Genotype Effects and Gene x Environment Interactions on Inter-Individual Licking Received and Licking Provisioning in Female Rat Offspring

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Abstract

In most mammals, mothers exhibit natural variations in care that propagate between generations of female offspring. However, there is limited information on genetic variation that influences this propagation. We assessed early-life maternal care received by individual female rat offspring in relation to genetic polymorphisms linked to dopaminergic activity, maternal care provisioning, and dopamine levels in the maternal brain. We also conducted a systematic analysis of other genetic variants potentially related to maternal behavior in our Long-Evans rat population. We found that dopamine receptor 2 (rs107017253) variation interacted with the relationship between early-life maternal care received and dopamine levels in the nucleus accumbens shell which, in turn, were associated with later-life maternal care provisioning. We also discovered and validated new variants that were predicted by our systematic analysis. Our findings suggest that genetic variation influences the relationship between maternal care received and maternal care provisioning, similar to findings in human populations.

Keywords: Maternal Care, Genotype, Gene x Environment Interaction, Dopamine Receptor D2, Long-Evans, Intergenerational

1. Introduction

The maternal environment has a substantial role in influencing offspring phenotype in adulthood, including the intergenerational transmission of maternal care through the female lineage (Fleming et al., 2002). Both maternal care received and changes in the brain that occur when the mother is preparing for postnatal offspring care involve alterations in the dopaminergic (Champagne, 2004; Peña, Neugut, Calarco, & Champagne, 2014) and oxytocinergic systems (Beery, McEwen, MacIsaac, Francis, & Kobor, 2016; Francis, Young, Meaney, & Insel, 2002; Toepfer et al., 2017), as well as changes in estrogen signaling (Cameron et al., 2008; Champagne, 2008). Maternal care provisioning also involves neuropeptides aside from oxytocin, including prolactin and arginine vasopressin (Bridges, 2015). These mechanisms have been well-characterized in rodents and studies with humans show similar findings (Rosenblatt, 1994), suggesting that the factors involved in the intergenerational transmission of maternal care are evolutionarily conserved.

Rat pups with early-life adversity induced by maternal and sibling deprivation are less attentive to foster pups and their own offspring later in life than rat pups maternally reared (Gonzalez, Lovic, Ward, Wainwright, & Fleming, 2001; Palombo, Nowoslawski, & Fleming, 2010). Higher licking-like tactile stimulation during the deprivation period partially mitigates the later-life impairments in maternal care provisioning (Gonzalez et al., 2001; Palombo et al., 2010). In addition, naturally occuring variation between litters in maternal licking/grooming received can transmit between generations of offspring. High licking/grooming and low licking/grooming mothers (defined as +/- one standard deviation from the average) tend to produce female offspring that become high licking/grooming and low licking/grooming mothers to their offspring, respectively (Champagne, Francis, Mar, & Meaney, 2003; Francis, Diorio, Liu, & Meaney, 1999). This effect persists in cross-fostering experiments, suggesting a prominent role of maternal behavior in the intergenerational transmission of maternal care (Francis et al., 1999).

Previous work on the intergenerational transmission of maternal care in rats has mainly focused on proxies of maternal neglect or the tail ends of the normal distribution of maternal care received. We have more limited knowledge of factors that affect the intergenerational transmission of maternal care in the average rat mother. Seminal research examining natural variations in maternal care received between litters found that there is also substantial within-litter variation in later-life maternal care provisioning (Champagne et al., 2003). In addition, studies with human cohorts in nonclinical populations have shown a modest relationship between parental care received and parental care provisioning (Van Ijzendoorn, 1992) and found that the genotype of the individual can interact with the early-life environment or directly affect maternal care provisioning (Fleming & Kraemer, 2019). Studies in mice also show an important role of offspring genotype in maternal care received (Ashbrook, Sharmin, & Hager, 2017; Ashbrook, Gini, & Hager, 2015).

Findings from our group and others have shown effects of early-life inter-individual maternal care received on later life behaviour (Lauby, Chatterjee, Pan, McGowan, & Fleming, 2019; Pan,

62 Fleming, Lawson, Jenkins, & McGowan, 2014; Pan et al., 2018; Ragan, Loken, Stifter, & Cavigelli,
63 2012; van Hasselt et al., 2012). We have also reported gene x environment interactions
64 involved in some of these effects (Lauby et al., 2019; Pan et al., 2018). For example, we recently
65 reported an interaction between a dopamine receptor 2 (*Drd2*) single nucleotide polymorphism
66 (rs107017253) and early-life average licking duration per bout (a proxy of maternal care quality)
67 on strategy shifting and sucrose preference, two dopamine-related phenotypes (Lauby et al.,
68 2019). Executive function measured by strategy shifting performance has previously been
69 shown to be related to maternal care provisioning in human cohorts (Gonzalez, Jenkins, Steiner,
70 & Fleming, 2012). However, studies on the effect of genotype in the Long-Evans rat model
71 commonly used in these experiments have been limited by the lack of knowledge of genetic
72 variation in this outbred strain. Although there are now data available on genetic variation
73 within wild rat populations (Ness et al., 2012) and between inbred rat lines (Hermsen et al.,
74 2015; STAR Consortium et al., 2008), no mapping has been carried out within the Long-Evans
75 population.
76
77 The purpose of this study was to investigate the role of genotype and gene x environment
78 interactions in the relationship between early-life maternal licking received and later-life licking
79 provisioning, with a specific focus on the dopaminergic system of the maternal brain. We also
80 aimed to investigate other genes relevant to maternal behaviour using a more systematic
81 approach to identify and verify genetic variants in our Long-Evans rat population. We measured
82 inter-individual maternal licking received within the first week of life and assessed later-life
83 female offspring maternal licking provisioning, maternal brain dopamine and its metabolite
84 levels, and variation in single nucleotide polymorphisms (SNPs) in genes involved in dopamine,
85 estrogen, and neuropeptide function. We predicted that higher early-life licking received would
86 correspond to higher later-life licking provisioning in female offspring. In addition, we
87 hypothesized that this relationship would be indirectly affected by dopaminergic activity in the
88 maternal brain and interact with female offspring genotype. The hypothesized moderated-
89 mediation model is displayed in Figure 1.
90

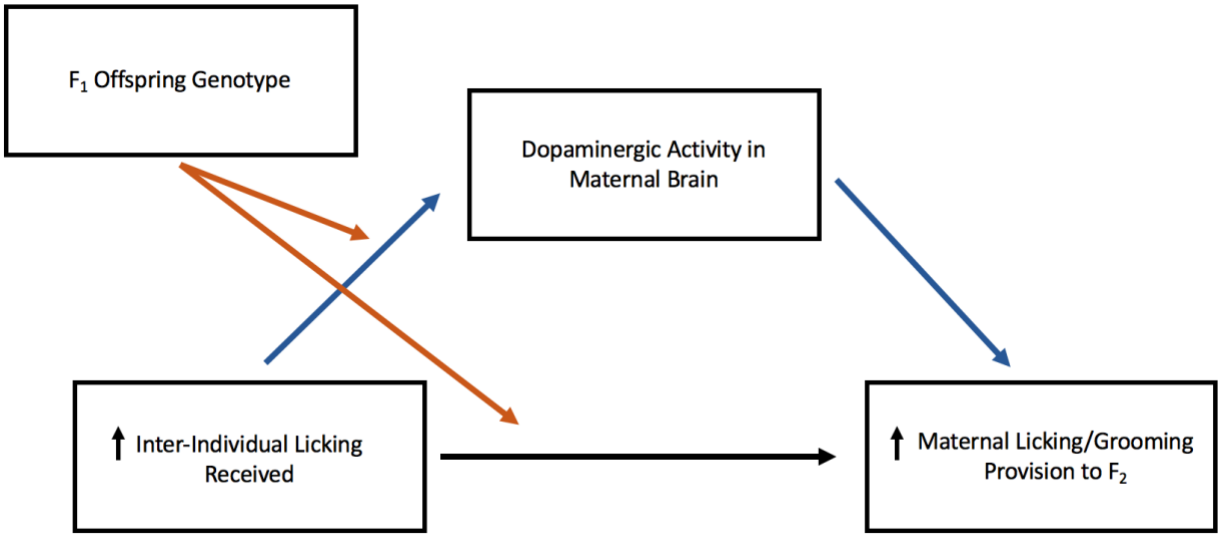


Figure 1. Hypothesized moderated mediation between early-life licking received and later-life licking provisioning. Dopaminergic activity in the maternal brain would mediate the association and offspring genotype would moderate licking provisioning and dopaminergic activity.

2. Results

2.1 Relationship between Early-Life Licking Received and Later-Life Licking Provisioning

Total licking duration received across all maternal care observation periods averaged 168.79 ± 18.6 seconds and ranged from 0 to 450.72 seconds (Figure 2A). Average licking duration per bout of licking received averaged 8.02 ± 0.56 seconds and ranged from 0 to 13.93 seconds (Figure 2B).

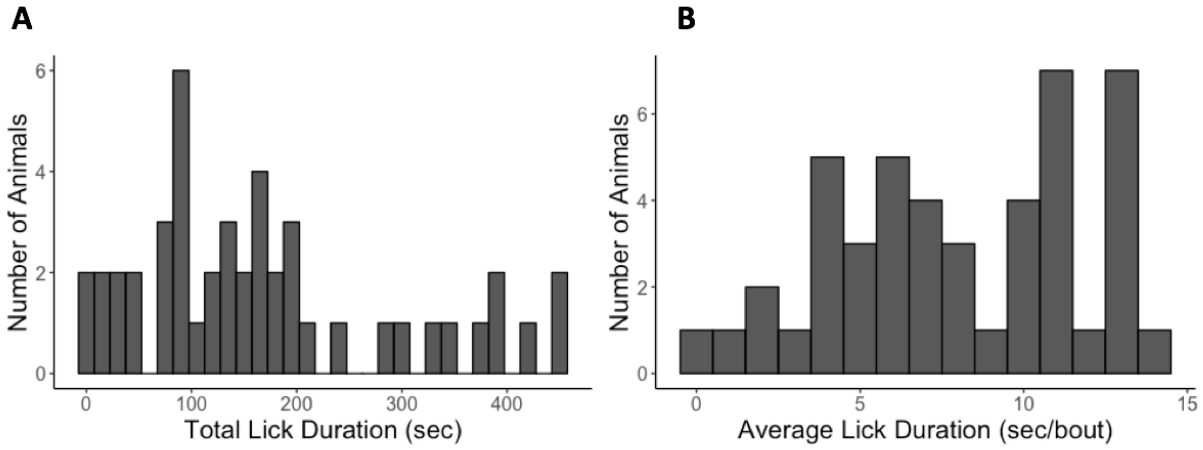


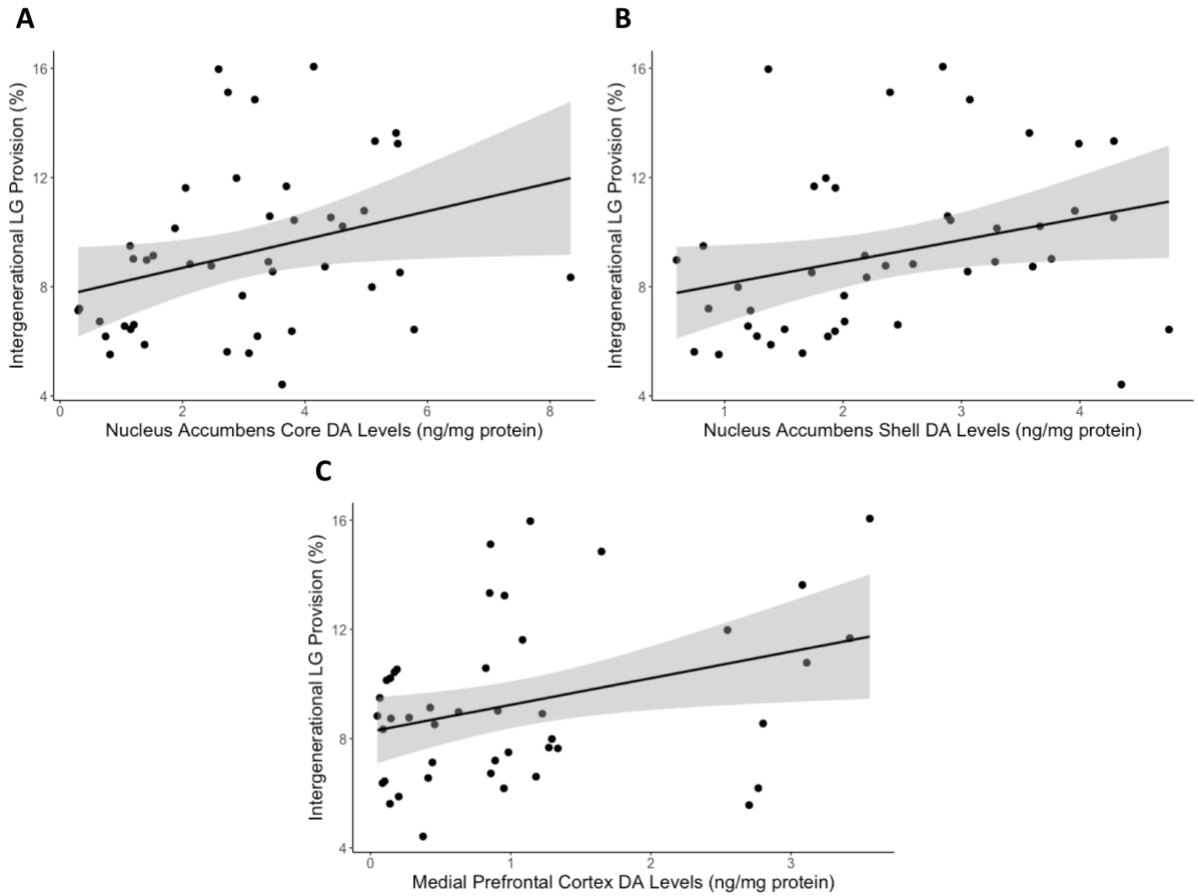
Figure 2. Maternal licking received within the first week of life. (A) Total licking duration (15-second bins) and (B) average licking duration per bout (1-second bins) received across all observation days for the female offspring tested (n = 46).

We examined the relationship between early-life maternal care received and later-life maternal care provisioning. There were no direct associations between early-life licking received and later-life licking provisioning. Neither total licking received (Pearson's r = -0.036, p = 0.812) nor average licking received (Pearson's r = -0.027, p = 0.861) significantly correlated with later-life licking provisioning.

2.2 Relationship between Early-Life Licking Received, Later-Life Licking Provisioning and Dopaminergic Activity in the Maternal Brain

We then examined relationships between maternal care received and provision with dopamine and 3, 4-dihydroxyphenylacetic acid (DOPAC) levels in the medial prefrontal cortex, nucleus accumbens core and shell, medial preoptic area, and ventral tegmental area in the maternal brain. There was a marginal positive correlation between average licking received with dopamine levels in the nucleus accumbens shell (Pearson's r = 0.259, p = 0.089). There were no

122 other associations between early-life licking received and dopamine or DOPAC levels in any
123 other brain regions.
124
125 There were significant positive correlations between later-life licking provisioning and
126 dopamine levels in the medial prefrontal cortex (Pearson's r = 0.339, p = 0.025; Figure 3A),
127 nucleus accumbens core (Pearson's r = 0.306, p = 0.043; Figure 3B), and nucleus accumbens
128 shell (Pearson's r = 0.302, p = 0.047; Figure 3C). There were no other associations between
129 later-life licking provisioning and dopamine or DOPAC levels in any other brain regions.
130



131 **Figure 3.** Dopamine levels in select areas in the maternal brain are associated with later-life
132 licking provisioning. Dopamine levels in the nucleus accumbens (A) core and (B) shell and (C)
133 medial prefrontal cortex positively correlate with percent licking/grooming observed from
134 postnatal day 2-9. Scatterplots are displayed with 95% confidence interval in grey.
135
136 **2.3 Effects of Genotype**
137
138 To carry out genetic or gene x environment analyses, we first need to know what variants exist
139 in the population. No whole-genome sequencing has been carried out in the Long-Evans
140 outbred population, and therefore genetic variation in Long-Evans is largely unknown. To
141 overcome this, we used 119 publically available RNA-seq datasets (Supplementary Table 1) and

142 reverse-transcribed the sequence information to identify genetic variants in expressed loci. We
143 detected 792,253 SNPs which segregate in the Long-Evans population. There variants are
144 available as a UCSC genome browser track at (http://genome.ucsc.edu/cgi-
145 bin/hgTracks?db=rn6&hgsid=781311681_LBwfWCEAejwRHF7U0aiK90vtLacl). However, given
146 the high multiple-comparisons burden enforced by genome-wide analyses, we chose seven
147 genes relevent to maternal care provisioning, and examined 16 variants within these genes.
148
149 Of the 16 variants in genes relevent to maternal provisioning detected, 11 showed variation in
150 our animals (Table 1). These SNPs were in dopamine receptor 1 (*Drd1*; rs105271298), dopamine
151 receptor 2 (*Drd2*; rs107017253), dopamine transporter (*Dat*; chr1:32354790 and rs13448119),
152 estrogen receptor alpha (*Esr1*; chr1:41325782 and chr1:41590586), estrogen receptor beta
153 (*Esr2*; rs8170138, rs13448055, and rs106738562), aromatase (*Cyp19a1*; chr8:58755644), and
154 oxytocin receptor (*Oxtr*; chr4:144398803). The one female offspring with the *Drd1* A/A
155 genotype was combined with the A/T genotype for analysis. In addition, since the three SNPs
156 within *Esr2* and the two SNPs within *Dat* were in complete linkage, only one SNP in each gene
157 (rs8170138 and rs13448119, respectively) was used for analysis.
158
159 **Table 1.** List of candidate single nucleotide polymorphisms assessed for variation, information
160 related to their location and function, and genotype distribution in our Long-Evans rat
161 population.

Gene	Coordinate	Location	Consequence	Accession Number	Genotype Distribution
Dopamine-Related					
Dopamine Receptor 1 (*Drd1*)	chr17:11100256	upstream		rs105271298	**33 T/T, 12 T/A, 1 A/A**
Dopamine Receptor 2 (*Drd2*)	chr8:53736747	exon	synonymous	N/A	No Variation
	chr8:53742670	intron		rs107017253	**34 A/A, 12 A/G**
Dopamine Transporter (*Dat*)	chr1:32354790	exon	synonymous	N/A	**12 A/A, 26 A/G, 8 G/G***
	chr1:32362114	exon	synonymous	rs13448119	**13 G/G, 25 G/A, 8 A/A***
Estrogen-Related					
Estrogen Receptor Alpha (*Esr1*)	chr1:41325782	exon	synonymous	N/A	**15 C/C, 31 C/T**
	chr1:41590586	exon	synonymous	N/A	**31 C/C, 5 C/T, 10 T/T**
Estrogen Receptor Beta (*Esr2*)	chr6:99180312	exon	synonymous	rs8170138	**37 A/A, 9 A/G****
	chr6:99184395	exon	synonymous	rs13448055	**37 G/G, 9 G/A****
	chr6:99204426	exon	synonymous	rs106738562	**37 C/C, 9 C/T****
Aromatase (*Cyp19a1*)	chr8:58755644	exon	synonymous	N/A	**13 T/T, 21 T/C, 12 C/C**

	chr8:58755702	exon	missense	rs197770329	No Variation
Neuropeptide-Related					
Prolactin Receptor (*Prlr*)	chr2:60285990	exon	synonymous	rs199216170	No Variation
Arginine Vasopressin (*Avp*)	chr3:123118892	intron		rs197835086	No Variation
Arginine Vasopressin Receptor 1a (*Avpr1a*)	chr7:67341790	intron		rs199089038	No Variation
Oxytocin Receptor (*Oxtr*)	chr4:144398803	downstream		N/A	**23 A/A, 23 G/G**

,* SNPs are in linkage disequilibrium

2.3.1 Dopamine-Related Genotypes (*Drd1, Drd2, Dat*)

There were no effects of dopamine-related SNPs (*Drd1, Drd2 or Dat*) on early-life maternal licking received or provisioning.

2.3.2 Estrogen and Oxytocin-Related Genotypes (*Esr1, Esr2, Cyp19a1, Oxtr*)

There was a main effect of *Esr2* (rs8170138) genotype ($F_{(1,44)} = 6.78$, $p = 0.014$), *Cyp19a1* (chr8:58755644) genotype ($F_{(2,43)} = 4.23$, $p = 0.021$), and *Oxtr* (chr4:144398803) genotype ($F_{(1,44)} = 9.14$, $p = 0.004$) on average licking received. For *Esr2*, heterozygous A/G female rat offspring received lower average licking per bout than homozygous A/A female rat offspring (Figure 4A). For *Cyp19a1*, homozygous C/C female rat offspring received lower average licking per bout than heterozygous C/T and homozygous T/T female rat offspring (Figure 4B). For *Oxtr*, homozygous G/G female offspring received lower average licking per bout than homozygous A/A female rat offspring (Figure 4C).

In addition, there was a main effect of *Esr1* (chr1:41590586) genotype ($F_{(2,43)} = 9.54$, $p < 0.001$), a marginal effect of *Cyp19a1* genotype ($F_{(2,43)} = 2.93$, $p = 0.064$), and a marginal effect of *Oxtr* genotype ($F_{(1,44)} = 3.31$, $p = 0.076$) on later-life licking provisioning. For *Esr1*, homozygous C/C and T/T female rat offspring provided lower licking/grooming than heterozygous C/T female offspring (Figure 4D). For *Cyp19a1*, heterozygous C/T female rat offspring provided lower licking/grooming than homozygous T/T female rat offspring (Figure 4E). For *Oxtr*, homozygous G/G female offspring provided lower licking/grooming than homozygous A/A female rat offspring (Figure 4F).

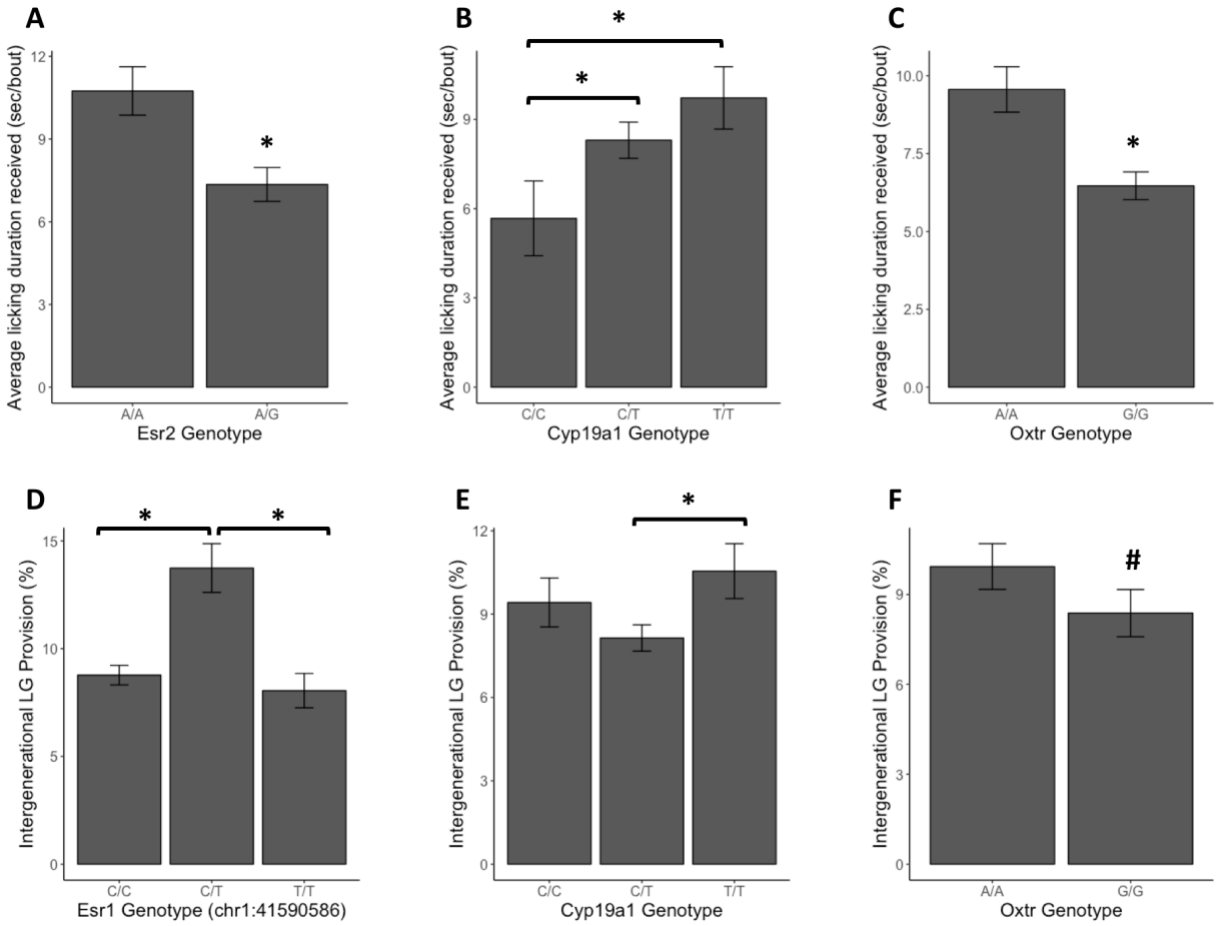


Figure 4. Genotype of estrogen- and oxytocin-related genes affects licking received and/or licking provisioning. (A) Heterozygous A/G female rat offspring for estrogen receptor beta (*Esr2*) received lower average licking per bout than homozygous A/A female rat offspring, (B) Homozygous C/C female rat offspring for aromatase (*Cyp19a1*) received lower average licking per bout than heterozygous C/T and homozygous T/T female rat offspring, and (C) Homozygous G/G female rat offspring for oxytocin receptor (*Oxtr*) received lower average licking per bout than homozygous A/A female rat offspring. (D) Homozygous C/C and T/T female rat offspring for estrogen receptor alpha (*Esr1*) provided lower licking/grooming than heterozygous C/T female rat offspring, (E) Heterozygous C/T female rat offspring for aromatase (*Cyp19a1*) provided lower licking/grooming than homozygous T/T female rat offspring, and (C) homozygous G/G female rat offspring from oxytocin receptor (*Oxtr*) provided lower licking/grooming than homozygous A/A female rat offspring. Barplots are displayed with mean +/- standard error. * $p < 0.05$, # $p < 0.10$ with post-hoc tests.

2.4 Gene x Environment Interactions

We restricted our gene x environment analyses to dopamine-related SNPs (*Drd1, Drd2, Dat*) because we hypothesized that the indirect relationship between early-life licking received and later-life licking provisioning would be mediated by dopaminergic activity in the maternal brain.

2.4.1 Later-Life Licking Provisioning

There was a marginal interaction of *Drd1* (rs105271298) genotype with total licking received on later-life licking provisioning (Coefficient β = -0.0152 $\pm$ 0.0085, $t_{(1,42)}$ = -1.801, p = 0.079). No other gene x environment interactions were observed with average licking received, other SNPs or brain regions.

2.4.2 Dopaminergic Activity in the Maternal Brain

Drd2 (rs107017253) genotype interacted with average licking received on dopamine levels in the nucleus accumbens (Coefficient β = 0.184 $\pm$ 0.086, $t_{(1,42)}$ = 2.1304, p = 0.0393; Figure 5). Specifically, there was a significant effect for the A/G genotype (Effect = 0.184 $\pm$ 0.066, t = 2.7886, p = 0.0081) but not the A/A genotype (Effect = -0.0003 $\pm$ 0.056, t = -0.0062, p = 0.9951). No other gene x environment interactions were observed with total licking received, other SNPs or brain regions.

2.5 Moderated Mediation of Early-Life Licking Received on Later-Life Licking Provisioning

Based on the reported results, we conducted a moderated mediation analysis in PROCESS (Model 8) using average licking received and percent licking/grooming provisioning as variables, dopamine levels in the nucleus accumbens shell as a mediator, and *Drd2* (rs107017253) genotype as a moderator.

We did not find a direct moderation of *Drd2* genotype between average licking received and later-life licking provisioning (Coefficient β = -0.2305, $t_{(1,39)}$ = -0.9193, p = 0.3636), as expected from our previous analyses. However, we found an indirect moderation of *Drd2* genotype between average licking received and later-life licking provisioning by dopamine levels in the nucleus accumbens. Specifically, there was a significant indirect effect for the A/G genotype (Effect = 0.198, Bootstrap CI [0.0173, 0.4135]) but not the A/A genotype (Effect = -0.0004 Boostrap CI [-0.1164, 0.1198]). This suggests that higher early-life average licking received is associated with higher dopamine levels in the nucleus accumbens shell which, in turn, are associated with higher later-life licking/grooming provisioning, but only in female rat offspring with the A/G *Drd2* genotype.

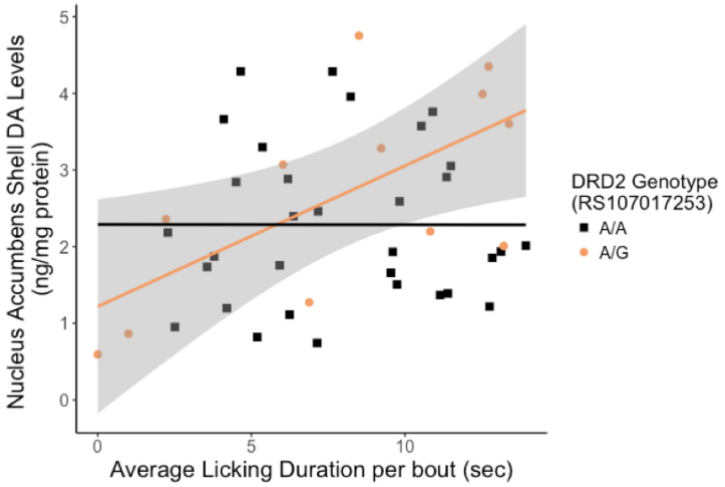


Figure 5. The association between average licking duration received and nucleus accumbens shell dopamine levels in the maternal brain are moderated by dopamine receptor 2 (*Drd2*) genotype. Heterozygous A/G female rat offspring had higher levels of dopamine in the nucleus accumbens shell with more early-life licking received. Homozygous A/A offspring do not show an association with early-life licking received. Scatterplots are displayed with regression lines for A/A (black) and A/G (orange) with the 95% confidence interval for the A/G genotype in grey.

3. Discussion

In this study, we investigated the role of genotype in the transmission of inter-individual maternal care across generations of female rat offspring. To our knowledge, this is the first study that has examined the relationship between inter-individual maternal care received and maternal care provisioning to the next generation of offspring and the genetic factors that could influence this relationship. We found that the relationship between early-life licking received and later-life licking provisioning was indirectly affected by dopamine levels in the nucleus accumbens shell and dependent on *Drd2* genotype. More specifically, female rat offspring with the A/G genotype showed a positive relationship between average licking received and dopamine levels in the nucleus accumbens of the maternal brain; there was no relationship with female rat offspring with the A/A genotype. The increase in nucleus accumbens dopamine corresponded to an increase in maternal licking/grooming provisioning from postnatal day 2-9. The updated moderated-mediation model is displayed in Figure 6. In addition, estrogen- and oxytocin-related SNPs affected both early-life licking received and later-life licking provisioning, suggesting that genotype can interact with the early-life maternal environment and influence later-life maternal care provisioning. Given that the early-life licking received we observed represented a continuous range of maternal care, these results suggest that genotype has an important role in the transmission of maternal care across generations in the average rat mother.

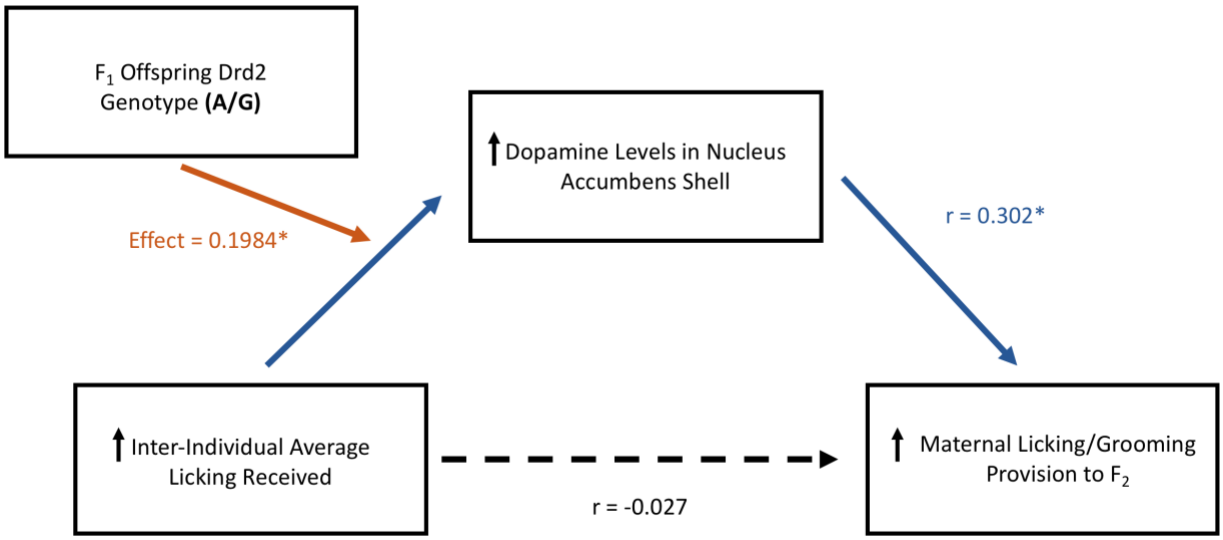


Figure 6. Updated moderated-mediation model between early-life licking received and later-life licking provisioning. Offspring *Drd2* genotype moderated the indirect relationship between average lick received and licking provisioning by dopamine levels in the nucleus accumbens shell. * $p < 0.05$

3.1 Relationship between Early-Life Licking Received and Later-Life Licking Provisioning

We first investigated the direct relationship between early-life licking received and later-life licking provisioning and did not find any correlation between the two measures. Previous work in rats has shown that inducing early-life adversity and, in studies observing natural variations in maternal care, the outliers of maternal care received (+/- one standard deviation) transmit across generations of offspring (Champagne et al., 2003; Francis et al., 1999; Gonzalez et al., 2001), demonstrating that early-life environmental influences are sufficient to alter maternal care to the next generation. However, there is substantial within-litter variation in the intergenerational transmission of maternal care, especially with female offspring born to Mid-Licking/Grooming mothers (Champagne et al., 2003). Since we did not select for the tail ends of the normal distribution in inter-individual licking received across all litters for the maternal care provisioning observations, our findings may reflect the substantial variation in intergenerational maternal care transmission within the normal distribution of maternal care received.

3.2 Relationship between Later-Life Licking Provisioning and Dopaminergic Activity in the Maternal Brain

We found that later-life licking provisioning was positively correlated with dopamine levels in the medial prefrontal cortex and the core and shell of the nucleus accumbens. Previous studies have shown links between maternal care provisioning and dopaminergic activity in the medial prefrontal cortex by lesions and pharmacological manipulations (Afonso, Sison, Lovic, & Fleming, 2007; Febo, Felix-Ortiz, & Johnson, 2010) and dopaminergic activity in the nucleus

accumbens, especially the nucleus accumbens shell (Afonso, Grella, Chatterjee, & Fleming, 2008; Afonso, King, Novakov, Burton, & Fleming, 2011; Champagne, 2004; Lee, Li, Watchus, & Fleming, 1999).

The relationship between early-life licking received and later-life licking provisioning was also mediated by levels of dopamine in the nucleus accumbens shell in the maternal brain, suggesting that the nucleus accumbens shell may be an important site of intergenerational transmission of maternal care. Artificially reared female rat pups show later-life elevated basal dopamine levels and lower pup-evoked dopamine levels in the nucleus accumbens and a reduction in maternal care (Afonso et al., 2011; Gonzalez et al., 2001) than normal maternally reared offspring. Additional studies are needed to explore the potential role of the nucleus accumbens shell as a brain area involved in the transmission of the effects of maternal care received at the early-life pup stage.

3.3 Systemic Analysis of Genetic Variants from RNA-seq data

We detected 792,253 genetic variants that appear to segregate in the Long-Evans population, overlapping 20,508 Ensembl gene annotations. This number is likely an underestimation due to RNA-seq data being enriched in reads from transcribed regions. Of sixteen variants of interest that we verified using massarray, five were not polymorphic. The two most likely reasons for this are that either a) the variant didn't exist in our population of Long-Evans, either due to genetic drift, different suppliers, or simple change; or b) they were false positive calls, due to the greater difficulty in calling variants from RNA-seq data than from DNA-seq data. However, the majority of our calls variants were confirmed (69%), and four are novel variants (25%). These findings show that this method is useful in understudied populations, which is important because a large amount of rat genetic variation is still undocumented. The data we have generated and publically shared on the genetic variation in the Long-Evans rat strain stands to be a useful resource for other researchers studying the effects of natural genetic variation on phenotype and epigenetic mechanisms.

3.4 Effects of Estrogen- and Oxytocin-Related SNPs on Early-Life Licking Received and Later-Life Licking Provisioning

We found that single nucleotide polymorphisms (SNPs) in candidate genes related to maternal care (estrogen receptor alpha and beta, aromatase, oxytocin receptor) affect early-life licking received, later-life licking provisioning, or both. However, the effect of most SNPs on early-life licking received and later-life licking provisioning did not show a consistent pattern (with the exception of the *Oxtr* chr4:144398803 SNP). Previous work in mice has demonstrated that the genotype of the offspring can affect the amount of care they receive, termed offspring genetic effects (Ashbrook et al., 2017; Ashbrook et al., 2015), which implies that the genetic profile of an organism can also affect its early-life experience and the organism is not simply a passive recipient of environmental exposures. Future work is needed to determine the how these genotypes affect interindividual differences in the solicitation of maternal care (e.g., via ultrasonic vocalizations, locomotor activity; Pan et al., 2018). Additional work is also needed to

determine biological consequences of these SNPs and it is likely that there are additive effects of different genotypes on maternal care received and provisioning that we were unable to elucidate in this study. The role of genotype in the male lineage may also be an important consideration when studying intergenerational transmission of maternal care, as the fathers provide half their genetic profile to the offspring and play an essential role in genomic imprinting (Wilkinson, Davies, & Isles, 2007).

3.5 Gene x Environment Interactions with *Drd2* Genotype

We found a gene x environment interaction with average licking received and *Drd2* genotype (rs107017253) on dopamine levels in the nucleus accumbens shell, as a mediator between early-life licking received and later-life licking provisioning. We previously reported similar interactions between average licking received and the same *Drd2* SNP in other dopamine-related behaviours (Lauby et al., 2019), but involving the A/A genotype, not A/G. It is possible that the nature of this interaction changes when the brain undergoes significant changes in the dopaminergic system, such as when a female rat is pregnant and preparing for postnatal care of her offspring (Akbari et al., 2013; Byrnes, Byrnes, & Bridges, 2001). Identifying other biological mechanisms that underlie these gene x environment interactions, such as linkage disequilibrium and gene expression changes, could help elucidate this discrepancy. Studies that investigate epigenetic mechanisms (e.g., DNA methylation) that would affect the expression of these genes would also be an important component in elucidating mechanisms underlying gene x environment interactions, as both maternal care received and the neural changes that prime mothers to provide care for offspring involve alterations in epigenetic mechanisms and gene expression (Beery et al., 2016; Cameron et al., 2008; Champagne, 2008; Stolzenberg, Stevens, & Rissman, 2014).

Studies of gene x environment interactions on maternal behavior to date have focused on human cohorts. These studies have identified SNPs in oxytocin- and dopamine-related genes that interact with early-life experiences on different components of mothering behaviour (Fleming & Kraemer, 2019; Jonas et al., 2013; Mileva-Seitz et al., 2012; Mileva-Seitz et al., 2013; Cost et al., 2017) and that can be mediated by executive function (Cost et al., 2017) or depressive symptoms (Jonas et al., 2013). A strength of our study is application of statistical models typically used in human cohorts to rat populations, where causal biological mechanisms can be more readily examined (Jenkins, McGowan, & Knafo-Noam, 2016). More specifically, we can look directly into the rat maternal brain for specific neurotransmitters, neuropeptides, and expression of relevant genes that would not be possible with human populations. This cross-species approach is important to refine hypotheses on factors important for maternal behavior that are evolutionarily conserved.

4. Materials and Methods

4.1 Rat Breeding

Seven-week-old female (n = 24) and male (n = 6) Long-Evans rats were obtained from Charles River Laboratories. They were housed in same-sex pairs on a 12:12 hour light-dark cycle (lights on at 7:00) with ad libitum access to standard chow diet and water. For breeding, one male was housed with two females for one week. Females were then housed separately and weighed weekly throughout pregnancy. All animal procedures were approved by the Local Animal Care Committee at the University of Toronto Scarborough and conformed to the guidelines of the Canadian Council on Animal Care.

Females were checked for parturition starting three weeks after breeding at 9:00 and 17:00. Postnatal day (PND) 0 was determined if the birth occurred between 9:00 and 17:00 or if pups were found at 9:00 but have not nursed yet. Pups found at 9:00 with a milk band were considered PND 1. At PND 1, litters were culled to five to six female pups and individually weighed. We focused on smaller litters in order to accurately measure maternal care received. Therefore, only female offspring were examined for this study to analyze a full range of maternal care within a litter. A total of 136 pups were assessed for maternal care.

4.2 Maternal Care Received Observations

At PND 1, 3, 5 and 7, maternal care was assessed as previously reported (Lauby et al., 2019). From 10:00 to 17:00, litters were briefly separated from their mother and individually marked using odorless and tasteless food coloring (Club House, London, Canada) to distinguish between siblings. The entire litter was then placed in the opposite corner of the established nest and maternal behavior was observed for 30 minutes using Observer XT 11.5 (Noldus Information Technology, Wageningen, The Netherlands). To establish inter-rater reliabilities on behavioral observations, three researchers coded the same mothers with an experienced coder until high reliability (>90%) was consistently met.

The order pups were retrieved was recorded manually. Duration and frequency of anogenital licking and body licking were coded for individual pups, meaning each pup had designated keys in the Observer software. Hovering, nursing (blanket and arched-back), and nest-building were coded as a litter, since these behaviors typically involve whole litters. Self-directed behaviors (feeding and self-grooming) by the mother were also coded. Cages were not changed throughout the maternal behavior observation period.

Total duration of licking (anogenital and body licking) and average duration of a lick bout (total duration of licking/number of licking bouts) across all four observation days for each pup were calculated as measures of maternal care. This was referred as “total licking duration” and “average licking duration”, respectively.

Female offspring were weaned at PND 22 and were pair-housed with siblings. They were weighed periodically until adulthood (PND 75).

4.3 Maternal Care Provisioning Observations

426 A subset of adult female offspring (n = 54) were bred with sexually experienced males (n = 6)
427 for one week following other behaviour tasks, some previously published (Lauby et al., 2019)
428 and others will be reported in subsequent publications. Six females were unable to get
429 pregnant after two rounds of breeding and two females failed to lactate following partuition,
430 with a total of 46 rat mothers being used for intergenerational maternal care analysis. Births
431 were checked starting three weeks after breeding at 9:00 and 17:00. At PND 1, cages were
432 changed with no culling of pups. Litter size did not correlate with maternal care provisioning
433 (Pearson's r = 0.001, p = 0.994).
434
435 From postnatal day 2 to 9, each litter was video recorded for one hour three times during the
436 light phase (9:00-10:00, 13:00-14:00, 17:00-18:00) and three times during the dark phase
437 (21:00-22:22, 1:00-2:00, 5:00-6:00). These videos were coded with Observer XT 10.5 (Noldus
438 Information Technology) for maternal behavior by four coders. Nursing, licking/grooming, nest-
439 building, and other self-directed behaviors were scored every three minutes based on previous
440 literature (Champagne, Francis, Mar, & Meaney, 2003). A total of 120 observations per mother
441 per day were coded and maternal licking provisioning was represented as a percentage of the
442 frequency of licking/grooming behavior coded over total observations.
443
444 After 10:00 on PND 9, the rat mother was separated from her pups and was sacrificed with CO_2
445 inhalation and decapitation. Liver and whole brain tissue were collected and placed in dry ice or
446 flash-frozen in isopentane, respectively. Tissue was stored in -80°C until further processing.
447
448 **4.4 High Performance Liquid Chromatography**
449
450 Forty-six maternal rat brains were sliced and microdissected using a Leica CM3050S cryostat
451 (Leica Microsystems, Wetzlar, Germany). Medial prefrontal cortex (+4.20 mm to +2.70 mm
452 Bregma), nucleus accumbens core and shell (+2.20 mm to +1.20 mm Bregma), medial preoptic
453 area (0.30mm to -0.80mm Bregma), dorsal hippocampus (control brain region; -2.30 mm to -
454 3.30 mm Bregma), and the ventral tegmental area (-5.20 mm to -5.60 mm Bregma) were
455 identified using an adult rat brain atlas (Paxinos & Watson, 1997). 5 µL of 1.0 M ascorbic acid
456 was added to each sample to stabilize the neurotransmitters followed by storage at -80°C.
457
458 To prepare the samples, the brain tissue was thawed on ice, suspended in 20 µl artificial
459 cerebrospinal fluid (Harvard Apparatus, Holliston, MA) and homogenized by four pulses of
460 sonication (2 seconds per pulse). 2 µl of brain homogenate from each sample was analyzed for
461 protein concentration using BioRad protein assay reagent (BioRad, Hercules, CA). 1 µl of 0.2 M
462 perchloric acid per sample was added to the remaining homogenate and was centrifuged at
463 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and stored at -80°C.
464
465 Dopamine (DA) and 3, 4-dihydroxyphenylacetic acid (DOPAC) levels were measured by high
466 performance liquid chromatography (HPLC) as described by previous studies (Chatterjee &
467 Gerlai, 2009; Lauby et al., 2019). A BAS 460 MICROBORE-HPLC system with electrochemical
468 detection (Bio-analytical Systems Inc., West Lafayette, IN) was used together with a Uniget C-18
469 reverse phase microbore column (#8949, Bio-analytical Systems Inc.). The mobile phase

470 contained a buffer [0.1 M monochloro acetic acid, 0.5 mM Na-EDTA, 0.15 g/L Na-octylsulfonate
471 and 10 nM sodium chloride, pH 3.1], acetonitrile and tetrahydrofuran at a ratio of 94:3.5:0.7.
472 The flow rate was 1.0 ml/min, and the working electrode (Uniget 3 mm glassy carbon, BAS P/N
473 MF-1003) was set at 550 mV vs. Ag/Ag/Cl reference electrode. Detection gain was 1.0 nA, filter
474 was 0.2 Hz, and detection limit was set at 20 nA. 5 µl of the sample supernatant was directly
475 injected into the column. External standards for DA and DOPAC (Sigma Aldrich, St. Louis, MO) of
476 known concentrations were used to quantify and identify peaks on the chromatogram. Under
477 these parameters, the retention times for DA and DOPAC were approximately 3.7 minutes and
478 5.5 minutes, respectively.
479
480 DA and DOPAC levels were normalized against total protein concentration for each sample and
481 expressed as ng/mg of protein as a measure of dopaminergic activity in the maternal brain.
482
483 **4.5 Genotyping**
484
485 To identify variants which segregate within the Long-Evans population, we took advantage of
486 large amounts of RNA-seq data available online in the population. We identified 119 RNA-seq
487 datasets on the Gene Expression Omnibus (GEO; Supplementary Table 1) which we could use
488 for variant calling. Fastq files were downloaded, aligned and variants called following the
489 Genome Analysis Toolkit (GATK) guidelines. In brief, adaptors were trimmed from the fastq files
490 using trim galore (version 0.4.1). The aligned files were aligned to the rn6 rat reference genome
491 using STAR (version 2.6.0.), using two-pass mode (Dobin et al., 2013). Read groups were added
492 and duplicates were marked using picard tools. NCigar reads were split using GATK (version
493 4.0.8.1), and variants were called using HaplotypeCaller. Joint-calling was carried out using all
494 119 samples using GenomicsDBImport and GenotypeGVCFs. Variant recalibration was carried
495 out, using a 'true positive' variant list from the rat genome database. This provided us with a list
496 of high quality SNPs and small INDELs.
497
498 To validate variation in candidate SNPs, liver DNA was extracted using an EZNA Tissue DNA
499 extraction kit (Omega Bio-tek, Norcross, GA) and assessed for SNPs in genes related to maternal
500 behaviour (Table 1). Purified DNA (35 ng/µl) was submitted for a multiplex assay at The Centre
501 for Applied Genomics (SickKids, Toronto, Canada).
502
503 **4.6 Statistical Analysis**
504
505 All statistical analyses were performed using SPSS (IBM Corporation). To examine the
506 relationship between early-life maternal licking received and later-life maternal licking
507 provisioning, a Pearson correlation was used between total or average licking duration received
508 and the percentage of licking provisioning observed. In addition, to examine the relationship
509 between later-life maternal licking provisioning and dopaminergic activity, a Pearson
510 correlation was used between percentage of licking provisioning observed and DOPAC and DA
511 levels in each brain area. To examine the effects of genotype, a general linear model was used
512 to compare offspring with each varying genotype to early-life maternal licking received and
513 later-life maternal licking provisioning. Significant effects of genotype were followed with a

post-hoc test using Fisher's Least Significant Difference (LSD). To examine gene x environment interactions and the mediating role of dopaminergic activity on later-life maternal licking provisioning, we used Hayes PROCESS module for SPSS (Version 3.2) using a simple moderation (Model 1) and a moderated mediation (Model 8; Hayes, 2013). A moderation shows an interaction between two independent variables where at least one variable is continuous and a mediation shows an indirect correlation with the independent and dependent variables by a third variable. PROCESS is a flexible modelling module that can conduct moderation and mediation analyses and probe conditional effects of a focal moderator and the indirect effects of a mediator. Multicategorical moderator variables (i.e., more than two genotypes) were analyzed using the indicator coding system. All effects were considered statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.

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Competing Interests

The authors of the manuscript have no conflicts of interest to declare.

Author Contributions

SCL, PP, ASF, and POM designed the study. SCL and PP conducted the maternal care received and maternal care provisioning observations. DGA ran the variant calling analysis from Long-Evans RNA-seq datasets and created the track for the UCSC genome browser. HRM did the brain cryosectioning and liver DNA extractions for genotyping. DC conducted the HPLC. SCL and HRM analyzed the data. ASF and POM and supervised the research. SCL, DGA, HRM, and POM wrote the manuscript. All authors contributed to this manuscript and approved the final version of this manuscript.

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Supplementary Table 1: Publically available Long-Evans RNA-seq samples used for variant calling. Data taken from the Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA). Each sample name (SRR) is shown (n = 119), the tissue that the sample was taken from, the sequencing type (RNA-seq or microRNA-seq), and the project that the sample belongs in (n = 5).

Sample name	Tissue	Sequencing type	Project
SRR2155693	brain	RNA-Seq	PRJNA292611
SRR2155694	brain	RNA-Seq	PRJNA292611
SRR2155695	brain	RNA-Seq	PRJNA292611
SRR2155696	brain	RNA-Seq	PRJNA292611
SRR2155697	brain	RNA-Seq	PRJNA292611
SRR2155698	brain	RNA-Seq	PRJNA292611
SRR2155699	brain	RNA-Seq	PRJNA292611
SRR2155700	brain	RNA-Seq	PRJNA292611
SRR2155701	brain	RNA-Seq	PRJNA292611
SRR2155702	brain	RNA-Seq	PRJNA292611
SRR2155703	brain	RNA-Seq	PRJNA292611
SRR2155704	brain	RNA-Seq	PRJNA292611
SRR2155705	brain	RNA-Seq	PRJNA292611
SRR2155706	brain	RNA-Seq	PRJNA292611
SRR2927560	hippocampus	miRNA-Seq	PRJNA301355
SRR2927561	hippocampus	miRNA-Seq	PRJNA301355
SRR2927562	hippocampus	miRNA-Seq	PRJNA301355
SRR2927563	hippocampus	miRNA-Seq	PRJNA301355
SRR2927564	hippocampus	miRNA-Seq	PRJNA301355
SRR2927565	hippocampus	miRNA-Seq	PRJNA301355
SRR2927566	hippocampus	miRNA-Seq	PRJNA301355
SRR2927567	hippocampus	miRNA-Seq	PRJNA301355
SRR2927568	hippocampus	miRNA-Seq	PRJNA301355
SRR2927569	hippocampus	miRNA-Seq	PRJNA301355
SRR2927570	hippocampus	miRNA-Seq	PRJNA301355
SRR2927571	hippocampus	miRNA-Seq	PRJNA301355
SRR2927572	hippocampus	miRNA-Seq	PRJNA301355
SRR2927573	hippocampus	miRNA-Seq	PRJNA301355
SRR2927574	hippocampus	miRNA-Seq	PRJNA301355
SRR2927575	hippocampus	miRNA-Seq	PRJNA301355
SRR2927576	hippocampus	miRNA-Seq	PRJNA301355
SRR2927577	hippocampus	miRNA-Seq	PRJNA301355
SRR2927578	hippocampus	miRNA-Seq	PRJNA301355
SRR2927579	hippocampus	miRNA-Seq	PRJNA301355
SRR2927580	hippocampus	miRNA-Seq	PRJNA301355
SRR2927581	hippocampus	miRNA-Seq	PRJNA301355

SRR2927582	hippocampus	RNA-Seq	PRJNA301356
SRR2927583	hippocampus	RNA-Seq	PRJNA301356
SRR2927584	hippocampus	RNA-Seq	PRJNA301356
SRR2927585	hippocampus	RNA-Seq	PRJNA301356
SRR2927586	hippocampus	RNA-Seq	PRJNA301356
SRR2927587	hippocampus	RNA-Seq	PRJNA301356
SRR2927588	hippocampus	RNA-Seq	PRJNA301356
SRR2927589	hippocampus	RNA-Seq	PRJNA301356
SRR2927590	hippocampus	RNA-Seq	PRJNA301356
SRR2927591	hippocampus	RNA-Seq	PRJNA301356
SRR2927592	hippocampus	RNA-Seq	PRJNA301356
SRR2927593	hippocampus	RNA-Seq	PRJNA301356
SRR2927594	hippocampus	RNA-Seq	PRJNA301356
SRR4031271	cortical neurons	RNA-Seq	PRJNA313218
SRR4031272	cortical neurons	RNA-Seq	PRJNA313218
SRR4031273	cortical neurons	RNA-Seq	PRJNA313218
SRR4031274	cortical neurons	RNA-Seq	PRJNA313218
SRR4031275	cortical neurons	RNA-Seq	PRJNA313218
SRR4031276	cortical neurons	RNA-Seq	PRJNA313218
SRR4031277	cortical neurons	RNA-Seq	PRJNA313218
SRR4031278	cortical neurons	RNA-Seq	PRJNA313218
SRR4031279	cortical neurons	RNA-Seq	PRJNA313218
SRR4039831	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039832	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039833	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039834	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039835	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039836	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039837	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039838	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039839	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039840	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039841	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039842	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039843	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039844	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039845	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039846	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039847	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039848	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039849	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395

SRR4039850	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039851	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039852	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039853	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039854	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039855	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039856	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039857	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039858	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039859	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039860	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039861	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039862	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039863	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039864	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039865	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039866	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039867	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039868	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039869	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039870	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039871	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039872	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039873	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039874	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039875	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039876	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039877	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039878	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039879	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039880	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039881	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039882	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039883	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039884	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039885	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039886	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039887	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039888	Medial entorhinal cortex layer II	RNA-Seq	PRJNA339395
SRR4039889	Medial entorhinal cortex deep layers	RNA-Seq	PRJNA339395
SRR4039890	Lateral entorhinal cortex layer II	RNA-Seq	PRJNA339395

SRR4039891	Lateral entorhinal cortex deep layers	RNA-Seq	PRJNA339395	693