Alterations of Epigenetic Mechanisms in post-mortem Autism Spectrum Disorder (ASD) subjects

BY

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THESIS

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This thesis is dedicated to my wonderful parents, sister, brother and girlfriend. Without their love and support none of this would have been possible.

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Contribution of Authors

Chapters 1, 2, 3 and 4 are literature review that places my thesis question in the context of the larger field and highlights the significance of my research question. Chapters 5, 6 and 7 represent my synthesis of the research presented in the thesis and my overarching conclusion.

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5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
CHIP	Chromatin immunoprecipitation assays
CpG	Cytosine-phosphate-guanine
DNMT1	DNA methyltransferase 1
DSM 5	Diagnostic and Statistical Manual of Mental Disorders 5th edition
GABA	Gamma-aminobutyric acid
GAD 1	Glutamate (Glutamic acid) Decarboxylase 67
GAD 2	Glutamate (Glutamic acid) Decarboxylase 65
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hMeDIP	Hydroxymethylated DNA immunoprecipitation
MDS	MeCP2 Duplication Syndrome
MeCP2	Methyl CpG binding protein 2
MeDIP	Methylated DNA immunoprecipitation
RELN	Reelin
RTT	Rett Syndrome
SHANK3	SH3 and multiple ankyrin repeat domains
TAB-seq	TET assisted bisulphite –Pyrosequencing
TET 1	Ten eleven translocation 1
TSS	Transcription Start Site
UBE3A	Ubiquitin –protein ligase E3A

I. INTRODUCTION

A. Background

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by impaired social communication and social interactions, as well as, restrictive or repetitive behaviors (American Psychiatric Association, 2013). ASD was described for the first time by Kanner in 1943. Based on large scale epidemiological studies, it is estimated that prevalence of ASD is about 1 % in the general population. ASD is more common in males than in females (4:1) (Baron-Cohen et al., 2009, Bailey et al., 1995; Veenestra-VanderWeele & Cook 2004; Abrahams & Geschwind 2008, O'Roak & State, 2008; Brugha et al., 2011). ASD is a clinically heterogeneous neurodevelopmental disorder with significant genetic implication. Complex etiopathogenesis of ASD is likely a result of dynamic interaction between genetic and environmental factors. Epigenetic mechanisms such as DNA methylation and histone modification operates at the interface of genetic and environmental factors.

II. INTRODUCTION TO EPIGENETICS

The term "*Epigenetics*" derives from the Greek word *Epi* (around, above), referring to changes "around the DNA " and was used for the first time by Conrad Waddington (1943) (Rangasamy et al., 2013). He defined epigenetics as the interactions of genes with their environment, which bring the phenotype into being. Different definitions were utilized over the years to conceptualize the broad aspects of epigenetics, but all of them overlap in defining epigenetics as sets of mechanisms which are dynamically involved in the regulation of gene expression without affecting the DNA base composition. These mechanisms are stable, yet reversible and activity-dependent. The aspect of reversibility makes them plausible target for intervention and modification. Epigenetic mechanisms are crucial in the development of neuronal function and structure. However, the alteration of these mechanisms could set the stage for the development of various neurodevelopmental disorders, including ASD. For a better understanding of epigenetic mechanisms it is worthwhile revisiting some basic structural and functional aspects of these mechanisms.

The nucleosome is the fundamental unit of chromatin and it is composed of the double stranded DNA (146 base pair), which is tightly wrapped around an octamer core of histone. Histone proteins are composed of globular structure and a pair of H3-H4 dimers and a pair of H2A-H2B dimers, connected by linker DNA. Traditionally, it was believed that DNA-histone interaction serves only for the packaging of DNA in the nuclei of the cells. But recent studies have expanded this concept into a more dynamic one, where accessibility of transcription factors to promoter

regions, required for the regulation of gene expression, is determined by the degree of chromatin compaction as a function of DNA-histone interplay (Graff et al., 2011).

Epigenetic modifications involve DNA (methylation or demethylation) as well as post-translational modifications of histone proteins.

A. DNA methylation and demethylation

DNA methylation is a covalent modification of DNA, which consists of the transfer of the methyl group from a methyl donor (S-adenosyl methionine –SAM) to position C-5 of cytosine of the recipient substrate. This process is catalyzed by members of DNA-methyltransferases (DNMTs) family, which includes DNMT1, DNMT3A and DNMT3B. DNA methylation is very important for epigenetically mediated X chromosome inactivation, imprinting and for suppression of foreign DNA. The most recent studies based on genome-wide analyses, as well as base-resolution analysis of DNA methylation in the human and mouse brain strongly implicate the role of DNA methylation process in brain development (Lister et al, 2013). Traditionally, it was believed that methylation largely affects CpG dinucleotides, but recent studies show that this phenomena also occurs at the CpH sites (H is A, C or T) in adult human brain (Lister et al., 2013; Kozlenkov et al., 2014). Genome wide studies have confirmed that CpG islands are sites with enriched content of CpG dinucleotides that are localized near promoters and enhancers of genes. CpG content plays a regulatory role in transcription as well as delineates the border to open chromatin, which facilitates transcription factor and RNA polymerase II access to open chromatin domains (Fenouil et al., 2014). DNA methylation is highly stable epigenetic mark

with predominantly repressive functions in the regulation of gene expression. In contrast, recent studies suggest that DNA methylation state represents a balance between two opposing actions: methylation, catalyzed by DNMTs and DNA demethylation pathway (Grayson and Guidotti 2013). DNA demethylation is catalyzed by members of the TET protein family and involves the conversion of 5-mC to 5-hmC through the enzymatic hydroxylation of the methyl group. Interestingly, regulation of TET 1 has been reported to be activity dependent, while TET 1 itself regulates expression of neuronal-activity regulated genes which are involved in learning and memory (Kaas et al 2013). TET family proteins (TET1, TET2 and TET3) function as oxyglutarate- and iron-dependent dioxygenases, in which 5- hmC serves as an intermediate product in an oxidative DNA demethylation pathway. The process of 5-hmC demethylation is achieved through the base excision repair (BER) pathway.

5 –hmC is detected in the Purkinje cells and granular cells of cerebellum (Zhubi et al., 2014) as well as the embryonic stem cells. The levels of 5-hmC in neurons is approximately 10 times higher than in peripheral tissues or embryonic stem cells (Kriaucionis & Heintz, 2009). 5-hmC is significantly distributed at promoter regions, gene body regions and distal regulatory elements (particularly in distal enhancers) (Szulwach & Jin 2014; Serandour et al 2012). More recent studies have shown that the enrichment of 5-hmC and depletion of 5-mC in the gene body regions are important phenomena which determine transcriptional activity (Mellen et al., 2012). The dynamic balance of methylation vs hydroxymethylation in the enhancers may represent an epigenetic code important for regulation of gene expression in a cell specific manner.

B. Histone modification

Post-translational modification of histones involve: acetylation, methylation, demethylation, phosphorylation, ubiquitination and sumoylation (Grayson & Guidotti 2013, Mersfelder & Parthun, 2006; Bannister & Kouzarides, 2011). Acetylation of histones occurs at lysine (K) residues and is catalyzed by histone acetyltransferases (HAT). These enzymes catalyze the transfer of acetyl group to lysine, which reduce the positive charge of histone and introduces repulsive forces between negatively charged DNA and histone tails, ultimately resulting in the opening of chromatin, allowing increased access to the transcription machinery. On the other hand, methylation of histone on lysines is catalyzed by histone methyltransferases (HMT) and is associated with both accessible (open) and repressed (close) chromatin, depending on the residue that has been modified, e.g. H3K4 (open), H3K9(close), H3K27 (close) etc. and it can occur as mono-,di- or tri- methylation .

III. EPIGENETICS OF ASD

ASD is a clinical condition characterized by a high heritability rate. Early twin studies showed that concordance rates between MZ twins is 70-90% and between DZ twins is 6-10%. (Bailey et al., 1995; Steffenburg et al., 1989). More recent studies by Hallmayer et al., (2011), showed that in male MZ twins the concordance rate is 77% and in DZ twins is 31%, while in female MZ twins, this rate is 50 % for MZ and 36% for DZ. Some of the factors that could help in explaining this phenotypic discordance between MZ twins are genetic (de novo mutations), non-shared environmental (prenatal, in utero and post-natal) and epigenetic factors. Lately, there has been increased interest in studying epigenetic mechanisms implicated in the regulation of ASD risk genes encoding proteins acting at the synaptic level. ASD risk genes known to be targets of epigenetic mechanisms (DNA methylation/demethylation and histone modifications) are : GAD1/2 and RELN (Veldic et al., 2005;2007, Ruzicka et al., 2007, Zhubi et al., 2014), GABA-A β3 (Thanseem at el., 2012), oxytocin (OXT) (Kususi et al., 2001, Gregory et al., 2009), ubiquitin –protein ligase E3A (UBE3A) (Jiang et al., 2004, Mabb et al., 2011, Flashner et al., 2013, Powel et al., 2013), engrailed (EN-2) (James et al., 2013), SHANK3 (Zhu et al., 2013). One potential target gene whose dysfunction is strongly associated with the ASD phenotype, based on the animal model studies and studies of human post-mortem brain is MeCP2. Interestingly, MeCP2 is found to be mutated in Rett syndrome, but also has epigenetic implication in the regulation of expression of other ASD risk genes.

A. Methyl CpG binding protein (MeCP2)

MeCP2 is a member of the methyl-binding domain (MBD) family of proteins and is a fundamental epigenetic modulator of genome architecture with pleiotropic functions in human brain. MeCP2 plays a crucial role in the regulation of synaptic and neuronal plasticity, development of motor skills, cognitive and social behaviors (Ebert et al 2013).

Normal brain functions are dependent on optimal levels of MeCP2 and function throughout brain development, more so during critical periods of neuronal development. Mouse studies have confirmed that when mutations of MeCP2 occur in the germline (representative of classic RTT), or MeCP2 is inactivated later during the juvenile or adult age, the result is a reduction of brain size, downregulation of synaptic protein expression, and overall reduction of dendritic arboriazation in neurons (Nguyen et al 2012). In addition, there are morphological and functional changes in astrocytes (Nguyen et al 2012). On the other hand, a severe neurologic phenotype ensues when human MeCP2 is overexpressed in transgenic mice lines (Collins et al 2004). In humans, loss or gain of MeCP2 function is represented by 2 syndromes: Rett (RTT) and MeCP2 duplication (MDS), respectively. RTT (loss of MeCP2 function) is an X-linked neurodevelopmental disorder affecting primarily females and that is characterized by an apparently normal developmental period, followed by the regression of previously acquired skills, deceleration of head circumference, autistic like features, seizures, ataxia and stereotypic hand movements. MDS (gain of function) which was first described by Van Esch et al., 2005 predominantly affects males and is characterized by infantile hypotonia, progressive spasticity,

intellectual disability, delayed or absent speech development, autism or autistic features, seizures and progressive encephalopathy/developmental regression (Schwoerer J et al. 2004).

MeCP2 is capable of binding genome wide and is structurally composed of several domains with distinct functions: a) an MBD, responsible for binding to methylated CpG dinucleotides; b) the transcriptional repression domain which mediates the binding of chromatin remodeling factors (HDACs, DNMT1, Sin3A etc.); c) an AT-hook domain that binds AT-rich DNA; and d) a C-terminal domain alpha and beta, which can bind to naked DNA and to RNA splicing factors (guy et al.,2011; Nan et al., 1998; Baker et al., 2013; Hite et al.,2009).

Traditionally, MeCP2 is regarded predominantly as a global gene repressor resulting in the downregulation of gene expression. However, increased evidence in the last years suggests that MeCP2 acts as a positive modulator of gene expression (Gonzales et al. 2012), actively involved in promoting gene imprinting (LaSalle et al., 2007), in modulating RNA-splicing (Young et al., 2005), in activating retrotransposon transcription in neurons (Muotri et al., 2010), in global alterations of chromatin condensation (Skene et al., 2010),in regulating the expression of miRNAs important for brain development and plasticity (Klein et al., 2007; Wu et al., 2010; Urdinguio et al., 2010).

Increased interest in investigation of the role of MeCP2 in recent years has enabled identification of target genes which are epigenetically regulated by MeCP2. Some of these MeCP2 target genes are Ube3A, Dlx5, Dlx6, BDNF, GABAergic (GAD1, GAD2, RELN) etc.

The importance of the MeCP2 in the function of GABAergic neurons has been documented in the study by Chao et al 2010. This study confirmed that cortical wild type GABAergic neurons

express ~50% more MeCP2 than non-GABAergic neurons. Global loss of MeCP2 in GABAergic neurons in mice produces autistic-like features (stereotyped behavior, deficits in social interaction, motor function, learning, memory and sensorimotor gating) (Chao et al 2010).

IV. GABAergic DYSFUNCTION IN ASD

ASD is conceptualized as a dysfunction of connectivity between different parts of the brain. There is a growing body of evidence implicating the cerebellum as one of the important areas involved in the pathophysiology of ASD. One of the most consistent neuropathological finding in the postmortem cerebellar samples is the Purkinje cells (PC) loss (Allen et al., 2005; Bailey et al., 1998, Palmen et al., 2004), which is observed in the vermis and particularly the cerebellar hemispheres. Unique morphological and functional features make PC one of the most striking cell types in the mammalian brain. PC are composed of the large dendritic arborizations which enhances significantly signal receptive areas and enables PC to receive more synaptic inputs than any other type of cells in the brain. PC are inhibitory GABAergic neurons and serve as the sole output of the cerebellar cortex. The GABAergic system provides inhibitory control over excitatory neurons in different brain areas and is thought to be implicated in the etiopathogenesis of ASD. These conclusions are based on several postmortem human brain studies which show dysfunction at different levels of GABA system organization, including 1) reduction in the number of GABAergic PC in cerebellar cortex (Bailey et al., 1998; Bauman & Kemper 1985; Whitney et al., 2004), 2) reduction by ~50% of the GABA synthesizing enzymes glutamic acid decarboxylase 65 (GAD2) in cerebellum and glutamic acid decarboxylase 67 (GAD1) in parietal cortex of autism patients (Fatemi et al., 2002; 2012), 3) reduction of GABA-A β3 receptor subunit (GABA-A β3) expression in the cingulate cortex and cerebellar vermis of ASD (Thanseem et al., 2011), reduction of GABA-A α1 receptor subunits in the superior frontal cortices (BA-9) of ASD subjects (Fatemi et al., 2009).

A. Glutamic acid decarboxylase 67 (GAD1) and 65 (GAD2)

GAD1 and GAD2 catalyze the decarboxylation of glutamate to form GABA. The GADs are two protein isoforms with different molecular weights (67kDa and 65 kDa) derived from two unlinked genes, with different cellular localization and cofactor requirements (Erlander et al., 1991). Epigenetic mechanisms are thought to be important in regulating GAD1/2 expression not only in ASD, but also in schizophrenia and bipolar disorder (Guidotti et al. 2000; Veldic et al., 2005; Grayson et al., 2005; Ruzicka et al., 2007). Studies have shown that GAD1 gene expression homeostasis is determined by the dynamic and bidirectional action of DNA-methyltransferases (DNMT) and an active DNA-demethylation pathway, which is preceded by the initial hydroxylation of 5-mC to form 5-hmC by members of the TET protein family. Moreover, our studies have shown that MeCP2 protein binds to GAD1 and GAD2 promoter and gene body regions.

B. REELIN (RELN)

RELN is a large extracellular matrix glycoprotein which plays fundamental roles in the neuronal migration and layer stratification of different brain areas during development, while in the mature brain it is involved in modulating synaptic function and connectivity. RELN is expressed in GABAergic interneurons in neocortex and hippocampus, but also in glutamatergic granule cells in cerebellum and pyramidal cells in entorhinal cortex. Postmortem human brain studies from patient with brain disorders (ASD, schizophrenia , bipolar disorder, Alzheimer's

disease) have revealed a reduction in the level of RELN expression and resultant changes in morphology of dendritic spines, likely followed by dysfunction at the synaptic level in neurons (Fatemi et al 2002, 2005,Costa et al., 2001; Levenson et al., 2008,Guidotti et al 2000). One plausible mechanism that may explain the dysfunction of RELN expression is hypermethylation of the promoter region of RELN with consequent downregulation of mRNA and protein levels (Guidotti et al., 2000; Veldic et al., 2005; Ruzicka et al., 2007; Grayson & Guidotti, 2013). Our studies show that RELN is another target of the MeCP2 protein and is subject to dynamic interactions between methylation and hydroxymethylation processes.

The focus of my investigation has been to explore the epigenetic mechanisms important for the regulation of GAD 1, GAD2 and RELN genes in cerebellum of ASD subjects. I specifically focused on exploring the roles of methylation and hydroxymethylation status, as well as the binding of MeCP2, in the regulation of gene expression of GAD1, GAD2 and RELN in postmortem cerebella.

V. RESULTS

This investigation was conducted in the cohort of 10 CON and 10 ASD human post mortem cerebellar samples which were obtained frozen at -80 °C from the Harvard Brain Tissue Resource Center, McLean Hospital (Belmont, MA, USA) with approval of Autism Speaks (Autism Tissue Program).

A. STUDY 1: The implication of MeCP2 protein in the regulation of the expression of GAD1, GAD 2 and RELN genes. Measurements of MeCP2 mRNA expression, it's protein level and it's binding to the promoter regions and gene bodies of GAD 1, GAD2 and RELN in cerebellum of ASD.

1. MeCP2 mRNA expression and protein levels in cerebella of ASD

Measurements of the expression of MeCP2 mRNA relative to the expression of housekeeping gene (GAPDH mRNA) showed a statistical significant increase (~ 70%) in ASD (0.78±0.095, *p=0.01*) compared to CON (0.40±0.09) in postmortem cerebellar samples (FIG 1a). Additionally, a positive correlation was observed between MeCP2 mRNA level and binding of MeCP2 protein to the RELN (Pearson r^2=0.54, *p=0.04*) and GAD1 promoters (Pearson r^2=0.59, *p=0.01*).On the other hand, differences in the mRNA expression between ASD and CON subjects were not associated with changes in the protein level. The mean value of MeCP2 protein in ASD subjects was not statistically different from the mean value in the CON subjects in cerebellar homogenates (FIG.1b).

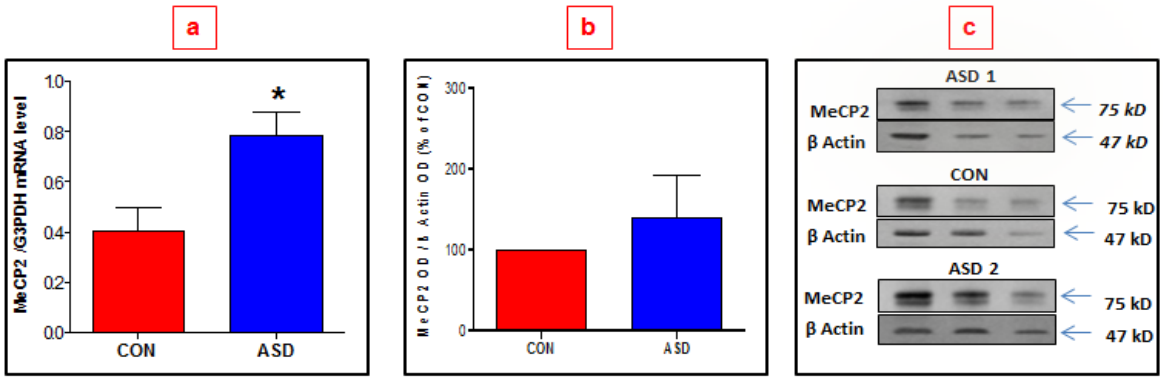

FIG.1 MeCP2 mRNA and protein expression in CON vs ASD.

(a) MeCP2 mRNA expression is increased in ASD vs CON (**p*=0.01). Values were corrected for expression of housekeeping gene GAPDH mRNA. All values are expressed as Means ± SEM. (b) MeCP2 protein levels fail to increase in ASD samples relative to CON (*p*=0.45, *n*=10). MeCP2 protein levels in ASD were expressed as percentage relative to CON (100%). (c) Representative immunoblots show a major band of 75 kDa for MeCP2 and 42 kDa for β-actin. ASD, autism spectrum disorder; CON, control.

2. MeCP2 protein binding to the promoter region and gene body of GAD1, GAD2 and RELN in cerebellum of CON and ASD

First, we tested if there is selectivity for the binding of MeCP2 to our target genes, then proceeded with measurement of the magnitude of the MeCP2 binding to these genes. Results showed that MeCP2 binds selectively to the RELN, GAD 1 and GAD 2 promoter and gene body, but only marginally (<0.01%) binds to the housekeeping gene GAPDH (−122 bp to +42 bp). Measurements showed that MeCP2 binds more efficiently to the gene body regions than to the CpG-rich promoter regions of RELN, GAD 1 and GAD 2. Quantitative analysis showed that MeCP2 binds significantly more (1.5-2 fold) to the promoter regions of GAD1 ($p=0.04$) and RELN ($p=0.03$) cerebellar samples of ASD compared to CON (FIG.2a). In contrast, MeCP2 binding to the GAD2 promoter region did not show significant changes in ASD vs CON ($p=0.32$) (FIG.2a). Furthermore, MeCP2 binding to RELN, GAD1 and GAD2 body regions failed to show significant changes in ASD compared to CON (FIG.2b).

Pearson's correlation analyses were conducted to identify possible association of the binding of MeCP2 to promoter region of GAD1 and RELN with corresponding mRNA level. Analysis showed that there is statistically significant ($p=0.04$) negative correlation between the binding of MeCP2 protein to the RELN promoter and the expression of RELN mRNA in ASD (FIG. 2c). However, due to variability and small sample size, the binding of MeCP2 to the GAD1 promoter showed only a trend towards statistical significance ($p<0.08$).

To establish if the observed changes in the level of MeCP2 binding to either the GAD1 or RELN promoters could be attributed to potential confounding factors (age, sex, brain pH, post-mortem interval), we analyzed the data using analysis of covariance. ANCOVA did not reveal a statistically significant correlation between MeCP2 binding to either the GAD1 or RELN promoters with changes in age (GAD1 vs MeCP2, Pearson r^2=−0.31, *p=0.19*; RELN vs MeCP2, Pearson r^2=−0.39, *p=0.09*), sex (GAD1 vs MeCP2, Pearson r^2=0.28, *p=0.25*; RELN vs MeCP2, Pearson r^2=−0.14, *p=0.56*), PMI (GAD1 vs MeCP2, Pearson r^2=−0.3, *p=0.23*; RELN vs MeCP2, Pearson r^2=−0.33, *p=0.17*) in CON and ASD patients considered either together or separately. Furthermore, we wanted to elaborate statistically the impact of the various groups of medication on the values of MeCP2 binding. There was no significant differences in MeCP2 binding between ASD subjects treated with antipsychotics, antidepressants or mood stabilizers (*n*=7), duration of treatment, and the few ASD (*n*=3) that apparently were non-treated at the time of death. In conclusion, increased binding of MeCP2 the GAD1 and RELN promoters in cerebellar cortex of ASD patients is predicted by the diagnosis of ASD, but not influenced significantly by confounding variables.

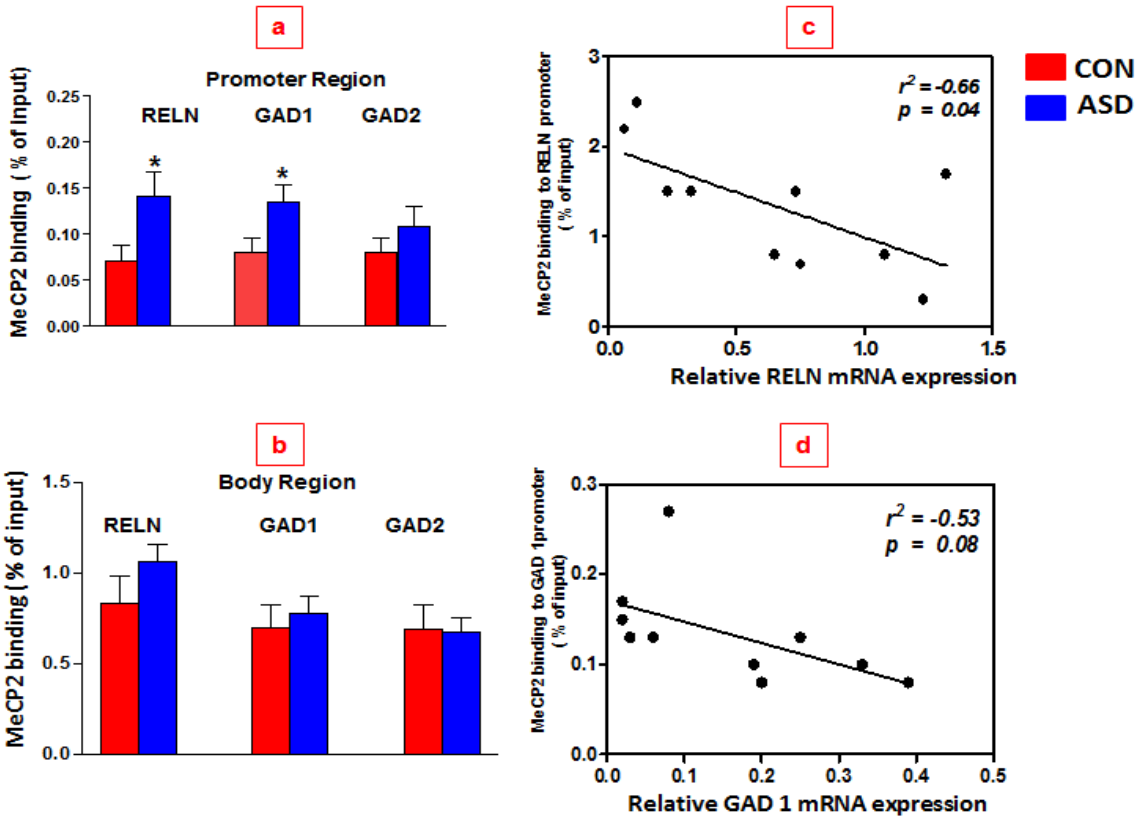

FIG. 2 MeCP2 binding to RELN, GAD1 and GAD2 promoters and gene bodies and correlations with corresponding mRNA in the cerebella of CON and ASD.

(a) MeCp2 binding to the promoter regions of RELN ((−220 to +70), (*$p=0.03$)) and GAD1 ((−55 to +121), (*$p=0.04$)) are increased in ASD vs CON, while GAD2 ((−1507 to +1310) ($p=0.32$)) is unchanged in ASD vs CON. (b) MeCP2 binding to gene body regions of RELN ((+562 to +763), ($p=0.2$)), GAD1 ((+656 to +856), ($p=0.6$)) and GAD2 ((+1293 to +1447), ($p=0.9$)) are unchanged in ASD vs CON. (c) Increased binding of MeCP2 to the RELN promoter was associated with reduced RELN mRNA (Pearson r^2=−0.66, *$p=0.04$, n=10) in ASD. (d) Increased binding of MeCP2 to the GAD1 promoter shows a trend to statistical significance with decreased GAD1 mRNA expression (Pearson r^2=−0.53; $p=0.08$, n=10) in ASD. ASD, autism spectrum disorder; CON, control; GAD1, glutamic acid decarboxylase 67; GAD2, glutamic acid decarboxylase 65; RELN, Reelin.

B. STUDY 2: Methylation and Hydroxymethylation enrichment of the promoters and gene body regions of GAD1, GAD2 and RELN

1. Measurement of TET 1 and DNMT 1 mRNA levels in the cerebella of CON and ASD

The level of TET 1 mRNA expression relative to the expression of housekeeping gene (GAPDH mRNA), showed a significant increase (*p=0.01)* in cerebellum of ASD (0.052±0.010) compared to CON (0.021±0.010). This finding is paralleled by the significant increase *(p=0.046)* in binding of TET1 protein to the GAD1promoter in ASD (0.100 ± 0.013) compared to CON (0.070 ± 0.011), as well as significant increase *(p=0.01)* in binding of TET1 protein to the RELN promoter in ASD (0.031 ± 0.064) compared to CON (0.011 ± 0.021). These observations strongly suggest that the increased expression of TET mRNA and TET1 binding are responsible for the 5-hmC enrichment observed at the GAD1 and RELN promoters (Fig 4a,b).

On the other hand, the level of DNMT1 mRNA expression corrected for the housekeeping gene (G3PDH mRNA) did not change in ASD (0.60 ± 0.11) (*p=0.6*) compared to CON (0.76 ± 0.25). Similarly, there were no differences in the binding of DNMT1 to the promoter regions of GAD1 [CON (0.11 ± 0.020) vs ASD (0.13 ± 0.020), *p=0.6*] and RELN [CON (0.20 ± 0.010) vs ASD (0.16 ± 0.030), *p=0.5*]. Failure of DNMT1 mRNA expression and DNMT1 binding to change in ASD samples was associated with depletion of 5-mC relative to 5-hmC in the promoter region of GAD1 and RELN (Fig 5a, b).

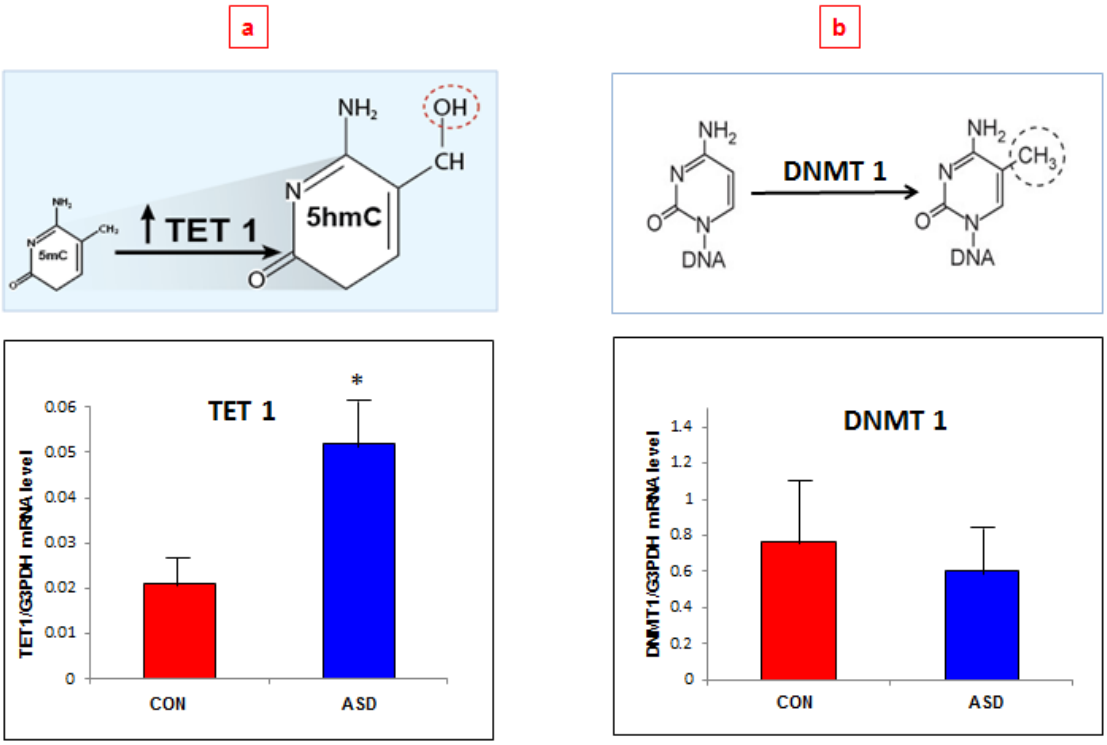

FIG.3 TET 1 and DNMT 1 mRNA expression in CON vs ASD.

(a) TET 1 mRNA expression is increased in ASD (0.052±0.010) vs CON (0.021±0.010) (*$p=0.01$). (b) DNMT 1 mRNA expression failed to increase in ASD (0.60 ± 0.11). vs CON (0.76 ± 0.25) (p=0.6) Values were corrected for expression of housekeeping gene GAPDH mRNA. All values are expressed as Means ± SEM. ASD, autism spectrum disorder; CON, control; TET 1, Ten eleven translocation 1, DNMT 1, DNA methyltransferase 1.

2. Enrichment of 5-hmC and 5-mC in the promoters and gene body regions of GAD1, GAD2 and RELN

5-hMeDIP measurement revealed significant enrichment of 5-hmC at the GAD1 (*p=0.05)* and RELN (*p=0.03*) promoters of ASD compared to CON (FIG. 4a, b), while the level of 5-mC enrichment failed to change among respective groups (FIG. 5a, b). Furthermore, correlation analysis showed statistically significant inverse correlation between the 5-hmC enrichment at the GAD1 *(p=0.01)* and RELN *(p=0.04)* promoters and their corresponding mRNA levels in ASD patients (FIG. 4d, e). A similar statistical approach did not show association between the GAD1 and RELN promoter 5-mC enrichment and the corresponding mRNA levels (FIG. 5a, b).We did not find significant enrichment in the level of 5-hmC and 5-mC in the gene body regions of RELN, GAD1 and GAD2 in ASD vs CON (FIG. 5a, b, c). From this study we concluded that the level of 5-hmC and 5-mC enrichment appears to be specific not only to some genes versus others, but also specific within a region of a given gene. This observation could potentially play a fundamental role in understanding alterations of gene function in ASD and related disorders.

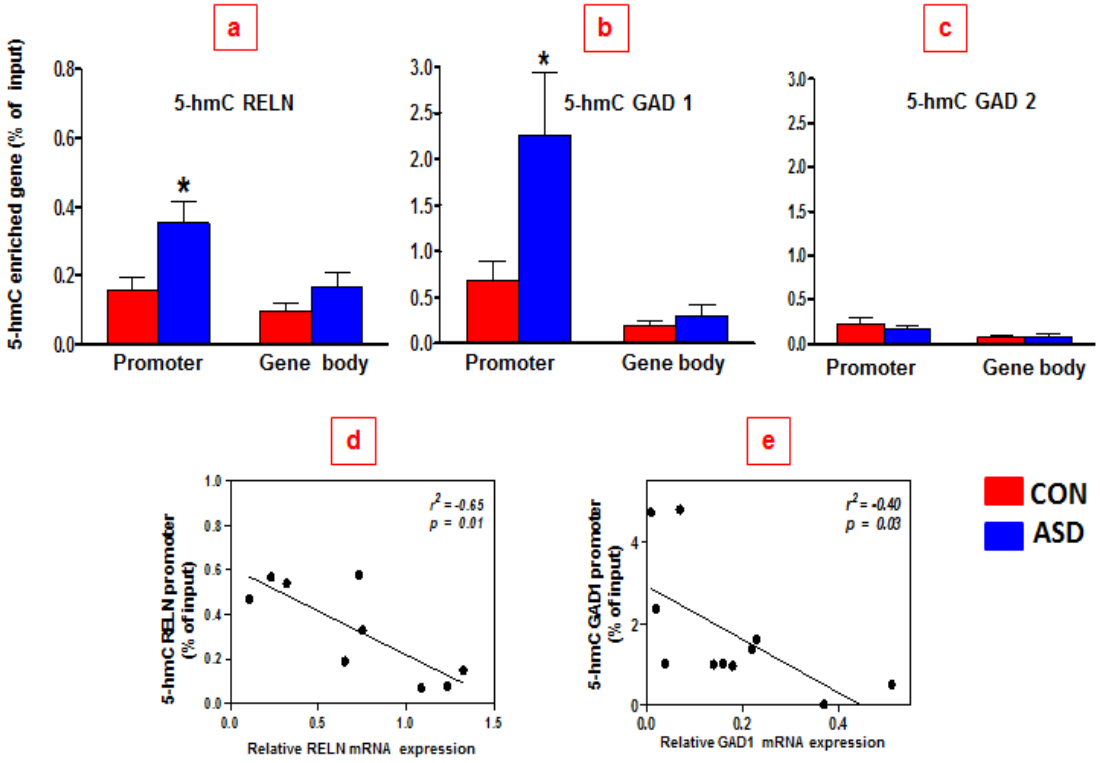

FIG.4 Comparison of 5-hmC enrichment in two different gene regions of the RELN, GAD1, GAD2 and correlations between 5-hmc enrichment in the promoter regions of RELN and GAD1 with the respective mRNA levels in the cerebella of CON and ASD.

(a) Increased enrichment of 5-hmC at the promoter of RELN ((−220 to +70 bp) (*$p=0.026$)) and (b) GAD1 ((−55 to +121 bp), (*$p=0.047$)), (c) no changes in the enrichment of 5-hmC at the promoter of GAD2 ((−1507 to −1310 bp), (P=0.36)) in cerebella of ASD vs CON. No changes in the enrichment of 5-hmC at the gene bodies of a) RELN ((+562 to +763 bp), ($p=0.22$)), b) GAD1 ((+562 to +763 bp), (P=0.53)) and c) GAD2 ((+1293 to +1447 bp), ($p=0.46$)) in cerebella of ASD vs CON. Statistically significant correlation of 5-hmC in the promoter of (d) RELN ((−220 to +70 bp), (Pearson r^2=−0.65, *$p=0.01$)) and (e) GAD1 ((−55 to +121 bp), (Pearson r^2=−0.40, *$p=0.036$)) and with corresponding mRNA levels in ASD samples. ASD, autism spectrum disorder; CON, control; GAD1, glutamic acid decarboxylase 67; GAD2, glutamic acid decarboxylase 65; RELN, Reelin.

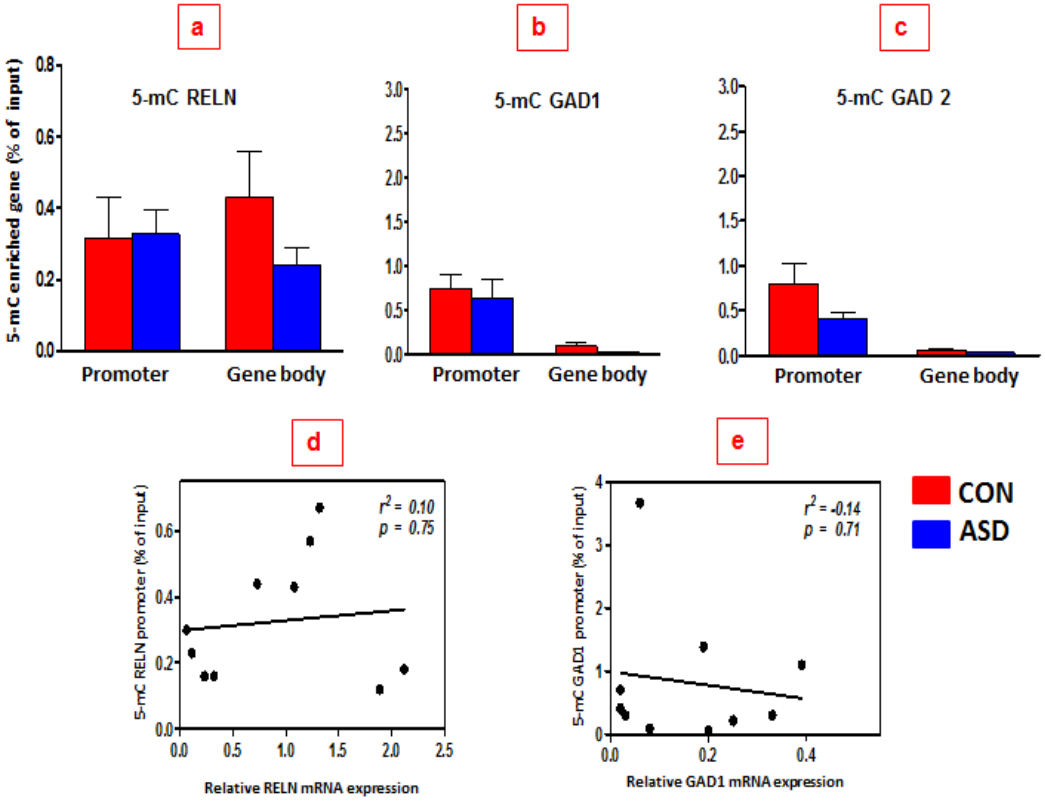

FIG.5 Comparison of 5-mC enrichment in two different gene regions of the RELN, GAD1, GAD2 and correlations between 5-mc enrichment in the promoter regions of RELN and GAD1 with the respective mRNA levels in the cerebella of CON and ASD.

No changes in the enrichment of 5-mC at the promoter of (a) RELN ((−220 to +70 bp), (*p=0.91*)), (b) GAD1 ((−55 to +121 bp), (*p=0.67*)) and (c) GAD2 ((−1507 to −1310 bp), (*p=0.36*)) in cerebellum of ASD vs CON. Also, there were no changes in the enrichment of 5-mC at body regions of (a) RELN ((+562 to +763 bp), (p=*0.16*)), (b) GAD1 ((+656 to +856), (*p=0.11*)) and (c)GAD2 body ((+1293 to +1447 bp), (*p=0.46*)).No significant correlations between 5-mC content in the promoters of (d) RELN ((−220 to +70 bp) (Pearson r^2=−0.10, *p=0.75*)) and (e) GAD1 ((−55 to +121 bp), (Pearson r^2=−0.14, *p=0.71*)) and with mRNA levels were evident in ASD (vs CON) samples. ASD, autism spectrum disorder; CON, control; GAD1, glutamic acid decarboxylase 67; GAD2, glutamic acid decarboxylase 65; RELN, Reelin.

3. Validation of the Methylation and Hydroxymethylation status of GAD1 promoter region by Tet-assisted bisulfite pyrosequencing with base resolution (TEB-seq)

Tet-assisted bisulfite pyrosequencing with base resolution was used to validate the results obtained from the MeDIP and hMeDIP assays. For this purpose we chose three representative samples for the analysis of GAD 1 promoter methylation and hydroxymathylation status.

Experiments showed that in CON subjects, 5-hmC is preferentially enriched at CpG position −2 of the GAD1 promoter (sequence relative to the TSS: −54 to +69 bp) (FIG. 6). In the same samples, the levels of 5-mC is low but detectable at positions −2, −1 and 3 and absent in other CpG sites studied. In ASD patients (as compared with CON), 5-hmC is preferentially enriched at CpG position −2 with smaller amounts at position 1 and 2. 5-mC is depleted at this site in the ASD samples but present at these positions in the CON samples (FIG. 6). These methylation changes result in a significant increase in the amounts of 5-hmC relative to 5-mC and the overall 5-hmC/5-mC ratio at the GAD1 promoter (−54 to +69 bp) is 1.2 in CON and 5.5 in ASD patient DNA.

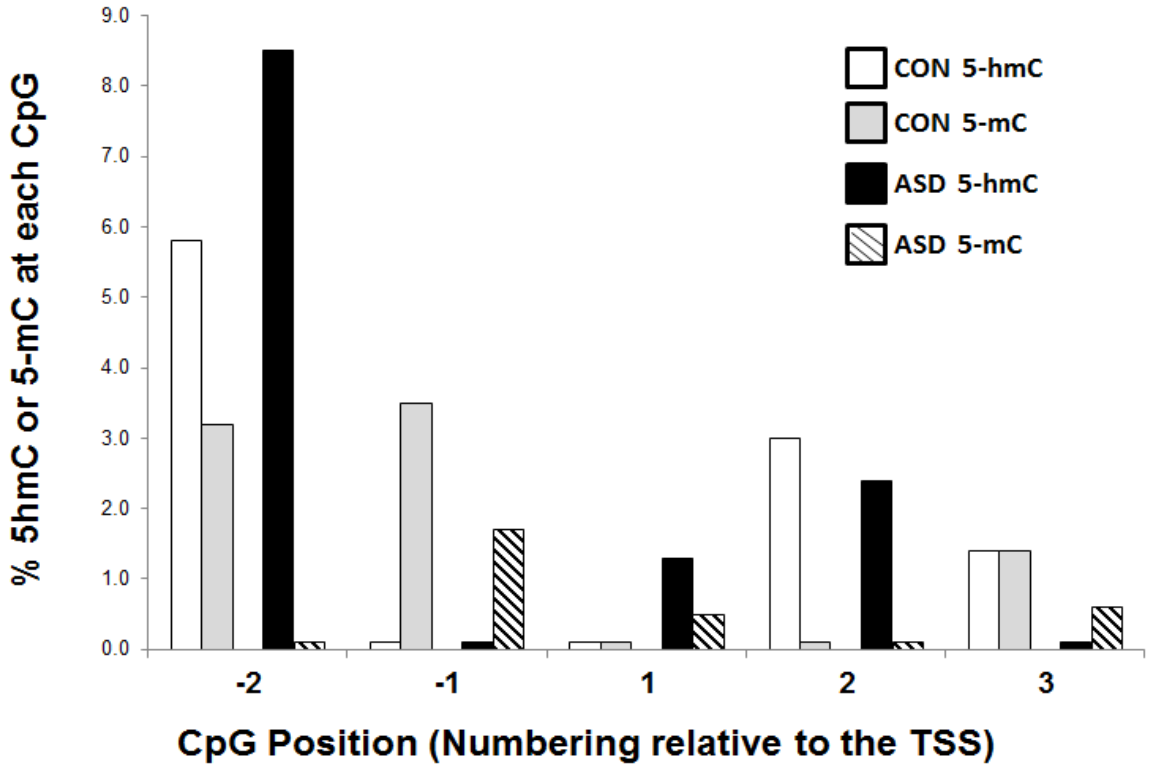

FIG.6 TAB-seq shows the differential distribution of 5-mC and 5-hmC along the GAD1 promoter.

The GAD1 promoter includes regulatory elements proximal to both sides of the transcriptional start site (TSS).32 Pyrosequencing was used to determine the % 5-hmC or 5-mC (y axis) at five CpG sites proximal to the GAD1 TSS. CpGs (x axis) are numbered relative to the TSS (NCBI Ref: NM_000817) and Cs correspond to the following positions: −2=−5 bp, −1=−1 bp, 1=+8 bp, 2=+27 bp and 3=+48 bp. Bisulfite-treated DNA from −54 bp to +69 bp of the GAD1 gene was amplified. While the fragment assayed is shorter, it contains the amplicon used to immunoprecipitate the 5-hmC and 5-mC containing GAD1 fragments (−55 bp to +123 bp, see Supplementary Figure 1). Each bar represents the mean of three sample measurements. Total 5-hmC and 5-mC content is determined by the number of Cs at each position following bisulfite conversion and amplification of the same region. Based on evaluations of the 5-hmC (pGEM1) and the 5-mC (λDNA), we estimate the efficiency of β-glucosylation (protection) to be >95%, while the bisulfite conversion efficiency was greater than 99%. CON, control; GAD1, glutamic acid decarboxylase 67 GAD1.

VI. METHODOLOGY

A. Subjects and cerebellar collection

This study was conducted in a cohort of 20 post-mortem cerebellar samples obtained from the Harvard Brain Tissue Resource Center, McLean Hospital (Belmont, MA, USA) with approval of Autism Speaks (Autism Tissue Program). Based on the neuropathological reports obtained from each subject, there were no signs of infarction, hemorrhages or inflammatory lesions, with the exception of one ASD subject, which had a large early intermediate stage infarct. Subjects were matched for age, sex, post-mortem interval (PMI) and brain mass (Table 1).In this study, subjects with conditions such as Asperger's syndrome, Fragile–X syndrome, RTT, pervasive developmental disorder not otherwise specified, and 15q11-q13 duplication, were not included. In addition, CON cases were free of neurological disorders, seizures, intellectual disability, dementia, and so on based on medical records.

CON

AGE	SEX	PMI	pH	Brain Mass	Ethnicity	Cause of Death
16	M	26	6.6	1500	unk	Heart Disease
19	M	19	6.5	1555	English-White	Pneumonia
24	M	19	5.9	1365	unk	unk
28	M	23	6.5	1580	unk	unk
31	M	33	6.4	1810	unk	Asphyxia
32	F	29	6.4	1360	unk	unk
39	M	unk	6.4	1350	unk	unk
48	M	30	6.0	1400	unk	myocardial infarction
50	M	20	6.5	1740	unk	unk
56	M	23	6.5	1500	unk	Heart Attack

	CON (n=12)	ASD (n=10)	Stat
M/F	11 / 1	8 / 2	
Age	37 ± 5	31 ± 5	t-test ,p=0.6
Brain Mass	1492 ± 52	1340 ± 80	t-test ,p=0.2
PMI	27 ± 2	21 ± 2	t-test ,p=0.07
pH	6.36± 0.1	6.34± 0.1	t-test ,p=0.9

ASD

AGE	SEX	PMI	pH	Brain Mass	Ethnicity	Cause of Death	Perinatal Condition	Medication
15	M	31	6.4	unk	unk	Head trauma		Z,Pri,K,Mel
19	M	15	6.6	1090	unk	Respiratory arrest		
20	M	24	6.3	1144	English-White	MV Accident	↑BP	Min,Bn
24	M	19	6.1	1400	N European White	ARDS		R,CEL,F,Flx,Li, D,L,Clo,G,Bus
27	M	8	6.4	1575	English-White	Drowning	Oxytocine jaundice, Prol delivery	Syn
29	F	18	6.4	unk	unk	Seizure d/o		R,F,Li,K,Carb
30	M	16	6.4	1800	unk	CHF,4th vent dermoid tumor		C,Zol
38	M	unk	6.3	unk	English-White	Aspiration		
50	M	23	6.0	unk	unk			
56	M	20	6.5	1630	English-White	Anoxic Encephalopathy	CS,prematu birth	H,TH,Li,Ben, Diph

H-Haldol,TH-Thorazine, Z-Ziprasidone , R-Risperidone,C-Clomipramine,Ser-Sertraline,Flx-Fluoxetine, Des-Desvenlafaxine,F-Fluvoxamine,Li-Lithium,D-Divalproex sodium ,Carb-Carbamazepine,Ben-Benztropine,Alp-Alprazolam,L-Lorazepam, Clo-Clonazepam,Bus-Buspirone,M-Melatonin,G-Gabapentin,Bn-Benzamycine,L-Levothyroxine,Dip-Diphenhydramine,Ox-Oxytocin

Table 1. Demographic data corresponding to the ASD and CON groups used in this study.

Individual data are listed under CON and ASD table headings. A summary, which includes mean values and standard errors, is shown in the upper right quadrant. Stat represents the results of a t-test analysis of the variables. Abbreviations of various medications used (when known) are listed in the Table.

B. RNA isolation and mRNA quantification using real-time PCR

Total RNA from post-mortem human samples was isolated using TRIzol reagent (Life Technology, Grand Island NY). 30 mg of human tissue was homogenized in 1 mL of TRIZOL solution and incubated for 5 min in 15-30°C to permit complete dissociation of nucleoprotein complex. 0.2 mL of Chloroform was added to each sample, than vigorously shaken for 15 sec and incubated for 2-3 min in 15-30°C. Samples were centrifuged at 12,000 x g for 15 min at 2-8°C. RNA containing aqueous phase located on the upper compartment of homogenate was transferred to a fresh tube and mixed with 0.5 mL isopropyl alcohol per 1 mL of TRIZOL solution. Samples were incubated at 15 to 30°C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 2 to 8°C. The final product of isolation is gel-like pellet on the bottom on the tube which contains RNA precipitate. The final step consists on the washing of pellet with 1mL of 75% ethanol, mixing and centrifugation at 7,500 x g for 5 minutes at 2 -8°C. Resultant pellet was air-dried for 5-10 min. and dissolved in 30 µl of RNase free water.

Quantification of mRNA expression was conducted using Applied Biosystems Real-Time PCR system with a SYBR green master mix (Fermentas, Glen Burnie, MD, USA). In our experiments we measured mRNAs corresponding to DNMT1, MeCP2, TET 1, GAD 1, GAD 2 and RELN, while GAPDH was used as internal controls for sample normalization (see table 2 for primer structure). The PCR products were subject to a melting curve analysis in order to confirm specificity of primers amplification, in which only one peak was observed. Each target was run in duplicate and the housekeeping genes were run in parallel. Values were calculated as relative abundance to the housekeeping gene after normalization.

	Primer used in ChiP Assays
Promoter	Amplicon = 297 bps, 42 CpGs
RELN -220 to +70	F: -220: 5′ CCGGGACACGTGTGGCGGCG 3′ R: +70: 5′-GGCGAGAAGAAGGCGGACGGG-3
	Amplicon = 164 bp, 10 CpGs
GAD1 -55 to+121	F: -43: 5′ GCGTGTTGAGTACGTTCTGGATTACT 3′ R: +121: 5′ GGAAATCACGAGGCCGTTGCT 3′
	Amplicon = 197 bps, 9 CpGs
GAD 2 -1507 to -1310	F: -1507: 5′ GCCTGGAACAGACTTCACCTAGC 3′ R: -1310: 5′ CTGTCCCTGGTCAATTCTCTCCAC 3′
Gene Body	Amplicon = 201 bp, 7 CpGs
RELN Intron 1: +562 to +763 bp	F: 5′ GTTGGGATTCATTCTCCATGTCCCC 3′ R: 5′ GGAGCAGGACAAAGGTCTGAAATCC 3′
	Amplicon = 200 bp, 10 CpGs
GAD 1 Intron 1: +656 to +856 bp	F: 5′ CCATGCTTACTTACCCCACCCTC 3′ R: 5′ CACAGTCACCTTGCACTCCAGAC 3′
	Amplicon = 154 bp, 12 CpGs
GAD 2 Intron1/2 +1293 to +1447	F: 5′ GTCTGCCTGCCTATTCTTCCTTGC 3′ R: 5′ GAGGTGAGAAGACTGCTTGGACC 3′

	Primer used in Gene Expression Assays
RELN	F: 5′ CGTCTAGAAAACGGGAACCAAGCAAG 3 R: 5′TCTAATCCCAGTTACTTGGGAGGCTG 3′
DNMT 1	F: 5′ TTCTTCGCAGAGCAAATTGA 3′ R: 5′ TGCCACCAAATTTAACCATGT 3′
GAD 1	F: 5′AGGCAATCCTCCAAGAACCT 3′ R: 5′GGTGGAGCGATCAAATGTCT 3′
GAD 2	F: 5′GTGTGATGGAGAAAGGCCCAC 3′ R: 5′AGTCTGCTGCTAATCCAACCATAT 3′
TET 1	F: 5′CCCGGGCTCCAAAGTTGTG 3′ R: 5′GCAGGAAACAGAGTCATT 3′
GAPDH	F: 5′ CGA GATCCCTCCAAAATCAA 3′ R: 5′TTCA CACCCATGACGAACAT 3′

Table 2. Primers Used to Measure Amounts of mRNA and Protein Binding (ChIP) to DNA.

Complete list of the primers used to measure mRNA levels (Gene Expression Assays) and to quantitate DNA amounts from ChIP assays (Promoter and Gene body). The relative locations of the latter primers are depicted in Supplemental Figure 1.

C. Western blot analysis

Around 10 mg of human tissue samples were homogenized directly in 100 ul of Leammli buffer Three aliquots of cerebellar cortex extracts (~ 40 ug of protein) for every sample were applied to SDS- PAGE to identify the linear range for protein quantification. Electrophoretic separation of proteins was done using NuPAGE 4-12 % Bis Tris gel (NOVEX ,Grand Island, NY), then followed by the transfer to nitrocellulose membranes (BIO RAD, Hercules, CA) .The membrane was blocked for 1 h at room temperature with 5% nonfat milk in PBS (10 mM PBS, pH 7.4) and exposed to anti-MeCP2 polyclonal antibody (1:2000) (DIAGENODE, Denville, NJ). The detection of immunoreactive bands was done using secondary antibody , anti-rabbit IgG produced in goat (BIORAD) at 1:2000 for 2 h, followed by the application of the Pierce ECL Plus chemiluminescence Western blotting substrate (Thermo Scientific, Hanover Park ,IL) on a STORM 860 fluorescent imager (Molecular Dynamics) and analyzed with IMAGEQUANT software. The MeCP2 antibody recognizes a major band of ~ 75kDa corresponding to the full length MeCP2 protein. The level of MeCP2 was calculated as a ratio of the optical density of MeCP2 versus optical density of β-Actin band. Changes in MeCP2 protein levels in ASD vs CON were determined by expressing the data as a percentage of the CON.

D. Chromatin immunoprecipitation assays (CHIP)

We used Chromatin Immunoprecipitation kits (Millipore) for CHIP measurements. About 30 mg of brain tissue was fixed in 500µl of 1% formaldehyde solution, then homogenized and incubated at room temperature for 15 min in a rotating platform. Following two washes with cold PBS, samples were added protease inhibitor and 0.5mL of cell lysis buffer, than incubated on ice for 15 min. Homogenates were spun down to obtain pellet, then added 0.5mL of nuclear lysis buffer to the pellet and proceeded to sonication. To obtain consistent chromatin fragmentation, the lysates were sonicated for 60 min on ice using Bioruptor (UCD-200; Diagenode, Liege, Belgium). Cross-linked chromatin was sheared to an average DNA fragment molecular length of 100– 200 bp. In 50µl of sheared cross-linked chromatin was added 450µl of dilution buffer, and from this mixture, 5µl was removed in order to quantify the total amount of DNA present in samples before immunoprecipitation (inputs). In the chromatin preparation was added 20µl of protein A/G magnetic beads and immunoprecitpitated with 3µg of Anti- DNMT 1 antibody (mouse monoclonal; Imagenex) and MeCP2 antibody (rabbit polyclonal; Diagenode), Anti-TET1 (Zymo, Irving, CA, USA) DNMT1 antibodies (Abcam, Cambridge, MA, USA) and Anti-MeCP2 polyclonal antibodies (1:2000 dilution, Diagenode, Denville, NJ, USA).Following washing and elution step, cross-linking between DNA and proteins was reversed by heating the samples at 65◦C overnight, followed by Proteinase K digestion at 45◦C for 1 h. DNA was purified and quantified using real-time qPCR. The percentage of immunoprecitpitated DNA were calculated using the following: % (gene−IP/total input)=$2^{(Ct(10\%\ input)-3.32)-Ct(gene-IP)} \times 100\%$.

E. Methylated and hydroxymethylated DNA immunoprecipitation (MeDIP and hMeDIP)

Genomic DNA from 10 mg of FC tissue was extracted using the Qiagen DNeasy Kit. After sonication to fragment DNA into nucleosomes, 4 μg of genomic DNA was incubated with 5-mC monoclonal mouse (Diagenode, Denville, NJ, USA) and 5-hmC monoclonal mouse (Diagenode) antibodies overnight at 4°C. The DNA-antibody complex was then harvested using protein A/G agarose beads (BioVision). The DNA precipitated by the antibody was treated with proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in 10 mM Tris·HCl (pH 8.5) and used for qPCR quantification.). The percent methylated vs. unmethylated promoter was calculated by the following equation: % (meDNA-IP/total input) = 2^[(Ct(10% input) − 3.32) − Ct (meDNA − IP)] × 100% (MagMeDIP kit instruction manual, Diagenode).

F. TET assisted bisulphite (TAB) –Pyrosequencing

*Validation of 5-hmC and 5-mC DNA Immunoprecipitation Assay using Amplicon-Specific TAB-Pyrosequencing***.** The distribution of 5-hmC and 5-mC in the GAD1 promoter amplicon was determined by a modification of TAB-seq (Yu et al., 2012a). In brief, this is a two-step method that first uses β -glucosyltransferase (β -GT) to add O-glucosylgroups (5-gmC) to the hydroxyl group, to protect existing 5-hmCs. In a second step, 5-mCs are oxidized to form 5-carboxycytosine (5caC) using recombinant mTet1 (Yu et al., 2012a, 2012b). Following bisulphite conversion, 5-gmC is converted to C while 5-caC residues are converted to T (Yu et al., 2012). To determine the efficiency of each enzymatic step, spike-in controls were added and

independently scored following bisulphite conversion and conventional Sanger DNA sequencing.

Spike-in Controls. Spike-in controls were generated to determine the efficiency of 5-mC conversion (to 5-caC) and 5-hmC protection as previously described (Yu et al., 2012b). Following bisulfite conversion, the 5-mC λ and 5-hmC pGEM1 templates were amplified (using appropriate bisulphite-designed primers) with Pfu Turbo Cx hotstart DNA polymerase (Agilent), cloned and sequenced by the UIC DNA Services Facility.

Oxidation (Conversion) control (5-mC λ): Unmethylated λ DNA (cI857 Sam7, Promega) was methylated *in vitro* with CpG Methyltransferase (M.SssI, New England Biolabs) using 160 λ M S-adenosyl methionine as described by the manufacturer. Lambda DNA (48,502 bp) contains 3112 CpG sites that can be methylated using CpG Methyltransferase. The methylated λ DNA (0.5% or 5 ng/µg genomic DNA) was added to genomic DNA prior to sonication (see ChIP protocol). In the 245 bp 5-mC control amplicon targeted for analysis, there are a total of 22 CpGs. In addition, this template contained 46 non-CpG Cs which were not methylated by CpG methyltransferase and were used to determine the efficiency of bisulphite conversion.

Protection 'spike-in' control (5-hmC pGEM1): A 275 bp segment corresponding to 1635 bp to 1810 bp of pGEM1 (Promega) was generated using Zymo Taq DNA Polymerase (Zymo, Irvine CA). The fragment was amplified using 1 ng of linear pGEM1 template and 5-hydroxymethyl-2'-deoxycytidine-5'-triphosphate (5-hm dCTP, Bioline, >99% based on HPLC as per manufacturer's data sheet) in place of 2'-deoxycytidine-5'-triphosphate during amplication with Zymo *Taq* DNA polymerase (Yu et al., 2012). The size and purity of the PCR product was

verified on 1.2% agarose gels and the amplified band was subsequently gel purified. The purified 5-hmC-containing template was added to genomic DNA (0.25% or 25 ng/µg genomic DNA) immediately following the sonication step. The forward strand contained 57 Cs while the reverse strand contained 50 Cs all of which would become 5-hmCs following PCR.

GAD1 Pyrosequencing. Determination of the 5-hmC and 5-mC content of the GAD1 genomic 5-hmC IP and 5-mC IP amplicons was measured after the λ-GT protection and mTet1 oxidation steps and following bisulfite conversion. DNA from three individual IPs from each patient were pooled and concentrated using DNA Clean and Concentrator Columns (Zymo Technologies, Irvine CA). In parallel, additional three independent 5-hmC and 5-mC IPs were prepared from each patient for bisulfite conversion alone to determine the total number of Cs present at each position. Percent methylation at each position along the GAD1 amplicon was measured with pyrosequencing (Epigendx, Hopington, MA) following amplification of treated (protection, oxidation and bisulfite conversion) and non-treated (bisulphite conversion only) genomic immunoprecipitated templates. The GAD1 bisulphite primers (Epigendex, Assay ADS3737) were tested to ensure the absence of methylation-based amplification bias. Two distinct (FS2 and FS3) primers were used in performing the pyrosequencing because of a stretch of 16 Ts that separate the second and third CpGs in the targeted region. This region extends from -54 bp to +69 bp relative to the GAD1 transcriptional start site whereas the region targeted for immune precipitation spans from -55 bp to +123 bp. This extended portion contains an additional eight CpG dinucleotides for which we have no information regarding methylation status.

Following Tet1-assisted bisulphite conversion, 5-hmC groups were converted to 5-glucosylmethylcytosines (5-gmC) with 5-hydroxymethylglucosyltranferase (λ -GT, obtained from Wisegen). After the conversion/oxidation reaction, using recombinant mTet-1 (Wisegen), and subsequent bisulphite conversion, GAD1 IP DNA was amplified using bisulphite-primer pairs and Pfu Turbo Cx hotstart DNA polymerase (Agilent). Amplified DNA was analyzed by pyrosequencing (Epigendx) to determine the perscent 5-hmC and 5-mC at each position along the target sequence.

G. Statistical analysis

The primary test of the difference between CON and ASD for each mRNA, protein, ChIP, hMeDIP and MeDIP assays was an independent two sample *t*-test for equal and unequal variance as appropriate. Additional analyses were conducted using analyses of variance and covariance controlling for factors such as gender, PMI, pH, presence of medication, type of medication using PASW v.18 software (SPSS, Armonk, NY, USA). Relationships between mRNA and protein expression variables and ChIP level variables were analyzed with Pearson correlations and scatter plots. Two-sided probability levels were used for statistical significance ($P<0.05$), or trends ($P<0.1$).

VII. CONCLUSIONS

This project supports the importance of the epigenetic regulation of the gene expression mediated by the MeCP2. The study shows that MeCP2 binding to the promoter regions of GAD1 and RELN is significantly increased in ASD compared to CON. In contrast, MeCP2 binding to the corresponding gene body did not show difference between ASD and CON. For further elaboration of the factors that could explain the increased binding of the MeCP2 to the promoter regions of GAD 1 and RELN we proceeded with measurements of MeCP2 mRNA and protein level. Although we found tendency to increase in the MeCP2 mRNA and protein, this observation cannot explain why the binding of MeCP2 increases selectively in the promoter regions, but not in the gene body regions of the corresponding genes. This conclusion prompted further investigation of methylation and hydroxymethylation processes as important determinants of the MeCP2 binding. Recent study by Mullen et al 2012, demonstrated that mouse MeCP2 binds to both 5-mC and 5-hmC with high affinity. Using MeDIP and hMeDIP approach we demonstrated enrichment of 5-hmC (but not 5-mC) in the same promoter regions of GAD 1 and RELN of the ASD, which were shown to bind more MeCP2 in ASD compared to CON. This association supports the idea that the enrichment of 5-hmC to the promoter region of GAD 1 and RELN facilitates selectively the binding of MeCP2. A possible explanation for the enrichment of 5-hmC at the promoter region of GAD 1 and RELN is the upregulation of TET1 mRNA expression and TET1 protein binding to the promoter regions of these genes. In addition, DNMT 1(methylation writer) is found to be unchanged in cerebellum of ASD, which could explain failure of 5-mC to change significantly in the promoter regions of GAD1and RELN.

In summary, the data suggest that the increased binding of MeCP2 to the GAD1 and RELN regulatory domains may be mediated by an enrichment of 5-hmC. Finally, in our study, MeCP2 acts as repressive factor, since its increased binding to the promoter region of GAD 1 and RELN is associated with downregulation of the expression of the corresponding mRNAs. FIG. 6 depicts our working hypothesis based on the experimental work described in the section above.

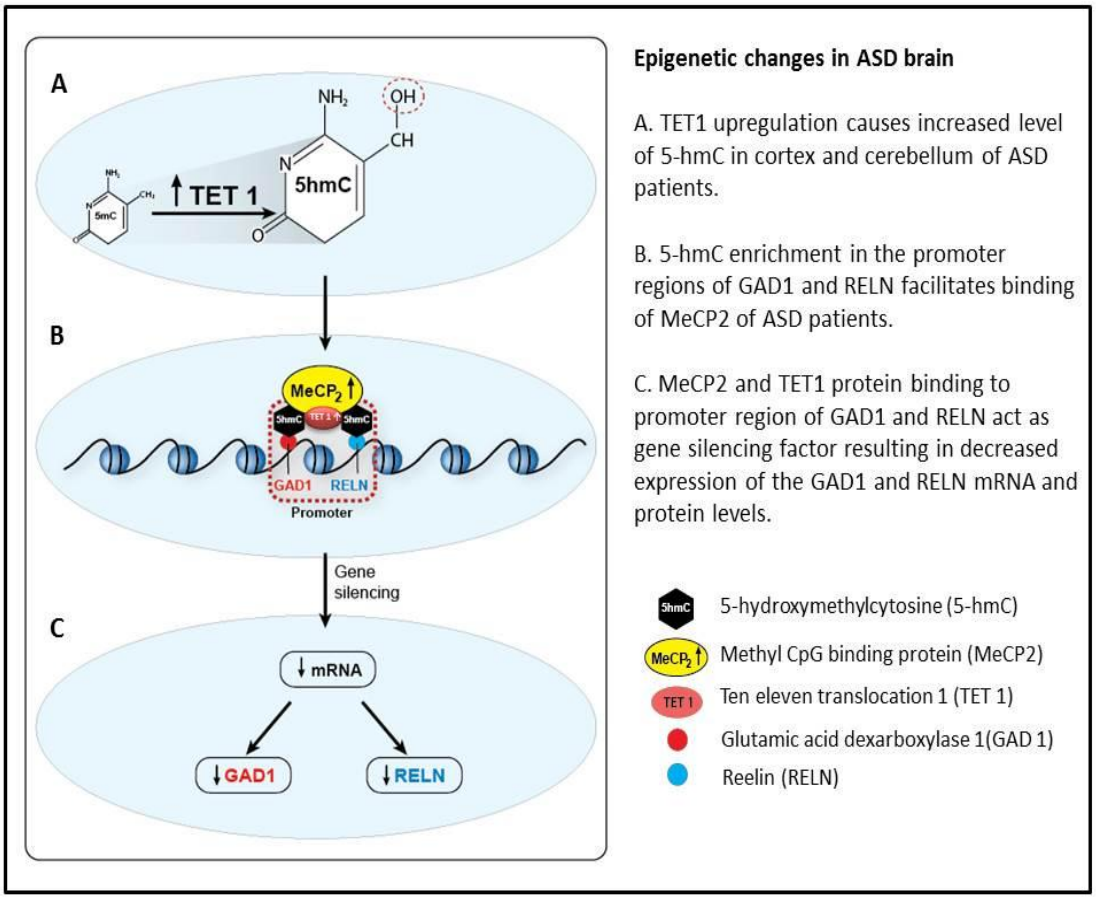

Fig. 7 Epigenetic changes in ASD brain**.**

As indicated, the slide depicts mechanisms described in the text regarding the down-regulation of gene expression in post-mortem brain of ASD subjects. We have depicted a description of TET1-mediated hydroxymethylation and binding of MeCP2 to the hydroxymethylated site proximal to the GAD1 and RELN promoters.

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Appendix

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