Behavioral and Brain Functions

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Assessing sociability using the Three-Chamber Social Interaction Test and the Reciprocal Interaction Test in a genetic mouse model of ASD

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Abstract

Autism Spectrum Disorder (ASD) is a group of neurodevelopmental disorders with heterogeneous symptomatology. Arguably, the most pervasive shortfall of ASD are the deficits in sociability and the animal models of the disorder are expected to exhibit such impairments. The most widely utilized behavioral task for assessing sociability in rodents is the Three-Chamber Social Interaction Test (SIT). However, SIT has been yielding inconsistent results in social interaction behavior across different rodent models of ASD, which could be pointing to the suboptimal methodology of the task. Here, we compared social behavior assessed in SIT and in another prominent sociability behavioral assay, Reciprocal Interaction Test (RCI), in a *SH3 and multiple ankyrin repeated domains 3* (SHANK3) mouse model of ASD. Head-to-head comparison showed no association ($p = 0.15, 0.25, 0.43$) and a fixed bias ($p = 0.01, < 0.001, < 0.001$) in sociability assessment between the behavioral assays in both wild-type (WT) controls and *Shank3B*$^{(-/-)}$ mice. Adult *Shank3B*$^{(-/-)}$ mice of both sexes displayed normative sociability in SIT when compared to the WT controls ($p = 0.74$) but exhibited less than half of social interaction ($p < 0.001$) and almost three times more social disinterest ($p < 0.001$) when compared to WT mice in RCI. At least in the *Shank3B*$^{(-/-)}$ mouse model of ASD, we presume RCI could be a preferable way of assessing social interaction compared to SIT. Considering the variability of animal models of ASD and the wide palette of tools available for the assessment of their behavior, a consensus approach would be needed for observational and interventional analyses.

Keywords Autism, Behavioral phenotyping, Phelan-McDermid syndrome

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder defined by deficits in social interaction, communication and stereotypical, repetitive behaviors [1]. Considerable portion of research is focused on developing an animal model which consistently exhibits behavioral phenotype relevant to ASD to study its causes and manifestations. Since a large part of the etiopathogenesis of the disorder is explained by genetic factors (~70%), transgenic mouse models that carry mutation in high-risk genes implicated in ASD are often used. Mouse model of *Shank3* deficiency (*Shank3*$^{(-/-)}$) is one of the most widely

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used genetic animal models in autism research. In human patients, difficulties with sociability are considered the most pervasive and disabling symptom of ASD and thus, the *Shank3*$^{(-/-)}$ mice were produced with defects in social behavior [2].

A critical element to the development and standardization of a proper animal model of ASD is using an optimal methodology for assessing relevant behavioral phenotype. The Three-Chamber Social Interaction (SIT) test is the most widely used rodent behavioral assay for the assessment of social behavior in the field of biomedicine and neuroscience [3]. Traditionally, SIT is conducted in a three-chambered apparatus with a wired cup placed in each of the side chambers, one containing a social-partner mouse, the other an inanimate object. Following an initial habituation period, the experimental animal is left to freely explore the apparatus, while its interaction with both social partner and object is quantified. Despite a rather unified methodology, modifications to the task have been made over the years and social interaction as measured by SIT was shown to produce inconsistent results across different mouse models of ASD. This shortcoming was recognized by various research teams [3, 4], who in turn proposed standardized protocols and automated approaches to the task, in hope to increase the reproducibility of results.

Another widely used behavioral assay for sociability assessment in rodents is the Reciprocal interaction test (RCI). Generally, following the initial social isolation and habituation, the experimental mouse is placed in a standard Open field arena together with a sex- and age-matched social partner mouse. Social behavior, such as nose-to-nose, nose-to-anogenital, or side sniffing, and non-social behavior, such as evading, escaping or freezing in contact are quantified [5].

Previously, in a *Shank3*$^{(-/-)}$ mouse model of ASD, social deficits were confirmed using RCI, while normative sociability was reported using SIT, indicating possible methodological disparities [6]. A method with sufficient sensitivity and specificity is crucial when evaluating social behavior in an animal model designed to simulate ASD symptomatology. Therefore, the present study aimed to investigate the methodological properties of SIT, evaluating its ability to assess social interaction in the *Shank3B*$^{(-/-)}$ mouse model of autism, comparing it to RCI, a different widely used behavioral method for social interaction assessment in rodents and thus, validating SIT for continuous use in animal research of ASD.

Material and methods

Animals

Breeding pairs of adult heterozygous *Shank3B* mice with pure C57BL/6 background obtained from Jackson Laboratories (JAX Stock No. #017688) were used to produce *Shank3B* WT and knock-out (KO, *Shank3B*$^{(-/-)}$) mice. Genotyping was conducted to determine the genotype of produced animals at the time of weaning. Adult (3-month old) female (WT = 20, KO = 20) and male (WT = 20, KO = 23) *Shank3B* mice were used in the study. Animals were housed in groups of 4–6 per cage and kept in a controlled environment of 24 ± 2 °C and 55 ± 10% humidity with ad libitum access to food and water on a 12-h light/dark cycle (lights on at 0700 – 1900). No animals were excluded from the study. The experiment was conducted with cages randomly selected from the in-house colony, matched for age and genotype, and the experimenters were blinded to the origin of the animals. The experiment was performed in accordance with the *Animal Research: Reporting of* In Vivo *Experiments 2.0 (ARRIVE 2.0)* guideline [7].

Behavioral testing

Each behavioral testing was conducted in the same dimly lit room with a room temperature of 24 ± 1 °C over the period of 3 consecutive days, during the light period of the day (1000 – 1500 h). Animals were weighed (Fig. S1) and habituated to the testing room 30 min prior to the assessment. Mouse handling and experiments were carried out consistently by the same experimenter throughout the study. Each testing apparatus was illuminated with 100 ± 3 lx. Behavioral assays were evaluated using EthoVision® XT (Noldus, Wageningen, Netherlands) software.

Three-chamber social interaction test

Plastic apparatus (60 cm × 40 cm × 20 cm) with an open top divided into 3 chambers (20 cm × 40 cm × 20 cm) by 2 transparent walls was used to assess social interaction. Each chamber was accessible by a retractable doorway upon opening. Experimental mouse was habituated to the apparatus for 5 min. Social-partner mouse was placed in a cylindrical wired cup (10 cm diameter) in one of the side chambers, alternating per trial to prevent side bias. An identical empty wired cup was placed in an opposite chamber. After the habituation period, the experimental mouse was left to freely explore the apparatus for 10 min. The cumulative time spent in each chamber, as well as the cumulative time spent interacting with an empty wired cup (object) or the wired cup containing the social-partner mouse was measured. Interaction with either was defined as when the experimental mouse was in close proximity (~ 1 cm) nose-oriented towards the cups.

Reciprocal interaction test

Animals were socially isolated for 24 h prior to the testing in a dedicated cage (1 per cage) and then randomly

paired with a socially novel WT animal of the same sex and age used as a social partner. Both animals were placed in the PhenoTyper 4500 cage (Noldus Information Technology, Wageningen, Netherlands) filled with 1 cm sawdust bedding and left to freely interact for 10 min while being recorded. Recording was manually scored by an observer for cumulative time spent nose-to-nose, nose-anogenital and side-sniffing as a measure of social interaction, and self-grooming, digging, lying flat, freezing in contact, or avoiding the social partner as a measure of social disinterest.

Statistical analysis

Power analysis was conducted using G*Power. Statistical analyses were carried out in IBM SPSS Statistics 23.0 (IBM, Armonk, NY, USA) and visualized using GraphPad Prism 8.4.0 (GraphPad Software, San Diego, CA, USA). As no differences between females and males were observed across all constructs (Fig. S2), the sample was sex-pooled for the analyses. Levene's test for Equality of Variances was used to assess homoscedasticity of data and in case of violation of this assumption, statistics designed to correct for unequal variances were employed. To compare the groups based on genotype in each task, Student's T-test was utilized based on the Shapiro–Wilk test of normality of distribution. To synchronize the outcomes of both behavioral assays, discrimination ratio (*DR*) was computed for each assessed construct. The *DR* reflects the duration of social interaction (T_{soc}) compared to the duration of non-social interaction (T_{nonsoc}) as a proportion of total time spent in interaction (T_{total}), as follows $DR = \frac{Tsoc-Tnonsoc}{Ttotal}$. To assess the level of agreement between the assays, the difference between the respective *DR*s (bias) was compared using One-Sample T-test with a test value of 0 and visualized using Bland–Altman plot. Data are presented as mean ± SEM. *P*-values of less than 0.05 were considered significant.

Results

Sociability in SIT

No differences in sociability were observed based on genotype in SIT [$t(81) = 0.32$, $p = 0.75$]. *Shank3B*$^{(-/-)}$ mice did not prefer interacting with a social partner over an object (17.6 ± 7.6 s) any less than the WT controls did (21 ± 7.2 s, Fig. 1A). Consistently, no differences were observed in preference for spending time in the social chamber based on genotype [$t(81) = -0.55$, $p = 0.585$]. *Shank3B*$^{(-/-)}$ mice chose to spend similar time in the social chamber versus the object chamber (74.5 ± 8 s) compared to the WT controls (67.6 ± 9.9 s, Fig. 1B). Evaluating the effect of genotype using DR in SIT did not show differences either [$t(81) = 1.66$, $p = 0.1$; WT = 13.3 ± 0.2%; *Shank3B*$^{(-/-)}$ = 7.1 ± 2.8%, Fig. 3A].

Sociability in RCI

Comparing the genotypes in social interaction behavior in RCI showed differences between the groups [$t(47.5) = 10.97$, $p < 0.001$]. *Shank3B*$^{(-/-)}$ mice spend less than half the time interacting with the social partner mouse (94.9 ± 3.1 s) than the WT controls (204.3 ± 9.4 s, Fig. 2A). Complementary to this finding, genotypes differed in social disinterest behavior as well [$t(51.06) = -11.33$, $p < 0.001$]. In this case, *Shank3B*$^{(-/-)}$ mice spend almost three fold longer time in social disinterest (130.8 ± 6.9 s) than the WTs (49 ± 2.2 s, Fig. 2B). The effect of genotype was further observed in DR of RCI as well [$t(71.7) = 20.58$, $p < 0.001$; WT = 60.2 ± 1.9%; *Shank3B*$^{(-/-)}$ = − 13.2 ± 3%, Fig. 3B].

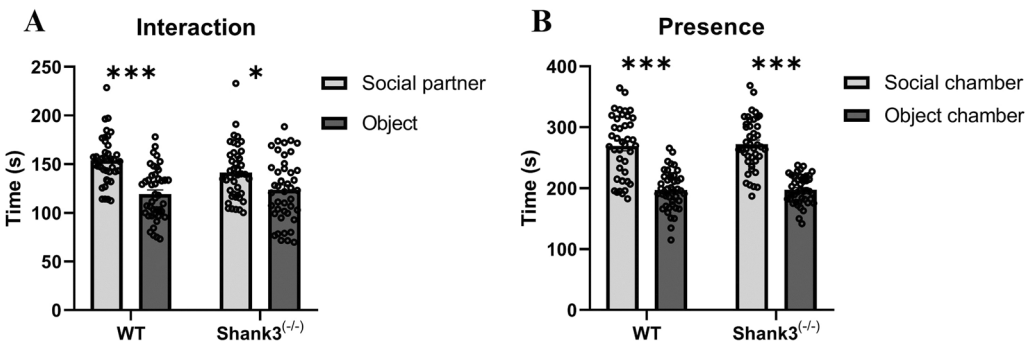

Fig. 1 **A** Cumulative time spent in interaction with a social partner animal and an inanimate object in the Three-Chamber Social Interaction Test. **B** Cumulative time spent in a chamber with a social partner animal and an inanimate object in the Three-Chamber Social Interaction Test. Asterisks indicate a within-subject comparison. No differences were recorded in group comparison. WT Wild-type animal; Shank3$^{(-/-)}$ Shank3-deficient animal; *$p < 0.05$; *** $p < 0.001$

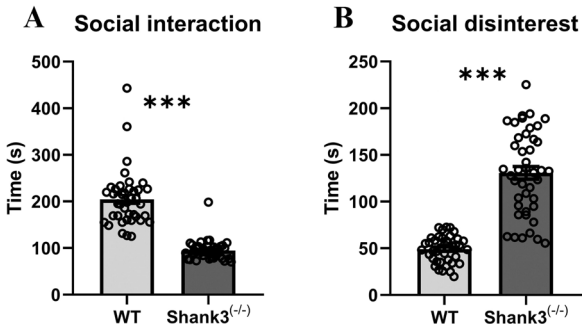

Fig. 2 **A** Cumulative time spent in interaction with a social partner animal in the Reciprocal Interaction Test. **B** Cumulative time spent without interest in social contact with a social partner animal in the Reciprocal Interaction Test. WT Wild-type animal; Shank3$^{(-/-)}$ Shank3-deficient animal; ***$p < 0.001$

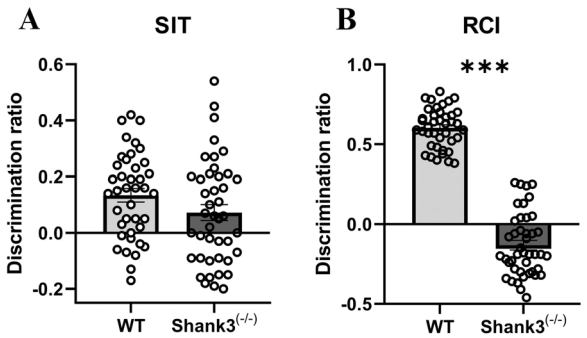

Fig. 3 **A** Discrimination ratio as computed in the Three-Chamber Social Interaction Test (SIT). **B** Discrimination ratio as computed in the Reciprocal Interaction Test (RCI). WT Wild-type animal; Shank3$^{(-/-)}$ Shank3-deficient animal; ***$p < 0.001$

Sociability comparison between SIT and RCI

No significant correlation was observed between DR of SIT and RCI assays in neither WT mice (r = − 0.18, $p = 0.25$, $R^2 = 0.03$, Fig. 4A), *Shank3B*$^{(-/-)}$ mice (r = − 0.23, $p = 0.15$, $R^2 = 0.05$, Fig. 4B) or when both genotypes were pooled (r = 0.09, $p = 0.43$, $R^2 < 0.001$, Fig. S3A). Subsequent Bland–Altman analysis in WT group revealed average difference for DR of both assays equal to -0.47 (-0.9 to -0.04 CI 95%, Fig. 5A), indicating a fixed bias [$t(39) = -13.7$, $p < 0.001$]. Bland–Altman analysis in *Shank3B*$^{(-/-)}$ mice showed average difference in DR of both assays to be 0.2 (-0.37 to 0.78 CI 95%, Fig. 5B), also indicating a fixed bias [$t(42) = 4.56$, $p < 0.001$]. Finally, with both genotype groups pooled, average difference in DR of both assays was -0.12 (-0.95 to 0.71 CI 95%, Fig. S3B), showing a fixed bias [$t(82) = -2.59$, $p = 0.01$].

Discussion

Deficient sociability is one of the most pervasive and disabling symptoms of ASD. Animal models are required to exhibit reduced sociability to allow proper research into the disorder. An important part of such effort is using a sensitive methodology. To our knowledge, present work is the first article which provides the evaluation of the methodological properties of SIT in the *Shank3B*$^{(-/-)}$ mouse model of ASD and compares it to another widely used rodent behavioral assay for measuring sociability, RCI. Social interaction of *Shank3B*$^{(-/-)}$ mice was normative as measured by SIT but testing in RCI revealed 50% less social interactions and almost three times more social disinterest than the WT mice. Considering the highly heterogeneous autistic phenotype and the wide variety of tools designed for its assessment in animal models, our results highlight the necessity for a unified approach in sociability testing.

Historically, testing social interaction in SIT produced opposite outcomes across studies in mouse [8–11] and rat models [12–14] of *Shank3* deficiency. Despite the fact that the *Shank3B*$^{(-/-)}$ mouse model was produced

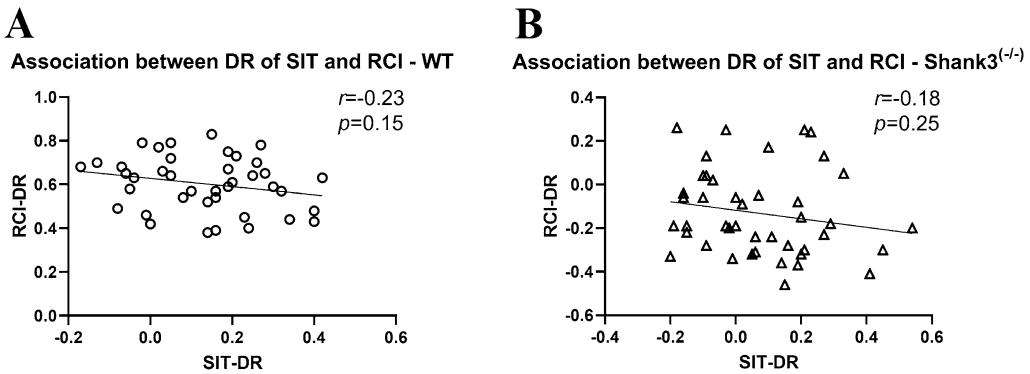

Fig. 4 **A** Correlation between Discrimination ratio (DR) as computed in the Three-Chamber Social Interaction test (SIT) and in the Reciprocal interaction test (RCI) in Wild-type animals (WT). **B** Correlation between DR as computed in the SIT and in the RCI in Shank3-deficient animals (Shank3$^{(-/-)}$). *r* Pearson correlation coefficient; *p* probability

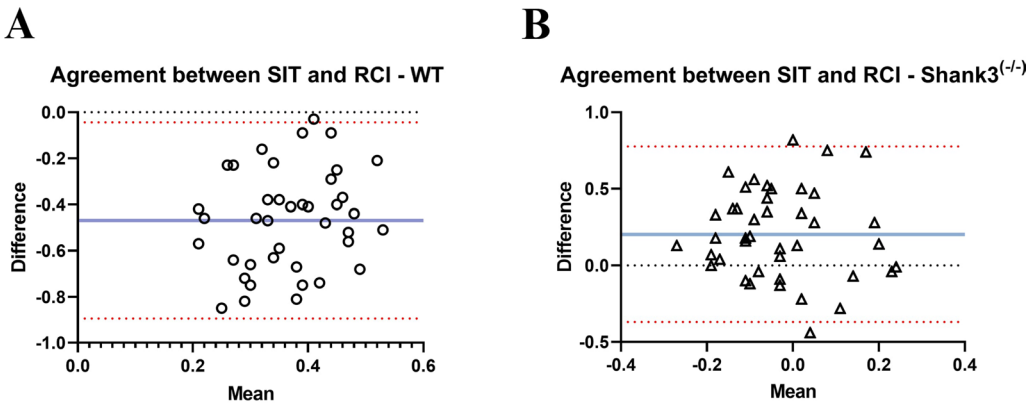

Fig. 5 **A** Bland–Altman plot computed to assess the agreement between the Discrimination ratio of the Three-chamber Social Interaction Test (SIT) and Reciprocal Interaction Test (RCI) in Wild-type animals (WT). **B** Bland–Altman plot computed to assess the agreement between the Discrimination ratio of the SIT and RCI in Shank3-deficient animals (Shank3$^{(-/-)}$). **- - -** Difference of 0 (black). **----** Mean difference (blue). - - - 95% Confidence intervals (red)

with deficits in sociability [2], we observed normative social interaction in the mutant strain when tested in SIT. Meanwhile, the same experimental mice were tested in RCI, and an extensively reduced sociability of *Shank3B*$^{(-/-)}$ mice was observed. Consistent with our results, two large phenotyping studies reported no abnormalities of *Shank3B*$^{(-/-)}$ mice in social interaction when using SIT, but pervasive deficits when conducting RCI with the same animals [15, 16]. Most of the behavioral assays designed to assess social interaction in rodents utilize unrestricted contact of the experimental animal and the social partner animal(s), similar to RCI [5]. While the experimental mouse can freely choose when and how to approach the social partner mouse in SIT, the social partner mouse is prevented from doing so by being restricted in the wired cup. In rodents, naturalistic direct social interaction is the main component of social behavior and includes the reciprocity of the contact [17]. Limiting the access of the social partner mouse to the experimental mouse fails to simulate a proper social situation for the animals, thus possibly putting the validity of the assessed social interaction to question. Despite the continuous efforts for the standardization of an accurate SIT testing protocol, the inconsistencies in outcomes produced by research teams across the field could be a result of its methodological design.

However, these properties might be specific to the *Shank3B*$^{(-/-)}$ mouse model of ASD. Previous studies with inbred BALB/c, BTBR, C58/J ASD mouse models [18, 19] and ASD-relevant genetic mouse models, such as *En2*$^{(-/-)}$, *Pten*$^{Y68H/+}$, *Tsc1*$^{(-/-)}$ or *Cntnap2*$^{(-/-)}$ mutants [20–23] reported deficient social interaction in both SIT and RCI. Head-to-head comparison with consistent methodology could provide additional evidence before conclusion in other models is confirmed. Lastly, our *Shank3*$^{(-/-)}$ mice were sex-pooled, since we observed no sexually dimorphic behavioral patterns in sociability, consistent with previously reported outcomes [15, 24]. Thus, it is unlikely that sex played any major role in reported differences of sociability measured by SIT and RCI.

In conclusion, widely utilized SIT behavioral assay produces opposite results in social interaction of *Shank3B*$^{(-/-)}$ mice. Comparing the ability to test social interaction in SIT to another widely used sociability behavioral task, RCI, revealed methodological shortcomings of SIT. The construct validity of SIT could be in question, at least in the *Shank3B*$^{(-/-)}$ mouse model of ASD, as it seems to be lacking an essential part of rodent sociability—reciprocity. When a wide selection of available behavioral methods for testing sociability in rodents is considered, a unified approach seems to be required to produce meaningful outcomes.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12993-024-00251-0.

Supplementary Material 1.

Author contributions

J.S., E.R. and V.B. conducted the experiment. J.S. prepared the draft of the manuscript. D.O. and P.C. provided the rationale for the experiment and corrected the draft. All authors reviewed the manuscript.

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Availability of data and materials
Used dataset is available on a reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate
All experimental procedures were approved by the Ethics Committee of the Institute of Molecular Biomedicine, Comenius University, Bratislava, and have been conducted in accordance with the EU Directive 2010/63/EU and Slovak legislation (approval number 3629/17-221).

Competing interests
The authors declare no competing interests.

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