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Dysregulation of the histone demethylase KDM6B in alcohol dependence is associated with epigenetic regulation of inflammatory signaling pathways

Andrea L. Johnstone[1,2,3] | Nadja S. Andrade[2] | Estelle Barbier[4] | Bohdan B. Khomtchouk[1,2,5] | Christopher A. Rienas[1,2] | Kenneth Lowe[1,2] | Derek J. Van Booven[6] | Esi Domi[4] | Rustam Esanov[1,2] | Samara Vilca[1,2] | Jenica D. Tapocik[7] | Keli Rodriguez[3] | Danielle Maryanski[3] | Michael Christopher Keogh[3] | Marcus W. Meinhardt[8] | Wolfgang H. Sommer[8] | Markus Heilig[4] | Zane Zeier[1,2] | Claes Wahlestedt[1,2]

[1] Center for Therapeutic Innovation, University of Miami Miller School of Medicine, Miami, Florida, USA

[2] Department of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, Miami, Florida, USA

[3] Division of Product Development, EpiCypher, Inc, Durham, North Carolina, USA

[4] Department of Clinical and Experimental Medicine, Division of Cell Biology, Faculty of Health Sciences, Linköping University, Linköping, Sweden

[5] Department of Medicine, Section of Computational Biomedicine and Biomedical Data Science, Institute for Genomics and Systems Biology, University of Chicago, Chicago, IL, USA

[6] John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Miami, Florida, USA

[7] Laboratory of Clinical and Translational Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland, USA

[8] Department of Psychopharmacology, Central Institute of Mental Health, Heidelberg University, Mannheim, Germany

Correspondence
Claes Wahlestedt and Zane Zeier, Department of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, 1501 NW 10th Ave, BRB #407 (M-860),

Abstract

Epigenetic enzymes oversee long-term changes in gene expression by integrating genetic and environmental cues. While there are hundreds of enzymes that control histone and DNA modifications, their potential roles in substance abuse and alcohol dependence remain underexplored. A few recent studies have suggested that epigenetic processes could underlie transcriptomic and behavioral hallmarks of alcohol addiction. In the present study, we sought to identify epigenetic enzymes in the brain that are dysregulated during protracted abstinence as a consequence of chronic and intermittent alcohol exposure. Through quantitative mRNA expression analysis of over 100 epigenetic enzymes, we identified 11 that are significantly altered in alcohol-dependent rats compared with controls. Follow-up studies of one of these enzymes, the histone demethylase KDM6B, showed that this enzyme exhibits region-specific dysregulation in the prefrontal cortex and nucleus accumbens of alcohol-dependent rats. KDM6B was also upregulated in the human alcoholic brain. Upregulation of KDM6B protein in alcohol-dependent rats was accompanied by a decrease of trimethylation levels at histone H3, lysine 27 (H3K27me3), consistent with the known demethylase specificity of KDM6B. Subsequent epigenetic (chromatin immunoprecipitation [ChIP]–sequencing) analysis showed that alcohol-induced changes in H3K27me3 were significantly enriched at genes in the IL-6 signaling pathway, consistent with the well-characterized role of KDM6B in modulation of inflammatory responses. Knockdown of KDM6B in cultured microglial cells diminished IL-6 induction in response to an inflammatory stimulus. Our findings implicate a novel

Andrea L. Johnstone and Nadja S. Andrade are co-authors.

Miami, FL 33136, USA.
Email: cwahlestedt@med.miami.edu; zzeier@med.miami.edu

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KDM6B-mediated epigenetic signaling pathway integrated with inflammatory signaling pathways that are known to underlie the development of alcohol addiction.

KEYWORDS

alcoholism, epigenetics, inflammation, JMJD3, KDM6B, nucleus accumbens, prefrontal cortex

1 | INTRODUCTION

By the time an affected individual seeks help for an alcohol use disorder, the condition is often quite advanced. The development of improved treatment strategies thus depends on knowledge of the long-term mechanisms that underlie continued maladaptive alcohol seeking behavior. Human and animal studies have shown that genetic predispositions interact with environmental risk factors to facilitate long-term transcriptional changes that rewire brain reward circuitry and promote alcohol dependence.[1-8] Between 30% and 70% of the risk for alcohol dependence can be attributed to genetic factors.[9] Much progress has been made in identifying the genetic risk variants that predispose individuals to alcohol abuse.[8,9] However, as with many complex psychiatric disorders, genetic studies suffer from small effect sizes (less than 5%) and poor replicability.[8,10] Understanding the dynamic mechanisms that translate experiences such as early life adversity, stress, and repeated alcohol exposure into persistent brain adaptations that underlie addiction and relapse is therefore a critical aspect of understanding and treating this complex disorder.

Epigenetic mechanisms are known to alter transcriptomic programs in an environmentally sensitive manner.[11] These mechanisms do not alter DNA sequence but rather affect gene expression through chemical modifications of DNA and histone tails. A diverse array of epigenetic enzymes oversee the addition, interpretation, and removal of these modifications.[12] Histone tail modifications include acetylation, methylation, and phosphorylation, among others. Histone methylation can either facilitate or repress transcription depending on the particular amino acid residue that is modified. Methylation at histone H3 lysine 4 (H3K4) is typically associated with gene induction, while methylation at H3K27 condenses chromatin and inhibits gene expression. This "histone code" is further complicated by the fact that lysine can be monomethylated, dimethylated, or trimethylated and the degree of methylation can differentially influence gene expression. There are over 50 human epigenetic enzymes that catalyze the addition of methyl groups to histones (histone methyltransferases) and approximately 30 enzymes that remove these methyl groups (histone demethylases).[13] Classically, these enzymes exhibit specificity toward a particular amino acid on a particular histone tail and further exhibit specificity toward the degree of methylation.

Despite the potential for epigenetic processes to contribute to the widespread gene expression changes, interaction of genetic and environmental risk factors, and long-term vulnerability for relapse that characterize substance abuse, this field is relatively underexplored. Several studies have identified changes in histone acetylation and DNA methylation that are associated with alcohol use in both humans and rodents.[14-20] Other epigenetic modifications that could influence gene expression, such as histone methylation, remain largely unexplored in the context of alcohol dependence.

Previously, we hypothesized that dysregulation of epigenetic regulatory networks could mediate alterations in transcriptional programs that underlie behaviors associated with alcohol dependence. By mining epigenetic enzyme expression from whole-transcriptome RNA-sequencing analysis in alcohol-dependent rats, we found several epigenetic enzymes that were dysregulated in the dorsomedial prefrontal cortex (dmPFC) as a consequence of chronic and intermittent ethanol exposure. Follow-up studies of one enzyme, the histone methyltransferase PRDM2, showed that this enzyme contributes to gene expression changes and behavioral phenotypes characteristic of alcohol dependence.[21] This study showed that epigenetic enzymes known to oversee histone methylation levels contribute to the development of alcohol addiction. In the present study, we sought to identify epigenetic enzymes that are associated with alcohol dependence in the nucleus accumbens (NAc). The PFC and NAc are key elements of the brain reward pathway, where the NAc encodes the perception of the rewarding properties of a stimulus and the PFC integrates this information by weighing immediate desires against perceived future consequences to exert executive decision making.[22,23] Thus, identification of epigenetic processes disrupted in these two brain regions is critical to understand the altered neurocircuitry that underlies impulsive alcohol intake and vulnerability to relapse.

In the present study, we explored the expression of epigenetic enzymes in a rat model of alcohol dependence where rats are subjected to long-term, intermittent exposures to alcohol vapor in order to mimic cycles of binge intoxication in human alcoholics. This model induces key transcriptomic, pathological, and behavioral features of alcohol dependence and exhibits high predictive validity with respect to effective treatment strategies.[24] We examined epigenetic enzyme expression in alcohol-dependent rats after 3 weeks of withdrawal ("postdependence"). We hypothesized that examining epigenetic signaling events that persist into protracted abstinence could help to understand the mechanisms that underlie chronic disruption of transcriptomic programs and mediate long-term increases in alcohol-seeking behavior and vulnerability for relapse.[25] Our expression analysis of epigenetic enzyme levels in postdependent rats implicated lysine (K)–specific demethylase 6B (KDM6B; also known as JMJD3), a Jumonji domain (JmjC)–containing histone demethylase with a well-characterized role in epigenetic modulation of inflammatory responses. Neuroimmune signaling pathways are potentiated by alcohol exposure and are increasingly implicated in neurobiological changes that contribute to alcohol addiction.[1] Progressive induction of neuroimmune signaling pathways over lifetime alcohol exposure creates a persistent state of inflammation that must be targeted to alleviate maladaptive drug-seeking behavior.[1] The intersection of epigenetic and inflammatory signaling pathways has only begun to be explored and has not previously been studied in the context of alcohol dependence. Our studies are the first to implicate dysregulation of the KDM6B signaling pathway in alcohol dependence and show that it is associated with epigenetic modulation of inflammatory response genes in alcohol-dependent brain reward regions.

2 | METHODS

2.1 | Alcohol-dependence induction

Dependence was induced using chronic intermittent alcohol vapor exposure as described.[26] Briefly, postdependent male Wistar rats were exposed to alcohol vapor for 14 hours each day (on at 7:30 PM and off at 9:30 AM) for 7 weeks, resulting in blood alcohol concentrations (BACs) between approximately 200 and approximately 300 mg/dL. Controls were kept in identical chambers with normal air flow. Once weekly, blood was collected from the lateral tail vein. BACs were assessed using quantitative gas chromatography.[27] Molecular and biochemical analyses in these studies were conducted using NAc (coordinates[28] relative to bregma: +0.84 to +2.76) collected 3 weeks after the end of the last exposure in order to assess persistent, rather than intoxication—or withdrawal-related effects of alcohol exposure.[29,30]

2.2 | Cell culture and lipopolysaccharide treatment

Microglial BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM Glutamax, 10% fetal bovine serum (FBS), 100-units/mL penicillin, and 100-µg/mL streptomycin (Life Technologies, Carlsbad, CA, USA). Prior to treatment, the cells (80% density) were left overnight in reduced FBS (2%) media. The following morning, the cells were treated with 10-ng/mL lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, USA) or PBS vehicle control in reduced FBS (2%) media. Cells were collected for simultaneous RNA isolation and protein extraction at the indicated hours after treatment.

2.3 | KDM6B knockdown, overexpression, and Cas9/CRISPR knockout

For siRNA knockdown, BV-2 cells were transfected with predesigned Silencer select siRNAs (Thermo Fisher Scientific) targeting mouse KDM6B or scrambled control using Lipofectamine 2000 CD (Invitrogen, Carlsbad, CA, USA). After 24 hours, transfected cells were treated with 10-ng/mL LPS or vehicle (PBS) and then collected for RNA isolation. Overexpression of KDM6B in BV-2 cells was achieved using a plasmid (OriGene, Rockville, MD, USA) and Lipofectamine 2000 CD (Invitrogen, Carlsbad, CA, USA). KDM6B knockout cell lines were generated using CRISPR/Cas9 expression plasmids (GeneScript U3789CF280_1,2,3). Three plasmids were tested, each expressing a puromycin resistance gene, Cas9, and unique guide RNA pairs (gRNAs: TCTCATGGCAGTAGCTCCGG; TCACGGGAAGTTGGAATCCC; TGGAGGAAGCTTCGCCGAGC) designed to eliminate KDM6B coding sequence by conferring sequence-specific exonuclease activity. Following transfection, BV-2 cells were selected using puromycin, and surviving colonies were manually picked and expanded. Clonal cell lines were propagated and analyzed for KDM6B gene editing using polymerase chain reaction (PCR) with primers (forward: TCTAGGATTGGAGGGAAATTGG; reverse: AAAGTACGGCCAAGGACA) within gRNA seed sequences.

2.4 | Human alcoholic cohort

Frozen microdissected human postmortem brain tissue samples of the anterior cingulate cortex (ACC) were obtained from the New South Wales Tissue Resource Centre (http://www.neura.edu.au/sydneybrainbank) at the University of Sydney, Australia. Tissues from 27 male subjects of European descent consisting of 13 chronic alcoholics and 14 control cases were used for this study (Table 1). Subject affiliation with the alcoholic or control group was confirmed postmortem using the Diagnostic Instrument for Brain Studies—Revised (DIBS-R), which is consistent with the criteria of the Diagnostic and Statistical Manual for Mental Disorders, 4th edition (DSM-IV).[31] All alcoholic subjects had consumed more than 80 g of ethanol per day while the control cases had an average daily consumption below 20 g. No blood alcohol or significant amounts of psychiatric medication (conc. > 1.0 mg/L) was detected at autopsy. There were no significant differences in age and postmortem interval (PMI) between alcoholics

TABLE 1 Summary statistics of the demographic characteristics for the postmortem alcoholic cohort (mean ± SEM)

Human Subjects	*N*	Age in Years	PMI in Hours	Brain pH	Smoking History
Controls	17	56.7 (2.6)	20.7 (1.6)	6.6 (0.05)	10/17
Alcoholic	16	59.6 (2.9)	25.9 (3.3)	6.4 (0.09)	10/11 available

and controls (58.1 ± 10.91 and 56.3 ± 9.92 y, t = 0.46; 25.3 ± 9.17 and 21.2 ± 6.44 h, t = 1.34, mean ± SD, respectively).

2.5 | RNA isolation and quantitative real-time PCR

For brain tissues, dmPFC, NAc, dorsal striatum (DS), and cerebellum (CB) were dissected as described[32] and flash frozen. Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) as previously described[33]; samples underwent a cleanup step using the RNeasy Mini Kit (Qiagen, USA) and were then treated with RNase-free DNase (Promega, USA) following manufacturer's instructions to mitigate contamination by genomic DNA. All RNA samples had 260/280 ratios between 1.8 and 2.1 and were evaluated for quality using an Agilent 2100 Bioanalyzer. RNA integrity numbers (RINs)[34] were 7.12 ± 0.23 and 7.37 ± 0.24 in alcoholics and controls, respectively, with no significant group difference (P = .78). Complementary DNA (cDNA) was generated using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) on a Veriti 96-well Fast Thermal Cycler (Life Technologies, Carlsbad, CA, USA). For in vitro experiments, medium was removed, and TRIzol reagent was added promptly to cells, and RNA was then isolated using the same procedures. Inventoried TaqMan Gene expression assay probes (KDM6B, Mm00801998, Rn01471506_m1, or HS 00996325_g1; IL-6, Mm00446190, or Rn01410330_m1; TNF-α, Mm00443258, or Rn01525859_g1; GAPDH, 4308313; GUSB, Rn00566655_m1; B2M, Mm00437762, or Rn00560865_m1; Life Technologies, Carlsbad, CA, USA) were used to examine the expression of target genes on a QuantStudio 6 Flex (Life Technologies, Carlsbad, CA, USA). Target gene expression was quantified relative to endogenous controls by calculating a relative quantification value using $2^{-\Delta\Delta CT}$ analysis.[35]

2.6 | NanoString analysis

NanoString nCounter analysis was performed by the Oncogenomics Core Facility (University of Miami Miller School of Medicine, Miami, FL, USA). nSolver Analysis Software (NanoString Technologies, Seattle, WA, USA)[36] was used to normalize counts by lane using the positive control samples. Data were then normalized to the reference genes HPRT, SDHA, and ATAT1. Genes that passed a nominal significance cutoff between control and postdependent rats were considered for further validation (P < .05, n = 4).

2.7 | Protein and western blot analysis

Tissue samples were homogenized in RIPA buffer containing 50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 1mM EDTA, and 1X Complete Mini EDTA-free protease inhibitor (Roche, Indianapolis, IN, USA). Samples were sonicated, and debris removed by centrifugation before protein levels were quantified using the BCA assay (Thermo Fisher Scientific Pierce, Rockford, IL, USA). Cell lysates were subjected to simultaneous RNA and protein extraction using the Qiagen AllPrep RNA/Protein kit (Qiagen, Louisville, KY, USA). Proteins were further purified using cold acetone as described by the manufacturer and resuspended in 20mM Tris-HCl (pH 7.5) and 500mM NaCl. A 5% SDS in Tris pH 8.0 was added to samples with low pH (pH < 3). For western blot analysis, protein (30 μg) was size separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to standard immunoblotting procedures using the following antibodies: KDM6B (OriGene #TA319844, Rockville, MD, USA), ACTB (Sigma-Aldrich #A1978, St. Louis, MO, USA), GAPDH (Santa Cruz #sc-47724, CA, USA), and Histone H3 or H3K27me3 (Cell Signaling #4499 and #9733, Danvers, MA, USA). Secondary HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz #sc-2318, CA, USA). Antibody staining was detected by chemiluminescence (Bio-Rad, Hercules, CA, USA) and visualized by C-DiGit Blot Scanner (Li-Core, Lincoln, Nebraska, USA).

2.8 | Chromatin immunoprecipitation sequencing analysis

Chromatin immunoprecipitation (ChIP), followed by massively parallel DNA sequencing (seq), and bioinformatics analysis was carried out as previously described.[21] Briefly, n = 4 control and n = 4 postdependent rat NAc were used to immunoprecipitate H3K27me3 (4-μg rabbit anti-H3K27me3, Cell Signaling #9733, Danvers, MA) or rabbit anti-IgG (Millipore #12-370, Billerica, MA). Specificity of the H3K27me3 antibody was confirmed by ChIP quantitative real-time PCR (qPCR), where the SNAP-ChIP K-MetStat Panel (EpiCypher #19-1001) of DNA-barcoded recombinant nucleosomes was spiked into a ChIP experiment prior to chromatin fragmentation.[37] Recovery of on-target nucleosomes (H3K27me3) compared with related lysine methylation states was deciphered by TaqMan qPCR using the SNAP-ChIP Dual Labeled Hydrolysis Probe (EpiCypher #18-6001) and the Full Panel Primer Set (EpiCypher #18-6101). DNA sequencing for the ChIP samples as well as each respective genomic DNA input was performed by the Center for Genome Technology Sequencing Core (Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Miami, FL). DNA concentration and shearing were determined by High Sensitivity DNA Assay (Agilent Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA). Seven nanograms of DNA was used for 2 × 100 paired-end (PE)–multiplexed library preparation (NEBNext Ultra DNA Kit, New England Biolabs, Ipswich, MA). Sequencing was done using an Illumina HiSeq2000 with a PE rapid flow cell. Bioinformatic analysis was performed according to our previously described pipeline[21] using SICER[38] to identify H3K27me3 regions of enrichment followed by edgeR[39] analysis to determine statistically significant differences between groups. H3K27me3 regions of enrichment were determined to be within a gene if they lie between 2000 BP upstream of the first exon to 500 BP downstream of the last exon, as determined by the geneXtendeR Bioconductor package.[40] Only genes passing a false discovery rate (FDR)–corrected P value less than .05 (Benjamini-Hochberg method) were used for pathway analysis in WebGestalt.[41] Alignment statistics are shown in Table 2, and full results are shown in Table S2.

TABLE 2 Alignment statistics for the H3K27me3 ChIP-seq analysis

Sample Name	Total Reads	Mapped Reads	Alignment Percentage
Input-control 1	53 543 238	51 732 761	96.62
Input-control 2	53 537 640	51 129 030	95.50
Input-control 3	47 315 024	45 169 753	95.47
Input-control 4	46 416 866	44 049 063	94.90
Input-postdependent 1	51 216 186	48 760 502	95.21
Input-postdependent 2	47 548 502	45 966 330	96.67
Input-postdependent 3	46 153 752	43 863 316	95.04
Input-postdependent 4	45 842 958	43 709 443	95.35
H3K27me3 ChIP-control 1	50 856 100	48 842 106	96.04
H3K27me3 ChIP-control 2	48 540 848	46 447 296	95.69
H3K27me3 ChIP-control 3	39 959 660	38 102 918	95.35
H3K27me3 ChIP-control 4	40 344 610	38 379 721	95.13
H3K27me3 ChIP-postdependent 1	41 866 428	40 016 347	95.58
H3K27me3 ChIP-postdependent 2	42 302 068	40 179 476	94.98
H3K27me3 ChIP-postdependent 3	41 304 560	39 218 849	94.95
H3K27me3 ChIP-postdependent 4	40 011 770	38 318 283	95.77

Abbreviation: ChIP-seq, chromatin immunoprecipitation sequencing.

2.9 | Statistical analysis

Statistics were done in GraphPad Prism (version 5.0d). The precise statistical tests are described alongside each result.

3 | RESULTS

3.1 | The histone demethylase KDM6B is dysregulated in alcohol-dependent rats

To examine mRNA expression of epigenetic enzymes in alcohol-dependent rats, we used NanoString nCounter analysis, a molecular barcoding technology that enables highly quantitative analysis of nucleic acids.[36,42] NanoString analysis in a small cohort of postdependent rats (n = 4) revealed that out of 114 epigenetic enzymes tested, 11 were dysregulated in the NAc during protracted abstinence (Figure 1A). Our previous whole transcriptome analysis[21] showed that one of these enzymes, the histone demethylase KDM6B, was also downregulated in the dmPFC of postdependent rats at the same time point (3 wk into withdrawal). qPCR validation from a larger cohort confirmed that KDM6B mRNA is significantly and persistently downregulated in both the NAc (1.49-fold decrease, t_{14} =3.12, P = .0038, n = 6 control, n = 10 postdependent) and the dmPFC (1.94-fold decrease, t_{12} = 3.24, P = .0035, n = 6 control, n = 8 postdependent) of postdependent rats (Figure 1B,C). To determine whether KDM6B downregulation in postdependent rats is region specific, we quantified KDM6B expression in the DS and CB. KDM6B expression was not altered in either region (Figure 1D,E), suggesting that KDM6B mRNA downregulation in alcohol dependence occurs in specific brain reward circuits rather than being a global effect of alcohol exposure.

3.2 | Alcohol-induced dysregulation of KDM6B protein is associated with altered enzymatic function

To determine whether dysregulation of KDM6B mRNA in alcohol-dependent rats could affect the epigenetic function of KDM6B, we used western blot analysis to quantify KDM6B protein levels as well the histone target modification catalyzed by the KDM6B enzyme. KDM6B is a histone demethylase that has been extensively characterized to specifically remove trimethylation on histone H3 at lysine 27 (H3K27me3) without affecting dimethylation at this site or methylation at other histone H3/H4 residues.[43] Surprisingly, we found that protein levels of KDM6B were significantly increased in alcohol-dependent rat NAc (Figure 2A) (1.64-fold increase, t_{14} = 2.21, P = .022, n = 7 control, n = 9 postdependent). This was accompanied by the expected decrease in H3K27me3 (Figure 2B) (1.33-fold decrease, t_{13} = 1.81, P = .047, n = 5 control, n = 10 postdependent), consistent with the known function of KDM6B. Due to the inconsistency between KDM6B mRNA downregulation compared with protein upregulation in postdependent animals, we repeated the western blot analysis in an independent cohort of rats subjected to the same chronic and intermittent alcohol exposure.

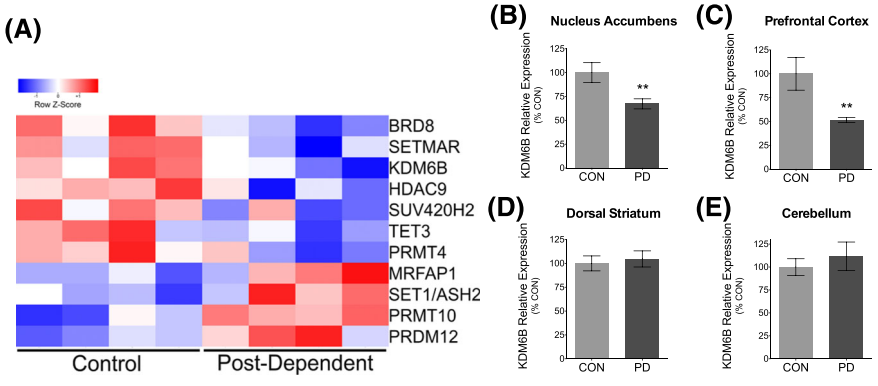

FIGURE 1 KDM6B mRNA is persistently dysregulated in a region-specific manner as a consequence of alcohol dependence induced by chronic and intermittent alcohol exposure. A, Heat map showing epigenetic enzyme mRNAs that were significantly dysregulated in NanoString nCounter analysis of alcohol-dependent ("postdependent [PD]") rat nucleus accumbens (NAc) (P < .05, n = 4). B-E, KDM6B-relative mRNA expression in reward-associated and control brain regions of PD rats relative to controls (CON) (**P < .01, n = 6 CON, n = 8-10 PD)

At the same time point of 3 weeks after cessation of alcohol exposure, we again found that KDM6B protein was upregulated in the rat NAc (1.60-fold increase, t_{13} = 2.02, P = .033, n = 7 control, n = 8 postdependent) and H3K27me3 levels were decreased (1.23-fold decrease, t_{12} = 4.11, P = .0007, n = 7 control, n = 7 postdependent) (Figure 2C-E).

To determine whether these findings translated to human subjects, we quantified KDM6B expression in postmortem brain tissue samples from alcohol-dependent patients and controls matched for age and postmortem interval.[44-47] In ACC, a region that is anatomically and functionally related to the rodent mPFC,[48] we found a significant 1.86-fold increase (P = .0386, t_{25} = 2.183) in KDM6B transcript levels in alcoholics compared with controls (Figure 3), suggesting that the KDM6B dysregulation observed in postdependent rats could reflect the pathophysiology of alcohol dependence in humans.

3.3 | KDM6B expression is dynamically regulated over the time course of an inflammatory response

We observed discrepancies in the direction of change for alcohol-associated KDM6B dysregulation (mRNA downregulation vs protein upregulation in postdependent rats and mRNA downregulation in

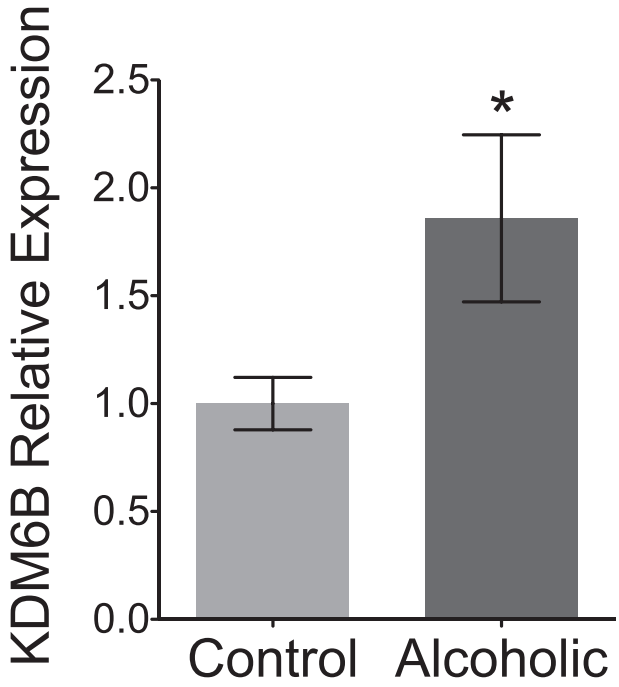

FIGURE 3 KDM6B mRNA is upregulated in postmortem alcoholic brain (n = 17 controls, n = 16 alcoholic, unpaired t test: P < .05)

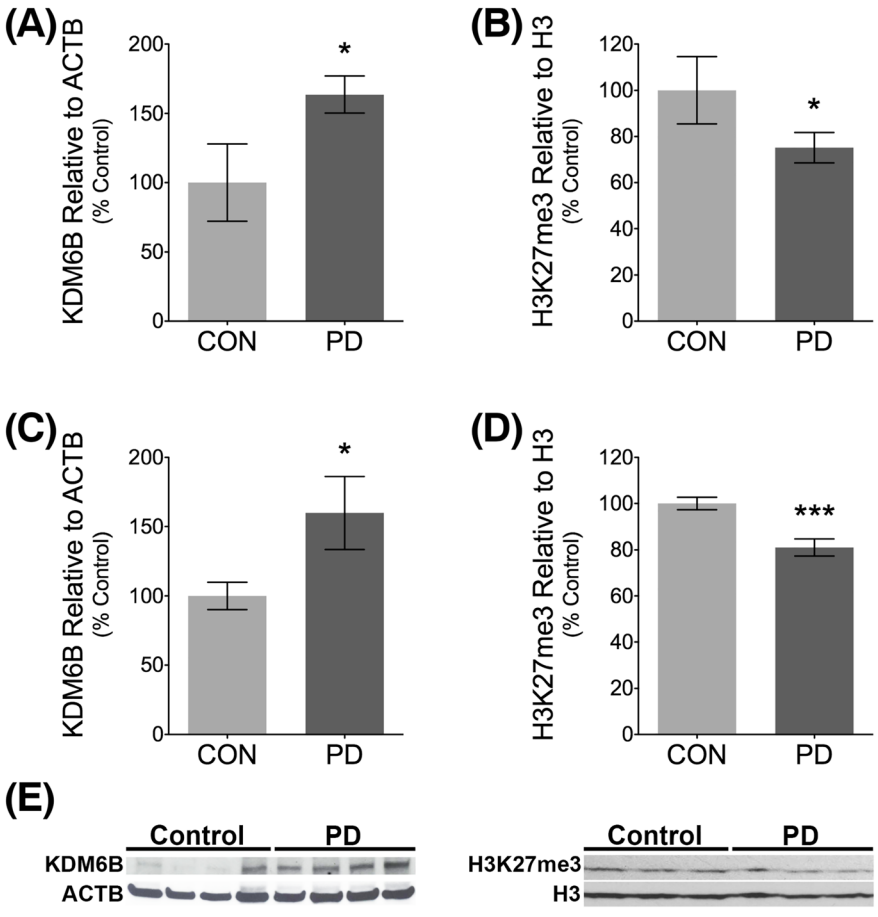

FIGURE 2 KDM6B protein levels are increased, and H3K27me3 levels are decreased in the nucleus accumbens of alcohol-dependent rats. A,B, KDM6B (A) and H3K27me3 (B) levels relative to ACTB and total histone H3, respectively, in control (CON) compared with postdependent (PD) rat nucleus accumbens (*P < .05, n = 5-7 CON, n = 9-10 PD). C,D, KDM6B (C) and H3K27me3 (D) levels in an independent group of rats subjected to the same dependence induction paradigm (*P < .05, ***P < .001, n = 7 CON, n = 7-8 PD). E, Representative western blot image

postdependent rats vs mRNA upregulation in human alcoholics). Therefore, we sought to further characterize the function of KDM6B in the nervous system and its response to environmental stimuli. The majority of KDM6B studies have demonstrated a pivotal role for this enzyme in modulation of macrophage inflammatory responses.[49] Comparatively little is known about the role of KDM6B in the brain besides a handful of studies showing its involvement in neuronal differentiation and survival.[50,51] According to FACS analyses from rodent brain,[52,53] including publicly available RNA-sequencing data[53,54] (data accessible at NCBI GEO database, accession number GSE52564[55]), KDM6B is significantly enriched (greater than twofold increase) in microglia (one-way ANOVA: $F_{6,13} = 11.89$, $P = .0023$, $n = 2$) (Figure 4A). These data suggest that KDM6B may play a specialized role in neuroimmune responses and make cultured microglial cells an ideal model system to investigate the function of this enzyme in the brain.

Due to the well-established role of KDM6B in inflammation, its enrichment in microglial cells, and the causal role of inflammation in alcohol dependence,[1] we first investigated in detail the KDM6B response to an inflammatory stimulus in cultured microglial BV-2 cells. Induction of an inflammatory response by treatment with LPS was verified by rapid and robust induction of two well-known inflammatory cytokines: TNF-α (two-way ANOVA revealed a significant effect of time: $F_{7,32} = 33.92$, $P < .0001$, treatment: $F_{1,32} = 337.1$, $P < .0001$, and interaction: $F_{7,32} = 36.15$, $P < .0001$; $n = 3$) and IL-6 (two-way ANOVA revealed a significant effect of time: $F_{7,32} = 82.33$, $P < .0001$, treatment: $F_{1,32} = 402.1$, $P < .0001$, and interaction: $F_{7,32} = 82.34$, $P < .0001$; $n = 3$) (Figure 4B and 4D). TNF-α mRNA peaked at 2 hours, while IL-6 mRNA peaked at 4 hours post-LPS treatment. Both cytokines returned to baseline levels (no significant differences between LPS and vehicle controls) by 20 hours posttreatment. KDM6B mRNA was also rapidly and significantly increased by LPS (two-way ANOVA revealed a significant effect of time: $F_{7,32} = 33.82$, $P < .0001$, and interaction: $F_{7,32} = 50.07$, $P < .0001$; $n = 3$) (Figure 4F). After 2 hours of treatment, KDM6B mRNA expression peaked at 4.2-fold increase compared with vehicle control. This was followed by a rapid decrease in KDM6B to vehicle control levels by 4 hours of treatment. Interestingly, by 8 hours of treatment, KDM6B mRNA levels dipped significantly below vehicle control and remained below controls during the full course of the 24 hours examined.

Western blot analysis of KDM6B protein levels over the same time course confirmed KDM6B upregulation by LPS, which peaked at a later time point of 8 hours ($F_{7,15} = 14.72$, $P < .0001$, $n = 3$) (Figure 4E). As with the mRNA, KDM6B protein also eventually

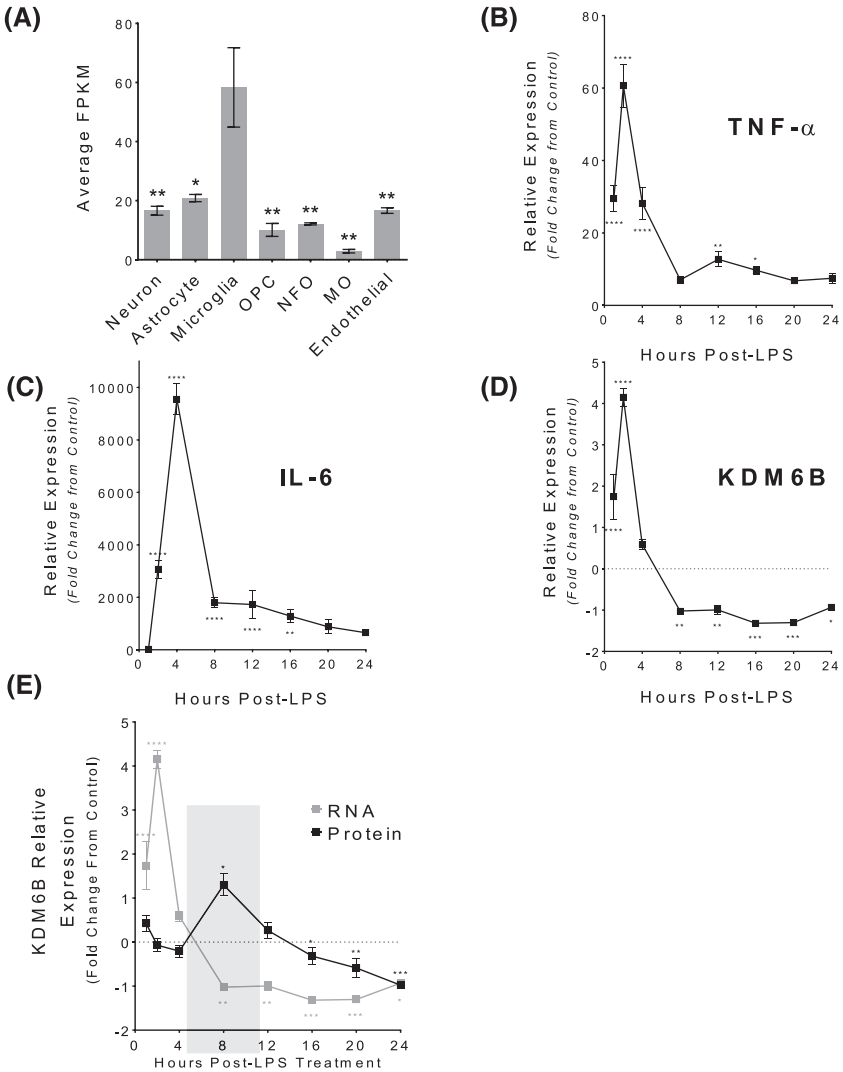

FIGURE 4 KDM6B expression is acutely increased but subsequently decreased compared with vehicle control as a consequence of an inflammatory stimulus. A, KDM6B expression is significantly enriched in microglia compared with other brain cell types according to RNA-sequencing data[53] (NCBI GEO accession number GSE52564) (asterisks indicate significant differences between microglia and the indicated cell type by one-way ANOVA with Bonferroni posttest, *$P < .05$, **$P < .01$, $n = 2$). B-D, A time course analysis of TNF-α (B), IL-6 (C), and KDM6B (D) mRNA expression after 10-ng/mL lipopolysaccharide (LPS) treatment in cultured BV-2 microglial cells (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$, $n = 3$). E, Comparison of KDM6B mRNA (gray) versus protein (black) in response to LPS treatment (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$, $n = 3$). Gray box indicates a time point at which the protein is significantly upregulated but the mRNA is significantly downregulated compared with vehicle control

decreased below control levels by 16 hours. At 8 hours, KDM6B protein was significantly elevated; however, the mRNA was significantly decreased below vehicle control (gray box in Figure 4E). These data show that there are time points during the course of an inflammatory response at which opposing directions of change in KDM6B mRNA as compared with protein levels can be observed. Furthermore, these data may indicate that KDM6B has a delayed mechanism of action in response to inflammation.

3.4 | Inflammatory signaling pathways are epigenetically regulated by H3K27me3 in the alcohol-dependent brain

In order to determine the genes that could be subject to alcohol-induced epigenetic dysregulation as a consequence of KDM6B/H3K27me3 signaling, we performed ChIP followed by whole genome sequencing (ChIP-seq). Similar to our previous approach,[21] we elected to immunoprecipitate genomic loci associated with H3K27me3, the modification demethylated by KDM6B, rather than performing ChIP for KDM6B directly. This allowed us to identify persistent epigenetic changes rather than trying to find the ideal time point to capture a more transient enzyme-substrate interaction between KDM6B and its target. In light of recent findings casting doubt on the specificity of ChIP-grade histone modification antibodies,[37] we performed extensive optimization experiments to ensure a clean ChIP signal for our exploratory and unbiased analysis. The H3K27me3 antibody demonstrated clear enrichment of the target Post translational modification (PTM) in a ChIP experiment without cross-reacting with H3K27me2, H3K27me1, or any methylation states at H3K4, H3K9, H3K36, or H4K20 (Figure 5A). Further, our protocol showed clear enrichment of the ChIP signal over IgG negative controls (Figure 5B). BRDT, a testis-specific gene that is silenced in the brain, was enriched for the transcriptionally repressive H3K27me3 modification. In contrast, H3K4me3, a modification associated with transcriptional activation, was more highly associated with ACTB, a gene that is highly expressed in the brain (two-way ANOVA revealed a significant effect of gene: $F_{1,10} = 11.13$, $P < .0075$, IP: $F_{2,10} = 88.50$, $P < .0001$, and interaction: $F_{2,10} = 65.37$, $P < .0001$; $n = 3$) (Figure 5B).

Using our optimized ChIP protocol, we isolated H3K27me3-enriched genomic regions in control compared with postdependent rat NAc. Of the SICER-identified H3K27me3-marked genomic regions, a greater proportion (71%) was more enriched in control rats as compared with postdependent rats (Figure 5C). This epigenetic signature is consistent with our western blot analysis showing upregulation of KDM6B protein and decreased H3K27me3 in postdependent NAc. These regions were subjected to edgeR analysis to determine statistically significant differences between control and postdependent rats ($n = 4$). In total, 93 regions of H3K27me3 enrichment exhibited FDR-corrected, statistically significant differences (Table S2). To identify the signaling pathways significantly overrepresented in this list, we performed a WebGestalt WikiPathways enrichment analysis.[41] In this analysis, the IL-6 signaling pathway was the top result (FDR $P = .0021$, Table 3).

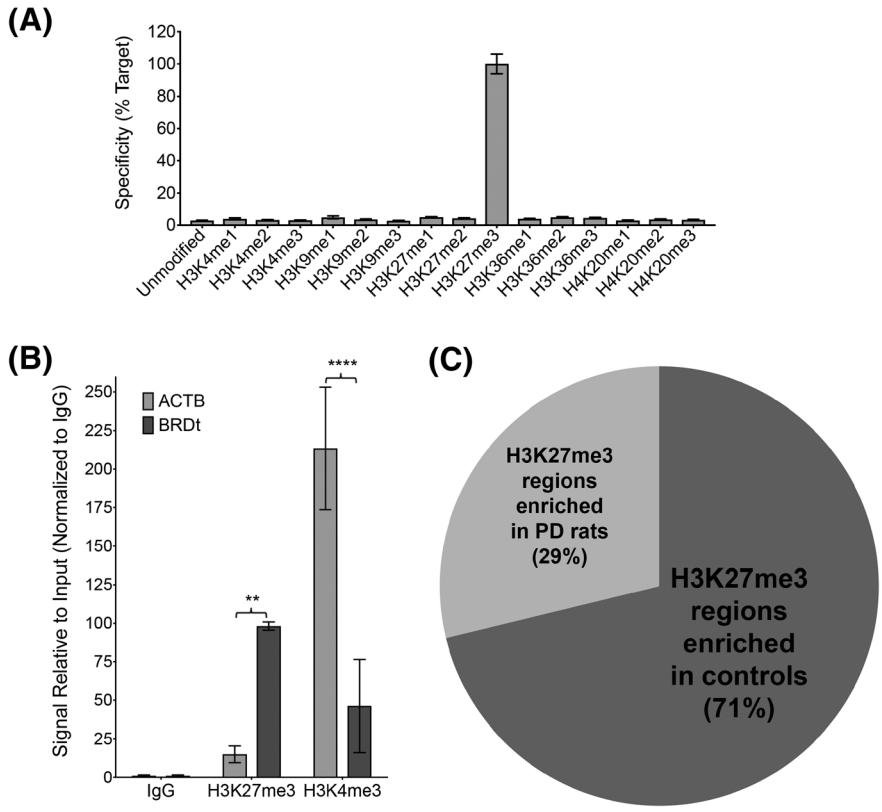

FIGURE 5 Chromatin immunoprecipitation sequencing (ChIP-seq) analysis of H3K27me3 in control compared with postdependent (PD) nucleus accumbens. A, A ChIP quantitative real-time polymerase chain reaction (qPCR) experiment demonstrated that the H3K27me3 ChIP antibody showed high specificity for the target PTM when tested against a panel of spike-in recombinant nucleosomes bearing related methylation states that were distinguished by primers specific to unique DNA barcodes. B, Optimization of ChIP-seq protocol in rodent brain shows enrichment of the antibody signals over IgG controls. H3K27me3, a repressive epigenetic modification, is highly associated with a gene that is suppressed in the brain (BRDT) and lowly associated with an actively transcribed gene (ACTB). In contrast, H3K4me3, a mark associated with transcriptional activation, is enriched for ACTB over BRDT. C, ChIP-seq analysis shows that the majority of H3K27me3-associated genomic regions (71%) are enriched in control rats as compared with PD rats ($n = 4$)

TABLE 3 Pathway analysis of the genes differentially enriched for H3K27me3 in control compared with postdependent NAc (ChIP-seq data, $n = 4$)

Pathway Name	Gene Names	No. of Genes vs Expected Number	FDR *P* Value
IL-6 signaling pathway	Stat5a, Fgr, Map 2k4	3/0.14	.0021
Adipogenesis	Cdkn1a, Stat5a, Ppard	3/0.16	.0021
Kit receptor signaling pathway	Stat5a, Fgr	2/0.09	.0042
TGF-beta signaling pathway	Eng, Zfp423	2/0.07	.0042
TGF-beta receptor signaling pathway	Cdkn1a, Eng, Rbx1	3/0.29	.0042
ErbB signaling pathway	Cdkn1a, Stat5a	2/0.08	.0042
Apoptosis	Irf4, Map 2k4	2/0.12	.006

Abbreviations: ChIP-seq, chromatin immunoprecipitation sequencing; FDR, false discovery rate; NAc, nucleus accumbens.

3.5 | Knockdown and genome editing of KDM6B suppress induction of IL-6 in vitro

To determine whether KDM6B causally impacts IL-6 signaling, we knocked down KDM6B levels in cultured BV-2 microglia. As is seen in previous reports,[49,56,57] treatment with LPS-induced KDM6B mRNA expression. However, expression was significantly reduced to 29.3% of scramble control levels by KDM6B siRNA (two-way ANOVA revealed a significant effect of siRNA: $F_{1,8} = 130.9$, $P < .0001$, treatment: $F_{1,8} = 163.1$, $P < .0001$, and interaction: $F_{1,8} = 67.68$, $P < .0001$; $n = 3$) (Figure 6A). IL-6 was also induced by LPS treatment but was significantly attenuated by KDM6B siRNA (two-way ANOVA revealed a significant effect of siRNA: $F_{1,8} = 13.64$, $P < .0061$, treatment: $F_{1,8} = 357.0$, $P < .0001$, and interaction: $F_{1,8} = 13.89$, $P < .0058$; $n = 3$) (Figure 6B). To further assess the role of KDM6B in microglial proinflammatory signaling, we used CRISPR/Cas9 genome editing to eliminate KDM6B coding sequence from BV-2 cells. Expression plasmids encoding Cas9, KDM6B gRNAs, and antibiotic resistance were used to transfect BV-2 cells. Clonal cells lines were selected using puromycin and manually isolated. To identify KDM6B deletion clones, genomic DNA was isolated and amplified by PCR using primers that anneal within the KDM6B gRNA seed sequences. Unedited DNA template produces a 456 base pair product while edited genomes are not compatible with amplification due to loss of complementary primer sequence. Several clones had diminished PCR yields and were chosen for further verification of KDM6B loss of function. Levels of KDM6B protein were quantified by western blot analysis revealing a pronounced depletion of KDM6B for clone 28 (Figure 6C). KDM6B RNA levels were reduced by approximately 50% for clone 28 as compared with the parent cell line, as determined by real-time PCR (Student's *t* test, $P < .005$) (Figure 6D). These data indicate that for clone 28, only one KDM6B allele was edited resulting in hemizygous expression of KDM6B. The fact that none of the clones were nullizygous indicates that complete loss of KDM6B is not compatible with cellular viability. This is consistent with the observation that KDM6B knockout in mice is lethal.[58] When exposed to LPS, there was a significant reduction (greater than 75-fold decrease) in IL-6 expression for clone 28 BV-2 cells as compared with wild-type BV-2 cells (one-way ANOVA, $F_{3,8} = 599.5$, $P < .0001$) (Figure 6E). The data indicate that depletion of KDM6B via genome editing attenuates proinflammatory cytokine signaling in response to LPS in BV-2 cells.

3.6 | Overexpression of KDM6B induces IL-6 expression in vitro

To assess whether increased KDM6B levels lead to IL-6 induction, BV-2 cells were transfected with a KDM6B expression plasmid. After 24 hours, IL-6 levels were robustly and significantly increased (greater than 300-fold increase) in KDM6B-transfected cells as compared with cells transfected with a control GFP expression plasmid (one-way ANOVA, $F_{2,6} = 284.2$, $P < .0001$) (Figure 7). To determine whether the KDM6B catalytic domain is required for the proinflammatory effects of overexpression in microglia, as has been shown in macrophage,[59] we mutated three amino acids to inactivate the JMJ domain (JMJΔ: H1388A, E1390G, and H1468A). Overexpression of KDM6B-JMJΔ in BV-2 cells significantly attenuated IL-6 induction (over 100-fold decrease) as compared with KDM6B-WT overexpression (one-way ANOVA, $F_{2,6} = 284.7$, $P < .0001$) (Figure 7). Taking these together, our data indicate that increased KDM6B catalytic activity induces IL-6 expression.

4 | DISCUSSION

Studies have shown that human alcoholics as well as animal models of alcohol abuse exhibit chronic alterations in gene expression that underlie neuroplastic and neuroimmune changes within the reward circuitry of the brain.[1-6] Despite these important insights, the precise mechanisms that lead to chronic disruption of gene expression profiles remain unclear. In the present study, we showed for the first time that a histone demethylase, KDM6B (also known as JMJD3), is persistently and region specifically dysregulated in alcohol-dependent rodent and human brains. Dysregulation of KDM6B in vivo was associated with altered epigenetic regulation of the IL-6 inflammatory signaling pathway in alcohol-dependent rat NAc. Knockdown of KDM6B in vitro suppressed IL-6 induction after an inflammatory stimulus, showing that KDM6B functionally contributes to the IL-6 inflammatory

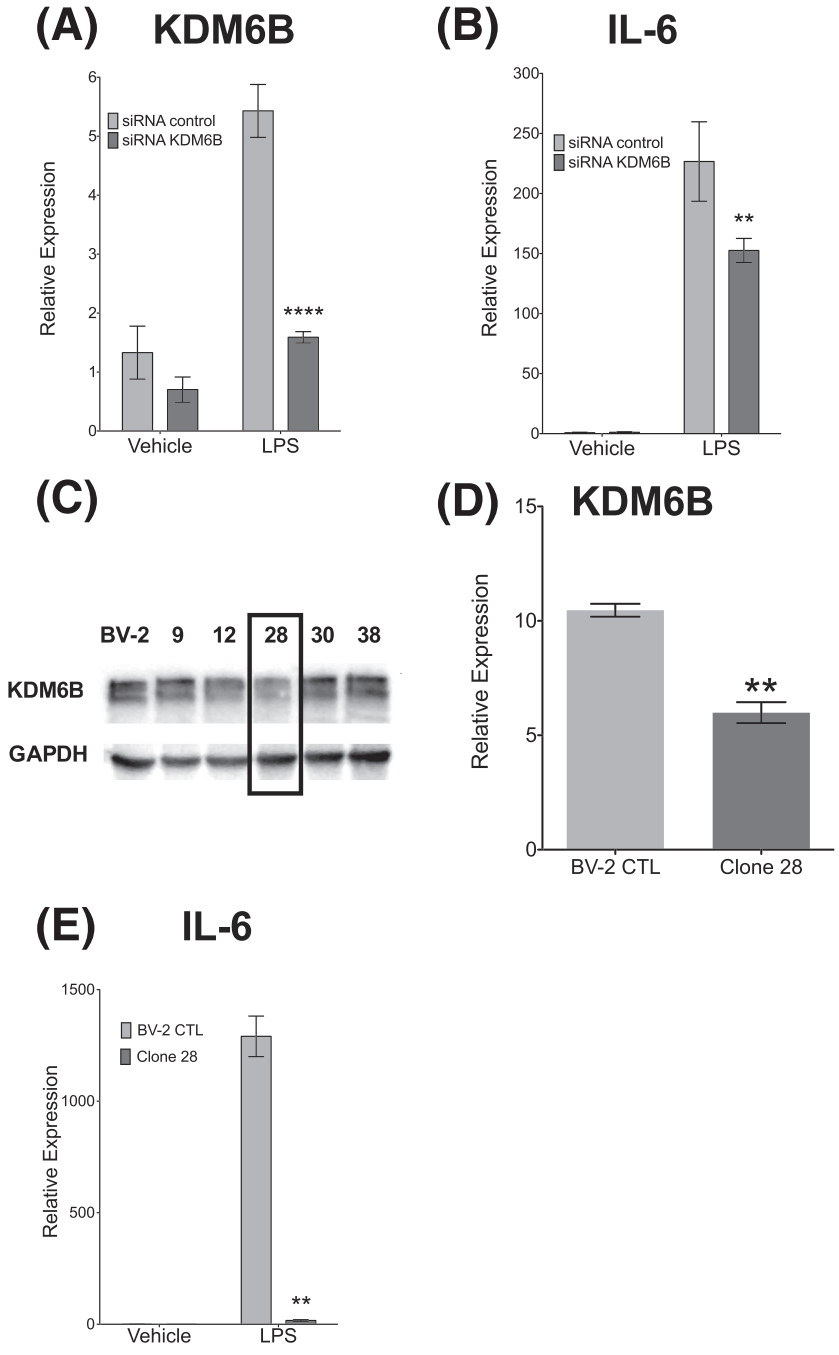

FIGURE 6 Knockdown and partial knockout of KDM6B suppress IL-6 upregulation in response to an inflammatory stimulus. (A) KDM6B and (B) IL-6 mRNA expression in scramble controls compared with KDM6B siRNA after lipopolysaccharide (LPS) treatment in cultured BV-2 microglial cells (**P < .01, ****P < .0001, n = 3). (C) Representative western blot from parent cell line and different clones after LPS induction. (D) KDM6B mRNA levels are reduced in clone 28 when induced with LPS (*P < .005, n = 3). (E) IL-6 mRNA levels in vehicle versus LPS-treated cells comparing BV-2 (control parent cell line) and clone 28 (partial knockout) (**P < .005, n = 3)

response in microglia. Moreover, genome editing of KDM6B using CRISPR/Cas9 further decreased IL-6 induction in vitro. Overexpression of KDM6B in BV-2 cells induces IL-6 expression while overexpression of mutant with three-point mutations within the catalytic domain did not, suggesting that the catalytic domain of KDM6B is necessary for IL-6 induction in microglia. Collectively, these findings indicate that cross talk between epigenetic and inflammatory signaling networks could contribute to the perpetuation of neuroimmune activation that has been implicated in compulsive alcohol seeking.

A series of groundbreaking recent studies have demonstrated a causal role for histone methyltransferase enzymes in neuroadaptations underlying cocaine and morphine addiction.[54,60-62] However, the roles of histone methylation networks in alcohol dependence are comparatively unexplored. Several studies have identified changes in histone acetylation and DNA methylation that are associated with alcohol use in both humans and rodents.[14-20] While such studies suggest that variations in ethanol-induced epigenetic responses could underlie vulnerability for alcohol dependence, little is known about the potential role of enzymes that control histone methylation and demethylation. Recently, our group showed that the histone methyltransferase PRDM2 contributes to behavioral and dmPFC transcriptomic changes underlying the development of alcohol addiction.[21] Although the present study uses a passive model of alcohol dependence (intermittent vapor exposure) that may not fully recapitulate compulsive alcohol intake, it results in behavioral traits similar to those seen in human alcoholics, including excess voluntary alcohol consumption, aversion-resistant

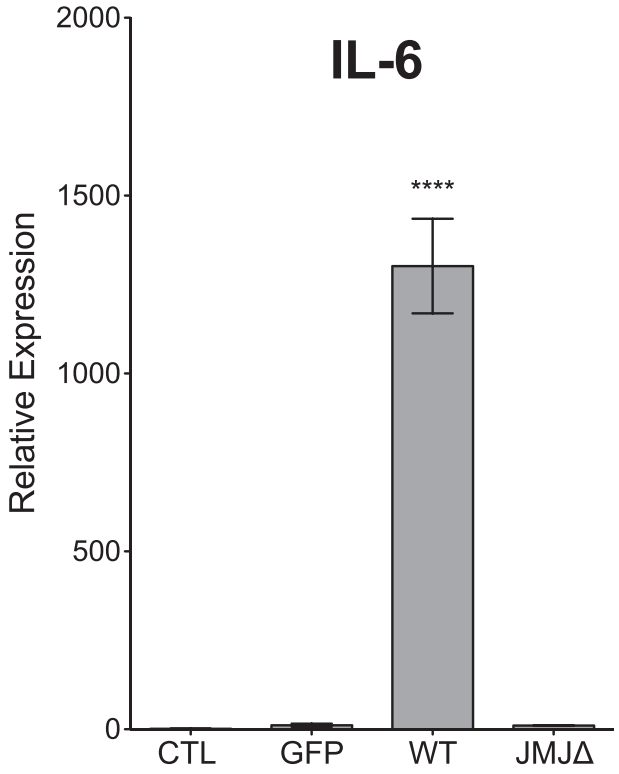

FIGURE 7 KDM6B expression increases IL-6 through a mechanism dependent on the JMJ demethylase domain. IL-6 mRNA expression levels are increased in KDM6B-transfected cells but not in JMJ mutant (catalytic domain deletion JMJΔ), GFP control plasmid, and control cells (****$P < .0001$, $n = 3$)

drinking despite adverse consequences, and stress-induced relapse.[21] Because KDM6B was specifically dysregulated in the prefrontal cortex and NAc but not in the DS or CB, KDM6B is not a global and nonspecific side effect of alcohol exposure. Rather, these findings are consistent with the notion that KDM6B could mediate changes in the neurochemical circuits that subserve altered reward perception and impulse control in alcohol addiction.

In our initial NanoString analysis, KDM6B mRNA was decreased 3 weeks after alcohol exposure in dependent rat NAc. This finding was confirmed by qPCR analysis in a larger cohort. However, we found that KDM6B protein was upregulated under the same conditions, a finding that was confirmed in two independent groups of animals. Both western blot and ChIP-seq analyses corroborated a decrease in H3K27me3, a finding consistent with increased enzymatic activity of KDM6B. Further, KDM6B mRNA was upregulated in postmortem brain from human alcoholics. In order to explore a potential mechanism that could explain these discrepant findings, we investigated KDM6B biochemical changes in cell culture. Although the function of KDM6B in the brain has only begun to be uncovered, several studies have shown that KDM6B is enriched in microglia, the resident immune cells in the brain, compared with other brain cell types.[52-54] Two studies of cultured microglial cells have shown that KDM6B modulates the induction of immune response genes in response to inflammatory stimuli,[56,57] consistent with the well-characterized role of KDM6B to mediate inflammatory responses in peripheral macrophages.[49] Because inflammation is increasingly recognized to underlie the development of alcohol addiction,[1] we chose cultured microglial cells as a model system and investigated the KDM6B response to an inflammatory stimulus.

KDM6B expression in BV-2 microglia was initially increased in response to LPS treatment during a similar time course as the proinflammatory cytokines IL-6 and TNF-α. However, at later time points, KDM6B mRNA levels dipped significantly below vehicle controls. Therefore, depending on the time point observed, the direction of KDM6B expression changes in response to inflammation could be opposite. Further, at 8 hours, KDM6B protein was significantly increased whereas the mRNA levels were significantly decreased. This study showed that in vitro KDM6B is dynamically regulated over the course of an inflammatory response and further, there is a window of time where KDM6B protein is upregulated and the mRNA is simultaneously downregulated. In vivo, in the context of prolonged alcohol exposure, it is possible that the KDM6B protein is similarly dynamically regulated during the course of acute alcohol exposure, the development of tolerance and dependence, acute withdrawal, and prolonged abstinence. Future studies could also investigate whether KDM6B protein induces regulatory mechanisms that inhibit transcription of its own gene through negative feedback. One potential limitation of our study is the use of LPS for induction of KDM6B in vitro, rather than ethanol. While ethanol has been shown to activate microglial cells in vitro,[1] acute exposure does not fully recapitulate the effects of chronic alcohol exposure in vivo. Moreover, LPS is a more potent inducer of KDM6B known to be mediated through TLR4 receptors[63-65] and downstream cell signaling pathways.[49,56,57]

In order to understand the potential role of KDM6B in addiction, we performed an unbiased ChIP-seq analysis of tissue from postdependent rat NAc to identify genes that exhibit an epigenetic signature consistent with KDM6B upregulation in alcohol dependence. Pathway analysis implicated IL-6 as the most significantly enriched pathway in the list of genes less enriched for H3K27me3 in alcohol-dependent rats compared with controls. Knockdown of KDM6B has previously been shown to suppress inflammation-induced IL-6 levels in cultured microglia,[56] a finding that we confirmed in vitro. In contrast, KDM6B knockdown did not affect TNF-α levels, consistent with previous studies that show KDM6B oversees specific and context-dependent aspects of the inflammatory response.[49] Moreover, we show that in vitro KDM6B catalytic domain is required for IL-6 expression and that partial knockout is sufficient to decrease IL-6 response almost completely.

Alcohol increases transcription of proinflammatory cytokines, including IL-6, TNF-α, IL-1β, and others.[1,66] IL-6 is upregulated, and the IL-6 signaling pathway is overrepresented in transcriptomic analysis of brain reward regions in rats bred to consume excess alcohol.[67,68] IL-6 knockout mice exhibited reduced alcohol consumption in a two-bottle choice test, showing for the first time that this gene modulates alcohol intake.[69] Positive feedback loops in immune response pathways and prolonged alcohol exposure create a progressive state of brain inflammation that underlies impulsive drug

seeking and negative affect.[1] Furthermore, sustained elevation of inflammatory cytokines has been shown to increase alcohol craving and severity of dependence.[70,71] Identifying epigenetic mechanisms that cross talk with inflammatory signaling pathways could therefore be essential to understand the underlying processes that contribute to the persistence of neurobiological and behavioral changes in alcoholism.

The precise mechanisms through which KDM6B modulates gene expression have been predominantly explored in the context of cancer and peripheral macrophage inflammatory responses. The preponderance of these studies showed that KDM6B couples with transcription factors in order to modulate transcription of inflammatory genes. For instance, KDM6B is known to directly interact with NF-κB, IRF4, SMAD3, T-bet, and p53 to mediate transcriptional coactivation of immune response genes.[49] One study in cultured microglia showed that KDM6B cooperated with STAT1 and STAT3 to regulate levels of inflammatory response genes, including IL-6 and others.[56] Future studies should seek to identify potential transcriptional cofactors that could cooperate with KDM6B in the context of alcohol dependence. Additionally, although KDM6B has been shown to remove trimethyl marks on H3 specifically at lysine 27 residues without affecting other H3/H4 methylation marks, KDM6B also has known roles outside of histone demethylation. For example, KDM6B is capable of recruiting transcriptional cofactors such as SWI/SNF chromatin remodeling complexes to inflammatory gene promoters.[49,72] Although our ChIP-sequencing study focused on the histone demethylase function of KDM6B, future studies could explore a potential role of demethylase-independent KDM6B functions in alcohol-mediated gene expression changes.

There is a strong emerging interest and increasing precedent of clinical success for targeting epigenetic enzymes as therapeutic strategies for complex human diseases such as cancer, diabetes, and neurodegenerative disease.[73-75] Further, compounds that suppress inflammation have a proven record of clinical trial success for alcohol addiction.[76] Future studies should address the question of whether KDM6B functionally contributes to alcohol-seeking behavior. If so, KDM6B-targeted compounds could find therapeutic utility in alcohol use disorders by simultaneously correcting epigenetic and neuroinflammatory signaling processes.

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CONFLICT OF INTEREST

The authors declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

ORCID

Zane Zeier https://orcid.org/0000-0003-0623-8610

REFERENCES

1. Walter TJ, Vetreno RP, Crews FT. Alcohol and stress activation of microglia and neurons: brain regional effects. *Alcohol Clin Exp Res*. 2017;41(12):2066-2081.
2. Wolen AR, Phillips CA, Langston MA, et al. Genetic dissection of acute ethanol responsive gene networks in prefrontal cortex: functional and mechanistic implications. *PLoS ONE*. 2012;7(4):e33575.
3. Ron D, Jurd R. The "ups and downs" of signaling cascades in addiction. *Science's STKE: Signal Transduction Knowledge Environment*. 2005; 2005(309):re14.
4. Rimondini R, Sommer WH, Dall'Olio R, Heilig M. Long-lasting tolerance to alcohol following a history of dependence. *Addict Biol*. 2008; 13(1):26-30.
5. Mayfield RD, Lewohl JM, Dodd PR, Herlihy A, Liu J, Harris RA. Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. *J Neurochem*. 2002;81(4):802-813.
6. Lewohl JM, Wang L, Miles MF, Zhang L, Dodd PR, Harris RA. Gene expression in human alcoholism: microarray analysis of frontal cortex. *Alcohol Clin Exp Res*. 2000;24(12):1873-1882.
7. Wong CC, Mill J, Fernandes C. Drugs and addiction: an introduction to epigenetics. *Addiction*. 2011;106(3):480-489.
8. Morozova TV, Mackay TF, Anholt RR. Genetics and genomics of alcohol sensitivity. *Mol Genet Genomics: MGG*. 2014;289(3):253-269.
9. Dick DM, Foroud T. Candidate genes for alcohol dependence: a review of genetic evidence from human studies. *Alcohol Clin Exp Res*. 2003;27(5):868-879.
10. Agrawal A, Lynskey MT. Are there genetic influences on addiction: evidence from family, adoption and twin studies. *Addiction*. 2008; 103(7):1069-1081.
11. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. *Nat Rev Genet*. 2011;13(2):97-109.
12. Borrelli E, Nestler EJ, Allis CD, Sassone-Corsi P. Decoding the epigenetic language of neuronal plasticity. *Neuron*. 2008;60(6):961-974.
13. Copeland RA, Solomon ME, Richon VM. Protein methyltransferases as a target class for drug discovery. *Nat Rev Drug Discov*. 2009;8(9):724-732.

14. Qiang M, Denny A, Lieu M, Carreon S, Li J. Histone H3K9 modifications are a local chromatin event involved in ethanol-induced neuroadaptation of the NR2B gene. *Epigenetics*. 2011;6(9):1095-1104.
15. Botia B, Legastelois R, Alaux-Cantin S, Naassila M. Expression of ethanol-induced behavioral sensitization is associated with alteration of chromatin remodeling in mice. *PLoS ONE*. 2012;7(10):e47527.
16. Zhao R, Zhang R, Li W, et al. Genome-wide DNA methylation patterns in discordant sib pairs with alcohol dependence. *Asia-Pacific Psychiatry: Official Journal of the Pacific Rim College of Psychiatrists*. 2013;5(1):39-50.
17. Zhang R, Miao Q, Wang C, et al. Genome-wide DNA methylation analysis in alcohol dependence. *Addict Biol*. 2013;18(2):392-403.
18. Ponomarev I, Wang S, Zhang L, Harris RA, Mayfield RD. Gene coexpression networks in human brain identify epigenetic modifications in alcohol dependence. *J Neurosci*. 2012;32(5):1884-1897.
19. Warnault V, Darcq E, Levine A, Barak S, Ron D. Chromatin remodeling—a novel strategy to control excessive alcohol drinking. *Transl Psychiatry*. 2013;3(2):e231.
20. Barbier E, Tapocik JD, Juergens N, et al. DNA methylation in the medial prefrontal cortex regulates alcohol-induced behavior and plasticity. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 2015;35(15):6153-6164.
21. Barbier E, Johnstone AL, Khomtchouk BB, et al. Dependence-induced increase of alcohol self-administration and compulsive drinking mediated by the histone methyltransferase PRDM2. *Mol Psychiatry*. 2017;22(12):1746-1758. https://doi.org/10.1038/mp.2016.131
22. Johnstone AL, Wahlestedt C, Silva JP. To eat or not to eat: The neurobiological substrates guiding maladaptive decision-making in obesity. *J Addict Med Ther*. 2013;1(1):1002.
23. Heilig M, Barbier E, Johnstone AL, et al. Reprogramming of mPFC transcriptome and function in alcohol dependence. *Genes Brain Behav*. 2017;16(1):86-100.
24. Gilpin NW, Richardson HN, Cole M, Koob GF. Vapor inhalation of alcohol in rats. *Curr Protoc Neurosci*. 2008. Chapter 9:Unit 9 29
25. Sommer WH, Rimondini R, Hansson AC, et al. Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. *Biol Psychiatry*. 2008;63(2):139-145.
26. Rimondini R, Arlinde C, Sommer W, Heilig M. Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. *FASEB J*. 2002;16(1):27-35.
27. Tapocik JD, Solomon M, Flanigan M, et al. Coordinated dysregulation of mRNAs and microRNAs in the rat medial prefrontal cortex following a history of alcohol dependence. *Pharmacogenomics J*. 2013;13(3):286-296.
28. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. Hard Cover ed. London, United Kingdom: Elsevier Academic Press; 2018.
29. Macey DJ, Schulteis G, Heinrichs SC, Koob GF. Time-dependent quantifiable withdrawal from ethanol in the rat: effect of method of dependence induction. *Alcohol*. 1996;13(2):163-170.
30. Meinhardt MW, Sommer WH. Postdependent state in rats as a model for medication development in alcoholism. *Addict Biol*. 2015;20(1):1-21.
31. American Psychiatric Association. *Diagnostic and statistical manual of mental disorders* (4th ed., Text Revision). Washington, DC: Author 2000.
32. Bjork K, Saarikoski ST, Arlinde C, et al. Glutathione-S-transferase expression in the brain: possible role in ethanol preference and longevity. *FASEB J*. 2006;20(11):1826-1835.
33. Sommer WH, Lidström J, Sun H, et al. Human NPY promoter variation rs16147:T>C as a moderator of prefrontal NPY gene expression and negative affect. *Hum Mutat*. 2010;31(8):E1594-E1608.
34. Schroeder A, Mueller O, Stocker S, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol*. 2006;7(1):3.
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta C(T)) method. *Methods*. 2001;25(4):402-408.
36. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol*. 2008;26(3):317-325.
37. Shah RN, Grzybowski AT, Cornett EM, et al. Examining the roles of H3K4 methylation states with systematically characterized antibodies. *Mol Cell*. 2018;72(1):162-177 e167.
38. Zang C, Schones DE, Zeng C, Cui K, Zhao K, Peng W. A clustering approach for identification of enriched domains from histone modification ChIP-seq data. *Bioinformatics*. 2009;25(15):1952-1958.
39. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol*. 2010;11(10):R106.
40. Khomtchouk B, Koehler W, Booven D, Wahlestedt C. Optimized functional annotation of ChIP-seq data. *bioRxiv*. 2018. https://doi.org/10.1101/082347
41. Wang J, Duncan D, Shi Z, Zhang B. WEB-based gene set analysis toolkit (WebGestalt): update 2013. *Nucleic Acids Res*. 2013;41(Web Server issue):W77-W83.
42. Malkov VA, Serikawa KA, Balantac N, et al. Multiplexed measurements of gene signatures in different analytes using the NanoString nCounter Assay System. *BMC Res Notes*. 2009;2(1):80.
43. De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell*. 2007;130(6):1083-1094.
44. Sheedy D, Garrick T, Dedova I, et al. An Australian Brain Bank: a critical investment with a high return! *Cell Tissue Bank*. 2008;9(3):205-216.
45. Meinhardt MW, Hansson AC, Perreau-Lenz S, et al. Rescue of infralimbic mGluR2 deficit restores control over drug-seeking behavior in alcohol dependence. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 2013;33(7):2794-2806.
46. Hirth N, Meinhardt MW, Noori HR, et al. Convergent evidence from alcohol-dependent humans and rats for a hyperdopaminergic state in protracted abstinence. *Proc Natl Acad Sci U S A*. 2016;113(11):3024-3029.
47. Hermann D, Hirth N, Reimold M, et al. Low mu-opioid receptor status in alcohol dependence identified by combined positron emission tomography and post-mortem brain analysis. *Neuropsychopharmacology*. 2017;42(3):606-614.
48. Uylings HB, Groenewegen HJ, Kolb B. Do rats have a prefrontal cortex? *Behav Brain Res*. 2003;146(1–2):3-17.
49. Salminen A, Kaarniranta K, Hiltunen M, Kauppinen A. Histone demethylase Jumonji D3 (JMJD3/KDM6B) at the nexus of epigenetic regulation of inflammation and the aging process. *J Mol Med (Berl)*. 2014;92(10):1035-1043.
50. Wijayatunge R, Chen LF, Cha YM, Zannas AS, Frank CL, West AE. The histone lysine demethylase Kdm6b is required for activity-dependent preconditioning of hippocampal neuronal survival. *Mol Cell Neurosci*. 2014;61:187-200.
51. Wijayatunge R, Liu F, Shpargel KB, et al. The histone demethylase Kdm6b regulates a mature gene expression program in differentiating cerebellar granule neurons. *Mol Cell Neurosci*. 2018;87:4-17.

52. Smith SM, Kimyon RS, Watters JJ. Cell-type-specific Jumonji histone demethylase gene expression in the healthy rat CNS: detection by a novel flow cytometry method. *ASN Neuro*. 2014;6(3):193-207.

53. Zhang Y, Chen K, Sloan SA, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci*. 2014;34(36):11929-11947.

54. Zhang Y, Sloan SA, Clarke LE, et al. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. *Neuron*. 2016;89(1):37-53.

55. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 2002;30(1):207-210.

56. Przanowski P, Dabrowski M, Ellert-Miklaszewska A, et al. The signal transducers Stat1 and Stat3 and their novel target Jmjd3 drive the expression of inflammatory genes in microglia. *J Mol Med (Berl)*. 2013; 92(3):239-254. https://doi.org/10.1007/s00109-013-1090-5

57. Tang Y, Li T, Li J, et al. Jmjd3 is essential for the epigenetic modulation of microglia phenotypes in the immune pathogenesis of Parkinson's disease. *Cell Death Differ*. 2014;21(3):369-380.

58. Burchfield JS, Li Q, Wang HY, Wang RF. JMJD3 as an epigenetic regulator in development and disease. *Int J Biochem Cell Biol*. 2015;67: 148-157.

59. Xiang Y, Zhu Z, Han G, Lin H, Xu L, Chen CD. JMJD3 is a histone H3K27 demethylase. *Cell Res*. 2007;17(10):850-857.

60. Maze I, Covington HE 3rd, Dietz DM, et al. Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. *Science*. 2010;327(5962):213-216.

61. Covington HE 3rd, Maze I, Sun H, et al. A role for repressive histone methylation in cocaine-induced vulnerability to stress. *Neuron*. 2011;71(4):656-670.

62. Sun H, Maze I, Dietz DM, et al. Morphine epigenomically regulates behavior through alterations in histone H3 lysine 9 dimethylation in the nucleus accumbens. *J Neurosci*. 2012;32(48):17454-17464.

63. Das A, Chai JC, Kim SH, et al. Transcriptome sequencing of microglial cells stimulated with TLR3 and TLR4 ligands. *BMC Genomics*. 2015; 16(1):517.

64. De Santa F, Narang V, Yap ZH, et al. Jmjd3 contributes to the control of gene expression in LPS-activated macrophages. *EMBO J*. 2009;28(21): 3341-3352.

65. Hirbec H, Marmai C, Duroux-Richard I, et al. The microglial reaction signature revealed by RNA seq from individual mice. *Glia*. 2018; 66(5):971-986.

66. Zou J, Crews F. Induction of innate immune gene expression cascades in brain slice cultures by ethanol: key role of NF-κB and proinflammatory cytokines. *Alcohol Clin Exp Res*. 2010;34(5):777-789.

67. Kimpel MW, Strother WN, McClintick JN, et al. Functional gene expression differences between inbred alcohol-preferring and -non-preferring rats in five brain regions. *Alcohol*. 2007;41(2):95-132.

68. Mulligan MK, Ponomarev I, Hitzemann RJ, et al. Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. *Proc Natl Acad Sci U S A*. 2006;103(16):6368-6373.

69. Blednov YA, Ponomarev I, Geil C, Bergeson S, Koob GF, Harris RA. Neuroimmune regulation of alcohol consumption: behavioral validation of genes obtained from genomic studies. *Addict Biol*. 2012;17(1): 108-120.

70. de Timary P, Starkel P, Delzenne NM, Leclercq S. A role for the peripheral immune system in the development of alcohol use disorders? *Neuropharmacology*. 2017;122:148-160.

71. Heberlein A, Kaser M, Lichtinghagen R, et al. TNF-alpha and IL-6 serum levels: neurobiological markers of alcohol consumption in alcohol-dependent patients? *Alcohol*. 2014;48(7):671-676.

72. Miller SA, Mohn SE, Weinmann AS. Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. *Mol Cell*. 2010;40(4):594-605.

73. Arrowsmith CH, Bountra C, Fish PV, Lee K, Schapira M. Epigenetic protein families: a new frontier for drug discovery. *Nat Rev Drug Discov*. 2012;11(5):384-400.

74. DeWoskin VA, Million RP. The epigenetics pipeline. *Nat Rev Drug Discov*. 2013;12(9):661-662.

75. Green EM, Gozani O. Everybody's welcome: the big tent approach to epigenetic drug discovery. *Drug Discov Today Ther Strateg*. 2012;9(2–3):e75-e81.

76. Vendruscolo LF, Estey D, Goodell V, et al. Glucocorticoid receptor antagonism decreases alcohol seeking in alcohol-dependent individuals. *J Clin Invest*. 2015;125(8):3193-3197.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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