Received: 26 December 2023 | Revised: 14 February 2024 | Accepted: 5 March 2024

DOI: 10.1111/gbb.12892

ORIGINAL ARTICLE

Genes, Brain and Behavior

Chd8 haploinsufficiency impacts rearing experience in C57BL/6 mice

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Funding information
National Institutes of Health, Grant/Award Number: R21MH118685; National Science Foundation, Grant/Award Number: DBI2011039; The Saban Research Institute Research Career Development Fellowship; Developmental Neuroscience and Neurogenetics Program

Abstract

Mutations in *CHD8* are one of the highest genetic risk factors for autism spectrum disorder. Studies in mice that investigate underlying mechanisms have shown *Chd8* haploinsufficient mice display some trait disruptions that mimic clinical phenotypes, although inconsistencies have been reported in some traits across different models on the same strain background. One source of variation across studies may be the impact of *Chd8* haploinsufficiency on maternal-offspring interactions. While differences in maternal care as a function of *Chd8* genotype have not been studied directly, a previous study showed that pup survival was reduced when reared by *Chd8* heterozygous dams compared with wild-type (WT) dams, suggesting altered maternal care as a function of *Chd8* genotype. Through systematic observation of the C57BL/6 strain, we first determined the impact of *Chd8* haploinsufficiency in the offspring on WT maternal care frequencies across preweaning development. We next determined the impact of maternal *Chd8* haploinsufficiency on pup care. Compared with litters with all WT offspring, WT dams exhibited less frequent maternal behaviors toward litters consisting of offspring with mixed *Chd8* genotypes, particularly during postnatal week 1. Dam *Chd8* haploinsufficiency decreased litter survival and increased active maternal care also during postnatal week 1. Determining the impact of *Chd8* haploinsufficiency on early life experiences provides an important foundation for interpreting offspring outcomes and determining mechanisms that underlie heterogeneous phenotypes.

KEYWORDS
autism, *CHD8* haploinsufficiency, development, early life experience, individual differences, macrocephaly, maternal care, trait heterogeneity

1 | INTRODUCTION

CHD8 haploinsufficiency is highly penetrant for an autism spectrum disorder (ASD) diagnosis and macrocephaly.[1–3] Between impacted individuals, however, there is heterogeneity in the occurrence and severity of highly penetrant trait and co-occurring trait disruptions, including intellectual disability, anxiety and attentional problems, due to *Chd8* haploinsufficiency. Pre-clinical research in mouse models of *Chd8* haploinsufficiency has been conducted almost entirely in a single inbred genetic background strain. The studies have showed variable trait disruptions that mimic those observed in humans, including macrocephaly and atypical social behaviors.[4–11] A recent study leveraged genetic reference panels, which vary genetic background in a controlled fashion, to investigate the impact of *Chd8*

haploinsufficiency on clinically relevant traits across 33 strains.[12] The study showed that genetic background impacts the manifestation of trait disruptions due to *Chd8* haploinsufficiency and accurately replicated the heterogeneity of reported clinical phenotypes compared with single strain studies. In addition to genetic mutations, offspring phenotypes also can be influenced by early experiences, including those that occur during rapid development, prior to weaning, in which maternal care occurs.[13–19] Thus, additional potential contributors to variable phenotypic outcomes due to *Chd8* haploinsufficiency across single strain studies and between genetically diverse strain backgrounds are reciprocal maternal-offspring and sibling interactions.

Maternal-offspring interactions are a primary source of sensory and social experience during early development, particularly in rodent models in which variability of other sources of sensory and social experience are highly controlled. Variation in maternal care contributes to development of the offspring and can have profound effects on trait outcomes of the offspring across species.[16,20–22] For example, in rats, increased frequency of pup licking and grooming and arched-back nursing by the dam is associated with reduced stress-evoked behaviors in the adult offspring, including reduced HPA responses to stress, compared with pups that received low levels.[22] Thus, pup sensory stimulation and optimal nursing impacts the development of brain and behavioral responses to stress. Moreover, variability in the frequency, duration and pattern of dam behaviors can be induced by limiting bedding and nesting material in a rodent model of early life adversity.[23–25] Limiting the dam's ability to nest induces variability in maternal behavior, particularly by reducing the frequency and patterns of nest exits.[23–25] This variability in maternal care disrupts offspring physical growth as well as cognitive and emotional behaviors and neurochemical systems.[26–29] As variation in maternal care can impact the development of the brain and behaviors of offspring, it is important to determine if there are differences in maternal behaviors during offspring development in models of neurodevelopmental disorders in order to fully ascertain underlying mechanisms.

Chd8 haploinsufficiency typically is examined using the C57BL/6 (B6) strain, with different studies reporting varying inheritance of the mutation for the offspring from the dam or sire. Thus, maternal inheritance results in pups being reared by a dam with *Chd8* haploinsufficiency, in contrast to paternal inheritance in which the offspring are reared by a wild-type (WT) dam. A previous study found reduced litter size at postnatal day (P) 2, but not at birth, in B6 mice that were reared by a dam with *Chd8* haploinsufficiency compared with litters reared by WT dams with *Chd8* haploinsufficient sires or with WT sires.[10] This suggests that the maternal care of *Chd8* haploinsufficient dams may be reduced, which could contribute to differences in pup survivability and litter size at P2. Maternal care was not directly quantified, and thus, it is currently unknown whether *Chd8* haploinsufficiency impacts maternal care and equally important, whether *Chd8* haploinsufficiency in the offspring litter impacts maternal care. Both of these variables may have consequences on offspring development and trait outcomes and may therefore help to inform discrepancies between studies as well as factors contributing to individual differences in phenotypes. In the present study, we sought to evaluate additional maternal behaviors that may contribute to differences in pup survival and development in B6 models of *Chd8* haploinsufficiency. We used an experimental strategy of systematic litter observation to determine if *Chd8* heterozygosity (*Chd8*$^{+/-}$) impacts maternal behavior in the B6 genetic background. The studies focused on maternal behavior similarities and differences between *Chd8*$^{+/-}$ and WT dams toward litters with *Chd8*$^{+/-}$ and WT offspring. Using quantitative observational analyses of care in the home cages, the first study determined if litters comprised of WT and *Chd8*$^{+/-}$ offspring impact maternal behaviors in B6 WT dams. Maternal behaviors of B6 WT dams that reared litters with only WT offspring were compared with B6 WT dams that reared litters with both WT and Chd8$^{+/-}$ offspring. The second study determined if *Chd8* haploinsufficiency impacts maternal behavior toward litters comprised of WT and *Chd8*$^{+/-}$ offspring. Maternal behaviors of B6- *Chd8*$^{+/-}$ dams were compared with B6 WT dams.

2 | METHODS

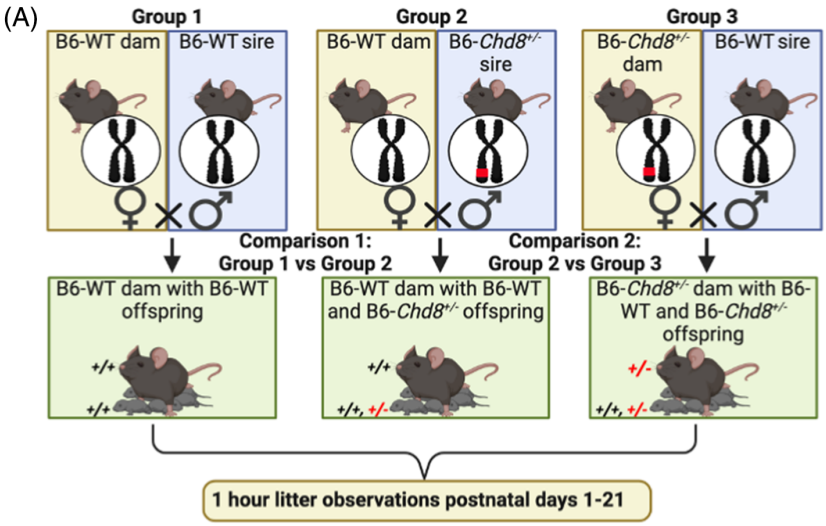
2.1 | Animals

Mice were house in the Ray R. Irani vivarium at the University of Southern California (USC) main campus from 2019 to 2021. Mice were housed in standard ventilated cages on a 12 h light/dark cycle (lights on at 6:00 AM) in a temperature (20–22°C) and humidity (40%–60%) controlled room with unrestricted access to standard rodent chow and filtered water. C57BL/6J (B6) mice that were heterozygous for *Chd8* (*Chd8*$^{+/-}$) were originally received from Dr. Feng Zhang (MIT). This B6-*Chd8* mouse line was generated through Cas9-mediated germline editing followed by germline transmission and inheritance. *Chd8*$^{+/-}$ mice in this study are descendants from one founder with germline transmission of a loss-of-function *Chd8* allele containing a 7-nucleotide deletion in exon 1, resulting in a 50% reduction in CHD8 protein expression at embryonic day 18 compared with WT littermates.[5] *Chd8*$^{+/-}$ mice were bred with C57BL/6J mice, all obtained from The Jackson Laboratory, to construct the groups detailed in Figure 1. Notably, the sire was removed from the home cage prior to litter births to control for individual differences in paternal care. Breeding pairs were checked daily for signs of pregnancy and sires were removed from breeding cages approximately 1 week prior to litter births. All mice were weaned at P21 and genotyped for *Chd8* with validated in-house genotyping protocols using approximately 1–1.5 mm long tail snips collected at weaning. All experimental procedures were approved by the USC institutional Animal Care and Use Committee under protocol 11844-CR011. In addition, all experimental procedures followed the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health.

2.2 | Litter observations

Pregnant dams were checked daily for litter births. The first day a litter was born was designated as P0 and the litter observations were conducted during P1-21. Each day of litter observations consisted of recording 12 snap shots of behavior conducted at 5-min intervals over

(A)



(B)

Number of litters observed each postnatal day (P)

Experimental group	Offspring Chd8 genotype	Dam Chd8 genotype	Sire Chd8 genotype	Total # of litters	Total # of observations	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21
Group 1: B6-WT x B6-WT	WT	WT	WT	30	185	14	13	12	11	10	5	3	5	8	2	9	8	5	10	14	7	10	13	11	6	9
Group 2: B6-WT x B6-*Chd8*$^{+/-}$	WT & Het	WT	Het	27	219	7	7	8	7	5	8	17	13	10	7	14	11	13	13	14	8	13	11	11	13	9
Group 3: B6-*Chd8*$^{+/-}$ x B6-WT	WT & Het	Het	WT	24	148	15	8	5	7	6	4	7	12	8	5	6	8	8	11	12	9	4	6	6	7	8

(C)

Number of litters included in means & mean ages

Experimental group	P1-21				PNW 1				PNW 2				PNW 3			
	N	Mean P	SD	Mode P	N	Mean P	SD	Mode P	N	Mean P	SD	Mode P	N	Mean P	SD	Mode P
Group 1: B6-WT x B6-WT	11	10.9	0.9	12	11	3.3	0.4	5	5	12.1	0.9	3	13	17.5	1.4	5
Group 2: B6-WT x B6-*Chd8*$^{+/-}$	10	10.3	1.6	11	9	3.6	1.3	5	15	11.1	1.2	4	14	18.0	0.6	4
Group 3: B6-*Chd8*$^{+/-}$ x B6-WT	8	10.9	1.1	15	8	3.5	1.0	5	11	10.9	0.8	5	10	17.7	0.7	5

FIGURE 1 Study design and groups: (A) Study design and experimental groups defined by mating strategies. In Group 1, B6-WT females mated with B6-WT males and produced all WT offspring. In Group 2, B6-WT dams were paired with B6-Chd8$^{+/-}$ sires to produce litters with B6-WT and Chd8$^{+/-}$ male and female progeny. To determine if litters containing *Chd8*$^{+/-}$ offspring impacts B6-WT dam behavior, B6-WT dam behaviors in Group 2 were compared with the behaviors of B6-WT dams in Group 1 (Comparison 1). In Group 3, B6-*Chd8*$^{+/-}$ dams were paired with B6-WT males to produce litters with B6-WT and Chd8$^{+/-}$ male and female progeny. To test if *Chd8* haploinsufficiency impacts maternal behaviors, B6-*Chd8*$^{+/-}$ dam behaviors from Group 3 were compared with B6-WT dams in Group 2 (Comparison 2). (B) The number of litters and number of observations are listed by experimental groups across postnatal days (P). (C) The number of litters (N) that met the inclusion criteria for analyses for group differences in mean behaviors during P1-21 and for each postnatal week (PNW) are specified per group in addition to the mean postnatal day (Mean P) and standard deviation (SD) as well as the mode for the number of postnatal days observed per litter within each time bin (Mode P).

a 1-h period and was based on established literature that shows multiple brief observation periods are sufficient to identify differences in rodent maternal care over preweaning development.[20,30–34] During each spot check, a researcher would observe a snapshot of the dam behaviors at that moment and record tally marks corresponding to observed behaviors in a laboratory notebook which was later transferred to an Excel document for analysis. Cages with litters were transferred from the main colony to standard tables and racks in a quiet behavioral suite, allowed to acclimate in the suite for 1 h prior to litter observations by the research observer with an unobstructed view of all sides of each cage. We were unable to observe cages in the housing room because the housing rack obstructed views of maternal behaviors. Multiple cages were observed per day and cage order was counterbalanced between days. All litter observations were conducted during the light cycle between 7:00 AM and 10:00 AM. The research observer was blind to the *Chd8* genotypes of the dam and litters during litter observations. Dam behaviors observed included passive nursing, active nursing, licking and grooming, nest building, out of the nest, resting, running and eating frequencies.[30–34] Passive nursing consisted of the dam resting with pups located underneath the dam. Active nursing included pups located ventral to the dam while the dam was engaged in an active behavior such as licking and grooming, eating, drinking, autogrooming and nest building. Passive and active nursing frequencies were also summed to analyze group differences in total nursing frequencies. Litters from all experimental groups were observed across P1-21 and only primiparous dam behaviors were included in analyses.

Litter composition variables were also measured at weaning and included litter size (i.e., the number of pups per litter), the percentage of males per litter, and the percentage of *Chd8*$^{+/-}$ offspring per litter.

Litter size and the percentage of males was calculated during the weaning of each litter and the percentage of *Chd8*$^{+/-}$ offspring per litter was calculated after genotyping tail samples harvested at weaning. Litter composition variables were measured at weaning because handling of pups preweaning adds an experimental variable that may impact maternal behaviors differently across experimental groups. We also did not genotype preweaning because this requires pup separation from the dam and tail clipping, which can stress the pups and dam.

2.3 | Statistical analyses

All statistical analyses were performed using SPSS Statistics software (IBM). Statistical significance was set at $p < 0.05$. Tally marks totaling spot checks per behavior were used for data analysis. Dam behavior was observed from all experimental groups for each day from after birth until weaning at P21 and this data is presented in the figures of dam behaviors across P1-21. Analyses that tested group differences in behaviors across P1-21 included average behavioral frequencies for litters that had at least 2 postnatal day observations during each postnatal week (PNW), to meet enough litters for statistical analyses. Analyses that tested group differences in behaviors across PNWs included average frequencies of dam behaviors per litter for litters that had at least three PND observations per PNW. The total number of observations as well as the number of litters included in the analyses of specific postnatal periods are listed in Figure 1.

Group differences in the percent of litters that survived to weaning were analyzed with Chi-squared tests. Group differences in litter composition variables were analyzed by one-way ANOVA. Group differences in mean dam behaviors and principal component scores were analyzed with linear mixed effects models with experimental group as a fixed factor and litter size, the percentage of males per litter and the percentage of *Chd8*$^{+/-}$ pups per litter covaried. Spearman's correlation coefficients were calculated from litter means across each postnatal week. Principal component analyses were conducted on observation frequencies standardized trait values (*z*-scored) with a rotated component matrix with Varimax rotation and Kaiser normalization. Regression scores were calculated based on each principal component. Cohen's *D* effect sizes were calculated by subtracting the mean trait values between groups and then dividing by the pooled standard deviation (SD). Cohen's *D* effect sizes were calculated as follows: (Mean1-Mean2)/√(((n1-1)*SD1^2 + (n2-1)*SD2^2)/(n1 + n2-2)). Figures and graphs were constructed with SPSS, Prism and BioRender.

3 | RESULTS

3.1 | Comparison 1: Impact of *Chd8* haploinsufficiency in the litter on WT-B6 maternal care

To determine if B6-WT maternal behavior is altered toward litters composed of all WT pups versus WT and *Chd8*$^{+/-}$ pups, differences in litter compositions and dam behavioral frequencies were compared between B6-WT dams that mated with B6-WT sires and gave birth to all WT litters versus B6-WT dams that mated with B6-*Chd8*$^{+/-}$ sires and gave birth to litters with WT and *Chd8*$^{+/-}$ pups (Figure 1A; Comparison 1). The number of litters observed during each day and the number of litters included in each analysis of time across groups are listed in Figure 1B,C. Notably, the average age during each time bin was comparable across groups and there were enough litters per group and a sufficient number of postnatal days observed for each litter for analyses across time bins (Figure 1C).

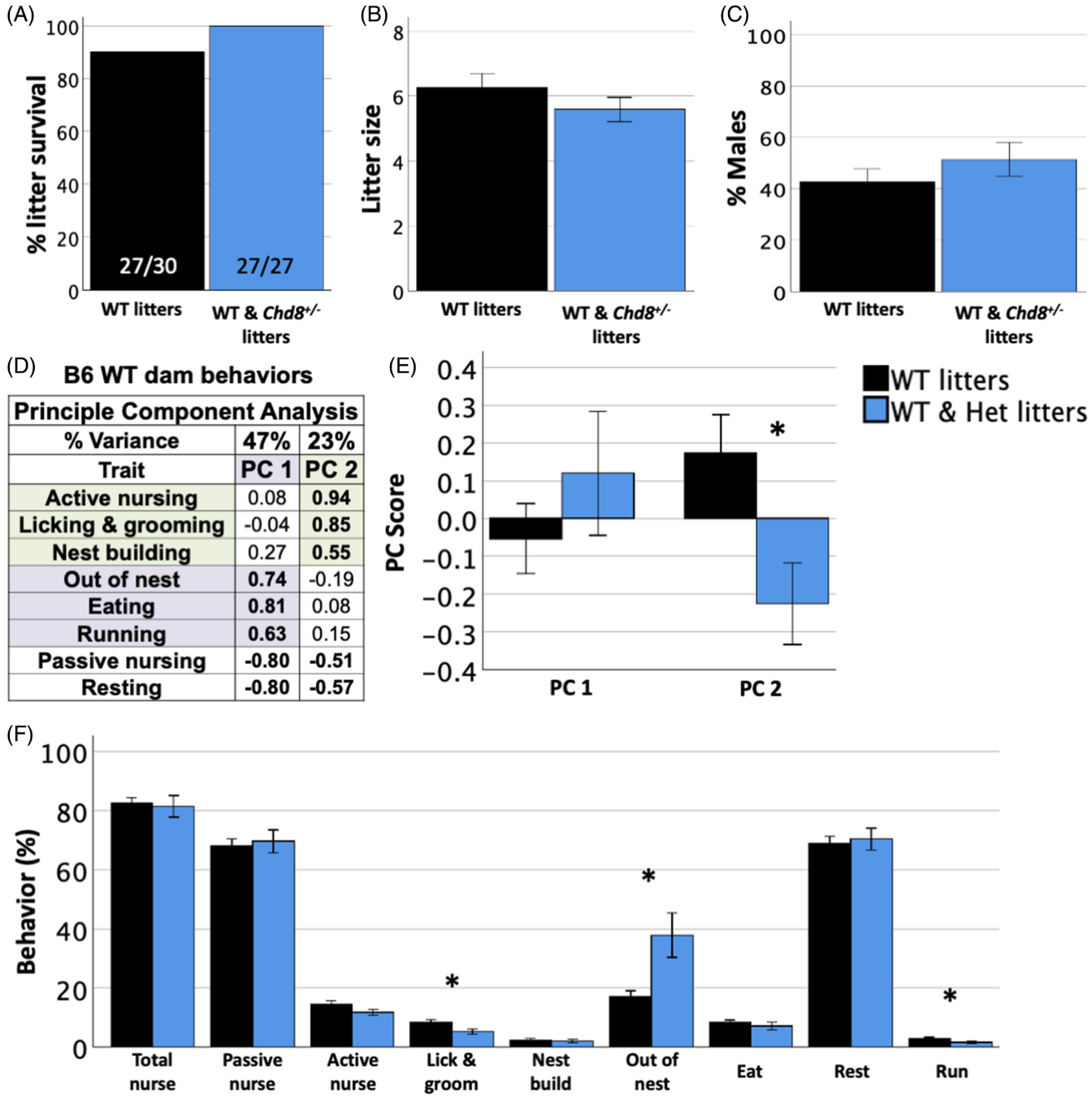
Chd8 haploinsufficiency in the litter did not impact the percentage of litters born to B6-WT dams that survived to weaning at P21 (Figure 2A). There were no differences in the mean number of pups per litter and in the percentage of males per litter, measured at P21, between litters with all WT pups compared with litters with mixed genotypes (Figure 2B,C).

A principal component analysis (PCA) was used to reduce eight behavioral frequencies of dams observed across P1-21 to meaningful factors that capture most of the behavioral variance. Two principal components (PCs) accounted for 70% of the total variance in behavior (Figure 2D). Kaiser–Meyer–Olkin Measure of Sampling Adequacy was 0.75. Out of the nest, eating and running frequencies loaded highly (i.e., >0.4) on PC 1 while active maternal care behaviors loaded highly on PC 2 including active nursing, licking and grooming, and nest building. B6-WT dams with WT and *Chd8*$^{+/-}$ pups had decreased mean PC 2 scores compared with B6-WT dams with only WT pups across P1-21, with a large effect size ($F_{1,19} = 6.381$, $p < 0.05$; $d = -1.1$; Figure 2E). There were no significant group differences in PC 1 scores. Thus, the data indicate that mixed *Chd8* genotype litters appear to selectively decrease active maternal care behaviors in B6-WT dams.

We next tested for differences in the mean frequencies of each behavior observed across P1-21. B6-WT dams with WT and *Chd8*$^{+/-}$ pups had decreased licking and grooming ($F_{1,19} = 5.818$, $p < 0.05$; $d = -1.0$), and running ($F_{1,19} = 5.291$, $p < 0.05$; $d = -0.9$) frequencies, and increased out of the nest frequencies ($F_{1,17.306} = 8.570$, $p < 0.01$; $d = 1.2$) compared with B6-WT dams with WT pups (Figure 2F). There were no differences in total nursing, passive nursing, active nursing, nest building, resting and eating frequency means across P1-21. Effect sizes of mean differences between B6-WT dam behaviors toward litters with mixed *Chd8* offspring compared with all WT offspring across P1-21 were large with the largest effect size in out of the nest.

Because the collection of dam behaviors changes over time prior to weaning, we next binned the data into separate postnatal weeks (PNW) 1–3 and correlations between litter compositions and dam behaviors as well as differences in PC scores and behavioral frequencies were determined for each PNW.

Litter size correlated with many dam behaviors depending on the *Chd8* genotype status of the litter starting at PNW2 and most prevalent at PNW3 (Figure S1). The data indicate that dam behaviors reflect more sensitivity to litter size during later PNWs and that litter size correlates with differences in the behaviors of B6-WT dams with WT and *Chd8*$^{+/-}$ offspring compared with B6-WT dams with only WT offspring.



Principle Component Analysis		
% Variance	47%	23%
Trait	PC 1	PC 2
Active nursing	0.08	**0.94**
Licking & grooming	-0.04	**0.85**
Nest building	0.27	**0.55**
Out of nest	**0.74**	-0.19
Eating	**0.81**	0.08
Running	**0.63**	0.15
Passive nursing	**-0.80**	**-0.51**
Resting	**-0.80**	**-0.57**

FIGURE 2 *Chd8* haploinsufficiency in the litter impacts B6 WT maternal care: (A) There were no differences in the percentage of litters that survived to weaning between litters with all WT pups compared with litters with WT and *Chd8*$^{+/-}$ pups. (B-C) The mean litter size (B) and the percentage of males per litter (C), both measured at weaning, did not differ between B6 litters comprised of only WT offspring compared with litters with WT and *Chd8*$^{+/-}$ offspring. (D) A principal component analysis on B6-WT dam behaviors showed two principal components (PCs) that accounted for 70% of the total variance. Bold text indicates dam behavior variables that loaded highly on each PC (i.e., >0.4). PC 1 comprised of high loadings of out of the nest behavior, including running and eating. In the nest and pup-directed behaviors, including nursing, nestbuilding and licking and grooming, loaded highly on PC 2. (E) PC 1 and PC 2 scores were calculated from respective loadings. B6-WT dams with WT and B6-*Chd8*$^{+/-}$ offspring (blue bars) had decreased mean PC 2 scores compared with B6-WT dams with WT litters (black bars) across P1-21. (F) During the P1-21 period, B6-WT dams with WT and B6-*Chd8*$^{+/-}$ offspring had decreased licking and grooming and running percent behavioral frequencies and increased out of the nest frequencies compared with B6-WT dams with WT litters. Bar graphs report group means and error bars represent plus/minus standard error of the mean. *≤0.05, LMM, litter size and percentage of males per litter covaried.

There were no differences in B6-WT dams PC 1 scores across PNWs (Figure 3A). B6-WT dams with litters containing WT and *Chd8*$^{+/-}$ offspring had decreased PC 2 scores ($F_{1,18} = 7.578$, $p < 0.05$; $d = -1.2$) during PNW 1, with a large effect size (Figure 3B). B6-WT dams with mixed genotype litters had reduced active nursing ($F_{1,16.718} = 6.763$, $p < 0.05$; $d = -1.1$; Figure 3E) and licking and

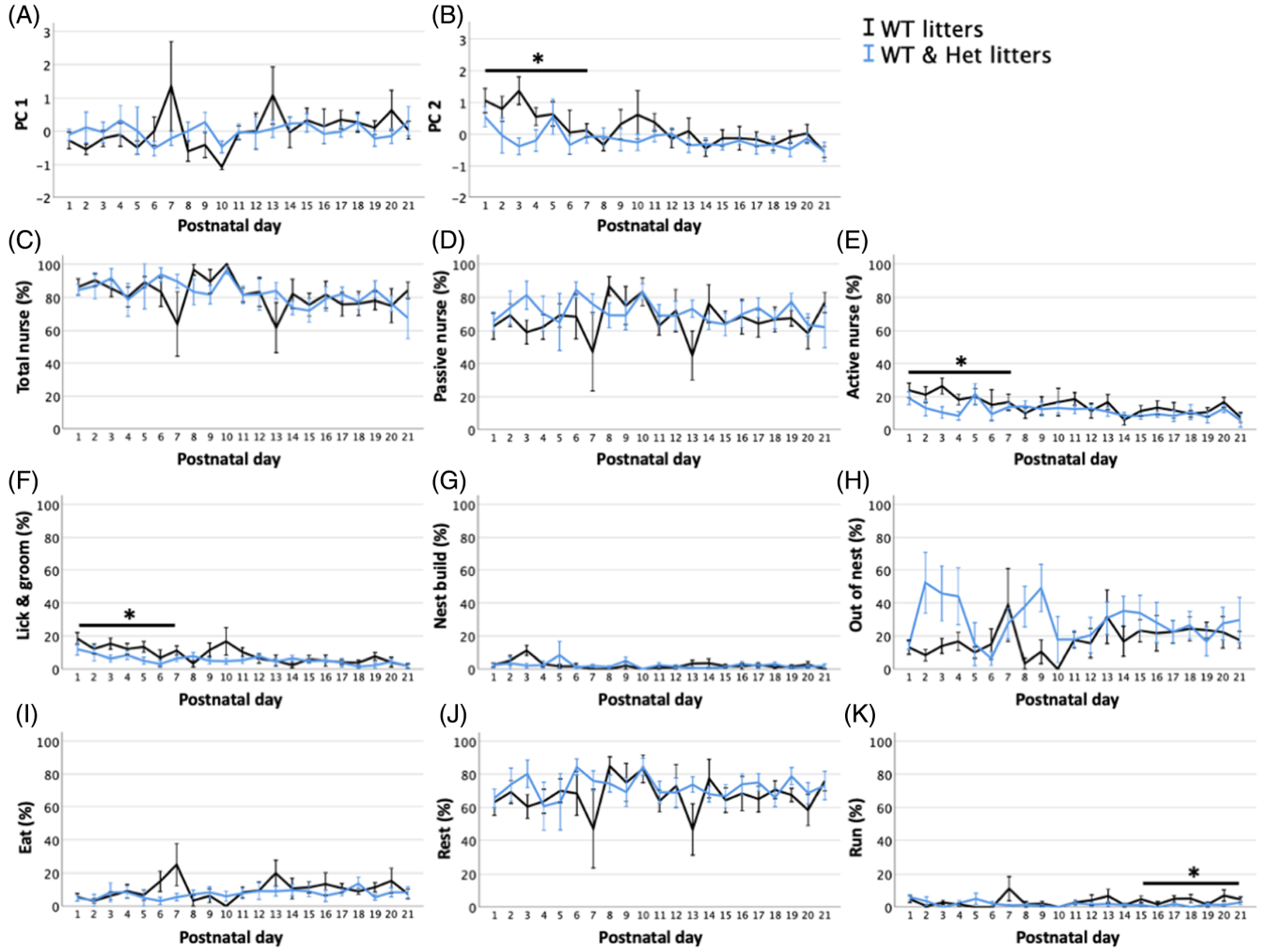


FIGURE 3 *Chd8* haploinsufficiency in the litter impacts B6 WT maternal care uniquely across postnatal weeks: Line graphs show B6-WT dams mean principal component (PC) scores (A, B) and percent behavioral frequencies (C–K) across each postnatal day for WT and *Chd8*$^{+/-}$ litters (blue lines) and WT litters (black lines). B6-WT dams with WT and *Chd8*$^{+/-}$ offspring had reduced PC 2 scores (B), active nursing (E) and licking and grooming frequencies (F) during PNW 1 and reduced running frequencies during PNW 3 (K) compared with B6-WT dams with only WT offspring. Lines and asterisks over line graphs represent significant differences between dam groups during PNWs 1, 2 and 3. Error bars represent plus/minus standard error of the mean. *≤0.05, LMM, litter size and percentage of males per litter covaried.

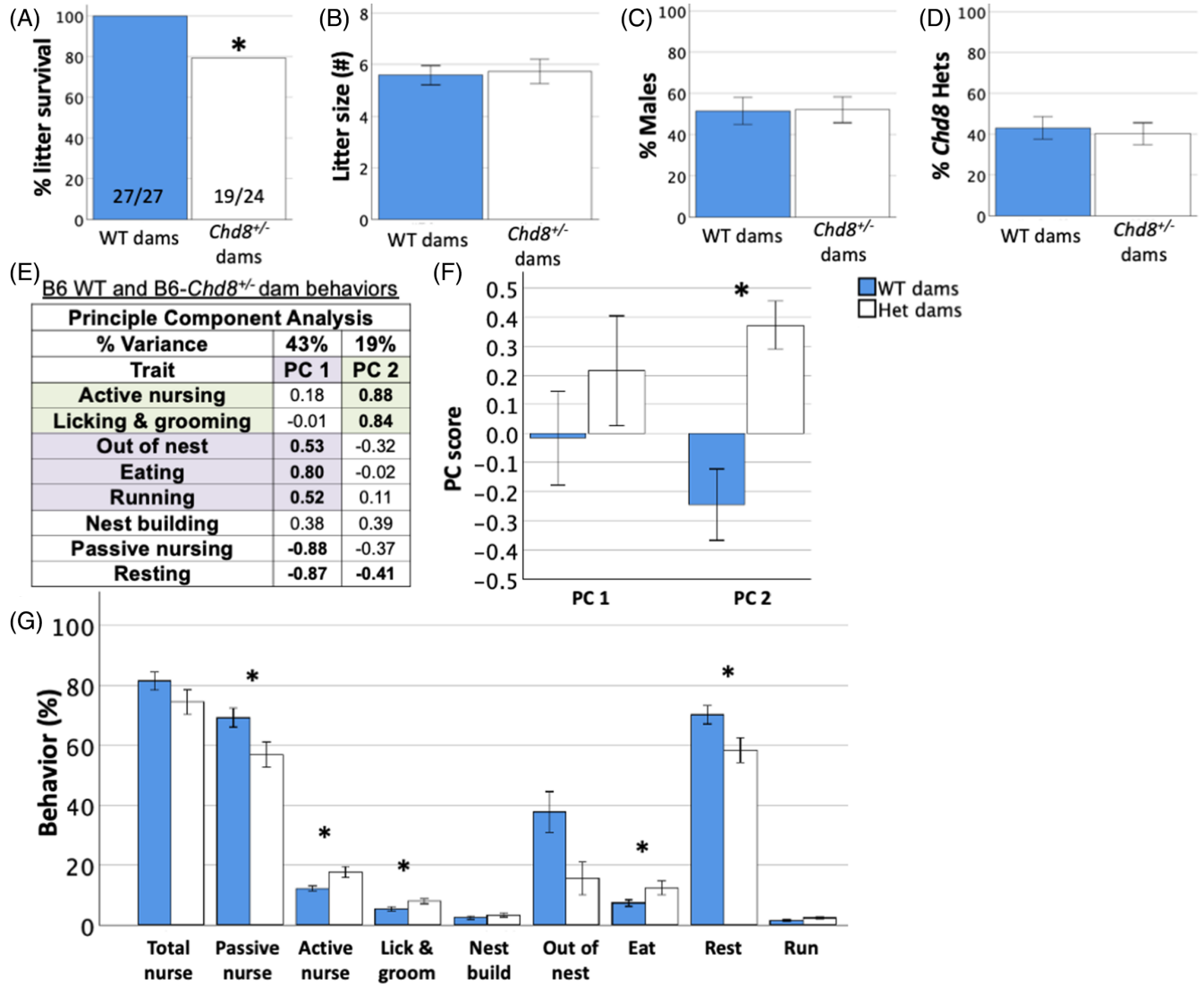
grooming ($F_{1,18} = 7.441$, $p < 0.05$; $d = -1.2$; Figure 3F) frequencies during PNW 1 as well as reduced running frequencies during PNW 3 ($F_{1,24.014} = 18.656$, $p < 0.001$; $d = -1.4$; Figure 3K), compared with B6-dams with only WT offspring. There were no differences in the mean percentage of behavioral frequencies between B6-WT dams mean total nursing, passive nursing, nest building, out of the nest, eating and resting during each PNW (Figure 3C,D,G–J). The data indicate that B6-WT maternal care behavior during PNW1 is more sensitive to *Chd8* haploinsufficiency in the litter than as the pups age prior to weaning.

3.2 | Comparison 2: Impact of *Chd8* haploinsufficiency on maternal behavior

To determine whether *Chd8* haploinsufficiency impacts B6 maternal care toward litters with B6 WT and *Chd8*$^{+/-}$ pups, maternal behavior frequencies were compared between B6-WT dams and B6-*Chd8*$^{+/-}$ dams that had mated with B6-*Chd8*$^{+/-}$ and B6-WT sires, respectively (Figure 1A; Comparison 2).

Chd8$^{+/-}$ dams had significantly fewer litters that survived to weaning compared with WT dams ($X^2_{1,46} = 6.236$, $p < 0.05$; Figure 4A). In those litters that survived to weaning, there were no differences in the mean number of pups, the percentage of males, and the percentage of *Chd8*$^{+/-}$ pups at weaning between litters born to B6-WT and B6-*Chd8*$^{+/-}$ dams (Figure 4B–D).

Next, a PCA was performed on B6-WT and B6-*Chd8*$^{+/-}$ dam behaviors observed across P1-21. Two PCs, comprised of the same measure variables as in Comparison 1, accounted for 62% of the total variance in dam behavioral frequencies (Figure 4E). Kaiser–Meyer–Olkin Measure of Sampling Adequacy was 0.73. PC 1 represented dam-centered behaviors and included high loadings (i.e., < 0.4) for out of the nest, eating and running frequencies, which were negatively related to passive nursing and resting. PC 2 represented active



Principle Component Analysis		
% Variance	43%	19%
Trait	PC 1	PC 2
Active nursing	0.18	**0.88**
Licking & grooming	-0.01	**0.84**
Out of nest	**0.53**	-0.32
Eating	**0.80**	-0.02
Running	**0.52**	0.11
Nest building	0.38	0.39
Passive nursing	**-0.88**	-0.37
Resting	**-0.87**	**-0.41**

FIGURE 4 *Chd8* haploinsufficiency impacts B6 maternal care: (A) B6-*Chd8*$^{+/-}$ dams (white bars) had fewer number of litters that survived to weaning compared with B6-WT dams (blue bars). (B–D) The mean litter size, percentage of males and the percentage of *Chd8*$^{+/-}$ offspring per litter did not differ between B6-WT and B6-*Chd8*$^{+/-}$ dams that reared litters with WT and *Chd8*$^{+/-}$ offspring. (E) A principal component analysis on B6-WT and B6-*Chd8*$^{+/-}$ dam behavior showed two principal components (PCs) that accounted for 62% of the total variance. Bold text indicates dam variables that loaded highly (i.e., >0.4) on each PC. (F) PC 1 and PC 2 scores were calculated from respective loadings. B6-*Chd8*$^{+/-}$ dams had increased PC 2 scores across P1-21 compared with B6-WT dams. (G) Over P1-21, B6-*Chd8*$^{+/-}$ dams had decreased passive nursing and resting percent behavioral frequencies compared with B6-WT dams and increased active nursing, licking and grooming and eating frequencies. Bar graphs show means and error bars represent plus/minus standard error the mean. *$p < 0.05$, LMM, litter size, percentage of males and percentage of *Chd8*$^{+/-}$ offspring per litter covaried.

maternal care behaviors including high loadings for active nursing and licking and grooming which were more modestly negatively related to resting frequencies. There were no differences in PC 1 scores between B6-WT and B6-*Chd8*$^{+/-}$ dams (Figure 4F). B6-*Chd8*$^{+/-}$ dams had increased PC 2 scores compared with B6-WT dams ($F_{1,16}$ = 14.267, $p < 0.01$; $d = 1.9$; Figure 4F), with a large effect size for behavioral differences in maternal care.

The percent frequency of dam behaviors averaged across P1-21 were each analyzed. B6-Chd8$^{+/-}$ dams had decreased passive nursing ($F_{1,16} = 5.785$, $p < 0.05$; $d = -1.1$) and resting ($F_{1,16} = 5.461$, $p < 0.05$; $d = -1.0$) percent frequencies compared with B6 WT dams, with large effect sizes (Figure 4G). B6-Chd8$^{+/-}$ dams also had increased active nursing ($F_{1,16} = 9.403$, $p < 0.01$; $d = 1.4$), licking and grooming ($F_{1,16} = 6.132$, $p < 0.05$; $d = 1.1$), and eating ($F_{1,16} = 4.728$, $p < 0.05$; $d = 0.9$) frequencies compared with B6-WT dams, with large effect sizes (Figure 4G). There were no differences in total nursing, nest building and running frequencies between B6-*Chd8*$^{+/-}$ dams and B6 WT dams. There was a trend for B6-Chd8$^{+/-}$ dams to leave the nest less frequently on average across P1-21 compared with B6-WT dams ($p = 0.075$; Figure 4G).

As for Comparison 1, differences in the correlation between dam behaviors and litter composition variables and mean differences in PC

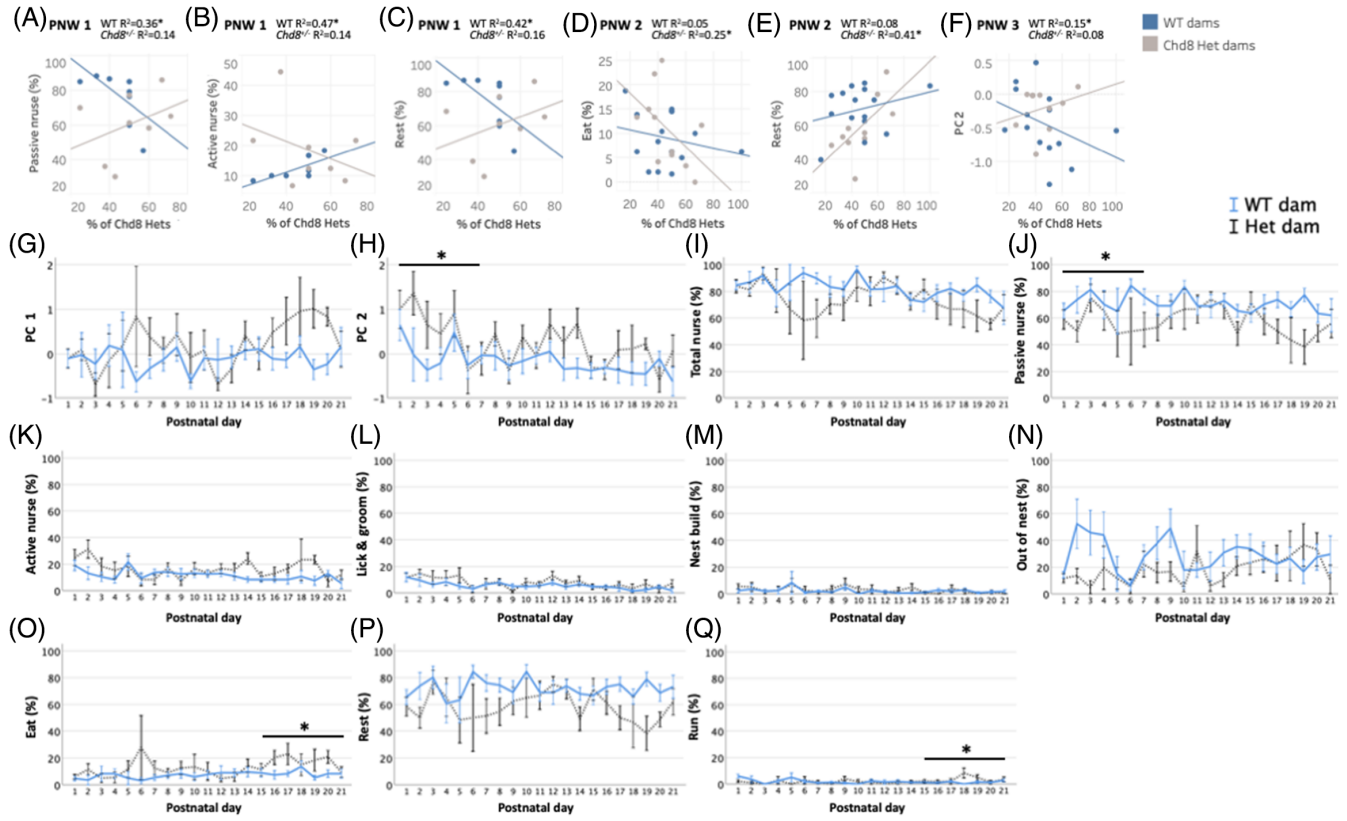


FIGURE 5 *Chd8* haploinsufficiency impacts B6 maternal care uniquely across postnatal weeks: (A–C) During postnatal week (PNW) 1, the percentage of *Chd8*$^{+/-}$ offspring per litter significantly correlated with B6-WT dams (blue circles) passive nursing (A), active nursing (B) and resting (C) frequencies, but not B6-*Chd8*$^{+/-}$ dams (gray circles). (D, E) During PNW 2, the percentage of *Chd8*$^{+/-}$ offspring per litter correlated with B6-*Chd8*$^{+/-}$ dams eating and resting behavioral frequencies, but not B6-WT dams. (F) During PNW 3, the percentage of *Chd8*$^{+/-}$ offspring per litter negatively correlated with B6-WT dams PC 2 scores, but not B6-*Chd8*$^{+/-}$ dams. (G–Q) Line graphs show mean PC scores (G, H) and mean dam behavioral frequencies (I–Q) across each postnatal day for B6-WT (blue lines) and B6-*Chd8*$^{+/-}$ (broken black lines) dams. B6-*Chd8*$^{+/-}$ dams had increased PC 2 scores (H) and decreased passive nursing percent frequencies (J) during PNW 1, compared with B6-WT dams. In addition, B6-*Chd8*$^{+/-}$ dams had increased eating (O) and running (Q) during PNW 3, compared with B6-WT dams. There were no differences in total nursing (I), active nursing (K), licking and grooming (L), nest building (M), out of nest (N) and resting (P) percent frequencies between B6-WT and B6-*Chd8*$^{+/-}$ dams during each PNW. Lines and asterisks over line graphs represent significant differences between dam groups during PNWs 1, 2 and 3. Error bars represent plus/minus standard error of the mean. *$p < 0.05$, LMM, litter size, percentage of males and percentage of *Chd8*$^{+/-}$ offspring per litter covaried.

scores and the percentage of dam behavioral frequencies were analyzed for each PNW bin during preweaning development.

Litter size was associated with increased eating frequencies during PNW 1 and PC 1 scores during PNW 2 in B6-*Chd8*$^{+/-}$ dams, but not in B6-WT dams (Figure S2). In B6-WT dams, litter size was associated with reduced licking and grooming at PNW 2. During PNW 3, litter size was associated with increased active nursing in B6-*Chd8*$^{+/-}$ dams but decreased total nursing and increased out of the nest in B6-WT dams (Figure S2). These data indicate that litter size impacts B6-WT and B6-*Chd8*$^{+/-}$ dams behavior differently across a subset of behaviors measured depending on the PNW. In addition, the percentage of males per litter was associated with reduced resting and increased nest building frequencies in B6-*Chd8*$^{+/-}$ dams at PNW 2, but not in B6-WT dams (Figure S2). This suggests that B6-*Chd8*$^{+/-}$ dam behavior may be more sensitive to the percentage of males per litter compared with B6-WT dams.

The percentage of *Chd8*$^{+/-}$ offspring per litter was associated with increased active nursing and decreased passive nursing and resting frequencies in B6-WT dams, but not B6- *Chd8*$^{+/-}$ dams during PNW 1 (Figure 5A–C). In contrast, for B6-*Chd8*$^{+/-}$ dams, the percentage of *Chd8*$^{+/-}$ offspring per litter was associated with increased resting and decreased eating during PNW 2 (Figure 5D,E). Lastly, the percentage of *Chd8*$^{+/-}$ offspring per litter was associated with decreased PC 2 scores in B6-WT dams at PNW 3 (Figure 5F). These data suggest that the percentage of *Chd8*$^{+/-}$ offspring per litter differentially impacts B6-WT and B6-*Chd8*$^{+/-}$ dam behaviors depending on the developmental period of maternal care.

Analyzing the data over each PNW revealed that B6-*Chd8*$^{+/-}$ dams had increased PC 2 scores during PNW 1 ($F_{1,15} = 6.617$, $p < 0.05$; $d = 1.2$), compared with B6-WT dams, with a large effect size (Figure 5H). There were no differences in PC 1 scores in B6-WT dams versus B6-*Chd8*$^{+/-}$ dams during each PNW (Figure 5G).

B6-*Chd8*$^{+/-}$ dams also had decreased passive nursing at PNW 1 ($F_{1,15} = 4.729$, $p < 0.05$; $d = -1.0$) compared with B6-WT dams (Figure 5J). In contrast, B6-*Chd8*$^{+/-}$ dams were observed to eat ($F_{1,22} = 5.181$, $p < 0.05$; $d = 0.9$), and run ($F_{1,25} = 8.412$, $p < 0.01$; $d = 0.9$) more frequently during PNW 3, compared with B6-WT dams, also with large effect sizes (Figure 5O,Q). Distinct from WT B6 mice, these data suggest that during PNWs 1 and 3, different aspects of dam-centered and maternal care behaviors are sensitive to *Chd8* haploinsufficiency.

4 | DISCUSSION

To better understand the contributing factors underlying trait disruptions due to mutations in the high-confidence autism risk gene, *CHD8*, we measured maternal behavior as a factor of offspring genotype and maternal genotype. We found that *Chd8* haploinsufficiency in the litter and the dam each contributed to differences in maternal behaviors and therefore early life experiences of the offspring. These differences may impact the development of offspring and susceptibility to outcomes in *Chd8* haploinsufficiency.

Here, in Comparison 1, we show that the presence of *Chd8* haploinsufficient offspring in the litter impacts B6-WT maternal care across development. B6-WT dams engaged in active maternal care behaviors less frequently toward litters with mixed *Chd8* genotypes compared with WT litters, including reduced PC 2 scores, and licking and grooming, particularly during PNW 1 but also on average across P1-21, with large effect sizes (Figure 6A). In addition, B6-WT dams with *Chd8* offspring also had increased out of the nest frequencies on average across P1-21, with a large effect size (Figure 6A). Differences in frequencies and the pattern of dam exits have been reported in mouse models of early life adversity and have been associated with the development of the brain and behaviors in the offspring.[24,25,35] Thus, future studies should further assess if increased nest exits in B6-WT dams with litters containing offspring with mixed *Chd8* genotypes, compared with all WT offspring, results in differences in susceptibility to trait disruptions due to *Chd8* haploinsufficiency.

It is unclear whether there are specific stimuli derived from the offspring that underlies the reduction in maternal behavior toward litters with mixed *Chd8* genotypes compared with litters with all WT offspring. Prior research has shown that B6-*Chd8* haploinsufficient male and female mice have reduced body weights at weaning and decreased locomotor behavior in adulthood, compared with WT mice.[12] In addition, B6-*Chd8*$^{+/-}$ males have increased social dominance whereas B6-*Chd8*$^{+/-}$ females have increased anxiety-like behavior in adulthood.[12] While it remains to be determined if these trait disruptions measured in adulthood exist during preweaning development, these differences in offspring behaviors may contribute to dam behavioral differences, potentially with a reciprocal impact on the pups. *Chd8* haploinsufficient male mice have been shown to emit more ultrasonic vocalizations upon brief separation from the dam, more quickly, and for longer durations compared with WT counterparts during the preweaning period.[4] Upon reunion with the dam, *Chd8* haploinsufficient male mice also spend more time with the dam, suggesting that male *Chd8* haploinsufficient offspring may already exhibit atypical social behaviors prior to weaning.[4] In addition, peer to peer interactions during development can impact adult sociability and neurochemical levels in mice.[16] Sibling interactions may be altered in litters comprised of all WT offspring versus litters with WT and *Chd8* haploinsufficient offspring and these differences may also contribute to maternal care variability across experimental groups. Furthermore, it is possible that factors other than offspring behavior may be driving the observed differences in dam behaviors such as differences in the placental and paternal *Chd8* genotype on priming maternal behavior.[36]

The reduction in B6-WT dam behaviors toward litters with WT and *Chd8*$^{+/-}$ offspring, compared with litters with only WT offspring,

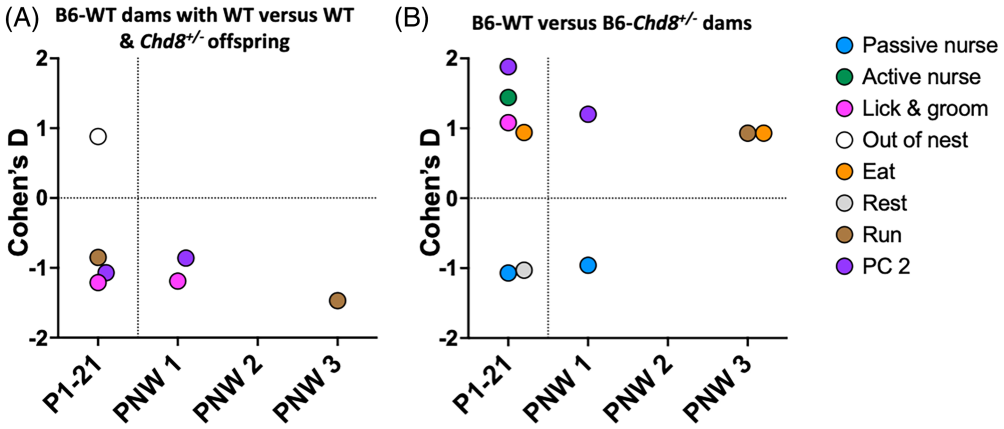


FIGURE 6 Summary of the impact of *Chd8* haploinsufficiency in the litter (A) and the dam (B) on B6 dam behaviors: (A, B) Cohen's *D* effect sizes were plotted for dam behavioral traits and principal component (PC) scores that significantly differed between groups. (A) Cohen's *D* effect sizes for significant trait differences between B6-WT dams that reared litters with all WT offspring compared with B6-WT dams that reared litters with WT and *Chd8*$^{+/-}$ offspring across postnatal day 1–21 (P1-21) and postnatal week (PNW) 1, PNW 2 and PNW 3. (B) Cohen's *D* effect sizes for significant trait differences between B6-WT dams and B6-*Chd8*$^{+/-}$ dams during P1-21 and PNW 1–3.

may be a contributing factor to the impact of *Chd8* haploinsufficiency on offspring outcomes. Most studies that investigate the impact of *Chd8* haploinsufficiency on trait outcomes use WT littermates to control for differences in maternal care. Yet, the data presented here show differences in B6-WT dam behaviors toward litters with mixed WT and *Chd8*$^{+/-}$ offspring, compared with only WT offspring. The altered maternal behaviors may contribute to trait disruption differences in the offspring in unexpected ways because differences in maternal care may impact offspring development and outcomes differently depending on the *Chd8* genotype of the offspring. It is unclear if maternal care differences are directed toward individual WT versus *Chd8*$^{+/-}$ offspring, but the percentage of *Chd8*$^{+/-}$ offspring per litter correlated with specific B6-WT dam behaviors, including passive and active nursing during PNW 1 and PC 2 scores during PNW 3.

Comparison 2 showed that maternal inheritance of *Chd8* haploinsufficiency, with offspring being reared by a B6-*Chd8*$^{+/-}$ dam, also results in large effect size differences in maternal care across development compared with paternal inheritance with rearing by a B6-WT dam (Figure 6B). Litter survival was reduced in *Chd8*$^{+/-}$ dams that bred with WT sires compared with WT dams that bred with *Chd8*$^{+/-}$ sires. Previous research has shown dams carrying mutations of neurodevelopmental risk genes impact pup survival in mixed genotype litters.[10,37,10,37] In a B6 mouse model of *Chd8* haploinsufficiency, a prior study found that pup survivability was reduced at P2 when litters were born to B6-*Chd8*$^{+/-}$ dams compared with litters born to WT dams with *Chd8* haploinsufficient sires or with WT sires.[10] Litter size at birth was not impacted.[10] We similarly found a decrease in pup survivability via a decrease in the percentage of litters that survived to weaning in litters born to *Chd8*$^{+/-}$ dams and WT sires compared with WT dams and *Chd8*$^{+/-}$ sires. We did not observe differences in litter size at weaning. Together, these data suggest that pup survival further decreases after P2 in litters born to B6-*Chd8*$^{+/-}$ dams and WT sires, compared with WT dams and B6-*Chd8*$^{+/-}$ sires, and that decreased pup survival in early postnatal days may predict a reduced likelihood of survival of the entire litter to weaning.

B6-*Chd8*$^{+/-}$ dams also had increased active maternal care behaviors compared with B6-WT dams, both with WT and *Chd8*$^{+/-}$ offspring. The increase in active maternal care may be a compensatory mechanism displayed by B6-*Chd8*$^{+/-}$ dams to potentially circumvent mechanisms underlying the increased risk for litter death. In addition, because B6-*Chd8*$^{+/-}$ females have increased anxiety-like behaviors in the bright open field task,[12] it is possible that increased active maternal behaviors and reduced passive nursing, observed in B6-*Chd8*$^{+/-}$ dams, is driven by differences in trait anxiety levels compared with B6-WT dams.

Non-maternal care behaviors also changed in B6-*Chd8*$^{+/-}$ dams compared with B6-WT dams including decreased resting and increased running and eating frequencies. We reported previously in this model that B6-*Chd8*$^{+/-}$ adult males and females have reduced locomotor activity compared with WT counterparts.[12] Moreover, B6-*Chd8*$^{+/-}$ males and females have reduced body weights from weaning to adulthood.[12] Thus, increased eating and running frequencies in B6-*Chd8*$^{+/-}$ dams, particularly during PNW 3, may be a compensatory mechanism for reduced body weights and activity in the dam and offspring during a time when offspring are beginning to eat solid food and increase exploratory activity, all under maternal guidance.

Our studies measured dam behaviors during the light phase of the diurnal cycle, which may minimize the potential differences observed across groups. It will be important to study group differences in dam behaviors as a function of maternal and offspring *Chd8* genotype during the dark phase, when mice are more active, to fully understand rearing experience differences which may contribute to adult outcomes in models of *Chd8* haploinsufficiency. Understanding how maternal behavior varies during both the night and day cycle is important for determining potential impact on offspring outcomes. Moreover, our studies measured snapshots of behavioral frequencies over 1 h per day observation periods. While this was sufficient to capture group differences in some dam behaviors with large effect sizes, group differences in behaviors that occur less frequently over specific postnatal weeks may have been missed. Future studies will provide additional insights by measuring the timing of behavioral frequencies and duration of dam behaviors to determine if maternal care is fragmented or differs in predictability across experimental groups. Longer and more detailed observation periods and analyses of dam behavioral differences over each postnatal day during preweaning development may provide additional contributing factors of early life experiences on susceptibility to *Chd8* haploinsufficiency.

5 | CONCLUSION

In a mouse model of *Chd8* haploinsufficiency, the detailed analyses show that both maternal and offspring *Chd8* genotype impacts maternal care, with large effect sizes (Figure 6A,B). Individuals with *CHD8* haploinsufficiency display heterogeneity in the penetrance, severity and expression of symptoms. It is therefore important to determine environmental and genetic contributions to this trait heterogeneity to fully understand underlying mechanisms. The current study does not imply that human maternal care is a factor in *CHD8* haploinsufficiency and ASD clinical impact. Rather, murine maternal care, as a key to pup development and one of the main sources of sensory experience for the offspring in mouse laboratory environments, is an example of an early environmental experience that can vary and may impact behavioral outcomes. Here, we suggest that genetic modifiers, as well as a variety of different environmental factors, are likely to combine with other risk factors to contribute to clinical heterogeneity for children with ASD.[38–40] Even for studies using basic research models of *Chd8* haploinsufficiency in the same genetic background strain, for which there is unprecedented control of the environment and genetic background, inconsistencies are reported in the impact of *Chd8* haploinsufficiency on phenotypes. Differences in maternal care due to maternal *Chd8* genotype may be one contributing factor to differences that have been reported across murine studies that vary maternal versus paternal inheritance of the mutation.[4,5,7–9,11] Moreover, it is important

to note that another level of variability across studies is the parental location of the mutation in the *Chd8* gene, which may also contribute to differences in reported phenotypes across studies. Future studies can aim to tease these factors apart by determining if there are differences in adult outcomes in *Chd8*$^{+/-}$ mice, compared with WT, that have been reared by a WT dam versus a *Chd8*$^{+/-}$ dam between litters with the same *Chd8* mutation. Most important, the differences described in dam behavior in this study result in different rearing experiences for the offspring and it will be critical to determine how these differences in early life experiences impact adult phenotypes and susceptibility to *Chd8* haploinsufficiency.

ACKNOWLEDGMENTS

The authors thank Amanda Whipple and Allison Knoll for their assistance with breeding and colony management. We are grateful to Allison Knoll for feedback on the experimental design and to Panteha Hayati Rezvan (The Saban Research Institute Biostatistic and Data Management Core) for feedback on data analyses and presentation. We also thank our funding sources: National Institute of Mental Health R21MH118685 (P.L. and A.K.), National Science Foundation Postdoctoral Research Fellowship in Biology DBI2011039 (M.T.), The Saban Research Institute Research Career Development Fellowship (M.T.), Simms Mann Chair in Developmental Neurogenetics (P.L.), Developmental Neuroscience and Neurogenetics Program, Children's Hospital Los Angeles and WM Keck Chair in Neurogenetics, Keck School of Medicine, University of Southern California (P.L.).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Mendeley at https://data.mendeley.com/, DOI: 10.17632/jcd8d8rkrk.2.

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How to cite this article: Tabbaa M, Levitt P. *Chd8* haploinsufficiency impacts rearing experience in C57BL/6 mice. *Genes, Brain and Behavior*. 2024;23(2):e12892. doi:10.1111/gbb.12892