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Autism Spectrum Disorder Traits in *Slc9a9* Knock-Out Mice

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Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders which begin in childhood and persist into adulthood. They cause lifelong impairments and are associated with substantial burdens to patients, families, and society. Genetic studies have implicated the sodium/proton exchanger (NHE) nine gene, *Slc9a9*, to ASDs and attention-deficit/hyperactivity disorder(ADHD). *Slc9a9* encodes, NHE9, a membrane protein of the late recycling endosomes. The recycling endosome plays an important role in synapse development and plasticity by regulating the trafficking of membrane neurotransmitter receptors and transporters. Here we tested the hypothesis that *Slc9a9* knock-out (KO) mice would show ADHD-like and ASD-like traits. Ultrasonic vocalization (USV) recording showed that *Slc9a9* KO mice emitted fewer calls and had shorter call durations, which suggest communication impairment. *Slc9a9* KO mice lacked a preference for social novelty, but did not show deficits in social approach; *Slc9a9* KO mice spent more time self-grooming, an indicator for restricted and repetitive behavior. We did not observe hyperactivity or other behavior impairments which are commonly comorbid with ASDs in human, such as anxiety-like behavior. Our study is the first animal behavior study that links *Slc9a9* to ASDs. By eliminatingNHE9 activity, it provides strong evidence that lack of *Slc9a9*-leads to ASD-like behaviors in mice and provides the field with a new mouse model of ASDs.

Key words: ASDs; ADHD; USVs; social behavior; repetitive behavior

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INTRODUCTION

Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders characterized by persistent deficits in social communication and social interaction, and restricted repetitive patterns of behavior [Association, 2013]. Twin studies show that genes mediate much of the etiology of ASDs [Rosenberg et al., 2009; Hallmayer et al., 2011]. The estimated heritability of ASD is more than 90% [Freitag, 2007]. Mutations in a single gene and chromosomal abnormalities such as translocations, inversions, and copy number variations (CNVs) have been identified in some ASDs cases [Liao et al., 2013; Nicholl et al., 2013; Zafeiriou et al., 2013].

The *Slc9a9* gene encodes a sodium/proton exchanger (NHE) protein which locates on the membrane of late recycling endosomes. It regulates the pH and maintains cation homeostasis of endosomes. NHE9 is predicted to have N-terminal 12 transmembrane helices that serve as a Na^+/H^+ transporter, and C-terminal domains that interact with various molecules serving as regulatory domains and signaling scaffolds [Orlowski and Grinstein, 2004; Slepkov et al., 2007; Donowitz et al., 2009].

Slc9a9 is a risk gene for ASDs. Three ASD-associated substitutions (V176I, L236S, and S438P) reside within the membrane domain of NHE9 [Kondapalli et al., 2013]. A stop codon mutation (R423X) was found in an individual with ASDs and epilepsy [Morrow et al., 2008]. Instead of producing a protein truncation, our group found that the protein expression was not detectable in cell cultures, suggesting that the R423X mutation causes lack or low level expression possibly due to nonsense-mediated mRNA decay [Zhang-James et al., In Revision]. A ~0.5 kb deletion compassing exon two of *Slc9a9* was reported in a patient with ASDs and epilepsy

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[Wagle and Holder, 2014b]. Abnormal expression of *Slc9a9* was observed in autism brains [Schwede et al., 2014]. *Slc9a9* is also a risk gene for ADHD, which is frequently comorbid with ASDs [Antshel et al., 2013]. (i) *Slc9a9* was first implicated in ADHD by a report of a large family with 22 family members, 11 of whom had features of ADHD and intellectual disability. For the affected members, *Slc9a9* was disrupted by a pericentric inversion [de Silva et al., 2003]. (ii) *Slc9a9* was significantly associated with ADHD in candidate gene studies [Brookes et al., 2006; Markunas et al., 2010]. (iii) In an ADHD genome-wide association study, *Slc9a9* achieved one of the lowest *P*-values [Lasky-Su et al., 2008]. (iv) In two rat models of ADHD, WKY/NCrl rats, and SHR/NCrl rats, there is increased NHE9expression in ADHD related regions in brain [Zhang-James et al., 2011, 2012]. In WKY/NCrlrats, two nonsynonymous mutations in *Slc9a9* were found [Zhang-James et al., 2011]. These genetic associations of *Slc9a9* in both ASDs and ADHD indicate that NHE9 may play an important role in neurodevelopmental processes.

By disrupting the normal function of endosomes, deficits in NHE9 may dysregulate the trafficking and recycling of transporters and receptors. For example, overexpressing NHE9 in cortical astrocytes leads to increased pH in recycling endosomes. Compared with controls, these astrocytes have more glutamate aspartate transporters (GLAST) and transferrin receptors, and have increased uptake of glutamate and transferrin [Kondapalli et al., 2013]. These increases were abolished by ASD associated mutations, L236S, S438P, and V176I [Kondapalli et al., 2013]. Abnormal NHE9 may also affect molecules that directly or indirectly interact with it. In a previous study, we showed that the NHE9 C-terminal interacts with calcineurin homologous protein (CHP) and Receptor for activated C kinase 1(RACK1) [Zhang-James et al., 2011]. In the brains of two rat models of ADHD, we found highly correlated gene expression between *Slc9a9* and numerous synaptic signaling molecules [Zhang-James et al., 2012]. Correlated gene expression of *Slc9a9* with synapse-related genes was also observed in human ASD brains recently [Schwede et al., 2014]. Altogether, we believe that deficits in NHE9 during neurodevelopment may cause abnormalities in synaptogenesis, maturation, and synaptic transmission, which result in long-term defects in brain function seen in ASDs and ADHD.

The present study sought to further test the relationship of NHE9 with ASD and ADHD behaviors using a KO mouse model. Our hypothesis is that the *Slc9a9 gene* KO will produce behavior deficits that resemble behaviors in ASDs, such as social communication and interaction deficits, restricted repetitive patterns of behavior, anxiety, as well as behaviors in ADHD, such as hyperactivity, and deficits in spatial learning and memory.

MATERIALS AND METHODS

Slc9a9 floxed lines were generated at Gene Targeting and Transgenic Facility of the University of Connecticut Health Center by electroporating ES cells with targeting vectors obtained from the Knockout Mouse Project (KOMP) Repository ($Slc9a9^{tm1a(KOMP)Wtsi}$, Clone ID PRPGS00082_B_C09). This is a promoter-driven targeting vector for the generation of "knockout-first allele" (detailed vector information is on the KOMP website https://www.komp.org/alleles.php#conditional-promoter-csd). Lox-p sites were flanking exon 2. Global KO mice were generated by crossing the floxed founder lines with Hprt-Cre mice (JaxMice, Bar Harbor, Maine), which resulted in a LacZ-tagged knockout allele without exon two and lead to a frame shift. The experimental animals were obtained by subsequent heterozygous–heterozygous mating. We used gender-matched littermates, including homozygous (Hom) KO, heterozygous (Het) KO, and wild-type genotypes (WT), for behavioral tests. Confirmation of gene knockout on protein expression levels was determined by western blot using an anti-*Slc9a9* antibody (Proteintech, Chicago, IL). On postnatal day (PND) 3, pups were identified with a foot tattoo and genotyped by PCR using DNA extracted from tail biopsies. Animals were handled daily and weighted weekly. All breeding and testing animals were group housed (2–5 animals/cage) in standard cages with paper beddings. Animals were kept at a 12 hr dark/light cycle (lights on 7:00–19:00) with ad lib food and water. All procedures were approved by the Institutional Animal Care and Use Committee of SUNY Upstate Medical University and were performed between 10:00 and 18:00.

We chose measurements based on prior studies. For ASD measures, deficits in communication were tested by recording and measuring the USVs of mouse pups [Scattoni et al., 2008; Gandal et al., 2010]. Impaired social interactions were tested by conducting three-chambered social interaction tests [McFarlane et al., 2008; Dufour-Rainfray et al., 2010; Roullet et al., 2010; Kim et al., 2011]. Stereotypic/repetitive patterns of behavior were tested by measuring the time that mice spent grooming themselves [Schneider and Przewlocki, 2005; McFarlane et al., 2008; Silverman et al., 2010b; Zhang et al., 2012]. Besides these core features of ASDs, we also tested ADHD-like behaviors in open field activity tests (OF) and several other ASD comorbid symptoms, such as anxiety-like behavior in elevated plus maze tests (EPM). We tested their spatial learning and memory ability, as well as reverse learning with the Barnes maze tests (BM). The detailed behavioral procedures are described below according to the order that animals were tested. All behavioral tests were performed by an experimenter who was blinded to the genotypes.

Maternal Separation and USVs Recording

We recorded USVs emitted by 90 mice (Hom = 25, Het = 39, WT = 26) at PND13. Individual pup was removed from its home cage and placed in a cage with clean bedding but without lid. Then the cage was transferred into a noise-attenuating chamber with an USV detector (Med Associates) mounted inside directly over the open cage. The recording lasted for 10 min for each mouse. The number of calls and total call duration were measured.

EPM

Seventy-nine mice (Hom = 24, Het = 32, WT = 23) were tested on the EPM on PND21.The apparatus consists of two closed arms ($110 \times 10\,cm^2$, LxW) with 30 cm tall non-transparent walls on both sides and two open arms ($110 \times 10\,cm^2$, LxW). The apparatus is 50 cm elevated from the floor and visually isolated by screens.

Individual mouse was placed in the center of the maze with its head toward an open arm and was allowed to explore the maze freely for 10 min under regular room light. The activities of the mice were recorded by a camera hanging above the maze and tracked by Any Maze Software (Steolting, Wood Dale, IL). The time spent in each arm was measured. Entries were recorded if all four paws entered the arm.

OF

Seventy-eight mice (Hom = 25, Het = 30, WT = 23) were tested in OF chambers on PND 22. We used four square non-transparent OF boxes ($50 \times 50 \times 38\ cm^3$, WxLxH). Each animal was placed in the center of the box and was allowed to move freely for 20 min under dim light. The activities of the mice were recorded by a camera hanging above the maze and tracked by Any Maze Software (Steolting, Wood Dale, IL). Each box field was divided into four zones by a grid drawn on the screen: center, middle, edge and corner zones (Suppl. Fig. S1). Overall distance and average speed, as well as the distance traveled and time spent in different zones were assessed.

SHIRPA Tests

A modified SmithKline Beecham, Harwell, Imperial College and Royal London Hospital phenotype assessment (SHIRPA) protocol was used to assess the general behavioral and morphologic phenotypes for the animals [Rogers et al., 1997]. Seventy-eight mice (Hom = 25, Het = 30, WT = 23) were tested in SHIRPA on PND 22. A total of 22 items including tremor, coat appearance, locomotor activity, corneal reflex, etc, as described in the protocol (http://empress.har.mrc.ac.uk/browser/?sop_id=10_002_0) were assessed by a genotype-blinded experimenter. Results for each item was recorded in an excel sheet (Suppl. Table S1) and analyzed using appropriate regression models independently.

BM

Fifty-seven (Hom = 19, Het = 21, WT = 17) mice were tested for BM during PND29-41. The BM consists of a circular platform (92 cm of diameter) with 20 equally placed holes along the perimeter 91 cm above the floor. Among the 20 holes, one is the entry of a dark recessed chamber underneath the platform called the "target box" (Suppl. Fig. S2). The target box is 5 cm in depth and 15.4 cm in length. The non-target box is 2 cm in depth and 6.2 cm in length. The platform is evenly illuminated by overhead fluorescent white lighting. The room has several visual cues on the wall, such as triangles, rectangles, and circles. These spatial visual cues, including the cues endogenous to the room, a door, a sink, and a speaker for instance, remained in the same place during the whole experiment as they provided reference points for the animals to locate the target box. To begin each trial, individual mouse was placed in an opaque cylinder in the center of the platform for 10 sec. The chamber was then lifted and a high-pitched (110 Hz) buzzer was turned on. On day one (PND 29), the mouse was guided gently to the target box. We turned off the buzzer immediately and allowed the animal to stay in the box for 2 min. Each animal received one trial on day 1. On days 2–4 (PND 30–33), the animals were tested for four trials per day with an inter-trial interval of 1 hr for spatial acquisition.

For each acquisition trial, the animal explored the maze freely for up to 3 min. Once the mouse found and entered the target box, the buzzer was turned off and the mouse was allowed to stay in the box for 1 min. If the mouse did not find the target within 3 min, it was guided to the target box to stay for 1 min. The latency for the mouse to locate the target box (3 min or less) and the average speed were recorded by AnyMaze Software.

Twenty-four hours after the completion of 16 trials of spatial acquisition, the animals were tested on a probe trial (PND 34). The target box was replaced with a non-target box so that it was closed. The mouse was allowed to explore the maze freely for 90 sec. A second probe trial was conducted on PND 41. The latency of first head entry to the old target box (indicating that the animal found the old target box) and the time spent in the old now closed target box zone was measured.

Three-Chambered Social Interaction Test

Fifty-three adult mice (Hom = 17, Het = 17, WT = 19, PND60-110) were tested on a modified three-chambered social interaction test [Nadler et al., 2004; Zhang-James et al., 2014]. The apparatus has three chambers ($114 \times 51 \times 51\ cm^3$, WxLxH) with retractable door between two chambers. In one side chamber, there is a plastic cage which contains a novel stranger mouse (novel mouse-1) of the same gender and approximately the same age as the subject mouse. The other side chamber contains an empty plastic cage (Suppl. Fig. S3). There are holes on one side of the plastic cage that allow the subject and stranger mouse to have visual, olfactory, auditory, and tactile contact. Novel mouse-1 had had no previous contact with the subject mouse. The social preference and memory tests consisted of four repeated trials (once/day) with the same novel mouse. Briefly, the subject mouse was placed in the center chamber with the door closed for a 10 min habituation period. Then the doors between each chamber were raised and the subject mouse was allowed to move freely across all three chambers for 10 min. A camera controlled by AnyMaze software recorded the number of entries of the subject mouse into each chamber and the time spent in each chamber. Because the novel mouse was contained in the cage, the social preference and interactions were initiated only by the subject mouse.

On the last day, immediately following the social preference and memory test, another novel stranger mouse (novel mouse-2) was introduced into the three-chambered apparatus and replaced the empty cage. Novel mouse-2 also had no previous contact with the subject mouse, whereas the novel mouse-1 had become a now-familiar mouse. The animals were allowed to explore the three chambers for another 10 min and the same measurements were recorded.

Social Behavioral Observations

One to two weeks following the three chambered test, 48 adult mice (Hom = 15, Het = 16, WT = 17) were further analyzed for more detailed social behavioral evaluation by a blinded experimenter. We utilized the differential familiarity with the two novel mice

from the three chambered tests and allowed each test mouse to directly interact with the novel mouse-1 for 10 min followed by direct interaction with the novel mouse-2 for another 10 min in the center chamber ($38 \times 51 \times 51 cm^3$, WxLxH). The videos were imported to AnyMaze software to help the rater to record the time for eight different categories of behaviors to evaluate social behavior and stereotypical/repetitive patterns of behavior including investigating, being investigated, chasing, being chased, self-grooming, reciprocal grooming, fighting, and rearing. We consider the total social contact time as the sum of time spend in investigating, being investigated, chasing, being chased, reciprocal grooming, and fighting.

Statistical Analysis

We used regression analyses and paired Student's t-tests to assess behavior measurements in STATA 12. Significance was set as P-value <0.05. To assess the effects of genotype and covariates on behavior, we used the general linear model (GLM) in STATA 12.0 [StataCorp, 2011]. The GLM allows us flexibility in modeling the outcome variable by choosing the appropriate distribution and link function for each outcome. We report results as F-tests or χ^2 tests depending on the model chosen. When there were statistically significant differences between genders, we reported the gender effects and analyzed genotype effects in males and females separately. Otherwise, we analyzed the results with males and females together. The genotype was treated as a categorical variable first. If this analysis was not significant, to potentially increase power, we either (i) analyzed genotype as a dose effect to reflect the copy number of the gene (2 = WT, 1 = Het, 0 = Hom); or (ii) when no difference was found between Hom and Het mice, we combined them into one gene KO group. For count variables such as numbers of entries, we used Poisson or negative binomial regression models.

RESULTS

Gene Knockout Produced Loss of Protein Expression

We used olfactory bulb to confirm the loss of the protein expression because it is one of the few brain regions express moderate levels of *Slc9a9* according to the Allen Brain Atlas (http://mouse.brain-map.org/). Supplementary Figure S5 showed that protein expression was significantly reduced or lost in Het and Hom mice compared with the littermate WT mice. Endogenous *Slc9a9* protein is very difficult to detect, not only because of the low levels of expression in many tissues, but also because that the protein dimerizes and aggregates due to its high hydrophobicity regions [Zhang-James et al., 2011]. The identity of the high molecular weight immunoreactive bands, composed of mostly protein aggregates, was confirmed to be *Slc9a9* (Suppl. Fig. S5).

General Phenotype

The animals produced normal sized litters ranging from 4 to 10 pups. We examined all the animals' weights from PND 5 up to 100 days, no growth rate difference was observed for the different genotypes ($\chi^2(2) = 1.3$, $P = 0.52$). The gene dose effect was not significant either ($\chi^2(1) = 0.26$, $P = 0.6$) (Suppl. Fig. S4). For the SHIRPA tests, no genotype effects were detected for all the phenotype assessments (data not shown) except the locomotor activity. One animal at a time was dropped from ~25 cm above an arena floor, and number of squares ($11 \times 11 cm^2$) they traveled in 30 sec were counted immediately. There was a significant gender effect ($\chi^2(1) = 10.0$, $P = 0.002$) and a marginally significant genotype effect $\chi^2(2) = 5.26$, $P = 0.07$) due to primarily the Het KO males who traveled across significantly more squares in 30 sec than the other two genotypes. For males, the genotype effect was significant ($\chi^2(2) = 11.04$, $P = 0.004$). For females, no difference was observed among genotypes (Fig. 1).

USVs. For band 1, the lower frequency bandwidth (20–50 kHz), we used negative binomial model and Gamma regression models to assess the effect of genotype on number of calls and call duration respectively. In females, we found a significant effect of genotype on number of calls ($\chi^2(2) = 15.25$, $P = 0.0005$). Although the genotype effect for call duration was not significant ($\chi^2(2) = 5.01$, $P = 0.08$), the gene dose effect for call duration was significant ($\chi^2(1) = 5.34$, $P = 0.02$). In males, genotype did not have any effect on number of calls ($\chi^2(2) = 0.93$, $P = 0.6$), or call duration ($\chi^2(2) = 0.03$, $P = 0.9$). The gene dose effect was not significant for number of calls ($\chi^2(1) = 0.88$, $P = 0.35$) or call duration ($\chi^2(1) = 0.01$, $P = 0.9$) (Fig. 2A and B).

For band 2, the higher frequency bandwidth (50–100 kHz), we used negative binomial regression and Gaussian linear regression to model the effect of genotype on number of calls and call duration

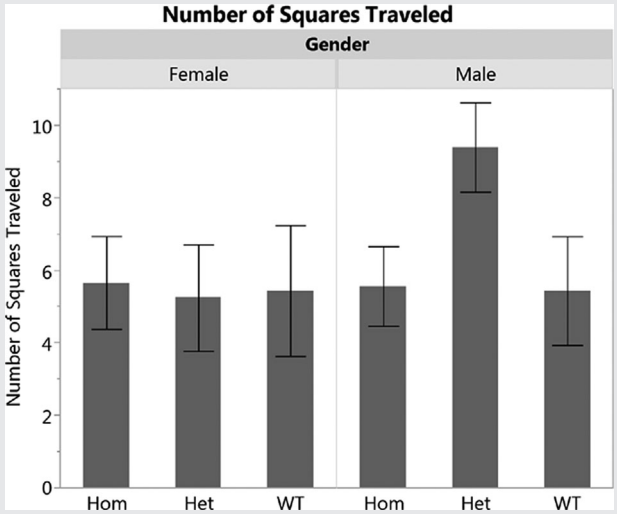

FIG. 1. Number of squares traveled in SHIRPA. Animals were dropped from 25 cm height above the arena floor and the number of squares they traveled in 30 sec were counted. There were no differences among the three genotypes for females. But there were significant genotype difference for males. Het KO males traveled across more squares than Hom KO and WT males. Data are expressed as means ± s.e.m.

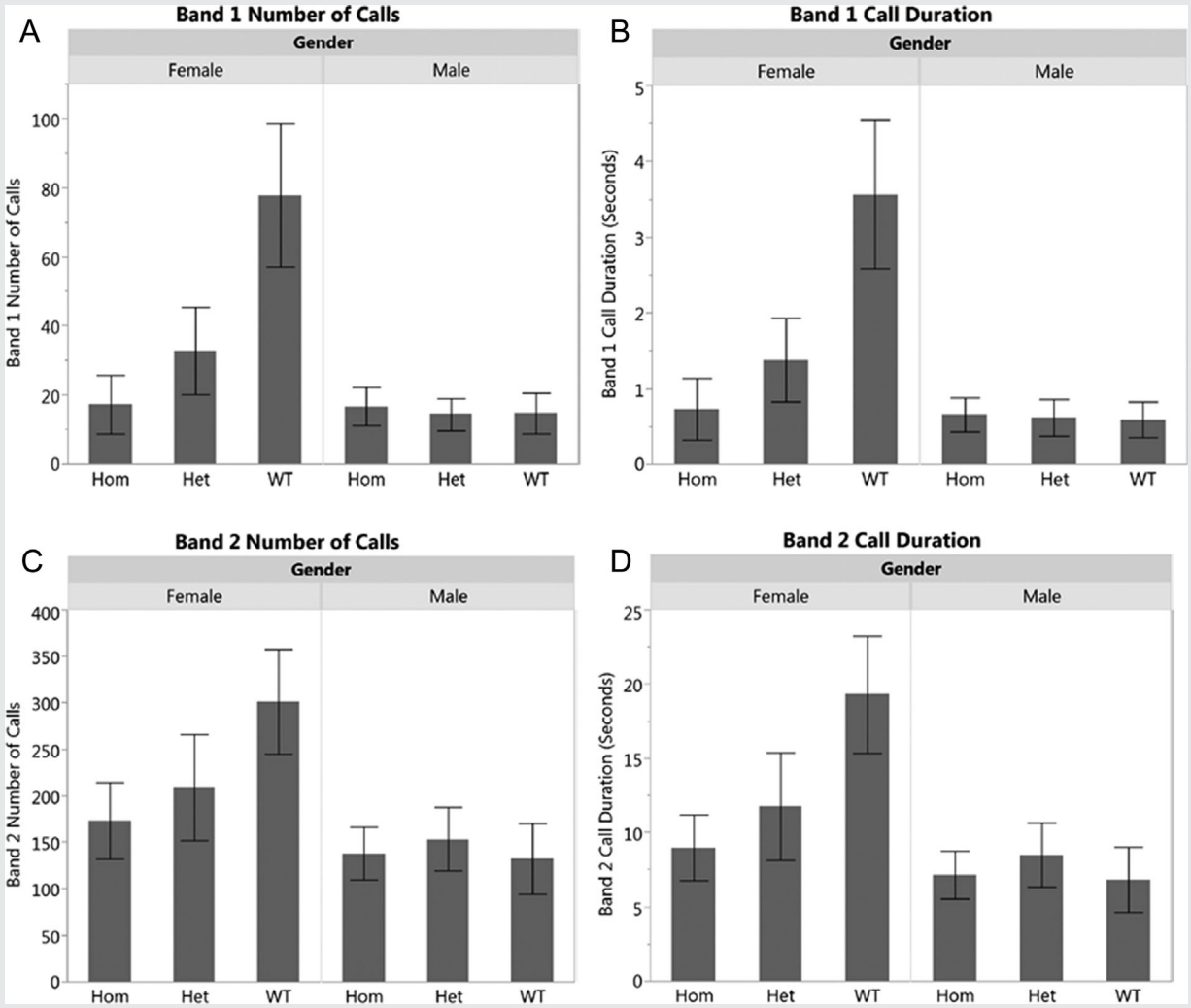

FIG. 2. USV results. For band 1 (20–50 kHz), the number of calls (A) and call duration (B) were plotted over genotype for females and males separately. In females, there was a genotype effect on number of calls, and a gene dose effect for call duration. No differences were observed for males. For band 2 (50–100 kHz), the number of calls (C) and call duration (D) were plotted over genotype for females and males separately. The results were similar to the band 1 results. A significant genotype effect on number of calls and a gene dose effect for call duration were observed for females. No differences were observed for males. Data are expressed as means ± s.e.m.

respectively. In females, a significant genotype effect was detected for number of calls ($\chi^2(2) = 6.11$, $P = 0.047$). Although the genotype effect was not significant for call duration ($F(2.24) = 2.27$, $P = 0.13$), the gene dose effect was significant ($F(1, 25) = 4.24$, $P = 0.050$). In males, no genotype effect was detected for number of calls ($\chi^2(2) = 0.29$, $P = 0.9$) or call duration ($F(2, 51) = 0.09$, $P = 0.9$). The gene dose effect was not significant for number of calls ($\chi^2(2) = 0.05$, $P = 0.8$) or call duration ($F(1,52) = 0.16$, $P = 0.7$) for males either (Fig. 2C and D).

EPM. We used Gaussian linear regression to model the effect of genotype on total distance traveled, time in the open and closed arms, and time in the center and probing zones. We used Poisson regression to model the effect of genotype on total arm entries, open arms, and closed arms entries. We found a significant genotype effect on time spent in the open ($F(2, 66) = 5.08$, $P = 0.009$) and closed arms ($F(2, 66) = 3.52$, $P = 0.04$). Compared with Het KO mice, Hom KO mice spent less time in the open arms ($F(1, 66) = 10.14$, $P = 0.002$) (Fig. 3A) and more time in the closed arms (Fig. 3B) ($F(1, 66) = 6.85$, $P = 0.01$). Hom KO mice and Het KO mice did not significantly differ from WT mice in these two variables. The time that mice spent exploring the edge of the open arms (probing zone) did not differ among genotypes ($F(2, 65) = 0.57$, $P = 0.6$). The gene dose effect was not significant for the time that mice spent exploring the probing zone ($F(1, 66) = 1.02$, $P = 0.3$) either.

To evaluate if overall locomotor activity affected behavior in the EPM, we analyzed the distance traveled and total number of entries. Neither genotype ($F(2, 65) = 1.5$, $P = 0.20$) nor gene dose ($F(1, 66) = 2.98$, $P = 0.09$) affected total distance traveled. The three genotypes differed in their total number of entries into

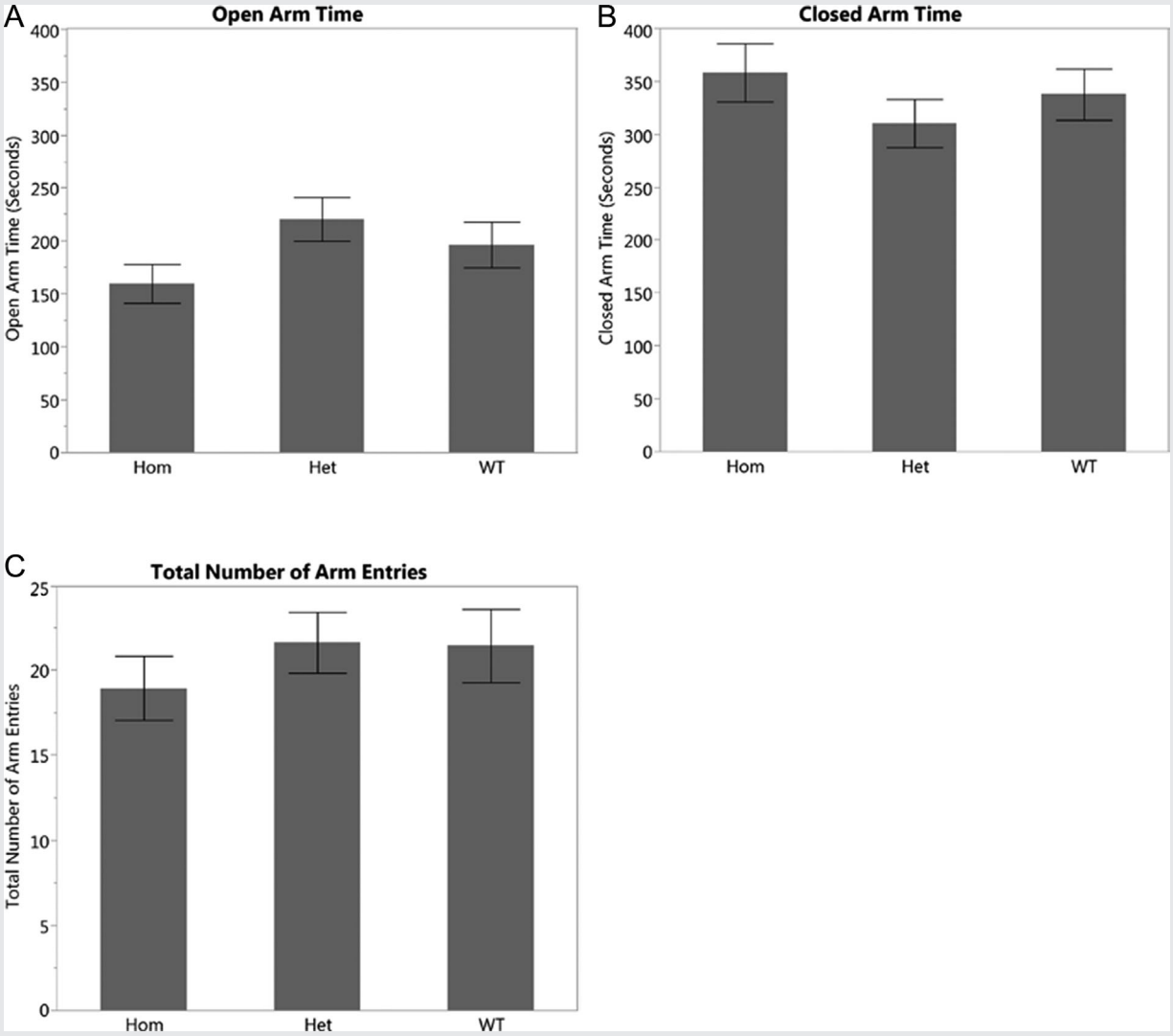

FIG. 3. EPM results. The time spent in the open (A) and closed arms (B) was plotted. Total arm entries (C) were plotted. Compared with Het KO mice, Hom KO mice spent less time in the open arms and more time in the closed arms. Hom KO mice and Het KO mice did not significantly differ from WT mice in these two variables. Hom KO mice also had fewer total numbers of entries into the arms. Data are expressed as means ± s.e.m.

the arms ($\chi^2(2) = 7.3$, $P = 0.026$), with Hom KO mice showing fewer entries (Fig. 3C).

OF. We used Gaussian linear regression to model the effect of genotype on total distance traveled and time spent in each zone. We did not observe a genotype difference in total distance traveled ($F(2, 64) = 0.58$, $P = 0.6$) or time spent in the center zone ($F(2, 65) = 0.69$, $P = 0.5$), middle zone ($F(2, 74) = 0.04$, $P = 0.96$), edge zone ($F(2, 75) = 1.29$, $P = 0.28$), corner zone ($F(2, 64) = 0.34$, $P = 0.71$), or outer area (edge zone plus corner zone time, $F(2, 64) = 0.03$, $P = 0.97$). Gene dose effect was also not significant for the observations mentioned above (all P's >0.05).

BM. We used negative binomial regression for the analysis. In the acquisition phase, a significant interaction between genotype and trial was observed for the latency to locate the target box ($\chi^2(2) = 8.38$, $P = 0.02$). To assess the non-linear effect of trial, we added a quadratic term for trial to our statistical model. The interaction between genotype and this quadratic term was significant for the latency to locate the target box ($\chi^2(2) = 7.75$, $P = 0.02$). Across the 16 training trials, all three genotypes needed less and less time to locate the target box with increasing numbers of trials ($\chi^2(1) = 4.25$, $P = 0.04$). However, this decreasing trend was the most dramatic for Het KO mice. Compared with WT, Het KO mice spent less time locating the target box ($\chi^2(1) = 8.21$, $P = 0.004$) (Fig. 4A). The Hom KO mice did not differ from WT ($\chi^2(1) = 2.7$, $P = 0.1$) or Het KO mice ($\chi^2(1) = 1.18$, $P = 0.3$) (Fig. 4A).

To explain why Het KO mice appeared to have enhanced spatial learning and memory, we analyzed the speed of all the mice across the training trials using a Gaussian linear regression model. We

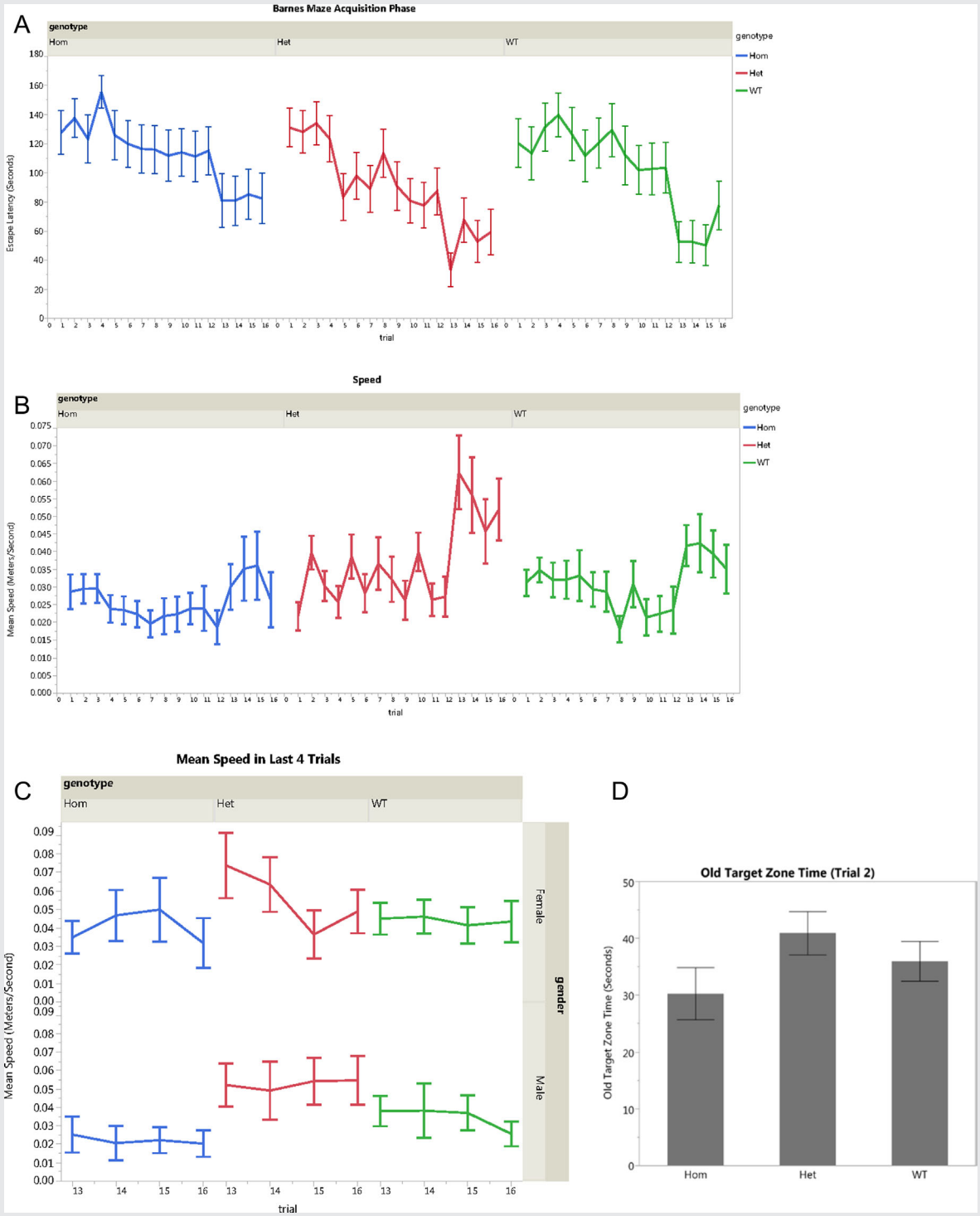

FIG. 4. BM results. In the acquisition phase, latency to locate the target box (A), and mean speed were plotted over trials (B). The amount of time needed to locate the target box decreased for all three genotypes with increasing numbers of trials across a total of 16 training trials. This decreasing trend was most dramatic for Het KO mice. The Het KO mice also had significantly increased speed across the trials. Mean speed for the last four trials was plotted (C). For the last four trials, the male Het KO mice had significantly faster speed compared with Hom but they did not differ from WT mice. Females showed no genotype or gene dose effects on speed for the last four trials. In the probe tests performed eight days after the last training session, the time spent in the old escape zone was plotted (D). Het KO mice spent more time in the old escape zone than Hom KO mice even when the learned target box had been removed. Data are expressed as means ± s.e.m. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb].

found that Het KO mice also had significantly increased speed across the trial ($\chi^2(1) = 15.53$, $P = 0.0004$) (Fig. 4B). When we included speed in our negative binomial regression model, the speed effect was significant ($\chi^2(2) = 91.88$, $P < 0.0001$). However, the effect of the interaction between genotype and trial was no longer significant ($\chi^2(2) = 5.29$, $P = 0.07$), and the interaction between genotype and quadratic trial was not significant ($\chi^2(2) = 4.59$, $P = 0.1$) either. This increase in speed accounted for the decreased latency to locate the target box in Het KO mice. So there was not enhanced spatial learning and memory in Het KO mice, just increased locomotor activity. We also noticed that compared with previous trials, in the last four trails (on day 4) there was a dramatic increase of speed in Het KO mice (Fig. 4B). The male Het KO mice had significantly faster speed compared with Hom ($\chi^2(1) = 10.32$, $P = 0.001$) but they did not differ from WT mice ($\chi^2(2) = 1.52$, $P = 0.22$). In females, neither the genotype effect ($\chi^2(2) = 1.55$, $P = 0.46$) nor the gene dose effect ($\chi^2(1) = 0.46$, $P = 0.5$) on speed were significant (Fig. 4C).

In both probe tests, neither genotype nor gene dose affected latency to first entry to the old target box (all P's >0.05) using Poisson regression. In the probe test performed 24 hr after the last training session, no genotype effect ($F(2, 45) = 0.17$, $P = 0.8$) or gene dose effect ($F(1, 55) = 0.34$, $P = 0.56$) on time spent in the old target zone was detected by using Gaussian linear regression. But in the probe tests performed eight days after the last training session, a significant genotype difference was observed in time spent in the zone where the target box had been placed ($F(2, 44) = 3.35$, $P = 0.04$). Het KO mice spent more time in the zone where the target box had been placed compared to Hom KO mice ($F(1, 44) = 6.33$, $P = 0.02$) (Fig. 4D).

Three-chambered social interaction test. We used Gaussian regression. During the social memory/sociability test from days 1 to 4, all mice spent similarly more time in the mouse chamber than in the center chamber or the empty cage chamber. No genotype, gene dose, or trial effects were observed (all P's >0.05).

During the social novelty test, we did not observe significant effect of genotype ($F(2,40) = 2.63$, $P = 0.08$) or gene dose ($F(1,41) = 2.15$, $P = 0.2$) on time spent in the novel animal chamber. Because Hom and Het KO mice were not different ($F(1,40) = 0.63$, $P = 0.4$), we combined Hom and Het KO mice into one group, KO mice. The KO mice spent less time in the novel animal chamber compared with WT mice ($F(1, 41) = 4.66$, $P = 0.04$). In contrast, KO mice spent more time in the center chamber compared with WT mice ($F(1, 51) = 4.37$, $P = 0.04$). Genotype did not have an effect on the time spent in the familiar mouse chamber ($F(1, 51) = 1.24$, $P = 0.3$). We used Student's paired t-tests to examine the novelty preference. We found that WT mice preferred to spend more time in the novel mouse chamber than the familiar mouse chamber ($t(18) = 2.14$, $P = 0.047$), whereas KO mice spent similar amount of time in novel mouse chamber and familiar mouse chamber ($t(33) = 0.2$, $P = 0.8$).

Observation of social behavior. No significant genotype effect ($F(2, 27) = 0.69$, $P = 0.51$) or gene dose effect ($F(1, 28) = 1.42$, $P = 0.24$) was detected for total social contact time, or each individual social behavioral category. We also did not detect a genotype difference ($F(2, 45) = 0.35$, $P = 0.7$) nor gene dose effect ($F(1, 46) = 0.71$, $P = 0.4$) in time spent on rearing. We did not detect genotype effect ($F(2, 45) = 1.31$, $P = 0.28$), but detected a significant gene dose effect on time spent self-grooming ($F(1, 19) = 6.54$, $P = 0.02$) (Fig. 5). Hom KO and Het KO mice spent more time in self-grooming than WT mice.

DISCUSSION

Our study is the first study to show the behavioral phenotypes in mice lacking NHE9. We tested traits that resemble the behaviors in ASDs: deficits in social communication, deficits in social interaction, and restricted repetitive behavior. The *Slc9a9* KO mice emitted fewer calls of shorter call duration; *Slc9a9* KO mice lacked preference for social novelty, but did not show obvious deficits in sociability and social memory; *Slc9a9* KO mice spent more time on self-grooming, which is a proxy for the stereotypic, repetitive behaviors seen in ASDs. We did not observe spatial learning and memory deficits in *Slc9a9* KO mice. Although both groups of KO mice were not different from WT littermates in the anxiety measures of the EPM and overall open-field activities, the Het KO mice appeared to be less anxious and more responsive with a faster response speed than the Hom mice across several test platforms including SHIRPPA and BM.

Effect of Genotype on USVs

Rodent pup calls serve functions that are similar to the human babies' crying. By initiating maternal behaviors, such as searching, retrieving, nest building, licking, and feeding behavior, USVs serve an important communicative function between pups and their mother [Sewell, 1970; Brunelli et al., 1994; Geissler and Ehret, 2004; D'Amato et al., 2005].

When separated pups from their mother and littermates on PND13, *Slc9a9* KO female pups emitted fewer calls of shorter

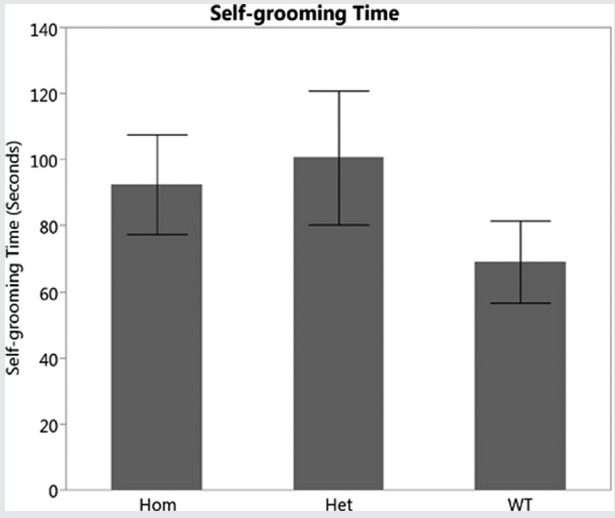

FIG. 5. Time spent on self-grooming. Hom KO and Het KO mice spent more time self-grooming than WT mice. Data are expressed as means ± s.e.m.

duration than WT female pups. This result is consistent with what has been reported for other ASD mouse models, including serotonin receptor-1A (*Htr1a*) KO pups [Weller et al., 2003; van Velzen and Toth, 2010], serotonin receptor-1B (*Htr1b*) KO pups [Brunner et al., 1999; El-Khodor et al., 2004], oxytocin neuropeptide (*Oxt*) KO pups [Winslow et al., 2000], *Shank1* KO pups [Wohr et al., 2011], human neuroligin-3 mutation male pups (*Nlgn3-R451C*) [Chadman et al., 2008]; oxytocin neuropeptide receptor (*Oxtr*) KO male pups [Takayanagi et al., 2005], reelin (Reln) KO male pups [Ognibene et al., 2007], forkhead box P2 (*Foxp2*) KO pups [Shu et al., 2005] and μ-opioid receptors (*Orpm*) KO pups [Moles et al., 2004]. The reduced level of USVs during maternal separation and isolation from littermates could be an indicator for early communication impairments.

In contrast with these USV findings, some mouse models of ASDs reported increased USVs calls. For example, BTBR T+tf/J (BTBR) mice called more frequently and the durations of those calls were longer compared with controls on PND8 [Scattoni et al., 2008]. Fragile X mental retardation 1 (*fmr1* KO*)* KO mice is a mouse model of fragile X syndromes (FXS), which is associated with ASDs. These KO mice showed an increased number of calls on PND7 but not on PND4 and PND10 [Lai et al., 2014]. Methyl CpG binding protein 2 (*Mecp2*) Hom KO males and Het females showed a dramatic increase of USVs calls on some PND [Picker et al., 2006]. The *Mecp2* mutant mouse is a model for Rett Syndrome which is also associated with ASDs. These increased USVs calls in mouse models relevant to ASDs have been attributed to higher anxiety and fear [Kalueff et al., 2007; Nakatani et al., 2009; Laloux et al., 2012].

For male pups, we did not observe genotype difference in USVs. We noticed that the overall USV levels for males were significantly lower than for females. This result was consistent with another study which reported that during maternal separation, male inbred mouse pups had lower rate of calling and shorter call length than female inbred mouse pups on PNDs 6–12 and 11–12, respectively [Hahn et al., 1998]. It is possible that we could not observe any genotype differences for male pups because of their low levels of USVs at the time of testing. Future studies of male pups at earlier ages (e.g., PND 5–10) may be helpful because they produce more USVs at earlier ages following maternal separation [Hahn et al., 1998].

We previously reported some behavioral phenotypes in the WKY/Ncrl and WKY/NHsd inbred rats [Zhang-James et al., 2014]. These two substrains are genomically similar, having only a 2.5% difference as detected by a 10 K genome-wide SNP array [Zhang-James et al., 2013]. There are two nonsynonymous mutations in *Slc9a9* in WKY/NCrl rats [Zhang-James et al., 2011]. Male beddings were used to stimulate adult females to emit USVs. We also reported that WKY/NCrl females had fewer USV calls compared with WKY/NHsd females [Zhang-James et al., 2014]. But because the *Slc9a9* mutations are not the only genetic differences between the WKY/Ncrl and WKY/NHsd substrains, we could not definitively implicate *Slc9a9* mutations in communication impairments as we could in the present study. Male animals were not studied in the WKY/NCrl rats, therefore, we do not know if adult male would emit different USVs than the control strain WKY/NHsd rats.

Effect of Genotype on Preference for Social Novelty

Lower social interest should be a major feature of ASD models. However, our data about this trait were not consistent. We found no effect of genotype on direct physical contact of the test and novel mice. But the *Slc9a9* KO mice spent the same amount of time in the novel mouse chamber and the familiar mouse chamber, while the WT mice spent significant more time with the novel mouse than the familiar mouse.

The mechanism underlying social novelty behaviors are complex. On one hand, social novelty preference demonstrates that the mice prefer to have physical contact or spend more time with a stranger rather than a familiar individual. On the other hand, social novelty preference is an indicator for social recognition and social memory [Silverman et al., 2010b]. In our experiments, when exposed to both familiar and novel mice, the subject mice, based on their previous experience with the familiar mouse, should be able to discriminate between the two mice and recognize the one that had no contact before. In our study, the *Slc9a9* KO mice did not show social novelty preference. This suggests that the *Slc9a9* KO mice were either not interested in exploring the novel mice, or not able to discriminate between the novel and familiar mice.

Some other ASD mouse models also show an absence of social novelty preference. Mice lacking the integrin β3 receptor subunit, which is related with brain serotonin levels, showed no social novelty preference [Carter et al., 2011]. *Oxtr* KO male mice showed deficiency in social novelty preference [Takayanagi et al., 2005]. *Oxt* KO mice [Ferguson et al., 2000] and oxytocin-regulating protein CD38 KO mice also had difficulty discriminating between a familiar mouse and a novel mouse [Jin et al., 2007].

Like *Slc9a9* KO mice, NL-3 KO mice show no altered performance in sociability or social interaction but do show deficits in social novelty preference [Radyushkin et al., 2009]. NL-3 KO mice also have olfactory deficits. Because olfactory cues play an important role in social behavior, olfactory malfunction may cause the NL-3 KO mice to fail to discriminate between the familiar mouse and the novel mouse [Radyushkin et al., 2009]. Interestingly, impairments in olfactory identification have also been reported in patients with ASDs [Suzuki et al., 2003; Bennetto et al., 2007]. Future studies should study olfaction in *Slc9a9* KO mice to determine if olfactory deficits mediate the effects of genotype on social novelty.

Our previous behavioral studies of rats reported that the WKY/NCrl and WKY/NHsd substrains behaved similarly in the social novelty preference tests, but that the WKY/NCrl substrain (which carries two nonsynonymous mutations in *Slc9a9*) had lower social interests compared with the WKY/NHsd substrain [Zhang-James et al., 2014].

The diagnostic criteria for ASDs in DSM-5 require "persistent deficits in social communication and social interaction" which may include "failure to initiate or respond to social interactions," and "absence of interest in peers." DSM-5 does not mention or give examples of lack of social novelty preference in individuals with ASDs, although it does state that its examples are illustrative, not exhaustive. Thus lack of sociability seems to be a more straightforward and powerful level of evidence than lack of social novelty preference for ASD rodent models. Many ASD mouse studies have

focused on sociability testing as defined by preferring other mice over objects [Kwon et al., 2006; McFarlane et al., 2008; Moy et al., 2009; Tabuchi et al., 2007]. Many ASD mouse model, BTBR for example, showed absence of preference for mouse over object [McFarlane et al., 2008].

Effect of Genotype on Repetitive Self-Grooming Behavior

Stereotypic/repetitive movement is a diagnostic symptom of ASDs. Behaviors that appear to be normal patterns but persist for unusually long periods are considered stereotypic/repetitive movements [Silverman et al., 2010a]. *Slc9a9* KO mice spent longer periods of time self-grooming than WT. Their high level of self-grooming is consistent with other ASD mouse models: BTBR mice [McFarlane et al., 2008; Silverman et al., 2010a], *fmr1* KO mice [McNaughton et al., 2008], *neuroligin-1* KO mice [Blundell et al., 2010], C58-J inbred mice [Ryan et al., 2010], *Shank2* KO [Schmeisser et al., 2012], and *Shank3* KO mice [Peca et al., 2011; Wang et al., 2011]. However, in our previous study of ASD phenotypes in rats, we did not find any difference in the time spent self-grooming between WKY/Ncrl rats and controls [Zhang-James et al., 2014].

ASDs Comorbid Phenotypes

Both groups of *Slc9a9* KO mice did not differ from WT littermates in measures of anxiety (EPM) and hyperactivity (OF), symptoms which are commonly comorbid with ASDs in humans and in some ASD mouse models, although Hom mice were more anxious than the Het mice and spent more time in closed arms than in the open arms. We also found no spatial learning or memory deficits in the KO mice, but did observe increased locomotor activity and response speed in Het KO mice for the BM tests, especially for the last four trials. These are consistent with the SHIRPA test, in which Het KO mice also showed increased locomotor activity and speed compared with the Hom mice. In both the BM and SHIRPA tests, animals must respond to stressors, that is, bright light and noise in BM, drop from height in SHIRPA. We speculate that the Het KO mice are not hyperactive, nor do they have learning deficits. Instead, they show a faster response speed while escaping. One study reported that, compared with children without ASD, children with ASD had enhanced performance in perceiving the direction of a high-contrast, fast moving bar [Foss-Feig et al., 2013]. This hypersensitive perception may lead them to over-react to sensory input. In fact, individuals with ASD tend to escape or avoid negative stimuli. Stereotypical behaviors have been hypothesized to allow persons with ASDs to escape from negative reinforcement [Kennedy et al., 2000]. For Het KO mice, such hypersensitivity may cause them to quickly respond to escape from aversive stimuli. Hom KO mice, although not differ in anxiety from WT, but are more anxious than Het KO mice as shown in the EPM test. This anxiety may counteract the intensified response by causing Hom KO mice to freeze. We note that in our previous study of rat models, although WKY/Ncrl rats showed similar motor activity behaviors to WKY/NHsd rats, they were significantly more anxious than the WKY/NHsd rats in EPM test [Zhang-James et al., 2014]. The EPM and OF tests are among the most commonly used tests to detect anxiety towards open spaces in rodents. Other behavioral paradigms such as marble burying and the light/dark box test should be conducted in the future to further elucidate anxiety phenotypes in our *Slc9a9* KO mice.

Potential Limitations and Future Directions

Our KO mice line was generated from C57Bl/6 × 129SVEV F1 hybrid ES cell lines. Backcrossing to C57Bl/6 for ten or more generations is needed to create an isogenetic C57Bl/6 background [Markel et al., 1997]. KO mice used in this study have not been backcrossed fully to remove the 129 background, which may have added noise to our measurements. However, this extra noise cannot explain our statistically significant findings. This is also supported by consistent findings, such as hyper-response speed, across several platforms. Future studies using backcrossed mice with isogenic background are needed to confirm the current findings.

Several deleterious mutations in *Slc9a9* were found in human patients with ADSs and epilepsy, including a nonsense mutation and a ~0.5 kb deletion compassing exon two of *Slc9a9*, which would produce an frame-shift premature stop codon [Morrow et al., 2008; Wagle and Holder, 2014a]. In both cases, premature stop codons may result in loss of protein due to nonsense-mediated mRNA decay, as we observed in vitro with the R423X mutation [Zhang-James et al., In Revision]. Thus, these mutations are similar to an *Slc9a9* knockout effect. In the current study, we did not observe obvious motor epilepsy phenotypes in our KO mice. Future studies should examine the propensity of seizures due to the loss of *Slc9a9* through study of electroencephalogram (EEG) and behavior.

Although we did not observe hyperactivity in *Slc9a9* KO mice, since *Slc9a9* has been associated with ADHD in humans, future studies should test other ADHD phenotypes in these mice, such as inattention and impulsivity. It will also be interesting to study other ASD associated symptoms in *Slc9a9* KO mice, including sleep disruption, gastrointestinal disturbances, mental retardation, hypersensitivity to auditory and visual stimuli, impaired disengagement of attention, and abnormal brain volumes [Stanfield et al., 2008; Taylor et al., 2012; Carbone, 2013; Clery et al., 2013; Keehn et al., 2013; Thabet and Zaghloul, 2013; Anderson, 2014].

ASDs are collection of neurodevelopmental disorders, and function and plasticity of synapse can play an important role in ASDs. *Slc9a9* mutations could dysregulate transcription, translation, and protein trafficking at the synapse, which could, in turn, impact dendritic branching, synapse maturation, and synapse elimination. These processes have been implicated in neurodevelopmental disorders, such as mental retardation and ASDs [Flavell and Greenberg, 2008]. Future studies should examine *Slc9a9* activity in pre- and post-synaptic neurons and glial cells. By doing so, the potential role of *Slc9a9* on synaptic development and plasticity may be uncovered.

Finally, effective and safe treatments for ASDs are an urgent need. Since one of the main functions of NHE9 is to regulate the pH of endosome, it should be possible to use medications which can acidify or alkalinize endosome pH to compensate for the abnormal endosomal pH level in ASDs patients. Screening and searching the potentiators and inhibitors that target NHE9 and regulate its

function can be helpful for the development of ASDs drugs [Kondapalli et al., 2014]. We previously suggested, using an in silico approach, that NHE inhibitors might be useful for neurodevelopmental disorders [Faraone and Zhang-James, 2013]. Further studies can also study the similar drug repositioning potential using *in vitro* and *in vivo* approaches.

In conclusion, our study is the first animal behavior study that uncovers ASD-relevant behavioral abnormalities due to *Slc9a9* gene knockout. By modeling a human mutation that eliminates NHE9 activity, our work provides strong evidence that lack of NHE9 leads to ASD-like behaviors in mice, including deficits in social communication and social interaction, and restricted repetitive patterns of behavior. This provides the field with a new genetic mouse model of ASDs.

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