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AUTISM SPECTRUM DISORDER TRAITS IN *SLC9A9* KNOCK-OUT MICE

Lina Yang

A Dissertation in the Departments of Neuroscience & Physiology

Submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Graduate Studies of State University of New York,Health Science Center.

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Abstract

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 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 eliminating NHE9 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.

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LIST OF ABBREVIATIONS

ASD	Autism spectrum disorder
ADHD	Attention-deficit/hyperactivity disorder
NHE	Sodium/proton exchanger
KO	Knock-out
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
FDA	Food and Drug Administration
CNV	Copy number variation
GWAS	Genome-wide association study
MRI	Magnetic resonance imaging
fMRI	Functional magnetic resonance imaging
PGC	Psychiatric Genomics Consortium
SNP	Single-nucleotide polymorphism
RACK1	Receptor for activated C-kinase 1
CHP	Calcineurin homologous protein
CaM	Calmodulin
PIP2	Phosphatidylinositol 4,5-bisphosphate
SYP	Synaptophysin
PPP1R10	Protein phosphatase 1 subunit 10
PKCm	Protein kinase C, mu
CaMKI	Calcium/calmodulin-dependent protein kinase 1
NR2B	Glutamate NMDA receptor 2B subunit

PLCb1	Phospholipase C, beta 1
DAT	Dopamine transporters
LTP	Long-term potentiation
VPA	Valproic acid
USV	Ultrasonic vocalization
HX	Homozygous
Het	Heterozygous
WT	Wild type
PND	Postnatal day
EP	Elevated plus maze
OF	Open field
BM	Barnes maze
GLM	General linear model
SHIRPA	SmithKline Beecham, Harwell, Imperial College and Royal London Hospital phenotype assessment
Htr1a	Serotonin receptor-1A
Htr1b	Serotonin receptor-1B
Oxt	Oxytocin neuropeptide
Nlgn3	Neuroligin-3
Oxtr	Oxytocin neuropeptide receptor
Reln	Reelin
Foxp2	Forkhead box P2

Orpm	μ-opioid receptors
fmr1	Fragile X mental retardation 1
FXS	Fragile X syndromes
Mecp2	Methyl CpG binding protein 2
NL-3	Neuroligin-3

Introduction:

Part Ⅰ: ASDs background

1. Introduction of autism spectrum disorders

Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders. According to the 2013 released Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) [1], patients diagnosed with ASDs must present syndromes of 1) persistent deficits in social communication and social interaction, and 2) restricted repetitive patterns of behavior, and these syndromes should present in early childhood and cause clinically significant impairment in occupational, social, or other important functioning. ASDs are among the most common developmental disorders [2]. According to the recently released (2014 March) data from US Centers for Disease Control and Prevention, approximately 1 in 68 8-year-old US children were diagnosed with ASDs, and the ratio of ASDs in boys to girls is 5 to 1 [3]. The ASDs prevalence has increased significantly over the past decades. The estimate for ASDs prevalence in US is 1 in 150 in year 2000, 1 in 110 in year 2006, and 1 in 88 in year 2008 [3]. The cause of this increase is unknown, but it may be partially due to better screening and diagnosis in local communities, the changes in DSM criteria, and the increase in overall public and professional awareness [3, 4]. The up to date worldwide ASDs prevalence varies among studies. According to the nationwide studies in several European countries, Asian countries, and western Australia, the estimates have been made of 0.3-2.6% with significant increasing trends over years [5-12].

ASDs begin in childhood and generally persist into adulthood causing lifelong impairments to people with ASDs. Few people with ASDs have close friends, and permanent employment [13]. They tend to be more dependent on their families in adulthood than others [13]. In addition, they need more resources from the health care systems and the costs of lost productivities are significant [14]. ASDs not only affect life of individuals and families, the social and economic burden of ASDs are also substantial.

There is currently no cure for ASDs, nor effective pharmacological or non-pharmacological treatments [15]. Up to now, some psychotropic medications can alleviate various behavior impairments in ASDs individuals. Educational interventional therapy and dietary intervention are also found to be helpful for some ASDs children [15]. However, most pharmacological treatment to ASDs focus on targeting specific associated symptoms of ASDs, such as hyperactivity, irritability, anxiety, and compulsivity, while the core symptoms of ASDs are not directly addressed [16]. In addition, the side effects of the psychotropic medications to children and young people can sometimes be intolerable[16]. For example, when treated with antidepressants, commonly prescribed off-label to children with ASDs to alleviate compulsive and repetitive behaviors, children with ASDs experience significant side effects such as hyperactivity, agitation, aggression and suicide ideation [17]. Methylphenidate, prescribed to children of ASDs with comorbid

attention-deficit/hyperactivity disorder (ADHD) symptoms, causes adverse side effects including social withdrawal and irritability [18]. Even the only two US Food and Drug Administration (FDA) approved medications treating children with ASD, risperidone and aripiprazole, can have significant side effects such as weight gain and somnolence for risperidone [19, 20], and sedation, drooling, weight gain and extrapyramidal symptoms for aripiprazole [21-24].

In summary, ASDs are among the most common developmental disorders of childhood, and the prevalence has increased drastically over years. ASDs not only cause impairment in individuals' life and functioning, but are also associated with substantial burden to families and the society. There are currently no effective treatments for ASDs.

2. Genetic studies on ASDs

What causes ASDs is still not fully understood. It is commonly accepted that genetics plays a major role in ASDs. In twin studies, the ASD concordance in dizygotic twins is about 30%, whereas the ASD concordance in monozygotic twins can be up to 80% - 90%[25, 26]. Because both dizygotic and monozygotic twins usually share similar environment, the large difference of concordance ratios between dizygotic twins and monozygotic twins suggests that ASDs are mainly related with genes.

There are numerous genetic studies on ASD. Mutations in a single gene have been identified in some cases of ASD, such as ASDs comorbid with Rett syndrome (*MECP2* mutation) [27], Fragile X syndrome (*FMR1* mutation) [28], tuberous sclerosis (*TSC1* mutation) [29], neurofibromatosis (*NF1* mutation) [30], PTEN hamartoma tumor syndromes (*PTEN* mutation) [31], Phelan–McDermid syndrome (*SHANK* mutation) [32], and Duchenne/Becker muscular dystrophy (*DMD* mutation) [33]. Besides single gene mutation, chromosomal abnormalities such as translocation, inversion and copy number variation (CNV) of chromosomes have also been identified in some ASD cases [34]. For instance, it has been found that CNV is more frequent in patients with ASD than in controls [34-38]; several CNV candidate loci have been identified, such as 16p11.2 [34], 16p12.1 [39], 22q11.2 [40], 1q21.1 [41], 15q11-q13 [42] and 7q11.23 [38]. Besides DNA, noncoding RNA, including microRNA and long noncoding RNA, are also found to play a role in ASD [43, 44].

These genetic variations can only explain a part of the ASD cases. The cause of the majority of cases of ASD still needs to be explored. As the data piled up, researchers hope to find the connections between various genetic risk factors through bioinformatic methods. One research group integrated known rare mutations in ASD with results of an ASD genome-wide association study (GWAS) and with gene co-expression network of human brain [45]. They found that many rare and common mutations in ASD are involved in synaptic and neuronal plasticity. In another study, researchers linked ASD associated rare de novo CNVs with biological function analysis, and found synapse formation and function are significant affected by CNVs

[46]. In a review paper, the authors made a general genetic landscape of ASDs, in which molecular pathways involving postsynaptic translational regulation, neuronal cell adhesion, neuronal activity modulation and excitatory and inhibitory function imbalance are highly related with suspected ASD genes [47]. There exists the possibility that although hundreds of genes are associated with ASDs, those mutated genes can be clustered into subgroups and be encompassed by neuronal signaling pathways, synapse structure and/or synapse functions. A group of ASD associated mutations involved in synapse structure formation can be used as an example. Two of the most convincing reported and well-studied risk genes for ASD, *SHANK3* and *NLGN 3/4*, are both involved in synapse formation: *SHANK3* on chromosome 22q13 encodes a synaptic scaffolding protein, and *NLGN 3/4* on chromosome X encodes neuroligins which can induce the formation and reformation of synapses [34]. CNV at 15q11.2 region can alter CYFIP1 expression levels in neurons which influence dendritic complexity, the post-synaptic end of a synapse [48]. Besides DNA mutation, microRNA-132, dysregulation of which was reported in a family with ASD, has also been found to regulate dendritic structure and density [44]. Thus, by investigating these shared functional pathways, the relationships of hundreds of ASD-associated mutations can be discovered, and the mechanisms underneath them might be unearthed. It will further help the development of medications that treat ASDs, because amenable medication targets among these pathways may be exposed.

Besides genetic factors, environmental factors, especially exposure to chemicals during early pregnancy can also cause ASDs. The environmental risk factors include medications (such as valproic acid, misoprostol and thalidomide), insecticides (such as chlorpyrifos), cigarette smoke, maternal hypothyroxinemia and maternal rubella infection [49-51]. Epigenetic modifications of DNA may also contribute to ASDs. Although the DNA sequence is not changed, the expression level is modified. A recently published study showed significant site-specific DNA methylation difference between ASD affected and their unaffected monozygotic co-twins [52]. Genomic imprinting, another example of epigenetic modification, is also found to be associated with some cases of ASD [53]. These results provide additional strong evidence of a possible role of environmental factors in ASDs. The genetic and environmental factors may contribute to ASDs by their own, but the interaction of genetic and environmental factors are reported to increase the likelihood to develop ASDs [54].

Although ASDs are highly heritable disorders and genetic variations seem to play a major role in ASDs, in a most recently published paper, researchers believed genetic factors and environmental factors affect ASDs equally. They investigated 2,049,973 children born in Sweden between 1982 and 2006 to estimate the probability of the genetic and environmental factors that can be associated with ASD [55]. Among these children, 14,516 children were diagnosed with ASD. By comparing the correlation of ASD diagnosis among monozygotic and dizygotic twins, full siblings, paternal half siblings, maternal half siblings, and cousins, researchers made the estimation that heritability of ASD was 50%, which means the genetic factors accounted for half risk

of ASD while non-shared environmental factors accounted for the rest.

Taken together, there is no doubt that ASDs are among the most heritable and complex of psychiatric disorders. No single pathology accounts for all ASDs cases. Genetic mutations including single gene mutation and chromosome abnormalities (CNV, translocation and inversion) are highly related with ASDs; environment factors also have significant effects on ASDs. By integrating multiple ASDs related factors, several possible neuronal pathways have been suggested to be affected in ASDs. But from my point of view, comparison studies need to be done to see if those neuronal pathways involved in ASDs can be discriminated from those in other neurodevelopmental disorders. Thus in the future more studies besides ASD studies may be integrated together to help unravel the complexity of the genetics of ASDs.

3. Neuroimaging studies on ASD

Besides the abnormal behavior syndromes of ASD, researchers hope to find valid biomarkers for ASD diagnosis. Efforts have been put on neuroimaging studies to investigate the possible common brain architecture in individuals with ASD.

Structural magnetic resonance imaging (MRI) studies showed that in ASD, cerebral hemispheres, cerebellum and caudate nucleus are enlarged, whereas the corpus callosum area is decreased [56]. The overall brain volume in ASD is increased [56]. The global gray matter and white matter were not found different between ASD and controls, but regional differences were found between ASD and matched controls: smaller gray matter volumes in the amygdala-hippocampus complex and medial parietal regions, larger white matter volumes in regions relevant to language and social cognition in ASD [57].

Functional magnetic resonance imaging (fMRI) studies found both hypo- and hyper-connectivity of brain activity in ASD. Hypo-connectivity is more dominated particularly for cortico-cortical connections and interhemispheric connections; hyper-connectivity is more limited, affecting subcortical nuclei and parietal cortex [58]. Overall, MRI and related studies suggest increased brain size, regional altered gray and white matter volumes, and primarily decreased connectivity are associated with ASD. However, inconsistence of MRI results within the ASD exists among the results from different studies, and various factors such as population age, MRI acquisition parameters among different studies may confound the overall conclusion [59, 60]. There is currently no valid diagnostic criteria for ASDs based on MRI or its associated techniques, which reflects its complexity and inconsistence of results of ASDs [61].

4. ASD comorbidity, and relationship with ADHD

ASDs often co-occur with other disorders, such as epilepsy, depression, anxiety, attention deficit/hyperactivity disorder (ADHD), intellectual disability, gastro-intestinal problems[62, 63], and the monogenetic disorders mentioned in "genetics studies on ASD" section above. Among them ADHD is one of the most common comorbid disorders in individuals with ASDs [64].

ADHD overlaps many aspects with ASD. There are behavioral, biological and neuropsychological similarities between these two disorders [65].

ADHD is characterized by persistent pattern of inattention and/or hyperactivity-impulsivity that cause impairments in daily functioning [1]. Many studies reported that children with ASD are known to have inattentive and hyperactive behaviors, whereas children with ADHD often show social difficulties with peers [64, 66]. 31-95% of children with ASDs also show significant symptoms of ADHD [67-69]. This prevalence far exceeds 5-8% prevalence of ADHD in children and adolescent [70, 71], and 4-5% prevalence in adults [72]. There is currently no population based study of the prevalence of ASD diagnoses in children with a primary diagnosis of ADHD, but in a study based on families that had at least one ADHD proband, ASD symptoms were significantly higher in probands than in healthy siblings or controls [73].

Both ASD and ADHD are highly heritable and complex with multifactorial etiology. ASD and ADHD share many genetic risk factors [74, 75]. According to genetic linkage studies, several regions on chromosome, 16p13 [76], 2q24 [77], 15q [75, 78], 17p11 [79] have been associated with both ASD and ADHD. Some single gene mutation syndromes, for instance Fragile X syndrome (*FMR1* mutation) and neurofibromatosis (*NF1* mutation), are comorbid with both ASD and ADHD [80, 81]. When the GWA data from the Psychiatric Genomics Consortium (PGC) for five disorders schizophrenia, bipolar disorder, major depressive disorder, ASD and ADHD were pooled together, the calculated genetic correlation between ASD and ADHD using common SNPs in GWA data from the Psychiatric Genomics Consortium is not significant [82], however, SNPs at four loci reached genome-wide significance ($p<5\times10^{-8}$) for all five disorders: regions on chromosomes 3p21 and 10q24, and SNPs within two L-type voltage-gated calcium channel subunits, CACNA1C and CACNB2 [83]. There is also evidence show that de novo CNVs are significant risk factors for both ASD and ADHD [35, 84]. Besides sharing genetic variations, ASDs and ADHD were also found to be involved in similar pathways including synaptogenesis, neuronal growth and differentiation [66]. Similar environmental risk factors (e.g., cigarette smoke during early pregnancy) were also implicated to be shared by both disorders.

There are also neuropsychological features that overlap between ASD and ADHD. For example, children with ASD and children with ADHD both have executive dysfunction [85]. Children with ASD have more difficulties with planning and cognitive flexibility and children with ADHD have more difficulties with inhibition and spatial working memory [86-89]. Besides executive dysfunction, social cognition impairment (especially theory of mind) is also found to be associated with both ASD and ADHD [90]. Children with ASD and children with ADHD both performed worse in theory of mind tasks than controls [90-92].

In summary, ASD and ADHD share behavioral, biological and neuropsychological

features, which makes it meaningful to study both ASD behavior features and ADHD behavior features (especially hyperactivity) in our study.

Part Ⅱ: *SLC9A9* background

1. Introduction of *SLC9A9*

SLC9A9, also called sodium/proton exchanger 9, belongs to the family of Na^+/H^+ exchangers (NHEs or *SLC9*). The NHEs are ATP-independent membrane proteins that mediate the simultaneously efflux of proton ions and influx of sodium ions. They utilize the electrochemical gradient of one ion to allow the other ion to be transported against its gradient [93].

There are currently 13 known isoforms of NHE in mammals [94-96]. The *SLC9* gene family contains 3 subgroups: *SLC9A*, *SLC9B*, *SLC9C*. There are 9 isoforms in SLC9A in human. NHE1-5 (*SLC9A1-5*) exist on plasma membrane, whereas NHE6-9 (*SLC9A6-9*) exist on intracellular organelles [94]. There are 2 isoforms in *SLC9B*, NHA1 and NHA2 (*SLC9B1* and *SLC9B2*, respectively). The *SLC9C* contains a sperm specific plasmalemmal NHE *SLC9C1*, and *SLC9C2* with no currently functional information about it [95]. Among all the NHE isoforms, as well as the Nhx1, the NHE9 ortholog in yeast, and NhaA, the NHE9 ortholog in bacteria, the only available crystal structure for this family is NhaA [97]. Based on the crystal structure of NhaA and sequence alignment analysis, the structure of NHE 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 [96, 98, 99]. The NHE binding molecules include calcineurin homologous protein (CHP) [100], receptor for activated C-kinase 1 (RACK1) [101], calmodulin (CaM) [102] and phosphatidylinositol 4,5-bisphosphate (PIP2) [103]. They participate in various signaling pathways, such as Ca^{2+} signaling cascades and protein phosphorylation/dephosphorylation which may involve in synaptic transmission and plasticity.

SLC9A9 locates on the membrane of late recycling endosomes. It regulates the pH and maintains cation homeostasis of endosomes. As the other NHEs, *SLC9A9* has a transmembrane domain and a long C-terminal domain. Genetic mutations associated with ASD and ADHD reside on last transmembrane domain and C-terminal domain of *SLC9A9* [104]. By constructing the detailed structural model of *SLC9A9*, researchers indicated that two ASD-associated mutations, L236 and S438, change the physiocochemistry and sterochemistry of the amino acid side chains significantly, whereas the effect of V176I, another ASD-associated mutation, is moderate [105].

In a previous study, members in our laboratory showed that *SLC9A9* C-terminal interacts with CHP and RACK1 [104]. They also examined the expression and relationship of *SLC9A9* and its potential binding molecules in two ADHD rat models (WKY/NCrl and SHR) [106]. They found that the expression levels of *SLC9A9*, CHP, RACK1, CaM, synaptophysin (SYP), a type B calcineurin (protein phosphatase

3, regulatory subunit B, alpha isoform, PPP*3R1*), protein phosphatase 1 subunit 10 (PPP1R10), protein kinase C, mu(PKCm), calcium/calmodulin-dependent protein kinase 1 (CaMKI), glutamate NMDA receptor 2B subunit (NR2B), and phospholipase C, beta 1 (PLCb1) in brain are highly correlated [106]. These molecules are involved in signaling pathways that play a critical role for synaptic plasticity and learning and memory [106].

2. Genetic association of *SLC9A9* with ASD and ADHD

SLC9A9 is a risk gene for ASD. Three ASD-associated substitutions, namely V176I, L236S and S438P, reside within the membrane domain of *SLC9A9* [105]. A protein truncation in the extracellular loop (C-terminal) of *SLC9A9* caused by a stop codon mutation (R423X) was found in individuals with ASD and epilepsy [107]. Because C-terminal provides binding regions for regulatory protein, loss of C-terminal may completely remove all the interactions of the *SLC9A9* C-terminal with its regulatory protein, such as CHP and RACK1 [104, 106].

SLC9A9 is also a risk gene for ADHD. Several pieces of evidence show its association with ADHD. 1) *SLC9A9* was first implicated in ADHD in a clinical report [108]. In a large family with 22 family members, 11 of them presented features of ADHD and intellectual disability. Cytogenetic analysis revealed that these affected members carried an abnormal copy of chromosome 3 which has a *SLC9A9* gene disrupted by pericentric inversion [108]. Thus in this family ADHD coexisted with a pericentric inversion of chromosome 3 disrupted *SLC9A9* gene [108]. 2) *SLC9A9* significant associated with ADHD was also found in candidate gene studies [109, 110]. 3) In an ADHD genome-wide association study, *SLC9A9* achieved one of the lowest P-values of the candidate genes [111]. 4) In two rat models of ADHD, WKY/NCrl rats and SHR/NCrl rats, increased *SLC9A9* expression in ADHD related regions in brain was found [104, 106]. 5) There are two nonsynonymous mutations in *SLC9A9* in WKY/NCrl rats which may influence the regulatory protein binding with *SLC9A9* [104]. For example, the mutations significant changed the interaction of CHP with *SLC9A9* [104].

These genetic associations of *SLC9A9* in both ASD and ADHD indicate *that SLC9A9* may play an important role in neurodevelopmental processes.

Deficits in *SLC9A9* may cause series of problems. For example, by disrupting the normal function of endosomes, deficits in *SLC9A9* may cause trafficking and recycling problems of transporters and receptors that may be essential to ADHD and ASD syndromes, such as glutamate receptors and the dopamine transporters (DAT) [112, 113]. Endosomal trafficking of glutamate receptors, especially AMPA-type and NMDA-type glutamate receptors, is critical to long-term potentiation (LTP) [113, 114]. Blocking the endosomal trafficking may inhibit LTP. Endosomal trafficking of DAT is critical to regulate the DAT on presynaptic membrane [115]. Abnormal DAT, one of the targets of ADHD stimulate medications, may result in neurodevelopmental disorders.

Since *SLC9A9* may also involve in various synaptic signaling cascades, deficits in *SLC9A9* may cause synaptogenesis and pruning problems which may result in long term defects in brain function. Generation of *SLC9A9* knock-out mice and studying their ASD- and ADHD-associated behavioral phenotypes will clarify the direct relationship of *SLC9A9* with these two neurodevelopmental disorders.

Part III. Introduction of valproic acid (VPA) model, an existing animal model for ASD and ADHD, leading to the methods of behavioral phenotyping

One of the most extensively used animal models for ASD is maternal exposure to valproic acid (VPA) in mice. VPA is a widely used anti-epilepsy and mood-stabilizer. However, exposure to VPA in utero increases the risk of ASD. Behavior studies show that VPA administration to pregnant mice and rats leads to autistic-like behaviors in the offspring, including the three core signs of ASD: deficit in communication, impaired social interaction, and stereotypic/repetitive patterns of behaviors (DSM-IV, 1994). Many specific tasks have been designed to unravel the autistic-like behaviors in mice and rats. In a review paper, the authors summarized the behavior works done in the VPA model that related to the three core signs of ASD [116]:

1) Deficits in communication

Reduced pup distress calls and adult 70-kHz premating vocalizations are indicators for deficit in communication in VPA mice model. They were measured by the density and latency to initiate ultrasonic vocalizations (USVs) as well as the spectral content of the USVs [117].

2) Impaired social interaction

In three-chambered social interaction test (the detailed information about three-chambered social interaction test can be found in the methods part), VPA mice or rats spent less or no different time in the chamber that contained an stranger compared to the empty chamber, whereas the control mice or rats spent significant more time in the chamber that contained an stranger compared to the empty chamber. When one familiar and one novel animal were put in the chamber at the same time, the VPA mice or rats did not show preference to the novel animal as the control did. These results indicated a decreased level of sociability and preference for social novelty in VPA mice or rats [118-120].

Another experiment to test the social behavior is to put pairs of mice or rats in the open field and observe their social interactions, such as chasing, pinning, reciprocal grooming, sniffing, aggressive behaviors, social exploration and avoidance. Although not all of the social behaviors showed difference in VPA mice or rats compared with controls, the VPA mice or rats had an overall reduced level of social interactions [121-124].

3) Stereotypic/repetitive patterns of behaviors

Stereotypic/repetitive movements, self-grooming, marble burying in an open field and alternation between the apparatus arms in the Y-maze are all measurable indicators for stereotypic/repetitive behaviors. Compared with control, VPA mice or rats spent more time on repetitive/stereotyped movements in open field [121, 125], and self-grooming in clean empty mouse cage [117, 126]. VPA mice or rats also buried more marbles under bedding [126], and showed reduced alternation in Y-maze tests [123]. All these evidence are indicators for the enhanced stereotypic/repetitive patterns of behaviors in VPA mice or rats.

Taken together, after testing all these three core symptoms of ASD, VPA model is proved to be a valid model for ASD. Besides VPA model, BTBR *T+tf*/J (BTBR) mouse also displays behaviors consistent with those three core ASD related symptoms, so BTBR mouse is another commonly used ASD animal model.

Those tasks mentioned above are also proved to be valid methods to test ASD behaviors in rodents. That is how we designed USVs recording, three-chambered social interaction tests, and behavior observation in our experiments to test ASD phenotypes in *SLC9A9* knock-out mice. Besides the three core symptoms, we will also test several ASD comorbid symptoms, such as ADHD-like behavior (especially hyperactivity) in open field activity tests, anxiety-like behavior in elevated plus maze tests, and resistance to change in Barnes maze tests. There might be also other deficits revealed by our experiments such as deficits in spatial learning and memory in Barnes maze tests, which can be supportive evidence for *SLC9A9* may play a role in neurodevelopment and synaptic transmission.

In summary, ASDs are one of the most common developmental disorders in the world with no cure nowadays. Although MRI studies showed altered brain structure and function in some cases of ASDs, the diagnosis of ASDs are mainly based on behavior symptoms, including persistent deficits in social communication and social interaction, and restricted repetitive patterns of behavior. The cause of ASDs is still not fully understood. Genetics seems to play a major role in most cases of ASDs. Genetic mutations including single gene mutation and chromosome abnormalities are related with ASDs. Among the risk genes for ASDs, *SLC9A9*, a sodium/proton exchanger on endosome membrane, shows its strong association with ASDs, which inspires us to study the neurobehavioral phenotypes in mice expressing no *SLC9A9*.

Our hypothesis is that the *SLC9A9* knock-out mice have behavior deficits that resemble the behaviors in ASD, such as social communication and interaction deficits, stereotype of behavior, resistance to change, anxiety, etc. Because ASD often comorbid with ADHD and share behavioral, biological and neuropsychological features, we also expect to observe the ADHD behavior features especially hyperactivity in the knock-out mice.

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. Global KO mice were generated by crossing the floxed line with Hprt-Cre mice (JaxMice, Bar Harbor, Maine). The experimental animals were obtained by heterozygous-heterozygous matings. We used gender-matched littermates, including homozygous (HX)KO, heterozygous (Het)KO and wild type genotypes(WT), for behavioral tests. 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 ata 12hrdark/light cycle (lights on 7:00 to 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 10:00-18:00.

We chose measurements based on prior studies. For ASD measures, deficits in communication were tested by recording and measuring the ultrasonic vocalizations (USVs) of mouse pups [117, 127]. Impaired social interactions were tested by conducting three-chambered social interaction tests [118-120, 128]. Stereotypic/repetitive patterns of behavior were tested by measuring the time that mice spent grooming themselves [121, 125, 128, 129]. Besides these core features of ASDs, we also tested ADHD-like behaviors in open field activity tests and several other ASD comorbid symptoms, such as anxiety-like behavior in elevated plus maze tests. We tested their spatial learning and memory ability with the Barnes maze tests because deficits in spatial learning and memory would provide supportive evidence for NHE9 playing a role in neurodevelopment and synaptic transmission.

1. Maternal separation and USVs recording

We recorded USVs emitted by 90 mice (HX=25, Het=39, WT=26) at PND13. One pup at a time was removed from its home cage and put it in a cage with clean bedding but without lid covering. Then the cage was transferred into a box which has an ultrasonic vocalization detector (Med Associates) attached inside on the top. The recording lasted for 10min for each mouse. The number of calls and total call duration during the maternal separation were measured.

2. Elevated plus maze test (EP)

Each mouse was put on the elevated plus maze and recorded for 10 min on PND21. 79 mice (HX=24, Het=32, WT=23) were used. The apparatus consists of two closed arms (110x10cm, LxW) with 30cm tall non-transparent walls on both sides and two open arms (110x10cm, LxW) without walls. The apparatus is 50cm elevated from the

floor and visually isolated by screens. On PND 21, the mouse was put in the center zone 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 (MED Associates). The time spent in each arm was measured. Entries were recorded if all four paws entered the arm.

3. Open field activity (OF)

We used four square non-transparent open field boxes (50x50x38cm, WxLxH). 78 mice (HX=25, Het=30, WT=23) were used on PND 22.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 (MED Associates). Each box field was divided into four zones by a grid drawn on the screen: center, middle, edge and corner zones. Overall distance and average speed, as well as the distance traveled and time spent in different zones were assessed.

4. 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 [130].78 mice (HX=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 and analyzed using appropriate regression models independently.

5. Barnes maze test (BM)

57 (HX=19, Het=21, WT=17) mice were used. The Barnes maze 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". 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.

On PND 29, the adaptation period, each mouse was placed in a cylindrical start chamber in the center of the platform. After 10s habituation, the chamber was lifted, the buzzer was turned on and the mouse was guided gently to the target box. Once the mouse got into the target box, the buzzer was turned off immediately and the mouse stayed in the box for 2 min.

On PND 30, the spatial acquisition period, each mouse went through the same steps as the adaptation period, but instead of guiding the mouse to the target, it was allowed to explore the maze freely for 3 min. Once the mouse found the target box and hid in it, 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 and was left inside for 1 min. Each mouse was tested four times every day with an inter-trial interval of 1 h from PND 30 to PND 33. The latency for the mouse to locate the target box and the average speed were recorded by Any Maze Software.

After the four days spatial acquisition, the first probe trial was conducted on PND 34. The target box was replaced with a non-target box so that it was closed. The mouse was placed in the cylindrical start chamber in the center of the platform. After 10s habituation, the chamber was lifted, the buzzer was turned on and the mouse was allowed to explore the maze freely for 90s. The time spent in the old now closed target box zone was measured. The second probe trial was conducted on PND 41 with the same procedures as the first probe trial.

6. Three-chambered social interaction test

The three-chambered social interaction test was applied after the mice became adults (more than 60 days old). 53 mice (HX=17, Het=17, WT=19) were used.The apparatus has three chambers with retractable door between two chambers. In one side chamber, there is a plastic cage which contains a novel stranger mouse (novel mouse-1) approximately the same age as the subject mouse. The other side chamber contains an empty plastic cage. 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-1had had no previous contact with the subject mouse.

In the first period of testing, the subject mouse was placed in the center chamber with the door closed for a 10min habituation period. Then the doors between each chamber were raised and the subject mouse was able to move freely about the three-chambered apparatus. The social interaction test lasted for 10min with a camera recording 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 interaction was initiated only by the subject mouse.

The same testing procedures were repeated once a day for the next three days. On the last day, right after the 10 min testing, the test mouse proceeded to the next 10min testing. In this second 10 min period of testing, another novel stranger mouse (novel mouse-2) was introduced into the three-chambered apparatus. It was placed in the empty plastic cage that was in the side chamber. Novel mouse-2 had had no previous contact with the subject mouse. Novel mouse-1 in the first testing period would have become a now-familiar mouse and was placed in the plastic cage in the other side chamber. In this second period of testing, the same observations were recorded and

measured as in the first period of testing.

7. Behavioral observations

48 adult mice (HX=15, Het=16, WT=17) were used for behavioral observation. We put the test mouse in the center chamber with both doors closed. After 10 mins of habituation, we put novel mouse-1 in the center chamber and video recorded behavior for 10 min. After that, we took out novel mouse-1 and put novel mouse-2 in the center chamber and video recorded for 10 min. The videos were scored for 8 different categories of behaviors to evaluate social behavior and stereotypical/repetitive patterns of behavior. Eight different categories of behaviors were studied: investigating, being investigated, chasing, being chased, self-grooming, reciprocal grooming, fighting and rearing. Social contact is the sum of investigating, being investigated, chasing, being chased, reciprocal grooming, and fighting.

Statistical Analysis

To assess the effect of genotype and covariates on behavior, we used the general linear model (GLM) in STATA 12.0 [StataCorp, 2011 #25856]. 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 chi-square tests depending on the model chosen. We first analyzed the effect of genotype as a categorical variable. If the prior analyses were not significant, to potentially increase power, we either a) analyzed genotype as a dose effect (0 = WT, 1 = Het, 2 = HX) or b) when no difference was found between HX and Het mice, we combined them into one group. Significance was set as p-value<0.05.

Results

General Phenotypes

We examined all the animals' weight from PND 5 to up to 100 days, no growth rate difference was observed for different genotypes ($X^2(2) = 1.3$, $p = 0.52$). The gene dose effect was not significant either ($X^2(1) = 0.26$, $p = 0.6$) (Figure 1).

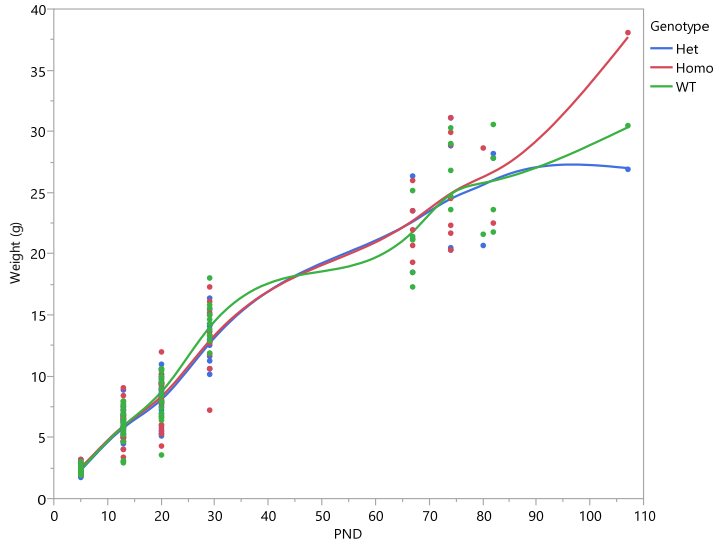

Figure 1
Weight plotted over PND to show animal general growth.

In SHIRPA, no difference was detected among animals for all the phenotype assessments (data not shown) except the locomotor activity. For males, genotype effect was significant ($X^2(2) = 11.04$, $p=0.004$) with Het KO males traveled more squares in 30s than other two genotype mice. For females, genotype effect was not significant ($X^2(2) = 5.89$, $p= 0.053$), but gene dose effect was significant ($X^2(1) = 5.17$, $p= 0.023$) (Figure 2).

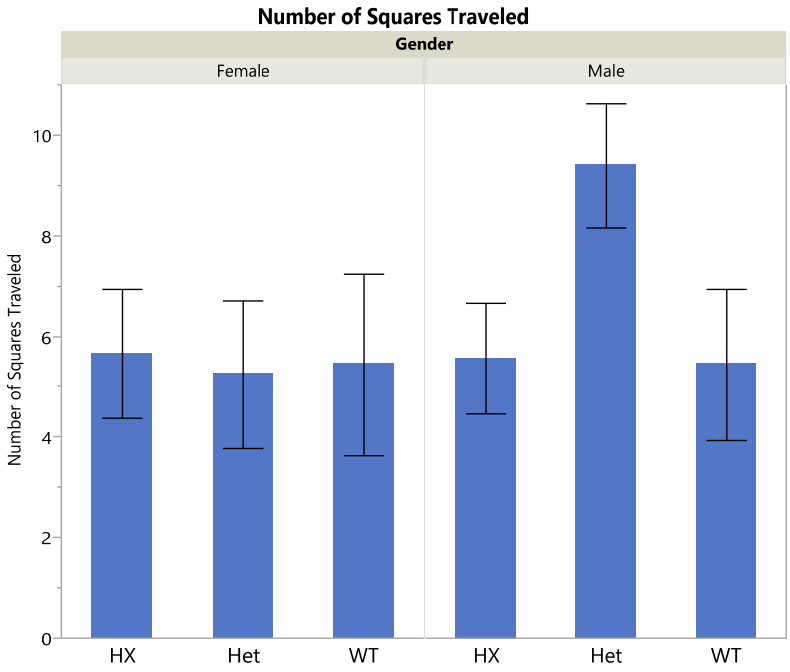

Figure 2. Number of squares traveled in SHIRPA
Animals were dropped from 25 cm height above the arena floor and number of squares they traveled in 30 s were counted.

1. Ultrasonic Vocalizations (USVs)

For band 1, the lower frequency bandwidth (20Hz - 50Hz), we used negative binomial regression model and GLM to assess the effect of genotype on number of calls and call duration respectively. In female, we found a significant effect of genotype on number of calls ($X^2(2) = 15.25$, $p = 0.0005$). Although genotype effect for call duration was not significant ($X^2(2) = 5.01$, $p = 0.08$), gene dose effect for call duration was significant ($X^2(1) = 5.34$, $p = 0.02$). In male, genotype did not have any effect on number of calls ($X^2(2) = 0.93$, $p = 0.6$) or call duration ($X^2(2) = 0.03$, $p = 0.9$) (Figure 3. A and B).

For band 2, the higher frequency bandwidth (50 Hz - 100 Hz), we used negative binomial regression model and Gaussian linear regression model to assess the effect of genotype on number of calls and call duration respectively. In female, significant genotype effect was detected for number of calls ($X^2(2) = 6.11$, $p=0.047$). Although the genotype effect was not significant for call duration ($F(2.24) = 2.27$, $p = 0.13$), gene dose effect was significant ($F(1, 25) = 4.24$, $p = 0.050$). In male, no genotype effect was detected for number of calls ($X^2(2) = 0.29$, $p=0.9$) or call duration ($F(2, 51) = 0.09$, $p = 0.9$) (Figure 3.C and D).

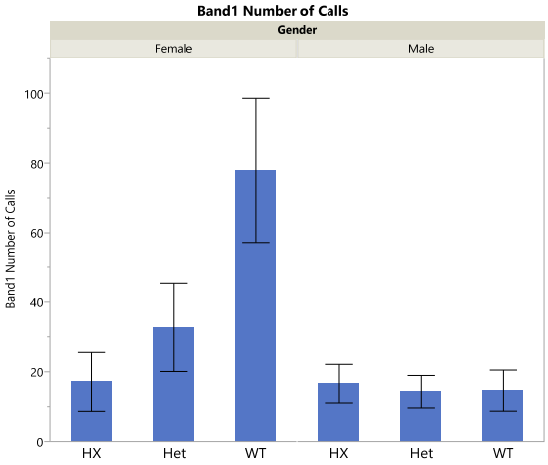

Fig. 3. A

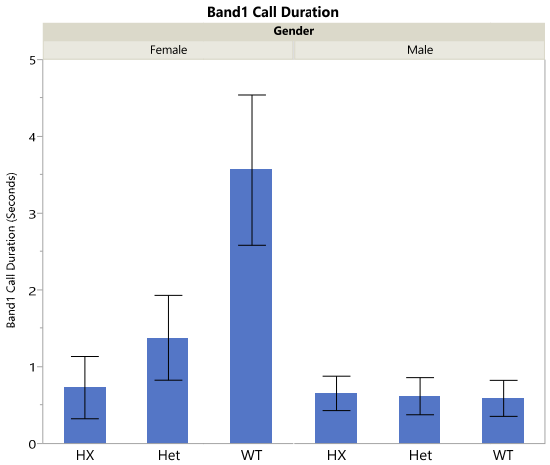

Fig. 3. B

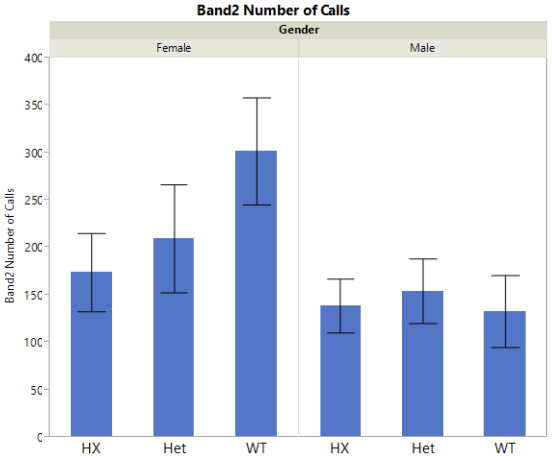

Fig. 3. C

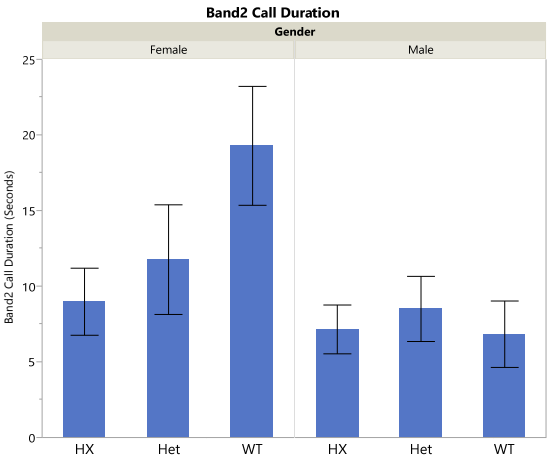

Fig. 3. D

Figure 3. USVs results
For band 1 (20Hz - 50Hz), number of calls (A) and call duration (B) were plotted over genotype for female and male separately. For band 2 (50Hz - 100Hz) number of calls (C) and call duration (D) were plotted over genotype for female and male separately.

2. Elevated-Plus Maze Test (EPM)

We used Gaussian linear regression model to assess the effect of genotype on total distance traveled, time in open and closed arms, time in center zone and probing zone. We used Poisson regression model to assess 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, HX KO mice spent less time in the open arms ($F(1, 66) = 10.14$, $p = 0.002$) (Figure 4. A) and more time in the closed arms (Figure 4. B) ($F(1, 66) = 6.85$, $p = 0.01$). HX 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 ($F(2, 65) = 0.57$, $p = 0.6$).

To evaluate the overall locomotor activity, we analyzed the distance traveled and total number of entries. Genotypes did not affect total distance traveled ($F(2, 65) = 1.50$, $p = 0.20$). The three genotypes differed in their total number of entries into the arms ($X^2(2) = 7.27$, $p = 0.026$), with HX KO mice showing fewer entries (Figure 4.C).

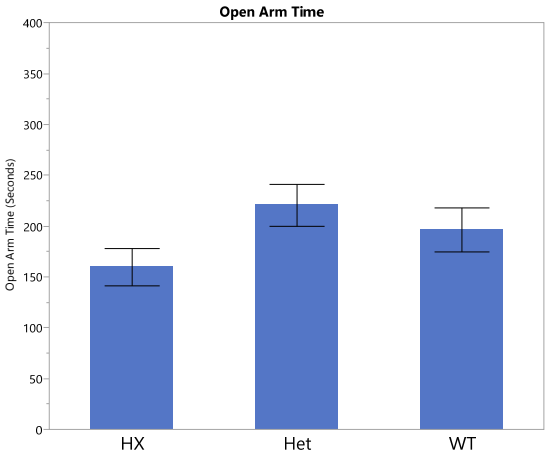

Fig. 4. A

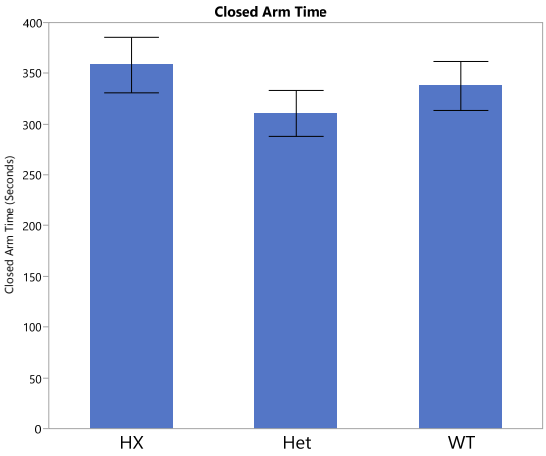

Fig. 4. B

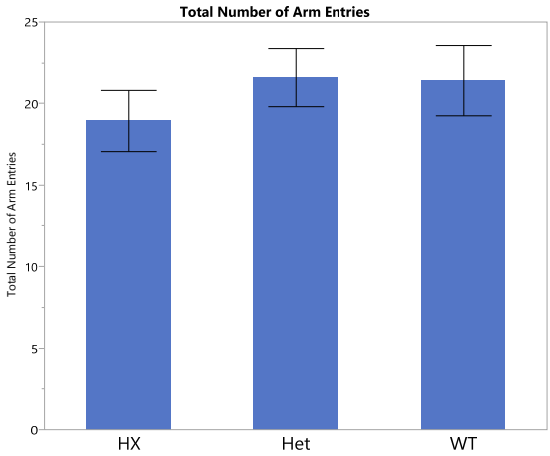

Fig. 4. C

Figure 4. EPM results

Time spent in open arms (A) and closed arms (B) was plotted. Total distance traveled (C) and total arm entries (D) was plotted.

3. Open Field Test (OF)

The experimental design for the open field box in open field test is showed in Figure 5. We used Gaussian linear regression model to assess 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, $F(2, 64) = 0.03$, $p = 0.97$).

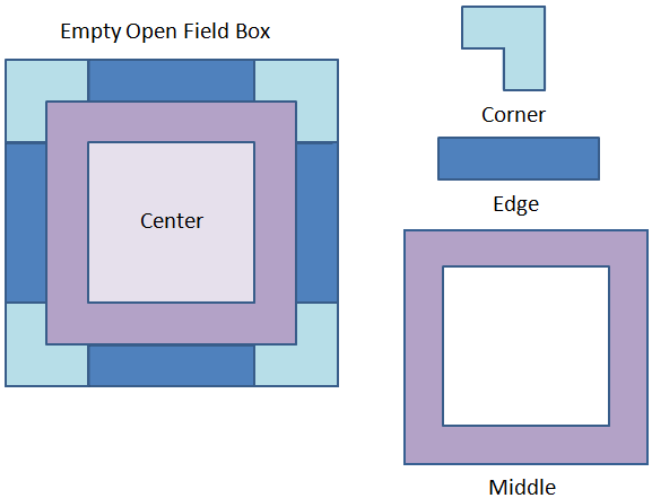

Figure 5. Open field

The experimental design for the open field box in open field test.

4. Barnes Maze Test (BM)

The experimental design for the Barnes maze is showed in Figure 6. We used GLM for the analysis. In the acquisition phase, a significant interaction between genotype

and trial was observed for the latency to locate the escape box ($X^2(2) = 8.38$, $p = 0.02$). 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 escape box ($X^2(2) = 7.75$, $p = 0.02$). Across the 16 training trials, all three genotypes needed less and less time to locate the escape box with increasing numbers of trials ($X^2(1) = 4.25$, $p = 0.04$). However, this decreasing trend was the most dramatic for Het KO mice. Compared with WT, Het KO mice spent less time locating the escape box ($X^2(1) = 8.21$, $p = 0.0042$) (Figure 7. A). The HX KO mice did not differ from WT ($X^2(1) = 2.72$, $p = 0.0993$) or Het KO mice ($X^2(1) = 1.18$, $p = 0.28$) (Figure 7. A).

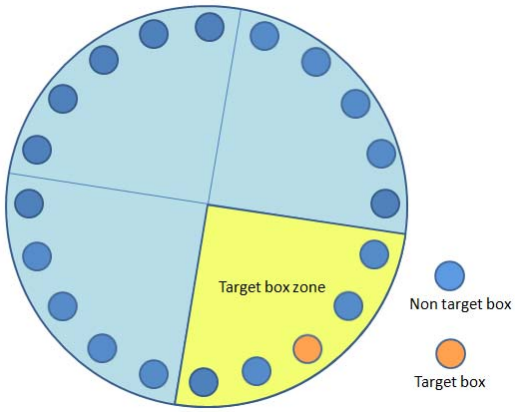

Figure 6. Barnes maze
The Barnes maze is a circular platform with 20 equally placed holes along its perimeter. One hole is "target hole" that is the entries of a dark recessed "target box". The animal is expected to find the target box and hide in it.

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 found that Het KO mice also had significantly increased speed across the trial ($X^2(1) = 15.53$, $p = 0.0004$) (Figure 7.B). When we included speed in our GLM model, the speed effect was significant ($X^2(2) = 91.88$, $P < 0.0001$). However, the effect of interaction between genotype and trial was not significant any more ($X^2(2) = 5.29$, $p = 0.07$), and the effect of interaction between genotype and quadratic trial was not significant ($X^2(2) = 4.59$, $p = 0.1$) either. This increase in speed accounted for the decreased latency to locate the escape box in Het KO mice. So there was not enhanced spatial learning and memory in Het KO mice, but just increased locomotor activity.

In the probe tests performed 24 h after the last training session, no genotype effect was detected by using a Gaussian linear regression model (Genotype $F(2, 45) = 0.17$, $p = 0.8$). But in the probe tests performed 8 days after the last training session, a significant genotype difference was observed in time spent in the zone where the escape box had been placed ($F(2, 44) = 3.35$, $p = 0.04$). Het KO mice spent more time

in the zone where the escape box had been placed compared to HX KO mice (F(1, 44) = 6.33, p = 0.02) (Figure 7. C).

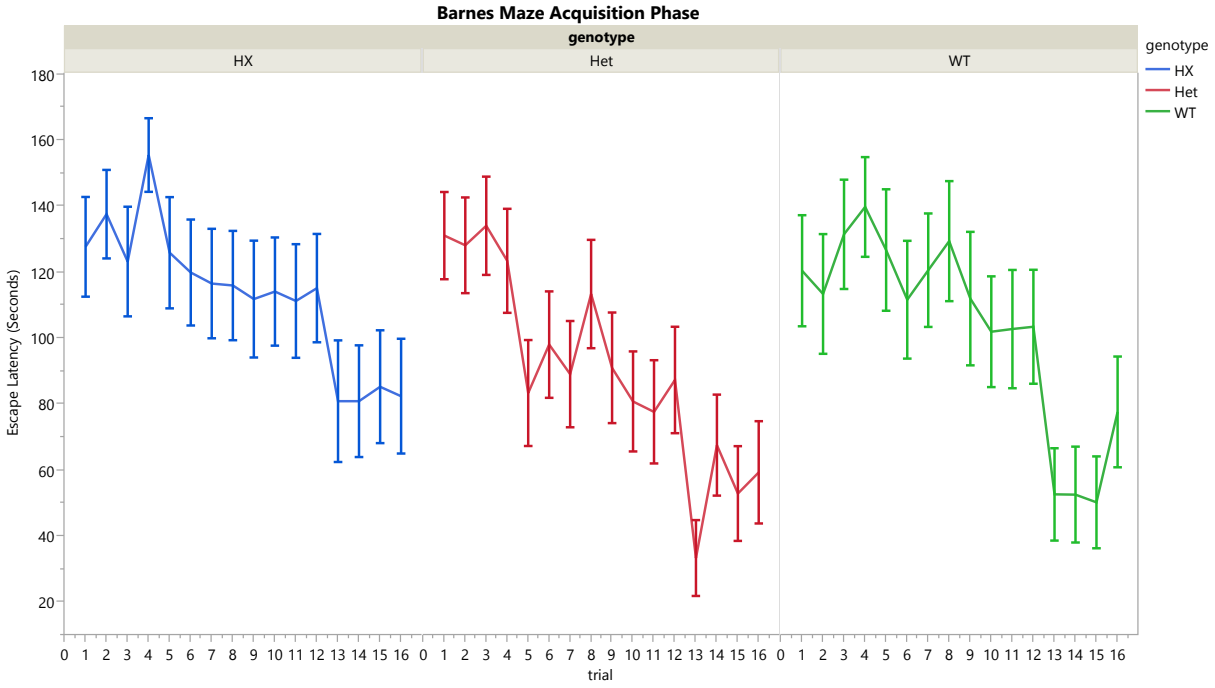

Fig. 7. A

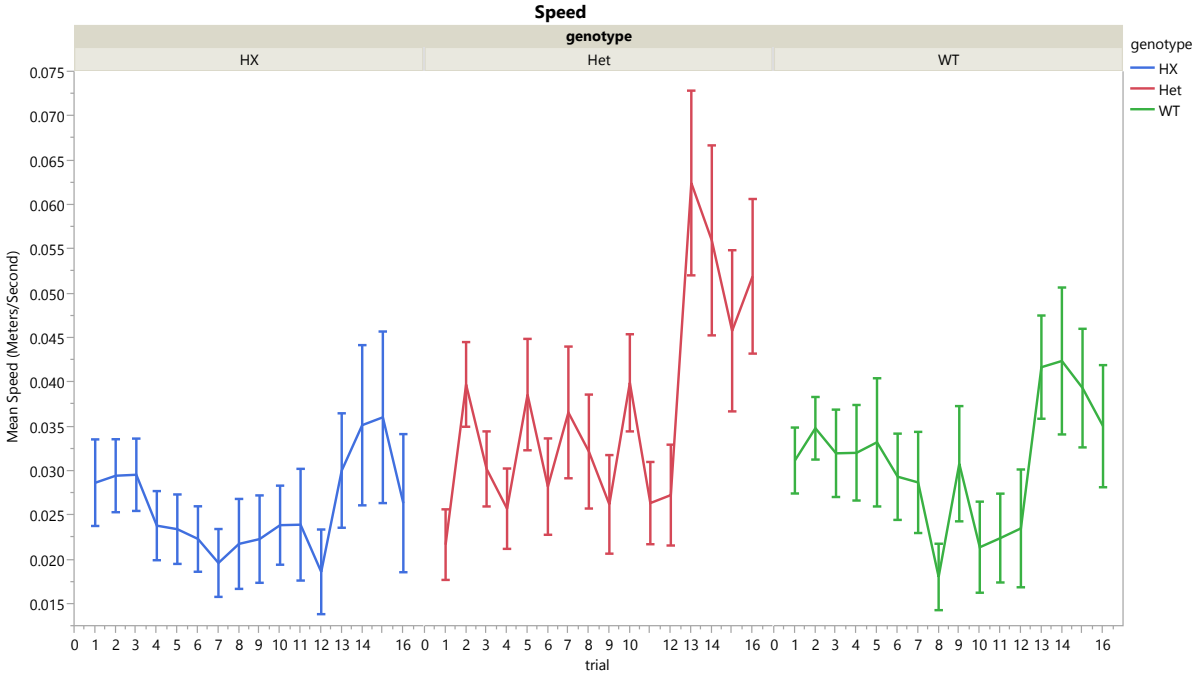

Fig. 7. B

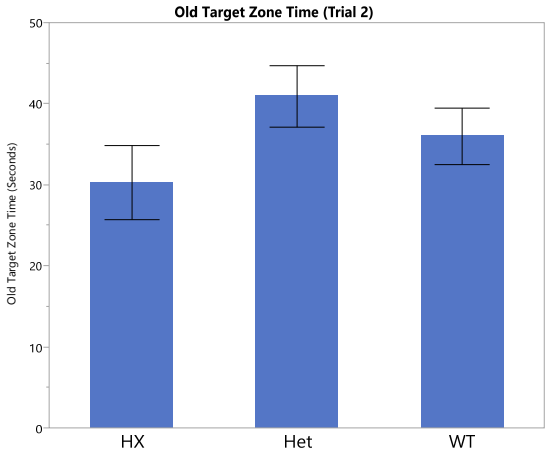

Fig. 7. C

Figure 7. BM results

In the acquisition phase, the latency to locate the escape box was plotted over trials to show genotype difference (A), and average speed was plotted over trials (B). In the probe tests performed 8 days after the last training session, time spent in old escape zone was plotted (C).

5. Three-chambered social interaction test

The experimental design for the three-chambered apparatus in social interaction test is showed in Figure 8. We used Gaussian regression model to do the analysis. During the social object habituation/sociability test from day 1 to day 4, all mice showed social approach behavior as all mice spent more time in the mouse chamber than in the center chamber or the empty cage chamber. No genotype effects 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 HX and Het KO mice were not different ($F(1,40) = 0.63$, $p = 0.4$), we combined HX 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 *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 ($t(33)=0.2$, $p=0.8$).

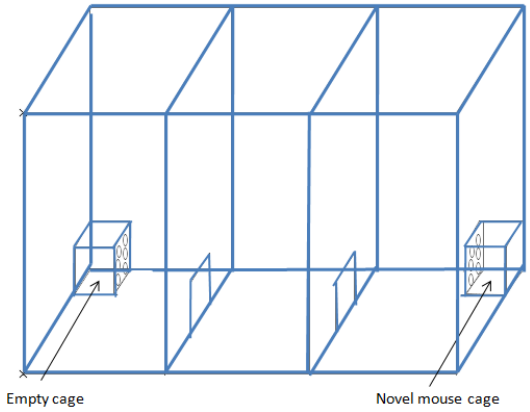

Figure 8. Three-chambered apparatus

The experimental design for the three-chambered apparatus used in the three-chambered social interaction test. A novel mouse is enclosed in a cage which has holes on one side. This cage and another similar empty cage are then placed in the right and left chambers respectively. The subject mouse is placed in the center chamber and can enter side chambers through the doorway in each dividing wall.

6. Observation of social behavior

No significant genotype difference was detected for social contact time ($F(2, 27) = 0.69$, $p = 0.51$).We did not detect genotype effect on time spent self-grooming ($F(2,45) = 1.31$, $p = 0.28$), but detected a significant gene dose effect on time spent self-grooming ($F(1, 19) = 6.54$, $p = 0.02$) (Figure. 9). HX KO and Het KO mice spent more time self-grooming than WT mice. We did not detect a genotype difference in time spent on rearing ($F(2, 45) = 0.35$, $p = 0.7$).

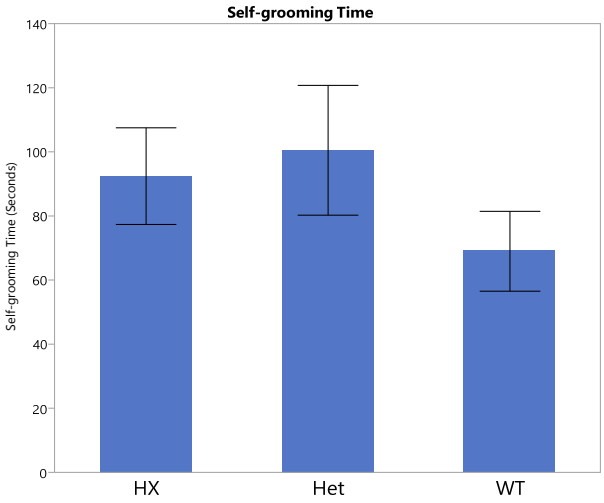

Figure 9.

Time spent on self-grooming in different genotype mice.

Discussion

To my knowledge, our study is the first study to show the behavioral phenotypes in mice lacking *SLC9A9*. Our study focused on testing the main deficits that resemble

the behaviors in ASD, such as deficits in social communication, deficits in social interaction, and restricted repetitive behavior. We observed that *SLC9A9* KO mice emitted less number of calls and shorter call duration; *SLC9A9* KO mice lacked preference for social novelty, but did not show deficits in social approach; *SLC9A9* KO mice spent more time on self-grooming. Beside these, we did not observe behavior impairments which are commonly comorbid with ASD in human: no anxiety-like behavior, hyperactivity or spatial learning and memory deficits were detected in *SLC9A9* KO mice.

1. Effect of genotype on USVs

Rodent pup calls serve functions that could be similar to the human babies' crying. By initiating the maternal behaviors of the mothers, such as searching, retrieving, nest building, licking and feeding behavior, USVs serve an important communicative function between pups and their mother [131-134].

In our study, when separated from their mother and littermates on PND13, *SLC9A9* KO pups emitted less number and duration of calls than WT pups. This result is consistent with the majority of the ASD mouse models. For example, in the studies that reported USV calls during maternal separation in pups of ASD mouse models, serotonin receptor-1A (*Htr1a*) KO pups [135, 136], serotonin receptor-1B (*Htr1b*) KO pups [137, 138], and oxytocin neuropeptide (*Oxt*) KO pups [139] had reduced USV on PND7-8; *Shank1* KO mice [140] and male mice pups with the human neuroligin-3 mutation (*Nlgn3-R451C*) showed reduced USV calls on PND8 [141]; oxytocin neuropeptide receptor (*Oxtr*) KO male pups [142] and reelin (Reln) KO male pups showed reduced USV calls on PND7 [143]; forkhead box P2 (*Foxp2*) KO pups had reduced USV calls on PND6 [144]; μ-opioid receptors (*Orpm*) KO pups emitted less USV on PND4, 8, 12 [145]. The reduced level of USV during maternal separation and isolation from littermates could be an indicator for early communication impairments.

However, in some other mouse models relevant to ASDs, the researchers reported an increased USV calls. For example, BTBR T+tf/J (BTBR) mice, a commonly used ASD model, were observed to call more frequently and the duration of those calls were longer compared with controls on PND8 [127]; fragile X mental retardation 1 (*fmr1* KO*)* KO mice, a mouse model to study the fragile X syndromes (FXS) which are associated with ASDs, showed an increased number of calls on PND7 but not on PND4 and PND10 [146]; methyl CpG binding protein 2 (*Mecp2*) HX KO male and Het female showed a dramatic increase of USV calls on some PND [147]. The *Mecp2* mutant mouse is a model for Rett Syndrome which is also associated with ASDs. These increased USV calls in mouse models relevant to ASDs could be due to higher anxiety and fear [148-150].

In a previous published paper, we reported some behavioral phenotypes in the inbred rats, WKY/Ncrl and WKY/NHsd substrains [151]. WKY/Ncrl and WKY/NHsd substrains were extremely similar in genome with only 2.5% difference as detected by

a 10K genome-wide SNP array [152]. There are two nonsynonymous mutations in *SLC9A9* in WKY/NCrl rats [104]. We observed that when exposed to male bedding, females from WKY/NCrl strain also had fewer USV calls compared with the WKY/NHsd strain as control [151]. But because *SLC9A9* mutations are not the only genetic variations between WKY/Ncrl and WKY/NHsd substrains, we could not make the straightforward association between *SLC9A9* mutations and communication impairments. Our present results in behavioral phenotypes study of *SLC9A9* KO mice compared with their WT littermates provide a strong evidence that lack of *SLC9A9* can lead to communication impairments.

Besides the numbers and duration of calls, spectral patterns of calls were also studied in a number of genetic mouse models of ASD. Abnormal patterns of calls, such as calls emitted at lower frequencies [150, 153], call-type specific deficits [154], and impaired complex patterns of USVs [155] were reported in ASD mouse models. Unfortunately, the USV detector and analyzer from Med Associates, the one we used in our experiments, has very limited functions. We are not able to analyze detailed patterns of USV calls.

2. Effect of genotype on preference for social novelty

Lower social interest should be a major feature of ASD models. However, we did not observe an effect of genotype on social interests. The *SLC9A9* KO mice spent similar an amount of time with the novel mouse as the WT mice. This result was consistently seen when we put subject mouse and novel mouse together in the center chamber. There was no effect of genotype on the direct physical contact of subject mouse with novel mouse either. However, we did observe that the *SLC9A9* KO mice lacked social novelty preference. 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.

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 [156]. In our experiments, the tester mice were enclosed in a cage with holes on one side which allowed the subject mouse to have visual, olfactory, auditory and some tactile contact with the tester mouse. When exposed to both familiar and novel tester 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.

In some other behavior studies of ASD relevant mutant mice, an absence of social novelty preference was also detected. Mice lacking the integrin β3 receptor subunit,

which is related with serotonin level (biomarker in ASD patients) in brain, showed no social novelty preference [157]. *Oxtr* KO male mice showed deficiency in social novelty preference [142]. *Oxt* KO mice [158] and oxytocin-regulating protein CD38 KO mice [159] also had deficiency in discrimination between a familiar mouse and a novel mouse.

In another ASD related study, same sociability and social novelty preference tests were conducted in the neuroligin-3 (NL-3) KO mice [160], an ASD relevant mouse model. Similar to the *SLC9A9* KO mice, NL-3 KO mice showed no altered performance in sociability or social interaction but did show deficits in social novelty preference. In the same study, the researchers also observed an olfactory deficiency in NL-3 KO mice. 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 [160]. Interestingly, impairments in olfactory identification have also been reported in patients with ASD [161, 162]. Future studies will be designed to study the olfactory function of *SLC9A9* KO mice. For example, latency to locate the buried food and response to social or non-social odor can be an indicator for olfactory function in rodent animals [141, 163].

In our previous behavioral studies in rats, we reported that the WKY/NCrl substrain and the WKY/NHsd substrain behaved similarly in the social novelty preference tests, but WKY/NCrl substrain had lower social interests compared with WKY/NHsd substrain [151].

The diagnostic criteria of ASD in DSM-5 indicate that individuals with ASD should have "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". But it does not mention or give examples of lack of social novelty preference in individuals with ASD, although it does state that 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 related mouse studies tended to focus on sociability testing or preference for mouse over object [128, 164-166]. Many ASD mouse model, BTBR for example, showed absence of preference for mouse over object [128]. However, although one of the criteria of ASD diagnosis states that individuals with ASD may have "highly restricted, fixated interests that are abnormal in intensity or focus (e.g, strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interest)", in DSM-5 no criteria of diagnosis is based on lack of preference for another individual over an object [1]. An eye tracking study was conducted among preschool children with ASD and their age and gender matched controls [167]. Children with ASD showed similar eye attraction to faces presented with object stimuli as controls. But when presented with objects related to circumscribed interests (clocks, blocks, trains and vehicles in this test), children with ASD showed significantly less attention to faces compared with controls. For our

SLC9A9 KO mice, we observed that they were similar to WT in preference for a mouse contained in a cage over an empty cage. For future studies, we can change the empty cage to other novel object related to "circumscribed interests" to mice.

3. Effect of genotype on repetitive self-grooming behavior

Stereotypic/repetitive movement is an essential diagnostic symptom of ASD. Behaviors that appear to be normal patterns but persist for unusually long periods are considered stereotypic/repetitive movements [156]. *SLC9A9* KO mice spent longer periods of time self-grooming than WT. The high level of self-grooming is consistent with other ASD mouse models: BTBR mice [128, 129], *fmr1* KO mice [168], *neuroligin-1* KO mice [169], C58-J inbred mice [170], *Shank2* KO [171] and *Shank3* KO mice [172, 173] were all reported to have increased level of self-grooming. However, in our previous study of ASD phenotypes in rats, we did not find any difference of time spent in self-grooming between WKY/Ncrl rats and their control rats, WKY/NHsd [151].

4. ASD comorbid phenotypes

We did not observe any anxiety, hyperactivity, spatial learning and memory deficits, symptoms which are commonly comorbid with ASD in humans and in ASD mice models, in our *SLC9A9* KO mice. Interestingly, we did observe an increased locomotor activity in Het KO mice in SHIRPA, and in BM, especially the last 4 trials. In both cases, animals responded to escape. We speculate that for Het KO mice, they are not hyperactive, nor have learning deficits, but show faster response speed to escape. One study reported that children with ASD perceived better vision of motion than children without ASD. Compared with children without ASD, children with ASD had enhanced performance in perceiving the direction of a high-contrast, fast moving bar [174]. This hypersensitive perception may lead to over-react to sensory input in children with ASD. In fact, individuals with ASD tend to escape or avoid negative stimulus. Stereotypical behaviors, one of the criteria in ASD diagnosis, are functioned in individuals with ASD primarily for escape from negative reinforcement purpose [175]. For Het KO mice, this hypersensitivity may cause them quick respond to escape from aversive stimulus. For HX KO mice, although they are not different in anxiety measurements from WT, they are more anxious than Het KO mice as shown in EP test, which may counteract the intensified response by causing HX KO mice to freeze. The absence of anxiety, abnormal motor activity and spatial learning and memory deficits in *SLC9A9* KO mice suggests that *SLC9A9* KO mice may be a highly specific mouse model for ASD-traits. In our previous study in rats, WKY/Ncrl rats showed similar motor activity behaviors to WKY/NHsd rats, but WKY/Ncrl rats were significantly more anxious than the WKY/NHsd rats [151].

In human studies, a nonsense mutation in *SLC9A9* was found in patients who had ASD as well as epilepsy [107]. This nonsense mutation causes the loss of the final extracellular loop of *SLC9A9*. In another human study, a ~0.5 kb deletion compassing exon 2 of *SLC9A9* was reported in a patient with ASD and epilepsy [176]. In our study,

we did not observe obvious epilepsy symptoms in our mice. There were several mice from HX, Het and WT genotypes that died of unexplained reason during housing. It was possible that some of them may have died from epilepsy.

Interestingly, another genetic mouse model which lacks *SLC9A6*, another NHE that locates in early recycling endosomes, also had high potential to seizures. Similar to *SLC9A9*, in recent years, many studies have implicated mutation or deletion of *SLC9A6* in ASD [177-179]. A *SLC9A6* KO mouse model was generated by Jackson Labs (B6.129P2-*SLC9A6*^{tm1Dgen}/J) with increased susceptibility to drug induced seizures. Behavior studies showed that the *SLC9A6* KO is hyperactive in general locomotor activities and has motor coordination deficits, but not has anxiety-like behaviors or impairments in spatial memory [180]. Unfortunately, no straightforward ASD related behavioral testing, such as sociability, USV, restricted behavior testing, have been done in *SLC9A6* KO mice so far.

In the future, studies will be designed to test epilepsy symptoms in *SLC9A9* KO mice. 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 volume [56, 181-186].

5. Potential limitations

Traditionally, developing a congenic strain requires ten generations of backcross matings so that the mice can have 99.90% recipient genetic background [187]. In our study, we did not backcross our mice to generate a congenic strain, but directly bred the offspring by mating the *SLC9A9* KO mice which we bought from the Jackson Laboratory. Because the Jackson Laboratory produced *SLC9A9* KO mice by crossing two different inbred strains, C57BL/6 and 129, the *SLC9A9* KO have a mixed C57BL/6 x 129 genetic background. Thus all of the offspring we bred have a random genetic background mixture of alleles from C57BL/6 and 129. The varied genetic background of our experimental mice may confound their behaviors. However, we have used their WT littermates as control, which minimized the varied genetic background effect. From this perspective, our behavioral results and the genotype effects on phenotypes are valid and meaningful.

As mentioned in the section about social novelty preference tests, we did not test the olfactory function in *SLC9A9* KO mice. Deficits in olfactory function may confound social behaviors in rodents. Future studies should be designed to test olfactory function in *SLC9A9* KO mice.

6. Future directions

6.1 ADHD features in *SLC9A9* KO mice

SLC9A9 mutation was first reported in a large family. 11 out of 22 family members presented features of ADHD and intellectual disability. In this family, ADHD coexisted

with a pericentric inversion of chromosome 3 that disrupted *SLC9A9* [108]. After that, a number of genetic association studies also implicated *SLC9A9* in ADHD. Future studies should test ADHD phenotypes in *SLC9A9* KO mice.

6.2 Dopamine transporter (DAT) in *SLC9A9* KO mice

Decreased level of dopamine is one of the hypothesized causes of ADHD, and the central dopaminergic system is also strongly related with ASD pathogenesis [188]. For example, medications acting on dopaminergic system have been found to be effective for ASD treatment [189, 190]. Several genetic mutations on DAT [191], dopamine receptors [192-194], and enzymes involved in dopamine synthesis [195] and catabolism [196, 197] have been linked to ASD.

DAT is involved in dopaminergic system. It controls the dopamine concentration by pumping dopamine out of synapse back into the cytosol. Elevated striatal DAT density can decrease the normal dopamine concentration in synapse, which can cause psychiatric disorder. In fact, various dopaminergic drugs that target the DAT, such as MPH and AMPH, can alleviate ADHD symptoms, both clinically and in experimental animal models [198, 199]. There are also many studies showing that striatal DAT density is increased in human ADHD and in the SHR (a rat model of ADHD) [104, 200-202]. It is possible that *SLC9A9* resides on the membrane of late recycling endosomes and plays an important role in trafficking DAT to the pre-synaptic membrane. No study has yet assessed the effect of *SLC9A9* on DAT. Future studies can be designed to examine DAT density in *SLC9A9* KO mice. First, studies can be designed to examine the expression level of genes involved in dopamine signaling and metabolism in the striatum of *SLC9A9* KO mice. Second, the striatal DAT density can be examined by ligand binding assays. It will give us clues on the mechanisms of *SLC9A9* in ASD and ADHD by comparing DAT in *SLC9A9* KO mice with that in WT.

6.3 Conditional *SLC9A9* KO strategies

As the behavioral studies were done in mice with global KO of *SLC9A9*, future studies can be designed to study phenotypes in conditional, dopaminergic neurons group (exits in substantial nigra, ventral tegmental area and striatum) *SLC9A9* KO mice, so that we can study the potential effect of *SLC9A9* on dopamine system in ASD.

6.4 Alteration in particular brain regions

Since we have observed behavioral alterations in *SLC9A9* KO mice, further studies can be designed to evaluate the alterations in brain regions underlying the behavioral changes. For example, because we observed deficits of social novelty preference in *SLC9A9* KO mice, future work should evaluate hippocampus, amygdala and the olfactory bulb, the social memory related brain regions in these mice [203, 204]. Because we observed increased grooming in *SLC9A9* KO mice, we should examine the corticostriatal circuit which has been found to be related to obsessive-compulsive-like behaviors in mice [205, 206]. Some significant structural and functional alterations in brains of patients with ASD have already been reported. It

will be interesting to check if similar alterations also exit in *SLC9A9* KO mice.

6.5 *SLC9A9* role in synaptic neurons

ASD is a collection of neurodevelopmental disorders, so function and plasticity of synapse can play an important role in ASD. Any dysregulation of transcription, translation, modification, and trafficking at the synapse can impact dendritic branching, synapse maturation, and synapse elimination, which can lead to neurodevelopmental disorders, such as mental retardation and ASDs [207]. In further studies, researchers should examine *SLC9A9* activity in pre- and post-synaptic neurons and astrocytes. By doing so, the potential role of *SLC9A9* on synapse development and plasticity may be uncovered.

6.6 Medication development

Finally, effective and safe treatments for ASD are an urgent need. Since one of the main functions of *SLC9A9* 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 ASD patients. Screening and searching the potentiators and inhibitors that target *SLC9A9* and regulate its function can be helpful for the development of ASD drugs [208]. Researchers in our group have evaluated the potential for respositioning inhibitors against well-known NHE for against *SLC9A9* using an *in silico* approach [209]. Further studies can also study the similar drug repositioning potential using *in vitro* and *in vivo* approaches.

In conclusion, our study has provided a strong evidence that lack of *SLC9A9* can lead to ASD-like behaviors in mice, including deficits in social communication and social interaction, and restricted repetitive patterns of behavior. Although several genetic association studies have implicated *SLC9A9* in ASD, our study is the first behavioral study that links *SLC9A9* to ASD. It would be a helpful foundation for future studies of *SLC9A9* in ASD. *SLC9A9* KO mice could be used as a genetic mouse model for ASD in future studies.

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