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Inter-individual Maternal Care Received and Genotype Interactions Affect Dopaminergic Phenotypes in Female Rat Offspring

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Running Head: Maternal Care and Dopamine Genotype Interactions

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Abstract
Rat mothers exhibit natural variations in care and can shape offspring adult behavior and their maternal care by affecting the dopaminergic system. We explored whether genotype and gene x environment interactions are involved in these processes in nulliparous female offspring. We assessed maternal licking/grooming toward individual female pups during the first week postpartum and dopamine-related behavior of the offspring in adulthood. Behaviors explored included strategy shifting, impulsive action, and sucrose preference. Single nucleotide polymorphisms in the dopamine receptor 2, dopamine transporter, and

catechol-O-methyltransferase genes were examined in relation to offspring behavior and baseline dopamine turnover in select brain regions. Dopamine receptor 2 (RS107017253) variation moderated, or interacted with, the relationship between early-life licking received and behavior. Specifically, offspring with the A/A genotype showed a significant correlation between early-life licking received and behavior. Offspring with the A/G and G/G genotypes did not show this relationship. Dopamine transporter gene variation affected offspring behavior regardless of early-life licking received. Our findings suggest that genotype can directly affect dopamine-related behaviors and alter the offspring's sensitivity to the maternal environment. This could be informative on how maternal care is transmitted between generations of female offspring.

Key Words: Maternal Care, Genotype, Gene x Environment Interaction, Dopamine Receptor D2, Rat, Female

1. Introduction

The maternal environment can shape offspring behavior later in life, including how female offspring themselves provide maternal care[1]. This phenotypic plasticity could be an adaptive response to maximize offspring's chances of survival to environmental challenges[2]. The maternal brain involves coordination of several neural systems, including oxytocin and dopamine, to approach and respond to infant cues[3–5]. In rat models, disruption of maternal care through early-life isolation has been shown to affect offspring behavior in adulthood, including their maternal behavior and other dopamine-dependent behavior such as impulsive action, strategy shifting, and baseline reward sensitivity to drugs and sucrose[6–12]. This impairment in adulthood by early isolation from the mother can be rescued with early-life supplemental tactile stimulation[7,10].

Previous research has established that there is natural variation in how rodent mothers respond and care for their pups, measured by the total licking/grooming (LG) of the entire litter during the first postpartum week[13]. The quality of maternal care may also be determined by the average duration of licking per bout mothers provide to the entire litter. This has been shown in rat mothers who show natural variations in maternal care across time[14,15], where high LG mothers show a higher duration of licking per bout rather than a higher frequency of licking bouts or a higher licking total. In addition, inducing disruption and fragmentation of maternal care by limiting nesting material produces anxiety- and depressive-like behavior in offspring even when the total duration of maternal LG is not affected[16–18].

Other studies, including our own, have demonstrated that mothers also show variation in their maternal licking to different pups within the litter[19–24]. We found that total inter-individual LG received is associated with differences in stress reactivity at adulthood[23,24]. The effects of average licking bout length received by individual pups on their later-life behavior has not been explored.

However, the effects of early-life maternal care on later-life behavior could be influenced by offspring genotype. We recently reported that adult stress reactivity depends on an interaction between LG received and single nucleotide polymorphisms (SNPs) in genes

coding for FK506-binding protein, glucocorticoid receptor and serotonin transporter[23]. Gene x environment interactions are also known to exist for genes involved in dopamine signaling. For example, early-life adversity by artificial rearing increases dopamine receptor 2 (DRD2) expression in the nucleus accumbens shell of adult rats, but is dependent on the genotype for a DRD2 SNP (RS13448058)[25]. Studies in human populations have found gene x environment interactions with dopamine transporter (DAT), prenatal adversity and ADHD symptoms in children[26,27]. Dopamine transporter genotype has been associated with its availability in human striatum to metabolize dopamine[28], influencing the rate dopamine is cleared from the synapse. Like the dopamine transporter, catechol-O-methyltransferase (COMT) metabolizes dopamine and a common functional SNP is associated with its activity in the prefrontal cortex of humans[29], but gene x environment studies on these mechanisms have not been performed to our knowledge. In addition, we could not find studies on the effects of both DAT and COMT SNPs on phenotype in rat populations.

We have a limited understanding whether inter-individual licking/grooming received is associated with dopamine-dependent behavior and the extent to which genotypes involved in dopaminergic activity would moderate this relationship. Our goal is to understand if genetic variation would contribute to offspring adult behaviors and hence to maternal care transmission between generations. We used nulliparous female offspring to explore this question and assessed strategy shifting, impulsive action, and sucrose preference. Early-life maternal care received between litters has been demonstrated to affect dopamine-related phenotypes in female rat offspring[30]. Strategy shifting assesses how quickly individuals switch strategies when a previous strategy no longer produces a reward. This task has been associated with medial prefrontal cortex functioning[31]. Both strategy shifting and impulsive action have been shown to mediate early-life experiences on later-life provisioning of maternal care[32–34]. Sucrose preference, typically used to measure anhedonia, naturally varies between individuals and correlates with dopamine receptor 2 function in the basal ganglia[35].

The purpose of this study was to investigate dopamine gene x maternal licking interactions on (a) strategy shifting, (b) impulsive action, (c) sucrose preference, and (d) baseline dopamine turnover in key brain areas of the dopamine system in female offspring. We measured inter-individual maternal licking within the first week of life and assessed adult female offspring behavior, dopamine and its metabolite levels, and variation in SNPs in the DRD2, DAT, and COMT genes.

We predicted that higher levels of inter-individual maternal licking received would facilitate strategy shifting and decrease impulsive action and sucrose preference. We hypothesized this would be mediated by differences in baseline dopamine turnover. In addition, we hypothesized both behavior and dopamine turnover would be moderated by genotype. The hypothesized moderated-mediation is visualized in Figure 1.

2. Materials and Methods

2.1 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 in Scarborough and conformed to the guidelines of the Canadian Council on Animal Care.

Births were checked 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.

2.2 Maternal Care Observations

At PND 1, 3, 5 and 7, maternal care was assessed as previously reported[24]. 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, Ontario, 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 all offspring were pair-housed, the majority with siblings. Female offspring were weighed periodically until adulthood (PND 75).

2.3 Dopamine-Related Behavior Tasks

A subset of PND 90+ female offspring (n = 55) were tested for either strategy shifting (n=28) or a Differential Reinforcement of Low Rates (DRL-20 seconds) schedule (n=27), followed by a sucrose preference task (n=55). Other female offspring were assessed for distinct behavioral phenotypes in adulthood and will be reported elsewhere. We did not assess estrous cycle during these tasks; however, previous work has shown that variability across the cycle is not substantial enough to change the interpretability of several phenotypes[36]. The testing occurred over two cohorts; the first cohort included one to two pups from 20 litters and the second cohort included five to six pups from four litters. There were no

significant differences between cohorts in total litter size or any of the behavior measures (all p's > 0.10) and were therefore analyzed together.

Sound-attenuating operant chambers (MED Associates, St. Albans, VT, USA) connected to a Dell desktop PC with MED-PC-IV software installed were used for the strategy shifting and DRL-20 tasks. Each operant chamber was equipped with a ventilation fan, two retractable levers, two stimulus lights above the levers, a house light, and a food magazine that dispensed plain 45 mg sucrose pellets (#F06233, Bio-Serv). Each animal was gradually reduced to 85-90% of their ad libitum feeding weight and handled daily (two minutes per day) six days before chamber acclimation. The day before chamber acclimation, offspring received 20 sucrose pellets in their home cages. All female offspring acclimated to the operant chamber for one day with exposure to the ventilation fan and house light for 30 minutes. Offspring completed an auto-shaping procedure for five days and went through a Fixed Ratio-1 schedule until they made over 100 lever presses over three consecutive days. To account for potential circadian rhythm effects, the two operant tasks were counter-balanced between the morning (9:00-11:00) and afternoon (15:00-17:00) over the two cohorts.

2.3.1 Strategy Shifting

Twenty-eight female offspring completed the strategy shifting task, a measure of behavioral flexibility, as described by Brady and Floresco[37]. To reduce the number of omitted responses, offspring were required to press a randomly extended lever within ten seconds at least 85 times out of 90 trials. After meeting this requirement, offspring were reinforced to press the lever on the illuminated side of the chamber (cued task) until they made 10 consecutive correct responses. The illuminated side was random between trials. Each day involved 150 trials and offspring were disqualified if criterion was not met after three days (450 trials). After reaching criterion on the cued task, offspring were reinforced to press a randomly assigned lever regardless of illuminated side (response task) until they made 10 consecutive correct responses. Trials to criterion on the response task, adjusting for the number of trials with no responses, was used as a measure of behavioral flexibility. Fewer trials reflected greater flexibility. Two offspring were disqualified for not reaching criterion in the cued task after 450 trials and one offspring was removed for completing the response task after 11 trials, leaving a total of 25 offspring reaching all criteria.

2.3.2 Differential Reinforcement of Low Rates (DRL-20)

Twenty-seven female offspring completed a Differential Reinforcement of Low Rates (DRL-20 seconds) schedule for 30 minutes over eighteen days as a measure of impulsive action[38]. A DRL-20 schedule reinforced rats if they pressed a lever after 20 seconds has passed. Premature responses reset the timer with no reward dispensed. The first response in a session was always rewarded. Percent efficiency (number of rewarded presses / number of total presses * 100) was averaged over the last three days and used as a measure of impulsive action. Percent efficiency and average inter-response time were highly correlated in this task (Pearson's r = 0.966). Higher efficiency reflected lower impulsivity.

2.3.3 Sucrose Preference

All female offspring (n = 55) were individually housed and water-deprived for 10 hours before a two-choice bottle task. Tap water and tap water with 1% sucrose were randomly placed between offspring. Bottles were weighed before and after one hour in the dark phase (19:00 – 20:00). Nine offspring were excluded due to excessive liquid in the bedding, leaving a total of 46 offspring being analyzed. Sucrose preference (amount of sucrose water consumed / total liquid consumption * 100) was calculated and used as a measure of baseline reward sensitivity.

Forty-three offspring were sacrificed with CO_2 inhalation and decapitation following the sucrose preference task. Twelve offspring were sacrificed after further testing on intergenerational maternal care, which will be published in a subsequent report. Liver and whole brain tissue were collected and placed in dry ice or flash-frozen in isopentane, respectively. Tissue was stored in -80°C until further processing.

2.4 Genotyping

Liver DNA was extracted using an EZNA Tissue DNA Extraction kit (Omega Bio-Tek, Norcross, GA, USA) and assessed for single-nucleotide polymorphisms (SNPs) at two sites in the dopamine receptor 2 (DRD2) gene (RS107017253 and RS13448058), one site in the dopamine transporter (DAT) gene (RS13448119), and two sites in the catechol-o-methyltransferase (COMT) gene (RS107501401 and RS13451556). Polymerase Chain Reaction (PCR) was used with custom primer sets (Table 1) to amplify the region of interest. Target amplicons were verified with gel electrophoresis and the PCR products were purified using a QIAmp PCR Clean-Up kit (Qiagen, Hilden, Germany). Purified DNA (20 ng) was submitted for sanger sequencing (The Centre for Applied Genomics, Sickkids, Toronto, ON).

2.5 High Performance Liquid Chromatography

Forty-two female offspring brains were sliced and microdissected for specific brain areas in a Leica CM3050S cryostat. Medial prefrontal cortex (mPFC; +4.20mm to +2.70mm Bregma), nucleus accumbens core and shell (NAcc; +2.20mm to +1.20mm Bregma), medial preoptic area (MPOA; -0.30mm to -0.80mm Bregma), dorsal hippocampus (control brain region; -2.30mm to -3.30mm Bregma) and ventral tegmental area (VTA; -5.20mm to -5.60mm Bregma) were identified using an adult rat brain atlas[39]. 5 µl of 1.0 M ascorbic acid was added to each sample to stabilize the neurotransmitters and were stored at -80°C.

To prepare the samples, the brain tissue was thawed on ice, suspended in 20 µl artificial cerebrospinal fluid (ACSF; Harvard Apparatus) and homogenized by four pulses of sonication (2 seconds per pulse). 2 µl of brain homogenate from each sample was analyzed for protein concentration using BioRad protein assay reagent (BioRad, Hercules, CA, USA). 1 µl of 0.2 M perchloric acid per sample was added to the remaining homogenate and was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and stored at -80°C.

Dopamine (DA) and 3, 4-dihydroxyphenylacetic acid (DOPAC) concentrations were measured by high performance liquid chromatography (HPLC) as described by Chatterjee and Gerlai[40]. A BAS 460 MICROBORE-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA) was used together with a Uniget C-18 reverse phase microbore column (#8949, BASi). The mobile phase contained a buffer [0.1 M

monochloro acetic acid, 0.5 mM Na-EDTA, 0.15 g/L Na-octylsulfonate and 10 nM sodium chloride, pH 3.1], acetonitrile and tetrahydrofuran at a ratio of 94:3.5:0.7. The flow rate was 1.0 ml/min, and the working electrode (Uniget 3 mm glassy carbon, BAS P/N MF-1003) was set at 550 mV vs. Ag/Ag/Cl reference electrode. Detection gain was 1.0 nA, filter was 0.2 Hz, and detection limit was set at 20 nA. 5 µl of the sample supernatant was directly injected into the column. External standards for DA and DOPAC (Sigma) of known concentrations were used to quantify and identify peaks on the chromatogram. Under these parameters, the retention times for DA and DOPAC were approximately 3.7 minutes and 5.5 minutes, respectively.

DA and DOPAC concentrations were normalized against total protein concentration for each sample and expressed as ng/mg of protein. DOPAC divided by DA, or DOPAC/DA ratio, was calculated for baseline dopamine turnover, a measure of dopaminergic activity, in each brain area. We were unable to cryosection one brain due to extensive damage during collection and analyze two samples (one MPOA and one VTA) due to lost tissue.

2.6 Statistical Analysis

All statistical analyses were performed using SPSS (IBM Corporation). To examine the relationship between maternal care and dopamine-related phenotypes, a Pearson correlation was used between total or average licking duration and the behavior measures and DOPAC/DA ratio in each brain area. In addition, to examine the relationship between behavior and dopamine turnover, a Pearson correlation was used between the behavior measures and DOPAC/DA ratio in each brain area. To examine the effects of genotype, a linear mixed model was used to compare offspring with each varying genotype to licking received and adult measures. Litter ID was used as a random effect to reduce between-litter effects [41]. Significant effects of genotype were followed with a post-hoc test using Fisher's Least Significant Difference (LSD). To examine gene x environment interactions, a multiple regression was used with total lick duration or average lick duration (X_1) and each varying genotype (X_2; Model A) with an interaction term (X_1*X_2; Model B) for each behavior and brain area DA/DOPAC ratio:

Model A: $\hat{y} = b_0 + b_1X_1 + b_2X_2 + \varepsilon$
Model B: $\hat{y} = b_0 + b_1X_1 + b_2X_2 + b_3(X_1*X_2) + \varepsilon$

Regression models with a significant R^2 change when adding the interaction term were further analyzed with Hayes PROCESS module for SPSS (Version 3.1) using a simple moderation (Model 1)[42]. PROCESS is a flexible modelling module that can conduct moderation analyses and probe conditional effects of a focal moderator. All effects were considered statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.

3. Results

3.1 Correlations between Early-Life Licking Received, Baseline Dopamine Turnover and Behavior

Within litters, the two highest and two lowest licked pups consistently received high and low levels of licking, respectively, while the one to two remaining pups received mid-levels of licking. This was demonstrated with both total licking duration (Figure 2A) and average

licking duration (Figure 2B). For the female offspring tested, both total licking duration (Figure 2C) and average licking duration (Figure 2D) showed a normal distribution. Total licking duration across all maternal care observation periods averaged 123.06 ± 8.43 seconds and ranged from 0 to 285.69 seconds. Average licking duration averaged 7.83 ± 0.47 seconds and ranged from 0 to 16.65 seconds per bout of individual licking.

Total licking duration positively correlated with baseline DOPAC/DA ratio in the nucleus accumbens (Pearson's r = 0.383, p = 0.012; Figure 3A) and was not associated with baseline DOPAC/DA ratio in other brain areas or any behavior measures. Average licking duration negatively correlated with sucrose preference (Pearson's r = -0.372, p = 0.012; Figure 3B) and was not associated with trials to criterion in the strategy shifting task (Pearson's r = -0.276, p = 0.192), percent efficiency in the DRL-20 schedule (Pearson's r = -0.024, p = 0.907), or baseline DOPAC/DA ratio in any brain area.

3.2 Correlations between Behavior and Baseline Dopamine Turnover

Baseline DOPAC/DA ratio in the ventral tegmental area negatively correlated with percent efficiency in the DRL-20 schedule (Pearson's r = -0.534, p = 0.023; Figure 4A) and trials to criterion in the strategy shifting task (Pearson's r = -0.564, p = 0.015; Figure 4B). In addition, baseline DOPAC/DA ratio in the medial preoptic area negatively correlated with percent efficiency in the DRL-20 schedule (Pearson's r = -0.629, p = 0.005; Figure 4C). No other significant correlations were observed.

3.3 Effects of Genotype

One site in the DRD2 gene (RS107017253) and the DAT gene (RS13448119) were polymorphic in female offspring. For DRD2, 45 offspring were A/A, 7 were A/G, and 3 were G/G. The A/G and G/G groups were combined for statistical analysis. For DAT, 20 offspring were A/A, 22 were A/G, and 13 were G/G. The other DRD2 site (RS13448058) exhibited low variation (52 G/G offspring and 3 A/G offspring) and the COMT sites exhibited no variation in female offspring.

3.3.1 DRD2 (RS107017253)

DRD2 genotype had a marginal effect on baseline DOPAC/DA ratio in the nucleus accumbens ($F_{(1,32.676)} = 3.965$, p = 0.055). Offspring with the A/A genotype had lower DOPAC/DA ratio than offspring with A/G or G/G genotypes. With the DRD2 genotype there were no significant differences in baseline DOPAC/DA ratio in other brain areas, any behavior measures, or licking received between female offspring.

3.3.2 DAT (RS13448119)

DAT genotype had no effect in any brain site on baseline DOPAC/DA ratio. However, DAT genotype was associated with significant differences in sucrose preference ($F_{(2,40.527)} = 3.804$, p = 0.031; Figure 5A). Heterozygous A/G offspring had the lowest sucrose preference, were comparable to offspring with the A/A genotype (Mean difference = -3.308 ± 4.364, LSD p = 0.453) and were significantly different from offspring with the G/G genotype (Mean difference = -12.797 ± 4.706, LSD p = 0.010). Offspring with the A/A genotype and G/G genotype were marginally different in sucrose preference (Mean difference = -9.489 ± 4.843, LSD p = 0.057).

In addition, we observed a marginal effect of DAT genotype on percent efficiency in the DRL-20 schedule ($F_{(2,21.133)} = 3.324$, $p = 0.056$; Figure 5B). Again, heterozygous A/G offspring had the lowest percent efficiency and were comparable to offspring with the A/A genotype. Offspring with the G/G genotype had the highest percent efficiency. There were no significant differences in trials to criterion in the strategy shifting task, or licking received between female offspring with varying DAT genotype.

3.4 Gene x Environment Interactions

Since female offspring with A/A and A/G DAT genotypes performed similarly in the behavior measures tested (all p's > 0.05), we grouped them together for multiple regression. We did not observe any total licking duration x genotype interactions and only report average licking duration x genotype interactions.

3.4.1 Strategy Shifting

DRD2 genotype interacted with average licking duration on performance in the strategy shifting task. Specifically, we found a significant R^2 change when the average licking x DRD2 interaction term was added to the regression model ($\Delta R^2 = 0.312$, $F_{(1,20)} = 10.312$, $p = 0.004$). Analysis with PROCESS also showed this interaction (Coefficient $\beta = 12.9739 \pm 4.0401$, $t_{(1,20)} = 3.2112$, $p = 0.0044$) and found a significant effect at the A/A genotype (Effect = -7.4747 ± 2.3497, $t = -3.1811$, $p = 0.0047$) but not the A/G or G/G genotypes (Effect = 5.4922 ± 3.2866, $t = 1.6732$, $p = 0.1099$). For female offspring with the A/A genotype, higher average licking duration was associated with fewer trials to criterion on the response task, corresponding to higher behavioral flexibility in adulthood (Figure 6A). We observed a marginal interaction with the DAT genotype ($\Delta R^2 = 0.128$, $F_{(1,20)} = 3.466$, $p = 0.077$) on the behavioral flexibility task.

3.4.2 DRL-20

We did not observe an interaction with the DRD2 genotype ($\Delta R^2 = 0.045$, $F_{(1,22)} = 1.226$, $p = 0.280$) or the DAT genotype ($\Delta R^2 = 0.039$, $F_{(1,22)} = 1.266$, $p = 0.273$) with percent efficiency on the DRL-20 schedule.

3.4.3 Sucrose Preference

DRD2 genotype interacted with average licking duration on sucrose preference. Specifically, we found a significant R^2 change when the average licking x DRD2 interaction term was added to the regression model ($\Delta R^2 = 0.087$, $F_{(1,41)} = 4.687$, $p = 0.036$). Analysis with PROCESS also showed this interaction (Coefficient $\beta = 2.8812 \pm 1.39090$, $t_{(1,41)} = 2.2011$, $p = 0.0334$) and we found a significant effect at the A/A genotype (Effect = -2.0189 ± 0.6488, $t = -3.1118$, $p = 0.0034$) but not the A/G or G/G genotypes (Effect = 0.8623 ± 1.1369, $t = 0.7585$, $p = 0.4525$). For female offspring with the A/A genotype, higher average licking duration was associated with lower sucrose preference, corresponding to lower baseline reward sensitivity in adulthood (Figure 6B). We did not observe an interaction with the DAT genotype ($\Delta R^2 = 0.030$, $F_{(1,41)} = 1.100$, $p = 0.300$).

3.4.4 Baseline Dopamine Turnover

We did not observe any interactions with the DRD2 genotype or DAT genotype for baseline DOPAC/DA ratio in any of the brain areas tested (Table 2).

4. Discussion

To our knowledge, this is the first study to investigate the effects of inter-individual maternal care on female offspring dopaminergic phenotypes and the moderating role of genotype. Our results show that a SNP in the DRD2 gene moderates the effect of average licking per bout received on two behavior measures, strategy shifting and sucrose preference, with a minimal role on baseline dopamine turnover. The updated moderation model is displayed in Figure 7. Specifically, early-life licking received affects female offspring carrying the A/A genotype and does not affect female offspring carrying the A/G or G/G genotypes. In addition, a SNP in the DAT gene, regardless of licking received, has an effect on sucrose preference and a marginal effect on impulsive action. This suggests that offspring's sensitivity to early-life environments can be influenced by genetic variation in dopamine-related genes. This idea has also been discussed in the context of sensory processing sensitivity in the human literature with serotonin- and dopamine-related genes[43]. Sensory processing sensitivity is a stable trait within populations of multiple species that increases an individual's responsiveness to early-life environments[44].

4.1 Relationships Between Early-life Maternal Licking Measures and Phenotype

We examined two different methods to measure maternal licking/grooming, total duration and average duration per bout, to see if quality of maternal care plays a role in later-life offspring behavior and baseline dopamine turnover. We found each maternal care measure correlated with a different outcome; specifically, total licking duration positively correlated with nucleus accumbens dopamine turnover and average licking duration negatively correlated with sucrose preference. In addition, average licking duration showed stronger gene x environment interactions. Therefore, the average duration a mother licks a pup per bout may be a useful measure of inter-individual maternal care, similar to its use in whole-litter paradigms[14,15], and can be a compliment measure to the total duration of licking received.

4.2 Relationships Between Behavior and Baseline Dopamine Turnover

We only found few correlations between the behavior measures and baseline dopamine turnover, which does not support our hypothesis that early-life licking effects on behavior are mediated by dopaminergic activity. However, baseline dopamine turnover does not necessarily predict stimulus-induced dopamine turnover. For example, a study that used maternal isolation found that female offspring had a blunted stimulus-induced dopamine response to pups than undisturbed controls, even though their baseline dopamine levels were higher[45]. Investigating the relationships between inter-individual maternal licking received, behavior, and stimulus-induced dopamine turnover is a potential avenue for future work.

4.3 Effects of Genotype and Gene x Environment Interactions

Offspring with a heterozygous genotype for a SNP in the DAT gene had decreased sucrose preference and had a marginal decrease in DRL-20 efficiency. How this specific SNP affects dopamine transporter expression or function is unknown. While there is information on a DAT variable number tandem repeat and its effects on expression and behavior in human populations[28], we did not find any studies of DAT variation with rat populations.

We found gene x environment interactions with a SNP in the DRD2 gene for strategy shifting and sucrose preference. DRD2 gene variation has been shown to interact with the effects of early-life environment on dopamine receptor 2 expression in Sprague-Dawley rats[25], though the specific SNP in that study (RS13448058) showed low variation in our Long-Evans rat population (with three heterozygous offspring). Nevertheless, the pattern is similar: early-life events affect offspring with a certain genotype while others show a minimal association. We[23] also reported this pattern with SNPs in the FK506-binding protein, glucocorticoid receptor and serotonin transporter genes using rat siblings that received differential maternal licking. We currently do not know the specific transcriptional regulatory mechanisms involved in these interactions.

Sucrose preference showed a significant negative correlation with average licking duration with all offspring, but the relationship was stronger for female offspring with the A/A DRD2 genotype. This relationship between sucrose preference and maternal care is consistent with previous findings on maternal isolation[12]; however, it is counter-intuitive if sucrose preference is viewed as a measure of depressive-like behavior. We tested sucrose preference after the other tasks in the operant chamber which used sucrose pellets as a reward. Familiarity with the taste of sucrose could help explain the phenotype this task measured, but further experiments are required to confirm this explanation. In addition, as we only tested the female offspring once in the dark phase, we do not know if multiple testing would alter the results. However, a previous study demonstrated that sucrose preference scores are stable across days, especially during the dark phase[35].

Strategy shifting only showed a negative correlation with average licking duration for female offspring with the A/A DRD2 genotype. The relationship between strategy shifting and maternal care is consistent with previous findings on maternal separation and isolation[8,9,11]. These studies tested rats using an attentional set-shifting task, while we used a recently developed operant procedure to reduce training and extensive human handling during the task. The automated procedure would reduce individual variation from human error. The consistency indicates construct validity between the two testing paradigms and a robust relationship between early-life experiences and executive functioning. Testing the female offspring with a reversal task could provide additional insights regarding underlying neural circuitry, since strategy shifting and reversal learning involve different areas of the prefrontal cortex and can be dissociable[31,46].

We did not find any relationships between maternal licking and impulsive action, which has been found with maternal isolation[7]. Our data indicated that efficiency for a few female offspring did not increase from the first three sessions to the last three sessions of the DRL-20 schedule, indicating that the inability to learn the task in some subjects could be a confound. It is difficult to conclude whether these offspring were highly impulsive or did not learn the association required for the sucrose reward. In future studies, the inclusion of several sessions with DRL-5 and DRL-10 schedules may make the variations in DRL-20 performance easier to interpret.

4.4 Limitations

A caveat in this study is that the maternal care observations occurred immediately after handling the pups. Removing the pups from the nest was necessary to mark and differentiate siblings, but temperature changes in the pups and some tactile stimulation from the handler was unavoidable. Handling can induce changes in maternal care by increased solicitation from the pups[47], though it is unknown whether it can change maternal care between siblings. While our research group has used this paradigm in the past and found differences in female offspring stress reactivity[23,48], inter-individual variation in maternal licking could be verified with an undisturbed observation period.

Another caveat is that our study design consisted of two cohorts and we analyzed as few as one to two female offspring from a litter. Therefore we did not analyze differences in behavior using within-litter pup rankings as we have done previously with a minimum of five female pups in a litter[23,48]. Nevertheless, our correlational analysis of maternal licking identified findings that were in the direction we predicted from the literature and the interaction between average licking duration and DRD2 genotype was consistent between two different tasks. The external and internal replicability of our results provide evidence of a robust effect of maternal licking on dopamine-related behavior. However, a more comprehensive assessment of gene x environment interactions, with additional SNPs and behavior tasks, is needed to further support our interpretations.

In this study, we investigated the role of genotype in maternal care transmission of later-life dopamine-dependent behavior. Our findings suggest that the relationship between maternal licking and dopamine-related behavior depend on genotype for the DRD2 gene. These findings could help inform future work on the transmission of maternal care between generations, which is currently underway. Previous work focusing on the maternal brain has highlighted the importance of experience and hormonal priming via DNA methylation of estrogen and oxytocin receptor genes[49,50]. Overall, our findings suggest that variation in the DRD2 gene, likely along with other genes, could influence intergenerational maternal care by altering the female offspring's sensitivity to the maternal environment.

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Conflict of Interest Statement

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

Author Contributions

SCL, PP, POM, and ASF designed the study. SCL and PP conducted the maternal care observations. SCL conducted the behavioral testing and genotyping. DC conducted the HPLC. SCL and ASF analyzed the data. POM and ASF supervised the research. SCL, POM, and ASF wrote the manuscript. All authors contributed to this manuscript and approved the final version of this manuscript.

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Figure Legends:

Figure 1. Hypothesized moderated-mediation between early-life licking received and dopamine-dependent behavioral outcomes. Baseline dopamine turnover would mediate the association and offspring genotype would moderate behavior and baseline dopamine turnover.

Figure 2. Maternal licking received by female pup offspring in the first week of life. For both (A) total licking duration and (B) average lick duration, the highest lowest licked pups in a litter consistently received high and low levels of licking, respectively, while the remaining pups received mid-levels of licking. (C) Total licking duration (15-second bins) and (D) average licking duration (1-second bins) across all observation days are normally distributed for the female offspring tested (n = 55).

Figure 3. Maternal licking received correlated with some adult offspring outcomes. (A) Total licking duration positively correlated with baseline dopamine turnover in the nucleus accumbens. (B) Average licking duration negatively correlated with sucrose preference. Scatterplots are displayed with 95% confidence interval in gray.

Figure 4. Baseline dopamine turnover in select brain areas correlated with performance in the DRL-20 and strategy shifting tasks. Baseline DOPAC/DA ratio in the ventral tegmental area negatively correlated with (A) percent efficiency in the DRL-20 schedule and (B) trials to criterion in the strategy shifting task. (C) Baseline DOPAC/DA ratio in the medial preoptic area negatively correlated with percent efficiency in the DRL-20 schedule. Scatterplots are displayed with 95% confidence intervals in gray.

Figure 5. Dopamine Transporter genotype affected sucrose preference in adult offspring. (A) Heterozygotes had significantly reduced sucrose preference compared to homozygous G/G offspring and were similar to homozygous A/A offspring. A/A and G/G offspring were not significantly different from each other. (B) A similar pattern was observed for efficiency in the DRL-20 schedule but was not statistically significant. Bar plots are displayed with Estimated Marginal Means +/- standard error. * *Post-Hoc* $p < 0.05$ and ✢ $p < 0.10$ with Fisher's Least Significant Difference.

Figure 6. Association of average lick duration with strategy shifting and sucrose preference were moderated by dopamine receptor 2 genotype. Homozygous A/A offspring had (A) fewer trials to criterion in the strategy shifting task and (B) lower sucrose preference with more early-life licking received. Heterozygous and homozygous G/G offspring did not show an association with licking received. Scatterplots are displayed with linear regression lines for the A/A genotype (black) and A/G + G/G genotype (orange), with the 95% confidence interval for the A/A genotype in gray.

Figure 7. Updated moderation model between early-life licking received and dopamine-dependent behavioral outcomes. Offspring DRD2 genotype moderated the relationship between average lick received and later-life strategy shifting and sucrose preference.

Table 1. List of PCR Primers for Genotyping

Gene Name	SNP Accession	Primer	Sequence (5' to 3')
DRD2[1]	RS107017253	Forward	CAACATCGAGTTCCGCAAGG
		Reverse	GCATCGAGCCAAGCTAACAC
	RS13448058	Forward	TGAGTGGGTGGACAAGTGA
		Reverse	TTTCAAGGCATGCTTCCTCT
DAT[2]	RS13448119	Forward	CACTACTGCACCCCCAAATC
		Reverse	CTGACCAACTCCACCCTCAT
COMT[3]	RS107501401	Forward	TGTTAAAACCCGTGTCTGCGG
		Reverse	AGTCCCAGTTCCGTGTTTGC
	RS13451556	Forward	CCACATGCTTCTCTAGGGCG
		Reverse	GCTGCTCCCTCTCACATACG

[1]DRD2 – Dopamine Receptor 2, [2]DAT – Dopamine Transporter, [3]COMT - Catechol-O-Methyltransferase

Table 2. Results of Genotype x Average Lick Duration Multiple Regression Analysis

Dependent Variable	Genotype	R^2 change	p-value[1]
Strategy Shifting (Trials to Criterion)	*DRD2*	**0.312**	**0.004**
	DAT	*0.128*	*0.077*
DRL-20 (Percent Efficiency in Last Three Sessions)	*DRD2*	0.045	0.280
	DAT	0.039	0.273
Sucrose Preference	*DRD2*	**0.087**	**0.036**
	DAT	0.020	0.300
Medial Prefrontal Cortex DOPAC/DA Ratio	*DRD2*	< 0.001	0.959
	DAT	0.045	0.181
Nucleus Accumbens DOPAC/DA Ratio	*DRD2*	0.031	0.228
	DAT	0.001	0.815
Medial Preoptic Area DOPAC/DA Ratio	*DRD2*	0.043	0.198
	DAT	0.005	0.665
Dorsal Hippocampus DOPAC/DA Ratio	*DRD2*	0.002	0.773
	DAT	0.009	0.544
Ventral Tegmental Area DOPAC/DA Ratio	*DRD2*	0.001	0.889
	DAT	0.028	0.298

[1]p **< 0.05**, *p < 0.10*

Figure 1

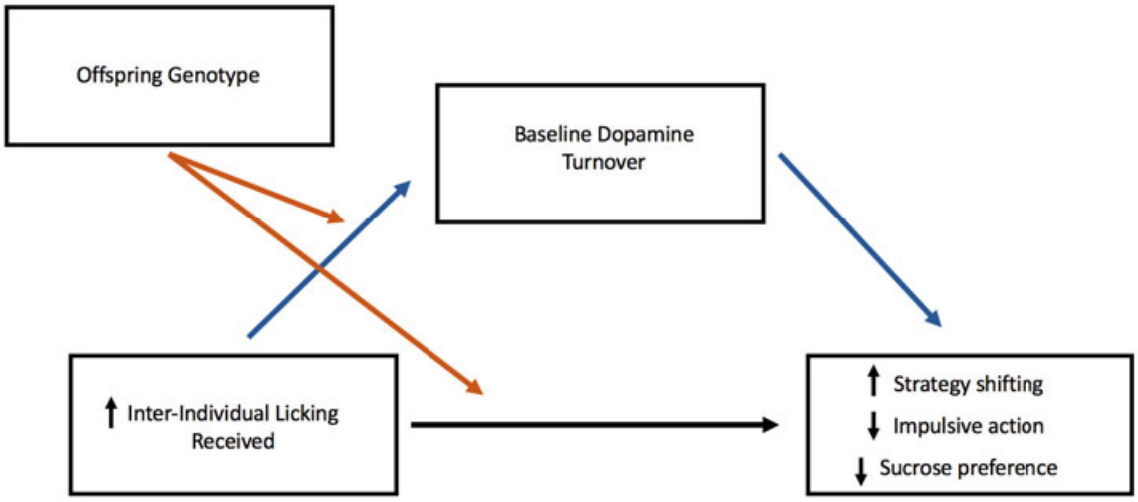

Figure 2

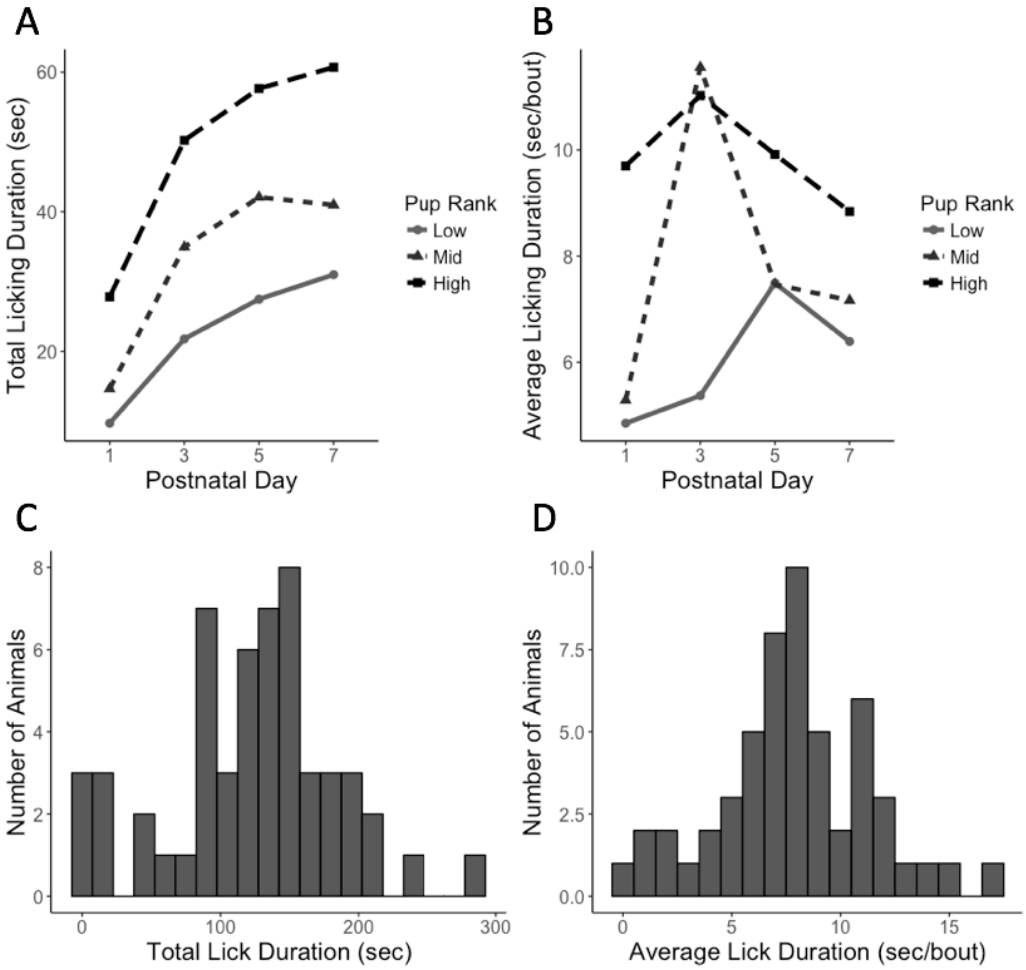

Figure 3

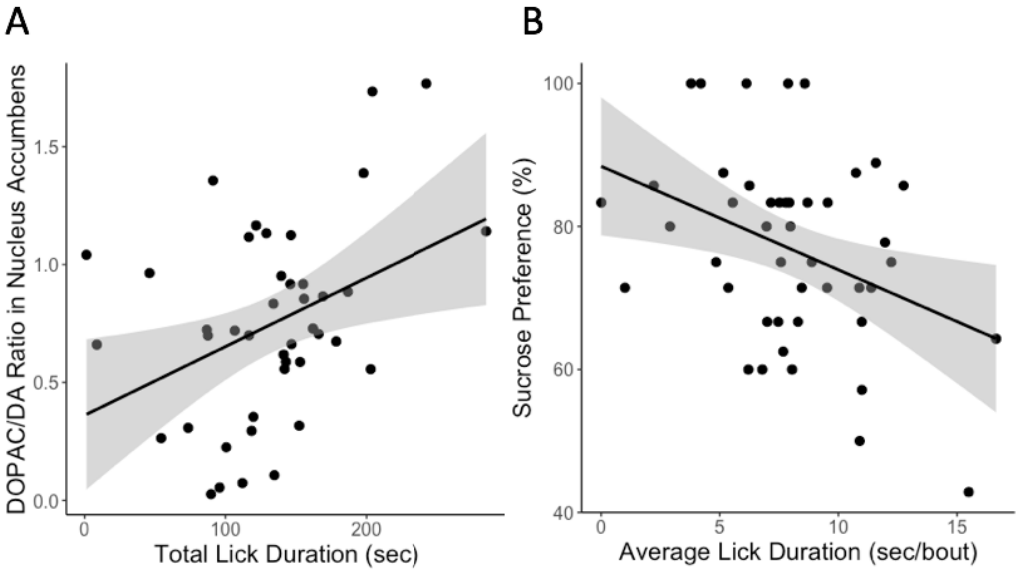

Figure 4

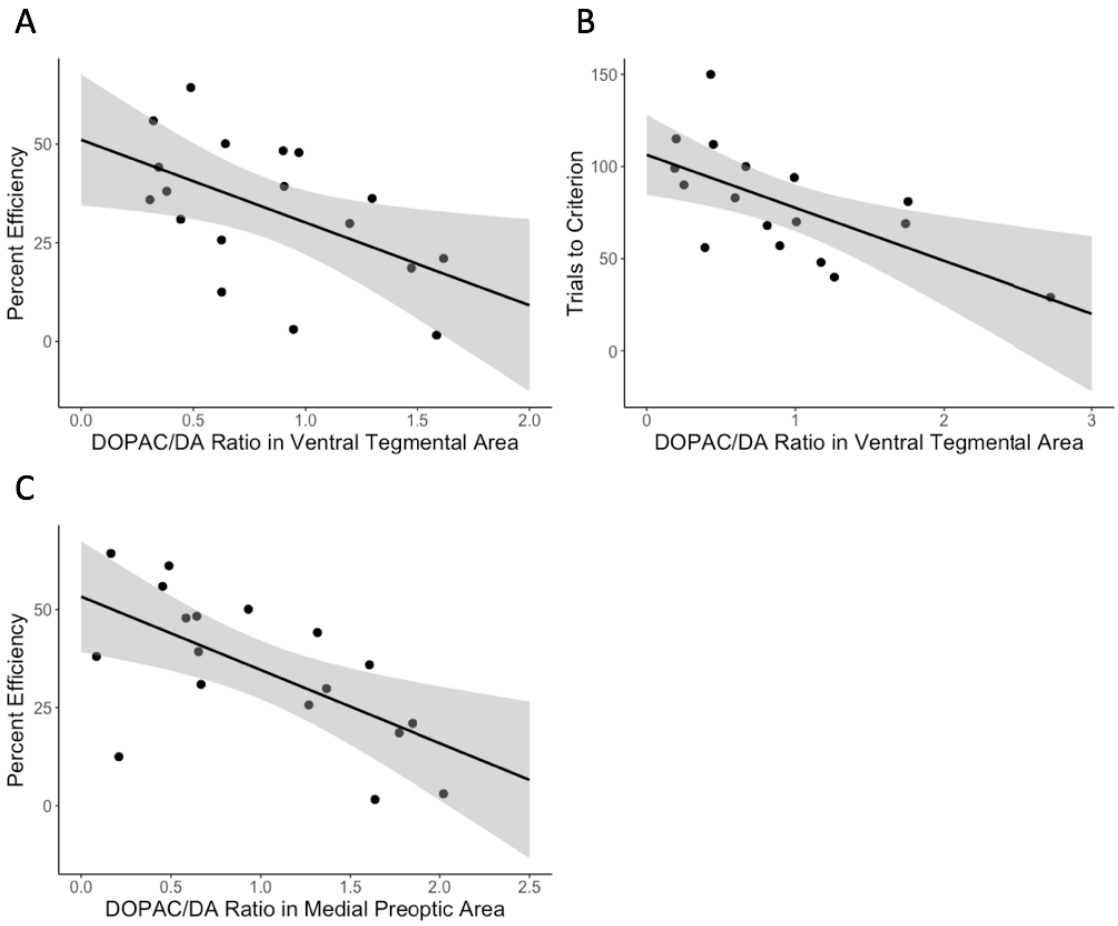

Figure 5

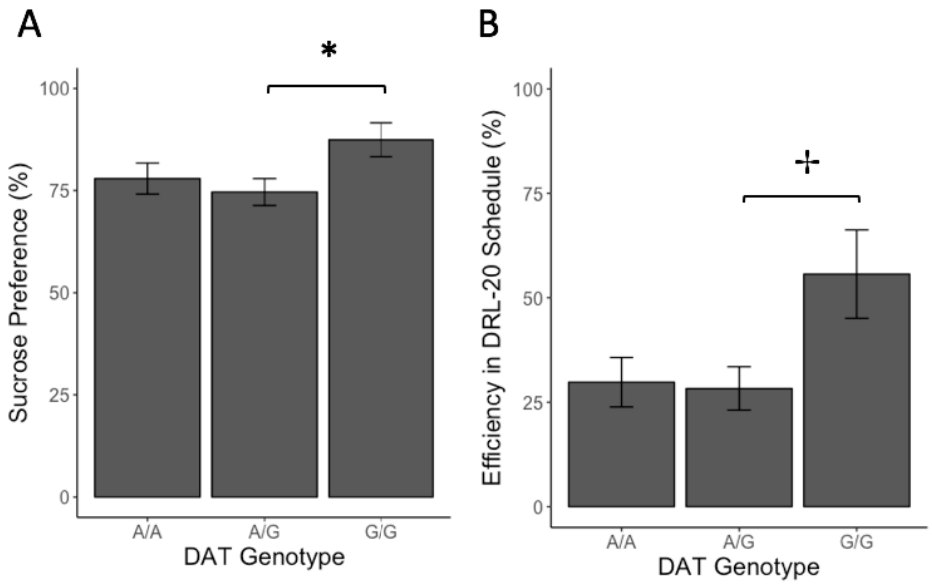

Figure 6

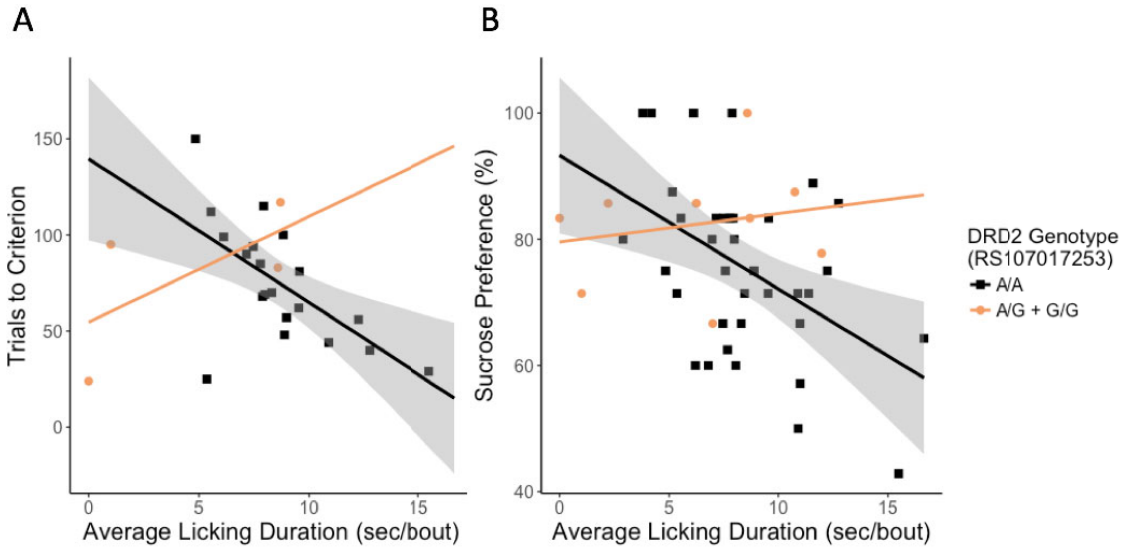

Figure 7