EPSTEIN-BARR VIRUS AND EPIGENETIC MODIFICATIONS: INSIGHTS INTO NEURODEGENERATION AND DEHYDROEVODIAMINE AS A NEUROPROTECTIVE AGENT

M.Sc. Thesis

By

CHAITALI VORA

DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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EPSTEIN-BARR VIRUS AND EPIGENETIC MODIFICATIONS: INSIGHTS INTO NEURODEGENERATION AND DEHYDROEVODIAMINE AS A NEUROPROTECTIVE AGENT

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Epstein-barr virus and epigenetic modifications: insights into Neurodegeneration and Dehydroevodiamine as a Neuroprotective agent** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2024 to May 2025 under the supervision of Dr. Hem Chandra Jha, Associate Professor, Indian Institute of Technology Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Chaitali Vora 22/05/2025

Signature of the student with date

(CHAITALI VORA)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

22/5/2025

Signature of MSc thesis supervisor

(DR. HEM CHANDRA JHA)

CHAITALI VORA has successfully given her M.Sc. Oral Examination held on **May 2025**

Signature(s) of Supervisor(s) of MSc thesis

Date: 22/5/25

P.V. Kodgire

Convener, DPGC

Date: 22/05/2025

MAY-2025

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DEDICATED TO MY FAMILY

ABSTRACT

Neurodegenerative diseases, including Alzheimer's, are complex disorders influenced not only by genetic and environmental factors but also by viral infections that can profoundly disrupt the brain's regulatory systems. Epstein-Barr Virus (EBV), a widely prevalent neurotropic herpesvirus, has emerged as a potential contributor to neurodegeneration through its capacity to induce chronic inflammation and epigenetic remodeling of host genes. This study investigates how EBV infection modulates the epigenetic status of critical neurological genes—such as *BACE1*, *APP*, *LDLR*, and *GFAP*—focusing on histone methylation and DNA methylation at splice sites that govern isoform expression. Using a neural tri-culture system comprising neurons, astrocytes, and microglia, we observed that EBV infection resulted in increased repressive histone marks (H3K9me3 and H3K27me3), altered DNA methylation patterns, elevated oxidative stress, and dysregulated expression of disease-associated genes and neurotrophic factors. Methylation-specific PCR (MSP) revealed hypomethylation near splice junctions in disease-relevant genes, correlating with isoform shifts that promote amyloidogenic and inflammatory responses. Western blotting further validated changes in isoform expression at the protein level. Importantly, treatment with the natural compound Dehydroevodiamine (DHE) reversed several EBV-induced epigenetic changes, restored histone balance, and attenuated disease markers, underscoring its therapeutic potential. Collectively, these findings suggest that EBV-driven epigenetic modifications may play a critical role in the initiation and progression of neurodegenerative processes and highlight the need for future research targeting viral-epigenetic interactions in the brain.

LIST OF PUBLICATIONS

Publications from Thesis

- **Vora C**,……Jha, H.C., From Latency to Neuroinflammation: How Viruses Reprogram Host Epigenomes. *Review manuscript*, Submission ID: JVI00802- 25.

- **Vora C**,……Jha, H.C., Epstein-Barr virus and Epigenetic Modifications: Insights into Neurodegeneration and Dehydroevodiamine as a Neuroprotective Agent. *Thesis Manuscript under Preparation.*

Additional Projects

- *In silico* assessment of *Lactobacillus*-proteins on *H. pylori*-mediated oncogenesis (Under CRDT, IIT Indore).
- Investigation of synaptic modulations in coinfected Gut-brain axis.

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ACRONYMS

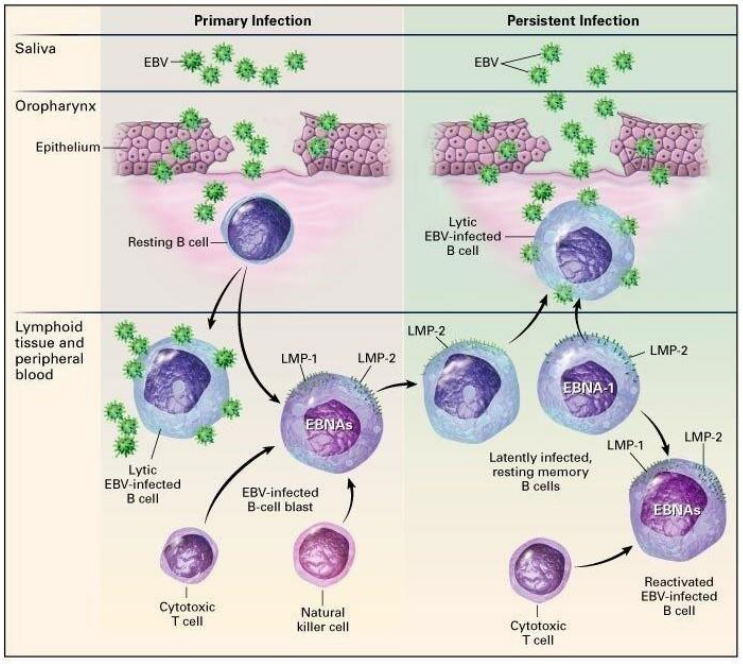
Aβ	Amyloid Beta
APP	Amyloid Precursor Protein
BACE1	Beta-site APP Cleaving Enzyme 1
LDLR	Low-Density Lipoprotein Receptor
GFAP	Glial Fibrillary Acidic Protein
EBV	Epstein-Barr Virus
DHE	Dehydroevodiamine
KPI	Kunitz Protease Inhibitor
ROS	Reactive Oxygen Species
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
MSP	Methylation-Specific PCR
H3K9	Histone H3 at Lysine 9
H3K27	Histone H3 at Lysine 27
H3K9me3	Trimethylation of Histone H3 at Lysine 9
H3K27me3	Trimethylation of Histone H3 at Lysine 27
hnRNPs	Heterogeneous Nuclear Ribonucleoproteins
MeCP2	Methyl-CpG Binding Protein 2
MBD2	Methyl-CpG Binding Domain Protein 2
IC10	Inhibitory Concentration for 10% of Cells
MBP	Myelin Basic Protein
BDNF	Brain-Derived Neurotrophic Factor
VEGF	Vascular Endothelial Growth Factor
NGF	Nerve Growth Factor
GDNF	Glial Cell Line-Derived Neurotrophic Factor

Chapter 1: Introduction

1.1 Epstein Barr Virus

Epstein-Barr Virus (EBV), also known as human herpesvirus 4 (HHV-4), is a widespread gammaherpesvirus that infects over 90% of the global population and establishes lifelong latency primarily in B lymphocytes [1]. The virus enters host cells via interaction between its major envelope glycoprotein gp350 and the CD21 receptor on B cells, with membrane fusion facilitated by gp42, gH/gL, and gB. Once inside, EBV follows either a lytic or latent cycle. Latency is categorized into types I–III and is characterized by the expression of specific viral proteins such as Epstein-Barr Nuclear Antigens (EBNAs), Latent Membrane Proteins (LMP1, LMP2A), and non-coding RNAs (EBERs), which help the virus persist by subverting apoptosis and immune responses. LMP1 mimics CD40 signaling, while LMP2A mimics B-cell receptor activity, promoting cell survival and immune evasion [2]. EBV is strongly linked with multiple malignancies including Burkitt lymphoma, Hodgkin lymphoma, and nasopharyngeal carcinoma, and it plays a critical role in autoimmune diseases such as multiple sclerosis (MS) and systemic lupus erythematosus (SLE), likely through molecular mimicry and chronic immune activation. The virus has also been increasingly implicated in neurodegenerative diseases like MS and Alzheimer's disease, where EBV-infected B cells infiltrate the central nervous system, triggering microglial activation, astrocyte reactivity, blood-brain barrier disruption, and oxidative stress [3]. Viral proteins and exosomal miRNAs contribute to mitochondrial dysfunction and altered neuroimmune signaling. Moreover, EBV induces epigenetic modifications, such as DNA methylation and histone alterations, which further promote immune evasion and pathogenesis. Diagnosis is typically based on serological markers (e.g., VCA-IgM, EBNA-IgG)

and PCR-based detection of viral [4]. Current therapies like acyclovir are effective only against the lytic phase, whereas latent infections remain difficult to eradicate. Therefore, emerging strategies include B-cell depletion therapies, latency-disrupting agents, and gene-editing technologies aimed at controlling EBV's role in cancer, autoimmunity, and neurodegeneration [5]



.Figure 1. The Epstein-Barr Virus (EBV) life cycle alternates between latent and lytic phases to ensure persistence and viral propagation. (Source: Karen H Costenbader, Elizabeth W Karlson et al; *Arthiritis Research and Therapy, Janumary 2006)*

1.2 Neurodegeneration

Neurodegeneration encompasses a group of progressive, debilitating conditions marked by the gradual dysfunction and death of neurons, commonly affecting the central and peripheral nervous systems. Disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Multiple Sclerosis (MS), and Amyotrophic Lateral Sclerosis (ALS) are prime examples, each with distinct clinical features but sharing overlapping pathogenic mechanisms [6]. These mechanisms include chronic neuroinflammation, mitochondrial

dysfunction, oxidative stress, excitotoxicity, protein aggregation, impaired autophagy, and dysregulated immune responses. A critical early event in many neurodegenerative diseases is the activation of microglia and astrocytes, which, upon sensing neuronal injury or misfolded proteins, release pro-inflammatory mediators such as IL-1β, TNF-α, and IL-6. This sustained inflammatory response leads to a toxic environment that exacerbates neuronal injury. Concurrently, mitochondrial damage impairs ATP production and increases the generation of reactive oxygen species (ROS), leading to oxidative damage of proteins, lipids, and DNA [7]. Proteinopathies, including amyloid-beta plaques and neurofibrillary tangles in AD, α-synuclein inclusions in PD, or mutant huntingtin in HD, disrupt intracellular trafficking and synaptic function, contributing to neuronal dysfunction. In MS, immune-mediated demyelination and axonal injury occur due to both peripheral immune infiltration and resident glial activation, further supported by blood-brain barrier (BBB) breakdown. Autophagy, the process responsible for clearing damaged organelles and proteins, is often impaired, resulting in accumulation of toxic aggregates [8]. Moreover, epigenetic changes such as aberrant DNA methylation, histone modifications, and non-coding RNA expression are increasingly recognized as contributors to disease pathogenesis by altering neuronal gene expression and glial reactivity. As these pathological processes are typically interlinked, they form self-reinforcing cycles that culminate in synaptic failure, loss of neural connectivity, and eventual neuronal death [9]. Due to the multifactorial nature of neurodegeneration, current therapeutic strategies are largely symptomatic or palliative, with ongoing research aimed at identifying molecular targets that can halt or slow the progression of these devastating disorders [10]

1.3 EBV pathogenesis

Epstein-Barr Virus (EBV), a ubiquitous human herpesvirus, is a major

etiological agent of various diseases, including infectious mononucleosis, Burkitt lymphoma, nasopharyngeal carcinoma, and autoimmune and neurodegenerative disorders. The virus primarily targets B lymphocytes, though it also infects epithelial cells and can establish lifelong latency in these cell types [11] During latency, EBV expresses a limited set of viral genes, including the Latent Membrane Protein 1 (LMP1), Epstein-Barr Nuclear Antigen 1 (EBNA1), and other latency-associated proteins, which play crucial roles in immune evasion and the transformation of infected cells. LMP1, in particular, mimics the signaling of tumor necrosis factor receptors, activating key cellular pathways such as NF-κB, PI3K/AKT, and MAPK, leading to enhanced cell survival, proliferation, and immune tolerance [12]. These viral proteins contribute to the persistence of EBV and are implicated in the development of EBV-associated malignancies. The virus is also capable of inducing both lytic and latent phases, with the transition between these stages being tightly regulated by host factors and viral proteins. The ability of EBV to modulate host immune responses and cellular pathways is a major factor in its association with malignancies and autoimmune diseases, such as systemic lupus erythematosus and multiple sclerosis [13]. Recent studies have highlighted the role of EBV in neurodegeneration, particularly in conditions such as Alzheimer's disease and MS, where the virus has been shown to induce epigenetic modifications. These modifications include alterations in DNA methylation patterns, histone modifications, and chromatin remodeling, which can disrupt the expression of critical genes involved in neuronal function and immune regulation. Notably, EBV infection has been linked to increased expression of genes involved in neuroinflammation and immune responses, such as the pro-inflammatory cytokines TNF-α and IL-6, which are known to exacerbate neurodegeneration in diseases like Alzheimer's [14]. The virus's ability to alter the epigenome of host cells is thought to contribute to long-term changes in gene expression,

potentially leading to chronic inflammation and neuronal dysfunction, hallmark features of many neurodegenerative diseases. Furthermore, the interaction between EBV and the host immune system is complex, as the virus can both stimulate and evade immune responses [15]. In MS, for instance, EBV infection has been shown to trigger the activation of autoreactive T cells and the subsequent damage to the myelin sheath in the central nervous system (CNS), a process that may be facilitated by epigenetic changes induced by the virus. Thus, EBV's role in both oncogenesis and neurodegeneration highlights its significance as a modulator of cellular and immune responses, contributing to a range of chronic diseases through its ability to persist in the host, induce epigenetic modifications, and dysregulate immune responses [16].

1.4 EBV and Neurodegenretion

Epstein-Barr Virus (EBV), a ubiquitous gamma-herpesvirus infecting over 90% of the global population, has emerged as a significant contributor to neurodegenerative disorders such as Multiple Sclerosis (MS), Alzheimer’s Disease (AD), Parkinson’s Disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Huntington’s Disease (HD). Following primary infection via epithelial cells and B lymphocytes—mediated by glycoprotein gp350 binding to the CD21 receptor and fusion facilitated by gp42, gH/gL, and gB-EBV establishes latency predominantly in memory B cells. During latency, minimal gene expression (e.g., EBNA and LMP families) ensures immune evasion while maintaining viral persistence, with periodic reactivation under immunosuppressive or stress-induced conditions. A growing body of evidence implicates EBV in chronic neuroinflammation, blood-brain barrier (BBB) dysfunction, and immune dysregulation, all of which are pivotal drivers of neurodegeneration [17]. In MS, EBV-infected B cells infiltrate the CNS and form ectopic lymphoid follicles, perpetuating inflammation

through cytokine release (TNF-α, IL-6, IFN-γ) that triggers microglial activation, reactive astrogliosis, and demyelination. Molecular mimicry between EBV antigens and neural proteins like myelin basic protein (MBP) may induce autoimmune cross-reactivity, exacerbating tissue damage. In AD, chronic EBV infection promotes neuroinflammatory cascades that enhance amyloid-beta aggregation, tau hyperphosphorylation, and synaptic dysfunction [18]. Additionally, EBV can compromise the BBB, facilitating the entry of peripheral immune cells and viral miRNAs, which modulate host gene expression related to apoptosis and neurodegenerative pathways. In PD, EBV-induced oxidative stress and mitochondrial impairment in dopaminergic neurons—via LMP1 and LMP2A—lead to ATP depletion, ROS overproduction, and neuronal apoptosis. EBV-derived exosomal miRNAs further contribute by altering host epigenetic regulation and promoting chronic neurotoxicity. The virus's impact on mitochondrial dynamics, particularly via interaction with NF-κB and PI3K/Akt signaling, links viral persistence with metabolic failure in neurons. In ALS and HD, EBV-driven neuroinflammation and mitochondrial destabilization have been associated with motor neuron degeneration and accelerated disease progression. Beyond direct infection, EBV epigenetically reprograms host chromatin architecture by inducing histone modifications and aberrant DNA methylation patterns, especially in genes governing immune responses and neural function [19]. These viral epigenetic footprints may serve as persistent 'molecular scars' that rewire transcriptional landscapes in neurons and glia. Given the complexity of EBV's neuropathogenic mechanisms—including its ability to hijack immune signaling, disrupt redox homeostasis, and induce chronic inflammation— therapeutic strategies targeting EBV's latent reservoir, B-cell activity, and downstream inflammatory responses hold promise. Approaches under investigation include latency-reversing agents, CRISPR-Cas9-based gene editing, B-cell depletion therapies like ocrelizumab, microglial

modulators, and antioxidants aimed at restoring mitochondrial health. Understanding the interplay between EBV latency, immune modulation, and neuroepigenetic reprogramming is thus essential for developing targeted therapies that mitigate or prevent virus-associated neurodegeneration [20].

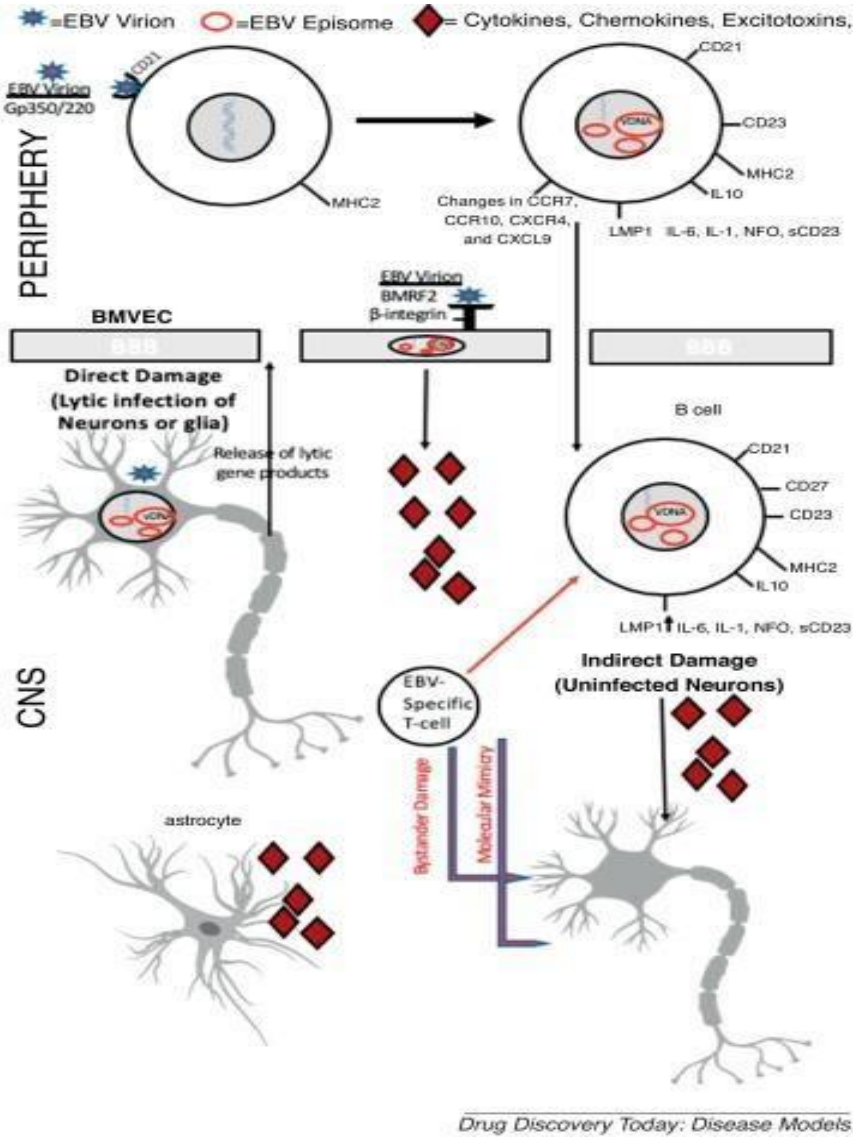


Figure 2. Epstein Barr virus and Neurological disorders. (Source: Samantha S. Soldan, *et. al*; Drug Discovery Today: Disease Models 2020)

1.5 Epigenetic modifications

Epigenetic modifications refer to heritable changes in gene expression

or cellular phenotype that do not involve alterations in the underlying DNA sequence but are instead driven by chemical modifications to DNA and histones, or by the regulation of chromatin structure. Key epigenetic mechanisms include DNA methylation, histone modification, and non-coding RNA-mediated regulation, each of which plays a pivotal role in the control of gene expression. DNA methylation, typically occurring at the 5' position of cytosine residues within CpG dinucleotides, leads to gene silencing by inhibiting transcription factor binding and promoting the recruitment of repressive chromatin remodeling complexes [21]. Histone modifications, including acetylation, methylation, phosphorylation, and ubiquitination, alter chromatin structure by either promoting a more open (euchromatin) or closed (heterochromatin) conformation, thereby influencing gene accessibility to transcriptional machinery. For example, histone acetylation generally correlates with gene activation by neutralizing the positive charge of histones, reducing their affinity for negatively charged DNA, and allowing for a more relaxed chromatin structure. In contrast, histone methylation can either activate or repress transcription depending on the specific histone residue modified and the type of methylation (mono-, di-, or tri-methylation). Non-coding RNAs, particularly microRNAs and long non-coding RNAs, can also regulate gene expression by interacting with mRNA transcripts or chromatin, affecting both transcription and translation processes [22]. The dynamic regulation of these epigenetic marks is crucial for cellular differentiation, development, and response to environmental signals. Dysregulation of epigenetic mechanisms has been implicated in various diseases, including cancer, neurological disorders, and autoimmune diseases, where aberrant DNA methylation patterns or histone modifications lead to inappropriate gene activation or silencing, thus contributing to disease pathogenesis. Moreover, recent studies have highlighted the interplay between viral infections, such as Epstein-Barr virus (EBV), and host epigenetic landscapes,

suggesting that viruses may induce lasting epigenetic modifications that contribute to the development of chronic conditions and malignancies [23].

1.6 EBV modulated Epigenetics modifications in Neurodegenration.

Epstein - Barr Virus (EBV) is a highly versatile virus that not only contributes to the development of cancers, such as Burkitt lymphoma (BL) and Hodgkin lymphoma (HL), but also plays a significant role in immune modulation and neurodegeneration through epigenetic reprogramming. In BL, EBV alters methylation patterns of critical genes such as ID3, TCF3, and RUNX1, with EBV-encoded Latent Membrane Protein 1 (LMP1) driving promoter hypermethylation and activating DNA methyltransferase enzymes, which distinguishes EBV-positive BLs from EBV-negative. These epigenetic shifts may also extend to central nervous system (CNS) disorders, indicating a potential link between lymphomagenesis and neurodegenerative processes. Notably, the methylation of ID3 and TCF3, genes crucial for synaptic plasticity and neuronal survival, suggests that EBV-driven epigenetic changes could bridge the gap between cancer development and neurodegeneration [17]. In nasopharyngeal carcinoma (NPC), two distinct epigenetic subtypes emerge: HyperNPC, characterized by DNMT1/3A-driven hypermethylation and CTCF loss, and HypoNPC, marked by APOBEC3A-mediated hypomethylation. Loss of CTCF destabilizes methylation patterns and impairs transcription factor binding, further supporting the notion that EBV-induced chromatin alterations can affect both cancer progression and neuronal dysfunction. EBV also plays a pivotal role in immune evasion by upregulating CD74 and inducing T cell exhaustion, underscoring its capacity to disrupt both the immune system and epigenetic regulation. In HL, promoter hypermethylation of key

cancer-related genes, such as P16, reveals distinct epigenetic patterns based on EBV status, with EBV-positive HLs exhibiting strong LMP1 expression and virus-driven epigenetic modifications [24]. In contrast, EBV-negative HLs show alternative methylation patterns affecting genes like DAPK and SHP1 (Shilatifard, 2012). Moreover, multiple gene hypermethylation is more common in females, impacting genes like GSTP1 and RASSF1A, which are involved in oxidative stress responses in the brain, suggesting that similar mechanisms may influence neuronal health and contribute to neurodegeneration. Further complicating the landscape, BART lncRNA-driven immune signaling, which is induced by EBV, could exacerbate neurodegeneration by enhancing inflammation, a process that may be amplified in the context of EBV-associated CNS autoimmunity [25]. In the MUN14 EBV^{+} Burkitt lymphoma model, epigenetic reprogramming increases the expression of neuroinvasion-associated molecules, such as CXCR4 and osteopontin (SPP1), both of which are implicated in B-cell migration and neuroinvasion. Neutralizing osteopontin reduces this effect, suggesting that these molecules contribute to CNS pathology in primary CNS lymphoma (PCNSL) and multiple sclerosis (MS). These findings emphasize the complex and multifactorial influence of EBV in both oncogenesis and neurodegeneration, highlighting epigenetic reprogramming as a critical mediator of its effects on the immune system and neuronal function. [26].

Chapter 2: Review of Past Work and Problem Formulation

2.1Review of Past Work

Epstein-Barr virus (EBV) is a pivotal etiological factor in various cancers, including Burkitt lymphoma (BL), Hodgkin lymphoma (HL), and nasopharyngeal carcinoma (NPC), primarily through its ability to induce epigenetic modifications in key regulatory genes such as ID3, TCF3, and RUNX1. EBV-driven alterations in DNA methylation and histone modifications, facilitated by viral proteins like Latent Membrane Protein 1 (LMP1), lead to the silencing of tumor suppressor genes and the activation of oncogenes, contributing to tumorigenesis and immune evasion. In addition to its oncogenic properties, EBV has been implicated in the pathogenesis of autoimmune diseases and neurodegenerative disorders such as multiple sclerosis (MS) and Alzheimer's disease (AD). [27] In these conditions, EBV mediates significant epigenetic reprogramming by altering the expression of genes involved in immune regulation, synaptic plasticity, and neuronal survival. This reprogramming results in dysregulated immune responses, sustained chronic inflammation, and neuronal damage. Specifically, EBV-induced epigenetic changes in genes critical for synaptic function, such as ID3 and TCF3, and in neuroinflammatory molecules like osteopontin, exacerbate neurodegeneration. Osteopontin, in particular, facilitates the migration of EBV-infected B-cells into the central nervous system (CNS), promoting chronic neuroinflammation and amplifying neurodegenerative processes [28]. Additionally, EBV infection alters chromatin structure and transcription factor binding, further contributing to the disruption of normal neuronal function and promoting neuroinflammatory cascades. Despite the increasing recognition of EBV's role in neurodegenerative diseases, the precise molecular mechanisms by which EBV- induced

epigenetic modifications drive neuronal dysfunction and pathology remain incompletely understood. This underscores the need for continued research to elucidate the viral entry pathways, epigenetic alterations, and their downstream effects on neuroinflammation, with the goal of identifying potential therapeutic targets to mitigate EBV-associated neurodegeneration [29].

2.2 Problem Formulation

EBV, a neurotropic gammaherpesvirus, has been increasingly linked to the onset and progression of neurodegenerative diseases such as MS, AD and PD. Its ability to establish lifelong latency in host cells and trigger chronic neuroinflammation positions it as a critical environmental risk factor in neurological disorders. Recent studies suggest that EBV contributes to disease pathology by altering the host epigenome, particularly through changes in DNA methylation and histone methylation, which regulate key genes involved in immune signaling, neuroplasticity, and DNA repair pathways. However, the mechanisms by which EBV crosses into the central nervous system (CNS), infects neural cells, and reprograms their epigenetic landscape remain poorly understood. Notably, membrane cholesterol has been identified as a facilitator of EBV entry via lipid raft-mediated endocytosis, highlighting a potential target for disrupting viral infection. To investigate these mechanisms in a physiologically relevant environment, this study employs a tri- culture model consisting of neurons, astrocytes, and microglia in a 5:2:1 ratio, simulating the complex cellular interactions of the brain. This model allows for a comprehensive assessment of EBV-induced epigenetic modifications and cellular responses in a controlled in vitro setting. Moreover, the therapeutic potential of natural phytocompounds such as Dehydroevodiamine (DHE), demethoxycurcumin (DMC), and rosmarinic acid (RA) is explored, with a focus on their ability to reverse or mitigate EBV-induced DNA and histone methylation

changes, thus offering a possible strategy to protect against or slow the progression of neurodegeneration.

Chapter 3: Aim and Objectives

3.1 Aim

The aim of this study, *"Epstein-Barr Virus and Epigenetic Modifications: Insights into Neurodegeneration and Dehydroevodiamine as a Neuroprotective Agent"*, is to explore how Epstein-Barr Virus (EBV) contributes to the development of neurodegenerative diseases through alterations in the host's epigenetic landscape. Specifically, the research investigates changes in DNA and histone methylation patterns within a neural tri-culture system (astrocytes:neurons:microglia in 5:2:1 ratio), simulating the brain environment. By examining both EBV's latent and lytic activity and its influence on neurodegenerative markers, the study aims to uncover mechanisms of virus-induced neural damage. Additionally, it evaluates the therapeutic potential of Dehydroevodiamine (DHE), a natural compound, in reversing or mitigating these epigenetic changes, thus offering insights into novel neuroprotective strategies.

3.2 Objectives

This study aims to investigate the epigenetic modulations induced by EBV infection in a neural tri-culture model composed of astrocytes, neurons, and microglia in a 5:2:1 ratio. Special emphasis is placed on analyzing changes in DNA methylation and histone methylation that may contribute to virus-mediated neurodegeneration. By examining alterations in both EBV latent and lytic markers alongside host neurodegenerative markers, this work seeks to elucidate the potential mechanisms by which EBV influences neural homeostasis. Furthermore, the study evaluates the neuroprotective role of DHE against EBV dUTPase, aiming to understand how this compound may counteract epigenetic dysregulation and offer therapeutic potential in neuroinflammatory conditions.

- Alteration in EBV latent, lytic markers, and host neurodegenerative markers in tri-culture model

- To elucidate epigenetic modulations upon EBV infection and their plausible role in virus-mediated neurodegeneration.

- To demonstrate neuroprotective effect of Dehydroevodiamine on EBV dUTPase to understand host epigenetic dynamics.

Chapter 4: Materials and Methods

4.1 Materials

Biosafety level 2A cabinet (cell culture), Laminar airflow hood (for working on bacteria), Centrifuge, Refrigerator (-80ºC, -20ºC, 0ºC, 4ºC), Heat block, pH meter, Vortex shaker, CO2 incubator, Liquid Nitrogen Container, Pipette with tips, Serological pipettes, Sterile disposable Culture dish (60mm and 100 mm), 15 ml screw-cap centrifuge tubes, 1.5 ml micro centrifuge tubes, 6/ 12/ 24 well plates, Glass slides and coverslip, Homogenizer, 0.22µm filter, Probe sonicator, Microplate reader, Trans illuminator, Ethanol, PBS, Phenol, Chloroform, Sodium Acetate, Loading dye, 2.5% Glutaraldehyde, triton X100, Trypsin 0.05%, tris-HCl, Sodium dodecyl sulfate, BHI agar, DMSO, Isopropanol, 100 and 70% Ethanol, Ponceau, Glycerol, Tris free base, Paraformaldehyde, methanol.

4.2 Methods

4.2 1 Mammalian cell culture

The following cell lines were obtained from the National Centre for Cell Science, Pune, India – IMR-32, U87-MG, and CHME-3. For virus purification, HEK 293T cells were used, which contain stably transfected bacterial artificial chromosome (BAC) green fluorescent protein (GFP)-EBV. The cells were cultured in Dulbecco's modifed Eagle's medium (DMEM; South America origin, Gibco, New York, USA) containing 10% fetal bovine serum (FBS; South America origin, Gibco, New York, USA) with 100U/mL penicillin/streptomycin (FBS; South America origin, Gibco, New York, USA). The cells were incubated in 5% CO2 and humidified air at 37 °C (Forma, Steri-cycle i160, Thermo Scientifc, Waltham, USA).

4.2.2 Virus Isolation and Purification

The BAC EBV GFP was stably transfected into HEK-293T cells and were grown in complete DMEM with puromycin selection. EBV particles were obtained by using protocol illustrated previously.

4.2.3 Cell Cytotoxicity Assay

The MTT assay was performed to determine the cytotoxic activity of the drug compounds Dehydroevodiamine and Acyclovir as a positive control toward Tri- culture cells. The cells were counted and seeded in a 96-well plate according to 10,000 cells per well density. The cells were cultured in 200 μL of growth medium containing DMEM and 10% fetal bovine serum supplied with 0.5% antibiotics solution (Pen-Strep). After 24h of culture, the cells were treated with different dilutions of both the Test drug and positive control from a range of 250um to 0um and incubated for 24hrs. After the completion of the respective incubation period, MTT (MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) dye was added to the cells and kept at 37 °C for 3 h in a CO2 incubator. Thereafter, the solution was removed, and to dissolve the formazan crystals formed, 100 μL of DMSO/well was added and shaken for 2 h on the rocker. The optical density was then recorded on a multiplate reader.

4.2.4 Infection Methodology

Cells were seeded and allowed to adhere for 24 hours prior to infection. Epstein-Barr Virus (EBV) infection was then administered. Twelve hours post-infection, cells were treated with Acyclovir (ACV) and Dehydroevodiamine (DHE). Following a total of 24 hours of EBV exposure, all samples were harvested by gentle scraping for downstream analysis.

4.2.5 qRT-PCR

qRT-PCR was performed to analyze the transcriptomic profiles of various host genes in infected and treated Tri-culture cells. The cell pellets were washed with 1X phosphate buffer saline (PBS) for RNA isolation. Total RNA was extracted using TRIzol reagent (Invitrogen, Inc., Carlsbad, CA) [78], and cDNA was synthesized using a reverse transcription kit (PrimeScriptTM RT Master Mix; Takara, India) according to the manufacturer's instruction. cDNA was subjected to qRT-PCR using SYBR green real-time master mix (Thermo Scientific, USA) on Agilent AriaMX, following the instrument's standard cycling conditions. The primers used for qRT- PCR are listed in the supplementary table. The relative gene expression of the target genes was analyzed using the 2-ΔΔCt method. Briefly, delta Ct (ΔCt) is the difference obtained after subtracting the cycle threshold (Ct) value of the gene of interest and GAPDH. Further, delta Ct (ΔΔCt) is the difference between the delta Ct (ΔCt) of the sample after transfection/infection and the control sample [79]. These values we finally used to calculate the fold change. All reactions were performed in triplicate and repeated at least twice.

4.2.6 Western blot

The cells upon completion of the incubation period were harvested, washed with ice- cold PBS, and lysed in radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris (pH 7.5), 150-mM sodium chloride, 2 mM EDTA, 1% NP-40] containing protease and phosphatase inhibitors. Proteins in the supernatant were quantified using Bradford protein assay reagents (Pierce, Rockford, IL). Equal quantities of protein from each sample were separated using SDS-PAGE and transferred onto 0.45 μm nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked with 4.5% BSA and incubated with primary antibodies specific to GAPDH (14C10, 1:2000, Cell Signaling Technology, Danvers, MA, USA). Following

incubation and washing, the membrane was treated with a 1:3000 dilution of horseradish peroxidase-conjugated anti- rabbit/anti-mouse antibodies for 1 h. The chemiluminescent detection was based on the Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL). Image analysis and quantification were performed using Image J software (National Institutes of Health, Bethesda, MA, USA).

4.2.7 Methylation sensitive PCR

Genomic DNA was extracted from control and experimental groups using a standard phenol-chloroform method. The purity and concentration of DNA were assessed using spectrophotometry. For methylation analysis, 3 μg of genomic DNA was digested with the methylation-sensitive restriction enzyme HpaII, which cleaves unmethylated CCGG sites but leaves methylated sites intact.Digestion was performed in a 20 μL reaction volume containing HpaII enzyme, appropriate buffer, and genomic DNA, incubated at 37°C for 4–6 hours. Parallel reactions without enzyme treatment were also set up as undigested controls. Following digestion, PCR was performed using primers flanking the HpaII recognition sites within the promoter or regulatory regions of genes of interest. PCR amplification conditions included an initial denaturation at 95°C, followed by 35–40 cycles of denaturation, primer annealing, and extension. Amplified products were resolved on a 2% agarose gel stained with ethidium bromide and visualized under UV light. A clear band in the HpaII-digested sample indicated methylation protection (i.e., methylated site), while absence of a band indicated unmethylated DNA cleaved by HpaII [30].

4.2.8 Histone Isolation

Cells or tissue samples were harvested, washed with cold PBS, and lysed in a buffer containing Tris-HCl, EDTA, Triton X-100, NaCl, PMSF, and protease inhibitors. The lysate was incubated on ice for 10 minutes with intermittent vortexing, followed by centrifugation at

10,000 × g to isolate nuclei. The nuclear pellet was resuspended in 0.2 N HCl and incubated overnight at 4°C to extract histones. The mixture was then centrifuged at 16,000 × g, and the histone-containing supernatant was precipitated with four volumes of cold acetone and incubated at –20°C. The resulting pellet was washed with chilled ethanol, air-dried, and resuspended in either distilled water or 0.1 M NaOH for further analysis. Histones were stored at –80°C in aliquots or at –20°C in acid [31].

4.3 Experimental model

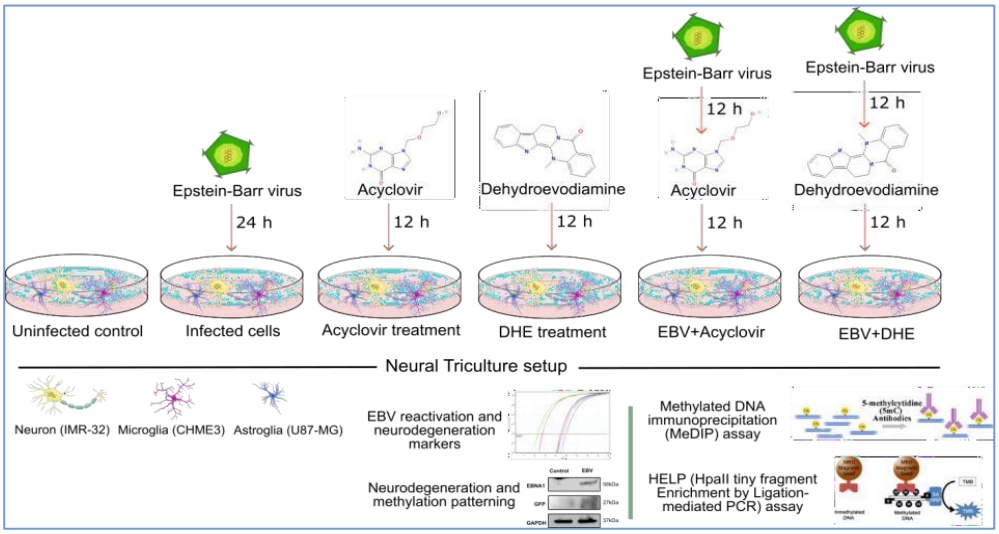


Figure 3. Experimental Methodology

Chapter 5: Results and Discussion

5.1 Alterations in EBV markers at transcript and protein levels

The analysis of EBV-associated markers in the neural tri-culture model revealed significant transcript and protein-level modulations upon infection. qRT-PCR analysis showed upregulation of viral EBV genes such as EBNA1, GFP, EBNA-LP, LMP1, BRLF1 indicating active transcription following infection. Correspondingly, western blot analysis confirmed changes in protein expression of EBNA further validating viral gene expression at the post-translational level (**Figure 4**) p value < <0.05, <0.01 and <0.0001 were represented with *, ** and *** respectively. These findings suggest that EBV actively modulates gene and protein expression in the tri-cultured system, potentially influencing downstream neuroinflammatory and neurodegenerative pathways.

5.2 Perturbations in disease markers upon EBV infection

EBV infection in neural tri-cultured cells led to significant dysregulation of disease- associated markers such as APP, BACE1, PS1, LRP, GFAP, MBP, and neurotropic markers BDNF, CSF2, CTNF, FDF2, GDNF, NGF, VEGF, as observed through qRT- PCR analysis. Further disease markers including MBP, GFAP, APP, APOE4 were also observed to increase through western blot (**Figure 5**). Notably, disease-related genes exhibited a significant increase in expression suggesting a potential role of EBV in exacerbating neurodegenerative pathways. Conversely, neurotropic markers showed a marked decrease indicating possible impairment of neuronal function and homeostasis. Statistical analysis using an unpaired T-test confirmed these infection- induced molecular alterations, highlighting EBV's potential impact on neurodegeneration by shifting the balance between protective neurotropic factors and pathogenic disease-

associated genes

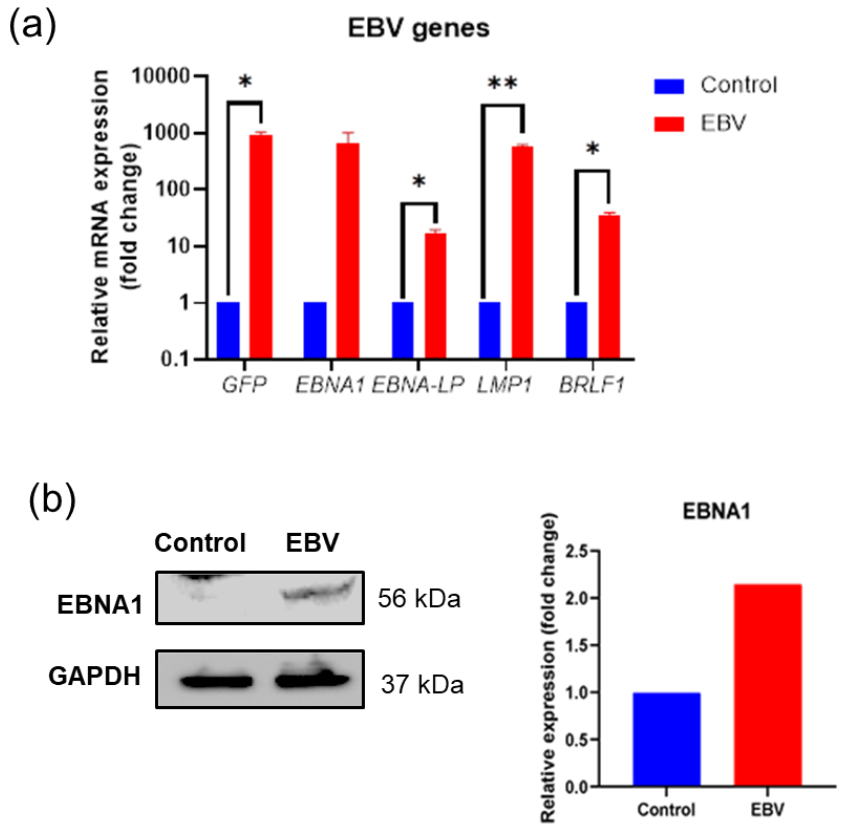


Figure 4. Transcript and protein level modulations in EBV-associated markers upon infection to tri-cultured setup (a) Transcript expression was checked through qRT- PCR, (b) changes at protein level were observed through western blotting. Unpaired T test were applied to determine the statistical significance. p-values of <0.05, <0.01 and <0.0001 were represented with *, ** and *** respectively for significant upregulation and #, ## and ### for siginificant downregulation.

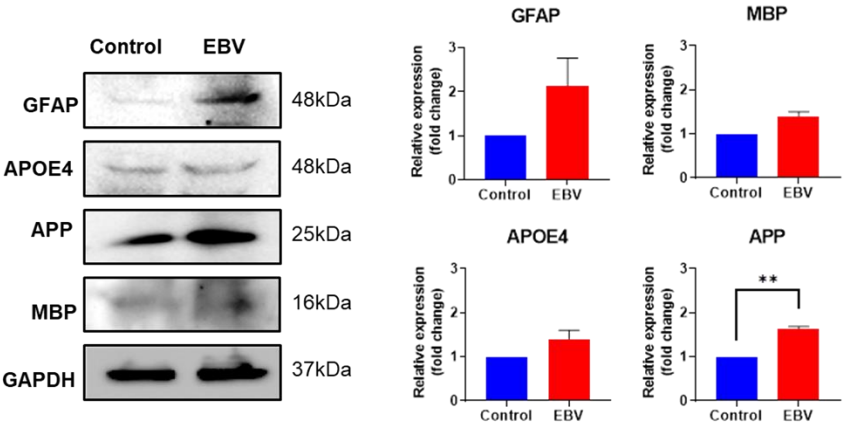


Figure 5. Dysregulation of disease markers upon EBV infection to tri-cultured cells Altered expression of neurotropic and disease marker at qRT-PCR. Unpaired T test were applied to determine the statistical significance. p-values of <0.05, <0.01 and <0.0001 were represented with *, ** and *** respectively for significant upregulation and #, ## and ### for siginificant downregulation.

5.3 Cell viability assay on Neural tri-culture cells

The cell viability assay was performed to determine the optimal non-toxic drug concentrations for neural tri-cultured cells, ensuring minimal cytotoxic effects while maintaining therapeutic efficacy. The IC10 values, representing the concentration at which 10% of cell viability is inhibited, were calculated for Acyclovir (44.29 nM) and Dehydroevodiamine (5.14 µM) (**Figure 6**). These values help establish the lowest effective drug concentration that can be used for further experiments without significantly affecting cell survival, allowing the investigation of their neuroprotective or antiviral effects in EBV-infected neural cultures.

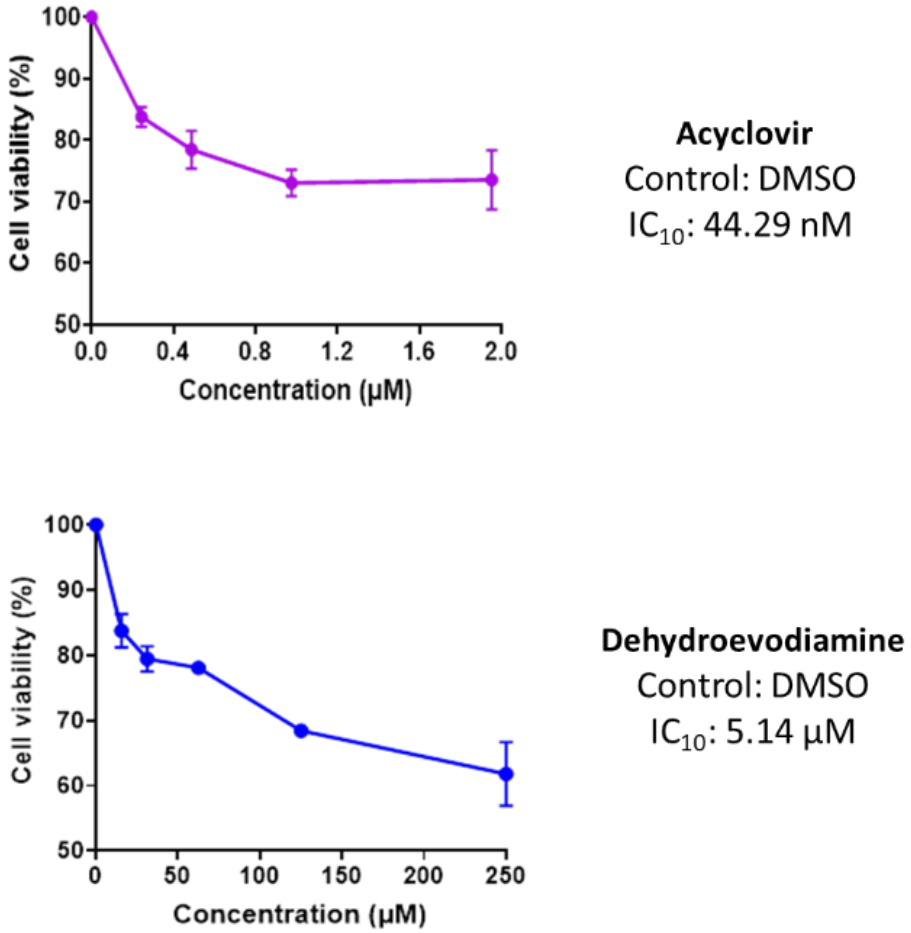


Figure 6. Cell viability assay to measure optimal drug concentration to be exposed in neural tri-culture setup.The IC10 value of Acyclovir and Dehydroevodiamine were 44.29 nM and 5.14 µM respectively.

5.4 Tri-methylated H3K9 and H3K27 histone alterations upon EBV infection

Western blot analysis of histone methylation revealed significant alterations in H3K9me3 and H3K27me3 levels upon EBV infection in the neural tri-culture model. Infection led to an increase in these repressive histone marks, suggesting enhanced chromatin compaction and transcriptional silencing of key neuronal genes. However, treatment with Dehydroevodiamine (DHE) resulted in a marked reduction in H3K9me3 and H3K27me3 levels, indicating its potential therapeutic role in reversing infection-induced epigenetic repression.

(**Figure 7**). These findings suggest that EBV-driven histone modifications contribute to neurodegenerative pathways, while DHE may help restore normal gene expression by modulating histone methylation.

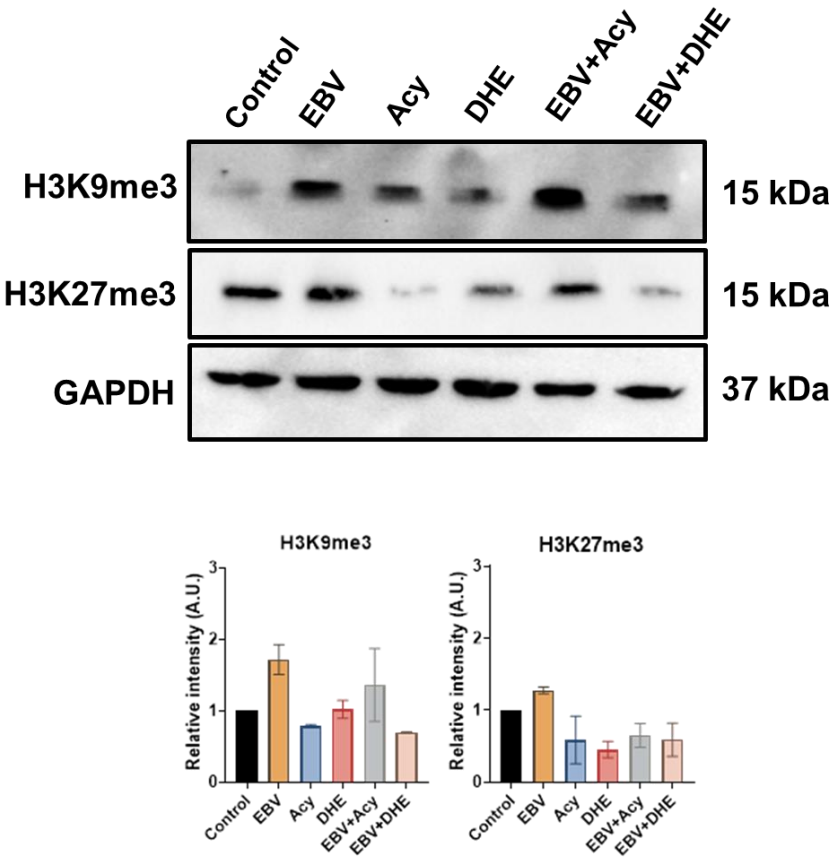
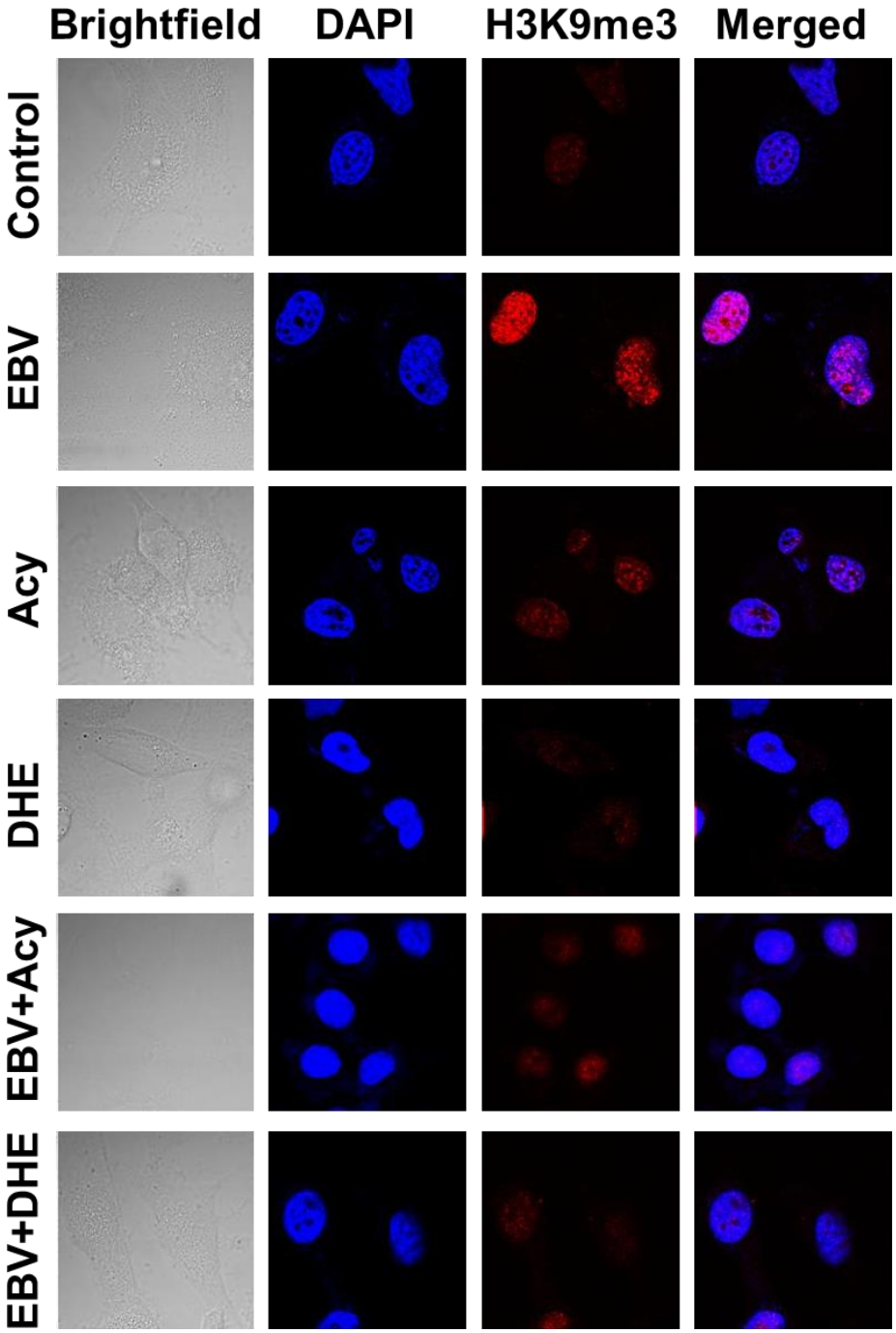
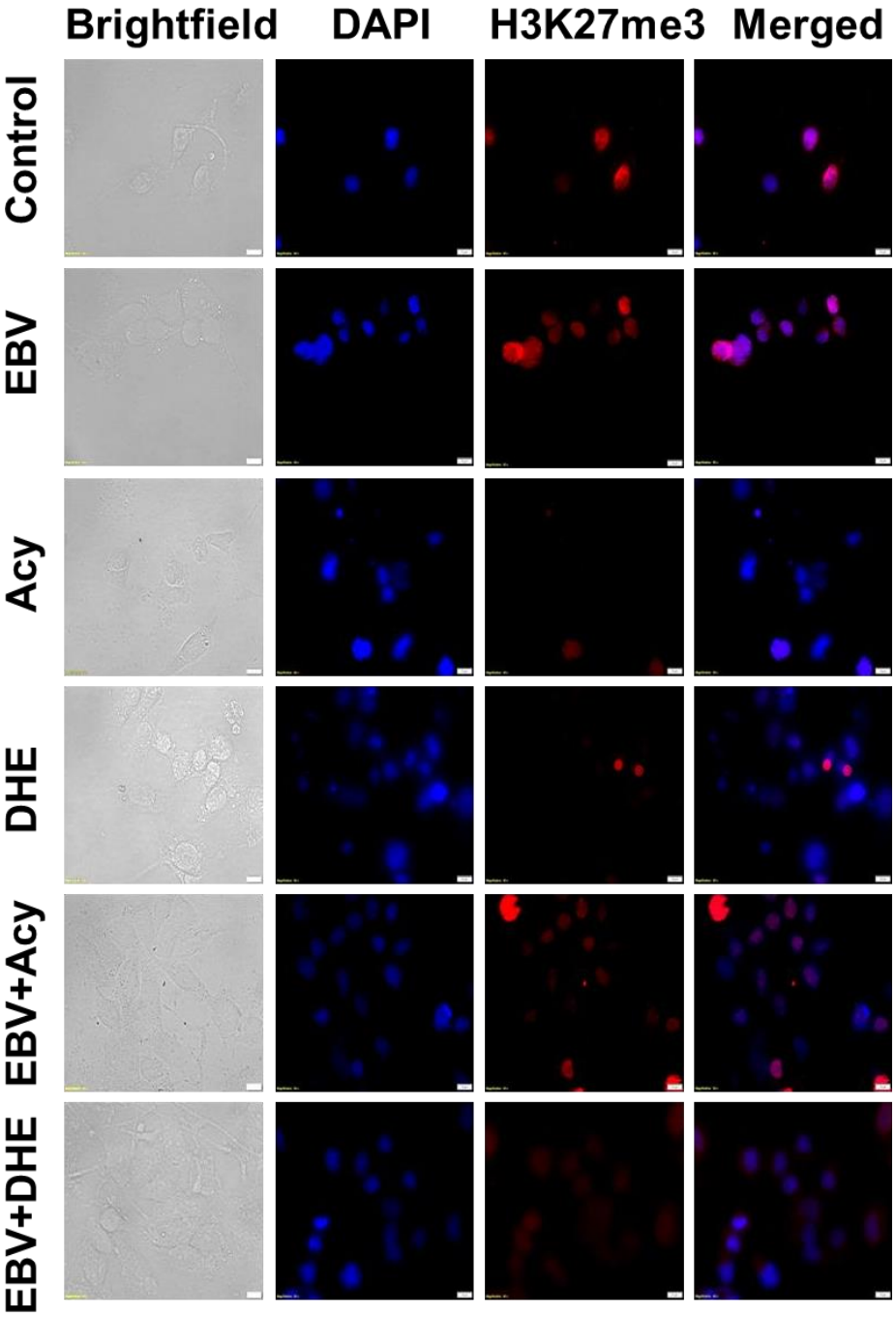


Figure 7. Histone methylation analysis through western blot revealed alterations in H3K9me3 and H3K27me3.


Brightfield
DAPI
H3K9me3
Merged
Control
EBV
Acy
DHE
EBV+Acy
EBV+DHE


Brightfield
DAPI
H3K27me3
Merged
Control
EBV
Acy
DHE
EBV+Acy
EBV+DHE

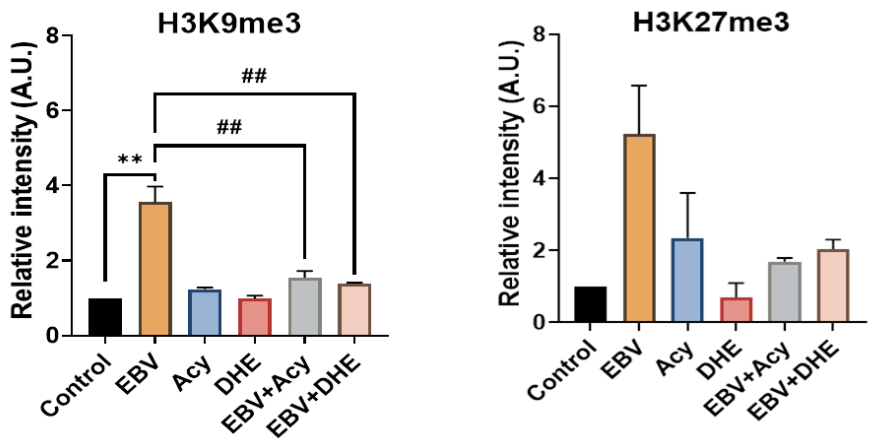


Figure 8. Histone methylation analysis through immunostaining revealed alterations in H3K9me3 and H3K27me3. Tri-methylated H3K9 and H3K27 Histone alterations upon EBV infection Disease marker dysregulation due to Histone methylation upon EBV infection. One-way ANOVA followed by Tukey's post-hoc tests was used to determine statistical significance. *p<0.05, **p<0.01,***p<0.001.

5.5 Disease marker dysregulation due to Histone methylation upon EBV infection.

EBV infection in the neural tri-culture model led to a significant upregulation of disease-associated markers, indicating its role in promoting neuroinflammatory and neurodegenerative pathways. The increase in these markers suggests potential disruptions in neuronal homeostasis and immune signaling, contributing to disease progression. However, treatment with Dehydroevodiamine (DHE) effectively mitigated these changes, reducing disease marker expression and suggesting its neuroprotective potential **(Figure 9**). These findings highlight EBV-induced molecular alterations in neurodegeneration and demonstrate DHE's therapeutic promise in counteracting these pathogenic effects.

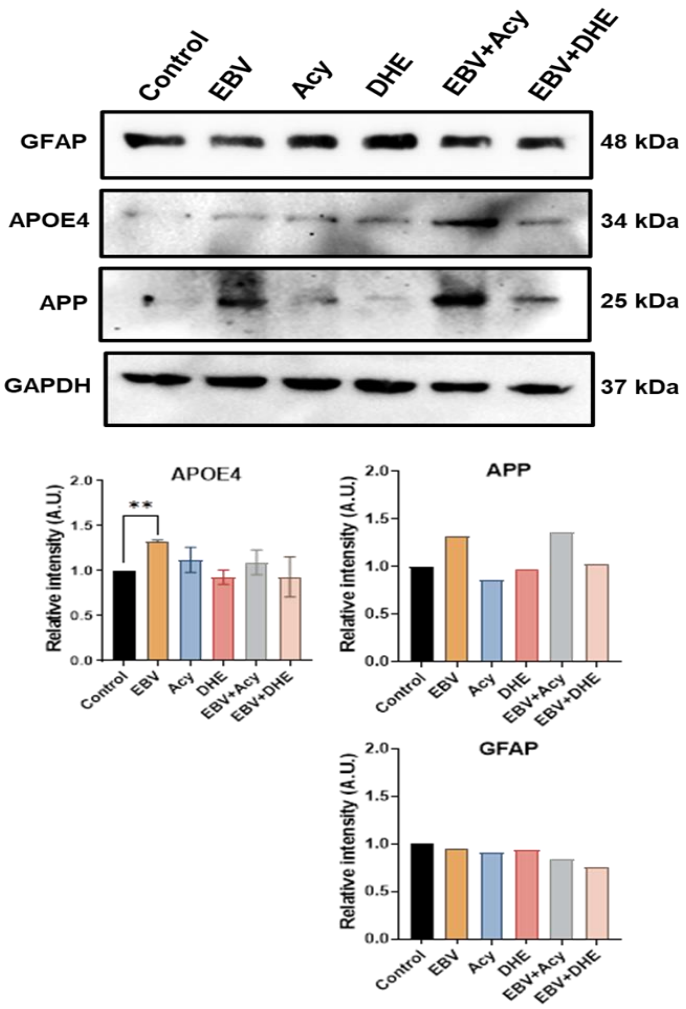


Figure 9. Dysregulation observed in disease markers due to EBV infection in tri- culture models. One-way ANOVA followed by Tukey's post-hoc tests was used to determine statistical significance. *p<0.05, **p<0.01,***p<0.001.

5.6 Methylation-specific PCR for disease markers.

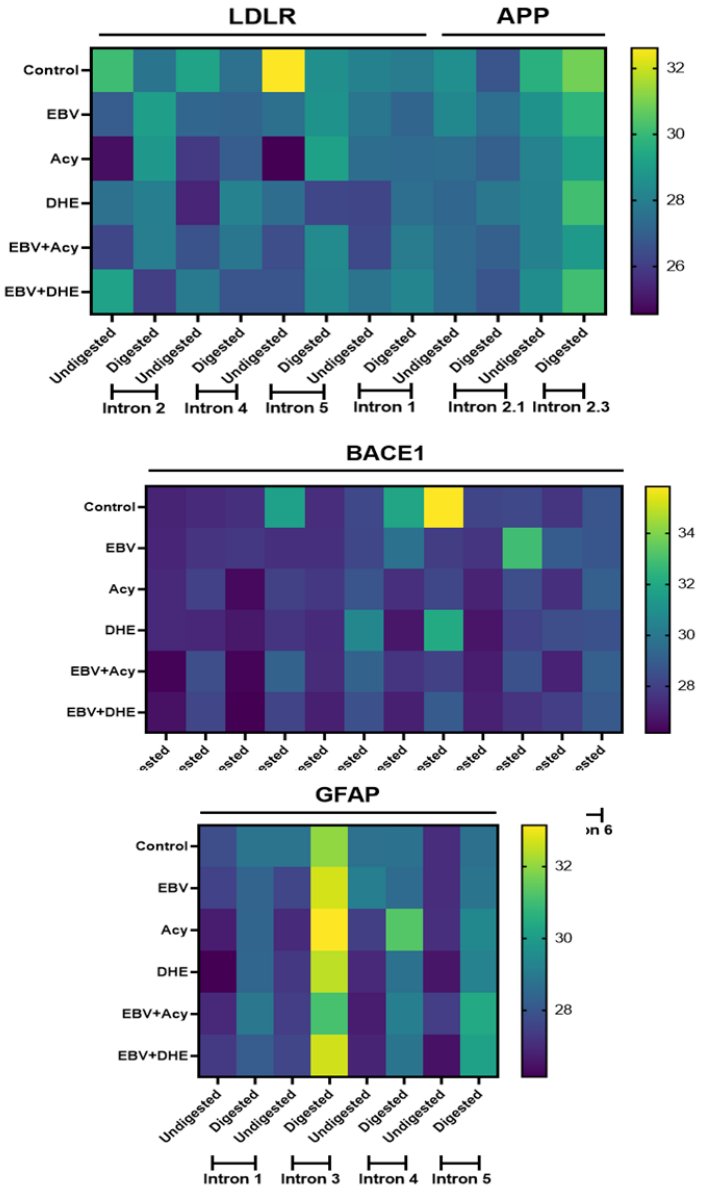


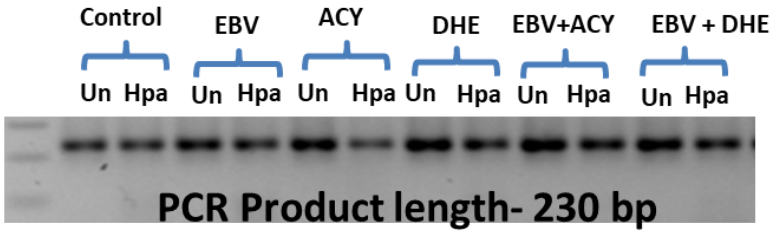
Figure 10. Dysregulation observed in methylations of disease markers due to EBV infection in tri-culture of neural microenvironment upon treatment of Acyclovir and Dehydroevodiamine.

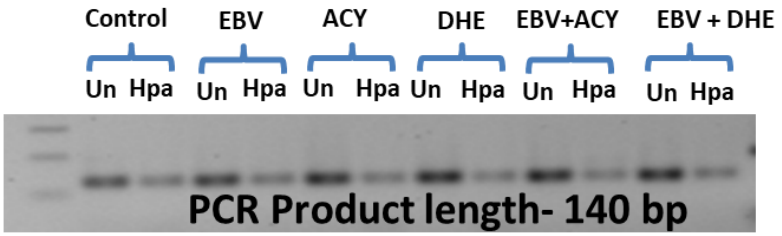
5.7 Methylation-specific PCR for disease markers. – GFAP

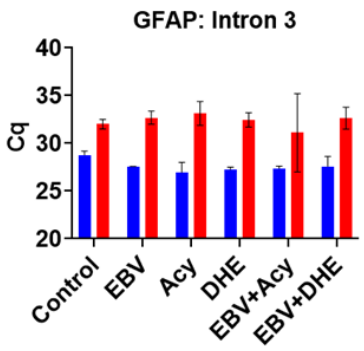
GFAP is a key cytoskeletal protein in astrocytes, and its alternative splicing leads to multiple isoforms, including GFAP-α and GFAP-δ.

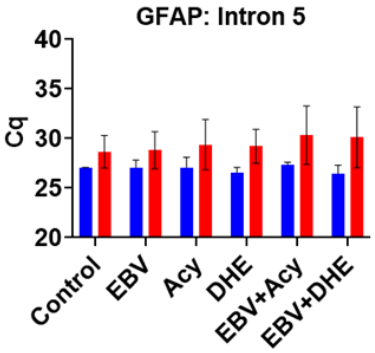
GFAP-δ, in particular, has been linked to reactive astrocyte phenotypes and neuroinflammatory states. Hypomethylation at splice regulatory regions near exon 7a promotes the inclusion of this exon, leading to increased production of GFAP-δ. This isoform alters the structural and functional dynamics of astrocytes, enhancing their responsiveness to injury and inflammation. In chronic neurodegenerative disorders like Alzheimer's and Parkinson's, such splicing shifts can amplify glial reactivity, contributing to persistent neuroinflammation and impaired neuronal support (**Figure 11**). Thus, methylation-sensitive splicing of GFAP represents a key epigenetic mechanism driving astrocyte dysfunction in neurodegeneration.

(a)









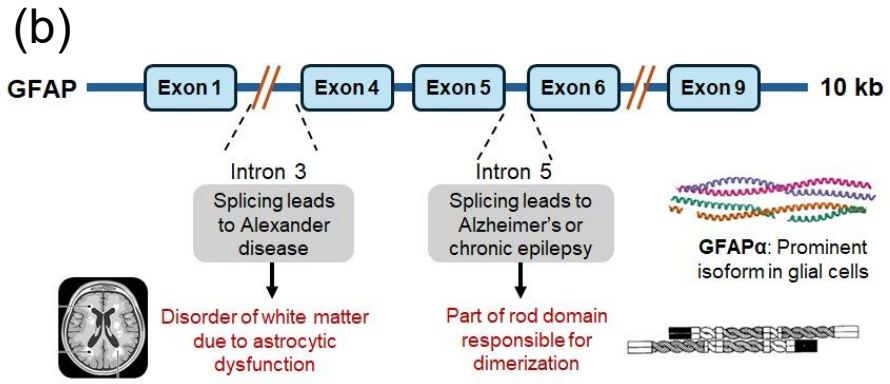
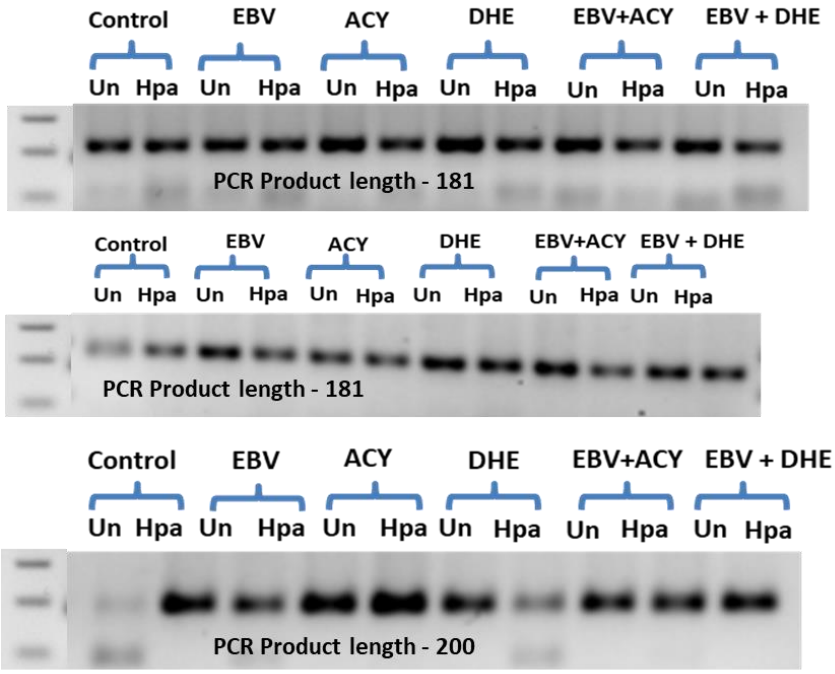


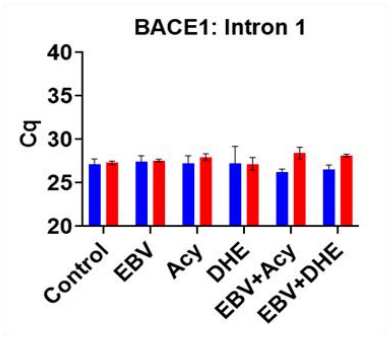
Figure 11. Dysregulation observed in methylations of disease markers – GFAP due to EBV infection in tri-culture of neural microenvironment upon treatment of Acyclovir and Dehydroevodiamine through (a) Methylation specific PCR and its subsequent (b) Agarose gel electrophoresis. Functional importance of the hypomethylations observed in the introns.

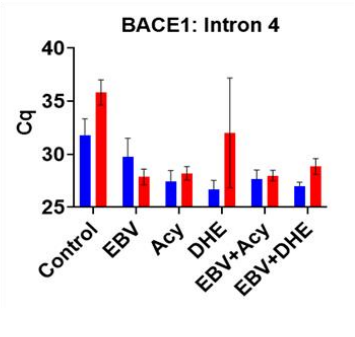
5.8 Methylation-specific PCR for disease markers. – BACE-1

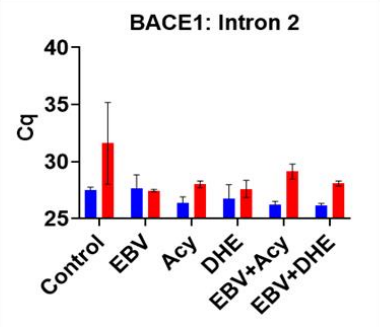
BACE1 plays a central role in Alzheimer's disease by initiating the cleavage of amyloid precursor protein (APP) to produce amyloid-β (Aβ) peptides. This gene undergoes alternative splicing to generate multiple isoforms, with the full-length variant being most active in amyloidogenic processing. Hypomethylation at the splice donor or acceptor sites near exon- intron boundaries can promote the inclusion of exons critical for producing the full-length, catalytically active BACE1 isoform. This occurs because low methylation levels allow for the proper binding of splicing enhancers and regulatory factors such as SR proteins, enhancing exon recognition and inclusion **(Figure 12)**. As a result, neurons may exhibit increased BACE1 activity, leading to elevated Aβ generation and contributing to plaque formation in the Alzheimer's brain.

(a)


Control
EBV
ACY
DHE
EBV+ACY
EBV + DHE
Un Hpa Un Hpa Un Hpa Un Hpa Un Hpa Un Hpa
PCR Product length - 181
Control
EBV
ACY
DHE
EBV+ACY
EBV + DHE
Un Hpa Un Hpa Un Hpa Un Hpa Un Hpa Un Hpa
PCR Product length - 181
Control
EBV
ACY
DHE
EBV+ACY
EBV + DHE
Un Hpa Un Hpa Un Hpa Un Hpa Un Hpa Un Hpa
PCR Product length - 200


BACE1: Intron 1
Cq
40
35
30
25
20
Control
EBV
Acy
DHE
EBV+Acy
EBV+DHE


BACE1: Intron 4
Cq
40
35
30
25
Control
EBV
Acy
DHE
EBV+Acy
EBV+DHE


BACE1: Intron 2
Cq
40
35
30
25
Control
EBV
Acy
DHE
EBV+Acy
EBV+DHE

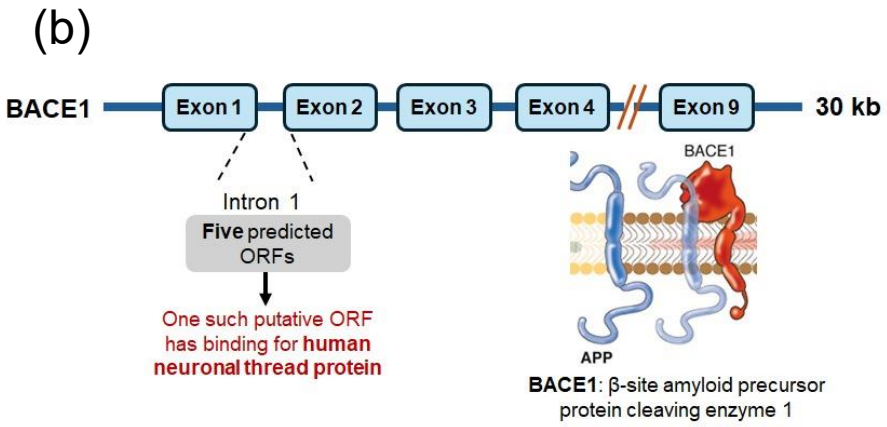


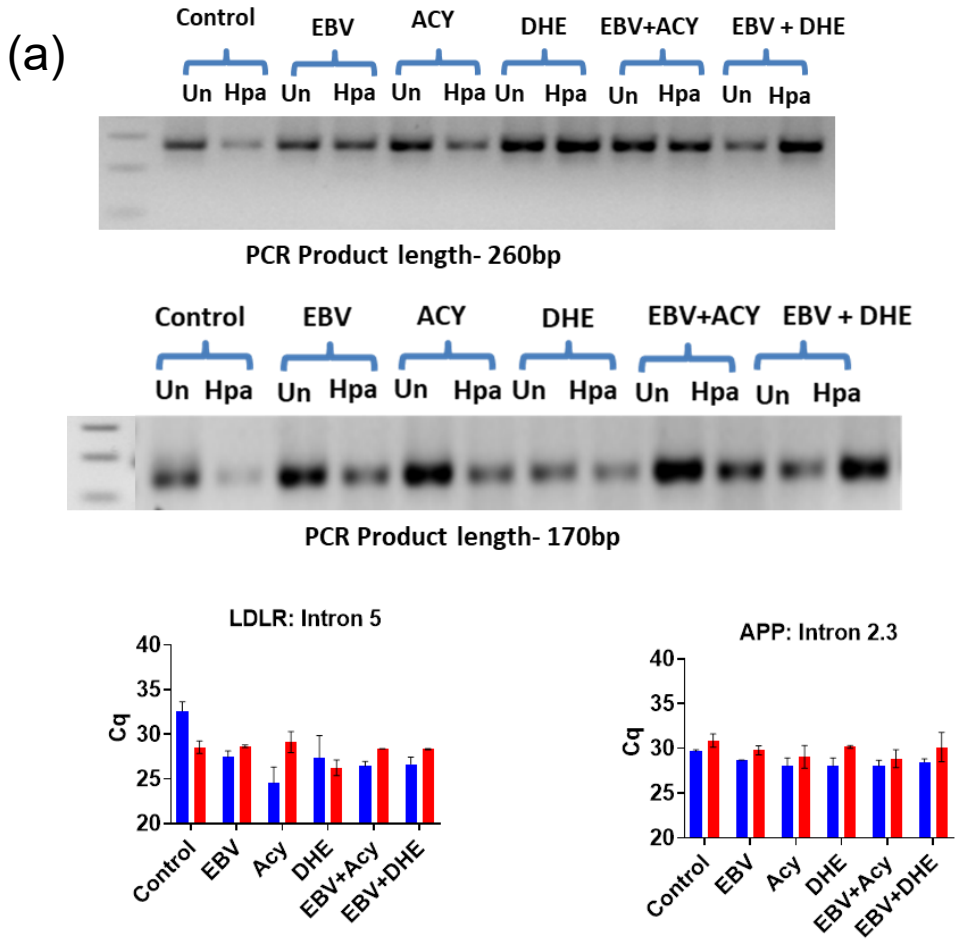
Figure 12. Dysregulation observed in methylations of disease markers - BACE 1 due to EBV infection in tri-culture of neural microenvironment upon treatment of Acyclovir and Dehydroevodiamine through (a) Methylation specific PCR and its subsequent Agarose gel electrophoresis (b) Functional importance of the hypomethylations observed in the introns.

5.9 Methylation-specific PCR for disease markers. – LDLR and APP

The APP gene gives rise to several isoforms—APP695, APP751, and APP770— through alternative splicing, with differential expression in neuronal versus glial cells. APP695, predominant in neurons, lacks exons 7 and 8, while the KPI-containing isoforms APP751 and APP770 are more amyloidogenic and expressed in glial cells. Hypomethylation near the splice sites flanking exons 7 and 8 facilitates their inclusion by allowing access to splicing machinery, resulting in increased expression of KPI- domain-containing isoforms **(Figure 13**). These isoforms enhance Aβ production, particularly under neuroinflammatory conditions or in disease states. Therefore, hypomethylation driven alternative splicing of APP contributes to the amyloidogenic shift that underlies Alzheimer's pathology.

LDLR is essential for maintaining cholesterol homeostasis in the brain, influencing processes such as synaptic plasticity and Aβ clearance. The

gene undergoes alternative splicing that can modify its ligand-binding domain and internalization efficiency. Hypomethylation at exon-intron junctions supports the inclusion of exons like 12 or 17, resulting in functionally intact LDLR isoforms that efficiently mediate cholesterol uptake and contribute to Aβ clearance from the extracellular space. In this context, hypomethylation has a protective role, preserving neuronal lipid balance and mitigating the buildup of neurotoxic amyloid species **(Figure 13)**. However, if the Aβ burden is high due to upregulation of BACE1 and KPI-containing APP isoforms, even a functional LDLR system may not be sufficient to maintain homeostasis.



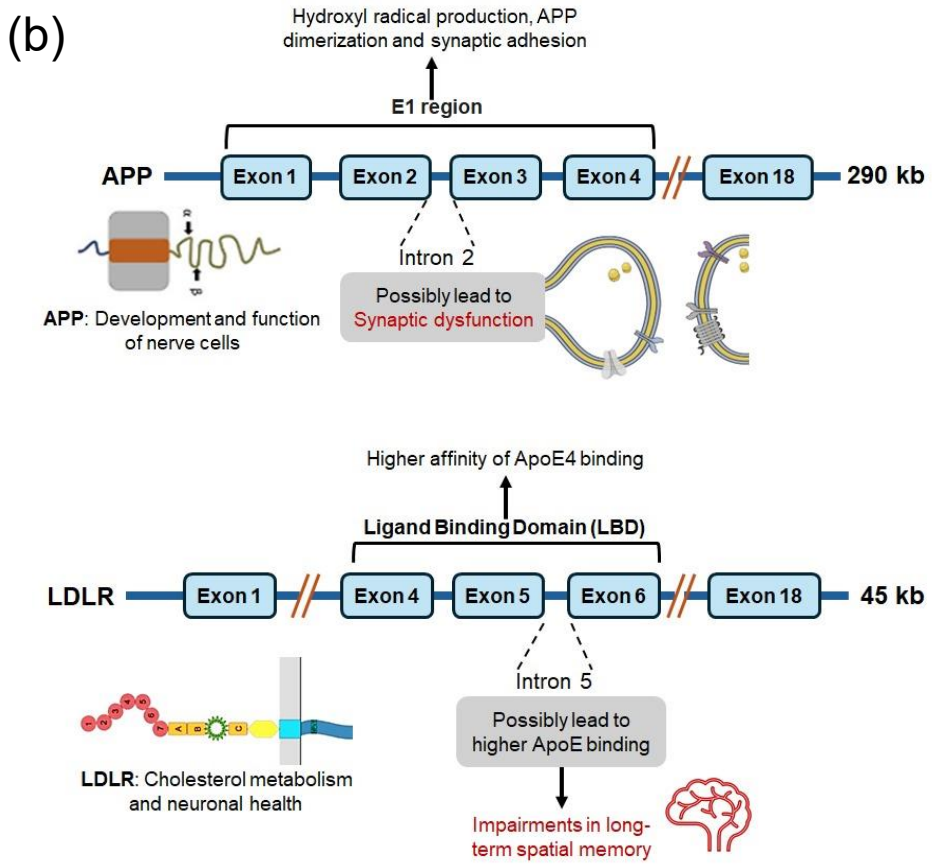


Figure 13. Dysregulation observed in methylations of disease markers – LDLR and APPdue to EBV infection in tri-culture of neural microenvironment upon treatment of Acyclovir and Dehydroevodiamine through (a) Methylation specific PCR and its subsequent Agarose gel electrophoresis (b) Functional importance of the hypomethylations observed in the introns.

Chapter 6: Discussion

Histone methylation is a key post-translational modification that plays a pivotal role in the regulation of chromatin structure and gene expression. Methylation occurs on specific lysine residues within the histone tails, with each modification having distinct effects on gene activity. For example, H3K4me3 is commonly associated with active transcription and is found near the promoter regions of actively transcribed genes, while H3K9me3 and H3K27me3 are generally associated with transcriptional repression and heterochromatin formation. These modifications are dynamically regulated by a variety of histone methyltransferases (e.g., SETD1A for H3K4me3) and demethylases (e.g., KDM1A for H3K9me3). In the context of neurodegenerative diseases like Alzheimer's, histone methylation can lead to the silencing of genes that are critical for neuronal survival, synaptic plasticity, and neuronal repair. An increase in repressive histone marks, such as H3K9me3 and H3K27me3, has been observed in the brains of Alzheimer's patients, contributing to the dysregulation of neurotrophic factors and exacerbating neuroinflammation. These changes in histone methylation patterns can silence essential genes, hindering the neuronal capacity for regeneration and repair, and thus play a key role in the progression of neurodegenerative diseases.

DNA methylation is another fundamental epigenetic modification that involves the addition of a methyl group to the 5' position of cytosine residues, typically within CpG dinucleotides. This modification represses gene expression by preventing the binding of transcription factors or by recruiting methyl-binding proteins that facilitate chromatin condensation. DNA methylation can occur at the promoter region of genes, silencing transcription, or at the exonic or splice site regions, influencing alternative splicing and isoform production. The methylation status at splice sites is particularly important in diseases

where isoform switching can alter the function of critical genes, such as in neurodegenerative disorders like Alzheimer's and Parkinson's disease. Methylation-Specific PCR (MSP) is a powerful technique used to assess DNA methylation at specific loci. In this method, DNA is treated with sodium bisulfite, which deaminates unmethylated cytosines to uracil, leaving methylated cytosines unaffected. PCR amplification then distinguishes between methylated and unmethylated alleles, allowing for precise detection of methylation patterns in specific regions. This technique is widely used to investigate the epigenetic regulation of genes involved in various diseases, particularly in understanding how methylation influences alternative splicing and isoform production, which can have profound implications for disease susceptibility.

GFAP is a key structural protein in astrocytes that helps maintain the shape and strength of brain cells and supports nearby neurons and the blood–brain barrier. Its main form in the brain is GFAP-α, while another version, GFAP-δ, appears more in stem-cell-like astrocytes and is created through alternative splicing. Changes in how this gene is spliced have been linked to neurological conditions like Alexander disease. BACE1 is an enzyme that starts the process of forming amyloid-beta, a protein involved in Alzheimer's disease. Its levels can increase when its gene becomes less methylated, which often happens in Alzheimer's. LDLR is a receptor that helps manage cholesterol in the brain and interacts with different forms of the ApoE protein—especially ApoE4, which increases the risk of both heart and brain diseases. How much LDLR is made can also be affected by changes in gene methylation. The APP gene produces different forms of the amyloid precursor protein through splicing. The version made mostly in neurons (APP695) lacks certain domains found in other forms like APP751 and APP770, which are more common in other brain cells. These differences influence how amyloid-beta is formed. Both splicing

changes and gene methylation play an important role in how these genes function in the brain and may contribute to neurodegenerative disease development.

Chapter 7: Conclusion and future work

7.1 Conclusion

In conclusion, both histone methylation and DNA methylation play critical roles in the regulation of gene expression, with significant implications for neurodegenerative diseases. Histone methylation, through the addition or removal of methyl groups on specific histone residues, can either promote or repress gene activity, influencing neuronal survival, synaptic plasticity, and neuroinflammation. In diseases such as Alzheimer's, altered histone methylation patterns, particularly the upregulation of repressive marks like H3K9me3 and H3K27me3, contribute to the silencing of essential neuroprotective genes, exacerbating disease progression. Similarly, DNA methylation, especially at splice sites, regulates the alternative splicing of critical genes, influencing isoform production and impacting neurodegeneration. Techniques like Methylation-Specific PCR (MSP) provide valuable tools to dissect these modifications at a molecular level, allowing for the identification of epigenetic changes that drive pathological shifts in gene expression. By understanding the interplay between histone and DNA methylation, particularly in genes such as BACE1**,** APP**,** and GFAP, we gain deeper insights into the molecular mechanisms that underpin neurodegenerative diseases. This knowledge could lead to the development of novel therapeutic strategies aimed at reversing or modulating these epigenetic alterations, offering hope for the treatment and prevention of these debilitating conditions.

7.2 Future work

Building on the current findings, future research should focus on high-resolution, genome-wide mapping of histone and DNA methylation changes in neural cells following Epstein-Barr Virus (EBV) infection, using techniques such as ChIP-seq for histone modifications and

whole-genome bisulfite sequencing (WGBS) for DNA methylation. This would help identify novel regulatory elements and gene networks disrupted by epigenetic reprogramming in the context of neurodegeneration. Additionally, investigating cell-type-specific methylation dynamics using single-cell epigenomics could clarify how individual neural populations—neurons, astrocytes, and microglia—respond differently to EBV-induced stress and contribute to the disease phenotype.

Further exploration of the impact of methylation at splice junctions should include RNA-seq isoform quantification coupled with bisulfite-converted RNA analysis to directly correlate methylation with isoform expression. This would provide deeper insight into how specific methylation events at intron-exon boundaries influence alternative splicing and functional protein diversity in genes such as BACE1, APP, LDLR, and GFAP. Moreover, future studies should assess how these splicing events affect protein-protein interactions, trafficking, and function in the neural microenvironment, particularly under viral or inflammatory stress.

On the therapeutic front, continued evaluation of epigenetic modulators like Dehydroevodiamine (DHE) is essential. These studies should be expanded to include in vivo models to validate DHE's ability to reverse histone and DNA methylation alterations and rescue cognitive deficits. Combinatorial treatments that target both epigenetic and oxidative stress pathways could be tested for synergistic neuroprotective effects. Long-term, integrating these approaches may help develop precision therapies aimed at mitigating EBV-induced neurodegenerative damage through targeted epigenetic reprogramming.

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