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# Intermittent Fasting Enhances Genome Integrity and Cytoprotective Pathways via (BHB) $\beta$ -Hydroxybutyrate Signaling and Chromatin Remodeling

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## ABSTRACT

DNA damage and oxidative stress are key drivers of cellular aging and brain dysfunction, and enhancing cytoprotective pathways is therefore a promising strategy to preserve neuronal genome integrity. Intermittent fasting (IF) elevates the ketone body  $\beta$ -hydroxybutyrate (BHB), a signaling metabolite implicated in cytoprotective pathways and, more recently, in chromatin regulation. Yet the mechanisms by which repeated fasting reshapes hippocampal epigenetic programs and influences genome maintenance remain poorly defined. Here, we compared a single 24-h fast versus a month-long IF regimen in adult female mice, focusing on oxidative stress defense and DNA repair pathways, and tested whether protective states persist after refeeding. During a single 24-h fast, hippocampal nuclear BHB increased modestly and coincided with elevated HDAC2 activity, consistent with a transient metabolic stress response. In parallel, acetyl-CoA levels remained unchanged, potentially limiting broader EP300-driven acetylation. Under these conditions, we observed a brief enrichment of H3K9bhb at promoters of cytoprotective genes, suggesting an early priming phase. In contrast, recurrent IF was associated with robust nuclear BHB accumulation and reduced HDAC2 activity. Together with increased hippocampal acetyl-CoA availability and enhanced EP300 interaction with chromatin, recurrent IF shifted promoter regulation toward sustained H3K27 acetylation and robust induction of cytoprotective transcriptional programs. Functionally, IF and IF-refed mice exhibited reduced nuclear 8-oxo-dG accumulation and accelerated resolution of  $\gamma$ H2AX foci following hippocampal activation by contextual fear conditioning, indicating enhanced genome stability and improved DNA repair capacity. Collectively, these findings support a model in which IF drives a coordinated metabolic-epigenetic transition from an early, transient priming state to a more sustained cytoprotective program that promotes genome maintenance in the hippocampus.

## 1 | Introduction

DNA damage and oxidative stress are central drivers of cellular aging, brain dysfunction, and neurodegeneration [1, 2]. These insults represent distinct yet interconnected threats to genome stability. To counteract them, neurons engage cytoprotective pathways that both limit oxidative burden and preserve DNA integrity.

Mitochondrial respiration and high metabolic demand render neurons particularly prone to oxidative stress, where excessive reactive oxygen species (ROS) exceed the capacity of antioxidant defenses such as superoxide dismutase (SOD) and glutathione peroxidases (GPX) [3]. This imbalance generates oxidative DNA lesions, most notably 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), a mutagenic and cytotoxic base modification [4, 5]. In neurons, 8-oxo-dG is primarily repaired

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through base-excision repair (BER), initiated by the glycosylase OGG1 and completed by APE1 and DNA polymerase  $\beta$ . Another major form of DNA lesion is the double-strand break (DSB), which may arise either from the misrepair of clustered oxidative damage or as a consequence of physiological bursts of neuronal activity, where rapid, locus-specific breaks relieve torsional stress and permit transcription of immediate-early genes [6, 7].

Under healthy conditions, DSBs are swiftly detected by ATM kinase, which phosphorylates histone H2AX. The resulting  $\gamma$ H2AX spreads over megabase-sized domains to recruit high-fidelity repair machinery [8]. Chief among these is the MRN complex, comprising Mre11, Rad50, and Nbs1, which clamps DNA ends, processes termini, and channels repair through non-homologous end-joining (NHEJ) or homologous recombination (HR) [8]. The essential role of this system is underscored by the embryonic lethality of Rad50 knockout mice [9].

Although most DSBs and oxidative base damage are promptly and accurately repaired, this process is not infallible. Aging, sustained oxidative stress, or metabolic imbalance can shift the balance toward higher lesion incidence or diminished repair efficiency, resulting in the persistence of unrepaired or mis-repaired DNA [10]. Because neurons are long-lived, highly transcriptionally active, and metabolically demanding, they are especially vulnerable to persistent DNA damage, which can ultimately lead to neuronal loss. Consequently, recent therapeutic strategies [11–13] aim not only to strengthen antioxidant defenses but also to augment neuronal DNA-repair capacity. In this context, nutrient-sensing pathways have emerged as powerful orchestrators that simultaneously remodel chromatin, adjust cellular metabolism and energy balance, and strengthen antioxidant defenses, making metabolic interventions particularly compelling for preserving genome integrity.

By cycling between periods of fasting and feeding, intermittent fasting (IF) engages these nutrient-sensing networks and has emerged as a straightforward, non-pharmacological strategy that promotes brain health and confers neuroprotection. Accumulating evidence [14–17] suggests that IF improves cognitive function, mood, and synaptic plasticity while reducing neuroinflammation and the risk for neurodegenerative disorders. Mechanistically, fasting triggers a suite of intrinsic cytoprotective pathways, augmenting antioxidant defenses, enhancing autophagy, and improving mitochondrial bioenergetics [18]. These adaptations are tightly linked to the rise of circulating ketone bodies—acetoacetate, acetone, and, most notably,  $\beta$ -hydroxybutyrate (BHB)—a metabolic hallmark of prolonged energy restriction [19]. Beyond its traditional role as an alternative metabolic fuel, BHB also acts as a potent epigenetic signaling molecule that can reshape chromatin structure. BHB has been shown to directly modify chromatin structure through lysine  $\beta$ -hydroxybutyrylation (k-bhb), a novel post-translational modification on histone tails [20]. This modification is catalyzed by the CBP/p300 complex, which also governs canonical histone acetylation. Indirectly, BHB influences chromatin organization by increasing intracellular acetyl-CoA levels (fueling histone acetylation) and potentially inhibiting class I histone deacetylases (HDACs), thereby shifting the balance toward a more

open, transcriptionally active chromatin state [21]. Remarkably, recent evidence [22] indicates that HDAC2 can “write” lysine  $\beta$ -hydroxybutyrylation. Additionally, BHB may modulate DNA methylation and histone methylation dynamics, influence microRNA expression, and promote histone demethylase activity [23], suggesting that BHB acts as a central node linking metabolic state to the epigenetic regulation of gene expression.

Despite this promise, the link between IF, BHB-signaling, chromatin plasticity, and DNA-damage control remain poorly defined. Moreover, it is unclear whether any benefit persists once feeding resumes. To address these gaps, we compared the effects of a single 24-h fasting bout with those of a prolonged IF regimen on hippocampal epigenetic landscapes and gene-expression programs, concentrating on BHB-dependent chromatin changes and transcripts governing oxidative-stress defense and DNA repair. C57BL/6J female mice were divided into five groups: a control group with continuous ad libitum feeding; two groups subjected to a single 24-h fast, with or without 24-h refeeding (Fast-Single and Refed-Single); and two groups exposed to 15 cycles of intermittent fasting, euthanized either immediately after the final fast (IF) or after 24 h of refeeding (IF-Refed).

Our results show that repeated IF induced a markedly greater rise in circulating BHB compared to a single fast. In parallel, an acute single fasting bout was characterized by a modest increase in hippocampal nuclear BHB that coincided with elevated HDAC2 activity and a near-significant increase in its association with k-bhb–marked chromatin, while acetyl-CoA levels remained unchanged. Under these conditions, a single fasting bout unexpectedly promoted the transient enrichment of the histone mark H3K9  $\beta$ -hydroxybutyrylation (H3K9bhb) at promoters of genes governing oxidative stress defense (*Gpx1*, *Sod1*), autophagy (*Sqstm1*), and DNA repair (*Xrcc6*, *Rad17*, *Rad50*, *Nbs1*, and *Recql*), consistent with a priming phase marked by limited transcriptional induction.

In contrast, recurrent IF was associated with robust nuclear BHB accumulation, reduced HDAC2 activity, and increased acetyl-CoA availability. These metabolic shifts coincided with enhanced EP300 engagement and a shift toward sustained H3K27 acetylation, driving the strong upregulation of these cytoprotective transcriptional programs. Notably, promoter-level chromatin and mRNA responses largely returned to baseline after 1 day of refeeding, indicating dynamic regulation by metabolic state. However, nuclear BHB remained significantly elevated in both IF and IF-Refed hippocampus despite the normalization of serum levels, suggesting persistent intracellular ketone signaling. This sustained nuclear BHB enrichment may support ongoing cytoprotective and genome-stabilizing effects after refeeding, consistent with the reduced 8-oxo-dG accumulation and accelerated  $\gamma$ H2AX resolution observed following contextual fear conditioning in IF-Refed mice.

Finally, both IF and IF-refed groups exhibited reduced nuclear 8-oxo-dG and accelerated resolution of  $\gamma$ H2AX foci following hippocampal activation by contextual fear conditioning, indicating enhanced genome stability and improved DNA repair capacity. Collectively, these data position BHB-linked chromatin remodeling as a central mechanism through which repeated IF strengthens cytoprotective responses in the hippocampus.

## 2 | Methods

### 2.1 | Animals

All animal trials were conducted following the National Council for Animal Experimentation guidelines and were subjected to approval by the Hebrew University of Jerusalem's Ethics Committee, approval No. AG-24657-01. Female C57BL/6J mice were used in this study. Mice were housed in conventional cages, 6 animals per cage, with bedding, and were kept under a 12-h dark: 12-h light cycle, with ad libitum access to water. C57BL/6J female mice were divided into five experimental groups. The control group (Con) had continuous ad libitum access to standard chow (Mucedola 4RF25). Two groups underwent a single 24-h fasting period: one group was euthanized immediately afterward (Fast-Single), while the other was refed for 24 h before euthanasia (Refed-Single). Two additional groups were subjected to a recurrent intermittent fasting regimen consisting of 15 cycles of alternating 24-h feeding and 24-h fasting over 30 days. One of these groups was euthanized immediately after the final fasting cycle (IF), and the other after 24 h of ad libitum refeeding (IF-Refed).

All animals were weighed throughout the experiment to make sure they remain at a healthy weight, following the ethics protocols. On the day of sacrifice, the animals were weighed again, and blood was taken to measure the levels of ketone bodies. Ketone blood levels were measured with FreeStyle Optium Neo, blood glucose, and ketone monitoring system (Abbott, Lake County, IL) using B ketone sticks (Abbott, Lake County, IL) according to the manufacturer's protocol. Additionally, the PFC, hypothalamus, and hippocampus were obtained from both hemispheres of each animal. One hemisphere of each brain region was used to extract RNA and measure gene expression. The other hemisphere of each brain region was utilized for chromatin immunoprecipitation assays.

### 2.2 | BHB Measurement

Single hippocampal hemispheres were lysed in ice cold PBS supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher, Waltham, MA, cat# 78446). An aliquot of the total lysate was reserved for protein quantification by BCA (Pierce Rapid Gold BCA Protein Assay Kit, Thermo Fisher, Waltham, MA, cat# A53225). The remaining lysate was centrifuged at 800g for 10 min at 4°C to separate the supernatant (cytosolic fraction) from the nuclear and large debris pellet. The cytosolic fraction was collected for BHB quantification using the BHB Glo (Ketone Body) Enzyme Pack (Promega, Madison, WI, cat# J4141). The pellet was resuspended in 10 mM EDTA and 20 mM Tris HCl (pH 8.0) supplemented with protease inhibitor cocktail, incubated on ice for 30 min with occasional vortexing, and then centrifuged at 16 000g for 10 min. The resulting supernatant was collected as the nuclear fraction for BHB measurement. For BHB quantification, cytosolic and nuclear fractions were acidified with 0.6 M HCl and neutralized using the kit neutralization solution, diluted 5-fold, and normalized to protein content measured by BCA. BHB levels were then quantified according to the manufacturer's instructions (Promega, Madison, WI, cat# J4141).

### 2.3 | HDAC2 Assay

80 µg of protein was taken from each sample and measured using the HDAC Activity Assay Kit (colorimetric) (Abcam, Cambridge, UK, cat# ab1432) according to the manufacturer's instructions.

### 2.4 | Acetyl-CoA

According to the Acetyl-CoA Assay Kit (Colorimetric) (Novus Biologicals, Centennial, CO, cat# NBP3-24455), 20 mg of hippocampal tissue was lysed in the kits extraction buffer and measured according to the manufacturer's instructions.

All assays were measured and quantified in BioTek Synergy H1 Multimode Reader (Agilent, Santa Clara, CA).

### 2.5 | Co-Immunoprecipitation

PFC tissue was cross-linked with 1% formaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min at room temperature and quenched with 0.125 M glycine (Cell Signaling, Danvers, MA, cat# 7005S). The samples were homogenized on ice in RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 7.4, 1% (v/v) TritonX100, 0.5% (w/v) deoxycholic acid, 0.05% SDS, full protease inhibitor, Roche) and then incubated overnight with EP300 antibody (1:500, Abcam, Cambridge, UK, cat# ab275378, RRID: [AB\\_2935873](#)). For background, normal rabbit IgG antibody (3 µg/sample) was used. Antigen-antibody complexes were separated using Magna ChIP Protein A + G Magnetic Beads (Sigma-Aldrich, St. Louis, MO).

### 2.6 | SDS-PAGE and Western Blot

PFC tissue was lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 7.4, 1% (v/v) TritonX100, 0.5% (w/v) deoxycholic acid, 0.05% SDS, full protease inhibitor, Roche) and diluted in 2× Leamly sample buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol). Protein extracts/immunoprecipitants were separated on a 10% or 15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline with Tween 20 (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) + 5% bovine serum albumin (BSA) (Sigma Aldrich, cat# A3912-100G) for 1 h at room temperature and incubated overnight with H3K9bhb (1:1000; PTM Biolab, Chicago, IL, cat# PTM-1250, RRID: [AB\\_3076399](#)), H3K27ac (Cell Signaling, Danvers, MA, cat# 8173, RRID: [AB\\_10949503](#)), rabbit anti-Hif1a (1:1000; Invitrogen, Carlsbad, CA, cat# PA1-16601, RRID: [AB\\_2117128](#)), Anti-DNA/RNA Damage antibody (8-oxo-dG) (1:1000; Sigma-Aldrich, St. Louis, MO, cat# SAB5200010-100UG, RRID: [AB\\_3713037](#)), H3K27bhb (1:500; PTM Biolab, Chicago, IL, cat# PTM-1293, RRID: [AB\\_2927632](#)), GAPDH Antibody (Santa Cruz, Dallas, TX, cat#: sc-25778, RRID: [AB\\_10167668](#)), and histone 3 (Cell Signaling, Danvers, MA, RRID: [AB\\_331563](#)) antibodies at 4°C. The membranes were

**TABLE 1** | Primers used for real-time PCR.

| Gene name    | Forward sequence       | Reverse sequence          |
|--------------|------------------------|---------------------------|
| Tubulin [27] | AGCAACATGAATGACCTGGTG  | GCTTTCCTAACCTGCTTGG       |
| RAD17        | GTGCAGATTGGCCGGTGT     | GGGAAGAGTAGCCGCTGATTATTGT |
| RAD50        | GTGCGTCAGACACAGGGTCA   | CGGATCTCACACGCTTCTCC      |
| GPx1         | GGTTCGAGCCCAATTTTACA   | CCCACCAGGAAGTTCTCAAA      |
| SOD1         | TGCTGGAAAGGACGGTGTG    | ACGGCCAATGATGGAATGCT      |
| XRCC6        | ACAGGGACATCATCACCACCG  | GTCTCCTTGGCTCGAACCTTCC    |
| SQSTM1       | TGTGGTGGGAAGTCTGCTATAA | CAGCGGCTATGAGAGAAGCTAT    |
| NBS1         | CGAGCCCTTGTTGTTTGTCTT  | TGCGGTAAGTCCTCCAAGCTGT    |
| RECQL        | AGACGGCTCCCATCATGC     | GCTGGGAAAATACAACGCACA     |

washed and then incubated with HRP conjugated anti-mouse and anti-rabbit secondary antibodies (1:10000; GE Healthcare) at room temperature for 1 h. A chemiluminescent signal was detected using Pierce™ ECL Western blotting Substrate (Pierce Biotechnology, Waltham, MS, cat# 32106) by the ChemiDoc™ XRS + molecular imager (BIO-RAD, Hercules, CA, USA), and densitometric analysis was performed using ImageJ open-source software. As described previously [24–26], co-IP experiments k-bhb and H3K27ac signals were normalized to total histone H3, allowing direct comparison of chromatin modification states across experimental groups while controlling for histone content and chromatin input.

## 2.7 | RNA Isolation and cDNA Synthesis and Quantitative Real-Time PCR (qPCR)

Total RNA was isolated with the RNA/DNA Purification Micro Kit (Norgen Biotek, Thorold, Canada) according to the manufacturer's instructions. Isolated RNA was reverse-transcribed to single-stranded cDNA by SuperScript IV Reverse Transcriptase and oligo (dT) plus random primers (Invitrogen, Carlsbad, CA). Real-time PCR (qPCR) was performed with 10-ng cDNA in CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with PerfeCTa SYBR Green FastMix, ROX (Quanta BioSciences, Gaithersburg, MD). Dissociation curves were analyzed following each real-time PCR to confirm the presence of only one product and the absence of primer dimer formation. The threshold cycle number (C<sub>q</sub>) for each tested gene (X) was used to quantify the relative abundance of that gene using the formula  $2^{(C_t \text{ gene X} - C_t \text{ standard})}$ . Tubulin was used as the standard for mRNA expression. The primers used for real-time PCR are listed in Table 1.

## 2.8 | Chromatin-Immunoprecipitation (ChIP) and qPCR

Following a protocol previously published [28], cells were cross-linked with 1% formaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min at room temperature and quenched with glycine quenching solution (Cell Signaling, Danvers, MA, cat# 7005S). Crosslinked samples were

resuspended in a ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH8, protease inhibitor) and sonicated (Bioruptor Plus, Diagenode) to release 500–1000 bp fragments. Samples were diluted 1:5 with a ChIP dilution buffer (167 mM NaCl, 16.7 mM Tris-HCl pH8, 1.2 mM EDTA, Tritonx1.1, 0.01% SDS). Antibodies (4 μL per 200 μL sample) against H3K9bhb (PTM Biolab, Chicago, IL, cat# PTM-1250, RRID: [AB\\_3076399](#)) or H3K27ac (Cell Signaling, Danvers, MA, cat# 8173, RRID: [AB\\_10949503](#)) were immunoprecipitated at 4°C overnight at an end-over-end mixer. The samples were then conjugated to Magna ChIP Protein A + G Magnetic Beads (Sigma-Aldrich, St. Louis, MO) or Sera Mag SpeedBeads Protein A/G (Cytivia, Marlborough, MA) at 4°C overnight. Immunocomplexes were washed sequentially with the following buffers: low-salt buffer (0.1% SDS, Tritonx1, 1 mM EDTA, 20 mM Tris-HCl pH8, 0.15 M NaCl), high-salt buffer (0.1% SDS, Tritonx1, 2 mM EDTA, 1 M Tris-HCl pH8, 0.5 M NaCl), and two washes with TE buffer (10 mM Tris-HCl pH8, 1 mM EDTA). The samples were then eluted with the following buffer: (1% SDS, NaHCO<sub>3</sub> 90 mM), coupled with RNase A (Cell Signaling, Danvers, MA) for 20 min at 37°C. Chromatin was deproteinized and de-crosslinked with proteinase K (Thermo Fisher, Waltham, MA) and NaCl 4 M for 4 h at 62°C. For de-conjugation, samples were heated for 10 min at 95°C. DNA was purified and precipitated using DNA Purification Buffers and Spin Columns (ChIP, CUT&RUN) 1 Kit (Cell Signaling, Danvers, MA) or MinElute PCR Purification Kit (QIAGEN, Venlo, The Netherlands). The primers used for real-time PCR are listed in Tables 2 and 3.

## 2.9 | Contextual Fear Conditioning (CFC)

Mice were subjected to a contextual fear conditioning (CFC) protocol to induce hippocampus-dependent neuronal activation. Each mouse was placed in the conditioning chamber (Med Associates, USA) and allowed to freely explore the environment for 3 min. Following habituation, a single foot shock (0.7 mA, 1-s duration) was delivered through the grid floor. Mice remained in the chamber for an additional 30 s before being returned to their home cages. Animals were euthanized either 30 min or 4 h after conditioning, and brains were immediately perfused with 4% paraformaldehyde (PFA) for subsequent immunofluorescence analysis.

**TABLE 2** | Primers for gene promoters used for ChIP k-bhb.

| Gene promoter | Forward sequence      | Reverse sequence      |
|---------------|-----------------------|-----------------------|
| RAD17         | CCGCGGCGTCCTTACTTTC   | GGCTGGGTGGCGAAGTACA   |
| RAD50         | ACATTCGCGCTCGCACG     | ACTCCCACTTCCCGGCATGC  |
| GPx1          | GAGCGCTAGTACGGATTCCA  | AGAAGGCATACACGGTGGAC  |
| SOD1          | CGGGCCTCGTGTTCTCGGTT  | CCTGGAGCGTGGGACTGA    |
| XRCC6         | CCCCTTCCCCTGCTCCTG    | ACTGCAATGGGAAAAGCGACC |
| SQSTM1        | CAGCTGTTTCGTCCGTACCT  | GGCTGAAGCAGAAGCTGAAG  |
| NBS1          | ATTCGATGCCCTCTTCTGGTG | ACCGTGAGCCATCCATCT    |
| RECQL         | ATGCAGCGCCACCTTC      | CGCGCTCTTGTTCGGATGTG  |

**TABLE 3** | Primers for gene promoters used for ChIP k-Ac.

| Gene promoter | Forward sequence       | Reverse sequence        |
|---------------|------------------------|-------------------------|
| RAD17         | GGAGTTCTTGGTGCCTGC     | CTGTACGCGGCTAGGTTCCC    |
| RAD50         | GCTGCCACCTATCACTGTTTGC | CGACGCTTGAAGACTGAGG     |
| GPx1          | GAGCGCTAGTACGGATTCCA   | AGAAGGCATACACGGTGGAC    |
| SOD1          | CCGTCGGCTTCTCGTCTTGCT  | ACCGGACCGTCGCCCTTC      |
| XRCC6         | GCAATCCCTGAGGCTTCCA    | CCCAGGGCCCTATCTAAGTACCA |
| SQSTM1        | CAGCTGTTTCGTCCGTACCT   | GGCTGAAGCAGAAGCTGAAG    |
| NBS1          | CTTCCGCGCTGCACTGC      | GTGCGCGTTCCAGGACTC      |
| RECQL         | CTAACCTGAGCGCATCCC     | GATAGGAGCTGGGCGTCGT     |

## 2.10 | Immunofluorescence

Mice were deeply anesthetized with an intraperitoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed in 4% PFA at 4°C for 12–16 h, washed in PBS, and cryoprotected in 30% sucrose in PBS at 4°C until equilibrated (24–48 h). Coronal brain sections (40 µm) were prepared using a vibratome (Leica VT1000S) in cold PBS and stored at 4°C in PBS containing 0.01% sodium azide. Free-floating sections were processed for immunofluorescence to ensure optimal antibody penetration. After PBS washes, sections were incubated for 1 h at room temperature in blocking solution (PBS with 4% normal bovine serum and 0.1% Triton X-100). Primary antibodies were diluted in blocking buffer and applied overnight at 4°C on a gentle shaker. The following primary antibodies were used: anti-γH2AX (phospho S139, 1:750; Abcam, Cambridge, UK, cat# ab22551, RRID: [AB\\_447150](#)) and Monoclonal Anti-DNA/RNA Damage antibody (8-oxo-dG) (1:1000; Sigma-Aldrich, St. Louis, MO, cat# SAB5200010-100UG, RRID: [AB\\_3713037](#)). After washing, sections were incubated for 2 h at room temperature with a fluorescent secondary antibody (Goat anti-Mouse IgG H&L, Alexa Fluor 488; Abcam, Cambridge, UK, cat# ab150113, RRID: [AB\\_2576208](#)). Nuclear counterstaining was performed with DAPI (Sigma-Aldrich, cat# D9542, 1 µg/mL) for 10 min. Sections were then washed, mounted on glass slides, air-dried, and coverslipped

with antifade mounting medium (VECTASHIELD, Vector Laboratories, cat# H-1000–10).

## 2.11 | Imaging and Quantification of γH2AX

Coronal hippocampal sections were imaged using an Andor BC43 microscope (Oxford Instruments) equipped with a 60× oil-immersion objective. Image stacks were processed in FIJI (ImageJ, NIH). The γH2AX (Alexa Fluor 488) and DAPI channels were separated, and a light Gaussian blur ( $\sigma = 1$ ) was applied to reduce background noise. The dentate gyrus was manually outlined in the DAPI channel to generate a region of interest (ROI) mask. Within this mask, each channel was thresholded, binarized, inverted, and processed with a watershed algorithm to separate adjacent signals. Individual γH2AX foci were then detected using the “Analyze Particles” tool, which recorded area, centroid, and fluorescence intensity. Foci counts and mean-intensity measurements were restricted to the DAPI-defined ROI. Quantitative data were exported for subsequent statistical analysis.

## 2.12 | Imaging and Quantification of 8-Oxo-dG

Confocal images were acquired using an Andor BC43 microscope (Oxford Instruments) equipped with a 60× oil-immersion objective. Image stacks were processed in FIJI (ImageJ, NIH). The 8-oxo-dG (Alexa Fluor 488) and DAPI channels were

separated, and a light Gaussian blur ( $\sigma=1$ ) was applied to reduce background noise. The CA1 region of the hippocampus and the anterior cingulate cortex (ACC) were identified based on anatomical landmarks, and one representative field was captured per region. Nuclear segmentation was performed on the DAPI channel with the StarDist 2D plugin to generate nuclear ROIs. These ROIs were overlaid onto the 8-oxo-dG channel to quantify nuclear-localized signals. Oxidative DNA damage was assessed by detecting discrete 8-oxo-dG immunoreactive puncta within each nuclear ROI using the “Find Maxima” function. To account for differences in cellular density, the total puncta count per image was normalized to the number of DAPI-positive nuclei, yielding a puncta-per-nucleus ratio. Quantitative data were exported for subsequent statistical analysis.

### 2.13 | Statistical Analysis

For the statistical analysis, we used GraphPad Prism 10.1.2 software. All data were examined for normality by the Shapiro–Wilk normality test and by the Kolmogorov–Smirnov normality test. As the distribution was normal, the parameters were not transformed. Sample number ( $n$ ) included the number of individual mice in each treatment group (indicated in the figure legends). One-way ANOVA followed by Fisher's LSD multiple comparison tests were used. Figure data are presented as mean  $\pm$  standard error mean (SEM), with exact  $n$ - and  $p$ -values for effects, as well as  $p$ -values for multiple comparisons reported in the legends.

## 3 | Results

### 3.1 | Repeated Intermittent Fasting Regimen Induced a Significantly Greater Rise in Circulating BHB Compared to a Single Fast

C57BL/6J female mice were assigned to five experimental groups. The control group (Con) had continuous ad libitum access to food. Two groups underwent a single 24-h fasting period: one group was euthanized immediately afterward (Fast-Single), while the other was refed for 24h before euthanasia (Refed-Single). An additional two groups followed a recurrent intermittent fasting regimen consisting of 15 cycles of alternating 24-h fasting and 24-h ad libitum feeding. Among these, one group was euthanized immediately following the final fasting period (IF), while the other was euthanized after 24h of ad libitum re-feeding (IF-Refed) (Figure 1A).

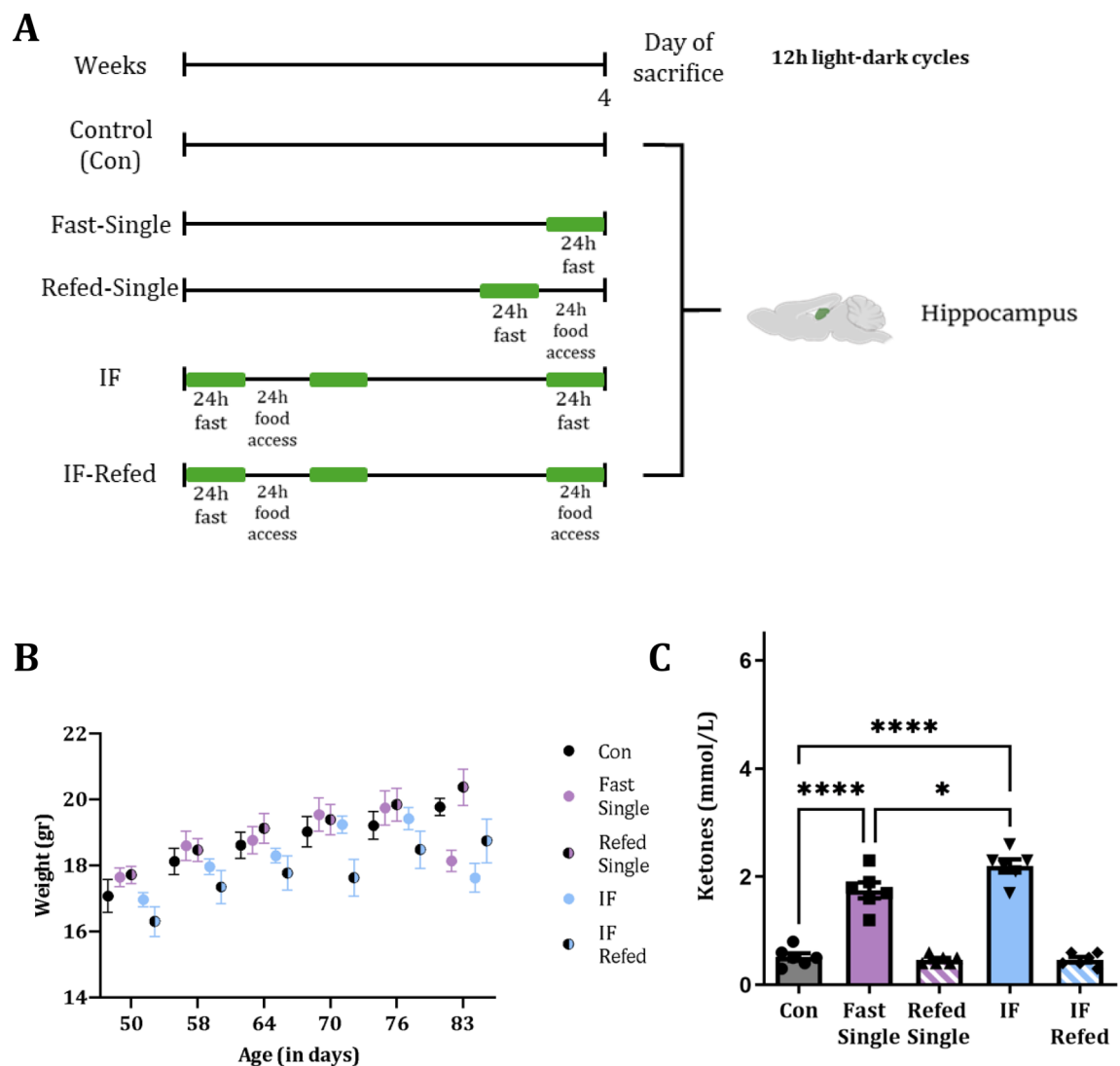
Throughout the experiment, body weights remained comparable across all groups (Figure 1B). As expected, blood ketone levels were significantly elevated in both Fast-Single and IF groups compared to controls ( $F_{(4,25)}=77.03$ ,  $p<0.0001$ , Figure 1C). Notably, the IF group exhibited significantly higher blood ketone levels than the Fast-Single group ( $p=0.0025$ ), indicating an enhanced adaptive response to recurring fasting cycles. In alignment with previous reports [29], these findings suggest that mice subjected to repeated fasting developed improved metabolic efficiency, with an enhanced capacity of the liver for ketone body production.

### 3.2 | IF Modulates Histone Modifications Through Dynamic EP300 and HDAC2 Interactions

In parallel with monitoring circulating BHB, we examined BHB dynamics within the hippocampus. To better distinguish systemic versus intracellular contributions, and to separate potential cytosolic and chromatin-associated roles of BHB, we performed biochemical fractionation of hippocampal tissue into cytosolic and nuclear compartments (Figure 2A,B). Cytosolic BHB was significantly elevated in the Fast-Single group ( $p=0.0331$ ) and moderately in the IF group ( $p=0.0983$ ), compared to control ( $F_{(4,20)}=1.637$ ,  $p=0.2040$ , Figure 2A). In contrast, recurrent IF induced a robust and significant accumulation of nuclear BHB, which persisted even after 24h of refeeding in the IF Refed group ( $F_{(4,20)}=8.817$ ,  $p=0.0003$ , Figure 2B). These results suggest that circulating BHB normalization may not fully capture intracellular signaling states and support the possibility that BHB-dependent regulation can extend beyond the fasting window.

Because ketone utilization is tightly coupled with mitochondrial oxidation and can influence the availability of nuclear acyl donors, we next examined acetyl-CoA levels (Figure 2C). Acetyl-CoA levels were unchanged in Fast Single animals compared with controls. In contrast, both IF and IF Refed groups ( $p=0.0214$ ,  $p=0.0065$ , respectively) exhibited a significant increase in acetyl-CoA levels ( $F_{(4,16)}=3.873$ ,  $p=0.0219$ , Figure 2C), indicating that recurrent fasting promotes a distinct metabolic environment that supports acetyl donor availability. To functionally complement these metabolic readouts, we directly measured HDAC2 enzymatic activity in hippocampal lysates (Figure 2D). Consistent with an acute metabolic stress response, the Fast-Single group exhibited a significant increase in HDAC2 activity compared with controls ( $p=0.0023$ ). In contrast, recurrent IF was associated with reduced HDAC2 activity relative to Fast-Single, although this effect did not reach statistical significance compared with controls ( $F_{(4,20)}=3.944$ ,  $p=0.0161$ , Figure 2D).

In light of the robust nuclear BHB accumulation, increased acetyl-CoA availability, and altered HDAC2 activity observed during recurrent fasting (Figure 2A–D), we next asked whether these metabolic shifts translate into measurable changes in chromatin modifier engagement and histone mark dynamics in the hippocampus. To address this, we performed co-immunoprecipitation (co-IP) assays across all experimental groups, focusing on EP300 interactions with two major lysine-based post-translational histone modifications linked to fasting and caloric restriction [20, 30], namely  $\beta$ -hydroxybutyrylation (k-bhb) and acetylation (k-Ac). Despite the elevated ketone state in the IF and IF Refed groups, EP300 association with H3K9bhb was unexpectedly reduced in both conditions, suggesting that H3K9bhb may represent a transient chromatin state that is not maintained during recurrent fasting ( $F_{(4,24)}=5.054$ ,  $p=0.0042$ , Figure 2E). In contrast, EP300 interaction with H3K27ac showed a significant increase in the IF group ( $p=0.0280$ ) consistent with a progressive shift toward an acetylation-enriched chromatin configuration during repeated fasting cycles ( $F_{(4,24)}=2.146$ ,  $p=0.1062$ , Figure 2F).



**FIGURE 1** | (A) Schematic representation of the experiment and different groups. (B) Animals were weighed at the beginning of the experiment (50 days old), at several time points throughout their development, and on the day of sacrifice. (C) On the day of sacrifice, truncated blood was taken from each animal to assess their current levels of ketones. Values are mean  $\pm$  SEM; One-way ANOVA,  $n = 6$ ,  $*p < 0.05$ ,  $****p < 0.0001$ .

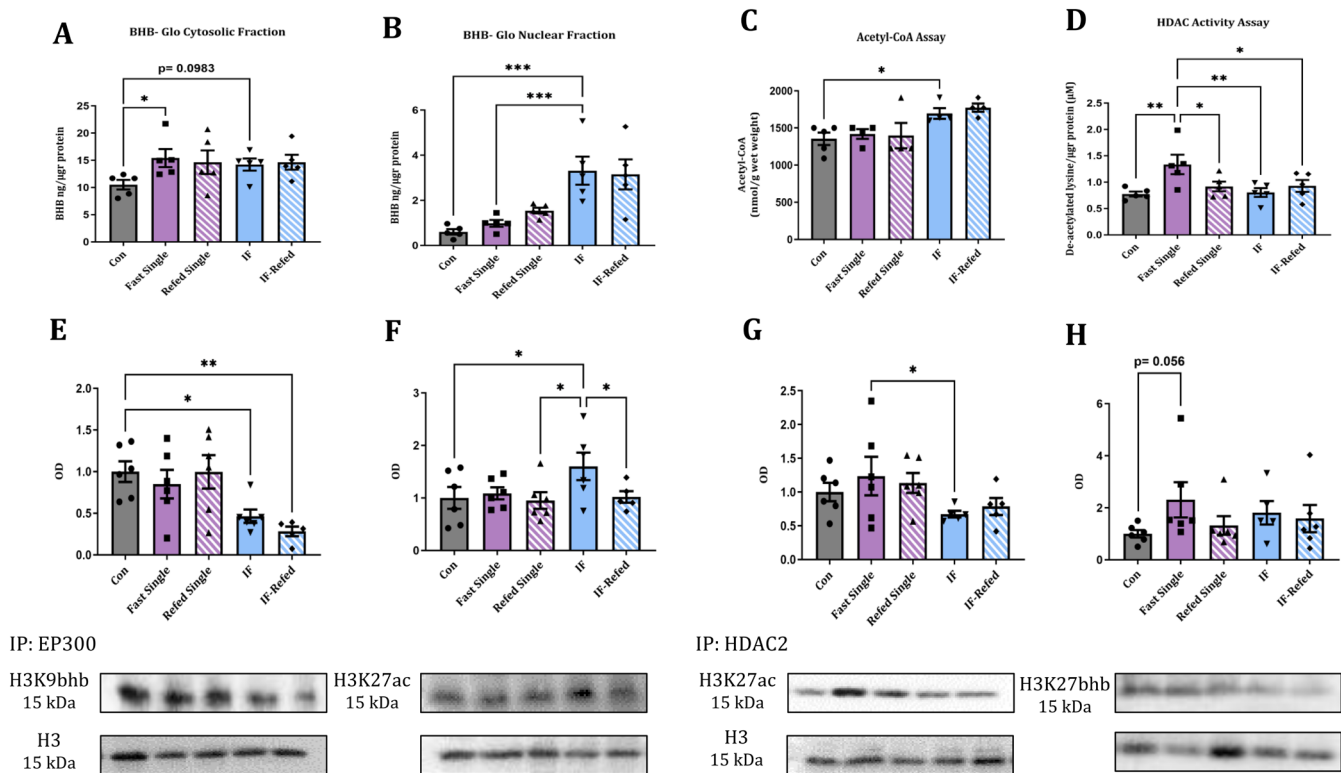
Next, we examined the “eraser” axis of chromatin regulation. BHB has been reported to inhibit class I HDAC activity [21, 31], and recent evidence [22] further suggests that HDAC2 can catalyze lysine  $\beta$ -hydroxybutyrylation under specific metabolic conditions. In this context, we observed a significant reduction in HDAC2–H3K27ac interaction in the IF group, compared to the Fast-Single group ( $F_{(4,23)} = 1.719$ ,  $p = 0.1801$ , Figure 2G). Furthermore, we examined the association between HDAC2 and k-bhb–marked chromatin. We observed a near-significant increase in the Fast-Single group ( $F_{(4,24)} = 1.150$ ,  $p = 0.3573$ , Figure 2H), which is directionally consistent with the newly described enzymatic capability of HDAC2 to engage with k-bhb substrates under acute metabolic shifts [22].

Collectively, our findings provide a biochemical framework for the transition from single to recurrent fasting chromatin states. An acute 24-h fast elevates circulating and nuclear BHB alongside increased HDAC2 activity, consistent with a transient

metabolic stress state that does not broadly favor acetylation. In contrast, IF is marked by sustained nuclear BHB accumulation and increased acetyl-CoA availability, coinciding with a shift toward a more permissive chromatin environment characterized by increased EP300 engagement and reduced HDAC2 interaction.

### 3.3 | Single Fasting Transiently Enriches Promoter H3K9 $\beta$ -Hydroxybutyrylation at Cytoprotective Genes

Building on the observed metabolic and chromatin-modifier changes, we next examined whether BHB signaling drives gene-specific histone remodeling under different fasting regimens. We began by reanalyzing a published ChIP-seq dataset [32] that mapped H3K9bhb enrichment after a 48-h fast (Figure 3A). Gene ontology (GO) analysis of the enriched promoter regions revealed significant involvement in biological



**FIGURE 2** | Hippocampal hemispheres were biochemically fractionated into cytosolic (A) and nuclear (B) compartments. BHB levels were quantified using the BHB-Glo assay after acidification/neutralization and 5× dilution, with results normalized to total protein content (BCA). (C) Acetyl-CoA levels were measured from 20mg of hippocampal tissue using a colorimetric assay. (D) HDAC2 enzymatic activity was quantified in hippocampal lysates (80μg protein) using a colorimetric activity assay. (E–H) Co-immunoprecipitation (co-IP) assays were performed to assess protein–chromatin interactions in the hippocampus. Representative blots and quantification show interactions between EP300 (IP) and H3K9bhb (E) or H3K27ac (F), and between HDAC2 (IP) and H3K27ac (G) or k-bhb–marked chromatin (H). All co-IP signals were normalized to total histone H3 to account for substrate abundance and are presented as mean fold change relative to the control group ± SEM. Values are mean ± SEM; One-way ANOVA,  $n = 5–6$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

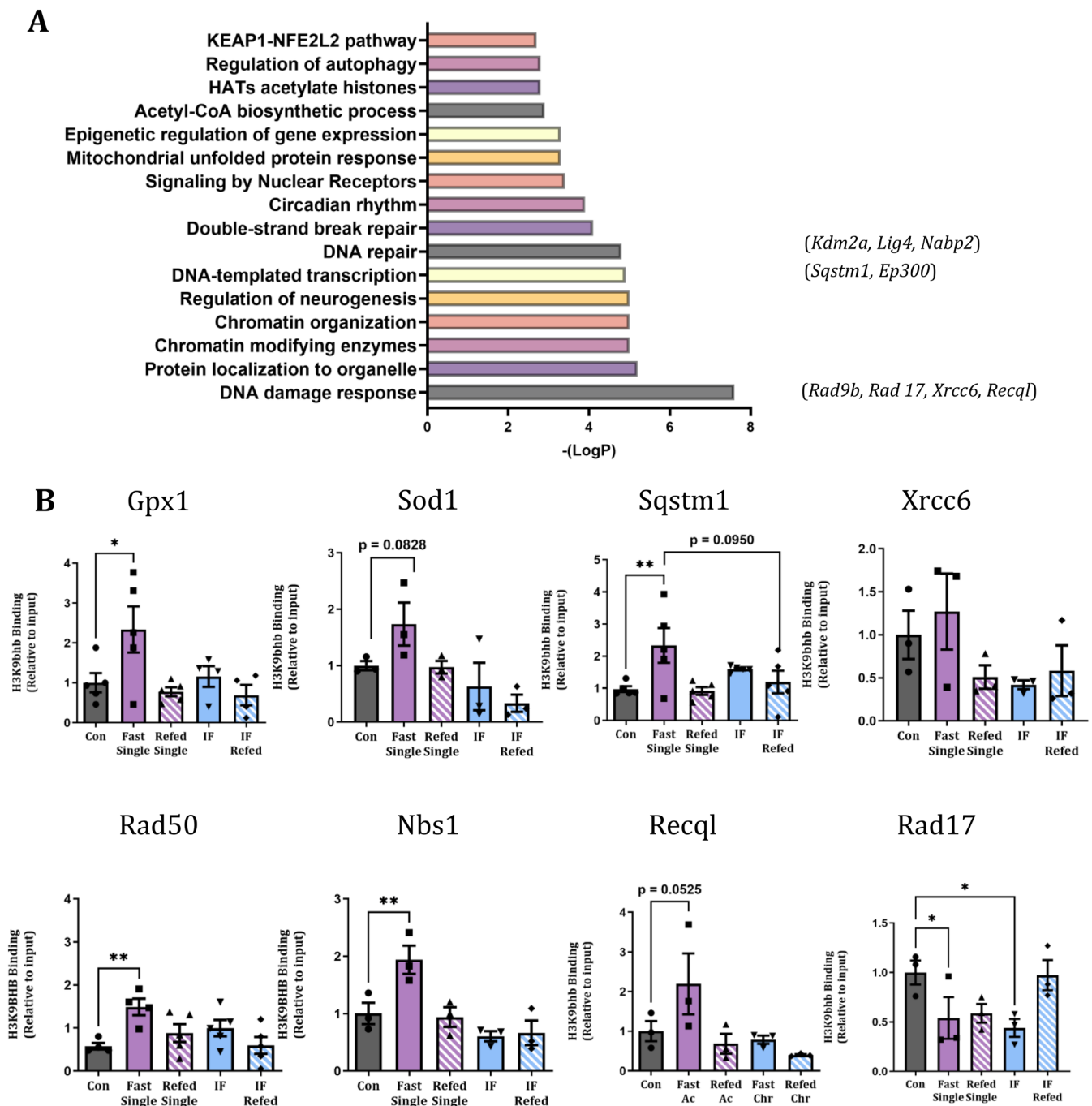
pathways associated with autophagy, oxidative stress response, and DNA damage repair (Figure 3A). Based on these findings, we curated a panel of genes with key regulatory roles in these processes: (i) oxidative stress response—*Gpx1* and *Sod1*; (ii) autophagy—*Sqstm1*; and (iii) DNA damage response—*Xrcc6*, *Rad17*, *Rad50*, *Nbs1*, and *Recql*. H3K9bhb enrichment at promoter regions of selected genes was assessed across all experimental groups, using chromatin-immunoprecipitation followed by quantitative PCR (ChIP-qPCR).

In the Fast-Single group, we observed a significant increase in H3K9bhb binding at the promoters of *Gpx1* ( $F_{(4,18)} = 3.977$ ,  $p = 0.0175$ ), *Sod1* ( $F_{(4,10)} = 3.777$ ,  $p = 0.0402$ ), *Sqstm1* ( $F_{(4,20)} = 3.874$ ,  $p = 0.0173$ ), *Rad50* ( $F_{(4,18)} = 3.657$ ,  $p = 0.0238$ ), *Nbs1* ( $F_{(4,10)} = 8.051$ ,  $p = 0.0036$ ), and *Recql* ( $F_{(4,10)} = 3.291$ ,  $p = 0.0576$ ), compared to the control group, indicating active chromatin association by BHB during acute fasting (Figure 3B). In contrast, H3K9bhb enrichment in chronic and refed groups was comparable to controls (Figure 3B). These findings suggest that in acutely fasted animals, BHB is capable of transiently associating with the promoter regions of cytoprotective genes. However, this interaction does not appear to persist following repeated fasting or refeeding, indicating a temporal window of chromatin engagement by BHB during early fasting states.

### 3.4 | Recurrent Fasting Regimen Increases Promoter H3K27ac Levels and Transcriptional Activation mRNA Expression of Hippocampal Cytoprotective Genes

Based on our biochemical and interaction analyses in Figure 2, which support increased EP300 engagement together with reduced HDAC2 association under recurrent fasting, we next asked whether these shifts translate into altered histone acetylation at cytoprotective loci. Hence, we quantified H3K27ac enrichment across all experimental groups. Interestingly, the Fast-Single group exhibited a mild increase in H3K27ac levels at several promoter regions, including *Gpx1* ( $F_{(4,25)} = 3.914$ ,  $p = 0.0133$ ), *Sqstm1* ( $F_{(4,30)} = 4.029$ ,  $p = 0.0099$ ), and *Xrcc6* ( $F_{(4,28)} = 3.956$ ,  $p = 0.0114$ ), compared to the control group (Figure 4A). In contrast, the IF group displayed a robust and significant increase in H3K27ac enrichment at the promoter regions of all tested genes, excluding *Rad17*, relative to controls (Figure 4A,D).

To determine whether this transcriptionally permissive chromatin state also corresponds with increased gene expression, we next assessed hippocampal mRNA levels of the selected genes. We identified two patterns of gene expression. The first, a “responsive” pattern, was characterized by a modest

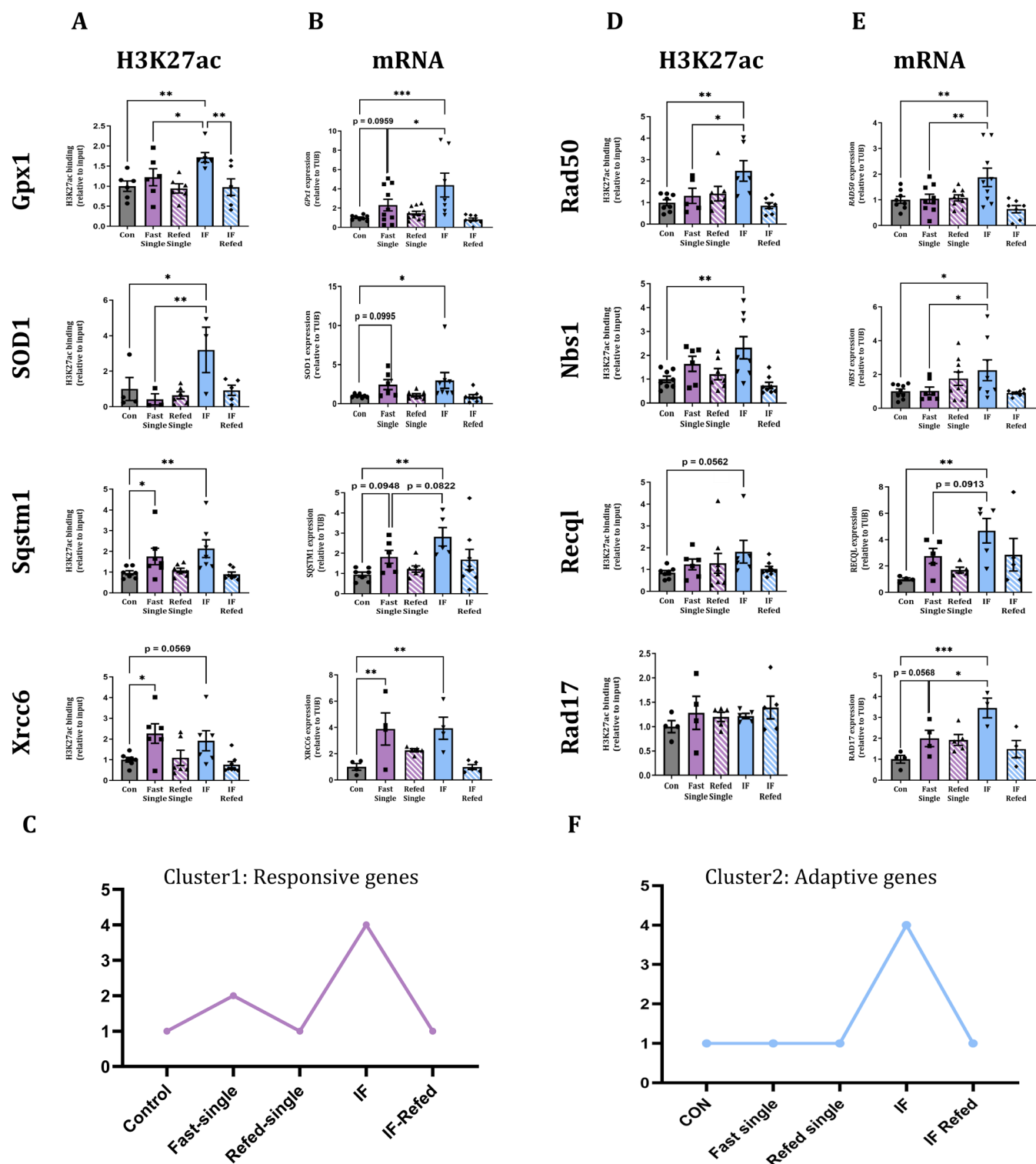


**FIGURE 3** | (A) Gene ontology (GO) analysis of the promoter regions enriched in H3K9bhb following a 48-h fast taken from Cornuti et al. [32] (B) On the day of sacrifice, the hippocampus of each animal was obtained. DNA fragments bound to H3K9bhb were extracted, and qPCR with designated primers to selected genes' promoters was used to determine enrichment levels, relative to input. Values are mean of fold change relative to control  $\pm$  SEM; one-way ANOVA,  $n = 3-5$ , \* $p < 0.05$ , \*\* $p < 0.01$ .

increase in gene expression during a single fast, followed by a more pronounced elevation in the IF group (Figure 4B,C). This pattern mirrored the dynamics of H3K27ac enrichment and was primarily observed in genes associated with the antioxidant response, including *Gpx1* ( $F_{(4,39)} = 5.978$ ,  $p = 0.0008$ ), *Sod1* ( $F_{(4,32)} = 2.932$ ,  $p = 0.0358$ ), *Sqstm1* ( $F_{(4,28)} = 3.539$ ,  $p = 0.0185$ ), and *Xrcc6* ( $F_{(4,17)} = 5.453$ ,  $p = 0.0052$ ).

The second pattern reflected an adaptive response to repeated fasting, wherein significant increases in mRNA expression

were detected exclusively in the IF group, with no appreciable changes in the Fast-Single or control groups (Figure 4E,F). This expression pattern also corresponded with the marked elevation of H3K27ac at the promoters of these genes, which were predominantly involved in DNA damage repair, such as *Rad50* ( $F_{(4,37)} = 4.429$ ,  $p = 0.0050$ ), *Nbs1* ( $F_{(4,36)} = 2.789$ ,  $p = 0.0392$ ). Although *Rad17* mRNA levels were significantly elevated in the IF group ( $F_{(4,15)} = 6.038$ ,  $p = 0.0042$ ), we did not detect any changes in H3K27ac enrichment at its promoter ( $F_{(4,18)} = 0.5583$ ,  $p = 0.6957$ ), suggesting alternative regulatory mechanisms may



**FIGURE 4** | On the day of sacrifice, the hippocampus of each animal was obtained. DNA fragments bound to (A) and (D) H3K27ac were extracted, and qPCR with designated primers to selected genes' promoters was used to determine enrichment levels, relative to input. (B) and (E) RNA was extracted, and qPCR was used to quantify the expression levels of selected genes. (C) and (F) Schematic representation of the patterns viewed in H3K27ac binding levels and mRNA expression. Values are mean of fold change relative to control  $\pm$  SEM; One-way ANOVA,  $n = 4-8$  for ChIP,  $n = 4-11$  for mRNA, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

be involved in its transcriptional activation. Consistent with the dynamics of PTHMs, both refed groups exhibited basal mRNA expression levels comparable to those of the control group. These data imply that the observed chromatin and transcriptional responses are associated with metabolic shift.

Collectively, our data suggests a potential immediate response mechanism activated by a single-fasting event and marked by H3K9bhb. However, this response appears limited in scope, as many genes in the Fast-Single group exhibited only modest and mostly nonsignificant increases in transcription. In contrast,

repeated fasting triggered broad transcriptional activation coupled with enriched H3K27ac at gene promoters, indicating an adaptive response to sustained IF. Notably, this adaptation centered on genes governing antioxidant and DNA-repair pathways. In addition, refeeding rapidly dampened both gene expression and H3K27ac, underscoring a reversible epigenetic switch that allows the brain to toggle between protective and basal states as energy availability changes.

### 3.5 | Repeated IF Accelerates Resolution of Activity-Induced DNA Damage in the Hippocampus

To determine whether fasting-induced molecular alterations translate into functional protective effects *in vivo*, we next examined key markers of stress adaptation and DNA damage. To differentiate acute “responsive” from long-term “adaptive” programs, we first assessed Hif1a, a master regulator of hypoxic and oxidative stress responses closely tied to mitochondrial metabolism. Western blot analysis revealed a significant increase in Hif1a protein in the Fast-Single group compared to controls ( $F_{(4,23)} = 3.401$ ,  $p = 0.0252$ ; Figure 5A), aligning with the elevated HDAC2 activity in this group (Figure 2G) and supporting an acute metabolic stress-like response to fasting. In contrast, Hif1a expression was significantly reduced in both IF and IF-Refed groups, as compared to Single-fast, aligning with broader transcriptional and epigenetic adaptations and suggesting the emergence of a less reactive, adaptive state under repeated fasting. Given the strong link between oxidative stress and DNA integrity, we next analyzed levels of hippocampal 8-oxo-dG, a mutagenic oxidative DNA lesion marker. WB analysis showed lower 8-oxo-dG levels in the IF and IF-Refed groups ( $p = 0.1353$ ,  $p = 0.048$ ; Figure SS1). Notably, this reduction in the IF-Refed group occurs despite the normalization of several chromatin and transcriptional signatures after refeeding. As our analyses focused on a defined set of candidates’ cytoprotective genes (prioritized from published ChIP-seq datasets [32]), we cannot exclude the possibility that additional protective programs, regulatory elements, or alternative DNA repair pathways remain engaged after refeeding and contribute to this phenotype.

In the next phase, we sought to evaluate DNA damage and repair mechanisms under physiologically relevant challenge. To achieve that, mice from all experimental groups were subjected to contextual fear conditioning (CFC), a hippocampus-dependent behavioral paradigm that elicits robust neuronal activation and corresponding increases in oxidative DNA lesions and DSBs [5, 33]. Following exposure to a mild foot shock in a novel context (i.e., a novel cage), brain tissues were collected at 0.5 h and 4 h post-conditioning (Figure 5B), time points previously shown to capture the induction and subsequent resolution of DNA damage and DSBs [4].

First, we measured 8-oxo-dG intensity in the dentate gyrus (DG) and CA1 subregions of the hippocampus using immunofluorescence staining. At 0.5 h post-conditioning, nuclear 8-oxo-dG intensity was significantly lower in IF and IF-Refed mice compared to controls (Figure 5C, Figure SS2), indicating that repeated fasting attenuates the oxidative burden induced by neuronal

activation. To complement this measure of oxidative lesions, we next examined  $\gamma$ H2AX foci as a marker of double-strand break (DSB) recognition and repair. As expected,  $\gamma$ H2AX foci were markedly elevated across all groups 0.5 h post-conditioning, reflecting the induction of DSBs in response to heightened neuronal activation (Figure 5D, Figure SS3). By 4 h,  $\gamma$ H2AX levels declined in all groups, consistent with ongoing DNA repair activity. Importantly, IF and IF-Refed mice displayed significantly lower  $\gamma$ H2AX levels at this later time point compared to controls (Figure 5D, Supplementary Figure S3), suggesting more efficient resolution of DSBs. Next, we quantified the decline in  $\gamma$ H2AX signal between 0.5 and 4 h (Figure 5E). This analysis revealed a significantly steeper reduction in the IF and IF-Refed groups (Figure 5E), indicating enhanced DNA repair capacity and more efficient resolution of activity-induced damage. These findings support the conclusion that repeated fasting enhances the brain’s capacity to resolve activity-induced DNA damage.

## 4 | Discussion

Our data places chromatin reorganization and cytoprotective enhancement at the core of the adaptive response to intermittent fasting. Repeated fasting initiates a chromatin program that promotes transcription of genes involved in both antioxidant defense and DNA repair. These molecular changes were reflected in enhanced genomic stability following physiologically induced hippocampal activation. Together, these findings support the view that IF fosters an adaptive cytoprotective state, enabling the brain to better withstand metabolic and oxidative challenges.

During a single 24 h fast, circulating and nuclear BHB levels increase and coincide with elevated HDAC2 activity (Figures 1C and 2B,D), consistent with a transient metabolic stress response, also supported by increased Hif1a (Figure 5A). Notably, recent evidence [22] indicates that HDAC2 can catalyze lysine k-bhb under specific metabolic conditions, which is conceptually consistent with our observation of a mild increase in HDAC2–k-bhb association in the acutely fasted state (Figure 2H). In this acute state, we observed a brief enrichment of H3K9bhb at promoters of cytoprotective genes without a corresponding transcriptional response (Figures 3 and 4), consistent with an early priming phase. In addition, acetyl-CoA levels remained unchanged relative to controls (Figure 2C), suggesting that the increased energetic demand of single fasting may preferentially divert acetyl-CoA toward mitochondrial oxidation, thereby limiting broader EP300-driven acetylation.

In contrast, recurrent IF was associated with higher nuclear BHB levels, which in this state may contribute to moderate HDAC2 activity (Figure 2B,D). Together with increased acetyl-CoA availability (Figure 2C), this metabolic configuration is consistent with enhanced histone acetyltransferase capacity, reflected by increased EP300 engagement with H3K27ac marked chromatin and reduced HDAC2 association (Figure 2E,G). Accordingly, at the promoters of key cytoprotective genes, the transient H3K9bhb signature was accompanied by a significant enrichment of H3K27ac in the IF group compared with controls (Figures 3 and 4). These findings suggest that BHB influences

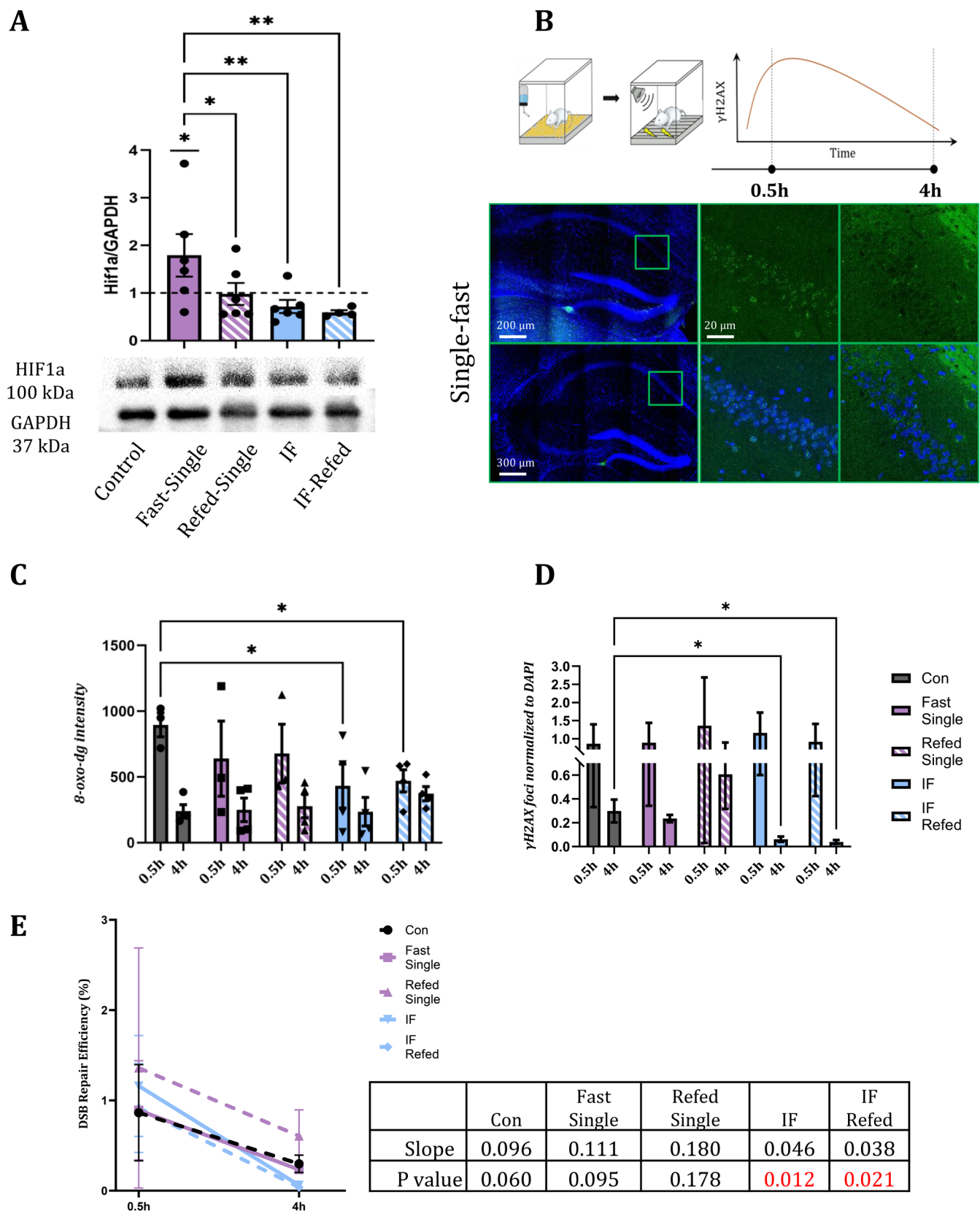


FIGURE 5 | Legend on next page.

chromatin architecture through both direct and indirect mechanisms, promoting a more open and transcriptionally permissive state during repeated fasting.

Importantly, these epigenetic modifications were reflected in the transcriptional upregulation of genes involved in key cytoprotective pathways (Figure 4), including antioxidant defense

**FIGURE 5** | (A) HIF1a Protein levels were measured by Western blot and normalized to Actin. (B) Schematic of the experimental design: All experimental groups were subjected to contextual fear conditioning test. Brains were collected at 0.5 h and 4 h post-CFC for  $\gamma$ H2AX immunostaining. Illustrative model of  $\gamma$ H2AX dynamics over time post-CFC, with peak DNA damage expected around 0.5 h and resolution by 4 h indicating repair efficiency. Lower panel show representative confocal images of hippocampal sections immunostained for  $\gamma$ H2AX (green) and DAPI (gray). Quantification of (C) mean 8-oxo-dG intensity within DAPI boundaries and (D)  $\gamma$ H2AX foci normalized to DAPI across experimental groups at 0.5 h and 4 h post-CFC. (E) Calculation of DSB repair efficiency (% decline in  $\gamma$ H2AX signal from 0.5 h—4 h). *p*-value was calculated by simple linear regression. Data represent mean  $\pm$  SEM; One-way ANOVA, *n* = 4–8, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

(e.g., Gpx1 and Sod1), autophagy (Sqstm1), and DNA damage response (Xrcc6, Rad50, Nbs1, and Recql). The gene expression patterns revealed distinct modes of regulation: while some genes showed a modest increase following a single-fasting episode, robust transcriptional activation was observed predominantly under repeated IF conditions. Prior studies [34, 35] have suggested that fasting can transiently elevate oxidative stress, but typically at mild and physiologically manageable levels rather than pathological extremes. Consistent with this notion, we observed that only a limited subset of cytoprotective genes was modestly upregulated following a single 24 h fast. Two plausible explanations might account for this subdued response. First, a brief fasting period may not produce sufficient oxidative stress to robustly activate the full stress response pathways. Second, essential metabolic or epigenetic mechanisms, such as sufficient accumulation of BHB or extensive chromatin remodeling, may remain incompletely engaged after a single fast, thus limiting gene activation. In contrast, the robust and coordinated induction of cytoprotective genes following repeated IF strongly supports the establishment of an adaptive transcriptional response. Crucially, we propose that this adaptive state is not solely driven by oxidative stress; rather, it critically depends on the progressive accumulation of BHB (Figure 2) and the remodeling of chromatin toward a transcriptionally permissive configuration (Figures 2–4).

Interestingly, refeeding was sufficient to reverse both the transcriptional and chromatin level effects of IF (Figure 4). Because only a single refeeding time point was examined, the kinetics of this reversal remain unresolved and will require longitudinal profiling to define how rapidly chromatin states reset following nutrient restoration. While these findings suggest that the cytoprotective state induced by fasting is dynamic and tightly linked to metabolic cues, they also imply the existence of an underlying ‘fasting molecular memory’. In other words, this reversibility supports a model in which ketone body signaling and epigenetic plasticity enable the brain to flexibly toggle between protective and basal states in response to shifting energetic demands. At the same time, the persistence of nuclear BHB after refeeding raises (Figure 2B) the possibility that intracellular BHB signaling extends beyond the fasting window and contributes to sustained genome-stabilizing effects. In addition, we cannot exclude that other protective programs, alternative repair pathways, or distal regulatory elements not examined here remain engaged after refeeding.

In alignment with our transcriptional data and the concept of a transient stress response, we observed a notable increase in Hif1a expression following a single fasting episode (Figure 5A). In contrast, Hif1a levels were significantly reduced in the IF group, despite repeated metabolic challenges. This inverse

pattern further strengthens the hypothesis that repeated IF induces an adaptive physiological state, one in which oxidative stress is more efficiently managed and stress-related signaling pathways are attenuated, reflecting a shift toward a refined, less reactive cellular program. Moreover, the downstream consequences of this adaptation were evident in our functional assays (Figure 5). Following hippocampal activation via contextual fear conditioning, animals in the IF and IF-Refed groups exhibited enhanced DNA repair efficiency, as demonstrated by the accelerated resolution of  $\gamma$ H2AX signals and reduced 8-oxo-dG nuclear accumulation [36–38]. Nevertheless, we acknowledge an apparent discrepancy whereby promoter-level chromatin and transcriptional signatures largely normalize after refeeding, whereas functional DNA damage markers remain improved (Figure 5, Figures S1–S3). One possibility is that protective programs outside the candidate gene set analyzed here contribute to the sustained functional phenotype. Another possibility is that IF-Refed animals retain an enhanced ability to rapidly re-engage cytoprotective chromatin states upon neuronal activation, which was not captured by basal homeocage profiling (Figure 4).

Taken together, our findings support a model in which repeated IF enhances hippocampal resilience by orchestrating a coordinated transcriptional and epigenetic response. Through dynamic BHB-linked chromatin remodeling and activation of cytoprotective gene networks, recurrent fasting promotes a genome-maintenance state that improves tolerance to metabolic and oxidative challenge. These results contribute to a growing understanding of how dietary interventions can modulate brain health and suggest new avenues for leveraging metabolic epigenetic interactions in the prevention of neurodegenerative diseases.

#### 4.1 | Limitations

Although the results of this work represent progress in understanding the interplay between metabolism, epigenetic regulation, and cytoprotective gene networks, several limitations warrant further investigation. First, because chromatin mapping was restricted to selected promoters, future genome-wide profiling will be important to define the full regulatory landscape underlying this adaptation.

In this study, experiments were conducted exclusively in female mice, selected for their robust ketogenesis and unique IF-induced transcriptional sensitization often absent in males [29, 39, 40]. While this strategy maximized sensitivity for detecting BHB-linked chromatin responses in the hippocampus, we acknowledge that it limits generalizability given documented

sexual dimorphism in fasting physiology, ketone metabolism, and chromatin regulation. Future studies are required to determine whether similar chromatin remodeling and genome-maintenance adaptations occur in males, and to assess potential differences in the magnitude or kinetics of these responses.

Finally, although our functional assays indicate faster resolution of activity-induced  $\gamma$ H2AX and reduced 8-oxo-dG accumulation, we did not directly quantify pathway-specific repair flux (e.g., BER or NHEJ). We therefore interpret these findings as evidence for improved genome maintenance rather than definitive enhancement of a particular repair pathway. Future studies combining pathway-selective reporter systems with targeted pharmacological or genetic perturbations will be needed to identify which repair modules are preferentially strengthened by IF. Importantly, contextual fear conditioning was used here as a physiological trigger for hippocampal activation and DNA lesion formation, rather than to quantify memory performance. Nevertheless, determining whether fasting modulates behavioral outcomes, including memory strength, consolidation, or extinction, remains an important direction for future work.

### Author Contributions

H.P.: conceptualization, data curation, formal analysis, investigation, methodology, resources, software, validation, visualization, writing—original draft, writing—review and editing; J.B.: conceptualization, data curation, formal analysis, visualization, writing—review and editing; T.R.: data curation, formal analysis, investigation, methodology, project administration, resources, supervision, validation, writing—review and editing, writing—original draft; R.Y.: conceptualization, data curation, formal analysis; N.G.: conceptualization, data curation, formal analysis; A.M.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing—original draft, writing—review and editing.

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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

All data supporting the findings of this study are included within the article. Additional raw datasets or relevant materials are available from the corresponding author upon reasonable request.

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### Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** fsb271681-sup-0001-FigureS1.pdf. **Figure S2:** fsb271681-sup-0002-FigureS2.pdf. **Figure S3:** fsb271681-sup-0003-FigureS3.pdf. **Data S1:** fsb271681-sup-0004-FigureLegends.docx.