REGULATION OF RGS10 IN BV-2 MICROGLIA BY HDAC ISOFORMS

by

PRIYANKA KOTA

(Under the Direction of Shelley Hooks)

ABSTRACT

RGS10 (regulator of G-protein signaling) is a GTPase accelerating protein which increases the rate of hydrolysis of GTP bound to Gα subunit (active) into GDP bound form (inactive) thereby inactivating G-protein signaling. RGS10 is enriched in microglia and is a key regulator of pro-inflammatory cytokine production. Previous studies suggest that epigenetic mechanisms involving histone deacetylases (HDACs) regulate the expression of RGS10. HDAC1 has been shown to be bound with RGS10 promoter region and histone H3 proteins are deacetylated at the RGS10 proximal promoter when microglia are stimulated with LPS. Pharmacological inhibition of HDACs using broad spectrum HDAC inhibitors has been shown to block LPS induced suppression of RGS10 in microglia. The aim of this study is to identify the specific HDAC isoforms involved in the epigenetic regulation of RGS10 in microglia by using selective HDAC inhibitors and evaluate the role of HDAC1 in regulation of RGS10 by using HDAC1 siRNA.

INDEX WORDS: Regulator of G-protein Signaling, Microglia, HDAC, epigenetics.

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A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2017

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May 2017

DEDICATION

I would like to dedicate this work to my family and friends. I would not have been able to complete my masters without their support and encouragement.

To my dearest husband, Bhanu Vanteru, I owe you many thanks for your enormous support throughout my studies at UGA. You have been a wonderful friend in encouraging me to pursue my education, and have given me great support all the time.

To my parents and parents in law for supporting me and having the trust in me all the time.

To my friends, sharing my hard and good times and making me smile and filling with enthusiasm.

ACKNOWLEDGEMENTS

I would like to thank my husband for his support and encouragement in pursuing masters at UGA. I would not have been able to earn this degree without his confidence in me.

I would like to thank Dr. Shelley Hooks for guiding and supporting me. She has been a great teacher and a good friend.

I would also like to thank my committee members Dr. Jason Zastre and Dr. Arthur Roberts for their valuable suggestions.

I am thankful to all my lab members for their friendship and support, especially Mohammad for teaching me various techniques with patience and for his friendly gesture. I would also like to thank Pooja for her care and support. Thanks to Ben, Membi, Faris, Parth and Vitoria for their support.

Thanks to graduate students of Dr. Brian Cummings lab Ramya, Sukhneeraj, Wided and Nhat for their help. Thanks to Murph, Cumming and Beedle labs for allowing me to use their reagents and instruments.

Finally, I would like to thank my parents, parents in law and friends for their trust and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. G-protein coupled receptors (GPCR)

GPCRs are seven transmembrane proteins which when activated by ligands (eg: histamine, norepinephrine, endothelin, vasopressin, prostaglandin etc) cause intracellular activation of heterotrimeric G-proteins [1]. Upon activation GDP is exchanged for GTP and the heterotrimeric G-proteins dissociate into their alpha and beta-gamma subunits causing activation of various downstream signaling pathways and second messengers (eg: inositol triphosphate, diacylglycerol, calcium, cyclic AMP, cyclic GMP etc) [2]. Large number of GPCRs for chemokines and chemo attractants are expressed on the surface of inflammatory cells which play an important role in regulating inflammation. GPCRs have been known to be involved in regulation of inflammatory genes mediated through transcription factors such as NF-kB, CREB and STATs [1, 3]. GPCRs mediate functions that can both promote and resolve inflammation depending on the type of G-protein that is activated.

1.2. Regulator of G-protein signaling (RGS) proteins

RGS proteins were first identified as negative regulators of G-protein signaling in nematodes and fungi [4]. RGS proteins accelerate the rate of hydrolysis of GTP bound to α-subunit of heterotrimeric G-proteins and thus serve to terminate their signaling by acting as GTPase accelerating proteins (GAP) [4]. RGS proteins are found to be abundant in brain, retina,

heart and immune tissues [5]. About 20 RGS proteins have been identified in eukaryotes. They share a conserved 120 amino acid sequence called the RGS domain. The integrity of the amino acid sequence in the RGS domain is essential for the catalytic (GAP) activity of these proteins [6]. However, in addition to the RGS domain, some RGS proteins like RGS4 and RGS16 contain other domains that mediate GAP-independent functions [7]. RGS proteins can alter the intensity and duration of a signal generated by heterotrimeric G-proteins and cause fine tuning of GPCR signaling [1].

Figure 1 shows activation of G - protein coupled signaling by a ligand and role of RGS proteins in accelerating the GTPase activity of α-subunit.

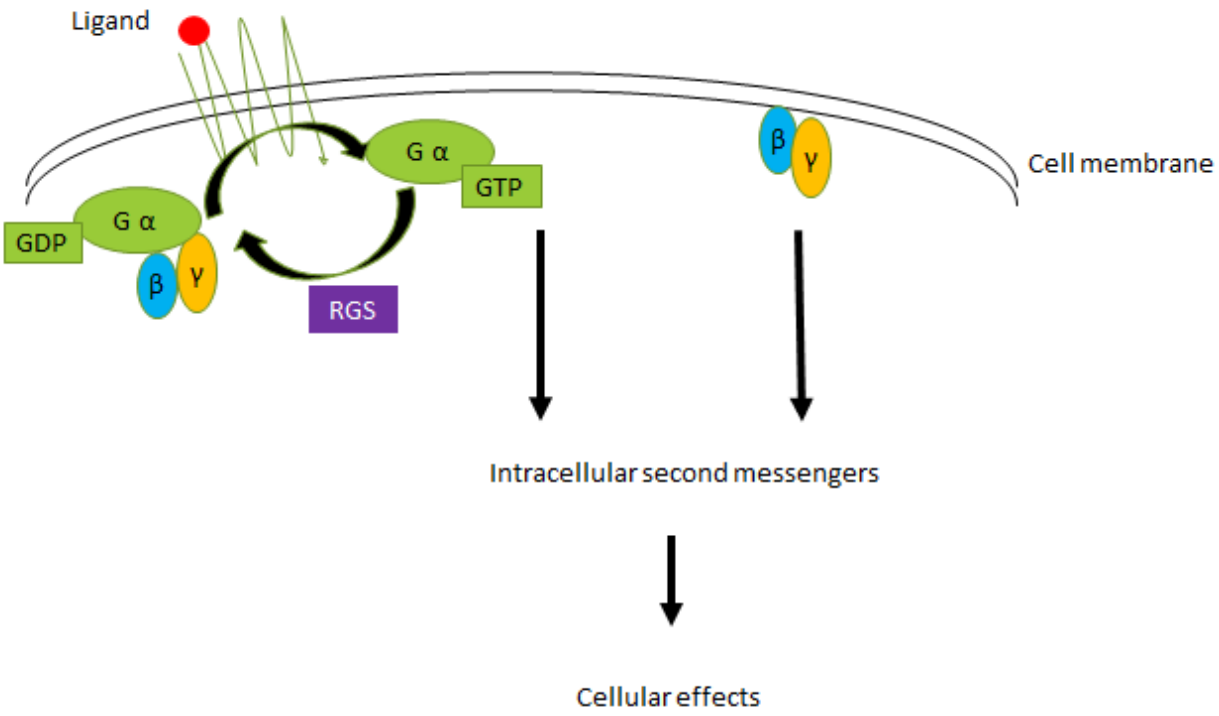

Figure 1: G - protein coupled receptor signaling and role of regulator of G - protein signaling (RGS) proteins.

1.3. Microglia and neuroinflammation

Microglia are the brain's resident immune cells which are in constant surveillance of central nervous system (CNS) and maintain homeostasis. They regulate CNS innate immunity by producing inflammatory responses which are fundamental for exerting its protective action. Prolongation of this inflammatory response causes cellular damage or tissue destruction. This chronic microglial activation manifested as neuroinflammation transforms into a vicious cycle causing long term disastrous effects leading to neurodegenerative conditions [8, 9].

1.4. RGS10 in microglia

RGS10 is a GTPase accelerating protein (GAP) which negatively regulates signaling pathways associated with Gαi (subunit of heterotrimeric G-protein) and to a much lesser extent Gαq [4, 6]. It belongs to the D/R12 subfamily and is highly expressed in brain and immune tissues [5, 10].

Microglia are important for maintaining the integrity of the brain and are enriched with RGS10. RGS10 was shown to have a neuroprotective role by reducing microglial inflammatory in the CNS and preventing dopaminergic neuronal damage [11]. Knockdown of RGS10 in BV-2 microglia increased production of TNF-α (a pro-inflammatory cytokine) and enhanced cytotoxic effects in these cells [11]. NF-kB is a transcription factor involved in the transcription of pro-inflammatory genes. RGS10 can negatively regulate NF-kB and lentiviral mediated restoration of RGS10 has been shown to reverse the pro-inflammatory and cytokine response in RGS10 null mice [12]. Thus, the anti-inflammatory properties of RGS10 was proved in various studies and mechanisms controlling RGS10 expression will indirectly regulate neuroinflammation.

1.5. Regulation of RGS proteins

RGS proteins are known to be regulated by post translational mechanisms such as phosphorylation and palmitoylation which are known to regulate cellular localization and thus affect the GAP function of RGS proteins. However, these modification depend on the type of RGS protein and their associated cell type [2, 13]. Agonists of Gq/Gi coupled receptors are known to regulate expression of RGS proteins suggesting that they serve as feedback mechanisms and for cross-desensitization of other GPCRs [13]. Post translational mechanisms such as palmitoylation, myristoylation and phosphorylation are known to regulate RGS proteins. Palmitoylation at cysteine residues is important for its membrane localization of these proteins. RGS proteins contain myristoylation sites in their N-terminus and its role in the attachment of the protein to membrane is not yet known. RGS proteins have phosphorylation sites in their N-terminus and RGS domain [2]. Phosphorylation has also been shown to impact localization, degradation and stability of certain RGS proteins. Proteosomal degradation and N-end rule of degradation also affects the stability of RGS proteins [14]. RGS proteins are also known to be regulated by bacterial lipopolysaccharide (LPS) and pro-inflammatory cytokines like TNF-α in immune cells [10, 13] and in response to stress conditions induced by cytotoxic drugs [13, 15]. Epigenetic mechanisms involving histone deacetylases (HDACs) and DNA N-methyl transferases (DNMTs) are known to be involved in regulation of RGS10 and RGS2 in chemoresistant cancer cells [16, 17]. Since RGS proteins can be differentially regulated by various mechanisms depending on the cell type, it is important to explore the details of their regulation in various disease pathologies.

1.6. Epigenetic overview: Histone acetylation and DNA methylation

Lysine residues of histones are acetylated and deacetylated by the catalytic activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Acetyl groups impart negative charge to histones and cause repulsion between histones and DNA. This causes a more relaxed or open confirmation of DNA and allows easy access of various transcription factors to promoter region of genes that are to be transcribed. Removal of acetyl groups causes condensation or closed confirmation of DNA limiting access to transcription factors and silencing gene expression [18, 19]. The balance between activities of HATs and HDACs is crucial in regulating gene expression [20]. Altered activity could lead to diseased states because of disrupted gene expression profiles [18, 19].

Mammalian HDACs are classified into four different classes based on their homology to yeast HDACs [18, 21]. HDACs in class I, II and IV are Zinc dependent enzymes whereas class III HDACs are dependent on NAD+ for exerting their enzymatic activity [18, 21, 22]. Table 1 represents the classification of different subtypes of HDACs, isoforms, localization in a cell and the pharmacological HDAC inhibitors used in this study [23].

HDAC class	HDAC isoforms	Localization of HDAC	HDAC inhibitors		
			TSA	VPA	MS-275 (Entinostat)
Class I	1 2 3 8	Nucleus	xx xx xx xx	xx xx xx xx	xx - x -
Class IIa	4 5 7 9	Nucleus/cytoplasmic	xx xx xx xx	x x x x	- - - -
Class IIb	6 10	Mainly cytoplasmic	xx xx	- -	- -
Class III	Sirtuins 1-7	Nucleus/cytoplasmic/mitochondria	-	-	-
Class IV	11	Nucleus/cytoplasmic	xx	-	-

Table 1: Classification of HDAC classes, isoforms and inhibitors [23]. xx indicates inhibition at concentration used in the study, x indicates inhibition at higher concentration or partial inhibition.

HDAC inhibitors are potent compounds capable of inhibiting the deacetylating property of HDAC enzymes thereby acetylating histones and causing cellular and systemic effects by altering the overall gene expression profile [21]. The therapeutic benefits of HDAC inhibitors in various diseases has been known to be the result of transcriptional reprogramming [22].

Epigenetic mechanisms involving histone deacetylation and DNA methylation have known to be altered in various cancers. The Hooks lab identified that there is reduced expression of RGS10 in acquired chemoresistant ovarian cancer cells. They identified the association of HDAC1 and DNMT1 in the RGS10-1 promoter region suggesting their role in epigenetic regulation of RGS10 in ovarian cancer cells [16]. Over expression of HDAC1 in the

chemoresistant ovarian cancer cells reduced the expression of RGS10 in these cells. Knock down of HDAC1 or DNMT1 in chemoresistant ovarian cancer cells increased expression of RGS10 and enhanced their sensitivity to chemotherapeutic agents [17]. Use of pharmacological inhibitors of DNMT1 and HDAC1 enhanced the transcription and expression of RGS10 reversing the acquired chemoresistance in ovarian cancer cells thereby increasing their sensitivity to chemotherapeutic agents [16, 17]. This is consistent with other reports where HDAC1 has been identified to play a major role in acquired chemoresistance in various cancers. In esophageal carcinoma cells EC109, HDAC1 knockdown enhanced chemosensitivity to chemotherapeutic drugs and also inhibited metastasis [24]. Other RGS proteins such as RGS2 are known to be epigenetically suppressed by DNA methylation in prostate cancer cell lines and human prostate cancer. Pharmacological inhibition of DNMT enzymes by 5-Aza-dC reversed this epigenetic silencing and increased expression of RGS2 and suppressed androgen-independent prostate cancer cell growth [25]. These studies suggest that RGS proteins are epigenetically regulated by histone deacetylation and DNA methylation mechanisms in cancer cells.

HDAC inhibitors have been originally developed for their anti-tumor properties but are now gaining importance for their anti-inflammatory and neuroprotective effects. Several HDAC inhibitors have been tested for their neuroprotective/modulatory effects and are known to alter the transcriptional activity of pro-inflammatory genes. HDACs also alter the acetylation of various non-histone proteins which might be important in regulation of inflammation. However, the responses of HDAC inhibitors are specific to different cell types. Exploring the neuroprotective activity of HDAC inhibitors already in the market or in clinical trials for treating

cancer might accelerate their applicability to treat neuroinflammatory and neurodegenerative diseases.

The innate immune activation of microglia is known to be modulated by HDAC inhibitors by their effect on HDAC enzymes. Various studies have reported that HDAC inhibitors modulate inflammation and immune cell activation [26]. Class I and II HDAC inhibitors SAHA and ITF2357 were shown to reduce transcripts of iNOS, COX-2 and IL-1β in primary glial cells. Also, these HDAC inhibitors reduced LPS dependent release of TNF-α in cultured medium and had long lasting anti-inflammatory effects against LPS induced microglial activation. They reported that the observed anti-inflammatory effect was not due to expression of immunosuppressant proteins [27].

Given that HDACs and DNMTs regulate RGS10 in chemoresistant ovarian cancer cells [16, 17] and that RGS10 is suppressed in microglia [5, 11], our lab set out to define the role of these epigenetic mechanisms in microglia. The research from Hooks lab revealed that RGS10 is suppressed in both in-vitro and in-vivo models of inflammation and suggested that epigenetic mechanisms involving histone acetylation play a major role in regulating RGS10 expression in microglia [10]. The broad spectrum HDAC inhibitor Trichostatin A (TSA) was shown to reverse LPS induced suppression of RGS10 in microglia suggesting that HDACs mediate epigenetic suppression of RGS10 in microglia. Chromatin immunoprecipitation (ChIP) assays showed the association of HDAC1 and the reduction in acetyl histone H3 at RGS10 promoter region in LPS stimulated BV-2 microglial cells. Treatment of BV-2 microglia with 5-Aza, a pharmacological inhibitor of DNMT enzymes, did not block LPS induced suppression of RGS10 suggesting that DNMTs do not have a role in regulating RGS10 expression in microglia [10]. The mode of

RGS10 suppression by inflammation must be explored in detail to further understand the importance of RGS10 in neuroinflammation.

Pro-inflammatory or anti-inflammatory activity of HDAC inhibitors depends on the cell type and the differential expression of HDACs [23]. There has been various reports highlighting the importance of HDAC inhibitors as therapeutic agents in neuro-inflammatory and neurodegenerative conditions [18, 22]. Pan HDAC inhibitors/non-selective inhibitors such as Trichostatin A cause robust acetylation of histones and may have off target effects. For example, non-selective inhibitors of HDACs were known to cause acetylation of high molecular mass proteins like tubulin by their activity towards HDAC6 [22]. HDAC class I or II selective inhibitors have been shown to have less off target effects.

To date there is no clear understanding of the epigenetic mechanisms involving histone deacetylases and how they regulate RGS10 expression in microglia. Given that RGS10 has anti-inflammatory properties and that HDAC inhibitor TSA can block LPS induced suppression of RGS10, there is a need for further exploration to identify the importance of specific HDAC isoforms regulating RGS10 expression. The potential of isoform selective HDAC inhibitors in eliciting their neuroprotective effect must be investigated for the development of therapeutic strategies in treating neuroinflammatory disorders.

We hypothesize that RGS10 is epigenetically regulated by a subset of histone deacetylases (HDACs) and aim to identify which class of HDACs are involved in regulating RGS10 expression by use of selective HDAC inhibitors and investigate the role of HDAC1 isoform in regulating RGS10 expression by use of HDAC1 selective inhibitor and knockdown of HDAC1 using small interfering RNA (siRNA).

CHAPTER 2

MATERIALS AND METHODS

2.1. Cells and Reagents

BV-2 microglial cells were obtained as a generous gift from G. Hasko, University of Medicine and Dentistry of New Jersey (Newark, NJ). BV-2 microglia were maintained in Dulbecco's modified Eagle's medium (American Type Culture Collection, Manassas, VA) supplemented with 10% fetal bovine serum (GE Healthcare, Pittsburg, PA). Lipopolysaccharide (LPS), Trichostatin A (TSA), Valproic acid (VPA) and Entinostat (MS-275) were obtained from Sigma-Aldrich (St. Louis, MO).

For Western blot analysis, the following antibodies were used: anti-RGS10 and anti-HDAC1 antibodies (Santa Cruz Biotechnology), anti-acetylated histone H3 and anti-total histone H3 antibodies (Cell Signaling Technologies), anti-GAPDH (Millipore Technologies).

2.2. Quantitative Real-Time Polymerase Chain Reaction

mRNA was isolated using Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA) and cDNA was synthesized from 2 µg total RNA using High Capacity Reverse Transcriptase cDNA kit (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA). Quantitative real-time polymerase chain reaction (PCR) was performed using Superscript III kit for RT-PCR (Invitrogen/Life Technologies) and Power SYBR Green reagent (Life Technologies/Thermo Fisher Scientific). Reactions were normalized using the housekeeping gene (actin) and 2-ΔΔCT

method was used to perform the calculations. Primers used were based on algorithm-generated sequences from Primer Bank (http://pga.mgh.harvard.edu/primerbank/). Primers for RGS10 were purchased from Integrated DNA Technologies, IDT (Coralville, IA) and for actin, HDAC1,2, 3, 5, 8 from Sigma. The sequence of forward and reverse primers for the genes used in the present study are presented in Table 2.

Gene	Sequence of primers	
	Forward (5'-3')	Reverse (5'-3')
RGS 10	CCTGGAGAATCTTCTGGAAGACC	CTGCTTCCTGTCGTAG
ACTIN	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
HDAC1	GGACACGCCAAGTGTGTGG	GAGCAACATTCCGGATGGTG
HDAC2	CAACAGATCGCGTGATGACC	CCCTTTCCAGCACCAATATCC
HDAC3	GACGTGCATCGTGCTCCAGT	ACATTCCCCATGTCCTCGAAT
HDAC5	ACGCCTCCCTCCTACAAATTG	TAAGTTGGGTTCCGAGGCC
HDAC8	GGGTGGCATCATGCAAAGAA	CAAATTTCCGTCGCAATCGT

Table 2: Sequence of various gene primers used for RT-PCR.

2.3. Western Blot Analysis

Evaluation of protein expression in BV-2 microglia was done by lysing 3 x 10^5 cells and 2 x 10^5 cells in SDS-PAGE lysis buffer for VPA experiments and Entinostat experiments respectively. For knock down experiments in BV-2 cells using siRNA, 10^5 cells were harvested in lysis buffer. The cell lysates were boiled for 5 minutes and analyzed using SDS-PAGE. Membranes were blocked with milk for one hour and incubated with primary antibodies overnight, washed and incubated with appropriate HRP-conjugated goat, rabbit, mouse secondary antibodies for one hour and imaged to visualize the proteins using enhanced

chemiluminescence reagent (Thermo Fisher Scientific; Pierce). Membranes were subsequently blotted with anti-GAPDH antibody (Millipore Technologies) as a loading control.

2.4. siRNA constructs and transient transfection

Short interfering RNA (siRNA) knockdown experiments were conducted in 24-well plates. BV-2 cells were plated (10^5 cells/well) and transfected with 10 nM of mouse HDAC1 siRNA or 60 nM control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine LTX transfection reagent (Life Technologies, Carlsbad, CA). 24 hours after the transfection, cells were treated with LPS (10ng/ml) for another 24 hours and protein analysis was carried out. To determine the transcript levels, 42 hours after the transfection, cells were treated with LPS (10ng/ml) for 6 hours and harvested in trizol for further analysis using qRT-PCR.

2.5 Statistical Analysis

Data were analyzed by ANOVA followed by Tukey's test for some experiments and by t-tests between selected groups for some experiments. Statistical method and criteria are listed in the figure legends.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Evaluating the effect of VPA (Class I and IIa specific HDAC inhibitor) on RGS10 expression and screening for the expression of class I HDACs in BV-2 microglia

Previous reports from our lab suggest that RGS10 is epigenetically regulated by HDACs in both chemo-resistant ovarian cancer cells and microglial cells [10, 16, 17]. Pharmacological inhibition of HDAC enzymes by TSA, a pan HDAC inhibitor, was able to block LPS induced RGS10 suppression in BV-2 and primary microglial cells [10]. TSA is a non-selective HDAC inhibitor and inhibits class I, II and IV HDACs.

To determine which HDAC subtypes are required for regulating RGS10 expression, class I and IIa selective HDAC inhibitor valproic acid (VPA) was selected.

Figure 2 shows the schematic representation for evaluating the effect of VPA on regulation of RGS10 expression.

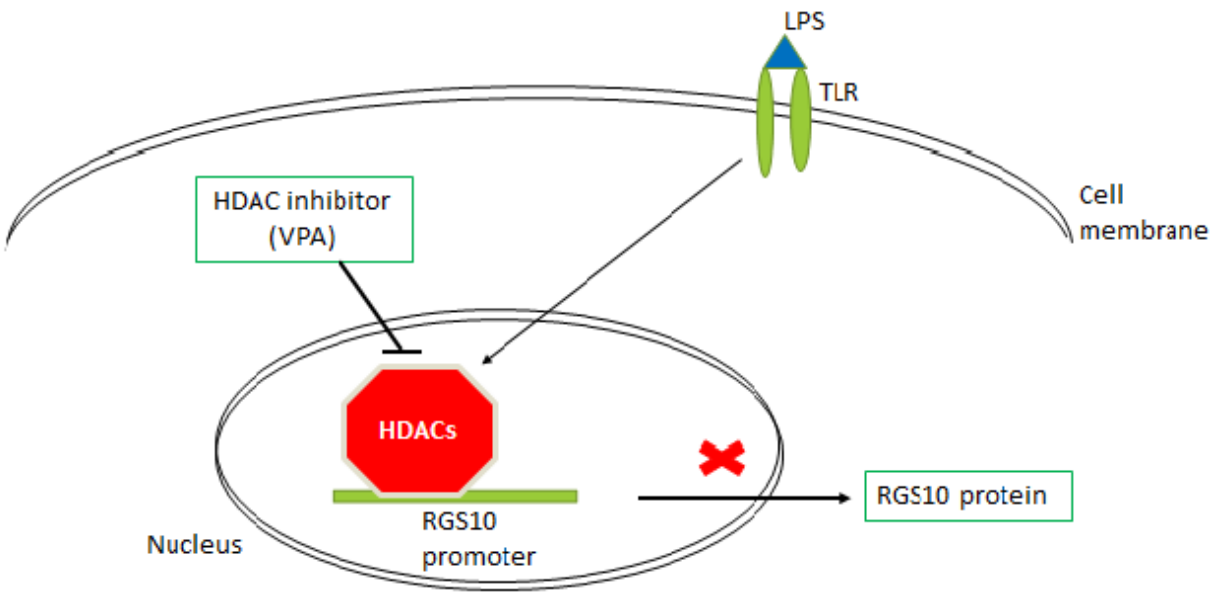

Figure 2: Schematic representation for evaluating the effect of VPA on regulation of RGS10 expression.

Experiments were performed to test if VPA can block LPS induced suppression of RGS10 like TSA. To test this, we pre-treated BV-2 microglial cells with VPA (1mM) and TSA (25mM and 10mM) for 1 hour followed by LPS (10 ng/mL) treatment for 24 hours and analyzed the protein expression of acetylated histone H3, total histone H3 and RGS10 by Western blot analysis. GAPDH was used as loading control.

As shown in figure 3, both VPA and TSA blocked the LPS induced suppression of acetyl histone H3, confirming that both compounds have functional histone deacetylase inhibitory activity in this system. There was no change in the level of total histones with the compound treatment confirming that the HDAC inhibitors only cause histone acetylation and do not alter the level of total histone.

Statistical analysis of data did not show any significance because there was more variability between experiments but there appears to be a trend. Data was quantified by normalizing acetyl histone to GAPDH, total histone to GAPDH and then acetyl histone was normalized to total histone. Since there were multiple levels of normalizing the data, the error bar was high.

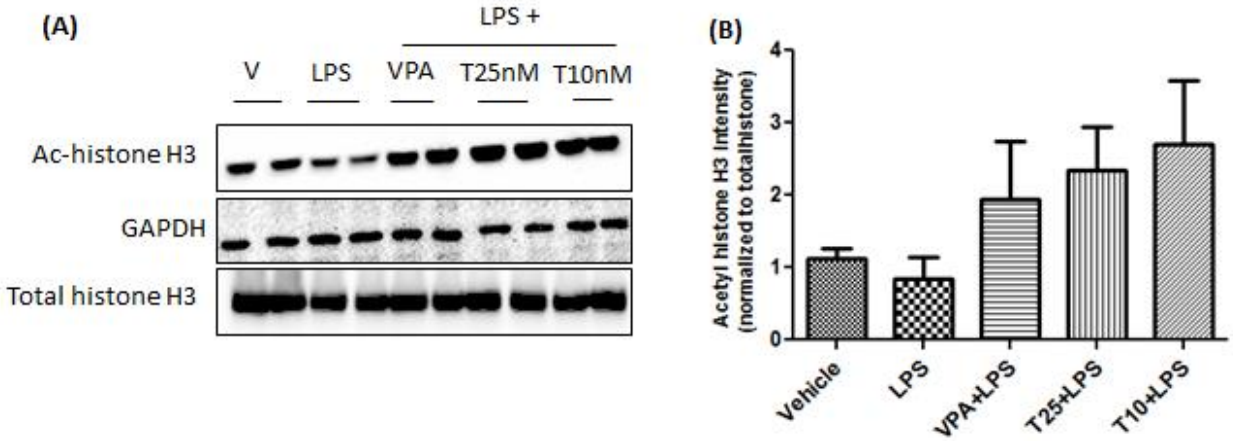

Figure 3: Effect of VPA and TSA on acetylated histone level. A) BV-2 cells were plated in a 6 well plate, allowed to adhere overnight and treated with vehicle (serum free media), LPS (10ng/ml) and pre-treated with HDAC inhibitors VPA (1mM), TSA (25nM and 10nM) for 1 hour before treatment with LPS. Cells were harvested after 24 hours and analyzed for protein levels by Western blot analysis. GAPDH was used as a loading control. B) Data quantified from western blotting images by measuring the intensity of bands. Acetyl histone H3 was normalized to total histone H3. Statistical analysis of data by two-tailed t-test did not show significance but there appears to be a trend.

Similarly, to test the change in RGS10 transcript levels, BV-2 microglial cells were pretreated with VPA (1mM) for 1 hour followed by LPS (10ng/mL) for 6 hours and transcript levels were analyzed by qRT-PCR.

Valproic acid blocked LPS induced suppression of RGS10 and this was observed by increase in RGS10 protein expression and transcript levels in Figure 4 and 5 respectively.

As shown in figure 4, VPA significantly blocked LPS induced reduction in RGS10 protein expression. In figure 5, VPA significantly blocked LPS induced reduction in RGS10 transcript levels and increased basal level of RGS10 as seen from qRT-PCR analysis. Statistical analysis of LPS vs VPA+LPS groups in the qRT-PCR analysis did not show significance because there was high error bar in the treatment group. Repetition of the qRT-PCR experiment might be useful to obtain statistical significance.

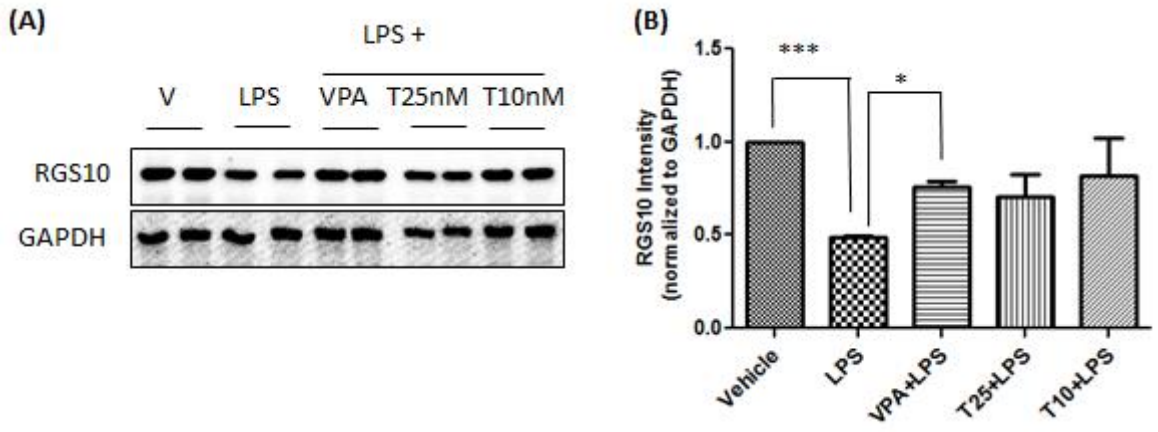

Figure 4: Effect of VPA and TSA on RGS10 expression. A) BV-2 cells were plated in a 6 well plate, allowed to adhere overnight and treated with vehicle (serum free media), LPS (10ng/ml) and pre-treated with HDAC inhibitors VPA (1mM), TSA (25nM and 10nM) for 1 hour before treatment with LPS. Cells were harvested after 24 hours and analyzed for protein levels by Western blot analysis. GAPDH was used as a loading control. B) Data quantified from western blotting images by measuring the intensity of bands. RGS10 was normalized to GAPDH. Data was analyzed by two-tailed t-test. *** indicates $p<0.001$, * indicates $p<0.05$.

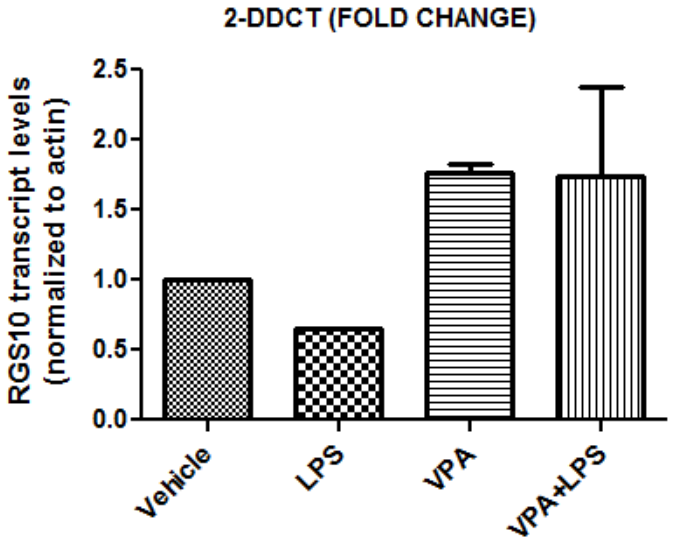

Figure 5: Effect of VPA on RGS10 transcript levels. BV-2 cells were plated in a 6 well plate, allowed to adhere overnight and treated with vehicle (serum free media), LPS (10ng/ml) and pre-treated with HDAC inhibitor VPA (1mM) for 1 hour before treatment with LPS. Cells were harvested after 6 hours in trizol, RNA was isolated, cDNA was synthesized. RGS10 transcript levels were quantified using qRT-PCR relative to actin.

The results demonstrate that class I and IIa HDAC subtypes are involved in regulating RGS10 expression. However, literature suggests that class IIa HDACs acetylate non-histone proteins rather than histone proteins and are also not inhibited by VPA at pharmacologically relevant concentration. Also, there are reports suggesting that VPA inhibited class I HDACs 1, 2, 3 and 8 in the millimolar concentration but did not have any effect on class IIa at this concentration [28]. This suggest that class IIa are not of much significance in regulating RGS10 expression by modulating histone acetylation and class I HDACs might be involved in regulating RGS10 expression in microglia.

To further define which Class I HDACs may be involved in regulation of RGS10 in BV-2 microglia, we screened for the expression levels HDAC 1, 2, 3 and 8 isoforms by qRT-PCR. The transcripts were analyzed in both vehicle and LPS treated BV-2 cells to see if there is a difference in the expression of HDAC isoforms in response to LPS treatment. HDAC5 which belongs to HDAC Class IIa has been shown to be expressed at significantly high level in microglia when compared to other isoforms from Class II [26]. So, we also analyzed HDAC5 along with Class I HDACs to examine for their expression.

Figure 6 shows expression levels of Class I HDAC isoforms in microglia in vehicle and LPS treated group. There was no noticeable difference in the expression of Class I HDACs between vehicle and LPS treated group. Based on CT values, we expect HDAC1 to be highly expressed in BV-2 cells, but our current data does not allow quantitative comparison of expression.

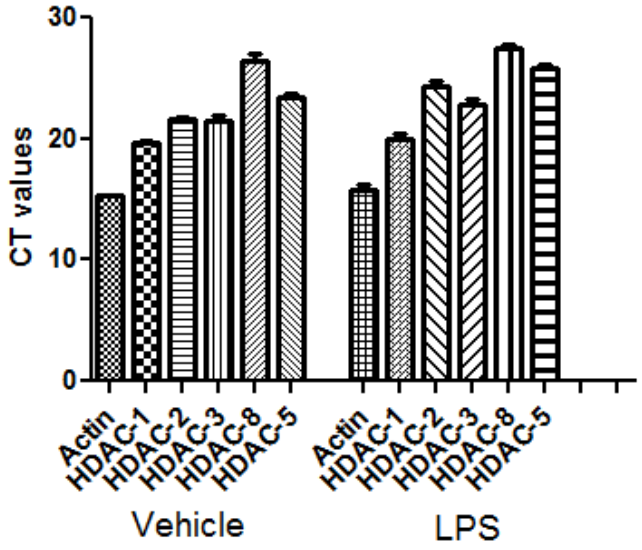

Figure 6: Expression levels of HDAC isoforms in microglia. BV-2 cells were plated in a 12 well plate, allowed to adhere overnight and treated with vehicle (serum free media) or LPS (10ng/ml). Cells were harvested after 6 hours in trizol, RNA was isolated and cDNA was synthesized. Actin, HDAC1, 2, 3, 5 and 8 transcript levels were analyzed using qRT-PCR.

Epigenetic mechanisms involving histone modifications are known to be involved in regulating gene expression. Drugs such as valproic acid (VPA) have been shown to have anti-inflammatory and neuroprotective activity owing to their HDAC inhibitory activity. Several HDAC inhibitors have been tested for their neuroprotective/modulatory effects and are known to alter the transcriptional activity of pro-inflammatory genes [18]. HDACs also alter the acetylation of various non-histone proteins which might be important in regulation of inflammation. However, the responses of HDAC inhibitors are specific to different cell types. HDAC inhibitors have been originally developed for their anti-tumor properties but are now gaining importance for their neuroprotective effects. Exploring the neuroprotective activity of HDAC inhibitors already in market or in clinical trials for treating cancer might accelerate their applicability to treat neuroinflammatory and neurodegenerative diseases [29]. VPA has been used to treat epilepsy, bipolar disorder and crosses BBB. Its HDAC inhibitory activity could be explored to alter gene expression and modulate neuroinflammatory conditions.

3.2. Evaluating the role of HDAC1 in regulating RGS10 expression in BV-2 microglia

Previous studies from our lab suggest that HDAC1 binds to RGS10 promoter when microglia are activated by LPS and in chemoresistant ovarian cancer cells compared to chemosensitive cancer cells causing epigenetic silencing of RGS10 [10, 17]. Also, HDAC1 knockdown increased RGS10 expression in chemoresistant ovarian cancer cells [17]. However, there are no studies indicating that HDAC1 is a major player in regulating RGS10 expression in microglia. To identify the role of HDAC1 in suppression of RGS10 in BV-2 microglia, HDAC1 was pharmacologically inhibited by using a HDAC1 selective inhibitor Entinostat and knocked down using siRNA.

Figure 7 shows the schematic representation for evaluating the role of HDAC1 in regulating RGS10 expression.

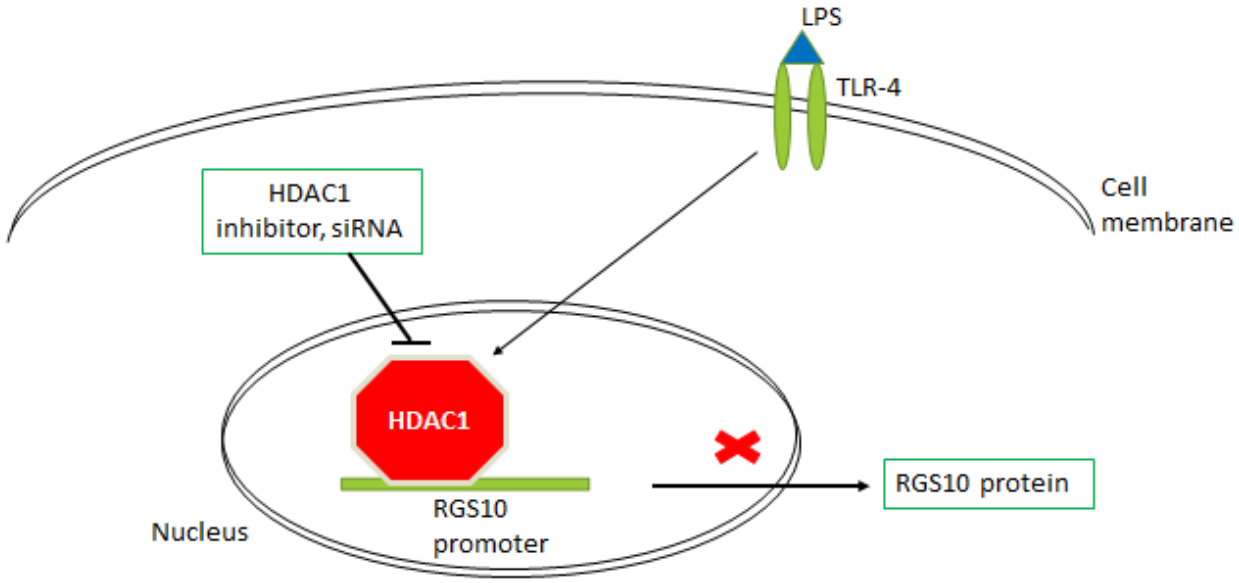

Figure 7: Schematic representation for evaluating the role of HDAC1 in regulation of RGS10 expression.

3.2.1 Effect of Entinostat (HDAC1 selective inhibitor) in BV-2 microglia

To determine if Entinostat HDAC1 selective inhibitor can block LPS induced suppression of RGS10 we pre-treated BV-2 microglial cells with Entinostat (200 nM and 1 uM) for 1 hour followed by LPS (10 ng/mL) treatment for 24 hours and analyzed the protein expression of RGS10 by Western blot analysis.

As shown in Figure 8, Entinostat did not block LPS induced reduction in RGS10 levels at both concentrations tested. This suggests that HDAC1 is not required for regulating RGS10 expression in BV-2 microglia.

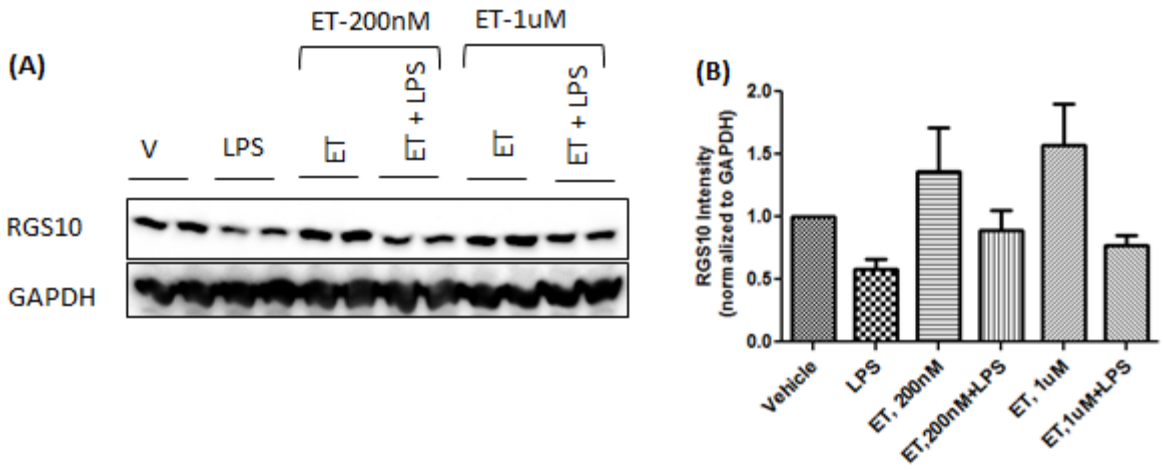

Figure 8: Effect of MS-275 (Entinostat) on RGS10 expression. A) BV-2 cells were plated in a 12 well plate, allowed to adhere overnight and treated with vehicle (serum free media), LPS (10ng/ml) and pre-treated with HDAC inhibitor Entinostat (200nM and 1uM) for 1 hour before treatment with LPS. Cells were harvested after 24 hours and analyzed for protein levels by Western blot analysis. GAPDH was used as a loading control. B) Data quantified from western blotting images by measuring the intensity of bands. RGS10 was normalized to GAPDH. Statistical analysis by two-tailed t-tests showed no significance.

To test the change in RGS10 transcript levels, BV-2 microglial cells were pretreated with Entinostat (200 nM and 1 uM) for 1 hour followed by LPS (10 ng/mL) for 6 hours and RGS10 transcript levels were analyzed by RT-PCR.

Figure 9 shows that the HDAC inhibitor Entinostat did not block LPS induced suppression of RGS10 transcript level at the tested conditions. This was similar to that observed by analysis of RGS10 proteins levels by Western blotting. This result suggests that HDAC1 is not required for regulating RGS10 expression in BV-2 microglia.

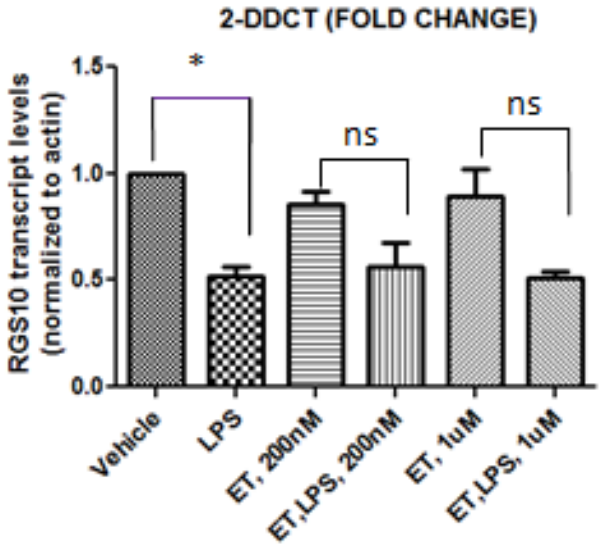

Figure 9: Effect of MS-275 (Entinostat) on RGS10 transcript levels. BV-2 cells were plated in a 12 well plate, allowed to adhere overnight and treated with vehicle (serum free media), LPS (10ng/ml) and pre-treated with HDAC inhibitor Entinostat (200 nM and 1 uM) for 1 hour before treatment with LPS. Cells were harvested after 6 hours in trizol, RNA was isolated, cDNA was synthesized. RGS10 transcript levels were quantified using qRT-PCR relative to actin. Data was statistically analyzed by one-way ANOVA followed by Tukey's test from 2 independent experiments performed in duplicates: *$p<0.05$.

3.2.2 Effect of HDAC1 knockdown in BV-2 microglia

HDAC1 knockdown in chemo-resistant ovarian cancer cells increased the expression of RGS10 and restored chemo-sensitivity to chemotherapeutic agents [17]. To evaluate if HDAC1 knockdown would block LPS induced RGS10 suppression in BV-2 microglia, cells were transiently transfected with 10nM HDAC1 siRNA and control siRNA in a 24-well plate. After 24 hours, BV-2 cells were treated with LPS (10ng/mL) for 24 hours and harvested in SDS sample buffer. Western blot analysis was carried out to determine the protein expression of HDAC1 and RGS10.

Figure 10 shows successful knock down of HDAC1 using 2 different concentrations of siRNA. As 10nM HDAC1 siRNA caused a significant knock down, the same concentration was used for rest of the experiments with HDAC1 siRNA.

The effect of LPS on HDAC1 levels in knock down groups is shown in figure 11 and RGS10 expression levels in figure 12. As seen in figure 11, HDAC1 levels were not altered in LPS treated control and HDAC1 knock down groups indicating that HDAC1 expression is not changed with LPS treatment instead its association with various gene promoters including that of RGS10 promoter is altered.

As shown in figure 12, in contrast to the expectations, HDAC1 knock down did not block LPS induced suppression of RGS10 suggesting that HDAC1 is not required for the regulation RGS10 expression consistent with the result from Entinostat experiments.

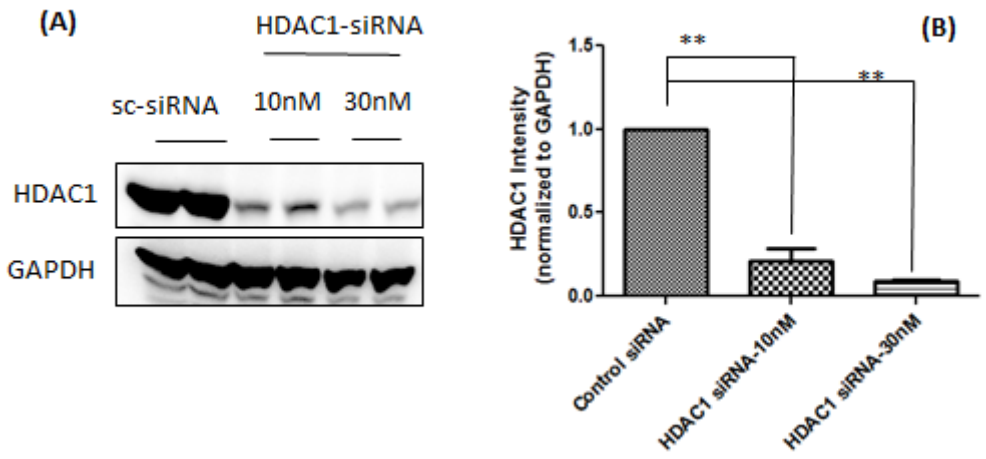

Figure 10: Knock down of HDAC1 using siRNA. A) HDAC1 was knocked down in BV-2 microglia in a 24 well plate using control siRNA and 2 different concentrations of HDAC1 siRNA (10nM and 30nM). Cells were harvested after 48 hours and protein levels were analyzed by Western blot analysis. GAPDH was used as a loading control. B) Data quantified from western blotting images by measuring the intensity of bands. HDAC1 was normalized to GAPDH. Data was statistically analyzed by one-way ANOVA followed by Tukey's test from 2 independent experiments performed in duplicates: **$p<0.01$.

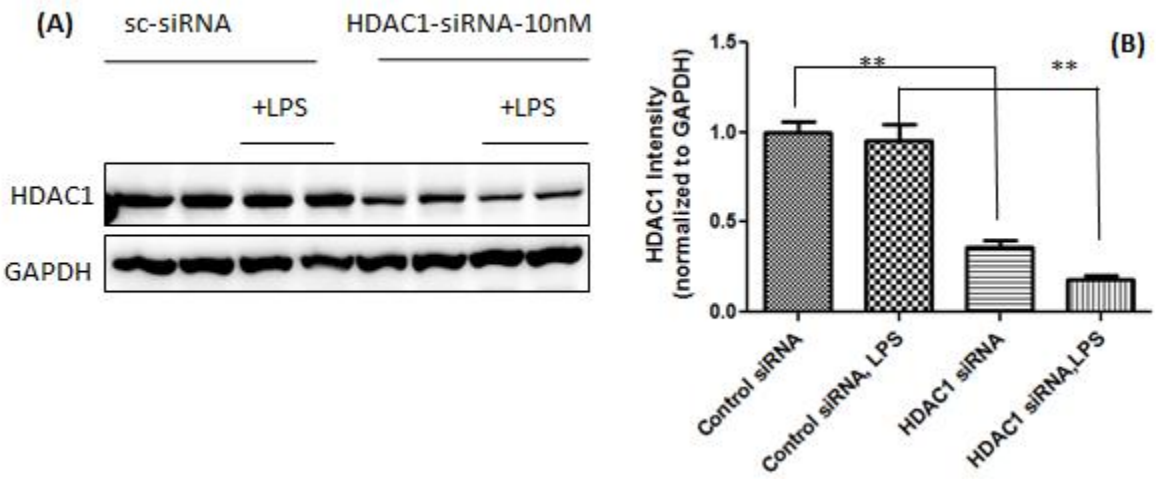

Figure 11: Effect of HDAC1 knock down in control and LPS treated groups. A) HDAC1 was knocked down in BV-2 microglia in a 24 well plate using control siRNA and 10nM HDAC1 siRNA. Cells were treated with LPS (10ng/ml) after 24 hours of transfection. After 24 hours of LPS treatment, cells were harvested and protein levels were analyzed by Western blot analysis. GAPDH was used as a loading control. B) Data quantified from western blotting images by measuring the intensity of bands. HDAC1 was normalized to GAPDH. Data was statistically analyzed by one-way ANOVA followed by Tukey's test: **$p<0.01$.

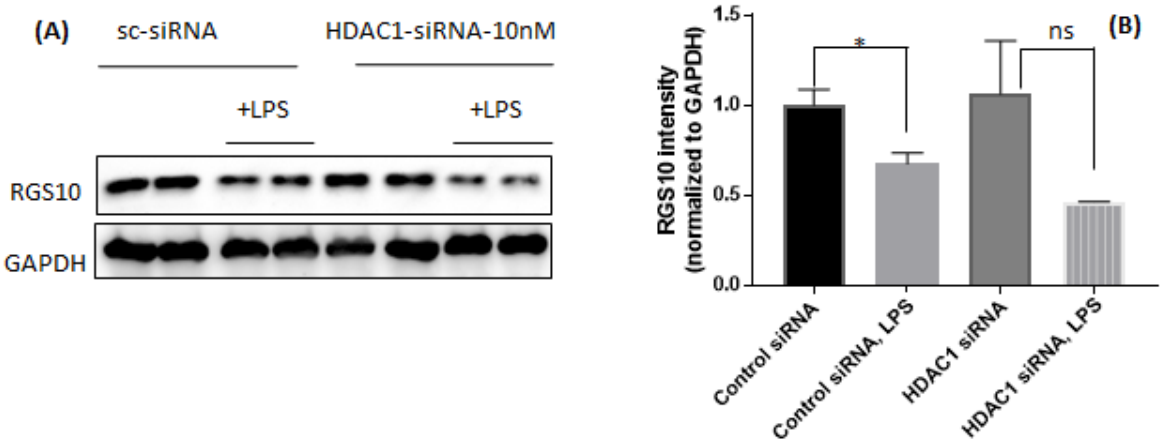

Figure 12: Effect of control and HDAC1 siRNA on RGS10 expression in vehicle and LPS treated group. A) HDAC1 was knocked down in BV-2 microglia in a 24 well plate using control siRNA and 10nM HDAC1 siRNA. Cells were treated with LPS (10ng/ml) after 24 hours of transfection. After 24 hours of LPS treatment, cells were harvested and protein levels were analyzed by Western blot analysis. GAPDH was used as a loading control. B) Data quantified from western blotting images by measuring the intensity of bands. RGS10 was normalized to GAPDH. Data was analyzed by two-tailed t-test: *$p<0.05$.

To estimate RGS10 transcript levels, cells were transfected with HDAC1 siRNA, control siRNA and treated with LPS after 42 hours for six hours and harvested in Trizol. RGS10 transcript levels were determined by RT-PCR.

Figure 13 shows HDAC1 and RGS10 transcript levels in vehicle and LPS treated groups. In figure 13(A), HDAC1 transcripts were significantly reduced in control vs HDAC1 knock down group and in control group with LPS vs HDAC1 knock down with LPS. Also, HDAC1 transcripts were not increased with LPS treatment in comparison to their control or HDAC1 knock down groups. This suggests that the overall expression of HDAC1 is not affected with LPS.

In figure 13(B), LPS caused reduction in RGS10 transcript levels and HDAC1 knock down did not block LPS induced suppression of RGS10.

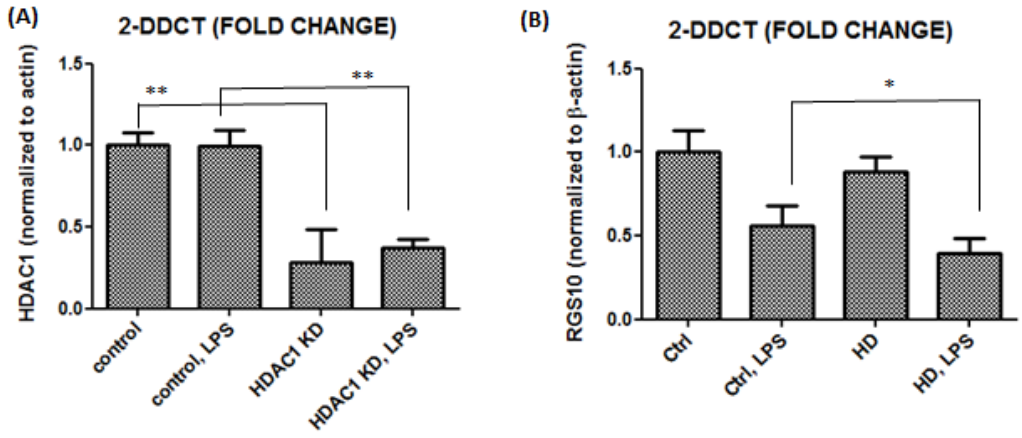

Figure 13: Effect of knock down on HDAC1 and RGS10 transcript levels in vehicle and LPS treated groups. A) HDAC1 was knocked down in BV-2 microglia in a 24 well plate using control siRNA and 10nM HDAC1 siRNA. Cells were treated with LPS (10ng/ml) after 42 hours of transfection. After 6 hours of LPS treatment, cells were harvested in trizol, RNA was isolated, cDNA was synthesized. HDAC1 and RGS10 transcript levels were quantified using qRT-PCR relative to actin. Data was analyzed by one-way ANOVA followed by Tukey's test: **$p<0.01$, *$p<0.05$

Previous studies reported that HDAC1 forms homo/heterodimers or works in complex with HDAC2 or HDAC3 [30]. It has been reported that HDAC1 has a high degree of homology with HDAC2 and overlap for various biological functions [30]. From the results, it is evident that HDAC1 is not required for RGS10 silencing in microglia. It could be possible that other HDAC isoforms may be compensating for HDAC1 activity at the RGS10 promoter to regulate its expression or that HDAC1 may require interaction with other HDAC isoforms to suppress RGS10 expression in response to inflammation. Accordingly, it could be that class I HDACs work in association with each other and regulate histone acetylation and gene transcription regarding RGS10 expression.

Relative expression of HDAC 1 and 2 vary with cell type. Also, heterodimer levels depend on cell type. Abundance of HDAC1 has been reported in glial cells. HDAC 1 and 2 form multiprotein complexes with other proteins and are recruited to chromatin by transcription factors and have diverse cell-specific roles [31]. HDAC1 hetero/homodimer may have different properties and substrate preferences. Understanding of mechanisms regulating homo/heterodimer formation will further help to understand the biology of theses enzymes and their activity.

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

The results from this study suggest that HDAC Class I selective inhibitor valproic acid is capable of blocking LPS induced suppression of RGS10 by its ability to cause histone hyperacetylation and induce gene transcription. Considering HDAC1 association at RGS10 promoter region when microglia were stimulated with LPS in previous studies, we expected that HDAC1 isoform could be regulating expression of RGS10. In contrast to our expectations, the obtained results suggest that HDAC1 isoform is not responsible for regulating expression of RGS10 under the tested conditions. This suggests that other HDAC isoforms may be regulating RGS10 expression in the absence of HDAC1 activity. Given the ability of HDAC1 to form homo/heterodimers with other isoforms of HDACs, it is also possible that HDAC1 could be working with other isoforms of Class I HDACs to regulate expression of RGS10 in microglia.

Further experiments are needed to fully understand which isoforms of histone deacetylases are involved in regulation of RGS10 expression. Pull down assays can be carried out to identify HDAC1 association with other HDAC isoforms in normal and activated microglia. ChIP assays could be carried out to identify if other HDAC isoforms bind to the RGS10 promoter region.

BV-2 microglial cell line has been well established to study microglial activation. Since these are immortalized cells there could be some changes in the epigenetics when compared to primary microglia.

Understanding of epigenetic regulation of RGS10 by HDACs helps to develop therapeutics to restore RGS10 in microglia and treat neuroinflammation. Owing to the anti-inflammatory properties of HDAC inhibitors and their ability to restore RGS10 in microglia which has shown to have a neuroprotective role, HDAC inhibitors have the potential to be used to treat neuroinflammatory disorders.

Exploring the epigenetic mechanisms regulating RGS proteins will help to further investigate their role in various pathological conditions such as cardiovascular disorders, neurodegenerative diseases and several types of cancers.

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