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Associations among within-litter differences in early mothering received and later emotional behaviors, mothering, and cortical tryptophan hydroxylase-2 expression in female laboratory rats

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Abstract

The effects of differential maternal care received on offspring phenotype in rodents has been extensively studied between litters, but the consequences of differential mothering within litters on offspring neurobehavioral development have been rarely examined. We here investigated how variability in maternal care received among female rat siblings (measured four times daily on postnatal days 4, 6, 8, and 10) relates to the siblings' later emotional and maternal behaviors. As previously reported, we found that some female pups received up to three times more maternal licking bouts compared to their sisters; this difference was positively correlated with the pups' body weights. The number of maternal licking bouts females received was negatively correlated with their later neophobic behaviors in an open field during periadolescence, but positively correlated with their anxiety-related behavior in an elevated plus maze during adulthood. Licking received was also positively correlated with females' later likelihood to retrieve pups in a maternal sensitization paradigm. In addition, females' neophobia during adolescence and anxiety-related behavior during adulthood predicted some aspects of both postpartum and sensitized maternal responsiveness. Medial prefrontal cortex expression of tryptophan hydroxylase-2 (TPH2; enzyme necessary for serotonin synthesis) was negatively associated with early maternal licking received. Interestingly, cortical TPH2 was positively associated with the maternal responsiveness of sensitized virgins but negatively associated with it in postpartum females. These results indicate that within-litter differences in maternal care received is an often neglected, but important, contributor to individual differences in offspring socioemotional behaviors and the cortical serotonin neurochemistry that may influence these behaviors.

Keywords

anxiety; maternal behavior; neophobia; tryptophan hydroxylase-2; within-litter

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Introduction

It is well known that there are differences between family groups in the interactions between mothers and offspring. These differences provide the foundation for variation between families in offspring developmental trajectories and thereby have long-term consequences for many aspects of offspring physiology and behavior (reviewed in Champagne and Curley, 2009; Fleming, O'Day, and Kraemer, 1999). An excellent example of this in laboratory rats and mice is that females originating from litters that receive more frequent maternal licking develop into adults that are themselves high licking mothers (Champagne et al., 2003a; Champagne, 2008; Curley, et al., 2009; Fleming et al., 2002; Francis et al., 1999a, b; Gonzalez et al., 2001) or more maternally responsive when tested as virgins in a maternal sensitization paradigm (Champagne et al., 2001). Furthermore, these high-licked litters show less anxiety-related behavior in numerous paradigms when compared to the offspring in low-licked litters (Caldji et al., 1998; Francis et al., 1999b; Weaver et al., 2004; Pedersen et al., 2011). These differences between high- and low-licked litters in their later socioemotional behaviors are associated with differences in central expression of receptors for estrogen (Champagne et al., 2003b; Champagne et al., 2006; Peña, Neugut, and Champagne, 2013), oxytocin (Champagne et al., 2001), dopamine (Peña et al., 2014), benzodiazepines (Fride et al., 1985; Cadji et al., 1998, 2004) glucocorticoids (Kaffman and Meaney, 2007; Liu et al., 1997; Liu et al., 2000) and corticotropin releasing hormone (Liu et al., 1997; Caldji et al., 1998) in the brain sites relevant for these behaviors.

Although it is rarely studied or even recognized, there are also within-family differences in mother-offspring interactions in many species and these differences significantly contribute to variation in the developmental trajectories of the siblings within those families (Birnie et al., 2013; Cavigelli et al., 2010; Moore and Power, 1992; Pan et al., 2014; Plotsky and Meaney, 1993; Ragan et al., 2012; Rubin, Cheah, and Fox, 2001; Stanton and Levine, 1990; van Hasselt et al., 2012). Indeed, Ragan and Cavigelli have reported that the within-litter differences in maternal licking received by rat and mouse pups are of the same magnitude as those found between litters, with the highest-licked pups receiving up to three times as many licking bouts as their lowest-licked siblings (Cavigelli et al., 2010; Ragan et al., 2010; Ragan et al., 2012). For daughters, the consequences of such differences include that the highest-licked sisters are more neophobic (Ragan et al., 2012) yet have lower basal plasma corticosterone than their lower-licked sisters (Ragan et al. 2011). Notably, these correlations for the females within litters are in opposite directions of what is found for males when these relationships are compared across litters (Weaver, Diorio, Seckl, Szyf, and Meaney, 2004).

The influence of within-litter variation on development of these behaviors is particularly important to understand because, even though there are genetic contributions to parental and emotional behaviors that explain some of the variability in their display across rodent and primate families, the transmission of these behaviors is more strongly related to early-life environmental factors and gene by environment interactions (Berman, 1990; Fairbanks 1996; Francis, Diorio, Liu, and Meaney, 1999; Gonzalez et al., 2001; Kendler and Baker, 2007; Klahr and Burt, 2013; Kovan, Chung, and Sroufe, 2009; Maestripieri, 1999). Part of the non-heritable, environmentally-driven contributions to differences among siblings in their neurobehavioral development is that mothers selectively invest attention and resources in

some offspring more than others, even when the offspring are genetically identical. Both the mother and the offspring contribute to these non-heritable effects because their interactions are dyadic and a host of characteristics about each participant greatly influences the behavior of the other (Britton, 2011; Jenkins, Dunn, O'Connor, Rasbash, and Behnke, 2005; Polan, Milano, Eljuga, and Hofer, 2002; Stern, 1997). For example, offspring body weight affects the amount of maternal care received in animals ranging from birds (Gottlander, 1987) to humans (Beaulieu and Bugental, 2008; Feldman and Eidelman, 2007; Singer et al., 2003) with heavier offspring receiving higher priority for maternal care. Furthermore, differences among offspring in behaviors that solicit maternal attention (which in rats include ultrasonic vocalization, moving close to the mother, and probing her with their snouts and paws; Polan and Hofer, 1999; Shair et al., 1997) greatly contributes to how much care they receive (Stern, 1997). Even human infants differ in the pitch and frequency of their crying, amount of physical activity, and degree of demandingness for attention - all of which affect their mother's responses to them (Bornstein and Manian, 2013; Lester, Boukydis, Garcia-Coll, Hole, and Peuker, 1992; McGuire and Dunn, 1994) - and contributes to later differences between human siblings in their prosocial behavior (Stocker, Dunn, and Plomin, 1989; Brody et al., 1987) and emotional regulation (Eley, Liang, Plomin, Sham, Sterne, Williamson, and Purcell, 2004; Shanahan, McHale, Crouter, and Osgood, 2007a).

The primary purpose of the present study was to examine the relationships between differences in mother-offspring interactions within litters of laboratory rats and later differences in the female siblings' maternal behavior during adulthood. We also examined the females' anxiety-related behavior both during periadolescence and adulthood because emotional reactivity can positively or negatively influence maternal responsiveness (Fleming and Luebke, 1981; Lonstein, 2007; Ragan and Lonstein, 2014). To accomplish our purpose, we first determined the degree to which maternal care received by pups varies among Long-Evans female rat siblings within litters, as previously demonstrated in other laboratory rat strains and in laboratory mice (Cavigelli et al., 2010; Pan et al., 2014; Ragan et al., 2012; Ragan et al. 2010; van Hasselt et al., 2012a, b). We then examined if this variability in maternal care received was associated with some relevant physical characteristics of the pups and their display of maternal solicitation behaviors. We then determined the relationships among the within-litter differences in licking received and the daughters' later neophobic behaviors, anxiety-like behaviors, and maternal responsiveness to pups (postpartum or sensitized). Lastly, we determined if there was a relationship between within-litter differences in the frequency of licking that females received and later tryptophan hydroxylase-2 (TPH2) in their medial prefrontal cortex (mPFC). TPH2 is found in all serotonergic somata and proximal axons (Donner ff Handa 2009; Hale, Shekhar, and Lowry, 2011; Walther, 2003) and its levels are highly correlated with the amount of serotonin that the cells produce and their rate of activity (Calcagno et al., 2007; Cervo et al., 2005; Gutknecht et al., 2012; Patel et al. 2004; Zhang et al., 2004). TPH2 mRNA and protein also exist in the cytoplasm of serotonergic terminals, including the prefrontal cortex (Carkaci-Salli et al., 2011; Sakowski et al., 2006; Perroud et al., 2010; Zill et al., 2007). This distal expression in the rat brain overlaps with serotonin immunoreactivity (Cohen et al., 1995; Weissmann et al., 1987), correlates with the local extracellular serotonin release (Calcagno et al., 2007; Chen and Miller, 2012), and for many years has also been suggested to indicate

the capacity for local serotonin synthesis (Pickel, 1976; Weissmann et al., 1987; Perroud et al., 2010). Central serotonin systems are tremendously sensitive to developmental events in male rodents (reviewed in: Albert, Vahid-Ansari, and Luckhart, 2014), are implicated in the display of maternal behaviors in female rats (Barofsky et al., 1983; Pawluski et al., 2012; Zhao and Li, 2010), and when released in the mPFC is associated with anxiety-related behaviors (see Albert, Vahid-Ansari, and Luckhart, 2014). Thus, we hypothesized that females' mPFC levels of TPH2 would be associated with the within-litter differences in all of our behaviors of interest.

Materials and Methods

Subjects

Subjects were the daughters of six unrelated postpartum Long-Evans rats, descended from rats purchased from Harlan Laboratories (Indianapolis, IN) and housed in our breeding colony as previously described in detail (Ragan and Lonstein, 2014; Smith and Lonstein, 2008). Lights were on from 0700 to 1900 h with food and water provided *ad libitum*. Litters were culled to contain 8 pups (3–4 females) on the day of birth (PND 0). On PND 21, female offspring were weaned and housed with their same-sex littermates. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at Michigan State University.

Observations of Mother-Offspring Interactions

On postnatal days 4, 6, 8, and 10, undisturbed observations of the mothers and their litters were conducted 4 times/day (1200, 1500, 1800, 2000 hr - i.e., twice during the light photophase and twice during the dark photophase) for 30 min each, yielding 16 observations and 8 hours of data per litter. Observations began on PND 4 rather than earlier because we and others have found that rat maternal licking begins to peak around this day (Cavigelli et al., 2010; Champagne et al., 2003a; Fleming and Rosenblatt, 1974; Liu et al., 2000; Ragan et al., 2012) and a re-analysis of previous studies by Ragan et al. (2012, and unpublished data) had revealed that differences among sisters in the frequency of licking they received between PND 4–8 most strongly affected their adult neophobia (Ragan et al., 2012) and anxiety (Ragan et al., 2010). One litter was observed at a time to ensure that observers gave adequate attention for tracking each of the eight pups in the litter, and behaviors were live-scored as they occurred. Maternal behaviors recorded included body and anogenital licking of pups, nursing in any posture, and some non-pup-directed behaviors (i.e., nest building, self-grooming, and eating/drinking). Pup behaviors recorded included the frequency that each pup initiated contact with the dam by touching her face (perioral contact) or ventrum with their snout (ventral probing). Daughters were later categorized as high- or low-licked based on median splits of the total number of licking bouts received within each litter, resulting in up to two high-licked and two low-licked females per litter. These categorical designations were only used to assign high-licked and low-licked females to be mated or undergo maternal sensitization (see below), and were not used for statistical analyses.

At 0900 h each day (i.e., three hours before the first observations of maternal behavior began), we measured ultrasonic vocalizations, weighed, and marked pups. During litter handling, dams were placed in clean holding cages for 15 min to ensure equal separation time for all pups. During this time, each pup was placed in a 100 mL glass beaker while the handler held the microphone at the top of the beaker and for 60 sec counted the pup's 45-kHz ultrasonic vocalizations, which are emitted when pups are separated from their dam (Shair, Masmela, Brunelli, and Hofer, 1997), with an ultrasound detector (D230 Ultrasound Detector, Pettersson Elektronik, Uppsala, Sweden). To identify individual pups in each litter, pups were then weighed and then marked with a unique number with a non-toxic Sharpie™ on their back, head, and abdomen as described previously (Cavigelli et al., 2010; Ragan et al., 2012). Weighing and marking took ~30 sec for each pup. After the 15 min necessary for ultrasound recording and pup marking, mothers and litters were returned to their home cages.

Response to Novel Object

In periadolescence (PND 30), we observed daughters' responses to a novel object placed in an open field. The 120 cm × 120 cm arena had 46-cm high white polypropylene walls and a novel pink rubber ball with a circumference of 16.5 cm was placed in the center. Illumination in the center of the arena was 31 lux. Between 1400 and 1600 h, animals were carried in their home cage to the testing room, gently placed into the arena, and videotaped for 5 min by a camera aimed at a mirror hanging above the arena. The subjects' latencies to contact the novel object were recorded. Frequencies of self-grooming, rears, and physical interactions with the novel object were also recorded, but were not significantly associated with any of the other behaviors we measured, so are not reported. After testing, the novel object and arena were cleaned with 70% ethanol and allowed to dry between subjects.

Elevated Plus-Maze Behavior

Starting on PND 70, the adult daughters were vaginally smeared daily and on the first day of diestrus detected their anxiety-related behavior was observed on an elevated plus maze for 10 min between 1400 and 1600 h as described in detail previously (Ragan and Lonstein, 2014). The percentage of time spent in open arms and the frequency of entries made into the open arms were measured as primary indicators of anxiety-related behavior (Pellow et al. 1985). The apparatus was cleaned with 70% ethanol and allowed to dry between subjects.

Maternal Sensitization

Starting at approximately PND 120, one high-licked and one low-licked female from each litter underwent a maternal sensitization paradigm using methods similar to those previously described (Lonstein, Wagner, and De Vries, 1999; Smith, Holschbach, Olszewicz, and Lonstein, 2012). In litters with only three females, the middle sister was randomly assigned to either be mated or sensitized. Briefly, females were chronically exposed in their home cages to groups of 3 freshly-fed young pups and their behavior observed every 10 sec for 15 min at 0900 h daily. Females were categorized as maternal if they retrieved all 3 pups to a single location and crouched over them on two consecutive days within the 9 days of testing. Females that never fully sensitized were given a latency of 8 days.

To collect brains for TPH2 analysis (see below), females remained alone in their home cages after the completion of sensitization testing and were vaginally smeared daily. On the first day of diestrus detected (which was 4.8 ± 1.4 days after completion of sensitization) females were narcotized by exposure to carbon dioxide for up to 2 min. After rapid decapitation, brains were removed and stored at -80°C until Western blotting.

Postpartum Maternal Behavior

Between PND 90–100, the remaining high-licked and low-licked sisters from each litter had their estrous cycles monitored daily with a vaginal impedance meter (Fine Science Tools, Foster City, CA) and on a day of proestrus were housed with sexually-experienced males from our colony for two days. Females were then housed with another pregnant female until being singly housed starting 5–7 days before the expected day of parturition. After giving birth, their maternal behavior was observed on postpartum days (PPD) 4, 6, 8, and 10 using the same methods as those used to study their own mothers described above, although their pups were not marked. In addition, a 45-min observation of maternal behavior was conducted on postpartum day 7 after separating the mothers from their pups for 1 hr. The pups were removed from the home cage at 1300 h and placed in an incubator set at nest temperature. After incubation, pups were expressed of feces and urine and scattered in the home cage away from the nest, and maternal behaviors recorded (Ragan et al., 2012). On PPD 11, the dams were narcotized with carbon dioxide for up to 2 min and rapidly decapitated. Their brains were collected, flash-frozen in cold isopentane, and stored at -80°C until analysis for TPH2 (see below).

Western Blot Analysis of TPH2 in the mPFC

Forebrains were cut coronally into 500- μm thick sections using a cryostat (Leica CM1950, Nussloch, Germany) and three sections encompassing the prefrontal cortex (PFC) (corresponding to plates 8–10 from Swanson, 1998) were collected. Homogenization, determination of protein concentrations, and Western blotting procedures were conducted as previously described (Ragan and Lonstein, 2014) using a TPH2 primary antiserum (1:1000, #PA1-778, Lot: PB195015, Thermo Fisher Scientific, Rockford, IL) with a Super Signal Western Blot Enhancer kit (#46640, Lot: PC197951A, Thermo Fisher Scientific, Rockford, IL) and a peroxidase-conjugated anti-rabbit IgG secondary antiserum (1:2500, #7074, Lot: 24, Cell Signaling Technology, St. Louis, MO). After stripping the membranes, GAPDH was blotted for as the “housekeeping” protein to be used as a loading control (1:500; MAB374; Lot: 2145925, Millipore, Billerica, MA) with a peroxidase-conjugated rabbit anti-mouse secondary antiserum (1:80,000; #A9044, Lot: 010M4797, Sigma-Aldrich, St. Louis, MO). ImageJ (National Institutes of Health, Bethesda, MD) was used to determine the integrated density of the immunoreactive bands. The TPH2 integrated densities were standardized using the subjects’ corresponding GAPDH integrated density measurements.

Data Analyses

Non-normal data were log-transformed before parametric analyses. Spearman correlations were used to examine the relationships among daughters’ maternal behavior received, their own neonatal physical characteristics and behaviors, their maternal sensitization latency (virgins) or postpartum maternal behavior given to their own pups, their latency to approach

novelty, elevated-plus maze behavior, and mPFC TPH2 protein expression. Multiple regression analysis was used to examine how five pup characteristics (birth weight, weight on the first day of observation [PND 4], frequency of perioral contact with dam, frequency of ventral probing of the dam, and frequency of ultrasonic vocalizations emitted during the brief separations on PND 4–10) predicted the frequency of maternal licks that daughters received, and their later maternal behaviors. Independent *t*-tests were used to compare the daughters that successfully sensitized to those that did not in their maternal behavior received, neonatal characteristics, neophobia, anxiety-related behavior, and mPFC TPH2 expression. Statistical significance was indicated by $P \leq 0.05$.

Data from the maternal behavior observations after brief separation was lost for one of the mated daughters. Two subjects (both from the mated daughter group) were revealed by SPSS on quantile-quantile and boxplots as significant outliers (defined as >1.5 interquartile ranges) for their behavior in the novel object test and TPH2 protein expression in the mPFC, respectively, so were removed from the analyses of those data.

Results

Within-litter variability in maternal care received and its relationship with the pups' characteristics

As previously reported in Sprague-Dawley rats (Cavigelli et al., 2010; Ragan et al., 2012) and Agouti viable yellow mice (Ragan et al. 2010), we here found in Long-Evans rats that the most-licked female pups received up to three times as many licking bouts as their least-licked sisters ($t_{19} = 2.85$; $P = 0.01$; Fig. 1A).

Regression analysis revealed that 36% of the variability among sisters in the frequency of maternal licking they received was explained by the five neonatal variables we assessed. While the full model was not statistically significant ($r^2 = 0.60$, $F = 1.69$, $P > 0.05$), there was a significant association between pup weight on the first day of observations (PND 4) and the total frequency of licks they received across the entire the observation period (PND 4–10) ($r_{21} = 0.44$; $P > 0.05$; Fig 1B).

Relationship between within-litter variability in maternal care received and emotion-related behaviors

The number of maternal licking bouts received was negatively associated with the daughters' later latency to approach a novel object when tested during periadolescence ($r_{21} = -0.56$, $P = 0.009$, Fig. 2A). The frequency of daughters' ventral probing of their mothers was also negatively associated with the daughters' later latency to approach the novel object ($r_{21} = -0.53$, $P = 0.01$, Fig. 2B).

Notably, and consistent with previous work that found strong associations between licking and neophobia among sisters (Cavigelli et al., 2010; Ragan et al., 2012), the number of licking bouts females received from their mothers was negatively associated with the percentage of time they later spent in the open arms of the elevated plus maze ($r_{21} = -0.56$, $P = 0.008$; Fig. 3). The number of licking bouts received was not associated with the

percentage of entries they made into the open arms ($r_{2I} = -0.34$, $P > 0.05$) nor the number of closed arm entries made ($r_{2I} = 0.07$, $P > 0.05$).

Relationship between within-litter variability in maternal care received and later maternal care given

In the maternal sensitization paradigm, after categorizing the virgin females as either retrieving all three pups or failing to retrieve all three pups on any day of testing, we found that high-licked females were more likely to retrieve pups than low-licked females ($t_8 = 6.83$, $P = 0.03$; Fig. 4). Virgin females' latency to show full maternal behavior was not significantly correlated with the number of licking bouts they had received as pups, though (median latency = 7 days; $r_{10} = 0.15$; $P > 0.05$).

For the postpartum females, the frequency of licking bouts received as a pup tended to be correlated with the time they later spent licking their own offspring after a brief separation from them on PPD7 ($r_9 = 0.62$; $P = 0.07$). There was no relationship between the frequency of licking bouts the females received and the number of licking bouts they later gave their own pups during the undisturbed maternal behavior observations ($r_{10} = 0.18$; $P > 0.05$) nor after a brief separation from them ($r_9 = 0.53$; $P > 0.05$).

Relationship between adult daughters' emotion-related behavior and maternal care given

Sensitized Maternal Behavior—Virgin females with the shortest latencies to approach a novel object during periadolescence had the shortest latencies to show full maternal behavior during adulthood ($r_{10} = 0.62$, $P = 0.05$; Fig. 5). Similarly, the virgin females that spent a greater percentage of time in the open arms of the elevated plus maze were more likely to retrieve all three pups at least once during observations (but did not crouch) compared to virgins that spent a lower percentage of time in the open arms ($t_8 = 3.31$, $P = 0.01$). Neither the frequency of open arm entries, nor the number of closed arm entries they made were significantly correlated with their latency to retrieve all three pups (P 's > 0.05).

Postpartum Maternal Behavior—Females with a faster latency to approach the novel object during periadolescence spent less time licking their own pups after a separation from them ($r_7 = -0.70$; $P = 0.05$; Fig 6). However, there was no association between the females' latency to approach the novel object and the number of licking bouts they later gave their pups after a brief separation ($r_9 = -0.40$; $P > 0.05$) nor during the undisturbed observations across PND 4–10 ($r_{10} = 0.03$; $P > 0.05$). The percentage of entries that dams made into the open arms tended to be positively associated with the duration of time that they licked their offspring after the separation ($r_9 = 0.60$; $P = 0.08$); no other relationships between elevated plus-maze behavior and undisturbed and post-separation maternal behavior approached significance (P 's > 0.05).

Relationship between variability in maternal behavior received, emotion-related behavior, and TPH2 in the mPFC

The number of licking bouts females received during the early postnatal period was negatively correlated with TPH2 expression in the mPFC ($r_{18} = -0.48$, $P = 0.04$ Fig. 7). There was no association between females' latency to approach a novel object during

periadolescence and their adult TPH2 expression in the mPFC ($r_{19} = -0.19$, $P > 0.05$) and no significant relationship between any elevated plus-maze behavior and TPH2 expression in the mPFC (all P s > 0.05).

Relationship between maternal care given and TPH2 expression in the mPFC

Sensitized Females—Maternal responsiveness was significantly associated with TPH2 expression in the mPFC in virgins. Specifically, their latency to become fully maternal was negatively correlated with TPH2 expression in their mPFC ($r_{10} = -0.84$, $P = 0.002$; Fig. 8A).

Postpartum Mothers—The frequency of licking bouts females gave to their own pups in the undisturbed home cage was negatively correlated with the subjects' TPH2 in the mPFC ($r_{18} = -0.71$, $P = 0.05$; Fig. 8B). There was no significant relationship between the frequency ($r_7 = 0.85$, $P < 0.05$) or the duration ($r_8 = -0.06$, $P > 0.05$) of licking their own offspring after a brief separation from the litter, and TPH2 expression in the mPFC.

Discussion

In mammals, maternal care that offspring receive can vary greatly between and within litters and this variability contributes to differences among offspring in their later physiology and behavior. These differences in offspring outcomes are thought to be important for maternal fitness because they increase offspring behavioral and physiological adaptability to a broader range of environments (Boyce and Ellis 2005; Clutton-Brock, 1991; Philipi and Seger, 1989). In the present study of within-litter differences, we found that variability among female rat siblings in the frequency of maternal licking they received was: 1) associated with the pups' body weight at the initiation of behavior observations, 2) negatively associated with the daughters' periadolescent neophobia and adult TPH2 expression in the mPFC, 3) positively associated with some aspects of the daughters' own maternal responsiveness and anxiety-related behavior. Furthermore, analyses of the relationships among the numerous measures taken during adulthood showed that: 4) TPH2 in the mPFC was positively associated with aspects of maternal responsiveness in sensitized females, but negatively associated with it in postpartum females. These data further demonstrate that variations in the early mother-pup environment is not only associated with behavioral and physiological outcomes when measured between litters, but also within them.

Relationship between within-litter variability in maternal licking and offspring behavior

We found that the highest-licked female pups within their litters received up to three times as many licking bouts as their lowest-licked sisters, which is congruent with previously-reported ranges both between-litters (Caldji et al., 1998; Champagne et al., 2003a) and within-litters (Cavigelli et al., 2010; Ragan et al. 2010; Ragan et al., 2012; van Hasselt et al., 2012b). We do not believe this difference within litters is due to any major abnormality in maternal care resulting from the handling and marking of pups, because contemporaneous to the current study, we analyzed the overall frequency of maternal care given to a group of six unmarked litters using similar observation methods and found a similar total frequency of licking (290 ± 47 vs. 199 ± 18 ; $t_{10} = 1.83$, $P > 0.05$) and nursing (4235 ± 538 vs. 4118 ± 311 ; $t_8 = 0.19$, $P > 0.05$) across days. The unmarked and marked litters did differ in the

frequency they probed their dam's ventrum (379 ± 39 vs. 242 ± 10 ; $t_{10} = 3.40$, $P = 0.007$), but not their frequency of making perioral contact with their dams (29 ± 3 vs. 18 ± 4 ; $t_{10} = 2.02$, $P > 0.05$). Numerous studies have found differences in maternal behaviors given to handled and unhandled pups, but it is relevant to note that these studies involve observations immediately after reunion with the litter (Denenberg, 1964; Levine, 1975; Lee and Williams, 1974; Liu et al., 1997). The effects of pup handling on maternal care are transient each day (Champagne et al., 2003a; Claessens, Daskalakis, Oitzl, and de Kloet, 2012; Sherrod, Connor, and Meier, 1974) and dissipate as quickly as three hours post-handling (Reis et al., 2014). In our experiments, we intentionally avoided observing the dams and litters within the first three hours after handling to obtain a better impression of how their undisturbed relationship affects development of the offspring, so it is not surprising that we found no differences in total licking bouts between handled and non-handled litters. Whether meaningful individual differences exist among siblings in the licking they receive immediately after a brief separation and reunion with the mother remains to be determined.

Mother-infant interactions are dyadic and regulated by mothers' and pups' exquisite responsiveness to each other's cues (Polan, Milano, Eljuga, and Hofer, 2002; Stern and Johnson, 1989, 1990). Previous research has shown that maternally-directed solicitation behaviors, including making perioral contact with the dam and probing her ventrum (Ragan et al., 2012; Ragan et al. 2010), were positively associated with the frequency of licking bouts that the pups received. We did not find this in the current study and this may be because the previous studies did not cull litters to a uniform number, so the pups' behavior may have been affected by limited availability of nipples. In the current study, the eight pups had greater opportunities for access to nipples and this probably reduced how often they were not attached to a teat. Thus, the potential for perioral contact with the dam and the need for pups to persistently probe her ventrum may have been reduced. We did find, however, that heavier pups at the beginning of testing received more maternal licking throughout the early postnatal period. As previously mentioned, mothers often provide more attention to heavier offspring because they are more likely to survive than smaller offspring, particularly when resources are limited (Feldman and Eidelman, 2007; Singer et al., 2003; Nakamichi, 1986; Wheelwright, 2003), and this may have consequence for maternal fitness (Philippi and Seger, 1989).

Relationship between within-litter variability in maternal licking and offspring emotional behavior

A number of between-litters studies have found that high-licked litters show less emotional reactivity later in life compared to low-licked litters (Caldji et al., 1998; Francis et al., 1999; Weaver et al., 2004; although see Starr-Phillips and Beery, 2014). Our current experiment is partly consistent with these studies in that our higher-licked sisters had a shorter latency to approach a novel object at periadolescence compared to their lower-licked sisters (i.e., were less neophobic). However, we also found that the highest-licked females showed more anxiety-like behavior when tested in an elevated plus maze during adulthood. This association between more licking received and higher anxiety-related behavior in adulthood mimics what we previously reported in a similar within-litter study (Cavigelli et al., 2010). However, Pan et al., (2014) recently reported less emotion-related behavior in high- vs. low-

licked sisters in their analysis of all-female litters that were observed for the first time each day immediately after marking/handling. Also, van Hasselt et al. (2012a) reported no relationship between licking received and later elevated plus-maze behavior in unhandled female rats. Handling does not explain these discrepancies because we found no significant differences between our unmarked and marked litters in the females' later latency to approach the novel object (173 ± 19 vs. 174 ± 25 sec; $t_{49} = 0.003$, $P > 0.05$) or the percentage of time they spent in the open arms of the elevated plus maze (0.05 ± 0.01 vs. 0.10 ± 0.02 ; $t_{47} = 0.82$, $P > 0.05$). In any case, the association we found between licking received and later anxiety are consistent with studies in non-human primates have found that highly protective mothers that have the most contact with their infants produce offspring that are the least exploratory (Fairbanks and McGuire, 1993; Fairbanks, 1996). Similar outcomes on offspring emotional responses are found in human mothers who display overprotective maternal care (Hudson and Rapee, 2005; Hane and Fox, 2006).

It is particularly interesting that a negative relationship between maternal licking received and daughters' emotional behavior during adulthood has now been found in three different studies (Cavigelli et al., 2010; Ragan et al., 2012; present study), but that a positive relationship has more often found in some of the between-litter studies. Within- vs. between-group differences arise from completely different processes and their data are subjected to statistical analysis that make them more difficult to compare (Molenaar, 2004; McCartney, Burchinal, Bub, and Burchinal, 2014; van de Pol and Wright, 2009). Importantly, the "ecological fallacy" states that making inferences about individuals based on group outcomes is not only possibly erroneous (Robinson, 1950) but may lead to conclusions that are the opposite of what would be predicted (e.g., Menotti, et al., 1997). Furthermore, the within- and between-litter studies are also not easily compared because of differences in methodology - many of the between-litter studies examined emotional behavior only in the male offspring, studies vary in the number of observations of maternal care received, they differ in which postnatal days maternal care was observed, some may not have controlled for the daughters' estrus cycle during adult emotional behavior testing, and the behavioral paradigms used to assess emotional behaviors have varied (Pan et al., 2014; Uriarte, 2007; van Hasselt, 2012b).

Relationship between within-litter variability in maternal licking and offspring maternal behavior

We found that maternal licking received predicted some aspects of the daughters' later maternal behavior. In the sensitized virgin females, maternal licking received positively predicted how likely they were to retrieve all three pups (although not the latency to show full maternal behavior) and these results are congruent with a between-litter study showing similar effects in a sensitization paradigm (Champagne et al., 2001). In the postpartum females, we found a trend for the high-licked daughters to spend more time licking their pups after being separated from them compared to low-licked daughters. Again, this is generally in agreement with what is found in between-litter studies (reviewed in Champagne and Curley, 2009). Perhaps this early experience of receiving higher maternal care primes females to be more maternally responsive to pups later in life, true both at the group and

individual level, and may do so in both cases by altering expression of the receptors for estradiol, oxytocin, and other neurochemicals influencing caregiving behaviors.

Relationship between daughters' emotional behavior and maternal behavior

Neophobia and anxiety that are neither too high nor too low are probably ideal for successful maternal caregiving (Fleming and Luebke, 1981; Leckman et al., 2004; Ragan and Lonstein, 2014). We found that virgins that were most neophobic during periadolescence and anxious during adulthood made fewer pup retrievals during sensitization compared to their less emotionally reactive sisters, suggesting that high emotionality is particularly inconsistent with maternal responsiveness in nulliparous females. We found a similar relationship between neophobia during periadolescence or anxiety during adulthood on the postpartum females' licking of their own pups. These results are consistent with previous studies showing that high-licking dams are less neophobic than low-licking dams (Francis, Champagne, and Meaney, 2000) and that, more generally, postpartum rats display less anxiety-related behavior than nulliparous females (reviewed in Lonstein, 2007). This lower "trait-level of emotional reactivity observed in the more maternally responsive females may be what allows them to be more accepting of and attentive to pups, and may be regulated by differences in their central GABA, oxytocin, and corticotropin releasing hormone receptor signaling (Walker et al., 2001; Klampfl et al., 2013; Hansen, Ferreira, and Selart, 1985; Lonstein, Maguire, Meinschmidt, and Neumann, 2014).

Relationships among licking received, adult offspring behavior, and TPH2 in the mPFC

Early mother-pup interactions were associated with the daughters' later TPH2 protein expression in the mPFC. Specifically, we found that female pups that received more maternal licking bouts compared to their sisters had lower TPH2 expression in their mPFC in adulthood. Because TPH2 expression is positively correlated with the amount of serotonin produced by cells (Patel et al. 2004; Zhang et al., 2004;) and its presence in terminals may indicate the capacity for local serotonin synthesis (Pickel, 1976; Weissman et al., 1987; Perroud et al., 2010), this would suggest that the high-licked females have reduced capacity for serotonin signaling in the mPFC. Previous studies have found that the highest-licked males within a litter had lower serotonin transporter (SERT) expression in the midbrain compared to their low-licked brothers (Ragan et al., 2011), and that while long separations from the mother can increase TPH2 mRNA in the offspring dorsal raphe (Gardner et al., 2009) serotonin levels are decreased in brain areas related to emotional behavior (Daniels et al., 2004; Matthews et al., 2001; Matthews and Robbins, 2003). Based on studies of mPFC serotonin signaling and emotional behaviors in male rats (Hui et al., 2015; Pum et al., 2009; Solati et al., 2011), we had proposed that any relationship between development of the serotonin system and the amount of maternal care received would have consequences for similar behaviors in females. We found no correlations between TPH2 in the mPFC and females' neophobia or anxiety-related behaviors, though. Perhaps the state of the highly malleable pre-weanling serotonin system, rather than its function during adulthood, would be more strongly associated with females' anxiety (see Alberts, Vahid-Ansari, and Luckhart, 2014).

In contrast to females' emotional behaviors, we did find relationships between their TPH2 expression in the mPFC and some aspects of maternal responsiveness. Central serotonin signaling is required for normal postpartum maternal behaviors in rodents (Angoa-Perez, 2014; Barofsky et al., 1983; Lerch-Haner et al. 2008; Pawluski et al., 2012; Zhao and Li, 2010) and the mPFC may be a site where it acts for these effects (see Afonso et al. 2007; Febo, Felix-Ortiz, and Johnson, 2010; Pereira and Morrell, 2011; Veiga, Miczek, Lucion, and Alegre, 2007). Interestingly, we found that TPH2 expression in the mPFC was associated with maternal responsiveness in both nulliparous and postpartum females, but in opposite directions. TPH2 in the mPFC was positively associated with retrieval in nulliparae, but negatively associated with the frequency of licking in the postpartum females. Differences between the female reproductive states in basal mPFC serotonergic activity may help explain the opposite directions of effects, because dams already have higher serotonin metabolism in the cortex and some other brain sites compared to virgins or pregnant females (e.g., Desan et al., 1988; Lonstein, Dominguez, Putnam, Vries, and Hull, 2003; Smith, Piasecki, Weera, Olszewicz, and Lonstein, 2013), so perhaps serotonergic activity in the mPFC that is neither too high nor too low is optimal for maternal responsiveness - lower levels in dams but higher levels in virgins meet in the middle to promote maternal responsiveness in both groups. It should also be recognized that while sensitized and postpartum maternal behaviors appear quite similar, the behaviors do not necessarily emerge via similar neurochemical processes. For example, glucocorticoids promote maternal behavior in postpartum rats but disrupt it in sensitized virgins (Rees et al., 2004, 2006) and sensitized nulliparous rats have higher dopamine content in their medial preoptic area compared to non-sensitized rats, but postpartum mothers do not show this difference from non-maternal virgins (Olazábal et al., 2004).

Conclusions

The results of the current study further expand the literature on the long-term effects of differences in maternal care received on offspring socioemotional behaviors and neurochemistry, and particularly the increasing literature demonstrating significant within-family effects. Studying these within-family effects can answer unique questions about the consequences of differential maternal care at an individual level that cannot be addressed in between-litter studies. Future studies comparing siblings in their neurochemistry, including expression of other monoamines, and differential gene and protein expression of target molecules will be necessary to clarify the biological contributors to differences in their behavior and physiology, and whether or not those contributors are the same as those known to be associated with the between-family effects of differential mothering. Manipulating neonate phenotypes, like weight, may also clarify possible contributors to differential maternal care. Furthermore, transgenerational studies that include examining effects on the individual level can continue to examine the long-term effects of differential care. Importantly, these findings could have implications for better understanding within-family differences in the socioemotional behaviors of human siblings.

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Highlights

- Females pups received about three times more maternal licking bouts vs. sisters
- Licking bouts females received was correlated with pups' later emotional behavior
- Licking received was correlated with mothering in sensitized virgin females
- TPH2 was associated with mothering in virgins and postpartum dams in opposite ways

Figure 1.

A) Number of maternal licking bouts received by the female rat pups in six litters (indicated by different bar patterns/colors) during 30-min observations four times daily on PND 4, 6, 8, and 10. The most-licked females received up to three times more licks than their least-licked sisters. *B)* Correlation between female pups' body weight on the first day of mother-litter observations (PND4) and the licking bouts the females received on PND 4–10. Female pups that weighed more received more licking bouts from their mothers.

Figure 2.

Correlations between *A*) the number of maternal licking bouts that female pups received as pups and their later latency to approach a novel object during periadolescence and *B*) the frequency of ventral probing female pups made and their later latency to approach a novel object during periadolescence. Female pups that received more maternal licking or probed their mother's ventrums more often later had shorter latencies to approach the novel object.

Figure 3.

Correlation between maternal licking bouts that female pups received and later percentage of time they spent in the open arms of the elevated plus maze as an adult. Females that received more licking bouts than their sisters spent a lower percentage of time in the open arms of the elevated plus maze.

Figure 4.

Comparison of virgins that retrieved all three pups or did not retrieve all three in a sensitization paradigm and their mean licking bouts received as a pup. Uncentered, raw data are presented for clarity. (Mean $\pm$ SEM; * $P < 0.05$).

Figure 5.

Correlation between periadolescent female rats' latency to approach a novel object and their later latency to display full maternal behavior in sensitization paradigm. The females that had longer latencies to approach novelty during periadolescence later showed longer latencies to show full maternal behavior.

Figure 6.

Correlation between periadolescent female rats' latency to approach a novel object and time spent licking their pups in the home cage during adulthood. Females that had longer latencies to approach novelty later spent less time licking their pups.

Figure 7.

Correlation between the number of maternal licking bouts that female pups received and their TPH2 expression in the prefrontal cortex during adulthood. Females that received more licking bouts expressed lower TPH2 in the mPFC compared to females that received less licking.

Figure 8.

Correlations between TPH2 expression in the mPFC and *A*) the latency to display full maternal behavior in a virgin sensitization paradigm and *B*) the number of licking bouts postpartum female rats gave to their pups. Less maternally responsive females had lower levels of TPH2. Females that licked their pups more than their sisters had less TPH2 in the mPFC.