ELUCIDATING THE PATHOPHYSIOLOGY OF BIPOLAR DISORDER

BLOOD BRAIN BARRIER DISRUPTION AND MYC-ASSOCIATED FACTOR X (Max) GENE EXPRESSION IN THE PATHOPHYSIOLOGY OF BIPOLAR DISORDER

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

Master of Science (2017)
Neuroscience (MiNDS)

McMaster University
Hamilton, Ontario

TITLE: Blood-Brain Barrier Disruption and Myc-Associated Factor X (Max) Gene Expression in Bipolar Disorder

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NUMBER OF PAGES: xi,75

Abstract

Bipolar Disorder (BD) is a debilitating mental illness that presents as mood alterations between manic and depressive states. There remain large gaps in the knowledge surrounding the disease, due to three main issues in understanding the illness. First is a lack of an appropriate animal model that mimics both manic and depressive symptoms. Second is a lack of knowledge on the biological cause of the disease. Finally, a lack of knowledge on the precise mechanism of action of lithium (Li), the main treatment for BD prevents more progressive research into the disease.

Inflammation and a subsequent disruption of the blood-brain barrier (BBB) have recently been demonstrated in other psychiatric conditions, such as Alzheimer's Disease (AD) and Schizophrenia (SZ). This mechanism remains to be fully investigated in BD. This thesis presents an inflammatory model of BBB disruption in rodents.

A study examining gene expression in discordant sibling pairs with SZ or BD discovered that the Max gene was elevated two-fold in bipolar patients as compared to their non-BD siblings. We aim to elucidate on these findings and examine the effect of common BD treatments on Max gene expression.

The first study utilized lipopolysaccharides (LPS) to induce an inflammatory response in the BBB, and sodium fluoroscein (NaF) to measure the levels of resulting disruption. It was shown that Li is unable to attenuate disruption of the BBB, and an LPS administration with Li pretreatment causes higher disruption than either substance alone in several brain regions.

The second study examined Max gene expression levels in naïve rats as a result of Li or valproate (VPA) treatment. VPA was shown to significantly downregulate the expression of Max in a rodent model.

These studies may provide insight into understanding the pathophysiology of BD, leading to better, more accurate animal models and more targeted therapies for the disorder.

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Ram Mishra, for all his support and guidance over the past 2 years. Your constant encouragement, both in lab endeavours and in my career goals and further education, has been indescribably valuable. The students in the Mishra Lab are lucky to have your vast wealth of knowledge and your mentorship. Also thank you to Dr. Benicio Frey, for his much appreciated assistance, support, and input at every stage along this project. Your time and knowledge was greatly appreciated. Thank you to Dr. Laurie Doering for being an invaluable member of my committee, offering encouraging feedback and support along the way.

I am so appreciative for Jay Patel, Aaron Edward and Ritesh Daya, for taking me under their wing and teaching me everything there is to know about early morning injections, “textbook” perfusions, and how to curate the perfect soundtrack for late night analyses. Your expertise, work ethic, and patience were a joy to work with.

Thank you to the entirety of the Mishra Lab, both present and graduated members. To Ashley, Shreya, Luke, Jay, Omar, Sharon, Joella, Kos, Raphaela, Heta, Dima and Brett, it has been amazing working with all of you over the past 2 years. I could never ask for better lab partners or coffee run buddies. Thank you to Manpreet Sehmbi for being the most fun! We’ve had so many amazing times, and my entire grad school experience is better because of it.

Just as they impact every part of my life, I could never have done this without Annie Shi and Adrienne Meyers. Thank you for being the best

friends/entertainers/supporters/sisters I could ever ask for. I am forever grateful that you are in my life.

Finally, thank you to my family. To my amazing parents, Kavita and Pardeep, my supportive brother, Rohan, my loving grandparents, amazing uncle and aunt, Himanshu and Martine, and of course, my babies, Kiara and Nikhil, I will always be indebted to you. Thank you for all your sacrifices, love, and support that you have given to me. Thank you for keeping me grounded and on track. I love you all.

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List of Abbreviations

AD, Alzheimer's Disease

AJ, Adheren Junction

ANOVA, Analysis of Variance

AREB, Animal Research Ethics Board

BBB, Blood-Brain Barrier

BD-2, Bipolar Disorder 2

BD-I, Bipolar Disorder 1

BD, Bipolar Disorder

bHLHLZ, basic Helix-Loop-Helix Leucine Zipper

BL, Burkitt's Lymphoma

BLAST, Basic Local Alignment Search Tool

CCAC, Canadian Council on Animal Care

cDNA, complementary DNA

CNS, Central Nervous System

CREB, cAMP Response Element-binding Protein

Ct, Threshold Cycling

dlPFC, Dorsolateral Prefrontal Cortex

DNMT1, DNA Methyltransferase 1

EDTA, Ethylenediaminetetraacetic Acid

ERK, Extracellular Signal-Related Kinase

GABA, Gamma-aminobutyric acid

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

GSK3β, glycogen synthase kinase 3 beta

HAT, Histone Acetyl Transferase

HDAC, Histone Deacetylase

HSF1, Heat shock protein 1

IL-1B, Interleukin 1 beta

IMPA1, Inositol monophosphatase 1

IMPA2, Inositol monophosphatase 2

INPP1, Inositol polyphosphate 1-phosphatase

IP_3, Inositol triphosphate

JAM, Junction Adhesion Molecules

K/X, Ketamine/Xylazine

Li, Lithium

LiCl, Lithium Chloride

LPS, Lipopolysaccharides

MAPK, Mitogen-Activated Protein Kinase

Max, MYC-Associated Factor X

Mg^{2+}, Magnesium

mPFC, Medial Prefrontal Cortex

mRNA, messenger RNA

MS, Multiple Sclerosis

NaF, Sodium Fluorescein

OFT, Open Field Locomotor Task

PBS, Phosphate Buffered Saline

PFC, prefrontal cortex

PI-3K, Phosphoinositide 3-Kinase

PPI, Prepulse Inhibition

PVDF, Polyvinylidene difluoride

RELN, Reelin

RT-qPCR, Reverse Transcription – Real-time Polymerase Chain Reaction

SDS, Sodium dodecyl sulfate

SEM, Standard Error of Mean

SZ, Schizophrenia

TBI, Traumatic Brain Injury

TBS-T, Tris-buffered saline + Tween 20

TCA, Trichloroacetic Acid

TJ, Tight Junction

TNF-α, Tumour Necrosis Factor alpha

VPA, Valproate/Valproic Acid

ZO, Zona Occludin

CHAPTER 1: INTRODUCTION

1.1 Bipolar Disorder

Bipolar Disorder (BD) is a debilitating mental illness that affects more than 2% of the global population (Geddes & Miklowitz, 2013). There is a sex difference in the manifestation of BD, with women being twice as likely to be diagnosed as men. The estimated lifetime prevalence of BD is approximately 1% for Bipolar Disorder I (BD-I), and 1.1% for Bipolar Disorder II (BD-II) globally (Merikangas, Akiskal, Angst, Greenberg, Hirschfield, 2007). However, it has been suggested that the reported prevalence may underrepresent the true nature and epidemiology of the disorder. Diagnosing the disease has proven difficult, particularly in early stages, when the disorder first begins to emerge. BD begins to manifest in young adulthood, typically between 18-22 years of age. Due to the constraints in diagnosis, the true lifetime prevalence of the disease may be as high as 6%. Despite its relatively low occurrence, BD is a globally leading cause of premature mortality, reducing the lifespan of individuals by an average of nine years (Patel & Frey, 2015). Individuals with BD have a higher risk of suicidal behaviour when compared to both the general population and individuals with different psychiatric conditions (Song, Sjölander, Joas, Bergen, Runeson et al., 2017). When compared with other psychiatric conditions, BD is the 1st and 2nd leading cause of death by suicide in men and women, respectively (Nordentoft et al., 2011).

Additionally, BD is comorbid with a number of secondary medical and psychiatric conditions, including diabetes mellitus, cardiovascular disease, anxiety disorders,

impulse-control disorders, and substance abuse issues (Merikangas, Akiskal, Angst, Greenberg, Hirschfield, 2007). Comorbid diagnoses in BD are associated with a number of complications in the prognosis of the disease (Eisner, Johnson, Youngstrom & Pearlstein, 2017). A higher number of comorbidities are proportional to the severity of the disorder, time to recover from each episode, suicidal ideations, and risk of substance abuse. The number of comorbidities is also associated with a younger age of disease onset, poorer quality of life, and lower treatment responsivity. The recurrent, fluctuating nature of the disease makes it susceptible to high direct and indirect social and economic costs, which may be partially accounted for by patients with psychiatric and medical comorbidities (Merikangas, Akiskal, Angst, Greenberg, Hirschfield, 2007; Eisner, Johnson, Youngstrom & Pearlstein, 2017). There remain large gaps in the knowledge surrounding the disease, due to three main issues in understanding the illness. First is a lack of an appropriate animal model that mimics both manic and depressive symptoms. Second is a lack of knowledge surrounding the biological cause of the disease. Finally, a lack of knowledge on the precise mechanism of action of lithium (Li), the main treatment for BD, prevents more progressive research into the disease.

1.1.1 Symptomology & Pathophysiology

BD presents as fluctuations and cycling between manic and depressed states in a single patient, and much like other psychiatric disorders, has proven to be a difficult disease to treat (Patel & Frey, 2015). Manic states are characterized by a hyper-aroused state, increases in motor activity, impaired judgement and decreased sleep (Gould, Quiroz, Singh, Zarate & Manji, 2004). Depressed states mirror those of major depressive

disorder, presenting with a depressed mood, cognitive changes, and clinically significant impairments in social, occupational, and executive functioning (Hasin, Goodwin, Stinson, Grant, 2005). There are two main categories of BD diagnoses, BD-I and BD-II. BD-I is characterized by equally cycling episodes of mania and depression, while BD-II consists of predominantly depressive episodes, paired with occasional hypomanic periods (Moretti, Fraga & Rodrigues, 2017).

Underlying the difficulty in treating this disease is the mystery that still surrounds its pathophysiology (Kim et al. 2007). Like many other psychiatric disorders, bipolar disorder can present with diagnostic heterogeneity across patients. Psychotic episodes may feature during the manic episode of the disease, indicating a connection between BD and schizophrenia (SZ) (Goes, Sanders, Potash, 2008). In its early stages, BD patients present with excessive dopaminergic and glutamatergic signalling, mirroring the pathophysiology of schizophrenia (Moretti, Fraga & Rodrigues, 2017). Additionally, family studies point to a partial overlap in the genetics of SZ and BD (Craddock, O'Donovan & Owen, 2006; Berrettini, 2000). The Reelin (RELN) gene codes for a protein necessary for proper neurodevelopment (Guidotti, Auta, Davis, Gerevini, Dwivedi, et al., 2000). It has been shown that RELN is expressed at lower levels in SZ and BD patients as compared to non-psychiatric controls. This decrease may be due to epigenetic controls through methylation silencing in the gene's promoter region. Mouse models injected with methionine, a methyl group donator, demonstrated reduced RELN levels, as well as behavioural deficits in the pre-pulse inhibition (PPI) task, which measures sensorimotor gating abilities (Tremolizzo, Carboni, Ruzicka, Mitchell, Sugaya,

et al., 2002). Postmortem cortical samples of BD patients show an overexpression of DNMT1, the gene coding for DNA methyltransferase 1 (Veldic, Guidotti, Maloku, Davis, Costa, 2005). This enzyme is responsible for causing hypermethylation and a downstream effect of reduced promoter functioning. Valproate (VPA), a prominent treatment for BD, influences the effect of methylation through its targeted role on histone deacetylase (HDAC).

Previous efforts to elucidate on the pathophysiology of BD have addressed theories such as the disruption of monoamine signalling in the hypothalamic-pituitary-adrenal axis as a possible target (Manji & Lenox, 2000). Recent discoveries on the role of inflammation as a causal mechanism for a number of psychiatric and non-psychiatric disorders has created interest in the causal link between inflammation, specifically of the blood-brain barrier (BBB), and bipolar disorder (Kim et al. 2007). Other psychiatric conditions, such as schizophrenia and Alzheimer's Disease (AD) have been shown to present with increased inflammatory markers. Lipopolysaccharides (LPS) are a potent recruiter of inflammatory cytokines and microglial responses. LPS is a component of the cell wall in gram-negative bacteria (Glass, Saijo, Winner, Marchetto, Gage, 2010). LPS is a potent recruiter of microglia, and has been shown to produce an acute immune, inflammatory response. LPS has been shown to increase the blood-to-brain transport of amyloid-beta protein, and decrease the brain-to-blood transport of the same protein (Jaeger, Dohgu, Lynch, Fleegal-DeMotta & Banks, 2009). Amyloid-beta is heavily involved in the pathogenesis of AD, lending credibility to the theory of LPS' role in BBB inflammation.

The recent, controversial shift in focus from monoamines to changes in inflammatory cytokines, neurotrophins and oxidative stress shows potential in contributing to a more robust understanding of the pathophysiology of BD (Berk et al. 2010).

1.2 Treatments in BD

1.2.1 Lithium (Li)

Current treatment for bipolar disorder primarily involves the use of lithium and valproic acid, both of which are known mood stabilizers (Manji & Lenox, 2000). Lithium is a monovalent cation with powerful anti-inflammatory and anti-oxidant effects (Patel & Frey, 2015). Since its discovery in the 1950s, lithium remains the frontline treatment for BD. To date, it is the only treatment for BD that has shown efficacy for modulating both acute mania and depression (Young, 2009). On a macro level, lithium has been shown to attenuate clinical symptoms due to its unique anti-suicidal properties. BD patients are 10 times more likely to attempt suicide as compared to control populations, and significantly more likely to attempt suicide than any other psychiatric disorder. Lithium has been shown to reduce this risk of suicide by 60%. Lithium has also been shown to increase the volume of brain regions associated with emotion regulation, such as the prefrontal cortex, hippocampus and amygdala (Malhi, Tanious, Das, Coulston & Berk, 2013). Lithium peaks in serum levels approximately 4 to 5 hours after administration (Timmer & Sands, 1999). Lithium-responsive patients have been shown to have better executive functioning skills than non-responding patients (Malhi et al., 2013). On a micro scale, lithium alters

cellular, intracellular, and molecular processes. Lithium has been shown to attenuate pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 1-beta (IL-1β). Lithium acts to reduce oxidative stress by up-regulating mitochondrial complexes I and II (Malhi et al., 2013).

Lithium is thought to have direct effects on four main proteins (Drago, Crisafulli, Sidoti, Calabrò, Serretti, 2016). Three of these (IMPA1, IMPA2 and INPP1) are directly involved in the phosphatedylinositol signaling pathway. Li's inhibition of this pathway reduces inositol levels, influencing inositol triphosphate (IP_3) levels. This is an important effector which controls neuronal growth and stress-induced cognitive impairment related mechanisms (Drago et al., 2016). Although the exact mechanism of lithium's actions are yet unknown, it has a downstream effect on several pathways, including the inhibition of glycogen synthase kinase 3 beta (GSK3β) (Drago, Crisafulli, Sidoti, Calabrò & Serretti, 2016). Lithium has been shown to directly inhibit GSK3β activity through compeition with magnesium (Mg^{2+}) binding, which is required for its activity (Di Daniel, Budge, Maycox, 2005). GSK3β is a negative regulator of glutamate homeostasis. Glutamate, an excitatory neurotransmitter is elevated during manic episodes. Inhibition or silencing of GSK3β through phosphorylation suppresses glutamate excitotoxicity *in vitro* (Yu, Wang, Tchantchou, Chiu, Zhang et al., 2012). This inhibition promotes the activation of cell survival transcription factors, and further inhibits pro-apoptotic protein functioning, such as p53 and Bax (Yu et al., 2012). Lithium's effects on GSK3β have been shown to rescue neurons from apoptosis and other cell death stimuli (Di Daniel, Budge & Maycox, 2005). The vast array of GSK3β's downstream effects makes it a powerful target for lithium's

therapeutic actions. GSK3β is a vital component to multiple intracellular signalling pathways (Li, Bijur & Jope, 2002). Several other pathways also thought to be targets of lithium's therapeutic action can inhibit GSK3β. Phosphorylation by the phosphatidylinositol 3-kinase (PI-3K) and protein kinase B (Akt) pathways also act to inhibit GSK3β activity. GSK3β, as a pro-apoptotic enzyme, inhibits several transcriptional factors vital to cell survival, such as heat shock protein 1 (HSF1), cAMP response element-binding protein (CREB), and Myc. GSK3β is able to phosphorylate Myc at multiple sites, causing a decrease in cell growth and differentiation. Lithium's strong role in inhibiting the effects of GSK3β are vital to its therapeutic benefits in BD. This Li-induced GSK3B inhibition causes an overall reduction in microglial activation, cytokine release, and neurotoxicity (Yu, 2012). Increasingly, bipolar disorder is being recognized as a neurodegenerative process. Disease-related stressors, such as GSK3B-induced excitotoxicity, activate apoptotic pathways (Malhi, Tanious, Das, Coulston, Berk, 2013). By inhibiting GSK3B pathways, lithium is able to promote cell viability and longevity. Despite its efficacy in treating bipolar disorder, there remain a number of concerns regarding the use of lithium, one of which is that patients who are lithium-responders still show high rates of relapse (Gould et al., 2004).

1.2.2 Valproate

Valproate (VPA) is an anti-covulsant drug used in the maintenance treatment of BD (Macritchie, Geddes, Scott, Haslam, Goodwin, 2001). The utilization of anti-convulsant mood stabilizers in the treatment of BD has been established as a vital part of the BD treatment plan (Li, Bijur & Jope, 2002). VPA has been shown to be highly

effective in ameliorating manic episodes in BD, and may even be more effective than lithium in treating rapid cycling BD. However, VPA does not show the same protective effects as lithium in preventing suicidal tendencies and ideations (Song, Sjölander, Joas, Bergen, Runeson et al., 2017). The efficacy of valproate in ameliorating both manic and depressive symptoms is due to its ability to enhance GABA neurotransmission and inhibit enzymes involved in GABA metabolism (Chiu et al., 2013). VPA is a potent inhibitor of histone deacetylase. Histone proteins are responsible for organizing DNA in nucleosomes – structured, regular repeats of chromatin. HDACs are responsible for removing acetyl groups from these histone proteins, resulting in a transcriptionally inactive chromatin conformation. The inhibition of HDACs by VPA promotes a transcriptionally active chromatin conformation, affecting synaptic plasticity, learning and memory (Chiu et al., 2013). In vitro, VPA has been shown to robustly induce the activation of extracellular signal-regulated kinases (ERK) and mitogen-activated protein kinases (MAPK) (Di Daniel, Budge & Maycox, 2005). VPA is associated with a number of adverse side effects, including gastro-intestinal discomfort, development of tremors, ataxia, and in extreme cases, haematological dysfunction (Macritche et al., 2001). The discovery and utility of VPA in BD has opened the doors for further research into different anti-convulsant drugs in treating psychiatric conditions.

1.3 Blood-Brain Barrier (BBB)

The blood-brain barrier (BBB) is a protective barricade between the peripheral blood and central nervous system (Patel & Frey, 2015). It is responsible for regulating the transport of molecules, in and out of the central nervous system (CNS), as well as

protecting against various neurotoxins. By tightly regulating the movement of micro- and macromolecules in and out of the central nervous system, the BBB plays a protective role in ensuring the health of the human body's most complex system. The BBB is comprised of connective cells, transporting proteins, and immuno-specific cells. The BBB acts as a diffusion barrier, and is composed of brain endothelial cells, astrocytes, macrophages, and a basal membrane (Patel & Frey, 2015). These structures join together to form a monolayer barrier connected by tight junction (TJ) proteins and adherens junctions (AJ) (Abbott et al., 2010). Tight junction proteins are composed of a variety of protein families, notably the occludins, claudins, and junction adhesion molecules (JAMs). These tight junctions between endothelial cells of the BBB act to allow only essential, water-soluble nutrients and molecules to enter the CNS, while barring apoptotic neurotoxins. Transmembrane proteins such as occludin and claudin-5 are necessary for regulating the integrity and functioning of the BBB (Jiao, Wang, Liu, Wang & Xue, 2011). Zona-occludins (ZO) are located in the endothelial cell, and act to provide proper TJ placement.

The "bipolar" nature of the BBB influences both the CNS and the peripheral blood system (Banks & Erickson, 2009). Patients with bipolar disorder present with pro-inflammatory cytokine hyperactivity, resulting in increased levels of large, invasive molecules in the central nervous system (Kim et al. 2007). This suggests that disruptions in the BBB could be implicated in psychiatric conditions. Disruptions in BBB functions can arise from modifications in any of three areas: alterations in TJ presence, decrease in macropinocytosis functioning, or a loss of fenestrae (Banks, Gray & Erickson, 2015). Inflammation, through the administration of LPS can induce disruptions in the BBB by

altering TJ function and inducing leakage. The BBB is not a static entity – rather, components of the BBB change in response to the needs of the CNS (Banks & Erickson, 2009). The incredibly vital function that the BBB plays in the maintenance of health has given rise to an increasing interest in the role of the blood-brain barrier in the pathophysiology and development of a number of neuropsychiatric disorders – such as bipolar disorder. Recent studies looking at the role of cytokines in BD have produced inconsistent findings, and the role of inflammation remains controversial (Kim et al, 2007).

1.4 Myc-Associated Factor X (Max)

The Max gene codes for a basic helix-loop-helix-leucine zipper (bHLHLZ) protein that can form either hetero- or homodimers (Shichiri, Kato, Doi, Marumo, Hirata, 1999). The bHLHLZ family of transcription factors contains many proteins, notably Max, Myc and Mad (Naud et al., 2005). Max contributes heavily to a cell's ability to rescue itself from apoptosis, through its homodimeric activity. The Max-Max homodimer is a competitive antagonist of the Max-Myc heterodimer for the E-box DNA sequence (CACGTG) in target gene promoters. Most importantly, Max gene expression remains stable throughout the cell cycle, and affects cell proliferation, either activating or repressing the process, depending on its protein's dimerization partner (Shichiri et al., 1999). Max-Myc activates transcription, thereby promoting downstream cell proliferation. Due to its lack of a transcription activation domain, present only in Myc, the Max-Max homodimer represses transcription through its inability to bind the DNA E-box binding site. These Max dimerizations derive their activity from their ability to recruit

chromatin-modifying complexes to bind DNA and activate or repress transcription processes (Orian et al., 2003).

An up-regulation of Max may result in opposing physiological effects, dependant on the presence and availability of the c-Myc protein. An increase in Max messenger ribonucleic acid (mRNA) levels without a corresponding increase in c-Myc may indicate overall transcriptional inhibition by the max-max homodimer.

A study examined gene expression in blood leukocyte samples of discordant sib-pairs with schizophrenia or bipolar disorder (Middleton, Pato, Gentile, McGann, Brown, et al., 2005). The study provided a list of genes that were up- or downregulated in either disease state, and newly noted Max as an anomaly in BD. While the study did not disclose what, if any, drugs the patients were taking, they identified Max as having a two-fold increase in expression in bipolar patients as compared to normal siblings. This study aims to elucidate on these findings, and investigate the role of common BD drugs on Max expression.

1.5 c-Myc

Inherent to the regulatory role of Max is the interaction between Max and c-myc proteins. C-myc belongs to a family of potent oncogenes, acting as master regulators of cellular growth regulation and metabolism (Miller, Thomas, Islam, Muench, Sedoris, 2012). The c-Myc gene is necessary for development; deletions of the c-myc gene in murine models result in embryonic death (Davis, Wims, Spotts, Hann & Bradley, 1993). The Myc oncoprotein is a potent transcription factor implicated in a number of human cancers (Kim, Lee & Iyer, 2008). In normal, non-cancerous cells, Myc's activity is

induced by mitotic signals, and acts to regulate the activity and expression of downstream gene targets. Myc is capable of targeting a variety of downstream functions, including DNA and RNA metabolism, cell apoptosis, cell proliferation, signal transduction, and prolonging the cell cycle (Kim, Lee & Iyer, 2008). The Myc oncogene is vital in regulating these activities. However, when Myc is deregulated, high levels of Myc cause uncontrolled cell proliferation and an inhibition of cell differentiation. Thus, Myc deregulation is closely associated with tumourigenesis (Kim, Lee & Iyer, 2008; Dang, 1999). The ability of C-Myc to promote uncontrolled cell proliferation is highly suggestive of its ability to cause deregulated DNA synthesis and subsequent genomic instability (Mai, Fluri, Siwarski & Huppi, 1996). C-Myc's activity combines with activated oncogene Ras to cause cell proliferation. C-Myc is able to inhibit cellular responses that would provide inhibitory feedback to Ras. This inhibition results in Ras becoming a growth-promoting gene. Alterations in C-Myc activity are responsible for close to 15% of cancer-related deaths (Dang, 1999). C-Myc degregulation has been implicated in a variety of cancers, including Burkitt's Lymphoma (BL), breast cancer, gastrointestinal cancer and prostate cancer (Nesbit, Tersak & Prochownik, 1999). C-Myc amplification is more prominent in larger tumours, and is correlated with the progression of the disease (Nesbit, Tersak & Prochownik, 1999).

The Myc protein is unable to form homodimers or bind to DNA alone (Amati, Littlewood, Evan & Land, 1993). It is dependant on binding with Max in order to bind to the DNA E-box sequence and influence downstream targets. This dimerization is a

prerequisite for Myc's downstream activity of either inducing apoptosis or cell cycle progression.

Conversely, c-Myc can also act as a proapoptotic factor (Evan, Wyllie, Gilbert, Littlewood, Land et al., 1992; Juin, Hunt, Li, Guessous, Johnson, Eberhart, Li et al, 2007). In drug-naïve BD patients, proapoptic factors including p53 and c-myc show an increase in mRNA levels (Benes, MAtzilevich, Burke & Walsh, 2006). This upregulation disappears under treatment with neuroleptics. It is suggested that this apoptosis is potentiated by an increase in oxidative stress, as is seen in a number of psychiatric conditions.

This research project was focused on better understanding the cause and development of bipolar disorder. By creating a better model for understanding the biological mechanisms of bipolar disorder, true advances in the treatment of the disease can finally be made. The mystery shrouding the pathophysiology of this disease since its discovery more than a century ago is baffling, and must immediately be addressed. Our novel approach to the biology behind this disease shows to be a promising avenue for both better understanding its pathology, and also in creating a more accurate model to allow for future research into treatments for this devastating illness.

CHAPTER 2: METHODS AND MATERIALS

2.1 LPS Model of Inflammatory BBB Disruption

Hypothesis:

The hypothesis of this study is three-fold. First is that increased levels of sodium fluorescein (NaF) will be detected in rats treated with lipopolysaccharides (LPS), clearly indicating disruption of the BBB. Secondly, these disruptions of the BBB will be reflected in downregulated levels of significant tight-junction proteins of the BBB, specifically Claudin-V. Finally, pre-treatment with lithium will be able to rescue and diminish the disruption of the BBB.

Specific Aims:

Aim #1: To assess permeability of the BBB in various areas of the brain to determine if there is a clear connection between inflammation of the BBB and consequent development of bipolar disorder.

Aim #2: To determine if lithium can significantly prevent or rescue inflammation of the BBB.

Aim #3: To determine the effect of inflammatory agents on BBB tight junction protein expression.

2.1.1 Winter 2015 Cohort

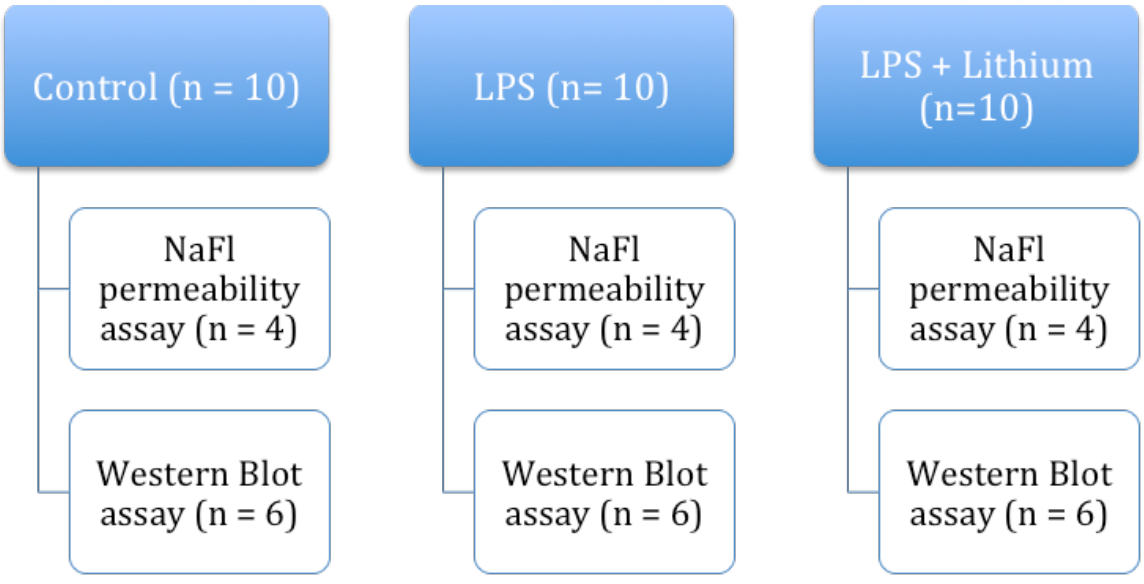
Animals:

Male Sprague-Dawley rats were obtained from Charles-River Laboratories (Wilmington, MA) weighing between 125-150 g. Rats were singly housed in standard

cages on a reverse 12-hour light/dark cycle, with access to food and water *ad libitum*. Rats were allowed to habituate to the McMaster Central Animal Facility for 2 days, and then handled for 5 days prior to their first treatment. All animal procedures were carried out in accordance with the Canadian Council on Animal Care (CCAC) and McMaster University Animal Research Ethics Board (AREB).

Treatment Protocol:

Animals were randomly assigned to one of three treatment groups (n = 10 per group): saline (0.9%; Baxter, Mississauga, ON, Canada), Lithium (LiCl) (47 mg/kg; Sigma Aldridge, St. Louis, MO, USA), or LPS (5mg/kg; Sigma, St. Louis, MO, USA). Treatments were administered intraperitoneally at a dose of 1mg/kg once a day for 8 days. 24 hours before sacrifice, animals in the LPS and Lithium groups were administered a one-time challenge of 5mg/kg LPS.



Sacrifice:

10% Sodium fluorescein (NaF; Sigma, St. Louis, MO, USA) was administered at 0.6ml/kg via tail vein injection 30 minutes prior to perfusion and sacrifice. Animals were administered a ketamine/xylazine (K/X) anaesthetic injection approximately 10 minutes prior to sacrifice. Once animals reached a surgical plane of anaesthesia, whole animal perfusion fixation through the circulatory system was performed, perfusing saline through the body for 30 minutes (Gage, Kipke & Shain, 2012). Animals were then decapitated using a standard guillotine, and brains were sectioned. Brain areas isolated and collected were the striatum, prefrontal cortex (PFC), cortex, hippocampus, cerebellum, and remaining whole brain samples. Samples were immediately flash frozen on dry ice. Samples were stored at -80°C until processing.

Blood Collection:

Blood collection was done through an incision in the right atrium prior to saline perfusion in SST Blood Collection Vacutainer tubes coated with ethylenediaminetetraacetic acid (EDTA). Samples were inverted five times, and kept at room temperature for 30 mins. Tubes were then spun at 2000 rpm for 10 minutes, and plasma was isolated into Eppendorfs. Samples were stored at -80°C until processing. Plasma levels were analyzed at St. Joseph's Healthcare Hamilton (Charlton Campus) using the Easylyte machine (Medica Vendo Cypress Diagnostics Inc., Belgium). Samples were tested for plasma concentrations of Li to ensure that levels fall within the therapeutic range for the drug.

Sodium Fluorescein Assays:

Brain tissue samples were first weighed to determine their weight. Samples were mechanically homogenized in glass homogenizers with 500uL of phosphate-buffereed saline (PBS). Proteins were precipitated with the addition of 500 uL of 50% tricholoroacetic acid (TCA). Supernantants were isolated, cooled on ice for 15 minutes, and then spun for 15 minutes at 10,000g. Samples were plated on 96-well plates along with a standard curve made with NaF dissolved in PBS with half-step dilutions. NaF levels were quantified on a Synergy 2 Microplate reader (Biotek, Winooski, VT, USA). Tissue fluorescence was normalized to serum fluorescence levels and tissue weight using the following formula:

$$\text{Relative fluorescence} = \frac{\text{Tissue fluorescence}}{\text{Serum fluorescence x brain tissue weight (g)}}$$

Western Blotting:

Western Blot analysis was conducted on a vital tight junction protein, Claudin-5. This was completed using the Bio-Rad ChemiDoc Western Blotting system and protocol (Bio-Rad). In short, tissue samples were homogenized using mechanical agitation and sonication in PBS. Initial concentrations of homogenized samples were determined using a Bradford Assay. 2X sodium dodecyl sulfate (SDS) sample buffer (Bio-Rad) and distilled water were added, and samples were stored at -20°C. Samples were separated by electrophoresis on 4-20% precast gradient gels (Bio-Rad). The semi-dry Trans-Blot Turbo system (Bio-Rad) conducts a 7-minute transfer of the separated proteins from the

gels to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Stain-free, total protein images were taken using the Bio-Rad ChemiDoc^{TM} MP Imaging System. PVDF membranes were blocked in 5% non-fat milk dissolved in TBS-T (0.1% Tween 20 from VWR and 1X Tris-buffered saline from BioShop Canada Inc., pH 8.5) to minimize background readouts in later imaging. PVDF membranes were incubated in primary antibody, Claudin-5 (1:1000) overnight at 4°C with gentle shaking on a nutator. Membranes were washed for two 5-minute and one 10-minute intervals the next morning in TBS-T. Membranes were then incubated in secondary antibody, anti-mouse (1:10000) for 1.5 hours. Membranes were then washed again, and imaged using the Bio-Rad ChemiDoc^{TM} MP Imaging System after activation with ECL (Bio-Rad). Colorimetric and Chemi Hi-Sensitivity images were captured and saved using the imaging system. Bands were identified and intensities were normalized to the corresponding stain-free total protein images using ImageLab software (Bio-Rad). Percent expression in relation to the average normalized intensity of the saline group was calculated.

Statistical Analysis:

All data are presented as a mean ± SEM. Comparisons of LPS treatment and NaF permeability was carried out using ANOVA and post-hoc Bonferroni's test. P-values <0.05 were considered statistically significant. GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses.

2.1.2 Summer 2016 Cohort

The methods in this cohort are identical to the Winter 2015 cohort, with the addition of a Li-only treatment group.

Saline	LPS	Li & LPS	Li
•n = 5	•n = 5 •Dose = 5 mg/kg	•n = 5 •Dose = 47.5mg/ kg LiCl + 5mg/kg LPS	•n = 3 •Dose = 47.5 mg/ kg

2.2 Li/VPA Administration Study

Hypothesis:

This study hypothesizes that the administration of mood stabilizers lithium and valproate will cause a reduction in the gene expression of Max and Myc in unchallenged rats, as compared to control animals.

Specific Aims:

Aim #1: To investigate the effects of mood stabilizers lithium and valproate on Max gene expression across various brain regions.

Aim #2: To investigate the effects of mood stabilizers lithium and valproate on Max and c-Myc gene expression in an *in vitro* model.

2.2.1 Winter 2016 Cohort

Animals:

Male Sprague-Dawley rats were obtained from Charles-River Laboratories (Wilmington, MA) weighing between 125-150 g. Rats were singly housed in standard cages on a reverse 12-hour light/dark cycle, with access to food and water *ad libitum*. Rats were allowed to habituate to the McMaster Central Animal Facility for 2 days, and then handled for 5 days prior to their first treatment. All animal procedures were carried out in accordance with the CCAC and McMaster University AREB.

Treatment Protocol:

Saline	LiCl	VPA
• n = 6	• n = 8 • Dose = 47.5 mg/kg	• n = 8 • Dose = 200mg/kg

Animals were randomly assigned to one of three treatment groups (n = 6-8 per group): saline (0.9%; Baxter, Mississauga, ON, Canada), Lithium (LiCl) (47 mg/kg; Sigma Aldridge, St. Louis, MO, USA), or Valproic Acid (200mg/kg; Sigma Aldridge, St. Louis, MO, USA). Treatments were administered intraperitoneally at a dose of 1mg/kg. LiCl and VPA were dissolved in saline to their final concentrations. The first injection was given in the evening on day 1, and the final injection was delivered in the morning on day 15. Animals were weighed before injections, and treated twice daily at 0900 and 1800 hrs for 2 weeks.

Sacrifice:

Animals were sacrificed 6 hours after their final morning injection on day 15. Rats were anesthetized (Isoflurane, Pharmaceutical Partners of Canada Inc., Richmond Hill, ON) and immediately decapitated. The brain was isolated, and the following brain regions were sectioned: prefrontal cortex (PFC), cortex, striatum, hippocampus, cerebellum and remaining whole brain tissues. Brain tissues were flash frozen on dry ice, and stored at –80°C until processed.

Blood Collection:

Blood collection was done at the decapitation site in SST Blood Collection Vacutainer tubes coated with EDTA. Samples were inverted five times, and kept at room temperature for 30 mins. Tubes were then spun at 2000 rpm for 10 minutes, and plasma was isolated into Eppendorfs. Samples were stored at -80°C for 2 days until processing. Plasma levels were analyzed at St. Joseph's Healthcare Hamilton (Charlton Campus) using the Easylyte (Medica Vendo Cypress Diagnostics Inc., Belgium). Samples were tested for plasma concentrations of LiCl and VPA to ensure that levels fall within the therapeutic range for each drug.

RNA Extraction:

Frozen brain samples were thawed in 1 mL of TRIzol. RNA isolation was carried out according to the TRIzol Reagent protocol (ThemoFisher Scientific, Oakville, Canada). RNA yield determination was done using the NanoDrop 2000 (ThermoFisher

Scientific, Oakville, Canada), and the purity of the sample was determined using the 260/280 absorbance ratios. Ratios between 1.8 and 2.2 were considered to be pure samples. RNA samples were DNase treated (ThermoFisher Scientific, Oakville, Canada), and diluted down to uniform RNA levels per brain region for complementary DNA (cDNA) preparation. RNA samples were reverse transcribed to generate cDNA using the QuantiFast cDNA Supermix kit.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR):

Max gene expression was determined by RT-qPCR. 2 µg of SH-SY5Y RNA was reverse transcribed with cDNA Superscript III (Invitrogen). The reverse transcription reaction was carried out at 25°C for 5 minutes, 50°C for 50 minutes, and 70°C for 15 minutes. Reaction master mixes for quantitative real-time PCRs were prepared, containing 12.5µL of SYBR Green qPCR Supermix UDG (Invitrogen, Oakville, Canada), 5µM of forward and reverse primers, and 0.5µL of nuclease free water for a total volume of 23µL of mastermix with 2µL of cDNA. Primers were designed to produce amplicons of the appropriate base pair length for each gene investigated. Primer pairs were optimized for complimentary annealing and melting temperatures. Primers were assessed by gel electrophoresis, and sequenced to confirm gene specificity (IDTMobix Lab, McMaster University, Hamilton, ON). Primer amplicons were excised from the gel using the Freeze ‘N Squeeze DNA Gel Extraction Spin Column (Bio-Rad). Amplicons were diluted down to a concentration of 1pg, and used to create a serial standard dilution. Standards were run on each plate. Primer sequences are described in Table 1. Samples,

including standards and negative controls (No-RT and no-template control), were run in triplicate technical replicates. Threshold cycling (Ct) values were normalized to the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the ΔΔCt method. The formula for this normalization is as follows: $2^{-\Delta\Delta Ct}$, where ΔΔCt = $(Ct \text{ gene of interest} - Ct \text{ internal control})_{\text{sample A}}$ - $(Ct \text{ gene of interest} - Ct \text{ internal control})_{\text{sample B}}$ (Schmittgen & Livak, 2008).

Statistical Analysis:

All data are presented as a mean ± SEM. Comparisons of treatment and gene expression was carried out using analysis of variance (ANOVA) and post-hoc Bonferroni's test. P-values <0.05 were considered statistically significant. GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	NCBI (Reference Sequence)	Amplicon Size (bp)
Max	CTC AGA CAA CAG CCT CTA CAC	AAG AAA CTG CGG ATG GAG G	NC_005105.4	128
GAPDH	CAA CTC CCT CAA GAT TGT CAG CAA	GGC ATG GAC TGT GGT CAT GA	NC_005103	210

Table 1: List of Winter 2016 primer sequences used for rat brain samples. Sequences were designed in lab, and verified through Basic Local Alignment Search Tool (BLAST).

2.2.2 Li/VPA Administration Study – Spring 2017 Cohort

The methods and materials in this cohort are identical to those in the Li/VPA Winter 2016 cohort, with exceptions made in the following areas.

Treatment Protocol:

Saline	LiCl	VPA
• n = 6	• n = 6 • Dose = 47.5 mg/kg	• n = 4 • Dose = 200mg/kg

Sample sizes were altered for the lithium and valproate groups, although dosing remained the same.

Locomotor Activity:

The open-field locomotor task (OFT) was used to assess locomotor activity. The task was performed two hours after the final injection on day 15. The task was conducted in an 80 cm by 100 cm enclosure with 50 cm high walls. The floor of the open field was covered in benchcoat marked with 10 cm^2 squares. Animals were placed in the front left corner of the enclosure and allowed to explore the arena. After a 5-minute habituation period, crossings of the lines and number of rearings were tallied for a 5-minute period (Frey, Valvassori, Réus, Martins, Petronilho, et al., 2006).

Sacrifice:

Animals were sacrificed 2.5 hours after their final morning injection on day 15. The brain was isolated, and the following brain regions were sectioned: prefrontal cortex (PFC), medial prefrontal cortex (mPFC), cortex, striatum, hippocampus, amygdala, cerebellum, and remaining whole brain tissues.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR):

C-Myc, one of the dimerizing partners of Max was also investigated in this cohort. The RT-qPCR primers are listed in Table 2. A different housekeeping gene was also tested in order to account for the difference in GAPDH expression seen in the cortex in the previous cortex. Two sets of 18S primers were investigated for this purpose.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	NCBI (Reference Sequence)	Amplicon Size (bp)
C-Myc	GAA GAA CAA GAT GAT GAG GAA	GCT GGT GAG TAG AGA CAT	NM_012603.2	160
18S (a)	TAC CAC ATC CAA GGA AGG CAG CA	TGG AAT TAC CGC GGC TGC TGG CA	NC_005113.4	180
18S (b)	GGC TCA TTA AAT CAG TTA TGG TTC CT	GTT GGT TTT GAT CTG ATA AAT GCA CG	NC_005113.4	147

Table 2: Spring 2017 list of rat gene primers for RT-qPCR analysis. C-Myc primers were retrieved from Begue, Douillard, Galbes, Rossano, Vermus, Candau & Py (2013). 18S primers were retrieved from Zhu & Altmann (2005).

CHAPTER 3: RESULTS

3.1 LPS Study

3.1.1 Winter 2015 Cohort

The results in Figure 1 clearly show an enhanced disruption of the BBB as a result of pretreatment with lithium and an LPS challenge, as compared to control saline or LPS-only treatment groups. The combination treatment causes a significantly higher disruption than control saline animals in the cortex and hippocampus. These results suggest that the combination of LPS and Li are more disruptive and toxic to the functionality of the BBB than an inflammatory challenge of LPS alone. The LPS-only treatment group demonstrated levels of disruption comparable to the saline control group in all brain areas, suggesting that either LPS does not cause BBB disruption as predicted, or that the NaF method utilized is not sufficient to detect this disruption.

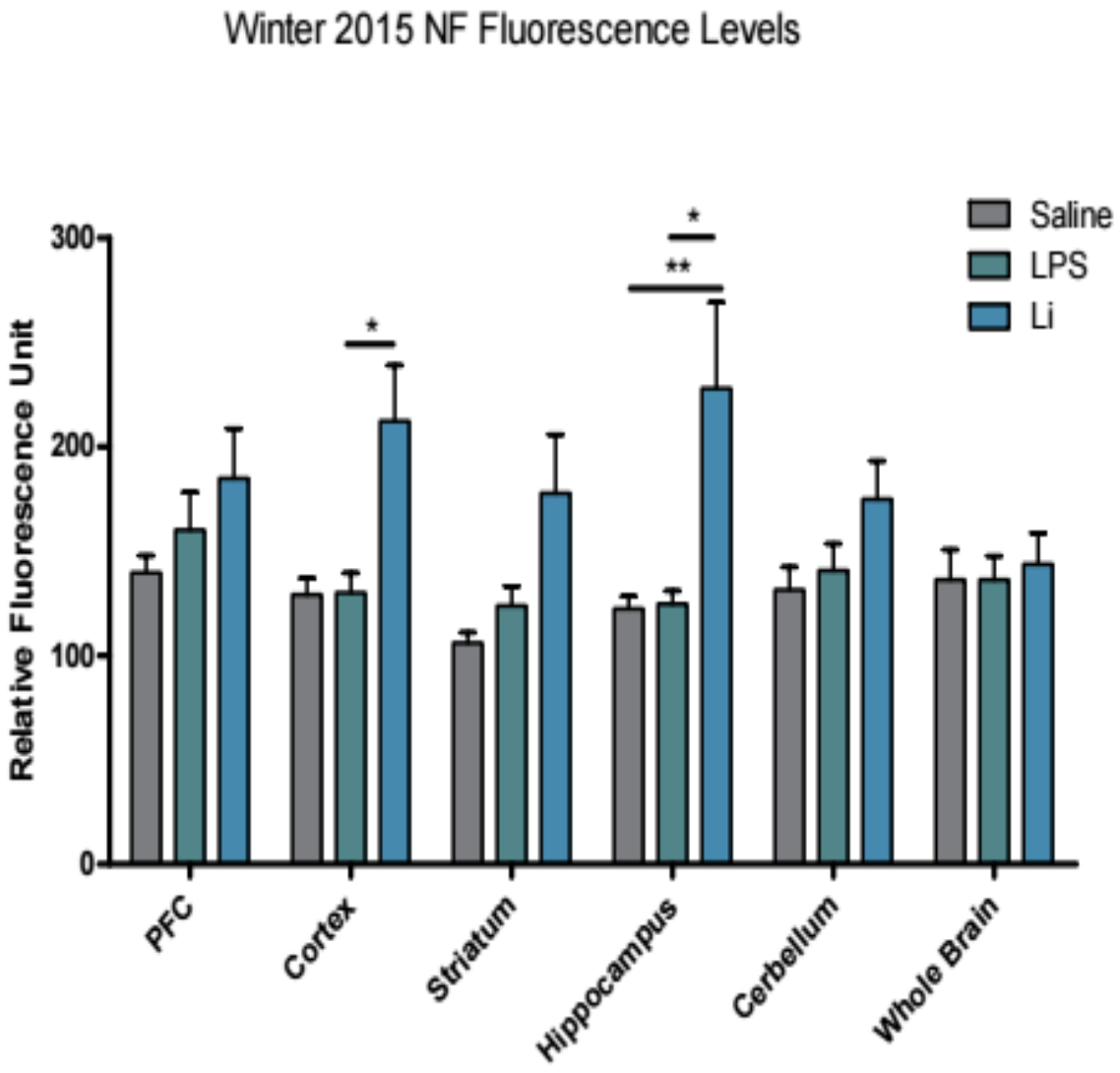


Figure 1: Winter 2015 NaF Fluorescence Levels. Levels of fluorescence in different brain regions were detected using a microplate fluorescence reader. Levels of NaF were normalized to the serum concentrations and tissue weights of each brain for each rat. Samples were compared to a standard with half-step dilutions of NaF on each plate. Samples were run in technical triplicates.

The results of the Li plasma drug concentration assays (Table 3) suggest that a 6-hour delay between the final treatment injection and sacrifice is successful in only 20% of cases in producing concentrations within the human therapeutic range of the drug. The human therapeutic range for humans is between 0.6-1.2 mmol/L, with lithium toxicity possible at the higher end of the range. Two animals in the Li treatment group demonstrated levels just below the therapeutic range, with concentrations of 0.5 mmol/L each. This suggests that decreasing the delay between final treatment and sacrifice by an hour would produce a more accurate model of lithium's therapeutic benefits in an LPS-challenged model.

Saline Group (mmol/L)		**LPS Group**		**LPS & Li Group**	
Study ID	**Lithium Level**	**Study ID**	**Lithium Level**	**Study ID**	**Lithium Level**
S001	<0.2	LP11	<0.2	L021	0.4
S002	<0.2	LP12	<0.2	L022	0.3
S003	<0.2	LP13	<0.2	L023	0.2
S004	<0.2	LP14	<0.2	L024	0.5
S005	<0.2	LP15	<0.2	L025	0.4
S006	<0.2	LP16	<0.2	L026	0.5
S007	<0.2	LP17	<0.2	L027	0.6
S008	<0.2	LP18	<0.2	L028	0.3
S009	<0.2	LP19	<0.2	L029	0.3
S010	<0.2	LP20	<0.2	L030	0.7

Table 4: Winter 2015 LPS Study Plasma Li Concentrations. Blood samples were collected prior to perfusion and assessed for whether or not plasma levels were within a human therapeutic range. The human therapeutic range for lithium is from 0.6-1.2 mmol/L. Two of the 10 animals in the lithium treatment group fell within this range.

In order to understand the mechanism of BBB disruption, the protein expression of a tight junction protein, Claudin-5, was examined through Western Blotting (Figure 2). Protein expression was examined in three brain regions: the hippocampus, PFC, and striatum. Claudin-5 expression did not change as a result of treatment in any of the brain regions examined. Protein expression was slightly increased in the striatum, as a result of the LPS+Li treatment as compared to saline control levels ($p = 0.16$).

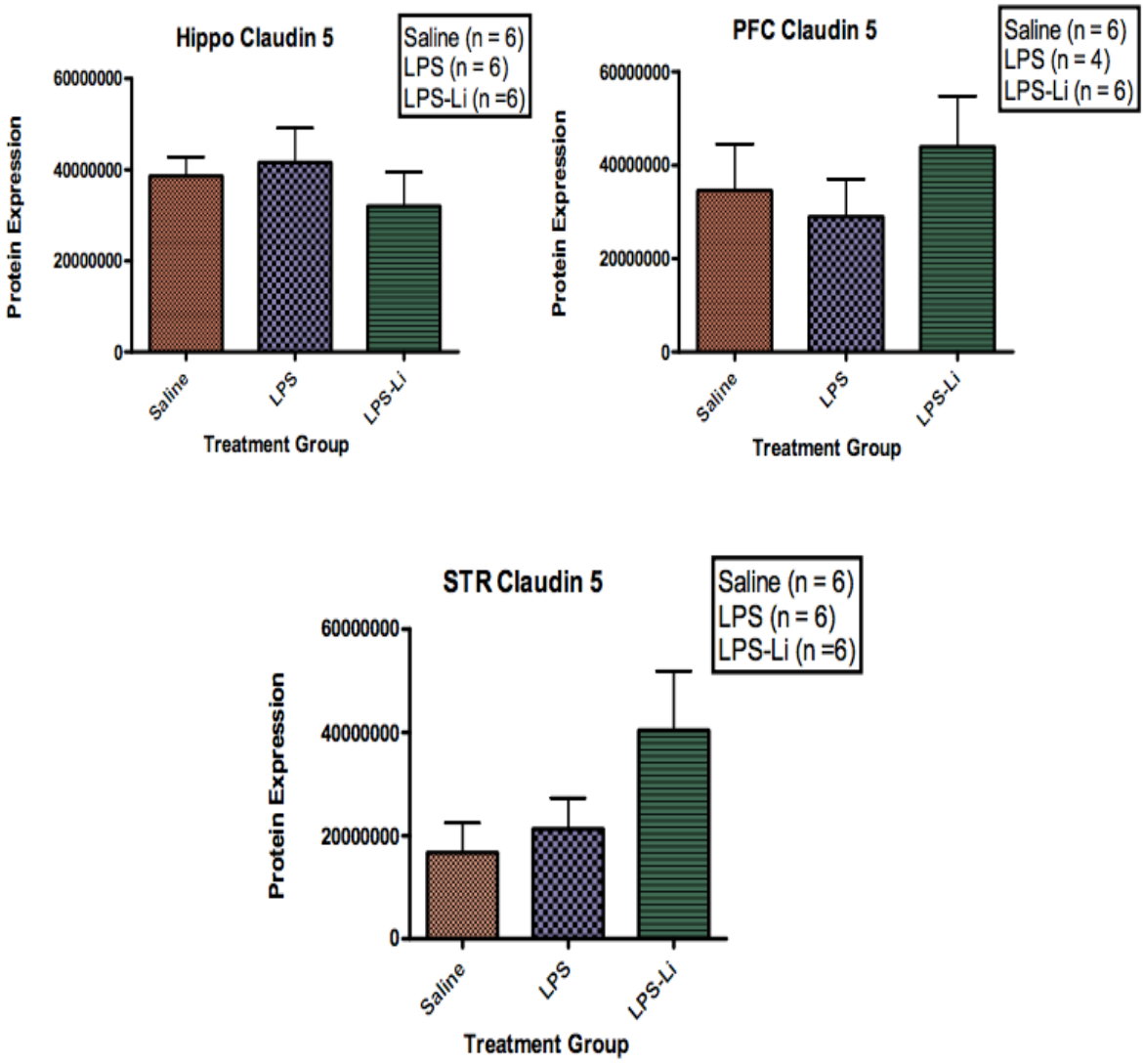


Figure 2: Winter 2015 Protein Expression of Claudin-5. The protein expression levels of Claudin-5 following an LPS challenge, and pretreatment with lithium were detected by Western Blot in three brain regions. Samples were normalized to a total protein expression of each sample. No significance is seen in any region as a result of treatment. 1-way ANOVA (p-value > 0.05 in all groups).

3.1.2 Summer 2016 Cohort

The results of the 2016 NaF assay (Figure 3) show a similar trend to the Winter 2015 cohort in the LPS+Li treatment group. The BBB shows increased disruption through permeability of NaF in the LPS+Li combination treatment group as compared to the saline control group. The hippocampus, as in the previous cohort, shows a high level of NaF permeability in this group. In this cohort, the PFC and striatum showed significant increases in BBB disruption, but not the cortex, as previously demonstrated. These results may suggest that with the exception of the hippocampus, BBB disruption and the subsequent NaF permeability is fluid across the brain when presented with an identical LPS+Li challenge. This cohort was able to produce a higher level of disruption with a 1-time administration of LPS as compared to the control saline level. LPS caused a higher level of disruption, as evidenced by increased detection of NaF, in the PFC, striatum, and hippocampus.

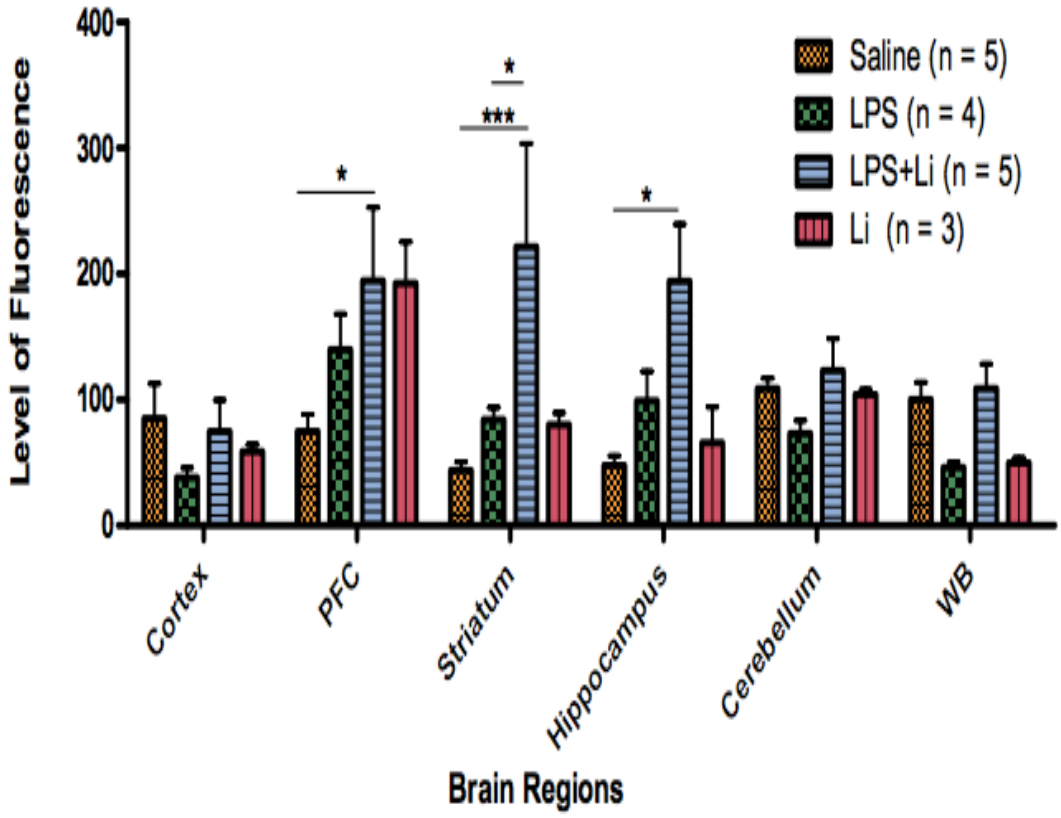


Figure 3: Summer 2016 NaF Assay Results. Levels of fluorescence in different brain regions were detected using a Synergy microplate fluorescence reader. Levels of NaF were normalized to the serum concentrations and tissue weights of each brain for each rat. Samples were compared to a standard with half-step dilutions of NaF on each plate. Samples were run in technical triplicates. 2-way ANOVA with Bonferroni post-hoc test (p-value <0.05).

Animals were weighed prior to each injection. While animals in the control group continued to gain weight, animals in the lithium pre-treatment groups consistently

weighed less, though not to a significant level. The addition of an LPS treatment on day 7 resulted in extreme weight loss.

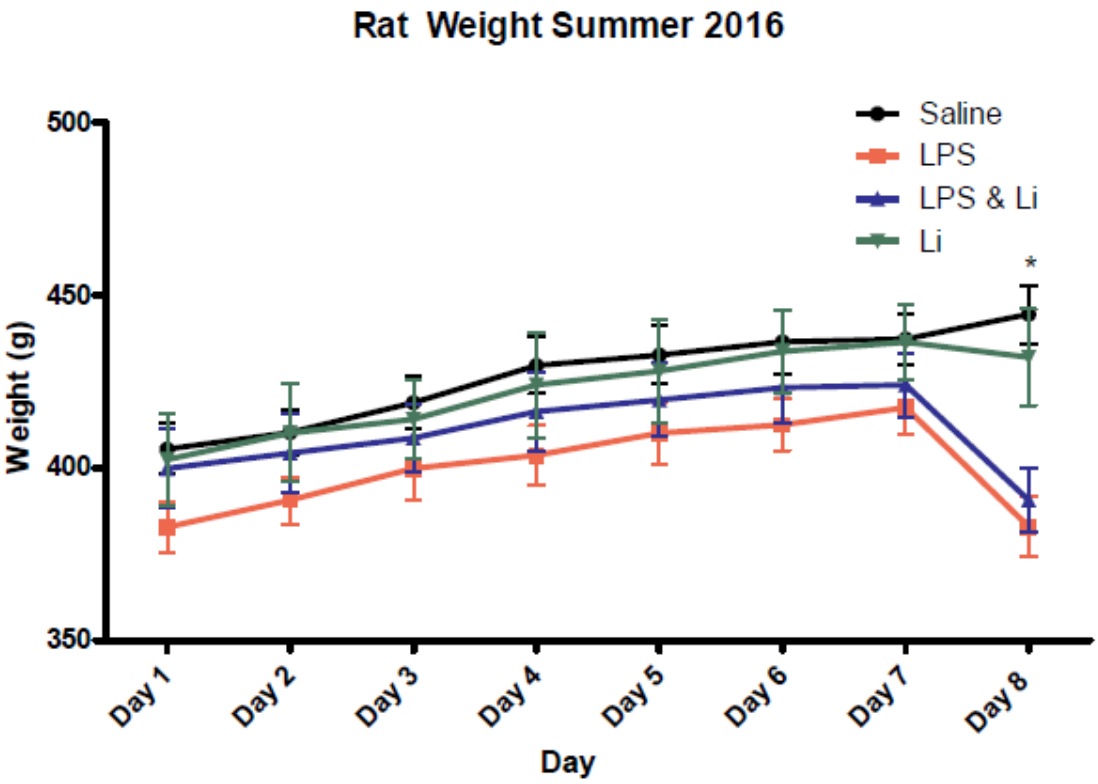


Figure 4: Animal weights as a result of treatment. Animals in the LPS-treated groups showed a significant weight loss following LPS-administration on day 7. Li-pretreatment was not sufficient in ameliorating this sudden weight loss. Saline (n = 5), LPS (n = 4), LPS+Li (n = 5), Li (n = 3). 1-way ANOVA of treatment was significant in the LPS-treated groups (p-value < 0.0001).

3.2 Li/VPA Administration Studies

3.2.1 Li/VPA Winter 2016 Cohort

GAPDH, a commonly used housekeeping gene was used to normalize the gene expressions of Max for the Winter 2016 cohort. It is expected with housekeeping genes that the expression of these essential genes should be unaffected by treatment. Significance between treatment groups in a brain region was not seen in any brain region with the exception of the cortex (Figure 4). 1-way ANOVAs were conducted on each brain region separately to determine if there is an effect of treatment. In the cortex, the p-value is 0.04.

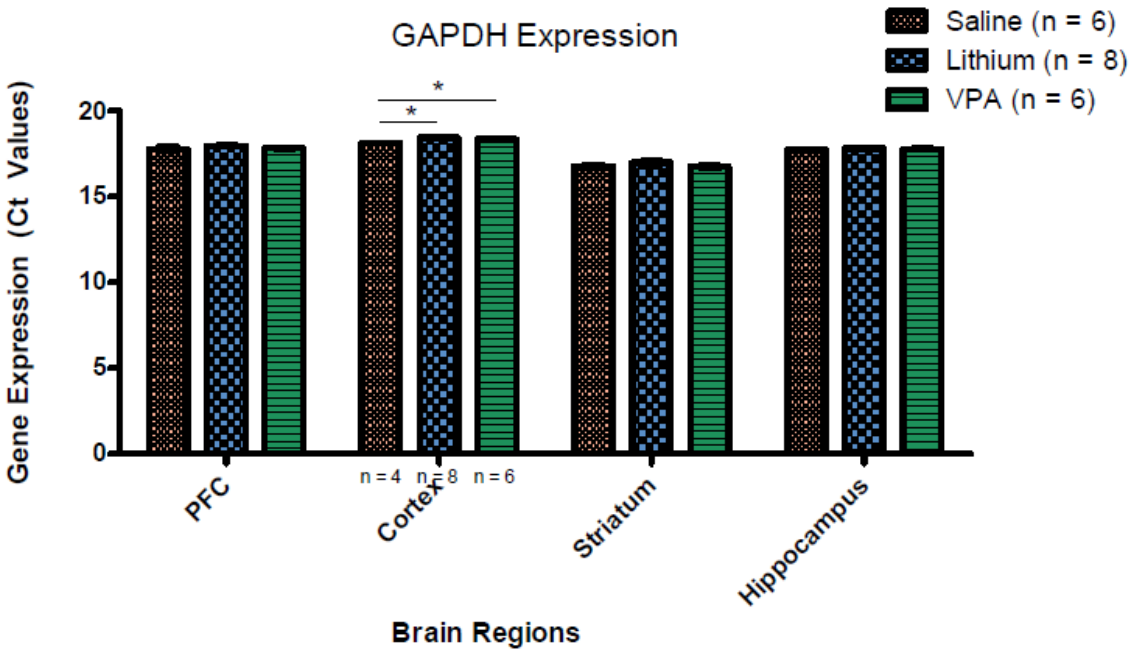


Figure 5: Li/VPA Winter 2016 - GAPDH Expression. GAPDH Ct values were determined through RT-qPCR. Ct values for these samples were used to normalize Max gene expression values. Differences in gene expression were not significant, with the notable exception of the cortex ($p > 0.05$).

The results of the Max RT-qPCRs (Figure 5) show that VPA significantly decreases Max gene expression across all brain regions studied, with the exception of the hippocampus. Lithium treatment produced Max gene expression levels comparable to saline in all brain regions studied. Threshold cycling (Ct) values for Max was normalized to Ct values of GAPDH in each brain region using the ΔΔCt method. These findings suggest that VPA's mechanism of action in BD may be associated with its important effect on downregulating Max gene expression.

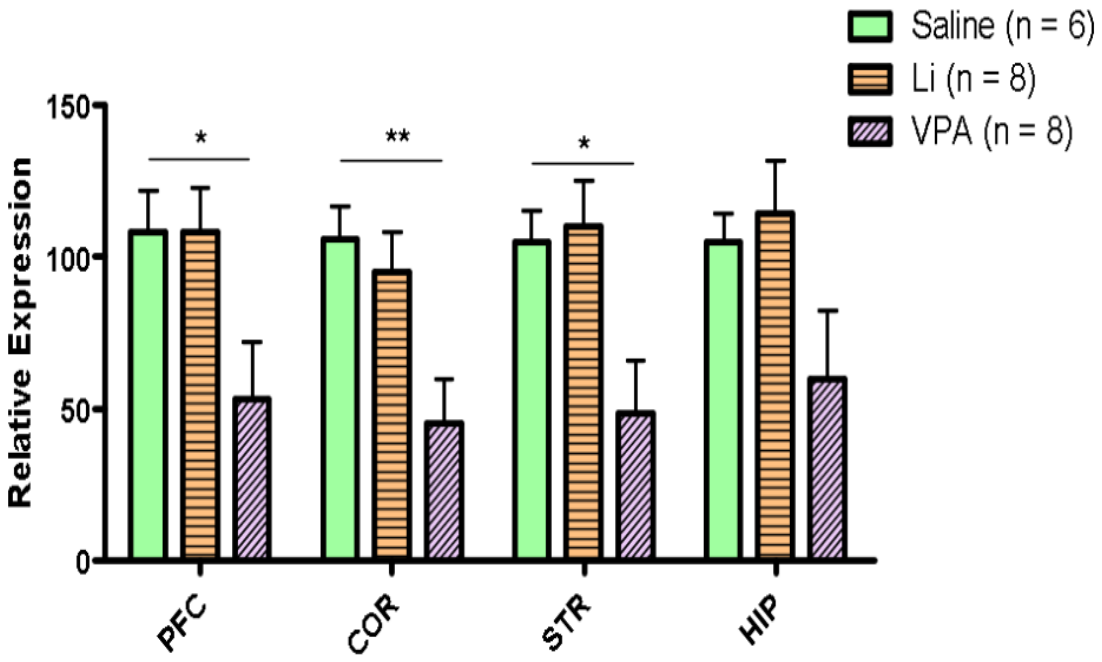


Figure 6: Li/VPA Winter 2016 Max Expression. Max gene expression was detected using a 2-step RT-qPCR. Levels of Max gene expression were normalized to the gene expression of a housekeeping gene, GAPDH.

The results of the 2016 Li plasma drug concentration assays (Table 4) suggest that a 6-hour delay between the final treatment injection and sacrifice is unsuccessful in producing plasma levels within the human therapeutic range for the drug. These results contradict the plasma results of the Winter 2015 BBB cohort, which showed a 20% success rate in achieving levels within the therapeutic range. These results also demonstrate that a 6-hour delay between final treatment and sacrifice is insufficient to produce VPA plasma levels within the therapeutic range. Rat #V016, although demonstrated VPA levels close to the range, was excluded from analysis due to the outlier nature of the plasma reading.

Saline (mmol/L)			Lithium Levels (mmol/L)		VPA Levels (µmol/L)	
Study ID	**Li Level**	**VPA Level**	**Study ID**	**Li Level**	**Study ID**	**VPA Level**
S001	<0.2	<42	L007	0.34	V015	80
S002	<0.2	<42	L008	0.40	V016	336
S003	<0.2	<42	L009	0.36	V017	79
S004	<0.2	<42	L010	0.43	V018	73
S005	<0.2	<42	L011	0.40	V019	77
S006	<0.2	<42	L012	0.30	V020	63
			L013	0.50	V021	51
			L014	0.40	V022	100

Table 5: Li/VPA Winter 2016 Plasma Drug Analysis. Plasma drug concentration results to determine if treatments were in the human therapeutic range for their respective drug. The therapeutic range for VPA is between 350-700µmol/L. Rat #16 was excluded from analysis due to abnormally high VPA plasma concentrations.

Animals were weighed daily prior to injections. At the end of the 14-day treatment regimen, animals in the control saline group weighed significantly more than those in the lithium-treated group (Figure 6).

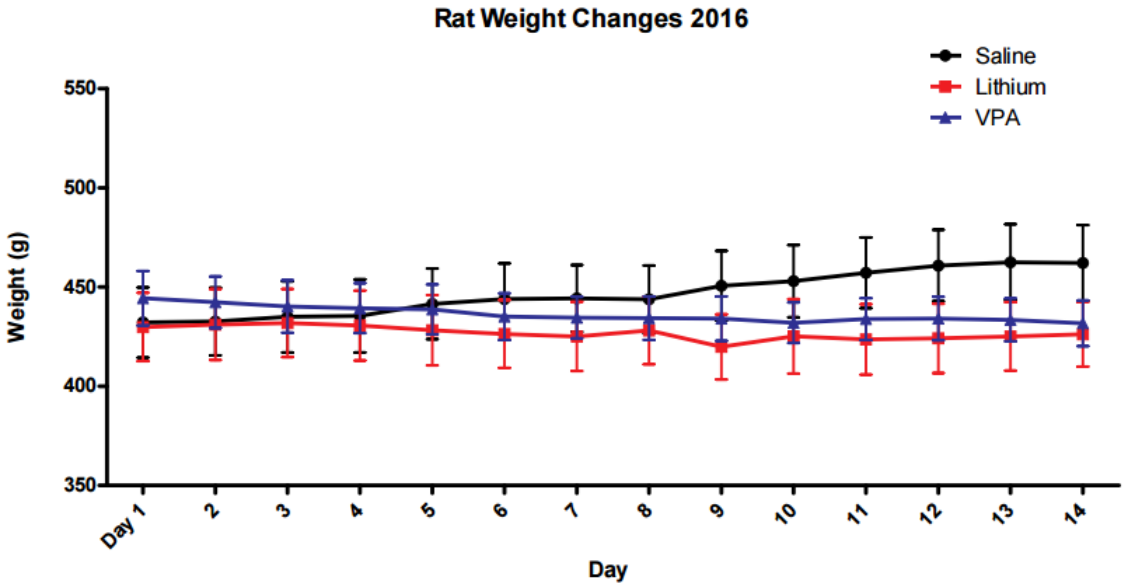


Figure 7: Rat weights over a 14-day treatment period. Animals were weighed prior to each injection. On day 14, there is a significant difference between animal weight as a result of treatment. 1-way ANOVA, p-value < 0.0001.

3.2.2 Li/VPA Spring 2017

This set of results was disregarded, as the animals in the cohort were physiologically unwell for the duration of the study.

CHAPTER 4: DISCUSSION

4.1 BBB-Disruption Study

In the LPS-BBB Winter 2015 cohort, we attempted to reproduce and verify previous work done in the summer of 2015 in collaboration with the Frey lab. We investigated the role of inflammation in the BBB, and more specifically, lithium's role in ameliorating this disruption. LPS is a potent recruiter of inflammatory and oxidative stress pathways, and is a promising method of causing disruptions in the BBB in murine models (Nisioku, Dohgu, Takata, Eto, Ishikawa, et al., 2008). LPS has previously been shown to have a significant effect in causing disruptions of the BBB, allowing for the movement of toxic and damaging neurotoxins across the BBB into the CNS (Wispelway, Lesse, Hansen & Scheld, 1988). It was also shown in our lab that lithium pretreatment could attenuate this disruption, and partially prevent the damaging effects of an inflammatory LPS challenge. The Winter 2015 cohort was run in an attempt to improve upon the small sample size of this previous cohort, and increase the reliability and veracity of this test. Animals were run under the same conditions, using the same protocol, materials and methods. Interestingly, this cohort gave contradicting results to the precursor study. A significant main effect of treatment on BBB disruption was observed, but in an opposite trend to the encouraging results of the previous cohort. A significantly larger BBB disruption was seen in the LPS + Li treatment group than both the saline group in the cortex and hippocampus. This indicates that the combination of lithium and LPS is somehow more inflammatory and disruptive than the application of

LPS alone. Although LPS has been reported to disrupt the BBB, more recent literature has shown that it is difficult to reliably and consistently disrupt the BBB with the use of LPS (Banks & Erickson, 2009). It has also been shown that inflammation in the BBB can be induced under disease states, as in rat models of multiple sclerosis (MS), but not necessarily through a systematic administration of LPS (de Boer & Gaillard, 2005). Although our study used a high dosage of LPS (5mg/kg), it may be possible that the 24-hour delay between the LPS injection and sacrifice may have been sufficient for natural immune system functioning to recover some of the damage done by the LPS. Lithium has reliably shown to attenuate neuroinflammation and oxidative stress in rodent models of traumatic brain injury (TBI), and mania (Yu, Wang, Tchantchou, Chiu, Zhang, et al., 2012; Frey, et al., 2006). The pathways by which the combination of LPS and Li cause an increase in BBB disruption is unclear, and remains to be elucidated.

In this cohort, we also investigated the effect of LPS and a pre-treatment of lithium on the protein expression of tight junction protein, Claudin-5. Claudin-5 is a member of the claudin family, and is implicated in maintaining BBB integrity. There was no significance of treatment seen in any of the three brain regions studied, the striatum, PFC and cortex. In the BBB, there are a number of other important protein families that maintain the TJs of the brain endothelial cells. These include occludin, zona occludins, and junction adhesion molecules. As stated before, the BBB is not a static entity, but rather can change in accordance with the CNS' needs. It is possible that claudin 5 is able to remain steady when presented with LPS, and other protein families are downregulated by the inflammation. Vascular endothelial growth factor (VEGF) is a growth factor which

is induced by inflammation (Morin-Brureau, Lebrun, Rousset, Fagni et al., 2011). The VEGF receptor has been shown to cause a downregulation in ZO expression, clearly indicating a correlation between inflammation and decreased or mislocalized TJ functioning. To fully understand the role of inflammation on the BBB, as well as lithium's role in rescuing the subsequent disruption, other protein families in the BBB could be investigated.

As seen in Table 3, only two of the ten animals pretreated with lithium were within the therapeutic plasma drug concentration range. Decreasing the time between the final lithium injection and sacrifice to around two hours would place animals within the therapeutic range for the drug, and would give a clearer picture of lithium's true effects on preventing BBB disruption (Wood, Goodwin, De Souza & Green, 1986).

The summer 2016 cohort was created to replicate the methods and protocols of the precursor study, as well as the winter 2015 cohort. The goal was to determine the correct trend in results, and verify which previous cohort provided more accurate results. The outcomes from this study corroborated the results of the Winter 2015 study in the LPS+Li treatment group. This combination treatment was shown to produce significantly higher disruption in the hippocampus in both cohorts. The Summer 2016 cohort was successful in producing a higher level of disruption in the LPS group than controls in the PFC, striatum and hippocampus, an effect not seen in the Winter 2015 cohort. This may be an indication of procedural errors in the Winter 2015 cohort. Of note in both cohorts, as well as the preceding cohort in the summer of 2015, is the effect of a joint LPS+Li treatment. It was anticipated that this combination would cause a reduction in the amount of

disruption as caused by LPS. However, across most brain regions in both cohorts, this combination causes an increased level of BBB disruption. LPS is a potent inducer of neuroinflammatory response (Banks & Robinson, 2009). It binds to toll-like receptors (TLRs), and is responsible for causing a neuroimmune response. In rodents, LPS administration causes significant reductions in body weight, diarrhea, suppression of locomotor, exploratory and social behaviours, fatigue, impairment of cognitive abilities, and anhedonia (Yirmiya, Pollak, Barak, Avitsur, Ovadia et al., 2000). LPS has been shown to mimic the behavioural and neuroendocrine symptom set of depression (Yirmiya et al., 2000). Chronic lithium usage can have severe side effects, the most common of which is polyuria-polydipsia syndrome (Henry, 2002). In our rodent studies, we observed this polyuria-polydipsia syndrome, as well as less weight gain over the treatment period as compared to control saline animals. Although chronic lithium use is associated with weight gain in a clinical population, the opposite trend has been seen in all our rat cohorts (Henry, 2002). The combination of negative symptomology associated with LPS and lithium, such as weight loss, fatigue, diarrhea and polyuria-polydipsia syndrome may combine to exacerbate, rather than alleviate inflammatory disruptions across the body and in the BBB.

4.2 Li/VPA Max Study

The effects of mood stabilizers lithium and valproate on the gene expression of Max hold several interesting implications for understanding the pathophysiology of bipolar disorder. As the Middleton et al. study demonstrated, Max gene expression levels are elevated 2-fold in bipolar patients as compared to their discordant, matched sibling-pairs (2005). Lithium is the most commonly prescribed treatment for bipolar disorder, as it has an effect on stabilizing both manic and depressive episodes. VPA is a secondary treatment, but has also proven to be therapeutically beneficial in treating the symptomology of BD. In this study, lithium's ability to produce Max mRNA levels similar to saline control animals could be indicative of its therapeutic benefits. Max mediates both positive and negative transcriptional effects on E-box-containing target genes (Ayer & Eisenman, 1993). Lithium has long been the first-line treatment option for bipolar disorder, with the caveat that its underlying mechanisms remain unknown. The results of our study suggest that lithium's effect on ameliorating elevated Max gene expression levels back to normal, control levels may play a role on its therapeutic role in treating bipolar disorder. Even at sub-therapeutic levels, this effect can be seen with a chronic, 2-week treatment.

More interesting are our findings on VPA's effect on Max expression. VPA's ability to significantly downregulate Max gene expression in the cortex, striatum and PFC could also be indicative of its therapeutic effects. VPA's therapeutic role in BD and epileptic disorders revolves around on its role as a HDAC. C-Myc, one of Max's dimerizing partners, has been shown to enhance histone acetylation (Fernandez, Frank,

Wang, Schroeder, Liu et al., 2003). As stated before, C-myc's functionality depends on its dimerization with Max and the dimer's subsequent binding to a DNA E-box. C-myc further increases the acetylation of histones that are already pre-acetylated, causing substantial net increases in acetylation (Fernandez et al., 2003). VPA's role as an HDAC allows for continued histone acetyl transferase (HAT) activity, causing increased acetylation at the histone site. The combination of two potent acetylation activators should theoretically lead to over-acetylation and deregulation of cell functions. However, VPA's effect of significantly reducing Max expression corrects for this deregulation. A reduction of Max expression would reduce the functional activity of C-myc, due to a lowered dimerizing potential.

Spring 2017 Cohort:

A few adjustments were made to the protocol of this study. We aimed to improve upon the drug dosing in order to get animals into the human therapeutic range for their respective drug treatment groups. To address this, the time between final treatment injection and sacrifice was reduced from 6 hours to 2.5 (Zanni et al., 2017). We also aimed to study the expression of C-myc in this cohort, to elucidate the functions of Max. Our third goal for this cohort was to add two brain regions to our analysis: the mPFC and amygdala. The rodent mPFC correlates to the human dorsolateral prefrontal cortex (dlPFC) (Uylings, Groenewegen & Kolb, 2003). The dlPFC of bipolar patients shows reduced neuronal, pyramidal and glial cell densities (Rajkawska, Halaris & Selemon,

2001). Amygdalar volume reductions are also shown in BD patients as compared to controls (Blumberg, Kaufman & Martin, 2003).

Unfortunately, the results from this cohort were excluded, due to issues with the animals. Two rats were excluded from the study due to early complications, which arose at the beginning of treatment. These rats were physically unwell, and appeared to be urinating dilute blood before their first injection. Although all the other rats appeared physically well and did not show any of the same symptoms, it may be possible that the cohort as a whole was not ideal for the study, due to physiological issues.

CHAPTER 5: LIMITATIONS AND FUTURE DIRECTIONS

5.1 BBB Study

The results of this study, while promising, hold room for improvement and optimization. One such limitation in our study was the sub-therapeutic levels of lithium detected in our cohorts. This can be remedied by altering the time before sacrifice, in order to ensure that animals have plasma drug concentrations within the therapeutic range for the drug of interest. While there is a difference of effect seen between the treatment groups, it is imperative to build upon these results by placing lithium within its therapeutic range. With regards to the heightened levels of disruption seen in the LPS+Li treatment group across cohorts, this elevation in BBB disruption may be the result of sub-optimal Li dosing. Increasing the pre-treatment schedule of lithium from 8 days to 14 would allow for a chronic lithium effect in the animal, and give better insight into the role of lithium in ameliorating the symptoms of LPS inflammation.

Examining other TJ proteins is also vital to building a profile of inflammatory disruptions in the BBB. Other important TJ proteins, such as occludin, may be affected by LPS treatment, and may show the preventative effects of lithium. Studying the expression of ZO's would also provide insight into the placement and expression of proteins located outside of the endothelial cells that make up the BBB. Examining transport proteins such as aquaporin would provide better information on the mechanism of LPS's neurotoxic effects. The gene expressions of these proteins could also be examined, in order to determine the effects of these treatments on a nuclear level.

5.2 Li/VPA Study

For the Li/VPA animal study, it is imperative to re-run the study, with animals within the therapeutic range for each drug. Our attempt at replicating the study was unsuccessful due to complications with the animals themselves, preventing further insight into the role of Li and VPA on max gene expression and its subsequent effects on c-myc and downstream transcriptional factors. It would also be beneficial to introduce a behavioural paradigm to the study, examining the cognitive, social and motor abilities of the animals as a result of treatment. A task such as Novel Object Recognition would allow for insight into learning and memory consolidation as a result of treatment with lithium and VPA, and their correlating effects on Max and c-Myc gene expression (Grayson, Idris & Neill, 2007).

Our attempts at examining c-Myc expression in relation to Max were also unsuccessful as a result of the suboptimal animals in the Spring 2017 study. It is vital to examine the effects of Li and VPA on c-Myc in order to further elucidate Max's role in the brain, and the overall effect on downstream transcriptional targets. As in the BBB study, it is imperative to reduce the pre-sacrifice time from 6 hours to 2 hours, in order to see the true effects of these drugs in their known therapeutic ranges.

An *in vitro* study should also be run, in order to understand the role of mood stabilizers on oncogene expression in a human cell line. While VPA is a known down regulator of c-myc, lithium's effects on the gene have not been studied in detail. Doing so may deliver insight into lithium's mechanism of action, and help build the puzzle of lithium's effects on downstream effectors.

CHAPTER 6: CONCLUSIONS

The present studies aimed to elucidate the role of BBB disruption and permeability in BD, as well as establish the effects of mood stabilizers on Max gene expression.

LPS was utilized to model the inflammation seen in BD, while lithium, a potent BD treatment, was used to prevent this permeability. Interestingly, we saw the highest levels of BBB permeability in the LPS+Li treatment group. This suggests that the combination of treatments causes significantly greater disruptions in the BBB than a separate treatment of LPS or lithium alone. These results were seen in both our Winter 2015 and Summer 2016 cohorts, and corroborate the effects seen in the precursor study run in the summer of 2015.

Our study on Li and VPA's effects on Max gene expression show some promising results for expanding on the pathophysiology of BD. Our results suggest that VPA's significant downregulation of Max may help elucidate its therapeutic benefits in BD. Upregulating Max gene expression may aid in the creation of an effective BD animal model, and allow us to better study the pathophysiology of the disease.

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