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Rescue of altered HDAC activity recovers behavioural abnormalities in a mouse model of Angelman syndrome

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ABSTRACT

Angelman syndrome (AS) is a neurodevelopmental disorder characterized by severe intellectual and developmental disabilities. The disease is caused by the loss of function of maternally inherited *UBE3A*, a gene that exhibits paternal-specific imprinting in neuronal tissues. *Ube3a*-maternal deficient mice (AS mice) display many classical features of AS, although, the underlying mechanism of these behavioural deficits is poorly understood. Here we report that the absence of Ube3a in AS mice brain caused aberrant increase in HDAC1/2 along with decreased acetylation of histone H3/H4. Partial knockdown of Ube3a in cultured neuronal cells also lead to significant up-regulation of HDAC1/2 and consequent down-regulation of histones H3/H4 acetylation. Treatment of HDAC inhibitor, sodium valproate, to AS mice showed significant improvement in social, cognitive and motor impairment along with restoration of various proteins linked with synaptic function and plasticity. Interestingly, HDAC inhibitor also significantly increased the expression of Ube3a in cultured neuronal cells and in the brain of wild type mice but not in AS mice. These results indicate that anomalous HDAC1/2 activity might be linked with synaptic dysfunction and behavioural deficits in AS mice and suggests that HDAC inhibitors could be potential therapeutic molecule for the treatment of the disease.

1. Introduction

Angelman syndrome (AS) is a neurodevelopmental disorder characterized by severe developmental delay, cognitive and motor deficits, lack of speech and epileptic seizures along with multiple other associated features particularly excessive laughter and sleep disturbances (Williams et al., 2006; Williams et al., 2010). Genetic studies have revealed that the AS is caused by the loss of function of the maternally inherited *UBE3A* allele (Fang et al., 1999; Kishino et al., 1997; Matsuura et al., 1997). Because the paternally inherited *UBE3A* is epigenetically silenced in the neuronal tissue through cell type specific imprinting, the defect in maternally inherited *UBE3A* results its loss of function in the brain (Albrecht et al., 1997; Mabb et al., 2011; Yamasaki et al., 2003). The *UBE3A* gene encodes a 100 kDa protein that has been characterized as an E3 ubiquitin ligase (involved in targeting proteins for ubiquitination) and transcriptional co-activator for steroid hormone receptors (Huibregtse et al., 1995; Ramamoorthy and Nawaz, 2008). Therefore, it is hypothesized that loss of ubiquitin ligase activity or co-activator function of Ube3a might be linked with the AS phenotypes.

To gain deeper insight into the AS patho-mechanism, several mouse models has been generated by disrupting the maternally inherited Ube3a (Jana, 2012). Mouse model generated by Jiang et al. reproduced many characteristic features of AS and is widely used to understand the disease pathogenesis (Jiang et al., 1998). These mice not only exhibit classical cognitive and motor deficits, but also display audiogenic seizure, anxiety-like behaviour, disturbance in circadian clock and sleep homeostasis (Godavarthi et al., 2012; Heck et al., 2008; Jiang et al., 1998; Mulherkar and Jana, 2010; Shi et al., 2015). Moreover these AS mice also become obese (Meng et al., 2015; Shi et al., 2015). In depth study in this mouse model further demonstrates defect in hippocampal calcium/calmodulin dependent protein kinase-II and long-term potentiation, experience-dependent synaptic plasticity and imbalance of excitatory/inhibitory circuitry (Jiang et al., 1998; Sato and Stryker, 2010; Wallace et al., 2012; Weeber et al., 2003; Yashiro et al., 2009). These results strongly indicate that Ube3a plays a crucial role in regulating synaptic function.

Although there have been considerable progress in understanding AS pathogenesis, currently there is no effective therapy. It has long been thought that activation of silenced paternal allele of *UBE3A* could be an attractive therapeutic strategy. Because the silencing is mediated by the expression of large noncoding antisense RNA transcript (UBE3A-ATS), several attempts have been made to suppress the expression of UBE3A-ATS. Recently, antisense oligonucleotide of UBE3A-ATS

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has been shown to activate the paternal Ube3a and consequently improve the behavioural abnormalities in AS mice (Meng et al., 2015). Furthermore, topoisomerase inhibitors also have been reported to unsilence the paternal Ube3a expression by inhibiting the UBE3A-ATS (Huang et al., 2012). However, the therapeutic benefit of these inhibitors in animal models have not been established so far.

Histone acetylation and deacetylation regulated by histone acetyl transferase (HAT) and histone deacetylase (HDAC) plays a significant role in regulating the gene transcription (Delcuve et al., 2012; Haberland et al., 2009). Differential histone acetylation has been reported in the key imprinted gene locus in Prader-Willi Syndrome (PWS), but not in the AS imprinting centre or promoter region of *UBE3A* (Saitoh and Wada, 2000). Therefore, we aimed to investigate the role of HDAC inhibitor in the regulation of Ube3a expression using AS mouse model. We found that HDAC inhibitors significantly increased the expression of Ube3a in the brain of wild type mice, but had no effect on the expression of Ube3a in AS mice brain. During our study, we surprisingly noticed that the acetylation of histones H3(K9) and H4(K12) was significantly lower along with increased levels of HDAC1/2 in AS mice brain compared to the wild type counterpart. Finally, we demonstrate significant improvement of various behavioural abnormalities upon treatment with the HDAC inhibitor, sodium valproate to AS mice.

2. Materials and methods

2.1. Materials

Sodium valproate, sodium butyrate, trizol reagent and mouse monoclonal antibody against β-actin and synaptophysin were purchased from Sigma. Rabbit polyclonal anti-Ube3a, and anti-BDNF, anti-phosphoThr286 CaMKIIα and anti-CaMKIIα antibodies were purchased from Santa Cruz Biotechnology. Mouse specific Ube3a siRNA oligoneucleotides (a pool of 3 target specific 20–30 nucleotide siRNA) and control siRNA (scrambled sequences) were also purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-HDAC1 and HDAC2, PSD95, anti-H2A, H2B, H3, H4 and their acetylated antibodies were procured from Cell Signalling Technology. Rabbit polyclonal GluR1 and GluR2 antibodies were obtained from Millipore and mouse monoclonal anti-Ube3a was from BD Biosciences. Biotinylated anti-rabbit IgG, HRP-conjugated anti-rabbit and anti-mouse IgG, VECTASTAIN-Elite ABC reagent and ImmPact Novared staining kit were purchased from Vector Laboratories.

2.2. Ethic statement

All animal experiments were approved by the Institutional Animal and Ethics Committee (IAEC) of the National Brain Research Centre. Mice were handled strictly according to guidelines defined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forestry, Government of India.

2.3. Animal treatment

Heterozygous mice for *Ube3a* gene were obtained from the Jackson Laboratory (Jackson code: 129-*Ube3a*tm1Alb/J) and maintained in animal house facility of the Institute. The test for genotyping was performed from the isolated genomic DNA of mice tails using PCR as described previously (Jiang et al., 1998). *Ube3a* heterozygous female (Ube3am−/p+) mice were crossed with wild type male (Ube3am+/p+) to obtain the wild type as well as Ube3a-maternal deficient or AS mice (Ube3am−/p+). Just after weaning (at 21 days), male wild type and AS mice were taken to experimental room, divided into different groups and acclimatized for about one week. Animals were then intraperitoneally injected sodium valproate (300 mg/kg body weight daily) for 60 days. Control group received same volume of saline. Each group was having 10–12 animals. Behavioural studies were conducted between 50 and 60 days of drug treatment.

2.4. Animal behavioural study

2.4.1. Test for social interaction

Crawley's sociability and preference for social novelty test protocol was used to study the social interaction (Kaidanovich-Beilin et al., 2011). The test used a 3-chamber rectangular box (20 × 45 cm) with removable dividing walls (made in plexiglass) and two identical cup-like container made up of metal wires to hold a single mouse that can freely breathe and move around. These cups were placed inside the left or right side chamber of the rectangular box to keep the naive mice. Initially, the test mouse was placed at the center of the middle chamber with closed left and right chamber and allowed to familiarize for 5 min. Subsequently, one of the wild type mice (stranger 1) was kept inside the cup of left side chamber (keeping empty cup in the right side chamber) and plexiglass walls between the compartments were removed. Interaction time of test mice with the stranger 1 as well as with empty cup was monitored for 10 min using a digital camera mounted above the apparatus. This experiment was conducted to evaluate the sociability of the test mice which is directly proportional with its interaction time on stranger. In the second session, another wild type mouse (stranger 2) was placed in the right side chamber that was empty during session 1 and the interaction time of the test mice with both strangers was monitored for 10 min duration. The tendency of the test mice to spend time with stranger 2 (previously un-encountered mouse) with regard to stranger 1 (familiar mouse) was used to assess social novelty. The experiment for session 1 and session 2 for each test mouse (from saline and valproate treated wild type, AS mice) were conducted in similar manner, interaction time for stranger 1 and stranger 2 were analyzed and plotted. After each trial all chambers were cleaned with 70% ethanol to prevent olfactory cues bias.

2.4.2. Test for motor function

Balance beam test was used to assess the fine motor skill of AS mice (Mulherkar and Jana, 2010). Mice were allowed to walk 80 cm long beam (either square or round with 8, 12 and 20 mm diameter) that was kept 50 cm above base and at the end of the beam, a dark wooden box was kept to rest the mice. Mice were initially trained on 20 mm diameter round or square beam for two days and on the following day, they were tested for round or square beam walk on 8 and 12 mm diameter beam. Each mouse was used for 3 trials. Round bean and square beam experiments were conducted on different days. All experiments were recorded using a digital camera. Amounts of time to cross the beam for each mice was calculated and average values were used for statistical analysis.

2.4.3. Test for cognitive function and anxiety

Novel object recognition test was used to assess the cognitive function in wild type and AS mice. The test for anxiety was conducted using light/dark box test. Detailed procedures for these tests have been described in our earlier paper (Godavarthi et al., 2012).

2.5. Neuronal cell culture and transfection

Mouse neuro 2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics penicillin/streptomycin. For experimental purpose, cells were plated into 6-well tissue culture plate at sub-confluent density. After 24 h of plating, cells were transfected with Ube3a and control siRNA oligonucleotides using Lipofactamine® 2000 (Invitrogen) according to the instruction of the manufacturer. Cells were harvested 48 h of post transfection, lysates were made and then processed for immunoblot analysis using various antibodies. In another experiment, cells were treated with different doses of either sodium valproate or

sodium butyrate for 24 h and then the collected cells were subjected to immunoblot analysis.

2.6. Immunoblot analysis

Mice were sacrificed by cervical dislocation, Cortical and hippocampal regions of the brain were carefully separated out, snap frozen in liquid nitrogen and stored at −80 °C. Collected brain tissues were homogenized in the ice cold RIPA lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10 mM NaF, 5 mM $Na_4P_2O_7$, 0.1 mM Na_2VO_5, complete protease inhibitor cocktail), lysates were sonicated for a short time and centrifuged for 10 min at 15000 × *g* at 4 °C. Supernatants were collected, protein amounts in the supernatant were estimated by BCA method and stored at −80 °C. Brain samples were then boiled with sample buffer and equal amounts of proteins were separated through SDS-PAGE followed by immunoblot analysis as described earlier (Rao et al., 2010). BDNF, Ube3a and HDAC1 antibodies were used at 1:1000 dilutions while antibodies against all histones and their acetylated derivatives, HDAC2, synaptophysin, PSD95, GluR1 and GluR2 were used at 1:5000 dilutions.

2.7. Immunohistochemical staining

Mice were anaesthetized with Xylazine (10 mg/kg body weight) and Ketamine (100 mg/kg body weight and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (*w*/*v*) in PBS. Collected brains were further placed in 4% paraformaldehyde for 24 h and then subjected to 10, 20 and 30% sucrose (in PBS) treatment followed by sectioning in freezing microtome (20 μm thickness). Serial brain sections were kept in PBS with 0.02% sodium azide and stored at 4 °C. For immunostaining, brain sections were first treated with antigen unmasking solution at 70 °C for 1 h in a water bath followed by blocking in endogenous peroxidase activity using hydrogen peroxide (3% *v*/*v*). Sections were washed with PBS, blocked with 2% BSA along with normal goat serum for 2 h and then incubated overnight with primary antibody. Ube3a antibody (BD Bioscience) was used at 1:150 dilution and acetylated H3/H4 antibodies were used at 1:200 dilutions. Sections were subsequently washed, treated with biotinylated secondary antibody for 2 h, washed and incubated with VECTASTAIN-Elite ABC reagent following the protocol provided in the kit. The color was developed using ImmPACT Novared kit. Images were taken using bright field microscope (Leica DM RXA2 microscope) and organized using Adobe Photoshop.

2.8. Quantitative real-time RT-PCR

RNA was extracted from neuro2a cells using trizol reagent according to the manufacturer protocol. The cDNA was synthesized from total RNA and then subjected to real-time RT-PCR for Ube3a using power SYBR Green PCR master mix (Applied Biosystems). PCR reaction was conducted in ViiA7 real time PCR system, and data were analyzed and expressed as fold change. RT-PCR products for Ube3a were normalized with that of 18S RNA as an internal control. Primer sequences for Ube3a and 18S RNA are as follows: Ube3aF, 5′-CATACCTGAGTCCAGCGAATTA-3′; Ube3aR, 5′-ACGCCAAGTTCGGTTTCT-3′; 18SF, 5′-GAGGGA GCCTGAGAAACGG-3′; 18SR, 5′-GTCGGGAGTGGGTAATTTGC-3′.

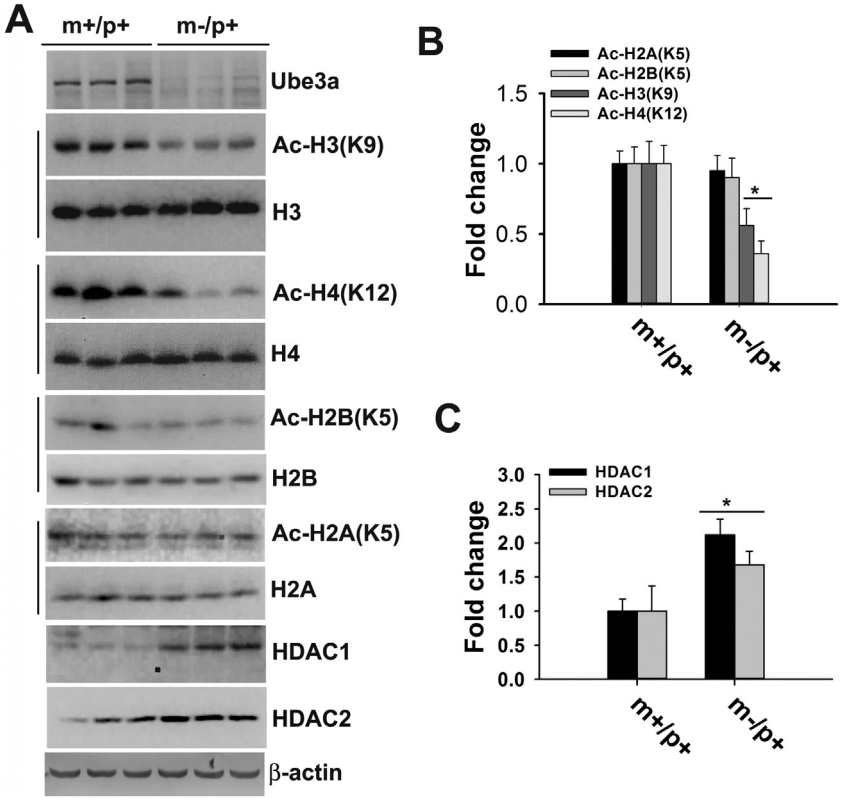

Fig. 1. AS mice display increased levels of HDAC1/2 accompanied with decreased acetylation of histones H3(K9) and H4(K12) in their brain. A) Brain samples of cortical region obtained from two months old AS (m−/p+) and wild type (m+/p+) mice were subjected to immunoblot analysis using antibodies against various histones and their acetylated (Ac) derivatives as well as HDAC1/2. Quantitation of band intensities of histones, their acetylated form and HDAC1/2 were carried out using NIH image analysis software, normalized and expressed as fold change. Each band represent sample from different mouse. B) Comparison of fold change of acetylated histones H3(K9), H4(K12), H2A(K5) and H2B(K5) among wild type and AS mice. Values were normalized against respective total histone. C) Increased levels of HDAC1/2 in the cortex of AS mice cortical sample. Values were normalized against β-actin. Values are mean ± SD of 5 different mice in each group. *$p < 0.001$ (Student's *t*-test) as compared to wild type group.

2.9. Statistical analysis

SigmaStat software was used for statistical analysis of the data. Values were expressed as mean ± SD. One-way or two-way analysis of variance (ANOVA) followed by Holm-Sidac *Post-hoc* test was used for inter-group comparison of animal behavioural experiments and some immunoblot quantitation data. Student's *t*-test was also used for inter group comparison in some experiments. $P < 0.05$ was considered statistically significant.

3. Results

3.1. AS mice exhibit increased levels of HDAC1/2 along with decreased acetylation of histones H3 and H4 in their brain

Histone acetylation was not shown in AS imprinting centre or promoter region of *UBE3A* gene. Since histone acetylation and deacetylation plays a prominent role in regulating gene expressions, we attempted to explore the role of HDAC inhibitors in regulating the expression of Ube3a. During our exploratory study, we surprisingly noticed that some histones were hypo-acetylated in the cortex and hippocampus of AS mice. In order to dissect out the role of Ube3a in regulating acetylation of the specific histone molecule and the underlying cause, we first analyzed the level of various acetylated histones in the cortical sample of adult AS mice along with age-matched wild type control. As shown in Fig. 1, acetylation levels of the histone H3(K9) and H4 (K12) were significantly reduced (about 50% decrease) in the cortical lysate of AS mice as compared to control. Acetylation levels of H2A(K5) and H2B(K5) were unaltered between wild type and AS mice. Since acetylation of histones are negatively regulated by HDAC, we further analyzed the level of HDAC1 and HDAC2 in the same cortical sample. We detected about 1.8 to 2-fold higher levels of both HDAC1 and HDAC2 in the cortex of AS mice when compared with wild type control (Fig. 1A and C). To further confirm reduced acetylation of histones H3(K9) and H4(K12) in different brain regions of AS mice, we did immunohistochemical staining of these markers using their specific antibodies. In wild type mice, both acetylated histones H3(K9) and H4(K12) showed specific nuclear localization and were widely distributed all over the brain. However, acetylation levels of these histones were considerably reduced in most cortical and hippocampal regions of AS mice brain (Fig.2). Quantification of the immunohistochemical staining showed about 45–55% decrease in acetylation of histones H3(K9) and H4(K12) in somatosensory cortex and hippocampal CA3 region of AS mice with respect to control (Supplementary Fig.S1). Immunohistochemical staining also confirmed that the acetylation levels of H2A(K5) and H2B(K5) were unaffected in the cortical and hippocampal region of AS mice brain (Supplementary Fig. S2). These result confirms that specific acetylation level of at least histones H3 and H4 are significantly decreased in AS mice brain and this effect could be due to increased level of HDAC1/2.

3.2. Knockdown of Ube3a increases HDAC1/2 levels and decreases acetylation of histones H3 and H4 in the cultured neuronal cell

To understand the role of Ube3a in regulating HDAC1/2 levels and hypo-acetylation of histones H3 and H4, we performed siRNA-mediated knockdown of Ube3a in mouse neuro2a cell line and then checked the

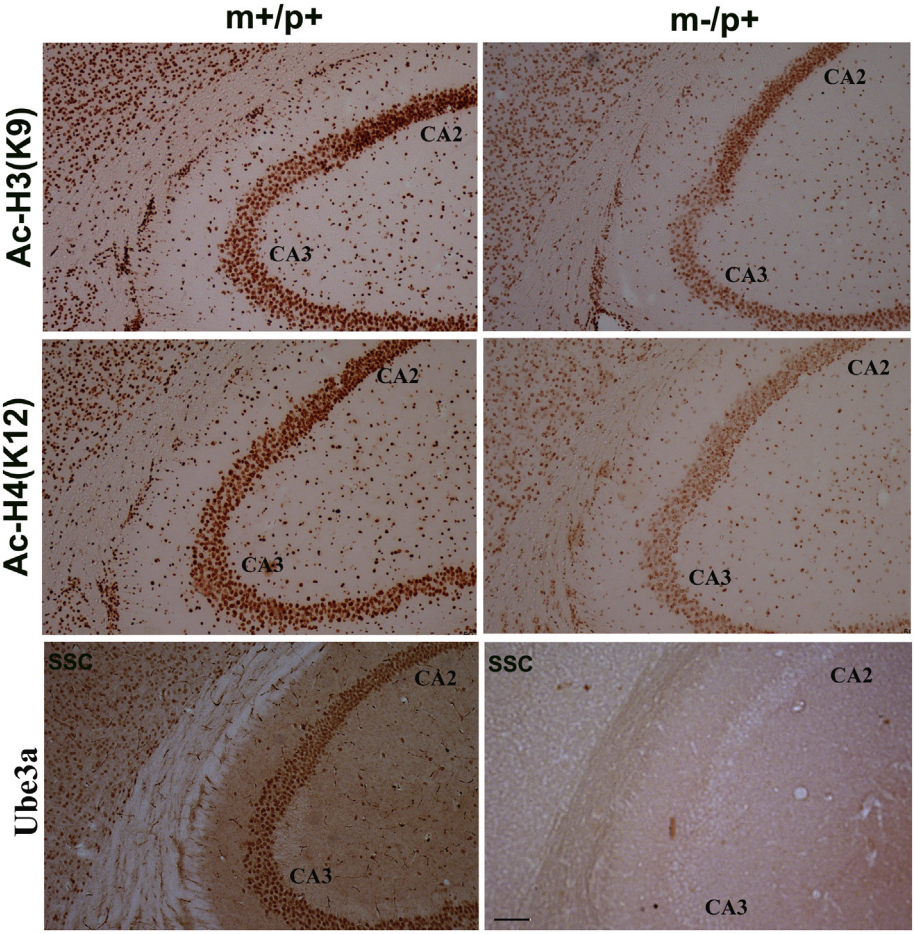

Fig. 2. Representative immunohistochemical staining showing decreased levels of acetylated histones H3(K9) and H4(K12) in the cortex and hippocampus of AS mice. Brain sections (20 μm thickness) obtained from four wild type (m+/p+) and AS (m−/p+) mice were kept on the same slide and processed for immunostaining using antibodies against acetylated histones H3(K9) and H4(K12) as well as Ube3a. Hippocampal CA3, CA2 and somatosensory cortex (SSC) areas are shown in the images. Scale bar, 100 μm.

level of these markers. Transient transfection of Ube3a siRNA caused about 70–80% reduction in the mRNA (control, 1 ± 0.13; Ube3a siRNA, 0.35 ± 0.11; $P < 0.01$) and the protein level of Ube3a (Fig.3). As shown in Fig. 3, partial knockdown of Ube3a significantly increased the level of HDAC1 and HDAC2 along with decreased acetylation of histones H3(K9) and H4(K12). There was about 2-fold increase in the level of HDAC1/2 and 50% decrease in acetylation of histones H3(K9) and H4(K12). Acetylation levels of histones H2A(K5) and H2B(K5) were unaffected upon knockdown of Ube3a. These results clearly indicate that Ube3a is involved in regulating the level of HDAC1/2.

3.3. Treatment of sodium valproate rescues behavioural abnormalities and normalizes altered HDAC1/2 levels and hypo-acetylation of histones H3/H4 in AS mice brain

HDAC2 has been shown to negatively regulate the memory formation and HDAC inhibitors are widely known to improve cognitive and other behavioural deficits in various neurodegenerative disease models (Chuang et al., 2009; Graff et al., 2012; Guan et al., 2009). Because HDAC1/2 levels are regulated by Ube3a and are increased in multiple regions of AS mice brain, we presumed that aberrant HDAC1/2 activities might be linked at least in part with the AS phenotype. Therefore, we tested the role of HDAC inhibitor, sodium valproate in the reversal of behavioural deficits in AS mice. AS mice along with their wild type counterpart were treated with sodium valproate for 60 days and behavioural tests were conducted between 50 and 60 days of the drug treatment. In the social interaction test, AS mice exhibited significant deficits in sociability (interaction with stranger 1 with respect to empty cup) and social novelty (interaction with stranger 2 with regard to stranger 1), while valproate treatment considerably reversed these abnormalities (Fig.4A and B). Next we performed novel object recognition test to evaluate visual recognition memory that depends on hippocampal function. This test is based on the usual tendency of the rodent to explore novel object and preference for novel object over familiar one is considered recognition memory. Importantly, locomotor activity is not very critical for this test. In novel object recognition test, AS mice showed reduced preferences for novel object compared to wild type and treatment of valproate significantly rescued this deficit (Fig.4C). In light/dark box test (test for anxiety), AS mice was found to spent more time in dark compartment than wild type and this abnormality was considerably improved upon prolonged treatment of valproate (Fig.4D). Interestingly, prolonged treatment of sodium valproate also significantly improved the fine motor skill of AS mice in a balance beam test that allows mice to walk in a round or square beam of different diameter (Fig.5). Thus prolonged treatment of sodium valproate has significant beneficial effect not only on learning and memory related behavioural deficits but also on motor abnormalities and anxiety-like behaviours in AS mice.

After completion of behavioural tests, animals were sacrificed and brain samples were analyzed for HDAC1/2 and acetylated histones H3(K9) and H4(K12) using immunoblot analysis and immnohistochemical staining. Fig. 6 showed that decreased acetylation of histones H3(K9) and H4(K12) in the cortical sample of AS mice was restored to control mice level upon treatment with sodium valproate. Similarly, sodium valproate treatment also normalized aberrantly increased levels of HDAC1/2 in the cortex of AS mice (Fig.6A and C).

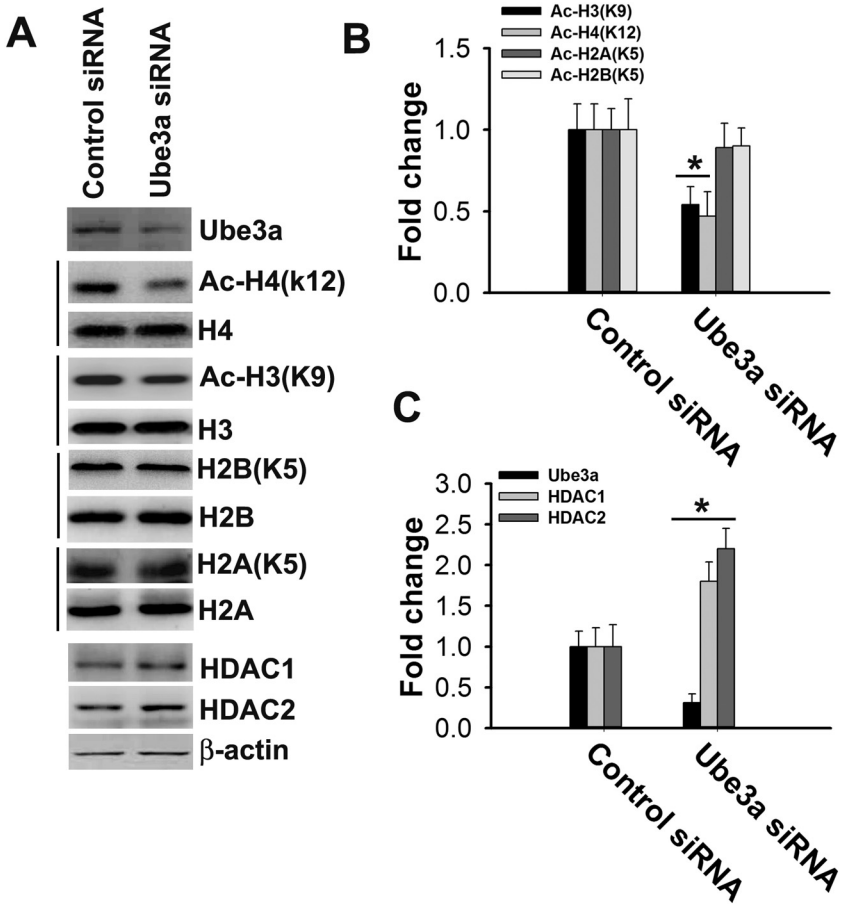

Fig. 3. Partial knockdown of Ube3a increased the level of HDAC1/2 along with decreased acetylation of histone H3(K9) and H4(K12). A) Mouse neuro2a cells were transiently transfected with either control or Ube3a-specific siRNA and after 48 h, cells were collected and processed for immunoblotting using various antibodies shown in the figure. Quantitation of band intensities of acetylated histones H2A/H2B/H3/H4 (B), HDAC1/2 (C) and Ube3a (C) using NIH image analysis software. Normalization and fold change was calculated as shown in Fig. 1. Values are mean ± SD of 3 independent experiments. *$p < 0.01$ (Student's *t*-test) as compared to control siRNA transfected group.

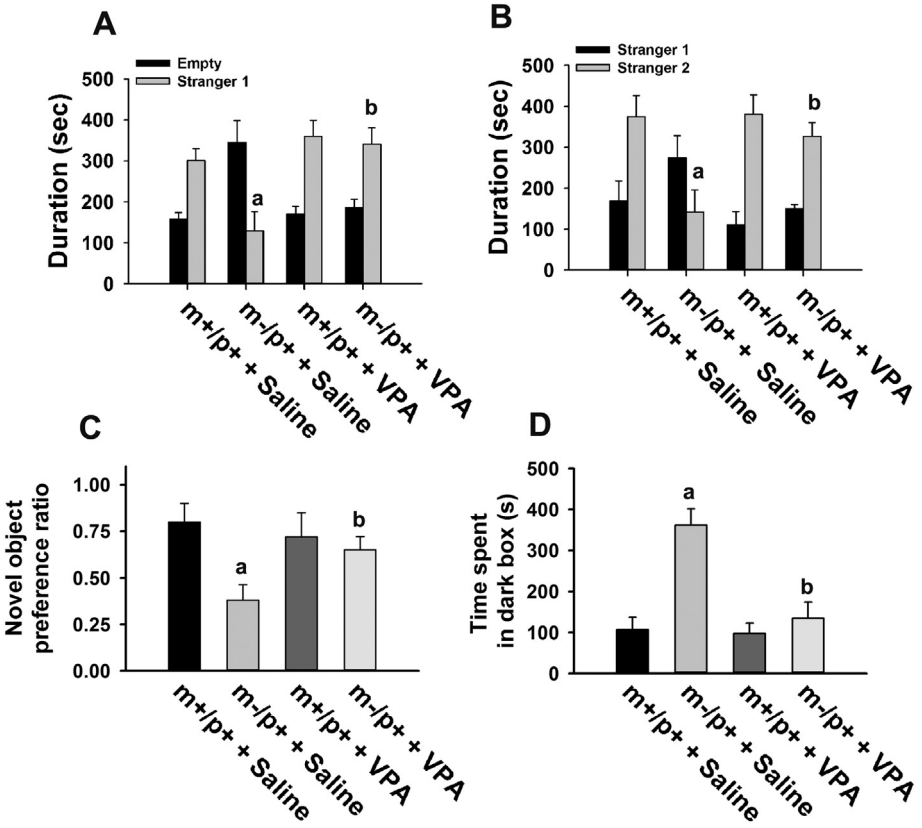

Fig. 4. Treatment of sodium valproate to AS mice improved their social interaction, memory deficit and anxiety-like behaviour. Wild type (m+/p+) and AS (m−/p+) mice were intraperitoneally injected sodium valproate for 60 days and behavioural studies were conducted between 50 and 60 days of drug treatment. A and B) Social interaction test showing significant improvement of sociability (A) and social novelty (B) of AS mice upon treatment of the drug. C) Novel object recognition test. D) Light/dark box test. Values are mean ± SD with 10 animals in each group. The "a" indicates $p < 0.001$ compared to saline treated wild type group and "b" denotes $p < 0.001$ compared to saline treated AS mice group. Data was analyzed by two-way (A and B) or one-way (C and D) ANOVA followed by Holm-Sidak *post- hoc* test.

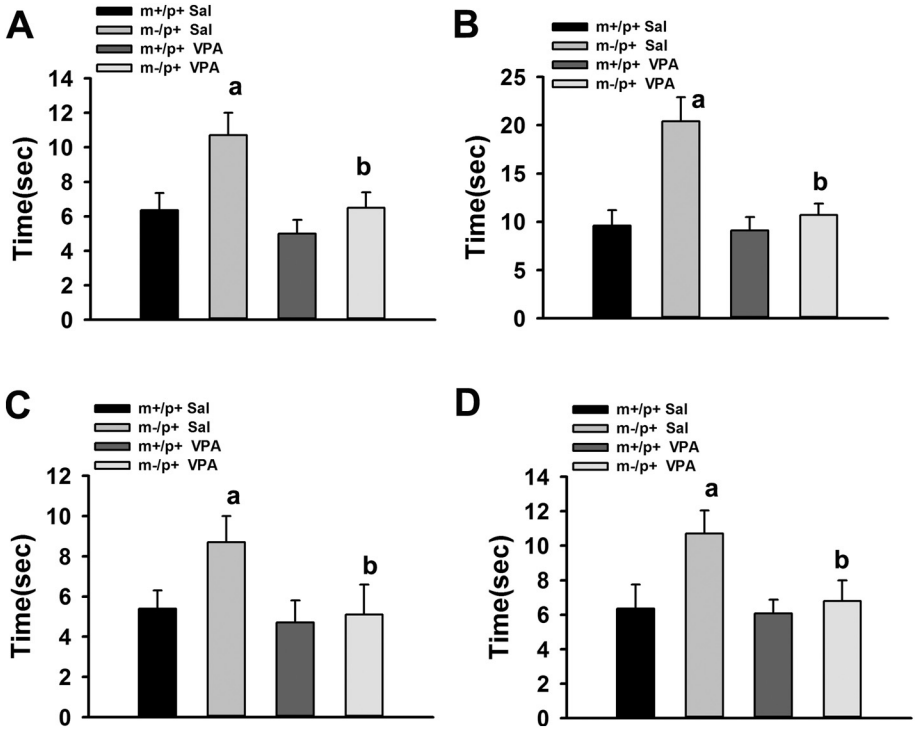

Fig. 5. Improvement of motor abnormality of AS mice upon prolonged treatment of sodium valproate. Balance beam test was conducted to assess the fine motor skills of mice. Mice were allowed to walk on either a round beam (top panel) or a square beam (bottom panel) with 12 mm (A and C) and 8 mm (B and D) diameter and the time taken to complete the task was recorded. Values are mean ± SD with 10 mice in each set. The "a" indicates $p < 0.01$ compared to saline treated wild type group and "b" denotes $p < 0.01$ compared to saline treated AS mice group. Data was analyzed by one-way ANOVA followed by Holm-Sidak *post- hoc* test.

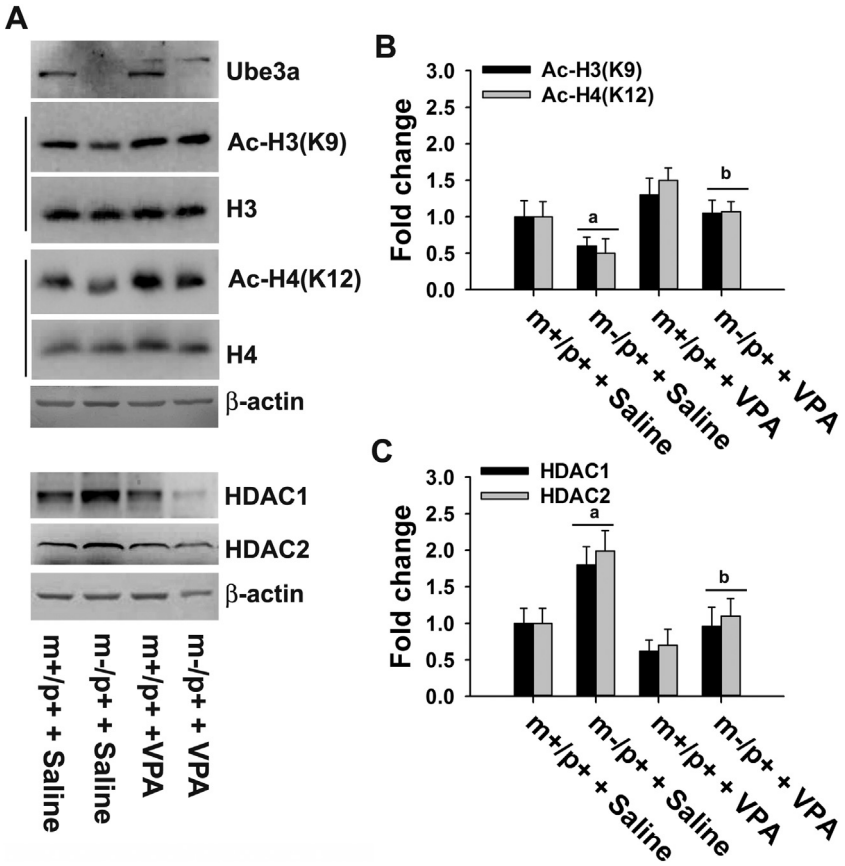

Fig. 6. Treatment of sodium valproate normalizes altered levels of HDAC1/2 and hypo-acetylation of histones H3/H4. A) Cortical regions of the brain sample obtained from saline and valproate treated wild type and AS mice were processed for immunoblot analysis using various antibodies shown in the figure. B) Band intensities of acetylated histones H3(K9) and H4(K12) as well as HDAC1/2 were quantified, normalized and expressed as fold change in the similar way described in Fig. 1. Values are expressed as mean ± SD with 4 mice in each group. The "a" designates $p < 0.01$ compared to saline treated wild type group and "b" represents $p < 0.05$ compared to saline treated AS mice group. One-way ANOVA followed by Holm-Sidak *post- hoc* test was used to analyse the data.

Interestingly, treatment of sodium valproate significantly increased (about 1.7-fold) the expression of Ube3a in the cortex and hippocampus of wild type mice (Fig.6A and Supplementary Fig. S3 for immnohistochemical staining). However, in AS mice brain, Ube3a expression was negligible and valproate treatment did not result in any increase in its expression. Because AS mice harbor null allele for maternal *Ube3a*, for any increase in Ube3a expression, paternal *Ube3a* would have to be unsilenced in the brain. In the neuronal cell culture, treatment of sodium valproate as well as sodium butyrate also dose-dependently increased the expression of Ube3a (Supplementary Fig.S4). Decreased acetylation of both histones H3(K9) and H4(K12) in the hippocampal region of AS mice and their restoration by valproate treatment were also confirmed through immunohistochemical staining (Fig.7).

3.4. Sodium valproate treatment restores altered levels of some of HDAC2-dependent genes in the hippocampus of AS mice

HDAC2 was shown to recruit to the promoter region of many genes linked with synaptic function and plasticity and negatively regulates their expression (Guan et al., 2009). This prompted us to explore the expression level of few synaptic function and plasticity related proteins in the hippocampus of AS mice and the effect of sodium valproate on them. As shown in Fig. 8, expression levels of synaptophysin and BDNF were significantly reduced in the hippocampus of AS mice and prolonged valproate treatment restored them to almost normal level. GluR1 and GluR2 levels were unaffected in AS mice hippocampus. These results indicate that sodium valproate or other HDAC inhibitors could be able to restore altered expression of various neuroplasticity-related proteins in AS mice brain, which could be linked with the reversal of behavioural phenotypes in these mice.

4. Discussion

Here we provide strong evidence that the epigenetic blockade of gene transcription through altered HDAC1/2 is one of the mechanisms by which loss of Ube3a could lead to AS pathogenesis. We also demonstrate that the HDAC inhibitor could potentially ameliorate various behavioural deficits observed in AS mice. First, we have shown increased levels of HDAC1/2 along with the decreased acetylation of histones H3(K9) and H4(K12) in multiple brain regions of AS mice in comparison with their wild type counterpart. Partial knockdown of Ube3a in cultured neuronal cells also leads to increased level of HDAC1/2 along with reduced acetylation of histones H3(K9) and H4(K12) indicating a prominent role of Ube3a in regulating HDAC1/2 levels.

Histone acetylation is a dynamic process that is tightly regulated by the antagonistic function of HAT and HDAC. Balance between the activity of these two groups of enzymes is crucial in regulating the gene expression and directs several physiological functions. Imbalance of their activities are linked with various disease states (Graff et al., 2011; Haberland et al., 2009; Penney and Tsai, 2014). HDAC1/2 are highly homologous and belong to class I HDAC family member. They regulate multiple biological pathways that are sometimes redundant or specific depending on cell types or in response to various stimuli (Jurkin et al., 2011). HDAC2 has been shown to negatively regulates synaptic plasticity and memory formation. It recruits to the promoter region of several neuronal activity, synaptic plasticity and memory related genes and regulates their expression (Guan et al., 2009). Some of those include BDNF,

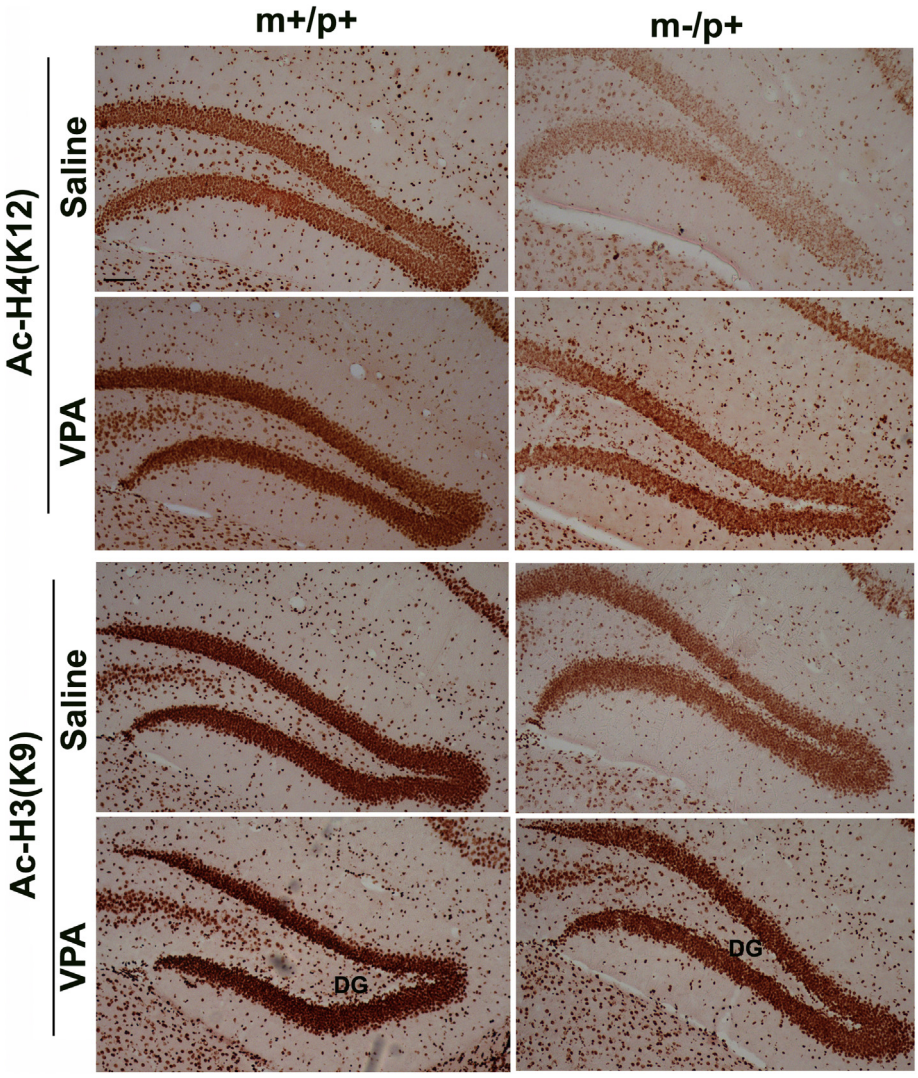

Fig. 7. Representative immunohistochemical staining of acetylated histones H3(K9) and H4(K12) in the hippocampal dentate gyrus (DG) region of wild type and AS mice received saline or sodium valproate (VPA) treatment. Brain sections obtained from all 4 groups of mice were placed on the same slide and processed for immunostaining. Three mice in each experimental group were evaluated. Scale bar, 100 μm.

Arc, Egr1, PSD95, synaptophysin, shank3, GluR1, GluR2, NR2A, NR2B etc. Aberrant high level of HDAC2 as well as HDAC1 also have been reported in the brain of various neuro-psychiatric disorders or their model systems having cognitive deficits (Bator et al., 2015; Graff et al., 2012; Subburaju et al., 2016). Moreover, HDAC inhibitors are very well known to rescue cognitive and other behavioural dysfunctions in several animal models of neurodegenerative disorders like Alzheimer's disease (AD) and Huntington's disease (HD) (Chuang et al., 2009; Mielcarek et al., 2011). These reports strongly suggest that the aberrant increased levels of HDAC1/2 observed in AS mice brain might be linked with synaptic and cognitive dysfunctions in these mice. Decreased expression of some HDAC2 regulatory genes like BDNF and synaptophysin in the hippocampus of AS mice further supports our conclusion. However, the exact consequences of aberrant high levels of HDAC1/2 in AS mice brain could be much broader and need further investigation. Mechanistic basis of altered synaptic plasticity in AS mice also have been demonstrated based on Ube3a-dependent ubiquitination and regulation of Arc and small conductance calcium-activated potassium channel (SK2) (Greer et al., 2010; Sun et al., 2015). These studies are further supported by the fact that AS mice exhibit defects in experience-dependent synaptic plasticity (Sato and Stryker, 2010; Yashiro et al., 2009). Presently we do not know how Ube3a regulates the level of HDAC1/2. There could be several possibilities. Ube3a, being an ubiquitin ligase, could directly regulates the turnover of HDAC1/2 by ubiquitinating and targeting them for proteasomal degradation. HDAC2 is also known to be degraded via proteasome(Kramer et al., 2003). Alternately, Ube3a might negatively regulates the expression of HDAC1/2 through direct regulation of its promoter. This aspect need further investigation.

Aberrantly increased HDAC1/2 activity in AS mice brain led us to test the effect of HDAC inhibitor, sodium valproate (Class I HDAC inhibitor), on the behavioural outcome in these mice. Interestingly, we have found that prolonged treatment of sodium valproate significantly improved various behavioural deficit in AS mice. Valproate treatment also restored increased HDAC1/2 levels and hypo-acetylation of histones H3 and H4. Mechanistic basis of the valproate-induced inhibition of HDAC1/2 activity seems very complex. Valproate is demonstrated not only to directly inhibit the HDACs activity through binding to the catalytic centre of HDACs (Gottlicher et al., 2001), but also reported to induce proteasome-mediated degradation of HDAC2 (Kramer et al., 2003) that are in line with our observation. Valproate is one of the most widely used drug in the long-term treatment of different kinds of seizure in adults and children (Loscher, 2002). It is also commonly used to treat seizure in AS patients (Guerrini et al., 2003). Although, sodium valproate is well tolerated in adult and children, its prenatal exposure increases the risk of birth defect and childhood autism

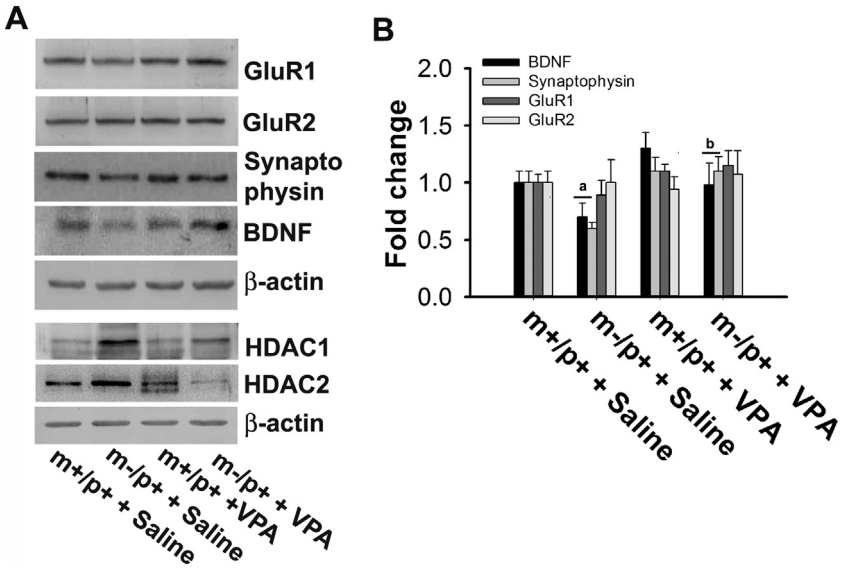

Fig. 8. Comparative analysis of the levels of various neuroplasticity-related proteins in the hippocampus of saline and valproate treated wild type and AS mice. A) Representative immunoblots of various neuroplasticity-related proteins. B) Quantitative assessment of BDNF, and synaptophysin, GluR1 and GluR2. Data were normalized against β-actin. Values are expressed as mean ± SD with 3 mice in each group. BDNF and synaptophysin showed significant reduction in AS mice hippocampus compared to wild type control, which was reversed back to nearly normal level upon treatment of valproate("a" and "b" denotes $p < 0.05$ as assessed by one-way ANOVA).

(Christensen et al., 2013). In addition to control seizure, it has been shown to improve cognitive function and produce neuroprotective effect in various animal models of neurodegenerative disorders (Chuang et al., 2009; Nalivaeva et al., 2012; Yao et al., 2014). In the animal model of HD, HDAC inhibitors reported to improve a number of motor abnormalities as well as survival rate (Ferrante et al., 2003; Hockly et al., 2003; Mielcarek et al., 2011). Beneficial effect of HDAC inhibitors also implicated in mood and anxiety disorders and some neurodevelopmental disorders (Penney and Tsai, 2014). These evidences could explain why sodium valproate improved not only cognitive function but also motor and other behavioural abnormalities in AS mice. Besides HDACs inhibition, sodium valproate also known to affects GABA metabolism and ion channel function (Loscher, 2002) and these mechanisms also could be linked with the recovery of behavioural deficits in AS mice. Although valproate is being used to treat seizure in AS patients, its involvement in improving cognitive function is not established so far and warrant further investigation.

Another interesting aspect of our study is that sodium valproate increased the expression of Ube3a in the brain of wild type but not in AS mice indicating HDACs might not be involved in regulating the expression UBE3A-ATS. Therefore, valproate-dependent behavioural improvement in AS mice is not linked with the activation of paternally silenced Ube3a. Valproate-induced expression of Ube3a in normal mice indicate HDACs might be playing an important role in regulating the promoter of Ube3a and increased Ube3a could be implicated in the HDAC inhibitor mediated regulation of cognitive function.

Altogether, our study concludes that the aberrant increase in HDAC1/2 in the brain of AS mice might be linked with synaptic dysfunction and associated behavioural anomalies in these mice. Our findings also suggest that the HDAC inhibitor could be promising drug to treat AS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2017.05.010.

References

Albrecht, U., et al., 1997. Imprinted expression of the murine Angelman syndrome gene, Ube3a, in hippocampal and Purkinje neurons. Nat. Genet. 17, 75–78.
Bator, E., et al., 2015. Valproic acid (VPA) reduces sensorimotor gating deficits and HDAC2 overexpression in the MAM animal model of schizophrenia. Pharmacol. Rep. 67, 1124–1129.
Christensen, J., et al., 2013. Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. JAMA 309, 1696–1703.
Chuang, D.M., et al., 2009. Multiple roles of HDAC inhibition in neurodegenerative conditions. Trends Neurosci. 32, 591–601.
Delcuve, G.P., et al., 2012. Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clin. Epigenetics 4, 5.
Fang, P., et al., 1999. The spectrum of mutations in UBE3A causing Angelman syndrome. Hum. Mol. Genet. 8, 129–135.
Ferrante, R.J., et al., 2003. Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. J. Neurosci. 23, 9418–9427.
Godavarthi, S.K., et al., 2012. Defective glucocorticoid hormone receptor signaling leads to increased stress and anxiety in a mouse model of Angelman syndrome. Hum. Mol. Genet. 21, 1824–1834.
Gottlicher, M., et al., 2001. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J. 20, 6969–6978.
Graff, J., et al., 2011. Epigenetic regulation of gene expression in physiological and pathological brain processes. Physiol. Rev. 91, 603–649.
Graff, J., et al., 2012. An epigenetic blockade of cognitive functions in the neurodegenerating brain. Nature 483, 222–226.
Greer, P.L., et al., 2010. The Angelman syndrome protein Ube3A regulates synapse development by ubiquitinating arc. Cell 140, 704–716.
Guan, J.S., et al., 2009. HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459, 55–60.
Guerrini, R., et al., 2003. Angelman syndrome: etiology, clinical features, diagnosis, and management of symptoms. Paediatr. Drugs. 5, 647–661.
Haberland, M., et al., 2009. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat. Rev. Genet. 10, 32–42.
Heck, D.H., et al., 2008. Analysis of cerebellar function in Ube3a-deficient mice reveals novel genotype-specific behaviors. Hum. Mol. Genet. 17, 2181–2189.
Hockly, E., et al., 2003. Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. Proc. Natl. Acad. Sci. U. S. A. 100, 2041–2046.
Huang, H.S., et al., 2012. Topoisomerase inhibitors unsilence the dormant allele of Ube3a in neurons. Nature 481, 185–189.

Huibregtse, J.M., et al., 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc. Natl. Acad. Sci. U.S.A. 92, 5249.
Jana, N.R., 2012. Understanding the pathogenesis of Angelman syndrome through animal models. Neural Plast. 2012, 710943.
Jiang, Y.H., et al., 1998. Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. Neuron 21, 799–811.
Jurkin, J., et al., 2011. Distinct and redundant functions of histone deacetylases HDAC1 and HDAC2 in proliferation and tumorigenesis. Cell Cycle 10, 406–412.
Kaidanovich-Beilin, O., et al., 2011. Assessment of social interaction behaviors. J. Vis. Exp.
Kishino, T., et al., 1997. UBE3A/E6-AP mutations cause Angelman syndrome. Nat. Genet. 15, 70–73.
Kramer, O.H., et al., 2003. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. EMBO J. 22, 3411–3420.
Loscher, W., 2002. Basic pharmacology of valproate: a review after 35 years of clinical use for the treatment of epilepsy. CNS Drugs 16, 669–694.
Mabb, A.M., et al., 2011. Angelman syndrome: insights into genomic imprinting and neurodevelopmental phenotypes. Trends Neurosci. 34, 293–303.
Matsuura, T., et al., 1997. De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. Nat. Genet. 15, 74–77.
Meng, L., et al., 2015. Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. Nature 518, 409–412.
Mielcarek, M., et al., 2011. SAHA decreases HDAC 2 and 4 levels in vivo and improves molecular phenotypes in the R6/2 mouse model of Huntington's disease. PLoS One 6, e27746.
Mulherkar, S.A., Jana, N.R., 2010. Loss of dopaminergic neurons and resulting behavioural deficits in mouse model of Angelman syndrome. Neurobiol. Dis. 40, 586–592.
Nalivaeva, N.N., et al., 2012. Effect of sodium valproate administration on brain neprilysin expression and memory in rats. J. Mol. Neurosci. 46, 569–577.
Penney, J., Tsai, L.H., 2014. Histone deacetylases in memory and cognition. Sci. Signal. 7, re12.
Ramamoorthy, S., Nawaz, Z., 2008. E6-associated protein (E6-AP) is a dual function coactivator of steroid hormone receptors. Nucl. Recept. Signal. 6, e006.
Rao, S.N., et al., 2010. Co-chaperone CHIP stabilizes aggregate-prone malin, a ubiquitin ligase mutated in Lafora disease. J. Biol. Chem. 285, 1404–1413.
Saitoh, S., Wada, T., 2000. Parent-of-origin specific histone acetylation and reactivation of a key imprinted gene locus in Prader-Willi syndrome. Am. J. Hum. Genet. 66, 1958–1962.
Sato, M., Stryker, M.P., 2010. Genomic imprinting of experience-dependent cortical plasticity by the ubiquitin ligase gene Ube3a. Proc. Natl. Acad. Sci. U. S. A. 107, 5611–5616.
Shi, S.Q., et al., 2015. Ube3a imprinting impairs circadian robustness in Angelman syndrome models. Curr. Biol. 25, 537–545.
Subburaju, S., et al., 2016. Toward dissecting the etiology of schizophrenia: HDAC1 and DAXX regulate GAD67 expression in an in vitro hippocampal GABA neuron model. Transl. Psychiatry 6, e723.
Sun, J., et al., 2015. UBE3A regulates synaptic plasticity and learning and memory by controlling SK2 channel endocytosis. Cell Rep. 12, 449–461.
Wallace, M.L., et al., 2012. Maternal loss of Ube3a produces an excitatory/inhibitory imbalance through neuron type-specific synaptic defects. Neuron 74, 793–800.
Weeber, E.J., et al., 2003. Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. J. Neurosci. 23, 2634–2644.
Williams, C.A., et al., 2006. Angelman syndrome 2005: updated consensus for diagnostic criteria. Am. J. Med. Genet. A 140, 413–418.
Williams, C.A., et al., 2010. Clinical and genetic aspects of Angelman syndrome. Genet. Med. 12, 385–395.
Yamasaki, K., et al., 2003. Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of Ube3a. Hum. Mol. Genet. 12, 837–847.
Yao, Z.G., et al., 2014. Valproate improves memory deficits in an Alzheimer's disease mouse model: investigation of possible mechanisms of action. Cell. Mol. Neurobiol. 34, 805–812.
Yashiro, K., et al., 2009. Ube3a is required for experience-dependent maturation of the neocortex. Nat. Neurosci. 12, 777–783.