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

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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
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 transcriptional 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 dorsolateral prefrontal cortex (dPFC) 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. 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. 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. 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. 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. 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. Molecular and biochemical analyses in these studies were conducted using NAc (coordinates 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.

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: TCTCATGCGAGTACGCTCCGG; TACACGGGAAGTGGGACGTGC; TGGAGGAAGCTTGCCGAGC) 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: TCTAGATTGGAGGGAAATTGG; reverse: AAAGTACCAGCAAGAGA) 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). 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** | 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 and flash frozen. Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) as previously described; samples underwent a cleanup step using the RNeasy Mini Kit (Qiagen, 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) were 7.12 ± 0.23 and 7.37 ± 0.24 in alcoholics 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). 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-Cor, 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. 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. 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 x 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 using SICER to identify H3K27me3 regions of enrichment followed by edgeR 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. Only genes passing a false discovery rate (FDR)-corrected P value less than .05 (Benjamini-Hochberg method) were used for pathway analysis in WebGestalt. Alignment statistics are shown in Table 2, and full results are shown in Table S2.
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. 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 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. Surprisingly, we found that protein levels of KDM6B were significantly increased in alcohol-dependent rats 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.
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. In ACC, a region that is anatomically and functionally related to the rodent mPFC, 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...
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. 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. According to FACS analyses from rodent brain, including publicly available RNA-sequencing data (data accessible at NCBI GEO database, accession number GSE52564), 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, 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 and IL-6 (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 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 (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, 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, 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. In this analysis, the IL-6 signaling pathway was the top result (FDR $P = .0021$, Table 3).
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: \( F_{1,8} = 13.64, P < .0001 \), 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 cell 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 resulting in hemizygous expression of KDM6B. The fact that KDM6B functionally contributes to the IL-6 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, \( F_{2,8} = 284.2, P < .0001 \)) (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 (JMJD: H1388A, E1390G, and H1468A). Overexpression of KDM6B-JMJD in BV-2 cells significantly attenuated IL-6 induction (over 100-fold decrease) as compared with KDM6B-WT overexpression (one-way ANOVA, \( F_{2,8} = 284.7, P < .0001 \)) (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

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**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.
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. 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. 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. 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...
inflammation is increasingly recognized to underlie the development of alcohol addiction, 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, 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 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 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

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, consistent with the well-characterized role of KDM6B to mediate inflammatory responses in peripheral macrophages.49 Because 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 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 Δ (catalytic domain deletion JMJΔ), GFP control plasmid, and control cells (****P < .0001, n = 3)
seeking and negative affect.\textsuperscript{1} Furthermore, sustained elevation of inflammatory cytokines has been shown to increase alcohol craving and severity of dependence.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{73-75} Further, compounds that suppress inflammation have a proven record of clinical trial success for alcohol addiction.\textsuperscript{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.

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

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