BLOOD BRAIN BARRIER DISRUPTION IN BIPOLAR DISORDER

CHARACTERIZING THE ROLE OF BLOOD BRAIN BARRIER
DISRUPTION IN BIPOLAR DISORDER

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Abstract

Bipolar disorder (BD), previously known as manic depressive disorder, is associated with recurring episodes of depression and mania/hypomania. Currently, no definitive biological mechanisms have been pinpointed with regards to the origin and progression of BD, however, inflammation and oxidative stress have been shown to present in the brains of individuals with BD. Notably, other neurodegenerative disorders which also contain an inflammatory component including Alzheimer's disease and Multiple Sclerosis display with disruption of the brain blood barrier (BBB). We thus propose a model of BD wherein BBB disruption facilitates inflammation and oxidative stress induced neural damage.

This study looked to utilize amphetamine (AMPH) induced mania model and lipopolysaccharide (LPS) induced inflammatory model to represent BD like conditions in rats and to assess BBB permeability. We observed elevated locomotor data in response to AMPH administration and a trend of increased BBB permeability across regions following low dose chronic AMPH injections. In the LPS induced BBB permeability model, we did not detect any elevated serum C-reactive protein (CRP) or tumour necrosis factor alpha (TNF-α) levels but did see significantly elevated BBB permeability in the LPS group and lithium pre-treatment providing some protection against BBB permeability in one of our cohorts. These trends were further corroborated by a follow-up study and thus warrant further investigation into the mechanistic nature of BBB breakdown in

this model. This may provide a breakthrough in understanding the pathophysiology of BD and the underlying mechanistic effects of lithium, paving the way for new more target therapeutic remedies to combat this debilitating disorder.

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List of Abbreviations

AD, Alzheimer's disease

AJ, Adherens junction

AMPH, Amphetamine

ANOVA, Analysis of variance

BBB, Blood Brain Barrier

BCA, bicinchoninic acid assay

BCSFB, Blood-cerebrospinal fluid barrier

BD, Bipolar Disorder

CAT, Catalase

CNS, Central nervous system

CRP, C-reactive protein

CSF, Cerebrospinal fluid

DCE-MRI, Dynamic enhanced contrast magnetic resonance imaging

EB, Evans blue

ELISA, Enzyme-linked immunosorbent assay

FL, Sodium Fluorescein

GABA, γ-Aminobutyric acid

IL-1, Interleukin 1

IL-6, Interleukin 6

i.p., intraperitoneal

JAM, Junctional adhesion molecules

Li+LPS, Rats pretreated with lithium and challenged with lipopolysaccaride

LPS, Lipopolysaccaride

MADRS, Montgomery-Asberg depression scale

MMP, Matrix metallopeptidase

MS, Multiple sclerosis

NGF, Nerve growth factor

NMDA, N-methyl-D-aspartate

NVU, Neurovascular unit

PBS, Phosphate-buffered saline

PCR, Polymerase chain reaction

PDQ, Perceived deficits questionnaire

PET, Positron emission tomography

ROS, Reactive oxygen species

SCID, Structured clinical interview for DSM disorders

SOD, Superoxide dismutase

SCZ, Schizophrenia

TBARS, Thiobarbituric acid reactive substances

TCA, Trichloroacetic acid

TNF-α, Tumour necrosis factor α

TJ, Tight junctions

YMRS, Young mania rating scale

CHAPTER 1:
Disruption in the Blood-Brain Barrier: The Missing Link Between Brain and Body Inflammation in Bipolar Disorder?

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1.1 Physiology of the Blood-Brain Barrier

1.1.1 Structure of the BBB

The characterization of blood-brain barrier (BBB) began in 1885 with Paul Ehrlich's reports that various water-soluble dyes failed to stain the brain and spinal cord upon injection into the circulatory system, which he attributed to the lower affinity for the dye by the CNS (Ribatti, Nico, Crivellato, & Artico, 2006; Wong et al., 2013). Later in 1898, Biedl and Kraus demonstrated that only injection of bile acids directly into the brain caused symptoms including seizures and coma, but not when injected into the circulatory system (Wong et al., 2013). In 1900, Lewandowsky demonstrated a similar effect using potassium ferrocyanide, and attempted to describe this with the term *bluthirnschranke* (blood-brain barrier) (Ribatti et al., 2006). Further experimentation by Goldmann, a student of Ehrlich, demonstrated that trypan blue when injected into the cerebrospinal fluid (CSF) stained CNS tissue, contradicting Ehrlich's dye affinity hypothesis and lending support to the notion that there is a barrier between the circulatory system and the CNS (Wong et al., 2013). Then in 1967, with newly available electron microscopy technology, Reese and Karnovsky demonstrated at the ultrastructural level that horseradish peroxidase (HRP) was unable to enter the CNS due to the presence of tight junctions (TJ) (Bradbury, 1993). This showed the continuous nature of the BBB in the CNS and led Reese and Karnovsky to conclude that the BBB existed at the level of the endothelial cells.

Acting as a diffusion barrier, the BBB is composed primarily of brain endothelial cells, astrocyte end-feet, pericytes, perivascular macrophages, and a

basal membrane. Its barrier is a result of a tightly sealed monolayer of endothelial cells with TJ and adherens junctions (AJ) forming the seal between cells at junctional complexes. The basal membrane and astrocyte end-feet contribute to BBB function and integrity by regulating the expression of specific TJ proteins and other BBB transporters. Essentially, the TJ are the result of ostensible fusion between the outer lipid bilayers of neighbouring endothelial cells. Claudin, occludin and junction adhesion molecules primarily form the composition of TJ, which serve to limit permeability between cells and to increase the barrier's electrical resistance. As a class of transmembrane proteins, two claudin extracellular loops undergo homophilic binding to loops from claudins on adjacent endothelial cells, forming the primary seal of the TJ. Distinct claudins isoforms regulate the diffusion of different sizes of molecules. To date, claudin -3, -5, and -12 are thought to be incorporated in the BBB (Nitta et al., 2003; Berislav V Zlokovic, 2008), while the presence of claudin-1 is still in debate (Lee et al., 2003). For instance, claudin-5 knockout mice display abnormal endothelial cell TJs, increased BBB permeability to small molecules (<800 Da) and die shortly after birth (Nitta et al., 2003). Another transmembrane protein, occludin, is also implicated in the foundation of TJ. Similar to claudins, two occludin extracellular loops homophilically bind to occluding loops on a neighbouring cell, abetting in the formation of the TJ. In an occludin construct lacking the N-terminus and extracellular domains, an efficient permeability barrier failed to take shape with unblocked diffusion of several small markers and the presence of gaps – thus

establishing the underlying significance of occludin proteins in the formation of TJ. Belonging to the immunoglobulin superfamily, junctional adhesion molecules (JAMs) with their single transmembrane domain are thought to contribute to the sealing capacity of TJ. However, the exact role of JAMs in the function of the BBB is still not fully understood. Adherins junctions (AJ) are typically found to be intermixed with TJ in the BBB. AJ are composed of the membrane protein cadherin whose extracellular domain homophilically binds cadherin on adjacent cells while the cytosolic domain in bound to catenins, which in turn are bound to the actin cytoskeleton of the cell, effectively joining neighbouring cells.

Unlike in the BBB where the barrier is localized at the level of the endothelial cells, the blood-cerebrospinal fluid (CSF) barrier (BCSFB) is established by choroid plexus epithelial cells (Engelhardt & Sorokin, 2009). The choroid plexus is connected by apical TJs and consists of a capillary network, which is enclosed, in a single layer of epithelium cells (Engelhardt & Sorokin, 2009; Johanson, Stopa, & McMillan, 2011). The choroid plexus epithelial cells limit paracellular diffusion, and contains a secretory function producing the CSF. While the BBB may be predominant site of transport for O_2, glucose and amino acids, the BCSFB plays a critical role in maintain brain Ca^{2+} homeostasis (Laterra, Keep, Betz, & Goldstein, 1999). The choroid plexus is also responsible for the entry of certain hormones into the CSF, and also secretes insulin like growth factor-II (IGF-II) into the CSF (Nilsson, Lindvall-Axelsson, & Owman, 1992). The BCSFB also boasts of other active transport systems which aid in the efflux

of certain solutes including iodide, thiocyanate and the penicillin and neurotransmitter metabolites homovanillic acid and 5-hydroxyindoleacetic acid (Lorenzo, 1977).

1.1.2 Functions of the BBB

The BBB limits the passage of large and hydrophilic solutes, while allowing small lipophilic molecules (O_2, CO_2, hormones) to freely diffuse following concentration gradients. The BBB possesses specific transporters which are used to move complex nutrients such as glucose and amino acids into the brain. The BBB can also use receptor-mediated endocytosis to transport certain proteins such as insulin, leptin and iron transferrin into the brain (Pardridge, Eisenberg, & Yang, 1987; Pardridge, 2005).

1.1.3 Regulation of Ion and Neurotransmitter Systems

The BBB plays a critical role not only in regulating the transport of macro and micromolecules as mentioned above, but also in the management of ion and neurotransmitter levels in the CNS, and is the primary defence against neurotoxins. For instance, neuronal function and synaptic signalling relies on stable environment containing optimal concentrations of specific ions such as potassium $[K^+]$. In spite of a higher and fluctuating $[K^+]$ in the plasma akin to ~ 4.5 mM, the BBB helps maintain $[K^+]$ at ~ 2.4-2.9 mM in the CNS. Other major ions and systems regulated by the BBB includes calcium (Ca^{2+}), magnesium (Mg^{2+}) and pH levels. The BBB also plays a major role maintaining physiological

levels of certain neurotransmitters, such as glutamate via excitatory amino acid transporters (EAATs), in the CNS. Additionally, the betaine/GABA transporter 1 (BGT1, SLC6A12), present in the brain microvessels, may play a role in the regulation of γ-aminobutyric acid (GABA) in the CNS (Shawahna et al., 2011; Uchida et al., 2011). Notably, this compartmentalization of central and peripheral neurotransmitter pools by the BBB is important in the minimization of "cross talk" between the separate systems.

1.1.4 Neurotoxins, Macromolecules & Essential Nutrients

Taking into account that in the adult CNS steady neurodegeneration greatly overshadows neurogenesis (Gould & Gross, 2002), the sheltering of the CNS from endogenous and foreign toxins is of paramount importance. The TJs of the BBB provide an effective and stable barrier from potential toxins circulating in the peripheral blood, while a family of ATP-binding cassette (ABC) transporters in the BBB actively pump such toxins out of the brain. Total protein content in the CNS is inherently lower than plasma levels given the highly selective permeability of the BBB. Consequently, many plasma macromolecules such as albumin, pro-thrombin and plasminogen, which can cause irreversible damage to nervous tissue resulting in apoptosis, are excluded. Furthermore, specific transporter systems such as the glucose transporter 1 (GLUT1) which is exclusive to the BBB, and monocarboxylate transporter 1 (MCT1) facilitate the transport of glucose and monocarboxylates (i.e. lactate), respectively, as fuel for the brain (Simpson, Carruthers, & Vannucci, 2007). The L1 and y^+ systems,

present ubiquitously in the BBB, provide transport for all essential amino acids into the CNS (R. A. Hawkins, O'Kane, Simpson, & Viña, 2006). Five sodium dependent systems – ASC, A, LNAA, EAAT, N – facilitate the efflux of nonessential AA (ASC, A), essential AA (LNAA), the excitatory acidic AA (EAAT), and nitrogen-rich AA (N) from the brain (Zlokovic, 2008). Larger neuroactive peptides and proteins including enkephalins (Zloković, Lipovac, Begley, Davson, & Rakić, 1987), arginine-vasopressin (AVP) (Zlokovic et al., 1990), and luteinizing-hormone releasing hormone (LHRH) can generally not pass the BBB and thus rely upon highly specific transporter systems to move from blood to brain and vice versa. Peptide transport system 1 (PTS-1) and PTS-2 mediate the efflux of enkephalins and AVP, respectively (Banks, 2006), from the brain while PTS-4 facilitates bidirectional transport of LHRH (Berislav V Zlokovic, 2008). Other large proteins such as leptin (Zlokovic et al., 2000), insulin & insulin-like growth factor (W. M. Pardridge, 2005), low-density lipoproteins (LDL) (Méresse, Delbart, Fruchart, & Cecchelli, 1989), and immunoglobin-G (IgG) (Deane et al., 2005) also rely on receptor-mediated transport systems to cross the BBB.

In sum, the BBB serves two main functions: (i) establishment and maintenance of a specific and stable fluid environment to meet the rigorous needs of the CNS, and (ii) protection of the CNS from potentially damaging material originating from both within and outside the confines of the body. The slightly imperfect nature of the BBB allows for the free diffusion of certain small essential

water-soluble nutrients, while other complex nutrients rely on highly selective transport systems to enter the brain. Therefore, considering the central role of the BBB in protecting the CNS against neurotoxic compounds, there has been growing interest in the understanding of the BBB function in neuropsychiatric disorders.

1.1.5 Neurovascular Unit

The neurovascular unit (NVU) was initially defined as "interactions between circulating blood elements and the blood vessel wall, extracellular matrix, glia, and neurons" (Grotta et al., 2002) but has recently developed to incorporate other structures including pericytes and microglia (detailed anatomy and organization is reviewed in (Abbott, Rönnbäck, & Hansson, 2006), (Neuwelt et al., 2011)). Due to the amalgamation of these structures, the NVU is considered the site of the coupling of neuronal activity and cerebral blood flow (Abbott & Friedman, 2012; Najjar et al., 2014). The various components of the NVU are intricately link to one another, and this relationship is facilitated by adhesion molecules (including cadherins and integrins) and gap junctions (del Zoppo, 2010; Figley & Stroman, 2011; Simard, Arcuino, Takano, Liu, & Nedergaard, 2003), which in conjunction with ion channels aid in the movement of various ions such as calcium and potassium, and also other neuromodulators (ATP) (Filosa, 2010; Gordon, Mulligan, & MacVicar, 2007). The interlink between neuronal and vascular components is genetically tied as during early embryogenesis neural progenitor cells (originate from neural tube) and vascular progenitor cells

(originate from neural plate) are positioned in close proximity (Kraemer & Hempstead, 2003; Muoio, Persson, & Sendeski, 2014; Ward & Lamanna, 2004). Due to their position relative to one another, both neural and vascular cells are exposed to similar factors and both respond to vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) (Soker, Miao, Nomi, Takashima, & Klagsbrun, 2002), (Sanchez et al., 2013). The various components of the NVU all play a distinct and specific role in maintaining the functionality of the NVU (Simard et al., 2003), (Fields & Stevens-Graham, 2002) however the exact role of each component is still yet to be elucidated (Muoio et al., 2014), (Kowiański, Lietzau, Steliga, Waśkow, & Moryś, 2013).

Exploring the relationship between neurological conditions and NVU dysfunction is still in its infancy, however indirect and epidemiological data does suggest a role for NVU dysfunction in psychiatric conditions such as major depressive disorder (MDD). A study examining endothelial dysfunction via the relative uptake ratio (RUR) of blood flow in the brachial artery following hyperemic challenge found a significantly lower RUR in patients with MDD or minor depressive disorder as compared to healthy controls, implying impaired vascular endothelial function (Lavoie, Pelletier, Arsenault, Dupuis, & Bacon, 2010). Another study exploring apoptotic activity in the endothelium (% of apoptotic nuclei in human unbilical vein endothelial cells) found a significantly greater amount of apoptotic nuclei in patients with MDD when compared to healthy controls (Politi, Brondino, & Emanuele, 2008). Epidemiological studies

also point towards a role for vascular endothelial impairment in MDD. A meta-analysis encompassing 16,221 studies found an increased risk for MDD in those with major vascular diseases including diabetes, cardiovascular disease, and stroke (Valkanova & Ebmeier, 2013).

1.2 Models of BBB Disruption in Neuropsychiatric Disorders

1.2.1 Alzheimer's disease

Alzheimer's disease (AD) is characterized by a progressive decline in cognitive function with an onset of >65 years old in most cases. Biologically, AD has been associated with defects in the neurovascular system, accumulation of amyloid-β (Aβ; neurotoxin) on and around blood vessels as well as in the brain parenchyma, and the presence of neurofibrillar tangles (NT) (Takeda, Sato, & Morishita, 2014; Berislav V Zlokovic, 2008, 2011) and hyperphosphorylated tau (Phillips et al., 2014). The role of Aβ in AD is the most studied and well understood. Notably, it has been recently shown that peripheral circulating Aβ is transported into the brain via the receptor for advanced glycation end products (RAGE) (Deane et al., 2003). Normally expressed in relatively low levels, the expression of RAGE in the BBB is greatly amplified in response to the accumulation of RAGE ligands including Aβ (Deane et al., 2003; Donahue et al., 2006). This Aβ/RAGE interaction in the BBB may lead to increased transportation of circulating Aβ into the CNS, resulting in a NF-κB mediated activation of endothelial cells and the release of pro-inflammatory cytokines. It

has been demonstrated that binding of Aβ/RAGE at the luminal membrane of the BBB can destroy RAGE expressing neurons through oxidative damage. The clearance of Aβ from the brain is facilitated by lipoprotein receptor-related protein 1 (LRP1). Numerous studies using both animal models and human patients with AD show that Aβ clearance is impaired in these cases (Bell et al., 2009; Deane et al., 2004; Donahue et al., 2006; Erickson et al., 2012; Shibata et al., 2000). For instance, LRP1 functions to transport Aβ into the periphery vascular system whereupon soluble LRP1 (sLRP1) facilitates the total systematic clearance of Aβ from the body via the kidney and liver. The role of LRP2 is not well understood but is hypothesized to utilize apolipoprotein J (APOJ) to facilitate the transfer of Aβ out of the brain (Bell et al., 2007). Moreover, the ATP-binding cassette (ABC) family of transporters have also been implicated in Aβ clearance. ABCB1 (P-Glycoprotein, P-gp), the product of the MDR1 gene, is the best known and best studied of these transporters. Most commonly found in the BBB, several *in vitro* and *in vivo* studies have found ABCB1 to clear Aβ from the albuminal to the luminal side of the membrane (Abuznait & Kaddoumi, 2012). In MDR1 transfected pig kidney epithelial cells, the transport of $A\beta_{40}$ and $A\beta_{42}$ was significantly decreased in the presence of cyclosporine A (ABCB1 inhibitor) (Kuhnke et al., 2007). Additionally, following the injection of labelled $A\beta_{40}$ and $A\beta_{42}$ into ABCB1 knockout mice, the clearance rate of Aβ was found to be half that of the wild type (Cirrito et al., 2005). In addition to faulty BBB clearing mechanisms in the pathology of AD, recent evidence using APP23 transgenic

mice overexpressing mutant human APP, the precursor of Aβ, suggests that the BBB may be susceptible to peripherally induced inflammation (Takeda et al., 2014). For instance, Takeda et al. administered a peripheral LPS injection to APP transgenic mice and observed a greater increase in inflammatory levels in the brain interstitial fluid, which was accompanied by abnormalities in food intake, social behaviour and basal activity. In summary, various models of AD suggest that BBB dysfunction is associated with abnormal Aβ clearance and increased permeability and subsequent influx of pro-inflammatory substances into the CNS.

1.2.2 Multiple Sclerosis

Multiple Sclerosis (MS), a brain disorder characterized by extensive damage to the myelin sheath, presents a wide host of symptoms including but not limited to: numbness and weakness in limbs, visual impairments, electric shock sensations, tingling and/or pain across the body and cognitive impairment. While the exact cause of MS remains unknown, there is still a debate whether or not MS is an autoimmune disease, as classically held, or if it is in reality a neurodegenerative disorder (Lassmann, 2007; Trapp & Nave, 2008). With respect to the autoimmune aspect of MS, the BBB is responsible for the regulation of immune cell transport and inflammatory pathway mediator activity from the periphery into the CNS. Under physiological conditions, few leukocytes are present in the CNS, but in response to injury and/or disease peripheral leukocytes are thought to enter the cerebral spinal fluid (CSF), the parenchymal perivascular space, and the subarachnoid space(Engelhardt & Ransohoff, 2005; Ransohoff,

Kivisäkk, & Kidd, 2003; Berislav V Zlokovic, 2008). In the experimental autoimmune encephalomyelitis (EAE) model of MS, it has been shown that aggressive CD4+ T lymphocytes accumulate in the brain via the BBB and blood-CSF barrier (Engelhardt & Ransohoff, 2005; Man, Ubogu, & Ransohoff, 2007; Ransohoff et al., 2003). A subset of these T lymphocytes have been reported to exert immunosurveillance in the CNS while another subset is implicated in the destruction of neurons. The regulation and transport of immune cells and other mediators across the BBB and blood-CSF barrier is thus thought to be implicated in the pathophysiology of MS. An imaging study using dynamic contrast-enhanced MRI (DCE-MRI) noted an increase in BBB permeability, as measured by K^{trans}, in the periventricular normal appearing white matter (NAWM) in patients with MS (Cramer, Simonsen, Frederiksen, Rostrup, & Larsson, 2014). Notably, immunomodulatory treatment (with β-interferon or glatiramer acetate) aided in the gradual decrease of BBB permeability following a relapse episode. Considering that β-interferon has been shown to the stabilize the barrier on brain capillary endothelial cells *in vitro* (Kraus et al., 2008), this study provided strong evidence that abnormalities in the BBB function may be associated with the neurobiology of MS. Notably, a recent *in-vitro* study that exposed human brain microvascular endothelial cells (BMECs) to serum from patients with relapse-remitting MS (RRMS) found that serum from patients with RRMS lowered claudin-5, an integral TJ protein expression, and decreased transendothelial electrical resistance (Shimizu et al., 2014). Together, these clinical and pre-

clinical studies indicate that an increase in BBB permeability may occur soon after the flair-ups observed in MS. In addition, preliminary yet encouraging data suggest that successful anti-inflammatory treatment may speed up the rate of closing of the BBB.

1.2.3 The Role of Matrix metalloproteinase-9 on BBB Function

Matrix metalloproteinases (MMPs) encompass a large family of proteases which are typically produced in a latent form and upon activation by inflammatory stimuli regulate pathophysiological pathways including the regulation of growth factors, death receptors and various other signalling molecules (Cauwe, Van den Steen, & Opdenakker, 2007; Gary A Rosenberg, 2009). The effects of MMPs are diverse and depend on a host of factors such as location, time, and surrounding environment and thus some MMPs can engage in opposite functions at different points in time. For instance, MMPs have been implicated in angiogenesis, neurogenesis, axon growth, tissue repair, myelinogenesis and apoptotic protection (Gardner & Ghorpade, 2003; Stomrud, Björkqvist, Janciauskiene, Minthon, & Hansson, 2010; Yong, Power, Forsyth, & Edwards, 2001). Notably, the promoter region of MMP9 includes a binding region for activator protein-1 (AP1) and NF-κB, both of which are involved in key inflammatory pathways and thus linking neuroinflammation and MMP9 (Gary A Rosenberg, 2009). Upon the induction of the neuroinflammatory pathway, MMP9, along with MMP2 and MMP3 can facilitate the proteolysis of the basal lamina, TJ, and extracellular matrix resulting in increased BBB

permeability (Hamann, Okada, Fitridge, & del Zoppo, 1995; Heo et al., 1999). Inhibitors of MMPs have been shown to restore BBB integrity (Gasche, Copin, Sugawara, Fujimura, & Chan, 2001). In individuals experiencing an exacerbation of MS, MMP9 was found to be elevated in the CSF (Gijbels, Masure, Carton, & Opdenakker, 1992) and treatment with prednisolone was found to restore BBB integrity resulting in a decrease of MMP9 levels in the CSF (G A Rosenberg, Dencoff, Correa, Reiners, & Ford, 1996). Furthermore, in an EAE model of MS in which demyelination is associated with neuroinflammation, treatment with the MMP inhibitor GM-6001 halted the progression of EAE in mice (Gijbels, Galardy, & Steinman, 1994).

Accumulation of Aβ endogenously induces the secretion of MMPs in microglia and astrocytes as a part of the neuroinflammatory pathway (Reitz et al., 2010; Gary A Rosenberg, 2009). Plasma MMP9 levels are elevated in patients with AD (Lorenzl et al., 2003). PCR and immunohistochemistry data show accumulation of a latent/inactive form of MMP9 in the hippocampus of patients with AD (Backstrom, Lim, Cullen, & Tökés, 1996), which is postulated to be associated with less degradation of Aβ plaques in the brain. In addition, Aβ-induced cognitive impairment and neurotoxicity were significantly alleviated in MMP9 homozygous K/O mice and with administration of MMP inhibitors (Mizoguchi et al., 2009). Together, these studies indicate an important role of MMP9 in AD and MS via BBB dysfunction.

1.2.4 BBB in Schizophrenia

The role of BBB dysfunction in psychiatric conditions has been far less studied. Some studies have investigated "blood–CSF barrier dysfunction" as measured by CSF-to-serum albumin ratio. Evidence of increased CSF-to-serum albumin ratio has been reported in individuals with schizophrenia (SCZ) (Bauer & Kornhuber, 1987; Kirch, Kaufmann, Papadopoulos, Martin, & Weinberger, 1985; Severance et al., 2014), bipolar disorder (BD) (Zetterberg et al., 2014) and a mixed sample of inpatients with mood and SCZ spectrum disorders (Bechter et al., 2010). Given that albumin is not synthesized in the CSF, all albumin present in the CSF originated from the peripheral blood compartment. Thus, these findings of elevated CSF-to-serum albumin in mood and SCZ subjects have been interpreted as potential blood-CSF or blood-brain barrier dysfunction. A recent controversial study (Hammer et al., 2013) proposed a link between BBB dysfunction and SCZ based on two indirect findings: (a) worse scores in the Cambridge Neurological Inventory in SCZ subjects who were positive for anti-NMDA receptor autoantibodies and had past history of birth complications or head trauma (used as proxies of BBB disruption), and (b) behavioural changes in ApoE -/- mice (known to display BBB deficiency) after injection of Ig fractions from NMDAR-AB seropositive (IgM, IgG, IgA) subjects compared to serum from control subjects. However, this study has been criticized (Titulaer & Dalmau, 2014) by (i) using retrospective data to determine birth complications and history of head trauma and assuming that these retrospective events disturbed BBB integrity; (ii) by providing no confocal microscopy images pertaining to

their NMDA receptor immunostaining in the presence of NMDAR-AB, thus calling into question their immunostaining results by pointing to other studies (Hughes et al., 2010; Moscato, Peng, Parsons, Dalmau, & Balice-Gordon, 2012) which utilized anti-NMDR encephalitis antibodies to visualize NMDAR internalization with confocal microscopy and could not draw the same conclusions; and (iii) by suggesting that the study needed to prove that the injection of patients' IgG reached the brain, bound to NMDAR and altered receptor levels and functions before drawing strong conclusions using the ApoE -/- mice data.

Therefore, while the study of BBB in psychiatric disorders is still in its infancy, there is converging data showing that SCZ and BD are associated with increased CSF-to-serum albumin ratio.

1.3 Why BBB Disruption may be Associated with Bipolar Disorder?

Like most major neuropsychiatric disorders, BD has also been heavily linked with inflammatory processes. In fact, increased neuroinflammation is thought to mediate, at least in part, the cognitive decline observed over the course of BD, as well as the abnormalities observed in gray and white matter content in individuals with BD. In addition, several cohort studies have now demonstrated that BD is associated with excessive mortality rates (Angst, Stassen, Clayton, & Angst, 2002; Fiedorowicz et al., 2009; Ramsey et al., 2013). Compared to the general population, individuals with BD die on average 9 years younger (Crump,

Sundquist, Winkleby, & Sundquist, 2013), but, more importantly, these striking elevated mortality rates are primarily due to death from natural causes including cardiovascular, respiratory, diabetes and infectious diseases, all of which have been associated with increased inflammation (Crump et al., 2013; Hoang, Stewart, & Goldacre, 2011; Kupfer, 2005). Below, we propose a novel model where disruption in the BBB is associated with less protection and subsequent more influx of inflammatory material from the periphery to the brain of individuals with BD.

1.3.1 Inflammation & Oxidative Stress in Bipolar Disorder

Several lines of evidence indicate that BD is associated with increased inflammation and oxidative stress. For instance, the monocyte-T cell theory of mood disorders implicates the inflammatory response system (IRS) as a primary contributor to the neurobiology of BD (Maes, Smith, & Scharpe, 1995). This theory is supported in part by evidence of increased levels of pro-inflammatory cytokines including IL-1, IL-6, and TNF-α in plasma (B. C. M. B. Haarman et al., 2014; Stertz, Magalhães, & Kapczinski, 2013), abnormal expression of pro-inflammatory genes in circulating monocytes (Padmos et al., 2008a), and evidence that psychotropics can modulate the immune system (Drzyzga, Obuchowicz, Marcinowska, & Herman, 2006; B. C. M. B. Haarman et al., 2014; B. C. M. Haarman et al., 2014; Padmos et al., 2008a). Activation of the immune system is linked with neuroinflammation through activation of microglia which is a central player in neuroinflammatory pathways (Stertz et al., 2013). A recent

PET imaging study using [^{11}C]-(*R*)-PK11195 found greater [^{11}C]-(*R*)-PK11195 binding potential in the right hippocampus and a similar non-significant trend in the left hippocampus of bipolar type I subjects, suggesting increased microglial activity and neuroinflammation in these brain areas. Notably, oxidative damage to RNA (Che, Wang, Shao, & Young, 2010) and decreased expression of growth associated proteins (Tian, Wang, Bezchlibnyk, & Young, 2007b), both believed to be involved in neuroinflammation (Kato, Nemoto, Arino, & Fujikawa, 2003), have been observed in post-mortem hippocampal samples from BD subjects. Disruption of mitochondria, responsible for the regulation of apoptosis and intracellular calcium levels, has been increasingly implicated as a contributing factor in the oxidative stress facet of BD perhaps through decreased activity of mitochondrial complex I (Scola, Kim, Young, & Andreazza, 2013). Moreover, studies conducted in the peripheral blood have consistently found increased markers of oxidative damage to lipids, RNA and DNA in BD (Ana C Andreazza et al., 2008; Brown, Andreazza, & Young, 2014).

1.3.2 Oligodendrocyte & Myelin Damage in Bipolar Disorder

Oligodendrocytes facilitate the formation and stability of neural circuits by insulating axons with myelin sheath. In the last several years, there has been increasing attention to changes in white matter and oligodendrocyte structure/function in BD. For instance, oligodendrocyte-specific mRNA markers including OLIG2, SOX10, GALC, MAG, PLP1, CLDN11, MOG, ERBB3, TF were found to be downregulated in the brain of individuals with BD (Konradi,

Sillivan, & Clay, 2012). Uranova et al. used electron microscopy to analyze ultrastructural altercations in oligodendrocytes in the prefrontal cortex of individuals with BD (Uranova et al., 2001). The oligodendrocyte cells in BD were found to be surrounded by astroglial cells and displayed strong signs of apoptosis and necrosis. In this qualitative study, apoptosis was characterized by nuclear chromatin aggregation, cell shrinkage and the preservation of organelles while necrosis was characterized by chromatin condensation, cell swelling, and membrane lysis of organelles. Previously, this group described a decrease in oligodendrocyte density in layer VI of BD patients (31%) (Orlovskaya, Vostrikov, Rachmanova, & Uranova, 2000), further implicating oligodendrocyte disruption in the pathophysiology of BD. Furthermore, several imaging, genetic and post-mortem tissue analyses have shown myelin abnormalities in BD subjects (Aston, Jiang, & Sokolov, 2005; Heng, Song, & Sim, 2010; Herring & Konradi, 2011; Konradi et al., 2012; Sokolov, 2007), establishing a link between oligodendrocyte dysfunction and myelin damage in BD.

1.3.3 Implication of Inflammation & Oxidative Stress in the Treatment of Bipolar Disorder

One of the key questions in BD research has been the extent to which available treatments may reverse/prevent inflammation and oxidative stress. While an extensive review of the effects of pharmacological and non-pharmacological treatments on inflammation and oxidative stress is beyond the objective of the present article, there is growing evidence that mood stabilizing

and antidepressant agents possess anti-inflammatory and antioxidant properties (as reviewed in (Behr, Moreira, & Frey, 2012; Diniz, Machado-Vieira, & Forlenza, 2013). Lithium, the hallmark treatment of BD, was shown to aid in the defense against oxidative stress by upregulating mitochondrial complexes I and II (Maurer, Schippel, & Volz, 2009). Relevant to the notion that lithium can protect against ROS-induced damage, previous studies have shown that oxidative stress can effect BBB permeability, particularly by affecting the integral TJ protein occludin (Cummins, 2012; González-Mariscal, Tapia, & Chamorro, 2008). Administration of tempol, a ROS scavenger, to λ-carrageenan-induced peripheral inflammatory pain (CIP) rats attenuated (14)C-sucrose and (3)H-codeine uptake in the brain and provided protection to occludin, and thus preserving BBB integrity (Lochhead et al., 2012). Future studies investigating the ability of lithium to protect against BBB disruption are warranted.

Lithium also downregulates the AA – prostaglandins (PGs) pathway (Karmazyn, Manku, & Horrobin, 1978; Murphy, Donnelly, & Moskowitz, 1973) which has been implicated with neuroinflammation (Chang et al., 1996; Rao, Lee, Rapoport, & Bazinet, 2008). More specifically, chronic lithium treatment resulted in decreased AA to PGs turnover, decreased activity of cyclooxygenase-2 (COX-2), the enzyme responsible for converting AA to PGs, and PG-E$_2$ concentration in rat brain (Bosetti et al., 2002). Another preclinical study showed that lithium treatment significantly increased levels of 17-hydroxy-docosahexanoic acid (Basselin et al., 2010), which possesses known anti-inflammatory properties

(González-Périz et al., 2006; Hong, Gronert, Devchand, Moussignac, & Serhan, 2003). Furthermore, several *in vitro* an *in vivo* studies have shown that lithium treatment results in the attenuation of pro-inflammatory cytokines including TNF-α (Tan et al., 2010; H.-M. Wang et al., 2013; Y. Wang et al., 2009), IL-1β (Green & Nolan, 2012; Himmerich et al., 2013; Tay et al., 2012), IL-6 (Beurel & Jope, 2009; Chen et al., 2013; Zhang, Katz, & Michalek, 2009), and interferon-γ (INF-γ) (Agrawal, Gollapudi, Gupta, & Agrawal, 2013; Boufidou, Nikolaou, Alevizos, Liappas, & Christodoulou, 2004; Rowse et al., 2012) while increasing the secretion of the anti-inflammatory cytokines IL-2 (Kucharz, Sierakowski, & Goodwin, 1993; Rapaport, Guylai, & Whybrow, 1999; Sztein et al., 1987) and IL-10 (Agrawal et al., 2013; Ballanger, Tenaud, Volteau, Khammari, & Dréno, 2008; Tay et al., 2012). With respect to oligodendrocyte function, lithium treatment has been shown to increase oligodendrocyte proliferation and increase myelination of optic nerves in mice (Azim & Butt, 2011).

In summary, there is overwhelming data pointing towards inflammatory and oxidative stress modulation by lithium and other psychotropic agents (as reviewed in (Behr et al., 2012)). Given that inflammation and oxidative stress have been associated with disruption in the BBB integrity, a natural next step for future studies is to test whether lithium and/or other mood stabilizing agents used in the treatment of BD may protect against BBB damage.

1.3.4 A Novel Model of BBB Disruption in Bipolar Disorder

Decades of research has implicated increased peripheral inflammation and oxidative stress, as well as oligodendrocyte and white matter changes in the pathophysiology of BD. This is in line with a number of cohort studies showing increased mortality rates due to general medical conditions associated with inflammation and oxidative stress. Further evidence is provided by studies showing that first-line treatments for BD, such as lithium, can modulate inflammatory and oxidative stress pathways. More recently, imaging and post-mortem studies have provided evidence of increased neuroinflammation in BD through excessive microglial activation. Considering the close anatomical proximity of microglia, oligodendrocytes, and astrocytes to the BBB, and the increasing attention of BBB disruption in other neuropsychiatric conditions, such as AD, MS and SCZ, we propose a novel model of BBB dysfunction in BD wherein transient or persistent loss of BBB integrity is associated with decreased CNS protection and increased permeability of pro-inflammatory (e.g. cytokines, reactive oxygen species) substances from the peripheral blood into the brain. This will trigger the activation of microglial cells and promote localized damage to oligodendrocytes and the myelin sheath, thereby compromising myelination and neural circuit integrity (**Figure 1**).
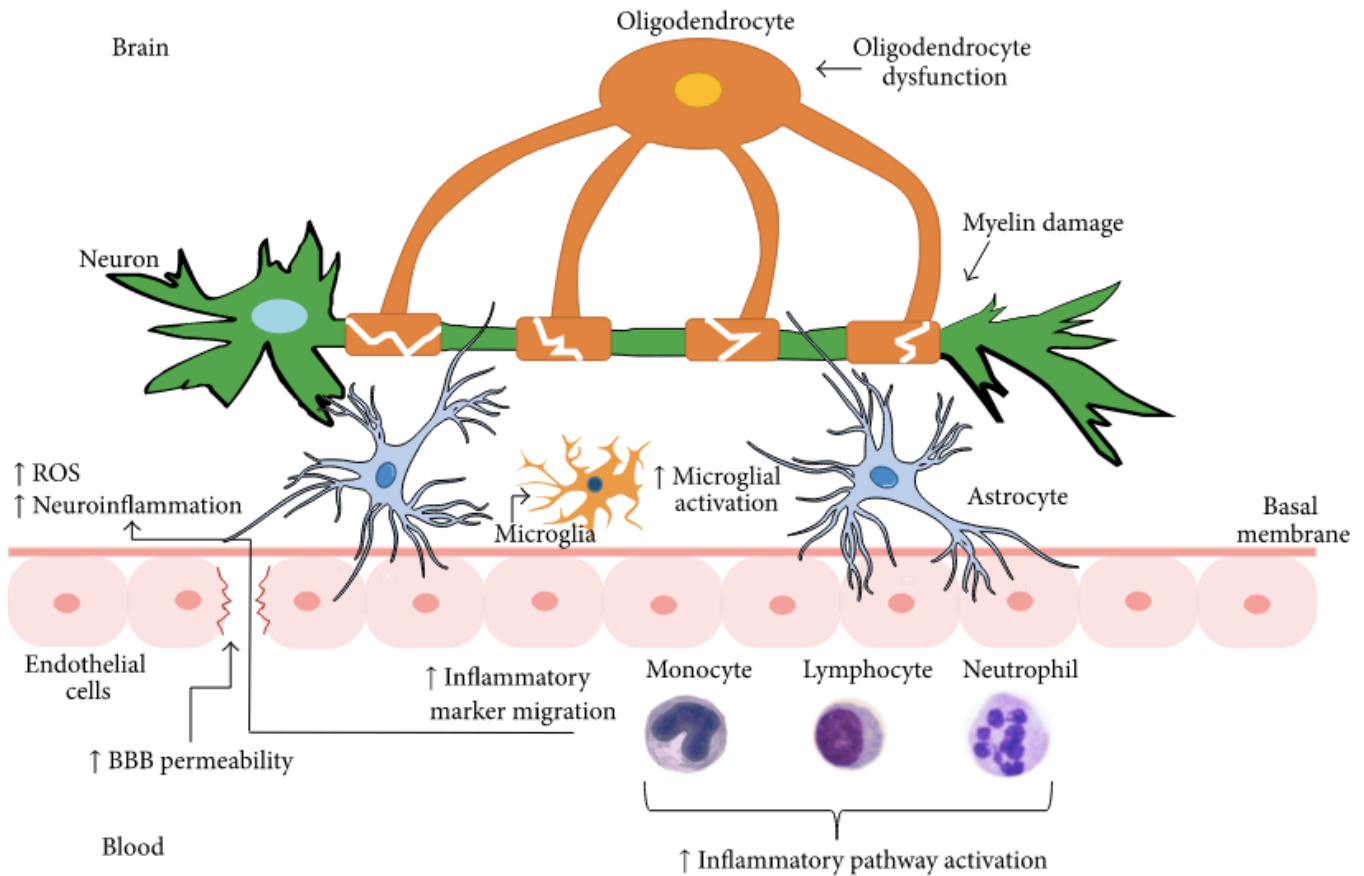
Figure 1. Proposed model of blood-brain barrier (BBB) disruption in bipolar disorder. Increased BBB permeability through the endothelial cells (pink) and basal membrane (dark pink) may facilitate increased migration of inflammatory molecules into the brain. Activation of microglial cells (light orange) and an increase in reactive oxygen species (ROS) would amplify neuroinflammatory processes and ultimately induce damage in the myelin sheath, either directly via lipid/protein oxidation or indirectly via oligodendrocyte dysfunction (dark orange).

While we could not identify a study that directly examined the BBB integrity in BD, a recent study found increased levels of MMP9, which increases BBB permeability during pro-inflammatory states (see section 2.1.3. above), in bipolar depression (Rybakowski et al., 2013). In addition, both manic and depressive episodes are associated with increased levels of pro-inflammatory cytokines (Goldstein, Kemp, Soczynska, & McIntyre, 2009) and, therefore, it is conceivable that BD subjects may experience a transient increase in the BBB permeability during major mood episodes. Also, it is well established that most drugs of abuse disrupt the BBB integrity (Kousik, Napier, & Carvey, 2012; O'Shea, Urrutia, Green, & Colado, 2014a). Given the exceeding rates of drug abuse in individuals with BD, it is possible that excessive drug use can contribute to the disruption in BBB permeability in a proportion of individuals with BD.

1.3.5 Future Directions

It is imperative to test this model by further analyzing the role of the BBB in BD. Currently, at least a couple of brain imaging techniques are available to test the hypothesis of disrupted BBB structure or function directly in individuals with BD. One possibility would be the use of dynamic contrast-enhanced MRI (DCE-MRI) as a method for studying BBB disruption *in vivo* (Heye, Culling, Valdés Hernández, Thrippleton, & Wardlaw, 2014). Another available technique is the use of $[^{11}C]$-verapamil to study the function of the P-glycoprotein (Pgp) transporter at the blood-brain barrier (BBB) with PET (Hendrikse & Vaalburg, 2002). Finally, the use of *in vivo* and *in vitro* preclinical models may be

particularly useful to test whether lithium and other medications commonly used in the treatment of BD can reverse and/or prevent BBB damage. If a link between BD and BBB disruption is established, this would not only advance the knowledge on the neurobiology of BD, but also open numerous possibilities to investigate new treatment pathways (e.g. MMP inhibitors (Candelario-Jalil, Yang, & Rosenberg, 2009), ROS scavengers (Lochhead et al., 2012)) for this devastating major mental illness.

CHAPTER 2: Introduction

2.1 Rat Models of Bipolar Disorder and BBB Disruption

The first step in determining whether a link exists between bipolar disorder and blood brain barrier permeability we sought to establish an animal model encompassing both the behavioral and biological components of bipolar disorder. The use of acute amphetamine (AMPH) administration in rats has been used as a model of mania by many groups (B. N. Frey et al., 2006; Machado-Vieira, Kapczinski, & Soares, 2004; Steckert et al., 2012). This model has been shown to present with elevated oxidative stress, by the way of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), and catalase (CAT) activity, along with monoamine depletion, neurofilament loss, and neurite deterioration (B. N. Frey et al., 2006). Moreover, rats treated with AMPH displayed increased locomotor activity, all of which is right in line with the elevated markers of inflammation & oxidative stress and hyperactivity commonly associated with bipolar mania (Cosgrove, Kelsoe, & Suppes, 2016; Zhou et al., 2015). Another commonly utilized model in the scope of BBB research is that of lipopolysaccaraide (LPS) induced BBB permeability (Banks et al., 2015; Dénes, Ferenczi, & Kovács, 2011). This model aims to exploit the underlying mechanism of systematic inflammation, wherein significant amounts of LPS are released into circulation causing severe BBB dysfunction (Banks et al., 2015). Acute LPS administration has been used in a range of studies encompassing sepsis models

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(Kwon et al., 2015; Thomas, Bath, Stover, Lambert, & Thompson, 2014), blood-cerebrospinal fluid barrier studies (D'Angelo, Ek, Sandberg, & Mallard, 2013) and other endothelial protein level research (Barabutis et al., 2015; Meng et al., 2015; Reho, Zheng, Asico, & Fisher, 2015).

2.1.1 Study 1 – Rat Model: Hypothesis and Specific Aims

Amphetamine Model of BBB Disruption
Hypothesis:

i.   The AMPH treated rats will present with an elevated concentration of Evans Blue (EB) dye in the brain when compared to the saline group, indicating greater BBB permeability.

ii.   The AMPH treatment group will show increased locomotor activity as compared to saline treated rats.

Specific Aims:

i.   To determine the presence and extent of BBB permeability with varying AMPH doses (15 mg/kg and 2 mg/kg) and treatment periods (single administration, 7-days, 14-days).

ii.   To determine the regions of the brain that show the greatest susceptibility to AMPH induced BBB permeability.

iii.   To elucidate the effect of a withdrawal period following a 14-day AMPH treatment regime on BBB permeability and locomotor activity.

LPS Induced BBB Disruption

Hypothesis:

i.   Rats treated with LPS will have an increased concentration of sodium fluorescein (FL) dye, a marker of BBB disruption, in the brain as compared to controls.

ii.   Lithium pretreatment will attenuate or prevent the LPS induced disruption of the BBB.

Specific Aims:

i.   To establish whether exists a relationship between LPS induced inflammation and BBB disruption in regions of interest (cortex, prefrontal cortex, hippocampus, striatum, cerebellum, whole brain).

ii.   To determine if lithium pretreatment can rescue LPS induced BBB disruption and mitigate LPS induced inflammation and oxidative stress.

2.2 DCE-MRI Imaging of BBB Disruption in a Bipolar Population

Bipolar disorder is associated with recurring episodes of depression and mania. Globally, the lifetime prevalence estimate for Bipolar I disorder is 0.6%, 0.4% for Bipolar II disorder and 1.4% for sub-threshold Bipolar disorder (Merikangas et al., 2011). Life expectancy of patients with BD is almost a decade shorter than the average, while their quality of life can also be severely diminished (Crump et al., 2013; Ustün, Ayuso-Mateos, Chatterji, Mathers, &

Murray, 2004). Apart from severely impacting the quality of life of patients across all age groups (Depp, Davis, Mittal, Patterson, & Jeste, 2006; Freeman et al., 2009; Ishak et al., 2012), BD and other mental illnesses cost the Canadian economy over $51 billion in 2003 and has been increasing ever since (Health Canada, 2002; Heijink, Noethen, Renaud, Koopmanschap, & Polder, 2008; Lim, Jacobs, Ohinmaa, Schopflocher, & Dewa, 2008).

Currently, no definitive biological mechanisms have been pinpointed with regards to the origin and progression of BD. However, inflammation and oxidative stress have been shown to present in the brains of individuals with BD (Berk et al., 2011; Padmos et al., 2008b). Numerous *in vitro* studies have shown pro-inflammatory cytokine levels to be higher in depressed, manic and euthymic BD patients (Hamdani, Tamouza, & Leboyer, 2012; Kapczinski et al., 2011; Padmos et al., 2008b; Ryan et al., 2006). Post-mortem studies conducted in individuals with BD have shown increased levels of inflammation linked apoptotic genes in hippocampal RNA along with a decrease in growth associated protein (Padmos et al., 2008b; Tian, Wang, Bezchlibnyk, & Young, 2007a).

Oxidative stress, in the form of free radicals, has been shown to disrupt neural signal transduction pathways, synaptic plasticity and neural cellular resistance in patients with BD (Grintzalis, Zisimopoulos, Grune, Weber, & Georgiou, 2013; Mahadik, Evans, & Lal, 2001; Soeiro-de-Souza et al., 2013).

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Oxidative stress related DNA damage was reported to be correlated with the severity of depressive symptoms in patients with BD (Ana Cristina Andreazza et al., 2007).

Oligodendrocytes facilitate the formation of white matter neural circuits by insulating axons with myelin sheath. Oligodendrocyte specific mRNA is found to be downregulated in patients with BD (Konradi et al., 2012). Furthermore, several imaging, genetic and post-mortem tissue analysis show myelin abnormalities in BD patients (Aston et al., 2005; Heng et al., 2010; Herring & Konradi, 2011; Konradi et al., 2012; Sokolov, 2007).

2.2.1 Study 2 – Human Imaging: Hypothesis and Specific Aims

Hypothesis:

i. We will visualize increased BBB permeability in patients with BD as compared to healthy controls

ii. We will see a greater level of BBB permeability in patients with a longer history of BD and in patients with a larger number of bipolar episodes

iii. BD patients with more bipolar episodes and a longer history of BD will display worse cognitive function

Specific Aims:

i. To quantify and locate any BBB permeability present in BD patients

ii. Uncover the relationship between BBB permeability and a) total number of bipolar episodes and b) time since first bipolar episode and c) cognitive function (using cognitive scales and objective inflammation and myelin damage markers)

CHAPTER 3
Study 1: Rat Models – Materials and Methods

3.1 Amphetamine Induced Model of Bipolar Mania & BBB Disruption

3.1.1 Single 15mg/kg Dose

Male Sprague-Dawley rats weighing 250-300g (Charles River, QC, Canada) were housed individually in the McMaster University Central Animal Facility on a reverse 12:12 light/dark cycle and were provided food and water *ad libitum*. All animal handling procedures and protocols were in accordance to the McMaster University Animal Research Ethics Board along with the guidelines provided by the Canadian Council on Animal Care. Following a 3-day habituation period, the rats were handled for 5 days wherein they were sensitized to the handler's gloves & scent and were wrapped to mimic injection conditions. Split into two groups (4 saline; 6 AMPH), the rats were administered a single 15 mg/kg dose of amphetamine (AMPH) or saline through intra-peritoneal (i.p) injection.

BBB disruption was analyzed using Evans blue (EB) dye. Normally, EB binds albumin in the blood and thus cannot cross the BBB (B. T. Hawkins & Egleton, 2006). In the event of BBB disruption, however, the EB-albumin complex may pass through the BBB and can thus be used to quantify BBB dysfunction (B. T. Hawkins & Egleton, 2006). EB was injected into the rats via

the tail vein one hour after AMPH administration and was allowed to circulate for 3 hours prior to sacrifice and brain tissue collection. Rats were administered 0.6 mL of a 75mg/kg ketamine (Ketaset; Zoetis, Kirkland, QC, Canada) & 10mg/kg xylene (Rompun; Bayer, Mississauga, ON, Canada) solution. Upon lack of response following a toe pinch, confirming unconsciousness, the rats were perfused through the right ventricle using a 0.9% saline solution (Baxter, Mississauga, ON, Canada). All rats were administered 0.2 ml of a 1000U/ml Heparin (Sigma, St. Louis, MO, USA) was used as an anticoagulant. Following 20 minutes of perfusion, the rats were decapitated via guillotine and samples of the hippocampus, cortex, prefrontal cortex, cerebellum, striatum and whole brain were extracted and stored at -80°C.

In order to quantify [EB] in the brain, the samples were homogenized in 500 ul of phosphate-buffered saline (PBS). The homogenized product was split into two aliquots to measure i) total protein content of sample and ii) [EB] in the sample. Total protein content in the PBS homogenized sample was measured using a bicinchoninic acid assay (Thermo Fisher, Mississauga, ON, Canada), reading the sample florescence at 750 nm on a Synergy 2 microplate reader (Biotek, Winooski, VT, USA). To measure [EB], the second PBS homogenized aliquot was treated with 50% trichloroacetic acid (TCA) in a 1:1 ratio with the homogenized solution followed by a 1:1 100% ethanol treatment before reading sample florescence at 650 nm.

3.1.2 Seven-Day AMPH Challenge

Male Sprague-Dawley rats (2 saline; 8 AMPH) received a daily dose (2 mg/kg) of AMPH or saline through intra-peritoneal injection for 7 days. Additionally, 4 AMPH treated rats were subject to a 3-day withdrawal period with no injections of any kind. Subsequently, one hour after the final AMPH injection, EB was injected via tail vein and the brain tissue was collected and analyzed as previously described in 2.2.1.

3.1.3 Fourteen-Day AMPH Challenge

Male Sprague Dawley rats were weighed day-to-day and administered a daily 2mg/kg dose of AMPH or saline i.p. for 14 days. At the end of the injection period, the rats i) were sacrificed with no withdrawal period (2 saline, 4 AMPH), or ii) underwent a 3 day withdrawal period with no injections and subsequently sacrificed (3 AMPH+withdrawal), or iii) underwent a 3 day withdrawal period and were re-challenged with a dose of AMPH on the fourth day, prior to being sacrificed (3 AMPH+withdrawal+re-challenge). Locomotion data was gathered on days 2, 4, 7, 10, 13, and 14 using computerized chambers (AccuScan Instruments, Columbus, OH, USA), wherein infrared sensors tracked the movement of the rat over a three-hour period. One hour after the final saline or AMPH treatment, EB tail vein injections were administered. Brain collection and tissue analysis was completed as previously described in 2.2.1.

3.2 LPS Model of Inflammation and BBB Disruption

3.2.1 Summer and Winter 2015 Cohorts

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                                    ┌──────────────────────────┐
                                    │    Saline Challenge       │
                    ┌───────────────┤ (Summer n=3; Winter n=10) │
┌──────────────────┐│               └──────────────────────────┘
│                  ││
│ Saline Pretreatment│              ┌──────────────────────────┐
│                  ││               │     LPS Challenge         │
└──────────────────┘└───────────────┤ (Summer n=5; Winter n=10) │
                                    └──────────────────────────┘

┌──────────────────┐               ┌──────────────────────────┐
│                  │               │     LPS Challenge         │
│ Lithium Pretreatment│────────────│ (Summer n=6; Winter n=10) │
│                  │               └──────────────────────────┘
└──────────────────┘

        7 Days                        Following the last daily
                                      pretreatment on Day 7
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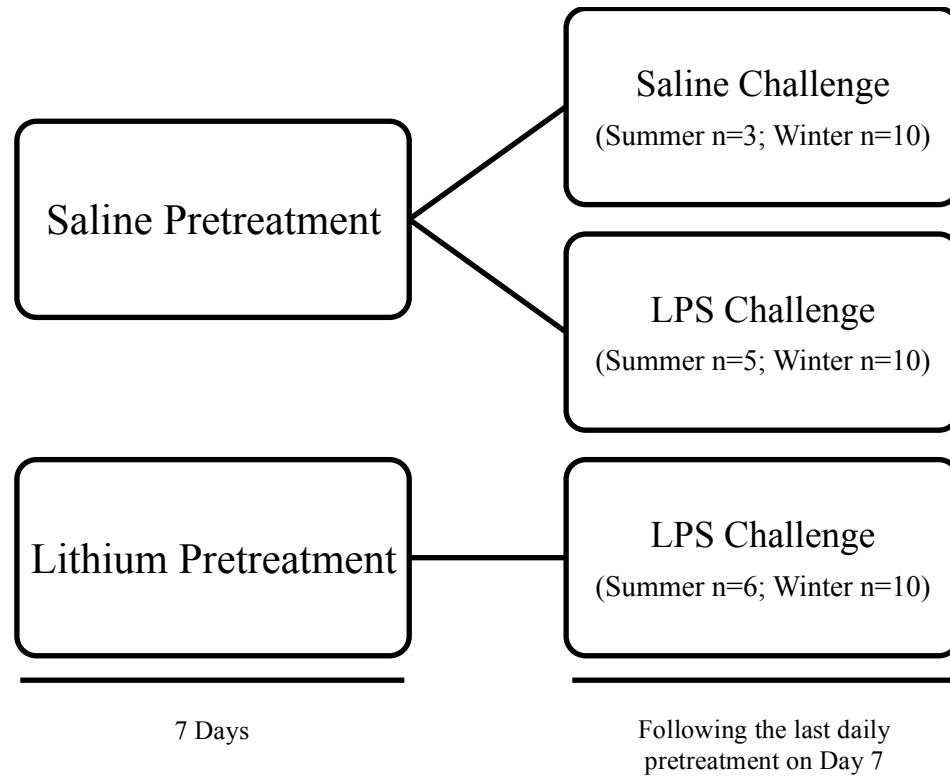
Figure 2. Study design of the LPS model of inflammation and BBB disruption for the summer and winter 2015 cohorts.

Male Sprague-Dawley rats weighing 250-300g (Charles River, QC, Canada) were housed individually in the McMaster University Central Animal Facility on a reverse 12:12 light/dark cycle and were provided food and water *ad libitum.* All animal handling procedures and protocols were in accordance to the McMaster University Animal Research Ethics Board along with the guidelines

provided by the Canadian Council on Animal Care. Following a 3-day habituation period, the rats were handled for 5 days prior to the start of the experiment. Rats were administered saline (0.9%; Baxter, Mississauga, ON, Canada) or lithium (47 mg/kg; Sigma Aldridge, St. Louis, MO, USA) intraperitoneally at 1 mL/kg for 8 days. A full 24 hours prior to sacrifice, rats received a one time challenge of saline or LPS (Sigma, St. Louis, MO, USA) at 5 mg/kg. 30 minutes prior to perfusion and sacrifice, all rats were administered 10% FL (MW ~ 376.3 Da; Sigma, St. Louis, MO, USA) at 0.6ml/kg via tail vein injection. A 0.6 ml injection of ketamine (75 mg/kg) & xylene (10 mg/kg) was given 10 minutes preceding the perfusion. Blood was collected in an ethylenediaminetetraacetic acid (EDTA) coated serum separator tube immediately preceding the perfusion through an incision in the right ventricle. Saline was perfused through the body via whole animal perfusion fixation through the circulatory system. Following a 30-minute perfusion, the rats were decapitated using a guillotine and brain sections of the cortex, prefrontal cortex, hippocampus, striatum, cerebellum, and whole brain were collected. All samples were stored at -80°C until assayed.

FL Quantification

Brain tissue was homogenized mechanically in 500ul of PBS before the addition of an additional 500 ul of 50% TCA to precipitate out protein content. Serum samples were diluted 1:10 in PBS before a 1:2 dilution with 50% TCA. All samples were left on ice for 15 minutes and then spun at 10,000g for 15 minutes.

FL (excitation 475-490 nm, emission 510-520 nm) quantification was read on a 96-well fluorescence synergy 2 microplate reader against an in-house generated standard curve consisting of FL dissolved in a PBS background. Tissue [FL] was normalized to serum [FL] and amount of tissue homogenized as follows:

$$\text{Relative Fluorescence units} \ = \frac{\text{Tissue [FL]}}{\text{Serum [FL]}} \div tissue\ weight\ (g)$$

Serum Collection & Assays

Collected blood was left at room temperature for 45 minutes, after which it was spun at 10,000g for 10 minutes at 4°C. The serum supernatant was aliquoted and stored at -80°C. Serum lithium concentration was assessed using an EASYLYTE machine (Medica Vendo Cypress Diagnostics Inc., Bedford, MA, USA) using manufacturer provided standard calibrations. Cytokine ELISA kits were used to quantify serum C reactive protein (CRP; RayBiotech, Norcross, GA, USA) and tumor necrosis factor alpha (TNF-α; eBioscience, San Diego, CA, USA) concentrations.

3.2.2 Summer 2016 LPS Cohort
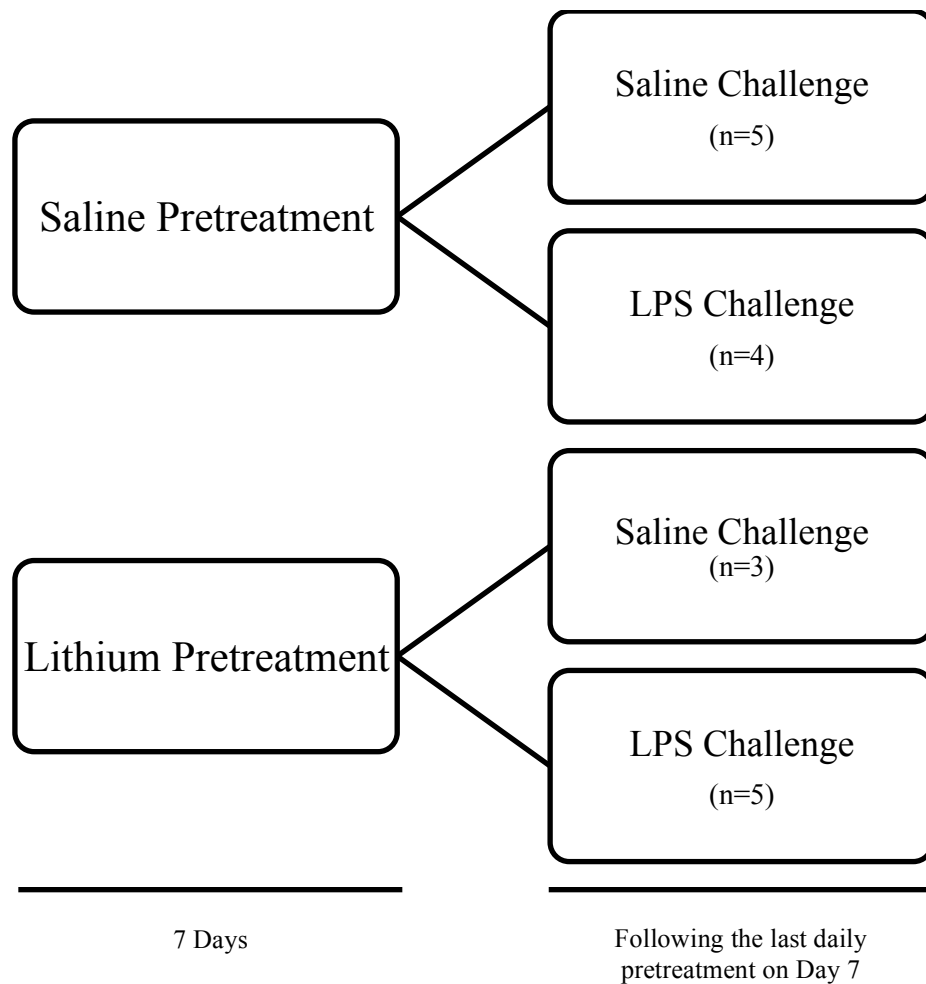
Figure 3. Study design outlining seven day pretreatment and challenge groups – Summer 2016 cohort.

Animal Handling, brain tissue collection & FL quantification

All rat handling and tissue collection protocols remained unchanged from

3.2.1. FL quantification followed the same protocol, but instead a standard curve

of FL dissolved in a PBS + TCA background was adopted. All tissue samples were spiked with 0.028125 μmol of FL to ensure samples fell within the generated standard curve. Tissue [FL] was again normalized to serum [FL] and weight of tissue homogenized as previously described.

3.3 Statistical Analysis

The effect of AMPH and LPS treatments on EB and FL permeability, respectively, was assessed using a two-way ANOVA with treatment and brain region as factors applying post-hoc Bonferonni corrections and Tukey's multiple comparisons test for the summer 2016 cohort only. Locomotor activity was analyzed using a one-way ANOVA and Tukey's multiple comparisons test. GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used to perform all stats pertaining to all AMPH-[EB] setups and the summer 2015 & winter 2015 LPS setups while R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used to analyze the summer 2016 LPS cohort.

CHAPTER 4
Study 1: Rat Models – Results

4.1 AMPH-Induced BBB Disruption

4.1.1 Single Dose AMPH at 15 mg/kg

Evans Blue dye leakage into the brain was used as a proxy to determine BBB permeability in the AMPH model. EB quantification (ug) was normalized to total protein concentration (mg total protein) to take into account fluctuating albumin levels across the individual rats, as EB is known to cross the BBB as a part of an EB-albumin complex. A two-way ANOVA revealed no significant main effect of brain region (F=0.84, p>0.05) and no significant interaction between treatment and brain region (F=1.04, p>0.05); however, a significant main effect of treatment (F=8.23, p=0.0001) was found (**Figure 4**). Furthermore, post-hoc Bonferonni correction indicated a significant difference in the cortex between EB quantification in the saline and AMPH groups (p<0.05).
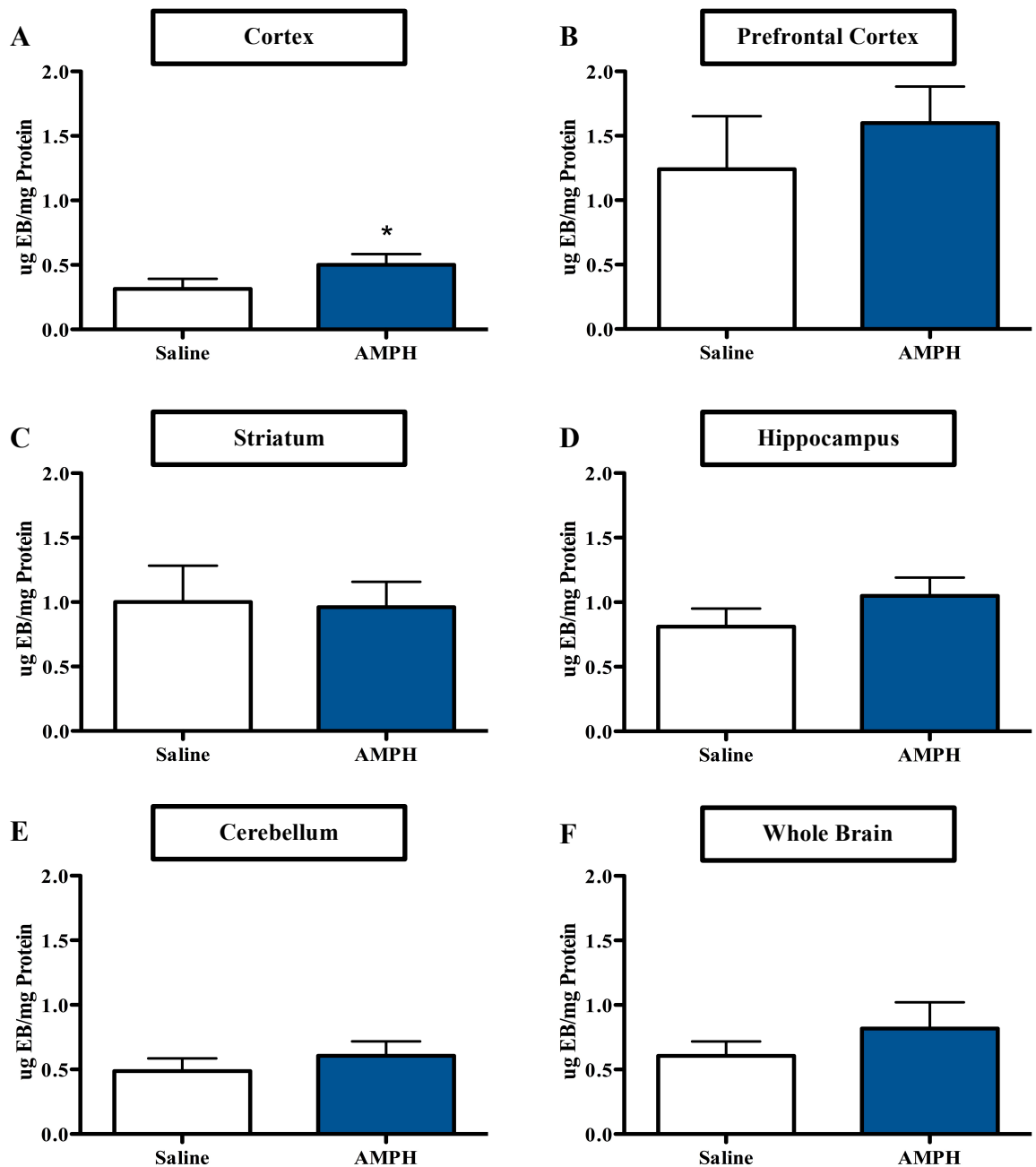
Figure 4. EB permeability 4 hours following a one-time AMPH challenge. All rats were administered a 15mg/kg i.p. injection of AMPH (blue). The brain structures were collected 4 hours following the AMPH injection (A-F). A two-factor ANOVA showed no significant main effect of brain region (F=0.84, p>0.05), or interaction between treatment and region (F=1.04, p>0.05). However, a significant main effect of treatment was found (F=8.23, p<0.0001), with post-hoc Bonferonni correction revealing a significant difference between the treatment groups in the cortex (* - p<0.05). Data represents mean ± standard error of mean.

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4.1.2 Seven Day AMPH treatment (2 mg/kg) & three day withdrawal period

Evans Blue permeability into the brain was again used to represent BBB permeability. Total protein quantification was used to normalize EB levels in the brain, as previously described in 3.1.1. A two-way ANOVA revealed a significant main effect of treatment (F=8.83, p=0.005), but no significant main effects for brain region (F=0.65, p>0.05) or interaction between treatment and region (F=1.14, p>0.05), as seen in **Figure 5**. Additionally, post-hoc Bonferroni correction did not show any differences between the treatment groups.
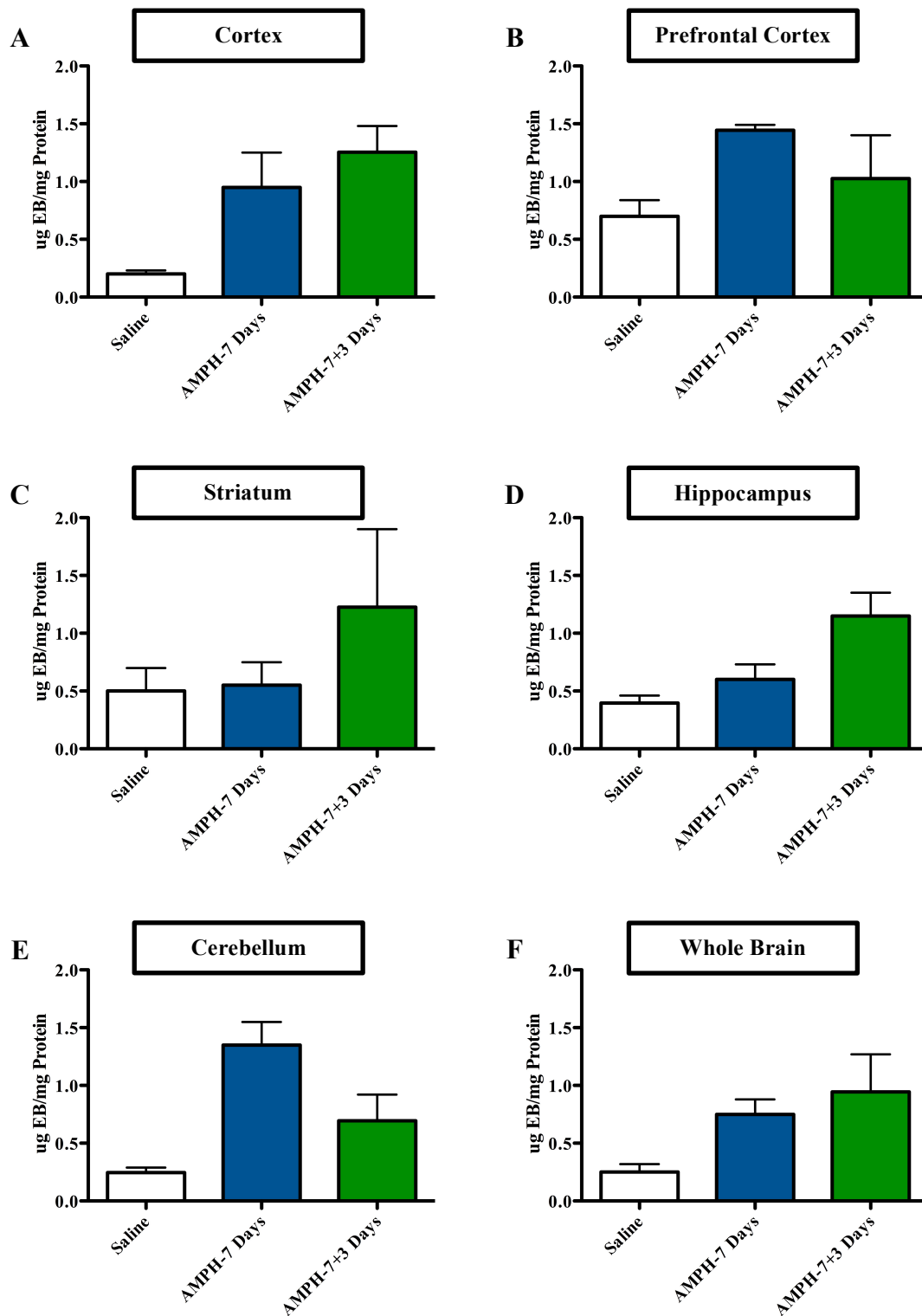
Figure 5. EB permeability following a 7-day AMPH treatment and withdrawal. Rats were injected with 2mg/kg AMPH i.p. (blue) while a subset underwent a 3 day withdrawal period (green). A two-factor ANOVA showed a significant main effect of treatment (F=8.83, p=0.005), but no significant main effects were found for brain region (F=0.65, p>0.05) or interaction between treatment and region (F=1.14, p>0.05). Post-hoc Bonferonni correction did not find any differences between the groups. Data represents mean ± standard error of mean.

4.1.3 Two Week AMPH challenge Preceding a Withdrawal & AMPH Re-challenge

This setup consisted of 4 distinct treatment groups: Saline (white), AMPH with no withdrawal (red), AMPH with withdrawal (green), and finally AMPH with withdrawal and re-challenege (yellow). The AMPH group (blue) represents the aggregated data of all the AMPH groups as a whole, as shown in **Figure 6**. A two-way ANOVA pointed towards a significant main effect for brain region (F=35.06, p<0.0001). However, no significant main effects were found for treatment (F=0.91, p>0.05) or interaction between brain region and treatment (F=1.34, p>0.05). Furthermore, post-hoc Bonferonni corrections did not show any significant differences between the treatment groups.

In the midst of the 14-day AMPH treatment period, locomotion data was collected on days 2, 4, 7, 10, 13, and 14 using a rotating sample set, consisting of eight of the ten AMPH treated rats. **Figure 8** illustrates the locomotor activity, measured in centimeters travelled, of the AMPH group through the duration of the treatment period. A one-way ANOVA found a significant difference in locomotor activity across the days (F=9.14, p<0.0001). Tukey's multiple comparison test was performed to highlight where the difference in daily locomotor activity lie and is summarized in **Table 1**. We continued to collect locomotor data during the 3-day withdrawal period, displayed in **Figure 9**. A one-way ANOVA found a significant difference in mean locomotor activity during the withdrawal period (F=3.77, p<0.05) (**Figure 10)**. However, Tukey's multiple comparison test revealed no

significant differences with regards to locomotor activity during the withdrawal period. Subsequently, locomotor activity was gathered from rats who, following the withdrawal period, were re-challenged with saline or AMPH (**Figure 11**). A two-tailed t-test revealed a significant difference between the saline challenged and AMPH challenged groups (t=6.09, p<0.01).
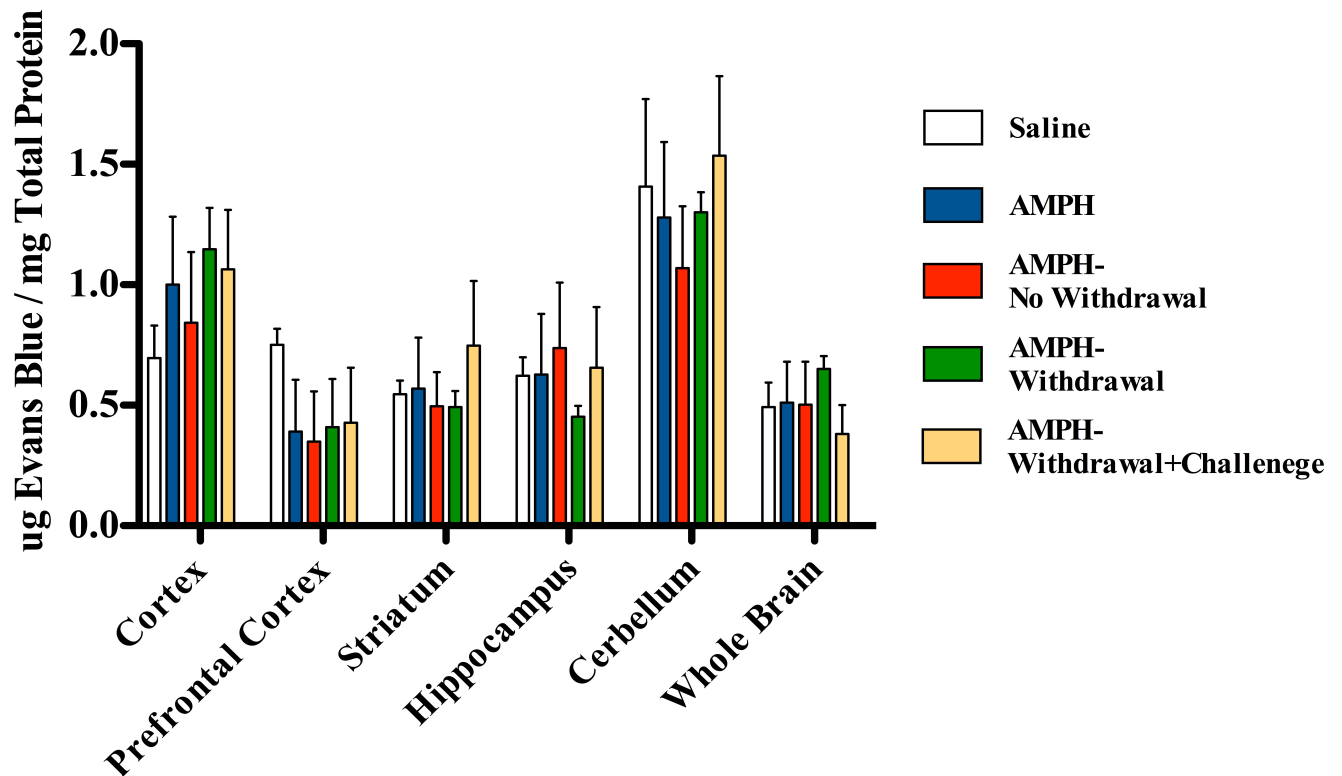
Figure 6. EB permeability in a 14-day model of AMPH treatment, withdrawal, and re-challenge in the whole brain. After a 14-day treatment period consisting of daily 2mg/kg i.p. AMPH injections (red), a subset of rats were subject to a 3-day withdrawal period (green) of which another subset was re-challenged with a subsequent dose of AMPH (yellow) prior to whole brain collection. Aggregated AMPH results are shown in blue. A two-way ANOVA found a significant main effect of brain region (F=35.06, p<0.0001) but no significant main effects were observed for treatment (F=0.91, p>0.05) or interaction (F=1.34, p>0.05). Post-hoc Bonferonni correction did not reveal any significant differences between the treatment groups. Data represents mean ± standard error of mean.
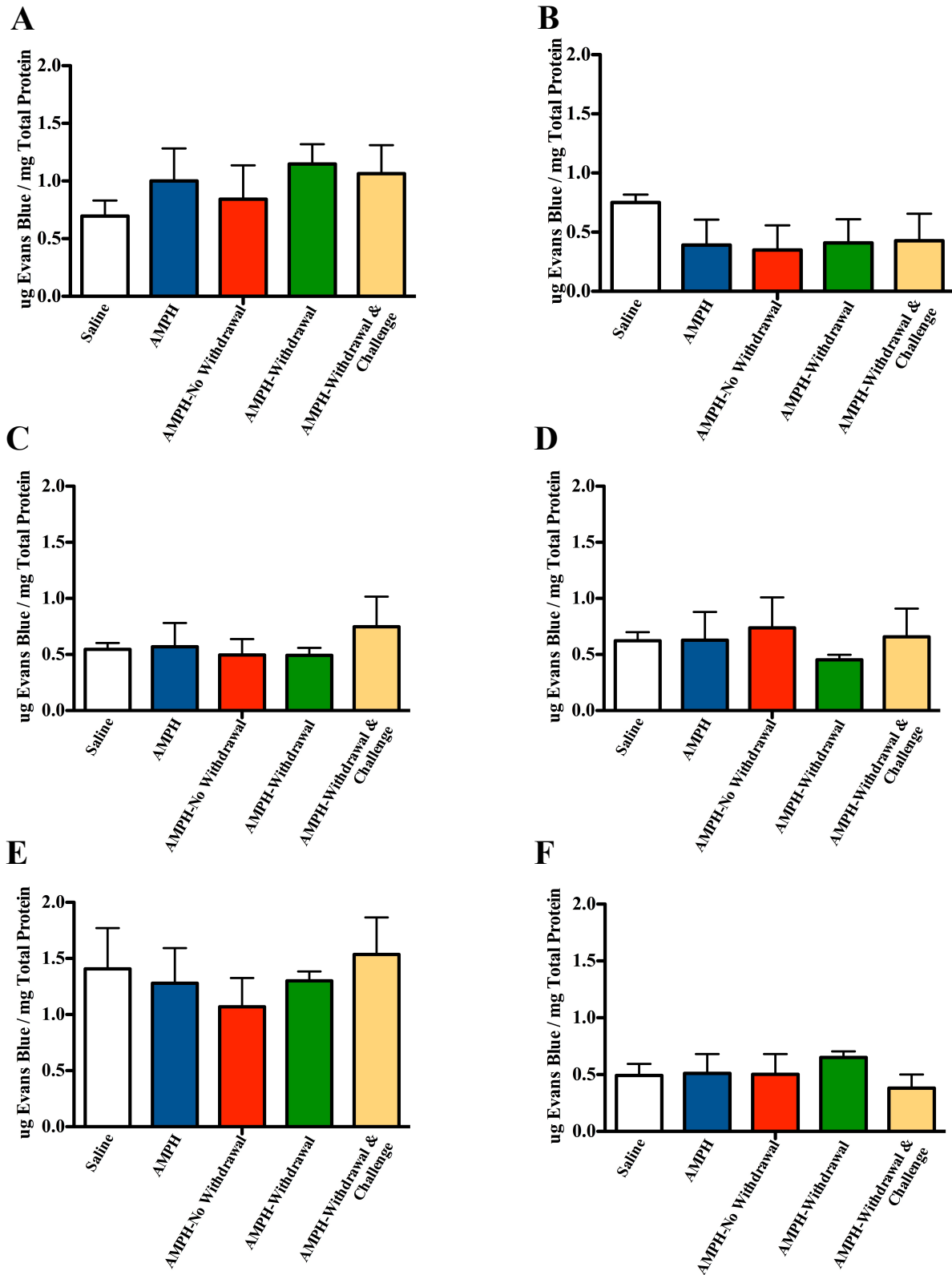
Figure 7. EB permeability in all AMPH treatment groups sorted by region (A-cortex; B-prefrontal cortex; C-striatum; D-hippocampus; E-cerebellum; F-whole brain). Data represents mean ± standard error of mean.
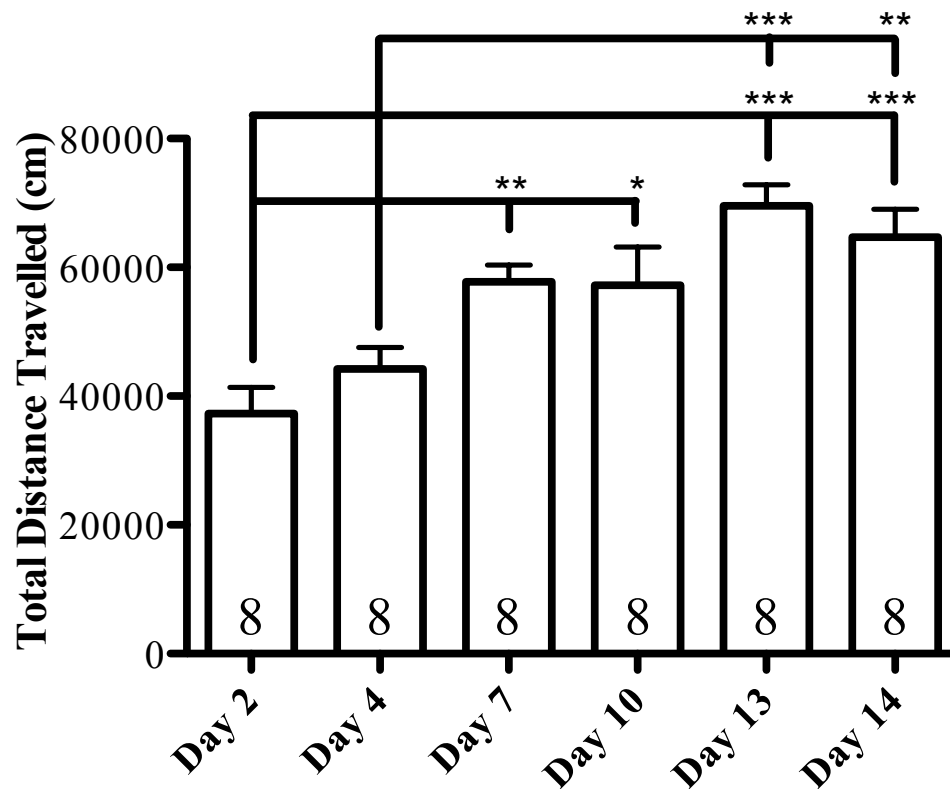
Figure 8. Locomotor activity of AMPH treated rats. We measured total distance travelled over a 3-hour period following daily AMPH injections over 14 days in our rat cohort. We rotated through a different subset of 8 AMPH treated rats for each locomotor collection day. A one-way ANOVA revealed a significant difference in mean locomotor activity across the days (F=9.14, p<0.0001). Tukey's multiple comparison test was used to compare each pair of days individually. * - p<0.001, ** - p<0.01, * - p<0.05. Data represents mean ± standard error of mean.**

Table 1. Tukey's multiple comparison test of locomotor activity of AMPH treated rats through the 14-day treatment period.

	Mean Diff.	p Value
Day 2 vs Day 4	-6957	p>0.05
Day 2 vs Day 7	-20490	p<0.01
Day 2 vs Day 10	-19940	p<0.05
Day 2 vs Day 13	-32320	p<0.001
Day 2 vs Day 14	-27430	p<0.001
Day 4 vs Day 7	-13530	p>0.05
Day 4 vs Day 10	-12980	p>0.05
Day 4 vs Day 13	-25360	p<0.001
Day 4 vs Day 14	-20470	p<0.05
Day 7 vs Day 10	550.4	p>0.05
Day 7 vs Day 13	-11830	p>0.05
Day 7 vs Day 14	-6939	p>0.05
Day 10 vs Day 13	-12380	p>0.05
Day 10 vs Day 14	-7489	p>0.05
Day 13 vs Day 14	4892	p>0.05
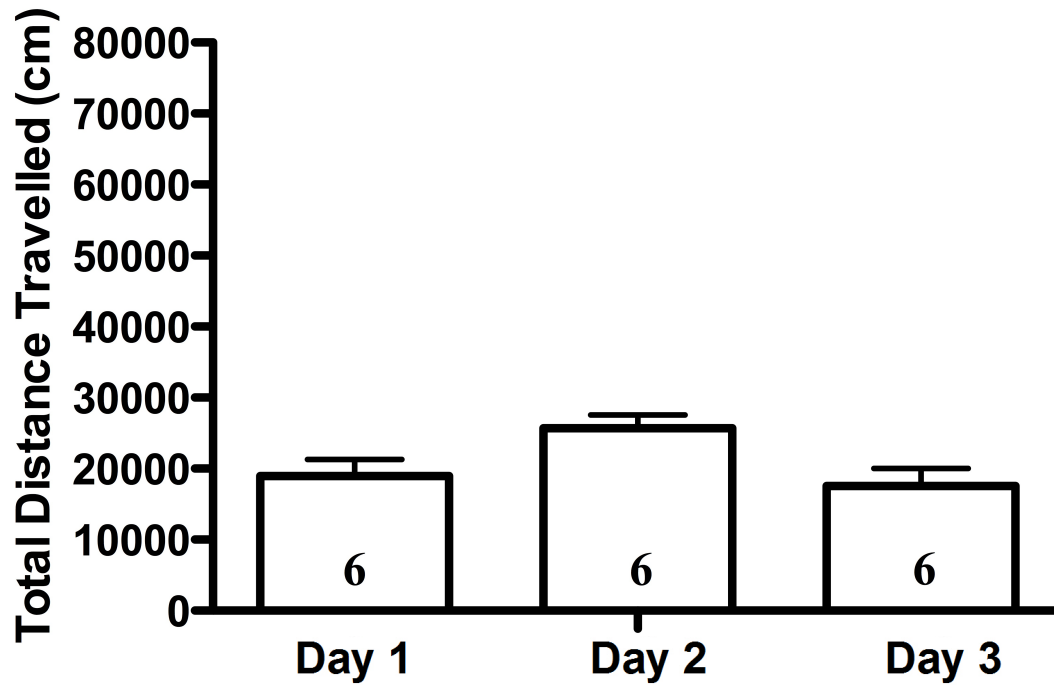
Figure 9. Locomotor activity of AMPH treated rats during withdrawal period. Total locomotor activity, collected over 3 hours, was measured in rats over a 3-day withdrawal period following a 14-day AMPH treatment regime. A one-way ANOVA found a significant difference in mean locomotor activity during the withdrawal period (F=3.77, p<0.05). Interestingly, a post-hoc Tukey's HSD test did not point to a significant difference in locomotor activity between any of the withdrawal days. Data represents mean ± standard error of mean.
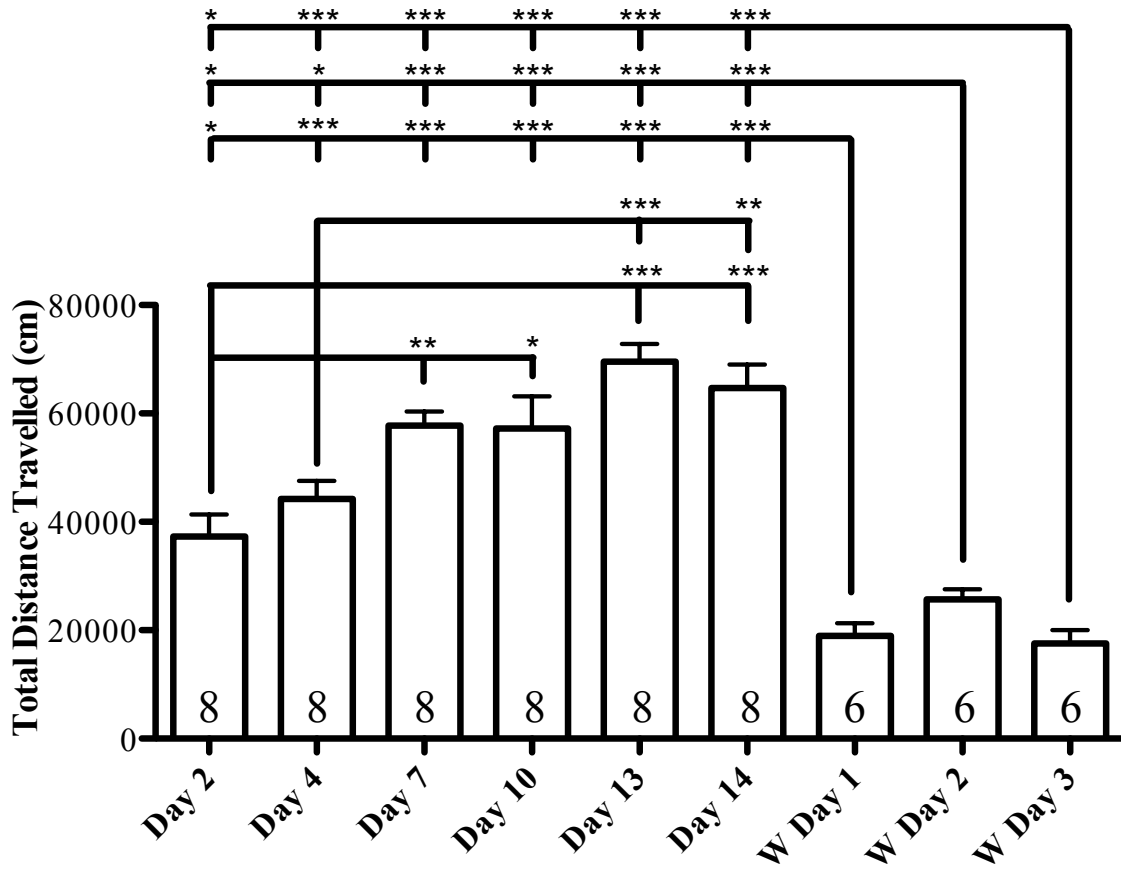
Figure 10. Aggregated locomotor data of rats during a 14-day AMPH treatment period and an ensuing 3-day withdrawal phase. Tukey's multiple comparison test was used to compare each pair of days individually. * - p<0.001, ** - p<0.01, * - p<0.05. Data represents mean ± standard error of mean.**
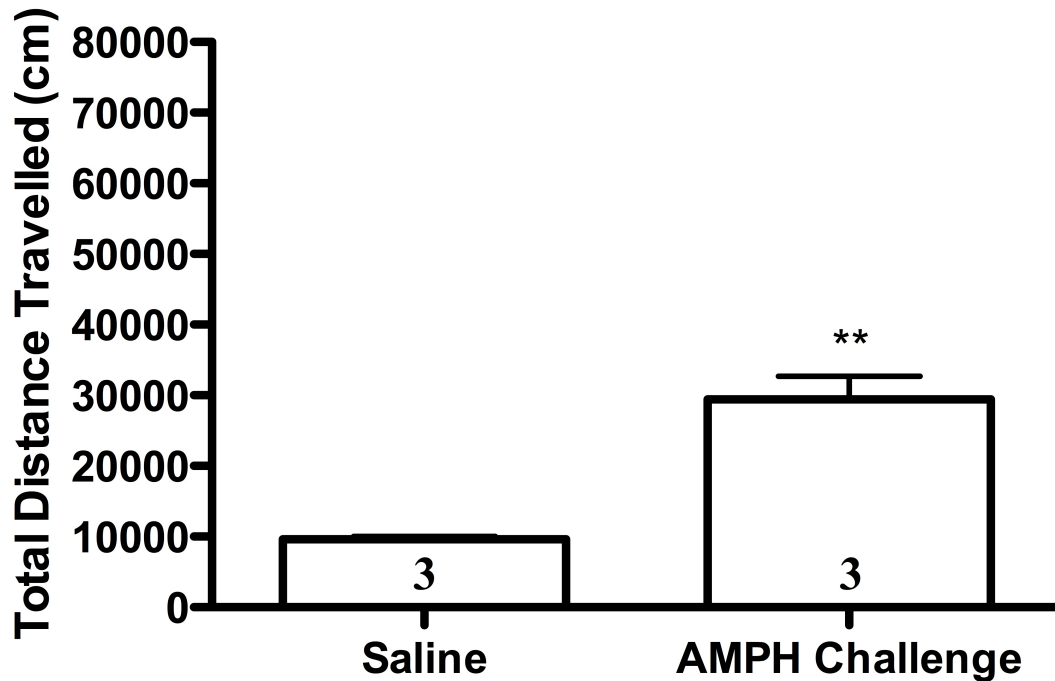
Figure 11. Locomotor activity of AMPH treated following a withdrawal period and subsequent re-challenge. Total locomotor activity over 3 hours was measured in rats who were re-challenged with saline or AMPH following a 14-day AMPH regiment and 3-day withdrawal period. Using a two-tailed t-test, it was found that locomotor activity differed significantly between the saline challenged and AMPH challenged groups (t=6.09, **p<0.01). Data represents mean ± standard error of mean.

4.2 LPS Model of Inflammation and BBB Disruption

4.2.1 Summer and Winter 2015 Cohorts

LPS treatment was used to induce systematic inflammation and BBB opening that was quantified using a small FL dye. Considering just the summer 2015 cohort, a two-way ANOVA pointed towards a significant main effect of treatment (F=3.90, p=0.02), but not for brain region (F=0.52, p>0.05) or interaction (F=0.83, p>0.05), shown in **Figure 12A**. However, no differences between the treatment groups were found using post-hoc Bonferroni corrections. On the other hand, performing a two-way ANOVA on the winter 2015 cohort yielded a significant main effect of brain region (F=2.42, p=0.04), but not of treatment (F=2.59, p>0.05) or interaction (F=0.22, p>0.05), represented in **Figure 12B**. Once again, no significant differences were found between the treatment groups using post-hoc Bonferroni corrections. Considering the fact that both the summer and winter 2015 cohorts followed all the same protocols and used the same materials, we consolidated the data to increase power (**Figure 12C**). A two-way ANOVA containing the summer & winter 2015 data set pointed towards a significant main effect for brain region (F=3.45, p<0.005) but not for treatment (F=1.94, p>0.05) or interaction (F=0.74, p>0.05). Post-hoc Bonferroni corrections did not uncover any significant differences between the treatment groups.

Furthermore, to test whether any differences persisted between the summer and winter 2015 data sets themselves, a 3-way between groups factorial

ANOVA (treatment, brain region, and cohort) showed a significant main effect of cohort (F=12.24, p=0.0007) but not treatment or brain region, as summarized in **Table 1**.
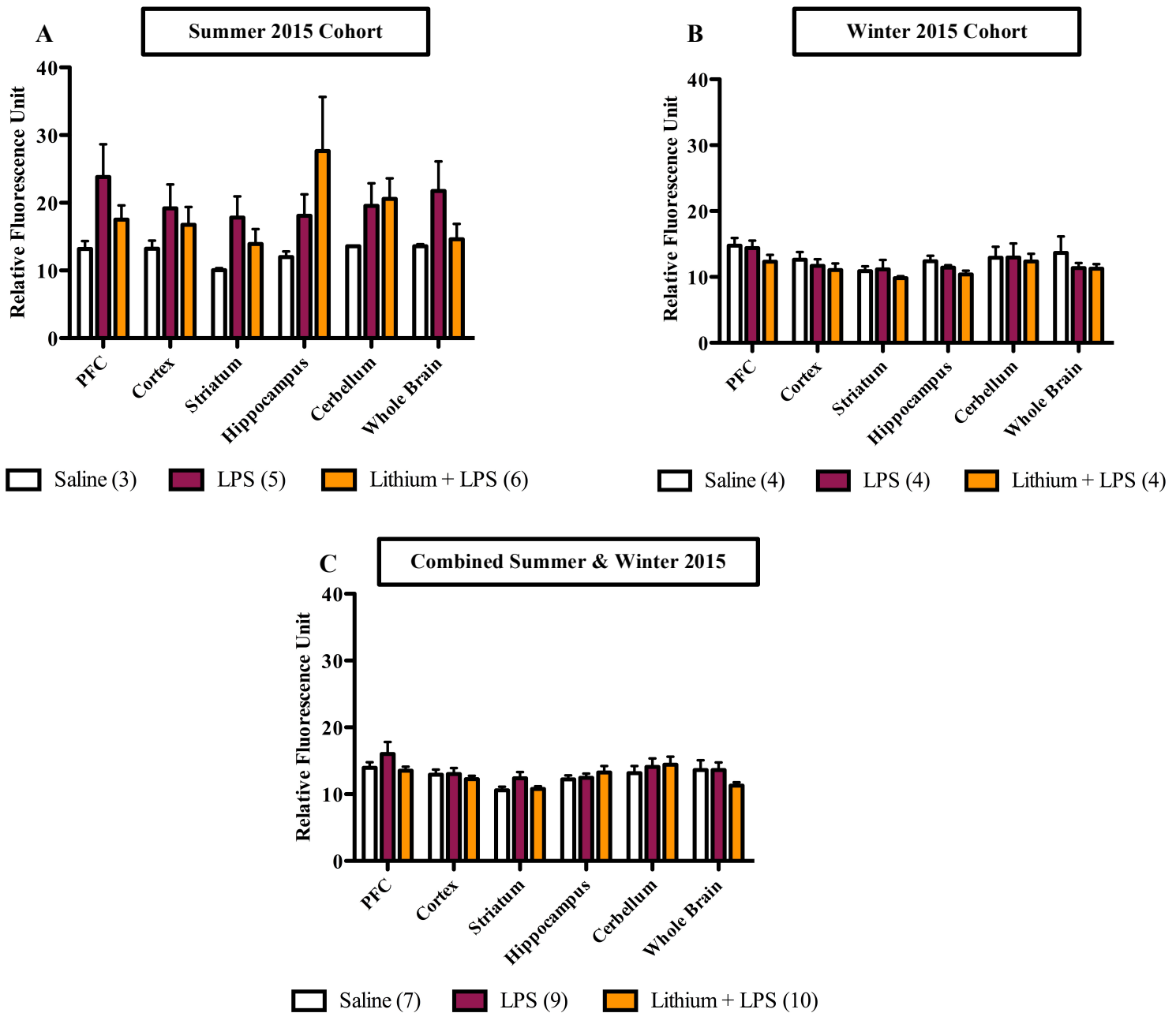
Figure 12. Relative FL quantification in LPS and lithium treated rats. Rats who underwent a 7-day regime of saline or lithium were then given a saline (white) or LPS challenge (saline+LPS=Maroon, Lithium+LPS=Orange). A two-factor ANOVA of the combined summer and winter 2015 cohorts pointed towards a significant main effect for brain region (F=3.45, p<0.005) but not for treatment (F=1.94, p>0.05) or interaction (F=0.74, p>0.05). Post-hoc Bonferonni correction did not uncover any differences between the treatments. Data represents mean ± standard error of mean.

Table 2. Three-factor ANOVA comparing the summer and winter 2015 cohorts (*** - p<0.001).

Source of Variation	F Value	p value
Treatment	1.799	0.17
Brain Region	0.492	0.78
Cohort	12.242	0.0007 ***
Interaction (Treatment:Brain Region)	0.193	0.96
Interaction (Treatment:Cohort)	0.348	0.96
Interaction (Brain Region:Cohort)	2.527	0.09
Interaction (Treatment:Brain Region:Cohort)	0.265	0.99

4.2.2 Serum Assays

Quantification of serum CRP via commercially available kits resulted in no significant differences between the saline, LPS and Li+LPS groups in both the summer (F=0.31, p=0.74) and winter (F=1.29, p=0.29) 2015 cohorts, as determined using a one-way ANOVA (**Figures 13A-B**). As with FL quantification in our LPS model, data from the summer and winter 2015 cohorts was combined to increase power. A one-way ANOVA using the combined 2015

data does not show any differences between treatment groups (F=1.34, p=0.27), seen in **Figure 13**C.
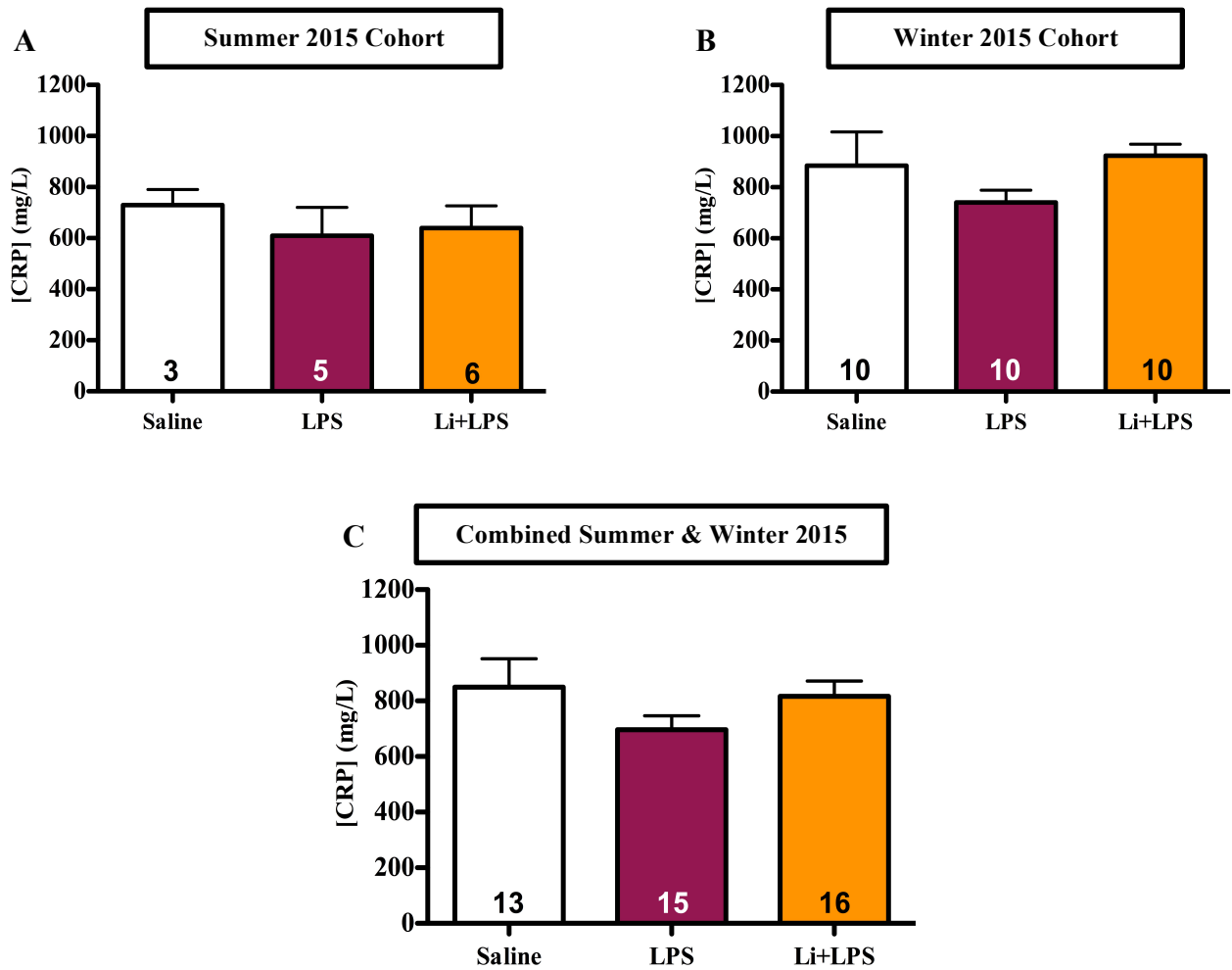
Figure 13. Serum CRP in LPS and lithium treated rats. A single-factor ANOVA with the combined summer and winter 2015 data yielded no significant differences in serum CRP concentrations between the treatment groups (p>0.05). Data represents mean ± standard error of mean.

To gather a more complete serum cytokine profile in the LPS model, we also measured TNF-α levels. All samples fell below the lowest standard provided by the manufacturer and thus, a relative approach was adopted. All individual readings were divided by the mean encompassing all saline readings, yielding an artificially established saline group mean of "1" and providing relative TNF- α
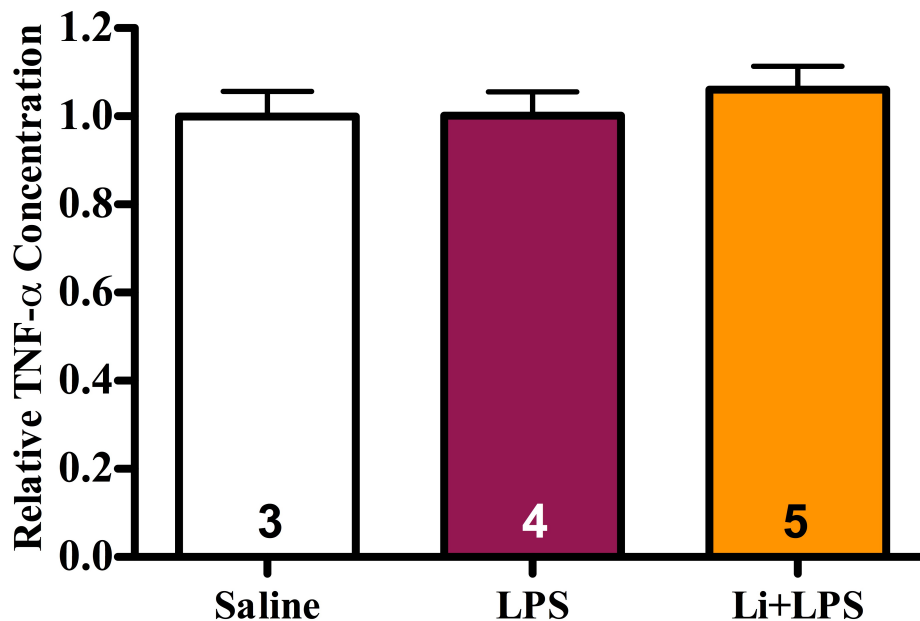
in LPS and lithium treated rats. Samples from both the summer and winter 2015 cohorts were used. The saline group was assigned a value of "1" while the LPS and Li+LPS groups were normalized to the saline. A single-factor ANOVA yielded no significant differences in relative TNF-α concentrations between the treatment groups (p>0.05). Data represents mean ± standard error of mean.

levels in the LPS and Li+LPS groups as compared to controls (**Figure 14**). A one-way ANOVA did not show a significant difference between treatment groups (F=1.78, p=0.22).

To ensure the efficacy of lithium treatment, serum lithium concentration was assessed. Previous studies show serum lithium concentrations to be therapeutic from 0.6-1.2 mmol/L (B. N. Frey et al., 2006). In the summer 2015 cohort, all non-lithium treated rats presented with undetectable (<0.2mmol/L) serum lithium concentrations, while 5/6 lithium treated rats displayed measurable (>0.4mmol/L) lithium levels in serum. Of the 5 aforementioned rats, 3 rats fell in the effective range of 0.6-1.2mmol/L (**Table 2**). As for the winter 2015 cohort, again all non-lithium treated rats did not show quantifiable levels of lithium in serum (**Table 3**). We were able to quantify serum lithium in all 10 rats that received lithium pre-treatment, (>0.2mmol/L), with 3/6 falling in the effective range.

Table 3. Serum lithium concentration of saline, LPS, and lithium+LPS treatment groups – Summer 2015 cohort.

Treatment Group	Serum Lithium Concentration (mmol/L)
Saline	<0.2
Saline	<0.2
Saline	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
Li+LPS	0.4
Li+LPS	<0.2
Li+LPS	0.4
Li+LPS	0.8
Li+LPS	0.8
Li+LPS	0.8

Table 4. Serum lithium concentration of saline, LPS, and lithium+LPS treatment groups – Winter 2015 cohort.

Treatment Group	Serum Lithium Concentration (mmol/L)
Saline	<0.2
Saline	<0.2
Saline	<0.2
Saline	<0.2
Saline	<0.2
Saline	<0.2
Saline	<0.2
Saline	<0.2
Saline	<0.2
Saline	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
LPS	<0.2
Li+LPS	0.4
Li+LPS	0.3
Li+LPS	0.2
Li+LPS	0.5
Li+LPS	0.4
Li+LPS	0.5
Li+LPS	0.6
Li+LPS	0.3
Li+LPS	0.3
Li+LPS	0.7

4.2.3 Summer 2016 Cohort

In this cohort, a replication of the summer and winter 2015 setups, a two-way ANOVA showed a significant main effect for treatment (F=8.457, p<0.0001), region (F=4.743, p<0.0005) and interaction (F=2.347, p<0.005), illustrated in **Figure 15**. A post-hoc Tukey's HSD test revealed a significant difference between saline and lithium+LPS hippocampus (p<0.001) as well as between lithium hippocampus and lithium+LPS hippocampus (p<0.01).
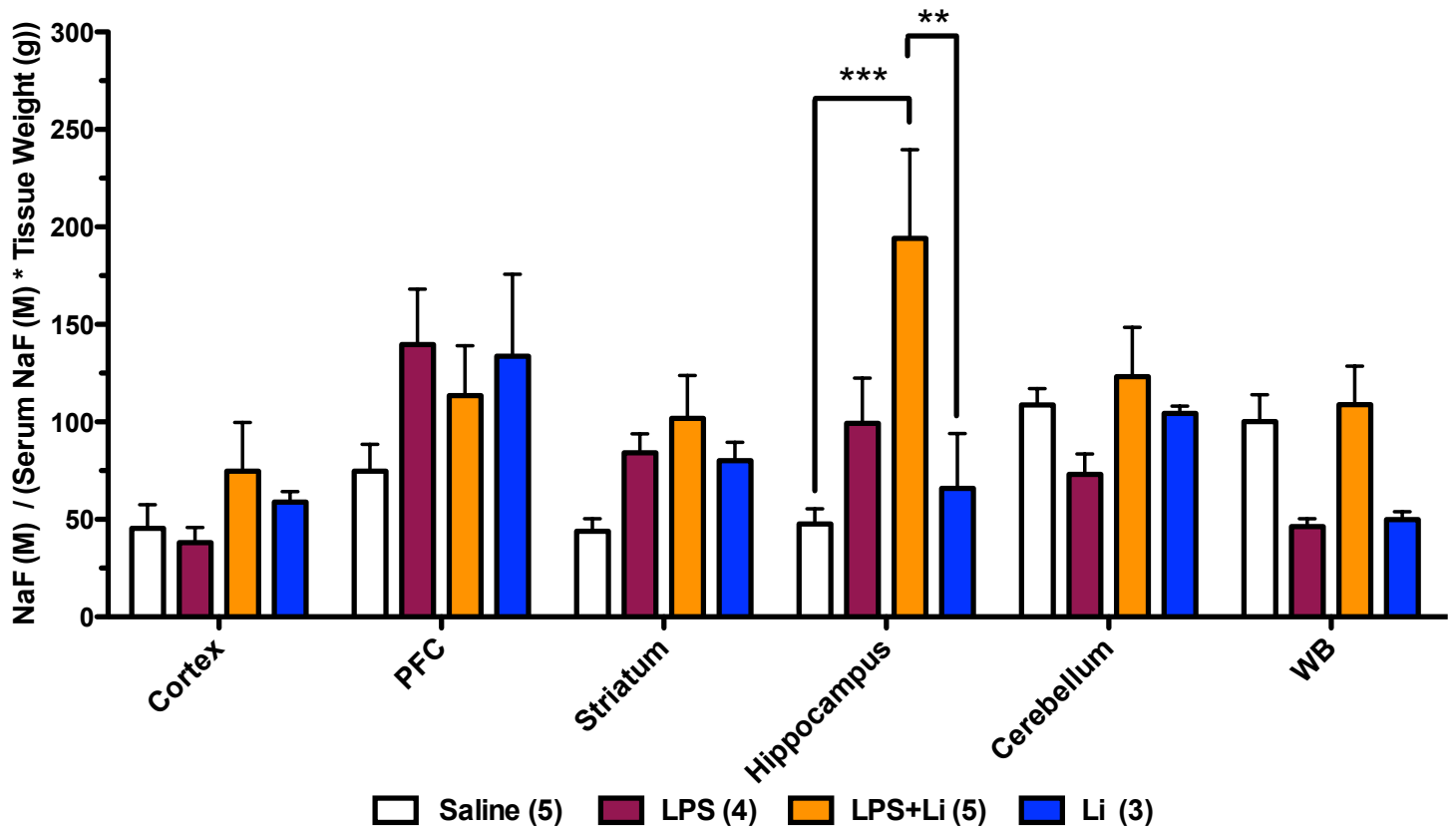
Figure 15. Normalized FL quantification in saline, LPS, and lithium treated rats. Rats which underwent a 7-day pre-treatment with saline or lithium were then given a saline (saline+saline=white, lithium+saline=blue) or LPS challenge (saline+LPS=maroon, lithium+LPS=orange). A two-factor ANOVA yielded a signification main effect for treatment (F=8.457, p<0.0001), region (F=4.743, p<0.0005) and interaction (F=2.347, p<0.005). Difference using a post-hoc Tukey's HSD test are indicated with ** - p<0.01 and *** - p<0.001. Data represents mean ± standard error of mean.

CHAPTER 5
Study 2: Human Imaging – Materials and Methods

5.1 DCE-MRI Imaging of BBB Disruption in a Bipolar Population

5.1.1 Ethics

All experimental procedures and protocols were reviewed and approved by the Imaging Research Center (IRC) at St. Joseph's Healthcare Hamilton and the Hamilton Integrated Research Ethics Board (HiREB). All participants provided informed consent, signed a consent form, and were given the opportunity to drop out of the study at any time. Participant information was kept confidential and no identifying information was used on any study materials.

5.1.2 Study Design

We wish to use gadolinium contrast MRI imaging to compare BBB structural integrity across BD patients (30) in all states (manic, depressive and euthymic) and healthy controls (30) in participants aged 16-45. In this cross-sectional study, all potential participants will undergo a structured clinical interview for DSM disorders (SCID) to determine their clinical status. Clinically diagnosed bipolar type I participants will be classified to be in a manic state provided they score 15 or above on the Young Mania Rating Scale (YMRS) while depressed patients will be ascertained through a score of 18 or above on the Montgomery-Asberg Depression Scale (MADRS). All participants will then undergo clinical subjective (scales) and objective (computerized battery CNSVS)

cognitive testing. Participants will be administered the perceived deficits questionnaire (Sullivan, Edgley, & Dehoux, 1990) (PDQ) and cognitive failures questionnaire (Alloy et al., 2000) (CFQ) followed by a computerized neurocognitive testing using CNS vital signs. Five domains of the CNSVS will be used: verbal memory, finger tapping, symbol digit coding, stroop test, and continuous performance.

Following the cognitive assessment, all participants will undergo a blood test to assess kidney function. A blood creatinine and GFR test will be conducted to ensure suitability of gadovist contrast agent administration. Only those with normal kidney function will be allowed to proceed with the DCE-MRI scan. Subjects will be scanned in using a 3Tesla GE MR750 in the Imaging Research Centre. Prior to scanning a 20 Gauge catheter will be inserted into the left antecubital vein. Following a 3-plane localizer and ASSET calibration scan a 3D high resolution T1-weighted scan, 3D-CUBE (T2-weighted) and multi-slice 2D whole brain T1-map (using mutiple flip angles and B1 correction) will be acquired. The DCE-MRI scan will involve a rapid image acquisition (whole brain every 6s) with a fast 3D fSPGR sequence, over 6 minutes of scanning. Gadolinium contrast (Gadovist) will be injected intravenously at a rate of 5cc/second using a Spectris Solaris MRI compatible power injector. Modeling of BBB permeability (Ktrans), extravascular leakage space (Ve) and cerebrovascular

blood flow (i.e. perfusion, CBF) will be done using the validated Tofts model. BBB modeling will be done using software written in-house using Matlab.

Finally, 15ml of blood will be drawn from all patients to test for blood brain barrier proteins (as a result of BBB disruption) including metallomatrix protein 9 (MMP-9)(Shigemori, Katayama, Mori, Maeda, & Kawamata, 2006) and S100 calcium binding protein B (S100B)(Koh & Lee, 2014) and myelin damage marker proteins(Lamers et al., 2003) S100B and myelin basic protein (MBP). This blood data will then be correlated to BBB permeability, mood status and cognitive function to determine the relationship.

CHAPTER 6: DISCUSSION

6.1 AMPH Induced Model of Mania

The role of the BBB is well characterized in the pathophysiology of several neurodegenerative diseases, but its role in BD has yet to be studied. We used an AMPH induced mania model in rats to tease out any alterations in BBB permeability. Initially, as this study was more exploratory in nature, a high single challenge of AMPH (15 mg/kg) was used. We assumed that if there was no BBB disruption with acute AMPH administration at this dose, there may be no effect of acute AMPH on BBB integrity. Analyzing EB permeability into the brain (as ug EB per mg of total protein) between the groups, we observed a significant increase in the cortex, with a similar trend visible in the prefrontal cortex and hippocampus. These results point towards the presence of a relationship between acute AMPH treatment and BBB disruption. However, utilizing such a high dose of AMPH (15 mg/kg) can prove to be toxic to the rat by inducing elevated inflamation and oxidative stress, altered dopamine integrity, and a global increase in serotonin and dopamine release (Cadet & Brannock, 1998; B. Frey et al., 2006; Gluck et al., 2008; Ohmori, Koyama, Muraki, & Yamashita, 1993). Furthermore, the use of AMPH at this dose caused measureable damage to the health of the rats in this cohort, as evidenced by one rat falling unconcious 2 hours post AMPH injection. Given that we observed a trend of elevated BBB permeability across the cortex, prefrontal cortex and hippocampus in the AMPH treated rats, we decided

to go forward with an acute long-term AMPH treatment protocol. Some studies have used a similar AMPH dose to that used in this cohort and have assessed BBB permeability (Bowyer, Thomas, Schmued, & Ali, 2008; Martins et al., 2011; O'Shea, Urrutia, Green, & Colado, 2014b). Bowyer et al. showed that at such high doses, BBB permebility as quantified via Fluoro-Jade C staining as a marker for nerual damage, is maximal 2 days post the single AMPH administration (Bowyer et al., 2008). Moreover, previous work assessing behvioural and biological paradigms with the aid of an AMPH model opted for treatment courses lower in dosage and more chronic in nature. Behavioural components such as locomotion, decision making, and social play alongside biochemical markers such as ROS, inflammatory markers, and neurotransmitter fluctuations are better represented in an acute and chronic AMPH model.

We thus moved to a 2 mg/kg dose of AMPH administered daily for 7-days. This cohort also featured a three day withdrawal period, characterized by the absence of injections of any kind. With only 2 saline controls, 4 rats in the seven day AMPH treatment group, and 4 in the AMPH plus three day withdrawal group, no statistical difference in [EB] was observed between the groups in any of the brain regions. A point of interest was the large error bars, especially in the AMPH groups. This may point to a less than optimal setup with regards to the EB dye and subsequent tissue analysis.

To further investigate the validity of using EB as a marker for BBB permeability, a 14-day AMPH treatment period with a 3-day withdrawal and subsequent re-challenge was adopted in the hopes of detecting more stable and constant BBB permeability changes within the individual treatment groups. Given the disparity in sample size between the saline (2) and AMPH (10) groups achieving statistical significance was a challenge. The locomotion data points towards an interesting trend in distance travelled over the 14-day AMPH period, with increases in locomotion occurring until the very end of the treatment period (different letters represent significance; i.e. bar labeled "A" is significantly different from bar labeled "B"). This points towards an increased AMPH induced effect throughout the two week period, suggesting a low dose chronic injection period may be more representative of BD rather than a large single dose as we previously attempted. As expected, distance travelled decreased significantly ($P<0.05$) in the withdrawal period providing some physical data corresponding to our AMPH administration schedule. Our locomotion data falls in line with previous AMPH studies, showing a rise in locomotor activity within the first 10 days followed by a plateau and subsequent decline upon AMPH withdrawal (J. W. Young, Minassian, & Geyer, 2016).

Looking at the EB data, no strong trends are apparent, partially due to the very large error bars. Literature (reviewed by Wang & Lai 2014) indicates that the use of EB has been shown to in some cases lead to high variability, and can

explain a portion of the large variance observed in our cohort. In light of the difficulties which arose with EB, using another florescence tag such as fluorescein (synthetic fluorophore) would allow for much better quantification of BBB permeability. Fluorescein has been extensively used in stroke models to assess BBB disruption (B. T. Hawkins & Egleton, 2006; B. T. Hawkins, Ocheltree, Norwood, & Egleton, 2007) and provides much more consistency post tissue treatment and during subsequent quantification. Once entering the circulatory system, EB binds albumin and the complex infiltrates the CNS in the event of BBB disruption. Thus, in the tissue processing phase, the EB-albumin complex must be separated leading to some loss of EB, which may be causing some of the variability observed in our dataset. Another source of variance in our EB data may be through our serum/tissue normalization process. To control for total EB injected into each rat and the varying rates of EB clearance in individual rats, we normalized measured [EB] in the brain to [EB] in the serum. And furthermore, given the movement of EB into the brain is a direct function of albumin movement in the same direction, it was vital to account for total [albumin] in each individual rat. We thus assessed total protein concentration in all the AMPH treatment protocols, with the assumption that the [albumin]:[total protein] for each rat was consistent. This may introduce another source of variance when examining EB data. As fluorescein does not form a complex when injected, this model may provide more sensitivity in detecting BBB alterations. Furthermore, the significantly smaller size of fluorescein vs. the EB-albumin complex allows

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for a greater "resolution" in detecting BBB disruption. Fluorescein also allows for a more robust setup down the line as it can easily be used in conjunction with a variety of antibodies in order to facilitate targeted visualization of specific BBB proteins, which may be implicated in its disruption.

Furthermore, we chose to adopt a LPS induced model of BBB disruption as this model has been shown to cause pronounced alterations to BBB structure and function including various transport systems and immune cell trafficking (Banks & Erickson, 2010). LPS injections lead to a state of systematic inflammation similar to the inflammatory environment described in BD (Dantzer, 2001; Gasparotto, Carobrez, & Bohus, 2007; Miller, Capuron, & Raison, 2005; Simmons & Broderick, 2005).

6.2 LPS Induced BBB Permeability

Moving away from the aforementioned AMPH + EB model, we investigated whether LPS, through activation of inflammatory and oxidative stress pathways, not unlike the activation seen in BD, can disrupt BBB integrity. Moreover, we also sought to determine whether pretreatment with lithium – the only known BD specific medication in use today (Shorter, 2009; A. H. Young & Hammond, 2007) – could attenuate any possible BBB disruption. In our first

cohort, summer 2015, we observed a significant main effect of treatment on BBB permeability. This indicates that a one-time dose of LPS altered BBB organization and that lithium pretreatment provided protection against BBB damage. Interestingly, in the hippocampus we observe significantly increased BBB permeability in the rats pretreated with lithium and challenged with LPS unlike in the other brain regions. One possible reason for this could be the proximity of the hippocampus to the circumventricular organs many of whom are key constituents in hormone regulation and thus are left unprotected by the BBB (Ballabh, Braun, & Nedergaard, 2004). With no BBB present in nearby proximity, lithium pre-treatment may not confer the same benefits as it does to other structures and the interaction between LPS and lithium in such an environment may be causing the elevated [FL] we see.

Given the low powered nature of the study, we aimed to repeat it with an identical protocol, giving rise to the winter 2015 cohort. In this cohort, we found a significant main effect of brain region but not treatment. Although the summer and winter 2015 cohorts were run under the same conditions, using the same techniques, and materials sourced from the same companies, the [FL] results, especially in the treatment groups, do not match. A three-way ANOVA indicated a significant main effect of cohort among the summer and winter 2015 cohorts. Much lower [FL] was observed in the winter 2015 sample set along with a greatly reduced SEM. Interestingly, the control groups in both cohorts displayed a

comparable level of [FL] in all the regions of interest. The presence of a stable control across the cohorts lends credibility to the setup parameters of the model, however an alternate method of [FL] analysis must be considered as the SEM between cohorts is unusual, especially the complete lack of variability in each treatment group of the winter 2015 cohort. To better approximate the background noise in which the samples are homogenized in, a FL standard in a different background was considered. An equal amount of FL was added to a PBS/TCA mixture and whole brain homogenate of a rat which underwent no treatments of any kind. We found minimal variance between the two sets of standards, indicating the better suitability of a PBS/TCA standard in approximating the background condition of the tissue homogenates (**Appendix Figure 1**).

The summer 2016 cohort was undertaken as a replication of the summer and winter 2015 cohorts in order to tease out the underlying trend in a LPS induced model of BBB disruption. FL results from this setup fell more in line with our summer 2015 results, indicating an underlying technical flaw in the winter 2015 cohort. As in our summer 2015 run, we see an increase in BBB permeability with LPS treatment in the PFC, striatum, and hippocampus in the summer 2016 cohort. Interestingly, following a trend observed in the summer 2015 group, we again see a significant rise in BBB permeability in the hippocampus in the lithium+LPS group as compared to the saline and LPS groups in the summer 2016 setup (further discussed below). Considering the summer 2015 and 2016 cohorts

as a whole, a clear trend of LPS induced BBB permeability is visible in the PFC, striatum, and hippocampus. Many mood and cognitive domains are found in these structures and as such these structures are heavily linked to BD (Fusar-Poli, Howes, Bechdolf, & Borgwardt, 2012; Hanford, Nazarov, Hall, & Sassi, 2016; Hozer & Houenou, 2016; Nery, Monkul, & Lafer, 2013; Otten & Meeter, 2015). Altered BBB integrity in the aforementioned regions in response to systematic inflammation could provide a clue about the biological underpinnings governing these key nodes in BD.

We then looked to confirm the presence of an inflammatory state in rats treated with LPS. Surprisingly, we observed no significant differences across the treatment groups for serum concentrations of CRP or TNF-α. This is in direct contrast to other work, which suggests LPS administration at doses lower or comparable to that which was used in our study illicit vastly elevated pro-inflammatory cytokine levels (Gonzalez-Rey, Chorny, Robledo, & Delgado, 2006; Kinoshita et al., 2014; Xiao et al., 2015). While we were able to quantify CRP levels in our rats, [TNF-α] was quantified relatively as all read samples fell below the lowest standard concentration provided by the manufacturer (31.25 pg/ml). Published literature indicates serum TNF- α concentrations ranges from 8-30 pg/ml (Margoni et al. 2011; Yan et al. 2010), indicating a need for a high sensitivity kit (Bakacak et al., 2015; Simsek et al., 2014) allowing for the detection of lower cytokine concentrations in the serum. LPS acts through the

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TLR4 / IKK / NF-κB pathway (TLR4 - toll-like receptor 4; IKK - IκB kinase; NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells) (Dokladny, Lobb, Wharton, Ma, & Moseley, 2010; Molinaro et al., 2015). Activation of this pathway elicits a drastic rise in cytokine levels. Behaviourally, LPS induces general sickness symptoms including fever, lethargy, reduced food and water intake, ptosis, and diarrhoea (Cross-Mellor, Kent, Kavaliers, & Ossenkopp, 2000; Lacosta, Merali, & Anisman, 1999; Meltzer, Serpa, & Moos, 1989; Weil, Bowers, Pyter, & Nelson, 2006; Yirmiya et al., 2001). While not objectively assessed, many of these symptoms were observed in our cohorts, including lethargy, diarrhoea, and ptosis. We thus would expect to see a corresponding rise in serum cytokine levels in these LPS treated rats and an attenuation of pro-inflammatory cytokine levels in the lithium pre-treatment group. Freeze/thaw cycles and age of samples prior to cytokine quantification may have compromised sample integrity (reviewed by Guo, Dong, Yuan, Dong, & Tian, 2013). While the exact mechanistic effects of lithium with respect to BD remain unclear, it has been repeatedly shown that lithium pre-treatment can provide protection against inflammation and oxidative stress induced damage in both humans and rodents (Albayrak et al., 2013; Forlenza, De-Paula, & Diniz, 2014; Kang et al., 2012; Kaplanski et al., 2014; Nassar & Azab, 2014; Valvassori et al., 2015a). We analyzed serum lithium concentration to ensure it fell within the effective range (0.6-1.2 mmol/L) often prescribed to BD patients (Li et al., 2010; Machado-Vieira et al., 2014; Yu et al., 2015). Of the 16 lithium treated rats across both cohorts, 5

fell within this range, while another 6 presented with 0.4-0.5 mmol/L of serum lithium. We observed higher serum lithium levels in all but one lithium pre-treated rat as compared to the other groups. Final lithium injections were given 6-8 hours prior to sacrifice as this timeframe has been shown to correspond best with the aforementioned effective lithium dose range (Hillert, Zimmermann, & Klein, 2012). Of note with regards to the lithium pre-treatments is the fact that across the 2015 and 2016 LPS cohorts, lithium was not able to provide any protection against LPS induced BBB disruption in any of our regions of interest and even exacerbated BBB opening in the hippocampus when administered in conjunction with LPS. This is interesting considering the well documented anti-inflammatory properties of lithium and the complete protection against oxidative stress induced damage in an AMPH mania model (Frey et al., 2006). Quantifying relative TJ and AJ protein concentrations and mRNA levels of BBB structural proteins in the hippocampus can help shed light onto the mechanism by which lithium is acting to open up the BBB. And while a large section of the literature highlights the anti-inflammatory properties of lithium, there are some studies which suggest lithium can in fact raise inflammation levels in certain situations (Guloksuz et al., 2010; Merendino et al., 1994; Valvassori et al., 2015b), potentially explaining the amplified nature of BBB opening in response to lithium pre-treatment specifically in the hippocampus. In future studies, a more stringent protocol should be put in place regarding the timing of the final lithium injection

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as well as greater precaution to ensure consistency between the time between serum separation and serum analysis.

In conclusion, we detected an encouraging trend of increased BBB permeability in the LPS group, with lithium pre-treatment providing at least partial protection against the aforementioned BBB opening in the summer 2015. This was in line with our hypothesis, but we were not able see identical findings in our repeated winter 2015 cohort. A more consistent FL quantification methodology emphasizing absolute quantification with a more representative FL standard curve (as described above) was used to analyze the new summer 2016 cohort wherein LPS induced greater BBB permeability in the PFC, striatum, and hippocampus while lithium pre-treatment led to partial protection against said BBB opening in the PFC. A more thorough protein and mRNA profile of key BBB structural components is needed to understand the varying of effects of LPS and inflammation on BBB integrity in these brain regions and help to move one step closer to establishing a more concrete model of BBB permeability in BD.

CHAPTER 7: LIMITATIONS AND FUTURE DIRECTIONS

The first and most foremost limitation we encountered in our AMPH setup was the relatively small sample size across the treatment groups. This may explain the large absence of statistically significant results despite apparent trends, especially in the single dose and seven-day AMPH setup. However, given the exploratory nature of the study, these studies have served a useful purpose in elucidating the effects of AMPH on BBB permeability, and helped shed light on the usefulness of using EB as a marker to quantify changes in BBB permeability in this setup.

Bolstered by encouraging trends seen in the single dose and 7-day AMPH setups, a 14-day AMPH treatment course with a larger sample size was conducted but large intra-group variability, perhaps stemming from the use of EB as an indicator of BBB permeability, made drawing any concrete conclusions difficult. EB is a popular choice in various rat models which contain a component of BBB disruption analysis (Chelluboina et al., 2015; Sharma et al., 2015; Uzum, Bahçekapılı, Baltaci, Mogulkoc, & Ziylan, 2015), however evidence suggests that higher variance in not uncommon with the use of EB.

Looking for alternative methods to quantify BBB permeability, we settled on using FL. When quantifying FL in our summer and winter 2015 cohorts, we

utilized a FL standard curve in a PBS background. Tissue samples were homogenized in PBS while a TCA treatment followed to precipitate out any proteins, membranes and other non-fluorescein components. Our rationale for using only a PBS background in our standard was that the fact that the acidic component of TCA reacts with the various cellular protein components to precipitate them out, leaving on the FL in a PBS background once the supernatant is separated from the precipitated pellet (Jiang, He, & Fountoulakis, 2004; Sivaraman, Kumar, Jayaraman, & Yu, 1997). However, considering our vastly different results in the summer and winter 2015 cohorts despite both boasting identical procedures and protocols, we looked to optimize the FL standard curve. A FL standard curve in a background containing both PBS and TCA better approximates the background in which the tissue samples are read. The summer 2016 cohort utilizes this improved FL standard, limiting external factors affecting the quantification of BBB permeability. Additionally, the AMPH and LPS models used in our studies are not globally encompassing models of BD, and so any conclusions drawn from this setup must be carefully considered when talking about BD as a whole (Einat, n.d.; Slattery & Cryan, 2014).

Most importantly, it was imperative to re-run the LPS induced BBB disruption model to established whether the results found in the summer 2015 cohort are reproducible or not. Using the improved [FL] reading methodology along with a larger sample size, our summer 2016 data supports that of the

summer 2015 cohort and as such, protein analysis of key TJ proteins including claudin-5, occludin, and ZO-1 and mRNA analysis of a wide variety of BBB structural and functional pathways can provide a complete picture of the mechanistic effect of LPS on BBB integrity. In the event of BBB permeability attenuation in the lithium pre-treatment group, a similar analysis can provide valuable insight into the mechanistic workings of lithium whose exact method of effect with relation to BD has not been elucidated.

Furthermore, there a variety of brain imaging techniques to test the hypothesis of disrupted BBB structure or function directly in individuals with BD. One possibility would be the use of dynamic contrast-enhanced MRI (DCE-MRI) as a method for studying BBB disruption in vivo (Heye et al., 2014). Another available technique is the use of $[^{11}C]$-verapamil to study the function of the P-glycoprotein (Pgp) transporter at the blood-brain barrier (BBB) with PET (Hendrikse & Vaalburg, 2002). Finally, the use of in vivo and in vitro preclinical models may be particularly useful to test whether lithium and other medications commonly used in the treatment of BD can reverse and/or prevent BBB damage.

CHAPTER 8: CONCLUSIONS

The present studies aimed to investigate the role of BBB permeability in BD. The AMPH based setups looked to model mania in rats while the LPS studies sought to mimic the inflammatory state of BD. We saw encouraging trends pointing to elevated BBB permeability following the single dose and 7-day AMPH treatment protocols. However, given the underpowered nature of the studies, no firm conclusion can be drawn.

We then proceeded with 14-day AMPH treatment period and a larger sample size. Locomotor data collected from this cohort saw a steady rise in activity over the first ten days followed by a subsequent plateau for the remainder of the 14-day treatment period. AMPH withdrawal attenuated locomotor activity, while a subsequent AMPH re-challenge once again elicited an elevated locomotor response. However, when analyzing tissue [EB], significant intra-group variability led to a complete overhaul of our study design.

In our LPS induced BBB disruption model we utilized FL to quantify BBB disruption. We saw significantly elevated [FL] in the brain in response to LPS administration in our summer 2015 cohort, but not in the identically ran winter 2015 cohort. In our follow-up study, the summer 2016 data supports the trends observed with our summer 2015 cohort which warrants further

investigation into the nature of BBB disruption in the PFC, striatum and hippocampus. With that said, neither setups are a true representation of BD and the conclusions drawn from these studies must be approached with caution. If a link between BD and BBB disruption is established, this would not only advance the knowledge on the neurobiology of BD but also open numerous possibilities to investigate new treatment pathways for this devastating major mental illness.

APPENDIX

A

B
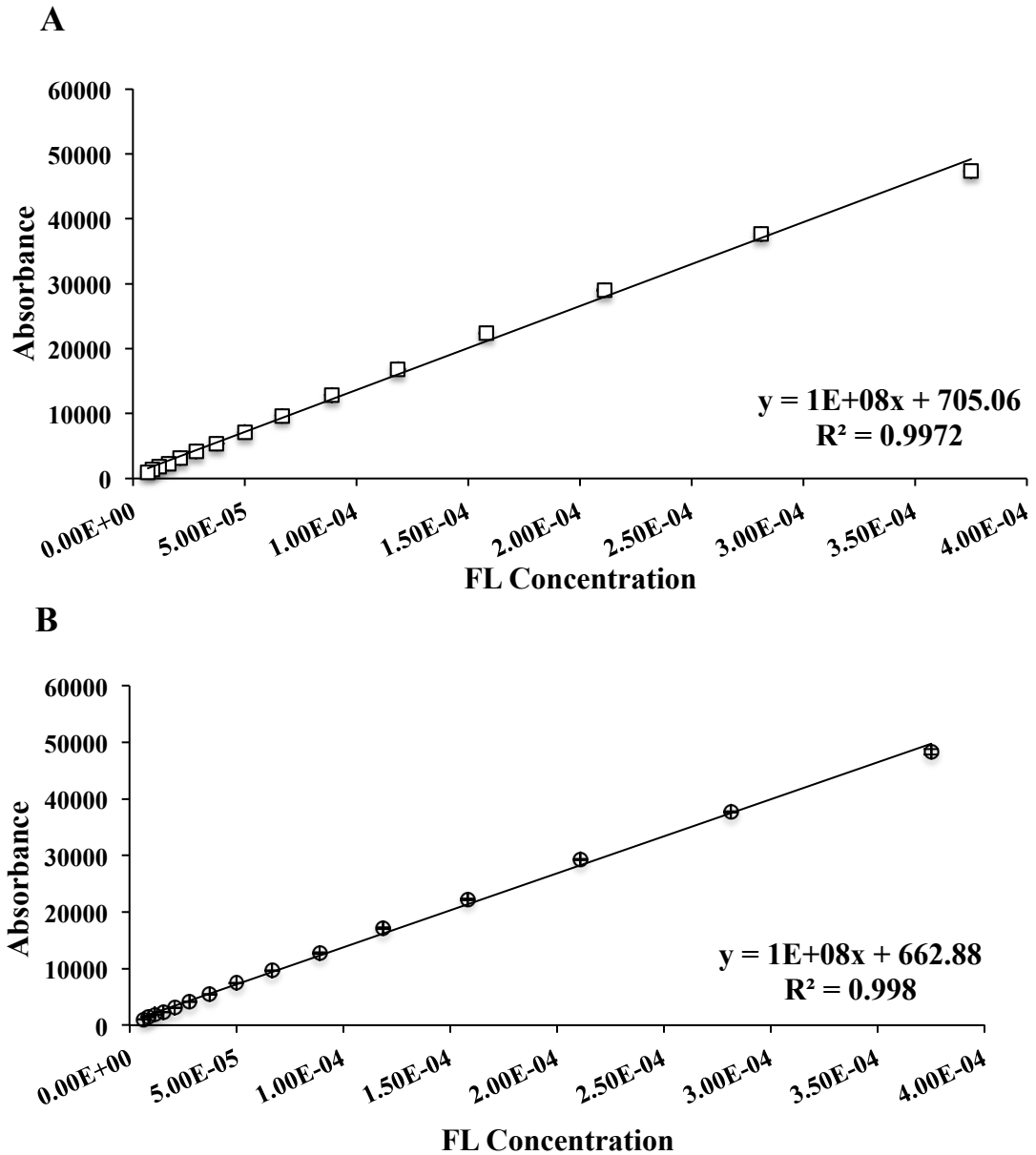
Appendix Figure 1. In-house generated FL standard curves. Comparison of FL standard curves in a supernatant (A) and PBS/TCA background (B).

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