GUANABENZ REDUCES HYPERACTIVITY AND NEUROINFLAMMATION CAUSED BY LATENT TOXOPLASMOSIS IN MICE

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DEDICATION

To Dad. Cancer sucks. You were taken from us too soon but I know that you're looking out for me. You always supported what I did and bragged about me to your friends, even when you didn't understand what I was doing. You only got to see a small piece of this bigger puzzle but I'm thankful for what I did get to share with you. You are missed. You are loved. This one's for you.

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Jennifer Marie Martynowicz

GUANABENZ REDUCES HYPERACTIVITY AND NEUROINFLAMMATION CAUSED BY LATENT TOXOPLASMOSIS IN MICE

Toxoplasma gondii is an intracellular parasite that causes persistent, lifelong infection in one-third of humans worldwide. The parasite converts from a lytic, actively replicating form (tachyzoite) into a latent tissue cyst form (bradyzoite) that evades host immunity and is impervious to current drugs. While acute infection can be life threatening to immunosuppressed individuals, chronic infection has been linked to behavioral changes in rodents and neurological disease in humans. Notably, chronic infection in mice leads to hyperactivity in an open field. Whether these behavioral changes are due to parasite manipulation of the host or the host response to infection remains an outstanding question. We have previously shown that the anti-hypertensive drug guanabenz reduces *Toxoplasma* cyst burden in the brains of BALB/c mice, providing a means to examine whether brain cyst depletion reverses behavioral changes. We used two mouse strains (BALB/c and C57BL/6) differing in their susceptibility to infection. Following drug treatment of chronically infected mice, locomotor activity in an open field was assessed. In both mouse strains, the increased hyperactivity seen during chronic infection returned to normal levels following guanabenz treatment. Guanabenz reduced brain cyst burden ~70% in BALB/c mice as expected, but it increased cyst burden 49% in C57BL/6 mice. Examination of the brains showed that guanabenz decreased inflammation and perivascular cuffing in both infected mouse strains. Our study shows for the first time that it is possible to reverse a key behavioral change associated with chronic *Toxoplasma* infection. Surprisingly, the rescue from parasite-

induced hyperactivity correlates with a decrease in neuroinflammation instead of cyst counts, suggesting that some behavioral changes arise from host responses to infection rather than a parasite-driven process.

William J. Sullivan, Jr., PhD, Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CNS	central nervous system
AAALAC	American Association for Accreditation of Laboratory Animal Care
AIDS	acquired immunodeficiency syndrome
BBB	blood brain barrier
BSA	bovine serum albumin
CDPK1	calcium-dependent protein kinase 1
DHFR	dihydrofolate reducatase
eIF2α	eukaryotic translation initiation factor 2 alpha
ELQs	endochin-like quinolones
FACE-SZ	FondaMental Academic Centers of Expertise for Schizophrenia
FBS	fetal bovine serum
FDA	Food and DrugAdministration
GABA	γ-aminobutyric acid
GLT-1	astrocytic glutamate transporter
HFF	human foreskin fibroblast
HIV	human immunodeficiency virus
i.p.	intraperitoneal
IACUC	Institutional Animal Care and Use Committee
IDO	indolamine 2,3-dioxyganse
IFA	immunofluorescence assay
IFN	interferon
IHC	immunohistochemistry
iNOS	inducible nitric oxide synthases
IRGs	immune related GTPases
LARC	Laboratory Animal Research Center
NBF	neutral buffered formalin
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with 0.5% Tween
PCR	polymerase chain reaction
PP1	protein phosphatase 1
Pru	Prugniad
SAR	structure activity relationship
SARC	Science Animal Research Center
TLR	toll-like receptor
WT	wild type

Chapter One: Introduction

Toxoplasma gondii is an obligate intracellular parasite in the phylum Apicomplexa that currently infects approximately one third of the human population with seroprevalence as high as 77% in some regions (Pappas, Roussos, & Falagas, 2009). The success of *Toxoplasma* is largely associated with the parasite's ability to transition between multiple life stages and cause longstanding latent infections in a wide variety of hosts.

Chronic infection in rodents leads to an array of central nervous system (CNS) dysfunctions, including alterations in neurotransmitter levels, protein expression, immune responses, and behavior (Ihara et al., 2016; Kannan et al., 2017; Li et al., 2018; Martynowicz, Augusto, Wek, Boehm, & Sullivan, 2019; McFarland et al., 2018; Tyebji, Seizova, Garnham, Hannan, & Tonkin, 2019; Wohlfert, Blader, & Wilson, 2017; Xiao et al., 2016). Behavioral changes include hyperactivity, cognitive deficits, and altered anxiety and fear responses (C. Afonso et al., 2017; David et al., 2016; Hermes et al., 2008; McFarland et al., 2018). These behavioral changes coalesce to form the basis of a popular behavior modification hypothesis that proposes *Toxoplasma* manipulates its rodent hosts to become easy prey for cats (Berdoy, Webster, & Macdonald, 2000). By driving rodent hosts into the gut of feline predators, the definitive host of the parasite, *Toxoplasma* secures progression to the sexual stage of its life cycle and widespread transmission as oocysts in cat feces.

Given the reported behavioral changes in rats and mice, several groups have begun to examine whether latent toxoplasmosis is linked to neurological anomalies in human hosts. Chronic toxoplasmosis is associated with schizophrenia, bipolar disorder,

epilepsy, rage disorder, and sudden onset psychosis (Brooks et al., 2015; de Barros et al., 2017; Kannan et al., 2017; Monroe, Buckley, & Miller, 2015). Given the complex, multifactorial nature of these diseases, their differences in clinical manifestations, and a lack of direct causation, *Toxoplasma* is postulated to be an environmental insult that contributes to neuropathology (Dupont, Christian, & Hunter, 2012; Solvsten Burgdorf et al., 2019; Tyebji, Seizova, Hannan, & Tonkin, 2019). Additionally, there is evidence that treating seropositive schizophrenic patients with antipsychotic drugs that also possess anti-*Toxoplasma* activity improves control of some of the neurological symptoms (Fond et al., 2018).

I. *Toxoplasma* Life Cycle and Transmission

A. Life Cycle

Toxoplasma is capable of transitioning between multiple life cycle stages. Several of these stages are found exclusively within the feline, which is the definitive host of *Toxoplasma*, because cats support the sexual reproductive stages within the gut (Delgado Betancourt et al., 2019). Following parasite sexual reproduction, the feline releases highly infectious oocytes into the environment in their feces. Theses oocytes are stable and can persist within the environment for months to years (Yan, Liang, Zheng, & Zhu, 2016). Once ingested by a host, oocytes will rapidly transition into tachyzoites.

The tachyzoite is the fast replicating, lytic form of the parasite that is responsible for acute disease. A single tachyzoite will invade a host cell, replicate within that host cell and then egress, causing cell lysis. This process causes the extensive tissue destruction seen in life-threatening infection if left unchecked. While the tachyzoite is

motile, extensive host dissemination is believed to be the product of tachyzoites infecting motile cells, such as dendritic cells, NK cells, and macrophages, which then travel throughout the host's body and accelerate the spread of infection. In fact, tachyzoites are found at peripheral sites following intraperitoneal infection (i.p), such as the brain, heart, lungs, spleen, and prostate (Belal et al., 2016; Colinot et al., 2017; Wohlfert et al., 2017).

Under various conditions, the tachyzoite transitions into a second life stage, the bradyzoite. This life stage, sometimes referred to as a tissue cyst because it forms a cyst wall within the host cell, causes the long lasting chronic stage of infection. Tissue cysts can be found in multiple organs, including but not limited to the heart, lungs, muscle, kidneys, liver, and prostate, but are found in large numbers in the brain (Belal et al., 2016). Tissue cysts are capable of evading the immune system and persist for the life of the host. Therefore, once an individual has become infected, the long lasting bradyzoites ensure that they remain infected for the rest of their life.

While *Toxoplasma* has been shown *in vitro* to be capable of infecting astrocytes and other CNS cell types, *in vivo* within the brain bradyzoites have been shown to be exclusively found in neurons (Koshy et al., 2012). The reason for this is still not understood. Other cell types that show high quantities of bradyzoites include cardiac muscle, which can lead to life threatening myocarditis, and skeletal muscle, which aids in transmission due to the consumption of contaminated meat (Delgado Betancourt et al., 2019; Yan et al., 2016).

B. Transmission

Both horizontal and vertical transmission are possible for *Toxoplasma*. If a pregnant mother is infected with the parasite for the first time and does not have an existing programmed adaptive immune response (neutralizing antibodies) she is usually unable to control the acute infection before it reaches the placenta (Piao et al., 2018). Tachyzoites, the lytic form of infection, are also capable of crossing the placenta and infecting the fetus, thereby allowing for vertical transmission from a mother to child.

Horizontal transmission is the most common method of acquisition of *Toxoplasma*. Ingestion of oocysts from the environment or tissue cysts from improperly prepared meat are the two most frequently observed methods. Oocysts can also contaminate produce that is not properly washed before serving or directly contaminate water supplies. Additionally, household cats can shed oocysts into litter and if an individual is not careful they can become exposed while cleaning a contaminated litter box (Delgado Betancourt et al., 2019; Yan et al., 2016).

Bradyzoites are transmitted when meat is consumed that was not cooked at the appropriate temperature or duration. This was tested in the early 1960s where orphans in a Paris sanatorium were fed raw meat from various sources and seroconversion was recorded. In cases where meat from horses or cows was used, seroprevalence rose from 10% to 50%, but then reached 100% with the addition of lamb, likely due to the very high infection rate of sheep as compared to cows or horses (Desmonts et al., 1965; Dubey, 2008). The consumption of raw meat as part of cultural norms can help explain some of the high global seroprevalence of *Toxoplasma*.

While rare, horizontal transmission is possible from one human to another in the form of solid organ transplants. If an uninfected transplant recipient is given an organ from an infected donor, there is the possibility that the organ contains bradyzoites and the recipient will become infected. The rate at which this happens varies depending on the organ which is transplanted. Higher incidence is reported in heart and lung transplants than in kidney transplants. However, due to the immunosuppressants that transplant recipients receive in order to prevent rejection of the new organ, there is the possibility of life threatening acute toxoplasmosis. Furthermore, an active acute infection in the new organ can facilitate organ rejection in the recipient. This problem has become less of an issue since the adoption of prophylactic treatment for the prevention of pneumocystis pneumonia (*Pneumocystis jirovecii*) with trimethoprim/sulfamethoxazole (TMP/SMX), which has efficacy against *Toxoplasma* (Dunay, Gajurel, Dhakal, Liesenfeld, & Montoya, 2018).

II. Pathogenesis of *Toxoplasma* Infection

A. Acute Infection

Toxoplasma is a lytic parasite. When the tachyzoite invades a host cell, it will rapidly replicate within that cell through an asexual processes called endodyogeny where two daughter cells form within the mother cell before emerging as individual parasites. This process continues until the host cell can no longer contain the parasites and they lyse out of the host cell through a process called egress. Egress from a host cell destroys the cell and releases the tachyzoites, allowing them to invade the neighboring cells and perpetuate the lytic cycle. This can cause extensive damage in the infected organ or tissue

the parasite resides within and causes the life-threatening condition of acute toxoplasmosis. Unchecked acute infection will lead to extensive organ damage and organ failure.

Acute toxoplasmosis can be the result of the initial infection and dissemination of the parasite or it can be the result of reactivation of existing bradyzoites. Either case can be life-threatening if the host is unable to mount an effective immune response. For most individuals, symptoms are flu-like or are mistaken for a minor illness. The most common symptoms include headache, lymphadenopathy, myalgia and fever (Teutsch, Juranek, Sulzer, Dubey, & Sikes, 1979). Symptoms persist for several days and then resolve and the individual is often unaware that they have been infected with *Toxoplasma*. These individuals are able to safely transition to being chronically infected. However, if an individual becomes severely immune suppressed, they cannot effectively control the infection on their own and require additional support through anti-parasitic drugs.

Symptoms of severe acute toxoplasmosis vary depending on the organ system(s) that is affected. The most common symptoms associated with acute toxoplasmosis are diffuse, poorly localized neurological symptoms. These can vary but generally can include seizure, paralysis, cranial nerve involvement, and headache. This is caused by the impact of extensive parasite replication, lysis, and damage to various regions within the CNS.

Ocular disease is another common manifestation of *Toxoplasma* infection, which is the most common etiology of posterior uveitis in the world (Pappas et al., 2009). Due to the lytic nature of the parasite, if *Toxoplasma* manages to seed the eye it can causes extensive damage that may lead to blindness. The inability to eliminate chronic infection

also means that focal reactivation of encysted parasites can lead to progressive damage and loss of sight. Additionally, chorioretinitis is the most common symptom associated with congenital *Toxoplasma* infection with an incidence rate over 75% (Stagno et al., 1977).

B. Reactivation

Reactivated toxoplasmosis often causes extensive CNS and cardiovascular damage due to presence of tissue cysts that reside in these regions during chronic infection (Wohlfert et al., 2017). Patients will often present with diffuse neurological symptoms and/or seizures and show multiple ring-enhancing lesions on a CT scan indicating where the parasite has destroyed tissue in the brain. As discussed in later sections of this chapter, aggressive anti-parasitic drug treatment is necessary to control the infection and prevent further neurologic deficits.

Reactivated infections occur almost exclusively in immunosuppressed patients. However, there are infrequent case studies which show reactivation pathology discovered upon autopsy of relatively immunocompetent individuals, but these are usually in the context of severe disease like sepsis or lupus (Murro, Novo, & Arvanitis, 2016; Pusch, Romeike, Deckert, & Mawrin, 2009). The two most common patient populations with reactivated *Toxoplasma* infections are HIV/AIDS (human immunodeficiency virus/acquired immunodeficiency syndrome) patients and organ transplant patients taking immunosuppressive drugs to prevent rejection.

Early in the AIDS epidemic, *Toxoplasma* encephalitis was a major cause of death in HIV patients. This was in part because patients already had existing *Toxoplasma*

infections and the parasite itself is sensitive to CD4+ T cell numbers. Most patients develop reactivation when their CD4+ T cell count drops below 200/$mm^{3.}$

Reactivated toxoplasmosis has also been reported in patients with solid organ, bone marrow, and hematopoietic stem cell transplantation. For solid organ transplants, it is possible for a naive recipient to receive an infected organ and acquire infection. Reactivation is likely to cause extensive damage to the new organ. Particularly with heart transplant, the lytic damage from the parasite can lead to organ failure or rejection. Recrudescence is more prevalent in bone marrow transplants (up to 80% of cases in seropositive individuals) and is rarely the result of a new infection (Dunay et al., 2018). Clinical manifestations are not limited to encephalitis and can include retinal, myocardial, and pulmonary involvement as seen with other forms of disseminated acute infection.

If a patient is able to return to immunocompetent status, such as with treatment with anti-retroviral therapy to restore CD4 T cell counts for individuals with HIV/AIDS, the individual is typically able to maintain chronic or latent infection. However, individuals with continued immune suppression are placed on prophylactic drug treatment, often for the rest of their life.

C. Congenital Infection

Congenital infection occurs following vertical transmission from an infected mother to her fetus across the placenta. Depending on the trimester, the outcomes can vary substantially. Generally, the earlier the onset of infection, the more severe the outcome. If the infection occurs during the first or second trimester, it will often result in miscarriage or stillbirth. Additional outcomes include an array of birth defects and

complications such as hydrocephaly, blindness, seizures, and mental retardation. If the infection occurs late in the third trimester there may not be obvious symptoms present at birth, which means a neonate can go days to weeks before being diagnosed, which complicates treatment. Early identification of congenital toxoplasmosis along with treatment modestly improves outcomes for infected neonates (Gollub, Leroy, Gilbert, Chene, & Wallon, 2008; Thalib et al., 2005).

D. Chronic Infection

Most of our knowledge of chronic *Toxoplasma* infection comes from animal models. Until recently, it was believed that these infections were benign occurrences that had the potential to evolve into life threatening infections in the case of immunosuppression. *Toxoplasma* has been noted in incidental autopsy findings, but these were undiagnosed reactivation events and can no longer be described as latent chronic infection (Habek, Ozretic, Zarkovic, Djakovic, & Mubrin, 2009; Murro et al., 2016; Pusch et al., 2009). Therefore, there is a substantial knowledge gap in our understanding of these lifelong chronic infections and the impact they have on the brain.

In rodents, chronic toxoplasmosis has been shown to cause widespread CNS dysfunction. These changes include alterations to neurotransmitter levels, astrocyte dysfunction, damage to the blood brain barrier (BBB), increased inflammation, and behavioral changes.

Several different groups of neurotransmitters, the major communication molecules within the brain, have been shown to be dysregulated upon chronic infection. While dopamine was historically believed to decrease (Stibbs, 1985), there does not

appear to be consensus in the more recent literature (Ihara et al., 2016; Z. T. Wang, Harmon, O'Malley, & Sibley, 2015). Glutamate, which is a major excitatory neurotransmitter, is increased in chronic infection through dysregulation of the major reuptake transporter GLT-1 (David et al., 2016). The major inhibitory neurotransmitter γ-aminobutyric acid (GABA) is also downregulated within the brain through mis-localization of glutamate decarboxylase, the key enzyme for GABA formation (Brooks et al., 2015). Taken together, increased activating neurotransmitters and decreased inhibitory neurotransmitters are believed to prime the brains of infected mice into a more active state.

Chronic infections have been shown to lead to dysfunction in astrocytes, which are a major component of the BBB. Astrocyte dysfunction contributes to the increased permeability of the BBB, which is normally highly selective in a healthy brain. The astrocyte's endfoot processes wrap around the blood vessels to maintain the CNS component of the barrier. While *Toxoplasma* is found throughout the brain and in multiple cell types during acute infection, it is found exclusively in neurons during chronic infection. Therefore, the astrocyte dysfunction is not a direct effect of the presence of the intracellular parasite within the astrocytes. Transmission electron microscopy images show a swelling of the astrocyte endfoot processes (David et al., 2016) and immunohistochemistry (IHC) shows activation markers on the astrocytes (T. Wang et al., 2019). Additionally, a large influx of immune cells in the perivascular space between the astrocytes and the endothelial cells are believed to contribute to compromising the BBB.

III. Immune Response to *Toxoplasma*

Though the brain has been classically considered an immune privileged site, how the host responds to a lifelong parasitic infection has an impact on disease progression and potential reactivation. There is overwhelming evidence that the host must maintain a functional adaptive immune response within the brain in order to sustain latency of the parasites and an innate immune response to control the initial infection to prevent host death. The status of *Toxoplasma* as an opportunistic pathogen in AIDS, transplant, and other immunosuppressed patients highlights the need for a functional immune system. However, the longstanding immune response to chronic infection is a complex balance between both pro-inflammatory and anti-inflammatory immune responses.

A. Initial Immune Response

Initial infection with *Toxoplasma* triggers an innate immune response in the host, which is necessary to control the infection. Initially, monocytes, neutrophils and dendritic cells respond to the site of infection where they use toll-like receptors (TLRs) to sense the presence of *Toxoplasma*. The TLRs which have been implicated are 2, 4, 9, and 11. TLRs 2 and 4 are believed to recognize the glycosylphosphatidylinositols on the surface of the parasite, while TLR 9 recognizes un-methylated CpG motifs in *Toxoplasma* DNA (Santamaria, Perez Caballero, & Corral, 2016). TLR 11 responds to parasite derived profilin (Neal & Knoll, 2014).

Monocytes, dendritic cells, and neutrophils promote a Th1 immune response through the production of cytokine IL-12. Natural killer (NK) cells and T cells to produce interferon-gamma (IFN-γ) upon stimulation with IL-12. IFN-γ is responsible for

activating the major intracellular mechanisms t inhibit parasite replication. Deficits in IL-12 or IFN-γ will result in overwhelming parasite burden and death of the host (Gazzinelli et al., 1994; Suzuki, Orellana, Schreiber, & Remington, 1988).

One mechanism through which IFN-γ signaling can be anti-parasitic is through increased synthesis of Nitric Oxide (NO). IFN-γ promotes synthesis of NO through inducible nitric oxide synthases (iNOS). NO is shown to inhibit replication of *Toxoplasma* in various cell types (Chao et al., 1993; Dunay & Sibley, 2010; Langermans et al., 1992). Another mechanism, which appears critical for murine toxoplasmosis control, is the upregulation of immune related GTPases (IRGs), which are increased with IFN-γ stimulation (Howard, Hunn, & Steinfeldt, 2011). IRGs appear to localize to the parasitophorous vacuole within the host cell and disrupt it, either allowing the parasites to be killed intracellularly or egress prematurely (Ling et al., 2006). A final proposed mechanism for parasite control through IFN-γ signaling is through the decrease in available intracellular tryptophan by upregulation of indolamine 2,3-dioxyganse 1 and 2 (IDO-1 and IDO-2) (Divanovic et al., 2012). Both enzymes catalyze the degradation of tryptophan, which *Toxoplasma* needs due to being a tryptophan auxotroph. Reduced levels of tryptophan inhibits parasite growth through starvation.

Dissemination throughout the host is an important aspect of acute infection. The parasite is believed to hijack mobile immune cells, which then develop a hyper-migratory phenotype, in a Trojan horse like process which allows the parasite to travel around the host (Lambert, Hitziger, Dellacasa, Svensson, & Barragan, 2006). The two cell types most often implicated in this mechanism are monocytes and dendritic cells. The Trojan horse model is also proposed as a possible mechanism for the parasite to cross the BBB

and enter the CNS (Courret et al., 2006). Others argue that the parasite directly infects endothelial cells and crosses the BBB following egress, where lysis of the endothelial cells compromises the barrier (Wohlfert et al., 2017).

B. Chronic Immune Response

A key histological finding for chronic *Toxoplasma* infection is the presence of extensive immune cell infiltrate throughout the brain. These cells do not localize to where tissue cysts are present, but instead aggregate in plaques, occupy the perivascular space, and are found diffusely throughout the brain (Hermes et al., 2008). The exact reasoning for the poor localization is not known, but could be due to the intracellular localization of the tissue cyst and the influence of effector proteins on host cell function. While a large influx of immune cells is not uncommon during acute diseases of the brain, it is unusual for a chronic infection to cause such an extreme response. Additionally, the makeup of these cells highlights a balance between both the Th1, the more pro-inflammatory, and the Th2, the more anti-inflammatory immune responses. Both CD8+ and CD4+ T cells are required for maintaining chronic infection (David et al., 2016; Hermes et al., 2008). Upregulation of the chemokines CCL21 and CXCL10 help recruit T cells into the brain parenchyma and facilitated migration across the endothelium. The presence of activated T cells within the brain is believed to help control any reactivation that might occur, as these cells are highly responsive to *Toxoplasma* antigens and localize to reactivated cysts rapidly.

Other cell types found in infected brains include plasma cells, which offer a source of antibodies, and microglia, which are considered brain resident macrophages.

Microglia play a key role in control of acute infection as well as being a major source of IFN-γ. Activated microglia remain poised to target any reactivated parasites within the brain during chronic infection, but in lower numbers than in acute infection.

The most important cytokine for preventing reactivation of encysted parasites is IFN-γ. Within the brain, major sources of IFN-γ include infiltrating T-cells, dendritic cells, and macrophages, while the cells which typically respond include microglia, astrocytes, and neurons. IFN-γ is responsible for activating anti-microbial processes such as iNOS, IDO, and induction of IRGs, all of which continue to play a role during chronic infection. The loss of IFN-γ leads to life-threatening, extensive reactivation of tissue cysts (Suzuki et al., 1988).

An important counter regulatory cytokine to IFN-γ is IL-10, which has anti-inflammatory properties and is critical in preventing autoimmune-like pathology. Mice infected with *Toxoplasma* but lacking IL-10 or IL-10 receptors die, not from parasite mediated processes, but from the overwhelming and destructive immune response (Khan, Matsuura, & Kasper, 1995).

IV. Behavioral Changes

A. Rodent Models

The earliest published study on the effect of latent *Toxoplasma* infection on rodents was in 1978 (Piekarski, Zippelius, & Witting, 1978). Since then, studies in both mice and rats have shown various behavior modifications, but some have been inconclusive. Tyebji et al recently published a comprehensive review of the last ten years worth of studies in mice and rats and listed their outcomes (Tyebji, Seizova, Hannan, et al., 2019). A

shortcoming of these studies is the lack of consensus in experimental design, which makes comparing the results difficult. Variables that change between studies include species, sex, rodent strain, *Toxoplasma* strain, age of animal, time from infection, inoculum dose, sample size and type of assay used. These differences make repeating experiments difficult and evaluation of overall trends complicated.

There are several behavioral changes that have similar outcomes across multiple trials. Locomotor activity in an open field increases in chronic infection (Cristina Afonso, Paixão, & Costa, 2012; C. Afonso et al., 2017; Hodkova, Kodym, & Flegr, 2007; Kannan et al., 2010). Infected mice not only display hyperactivity, but also do not seem to show a preference for the periphery of the testing chamber. When observing anxiety like behavior, *Toxoplasma* infected mice show a reduced level of anxiety and appear to spend increased amounts of time in exposed areas such as the center of a locomotor chamber or the exposed arms of elevated plus mazes (Cristina Afonso et al., 2012; Kannan et al., 2010; Machado et al., 2016). Finally, cognitive defects also appear to be common in chronic infection, and these branch across multiple forms of memory. There is reduced recognition memory where mice fail to recognize a novel object (Machado et al., 2016; Xiao et al., 2016). There is reduced spatial memory in the Y-maze (Hay, Aitken, Hair, Hutchison, & Graham, 1984; Hutchison, Aitken, & Wells, 1980), Morris water maze (Daniels, Sestito, & Rouse, 2015), and the radial arm maze (Hodkova et al., 2007). Finally, infected rodents appear to lose the innate fear of cat odor (Hari Dass & Vyas, 2014; Vyas, Kim, Giacomini, Boothroyd, & Sapolsky, 2007; Vyas, Kim, & Sapolsky, 2007).

These behaviors are believed to contribute to what is referred to as the 'fatal feline attraction' hypothesis (Berdoy et al., 2000). *Toxoplasma* is believed to have evolved to manipulate certain hosts in order to increase predation. By altering the risk/reward for certain actions, such as reducing the fear of a predator's scent or increasing the time spent in exposed areas, the parasite makes it more likely that the host it is inhabiting will be eaten and continue its way up the food chain until it reaches its definitive host, the cat.

B. Attempts to Reverse *Toxoplasma*-Induced Behavior Modification

Previous efforts to restore normal behaviors altered by *Toxoplasma* have been unsuccessful. Many of these attempts have focused on neurotransmitters, which are the major signaling molecules within the brain. Ceftriaxone, an antibiotic known to increase astrocytic glutamate transporter (GLT-1) expression, can restore the altered glutamate levels and neuronal morphology in chronically infected mice, but failed to reverse infection-induced changes in anxiety behavior on an elevated plus maze (David et al., 2016). Attempts to normalize acetylcholinesterase activity in the CNS of infected mice using diphenyl diselenide also did not restore altered behaviors in locomotor activity or elevated plus maze (Machado et al., 2016).

To address possible dopamine level manipulation, labs have tried deleting parasite proteins such as the *Toxoplasma* AaaH2 tyrosine hydroxylase. It was hypothesized that the parasite used its own tyrosine hydroxylase to manipulate host dopamine levels, however deletion of AaaH2 did not impact the parasite's influence on locomotor activity,

altered amphetamine response, or risk assessment (C. Afonso et al., 2017; McFarland et al., 2018).

Finally, researchers looked to see if non-cyst forming parasite strains could still alter behavior. Mice infected with an attenuated RH strain incapable of cyst formation continued to display altered fear responses, even though parasites could no longer be detected (Ingram, Goodrich, Robey, & Eisen, 2013).

These studies suggest that the behavioral control that *Toxoplasma* appears to have over its rodent host is long lasting and resilient.

V. *Toxoplasma* in Mental Illness

There have been many studies correlating *Toxoplasma* infection with various neuropsychiatric disorders, such as schizophrenia, bipolar disorder and suicide. Some of the earliest work include connecting psychiatric symptoms in 21.1% of individuals who had documented acquisition of *Toxoplasma* across a 24 year period (Kramer, 1966).

A. Schizophrenia

The mental illness that is mentioned most frequently in the literature as being associated with chronic toxoplasmosis is schizophrenia. A large meta-analysis of 38 studies shows a combined odds ratio of 2.71 (95% CI 1.93-3.8), which places it as an intermediate risk factor (Torrey, Bartko, & Yolken, 2012). Other risk factors with higher correlations include first degree relatives (mother, father, and sibling) with schizophrenia and being an immigrant from selected countries (Torrey et al., 2012). While the majority of the studies done have been retrospective, there is evidence that *Toxoplasma* IgG

antibody levels can be predictive of schizophrenia development. In a prospective cohort study of mothers in Denmark, there was a significant positive association between *Toxoplasma* IgG antibody level and development of schizophrenia spectrum disorders. Mothers with the highest antibody titers had a statistically higher risk of developing schizophrenia spectrum disorders (Pedersen, Stevens, Pedersen, Norgaard-Pedersen, & Mortensen, 2011) than those with no or lower levels of antibodies. The differences in antibody titer suggests a possible connection with severity of infection and the development of neuropsychiatric symptoms.

Another connection between *Toxoplasma* and schizophrenia is the impact of infection on N-methyl-D-aspartate receptors (NMDAR), which is a glutamate receptor within the brain that is dysregulated in schizophrenia patients (Javitt, 2010). There is evidence that *Toxoplasma* infection compromises the BBB and allows for formation and transmission of antibodies against the NMDAR in a subset of schizophrenia patients (Kannan et al., 2017). This antibody formation is proposed to be the result of extensive peptide overlap between the NMDAR subunits and the *Toxoplasma* genome, suggesting that cross-reaction of epitopes for a parasite specific factor could account for the anti-NMDAR antibody activity (Lucchese, 2017). This work continued to show that infection itself is sufficient to generate anti-NMDAR antibodies in a mouse model, with subsequent behavioral changes in those mice (Li et al., 2018).

B. Other Associations

Other neuropsychiatric associations span a broad range of etiologies. *Toxoplasma* specific antibodies correlate with recent onset psychosis (Monroe et al., 2015; Yolken,

Torrey, & Dickerson, 2017). A meta-analysis of eight studies showed an odds ratio of 1.26 (95% CI 1.08-1.47) for a positive association between *Toxoplasma* infection and bipolar disorder (de Barros et al., 2017). Suicide and risky behavior are other serious psychiatric behaviors associated with *Toxoplasma* infection. For example, high speed traffic accidents have been used as a metric for risky behavior. Another meta-analysis of thirteen studies and eleven studies, respectively, showed an odds ratio of 1.39 (95% CI 1.10-1.76) for suicides and 1.69 (95% CI 1.20-2.38) for traffic accidents (Sutterland et al., 2019).

C. Environmental Factor and Disease Modifier

While *Toxoplasma* statistically correlates with various forms of CNS dysfunction, the number of individuals worldwide who carry the parasite without any noticeable side effects suggests that the infection by itself is not enough to cause mental illness. In fact, a key argument against the association between *Toxoplasma* and schizophrenia has been that countries such as France, which have historically have a high seroprevalence, do not have particularly high rates of schizophrenia (Pappas et al., 2009). Additionally, decreasing rates of *Toxoplasma* infection as food safety improves have not had a remarkable impact on schizophrenia rates (Kirkbride et al., 2012). However, the recent FACE-SZ (FondaMental Academic Centers of Expertise for Schizophrenia) study, a ten-center collaboration in France, showed that young individuals with schizophrenia were three times as likely to have *Toxoplasma* than the community controls (73.6% compared to 27%). This suggests that the age of infection potentially plays a role in disease

development, which might be masked in large scale population studies (Fond et al., 2018).

Taken together, the data suggest that *Toxoplasma* could be acting as an environmental factor that could exacerbate an existing disease condition or trigger disease onset. A major argument for the idea of *Toxoplasma* as an environmental factor is that the risk associated with latent *Toxoplasma* infection is higher than any schizophrenia associated gene variant (Purcell et al., 2009). The lack of strong genetic markers contrasts with the high risk associated with an immediate family member with the disease, suggesting a common environmental factor to which the family has been exposed (Torrey et al., 2012).

Sudden onset psychosis is also linked with chronic toxoplasmosis, however there is also strong evidence that it is a recently acquired infection that is the trigger. A significant increase in *Toxoplasma* specific IgM antibodies, which are the first antibodies in response to infection, were found in acute psychosis patients, especially those with schizophrenia (Monroe et al., 2015). Elevated *Toxoplasma* IgM antibodies have also been found in patients who attempted suicide while receiving treatment for existing schizophrenia, bipolar disorder, or major depression (Dickerson et al., 2017).

Additionally, *Toxoplasma* can influence other disease models such as Huntington's disease and Alzheimer's disease. Infection with *Toxoplasma* in the mouse model for Huntington's disease lead to activation of indoleamine-2,3-dioxygenase, which contributed to more severe disease and early death (Donley, Olson, Raisbeck, Fox, & Gigley, 2016). The results for Alzheimer's disease have been mixed. Some show a protective effect (Jung et al., 2012; Mohle et al., 2016) while others suggest that the infection exacerbates the condition (Montacute et al., 2017; Torres et al., 2018).

Differences in models, strains of mouse and parasites could potentially account for the conflicting results.

VI. Drug Treatments for *Toxoplasma* Infection

A. Acute Infection

Standard of care for acute *Toxoplasma* infection is almost exclusively a combination therapy. Pyrimethamine with a sulfonamide is considered the gold standard drug treatment for acute toxoplasmosis and has remained unrivaled for treatment for nearly sixty years (Eyles & Coleman, 1953). Pyrimethamine, the most effective of the anti-parasitic drugs, is a dihydrofolate reducatase (DHFR) inhibitor while sulfonamides are dihydropteroate synthetase inhibitors, both of which fall under the classification of folic acid synthesis inhibitors. They therefore inhibit parasite growth by starving them of purines and inhibiting DNA replication. Due to the high prevalence of sulfa drug allergies in the population, second line drug combinations include pyrimethamine with clindamycin, atovaquone, clarithromycin or azithromycin.

Pyrimethamine with sulfonamide, while efficacious, is not without therapeutic problems. There is a high rate of adverse effects with drug treatment, most notably hematologic toxicity. These adverse events are noted in up to 60% of *Toxoplasma* encephalitis patients, which leads to discontinuation of therapy in up to 45% of patients (Cohn, McMeeking, Cohen, Jacobs, & Holzman, 1989; Dunay et al., 2018; Haverkos, 1987). Additional side effects include leukopenia, thrombocytopenia, cutaneous rash and fever (Haverkos, 1987). Efforts to overcome the hematologic toxicity associated with pyrimethamine include supplementation with folinic acid.

Additional hurdles that must be overcome include the need for maintenance therapy for individuals at risk for reactivation. Many patients at risk for *Toxoplasma* encephalitis cannot be discontinued from therapy due to their immune compromised status and risk for reactivation. These patients are therefore placed on a prophylactic therapy that must be maintained for the duration of the time that they are immunosuppressed, which for many patients is the rest of their life. There are currently no available treatments to eliminate the infection and allow these individuals to stop treatment.

In an effort to address toxicity concerns when targeting the same pathway with two drugs, additional combination therapies have also been explored. Multiple trials (B. Dannemann et al., 1992; B. R. Dannemann, Israelski, & Remington, 1988) show efficacy with the combination of pyrimethamine with clindamycin, with similar response to pyrimethamine with sulfonamide (70% and 65% clinical response). However, there were issues with the pyrimethamine/clindamycin group maintenance doses and relapse was more common with these patients (Katlama, De Wit, O'Doherty, Van Glabeke, & Clumeck, 1996). While pyrimethamine/clindamycin did not outperform pyrimethamine/sulfonamide, it does provide a viable second line option for patients experiencing adverse reactions to the first line option.

Pregnant woman and their fetus are at particular risk due the vertical transmission of infection and presents a unique problem due to toxicity concerns for the developing fetus. Outside of the United States, the typical drug regiment it replaced with spiramycin, which is nontoxic and does not cross the placenta (Montoya & Remington, 2008). Though it is still considered experimental within the United States, spiramycin can be

obtained for treatment during the first trimester with special request of the Food and Drug Administration (FDA) if certain laboratory criteria are met. Spiramycin treatment of infected pregnant women is meant to specifically prevent maternal-fetal transmission of infection and prevent the number of adverse outcomes for the fetus. However, if the fetus is confirmed to be infected, spiramycin is typically switched to the first line pyrimethamine combination treatment.

B. Chronic Infection

There are currently no FDA approved therapies for chronic toxoplasmosis. There are, however, several experimental compounds that have shown efficacy against chronic infections in mouse models and provide evidence that it is possible to reduce cyst burden in chronic infections. None of these compounds have been able to completely eliminate cyst burden and thereby eliminate reactivation in immunosuppressed individuals, but they do provide evidence that it is possible to target latent parasites.

Guanabenz is an anti-hypertensive drug that shows efficacy both *in vitro* and *in vivo* against *Toxoplasma* (Benmerzouga et al., 2015; Konrad, Queener, Wek, & Sullivan, 2013). Originally designed as an alpha adrenergic receptor agonist, guanabenz is a small molecule that rapidly disseminates throughout the body and crosses the BBB in micromolar concentrations. Recent studies have shown off target effects of the drug that include inhibition of a regulatory subunit of protein phosphatase 1 (PP1) called GADD34 when the drug reaches these concentrations. GADD34 is upregulated under stress conditions and increases the dephosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) in mammalian cells. Phosphorylation of eIF2α slows mRNA translation

as part of the integrated stress response. GADD34 is one of the negative regulators and inhibiting its function maintains the integrated stress response, thereby inhibiting protein synthesis. While *Toxoplasma* does not have a clear homologue of GADD34, guanabenz does inhibit dephosphorylation of the *Toxoplasma* IF2α in a dose dependent manner (Konrad et al., 2013). It also inhibits parasite growth and prolongs life during lethal acute infection in mice (Benmerzouga et al., 2015; Konrad et al., 2013). When administered to chronically infected mice, guanabenz decreases brain cyst burden 69% (Benmerzouga et al., 2015).

Another class of compounds that show efficacy against chronic *Toxoplasma* infection are the endochin-like quinolones (ELQs). These drugs are based on the endochin scaffold and similar to 4-(1H)-quinolone, which showed efficacy against malaria. A library was formed and screened with several compounds identified with an IC_{50} in the low nanomolar range, specificity to *Toxoplasma*, and minimal toxicity to human cells in culture (Doggett et al., 2012). The mechanism of action for ELQs is believed to be inhibition of the parasite cytochrome bc_1 complex, which is located in the inner mitochondrial membrane and contributes to pyrimidine biosynthesis and oxidative phosphorylation as part of the electron transport chain (Alday et al., 2017). When administered to chronically infected mice, the ELQs decrease brain cyst burden 76-88% (Doggett et al., 2012).

Calcium-dependent protein kinase 1 (CDPK1) is required for invasion and egress from host cells and was identified as a promising drug target for anti-*Toxoplasma* compounds. Optimization of inhibitors of CDPK1 identified a small molecule (compound 32) with strong efficacy (IC_{50} 0.060μM) and limited toxicity (Vidadala et al., 2016).

Compound 32 is effective against acute infection as well as capable of lowering cyst burden 88% (Vidadala et al., 2016).

VII. Hypothesis and Aims

Toxoplasma is a highly successful eukaryotic pathogen that is commonly found in humans as a latent, chronic infection. The long-term consequences of these lifelong infections and the potential changes they cause within the host are poorly understood. However, latent *Toxoplasma* infection causes behavioral changes in rodent models and correlates with several mental illnesses in humans. Additionally, chronic infection provides a long-standing reservoir within a host that can reactivate into a life-threatening acute infection upon severe immune suppression.

Previous studies have shown behavioral changes associated with chronic toxoplasmosis in rodents to be resilient and there is precedent that these changes could be permanent. Past efforts to reverse changes through targeting neurotransmitter levels have been unsuccessful. Recent advancements in compounds that show efficacy against encysted parasites provide me with a means of reducing brain cyst burden in established chronic infections. These new tools will allow me to evaluate the role of cyst burden on behavior. The mouse model for chronic infection will provide me with a model system to evaluate the characteristics of chronic infection. Additionally, inbred mouse strains respond to *Toxoplasma* differently so investigating mice strains with different responses, both resilient (BALB/c) and susceptible (C57BL/6), will help to further our understanding of host-parasite interactions. **I hypothesize that reducing cyst burden**

will restore normal behavior in mice with latent *Toxoplasma* infections. This hypothesis will be dissected using the following aims:

1) Evaluate the efficacy of the next generation guanabenz compound Sephin1 against *Toxoplasma* both *in vitro* and *in vivo*.
2) Optimize the *in vivo* experimental design for the use of guanabenz against latent toxoplasmosis.
3) Determine the effect of guanabenz treatment on activity levels of chronically infected mice by recording locomotor activity in an open field.
4) Examine the histology of guanabenz treated brains using hematoxylin and eosin staining and IHC.
5) Evaluate the influence of genetic background on brain cyst burden and behavior following guanabenz treatment by using two non-isogenic mouse strains (BALB/c and C57BL/6).

The long-term goal of this thesis is to evaluate the plasticity of behavioral alteration, specifically the induced hyperactivity, observed in mice with latent *Toxoplasma* infections. Optimization of the experimental designs will provide me with a robust system to evaluate my hypothesis. Examining the histology of the brains at the conclusion of the experiments will provide insight into the health of the brains as well as provide information on additional long-term changes associated with chronic *Toxoplasma* infection such as neuroinflammation. The use of two non-isogenic mouse strains will help further clarify the role of host biology on my system, which is a crucial factor when observing complex processes such as behavior.

Chapter Two: Materials and Methods

I. Tissue Culture Techniques

A. Parasite Strains and Culture

Toxoplasma gondii parasites were either Type I RH or Type II Prugnaid (Pru). Pru parasites were collected as tissue cysts from chronically infected BALB/c mice and used to infect human foreskin fibroblasts (HFF). RH parasites were thawed from frozen stocks available in the lab. The infected cultures were maintained in Dulbecco's medium supplemented with 1% heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. To ensure their developmental capacity, tachyzoites were maintained in culture no more than 15 passages. Parasites were tested for mycoplasma using polymerase chain reaction (PCR) as previously described prior to use in mice (Uphoff & Drexler, 2002).

B. Host Cell and Parasite Culturing

Parasites were cultivated in confluent monolayers of HFFs grown in T25cm^2 or T175cm^2 flasks. HFFs were cultured in host medium that consisted of Dulbecco's medium supplemented with 10% heat-inactivated FBS. The HFF cultures were grown at 37ºC in a humidified incubator with 5% CO_2. Passage number used were between 4 and 20, due to the age effects on HFFs and to maintain consistency between experiments. All host cell and parasite culturing was performed under sterile conditions in a laminar flow hood.

Type II parasites exhibit a sensitivity to remaining extracellular for extended periods of time and therefore require fresh HFFs to maintain viability and avoid

adaptation to culture conditions, which increases virulence. For maintaining passages, a volume culture medium from a freshly lysed flask, or from a manually lysed flask following scraping and syringe lysis, was added to fresh HFF monolayer.

C. Parasite Doubling Assay

Doubling assays were used to quantify parasite growth *in vivo*. Glass coverslips were placed into the wells of a 12 well tissue culture plate. HFF cells were seeded onto the coverslips and allowed to mature to confluence (typically 2-4 days) but not allowed to overgrow. For Type I parasites, 10^4 parasites from a freshly lysed T25cm^2 flask were plated into each well and allowed to invade for two hours. After those two hours, the coverslips were washed three times with warm, sterile PBS before medium with various drug concentrations were added to the wells, in technical triplicate. After 24 hours, the cultures were fixed with 4% PFA for 20 minutes, permeablized with 0.3% Triton X-100 (Sigma No. 9002-93-1) blocked with 1.5% BSA (Bovine Serum Albumin) overnight. Coverslips were incubated in primary mouse anti-p30(SAG1) (Invitrogen No. MA1-83499 *Toxoplasma gondii* P30 monoclonal antibody (p/30/3)) at 1:2000 in blocking buffer for one hour, followed by three washes in PBS. Slides were then incubated in secondary goat anti-mouse (Invitrogen No. A11005) at 1:2000 in blocking buffer for one hour followed by three washes in PBS before twenty minutes of DAPI staining and plating with mounting medium (Vector Laboratories). Fifty random vacuoles were counted for each coverslip. Data are presented as the percentage of each vacuole size for the total number of vacuoles counted for each condition.

For Type II parasites, the procedure was the same as above with the exception of $5.0x10^3$ parasites in each well which were allowed to invade for 4 hours and being allowed to grow for 36 or 48 hours before fixation.

II. Mouse Model of Infection

A. Acquisition, Housing and General Care of Mice

The mice used were housed in American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facilities at either the Indiana University School of Medicine Laboratory Animal Research Center (LARC) or the Indiana University Purdue University Science Animal Research Center (SARC). The Institutional Animal Care and Use Committee (IACUC) at Indiana University School of Medicine approved the use of all animals and procedures (IACUC protocol numbers 10852 and 11376).

Mice were sourced from two different vendors. Early experiments performed with five/six week old BALB/c mice were purchased from Envigo. All experiments were then repeated with five week old BABL/c mice from Jackson Laboratories. C57BL/6 mice were purchased exclusively from Jackson Laboratories at five weeks old.

Upon arrival, mice were randomly grouped in four or five mice per cage and allowed to acclimate to the facility for one week. Mice were given free access to dry chow pellets which were available through wire feed hopper over the cage. During acute infection and subsequent drug treatment, mice were provided with chow pellets soaked in water which were placed on the cage floor. To prevent mold growth, wet feed was made fresh every other day and any uneaten food was removed from the cage daily. Water was

provided through a self-use spigot in the LARC facility or a metal sipper water bottle in the SARC facility.

Mice in each cage were randomly assigned a color/letter designation. A small mark was made at the base of the tail with a permanent marker to indicate their identification. Yellow/A, purple/B, blue/C, green/D and red/E. This allowed for a low stress, simple identification without the pain of toe cutting or ear tags. Colors were refreshed once per week to combat fading.

B. Preparation of Parasites for Mouse Infection

All mouse infections were done with a Pru WT strain of parasites. Two days prior to infection, three different T25cm^2 flasks of confluent HFF cells were infected with different volumes of parasites to ensure adequate parasite number without host cell lysis. On the day of infection, whichever flask that had the most number of intracellular tachyzoites without host cell lysis was selected for use. This prevents any delays if the parasites in the low volume flask differentiate into bradyzoites or the host monolayer has been lysed due to parasite egress. All parasites were intracellular tachyzoites. Following a single wash of the monolayer with 37°C autoclaved, filter-sterilized PBS, the parasites were harvested by scraping an infected HFF monolayer, passing the culture through 25g syringe needle, and then purified from HFF debris using a sterile 3 micron filter. Purified parasites were quantified using a hemocytometer before being diluted into 10mL of 37°C autoclaved, filter-sterilized PBS at the appropriate inoculum for the experiment (either 10^3 or 10^4 parasites per 100µL of autoclaved, filter-sterilized PBS). To maintain parasite viability, it is important to keep the temperature as close to 37 °C as possible. Therefore, flasks containing parasites were placed

in a Styrofoam container with several 50mL conical flasks filled with 37°C water to help maintain flask temperature while being transported to the animal facility for infection.

C. Infection and Monitoring of Mice

BALB/c mice were i.p. infected with 10^4 Pru tachyzoites suspended in 100µL of autoclaved, filter-sterilized PBS. C57BL/6J mice were infected with 10^3 Pru tachyzoites. Different parasite inoculums were necessary due to the differences in survivability between the mouse strains. Mice were routinely observed multiple times a day throughout the course of acute infection. Mock-infected animals were handled in an identical manner and injected with the same volume of sterile PBS (100µL). Weight, in grams, was recorded every other day.

D. Group Randomization

Groups were randomized the day before drug administration was scheduled to start (21 days post infection). Since the percent weight lost is a good indicator of severity of infection, this was used as the metric to randomize the groups. The percent weight was calculated as (Weight on the current day/Weight on the day of infection)x100%. Mice were arranged in ascending order based on the percent weight loss. A treatment group was then assigned to each mouse in first ascending and then descending order (i.e. vehicle four weeks, guanabenz four weeks, vehicle six weeks, guanabenz six weeks, guanabenz six weeks, vehicle six weeks, guanabenz four weeks, vehicle four weeks, vehicle four weeks, vehicle four weeks, etc.). This allowed for a distribution of mice with severe infections across all of the groups and allowed a near average percent weight between groups. Each

experiment required a vehicle control due to the variability between infections. Data were not pooled from different experiments due to this phenomenon.

E. Intraperitoneal Drug Administration

Guanabenz (Sigma Guanabenz acetate salt, G110) was administered intraperitoneally unless otherwise stated. A stock solution of 2 5mg/mL in filter sterile saline was made at the start of each experiment and aliquoted before being stored at -20°C. Aliquots were thawed the morning of drug administration. 5mg/kg/day was administered i.p. at the same time every day for 3 to 6 weeks depending on the experiment.

F. Oral Gavage Drug Administration

A 2mg/mL stock solution of Sephin1 (APExBIO Sephin1 A8708) in saline was made for oral gavage. Mice were given 10mg/kg/day by oral gavage with a flexible plastic gavage needle. Vehicle controls were given the same volume of saline.

For guanabenz oral gavage administration, a stock solution of 10mg/kg/day in saline was made at the beginning of the experiment. The dosage was increased from the i.p. dosage to account for the likelihood of first pass effects on systemic drug concentrations. Each mouse was gavaged with a flexible plastic gavage needle with 100µL of solution, either vehicle or drug.

G. Medicated Food Drug Administration

For the medicated feed group, fresh feed was made every other day and old feed was removed when fresh feed was added. To minimize the influence of feed buried in the cage bedding on fresh feed consumption, the cages for this group were changed every 4 days. Feed (5g/mouse/day) was weighed out, placed in a metal bowl, and combined with 5.5mL water per gram of feed which was allowed to absorb for 15 minutes. Separate bowls were used for the vehicle group and drug treatment group. The calculated dose of guanabenz (10mg/kg/day/mouse) was suspended in 10mL of corn oil (commercially available from the grocery store), or 10mL of corn oil alone for the vehicle control, and added to the wet feed before being mixed with a potato masher and placed in a plastic weigh boat. The weigh boat was placed on the floor of the cage where the mice could easily reach it.

H. Dough Treat Drug Administration

Edible dough consisted of flour, sugar, salt, and glycerol (all commercially available from the grocery store) using the ratio 30g : 4g : 1g : 20ml respectively. Following the weighing and mixing of all of the dry ingredients, glycerol for the vehicle or guanabenz with glycerol for the drug treatment was pipetted into the dry ingredients. All ingredients were thoroughly combined using a spatula to create a batch of dough. A single dough serving is portioned for each mouse (5 mg/kg/day) and placed in a clean, empty mouse cage. Mice were moved individually from their home cage to an empty cage containing the single dough serving for that mouse at a set time each morning. During acclimation prior to infection, the mice were given access to vehicle dough for 2

hours on the first day, and the time was subsequently decreased by half hour intervals each following day until it was limited to 30 min each day. All of the mice were acclimated to the dough because I did not know what mice would be assigned the dough treatment group until the chronic infection stage. Dough administration was then discontinued during acute infection.

Following group randomization, mice in the dough administration groups, both vehicle and drug, were give a single dose of vehicle dough the day before drug administration was scheduled to start to re-acclimate them to the dough. Once treatment began, the mice in the drug treatment group were given dough with guanabenz while the vehicle control mice were given dough without drug. Any residual dough left in the cage following the thirty minute treatment time was collected and quantified to determine percent consumed and therefore dose each mouse consumed.

I. Euthanasia and Tissue Extraction

At the conclusion of the mouse experiment, mice were euthanized using CO_2 asphyxiation followed by cervical dislocation. Blood was immediately collected by cardiac puncture using a 28g needle (EXCEL No. 26046) and placed in an Eppendorf tube on ice for later serum collection. The neck of the mice was disinfected with 70% ethanol before they were decapitated with a curved pair of scissors. The skin was cut from the subcutaneous fat and the tissues connecting the ears were cut to allow the skin to be folded back over the head. Excess fat, muscle and any residual spinal cord was removed from the base of the skull before the base of the skull was removed to visualize the brainstem. Two shallow cuts were made along the skull from the cut opening at the

base to the eye socket while care was made to limit the damage to the brain tissue underneath. A pair of curved tweezers were used to peel back the skull flap and visualize the brain. The curved tweezers were then run along the inner edge of the skull and underneath the brain to sever the cranial nerves and allow the brain to be transferred to a clean plastic weigh boat. A razorblade was then used to bisect the brain in the sagittal plane along the longitudinal fissure, with the cut continuing through the cerebellum, resulting in 2 brain hemispheres.

III. Tissue Processing and Staining

A. Preparation of Brain Samples Following Euthanasia

If a hemisphere was used for parasite quantification, it was placed in an Eppendorf tube on ice. Later, 650µL of autoclaved sterile PBS was added to the tube and the entire contents were emptied into a clean mortar and pestle where it was homogenized thoroughly. The brain homogenate was then placed back into the Eppendorf tube for allocation to different quantification techniques. If a hemisphere was used for histological examination, it was placed in a tissue cassette and the cassette was deposited into 10% Neutral Buffered Formalin (NBF) (ThermoScientific 10% Neutral Buffered Formalin No. 6701).

B. Preparation of Immunofluorescence Assay Samples for *in vivo* Cyst Burden Quantification

To prepare immunofluorescence assay (IFA) samples from brain homogenates, 250µL of brain homogenate was aliquoted from the hemisphere preparation and spun

down at 3000xg for 5 minutes. All centrifugation steps were performed with this speed and time to prevent damage to the tissue cysts. The supernatant was removed and the pellet was fixed by suspending in 150µL of 3% methanol-free formaldehyde (prepared from 16% stock, Electron Microscopy Sciences No. 50980487) for 20 minutes in a sterile fume hood. Following three washes with sterile PBS and subsequent removal of the supernatant, blocking was performed with 3% BSA in PBS in 0.2% Triton X-100 (Sigma No. 9002-93-1) overnight at 4°C.

To stain the cyst walls of any bradyzoites, rhodamine-conjugated *Dolichos bifluorus* lectin (Vector Laboratories) was applied at 1:250 for one hour at room temperature. The sample was washed three times with sterile PBS. After the final wash, 5µL of the pellet was deposited onto a glass microscope slide (Fisherbrand Superfrost precleaned microscope slide 3"x1"x1.00mm No. 12-550-123) and covered with a circular coverslip (Fisherbrand Microscope coverglass No. 12-545-83 15CIR-1D). Three samples of 5µL each were prepared per mouse brain homogenate. The slides were sealed on the outer edge with nail polish before being allowed to dry overnight in a bench drawer. Slides were then stored at 4°C.

C. Quantification of Mouse Brain Cysts

For each mouse brain, all three IFA samples were scored for the number of cysts using the Nikon Eclipse 80i 37 fluorescent microscope. Structures within the brain homogenates that were lectin-positive and spherical or ovoid with distinct smooth outer "lectin rims" were counted as cysts. Total cyst burden approximates per brain were calculated from individual slide counts by taking the summation of the total cysts counted

and multiplying by twenty-six to account for the aliquoting of the brain material (Benmerzouga et al., 2015).

D. Mouse Brain Tissue Processing

Following a week in 10% NBF, the fixed tissue was processed using dehydration through graded ethanol and xylene before being embedded into paraffin blocks. Briefly, tissue cassettes were rinsed for one hour in ddH_2O on a shaker. They were then rinsed briefly with 70% ethanol in water before being stored in 70% ethanol until the rest of the processing protocol could be completed. The following steps were all completed in succession. Brains were dehydrated in 80% ethanol for 45 min, 95% ethanol for 45 minutes twice, 100% ethanol for 40 minutes twice, 50% ethanol/50% xylenes for twenty minutes twice and finally 100% xylenes for thirty minutes twice. Next, the cassettes were placed in 50% xylenes/50% paraffin at 58°C on a rotating oven rack for 40 minutes. Then the cassettes were treated with 100% paraffin at 58°C for 40 minutes three times before finally embedding them into blocks on a warm hotplate and placing them in the freezer for 20 minutes to set. Blocks were then stored in cardboard boxes until sectioning.

E. Mouse Brain Tissue Sectioning

Blocks were cut into 5 μm sections using a microtome and ribbons were placed in a warm (40°C) water bath before being transferred to glass slides (Fisherbrand Superfrost Plus precleaned microscope slides 25x75x1mm No. 12-550-15.) The slides were allowed to dry at room temperature overnight before being heat fixed for two hours at 65°C.

F. Hematoxylin and Eosin Staining

Slides were stained with hematoxylin and eosin. Briefly, slides were rehydrated stepwise through xylenes and ethanol. First, for 3 minutes three times in 100% xylenes, then 3 minutes in 100% ethanol, 95% ethanol, 70% ethanol and finally 2 minutes in 50% ethanol before being placed in tap water for 30 seconds. Once rehydrated, the slides were placed in hematoxylin for 10 minutes, rinsed with tap water until the slides were no longer blue/purple and then acid fixed for one second in acid alcohol before being placed immediately back in water. The slides were then rinsed in water for one more minute before being placed in lithium carbonate solution for 30 seconds. After washing in running tap water for 3 minutes, the slides were stained with eosin for 10 minutes. The slides were dehydrated through ethanol, 30 seconds in 70%, 60 seconds in 95% and 60 seconds in 100% ethanol before three 30 seconds washes in 100% xylenes. The slides were then mounted with coverslips using a mounting medium (Cytoseal 60, ThermoScientific N0. 8310-4) and allowed to dry overnight. They were then viewed on a Leica DM 2500 microscope. Representative images were taken from each treatment and infection status group, with particular focus given to the perivascular regions.

G. Antigen Retrieval for Immunohistochemistry

Antigenic retrieval is important for epitope recognition by antibodies. To start, slides were rehydrated stepwise through xylenes and methanol. First, 10 minutes in 100% xylenes and then 5 minutes in fresh 100% xylenes, twice. Then the slides were washed in 100% methanol first for 10 minutes and then 5 minutes before being placed in distilled water for 5 minutes. Slides were then placed in boiling citric buffer for 10 minutes

before the beaker was removed from the heat for a further 10 minutes. Slides were then washed in distilled water for 5 minutes and then transferred to phosphate buffer solution with tween (PBS-T) for 5 minutes.

H. Anti-CD45 Staining Using Immunohistochemistry

Sections were then blocked using 10% normal goat serum, 1% bovine serum albumin in PBS-T for two hours at room temperature. Rat anti-mouse CD45 antibody (Stem Cell technologies Lot #BX30892) diluted 1:400 in blocking buffer was added for 1 hour before repeat washes in PBS-T. Goat anti-rat secondary antibody (Invitrogen AlexaFluor 488 Lot # 1928689) diluted 1:200 in blocking buffer was added for 1 hour in the dark. After serial washes with PBS-T, sections were incubated with 20 μg/mL Hoechst 33342 in PBS-T for 10 min to visualize nuclei. Coverslips were mounted using PermaFluor Mountant media (#TA-030-FM, Thermo). Slides were viewed using a Nikon Eclipse E100080i microscope and digital images were captured with Hamamatsu C4742-95 charge-coupled device camera using NIS elements software.

I. CD-45 Positive Cell Quantification

Five random fields of view were selected and captured while viewing nuclei. Images were blinded and randomized before CD45+ cells were counted in each image. The average number of CD45+ cells were calculated for each mouse.

J. Cyst Wall Staining Using Immunohistochemistry

Brain tissue cysts were visualized in tissue sections using IHC. Following the standard preparation after the PBS-T washes, the slides were incubated for one hour in 1:250 rhodamine-conjugated *Dolichos bifluorus* lectin (Vector Laboratories) in blocking buffer. After serial washes with PBS-T, sections were incubated with 20 µg/mL Hoechst 33342 in PBS-T for 10 min to visualize nuclei. Coverslips were mounted using PermaFluor Mountant media (#TA-030-FM, Thermo). Slides were viewed using a Nikon Eclipse E100080i microscope and digital images were captured with Hamamatsu C4742-95 charge-coupled device camera using NIS elements software.

K. Infection Confirmation From Serum Using Dot Blots

There remains a possibility of not identifying tissue cysts in brain homogenate following i.p. injection of parasites and drug treatment. If I identify any mice that do not have visible brain tissue cysts upon quantification, then I need to confirm that they were exposed to the pathogen. It is possible that a syringe can fail during inoculation and that a mouse is never exposed to the parasite. It is also possible that drug treatment can lower the cyst burden below my level of detection. To account for this, I use dot blots to detect IgG antibodies against *Toxoplasma* antigens.

After blood was collected by cardiac puncture, it was allowed to coagulate at room temperature for thirty minutes before being spun down at 32000xg 4°C for 25 minutes. Serum was pipetted into a fresh Eppendorf tube and stored at -80°C.

Protein lysate was prepared by infecting a confluent T175cm^2 flask of HFF cells with Pru wild type (WT) parasites. Once the monolayer was near lysis, it was scraped,

syringe lysed and filtered through a 3 micron filter. The filtered parasites were centrifuged at 1500xg for 10 minutes at room temperature before medium and cellular debris were aspirated and the resulting pellet was suspended in 1mL of sterile PBS. The parasites were then sonicated for 15 seconds three times, with 30 seconds on ice between each sonication. The resulting lysate was stored at -80°C until needed for dot blots.

If a mouse's infection status was questioned due to no observable cysts during quantification, serum samples and the parasite lysate were thawed on ice. Small strips of Nitrocellulose membrane (GE Healthcare Life Sciences, No. 10600004) were cut and 10µL of parasite protein lysate was placed on the center of each strip. The membranes were blocked with 4% milk/TBST for one hour at room temperature. 1:500 dilutions of serum samples in blocking buffer were applied to the membranes for 1 hour at room temperature. A positive and negative control serum sample were included along with the test samples. Following three washes with blocking buffer, anti-mouse antibody conjugated to horseradish peroxidase (Amersham ECL Anti-mouse IgG No. NA931V) at 1:2000 dilution was added for 1 hour. The membranes were washed three times with blocking buffer to remove excess antibody. The presence of mouse antibodies specific to *Toxoplasma* were identified as positive signals where *Toxoplasma* protein lysate were dotted on the membranes. These signals were visualized using chemiluminescence catalyzed by horseradish peroxidase on SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisherScientific, #34094) and detected the signals using the FluorChem E Imager and AlphaView® software (Protein Simple).

IV. Behavioral Assays

A. General Considerations for Behavioral Assays

It became apparent early on in the drug treatment segment of the experiment that mice receiving guanabenz developed a strong fear response to the individual responsible for administering the drug. Animals would crowd into the corner of the cage or try to bury themselves in the bedding. By week two of drug treatment, mice were actively trying to escape the cage when the person doing injections opened it. This presented a concern that if the individual who administered the drug was present during behavioral data collection, then it would interfere with the behaviors. Therefore, two other students were asked to come into the animal facility and handle the mice briefly each day without negative consequences. As a result, the mice were not stressed when these individual were present and they could successfully carry out the behavioral assays. This individual varied throughout the course of the project and depended on availability. They were always blind to group conditions. The one time that the individual who was administering the drug performed a behavior test, the activity of the control groups was drastically out of alignment with past experiments. The experimental outcomes were unable to be interpreted, thereby stressing the importance of the two person behavior assay system.

B. Order of Behavioral Assays

Several of the behaviors tested have the potential to influence the outcomes of other behavioral assays. Due to this phenomenon, the order in which I chose to administer behavioral assays became important. I measured anxiety phenotypes first (either light/dark box or elevated plus maze), then locomotor activity, followed by

cognitive tasks (Morris water maze, spontaneous alteration, or T maze), and ended with the fear assay (cat odor).

C. Exclusion Criteria and Body Scores

Some of the C57BL/6 mice developed motor deficits that drastically hindered their ability to perform tasks. To account for this, the day before testing mice were evaluated by an individual blinded to the experimental group. The scoring criteria can be found in Table 1. All animals underwent behavioral testing to avoid researcher bias but mice that showed signs of severe illness or had a mobility deficit (such as limb paralysis) were excluded from the analysis.

Table 1- Body Scores for Infected Mice		
Fur		
	0	Shiny and well kept
	1	Scruffy and signs of lack of grooming
Gait		
	0	Normal gait and exploratory behavior
	1	Roached gait (hunched)
	2	Motor deficit, but still able to travel around the cage
	3	Extremely limited mobility, laying on side
Balance		
	0	Normal
	1	Head tilt
	2	Rolling through cage (alligator roll)

D. Locomotor Activity in an Open Field

The day before testing, twenty to twenty-four hours after the last drug treatment, mice were moved to the testing room and allowed to acclimate for 30 minutes. Individual mice were then placed in a Versamax locomotor activity testing chamber (AccuScan

Instruments) and locomotion was recorded for 30 minutes. The activity chamber measured 40 cm x 40 cm and was housed within a sound-attenuating chamber with a house light and fan for ventilation. The chamber was equipped with photocell beams located 2 cm above the Plexiglas floor to record locomotor activity. Mice were returned to their home cage after the 30 min test session. Mice marked for mobility deficits were excluded during data processing.

V. Gene Expression Analysis

A. Purification of Total RNA

$1x10^5$ J774.1 cells were seeded into 6-well plates and allowed to adhere overnight. Cells were stimulated with lipopolysaccharide (10ng/mL) for 6 hours in the presences or absence of guanabenz (50μM) as previously reported (Takigawa et al., 2016). Total RNA was isolated from the cells using TRIzol LS (Invitrogen) and cDNA was generated using Omniscript (Qiagen).

B. RT-qPCR of Total RNA

RT-qPCR was carried out using SYBR® Green Real-Time PCR Master Mixes (Invitrogen) and StepOnePlus Real System (Applied Biosystems). Relative levels of transcripts were calculated with the *ΔΔCt* method using GAPDH as the internal control. The relative levels of the target mRNAs from the untreated cells were adjusted to 1 and served as the basal control value. Each experiment was performed three times, each with three technical replicates. The primers used are listed in the Table 2.

Table 2 - List of Primers Used for RT-qPCR		
Gene	Forward Primer	Reverse Primer
IL-1β	5'-CCCATCCTCTGTGACTCAT-3'	5'-AGGCCACAGGTATTTTGTCG-3'
IL-6	5'-TCCATCCAGTTGCCTTCTT-3'	5'-TCCACGATTTCCCAGAGAAC-3'
TNF-α	5'-GAACTGGCAGAAGAGGCACT-3'	5'-AGGGTCTGGGCCATAGAACT-3'
COX2	5'-CCCCCACAGTCAAAGACACT-3'	5'-CTCATCACCCCACTCAGGAT-3'
GAPDH	5'-TGCACCACCAACTGCTTAG-3'	5'-GGATGCAGGGATGATGTTC-3'
IFN-γ	5'-TTCTTCAGCAACAGCAAGGC-3'	5'-TCAGCAGCGACTCCTTTTCC-3'

Chapter Three: Results

Our hypothesis is that reducing cyst burden will restore normal behavior in mice with latent *Toxoplasma* infections. Previous studies show that guanabenz reduces *Toxoplasma* brain cyst burden in chronically infected female BALB/c mice 69% (Benmerzouga et al., 2015) and the *in vitro* IC_{50} is 6μM against tachyzoites.

I. Optimization of Guanabenz Treatment of Chronic Toxoplasmosis in Mice

The cyst burden for previous studies was determined using whole brain homogenate (Benmerzouga et al., 2015). I compared the cyst burden between hemispheres of the brain to determine if it possible to hemi-sect the brain and use one half for cyst quantification and the other half for the histological studies. The possible difference in cyst count between the two hemispheres was of interest both with and without drug treatment. To test whether there were differences in cyst counts before homogenization, the brain was cut in half, sagittal along the longitudinal fissure with a razorblade, continuing through the cerebellum. Both halves were processed separately and cyst burden was quantified from blinded samples using immunofluorescence to identify cyst wall (Figure 1). There was no statistically significant difference in brain cyst burden between the right and left hemispheres for either the vehicle or drug treated mice. Therefore, I could select either hemisphere for cyst quantification and use the other for histological analysis without concern for bias.

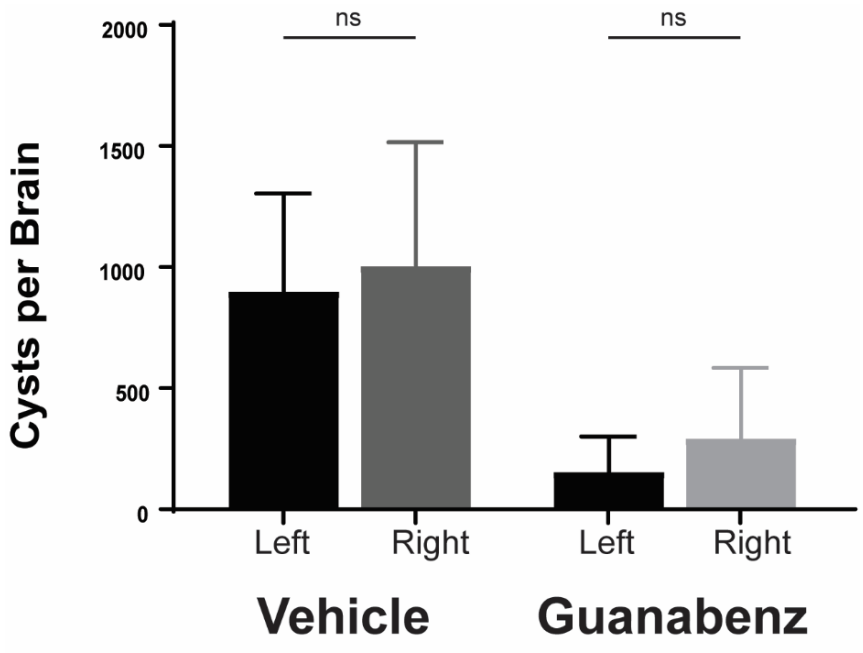

Figure 1 - No Difference in Cyst Burden Between Hemispheres. Following establishment of chronic infection, female BALB/c mice were randomized into vehicle or guanabenz (5mg/kg/day) treatment by i.p. injection. After 3 weeks of drug treatment, the mice were euthanized and the brains were extracted. Using a razorblade, a sagittal cut was made along the longitudinal fissure and both sides were homogenized in sterile PBS. Cyst burden was quantified by counting spheroid dolichos positive structures under immunofluorescence from blinded slides. Unpaired, two-tailed Student's t test, ns $p>0.05$. Error is standard deviation of the mean. N=3

The original experimental design for examining the efficacy of guanabenz *in vivo* was performed exclusively with female BALB/c mice (Benmerzouga et al., 2015; Konrad et al., 2013). To examine whether sex influences experimental outcome, I directly compared brain cyst burdens in male and female mice following guanabenz treatment. To this end, both male and female BALB/c mice were infected, allowed to transition to chronic infection, and then administered guanabenz 5 mg/kg/day i.p. for three weeks (Figure 2A). Following drug treatment, the brains were extracted and cyst burden was quantified from blinded samples by dolichos staining of cyst wall. My findings establish that guanabenz treatment yields an ~75% reduction of brain cysts in both male (Figure

2B) and female (Figure 2C) BALB/c mice. A 73% reduction of brain cysts is observed when data from both sexes are combined (Figure 2D). While the overall cyst burden in male mice is lower than in female mice, the percent reduction shows is the same between sexes. As a result, I chose to use only female mice moving forward.

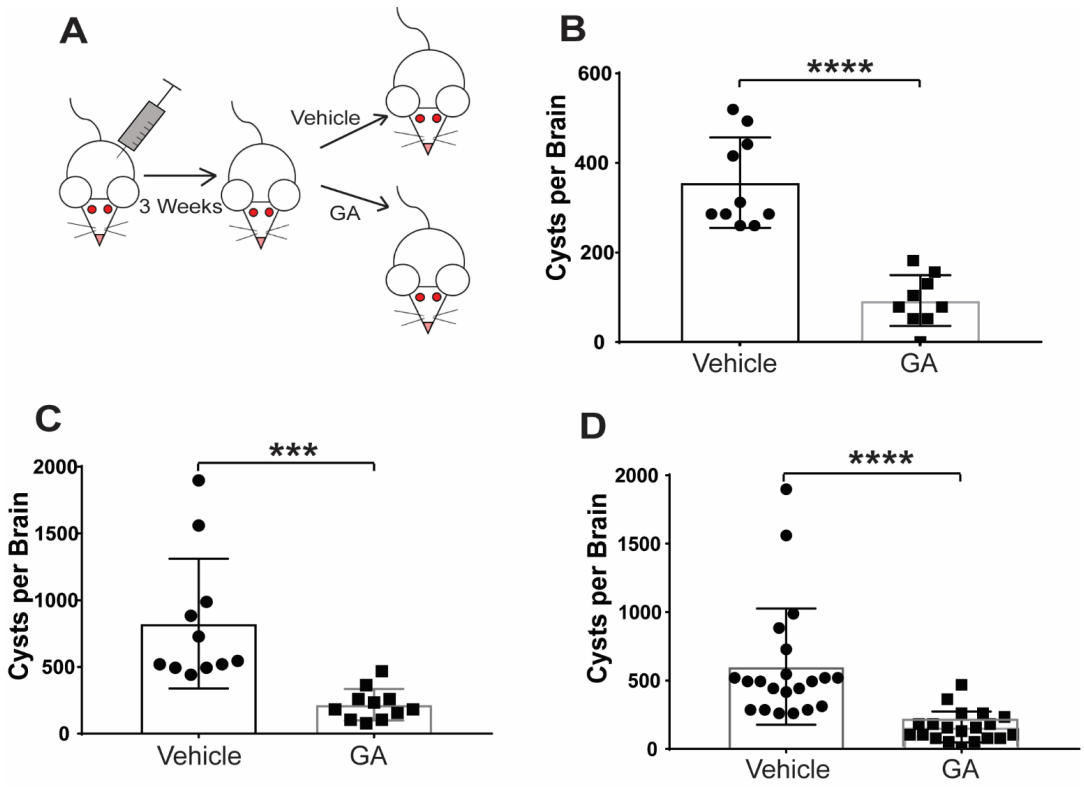

Figure 2 - Guanabenz Reduces Cyst Burden in Male and Female Mice. (A) BALB/c mice were infected i.p. with 10^4 Pru WT (grey syringe) parasites and allowed to progress to chronic infection over three weeks. The mice were then randomized into either vehicle or guanabenz (GA) treatment (5mg/kg/day i.p.) for a further 21 days. Mice were then euthanized and cyst burden was quantified by counting spheroid dolichos positive structures under immunofluorescence from blinded slides. Both male (B) and female (C) showed a 75% reduction in cyst burden, which was maintained when the sexes were pooled together (D). Unpaired, two-tailed Student's t test ***$p<0.001$, ****$p<0.0001$. Error is standard deviation of the mean.

Previous work in our lab has established that increasing the dosage of guanabenz to 10 mg/kg/day does not further reduce cyst burden (Benmerzouga et al., 2015). To determine if prolonged treatment reduces cyst burden further, I examined whether

extending guanabenz treatment from 3 weeks to 4 or 6 weeks would reduce cyst counts beyond the previously reported ~75% (Figure 3A). I observed that cyst counts remained reduced for the extended drug treatment times, but no further reduction was noted with prolonged treatment (Figure 3B).

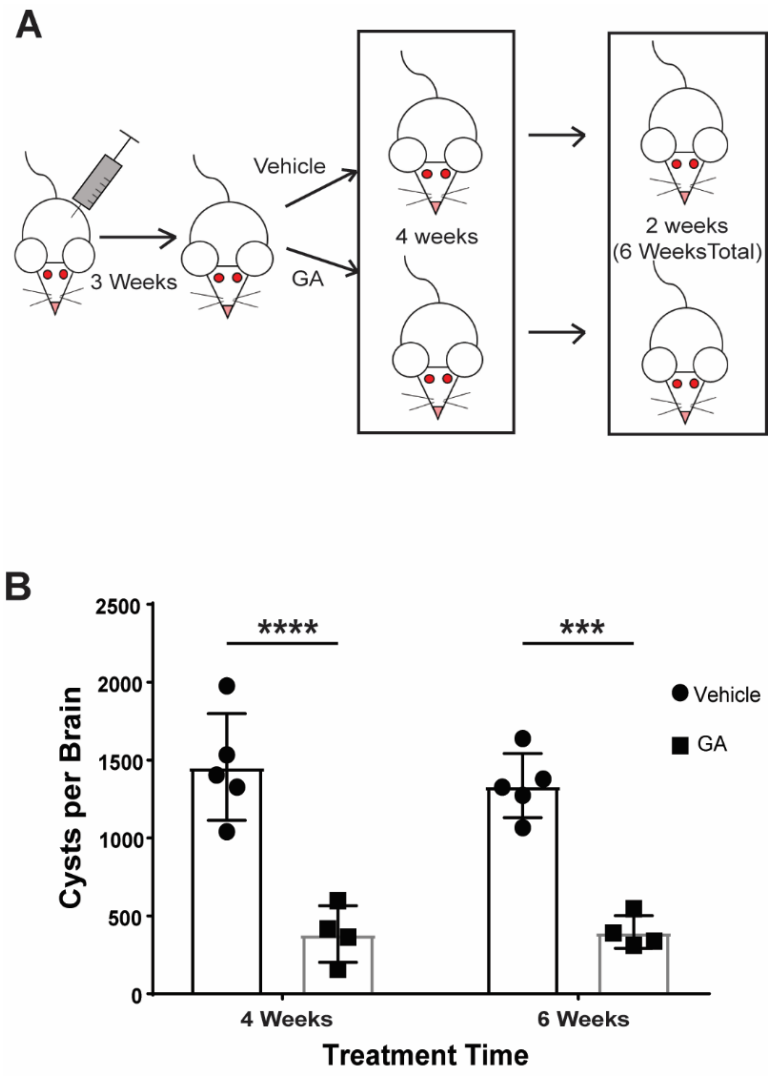

Figure 3 - Prolonged Guanabenz Treatment Does Not Improve Reduction in Brain Cyst Burden. (A) Female BALB/c mice were infected i.p. with 10^4 Pru WT (grey syringe) parasites and allowed to progress to chronic infection over three weeks. The mice were then randomized into either vehicle or guanabenz (GA) treatment (5mg/kg/day i.p.) for a further 28 days. (B) A group of Mice were then euthanized and cyst burden was quantified using immunofluorescence of blinded slides (4 weeks). Following two more weeks, for a total of six weeks of drug treatment, the second group of mice were euthanized and their cyst burden was quantified (6 weeks). Unpaired, two-tailed Student's t test ***$p<0.001$, ****$p<0.0001$. Error is standard deviation of the mean.

II. The Anti-Parasitic Activity of Sephin1 *in vitro* and *in vivo*

We determined whether new derivatives of guanabenz had anti-*Toxoplasma* activity. Structure activity relationship (SAR) studies show that the guanidine component of guanabenz is the crucial piece for GADD34 inhibition, while the chlorines on the aromatic ring are responsible for the alpha adrenergic receptor agonist properties (Das et al., 2015; Nguyen et al., 2014). Sephin1 was designed to limit the alpha adrenergic receptor activity, which is responsible for the main side effect of hypotension when guanabenz is used in normotensive animals (Figure 4A). My hope was that this would provide an effective clinical treatment for chronic toxoplasmosis and eliminate the side effects of guanabenz. An *in vitro* doubling assay showed that Sephin1 has stepwise growth inhibition at concentrations similar to guanabenz (Figure 4B). The IC_{50} of guanabenz against the Type I parasite strain RH is 6μM and Sephin1 is 5.4 μM.

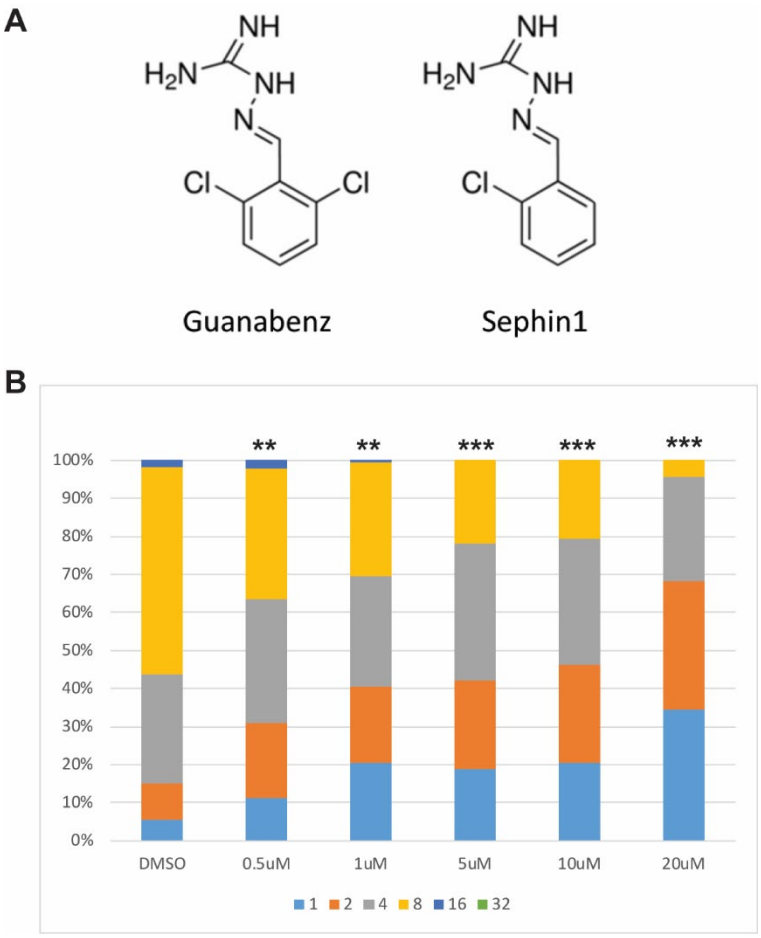

Figure 4 - Sephin1 Shows a Dose Dependent Inhibition of Parasite Growth *in vitro*. (A) The chemical structures of guanabenz and Sephin1. (B) Quantification of parasite number per vacuole of fifty randomly selected vacuoles 24 hours after start of Sephin1 treatment following 2 hours of invasion. Representative of two independent replicates done in biological triplicate. One way ANOVA $p<0.0001$. Followed by an unpaired, two-tailed Student's t-test, $**p<0.01$, $***$ $p<0.0001$

Following the success of Sephin1 *in vitro* I tested its efficacy *in vivo* against lethal acute infection with RH parasites (Figure 5). Guanabenz prolongs life for three to four days when used to treat lethal RH infections, therefore I used the same experimental setup to test the efficacy of Sephin1 (Konrad et al., 2013). Consequently, I administered Sephin1 by i.p. injection as opposed to the published method, oral gavage (Das et al., 2015). The decision to change the route of administration was meant to facilitate the comparison between guanabenz and Sephin1. In addition to a vehicle control, a mock

infected group which was given the maximum dose of Sephin1 (10mg/kg 2x per day) was included to account for possible toxicity of Sephin1.

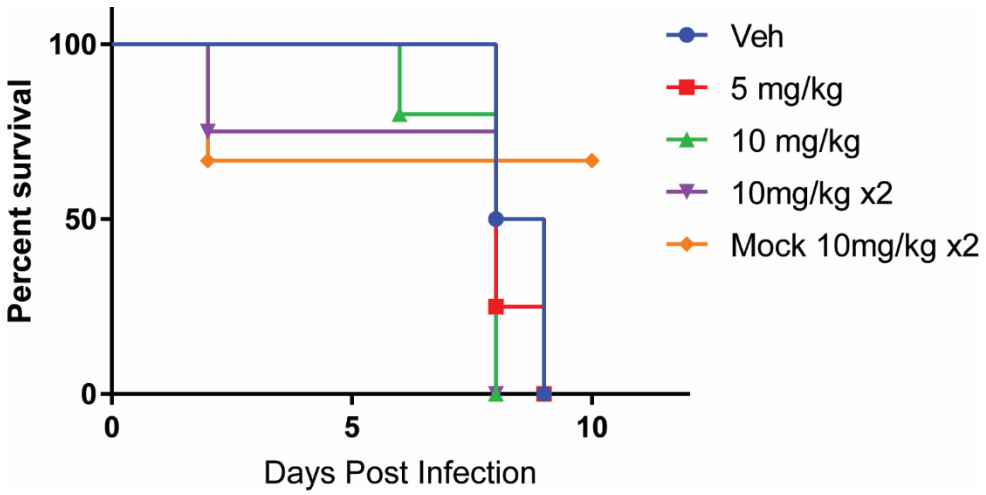

Figure 5 - Sephin1 by i.p. Injection is Not Effective Against Acute Toxoplasmosis. Female BALB/c mice were infected with 100 RH parasite i.p. on day 0, except for a mock infected group which was only administered vehicle. The following day, mice were dosed with either vehicle, 5mg/kg or 10mg/kg either once or twice per day.

What I observed was that several of the mock infected and infected mice who were receiving the highest does (10mg/kg two time per day) died on the first day of drug treatment, thereby indicating a toxicity issue when Sephin1 is administered i.p. I suspect that this was due to the rapid absorption of Sephin1 into the brain. Mice administered any dose of Sephin1 developed spastic paralysis within minutes of injection, with varying severity with dose. All treatment groups died at a faster rate than the vehicle control, suggesting that the toxicity of Sephin1 at these doses outweighed any positive anti-parasitic effects when give i.p.

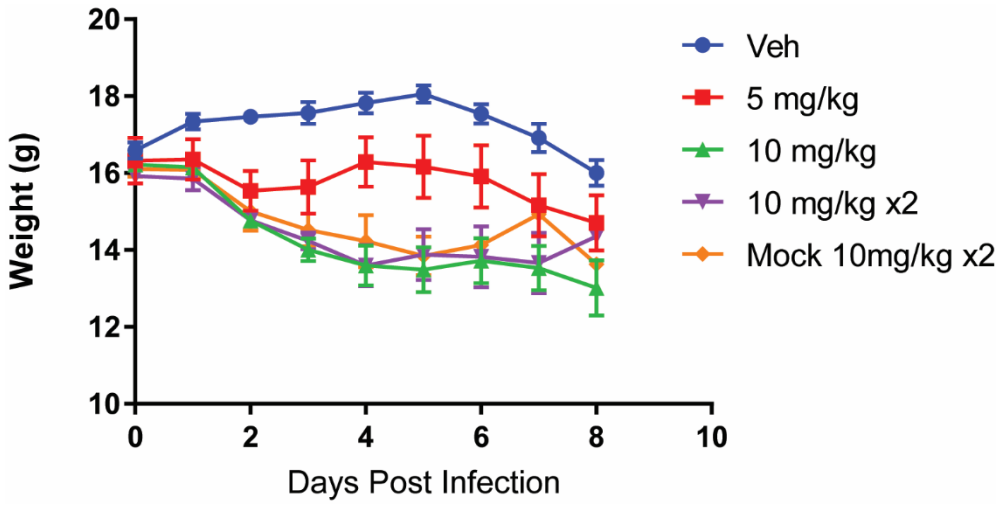

Figure 6 - Sephin1 Decreases Weight, Regardless of Infection Status. Mice were weighed in the morning each day. All mice that received Sephin1 showed a dose dependent weight loss, even before the vehicle control mice started to get sick. Error is standard error of the mean.

The toxicity of Sephin1 is readily apparent in the weight loss seen in the treated mice, regardless of dose (Figure 6). While infected, vehicle treated mice do not start to lose weight until day 5. All mice given Sephin1 immediately began losing weight. The weight loss is more pronounced for the 10 mg/kg and the 10 mg/kg twice per day groups than for the lower 5mg/kg group. There was no difference in the weight loss between the mock infected and *Toxoplasma* infected mice receiving the maximum dose. I conclude that the toxicity of Sephin1 when administered i.p. outweighs any potential beneficial anti-parasitic effects.

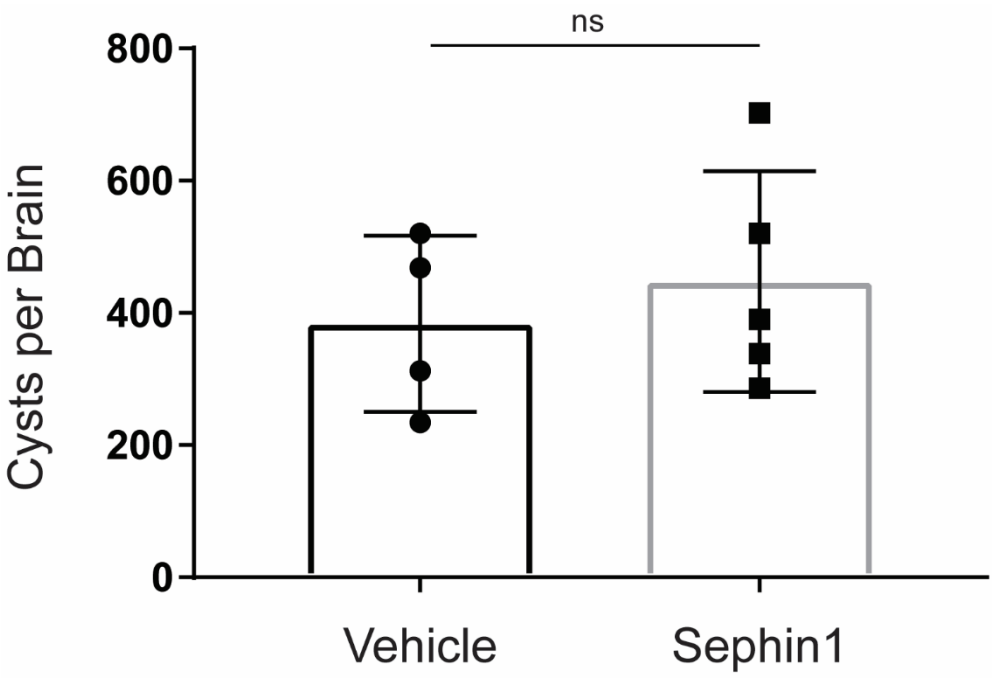

Figure 7 - Sephin1 Does Not Impact Cyst Burden. Female BALB/c mice were infected with 10^4 Pru WT parasites and allowed to transition to chronic infection. Groups were then randomized into vehicle or Sephin1 at 10mg/kg/day by oral gavage. Mice were treated for three weeks before euthanasia and brain collection. Cyst burdens were quantified using immunofluorescence on blinded slides. Unpaired, two-tailed Student's t test. ns $p>0.05$. Error as standard deviation of the mean.

Since no toxicity was previously reported when Sephin1 was administered by oral gavage, I chose to test the efficacy of Sephin1 against chronic toxoplasmosis using gavage instead of i.p. injection (Das et al., 2015). Following the establishment of chronic infection, BALB/c mice were administered Sephin1 by oral gavage at 10mg/kg/day for three weeks. No adverse effects or toxicity was observed in the treated mice. When the brains were collected and cyst counts were calculated I observed no difference between the Sephin1 treated mice and the vehicle control (Figure 7). Due to this failure to reduce cyst burden by oral gavage administration and the toxicity with i.p. administration, I decided not to pursue Sephin1 for my future studies.

III. Guanabenz Returns Normal Locomotor Behavior in Latently Infected BALB/c Mice

With my established *in vivo* model for latent brain cyst reduction, I interrogated the effect of guanabenz treatment on locomotor activity in female BALB/c mice. Hyperactivity is a well-established behavioral change that occurs in chronically infected mice. I reasoned that since guanabenz could reduce cyst counts then parasite-induced behaviors may also be attenuated. To minimize potential effects of guanabenz itself on behavior, assessment of locomotor activity was performed in the 4-h window before daily drug dosing, when the literature suggests that there is no detectable drug in circulation or brain for the dosage used (Way et al., 2015). Locomotor activity was recorded as total distance traveled (cm) in an open field over 30 min. Chronically infected or mock-infected BALB/c mice were treated with 5/mg/kg/day guanabenz, as previously established, or vehicle daily for 4 weeks (Figure 8A). I observed no change in locomotor activity in mock-infected mice given guanabenz compared to mock infected mice given vehicle. The *Toxoplasma*-infected vehicle treated mice displayed the expected hyperactivity; strikingly, the group of chronically infected mice treated with guanabenz exhibited significantly reduced hyperactivity compared to vehicle-treated infected mice, nearly reverting to the baseline recorded for uninfected mice (Figure 8B).

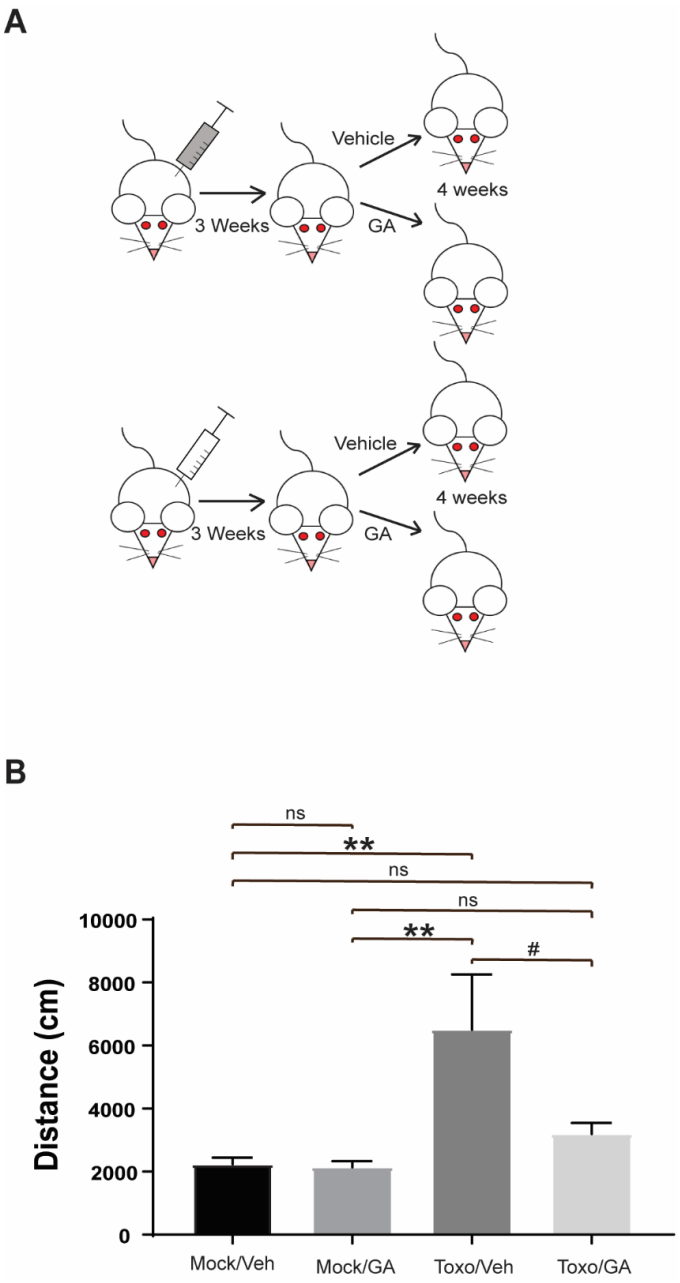

Figure 8 - Guanabenz Treatment Restores Normal Locomotor Activity. (A) Female BALB/c mice were infected i.p. with 10^4 Pru WT (grey syringe) parasites while a second group were mock infected with vehicle (white syringe) and allowed to progress to chronic infection over three weeks. The mice were then randomized into either vehicle or guanabenz (GA) treatment (5mg/kg/day i.p.) for a further 28 days. (B) After 4 weeks of treatment, locomotor activity was recorded in an unfamiliar 40cm x 4cm x 30cm Plexiglas box and the total distance traveled over 30 minutes was compared. Error bars indicate standard error of the mean. One-way ANOVA p=0.0019, followed by an unpaired, two-tailed Student's t-test, **p<0.001, #p<0.1, ns p>0.1. N=10

IV. Evaluation of the Route of Administration on Guanabenz Activity

Guanabenz was originally designed as a *per oral* anti-hypertensive drug. As such, I decided to investigate the efficacy of guanabenz against *Toxoplasma* cyst burden and locomotor activity when given orally. Concerns for experimental design included the impact of the stress of oral gavage on behavior and the willingness of the mice to consume the drug. In order to overcome these concerns, I devised a four branch dosing strategy (Figure 9). The first branch was vehicle and guanabenz given i.p. at 5mg/kg/day. The second branch provided either vehicle or guanabenz infused feed. I was concerned about altered drug absorption and its impact on pharmacokinetics; So I increased the dosage to 10mg/kg/day. I added a third arm of oral gavage administration at 10mg/kg/day. The fourth branch utilized a dough treat to administer either guanabenz or vehicle.

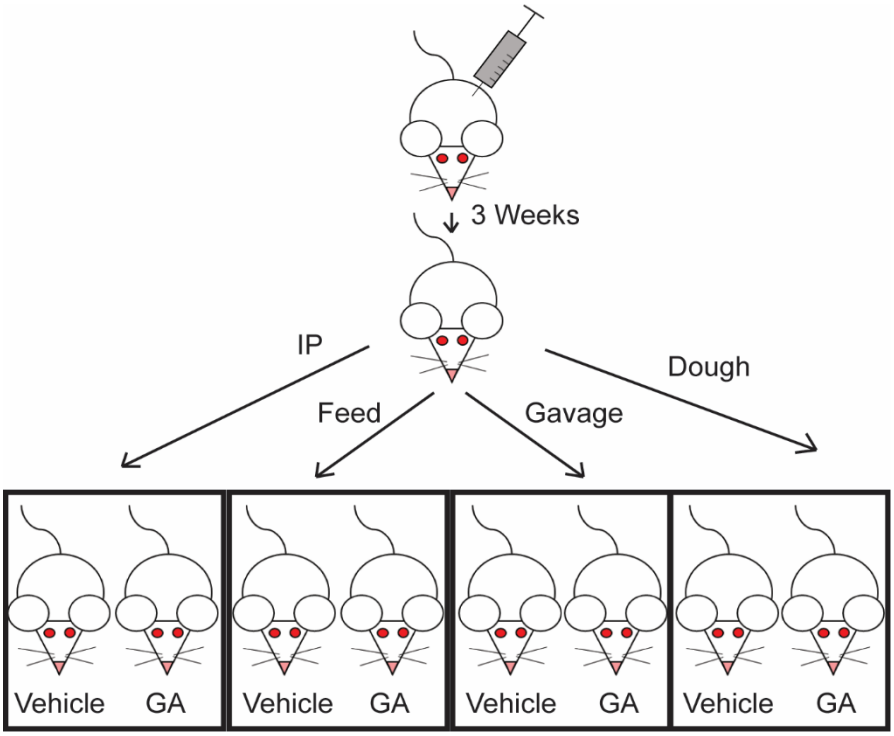

Figure 9 - Experimental Design for Multiple Routes of Drug Administration. Female BALB/c mice were infected i.p. with 10^4 Pru WT (grey syringe) and allowed to progress to chronic infection over the course of three weeks. Mice were then randomized into eight different groups. Each route of drug administration was divided into a vehicle and a guanabenz (GA) treatment group.

Prior to infection, all mice were conditioned to eat the dough treat. This involved access to the dough treat for two hours before the time was decreased to half an hour over the course of a week while the mice were acclimated to the animal facility. By the end of the acclimation period, all mice consumed all of the dough. During acute infection, the mice were not given access to dough. Groups were randomized the day before drug treatment began and after the mice had transitioned to chronic infection. Those mice that were selected for dough treat drug administration were given a dough ball containing 10mg/kg of guanabenz. The first day of dough access, each mouse consumed 100% of the dough, indicating that there is not a taste aversion to guanabenz and that mice were willing eat it (Figure 10). On day two, several of the guanabenz treated mice did not

finish the dough while vehicle treated mice continued to finish all of it. All of the vehicle treated mice consumed all of their dough every day they were tested. By day three, the majority of the guanabenz treated mice were not finishing the guanabenz containing dough. I was concerned that the guanabenz treated mice were developing a conditioned taste aversion due to the drowsiness and dizziness that guanabenz causes as a result of hypotension. To test this, I provided drug free dough to the guanabenz group on day four and the mice continued to refuse to eat it. Because the mice willingly ate the guanabenz in the dough at the beginning and the vehicle treated mice always ate there dough, it suggests a conditioned taste aversion to the dough itself once it has been associated with the side effects of guanabnez. Because the mice were refusing the drug-containing dough, it is not possible to draw conclusions from this group, consequently this branch of the study was terminated early.

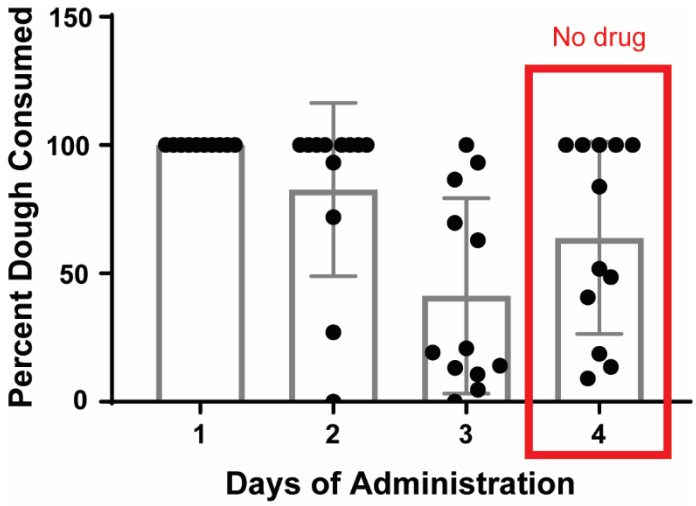

Figure 10 - Mice Develop Conditioned Taste Aversion to Guanabenz. Prior to infection, all mice were acclimated to the dough administration procedure. Following group randomization, infected BALB/c mice were presented with a dough ball that was laced with guanabenz (10mg/kg/day) for the first three days of administration. On the fourth day, the mice were presented with a control dough ball without any drug. Error bars indicate standard deviation of the mean.

At the conclusion of drug treatment using different routes of administration, locomotor activity was assessed in an open field across the remaining six groups (Figure 11). As seen previously, guanabenz administration reduced hyperactivity when it was given i.p. The other two routes of administration that were tested (gavage and feed) also showed a statistically significant reduction in *Toxoplasma* induced hyperactivity, concluding that guanabenz reduces hyperactivity when given orally as well as i.p.

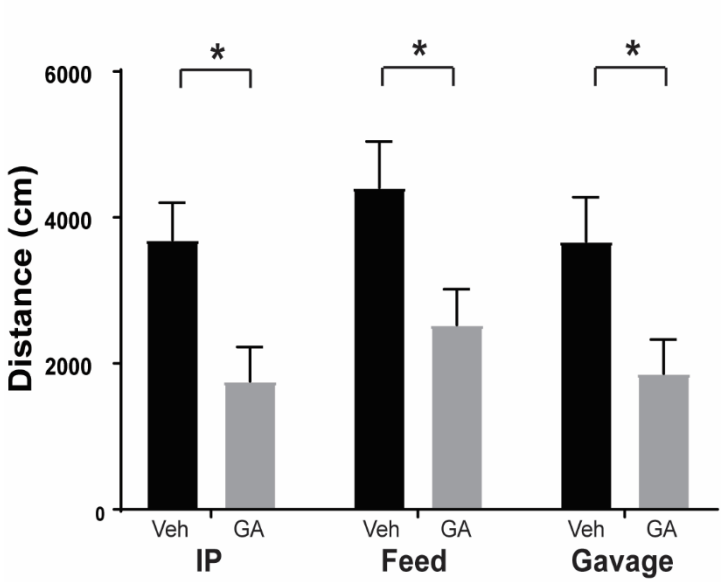

Figure 11 - Guanabenz Reduces Hyperactivity Through Multiple Methods of Drug Administration. After 3 weeks, guanabenz (GA) or vehicle (Veh) treatment locomotor activity was recorded in an unfamiliar 40cm x 4cm x 30cm Plexiglas box and the total distance traveled over 30 minutes was compared. Error bars indicate standard error of the mean. Single replicate with one-way ANOVA $p=0.0022$, followed by unpaired, two-tailed Student's t-test, $*p<0.05$. N=12

Cyst burden was quantified in the different dosing strategies (Figure 12). Surprisingly, cyst counts were not significantly altered in the latently infected mice that received guanabenz by gavage or medicated feed, while the cyst burden of mice receiving guanabenz i.p. was significantly lowered. This does not correlate with the

hyperactivity data, which was reduced across all groups, thereby suggesting that rescue from parasite-induced hyperactivity occurs independently of cyst burden.

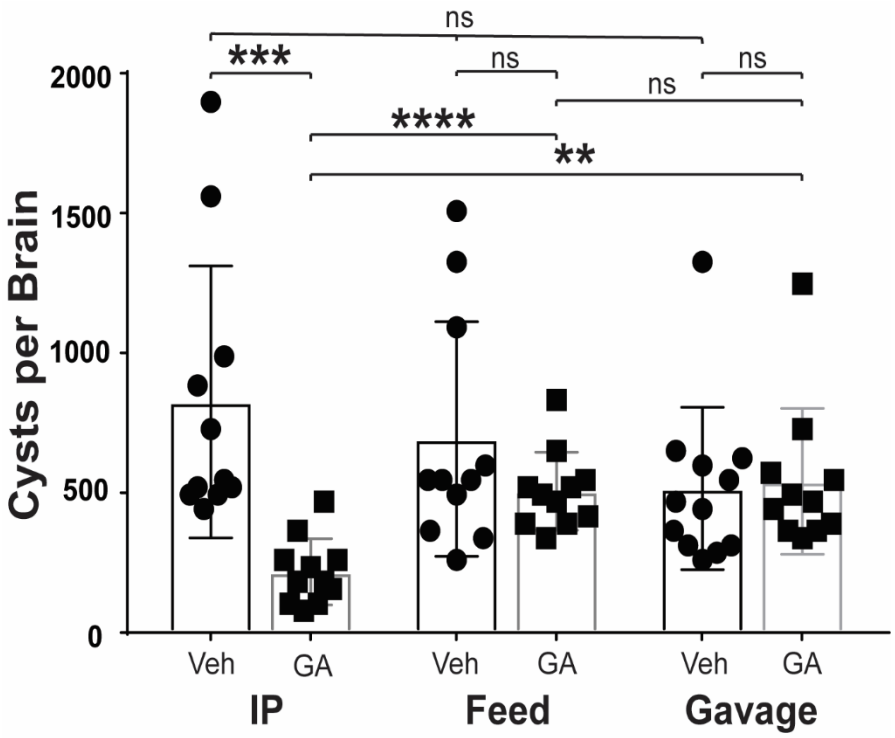

Figure 12 - Guanabenz Does Not Decrease Cyst Burden When Administered Per Oral. After 3 weeks guanabenz (GA) treatment, samples were blinded and brain cysts were enumerated. Error bars indicate standard deviation of the mean. Single replicate with one-way ANOVA p=0.0012 followed by an unpaired, two-tailed Student's t-test, ns p>0.5, **p<0.1, ***p<0.001, ****p<0.0001.

V. Guanabenz Reduces Inflammation *in vitro* and *in vivo*

As my previous experiment showed that reduced brain cyst burden is not correlated to the reversal of hyperactivity, I examined other aspects of neurophysiology that may explain the effect of guanabenz. It is well established that guanabenz exhibits anti-inflammatory properties *in vitro* and *in vivo* (Chang, Blazek, Skowronek, Marinari, & Carlson, 1987; Perego et al., 2018; Takigawa et al., 2016; Way et al., 2015). *In vitro*, guanabenz has been shown to decrease the expression of pro-inflammatory cytokines, including IFN-γ, IL-6, and TNF-α (Takigawa et al., 2016). Using RT-qPCR, we determined the mRNA expression levels of these inflammatory cytokines in J774.1 cells, a BALB/c-derived macrophage line (Figure 13 A-E). Stimulation of these cells using LPS resulted in increased expression of IFN-γ, TNF-α, IL-6, IL-1β, and COX-2; however, guanabenz blocked this effect. These results are consistent with the aforementioned studies showing that guanabenz has immunomodulatory properties.

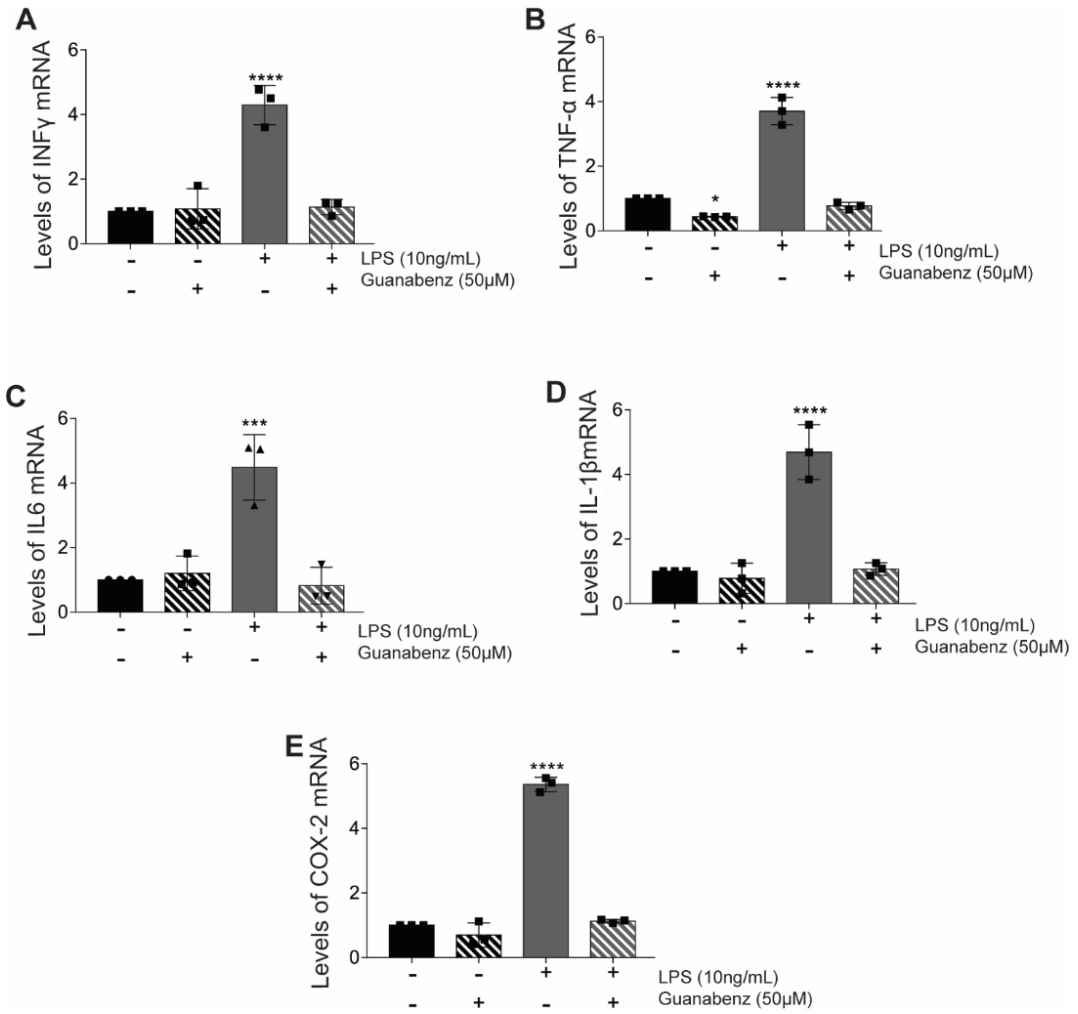

Figure 13 - Guanabenz Reduces Expression of Pro-Inflammatory Cytokines *in vitro*. J774.1 cells were stimulated with LPS (10ng/mL) in presence or absence of guanabenz (50 μM) for 6 h. Levels of (A) *IFN-γ,* (B) *TNF-α,* (C) *IL-6,* (D) *IL-1β* and (E) *COX-2* mRNA were then measured by RT-qPCR. Values of infected cells were normalized to untreated cells. Error bars indicate standard deviation of the mean; n = 3, Student's t-test *p<0.05, ***p<0.005, ****p<0.0001.

Following the reduction in cytokine signaling *in vitro*, I examined the brains from my previous experiment to determine if there were global changes observable within my samples. I performed histological examinations of brain sections from BALB/c mice from my i.p., gavage, and feed dosing strategies using hematoxylin and eosin (H&E) staining (Figure 14). In mock-infected control mice, I found that guanabenz had no effect on baseline inflammation. Consistent with findings reported by others (Hermes et al.,

2008; Mahmoudvand et al., 2016), I observed increased neuroinflammation in mice with chronic toxoplasmosis. However, chronically infected mice treated with guanabenz consistently showed reduced neuroinflammation. Reduced inflammation was especially noticeable in perivascular region where the cuff of immune cells is characteristic of chronic infection and the difference was present regardless of the drug delivery method used.

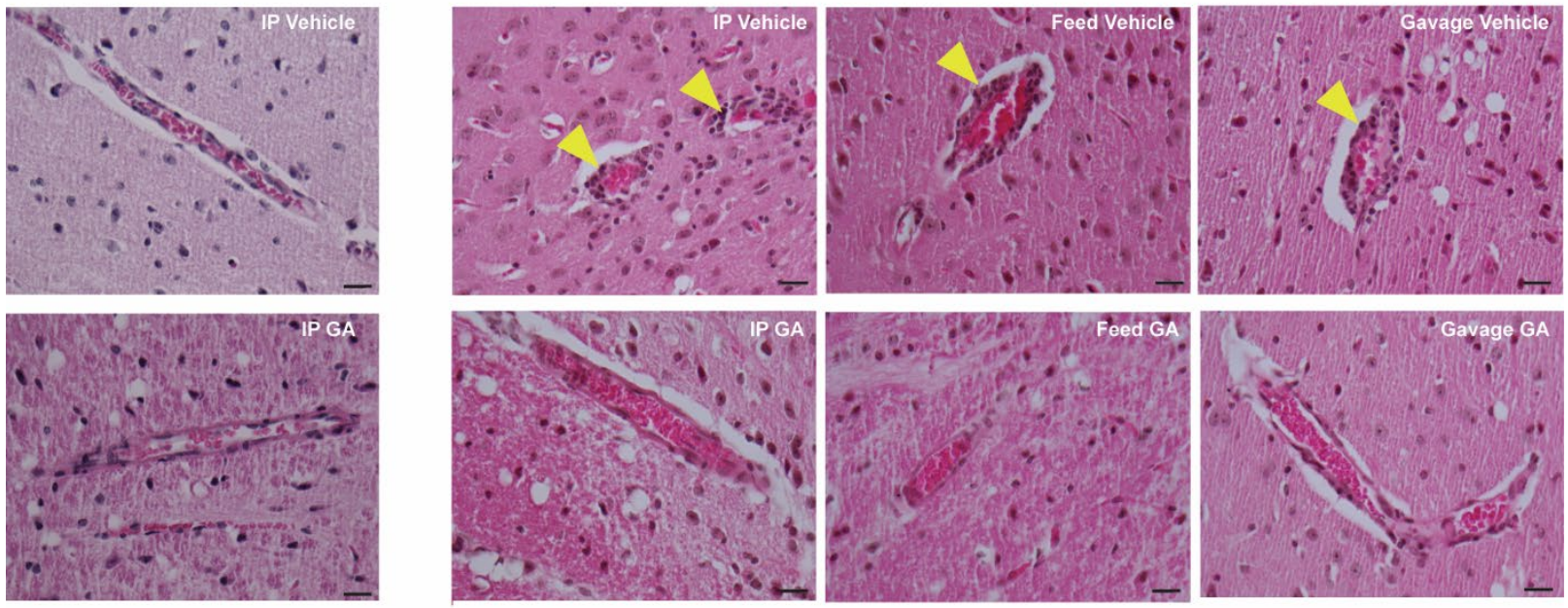

Figure 14 - Guanabenz Perivascular Cuffing in Infected BALB/c Mice. Representative images of H&E stained brain sections from (left) mock-infected and (right) *Toxoplasma*-infected BALB/c mice treated with vehicle or guanabenz (GA) as indicated. Scale bar represents 20µm. Yellow arrowheads highlight the perivascular cuffing associated with chronic *Toxoplasma* infection, which is not as pronounced in GA-treated mice.

While H&E is effective in showing structural qualities, it is difficult to identify cell populations without a specific marker. To quantify the reduction in neuroinflammation, I used IHC. I labeled sections of brain with anti-CD45 antibody, which serves as a general marker for immune cells by identifying leukocytes (Figure 15).

As observed with H&E, there is increased inflammation, as indicated by the abundance of CD45+ cells in *Toxoplasma* infected brains. Regardless of the administration route, guanabenz caused a reduction in $CD45^{+}$ cells in chronically infected BALB/c mice.

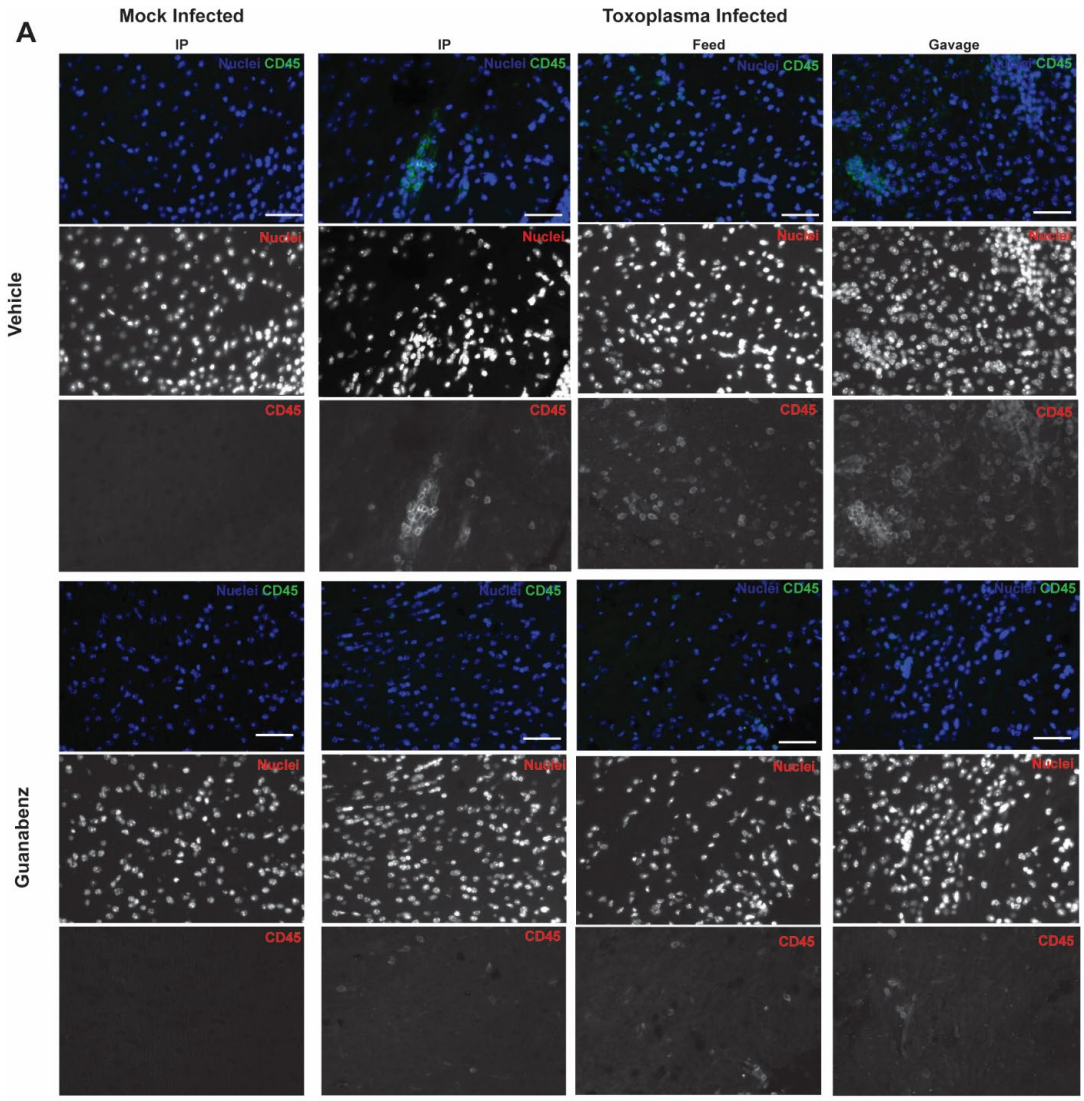

Figure 15 - CD45+ Cells in Vehicle and Guanabenz Treated Brains. Brain tissue sections were stained with anti-CD45 antibody (green). Hoechst was used to label nuclei (blue). Representative images for each group are shown. Scale bar = 50µm.

These results were further quantified by counting blinded random fields of view (Figure 16). There is a statistically significant reduction in CD45+ cells within the brain following guanabenz treatment of *Toxoplasma* infected mice. The inflammation is not eliminated, but significantly reduced without adverse effects on the host. No difference in $CD45^{+}$ cells was detected in the brains from mock-infected mice. These results associate

better to the activity data as compared to the cyst count data, suggesting a possible neuroinflammation mediated mechanism for behavior alteration.

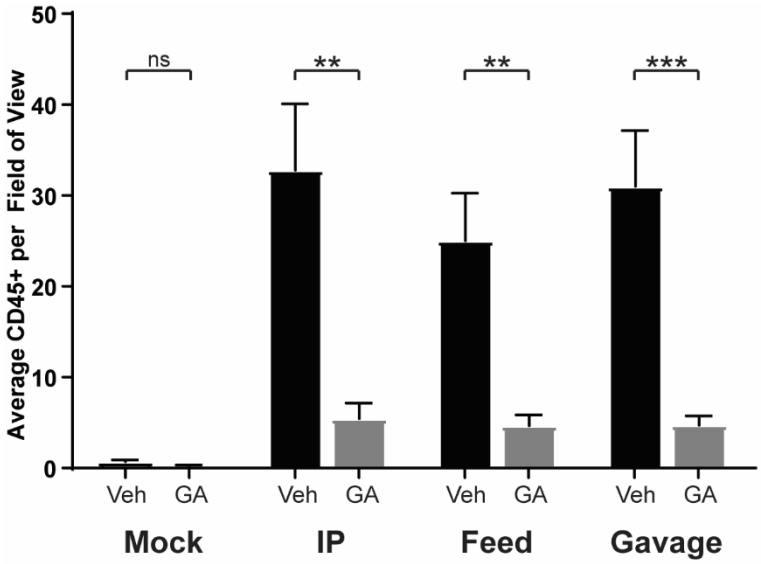

Figure 16 - Guanabenz Reduces the Average Number of CD45+ Cells in Latently Infected Mice. Five random fields of view were selected under Hoechst staining for each mouse. Images were then randomized and blinded before CD45+ cells were counted. Average number of CD45+ cells per field for each mouse was calculated and visualized. Error bars indicate standard deviation of the mean. One-way ANOVA $p<0.0001$ followed by an unpaired, two-tailed Student's t-test, ns $p>0.5$, $^{**}p<0.01$, $^{***}p<0.001$. N=12

VI. Differences in Drug Response Based on Mouse Model

To further examine the mechanism of guanabenz against *Toxoplasma in vivo*, I performed a series of similar experiments in C57BL/6 mice. These experiments would determine if the effects of guanabenz were consistent in an independent, nonisogeneic mouse strain. Moreover, C57BL/6 mice are commonly employed when investigating the immune response to *Toxoplasma*. C57BL/6 mice are highly susceptible to *Toxoplasma* infection, displaying enhanced pathology and higher cyst burdens than more resilient strains like BALB/c. Both male and female C57BL/6 mice were injected with a nonlethal dose of *Toxoplasma* to establish chronic infection for 3 weeks (Figure 17A). Mice were then randomized into groups before being treated with either guanabenz or vehicle i.p. for 3 weeks. Unexpectedly, guanabenz caused nearly half of the chronically infected mice to die within the first 10 days of drug treatment (Figure 17B). Mice that succumbed to the guanabenz treatment developed classic symptoms of *Toxoplasma* encephalitis, including lethargy, tremors, paralysis and seizure. None of these mice showed signs of active infection at the start of guanabenz treatment. These results suggest that the mice experienced reactivation of encysted parasites rather than continuation of a severe acute infection. The mice that survived the full drug treatment did not display symptoms of *Toxoplasma* encephalitis. Stratifying the groups by sex shows that the effects were equal in severity among both male and female C57BL/6 mice (Figure 17C).

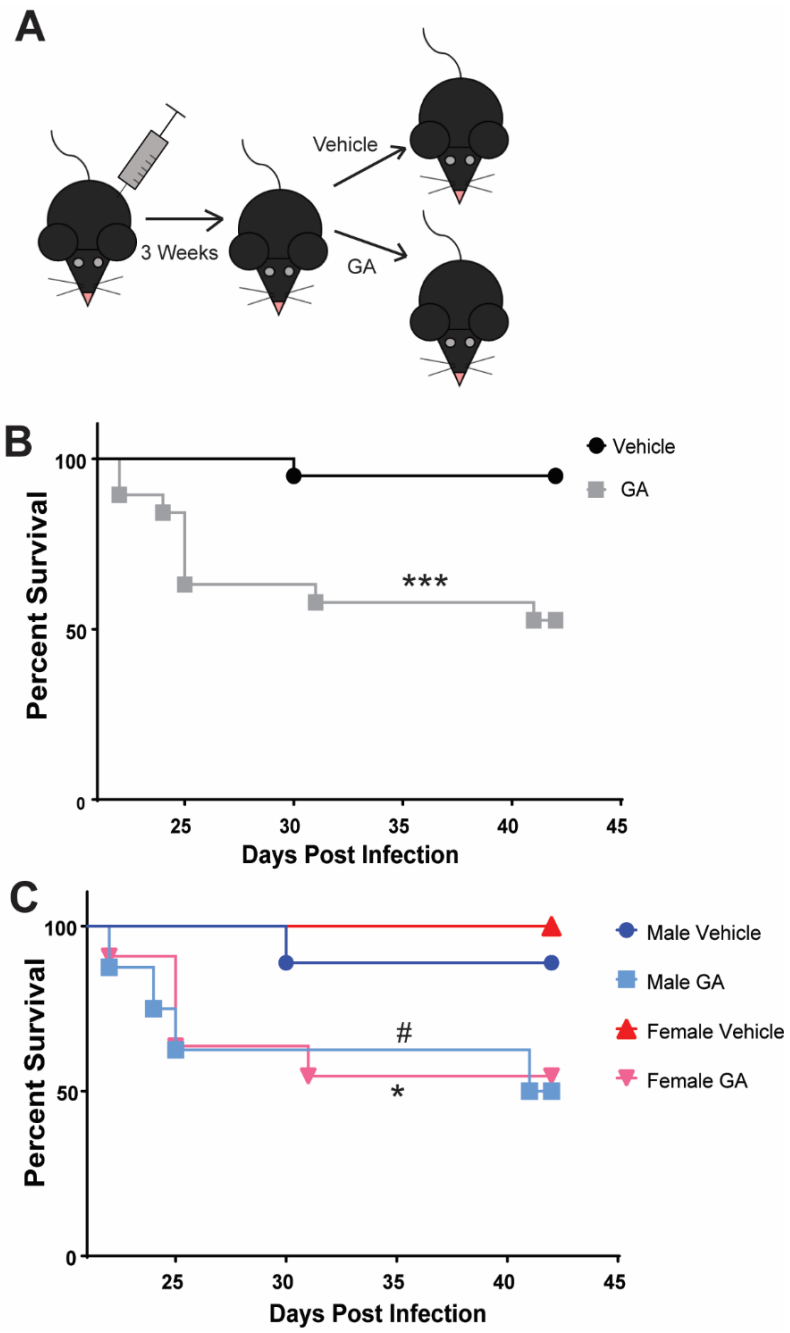

Figure 17 - C57BL/6 Mice Develop Lethal Reactivated *Toxoplasma* Encephalitis with Guanabenz Treatment. (A) Male and female C57BL/6 mice were infected i.p. with 10^3 Pru tachyzoites. Following establishment of chronic infection at 21 days, groups were randomized to receive vehicle or guanabenz (GA 5mg/kg/day i.p.). (B) Survival of mice in designated treatment groups. Single replicate with both sexes, log rank test p=0.00232; ***p<0.001. (C) When the sexes are split, both trend together, showing that the lethal drug effects are not sex-dependent. Single replicate, log rank test, male p=0.072, female p=0.0128. *p<0.05, #p<0.08. Male N=8, Female N=12

Once I identified a negative effect of guanabenz treatment in T*oxoplasma* infected C57BL/6 mice, I was concerned that the side effects of drug treatment could impact the behavior assays. Therefore, I endeavored to quantify the physical condition of all the mice to identify those mice who could not properly perform the behavior assays due to a deficit. I planned to exclude these mice from the analysis of locomotor activity after testing was complete. An individual blind to experimental design was asked to assign a body score to each mouse the day that behavior testing occurred. Table 1 lists how these scores were assigned. The higher the score, the more deficits that the mouse displays. Male mice (Figure 18A) showed a statistically significant worse score with drug treatment but females did not (Figure 18B). When the two groups were combined (Figure 18C), the differences remained significant, mirroring the negative effects that were seen with survival.

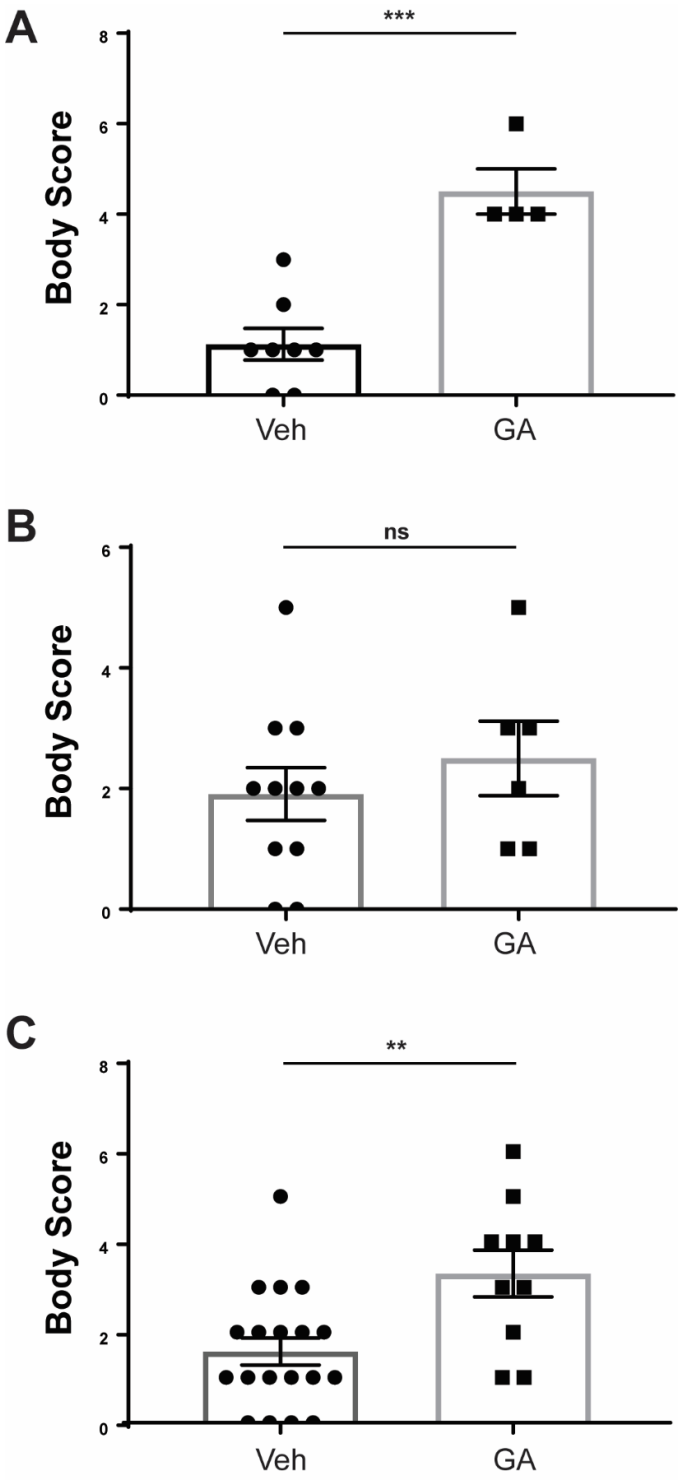

Figure 18 - Guanabenz Negatively Impacts Wellness in Male C57BL/6 Mice. An individual blind to study design scored the mice prior to behavioral testing. (A) Male mice show more severe deficits than (B) female mice. (C) The difference remained significant when both sexes were pooled. Unpaired, two-tailed Student's t-test, ns $p>0.5$, $^{**}p<0.01$, $^{***}p<0.001$. Error bars indicate standard deviation of the mean.

At the completion of the experiment, the surviving mice were euthanized and brain cyst burden was quantified. In contrast to its effect in BALB/c mice, i.p. guanabenz treatment caused an increase in cyst burdens in the surviving C57BL/6 mice. Male mice (Figure 19A) and female mice developed a higher cyst burden (Figure 19B); drug treatments with both sexes combined also led to statistically higher cyst burden (Figure 19C).

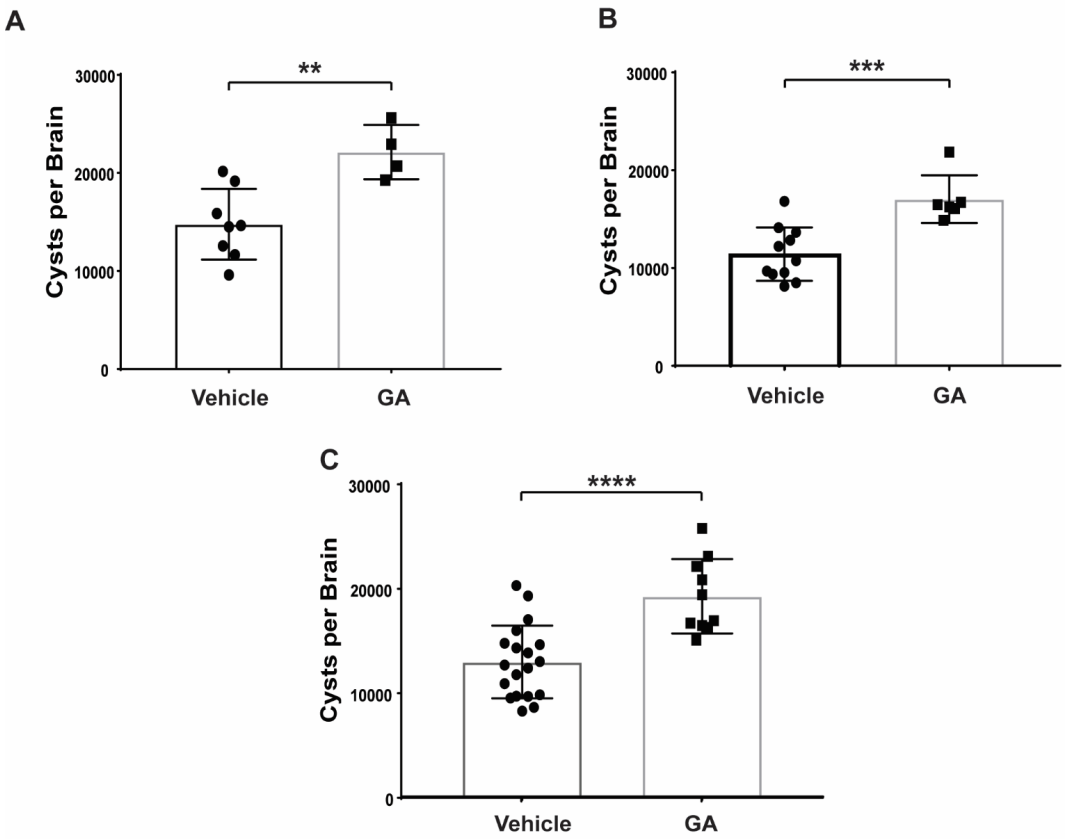

Figure 19 - Guanabenz Treatment Increases Cyst Burden in C57BL/6 Mice. Blinded brain cyst quantification was performed on the vehicle and GA-treated C57BL/6 mice. Both (A) male and (B) female mice show an increase in cyst burden. This held true when both sexes were (C) combined. Error bars indicate standard deviation of the mean, p values determined by Student's t-test: **p<0.01, ***p<0.001, ****p<0.0001.

Locomotor activity was also recorded for the surviving mice. Surprisingly, despite the increase in brain cyst counts in the guanabenz-treated C57BL/6 mice, the drug still reversed *Toxoplasma*-induced hyperactivity. Any mice that had an obvious motor deficit (one male vehicle and one male guanabenz treated) as identified in the body score were excluded from the locomotor analysis. Both chronically infected male (Figure 20A) and female (Figure 20B) mice showed a statistically significant reduction in hyperactivity following guanabenz treatment. The difference held when the two sexes were combined (Figure 20C), again suggesting that locomotor changes are not driven by brain cyst burden.

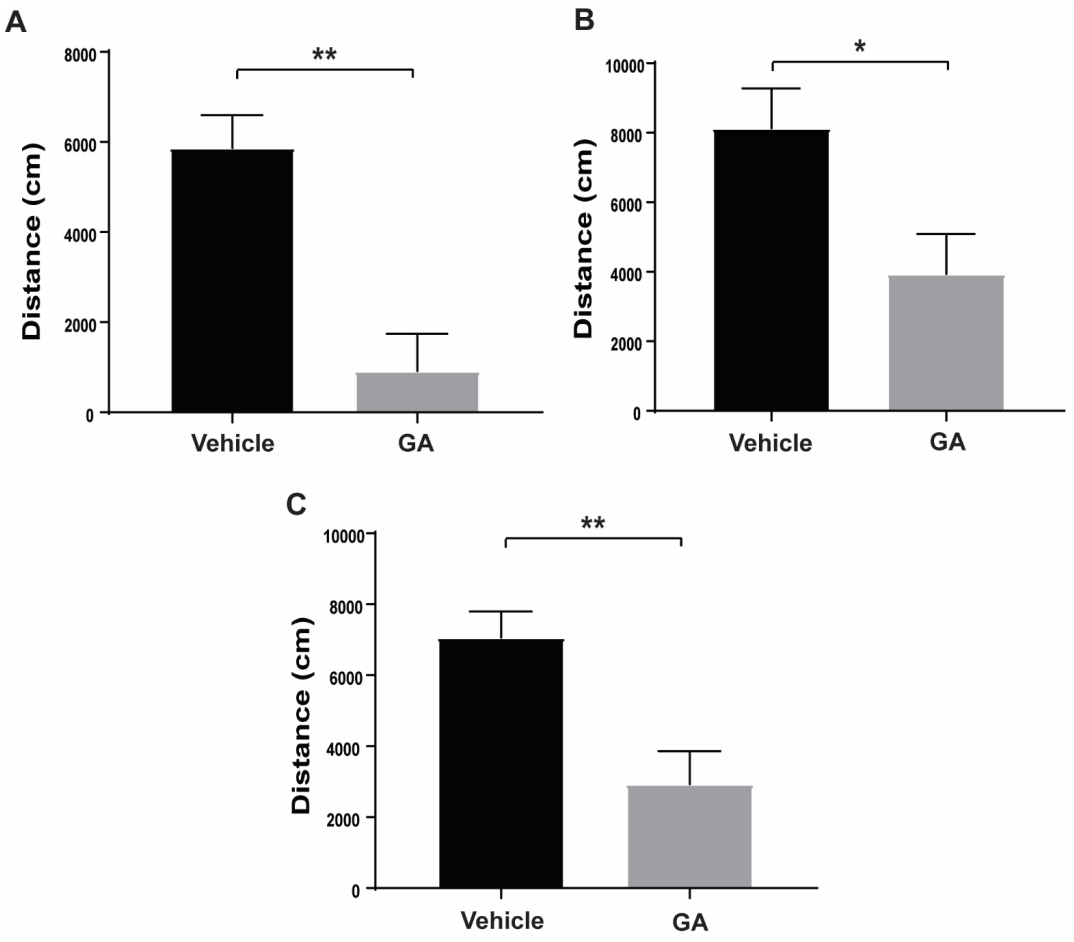

Figure 20 - Hyperactivity is Reduced in Both Chronically Infected Male and Female C57BL/6 Mice Following Guanabenz Treatment. After 3 weeks guanabenz (GA) or vehicle (Veh) treatment, locomotor activity was recorded in an unfamiliar 40cm x 4cm x 30cm Plexiglas box and the total distance traveled over 30 minutes was compared. Both (A) male and (B) female mice show an increase in cyst burden. Results held when both sexes were (C) combined. Error bars indicate standard error of the mean with p values determined by two-tailed Student's t-test, *p<0.05, **p<0.01. Male vehicle N=7, male GA N=3, female vehicle N=12, Female GA N=6

To confirm my findings, the experiment was repeated with the inclusion of mock-infected C57BL/6 mice (Figure 21A). The decrease in hyperactivity was again observed in guanabenz treated chronically infected mice, but there was no effect on activity level in the mock-infected mice receiving guanabenz (Figure 21B). This suggests my results

were reproducible with independent C57BL/6 mouse experiments and with a non-isogenic mouse strain since the data match the BALB/c experiments.

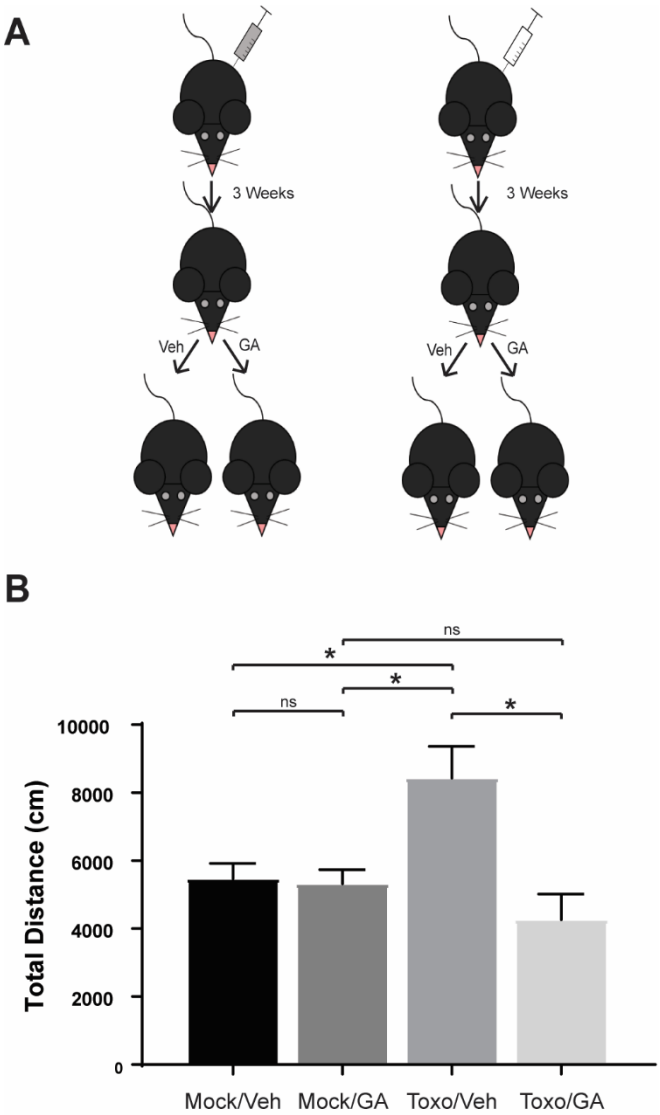

Figure 21 - Guanabenz Treatment of C57BL/6 Mice with Latent Toxoplasmosis Restores Returns Normal Activity Levels. (A) Female C57BL/6 mice were infected i.p. with 10^3 Pru tachyzoites (Toxo, grey syringe) or were mock infected with sterile PBS (Mock, white syringe). Following establishment of chronic infection at 21 days, groups were randomized to receive vehicle or guanabenz (GA 5mg/kg/day i.p.). (B) After 3 weeks guanabenz (GA) or vehicle (Veh) treatment, locomotor activity was recorded in an unfamiliar 40cm x 4cm x 30cm Plexiglas box and the total distance traveled over 30 minutes was compared. Error bars indicate standard error of the mean. one-way ANOVA p=0.0024 followed by an unpaired, two-tailed Student's t-test *p<0.05. Mock N=8, Toxo Veh N=7, Toxo GA N=3

Given the previous correlation between neuroinflammation and behavior established with the BALB/c mice, I examined the immune response in the C57BL/6 brains. Histological examination of the brains from latently infected C57BL/6 mice treated with vehicle showed the overwhelming level of inflammation that is characteristic of latent toxoplasmosis in this mouse strain, with pronounced perivascular cuffing and hypercellularity (Figure 22). As previously reported, this inflammation does not localize to tissue cysts, but remains concentrated around the vasculature and diffuse throughout the cortex.

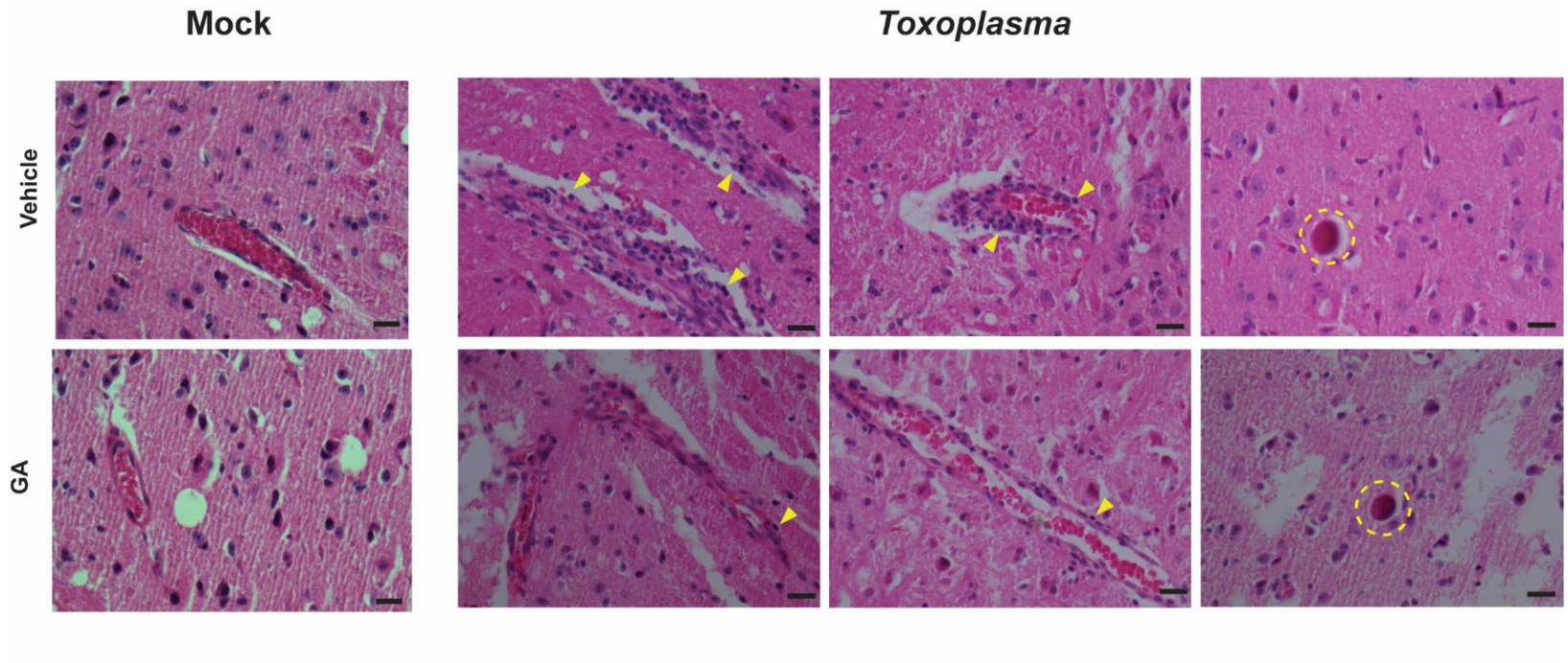

Figure 22 - Guanabenz Reduces Perivascular Cuffing in Latently Infected C57BL/6 Mice. Representative images of H&E stained brain sections from (left) mock-infected and (right) *Toxoplasma*-infected C57BL/6 mice treated with vehicle or guanabenz (GA) as indicated. Scale bar represents 20µm. Yellow arrowheads highlight the perivascular cuffing associated with chronic *Toxoplasma* infection, which is not as pronounced in GA-treated mice. The dashed yellow circle indicates a tissue cyst with minimal inflammation in both treatment groups.

I repeated the same IHC analysis with the C57BL/6 brains as I did with the BALB/c brains. CD45 staining shows extensive leukocyte presence within the *Toxoplasma* infected brains, which remains present, but with a lower frequency, in the guanabenz treated mice (Figure 23). When the brains were examined using IHC with anti-CD45 antibody, the increased inflammation following *Toxoplasma* infection was confirmed.

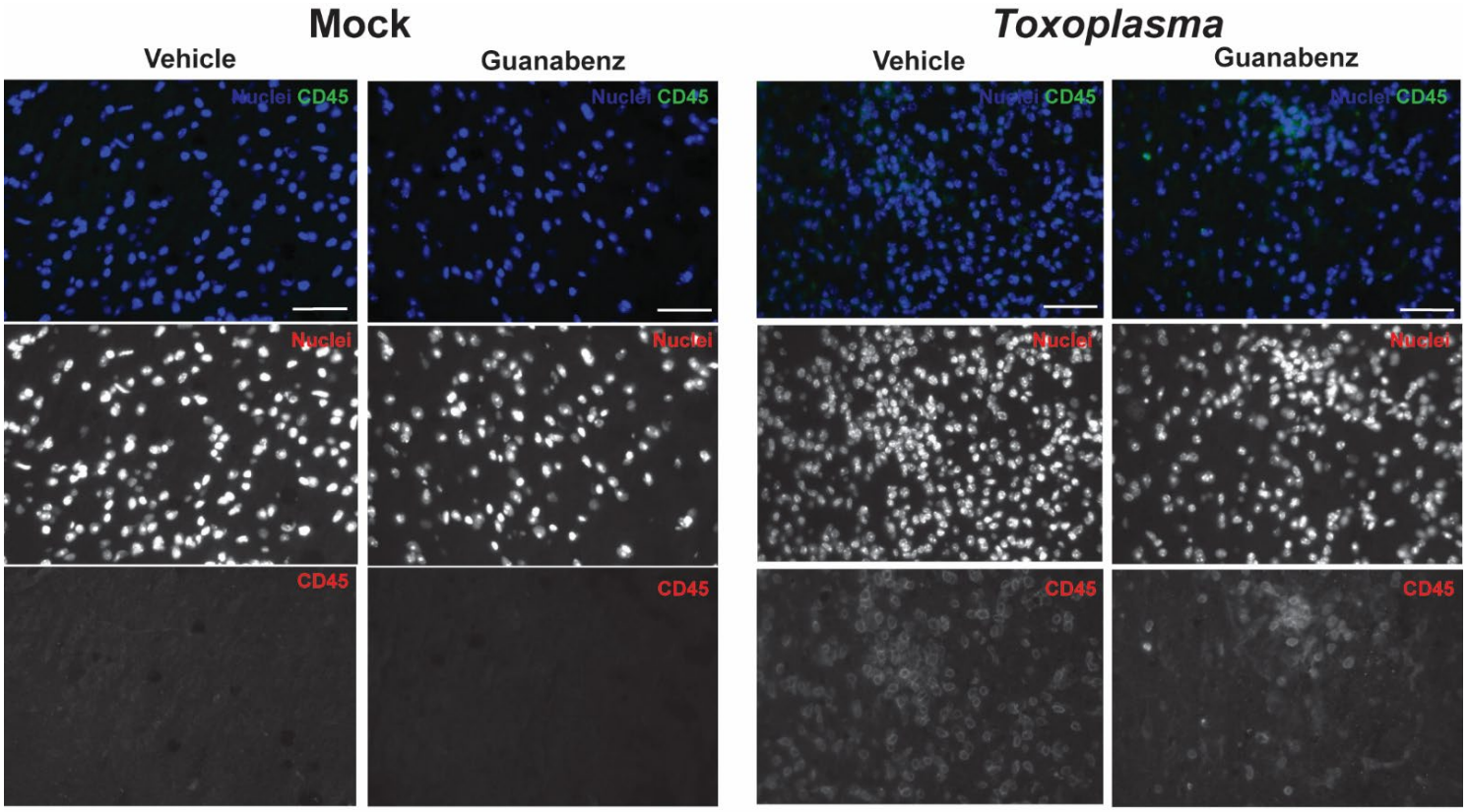

Figure 23- CD45+ Cells in *Toxoplasma* Infected and Mock Infected Brains, With and Without Guanabenz Treatment. Brain tissue sections were stained with anti-CD45 antibody (green). Hoechst was used to label nuclei (blue). Representative images for each group are shown. Scale bar = 50μm.

Blinded quantification of random fields of view showed a statistically lower number of CD45+ cells in the guanabenz-treated mice (Figure 24). While the decrease in CD45+ cells was not as drastic as with the BALB/c mice, only the survivors were able to be quantified and the mice that died could have altered the results. These findings bolster the idea that reversal of the hyperactive behavior depends more on controlling neuroinflammation than cyst burden.

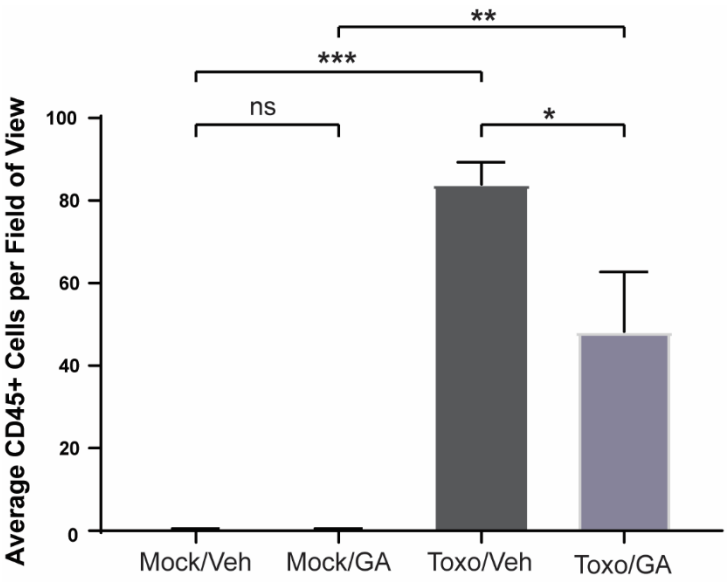

Figure 24 - Guanabenz Treatment Lowers the Average Number of CD45+ Cells per Field of View in *Toxoplasma* Infected C57BL/6 Mice. Five random fields of view were selected under Hoechst staining for each mouse. Images were then randomized and blinded before the number of CD45+ cells were counted. Average number of CD45+ cells per field for each mouse was calculated and visualized. Error bars indicate standard error of the mean. Scale bar = 50μm. One-way ANOVA $p<0.0001$ followed by an unpaired, two-tailed Student's t-test, ns $p>0.5$, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Mock N=8, Toxo Veh N=7, Toxo GA N=3

VII. Combination Therapy with Pyrimethamine Improves C57BL/6 Outcomes

The development of symptoms of *Toxoplasma* encephalitis following guanabenz treatment in previously healthy mice, combined with the expansion of the cyst burden, suggests that drug treatment in C57BL/6J mice causes reactivation of encysted parasites. In an attempt to prevent the death of guanabenz treated mice, I co-administered the tachyzoite-specific drug pyrimethamine (Figure 25A). Administration of guanabenz and pyrimethamine together prevented the death of mice in this group, while monotherapy again caused loss of half of guanabenz treatment group (Figure 25B). The effectiveness of pyrimethamine with guanabenz suggests that the detrimental effects of guanabenz treatment can be diminished with combination therapy.

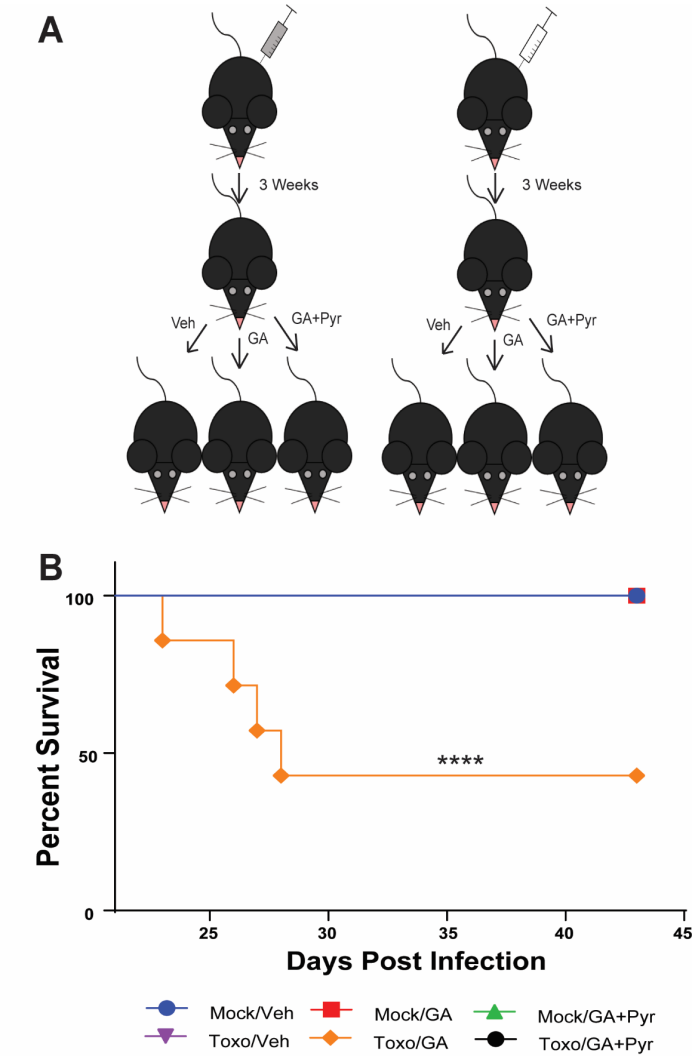

Figure 25 - Combination Therapy with Guanabenz and Pyrimethamine Prevents Death in Chronically Infected C57BL/6 Mice. (A) Female C57BL/6 mice were infected i.p. with 10^3 Pru tachyzoites (Toxo, grey syringe) or mock infected with an equal volume of sterile PBS (Mock, white syringe). Following establishment of chronic infection at 21 days, groups were randomized to receive vehicle, guanabenz (GA 5mg/kg/day i.p.) or guanabenz plus pyrimethamine (GA+Pyr, 5mg/kg/day and 20mg/kg/day i.p.). (B) Survival of mice in designated treatment groups. Single replicate, log rank test; ****p<0.0001. N=7

Following euthanasia at the conclusion of the study, brains were collected and cyst burden was blindly quantified (Figure 26). Interestingly, combination therapy with guanabenz and pyrimethamine restored the cyst lowering capabilities of guanabenz in

C57BL/6 mice, again supporting the possibility for an effective combination therapy in a susceptible mouse strain.

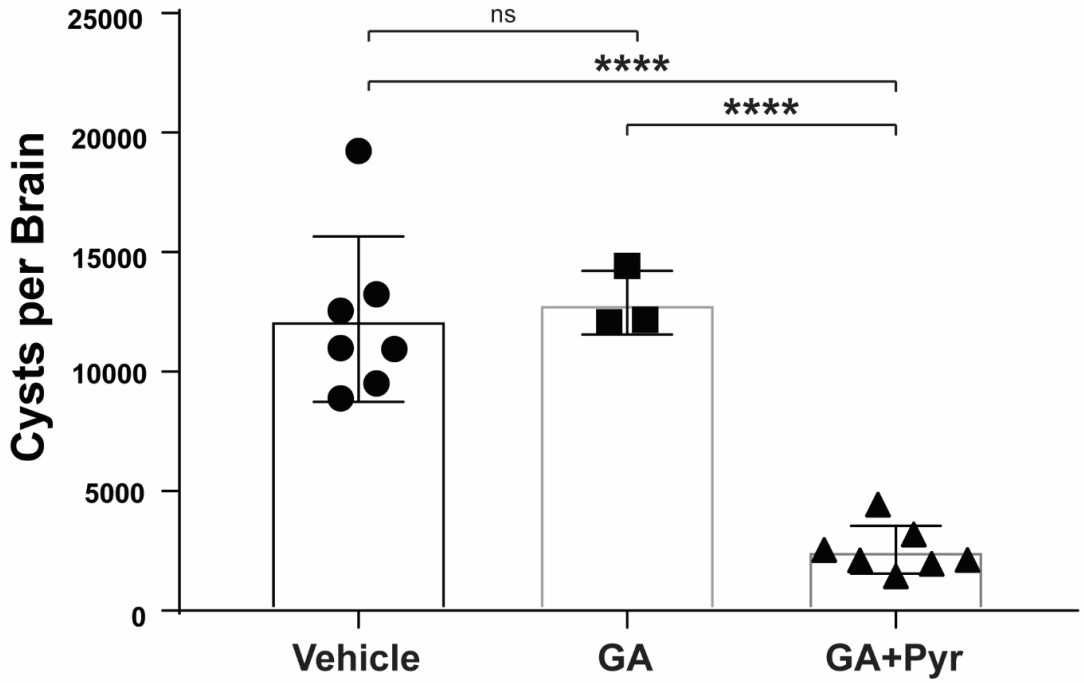

Figure 26 - Combination With Pyrimethamine Restores the Cyst Lowering Capability of Guanabenz in C57BL/6 Mice. Blinded brain cyst quantification was performed on the vehicle, guanabenz (GA) and guanabenz with pyrimethamine (GA+Pyr) treated C57BL/6 mice chronically infected with *Toxoplasma*. Single replicate with one-way ANOVA $p<0.0001$ followed by an unpaired, two-tailed Student's t-test, ns $p>0.5$, ****$p<0.0001$.

A concern I noted with the C57BL/6 hyperactivity reduction seen in figure 21 was that the reduction in hyperactivity could have been spurious due to loss of half of the drug treatment group. Since pyrimethamine and guanabenz restored survival of the mice, I was able to address this concern. Locomotor activity of all groups was analyzed and both the guanabenz alone and guanabenz with pyrimethamine groups showed restoration of activity levels to those seen in mock-infected mice (Figure 27). I thereby conclude that both guanabenz monotherapy and guanabenz + pyrimethamine combination therapy were

effective in restoring normal activity levels in mice with latent toxoplasmosis, regardless of cyst burden.

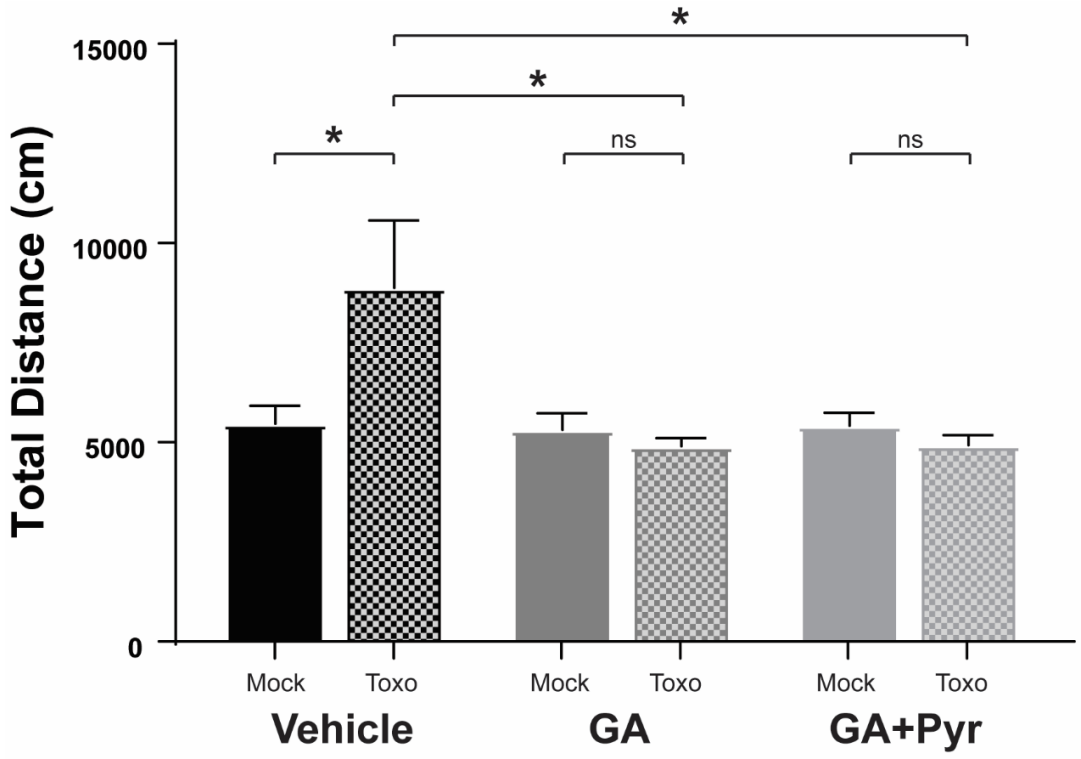

Figure 27 - Combination Therapy Restores Normal Behavior. Following three weeks of drug treatment, locomotor activity was recorded in an unfamiliar 40cm x 4cm x 30cm Plexiglas box and the total distance traveled over 30 minutes was compared. No mice were excluded. Error bars indicate standard error of the mean. one-way ANOVA $p=0.0044$ followed by an unpaired, two-tailed Student's t-test *$p<0.05$, ns $p>0.05$. All except Toxo GA N=7, Toxo GA N=3.

Chapter Four: Discussion

The focus of this thesis is the impact of drug treatment on the long term changes associated with chronic toxoplasmosis, which is an understudied disease. *Toxoplasma* induces behavioral changes in chronically infected mice and rats. These alterations are proposed as manipulation of the rodent as a means to facilitate capture and predation by *Toxoplasma's* definitive feline host. Changes include hyperactivity, cognitive impairment, and diminished fear of cat odors (Hutchison et al., 1980; Ihara et al., 2016; Vyas, Kim, Giacomini, et al., 2007).

A key strategy that many pathogens use to facilitate transmission involves behavioral modification of its host. For example, the rabies virus, which is spread through the saliva, makes its host more aggressive and likely to bite. A parasitic fungus (*Ophiocordyceps unilateralis*) infects ants and forces them to leave the forest floor to facilitate growth and spread of spores in the high humidity of the trees (Sandra B. Andersen et al., 2009). A bright green worm (*Leucochloridium paradoxum*) will infect the eye stalks of snails in order to mimic caterpillars and force the snail into exposed areas to attract birds (Wesołowska & Wesołowski, 2014). The evolutionary practice of a pathogen manipulating its host is not uncommon, but the mechanisms by which pathogens accomplish this vary and are not well understood in the case of *Toxoplasma.* The established rodent models of behavioral changes with *Toxoplasma* infection provides me with a model system for my studies while guanabenz offers a much needed tool used to interrogate the mechanisms behind parasite-induced changes in behavior.

Several hypotheses have emerged to explain how *Toxoplasma* might manipulate its host. The parasite may cause structural damage to the brain that affects neurobiological processes, but to date there is no consensus on the parasite's distribution within the brain (Daniels et al., 2015; Hermes et al., 2008; Vyas, Kim, Giacomini, et al., 2007). The release of parasite effector proteins may lead to altered levels of neurotransmitters in the host, but attempts to restore neurotransmitter levels through pharmacological intervention thus far have failed to reverse behavioral changes (David et al., 2016; Machado et al., 2016). Finally, behavioral changes could be an indirect consequence of the host immune response, such as neuroinflammation (Lang et al., 2018; Tyebji, Seizova, Hannan, et al., 2019). It is important to note that these hypotheses are not necessarily exclusive and likely exhibit some overlap. My original hypothesis for this project was that reducing the cyst burden in chronically infected mice would restore normal behavior. I endeavored to use pharmacological means to reduce cyst burden in chronically infected mice and evaluate their behavior. However, I instead show that restoration of normal behavior can be independent of cyst burden and that observed behavioral changes could be in part host driven through the host immune response.

I. Sephin1 has Anti-Parasitic Effects *in vitro* But Not *in vivo*

In the interest of improving the anti-parasitic effects of my system, I initially explored the possible use of alternative compounds to guanabenz, which our lab had previously shown to lower cyst burden. SAR studies have indicated the importance of the guanidine group in maintaining GADD34 inhibition while positioning of the chlorines within the aromatic ring is important for the alpha adrenergic agonist properties (Das et

al., 2015; Nguyen et al., 2014). Because guanabenz was originally designed as an anti-hypertensive drug, the alpha adrenergic agonist aspect of the treatment results in hypotension as the most common side effect for normotensive individuals given the drug. In fact, a clinical trial for the treatment of multiple sclerosis with guanabenz was terminated due to this side effect (NCT02423083). The goal of identifying a functional, similar structure of guanabenz without alpha adrenergic agonist properties was intended to eliminate one of the major side effects of guanabenz.

Sephin1 was one of the most promising of the compounds to examine due to its favorable results from the SAR study (Das et al., 2015) which showed no alpha adrenergic agonist activity. It has also been granted orphan drug status in Europe, was recently announced to have positive results for a Phase 1 clinical trial (NCT03610334), and is currently being proposed for a Phase 2 trial for Charcot Marie Tooth disease.

Our initial *in vitro* studies showed similar IC_{50} between guanabenz and Sephin1. Previous studies in mice with Sephin1 reported no signs of hypotension, as measured by lack of deficits on rotorod testing or locomotor activity (Das et al., 2015), when dosing Sephin1 by oral gavage. However, in an effort to better compare guanabenz to Sephin1, I chose to use the same route of administration (i.p. injection) for the acute treatment study.

Toxicity was immediately apparent when Sephin1 was given by i.p. injection. The mice developed worsening spastic paralysis with increasing doses, lost substantial amounts of weigh immediately, leading to several deaths among the group receiving the maximum dose, irrespective of infection status. The toxicity and weight loss, which likely outweighed potential anti-parasitic effects of the compound, likely led to the Sephin1 treated mice dying before the vehicle treated mice.

For evaluation of the efficacy of Sephin1 against latent toxoplasmosis, I chose to use oral gavage as my method of administration in an effort to prevent the toxicity observed with i.p. injection. I did not observe the toxicity previously noted for the Sephin1 i.p. treated mice and were able to complete a three-week drug treatment regimen. This is potentially due to different pharmacokinetics between the two dosing strategies and a typically slower absorption when compounds are consumed. Additionally, I observed no reduction in the number of cysts in Sephin1 treated mice at the designated dosages and chose not to purse continued studies with Sephin1.

There are several possibilities as to why Sephin1 did not reduce the brain cyst burden in my study. One possible explanation is that Sephin1 is not effective against bradyzoites. My *in vitro* data looked exclusively at tachyzoites and did not examine the response of bradyzoites to Sephin1. I could potentially address this by examining the morphology of Sephin1 treated cysts *in vitro*. Guanabenz treated tissue cysts show abnormal morphology and have empty space not taken up by parasites within the cyst wall (Benmerzouga et al., 2015). I could also use a reactivation assay to assess the viability of bradyzoites following drug treatment. In this experiment, differentiated bradyzoites are treated at various concentrations for several days and then washed and grown under normal growth conditions with standard medium. The monolayers are then allowed to form plaques as the parasites reactivate and transition into the fast replicative tachyzoites. Quantifying the number of plaques and plaque area provide evidence for the efficacy of the drug against bradyzoites.

Another possible explanation for the lack of efficacy against brain cyst burden could be the changes in pharmacokinetics when a drug is administered by oral gavage. I

showed that guanabenz is no longer capable of reducing cyst burden when it is given by oral gavage at 10mg/kg/day. I chose to dose Sephin1 by oral gavage for my chronic study due to the toxicity issues that I was facing when it was administered by i.p. injection. It could be that the slower absorption through the gut and/or first pass effects which could be contributing to a reduced efficacy. Drug quantification using chromatography from blood and brain samples taken from various time points following drug administration by oral gavage or by i.p. injection would allow me to evaluate the pharmacokinetic profiles and confirm this possible explanation.

However, my later experiments showed that the cyst burden did not associate with the reversal of hyperactivity caused by *Toxoplasma*. As a result, the inability of Sephin1 to lower cyst burden should not eliminate it from use against behavioral alterations. There is currently no information on possible anti-inflammatory effects of Sephin1. Further studies should examine Sephin1 in behavioral studies and chronic inflammatory characterizations.

II. Guanabenz Optimization

Our initial studies addressed whether guanabenz could be more effective at lowering cyst burden if administered through different routes or for a longer period. I also explored if guanabenz was effective in male mice, which had yet to be examined. I found no differences between the sexes in BALB/c mice and achieved a reproducibly lower brain cyst burden of 70-80% when using i.p. injection, which allowed me to focus exclusively on female mice. Additionally, prolonged drug dosing (6 weeks) had no effect on cyst burden but did provide me with a reproducibly lowered cyst burden that was

maintained for several weeks. The consistent reduction of cyst burden in guanabenz treated mice provided a novel model that could be used for behavioral testing and interrogation of my hypothesis.

A residual population of cysts remains present in the brains of treated mice, even with prolonged dosing. Interestingly, the residual population which I observed appears to be a common shortfall of the drugs that have shown efficacy against chronic toxoplasmosis. Similar results were reported with endochin-like quinolones, which inhibit the parasite cytochrome bc(1) complex, and an inhibitor of *Toxoplasma gondii* calcium-dependent protein kinase 1 (compound 32). It remains unclear why, in all cases, brain cyst counts could not be lowered beyond ~80%.

A possible explanation for the residual brain cyst population could be their location within the brain. There could be a specific region that these brain cysts reside in which makes them harder to reach with my treatments, such as areas with lower perfusion. My current method for cyst quantification looks at brain homogenate, so I lose the spatial component of the analysis. I could overcome this by subdividing the brains into different structures for quantification or by quantifying cyst location through tissue sections.

Another possible explanation could be that the residual cyst population has developed drug resistance and my treatment is no longer effective. I could test this by extracting parasites from drug treated brains and then quantifying their response to the treatment.

III. Guanabenz Reduces Hyperactivity in Chronically Infected Mice

We examined multiple reported behavioral changes in rodent models. These included anxiety, working memory, odor aversion, and hyperactivity, but chose to focus on hyperactivity as it was the most consistent and reproducible behavioral change observed in my hands. The other behaviors tested proved inconsistent between experiments, with untreated *Toxoplasma* infected mice only exhibiting statistically significant behavioral alteration in some of the experiments. Guanabenz treated mice were never statically different from the vehicle controls. I therefore chose to move forward with the hyperactivity as my metric for behavioral changes.

Three week treatment of chronically infected BALB/c mice with guanabenz consistently reversed *Toxoplasma*-induced hyperactivity without affecting the baseline activity of the mock infected control. This led me to believe that cyst burden correlated with locomotor changes, which appeared to support my hypothesis. Additionally, it provides me with the first evidence that an established behavioral change in a chronic *Toxoplasma* infection can be reversed.

IV. Hyperactivity Correlates with Neuroinflammation Instead of Cyst Burden

An identified flaw of my experimental set up for behavior testing with guanabenz was the impact of stress from repeat i.p. injections. Stress can influence behavior and I wanted to ensure that the restoration of normal activity levels was due to my drug treatment and not an artifact of stress (DeVallance et al., 2017). I devised two different methods to reduce stress from my experimental design, but both involved intragastric methods of drug administration, which alters the pharmacokinetics and necessitated proof

of *per oral* efficacy. I therefore added an oral gavage control group to account for any altered metabolism and absorption. However, oral gavage id stressful as well so it did not eliminate this stress as a variable in the behavior.

The first and simplest method for low stress dosing that I interrogated was the addition of guanabenz to the feed. This allowed the mice to freely consume the drug throughout the day with no additional handling or restraint. The drawback to this method is the lack of control on the timing and the amount of drug that each mouse received. Since the mice were group housed, there was no way to calculate the amount of drug each individual mouse was receiving and I based dosing on the average intake of the cage as a whole.

The second low stress method that I explored was the use of a dough treat, which had been previously devised by our collaborator for the use of self-administration of oral THC (Smoker, Mackie, Lapish, & Boehm, 2019). This route of administration also removes the stress of restraint and injection while providing a controlled dosing. Once acclimated to the treat, my mice would eat the entire un-medicated dough ball within 30 minutes. The dough treat allows for a tailored dose of drug for each individual mouse to be given in a bolus amount. Any residual dough after the 30 minutes can be quantified so that I can calculate exact doses for each mouse. The drawback of this particular method is that it requires the mouse to willingly eat the dough in the allotted time, which I found to be problematic.

The mice given guanabenz dough almost immediately stopped eating it and likely developed a conditioned taste aversion to the dough, suggesting that the side effects of the drug were too much to overcome despite the dough incentive. The fact that the mice

ate the dough in its entirety the first day of the experiment shows that it is not the taste of the drug that deters them from eating the dough. I further showed that the taste of guanabenz is not the problem when I tried un-medicated dough on the fourth day and the mice continued to reject the treat. Due to the inability to drug dose this group effectively, I chose to terminate this arm of the experiment early, but maintained the other treatment groups.

Analysis of the activity data shows that guanabenz treatment, regardless of the route of administration, was sufficient to eliminate hyperactivity and return normal locomotor activity. However, examination of the brains showed that oral routes had no impact on the cyst burden. This presented me with direct evidence that my hypothesis, which was that reducing cyst burden would return normal behavior, was incorrect. Consequently, I explored other possible explanations for the restoration of normal behavior with guanabenz treatment.

Guanabenz has wide-ranging anti-inflammatory properties, especially in the CNS. These anti-inflammatory attributes have been shown both *in vitro* and *in vivo* (Chang et al., 1987; Perego et al., 2018; Takigawa et al., 2016; Way et al., 2015). Guanabenz decreases cytokine signaling of stimulated immune cells, which I also observed using LPS stimulated J774.1 cells (Takigawa et al., 2016). While an argument can be made that GADD34 inhibition leads to a global decrease in cytokine production, additional work has shown a GADD34 independent anti-inflammatory pathway. An additional TLR inhibitory mechanism has been proposed, specifically the inhibition of TLR9 through the loss of signaling intermediates, with the potential to inhibit other TLRs' activities (Perego et al., 2018).

In vivo, guanabenz is effective at reducing the symptoms of the neuroinflammation using the experimental autoimmune encephalitis (EAE) mouse model (Way et al., 2015) as well as the pristine injection model, which is used to simulate lupus (Perego et al., 2018). Other models that have shown positive outcomes with guanabenz treatment include tuberous sclerosis (Jiang et al., 2016) and traumatic brain injury (Dash et al., 2015).

We therefore proposed an alternative possibility: the behavioral changes induced by latent toxoplasmosis could arise from chronic neuroinflammation. To test this idea in my system, I examined brain tissue harvested from guanabenz-treated or vehicle-treated mice chronically infected with *Toxoplasma*. I initially used H&E staining to assess the presence of inflammation and then used anti-CD45 to quantify the degree of immune presence into the brain.

Consistent with other reports (David et al., 2016; Hermes et al., 2008), the latently infected brains in my study continued to show inflammation despite the absence of acute toxoplasmosis symptoms. The perivascular cuffing associated with chronic *Toxoplasma* infection was clearly present in my infected, vehicle treated mice while the cuff was virtually nonexistent in the infected, guanabenz treated group, suggesting reduced neuroinflammation. Due to a lack of markers when using hematoxylin and eosin staining, it is difficult to distinguish immune cells from the rest of the cellular milieu of the brain; So I chose to use IHC to establish the presence of a leukocyte specific marker, CD45. I was able to establish that while there were abundant CD45+ cells in my vehicle controls, there quantity was significantly reduced in all of my drug treated groups, regardless of the route guanabenz was given. My data show that the return to baseline of *Toxoplasma*-

induced hyperactivity is associated with reduced neuroinflammation rather than brain cyst burden.

Neuroinflammation has been repeatedly suggested as a major player in global CNS dysfunction. The brain has long been considered an immune privileged site which maintains its own resident macrophage (microglia) to sustain homeostasis. Elevated levels of inflammation have been associated with behavioral changes in various rodent models and have even been linked with some of the neurological diseases including schizophrenia, for which there are consistent correlations in patients seropositive for *Toxoplasma* (Fond et al., 2018; Kannan et al., 2017). Schizophrenic patients given antipsychotics with anti-*Toxoplasma* activity show a decrease in symptoms along with a decrease in inflammatory markers (Fond et al., 2018; Fond et al., 2019).

The connection between a pathogen and long-term CNS dysfunction is not limited to *Toxoplasma* infection. Other pathogens, including herpes simplex virus, are proposed to be a factor in the development of neuropathological diseases, in particular Alzheimer's disease (Ashraf et al., 2018).

Our findings lend support to the idea that neuroinflammation plays a role in pathogen-induced change in the host. It has been proposed that extensive cytokine secretion and NF-κB activation induced by the parasite mediates neurobehavioral effects (T. Wang et al., 2019). An analysis of synaptosomal protein composition found that inflammation-related responses are upregulated during chronic toxoplasmosis in mice (Lang et al., 2018). Multiple transcriptomic analyses show immune-specific transcripts to be among the most highly upregulated in chronic infection (Hermes et al., 2008; Mahmoudvand et al., 2016; Pittman, Aliota, & Knoll, 2014). Immune infiltration in the

brain was also found to persist for weeks in mice who overcame infection with an attenuated strain of *Toxoplasma* that formed no detectable cysts *in vivo* (Ingram et al., 2013).

V. Mouse Strain Effects of Guanabenz Treatment

To further confirm my new findings, I chose to examine guanabenz treatment in a different mouse strain. All previous work was completed in the inbred BALB/c mouse model, which is typically used for long term drug studies due to its high resilience and stable cyst burden once chronic infection is established. The inbred C57BL/6 mouse model is predominantly used to answer immunologic questions. C57BL/6 mice are much more susceptible to infection. This susceptibility is characterized by an increased cyst burden and inflammatory response, and the mice will ultimately succumb to infection. The comparison between the two strains becomes an important confirmation of my new hypothesis because BALB/c and C57BL/6 exhibit important differences in their immune response to *Toxoplasma* infection. The enhanced pathology in C57BL/6 is due to differences mapped to the major histocompatibility complex (MHC) class II haplotype, which is found on antigen presenting cells (Suzuki, Joh, Orellana, Conley, & Remington, 1991). Given the more severe pathology, I reasoned that this system would be a more rigorous evaluation of my hypothesis. I proposed that the reversal of behavior and reduction in neuroinflammation would support my proposed mechanism of a host driven means of hyperactivity induction.

I was surprised to find that when C57BL/6 mice were treated with guanabenz, almost half of the drug-treated group died. The mice that did not survive displayed signs

of reactivated toxoplasmosis, including weight loss, paralysis, tremors, and seizures. With both male and female mice dying at the same rate, this lead me to conclude that sex differences do not account for the decreased survival. Due to the critical role of IFN- γ in control of infection, I suspected that the anti-inflammatory effects of guanabenz reduced the ability of the more susceptible host to control reactivated parasites. The fact that I observed vastly different effects of guanabenz on two different strains of mice highlights that *in vivo* studies of *Toxoplasma* should be performed in multiple non-isogenic strains of mice before firm conclusions can be drawn.

Due to the suspected reactivation events, it was unsurprising that the C57BL/6 mice which survived guanabenz treatment had a higher cyst burden than the vehicle control. This finding is in contrast to the previous evidence of the cyst lowering capabilities of guanabenz in BALB/c mice.

Interestingly, even with a higher cyst burden, guanabenz treated C57BL/6 mice had reduced hyperactivity. This held true even after correction for any locomotor deficits that could have skewed results, as identified by an individual blind to experimental design, and was consistent across multiple experiments.

VI. Combination Therapy Provides an Improved Treatment Regimen

Current standard of care for acute *Toxoplasma* infection is a combination therapy of pyrimethamine and sulfadiazine. Furthermore, second line treatments are also combination therapies. Therefore I hypothesized that I could overcome the detrimental effects of guanabenz treatment in C57BL/6 mice using a combination approach. I combined guanabenz with pyrimethamine as a possible means to prevent death from

reactivation. While pyrimethamine is not effective against chronic infections, it is the most potent approved first line clinical treatment for acute toxoplasmosis. Additionally, I was concerned that the loss of half of the guanabenz treated mice could be biasing the results if the most active mice were the ones dying. Combination therapy could help address the possible influence of reduced samples size on hyperactivity.

As I anticipated, the addition of pyrimethamine prevented guanabenz induced encephalitis. All of the combination therapy mice survived the entirety of treatment and the guanabenz monotherapy repeated my earlier outcome and half of the group died. I was therefore able to assess the full population for activity levels. I observed no difference between the activity levels of the guanabenz alone and the guanabenz with pyrimethamine groups, while neither of them were statistically different from the mock infected controls. Therefore, I conclude that the return of normal activity with guanabenz treatment is not due to the loss of mice from the guanabenz monotherapy group.

Surprisingly, I observed a restoration of the cyst lowering capability of guanabenz in C57BL/6 mice when it was combined with pyrimethamine. While surviving mice given guanabenz saw a slight increase in cyst burden, those given combination therapy saw a significant reduction in cyst burden, similar to what is seen in latently infected BALB/c mice treated with guanabenz. The ability of pyrimethamine to reduce cyst burden when combined with guanabenz in C57BL/6 mice suggests that the expansion of cyst burden in guanabenz monotherapy is due to the presence of tachyzoites. Pyrimethamine is likely able to target these tachyzoites and eliminate them before they can re-encyst, thereby preventing the expansion of cyst burden.

When combined with the prevention of death and lowering of cyst burden, guanabenz and pyrimethamine combination therapy provides a promising precedent for other potentially more efficacious combinations.

VII. Conclusions and Future Studies

This study provides the first evidence that *Toxoplasma* induced hyperactivity is reversible in a mouse model, and that it can be reversed in both sexes across two different mouse strains. Additionally, my work provides evidence that some of the behavioral manipulation induced by *Toxoplasma* during latent infection may be driven indirectly through the host, likely mediated by the immune response. Depending on the method of drug administration, I was able to decrease, increase or keep the cyst burden constant, yet consistently restored normal locomotor behavior. Thorough examination of the brains from guanabenz treated mice showed an association between CD45+ cells and behavior, thereby providing evidence that the host immune response could be the driving force for this behavioral alteration.

Interrogation of the immune response following guanabenz treatment could provide greater insight into *Toxoplasma*-induced behavioral changes. My analysis shows reduction in CD45+ cells, but identifying different cell populations could provide further understanding. For example, recent work (Carrillo et al., 2019) suggests the importance of reactive microglia in remodeling of perisomatic synapses and synaptic stripping in chronic infection. These processes have been implicated in the loss of inhibitory signals that could lead to overstimulation within the CNS and provide potential mechanisms for seizures and psychiatric problems.

Analysis using flow cytometry could provide an unbiased approach to identifying the differences in cell populations upon guanabenz treatment. While flow cytometry could help address the number and types of cells, it relies on single cell suspensions and cannot address the impact of location. I could then use IHC to localize these populations within my tissue sections to see if there is a special component that could be of importance.

There is also a temporal aspect to drug treatment. My treatments were started at three weeks post infection, which is considered the early chronic phase of infection. An argument could be made that the long-term effects of the prolonged immune presence within the brain have not yet become irreversible, and that I am treating the mice in a window of time that permits return of normal behavior. There could be differences in response to guanabenz if I treated the mice later in chronic infection, after the brains have been exposed to the immune response for an extended period of time. Considering that mice exposed to the parasite strain that does not encyst but still elicits an immune response weeks after infection still develop behavioral changes, I could argue that the timing and degree of the immune response play a role in behavior alteration. It would be interesting to see if I can still reverse hyperactivity using guanabenz weeks to months after infection occurs or if guanabenz can reverse the behavioral changes in the mice infected with the attenuated parasites.

Despite longer treatment times and various methods of administration, guanabenz was unable to lower cyst burden further or eliminate cysts altogether. Why these cysts remain is an outstanding question that could be related to location or a quality of the cysts themselves. Additionally, the conflicting results in the susceptible C57BL/6 mice showed

an increase in cyst burden with monotherapy, which was corrected with combination of guanabenz and pyrimethamine. While I showed a dissociation between cyst burden and behavior, cyst eradication remains a vital area for continued study with important clinical ramifications. Not only do tissue cysts give rise to chronic infection in humans, but they also cause life-threatening reactivation of acute infection in immunocompromised patients. These patients remain at risk as long as the tissue reservoir remains present.

Our experiment investigating combination therapy with guanabenz and pyrimethamine provides promising precedent for the exploration of novel combination therapies. I am interested in exploring multiple mechanisms of action and introducing other compounds that have shown efficacy against chronic infection, such as the endochin-like quinolones, with the goal of further cyst reduction and the potential to eliminate the tissue cyst reservoir within the host.

Latent *Toxoplasma* infection has also been linked to neuropsychiatric disorders such as schizophrenia, bipolar disorder, and suicide (Martinez, de Mendonca Lima, de Carvalho, & Menezes-Filho, 2018; Tyebji, Seizova, Hannan, et al., 2019). Aside from cyst reduction, my studies also suggest that certain neuropsychiatric disorders, if linked to latent toxoplasmosis or other pro-inflammatory states, might be controlled through anti-inflammatory therapies. Since *Toxoplasma* is statistically correlated with diverse neuropathological diseases, further clarification of the impact of guanabenz on *Toxoplasma* mediated neuroinflammation could provide a means to explore the association between chronic toxoplasmosis and CNS dysfunction in humans.

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Yolken, R., Torrey, E. F., & Dickerson, F. (2017). Evidence of increased exposure to Toxoplasma gondii in individuals with recent onset psychosis but not with established schizophrenia. *PLoS Negl Trop Dis, 11*(11), e0006040. doi:10.1371/journal.pntd.0006040

CURRICULUM VITAE

Jennifer Marie Martynowicz

Education

2022	Indiana University School of Medicine. Indianapolis, IN 46202. MD
2020	Indiana University. Indianapolis, IN 46202. PhD, Microbiology and Immunology
2014	University of Notre Dame. South Bend, IN 46556. BS, Chemistry and French

Positions and Appointments

2017-2020	Graduate Student Researcher, Sullivan Lab, Indiana University School of Medicine, Indianapolis, IN
2013	Undergraduate Research Intern, Eli Lilly & Co. Indianapolis, IN
2011-2014	Undergraduate Student Researcher, Miller Lab, University of Notre Dame, South Bend, IN
2011-2014	Undergraduate Tutor, University of Notre Dame, South Bend, IN

Honors and Awards

2019	Erica Daniel Kepner Award for Scientific Achievement
2019	Indiana University Representative to the MD/PhD National Student Conference in Copper Mountain CO
2019	Harold Raidt Graduate Student Teaching Award
2018	Indiana University School of Medicine Graduate Student Travel Award
2017-2020	Predoctoral Fellowship on the Immunology and Infectious Disease Training Program Departmental T32 grant
2016-2020	Joseph and Lucille Madri Scholarship
2014	Pi Delta Phi National French Honor Society
2012	Norbert L. and Linda W. Wiech Endowment for Excellence for Rare Disease Research in the Drug Discovery Core

Academic Service

2017-2018	Combined Degree Student Council Co-President, Indiana University School of Medicine, Indianapolis, IN
2015-present	Student Interest Group in Neurology Member, Indiana University School of Medicine, Indianapolis, IN
2014-present	Combined Degree Student Council Member, Indiana University School of Medicine, Indianapolis, IN

Peer- Review Publications

* Co-First Author

Martynowicz J, Augusto L, Wek RC, Boehm SL 2nd, Sullivan WJ Jr. Guanabenz Reverses a Key Behavioral Change Caused by Latent Toxoplasmosis in Mice by Reducing Neuroinflammation. *MBio*. 2019;10(2):e00381-19. Published 2019 Apr 30. doi:10.1128/mBio.00381-19

Augusto L, **Martynowicz J**, Staschke KA, Wek RC, Sullivan WJ Jr. Effects of PERK eIF2α Kinase Inhibitor against *Toxoplasma gondii*. *Antimicrob Agents Chemother*. 2018;62(11):e01442-18. Published 2018 Oct 24. doi:10.1128/AAC.01442-18

Harris MT*, Jeffers V*, **Martynowicz J**, True JD, Mosley AL, Sullivan WJ, Jr. A novel GCN5b lysine acetyltransferase complex associates with distinct transcription factors in the protozoan parasite *Toxoplasma gondii*. *Mol Biochem Parasitol.* 2019;232:111203.

In Preparation

Martynowicz J, Wek RC, Sullivan WJ Jr. The Impact of Guanabenz Combination Therapy on Brain Cyst Burden in Chronic *Toxoplasma gondii* Infection

Martynowicz J Wek RC, Sullivan WJ Jr. Discontinuation of Guanabenz Treatment Causes *Toxoplasma gondii* Cyst Burden Rebound in Mice

Augusto L, **Martynowicz J**, Wek RC, Sullivan WJ Jr. TGIF2K-B: A Unique Kinase in *Toxoplasma gondii*

Augusto, L.; Amin, P.H.; **Martynowicz, J**.; Alakhras, N.S.; Kaplan, M. H.; Wek, R.C.; Sullivan, W.J. Jr. UPR controls the cell migration during Toxoplasma gondii infection.

Scientific Conferences

* Presenter

2019 Molecular Parasitology Meeting. September. Woods Hole, MA "Guanabenz Reverses a Key Behavioral Change Caused by Latent Toxoplasmosis in Mice by Reducing Neuroinflammation" **Martynowicz J***, Augusto L, Wek RC, Boehm SL 2nd, Sullivan WJ Jr. *Poster*.

2019 MD/PhD National Student Conference. July. Copper Mountain, CO. "Guanabenz Reverses a Key Behavioral Change Caused by Latent Toxoplasmosis in Mice by Reducing Neuroinflammation" **Martynowicz J***, Augusto L, Wek RC, Boehm SL 2nd, Sullivan WJ Jr. *Selected speaker*.

2019 MD/PhD National Student Conference. July. Copper Mountain, CO. "Guanabenz Reverses a Key Behavioral Change Caused by Latent Toxoplasmosis in Mice by Reducing Neuroinflammation" **Martynowicz J***, Augusto L, Wek RC, Boehm SL 2nd, Sullivan WJ Jr. *Poster*.

2018 Biology of Intracellular Pathogens Retreat. June. Indianapolis, IN. “Targeting Translational Control as a Means to Reduce *Toxoplasma* Tissue Cyst Burden". **Martynowicz, Jennifer***; Wek, Ronald C; Sullivan Jr, William J. *Selected speaker*.

2018 Biology of Intracellular Pathogens Retreat. June. Indianapolis, IN. “A PERK eIF2 kinase inhibitor has activity against *Toxoplasma gondii*” Augusto, Leonardo*; **Martynowicz, Jennifer**; Wek, Ronald; Sullivan Jr, William J. *Poster*.

2018 Biology of Intracellular Pathogens Retreat. June. Indianapolis, IN. “The GCN5b Chromatin Remodeling Complex in *Toxoplasma gondii*” Harris, Michael T.*; **Martynowicz, Jennifer**; Sullivan Jr, William J. *Poster.*

2018 Gordon Research Seminar: Biology of Host-Parasite Interactions. June. Newport, RI. “Targeting Translational Control as a Means to Reduce *Toxoplasma* Tissue Cyst Burden". **Martynowicz, Jennifer***; Wek, Ronald C; Sullivan Jr, William J. *Selected speaker*.

2018 Gordon Research Conference: Biology of Host Parasite Interactions. June. Newport, RI. “Targeting Translational Control as a Means to Reduce *Toxoplasma* Tissue Cyst Burden". **Martynowicz, Jennifer***; Wek, Ronald C; Sullivan Jr, William J. *Poster*.

2018 Gordon Research Conference: Biology of Host Parasite Interactions. June, Newport, RI. “A PERK eIF2 kinase inhibitor has activity against *Toxoplasma gondii*” Augusto, Leonardo*; **Martynowicz, Jennifer**; Wek, Ronald; Sullivan Jr, William J. *Poster*.

2017 Molecular Parasitology Meeting. September. Woods Hole, MA “Targeting translational control as a means to reduce *Toxoplasma* tissue cyst burden” **Martynowicz, Jennifer***; Wek, Ronald; Sullivan Jr, William J. *Poster*.

2017 Molecular Parasitology Meeting. September. Woods Hole, MA “The GCN5b Chromatin Remodeling Complex in *Toxoplasma gondii*” Harris, Michael T.*; **Martynowicz, Jennifer**; Sullivan Jr, William J. *Poster*.

2017 Midwest Neglected Infectious Diseases Meeting. August. South Bend, IN. “The GCN5b Chromatin Remodeling Complex in *Toxoplasma gondii*” Harris, Michael T*.; **Martynowicz, Jennifer**; Sullivan Jr, William J. *Poster*.

2017 Biology of Intracellular Pathogens Retreat. June. Indianapolis, IN. “The GCN5b Chromatin Remodeling Complex in *Toxoplasma gondii*” Harris, Michael T.*; **Martynowicz, Jennifer**; Sullivan Jr, William J. *Poster*.

Teaching Experience

2019	Harold Raidt Graduate Student Teaching Award
2019	Jumpstart to Teaching Certification Program, Indiana University School of Medicine Graduate Division, May 13th-17th
2019	Teaching Assistant. Indiana University Purdue University, Indianapolis, IN. Spring Semester Nursing Microbiology and Immunology Laboratory (J210)
2011-2014	Chemistry and French Undergraduate Tutor. University of Notre Dame College of First Year of Studies, South Bend, IN