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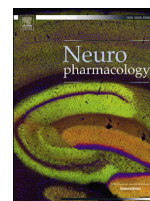

Sodium butyrate attenuates social behavior deficits and modifies the transcription of inhibitory/excitatory genes in the frontal cortex of an autism model

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ABSTRACT

The core behavioral symptoms of Autism Spectrum Disorders (ASD) include dysregulation of social communication and the presence of repetitive behaviors. However, there is no pharmacological agent that is currently used to target these core symptoms. Epigenetic dysregulation has been implicated in the etiology of ASD, and may present a pharmacological target. The effect of sodium butyrate, a histone deacetylase inhibitor, on social behavior and repetitive behavior, and the frontal cortex transcriptome, was examined in the BTBR autism mouse model. A 100 mg/kg dose, but not a 1200 mg/kg dose, of sodium butyrate attenuated social deficits in the BTBR mouse model. In addition, both doses decreased marble burying, an indication of repetitive behavior, but had no significant effect on self-grooming. Using RNA-seq, we determined that the 100 mg/kg dose of sodium butyrate induced changes in many behavior-related genes in the prefrontal cortex, and particularly affected genes involved in neuronal excitation or inhibition. The decrease in several excitatory neurotransmitter and neuronal activation marker genes, including cFos Grin2b, and Adra1, together with the increase in inhibitory neurotransmitter genes Drd2 and Gabrg1, suggests that sodium butyrate promotes the transcription of inhibitory pathway transcripts. Finally, DMCM, a GABA reverse agonist, decreased social behaviors in sodium-butyrate treated BTBR mice, suggesting that sodium butyrate increases social behaviors through modulation of the excitatory/inhibitory balance. Therefore, transcriptional modulation by sodium butyrate may have beneficial effects on autism related behaviors.

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1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by social interaction and communication deficits, and repetitive or stereotyped behaviors. ASD affects approximately 1 in 68 children with a ratio of 4:1 affected male to female (Liu and Takumi, 2014). However, the determination of specific molecular mechanisms in the brain that are involved in the development of autistic behavior has remained elusive. Dynamic changes in histone modification are considered to play a primary role in the development of the nervous system and the development of mammalian behaviors (Rangasamy et al., 2013). Histone modifications and DNA methylation are the main epigenetic mechanisms that modify gene transcription. Different subtypes of histone modifications, including acetylation, methylation, and ubiquitination, define a specific epigenetic state that balances between gene silencing and gene activation (Sailaja et al., 2012). Histone acetylation in promoter regions is usually associated with activation of gene transcription, while the effect of histone methylation is highly dependent on the histone region which is modified. Histone acetylation is performed by Histone acetylases, while Histone Deacetylases (HDACs) removes acetyl groups from the histones.

Recently, several lines of evidence have led to the conclusion that both DNA methylation and histone modifications are involved in the etiology of ASD. Neuronal H3K4 methylation was found to be dysregulated in the brain of individuals with autism (Shulha et al., 2012). HDAC4 gene expression levels were also found to be upregulated in the prefrontal cortex of individuals with autism (Nardone et al., 2014). DNA methylation levels were also found to be altered in the brain of individuals with autism (Ladd-Acosta

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et al., 2014; Nardone et al., 2014).

Several studies suggest that HDAC inhibitors (HDACis) have potential as a therapeutic agent against cognitive impairments (Gräff and Tsai, 2013a, 2013b; Takuma et al., 2014). HDACi administration has beneficial effects in models of neurodegenerative diseases, such as Alzheimer's Disease (Guan et al., 2009) and Huntington's Disease (Gundersen and Blendy, 2009), and HDAC inhibitors increased social behaviors in prairie voles (Wang et al., 2013). Sodium butyrate (SB) is a widely known HDAC inhibitor which is often used in psychobiology studies, due to its ability to cross the blood brain barrier. SB attenuates memory deficits and improves cognitive function in several animal models (Fischer et al., 2007; Takuma et al., 2014). In addition, multiple studies have determined a memory-enhancing effect of SB in Alzheimer's Disease mice models (Govindarajan et al., 2011; Kilgore et al., 2010), and a separate study determined a similar effect in a rat model aging-related memory decline (Reolon et al., 2011). In addition, SB has antidepressant effects (Steckert et al., 2013), and may attenuate anxiety like behavior (Gundersen and Blendy, 2009). SB has an established role in the inhibition of most class I and II (Wei et al., 2014) HDACs.

The frontal cortex has primary roles in high-order cognitive and emotional behaviors, including social behavior, and recent studies have highlighted morphological, transcriptional, and epigenetic dysregulation in the frontal cortex of individuals diagnosed with autism. Separate studies reported an enlarged frontal cortex (Carper and Courchesne, 2005) and an increase in neuronal number in the frontal cortex (Courchesne et al., 2011). Converging evidence also suggests over connectivity within the frontal cortex, with parallel decreases in connectivity in other brain regions (Courchesne and Pierce, 2005). In a whole throughput gene transcription study, Voineagu et al. documented an increase in immune response gene expression, and a decrease in synaptic gene expression, in the frontal cortex of individuals with autism (Voineagu et al., 2011). In epigenetic studies, dysregulation in both H3K4 methylation patterns and DNA methylation patterns have been identified in the frontal cortex (Nardone et al., 2014; Shulha et al., 2012).

Animal models of autism include BTBR T+tf/J (BTBR), which exhibits several autism-like behavioral phenotypes, including social interaction deficits, impaired communication and repetitive behavior (Langley et al., 2015; Martin et al., 2014; McFarlane et al., 2008). While no animal model can fully recapitulate all symptoms of a human neurodevelopmental disorder, BTBR mice strain displays phenotypic traits of all diagnostic symptoms of autism. BTBR is an inbred mouse strain, and unlike genetic models, it is not clear what is the exact genetic aberration in this strain that is responsible for the autism-like phenotype. BTBR mice have a mutation in the autism-related gene Disc1, and a nonsynonymous SNP in the geneKmo (Meyza et al., 2013). However, it is not clear if these genetic changes have any relation to their autism-like behavior. Due to the consistent replication of the autism-like phenotype in several laboratories, BTBR mice are often used in pharmacological studies related to autism. In early work on BTBR mouse, researchers also found an abnormality of the corpus callosum, which is an anatomical anomaly also present in some autistic individuals (McFarlane et al., 2008).

In this study we test the hypothesis that chronic treatment with the HDAC inhibitor sodium butyrate will improve behavior deficits in the BTBR mouse model for ASD. Here we show that chronic treatment with SB during adulthood can attenuate social deficits in the three chamber test and social odor test, while having no side-effects on locomotor or anxiety-like behaviors. Whole genome gene expression has been employed to reveal molecular mechanisms in the frontal cortex which are likely to be responsible for the effects of sodium butyrate on behavior.

2. Materials and methods

2.1. Mice handling

Mice were housed according to the FELSA guidelines. All mice were bred and maintained in a vivarium at 22 °C and 50% humidity in a 12 h light/dark cycle, with food and water available *ad libitum*. BTBR T+tf/J strain was donated by Dr. Tali Kimchi (Weizmann Institute of Science). All experimentation performed in this study was approved by the Bar Ilan University Institutional Animal Care and Use Committee (IACUC) in protocol number 27-7-2013.

2.2. Drug administration

Sodium butyrate (Sigma and Cayman chemical company) was suspended in PBS and administrated in two concentrations: 100 mg/kg and 1200 mg/kg. PBS was administered as control. Drugs were administered by an intraperitoneal injection once a day for 10 days. Last injection was given 60 min before the beginning of the experiment.

Separate groups of mice were used for each behavioral test and for each molecular test (RNA-seq, cFos, etc.). Separate groups were necessary to ensure that each test was carried out at the exact same time point, 60 min following the tenth administration of sodium butyrate.

DMCM (methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate, Santa Cruz) was dissolved in 100% DMSO (Dimethyl Sulfoxide, Sigma) and diluted further with PBS. All experimental mice received 9 days injection of SB 100 mg/kg and on the day of the experiment the mice were divided in to two groups. The first group injection containing both SB and DMCM. The final injection was administrated 30 min before the beginning of the experiment.

2.2.1. Social interaction

The three chamber paradigm was performed as previously described (McFarlane et al., 2008). Apparatus is a Non-Glare Perspex box (60 × 40 cm) with two partitions that divide the apparatus to three chambers, Left, Center and Right (20 × 40 cm). The mouse is placed in the middle chamber for habituation (5 min) when the entry for both side chambers is barred. Test mouse was then allowed to explore the whole arena for ten minutes, where they freely choose between interacting with a novel mouse in one chamber, or stay in an empty chamber (social test). Immediately following this test, a second stranger mouse is introduced to the empty chamber, and the test mouse is allowed ten minutes to freely choose between interacting with the novel or familiar mouse. Time spent in each chamber is measured by Ethovision. The experiments were recorded with the Panasonic WV-CL930 camera and with the Ganz IR 50/50 Infrared panel. The recorded movement of the mice was analyzed by the Ethovision XT 10/11 (Noldus) software.

2.2.2. Social odor test

The test was performed in the same apparatus as the social interaction test. The mouse is placed in the middle chamber for habituation (5 min) when the entry for both side chambers is barred. Test mouse was then allowed to explore the whole arena for ten minutes, where they freely choose between the odor of unfamiliar bedding in one chamber, or stay in an empty chamber with clean bedding (social odor test). Immediately following this test, novel bedding was introduced to the empty chamber, and the test mouse is allowed ten minutes to freely choose between the novel or familiar odor. Time spent in each chamber is measured by Ethovision.

2.2.3. Open field test

50 × 50 cm and illuminated at 40 lux. A square arena made from a Non-Glare Perspex was used in this study. During the 10 min trial we measured total distance moved and time in center (25 × 25 cm).

2.2.4. Self-grooming

Mice were scored for spontaneous self-grooming as previously described (Silverman et al., 2015). Each mouse was placed individually into a 50 × 50 cm square arena made from a Non-Glare Perspex, illuminated at 40 lux. After 10 min habituation period, each mouse was scored using parameters of cumulative time spent grooming during a 20 min session.

2.2.5. Marble burying test

Repetitive marble burying was measured as previously described (Gould et al., 2012). The apparatus is a Non-Glare Perspex (20 × 40 cm). Twenty green glass marbles (15 mm in diameter) were arranged in a 4 × 5 grid that covered 2/3 of the apparatus on top of 5 cm clean bedding. Each mouse was placed in the corner that did not contain the marbles and was given 30 min exploration period, after which the number of marbles buried, was counted. "Buried" was defined as 2/3 covered by bedding. Testing was performed under dim light (25 lux).

2.2.6. Western blot analysis

Western blot analysis was carried out as previously described (Takuma et al., 2014). Mice were sacrificed by rapid decapitation and brains were quickly removed. The PFC was isolated on dry ice and homogenized in a lysis buffer (1 M Tris, pH 7.4, 0.4 M EDTA, and 0.3 M sucrose) using a hand homogenizer. The protein concentration was determined using Bradford or Pierce® BCA Protein Assay Kit (Thermo Scientific Inc., Rockford, IL, USA). After homogenization samples were mixed with sodium dodecyl sulfate (SDS) sample loading buffer (1 M Tris–HCl pH 6.8, 0.5 M SDS,0.18 M Bromophenol Blue, 6.5 M glycerol, 14.3 M β-mercaptoethanol) in a ratio 1:5 and boiled for 3 min. Equal amounts of protein (25 μg) were separated by SDS-polyacrylamide gel electrophoresis. The membrane was probed with primary antibodies, Acetyl-Histone H2B (1:500), Histone H2B (1:1000), Acetyl-Histone H3 (1:3000), Histone H3 (1:1000), Acetyl-Histone H4 (1:1000) and Histone H4 (1:1000) (Acetyl-Histone Antibody Sampler Kit #9933, Cell Signaling Technology) overnight, followed by IRDye 800CW/680RD secondary antibodies (LI-COR). The immune complexes were visualized with infrared fluorescence (ODYSSEY CLx, LI-COR).

2.2.7. cFos immunostaining

Mice were anaesthetized with pentobarbital and transcardially perfused with 100 ml of ice cold phosphate buffered saline (PBS) solution, followed by 100 ml freshly prepared ice cold paraformaldehyde (PFA) 4%. Brains were removed, immersed in PFA for 24 h at 4 °C and transferred into 30% sucrose. 30 μM coronal sections were cut in a sliding microtome. Sections were stained for two days with a primary antibody against cFos (1:500, Santa Cruz Biotechnology), followed by one hour incubation with a Biotin-SP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, INC), followed by Cy3 conjugated Streptavidin (Jackson ImmunoResearch Laboratories, INC), and counterstained with Hoechst. The slices were examined by florescence microscopy (HITACHI HV-F22 color camera and Zeiss AxioCamMRm) and total number of cFos positive cells in the PFC was analyzed by ImageJ and Imaris 8.0.

2.2.8. RNA-seq library preparation

Mice were sacrificed by rapid decapitation and brains were quickly removed. The PFC was isolated on dry ice. Total RNA was produced using RNeasy® Mini Kit (QIAGEN). RNA integrity of all samples was checked by Bioanalyzer (Agilent), and all samples had a minimum RIN number of 8. Sequencing libraries were prepared using Illumina® TruSeq® RNA Sample Preparation Kit v2. RNA was extracted from the PFC of 18 BTBR mice (9 for each group) and pooled for 3 libraries per treatment with an average of 70 million reads per library.

2.2.9. Sequence analysis

Reads were mapped to the Mus Musculus reference genome (mm9) using the Tophat2 software (release Tophat2.0.12). The heatmap (Fig. 3) was produced by calculating isoform expression in FPKM units using cufflinks (release cufflinks2.2.1) (Trapnell et al., 2010) (STable 1). For differential expression analysis, we used the script HTSeq-count (Anders et al., 2014) (version 0.6.1) to retrieve read count from each library, followed by analysis with DEseq (Anders and Huber, 2010). Statistics for each gene, including FDR corrected p-values are found in STable 2. Each observed alternation in expression with p value <0.01 was considered significant.

2.2.10. Real time PCR

Mice were sacrificed by rapid decapitation and brains were quickly removed. The PFC was isolated on dry ice. Total mRNA was purified using PerfectPure tissue RNA kit (5 Prime). cDNA was produced using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Real time PCR was performed using FastStart Universal SYBR Green Master (Rox) (Roche) and analyzed with ViiA™7 Real-Time PCR System (Applied biosystems). Primers used are described in STable 3.

2.2.11. Statistical analysis

Data are presented as mean ± SEM. Analysis between two groups is performed by two-tailed T-tests. Analysis performed by more than two groups is performed by one way ANOVA, followed by two-tailed T-tests.

3. Results

3.1. Social behavior tests after chronic treatment with sodium butyrate

The effect of sodium butyrate (SB) on social behavior in BTBR mice was evaluated by the three-chamber social interaction test, social novelty test, and social odor test. This test is designed in order to evaluate social behavior in mice and to assess social deficits (McFarlane et al., 2008). We tested both a low dose (100 mg/kg) and high dose (1200 mg/kg) of SB in all of our experiments. Both of these doses have been administrated in multiple studies (Fischer et al., 2007; Gundersen and Blendy, 2009; Sailaja et al., 2012), and one previous study found differences in hormonal activity and anxiety-like behavior between administration of these two doses (Gundersen and Blendy, 2009). Mice which received SB at a low dose displayed a significant preference for the chamber containing the stranger mouse compared to the chamber with the empty cage, unlike mice which received injections of PBS (Fig. 1A). Interestingly, the high dose SB group did not exhibit any significant preference for social interaction (Fig. 1A). In the social novelty test, the low dose SB group significantly preferred the novel mouse to a familiar mouse, unlike the PBS and high dose SB groups (Fig. 1B). Therefore, the low dose of sodium butyrate induced increased social behaviors in the social interaction and social novelty tests.

A social odor test was used to further examine the effects of SB on social behavior. In this test, mice choose to spend time in a chamber with male-soiled bedding, and therefore contain social odors, or a chamber with clean bedding. The low dose of SB induced

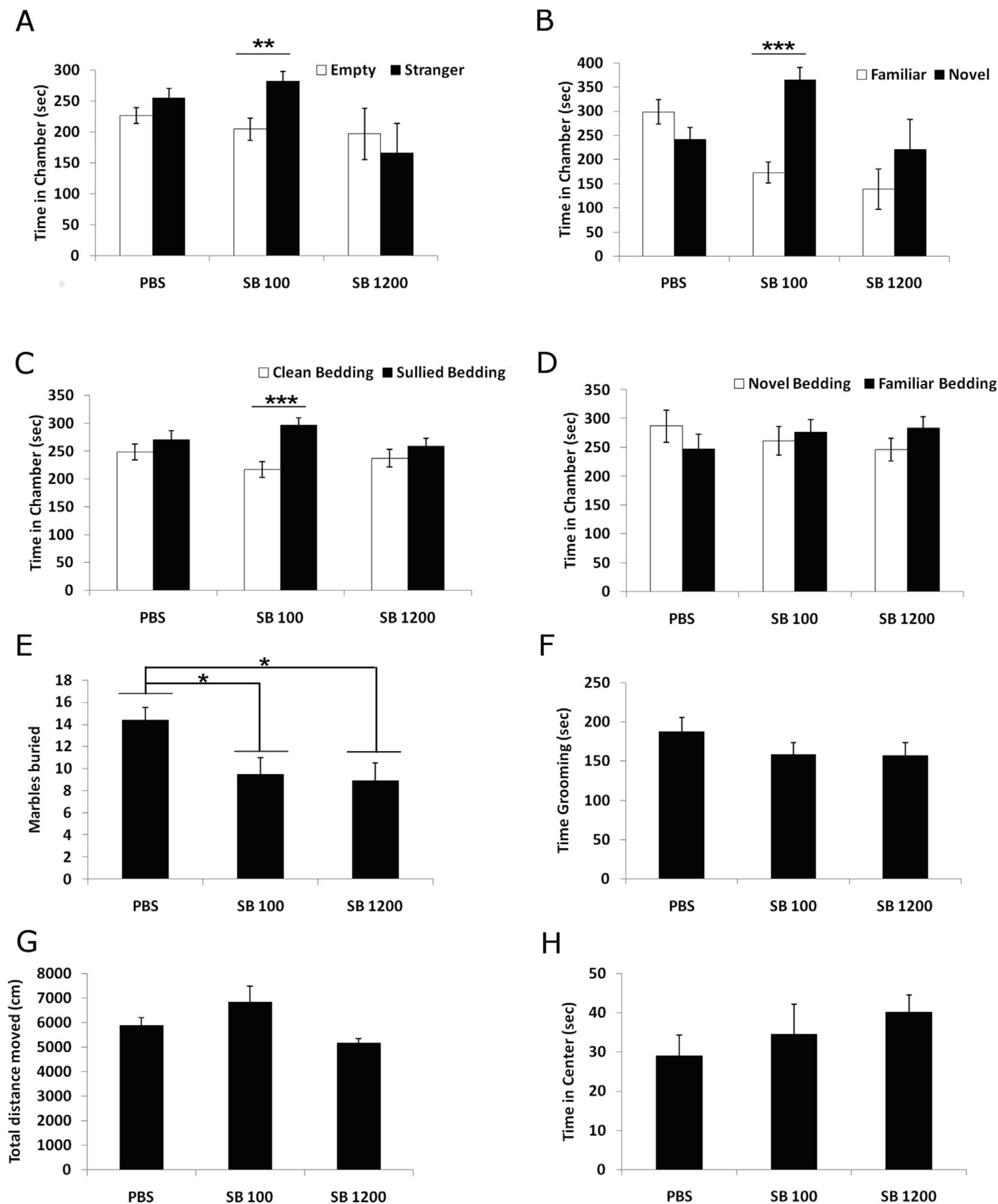

Fig. 1. Specific dose of sodium butyrate increases social behavior in the BTBR autism model. BTBR mice were treated for ten days by i.p. injection of PBS, 100 mg/kg sodium butyrate, or 1200 mg/kg sodium butyrate. Low dose of sodium butyrate induced a preference for spending time in chamber with stranger mouse (paired two tailed t-test, $^{**}p = 0.0058$, $n = 11$–12 per group) (A) and preference for spending time in chamber with a novel mouse, in comparison to familiar mouse ($n = 11$–12 per group, paired two tailed t-test, $^{***}p = 0.000015$) (B). In addition, low dose induced preference for social odor (paired two tailed t-test, $^{***}p = 0.00067$, $n = 11$–12 per group) (C), but no significant effects were observed in preference for novel or familiar social odor (D). Both doses of sodium butyrate decreased marble burying (One Way Anova, $F_{2-16} = 6.156$, $p = 0.011$, Tukey's Test, $^{*}p < 0.05$, $n = 6$–7 per group) (E), but neither had a significant effect on grooming (F). Sodium butyrate didn't effect locomotor behavior in open field, as measured by distance traveled ($n = 11$–12 per group) (G), or anxiety-like behavior, as measured by time in center of open field $n = 11$–12 per group (H).

a preference for the social odor chamber, while no preference was seen in PBS or high dose injected groups (Fig. 1C). However, no experimental groups displayed a differential preference for novel social odors, in comparison to familiar social odors (Fig. 1D).

3.2. Marble burying and self-grooming tests

It is well established that BTBR mice display repetitive and stereotyped patterns of behavior (Langley et al., 2015; Martin et al., 2014; McFarlane et al., 2008; Shpyleva et al., 2014; Stephenson et al., 2011). The effect of SB on repetitive behaviors was determined using the marble burying and self-grooming paradigms. Both the high dose and low dose SB treatments induced a decrease in marble burying, in comparison to PBS treated mice (Fig. 1E), which suggests a decrease in repetitive behaviors. In the grooming test, however, there was no significant difference in grooming between all experimental conditions (Fig. 1F). Therefore, sodium butyrate may regulate only specific types of repetitive behaviors.

3.3. Anxiety and locomotion

Considering that locomotor and anxiety behavior may be cofounders in the behavioral tests we have employed, we determined if SB regulates these behaviors in the open field. There were no

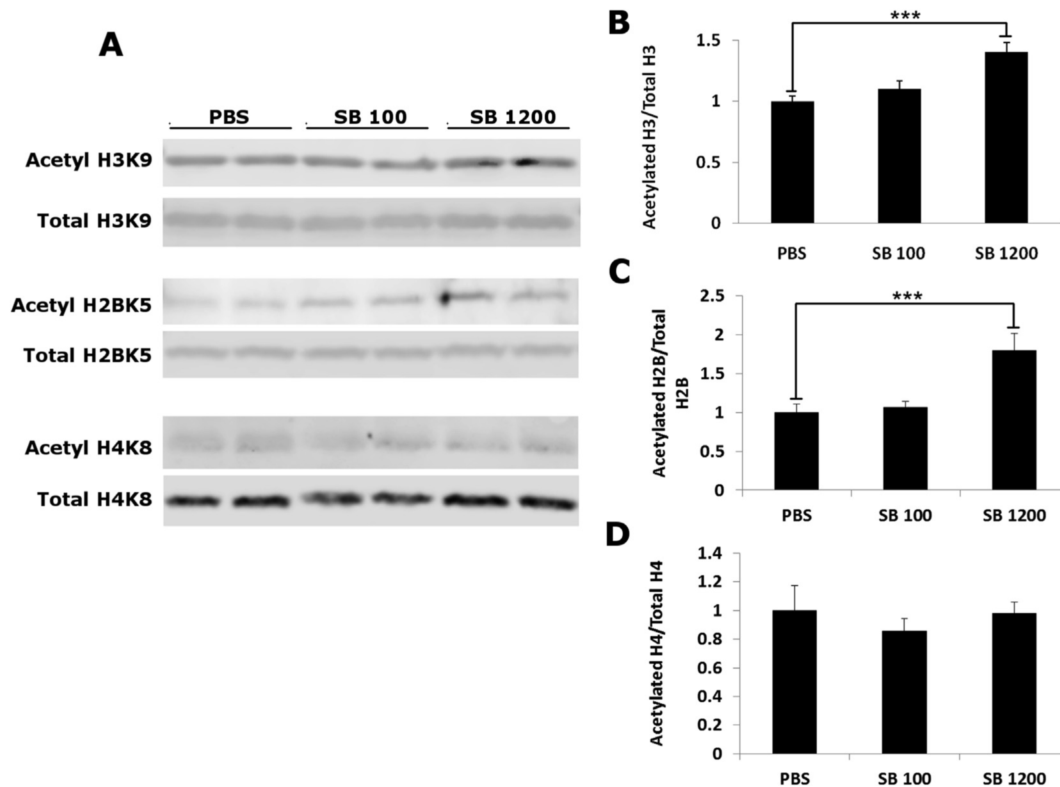

Fig. 2. Sodium butyrate-induced changes in global histone acetylation levels are dose dependent. (A) Western Blot analysis was performed on the prefrontal cortex with antibodies targeting acetylated and total histones. High dose (1200 mg/kg) administration of sodium butyrate induced an increase in acetylation levels of H3K9 (One Way Anova, $F_{2,21} = 10.24$, $p = 0.000787$, Tukey's Test, $^{***}p = 0.001$, $n = 8$ per group) (B) and H2BK5 (One Way Anova, $F_{2,15} = 7.808$, $p = 0.008$, Tukey's Test, $^{***}p = 0.001$, $n = 6$ per group) (C), but not H4K8 (D).

differences between experimental groups in total distance moved in a ten minute period in the open field (Fig. 1G). In addition, there were no significant differences in anxiety behavior, as measured by time spent in the center zone of the open field (Fig. 1H). Therefore, it is unlikely that the sodium butyrate-induced changes are related to locomotor function or general changes in anxiety-levels.

3.4. Effects of sodium butyrate on histone acetylation in the PFC

We next determined if sodium butyrate induces global changes in histone acetylation in the prefrontal cortex of BTBR mice, at the dosage used in our experimental studies. The high dose of sodium butyrate induced increases in histone acetylation at Histone 2BK5 and Histone 3K9, but not at Histone 4K8 (Fig. 2). Increase in H3 acetylation after high dose treatment of SB was also previously observed in Takuma et al. (Takuma et al., 2014). However, the low dose of sodium butyrate, which had induced changes in social behavior, did not induce global histone acetylation differences that could be detected by the western blot assay. Therefore, the behavioral effects of low dose SB are not likely to be due to large global changes in histone acetylation.

3.5. Changes in expression of neuron activity-related genes in the prefrontal cortex after low dose SB treatment

Considering that the low dose of sodium butyrate doesn't regulate global acetylation levels, we hypothesized that it may regulate the expression of a subset of relevant genes. Therefore, to determine the effect of low dose SB treatment on gene expression in the prefrontal cortex of BTBR mice we conducted RNA-seq assay.333 genes were differentially expressed ($FDR < 0.01$) between SB treatment and control, including 133 genes that were down regulated and 200 that were up regulated (Fig. 3A, STable 2). Of the 333 genes, 30 of them are listed in the SFARI list of autism-related genes, which represents a highly significant overlap ($p < 0.00001$ hypergeometric probability test) (Fig. 3B). Gene ontology analysis of the upregulated and downregulated genes revealed several overlapping categories, including transmission of nerve impulse, cell–cell signaling, synaptic transmission, and neuron differentiation (Fig. 4C,D). A closer examination of the genes that were differentially expressed revealed that many genes encoding voltage-gated ion channels and excitatory neurotransmitter receptors were decreased, while a few inhibitory receptors were increased. We performed real time PCR, and validated the decrease in neuronal activation markers cFos and Fosb (Fig. 4A), as well as the decrease in several voltage gated ion channel genes (Fig. 4B). In addition the real time PCR analysis validated the decrease in excitatory receptor genes, such as Adra1a, Adra1d, Grin2b, and Grm2 (Fig. 4C), and the increase in inhibitory receptor genes DRD2 and Gabrg1 (Fig. 4D). These results suggest that sodium butyrate changes the expression in genes involved in the excitatory/inhibitory balance in the prefrontal cortex. The dysregulation of this balance towards excitation has previously been implicated in the etiology of autism spectrum disorders (Han et al., 2014; Yizhar et al., 2011).

3.6. Immunohistochemistry of cFos positive cells in the PFC of BTBR mice after SB treatment

Considering the sodium butyrate-induced changes in cFos and genes involved in neuronal activation or inhibition, we used immunohistochemistry to determine if sodium butyrate regulated the level of cFos protein. SB induced a significant decrease in the number of cFos-positive cells in the prefrontal cortex of the BTBR

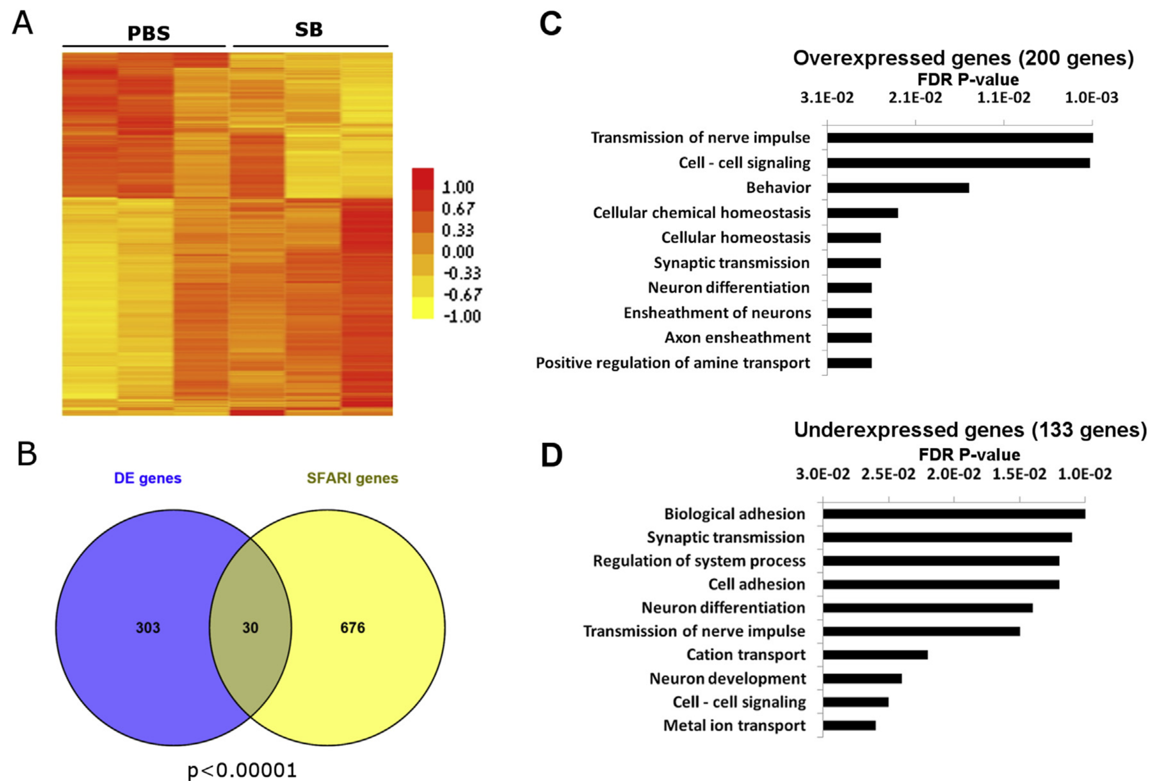

Fig. 3. Whole transcriptome analysis of sodium butyrate-induced changes in gene expression in prefrontal cortex. (A) Heat map of 333 differentially expressed genes in frontal cortex of mice treated with 100 mg/kg sodium butyrate. (FDR < 0.01). This includes 200 upregulated genes and 133 downregulated genes. Color Key is in logarithmic scale. (B) Venn diagram representing the overlap between the differentially expressed genes and the SFARI database of autism-related genes. (C,D) Gene ontology categories that are enriched in the upregulated genes (C) and in the downregulated genes (D).

mice (Fig. 4E,F). These results strengthen the finding that sodium butyrate decreases neuronal activity in the PFC.

3.7. Social behavior test after DMCM administration

Previous studies had indicated that the GABAergic system in the prefrontal cortex is dysregulated in individuals with autism (Braat and Kooy, 2015; Mori et al., 2012). In addition, studies have suggested that dysregulated GABAergic function may be partly responsible for the autism-like behavior in the BTBR mice (Braat and Kooy, 2015; Han et al., 2014; Harada et al., 2011; Silverman et al., 2015). In order to determine if excitatory/inhibitory signaling may be partly responsible for the effects of sodium butyrate on social behavior, we administered DMCM (methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate) to SB-treated BTBR mice. Since DMCM works to downregulate GABAergic inhibitory signaling, we hypothesized that DMCM would decrease social behaviors in SB-treated mice. cFos levels were increased by approximately 50% in the frontal cortex of sodium butyrate treated mice at 30 min after administration (Fig. 5A). In the social interaction test the BTBR mice which received SB alone displayed social preference in the three-chambered test, while those treated with SB and DMCM displayed no social preference (Fig. 5B). Similar results were present in the social novelty test. The BTBR mice which received SB displayed preference for social novelty while the DMCM group displayed no preference for social novelty (Fig. 5C). In contrast, DMCM did not affect the number of marbles buried by the sodium butyrate treated mice (Fig. 5D). Therefore, GABAergic signaling may be involved specifically in the SB-induced sociability, but not related to the effects on the marble burying test. These results support the hypothesis that sodium butyrate-induced social behavior is specifically mediated through changes in the excitatory/inhibitory signaling pathways.

4. Discussion

The present study suggests that sodium butyrate has positive effects on social behavior in an autism mouse model, and these effects are partly due to modulation of the excitation/inhibition balance in the prefrontal cortex. Our findings add to current literature suggesting that HDAC inhibitors, in general, and sodium butyrate, in particular, have beneficial effects on behaviors related to neurodevelopmental and neurocognitive disorders. Previous studies suggest that HDAC inhibitors have potential as a therapeutic agent for treating learning and memory deficits (Takuma et al., 2014), have antidepressant like effects (Wei et al., 2014) and improve synaptic plasticity and cognition deficits (Gräff and Tsai, 2013a).

A low dose of sodium butyrate, but not a higher dose, had positive effects on social behavior. In a previous work, Gagliano et al. found that the same high dose of sodium butyrate (1200 mg/kg) induced an increase of anxiety-like behavior, while a 200 mg/kg dose did not induce anxiety-like behavior (Gagliano et al., 2014). In addition, only the high dose of SB induced an increase in stress hormone levels in the blood and markers of physiological stress in the brain. Considering that social behavior is strongly effected by blood hormones, it is possible that these high dose associated hormonal changes inhibit the beneficial effects of SB on social behavior. Our finding that the high dose, but not low dose, of sodium butyrate induces global changes in histone acetylation,

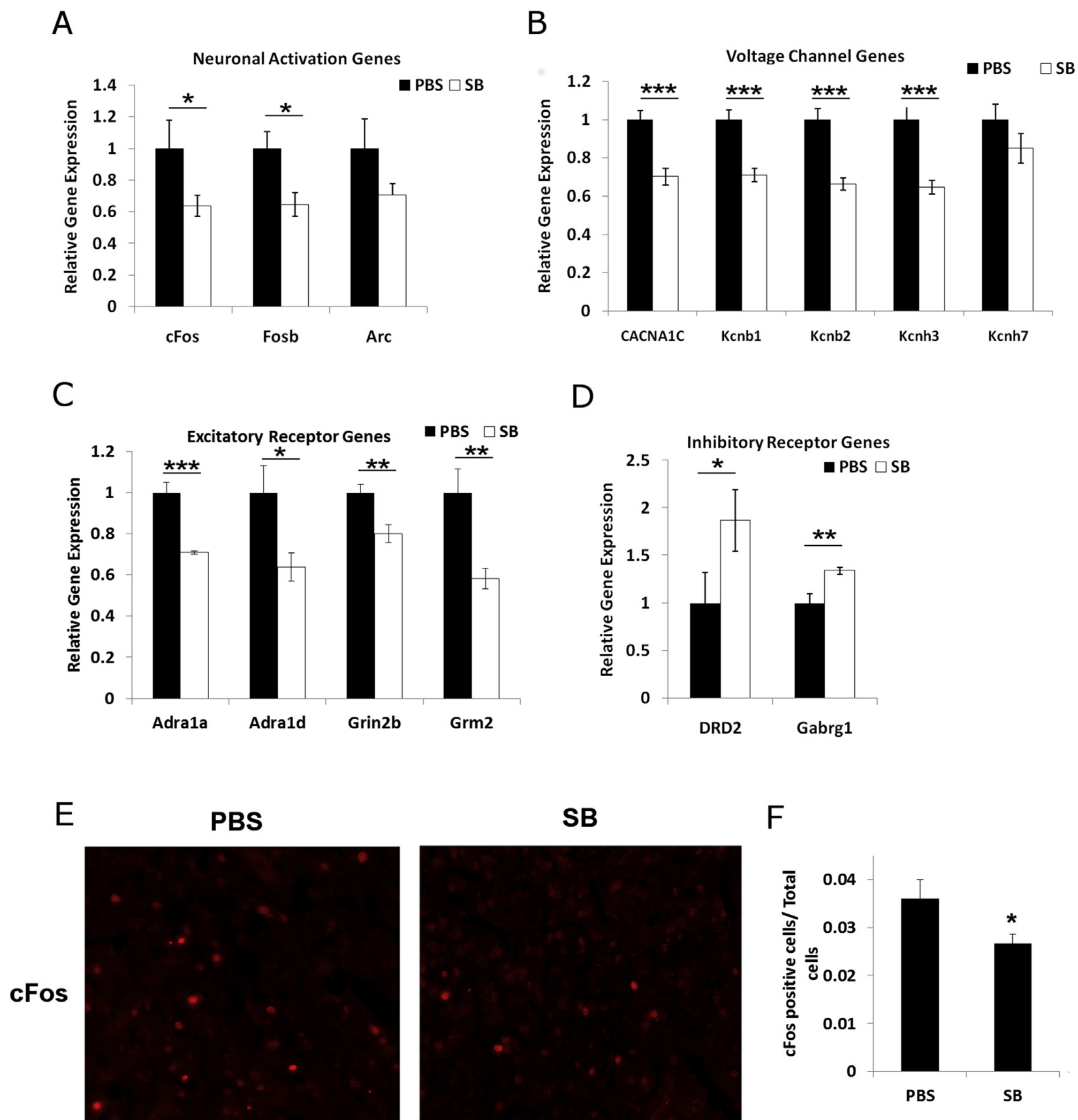

Fig. 4. Sodium butyrate affects the gene transcription of genes involved in excitatory/inhibitory balance and neuronal activation. Real Time PCR analysis validating differential expression among neuronal activation marker genes (A), voltage gated channel genes (B), excitatory neurotransmitter receptor genes (C), and inhibitory neurotransmitter receptor genes (D) n = 9 per group. Two-tailed t-tests were performed. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. (E) Immunohistochemistry of cFos peptide in the prefrontal cortex of PBS and sodium butyrate (100 mg/kg) treated mice. (F) Cell count of cFos positive cells reveals significant decrease in cFos staining in sodium butyrate treated animals. One tail T-test, *$p < 0.05$.

supports the view that the high dose had more global and wide-ranging molecular effects. In addition, it is possible that non-histone related effects of the high dose mask the beneficial properties of SB treatment on social behavior.

Social and cognitive deficits have been connected to increased ratio of excitatory to inhibitory synaptic transmission in the frontal cortex (Braat and Kooy, 2015; Han et al., 2014; Harada et al., 2011; Silverman et al., 2015). A decrease in GABA receptor expression and GABA receptor function has been reported in the brain of individuals diagnosed with autism (Braat and Kooy, 2015; Mori et al., 2012). In an optogenetic study, activation of PFC excitatory neurons in mice leads to impairments in social function and cognition without any abnormality in motor function or increased anxiety (Yizhar et al., 2011). In addition, treatment of the BTBR model with an inverse agonist of GABA receptors has previously been shown to attenuate autistic-like behavior. In our study, we determined that low dose of sodium butyrate induced a change in the expression of genes involved in excitatory and inhibitory pathways, including an increase in inhibitory receptor genes as Gabrg1 and DRD2, as well as decrease in activatory receptor genes as Grin2b and Grm2. Our results correlate with what is known so far on the GABAergic/Glutamergic balance in cognition impairments and social deficits in ASD individuals. Our further finding that SB-induced social behavior in BTBR mice can be reversed by a GABA reverse agonist strengthens the hypothesis that these transcriptional changes are involved in social behavior. Therefore, the excitatory/inhibitory balance in the prefrontal cortex may be a mechanism for the therapeutic effect of sodium butyrate, and may be a potential target for other therapeutics as well.

Previous links have been drawn between epigenetic processes

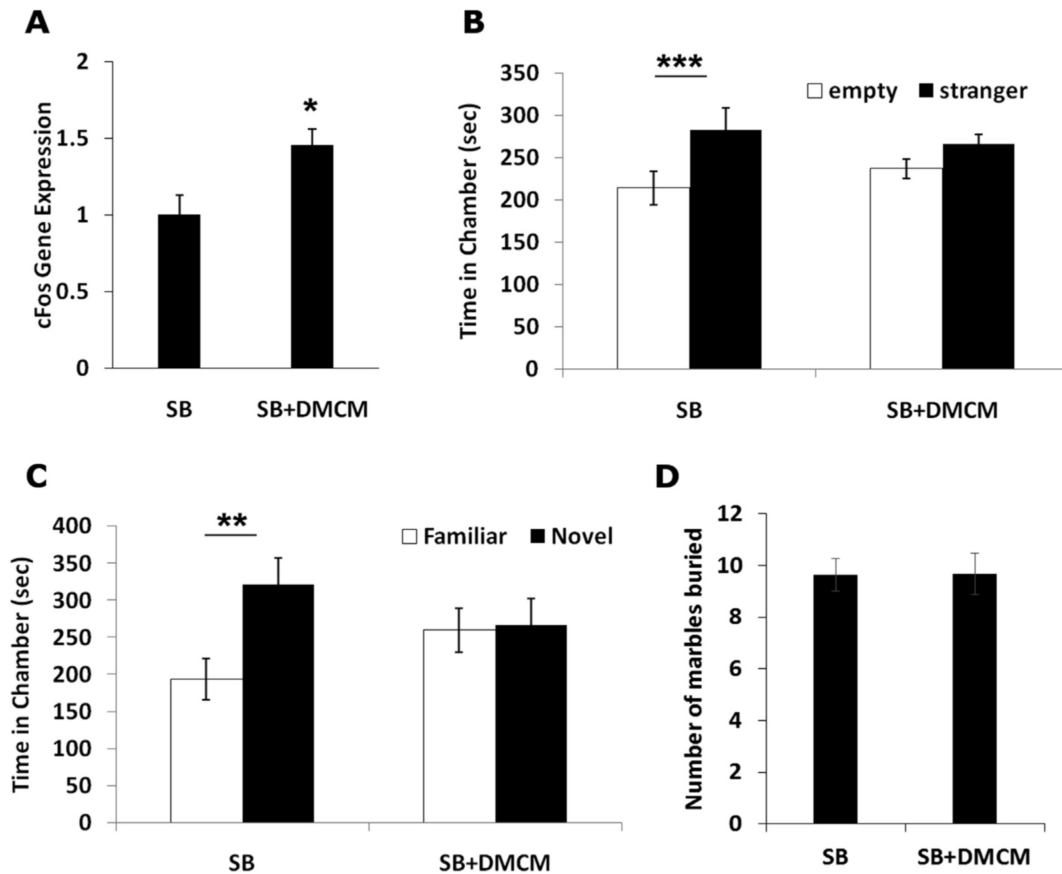

Fig. 5. Sodium butyrate decreases levels of cFos protein and reversal of sodium butyrate-induced social behavior by a reverse agonist of GABA. (A) Real Time PCR analysis of effect of cFos expression in the prefrontal cortex 30 min after administration with DMCM or vehicle. (Two tailed t-test, $^{*}p = 0.032$, $n = 6–7$ per group) (B, C) GABA reverse agonist DMCM decreased social behavior in sodium butyrate treated mice (paired two tailed t-test, $^{***}p = 0.000617$, $n = 12$ per group) (B), and decreased their preference for novel social interaction (paired two tailed t-test, $^{**}p = 0.007$, $n = 12$ per group) (C). (D) DMCM did not affect marble burying ($n = 12$ per group).

and neurophysiology. HDAC2 knockout increases long term potentiation (Guan et al., 2009) and inhibition of histone deacetylases with Trichostatin A in hippocampal slices induced an increase in long term potentiation in hippocampal slices (Levenson et al., 2004). However, in our studies, sodium butyrate treatment induced a decrease in the expression of neuronal activity related genes. Since the classic mechanism of action of sodium butyrate is the hyperacetylation of genes and increase of gene expression, the decrease in neuronal activity and related gene expression is likely due to downstream effects. This may be through the increase of transcription factors that downregulate the expression of activity-related genes. Alternatively, the increase of inhibitory genes, such as DRD2 and Gabrg1, may induce downstream pathways which inhibit neuronal activation and the expression of activatory genes. It is worthy to note that DNA methyltransferases, which are often involved in inhibition of gene expression, similar to HDACs, induce an increase in neuronal activity. DNA methylation inhibitors attenuate long term potentiation in vitro (Levenson et al., 2006) and conditional DNMT1/DNMT3a knockout in excitatory neurons also display a decrease in learning and memory (Feng et al., 2010), in parallel to decreased long term potentiation. Therefore, there appears to be a more complex relationship between epigenetic factors and neuronal activity.

In our study, we show that sodium butyrate has major effects on gene transcription in the prefrontal cortex. Our choice of sodium butyrate was due to its ability to cross the blood brain barrier (Banks et al., 1997), and be administered by intraperitoneal injection, which more closely resembles therapeutically-relevant systems of administration. However, we cannot rule out the possibility that sodium butyrate may also be affecting social behavior through gene transcription modifications in other brain regions.

There are still open questions in regards to the exact mechanism through which sodium butyrate influences social behavior and neuronal activity associated gene expression. Sodium butyrate targets all Class I HDACs and most class II HDACs, except for HDAC6 and HDAC10 (New et al., 2012). Therefore, inhibition of any of these HDACs may be involved in the molecular and behavioral effects. In addition, some HDACs, such as HDAC5, have a role in acetylation of cytoplasmic proteins, including tubulin (Cho and Cavalli, 2012). Acetylation of tubulin positively regulated axonal guidance. Therefore, we cannot rule out the role of non-histone acetylation in the effects of sodium butyrate. Our choice of sodium butyrate was mainly due to its ability to cross the blood brain barrier (Banks et al., 1997), however intracerebral administration of more specific HDAC inhibitors can uncover the more specific roles of HDAC inhibition in the attenuation of social deficits.

It is well established that administration of Valproic Acid (VPA), an HDAC inhibitor, during pregnancy increase the risk for autism in the offspring (Mbadiwe and Millis, 2013). In addition, prenatal exposure to VPA in animal models induces autism-like behavior in the offspring (Kataoka et al., 2011; Mbadiwe and Millis, 2013; Takuma et al., 2014). Multiple studies have determined that treatment of the offspring with HDAC inhibitors attenuates the behavioral deficits induced by VPA exposure in the prenatal period (Foley

et al., 2014, 2012). Therefore, the effects of histone deacetylase treatment on autism-like behavior is dependent on the time of exposure (Takuma et al., 2014), which may explain why sodium butyrate, at postnatal periods, has positive effects on social behavior. While these previous studies determined that HDAC inhibition can have beneficial effects in environment-induced autism mouse models, our study determined that HDAC inhibition has an effect on a more general autism mouse model, in which autism-like behavior isn't induced by a previous exposure to HDAC inhibitors. Therefore, HDAC inhibition may not only be helpful in the rare cases of previous VPA exposure, but may have potential benefits for a more wide ranging population.

In conclusion, our results indicate that sodium butyrate can affect social behaviors by modifying the transcriptional regulation of neuronal excitation and inhibition. Therefore, epigenetic pathways are a possible avenue for research into pharmacological treatments for autism spectrum disorders.

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The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2015.11.003.

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