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Research report

Long term beneficial effect of neurotrophic factors-secreting mesenchymal stem cells transplantation in the BTBR mouse model of autism

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ABSTRACT

Autism spectrum disorders (ASD) are neurodevelopmental disabilities characterized by severe impairment in social communication skills and restricted, repetitive behaviors. We have previously shown that a single transplantation of mesenchymal stem cells (MSC) into the cerebral lateral ventricles of BTBR autistic-like mice resulted in an improvement across all diagnostic criteria of ASD. We suggested that brain-derived neurotrophic factor (BDNF), a protein which supports the survival and regeneration of neurons secreted by MSC, largely contributed to the beneficial behavioral effect. In this study, we investigated the behavioral effects of transplanted MSC induced to secrete higher amounts of neurotrophic factors (NurOwn®), on various ASD-related behavioral domains using the BTBR mouse model of ASD. We demonstrate that NurOwn® transplantation had significant advantages over MSC transplantation in terms of improving communication skills, one and six months following treatment, as compared to sham-treated BTBR mice. Furthermore, NurOwn® transplantation resulted in reduced stereotypic behavior for as long as six months post treatment, compared to the one month improvement observed in the MSC treated mice. Notably, NurOwn® treatment resulted in improved cognitive flexibility, an improvement that was not observed by MSC treatment. Both MSC and NurOwn® transplantation induced an improvement in social behavior that lasted for six months. In conclusion, the present study demonstrates that a single transplantation of MSC or NurOwn® have long-lasting benefits, while NurOwn® may be superior to MSC treatment.

1. Introduction

Autism spectrum disorder (ASD) is a debilitating neurodevelopmental disability characterized by deficits in social communication and stereotypical patterns of behavior, interests, or activities [1]. A recent survey demonstrated that ASD has an estimated prevalence of 1 in 68 children in the United States [2]. The etiology and pathophysiology of ASD is still largely unclear, and despite major efforts, there are no known efficacious pharmacologic treatments for the core symptoms of autism [3].

Owing to their essential role in cellular proliferation, migration, differentiation and integrity, and thus in regulation of neurodevelopment, neurotrophic factors have gained increasing attention in ASD research [4]. One of the most studied neurotrophic factors is brain-derived neurotrophic factor (BDNF). A growing body of evidence indicates that alterations in the BDNF/tyrosine kinase B (TrkB) signaling pathway increase vulnerability to autism [4–6].

The BTBR T + Itpr3tf/J (BTBR) inbred strain demonstrates autistic-like behavioral phenotypes consistent with the diagnostic criteria for ASD, and is therefore considered a widely accepted animal model for ASD [7]. BTBR mice display impaired social interaction and communication as well as cognitive rigidity [8,9]. Moreover, BTBR mice show biochemical disturbances consistent with findings seen in autistic patients, such as alterations in transcription and protein expression of

Abbreviations: ASD, autism spectrum disorders; MSC, mesenchymal stem cells; NurOwn®, MSC induced to secrete higher amounts of neurotrophic factors; BDNF, brain-derived neurotrophic factor; TrkB, tyrosine kinase B; BTBR, BTBR T+ Itpr3tf/J; B6, C57bl/6

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BDNF in the hippocampus and decreased hippocampal neurogenesis [9–11].

Stem cells, and mesenchymal stem cell (MSC) in particular, hold great potential for treatment of ASD due to their neurotrophic and immunomodulatory paracrine effects [12–14]. Our group has recently shown that transplantation of MSC into the lateral ventricles of BTBR mice resulted in a reduction in stereotypical behaviors and cognitive rigidity and an improvement in social behavior 4–6 weeks post transplantation. The behavioral effects were associated with elevated BDNF protein levels in the hippocampus accompanied by increased hippocampal neurogenesis in the MSC-transplanted mice [15]. Encouraged by these results, we aimed at enhancing the therapeutic effect by using MSC induced to secrete higher amounts of neurotrophic factors.

We have developed a protocol for differentiating MSC into cells secreting higher levels of neurotrophic factors (NurOwn®) [16]. Specifically, these cells produce 2 and 5 fold increased levels of BDNF and Glial cell-Derived Neurotrophic Factor (GDNF) respectively, compared to MSC from the same donor [17]. In preclinical studies, NurOwn® were found efficient in treating a rat model of Parkinson's disease [18] and a mouse model for Huntington's disease [19]. Importantly, recent work has shown that administration of NurOwn® cells in Amyotrophic Lateral Sclerosis (ALS) patients is safe with early indications of clinical benefits [20].

In the current study, we aimed to evaluate whether NurOwn® treatment results in augmented beneficial effect compared to MSC in terms of social interaction, communication skills, repetitive behaviors and cognitive rigidity, and whether MSC and NurOwn® provide a long lasting effect following a single stem-cell transplantation.

2. Materials and methods

2.1. Animals

BTBR mice were bred from adult pairs originally purchased from The Jackson Laboratory (Bar Harbor, ME). C57bl/6 (B6) mice, bred from adult pairs originally purchased from the Jackson Laboratory (Bar Harbor, ME), underwent sham operation and were used as controls (n = 10). BTBR male mice (6–8 weeks of age) were randomly assigned to sham operated (n = 10), MSC transplanted (n = 11) and NurOwn® transplanted (n = 10) groups. Mice were housed in groups of 3–5 littermates per cage and allowed to recover for 3 weeks following operation and prior to behavioral examinations. Social stimulus mice in the dyadic reciprocal social interaction test were 5 weeks old Hsd:ICR [CD-1] mice (Harlan Laboratories, Rehovot, Israel). All experimental procedures were approved by and conducted in strict compliance with the Institutional Animal Care and Use Committees of the Tel-Aviv University.

2.2. Preparation of cells

Bone Marrow of healthy donors was purchased from Lonza (US). MSC were isolated and cultured and expanded in growth media. Differentiation into NurOwn® was induced using a medium containing 1 mM dibutyrylcyclic AMP (cAMP), 20 ng/ml human Basic Fibroblast Growth Factor (hbFGF), 5 ng/ml human platelet derived growth factor (PDGF-AA), and 50 ng/ml human Heregulin β1 as previously described [17].

On the day of treatment, the cells were harvested, washed, and prepared for transplantation at a concentration of 50,000 cells/μL suspended in DMEM.

2.3. Cell transplantation

Under ketamine/xylazine anesthesia, the mice were placed in a stereotactic frame (Kopf, Tujunga, CA), and either cells or excipient (1 μl per injection site) were injected into the cerebral lateral ventricles bilaterally at 0.5 μL/min (Hamilton 701N syringe) to the following coordinates (relative to the bregma): anterior-posterior, −0.35 mm; medial-lateral, ± 0.85 mm; dorsal-ventral, −2.3 mm. The needle was withdrawn after 5 min. To suppress the possible immune response animals received 15 mg/kg cyclosporine (Novartis, Basel, Switzerland) subcutaneously for 3 days around transplantation. Thereafter, cyclosporine was added to drinking water (15 mg/kg according to expected daily drinking volume per mouse) throughout the experiment up until the animals were sacrificed.

2.4. Behavioral tests

All behavioral tests were conducted one month and six months post treatment to evaluate the long-lasting effect of the treatment.

2.4.1. Reciprocal dyadic social interaction test

The reciprocal dyadic social interaction test was conducted as previously described [15]. A 5-week-old male Hsd:ICR[CD-1] stranger mouse was used as the social stimulus. The stranger mouse was placed in a 40 × 40 × 20 cm cage together with the test mouse, and both mice were videotaped for 20 min. The last 10 min were quantified by an observer blinded to treatment. Both mice were isolated for 1 h prior to the test. Social contact initiated by the test mouse was scored using Cowlog video coding software and post analyses were done using MATLAB software. The duration of social engagement initiated by the test mouse was measured and summed as well as the time spent in repetitive digging behavior. In terms of social behavior, we measured the following parameters: nose to nose sniffing (i.e. approach to the front of the stranger), nose to genital sniffing (i.e. approach to the back of the stranger), attacking (resident attacking the stranger mouse) and avoiding (resident mouse avoiding interaction initiated by the stranger).

2.4.2. Ultrasonic vocalizations

Both BTBR and C57bl/6 males interacted with C57bl/6 females. Each male was placed in a separate cage for 1 h before encountering the female. Ultrasonic vocalizations were recorded for the first 5 min of encounter to prevent extremely high sexual arousal and mating behaviors. The encounters were filmed in order to analyze duration of social interaction initiated by the male mouse. All males and females were sexually naïve. Females were placed in the same cage to synchronize their estrus cycle and met the males on the same day. Vocalizations were recorded with Avisoft-RECORDER v. 4.2.21 recording program. The settings included a sampling rate of 250 kHz and a format of 16 bit. For spectrogram generation, recordings were transferred to Avisoft-SASLab Pro Version 5.2.07 and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT-length of 256 points and a time window overlap of 50% (100% Frame, FlatTop window).

2.4.3. Repetitive self-grooming behavior

Assessment of repetitive self-grooming behavior was conducted as previously described [15]. Each mouse was placed in a 40 × 40 × 20 cm cage for 20 min. The last 10 min were quantified by an observer blind to the treatment. Repetitive self-grooming behavior was scored using Cowlog video coding software and post analysis were conducted using MATLAB software.

2.4.4. Water T-maze assay

Mice were placed in a water filled T- shaped Plexiglas chamber (15 cm water depth, kept on 25 ± 1 °C), with three arms sized 22 × 11 cm and a center zone sized 11 × 11 cm [21]. An escape platform (diameter = 8 cm) was submerged to 0.5 cm below water level. Animals underwent 10 trials on 2 consecutive days to learn on which side the platform was present. The animals were placed in the starting arm facing the wall, and were allowed to swim until they found

the platform, or until 90 s had passed. When the animal mounted the platform, it was allowed to stand on it for 15 s. If a mouse did not find the platform within 90 s, it was gently guided to the platform and allowed to stand on it for 15 s. Inter-trial interval was > 5 min. On the first and second day, the platform was located in one arm, while on the third day it was relocated to the opposite arm. Starting arm was identical on all days. Latency to climb on the platform, swimming distance and velocity, as well as number of entrances to each arm were measured from the second the mouse was released into the water until it stood on the platform. Due to significant differences in swimming capacities between B6 and BTBR mice (data not shown), the B6 group was excluded from the test.

2.5. Statistical analysis

All data are expressed as the mean + SEM. Statistical analyses were performed using a commercial software (GraphPad Prism 7). Comparisons between four treatment groups (B6, Sham-treated BTBR, MSC-treated BTBR, NurOwn®-treated BTBR) were conducted using a one-way ANOVA, followed by Tukey's post hoc test. In all statistical analyses $p < 0.05$ was considered statistically significant.

3. Results

3.1. MSC and NurOwn® transplantation improved social behavior in BTBR mice

In the dyadic reciprocal social interaction task NurOwn®- and MSC-treated BTBR mice spent significantly more time initiating nose to nose social interaction with a stranger male mouse, as compared to sham-treated BTBR mice at one month post transplantation (Fig. 1A, $F(3, 37) = 14.91$, Sham vs. MSC: $p = .0006$; Sham vs. NurOwn®: $p = .0019$). This effect was maintained six months post treatment (Fig. 1B, $F(3, 28) = 8.478$, Sham vs. MSC: $p = .0023$; Sham vs. NurOwn®: $p = .0009$). The duration of nose to nose sniffing in NurOwn®- and MSC-treated BTBR mice was not statistically different from the pro-social B6 control group at both time points (one month post transplantation: B6 vs. MSC: $p = .0987$, B6 vs. NurOwn®: $p = .0571$; Six months post transplantation: B6 vs. MSC: $p = .8071$; B6 vs. NurOwn®: $p = .9756$).

Furthermore, during the dyadic reciprocal interaction test, at one month post transplantation, BTBR mice transplanted with MSC and NurOwn® engaged in much shorter sessions of repetitive digging than sham treated BTBR mice (Fig. 1A, $F(3, 37) = 6.591$, Sham vs. MSC: $p = .0158$; Sham vs. NurOwn®: $p = .0022$). At six months post treatment, NurOwn® transplantation resulted in significantly shorter bouts of repetitive digging compared to sham treated BTBR mice, whereas MSC treated mice exhibited a very strong trend toward significance (Fig. 1B, $F(3, 28) = 6.325$, Sham vs. MSC: $p = .0501$; Sham vs. NurOwn®: $p = .0059$).

3.2. NurOwn® but not MSC transplantation increased the number of male-to-female vocalizations in BTBR mice

NurOwn® transplantation resulted in a significantly higher number of male-to-female ultrasonic vocalizations compared to sham operated BTBR mice. This improvement was evident at both one month (Fig. 2B, $F(3, 35) = 4.185$, Sham vs. NurOwn®: $p = .0214$) and six months (Fig. 2C, $F(3, 29) = 4.873$, Sham vs. NurOwn®: $p = .0401$) post treatment. Importantly, there was no significant difference between the duration of interaction initiated by the males toward the females in any of the groups (data not shown). The number of ultrasonic vocalizations of NurOwn® treated BTBR mice was comparable to those of the B6 control group. MSC-transplanted BTBR mice did not exhibit a significant increase in the number of male-to-female ultrasonic vocalizations compared to sham operated BTBR mice both at one month (Fig. 2B, Sham vs. MSC: $p = .4770$) and six months post treatment (Fig. 2C, Sham vs. MSC: $p = .3419$). Inter syllable interval distribution became comparable to that of sham treated B6 mice following both MSC (Fig. 2E) and NurOwn® (Fig. 2F) treatment, indicating that MSC- and NurOwn®-treated BTBR mice tend to vocalize at shorter intervals between the syllables compared to sham-operated BTBR mice.

We also compared the duration and amplitude of syllables between the experimental groups. We found that B6 mice tend to make significantly longer syllables compared to BTBR mice (Supplementary Fig. S1, A:1-month post treatment: $F(3, 24307) = 188$, B6 vs. Sham: $p < .0001$; B:6-months post treatment: $F(3, 35689) = 548.9$, B6 vs. Sham: $p < .0001$). One month following transplantation, the duration of syllables of both MSC and NurOwn® treated groups became significantly longer compared to sham-treated mice (Supplementary Fig. S1A, Sham vs. MSC: $p < .0001$, Sham vs. NurOwn®: $p < .0001$). This effect was maintained six months post treatment for both MSC and NurOwn® treated groups (Supplementary Fig. S1B, Sham vs. MSC: $p < .0001$, Sham vs. NurOwn®: $p < .0001$). Moreover, we found that B6 mice tend to produce syllables with larger amplitudes (measured in bandwidth) compared to BTBR mice (Supplementary Fig. S1, C:1-month post treatment: $F(3, 24307) = 403.3$, B6 vs. Sham: $p < .0001$; D:6-months post treatment: $F(3, 35689) = 873.4$, B6 vs. Sham: $p < .0001$). One month after treatment, the amplitude of syllables of both MSC and NurOwn® treated groups became significantly higher compared to sham treated mice (Supplementary Fig. S1C, Sham vs. MSC: $p < .0001$, Sham vs. NurOwn®: $p < .0001$). This effect persisted six months after treatment for both MSC and NurOwn® groups (Supplementary Fig. S1D, Sham vs. MSC: $p < .0001$, Sham vs. NurOwn®: $p < .0001$).

3.3. NurOwn® and MSC transplantation ameliorated repetitive behaviors in BTBR mice, but only the effect of NurOwn® transplantation persisted for six months

BTBR mice transplanted with MSC and NurOwn® exhibited significantly lower levels of stereotypical behavior as determined by decreased self-grooming compared to their sham-treated littermates one month post treatment (Fig. 3A, $F(3, 37) = 9.604$, Sham vs. MSC: $p = .0233$; Sham vs. NurOwn®: $p = .0205$). While NurOwn® transplanted BTBR mice maintained lower levels of self-grooming six months post transplantation, MSC treated BTBR mice did not significantly differ from sham treated BTBR mice (Fig. 3B, $F(3, 25) = 5.152$, Sham vs. MSC: $p = .9948$; Sham vs. NurOwn®: $p = .0442$).

3.4. NurOwn®, but not MSC, transplantation decreased cognitive rigidity in BTBR mice

BTBR mice transplanted with NurOwn® showed significant improvement in their flexibility to adjust to changes in the environment one month post treatment, manifested by shorter latency to reach the platform in the wet T-maze test on the first trial of the day in which the platform location was switched. MSC treated mice showed an improvement but it did not reach statistical significance (Fig. 4A, $F(2, 26) = 4.634$, Sham vs. MSC: $p = .085$; Sham vs. NurOwn®: $p = .02$). No difference in latency to reach the platform was observed six months post treatment in either treatment groups compared to sham treated BTBR mice (Fig. 4B, $F(2, 21) = 0.2775$, $p = .7604$).

4. Discussion

In the current study, we report that a single MSC or NurOwn® transplantation in young adult BTBR mice induces a significant improvement in various autism-related behavioral domains that is sustained for six-months post treatment.

We previously demonstrated that a single MSC transplantation induces a reduction in repetitive behaviors, increases cognitive flexibility and improves sociability in young adult BTBR mice. In addition,

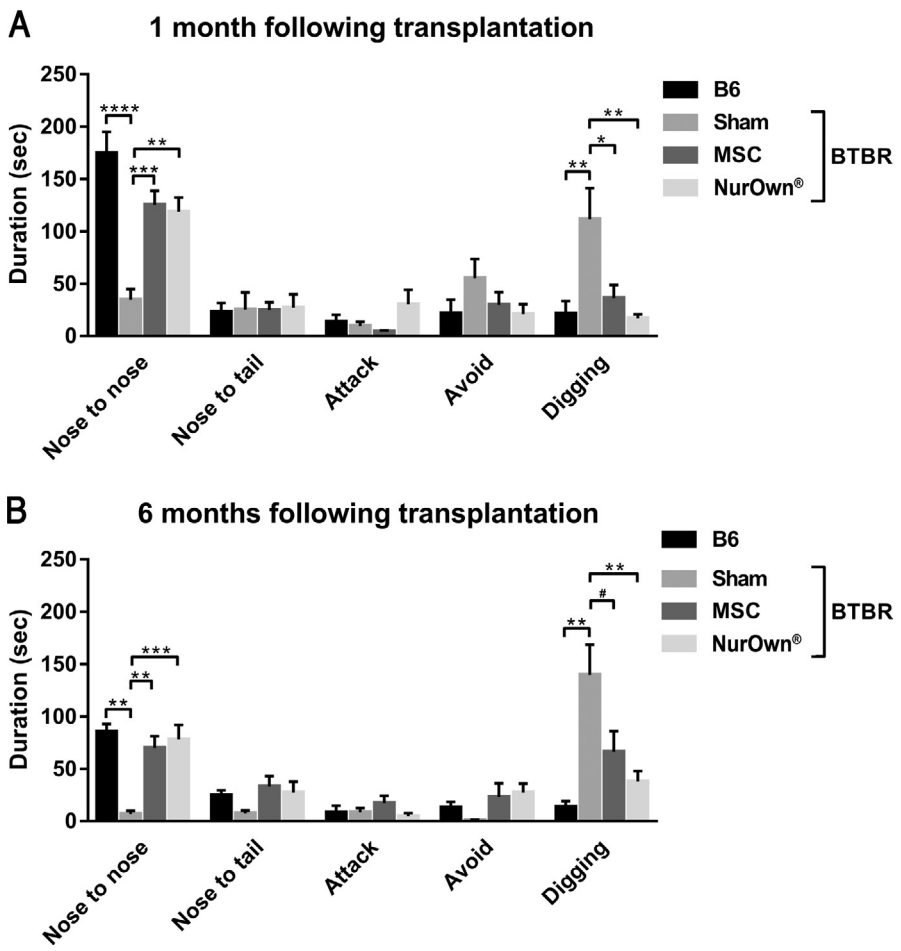

Fig. 1. MSC and NurOwn® transplantation improved social behavior in BTBR mice. Quantification of multiple parameters during the dyadic reciprocal social interaction test one month post treatment (A) and six months post treatment (B). Behavioral parameters tested from left to right: nose to nose and nose to tail contact initiated by the test mouse; bouts of attacks of the test mouse; sessions of avoidance of the test mice; bouts of repetitive digging behavior. Data is presented as mean + SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.000$, $^{\#}P = 0.0501$ in 1-way ANOVA with Tukey's post hoc. B6: sham-treated C57bl/6 mice; Sham: sham operated BTBR mice; MSC: human mesenchymal stem-cells transplanted BTBR mice; NurOwn®: MSC differentiated into neurotrophic factor secreting cells transplanted BTBR mice.

MSC-treated mice revealed increased hippocampal BDNF levels and enhanced neurogenesis compared to sham-treated mice [15]. The current findings confirm our hypothesis that the effect of a single cell transplantation is maintained for longer period than previously reported, and that the use of cells that secrete higher levels of neurotrophic factor contributes to the efficacy of treatment.

We found that intraventricular transplantation of NurOwn® is superior to MSC in several aspects; First, solely NurOwn® induced an increase in male-to-female ultrasonic vocalizations. Second, NurOwn® triggered a decrease in repetitive digging and self-grooming behaviors that sustained for six months while the effect of MSC in ameliorating these stereotypical behaviors did not last six months following transplantation. Third, NurOwn® improved cognitive flexibility in the wet T Maze paradigm whereas MSC did not. These results suggest that neurotrophic factors hold a critical role in mediating the beneficial effect of MSC in ameliorating autistic-like behaviors of the BTBR mouse model. One plausible mechanism is that multiple neurotrophic factors secreted by the NurOwn® induce a long-lasting modification thorough the induction of neurogenesis [15] and the formation of new neural networks.

It was previously reported that BTBR mice display an unusual repetoir of male-to-female ultrasonic vocalizations compared to B6 mice, indicating impared social communication, which is a core diagnostic criteria of ASD [22]. In the current study we show the benefical effect of MSC and NurOwn® transplantation on male-to-female ultrasonic vocalizations. This effect was comprehensively evaluated by analyzing several features of the syllable. To our knowledge, this is the first study to show increased ultrasonic vocalizations following treatment, indicating improved communication in BTBR mice. Importantly, the improvement in the number of male-to-female ultrasonic vocalizations did not seem to be caused by higher sexual arousal as there was no significant difference between the duration of interaction initiated by the males toward the females in any of the groups. Considering the growing recognition that some adolescents and young adults with ASD may exhibit inappropriate sexual behaviors [23,24], the fact the MSC and NurOwn® transplantation does not enhance sexual arousal is an advantage.

Several pre-clinical studies showed improvement in more than one domain of autistic-like behaviors in BTBR mice, including social interaction, repetitive behaviors and cognitive rigidity [21,25–27]. However, our study is the first to show improvement across all behavioral phenotypes measurable in mice and the first to demonstrate a long-lasting effect of a single treatment for six months.

Indeed, two recent clinical studies provide encouraging evidence for the beneficial influence of MSC on ASD patients. Sharma et al. performed intrathecal transplantation of autologous bone marrow mononuclear cells, that contain MSC, in 32 patients with ASD. Most of the patients showed improved scores in various behavioral scales after a 26 months follow up [28]. Lv et al. showed in a smaller set of ASD patients that intravenous and intrathecal infusions of human cord blood mononuclear cells and umbilical cord-derived MSC result in improved scores in different ASD scales 24 weeks post-treatment [29].

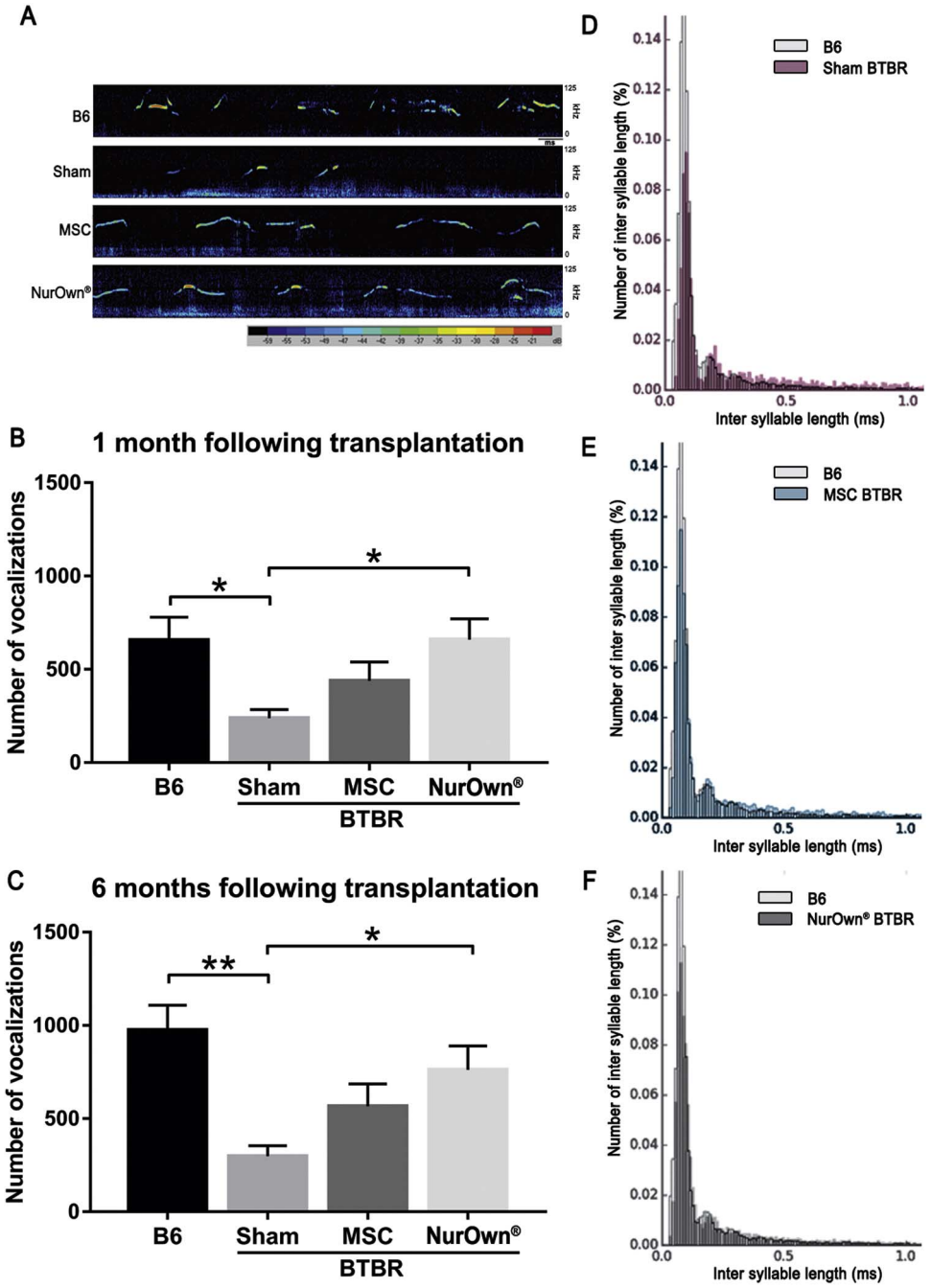

Fig. 2. NurOwn® but not MSC transplantation increased the number of male-to-female vocalizations in BTBR mice. (A) Representative spectrograms of male-to-female ultrasonic vocalizations of each group. (B) Number of syllables one month post treatment. (C) Number of syllables six months post treatment. (D-F) Inter syllable interval distributions. (D) Sham operated BTBR mice vs. sham operated B6 mice. (E) MSC-transplanted BTBR mice vs. sham operated B6 mice. (F) NurOwn®-transplanted BTBR mice vs. sham operated B6 mice. Data is presented as mean + SEM. $*P < 0.05$, $**P < 0.01$ in 1-way ANOVA with Tukey's post hoc. B6: sham-treated C57bl/6 mice; Sham: sham operated BTBR mice; MSC: human mesenchymal stem-cells transplanted BTBR mice; NurOwn®: MSC differentiated into neurotrophic factor secreting cells transplanted BTBR mice.

Additional large-scale well-controlled studies are warranted in order to prove the efficacy and a long term beneficial effect of MSC therapy in ASD patients. Taken together, our results highlight the potential beneficial effects of MSC and NurOwn® as possible treatment options for autistic patients.

Conflict of interest

Gothelf Y. and Abramov N. are BrainStorm Cell Therapeutics employees and Offen D. is the chief scientific advisor of BrainStorm Cell Therapeutics. All of the other authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2017.03.047.

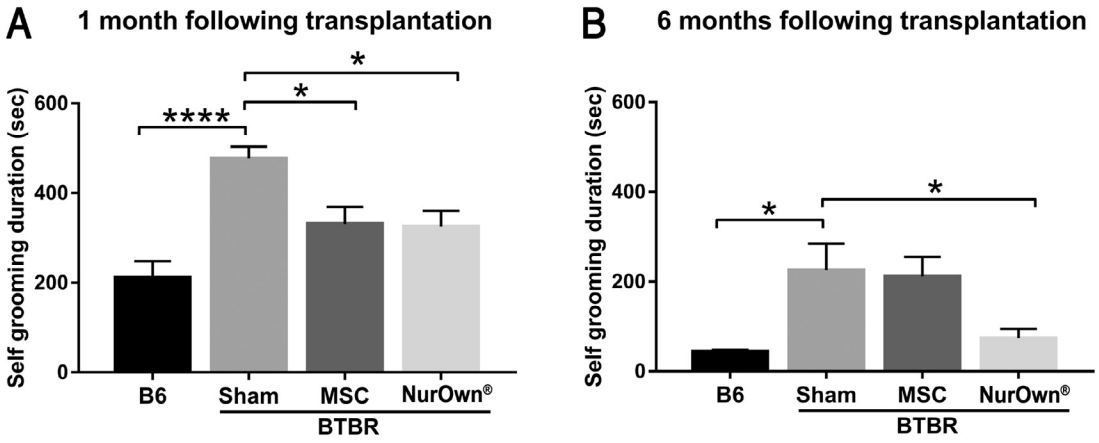

Fig. 3. NurOwn® and MSC transplantation ameliorated repetitive behaviors in BTBR mice, but only the effect of NurOwn® transplantation maintained following six months. Total duration of self-grooming was quantified in a 10-minute period one month post treatment (A) and six months post treatment (B). Data is presented as mean + SEM. $*P < 0.05$, $****P < 0.000$, in 1-way ANOVA with Tukey's post hoc. B6: sham-treated C57bl/6 mice; Sham: sham operated BTBR mice; MSC: human mesenchymal stem-cells transplanted BTBR mice; NurOwn®: MSC differentiated into neurotrophic factor secreting cells transplanted BTBR mice.

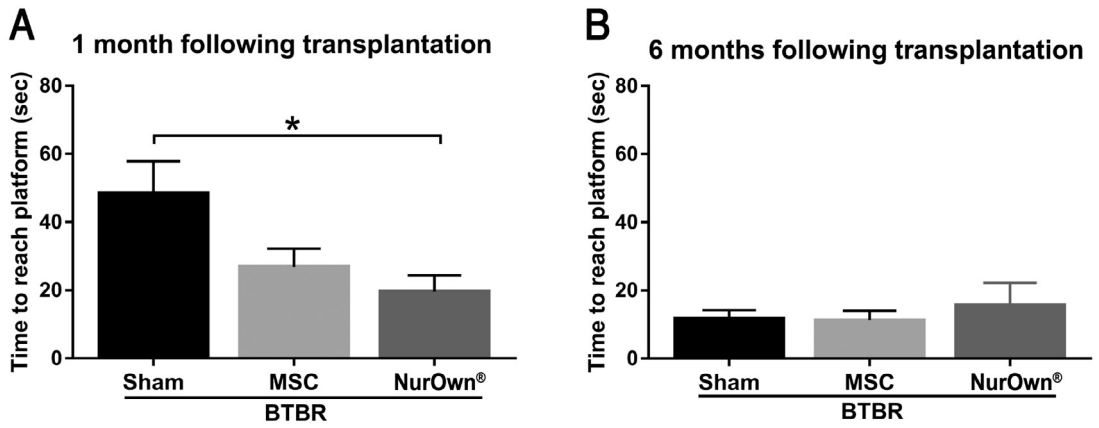

Fig. 4. NurOwn®, but not MSC, transplantation decreased cognitive rigidity in BTBR mice. Latency to reach the escape platform in the first trial on the reversal-learning day one month post transplantation (A) and six months post transplantation (B). Data is presented as mean + SEM. $*P < 0.05$, in 1-way ANOVA with Tukey's post hoc. Sham: sham operated BTBR mice; MSC: human mesenchymal stem-cells transplanted BTBR mice; NurOwn®: MSC differentiated into neurotrophic factor secreting cells transplanted BTBR mice.

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