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Research report

# Systematic autistic-like behavioral phenotyping of 4 mouse strains using a novel wheel-running assay

Golan Karvat, Tali Kimchi*

*Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel*

## HIGHLIGHTS

- Autism-like behavioral phenotype can be measured by the jammed/running wheel paradigm.
- ICR and C57 mice were found behaviorally typical.
- BTBR mice exhibited autistic-like phenotype.
- No autistic-like behavioral phenotype was found in the NLGN3-KI mice.

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## ABSTRACT

Three core symptoms of autistic spectrum disorders are stereotypic movements, resistance to change in routines and deficits in social interaction. In order to understand their neuronal mechanisms, there is a dire need for behavioral paradigms to assess those symptoms in rodents. Here we present a novel method which is based on positive reward in a customized wheel-running apparatus to assess these symptoms. As a proof of concept, 4 mouse strains were tested in the new behavioral paradigm; 2 control lines (C57BL/6 and ICR) and 2 mouse-models of autism (BTBR T+ tf/J and *Nlgn3*$^{tm1Sud}$). We found that the C57BL/6, ICR and *Nlgn3*$^{tm1Sud}$ mice showed a significant reduction in stereotypical behavior in the presence of the running wheel, ability to forfeit the running habit when the running-wheel was jammed, and preference of interacting with a social stimulus over the jammed running-wheel. No difference was found between genotypes of the *Nlgn3*$^{tm1Sud}$ mice. On the other hand, the BTBR mice exhibited persistent, elevated levels of stereotypical behavior. In addition, they presented a deficit in their ability to adjust to a changing environment, as manifested in persistence to interact with the wheel even when it was jammed. Lastly, the BTBR mice exhibited no significant preference to interact with the stranger mouse over the jammed running-wheel. These results were validated by a set of commonly used behavioral tests. Overall, our novel behavioral paradigm detects multiple components of autistic-like phenotypes, including cognitive rigidity, stereotypic behavior and social deficiency.



## 1. Introduction

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by persistent deficits in social communication and social interaction across contexts and restricted, repetitive patterns of behavior, interests or activities [1,2]. The repetitive symptom can be divided into 'lower-order', including stereotypic movements and self-injury, and 'higher-order' responses reflecting general cognitive rigidity, such as restricted, obsessive interests and habits alongside strong resistance to environmental change [3–5]. Autism is defined by both aspects of stereotypical behavior [1].

Twin studies have demonstrated a clear genetic linkage for the disorders [6,7] and large-scale genetic screenings have highlighted more than 130 genes as associated with autism [8]. Unfortunately, none of these genes has been reported to account for more than 3% of ASD cases [9]. Therefore, disease etiology is thought to involve an interaction between genetic susceptibility and environmental factors [9,10]. Nevertheless, various promising mouse lines genetically manipulated in genes associated with the disorder have been generated [11,12].

Any mouse model of a mental disorder must demonstrate behavioral similarities to the human symptoms [13]. A great improvement has been achieved in recent years in standardizing the "phenotyping tool-box" for mouse models of autism [14–16]. However, while standardized tests exist for communication and

* Corresponding author. Tel.: +972 8 9346216; fax: +972 8 9346357.
*E-mail addresses:* Golan.karvat@weizmann.ac.il (G. Karvat), tali.kimchi@weizmann.ac.il (T. Kimchi).

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social approach (reviewed in Refs. [11,12]), there is a need to broaden the repository of tests for assessment of the stereotypical (repetitive) behavior, insistence on sameness and social interaction symptoms.

In mice, lower-order stereotypies are mostly measured by the marble burying test [17] and video-taped observations of the animals' behaviors in their home-cages, such as continuous self-grooming, jumping, obsessive digging and hatching on the cage's grid [18].

The approach to assess higher-order rigidity is based on the rationale that mice could be trained to accomplish a task, and then forced to make a change in it. To this end, modifications of the stressful tests aimed to measure spatial learning and memory such as the T- and Y-mazes [16] and Morris water maze [19] are used. However, it must be taken into account that success in the task is affected also by vision [20] and reaction to stress [21].

Social preference is most widely tested using the 3-chamber test [22], in which the subject mouse is to choose between two rewarding stimuli: an empty wire-cage or a cage containing an unfamiliar mouse. Social interaction is also studied by observation of the play in cohorts of juvenile mice, and of the behavior of the adult following introduction of a freely behaving mouse to its home-cage, with no additional stimuli [23,24].

Phenotyping of several inbred strains of mice using the above experimental paradigms revealed that BTBR T+ tf/J (BTBR) mice exhibit robust autistic-like behavior manifested in all three core symptoms of the syndrome: they play less as juvenile, lack social preference as adult, emit fewer ultra-sonic vocalizations and display high levels of repetitive behavior such as self-grooming and marble-burying. Importantly, the strain's general health is normal and these autistic-like characteristics were reproduced in different laboratories [12,16,23,25–29].

Dozens of genetic modified mouse models for autism have been generated and behaviorally tested in the recent years. Among them is the B6;129-*Nlgn3*$^{tm1Sud}$/J (NL3-KI) mouse model which carries an R451C mutation in the Nlgn3 (neuroligin 3) gene, that was found in a family affected with ASD [30]. The gene is located on chromosome X and encodes a cell adhesion protein present at the postsynaptic side of the synapse. It connects to neurexin in the presynaptic side to form a gap junction [31,32], and thus may play a crucial role in the formation of functional synapses [33]. Interestingly, discrepancy exists as for this model behavioral resemblance of the autistic phenotype between different laboratories [34–36]. Expanding the scope of relevant behavioral tests may help settling this disagreement.

In the current study we set to broaden the tool-box of the behavioral tests relevant to mouse models of autism by establishing a novel, easy to conduct approach specifically designed to assess the stereotypical behaviors, rigidity to change habits and social interactions of mice utilizing positive reinforcement and by exposing the subject animals to a relatively non stressful environment. Specifically, in order to make the mice gain a habit we took advantage of their innate wheel-running tendency [37], a highly rewarding activity [38–40]. Although the initiating mechanisms of the wheel-running behavior are not fully understood, mice learn within minutes to run on wheels, and reach a stable, 'automatic' behavior in response to the wheel within few days (reviewed in Ref. [37]). Habits were defined as "learned sequences of acts that have become automatic responses to specific cues, and are functional in obtaining certain goals or endstates" ([41], p. 104). Since wheel-running is an automatic response to the presence of the wheel, and the reward can serve as an endstate, it can be referred to as a habit.

Analysis of the latency to start running may serve as a measure for positively motivated learning and habit gaining. A change in the habit may be achieved by jamming the running-wheel. Measurement of the time dedicated to behaviors such as digging when the wheel is running or jammed may be an intrinsic measure of stereotypical behaviors. Finally, social interaction can be assessed by introducing a stranger mouse into the running-wheel chamber and measuring interaction with the jammed wheel (object) versus the mouse.

The present study comes to validate the utility of the wheel-running test to evaluate autistic-like phenotype by addressing it to 4 mouse strains. The strains chosen for this validation were inbred (C57BL/6) and outbred (ICR) wild-type mice, commonly used as controls in neurobehavioral studies, as well as 2 mouse strains that were reported to exhibit traits of the autistic phenotype (BTBR and NL3-KI mouse-lines), as described above. All mouse strains were tested in our novel running wheel assay and the results were compared to a set of commonly used autism-related assays.

This study demonstrates that the running-wheel test can be used as a robust and easy to use tool to assess the 3 core symptoms of autism in mouse models. In addition, it supports and adds to the previous reports [26,42–44] of the usefulness of the BTBR strain as a mouse model for autism.

## 2. Materials and methods

### 2.1. Animals

All subjects in this study were male, in accordance with the strong male:female gender bias (at least 4:1) in ASD [2]. Six C57BL/6JOlaHsd (C57) inbred and 6 Hsd:ICR[CD-1] (ICR) outbred mice (Harlan Laboratories, Rehovot, Israel) served as control groups in the preliminary experimentation of the running-wheel assay. Later on, additional groups of 6 C57 and 6 ICR mice were examined for reproducibility of the test, alongside a group of 8 BTBR, 7 B6;129-*Nlgn3*$^{tm1Sud}$/J knock-in (NL3-KI) and 7 of their wild type littermates (NL3-WT) (The Jackson Laboratory, Bar Harbor, ME). These mice were used for the running wheel assay and the set of validation tests. Since the BTBR mice dag extensively in the bedding and did not gain the running habit (see Section 3.1.2), an additional group of 8 BTBR mice was tested in the same apparatus but without any bedding.

Animals were housed separately by strain, with three to five mice per cage. At sexual maturity (7 weeks of age) mice were housed separately and maintained on a reverse 12/12 h light/dark cycle. Behavioral assays began when mice were at 8 weeks of age, and lasted 2 weeks. All animals were sexually naïve. All experimental procedures were approved by and conducted in strict compliance with the policies on animal welfare of the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

### 2.2. Test procedures

The animals were subjected to the following battery of behavioral assays: (1) jammed running wheel series of assays (three consecutive stages; see below); (2) 3-chamber sociability assay; (3) general locomotion; (4) elevated plus-maze, and (5) "Wet" T-maze. All tests took place in the same room dedicated for behavior only, during the dark active period of the animals. For all but the "wet" T-maze the room was dimly lit with red light. "Wet" T-maze was conducted under white fluorescent lighting. The experiments were recorded by low-light sensitive video cameras connected to a digital video recording (DVR) unit under infra-red illumination. At the beginning of each experiment day, animals were moved to the experiment room and were allowed 30 min to acclimate. All mice appeared to be healthy at the conclusion of the testing sequence.

### 2.3. The jammed running wheel test

#### 2.3.1. Apparatus

The running-wheel apparatus was a transparent Plexiglas cage, sized 30 cm × 30 cm × 25 cm, covered with ~0.5 cm standard soil bedding and included ad libitum water and rodent food pellets. For the BTBR mice, two groups were tested-one with bedding and the other without bedding (see Section 3.1.2) (Fig. 1A and B). A standard plastic mouse running wheel, 14 cm in diameter, was attached to one of the walls and could either turn or be jammed by a metal pin (Fig. 1C).

#### 2.3.2. Gaining and maintaining the running on a wheel routine

At the first 4 days of the experiment, the mice were put in the chamber and allowed to run freely on the wheel, for 30 min. Routine (habit) gaining was assessed by measuring the latency to start running in each day.

#### 2.3.3. Stereotypical behavior assessment

Previous studies showed that a freely running, but not a jammed wheel, leads to reduction in stereotypical behaviors [45–48]. Therefore, time spent by the subject being involved in a stereotypical behavior (digging in the bedding) while the wheel

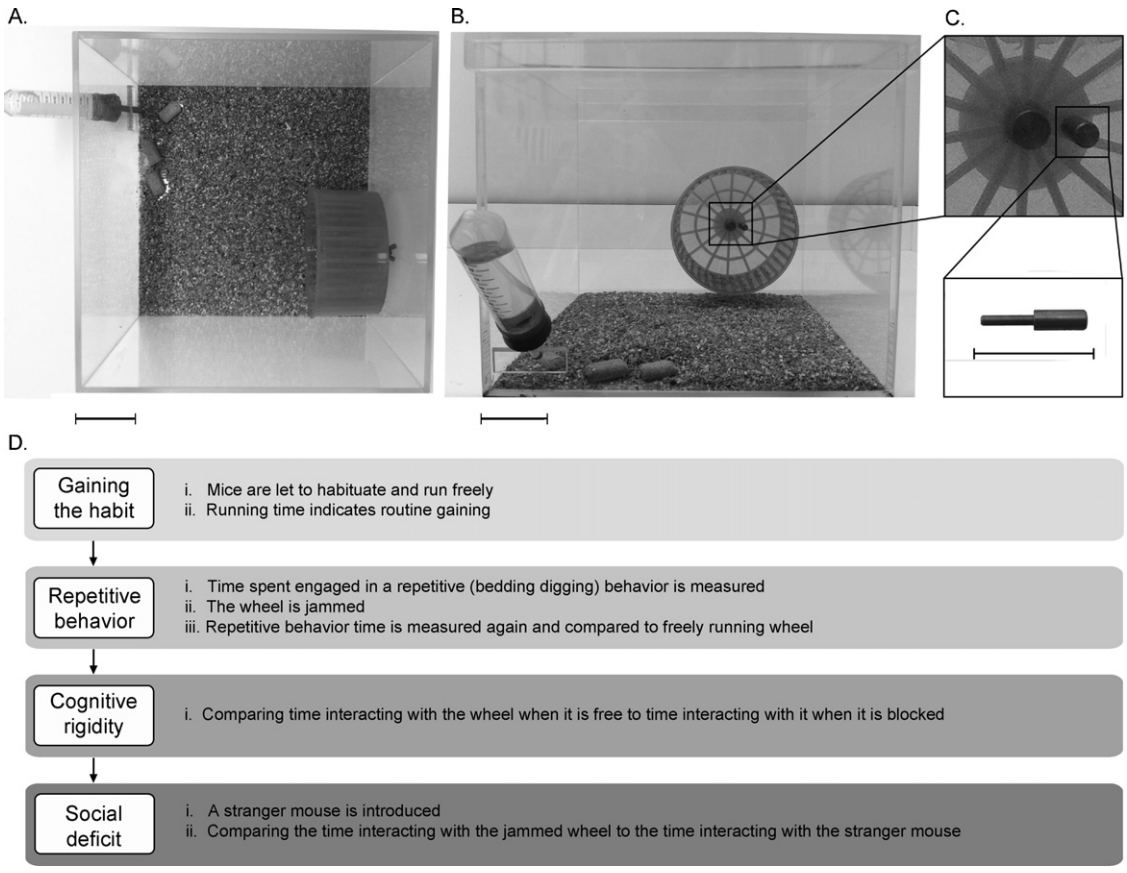


**Fig. 1.** The jammed running wheel assay. Experimental apparatus: (A) top and (B) side view of the apparatus. (C) Magnification of the jamming pin that prevented movement of the wheel when inserted (scale bars = 5 cm). Methodology: (D) scheme of the experimental procedure.

was freely running (on day 4) was compared to the time spent when the wheel was jammed by the metal pin (on day 5). The working hypothesis stated that time dedicated by a neurotypical animal to stereotypical behavior should be lower when the wheel in free.

#### 2.3.4. Cognitive rigidity measurement

Following acquisition of the wheel running routine, it was jammed for two consecutive days, in which the mice spent 15 min in the chamber. The ability to forfeit a habit was measured by comparing time spent in interaction with the wheel in the last running day (day 4) and the two jammed days (day 5 and day 6). The working hypothesis stated that a neurotypical mouse should decrease interaction time between the days.

#### 2.3.5. Social interaction evaluation

In the last day of the running wheel test (day 7) a freely behaving novel stranger mouse (5 weeks old male, ICR mouse strain) was introduced to the running-wheel apparatus. Since running on a wheel is a highly motivating activity (reviewed in Ref. [37]), the wheel was still jammed in order to avoid motivational conflict with social seek. A comparison between duration of time spent interacting with the mouse and with the jammed running-wheel in a 10 min trial served to measure social interaction. A mouse with a robust autistic-like social deficit was hypothesized to spend more time interacting with the wheel (object) versus the mouse.

#### 2.3.6. Scoring

Interaction time with the wheel in days 1 through 4 was scored when the mouse turned the wheel. In days 5 through 7, interaction was scored as any movement of the mouse that would have made the wheel move, if it was free. Time the mouse spent on top of the wheel was excluded. Preliminary studies showed that analysis of the initial 10 min of each trial were sufficient to detect the main effects (data not shown), thus this period of time was analyzed. Social interaction was considered as any physical engagement initiated by the subject. Digging was scored when pieces of the bedding were intentionally moved by the mouse. Behaviors were recorded using the Observer® software (Noldus, Wageningen, the Netherlands) by an experienced evaluator, who was blind to genotype for the NL3 mice. Blind measurement was not possible for the other strains due to different coating colors.

### 2.4. General locomotion

The apparatus was the cage of the running-wheel excluding the wheel. Mice were put in the cage for 5 min. Total distance traveled was measured by the Ethovision software (Noldus).

### 2.5. "Wet" T-maze

This test served to validate the cognitive rigidity, according to Guariglia et al. [49]. The mice learned to find a hidden platform in a T-shaped water pool. After learning and habituating that the platform is located in one arm of the maze, the platform was hidden in the opposite arm. The size and the shape of the pool allowed a relatively fast acquisition in the learning stages. A cognitively rigid mouse was expected to have difficulties in reversal learning with no deficiency in learning the task.

The water maze consisted of a rectangular Plexiglas cage, sized 40 cm × 40 cm × 20 cm, divided into 4 chambers and located in a room with numerous high contrast visual cues. One chamber was closed to form a "T" shape. The cage was filled with water 15 cm in depth, kept on 25 ± 1 °C. An escape platform (diameter = 8 cm) was submerged 0.5 cm below water level. Subjects had 5 trials during each of the 4 experiment days. The animals were put in the starting chamber facing the wall, and were allowed to swim for 90 s until finding the platform. If the mouse found and mounted the platform, it was allowed to stay on it for 15 s. If not, it was guided to the platform by the experimenter and allowed to stay there for 15 s. After each trial, mice were gently wiped and put into a previously heated standard mouse cage. The mice stayed in the warm cage until their fur dried, and then placed back in their home-cage until the next trial. Inter-trial interval was longer than 5 min. On the first and second days, the platform was located in one arm, while on the third and fourth days it was located in the opposite arm.

Latency to mount the platform was measured using stop-watch from the moment the mouse was released in the water until it stood safely on the platform. If a mouse did not find the platform within 90 s, a value of 90 s was recorded, and the mouse was guided to the platform. In addition, the first arm the mouse entered was recorded as correct/wrong turn, depending on the current location of the platform. An exclusion criterion was set to 80% correct turns in the second day. Only mice that met the criterion were included in analysis.

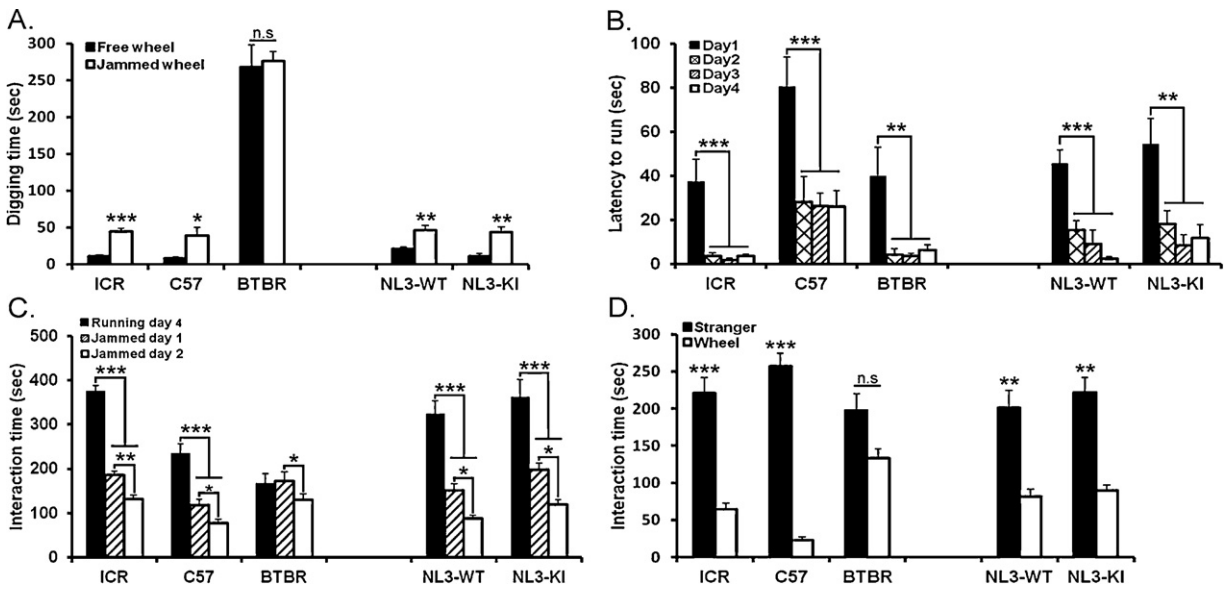


**Fig. 2.** Assessing the symptoms of ASD with the running-wheel assay. (A) Stereotypical behavior. Duration of digging activity in the last running day and the first jammed day. (B) Gaining the running habit. Latency to start running in each of the running days. (C) Cognitive rigidity. Time interacting with the wheel in the last running day and the two jammed days. (D) Social interaction. Time interacting with either the jammed wheel or the stranger mouse. For the BTBR strain, the data presented in (A) are for the group tested with bedding, while the data in (B–D) are for the group tested without bedding. All other strains were tested with bedding throughout all examinations. Data are presented in mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s. = not significant, within group ANOVA for repeated measures with Fisher's LSD post hoc correction. ICR: $n = 12$, C57: $n = 12$, BTBR: $n = 8$, NL3-WT: $n = 7$, NL3-KI: $n = 7$.

### 2.6. Three-chamber social and social novelty preference test

The apparatus was designed according to Moy et al. [22] and consisted of a rectangular, three-chambered box fabricated from polycarbonate, sized 70 cm × 24 cm × 29 cm. Side chambers were 25 cm × 24 cm × 29 cm and central chamber was 15 × 24 × 29 cm. Dividing walls had retractable doorways allowing access into each chamber through an opening sized 6.5 cm × 6.5 cm. For the sociability trial, in-house-made wire cages 14 cm in height, with a bottom diameter of 13 cm and bars spaced 1 cm apart were used. A weighted cup was placed on the top of each wire cage to prevent climbing by the subject mice.

The test consisted of 3 consecutive stages, 10 min each: habituation, social preference and preference for social novelty. In the habituation trial, the subject was placed in the middle chamber, the doors were opened and the mouse was allowed to explore the apparatus. Thereafter, the test mouse was enclosed in the center compartment, and an unfamiliar mouse (stranger 1; adult sexually naïve female C57), was enclosed in one of the wire cages and placed in one of the side chambers. An empty wire cage was put in the opposite chamber. The location of the stranger mouse alternated between the left and right sides of the social test box systematically across subjects. The doors were re-opened, and the subject was allowed to explore the entire social test box. Next, the subject was again enclosed in the center compartment and a new unfamiliar mouse (stranger 2; sexually naïve female littermate of stranger 1) was placed in the wire cage that had been empty during the previous session. The doors were re-opened and the subject had a choice between the first, already-investigated, now-familiar mouse (stranger 1) and the novel unfamiliar mouse (stranger 2). The chambers of the apparatus and wire-cages were cleaned with 15% ethanol and dried with paper towels between each subject.

Time spent in each chamber as well as the time in physical contact with each of the wire cages was scored using the Observer software (Noldus). Side preference in the habituation trial was measured using the Ethovision software (Noldus).

### 2.7. Elevated plus-maze

Anxiety is considered a secondary symptom of the autistic spectrum [1], and may lead to worsening of other autistic symptoms [10]. The elevated plus-maze [50] is a conflict test based on the tendency of mice to explore a novel environment versus their tendency to avoid the aversive properties of an open, elevated space.

The maze consisted of four alleys sized 30 cm × 5 cm, at right angles from each other, forming the shape of a plus, raised to a height of 26 cm from the floor. Two of the alleys were open runways ("open-arms"), which allow the animal to see the edge. The other two alleys were closed runways ("closed-arms"), with 30 cm high, dark side walls, which provided an enclosed environment. The animals were placed on the 5 cm × 5 cm center section, and allowed to freely explore the maze for 5 min. Measures were taken of time on and entries to the open and closed arms, according to center of mass location, using the Ethovision software. Percent open arms time was calculated as: (time spent on open arms)/(time spent on open arms + time spent on closed arms) × 100. Percent entries to the open arms was calculated similarly.

### 2.8. Statistical analysis

Data from each strain were analyzed separately, using within-strain comparisons relevant to the behavioral parameter(s) of the specific task. For multiple within-group comparisons, Fisher's post hoc tests were applied. Statistical significance was determined by repeated measures analysis of variation (ANOVA) and set at $\alpha < 0.05$, when $p < 0.05$ was considered significant, using the Statistica software (Statsoft, Tulsa, OK). For the NL3-KI and WT lines, as well as for the repeated groups of control mice, additional between groups ANOVA was conducted. Direct comparison of genotypes in the plus-maze test was performed with the non-parametric Mann–Whitney test.

## 3. Results

### 3.1. The running wheel assay

#### 3.1.1. Reproducibility

Similar results were obtained by the repeating 2 groups of ICR and 2 groups of C57 mice in latency to start running in each of the running days (ANOVA for effect of cohort group, $F_{1,10} = 1.04$, n.s. for ICR, $F_{1,10} = 3.48$, n.s. for C57), interaction time with the wheel ($F_{1,10} = 0.38$, n.s. for ICR, $F_{1,10} = 0.62$, n.s. for C57), digging duration ($F_{1,10} = 3.68$, n.s. for ICR, $F_{1,10} = 0.38$, n.s. for C57) and social interaction ($F_{1,10} = 4.66$, n.s. for ICR, $F_{1,10} = 0.35$, n.s. for C57). Therefore, both groups were conjoined into one group of 12 animals in each strain for the running wheel tests.

#### 3.1.2. Stereotypical behavior

As illustrated in Fig. 2A, ICR, C57, NL3-WT and NL3-KI mice spent significantly less time digging in the bedding when the wheel was running in comparison to a jammed wheel ($F_{1,11} = 79.23$, $p < 0.001$ for ICR, $F_{1,11} = 7.66$, $p < 0.05$ for C57, $F_{1,6} = 15.73$, $p < 0.01$ for NL3-WT and $F_{1,6} = 17.4$, $p < 0.01$ for NL3-KI). No effect for genotype was found in comparison of the NL3 groups ($F_{1,12} = 0.98$, n.s.). BTBR mice, however, spent similar time digging in the bedding when the wheel was running in comparison to a jammed wheel ($F_{1,7} = 0.076$, n.s.). Rank order of digging time was BTBR ≫ NL3-WT > ICR > NL3-KI > C57.

In fact, the digging compulsiveness of BTBR was so robust, that animals from this strain did not gain the running habit (day 4 mean

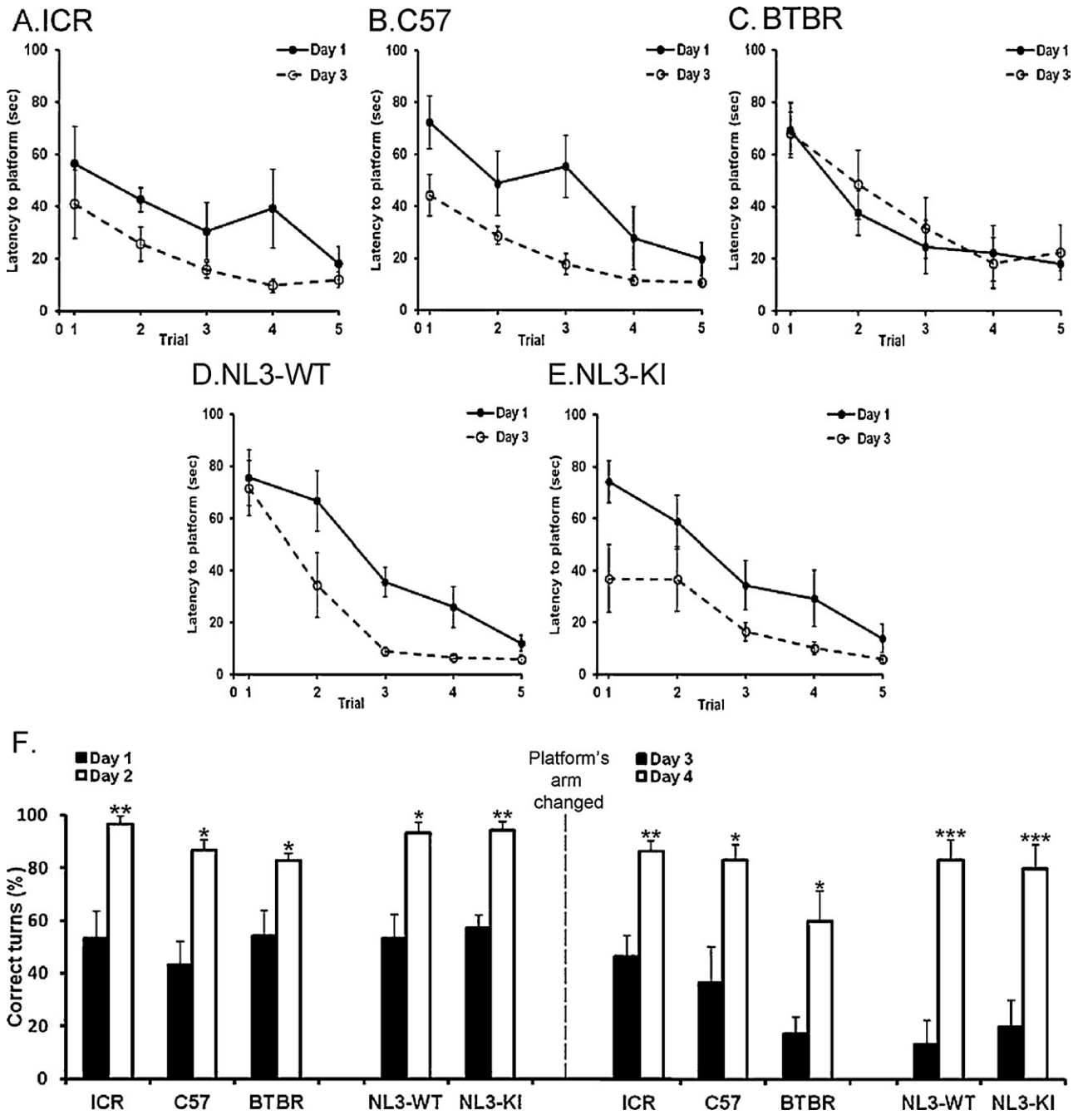


**Fig. 3.** Spatial learning and reversal learning in the "wet" T-maze. (A–E) The latency to mount the platform in the 1st and 3rd days of the test. (A) ICR, $n = 6$. (B) C57, $n = 6$. (C) BTBR, $n = 7$. (D) NL3-WT, $n = 6$. (E) NL3-KI, $n = 7$. (F) Memory of the platform spatial position before (left side) ad after (right side) switching the platform position. Percentage of correct first turn taken in 5 trials in each day of the test. Data are presented in mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, within group ANOVA for repeated measures.

running time of 19.7 ± 4.4 s, <3.5% of total trial duration), while in the other strains all mice quickly gained it. Therefore, the test was conducted on an additional group of 8 BTBR mice with no bedding in the chamber. In these conditions the mice did run on the wheel (day 4 mean running time of 167.2 ± 21.1 s, 28% of total trial duration), and that group was used for the rest of the study. No other stereotypical behavior was observed in any of the strains throughout the experiment.

### 3.1.3. Gaining the habit

As illustrated in Fig. 2B, for all strains the latency to start running on the wheel was significantly higher in the first day of the assay in comparison to the following days (days 2–4) (effect of day: $F_{3,33} = 10.72$, $p < 0.001$ for ICR, $F_{3,33} = 7.35$, $p < 0.001$ for C57, $F_{3,21} = 6.98$, $p < 0.01$ for BTBR, $F_{3,18} = 13.17$, $p < 0.001$ for NL3-WT and $F_{3,18} = 6.86$, $p < 0.01$ for NL3-KI. Fisher's post hoc test between day 1 and each of days 2–4: $p < 0.001$ for ICR, C57 and NL3-WT and $p < 0.01$ for BTBR and NL3-KI. Not significant for any of the other comparisons). No effect for genotype was found in comparison of the NL3 groups ($F_{1,12} = 1.13$, n.s.).

### 3.1.4. Rigidity to change in habit

A comparison between durations of interaction with the wheel in the last running day (day 4) and the two consecutive days in which the wheel was jammed (Fig. 2C), revealed a significant difference between days for ICR ($F_{2,22} = 149.4$, $p < 0.001$),

C57 ($F_{2,22}$ = 33.77, $p$ < 0.001), NL3-WT ($F_{2,12}$ = 46.16, $p$ < 0.001) and NL3-KI ($F_{2,12}$ = 32.56, $p$ < 0.001) but not for BTBR ($F_{2,14}$ = 2.80, n.s.). No effect for genotype was found in comparison of the NL3 groups ($F_{1,12}$ = 2.4, n.s.). Fisher's post hoc analysis revealed that the difference between interaction times with a running wheel in comparison to a jammed wheel was significant ($p$ < 0.001) for all groups but BTBR. The time interacting with the wheel in the second jammed day was significantly lower than in the first jammed day for all strains ($p$ < 0.01 for ICR, $p$ < 0.05 for C57, BTBR, NL3-WT and NL3-KI).

#### 3.1.5. Social interaction

The ICR, C57, NL3-WT and NL3-KI mice spent significantly more time interacting with the unfamiliar mouse than with the jammed running-wheel ($F_{1,11}$ = 28.95, $p$ < 0.001, $F_{1,11}$ = 113.61, $p$ < 0.001, $F_{1,6}$ = 15.35, $p$ < 0.01 and $F_{1,6}$ = 25.83, $p$ < 0.01, respectively). No effect for genotype was found in comparison of the NL3 groups ($F_{1,12}$ = 0.9, n.s.). Interestingly, the BTBR strain did not exhibit a significant preference for the social stimulus over interacting with the wheel, although a trend existed ($F_{1,7}$ = 3.29, n.s.) (Fig. 2D).

### 3.2. Validation assays

#### 3.2.1. "Wet" T-maze

The majority of ICR (100%), C57 (100%), BTBR (87.5%), NL3-WT (86%) and NL3-KI (100%) mice reached the criterion of ≥80% correct turns in the 2nd day, and thus included in the analysis. All exhibited a learning process, as expressed in a significant effect for trial in latency to climb on the platform in the first day ($F_{4,20}$ = 9.42, $p$ < 0.001 for ICR, $F_{4,20}$ = 6.56, $p$ < 0.01 for C57, $F_{4,24}$ = 4.25, $p$ < 0.01 for BTBR, $F_{4,20}$ = 8.35, $p$ < 0.001 for NL3-WT and $F_{4,24}$ = 8.52 for NL3-KI, Fig. 3A–E) and significantly more correct turns taken in the second day compared to the first ($F_{1,5}$ = 20.61, $p$ < 0.01 for ICR, $F_{1,5}$ = 10.97, $p$ < 0.05 for C57, $F_{1,6}$ = 8.82, $p$ < 0.05 for BTBR, $F_{1,5}$ = 15.0, $p$ < 0.05 for NL3-WT and $F_{1,6}$ = 29.82, $p$ < 0.01 for NL3-KI, Fig. 3F, left).

When comparing the learning curve of the first day to the curve of the third (when the location of the platform was changed), the ICR, C57, NL3-WT and NL3-KI mice showed a significantly better performance in the latter (effect of day in a 2-way ANOVA for trial and day, $F_{1,10}$ = 7.11, $p$ < 0.05 for ICR, $F_{1,10}$ = 5.4, $p$ < 0.05, for C57, $F_{1,10}$ = 8.2, $p$ < 0.05 for NL3-WT and $F_{1,12}$ = 5.45, $p$ < 0.05 for NL3-KI; Fig. 3A, B, D, E). No effect for genotype was found in comparison of the NL3 groups (3-way ANOVA: $F_{1,22}$ = 0.2, n.s.). The BTBR strain, however, exhibited similar learning performance for both days ($F_{1,12}$ = 0.1, n.s., Fig. 3C). The improvement in percent of correct turns taken in the third and fourth days was significant for all strains ($F_{1,5}$ = 20.0, $p$ < 0.01 for ICR, $F_{1,5}$ = 12.25, $p$ < 0.05 for C57, $F_{1,6}$ = 10.23, $p$ < 0.05 for BTBR, $F_{1,5}$ = 66.82, $p$ < 0.001 for NL3-WT and $F_{1,6}$ = 47.25, $p$ < 0.001 for NL3-KI; Fig. 3F, right). No effect for genotype was found in comparison of the NL3 groups: $F_{1,11}$ = 0.02, n.s.

#### 3.2.2. Sociability and social novelty

In the 3-chamber sociability assay, no side preference was recorded for any of the strains in the habituation trial (data not shown). Fig. 4A presents the strain distributions for duration of time interacting with each cage for the social preference trial. ICR, C57, NL3-WT and NL3-KI mice spent significantly more time in interaction with the cage containing the unfamiliar stranger mouse, versus the empty wire cage ($F_{1,5}$ = 11.72, $p$ < 0.05, $F_{1,5}$ = 11.76, $p$ < 0.05, $F_{1,6}$ = 43.91, $p$ < 0.001 and $F_{1,6}$ = 26.9, $p$ < 0.01, respectively. Effect for genotype among the NL3 mice: $F_{1,12}$ = 2.86, n.s.). BTBR mice, however, spent similar amount of time interacting with the empty cage and with the social stimulus ($F_{1,7}$ = 0.11, n.s.). Rank order of strain means for time spent with the stranger mouse was: ICR > C57 > NL3-WT > NL3-KI ≫ BTBR. In the preference for social novelty trial (Fig. 4B), all strains showed a significant preference for the novel stranger ($F_{1,5}$ = 17.6, $p$ < 0.01 for ICR, $F_{1,5}$ = 12.69, $p$ < 0.05 for C57, $F_{1,7}$ = 6.01, $p$ < 0.05 for BTBR, $F_{1,6}$ = 65.63, $p$ < 0.001 for NL3-WT and $F_{1,6}$ = 12.06, $p$ < 0.05 for NL3-KI). No effect for genotype was found in comparison of the NL3 groups: $F_{1,12}$ = 0.002, n.s. Rank order of strain means for time spent with the novel stranger mouse was: C57 > NL3-KI > NL3-WT > ICR > BTBR.

#### 3.2.3. General locomotion and anxiety

In order to determine whether effects of the running and digging behaviors were due to hyperactivity, we measured the distance the subject traveled in a 5 min period. Means ± SEM for total distance traveled were: ICR (34 ± 2 m) > BTBR (26 ± 2 m) > NL3-KI (25 ± 3 m) > C57 (23 ± 2 m) > NL3-WT (23 ± 2 m).

The time spent in the open arms and the number of entries to the open arms of the elevated plus-maze, suggest similar anxiety levels for all strains (Fig. 5A and B), with no significant difference between the NL3 genotypes (Mann–Whitney $U$ = 18.0, n.s. for duration, $U$ = 21.0, n.s. for entries).

## 4. Discussion

In this study we presented, for the first time to the best of our knowledge, a method for evaluating three core symptoms of autism in the same system utilizing a positive-rewarding context and minimal stress. The test was found reproducible as similar results were obtained from two independent groups of mice usually used in neurobehavioral assays (ICR and C57). Furthermore, the system was sensitive enough to detect the well reported autistic-like phenotypes of the BTBR strain, as well as adding some new behavioral characteristics to be discussed below. Interestingly, no difference was found between the NL3-KI and their wild type littermates in all our behavioral assays.

### 4.1. Stereotypical behavior

The reduction found in stereotypical behavior of ICR and C57 mice in the presence of a running, but not a jammed wheel (Fig. 2A) is in line with previous findings [46,47]. In contrast, BTBR mice showed low levels of wheel running in comparison to the other groups, concomitant with similar, much higher levels of digging, whether the wheel was free or jammed.

The elevated stereotypical behavior of BTBR mice, manifested especially in excessive self-grooming, is well documented [26,43,51]. Its digging activity as measured in the marble-burying test was also found significantly higher than that of C57 [25]. In the hole-board test [5], BTBR mice preferred clean bedding over a rewarding appetitive stimulus, although comparable measures of motivational food-seeking and olfaction [42]. Thus, it is possible that highly rewarding stimuli for other strains, such as food and wheel-running, are less rewarding for BTBR mice. Since the digging measurement was done in the 4th and 5th days of our test a new environment as an initiator of the digging behavior can be ruled out. Therefore, it can be argued that the excessive digging is a predisposition of this strain that is not reduced in the presence of other stimuli, and as such can be considered as a stereotypical behavior.

The wheel-running per se cannot be attributed for as a stereotypical behavior. Stereotypical behavior has been defined as "a highly repeated, relatively invariant behavior that has no obvious function" [52]. Although stereotypical behaviors can be considered beneficial to the organism when appearing in a proper context, it becomes abnormal when fixated. Wheel running activity, however, has the function of reward-achieving and is plastic; it is highly influenced by environmental factors as well as internal properties of

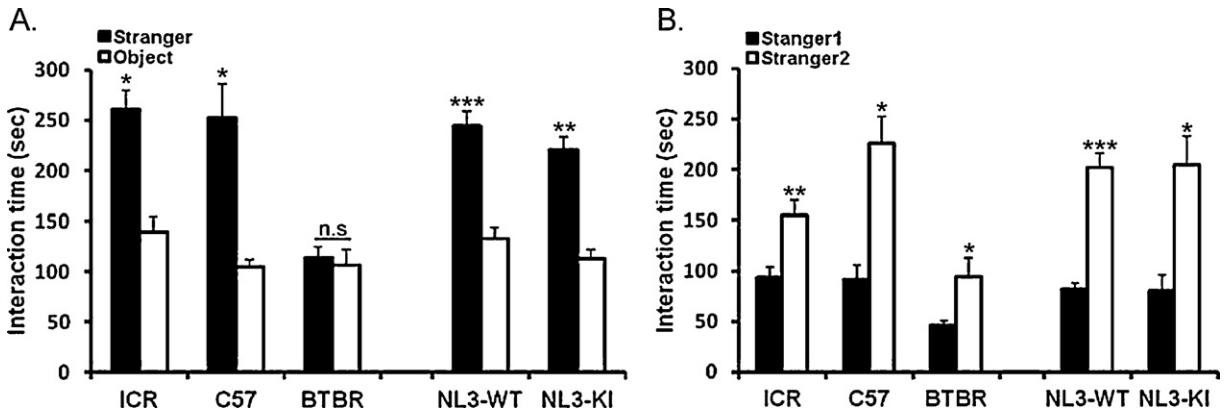


**Fig. 4.** The 3-chamber sociability assay. (A) Sociability and (B) social novelty preference. Interaction time with each cage is presented as mean + SEM for each mouse strain. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, within group ANOVA for repeated measures. ICR: $n = 6$, C57: $n = 6$, BTBR: $n = 8$, NL3-WT: $n = 7$, NL3-KI: $n = 7$.

the wheel and is very variable in its performance [53–59]. Therefore, the method allows us to measure how fixated a stereotypical behavior is, enhancing the robustness of the test.

### 4.2. Rigidity to change in habit

Previous studies showed that BTBR mice were able to learn a task and then make a shift in the learned strategies over days, in both the visual and olfactory modalities [25,42,60]. However, Amodeo et al. [25] reported recently of differences in reversal learning in probabilistic spatial discrimination task between C57 and BTBR mice. These reports are in line with our findings. The learning and memory capabilities of BTBR were found intact, as expressed in the reduced latency to start running on the wheel (Fig. 2B), improvement in correct turns taken between days (Fig. 3F) and reduced latencies between trials in the T-maze (Fig. 3C).

Interestingly, a deficit in their ability to adjust to a changing environment was found, as manifested in similar time of interaction with the wheel in the 4th running day and 1st jammed day (Fig. 2C). It can be argued that this finding stems from the lowered motivation of BTBRs to run compared to other tested strains (Section 4.1). However, this strain was able to gain the habit of wheel-running, as can be inferred from the reduction in latency to start running on the wheel (Fig. 2B) and from the substantial duration spent running on the wheel (28% of total time in day 4) (Fig. 2C). Furthermore, a 25% reduction in interaction time was evident in the second jammed day. This indicates that our finding appears not to be due to relatively low running levels of BTBR mice. Further support for the existence of cognitive inflexibility of the BTBR strain can be found in the result of the T-maze test. While BTBR mice exhibited similar learning curves in the first and third days of the assay (Fig. 3C), other tested strains adjusted to the new location of the platform and exhibited an improved learning curve.

The distinction between immediate adjustment to change and memory of a change may suggest of different neuronal mechanisms underlying them. Such neuronal differentiation was supported by discrimination studies on rodents. The orbitofrontal cortex has been shown to be important in suppressing the previous acquired choice strategies (thus preventing "perseverative" errors), and this process is dopamine dependent [61–63]. Maintaining the change (and preventing "regressive" errors), however, is depended on acetylcholine projection from the centromedian-parafascicular thalamic nucleus to M1-type muscarinic cholinergic receptors in the medial striatum [64–66].

### 4.3. Sociability levels

While ICR and C57 mice exhibited a significant preference of the social stimulus over the object in both the jammed running-wheel (Fig. 2D) and the 3-chamber assays (Fig. 4A), BTBR did not. These results are in agreement with comparisons of the C57 and

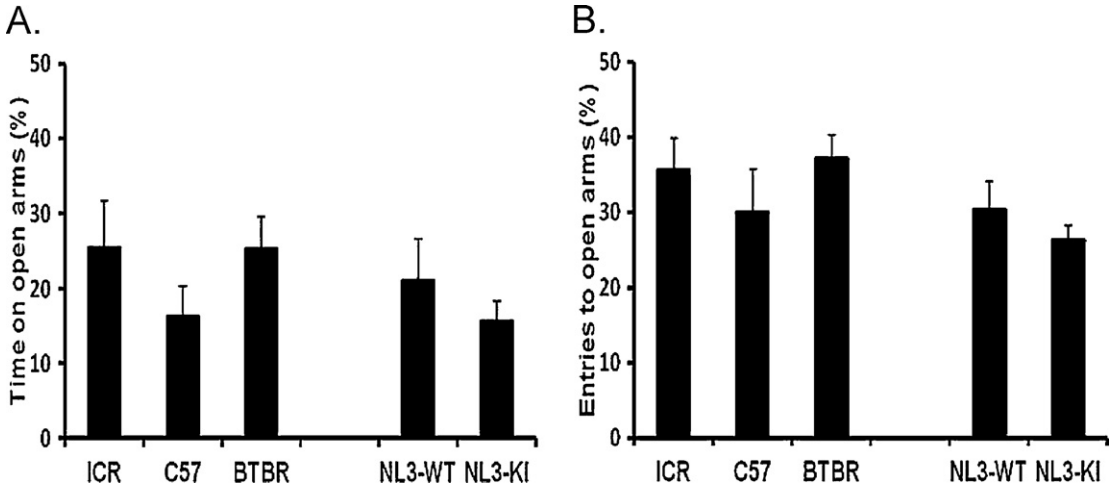


**Fig. 5.** Elevated plus-maze. Mean + SEM of the percentage of (A) time spent in the open arms, and (B) number of entries to the open arms, from the total of all 4 arms. ICR: $n = 6$, C57: $n = 6$, BTBR: $n = 8$, NL3-WT: $n = 7$, NL3-KI: $n = 7$.

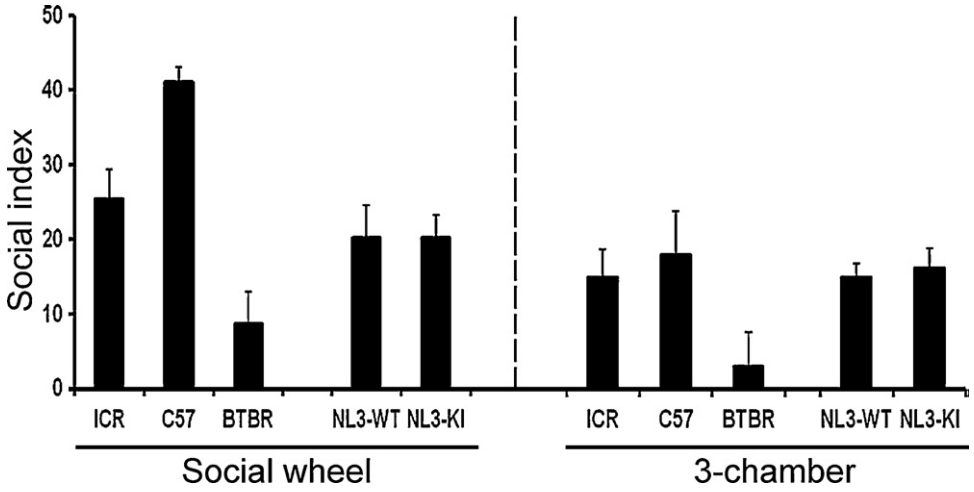


**Fig. 6.** Comparison of the social wheel and 3-chamber assays. Mean + SEM of the social indices in the two assays. Social index was calculated as ((interaction with stranger)/(interaction with stranger + interaction with object) × 100) − 50. ICR: $n = 6$, C57: $n = 6$, BTBR: $n = 8$, NL3-WT: $n = 7$, NL3-KI: $n = 7$.

BTBR strains in the 3-chamber test conducted in several laboratories [26,29,44,67,68].

However, all strains showed a significant preference of the novel stranger (Fig. 4B). In previous studies, C57 consistently showed such preference [26,29,44,67,68] (but see [69] for lack of such preference when the novel stranger was put in the chamber that previously contained the first stranger). As for BTBR, however, a controversy exists – while some groups reported of a deficit in this property [26,42], others reported it as intact [51,67]. Our results better support the latter; although displaying the lowest overall interaction time with the intruder mice among the strains studied, BTBR mice significantly preferred the novel stranger mouse over the familiar mouse.

To the best of our knowledge, no other laboratory has performed the sociability and social novelty test on the outbred strain ICR. However, one study has performed the sociability trial of the test [49] and found a social preference. Another study [70] showed that ICR mice remember social stimuli. Taken together, these results agree with our findings of social and social novelty preferences of this strain, resembling those of the C57 mouse strain.

Interestingly, all mouse strains exhibited higher social preference in the social running wheel test when compared to the 3-chamber assay (Fig. 6). Three reasons can contribute to this phenomenon: (a) in the social running-wheel test more physical interaction is possible; (b) less stress is posed on the subject, for it has been familiarized with the test chamber for 6 days prior to the test [71], and the stranger is not trapped in a cage [72]; (c) the jammed wheel is already familiar to the subject mouse, while in the 3-chamber assay the whole environment is novel. Since the running wheel test encourages the mouse to a social behavior, autistic-like phenotype found in it, as is the case of BTBR, is more robust and less prone to environmental effects.

### 4.4. Absence of autistic-like phenotype in NL3-KI mice

No significant behavioral difference was found between NL3-KI mice and their WT littermates, in the running-wheel tests as well as in the validation assays, in support of the findings of Chadman et al. [34]. These findings are despite an aberrant hippocampal and cortical synaptic function and striatal neurons anatomy differences found in these mice [73]. These findings emphasize the understanding that ASD is a heterogeneous syndrome, influenced by a group of genes and environmental factors [9]. Furthermore, it highlights the importance of testing the autistic phenotype in a variety of experimental systems.

### 4.5. Advantages of the system

#### 4.5.1. Positive reinforcement with minimal stress

Classical tests of learning and memory rely on stressful conditions: food deprivation in the appetitive mazes and life-threatening contact with water in the water mazes. It is broadly agreed that stress influences the emotional and cognitive states of animals [74–77]. Specifically, food deprivation has been shown to have a specific effect on cognitive tasks in rodents [78,79], and forced swimming has been shown to alter levels of neurotransmitters [80] and hormones [81] directly associated with stress. Forced swimming is stressful to rodents to such extent that it is widely used as a paradigm to induce stress [82,83].

In the jammed-running paradigm, however, the routine is gained by the introduction of a positive reinforcement. In an elegantly controlled study, Lawson and Watson [84] demonstrated that "positive reinforcement is a more efficient method of developing a habit than is negative reinforcement" (p. 88), and that the value of reinforcement influences the habit gaining curve. Thus, utilizing a highly rewarding reinforcement such as running on a wheel is a solid mean to induce a habit.

It should be noted, that although protracted (>4 weeks) social isolation is stressful and leads to reduced social behavior in mice [85], a one week isolation, as used in our study, is a common means to increase exploration and social behaviors [71,86].

#### 4.5.2. High animals' welfare

Good laboratory practice requires the reduction of suffer caused to animals to the minimum necessary [87]. Since very little handling is needed and no anxious or aversive procedures are used, suffer caused in our method is very low, especially in comparison to other learning and memory assays.

## 5. Summary and conclusion

The present study revealed that the jammed running wheel paradigm can measure fixation of stereotypic behaviors, cognitive rigidity and social interaction, in one easy to conduct assay, utilizing a rewarding stimulus and minimal stress. Also, it was sensitive enough to distinguish between immediate response to change and

lasting memory of the change in habit. Importantly, it was able to confirm the reported autistic-like phenotypes of the BTBR strain.

Lastly, we believe that further utility of the new set-up may lead the way for a better and standardized establishment of mouse models of human conditions such as obsessive–compulsive disorder [88], autism spectrum disorders, attention deficit hyperactivity disorder [89,90], schizophrenia [91] and frontal lobe lesions [63].

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