring. However, we found that GR expression levels were negatively and significantly correlated with the CORT response to stress as indexed using AUCg analysis, supporting previous evidence of stress-induced CORT regulation as a function of GR abundance in the hippocampus (F. A. Champagne et al., 2006).

To further investigate GR regulation, we quantified gene expression levels of GR₁₅, GR₁₆, GR₁₀, and GR₁₁, additional splice variants of GR known to be expressed in the hippocampus (McCormick et al., 2000). There was a significant correlation between GR₁₆, GR₁₀, and GR₁₁ abundance and total GR expression, suggesting the involvement of other exon GR exon 1 splice variants in total transcript abundance of GR in the hippocampus. There was no significant relationship between levels of expression of each of these additional splice variants and differential licking, indicating a complex relationship between the various expressed splice variants of GR in female offspring and maternal care. It is possible that a specific combination of alterations in the GR₁₆, GR₁₀, and GR₁₁ and perhaps other splice variants contribute to the overall regulation of the GR gene as a function of differential licking relative to other environmental factors, as GR splice variant usage was shown to be context-specific (Turner, Schote, Macedo, Pelascini, & Muller, 2006). Our results show evidence of coregulation of GR1 splice variants, as we have reported previously in the context of between-

litter differences in maternal care in adult male offspring (McGowan et al., 2011).

We tested the hypothesis that DNA methylation in the promoter region of the GR₁₇ splice variant is significantly affected by litter LG status, as several CpG sites in male offspring reared in high LG litters were shown to be hypomethylated when compared with those reared in low LG litters (Weaver et al., 2004; Weaver et al., 2005). Contrary to our prediction that higher levels of LG would predict lower GR₁₇ DNA methylation, we found that there was a significant effect of litter LG status on percent methylation at CpG site 7 of GR₁₇ promoter and a significant effect of within-litter LG rank differences as an effect of LG in sites 7 and 17 in our cohort of female offspring. Specifically, these CpG sites were hypermethylated among the highest licked offspring of high licking dams relative to the lowest licked offspring among low licking dams. For CpG 7, the highest licked offspring of low licking dams showed hypermethylation compared with the lowest licked offspring from the same litters. These CpG sites were previously characterized as binding sites for the transcription factors specificity protein 1 (Sp-1) and nerve growth factor inducible factor-a (Ngfi-a), respectively (Chen, Ou, Wu, & Shih, 2011; Hellstrom et al., 2012; Meinel et al., 2013). Our results suggest that the specific CpG sites contributing to the regulation of GR₁₇ promoter may differ both between and within maternal environments. Interestingly, recent evidence indicates that GR exon 1 splice variant expression also shows coregulated expression in human brain in association with variations in DNA methylation across multiple first exons (Cao-Lei et al., 2013). Together with our data, these results point to the potential involvement of DNA methylation in the regulation of additional splice variants of GR through variations in maternal care received. In addition, in future studies, time-course and protein-level analyses will be important in understanding the stability of the changes in GR associated with within-litter variation in maternal care observed in this study.

Most studies to date have focused on male rat offspring; we focused on female offspring. Females have been shown to have higher baseline levels of CORT and CRF neuron activation, and are generally observed to be more anxious than males (Babb, Masini, Day, & Campeau, 2013; Kokras et al., 2012; Simpson, Ryan, Curley, Mulcaire, & Kelly, 2012). In addition, as maternal behavior is transmitted across generations and manipulations during early development can alter the transmitted maternal behavior, females can serve as the basis for the behavioral transmission of individual differences in stress reactivity across generations (F. A. Champagne, Francis, et al., 2003; Francis, Diorio, et al., 1999). Differences between our study of the effects of both between- and within-litter variation in maternal care compared with previous studies of between-litter effects on male offspring could also indicate that within-litter differences in LG are a potent contributor to adult phenotype differences, perhaps even more important than between litter differences, at least in our all-female cohort.

Research in humans shows several patterns of relevance to understanding the animal data. First, within and between family processes are independently associated with children's mental health outcomes (Boyle et al., 2004; Meunier et al., 2013) and self-esteem (Feinberg et al., 2000). Second, individual characteristics of children drive the start of differential parenting, but differential parenting subsequently changes child behavior (Jen-

kings, Dunn, O'Connor, Rasbash, & Behnke, 2005; Plamondon & Jenkins, 2013). The findings in the current study suggest the same pattern of reciprocal influence: LG influences offspring behavior, gene expression, and DNA methylation but characteristics of pups may also elicit differential LG. There are also parallels between human and animal research related to the effects of absolute levels of parental care. For instance, adults with a history of childhood abuse and neglect display lower total GR gene expression in the hippocampus than those without a history of childhood adversity (McGowan et al., 2009), and gene expression is in turn mediated by epigenetic processes including DNA methylation. Low LG in rats and early life abuse and neglect in humans are correlated with increased DNA methylation within the GR promoter and lower expression of the gene in the hippocampus (McGowan et al., 2011; Suderman et al., 2012; Weaver et al., 2004; Weaver et al., 2005; Weaver et al., 2007).

In this study we have demonstrated within-litter and between-litter variations in maternal LG that influence the behavioral phenotypes of female offspring. These influences are associated with complex gene regulatory mechanisms. Future work should endeavor to describe the specific mechanisms that mediate the effects of maternal care on phenotypic outcomes and also examine additional epigenetic mechanisms that may underlie changes in gene expression.

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