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RESEARCH ARTICLE

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Both maternal care received and genotype influence stress-related phenotype in female rats

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Abstract

Rat dams differ naturally in the level of maternal care they provide to their offspring within the same litter. We explored possible mechanisms of differential maternal care focused on genetic variation. We examined single nucleotide polymorphisms in the glucocorticoid receptor, FK506-binding protein, and serotonin transporter genes in two separate cohorts, and the relationship between differential maternal care received, genotype, and offspring phenotype. Allelic variation in all three genes was significantly associated with levels of maternal care received by offspring and behavioral and endocrine stress responses in adulthood. Differences in pup behavior were also associated with allelic variation in these genes. Together, these results indicate that the dam/pup interaction is dynamic and implicate the genotype of the offspring in influencing the level of maternal care received. They further suggest that some genotypes may have a dampening effect on the impact of maternal care on stress-related phenotypes in adulthood.

KEYWORDS

anxiety, behavior, corticosterone, FK506-binding protein, genotype, glucocorticoid receptor, hypothalamic–pituitary–adrenal Axis, maternal care, rat, serotonin, single nucleotide polymorphism, stress response

1 | INTRODUCTION

In rodents and other mammals, the quality of maternal care relies on the integration of multiple physiological and behavioral systems and on early experiences that impact how a mother mothers (Barrett & Fleming, 2011). Many studies have now established that in rodents there is natural variation in the amount of maternal care—typically assessed by measuring the licking and grooming (LG) of pups—that dams provide to their litters (Champagne, Francis, Mar, & Meaney, 2003; Francis, Diorio, Liu, & Meaney, 1999; Weaver et al.., 2004). More recently, our studies and others have shown that mothers also provide differential levels of care to individual offspring within the same litters (Cavigelli, Ragan, Barrett, & Michael, 2010; Pan, Fleming, Lawson, Jenkins, & McGowan, 2014; van Hasselt et al., 2012). However, we understand very little about why some pups in a litter receive more or less care than their siblings from the same

Abbreviations: 5htt, serotonin transporter; BEST, behavioral evaluation strategy and taxonomy software; CORT, corticosterone; EPM, elevated plus maze; Fkbp5, FK506-binding protein; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal axis; KW, Kruskall–Wallis nonparametric test; LG, licking and grooming; MWU, Mann–Whitney U non-parametric test; NR3C1, nuclear receptor subfamily 3 group C 1; OFT, open field test; PND, postnatal day; SLc6a4, solute carrier 6 a 4; SNP, single nucleotide polymorphism; USV, ultrasonic vocalization.

mothers. The current studies are part of a series we have undertaken to explore this question. To do so, we used outbred Long-Evans rats to focus on the dynamics of mother–pup interactions at both the behavioral and genetic level by studying the relationship between natural genetic variation in female offspring and variation in their behaviors as neonates and adults. The experiments described here consist of two separate studies. In Study 1, we investigated the influence of single nucleotide polymorphisms (SNPs) in the nuclear receptor subfamily 3 group C member (*Nr3c1*), FK506-binding protein (*Fkbp5*) and serotonin transporter (*5htt*) genes in offspring on their adult stress-related behaviors in relation to the individual maternal care received as neonates. In Study 2, we examined, in addition, the influence of allelic variation in the *Nr3c1*, *Fkbp5*, and *5htt* genes on the behavior of pups during the neonatal period prior to weaning that may explain the differential care received, and perhaps be precursors to their later adult behaviors.

The rationale for the choice of the SNPs of interest derives from what we already know about the effects of maternal licking on offspring hypothalamic–pituitary–adrenal (HPA) function and on the regulation of serotonin-related behaviors (e.g., Belay et al., 2011; Zoratto, Fiore, Ali, Laviola, & Macrì, 2013). We explored SNPs within three genes associated with HPA and serotonin function.

1.1 | Glucocorticoid receptor

The first gene SNP of interest was *Nr3c1*, encoding the glucocorticoid receptor (GR), a transcription factor that, when bound to glucocorticoids, binds to genomic GR response elements and acts as a regulator of other transcription factors. *Nr3c1* was chosen because there is now ample evidence that maternal licking impacts both the release of the stress hormone corticosterone (CORT) in response to stress and the GR-related negative feedback mechanism in the hippocampus that terminates the stress response (Kaffman & Meaney, 2007). Reduced activity of GR in the hippocampus has also been associated with stress-related behaviors, including reduced locomotor activity and thigmotaxic responses in the elevated plus maze (EPM) and open field tasks (OFT) (Kaffman & Meaney, 2007). In humans, SNPs in the glucocorticoid receptor are risk factors for multiple phenotypic outcomes, including metabolic, immune, cardiovascular and psychiatric disease (Koper, van Rossum, & van den Akker, 2014; Wüst et al., 2004).

1.2 | FK506-binding protein

Another HPA-related gene is *Fkbp5*, a gene encoding the FK506-binding protein (FKBP5), a co-chaperone of steroid receptors, including the glucocorticoid receptor (Baughman, Wiederrecht, Chang, Martin, & Bourgeois, 1997). FKBP5 inhibits GR activation and, conversely, is activated by the binding of GR to GR response elements in the *Fkpb5* promoter (Jääskeläinen, Makkonen, & Palvimo, 2011). In humans, SNPs in *Fkbp5* alter the risk for post-traumatic stress disorder, suicide and depression, implicating allelic variation in *Fkbp5* in stress-related illness (Binder et al., 2008, 2009). These data indicate a close association between genetic variations in *Fkbp5* and *Nr3c1* in the emergence of stress-related phenotypes.

1.3 | Serotonin transporter

The *Slc6a4* (*5htt*) monoamine transporter gene belongs to the solute carrier 6 (*Slc6*) gene family of ion-coupled cotransporters, which includes transporters of dopamine and serotonin neurotransmitters, amino acids, creatine, and osmolytes, that regulate the temporal and spatial effects of released neurotransmitters (Hahn & Blakely, 2007). *5htt* genotype was a focus of interest in our studies because it has been shown to interact with maternal environment to affect serotonin and norepinephrine levels in the mouse brain (Carola, Pascucci, Puglisi-Allegra, Cabib, & Gross, 2011). The high activity version of rh5-HTTLPR predicts a high correlation between maternal and infant temperaments in non-human primates (Sullivan, Mendoza, & Capitanio, 2011). We previously reported that allelic variation in *5htt* in human mothers predicts maternal sensitivity, behavior, and attitudes toward 6-month-old infants (Mileva-Seitz et al., 2011).

Overall, the rationale behind the choice of a focus on *5htt*, *Nr3c1*, and *Fkbp5* genes in the same samples is strengthened by the findings that *5htt* gene variation is known to interact with early life stress and adversity, and associates with altered transcription of GR receptors, its co-chaperone FKBP5 (van der Doelen, Calabrese, et al., 2014), plasma levels of CORT, mRNA levels of adrenocorticotropin receptor (van der Doelen, Calabrese, et al., 2014), and DNA methylation of the corticotropin-releasing factor gene (van der Doelen et al.., 2015) in rats.

In the present experiments, we explore further the extent to which *Nr3c1*, *Fkbp5*, and *5htt* SNPs, known to vary in populations of outbred rats, are associated with variations in (a) licking that mothers provide their offspring and affects on individual pups within litters, (b) behaviors that pups exhibit during neonatal life that may elicit differential mothering behavior, and (c) offspring anxiety behaviors in adulthood that we know are affected by early differential maternal care.

2 | METHODS

2.1 | Animals and housing

All procedures in this study conformed to the guidelines set by the Canadian Council on Animal Care and were approved by the University of Toronto Mississauga Local Animal Care Committee. The rats were born and raised at the University of Toronto Mississauga from stock originally obtained from Charles River Farms (St. Constant, Quebec, Canada). The colony was maintained on a 12:12 hr light:dark cycle with lights on at 0,800, at approximately 22°C, 50%–60% constant humidity, and ad libitum access to rodent chow and tap water.

2.2 | Study 1: Genotype, maternal care received, and adult stress-related phenotype

2.2.1 | Mating and parturition

A total of 19 virgin adult female Long-Evans rats (55–65 days of age) obtained from Charles River Farms (St. Constant, Quebec) were mated 1:1 with sexually experienced adult male Long-Evans rats. At mating, each male was paired with one virgin female for 7 days. Females were single-housed after mating and for the duration of gestation. Females were monitored for parturition beginning on gestation day 18. For each litter, if pups were delivered before 1,500 hr, that day was designated as postnatal day (PND) 0. At PND 1, pups were sexed and culled to same-sex litters of six females to control for and eliminate the variability that occurs when animals are in mixed-sex litters (Cavigelli et al., 2010). Litters were included in the study if they contained at least four female offspring. Litters were excluded from the study because of cannibalism (n = 4), the presence of male pups that were mistaken for female pups (n = 2), or unsuccessful impregnation (n = 1). In total, 72 pups in 12 litters were assessed individually for maternal LG received.

2.2.2 | Maternal behavior

Maternal behavior was assessed as previously described (Pan et al., 2014). Briefly, maternal behavior was observed for 30 min daily from PND 1 to PND 10 between 900 hr and 1,300 hr (Figure 1). During this time, cage bedding was not changed to minimize disruptions of the nest and associated odor cues. Individual pups were taken from litters for 10 min. Upon removal from the nest, pups were placed in a baby cage that was situated on a heating pad maintained at approximately 37°C across all days, with bedding added from the home cage (i.e., the "warm pup cage"). During this time, they were handled and marked with a small paintbrush and nontoxic, odorless food coloring (Club House, London, Ontario, Canada). Pups were reintroduced to the litters by being placed in the opposite corner from where the nest was established. Behavior scoring occurred immediately after the pups are placed back into the nest. The specific color used to identify pups within the same litters (i.e., coloring) was identical to those that we previously found did not alter the level of licking received by individual pups (Pan et al, 2014). Similar methods of coloring pups have been used in previous studies (Cavigelli et al., 2010; van Hasselt, et al., 2012) and our methods were chosen to ensure accuracy when identifying pups in the nest, especially if dams were crouching over them.

Two experimenters with high inter-rater reliability (>90%) coded the dams' behaviors in real time using Behavioral Evaluation Strategy and Taxonomy (BEST) software (Educational Consulting Inc., Florida). Anogenital licking and body licking received were recorded individually for each pup; hovering and nursing over the pups were coded for each litter as a whole. The duration of combined anogenital licking, body licking, and nursing was used as an index of maternal care (Champagne et al., 2003). The absolute amount of LG received during the post-natal period in which maternal care was recorded as well as their percentage deviation from the litter mean was analyzed for each offspring within each litter (Pan et al., 2014; van Hasselt et al., 2012). Pups' ears were notched for future identification purposes at PND15, and all pups are weaned at PND 21. The handling and ear notching were consistent across all litters and pups. Efforts were taken to minimize any discomfort to the animals including the application of a local anesthetic (EMLA cream) prior to notching.

2.2.3 | Behavioral testing, endocrine response to stress, and genotyping in adult offspring

Behavioral testing and genotyping were performed on the two highest LG and the two lowest LG offspring from each litter (n = 4 × 12 = 48) in adulthood (PND75–PND105). The exceptions were the open field and stress tests, where the highest LG and lowest LG offspring in each litter (n = 2 × 12 = 24) were examined. The order of testing was: locomotor activity, EPM, OFT, and stress response test. All testing occurred in the subjective light phase of the circadian cycle (900–1,500) with intervals of at least 24 hr between each test.

Locomotor activity

Adult female offspring were tested over a 30-min session in a locomotor activity box (47 × 26 × 20 cm). Activity levels were measured by an automated monitoring system that consisted of 16 parallel test boxes with infrared photocells mounted on a metal assembly into which a standard cage without bedding was placed. Activity levels were quantified as the number of total photocell interruptions (i.e., "beam breaks") over the test session (Lynch, Castagné, Moser, & Mittelstadt, 2011). The test boxes were cleaned with 70% ethanol

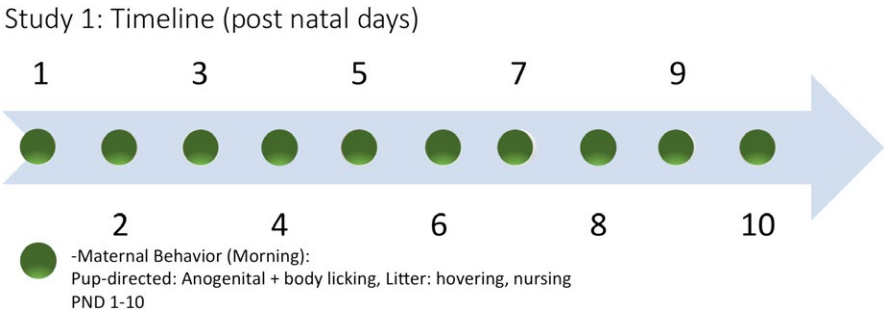

FIGURE 1 Timeline (in days) of postnatal assessments in rat pups in Study 1. PND: postnatal day

and dried before each trial. All female offspring were tested between 11 a.m. and 3 p.m. in a pseudo-randomized order by experimenters blinded to litter and offspring high/low status.

Elevated plus maze

Anxiety-like behavior was tested using a standard EPM. It consisted of two open arms (10 × 50 × 45 cm) and two closed arms (10 × 50 × 45 cm) that extended from a central platform (10 × 10 cm). The maze was constructed from wood painted in black and elevated 50 cm from the floor. The apparatus was cleaned with 70% EtOH between each test. Female offspring were transferred to a testing room between 1,000 and 1,300 hr. Prior to testing, dams were allowed to habituate in their home cages for 5 min. They were then placed in the center of the EPM apparatus and their activity was recorded for 20 min, for two consecutive days. Using BEST (Sharpe & Koperwas, 1999), duration and frequency of time spent in open and closed arms, the center, as well as grooming and rearing were recorded.

Open field task

We used the OFT as an additional validated measure of anxiety-related behavior (Belzung & Griebel, 2001) that has been used in many previous studies of the effects of interlitter and intralitter differences in maternal care (e.g., Caldji, Diorio, & Meaney, 2000; Champagne & Meaney, 2007; Weaver, Meaney, & Szyf, 2006;). Behaviors in the OFT were examined in a five-minute session in a 100 × 100 × 35 cm arena. The arena was divided into 49 equal grid squares. The nine central squares were designated as the "center," four squares situated in the corners of the box were designated as "corners," and six squares between two adjacent corners were designated as "sides." Adult female offspring were placed in the center facing the experimenter and allowed to explore the apparatus. The experimenter recorded the time spent in the center, corners, and sides using BEST software. The arena was cleaned with 70% ethanol and dried before each trial and the number of boli produced by each adult female offspring was recorded for each test session. Anxiety levels were quantified as the proportion of total time animals spent in the center of the apparatus (Hall & Ballachey, 1932). All adult female offspring were tested between 11 a.m. and 3 p.m. in a pseudo-randomized order by experimenters blinded to litter and offspring high/low status.

Stress response test

Adult female rats were handled for 5 days to habituate them to the experimenters prior to testing the stress response. The same room was used for habituation, the stress response test and blood collection. For stress response testing, animals were hand-restrained under a cotton towel and blood was collected from a nick in the tail as we have described previously (Belay et al., 2011) into a non-heparinized 0.6-ml centrifuge tube (baseline, approximately 100 μl per rat) and immediately placed on ice. Rats were then placed into Plexiglas restrainers (8 cm diameter 20 cm length), and 20 min later, a second sample of blood (peak, approximately 100 μl per rat) was collected as described above immediately before rats were released from the restraint. Rats were then returned to their home cages without their cage mates and left undisturbed for 70 min in the same room where the stress and blood collection occurred. Ninety minutes after the first blood collection, a third sample of blood was collected (return to baseline, approximately 100 μl per rat). Blood was left on ice for at least 30 min before the samples were centrifuged at 4°C and 4,000 rpm for 25 min. Blood serum was extracted and stored at 80°C. Serum levels of CORT for each female offspring at each of the three time points were measured using a commercial rat/mouse corticosterone ELISA kit (ALPCO Diagnostics, Cat # 55-CORMS-E01, Salem, NH; sensitivity 7.7 ng/ml and intraassay coefficient of variation 7.1%).

DNA purification and genotyping

Hippocampal genomic DNA was extracted using the QIAmp DNA Mini Kit (Cat no. 51304, Qiagen, Toronto, ON) and eluted with MilliQ ultrapure H2O. Purified genomic DNA samples were adjusted to a final volume of 40 μl at a concentration of 10 ng/μl. Multiplex SNP genotyping was performed using Sequenom's MassARRAY MALDI-TOF Platform at the Clinical Genomics Center at Mount Sinai hospital (in Toronto, ON) using custom primers. Six single nucleotide polymorphisms (SNPs) from this cohort were analyzed for their relationship to individual maternal care differences and adult female offspring behavior. This included four SNPs of the *Nr3c1* gene (encoding GR): (a) rs197359914, located within exon 8, at chr 18:31729455 with possible G/A alleles; (b) rs198255755, located within exon 8 at chr 18:31731734, with possible A/G alleles; (c) rs198862086, located within exon 8 at chr 18:31732120 with possible T/A alleles; and (d) rs198873320, located downstream of exon 1 at chr 18:32675926 with possible T/A alleles; one SNP of the *Fkbp5* gene (encoding FKBP5), rs8161939, located within exon 11, at chr 20:7975923 with possible T/C alleles; and one SNP of the *5htt* gene (encoding serotonin transporter): rs8154473, located within exon 3, at chr 10:63171823 with possible T/C alleles and synonymous amino acid coding.

2.3 | Study 2: Genotype, maternal care received, pup phenotype, and adult stress-related behavior

2.3.1 | Mating and parturition

Sixteen virgin adult female Long-Evans rats obtained from Charles River Farms (St. Constant, Quebec) were mated 1:1 with males in a manner similar to Study 1. Two females did not become pregnant and were exclude from the study. At PND 1, each mixed sex litter was culled to six female pups. In total, 84 pups in 14 litters were assessed individually for maternal LG received.

2.3.2 | Maternal behavior

Maternal behavior was observed for each litter in the morning on PNDs 2, 4, 6, and 8 to allow for pup assessments on alternate days

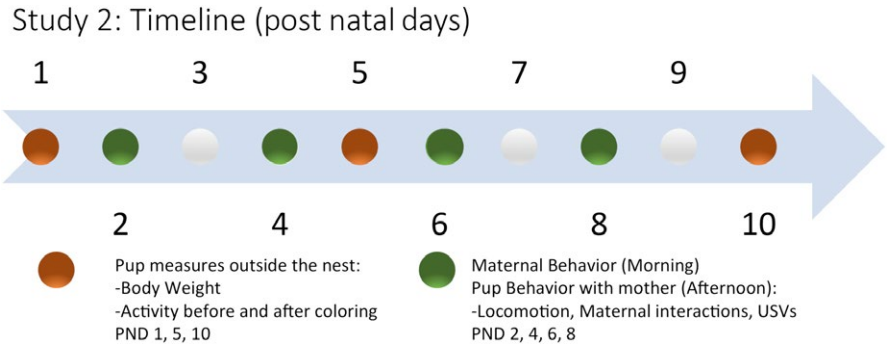

FIGURE 2 Timeline (in days) of postnatal assessments in rat pups in Study 2. PND: postnatal day; USVs: ultrasonic vocalizations

(Figure 2). Maternal behavior was observed in a manner identical to Study 1, except that a 15-min retrieval test was used in order to facilitate additional pup testing in this Study. Upon removal from the nest, pups were placed in a baby cage that was situated on a heating pad maintained at approximately 37°C across all days and tests, with bedding added from the home cage (i.e., the "warm pup cage"). The dams' behavior was coded and high and low pups were defined as described above in Study 1. The absolute amount of LG received during the postnatal period in which maternal care was recorded as well as the pups' percentage deviation from the litter mean was analyzed for each offspring within each litter (Pan et al., 2014; van Hasselt et al., 2012). Pups' ears were notched for future identification purposes at PND15, and all pups are weaned at PND 21.

2.3.3 | Behavioral testing and genotyping in offspring

Pup weights and behavioral profiles were examined in all pups between PNDs 1 and 10. Behavioral testing and genotyping were performed on the two highest LG and the two lowest LG offspring from each litter ($n = 4 \times 14 = 56$) in adulthood (PND75-PND105).

Pup body weight and activity recording independent of mother

Pup activity and body weight, independent of mother and littermates, were measured on PNDs 1, 5, and 10. The litter was removed from the nest and placed in the "heated pup cage." Individual pups were then removed from the "heated pup cage" and placed in a plastic cup (filled with bedding and nesting material from the corresponding maternal cage to avoid extra stress to the pups) situated on a digital weight scale, where activity and body weight were measured. Activity was measured upon placing the pup in the cup for 20 s, and scored as the frequency of movements in the limbs, head, and torso as the pups carried out pivoting (which includes punting and treading) and, later in development, crawling movements (Altman & Sudarshan, 1975). Activity was measured both before and after coloring. Activity after could provide insight into how pups respond to tactile stimulation (which would naturally be maternal). Body weight was measured after coloring.

Pup behavior in nest

Pup behavior, in the home cage with mother but independent of littermates, was observed for 5 min in the afternoon, after maternal behavior (see maternal behavior section above) on PNDs 2, 4, 6, and 8. Pups were separated from their mothers and placed in the "warm pup cage." A pup was then randomly selected and placed back in the home cage in the nest with the mother. The pup that was tested was placed back in the warm cage and another pup was selected at random for testing. Then, pup behaviors (locomotion and maternal interaction) were coded live using BEST software for 5 min. This was repeated for each pup individually in their home cage environment to avoid novelty and environmental stressors. The pup examined pup behaviors included crawl, not restricted to backward and forward movement (which typically emerges after PND 2) where the pup can appear as if it is swimming by making crawling movements, movement where the pup is not attempting to get anywhere; the pup may be trying to flip or establish contact with its head to mom, flip, under mom, attached to nipple, trying to find nipple, and maternal care. The maternal care components encompassed body- and anogenital licking.

Pup ultrasonic vocalizations (USVs)

USVs were monitored on PNDs 2, 4, 6, and 8 in the afternoon in the home cage after brief separation from the mother. The brief separation consisted of all pups being removed from the home cage and placed into baby cages with home cage bedding on heating pads. The first pup was placed back into the maternal home cage within ~2 to 3 min to resume pup behavior scoring and USV recordings, with the next pup following approximately 1 min later in sequential order. Recordings took place simultaneously along with the live scoring of pup behavior in the home cage with the mother present. To minimize and account for USVs produced as a result of lowered temperatures, baby cages were placed on heating pads as maintained approximately at 37°C for all days during the testing procedures. USVs were recorded by a bat detector (Batbox III, Stag Electronics) tuned to 40 kHz to discriminate pup calls from the mother, which tend to be at a much lower frequency. The detector was suspended above the home cage for 5 min and USVs were captured by a recorder (Olympus VN-120) connected to the detector.

TABLE 1 Summary of results obtained in Study 1

Gene	Genotype	LG received	Locomotor activity	Elevated plus maze	Open field task	Stress response
GR	TA/AA n = 10 TT n = 38	n.s.	n.s.	TT > TA/AA in % time in open arms, p = 0.032 (Figure 3a)	n.s. Time in centre correlates with pup LG as % of litter LG mean, p = 0.036 (Figure 3b)	TT < TA/AA CORT levels at 20 min, p = 0.031 (Figure 3c)
FKBP5	CC n = 11 CT n = 23 TT n = 14	CC > CT > TT, p = 0.031 (Figure 4a)	n.s.	n.s.	n.s.	TT > CT > CC CORT, p = 0.041 (Figure 4b)
5HTT	TC n = 24 TT n = 24	TC < TT, p < 0.01 (Figure 5a)	n.s.	n.s.	n.s. Time in centre correlates with pup LG as % of litter LG mean, p = 0.003 (Figure 5b)	n.s.

Behavioral testing in adult Offspring

Behaviors in the locomotor activity box, EPM, and OFT were assessed as described in Study 1.

DNA purification and genotyping

Liver genomic DNA was extracted using the QIAmp DNA Mini Kit (Cat no. 51304, Qiagen, Toronto, ON) and eluted with MilliQ ultrapure H_2O. Purified genomic DNA samples were adjusted to a final volume of 20 µl at a concentration of at least 8 ng/µl. Samples containing at least 8 ng of DNA per 10 µl volume were further processed for sequencing at the Center for Applied Genomics at the Hospital for Sick Children in Toronto via Sanger sequencing.

2.4 | Statistical analyses

Statistical analyses for both studies were conducted using IBM SPSS Statistics v.23 for Mac. For uniformity across the two studies, we employed the same statistical procedures for both. For each of the SNPs, the number of genotypes was established and two or three group comparisons on each of the behavioral, body weight, and corticosterone responses were analyzed with nonparametric statistics using either Mann–Whitney *U* tests (MWU, 2 group) or Kruskall–Wallis (KW, 3 group) comparisons. Where relevant, spearman correlations were also included. The choice of nonparametric, as opposed to parametric statistics was based on the fact that in some cases, there was an unequal distribution of genotypes represented in the sample, sample sizes of some genotypes were low and where sample sizes were small, variances were not homogeneous. However, to ensure that the more conservative nonparametric approach did not eliminate important effects, where appropriate, subsequent ANCOVAs were computed on some of the same data, also including dam ID as a covariate (to control for potential sibling effects). Litter ID made no contribution to any analysis and the overall significant effects and outcomes were similar to what we found with the nonparametric analyses. The nonparametric tests are therefore reported herein.

3 | RESULTS

Summaries of the results obtained in Study 1 and Study 2 are shown in Tables 1 and 2, respectively.

3.1 | Study 1: Genotype, maternal care received, and adult stress-related phenotype

3.1.1 | Glucocorticoid receptor

We examined 48 animals for *Nr3c1* genotype of four SNPs; all four resulted in the same distribution of genotypes. RS197359914: AA = 38, GG = 2, AG = 8; RS198255755: AA = 38, GG = 2, AG = 8; RS198862086: TT = 38, AA = 2, TA = 8; and RS198873320: TT = 38, AA = 2, TA = 8. As all four SNPs examined showed the same SNP distribution within subjects, we report results analyzed using the last SNP (RS198873320) as a representation of the data. Mann–Whitney U tests were computed after animals in the TA and AA groups were combined and compared to TT animals.

Maternal care received

Pups carrying different genotypes for the *Nr3c1* gene did not receive different amounts of LG from their mothers.

Adult behavior and endocrine response to stress

Adult offspring carrying different genotypes of the *Nr3c1* gene (RS19862086) did not display differential locomotor activity overall. Of the 48 animals tested in the EPM, data for five animals (n = 4 TT and n = 1 AT/AA were lost due to a technical error. Offspring carrying the TT allele for *Nr3c1* spent significantly more time in the open arms of the EPM than offspring carrying the AT/AA alleles (MWU = 78.5, n1 = 34, n2 = 9, p = 0.032; Figure 3a). In the OFT, offspring carrying the TT genotype showed a significant positive correlation between maternal care received and the time they spent in the center of the open field (rho = 0.48, p = 0.036, n = 19) while offspring carrying the AT/AA alleles (n = 5) showed

TABLE 2 Summary of results obtained in Study 2

Gene	Genotype	LG received	Pup weight	Pup activity independent of mother	Pup behavior in nest	Pup USVs	Locomotor activity	Elevated plus maze	Open field task
GR	TA/AA *n* = 2 TT *n* = 54	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
FKBP5	CC *n* = 24 CT *n* = 32	CC > CT, $p < 0.001$ (Figure 6a)	CT > CC on PND 1, $p = 0.003$ (Figure 6b)	CC > CT before their interactions with dams, $p = 0.007$ (Figure 6c)	CC < CT activity in nest, $p = 0.015$ (Figure 6d)	n.s.	n.s.	CC < CT, $p = 0.011$ (Figure 6e)	CC < CT, $p = 0.055$ (Figure 6f)
5HTT	TC *n* = 45 TT *n* = 11	n.s.	n.s.	n.s.	n.s.	TC > TT, $p = 0.055$ (Figure 7a)	TC > TT, $p = 0.035$ (Figure 7b)	n.s.	n.s.

no significant correlation between maternal care received and behavior in the open field (Figure 3b). For the stress response test, two animals (both AT/AA genotype) had insufficient serum for the corticosterone assay. Offspring carrying the TT allele had a significantly dampened endocrine response to restraint stress compared to offspring carrying the AT/AA alleles, as measured by CORT levels observed immediately following restraint (MWU = 70, n1 = 5, n2 = 17, $p = 0.031$; Figure 3c).

3.1.2 | FK506-binding protein 5

Of 48 adult female offspring genotyped for the RS8161939 SNP of the *Fkbp5* gene, 11 carried the CC genotype, 23 carried the CT genotype, and 14 carried the TT genotype.

Maternal care received

There was a significant effect of genotype on the amount of maternal care received. Pups carrying the CC genotype received the highest levels of maternal care, while pups carrying the TT genotype received the lowest levels of maternal care (KW = 6.94, $N = 48$, $p = 0.031$, Figure 4a).

Adult behavior and endocrine response to stress

Adult offspring carrying different genotypes of the *Fkbp5* gene (RS8161939) did not display different levels of activity in the locomotor activity box, EPM, or OFT. However, offspring carrying different SNPs of the *Fkbp5* gene showed significantly different endocrine responses to restraint stress. While CORT levels before and after restraint were relatively similar between offspring carrying different genotypes, CORT levels at peak stress differed as a function of genotype, with offspring carrying the TT genotype ($n = 5$) displaying a much more pronounced stress response to restraint than offspring with CC ($n = 5$) or CT ($n = 12$) genotypes (KW = 6.38, $N = 22$, $p = 0.041$, Figure 4b). Animals carrying the CC group did not show an elevated CORT response to stress compared to baseline ($p = 0.855$), whereas those in the CT and TT groups were elevated at 20 min relative to baseline ($ps < 0.05$). Two animals ($n = 1$ CC and $n = 1$ TT genotype) had insufficient serum for the corticosterone assay.

3.1.3 | Serotonin transporter

There were 24 offspring with the TC genotype and 24 offspring with the TT genotype in the cohort of 48 assessed for the RS8154473 SNP of the *5htt* gene.

Maternal care received

There was a significant effect of *5htt* genotype on total maternal care received by pups (MWU = 410, n1 = 24 n2 = 24; $p < 0.01$); pups with the TC genotype received significantly less maternal care than those with the TT allele (Figure 5a).

Adult behavior and endocrine response to stress

Offspring carrying different genotypes of the *5htt* gene did not behave differently in the locomotor activity box or the EPM. In the OFT, offspring carrying the TT genotype showed a significant positive correlation between maternal care received and % of total time spent in the center of the OFT ($r^2 = 0.302$, $p = 0.003$, $n = 13$; Figure 5b), whereas offspring with the TC genotype did not show a significant correlation between maternal care received and behavior in the open field. Offspring carrying different genotypes of the *5htt* gene did not display significantly different CORT responses to physical restraint.

3.2 | Study 2: Genotype, maternal care received, pup phenotype, and adult stress-related behavior

3.2.1 | Glucocorticoid receptor

We examined 56 female offspring for allelic polymorphisms in the *Nr3c1* gene in study 2. Of these, 54 female offspring carried the TT allele and only 2 offspring carried the TA allele at RS198873320. Due

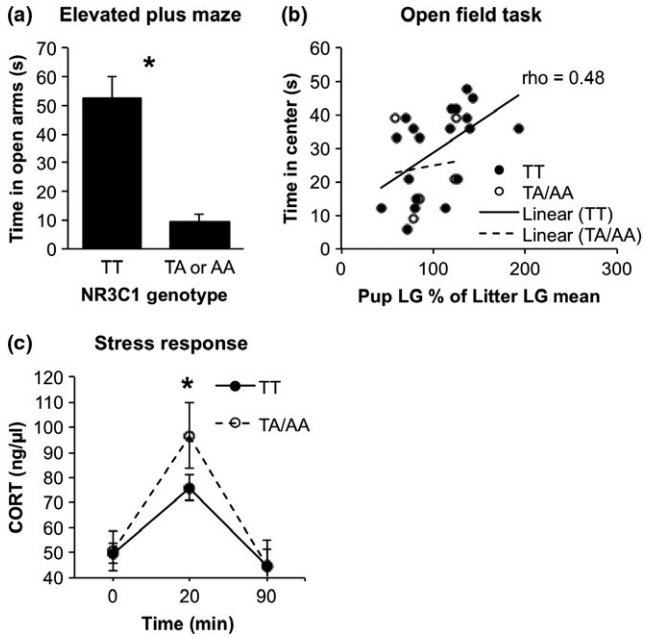

FIGURE 3 Glucocorticoid receptor genotype and anxiety in adulthood in Study 1. (a) Pups carrying the TT allele for *Nr3c1* spent significantly more time in the open arms of the EPM than animals carrying the AT or AA alleles. (b) Animals carrying the TT allele showed a significant positive correlation between differential LG levels received by pups and the time they spent in the center of the OFT while animals carrying the AT or AA alleles showed no significant correlation between maternal care and behavior in the OFT. (c) Animals carrying the TT allele had a significantly less active HPA axis in response to a restraint stressor than animals carrying the AT or AA alleles . *$p < 0.05$

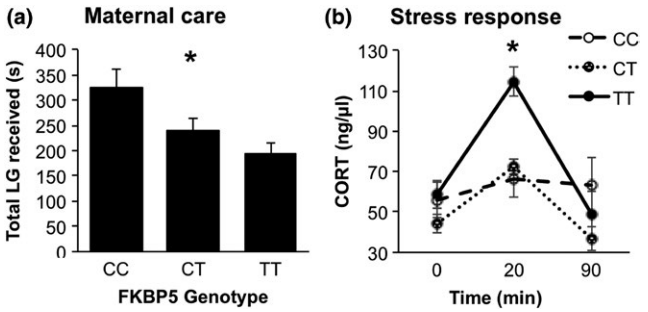

FIGURE 4 FK506-binding protein 5 genotype and anxiety in adulthood in Study 1. (a) There was a significant effect of genotype on the amount of LG that pups received. Animals carrying the CC genotype received the most LG while animals with the TT genotype received the least. (b) Variation of Fkbp5 genotype was associated with significantly different physiological responses to restraint stress. While CORT (ng/µl) levels immediately before and 90-min after the application of restraint were relatively similar between animals carrying different genotypes, animals carrying the TT genotype ($n = 5$) had a much more pronounced stress response to restraint than animals with the CC ($n = 5$) or CT ($n = 12$) genotypes. *$p < 0.05$

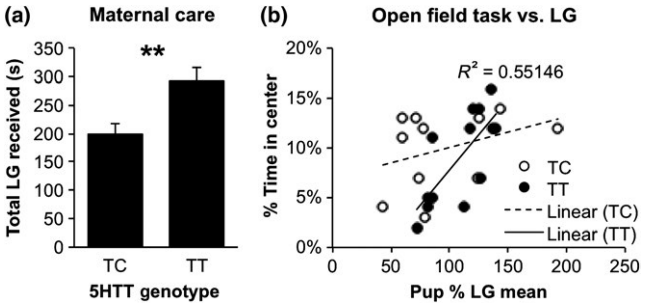

FIGURE 5 Serotonin transporter genotype and anxiety in adulthood in Study 1. (a) Pups with the TC genotype received significantly less maternal care than those with the TT allele. (b) In offspring carrying the TT but not the TC genotype, there was a significant positive correlation between pups' relative LG received and % of total time spent in the center of the OFT in adulthood. **$p < 0.01$

to the extremely small sample size of the TA group and the disproportionate distribution of allelic variations of the *Nr3c1* SNP in this cohort, we did not pursue further analysis of offspring phenotype in the context of this SNP.

3.2.2 | FK506-binding protein 5

Of 56 female offspring genotyped for allelic variation at RS8161939 in the *Fkbp5* gene in this cohort, 24 carried the CC allele, and 32 carried the CT allele.

Maternal care received

Pups carrying different alleles of the *Fkbp5* gene received significantly different levels of maternal care during the first 8 postnatal days; offspring carrying the CC genotype received significantly more maternal care than offspring carrying the CT genotype (MWU = 182, n1 = 24, n2 = 32, $p < 0.001$, Figure 6a).

Pup weight

We measured pup weights on PNDs 1, 5, and 10, and found that pups carrying the CC genotype weighed significantly less on PND 1 than pups carrying the CT genotype, (MWU = 562, n1 = 24, n2 = 32, $p = 0.003$; Figure 6b). Differences in pup weight between offspring carrying these different genotypes of *Fkbp5* were not different on PND5, PDN10, or the mean of the three time points (not shown).

Pup behavior

Pups carrying the two genotypes differed in their activity levels before, but not after, the coloring procedure when initially placed into the warm nest chamber (MWU = 221, n1 = 24, n2 = 32, $p = 0.007$; Figure 6c). Specifically, pups carrying the CC genotype were significantly more active than pups carrying the CT genotype when separated from their dams. However, in pup assessments during interactions with their dams in the nest, those with carrying the CC genotype showed lower levels of crawling behavior than those carrying the CT genotype (MWU = 531, n1 = 24, n2 = 32, $p = 0.015$; Figure 6d). Pups carrying different genotypes of *Fkbp5* did not differ in the number of ultrasonic vocalizations they emitted when separated from their dams and showed no other differences in behaviors that were measured in the nest.

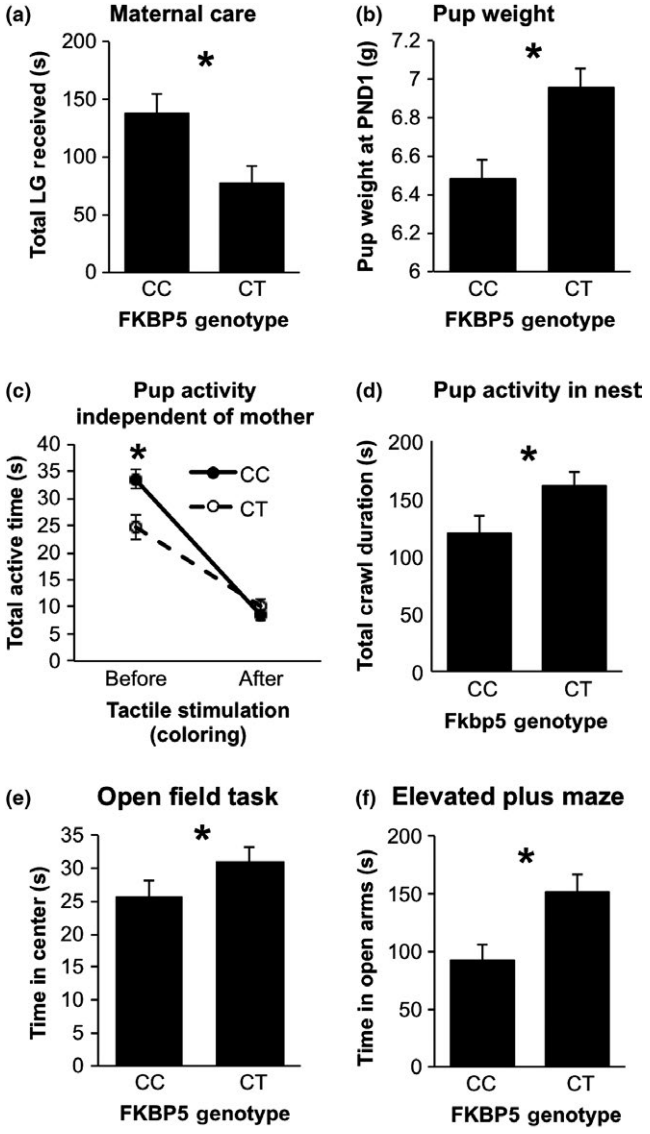

FIGURE 6 FK506-binding protein 5 genotype, neonatal pup profile and anxiety in adulthood in Study 2. (a) There was a significant effect of Fkbp5 genotype on the level of LG pups received; CC animals received significantly more LG than CT animals. (b) There was a significant effect of genotype on pup weights on PND1, but pup weights were not different on PNDs 5 or 10 (not shown). (c) When initially separated from their dams, pup activity was significantly higher in animals carrying CC than CT genotypes. (d) During interactions with their dams in the nest, pups carrying the CC genotype showed lower levels of crawling behavior than those carrying the CT genotype. (e) Adult offspring carrying the CC genotype spent significantly less time in the center of the OFT and (f) in the open arms of the EPM than adult offspring with the CT genotype. $^*p < 0.05$

Adult behavior

In adulthood, offspring carrying different genotypes of the *Fkbp5* gene did not differ in their adult locomotor activity. However, offspring carrying the CC genotype spent significantly less time than those carrying CT alleles in the center of the OFT (MWU = 500, n1 = 24, n2 = 32, $p = 0.055$; Figure 6e) and in the open arms of the EPM (MWU = 538, n1 = 24, n2 = 32, $p = 0.011$; Figure 6f).

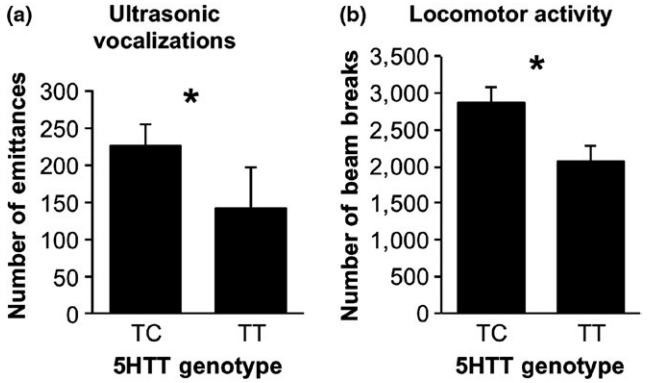

FIGURE 7 Serotonin transporter genotype, pup profile and adult activity in Study 2. (a) Animals with the TC genotype also emitted more ultrasonic vocalizations than animals carrying the CC genotype. (b) Animals with the carrying the TC genotype were significantly more active in the locomotor activity test in adulthood. $^*p < 0.05$

3.2.3 | Serotonin transporter

There were 45 offspring that carried the TC genotype and 11 offspring that carried the TT genotype for RS8154473 of the *5htt* gene.

5.1.1. Maternal care received

Pups carrying different genotypes of the serotonin transporter did not receive different levels of maternal care.

5.1.1. Pup weight

Pups carrying different genotypes of the serotonin transporter did not show different preweaning body weights.

Pup behavior

Pups carrying different genotypes did not display different levels of activity before or after tactile stimulation associated with the coloring procedure, nor did they display differences in behavior in the nest during interactions with their dams. However, pups carrying the TC genotype emitted significantly more USVs when separated from their dams than pups carrying the TT genotype (MWU = 155, n1 = 45, n2 = 11, $p = 0.055$, Figure 7a).

Adult behavior

In adulthood, offspring carrying the TC genotype were significantly more active than animals with the TT genotype in the locomotor activity test (MWU = 145, n1 = 45, n2 = 11, $p = 0.035$, Figure 7b). However, offspring carrying different genotypes did not show different behaviors in the EPM or the OFT.

4 | DISCUSSION

To our knowledge, this is the first study to examine the relationship between naturally occurring genetic variation and early licking experience on offspring phenotypes in rodents. Both genotype

and maternal care were associated with differential behavioral responses in the early postnatal period as well as stress-related behavioral responses in adulthood. This study provides an important analysis of the effects of maternal behavior and genotypic variation on behavior in female rodents, which are understudied compared to males (Becker, Prendergast, & Liang, 2016; Beery & Zucker, 2011), though some responses are likely to be sexually dimorphic (Beery & Kaufer, 2015; Taylor et al., 2000).

4.1 | Glucocorticoid receptor

We examined four polymorphic gene variants in the *Nr3c1* gene; three variants in the 3' untranslated region and one variant in the *Nr3c1* promoter region 2 kb upstream of the transcription start site. All four variants showed the same SNP distribution within female subjects. Information about haplotypes is unavailable for the Long-Evans strain and ancestral haplotypes for this strain do not exist; however, these data appear to indicate that the regions assessed are in linkage disequilibrium. Given the importance of rat models in behavioral studies (e.g., (Nestler, Gould, & Manji, 2002) and related efforts in epigenetic mapping (McGowan et al., 2011), comparative analyses with large-scale DNA sequencing data from outbred strains bred for quantitative genetic studies of psychological phenotypes are needed (see: (Ellenbroek & Youn, 2016). Female offspring from Study 1, but not Study 2, showed genotypic variation at the *Nr3c1* locus, with 10/48 (~21%) of female offspring carrying at least one copy of the A allele and 38/48 of female offspring carrying the TT genotype at RS198873320 in the *Nr3c1* promoter. TT offspring showed significantly reduced anxiety in the OFT with increasing levels of maternal care. Overall, TT offspring also showed decreased stress-related behavior in the EPM and lower endocrine stress reactivity in response to physical restraint than offspring carrying the A allele.

Although this study is the first to examine the association between natural variations in *Nr3c1* genotype and behavior in rodents, a number of findings in humans have indicated that genetic variations in *Nr3c1* contribute to modifications of the endocrine stress response (de Kloet, Derijk, & Meijer, 2007; DeRijk & de Kloet, 2008). For example, several polymorphisms in the 3' untranslated region of human *Nr3c1* have been described that destabilize mRNA, altering levels of GR transcription. Other polymorphisms have been associated with differences in the function of the HPA axis and stress-related behavioral responses. These include a variant in the *Nr3c1* promoter region that associated with higher levels of circulating cortisol (Rosmond et al., 2000), although it is not clear whether other SNPs within the same haplotype contribute to the effect (DeRijk & de Kloet, 2008). Other work has indicated that cognitive tasks performed during a stressor (public speaking and mental arithmetic; (Wüst et al., 2004) and disease states characterized by altered HPA function (e.g., myalgic encephalomyelitis/chronic fatigue syndrome; (Rajeevan et al., 2007)) may be at least partly explained by variation in *Nr3c1* genotype (DeRijk & de Kloet, 2008). At this time, we do not know whether genetic variation in regulatory elements of the *Nr3c1* locus modifies GR activity by, for example, altering mRNA stability, as described previously in humans (R H Derijk et al., 2001). However, our findings in rodents are consistent with these previous studies, and provide evidence that polymorphic variations in the *Nr3c1* locus in rodents likely contribute to endocrine responses and stress-related behavior in adulthood in female rats.

4.2 | FK506-binding protein

In both studies, female offspring carrying at least one copy of the T allele of *Fkbp5* received less maternal care compared to CC homozygotes. CC offspring also showed lower body weight at PND1, increased postnatal activity upon initial separation from their dams and decreased activity in the nest compared to animals carrying the T allele. Taken together, these results may indicate that female rat offspring carrying the CC genotype interact with dams in a way that encourages the dams provide them with more maternal care, possibly through a higher level of postnatal activity when not in physical contact with their dams. It is possible that altered maternal interaction among CC offspring is at least partly responsible for a catch-up in growth, as differences in body weight between the genotypes normalized by the first 5 postnatal days. Future studies should examine home orientation, which was shown to be a sensitive measure of developmental deficits in rat pups (Fischer et al., 2016).

In adulthood, differences in locomotor activity between genotypes were no longer apparent. Differential stress-related behaviors in adult offspring carrying the T allele in Study 1 were also not present; however, TT homozygotes, who had received the lowest levels of maternal care, displayed the most robust increase in CORT in response to restraint stress. We are unsure why offspring carrying the CC genotype did not mount a significant CORT response to restraint stress relative to baseline levels. However, we note that these offspring continued to show relatively high levels of CORT at the 90-min recovery period compared to CT or TT offspring, though this difference failed to reach significance. Replication of these findings in a larger cohort may help elucidate the nature of this response in CC homozygotes. In Study 2, CC homozygotes spent less time in the center of the OFT and in the open arms of the EPM, suggesting increased anxiety, even though they received the most LG from dams. Of note, while higher overall levels of maternal care in a litter have consistently been associated with lower levels of anxiety behavior in males (Curley & Champagne, 2016), the results of a limited number of studies assessing individual pup-directed care have been more equivocal (Cavigelli et al., 2010; Pan et al., 2014; Ragan, Harding, & Lonstein, 2016). It is interesting to note that the loss of *Fkbp5* by genetic deletion does not appear to alter locomotor activity or stress-related behavior in adult mice, however exposure to acute stressors alters the HPA response to stress in these animals (Touma et al., 2011). As such, the influence of *Fkbp5* variation on anxiety-related behavior may depend on the degree to which the task is stressful, though this would not appear to explain the discrepancy in the relationship between *Fkbp5* genotype and anxiety behavior observed between Study 1 and Study 2. It is also possible

that the effect of *Fkbp5* variation is dependent upon genetic variation in other as yet unknown genetic loci or additive or interactive effects of SNPs. In short, we cannot easily explain the differences in "emotional responsivity" in relation to Fkbp5 across the two studies based on the methodological differences. In both studies, however, performance on the task was sensitive to variations in this SNP.

Our data showing genotype effects on stress reactivity in female rodents support findings from human studies, where genetic variation of *Fkbp5* was associated with cortisol reactivity in the Trier Stress Test (Ising et al., 2008). Other work in humans has provided evidence that gene-environment interactions between SNPs of *Fkbp5* and severity of childhood abuse predict symptoms of post-traumatic stress disorder (Binder et al., 2008). Our findings of differential activity and stress-related behavior as function of *Fkbp* genotype may indicate an association between *Fkbp5* genotype and stress-related behavior in the absence of pathology. Interestingly, both our data from female rats and those from human studies suggest that modifications in the 3' untranslated region (RS8161939 in our study and rs3800373 in humans (Lukic et al., 2015) contribute to these effects, indicating a potential role for regulation of FKBP5 mRNA in these effects, perhaps through processes involved in alternative splicing events. Functional genomic studies of the effects of these variations are needed to test this hypothesis.

4.3 | Serotonin transporter

In Study 1, TT homozygotes received higher levels of maternal care than TC heterozygous offspring. This difference in maternal care was associated with reduced arm entries in the EPM and a positive and significant correlation between the amount of LG received and the time they spent in the center of the OFT. However, genotypic variation did not replicate in Study 2, though genetic variation in *5htt* was also associated with differential levels of USVs in early life, and differential levels of locomotor activity in adulthood. These results are perhaps not surprising given that *5htt* genotype has been shown to affect GR, MR, FKBP5 activity as well as DNA methylation in the CRF gene, all thought to underlie the expression of anxiety behavior (van der Doelen, Calabrese, et al., 2014; van der Doelen, Deschamps, et al., 2014; 2015). Taken together, our findings suggest that the influence of higher levels of maternal care received on offspring anxiety behavior from early life throughout adulthood depend upon *5htt* genotype.

5 | CONCLUSION

In this study, we investigated the impact of genetic variation in *Nr3c1*, *Fkbp5*, and *5htt* on female offspring in two independent cohorts. We observed an association between LG received and time in center of the OFT for offspring carrying select genotypes of *Nr3c1* and *5htt* in Study 1. In both cases, there was no overall difference in the time in the center of the OFT between genotypes, but only one genotype showed an association between LG received and time in center. In the case of the *5htt*, the genotype that received the highest levels of maternal care overall (TT homozygotes) showed an association between LG and time in center of the OFT. In comparing the effects of polymorphic variations examined in *Fkbp5* and *5htt*, the *direction* of the relationship between the absolute levels of maternal care received and later life stress-related outcomes depended on genotype. Our data suggest that these effects may, at least to some extent, be affected by alterations in the behavior of pups, including activity and USVs, in early life. Collectively, the impact of allelic variation of these genes depended on maternal care received by offspring individually overall and relative to their sibling, and affected stress-related behaviors in offspring during the early postnatal period and in adulthood.

It has long been known that the response to stress is stressor-specific (Jean Kant et al., 1985). The CORT response to stress also critically depends upon the timing and duration of the stressor (McGowan & Matthews, 2018). Stressors such as restraint or footshock stress have been used to quantitatively assess these parameters, which can be manipulated in a highly controlled manner in laboratory settings. However, these stressors are limited in their ability to inform on challenges that animals face in natural settings. Alternatively, chronic social defeat stress has helped elucidate the significant contribution of HPA dysfunction in development to anxiety-like phenotypes in female mice (Schmidt et al., 2010). In addition, stressors present over the evolutionary life history of the animal, such as cues that predict the threat of predation, are known to recruit specific endocrine and behavioral systems in response to the stressor (St-Cyr & McGowan, 2018). The use of these paradigms in future studies may be informative in understanding the impact of early life social interactions on responses to stress throughout the lifespan.

There are noted sex-specific responses to stress that are sensitive to differences in maternal care early in life. For example, neonatal rats show sex-dependent effects of maternal deprivation on responses to stressors (Viveros et al., 2009). In addition, organizational effects of steroid hormone exposure interact with later life changes in sex steroid levels, particularly in adolescence when sex differences in behaviors linked to affective disorders emerge (Bale & Epperson, 2015; McCormick & Mathews, 2007). Thus, given the preponderance of evidence that sex steroids influence HPA axis activity, there are likely sex differences in the manner in which genes such as GR, FKBP5, and 5HTT modulate stress-related responses, though these remain to be examined.

This study had several limitations. The sample size in this study limited our ability to identify the range of variation in genotypes and to examine possible gene–gene interactions. Larger sample sizes would also allow investigations not only the role of within-litter variation (the focus of this paper and others (Pan et al., 2014; van Hasselt et al., 2012)), but the contribution of the dam to variation in pup behavior, which has been the focus of a larger number of studies (e.g., Meaney, 2001). In addition, the coloring procedures necessitated handling the animals. We also note that although ear notching is standard practice in our laboratory, it is possible that this differentially affected pups' behavior as a function of the level of licking

received. Measuring maternal behavior once per day has been used in many other studies (Afonso, King, Chatterjee, & Fleming, 2009; Lovic et al., 2013; Pan et al., 2014) and all our observations were performed in the light phase of the cycle. In future studies, it would be informative to examine possible diurnal changes in mothering and additional measures of offspring behavior.

We previously reported seemingly "protective" effects of genetic variation in the dopamine system in a model of maternal neglect. For instance, Lovic et al (2013) found a significantly higher dopamine D2 receptor expression in isolated (vs. mother-reared rats) if they also possessed one SNP variant of the dopamine D2 receptor gene but not if they possessed the other, possibly explaining why there are significant individual differences in the impact of early life adversity on dopamine-dependent processes. The data reported here indicate that naturally occurring genetic variation, along with previously reported epigenetic variation (Pan et al., 2014), may constitute a mechanism permitting phenotypic plasticity in early life that is mediated by the maternal context. Viewed from an evolutionary perspective (Beery & Francis, 2011; Frankenhuis & Del Giudice, 2012), it is probable that this form of early life plasticity can confer either protective or pathological consequences for offspring phenotype depending on specific environmental conditions. The study of genetic variation in dams and pups, as well as detailed analysis of their behaviors during the postpartum period will aid in elucidating mechanisms involved in these complex interactions.

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