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Epstein-Barr virus dUTPase induces neuroinflammatory mediators: Implications for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

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Abstract

Purpose: Neuroinflammation is a common feature in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) affecting 85-90% of all patients yet the underlying mechanism(s) responsible for the initiation and/or promotion of this process is largely unknown. Multiple reports, however, have suggested a role for Epstein-Barr virus (EBV), in particular, in ME/CFS but its potential role, if any, in the neuroinflammatory process has not been addressed. In support of this premise, studies by our group have shown that the EBV protein deoxyuridine triphosphate nucleotidohydrolase (dUTPase), induces anxiety and sickness behaviors in female mice. We also found that a small subset of ME/CFS patients exhibited prolonged and significantly elevated neutralizing antibodies against EBV dUTPase protein in serum, which inversely correlated with ME/CFS symptomology. A larger ME/CFS case/control cohort study further confirmed that a significant percentage of ME/CFS patients (30.91-52.7%) were simultaneously producing antibodies against multiple human herpesviruses-encoded dUTPases and/or human dUTPase. Altogether, these findings suggest that EBV dUTPase protein may be involved in the neuroinflammatory process observed in ME/CFS. Thus, the aim of the present study was to determine whether the EBV dUTPase protein could contribute to neuroinflammation by altering the expression of genes involved with maintaining blood brain barrier (BBB) integrity and/or modulating synaptic plasticity.

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AUTHOR CONTRIBUTION

M.V.W. analyzed data and wrote paper; B.C., and W.P.L. conducted the experiments, analyzed data and edited paper; M.E.A designed and conducted the experiments, analyzed data and wrote paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

Declarations of interest: None

Methods: Using human immortalized astrocytes, microglia and cerebral microvascular endothelial cells, we conducted time-course (0-24 h) experiments with EBV dUTPase protein (10 μg/ml) to determine what effect(s) it may have on the expression of genes involved with BBB permeability, astrocytes and microglia cell function, tryptophan metabolism and synaptic plasticity by qRT-PCR. In parallel, *in vivo* studies were conducted in female C57Bl/6 mice. Mice were injected via intraperitoneal (i.p) route with EBV dUTPase protein (10 μg) or vehicle daily for 5 days and the brains collected and processed for further qRT-PCR analysis of the *in vivo* effect of the dUTPase on the dopamine/serotonin and GABA/Glutamate pathways, which are important for brain function, using RT^2 Profiler PCR Arrays.

Findings: EBV dUTPase protein altered the expression *in vitro (*12 of 15 genes and 32 of 1000 proteins examined) and *in vivo* (34 of 84 genes examined), of targets with central roles in BBB integrity/function, fatigue, pain synapse structure and function as well as tryptophan, dopamine and serotonin metabolism.

Implications: The data suggest that in a subset of patients with ME/CFS, the EBV dUTPase could initiate a neuroinflammatory reaction, which contributes to the fatigue, excessive pain and cognitive impairments observed in these patients.

Keywords

Epstein-Barr virus (EBV); deoxyuridine triphosphate nucleotidohydrolase (dUTPase); Toll-like receptor 2 (TLR2); Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS); synaptic plasticity

INTRODUCTION

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a chronic multisystem illness of unconfirmed etiology. ME/CFS has largely been diagnosed based upon Fukuda CFS criteria[1] and/or the Canadian ME/CFS criteria.[2] The Institute of Medicine in 2015 proposed, in addition to a new name “Systemic Exertion Intolerance Disease” (SEID), a new case criteria focused upon chronic fatigue, post-exertional fatigue and orthostatic intolerance/cognitive deficits for patients afflicted with this disease.[3] Neurocognitive dysfunction has been reported to occur in 85-90% of patients with ME/CFS. Self-reporting and assessment by objective task performance tests have shown that the cognitive problems include declining attention and concentration, slow information processing, as well as declining memory and learning of complex information.[4]

Several neuroimaging studies using magnetic resonance imaging (MRI), functional magnetic resonance imaging, magnetic resonance spectroscopy (MRS), positron emission tomography (PET) and single photon emission computerized tomography have demonstrated structural and functional alterations in the brains of patients with ME/CFS.[5-9] Recent studies using PET[10] and whole-brain MRS[11] showed evidence of widespread neuroinflammation in the brains of patients with ME/CFS, which was associated with the severity of neuropsychologic symptoms. While numerous models and hypotheses have been proposed to explain the neuroinflammation observed in patients with ME/CFS, the underlying mechanisms that contribute to this neuroinflammatory reaction(s) remain largely undefined.[8, 12-15]

In over half of ME/CFS cases, onset is associated with acute “flu-like” symptoms[16] and multiple reports in the literature have suggested a role for viruses in ME/CFS.[17-19], particularly Epstein-Barr virus (EBV). Yet further mechanistic studies to address a causal relationship between a virus and ME/CFS are missing and would be of high importance. In support of this premise, several studies have shown that EBV can infect and undergo lytic replication in neuronal cell lines as well as primary human neurons[20] and abortively replicates in human astrocytes.[21] While acute infections of EBV are known to cause neurological complications in immunocompetent patients with infectious mononucleosis, generally, they are benign. Conversely, in patients co-infected with human immunodeficiency virus (HIV-1), EBV is associated with primary CNS lymphoma, which has a poor prognosis.[22] EBV is also a risk factor in the development of multiple sclerosis [23,24], a claim further supported by a recent study which found significant levels of EBV genomic DNA in B cells, astrocytes and microglia cells in the brains of patients with multiple sclerosis.[24] These studies focused on the virus and none have addressed the possibility of a virus encoded protein rather than the virus itself, as the cause or driver of the pathological features observed in these diseases.

We have previously demonstrated that the EBV deoxyuridine triphosphate nucleotidohydrolase (dUTPase) protein, which is encoded by the BLLF3 gene and expressed during lytic/abortive-lytic replication of the virus, possesses novel functions acting as a pathogen-associated molecular pattern (PAMP) for toll-like receptor (TLR) 2. Engagement of TLR2 by EBV dUTPase leads to the activation of NF-κB and subsequent modulation of downstream genes involved in several cellular processes including chronic inflammation, effector T-cell function and neurotransmitter function.[25-30] We have also shown that the EBV dUTPase can be secreted from B cells in exosomes[28], which function as intracellular messengers and can cross the BBB. More importantly, we have shown that EBV dUTPase induces anxiety and sickness behaviors in mice[31, 32] and that patients with ME/CFS have, increased serum levels of antibodies to the EBV dUTPase.[19] The present study explored the contribution of EBV dUTPase protein, if any, in the neuroimmune dysfunction associated with ME/CFS using *in vitro* and *in vivo* model systems.

METHODS

Reagents.

Applied cell extracellular matrix (Cat#G422), Prigrow I medium (Cat#TM001), Prigrow III medium (Cat#TM003), Prigrow IV medium (Cat#TM004), and PriCoat T25 flasks (Cat#G299) were purchased from Applied Biological Materials Inc (Richmond, BC, Canada). X-vivo 15 serum-free medium (Cat#04-418Q) was purchased from Lonza Inc. GM-CSF and IL-4 were purchased from Peprotech (Rocky Hill, NJ). FBS and Superscript IV First-Strand Synthesis Kit were purchased from Invitrogen (Carlsbad, CA). Trizol reagent was purchased from Ambion. TaqMan Gene Expression Master Mix was purchased from Applied Biosystems. RNeasy Mini Kit, RNase-Free DNase Set, mouse Dopamine/ Serotonin (Cat#PAMM-158Z), mouse GABA/Glutamate (Cat#PAMM-152Z) RT^2 Profiler PCR Arrays and SYBR green reaction master mix were purchased from Qiagen. Ketamine (100 mg/ml) was obtained from the Wexner Medical Center Pharmacy at The Ohio State

University and xylazine hydrochloride was purchased from Sigma. The Human L-1000 Antibody Arrays were purchased from RayBiotech.

Purification of recombinant EBV dUTPase.

Subcloning and purification of recombinant dUTPase protein was performed as previously described [25,29,30]. Recombinant dUTPase protein preparations were tested for the presence of contaminants as described previously [25, 29] and were free of detectable levels of LPS, peptidoglycan (SLP-HS), DNA and RNA. Protein concentration was determined using the Qubit fluorimeter (Invitrogen Carlsbad, CA). The purified recombinant EBV dUTPase protein used in these studies was stored at −80°C in elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole; pH 7.4) until further use. Vehicle used in *in vitro* and *in vivo* experiments was elution buffer.

Cells.

Immortalized human astrocytes (fetal – SV40 large T antigen, Cat#T0280), microglia cells (adult; Cat#T0251) and cerebral microvascular endothelial cells (Cat#T0259) were obtained from Applied Biological Materials Inc., and maintained in Prigrow IV (Astrocytes), Prigrow III (Microglia) and Prigrow I (cerebral microvascular endothelial cells) medium supplemented with 10% FBS in a humidified atmosphere at 37°C and 5% CO_2. Human dendritic cells from healthy female donors were obtained from Astarte Biologics and cultured in X-Vivo serum-free medium supplemented with 500 U/ml GM-CSF and IL-4.

Human immortalized Cell treatments.

Cells were seeded at a density of 8×10^4 in 6-well plates coated with applied cell extracellular matrix and cultured in the appropriate Prigrow supplemented medium until they were 70-90% confluent. Media was then replaced and cells were treated with EBV dUTPase protein (10 μg/ml) or vehicle for various time points (0, 0.5, 1, 2, 3, 4, 6 and 24h). Following treatments, cells were collected for further processing and mRNA gene expression analysis by qRT-PCR. Studies were performed in triplicate a minimum of three times.

Human dendritic cell treatment and protein array.

Cells were seeded at a density of 2.5×10^5 in 24-well plates and cultured in X-Vivo serum-free medium supplemented with 500 U/ml GM-CSF and IL-4. The next day, hDCs were stimulated with EBV dUTPase protein (10 μg/ml), or vehicle for 24 h, as described previously[27-29]. Following treatments, cell culture supernatants were collected and the concentration of select immune mediators was measured using a Human L-1000 Antibody Array (RayBiotech) and the fluorescence captured with an Axon GenePix laser scanner. Positive control spots on the array are standardized amounts of biotinylated IgGs printed directly onto the array. Negative control spots on the arrays contain antibody diluent buffer and their signal intensities represent nonspecific binding of the Cy3-Conjugated Streptavidin (background signal). Normalized signal intensity data represent values in which the background signal of negative control spots has been subtracted out and normalized to the mean signal intensity of positive control spots. Following normalization, any ≥1.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte between samples is

considered a measurable and significant difference in expression, provided that both sets of signals are well above background (Mean background + 2 standard deviations, accuracy ≈ 95%). Data represent an n =2 experiments (2 biological replicates/experiment) are expressed as the fold-change signal intensity for each analyte in dUTPase treated samples relative to the vehicle control samples. Studies were performed in triplicate a minimum of three times.

Animals

Mice.—Female wild-type C57BL/6J mice (6 to 8 weeks of age) were purchased from The Jackson laboratory and housed for 7 days for acclimatization prior to use. Mice were housed in a Biosafety Level 2 barrier facility on a 12-hour light/dark cycle and given chow and water *ad libitum*. The facilities are maintained at 22–23°C and 30–50% relative humidity. All experiments were conducted in accordance with the Institutional Animal Care and Use Guidelines of The Ohio State University (OSU). Mice (n= 40, 10 mice per treatment group) were injected daily with EBV dUTPase protein (10 μg/ml) or vehicle control (100 μl total volume) for 5 days. This dose was selected based upon our previous studies.[27-32] Mice were then deeply anesthetised with a ketamine/xylazine mixture (ketamine 90 mg/kg & xylazine 8 mg/kg; in 200 μl) administered via intraperitoneal injection. Mice were observed for response to hind paw pinch and when no response was observed, surgery was performed to perfuse the brain. The thoracic cavity was opened to expose the heart and the perfusion needle inserted into the left ventricle. The mouse was perfused with PBS for 3-5 min using a perfusion pump. After perfusion surgery, mice were euthanized by decapitation and removal of the brain. Brains were stored in RNALater solution for further analysis by qRT-PCR.

Quantitative RT-PCR analysis.—Total RNA was isolated from human cells using Trizol reagent and further cleaned up with RNeasy Mini kit and on column DNAse I treatment. Whole mouse brain tissue samples were homogenized using the Bead Ruptor 12 (Omni International, Inc) in 7 ml screw cap tubes containing DNase/RNase-free1.4 mm ceramic beads at high speed for 20 seconds followed by a centrifugation step at 12,000 x g for 10 min at 4°C, according to the manufacture's protocol. Total RNA was then isolated and cleaned up as described above for human cells and RNA concentration and purity determined using the NanoDrop 2000. cDNA was synthesized using the SuperScript IV First-Strand Synthesis Kit and qRT-PCR was performed on a QuantStudio 6 Flex instrument (Applied Biosystems) using Qiagen dopamine/serotonin (Cat#PAMM-158Z) and GABA/Glutamate (Cat#PAMM-152A) RT^2 pathway arrays (96-well plate format) and SYBR green chemistry (mouse brain samples) or TaqMan chemistry (immortalized human cell samples) using custom made 96-well plates containing the following primer/probe target sets. Blood brain barrier target genes: TJP2-Hs00910543_m1, CGN-Hs00430426_m1, CLDN5-Hs00533949_s1, OCLN-Hs00170162_m1, RAPGEF6-Hs00255483_m1, CDH5-Hs00901465_m1, IL1B-Hs00174097_m1, IL6-Hs00985639_m1, TNF-Hs01113624_g1, TLR2-Hs02621280_s1, NFKB1-Hs00765730_m1, CTNNB1-Hs00355049_m1 and TBP-Hs00427620_m1. Astrocytes/Microglia target genes: IDO1-Hs00984148_m1, KMO-Hs00175738_m1, AADAT-Hs00212039_m1, PTGS2-Hs00153133_m1, TNC-Hs01115665_m1, GPR84-Hs01874713_s1, IL1B-Hs00174097_m1, IL6-Hs00985639_m1, TNF-Hs01113624_g1, TLR2-Hs02621280_s1, EGR1-Hs00152928_m1, VEGFA-Hs00900055_m1 and TBP-Hs00427620_m1. All reactions were run in triplicate in a final

volume of 20 μl and repeated at least twice. PCR values were normalized to internal standards (B2m and Gus B - Mouse brain samples), or (GUSB-Hs00939627_m1 and HPRT1-Hs01003267_m1 - human samples) and expressed as the mRNA expression levels relative to the control (vehicle control treated samples). The fold-change/fold-regulation of the expression for each target gene was calculated using threshold cycle (Ct) values as follows. Fold-Change (2^{Λ}(- Delta Delta CT)) is the normalized gene expression (2^{Λ}(- Delta CT)) in the Test Sample divided by the normalized gene expression (2^{Λ}(- Delta CT)) in the Control Sample. Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change. The p values are calculated based on a Student's t-test of the replicate 2^{Λ}(- Delta CT) values for each gene in the control group.

Statistical analysis.—GraphPad Prism 7 software (GraphPad Software, La Jolla, CA) was used for all statistical analyses. Values of $P < 0.05$ were considered statistically significant. To compare two groups a Mann-Whitney test was employed. RNA and protein experiments were not corrected for multiple comparisons.

RESULTS

Effect of EBV dUTPase on gene expression in human cerebral microvascular endothelial cells, astrocytes and microglia cells.

We have previously reported that the EBV dUTPase protein increased the expression and secretion of IL-1β, IL-6 and TNF-α from human dendritic cells (hDCs) and PBMCs) in a TLR2-dependent manner.[29] Since IL-1β and IL-6 can disrupt the BBB[3334] and affect neurocognitive functions in patients with ME/CFS[35-38], we asked the question could EBV dUTPase protein modulate the expression of genes important in maintaining BBB integrity and/or synaptic plasticity in immortalized human cerebral microvascular endothelial cells, astrocytes and microglia cells? As shown in Table 1, treatment of human cerebral microvascular endothelial cells with EBV dUTPase protein resulted in a rapid increase in IL-1β (10-fold) and IL-6 (3.5-fold) mRNA expression beginning at 30 min following treatment and reaching maximum induction levels at 2 h (32-fold and 43-fold for IL-1β and IL-6, respectively), when compared to vehicle-treated control. The increase in IL-1β and IL-6 mRNA expression levels was accompanied by a parallel increase in TLR2 and NF-κB gene expression in these cells. Conversely, EBV dUTPase down-regulated the expression of the genes encoding the tight junction proteins occludin (OCLN; 1.62 - fold), claudin-5 (CDH5; 1.80-fold) and cingulin (CGN; 2.24-fold), which reached maximum levels 6 h after treatment.

EBV dUTPase stimulation of microglia cells resulted in the induction of IL-1β and IL-6 mRNA expression, which reached their maximum level of expression at 6 h post-treatment (22-fold and 12-fold for IL-1β and IL-6, respectively). Conversely, while there was an induction of IL-1β and IL-6 mRNA expression in astrocytes, it was not as robust as that observed in cerebral microvascular endothelial cells or microglia cells. The expression of

TLR2 was also up-regulated in cerebral microvascular endothelial cells and astrocytes with maximum induction occurring at 24 h post-treatment (4.86- and 5.05-fold respectively). Whereas no changes in TLR2 expression were observed in microglia cells. Furthermore, the dUTPase protein up-regulated TNF-α mRNA expression in astrocytes and microglia with maximum expression occurring at 2 h post-treatment. The early growth response gene 1 (Egr-1) was significantly down-regulated in both astrocytes (−2.539-fold) and microglia cells (−5.138-fold), beginning at 3 h post-treatment. Interestingly, Egr-1 encodes for a transcriptional regulator reported to be involved in neuronal plasticity.[39, 40] A significant up-regulation of PTGS2, which encodes for cyclooxygensase 2 (COX2), was observed in astrocytes as early as 0.5 h posttreatment, which peaked (6.646-fold) at 2 h post-treatment. Whereas in microglia cells, the dUTPase protein strongly induced the expression of PTGS2 by 32.969-fold at 2 h following stimulation and it remained significantly increased throughout the treatment period. Similarly, a significant up-regulation in the expression of VEGFA (vascular endothelial growth factor) and TNC (brain extracellular matrix protein tenascin C) was observed in microglia cells but not in astrocytes. Enzymes involved in kynurenine metabolism were differentially modulated in astrocytes and microglia cells with kynurenine aminotransferase II (AADAT) and indoleamine 2.3-dioxygenase (IDO1) being unchanged and kynurenine monooxygenase (KMO) down regulated by 2.93-fold at 4 h post-treatment in astrocytes. In microglia cells, there was a significant increase in KMO mRNA expression by 2.474-fold at 3 h post-treatment and in IDO1 at 6 (2.898-fold) and 24 h (3.162-fold) post-treatment. Whereas no significant changes in the expression of AADAT were observed at any of the time points examined.

EBV dUTPase induces hDC to produce proteins with neuroimmune modulatory functions.

Under physiological conditions, the presence of DCs in the brain parenchyma is minimal but their numbers increase during neuroinflammation.[41] We have previously demonstrated that the EBV dUTPase protein increased the expression and secretion of proinflammatory cytokines.[25, 28-30] Thus, we next determined whether the EBV dUTPase induced the secretion of additional proteins or other soluble factors in hDCs that could contribute to a neuroinflammatory microenvironment. Briefly, primary hDCs were treated with the EBV dUTPase protein for 24 h and culture supernatants were collected and analyzed using a human immune Antibody Array (RayBiotech), as described in Material and Methods. As shown in Table 2, EBV dUTPase increased the production of over 30 proteins, including several neurotrophic factors involved with synaptic transmission, proteins that modulate inflammation and extracellular matrix, compared to vehicle control. Not surprisingly, these proteins have been associated with several neurological conditions including anxiety and sickness behaviors, depression, multiple sclerosis, schizophrenia and Alzheimer's disease.

Effects of EBV dUTPase on gene expression in female C57Bl/6 mouse brain

To better understand the biological effects of the EBV dUTPase protein on gene modulation in the central nervous system, C57Bl/6 female mice were injected with dUTPase protein or vehicle daily for 5 day. Mice were then sacrificed and brains harvested for further processing and gene expression analysis by qRT-PCR, as described in Materials and Methods. The results of this study identified 33 differentially expressed genes between control and EBV dUTPase protein injected mice for each normalized data set using the criteria fold change of

≥1.5 and $p < 0.05$. As shown in Table 3, the EBV dUTPase downregulated significantly the mRNA expression of blood brain barrier genes Cgn (cingulin), Tjp2 (Tight junction protein 2), Rapgef6 (Rap guanine nucleotide exchange factor 6) and Mmp15 (Metalloprotease 15); the synaptic plasticity genes Synpo (synptopodin), Lin 7B (Lin 7 homolog B), Rgs20 (Regulator of G protein signaling) and Rab33a (RAB33A member of Ras oncogene family), whereas it upregulated the mRNA expression of Egr1 (Early growth response 1), when compared to vehicle control. The dUTPase protein also significantly upregulated the expression of Gch1 (GTP cyclohydrolase) while it downregulated Gpr84 (G protein-coupled receptor 84), which are genes involved in pain. A down-regulation in the expression of Kmo (Kynurenine-3-monooxygenase), a key gene product in the tryptophan metabolism pathway, Gpr171 (G protein-coupled receptor 171) and Tbc1d1 (TBC1 Family domain member 1) genes was also observed. Both Gpr171 and Tbc1d1 are important proteins involved in metabolism/energy pathways. More importantly, the EBV dUTPase affected the dopamine and serotonin as well as GABA and glutamate pathways in the mouse brain, which are very important for brain development and the central nervous system (CNS) functions, including cognition, emotion processing and movement. Interestingly, among the genes downregulated by the dUTPase in the brain belonging to the dopamine/serotonin pathways were Alox12 (Arachidonate 12-lipoxygenase), Arrb1 (β-arrestin 1), Dbh (dopamine β-hydroxylase), Fos, Nr4a1/Nur77 (Nuclear receptor 77), Th (tyrosine hydroxylase), Tph2 (tryptophan hydroxylase 2), Slc6a3, Slc6a4, Drd1 (dopamine receptor D1), Drd5, Grk6, Pde10a (Phosphodiesterase 10A), Pik3cg and Plcb1 with the three most downregulated genes being Slc6a4 (dopamine transporter) followed by Tph2 and Th. Interestingly, Th and Tph2 are the rate-limiting enzymes in catecholamine synthesis and serotonin biosynthesis, respectively. Also single nucleotide polymorphisms (SNP) in the Alox12 gene have been associated with slower activity, loss of energy and tiredness, loss of pleasure and diminished libido in schizophrenia cohorts.[42] β-arrestin 1 has been shown to have a neuroprotective role and KO of Arrb1 was shown to exacerbate brain infarction and neurological deficit in a mouse model of cerebral ischemia.[43] Similarly, several studies have shown the important role of the nuclear receptors Nr4a, including Nur77/ Nr4a1 in dopamine (DA) neurotransmission in the developing and mature brain.[44] Most recently, decreased cortical expression of the Nur77/ Nr4a1 has been found in patients with schizophrenia. Furthermore, the dUTPase upregulated the mRNA expression of Gabrd ($GABA_A$ receptor subunit δ), Grik4 (Kainic acid receptor subunit KA1), Grik5 (Kainic acid receptor subunit KA2) genes belonging to the GABA/glutamate pathways as well as IL-1β mRNA expression. Increased expression of Grik4 and Grik5has been observed in the hippocampus of patients with refractory temporal lobe epilepsy.[45] These results indicate that EBV dUTPase primarily targeted genes involved in dopamine and serotonin biosynthesis causing a significant downregulation in the expression of these genes and thus, suggest aberrant neurotransmission *in vivo*. EBV dUTPase protein also modulated the expression of genes with key roles in blood brain barrier permeability, metabolism/energy and pain in mouse brain.

DISCUSSION

Numerous studies have proposed various hypotheses and models to explain how neuroinflammation could contribute to the chronic fatigue, post-exertional fatigue and cognitive deficits observed in patients with ME/CFS.[12-15] While neuroimaging studies have demonstrated structural and functional alterations in the brains of patients with ME/CFS, only a single study has presented evidence of an increased activation of astrocytes and microglia in the brain of ME/CFS patients, suggesting that widespread neuroinflammation was occurring.[10] However, the underlying mechanisms that contribute to this neuroinflammatory reaction(s) in ME/CFS patients remain undefined.

We have previously shown that a subgroup of patients diagnosed with ME/CFS exhibited a statistically significant elevation in antibodies against the EBV dUTPase protein.[19] In the present study we provide further evidence supporting a mechanism by which abortive-lytic reactivation of a systemic latent infection of EBV and subsequent production of dUTPase protein, which occurs in a subgroup of patients with ME/CFS, could contribute to the development of a neuroinflammatory microenvironment in the brain by modulating BBB, microglia cells and astrocytes gene expression/function, tryptophan, dopamine and serotonin metabolism and synaptic plasticity, which in turn may contribute to the increased pain, post-exertional fatigue and cognitive impairments observed in some patients with ME/CFS.

The BBB is composed of endothelial cells of the capillary wall, astrocytes end-feet ensheathing the capillary and pericytes embedded in the capillary membrane.[46] The function of the BBB is to prevent the free diffusion of substances and the movement of cells from the systemic circulation into the central nervous system (brain and spinal cord) and thus, prevent unwanted activation of brain cells. We have previously demonstrated that the EBV dUTPase protein induces the secretion of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in human dendritic cells (hDCs) and PBMCs.[28-30] In this study we show that the EBV dUTPase protein strongly induces the expression of IL-6 and IL-1β in cerebral microvascular endothelial cells and microglia cells as well as TNF-α in both astrocytes and microglia cells. These pro-inflammatory cytokines and IFN-γ have been reported to disrupt BBB integrity.[34] The EBV dUTPase also down-regulated *in vitro* or *in vivo* the expression of genes encoding for the proteins which have critical and direct implications for both forming and maintaining tight junctures between endothelial cells in capillaries comprising the BBB as well as modulating cellular adhesion and the extracellular matrix. Altogether, these data suggest that the EBV-dUTPase protein has the capacity to disrupt the BBB, which could result in neuroinflammation and/or neurodegeneration.[46]

In addition, the EBV dUTPase protein also induced a transient increase in the expression of PTGS2/COX-2 in astrocytes while in microglia cells, the dUTPase induced a strong and sustained PTGS2 expression, suggesting that microglia cells were the primary source of this pro-inflammatory enzyme. Cox-2 catalyzes the formation of prostaglandin E_2 (PGE_2), which is a key mediator of inflammatory responses. While COX-2 is generally considered to be inducible, it is constitutively expressed in some glutamatergic neurons in the cortex and hippocampus[47], astrocytes[48] and microglia.[49] The role of COX-2 as a contributor to neuroinflammatory toxicity in neurodegenerative disorders is well established.

Our data also demonstrate that the EBV dUTPase altered the expression of genes involved with pain (GPR84[51] and GCH1[52]) and fatigue (TBC1D1[53]). Chronic fatigue and pain are characteristic symptoms in patients with ME/CFS.[1-3]

In addition to disrupting the integrity of the BBB and modulating genes involved with inflammatory processes, pain and fatigue, our data suggests that the EBV dUTPase may alter synaptic plasticity *in vivo*, which is important in learning and memory processes, as demonstrated by the ability of the dUTPase protein to down-regulate the expression of LIN7b, SYNPTO and RAB33A and up-regulate Egr-1 in mouse brain. These genes have critical functions in (1) ensuring proper localization of the GRIN2B subunit of the N-methyl-D-aspartate receptor (NMDAR),[54] (2) long-term potentiation,[55, 56] (3) mediating antegrade axonal transport of post-Golgi synaptophysin-positive vesicles and their fusion at growth cones[57] as well as (4) NMDAR mediated down-regulation of PSD95 and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR) trafficking[39] all of which are very important for synaptic development, plasticity and functions. AMPAR and NMDAR play critical roles in the plasticity of most excitatory synapses, as demonstrated by the fact that a number of neurological disorders associated with synaptic dysfunction have altered NMDAR and AMPAR expression, trafficking and signaling. These data suggest that the EBV dUTPase is capable of altering synaptic structure and function as well as neuronal communication, which would affect cognitive processes.

Lastly, our data show that the EBV dUTPase protein modulates tryptophan, serotonin and dopamine metabolism and utilization in *vitro* and *in vivo*. The EBV dUTPase may alter kynurenine catabolism in microglia *in vitro*, suggesting that there is an increase synthesis of quinolinic acid (QUIN). QUIN, an agonist of NMDAR, can cause overstimulation resulting in neuronal toxicity.[58] The dopaminergic and serotonergic neurotransmitter systems are reported to play a critical role in the regulation of emotion and mood, and have been implicated in a spectrum of neuropsychiatric disorders.[59, 60]

EBV replicates primarily in the tonsils/nasopharynx and sheds in the saliva[61] There are also numerous reports demonstrating that physical and/or psychosocial stress induces the reactivation of latent EBV. Burning mouth syndrome (BMS) is a chronic condition characterized by a burning sensation of the oral cavity that affects menopausal or postmenopausal women (50-70 years of age). In addition, these patients exhibit high levels of anxiety and depression.[62,63] Idiopathic BMS can occur spontaneously and without any identifiable precipitating factors. Although the exact mechanism(s) involved in the pathophysiology of idiopathic BMS is unknown, there is evidence that it may be a neuropathic condition affecting the peripheral and central nervous systems. Clonazepam, a member of the benzodiazepine family, which is used for treatment of anxiety, is the preferred treatment option for patients with BMS because of its effect on the peripheral γ-aminobutyric acid (GABA) A receptor. It has been shown that nerve fibers on the tongue have high expression of GABA A receptors[64]. Our study demonstrates that EBV dUTPase upregulates a subunit of the GABA A receptor and suggests a possible involvement of EBV in this syndrome, especially in an older population with a decreased immune capacity to control the virus.

In summary, we are proposing that in a subset of patients with ME/CFS there is an increase in abortive lytic replication of EBV, especially in those patients exhibiting a diminished EBV-specific B and T cell response,[18] resulting in the increased release of EBV dUTPase possibly in exosomes.[29] Activation of TLR2 by the EBV dUTPase in cerebral microvascular endothelial cells can disrupt the integrity of the BBB by inducing the down-regulation of genes in cerebral microvascular endothelial cells that encode for products important for maintaining tight junctions between these cells and simultaneously inducing the up-regulation IL-1β, IL-6 and TNF-α proinflammatory cytokines that disrupt BBB. Disruption of the BBB allows the dUTPase protein to enter the central nervous system where ligation and activation of TLR2 by the dUTPase on astrocytes[65], microglia[66], mast cells[67] and possibly neurons[68] would result in altered excitatory glutamatergic synapses and expression of genes whose products are involved in fatigue, pain and cognitive responses all of which are altered in patients with ME/CFS. Recent studies have also shown that secretion of IL-33 by microglia cells activates mast cells resulting in the rapid secretion of TNF and sensitization of meningeal nociceptors.[69] This crosstalk between microglia and mast cells may be an important process for explaining stress-induced neuroinflammation, a common feature in many neurological conditions such as depression and anxiety.[70] While these findings are exciting further studies are necessary to confirm that changes in gene expression equates with changes in protein levels and altered functionality. Also multi-test correction was not employed which may affect statistical comparisons. The results of this study provide exciting new data at the molecular level suggesting a novel mechanism by which EBV dUTPase may modulate immune activation, disrupt the BBB and alter the structure/function of neurological synapses resulting in loss of neurocognitive functions in a cohort of patients with ME/CFS.

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REFERENCES

1. Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, and Komaroff A. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. Ann Intern Med. 1994; 121:953–940. [PubMed: 7978722]
2. Carruthers BM, Jain AK, De Meirleir DL, Peterson DL, Klimas NG, Lerner AM, Bested AC, Flor-Henry P, Joshi P, Powles AP and Sherkey JA. . 2003, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: clinical working case definition, diagnostic and treatment protocols. J CFS. 2003; 11:7–115.
3. Institute of Medicine 2015 Beyond Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: Redefining an Illness. Institute of Medicine: Washington WA USA ISBN 978-0-30931689-7.
4. Cockshell SJ and Mathias JL. Cognitive functioning in chronic fatigue syndrome: a meta-analysis. Psychological Medicine 2010; 40:1253–1267. [PubMed: 20047703]
5. Shan ZY, Kwiakek R, Burnet R, Del Fante P, Staines DR, Marshall-Gradisnik SM and Barnden LR. Progressive brain changes in patients with chronic fatigue syndrome: a longitudinal MRI study. J. Magn. Reson. Imaging. 2016; 44:1301–1311. [PubMed: 27123773]
6. Boissoneault J, Letzen J, Lai S, O'Shea A, Craggs J, Robinson ME and Staud R. Abnormal resting state functional connectivity in patients with chronic fatigue syndrome: an arterial spin-labeling fMRI study. J. Magn. Reson. Imaging. 2016; 34:603–608.

7. Morris G, Berk M and Puri BK. A comparison of neuroimaging abnormalities in multiple sclerosis, major depression and chronic fatigue syndrome (myalgic encephalomyelitis): is there a common cause? Mol. Neurobiol. 2018; 55:3592–3609. [PubMed: 28516431]
8. Shan ZY, Finegan K, Bhuta S, Ireland T, Staines DR, Marshall-Gradisnik SM and Barnden LR. Brain function characteristics of chronic fatigue syndrome: a task fMRI study NeuroImage: Clinical. 2018; 19:279–286. [PubMed: 30035022]
9. Zinn MA, Zinn ML, Valencia I, Jason LA and Montoya JG. Cortical hypoactivation during resting EEG suggests central nervous system pathology in patients with chronic fatigue syndrome. Biological Pathology. 2018; 136:87–99.
10. Nakatomi Y, Mizuno K, Ishii A, Wada Y, Tanaka M, Tazawa S, Onoe K, Fukuda S, Kawabe J, Takahashi K, Kataoka Y, Shiomi S, Yamaguti K, Inaba M, Karatsune H and Watanabe Y. Neuroinflammation in patients with chronic fatigue syndrome/myalgic encephalomyelitis: an ^{11}C-(*R*)-PK11195 PET study. J. Nuclear Med. 2014; 55:945–950.
11. Mueller C, Lin JC, Sheriff S, Maudsley AA and Younger JW. Evidence of widespread metabolite abnormalities in Myalgic encephalomyelitis/chronic fatigue syndrome: assessment with whole-brain magnetic resonance spectroscopy. Brain Imaging and Behavior. 2019; 10.1007/s11682-018-0029-4.
12. Glassford JAG. The neuroinflammatory etiopathology of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Front. Physiol. 2017; 8:88 doi:10.3389/fphys.2017.00088. [PubMed: 28261110]
13. Morris G, Berk M, Galecidi P, Walder K and Maes M. The neuro-immune pathophysiology of central and peripheral fatigue in systemic immune-inflammatory and neuro-immune diseases. Mol. Neurobiol. 2016; 53:1195–1219. [PubMed: 25598355]
14. Morris G, Berk M, Klein H, Walder K, Galecidi P and Maes M. Nitrosative stress, hypernitrosylation and autoimmune responses to nitrosylated proteins: new pathways in neuroprogressive disorders including depression and chronic fatigue syndrome. Mol. Neurobiol. 2017; 54:4271–4291. [PubMed: 27339878]
15. Monro JA and Puri BK. A molecular neurobiological approach to understanding the Aetiology of chronic fatigue syndrome (myalgic encephalitis or systemic exertion intolerance disease) with treatment implications. Mol. Neurobiol. 2018; 55:7377–7388. [PubMed: 29411266]
16. Klimas NG, Ironson G, Carter A, Balbin E, Bateman L, Felsenstein D, Levine S, P eterson D, Chiu K, Allen A, Cunningham K, Gottschalk CG, Fletcher M, Hornig M, Canning C, and Komaroff AL. Findings from a clinical and laboratory database developed for discovery of pathogenic mechanisms in myalgic encephalomyelitis/chronic fatigue syndrome. Fatigue Biomed Health Behav. 2015; 3:75–96. doi:10.1080/21641846.2015.1023652.
17. Cameron B, Flamand L, Juwana H, Middeldorp J, Naing Z, Rawlinson W, Ablashi D and Lloyd A. Serological and virological investigation of the role of the herpesviruses EBV, CMV and HHV-6 in post-infective fatigue syndrome. J. Med. Virol. 2010; 82:1684–1688. [PubMed: 20827765]
18. Loebel M, Strohschein K, Giannini C, Koelsch U, Bauer S, Doebis C, Thomas S, Unterwalder N, von Baehr V, Reinke P, Knops M, Hanitsch LG, Meisel C, Volk HD, and Scheibenbogen C. Deficient EBV-specific B- and T-cell response in patients with Chronic Fatigue Syndrome. PLoS ONE. 2014; 9(1): e85387. doi:10.1371/journal.pone.0085387. [PubMed: 24454857]
19. Halpin P, Williams MV, Klimas NG, Fletcher MA, Zachary Barnes Z, and Ariza ME. Myalgic encephalomyelitis/chronic fatigue syndrome and gulf war illness patients exhibit increased humoral responses to the herpesviruses-encoded dUTPase: Implications in disease pathophysiology J. Med. Virol. 2017; 89:1636–1645. [PubMed: 28303641]
20. Jha HC, Mehta D, Lu J, El-Naccache D, Shukla SK, Kovacsics C, Kolson D and Robertson E. Gammaherpesvirus infection of human neuronal cells. mBio. 2015; 6(6):e01844–15. doi:10.1128/mBio.01844-15. [PubMed: 26628726]
21. Menet A, Speth C, Larcher C, Prodinger WM, Schwendinger MG, Chan P, Jagar M, Schwarzmann F, Recheis H, Fontaine M and Dierich MP. Epstein-Barr virus infection of human astrocyte cell lines. J. Virol. 1999 73:7722–7733. [PubMed: 10438862]
22. Brandsma D and Bromberg JEC. Primary CNS lymphoma in HIV1 infection. In Handbook of Clinical Neurology 2018 152: 177–186. [PubMed: 29604975]

23. Fernandez-Menendz S, Fernandez-Moran M, Fernandez-Vega I, Perez-Alvarez A and Villafani-Echazu J. Epstein Barr virus and multiple sclerosis: from evidence to therapeutic strategies. J Neurological sciences. 2016; 361: 213–219.
24. Hassani A, Corboy JR, Al-Salam S and Khan G. Epstein-Barr virus is present in the brain of most cases of multiple sclerosis and may engage more than just B cells. PLoS ONE 2018; 13(2):e0192109 10.1371/journal.pone.0192109. [PubMed: 29394264]
25. Glaser R, Litsky ML, Padgett DA, Baiocchi RA, Yang EV, Chen M, Yeh PE, Green-Church KB, Caligiuri MA and Williams M. EBV-encoded dUTPase induces immune dysregulation: Implications for the pathophysiology of EBV-associated malignant disease. Virology. 2006; 346:205–218. [PubMed: 16321417]
26. Waldman WJ, Williams MV, Lemeshow SA, Binkley P, Guttridge D, Kiecolt-Glaser JK, Knight DA, Guttridge K, and Glaser R. Epstein-Barr virus encoded dUTPase enhances proinflammatory cytokine production by macrophages in contact with endothelial cells: evidence for depression-induced atherosclerotic risk. Brain Behavior and Immunity 2008; 22:215–223.
27. Ariza ME, Glaser R, Kaumaya PTP, Jones C and Williams M. The Epstein-Barr Virus (EBV)-Encoded dUTPase Activates NF-κB through the TLR2 and MyD88-dependent Signaling Pathway. J Immunol. 2009; 182:851–859. [PubMed: 19124728]
28. Ariza ME, Rivailler P, Glaser R, Chen M and Williams MV. Epstein-Barr virus encoded dUTPase containing exosomes modulate innate and adaptive immune responses in human dendritic cells and peripheral blood mononuclear cells. PLoS One 2013; 8: e69827 doi: 10.1371/journal.pone. 0069827. [PubMed: 23894549]
29. Ariza ME, Glaser R and Williams MV. Human herpesviruses encoded dUTPases: A family of proteins that modulate dendritic cells function and innate immunity. Frontiers in Microbiol 2014; Vol 5 Article 504.
30. Williams MV, Cox B and Ariza ME. Herpesviruses-encoded dUTPases: A new family of Pathogen-Associated Molecular Pattern (PAMP) proteins with Implications in human disease. Pathogens 2017; 6, 2; doi:10.3390/pathogens6010002.
31. Padgett DA, Hotchkiss AK, Pyter LM, Nelson RJ, Yang E, Yeh PE, Litsky M, Williams M, and Glaser R. Epstein-Barr virus-encoded dUTPase modulates immune function and induces sickness behavior in mice. J. Med. Virol. 2004; 74:442–448. [PubMed: 15368518]
32. Aubrecht TG, Zachary M. Weil ZM, Ariza ME, Williams M, Reader B, Glaser R, Sheridan J and Nelson RJ. Epstein-Barr virus (EBV)-encoded dUTPase and chronic restraint induce impaired learning and memory and sickness responses. Physiology & Behavior. 2014; 137:18–24. [PubMed: 25034413]
33. Zhao Z, Nelson AR, Betsholz C and Zlokovic BV. Establishment and dysfunction of the blood-brain barrier. Cell 2015; 163:1064–1078. [PubMed: 26590417]
34. Miner JJ and Diamond MS. Mechanisms of restriction of viral neuroinvasion at the blood-brain barrier. Current Opinion Immunol. 2016; 38:18–23.
35. Ifuku M, Hossain SM, Noda M and Katafuchi T. Induction of interleukin-1β by activated microglia is a prerequisite for immunologically induced fatigue. Eur. J. Neuroscience 2014; 40:3253–3263.
36. Patterson SL. Immune dysregulation and cognitive vulnerability in the aging brain: interactions of microglia, IL-1β BDNF and synaptic plasticity. Neuropharmacology 215; 96:11–18. [PubMed: 25549562]
37. Gruol DL. Impact of increased astrocyte expression of IL-6, CCL2 or CXCL10 in transgenic mice on synaptic function. Brain Sci. 2016; 6: 19; doi:10.3390/brainsci6020019.
38. Pozzi D, Menna M, Canzi A, Desiato G, Mantovani C and Matteoli M. The communication between the immune and nervous systems: the role of IL-1b in Synaptopathies. Front. Mol. Neurosci. 2018; 11:111. doi: 10.3389/fnmol.2018.00111. [PubMed: 29674955]
39. Qin X, Jiang Y, Tse YC, Wang Y, Wong TP and Paudel HK. The early growth response 1 (EGR-1) regulates N-methyl –D- aspartate receptor (NMDAR)-dependent transcription of PSD-95 and a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) trafficking in hippocampal primary neurons. J Biol. Chem. 2015; 290:29603–29616. [PubMed: 26475861]

40. Duclot F and Kabbaj M. The role of early growth response 1 (EGR1) in brain plasticity and neuropsychiatric disorders. Front. Behav. Neurosci. 2017; 11:35. doi: 10.3389/fnbeh.2017.00035. [PubMed: 28321184]
41. Ludewig P, Gallizioli M, Urra X, Behr S, Brait VH, Gelderblom M, Magnus T and Planas AM. Dendric cells in brain diseases. Biochimica et Biophysica ACTA. 2016; 1862:352–367. [PubMed: 26569432]
42. Kim T, Kim HJ, Park JK, Kim JW and Chung JH. 2010 Association between polymorphisms of arachidonate 12-lipoxygenase (ALOX12) and schizophrenia in a Korean population. Behavior and Brain Function 6:44.
43. Wang P, Xu TY, Wei K, Guan YF, Wang X, Xu H, Su DF, Pei G and Miao CY. 2014 ARRB1/β-arrestin −1 mediates neuroprotection through coordination of BECN1-dependent autophagy in cerebral ischemia. Autophagy 10:1535–1548. [PubMed: 24988431]
44. Decressac M, Volakakis N, Bjorklund A and Perlmann T. 2013 NURR1 in Parkinson disease-from pathogenesis to therapeutic potential. Nature Review Neurol 9: 628–636.
45. Das A, Wallace GC IV, Holmes C, McDowell ML, Smith JA, Marshall JD, Bonilha L, Edwards JC, Glazier SS, Ray SK and Banik NL. 2012 Hippocampal tissue of patients with refractory temporal lobe epilepsy is associated with astrocyte activation inflammation, and altered expression of channels and receptors. Neuroscience 220: 237–246. [PubMed: 22698689]
46. Obermeier B, Daneman R, and Ransohoff RM. 2013 Development, maintenance and disruption of the blood-brain barrier. Nature Medicine 19: 1584–1596.
47. Hewett SJ, Shi J, Gong Y, Dhandapani K, Pilbeam C and Hewett JA. Spontaneous glutamatergic synaptic activity regulates constitutive COX-2 expression in neurons. J. Biol. Chem. 2016; 291:27279–27288. [PubMed: 27875294]
48. Font-Nieves M, Sans-Fons MG, Gorina R, Bonfill-Teixidor E, Salas-Perdomo A, Marquez-Kisinousky L, Santalucia T and Planas AM. Induction of COX-2 and down-regulation of COX-1 expression by LPS control prostaglandin E_2 production in astrocytes. J. Biol. Chem. 2012; 287:6454–6468. [PubMed: 22219191]
49. Xia Q, Hu Q, Wang H, Yamg H, Gao F, Ren H, Chen D, Fu C, Zheng L, Zhen X, Ying Z and Wang G. Induction of COX-2-PGE2 synthesis by activation of the MAPK/ERK pathway contributes to neuronal death triggered by TDP-43 depleted microglia. Cell Death Disease. 2015; 6, e1702; doi: 10.1038/cddis.2015.69. [PubMed: 25811799]
50. Aid S and Bosetti F. Targeting cyclooxygenases-1 and −2 in neuroinflammation: therapeutic implications. Biochimie, 2011; 93:46–51. [PubMed: 20868723]
51. Nicol LSC, Dawes JM, La Russa F, Didangelos A, Clark AK, Gentry C, Grist J, Davies JB, Malcanglo M and McMahon SB. The role of G-protein receptor 84 in experimental neuropathic pain. J. Neurosci. 2015; 35:8959–8969. [PubMed: 26063927]
52. Nasser A, Bjerrum OJ, Heegaard AM, MØller AT, Larsen M, DalbØge LS, Dupont E, Jensen TS, MØller LB. 2013. Impaired behavioural pain responses in hph-1 mice with inherited deficiency in GTP cyclohydrolase 1 in models of inflammatory pain. Mol. Pain. 2013; 9:5. [PubMed: 23421753]
53. Whitfield J, Paglialunga S, Smith BK, Miotto PM, Simnett G, Robson HL, Jain SS, Herbst EAF, Desjardins EM, Dyck DJ, Spriet LL, Gregory R. Steinberg GR and Holloway GP. Ablating the protein TBC1D1 impairs contraction-induced sarcolemmal glucose transporter 4 redistribution but not insulin-mediated responses in rats. J. Biol. Chem. 2017; 292:16653–16664. [PubMed: 28808062]
54. Mizuno M, Matsumoto A, Hamada N, Ito H, Miyauchi A, Jimbo EF, Momoi MY, Tabata H, Yamagata T and Nagata KI. Role of an adaptor protein Lin 7B in brain development: possible involvement in autism spectrum disorders. J. Neurochemistry. 2015; 132:61–6
55. Korkotian E, Frotscher M and Segal M. Synaptopodin regulates spine plasticity: mediation by calcium stores. J. Neurosci. 2014; 34:11641–11651. [PubMed: 25164660]
56. Jedlicka P and Deller T. Understanding the role of synaptopodin and the spine apparatus in Hebbian synaptic plasticity-new perspective and the need for computer modeling. Neurobiology Learning Memory. 2017; 138:21–30.

57. Nakazawa, Sada T, Toriyama M, Tago K, Sugiura T, Fukuda M and Inagaki N. Rab33a mediates anterograde vesicular transport for membrane exocytosis and axon growth. J. Neurosci. 2012; 32:12712–12725. [PubMed: 22972995]
58. Parrott JM and O'Connor JC. Kynurenine 3-monooxygenase: an influential mediator of neuropathology. Front. Psychiatry. 2015; 6: 116. doi: 10.3389/fpsyt.2015.00116. [PubMed: 26347662]
59. Liu Y, Zhao J and Guo W. 2018 Emotional Roles of mono-aminergic neurotransmitters in major depressive disorder and anxiety disorders. Front Psycho. 9:2201. doi: 10.3389/fpsyg.2018.02201.
60. Palacios-Filardo J and Mellor JR. 2019 Neuromodulation of hippocampal long-term synaptic plasticity. Current Opinion Neurobiol. 54:37–43.
61. Hadinoto V, Shapiro M, Sun CC and Thorley-Lawson DA. 2009 The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. PLoS Pathog 5(7): e1000496. doi:10.1371/journal.ppat.1000496. [PubMed: 19578433]
62. Malik R, Goel S, Misra D, Panjwani S, and Misra A. 2012 Assessment of anxiety and depression in patients with burning mouth syndrome: A clinical trial. J Midlife Health. 3: 36–39. [PubMed: 22923978]
63. Di Stasio D, Candotto V, Serpico R, Migliozzi R, Petruzzi M, Tammaro M, Maio C, Gritti P, Lauritano D, Lucchese A. 2018 J Biol Regul Homeost Agents 32 (2 (Suppl 1): 91–95.
64. Teruel A and Patel S. 2019 Burning mouth syndrome: a review of etiology, diagnosis, and management. Gen Dent 67:24–29. [PubMed: 30875303]
65. Min H, Hong J, Cho IH, Jang YH, Lee H, Kin D, Yu SW, Lee S and Lee SJ. TLR2-induced astrocyte MMP9 activation compromises the blood brain barrier and exacerbates intracerebral hemorrhage in animal models. Mol. Brain 2015; 8: 23 doi 10.1186/s13041-015-0116-z. [PubMed: 25879213]
66. Tang SC, Arumugam TV, Xu X, Cheng A, Mughal MR, Jo DJ, Lathia DJ, Siler DA, Chigurupati S, Ouyang X, Magnus T, Camandola S, and Mattson MP. Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. Proc. Natl. Acad. Sci. USA. 2007; 104: 13798–13803. [PubMed: 17693552]
67. Sandig H and Bulfone-Paus. 2012 TLR signaling in mast cells: common and unique features. Front Immunol 3:185 doi: 10.3389/fimmu.2012.00185. [PubMed: 22783258]
68. Hoffmann O, Braun JS, Becker D, Halle A, Dorette Freyer D, Dagand E, Lehnardt S and Weber JR. TLR2 mediates neuroinflammation and neuronal damage. J. Immunol. 2007; 178:6476–6481. [PubMed: 17475877]
69. Caraffa AI, Conti C, D'Ovidio C, Gallenga CE, Tettamanti L, Mastrangelo F, Ronconi G, Kritas SK and Conti P. 2018 New concepts in neuroinflammation: mast cells pro-inflammatory and anti-inflammatory mediators. J Biol Regul Homeost Agents 32:1345–1347. [PubMed: 30574737]
70. Theoharides TC and Kavalioti M. 2018 Stress, inflammation and natural treatments. J Biol Regul Homeost Agents 32:1345–1347. [PubMed: 30574737]

Table 1.

Time course gene expression analysis of EBV dUTPase treatments of immortalized human endothelial cells, astrocytes and microglia cells by qRT-PCR.

Immortalized human Endothelial Cells							
Gene Symbol	**0.5 h**	**1h**	**2h**	**3h**	**4h**	**6h**	**24h**
IL-1b	10.0364*	15.1413*	31.7606*	18.2101*	16.3126*	13.2937*	10.9182*
IL-6	3.5231*	16.9808*	43.3237*	18.653*	10.8122*	10.829*	13.0713*
NFkB	0.9741	1.027	3.2147*	5.2695*	4.1351*	1.7161*	2.818*
TLR2	0.7067	1.027	1.407	2.577*	2.1737*	2.472*	4.8573*
CGN	−1.0639	−1.0492	−1.04	−1.6232	−1.8893*	−2.2359*	−1.2709
OCLN	1.2188	−1.0073	1.4671	−1.1103	−1.2797	−1.6228*	1.5438*
CDH5	−1.763*	−1.5308*	−1.1593	−1.2444	−1.2874	−1.8006*	1.0108
Immortalized human Astrocytes							
Gene Symbol	**0.5 h**	**1h**	**2h**	**3h**	**4h**	**6h**	**24h**
KMO	−1.0807	−1.1204	−1.1716	−1.0948	−2.9312*	−1.057	1.6771*
PTGS2	2.0392*	2.733*	6.6461*	2.9921*	3.0876*	1.9541*	1.5637
IL-1b	1.1165	1.1447	2.619*	1.7597*	2.16*	1.6313*	3.1788*
IL-6	1.4196	1.5889*	1.279	2.2214*	2.2207*	1.7734*	−1.4954
AADAT	−1.0021	−1.0094	−1.0007	1.074	1.0497	−1.0461	−1.129
EGR1	2.8501*	4.1641*	−1.0007	−2.5394*	−2.3958*	−2.4906*	1.1173
TLR2	1.0147	1.1282	1.9759*	1.9998*	1.5735*	1.2561	5.0543*
IDO1	−1.1676	−1.0688	1.1451	−1.2108	1.2509	−1.1603	1.0024
TNF	1.2614	4.9161*	1.7777*	1.1527	−1.401	1.4575	−1.1173
VEGFA	1.1732	1.4449	1.3985	1.429	1.0136	1.4687	1.1337
TNC	1.0007	1.1507	1.1822	1.0658	−1.454	−1.2897	−1.0331
Immortalized human Microglia Cells							
Gene Symbol	**0.5 h**	**1h**	**2h**	**3h**	**4h**	**6h**	**24h**
KMO	1.3599	1.3599	1.6121*	2.4753*	1.5756*	1.6262*	1.9897*
PTGS2	1.2146	1.2146	32.9696*	28.7065*	31.8242*	28.6373*	5.5168*
IL-1b	1.0673	11.4468*	17.9898*	11.5652*	7.7246*	22.7129*	4.4798*
IL-6	−1.0411	4.5281*	12.2746*	10.2858*	8.5455*	12.435*	1.3081
AADAT	1.0244	−1.043	−1.1827	−1.2233	−1.1061	−1.2476	−1.339
EGR1	−1.5767*	−1.1995	−1.0081	−5.1383*	−5.2705*	−6.2691*	−1.1319
TLR2	1.1128	−1.0849	1.6425*	1.7396*	1.6936*	1.1975	1.3081
IDO1	1.2555	−1.5851	−1.1672	1.149	1.0078	2.8985*	3.1619*

Immortalized human Endothelial Cells							
Gene Symbol	**0.5 h**	**1h**	**2h**	**3h**	**4h**	**6h**	**24h**
TNF	−1.0803	2.0305 *	3.8838 *	3.1083 *	2.0876 *	1.2817	1.5184
VEGFA	1.3083	3.0389 *	3.071 *	2.0312 *	1.6698 *	3.2235 *	1.528
TNC	−1.1904	1.8138 *	2.7783 *	3.2988 *	2.8257 *	2.7879 *	2.2279 *

Data represents mRNA expression levels relative to the vehicle-treated control and expressed as fold-regulation.

* indicates mean fold-change in expression significantly different from control by ≥ 1.5 with $P < 0.05$, n=3.

Table 2.

Major proteins modulated by EBV dUTPase in human primary dendritic cells* that alter brain function.

Protein	Fold-Change†	Function	Disease/Symptom Association
ACE-1/−2 (angiotensin-converting enzyme 1 & 2)	3.00/3.00	Zinc metallopeptidases	Alzheimer's disease
ADAMTS-15	330.00	Zinc metallopeptidases	Alzheimer's disease
APP	2.00	Amyloid precursor protein	Alzheimer's disease
BACE-1	4.00	β-site APP cleaving enzyme 1 or β-secretase	Alzheimer's disease
Brain-derived neurotrophic factor (BDNF)	2.00	Supports differentiation, maturation and survival of neurons and synaptic transmission	Alzheimer's disease Depression Schizophrenia
Chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2; GPR44)	3.00	Receptor for prostaglandin D2	Sickness behavior
COX-2	13.00	Cyclooxygenase 2	Inflammation
Dickkopf3 (DKK3)	3.00	Acts as a repressor/activator of WNT/β-catenin signaling	Alzheimer's disease
EN-RAGE (S100A12)	3.00	Ligand for RAGE; pro-inflammatory	Alzheimer's disease
EphB4/A1/A2/B6 (erythropoietin-producing hepatoma receptors)	45.00/3.00/3.00/3.00	Receptor tyrosine kinases; neural stem cell differentiation	
Fetulin A	3.00	Pro- and anti-inflammatory	Multiple sclerosis
Frizzled 5	35.00	Receptor for WNT ligands, establishment of neuronal polarity	Alzheimer's disease
Galanin	3.00	Neuropeptide	Nociception; Alzheimer's disease, epilepsy
Growth/differentiation factor 15 (GDF-15)	5.00	Neurotrophic factor	Oral cavity cancer
Glypican-5	8.00	Unknown; reported to regulate WNT andhedgehog pathways	B-cell lymphoma
IDE (insulin degrading enzyme)	2.74	Amyloid-β degradation	Alzheimer's disease
ITM2B	3.00	Regulator role in processing amyloid-β A4	Alzheimer's disease
LDLR (low-density lipoprotein receptor)	1.37	Expressed by adult neurons; binds ApoE	Alzheimer's disease
Lin41/TRIM71	3.00	E3 ubiquitin protein ligase; inhibits translation of EGR1	
MMP 2 (matrix metalloproteinase)	2.00	Calcium-dependent zinc endopeptidase	Alzheimer's disease
MMP 9 (matrix metalloproteinase)	6.00	Calcium-dependent zinc endopeptidase	Alzheimer's disease
Netrin-4	4.00	Ligand for Unc-5 homologue 5; promotes terminal branching of axons	
Neuritin (candidate plasticity gene 15; CPG15)	177.00	Neurotrophin synaptic plasticity	Depression
Orexin A/B	130.00/4.00	Neuropeptides important role in hippocampal neurogenesis spatial learning and memory	Depression, learning and memory deficiencies, inflammation
Presenilin 1	2.00	Component of γ-secretase complex	Alzheimer's disease

Protein	Fold-Change†	Function	Disease/Symptom Association
Presenilin 2	21.00	Component of γ-secretase complex	Alzheimer's disease
ProSAAS	3.00	Precursor protein processed to yield SAAS, GAV, PEN bigLEN, littleLEN	Behaviors, including anxiety, feeding, and stress
RAGE	2.00	Receptor for advanced glycation products	Alzheimer's disease
THY-1 (CD90)	4.00	T cell activation	Tumor suppressor nasopharyngeal carcinoma; axonal regeneration
TIMP-1, 2, 3, 4 (tissue inhibitor of metalloproteinase)	243.00/128.00/216.00 6.00	Major endogenous inhibitors of metalloproteinases in tissue	
TMEFF1 (tomorregulin-1)	2.00	Addicsin-associated factor	Schizophrenia
WIF-1	13.00	Inhibitor of WNT signaling pathway	Alzheimer’s disease

ADAMTS-15 = ADAM metallopeptidase with thrombospondin type 1 motif 15; APP = β-amyloid precursor protein; BACE = β-secretase 1; bigLEN = synonym for *PCSK1N*; dUTPase = deoxyuridine triphosphate nucleotidohydrolase; EBV = Epstein-Barr virus; EGR1 = early growth response 1; EN-RAGE = extracellular newly identified receptor for advanced glycation end-products binding protein; GAV = Gill-associated virus; GPR44 = putative G protein-coupled receptor 44; ITM2B = integral membrane protein 2B; littleLEN = synonym for *PCSK1N*; PEN = synonym for *PCSK1N*; ProSAAS = synonym for *PCSK1N*; RAGE = receptor for advanced glycation endproducts; SAAS = synonym for *PCSK1N*; TH2 = T helper type 2; THY-1 = Thy-cell surface antigen; TRIM71 = tripartite motif containing 71; WIF-1 = WNT inhibitory factor 1.

*Human primary dendritic cells were stimulated with EBV dUTPase protein (10 μg/mL) or vehicle for 24 h. After treatment, the levels of multiple immune proteins in the culture supernatant was measured with the Human L-1000 Antibody Array (RayBiotech), as described in Methods.

†Normalized signal intensity data for each analyte in EBV dUTPase-treated cells and expressed as fold-change relative to the vehicle control. Normalized data represent values in which the background signal of negative control spots on the array has been subtracted out and normalized to the mean signal intensity of positive control spots. After normalization, any ≥ 1.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte between dUTPase and control samples was considered a measurable and significant difference in expression, provided that both sets of signals are well above background (mean background [2]; accuracy ≈ 95%).

Table 3.

Major pathways/genes modulated by EBV-dUTPase protein in female C57Bl/6 mouse brain.

Gene Symbol	Gene Name	Fold-Regulation*	*P*	Function
Alox12	Arachidonate 12-lipoxygenase	−1.91324	0.030	Signal transduction phospholipase A2 pathway
Arrb1	Beta-ARRESTIN 1/negative regulators of GPCR	−1.60338	0.012	Signal transduction G-protein coupled receptor regulation
Cgn	cingulin	−1.62	0.049	Blood—brain barrier
Dbh	dopamine β-hydroxylase (DβH) enzyme	−1.7011	0.030	Dopamine metabolism
Drd1	Dopamine receptor D1	−1.75465	0.025	Dopamine receptor
Drd5	Dopamine receptor D5	−1.70713	0.032	Dopamine receptor
Egr1	Early growth response 1	1.74	0.046	Synaptic plasticity; pain
Fos	Transcription factor/essential role in stress resilience	−1.61098	0.033	Signal transduction; cAMP & protein kinase A signaling
Gabrd	$GABA_A$ receptor subunit δ	1.58754	0.017	Neurotransmitter receptor: GABAergic synapse
Gch1	GTP cyclohydrolase	2.94	0.016	Pain; dopamine biosynthesis
Grik4	Glutamate receptor, ionotropic, kainite subunit KA1	2.91804	0.016	Neurotransmitter receptor glutamatergic synapse
Grik5	Glutamate receptor, ionotropic, kainite subunit KA2	1.74256	0.024	Neurotransmitter receptor glutamatergic synapse
Grk6	G protein-coupled receptor kinase 6	−1.5332	0.033	Regulation of dopamine receptors
Gpr84	G protein-coupled receptor 84	−3.00	0.015	Pain
Gpr171	G protein-coupled receptor 171	−1.73	0.022	Energy/metabolism
Il1b	Interleukin-1β	2.0868	0.016	Immune
Kmo	Kynurenine-3-monooxygenase	−2.18	0.050	Tryptophan metabolism
Lin 7B	Lin 7 homolog B	−3.55	0.045	Synaptic plasticity
Mmp15	Metalloprotease 15	−1.83	0.049	Blood—brain barrier
Nr4a1	Nuclear receptor Nur77	−1.58885	0.023	Dopamine & serotonin target
Pde10a	Phosphodiesterase 10A	−1.51367	0.024	Signal transduction Phospholipase A2 pathway
Pik3cg	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	−1.52318	0.031	Signal transduction AKT andPI3 kinase signaling
Plcb1	Phospholipase C β1	−1.52803	0.031	Signal transduction phospholipase C signaling
Rab33a	RAB33A member of Ras oncogene family	−3.67	0.018	Synaptic plasticity
Rapgef6	Rap guanine nucleotide exchange factor 6	−1.61	0.019	Blood—brain barrier
Rgs20	Regulator of G protein signaling	−1.65	0.029	Synaptic plasticity
Slc6a3	Dopamine transporter (DAT1)	−1.5978	0.042	Dopamine transporter
Slc6a4	Solute carrier family 6α member 4/serotonin transporter	−5.76338	0.033	Serotonin transporter
Synpo	Synptopodin	−2.25	0.036	Synaptic plasticity
Tbc1d1	TBC1 family domain member 1	−1.55	0.042	Energy/metabolism
Th	Tyrosine hydroxylase/involved in dopamine synthesis	−2.23353	0.013	Dopamine metabolism
Tjp2	Tight junction protein 2	−2.68	0.023	Blood—brain barrier
Tph2	tryptophan hydroxylase 2	−3.77918	0.010	Serotonin metabolism

Data represent mRNA expression levels relative to the control and expressed as fold-regulation.

AKT = protein kinase B; cAMP = cyclic adenosine monophosphate; dUTPase = deoxyuridine triphosphate nucleotidohydrolase; EBV = Epstein-Barr virus; GABA = γ-aminobutyric acid; GPCR = G protein-coupled receptor; IL = interleukin; PI3 = phosphoinositide 3; TBC1 = TBC1 family domain member 1.

* Mean fold-change in expression significantly different from control by ≥ 1.5, n = 3.