Toxoplasma infection and dopamine signaling-dependent behaviors

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Abstract

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Toxoplasma gondii is an apicomplexan obligate intracellular parasite, capable of infecting a wide range of hosts and cell types. Human infection with the parasite involves an acute replicative phase followed by long term chronic neurotropic infection that may persist for decades and is generally observed as asymptomatic. Clinical interest in the parasite and the accompanying disease of toxoplasmosis, generally centers around infection of immune naïve or immune compromised patients, such as in utero infections and late stage AIDS cases. However, comprehensive examinations of patient serology suggest that antibodies to T. gondii are significantly correlated with some psychiatric disorders. Hampering the study of the putative link between infection and psychiatric disease, animal models of T. gondii infection have suffered from inconsistent behavioral phenotypes. Robust and reproducible behavioral phenotypes consistently altered during parasitic infection have been largely lacking in the field.

The thesis presented here centers around my work to characterize a consistent and significant behavioral change associated with T. gondii infection in mice. We have pioneered a stimulant induced activity assay wherein infected mice show significantly blunted response to cocaine or amphetamine administration, two stimulants that produce hyperactivity by increasing the release of dopamine from pre-synaptic
terminals. We have established that blunted response to stimulants are independent of mouse sex or strain, the duration of infection or the strain of the parasite. The failure of these drugs to induce hyperactive in infected mice led us to hypothesize that infection affects host dopamine signaling.

Consistent with this hypothesis, we have found that chronic *T. gondii* infection is associated with a significant decrease in expression of vesicular monoamine transporter and dopamine transporter, host factors that mediate the behavioral and synaptic effects of psychostimulants. These molecular changes are not associated with gross abnormalities in the brain morphology, cortical layering or overall architectonics. In addition, we found no synaptic damage, and no significant damage to brain morphology through inflammation or parasitic proliferation.

Along with the characterization of the behavioral effects described in this thesis, I also work to answer a persistent question about the role of the parasite in dopamine signaling which has existed in the field of *T. gondii* biology and host behavior. This question stems from the observation that the *T. gondii* parasite genome contains two genetic homologues of tyrosine hydroxylase (TH), the rate limiting enzyme in the production of dopamine in mammalian brains. Previous studies have shown that TH expression in the host brain is increased in and around the encysted parasites, along with levels of extra-cellular dopamine. Considerable speculation persists in the field about whether the Toxoplasma TH that is upregulated during brain specific infection is responsible for a direct impact of infection on host brain function, leading to behavioral change. In order to address this question, a collaborating lab provided me with parasites
deleted for the TH homolog gene. Applying these mutant parasites to my model of behavioral change, I was able to conclusively show that the impact of infection on reaction to dopaminergic drugs is wholly independent of Toxoplasma TH expression. Additionally, TH expression by the host, not the parasite, is responsible for any measurable changes in \textit{in vivo} dopamine release in the striatum.

Together, the thesis work represents a thorough analysis of the behavioral impacts that \textit{T. gondii} infection has on host response to dopaminergic drugs, and then the role that pre-synaptic dopamine signaling has in creating those impacts. The results provide a strong indication that host brain functions, particularly dopamine signaling, is significantly impacted by infection. Those impacts appear to be linked to expression of the traditionally defined markers of presynaptic dopamine release, reuptake and vesicular transport. These results highlight how the parasite may directly or indirectly change host brain function, resulting in perturbation of human biology toward a psychiatric disease state.
ii. Acknowledgments

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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine/Serotonin</td>
</tr>
<tr>
<td>Actin</td>
<td>beta-actin cytoskeletal protein</td>
</tr>
<tr>
<td>AMPH</td>
<td>D-amphetamine</td>
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<tr>
<td>Ctx</td>
<td>cortex</td>
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<tr>
<td>C4</td>
<td>Complement component 4</td>
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<td>D2</td>
<td>dopamine receptor 2</td>
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<td>DAT</td>
<td>dopamine transporter</td>
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<td>dpi</td>
<td>days post infection</td>
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<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>glutamate</td>
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<tr>
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<td>ionized calcium-binding adapter molecule 1</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate, neurotransmitter</td>
</tr>
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<td>Pru</td>
<td>Prugniaud strain <em>Toxoplasma</em></td>
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<td>PV</td>
<td>parasitophorous vacuole</td>
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<tr>
<td>RH</td>
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<td>SNAP-25</td>
<td>synaptosomal-associated protein 25</td>
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<td>TgTHAAH1</td>
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</tr>
<tr>
<td>TgTHAAH2</td>
<td><em>Toxoplasma gondii</em> tyrosine hydroxylase gene 2</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>VMAT</td>
<td>vesicular monamine transporter</td>
</tr>
<tr>
<td>VMAT2</td>
<td>vesicular monamine transporter 2</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
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<tr>
<td>wpi</td>
<td>weeks post infection</td>
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1. Introduction

1.1. *Toxoplasma gondii*

*Toxoplasma gondii* is a eukaryotic protozoan pathogen. It is a cosmopolitan microorganism, capable of infecting a wide range of hosts, and is found across the planet in the environment and among humans and our domestic animals. Current estimates place the percentage of human beings infected with *T. gondii* at between 10-50%, depending on location, making it one of the most prolific human pathogens worldwide (CDC-Biology). *T. gondii* belongs to the protozoal phylum of Apicomplexa, a group characterized by the presence of the apicoplast organelle. This organelle is believed to be a relict algal body, internalized during early evolution and retained within the cellular structure of these species (Maréchal and Cesbron-Delauw 2001). Many of the members of the phylum are parasitic and like other Apicomplexan parasites, *T. gondii* is an obligate intracellular pathogen, requiring host cells to survive and replicate. The closely related apicomplexan *Plasmodium spp.*, responsible for malarial disease, typically exhibit the ability to infect and replicated in only one or two vectoring mosquito species and only one mammalian host. In contrast, the *T. gondii* parasite is capable of invading and living inside any nucleated mammalian or avian cell, making it one of the most prolific and unspecific intracellular pathogens known. This large dispersion of available hosts means that the pathogen can persist in a wide variety of environments, and remain in reservoirs outside the human population with little difficulty (CDC-Biology). However, though the *T. gondii* parasite is widely spread and capable of prolific infection in many host species, like its relatives, it is also confined in a very particular life cycle.
1.2. Parasite life cycle

*T. gondii* has a complex life cycle with a definitive host and many possible intermediate hosts. Humans, mammals or other warm-blooded animals function as the intermediate species in the life cycle. The definitive host, however, is any of a wide variety of feline species. Typically moving between infected rodents and their feline predators (Fig. 1), the cats contract the parasite by consuming infected intermediate hosts. Once the parasite is inside the intestinal tract of the feline, it can undergo a form of sexual replication: mating with another parasite to form genetically distinct offspring. These offspring are then released from the intestinal tract of the feline into the environment in the form of an oocyst. This structure is a hard walled and environmentally resistant capsule containing infectious *T. gondii* (CDC-Biology).

Once passed out into the environment, the parasite is then consumed by an intermediate host. This consumption can come from direct contact with cat feces, or through consumption of contaminated water or plant material grown on contaminated soil. Then, inside an intermediate host, the parasite begins growing rapidly and invading a variety of cells. Invasion occurs though the binding of vertebrate cellular surface factors, and adhesion of the apical end of the parasite using molecules secreted from organelles called micronemes inside the parasite (Carruthers 2002). Pushing inside the host cell, *T. gondii* then exploits host cellular function via recruitment of host organelles to avoid cellular clearance, surrounding itself in a structure referred to as the parasitophorous vacuole (PV) (Coppens 2017). This invasion occurs when the *T. gondii* in
this rapid proliferative phase is referred to as tachyzoites, from the Greek meaning rapid organisms. In cases where host immune response is compromised, the parasite can overwhelm almost a mammalian host in a matter of days. However, if the intermediate host has an adequate adaptive immune response following infection, the parasite is triggered to enter a quiescent form, called the bradyzoite, from the Greek meaning slow organisms. In this chronic phase the parasite remains dormant inside the cells of its intermediate host (Dubey, Lindsay, and Speer 1998). Over time, host response to the slowly replicating parasites induces the accumulation of cyto-skeletal proteins, golgi, and endoplasmic reticulum to the PV. This accumulation forms a cyst structure that, as it grows, can eventually far exceed the original size of the host cell that had previously contained that PV (Paredes-Santos et al. 2017).

The infection continues to elicit a low level inflammatory response from the host but significantly diminished compared to the proliferative phase. Eventually the slower immune response removes most of the encysted bradyzoites from all cell types outside of immune privileged areas. Typically leaving behind parasites in lectin encased cyst structures in those privileged areas, though parasites may remain in muscular, ocular and other tissues as well. For this reason, the latent disease form of T. gondii infection is typically referred to as ‘neurotropic’ since it is most often observed in the CNS (Dubey, Lindsay, and Speer 1998). The predicted life cycle of the parasite involves this back and forth exchange of feline derived oocysts into the environment where prey species encounter them, become infected, retain a chronic infection, and are then are consumed by cats where the parasitereactivates and the cycle starts again.
Figure 1. *Toxoplasma* life cycle

This image, taken from the Centers for Disease Control summary of *Toxoplasma* biology (CDC-Biology), show a broad overview of the *T. gondii* life cycle. Moving between rodent intermediate hosts, and feline definitive hosts in the wild, the parasite can enter the human population through a variety of means, and potentially cause disease in humans, much the same way as in other intermediate host species.
Figure 2. *Toxoplasma cyst.*
A rat brain sample, taken at 14 months post infection, shows the unique *T. gondii* cyst structure. Surrounded by a thin outer layer of lectin (Indicated), the large number of bradyzoite parasites are encysted in the tissue of the CNS, remaining quiescent and largely isolated from immune response (Dubey, Lindsay, and Speer 1998).

1.3. **Human infection**

Humans enter into the *T. gondii* life cycle the same way as any other intermediate host. We can be infected either from contact with cat litter containing shed oocysts, or through the consumption of raw or undercooked animal tissue containing the bradyzoite cysts from another infected intermediate host (CDC). Once inside the human body, *T. gondii* replicates the same way it does in rodent intermediate
hosts, rapidly expanding and invading, encysting and remaining dormant to escape immune discovery (Fig. 2). As in rodents, human infection is typically characterized by a long term chronic infection that is largely asymptomatic. This similarity in infection profiles between rodent and human hosts is a primary advantage allowing the use of rodents as an adequate animal model system. Strains of parasite can be taken from human tissue and will readily infect mice without any adaptation, and that infectious profile will be largely similar to the human form (Szabo and Finney 2017). Particularly, the formation and distribution of the encysted parasite in the CNS, and the host immune response to it are highly conserved between mammal hosts of *T. gondii*.

In describing *Toxoplasma* strains, both in research and wild-type infectious settings, there are three broadly described strains, and several further subdivisions within those strain types. Generally, type i strains are identified as highly virulent and considered fatal to most research mouse strains (Reikvan and Lorentzen-Styr 1976). Type ii is described as the original strain family isolated from European patients (Fuentes et al. 2001) and is known to reliably produce a survivable latent infection in animal models, most notably with the Prugniaud and ME49 strains (Zenner et al. 1999). Type iii parasites can also be found in human infections and are less commonly applied to experimental systems (Jokelainen, Murat, and Nielsen 2018)

1.4. *Toxoplasma* effects on host brain function and behavior

Among infectious agents, *T. gondii* has a particularly dangerous potential as an environmental risk factor for human psychiatric disease. The parasite lifecycle results in a long lasting neurotropic infection where the parasite is encysted in the host CNS
without immune clearance, while simultaneously precipitating constant immune activation. Numerous studies have associated serologic response to *T. gondii* with the increased risk for the development of psychiatric disease (Vlatkovic et al. 2017; Yolken, Torrey, and Dickerson 2017; Yolken, Dickerson, and Torrey 2009) (Fig. 3), although negative findings have been reported as well (Sugden et al. 2016).

**Figure 3. Visual plot of the association between infection and psychosis.**

In this plot, taken from Torrey EF, et al. 2012, we can see a combined view of the odds ratio relationship between seropositivity to *T. gondii* and risk of developing schizophrenia. Each point and corresponding bar show the respective average and standard deviation for an individual study comparing *Toxoplasma* seropositivity and the development of schizophrenia in a given population. Cumulatively, across 38 studies of serology in psychiatric study cohorts worldwide, there is an approximate 2.8 fold increase in psychosis diagnosis among those that have been exposed to *T. gondii*, over those that have not.
In attempting to explore the link between infection and brain function, research has focused on establishing an animal model of infection and characterizing neurobehavioral consequences of chronic infection. Studies from the Pletnikov lab have, for example, demonstrated host sex-, time- and parasite strain-dependent effects of infection on pre-pulse inhibition (PPI) of the acoustic startle response, novelty-induced activity and learning and memory (Geetha Kannan et al. 2010). Other groups have shown that infection alters fear perception (Gonzalez et al. 2007) and affects host perception of predator urine scent (A Vyas, Kim, and Sapolsky 2007; Ajai Vyas et al. 2007; Lamberton, Donnelly, and Webster 2008). Curiously, some have suggested that infection could increase ethanol consumption in rodents (Samojłowicz, Borowska-Solonyko, and Kruczyk 2017).

The interest in *T. gondii* as an environmental risk factor in human psychiatric disease has existed for some time (Torrey and Yolken 2003). The development and analysis of animal models of infection to probe the links between infection and brain function has been ongoing, and significant findings have come from these model systems. Particularly, associative studies have indicated that infection may impact the way in which the rodent brain interprets signals of fear and olfactory recognition, causing the host animal to behave ‘recklessly’ and come into contact with predatory felines more often (Ingram et al. 2013). From these results, there has even come the suggestion that infection with *T. gondii* may induce rodent hosts to further the parasitic lifecycle by improving the chances that the host is consumed by a cat, the definitive parasitic host (Ajai Vyas et al. 2007). These studies and associative observations have
furthered interest in establishing the mechanistic links between infection and behavioral change. However, dependence of the reported behavioral effects on mouse strain, strain of parasite and/or the minute details of behavioral protocol have raised serious skepticism among investigators in translational significance of the pre-clinical studies.

In experimentally approaching behavioral change, one of the most substantial impediments is the fact that different laboratories have published different behavioral results following infection. This has resulted in a persistent lack of consensus about what the actual behavioral impacts of infection are. Groups have published on diminished aversion to cat urine (Ajai Vyas et al. 2007), and reduced anxiety in infected mice (Golcu, Gebre, and Sapolsky 2014). Other attempts to recreate the same experiments in other laboratory conditions have yielded no changes related to infection (Machado et al. 2016). In my own lab, work demonstrated that infection results in host deficits in pre-pulse inhibition of acoustic startle (Geetha Kannan et al. 2010) but that this effect is entirely dependent on when during development the animals are infected. These inconsistencies have prompted a continuing search for a robust behavioral assay that would serve as a robust and reproducible measure of infection for mechanistic studies and cysts elimination drug discovery efforts. The need for a strong, robust, and repeatable behavioral phenotype of chronic T. gondii infection is a critical need in the field.

1.5. Direct vs indirect mechanisms of impact

In trying to determine the impacts of infection on animal behavior, the theoretical mechanistic links come from two broad categories. They are direct and
indirect means of parasitic influence. Indirect forms encapsulate all impacts the parasite has on inflammation and other host reactions to infection which can influence brain function and behavior. Direct forms of impact are those where the parasite itself is secreting or expressing one or more factors which are directly influencing brain function and behavior in the host. In this thesis work, I detail experimental work to determine the role of putative mechanisms in both groups.

1.5.1. Indirect: Inflammation

The most prominent putative indirect impact of infection on behavior is the immune response to the parasite. Immune response to the *T. gondii* parasite involves many of the same factors common to a wide variety of intracellular pathogens, such as viruses and bacteria. Unlike many of these infectious agents, however, the ability of the parasite to form long lasting immune refractory cyst structures allows for a chronic infectious stage. A significant body of work has gone into describing and characterizing the innate and adaptive immune responses to both the proliferative acute *T. gondii* infection as well as to the latent encysted form of the parasite (Munoz, Liesenfeld, and Heimesaat 2011; Blanchard, Dunay, and Schlüter 2015). The work detailed in this thesis is based in large part on the relationship between infection and immune response in the host brain, focusing on observations in chronic infection models.

1.5.1.1. Innate

During the rapidly replicating tachyzoite phase, host animals show significant increases in classical markers of innate inflammatory response. The toll-like receptors(TLR)-2 and 4, which are found on host cellular membranes, are known to be
activated by parasitic glycosylphosphatidylinositol (GPI) anchored proteins present on the parasite surface (Debierre-Grockiego et al. 2007). The activation of these TLRs leads to downstream inflammatory response including increases in Tumor Necrosis Factor (TNF) and interleukin-12 (IL-12), factors which promote inflammation and the response of natural killer cells (Fig. 5) (Takeuchi et al. 2002). Membrane bound receptors such as the above mentioned TLRs are activated in the presence of extracellular *T. gondii*, found during initial infection and replication. Another critical TLR response to *T. gondii* occurs through the activation of TLR-11, which is found on endosomes of macrophages and other innate immune cells (Pifer et al. 2011). Specifically, TLR-11 responds to the binding of parasitic profilin, a key protein that assists in invasion by the parasite. The activation of this receptor occurs after intracellular invasion or uptake of the parasite by innate immune cells. Figure 4 shows a diagram taken from the Kirk Jensen laboratory, showing the immune activation pathways associated with *T. gondii* infection. In this representation, activation of TLRs leads to interferon gamma (IFN-γ) production through NK cell proliferation, and activation of adaptive CD-4 and CD-8 T cell populations.

Relating specifically to neurological immunity, the innate response to *T. gondii* involves cytokine induced activation of brain resident macrophage (microglia) in the host, intended to control infection within the CNS (Haroon et al. 2012). Murine models of *T. gondii* infection indicate that direct infection of neurons and microglia by the parasite is commonly observed, but no evidence of astroglial infection has been seen (Melzer et al. 2010). This profile indicates an innate response common to a wide variety
of intracellular pathogens. Parasitic infiltration induces an inflammatory response through monocytes, dendritic cells (DCs), and others. The cytokine release associated with this activation crosses the blood brain barrier (BBB) at around the same time the parasite itself transits, resulting in infection and inflammation in the CNS. As an IFN-γ response takes place, driving host control of parasite replication and pushing the organism from the proliferative phase into the dormant bradyzoite form, the innate response changes but key components remain present during the subsequent chronic infection. Particularly, chronic T. gondii infection is characterized by chronic activation of microglia in both athymic mice lacking innate immunity and rodents with fully competent immune response (Schlüter et al. 1995). From a clinical perspective, chronic T. gondii infection is generally regarded as asymptomatic, but the presence of chronically activated innate immune cells is a clinically relevant observation.

Humoral immune response in the CNS is a significant innate immune factor associated with T. gondii infection that has been explored recently in our laboratory. Findings by JC Xiao, et al. (2016) show that the complement protein C1q is upregulated in cerebral tissue from chronically infected mice. The complement system is a highly conserved innate immune mechanism for targeting not only invasive organisms, but clearing cellular debris and extraneous cellular components. Upregulation in the CNS is associated with a variety of disease states, including encephalitis (Shu et al. 2018).

1.5.1.2. Adaptive

The adaptive response to T. gondii infection begins with a T-cell response, induced by cytokine release from innate DCs and macrophage, leading to CD-4+ T-cell activation
and subsequent CD-8+ response to infected cells (Lütjen et al. 2006). The importance of a CD-4+ response in host immunity to *T. gondii* is most apparent when depletion of these cells occurs following HIV infection. Early analysis during the AIDS epidemic during the 1980’s indicated that activation of latent *T. gondii* parasites occurred concurrently in patients with falling CD-4+ T-cell populations (Luft et al. 1984). Removing CD-4+ T-cells causes an inability by the host to maintain long term IFN-γ upregulation, which in turn causes reactivation of the parasites and a return to proliferative tachyzoite growth. A healthy adaptive immune response, involving both cytotoxic T-cell activation against the parasite, as well as antibody generation, is required for proper control of parasitic proliferation and the maintenance of a chronic and asymptomatic infection (Suzuki et al. 2011).

In addition to the on-target effects of the host immune response to *T. gondii*, there are a variety of potentially detrimental adaptive immune responses. Our lab previously explored anti-self-antibody generation associated with infection, and found evidence that *T. gondii* infection induced considerable responses against subunits of the N-methyl-d-aspartate receptor (NMDAr) (Geetha Kannan et al. 2016). Those results suggest that parasitic infection in the brain causes significant inflammatory response, inducing the activation of B-cell responses to portions of host cellular debris that may be present in context with the parasite.
Figure 4. Immune response to Toxoplasma.

In this figure, adapted from Kirk Jensen’s work (Jensen n.d.), we see an overview of the host response to the parasite. Activation of innate cells, most notable dendritic cells, induces an IL-12 response, cascading into the production of IFNγ by a variety of cell types. Eventually, T and B cell activation induces an antibody response and killing via activated CD-8+ T-cells. As discussed in the introduction, the cytotoxic activity of that response is limited against cells of the CNS, due to their immune privileged state.

1.5.2. Direct: Dopamine and Toxoplasma

Beyond the immunologic effects of *T. gondii* infection on brain and behavior, there is also interest in understanding the role of *T. gondii* in directly impacting dopamine (DA) synaptic transmission. This interest stems from the so-called dopamine hypothesis of psychoses, a theoretical link between changes in striatal and cortical dopamine levels and the precipitation of psychosis (Howes et al. 2017). The link between the action of the effective typical antipsychotic drugs against dopamine receptor 2 (DR2) and resolution of schizophrenia in some patients has been the long-
standing keystone argument linking increases in DA signaling to psychosis (Seeman et al. 1975). Connecting this theory to Toxoplasma infection, a software based analysis of the T. gondii genome uncovered two genetic elements, TgthAah1 (Toxoplasma gondii aromatic amino acid hydrolase 1) and TgthAah2, which are homologues of mammalian tyrosine hydroxylase (TH), the rate limiting enzyme in DA production (Gaskell et al. 2009). Expression of one or both enzymes by the parasite has been shown to be capable of catabolizing tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine), the precursor to the active signaling monoamine, resulting in increased DA expression in and around the T. gondii cyst in vivo (Prandovszky et al. 2011) (Figure 5). For the purposes of examining the impacts of T. gondii derived TH and DA on behavior, the genetic element of most interest is TgthAah2. This genetic form is upregulated during the bradyzoite phase of latent infection, meaning that its impacts are imparted only during chronic infection when the parasite is in brain tissue. Determining what role this genetic element has on host behavior, through increased synthesis of DA in infected brains, has been a primary goal in the T. gondii research community for some time. Recent advances in CRISPR based genetic manipulation, and the help of my collaborators in Dr. David Sibley’s lab at Washington University in St. Louis, have provided me with a strain of T. gondii that is deficient in the TgthAah2 element, allowing for direct analysis of what behavioral impacts this gene is responsible for.
Figure 5. DA response in and around the cyst
These images, adapted from Prandovszky, et al. 2011, show evidence of *T. gondii* derived DA and TH in and around the parasitic cyst in a mouse brain. **A)** depicts DA stained in green, the parasitic lectin based cyst wall in red and DAPI showing a high concentration of parasitic nuclei in the cyst. In **B)** we see TH in green and lectin again in red, demonstrating the association of TH and DA with the latent form of the parasite inside a host brain.
1.6. Specific aims

1.6.1. Specific Aim 1 - Developing a DA linked behavioral assay

Specific Aim 1 is to address the lack of experimentally consistent behavioral changes related to *Toxoplasma* infection. As detailed in the introduction to this thesis, the lack of a generalizable behavioral model demonstrating a consistent effect of infection on host behavior has made the determination of mechanisms behind parasite induced behavioral change difficult. Additionally, in order to determine the role of the parasite in changes to host DA signaling, Aim-1 also proposes the establishment of a behavioral model of infection linked to DA signaling. Prior investigations reported behavioral alterations that could be linked to abnormal DA levels in specific brain regions (Geetha Kannan et al. 2010; Geetha Kannan and Pletnikov 2012), these tests are not sufficiently robust and consistent to be used for mechanistic studies. In order to overcome this, I explored the effects of *T. gondii* infection on behaviors produced by pharmacological compounds that strongly influence the DA system in the host. Among such compounds, amphetamine (AMPH) and cocaine (Shanks et al. 2015; Wheeler et al. 2015) formed the basis of my stimulant induced activity assays.

1.6.2. Specific Aim 2 - Biochemical impacts of infection on DA transmission

After the identification of a definitive behavioral phenotype, linking mechanistic changes to biochemical markers will be necessary to determine how host brain function is impacted by infection. Specific Aim 2 is to determine any neurochemical alterations in the DA system of infected hosts, providing a mechanistic explanation of identified
behavioral alterations. After identifying blunted response to stimulants in infected mice, I analyzed the effects of chronic infection on neuroinflammation, pre- and postsynaptic markers, markers of vesicular transport, DA release and reuptake.

1.6.3. Specific Aim 3 - determining the role of TgthAah2 in behavioral change

Mechanistic exploration of the link between infection and behavioral change requires assessing the role of direct parasitic impacts on DA neurotransmission and downstream behavioral change. Determining the behavioral impact of parasitic expression of TH in vivo is necessary to understanding the role of this genetic element. Specific Aim 3 is to determine if the Aah2 gene is critical for infected mice to exhibit blunted response to psychostimulants. Using Aah2 knockout (Δaah2) parasite strain generated and kindly provided to us by Drs. ZI Teng Weng and David Sibley (Washington University, St. Louis, MO), I characterized behavioral responses of the Δaah2 parasite strain to psychostimulants and analyzed the same markers of the DA system as described in SA2. This work ruled in or out the direct effects of parasite derived DA on behavioral change.
Chapter 2. Developing a reliable DA linked behavioral assay

2.1. Abstract

Infection with the *T. gondii* parasite has an observed relationship with human mental health and brain function (Yolken, Dickerson, and Torrey 2009) (Yolken RH, et al. 2009). Analyzing this relationship in an animal model to determine a mechanistic association between infection and behavior has proved problematic (Ajai Vyas 2015). Particularly, inconsistent behavioral phenotypes between laboratories have demonstrated that subtle factors of infection and host conditions can have significant impacts on behavioral outcomes (Worth, Andrew Thompson, and Lymbery 2014). In the work detailed below, I demonstrate a newly developed animal model of *T. gondii* infection and behavioral outcome that is independent of parasite strain, animal host background and remains constant and repeatable throughout the life of the host.

Using the dopaminergic drugs amphetamine and cocaine, I demonstrate that latent *T. gondii* infection results in significant impacts on mouse response to stimulant drugs. The complete lack of response in infected mice to an otherwise powerful stimulant suggests significant impacts of infection on brain function. It also suggests a strong associative link between infection and DA signaling, the first time such a link has been demonstrated experimentally.

2.2. Introduction

2.2.1. Previous inconsistencies in behavioral assays

As detailed in the introduction to this thesis, the field of *T. gondii* behavioral science has suffered from a lack of consistent behavioral phenotypes, and in the
experimental work detailed in this chapter, I describe a behavioral assay that addresses this need. Having a single reliable behavioral phenotype associated with *T. gondii* infection allows for a solid foundation for the exploration of mechanistic explanations behind the infection/behavior system.

### 2.2.2. Relating infection to DA system in behavior

In addition to the need to establish a behavioral assay that is consistently impacted by *T. gondii* infection, I have pursued the establishment of a behavioral testing paradigm that can link animal performance to dopamine release. Using cocaine and amphetamine to probe at the behavioral impacts of infection, allows for the analysis of behavior in conditions where extreme super-physiological levels of dopamine are present at the synapse. If differences in behavior were to arise during these conditions, I am able to link the effects of infection to components of the DA signaling system.

### 2.2.3. Psychostimulants and mechanisms of behavior

Amphetamine and cocaine operate by different mechanisms, but both have direct impacts on dopaminergic neuronal signaling. The mechanistic explanations of both drugs and the implications of what interference on the effects of each suggests is detailed below.

#### 2.2.3.1. Amphetamine

D-amphetamine (Amphetamine, AMPH) is a synthetic drug, used recreationally, illicitly, and medicinally. The effects in humans range from feelings of reward, increased concentration, alertness, euphoria, and even temporary psychosis at high doses (Dalmau, Bergman, and Brismar 1999). The specific effects of AMPH on DA release are
well understood, and act on a wide variety of targets. The pharmacodynamics of amphetamine are broad acting, interfering with an extensive list of DA release and reuptake molecular machinery (Overton et al. 2000) (Fig. 6b).

AMPH works by competitively binding dopamine transporter (DAT), a molecular pump directly responsible for the reuptake of DA from the synaptic cleft, and returning it to the pre-synaptic cytoplasm (Sandoval, Hanson, and Fleckenstein 2000). The consequences for this competitive action is that DAT is directly impeded from binding and transporting DA in the synaptic cleft, further allowing accumulation of the monoamine there.

AMPH also binds and inhibits the action of vesicular monoamine transporters 1 and 2 (VMAT), which are protein elements involved in ‘repackaging’ any monoamines collected from the synaptic cleft via reuptake, and containing them in new vesicles (Pifl et al. 1995). By inhibiting these factors, AMPH promotes the accumulation of free DA in the cytoplasm, which in turn escapes from the presynaptic terminal and ‘leaks’ into the synaptic cleft.

The collective result of all of the effects of AMPH is a profound and long-lasting increase in the amount of DA in the synaptic cleft. This results in a chronic excitatory state for dopaminergic neurons, particularly in the striatum. Because of the indiscriminate inhibitory activity of the drug on monoamine transport proteins, there is also an observed increase in accumulated serotonin, GABA, and other signaling factors across the brain (Karler et al. 1995). Due to this wider phenomenon, the relevance of differences in animal response to amphetamine cannot be tied to differences in DA
signaling alone. In animal models of drug administration, a dose of between 1-7mg/kg of
AMPH ip produces a 5-fold or more increase in locomotor activity lasting for up to
45mins post administration (McNamara et al. 2006).

2.2.3.2. Cocaine

Cocaine is a well-known recreational and illicit drug, with infrequent
medicinal use potential. Canonically, the drug is categorized as a DAT blocker, working in
a way similar to one of the functions of amphetamine at the synapse (Omenn 1976) (Fig.
6a). Cocaine binds to and blocks DAT function, precipitating the rapid accumulation of
DA at the synaptic cleft and subsequent increases in the sensation of reward, focus, and
also similar to AMPH, a large increase in heart rate and activity. In animal models, the
effects of cocaine occur at doses between 3-10 times that of amphetamine, but only
persist for a short period: typically, 15mins post injection or less (D’Mello and Stolerman
1977). With a much shorter period of action, and a mechanistic effect that is
significantly more refined than that of AMPH, cocaine is viewed as a drug that acts
specifically on DA signaling rather than the broader effects of AMPH.
Figure 6. Biochemistry of stimulant drug administration.

This image, adapted from CNSForum.com, illustrates the mechanisms of action for cocaine (a) and Amphetamine (b). Both drugs bind and inhibit the action of DAT and prevent the reuptake of DA from the synaptic cleft. Amphetamine also binds and inhibits several other targets, leading to both increased release and reduced reuptake of DA and noradrenaline.

2.3. Methods

All procedures were done in accordance with the National Institutes of Health's Guide for the Care of Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

2.3.1. Animals

Male BALB/c mice (Charles River Laboratories) arrived at 4 weeks old and were infected or given a vehicle (saline) injection at 5 weeks of age. Mice were kept on a 14hr/10hr light/dark pattern and all behavioral experimentation was conducted during
light cycle. Mice were housed in cages containing 5 animals except when fighting between cage mates necessitated separation, in which case, animals from all groups were separated into cages of equivalent populations.

### 2.3.2. Serology

Blood taken at sacrifice was immediately centrifuged at 10,000rpm for 5mins. Host serological response to *T. gondii* was measured in sera via a polyclonal commercial serum ELISA antibody assay (IBL-America, EG-127). (Results shown in Figure 16) All infected animals showed consistent 10-fold or greater expression of anti- *T. gondii* IgG than control animals at 5 wpi or greater, with no individuals experimentally excluded for evidence of insufficient infection.

### 2.3.3. Parasites/Infection

Type II Prugniaud(Pru) *T. gondii* parasites of low cellular passage number (between 7-10) were seeded onto Human foreskin fibroblast (HFFs; ATCC) cells grown in sterile Dulbecco’s modified eagle medium (DMEM; Gibco scientific) with the addition of PenStrep antibiotic and 10% fetal bovine serum. After 2 generations of tachyzoite replication in culture, parasites suspended in cellular medium were passed through a 3uM filter to remove any cellular debris and purified for infection. 400 *T. gondii* tachyzoites per mouse suspended in 200uL of Dulbecco’s phosphate buffered saline were injected intraperitoneally (ip) into the mice at 5 weeks of age, and control mice were given injections of 200uL sterile DPBS. 5 weeks were allowed to elapse for the establishment of a chronic infection before behavioral experimentation and any biochemical analysis.
2.3.4. Behavioral testing

Novelty-induced activity in the open field was assessed over a 30-minute period using activity chambers with infrared beams (San Diego Instruments Inc., San Diego, CA, USA). Horizontal activity was measured by beam breaks, indicating deliberative movement by the mouse in the chamber.

In amphetamine induced activity testing, 3-month and 8-month-old male uninfected and infected mice were given a single injection of amphetamine (5 mg/kg ip) and their locomotor activity was recorded in the activity chamber. Mice were initially habituation to the activity chamber (the first 30-minutes; the habituation session) followed by single ip injection with saline (30 mins; the saline session) to exclude possible group-related differences in responses to injection per se. Immediately afterwards, mice were injected with amphetamine and their locomotor activity was monitored for 90 minutes (the amphetamine session).

Cocaine induced activity was assessed with the same periods of observation, less the administration of a saline injection, on the previous result that host response to saline injection was not changed by infection status. Following a 30-minute habituation session, animals were administered cocaine via ip injection and monitored for 90-minutes in a cocaine session.

Pre-pulse inhibition of acoustic startle (PPI) was used to assay sensorimotor gating in the mouse model. Mice first adapted to the testing chamber with a 5min period of 78db background noise. 10 iterations of a 120db pulse and 10 of no pulse then followed. 5 pulse trials in randomized order presented 120dp pulses with pre-pulses of
0, 77, 78, 80, 84, 88, and 120db. Intervals between sessions ranged between 10-19 seconds.

2.3.5. Drugs

Experimentation was done using D-Amphetamine hemisulfate and Cocaine hydrochloride (AMP, Cocaine; Sigma Aldrich). Both drugs were dissolved in 200uL sterile DPBS and injected intraperitoneally, at a concentration of 5mg/kg and 30mg/kg respectively. Dosages were determined from comprehensive literature review indicating dosages responsible for the most significant increases in activity in BALB/c mice (Thomsen M, Caine SB 2011; Saylor AJ, McGinty JF 2008).

2.3.6. Statistics

Novelty-induced or psychostimulant-induced locomotor activity was analyzed with two-way repeated measures ANOVA, with infection status and time as independent variables, and locomotor activity as a dependent variable. PPI was analyzed with two-way repeated measures ANOVA, with infection status and pre-pulse intensities as independent variables, and the percentage of PPI as a dependent variable. Other behavioral data and Western blotting data were analyzed with one-way ANOVA or the Student two-tailed t-test. A value of \( P \leq 0.05 \) was considered significant. All data are presented as means ± Standard Error of Means (SEM).

2.4. Results

2.4.1. Startle and PPI

Chronic \( T. \ gondii \) infection significantly impaired pre-pulse inhibition (PPI) of the acoustic startle in mice. Figure 7a shows that infected mice exhibited decreased PPI at
all pre-pulse intensities, statistically significant differences at pre-pulse intensities above 82db. Notably, impaired PPI in infected mice was unlikely related to alterations in startle responsiveness as no group-dependent differences were detected in startle responses to the different magnitudes of the startle stimulus (Figure 7b).

2.4.2. Amphetamine

After an exhaustive behavioral testing of potential abnormalities in *Toxoplasma*-infected mice, we also evaluated amphetamine-induced hyperactivity in mice. This test provides a direct behavioral pharmacological assay to evaluate the status of DA neurotransmission in the brain. 8-month-old male uninfected and infected mice were given a single injection of amphetamine (5 mg/kg ip) and their locomotor activity was recorded in the activity chamber. Mice were initially habituation to the activity chamber (the first 30 mins; the habituation session) followed by single ip injection with saline (30 mins; the saline session) to exclude possible group-related differences in responses to injection *per se*. Immediately afterwards, mice were injected with amphetamine and their locomotor activity was monitored for 90 mins (the amphetamine session).

The introduction of amphetamine (5mg/kg ip, indicated by a red arrow) after 30-minutes of acclimatization in the open field, and an additional 30-minute observation period after an injection of saline (indicated by a green arrow), demonstrates a clear and significant difference between infected and uninfected mice (Figure 8). As I observed blunted responses in 8-month-old infected mice, I could not completely rule out that aging could confound this behavioral phenomenon. In order to address this question, I assessed amphetamine-induced activity in 3-month-old mice, 8wpi, Remarkably, the
blunted response to amphetamine was also observed in young infected mice and appeared more pronounced compared to older infected mice (Figure 9). Thus, my studies clearly demonstrated that chronic infection with *T. gondii* produces blunted response to amphetamine in age-independent manner. However, whether this behavioral phenomenon was specific to amphetamine or could be generalized to other stimulants remained unclear. Additionally, the broad acting effects of AMPH, described above, are not isolated to DA neurotransmission, whereas the stimulant cocaine has a more targeted effect on DA reuptake alone.

### 2.4.3. Cocaine

Thus, I also assessed the effects of cocaine on locomotor activity in mice. Control and infected 3-month-old mice were given single cocaine injections (30mg/kg ip) and locomotor activity was assessed as above except for saline injections that were not used for this experiment, as my prior studies had found no group-related effects of saline injections. Similar to the amphetamine results, I also observed blunted response to cocaine in infected mice (Figure 10). Animals infected with *T. gondii* do not have any measurable response in activity following cocaine injection (indicated by a red arrow), while uninfected animals show a rapid, and fleeting, 2-fold increase in activity that is consistent with the predicted effect of cocaine on animal behavior.
Figure 7. Chronic Toxoplasma infection impaired sensorimotor gating in mice.

The effects of chronic infection on sensorimotor gating were evaluated with pre-pulse inhibition (PPI) of the acoustic startle test. PPI was measured on control (vehicle) and infected (PRU) mice at different pre-pulse intensities (74-90 dB); the background noise was 70 dB, and the startle stimulus was 120 dB. The Y-axis depicts the percentage of PPI; the X-axis shows different intensities of pre-pulses (6a).

Two-way repeated measures ANOVA shows a significant effect of pre-pulse intensity, F(4,84)=25.8, p<0.001 and the group by pre-pulse intensity interaction, F(4,84)=3.34, p=0.016. Post-hoc Bonferroni t-test shows significant group-related differences for 87 dB and 91 dB pre-pulses, p<0.05; * denotes p<0.05 vs. PRU mice; n=10 mice/group.

These impacts are not linked to significant changes in amplitude of startle response in infected over uninfected mice (6b). Startle responses were measured at three different intensities of the startle stimulus (100, 110 and 120 dB); the background noise was 70 dB. The Y-axis depicts the startle magnitudes (artificial units); the X-axis shows different intensities of the startle stimulus.

No group differences were found in responses to the increasing intensities of the startle stimulus, p>0.05; n=10 mice/group.
Figure 8. Blunted response to amphetamine in 8-old-month-infected mice
Responses to single amphetamine injection were evaluated in control (vehicle) or infected (PRU) mice during the habituation session (intervals 1-6), the saline session (intervals 7-12) and the amphetamine session (intervals 13-30). The Y-axis depicts locomotor activity as assessed by counting beams breaks; the X-axis shows 5-min intervals for each session. The green arrow indicates the time of saline injection, red arrow indicating the time of amphetamine injection.
Two-way repeated measures ANOVA shows a significant effect of time, $F(29,599)=14.84$, $p<0.001$ and the group by time interaction, $F(29,599)=3.06$, $p=0.016$. Post-hoc Holm-Sidak test showed significant group-related differences at intervals 13 and 14, $p<0.05$; * denotes $p<0.05$ vs. PRU mice; n=10 mice/group
Figure 9. Blunted response to amphetamine in 3-month-old infected mice

Responses to single amphetamine injection were evaluated in control (vehicle) or infected (PRU) mice during the habituation session (intervals 1-6), the saline session (intervals 7-12) and the amphetamine session (intervals 13-30). The Y-axis depicts locomotor activity as assessed by counting beams breaks; the X-axis shows 5-min intervals for each session. Green arrow points to the time of saline injection, red arrow points to the time of amphetamine injection.

Two-way repeated measures ANOVA shows a significant effect of time, $F(29,569)=4.56$, $p<0.001$ and the group by time interaction, $F(29,569)=2.03$, $p=0.001$. Post-hoc Holm-Sidak test showed significant group-related differences at intervals 15-19, $p<0.05$; * - denotes $p<0.05$ vs. PRU mice; $n=10$ mice/group
Responses to single cocaine injection were evaluated in control (vehicle) or infected (PRU) mice during the habituation session (intervals 1-6) and the cocaine session (intervals 7-24). The Y-axis depicts locomotor activity as assessed by counting beam breaks; the X-axis shows 5-min intervals for each session. Red arrow points to the time of cocaine injection.

Two-way repeated measures ANOVA shows a significant effect of time, $F(23,161)=2.32$, $p=0.001$ and the group by time interaction, $F(23,161)=1.62$, $p=0.045$. Post-hoc Holm-Sidak test showed significant group-related differences at intervals 7-8 and 10-13, $p<0.05$; * denotes $p<0.05$ vs. PRU mice; n=10 mice/group

### 2.5. Discussion

#### 2.5.1. New and robust test

The finding that *T. gondii* infection creates a significant and sustained impact on the response to stimulant drugs is an important advancement for the field. This behavioral test represents a significant advancement in experimentation, allowing for a definitive test, whereby infected animals show a complete lack of response to drug administration, not a degree of attenuation or a fleeting behavioral phenotype.
Combining this work with previously described interactions of drug administration and infection in PPI (Geetha Kannan et al. 2010, 2016) and gene/infection interactions (Eells et al. 2015) this experimental protocol adds to the evidence that *T. gondii* infection has specific and definable impacts on brain function. Applying the stimulant induced activity model as a tool to understand the mechanistic associations between parasitic infection and behavior will allow the field to advance more rapidly.

### 2.5.2. Implications of blunted stimulant response

Beyond the experimental benefits of the protocol described in this work, there are also strong implications as to what a blunted response to stimulant drugs indicates about the effects of infection on the host brain. The complete oblation of the hyperactivity that is associated with both AMPH and cocaine administration indicates that significant changes have occurred in dopamine, or other monoamine neurotransmitter signaling systems in infected animals. Importantly, the effects of infection on locomotion and activity prior to the administration of stimulant drugs are not significant. This indicates that the effects of infection are only significantly present at extreme levels of DA release. I can also narrow the scope of interest to DA relevant neurobiology, since the blunting effects of infection were seen equally in AMPH, a broad-spectrum stimulant of a variety of monoamines, and cocaine, a more targeted DAT antagonist.

Even in mouse models of extreme DA dysregulation, such as DAT deficient mice (Rao, Sorkin, and Zahniser 2013), the effects of cocaine on mouse activity are not completely diminished. Primarily in models of complete DAT+VMAT2 KO is there
evidence for substantial oblation of the effects of AMPH and cocaine (Hall et al. 2014). These studies suggest that the effects of infection in this experimental design are extremely strong but visible in DA systems particularly at times of super physiological release.

2.5.3. Further analyses needed

As described above, the effect of AMPH on behavior and particularly on locomotion suggest a strong impact of infection on DA neurotransmission. Relevant to this observation, the dorsal striatum is the brain region associated with the DA neurotransmission and increased locomotion seen during stimulant administration (Yamamoto et al. 2013). Because of this, the follow up experimental steps require the analysis of DA release and reuptake machinery in the striatum. I proceeded with an analysis of striatal samples from infected and control animals, quantifying relevant protein expression in order to link infection to mechanistic impacts within the brain.
Chapter 3. Biochemical analysis of *Toxoplasma* effects on host brain function

### 3.1. Abstract

Having established a reliable behavioral assay of the effects on *T. gondii* infection on host behavior, I sought to determine any neurochemical alterations in the DA system of infected hosts. Linking these differences to the behavioral observations from chapter 1 of this thesis would provide a mechanistic explanation of the described behavioral alterations. I analyzed the effects of chronic infection on neuroinflammation, pre- and postsynaptic markers, markers of vesicular transport, DA release and reuptake.

Staining for microglial and astrocytic activation proved that chronic innate inflammation persists during the duration of chronic *T. gondii* infection. Protein expression analysis also showed that infection is linked to decrease in expression of two critical factors: dopamine transporter and vesicular monoamine transporter 2. The dysregulation of these proteins suggests that infection leads to chronic issues of DA management in animals that have chronic *T. gondii*. The behavioral data suggests these issues are not significant enough to impact behavior generally, but are highlighted when levels of DA release are extreme, notably after stimulant drug administration.

### 3.2. Introduction

#### 3.2.1. Role of vesicular transport and reuptake in DA signaling

In attempting to find the most likely targets for DA synthesis, release and reuptake, that were changed by infection, I focused on which proteins are targeted by the stimulant drugs including VMAT2 and DAT. In addition, I sought to determine if
generalized damage to synaptic health and density were impacted by infection. Any significant synaptic injury associated with infection would be indicated by changes in the expression of synaptosomal associated protein-25 (SNAP-25), a protein marker of synaptic health in the striatum (Chen et al. 2013). Also included in the assay were tyrosine hydroxylase (TH), the rate limiting enzyme in the conversion of tyrosine to L-DOPA, the substrate for DA production. Changes in levels of TH would indicate infection impacting the synthesis of DA, rather than transmission mechanisms. And finally, I included quantification of NR2A, a protein sub-unit of the NMDA receptor complex. Previous work by Kannan G, et al. (2016; 2017) in our laboratory indicated that changes in NMDA receptor sub-units accompanied behavioral change associated with T. gondii infection, under limited conditions. Additionally, NR2A protein expression levels are linked to the polygenic risk score for psychiatric disease in human populations (International Consortium on Lithium Genetics (ConLi+Gen) et al. 2018).

3.2.2. Markers of inflammation

In addition to quantifying protein level expression differences, I also sought to characterize the impact that chronic infection had on inflammatory response in the brain. Previous studies have associated the degree of host immune response to T. gondii with severity of behavioral change (Xiao, Li, Prandovszky, et al. 2016). Evidence for chronic innate immune activation (Dellacasa-Lindberg et al. 2011) accompanying T. gondii infection is also relevant. To address the potential effects of chronic inflammation in my model, I stained brain sections of infected and control animals for ionized calcium-binding adapter molecule 1 (IBA1), a canonical indicator of microglial activation in the
brain (Soulet and Rivest 2003), and glial fibrillary acidic protein (GFAP), the definitive cell surface marker in activated astrocytes (Nakase et al. 2004). The relative visible expression of these proteins in the striata and substantia nigra (Figure 11) of infected mice over uninfected would serve as accurate indicators of infection derived inflammation in the chronic disease state.

**Figure 11. Representation of the mouse brain.** This image depicts the relative positions of brain regions, showing the striatum and the substantia nigra, two regions referenced in this paper. The dorsal striatum (near the upper anterior end of the brain) is most often associated with the basal portions of dopaminergic neurons, and is activated during locomotion, and reward following dopaminergic drug administration. The substantia nigra (located ventrally and nearer the posterior) is also heavily associated with reward and locomotion. Together these two regions encompass a large portion of the critical activity in the brain following stimulant drug administration. Image taken from the Rockefeller University GENSET brain atlas (GENSAT Project, Mouse Brain Atlas).

### 3.3. Methods

All procedures were done in accordance with the National Institutes of Health’s Guide for the Care of Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine.
3.3.1. Animals

Brain samples for western blot analysis and sectioning were taken from the same cohorts described in chapter 2 of this thesis. Treatment of animals matched the description detailed there, and termination of the mice occurred at 12wks post infection, 13wks of age. Male BALB/c mice (Charles River Laboratories) arrived at 4 weeks old and were infected or given a vehicle (saline) injection at 5 weeks of age. Mice were kept on a 14hr/10hr light/dark pattern and all behavioral experimentation was conducted during light cycle. Mice were housed in cages containing 5 animals except when fighting between cage mates necessitated separation, in which case, animals from all groups were separated into cages of equivalent populations.

3.3.2. Parasites/Infection

Type II Prugniaud(Pru) Toxoplasma parasites of low cellular passage number (between 7-10) were seeded onto Human foreskin fibroblast (HFFs; ATCC) cells grown in sterile Dulbeccos modified eagle medium (DMEM; Gibco scientific) with the addition of PenStrep antibiotic and 10% fetal bovine serum. After 2 generations of tachyzoite replication in culture, parasites suspended in cellular medium were passed through a 3uM filter to remove any cellular debris and purified for infection. 400 *Toxoplasma gondii* tachyzoites per mouse suspended in 200uL of Dulbecco’s phosphate buffered saline were injected intraperitoneally (ip) into the mice at 5 weeks of age, and control mice were given injections of 200uL sterile DPBS.
3.3.3. Tissue preparation and WB

Whole striatum protein expression was measured via Western blotting following standard protocols (5–6 mice per group). Isolated striatum tissue was sonicated for 30 s in RIPA buffer (Cell Signaling Technology, 9806) containing 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF and 1 μg/mL leupeptin. Tissue lysates were spun down at 10,000 × g for 10 min at 4 °C. The resulting supernatant was subjected to SDS/PAGE, and the separated proteins were transferred onto a nitrocellulose membrane. The membrane was washed in Tris-buffered saline solution containing 0.05% Tween 20 (TBST) and was blocked for 1 hour at RT in TBST containing 5% non-fat dry milk. The membrane was incubated overnight at 4 °C with a primary antibody. All primary and secondary antibodies used are listed in Table 1.

The optical density of protein bands on each digitized image was normalized to the optical density of the β-actin using the freely available ImageJ software program (version 1.49v).

3.3.4. Histology/IFA

Brain tissue prepared for histology by submersion perfusion in 4% PFA PBS overnight, cryoprotection in progressive overnight submersions of 10 and then 30% sucrose in PBS. Flash frozen at -100C and then prepared for cutting via microtome.

3.3.5. Western Blotting

For Western blot assays, 2-month-old control and DN-DISC1 mice were euthanized and striata were isolated on ice, immediately frozen and stored at -80 °C
until protein extraction. Isolated hippocampi were sonicated for 30 sec in cell lysis buffer (Cell Signaling Technology, 9803) containing 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF and 1 μg/mL leupeptin. Cell lysates were spun down at 10,000 × g for 10 min at 4 °C. The resulting supernatant was subjected to SDS/PAGE, and the separated proteins were transferred onto a nitrocellulose membrane. The membrane was washed in Tris-buffered saline solution containing 0.05% Tween 20 (TBST), and was blocked for 1 hour at RT in TBST containing 5% fatty acid free bovine serum albumin (BSA) fraction V (03117057001, ROCHE) for anti-dopamine transporter antibody or 5% non-fat dry milk for all other antibodies. The membrane was incubated overnight at 4 °C with a primary antibody. Membranes were probed with Anti-Tyrosine Hydroxylase antibody (1:500, ab6211, Abcam), Anti-VMAT2 antibody (1:500, ab191121, Abcam), Anti-Dopamine Transporter antibody (ab111468, 1:500, Abcam), Anti-NMDA Receptor NR2A Subunit N-terminus Antibody (1501-NR2A, PhosphoSolutions), anti-Synaptosomal-Associated Protein 25 (SNAP-25) Antibody (Ab5666, 1:500, Abcam) and mouse anti-β-actin (1:2000, A5441, Sigma-Aldrich). After three washes with TBST, membranes were incubated with HRP-conjugated donkey anti-rabbit IgG (1:1000, NA934V, GE Healthcare) or donkey anti-mouse IgG (1:1000, NXA931, GE Healthcare) secondary antibodies for one hour at room temperature. The immunoblots were visualized in Blu-Ray autoradiography films (NDA8803, Next Day Science) and Super Signal West Pico Chemiluminescent Substrate (34080, ThermoFisher SCIENTIFIC). The optical density of protein bands on each
digitized image was normalized to the optical density of the β-actin using free ImageJ software (version 1.49v).

3.4. Results

3.4.1. Western blot results

The results of the western blotting showed significant impacts of infection on expression of the DA signaling proteins DAT(Fig. 12b) and VMAT2 (Fig. 12c). Infected mice had significantly less expression of these factors in the dorsal striatum, indicating severely impaired DA signaling machinery in the brain region most associated with dopamine induced locomotor activity. Impaired expression of DAT and VMAT in infected mice, combined with the behavioral outcomes detailed in chapter 2, indicates that significantly decreased expression of these factors following infection, may be preventing the effects of stimulant drug binding to the same protein targets.

Differences in expression levels of SNAP-25, TH and NR2A were not apparent in the samples (Fig. 12a), indicating that striatal levels of these proteins were not impacted by infection. From these results, it can be inferred that the synthesis of DA and overall synaptic density in the striatum were not influenced by infection. These results allow us to rule out striatal damage following infection being the cause of impaired response to stimulant drugs, and focus on the effects of dysregulation in DA neurotransmission.

3.4.2. Markers of inflammation via microscopy

The visual results of IBA1 and GFAP staining of infected and uninfected animals indicate strong evidence for chronic inflammation associated with chronic *T. gondii* infection (Figure 13). We can see the structure of the substantia nigra at 4x, and a
globally increased level of IBA1 staining, denoting increased microglial activation. GFAP staining is similarly considerably more visible in and around the substantia nigra, demonstrating a coincident activation of astrocytes in infected brains. At 40x magnification, the increased dendritic processes of activated microglia indicate that both the density and activity of those cells are increased in infected mice. These results are consistent with previous observations of inflammation associated with *T. gondii* infection (Zhang et al. 2014), and they represent a potential mechanistic link between infection, inflammation and DA signaling in the substantia nigra.

![Image of Western Blot](image)

**Figure 12. DAT and VMAT expression are significantly impacted by *T. gondii* infection**

Representative images of Western Blot staining in **10a** indicate that no significant levels of expression differences resulting from infection were found for SNAP-25, TH and NR2A. Visual evidence for differences in DAT and VMAT expression is supported by quantification (**10b, 10c**) of samples from n=8 animals per group. These results demonstrate a statistically significant decrease in DAT and VMAT proteins in infected mice.
Fluorescent staining for GFAP and IBA1 in the substantia nigra of infected and control animals shows a profound increase in expression, denoting chronic inflammation associated with chronic *T. gondii* infection. Increased staining of activated microglia (IBA1) and visible evidence of increased extension of processes (40x), indicate that the infected mice are suffering from significant CNS inflammation during infection. Similarly, increase in GFAP staining indicated astrocyte activation.

40 uM sections, taken coronally at between 70-75 sections from anterior terminus of the brain.
3.5. Discussion

3.5.1. Indications of synaptic health but dysregulated DA transport expression

The quantitative protein analysis shown indicates that chronic *T. gondii* infection is not associated with major synaptic injury. SNAP-25 levels do not show any indication that infection causes significant damage to striatal synapses. Similarly, TH expression is not significantly impacted by infection, nor do we see evidence of changes to NR2A. Together these results show that the basic functions of the striatal neurons are largely intact during chronic infection. However, the Western blot results do indicate that expression of DAT and VMAT2 are significantly impacted by infection. These results are consistent with the behavioral assays described in chapter 2 of this thesis, showing that infection massively impacts the ability of the host to respond to dopaminergic stimulant drugs. Chronic under-expression of VMAT2 and DAT provides significantly less substrate for binding of both cocaine and AMPH, diminishing their activity (Isingrini et al. 2016; Hall et al. 2014).

3.5.2. Significant microglial and astrocytic activation associated with infection

Chronic infection with *Toxoplasma* is associated with visibly noticeable levels of inflammatory response in the brain. Quantification of microglial activation in infected and uninfected groups (n=10) supports a massive increase associated with chronic *T. gondii* infection as well (data not shown). This chronic response represents a promising indirect mechanist link between infection and behavioral change. However, the evidence currently presented does not rule out direct impacts of the parasite on DA
neurotransmission, only that such impacts would also accompany significant chronic inflammation during infection. Moving forward, the primary goal is to achieve conclusive evidence demonstrating what role, if any, direct impacts of parasitic infection have on host behavior.
Chapter 4. Understanding the role of parasitic direct effects on DA synthesis and release

4.1. Abstract

The presence of two functional homologues of tyrosine hydroxylase in the Toxoplasma genome has been associated with the production and release of dopamine in and around the cyst structure in infected animals ([Prandovszky et al. 2011; Martin et al. 2015]). This observation has fueled speculation that behavioral change associated with T. gondii infection may be related to direct interference in the DA signaling pathway by the parasite. To date, the particular TH homologue that is upregulated during chronic CNS infection is the most promising candidate mechanism, whereby the parasite would directly interfere with host brain function.

The work detailed in the previous two chapters of this thesis demonstrates that T. gondii infection causes a sustained downregulation in expression of DAT and VMAT2, observed alongside a significant blunting of the effects of dopaminergic stimulant drugs. This experimental system is ideal for determining if the exogenous extracellular DA produced by T. gondii may be responsible for all, or part, of the downregulation of DA transport proteins, and of the DA related behavioral phenotype. Using a CRISPR/Cas9 based KO of the TgthAah2 gene in type II Prugniaud Toxoplasma, the laboratory of Dr. David Sibley at Washington University, St. Louis, provided me with a parasite strain deficient in this genetic element. Comparison of the effects of infection with WT PRU parasites vs those that are Δaah2 revealed that the gene is not responsible for any of the observed impacts of infection on host performance during a stimulant induced activity assay. Furthermore, the parasitic derived DA release also does not contribute to
any measurable differences in protein expression in infected host animals. These findings definitively demonstrate that no substantial evidence exists for the direct interference of the *T. gondii* parasite on host behavior.

4.2. Introduction

4.2.1. TgTHAAH2 and DA

As described in chapter 1 of this thesis, one of the prevailing theories regarding how *Toxoplasma* may be directly influencing host behavior, is through the generation of parasite-derived dopamine inside the CNS (Marino and Boothroyd 2017; Martin et al. 2015; Prandovszky et al. 2011). Focusing on the TgthAah2 genetic element, there has been a consistent interest in isolating the role that parasitic TH expression has on the generation of physiologically meaningful levels of DA increase at the host synapse. Applying a TgthAah2 deficient parasite to a robust behavioral test of infection, and comparing the results to an in-tact pathogen, is the behavioral test that is most sought after.

4.2.2. Generation of KO strains

Our laboratory was approached by Dr. David Sibley at Washington University in St. Louis, and his graduate student Zi Teng Weng. They had been pursuing the generation of a TgthAah2 deficient parasite for some time, and had succeeded in generating a KO in a type 2 PRU background pathogen. They graciously offered our lab these parasites for behavioral analyses and I accepted.

The initial parasites I received were generated in a laboratory strain that was genetically distinct enough from the one used by our group, that behavioral comparison
became too inconsistent to be experimentally useful (data not shown). Recognizing that
the effect of infection on DA neurotransmission that the type II strain our laboratory
created was the most critical test for this experiment, I provided our parasites for Dr.
Sibley’s group to conduct CRISPR/Cas9 based TgthAah2 deletion in. They returned
deficient parasites to us, allowing us to compare Δaah2 parasites and our own
background strain of PRU parasites, without any additional genetic or culture
dissimilarity.

4.3. Methods

All procedures were done in accordance with the National Institutes of Health's
Guide for the Care of Use of Laboratory Animals and were approved by the Institutional
Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

4.3.1. Animals

Male BALB/c mice (Charles River Laboratories) arrived at 4 weeks old and were
infected or given a vehicle (saline) injection at 5 weeks of age. Mice were kept on a
14hr/10hr light/dark pattern and all behavioral experimentation was conducted during
light cycle. Mice were housed in cages containing 5 animals except when fighting
between cage mates necessitated separation, in which case, animals from all groups
were separated into cages of equivalent populations.

4.3.2. Strain generation

CRISPR/Cas9 based disruption of the TgthAah2 gene was completed by the Sibley
group, using a protocol described in Long S, et al. 2016. Parasites deficient in this genetic
element were shown to have no deficiencies in growth either in vitro or in vivo (Wang ZT, et al. 2015; 2017).

4.3.3. Parasites/Infection

At 5 weeks of age animals were separated into 5 groups of n=10, corresponding to infection status, and given either a saline vehicle injection (control), 400 unaltered PRU parasites ip (infected), or 400 TgthAah2 deficient parasites (Δaah2). All behavioral experimentation occurred between 6 and 10 weeks post infection. Additional internal control groups were sent by the Sibley group, and included Δhxg parasites which have a genetic disruption in the HXGPRT genetic element, a feature that allowed for drug selection of the TH modified parasites, but no interference with TH expression. The final parasite group included T. gondii that was deficient in TgthAah2 as well as a cDNA insertion to rescue expression of this element.

4.3.4. Behavioral testing

Animals were infected and behavioral testing was conducted using an identical protocol to that described in chapters 2 and 3 of this thesis. Behavioral analysis using AMPH induced activity was selected to determine if disrupting parasitic TH expression had any functional impact on recovering the blunted response to stimulant drugs seen previously.

4.3.5. Tissue preparation

After termination at between 10 and 15 weeks post infection, animals were sacrificed and brain tissue collected for analysis and histology using identical protocols to the description in chapters 2 and 3 of this thesis.
4.3.6. **Serology**

Blood was taken by survival draw during acute infection and again during sacrifice, centrifuged at 10,000 $\times$ g for 5 min to collect serum. Absorbance values on a polyclonal anti- *T. gondii* IgG serum ELISA kit (IBL America) were used for statistical analysis. *T. gondii* cyst burden was determined using a previously developed assay for antibodies to cyst matrix antigen 1 (MAG1) (Xiao et al., 2015)

4.4. **Results**

4.4.1. **AMPH induced activity**

Results from the AMPH induced activity assay (Figure 14) demonstrated that disruption of the parasitic TH had functionally no impact on host response to the amphetamine stimulant. Infection with *T. gondii*, irrespective of genotype, created a severely blunted host response to the drug. At time points 15-20, following the administration of amphetamine at 12, there are statistically significant differences ($p<0.001$) in activity between uninfected and infected groups. These findings conclusively indicate that direct interference of the parasite on my observed behavioral phenotype, via DA generation, is not occurring.
Figure 14. Deletion of Aah2 does not affect blunted response to amphetamine

Responses to single amphetamine injection were evaluated in control (vehicle) mice, mice infected with wild-type laboratory strain (PRU), or mice infected with the KO strain (PRU-KO) during the habituation session (intervals 1-6), the saline session (intervals 7-12) and the amphetamine session (intervals 13-30). The Y-axis depicts locomotor activity as assessed by counting beam breaks; the X-axis shows 5-min intervals for each session. Green arrow points to the time of saline injection, red arrow points to the time of amphetamine injection.

Two-way repeated measures ANOVA shows a significant effect of time, F(29,899)=4.13, p<0.001 and the group by time interaction, F(29,899)=1.69, p=0.001. Post-hoc Holm-Sidak test showed significant difference between control mice and PRU or PRO-KO mice at intervals 16-18, and control and PRU mice at intervals 15-19; p<0.05; * - denotes p<0.05 vs. PRU and PRU-KO mice; ** - denotes p<0.05 vs. PRU only; n=10 mice/group
Figure 15. Disruption of the TgthAah2 element has no impact on expression of DA signaling proteins following infection.

Representative images of Western results show that infection with in-tact *T. gondii* and TgthAah2 deficient parasites have identical impacts on the expression profile of host DAT and VMAT2. Both strains are responsible for significant decreases in expression of these factors, consistent with the behavioral deficits demonstrated in Fig. 13. Similarly, neither strain of parasite induces measurable expression differences for SNAP-25, TH or NR2A.

Statistical analysis of relative expression indicated a significant (n=6, p<0.05) difference in both DAT and VMAT2 expression for animals infected with either parasite strain and their uninfected counterparts.
### Table 1. Antibodies Used

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Application</th>
<th>Description</th>
<th>Source</th>
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<tbody>
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<td>WB</td>
<td>Rabbit polyclonal</td>
<td>Abcam ab6211</td>
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<tr>
<td>VMAT-2</td>
<td>WB</td>
<td>Rabbit polyclonal</td>
<td>Abcam ab191121</td>
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<td>DAT</td>
<td>WB</td>
<td>Rabbit polyclonal</td>
<td>Abcam ab111468</td>
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<tr>
<td>NMDA Receptor NR2A Subunit N-terminus</td>
<td>WB</td>
<td>Rabbit polyclonal</td>
<td>Phospho Solutions 1501-NR2A</td>
</tr>
<tr>
<td>β-Actin</td>
<td>WB</td>
<td>Mouse monoclonal</td>
<td>Sigma A5441</td>
</tr>
<tr>
<td>Donkey- α -rabbit</td>
<td>WB</td>
<td>HRP-2°</td>
<td>GE Healthcare NA934V</td>
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<td>Donkey- α -mouse</td>
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<td>WAKO 019-19741</td>
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<td>GFAP</td>
<td>IF</td>
<td>Goat polyclonal</td>
<td>Abcam ab53554</td>
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<tr>
<td>Goat α -Rabbit</td>
<td>IF</td>
<td>AlexaFluor594-2°</td>
<td>Life Technologies R37117</td>
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<tr>
<td>Donkey α -Goat</td>
<td>IF</td>
<td>TexasRed620-2°</td>
<td>Abcam ab6883</td>
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Figure 16. TgthAah2 disruption has no impacts on inflammatory markers in the striata of infected mice

In this figure, we can see that infection with either intact or TgthAah2 KO parasites creates largely the same profile of inflammatory response. Microglial activation, shown by IBA1 staining in TxRed, is significantly increased in infected mice, over uninfected, and that increase is not dependent on parasitic genetics. Similarly, FITC staining of activated astrocytes is increased in infected animals, but independent of parasitic genome.

40uM sections, taken between 10-15 sections from the anterior end of the brain.
Table 2. Polyclonal Anti- *T. gondii* IgG Response

<table>
<thead>
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Data represent absorbance values ± SEM, n=4 in each group, wpi – weeks post infection.

Figure 17. No differences seen in overall host IgG response, or against *T. gondii* cyst wall

The effects of chronic *T. gondii* infection on expression of the cyst marker, MAG1, in the striatum in uninfected (Vehicle), PRU-infected mice (PRU) and mice infected with PRU-KO strain (PRU-KO). The Y-axis shows the relative units of absorbance in the ELISA test as described in the Methods; n=5 per group; * denotes P<0.05 vs. PRU or PRU-KO; one-way ANOVA.
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4.4.2. Histology and WB results

Consistent with the behavioral data already presented, the results of quantitative Western blotting demonstrate that changes in parasitic expression of TH do not have an impact on host protein expression (Fig. 14). In animals that received both intact and TgthAah2 KO parasites, there are significantly lower expression levels of DAT and VMAT in the striatum, while the remaining markers of synaptic health and neurotransmission appear unchanged. No factors are significantly affected by the genotype of parasite used for infection, ruling out the disrupted genetic element as a causative factor in the generation of this protein expression phenotype.

Histological analysis shows an identical profile and conclusion to that described in the Western data. Infection with *Toxoplasma*, irrespective of genotype, induced a visible increase in IBA1 and GFAP expression in the striata of infected animals. No impact of parasitic genotype appeared in any visible identifiers of inflammation. These results together demonstrate that the inflammatory and brain physiological impacts of *T. gondii* infection are completely independent of the generation of parasitic TH.

4.4.3. Serology and cyst burden

I found no significant differences between mice infected with wild-type or Δaah2 strain the levels of Mag1 protein, a brain cysts marker (Table 1) or polyclonal anti- *T. gondii* IgG response (Table 2). These findings, combined with work done by Dr. Sibley’s lab during initial culture of the parasite (Wang et al. 2015), demonstrate that the Δaah2 parasite has no defects in growth or cyst formation either *in vitro* or *in vivo.*
4.5. Discussion

4.5.1. Parasitic TH is unnecessary for behavioral change, inflammation or DA signaling protein expression

These behavioral, histological, and expression level analyses clearly demonstrate that infection with *Toxoplasma* consistently impacts behavioral response to dopaminergic stimulant drugs, and that this behavioral change is linked with increased CNS inflammation and decreased expression of DAT and VMAT2. However, all of these changes are completely independent of the production of TH by the parasite itself, effectively proving that the parasite is not directly impacting behavior directly via the exogenous production of TH.

The results detailed here are consistent with recent work that has been published demonstrating that parasitic TH may have a role during infection of the definitive feline host, rather than any impact during intermediate infection. The hydroxylase activity of the enzyme was found to have a role in forming dityrosine crosslinks, which are a component of the oocyst structure (Wang et al. 2017b). Disruption of that genetic element led to decreased oocyst production from feline hosts, without any impact on function or viability in intermediate hosts. Similarly, another laboratory pursuing similar questions regarding the role of parasitic TH in behavior, has shown that in a mouse model of risk aversion, genetically excluding the effects of parasitic TH has no impact on behavioral outcomes (Afonso et al. 2017). My results are part of recently expanding literature demonstrating that direct impact of the *T. gondii* parasite on behavior is increasingly unlikely. This work indicates that indirect effects
surrounding inflammation, or other factors driven by infection, may be most responsible for behavioral change during infection.
Chapter 5: General Discussion

5.1. Chronic *Toxoplasma* infection blunts host response to dopaminergic drugs

The results of the work detailed in this thesis clearly show that in an animal model of chronic infection, *Toxoplasma gondii* induces a significant change in host response to dopaminergic stimulant drugs. Resulting in an almost complete oblation of the locomotor response to amphetamine and cocaine, the behavioral testing indicates a strong and robust behavioral phenotype. Importantly for the field of *T. gondii* behavioral research, this novel testing paradigm persists through the course of infection, with a predictable response observed even 30 weeks post infection. Additionally, after instructing another laboratory on the potential usefulness of this assay in *T. gondii* behavioral analysis, they repeated the findings using outbred mice and a type 1 strain of parasites (Xiao, Li, Gressitt, et al. 2016). They observed a nearly complete blunting of response to AMPH in mice showing serological evidence of high cyst burden. Significantly, this appears to be the only published example of a behavioral assay reliably impacted by *T. gondii* infection across significantly different strains of parasites and of host rodents.

5.2. *Toxoplasma* induced behavioral change is linked to changes in DAT and VMAT expression

Western blot analysis of striata taken from my experimental animals show significant changes in the expression levels of DAT and VMAT2, two canonical proteins involved in pre-synaptic DA signaling. This observation suggests several potential mechanistic links between infection and the observed behavioral change after stimulant administration. Any lowering in DAT and VMAT2 expression in the striatum would result
in fewer targets for cocaine and AMPH to bind to, and cause a blunted locomotor response. Considerable published evidence indicates that in animal models of diminished or removed DAT and VMAT, the physiological (Perles-Barbacaru et al. 2011) and behavioral (Hall et al. 2014, 2009) impacts of stimulant administration are significantly impacted. In this regard, my behavioral model system indicates that *T. gondii* infection causes a reduction in expression of key signaling proteins, precipitating a highly significant blunting of host response to dopaminergic drugs.

**5.3. Indications of chronic inflammation are present in chronic infected mice**

Analysis of microglial and astrocytic activation in my animal model of infection also demonstrate that chronic brain specific inflammation is present during the course of *T. gondii* infection. Significant activation of brain specific monocytes is indicative of a chronic innate inflammatory state that accompanies infection. This finding is consistent with recent indications of large scale upregulation of innate pro-inflammatory factors in the brain during *T. gondii* infection (Umeda et al. 2017) and microglial activation contributing to neurodegenerative (Zhang et al. 2014) and behavioral (Evans et al. 2014) phenotypes. Furthermore, innate immune activation contributes to the induction and maintenance of adaptive immune responses. Work by our laboratory and others has demonstrated that adaptive immune responses to *T. gondii* in chronic infection may drive generation of anti-self-brain peptide antibodies, interfering with brain function (Geetha Kannan et al. 2016; G Kannan et al. 2017; Dickerson et al. 2013). These connections demonstrate that the immune activation observed in my model system has a critical mechanistic association with brain function and behavior.
Most relevant to the behavioral effects we observe in *T. gondii* infected mice, there has also been recent work indicating that innate inflammatory activation impedes the effects of dopaminergic stimulant drugs via interference in upstream GABA signaling. Taylor AM et al. (2015) demonstrated that in an animal model of chronic pain, microglial activation is associated with a complete lack of response to cocaine administration. This result is closely related to my own findings and indicates that the same effects of inflammation may be pushing the blunted locomotor response seen in my system. The impacts of infection on GABA signaling may also be occurring in my *T. gondii* model, and the effects on DAT and VMAT2 expression are in no way exclusive to impacts on other signaling systems.

5.4. Blunted response is entirely independent of parasitic tyrosine hydroxylase

The experimental data in this thesis also conclusively rules out the role of parasitically derived tyrosine hydroxylase in the behavioral changes I identified. This effectively closes discussion on the role of the TgthAah2 genetic element in direct interference with behavior. Using parasites manipulated by the CRISPR based KO system, I was able to experimentally prove that the removal of the TgthAah2 product alone had no impact on the behavioral outcome of infected hosts. Without having any further genetic manipulations of the parasite associated with *in vitro* parasitic culture, the protocol of infection with TH KO parasites could be as close as possible to those with intact TH expression.

Observations that the *Toxoplasma* genome contained homologues to TH (Gaskell et al. 2009), and an associated increase in DA expression in and around the cyst
(Prandovszky et al. 2011) provided an intriguing possibility that the direct synthesis of TH by chronic *T. gondii* may be impacting host brain function and behavior. Those observations required behavioral analysis to rule in or out the role of this genetic element, and I have successfully completed this analysis. My findings are also consistent with more recent work that has called into question the effects of parasitic TH on host DA metabolism (Wang et al. 2015) and indications that the TH elements have specific functions that promote transmission of the pathogen into its definitive host (Wang et al. 2017a). Similarly, my work is consistent with findings just published indicating that the TgthAah2 element has no role in behavioral change relating to risk avoidance (Afonso et al. 2017).
Figure 18. Overview of the link between infection and behavioral change.
This depiction shows the analysis of behavioral change conducted in this thesis work, linking behavior to mechanistic explanations of brain function following infection. Having identified that latent *Toxoplasma* infection results in a predictable behavioral outcome where dopaminergic stimulant drugs have little or no effect on host behavior, I sought to explain the changes via the analysis of both direct and indirect mechanisms. The analysis of the direct impact of *Toxoplasma* derived TH demonstrated that the expression of the TH homologue by the parasite has no significant impact on host response to DA stimulant drugs. Moving to explore the indirect effects of infection on behavior, I demonstrated that infection with the latent form of *Toxoplasma* results in chronic inflammation, consisting of activated microglia and astroglia within the brain. This activation is in turn linked to a significant downregulation in the expression of DAT in the host brain, demonstrating a connection between inflammatory response associated with infection, DA release and reuptake, and finally, behavior.

5.5. Future Directions

5.5.1. Experimental

There are several clear next steps in the experimental analysis of this animal model. The first is an experimental plan to assess the impact of *T. gondii* infection on real time DA release in the host striatum. This can be accomplished using microdialysis, sampling *in vivo* extracellular DA release before and after the administration of stimulant drugs (Hamilton, Redondo, and Freeman 2000). I hypothesize that levels of...
extracellular DA release will be impacted by infection, reflecting the blunted response to AMPH in cocaine in infected animals, and show a decrease in DA associated with infection. If the results demonstrate otherwise, then it may suggest a post-synaptic role for infection in DA signaling where levels of synaptic DA are not changed by infection, but host response to the DA is. Preliminary microdialysis has already been completed on the experimental cohorts shown in this work, but conclusions regarding DA levels could not be ascertained from this data. Preliminary indications that levels of the DA metabolite 3,4-Dihydroxyphenylacetic acid (DOPAC) are lower in infected animals (data not shown) after AMPH administration are consistent with my mechanistic hypothesis.

An additional experimental protocol is being developed to test one critical finding regarding inflammation and DA signaling in my model. In the chronic pain model described above (Taylor et al. 2015), the authors were able to link microglial activation with blunted response to cocaine, and rescue that effect by interfering with brain inflammatory signals in their model. They accomplished this by the administration of minocycline, a tetracycline class antibiotic that is also believed to cause depolarization of macrophage and microglia. I attempted to administer this drug, both ip and via water delivery, to T. gondii infected animals and assess if attenuation of the behavioral phenotype occurred, rescuing response to stimulant drugs. In all cases, handling and environmental changes associated with minocycline administration were too disruptive to the behavioral phenotype to make statistical assessment possible (data not shown). Recent studies showing depolarization and depopulation of microglia via other drug interventions (Pyonteck et al. 2013) and even irradiation (Acharya et al. 2016) offer
alternative means to disrupt brain specific inflammation. I hypothesize that effective blocking of this activation in my disease model will also rescue the host response to stimulant drugs, reflecting the same GABA-DA associative pathway outlined by Taylor et al.

5.5.2. Translational

Applying the findings of this thesis to human health, there is one particular next step that stands out experimentally. The association of T. gondii infection with human mental health has been well explored and appears variable based on population and disease severity factors. However, the behavioral effects in the animal model presented in this work are almost binary in their impact. Significant behavioral response to stimulant drugs in healthy animals, and completely blunted response in infected ones. The results speak to a potential impact in infected human subjects that is difficult to ignore. It has been established clinically that individual patients respond very differently, both in behavior (Pang et al. 2016) and in DA release (Smith et al. 2016) to d-amphetamine administration. These results can in be seen particularly in the highly variable response of ADHD patients to d-amphetamine treatment (Adderall) (Cherkasova et al. 2014). Comparing patient responsiveness to amphetamine treatment with T. gondii serology may indicated a potential new role of the parasite in treatment refractory ADHD. I predict that human serological positivity to Toxoplasma will be indicative of a diminished effectiveness of amphetamine-based medications.
5.6. Conclusion

My results demonstrating that parasitic TH has no observed role in behavioral change, combined with a new putative biological role for the synthesis of TH by the parasite, help to seal the case that direct interference of host behavior by the parasite via DA synthesis is not occurring. This significantly forwards the argument that indirect mechanisms are behind the observed behavioral changes associated with infection. I have also demonstrated that chronic inflammation and microglial activation are present in infected hosts, linking indirect effects of infection to changes in host neurobiological factors. These indications of chronic inflammation and changes in host expression of crucial DA transport machinery together suggest that *T. gondii* infection is interfering with how the mammalian host brain signals using dopamine. Those impacts have the directly observable effect that infected mice show significantly reduced response to dopaminergic stimulant drugs.

With these results, I think that we enter into a greater understanding of how *T. gondii* infection impacts behavior. Considering the impact of indirect factors such as inflammation, and drawing a more nuanced illustration of the interaction of parasite and host. It is exciting to consider the impacts of a neurotropic parasite as direct and purposeful, interfering with brain function to further its own biological needs. But the reality is likely much more subtle and indirect, and ultimately more interesting, as it links together risk factors for human disease, adding to a rapidly expanding view of how environmental factors perturb brain function and development toward a disease state.
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Curriculum Vitae

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Education

- **08/2012 – 01/2018 (Defense scheduled):** PhD candidate in the laboratory of Dr. Mikhail Pletnikov, MD, PhD at the **Johns Hopkins** Hospital, Department of Neurobiology. Thesis title: “Characterizing the effects of latent parasitic infection on dopamine signaling and brain function.” Thesis research on innate neuroimmunology, dopamine signaling, drugs of abuse, and impacts of infectious disease on brain function and behavior. Additional projects included cellular culture and in vivo analysis of a variety of viral pathogens, including Zika. Research on the relationship between the microbiome and brain function, probiotic treatment effects on behavior, and the effects of hormonal modulation on immune function.
  - Awarded the Carlton and Estelle Herman Award in Parasitology, Vector Biology and Animal Borne Diseases from Johns Hopkins SPH, 2013-2014.


Selected Research Skills

- DNA extraction, PCR and genetic analysis by several methods
- RNA purification, rt-PCR, and real time analytical methods for mRNA
- Animal behavioral work, including drug treatment, gavage, behavioral assays.
- Microsurgery, specializing in rodent neurosurgery and microdialysis.
- Protein quantification by Western, ELISA, and mass-spec
- High precision liquid chromatography (HPLC)
- Cell sorting, FACS
- Cell culture, maintaining human cell lines, passaging obligate pathogens
- BSL2 and 2+ handling in vitro and in vivo
- Collection and analysis of human tissue, blood and serum
- Bacterial culture, cloning and transformation
- Statistical analysis in MATLAB, R, proficiency in Excel, Adobe Illus. etc.
Selected Publications


- **06/2010 - 08/2012:** Research scientist in the laboratory of Dr. Lalita Ramakrishnan, MD at the *University of Washington,* Department of Immunology. Research on the immunology of tuberculosis infection, human genetics, and modeling immune response to the pathogen in an animal system.

- **09/2009 – 05/2010:** Field research technician in Lambir Hills National Park, Sarawak, *Malaysia.* Organizing an ongoing scientific study, observing tropical hardwood tree growth patterns in lowland rainforest for Dr. Sabrina Russo at the University of Nebraska, Lincoln.

- **02/2009 - 07/2009:** Field research technician in Khao Yai National Park, Thailand. Collecting entomological and mycological samples on behalf of the laboratory of Dr. Naomi Pierce, PhD at *Harvard University.* Specific research on the behavioral modifying mycological parasite of ants, *Ophiocordyceps unilateralis*.

- **09/2006 – 02/2009:** Laboratory technician in the laboratory of Dr. Charles Davis, PhD, *Harvard University.* During work study employment as an undergrad, and for several months after graduation, I worked conducting DNA extraction PCR and sequencing work for the group, researching the systematics of rare tropical plant species in the malpighiales clade.
Teaching Experience

- **09/2014-12/2014**: Teaching assistant for the *Johns Hopkins* Bloomberg School of Public Health course “Graduate immunology,” taught by Dr. Noel Rose. Duties included lecture presentations, email and web presence, and coordinating faculty participation.

- **07/2009 – 09/2009**: Teaching assistant for the *Harvard University* Summer School course, “The biodiversity of Borneo”, under professor Dr. Cambell Webb, based in Kota Kinabalu, Malaysia. Coordinated logistics, purchasing, email and web presence for the course, as well as lecturing on topics in entomology.

Additional Employment History


- **2005-2007**: Caller, Crimson Callers. Cambridge, MA. Calling alumni for development donations as college work study employment.

- **2005**: Deckhand, F/V Ocean Dancer. Sitka, AK. Full time deckhand aboard a commercial salmon fishing boat during a summer commercial season. Duties included landing, gutting and packing fish, cooking meals, cleaning and resupplying the vessel.

- **2006**: Paid Intern, Montana Water Trust. Missoula, MT. Full time summer work assessing and protecting trout habitat in western Montana. Work involved approaching landowners with requests for water rights transfers, writing legal documents, and grant requests for federal funding.

Additional Skills and Hobbies

- **USPA licensed skydiver (#A61844)** since 2013 with ~250 jumps. Experience in static line, freefly, and hot air balloon jumps.

- High proficiency in MIG, flux core welding, mild steel TIG, and moderate proficiency with stainless and aluminum TIG.

- Certified in fabrication, milling, lathe from the physics machine shops at Harvard University, University of Washington. Experience with CAD, Solidworks, etc.

- Experienced in fine woodwork, particularly wood turning.

- Highly experienced with auto and motorcycle repair. Projects have included a self-designed conversion of a 1993 Honda XR600R motorcycle to fully electric DC motor with a hand built lithium-polymer power pack ([https://www.instagram.com/p/BKB93lyBGEy/](https://www.instagram.com/p/BKB93lyBGEy/)). Also, the conversion of a 1985 Toyota 4x4 from gas motor to diesel, operating on biodiesel.
• **Fly-fisherman** since elementary school, including stream and ocean fishing across North America and the Caribbean.

• Rock and mountain **climber** with summits in Asia and Mexico, and have guided climbs, including Mt. Rainier, Adams, and others.

• Basic command of Spanish and Malay, fluency in English.