Role of EBV-dUTPase in Modulating Neuro-immune Dysfunction Associated with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in the undergraduate colleges of The Ohio State University

by

Julia Hasik

The Ohio State University

April 2020

Project Advisor: Dr. Maria Ariza, Department of Cancer Biology and Genetics

Abstract

Purpose: Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a debilitating chronic multisystem illness of unknown etiology characterized by profound fatigue and impaired neurocognitive, endocrine and immune functions. Currently, there are no tests or validated biomarkers for the definitive diagnosis of ME/CFS, which is based on exclusion of other medical explanations for symptoms.

While the cause is unknown, there are multiple reports in the literature suggesting a role for viruses, particularly human herpesvirus-6 (HHV-6) and Epstein-Barr virus (EBV) in ME/CFS. In support of this premise, recent studies by our group have demonstrated that 50% of patients with ME/CFS had high levels of antibodies to the deoxyuridine triphosphate nucleotidohydrolase (dUTPase) protein produced by EBV and HHV-6A/B. These results suggest that these viruses can and may contribute to the underlying pathology observed in some ME/CFS patients.

The objective of my project is to determine the mechanism(s) through which EBV-dUTPase may contribute to the neuro-immune dysfunction in ME/CFS using an *in vivo* model.

Methods: Gene expression analysis of 30 genes was conducted on brain tissue samples from wild-type and Toll-like receptor 2 (TLR2) knock-out C57Bl/6 mice injected via the intraperitoneal route for 5 days with either EBV-dUTPase protein or vehicle control. The effect of EBV dUTPase on the expression of genes with key functions in brain plasticity, pain, and fatigue as well as the involvement of TLR2 in EBV-dUTPase induced effects on gene expression was examined by qRT-PCR.

Findings: When compared to wild-type mice treated with EBV-dUTPase, TLR2-KO EBV-dUTPase treated mice showed a trend of up-regulation of the Drd1, Egr1, and Nr4a1 gene expression in the right hemisphere, and down-regulation of the Slc6a3, Slc6a4 and Tph2 gene expression in the left hemisphere, in addition to up-regulation of the Dbh gene in the left hemisphere. EBV-dUTPase also induced an up-regulation of the Gch1 in the left hemisphere and IL-1β genes in both hemispheres in the WT-EBV mice, though it appears to be TLR2 independent.

Implications: The data suggests that the EBV-dUTPase is capable of modulating the expression of genes (Dbh, Drd1, Egr1, Nr4a1, Slc6a3, Slc6a4, and Tph2) associated with early growth protein, nuclear receptor 77, and dopamine and serotonin pathways in a TLR2-dependent manner and may contribute to the cognitive impairments, neuroinflammation, and pain associated with ME/CFS.

INTRODUCTION

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a debilitating disease that involves unexplained and constant fatigue upon exertion, in addition to cognitive and immunological dysfunction (Rivera et al. 2019). Some studies suggest an estimated prevalence between 2.54% to 3.3% (Reeves et al. 2007, Johnston et al. 2013), with high variability based on factors including self-reported versus clinical assessment and geographical location of assessment. There is difficulty in diagnosing ME/CFS, as there are no clear biomarkers, so diagnosis is based upon exclusion of other conditions, which can account for the wide range in estimated prevalence. Regardless, the estimated annual cost of the disease, as reported by the Institute of Medicine in the United States, ranges from 17-24 billion USD per year with as many as 2.5 million Americans suffering from this condition (Rivera et al. 2019).

The symptoms of this complex disease were first described in 1934 as experiencing "general malaise, tender lymph nodes, sore throat, pain, and signs of inflammation of the brain and spinal cord" due to chronic infection, or encephalomyelitis. In 1955, more focus was brought to the possibility of the condition after over fifty doctors, nurses, and healthcare professionals were hospitalized with symptoms including gastrointestinal alterations, vertigo, sore throat, severe headache, muscle tenderness, and loss in function of the cranial nerves, following an upper respiratory infection. By 1959, the condition was given the name "myalgic encephalomyelitis" and described as having inflammation with severe muscular pain and damage due to the nervous system without resulting in mortality. The diagnostic criteria weren't expanded until 1980s, with a characterization of chronic muscle fatigue after minor exertion and several days needed to restore full strength. After two cases that resembled mononucleosis, the disease also became associated with the Epstein-Barr virus infection onset and was recognized by the US Center for

Disease Control and Prevention (CDC) as "chronic fatigue syndrome" (Rivera et al. 2019). In some patients, there are reports of activated Epstein-Barr virus (EBV) and human herpesvirus 6, however this is not present in most of the patient population. Even so, many patients do have a history of severe infection prior to development of their symptoms (Hickie et al. 2006, Stormorken et al. 2015).

It was not until 1994 that Fukuda proposed a clinical investigation to develop a "comprehensive and integrated approach to study ME/CFS". This resulted in a diagnosis that includes the absence of other fatigue-associated conditions, unexplained fatigue for at least six months, and at least four of eight other minor symptoms including: brain fog, sore throat, tender cervical or axillary nodes, muscle pain, multipoint pain without joint swelling, headaches, unrefreshing sleep, and malaise after exertion for more than 24 hours, as well as official recognition of Chronic Fatigue Syndrome in the medical classification of diseases for the US (Fukuda et al. 1994, Rivera et al. 2019).

Even though a clinical diagnosis was determined, there was still some doubt about the validity of the syndrome due to the lack of biomarkers or diagnostic criteria that doesn’t include exclusion of other disease components. However, evidence has shown that patients with ME/CFS show abnormalities in neuroendocrine, autonomic, neurological, bioenergetic, and immunological functions. Some groups have proposed that activation of inflammatory, immune, oxidative, and nitrosative (IO&NS) pathways due to a more permeable intestinal tract as a result of a pathogenic invasion, causes systemic inflammation, activating TLR2 and TLR4 receptors on antigen-producing cells, leading to a dysregulated immune system and exacerbated systemic and

neuroinflammation. This mechanism is believed by some to be a potential cause of the symptoms associated with ME/CFS (Maes and Twisk 2010, Brenu et al. 2011).

Our group, however, suggests an alternate pathway involving an Epstein-Barr virus protein, deoxyuridine triphosphate nucleotiodohydrolase (EBV dUTPase). A study in 2012 showed that a significant portion (44.2%) of ME/CFS patients have prolonged elevated antibody levels against the EBV dUTPase and DNA polymerase proteins (Lerner et al. 2012). Several studies in human primary immune cells demonstrated that the EBV dUTPase protein strongly induced the activation of NF-κB leading to the increased secretion of TNF-α, IL-1β, IL-6, and IL-10 cytokines through a TLR2-dependent mechanism. This response is believed to be mediated by the increased production of EBV dUTPase and its release in exosomes in response to EBV infection, which then activate the TLR2 signaling cascade through exosomes containing EBV-dUTPase (Ariza et al. 2013). Additionally, it has been shown that exosomes are able to cross the blood-brain barrier (BBB) under conditions of inflammation and TNF-α activation, as present in the previously described signaling cascade, through endocytosis by brain microvascular endothelial cells (BMECs) (Chen et al. 2017).

Further evidence for this mechanism was provided by Williams et al., in a study on EBV-dUTPase induction of neuroinflammatory mediators, which suggested that abortive-lytic reactivation of a latent EBV infection, resulting in the production of dUTPase protein, could modulate the BBB, microglia, and astrocyte gene expression, among other pathways, including those involving glutamate-GABA and serotonin/dopamine, to contribute towards neuroinflammation that may be the cause of increased pain, fatigue, and cognitive dysfunction observed in some patients with ME/CFS. The present project aims to further explore the

mechanism(s) by which EBV-dUTPase contributes to the neuro-immune dysfunction associated with ME/CFS, via the engagement of the TLR2 receptor.

METHODS

Reagents

Trizol reagent was purchased from Ambion. TaqMan Gene Expression Master Mix was purchased from Applied Biosystems. RNeasy Mini Kit and RNase-Free DNase Set 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. Superscript IV First-Strand Synthesis Kit was purchased from Invitrogen.

Purification of Recombinant EBV dUTPase

Subcloning and purification of recombinant dUTPase protein was performed as described in previous work (Ariza et. al 2014). Recombinant dUTPase protein preparations were tested for the presence of contaminants as described previously (Glaser, Litsky, Padgett et. al 2006) and were free of detectable levels of lipopolysaccharide, peptidoglycan (SLP-HS), DNA, and RNA. Protein concentration was determined with the Qubit fluorimeter (Invitrogen). The protein extraction and purification were performed by other members of the lab prior to the start of these experiments. The vehicle used was the protein elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 M imidazole; pH 7.4).

Mice

Mice Female wild-type C57BL/6 mice (6-8 weeks of age) and TLR2 Knockout C57BL/6 mice (6-8 weeks of age) were purchased from The Jackson Laboratory and housed for 7 days for

acclimatization before use. The wild-type mice (n = 10, 5 mice per treatment group) and TLR2 knockout mice (n=10, 5 per treatment group) were then injected daily with EBV dUTPase protein (10 μg/mL) or vehicle control for 5 days via the intraperitoneal route. This dose was selected based on previous studies. (Williams et. al 2019). Mice were then deeply anesthetized with a ketamine/xylazine mixture (ketamine 90 mg/kg and xylazine 8 mg/kg; in 200 μL) administered by intraperitoneal injection. Mice were observed for response to hind paw pinch, and, when no response was observed, surgery was performed to perfuse the brain. After perfusion surgery, mice were euthanized by decapitation, and the brain was removed. Brains were stored in RNALater solution for further analysis. All experiments were conducted in accordance with the Institutional Animal Care and Use Guidelines of The Ohio State University.

Quantitative RT-PCR

Brain tissue samples from the left and right hemispheres, representing approximately one-fifth of each hemisphere's mass, were homogenized with a Bead Ruptor 12 (Omni International, Inc) in 7-mL screw cap tubes containing DNase/RNase-free 1.4-mm ceramic beads at high speed for 30 s, followed by a centrifugation step at 12,000×g for 10 min at 4°C, according to the manufacture's protocol. Total RNA was then extracted using Trizol reagent, and further cleaned up using RNeasy Mini Kit and on-column DNase I treatment. RNA concentration and purity were determined with the NanoDrop 2000. cDNA was synthesized with the SuperScript IV First-Strand Synthesis Kit, and qRT-PCR was performed on a QuantStudio 6 Flex instrument (Applied Biosystems), using TaqMan chemistry and the following primer/probe target sets: CD33-Mm00491152_m1, Dbh-Mm00460472_m1, Drd1-Mm02620146_s1, Drd5-Mm04210376_s1, Egr1-Mm00656724_m1, Gabrd-Mm01266203_g1, Gch1-Mm01322973_m, Grik4-Mm00615472_m1, Grik5-Mm00433774_m1, Grk6-Mm00442425_m1, Gpr84-

Mm02620530_s1, Gpr171-Mm02620738_s1, Il1b-Mm00434228_m1, Kmo-Mm01321343_m1, Lin7B-Mm00457059_m1, Mmp15-Mm00485062_m1, Nr4a-Mm01300401_m1, Pik3cg-Mm00445038_m1, Rab33a-Mm00487874_m1, Rapgef6-Mm01288550_m1, Rgs20-Mm01228161_m1, Slc6a3-Mm00438388_m1, Slc6a4-Mm00439391_m1, Synpo-Mm03413333_m1, Tbc1d1-Mm00497989_m1, Th-Mm00447557_m1, Tjp2-Mm00495620_m1, Tph2-Mm00557715_m1, Trem 2-Mm04209422_m1. B2m-Mm00437762_m1. All reactions were run in a final volume of 20 μL, and PCR values were normalized to the internal standards (B2m). Fold regulation is expressed as the mRNA expression levels relative to the control group for each brain hemisphere. The fold-change/fold-regulation of the expression for each target gene was calculated with the threshold cycle (Ct) values as follows: fold-change ($2^{-\Delta\Delta Ct}$) is the normalized gene expression ($2^{-\Delta Ct}$) in the test sample divided by the normalized gene expression ($2^{-\Delta Ct}$) in the control sample. Fold-regulation represents fold-change results in a biologically meaningful way. Fold-change values > 1 indicate a positive- or an upregulation, and the fold-regulation is equal to the fold-change. Fold-change values < 1 indicate a negative or downregulation, 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 ($2^{-\Delta Ct}$) values for each gene in the control group. $P < 0.05$ values were reported as significant.

RESULTS

Previous research focused on the effects of EBV dUTPase on gene expression in female C57BL/6 mice brains identified 33 genes that showed a difference in expression between control groups and those injected with EBV dUTPase (Williams et al. 2019). The present study expands on these findings and explores the involvement of TLR2 as a potential mechanism by which the EBV dUTPase may be mediating its effects on gene expression in the mouse brain.

The present study found that EBV dUTPase induced differential mRNA expression in 10 of the 29 genes examined in the brain of wild-type mice compared to the control, including *Dbh, Drd1, Egr1, Gch1, Ilb1, Nr4a1, Slc6a3, Slc6a4, Th,* and *Tph2,* though not all values were statistically significant, potentially due to the low power of the study with only five animals per treatment group. As shown in Table 1, the addition of dUTPase suggested a trend of upregulation (1.62-fold) in the expression of *Gch1* (GTP cyclohydrolase), which is involved with pain, in the left hemisphere, although it was not statistically significant, and no significant fold change was seen compared to the EBV dUTPase-treated knockout mice (see Fig. 2 and 4). *Ilb1* (Interleukin-1), which is involved with the immune response, showed a trend of upregulation of 1.55-fold in the right hemisphere and 1.99-fold in the left hemisphere (see Table 1, Fig. 1 and 2) with the addition of EBV dUTPase in the wild-type, though these values were not statistically significant and there was not a change in expression compared to the EBV dUTPase-treated knockout mice (see Fig. 3 and 4).

The wild-type mice treated with EBV dUTPase also showed a change in the expression of genes involved with dopamine metabolism/utilization, including: an upregulation of *Dbh* (Dopamine β-hydroxylase enzyme), which is involved in dopamine metabolism, in the right (6.04-fold) and left (1.87-fold) hemispheres with only the right hemisphere being statistically significant (see Table 1, Fig. 1 and 2), a suggested trend in downregulation (-2.11-fold) of *Drd1* (Dopamine receptor 1) in the right hemisphere (see Table 1, Fig. 1), a trend of upregulation (1.62-fold) of *Th* (Tyrosine hydroxylase/dopamine synthesis), which is also involved with dopamine metabolism, in the right hemisphere (see Table 1, Fig. 1), and a trend upregulation of *Slc6a3* (Dopamine transporter 1) in both hemispheres (see Table 1) compared to the vehicle control wild-type mice, however, neither hemisphere expressed a statistical significance. compared to the vehicle control

wild-type mice. In the left hemisphere, the upregulation of Slc6a3 (7.78-fold) is significant (see Table 1), however, the lack of statistical significance may be due to the presence of only three data points within that group.

In addition, dUTPase also upregulated the expression of genes associated with serotonin metabolism/utilization including: *Slc6a4* (Solute carrier family 6α member 4/serotonin transporter) in the right (10.9-fold) and left (33.14-fold) hemispheres (see Table 1, Fig. 1 and 2), and *Tph2* (Tryptophan hydroxylase 2), which is involved in serotonin metabolism, in both the right (7.97-fold) and left (19.69-fold) hemispheres compared to the vehicle control with the left hemisphere being statistically significant for both genes (see Table 1, Fig. 1 and 2). The lack of statistical significance in the right hemisphere, even though significant fold-changes were present, may be due to the presence of outliers that skewed increased up-regulation, however, the removal of those outliers from the data set still maintains a fold-change greater than 1.5-fold. Futhermore, EBV dUTPase showed a trend of downregulation the mRNA expression in the right (-1.51-fold) and left (-2.0-fold) hemispheres of *Egr1* (Early Growth Response 1), which is associated with synaptic plasticity and pain, and in the right (-1.51-fold) and left (-2.04-fold) hemispheres of *Nr4a1* (Nuclear receptor 77), which is involved with the immune response as well as regulation of different components associated with dopamine synthesis and transmission (see Table 1, Fig. 1 and 2). However, only the upregulation of *Nr4a1* in the left hemisphere showed statistical significance (see Table 1). These results suggested that EBV dUTPase primarily targeted genes involved with serotonin and dopamine biosynthesis and transport, as well as altered the expression of genes associated with the immune response, synaptic plasticity, and pain.

As shown in Figures 3 and 4, which normalizes the EBV dUTPase-treated wild-type and knockout groups to their respective controls (vehicle treated wild-type and vehicle treated TLR2 knockout) then compares the fold-regulation between groups, TLR2 mediated the change in expression of 7 (*Dbh*, *Drd1*, *Egr1*, *Nr4a1*, *Slc6a3*, *Slc6a4*, and *Tph2*) of these 10 genes. In the EBV dUTPase-treated TLR2 knockout mice, there is an statistically significant upregulation of mRNA expression (4.66-fold) in the left hemisphere of *Dbh* (Dopamine β-hydroxylase enzyme) compared to the vehicle control (see Table 2, Fig. 4), which is a further upregulation (3.36-fold) from the mRNA expression of the EBV dUTPase-treated wild-type (see Table 3, Fig. 4), suggesting TLR2 is acting as a negative-mediator of gene expression. In the dopamine system, there is also a statistically significant upregulation (1.99-fold) of *Drd1* (Dopamine receptor 1) compared to the EBV dUTPase-treated wild-type (see Table 3, Fig. 3) in the right hemisphere to levels comparable to the vehicle control (see Table 2, Fig. 3). A similar trend is seen in the right hemisphere for *Egr1* and *Nr4a1* in which there is an upregulation (2.01-fold) of *Egr1* and an statistically significant upregulation (1.99) of *Nr4a1* in the EBV dUTPase-treated knockout mice compared to the EBV dUTPase-treated wild-type (see Table 3, Fig. 3) returning to levels that are comparable to the vehicle control mRNA expression values for both genes (see Table 2).

In the left hemisphere, as shown in Figure 4 and Table 2, there is trend of downregulation in mRNA expression of *Slc6a3*, which is statistically significant, *Slc6a4*, and *Tph2* for the EBV dUTPase-treated knockout group compared to the vehicle control, with these values representing a down-regulation from the EBV dUTPase-treated wild-type group for all three genes (see Table 3, Fig. 4) These results indicate that EBV dUTPase, through a TLR2 dependent mechanism, may induce an upregulation of Egr1 and Nr4a1, upregulation in genes associated with serotonin metabolism and transport (Slc6a3 and Tph2), as well as a dysregulation in the dopamine system.

Table 1: Gene Expression Analysis of WT EBV-treated compared to WT-VHC

Gene Symbol	Gene Name	RH Fold Regulation	*P*	LH Fold Regulation	*P*	Function
Cd33	Sialic Acid Binding Ig-like Lectin 3	-1.2	0.088	-1.16	0.276	Cascade inhibition of phagocytes
Dbh	Dopamine β-hydroxylase (DβH) enzyme	6.04*	0.020	1.87	0.458	Dopamine metabolism
Drd1	Dopamine receptor D1	-2.11	0.069	-1.17	0.781	Dopamine receptor
Drd5	Dopamine receptor D5	-1.12	0.514	-1.15	0.418	Dopamine receptor
Egr1	Early growth response 1	-1.51	0.240	-2.0	0.079	Synaptic plasticity; pain
Gabrd	$GABA_A$ receptor subunit δ	-1.02	0.784	1.11	0.481	Neurotransmitter receptor: GABAergic synapse
Gch1	GTP cyclohydrase	1.36	0.230	1.62	0.099	Pain; dopamine biosynthesis
Grik4	Glutamate receptor, ionotropic, kainite subunit KA1	-1.29	0.077	1.1	0.354	Neurotransmitter receptor glutamatergic synapse
Grik5	Glutamate receptor, ionotropic, kainite subunit KA2	-1.45	0.002	-1.27	0.080	Neurotransmitter receptor glutamatergic synapse
Grk6	G protein-coupled receptor kinase 6	-1.34	0.017	-1.2	0.032	Regulation of dopamine receptors
Gpr84	G protein-coupled receptor 84	-1.04	0.687	1.1	0.134	Pain
Gpr171	G protein-coupled receptor 171	-1.03	0.941	1.23	0.271	Energy/metabolism
Il1b	Interleukin-1	1.55	0.175	1.99	0.074	Immune
Kmo	Kynurenine-3-monooxygenase	1.44	0.314	1.29	0.851	Tryptophan metabolism
Lin7b	Lin 7 homolog B	-1.46	0.018	-1.31	0.126	Synaptic plasticity
Mmp15	Metalloprotease 15	1.07	0.802	1.14	0.548	Blood-brain barrier
Nr4a1	Nuclear receptor Nur77	-1.51	0.414	-2.04*	0.048	Immune Response; dopamine target
Pik3cg	Phosphotidylinositol-4,5-biphosphate 3-kinase catalytic subunit gamma	-1.27	0.003	-1	0.905	Signal transduction AKT andPI3 kinase signaling

Rab33a	RAB33A member of Ras oncogene family	-1.24	0.071	-1.16	0.159	Synaptic plasticity
Rapgef6	Rap guanine nucleotide exchange factor 6	-1.05	0.736	1.18	0.392	Blood-brain barrier
Rgs20	Regulator of G protein signaling	-1.49	0.004	-1.21	0.210	Synaptic plasticity
Slc6a3	Dopamine transporter (DAT1)	1.65	0.880	7.78	0.337	Dopamine transporter
Slc6a4	Solute carrier family 6α member 4/serotonin transporter	10.9	0.067	33.14*	0.021	Serotonin transporter
Synpo	Synptopodin	-1.06	0.002	-1	0.924	Synaptic plasticity
Tbc1d1	TBC1 family domain member 1	-1.22	0.139	1	0.989	Energy/metabolism
Th	Tyrosine hydroxylase/dopamine synthesis	1.62	0.391	1.44	0.738	Dopamine metabolism
Tjp2	Tight junction protein 2	-1.07	0.662	1.22	0.197	Blood-brain barrier
Tph2	Tryptophan hydroxylase 2	7.97	0.071	19.69*	0.024	Serotonin metabolism
Trem2	Triggering receptor expressed on myeloid cells 2	-1.33	0.025	-1.21	0.169	Prime myeloid cell response

*Mean fold-change in expression significantly different from control by ≥ 1.5, n = 5

Table 2: Gene Expression Analysis of KO EBV-treated compared to KO VHC

Gene Symbol	Gene Name	RH Fold Regulation	P	LH Fold Regulation	P	Function
Cd33	Sialic Acid Binding Ig-like Lectin 3	1.00	0.814	-1.10	0.623	Cascade inhibition of phagocytes
Dbh	Dopamine β-hydroxylase (DβH) enzyme	2.16	0.122	4.66*	0.004	Dopamine metabolism
Drd1	Dopamine receptor D1	1.60	0.367	-1.38	0.862	Dopamine receptor
Drd5	Dopamine receptor D5	1.08	0.486	-1.05	0.520	Dopamine receptor
Egr1	Early growth response 1	1.04	0.417	-2.22*	0.001	Synaptic plasticity; pain
Gabrd	$GABA_A$ receptor subunit δ	1.52*	0.011	1.43	0.002	Neurotransmitter receptor: GABAergic synapse
Gch1	GTP cyclohydrase	1.05	0.853	-1.25	0.299	Pain; dopamine biosynthesis

Grik4	Glutamate receptor, ionotropic, kainite subunit KA1	1.13	0.049	1.06	0.247	Neurotransmitter receptor glutamatergic synapse
Grik5	Glutamate receptor, ionotropic, kainite subunit KA2	1.07	0.199	-1.12	0.138	Neurotransmitter receptor glutamatergic synapse
Grk6	G protein-coupled receptor kinase 6	1.06	0.196	-1.17	0.002	Regulation of dopamine receptors
Gpr84	G protein-coupled receptor 84	1.14	0.058	-1.01	0.950	Pain
Gpr171	G protein-coupled receptor 171	-1.00	0.206	1.64*	0.011	Energy/metabolism
Il1b	Interleukin-1	1.66*	0.001	1.37	0.001	Immune
Kmo	Kynurenine-3-monooxygenase	1.32	0.021	-1.07	0.662	Tryptophan metabolism
Lin7b	Lin 7 homolog B	1.01	0.634	-1.24	0.007	Synaptic plasticity
Mmp15	Metalloprotease 15	-1.01	0.899	-1.30	0.001	Blood-brain barrier
Nr4a1	Nuclear receptor Nur77	1.35	0.131	-1.94*	0.001	Immune Response; Dopamine target
Pik3cg	Phosphotidylinositol-4,5-biphosphate 3-kinase catalytic subunit gamma	1.04	0.482	1.06	0.347	Signal transduction AKT andPI3 kinase signaling
Rab33a	RAB33A member of Ras oncogene family	1.17	0.027	-1.13	0.009	Synaptic plasticity
Rapgef6	Rap guanine nucleotide exchange factor 6	-1.04	0.337	1.39	0.007	Blood-brain barrier
Rgs20	Regulator of G protein signaling	1.01	0.828	-1.25	0.001	Synaptic plasticity
Slc6a3	Dopamine transporter (DAT1)	2.34	0.089	-2.40*	0.031	Dopamine transporter
Slc6a4	Solute carrier family 6α member 4/serotonin transporter	-1.11	0.558	-1.89	0.766	Serotonin transporter
Synpo	Synptopodin	-1.13	0.234	-1.22	0.132	Synaptic plasticity
Tbc1d1	TBC1 family domain member 1	1.14	0.030	1.18	0.018	Energy/metabolism
Th	Tyrosine hydroxylase/dopamine synthesis	1.18	0.669	-2.15*	0.001	Dopamine metabolism
Tjp2	Tight junction protein 2	1.13	0.028	1.19	0.029	Blood-brain barrier
Tph2	Tryptophan hydroxylase 2	-1.29	0.911	-1.69	0.886	Serotonin metabolism

Trem2	Triggering receptor expressed on myeloid cells 2	1.12	0.120	-1.13	0.124	Prime myeloid cell response

*Mean fold-change in expression significantly different from control by ≥ 1.5, n = 5

Table 3: Gene Expression Analysis of KO EBV-treated compared to WT EBV-treated

Gene Symbol	Gene Name	RH Fold Regulation	P	LH Fold Regulation	P	Function
Cd33	Sialic Acid Binding Ig-like Lectin 3	1.64*	0.001	1.42	0.032	Cascade inhibition of phagocytes
Dbh	Dopamine β-hydroxylase (DβH) enzyme	1.19	0.332	3.36	0.178	Dopamine metabolism
Drd1	Dopamine receptor D1	1.99*	0.039	-1.23	0.948	Dopamine receptor
Drd5	Dopamine receptor D5	1.26	0.100	-1.1	0.285	Dopamine receptor
Egr1	Early growth response 1	2.01	0.051	1.57	0.136	Synaptic plasticity; pain
Gabrd	$GABA_A$ receptor subunit δ	1.12	0.513	-1.0	0.893	Neurotransmitter receptor: GABAergic synapse
Gch1	GTP cyclohydrase	1.07	0.714	-1.17	0.705	Pain; dopamine biosynthesis
Grik4	Glutamate receptor, ionotropic, kainite subunit KA1	1.44	0.017	1.08	0.272	Neurotransmitter receptor glutamatergic synapse
Grik5	Glutamate receptor, ionotropic, kainite subunit KA2	1.38	0.055	1	0.975	Neurotransmitter receptor glutamatergic synapse
Grk6	G protein-coupled receptor kinase 6	1.24	0.092	-1.04	0.603	Regulation of dopamine receptors
Gpr84	G protein-coupled receptor 84	1.36	0.051	1.07	0.439	Pain
Gpr171	G protein-coupled receptor 171	-1.1	0.796	1.14	0.484	Energy/metabolism
Il1b	Interleukin-1	1.05	0.995	-1.35	0.012	Immune
Kmo	Kynurenine-3-monooxygenase	1.12	0.295	-1.23	0.234	Tryptophan metabolism
Lin7b	Lin 7 homolog B	1.17	0.253	-1.3	0.026	Synaptic plasticity
Mmp15	Metalloprotease 15	1.16	0.232	-1.08	0.435	Blood-brain barrier

Nr4a1	Nuclear receptor Nur77	1.99*	0.034	1.55	0.199	Immune Response; Dopamine target
Pik3cg	Phosphotidylinositol-4,5-biphosphate 3-kinase catalytic subunit gamma	1.38	0.001	1.15	0.180	Signal transduction AKT andPI3 kinase signaling
Rab33a	RAB33A member of Ras oncogene family	1.34	0.057	1.01	0.831	Synaptic plasticity
Rapgef6	Rap guanine nucleotide exchange factor 6	-1.13	0.877	1.06	0.602	Blood-brain barrier
Rgs20	Regulator of G protein signaling	1.51*	0.001	1.1	0.384	Synaptic plasticity
Slc6a3	Dopamine transporter (DAT1)	1.57	0.724	-3.08*	0.015	Dopamine transporter
Slc6a4	Solute carrier family 6α member 4/serotonin transporter	-1.22	0.905	-2.43	0.760	Serotonin transporter
Synpo	Synptopodin	-1.59*	0.002	-1.79*	0.0004	Synaptic plasticity
Tbc1d1	TBC1 family domain member 1	1.23	0.128	1.04	0.636	Energy/metabolism
Th	Tyrosine hydroxylase/dopamine synthesis	-1.42	0.054	-1.78*	0.0004	Dopamine metabolism
Tjp2	Tight junction protein 2	1.18	0.184	-1.03	0.805	Blood-brain barrier
Tph2	Tryptophan hydroxylase 2	-2.06	0.219	-3.13	0.260	Serotonin metabolism
Trem2	Triggering receptor expressed on myeloid cells 2	1.38	0.083	-1.05	0.632	Prime myeloid cell response

*Mean fold-change in expression significantly different from control by ≥ 1.5, n = 5

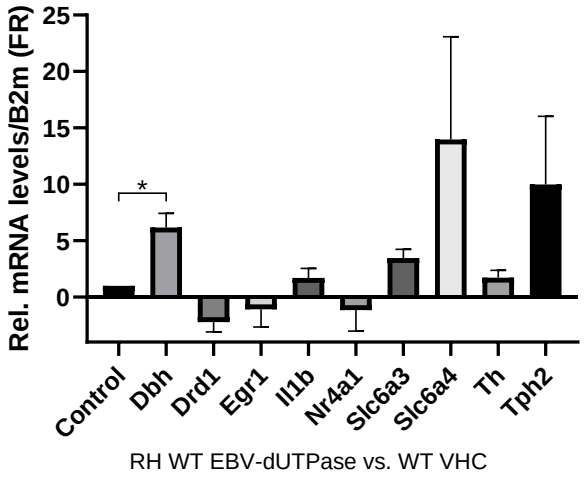

Figure 1: This figure represents the EBV dUTPase-treated wild-type mRNA levels, with at least a 1.5 mean-fold change, relative to the wild-type vehicle control in the **right hemisphere** expressed as fold regulation. An (*) denotes a statistically significant difference with P<0.05.

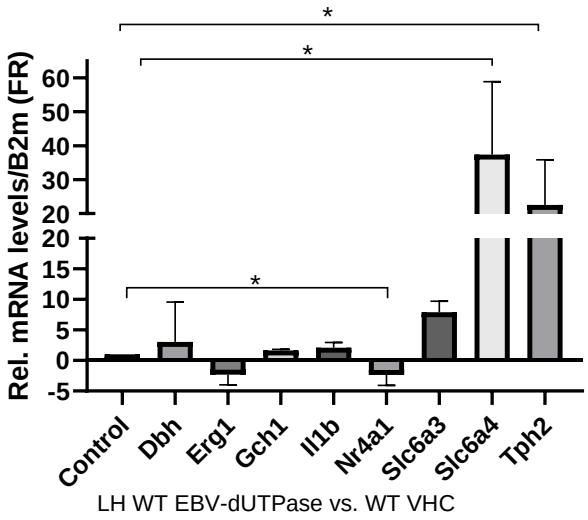

Figure 2: This figure represents the EBV dUTPase-treated wild-type mRNA levels with at least a 1.5 mean-fold change relative to the wild-type vehicle control in the **left hemisphere** expressed as fold regulation. An (*) denotes a statistically significant difference with a P<0.05.

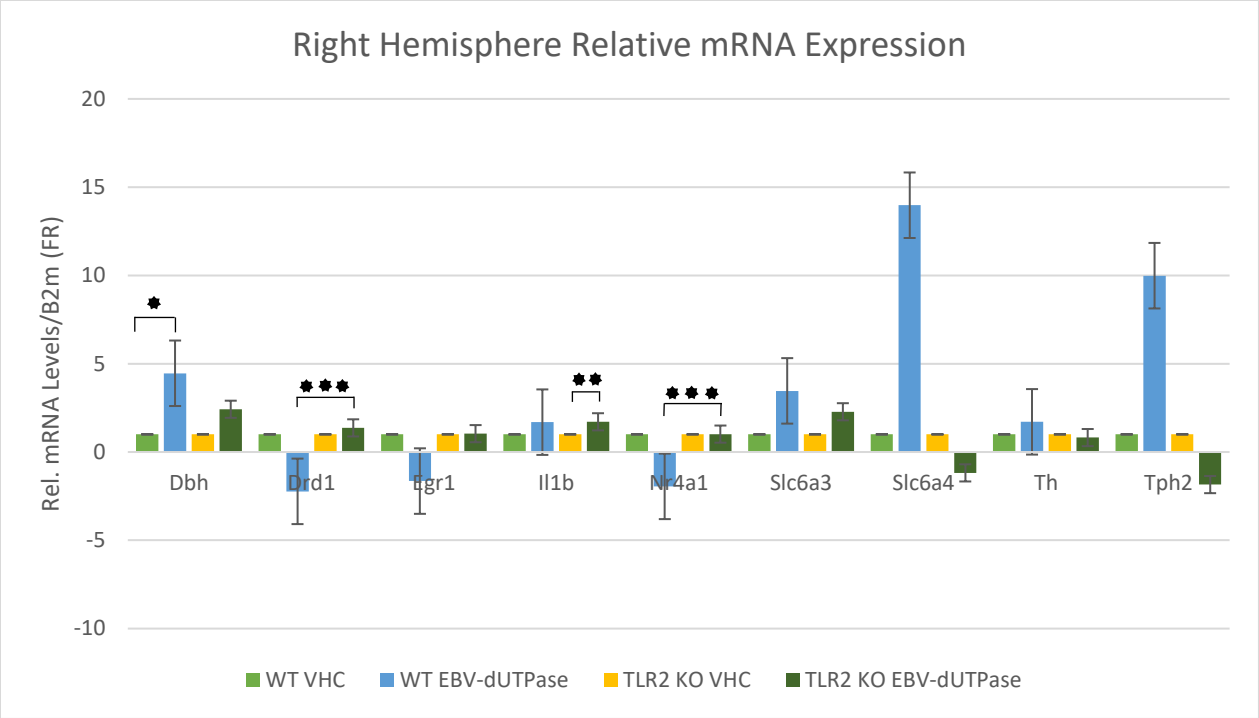

Figure 3: This graph represents a comparison between EBV dUTPase-treated wild-type mRNA levels compared to its control group (vehicle treated wild-type) and EBV dUTPase-treated TLR2 knockout mRNA levels compared to its control group (vehicle treated TLR2 knockout) in the **right hemisphere**. Genes included are those with at least a 1.5 mean-fold change for the EBV dUTPase-treated wild-type group relative to the vehicle treated wild-type group with mRNA expression changes represented as fold regulation. A (✹) denotes a statistically significant difference with P<0.05, with one (✹) representing a significant difference between WT EBV dUTPase-treated and WT VHC, two (✹✹) representing a significant difference between TLR2 KO EBV dUTPase-treated and TLR2 KO VHC, and three (✹✹✹) representing a significant difference between TLR2 KO EBV dUTPase-treated and WT EBV dUTPase-treated

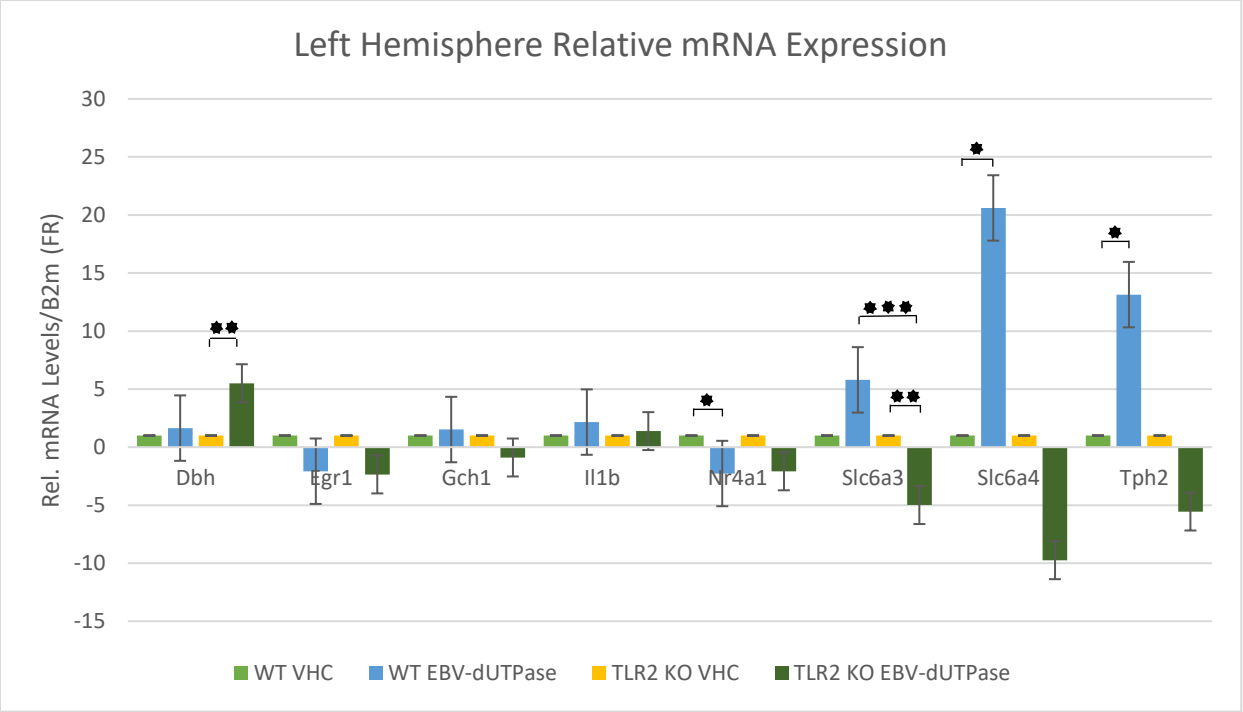

Figure 4: This graph represents a comparison between EBV dUTPase-treated wild-type mRNA levels compared to its control group (vehicle treated wild-type) and EBV dUTPase-treated TLR2 knockout mRNA levels compared to its control group (vehicle treated TLR2 knockout) in the **left hemisphere**. Genes included are those with at least a 1.5 mean-fold change for the EBV dUTPase-treated wild-type group relative to the vehicle treated wild-type group with mRNA expression changes represented as fold regulation. A (✹) denotes a statistically significant difference with $P<0.05$, with one (✹) representing a significant difference between WT EBV dUTPase-treated and WT VHC, two (✹✹) representing a significant difference between TLR2 KO EBV dUTPase-treated and TLR2 KO VHC, and three (✹✹✹) representing a significant difference between TLR2 KO EBV dUTPase-treated and WT EBV dUTPase-treated.

DISCUSSION

Our group has previously established that a significant percentage of patients with ME/CFS show an elevation in antibodies against the EBV dUTPase protein (Lerner et al. 2012; Halprin et al). The most recent study from our group, has provided further evidence to support the previously described mechanism in which reactivation of EBV results in the production of EBV dUTPase, which may contribute to peripheral and neural inflammation. This is able to occur through the induction of mRNA expression changes in genes that are associated with modulating the blood-brain barrier, dopamine and serotonin metabolism, pain, and synaptic plasticity which may contribute to many symptoms associated with ME/CFS including pain, fatigue, and cognitive impairments (Williams et al. 2019). The current study provides further evidence that EBV dUTPase may induce neuroinflammatory responses, among other changes in the brain, through a TLR2 dependent mechanism.

In the initial study, EBV dUTPase was shown to induce an upregulation in mRNA expression for *Gch1* (Williams et al. 2019), and the present study further suggests a similar trend in the left hemisphere, however, there was no evidence this occurs in a TLR2-dependent mechanism. This gene has been shown to modulate pain-like hypersensitivity, with decreases in expression being suggestive of reduced pain-like responses, leading to the conclusion that an increase in expression may induce an increase in pain-like responses, or potentially hyperalgesia (Nasser A, Bjerrum OJ, Heegaard AM et al. 2013). This may potentially be associated with the symptoms of ME/CFS, including muscle pain, multipoint pain without joint swelling, and malaise after exertion.

In addition, the current study showed the same pattern of upregulation in the mRNA expression for *Ilb1* (Interleukin-1) in both hemispheres, as in the previous study (Williams et al. 2019). This

cytokine has been known to play a role in disrupting blood-brain barrier (BBB) integrity (Miner JJ and Diamond MS 2016), which could contribute to neuroinflammation, among other immune responses, as well as allow EBV dUTPase to cross the BBB.

While the expression changes by EBV dUTPase in both *Ilb1* and *Gch1* have been suggested to be consistent across both studies, the present study provides preliminary data suggesting that this mechanism may occur in a TLR2-independent manner, as there is no significant change in mRNA fold regulation between the TLR2 knockout mice and the wild-type mice in the EBV dUTPase treated groups. More so, many results from the current study provide contradictory data compared to the results that were seen in the previous study (Williams et al. 2019), even though some of the results do support these previous findings. This may be due to the slices from brain samples containing a larger percentage of certain areas of the cortex as compared to others, given that changes in expression may have localized effects. Another possibility is that there is not enough power, since only an n = 5 animals were used in these preliminary studies, and a larger number of animals is needed.

Regarding the mRNA expression of *Egr1* (Early Growth Response 1), the present study found an approximate negative two-fold decrease in mRNA expression in the EBV dUTPase treated wild-type mice in the right hemisphere, as compared to the control (see Table 1). Then, in the TLR2 knockout mice treated with EBV dUTPase, there was evidence of an approximate two-fold increase in mRNA expression in comparison to the EBV WT mice (see Table 3), showing a return to levels near the control (see Table 2). This suggests that EBV dUTPase modulates Egr1 expression in a TLR2 dependent manner. The protein produced by Egr1, early growth response 1 protein, has been associated with depressive and anxiety-like symptoms in both mice and humans. In postmortem tissue of patients that are suffering from major depressive disorder, both

unmedicated and unresponsive to treatment, especially when stress is involved, there are decreased levels of Egr1 protein in the prefrontal cortex. It appears to rebound in the presence of effective medication, making it an ideal marker for positive response to antidepressant treatment. This same pattern is also observed in mice (Duclot and Mohamed 2017). The decrease in Egr1 protein, if seen specifically in the prefrontal cortex, may then contribute to the depressive and anxiety-like symptoms that many patients with ME/CFS experience.

This study found a significant upregulation in the mRNA expression of genes associated with serotonin in the EBV dUTPase treated WT mice, as compared to vehicle control, including *Slc6a4* (SERT) and *Tph2* (Tryptophan hydroxylase 2), which are associated with serotonin metabolism and transport (Table 1). This response is then attenuated with a significant downregulation in both genes in the TLR2 KO mice, as compared to the EBV dUTPase treated WT mice (Table 3), suggesting that this response to EBV dUTPase is TLR2 dependent. Within the rostral ventromedial medulla (RVM) in the brainstem, an increase in serotonin can be associated with sensitization of the transient receptor potential cation channel (TRPV1), or capsacin receptor, leading to hyperalgesia in the periphery, which is a state in which painful stimuli are perceived as more painful than normal (Kim et al., 2014). While chronic inflammation or upregulation of Gch1 could be the culprit behind the muscle pain, general malaise, and multipoint pain without joint swelling, given that patients with ME/CFS present with inflammatory markers including CCL11, CXCL10, IL-7, TNF-β-1, and TNF-α that can be associated with impaired cognitive processing and musculoskeletal pain (Josjö 2019), an increase in production of 5-HT as well as SERTs through an EBV dUTPase, TLR-2 mediated mechanism may contribute to a state of hyperalgesia, further exacerbating symptoms.

Additionally, the mechanism by which calcitonin gene-related peptide (CGRP), which is released around the brain, can cause intense inflammation of the meninges leading to migraines and headaches, involves an increase in serotonin binding to 5-HT3 receptors via perivascular nociceptive fibers that activate the trigeminal nociceptors, leading to intracranial blood vessels and meninges (Guerrero and Timonina et al., 2016). Given that a common symptom of ME/CFS is headaches or migraines, this may present a possible explanation for the occurrence.

Finally, the current study shows that the downregulation in the mRNA expression of Nr4a1 (Nuclear receptor 77) in brains of EBV dUTPase-treated WT mice, as compared to the controls (Table 1) was abrogated in TLR2 KO mice injected with EBV dUTPase to levels comparable to the vehicle control (Table 2). This suggests that EBV dUTPase is capable of modulating the expression of Nr4a1 through a TLR2-dependent mechanism. A decrease in Nr4a1 has been implicated in several neuroprotective measures including: increased dendritic cell function and T cell proliferation, NF-κB dependent inflammatory response, and subsequent induction of inflammatory cytokines, impaired immune regulation, and decreased apoptosis protection. All these changes would lead to an increase in neuroinflammation in the brain, as well as to several of the affects previously described, as well as potential destruction of neurons (Herring, Elison, and Tessem 2019). Depending upon the location of neuronal cell death, this could lead to cognitive dysfunctions seen in ME/CFS patients.

Nr4a1 has also been associated with neurotoxin-induced dopamine cell loss and is considered a regulator of the dopamine receptor 1 (Herruing, Elison, and Tessem 2019). Therefore, a decrease in Nr4a1 could lead to an increase in dopamine, as there would be less neurotoxin-induced cell loss, but a decrease in dopamine receptor 1, as the two are positively correlated. This may be a potential explanation for the dysregulation in the dopamine system that is seen both in the EBV-

dUTPase treated WT mice compared to controls, as well the irregularities that are seen when the EBV dUTPase-treated TLR2 KO mice are compared to the EBV dUTPase-treated WT group.

CONCLUSIONS

The present study provides preliminary data supporting a mechanism by which EBV dUTPase can modulate the expression of Egr1, Nr4a1, Slc6a4, Tph2, Scl6a3, Dbh, and Drd1 genes in the mouse brain in a TLR2-dependent manner and account for symptoms of hyperalgesia, headaches and depressive-like symptoms, all of which are common features associated with ME/CFS. While these are interesting preliminary results, further studies would be needed to confirm that the observed changes in mRNA expression induced by EBV dUTPase in the brain also translate into changes at the protein level. It would also be important to identify the specific areas of the brain where the dUTPase protein may localize and exert its effects.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Health (R01 A1084898) to Marshall V. Williams and Maria Eugenia Ariza.

REFERENCES

Ariza ME, Rivailler P, Glaser R, Chen M, Williams MV (2013) Epstein-Barr Virus Encoded dUTPase Containing Exosomes Modulate Innate and Adaptive Immune Responses in Human Dendritic Cells and Peripheral Blood Mononuclear Cells. PLoS ONE 8(7). https://doi.org/10.1371/journal.pone.0069827

Ariza, ME, Glaser, R, and Williams M.V. (2014) Human Herpesviruses-encoded dUTPases: A Family of Proteins that Modulate Dendritic Cell Function and Innate Immunity. Front Microbiol 5:504. https://doi.org/10.3389/fmicb.2014.00504

Brenu, Ekua et al. (2011) Immunological abnormalities as potential biomarkers in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. J Translational Medicine 9:81. https://doi.org/10.1186/1479-5876-9-81

Chen, C et al. (2017) Elucidation of Exosome Migration across the Blood-Brain Barrier Model in Vitro. Cell Mol Bioeng 9:509-529. https://doi.org/10.1007/s12195-016-0458-3

Duclot F and Kabbaj M (2017) The Role of Early Growth Response 1 (EGR1) in Brain Plasticity and Neuropsychiatric Disorders. Front Behav Neurosci 11:35. https://doi.org10.3389/fnbeh.2017.00035

Fukuda, Keiji et al. (1994) The Chronic Fatigue Syndrome: A Comprehensive Approach to Its Definition and Study. Ann Intern Med 121: 953-959. https://doi.org/10.7326/0003-4819-121-12-199412150-00009

Guerrero, C and Timonina, A et al. (2016) Facilitation of Serotonin-Induced Signaling by the Migraine Mediator CGRP in Rat Terminal Neurons. BioNanoScience 6:357-360. https://doi.org/10.1007/s12668-016-0241-x

Herring J, Elison W, and Tessem J (2019) Function of Nr4a Orphan Nuclear Receptors in Proliferation, Apoptosis, and Fuel Utilization Across Tissues. Cells 8(11):1373. https://doi.org/10.3390/cells8111373

Hickie, Ian et al. (2006) Post-infective and chronic fatigue syndromes precipitated by viral and non-viral pathogens: prospective cohort study. BMJ 333:575. https://doi.org/10.1136/bmj.38933.585764.AE

Huang J, Spier AD, and Pickel VM (2004) 5-HT3A receptor subunits in the rat medial nucleus of the solitary tract: subcellular distribution and relation to the serotonin transporte. Bain Research 1028:156-169. https://doi.org/10.1016/j.brainres.2004.09.009

Johnston, S et al. (2013) The prevalence of chronic fatigue sydomre/myalgic encephalomyelitis: a meta-analysis. Clinical Epidemiology 5:105-110. https://doi.org/10.2147/CLEP.S39876

Jonsjö, MA et al. (2019) The role of low-grade inflammation in ME/CFS (Myalgic Encephalomyelitis/Chronic Fatigue Syndrome) – associations with symptoms. Psychoneuroendrinology 113. https://doi.org/10.1016/j.psyneuen.2019.104578

Lerner, AM et. al (2012) Antibody to Epstein-Barr Virus Deoxyuridine Triphosphate Nucleotidohydrolase and Deoxyribonucleotide Polymerase in a Chronic Fatigue Syndrome Subset. PLoS 7:11. https://doi.org/10.1371/journal.pone.0047891

Maes, Michael and Twisk, Frank (2010) Chronic Fatigue Syndrome: Harvey and Wessely's (bio)psychosocial model versus a bio(psychosocial) model based on inflammatory and oxidative and nitrosative stress pathways. BMC Med 8:35. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2901228/

Miner, JJ and Diamond, MS (2016) Mechanisms of restriction of viral neuroinvasion at the

blood-brain barrier. Curr Opin Immunol. 38:18-23. https://doi.org/10.1016/j.coi.2015.10.008

Morris, Gerwyn et al. (2019) Myalgic encephalomyelitis or chronic fatigue syndrome: how could the illness develop? Metabolic Brain Disease 34:385-415. https://doi.org/10.1007/s11011-019-0388-6

Nasser A, Bjerrum OJ, Heegaard AM et al. (2013) Impaired behavioral pain responses in hph-1 mice with inherited deficiency in GTP cyclohydrolase 1 in models of inflammatory pain. Mol Pain 9:5. https://doi.org/10.1186/1744-8069-9-5

Reeves, William et. al (2007) Prevalence of chronic fatigue syndrome in metropolitan, urban, and rural Georgia. Population Health Metrics 5:5. https://doi.org/10.1186?1478-7954-5-5

Riviera, Mateo Cortes et al. (2019) Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: A Comprehensive Review. Diagnostics 9:91. https://doi.org/10.3390/diagnostics9039991

Stormorken, Eva et al. (2015) Fatigue in adults with post-infectious fatigue syndrome: a qualitative content analysis. BMC Nursing 14:64. https://doi.org/10.1186/s12912-015-0115-5

Williams M, Cox B, Lafuse W, and Ariza ME (2019) Epstein-Barr Virus dUTPase Induces Neuroinflammatory Mediators: Implications for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. Clinical Therapeutics 41:848-863. https://doi.org/10.1016/j.clinthera.2019.04.009

Yu, Shin Kim et al. (2014) Central Terminal Sensitization of TRPV1 by Descending Serotonergic Facilitation Modulates Chronic Pain. Neuron 81:873-887. https://doi.org/10.1016/j.neuron.2013.12.011