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Mania- and anxiety-like behavior and impaired maternal care in female diacylglycerol kinase eta and iota double knockout mice

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Abstract

Genome-wide association studies linked diacylglycerol kinase eta and iota to mood disorders, including bipolar disorder and schizophrenia, and both genes are expressed throughout the brain. Here, we generated and behaviorally characterized female mice lacking *Dgkh* alone, *Dgki* alone, and double *Dgkh/Dgki*-knockout (dKO) mice. We found that fewer than 30% of newborn pups raised by dKO females survived to weaning, while over 85% of pups survived to weaning when raised by wild-type (WT) females. Poor survival under the care of dKO mothers was unrelated to pup genotype. Moreover, pups from dKO dams survived when fostered by WT dams, suggesting the poor survival rate of dKO-raised litters was related to impaired maternal care by dKO dams. Nest building was similar between WT and dKO dams; however, some dKO females failed to retrieve any pups in a retrieval assay. Pups raised by dKO dams had smaller or absent milk spots and reduced weight, indicative of impaired nursing. Unlike WT females, postpartum dKO females showed erratic, panicked responses to cage disturbances. Virgin dKO females showed behavioral signs of anxiety and mania, which were not seen in mice lacking either *Dgkh* or *Dgki* alone. Our research indicates that combined deletion of *Dgkh* and *Dgki* impairs maternal behavior in the early postpartum period, and suggests female dKO mice model symptoms of mania and anxiety.

KEYWORDS

anxiety, bipolar disorder, Dgkh, Dgki, diacylglycerol kinase, female mice, knockout mice, mania, maternal behavior, mouse behavior, postpartum

1 | INTRODUCTION

Diacylglycerol kinases play an important role in mammalian physiology by altering the activity of the substrate diacylglycerol (DAG) and other effectors that control cellular functions.[1,2] All 10 mammalian *Dgk* genes are expressed in the brain, each with a distinct regional expression pattern.[3,4] Many *Dgk* genes have been implicated in the regulation of neuron physiology and mouse behavior.[3-6] *Dgkb* deletion and *Dgkz* knockdown were found to reduce spine formation and maintenance, respectively, and *Dgkk* knockdown reduced spine maturation and stability.[7-9] *Dgkb*, *Dgkz*, *Dgki* and *Dgkk* have each been shown to regulate synaptic plasticity.[9-12] Alterations in *Dgkb*, *Dgkz*, *Dgke* or *Dgkk* coincided with differences in lipid levels in various neuronal tissues.[9,10,13,14] On the behavioral level, seizure susceptibility was enhanced by *Dgkd* knockdown and *Dgkb* knockout[15,16] and reduced by *Dgke* loss.[14] *Dgkb*$^{-/-}$ and *Dgkh*$^{-/-}$ male mice showed neurological phenotypes including hyperactivity and reduced depression,[7,17] while *Dgkk*-deficient mice showed fragile X syndrome-like social deficits and stereotypic behaviors.[9] Therefore, changing the balance of lipids in this network by targeting various *Dgk* genes appears to influence neurophysiology.

Diacylglycerol kinase eta (*DGKH*) and diacylglycerol kinase iota (*DGKI*) are linked to mental and cognitive disorders, including schizophrenia, bipolar disorder (BD), depression, attention deficit hyperactivity disorder and autism spectrum disorders.[18-23] Recently, researchers examined the function of DGKH in male mice.[17] Overall, they found that *Dgkh*-knockout males displayed mania-like behaviors that could be reversed with lithium treatment. Using *Dgki*-knockout mice,[24] others found that DGKI loss caused no changes in learning, anxiety or motor phenotypes other than a slightly diminished habituation to an open field.[12] Note that the sex of the mice in this latter study was not indicated.

Here, using a new *Dgkh* mutant line, we show that double knockout (dKO) females lacking expression of both *Dgkh* and *Dgki* have deficits in maternal care, resulting in poor offspring survival. The dKO females also show mania- and anxiety-like behaviors. These phenotypes were not seen in mice missing only *Dgkh* or *Dgki*. Overall, this work highlights the impact of disrupting both *Dgkh* and *Dgki* on mood-disorder-related phenotypes and on maternal behavior.

2 | MATERIALS AND METHODS

2.1 | Mice

All procedures used in this study were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. We generated a *Dgkh*-knockout mouse using CRISPR/Cas9 technology.[25] To arrest translation at the start of the first catalytic domain of DGKH (Figure 1A), we designed a guide RNA (GTGTTCGTCAACTCTAAGAGTGG) and a homology-directed repair donor template (TTTCGTTCTGTGTCAGCCCCCTCTTGGTGTTCGTC AACTCTAA**ATAAAGGTACCTAGGATAAATA**GAGTGGAGATAATCA-GGGAGTGAAGTTCCTTCGTCGCTTTAAA) to insert a three-frame stop cassette (bold) and *KpnI* site (bold underlined) in exon 9 of *Dgkh* (Figure 1B). Pronuclear injections of the guide RNA and donor template produced founder mice from which we cloned and sequenced *Dgkh* alleles to show successful integration of the stop cassette. *Dgkh*$^{-/-}$ mice present no gross anatomical or overt motor phenotypes.

We acquired a *Dgki*$^{-/-}$ mouse line described previously,[24] which was on a mixed 129 and C57BL/6 background. All data presented here from both the *Dgki*$^{-/-}$ and dKO lines were from mice that were backcrossed with C57BL/6J mice for at least five generations. *Dgkh*-/- mice were generated on a C57BL/6J background.

Mice were maintained on a 12 hours:12 hours light:dark cycle (lights on 7 AM to 7 PM) and given food (Teklad 2020X; Envigo, Huntingdon, UK) and water ad libitum. For maternal behavior experiments, timed matings were set up in the evening within 2 hours of the start of the dark cycle, using one male mouse and one or two female mice per breeding cage. The male mouse was separated from the female mice after 48 or 72 hours. Female mice were single-housed at least 1 week prior to giving birth. Pup retrieval experiments and observations of pup survival, weight, and milk spots were conducted in the evening within 3 hours of the start of the dark cycle. When handling preweanling mice, care was taken to rub gloves with used bedding from the home cage before touching the animals, particularly when removing pups for weighing or testing in the retrieval assay.

Because of the large number of animals required for the tests of psychopathological behaviors, the three genetic models (*Dgkh*$^{-/-}$, *Dgki*$^{-/-}$ and dKO) were tested in separate cohorts, each with an age-matched wild-type (WT) cohort. For the elevated plus maze, open field, forced swim test, and acoustic startle and prepulse inhibition

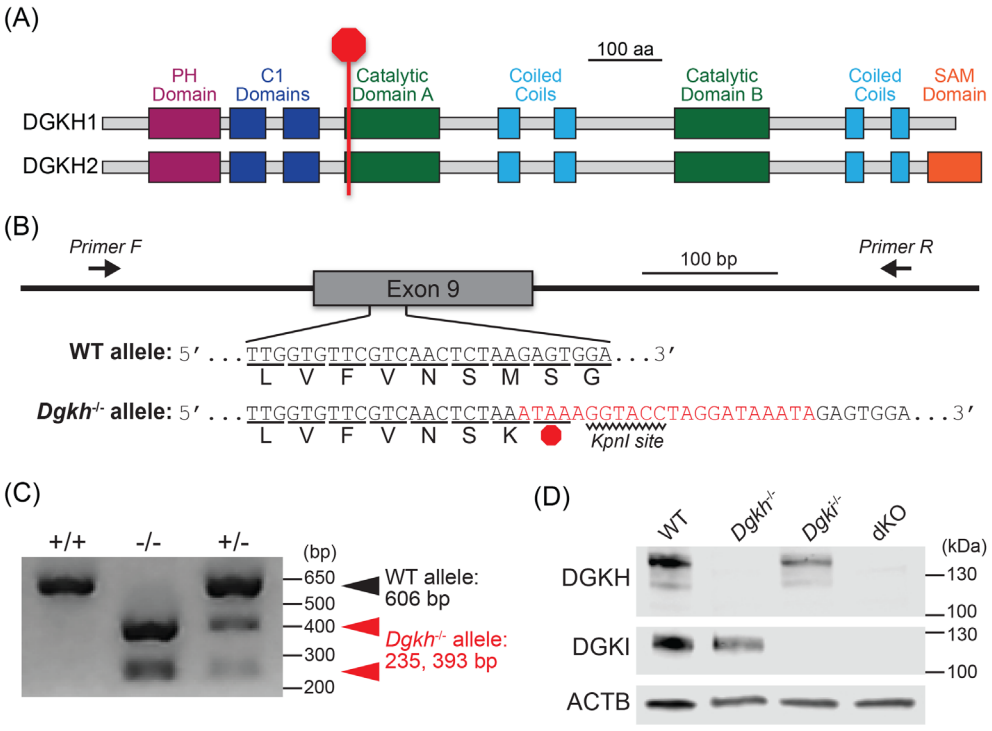

FIGURE 1 Generation of *Dgkh*-knockout mice using CRISPR/Cas9. A, Integration of a stop cassette arrests translation at the start of the first catalytic domain of DGKH. C1, cysteine-rich (diacylglycerol-binding); PH, pleckstrin homology; SAM, sterile alpha motif. B, The 22-base-pair cassette (red), containing a stop codon and *KpnI* restriction site, was inserted into Exon 9 of the *Dgkh* gene. C, PCR amplification of tail DNA and digestion with *KpnI*. Without *KpnI* digestion, the amplified fragment from the *Dgkh*$^{-/-}$ allele is 628 bp. D, Western blot using 30 μg of protein isolated from cerebral cortices of WT, *Dgkh*$^{-/-}$, *Dgki*$^{-/-}$ and dKO female mice

experiments (tested in that order), we tested 2- to 4-month-old virgin female mice during the second half of the light phase of their light: dark cycle. Mice were group-housed with three to five mice per cage. Mice were given at least 48 hours to recover after the elevated plus maze and open field assays, and at least 1 week to recover after the forced swim test. For these assays, 14 WT mice were tested with 14 *Dgkh*$^{-/-}$ mice; 15 WT mice were tested with 16 *Dgki*$^{-/-}$ mice; and 19 WT mice were tested with 19 dKO mice (only 14 mice of each genotype from the dKO cohort were tested in the acoustic startle and prepulse inhibition experiments). For the sucrose preference and sleep phenotyping experiments (tested in that order, separated by 1 week), we tested 2-month-old virgin female mice. The nine WT and eight dKO females were individually housed for both experiments; mice were group-housed (three to five mice per cage) before testing for sucrose preference and were again group-housed with their original cage mates for the 1 week before assessing sleep patterns. For each of these experiments, outliers were identified using the interquartile range (IQR = $Q_3 - Q_1$). Any data points that were 1.5 × IQR below Q_1 or 1.5 × IQR above Q_3 were excluded.

2.2 | Genotyping

We performed polymerase chain reaction (PCR) with genomic DNA isolated from tail clips to confirm genotypes of our mice. *Dgki* was analyzed as described previously.[24] A region of *Dgkh* containing exon 9 was amplified using primers 5′-GCAGATACTGAACCGTTTAGC CAG-3′ and 5′-CGCATGAGAGCAACAAAGATGTC-3′, on a PCR cycle that consisted of 35 rounds of 15 seconds of denaturing at 95°C, 15 seconds of annealing at 55°C, and 60 seconds of extending at 72°C. The products of this reaction were PCR purified and digested with *KpnI* (R0142; New England BioLabs, Ipswich, Massachusetts) and run on a 2% agarose gel containing SybrSafe (S33102; Invitrogen, Carlsbad, California; Figure 1C).

2.3 | Western blotting

Frontal cerebral cortex tissue was dissected from WT, *Dgkh*$^{-/-}$, *Dgki*$^{-/-}$ and dKO female mice and lysed on ice for 10 minutes in a buffer containing 50-mM Tris pH 7.4, 150-mM NaCl, 1-mM EDTA pH 8.0, 1% v/v Triton-X100, 1-mM phenylmethanesulfonyl fluoride, 1-mM sodium deoxycholate, 1× cOmplete Mini EDTA-free Protease Inhibitor Cocktail (4693159001; Roche, Basel, Switzerland). Following sonication on ice for 10 seconds, lysates were centrifuged at 10000*g* at 4°C for 15 minutes to separate the debris. Protein (30 μg) from each lysate was separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 4%-20% gel (456-1094; Bio-Rad, Hercules, California) and transferred to a 0.2-μm nitrocellulose membrane (1620095; Bio-Rad). Following 1 hour of blocking in a solution of 5% w/v milk (170-6404; Bio-Rad) in tris-buffered saline with Tween 20 (100-mM Tris pH 7.5, 165-mM NaCl, 0.1% v/v Tween 20; TBST) at approximately 23°C, membranes were washed for 15 to 20 hours at 4°C with primary antibodies for β-actin (1:3000 mouse anti-ACTB, ab6276; Abcam, Cambridge, UK) and either DGKH (1:1000 rabbit anti-DGKH, HPA040355; Sigma, St. Louis, Missouri) or DGKI (1:1000 rabbit anti-DGKI, LS-C118721; Lifespan Biosciences, Seattle, Washington) in a solution of 5% w/v bovine serum albumin (A3912; Sigma) in TBST. Blots were probed with secondary antibodies of 1:10000 IRDye 680RD-conjugated goat anti-mouse (925-68070; LI-COR Biosciences, Lincoln, Nebraska) and 1:10000 IRDye 800RD-conjugated donkey anti-rabbit (926-32213; LI-COR) in 5% w/v milk in TBST for 2 hours at approximately 23°C. Blots were imaged on an LI-COR Odyssey system and confirmed loss of DGKH and DGKI protein (Figure 1D).

2.4 | Fostering

Within an hour after the birth of the last pup from dKO female litters, the dKO dam was removed and replaced with a foster WT dam. Foster WT dams had given birth to a litter no more than 7 days prior to being used for fostering. The eight litters came from seven different dKO mothers and were fostered with eight different foster dams.

2.5 | Pup retrieval

To examine pup retrieval,[26-28] in a cage with a dam and her litter, the pups were removed from the home cage and kept warm on a SpaceGel heating pad (Braintree Scientific, Braintree, Massachusetts). With the nest in one corner of the home cage, one pup was placed in each of the three remaining corners. The mother was then placed in the cage at the site of the nest, and her latency to retrieve each pup and place them in the nest was timed. This assay was only performed when litters had at least three live pups. Mothers were tested on the day of or day after birth of the litter. After 15 minutes, all pups were returned to the nest by the experimenter. At P0, 22 WT mothers were assay with 25 litters, and 12 dKO mothers were assayed with 14 litters. At P1, 18 WT mothers were assay with 21 litters, and 8 dKO mothers were assayed with 9 litters. No mother was tested with more than 2 of her litters.

2.6 | Acoustic startle response and prepulse inhibition

To assess sensorimotor gating and startle reflex in these mice,[29,30] mice were placed into an acrylic tube (7 cm long × 3.75 cm inner diameter) that was paired with a piezoelectric transducer that measured flinch responses. The tube and transducer were housed in a 29-cm^3 sound-attenuating chamber with a light, fan and speaker. Responses to 40-ms, 120-dB acoustic stimulus was measured, alone or with a 20-ms prepulse tone played 100 ms preceding the 120-dB stimulus. Following a 5-minute acclimation to the startle chamber, testing sessions were 10 minutes and consisted of 42 randomized trials, 6 trials each of the following seven conditions: (a) no acoustic stimulus, (b) 120-dB startle tone alone, or (c) prepulse tone of 74, 78, 82, 86 or 90 dB followed by the 120-dB startle tone. The startle response was measured and analyzed with SR-LAB startle response system apparatus and software (San Diego Instruments, San Diego,

California). The degree to which the prepulse tone inhibited the startle response to the 120-dB tone was calculated as: 100 – [(response to startle stimulus post-prepulse)/(response to startle stimulus alone) × 100]. Mouse weights were measured at the end of the session; however, neither *Dgkh*$^{-/-}$, *Dgki*$^{-/-}$, nor dKO mice differed in weight from their simultaneously tested WT cohort, so we did not adjust the startle responses for weight in our analyses.

2.7 | Forced swim test

To model depression (despair behavior) or mania (goal-directed behavior),[31,32] activity was monitored in the forced swim test. Mice were placed into a 28-cm-tall cylinder of 20-cm diameter filled to approximately 15 cm with 24 to 26°C water for 6 minutes. Activity was video recorded. The time spent immobile during the final 4 minutes in the chamber was tracked and scored using EthoVision XT 7.0 software (Noldus Information Technology, Leesburg, Virginia). Floating without actively swimming was counted as immobility. Animals were monitored during the experiment period to ensure their head stayed above the water.

2.8 | Elevated plus maze

To model anxiety-like behavior,[33] mice were placed into the 7.5-cm^2 center of a 52-cm high elevated plus maze, the two closed and two open arms of which were each 30 cm long and 7.5 cm wide. The height of the walls of the closed arms was 20 cm, and the lip surrounding the open arms was 1.5 cm high. Activity of the freely exploring mouse was monitored by a human observer for 5 minutes. Time spent in the closed or open arms or the center of the maze was measured. Entry into the center or any arm was scored when three of the animal's paws crossed the threshold between each area.

2.9 | Open field

To test for exploratory behavior and activity levels,[34] mice were monitored in a 40-cm^2 open field of 28-cm depth, enclosed in a box with a light above the open field, for 60 minutes. Activity was analyzed using the VersaMax animal activity monitoring system (AccuScan Instruments, Columbus, Ohio) to determine the total distance moved horizontally in the entire arena, the number of vertical movements, and the total distance covered and the total time spent in the 25 cm × 25 cm center region, binned in 5-minute segments.

2.10 | Sucrose preference

To test for depressed (anhedonia) or manic (reward-seeking) behavior,[35,36] mice were assessed for their consumption of water and sucrose. Mice were individually housed in a cage with two identical water bottles for a 24-hour acclimation period. After 24 hours, each mouse cage and one control cage with no mouse was given one bottle of water and one bottle of 1% (w/v) sucrose in water. Each bottle was weighed before placing on the cages. The sucrose bottle was placed on the left or right at random. All cages were placed together on the same rack. Each bottle was weighed after 24 hours to determine the amount of liquid consumed by the mouse or lost in the control cage. The amount of water or sucrose lost in the control cage was subtracted from the water or sucrose consumption by each mouse to determine water or sucrose intake, respectively. Sucrose preference was calculated for each mouse individually as: (sucrose intake)/(sucrose intake + water intake) × 100.

2.11 | Sleep phenotyping

To survey sleep and wake patterns, mice were moved to a room with a 12 hours:12 hours light:dark cycle (lights on 8 AM to 8 PM) with a 1-hour shift in Zeitgeber time (ZT) relative to their previous housing. Mice were given two full dark phases to acclimate before recording sleep data. No other mice were housed in the room during the acclimation and test periods. Sleep and wake behavior were monitored continuously for 8 days with a noninvasive piezoelectric movement monitoring system described in detail previously.[37-39] Animals were individually housed with bedding, food and water in 15.5 cm^2 cages with a pressure-sensitive detector pad below each cage that transmitted activity signals to monitoring software (PiezoSleep 2.0; Signal Solutions, Lexington, Kentucky). Activity signals were analyzed in sliding 2-second intervals, with activity patterns of approximately 3 Hz—representing regular respiration consistent with sleep—coded as sleep and irregular patterns coded as wake. Sleep-wake thresholds were calculated automatically for each individual based on decision statistics. Sleep and wake behaviors were analyzed with customized statistics software (SleepStats; Signal Solutions).

2.12 | Statistics

After processing by the software programs mentioned above, data were analyzed with Prism version 7.04 (GraphPad Software Inc., La Jolla, California). Survival curves of WT and dKO pups were compared with a log-rank (Mantel-Cox) test. The proportion of mothers retrieving all three pups was compared between genotypes with a binomial test. Open field metrics were compared between WT and other genotypes using two-way repeated measures analysis of variance (ANOVA), with Sidak's multiple comparisons tests used for pairwise comparisons within 5-minute time bins. All other assays were tested for significance using two-tailed *t* tests with Welch's correction to compare WT and *Dgkh*$^{-/-}$, *Dgki*$^{-/-}$, or dKO, or to compare dKO and dKO Fostered, in the case of litter survival.

3 | RESULTS

3.1 | dKO mice lack DGKH and DGKI protein

We confirmed DGKH and/or DGKI protein loss in brain tissue from *Dgkh*$^{-/-}$, *Dgkh*$^{-/-}$ and dKO female mice via immunoblotting (Figure 1D). To address the potential for compensation, we examined protein levels of DGKH or DGKI in *Dgki*$^{-/-}$ or *Dgkh*$^{-/-}$ mice, respectively. We found no upregulation of DGKH in *Dgki*$^{-/-}$ mice and no upregulation of DGKI in *Dgkh*$^{-/-}$ mice relative to WT mice (data not shown).

3.2 | Poor survival rates of offspring raised by dKO mothers

While maintaining WT, *Dgkh*$^{-/-}$, *Dgki*$^{-/-}$ and dKO mice, we found that offspring of dKO mothers showed a significant decrease in survival within the first 2 days after birth (Figure 2A). Pups raised by WT mothers had a survival rate of 87.1%, whereas dKO-raised pups had a 29.5% survival rate (X^2_1 = 287.9, P < .0001). To evaluate the relative contribution of each *Dgk* gene to this phenotype, we examined survival rates of litters born from mothers of each genotype (Figure 2B). When raised by WT females, an average of 85.5% of the litter survived to weaning age, whereas dKO-raised litters had an average survival rate of 25.1% (t_{37} = 8.253, P < .0001). At 72.1% average survival, litters raised by *Dgkh*$^{-/-}$ did not significantly differ from WT litters. At 54.0%, litters raised by *Dgki*$^{-/-}$ mothers fared slightly worse than

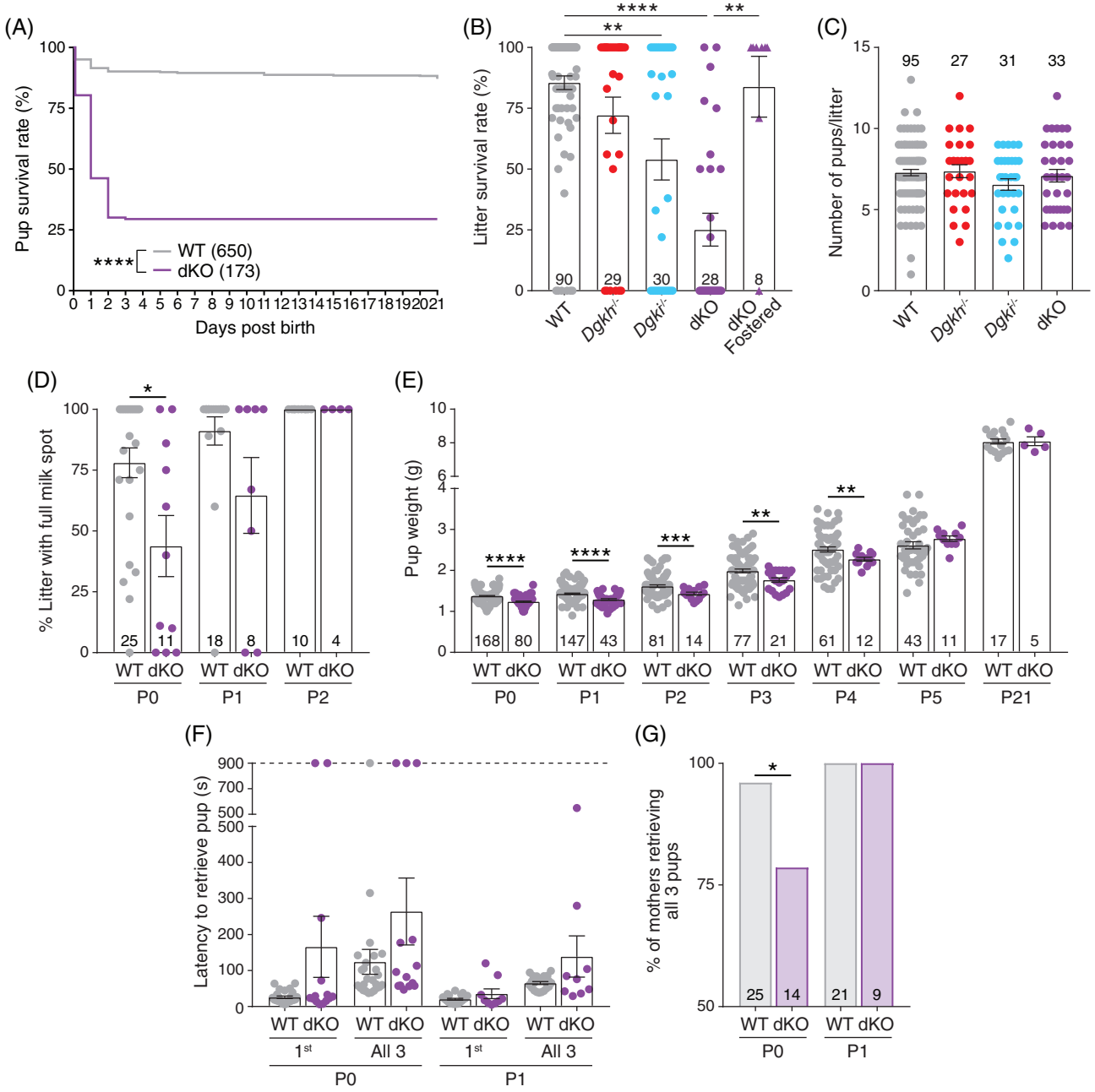

FIGURE 2 Poor survival of offspring raised by dKO females. A, Survival rate of pups born from dKO dams was significantly reduced after birth relative to those born from WT dams. B, The average proportion of each litter that survived to weaning was dependent on genotype of the mother. dKO fostered = litters born from dKO dams fostered with recently postpartum WT dams. C, Litter size on the day of birth based on the genotype of the mother. D, Percentage of each litter with a milk spot present, shown by genotype of the mother. E, Weight of pups raised by WT or dKO mothers. F, Time taken to retrieve the first pup and all three pups in the pup retrieval assay, based on the genotype of the mother. G, Percentage of mothers—tested in (F)—that retrieved all three pups to the nest. Number of pups indicated on graphs in (A) and (E). Number of litters indicated on graphs in (B-D). Bars in (B-F) represent mean ± SEM. P < *.05, **.01, ***.001, ****.0001

those raised by WT dams (t_{36} = 3.546, P = .0011), but the difference was most pronounced when both *Dgkh* and *Dgki* were deleted (dKO). The severity of this phenotype was not dependent on whether the dam was a new or experienced mother, the age of the mother, the genotype of the pup or the size of the litter (data not shown).

3.3 | dKO dams show signs of deficient postnatal care

To determine if the poor survival rate was due to deficiencies in prenatal development or postnatal care, we fostered pups from dKO dams to recently postpartum WT dams. When fostered to a WT mother, an average of 83.9% of dKO-born pups survived (dKO Fostered; Figure 2B), which was a significant improvement over dKO-raised litters (t_{11} = 4.134, P = .0015). These data suggest dKO-born pups can nurse and grow normally when under the care of a WT mother. Moreover, the normal litter size at birth (Figure 2C) ruled out the possibility that pup survival was impaired prenatally.

3.4 | Newborn offspring consume less milk when reared by dKO female mice

Since our data suggested poor pup survival was due to deficient maternal care, we next assayed an array of maternal behaviors. From observing their cages, we found that WT and dKO females made protective nests, with tall sides and top coverings that fully enveloped both the dam and the litter. The protective nests prevented the observation of nursing behavior directly, so we monitored the presence of milk spots in the pups to determine if the pups were consuming milk. Due to their transparent skin, milk in the stomach of newborn mice can be seen as a white spot on the abdomen. At P0, on average 43.8% of pups from dKO-born litters had visible milks spots, compared to litters from WT dams with an average of 78.0% (t_{15} = 2.451, P = .0270; Figure 2D). All dKO-raised pups had milk spots at P2, likely explaining why dKO pups that survived to P2 then survived to weaning. Moreover, the presence of milk spots ruled out the possibility that dKO females were unable to produce milk.

3.5 | Offspring of dKO mothers gain weight slowly in early postnatal period

This transient phenotype at birth had a measurable impact on the body weight of mice raised by dKO mothers (Figure 2E). Pups raised by dKO dams weighed significantly less than those raised by WT dams at P0 (1.24 and 1.38 g, respectively; t_{246} = 6.887, P < .0001), P1 (1.29 and 1.43 g; t_{95} = 5.568, P < .0001), and P2 (1.43 and 1.62 g; t_{35} = 3.83, P = .0005). The weight difference between dKO and WT pups began to normalize by P3 (1.77 and 1.99 g, respectively; t_{52} = 3.101, P = .0031) and P4 (2.28 and 2.51 g; t_{53} = 3.002, P = .0041). From P5 to P21 (typical weaning age), there were no significant differences in offspring weight based on maternal genotype. Weight gain was preceded by the presence of milk spots at P2 (Figure 2D) and coincided with a decrease in lethality (Figure 2A).

3.6 | dKO mothers show variable pup retrieval behavior

Dams retrieve pups that have strayed from the nest. In an assay of this maternal behavior, the latency to retrieve three stray pups was tested in WT and dKO moms on the day of (P0) or the day after (P1) birth of a new litter (Figure 2F). The average latency to return stray pups to the nest was higher in dKO dams at P0 (not significant) relative to WT (263.9 and 123.9 seconds, respectively) but was skewed by a subset of dKO mothers that failed to retrieve any pups during the entire 900-second assay period. Of the 14 P0 litters assayed with dKO mothers, 11 successfully retrieved all three pups (78.6%), compared to a success rate of 96.0% (24/25) in trials with WT mothers (P = .0167; Figure 2G). All dams retrieved all three pups at P1 (Figure 2G), with comparable latencies between WT and dKO dams (Figure 2F).

During the pup retrieval assay, as well as while working with dKO females in the animal housing facility, we noticed that dKO females would dart around the cage, rear frequently, and not settle down like WT females, especially after opening or disturbing the cage. And, during the pup retrieval assay, the dKO females that failed to return all three pups to the nest (Figure 2F) displayed this manic-like exploratory behavior for the entire testing period. We speculate that this manic-like behavior might make it difficult for newborn pups to nurse, and hence could contribute to poor pup survival.

3.7 | Loss of *Dgkh* and/or *Dgki* in females does not alter responses to startling acoustic stimuli

Given our findings above and the genetic linkage of *DGKH* and *DGKI* to mood disorders in humans, we next tested WT, *Dgkh*$^{-/-}$, *Dgki*$^{-/-}$ and dKO female mice with neuropsychiatric disorder-related behavioral assays. We used virgin females for these studies because it is not practical to generate large cohorts of age-matched and postpartum-time-matched females for behavioral studies. Additionally, since deficient maternal care of pups is known to impair behaviors of offspring in adulthood,[40-44] the dKO females used in these behavioral studies were raised by WT foster moms.

Deficits in prepulse inhibition are used to model sensorimotor gating, a symptom of schizophrenia.[45] We found that responses to a startling acoustic tone of 120 dB did not differ based on genotype (Figure 3A), nor did the inhibition of the startle response when paired with a softer prepulse tone of varying loudness (Figure 3B). This suggests that startle reflexes and sensorimotor gating are not dependent on *Dgkh* and/or *Dgki* expression in female mice.[29]

3.8 | dKO females exhibit decreased immobility in the forced swim test

In the forced swim test, neither *Dgkh*$^{-/-}$ nor *Dgki*$^{-/-}$ females differed from WT females, but the dKO females showed decreased immobility relative to WT female mice (195.7 and 216.4 seconds, respectively; t_{31} = 4.521, P < .0001; Figure 4A). Reduced immobility in this assay, that is, enhanced escape drive, models the goal-directed vigor domain of mania.[32]

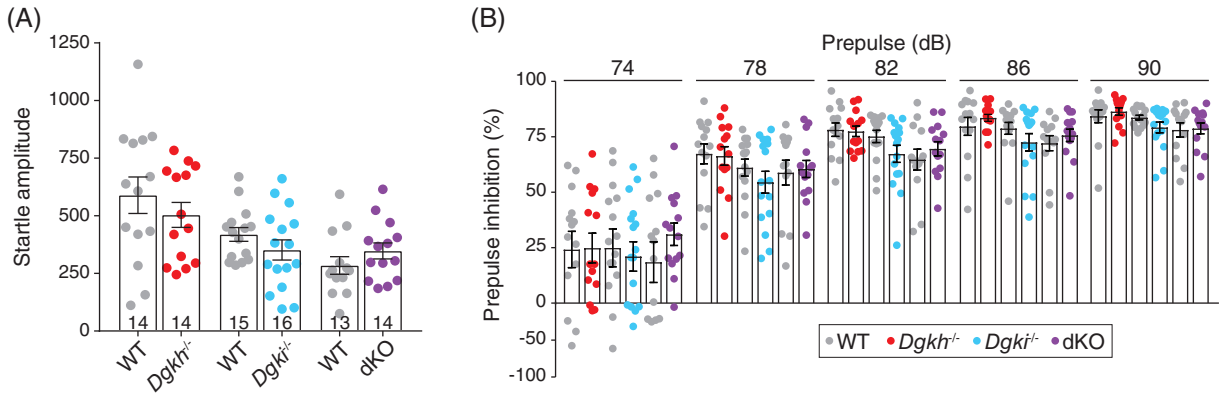

FIGURE 3 Deletion of *Dgkh* and/or *Dgki* does not alter responses or habituation to acoustic startle tone in female mice. A, Startle responses to a 120-dB tone. B, The percentage decrease in startle response from (A) when the 120-dB tone was preceded by a nonstartling tone of 74, 78, 82, 86 or 90 dB. Same mice were used in (A) and (B); number of mice indicated on graph in (A). Bars represent mean ± SEM

3.9 | dKO females prefer the closed arms in the elevated plus maze

The elevated plus maze was used to test for anxiety-like behavior. In this test, we measured how much time mice spent in the protective closed arms vs the aversive open arms (Figure 4B). Deletion of *Dgkh* or *Dgki* alone had no effect on behavior in this assay; however, females lacking both *Dgkh* and *Dgki* spent more time in the closed arms of the maze (187.0 seconds for dKO and 145.2 seconds for WT; t_{29} = 4.409, P = .0001) and less time in the open arms (74.7 seconds for dKO and 99.0 seconds for WT; t_{30} = 2.86, P = .0076), considered a representation of anxiety-like behavior.[33]

3.10 | Loss of *Dgkh* and/or *Dgki* in females does not affect behavior in an open field

In the open field test, none of the three genetic mouse models showed differences in horizontal (Figure 5A-C) or vertical (Figure 5D-F) locomotion relative to WT females, apart from a slight increase in dKO rearing activity in a single 5-minute bin (t_{420} = 3.306, P = .0123; Figure 5F). Additionally, the distance traveled (Figure 5G-I) and time spent (Figure 5J-L) in the center of the open field did not significantly differ with genotype. The behavior of dKO females in the open field suggested that their increased activity in the forced swim test (Figure 4A) was indicative of mania-like behavior, not general hyperactivity,[46] and the phenotype of the dKO females in the elevated plus maze (Figure 4B) represented anxiety and not simply hypo-exploratory behavior.[47]

3.11 | Loss of both *Dgkh* and *Dgki* in females enhances sucrose preference

We performed additional behavioral tests to evaluate mania- and anxiety-like phenotypes in dKO females. Because neither *Dgkh*$^{-/-}$ nor *Dgki*$^{-/-}$ females differed from WT in previous assays, subsequent analyses were performed with only WT and dKO female mice.

The degree to which mice prefer to drink a sucrose solution over water can indicate mood-related phenotypes. After monitoring water and sucrose consumption over 24 hours, we found dKO female mice have a significantly greater preference for sucrose over water than

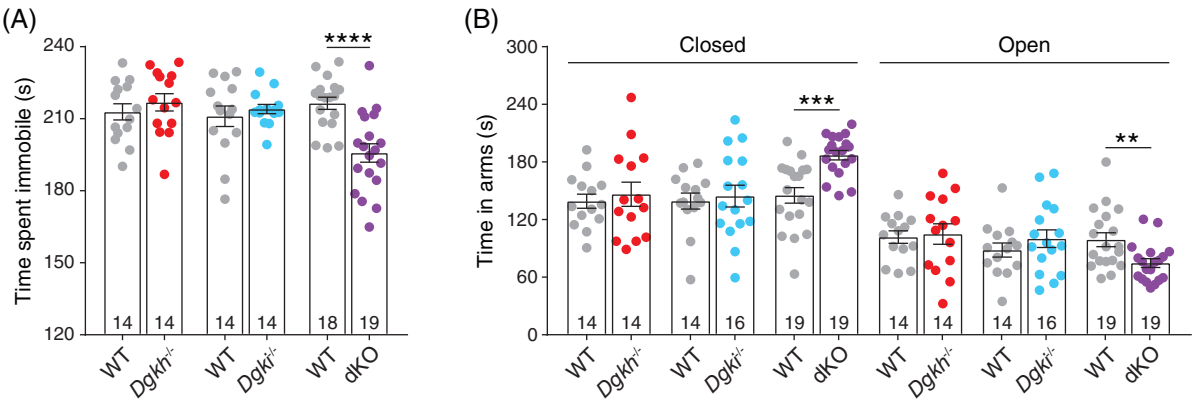

FIGURE 4 dKO females show mood-disorder-like phenotypes. A, In the forced swim test, mice were placed in a large cylinder of water for 6 minutes. Time spent immobile (ie, not swimming) was measured for the final 4 minutes. B, Time in closed arm vs aversive open arms of an elevated plus maze was measured in a five-minute period. Number of mice indicated on graphs. Bars represent mean ± SEM. **P < .01; ***P < .001; ****P < .0001

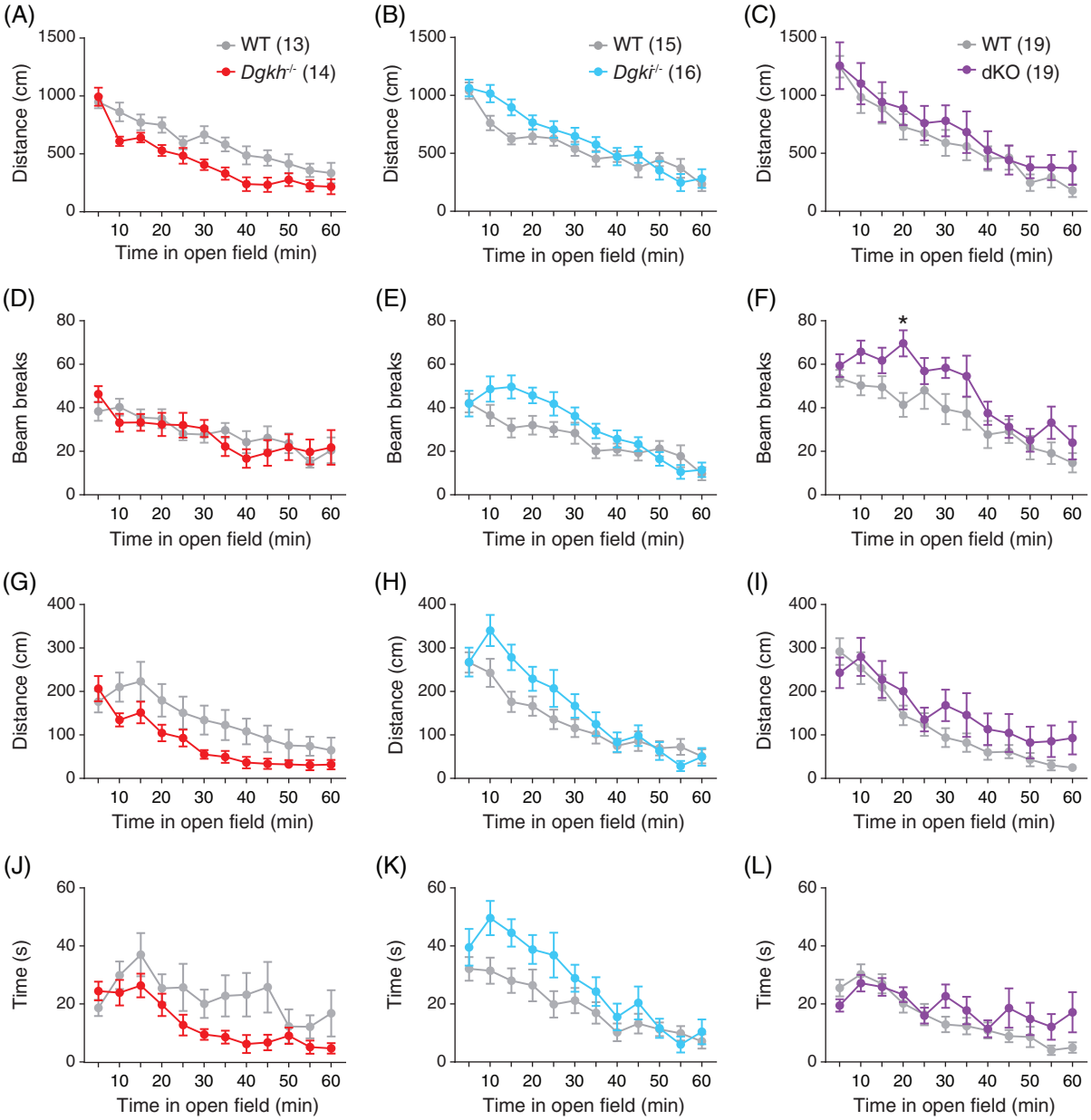

FIGURE 5 Locomotion and center behavior in an open field were unaffected by *Dgkh* and/or *Dgki* loss in female mice. In the 60-minute test, the total distance covered (A-C), vertical rearing activity (D-F), and the distance covered (G-I) and time spent (J-L) in the center of an open field were tracked. Number of mice indicated on graphs in (A-C). Data represent mean ± SEM. *$P < .05$

WT females (96.2% vs 87.5%, respectively; $t_{10} = 3.216$, $P = .0089$; Figure 6A), a phenotype analogous to the reward-seeking symptom of mania.[36]

3.12 | dKO females have disrupted sleep patterns

Sleep is impaired in anxious and manic human patients as well as in rodent models of mania and anxiety.[48-55] To determine the effect of *Dgkh* and *Dgki* loss on sleep, the patterns of wakefulness and sleep throughout the light and dark phases were measured continuously for 8 days in WT and dKO female mice. The proportion of time dKO female mice spend asleep differs from WT mice during multiple 1-hour blocks (Figure 6B) of both the light phase (ZT3, $t_{13} = 2.223$, $P = .0451$) and the dark phase (ZT13, $t_{13} = 2.849$, $P = .0138$; ZT15, $t_{13} = 2.306$, $P = .0385$; ZT17, $t_{14} = 2.491$, $P = .0260$; ZT20, $t_{13} = 3.131$, $P = .0082$). Larger trends in sleep patterns are visible in 6-hour blocks (Figure 6C). Relative to WT, dKO females spend less of their time asleep during the first half of the light phase (59.3% vs 63.2%, respectively, at ZT0-6; $t_{14} = 3.825$, $P = .0019$) but sleep more during the first half of the dark phase (19.7% vs 12.8% at ZT12-18; $t_{12} = 3.391$, $P = .0055$).

Although there are variations in the total amount of time spent asleep (Figure 6C), the average length of individual sleep bouts did not differ (Figure 6D). However, the average wake bout was longer for dKO than WT mice throughout the light phase (Figure 6E) at ZT0-6 (220.0 vs 172.7 seconds, respectively; $t_8 = 4.263$, $P = .0028$) and

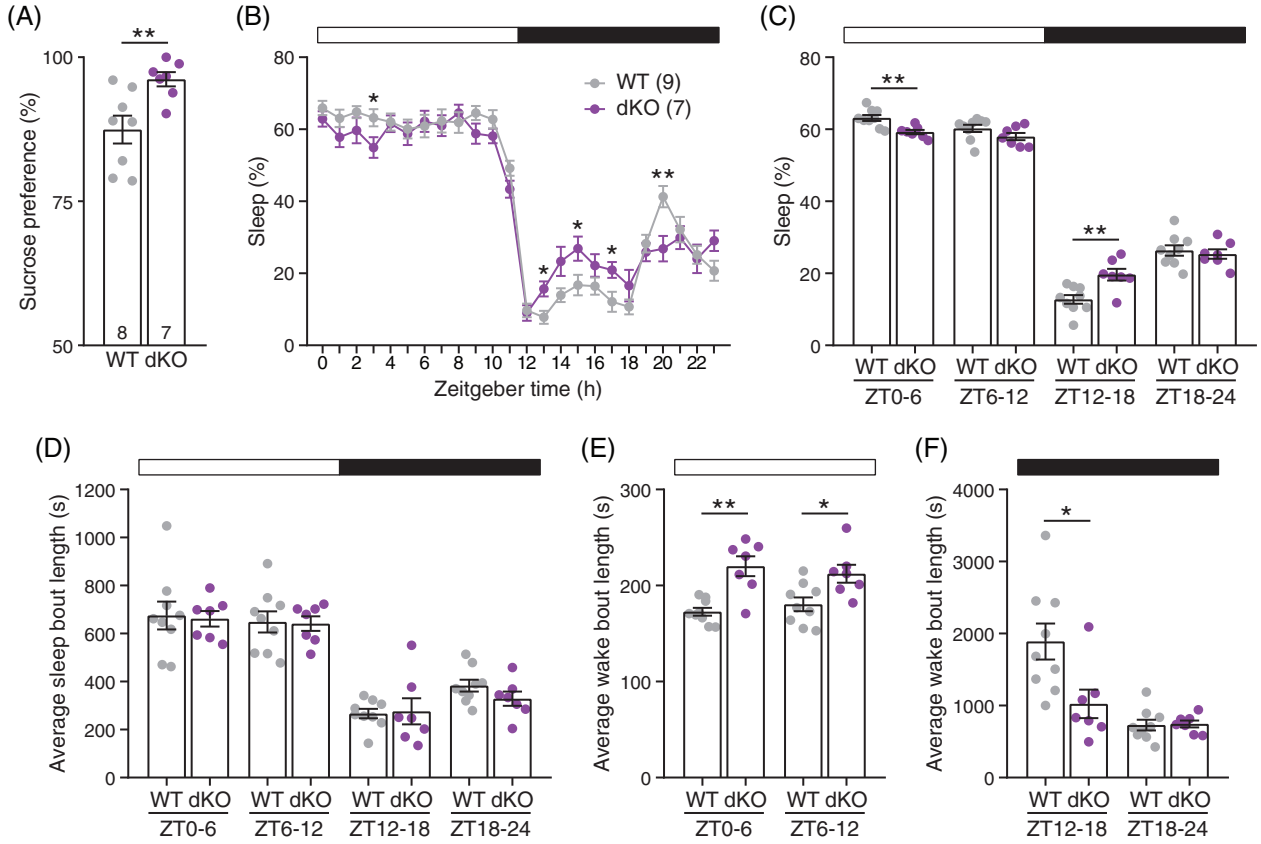

FIGURE 6 Sucrose preference and sleep patterns were disrupted in dKO female mice. A, The preference of a 1% sucrose solution over water was measured over 24 hours. B-F, Wake and sleep behaviors were measured continuously for 8 days, and the following measurements for each animal were calculated by averaging across days. The average percentage of time each mouse spent asleep was calculated in 1-hour (B) and 6-hour (C) bins over the 12-hour light and 12-hour dark phases. The average length of sleep bouts in the light and dark phases (D) and the average length of wake bouts in light (E) and dark (F) phases were calculated in 6-hour bins. Number of mice indicated on graphs in (A) and (B). Same mice from (B) are represented in (C-F). Bars represent mean ± SEM. *$P < .05$, **$P < .01$

ZT6-12 (212.3 vs 180.4 seconds; $t_{12} = 2.717$, $P = .0187$) and was shorter in the first half of the dark phase at ZT12-18 (1024 vs 1889 seconds; $t_{14} = 2.719$, $P = .0167$; Figure 6F). Together, these data suggest that WT and dKO mice differ in the total proportion of time spent asleep because of differences in how long the mice stay awake between sleep bouts.

Mania is strongly linked to reduced sleep in humans and rodents.[50,53,55] Disrupted sleep-related symptoms are connected to anxiety, although the direction of the disruption is ambiguous in anxious patients.[48,49,51,52,55] Both fatigue and restlessness are diagnostic criteria of anxiety,[55] and anxious patients report insomnia and hypersomnia.[48,51,52] In dKO females, reduced sleep in the light phase—the phase in which the most sleep occurs—could either represent a decreased need for sleep (a symptom of mania) or a decreased ability to sleep (a symptom of anxiety). The subsequent increase in sleep in the dark phase may reflect fatigue or a lack of sufficient rest during the light phase, suggesting a more anxiety-like phenotype. Indeed, a mouse model of high anxiety lacked a light-phase sleep phenotype, but slept more during the dark phase.[54] Overall, the light-phase sleep behavior more closely mimics rodent mania models,[50,53] while the dark-phase sleep behavior more closely mimics anxious rodents.[54]

4 | DISCUSSION

Here, we show that global loss of both *Dgkh* and *Dgki* in female mice causes anxiety- and mania-like behaviors—phenotypes not seen from loss of *Dgkh* or *Dgki* alone. These behavioral phenotypes were paired with a significant deficit in early maternal care. The results of the behaviors analyzed in the three genetic mouse models are summarized in Table 1. Poor offspring survival was paired with decreases in milk consumption and pup retrieval in dKO-raised litters. Anxious behaviors were seen in dKO female mice in the closed arm preference in the elevated plus maze and through disrupted sleep patterns. The dKO females showed mania-like behavior through reduced immobility in the forced swim test, enhanced sucrose preference and reduced light-phase sleep.

In human patients, the co-occurrence of mania and anxiety is debated. Some symptoms of manic episodes (eg, euphoric mood, high-energy and goal-directed behavior) oppose symptoms of anxiety (eg, worrying, fatigue and impaired concentration).[55] Additionally, many rodent models of mania have reduced anxiety.[56,57] On the other hand, some of the symptoms of mania and anxiety overlap, such as irritability and aberrant sleep patterns.[55,58] Anxiety disorders and BD

TABLE 1 Summary of behavioral results for *Dgkh*$^{-/-}$, *Dgki*$^{-/-}$ and dKO female mice

Phenotype	*Dgkh*$^{-/-}$	*Dgki*$^{-/-}$	dKO
Offspring survival	=	↓**	↓****
Offspring milk consumption at P0	NT	NT	↓*
Pup retrieval at P0	NT	NT	↓*
Acoustic startle response	=	=	=
Prepulse inhibition	=	=	=
Forced swim test: immobility time	=	=	↓****
Elevated plus maze: closed arm time	=	=	↑***
Open field: total distance	=	=	=
Open field: center distance	=	=	=
Sucrose preference	NT	NT	↑**
Light-phase sleep	NT	NT	↓**
Dark-phase sleep	NT	NT	↑**

Relative to WT females.
"=" symbolizes no difference from WT.
"NT" means mice of the indicated genotype were not tested for an individual assay.
*P < .05.; **P < .01.; ***P < .001.; ****P < .0001.

are highly comorbid[59-63] and have significantly greater co-occurrence than anxiety disorders and unipolar depression,[61,62] suggesting that mania may drive the BD-anxiety comorbidity. The prevalence of comorbid anxiety in BD patients in a manic state is ambiguous, as it has been found to be both higher[64] and lower[65] than in other BD patients. However, manic episodes strongly predicted the diagnosis of an anxiety disorder at a three-year follow-up.[66] Because of the inconclusive evidence for the co-occurrence of mania and anxiety, and without testing all features of both disorders, we hesitate to suggest that the dKO female mice are models of anxiety and/or mania. These are complex human conditions that would be difficult to wholly represent in a mouse. Instead, we argue that dKO female mice model some features of anxiety and mania. As the comorbidity of anxiety and mania is under debate, these mice may be useful tools for analyzing how phenotypes of both conditions could present in the same individual.

While a detailed exploration of the molecular, cellular and circuit-based mechanisms for these phenotypes is beyond the scope of this study, DGKH and DGKI are known to regulate multiple signaling pathways that have been linked to the phenotypes we uncovered. Activation of $G\alpha_q$-protein-coupled receptors ($G\alpha_q$-GPCRs) induces calcium release and production of DAG, together leading to neuronal activity.[3,67] We previously found that overexpression of *Dgkh* in HEK293 cells prolongs $G\alpha_q$-GPCR-stimulated calcium mobilization by attenuating the activation of protein kinase C (PKC).[68] Using hippocampal slices from neonatal (2 week old) *Dgki*-knockout mice, others found that metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD) was dampened, although they did not find this effect in adult tissue.[12] Impaired mGluR-LTD (a $G\alpha_q$-GPCR-dependent process) in the *Dgki*$^{-/-}$ mouse tissue required increased PKC activation, suggesting a mechanism analogous to that which we previously showed for DGKH. In addition to negatively regulating PKC, both DGKH and DGKI were found to positively regulate extracellular signal-regulated kinase (ERK) signaling.[24,69,70] Increased PKC activity has been shown in a mouse model of mania and anxiety[71,72] and in bipolar manic patients,[73] and PKC-null (*Prkcg*$^{-/-}$ and *Prkce*$^{-/-}$) mice show reduced anxiety.[74,75] ERK dysfunction leads to poor maternal care and mania- and anxiety-like behavior in rodents.[27,76,77] Mood stabilizers that manage mania in human patients can decrease PKC activity[73] or increase ERK signaling[78] in neurons. Thus, loss of DGKH and DGKI has the potential to affect behavior by enhancing PKC activity and/or attenuating ERK activity.

PKC and ERK have many signaling targets that could contribute to impaired behavior in dKO female mice. PKC modulates the function of $GABA_A$ and 5-HT_{2A} receptors,[79,80] both of which regulate maternal behavior[81,82] and anxiety.[83,84] Altered PKC or ERK function resulting from DGKH and DGKI loss may induce behavioral changes by modulating phosphorylation of GSK3β,[85,86] an enzyme implicated in the pathology of multiple mood disorders.[87,88] Indeed, other researchers found that phosphorylation (ie, inactivation) of GSK3β was decreased in brains of their *Dgkh*$^{-/-}$ male mice (a different mutant than the *Dgkh*$^{-/-}$ females used in this study), which showed manic behaviors.[17] Further investigations are needed to determine which signaling pathways are disrupted in the brains of dKO female mice.

Based on gene expression data from the Allen Brain Atlas,[89] *Dgkh* and *Dgki* have highly overlapping regional expression patterns in the brain.[3] *Dgkh* and *Dgki* expression are enriched in the cerebral cortex, hippocampus and striatum.[3,90] Human and animal studies connect the pathology of both mania and anxiety to cortical and hippocampal function,[91-96] whereas striatal function has been implicated in the regulation of nurturing behaviors in rodents.[97-99] Given the known links between different brain regions and the phenotypes we have shown here, it is possible that *Dgkh* and *Dgki* loss could affect mood by altering hippocampal and cortical activity, while their effects on maternal behavior may arise in the striatum. On the other hand, other mouse models of deficient maternal behavior have likewise shown comorbid psychopathological phenotypes, suggesting potential overlap in the pathology behind these behaviors. Mice lacking the δ subunit of $GABA_A$ receptors showed poor maternal care, anxiety, and depression.[81] Mice with loss of ephrin-A5 or central nervous system-specific ERK2 deletion had decreased nurturing behaviors and anxiety,[27,100] and mice bred for low anxiety were less maternal than their high-anxiety counterparts.[101] While these studies show variability in the phenotypes in each mouse model, they show that aberrant mood-related behaviors commonly coincide with disrupted maternal behavior in mice.

Parturition can induce depression and anxiety in mice and humans[81,102,103] and, importantly, frequently exacerbates symptoms in patients with prepartum mood disorders.[104] Having found that virgin dKO females show signs of anxiety and mania, an interesting future direction would be to investigate how parturition influences the mood-related phenotypes seen in dKO mice. Extensive research has showed that receiving poor maternal care as a young rodent

increases the likelihood of developing anxiety-like behaviors as an adult.[40-44] However, the influence of a rodent's mood on its ability to provide maternal care is underappreciated. One possible explanation of the poor survival of dKO-raised litters is that dKO females have exaggerated responses to stressful situations, including delivery or the presence of new pups in the cage, which cause them to neglect their offspring. Because the milk consumption and survival rate of dKO-raised pups improved after P2, the deficits in nurturing behavior by dKO dams may be specific to the early postpartum period. If symptoms of anxiety or mania impair maternal care, and mood-related symptoms worsen for a short period immediately after parturition, this may explain the apparent transience in the survival phenotype. Our data rule out the possibility that maternal neglect persists throughout the pre-weaning period, as survival of pups to dKO mice is not affected after P2.

Whereas the *Dgkh*$^{-/-}$ males tested by other researchers[17] were generated via a different mutation than the *Dgkh*$^{-/-}$ female mice presented here, differences between these mouse models are interesting to note. *Dgkh*$^{-/-}$ male mice presented with manic behavior in the tail suspension test, reduced anxiety in the elevated plus maze, and slightly increased activity in the open field.[17] None of these phenotypes were present in our *Dgkh*$^{-/-}$ female mice (using the forced swim test in place of tail suspension). Evidence of mutation- and sex-dependent variations in how *Dgkh* controls behavior suggests that both *Dgkh*$^{-/-}$ mouse lines may be useful tools for understanding the psychopathological conditions to which *DGKH* has been linked in human patients.

In summary, we found that dKO mothers showed poor nurturing behavior, evidenced by fewer milk spots, reduced weight and impaired survival of dKO-raised litters. Additionally, we observed that dKO females—but not *Dgkh*$^{-/-}$ or *Dgki*$^{-/-}$ females—showed mania- and anxiety-like behaviors, without disrupting activity or exploration of a novel environment, startle reflexes or sensorimotor gating. Our research using *Dgkh*$^{-/-}$, *Dgki*$^{-/-}$ and dKO female mice suggests that *Dgkh* and *Dgki* have functional compensation in each other's absence, perhaps due to the high degree of overlapping expression. Further experiments are needed to understand the mechanism by which loss of these genes induces deficits in maternal care and manic and anxious behavior. Moreover, defining how phosphorylation of DAG by DGKH and DGKI modulates behavior may help to identify new pharmacological treatments for symptoms of mania and anxiety, particularly during the postpartum period.

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