Heterozygous loss of epilepsy gene KCNQ2 alters social, repetitive and exploratory behaviors

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Abstract
KCNQ/Kv7 channels conduct voltage-dependent outward potassium currents that potently decrease neuronal excitability. Heterozygous inherited mutations in their principle subunits Kv7.2/KCNQ2 and Kv7.3/KCNQ3 cause benign familial neonatal epilepsy whereas patients with de novo heterozygous Kv7.2 mutations are associated with early-onset epileptic encephalopathy and neurodevelopmental disorders characterized by intellectual disability, developmental delay and autism. However, the role of Kv7.2-containing Kv7 channels in behaviors especially autism-associated behaviors has not been described. Because pathogenic Kv7.2 mutations in patients are typically heterozygous loss-of-function mutations, we investigated the contributions of Kv7.2 to exploratory, social, repetitive and compulsive-like behaviors by behavioral phenotyping of both male and female KCNQ2+/− mice that were heterozygous null for the KCNQ2 gene. Compared with their wild-type littermates, male and female KCNQ2+/− mice displayed increased locomotor activity in their home cage during the light phase but not the dark phase and showed no difference in motor coordination, suggesting hyperactivity during the inactive light phase. In the dark phase, KCNQ2+/− group showed enhanced exploratory behaviors, and repetitive grooming but decreased sociability with sex differences in the degree of these behaviors. While male KCNQ2+/− mice displayed enhanced compulsive-like behavior and social dominance, female KCNQ2+/− mice did not. In addition to elevated seizure susceptibility, our findings together indicate that heterozygous loss of Kv7.2 induces behavioral abnormalities including autism-associated behaviors such as reduced sociability and enhanced repetitive behaviors. Therefore, our study is the first to provide a tangible link between loss-of-function Kv7.2 mutations and the behavioral comorbidities of Kv7.2-associated epilepsy.

KEYWORDS
anxiety, epilepsy, excitability, KCNQ2, Kv7 channels, locomotion, seizures, social dominance, social interaction
INTRODUCTION

Neurodevelopmental disorders (NDDs) are a collection of heterogeneous disorders which include but are not limited to autism spectrum disorder (ASD), intellectual disability, developmental delay and epilepsy.1,2 These disorders can occur alone or together in the early childhood of NDD patients and persist throughout life.1 Among NDDs, ASD is estimated to occur in 1 out of 59 children (https://www.cdc.gov/ncbddd/autism/data.html). The main symptoms of ASD are behavioral abnormalities including difficulty with social interaction and communication as well as restrictive and repetitive behaviors.3 Additional ASD symptoms include obsessive interests, compulsive behavior, impulsivity, self-harm, aggression, hyperactivity, anxiety, hypersensitivity to auditory and tactile stimulation, sleep problems, learning disability and speech delay.4,9 Current available treatments for children with NDDs have limited efficacy alleviating the behavioral symptoms associated with ASD and seizures that are comorbid with some NDDs.20 To understand the etiology underlying NDDs, recent efforts have focused on next generation sequencing of whole genome and targeted sequencing of well-defined ASD cohorts.11,12 These efforts have dramatically accelerated discovery of genes affected by NDDs, providing key initial insights into the possible signaling pathways and mechanisms underlying NDDs.11,12

Among 35 genes with excess missense de novo mutations, significant numbers of recurrent mutations have been found in KCNQ2 and KCNQ3 genes,12 which encode the principle subunits of neuronal KCNQ/Kv7 potassium (K+) channels, Kv7.2 and Kv7.3.13 Kv7 channels are mostly heterotetramers of Kv7.2 and Kv7.3 subunits.14,15 Kv7.2 and Kv7.3 are found throughout the brain, including the hippocampus and cortex with overlapping distribution.16,17 Subcellularly, Kv7.2 and Kv7.3 are preferentially enriched at the plasma membrane of axonal initial segments (AIS) and distal axons17-19 where AP initiates and propagates.20 Because these slowly activating and noninactivating K⁺ currents are activated at the subthreshold potentials of action potential (AP) generation,12 they allow the firing of a single AP but effectively prevent repetitive and burst firing of APs.14,21-26 In contrast, inhibition of their currents by muscarinic agonists (hence, named “M-currents”) leads to profound increase in AP frequency.15 In addition, Kv7 currents regulate AP threshold and resting membrane potential.14,21-25

The physiological significance of neuronal Kv7 channels is underscored by the fact that more than 200 heterozygous mutations in KCNQ2 and KCNQ3 genes have been associated with early-onset epilepsy, including Benign Familial Neonatal Encephalopathy (BFNE [MIM: 121200]) and Epileptic Encephalopathy (EE [MIM: 613720]) (RIKEE database www.rikee.org). In BFNE, patients have inherited KCNQ2 and KCNQ3 mutations and display neonatal seizures that spontaneously remit after several weeks to months with benign psychomotor and intellectual outcomes.27 In EE, patients with de novo KCNQ2 and KCNQ3 variants have early-onset seizures, developmental delay and intellectual disability.28-38 Although a few gain-of-function EE mutations of Kv7.2 has been reported,39 experimentally-characterized epilepsy mutations mostly decrease current and surface expression of heteromeric Kv7.2/7.3 channels by 20% to 75%.27,32,40-43 Consistent with the loss-of-function mutations, the heterozygous Kv7.2 knockout mice and the heterozygous Kv7.2 knock-in mice for BFNE Y284C mutation display increased seizure propensity.44,45 Conditional homozygous deletion of KCNQ2 from excitatory pyramidal neurons during embryonic development in mice also results in cortical hyperexcitability, spontaneous seizures and early death.24 In contrast, the Kv7 channel opener ezogabine/retigabine suppresses seizures in rodents and humans.46

Despite its well-documented roles in dampening neuronal excitability and seizures,14,21-25,46,47 precise roles for Kv7 genes in behaviors other than seizures remain unclear. Anti-depressant efficacy by Kv7 channel opener ezogabine/retigabine was recently observed in humans with major depressive disorders48 and a social defeat stress model of depression in rodents.49 Although some patients with de novo EE mutations in KCNQ2 have ASD as a behavioral comorbidity,26,42,50 accumulating whole-exome or targeted sequencing studies on well-defined ASD cohorts have identified NDD variants in both KCNQ2 and KCNQ3 genes in patients who do not have epilepsy.12 The KCNQ2 and KCNQ3 transcripts are expressed early in the neocortex and hippocampus of the human brain prior to birth (http://hbatlas.org/) and in the embryonic neocortex of the mouse (http://hbatlas.org/mouseNCXtranscriptome/). The neonatal brain largely relies on Kv7 channels for neuronal inhibition24,51 when GABA acts as an excitatory rather than an inhibitory neurotransmitter.52 Therefore, disruption of Kv7 currents during the embryonic development of the brain may contribute to the onset of behaviors implicated in neuropsychiatric developmental disorders such as ASD. However, whether neuronal Kv7 channels contribute to ASD-associated behaviors is unknown.

Recurrent heterozygous de novo NDD mutations are enriched at the key functional domains in Kv7.2 and Kv7.3 important for voltage-dependent activation of Kv7 channels.12 Furthermore, pathogenic Kv7.2 mutations characterized to date are typically heterozygous loss-of-function mutations.27,32,40-43 Therefore, we hypothesize that heterozygous loss of Kv7 currents by genetic knockout of KCNQ2 gene would lead to the core ASD-associated behaviors in mice including reduced sociability and repetitive behaviors. In this study, we test this hypothesis by behavioral phenotyping both male and female heterozygous KCNQ2+/- mice and their wild type KCNQ2+/- littermates. Our results demonstrate for the first time a critical role of Kv7.2 in multiple behaviors in mice including circadian-dependent locomotor activity, as well as exploratory, social approach and repetitive behaviors, thereby offering a genotype-phenotype link between the heterozygous loss of Kv7 current and ASD-associated behaviors. Importantly, sex differences have been observed in social dominance and compulsive-like behaviors in KCNQ2+/- mice.

MATERIALS AND METHODS

2.1 Experimental animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana Champaign
and University of Connecticut. Heterozygous KCNQ2 gene knockout (KCNQ2+/−) mice on the C57BL/6J background were obtained from the Jackson Laboratory (Kcnq2+/−, Stock Number: 005830-20). KCNQ2−/− mice contain a deletion in the KCNQ2 gene from base 418 to 535 by insertion of a LacO-Sa-IRES-lacZ-Neo555G/Kan construct. These mice were originally produced by the Deltagene and were backcrossed at least five generations to C57BL/6J mice at the Jackson Laboratory. Upon arrival at our laboratory, these mice were backcrossed three more generations to C57BL/6J mice (Jackson Laboratory, Stock Number: 000664) before performing behavioral studies. For the tube dominance test and the three-chamber social interaction test, the outbred CD-1 (ICR) mice (Charles River Laboratory, Stock Number: 022) and the inbred C57BL/6J mice (Jackson Laboratory, Stock Number: 000664) were used as novel social targets, respectively. Both strains have been shown to be suitable for performing social behavioral tasks.53 Breeding pairs to generate experimental mice consisted of KCNQ2+/− mice crossed with C57BL/6J mice, yielding KCNQ2+/+ and KCNQ2−/−. Mice were bred and housed on a normal 14:10 light:dark cycle with food and water available ad libitum. At weaning, mice were group housed (up to five mice per cage) with littermates of the same sex. Genotyping of these mice was determined by Polymerase Chain Reaction (PCR) on their tail genomic DNA. The primers used in genotyping were the wild-type forward primer (5′-ATC GTG ACT ATC GTG GTA-3′) and the Neo forward primer specific for the target gene only (5′-GGG CCA GCT CAT TCC TTC AAT TC-3′). The reverse primer common to both target and truncated KCNQ2 gene that is present only in KCNQ2−/− mice (5′-GGG CCA GCT CAT TCC TTC AAT TC-3′). The dissected brain regions per mouse were homogenized in ice-cold homogenization buffer (solution A) containing (in mM): 320 sucrose, 1 NaHCO3, 140 sucrose, 6 Tris-HCl, 0.5% Triton-X (pH 8.0) and Halt protease inhibitors. The S2 fraction is enriched with cytosolic soluble proteins. The P2 fraction is enriched with transmembrane proteins and membrane-bound proteins. BCA assay (Pierce) analysis was performed to determine protein concentrations across samples, which were subsequently normalized to 0.5 mg/mL in Solution A (pH 7.4). The S1, S2 and P2 fractions were stored at −80°C until use. Hippocampal S2 and P2 fractions were immunoblotted with antibodies for K,7,2 (1:200, Neuromab cat# 73-079), K,7,3 (1:200, Alomone cat# APC-051) and GAPDH (1:1000, Cell Signaling) as described.54 After incubating in HRP-conjugated secondary antibodies, the blots were visualized with enhanced chemiluminescence substrate (ECL, Thermo Fisher Scientific), and developed with a Konica SRX-101A film processor.

2.3 | Slice electrophysiology

Kcnq2 heterozygous knockout and control littermates (P21–P25) were anesthetized with isoflurane and rapidly decapitated. Both male and female mice were used. The brain was quickly removed and placed in ice-cold sucrose based cutting solution consisting of the following: 25 mM NaHCO3, 200 mM sucrose, 10 mM glucose, 2.5 mM KCl, 1.3 mM NaH2PO4, 0.5 mM CaCl2 and 7 mM MgCl2. Transverse hippocampus slices were cut at 300 μm using a vibrating microtome (Leica VT1200S). Slices were then transferred to artificial cerebrospinal fluid (ACSF) consisting of the following (in mM): 125 NaCl, 26 NaHCO3, 2.5 KCl, 1 NaH2PO4, 1.3 MgCl2, 2.5 CaCl2 and 12 glucose and equilibrated at 35°C for 30 minutes, and then maintained at room temperature for at least 1 hour before electrophysiological recordings. Cutting solution and ACSF were saturated with 95% O2 and 5% CO2. All experiments were performed at near normal physiological temperature (32°C). Whole-cell recordings were obtained using borosilicate glass electrodes having resistances of 2 to 4 MΩ. For current-clamp whole cell recordings we used an internal solution consisted of the following (in mM): 130 potassium methylsulfate, 10 KCl, 5 Tris-phosphocreatine, 10 HEPES, 4 NaCl, 4 MgATP and 0.4 Na2GTP. The pH was adjusted to 7.2 to 7.3 with KOH. CNQX (4 μM), D-AP5 (10 μM) and picrotoxin (100 μM) were added in all slice experiments to block AMPA-mediated, NMDA-mediated, GABA-mediated synaptic transmission, respectively. CA1 pyramidal neurons were identified using 40x water-immersion objective lens on an upright microscope (BX51W, Olympus). Recordings were performed using a Multiclamp 700B amplifier (Molecular Devices Molecular Devices, Sunnyvale, CA), low pass-filtered at 2 kHz, sampled at 10 kHz, and analyzed offline using either Prism 7 (Graphpad) or Clamfit 10 (Molecular Devices, Sunnyvale, CA). All recordings were performed blind.

2.4 | Mice for behavioral studies

A total of 23 male mice were used (KCNQ2+/+ n = 12; KCNQ2−/− n = 11) and a total of 11 female mice were used (KCNQ2+/− n = 5; KCNQ2−/− n = 6). All behavioral tests were performed in a separate room from the colony, which were maintained on a "reverse" light:dark schedule with lights off at 10 AM and lights on at 10 PM. Under a "reverse" light:dark schedule, we were able to perform all behavioral tasks during the dark phase when the mice are more active. This behavioral test room contained a separate animal housing area which was far from the behavioral testing area. At 2 weeks before the first
behavioral test, all test mice as well as novel social target mice were moved from the initial housing room to the behavioral test room on a "reverse" 12:12 light:dark cycle where they were subsequently habituated to this new cycle for 2 weeks. All mice were tested on all described tests in the following order: open field, habituated home cage activity, elevated plus maze, rotarod, self-grooming, marble burying, social interaction test, social dominance tube and urine marking test. Each test was performed at least 2 days after the previous test. Between each test, mice were returned to the animal housing area within this behavior test room. The order of these tests was chosen to conduct in general from least invasive to most invasive tests, and to reduce the potential impact of one behavioral assay to the subsequent ones. All apparatus was cleaned with Clidox (Pharmacal Research Laboratories) after testing each mouse to eliminate any residual olfactory cues and mouse droppings. Mice were weighed at the time of each behavior study. Except for the habituated home cage activity which allows continuous video monitoring during 12:12 light:dark cycles (see the method description below), each test was always performed under red light during the dark phase starting at 10 AM as described. Mice were at 4 months old when behavioral studies began and at 6 months old when the last behavioral studies were conducted. After completing all the behavioral assays, all tested mice were used for scoring behavioral seizures induced by kainic acid except for one female KCNQ2+/− mouse because of its death.

2.5 | Open field test

The open field test was performed as described to evaluate exploratory and anxiety-like behavior in mice. Mice were placed in the center of an open field arena (26 in. × 26 in. × 12 in.) and allowed to explore for 5 minutes. Distance traveled throughout the arena, number of entries into and duration in the central square (14 in. × 14 in.), 6 in. away from the sides, were recorded by TopScan video-tracking software (CleverSystems, Reston, VA). After each trial, the mouse was returned to their home cage and the arena was cleaned before the next test mouse.

2.6 | Habituated home cage activity

Mice were placed individually into custom-made acrylic home cages (18.5 × 33.5 × 16 cm) with clear plastic lids that allow for continuous video tracking by TopScan, as described previously. Mice were measured continuously for 4 days with the normal light cycle of 12 hours of light and 12 hours of dark. After an extended habituation period of 3 days, the average distance traveled during the 4th day was compared between genotypes. Day-4 was chosen to represent behavioral patterns established in an acclimated environment of the home cage, without any confounding effects of novelty. After the test, the mice were individually housed in the animal facilities, and then left undisturbed for 4 days. The video tracking cages were cleaned prior to the next test mice.

2.7 | Elevated plus maze (EPM) test

The EPM test was performed as described to monitor open arm avoidance, exploration and general anxiety-like behavior. The maze is constructed of acrylic. There are two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm). The closed arms contain 20 cm-high walls, whereas the open arms have no walls. Arms emerge from the central zone platform (5 × 5 cm) and are arranged such that two pairs of identical arms are opposite to each other. The entire apparatus is raised to a height of 50 cm above floor. Each mouse was placed in the central zone platform of the maze and monitored for 5 minutes using the TopScan program while the experimenter sat 2 m away directly in line with the closed arms. The number of entries, the duration, the total distance traveled and the average velocity of the mice in the open and closed arms were compared between groups. After the test, the mouse was returned to their home cage and the EPM was cleaned before the next test mouse.

2.8 | Rotarod test

Rotarod testing was performed as described to assess motor coordination. Mice were placed on the rotarod apparatus starting at 0 rotation per minute (rpm). The start switch was then turned on to rotate the dowel at a constant acceleration rate (60 rpm/m). The latency of the mice to fall off the apparatus was recorded by photobeam counters as well as an experimenter using a stopwatch. Each mouse was subjected to three trials and the average latency from three trials was computed. The dowel was cleaned between each trial and dried with a paper towel.

2.9 | Self-grooming

Self-grooming behavior of the mice was observed to monitor repetitive behaviors in mice as previously described by video monitoring for 10 minutes when test mice were undisturbed in their home cages without bedding. The duration of grooming events and the total number of grooming events in 10 minutes in each individual mouse was quantified. Based on average grooming times previously exhibited in C57BL/6J mice, repetitive self-grooming was quantified as a period ≥2 seconds spent grooming with no more than a 5-second interval between grooming bouts.

2.10 | Marble burying test

Spontaneous burying of marbles by mice in a novel cage was observed to monitor compulsive-like behaviors in mice as previously described. In brief, 20 marbles were placed on top of the approximately 5 cm-deep bedding and arranged in a rectangular, 4 by 5 array in a cage that is half filled with bedding. The mouse was placed in this cage for 30 minutes. The mouse was then removed and the cage floor photographed. The total number of buried marbles was recorded. A marble that was two-thirds or more under bedding was considered
"buried" experimentally and counted. This procedure was repeated with a new cage with fresh bedding and marbles.

2.11 The three-chamber social interaction test

The three-chamber social interaction test was performed with modifications as described to measure affiliative behavior and social recognition. The three-chamber social testing rectangular arena was made with clear plexiglas. Each chamber is 20 × 40 × 25 cm in size with clear dividing walls which has rectangular openings (5 × 8 cm) allowing access into each chamber. The test was composed of three sessions: habituation, sociability and social novelty. Each session lasted 10 minutes. Between sessions, mice were removed from the chambers, and the chambers of the arena were thoroughly cleaned. For "habitation," the test mouse (KCNQ2−/− or KCNQ2+/+) was placed in the center chamber and allowed to freely explore the empty left and right chambers. After the habituation in the empty three-chamber apparatus for 10 minutes, the test mouse was reintroduced to the center chamber and allowed to explore the left and right chambers each of which contained an empty cylinder wire cage (15 cm diameter and 20 cm height). During this habituation period, the test mouse did not show any baseline side preference for either a left or a right chamber (Figure S2, Tables S6-7). In the "sociability" session, a C57BL/6J mouse (a stranger mouse-1 of the same sex which never encountered the test mouse before) was placed underneath the wire cage in the left chamber. The test mouse was then placed in the center chamber and allowed to freely explore the left chamber containing the novel social target and the right chamber containing an empty cage. The wire cage allows olfactory, visual, auditory and minimal tactile interaction but prevents fighting between the test mouse and the stranger mouse-1. This first session measures affiliative behavior of a test mouse which is defined as the propensity to spend time with a stranger mouse rather than with a familiar mouse.

2.12 Tube dominance test

The tube dominance test was performed as described to measure social approach-avoidance behavior and measure dominant vs submissive behaviors in mice. The tube apparatus is composed of clear plexiglas 30 cm in length and 4 cm diameter, a size just sufficient to permit one adult mouse to pass through without reversing direction and to restrict aggressive fights. Mice of the same sex but different genotypes (ie, the test mouse [KCNQ2−/− or KCNQ2+/+] against CD-1 mouse) were released into opposite ends of a tube at the same time. The subject mouse (KCNQ2−/− or KCNQ2+/+) was tested against an outbred CD-1 (ICR) mouse of the same sex purchased from the Charles River Laboratories. The CD-1 mice were used as social targets in this test because they have been shown to display more aggressive behavior than C57BL/6J mice. The test was repeated using a round-robin design such that each KCNQ2−/− or KCNQ2+/+ mouse was matched with every other CD-1 mouse 2 times. The two mice interacted in the center of the tube. The more dominant mouse will show greater aggression and force its opponent out of the tube. When one mouse has all four paws out of the tube, it is declared the loser, ending the match. The dominant mouse remaining inside the tube is declared the winner. The winner receives +1 point, whereas the loser gets −1 point. If the two mice stay or pass each other in the tube, both mice would receive 0. The number of wins is reported as a percentage (%) of total number of matches.

2.13 Urine marking test

The urine marking assay was performed as described to observe social dominance hierarchy. In this test, dominant mice mark larger territories than subordinate mice. The filter paper was placed in an empty cage. The cage was divided into two compartments of equal size by a wire mesh partition which allows mice in each compartment to see and smell each other but prevents their physical contact. Each pair of mice (ie, the test mouse KCNQ2−/− or KCNQ2+/+ against CD-1 mouse purchased from the Charles River Laboratories) was placed on opposite sides of a partition and allowed to interact through the wire mesh partition and mark on the filter paper for 30 minutes. After the end of each session, the mice were moved to their home cages. The urine-marked filter papers were dried overnight. To analyze the urine marks visualized by a UV light source, a transparent grid (each 1 × 1 cm) was placed over the dried filter paper. The number and the area of scent marks as well as the average distance of urine marking from the wire partition were measured.

2.14 Kainate-induced seizures

To measure seizure propensity, both male and female mice were subjected to intraperitoneal (i.p.) injection of vehicle control (saline) or kainate (Abcam, 15 mg/kg) as described. The mice were at 6-month old, and weighed 24 to 33 g when kainate injections were performed in the laboratory under the bright light. Mice were returned to their home cage and monitored for their behavioral seizures every 10 minutes using modified Racine, Pinal and Rovner scale, which consists of eight
stages: (stage-1) facial movements only, (stage-2) facial movements and head nodding, (stage-3) facial movements, head nodding and forelimb clonus, (stage-4) facial movements, head nodding, forelimb clonus and rearing, (stage-5) facial movements, head nodding, forelimb clonus, rearing, loss of balance and falling, (stage-6) a stage-5 terminating with multiple rearing and falling episodes, (stage-7) a stage-6 with a violent jumping and running fit, (stage-8) a stage-7 with periods of tonic and (stage-9) death. At 2 hours post injection, the mice were euthanized and their brains were stored at −80 °C until use.

2.15 | Statistical analysis

All analyses are reported as mean ± SEM. The n values indicate number of mice. Origin Pro 9.5 (Origin Lab) was used to perform statistical analyses. Data were analyzed using two-way ANOVA with genotype as one factor and sex as the other. For social interaction tests, data were analyzed using three-way repeated measures ANOVA with chamber entered as the within-subjects factor, and genotype and sex as between-subjects factors. In cases where the three-way interaction was significant, two-way repeated measures analyses were conducted separately for each sex. Tukey tests were used to establish post hoc pair-wise differences between means of >2 groups. The Student two-tailed t test was used to establish post hoc pair-wise differences between means of two groups. A priori value (P) < .05 was used to establish statistical significance.

3 | RESULTS

3.1 | Heterozygous loss of KcNQ2 increases exploratory behavior without affecting habituated home cage activity or motor coordination

To examine the effects of the heterozygous loss of KcNQ2 in behaviors, both male and female heterozygous KcNQ2+/− mice and their wild-type KcNQ2+/+ littermates at age 16 weeks were subjected to nine behavioral tests and then kainate-induced seizures (Figure 1A–C, Table S1). After confirming the genotype (Figure 1B,C), the mice were tested during the dark phase when they were most active because of their nocturnal nature (Figure 1A).

In the home cage, both male and female KcNQ2+/− mice displayed similar total 24 hours activity as the wild-type mice on day 4 (genotype: \( F_{(1, 30)}=0.02, P=0.889 \)) (Figure 2A,B). The KcNQ2+/− mice showed similar activity in their home cage during the dark phase compared with wild-type mice (genotype: \( F_{(1, 30)}=0.80, P=0.378 \)) (Figure 2A,C, Table S2). During the light phase when mice were generally less active, KcNQ2+/− mice displayed significantly greater activity compared with wild-type mice (genotype: \( F_{(1, 30)}=10.08, P=0.003 \)) (Figure 2A,D, Table S2). Both KcNQ2+/− and KcNQ2−/− mice were similar in their latency to fall from the rotarod (genotype: \( F_{(1, 30)}=0.13, P=0.726 \)), indicating no gross abnormality in motor coordination (Figure 2E, Table S2). In the open field test, the total distance traveled in the entire arena was not significantly different between KcNQ2+/− and KcNQ2−/− mice (genotype: \( F_{(1, 31)}=0.07, P=0.796 \)) (Figure 3A,B, Table S3). Taken together these results indicate that KcNQ2+/− and KcNQ2−/− mice show comparable levels of physical activity and motor coordination in tests of exploratory behavior and in the home cage under habituated conditions and motor coordination on the rotarod.

However, the KcNQ2−/− mice displayed a higher number of entries into the center of the open field arena compared with the wild-type littersmates (genotype: \( F_{(1, 31)}=6.38, P=0.017 \)) (Figure 3A,C, Table S3). Furthermore, both male and female KcNQ2−/− mice spent significantly more time within the center of the arena than the wild type mice (genotype: \( F_{(1, 31)}=12.57, P=0.001 \)) without increasing the total distance traveled within the center (genotype: \( F_{(1, 31)}=2.23, P=0.145 \)) (Figure 3D,E, Table S3). Interestingly, the male KcNQ2−/− mice traveled faster whereas the female KcNQ2−/− moved slower within the center of the arena than the wild type male mice (sex: \( F_{(1, 31)}=5.74, P=0.026 \), interaction:

FIGURE 1  An overview of behavioral assays performed with KcNQ2+/− and KcNQ2−/− mice. A, The order of behavioral assays performed on KcNQ2+/− (WT) and KcNQ2−/− (Q2+/−) mice. All behavioral assays mice were performed in the dark phase except for the home cage activity during a light-dark cycle (12 hours) each measured over 4 days. B, Sample genotyping results for the male (M) cohorts of WT and Q2+/− mice showing a 240-bp PCR product from the wild-type allele ("E" lanes) and a 427-bp product from the knockout allele ("T" lanes). C, Western blots of KcNQ2, KcNQ3 and GAPDH in the hippocampal membrane P2 fractions of WT and Q2+/− mice at age 3 and 5 months. The Q2+/− mice displayed reduced protein expression of KcNQ2 but not KcNQ3 in the hippocampi compared with the WT mice.
The KCNQ2+/− mice show increased home cage activity during the light phase. A-D, Home cage activity of male and female KCNQ2+/+ (WT) and KCNQ2+/− (Q2+/-) mice was examined using continuous video recording for 4 days. A, Sample traces of home cage activity of KCNQ2+/+ (WT) and KCNQ2+/− (Q2+/-) mice at the 4th day of recording. The locomotor activities of male mice and female mice were assessed by measuring the distance which they traveled per minute over a 12 hours dark phase (Light OFF) followed by a 12 hours light phase (Light ON). Quantifications of the average total distance traveled during a 24 hours period (B), a 12 hours dark phase (C) and a 12 hours light phase (D). The total distance traveled by Q2+/- mice during the light phase was significantly greater than that of WT mice for both sexes. E, The rotarod tests revealed no difference in latency to fall for WT and Q2+/- mice. Male mice (WT n = 12; Q2+/- n = 11). Female mice (WT n = 5; Q2 +/- n = 6). Data shown represent the Ave ± SEM. Student t test results are shown here (*P < .05). Table S2 shows two-way ANOVA test results with genotype as one factor and sex as the other.
In the EPM, the total distance traveled throughout the EPM was not different between KCNQ2+/− and wild-type mice (genotype: F(1, 30)=1.92, P=0.968) (Figure 4A,B, Table S4). However, both male and female KCNQ2+/− groups displayed significantly more entries into the open arms (genotype: F(1, 30)=10.52, P=0.003) (Figure 4A,C, Table S4). Compared with the wild-type group, the KCNQ2+/− mice traveled longer distances and spent more time in the open arms (genotype: F(1, 30)=6.99, P=0.013) (Figure 4D-F, Table S4). There was a trend in the distances traveled in the open arms (genotype: F(1, 30)=4.11, P=0.052) and a significant effect of sex in the latency to enter the open arms (genotype: F(1, 30)=5.24, P=0.029) (Figure 4E,F, Table S4). In contrast, no significant differences in the total number of entries, distance and duration in the closed arms were observed in the KCNQ2+/− mice compared with the wild type mice (Figure S1, Table S4).

These results together suggest that the heterozygous loss of Kv7.2 leads to enhanced exploratory behavior or decreased anxiety-like behavior, without affecting motor coordination or general activity levels.

### 3.2 | Heterozygous loss of Kv7.2 increases repetitive and compulsive-like behaviors

During the video recording of the home cage activity, we observed that the KCNQ2+/− mice have the tendency to groom more than the wild-type littermates. To test if this trend is significant, we quantified the grooming behavior of KCNQ2+/+ and KCNQ2+/− mice. The KCNQ2+/− mice displayed bouts of self-grooming that lasted between 10 and 25 seconds (Figure 5A), consistent with the reported duration of self-grooming in C57BL/6J strain for both sexes.72 Compared with the wild type littermates, the average duration of a grooming event was increased in KCNQ2+/− mice (genotype: F(1, 30)=8.79, P=0.006) (Figure 5A) and sex differences were observed (sex: F(1, 30)=13.77, P=0.001) (Table S5). The total number of grooming events in the 10 minutes test period was different between KCNQ2+/+ and KCNQ2+/− groups (genotype: F(1, 30)=5.19, P=0.03) (Figure 5B). Such elevation in the innate self-grooming behavior is suggestive of repetitive behaviors.60,61

To investigate this further, we performed a marble burying test which scores spontaneous burying of marbles in rodents as a measure of their compulsive-like behavior.62,63 In this test, the KCNQ2+/− mice buried more marbles than the wild-type mice (genotype: F(1, 30)=5.82, P=0.022). There was a significant interaction between sex and genotype (interaction: F(1, 30)=7.06, P=0.013). Post hoc tests indicated that the male KCNQ2+/− mice buried twice as many marbles as the wild-type mice (Tukey test, P=0.001) (Figure 5C,D, Table S5). This genotype difference in marble burying behavior was not observed in the females (Tukey test, P=0.999) (Figure 5C,D, Table S5). These findings
indicate that heterozygous loss of Kv7.2 increases compulsive-like behaviors in male mice but not female mice.

3.3 | Heterozygous loss of Kv7.2 decreases social interest

To examine social behaviors, we first used the three-chamber social interaction test which measures social motivation and approach (Figure 6A). After habituation in the empty three-chamber apparatus for 10 minutes, the subject mouse (KCNQ2+/− or KCNQ2−/−) was reintroduced to the center chamber of the apparatus that contained an empty wire cage in the left and right chambers. During this second habituation time, the subject KCNQ2+/− or KCNQ2−/− mouse spent similar amounts of time in the left and right chambers (Figure S2A), as indicated by no significant main effect of chamber ($F_{(1, 30)}=1.89$, $P = .179$) or interaction between chamber and genotype ($F_{(1, 30)}=0.19$, $P = .663$). Both genotypes also displayed similar sniffing duration toward the empty wire cages (chamber: $F_{(1, 30)}=0.52$, $P = .477$; interaction between chamber and genotype: $F_{(1, 30)}=1.73$, $P = .198$), and latency to enter the closed arms, latency to enter the closed arms and total distance traveled in the closed arms. Table S4 shows two-way ANOVA test results with genotype as one factor and sex as the other.
To test social motivation and approach, a novel social target C57BL/6J mouse (stranger mouse-1) was placed in the wire cage of the left chamber and the subject (KCNQ2+/− or KCNQ2+/+) mouse was introduced in the center chamber (Figure 6A). The three-way ANOVA indicated a trend for an interaction between chamber and genotype for duration spent on the left or right sides of the chamber (F(1, 30) = 4.14, P = .051). Hence, data were analyzed using separate two-way ANOVAs for each chamber. The two-way repeated ANOVA revealed that KCNQ2+/+ mice spent significantly more time in the left chamber containing the stranger mouse-1 than KCNQ2+/− mice (genotype: F(1, 30)=5.05, P = .032) whereas both genotypes spent similar duration at the right chamber containing an empty cage (genotype: F(1, 30)=0.93, P = .343) (Figure 6B, Tables S6-7). Interestingly, sex differences were observed for the duration at the left chamber (sex: F(1, 30)=23.87, P < .0001) and duration of sniffing at the stranger mouse-1 (sex: F(1, 30)=9.20, P = .005). Females spent longer time in the left chamber (Figure 6B) but sniffed a stranger mouse-1 for shorter time than males did (Figure 6C, Tables S6-7). In addition, KCNQ2+/+ males spent significantly more time sniffing at the stranger mouse-1 compared with KCNQ2+/− males (genotype: F(1, 21)=5.07, P = .035) (Figure 6C, Tables S6-7). Lastly, the KCNQ2+/− mice also displayed increased latency of sniffing at the novel social target compared with the wild-type mice (genotype: F(1, 30)=4.59, P = .040) (Figure 6C,D, Tables S6-7). These results together indicate that the KCNQ2+/− mice display reduced social approach and motivation than their wild type littermates.

To test the preference for social novelty and social memory, another novel target C57BL/6J mouse was placed in the wire cage of the right chamber (Figure 6A). In the second session of this three-chamber test, a mouse would normally recall its previous contact with the stranger mouse-1 in the left chamber and thus will spend more time with the newly introduced stranger mouse-2 in the right chamber (Figure 6A). A significant three-way interaction between sex, genotype and chamber was observed for duration at the chamber (interaction: F(1, 29)=8.60, P = .007) and duration sniffing (interaction: F(1, 29)=6.93, P = .014) (Table S6), hence sexes were analyzed separately (Figure 6E,F). Within males, a main effect of chamber was observed (chamber: F(2, 40)=28.82, P < .0001), but no significant effect of genotype, or interaction between genotype and chamber was detected. These data indicate that both KCNQ2+/+ and KCNQ2+/− males preferred the novel mouse (a stranger mouse-2) in the right chamber.

In females, a significant interaction was observed between genotype and chamber for duration (interaction: F(2, 19)=4.49, P = .026). Wild-type females spent a longer time in the right chamber containing a stranger mouse-2 than the left chamber containing a familiar mouse (Tukey test, P = .026) (Figure 6E, Tables S6-7), indicating that they display preference for social novelty. In contrast, KCNQ2+/− female mice
FIGURE 6  The KCNQ2+/− mice display reduced sociability. The KCNQ2+/− (WT) and KCNQ2+/− (Q2+/−) mice were subjected the three-chamber social interaction test. A, The design of the three-chamber social interaction test. The test mouse was first habituated in the empty apparatus for 10 minutes (step-1) and then in the apparatus containing an empty wire cage each in the left and right chambers for 10 minutes (step-2). During the “sociability” test, a stranger mouse-1 (age-matched C57BL/6J) was placed inside the wire cage of the left chamber, and the test mouse was introduced into the center chamber and allowed to explore for 10 minutes (step-3). During the “social novelty” test, a second stranger mouse-2 (another age-matched C57BL6) was placed inside the wire cage of the right chamber, and the test mouse was introduced into the center chamber and allowed to explore for 10 minutes (step-4). B-D, Quantifications of the time spent at each chamber (B) as well as the duration (C) and latency (D) of sniffing behavior during the “sociability” test. E-G, Quantifications of the time spent at each chamber (E) as well as the duration (F) and latency (G) of sniffing behavior during the “social novelty” test. Male mice (WT n = 12; Q2+/− n = 11). Female mice (WT n = 5; Q2+/− n = 6). Data shown represent the Ave ± SEM. Post hoc Tukey test results are shown here (*P < .05, ***P < .005). Figure S2 shows the quantification of the time spent at each chamber, as well as the duration and latency of sniffing behavior during the “habituation with 2 empty cages.” Table S6 shows three-way ANOVA test results and Table S7 shows two-way ANOVA test results with chamber as one factor and genotype as the other, or with genotype as one factor and sex as the other.
spent similar times in left and right chambers (Tukey test, $P = .546$) (Figure 6E, Tables S6-7). Similarly, in females, a significant interaction was observed between genotype and chamber for duration sniffing (interaction: $F_{1,9} = 7.12, P = .026$). Wild-type females spent more time sniffing a new stranger mouse-2 compared with a familiar mouse (Tukey test, $P = .049$) whereas KCNQ2+/- females did not (Tukey test, $P = .921$) (Figure 6F). KCNQ2+/- females spent less time sniffing a new stranger mouse-2 compared with their wild-type littermates (Tukey test, $P = .047$) (Figure 6F, Table S7). Finally, a significant interaction between genotype and chamber was observed for latency to visit the novel mouse in females (interaction: $F_{1,9} = 6.54, P = .031$). Post hoc tests indicated a trend for the KCNQ2+/- to display a longer latency to sniff the new stranger mouse-2 as compared with KCNQ2+/- (Tukey test, $P = .056$) (Figure 6G, Tables S6-7) whereas no genotype difference was observed for latency to sniff the familiar mouse. Taken together, these results on duration, sniffing and latency indicate that the KCNQ2+/- female mice display no preference for the novel social target compared with the familiar social target.

### 3.4 Heterozygous loss of Kv7.2 increases social dominance

To further examine social behaviors, we next performed the tube dominance test (Figure 7A), which provides a reliable measure of social dominance and social aggression in rodents.67-69 We found main effects of genotype for the average score (genotype: $F_{1,30} = 9.56, P = .004$) and % of wins (genotype: $F_{1,30} = 4.72, P = .038$). KCNQ2+/- displayed higher total score and greater % of wins compared with wild-type mice.

**FIGURE 7** The KCNQ2+/- mice display increased social dominance behavior. A-C, The KCNQ2+/- (WT) and KCNQ2+/- (Q2+/-) mice were subjected to the tube dominance test. A, The schematic of the tube dominance test. The test mouse (WT or Q2+/-) is placed at one end of the tube and the age matched control mouse (CD-1) is placed at the other end at the same time. A mouse which forces its opponent to retreat backward and exit the tube is deemed the winner and receives +1 point. The retreating mouse is deemed the loser and receives −1 point. If the mice stay in the middle of the tube or pass each other, they receive 0. B-C, Quantifications of average score (B) and % of win (C). The male and female Q2 +/- mice received higher scores and % of win than the WT mice against CD-1 mice. D-G, Male WT and Q2+/- mice were subjected to urine marking test for 30 minutes. D, Schematic of a urine test during which the test mouse and CD-1 mouse were separated by a wire mesh partition. Quantifications of the number of urine marks (E), the total area of urine marks (F) and the distance of urine marks from the partition (G). Both male and female Q2+/- mice displayed significantly more urine marks with larger areas near the partition compared with the WT mice. Male mice (WT n = 12; Q2+/- n = 11). Female mice (WT n = 5; Q2+/- n = 6). Data shown represent the Ave ± SEM. Student t test results are shown here (*P < .05, **P < .01, ***P < .005). Table S8 shows two-way ANOVA test results with genotype as one factor and sex as the other.
wins against male CD-1 mice than KCNQ2<sup>+/−</sup> (Figure 7B,C). We also observed sex differences for % of wins (sex: \( F_{1, 30} = 5.09, \ P = .032 \)). Compared with males, all tested female KCNQ2<sup>+/−</sup> mice retreated out of the tube whereas most female KCNQ2<sup>+/−</sup> mice stayed in the middle of the tube facing female CD-1 mice during the entire duration of this test (Figure 7B). Only a few female KCNQ2<sup>+/−</sup> mice forced the CD-1 mice out of the tube, resulting in a small % of wins and no genotype differences (Figure 7C, Table S8).

To confirm that the KCNQ2<sup>+/−</sup> mice are more socially dominant than the wild-type mice, we performed the urine marking test (Figure 7D), in which the socially dominant male mice mark larger territories than the subordinate mice for social communication and convey dominance status.\(^{69,70}\) We found a significant interaction between sex and genotype for the total number of urine marks (interaction: \( F_{1, 89} = 0.14, \ P = .032 \)). When novel CD-1 mice were introduced, both male and female KCNQ2<sup>+/−</sup> mice displayed a significantly larger number of urine markings compared with the wild type mice (genotype: \( F_{1, 89} = 0.13, \ P = .019 \)), although females marked far less than males (sex: \( F_{1, 89} = 0.12, \ P = .0003 \)) (Figure 7E, Table S8). KCNQ2<sup>+/−</sup> mice also displayed a greater area of urine markings compared with the wild type mice (genotype: \( F_{1, 89} = 9.50, \ P = .003 \)) and sex differences were observed (sex: \( F_{1, 89} = 20.60, \ P < .0001 \)) (Figure 7G). These results are consistent with the tube dominance test findings that heterozygous loss of K<sub>7.2</sub> enhances socially dominant behavior in mice.

### 3.5 Heterozygous loss of K<sub>7.2</sub> increases seizure propensity and hippocampal excitability

Heterozygous knockout of KCNQ2 gene has been previously shown to elevate sensitivity to pentylentetrazole-induced seizures.\(^ {44}\) To confirm whether KCNQ2<sup>+/−</sup> mice in our studies display heightened seizure propensity, we subjected KCNQ2<sup>+/+</sup> and KCNQ2<sup>+/−</sup> mice to i.p. injections of chemoconvulsant kainate (kainic acid) at a lower dose of 15 mg/kg. Systemic injections of kainate cause epileptiform seizures in the hippocampus which propagate to other limbic structures and induce hippocampus-restricted neuropathology comparable to those seen in patients with temporal lobe epilepsy (TLE).\(^ {73}\) The average cumulative behavioral seizure score for KCNQ2<sup>+/−</sup> mice was significantly higher than that of KCNQ2<sup>+/+</sup> sibling mice at 6-month of age (genotype: \( F_{1, 27} = 35.22, \ P < .0001 \)) (Figure 8A,B) although both groups had similar weight (genotype: \( F_{1, 27} = 0.16, \ P = .688 \)) (Figure 8C, Table S9). Sex differences were observed for seizure score (sex: \( F_{1, 27} = 6.05, \ P = .021 \)) and weight (sex: \( F_{1, 27} = 34.49, \ P < .0001 \)) (Table S9). These results indicate that heterozygous loss of K<sub>7.2</sub> increases seizure propensity at 6 month of age and eliminate the body weight as a confounding factor in our behavioral studies. Consistent with increased seizure susceptibility, deletion of one Kcnq2 copy significantly increased excitability of CA1 pyramidal neurons (Figure 8D).

We assessed CA1 pyramidal neuron excitability by injecting an increasing amount of current for 1 second in cells held at the resting membrane potential. We found that CA1 pyramidal neuron firing induced by 300 pA injection plateaued at 36 ± 2.1 APs in wild-type mice and at 43 ± 2.2 APs in KCNQ2<sup>+/−</sup> mice (\( t = 2.302, df = 16, \ P = .0351 \)). These results indicate that the KCNQ2<sup>+/−</sup> hippocampal CA1 neurons are more excitable than their wild-type counterparts.

### 4 DISCUSSION

Recent whole-exome or targeted sequencing studies on well-defined ASD cohorts have implicated heterozygous variants in KCNQ2 and KCNQ3 genes,\(^ {12}\) suggesting that disruption of K<sub>7.2</sub> currents may contribute to ASD. In this study, we provide strong evidence that neuronal K<sub>7.2</sub> channels containing K<sub>7.2</sub> contribute to multiple behaviors in mice including circadian-dependent activity, exploratory behavior, social behavior, repetitive and compulsive-like behavior, in addition to dampening seizure susceptibility (Figure 9). Our findings also reveal that genetic heterozygous loss of K<sub>7.2</sub> in mice leads to behavioral abnormalities reminiscent of clinical symptoms of ASD.\(^ {5,6,8,9,74,75}\)

#### 4.1 Role of K<sub>7.2</sub> channels in locomotor activity

We discover that KCNQ2<sup>+/−</sup> mice show increased home cage activity during the light phase compared with their wild-type siblings without grossly affecting their motor activity and coordination, although no differences were detected during the dark phase when mice are most active (Figure 2, Table S2). These results suggest that reduction in K<sub>7.2</sub> current leads to hyperactivity during the light phase when mice are generally less active. These findings are reminiscent of clinical reports that some ASD patients experience sleep problems.\(^ {5}\) K<sub>7.2</sub> is expressed in the reticular thalamic nucleus,\(^ {16}\) a brain area critical in regulating sleep wake cycle transitions.\(^ {76}\) Therefore, heterozygous loss of K<sub>7.2</sub> in reticular thalamic nucleus could also disrupt wake to sleep transition, leading to hyperactivity of mice during the inactive light phase.

Although speculative, hyperactivity of KCNQ2<sup>+/−</sup> mice during the light phase (Figure 2) may also result from altered circadian-dependent activity. K<sub>7.2</sub> is highly expressed in the hippocampus which projects directly to suprachiasmatic nucleus (SCN) in the hypothalamus,\(^ {77}\) the central coordinator of circadian rhythm.\(^ {78}\) The hippocampus expresses all clock genes necessary for circadian oscillation of neuronal activity that modulates hippocampal-dependent learning and memory.\(^ {79}\) Interestingly, human patients with TLE and rodent models of TLE display more seizures during the day.\(^ {80}\) Considering a very strong K<sub>7.2</sub> expression in the hippocampus and kainate-induced status epilepticus originates from hippocampal hyperexcitability,\(^ {73}\) it is tempting to speculate that increased seizure propensity and home cage activity of KCNQ2<sup>+/−</sup> mice during the inactive light period (Figures 2 and 8A,C) may result from reduced K<sub>7.2</sub> current and a subsequent increase in hippocampal excitability (Figure 8D) that may alter SCN rhythmicity in these mice.
4.2 Role of Kv7 channels in exploratory behavior, anxiety or perception of danger

Mice display aversions to environments that are new, brightly lit, open or large. They perceive these environments as dangerous and show anxiety responses. In our open field and EPM tests performed during the dark phase, KCNQ2+/− mice exhibited significant increases in the number of entries and the time spent in the center of the open field and the open arms in the EPM compared with the wild-type mice (Figures 3 and 4, Tables S3, S4). The increased exploratory behavior of KCNQ2+/− mice in these tests suggests that heterozygous loss of Kv7.2 reduces anxiety-related behavior in mice. This is somewhat contrary to the well-documented reports that patients with ASD experience greater anxiety than those without ASD. However, multiple mouse models of autism and Fragile X Syndrome (FXS) which accounts for about 2% to 3% of all ASD cases show reduced nonsocial anxiety measured by the same behavioral tests although they display typical autism-associated behaviors including social avoidance and repetitive behaviors. For example, FXS mouse model Fmr1...
knockout mice have been reported to spend more time in the open arms on the EPM test, and such behavior could be attributed to cognitive impairment.\textsuperscript{82,87}

Although the open field and EPM tests are widely used to examine anxiety in mice,\textsuperscript{81} increased entry and duration of KCNQ2\textsuperscript{+/−} mice to the exposed center or open arms could result from their hyperarousal to novel environments, especially considering that these tests were performed in the dark (Figures 3 and 4). K\textsubscript{\textit{r}}7 currents regulate intrinsic excitability of pyramidal neurons in the hippocampus and cortex including prefrontal cortex,\textsuperscript{14,21-25,47} the brain regions implicated in higher cognitive and executive functions.\textsuperscript{88,89} Therefore, our data may also reflect a decrease in the cognitive ability of KCNQ2\textsuperscript{+/−} mice to recognize the potential danger of open spaces and/or perceive fear, similar to Fmr1 knockout mice.\textsuperscript{82,87} Indeed, patients with ASD were slower to detect and respond to both social and nonsocial hazards in comparison to individuals without ASD.\textsuperscript{74,90} The underlying circuitry affected in KCNQ2\textsuperscript{+/−} mice is unclear. Elevated activity in the amygdala is associated with increased anxiety and perception of fear,\textsuperscript{91,92} whereas the medial prefrontal cortex (PFC) exerts an inhibitory effect on the amygdala.\textsuperscript{93} Considering the enrichment of K\textsubscript{\textit{r}}7,2 in the medial PFC but not the amygdala,\textsuperscript{94,95} reduced anxiety and/or perception of danger and fear could be attributed to inhibition of amygdala by increased medial PFC activity in KCNQ2\textsuperscript{+/−} mice.

### 4.3 Role of K\textsubscript{\textit{r}}7 channels in repetitive and compulsive-like behavior

Repetitive self-grooming behaviors in rodents are thought to recapitulate pathological repetitive behaviors seen in humans\textsuperscript{60} and are widely used to identify neural circuits and genes underlying repetitive behaviors in ASD.\textsuperscript{61} For example, the knockout mice for ASD-associated SHANK2-3 genes display increased frequency and/or duration of self-grooming in addition to social deficits.\textsuperscript{96,97} We discover that KCNQ2\textsuperscript{+/−} mice exhibited significant increases in the duration of self-grooming compared with the wild-type mice (Figure 5A,B, Table S5). Abnormal stereotypic grooming behavior is mediated by GABAergic output from the striatum\textsuperscript{98} and K\textsubscript{\textit{r}},7 channels are potent regulators of excitability in striatal presynaptic dopaminergic activity and GABAergic striatal medium spiny neurons.\textsuperscript{99,100} Therefore, our findings of increased self-grooming duration in KCNQ2\textsuperscript{+/−} mice (Figure 5A,B) may be attributed to the changes in presynaptic and/or postsynaptic striatal activity upon reduction in K\textsubscript{\textit{r}},7 current.

Another behavioral assay which we performed with the KCNQ2\textsuperscript{+/−} mice was marble burying test, which uses the tendency of mice to dig burrows or escape tunnels in nature.\textsuperscript{52,63} Although this test has been considered to assess the repetitive and compulsive-like behaviors of obsessive-compulsive disorder (OCD), this test has also been used to measure for autistic behavior or anxiety.\textsuperscript{61,101} OCD features disruptive thoughts (termed obsessions) that leads to behavioral compulsions such as repetitive behaviors often to reduce anxiety caused by the obsessions.\textsuperscript{62} In this study, we discover that the male but not female KCNQ2\textsuperscript{+/−} mice buried more marbles than the wild-type mice (Figure 5C,D, Table S5). These sex differences in marble-burying behaviors of KCNQ2\textsuperscript{+/−} mice are intriguing because of the sex differences seen in anxiety disorders and autism. At age 5 and younger, boys with autism have more restricted interests and repetitive behaviors than girls diagnosed with autism.\textsuperscript{102,103} In contrast, multiple anxiety disorders except for social anxiety disorder are more prevalent in women than men.\textsuperscript{104} Because the female KCNQ2\textsuperscript{+/−} mice show reduced anxiety in open field and EPM tests to a similar extent as male mice (Figures 3 and 4), increased marble-bearing behaviors in male KCNQ2\textsuperscript{+/−} mice (Figure 5C,D) may most likely reflect repetitive and compulsive-like behavior rather than increased anxiety.

### 4.4 Role of K\textsubscript{\textit{r}},7 channels in social interaction and motivation

Impaired social interaction is one of the core symptoms of autism in humans.\textsuperscript{105} Social interaction is driven by social motivation and social recognition. Social motivation reflects the interest to approach and interact with another conspecific social target, whereas social recognition reflects the ability to distinguish between different social targets. In the three-chamber social interaction test, KCNQ2\textsuperscript{+/−} spent longer time in the chamber sniffing the stranger mouse-1 than in the chamber containing the empty cage, but KCNQ2\textsuperscript{+/−} mice did not (Figure 6A-D, Tables S6-7). These results suggest deficits in social recognition, motivation or both. The impaired sociability of KCNQ2\textsuperscript{+/−} mice are reminiscent of the core clinical symptoms of ASD and suggest that reduced K\textsubscript{\textit{r}},7 current may contribute to ASD-associated behaviors in patients with de novo KCNQ2 mutation.

Furthermore, the female KCNQ2\textsuperscript{+/−} mice displayed reduced preference for novel social target compared with the female wild-type littermates (Figure 6E-G, Tables S6-7). Such behavior of KCNQ2\textsuperscript{+/−} females may reflect defects in the formation or retrieval of the memory of the familiar mice, just as children with autism struggle to form
working memories and autobiographical memories and show defects in social cognition. The male KCNQ2+/− mice exhibited preference for novel stranger C57BL/6J mice compared with familiar mice (Figure 6E-G, Tables S6-7) consistent with the previous report of the preference of the C57BL/6J males for novel males. Interestingly, the two-way ANOVA revealed that KCNQ2+/− males also exhibited preference for novel stranger C57BL/6J mice, revealing significant sex differences (Figure 6E-G, Tables S6-7).

Kv7 channels are highly expressed in the hippocampus and cortex including medial PFC, brain regions important for social approach and cognition. We speculate that hippocampal and cortical hyperexcitability (Figure BD) especially in the medial PFC of KCNQ2+/− mice may disrupt their social recognition and/or memory, resulting in altered social behaviors in these mice. Recent studies on MRI brain scans in children with autism have revealed that the mesolimbic reward pathway, the key reward circuit in the brain, is impaired in children with ASD and the degree of such structural impairment correlates with greater social impairment of the patients. This interesting study supports the notion that social interaction is generally perceived as rewarding in healthy individuals but not in patients with ASD. Therefore, altered Kv7 activity in the ventral tegmental area and nucleus accumbens within the mesolimbic dopamine system may also contribute to decreased sociability in KCNQ2+/− mice.

4.5 | Role of Kv7 channels in social dominance and aggression

As a major aspect of social hierarchy, social dominance can be observed in many social species across the animals, human and nonhuman primates. Socially dominant rodents are more aggressive and display reduced fear and stress responses. The increased social dominant behaviors of the KCNQ2+/− mice (Figure 7A-C, Table S8) are intriguing in the context of the prevalence of aggressive behaviors in children with ASD. Aggression positively correlates with the severity of social impairment and repetitive behavior. Functional involvement of PFC in social dominance and aggression has also been reported in humans and rodents. Socially, dominant mice display increased excitatory synaptic strength in layer V pyramidal neurons in the medial PFC where K7.2 is highly expressed (alleninstitute.org/). Because K7 current suppresses intrinsic excitability and postsynaptic transmission of PFC layer V pyramidal neurons, the heterozygous loss of K7.2 may likely contribute to the increased social dominant behaviors in KCNQ2+/− mice by increasing the activity of PFC layer V pyramidal neurons.

Enhanced social dominant behavior can also be viewed as a risk-taking behavior, because such behavior will increase the social challenges in rodents. Potential risk-taking behaviors of KCNQ2+/− mice are reflected in their behaviors during the open field and EPM tests (Figures 3 and 4). In these tests, mice are exposed to two conflicting choices. One choice is their interest to explore the novel environment. The second choice is to avoid exploration especially in the exposed area because such action is too risky and evokes fear. Interestingly, damages to the PFC are shown to impair decision making involving evaluations about risks and rewards and patients with damaged PFC make risky choices and decisions. Therefore, it is possible that increased excitability and synaptic strength in the PFC of KCNQ2+/− mice may alter their risk assessment and fear response, ultimately leading them to make risky decisions.

4.6 | Role of Kv7 channels in hippocampal and cortical hyperexcitability

Approximately 30% of autistic children display epilepsy, and conversely, several forms of epilepsy also display ASD as a comorbidity. Concerted effort of genome sequencing has identified de novo heterozygous mutations in K7.7 and K7.3 in patients with ASD. Two pathogenic heterozygous KCNQ2 variants (M1T and M1V) that disrupt the start codon ATG have been identified in patients with ASD. Interestingly, two pathogenic heterozygous KCNQ2 variants (M1T and M1V) that disrupt the start codon ATG have been identified in patients with ASD. Interestingly, two pathogenic heterozygous KCNQ2 variants (M1T and M1V) that disrupt the start codon ATG have been identified in patients with ASD. These variants essentially lead to heterozygote ablation of KCNQ2, supporting the use of the KCNQ2+/− mice for investigating how heterozygous loss of KCNQ2 affects seizure generation and behaviors. Although KCNQ2+/− mice did not display spontaneous seizures, we found that KCNQ2+/− mice show increased seizure susceptibility to chemically kainate and enhanced hippocampal excitability (Figure 8, Table S9).

Developmental delay and intellectual disability in ASD could result from abnormal hyperactivity in the hippocampus that express K7.2 and mediates rhythmic theta oscillation critical for learning and memory and synaptic plasticity. M-channels have been shown to facilitate hippocampal network resonance at the theta frequency in rat hippocampal neurons. Furthermore, pharmacological activation of K7 channels dampened stress-induced impairments in memory retrieval and hippocampal long-term potentiation (LTP). Conversely, K7 channel blocker XE991 enhanced learning and memory in healthy juvenile mice and alleviated kainite-induced cognitive impairment. Considering the early developmental expression of K7 subunits (http://hbatlas.org/), disruption of K7 function early in development may negatively impact the formation of circuitry and synaptic plasticity that underlie learning and memory in ASD.

5 | CONCLUSION

In this study, we subjected KCNQ2+/− and KCNQ2+/− mice to nine behavioral assays in the test battery. Although the order of these tests was chosen to conduct in general from least invasive to most invasive tests, it is possible that the behavioral responses of these mice in the social novelty test might have been affected by six prior tests. The potential impact of one behavioral assay to the subsequent ones...
is an important limitation to acknowledge in the behavioral test battery.\textsuperscript{55,56}

In summary, our findings provide original evidence that neuronal Kv7 channels containing Kv7.2 is involved in the modulation of multiple behaviors in mice including locomotor activity during the light phase, exploratory behavior, social behavior, repetitive and compulsive-like behavior (Figures 1–7 and 9). In addition, these mice show increased hippocampal excitability and seizure propensity (Figures 8 and 9). Some of the behavioral abnormalities observed in heterozygous Kv7.2 knockout mice are reminiscent of clinical symptoms of ASD, suggesting that reduced Kv7 current may contribute to ASD-associated behaviors. Furthermore, our data provides the pharmacological enhancement of Kv7 current\textsuperscript{135–137} as a potential therapeutic means for treating ASD-associated behaviors.

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AUTHOR CONTRIBUTIONS

H.J.C. conceived of the study and participated in its design and coordination. E.C.K. J.P. and A.T. carried out the experiments and statistical analyses. J.S.R. provided critical expertise for behavior experiments and statistical analyses. H.J.C., E.C.K., J.P. and J.Z. drafted the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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