JUVENILE *SHANK3B* DEFICIENT MICE PRESENT WITH BEHAVIORAL PHENOTYPE CONSISTENT WITH AUTISM SPECTRUM DISORDER

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ABSTRACT

Autism spectrum disorder (ASD) is a pervasive, multifactorial neurodevelopmental disorder diagnosed according to deficits in three behavioral domains: communication, social interaction, and stereotyped/repetitive behaviors. Mutations in *Shank* genes account for ~1% of clinical ASD cases with *Shank3* being the most common gene variant. In addition to maintaining synapses and facilitating dendritic maturation, *Shank* genes encode master scaffolding proteins that build core complexes in the postsynaptic densities of glutamatergic synapses. Male mice with a deletion of the PDZ domain of *Shank3* (Shank3B KO*)* were previously shown to display ASD-like behavioral phenotypes with reported self-injurious repetitive grooming and aberrant social interactions. Our goal was to extend these previous findings and use a comprehensive battery of highly detailed ASD-relevant behavioral assays including an assessment of mouse ultrasonic communication carried out on key developmental days in male and female Shank3B KO mice. We demonstrate that ASD-related behaviors, atypical reciprocal social interaction and indiscriminate repetitive grooming, are apparent in juvenile stages of development of Shank3B KO mice. Our findings underscore the importance of utilizing *Shank* mutant models to understand the impact of this gene in ASD etiology, which may enable future studies focusing on etiological gene-environment interactions in ASD.

PUBLICATIONS ARSING FROM THIS THESIS

Journal Publications

2017 Chantell Balaan, Michael J. Corley, Tiffany Eulalio, Ka'ahukane Leite-ahyo, Alina P.S. Pang, Alika K. Maunakea, Monika A. Ward. *Juvenile Shank3b deficient mice present with behavioral phenotype consistent with autism spectrum disorders*. (Resubmission in preparation)

Presentations and Abstracts

Talks

2017 Chantell Balaan, Michael J. Corley, Tiffany Eulalio, Ka'ahukane Leite-ahyo, Alina P.S. Pang, Alika K. Maunakea, Monika A. Ward. *Juvenile Shank3b deficient mice present with behavioral phenotype consistent with autism spectrum disorders*. 42nd Albert L. Tester Memorial Symposium; Honolulu, HI. April 2017

2016 Chantell Balaan, Michael J. Corley, Yasuhiro Yamauchi, Tiffany Eulalio, Ka'ahukane Leite-ahyo, Alina P.S. Pang, Caroline Blanchard, Alika K. Maunakea, Monika A. Ward. *Alterations in behavior relating to autism spectrum disorder in mice conceived by in vitro fertilization*. 41st Albert L. Tester Memorial Symposium; Honolulu, HI. April 2016

2016 Chantell Balaan, Alika K. Maunakea, Monika A. Ward. *Alterations in behavior relating to autism spectrum disorder in mice conceived by in vitro fertilization.* Developmental and Reproductive Biology Graduate Program semi-annual Graduate Student Retreat; Honolulu, HI. February 2016. (This presentation was awarded best presentation throughout beginning level students)

Posters

2017 Chantell Balaan, Michael J. Corley, Tiffany Eulalio, Ka'ahukane Leite-ahyo, Alina P.S. Pang, Alika K. Maunakea, Monika A. Ward. *Juvenile Shank3b deficient mice present with behavioral phenotype consistent with autism spectrum disorders*. JABSOM Biomedical Sciences and Health Disparities Symposium; Honolulu HI. April 2017

2016 Chantell Balaan, Michael J. Corley, Yasuhiro Yamauchi, Tiffany Eulalio, Ka'ahukane Leite-ahyo, Alina P.S. Pang, Caroline Blanchard, Alika K. Maunakea, Monika A. Ward. *Alterations in behavior relating to autism spectrum disorder in mice conceived by in vitro fertilization*. JABSOM Biomedical Sciences and Health Disparities Symposium; Honolulu, HI. April 2016

2015 Chantell Balaan. Michael J. Corley, Alika K. Maunakea, Amy Wassman, Psy.D. *The relationship between assisted reproductive techniques and the increased prevalence of autism spectrum disorder: a literature review*. JABSOM 2015 Department of Native Hawaiian Health Summer Internship Program; Honolulu HI, August 2015

2015 Chantell Balaan, Michael J. Corley, Yasuhiro Yamauchi, Ka'ahukane Leite-ahyo, Alika K. Maunakea. Monika A. Ward. *The effect of maternal obesity in the context of assisted reproduction on offspring ultrasonic communication in a mouse model*. JABSOM Biomedical Sciences and Health Disparities Symposium; Honolulu HI. April 2015

2015 Chantell Balaan, Michael J. Corley, Yasuhiro Yamauchi, Ka'ahukane Leite-ahyo, Alika K. Maunakea. Monika A. Ward. *The effect of maternal obesity in the context of assisted reproduction*

on offspring ultrasonic communication in a mouse model. 40th Annual Albert L. Tester Memorial Symposium; Honolulu HI. April 2015 (this poster was awarded best undergraduate poster)

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1. INTRODUCTION

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder characterized by deficiencies in three core behavioral categories: social interaction, communication, and motor stereotypies (Apa, 2013) that are usually discernible in the early developmental stages of a child's life (Charman & Baird, 2002) (Fig. 1). The prevalence of ASD has increased and the Centers for Disease Control and Prevention (CDC) currently estimates as many as 1 in 68 children in the United States are diagnosed with ASD (Developmental Disabilities Monitoring Network Surveillance Year Principal *et al.*, 2014). Notably, the diagnosis of ASD involves a complex range of behavioral deficits with a multifactorial etiology as well as a sex ratio bias skewed towards an increased male diagnosis as compared to females. Although the etiology of ASD includes a genetic component (Bailey *et al.*, 1995, Hallmayer *et al.*, 2011) many studies suggest that environmental factors contribute to its development (Conti *et al.*, 2013, Gardener *et al.*, 2011, Lyall *et al.*, 2014, Tordjman *et al.*, 2014), underscoring an etiological gene-environment interaction.

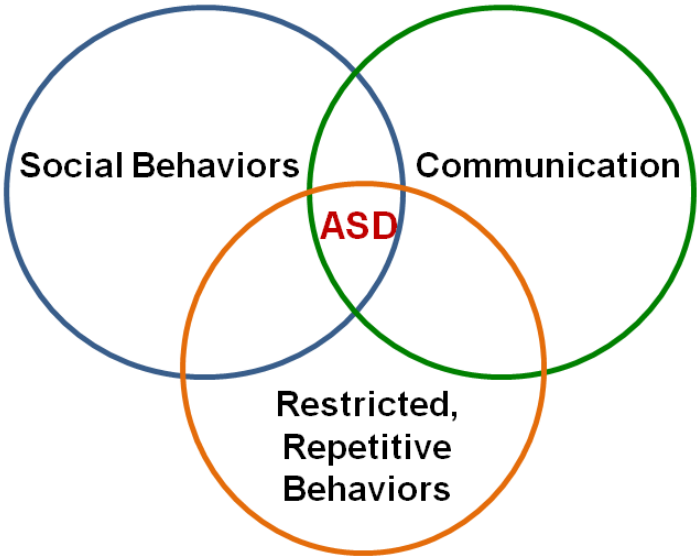


Fig. 1: ASD core behavioral symptoms. ASD is diagnosed based on the following core symptomatic categories during the earlier stages of a child's life where symptoms become apparent: communication, social interaction, and restricted/repetitive behaviors which range in severity and combinations in clinical ASD cases. This Venn diagram represents the overlapping combinations and complexities in terms of severity in clinical ASD cases.

Genetic mouse models capturing the behavioral features of ASD are part of a multidisciplinary approach to study the etiology and assess therapeutic treatments of ASD. One of the most well characterized ASD genes is *Shank3* (SH3/ankyrin domain gene 3) (Crawley, 2012, Jiang & Ehlers, 2013, Larsen *et al.*, 2016, Maunakea *et al.*, 2010, Mei *et al.*, 2016, Peca *et al.*, 2011, Wang *et al.*, 2011)

encoded on chromosome 22q13 in humans and chromosome 15 in mice. The *SHANK* gene family encodes proteins necessary to form core signaling complexes. *SHANK3* is responsible for assembling synaptic scaffolding proteins located in the post-synaptic density (PSD) of glutamatergic excitatory synapses in the central nervous system (CNS). Mutations within *SHANK3* have been implicated as a haploinsufficient monogenic cause of ~1% of human ASD cases (Boccuto *et al.*, 2013, Gauthier *et al.*, 2009, Leblond *et al.*, 2014, Moessner *et al.*, 2007) as well as have been described in patients with mental retardation, severe language delay, and Phelan-McDermid syndrome (also referred to as "22q13.3 deletion syndrome") (Wang *et al.*, 2011). While there are many candidate genes associated with ASD, the relatively high *SHANK3* mutation frequency and penetrance provides strong rationale for further research modeling ASD in animals by combining comprehensive behavioral profiling of mice with variable *Shank3* mutations.

The *Shank3* mutant mice were first established in 2011 using gene targeting to delete the highly conserved PDZ domain (exon 13-16) of the *Shank3* gene resulting in an absence of Shank3α and Shank3β isoform expression, and a reduction Shank3γ isoform expression (Fig.2) (Peca *et al.*, 2011).

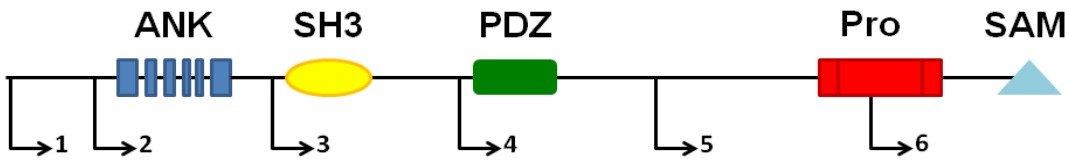


Fig. 2: Schematic representation of mouse *Shank3B* gene. Known promoters 1-6 (arrows) and protein binding domains are presented as specified: SH3 (SRC Homology 3) - involved in various signaling pathways; ANK (Ankyrin repeats) - mediates protein-protein interactions; PDZ (post synaptic density protein) - brain signaling; Pro (proline rich)- interacts with SH3; SAM (sterile alpha motif)- protein interaction modules present in many signal transducing proteins. *Shank3B* KO mice utilized in this project have a targeted deletion in the PDZ domain.

These mice, which we subsequently call Shank3B KO, were shown to display self-injurious grooming behaviors as well as abnormal social interactions, deficits in recognizing social novelty, and anxiety-like behaviors at adulthood. Biochemical, morphological, and electrophysiological tests revealed altered striatal and cortico-striatal neuronal networks in the PSD suggesting disruption of molecular pathways in

which SHANK3 is involved (Peca *et al.*, 2011). Since then, other studies focused on the role of the *Shank* gene family and ASD development using other *Shank* deficient mice produced by targeting various domain sites within the genes (Jiang & Ehlers, 2013).

To assess for an ASD behavioral phenotype, prior studies have characterized adult Shank3B KO mice. Here, we investigated juvenile Shank3B KO mice and assessed their ASD-related behavior on key developmental days, including scoring of micro-behaviors captured within standardized behavioral tests. Our findings provide unique and comprehensive characterization of juvenile Shank3B KO mice that could be used as a future reference.

2. MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise stated.

Animals

Shank3B heterozygous knockout mice (B6.129-Shank3^{tm2Gfng}/J) were purchased from the Jackson Laboratory (Stock No: 017688). Wild-type (WT) and homozygous knockout (KO) mice were generated by mating of heterozygotes. Genotypes of progeny were determined by PCR using the following primers: WT forward GAGACTGATCAGCGCAGTTG, WT reverse TGACATAATCGCTGGCAAAG; Shank3B KO forward TCTAACTCCCAGAGGCCAGA, Shank3B reverse TCAGGGTTATTGTCTCATGAGC. Age-matched CD-1 mice maintained 'in house' were used in several behavioral tests as stimuli. The mice were fed ad libitum with a standard diet and maintained in a temperature and light-controlled room (22ºC, 14h light/10h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawai'i and guidelines presented in National Research Council's (NCR) "Guide for Care and Use of Laboratory Animals" published by Institute for Laboratory Animal Research (ILAR) of the National Academy of Science, Bethesda, MD, 2011. The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawai'i.

Behavioral test battery analysis and general procedures

At least 9 adolescent (Brust *et al.*, 2015, Trezza *et al.*, 2011) mice per group (WT and KO of each sex) from 6 different litters underwent the behavioral test battery. The litter size prior to weaning was maintained at 6-10 pups. After weaning mice were housed at 5-6 per cage. To maintain the desired pup/mouse number heterozygotes were kept as littermates to KO and WT, if needed.

Each mouse underwent behavioral testing in the following sequence on specific post-natal days (PND) with at least 24 hour rest periods in between testing: PND 8, 10, & 12: Ultrasonic Vocalization (USV, communication); PND 25: 3-Chamber (social preference); PND 30: Self-Grooming (motor stereotypies); PND 31: Social Proximity (forced social interaction): PND 40: Elevated Mazes (anxiety) (Crawley, 2004, Crawley, 2007, Silverman *et al.*, 2010) (Fig. 3). The USV was performed when pups were

with their mother and the remaining tests were conducted after weaning. CD-1 stimulus mice adhered to the same schedule as the tested mice. Except for the USV, all behavioral tests required video recordings.

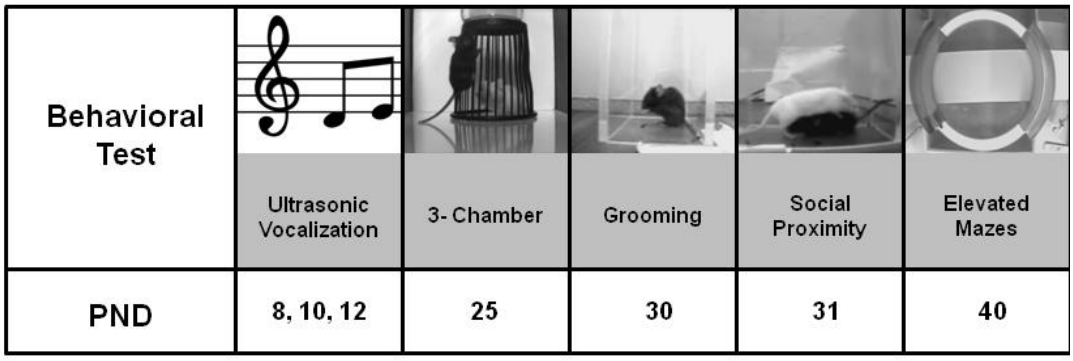


Fig. 3. ASD-relevant behavioral test battery. Schematics of comprehensive ASD-related behavioral test battery that Shank3B KO and WT mice underwent at specified postnatal days (PND). The battery covers core symptoms of ASD such as communication (ultrasonic vocalization), social behaviors (3-chamber and social proximity) and motor stereotypes (grooming). Anxiety-related tests (elevated mazes) were also administered to provide a more comprehensive behavioral phenotype of mice. The descriptions of tests are presented in Methods section.

Prior to the test, mice were moved to an experimental room with recording equipment and sufficient time was given to allow them to acclimate to the novel environment. In between testing the individual subjects, all testing apparatuses were wiped using 70% ethanol. For the USV test, tissue that lined the apparatus was replaced daily. In most ethological tests, subjects were naïve to the apparatus except for the 3-Chamber and Proximity tests, which required habituation prior to testing. Unless otherwise stated, trials were performed under sufficient fluorescent lighting during the light cycle (9 am to 5 pm) in temperature-controlled room (22°C).

Ultrasonic Vocalization (USV) test

The USV test involves isolation of neonatal mice from mother and exposure to a novel environment. This isolation elicits whistle-like distress calls ranging from ~30-90 kHz, demonstrating early communicative behavioral exchanges between the pup and its dam (Scattoni *et al.*, 2009). For USV testing, pups at PND 8, 10, and 12 were isolated from dam and placed in a polypropylene cage lined with bedding material, which was housed within a sound attenuating styrofoam container (Wohr *et al.*, 2011). Individual pups were recorded for 5 min with a condenser ultrasound microphone CM16/CMPA (Avisoft Bioacoustics, Berlin, Germany) placed ~10 cm from the bottom of the cage. Recordings were collected

via an Ultrasoundgate 116H base unit (Avisoft Bioacoustics) onto a laptop and displayed through the Recorder USGH software (Avisoft Bioacoustics, version 4.2). The condenser ultrasound microphone was sensitive to frequencies ranging from 10 kHz to 180 kHz. Program parameters included a sampling rate of 250 kHz with a 16-bit format. Recordings were later translated onto Avisoft SASLab Pro (version 5.2.09) and a fast Fourier transform was conducted (512 FFT length, 100% frame, Hamming window and 75% time window overlap). Similarly to that previously noted (Wohr *et al.*, 2011), spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. Call detection and average duration times were scored manually by a trained individual. False positive detection by the software was excluded from counts.

3-Chamber test

To assess general social behaviors the 3-Chamber test was employed. The 3-Chamber test measures social approach and preference for social novelty with a stimulus mouse (Moy *et al.*, 2004). At PND 25, tested mice were individually habituated in the arena of the 3-chamber apparatus for 10 min. After habituation, a CD-1 stimulus mouse of appropriate age and sex was placed in one of the inverted wired cups present in both outer chambers, marking it the “social chamber”; the remaining empty cup was considered the "non-social chamber". The time the tested mice spent in the social versus non-social chambers during a 10-min test period was measured (standard analysis); the mouse was considered to be within a specific chamber when all four paws entered the chamber. The behaviors of each mouse were video-recorded during the entire test for assessment of micro-behaviors (Rear, Contact, Sniff, Grooming, Stretch, Withdrawal, and Nose-to-Nose) (Fig. 4). Alternate placement of new CD-1 stimulus mouse in outer chambers occurred with each new introduction of a tested mouse.

Self-Grooming test

Another core symptom of ASD involves motor stereotypes modeled by self-grooming behaviors in mice (Lewis *et al.*, 2007). To assess their grooming behavior mice at 30 PND were placed in a holding

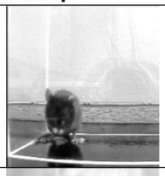
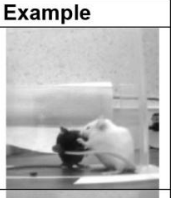
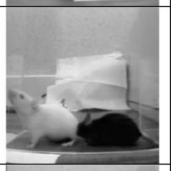
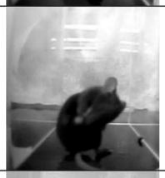
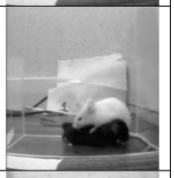
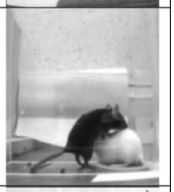
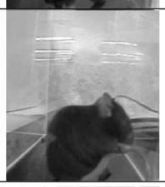
3-Chamber		Grooming		Social Proximity	
Micro-behavior	**Example**	**Micro-behavior**	**Example**	**Micro-behavior**	**Example**
Rear Subject is in reared, upright position on hindlimbs with nose tip oriented upwards		**Paw** Subject licks forelimbs		**Nose-to-Nose** Subject's nose tip/ vibrissae makes contact with nose tip/vibrissae of conspecific	
Contact Subject forelimbs grip metal wire cup and/or climbs onto metal wire cup		**Head** Subject grooms cranial region with paw		**Anogenital Sniff** Subject's nose tip/ vibrissae makes contact with anus or tail base of conspecific	
Sniff Subject's nose tip makes contact with metal wire up and/ or stimuli		**Body** Subject licks posterior and/or anterior trunk areas		**Crawl Under** Subject's cranio-thoracic region crosses conspecific's ventral surface	
Grooming Subject grooms itself		**Leg** Subject licks hindlimbs region as it is extended outwards		**Crawl Over** Subject's cranio-thoracic region crosses conspecific's dorsal surface	
Stretch Subject's trunk is extended with nose tip oriented towards stimuli or metal wire cup followed by a "frozen" posture		**Tail** Subject licks flexible extension of vertebrate, usually while holding it in paws		**Allogrooming** Subject grooms conspecific	
Withdrawal Subject swiftly darts away from stimuli in flight response		**Genitals** Subject's nose tip is in anogenital region either posteriorly (with hindlimbs extended upwards) or anteriorly (hyperflexion of vertebral column)		**Upright** Subject is in reared, upright position towards conspecific with vibrissae contact	
Nose-to-Nose Subject's nose tip/ vibrissae makes contact with nose tip/vibrissae of conspecific				**Self-grooming** Subject grooms itself	
				Nose-to-Head Subject's nose tip/ vibrissae makes contact with cranial region of conspecific	

Fig. 4. Micro-behaviors of 3-Chamber, Social Proximity and Self-grooming tests. Micro-behaviors were scored according to previously published criteria: 3-Chamber (Pearson *et al.*, 2010), Grooming (Pearson *et al.*, 2011), Social Proximity (Defensor *et al.*, 2011).

cage (14 x 7 x 30 cm) made of clear Plexiglas (Pearson *et al.*, 2011) for a total of 10 min. Two recording devices, placed at adjacent sides of the cage, captured both frontal and side views of mouse activities. Recordings were later assessed for grooming frequency, duration of grooming of various body parts (Paw, Head, Body, Leg, Tail, and Genitals) and behaviors unrelated to grooming marked as "other" (i.e. escaping/jumping, coprophagy, napping/stationary, etc) (Fig. 4).

Social Proximity test

Social proximity test enables the level of frontal contact and avoidance of an unknown mouse in forced social interaction to be measured (Defensor *et al.*, 2011). The apparatus employed for the grooming test was also utilized for this test. Tested mice at 31 PND were first habituated to the apparatus for 10 min. A CD-1 stimulus of appropriate sex and age was then introduced to the apparatus for a 10-min testing period. Video recordings from both frontal and side views were taken, allowing later assessment of reciprocal micro-behaviors that occurred between tested and stimulus mice such as Nose-to-Nose, Anogenital Sniff, Crawl Under, Crawl Over, Allogrooming, Upright, Self-grooming, Nose-to-Head, and other unspecified behaviors (i,e escaping/jumping, coprophagy, napping/stationary, aggression, etc) (Fig. 4).

Elevated Maze tests

Elevated Plus Maze (EPM)

Elevated Plus Maze (EPM) is a useful test to investigate the efficacy of anxiolytic compounds and to explore anxiety-like behaviors in mice (Handley & Mithani, 1984, Lister, 1987). In this test the previously described (Pobbe *et al.*, 2011) apparatus made of wood and acrylic containing 2 pairs of opposing arms (two open arms and two enclosed arms) extending outwards from a central intersection platform (5 cm x 5 cm) was used. The apparatus was raised 40 cm above the ground and was placed within a walled arena to prevent escape and to cushion accidental falls. Tested mice at 40 PND were individually placed on the central platform facing the left enclosed arm, initiating the start of the 10-min testing period. An overhead mounted video camera was used for video recordings. Recordings were later assessed for total time each mouse spent in each arm. Ethological measures involving risk assessments as well as number of entries into each arm were not included in the analysis.

Elevated Zero Maze (EZM)

Conceptually similar to the EPM, the elevated zero maze (EZM) offers less ambiguity as the center platform is eliminated (Shepherd *et al.*, 1994); the remaining features of the apparatus are as described for the EPM. At the start of the recording, tested mice were individually placed at the entrance of the left enclosed section of the circle maze, consistently facing towards the enclosed section. The mice were free to roam the circular maze for a total of 10 min. Similarly, to the EPM procedures, an overhead mounted video camera recorded mouse behavior for assessment of time spent in each open and enclosed section only.

Scoring of behavior

The behaviors were analyzed utilizing video recordings obtained from each test. Scoring was performed independently by two individuals (primary and secondary) blinded to the genotype, except for the USV test analysis. All tests were scored by the primary scorer and datasets chosen at random were validated by the secondary scorer. As a criterion of inter-rater reliability, scores were considered valid if at least 90% of measurements independently assessed between the two scorers were in agreement. Any discrepancies were re-evaluated and reconciled. All video recordings were scored using the Observer XT Software (Noldus Information Technology, The Netherlands, version 8.0.336). The individuals were trained to score based on 90% agreement.

Statistical Analysis

For most tests, statistical significance was defined using Excel and/or PRISM GraphPad (PRISM software 7.01) with two-tailed, unpaired t-test, and $P<0.05$ as a cut-off. Shank3B KO mice were compared against WT controls, with sexes scored independently. .

3. RESULTS

Juvenile Shank3B KO males display slightly altered communication behavior.

In the USV test, pups emit distress calls analyzed by Avisoft software, which produces a spectrogram image (Fig. 5) that is then quantified based on specific parameters. No significant differences in the duration and frequency of calls were observed between Shank3B KO and WT mice of either sex at any testing day (Fig. 6). Longitudinally, the frequency of calls is expected to peak at PND 8 and decrease

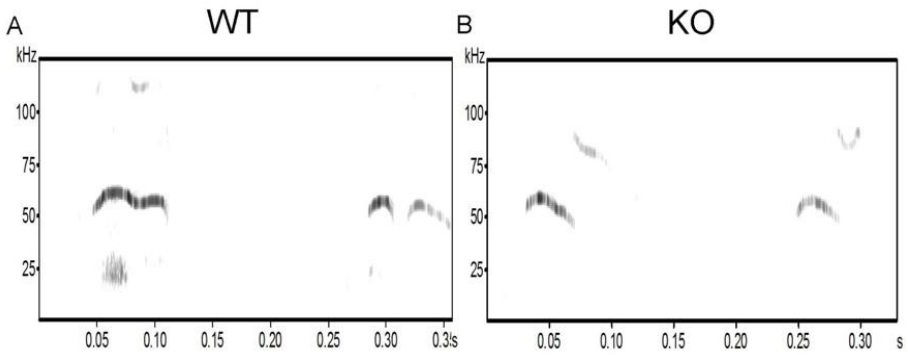


Fig. 5. USV test spectrograms. The exemplary spectrograms of 8 PND Shank3B KO (KO) and its wild-type littermate (WT). The acoustic recordings are plotted to show frequency (kHz) over time (s). Visual pup calls are referred to as tick marks shown in the spectrogram. The WT call pattern is more complex while the KO calls are simpler and the spectrogram shows frequency jumps.

thereafter (Branchi *et al.*, 2001, Elwood & Keeling, 1982). All tested groups followed this trend although the differences were not always significant.

The decrease in call frequency over examined developmental timepoints was less pronounced in Shank3B KO mice when compared to WT (Fig. 6C-D). In females, the difference in call frequency approached significance in WT ($P = 0.090$; PND 8 vs. 10 and $P = 0.077$; PND 8 vs.12) but not in Shank3B KO ($P = 0.297$ and $P = 0.296$). In males, the decrease in call frequency reached significance between PND 8 and 10 ($P = 0.048$; PND 8 vs. 10) and did not decrease further with Shank3B KO while with WT males the notable decrease was observed between PND 8 and 12. There were no differences in call duration between the examined timepoints for either genotype or sex.

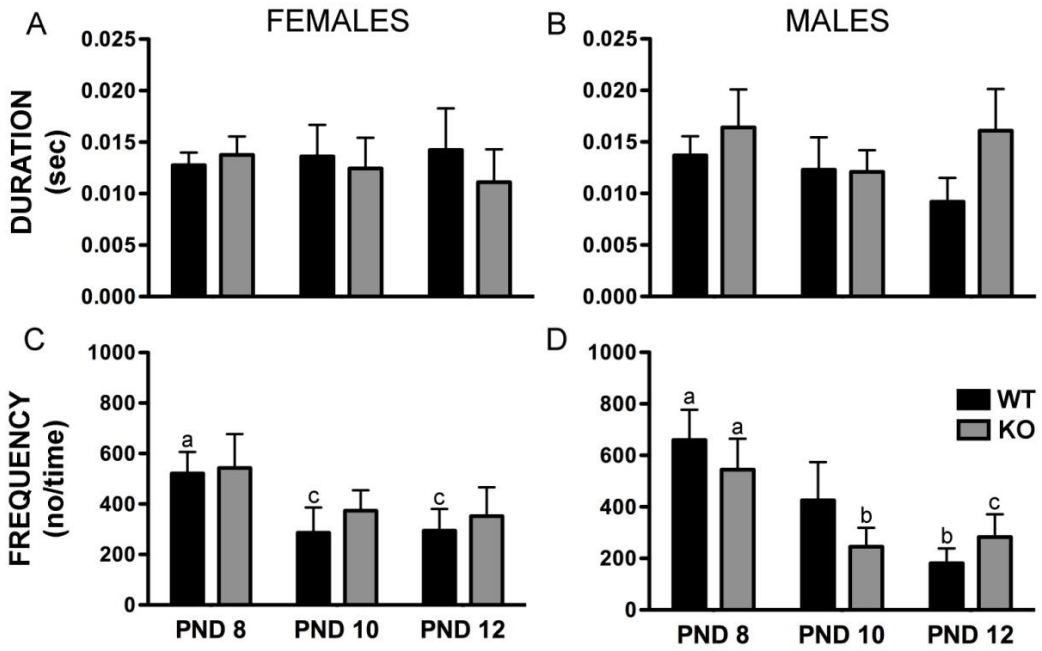


Fig.6. Ultrasonic Vocalization (USV). The Ultrasonic Vocalization test was performed for Shank3B KO (KO) mice and their wild-type (WT) littermates, grouped by sex (A,C: females; B,D: males) at postnatal days (PND) 8, 10 and 12. The data are expressed as call duration (A-B) and call frequency (C-D) during the test period. The graphs are averages ± SEM, with n=10 for WT and KO males and WT females, and n=9 for KO females. Statistical significance (t-test): a vs. b, $P < 0.05$; a vs. c, $P < 0.1$ (approaching significance) for within genotype comparison between PNDs. No differences between KO and WT were observed at any PND.

Juvenile Shank3B KO mice display impaired social interactions.

Social interaction was assayed using the 3-Chamber test for social preference and the Social Proximity test, in which response to forced social interaction is examined. During the 3-Chamber test, the duration of time spent by the examined mouse in social (containing a stimulus mouse) vs. non-social (empty) chamber (Fig. 7A-B) and the duration and the frequency of specific micro-behaviors (Fig. 7C-F) were scored. No differences were observed between Shank3B and WT females when considering the time, they spent in various chambers, with both genotypes demonstrating clear preference for the social chamber (Fig. 7A). Shank3B KO males, however, spent significantly less time in the social chamber when compared to WT males and presented with no preference for social interaction, spending similar amount of time in social and non-social environments; WT males demonstrated clear preference for the social chamber (Fig. 7B). The analysis of micro-behaviors revealed that Shank3B KO females spent more time

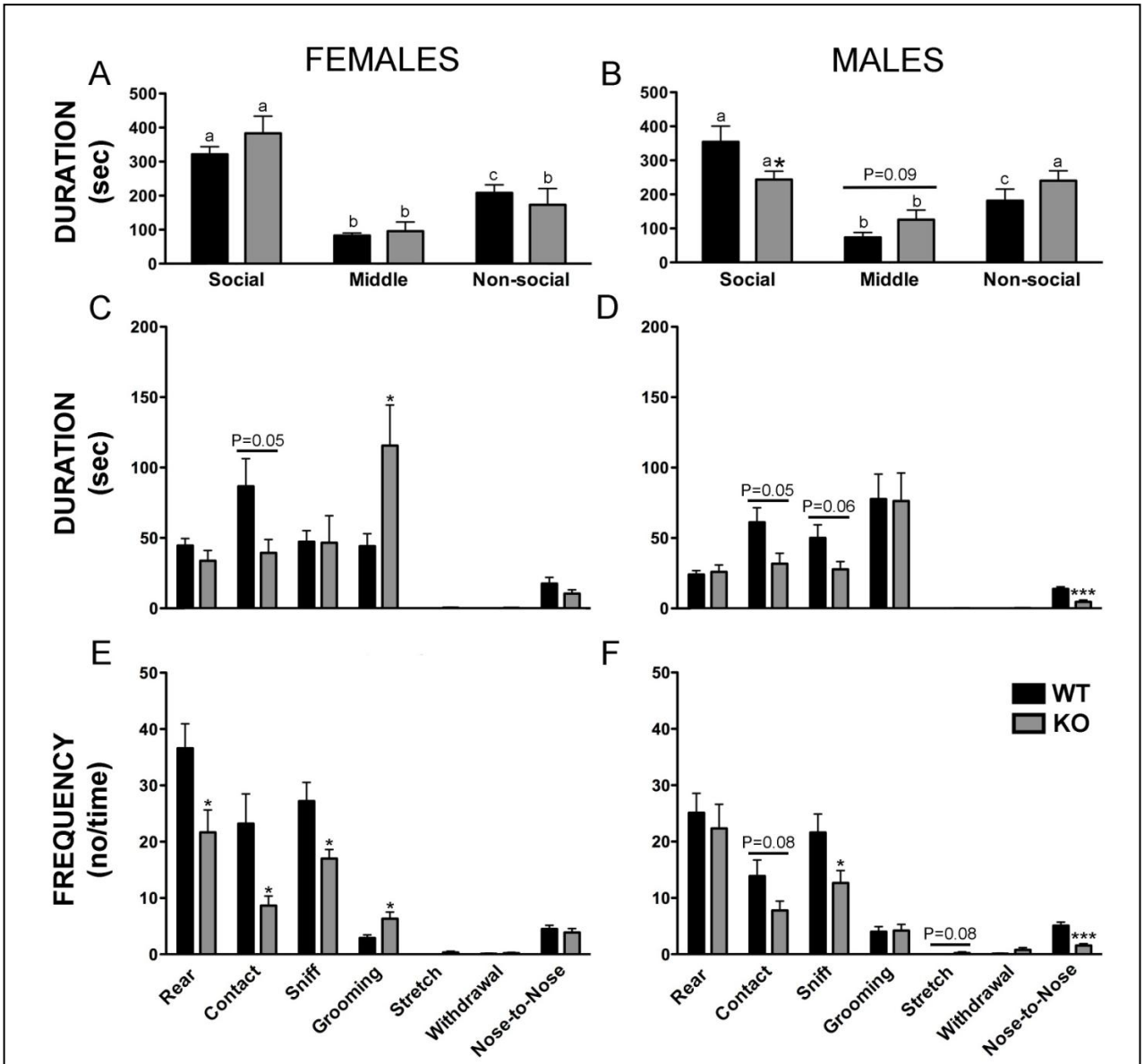


Fig. 7. 3-Chamber. The 3-Chamber test was performed for Shank3B KO (KO) mice and their wild-type (WT) littermates, grouped by sex (A,C,E: females; B,D,F: males). The duration spent in one of the three chambers (A-B) as well as the duration (C-D) and the frequency of 7 different micro-behaviors (E-F) were examined. The graphs are averages ± SEM, with n=10 for WT and KO males and WT females, and n=9 for KO females. Statistical significance (t-test): bars with different letters are different (A-B; within genotype comparison); * $P < 0.05$; *** $P < 0.001$, or as indicated in the figure (A-F, between genotype comparison).

Grooming in the presence of stimulus and did it more frequently than WT females. They also interacted less frequently with a stimulus mouse in Rear, Contact and Sniff micro-behaviors. Shank3B KO males self-groomed in the presence of stimulus similarly as WT males but spent less time in Contact, Sniff and Nose-to-Nose interaction with stimulus.

Forced social interaction assayed in Social Proximity test allows assessment of specific types of reciprocal interactions between mice. Shank3B KO females exhibited significantly increased duration and frequency of Nose-to-Nose interaction with stimulus when compared to WT (Fig. 8 A,C). In contrast, Shank3B KO males exhibited had fewer Nose-to-Nose and Nose-to-Head interactions than WT males (Fig. 8D).

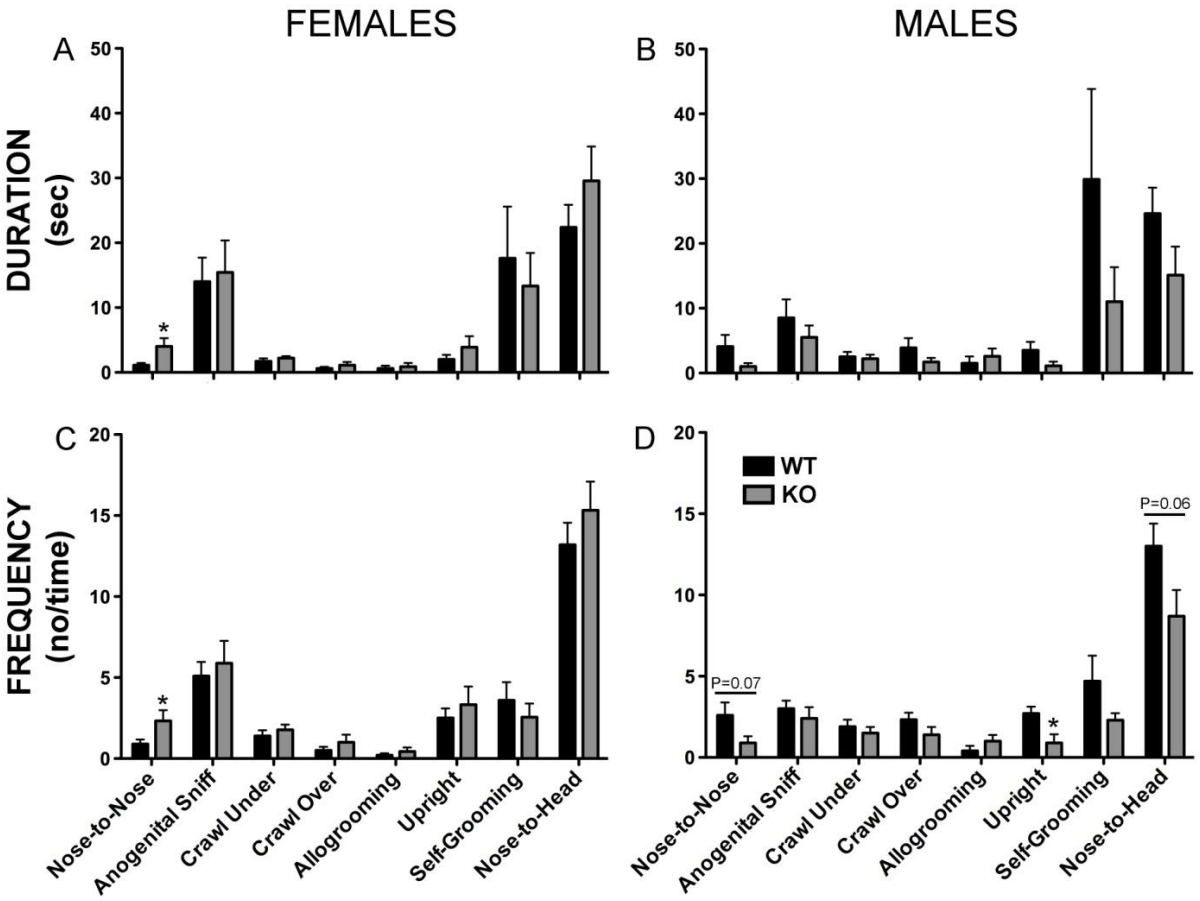


Fig. 8. Social Proximity. The Social Proximity **(**forced social interaction) test was performed for Shank3B KO (KO) mice and their wild-type (WT) littermates, grouped by sex (A,C: females; B,D: males). The duration (A-B) and the frequency (C-D) of eight different social interaction micro-behaviors were measured. The graphs are averages ± SEM, with n=10 for WT and KO males and WT females, and n=9 for KO females. Statistical significance (t-test): * $P < 0.05$ or as indicated in the figure.

Juvenile Shank3B KO mice display abnormal self-grooming behavior.

Restricted, repetitive behaviors are quantified in mice by assessment of self-grooming frequency and duration, with distinction between grooming of particular body parts. Shank3B KO mice spent overall

more time self-grooming when compared to WT mice, with males showing a more prominent difference compared to Shank3B KO females (Fig. 9A-B, Total).

The frequency of grooming was elevated with Shank3B KO females compared to WT females, and approaching significance with Shank3B KO males (Fig. 9C-D, Total). Significant increases in duration and/or frequency of specific self-grooming micro-behaviors were also observed, with Shank3B KO males more strongly affected (Fig. 9A-D). Reflective of elevated grooming activity Shank3B KO mice spent significantly less time exhibiting behaviors unrelated to grooming (i.e. escaping/jumping, napping/stationary, exploration, etc.) when compared to WT (mean ± SEM: 463.22 ± 14.57 vs. 507.20 ± 10.12, females, $P < 0.02$; 425.60 ± 12.32 vs. 506.60 ± , males, $P < 0.0001$).

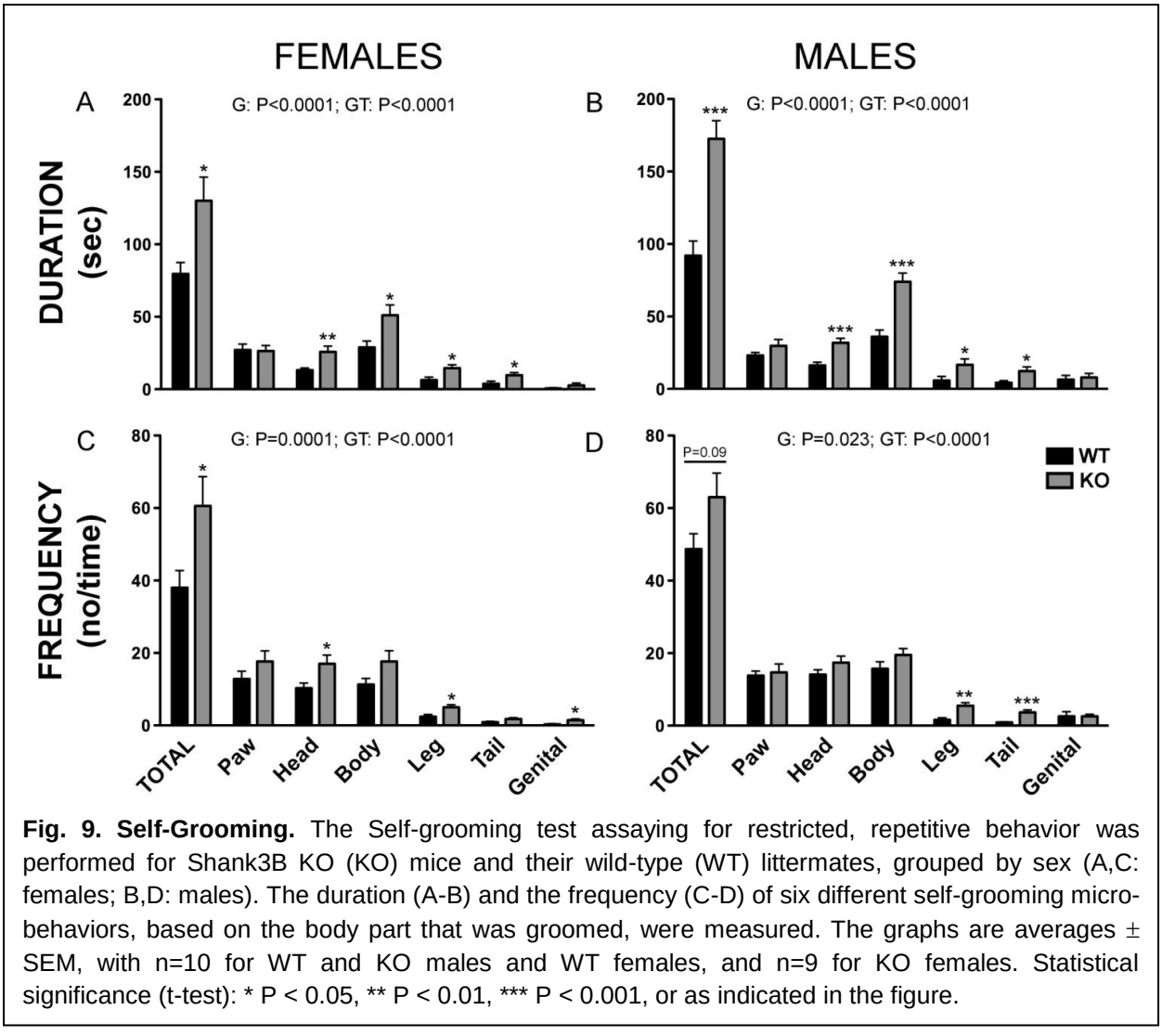


Fig. 9. Self-Grooming. The Self-grooming test assaying for restricted, repetitive behavior was performed for Shank3B KO (KO) mice and their wild-type (WT) littermates, grouped by sex (A,C: females; B,D: males). The duration (A-B) and the frequency (C-D) of six different self-grooming micro-behaviors, based on the body part that was groomed, were measured. The graphs are averages ± SEM, with n=10 for WT and KO males and WT females, and n=9 for KO females. Statistical significance (t-test): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, or as indicated in the figure.

Juvenile Shank3B KO mice do not display altered anxiety-like behaviors.

To interpret anxiety-like behaviors, mice were subjected to the Elevated Mazes test. Shank3B KO and WT mice scored similarly with longer duration in the enclosed quadrants; demonstrating preference for the enclosed quadrants over the open ones (Fig. 10.). No differences were noted between Shank3B and WT mice (Fig. 10) in both elevated maze tests when comparing genotype and sex across variables.

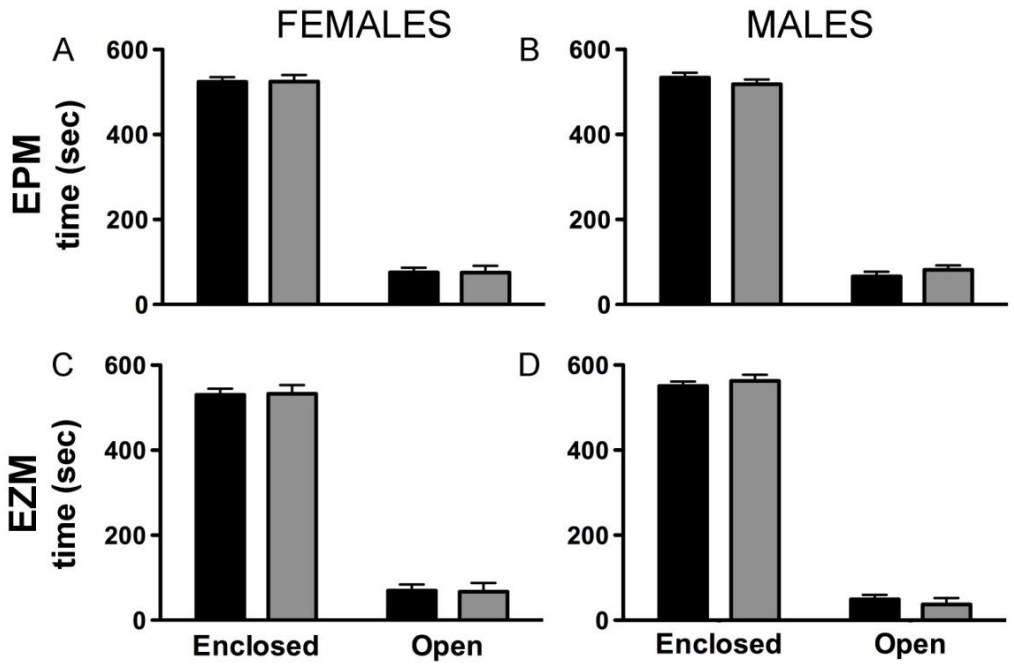


Fig. 10. Elevated Mazes. Elevated plus maze (EPM, A,B) and elevated zero maze (EZM, C,D) tests were performed to assess anxiety-like behaviors in Shank3B KO (KO) mice and their wild-type (WT) littermates, grouped by sex (A,C: females; B,D: males). The duration of time spent within enclosed or open area were measured. The graphs are averages ± SEM, with n=10 for WT and KO males and WT females, and n=9 for KO females. No differences between Shank3B KO and WT mice were noted.

4. DISCUSSION

Adult Shank3B KO's were previously shown to display the ASD-like behavioral anomalies, such as repetitive grooming behavior as well as aberrant social and anxiety-like behaviors consistent with ASD (Peca *et al.*, 2011). Here, we characterized juvenile Shank3B KO males and females utilizing an ASD-related behavioral test battery to measure abnormalities in the three core behavioral paradigms of ASD (communication, social behavior, and motor stereotypies) as well as anxiety-like behaviors and depression. As previously reported in adults, we observed that juvenile Shank3B KO mice display ASD-like behaviors, and that males are more strongly affected. We further characterized extended micro-behaviors of reciprocal social interaction of Shank3B KO mice, establishing detailed ASD-like behavioral phenotype not included in previously published standard analyses.

Early communicative behavior, specifically isolation-induced, illustrate dam-pup dyadic relationship that prompts maternal orientation, approach, and retrieval (Branchi *et al.*, 2001, Elwood & Keeling, 1982, Noirot, 1966, Scattoni *et al.*, 2009). Ethological evidence suggests that juvenile USV profiles may be indicative of anxiety profiles that may develop later in adulthood (Menuet *et al.*, 2011), providing an early screening for age-dependent neurodevelopmental deficits. In agreement with the previous study using a different *Shank3b* deficient mouse model (Wohr, 2014, Zhou *et al.*, 2016), we did not observe significant USV differences between Shank3B KO and WT mice at specific developmental timepoints. USV call frequencies follow an ontogenetic pattern, with continuous decrease from PND 8 to PND 12 (Branchi *et al.*, 2001, Elwood & Keeling, 1982, Scattoni *et al.*, 2009, Wohr, 2014). Shank3B KO males displayed less pronounced decrease in call frequency over time, suggesting a slight developmental delay and warranting additional investigations of anxiety-like behavior. An in-depth analysis involving decoding of various calls and group tick marks displayed on spectrograms (Lahvis *et al.*, 2011, Wohr, 2014) might reveal further discrepancies between genotypes. The current data, however, suggest that the Shank3B KO mice may not adequately model communication deficits observed in ASD.

The 3-Chamber test yielded results consistent with the previous study (Peca *et al.*, 2011) and demonstrated abnormal social interaction observed with Shank3B KO mice, with males more strongly affected. Sniff and Nose-to-Nose micro-behaviors, which were reduced with Shank3B KO males, are considered exploratory and pro-social behaviors (Pearson *et al.*, 2010), thus indicating social impairment.

Female Shank3B KO exhibited less Rear, Contact, and Sniff micro-behaviors compared to WT females but spent longer time Grooming, which underscores repetitive grooming habits and perhaps increased anxiety levels (Kalueff & Tuohimaa, 2005). Contradictory to what was observed with Shank3B KO males, Shank3B KO females displayed a preference for the social chamber, like that of WT females. This outcome is inconsistent with the micro-behaviors assessed and underscores the importance of including micro-behavioral assessment in addition to standard analyses. Although Shank3B KO females spent more time in the social chamber, they displayed disinterest towards the novel stimulus mouse, exhibiting potential anti-social behaviors previously unappreciated.

Unlike the 3-Chamber test, the Social Proximity test measures forced reciprocal social interaction within confined quarters. The readout of this test is not whether the examined mouse display social preference, but rather whether it engages in frontal oriented social interaction and/or social avoidance (Defensor *et al.*, 2011) with the stimulus. Shank3B KO males exhibited lower frequency in Upright as well as a tendency towards less frequent Nose-to-Nose interaction. Following behavior description outlined previously (Defensor *et al.*, 2011), Upright and Nose-to-Nose are considered examples of a frontal contact, synonymous to pro-social tendencies. Interestingly, Shank3B KO females displayed opposite behaviors, with increased duration and frequency of Nose-to-Nose interaction with the CD-1 stimulus. This is in agreement with previous studies showing sex-dependent differences, with males presenting with a more pronounced ASD-like behavior as compared to females (Blanchard *et al.*, 2012, Defensor *et al.*, 2011, Moy *et al.*, 2004, Pearson *et al.*, 2010, Peca *et al.*, 2011, Pobbe *et al.*, 2011). When the results of the 3-Chamber and the Social Proximity tests are interpreted together, our data show that when forced to interact with a stimulus mouse, Shank3B KO females will interact but when given the option they display anti-social behaviors with stimulus and engage in repetitive grooming habits.

Interpretation of grooming behaviors leads to a better understanding of mouse models with behavioral disorders (Smolinsky *et al.*, 2009). The microstructure of grooming patterns in mice has proven to be a useful tool in assessing anxiety and repetitive behaviors (Kalueff & Tuohimaa, 2005). We observed that Shank3B KO mice self-groomed for longer time and more frequently than WT while displaying excessive self-grooming of specific body parts. Surprisingly, we did not observe any self-injurious grooming resulting in dorsal cranial lesions (Peca *et al.*, 2011). However, we did observe slight

barbering and skin lesions (ulcerative dermatitis) in the dorsal-caudal region in male Shank3B KO mice. As this is a qualitative observation, we cannot determine whether these injuries were sustained due to aggressive behaviors or excessive grooming. Both scenarios are common occurrences in C57BL/6 strains (Burkholder *et al.*, 2012). Similarly, we cannot ascertain which genotype presented the most alopecia/skin lesions due to randomized housing of all Shank3B KO and WT genotypes. Mice grooming is orchestrated in a complex ordered pattern from cranial to caudal direction (Kalueff & Tuohimaa, 2005, Smolinsky *et al.*, 2009). Interruptions, or bouts, and divergence from this patterning constitutes anxiety-related grooming behaviors (Smolinsky *et al.*, 2009). Although Shank3B KO mice displayed consistent and repetitive grooming habits as compared to their WT counterparts overall, aberrant patterning and grooming bouts were not assessed and we therefore cannot attribute our data to anxiety-related grooming behaviors.

When analyzing the results of Elevated Maze, no significant differences between genotype and sex in terms of duration were observed. Both genotypes of either sexes displayed preference for the enclosed quadrants based on duration. However, certain observations were made during the testing period that warrants explanation. The Elevated Maze tests are standard behavioral tests utilized for the assessment of efficacy of anxiolytic drugs (Crawley, 2007, Lister, 1987, Rodgers & Dalvi, 1997). Mice placed in the arena made up of two enclosed quadrants and two open quadrants should display equal desire to explore all quadrants once acclimated to the novel environment. However, exploratory evasion of the open arms is related to higher levels of fear/anxiety (Lister, 1987, Rodgers & Dalvi, 1997). The apparatuses require that the open arms have a small barrier (~0.5 cm) to prevent mice from falling off (Komada *et al.*, 2008). Although these barriers were present on the apparatuses, it was observed that Shank3B KO, and not WT mice, tended to fall off the open arm as they were exploring. This may be indicative of neurodevelopmental deficiencies within primary brain regions. Throughout development, SHANK3B isoforms are differentially expressed in various brain regions, primarily the striatum, hippocampus, cerebellum, and neocortex (Maunakea *et al.*, 2010, Peca *et al.*, 2011), all of which are pivotal in sensorimotor functions and cognition (Jiang & Ehlers, 2013, Maunakea *et al.*, 2010, Peca *et al.*, 2011, Reig & Silberberg, 2014). Perhaps due to primary striatal dysfunction, sensorimotor activities, provided through tactile and visual stimuli, are disrupted causing Shank3B KO mice to have difficulties

recognizing depth (Reig & Silberberg, 2014). This is a contingent assumption as SHANK3 isoform-specific biochemical pathways and expression profiles within specific brain regions have yet to be fully characterized (Maunakea *et al.*, 2010).

The interpretation of Elevated Maze (falling off the platform) needs to be considered with caution because Shank3B KO mice are known to be prone to micro-seizures. Such micro-seizures were observed during routine husbandry of Shank3B KO mice; therefore, were triggered by a stressful situation (Peca *et al.*, 2011). If the mice undergoing testing in our study were affected by such seizures, this could have affected their performance in these tests; thus, posing a potential dilemma in interpreting the analyzed results.

ASD behavioral phenotypes are preferentially established utilizing male mice (Blanchard *et al.*, 2012, Defensor *et al.*, 2011, Kazdoba *et al.*, 2016, Moy *et al.*, 2004, Pearson *et al.*, 2010, Peca *et al.*, 2011, Pobbe *et al.*, 2011) because they display strong ASD-like phenotype. Here, we tested Shank3B KO mice of both sexes. Although the ASD-like behavioral phenotype was more apparent in males, as expected, the analyses of females yielded some observations, such as repetitive grooming and abnormal social micro-behaviors that warrant further investigations. The range and contrasting ASD-relevant symptoms that we observed with Shank3B KO females correlate to what can be observed in clinical cases with female patients (Frazier *et al.*, 2014, Lord *et al.*, 1982, Volkmar *et al.*, 1993).

In our study, we noted discrepancies between the results from standard analyses of Shank3B KO females in the social chamber during the 3-Chamber test and the in-depth micro-behavioral analyses utilizing the same test. Although standard analyses conferred normal sociability, with Shank3B KO females spending more time in the social chamber, the mice displayed hyperactive grooming behaviors instead of socializing with the stimulus female. Furthermore, in the Social Proximity test eliciting forced social interaction, Shank3B KO females exhibited pro-social behaviors more frequently than the wild-type counterparts. Unlike Shank3B KO males, which showed significant atypical behavior across most behavioral tests, Shank3B KO females demonstrated stronger ASD-like phenotype in specific tests. This discrepancy of social behaviors highlights the necessity to employ a behavioral test battery involving both comprehensive micro-behavioral analyses and standard analyses of ASD-relevant behavioral tests in

future studies to capture more nuances behavioral deficiencies. Although cognition is not a core symptom of ASD, it would also be interesting to see how Shank3B KO males and females perform on a battery of cognitive assays given the role of *Shank3* in memory and learning and considering that previous results from the Morris Water Maze test proved no distinction between Shank3B KO and wild-type males (Peca *et al.*, 2011). Further behavioral tests focusing on the cognitive paradigm may enhance and/or compliment our data and help us to further establish comprehensive behavioral phenotype of juvenile Shank3B KO mice.

5. CONCLUSION

In this study, we assessed ASD-related behavior in juvenile Shank3B KO males and females which has not been previously done. Our findings illustrate alterations in the 3 core behavioral symptoms (communication, social behaviors, and repetitive stereotypy) representative of human clinical ASD diagnosis. Like previous studies, stronger ASD-like phenotype was apparent in Shank3B KO males as compared to Shank3B KO females, paralleling human ASD clinical cases. The behavioral test battery that we used corresponds to specific timepoints that model ASD in humans and includes micro-behavioral analyses, allowing for comprehensive phenotypic classification. The results of our analyses support that the inclusion of in-depth analyses within canonical behavioral assays is essential when conferring phenotype of mice modeling neurodevelopmental disorders. The described behavioral data can be used as a reference in future studies focusing on gene-environment interaction in ASD etiology at initial stages of development.

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