Differential licking in early life alters stress behavior and brain gene expression in adult female rats

by

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A thesis submitted in conformity with the requirements for the degree of Master's of Science
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Abstract

We investigated licking and grooming (LG) levels received by each pup from their dams and the locomotor activity, anxiety-like behaviors, and stress reactivity in adult female offspring. We also investigated glucocorticoid receptor (GR) gene expression and its DNA methylation status in the hippocampus, comparing pups between and with-in litters. Rats that receive more LG than their siblings showed less anxiety-like behaviors and increased locomotor activity, regardless of their litter type. Higher licked pups also showed increased expression of the GR gene. Gene expression levels of the GR 17 splice variant were not significantly different as a function of dam LG or LG received, whereas DNA methylation levels at two CpG sites within GR17 promoter were significantly higher in high LG pups than low LG pups. Our results indicate that naturally occurring intra- and inter-litter differences in maternal LG have a lasting effect on the phenotypic outcomes of adult female offspring.
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Introduction

Variations in parental care during the early postpartum period are related to individual differences in the neurodevelopment and behavior of offspring (e.g. Weaver et al., 2004; McGowan et al., 2009). 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 young between litters (Champagne 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 licking dams, respectively. Animals reared in high and low LG litters show long-term changes in adult behavior, gene expression, and epigenetic signatures (Champagne et al., 2003; Weaver et al., 2004; McGowan 2011).

Pups reared in high LG litters spend more time in the center of an open field task, spend more time in the light compartment of a light/dark box compared to animals reared in low LG litters (Clinton et al., 2007), and less time being immobile in a forced swim test than pups reared in low LG litters (Weaver et al., 2006). These results suggest that pups in high LG litters generally display less anxiety- and depressive-like behaviors than pups in low LG litters. Animals reared in low LG litters are also more active compared to pups reared in high LG litters, an effect also seen among animal reared without their mothers in an artificial rearing paradigm (Burton et al., 2011). Animals from high LG litters also
exhibit a reduced hypothalamic-pituitary-adrenal (HPA) stress response, showing lower levels of corticosterone in plasma in response to a restraint stress (Weaver et al., 2005). These effects of licking on the HPA are thought to be mediated in part by changes in the expression of the Glucocorticoid Receptor (GR) gene, as high LG reared rat pups show higher mRNA expression of the GR gene in the hippocampus than do low LG reared pups (Meaney, 2001; Francis et al., 1999; Belay et al., 2011; in mice, Franklin et al., 2010; Chourbaji et al., 2011). Further, several studies have shown that the effects of elevated LG on behavior, the stress response, and gene expression are associated with reduced methylation of the promoter region of the GR17 untranslated splice variant (Weaver et al., 2004; McGowan et al., 2011; Liberman et al., 2012; Kosten et al., 2013).

Much of the existing literature in animal models examines the effects of variations in maternal care has focused on inter-litter differences in maternal care. However, recent findings show that significant variation exists in the level of maternal care received by individual rats within the same litter, and these differential LG levels result in differences in behaviour in adulthood between siblings (van Hasselt et al., 2011; 2012; Cavigelli et al., 2010; 2012; Ragan et al., 2011). Using dams who show levels of LG that are within one standard deviation of a given population’s mean LG (the middle group), one study has showed that pups that receive higher LG are more active (Cavigelli et al., 2010) and have
higher expression of the GR gene and greater dentate synaptic plasticity in the hippocampus (van Hasselt et al., 2011; 2012) than their low licked siblings.

What has not yet been done is to consider intra-litter effects in high and low litters. Differential parenting has also been studied in human families; and research in this area show evidence that children within a family receive different amounts and kinds of parental attention and, as a result, show different developmental trajectories (see Jenkins et al., 2013; Feinberg et al., 2000). An examination of the various changes in adult phenotypes of rat offspring may help us better understand the impact of differential parenting, particularly within litters in an animal model.

Given these observations, the present study had several aims. First, we investigated the effects of differential maternal care received by individual pups within litters on their activity, anxiety behaviors, HPA reactivity, gene expression and DNA methylation. We hypothesized that animals that receive higher LG than their siblings would show reduced anxiety-like behaviors, have less activation of the HPA axis in response to a stressor and will have higher GR gene expression and reduced DNA methylation levels in the GR17 splice variant. Based on the extant literature, predictions regarding activity were more ambiguous; however, we predicted that pups of high as opposed to low-licking dams would show reduced activity, but pups receiving more licking than their siblings, might show the opposite effect. We also predicted that higher levels of GR gene expression would be associated with reduced anxiety-like behaviours and a less reactive
stress response. Second, we investigated additive and interactive effects of intra-litter and inter-litter variations in maternal care. We predicted that the high vs. low LG effects would be greatest among the pups that received the highest licking across litters (highest licking received within litter and highest LG dam) in comparison to those that receive the lowest (lowest pup in lowest LG litter; Meunier et al., 2012; Burt et al., 2006).

**Methods**

**Subjects**

40 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 our facilities in the vivarium at 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 until mating. Animals were housed in standard Plexiglas cages (20 cm x 43 cm x 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 University of Toronto (UTM and UTSC) Animal Care Committees.
Maternal Behavior Observations

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 (21 - 26 days). 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 post-natal day (PND) 0. At PND 1, each litter was culled to six female pups whenever possible. Litters with fewer than four female pups were excluded from our study. Dams and remaining pups were transferred to large maternal observation cages (50 cm x 40 cm x 20 cm) where they remained for 21 days. Maternal behavior was observed in a 30-minute retrieval task after a 10-minute separation period from the pups daily for the first ten PNDs for each pup in each litter (modified from Rosenblatt, 1967). Pups were individually identified, as indicated below. Two experimenters with high inter-rater reliability (>0.9) coded the dams’ behaviors (frequency and duration) live using Behavioral Evaluation Strategy and Taxonomy (BEST) software (Educational Consulting Inc., Florida, USA). Behaviors coded differentially for each pup included of 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.

Each pup was marked using food coloring (Club House, London, Ontario, Canada) daily to differentiate them during observations. Each pup was covered with their designated color using a nylon paint brush (food colors were odorless
and tasteless). Experimenters were careful to ensure that pups remained their
designated colors throughout PNDs 1 to 10, and to avoid the anogenital region
of the pups when coloring. Observations were made every day immediately
following coloring. Litters were excluded from the experiment due to
cannibalism (n=4), the presence of male pups that were mistaken for female
pups (n=2), or unsuccessful impregnation (n=1); thus, a final number of 33 litters
(and 189 offspring) were included.

In total, 189 pups in 33 litters were assessed individually for maternal LG
received. Eight litters in the top 25% of the entire cohort’s total duration of LG
across 10 observed 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. Litters were identified this way using cumulative percentage and
quartile analysis of each dam’s total LG levels for all females in our cohort. The
remaining 17 litters in the middle 50% of the cohort’s LG were designated as
mid LG litters. The highest and lowest licked pups from each litter were tested
for the locomotor activity and open field task (n = 2 per litter x 33 litters = 66
animals).

Animals in the high 25% and low 25% LG litters were selected for
subsequent gene expression and DNA methylation analysis. In each of the high
25% and low 25% litters, one pup from the two highest licked pups and one
pup from the two lowest licked pups were randomly selected for behavioral
testing; and their counterparts were selected for physiological analysis of the
stress response. Animals in the latter cohort were subjected to a 20-min physical restraint test in addition to other behavioral tasks. All animals were sacrificed at the conclusion of behavioral testing, and their glucocorticoid receptor (GR) gene expression, mineralocorticoid receptor (MR) gene expression and GR17 promoter DNA methylation levels were determined (see below).

**Behavior and stress reactivity**

**Locomotor activity task.**

The highest and lowest licked pups from each litter (n = 2 x 33 litters = 66) were tested in adulthood (> PND 75) for general locomotor activity levels. Subjects were tested in a 30-minute session midway through the animals’ light phase of the circadian cycle (between 1100 and 1600 h) in a locomotor activity box (47 cm x 26 cm x 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 level was quantified as the number of total photocell interruptions (i.e. ‘beam breaks’) as a reliable measure of general locomotor activity by an automated testing system (Lynch et al., 2011). Test boxes were cleaned with 70% ethanol before each trial and the number of boli produced by each animal was recorded for each test session.
Open field task

The highest and lowest licked pups from each litter (n = 2 x 33 litters = 66) were tested in adulthood (> PND 75) for anxiety like behaviors. Subjects were tested in a five-minute session midway through the animals’ light phase of the circadian cycle (between 1100 and 1600 h) in an open field box (100 cm x 100 cm x 35 cm). The arena was divided into 49 equal grid squares. The nine central squares are 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’. Animals were placed in the center facing the experimenter and then allowed to explore the apparatus for five minutes. Time spent in the center, corners, and sides were recorded live using BEST software by a single experimenter, and the number of boli produced by each animal during the test session was also recorded. Anxiety levels were quantified as the proportion of total time animals spent in the center of the apparatus, shown to be a valid measure of anxiety in rodents (Hall & Ballachey, 1932).

HPA axis stress reactivity

One of the two most licked and one of the two least licked pups from the eight high LG and eight low LG litters (n = 2 x 16 litters = 32) were randomly selected to be tested in adulthood (> PND 75) for stress reactivity using physical
restraint. Subjects were tested in a 90-minute session midway through the animals’ light phase of the circadian cycle (between 1100 and 1600 h). Rats were transported from their home colony rooms into the testing room. They were hand-restrained under an ordinary cotton towel. Blood was collected from a nick in the tail as previously described (Fluttert et al., 2000; Belay et al., 2011) into a non-heparinized 0.6 ml centrifuge tube (baseline, approximately 100 μl per rat) and immediately placed on ice. Rats were then placed into Plexiglass restrainers (8 cm diameter x 20 cm length) and, 20 minutes later, a second sample of blood (peak, 1 tube, approximately 100 μl per rat) was collected as described above immediately before rats were released from the restraint. Rats 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. Blood was incubated on ice for at least 30 min before samples were centrifuged at 4°C and 4000 rpm for 25 min. Blood serum was extracted and stored at -80°C. Blood samples were processed for corticosterone (CORT) by ELISA assay using a commercial rat/mouse corticosterone ELISA kit (ALPCO Diagnostics, Cat # 55-CORMS-E01, Salem, New Hampshire, USA). HPA axis stress reactivity was measured by changes in the levels of CORT in response to a physical restraint stressor. Area under the curve with respect to the ground (AUCG) is calculated as AUCG = Σ (mi +1 + mi)/2, with mi denoting the individual measurement, and n the total number of measurements (Preussner et al., 2003).
Hippocampal gene expression and DNA methylation analysis

**Brain Extractions and Tissue Punches**

The highest licked and lowest licked pups from six high LG and six low LG litters (n = 2 x 12 litters = 24) that were tested for locomotor activity and open field task but not stress response were sacrificed via CO\textsubscript{2} inhalation and rapidly decapitated in adulthood. Whole brains were extracted, snap frozen in Isopentane on dry ice and stored at -80°C. Brains were cryo-sectioned using a Leica CM3050S cryostat and the hippocampus (HPC) was collected bilaterally for each subject using stereotaxic coordinates targeting the area between – 1.80 Bregma and – 5.20 Bregma (Paxinos and Watson, 5\textsuperscript{th} Ed.).

**Gene Expression**

Total RNA was extracted along with genomic DNA (DNA/RNA-Protein Mini kit, Qiagen, Toronto, Ontario, Canada). Total RNA was reverse transcribed into cDNA (High Capacity cDNA Reverse Transcription Kit, Life Technologies, Burlington, ON, Canada), and gene-specific real time quantitative PCR (RT-qPCR) was performed (StepOne Plus, Life Technologies, Grand Island, NY, USA). 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). 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 size was verified by the presence of a single peak. The following primer sets were used 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): MR – forward: 5’-ggcagctgcaagtctttct-3’, reverse: 5’-gacagtctttcgccgaaac-3’; GR – forward: 5’-ctcgaaggtctcccaagcaatgt-3’, reverse: 5’-gcaatgctttctccgaaag-3’; GR15 – forward: 5’-ctgagagggttttcgcatcg-3’, reverse: 5’-tcctcccctcaggctttttat-3’; GR16 – forward: 5’-cgggctcactaatattgccaatggac-3’, reverse: 5’-ggcgaagatgcagaaaccttgact-3’; GR17 – forward: 5’-ggagcctggagaagagaa-3’, reverse: 5’-ctaccaggggagctaagga-3’; GR110 – forward: 5’-cgcggtctgttttatctgg-3’, reverse: 5’-tcctccctcaggctttttat-3’: GR111 – forward: 5’-ggctttgatgggtggatg-ga-3’, reverse: 5’-taccaggggagctaaagat-3’.

**DNA methylation**

DNA methylation was quantified by sodium bisulfite mapping (Clark et al., 1994). Briefly, genomic DNA isolated as described above was bisulfite converted (Qiagen’s EpiTect Bisulfite Kit, Qiagen, Toronto, Ontario, Canada) and amplified by PCR using primers targeting 17 CpGs within the GR 17 promoter region (Weaver et al., 2004). Bisulfite-treated DNA was subjected to two cycles
of PCR (outside - forward: 5’-ttttttaggttttttaggc-3’, reverse: 5’-atttttaattttctttttcc-3’; nested – forward: 5’-ttttttttaggtgatatatttt-3’, reverse: 5’-ttctcccaacctcctcc-3’). The nested PCR product was subcloned, transformed, and at least ten different clones per plate were mini-prepped (Original TA cloning kit, Invitrogen). The ligated DNA fragments were then sequenced (Eurofins Operon MWG, Huntsville, Alabama).

**Statistical analysis**

Statistical analyses were conducted using SPSS v.21 and v.22 for Mac. Measures from the locomotor activity box, open field task, GR and MR gene expression, GR exon 1 splice variants expression, and GR17 DNA methylation were analyzed by repeated measure 2x2 or 2x3 (pup: low LG, high LG; litter: low 25%, high 25% or low 25%, mid 50%, high 25%) within x between Analysis of Variance (ANOVA), where appropriate. DNA methylation levels were assessed for each of the 17 CpG sites contained within GR17 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 pup’s specific deviation from litter’s LG mean, behavioral measures, stress reactivity (corticosterone levels at all time points and percent change across restraint stressor) and gene expression were
calculated using one-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 pups were also analyzed this way. 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 variability in the level of maternal care given by dams both between high and low LG litters \( F_{(1, 23)} = 931.481, p < 0.001 \), as well as given to individual pups within litters \( F_{(5, 115)} = 70.969, p < 0.001 \); see figure 1a) across the entire (10 d) observation period. To investigate whether differential treatment of siblings was a result of differences in overall licking, the amount of LG received by each pup was calculated as a percentage of their litters’ mean LG levels. Dams in all types of litters (high 25%, mid 50%, and low 25%) lick and groom their pups differentially, as pups’ percent LG levels were significantly different across different litter types \( F_{(5, 189)} = 104.562, p < 0.001 \); see figure 1b). To assess consistency in maternal levels of LG across days, LG was analyzed separately for each day during the post-natal observation period (PND 1-10).
This revealed that high, mid and low dams lick significantly differently from one another on each of PNDs 2, 3, 4, and 5; high dams lick significantly more than mid and low dams on each of PNDs 6, 7, and 8; (See figure 1c). Pups’ level of LG received across the 10 observation days was (in general) consistent, as pups’ LG ranking did not change significantly across days (see figure 1d). Using both parametric and non-parametric tests, we also found that between litter effects are comparable between analyses using either standard deviation characterized or quartile characterized LG statuses (data not shown).

Behavior and Stress Reactivity

Locomotor Activity

We found main effects of both litter and pup type on locomotor activity: higher licked siblings were significantly more active than their lowered licked siblings \( (F_{(1, 26)} = 4.368, p = 0.047) \) and pups reared by high licking dams were more active than pups reared by low licking dams \( (F_{(2, 26)} = 5.49, p = 0.01) \). Post-hoc analyses showed that animals in the low 25% litters and the mid 50% litters were significantly less active than animals in the high 25% litters (see figure 2a).

Open Field Task

We predicted that animals that receive higher levels of LG would be less anxious and that this effect would occur between and within litters. Results from this task showed a significant within litter pup effect, but no between litter effects. Higher licked siblings spent a significantly longer period of time in the
center of the arena than their lower licked siblings \( (F_{(1, 30)} = 5.439, p = 0.027) \) regardless of the type of litter in which they were reared (figure 2b). As well, higher LG pups spent a significantly higher proportion of the total time in the center of the arena than their lower LG siblings \( (F_{(1, 30)} = 5.415, p = 0.027; \) figure 2c). This effect persisted after co-varying general locomotor activity \( (F_{(1, 57)} = 4.249, p = 0.044); \) data not shown), which suggests that differences in open field task behavior are not a result of differences in general activity levels.

**Stress Response Test**

We predicted that animals receiving higher levels of LG would have a less reactive HPA axis in response to a physical restraint stressor. In fact, we found no significant effect of differential mothering between pups or between litters on CORT levels (ng/ml); however, there was a significant effect of time \( (F_{(2, 48)} = 32.696, p < 0.01), \) indicating a significant increase in CORT as a function of restraint. When CORT levels at 20 and 90 minutes were calculated as a percentage of baseline levels (min 0), there was a significant three-way interaction between time, litter type, and pup type \( (F_{(2, 50)} = 3.195, p = 0.049, \) see figure 2d). High licked pups from the high LG litters and low licked pups from the low LG litters had a significantly inhibited rise in CORT levels in response to restraint stress, whereas low licked pups from the high LG litters and high licked pups from the low LG litters had a more exaggerated response to restraint stress.
Gene Expression and DNA methylation

Gene expression of MR, GR, and GR exon 1 splice variants

We hypothesized that animals that receive higher levels of LG would display higher levels of GR expression. Analysis of GR gene expression in the hippocampus showed that pups who received higher LG than their siblings had higher levels of GR ($F_{(1,20)} = 4.862$, $p = 0.039$, figure 3a); but there was no effect of being reared in a low vs. high LG litter. In support of our predictions, there was a significant negative correlation between total GR expression and area under the curve (AUC_g) in the stress response test, ($R = -0.414$, $p = 0.028$; figure 3b). GR16, 110, and 111 expression levels were significantly and positively correlated with total GR expression levels (GR16: $R = 0.394$, $p = 0.028$, figure 4b; GR110: $R = 0.355$, $p = 0.044$, figure 4d; GR111: $R = 0.403$, $p = 0.026$, figure 4e). GR15 (data not shown) and GR17 (figure 4c) expression levels did not show a significant correlation with GR total expression levels. MR gene expression did not differ significantly between animals who received different levels of LG within or between litters (data not shown).

DNA methylation of GR17

We hypothesized that animals that received higher levels of LG would display lower levels of DNA methylation of the GR17 splice variant. There were no differences within ($F_{(1, 20)} = 0.021$, $p = 0.888$) or between litters ($F_{(1, 20)} = 0.151$, $p = 0.703$) in the overall percentage of methylated clones (figure 5a). However,
detailed analysis of percent methylation levels at each of the 17 GR17 CpG sites revealed differences in methylation as an effect of LG at sites #7 and #17 (see figure 5d). Methylation levels at site #7 were significantly different between high and low licked siblings ($F_{(1, 20)} = 5.603, p = 0.039$; post-hoc analysis showed that low LG pups from low LG litters had significantly lower methylation levels than high LG pups in low LG litters and than high LG pups from high LG litters, $p = 0.021$ and $0.039$ respectively; figure 5b). Methylation levels at site #17 were also significantly different between pups reared by low and high licking dams ($F_{(1, 20)} = 7.481, p = 0.021$; post-hoc revealed that low LG pups from low licking litters had significantly lower levels of methylation than high LG pups from high licking litters, $p = 0.05$; figure 5c). There was a significant positive correlation between the total LG pups received and their GR17 DNA methylation levels ($R = 0.345, p = 0.049$; data not shown).

**Discussion**

In this study, we investigated the effects of differential maternal care in the early developmental period on rat female offspring stress and anxiety phenotypes, as well as possible mechanisms for these changes. In addition, we sought to describe within-litter differences in maternal care. We found that dams differ in the level of maternal care they give to their litters, and as well, dams treat their individual pups differently. Animals who received more LG during early development are generally more active and less anxious than animals that received less LG. In addition, animals that received more maternal
care had higher levels of expression of the GR gene and had higher levels of methylation at GR1 CpG sites #7 and #17.

Maternal care results confirm previous results that there is naturally occurring variation in the level of LG dams give to their offspring (Champagne et al., 2003). As well, dams show substantial variation in levels of licking and grooming toward their individual female offspring, supporting previous findings in male offspring (van Hasselt et al., 2011; 2012; Cavigelli et al., 2010; 2012; Ragan et al., 2011). We also analyzed LG levels between low 25%, mid 50% and high 25% litters on each of the 10 PNDs. Dams behaved significantly differently between PNDs 3 and 8, with the high 25% dams licking significantly more than did the low and mid dams. These data support a number of previous studies of inter-litter differences in maternal care (e.g. Champagne et al., 2003; 2006). In addition, we ascertained that pups are licked fairly consistently across the 10-day observation period. That is, pups’ relative LG received ranking did not change significantly on a day-to-day basis and pups who were ranked as high LG consistently received significantly more LG than pups who were ranked as low LG. This finding suggests that the differential treatment siblings receive may be in part due to some characteristics of the individual pups, which elicits a specific level of care from their mothers. For example, maternal female rats show a particular responsiveness to pup vocalizations, which prompts them to actively orient themselves toward a vocalizing pup when it is in close proximity
(Farrell & Alberts, 2002a; b). It is possible then that different pups emit characteristically different levels of ultrasounds.

Although some previous studies have used one standard deviation above and below the cohort’s LG mean to determine the litter LG status, we characterized our litters by dividing the dams in our cohort into the highest 25%, middle 50%, and lowest 25%. However when we categorized groups according to the standard deviation criterion, the results were very similar to those reported herein. These data indicate that our results would be comparable to previous findings using standard deviations from the cohort LG mean to characterize high and low LG status (e.g. Champagne 2003; Weaver et al., 2004).

Within litters, pups that receive high LG are more active than their low LG siblings; in addition, pups reared in high LG litters are significantly more active than pups reared in low and mid LG litters (but pups reared in low and mid LG litters do not differ significantly). Generally, deficits in maternal care have been linked with hyperactivity, as seen in rats reared without their mothers artificially (Burton et al., 2006; 2011). However, our findings could suggest that within the normal range of varying maternal behaviors, those who receive more LG are more active (perhaps due to less anxiousness, as demonstrated from our open field task results).

We hypothesized that animals that receive higher LG than their siblings
would show reduced anxiety-like behaviors, and that animals receiving the highest absolute levels of LG (i.e., high LG pups reared in high LG litters) would be the least anxious. Consistent with this prediction, we find that higher LG pups are less anxious than their lower LG siblings within litters, as demonstrated by a significantly higher amount of time spent in the center of the open field arena, both in terms of absolute time and as a proportion of the total time spent in the arena. However, no differences between animals reared in high and low LG litters were found, as has been reported previously (Meaney et al., 2001). This within–litter, but not between-litter effect of LG on anxiety behaviors has been found before (Kurata et al., 2009; Sequeira-Cordero et al., 2012; Kosten et al., 2013) and indicates the effects of differential mothering between siblings, received individually, is a major contributor to the offspring’s adult stress phenotype. Total LG received was not significantly correlated with time spent in the center of the open field, which suggests that the absolute level of LG received is not a strong determining factor of behavior in the open field task.

We also predicted that high LG pups would show reduced activation of the HPA axis in response to physical restraint stress than their low LG siblings within litters, and that pups receiving the highest absolute levels of LG would have the least ‘reactive’ HPA axis. Our results demonstrated an unexpected relationship between the amount of LG received and their stress reactivity. Our results showed an interaction between litter LG status and within litter pup LG rank. Low licked pups from low LG litters and high licked pups from high LG
litters show a reduced stress response in comparison to high licked pups from low LG litters and low licked pups from high LG litters. Total LG received was not significantly correlated with percent change in CORT due to the stressor or area under the curve analysis (see Table 1), which suggests that absolute levels of licking is not driving HPA reactivity in this cohort of animals. This may suggest that simply receiving differential LG than one’s siblings can significantly influence the animal’s stress reactivity, regardless of whether an individual pups 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 (Feinberg et al., 2000; Burt et al., 2006; Meunier et al., 2012; 2013).

Although in general, studies have shown that male rats that receive more care are less reactive than rats that receive less care when stressed (Weaver et al., 2004; 2007), results from studies of females are less clear. For example, artificially reared females, who experience a significant deficit in maternal care during early development, do not show a significant increase in stress reactivity in comparison to maternally reared females (Burton et al., 2007).

We hypothesized that pups receiving higher LG would show higher expression of the GR gene than their lower LG siblings and that animals receiving the highest levels of absolute LG would show higher GR expression, as levels of GR in hippocampus are known to be important for the negative
feedback effects of CORT after a stress response (Sapolsky et al., 1984). We found that, within litters, high LG pups 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 gene expression in our cohort. However, we found that GR expression levels negatively and significantly correlated with stress response test AUCg analysis, supporting previous evidence of stress-induced CORT regulation as a function of GR abundance in the hippocampus (Champagne et al., 2006).

To further investigate GR regulation, we quantified gene expression levels of GR15, 16, 110 and 111, additional splice variants of GR known to be expressed in the hippocampus (McCormick, 2000). There was a significant correlation between GR16, 110, and 111 abundance and total GR expression, suggesting a complex relationship between various GR exon 1 splice variants and total transcript abundance of GR. It is possible that a combination of alterations in the GR16, 110, and 111 and perhaps other splice variants contribute to the overall expression of the GR gene as a function of differential licking, as GR splice variant usage was shown to be context-specific (Turner et al., 2006).

Previous studies have demonstrated that DNA methylation in the promoter region of the GR17 splice variant is significantly affected by litter LG status, and that several CpG sites in animals reared in high LG litters are
hypomethylated when compared to those reared in low LG litters (e.g. Weaver et al., 2004; 2005). Contrary to our prediction that higher levels of LG would predict lower GR17 DNA methylation, we found low but significant levels of methylation in GR, in support of several previous studies (Witzmann et al., 2012; Beery et al., 2013, personal comments). There was a significant effect of litter LG status on percent methylation at CpG site #7 of GR17 and a significant effect of within litter LG rank differences as an effect of LG in sites #7, which has been characterized as binding sites for the transcription factors specificity protein 1 or Sp-1 (Chen et al., 2011; Meinel et al., 2013) and #17, which has been identified as a binding site for nerve growth factor inducible factor-a Ngf-a (Hellstrom et al., 2012). Our results indicate a complex relationship between site-specific CpG methylation of GR17 and environmental context (Turner et al., 2008; Witzmann et al., 2012), and suggest that the specific CpG sites contributing to the regulation of GR17 promoter may differ between and within maternal environments.

The current study yielded some results that proved to be inconsistent with previous studies investigating the effects of differential LG on adult stress behavior and with our hypotheses. Despite the fact that we found great variations between dams of differing LG statuses, we could not replicate differences in gene expression, DNA methylation, and anxiety-like behaviors between offspring belonging to high and low LG litters. One reason for this discrepancy could be that our study employed a very different methodology for
quantifying maternal behavior. Previous studies have used time-sampling performed throughout the day to quantify the frequency of licking, grooming, and sometimes arch backed nursing (Meaney et al., 2001; Champagne et al., 2003; Weaver et al., 2004; McGowan et al., 2011), whereas in our study maternal behavior was recorded in one continuous 30 minute session at one time each day. 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, CRF neuron activation, and are generally observed to be more anxious than males (Kokras et al., 2012; Simpson et al., 2012; Babb et al., 2013). In addition, females may be more sensitive to differential maternal LG, as maternal behavior is transmitted across generations (Francis et al., 1999; Champagne et al., 2003). These differences 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 also indicates that there is differential effect of parental care on child development in and across individual children within the same family. Children (both males and females) who receive more negative maternal treatment than their siblings exhibit more aggressive behaviors than siblings who receive less negative maternal behaviors (Boyle & Jenkins, 2004). Additionally, siblings who receive more differential treatment report lower global self worth and higher emotionality than siblings who receive less differential
treatment (Feinberg et al., 2000). These differential parenting effects seen in humans suggest a possible frame of reference to interpret our results from this animal model of differential parenting. Postpartum studies indicate that 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 associated with increased DNA methylation within the GR and lower expression of the gene in the hippocampus (Weaver et al., 2004; 2005; 2007; McGowan et al., 2011, Suderman et al., 2012).

We have demonstrated in this study that not only do dams perform differently as mothers in a naturally varied manner; they also treat their individual offspring differentially. We have further shown that differential maternal care has significant and lasting effects on the behavioral phenotypes of rat offspring, and that these differences are mediated by complex gene regulatory mechanisms. Our study indicates that an important contributor to differential gene expression is the relative LG differences between siblings, in addition to any differences in LG due to being reared in low versus high LG litters. 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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