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ARTICLE

Genotype-dependent epigenetic regulation of *DLGAP2* in alcohol use and dependence

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Abstract

Alcohol misuse is a major public health problem originating from genetic and environmental risk factors. Alterations in the brain epigenome may orchestrate changes in gene expression that lead to alcohol misuse and dependence. Through epigenome-wide association analysis of DNA methylation from human brain tissues, we identified a differentially methylated region, DMR-DLGAP2, associated with alcohol dependence. Methylation within DMR-DLGAP2 was found to be genotype-dependent, allele-specific and associated with reward processing in brain. Methylation at the DMR-DLGAP2 regulated expression of DLGAP2 in vitro, and *Dlgap2*-deficient mice showed reduced alcohol consumption compared with wild-type controls. These results suggest that *DLGAP2* may be an interface for genetic and epigenetic factors controlling alcohol use and dependence.

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Introduction

Alcohol drinking is a major public health problem with an enormous societal impact [1]. It has been considered as one of the major risk factors for many diseases, including cardiovascular diseases, cancers, liver cirrhosism, and injuries [1]. According to the World Health Organization, 5.9% of

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all deaths and 5.1% of the economic burden of diseases worldwide were attributable to alcohol consumption in 2012. Despite the rapidly increasing understanding of the magnitude and significance of this problem, very little is known about molecular mechanisms underlying its neuropathology.

Like many other chronic diseases, one fundamental question is how genetic and environmental factors interact to determine the risk of disease etiology and development [2]. Much research, including twin, family, and adoption studies, case–control studies, linkage studies [3–8] and genome-wide association studies (GWASs) [9–11], have been carried out to understand the contribution of genetic factors related to alcohol misuse and dependence. Animal models have also been used to explore the role of genetic factors on alcohol consumption [12–14]. However, these genetic studies still cannot explain the role of environmental factors on disease susceptibility. Considering that epigenetic modifications, such as DNA methylation, play an important role in regulating gene expression in response to environmental factors, epigenetic mechanisms have started to come in focus in alcohol drinking. Since chronic ethanol consumption could induce global hypomethylation of DNA through reducing S-adenosyl methionine levels and inhibiting methionine synthase [15], some studies have observed associations between disease status and methylation changes in peripheral blood [16–18]. Others explored epigenetic changes associated with alcohol consumption using more representative human brain tissues [19–21]. In this study, we integrated analyses of both genetic and epigenetic variants in postmortem human brain tissues as well as blood, together with human neuroimaging result and an animal model, to address the role of the *DLGAP2* gene in alcohol consumption and dependence.

Materials and methods

Cohorts

Demographic characteristics of the cohorts are shown in the Supplementary Table 1. Cohort 1 included 39 male Caucasian cases with alcohol dependence and 47 matched controls. Tissues were collected at the New South Wales Brain Tissue Resource (NSW BTRC), University of Sydney, Australia (https://sydney.edu.au/medicine/pathology/btrc/). All subjects were males of European descent. Chronic alcoholic individuals were the subjects that met the Diagnostic and Statistical Manual for Mental Disorders, 4th edition (DSM-IV) criteria for Alcohol Abuse or Alcohol Dependence and consumed 206 ± 20 g of ethanol per day on average for the majority of their adult lives [22]. Dl-PFC samples were dissected from superior frontal gyrus/Brodmann area 9, and NAc samples were dissected from the region of the caudate–putamen junction located inferior to the internal capsule and anterior to the anterior commissure. Informed written consent for autopsy was obtained from the next-of-kin and collection was approved by the Human Research Ethics Committees of the Sydney Local Health District (X15-0199) and the University of Sydney. The study was approved by the Swedish Central Ethical Review Board.

Cohort 2 comprised 631 participants (mean age = 18.98 ± 0.75 years) (Supplementary Table 1), drawn from the IMAGEN cohort, a European multi-center imaging-genetics study of adolescents recruited across eights sites in England, France, Germany, and Ireland [23]. Participants were included if psychometric and genetic data were available. The European School Survey Project on Alcohol and Drugs (ESPAD) was used to assess the frequency of drunkenness (On how many occasions in your whole lifetime have you been drunk from drinking alcoholic beverages?). Subjects provided a self-report score based on a scale from 0 to 6, (1 = 1–2 times; 3 = 6–9 times; 6 = 40+ times). We divided participants into low alcohol and high alcohol groups by referring to a previous IMAGEN study in adolescents, which classified binge drinkers as those with a minimum of three lifetime binge drinking episodes leading to drunkenness [24]. The frequency distribution of drunkenness from cohort 2 is shown in Supplementary Fig. 1. Thus, in this study, the low alcohol group includes those individuals with score 0 (meaning zero occasion of drunkenness) and the high alcohol group includes those individuals with score of 2 or more (meaning three or more occasions of lifetime drunkenness). For the dosage analysis, we used the questionnaire data in the ESPAD that assessed the alcohol consumption on a typical day (How many drinks containing alcohol do you have on a typical day when you are drinking?). Subjects provided a self-report coded by 1 (1 or 2 drinks), 2 (3 or 4 drinks), 3 (5 or 6 drinks), 4 (7 to 9 drinks), and 5 (10 or more drinks).

Illumina methylation assay

For cohort 1, DNA was purified from human brain tissues or sorted nuclei using the DNeasy Blood & Tissue kit (Qiagen) and bisulfite converted using EZ DNA methylation Gold kit (Zymo) according to manufacturer's instructions. DNA methylation was evaluated using Infinium HumanMethylation450 BeadChip assay (Illumina) by the SNP&SEQ Technology Platform at Science for Life Laboratory (Sweden).

For cohort 2, DNA was extracted at the Hannover Medical School from whole blood samples (10 mL)

collected at the age of 19 and preserved in BD Vacutainer EDTA tubes (Becton, Dickinson and Company) using the Genomic DNA from blood (Macherey-Nagel). DNA methylation was quantified using the Illumina Infinium MethylationEPIC BeadChip on an Illumina HiScan System (Illumina) following the manufacturer's standard protocol. Hybridization was performed at the Genotyping Laboratory of the Institute of Clinical Molecular Biology (IKMB) in two waves.

Methylation analyses

The methylation data were preprocessed using the stratified quantile normalization implemented in the Bioconductor minfi package [25]. The neuronal proportions of each samples were estimated based on their methylation levels using CETS 3.0.3 package [26]. To identify the DMRs associated with alcohol drinking, we used the bumphunter function in minfi package [25] with adjustment for potential confounders: age, tissue type, smoking status, post mortem intervals (hours) and the estimated neuronal proportions. Regions satisfying the following criteria were identified as alcohol drinking-associated DMRs: (1) a bootstrapped (1000 permutations) family-wise error rate (FWER) <0.05; (2) must contain at least two probes; and (3) the maximum distance between two probes is <300 bp. To identify the DMPs associated with alcohol drinking, we fit a linear regression model predicting methylation at each CpG sites as a function of drinking status, adjusted for age, tissue type, smoking status, post mortem intervals (h), and estimated neuronal proportions. After excluding probes on sex chromosomes or containing SNPs in the probe sequence, 456,513 probes remained for downstream analyses. The DMPs associated with alcohol drinking were corrected for multiple testing using a stringent Bonferroni-adjusted threshold of $0.05/(456{,}513\ \text{CpGs}) = 1.10 \times 10^{-7}$.

To test the associations between methylation and genotype in brain, all seven CpGs within DMR-DLGAP2 were subsequently tested for cis-association with all available SNPs on chromosome 8 (91,431 SNPs) using an additive minor-allele dosage model. Genotype-methylation associations were corrected for multiple testing using a stringent Bonferroni-adjusted threshold of $0.05/(7\ \text{CpGs} \times 91{,}431\ \text{SNPs}) = 7.81 \times 10^{-8}$.

To test the associations between methylation in blood and behavioral data, we fit a linear regression model predicting methylation at each CpG site as a function of frequency of drunkenness (the high alcohol group vs. the low alcohol group). Potential confounders, such as gender, smoking status and recruitment sites, were adjusted for in the analyses. If $\beta_{j,\ i}$ is denoted the beta value for the methylation level at CpG site j for sample i, then the full model can be indicated as follows:

$$\beta_{j,i} = \mu_{j,1} * Z_{drunkenness,i} + \mu_{j,2} * X_{age,i} + \mu_{j,3} * X_{smoking,i} + \mu_{j,4} * X_{recruitment,i} + \varepsilon_{j,i}$$

where $Z_{\text{drunkenness, i}}$ represents the status of alcohol drunkenness for sample i; $X_{\text{age, i}}$, $X_{\text{smoking, i}}$, $X_{\text{recruitment, i}}$ indicate the covariate values for sample i; $\mu_{\text{j, k}}$ $(k = 1\text{–}4)$ represents regression coefficients; and $\varepsilon_{\text{j, i}}$ represents error.

Sorted nuclei from brain tissues

Isolation of neuronal nuclei was performed as described previously [27–29]. Briefly 1 g of thawed tissue from dl-PFC or NAc was disrupted in Dounce homogenizer in lysis buffer (0.32 M sucrose, 5 mM $CaCl_2$, 3 mM $Mg(CH_3COO)_2$, 0.1 mM EDTA, 10 mM Tris HCl (pH 8.0), 0.1% Triton X-100, 1 mM DTT), and the suspension was loaded onto a 15% sucrose cushion and centrifuged at 30,000 × *g* for 2.5 h at 4 °C. Nuclei pellets were resuspended in PBS and monoclonal anti-NeuN antibody (Millipore, Billerica, MA) conjugated with Zenon anti-mouse Alexa 647-IgG (Molecular Probes) was added to the suspension and incubated at 4 °C for 45 min. Neuronal, NeuN positive (NeuN(+)) nuclei were separated from nonneuronal, NeuN negative (NeuN(−)) nuclei using the FACSDiVa high-speed cell sorter (Becton Dickinson, Heidelberg, Germany). Nuclear suspension was centrifuged at 30,000×*g* for 3 min and nuclear pellets were resuspended in PBS and stored at −80 °C. To evaluate the quality of FACS sorting, the neuronal proportions of sorted nuclei fractions were estimated based on their methylation levels generated from the Infinium HumanMethylation450 BeadChip assay using the CETS 3.0.3 package [26]. The purity of NeuN(+) and NeuN(−) nuclei fractions were all above 90%.

Bisulfite pyrosequencing

Bisulfite pyrosequencing was performed on dl-PFC samples only. The CpG site, cg05041795, within DMR-DLGAP2 was selected for bisulfite pyrosequencing validation. Two-hundred nanograms of genomic DNA from each sample was bisulfite-converted using an EZ DNA methylation-Gold Kit (ZYMO) according to the manufacturer's recommendations. We amplified bisulfite-converted genomic DNA by PCR using unbiased nested primers and carried out quantitative pyrosequencing to measure DNA methylation using a PSQ HS96 (Biotage). The DNA methylation percentage was determined by the Q-CpG methylation software (Biotage). Genomic coordinates and primer sequences for all bisulfite pyrosequencing reactions are provided (Supplementary Table 2). For allele-specific methylation assessment, dl-PFC tissues from a subset of individuals

heterozygous for a SNP within the DMR-DLGAP2 (rs11785793) were used. Two separate PCR reactions were optimized to discriminate between the two alleles and allow for allele-specific methylation detection of the CpG located at chr8:1,365,715–1,365,716 (hg19) within DMR-DLGAP2 (this CpG was not detected on the Illumina 450 K array). Genomic coordinates and primer sequences are provided (Supplementary Table 2).

Methylation correlations between brain and blood

The online Blood Brain DNA Methylation Comparison Tool (http://epigenetics.iop.kcl.ac.uk/bloodbrain/) [30], in which methylation data from matched blood and brain tissues are available, was used to evaluate the methylation correlation across blood and brain. CpG sites with a correlation coefficient (r) > 0.7 are considered as highly correlated across blood and brain tissues.

MRI data acquisition and analysis

Functional MRI data were acquired with 3T MRI scanners (Siemens, GE and Philips) using the same scanning protocols across all sites. Full details of MRI acquisition protocols and quality checks have been previously described [23].

Monetary incentive delay (MID) task

Participants from cohort 2 performed a modified version of the MID task to examine neural responses to reward anticipation and reward outcome. During the task, participants were presented with one of three cues (a triangle, a circle with a single line, a circle with three lines) denoting the number of points that could be won (0, 2, and 10 respectively), followed by a target white square. Participants were instructed that they would receive monetary reward based on the number of points they received. For this study, the contrast anticipation of "large win vs no win" was used.

Neuroimaging analyses

To exclude the effect of genotype on DNA methylation and following quality control of the contrast maps derived from fMRI data, scans from 40 individuals (who had never smoked and possess the rs11785793 major allele homozygotes) were included for this analysis. We used a partial least squares (PLS) modeling analysis to explore the relationship between cg02490460 methylation, frequency of drunkenness, and brain function during the MID task (please see Supplementary materials and Supplementary Fig. 2 for more details). The PLS method simultaneously optimizes associations between MID, cg02490460 methylation, and frequency of drunkenness. Gender, site, and total intracranial volume were included as covariates of no interest. Significance was determined using 10,000 permutations.

Culture and transfection of 293T cells

HEK293T cells were maintained in a humidified 37 °C incubator with 5% CO_2 and cultured in complete medium containing Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin (Life Technologies) as previously described [31]. HEK293T cells were transfected with DNA constructs using Polyethylenimine Hydrochloride (PEI) (Polyscience, 24765-1) according to the manufacturer's instructions.

Luciferase reporter assays

The CpG-free Lucia luciferase reporter vector, pCpGfree-promoter (Invivogen), was used for detecting enhancer's activity. The DMR-DLGAP2 region was amplified from genomic DNA of the HepG2 cell line using PrimeSTAR GXL DNA Polymerase (Takara). The primer sequences to amplify this region were as follows: forward primer: 5′-AGGATCCAACATGTAACTAGTCGCATGTTCATAGAAGCCCTTG-3′ and reverse primer: 5′-AGCATGCTCTTCTCCACTAGTGTGGGCTTCTGTGTCTGGAGC-3′. The PCR product, validated by Sanger sequencing, was cloned into the *SpeI* site of pCpGfree-promoter plasmid using ClonExpress II One Step Cloning Kit (Vazyme). Methylation of luciferase reporter construct was generated by the treatment with M.SssI CpG methyltransferase (New England Biolabs). Methylated or unmethylated Lucia luciferase reporter was co-transfected with firefly luciferase reporter vector pCpGL-CMV [32] into HEK293T cells using PEI. Cells were harvested after 48 h transfection and measured for luciferase activity by the dual-luciferase reporter assay system (Promega). Enhancer activities were determined by calculating Lucia luciferase signal normalized to firefly luciferase signal. All assays were performed in triplicates.

5-Aza-2′-deoxycytidine treatment and quantitative real-time PCR

HEK293T cells were treated with 5 uM 5-Aza-2′-deoxycytidine (Selleck) for 5 days. Total RNAs were isolated using Trizol reagent (Invitrogen) followed by reverse transcription to generate cDNA using PrimeScript™ RT reagent Kit with the treatment of gDNA Eraser (Takara). Quantitative

real-time PCR was carried out using the LightCycler 480 (Roche) and 2X SYBR Green Master Mix (Bimake) according to the manufacturer instructions. The mRNA levels of *DLGAP2* were normalized to that of *GAPDH*.

Animals

All animal experiments were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Animal Care and Use Committee of School of Basic Medical Sciences, Fudan University, China.

Generation of *Dlgap2* KO mice

Dlgap2 KO mice used for the two-bottle preference experiment were generated by using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system to induce non-homologous end joining. In brief, the Cas9 mRNA and four sgRNAs, targeting both flanks outside exon 6 of *Dlgap2* were microinjected into the germ cells of C57BL/6J mice to generate F0 *Dlgap2* KO mice. The F0 mice containing the *Dlgap2* knockout mutation were screened and verified through PCR and sequencing. The F1 *Dlgap2* heterozygous (HE) mice were generated by crossing the male F0 *Dlgap2* KO mice into WT female C57BL/6J mice and confirmed by PCR. Then F1 *Dlgap2* HE mice were mated with the WT female C57/BL mice to generate enough F2 *Dlgap2* HE mice. The F3 *Dlgap2* KO mice and WT control littermates, which were used in the two-bottle preference experiment, were generated by mating F2 *Dlgap2* HE mice with each other.

Dlgap2 KO mice used for the drinking-in-the-dark (DID) experiments were generated by using homologous recombination principle and ES cell shooting method. In brief, JM8A3 ES cells from the C57BL/6N mouse strain were electrotransfected by the target vector of ES cells. The target vector contained a 5 kb 5′ homologous arm, a 1.7 kb flox region, a pgk-neo-polyA, a 5 kb 3′ homologous arm and a Mc1-tk-polya negative screening marker. Resistant clones were obtained by G418 and Ganc screening. The correct homologous recombination positive clones were identified by long fragment PCR. After amplification, positive ES cells were injected into blastocysts of C57BL/6 J mice to obtain C57BL/6 background chimeric mice. High proportion chimeric mice were mated with C57BL/6J-Dppa3-IRES-Cre (Dppa3-Cre can effectively exert Cre recombination enzyme activity in embryonic early stage and germ cell line) mice with C57BL/6J background to obtain F1 generation heterozygous mice. The chimeric mice were then backcrossed into the C57BL/6J inbred background for two generations. Subsequent mating strategies were the same as above.

The mice were genotyped by PCR using genomic DNA with the following primers: forward primer F1 targeting the upstream of exon 6 (5′-GGTATGTAGCAGAGGTTT TCCC-3′); Reverse primer R1 targeting the downstream of exon 6 (5′-ACTGCCACTTACAATAGGGACA-3′); Forward primer F2 targeting the upstream of exon 6 (5′-TA TATTTTCCTAACACCTACAAAG-3′); Reverse primer R2 targeting exon 6 (GCTGGTCTGTCAACAAAAACA GAA). For the primer pair of F1 and R1, the length of PCR product is described below: 2.5 kb for WT allele and 0.8 kb for KO allele. For the primer pair of F2 and R2, the length of PCR product was described below: 500 bp for WT allele and no product for KO allele. Adult *Dlgap2* KO and WT mice (2- to 6-month-old male littermates) were used for behavioral tests unless noted otherwise. All mice were maintained on a 12:12 h light/dark daylight cycle.

Dlgap2 KO mice were evaluated using RT-PCR and western blot (the antibody for DLGAP2: Santa Cruz Biotechnology, catalog #sc-87418; the antibody for GAPDH: PROTEINTECH GROUP, catalog # 60004-1-Ig.).

Two-bottle preference experiment

The experiment was performed as described previously [14] with minor modifications. Briefly, both *Dlgap2* KO male mice ($n = 12$) and WT control male mice ($n = 12$) were given access to two bottles: one containing water and the other containing either 2, 4, 8, 12, 16, 20, 26, 34, or 40% ethanol (vol/vol) in water. After acclimation to the two-bottle paradigm for 1 week, mice were exposed to ascending concentrations of ethanol solution, each for 4 days. Total liquid intake (water and ethanol-containing water) and body weight were measured each day. Alcohol consumption (grams) was calculated based on the density of ethanol (0.789 g/mL). To obtain the relative alcohol intake that corrected for individual differences in littermate size, alcohol consumption was normalized to body weight every day for each mouse. As a measurement of relative alcohol preference, the preference ratio was calculated at each alcohol concentration by dividing total consumed alcohol solution by total liquid volume. Two-bottle preference assays were also performed for sucrose (0.5 and 5%) and quinine (2 and 20 mg/dL) solutions. Alcohol experienced mice were used for this test 7 days after alcohol was replaced by water in the bottles. For all experiments, the positions of the two bottles were changed every 2 days to exclude position effects.

Drinking-in-the-dark (DID) experiment

The DID procedure was performed as described previously with minor modifications [33, 34]. In brief, on days 1–3, mice received access to the 30% ethanol solution (vol/vol) 3 h into the dark cycle by replacing water with the ethanol

solution. The mice had access to ethanol for 2 h, after which the ethanol bottles were replaced with water bottles. We also measured water intake of mice during the first 3 h into the dark cycle. Alcohol or water consumption was normalized to body weight for each mouse.

Estimation of the contribution of heritable and environmental influences on DNA methylation

We estimated the contribution of genetic and environmental influences on DNA methylation at 7 CpG sites within the DMR-DLGAP2 from a public available data set (http://www.epigenomicslab.com/online-data-resources). The proportion of variance in DNA methylation explained by additive genetic (A), shared environmental (C) and unshared or unique environmental (E) factors was calculated by fitting an ACE model using structural equation modeling implemented with functions from the OpenMx R package, as described previously [35]. The hypothesis underlying this model is that the additive genetic factors between MZ twins are completely correlated (i.e., genetic correlation = 1), while the correlation between DZ twins is only 50% (i.e., genetic correlation = 0.5) and the shared nongenetic effects between MZ and DZ twins being similar.

Results

Epigenome-wide analysis of alcohol dependence from human brain tissues

To identify differentially methylated regions (DMRs) associated with alcohol dependence, we performed an epigenome-wide association study (EWAS) using postmortem material from the Australian brain bank, including 39 male Caucasian cases with alcohol dependence and 47 matched controls (cohort 1, $n = 86$, Supplementary Table 1). Genome-wide DNA methylation was assessed using Illumina 450K array on total tissue DNA of the dorsolateral prefrontal cortex (dl-PFC) and nucleus accumbens (NAc) obtained from these samples. We identified two DMRs that are associated with alcohol dependence at a FWER less than 0.05. For both DMRs, diagnosis of alcohol dependence was associated with decreased DNA methylation and the methylation difference was consistent in both dl-PFC and NAc tissues (Fig. 1a and Supplementary Fig. 3). The two DMRs map to the upstream regions of the *zinc finger protein 57* (*ZFP57*) gene and the *Disk large-associated protein 2* (*DLGAP2*) gene, respectively (Table 1). The DMR associated with *DLGAP2* (designated as DMR-DLGAP2 hereafter) is of particular interest, considering that the *DLGAP2* gene encodes for a postsynaptic scaffold protein that has been shown to interact with *DLG4* (also known as postsynaptic density protein 95, *PSD-95*) [36], a core protein localized to the post-synaptic density in neurons and important for synapse function. Thus, we decided to focus on the DMR-DLGAP2 in this study.

We also identified differentially methylated positions (DMPs) associated with alcohol dependence. No obvious batch effect or other confounders were observed based on the estimation of inflation factor ($\gamma = 0.996$) and QQ plot (Supplementary Fig. 4a). Even though none of the CpG sites passed genome-wide significance (Supplementary Fig. 4b), six out of seven probes within the DMR-DLGAP2 showed decreased DNA methylation level among drinkers (Fig.1a and Supplementary Fig. 3a), with p values <0.01 (Supplementary Fig. 4c).

We evaluated the correlation of methylation at these CpGs and noticed that, although separated by one probe (cg06008419), the methylation levels of the first two probes and the last four probes are correlated (Supplementary Fig. 5), forming a pattern similar to that of genetically controlled methylation clusters known as GeMes [37]. Even though none of the seven CpG sites within the DMR-DLGAP2 contains a common single nucleotide polymorphism (SNP), we identified 126 SNP-CpG pairs, where methylation appears to be under genetic control (Fig. 1a and Supplementary Table 3). This suggests that the observed correlation of DNA methylation is indeed controlled by genotype, as in GeMes. The 126 SNP-CpG pairs comprised 33 unique SNPs and five CpGs. Among these 33 SNPs, 22 were associated with alcohol dependence when compared with controls (p value < 0.05) (Fig. 1a and Supplementary Table 4), suggesting that genotype may influence the association between methylation at DMR-DLGAP2 and alcohol dependence.

Replication with sorted neuron cells

One challenge in EWAS is the cellular heterogeneity in starting materials, which may act as a potential confounder [38]. We addressed this issue by adjustment for estimated neuron proportions. Nevertheless, to further confirm that the observed methylation difference is not a result of cellular heterogeneity, we replicated the findings using flow cytometry-sorted nuclei from neuronal and non-neuronal (glial) cells separated from the dl-PFC of a subset of individuals from cohort 1 (Supplementary Table 1). Consistent with what we had observed in the unsorted dl-PFC tissues, alcohol dependence was associated with decreased DNA methylation in both sorted neurons and glial cells (both with at least three probes at p value < 0.05) (Fig. 1b and Supplementary Table 5). In addition, methylation in sorted cells also formed GeMes (Supplementary Fig. 5), as in unsorted dl-PFC and NAc tissues.

Previous analyses have shown that estimates from the Illumina 450K array are highly consistent with whole genome

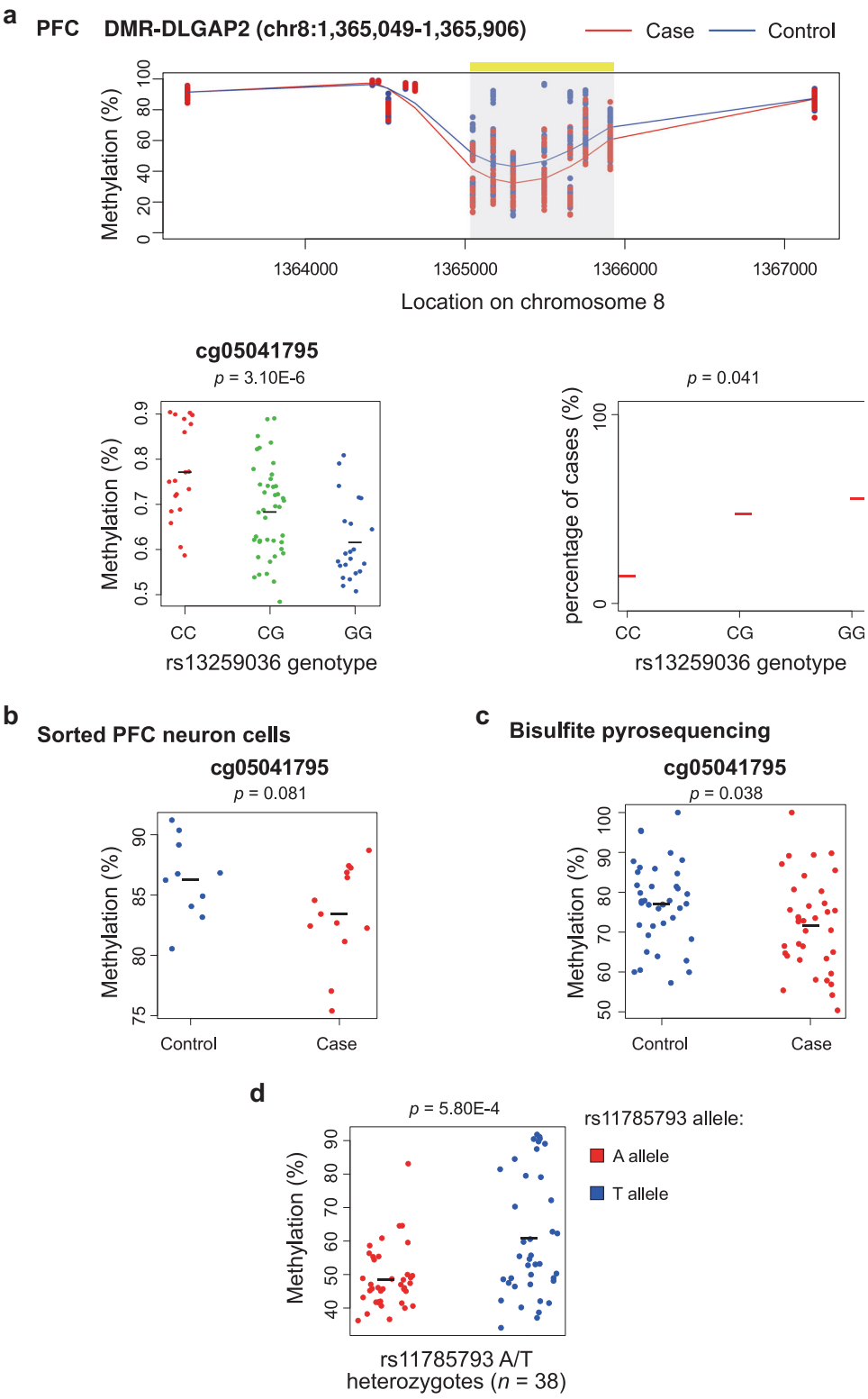

Fig. 1 Genotype-dependent DNA methylation on alcohol dependence in human brain tissues. **a** Top panel: associations between DNA methylation levels and alcohol dependence at DMR-DLGAP2, with red and blue colors representing cases and controls respectively, in human dl-PFC tissues. The plot is centered on DMR-DLGAP2 (highlighted with the yellow bar on top and the gray shadow). Bottom left panel: association between DNA methylation (cg05041795, chr8: 1365749) and genotype (rs13259036, chr8: 1405107). Black horizontal bars mark average DNA methylation levels. Bottom right panel: association between genotype and alcohol dependence. Red horizontal bars mark percentage of cases for each genotype. **b** Association between DNA methylation and alcohol dependence using FACS-sorted human dl-PFC neuron cells. **c** Validation of the association between DNA methylation and alcohol dependence using bisulfite pyrosequencing. **d** Allele-specific methylation (chr8: 1365715) within DMR-DLGAP2 quantified by bisulfite pyrosequencing from individuals heterozygous for a SNP within the DMR-DLGAP2 (rs11785793, chr8: 1365687, $n = 38$ individuals with A/T genotype) (red and blue colors representing A allele and T allele, respectively)

bisulfite sequencing [39]. We validated our array-based findings using bisulfite pyrosequencing and observed consistent methylation differences between cases and controls (Fig. 1c). In order to test whether genotype-dependent methylation of DMR-DLGAP2 is allele-specific, we evaluated the methylation level of DMR-DLGAP2 using the dl-PFC tissues from a subset of individuals heterozygous (A/T) for a SNP (rs11785793) within the DMR-DLGAP2. The

Table 1 Identified differentially methylated regions (DMRs)

Chr.	Start (hg19)	End (hg19)	Width (bp)	Number of CpGs	FWER	Value	Gene name	Description
chr6	29648161	29649092	932	25	0.021	−0.031	*ZFP57*	Upstream
chr8	1365049	1365906	858	7	0.043	−0.075	*DLGAP2*	Upstream

minor allele of rs11785793 (A allele) exhibited significantly lower methylation compared to the major allele (T allele) (p value = 5.80E−4) (Fig. 1d), indicating that methylation within DMR-DLGAP2 is allele-specific.

Associations between DMR-DLGAP2 methylation in blood and reward processing in brain

Recent findings show that, for certain CpGs, methylation levels in blood and brain tissues correlate [30, 40], potentially as a consequence of genetic influences. Using an online dataset with methylation levels from matched blood and brain tissues [30], we observed that, even though the mean methylation level of all CpG probes within DMR-DLGAP2 in blood was higher than that in PFC tissues (Supplementary Fig. 6), four out of seven probes within DMR-DLGAP2 showed significant methylation correlations between blood and PFC ($r > 0.7$) (Supplementary Fig. 7). Thus, for these four probes, methylation level in blood can likely be used as a surrogate measurement for that in brain. This made it possible for us to further evaluate the role of DNA methylation within DMR-DLGAP2 using other cohorts with blood DNA methylation measurements.

We investigated 19-year-old adolescents from the IMAGEN imaging-genetics study (cohort 2, Supplementary Table 1), in which blood DNA methylation was evaluated using the Illumina 850K BeadChip array. Among the four probes that are correlated between blood and PFC, three of them were available in this cohort. Consistent with our observations in brain tissues, individuals with higher frequency of drunkenness were significantly associated with decreased methylation levels (Fig. 2a, Supplementary Fig. 8a, 8b). Additionally, the association between the number of drinks on a typical day (questionnaire-based self-assessment) and methylation level at cg02490460 was marginally significant (effect size $r = -0.074$; p value = 0.094), suggesting this effect may be dose-dependent. The methylation level in blood was also genetically controlled (Fig. 2b), forming a GeMe (Supplementary Fig. 8c) similar to that in brain. These results corroborated our findings that hypomethylation within the DMR-DLGAP2 is associated with higher frequency of drunkenness, and that this methylation change is under genetic control.

To determine whether brain activity is associated with DNA methylation within DMR-DLGAP2 and frequency of drunkenness, we investigated Blood Oxygen Level Dependent (BOLD)-response during the Monetary-Incentive-Delay (MID) task, a measurement to access a person's neural response to the anticipation and receipt of a reward [41]. As methylation in cohort 2 is genotype dependent (Fig. 2b), we evaluated associations between methylation within DMR-DLGAP2 (cg02490460) and frequency of drunkenness stratified by rs11785793 genotype, in order to exclude the effect of genotype on DNA methylation. The relationships between cg02490460 methylation and frequency of drunkenness only remained significant for the major allele homozygotes (rs11785793_TT, p value = 0.040), but not for the heterozygotes (rs11785793_AT, p value = 0.109). In the neuroimaging analyses, we therefore only focused on individuals with rs11785793_TT genotype who had never smoked. Using a Partial Least Squares (PLS) method, we found 13 clusters to have significant positive associations with methylation within DMR-DLGAP2 (cg02490460) and negative associations with frequency of drunkenness (p value = 0.024) (Supplementary Table 6 and Fig. 2c, d), indicating reduced activation of these clusters in individuals reporting a history of high frequency of drunkenness. The largest cluster was found to be in the precuneus, previously implicated in alcohol use disorder [42] and alcohol dependence [43, 44]. In addition, the precuneus has been implicated to play a role in reward-related processing [45]. These results suggest that hypomethylation within DMR-DLGAP2 in adolescents harboring the rs11785793_TT genotype is related to both an altered reward system and an increased risk for high frequency of drunkenness.

DMR-DLGAP2 is a potential methylation-dependent enhancer, regulating *DLGAP2* expression

To understand the functional role of DMR-DLGAP2, we explored the epigenetic signatures of this region in human brain tissues using the Encyclopedia of DNA Elements (ENCODE) database [17, 46–48]. We observed relatively high intensity of DNase I hypersensitive sites (DHSs), H3K4me1, H3K27ac, and H3k27me3 (Fig. 3a) within this region, indicating that DMR-DLGAP2 is a potential enhancer. Analysis of the linkage disequilibrium (LD) maps showed that DMR-DLGAP2 belongs to a region separating two distinct LD blocks (Supplementary Fig. 9). Moreover, results from Hi-C [49] show that DMR-DLGAP2 interacts with the promoter region of *DLGAP2* in human hippocampus (Fig. 3a). All these data indicate that DMR-DLGAP2 may act as an enhancer regulating the expression of *DLGAP2*.

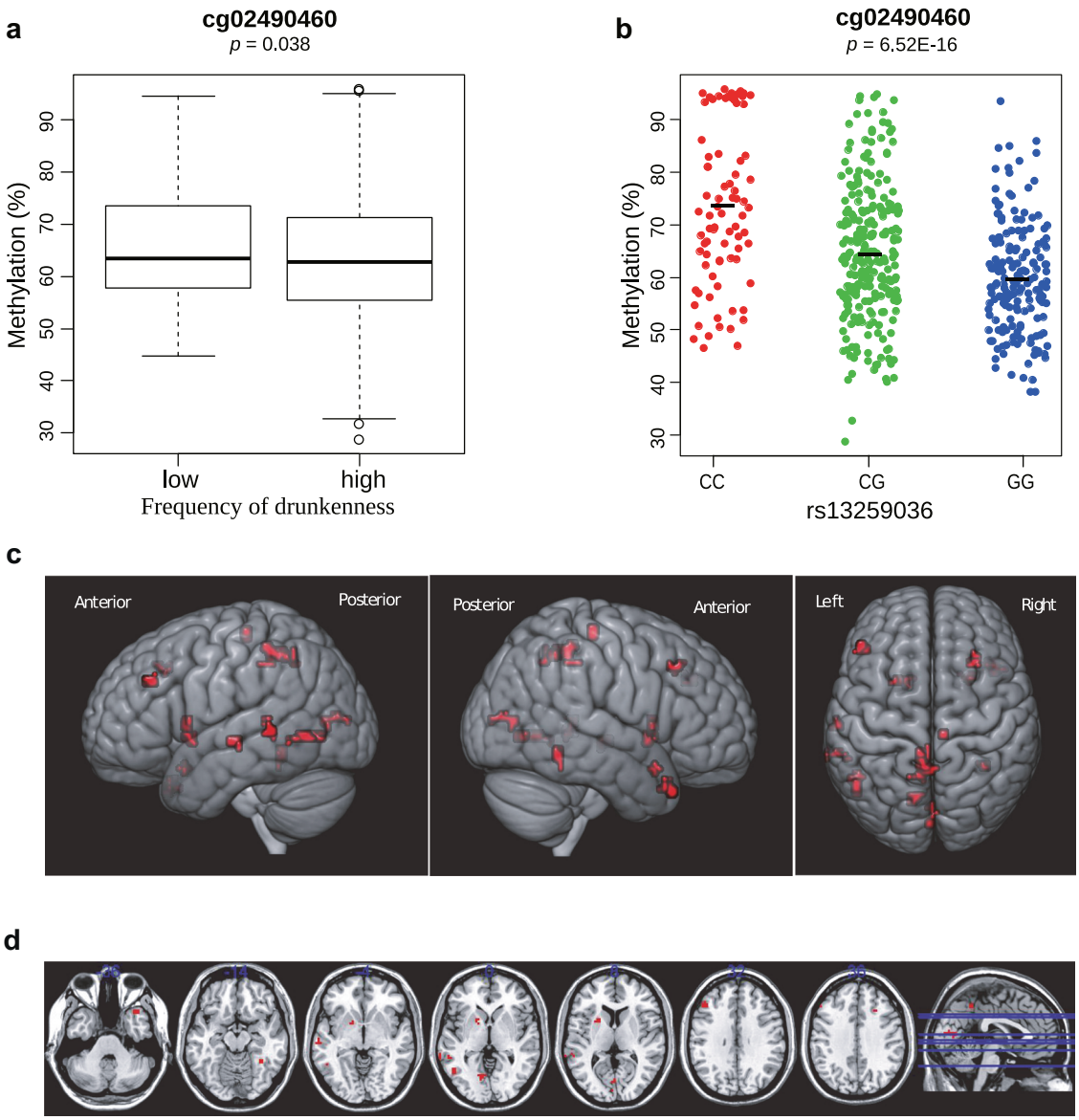

Fig. 2 Genotype-dependent DNA methylation at DMR-DLGAP2 in human blood is associated with frequency of drunkenness and brain activity. **a** Associations between DNA methylation at cg02490460 within DMR-DLGAP2 and frequency of drunkenness. The low alcohol group includes those individuals with zero occasion of frequency of drunkenness and the high alcohol group includes those individuals with 2 or more scales of frequency of drunkenness based on ESPAD. **b** The association between DNA methylation level at cg02490460 and genotype of rs13259036. **c**, **d** Spatial mapping of the associations between brain function during the MID task, DNA methylation at cg02490460 and frequency of drunkenness. 13 clusters associated with DNA methylation and frequency of drunkenness are illustrated using **c** 3-D and **d** multi-slice rendering from individuals with rs11785793_TT genotype. A partial least squares (PLS) path modeling was used for the analysis. Red regions represent 13 clusters

To test whether methylation in DMR-DLGAP2 can exert regulatory properties on gene expression, we performed a cellular-based dual-luciferase reporter assay and observed significantly increased gene expression when unmethylated DMR-DLGAP2 is present (p value = 0.015). However, this upregulation of gene expression was abolished when DMR-DLGAP2 on the luciferase reporter was in vitro methylated (p value = 0.0048) (Fig. 3b), suggesting that the regulatory feature of DMR-DLGAP2 on gene expression is methylation-dependent in vitro. Negative regulation of *DLGAP2* gene expression through methylation on DMR-DLGAP2 was further supported by the increase in *DLGAP2* expression in HEK293T cells treated with the demethylating drug 5-Aza-2′-deoxycytidine (Fig. 3c). Together, these findings suggest that DMR-DLGAP2 is a methylation dependent gene regulatory element, and that reduced DNA methylation of DMR-DLGAP2 can lead to increased *DLGAP2* gene expression.

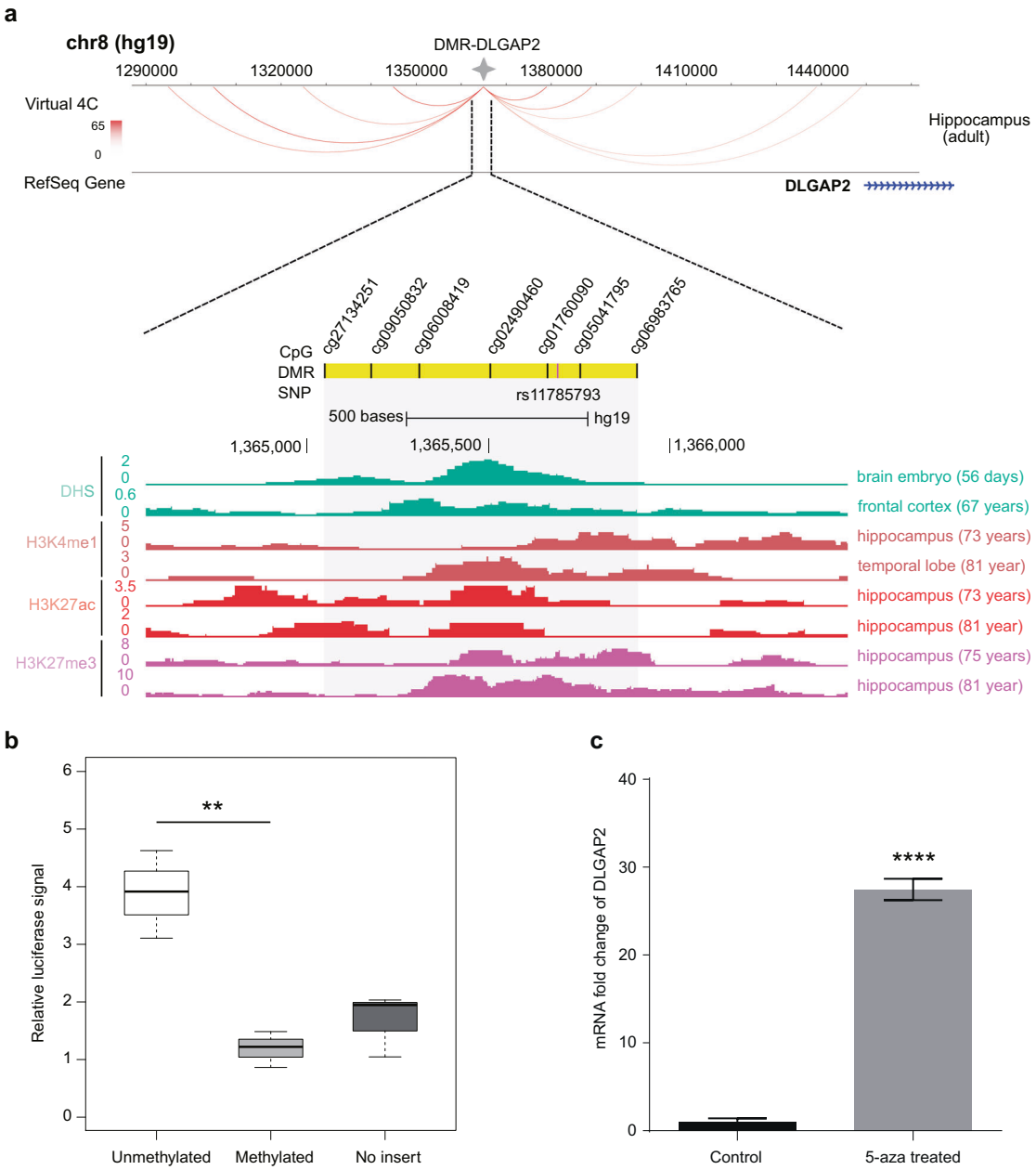

Fig. 3 DMR-DLGAP2 is a potential methylation-dependent enhancer regulating *DLGAP2* expression. **a** A virtual 4C plot generated from Hi-C data using the DMR-DLGAP2 region as the bait, showing potential interactions (red curve lines) in human hippocampus tissue. The DHS and ChIP-Seq tracks for histone modifications are visualized using the UCSC genome Browser. **b** DMR-DLGAP2 exhibited methylation-dependent enhancer activity on gene expression. Dual-luciferase reporter assays were performed using a CpG-free promoter-containing (Lucia) reporter gene vector to evaluate the transcriptional activity of the methylated or unmethylated DMR-DLGAP2 in HEK293T cells, with Lucia activity normalized to firefly activity. Data are represented as mean ± SEM (**p value < 0.01). **c** *DLGAP2* expression in HEK293T with or without the treatment of 5-Aza-2′-deoxycytidine (5-aza). The mRNA levels of *DLGAP2* were normalized to that of *GAPDH*. Data are represented as mean ± SEM (****p value < 0.0001)

Dlgap2-deficient mice demonstrate reduced alcohol consumption

We next wanted to determine whether the regulation of DMR-DLGAP2 on *DLGAP2* gene expression plays a role in alcohol drinking. Since DMR-DLGAP2 is not conserved in mice, we generated *Dlgap2* gene knockout (KO) mice instead (Supplementary Fig. 10). Hematoxylin and eosin (H&E) staining showed that *Dlgap2* KO mice develop normally (Supplementary Fig. 11). We then performed a voluntary two-bottle drinking experiment with water and increasing concentrations of alcohol (2, 4, 8, 12, 16, 20, 26, 34, or 40%, each given for 4 days) on both *Dlgap2* KO mice and wild-type (WT) control mice. There

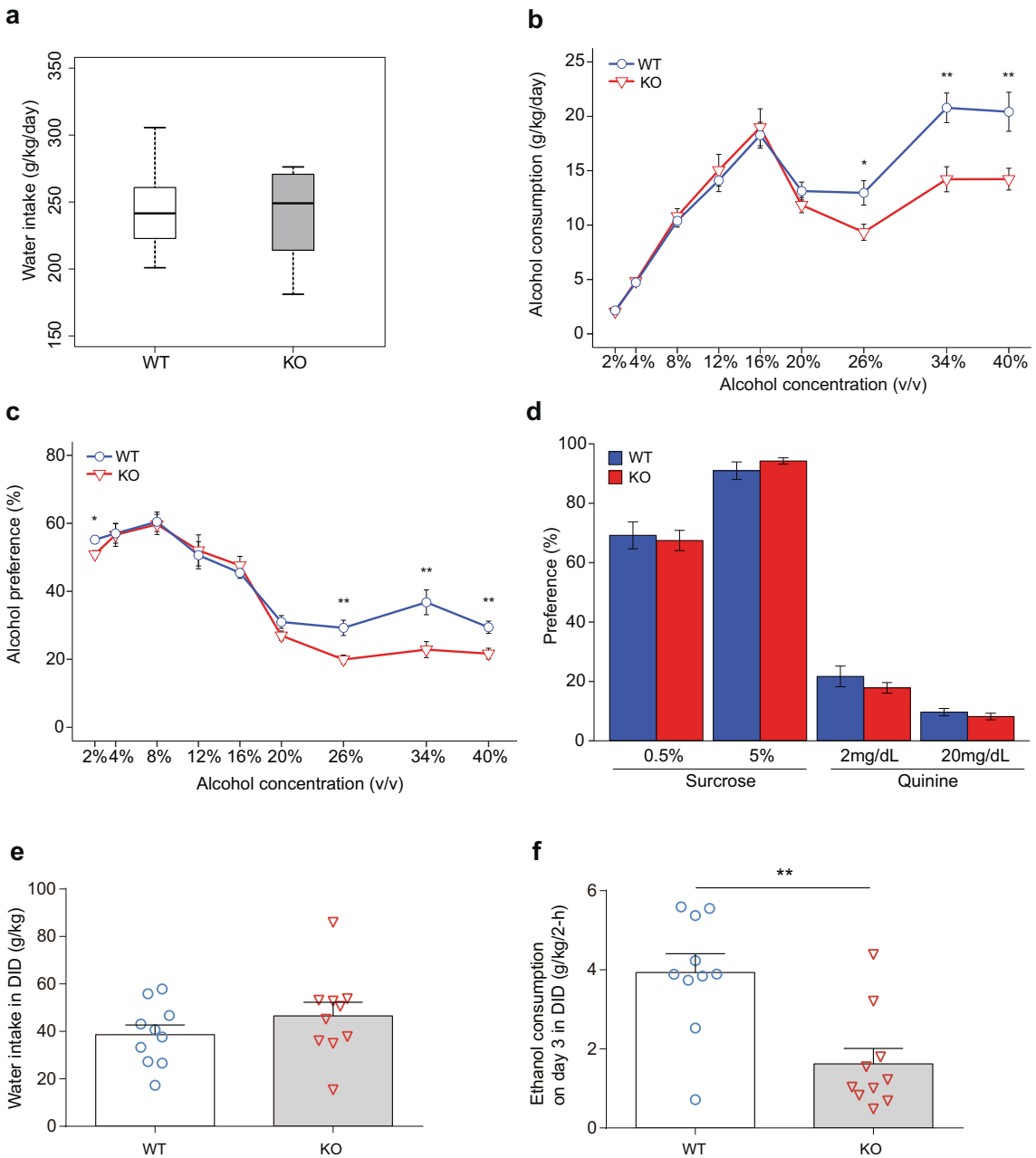

Fig. 4 Two-bottle preference and DID experiments in *Dlgap2*-deficient (KO) and wild-type (WT) mice. **a** No difference in daily water intake (g/kg/day) between *Dlgap2* KO mice and WT controls. **b**, **c** Alcohol consumption (g/kg/day) and alcohol preference ratio (%, volume of alcohol consumed/total volume of liquid consumed) at various alcohol concentration (v/v) between *Dlgap2* KO mice and WT controls. Mice were exposed to each concentration of ethanol solution for 4 days. **d** Preference ratio (%) for sucrose and quinine between *Dlgap2* KO mice (red) and WT controls (blue). **e** No difference in water intake (g/kg) during 3 h into the dark cycle between *Dlgap2* KO mice and WT controls. **f** Alcohol consumption (g/kg/day) of *Dlgap2* KO mice and WT controls at 30% alcohol concentration (v/v) in DID experiment on day 3. All data are represented as mean ± SEM (*p value < 0.05, **p value < 0.01). $n = 12$ per group in **a**–**d**. $n = 10$ per group in **e**, **f**

was no significant difference in daily water intake between *Dlgap2* KO mice and WT controls before the placement of the alcohol bottle (Fig. 4a). However, the *Dlgap2* KO mice showed significantly decreased alcohol consumption (Fig. 4b) and reduced preference (Fig. 4c) compared with WT mice at high alcohol concentrations (26, 34, or 40%). The reduction of alcohol consumption in *Dlgap2* KO mice is not a consequence of differential taste reactivity, since no significant difference was observed between *Dlgap2* KO mice and WT controls in the two-bottle drinking test with sucrose (sweet) or quinine (bitter) solutions (Fig. 4d).

"Drinking in the dark" (DID) is a well-established animal model of human binge-like ethanol drinking [33, 34]. We also performed a 3 days-DID experiment with a 30% (v/v) ethanol solution in *Dlgap2* KO mice (Supplementary Fig. 12). Consistent with the two-bottle drinking experiment, *Dlgap2* KO mice displayed significantly less alcohol drinking than WT mice on day 3 (Fig. 4f). No significant difference in water intake between *Dlgap2* KO mice and WT controls was observed during the first 3 h into the dark cycle (Fig. 4e).

Discussion

Through an epigenome-wide approach, we have identified two DMRs associated with alcohol dependence, with one of them mapping to the upstream region of the *DLGAP2* gene. *DLGAP2* is mainly expressed in brain [50] (Supplementary Fig. 13) and has been shown to interact with DLG4 [36], a core scaffold protein for forming a protein complex in the post-synapse [51]. SNPs controlling the DNA methylation level in DMR-DLGAP2 appear to be associated with alcohol dependence, even though none of them reached the genome-wide significant of 5×10^{-8} (Supplementary Table 4), probably as a consequence of the small sample size we have. Genetic variants in *DLGAP2* have previously been associated with autism spectrum disorder [52–54], schizophrenia [55–57], and Alzheimer's disease [58], but there have not yet been any reports on the association between *DLGAP2* and alcohol consumption and dependence [3–11]. It is worth further investigating whether SNPs in *DLGAP2* are associated with alcohol consumption and dependence in a larger independent cohort. Nevertheless, this work illustrates the power of an integrated genetic and epigenetic analysis which may augment the promise to identify hereditary risk that may otherwise be opaque when considering genetic variants in isolation [38, 59, 60]. Moreover, a recent study has shown that *Dlg4*-deficient mice consume less alcohol than WT controls [61], which is similar to what we observed in *Dlgap2*-deficient mice.

We found that DNA methylation in DMR-DLGAP2 is genotype-dependent in both brain and blood tissues, forming a correlated methylation cluster known as a GeMe. While methylation within DMR-DLGAP2 is regulated by genotype, we noticed that, even after adjusting for genotype rs13259036, individuals with higher frequency of drunkenness were still significantly associated with decreased methylation level on cg02490460 in cohort 2 (effect size $r = -0.145$; p value $= 0.002$) (for the association between cg02490460 methylation and alcohol dependence after adjusting for rs13259036 genotype in cohort 1: effect size $r = -0.044$; p value $= 0.062$). Combined with the fact that methylation levels within DMR-DLGAP2 in blood was significantly higher than that in dl-PFC tissues (Supplementary Fig. 6), these results suggest that, in addition to genotype, some other mechanisms may also influence the methylation level within DMR-DLGAP2. Moreover, the small effect size for the associations also indicates that, in addition to the methylation in DMR-DLGAP2, other genes/networks/mechanisms may affect the susceptibility of alcohol consumption and dependence.

In the evaluation of methylation correlations in blood using monozygotic and dizygotic twins [35], it was also shown that most of the CpGs within DMR-DLGAP2 are mainly influenced by additive genetic factors and highly heritable (Supplementary Fig. 14). Even though we do not know whether methylation in DMR-DLGAP2 is genetically imprinted in brain, as indicated in testis [62], its methylation is allele-specific (Fig. 1d). Since the brain has been shown to express *DLGAP2* biallelically [62], it is worth further investigating whether allele-specific methylation of DMR-DLGAP2 can lead to *DLGAP2* allele-specific expression in brain. In fact, the other DMR we identified in this study is associated to the gene *ZFP57*, whose product has been described as a master regulator of imprinting and mono-allelic expression through altering methylation in imprinting control regions [63]. It would also be intriguing to investigate any potential crosstalk between *ZFP57* and *DLGAP2*.

One challenge in interpreting EWAS results is to address whether identified epigenetic changes associated with disease phenotype are indeed causal. In human brain tissues, we observed that DMR-DLGAP2 is decorated with active epigenetic markers, such as DHSs, H3K4me1, H3K27ac, and H3k27me3, suggesting it is a potential enhancer. Moreover, HiC results show that DMR-DLGAP2 interacts with the promoter region of *DLGAP2*, indicating it may be a regulator for its gene expression. Consistent with this, we showed that decreased methylation levels in DMR-DLGAP2 in vitro (Fig. 3b, c) can lead to increased gene expression, which indicates that drinkers with DMR-DLGAP2 hypomethylation may have elevated expression of *DLGAP2* in brain, possibly in a cell type- and location-specific manner. Even though we cannot directly test the causal role of DMR-DLGAP2 using a mouse model, *Dlgap2*-deficient mice showed decreased alcohol drinking, supporting the causal role of *DLGAP2* in alcohol consumption susceptibility. Our in vitro and in vivo work supports the idea that hypomethylation in the DMR-DLGAP2 region in human brain may lead to increased expression of *DLGAP2* and, in turn, alcohol drinking. Considering DNA methylation being reversible, our work opens up new avenues for development of therapeutic strategies to treat alcohol drinking.

This research also makes a prediction that is beyond the scope of the current experiments. The glutamate homeostasis hypothesis of addiction suggests that reduced ability of drug

addicts to control their drug seeking is likely due to a loss of glutamate homeostasis, which in turn disrupts the prefrontal modulation of the striatum circuit [64]. It is evident that N-methyl-D-aspartate (NMDA) receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor are two major glutamate receptors required for alcohol-induced neuroplasticity and the susceptibility of alcohol drinking [65, 66]. Serving as scaffold proteins in the post-synaptic density (PSD), DLGAP family proteins play a vital role in supporting the formation of PSD through phase separation [67] and controlling synaptic scaling upon activation of NMDA and AMPA receptors. DLGAP family proteins exert their effect through direct interaction with postsynaptic scaffold proteins such as the DLG4 protein [55], that mediates the surface trafficking of NMDA and AMPA receptors [68, 69]. Similar to other Dlgap family genes knockout models [70–72], *Dlgap2* gene ablation reduces glutamate receptors in synapses along with deficits in synaptic transmission and ultrastructural defect in PSD in the orbitofrontal cortex [54]. Considering the direct interaction of DLGAP2 and DLG4 [36, 51], it is possible that the aberrant changes in *DLGAP2* lead to defects in synaptic functional defects as well via regulation of glutamate receptors. More detailed molecular and electrophysiological mechanisms on cellular and animal models remain to be further investigated.

An issue that may complicate the molecular mechanism studies of *DLGAP2* in alcohol dependence is that we do not know the primary target tissues or cell types related to the disease. Even though the methylation of DMR-DLGAP2 is genotype-dependent with strong correlations between blood and brain, the expression of DLGAP2 is mainly located in the brain. This suggests that methylation level on DMR-DLGAP2 is not the only determinant of DLGAP2 regulation in vivo, as we observed in vitro. In other words, the epigenome may be similar in many different cell types, but only have a functional significance for gene expression in selected ones. It is vital to determine how the expression of *DLGAP2* is regulated in the disease-related tissues or cell-types for the understanding of the molecular mechanism of *DLGAP2* in alcohol consumption and dependence.

Another limitation to our work is the use of different phenotypes and ages among different cohorts and the mouse model. Cohort 1 included adult subjects with alcohol dependence, while cohort 2 included 19-year old adolescents and, just like the mouse model, focused on non-pathological alcohol consumption. Even though alcohol consumption is a strong risk factor for adult alcohol dependence [73, 74], and thus may serve as a rough proxy for alcohol dependence, it is noteworthy to point out that a recent study reported that there was only modest genetic correlation between pathological and non-pathological alcohol drinking [11]. However, in our current study, we showed that *DLGAP2* is associated with both non-pathological and pathological alcohol drinking behavior, emphasizing its potential overlapping role in both alcohol consumption and progression to alcohol dependence.

In conclusion, we have identified a differentially methylated region, DMR-DLGAP2, in which genotype-dependent DNA methylation is associated with alcohol consumption and dependence. Furthermore, combining in vitro and in vivo studies, we could establish that DMR-DLGAP2 and its methylation pattern is regulating *DLGAP2* expression, and that lack of *Dlgap2* expression leads to diminished alcohol consumption in a mouse model. Together, our results implicate an important role for *DLGAP2* as an interface for genetic and epigenetic factors controlling alcohol consumption and dependence.

Data availability

The 450K array data from brain tissues is available from the corresponding author upon request and signature of data transfer agreement. Aggregated summary statistics are available upon request.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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