Mesenchymal Stem Cell Transplantation Promotes Neurogenesis and Ameliorates Autism Related Behaviors in BTBR Mice

Hadar Segal-Gavish, Golan Karvat, Noy Barak, Ran Barzilay, Javier Ganz, Liat Edry, Israel Aharony, Daniel Offen, and Tali Kimchi

Autism spectrum disorders (ASD) are characterized by social communication deficits, cognitive rigidity, and repetitive stereotyped behaviors. Mesenchymal stem cells (MSC) have a paracrine regenerative effect, and were speculated to be a potential therapy for ASD. The BTBR inbred mouse strain is a commonly used model of ASD as it demonstrates robust behavioral deficits consistent with the diagnostic criteria for ASD. BTBR mice also exhibit decreased brain-derived neurotrophic factor (BDNF) signaling and reduced hippocampal neurogenesis. In the current study, we evaluated the behavioral and molecular effects of intracerebroventricular MSC transplantation in BTBR mice. Transplantation of MSC resulted in a reduction of stereotypical behaviors, a decrease in cognitive rigidity and an improvement in social behavior. Tissue analysis revealed elevated BDNF protein levels in the hippocampus accompanied by increased hippocampal neurogenesis in the MSC-transplanted mice compared with sham treated mice. This might indicate a possible mechanism underpinning the behavioral improvement. Our study suggests a novel therapeutic approach which may be translatable to ASD patients in the future. ***Autism Res*** *2016, 9: 17–32.* © 2015 International Society for Autism Research, Wiley Periodicals, Inc.

Keywords: BTBR; animal model; MSC; BDNF; neurogenesis

Introduction

Autism spectrum disorders (ASD) are neurodevelopmental disabilities characterized by severe impairment in social communication skills and cognitive flexibility (Blenner, Reddy, & Augustyn, 2011). Although the pathophysiology underlying ASD is still unclear, recent evidences suggest that various molecular dysfunctions are involved. Those include neuroimmune processes (Ashwood et al., 2011; Li et al., 2009), neurogenesis (Wegiel et al., 2010), neurotrophic factor availability (Nickl-Jockschat & Michel, 2011), and glutamate balance (Choudhury, Lahiri, & Rajamma, 2012).

Stem cell transplantation holds therapeutic potential for patients affected with incurable brain disorders (Lindvall & Kokaia, 2006). Stem cell based regenerative therapy was proposed for the treatment of ASD (Ichim et al., 2007) as studies have shown that cell transplantation may affect molecular mechanisms associated with ASD pathophysiology. In light of the above mentioned processes, mesenchymal stem cells (MSC) represent an attractive cell source for regenerative therapy for ASD (Siniscalco, Bradstreet, Sych, & Antonucci, 2014), as studies have shown MSC capacity to improve behavioral endophenotypes of animal models of brain diseases through inducing immunomodulation (Barhum et al., 2010; Chao, He, & Tay, 2009; Kassis et al., 2008; Rafei et al., 2009; Sheikh et al., 2011; Stemberger et al., 2011), enhancing neurogenesis (Bao et al., 2011; Kan, Barhum, Melamed, & Offen, 2011; Snyder, Chiu, Prockop, & Chan, 2010; Tfilin et al., 2010), increasing neurotrophic factor levels (Barzilay et al., 2011; Sadan et al., 2012; Sasaki et al., 2009; Wakabayashi et al., 2010; Wang et al., 2010; Zhang et al., 2011), and improving glutamatergic balance (Bae et al., 2007; Barzilay et al., 2012). We have recently shown that MSC transplantation to the cortex (Barzilay et al., 2011) and to the hippocampus (Barzilay et al., 2012) protects from deficits in social behavior following repeated treatment with the NMDA antagonist phencyclidine.

In a translational perspective, there are significant advantages to utilize MSC, as they enable autologous transplantation thus reducing risks of immune rejection. Moreover, MSC engraft well into the brain, do not

From the Felsenstein Medical Research Center, Sackler Faculty of Medicine, Tel-Aviv University, Tel- Aviv, Israel(H.S., R.B., J.G., I.A., D.O.); Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel(G.K., N.B., L.E., T.K.); Geha Mental Health Center, Petah Tikva, Israel(R.B.)
Received December 14, 2014; accepted for publication July 17, 2015
Address for correspondence and reprints: Daniel Offen, Felsenstein Medical Research Center, Rabin Medical Center, Petah Tikva, Israel 49100. E-mail: danioffen@gmail.com and Tali Kimchi, Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel 76100. E-mail: tali.kimchi@weizmann.ac.il
Hadar Segal, Golan Karvat and Noy Barak contributed equally to this manuscript preparation.
Daniel Offen and Tali Kimchi are co-senior author.
Published online 10 August 2015 in Wiley Online Library (wileyonlinelibrary.com)
DOI: 10.1002/aur.1530

form tumors and fulfill safety criteria upon transplantation to the brain of non-human primates (Isakova, Baker, Dufour, Gaupp, & Phinney, 2006). Importantly, clinical studies evaluating MSC efficacy are currently underway for a variety of brain disorders (Tanna & Sachan, 2014). In the context of ASD, several studies have provided encouraging data on the potential of fetal stem cells as well as umbilical cord- and bone marrow- derived mononuclear cells, which include MSC, in children suffering from ASD (Bradstreet et al., 2014; Lv et al., 2013; Sharma et al., 2013).

Over the last few years, efforts were made to establish a valid animal model for the behavioral deficits observed in ASD (Silverman, Yang, Lord, & Crawley, 2010). Among these models, the BTBR T+tf/J inbred mouse strain is considered a reliable model due to its distinct behavioral patterns resembling ASD phenotypes including impaired social behavior (McFarlane et al., 2008; Moy et al., 2007; Pobbe et al., 2010; Weissbrod et al., 2013), aberrant communication (Scattoni, Ricceri, & Crawley, 2011; Wöhr, Roullet, & Crawley, 2011), increased repetitive behavior (Amodeo, Jones, Sweeney, & Ragozzino, 2012; Karvat & Kimchi, 2012; Pearson et al., 2011), and increased cognitive rigidity (Karvat & Kimchi, 2012; Moy et al., 2007; Rutz & Rothblat, 2012). Importantly, recent studies identified specific aberrations in biochemical phenotypes in BTBR mice, including immune state (Heo, Zhang, Gao, Miller, & Lawrence, 2011), neurogenesis (Stephenson et al., 2011), brain-derived neurotrophic factor (BDNF) signaling (Scattoni, Martire, Cartocci, Ferrante, & Ricceri, 2012), and glutamatergic signaling (Silverman et al., 2012) highlighting the relevance of this model in ASD research. In the current study, we examined the effect of intracerebroventricular MSC transplantation on behavioral and biochemical endophenotypes in the BTBR mouse model of ASD.

Materials and Methods

Animals

BTBR T+tf/J mice were bred from adult pairs originally purchased from The Jackson Laboratory (Bar Harbor, ME). At 6–8 weeks of age, first cohort of littermate male mice were randomly assigned to sham operated (Sham, $n = 13$) and MSC transplanted (MSC, $n = 14$) groups. Mice were housed in a group of 3–5 littermates per cage. The mice were allowed to recover for 3 weeks prior to behavioral examination. An additional cohort of littermate male mice was randomly assigned to sham treated ($n = 11$) and MSC treated ($n = 11$) groups to employ an additional test for social behavior for further validation of our results.

Stranger mice in the three-chamber sociability assay and in the running/ jammed wheel assay were 5 weeks old Hsd:ICR[CD-1] males (Harlan Laboratories, Rehovot, Israel). Social stimulus mice in the dyadic reciprocal social interaction test were 5 weeks old C57BL 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 Weizmann Institute of Science and Tel-Aviv University.

Preparation of Cells and Surgical Procedures

Human MSC were purchased from Lonza (Basel, Switzerland) and cultured and expanded as previously described (Sadan et al., 2012). On treatment day, the cells were harvested, washed, and prepared for transplantation at a concentration of 50,000 cells/μL suspended in 0.9% saline. Under ketamine/xylazine anesthesia the mice were placed in a stereotactic frame (Kopf, Tujunga, CA), and cells or saline for the sham operated group (1 μL/injection site) were injected bilaterally at 0.5 μL/min (Hamilton 701N syringe) to the following coordinates (relative to the bregma and dura): anterior-posterior, −0.35 mm; medial-lateral, ±0.85 mm; dorsal-ventral, −2.3 mm. The needle was withdrawn after 5 min. The lateral ventricles were chosen as the site of transplantation as we sought to enrich the CSF filling the ventricles with neurotrophic factors secreted by hMSC. In addition, this site was chosen due to its proximity to the dentate gyrus of the hippocampus, in which newly born neurons are incorporated as part of neurogenesis.We chose this approach as we hypothesized that the MSC transplantation will exert its therapeutic effect through enhancement of neurogenesis (Kan et al., 2011). Furthermore, intraventricular transplantation was chosen as it translates into intrathecal transplantation in humans, which is a clinically applicable strategy.

To prevent 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.

Behavioral Tests

Open field test. In the open field test, mice were placed in a 40 × 40 × 20 cm cage for 10 min. Total distance moved was measured using the Ethovision software.

Running/jammed wheel assay. This test was previously developed and validated in our lab (Karvat &

Kimchi, 2012; Karvat & Kimchi, 2013). Briefly, mice were put in a transparent Plexiglas cage, sized 30 × 30 × 25 cm and covered with ~0.5 cm soil bedding. A 14 cm diameter plastic running wheel was connected to one of the walls and could either turn or be jammed by a metal pin. In the first 3 days of the experiment, mice were put in the cage with a turning wheel for 25 min. Cumulative duration of digging at the bedding was quantified by an observer blind to treatment in the first 10 min of the first day. Due to high levels of digging preventing the running behavior, the bedding was taken out, and mice had two additional days with a turning wheel, 15 min per day. During the last (5th) running day, cumulative duration of running and self-grooming were quantified during the first 10 min. Next, the wheel was jammed for two consecutive days (Jam1 and Jam2), in which the mice spent 15 min in the chamber. In each day, an observer quantified the time mice spent in any interaction with the wheel that would have made it move was it not blocked. Previous studies of wild-type mice showed that analysis of the initial 10 min of each trial were sufficient to detect the main effects (Karvat & Kimchi, 2012; Karvat & Kimchi, 2013), thus this was the analysis duration. In the last day of the test, a 5 weeks old male Hsd:ICR[CD-1] stranger mouse was put in the apparatus, and both mice were recorded in the chamber for 10 min. Any kind of social contact initiated by the test mouse, as well as interaction with the wheel, were quantified by an observer blinded to the treatment group.

Water T-maze assay. The water T-maze (Guariglia & Chadman, 2013; Karvat & Kimchi, 2013) was a T-shaped Plexiglas chamber, with three arms sized 22 × 11 cm and a center zone sized 11 × 11 cm. The maze 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. Animals had 10 trials during each of the four experiment days. The animals were put in the starting arm facing the wall, and were allowed to swim until finding the platform, or until 90 sec have passed. When the animal mounted the platform, it was allowed to stay on it for 15 sec. If a mouse did not find the platform within 90 sec, it was gently guided to the platform and allowed to stay on it for 15 sec. Inter-trial interval was >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. Starting arm was identical in 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 of releasing the mouse in water until it stood on the platform.

Reciprocal dyadic social interaction test. The reciprocal dyadic social interaction test (Silverman et al., 2012) was conducted using a 5 week old male C57BL/6 stranger mouse 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 recorded for 20 min. The last 10 min were quantified by an observer blind to treatment. Both mice were isolated for 1 hr prior to the test. Social contact initiated by the test mouse was scored using the Observer 8.0XT software (Noldus, ageningen, the Netherlands), and defined by the duration of mice engaging the stranger mouse in social behaviors. Mice 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), push-crawl (i.e. physical contact, including pushing the snout or head underneath the stranger's body, and crawling over or under its body), and alo-grooming (i.e. mutual lick or rub with paws) were observed and quantified.

Three-chamber social assay. The three-chamber social approach and social novelty preference test was designed according to previous reports (Karvat & Kimchi, 2012; Moy et al., 2007). The test consisted of three consecutive stages, 10 min each: (a) Habituation, in which side-chambers were empty; (b) Social approach, in which a wire-cage with an unfamiliar mouse (stranger1) was situated in one side-chamber and an empty, similar cage (object) in the other, and; (c) Preference for social novelty, when an additional unfamiliar mouse (stranger 2; littermate of stranger 1) was placed in the wire cage that had been empty during the previous session. Time sniffing the two cages was scored using the Observer 8.0XT software Noldus, Wageningen, the Netherlands. Locations of different stimuli were counterbalanced between animals.

Computation of autism composite score. Based on a previous study (El-Kordi et al., 2012), scores of each mouse in 6 parameters (a pair of parameters per behavioral symptom) were *Z*-standardized such that higher values represent more severe autism-related behaviors. Relevant measures were selected based on reported abnormal phenotypes of the BTBR strain (Chadman & Guariglia, 2012), and included: (A) Sociability: social preference index in the 3-chamber test calculated as (time with stranger1)/(time with stranger1+time with object) and social preference index in the jammed-wheel test calculated as (time with stranger)/(time with stranger+time with jammed wheel). (B) Cognitive rigidity: ratio between day Jam1 and Run (adjustment to change) in the running/jammed wheel test and duration to reach the platform

in day 3 (reversal learning) of the water T-maze. (C) Stereotypical behavior: digging duration and self-grooming duration. Data from all mice were used with no exclusions. The data from the reciprocal dyadic social interaction test was not included as this test was conducted on a different cohort of mice. Each parameter was intercorrelated to all other readouts (pairwise Pearson correlations), and internal consistency was tested using Cronbach's α on the scores of all mice in the six parameters. Cronbach's $\alpha > 0.6$ (which support high internal consistency of the data) substantiated integration of all measures of each mouse into one score, designated as autism composite score.

Tissue Analysis

Dissection and tissue collection. At the end of the experiment, 8 mice from the sham treated group and 10 mice from the MSC treated group were sacrificed. Immediately thereafter, the prefrontal cortex and hippocampus tissues were dissected, divided into left and right hemispheres and cryopreserved at −80°C. Prefrontal cortex and hippocampus were chosen for examination as these brain areas are implicated in the pathophysiology of autism (Buxhoeveden et al., 2006; Courchesne & Pierce, 2005; Hasan, Walimuni, & Frye, 2013; Wegiel et al., 2010). Dissection of the mice brains was conducted using the Stainless Steel Zivic Adult Mouse Brain Slicer Matrix with 1.0 mm coronal section slice intervals (Zivic Instruments, PA). The frontal cortices were dissected from 2-mm thick slices. Tissues were randomly assigned for further analysis. A single mouse from the sham treated group suffered from severe hematomas in the brain, thus excluded from the analysis, leaving this group with $n = 12$.

BDNF protein level. For protein extraction, dissected brain tissues were thawed, and total protein was produced as previously described (Barzilay et al., 2011). Protein concentration was determined using the bicinchoninic acid kit (Thermo Scientific, Rockford, IL). Quantification of BDNF levels was conducted using a BDNF specific ELISA kit (R&D Systems) according to the manufacturer's instructions. The absorbance at 450 and 570 nm was recorded on a Microplate Reader (Labsystems Multiscan, MS). The results were normalized to total amount of protein. For statistical purposes, samples that were found below the threshold of detection were considered as the lowest detectable sample.

Immunohistochemistry and cell quantification. At the end of the experiment, four animals from each group were transcardially perfused, under ketamine/xylazine anesthesia, with cold phosphate-buffered saline (PBS), followed by paraformaldehyde 4% in phosphate buffer. The brains were immersed in 4% paraformaldehyde for 24 hr at 4°C followed by cryoprotection in 30% sucrose for an additional 48 hr. The brains were frozen in chilled 2-methylbutane (Sigma-Aldrich), stored at −80°C, and subsequently sectioned into slices measuring 10 μm.

For immunohistochemistry, slides underwent citrate buffer (pH = 6) antigen retrieval and were then incubated with blocking solution (5% goat/donkey serum, 1% BSA, 0.5% Triton X-100 in PBS) for 1 hr. Thereafter, slides were incubated overnight at 4°C with the following primary antibodies: mouse anti-human nuclear antigen (1:50 in blocking solution; MAB 1281; Millipore), rabbit anti-Ki67 (1:500 in PBS containing 0.25% TX-100; ab15580; Abcam), goat anti-doublecortin (1:100 in blocking solution; sc-8066; Santa Cruz). Accordingly, sections were incubated with secondary antibodies: goat anti-mouse Alexa 488 (1:500, Molecular Probes, Invitrogen), biotinylated goat anti rabbit followed by streptavidin Alexa 488 (1:300, Molecular Probes, Invitrogen) and donkey anti-goat Alexa 568 (1:300, Molecular Probes, Invitrogen). Secondary antibodies were applied for 1–2 hr at room temperature. Cells were counterstained with DAPI (1:500; Sigma-Aldrich). Finally, sections were mounted with fluorescent mounting solution (Fluoromount-G, SouthernBiotech), covered with a cover slide, and sealed with nail polish.

For microscopic analysis, fluorescence Olympus BX52TF microscope (Olympus, Lake Success, NY) was used. Ki67-positive and doublecortin-positive cells were quantified in six serial coronal sections 360 μm apart per mouse brain, covering the rostro-caudal extension of the dentate gyrus. To obtain an estimate of the total number of labeled cells per dentate gyrus, the total number of cells counted in the selected coronal sections from each brain was multiplied by the volume index (the ratio between the volume of the dentate gyrus and the total combined volume of the selected sections) as previously described (Cardon, Ron-Harel, Cohen, Lewitus, & Schwartz, 2010).

Statistical Analysis

All data sets were screened for normal distribution using Lilliefors test and for homogeneity of variance using the Levene test. Comparisons between treatment groups were conducted using two-tailed Student's *t*-tests or 2-way ANOVA, as appropriate for each assay. Tukey's multiple comparison post-hoc tests and Welch's corrections were applied, where appropriate. Between groups comparisons of non-normal data were conducted by Mann–Whitney tests. Statistical significance was set at $\alpha < 0.05$, when $P < 0.05$ was considered significant, using the Statistica software (Statsoft, Tulsa, OK).

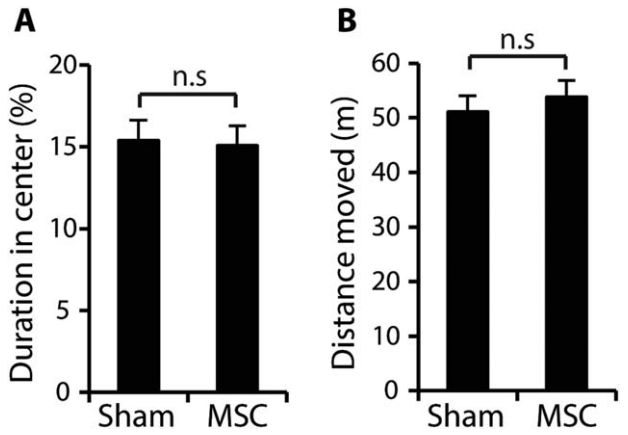

Figure 1. No differences in exploration and anxiety related behaviors following MSC transplantation in BTBR mice. (A) Percentage of time spent in the center of the arena. (B) Total distance traveled. n.s: not significant. Sham: saline-treated mice, $n = 12$. MSC: human mesenchymal stem-cells implanted mice, $n = 14$. Data presented as mean + SEM.

Results

No Differences in Anxiety-Related Behaviors and Locomotion

No differences were found between the MSC- and sham-treated groups in anxiety-related behavior (Fig. 1A, percentage of time in the center of the testing chamber: $t_{24} = 0.19$, $P = 0.85$) and basal locomotion (Fig. 1B, total distance moved, $t_{24} = 0.56$, $P = 0.58$). Thus, MSC transplantation did not affect gross motor activity and anxiety-like behavior.

Amelioration of Stereotyped Repetitive Behaviors

BTBR mice transplanted with MSC into the cerebral ventricles exhibited significantly lower levels of stereotypical behaviors compared to their sham-treated littermates, in both measures tested; 46% reduction in digging duration (Fig. 2A, $t_{24} = 2.99$, $P < 0.01$) and 60% reduction in self-grooming duration (Fig. 2C, $Z_{24} = 2.23$ in Mann-Whitney test, $P < 0.05$). Additionally, the latency to onset of repetitive behaviors was significantly longer in the MSC-treated group (digging: $t_{24} = 4.04$, $P < 0.001$, Fig. 2B; grooming: $t_{24} = 2.71$, $P < 0.05$, Fig. 2D).

Decrease in Cognitive Rigidity

MSC-transplanted mice showed improvement in their flexibility to adjust to changes in the environment as evaluated by two independent behavioral measures; the water T-maze assay which tested adjustment to reversal spatial learning (Fig. 3A), and the running/jammed wheel assay which tested, after 5 days of rewarding wheel-running activity, the ability to adjust to jamming of the wheel (Fig. 3D).

In the water T-maze assay, both groups showed similar degrees of spatial learning and memory, as manifested by similar durations to reach the escape platform in each trial of the first and second days of the task (Day 1: Sham: 7.91 ± 0.59 sec, MSC: 7.26 ± 0.41 sec, $F_{9,216} = 0.56$, $P = 0.832$, Day 2: Sham: 4.08 ± 0.34 sec, MSC: 4.49 ± 0.25 sec $F_{9,216} = 0.64$, $P = 0.765$). In the first trial of day 3, when the platform's location was changed to the opposite arm, all sham-treated (12/12) and most MSC-treated mice (12/13) took the first turn into the arm which contained the platform in day 2. However, MSC-treated mice re-entered the wrong arm significantly less times than sham-treated (MSC: 1.2 ± 0.1 re-entries, Sham: 2.3 ± 0.9, $F_{9,216} = 5.05$, $P < 0.01$, ANOVA for repeated measures with Tukey's correction) and swam for significantly shorter distance (MSC: 141.6 ± 33.9 cm, Sham: 251.5 ± 104.4, $F_{9,216} = 4.44$, $P < 0.05$). The swim velocity of both groups was comparable (MSC: 13.7 ± 1.0 cm/sec, Sham:

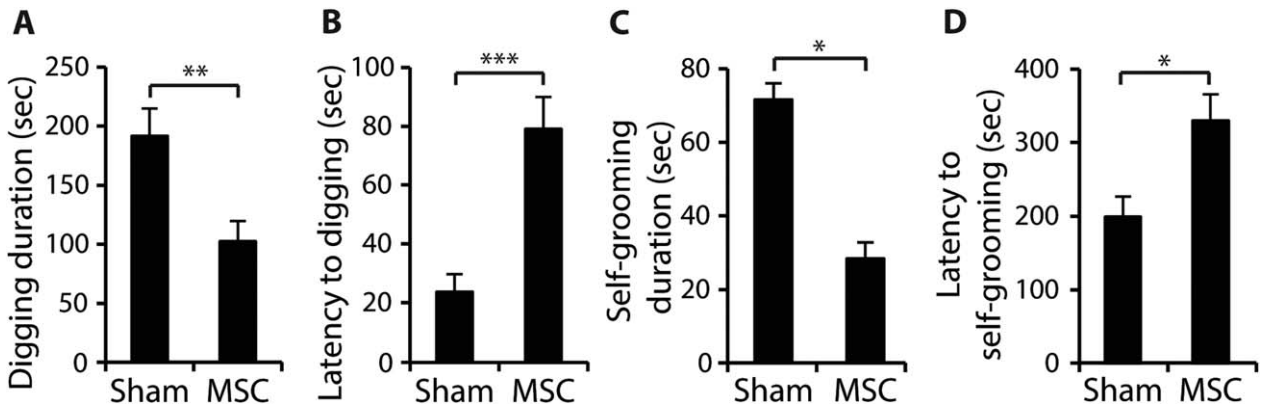

Figure 2. MSC transplantation ameliorated repetitive behaviors in BTBR mice. (A) Total duration and (B) latency to onset of digging at the bedding were quantified in a 10-min period. (C) Total duration and (D) latency to onset of self-grooming were quantified in a 10-min period. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, in Student's t-test (A, B, and D) or Mann-Whitney test (C). Sham: saline-treated mice, $n = 12$. MSC: human mesenchymal stem cells transplanted mice, $n = 14$. Data presented as mean + SEM.

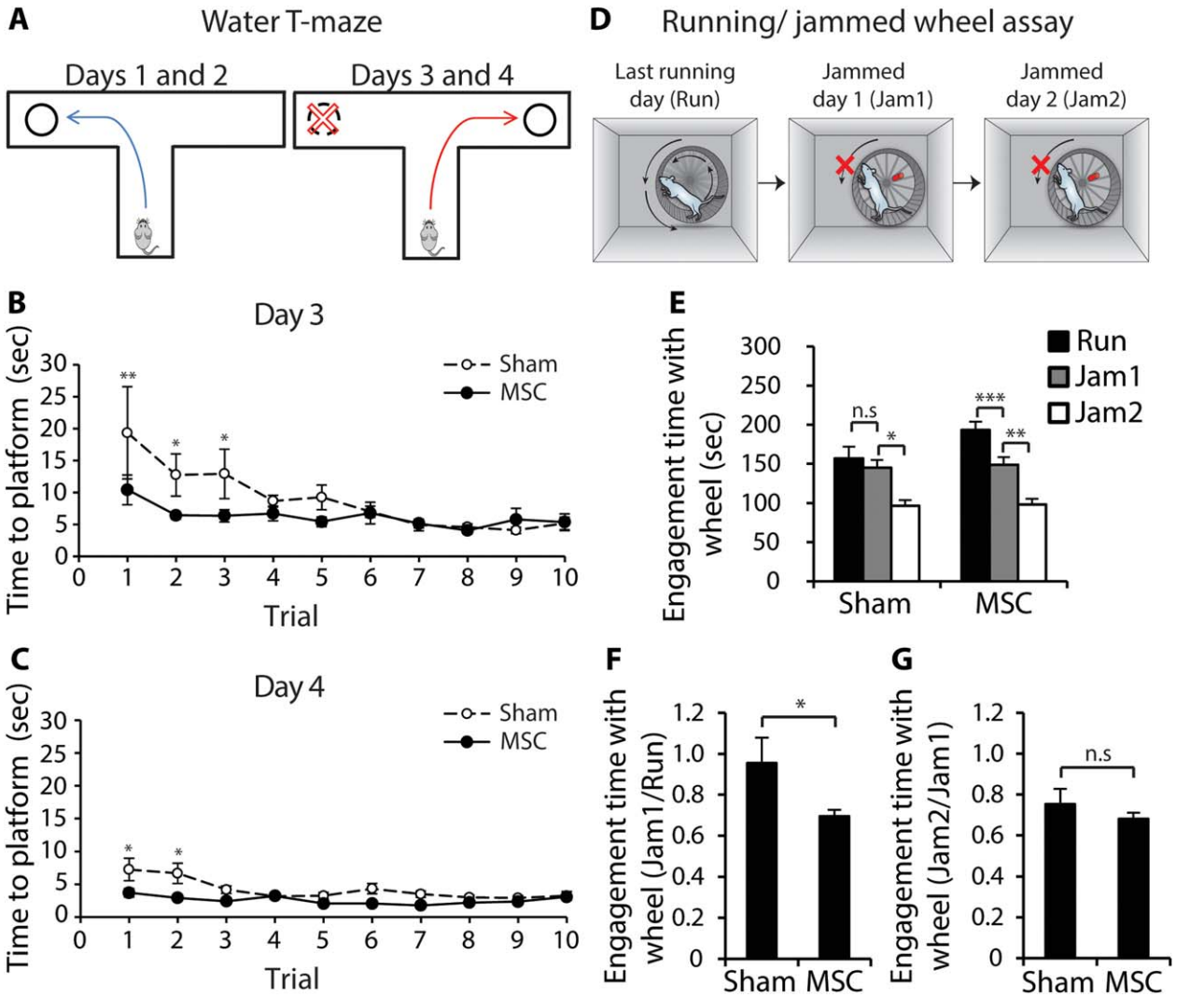

Figure 3. MSC transplantation decreased cognitive rigidity in BTBR mice. (A) Scheme of the water T-maze test. (B–C) Latency to reach the escape platform in each trial on the reversal-learning days. (D) Scheme of the running/ jammed wheel assay. Running duration during a 10-min period was quantified in the last running day (Run, E). Then, the wheel was jammed, and engagement time with the jammed-wheel within a 10-min period was quantified in two consecutive days (Jam1 and Jam2, E). (F) The ratio between engagement times in days Jam1 and Run represents adjustment to the change. (G) The ratio between engagement times in days Jam2 and Jam1 represents memory of the change. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s: not significant, in 2-way repeated measures ANOVA with Tukey's post-hoc analysis (B–C, E) or Student's *t*-test with Welch correction (F–G). Sham: saline-treated mice, $n = 12$. MSC: human mesenchymal stem-cells transplanted mice, $n = 14$. Data presented as mean ± SEM (B–C) or mean + SEM (E–G).

12.2 ± 0.6, $F_{9,216} = 1.49$, $P = 0.15$), yet in the first, second and third trials MSC-treated mice arrived significantly faster to the escape platform as compared to sham-treated mice (Fig. 3B, $F_{9,216} = 4.92$, $P < 0.001$). Taken together, these results indicate improved adjustment to change of the MSC-treated group.

On the 4th day of the task, when the platform's location was in the same location as on day 3, both groups showed a similar ability to remember the change with a minor advantage to the MSC-treated mice in the first two trials (Fig. 3C, $F_{9,216} = 2.20$, $P < 0.05$).

Improvement in adjustment to change was evident in MSC-treated mice also in the running/jammed wheel assay (Fig. 3D). In this test, cognitive rigidity is assessed by habituating mice to the innately rewarding wheel running behavior, and then blocking the wheel for two consecutive days. As previously reported (Karvat & Kimchi, 2012; Karvat & Kimchi, 2013), neurotypical mice are expected to reduce engagement time with the wheel when it is jammed. In the current study, sham-treated BTBR mice exhibited cognitive rigidity, as they spent similar periods of time engaging with the wheel in the last running day and the first jammed day (Fig. 3E). In contrast, MSC-treated mice exhibited a significant reduction in engagement time with the jammed wheel (Fig. 3E, significant effects for day: $F_{2,48} = 48.3$,

$P < 0.001$ and interaction between day and treatment: $F_{2,48} = 9.0$, $P < 0.001$). Indeed, sham-treated mice tended to be less engaged with the wheel in the Run day, most likely due to their increased grooming duration, as time mice from both groups engaged in behaviors other than running or grooming was similar (MSC: 357.0 ± 15.9 sec, Sham: 384.3 ± 15.8, $t_{24} = 1.16$, $P = 0.25$). Nonetheless, the ratio between engagement times in the last running day and the first jammed day of each mouse is smaller than 1 for neurotypical mice (Karvat & Kimchi, 2012; Karvat & Kimchi, 2013; Gordon & Corbitt, 2015), thus serves as an indicator of cognitive rigidity. This analysis revealed a significantly improved flexibility in MSC-treated mice (Fig. 3F, $t_{24} = 2.07$, $P < 0.05$ after Welch correction). Both groups showed a reduction in engagement times between the first and second jammed days, with no difference in the ratio between days (Fig. 3G, $t_{24} = 0.91$, $P = 0.366$ after Welch correction), indicating similar memory of the change.

Improvement in Social Behavior

MSC-transplanted mice showed improvement in sociability as evaluated by three independent behavioral measures.

In the dyadic reciprocal social interaction test (Fig. 4A), MSC-treated mice spent significantly more time initiating social interaction as compared with sham-treated mice (Fig. 4B, $t_{20} = 2.197$, $P < 0.05$).

In the three-chamber test, both groups spent similar amounts of time in each side-chamber during a 10 min habituation trial to the apparatus ($P > 0.45$ for each group). In the social approach trial (Fig .4C), sham-treated BTBR mice spent significantly more time in proximity to the empty cage compared with the stranger's cage (Fig. 4D, effect of interaction between treatment and stimulus: $F_{1,24} = 6.52$, $P < 0.05$), as previously reported (Blanchard et al., 2012). Post-hoc analysis revealed that MSC-treated mice spent significantly more time investigating the stranger's cage compared to sham-treated mice ($P < 0.01$, Fig. 4D), indicating enhanced social approach of the MSC-treated mice, but not enhanced social preference as they spent a similar amount of time in proximity to the stranger's cage and the empty cage.

Both treatment groups exhibited a preference toward social novelty (Fig. 4F, effect of stimulus (stranger 1 vs. stranger 2): $F_{1,23} = 17.26$, $P < 0.001$), with no significant effect of interaction between treatment and stimulus: $F_{1,23} = 0.58$, $P = 0.45$). However, total sniffing time of both mice (stranger 1 + stranger 2) was significantly higher for the MSC-treated group ($F_{1,23} = 5.20$, $P < 0.05$). Of note, one stranger mouse escaped from the wire-cage leading to omission of this trial from statistical analysis.

Social preference was also evaluated in the running/jammed wheel assay (Fig. 4G). Once a stranger mouse was introduced into the cage, social interaction duration with the stranger mouse was quantified and compared to the interaction duration with the jammed-wheel. Analysis of variation (ANOVA) revealed that interaction with the stimulus (the jammed wheel vs. the stranger) had a significant main effect ($F_{1,48} = 18.79$, $P < 0.001$), with Fisher LSD post-hoc analysis indicating a significant ($P < 0.001$) preference of the MSC-treated mice to interact with the stranger mouse over the object (Fig. 4H). In contrast, sham-treated BTBR mice did not prefer the social stimulus over the wheel ($P > 0.05$).

Improvement in Autism Severity Composite Score

In order to define the overall change in autism-related behavioral measures following the MSC treatment, we have first calculated a standardized score (Z-score) for all measured parameters. Following Z-score standardization for each measure all readouts could be integrated into a single value referred to as "autism composite score" (El-Kordi et al., 2012; Wöhr & Scattoni, 2013). In all Z-standardized behavioral readouts the MSC-treated mice were significantly lower than that of the sham-treated mice (i.e. lower severity in autistic symptoms in the MSC-treated group) (Fig. 5A). Furthermore, the overall autism composite score was significantly different between the treated and sham groups—indicating lower autism-related behavioral abnormality in the MSC-treated group as compared to the sham-treated group (Fig. 5B, Mann-Whitney $U = 5$, $P < 0.001$).

Elevation of BDNF Levels in the Hippocampus

BDNF levels in the hippocampus and frontal cortex were measured using ELISA. Analysis of hippocampal tissue revealed a significant increase of 36% in BDNF levels in the MSC-treated group compared to sham-treated mice ($t_8 = 2.311$, $P < 0.05$, Fig. 5C). The levels of hippocampal BDNF significantly correlated with the autism composite score of each mouse ($R = 0.799$, $P < 0.01$, Fig. 5D). In addition, there was a trend of increased cortical BDNF levels (102% increase) compared to the sham-treated group ($t_{11} = 2.16$, $P = 0.053$, Fig. 5E). The levels of cortical BDNF significantly correlated with the autism composite score ($R = 0.544$, $P < 0.05$, Fig. 5F).

Enhanced Hippocampal Neurogenesis

Immunofluorescence analysis revealed increased neural stem cell proliferation in the dentate gyri of the MSC-treated group compared to the sham-treated group as

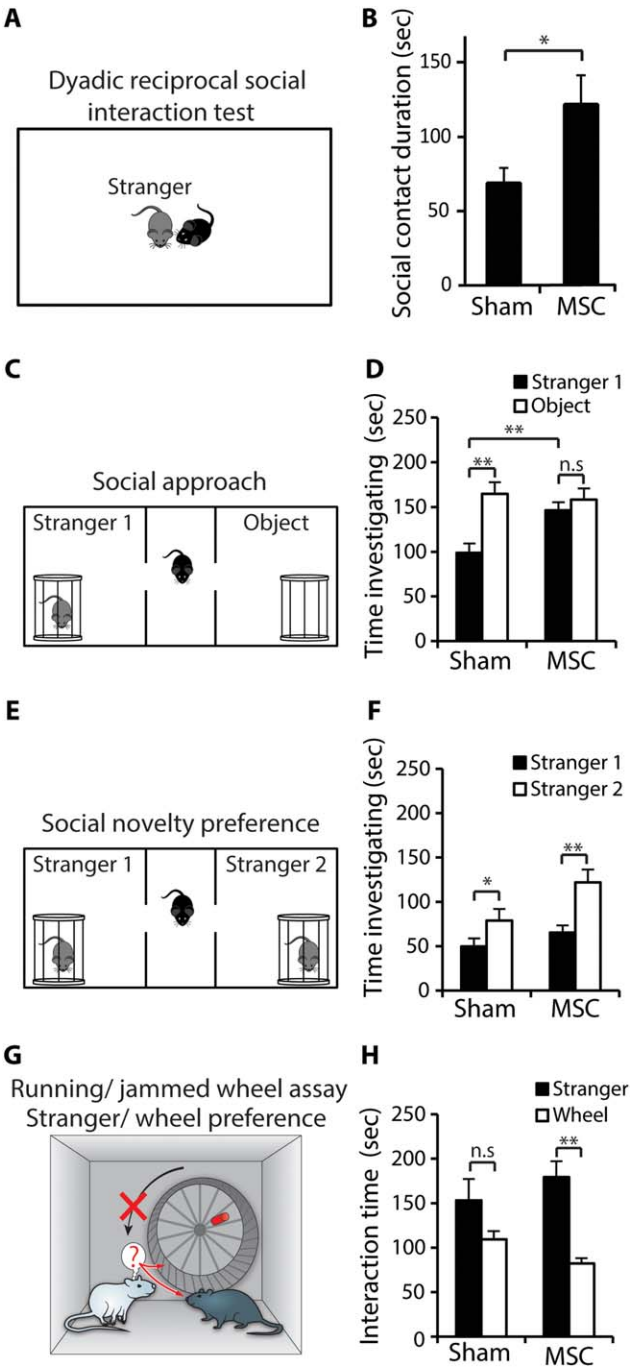

Figure 4. MSC transplantation improved social behavior in BTBR mice. (A) Scheme of the dyadic reciprocal social interaction test, (B) Quantification of the duration of social contact initiated by the test mouse. (C,E) Scheme of the 3-chamber assay. (D) Quantification of sniffing durations of each wire cage during the social approach trial, (F) Quantification of sniffing durations of each wire cage during the social novelty preference trial. In the running/jammed wheel assay (G) the preference of interaction with a stranger mouse over an object was measured by comparing interaction time with a stranger mouse to interaction time with the jammed-wheel (H). $*P < 0.05$, $**P < 0.01$, n.s: not significant in 2-way ANOVA with Tukey's post-hoc (D,F,H) or Student's *t*-test (B). Sham: saline-treated mice, $n = 12$ (D,F,H) or $n = 11$ (B). MSC: human mesenchymal stem-cells transplanted mice, $n = 13$ (F) or $n = 14$ (D,H), or $n = 11$ (B). Data is presented as mean + SEM.

observed by a 34% increase in Ki67-positive cells (Fig. 6A–C, $t_6 = 11.93$, $P < 0.001$). There was also an 19% increase in cells expressing the early neuronal differentiation marker doublecortin in the dentate gyri of the MSC-treated mice (Fig. 6E–G, $t_6 = 7.23$, $P < 0.001$). Importantly, Ki67-positive cells and doublecortin-

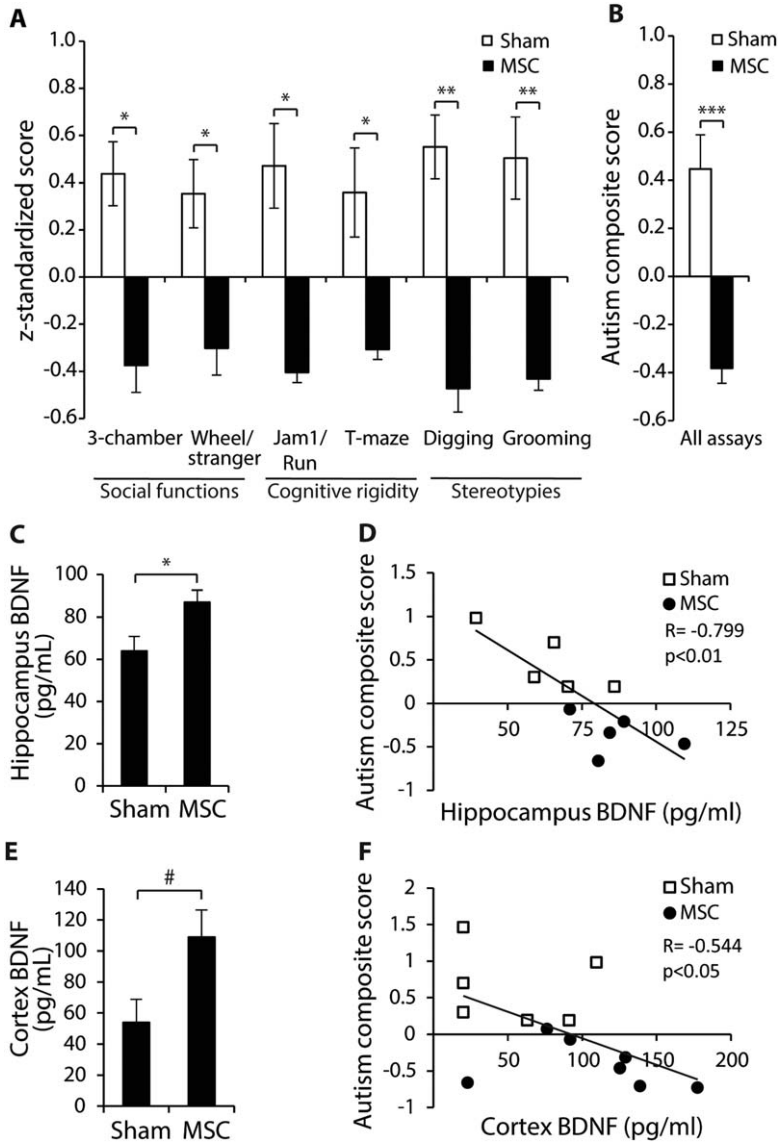

Figure 5. Effect of MSC transplantation on the autism severity auism score and correlation with hippocampal and cortical BDNF levels. (A) Two different measures of each autism symptoms were z-standardized, such that higher values represent more severe autism-related behavioral phenotype. All of the measures are described in detail in the 'materials and methods' section. (B) All measures of each mouse were integrated into a single value, the autism composite score. The higher the value the autism composite score the more severe the autistic-related behavioral phenotype. (C,E) Mean + SEM protein levels of BDNF in the hippocampus (C) and frontal cortex (E). (D,F) Scatter presentation of the correlation between hippocampal (D) and cortical (F) BDNF levels to the autism composite score of each mouse. #$P = 0.053$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Sham: saline-treated mice, $n = 12$ (A–B) or $n = 5$ (C–D) or $n = 6$ (E–F) . MSC: human mesenchymal stem-cells transplanted mice, $n = 14$ (A–B) or $n = 5$ (C–D) or $n = 7$ (E–F).

positive cell count also correlated with the behavioral autism composite score of each mouse (Fig. 6D, $R = 0.822$, $P < 0.05$ and Fig. 6H, $R = 0.855$, $P < 0.01$, respectively).

Detection of MSC Six Weeks After Transplantation

At 6 weeks post transplantation of human MSC, human nuclei (HuNu) -positive cells were identified adjacent to the wall of the dorsal third ventricle of transplanted BTBR mice (Fig. 7).

Discussion

In this study, we describe behavioral and molecular beneficial effects of MSC transplantation in an ASD animal model. We report that a single MSC transplantation in young adult mice induced significant

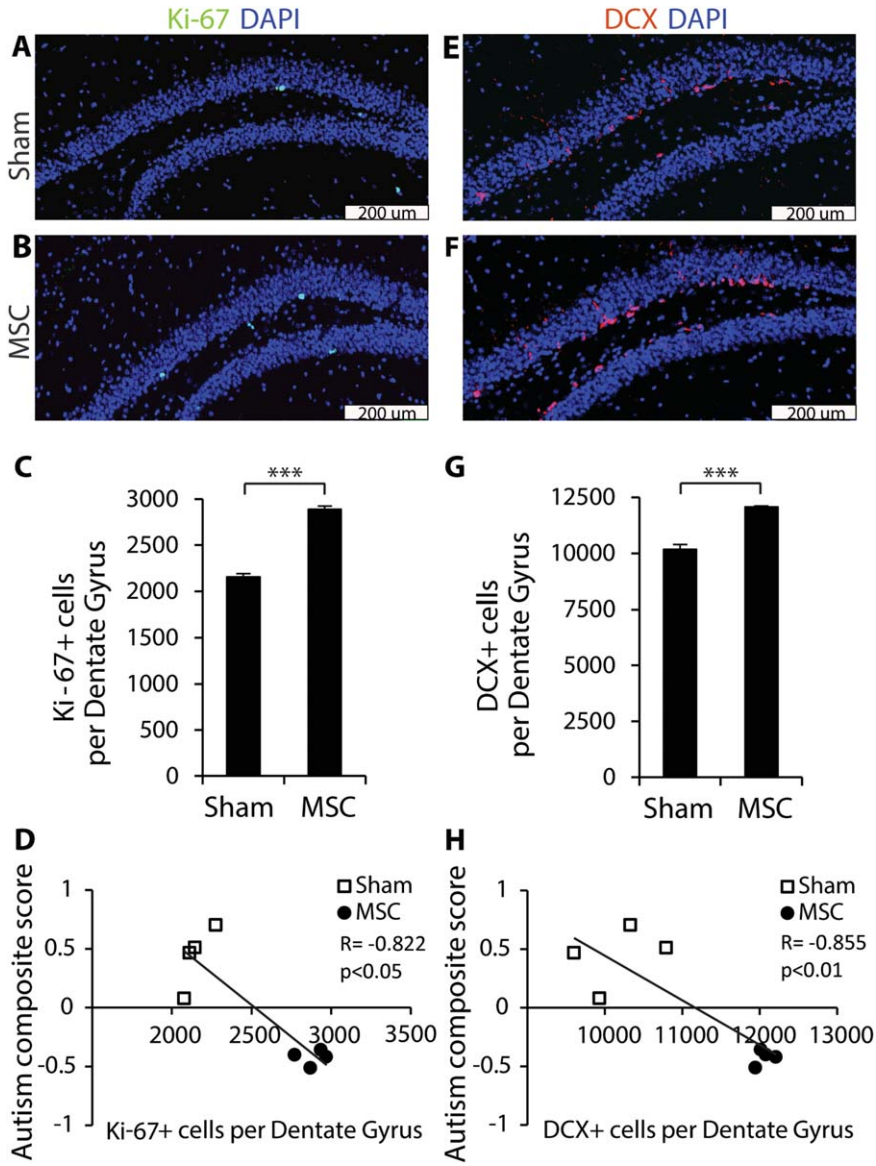

Figure 6. Effects of MSC transplantation on hippocampal neurogenesis and correlation with autism composite score. (A–B) Representative photos of Ki67-positive cells (green) and (C) mean+SEM number of Ki67-positive cells in the dentate gyrus of the hippocampus of sham and MSC treated mice. (E–F) Representative photos of doublecortin (DCX)-positive cells (red) and (G) mean + SEM number of doublecortin-positive cells in the dentate gyrus of the hippocampus of sham and MSC treated mice. (D, H) Scatter presentation of the correlation between Ki67-positive or doublecortin-positive cell count and the autism composite score of each mouse, respectively. $***P < 0.001$. Sham: saline-treated mice, $n = 4$ (C–D,G–H). MSC: human mesenchymal stem-cells transplanted mice, $n = 4$ (C–D,G–H).

improvement in three autism-related behavioral domains manifested as a reduction in repetitive behaviors, increased cognitive flexibility and improved sociability. Importantly, each ASD-like domain was tested in at least two distinct and complementary measures, contributing to the validity of our findings.

In autistic patients, lower-order repetitive behaviors include stereotyped motor movements and repetitive self-injurious behaviors (Lewis, Tanimura, Lee, & Bodfish, 2007). Here, we tested two complementary measures of this symptom- stereotypical digging in the bedding and repetitive self-grooming, both found elevated in BTBR mice (Amodeo et al., 2012; Karvat & Kimchi, 2013; McFarlane et al., 2008; Moy et al., 2008; Pearson et al., 2011). A significant reduction was found in both measures in mice treated with MSC. This reduction was not due to change in general activity, as no differences were found in the open-field test.

Autism is characterized also by the occurrence of higher-order cognitive rigidity, including insistence on sameness and circumscribed interests (Lewis et al., 2007). We have recently developed a test to measure

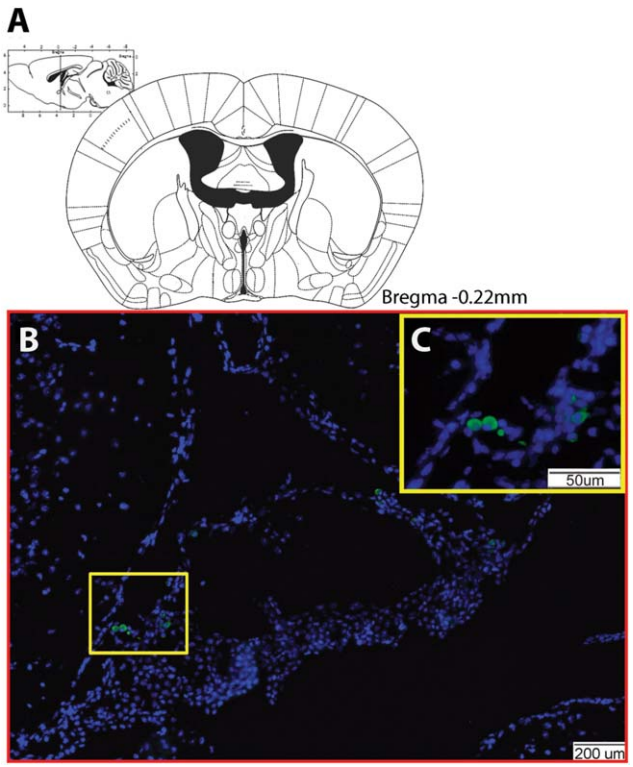

Figure 7. Identification of human MSC in the brain of mice 6 weeks after intracerebroventricular transplantation. (A) Illustration of the anatomic location of the coronal section of the mouse brain, modified from Franklin and Paxinos mouse brain stereotaxic atlas (1997). (B) Anti-human nuclei (HuNu) positive cells (green) counterstained for DAPI nucleic acid staining (blue) identified adjacent to the wall of the dorsal third ventricle of a transplanted BTBR mouse. (C) Higher magnification of human MSC found in the area marked with yellow rectangle in B. Scale bar in B = 200 μm and in C = 50 μm. DAPI: 4′,6-diamidino-2-phenylindole.

these behaviors, the running/jammed wheel assay, in which BTBR mice were found cognitively rigid (Karvat & Kimchi, 2012; Karvat & Kimchi, 2013). Treatment with MSC led to improvement in adjustment to change manifested in decreased interaction time with the no-longer-rewarding jammed wheel. Taking into consideration that this is a novel test, we also utilized a more validated approach to measure change in routine by reversal learning of a spatial task, the water T-maze assay, in which BTBR mice were found deficient (Crawley, 2007; Dong et al., 2005; Guariglia, Jenkins, Chadman, & Wen, 2011; Guariglia & Chadman, 2013). BTBR mice treated with MSC exhibited improved adjustment to change in this test as well, manifested in faster reversal learning. Importantly, these results cannot be attributed to learning and memory deficiencies, as shown previously (Amodeo et al., 2012; Guariglia & Chadman, 2013; Karvat & Kimchi, 2013; Moy et al., 2007), as both treatment groups exhibited normal capabilities in the initial learning stages of the test.

Evaluation of social behavior was assessed in three different tests. In the reciprocal dyadic social interaction test, possibly the most straightforward test of social behavior, MSC-treated mice demonstrated a significant increase in social contact duration. In the three-chamber social assay we found a decrease in aversive social behavior, while social preference per se was not observed in both treatment groups. Of note, in agreement with previous studies (Chadman, 2011; Karvat & Kimchi, 2012; McFarlane et al., 2008; Moy et al., 2007), both treatment groups showed no deficiency in social novelty preference. In the third test measuring preference for social interaction with a freely behaving stranger mouse over an object (the wheel)- MSC treatment resulted in increased social preference. It should be noted, that although the 3-chamber assay is considered today to be the "gold-standard" for testing sociability, as the stimulus stranger is confined in a cage, no reciprocal interaction between the mice is possible. As social reciprocity is severely impaired in ASD, we found it imperative to complement the behavioral assessment with tests allowing social interaction. Taken together, these results point to increased sociability following MSC transplantation.

To date, few studies have reported beneficial pharmacological interventions in BTBR mice. One study reported the beneficial effect of fluoxetine, a selective serotonin re-uptake inhibitor, on social behavior in BTBR (Chadman, 2011), without referring to the effect on repetitive behaviors and aspects of cognition. Silvermann et al. (Silverman, Tolu, Barkan, & Crawley, 2010) described an ameliorating effect of the mGluR5 antagonist MPEP on repetitive behavior but no improvement in sociability. In another study (Silverman et al., 2012), treatment with a negative allosteric modulator of mGluR5 resulted in a reduced self grooming and increased sociability in BTBR mice, with no reported effect on cognitive deficits. A more recent study (Silverman, Oliver, Karras, Gastrell, & Crawley, 2013) reported that administration of a positive modulator of the AMPA receptor, AMPAKINE, improves some aspects of social behavior as observed in the three-chamber assay and induces an improvement in novel object recognition – but had no effect on repetitive behavior. Other studies investigated the effect of risperidone, an atypical antipsychotic with multiple receptor actions that include blockade of serotonin [5-HT] 2A, 2C and 7, and dopamine 2 receptors. Risperidone improved reversal learning in BTBR mice (Amodeo, Jones, Sweeney, & Ragozzino, 2014), but failed to increase sociability and led to a decrease in repetitive self-grooming only in sedative concentrations (Silverman et al., 2010). A different approach showed that administration of low-doses of

benzodiazepines in order to increase inhibitory neurotransmission through positive allosteric modulation of postsynaptic $GABA_A$ receptors, improved social behavior, repetitive behavior and spatial learning without referring to the core symptom of cognitive rigidity (Han, Tai, Jones, Scheuer, & Catterall, 2014). Finally, we have recently shown that alleviation of available acetylcholine in the synaptic cleft results in improved cognitive flexibility and sociability, but does not affect repetitive behaviors (Karvat & Kimchi, 2013). This difficulty to find therapeutic agents capable of facilitating the three core symptoms (repetitive behavior, cognitive rigidity and social behavior) emphasizes the beneficial potential of MSC transplantation, since treated mice showed improvement in all criteria tested.

In the current work, we have utilized human MSC rather than murine MSC since bone-marrow derived MSC from the BTBR strain have not yet been characterized. This is a major limitation in working with mouse derived MSC as it was previously shown that the MSC properties largely differ across different strains (Peister et al., 2004). Moreover, in contrast to murine MSC, human MSC isolated from bone marrow are not contaminated by hematopoietic progenitors that overgrow the cultures (Meirelles Lda & Nardi, 2003), which makes them much more favorable to work with. Furthermore, our group has demonstrated in several studies that human MSC are beneficial in treating mouse models of various brain disorders (Barhum et al., 2010; Barzilay et al., 2011).

Importantly, in addition to the behavioral effect of MSC transplantation, tissue analysis of the MSC-treated mice revealed increased hippocampal BDNF levels compared to sham-treated mice. Notably, BDNF was linked to the pathophysiology of ASD in human patients (Raznahan et al., 2009). In animal models, studies have shown that BDNF plays a cardinal role in the formation of intact social recognition (Broad, Mimmack, Keverne, & Kendrick, 2002), and that decreased BDNF levels may explain in part the social deficit displayed by a mouse model of Rett syndrome (Schaevitz, Moriuchi, Nag, Mellot, & Berger-Sweeney, 2010), and by the valproic acid rodent model of autism (Roullet, Wollaston, deCatanzaro, & Foster, 2010). A recent study described mice with a mutant Ca^{+2}-dependent activator protein for secretion 2 (Caps2), which results in reduced BDNF secretion from axons, as a model for autism-like behavior (Sadakata et al., 2012). Importantly, reduced levels of BDNF mRNA (Stephenson et al., 2011), BDNF protein and BDNF receptor (Scattoni et al., 2012) were found in the hippocampus of BTBR mice. Although several autism rodent models exhibit reduced BDNF expression in adulthood, it is of interest to note that an increase of BDNF mRNA and protein levels was reported in the fetal brain of the valproic acid mouse model of autism (Almeida, Roby, & Krueger, 2014), suggesting that either a decrease or an increase in BDNF signaling could disrupt normal function of the developing and adult nervous system.

In the current study, we have utilized MSC capacity to enhance neurogenesis (Tfilin et al., 2010), as observed with increased Ki67-positive cells and doublecortin-positive neurons in the dentate gyrus of the hippocampus. Aberrant neurodevelopment stands at the base of ASD pathophysiology (Pescosolido, Yang, Sabbagh, & Morrow, 2012; Veenstra-VanderWeele & Warren, 2014). Post mortem studies have indicated that ASD patients display pathologic neuroanatomical changes pointing to defects in neurogenesis (Wegiel et al., 2010). Moreover, genetic studies conducted on human tissue have highlighted the role of neurogenesis as a key biological process involved in ASD pathophysiology (Hussman et al., 2011; Kumar et al., 2011; Tsigelny, Kouznetsova, Baitaluk, & Changeux, 2013). In that context, BTBR mice provide a useful model as recent studies describe extensive neuroanatomical differences from C57BL/6 mice (a commonly used control strain) (Ellegood, Babineau, Henkelman, Lerch, & Crawley, 2013) and alterations in connective tissue/vasculature and associated extracellular matrix in a neurogenic niche of these mice (Mercier, Cho Kwon, & Kodama, 2011), alongside with histopathological findings indicating reduced hippocampal neurogenesis (Stephenson et al., 2011). As BDNF signaling orchestrates neurogenesis (Bath, Akins, & Lee, 2012; Lee & Son, 2009), we postulate that MSC induced increase in BDNF levels may underpin the increase in neurogenesis of MSC-treated mice, thus leading to the behavioral improvement.

In light of recent clinical studies providing promising evidences for safety and efficacy of MSC transplantation in children suffering from autism, there is a necessity for preclinical models of MSC therapy for ASD to elucidate the restorative mechanisms involved in the beneficial outcome (Siniscalco et al., 2014). In this work, we demonstrated the potential of MSC treatment in an animal model for ASD and provided novel data relevant to the possible mechanisms in which MSC transplantation may act to trigger brain changes that could restore normal behavioral phenotype. Specifically, we showed that MSC-dependent BDNF increase is associated with enhanced neurogenesis. Future studies to investigate this mechanism and other possible mechanisms in ASD models are warranted in order to pave the way for the translation of MSC treatment to ASD patients.

Acknowledgments

We thank M. Dayan and I. Sofer for their assistant with the experiments. This research was supported by the National Institute for Psychobiology in Israel (R.B and

D.O.), Nella and Leon Benoziyo Center (T.K.) and Joan and Jonathan Birnbach foundation (T.K.). This work was performed in partial fulfillment of the requirements for a Ph.D. degree of Hadar Segal, Sackler Faculty of medicine, Tel Aviv University, Israel. All authors declare no potential conflicts of interests. D.O. serves as a consultant of Brainstorm Cell Therapeutics.

References

Almeida, L.E.F., Roby, C.D., & Krueger, B.K. (2014). Increased BDNF expression in fetal brain in the valproic acid model of autism. Molecular and Cellular Neuroscience, 59, 57–62. doi:10.1016/j.mcn.2014.01.007

Amodeo, D.A., Jones, J.H., Sweeney, J.A., & Ragozzino, M.E. (2012). Differences in BTBR T+ tf/J and C57BL/6J mice on probabilistic reversal learning and stereotyped behaviors. Behavioural Brain Research, 227, 64–72. doi:10.1016/j.bbr.2011.10.032

Amodeo, D.A., Jones, J.H., Sweeney, J.A., & Ragozzino, M.E. (2014). Risperidone and the 5-HT2A receptor antagonist M100907 improve probabilistic reversal learning in BTBR T + tf/J mice. Autism Research : Official Journal of the International Society for Autism Research, 7, 555–567. doi:10.1002/aur.1395

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I., & Van de Water, J. (2011). Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. Brain, Behavior, and Immunity, 25, 40–45. doi:10.1016/j.bbi.2010.08.003

Bae, J.S., Han, H.S., Youn, D.H., Carter, J.E., Modo, M., Schuchman, E.H., & Jin, H.K. (2007). Bone marrow-derived mesenchymal stem cells promote neuronal networks with functional synaptic transmission after transplantation into mice with neurodegeneration. Stem Cells (Dayton, Ohio), 25, 1307–1316. doi:10.1634/stemcells.2006-0561

Bao, X., Wei, J., Feng, M., Lu, S., Li, G., Dou, W., et al. (2011). Transplantation of human bone marrow-derived mesenchymal stem cells promotes behavioral recovery and endogenous neurogenesis after cerebral ischemia in rats. Brain Research, 1367, 103–113. doi:10.1016/j.brainres.2010.10.063

Barhum, Y., Gai-Castro, S., Bahat-Stromza, M., Barzilay, R., Melamed, E., & Offen, D. (2010). Intracerebroventricular transplantation of human mesenchymal stem cells induced to secrete neurotrophic factors attenuates clinical symptoms in a mouse model of multiple sclerosis. Journal of Molecular Neuroscience : MN, 41, 129–137. doi:10.1007/s12031-009-9302-8

Barzilay, R., Ben-Zur, T., Sadan, O., Bren, Z., Taler, M., Lev, N., et al. (2011). Intracerebral adult stem cells transplantation increases brain-derived neurotrophic factor levels and protects against phencyclidine-induced social deficit in mice. Translational Psychiatry, 1, e61. doi:10.1038/tp.2011.64

Barzilay, R., Ganz, J., Sadan, O., Ben-Zur, T., Bren, Z., Hinden, N., et al. (2012). Mesenchymal stem cells protect from subchronic phencyclidine insult in vivo and counteract changes in astrocyte gene expression in vitro. European Neuropsychopharmacology, 23, 1115–1123. doi:10.1016/j.euroneuro.2012.10.002

Bath, K.G., Akins, M.R., & Lee, F.S. (2012). BDNF control of adult SVZ neurogenesis. Developmental Psychobiology, 54, 578–589. doi:10.1002/dev.20546

Blanchard, D.C., Defensor, E.B., Meyza, K.Z., Pobbe, R.L., Pearson, B.L., Bolivar, V.J., et al. (2012). BTBR t+tf/J mice: Autism-relevant behaviors and reduced fractone-associated heparan sulfate. Neuroscience and Biobehavioral Reviews, 36, 285–296. doi:10.1016/j.neubiorev.2011.06.008

Blenner, S., Reddy, A., & Augustyn, M. (2011). Diagnosis and management of autism in childhood. BMJ (Clinical Research Ed.), 343, d6238. doi:10.1136/bmj.d6238

Bradstreet, J.J., Sych, N., Antonucci, N., Klunnik, M., Ivankova, O., Matyashchuk, I., et al. (2014). Efficacy of fetal stem cell transplantation in autism spectrum disorders: An open-labeled pilot study. Cell Transplantation, 23 Suppl 1, S105–S112. doi:10.3727/096368914X684916

Broad, K.D., Mimmack, M.L., Keverne, E.B., & Kendrick, K.M. (2002). Increased BDNF and trk-B mRNA expression in cortical and limbic regions following formation of a social recognition memory. The European Journal of Neuroscience, 16, 2166–2174.

Buxhoeveden, D.P., Semendeferi, K., Buckwalter, J., Schenker, N., Switzer, R., & Courchesne, E. (2006). Reduced minicolumns in the frontal cortex of patients with autism. Neuropathology and Applied Neurobiology, 32, 483–491. doi:NAN745 [pii]

Cardon, M., Ron-Harel, N., Cohen, H., Lewitus, G. M., & Schwartz, M. (2010). Dysregulation of kisspeptin and neurogenesis at adolescence link inborn immune deficits to the late onset of abnormal sensorimotor gating in congenital psychological disorders. Molecular Psychiatry, 15, 415–425. doi:10.1038/mp.2009.66

Chadman, K.K. (2011). Fluoxetine but not risperidone increases sociability in the BTBR mouse model of autism. Pharmacology, Biochemistry, and Behavior, 97, 586–594. doi:10.1016/j.pbb.2010.09.012

Chadman, K.K., & Guariglia, S.R. (2012). The BTBR t+tf/J (BTBR) mouse model of autism. Autism- Open Access, 01(S1), doi:10.4172/2165-7890.S1-009

Chao, Y.X., He, B.P., & Tay, S.S. (2009). Mesenchymal stem cell transplantation attenuates blood brain barrier damage and neuroinflammation and protects dopaminergic neurons against MPTP toxicity in the substantia nigra in a model of parkinson's disease. Journal of Neuroimmunology, 216, 39–50. doi:10.1016/j.jneuroim.2009.09.003

Choudhury, P.R., Lahiri, S., & Rajamma, U. (2012). Glutamate mediated signaling in the pathophysiology of autism spectrum disorders. Pharmacology, Biochemistry, and Behavior, 100, 841–849. doi:10.1016/j.pbb.2011.06.023

Courchesne, E., & Pierce, K. (2005). Brain overgrowth in autism during a critical time in development: Implications for frontal pyramidal neuron and interneuron development and connectivity. International Journal of Developmental Neuroscience : The Official Journal of the International Society for Developmental Neuroscience, 23, 153–170. doi:S0736-5748(05)00004-3 [pii]

Crawley, J.N. (2007). Mouse behavioral assays relevant to the symptoms of autism. Brain Pathology (Zurich, Switzerland), 17, 448–459. doi:10.1111/j.1750-3639.2007.00096.x

Dong, H., Csernansky, C.A., Martin, M.V., Bertchume, A., Vallera, D., & Csernansky, J. G. (2005). Acetylcholinesterase inhibitors ameliorate behavioral deficits in the Tg2576 mouse model of alzheimer's disease. Psychopharmacology, 181, 145–152. doi:10.1007/s00213-005-2230-6

El-Kordi, A., Winkler, D., Hammerschmidt, K., Kastner, A., Krueger, D., Ronnenberg, A., et al. (2012). Development of an autism severity score for mice using Nlgn4 null mutants as a construct-valid model of heritable monogenic autism. Behavioural Brain Research, 251, 41–49. doi:10.1016/j.bbr.2012.11.016

Ellegood, J., Babineau, B.A., Henkelman, R.M., Lerch, J.P., & Crawley, J.N. (2013). Neuroanatomical analysis of the BTBR mouse model of autism using magnetic resonance imaging and diffusion tensor imaging. NeuroImage, 70, 288–300. doi:10.1016/j.neuroimage.2012.12.029; 10.1016/j.neuroimage.2012.12.029

Gordon, E.A., & Corbitt,C. (2015). Investigation of prepubertal sex differences in wheel running and social behavior in three mouse strains. Journal of Ethology, 1–11. doi:10.1007/s10164-015-0429-5

Guariglia, S.R., & Chadman, K.K. (2013). Water T-maze: A useful assay for determination of repetitive behaviors in mice. Journal of Neuroscience Methods, 220, 24–29. doi:10.1016/j.jneumeth.2013.08.019

Guariglia, S.R., Jenkins, E.C.,Jr, Chadman, K.K., & Wen, G.Y. (2011). Chlorination byproducts induce gender specific autistic-like behaviors in CD-1 mice. Neurotoxicology, 32, 545–553. doi:10.1016/j.neuro.2011.06.008

Han, S., Tai, C., Jones, C.J., Scheuer, T., & Catterall, W.A. (2014). Enhancement of inhibitory neurotransmission by GABAA receptors having alpha2,3-subunits ameliorates behavioral deficits in a mouse model of autism. Neuron, 81, 1282–1289. doi:10.1016/j.neuron.2014.01.016

Hasan, K.M., Walimuni, I.S., & Frye, R.E. (2013). Global cerebral and regional multimodal neuroimaging markers of the neurobiology of autism: Development and cognition. Journal of Child Neurology, 28, 874–885. doi:10.1177/0883073812452917

Heo, Y., Zhang, Y., Gao, D., Miller, V.M., & Lawrence, D.A. (2011). Aberrant immune responses in a mouse with behavioral disorders. PloS One, 6, e20912. doi:10.1371/journal.pone.0020912

Hussman, J.P., Chung, R.H., Griswold, A.J., Jaworski, J.M., Salyakina, D., Ma, D., et al. (2011). A noise-reduction GWAS analysis implicates altered regulation of neurite outgrowth and guidance in autism. Molecular Autism, 2, 1-2392-2-1. doi:10.1186/2040-2392-2-1

Ichim, T.E., Solano, F., Glenn, E., Morales, F., Smith, L., Zabrecky, G., et al. (2007). Stem cell therapy for autism. Journal of Translational Medicine, 5, 30. doi:10.1186/1479-5876-5-30

Isakova, I.A., Baker, K., Dufour, J., Gaupp, D., & Phinney, D.G. (2006). Preclinical evaluation of adult stem cell engraftment and toxicity in the CNS of rhesus macaques. Molecular Therapy : The Journal of the American Society of Gene Therapy, 13, 1173–1184. doi:10.1016/j.ymthe.2005.12.014

Kan, I., Barhum, Y., Melamed, E., & Offen, D. (2011). Mesenchymal stem cells stimulate endogenous neurogenesis in the subventricular zone of adult mice. Stem Cell Reviews, 7, 404–412. doi:10.1007/s12015-010-9190-x

Karvat, G., & Kimchi, T. (2012). Systematic autistic-like behavioral phenotyping of 4 mouse strains using a novel wheel-running assay. Behavioural Brain Research, 233, 405–414. doi:10.1016/j.bbr.2012.05.028

Karvat, G., & Kimchi, T. (2013). Acetylcholine elevation relieves cognitive rigidity and social deficiency in a mouse model of autism. Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 39, 831–840. doi:10.1038/npp.2013.274

Kassis, I., Grigoriadis, N., Gowda-Kurkalli, B., Mizrachi-Kol, R., Ben-Hur, T., Slavin, S., et al. (2008). Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. Archives of Neurology, 65, 753–761. doi:10.1001/archneur.65.6.753

Kumar, A., Swanwick, C.C., Johnson, N., Menashe, I., Basu, S.N., Bales, M.E., & Banerjee-Basu, S. (2011). A brain region-specific predictive gene map for autism derived by profiling a reference gene set. PloS One, 6, e28431. doi:10.1371/journal.pone.0028431

Lee, E., & Son, H. (2009). Adult hippocampal neurogenesis and related neurotrophic factors. BMB Reports, 42, 239–244.

Lewis, M.H., Tanimura, Y., Lee, L.W., & Bodfish, J.W. (2007). Animal models of restricted repetitive behavior in autism. Behavioural Brain Research, 176, 66–74. doi:10.1016/j.bbr.2006.08.023

Li, X., Chauhan, A., Sheikh, A.M., Patil, S., Chauhan, V., Li, X., et al. (2009). Elevated immune response in the brain of autistic patients. Journal of Neuroimmunology, 207(1–2), 111–116. doi:10.1016/j.jneuroim.2008.12.002

Lindvall, O., & Kokaia, Z. (2006). Stem cells for the treatment of neurological disorders. Nature, 441, 1094–1096. doi:10.1038/nature04960

Lv, Y. T., Zhang, Y., Liu, M., Qiuwaxi, J. N., Ashwood, P., Cho, S. C., et al. (2013). Transplantation of human cord blood mononuclear cells and umbilical cord-derived mesenchymal stem cells in autism. Journal of Translational Medicine, 11, 196. doi:10.1186/1479-5876-11-196

McFarlane, H.G., Kusek, G.K., Yang, M., Phoenix, J.L., Bolivar, V.J., & Crawley, J.N. (2008). Autism-like behavioral phenotypes in BTBR t+tf/J mice. Genes, Brain, and Behavior, 7, 152–163. doi:10.1111/j.1601-183X.2007.00330.x

Meirelles Lda, S., & Nardi, N.B. (2003). Murine marrow-derived mesenchymal stem cell: Isolation, in vitro expansion, and characterization. British Journal of Haematology, 123, 702–711. doi:4669 [pii]

Mercier, F., Cho Kwon, Y., & Kodama, R. (2011). Meningeal/vascular alterations and loss of extracellular matrix in the neurogenic zone of adult BTBR T+ tf/J mice, animal model for autism. Neuroscience Letters, 498, 173–178. doi:10.1016/j.neulet.2011.05.014

Moy, S.S., Nadler, J.J., Poe, M.D., Nonneman, R.J., Young, N.B., Koller, B.H., et al. (2008). Development of a mouse test for repetitive, restricted behaviors: Relevance to autism. Behavioural Brain Research, 188, 178–194. doi:10.1016/j.bbr.2007.10.029

Moy, S.S., Nadler, J.J., Young, N.B., Perez, A., Holloway, L.P., Barbaro, R.P., et al. (2007). Mouse behavioral tasks relevant to autism: Phenotypes of 10 inbred strains. Behavioural Brain Research, 176, 4–20. doi:10.1016/j.bbr.2006.07.030

Nickl-Jockschat, T., & Michel, T.M. (2011). The role of neurotrophic factors in autism. Molecular Psychiatry, 16, 478–490. doi:10.1038/mp.2010.103

Pearson, B.L., Pobbe, R.L.H., Defensor, E.B., Oasay, L., Bolivar, V.J., Blanchard, D.C., & Blanchard, R.J. (2011). Motor and cognitive stereotypies in the BTBR t+tf/J mouse model of autism. Genes, Brain and Behavior, 10, 228–235. doi: 10.1111/j.1601-183X.2010.00659.x

Peister, A., Mellad, J.A., Larson, B.L., Hall, B.M., Gibson, L.F., & Prockop, D.J. (2004). Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood, 103, 1662–1668. doi:10.1182/blood-2003-09-3070

Pescosolido, M.F., Yang, U., Sabbagh, M., & Morrow, E.M. (2012). Lighting a path: Genetic studies pinpoint neurodevelopmental mechanisms in autism and related disorders. Dialogues in Clinical Neuroscience, 14, 239–252.

Pobbe, R.L.H., Pearson, B.L., Defensor, E.B., Bolivar, V.J., Blanchard, D.C., & Blanchard, R.J. (2010). Expression of social behaviors of C57BL/6J versus BTBR inbred mouse strains in the visible burrow system. Behavioural Brain Research, 214, 443–449. doi:10.1016/j.bbr.2010.06.025

Rafei, M., Campeau, P.M., Aguilar-Mahecha, A., Buchanan, M., Williams, P., Birman, E., et al. (2009). Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. Journal of Immunology (Baltimore, Md.: 1950), 182, 5994–6002. doi:10.4049/jimmunol.0803962

Raznahan, A., Toro, R., Proitsi, P., Powell, J., Paus, T., F Bolton, P., & Murphy, D. G. (2009). A functional polymorphism of the brain derived neurotrophic factor gene and cortical anatomy in autism spectrum disorder. Journal of Neurodevelopmental Disorders, 1, 215–223. doi:10.1007/s11689-009-9012-0

Roullet, F.I., Wollaston, L., deCatanzaro, D., & Foster, J.A. (2010). Behavioral and molecular changes in the mouse in response to prenatal exposure to the anti-epileptic drug valproic acid. Neuroscience, 170, 514–522. doi:http://dx.doi.org/10.1016/j.neuroscience.2010.06.069

Rutz, H.L., & Rothblat, L.A. (2012). Intact and impaired executive abilities in the BTBR mouse model of autism. Behavioural Brain Research, 234, 33–37. doi:10.1016/j.bbr.2012.05.048

Sadakata, T., Shinoda, Y., Oka, M., Sekine, Y., Sato, Y., Saruta, C., et al. (2012). Reduced axonal localization of a Caps2 splice variant impairs axonal release of BDNF and causes autistic-like behavior in mice. Proceedings of the National Academy of Sciences of the United States of America, 109, 21104–21109. doi:10.1073/pnas.1210055109

Sadan, O., Shemesh, N., Barzilay, R., Dadon-Nahum, M., Blumenfeld-Katzir, T., Assaf, Y., et al. (2012). Mesenchymal stem cells induced to secrete neurotrophic factors attenuate quinolinic acid toxicity: A potential therapy for huntington's disease. Experimental Neurology, 234, 417–427. doi: 10.1016/j.expneurol.2011.12.045

Sasaki, M., Radtke, C., Tan, A.M., Zhao, P., Hamada, H., Houkin, K., et al. (2009). BDNF-hypersecreting human mesenchymal stem cells promote functional recovery, axonal sprouting, and protection of corticospinal neurons after spinal cord injury. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 29, 14932–14941. doi:10.1523/JNEUROSCI.2769-09.2009

Scattoni, M.L., Martire, A., Cartocci, G., Ferrante, A., & Ricceri, L. (2012). Reduced social interaction, behavioural flexibility and BDNF signalling in the BTBR t+tf/J strain, a mouse model of autism. Behavioural Brain Research, 251, 35–40. doi:10.1016/j.bbr.2012.12.028; 10.1016/j.bbr.2012.12.028

Scattoni, M.L., Ricceri, L., & Crawley, J.N. (2011). Unusual repertoire of vocalizations in adult BTBR t+tf/J mice during three types of social encounters. Genes, Brain and Behavior, 10, 44–56. doi:10.1111/j.1601-183X.2010.00623.x

Schaevitz, L.R., Moriuchi, J.M., Nag, N., Mellot, T.J., & Berger-Sweeney, J. (2010). Cognitive and social functions and growth factors in a mouse model of rett syndrome. Physiology & Behavior, 100, 255–263. doi:10.1016/j.physbeh.2009.12.025

Sharma, A., Gokulchandran, N., Sane, H., Nagrajan, A., Paranjape, A., Kulkarni, P., et al. (2013). Autologous bone marrow mononuclear cell therapy for autism: An open label proof of concept study. Stem Cells International, 2013, 623875. doi:10.1155/2013/623875

Sheikh, A.M., Nagai, A., Wakabayashi, K., Narantuya, D., Kobayashi, S., Yamaguchi, S., et al. (2011). Mesenchymal stem cell transplantation modulates neuroinflammation in focal cerebral ischemia: Contribution of fractalkine and IL-5. Neurobiology of Disease, 41, 717–724. doi:10.1016/j.nbd.2010.12.009

Silverman, J.L., Oliver, C.F., Karras, M.N., Gastrell, P.T., & Crawley, J.N. (2013). AMPAKINE enhancement of social interaction in the BTBR mouse model of autism. Neuropharmacology, 64, 268–282. doi:10.1016/j.neuropharm.2012.07.013

Silverman, J.L., Smith, D.G., Rizzo, S.J., Karras, M.N., Turner, S.M., Tolu, S.S., et al. (2012). Negative allosteric modulation of the mGluR5 receptor reduces repetitive behaviors and rescues social deficits in mouse models of autism. Science Translational Medicine, 4, 131ra51. doi:10.1126/scitranslmed.3003501

Silverman, J.L., Tolu, S.S., Barkan, C.L., & Crawley, J.N. (2010). Repetitive self-grooming behavior in the BTBR mouse model of autism is blocked by the mGluR5 antagonist MPEP. Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 35, 976–989. doi:10.1038/npp.2009.201

Silverman, J.L., Yang, M., Lord, C., & Crawley, J.N. (2010). Behavioural phenotyping assays for mouse models of autism. Nature Reviews.Neuroscience, 11, 490–502. doi: 10.1038/nrn2851

Siniscalco, D., Bradstreet, J.J., Sych, N., & Antonucci, N. (2014). Mesenchymal stem cells in treating autism: Novel insights. World Journal of Stem Cells, 6, 173–178. doi: 10.4252/wjsc.v6.i2.173

Snyder, B.R., Chiu, A.M., Prockop, D.J., & Chan, A.W. (2010). Human multipotent stromal cells (MSCs) increase neurogenesis and decrease atrophy of the striatum in a transgenic mouse model for huntington's disease. PloS One, 5, e9347. doi:10.1371/journal.pone.0009347

Stemberger, S., Jamnig, A., Stefanova, N., Lepperdinger, G., Reindl, M., & Wenning, G.K. (2011). Mesenchymal stem cells in a transgenic mouse model of multiple system atrophy: Immunomodulation and neuroprotection. PloS One, 6, e19808. doi:10.1371/journal.pone.0019808

Stephenson, D.T., O'Neill, S.M., Narayan, S., Tiwari, A., Arnold, E., Samaroo, H.D., et al. (2011). Histopathologic characterization of the BTBR mouse model of autistic-like behavior reveals selective changes in neurodevelopmental proteins and adult hippocampal neurogenesis. Molecular Autism, 2, 7-2392-2-7. doi:10.1186/2040-2392-2-7

Tanna, T., & Sachan, V. (2014). Mesenchymal stem cells: Potential in treatment of neurodegenerative diseases. Current Stem Cell Research & Therapy, 9, 513–521. doi:CSCRT-EPUB-62473 [pii]

Tfilin, M., Sudai, E., Merenlender, A., Gispan, I., Yadid, G., & Turgeman, G. (2010). Mesenchymal stem cells increase hippocampal neurogenesis and counteract depressive-like behavior. Molecular Psychiatry, 15, 1164–1175. doi: 10.1038/mp.2009.110

Tsigelny, I.F., Kouznetsova, V.L., Baitaluk, M., & Changeux, J.P. (2013). A hierarchical coherent-gene-group model for brain development. Genes, Brain, and Behavior, 12, 147–165. doi:10.1111/gbb.12005

Veenstra-VanderWeele, J., & Warren, Z. (2014). Intervention in the context of development: Pathways toward new treatments. Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 40, 225–237. doi:10.1038/npp.2014.232

Wakabayashi, K., Nagai, A., Sheikh, A.M., Shiota, Y., Narantuya, D., Watanabe, T., et al. (2010). Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. Journal of Neuroscience Research, 88, 1017–1025. doi:10.1002/jnr.22279

Wang, F., Yasuhara, T., Shingo, T., Kameda, M., Tajiri, N., Yuan, W. J., et al. (2010). Intravenous administration of mesenchymal stem cells exerts therapeutic effects on parkinsonian model of rats: Focusing on neuroprotective effects of stromal cell-derived factor-1alpha. BMC Neuroscience, 11, 52-2202-11-52. doi:10.1186/1471-2202-11-52

Wegiel, J., Kuchna, I., Nowicki, K., Imaki, H., Wegiel, J., Marchi, E., et al. (2010). The neuropathology of autism: Defects of neurogenesis and neuronal migration, and dysplastic changes. Acta Neuropathologica, 119, 755–770. doi: 10.1007/s00401-010-0655-4

Weissbrod, A., Shapiro, A., Vasserman, G., Edry, L., Dayan, M., Yitzhaky, A., et al. (2013). Automated long-term tracking and social behavioural phenotyping of animal colonies within a semi-natural environment. Nature Communications, 4, 2018. doi:10.1038/ncomms3018

Wöhr, M., Roullet, F.I., & Crawley, J.N. (2011). Reduced scent marking and ultrasonic vocalizations in the BTBR t+tf/J mouse model of autism. Genes, Brain and Behavior, 10, 35–43. doi:10.1111/j.1601-183X.2010.00582.x

Wöhr, M., & Scattoni, M. L. (2013). Behavioural methods used in rodent models of autism spectrum disorders: Current standards and new developments. Behavioural Brain Research, 251, 5–17. doi:http://dx.doi.org/10.1016/j.bbr.2013.05.047

Zhang, M.J., Sun, J.J., Qian, L., Liu, Z., Zhang, Z., Cao, W., et al. (2011). Human umbilical mesenchymal stem cells enhance the expression of neurotrophic factors and protect ataxic mice. Brain Research, 1402, 122–131. doi:10.1016/j.brainres.2011.05.055