DNA methylation of Vesicular Glutamate Transporters in the mesocorticocolimbic brain following early life stress and ethanol exposure in adulthood

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Abstract

DNA methylation and gene expression can be altered by early life stress (ELS) and/or ethanol consumption. The present study aimed to investigate whether DNA methylation of the Vesicular Glutamate Transporters (Vglut) 1–3 is related to previously observed Vglut1-3 transcriptional differences in the ventral tegmental area (VTA), nucleus accumbens (Acb), dorsal striatum (dStr) and medial prefrontal cortex (mPFC) of adult rats exposed to ELS, modelled by maternal separation, and voluntary ethanol consumption. Targeted next-generation bisulfite sequencing was performed to identify the methylation levels on 61 5’-cytosine-phosphate-guanosine-3’ sites (CpGs) in potential regulatory regions of Vglut1, 53 for Vglut2, and 51 for Vglut3. In the VTA, ELS in ethanol-drinking rats was associated with Vglut1-2 CpG-specific hypomethylation, whereas bidirectional Vglut2 methylation differences at single CpGs were associated with ELS alone. Exposure to both ELS and ethanol, in the Acb, was associated with lower promoter and higher intronic Vglut3 methylation; and in the dStr, with higher and lower methylation in 26% and 43% of the analyzed Vglut1 CpGs, respectively. In the mPFC, lower Vglut2 methylation was observed upon ELS- or ethanol-only. The present findings suggest Vglut1-3 CpG-specific methylation signatures of ELS and ethanol drinking, underlying previously reported Vglut1-3 transcriptional differences in the mesocorticolimbic brain.

Introduction

Adversity during early life has been linked to later psychopathology, including alcohol use disorder (AUD) 1-3. Notably, early life stress (ELS) can lead to epigenetic modifications, such as alterations in DNA methylation patterns, which can in turn affect gene expression 4. DNA methylation, catalyzed by DNA (cytosine-5)-methyltransferases (DNMTs), is a chemical modification, suggested to serve as a signature of early life experiences that can imprint on the developing brain, explaining why those early experiences have a long-lasting effect 1.

In rats, ELS can be modeled via maternal separation (MS) daily for 60 to 360 minutes, whereas MS for only 15 minutes (MS15) is more naturalistic, thus serving as control condition 5. MS360 has been associated with higher alcohol consumption 5, while MS15 leads to hypo-reactive hypothalamic-pituitary-adrenal (HPA) axis to stress 6 and has a protective role towards alcohol intake 5. The effect of MS is mediated via disturbances in mother-pup interactions and maternal care towards the offspring 5,6. Differences in maternal care in rats and humans have been associated with altered HPA-axis response to stress and negative mental health via differential methylation of the promoter of the glucocorticoid receptors 7,8.

Not only ELS, but also ethanol consumption per se, can affect DNA methylation patterns 9. Chronic ethanol exposure has been associated with global DNA hyper-methylation in various brain regions of rodents 9, and specifically associated with increased DNMT1 expression in the nucleus accumbens (Acb) of adult mice 10 and the Acb and medial prefronal cortex (mPFC) of post-dependent rats after weeks of
abstinence to ethanol. On the contrary, though measured in blood, a recent epigenome-wide association study (EWAS) in humans showed greater alcohol intake to be associated with lower global DNA methylation. Yet, the effects of both ELS and ethanol on DNA methylation in the young adult non-dependent brain are largely unknown. Indeed, to disentangle the biological underpinnings of the initial effects of ethanol use can contribute to shed light on the potential neural mechanisms leading to the development of addiction.

Changes in glutamatergic neurotransmission have been implicated in various phases of the addiction cycle, from initial and voluntary to chronic and compulsory alcohol use. This is not of surprise considering that glutamate is the primary excitatory neurotransmitter in the brain. Via its interaction with the mesocorticolimbic dopaminergic system, glutamatergic activity mediates alcohol-related reward, whereas a hyperglutamatergic state is central to the development of alcohol dependence. Key regions of the mesocorticolimbic system are the ventral tegmental area (VTA) which projects to the Acb and the mPFC. Altered glutamatergic transmission is also seen upon both acute and chronic stress in the PFC and midbrain dopamine neurons. Furthermore, ELS has been shown to result in increased excitatory glutamatergic neurotransmission in the paraventricular nucleus of the hypothalamus, and in disturbed homeostasis of glutamatergic synapses, as well as in aberrant reward processing and drug-seeking behaviors, likely mediated by disturbances in dopaminergic and glutamatergic neurotransmission in the PFC and the Acb.

To date, the best markers for the glutamatergic phenotype are the Vesicular Glutamate Transporters (VGLUTs) 1-3 (encoded by the solute carrier superfamily genes Slc17a7/Vglut1, Slc17a6/Vglut2 and Slc17a8/Vglut3, respectively), as every neuron that expresses a Vglut gene has the ability to release glutamate. That is because VGLUTs actively package glutamate into presynaptic vesicles in neurons through an electrochemical proton-dependent gradient. Lately, a number of studies has shown a role of VGLUTs in reward and addiction, including alcohol-related phenotypes in both humans and rodents. Altered DNA methylation patterns of the Vglut2 promoter in the murine hippocampus have been observed as a result of prenatal ethanol exposure. A recent EWAS study found an association between alcohol intake and two CpGs within the body of VGLUT1 and the promoter of VGLUT2 in humans. Yet, the interplay between both ELS and adult ethanol consumption on Vgluts DNA methylation patterns in the brain of young adults has never been studied.

In fact, the ELS x ethanol interplay was only recently investigated on Vgluts gene expression in young adult outbred Wistar rats. More specifically, previous analysis provided evidence of differential Vglut1-3 expression in the mesocorticolimbic system [including the VTA, Acb, dorsal striatum (dStr)], and mPFC of adult outbred Wistar rats who had been previously exposed, during the first three postnatal weeks, to ELS as compared to control, as well as to voluntary consumption of ethanol or water during adulthood. Altered expression of Dnmt1 and Mecp2 (methyl-CpG-binding protein 2), two key regulatory genes of DNA methylation and the transcription machinery, was also observed as a result of the interaction between
ELS and ethanol. The effect was following the same direction as on Vglut3 in the Acb, but opposite direction than Vglut1 in the dStr, thus suggesting a potential involvement of the epigenetic machinery in the observed differences of striatal Vgluts expression \(^{30}\). Alterations in DNA methylation of potential regulatory regions within the promoter or gene body of Vglut1-3 could be a plausible epigenetic mechanism behind the abovementioned differences in gene expression, and therefore were here studied. The present study sought to investigate the effect of ELS and adult ethanol consumption on Vglut1-3 DNA methylation levels in the mesocorticolimbic young adult rat brain of relevance to the transcriptional differences previously observed in the same animals. Furthermore, correlations between DNA methylation, and expression, blood corticosterone levels as well as ethanol intake were tested to assess whether they reflect any observed stress or ethanol effects. Finally, moderation effects of DNA methylation on mRNA expression levels were explored.

**Materials And Methods**

**Animal experiment**

The present study is based on the animal experiment presented in \(^{30}\) and illustrated in Figure S1. Briefly, adult male Wistar rats (n = 50) were exposed to MS (MS15: control or MS360: ELS) during the first 3 postnatal weeks (PNW), and to water-only [MS15W (n = 10); MS360W (n = 10)] or voluntary alcohol (20%) exposure [MS15E (n = 10); MS360E (n = 20)] during the dark cycle in adulthood (PNW 10-16). At the end of each session, the ethanol and water intake was quantified by weighing the bottles. The rats were sacrificed at PNW16, immediately after the end of the last two-hour drinking session, and the brain as well as trunk blood for corticosterone analysis were collected and stored at -80°C. The study was approved by the Uppsala Animal Ethical Committee (C32/11) and followed the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Communities Council Directive (86/609/EEC).

Blood corticosterone levels (ng ml\(^{-1}\)) were measured using the ImmuChem Double Antibody Corticosterone 125I RIA kit for rats and mice (MP Biomedicals, Orangeburg, NY, USA), as described in \(^{38}\) and are reported in Table S8. DNA/RNA was isolated from the rat VTA, Acb, mPFC and dStr, using AllPrep DNA/RNA/miRNA Universal Kit according to the manufacturer's protocol (Qiagen AB Sollentuna, Sweden), and quantified using a Nanodrop ND 1000 spectrometer. RNA was converted to cDNA and used to assess the expression of Slc17a7/Vglut1, Slc17a6/Vglut2, Slc17a8/Vglut3, Dnmt1 and MeCP2 relative to three housekeeping genes (Actb, Gapdh and Rpl19) by real time PCR, as described in \(^{30}\). For the present study, the DNA was used for DNA methylation analyses. To minimize potential noise and cost of downstream DNA methylation analyses, for each gene and brain region, samples were selected on the basis of within-group homogeneity in terms of direction of gene expression differences compared to the respective control group [e.g. if MS360E group had significantly higher expression compared to MS15E group, rats within MS360E were assessed individually (by calculating the log2fold difference relative to the mean MS15E expression) to check whether their expression levels were higher (same direction) or
lower (opposite direction) than the mean MS15E mRNA levels]. Thus, high within-group heterogeneity led to individual-sample DNA methylation analysis for *Vglut1* and *Vglut2* in the VTA (n = 10/group for MS15W, MS360W, MS15E; n = 18 in MS360E), but pooled-sample DNA methylation analyses for *Vglut1* in the dStr (n = 10/group for MS15W, MS360W, MS15E; n = 17 in MS360E), *Vglut3* in the Acb (n = 10/group for MS15W, MS360W, MS15E; n = 17 in MS360E) and *Vglut2* in the mPFC [n = 10 (MS15W), n = 8 (MS360W), n = 9 (MS15E) and n = 20 (MS360E)], for which homogeneous within-group expression patterns were observed.

**Targeted next generation bisulfite sequencing:**

Targeted next generation bisulfite sequencing (tNGBS) was performed to identify differentially methylated CpG sites in target CpG regions on 48 samples for *Vglut1* and *Vglut2* in the VTA, and 4 pooled samples (one for each experimental group) for *Vglut1* in the dStr, *Vglut3* in the Acb and *Vglut2* in the mPFC. All methylation analyses were performed by EpigenDx Inc (Hopkinton, MA, USA). A flowchart of the procedure is shown in Figure 1.

**In silico Assay Design Process**

Each regulatory element of *Vglut1-3* was carefully evaluated before beginning the process of assay design. Gene sequences +/- 5000 bp containing the array target of interest were acquired from Ensembl genome browser and annotated. All genomic sequences were converted to a bisulfite sequence using EpigenDx's Bisulfite Sequence Converter with all annotations being conserved. The target sequences were re-evaluated against UCSC genome browser for repeat sequences including LINE, SINE, LTR elements, and other DNA repeats. Sequences containing repetitive elements, low sequence complexity, high thymidine content and overall CpG density were excluded for *in silico* design process. A list of all the assays designed are in Table S1; assays that failed PCR optimization were excluded from analyses.

The 41 designed assays [14 for *Vglut1* (VTA, dStr), 13 for *Vglut2* (VTA, mPFC) and 14 for *Vglut3* (Acb)] were grouped by gene in order to ensure that each PCR contained assays related to only one gene. Next, the assays of each gene were regrouped based on GC %, amplicon size, and design score. A gradient PCR was run on each group of assays at several different annealing temperatures using stock bsDNA. The most successful annealing temperature was chosen to be sequenced from each group. EpigenDx's custom library preparation method was performed on the chosen test samples, then the test samples were templated using the Ion Chef™ system (Thermo Fisher, CA, USA) and sequenced using the Ion S5™ sequencer (Thermo Fisher, CA, USA). Read counts from this sequencing run was used to regroup all the assays into final multiplex PCR conditions. The final number of successful assays (>30 reads) was 33 (11 for *Vglut1*, 10 for *Vglut2* and 12 for *Vglut3*).

More specifically, for *Slc17a7/Vglut1* (Ensembl ID: ENSRNOG00000020650, Chromosome 1), 61 CpG sites were targeted in a total of 11 regions of interest (ROIs) within 5’-upstream, exon7, intron 8 and 9, exon 9 and 12, and between exon 12 and 3’-untranslated region (UTR) (Figure 2a). For *Slc17a6/Vglut2* (Ensembl ID: ENSRNOG00000016147, Chromosome 1), 53 CpG sites were targeted in a total of 10 ROIs
within 5'-upstream, 5'-UTR, between 5'-UTR and exon1, and intron 1, 2 and 3 (Figure 2b). For Slc17a8/Vglut3 (Ensembl ID: ENSRNOG0000007581, Chromosome 7), 51 CpG sites were targeted in a total of 12 ROIs within 5'-upstream, 5'-UTR, intron 1, 2, and 10 and exon 12 (Figure 2c).

**DNA Bisulfite Modification**

300ng of extracted DNA samples were bisulfite modified using Zymo EZ- 96 DNA Methylation™ Kit (Zymoresearch, CA, USA) as per manufacturer's protocol with minor modification. The bisulfite modified DNA samples were eluted using M-elution buffer in 46 µl.

**Multiplex PCR**

All bisulfite modified DNA samples were amplified using 4 separate multiplex or simplex PCRs as listed in Table S1. PCRs included 0.5 units of Qiagen HotStarTaq (Qiagen, MD, Catalogue number 203205), 0.2µM primers, and 2 µl of bisulfite-treated DNA in a 20 µl reaction. All PCR products were verified and quantified using the QIAxcel Advanced System (Qiagen, Germany). Prior to library preparation, PCR products from the same sample were pooled and purified using QIAquick PCR Purification Kit columns (Qiagen, MD, USA).

**Library Preparation and Sequencing**

Libraries were prepared using a custom Library Preparation method created by EpigenDx Inc (Hopkinton, MA, USA). Next, library molecules were purified using Agencourt AMPure XP beads (Beckman Coulter Inc., CA, USA) and quantified using the Qiagen QIAxcel Advanced System (Qiagen, Germany). Barcoded samples were then pooled in an equimolar fashion before template preparation and enrichment were performed on the Ion Chef™ system (Thermo Fisher Scientific Inc., MA, USA) using Ion 520™ & Ion 530™ ExT Chef reagents. Following this, enriched, template-positive library molecules were then sequenced on the Ion S5™ sequencer using an Ion 530™ sequencing chip (Thermo Fisher Scientific Inc., MA, USA).

**Data Analysis**

FASTQ files from the Ion Torrent S5 server were aligned to the local reference database using open-source Bismark Bisulfite Read Mapper with the Bowtie2 alignment algorithm (https://www.bioinformatics.babraham.ac.uk/projects/bismark/) 39. Methylation levels were calculated in Bismark by dividing the number of methylated reads by the total number of reads.

**Statistical Analysis**

The methylation levels of the successfully analyzed CpG sites were tested for normality using the Shapiro-Wilk test. The grand majority was not normally distributed. Thus, between-group differences were investigated with the non-parametric Mann-Whitney U test. The modified Levene's test, based on medians, was used to test for homogeneity of variances. The methylation levels of Vglut1-2 CpGs showed homogeneity of variances ($p > 0.05$), except for Vglut1 CpGs 165, and -124 ($p < 0.05$). Thus, the
data were analyzed using a two-way ANOVA model to test for main and interactive effects between ELS and ethanol.

Correlations between methylation levels and gene expression as well as with corticosterone levels (Table 1) and ethanol intake during the last drinking week (PNW15) before decapitation, and during the last drinking week (PNW16) (Table S10b) were assessed by the non-parametric Spearman co-efficient. PNW15 was chosen for representing better the individual drinking pattern without any extra potential stress effect due to people entering the room to terminate the experiment. Based on PNW15, three subgroups were defined with distinct ethanol intake levels (i.e. high: >1.5g/kg/2h; moderate: 1 – 1.5g/kg/2h and low: < 1g/kg/2h drinkers; Table S10a. Among those subgroups, correlations between methylation and gene expression as well as ethanol intake during PNW15 and PNW16, which is likely more affected by the last ethanol consumption before decapitation, were also assessed (Table S10c).

Moderated moderation analysis was performed to assess the three-way interaction between rearing [MS15 vs. MS360 (ELS)] and drinking (water vs. ethanol) and Vglut2 methylation on Vglut2 expression, using three-way ANOVA and SPSS PROCESS macro v2.16. The Johnson-Neyman technique was used to determine the region of significance in the distribution of methylation levels where the interaction between ELS and ethanol on Vgluts expression was significant.

Differences in methylation of each CpG site are reported as absolute percentages (%), and relative (%) differences within brackets. For methylation data of the pooled samples (Vglut1 in the dStr, Vglut3 in the Acb and Vglut2 in the mPFC), for which no statistics could be performed (Table S5-7), CpGs with >5% difference in absolute values of methylation levels between the groups are reported. Statistical analyses were performed using SPPS (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp).

Transcriptional factor binding sites (TFBS) analysis

Potential TFBS were assessed using ALGGGEN PROMO which predicts TFBSs using TRANSFAC database version 8 (http://alggen.lsi.upc.es/cgi-in/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and are depicted in Figures 3 – 6.

Results

CpG methylation patterns in Vglut1-3 targeted regions

CpGs in potentially regulatory regions of Vglut1-3 were analyzed using tNGBS. Methylation data was available for 61 CpG sites for Vglut1, 53 for Vglut2, and 51 for Vglut3 (Table S2-4). In the dStr, methylation analysis failed in MS360E group for 15 CpGs (25%). Gene, but not brain region-, specific CpG methylation patterns were observed. In the control group (MS15W), CpGs in the promoter region of Vglut1 were virtually unmethylated (median: 0- 8%) in both the VTA and dStr, except for 3 CpGs with high methylation (65-86%). CpGs in intronic regions were highly methylated (65-88%) as well as in exonic regions (62-95%) with few exceptions with moderate methylation (23-56%) (Table S5). CpGs in the
promoter region, 5'-UTR and exon 1 of *Vglut2* were also virtually unmethylated (0-11%), while the range in methylation was wider in intronic regions (0-46%) in both the VTA and mPFC (Table S7). *Vglut3* in the Acb had the opposite pattern; CpGs in the promoter region were highly methylated (75-97%) as well as in exon 12 (83-98%), but more moderately in 5'-UTR (31-71%), while the range in methylation was wider in intronic regions (39-97%) with an exception of CpG316 that had low methylation (8%) (Table S6).

**Vglut1-3 CpG methylation associations of ELS and ethanol consumption**

CpG-specific methylation differences were noticed between the groups previously found to differ in their *Vglut* expression levels. In the VTA (Figure 3), ELS in ethanol-drinking rats (MS360E vs MS15E) was associated with hypo-methylation of *Vglut1* exon 9 and 12 (3 CpGs). ELS in water-drinking rats had a bidirectional effect on *Vglut2* CpG methylation (5 CpGs), while interaction between ELS and ethanol (driven by differences in MS360E vs. MS15E or MS360W) was associated with hypo-methylation of *Vglut2* promoter and gene body (6 CpGs). *Vglut2* CpG methylation did not moderate the previously found interactive effect of ELS and ethanol on *Vglut2* expression. The combination of ELS and ethanol (in MS360E) had bidirectional effects on *Vglut1* methylation in the dStr (Figure 4), leading to hypermethylation (>5%) in a total of 20% of the analyzed CpGs, the majority within intron 9, while 26%, most of them in exon 12, were hypomethylated, compared to both MS360W and MS15E. When considering smaller differences (1-5%) in the 5'-upstream, MS360E had a total of 26% of all analyzed CpGs hypermethylated, while 43% were hypomethylated, compared to both MS360W and MS15E. In the Acb, both factors were associated with lower promoter, but higher intronic *Vglut3* CpG methylation (Figure 5). Lastly, ELS- or ethanol-only were largely associated with lower *Vglut2* CpG-specific methylation in the mPFC (Figure 6). High correlations ($r > \pm 0.7$) between CpG-methylation and gene expression or corticosterone levels were observed in all groups, but they were sparse and moderate ($r < \pm 0.6$) in the ethanol-drinking ELS-rats (Table 1, S9). Correlations between CpG-methylation and ethanol intake during PNW15 and PNW16 and their directions were similar for the vast majority of CpGs for MS15E, MS360E and MS360E low drinkers, but not for MS360E moderate and high drinkers (Table S10b, c). However, none of the abovementioned differences or correlations survived Bonferonni correction.

**Discussion**

The present explorative study shows nominally-significant associations between ELS, voluntary ethanol consumption, and CpG-specific *Vglut1-3* methylation in the VTA, Acb, dStr and mPFC of outbred adult male rats, as well as correlations between *Vglut1-2* methylation and expression, blood corticosterone level and ethanol consumption in the VTA. *Vgluts* methylation varied in a gene- or brain region-dependent way, in the groups previously found to differ in their *Vglut* mRNA expression levels, whereas there was not a pattern of association between these effects and the gene targeted regulatory region (i.e. promoter or gene body).

**Vglut1**: In line with previous findings of higher *Vglut1* expression in the VTA of MS360E rats compared to MS15E, we observed hypomethylation of CpG149 (exon 9) and of CpG197 and 198 (exon 12). A
plausible mechanism could involve ELS- and ethanol-induced changes in transcriptional factor (TF) binding. TFs control the levels of gene expression \(^8\), while methylation in non-CpG islands, and in vicinity (±100bp) to the TFBS, could block the TFs binding \(^43\). Herein, the TF C/EBP-delta was predicted to bind to CpG198. It has been shown that C/EBP-delta is activated by glucocorticoids \(^44\), hormones (mainly corticosterone in rats) that mediate the physiological response to stress via activation of the HPA-axis \(^45\). Previous analysis showed that MS315E rats had higher corticosterone levels than MS360E, likely implying a stress-relieving effect of ethanol consumption in MS360E \(^38\). Interestingly, the higher corticosterone levels in MS15E group were positively correlated with methylation of CpG197, which is in close vicinity (~20bp) to CpG198. Higher methylation of CpG197 in MS15E, potentially due to high corticosterone levels, could lead to decreased binding of otherwise activated C/EBP-delta, and in turn to lower \(Vglut1\) expression in MS15E compared to MS360E rats. On the other hand, high ethanol intake (in MS360E) during the last week before decapitation was negatively correlated with CpG198 methylation (Table S10). Chronic ethanol consumption has also been related with increased C/EBP-delta in the brain \(^46\). Lower methylation of CpG198, likely due to ethanol drinking in MS360E rats, could potentially lead to increased C/EBP-delta binding contributing to higher \(Vglut1\) expression.

In the dStr, higher \(Vglut1\) expression was previously shown upon ELS in ethanol-drinking rats (MS360E vs. MS15E) and ethanol in ELS-rats (MS360E vs. MS360W) \(^30\). For the latter (MS360E vs. MS360W), the higher \(Vglut1\) expression was also accompanied by lower \(Dnmt1\) expression \(^30\). DNMT1 binds to hemi-methylated sites and maintains DNA methylation signatures during DNA replication \(^47\). Thus, lower \(Dnmt1\) expression was hypothesized to contribute to lower methylation of specific \(Vglut1\) CpGs in the MS360E compared to MS360W. Indeed, MS360E had lower methylation of 30% of all analyzed CpGs mainly within 5’-upstream, intron 9 and exon 12, compared to MS360W. Compared to both MS360W and MS15E, MS360E rats had a total of 43% of all analyzed CpGs, most of them in exon 12 and 5’-upstream, hypomethylated, while 26%, the majority in intron 9, were hypermethylated. Within the 5’-upstream, smaller differences (1 – 5%) were in fact observed for the majority of the analyzed CpGs. In the same region, small-in-magnitude hypermethylation of CpG −42 was previously observed in the promoter region of Monoamine Oxidase A (\(Maoa\)) of MS360E rats compared to MS15E as well as MS360W \(^48\), along with lower \(Maoa\) expression of MS360E rats compared to the same groups \(^38\). MAOA metabolizes monoamine neurotransmitters such as dopamine and serotonin and has been constantly implicated in behavioral regulation, stress- and alcohol-related phenotypes \(^49\). Drug-related epigenetic changes in \(Vglut1\) have been also demonstrated; with cocaine treatment leading to downregulation of \(Vglut1\) expression as well as hypermethylation of the promoter region of the gene in the Acb of mice \(^50\), while in a recent EWAS study, one CpG within the gene body of \(VGLUT1\) was associated with alcohol intake in humans \(^12\).

\(Vglut2\): Previous analysis showed an interactive ELS x ethanol effect on \(Vglut2\) expression \(^30\); driven by ELS rats (MS360W) displaying lower \(Vglut2\) expression in the VTA, while ethanol had a specular effect leading to higher \(Vglut2\) expression in ELS rats (MS360E), but lower in no-ELS rats (MS15E). Moderation analysis did not provide evidence that CpG methylation moderates the previously observed effect of ELS
x ethanol on \textit{Vglut2} expression in the VTA \cite{30}. However, and in line with this interactive effect, 6 CpGs within \textit{Vglut2} promoter and gene body were hypomethylated in ethanol-drinking ELS-rats (MS360E) compared to MS15E or MS360W. Our findings of ethanol effect in ELS-rats corroborate well with the study of Zhang \textit{et al.} that showed adult \textit{Vglut2} up-regulation due to prenatal ethanol exposure, further correlated with decreased DNA methylation of the promoter region in the murine hippocampus of males \cite{37}. However, herein, no-ELS rats showed the opposite pattern, i.e. hypermethylation if drinking ethanol (MS15E) compared to control (MS15W), but this hypermethylation was observed within introns 1-3, and not the promoter region. Lastly, the lower \textit{Vglut2} expression in MS360W rats compared to MS15W was accompanied by hypermethylation of CpG-36 (5'-upstream), 59 (intron 2), and 112 (intron 3), but hypomethylation of CpG2 (exon 1), and 110 (intron 3). Interestingly, CpG112 was positively correlated with corticosterone levels in MS360W group, further supporting an ELS-induced effect on its methylation. In the same animals, lower expression of \textit{Dnmt1} was previously observed \cite{30}, which may have contributed to the hypomethylation of CpG2, and 110, although methylation of these sites did not correlate with \textit{Dnmt1} expression (Table S9). On the contrary it is unlikely that DNMT1 has been implicated in the hypermethylation of CpG-36, 59 and 112 in MS360W. DNMT3a and 3b, with a role in \textit{de novo} methylation \cite{Moore2013}, might have contributed to the present hypermethylation instead, and should be investigated.

Furthermore, lower \textit{Vglut2} expression was found in the mPFC in ELS-only (MS360W) or ethanol-only (MS15E) compared to controls (MS15W) \cite{30}. ELS was associated with hypomethylation of CpG75 (intron 2) and CpG115-117 (intron 3), but hypermethylation of CpG59 (intron 2), similar to the ELS effect on the same CpG in the VTA. The TF USF-1 is predicted to bind to CpG59; higher methylation of this site could contribute to reduced binding of USF-1 and in turn to lower \textit{Vglut2} expression observed in both the mPFC and VTA of the same group (MS360W vs. MS15W). Ethanol resulted largely in lower CpG-specific methylation and especially within intron 3. This hypomethylation could potentially have contributed to the lower \textit{Vglut2} expression previously reported \cite{30}, in line with the notion that methylation in gene body is associated with higher gene expression \cite{43}. Ethanol also resulted in lower CpG-specific methylation in the promoter of the stress-related genes \textit{Pomc} (proopiomelanocortin), \textit{Avp} (arginine vasopressin) and \textit{Fkbp5} (FK506 Binding Protein 5) in the pituitary and hypothalamus of the same rats \cite{51}.

\textit{Vglut3}: In the Acb, lower \textit{Vglut3}, and \textit{Dnmt1} expression was previously found in ELS ethanol-exposed rats, whereas the opposite pattern was seen in the water-drinking counterparts \cite{30}. The lower \textit{Dnmt1} was hypothesized to contribute to lower methylation of specific \textit{Vglut3} CpGs in the MS360E group. Indeed, in the Acb of MS360E rats, \textit{Vglut3} methylation was lower in the majority of targeted CpGs within the 5'-upstream, but higher in most of analyzed CpGs within 5'-UTR, intron 1 and 2. Interestingly, CpG316 in intron 2 had more than 2-fold higher methylation in MS360E compared to MS15E. The TFs USF-2 and CREB were predicted to bind at CpG316. The CREB pathway has been implicated in addiction; especially in the Acb, chronic exposure to various drugs of abuse leads to activation of CREB \cite{52}, while upon chronic alcohol use, CREB has been suggested to modulate connectivity and synaptic plasticity \cite{9}. Herein, the effect of prolonged episodic ethanol consumption was not assessed on CREB itself, but its potential
binding to Vglut3 CpG316 could be blocked by higher methylation in MS360E, contributing to lower accumbal Vglut3 expression in that group.

The present exploratory study points to associations between differences in CpG-specific methylation in Vglut1-3 regulatory regions depending on ELS- as well as adult ethanol consumption. These DNA methylation differences could possibly serve as a mechanism behind the previously reported differences in Vgluts expression, via potential changes in binding of distinct transcriptional factors. Even though small-in-magnitude differences are ambiguous in regards to their biological significance, subtle differences (1 – 10%) in methylation of single CpG sites, such the ones found here, have a potential of functional relevance, not likely as on/off switch of gene expression, but rather in redistribution of the transcriptional landscape, affecting translational isoform production and the proteome. Correlations between Vgluts expression and methylation were present in all groups, but were sparse in the ethanol-drinking ELS-rats, suggesting that the molecular mechanisms regulating gene expression and DNA methylation are potentially different in the presence of two aversive environmental factors (ELS and ethanol) as compared to only one or none.

Moreover, in the VTA, CpG-specific methylation of both Vglut1 and 2 was correlated with ethanol intake levels in MS15E and MS360E, but also within the three MS360E subgroups during the last two drinking weeks. Few distinct correlations emerged or others were lost when assessing PNW16 compared to PNW15, especially within MS360E moderate and high drinkers, but not in low-drinking subgroup or when assessing MS360E as a whole. In fact, the vast majority of the correlations (or their direction) remained virtually the same between the two weeks in MS15E and MS360E, thus suggesting that the differences in methylation were not due to the acute effect of ethanol.

Overall, causality could not be assessed in the present study, thus it remains to be investigated whether these signatures precede or follow ELS- and/or ethanol-mediated effects on gene expression. Moreover, the potential confounding effect of single housing, which can be an extra social stressor in voluntary ethanol drinking paradigms, on DNA methylation patterns cannot be ruled out. Other epigenetic markers (e.g. histone modifications) that have been associated with ELS and/or ethanol-related phenotypes could have been implicated in the previously observed Vgluts expression differences, but they were not assessed herein. Nonetheless, DNA methylation is considered one of the principal interfaces between the genome and the environment. Lastly, the present findings should be interpreted with caution considering that bisulfite-treated DNA was analyzed, which although widely used, cannot distinguish between 5-methyl- and 5-hydroxymethylcytosine, limiting our understanding of each marker’s contribution and potential regulatory effect.

To our knowledge, this is the first study to assess the effect of ELS and subsequent voluntary adult ethanol drinking on Vglut1-3 DNA methylation patterns in the mesocorticolimbic system of outbred adult male rats, and calls for further investigation of the reported effects. Future functional studies on Vgluts as well as on downstream effects of ELS and ethanol on VGLUTs protein expression are guaranteed. Determining epigenetic signatures of key-neuronal markers such as VGLUTs, the best markers for the
glutamatergic phenotype, in key reward and stress-related brain regions as well as in different stages of ethanol consumption can shed light on the biological underpinnings of alcohol-related phenotypes and the AUD.

Declarations

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Conflict of Interest: LY is key shareholder of EpigenDx; all genetic analyses were performed in a blind manner. The other authors have no conflict of interest to declare.

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**Table**

Table 1 is available in the Supplementary Files.