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HIGH-FAT DIET EXACERBATES COGNITIVE RIGIDITY AND SOCIAL DEFICIENCY IN THE BTBR MOUSE MODEL OF AUTISM

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Abstract—The global increase in rates of obesity has been accompanied by a similar surge in the number of autism diagnoses. Accumulating epidemiological evidence suggest a possible link between overweight and the risk for autism spectrum disorders (ASD), as well as autism severity. In laboratory animals, several studies have shown a connection between various environmental factors, including diet-induced obesity, and the development of autism-related behaviors. However, the effect of high-fat or imbalanced diet on a pre-existing autism-like phenotype is unclear. In this study, we employed the BTBR inbred mouse strain, a well-established mouse model for autism, to assess the impact of inadequate fattening nutrition on the autism-related behavioral phenotype. Male mice were fed by high-fat diet (HFD) or control balanced diet (control) from weaning onward, and tested in a series of behavioral assays as adults. In addition, we measured the hypothalamic expression levels of several genes involved in oxytocin and dopamine signaling, in search of a possible neurobiological underlying mechanism. As an internal control, we also employed similar metabolic and behavioral measures on neurotypical C57 mice. Compared to control-fed mice, BTBR mice fed by HFD showed marked aggravation in autism-related behaviors, manifested in increased cognitive rigidity and diminished preference for social novelty. Moreover, the total autism composite (severity) score was higher in the HFD group, and positively correlated with higher body weight. Finally, we revealed negative correlations associating dopamine signaling factors in the hypothalamus, to autism-related severity and body weight. In contrast, we found no significant effects of HFD on autism-related behaviors of C57 mice, though the metabolic effects of the diet were similar for both strains. Our results indicate a direct causative link between diet-induced obesity and worsening of a pre-existing autism-related behavior and emphasize the need for adequate nutrition in ASD patients. These findings might also implicate the involvement of hypothalamic dopamine in mediating this effect.

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Abbreviations: ASD, autism spectrum disorders; HFD, high-fat diet.

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INTRODUCTION

In the last few decades, the prevalence of obesity has increased dramatically worldwide and has now reached epidemic proportions (Bastien et al., 2014). Extensive research has shown the dramatic effects of obesity and inadequate nutrition on cardiovascular diseases (Ritchie and Connell, 2007), diabetes (Guilherme et al., 2008) and feeding related behaviors (la Fleur et al., 2007). Several animal studies have also investigated behaviors that are not directly related to food, demonstrating that diet-induced obesity (Teodoro et al., 2014), may lead to anxiety (Andre et al., 2014) and depressive-like behaviors (Abildgaard et al., 2011). Exposure of rodents to high-fat diet (HFD) has also led to alterations in the function of reward-related circuitry in the brain (Sharma and Fulton, 2013) and to impairments of hippocampal plasticity (Grillo et al., 2011). Investigations into the effects of perinatal exposure to HFD and maternal obesity revealed that the offspring are more susceptible to developing mental health and behavioral disorders such as anxiety, depression, attention deficit hyperactivity disorder, and autism spectrum disorders (ASD) (reviewed by Sullivan et al. (2014)).

ASD are characterized primarily by marked impairment in social interactions and communication, increased repetitive behavior, and striking cognitive difficulties, chiefly in the form of cognitive rigidity (Hobson, 2012; Hall et al., 2015). Most of the primary symptoms of autism have been represented rather faithfully in rodents using several behavioral paradigms (Karvat and Kimchi, 2012; Kazdoba et al., 2016; Pasciuto et al., 2015). Moreover, various mouse models, including transgenic mice lines, have been developed to study the neurobiological basis of autism (reviewed in Crawley (2012), Banerjee et al. (2014) and Pasciuto et al. (2015)). One of the earliest and most studied mouse models of autism is the BTBR *T+tf*/J (BTBR) inbred mouse strain (McFarlane et al., 2008). BTBR mice display autism-related behavioral phenotype, including impaired social behavior (Pobbe et al., 2010; Weissbrod et al., 2013; Karvat and Kimchi, 2014), increased repetitive behavior (Pearson et al., 2011; Amodeo et al., 2012; Karvat and Kimchi, 2012) and increased cognitive rigidity (Moy et al., 2007; Rutz and Rothblat, 2012; Segal-Gavish et al., 2016). In addition, recent studies identified specific

biochemical alterations in BTBR mice that are consistent with findings from autistic patients, such as aberrant immune state (Heo et al., 2011), and reductions in both hippocampal neurogenesis (Stephenson et al., 2011) and in brain-derived neurotrophic factor signaling (Scattoni et al., 2013). Notably, due to its metabolic predisposition, the BTBR strain has also often been used to study obesity and metabolic-related disorders (Shedlovsky et al., 1993; Ranheim et al., 1997; Nadler et al., 2000; Clee et al., 2005). Thus, the BTBR mouse model appears to be an ideal tool to study the relationship between diet-induced obesity and ASD-related phenotype.

In the current research, we examined the ASD-related behavioral phenotype in BTBR and C57 male mice maintained from the age of weaning on either high-fat or control balanced diet. Mice were evaluated for their metabolic state and then tested in a series of behavioral assays representing the core symptoms of autism: cognitive rigidity, impaired social behavior and stereotypic behavior. Following behavioral testing, we measured hypothalamic mRNA expression levels of several genes involved in dopamine and oxytocin signaling, as these factors have been implicated both in obesity and feeding behavior (Meguid et al., 2000; Kelley et al., 2005; Baskerville and Douglas, 2010; Mason et al., 2013) as well as in social behavior, cognitive function and ASD-related phenotype (Alabdali et al., 2014; Beny and Kimchi, 2014; Nguyen et al., 2014; Gunaydin and Deisseroth, 2015; Scott et al., 2015; Yamasue, 2016).

EXPERIMENTAL PROCEDURES

Animals

BTBR T + tf/J mice were bred from adult pairs originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6JOlaHsd (C57) mice were purchased from Harlan Laboratories (Rehovot, Israel). At the age of weaning (22–26 days old), male mice were randomly assigned to either control diet (Control, $n_{BTBR} = 11$, $n_{C57} = 8$) or high-fat diet (HFD, $n_{BTBR} = 8$, $n_{C57} = 8$) groups. Mice were housed in groups of 2-4 littermates per cage and were given *ad libitum* access to food and water throughout the experiment.

Unfamiliar mice in the three-chamber sociability assay and in the running/jammed wheel assay were 5-week-old Hsd:ICR[CD-1] males (Harlan Laboratories).

All experimental procedures were approved by and conducted in strict compliance with the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Diets and metabolism

Mice were fed from weaning and throughout the experiment with either control low-fat diet (10% of kcal as fat, 20% kcal as protein) or HFD (60% kcal as fat, 20% kcal as protein), products D12450B and D12492, respectively; Research Diets, New Brunswick, NJ, USA) and weighed weekly. At 9 weeks of age, mice were tested individually in metabolic cages (LabMaster; TSE-Systems, Bad Homburg, Germany). Volume of oxygen uptake (VO2), carbon dioxide production (VCO2), food and water intake and locomotor activity (ESM Methods) were measured continuously and simultaneously over 3 days. The cages resemble the mice's home cage in their size, shape and bedding content, and the mice are allowed 2 days of adaptation prior to the metabolic measurements, in order to avoid any stressful influences. At 13–14 weeks of age, following behavioral assays, body composition was assessed using Echo-MRI (Echo Medical Systems, Houston, TX, USA).

Behavioral assays

Open field test. The assay was conducted as previously described (Karvat and Kimchi, 2012, 2014). Mice were placed in a 30 × 30 × 25-cm cage for 10 min. Locomotion parameters were measured using the Ethovision software (Noldus), while stereotypic (i.e. digging, self-grooming) behaviors and olfactory investigation were scored manually using the Observer software (Noldus, Wageningen, the Netherlands).

Three-chamber social assay. The three-chamber social approach and social novelty preference tests were designed as previously detailed (Moy et al., 2007; Karvat and Kimchi, 2012; Segal-Gavish et al., 2016). Briefly, the test consisted of 10 min habituation in an empty apparatus, followed by two consecutive phases of 10 min each: (i) social approach, with a wire-cage containing an unfamiliar mouse (mouse) situated in one side-chamber and an empty cage (object) on the opposite side, and; (ii) preference for social novelty, when an additional unfamiliar mouse was placed in the wire cage that had been empty during the previous session. Time spent in each chamber was scored using the Ethovision software (Noldus). Locations of different stimuli were counterbalanced between animals.

Running/jammed wheel assay. This test was previously developed and applied in our lab (Karvat and Kimchi, 2012, 2014; Segal-Gavish et al., 2016). Briefly, mice were placed in a transparent Plexiglas cage sized 30 × 30 × 25 cm, containing a 14-cm diameter plastic running wheel connected to one of the walls, which could either turn freely or be jammed by a metal pin. The wheel was free during the first 4 days (run1-4) and then jammed for two consecutive days (Jam1 and Jam2). For each day, an observer blind to the experimental groups quantified the time mice spent interacting with the wheel in an attempt to turn it. The impaired ability to adjust to change (i.e. cognitive rigidity) was calculated as the ratio between times spent on the wheel on day jam1 to day run4. In the last day of the test, an unfamiliar mouse was introduced to the apparatus, and the behavior of the resident mouse was recorded for 10 min. Social contacts initiated by the test mouse, as well as interaction with the wheel, were quantified by an observer blinded to the treatment groups.

Wet T-maze assay. The wet T-maze assay was conducted as previously detailed (Guariglia and Chadman, 2013; Karvat and Kimchi, 2014) using a T-shaped Plexiglas chamber filled with water with an escape platform submerged 0.5 cm below water level. Animals had 5 trials during each of the four experimental days, placed each trial in the starting arm facing the wall, and were allowed to swim until locating the hidden platform, or until 90 s have passed. 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. Latency to climb on the platform and the number of correct turns were measured manually. Animals with no correct turns on day 2 were not tested further.

Computation of autism composite score

Based on the scoring method developed by El-Kordi et al. (2013) and previously used in several autism-related animal studies (Dere et al., 2014; Segal-Gavish et al., 2016), the scores of each mouse in six parameters (a pair of parameters per core behavioral symptom of autism (American Psychiatric et al., 2013)) 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 (McFarlane et al., 2008), and included: (A) Social deficiency in the 3-chamber test: social preference index calculated as (time with unfamiliar mouse)/(time with unfamiliar mouse + time with object) and social novelty index calculated as (time with unfamiliar mouse)/(time with familiar mouse + time with unfamiliar mouse). (B) Cognitive rigidity: ratio between day Jam1 and day Run 4 (adjustment to change) in the running/ jammed wheel test and no. of correct turns on day 4 of the water T-maze. (C) Stereotypical behavior: digging duration and self-grooming duration. The average Z score of all six parameters was designated as the autism composite score.

Tissue dissection and real-time PCR

At the end of the behavioral assays mice were sacrificed and the hypothalamus was removed. Total RNA extraction, reverse transcription into cDNA and real-time PCR were conducted as previously described (Chalfin et al., 2014). Specific primer sequences are: Actin-beta, F: CTAAGGCCAACCGTGAAAAG, R: ACCAGAGGCATACAGGGACA; Tyrosine Hydroxylase (TH), F: TTGGATAAGTGTCACCACCTG, R: TGGCTCACCCTGCTTGTA; DA receptor D1, F: CGTGGTCTCCCAGATCGG, R: GCATTTCTCCTTCAAGCCCC; DA receptor D2, F: GACACCACTCAAGGGCAACT, R: TCCATTCTCCGCCTGTTCAC; Oxytocin, F: CTTGGCTTACTGGCTCTG, R: GAGACACTTGCGCATATCC; Oxytocin receptor, F: CATTGTTCTGGCCTTCATCG, R: GAAGGCAGAAGCTTCTTTGG.

Statistical analysis

All statistical analyses were performed using STATISTICA software (StatSoft, Tulsa, OK, USA). For all comparisons, we used either one-way ANOVA or repeated-measures ANOVA, followed by *post hoc* Fisher test. Correlation coefficients were calculated using pairwise Pearson's correlation. All results are displayed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

RESULTS

HFD in BTBR mice induces severe metabolic impairments

Mice were fed with either HFD or control diet, from weaning age and throughout the experiment (Fig. 1A). During the first 5 weeks of diet, we monitored their weights on a weekly basis. As expected, HFD in BTBR mice induced a massive weight gain compared to the control diet, noticeable already after 1 week of diet ($F = 25.6$, $p < 0.001$, Fig. 1B, C). At the end of the experiment, BTBR mice on HFD were significantly heavier with significantly higher fat mass and lower lean mass compared to control mice ($F = 48.0$, $p < 0.001$, Fig. 1D). The HFD consuming BTBR mice exhibited a significantly lower heat production, indicating lower energy expenditure, during both the dark ($p < 0.001$) and light ($p < 0.05$) phases of the day cycle (Fig. 1E). The reduced energy expenditure sustains the obese phenotype since the caloric intake at this stage as well as the locomotor activity were similar in both diet groups ($F = 0.063$, $p = 0.8$ and $F = 1.44$, $p = 0.25$, respectively, Fig. 1F, G). As expected by their main energy source, HFD consuming mice had also a significantly lower rate of respiratory exchange ratio ($F = 140.7$, $p < 0.001$), with no circadian cyclicality (light-dark main effect, $p_{control} < 0.001$, $p_{HFD} = 0.25$, Fig. 1H), indicative for fatty acid oxidation (Mitchell et al., 2014).

Similar metabolic effects were observed in C57 mice exposed to HFD compared to control diet (Fig. 6A, B).

BTBR mice fed with HFD display enhanced cognitive rigidity

To investigate the cognitive capabilities of the mice fed by different diets, we employed the T maze assay (Fig. 2A–E), and the running/jammed wheel assay developed in our lab (Fig. 2F–H). On days 1 and 2 of the T maze assay, mice learned to search and mount a hidden platform submerged under the water surface in one of the maze's arms. As shown in Fig. 2B, while control mice displayed a marginally significant increase in the percentage of correct turns from day 1 to day 2 of the assay ($p = 0.06$), HFD mice exhibited impaired learning, reflected in lack of improvement in the percentage of correct turns ($p = 0.7$). On days 3 and 4 of the assay, the location of the platform was replaced to the opposite arm, to evaluate the cognitive rigidity of the mice. Both control-fed and HFD BTBR mice presented a typical impairment in reversal learning manifested in a low percentage of correct turns on day 3 (control, 48.6 ± 10.6; HFD, 34.3 ± 15.6). HFD mice also showed an abnormal cognitive persistence,

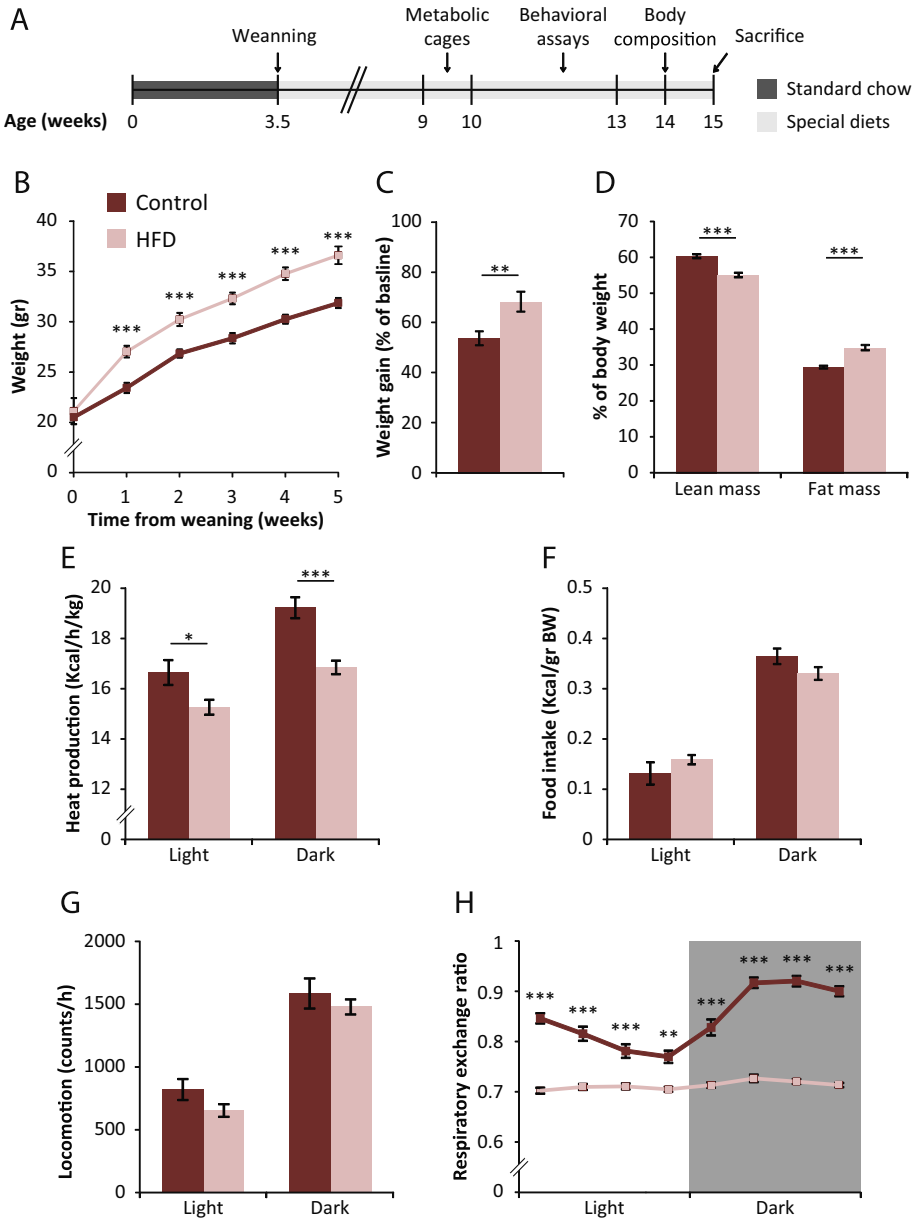

Fig. 1. Metabolic effects of high-fat diet in BTBR mice. (A) Experimental timeline. (B) Weight in grams and weight gain in percentage (C) during 5 weeks of special diets. (D) Body composition measurements, comprised of percentage lean and fat masses. (E) Average heat production, (F) food intake, (G) locomotion and (H) respiratory exchange ratio, measured in the metabolic cages, during the light and dark phases. Results are displayed as mean ± SEM; $n_{Control} = 11$, $n_{HFD} = 8$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for repeated measures ANOVA or one-way ANOVA followed by Fisher's *post hoc* analysis comparing each HFD measure to its respective control.

reflected by a significantly lower percentage of correct turns on day 4 ($p < 0.05$, Fig. 2C). In addition, we analyzed the basic reversal learning performance of both groups, by comparing the learning curves (i.e. latencies to reach the platform) of the animals on the first and third day. We found that HFD mice presented an impairment in reversal learning, as their learning pattern was similar on days 3 and 1 ($p = 0.57$), while control mice displayed shorter latencies to reach the platform on day 3 vs. day 1 ($p < 0.05$, Fig. 2D, E).

In the running/jammed wheel assay, both groups spent similar durations on the wheel during the first 4 days of running ($F = 0.95$, $p = 0.34$, Fig. 2G). However, similarly to the T-maze assay, HFD mice displayed enhanced cognitive rigidity compared to control, as the ratio of time spent on the wheel between day 1 of the jammed wheel and day 4 of the running wheel was significantly higher in the HFD group ($p < 0.05$, Fig. 2H). No difference was observed between the groups in the ratio between jam day 1 and jam day 2 ($p = 0.58$).

In contrast, C57 mice exposed to HFD did not differ significantly from control mice in both cognitive behavioral assays, though there was a slight trend

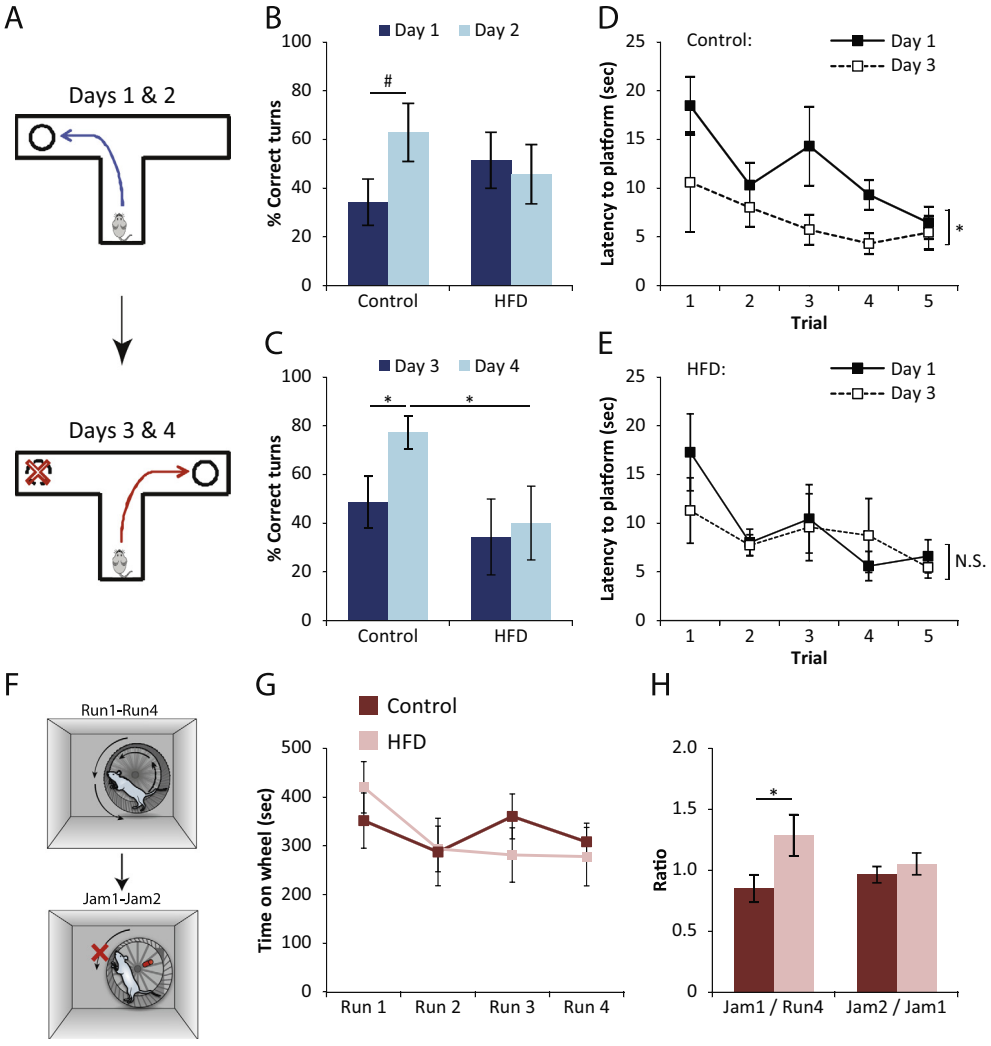

Fig. 2. HFD increases cognitive rigidity in BTBR mice. (A) Schematic illustration of the wet T-maze assay. (B and C) Percentage of correct turns during days 1 and 2 (B) and 3 and 4 (C) of the assay. (D-E) Learning curves, presented as the latencies to reach the platform during the first and third day of the assay in control (D) and HFD (E) mice. (F) Schematic illustration of the running/jammed wheel assay. (G) Total time spent on the wheel during the four days of running. (H) Ratios of the time durations spent on the wheel between the first jam day (day 5 of the experiment) and the last running day (day 4), and between the second and first jam days (days 6 and 5 of the experiment, respectively). Results are displayed as mean ± SEM; $n_{Control} = 7$, $n_{HFD} = 7$. $^{\#}p = 0.06$, $^{*}p < 0.05$ for repeated measures ANOVA followed by Fisher's *post hoc* analysis.

toward significant reduction in the percentage of correct turns on day 3 of the assay ($p = 0.1$, Fig. 6C), which might suggest a mild impairment in reversal learning.

HFD in BTBR mice impairs social memory

To evaluate the effects of nutrition on social behaviors, we measured social preference and social novelty preference in BTBR mice using the three-chamber assay (Fig. 3A–D), and social preference in the running/jammed wheel assay (Fig. 3E, F).

In the social preference phase of the three-chamber assay, both control and HFD groups displayed the typical BTBR autism-related phenotype and failed to present a significant preference toward the unfamiliar mouse compared to the object ($F = 1.52$, $p = 0.23$, Fig. 3B). In the social novelty preference phase, the control-fed BTBR mice significantly preferred spending more time with the novel unfamiliar mouse compared to the familiar mouse ($p < 0.01$, Fig. 3D), whereas the HFD mice spent only slightly more time (but not significantly different, $p = 0.07$) interacting with the unfamiliar mouse compared to the familiar one. Similar difference was noticed in the measurements of sniffing time, where significant preference for the novel over the familiar mouse was found only for the control (194 ± 22 vs. 86 ± 12.2, $p < 0.001$) but not for the HFD (168.5 ± 10.3 vs. 111.5 ± 17.2, $p = 0.06$) mice.

In the running/jammed wheel assay, both groups spent similar durations interacting with the unfamiliar mouse ($p = 0.31$, Fig. 3F). However, the HFD mice spent significantly less time interacting with the wheel compared to the control mice ($p < 0.05$, Fig. 3F).

In the C57 mice, similarly to the cognitive rigidity measures, HFD had no significant effect on social memory, as both control and HFD groups displayed a significant preference toward the social novelty (Fig. 6D).

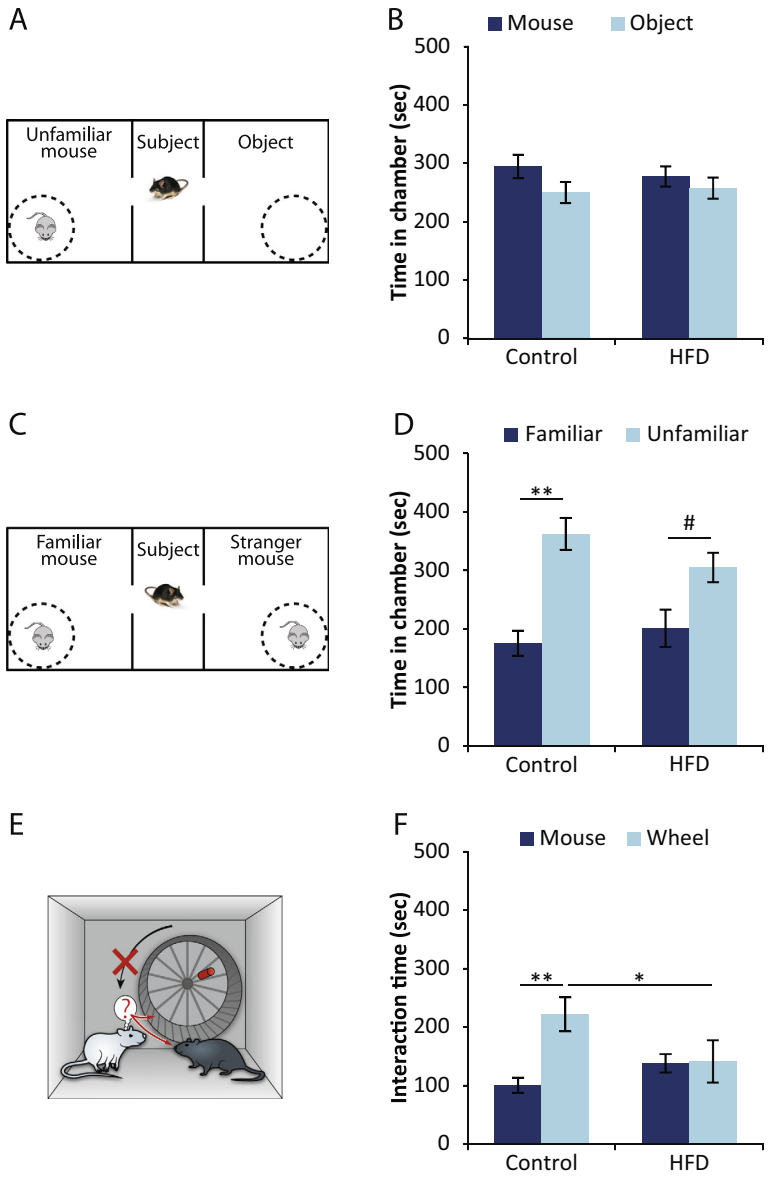

Fig. 3. HFD impairs social memory in BTBR mice. (A, C) Schematic illustrations of the 3-chamber assay: social preference phase (A) and social novelty preference phase (C). (B, D) Time spent in each chamber during the 10-minute assay: social preference (B) and social novelty preference (D). (E) Schematic illustrations of the social assay phase in the running/jammed wheel assay and interaction times with the wheel and novel mouse during the assay (F). Results are displayed as mean ± SEM; $n_{Control} = 10$, $n_{HFD} = 8$. $^{\#}p = 0.07$, $^{*}p < 0.05$, $^{**}p < 0.01$ for repeated measures ANOVA followed by Fisher's *post hoc* analysis.

HFD has no effect on stereotypic behavior or locomotor behavior in BTBR mice

We assessed stereotypic behavior by measuring durations of digging and self-grooming during a 10-min period in an open-field novel environment. In addition we quantified duration of time spent in the center of the arena, as a measure for exploration, and duration of olfactory investigation. No differences were observed between the HFD and control-fed groups in any of the behavioral parameters measured (Fig. 4). Likewise, no differences were noticed between HFD and control groups of C57 mice (Fig. 6E, F).

Importantly, all the behavioral effects described above cannot be attributed to any locomotor deficits, as HFD mice did not differ from control mice in the locomotor activity measured either in the open field assay (Fig. 4A) or by the metabolic cages system (Fig. 1G).

Autism-like severity in BTBR mice is associated with body weight and dopaminergic signaling in the hypothalamus

In order to define the overall difference in autism-related behavioral phenotype between the control and HFD mice, we calculated a standardized score for six behavioral parameters and integrated them into a single value referred to as "autism composite score" as described in the results section. The overall autism-like

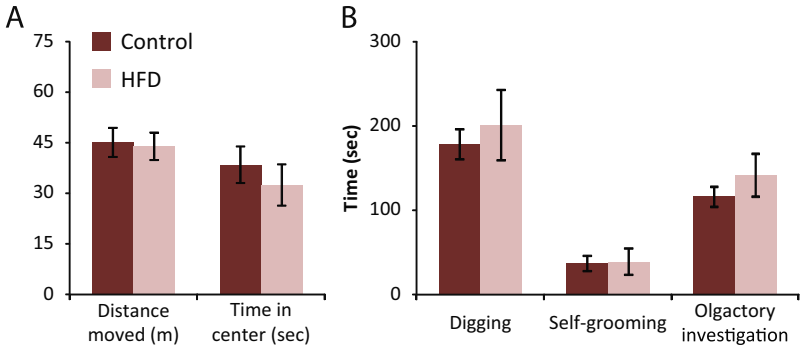

Fig. 4. No effect of HFD on locomotor or stereotypic behavior in BTBR mice. (A) Total distance traveled and time spent in the center of the arena during 10-min assay. (B) Total time spent digging, self-grooming and olfactory investigating the arena. Results are displayed as mean ± SEM; $n_{Control} = 11$, $n_{HFD} = 8$.

composite score was significantly higher in the HFD group compared to controls ($F = 6.4$, $p < 0.05$, Fig. 5A), indicating higher autism-related behavioral abnormality in mice fed by HFD. Moreover, the autism-like severity score was positively correlated with the weights of the mice following 5 weeks of diet assignment ($R^2 = 0.32$, $p < 0.01$, Fig. 5B).

At the end of the behavioral testing, we measured hypothalamic mRNA levels of several genes involved in dopaminergic and oxytocinergic signaling, as presented in Fig. 5C. No significant differences were found between control and HFD groups, though marginally significant increases of tyrosine hydroxylase (TH, $p = 0.097$) and dopamine receptor D1 (D1-R, $p = 0.06$) levels were noticed in the HFD group. Interestingly, we found significant negative correlations between the behavioral autism score and expression levels of TH ($R^2 = 0.27$, $p < 0.05$, Fig. 5D) and D1-R ($R^2 = 0.45$, $p < 0.01$, Fig. 5E). Consistently, the body weights of the mice were also negatively correlated with both TH ($R^2 = 0.43$, $p < 0.01$, Fig. 5F) and D1-R ($R^2 = 0.4$, $p < 0.01$, Fig. 5G) levels.

Consistently with the results of the behavioral assays, C57 mice fed by HFD did not differ in their autism-related severity score compared to control-fed mice, nor was there any correlation between the individual autism composite score and the weight of the mice following diet exposure (Fig. 6G, H).

DISCUSSION

In the past few decades, the world has been witnessing an emergence of an obesity pandemic (Flegal et al., 2010), which has been co-occurring alongside a gradual increase in worldwide rates of ASD (Baxter et al., 2015). Accumulating epidemiological data has been suggesting a link between the two (Kawicka and Regulska-Ilow, 2013; Broder-Fingert et al., 2014; Curtin et al., 2014; Suren et al., 2014), prompting us to explore the effects of nutrition on the ASD-related behavioral phenotype in BTBR mice.

Adult mice maintained on HFD from weaning onward presented a massive increase in body weight and fat accumulation compared to control-fed mice, while expending less energy. However, these metabolic changes were not due to differences in either food consumption or locomotion. These effects are consistent with the known literature regarding the effects of HFD on other rodent strains (Rousso-Noori et al., 2011; Pan et al., 2012; Shechter et al., 2013), and confirm the dramatic effect of HFD on metabolism.

When examining the effects of diet on cognitive behaviors, we noticed marked impairments in the performance of the HFD BTBR mice compared to the control-fed mice in all three behavioral measurements. HFD BTBR mice displayed enhanced cognitive rigidity in both the T-maze and the running/jammed wheel assays, whereas in the T maze poorer learning ability was observed as well. Additionally, C57 mice exposed to HFD displayed a marginally significant deficiency in reversal learning, compared to control mice. These findings are in line with previous studies demonstrating impaired cognitive function in the presence of obesity in humans (Elias et al., 2003; Waldstein and Katzel, 2006; Smith et al., 2011) and rodents (Winocur et al., 2005; Farr et al., 2008), and improved cognitive performance following dietary interventions (Farr et al., 2008; Cohen et al., 2011; Leidy et al., 2015) and physical exercise (Smith et al., 2010). Moreover, cognitive rigidity was increased in obese human individuals as measured by the Wisconsin card sorting test (Fagundo et al., 2012), while performance in the test was negatively correlated with weight and BMI (Cserjesi et al., 2007; Reinert et al., 2013). Additionally, in a modified version of this test adapted to primates, rhesus monkeys under moderately restricted diet displayed improved performance in adjusting to a change in the task conditions (Sridharan et al., 2012).

Next, we investigated the influence of diet on sociability and social memory using the three chamber assay. Both control and HFD BTBR mice spent similar durations with the social and non-social stimuli, consistent with previous studies performed in this line (McFarlane et al., 2008; Karvat and Kimchi, 2012, 2014). In the social-novelty preference test, when compared to the control group, we observed an exacerbation of BTBR social-impairments in HFD mice, with no significant display of preference to either novel or familiar, animal stimulus. In contrast, C57 mice in the HFD group displayed a clear significant preference toward the novel

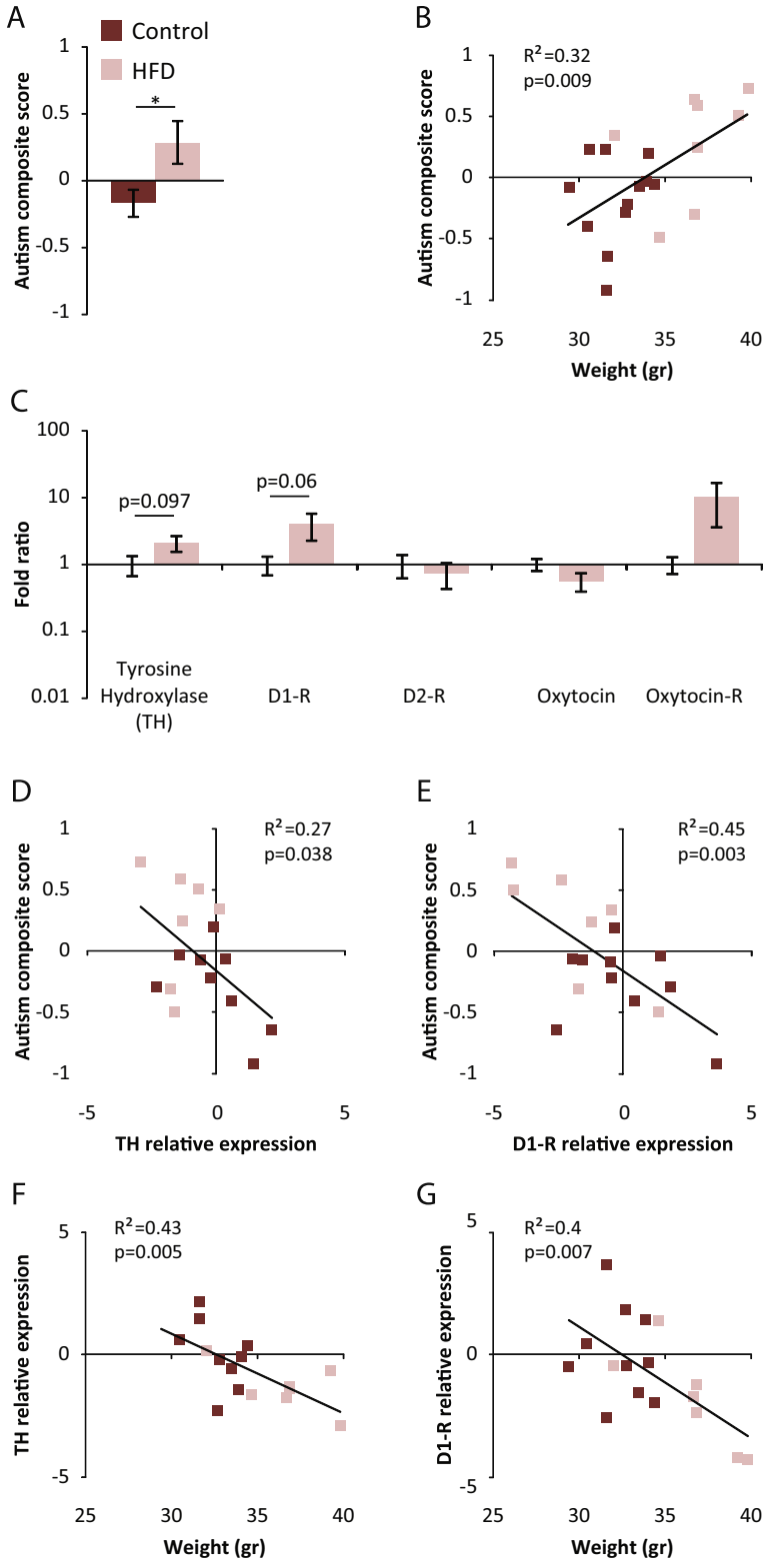

Fig. 5. Associations between autism-related severity, weight and dopaminergic markers in the hypothalamus of BTBR mice. (A) Autism composite score for control-fed and HFD BTBR mice. (B) Correlation between the autism composite score and the weight of each mouse. (C) Relative mRNA expression levels of selected genes in the hypothalamus region. Results are displayed as mean ± SEM; $^{*}p < 0.05$ for one-way ANOVA. (D–G) Correlations between hypothalamic levels of TH and D1-R to the autism composite score of each mouse (D, E), and to the weight of each mouse (F, G). $n_{Control}$ = 9–11, n_{HFD} = 7–8. TH, tyrosine hydroxylase; D1-R, dopamine receptor D1; D2-R, dopamine receptor D2; oxytocin-R, oxytocin receptor.

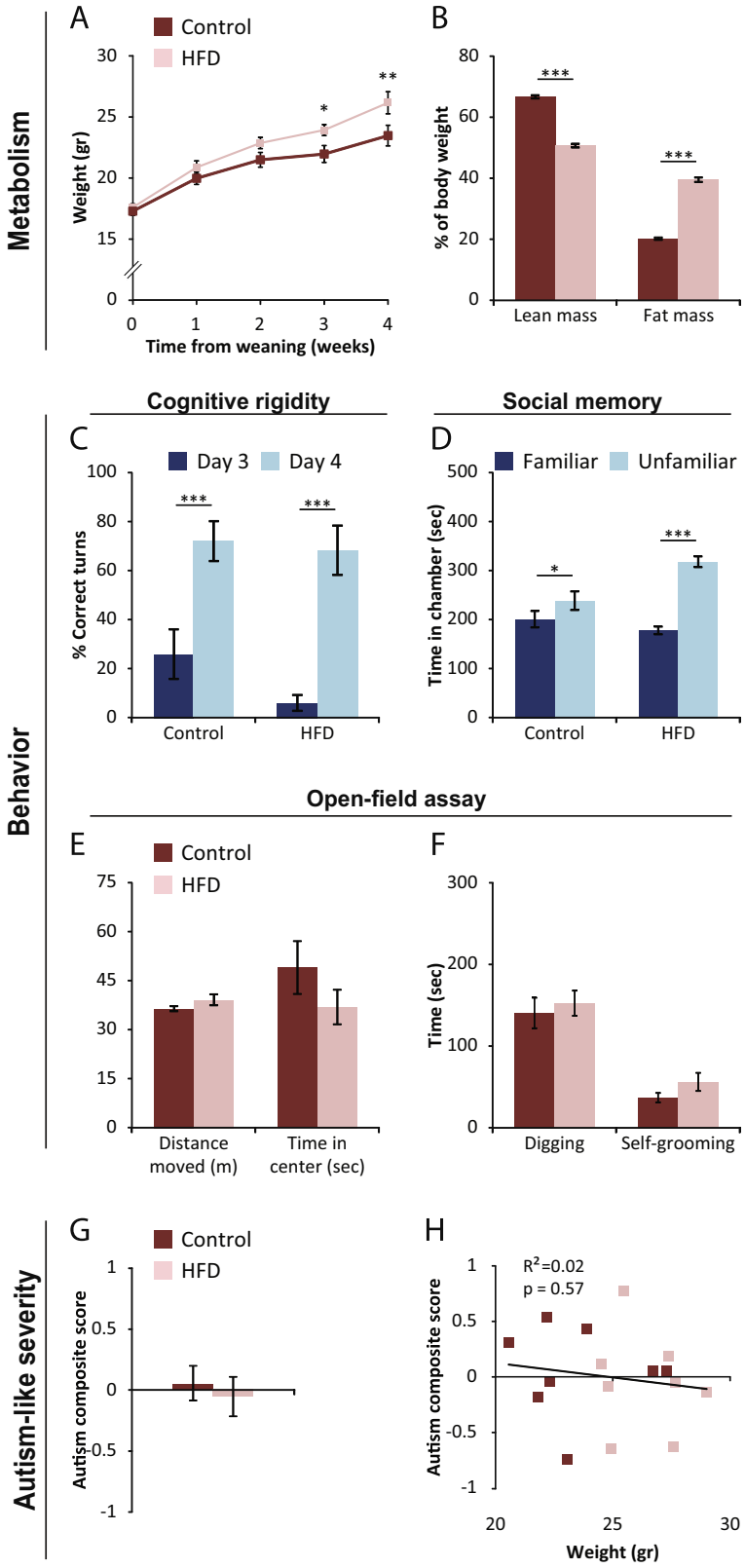

Fig. 6. Metabolic and behavioral effects of HFD in C57 mice. (A) Weight gain of control and HFD mice. (B) Body composition analysis, displayed as average lean and fat masses of each group. (C–F) Behavioral measurements. (C) T-maze assay, days 3–4. (D) Preference of social novelty assay. (E and F) open field assay: locomotor parameters (E) and stereotypical behaviors (F). (G) Autism composite score for control-fed and HFD C57 mice. (H) Correlation between the autism composite score and the weight of each mouse. Results are displayed as mean ± SEM. $n_{Control} = 8$, $n_{HFD} = 8$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for repeated measures ANOVA or a one-way ANOVA followed by Fisher's *post hoc* analysis.

mouse and did not demonstrate any social deficiency compared to the control group. In the social phase of the running/jammed wheel assay, both diet groups of BTBR mice spent similar durations of time with the unfamiliar mouse, while the control mice spent significantly more time with the wheel. This might suggest a lower level of motivation to turn the wheel in the HFD group. The literature regarding the effects of HFD on social behavior is inconclusive. Some researchers observed an increase in social behaviors in obese animals (Hilakivi-Clarke et al., 1996; Buchenauer et al., 2009; Haagensen et al., 2014), and a consistent reduction associated with weight loss (Koizumi et al., 2006), though in most cases the social parameter quantified was aggressive behavior. Other studies have found opposite effects of reduced social parameters in rats (Pohl et al., 2014), mice (Jones et al., 2013) and in humans (Taylor et al., 2013; Verdejo-Garcia et al., 2015). An analysis conducted on 5 measures of social skills and behavior for more than 13,000 ninth graders revealed no difference between overweight and non-overweight boys, while overweight girls displayed poorer social skills, but this effect was attributed, at least in part, to social discrimination from the other students (Judge and Jahns, 2007). Notably, neither HFD nor exercise had any effect on the behavior of C57 mice in the three-chamber social test (Kang et al., 2014), similarly to the results obtained in our cohort of C57 mice. Taken together, HFD appears to negatively affect some aspects of social behavior. Our results are for the most part in agreement, demonstrating an adverse effect of HFD on social memory, but only for a condition of pre-disposition to an autism-like phenotype in the BTBR mice.

High-fat diet in BTBR or C57 mice did not affect repetitive or stereotypic behavior, assessed by quantifying the duration of digging and self-grooming in a novel environment. Very few studies have investigated the influence of HFD or obesity on repetitive behavior, though one study showed that C57 female mice fed with HFD buried more marbles in the marble burring test (Krishna et al., 2015). The discrepancy compared to our results might be explained by the sex differences between the studies, though further research is required to assess the full interaction between the factors of sex and nutrition with regard to stereotypic behavior.

Taken together, our behavioral assessment suggest a general worsening in autism-related phenotype and specifically cognitive rigidity and social behavior, in BTBR mice fed by HFD continuously from their weaning. This effect is also manifested in the significant increase of their autism composite score. Notably, HFD in C57 mice produced only minor effects on autism-related behaviors, and the summarizing autism-related severity score was not associated with the individual weight of each mouse. This might indicate that the effect of HFD on autism-related behavior is specifically relevant in a condition of predisposition to an autism-like phenotype. These results correspond well with current epidemiological findings, demonstrating an association between autism and obesity (Curtin et al., 2014), and with reports on improved cognitive and behavioral symptoms of autism due to diet (Herbert and Buckley, 2013; Ruskin et al., 2013) or exercise (Srinivasan et al., 2014). Our results strengthen the assumption of causal link between obesity and autism, and further highlight the importance of balanced nutrition and weight monitoring in individuals with autism and other ASD.

An interesting question is regarding the physiological factor through which HFD enhances autism-related behavioral deficits. As in every study where high-caloric diet is administered *ad libitum*, it is not clear whether the behavioral outcome is due to exposure to certain ingredients in the diet itself or the resulting weight-gain (Coradini et al., 2013). It has been previously shown that HFD can alter social-related behaviors independently of weight gain (Michel et al., 2005; Finger et al., 2011). However, we assume that in our study, the factors of diet and weight were inter-connected, as autism-related severity score in BTBR mice was significantly correlated with the weights of the mice following 5 weeks of diet, suggesting some effect for the weight itself. It should be emphasized, though, that the effects of the weight were not due to any locomotor deficits, since we found no differences between the groups in two independent locomotion measurements. Further research is needed to assess the exact contribution of HFD compared to weight gain on autism-related behaviors in BTBR, possibly through restricting the amount of calories consumed by the mice in the HFD group to prevent excessive weight gain (Tauriainen et al., 2011).

Considering the major role of the hypothalamus in regulation of both social behavior (Goodson, 2005) and metabolism (Berthoud and Munzberg, 2011), we analyzed gene expression in this region following behavioral testing. We hypothesized that the effects of diet-induced obesity on social and autism-related behavior might be connected to alterations in dopamine or oxytocin signaling in the hypothalamus region (Love, 2014). Our results indicated marginally significant increases in the expression levels of TH and D1-R in the HFD mice. In addition, we found significant correlations between autism-related severity and weight to these two factors of dopamine transmission in the hypothalamus. Exposure of rodents to HFD has been shown to induce extensive alterations in dopamine signaling and dopamine down-stream response elements in several brain regions, including the prefrontal cortex (Vucetic et al., 2012; Grissom et al., 2015), nucleus accumbens (South and Huang, 2008; Labouesse et al., 2013), ventral tegmental area (Abizaid et al., 2006) (Teegarden et al., 2008) and the hypothalamus (Li et al., 2009; Vucetic et al., 2012; Kaczmarczyk et al., 2013). These alterations, in turn, have been associated with changes in behaviors related to various aspects of motivation and reward processing, such as executive function (Grissom et al., 2015), reward sensitivity (Davis et al., 2008; Carlin et al., 2013), learning and memory (Kaczmarczyk et al., 2013), anxiety-related behaviors (Sharma et al., 2013) and social interactions (Grissom and Reyes, 2013). Our findings are in line with these previous studies, suggesting a possible mechanism through which HFD exacerbates cognitive and social impairments in BTBR. In other words, the autism-related behavioral deficiencies induced by HFD might be mediated by the involvement of the dopaminergic system,

including reduction in dopamine signaling in the hypothalamus and perhaps also dysfunctions in additional regions of the brain reward system. However, extensive experimental research is needed to prove this relationship.

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