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Authors

Berg, Elizabeth L
Copping, Nycole A
Rivera, Josef K
et al.

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Developmental Social Communication Deficits in the *Shank3* Rat Model of Phelan-McDermid Syndrome and Autism Spectrum Disorder

Elizabeth L. Berg, Nycole A. Copping, Josef K. Rivera, Michael C. Pride, Milo Careaga, Melissa D. Bauman, Robert F. Berman, Pamela J. Lein, Hala Harony-Nicolas, Joseph D. Buxbaum, Jacob Ellegood, Jason P. Lerch, Markus Wöhr, and Jill L. Silverman

Mutations in the *SHANK3* gene have been discovered in autism spectrum disorder (ASD), and the intellectual disability, Phelan-McDermid Syndrome. This study leveraged a new rat model of *Shank3* deficiency to assess complex behavioral phenomena, unique to rats, which display a richer social behavior repertoire than mice. Uniquely detectable emissions of ultrasonic vocalizations (USV) in rats serve as situation-dependent affective signals and accomplish important communicative functions. We report, for the first time, a call and response acoustic playback assay of bidirectional social communication in juvenile *Shank3* rats. Interestingly, we found that *Shank3*-deficient null males did not demonstrate the enhanced social approach behavior typically exhibited following playback of pro-social USV. Concomitantly, we discovered that emission of USV in response to playback was not genotype-dependent and emitted response calls were divergent in meaning. This is the first report of these socially relevant responses using a genetic model of ASD. A comprehensive and empirical analysis of vigorous play during juvenile reciprocal social interactions further revealed fewer bouts and reduced durations of time spent playing by multiple key parameters, including reduced anogenital sniffing and allogrooming. We further discovered that male null *Shank3*-deficient pups emitted fewer isolation-induced USV than *Shank3* wildtype controls. Postnatal whole brain anatomical phenotyping was applied to visualize anatomical substrates that underlie developmental phenotypes. The data presented here lend support for the important role of *Shank3* in social communication, the core symptom domain of ASD. By increasing the number of *in vivo* functional outcome measures, we improved the likelihood for identifying and moving forward with medical interventions. ***Autism Res*** *2018, 0: 000–000.* © 2018 International Society for Autism Research, Wiley Periodicals, Inc.

Lay Summary: Clinically relevant outcomes are required to demonstrate the utility of therapeutics. We introduce findings in a rat model, and assess the impact of mutations in *Shank3*, an autism risk gene. We found that males with deficient expression of *Shank3* did not demonstrate typical responses in a bi-directional social communication test and that social interaction was lower on key parameters. Outcome measures reported herein extend earlier results in mice and capture responses to acoustic calls, which is analogous to measuring receptive and expressive communication.

Keywords: autism; shank; social; behavior; animal model; synapse; Phelan McDermid Syndrome; neurodevelopment

Introduction

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders with incomplete understanding of etiology and no biological markers. Diagnosis of the disease relies on behavioral criteria in the two distinct domains of (1) impairments in reciprocal social communication and (2) repetitive behaviors, with restricted interests and behavioral inflexibility. New diagnoses include a broader definition of the ASD phenotype and reflect the current consensus that the causes and clinical presentations are highly heterogeneous [American Psychiatric Association, 2013]. With such heterogeneity, studying clearly defined genetic

From the University of California, Davis, MIND Institute, School of Medicine, Sacramento, CA (E.L.B., N.A.C., J.K.R., M.C.P., M.C., M.D.B., R.F.B., P.J.L., J.L.S.); Mouse Imaging Centre, The Hospital for Sick Children, Toronto, Canada (J.E., J.P.L.); Department of Medical Biophysics, University of Toronto, Toronto, Canada (J.P.L.); Seaver Autism Center for Research and Treatment, Icahn School of Medicine at Mount Sinai, New York, NY (H.H.-N., J.D.B.); Behavioral Neuroscience, Experimental and Biological Psychology, Philipps-University of Marburg, Gutenbergstr. 18, Marburg, D-35032, Germany (M.W.); Marburg Center for Mind, Brain, and Behavior (MCMBB), Marburg, Germany (M.W.)
Markus Wöhr and Jill L Silverman are equally contributing last authors.
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Address for correspondence and reprints: Jill L Silverman, Ph.D., MIND Institute and Department of Psychiatry and Behavioral Sciences, University of California Davis School of Medicine, Room 1001B, Research II Building 96, 4625 2nd Avenue, Sacramento, CA 95817. E-mail: jsilverman@ucdavis.edu
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subgroups with known causal factors can lead to the discovery of underlying mechanisms of pathology and targeted treatment options. *In vivo* studies in preclinical model organisms with a high degree of genetic conservation relative to humans are indispensable for this strategy of therapeutic development.

Sophisticated tools for testing social communication across developmental windows are required for preclinical ASD research. For two decades, mouse models gained prevalence due to the widely applicable sophisticated genetic technologies. However, some complex behaviors and physiological processes are difficult to investigate in the mouse. The broader social behavioral repertoire in rats is obvious during social play, a behavior that is prominent in juveniles. In addition, the mouse acoustic communication system is less complex than that used by rats [Bigbee, Sharma, Gupta, & Dupree, 1999; Iannaccone & Jacob, 2009; Parker et al., 2014; Ellenbroek & Youn, 2016; Homberg, Wöhr, & Alenina, 2017]. Species-specific emissions of ultrasonic vocalizations (USV) in rats serve as situation-dependent affective signals and accomplish important communicative functions [Portfors, 2007; Brudzynski, 2009; Brudzynski, 2013; Wöhr & Schwarting, 2013]. There are three main categories of USV in rats. Pup 40-kHz "distress calls" are emitted when subjects are physically isolated from their mother and littermates. Deficits in pup USV emission are standard read-outs of anxiety-like behavior, stress responsivity, and/or social communication [Hofer, 1996; Shair, Masmela, Brunelli, & Hofer, 1997; Brudzynski, Kehoe, & Callahan, 1999; Hofer, Shair, & Brunelli, 2002; Bowers, Perez-Pouchoulen, Edwards, & McCarthy, 2013]. Juvenile and adult rats emit low frequency 22-kHz "alarm calls" in aversive situations [Blanchard, Blanchard, Agullana, & Weiss, 1991; Kroes, Burgdorf, Otto, Panksepp, & Moskal, 2007; Yee, Schwarting, Fuchs, & Wöhr, 2012] while high frequency 50-kHz "pro-social calls" occur in appetitive situations, such as rough-and-tumble play [Knutson, Burgdorf, & Panksepp, 1998]. These 50-kHz calls also elicit and result in extended periods of social play [Kisko, Himmler, Himmler, Euston, & Pellis, 2015b].

Mutations in the *SHANK3* gene are implicated in ASD, Phelan- McDermid Syndrome (PMS), and intellectual disability [Moessner et al., 2007; Gauthier et al., 2009; Betancur & Buxbaum, 2013; Leblond et al., 2014]. Reduced expression of *SHANK3*, which codes for a synaptic scaffolding protein, is hypothesized to lead to impairments in key brain functions underlying social communication and cognition [Durand et al., 2007]. To assess the neurobiological role of *Shank3*, numerous mutant mouse models were generated by targeting different biochemical domains and resulted in various degrees of reduction of Shank3 isoforms [Bozdagi et al., 2010; Peca et al., 2011; Yang et al., 2012; Kouser et al., 2013; Jaramillo et al., 2016; Mei et al., 2016; Jaramillo et al., 2017]. In addition, a complete null mouse model of *Shank3* with no detectable levels of expression has recently been generated [Wang et al., 2016]. Last year, Harony-Nicolas et al. [2017] reported the generation and initial characterization of a *Shank3*-deficient rat, a highly novel genetic model of PMS and ASD [Harony-Nicolas et al., 2017].

This rat model provided us the opportunity to investigate complex behaviors that have been difficult to capture with high signal sensitivity in mice, such as developmental and juvenile acoustic social communication and social play. The objectives of the present experiments were first to leverage the complex behavioral repertoire of rats to measure acoustic social communication using the species-specific and context-dependent pro-social 50-kHz USV emitted by rats. Second, to delineate and define categorical yes-no sociability via the standard three-chambered assay in rats, in parallel with a fine-grained, detailed analysis of social interactions during juvenile dyad play. Finally, we evaluated postnatal distress call emissions, alongside corroborative neuroanatomy using ex vivo neuroimaging. Given the relevance of *Shank3* deletions to human disease conditions, we hypothesized that *Shank3* deficiency would result in social communication deficits across our assays in a gene dose-dependent manner.

Materials and Methods

Subjects for Behavior and Neuroimaging

All animals were housed in a temperature-controlled vivarium maintained on a 12:12 light-dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California Davis and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were performed on *Shank3* wildtype (+/+), heterozygous (+/−), and null mutant (−/−) littermates that were offspring of *Shank3* +/− by +/− breeding pairs. To identify rats, pups were labeled by paw tattoo on postnatal day (PND) 2–3 using non-toxic animal tattoo ink (Ketchum Manufacturing Inc., Brockville, ON, Canada). The ink was inserted subcutaneously into the center of the paw through a 22-gauge hypodermic needle tip. Rats were also tail-marked with permanent marker at weaning to allow for additional identification. The tattoo and tail-marks for each rat were coded to allow investigators to run and score behaviors blind to genotype. At PND 2–7, tails of pups were clipped or at PND13–16 ears were hole-punched (1–2 mm) for genotyping, following the UC Davis IACUC policy regarding

tissue collection. Genotyping was performed in our laboratory by procedures outlined in Supporting Methods.

Behavioral Assays

Order of behavioral testing and description of cohorts. To minimize the carry-over effects from repeated testing and handling, 4 cohorts were tested. Each cohort was comprised of 5–16 litters. Cohort 1 were offspring of *Shank3* +/− × *Shank3* +/− breeding pairs, sampled from 9 litters, and tested in open field locomotion at PND 38–40 to confirm typical motor abilities. Pronounced locomotor deficits have been reported in *Shank3* mouse models [Peca et al., 2011; Kouser et al., 2013; Speed et al., 2015; Jaramillo et al., 2016; Mei et al., 2016; Copping et al., 2017a; Dhamne et al., 2017] and thus it was crucial that the social communication outcomes of this study not be confounded by motor deficiencies. Cohort 2 were offspring of *Shank3* +/− × *Shank3* +/− breeding pairs, sampled from 16 litters, and tested in acoustic playback of pro-social species-specific 50-kHz USV at PND 26–33. Cohort 3 were offspring of *Shank3* +/− × *Shank3* +/− breeding pairs, sampled from 5 litters, and tested in 40-kHz USV at PND 7 followed by perfusion for developmental neuroimaging. Cohort 4 were offspring of *Shank3* +/− × *Shank3* +/− breeding pairs, sampled from 10 litters, and tested in 40-kHz USV at PND 7, three-chambered social approach at PND 26–29, and social dyad play at PND 32–45.

Isolation-induced pup 40-kHz ultrasonic vocalizations. Ultrasonic vocalizations (USV) are emitted by rodent pups when separated from their mothers and littermates in the first 2 weeks of life. [Hofer et al., 2002; Wöhr & Schwarting, 2008]. On PND 7, pups were removed individually from the nest at random and gently placed into an isolation container (8 cm × 6 cm × 5 cm; open top) made of plastic, using methods outlined in Supporting Methods.

Three-chambered social approach. Social approach using a three-chambered apparatus was measured by methodology similar to protocols described previously for mice [Silverman, Yang, Lord, & Crawley, 2010; Yang, Silverman, & Crawley, 2011; Silverman et al., 2012; Sukoff Rizzo & Silverman, 2016; Dhamne et al., 2017] and rats [Ku et al., 2016] with modifications outlined in the Supporting Methods.

Juvenile social play. Two unfamiliar rats were placed together for 10 min in a clean, empty test arena (three opaque white walls, one transparent wall; 41.9 cm l × 41.9 cm w × 29.2 cm h) with a thin (0.5 cm) layer of clean corn cob bedding. Immediately prior to the test session, each rat was singly housed in a separate holding room for 30 min to facilitate and amplify total social play [Panksepp & Beatty, 1980; Panksepp, 1981], thereby permitting a behavioral assay that consists of brief observation periods. Subjects were PND 32–45, previously described to be the age range with maximal frequency of play behaviors in same sex dyads [Meaney & Stewart, 1981; Meaney, 1988]. Stimulus animals were strain-, sex-, and age-matched to subject rats. Behaviors were videorecorded through the test chamber's transparent front wall and later scored by a trained observer blinded to genotype was carried out based on historical literature [Thor & Holloway, 1983, 1984, 1986; Pellis & Pellis, 1998]. In order to minimize the influence of the stimulus rat, all stimulus rats were wildtype Sprague-Dawley and all behaviors were scored based on the actions/initiation of the subject rat, similar to the field's standard [Veeraragavan et al., 2016]. The amount of time in seconds that the subject spent exploring, nose-to-anogenital sniffing, social sniffing, following or chasing, rough-and-tumble playing, self-grooming, or allogrooming were scored as outlined in the Supporting Methods herein.

Open field locomotion. Exploratory activity in a novel open arena was evaluated as described previously [Ku et al., 2016], in order to control for potentially confounding effects of sedation or hyperactivity on the sociability assays.

Behavioral response to playback of pro-social 50-kHz ultrasonic vocalizations. In total $N = 109$ rats were tested between PND 26–33 (body weight: ~70–130 g), with the following group sizes: $N = 13$ male *Shank3* +/+ rats, $N = 19$ female *Shank3* +/+ rats, $N = 19$ male *Shank3* +/− rats, $N = 23$ female *Shank3* +/− rats, $N = 16$ male *Shank3* −/− rats, and $N = 19$ female *Shank3* −/− rats. All rats were handled for 2 days prior to testing in a standardized manner (5 min per rat per day). Social exploratory and approach behavior in response to playback of pro-social 50-kHz USV was assessed on an elevated radial eight-arm maze (Fig. 1A, right, 48.0 cm above floor; arms: 40.0 cm l × 10.0 cm w) surrounded by a black curtain under indirect dim white light (~8 lux) according to a modified protocol previously established [Wöhr & Schwarting, 2007]. Behavioral equipment was cleaned with 70% ethanol solution and thoroughly dried between subjects. Acoustic stimuli were presented through UltraSoundGate 116 Player (Avisoft Bioacoustics) connected to an ultrasonic loudspeaker (ScanSpeak, Avisoft Bioacoustics) placed 20 cm away from the end of one arm. An additional, but inactive loudspeaker was arranged symmetrically at the opposite arm as a visual control.

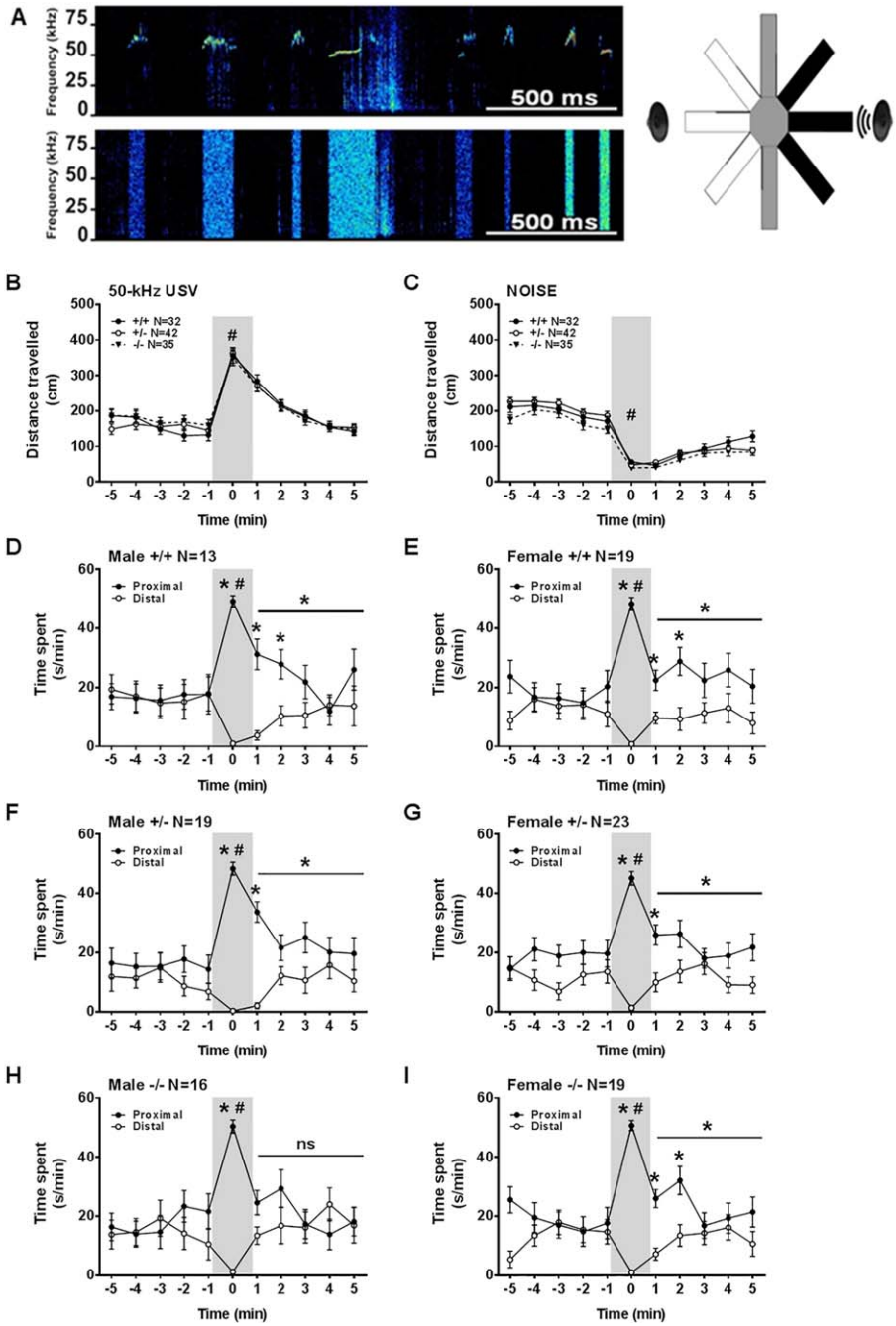

Figure 1. Reduced behavioral responses to pro-social 50-kHz USV in male Shank3 −/− rats. Acoustic stimulus presentation is highlighted in grey. (**A, left**) Exemplary spectrogram depicting 2-sec sections of pro-social 50-kHz USV (upper panel) and time- and amplitude-matched White Noise (lower panel) used for playback. (**A, right**) Schematic illustration of the radial maze used for playback depicting proximal (black), distal (white), and neutral arms (grey) relative to the active ultrasonic loudspeaker. Total distance travelled before (5 min; white), during (1 min; grey), and after (5 min; white) playback of (**B**) pro-social 50-kHz USV and (**C**) time- and amplitude-matched White Noise. In all experimental groups, the change in total distance travelled was time-locked to the minute of acoustic stimulus presentation. Social approach behavior in response to playback of pro-social 50-kHz USV, as reflected in time spent on proximal (black) versus distal (white) arms, for (**D**) male *Shank3* +/+, (**E**) female *Shank3* +/+, (**F**) male *Shank3* +/-, (**G**) female *Shank3* +/-, (**H**) male *Shank3* −/−, and (**I**) female *Shank3* −/− rats. Social approach behavior was evident in all groups of *Shank3* +/+ and *Shank3* +/- rats, with the exception of *Shank3* −/− males, which did not continue to explore after playback of pro-social 50-kHz USV ended. *$p < 0.05$ by repeated measures ANOVAs and Bonferroni or paired student t-tests.

Two acoustic stimuli were used: (1) pro-social 50-kHz USV and (2) White Noise (Fig. 1A, left); the latter serving as a time- and amplitude-matched acoustic stimulus control [Seffer, Schwarting, & Wöhr, 2014]. Pro-social 50-kHz USV used for playback were recorded from a naïve male rat during exploration of a cage containing scents from a recently separated cage mate. The 50-kHz USV stimulus consisted of 221 natural 50-kHz USV (total calling time: 15.3 sec), composed of a sequence of 3.5 sec, which was repeated for 1 min, that is, 17 times, to assure the presentation of a high number of frequency-modulated calls within a relatively short period of time. Each sequence contained 13 calls (total calling time: 0.90 sec). Out of these, 10 were frequency-modulated and 3 were flat, and had the following features: call duration 0.07 ± 0.01 sec (mean ± SEM); peak frequency: 61.24 ± 1.75 kHz; bandwidth: 4.63 ± 1.21 kHz; frequency modulation: 31.68 ± 4.62 kHz. After an initial 15-min habituation period, each rat was exposed to 1-min playback presentations of 50-kHz USV and White Noise, separated by a 10-min inter-stimulus interval. Stimulus order was counterbalanced to account for possible sequence effects. The session ended after an additional 10-min post-stimulus phase (total test duration: 37-min period). Behavior was monitored by a video camera (Polestar II EQ610 Surveillance/Network Camera, EverFocus Electronics; New Tapiei City, Taiwan) mounted 1.7 m centrally above the arena and analyzed using EthoVision XT 10 (Noldus, Wageningen, The Netherlands). Distance traveled served as a measure for locomotor activity. Number of arm entries proximal and distal to the active ultrasonic loudspeaker and time spent thereon served as measures for stimulus-directed locomotor activity [Seffer et al., 2014].

In the pro-social 50-kHz USV playback paradigm, USV were monitored with an ultrasonic condenser microphone (CM16, Avisoft Bioacoustics) placed centrally above the center of the maze at a height of 42 cm. It was connected via an UltraSoundGate 116H USB audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250,000 Hz in 16 bit format (recording range: 0–125 kHz) by Avisoft RECORDER USGH (Avisoft Bioacoustics). The microphone is sensitive to frequencies of 15–180 kHz with a flat frequency response (±6 dB) between 25 and 140 kHz. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 4.50; Avisoft Bioacoustics). High resolution spectrograms (frequency resolution: 488 Hz; time resolution: 0.512 ms) were obtained through a fast Fourier transformation (512 FFT length, 100% frame, Hamming window and 75% time window overlap). Call detection of pro-social and positive 50-kHz USV or frustration-related aversive 22-kHz USV emitted by juvenile rats during playback of pro-social 50-kHz USV was provided by an experienced observer blind to experimental conditions, who, through visual inspection of the spectrogram, who manually counted the numbers of USV in 1-min time bins. Identification of 22-kHz and 50-kHz USV emitted by the subject rat was obtained by matching the playback stimulus with the recording acquired during playback [Wöhr & Schwarting, 2007, 2009; Willadsen, Seffer, Schwarting, & Wöhr, 2014; Engelhardt, Fuchs, Schwarting, & Wöhr, 2017]. This study was the initial effort at establishing a finite frequency threshold in juvenile rats and thus the thresholds employed herein were based on established thresholds for adult rats [Portfors, 2007; Brudzynski, 2009; Brudzynski, 2013; Wöhr & Schwarting, 2013]. In adult rats, the 22-kHz USV are long, about 1–2 sec, and very low in frequency, that is, around 22 kHz. However, juvenile rats are smaller in size and lung capacity, and thus are not able to produce such long, low-frequency calls. USV emitted within a frequency range of 20–33 kHz were considered as 22-kHz USV and USV with peak frequencies higher than 33 kHz as 50-kHz USV. While 50-kHz USV are typically characterized by short call durations between 5 and 60 ms, 22-kHz USV are characterized by much longer call durations. If two 50-kHz USV elements were at least 10 ms apart, two independent 50-kHz USV were counted. In the playback of pro-social 50-kHz USV paradigm, the following periods were used for comparisons: the averaged 5-min baseline period before playback (PRE), the 1-min acoustic stimulus period (STIM), and the averaged 5-min post-stimulus period (POST). Behavioral parameters were analyzed using repeated-measures ANOVAs with genotype and sex as between-subject factors and time (PRE vs. STIM) or preference (proximal vs. distal) as within-subject factor. ANOVAs were followed by two-tailed paired *t*-tests when appropriate. $N = 1$ male *Shank3* +/− rat was excluded from USV analysis due to data loss.

Postnatal Ex Vivo Rat MRI Neuroimaging

A cohort of rats was sacrificed via perfusion at PND 7 for neuroimaging. Images were acquired and analyzed, as previously described for mice with modifications for postnatal rats outlined in the Supporting Methods [Copping et al., 2017b; Gompers et al., 2017].

Results

Behavioral Responses to Pro-social 50-kHz USV Are Reduced in Shank3 −/− Males

Consistent with previous findings [Wöhr & Schwarting, 2007, 2009, 2012; Seffer, Rippberger, Schwarting, & Wöhr, 2015], playback of pro-social 50-kHz USV induced social exploratory behavior, as reflected by an increase in total distance travelled, irrespective of genotype and sex

(Fig. 1B: time: $F_{(1, 103)}=211.570$; $P \leq 0.001$; time x genotype: $F_{(2, 103)}=0.678$; NS; time × sex: $F_{(1, 103)}=0.015$; NS; time × genotype × sex: $F_{(2, 103)}=1.340$; NS). Importantly, increased social exploratory behavior (i.e., distance travelled) was specifically seen in response to playback of pro-social 50-kHz USV, while the acoustic stimulus control (i.e., White Noise) causing inhibition of behavior (Fig. 1C: time: $F_{(1, 103)}=752.566$; $P \leq 0.001$; time × genotype: $F_{(2, 103)}=2.383$; NS; time × sex: $F_{(1, 103)}=0.037$; NS; time × genotype × sex: $F_{(2, 103)}=0.877$; NS).

Enhanced social exploratory activity in response to playback of pro-social 50-kHz USV was mainly driven by approach behavior toward the sound source, that is, the active ultrasonic loudspeaker, as reflected by a marked increase in proximal arm entries (entries: $F_{(1, 103)}=154.156$; $P<0.001$; entries × genotype: $F_{(2, 103)}=3.265$; $P \leq 0.05$; entries × sex: $F_{(1, 103)}=0.027$; NS; entries × genotype × sex: $F_{(2, 103)}=1.379$; NS). In contrast to proximal arm entries, distal arm entries decreased (entries: $F_{(1, 103)}=42.540$; $P \leq 0.001$; entries × genotype: $F_{(2, 103)}=0.518$; NS; entries × sex: $F_{(1, 103)}=0.441$; NS; entries × genotype × sex: $F_{(2, 103)}=0.219$; NS), resulting in a strong preference for proximal over distal arms during 50-kHz USV playback (preference: $F_{(1, 103)}=314.450$; $P \leq 0.001$; preference × genotype: $F_{(2, 103)}=2.902$; NS preference × sex: $F_{(1, 103)}=0.057$; NS; preference × genotype × sex: $F_{(2, 103)}=0.970$; NS). Social approach to 50-kHz USV was also reflected in time spent on arms. In all experimental groups, 50-kHz USV playback induced a substantial increase in the time spent in proximal arms (time: $F_{(1, 103)}=590.373$; $P \leq 0.001$; time × genotype: $F_{(2, 103)}=0.402$; NS; time × sex: $F_{(1, 103)}=1.462$; NS; time × genotype × sex: $F_{(2, 103)}=0.464$; NS), paralleled by a decrease in the time spent in distal arms (time: $F_{(1, 103)}=121.009$; $P \leq 0.001$; time × genotype: $F_{(2,103)}=0.870$; NS; time × sex: $F_{(1, 103)}=0.505$; NS; time × genotype × sex: $F_{(2, 103)}=0.268$; NS), resulting in a strong preference for proximal over distal arms during 50-kHz USV playback (Fig. 1D–I: preference: $F_{(1, 103)}=2131.375$; $P \leq 0.001$; time × genotype: $F_{(2,103)}=1.012$; NS; preference × sex: $F_{(1, 103)}=0.492$; NS; preference × genotype × sex: $F_{(2, 103)}=0.524$; NS).

In fact, a significant preference for proximal arms was seen in all individual experimental groups during playback of pro-social 50-kHz USV, both in males (*Shank3* +/+: $t_{(12)}=20.892$; $P \leq 0.001$; *Shank3* +/−: $t_{(18)}=22.31$; $P \leq 0.001$; *Shank3* −/−: $t_{(15)}=18.32$; $P \leq 0.001$) and females (*Shank3* +/+: $t_{(18)}=20.33$; $P \leq 0.001$; *Shank3* +/−: $t_{(22)}=15.88$; $P \leq 0.001$; *Shank3* −/−: $t_{(18)}=21.92$; $P \leq 0.001$). Interestingly, playback of pro-social 50-kHz USV produced sex-dependent genotype effects. Females of all genotypes continued exploring the side in proximity to the active ultrasonic loudspeaker after the auditory 50-kHz USV playback ended (Fig. 1E, G, I: *Shank3* +/+: $t_{(18)}=3.06$; $P \leq 0.008$; *Shank3* +/−: $t_{(22)}=3.73$; $P \leq 0.002$; *Shank3* −/−: $t_{(18)}=2.83$; $P \leq 0.02$), whereas in male *Shank3* +/+ and *Shank3* +/− rats explored rigorously (Fig. 1D, F: $t_{(12)}=3.07$; $P \leq 0.02$ and $t_{(18)}=3.26$; $P \leq 0.005$; respectively) but *Shank3* −/− rats (Fig. 1H: $t_{(14)}=0.48$; NS) did not. A more detailed temporal analysis revealed that male *Shank3* +/+ rats continued the preference by remaining in the proximal arms for another 2 min after playback of pro-social 50-kHz USV ($t_{(12)}=5.16$; $P \leq 0.001$ and $t_{(12)}=2.35$; $P \leq 0.04$; respectively). Male *Shank3* +/− rats continued for another 1 min ($t_{(18)}=8.12$; $P \leq 0.001$). Irrespective of genotype and sex, rats did not display a preference for the side in proximity to the active ultrasonic loudspeaker before playback of pro-social 50-kHz USV.

Response Calls to Pro-social 50-kHz USV Did Not Differ by Genotype

In addition to social exploratory and approach behavior, playback of pro-social 50-kHz USV induced USV emission in subject recipient rats. Irrespective of genotype and sex, recipient rats replied by emitting pro-social and positive 50-kHz USV (Fig. 2A: time: $F_{(1, 102)}=51.84$; $P \leq 0.001$; time × genotype: $F_{(2, 102)}=0.42$; NS; time × sex: $F_{(1, 102)}=1.51$; NS; time × genotype × sex: $F_{(2, 102)}=1.44$; NS). Besides 50-kHz USV, recipient rats also emitted frustration-related aversive 22-kHz USV, with emission rates not differing between genotypes but being higher in males than females (Fig. 2C: time: $F_{(1, 102)}=40.52$; $P \leq 0.001$; time × genotype: $F_{(2, 102)}=2.95$; NS time × sex: $F_{(1, 102)}=7.91$; $P \leq 0.006$; time × genotype × sex: $F_{(2, 102)}=1.184$; NS). Importantly, USV emission in recipient rats was specifically seen in response to playback of pro-social 50-kHz USV but not the acoustic stimulus control, White Noise (Fig. 2B, D).

Shank3 Deficient Rats −/− Exhibit Deficits in a Number of Key Juvenile Social Play Interactions

Social deficits on investigative and play parameters were observed in *Shank3* −/− when compared to *Shank3* +/+ littermates during the juvenile reciprocal dyad social interaction test. Multi-variate ANOVAs using every dependent parameter of our detailed examination of investigative and social play interaction during a 10-min session of reciprocal interactions indicated robust sex ($F_{(17, 52)}=2.10$; $P \leq 0.002$) and genotype effects ($F_{(34, 104)}=2.06$; $P \leq 0.003$).

Male but not female *Shank3* −/− spent less time engaged in social sniffing (Fig. 3A male: $F_{(2, 36)}=8.67$; $P \leq 0.05$, one-way ANOVA followed by Tukey's HSD $q=4.81$ +/+ versus +/− and $q=5.14$ +/+ versus −/− and Fig. 3B female: $F_{(2, 32)}=1.51$; NS). *Shank3* −/− exhibited fewer bouts of social sniffing in both sexes (male: $F_{(2, 36)}=11.21$; $P \leq 0.001$, one-way ANOVA followed by Tukey's HSD $q=5.31$ +/+ versus +/− and $q=5.97$ +/+ versus −/− and

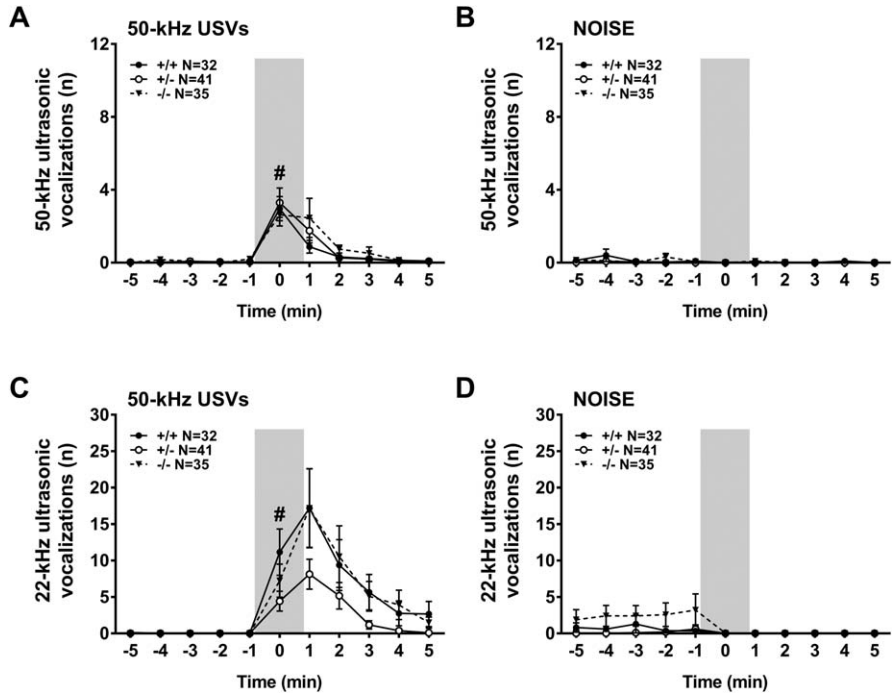

Figure 2. Normal playback-induced USV emission in Shank3 +/− and −/− rats. Acoustic stimulus presentation is highlighted in grey. Emission of 50-kHz USV by recipient rats before (5 min; white), during (1 min; grey), and after (5 min; white) playback of (**A**) pro-social 50-kHz USV and (**B**) time- and amplitude-matched White Noise. Emission of 22-kHz USV by recipient rats before (5 min; white), during (1 min; grey), and after (5 min; white) playback of (**C**) pro-social 50-kHz USV and (**D**) time- and amplitude-matched White Noise. All genotypes and sexes similarly emitted positive and pro-social 50-kHz USV and frustration-related aversive 22-kHz USV in response to pro-social 50-kHz USV playback. USV emission was not induced by the noise stimulus control. In all experimental groups, USV emission by recipient rats was specifically seen in response to playback of pro-social 50-kHz USV and time-locked to the minute of acoustic stimulus presentation. *p<0.05 by repeated-measures ANOVAs and Bonferroni or paired student t-tests.

female: $F_{(2,\ 32)}=4.97$; $P \leq 0.02$, one-way ANOVA followed by Tukey's HSD $q = 4.33$ +/+ versus −/−).

Nose-to-anogenital sniffing time, when initiated by the subject rat, was lower in male but not female *Shank3* −/− (Fig. 3C male: $F_{(2,\ 36)}=7.39$; $P \leq 0.002$, one-way ANOVA followed by Tukey's HSD $q = 5.28$ +/+ versus −/− and Fig. 3D female: $F_{(2,\ 32)}=1.70$; NS). *Shank3* −/− when compared to *Shank3* +/+ exhibited fewer bouts of nose-to-anogenital sniffing in males but not females (male: $F_{(2,\ 36)}=9.38$; $P \leq 0.005$, one-way ANOVA followed by Tukey's HSD $q = 4.51$ +/+ versus +/− and $q = 5.86$ +/+ versus −/− and female: $F_{(2,\ 24)}=4.90$; $P \leq 0.02$, one-way ANOVA followed by Tukey's HSD $q = 4.22$ +/− versus −/−). In females, the number of bouts of nose-to-anogenital sniffing was lower in the *Shank3* −/− but only as compared to *Shank3* +/−.

Shank3 −/− in both sexes trended to initiate fewer seconds of following or chasing behavior toward the stimulus rat (Fig. 3E male: $F_{(2,\ 34)}=1.89$; $P = 0.16$ and Fig. 3F female: $F_{(2,\ 32)}=2.1$; $P = 0.14$, one-way ANOVA). Following and chasing play bouts of behavior by *Shank3* −/− in both sexes also trended toward being less (male: $F_{(2,\ 35)}=1.77$; $P = 0.18$ and female: $F_{(2,\ 24)}=1.99$; $P = 0.16$, one-way ANOVA).

Bouts of allogrooming, also known as social grooming, initiated by the subject rat grooming the stimulus rat, were fewer in the *Shank3* −/− males but not females (Fig. 3G male: $F_{(2,\ 26)}=7.52$; $P \leq 0.003$, one-way ANOVA followed by Tukey's HSD $q = 4.83$ +/+ versus +/− and $q = 5.22$ +/+ versus −/− and Fig. 3H female: $F_{(2,\ 24)}=0.20$; NS). Time spent engaged in social grooming was less in the *Shank3* −/− males but not females (males: $F_{(2,\ 26)}=5.60$; $P \leq 0.01$, one-way ANOVA followed by Tukey's HSD $q = 4.17$ +/+ versus +/− and $q = 4.50$ +/+ versus −/− and female: $F_{(2,\ 24)}=0.22$; NS).

The internal control of subject exploration did not differ between genotype nor sex ($F_{(2,\ 71)}=1.27$; NS), which supported that any differences in social behavior in play were not confounded by motor abilities, hypo-, or hyper-exploration of arena exploration (Fig. 3I male: $F_{(2,\ 35)}=2.00$; NS and Fig. 3J female: $F_{(2,\ 32)}=3.93$; NS, one-way ANOVA).

Levels of investigation of these parameters were comparable and consistent with earlier findings using Sprague-Dawley rats at this age [Panksepp & Beatty, 1980; Meaney & Stewart, 1981; Panksepp, 1981; Thor & Holloway, 1984; Pellis & Pellis, 1998; Argue & McCarthy, 2015a,b].

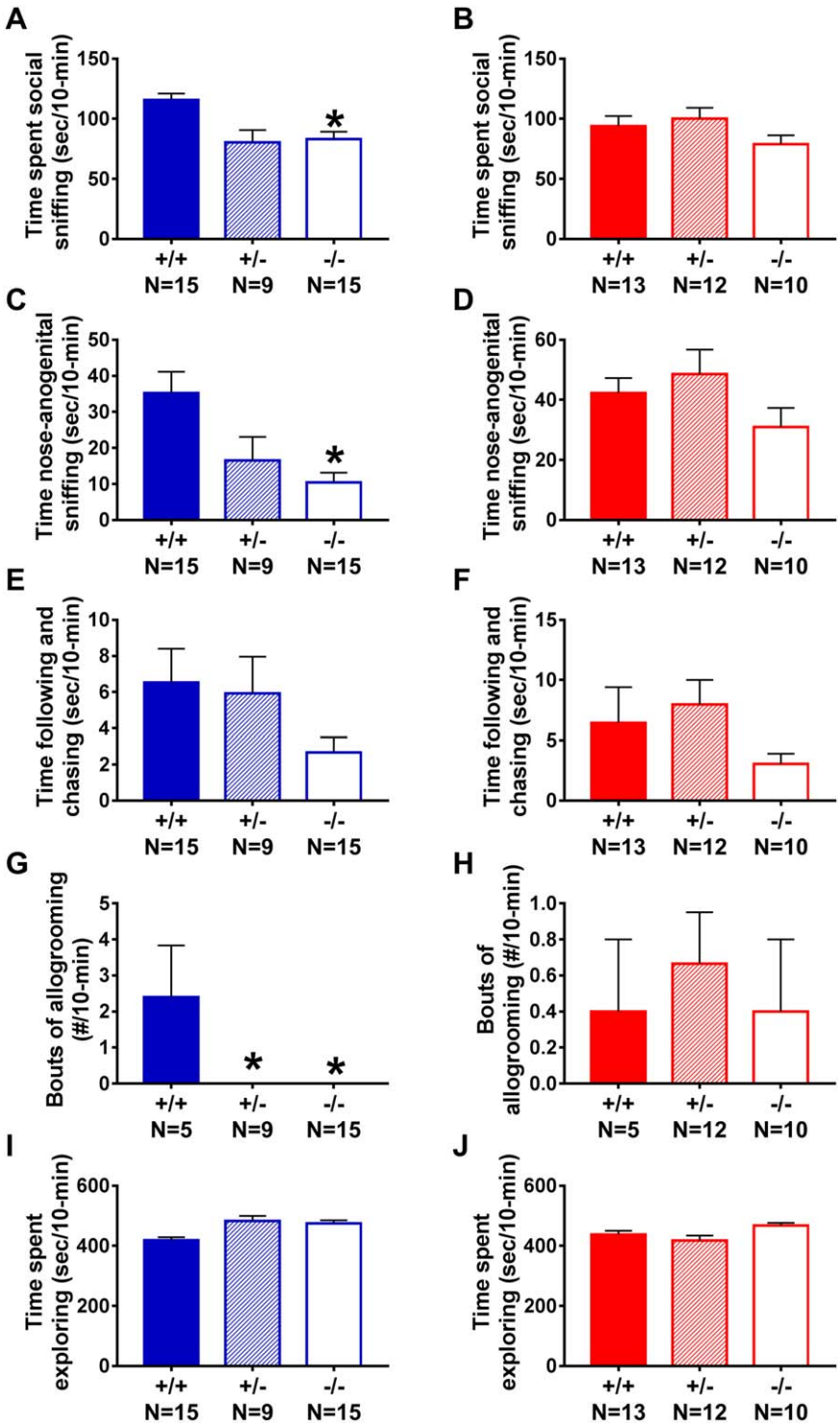

Figure 3. Shank3 −/− rats exhibit deficits in a number of key juvenile social play interactions. Subject initiated investigative and social play behaviors were collected on PND 32–45 in a test arena with a stimulus animal that was a novel wildtype, sex- and age-matched to the subject rat. The amount of time in seconds that the subject spent social sniffing, nose-to-anogenital sniffing, following and chasing, rough-and-tumble playing, exploring, or allogrooming were scored over a 10-min session. Male *Shank3* −/− exhibited less time spent engaged in **(A)** social sniffing, **(C)** nose-to-anogenital sniffing, and **(E)** following or chasing compared to +/+ and +/- littermates. Male *Shank3* +/- and −/− rats also had **(G)** fewer bouts of allogrooming during the juvenile reciprocal social interaction test. **(B, D, F, H)** Female *Shank3* rats did not demonstrate reductions in any of these behaviors. **(I, J)** No genotype differences in exploratory activity while in the play arena were observed in *Shank3* +/+, +/-, and −/− rats. *$p<0.05$ by one-way ANOVA and Tukey posthoc analysis.

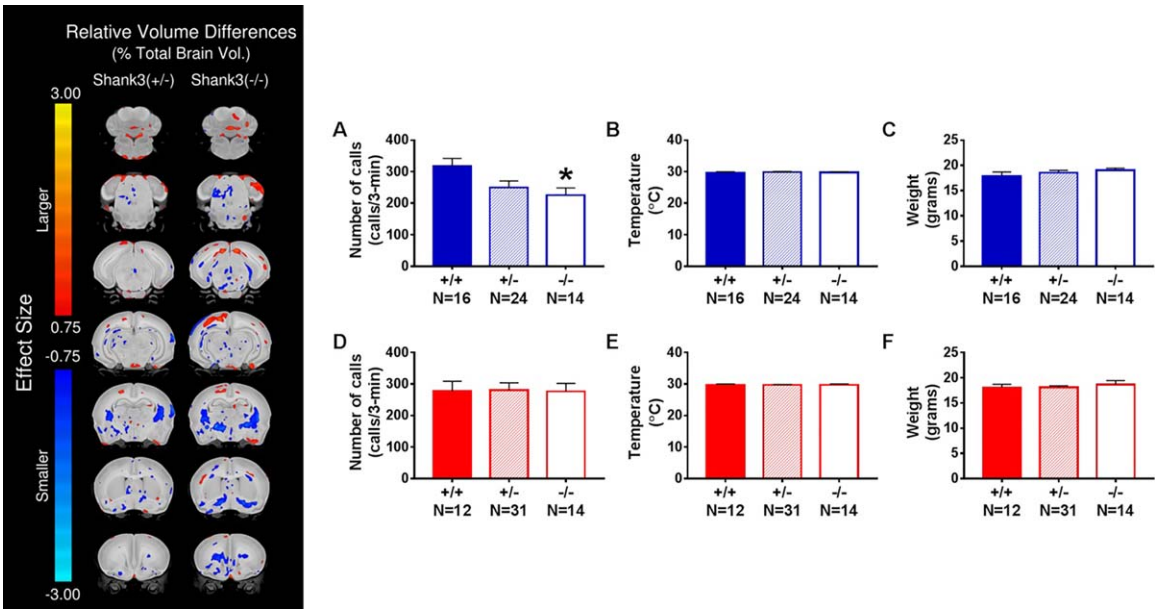

Figure 4. Reduced ultrasonic vocalizations (USV) emission in male *Shank3*-deficient −/− rat pups with neuroanatomical pathology. Number of isolation-induced ultrasonic vocalizations (USV) summed over a 3-min time period on postnatal day 7. **(A)** Fewer USV were emitted in male *Shank3*-deficient −/− than +/+ littermate controls. Neither **(B)** body temperature nor **(C)** pup weight differed between genotypes indicating typical growth and ability to thrive. No effect of genotype was observed in females by **(D)** pup USV, **(E)** core temperature, or **(F)** body weight. *Shank3* deficiency +/- or −/− did not cause major structural neuroanatomical phenotypes compared to +/+ sex- and age-matched littermate controls. The left panel illustrates representative coronal slice series, highlighting non-significant trends in absolute volume (mm^3). Less volume or smaller = dark blue-light blue. Increased volume or larger = red-yellow. Threshold for significance used an FDR of 5%.

Investigative behaviors that did not differ between genotypes included pushing past the stimulus rat to traverse the arena (bouts: $F_{(2,\ 53)}=0.1701$). Components of play behavior that did not differ between genotypes by one-way ANOVA include: bouts of rough-and-tumble play (male: $F_{(2,\ 36)}=3.21$; NS and female: $F_{(2,\ 32)}=0.90$; NS), time spent engaged in rough-and-tumble play (male: $F_{(2,\ 36)}=0.36$; NS and female: $F_{(2,\ 32)}=2.71$; NS), bouts of boxing (male: $F_{(2,\ 36)}=1.90$; NS and female: $F_{(2,\ 32)}=0.83$; NS), bouts of push under or crawl over (male: $F_{(2,\ 36)}=2.31$; NS and female: $F_{(2,\ 32)}=1.73$; NS), bouts of pinning (male: $F_{(2,\ 36)}=1.46$; NS and female: $F_{(2,\ 32)}=2.12$; NS), and bouts of pouncing (male: $F_{(2,\ 36)}=0.08$; NS and female: $F_{(2,\ 32)}=0.91$; NS). Given the lack of finding in the rough-and-tumble play parameter, it was not surprising to find its components also did not differ by genotype.

Shank3-Deficient −/− Rats Exhibit Typical Three-Chambered Social Approach

Typical sociability was evident using the three-chambered social approach assay in juvenile rats of all three genotypes and both sexes tested PND 26–29, Figure S1A-F. Detailed results are outlined in Supporting Results.

Shank3-Deficient −/− Rats Exhibit Typical Motor Activity in a Novel Open Field Arena

Figure S1G-L illustrates no hypo- or hyper-activating effects of *Shank3* genotype on open field exploratory locomotion in the rats, over a 30-min session, tested on PND 38–40, as outlined in Supporting Results.

Reduced Ultrasonic Vocalizations in Male Shank3 −/− Pups

Isolation-induced USV were collected for 3-min on PND 7 as social communication signals in rat pups [Hofer et al., 2002; Brudzynski, 2005; Wöhr & Schwarting, 2008]. Fewer USV were emitted in male *Shank3* −/− than +/+ littermate controls (Fig. 4A male: $F_{(2,\ 51)}=3.58$; $P \leq 0.05$, One-way ANOVA, Tukey's HSD $q = 3.56$ −/− versus +/+) but not females (Fig. 4D female: $F_{(2,\ 54)}=0.01$; NS). Body weight and core temperature were also collected, as these variables are known to alter pup USV emission [Oswalt & Meier, 1975; Hofer & Shair, 1991]. Neither temperature nor pup weight differed between genotypes, respectively, in both sexes (Fig. 4B male: $F_{(2,\ 51)}=0.83$; NS and Fig. 4C male $F_{(2,\ 51)}=0.082$; NS, one-way ANOVA and Fig. 4E female: $F_{(2,\ 54)}=0.36$; NS and Fig. 4F female: $F_{(2,\ 54)}=0.014$; NS, one-way ANOVA), indicating typical growth and ability to thrive.

Minimal Aberrant Reductions in Brain Volume in Shank3 +/- and −/− Pups

Figure 4, left panel, illustrates minimal reductions in brain volume attributable to *Shank3* deficiency +/− and −/− using Magnetic Resonance Imaging (MRI). Total brain volumes were assessed and no differences were found between groups, +/+ = 780 ± 50, +/− = 774 ± 37 ($P = 0.72$ vs. +/+), and −/− = 787 ± 62 ($P = 0.66$ vs. +/+). Sample sizes were *Shank3* +/+ N = 17 (12 male, 5 female), *Shank3* +/− N = 20 (11 male, 9 female) and *Shank3* −/− N = 14 (7 male, 7 female). Interestingly in the *Shank3* −/− rats, there were trends toward smaller volume in the posterior striatum and globus pallidus, the nucleus accumbens, and hypothalamus, which are all regions associated with rewarding behaviors and socially rewarding positive 50-kHz USV [Burgdorf et al., 2008; Trezza, Baarendse, & Vanderschuren, 2010; Dolen, Darvishzadeh, Huang, & Malenka, 2013; Willuhn et al., 2014]. Additional trends toward larger volumes were found in the dorsal cortex and cerebellum.

Discussion

Deletions, translocation, and breakpoint mutations in *SHANK3* are implicated in ASD and intellectual disabilities [Durand et al., 2007; Moessner et al., 2007; Gauthier et al., 2009; Betancur & Buxbaum, 2013] and account for ∼1% of individuals with ASD [Leblond et al., 2014]. *SHANK* genes encode scaffolding proteins in postsynaptic neurons that are crucial in the formation and stabilization of synapses [Qualmann et al., 2004; Grabrucker et al., 2011; Arons et al., 2012]. This study described the first measure of reciprocal social communication in a preclinical rat model of *Shank3*-deficiency and led to the discovery of a novel outcome measure, behavioral response to species-specific acoustic signals. For the first time, we captured response to playback of rat pro-social 50-kHz USV in a genetic model of a neurodevelopmental disorder, which is analogous to collection of receptive and expressive communication in a single task, and reflects the reciprocal nature of communication including both call emission and reception.

Our study identified a detrimental effect of reduced *Shank3* expression in male −/− rats on the response to social acoustic communication, which validates this bidirectional communication assay. Further, we found no sex or genotype difference in the acute response for preference for the arms associated with location of the USV speaker. Male *Shank3* −/− rats did not demonstrate the enhanced social approach behavior typically exhibited following playback of pro-social 50-kHz USV. Given intact acute responses, this selective deficit is unlikely due to impaired hearing or atypical auditory processing reported to exist in other models of *Shank3* mutations [Engineer et al., 2017]. Female *Shank3* rats of all genotypes exhibited intact behavioral responses.

High-frequency ultrasonic calls typically occurring in the range between 50 and 90 kHz, commonly referred to as 50-kHz USV, serve an important communicative function as social contact signals as shown by an elegant series of experiments using surgical devocalization. Kisko et al. [2015] described that play behavior is reduced in the absence of USV in devocalized rats [Kisko, Euston, & Pellis, 2015a]. It was also shown that 50-kHz USV occur more frequently immediately before playful contact than after contact and, relevant to our data, males were more likely to use 50-kHz USV during playful interactions [Himmler, Kisko, Euston, Kolb, & Pellis, 2014]. As shown, 50-kHz calls induce social approach behavior in the listening subject [Wöhr & Schwarting, 2007, 2009; Willadsen et al., 2014]. The biological mechanism underlying this playback behavior has been linked to the release of dopamine (DA) in the nucleus accumbens, evidence for 50-kHz USV as rewarding and an outcome of positively reinforcing events but not for the 22-kHz USV alarm calls [Willuhn et al., 2014]. Surprisingly, emission of USV in response to the playback calls did not differ by genotype and all groups (sex and genotype) emitted frustration-related 22-kHz USV. We suggest this phenomenon was due to the fact that subjects were not able to reach the sound source (i.e., juvenile rat understood to be emitting 50-kHz calls) and to establish contact with a conspecific [Wöhr & Schwarting, 2009]. These data suggest a deficit in receptive but not expressive communication in this *Shank3* rat model, probably linked to reduced social motivation.

Juvenile social play in rats is qualitatively rich with species-specific behavioral outcome measures and is composed of several moveable dependent variables. Incidence of social play can be markedly increased by situational variables, such as social isolation and sex of partner, and/or reduced by an increase in age and/or familiarity with the stimulus animal [Panksepp & Beatty, 1980; Meaney & Stewart, 1981; Panksepp, 1981; Thor & Holloway, 1984; Meaney, 1988; Argue & McCarthy, 2015a,b; Palagi et al., 2016]. Collectively, these studies indicate that social play can be efficiently studied in the laboratory rat, its levels are adjustable and quantifiable, and its functions may be involved in learning, establishing, and maintaining stable social relationships. This phenomenon is highly relevant to the clinical ASD syndrome, and thus we performed a careful, empirical analysis of juvenile social play. We discovered that male but not female *Shank3* −/− rats displayed reduced total social sniffing, nose-to-anogenital sniffing, and social grooming. The sex

difference is likely attributable to the greater frequency and vigor with which male juveniles engage in play as compared to females [Beatty, Dodge, Traylor, Donegan, & Godding, 1982; Thor & Holloway, 1984]. However, reductions on social play behavior were not global or all-encompassing. Rough-and-tumble play, boxing, pushing under or crawling over, pinning, and pouncing were not affected by genotype. No confounding genotype deficits on exploration were observed. Clinical cases of PMS and ASD are the result of *SHANK3* haploinsufficiency. We observed a gene dose effect on the important parameter of total time spent social sniffing in dyadic play but observed only trends for the *Shank3* +/− to exhibit phenotypes in acoustic playback and pup USV.

Inconsistent findings in the social behavioral domain in *Shank3* mouse models, the core pillar of an ASD diagnosis, re-emphasize the need to conduct more fine-grained analyses of social behavioral tasks, beyond the three-chambered assay, as shown here by acoustic social communication and dyadic play. This conclusion is supported by: (1) the current study illustrated *Shank3* +/− and −/− rats displayed typical sociability in the three-chambered task but impairments in acoustic playback and dyadic play when compared to *Shank3* +/+; (2) social deficits were detected in *Shank3* −/− male mice in dyad interactions, but no deficits were observed in the three-chambered assay [Dhamne et al., 2017]; and (3) an independent inter-laboratory study failed to replicate three-chambered social approach deficits in two *Shank3* mutant models [Kabitzke et al., 2017].

Shank3-deficient rats exhibited long term social memory and electrophysiological deficits but intact short term social memory [Harony-Nicolas et al., 2017], however, did not detect differences in the dyadic interactions which is likely due to procedural differences. For example, in the earlier study, rats were socially isolated for 24 hr before the juvenile social play test began while we isolated only for 30 min to facilitate and amplify total social play [Panksepp & Beatty, 1980; Panksepp, 1981] but not cause elevated stress. Subjects in our study were also older than in the original characterization (PND 32–45 versus PND 26–30). We carefully subdivided behavioral event by event, and scored for both event bouts and event duration time, as is the historical standard and observed in a rat model of Rett syndrome [Veeraragavan et al., 2016]. Both studies used sex-, age-, weight-matched, well rested wildtype stimulus rats over genotype/treatment matching controls for the influence of the stimulus rats' behavior on the social interaction has been widely described [Panksepp & Beatty, 1980; Meaney & Stewart, 1981; Panksepp, 1981; Thor & Holloway, 1984; Meaney, 1988; Argue & McCarthy, 2015a,b; Palagi et al., 2016]. Examiners conducting the Autism Diagnostic Observation Scale (ADOS) enter observation rooms or interactions with patients with a steady, standard level of play and enthusiasm. Therefore, effective translational modeling will use this analogous strategy during dyadic play and simulate standard testing conditions using wildtype partners.

Additional findings include that *Shank3* −/− rats exhibited normal growth in the first two weeks of postnatal life but emitted fewer isolation-induced 40-kHz USV than *Shank3* +/+ pups, which was supported by trends of reduced brain volume in the posterior striatum, globus pallidus, nucleus accumbens, and the white matter tracts surrounding these regions, neuroanatomical substrates of USV and social reward [Brudzynski, 2013; Dolen et al., 2013; Dolen & Malenka, 2014]. The trends of reduced brain volume were larger in the *Shank3* −/− versus *Shank3* +/−, supporting gene dose and underlying our pup USV data. *Shank3* +/− or −/− did not exhibit prominent structural neuroanatomical phenotypes compared to +/+ PND 7 brains, consistent with earlier reports of the *Shank3* mouse models [Ellegood et al., 2015].

Sequencing data shows that 12–24 millions of years of evolution separate the rat and mouse, and rats have 21 chromosome pairs while mice have 20 [Ellenbroek & Youn, 2016; Homberg et al., 2017]. The genetic analysis underscores the fact that while rats and mice look similar, there are significant genomic differences between the two rodents and rats possess important genes involved in immunity, pheromone production, and protein degradation that are not in the mouse [Gibbs et al., 2004; Tuzun, Bailey, & Eichler, 2004]. Another reason to embrace a rat model is that rats exhibit more behavior during critical developmental time periods [Thor & Holloway, 1984; Hamilton et al., 2014; Parker et al., 2014]. Rats have a much more complex behavioral repertoire and thus the discovered sophisticated social communication phenotypes presented herein could not have been detected in *Shank3* mouse models. The select behavioral deficits in social communication revealed herein corroborate and extend the previous *Shank3* mouse models research. The data lend support for the important role of *Shank3* in social communication.

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Author Contributions

Conceived and designed the experiments: MW and JLS; Performed the experiments: ELB, JKR, MCP, MC, JE, MW; Analyzed and interpreted the data: ELB, NAC, MCP, MDB, JE, MW, JLS; Contributed reagents/materials/analysis tools and resources: HHN, JDB, JPL, PJL, RFB; Drafted the article: JLS, MW, JE; Revised the article: All authors.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Summary of preclinical reports of social communication outcome measures in preclinical models of *Shank3* mutations.

Figure S1. Normal three-chambered social approach and locomotion in an open field arena. Sociability scores from the automated three-chambered social approach task. Time spent in the chamber in *Shank3* +/+, +/- and −/− showed typical, significant sociability in **(A)** males and **(D)** females. Time spent sniffing the novel rat versus the novel object in *Shank3* +/+, +/- and −/− showed typical, significant social sniffing in **(B)** males and **(E)** females. No significant difference was

identified between *Shank3* +/+, +/-, and −/− on the number of transitions between chambers for both **(C)** males and **(F)** females, confirming no locomotor confound during this behavioral readout. Normal exploratory locomotion in **(G-I)** male and **(J-L)** female *Shank3* +/+, +/- and −/− rats. Motor abilities were measured by total activity, vertical activity, and time in the center of the arena over the course of a 30-min trial. Data are shown in 5-min time bins. *$p<0.05$ by within genotype paired student t-tests for social approach. *$p<0.05$ by repeated-measures ANOVA for open field.