CHAPTER 2

Within-litter differences in individual mother-infant interaction predict stress phenotype in later-life

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Adapted from:

Development of individual differences in stress responsiveness:
an overview of factors mediating the outcome of early-life experiences

Psychopharmacology 2011 Mar; 214(1):141-54

ABSTRACT

Previous studies have shown that naturally-occurring variation in maternal care during the first week of life predicts stress responsiveness, cognitive performance and emotionality in adult offspring. These findings are based on the assumption that maternal care is distributed equally across pups sharing a litter, such that each individual pup inevitably develops a similar phenotype later in life. Therefore, the current study tests the hypothesis that the distribution of maternal care directed towards individual pups *within* a litter is homogenous and therefore results in a uniform 'stress phenotype' in later-life among individuals within a given nest.

Maternal care directed towards individual pups was observed for 5 h/day during the first week of life. Each pup was daily labeled and weighted, and examined at adolescence and adulthood for its endocrine responsiveness to a novelty stressor using an open field test. Blood samples were collected for analyses of basal and stress-induced corticosterone (CORT) levels. Our data show that: 1) distribution of maternal care is unequal *within* the litter, 2) males receive higher levels of maternal care than females, and 3) basal and stress-induced CORT levels were negatively correlated with the amount of maternal care received.

These findings reveal the impact of individual pup-oriented maternal care on the development of individual differences in stress responsiveness in later life and can help provide more insight into how very subtle early-life experiences within the family unit promote vulnerability or resilience throughout the lifespan.

INTRODUCTION

Both human epidemiological data and animal studies have consistently shown that adverse early-life events are associated with alterations in stress responsiveness as well as impaired cognitive and emotional development (1-6) which may increase the susceptibility to a wide array of both mental and physiological disorders later in life (7-13). The mechanisms underlying these long-term effects of early-life environmental adversity are largely unknown, but are thought to involve alterations in mother-infant interaction. Therefore, amount and/or quality of maternal care are seen as mediating factors for the effects of early environmental adversity on the development of the offspring.

Previous studies using a rodent model of naturally-occurring variation in maternal care indeed have shown a strong mediating effect of maternal care on the offspring's stress responsiveness, emotional and cognitive development (4, 14, 15). This model is based on individual differences among lactating rats in the frequency of maternal licking and grooming (LG) they provide to their pups and has shown that as adults, offspring raised by mothers that naturally display high levels of LG show decreased behavioural and endocrine responsiveness to acute stress compared with offspring of mothers that naturally show lower levels of LG (14, 15). Cross-fostering studies, in which the biological offspring of a high LG mother is cross-fostered to a low LG mother or vice versa, show that this procedure completely reverses the phenotype of the offspring (16), suggesting that maternal care transfers differences in stress responsiveness to the offspring in a non-genetic way.

In addition to stress responsiveness, development of emotionality and cognitive performance in the adult offspring also depends on the amount and quality of maternal care received during early-life. Offspring of high LG mothers showed substantially reduced behavioural fearfulness in response to novelty compared with offspring of low LG mothers (15). Additionally, relative to offspring of low LG mothers, offspring of high LG mothers showed greater hippocampal dendritic (and spine) complexity, synaptogenesis and synaptic plasticity, which are associated with enhanced long-term potentiation (LTP), spatial learning and memory function (4, 17, 18).

Interestingly, due to methodological considerations, the association between maternal care during early-life and phenotype of the offspring in later-life is based solely on maternal phenotype (i.e. high vs. low LG dam) and not on what is experienced by individual pups within the nest. However, recent studies have suggested that pups from the same litter (assumed to have received the same amount of maternal LG) display substantial variation in behavioural phenotype later in life (19). Additionally, we previously reported that a 24 hour maternal deprivation results in the amplification of individual differences in stress responsiveness, rather than having a generalized outcome (20). What makes some individuals more vulnerable or resilient to the impairing effects of maternal deprivation is largely unknown; however we propose that this phenomenon might be mediated by differences in individual mother-infant interactions during early life.

This study, using the Wistar rat as a model, provides a detailed description of the amount and quality of maternal care received by each individual pup *within* the litter during the first week of life. Secondly, we investigated whether variation in maternal care *within* the litter correlates to phenotypic differences in stress responsiveness in the offspring later in life, thus complementing previous studies on variation in maternal care *across* litters.

METHODS

Animals

20 female Wistar rats (6 weeks, 200 g) and 10 male Wistar rats (6 weeks, 250 g) obtained from Harlan (Horst, the Netherlands) were used as breeders. After a habituation period of one week, two females were mated with one male in type 4 polycarbonate cages (59x38x20cm) containing sawdust bedding and tissues. Food (RM3, Special Diet Services, Witham, Essex, UK) and water (8 ml 25% HCl /10 L tap water) were provided *ad libitum*. Animals were maintained on a 11-h light : 13-h dark cycle with light on at 08.30h, in a temperature (21+/- 2°C) and humidity (55 +/- 5%) controlled room. After breeding, pregnant females were individually housed in Plexiglass cages (55x30x44cm) that were customized to allow a clear view of all activity within the cage, despite the presence of sawdust bedding and tissues. Females were checked daily for presence of pups. If pups were present, the day of birth for that particular litter was defined as postnatal day 0 (pnd 0). On pnd 1, litters were culled to 8 pups (4 males and 4 females). Cages were not cleaned until pnd 10 and after that once weekly. After weaning (pnd 21), offspring was housed in same-sex, same-litter groups in type 4 polycarbonate cages (59x38x20cm) until time of testing.

Animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive (86/609/EEC).

Marking

In order to discriminate between individual pups within the litter, all pups were daily marked using a non toxic, odourless surgical marker (Codman, via Johnson & Johnson, Amersfoort, the Netherlands). The marking of the pups always took place between 08.30h and 10.30h in the morning. The procedure took about 7 min per litter and consisted of removing all the pups from the mother and marking them gently one by one after recording their body weight. A schematic of the marking system is depicted in Fig 1. Upon completion of the marking procedure, all pups were immediately returned to their mother.

Maternal Behaviour

The maternal behaviour of each dam was observed and scored for five 60 minutes periods per day during the first 7 days post partum using a procedure as described by Champagne and colleagues (21). Observations were performed

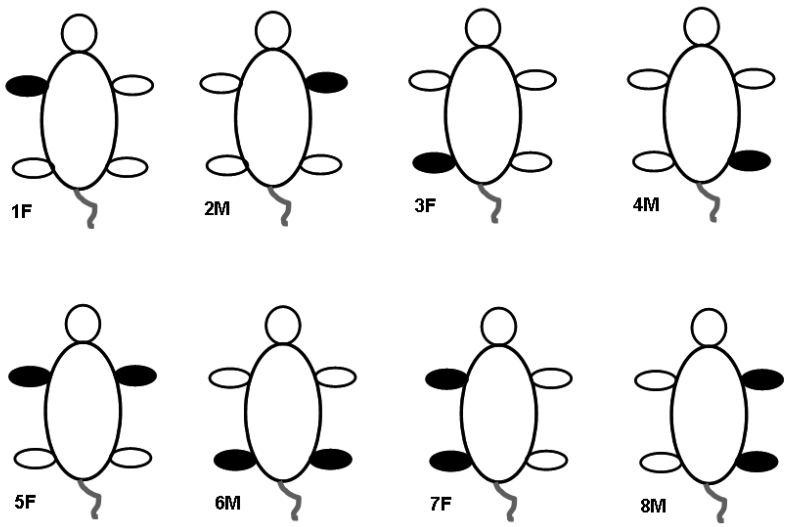

Fig 1. Marking system used to discriminate between individual littermates. Black areas indicate body parts that were marked. M = male, F = female.

at three periods during the light phase (10:00, 13:00, 17:00) and two periods during the dark phase (07:00 and 20:00) of the cycle. Within each 60 min observation period the behaviour of each mother was scored every 3 minutes (20 observations per period, 100 observations per day, 700 observations for the first 7 days postpartum, see Fig 2). The following maternal behaviours were scored: a) frequency of licking and grooming (LG) of the pups (whole body LG and anogenital LG), b) frequency of arched back nursing/blanket nursing/passive nursing, c) mother away from nest/no maternal contact. The data were analyzed as the percentage of observations in which animals displayed one of the behaviours described above. Furthermore, for every maternal behaviour scored, it was noted to which pup(s) it was directed. The data for these individual observations were analyzed as the percentage of observations in which each pup received one of the maternal behaviours described above. The total percentage of LG received by individual pups was used to categorize them. For each litter a LG 'family mean score' was calculated and pups that displayed a LG score of at least 1 standard deviation above or below the family mean were selected as high and low LG pups, respectively.

Endocrine responsiveness to novelty stress

Selected high and low LG animals (n = 13) were tested for their responsiveness to acute novelty stress in adolescence (pnd 28) and adulthood (4,5 months). All animals were individually housed two days prior to testing (to reduce the effect of testing-order within the cage) and returned to their home cage directly after testing. The novelty stressor consisted of 10 min exposure to a black circular open

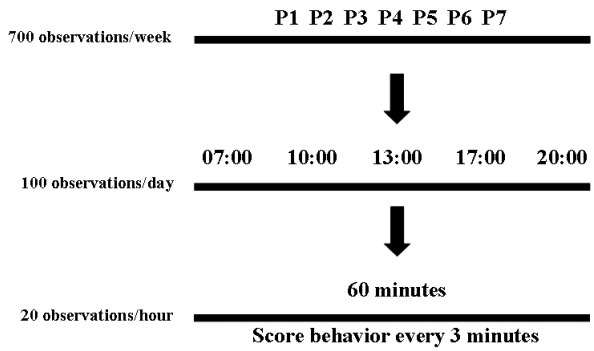

Fig 2. Observation schedule. Adapted from: Champagne *et al*, 2008. P1: postnatal day 1, P2 : postnatal day 2, etc.

field apparatus (diameter: 90 cm, height: 40 cm) in a dimly lit room (approximately 30 lux floor level). Two animals were tested at the same time and in the same testing room using two separate open field setups. All testing took place between 09.00 and 13.00h, and each test apparatus was cleaned thoroughly between animals using 70% ethanol.

Blood sampling for endocrine measurement

Blood samples were obtained from the tail vein, by a small incision with a razorblade at three different time points: a) basal, one day before novelty exposure, b) 10 min after onset of novelty stress, i.e. directly after termination of stressor, and c) 30 min after onset of novelty stress, i.e. 20 min after termination of stressor. Blood was collected in small EDTA coated tubes (Microvette CB 300 K2E, Sarstedt, Numbrecht, Germany). Plasma was obtained by 15 min centrifugation at 13000 rpm at 4°C and subsequently stored at -20°C until further analysis.

Plasma corticosterone determination

Plasma corticosterone (CORT) concentrations were measured in-duplo using radioimmunoassay (RIA) kits containing ^{125}Iodine labelled CORT (MP Biomedicals, Asse-Regelem, Belgium). All samples were analysed in one session to exclude inter-assay variability.

Data analysis

Statistics were performed using SPSS for Windows (version 14.0; SPSS, Chicago, IL). One-way ANOVA was performed to determine significant differences between high and low LG animals, and between males and females. Graphs were plotted using Prism Graph Pad software.

RESULTS

Maternal licking and grooming

Distribution of maternal LG received by individual pups in each of the 7 litters (with appropriate litter composition) studied is depicted in figure 3 and shows

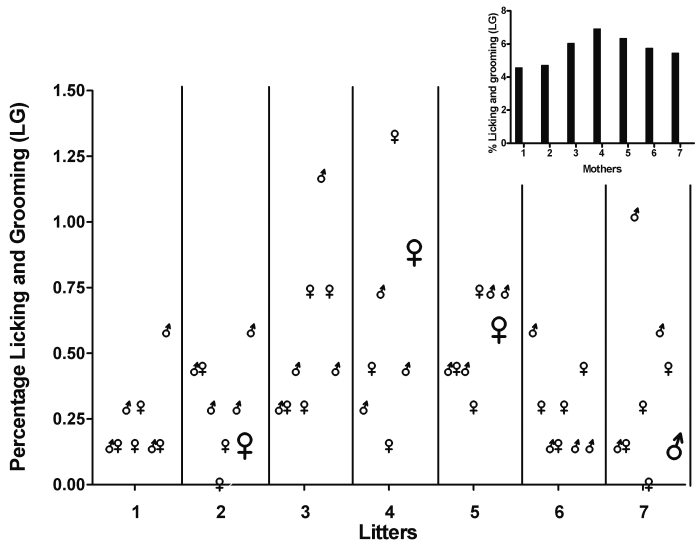

Fig 3. Distribution of maternal licking and grooming (LG) received by individual pups for each litter (1-7). Shown is percentage of observations (pnd 1-7) during which each individual pup received maternal LG. Male pups are indicated with ♂, female pups are indicated with ♀. Selected high (n = 9) and low LG (n = 4) offspring within the litter (that displayed a LG percentage of at least 1 standard deviation above or below the family mean, respectively) are indicated with enlarged symbols**.**
Inset: Total percentage of LG displayed by each mother.

that maternal LG is unequally distributed among pups *within* the litter and that particular pups clearly received higher levels of LG compared to their littermates. However there is variation in the amount of inequality between different litters. Mothers from litters 1, 2, 5, and 6 appear to distribute their care more equally compared to mothers from litters 3, 4, and 7, which show a more heterogeneous distribution of LG.

Gender preference

Additionally, we report that mothers show a preference for male over female offspring ($F = 4.31$; $p < 0.05$; $n = 55$; Fig 4). In the current study, 6 out of 7 mothers spend significantly more time providing LG to male compared to female offspring.

Endocrine responsiveness to novelty stress

Endocrine response to novelty stress, both at adolescence and adulthood was investigated in animals receiving either high or low levels of maternal LG during the first week of life (see Fig 5).

Both basal and stress-induced CORT levels in adolescent animals are associated with individual differences in maternal LG received during early-life. Thus, animals that received high levels of maternal LG *within* litter show significantly lower basal

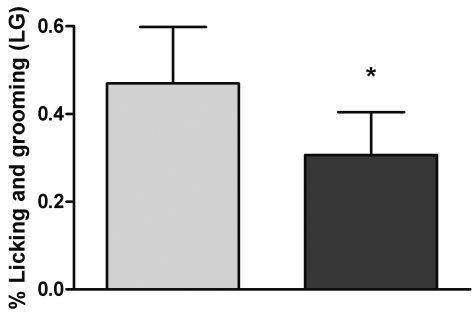

Fig 4. Percentage (mean ± SEM) of maternal licking and grooming (LG) received by male (light grey) and female (dark grey) offspring. In 6 out of 7 litters (86%), males (n = 31) received higher levels of maternal LG compared to females (n = 24). * = $p < 0.05$.

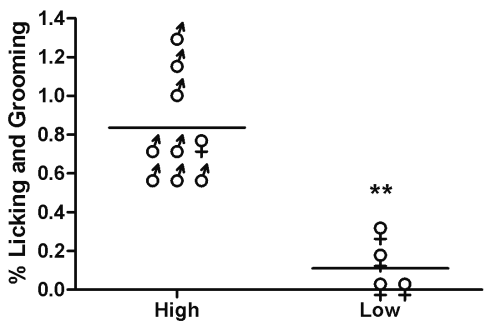

Fig 5. Percentage of licking and grooming (LG) received during the first 7 days postpartum for selected high and low LG pups taken from distinct litters. Male pups are indicated with ♂, female pups are indicated with ♀. The selected high and low offspring significantly differ in the mean percentage of LG ($p < 0.001$). Horizontal lines indicate the group mean. Mean ± SEM high LG group (n = 9: 8 males, 1 female) = 0.83 ± 0.09 and mean ± SEM low LG group (n = 4: all female) = 0.11 ± 0.07.

($F = 5.09$; $p < 0.05$) as well as stress-induced (10 min and 30 min after onset; $F = 4.93$; $p < 0.05$; $F = 5.56$; $p < 0.05$ respectively) CORT levels when compared to animals that received low levels of maternal LG (Fig 6a).

Similar to what is observed in adolescence; the amount of maternal LG received in infancy also appears to predict CORT levels in adulthood. Animals that received high levels of maternal LG *within* litter show significantly lower stress-induced (10 min and 30 min after onset; $F = 61,99$; $p < 0.001$; $F = 14,05$; $p < 0.01$ respectively) CORT levels when compared to animals that received low levels of maternal LG (Fig 6b). Due to technical issues, we are unable to show basal CORT levels of the animals in adulthood.

DISCUSSION

The present study provides a detailed description of the naturally-occurring variation in maternal care received by individual pups *within* a litter over the first week of life. The hypothesis was tested that maternal care is equally distributed among individual pups within the litter and therefore results in a uniform stress phenotype across littermates. We report that: 1) maternal care was unequally distributed among individual littermates, leading to the occurrence of high and

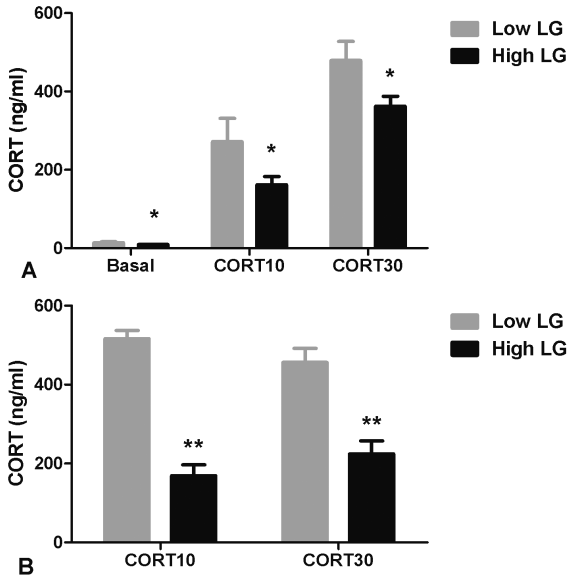

Fig 6. Plasma corticosterone (CORT) levels of rats characterised as high or low LG within the litter, expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$. Selected high (n = 9) and low LG (n = 4) animals were tested for their endocrine responsiveness to acute novelty stress in (A) adolescence (age pnd 28) and (B) adulthood (age 4.5 months). CORT levels were significantly more elevated in low LG compared to high LG offspring at 10 min (CORT10) and 30 min (CORT30) after onset of acute stress. Due to technical issues, we are unable to show basal CORT levels of the animals at adulthood; therefore in figure 6B only stress-induced CORT levels are displayed.

low LG offspring within a given litter; 2) LG was biased towards male offspring, leading to the fact that most high LG pups were of the male gender; 3) within-litter differences in maternal LG were negatively correlated with basal and stress-induced plasma CORT levels in adolescence and adulthood, although in a gender-dependent manner.

Individual differences in mother-infant interaction

The present study demonstrates that maternal care is not equally distributed across individual pups sharing a litter, meaning that the mother consistently spends more time caring for certain pups compared to others. However, in previous maternal care studies the behaviour of the mother was observed regardless of which pup the behaviour was directed at (4, 14, 15). In studies using this 'whole litter' paradigm it has been assumed that all pups sharing a litter receive the same amount of care, leading to the hypothesis that all individual littermates inevitably develop a similar phenotype in adulthood, although substantial variation in adult behavioural phenotype has been reported between littermates (19). A more recent study showed that individual differences in maternal LG were correlated with hippocampal synaptic plasticity and glucocorticoid receptor mRNA expression (22). These findings suggest that very subtle differences in *non-shared* maternal LG (up to 10 fold smaller than previously reported differences in LG used to characterize maternal behaviour directed towards the whole litter (21)) appear to have comparable predictive value for later-life phenotypic outcome. Although further studies are warranted to understand these observations, the findings suggest that results from whole litter observations should be carefully interpreted.

Underestimation of individual differences in LG

We showed that the amount of maternal LG is associated with basal and stress-induced CORT levels during adolescence and adulthood. This is reminiscent of previous studies using whole litter observations that have also shown the impact of naturally-occurring variation in maternal LG on endocrine response to acute stress (14, 23). The present study shows that even more subtle naturally-occurring *within-litter* differences in mother-infant interaction are associated with variation in stress responsiveness. However, the differences we observed using this paradigm might be too subtle to reveal significant interactions between LG received in infancy and behavioural responses to novelty stress in adulthood (data not shown); an effect that has been reported using the whole litter paradigm (15, 16).

Wistar dams display overall relatively low levels of maternal care compared to Long Evans dams that are frequently used for studies on the effects of maternal LG (21). Therefore the average levels of LG (i.e. 6%) displayed by Wistar dams in our study would be equivalent to levels displayed by low LG Long Evans dams. In our study, we observed that Wistar dams on average delivered 40 LG events over the 7 day observation period (700 observations). From the pup's perspective, this might represent on average 5 LG events per individual pup. Such scores might appear too low to reliably categorize individual littermates as low or high LG offspring. We acknowledge that this represents a limitation of our experimental approach, which leads to an underestimation of the occurrence of pup-directed LG events. In the future, this issue can be resolved by increasing the amount of observations per day to achieve a more reliable representation of the actual differences in *non-shared* maternal LG.

Gender preference

It is noteworthy that for most of the litters studied, dams displayed a strong gender preference for male over female offspring. Such a preference of the mother for male pups has been shown before (24, 25) and is proposed to be regulated by testosterone, pheromones, and urinary odour (26, 27). As a consequence, this gender preference resulted in a bias in the distribution of males and females over the selected low (exclusively females) and high LG (almost exclusively males) groups. Since glucocorticoid levels and corticosteroid binding globulin levels in the blood are modulated by fluctuations in sex hormone levels (28) the association between early-life maternal LG and later-life HPA axis activity has to be carefully interpreted. However, we report an association between neonatal LG and glucocorticoid levels as early as pnd 28, when the regulatory role of sex hormones on HPA axis functioning is assumed to be low compared to that in adulthood (29). Gender differences in endocrine responsiveness on pnd 28 have been shown in some (30, 31) but not all (32) studies. Because of the small sample size, we were unable to reveal a within-gender effect of maternal LG on glucocorticoid levels. Future studies using a higher number of animals and including ovariectomized female offspring could reveal the exact role of gender-dependent within-litter differences in maternal LG during early-life on the development of stress phenotype in later-life.

Introduction of early handling

In order to score individual LG, a daily marking procedure was used to label and identify individual littermates. Such a procedure inevitably introduces a substantial amount of handling. This implies that besides studying the impact of subtle differences in maternal care within the litter, we have to take into account the well-known effects of handling (33-36). The daily handling/marking procedure potentially altered maternal care directed towards the offspring. However we believe that this bias is distributed to a similar extent to all litters. Furthermore, since we observed a substantial amount of variation between littermates in terms of stress responsiveness, we suggest that *non-shared* experiences in LG rather than the *shared* experience of handling/marking mediate the effects observed here. This scenario is not unprecedented and has been previously proposed by Macri and co-workers for variation in *shared* maternal care (37, 38).

Early-life experiences within the family unit promote development of individual differences

It is well-known that the quality of early family environment can serve as a major source of vulnerability in later life. After both human and animal studies have reported that early-life adversity is associated with alterations in stress responsiveness (39, 40) which may increase the risk for developing various disorders later in life (41, 42), it has now been assumed that this effect is at least partly mediated by parental influence on the development of the stress-regulating system (43-45). Subtle differences in parenting style could lead to dysregulation of HPA responsiveness, which subsequently could promote the development of illness (46-48). Human studies have reported that parenting style not only varies between families but also within the family. These *non-shared* factors, such as variation in individual parent-child interaction might have higher predictive power for the development of stress-related pathologies in later-life compared to the *shared* family environment (49, 50).

The present study is to our knowledge the first to report the impact of within-family differences in parenting in rodents on the development of individual differences in stress responsiveness in later life and can help to provide more insight into how very subtle early-life experiences within the family unit promote vulnerability or resilience throughout the lifespan.

ACKNOWLEDGEMENT

The support from EU funded LifeSpan Network of Excellence (FP6-036894), IRTG NWO DN-95-420, Royal Netherlands Academy of Arts and Sciences, Eurostress, TI Pharma T5-209 and Smartmix Programme of the Netherlands Ministry of Economic Affairs, and the Netherlands Ministry of Education, Culture and Science is gratefully acknowledged.

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