Methylation of the Brain Derived Neurotrophic Factor Gene in Alcohol Use Disorder

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Abstract

Background: Alcohol use disorder (AUD) is a significant health problem in the U.S. Specifically, AUD is associated with memory problems and other cognitive defects. DNA methylation of CpG sites and other epigenetic factors have been found to play an important role in the development of Alcohol Use Disorders (AUD). Because of this, it was hypothesized that individuals with AUD would show differential methylation in neurotrophic factors, specifically, the BDNF gene, as compared to controls.

Methods: Precuneus brain tissue from 49 Alcohol Use Disorder cases and 47 controls were obtained from the New South Wales and Victorian Tissue Resource Centre. DNA from each sample was extracted, and methylation levels were analyzed using Infinium HumanMethylation450 BeadChip. 450,000 CpG sites were interrogated between AUD cases and controls; however, only 97 CpG sites in the Brain-derived neurotrophic factor (BDNF) gene were analyzed.

Results: Of the 97 CpG sites in the BDNF gene, only 4 displayed significant methylation differences between AUD cases and controls with the employment of a Bonferroni p-value correction. To obtain more accurate results of overall methylation, CpG sites were grouped according to gene loci (Promoter, 5'UTR, first exon, gene body, and 3'UTR). Only the gene body of the BDNF gene approached significance ($p=0.093$) for a difference in methylation between AUD cases and controls with AUD cases being slightly hypomethylated.

Conclusion: No BDNF gene regions were found to be differentially methylated in the AUD group. This could be due to the small and homogenous sample. However, one CpG region in the gene body approached statistical significance. Overall, gene body methylation is important for splicing kinetics. This may lead to different BDNF mRNA transcripts being produced between AUD cases and controls which can affect the half-life of BDNF mRNA. Future research is needed to examine this possibility in a larger study.

KEYWORDS: Alcohol Use Disorder, Epigenetics, BDNF, Methylation, Addiction

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Introduction

Alcohol use disorder (AUD) affects over 17 million adults over the age of 18 in the United States. It can also extend to adolescents in which 855,000 individuals aged 12-17 were affected by the disorder in 2012 (National Institute of Alcohol Abuse and Alcoholism, 2012). Alcohol is known to affect the function of several organs including the brain and nervous system which ultimately leads to cognitive dysfunction. Short-term alcohol use can cause slurred speech, breathing difficulties, impaired judgment, and memory lapses. However, long-term alcohol use can cause permanent brain damage that results in impairment of executive functions and working memory (Finn et al., 1999). AUD is currently diagnosed through specific criteria issued by the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) which looks at drinking habits and the effects of alcohol on social interactions. It should be noted that in the new edition of the DSM, an additional criterion for alcohol craving was added. With this, fMRI data also show differential activation of the thalamus, precuneus, and middle temporal gyrus between AUD and non-AUD phenotypes when presented with alcohol related stimuli (Schacht et al., 2013). This suggests activations in areas such as the precuneus are important in the manifestation of AUD.

Pathologically, AUD is complex but can manifest through several factors such as socioeconomic status, peer influence, and social perceptions. More recent research suggests an added epigenetic factor (Weng et al., 2014). Epigenetics as a whole can be described as the alteration in gene expression while the sequence of DNA bases remains unchanged. This alteration in gene expression is often associated with a change in chromatin structure or DNA-protein interactions via chemical modifications. These chemical modifications often include but

are not limited to acetylation, ubiquitination, and phosphorylation of histone proteins that aid in condensing DNA. In particular, this study looks at direct methylation of cytosine residues on CpG islands. The term CpG comes from an upstream cytosine residue adjacent to a guanosine residue in a given DNA sequence. The cytosine residue of this specific dinucleotide sequence can be methylated by DNA methyl-transferase to 5-methylcytosine. This chemical modification has been found to alter transcription factor association to DNA and can limit access of transcription factors to gene regulatory regions thus altering downstream gene expression. Promoter regions and other regulatory elements are especially important to observe methylation patterns as they are directly associated with transcriptional machinery (Deaton, Bird 2011). Therefore, a change in methylation pattern in one of these regions may significantly alter gene expression.

It is important to note that epigenetics is largely based on environmental cues. It allows the genome to turn on or off certain genes in order to adapt to current surroundings and needs. Thus, research shows that chronic alcohol consumption can induce differential methylation in AUD vs. non-AUD phenotypes (Choi et al., 1999). Furthermore, heavy alcohol consumption and general alcohol use disorders are associated with an increase in genomic methylation (hypermethylation) particularly in a gene promoter area and other regulatory regions (Bönsch et al. 2006). One study looked at 384 CpG sites in promoter regions of 82 candidate genes and found that there was a significant hypermethylation between the AUD cases and controls (Zhang et al., 2013). Other studies have demonstrated that hypermethylation of a promoter region leads to a decrease in gene expression (Ouku et al., 2009). This evidence seems to point towards a decrease in gene expression for AUD cases. Interestingly, gene expression levels of neurotrophic factors have been found to be lower in AUD cases vs controls (Han et al., 2015).

As a whole, neurotrophic factors aid in the survival of developing neurons and maintain mature neurons. In particular, brain-derived neurotrophic factor (BDNF) inflect dendritic complexity and spine density. Therefore, levels of BDNF can correlate to neuroadaptability and cognitive functioning. From a mechanistic standpoint, BDNF is released from postsynaptic vesicle and binds to tyrosine kinase receptors (Trk) on the postsynaptic membrane during neuronal depolarization. This causes an auto-phosphorylation of the Trk which in turn activates the MAP kinase pathway to alter the gene expression of the targeted neuron (Yoshii et al., 2010). These changes in gene expression are primarily implicated in the proliferation of new neurons and creation of new synaptic connections. Auto-phosphorylation of the Trk receptor via BDNF also activates CaMKII, a kinase involved in long-term potentiation which strengthens neuronal pathways (Yoshii et al., 2010). With this in mind, BDNF expression is implicated in neurological diseases, disorders, as well as cognitive disorders. For example, previous research has been done to show that changes in activities and levels of BDNF are associated with cognitive dysfunctions such as memory formation and storage (Mattson 2004). Because AUD is associated with some degree of cognitive defects, the role of BDNF in AUD was explored deeper at an epigenetic level. This was done to analyze one aspect of the environmental effects on the pathogenesis of AUD.

Overall, the aim of this study is to observe the methylation pattern of the BDNF gene in AUD cases vs. non-AUD controls. It is hypothesized that because AUD is associated with cognitive dysfunction that the BDNF gene will be differentially methylated when compared to controls. This is ultimately because BDNF is associated with cognitive function and neuroadaptability. Differences in the cognitive function of AUD could be explained, but may not be limited to a differential methylation of the BDNF gene. It is important to note that

aberrant methylation patterns are cell specific. It is imperative that areas of the brain which are affected by a certain disorder be studied directly. This puts a constraint on the current study in that brain tissue can only be collected post-mortem.

Methods and Materials

Samples

Samples of frozen precuneus brain tissue samples from the 49 cases that met a DSM-IV diagnosis of alcohol dependence and 47 controls were obtained from the New South Wales and Victorian Tissue Resource Centre. The precuneus was specifically targeted because fMRI analysis shows differential activation between AUD cases and controls in regions such as the precuneus during presentation of alcohol-related cues (Schacht et al., 2013). Each AUD case was matched to a control based on age, post mortem interval (PMI), and gender (table 1). It should be noted that of the tissue samples, 97.9 % of the AUD and 87.2% of the control group were Caucasian. Alcohol dependence was confirmed by review of hospital medical records, physician interviews, and questionnaires to next-of-kin. Reports of alcohol dependence from pathology, radiology and neuropsychology were also taken into account. Cause of death was determined by medical records and given to us along with the samples by the New South Wales and Victorian Tissue Resource Centre (table 2).

DNA Isolation and methylation analysis

Precuneus samples were stored initially at -80^0C. Precuneus tissue was treated with Qiagen®'s Puregene DNA prep kits in order to isolate DNA (Qiagen®, Venlo, Limburg). DNA yields were determined via PicoGreen® and fluorimetry (Qubit®, Life Technologies). The Illumina® Infinium® Assay Platform along with the Infinium HumanMethylation450 BeadChip

was used for whole genome methylation interrogation. The array interrogates 480,000 CpG sites which covers 99% of Reference Sequence genes. The array covers the promoter, 5'UTR, first exon, gene body, and 3'UTRof each gene. Zymo EZ DNA Methylation Kit was used to treat 600 ng of genomic DNA with sodium bisulfite. Sodium bisulfite converts unmethylated cytosines to uracil while methylated cytosines are not converted. The DNA was then purified and quantified by measuring absorbance at 260 nm in preparation for whole genome amplification. The purified DNA was then fragmented and precipitated using ethanol. Hybridization buffer was used to resuspend the DNA, which was then applied to the bead chip array. The chip was allowed to incubate overnight. Finally, the arrays were then washed to eliminate un-hybridized and non-specifically hybridized DNA. Next, the samples underwent single base extension and staining followed by more washing. The arrays were allowed to dry and then scanned using the Illumina iScan system.

Methylation was analyzed via two probe types. Type I probes incorporated florescent labels for detection at allele-specific single base extensions. Type II probes employed green dye colors to detect methylated (M) and red dye colors to detect unmethylated (U) signals using one probe per CpG locus. Illumina's GenomeStudio software was used to quantify methylation signals and annotate each site (e.g. transcription start site proximity, gene name, and presence of a single nucleotide polymorphism (SNP)). Summaries of the probe interrogations yield average signals for methylated (M) and unmethylated (U) alleles at each CpG site, which are used to compute a β-value, or ratio of overall methylation:

$$\beta = \text{Max}(M,0)/(M+U+100)$$

For example, a β-value of 0 would indicate an unmethylated CpG site while a β-value of 1 indicates a fully methylated site. The β-values of AUD cases and controls were then analyzed using SPSS statistics and univariate ANCOVA tests to co-vary out for age relate methylation changes.

Results

The Infinium HumanMethylation450 BeadChip contained 97 CpG sites in the BDNF gene. With 95% confidence, 4 out of the 97 CpG sites in the BDNF gene were found to display a statistical difference in methylation with a Bonferroni correction (table 3). CpG sites that approached significance ($0.05 < p < 0.1$) were also reported.

Methylation sites that are spread out over the gene do not seem to hold much statistical significance, so each CpG was grouped based on gene loci in the BDNF gene. These locations included CpG sites in the promoter, 5'UTR, first exon, gene body, and 3'UTR. β-values of each group were averaged to provide the overall methylation of a given gene location (table 4). The methylation of these gene locations were then compared via ANCOVA analysis with a Bonferroni p-value correction. Results show that when averaged by location, there was not a significant difference in methylation between AUD cases and controls, however, the gene body approached significance and displayed hypomethylation for AUD cases (Figures 1-5).

Discussion

Overall, the analysis does not provide evidence of a significant change in methylation between the AUD cases and controls. This could be due to the limited sample size (n=97) which was primarily comprised of Caucasian brains. If the sample size were increased and taken from

a more diverse population, perhaps it would be possible to see methylation differences indicative of a true population.

It is important to note that the change in methylation between AUD cases and controls approached significance in the gene body ($p=0.093$). If the study was extended, perhaps a significant value could be obtained for the difference in methylation between the AUD cases and controls. Due to this, it is important to speculate and discuss a significant gene body methylation pattern.

Methylation of CpG sites in the gene body has been shown to have important applications for mRNA splicing (Jones 2012). During transcription, RNA polymerase reads the template stand of DNA in order to build an RNA transcript. When RNA polymerase encounters a methylated CpG, the rate of transcription slows. This allows for spliceosomes to interact with the nascent RNA strand for a slightly extended period of time which may result in alternative splicing. Furthermore, alternative splicing produces different transcripts which may affect the stability of the mRNA or BDNF protein. Because of this, BDNF could be produced at different levels between AUD cases and controls depending on if splicing effects the cytosolic stability of the mRNA. Due to this, if splicing difference between AUD cases vs controls exist, then it would be important to sequence mRNA or to create a cDNA library for AUD cases vs controls. This could be used to compare mRNA transcript differences of not only BDNF, but other genes that may be differentially activated and spliced between the two conditions.

Furthermore, future research should analyze methylation of the BDNF gene in other areas of the brain. For example, AUD is associated with cognitive and memory defects. This points towards functional differences in the hippocampus and temporal lobes. Since methylation is tissue specific, these areas may exhibit different methylation patterns than those shown in the

precuneus (Davies et al., 2012). This would provide a greater insight as to if BDNF is differentially methylated throughout the brain and its implication in AUD.

Another aspect of the study that was not commented on was the presence of single nucleotide polymorphisms (SNPs) within the BDNF gene of AUD cases and controls. It is possible that a SNP could position a cytosine upstream from a guanosine or a guanosine downstream from a cytosine to create a CpG site. The reverse is also possible in that a SNP could change a cytosine or guanosine to eliminate a CpG site. This would create or destroy a CpG site that is not seen other AUD cases and controls to provide a slightly different methylation pattern which may influence gene expression. Because these SNPs may be present in AUD cases and controls, future research should examine these locations to see if they are also differentially methylated or to see if AUD cases tend to have common SNPs when compared to controls.

As a whole, the analysis of the BDNF gene in AUD cases vs controls provides some insight in to the epigenetic and environmental effects of alcohol use disorder. Overall, the nature of studying post-mortem brains limits available information. In the case of this study, the diagnosis of AUD had to be determined through a series of interviews and questionnaires. Although these sources are assumed to be credible, it is not a first-hand account of drinking habits. Finally, because brain tissue can only be collected post-mortem perhaps further research can establish a non-invasive procedure that correlates to the methylation patterns found in the brain. This would provide a more holistic approach towards CpG methylation epigenetics that can be studied in a living organism as its environment changes.

References

Bönsch, D., Lenz, B., Fiszer, R., Frieling, H., Kornhuber, J., & Bleich, S. (2006). Lowered DNA methyltransferase (DNMT-3b) mRNA expression is associated with genomic DNA hypermethylation in patients with chronic alcoholism. *Journal of Neural Transmission*, *113*(9), 1299–1304.

Choi, S., Stickel, F., Baik, H. W., Kim, Y., Seitz, H. K., & Mason, J. B. (1999). Biochemical and Molecular Action of Nutrients Chronic Alcohol Consumption Induces Genomic but Not p53-Specific DNA Hypomethylation in Rat Colon 1 , 2 , 3, (May), 1945–1950.

Deaton, A., & Bird, A. (2011). CpG islands and the regulation of transcription. *Genes & Development*, *25*(10), 1010–1022.

Davies, M. N., Volta, M., Pidsley, R., Lunnon, K., Dixit, a, Lovestone, S., … Mill, J. (2012). Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. *Genome Biology*, *13*(6), R43.

Fuchikami, M., Morinobu, S., Segawa, M., Okamoto, Y., Yamawaki, S., Ozaki, N., … Terao, T. (2011). DNA methylation profiles of the brain-derived neurotrophic factor (BDNF) gene as a potent diagnostic biomarker in major depression. *PLoS ONE*, *6*(8), 4–10.

Finn, P. R., Justus, A., Mazas, C., & Steinmetz, J. E. (1999). Working memory, executive processes and the effects of alcohol on Go/No- Go learning: Testing a model of behavioral regulation and impulsivity. *Psychopharmacology*, *146*(4), 465–472.

Han, C., Bae, H., Won, S.-D., Roh, S., & Kim, D.-J. (2015). The relationship between brain-derived neurotrophic factor and cognitive functions in alcohol-dependent patients: a preliminary study. *Annals of General Psychiatry*, *14*(1), 30.

Illumina, GenomeStudio Support, Questions & Answers, 2015. Available from: <https://support.illumina.com/array/array_software/genomestudio/questions.html> [2 February 2015].

Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics*, *33 Suppl*(march), 245–254.

Jones, P. a. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews. Genetics*, *13*(7), 484–92.

Kang, H. J., Kim, J. M., Lee, J. Y., Kim, S. Y., Bae, K. Y., Kim, S. W., … Yoon, J. S. (2013). BDNF promoter methylation and suicidal behavior in depressive patients. *Journal of Affective Disorders*, *151*(2), 679–685.

Kim, J.-M., Stewart, R., Park, M.-S., Kang, H.-J., Kim, S.-W., Shin, I.-S., … Yoon, J.-S. (2012). Associations of BDNF genotype and promoter methylation with acute and long-term stroke outcomes in an East Asian cohort. *PloS One*, *7*(12), e51280.

Kushner, M. G., Abrams, K., & Borchardt, C. (2000). The relationship between anxiety disorders and alcohol use disorders: A review of major perspectives and findings. *Clinical Psychology Review*, *20*(2), 149–171.

Lubin, F. D., Roth, T. L., & Sweatt, J. D. (2008). Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. *The Journal of Neuroscience*, *28*(42), 10576–10586.

Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., … Sun, Y. E. (2003). DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science*, *302*(5646), 890–893.

Martinowich, K., Manji, H., & Lu, B. (2007). New insights into BDNF function in depression and anxiety. *Nature Neuroscience*, *10*(9), 1089–1093.

Mattson, M. P., Maudsley, S., & Martin, B. (2004). BDNF and 5-HT: A dynamic duo in age-related neuronal plasticity and neurodegenerative disorders. *Trends in Neurosciences*, *27*(10), 589–594. http://doi.org/10.1016/j.tins.2004.08.001

Ouko, L. a., Shantikumar, K., Knezovich, J., Haycock, P., Schnugh, D. J., & Ramsay, M. (2009). Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes - Implications for fetal alcohol spectrum disorders. *Alcoholism: Clinical and Experimental Research*, *33*(9), 1615–1627.

Roth, T. L., & Sweatt, J. D. (2011). Epigenetic marking of the BDNF gene by early-life adverse experiences. *Hormones and Behavior*, *59*(3), 315–320.

Ruggeri, B., Nymberg, C., Vuoksimaa, E., Lourdusamy, A., Wong, C. P., Carvalho, F. M., … Schumann, G. (2015). Association of Protein Phosphatase *PPM1G* With Alcohol Use

Disorder and Brain Activity During Behavioral Control in a Genome-Wide Methylation Analysis. *American Journal of Psychiatry*, (June), appi.ajp.2014.1.

Schacht, J. P., Anton, R. F., & Myrick, H. (2013). Functional neurimaging studies of alcohol cue reactivity: A auantitative meta-analysis and systematic review. *Addict Biol*, *18*(1), 121–133.

Shukla, S. D., Velazquez, J., French, S. W., Lu, S. C., Ticku, M. K., & Zakhari, S. (2008). Emerging role of epigenetics in the actions of alcohol. *Alcoholism: Clinical and Experimental Research*, *32*(9), 1525–1534.

Wang, D., Yan, L., Hu, Q., Sucheston, L. E., Higgins, M. J., Ambrosone, C. B., … Liu, S. (2012). IMA: An R package for high-throughput analysis of Illumina's 450K Infinium methylation data. *Bioinformatics*, *28*(5), 729–730.

Weng, J. T., Wu, L. S., Lee, C. S., Hsu, P. W., & Cheng, a T. (2015). Integrative epigenetic profiling analysis identifies DNA methylation changes associated with chronic alcohol consumption. *Comput Biol Med*, *64*, 299–306.

Yoshii, A., & Constantine-Paton, M. (2010). Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. *Developmental Neurobiology*, *70*(5), 304–322.

TABLES

Table 1: Age and sex breakdown of post –mortem AUD brains (n=49) and controls (n=47). Post mortem index refers to the time between death and tissue collection (hours).

	AUD Case (n=49)	Controls (n=47)	P
AGE	Mean: 47.76 Standard Deviation: 7.78	Mean: 48.43 Standard Deviation: 8.38	.6859
SEX	Female: n = 11 Male: n = 38	Female: n = 12 Male: n=35	0.9088
POST MORTEM INDEX	Mean: 35.58 Standard Deviation: 13.98	Mean: 28.78 Standard Deviation: 12.09	0.032

Table 2: Cause of Death breakdown for AUD cases (n=49) and controls (n=47)

	Cardiac	Respiratory	Toxicity	Vascular	Hepatic	Neurological	Trauma	Obesity	Blood Loss	Other
Controls	37	4	1	1	0	0	0	0	0	3
AUD Cases	14	9	12	0	5	1	1	1	3	3

Table 3: Average methylation value of significant ($p<0.05$) and approaching significant ($0.05<p<0.1$) CpG sites in the BDNF gene. Reported numbers are averaged β-values. P values reported after Bonferroni correction.

CpG Site		**Methylation AUD Cases (Average β-value)**	**Methylation Controls (Average β-value)**	**P-Value**
Cg02723558	Mean	0.479	0.507	0.020
	Std. Deviation	0.065	0.065	
Cg233330212	Mean	0.460	0.480	0.033
	Std. Deviation	0.047	0.046	
Cg06991510	Mean	0.0233	0.0192	0.056
	Std. Deviation	0.0111	0.0091	

Cg22830701	Mean	0.189	0.143	0.086
	Std. Deviation	0.142	0.107	
Cg00298481	Mean	0.0774	0.0735	0.080
	Std. Deviation	0.0110	0.0099	
Cg25328597	Mean	0.0520	0.0475	0.020
	Std. Deviation	0.0099	0.0098	
Cg15688670	Mean	0.0369	0.0419	0.090
	Std. Deviation	0.0119	0.0158	
Cg16257091	Mean	0.213	0.193	0.070
	Std. Deviation	0.063	0.404	
Cg17413943	Mean	0.156	0.169	0.083
	Std. Deviation	0.034	0.039	
Cg21010859	Mean	0.0376	0.0309	0.060
	Std. Deviation	0.0177	0.0163	
Cg18867480	Mean	0.135	0.127	0.045
	Std. Deviation	0.022	0.017	

Table 4: Average methylation of gene regions in the BDNF gene. Reported numbers are averaged β-values while P values are reported after Bonferroni correction.

BDNF Gene Location		**Methylation AUD Cases (Average β-value)**	**Methylation Controls (Average β-value)**	**P-Value**
Promoter	Mean	0.0761	0.0718	0.187
	Std. Deviation	0.0184	0.0110	
5'UTR	Mean	0.190	0.190	0.957
	Std. Deviation	0.005	0.005	
First Exon	Mean	0.334	0.332	0.180
	Std. Deviation	0.009	0.007	
Gene Body	Mean	0.312	0.314	0.093
	Std. Deviation	0.006	0.006	
3'UTR	Mean	0.823	0.826	0.688
	Std. Deviation	0.042	0.035	

FIGURES

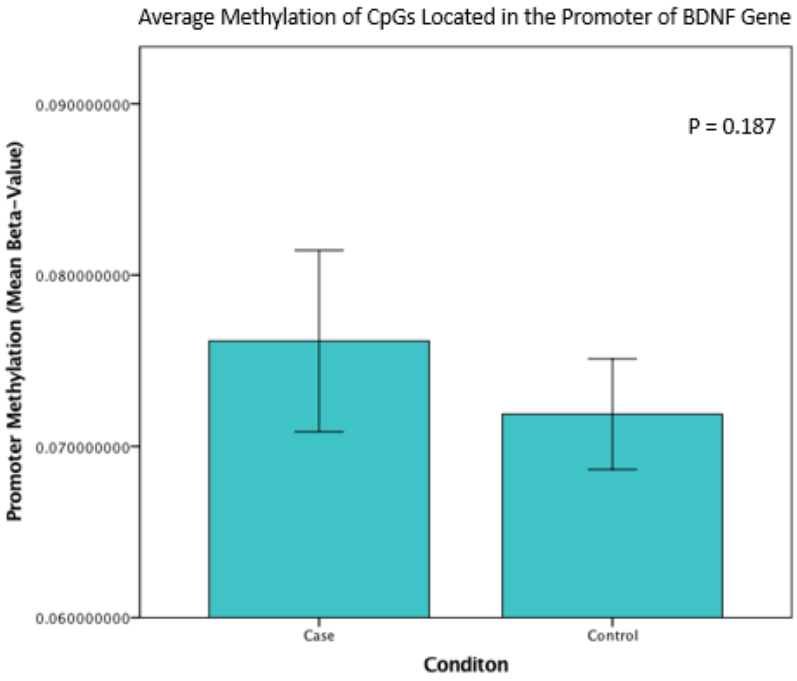

Figure 1: Average methylation of promoter region of the BDNF gene in AUD cases vs controls (p=0.187).

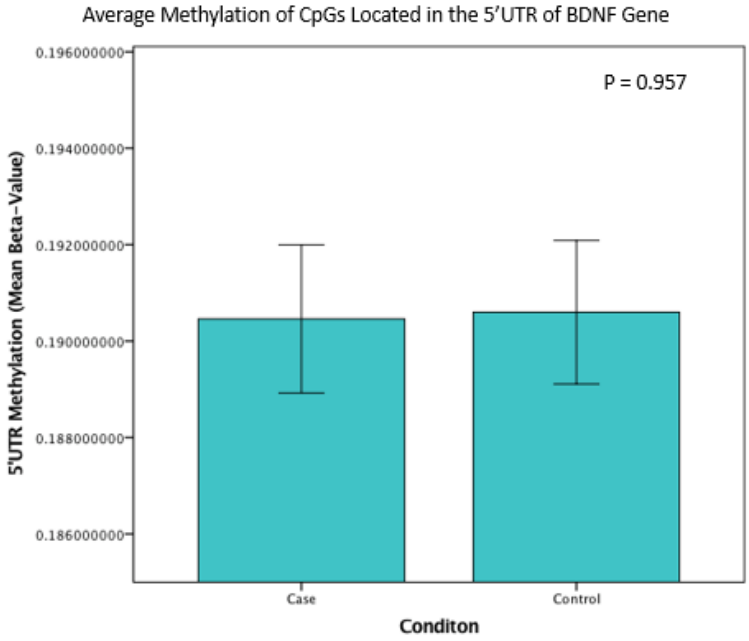

Figure 2: Average methylation of 5'UTR region of the BDNF gene in AUD cases vs controls (p=0.957).

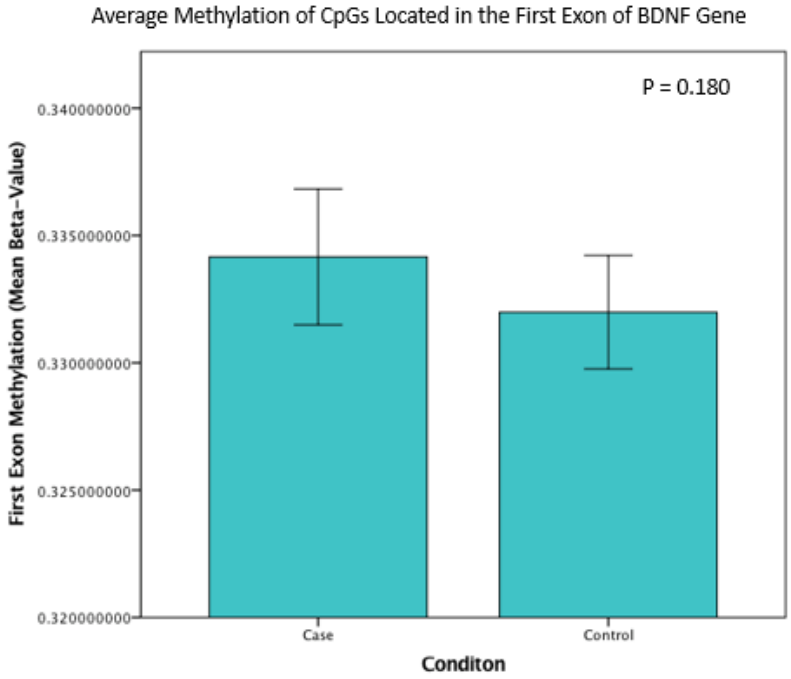

Figure 3: Average methylation of First Exon of the BDNF gene in AUD cases vs controls (p=0.180).

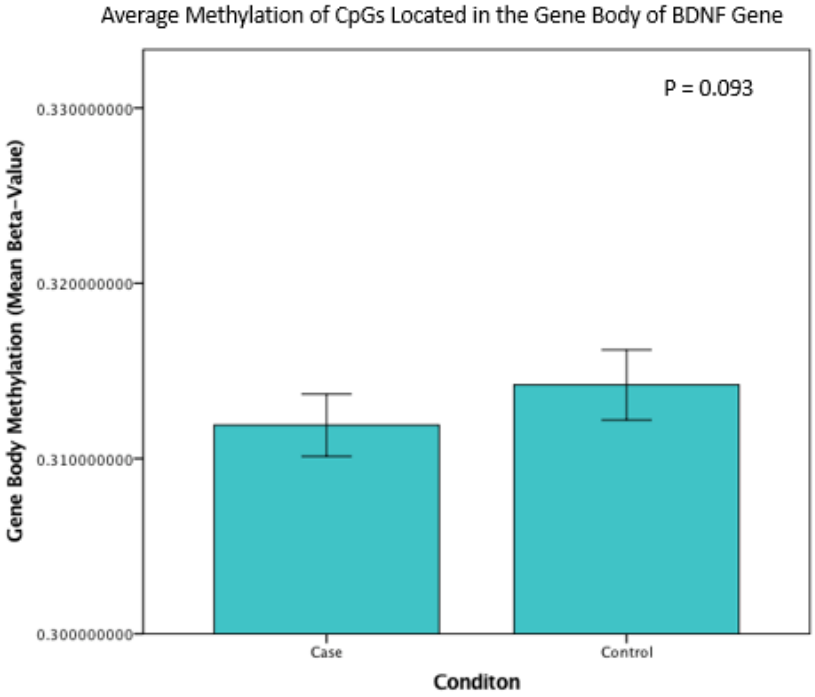

Figure 4: Average methylation of gene body of the BDNF gene in AUD cases vs controls (p=0.093)

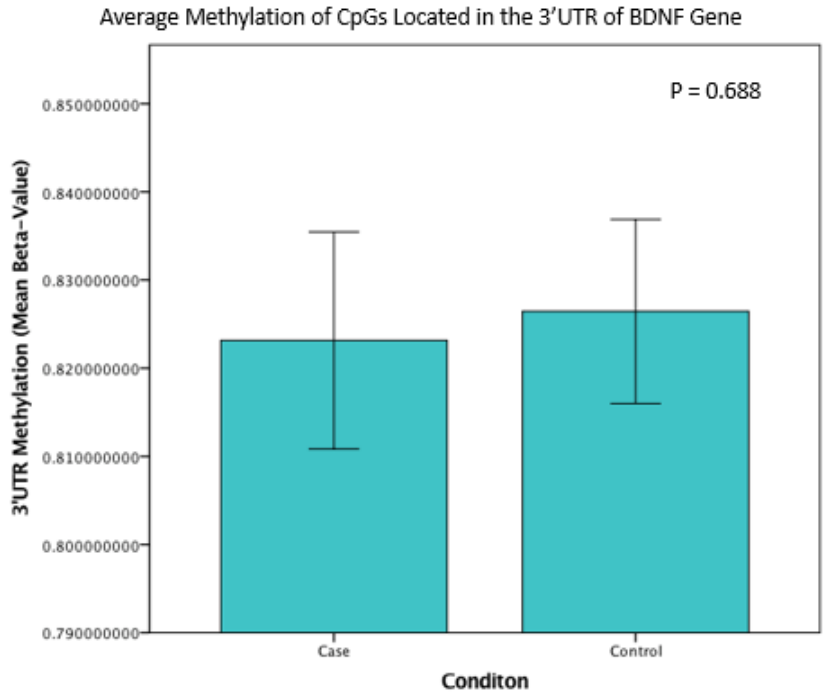

Figure 5: Average methylation of 3'UTR of the BDNF gene in AUD cases vs controls (p=0.688).